UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA
DEPARTAMENTO DE FISIOLOGÍA (FISIOLOGÍA ANIMAL)

TESIS DOCTORAL

Characterization of EGF and IGF-1 system in the ovary and its role in in vitro maturation of guinea pig oocytes

Caracterización del sistema EGF e IGF-1 en el ovario y su papel en la maduración in vitro de oocitos de cobaya

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Karina Esperanza Cañón Beltrán

DIRECTORES

María Árias Álvarez
Rosa María García García
Pedro Luis Lorenzo González

Madrid, 2018

© Karina Esperanza Cañón Beltrán, 2017

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA
Departamento de Fisiología (Fisiología Animal)

TESIS DOCTORAL

Characterization of EGF and IGF-1 system in the ovary and its role in *in vitro* maturation of guinea pig oocytes

Caracterización del sistema EGF e IGF-1 en el ovario y su papel en la maduración *in vitro* de oocitos de cobaya

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR:

Karina Esperanza Cañón Beltrán

Directores:

María Árias Álvarez
Rosa María García García
Pedro Luis Lorenzo González

Madrid, 2017

El Dr. Pedro Luis Lorenzo González, Profesor Titular de Universidad, la Dra. Rosa Mª García García, Profesora Titular del Departamento de Fisiología (Fisiología Animal) y; la Dra María Arias Álvarez Profesora Ayudante Doctor del Departamento de Producción Animal de la Facultad de Veterinaria de la Universidad Complutense de Madrid, hacen constar:

Que la Memoria presentada por la Licenciada en Veterinaria y Zootecnia Dª Karina Esperanza Cañón Beltrán, con el título: ***"*Characterization of EGF and IGF-1 system in the ovary and its role in *in vitro* maturation of guinea pig oocytes"**, ha sido realizada bajo nuestra dirección y que tras su revisión consideramos que tiene la debida calidad para su presentación y defensa.

Madrid, Abril de 2017

Fdo: D. Pedro Luis Lorenzo González

Fdo: Dª. Rosa Mª García García

Fdo: María Arias Álvarez

"Hay una fuerza motriz más poderosa
Que el vapor, la electricidad y la
Energía atómica: la voluntad"
Einstein

A mis padres.

A Andrés.

Agradecimientos

Quiero expresar mi agradecimiento a todas las personas que hicieron posible la realización y culminación de esta tesis doctoral.

A mi director de tesis, Dr. Pedro Lorenzo, por su confianza y por brindarme la oportunidad de ser parte de su equipo de investigación, por el apoyo para tener materiales indispensables para el desarrollo de esta tesis en el laboratorio de reproducción de Loja, sin tu apertura y visión no hubiera sido posible.

A mis dos directoras, Dra. María Arias Álvarez y Dra. Rosa María García, son un equipo increíble de profesionales, infinitas gracias por su constante e incondicional apoyo, dedicación y esfuerzo. Sin sus aportes esta tesis no hubiera sido posible. Son un gran ejemplo a seguir.

Al Departamento de Fisiología (Fisiología animal), al Dr. Luis Revuelta por su disponibilidad para ayudarme siempre en lo que fuera necesario y a mi compañera Ana, por su amistad y por adéntrame en el mundo de la biología molecular.

A la Dra. Pilar García Rebollar, por su amabilidad y disposición para ayudarme.

Al Departamento de Bioquímica y Biología Molecular IV, en especial al Dr. José Manuel Bautista, Amalia, Paloma y Sandra que me brindaron su apoyo y guia desinteresada para las PCR.

A Lola, del laboratorio de microscopia confocal de la Universidad autónoma de Madrid

A la Universidad Técnica Particular de Loja, es su representación al Rector Dr. José Barbosa Corbacho, a los vicerrectores Dr. Juan Pablo Suárez, Dr. Santiago Acosta Aide, por brindarme su confianza y facilidades para conjugar la labor docente e investigativa con la realización de este doctorado.

Al Departamento de Ciencias Biológicas, en especial a mis compañeros de trabajo de la sección de Biotecnología y Producción, por su apoyo en la parte experimental de esta tesis.

A mis estudiantes de las titulaciones de agropecuaría, biología y gestión ambiental, en especial a los que participaron como gestores productivos en el laboratorio de reproducción animal por sus palabras de aliento y preguntas acerca de esta tesis.

A la Dra. Catalina Rey y su esposo por su amistad y disponibilidad para explicarme conceptos y alternativas para manejar los datos estadísticos.

Al Dr. Julio de Fuente (Q.P.D), por sus consejos tan profundos sobre la vida científica y por inspirarme a luchar y conseguir lo que uno propone.

A Doña Dorarliza y su familia por su apoyo para obtener las muestras de cobaya, así como a todas las mujeres productoras de cobayas de la parroquia de Chuquiribamba-Loja-Ecuador por su amabilidad y disposición para ayudarme a conseguir las muestras.

Al Instituto Id de Cristo Redentor Misioneras y Misioneros identes, es especial a la Superiora General Mª del Carmen García Viyuela y las misioneras de la sede general, de Castilla y de Loja, por su cariño, apoyo y preocupación durante mis temporadas en Madrid y Loja para la realización de esta tesis.

A mis amigos Paulina y Wilson, por su apoyo y por toda la ayuda que me ha brindado durante mis estadías en Madrid.

A mi familia lojana, Doña Ángela Delgado, su esposo Don Gonzalo Suárez y Natasha, infinitas gracias por acogerme en su casa y brindarme sabios consejos, a mi gran amiga Yulia, por soportarme no solo con cosas del trabajo sobre todo con la tesis, por luchar conmigo para crear el laboratorio de reproducción y hacer que funcionara, quedó estupendo.

A mis amigos Anita, Glenda, Maricuchi, Francisco, Freddy, por su paciencia al escuchar una y otra vez el mismo tema de la cobaya.

A mi familia Stella, Yeimy, Diego, Valentina y a la pequeña María Elisa, gracias por ser un soporte familiar indispensable.

Y especialmente a mis padres Jaime y Esperanza y a mi hermano Andrés, gracias porque su apoyo incondicional y amor no conoce barreras geográficas, sin ustedes no hubiera sido posible comenzar este reto y mucho menos terminarlo. Ustedes son mi mejor todo. Los amo.

INDEX

Index

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

SUMMARY………………………………………………………………………… I

RESUMEN……………………………………………………………………….... XIII

1. INTRODUCTION………………………………………………………….…..... 1

1.1. Guinea pig as an experimental model…………………………………….. 1

1.2. Anatomical description of the guinea pig reproductive system………… 2

1.3. Ovarian physiology………………………………………………………..... 4

1.3.1. Estrous cycle: follicular and luteal phase……………………………... 4

1.3.2. Oogenesis and follicular development……………………………….. 5

1.3.2.1. Endocrine, paracrine and autocrine control of folliculogenesis.. 11

i. EGF system……………………………………………………….. 12

ii. IGF system……………………………………………….……….. 15

1.3.2.2. Follicular atresia…………………………………………………... 22

1.3.3. Oocyte maturation…………………………………….………………… 23

1.3.3.1. Nuclear maturation……………………………………………....... 24

1.3.3.2. Cytoplasmic maturation…………………………………………… 28

i. Cortical granules migration…………………………………….... 28

ii. Mitochondrial distribution………………………………………… 29

1.3.4. Ovulation and *corpus luteum* formation……………………………..... 31

1.3.5. Preimplantation embryo development………………………………… 33

1.4. Systems of oocyte maturation *in vitro*…………………………………….... 35

1.4.1. Definition and importance of *in vitro* maturation (IVM) in ARTs……. 35

1.4.2. IVM system…………………………………………………………..... 36

1.4.2.1. Ovary collection and oocyte selection ………………………….. 36

1.4.2.2. Maturation media………………………………………………….. 38

2. OBJETIVES AND THESIS OUTLINE………………………………………….. 45

3. MATERIALS AND METHODS………………………………………………….. 53

3.1. Animals and facilities…………………………………………………...... 53

3.2. Ovarian collection and transport…………………………………………….. 53

3.3. Gene expression of EGF, IGF-1 and their receptors (EGF-R and IGF-1R), and IGF- Binding Proteins 1-6 in ovaries, follicles and *corpus luteum*, and COCs in the guinea pig model………………………………… 54

3.3.1. Collection of ovaries, follicles and *corpus luteum*…………………….. 54

3.3.2. Collection and selection of cumulus-oocyte complexes…………….. 55

3.3.3. Extraction of total RNA, *mRNA* purification and Reverse Transcription…………………………………………………………. 55

3.3.3.1. RNA extraction in ovaries, follicles and *corpus luteum*…………. 55

3.3.3.2. RNA extraction in oocytes and cumulus cells…………………..... 56

3.3.3.3. *mRNA* purification in ovaries, antral and preovulatory follicles, *corpus luteum*, oocytes and cumulus cells………….............. 56

3.3.3.4. Reverse transcription……………………………………………… 57

3.3.4. Polymerase chain reaction…………………………………………….... 57

3.4. Immunolocalization of EGF-R and IGF-1R and IGFBPs 1-6 in the guinea pig ovary………………………………...…………………………............. 59

3.4.1. Ovary fixation……………………………………………………………….. 59

3.4.2. Immunohistochemistry of EGF-R and IGF-1R in guinea pig ovaries. 60

3.4.3. Immunohistochemistry of IGFBPs 1 – 6 in guinea pig ovaries……… 61

3.5. Immunolocalization of EGF-R and IGF-1R and IGFBPs 1-6 in cumulus-oocyte complexes and early embryos………………………………………. 62

3.5.1. Collection and selection of cumulus- oocyte complexes…………..... 62

3.5.2. Collection and selection of early embryos……………………………. 62

3.5.3. Immunocytochemistry of EGF-R and IGF-1R in cumulus-oocyte complexes and early embryos……………………………………….... 64

3.5.4 Immunocytochemistry of IGFBPs 1- 6 in oocytes and cumulus-oocyte complexes ……………………………………….................. 65

3.5.5. Quantification of the immunofluorescence intensity…………………. 65

3.6. Role of EGF and IGFI-1 on nuclear and cytoplasmic *in vitro* oocyte maturation……………………………………………………………………….. 65

3.6.1. Recovery, selection of cumulus- oocyte complexes and *in vitro* oocyte maturation systems.……………………………………………. 66

3.6.2. Assessment of nuclear and cytoplasmic maturation by confocal microscopy……………………………………………………………….. 67

3.6.2.1. Nuclear oocyte maturation……………………………………… 67

3.6.2.2. Cortical granule and mitochondrial distribution…………………. 67

3.6.3. Cumulus cell apoptosis………………………………………….......... 68

3.6.4. Steroidogenic production of cumulus- oocyte complexes…………… 69

3.7. Statistical analysis…………………………………………………………….. 69

4. RESULTS…………………………………………………………………….. 75

4.1. Experiment 1. Expression of mRNA encoding EGF, IGF-1 and their receptors (EGF-R and IGF-1R), and the IGFBPs 1-6 in the preantral and antral follicles, *corpus luteum* and cumulus-oocyte complexes in the guinea pig model……………………………………………………………. 75

4.1.1. Expression of mRNA encoding EGF and EGF-R in guinea pig ovaries, antral follicles, preovulatory follicles, *corpus luteum*, denuded oocytes and their cumulus cells……………………............ 75

4.1.2. Expression of mRNA encoding IGF-1 and IGF-1R in guinea pig antral follicles, preovulatory follicles, *corpus luteum*, denuded oocytes and their cumulus cells………………………………………… 77

4.1.3. Expression of mRNA encoding IGFBPs 1-6 in guinea pig ovaries, antral follicles, preovulatory follicles, *corpus luteum*, denuded oocytes and their cumulus cells…………………………………..…. 79

4.2. Experiment 2. Immunolocalization of EGF-R and IGF-1R and their ligands (IGFBPs 1-6) in ovaries, follicles, *corpus luteum*, cumulus-oocyte complexes and early embryos in the guinea pig model…………………… 82

4.2.1. Experiment 2.a. Immunolocalization of EGF-R and IGF-1R and their ligands (IGFBPs 1-6) by immunohistochemistry in ovaries of guinea pig………………………………………………………….................. 82

4.2.1.1. Immunolocalization of EGF-R in guinea pig ovaries……………. 82

4.2.1.2. Immunolocalization of IGF-1R in guinea pig ovaries……………. 83

4.2.1.3. Immunolocalization of IGFBPs 1-6 in guinea pig ovaries……… 85

4.2.2. Experiment 2.b. Immunolocalization of EGF-R and IGF-1R and their ligands (IGFBPs 1-6) by immunocytochemistry in cumulus-oocyte complexes and in the early embryos of guinea pig………………….. 94

4.2.2.1. Immunolocalization of EGF-R in cumulus-oocyte complexes and early embryos………………………………………………… 94

4.2.2.2. Immunocytochemical localization of IGF-1R in cumulus-oocyte complexes and early embryos ………………………………….. 96

4.2.2.3. Immunolocalization of IGFBPs 1-6 in cumulus-oocyte complexes……………………………………………………… 98

4.3. EXPERIMENT 3. Study the effect of EGF and IGF-1 on nuclear and cytoplasmic *in vitro* oocyte maturation, apoptosis and steroidogenic production by the cumulus-oocyte complexes in the guinea pig model… 101

4.3.1. Experiment 3a: Characterization of cortical granules and mitochondrial distribution patterns during *in vitro* oocyte maturation in the guinea pig……………………………………………………….. 101

4.3.1.1. Cortical granule patterns……………………………………….. 101

4.3.1.2. Mitochondrial patterns………………………………………….. 101

4.3.2. Experiment 3.b. Role of EGF on *in vitro* oocyte maturation system of guinea pig by the study of nuclear and cytoplasmic oocyte maturation, apoptotic rate in cumulus cells and steroidogenic response of cumulus-oocyte complexes…………………................... 103

4.3.3. Experiment 3.c. Role of IGF-1 on *in vitro* oocyte maturation of guinea pig by the study of nuclear and cytoplasmic oocyte maturation, apoptotic rate in cumulus cells and steroidogenic response of cumulus-oocyte complexes…………………………..................... 106

4.3.4. Experiment 3.d. Study of the effect of EGF and IGF-1 on *in vitro* oocyte maturation of guinea pig by the study of nuclear and cytoplasmic oocyte maturation, apoptotic rate in cumulus cells and steroidogenic response of cumulus-oocyte complexes………………. 108

5. DISCUSSION………………………………………………………………… 115

5.1. Gene expression and immunolocalization of EGF and EGF-R………… 116

5.1.1. Characterization of EGF and EGF-R system in the guinea pig ovary. 116

5.1.2. Characterization of EGF and EGF-R system in the guinea pig cumulus-oocyte complexes…………………………………………… 118

5.1.3. Characterization of EGF and EGF-R system in the guinea pig early embryos……………………………………………………………… 120

5.2. Expression and localization of IGF-1 and IGF-1R ……………………….. 120

5.2.1. Characterization of IGF-1 and IGF-1R system in the guinea pig ovary…………………………………………………………………….. 120

5.2.2. Characterization of IGF-1 and IGF-1R system in the guinea pig cumulus-oocyte complexes……………………………….............. 123

5.2.3. Characterization of IGF-1 and IGF-1R system in the guinea pig early embryos……………………………………………………………… 124

5.3. Expression and localization of IGFBPs 1 to 6…................................... 125

5.3.1. Expression and localization of IGFBPs 1 to 6 in guinea pig ovary…. 125

5.3.2. Expression and localization of IGFBPs 1 to 6 in guinea pig cumulus-oocyte complexes…………………………………………………… 132

5.4. Role of EGF and IGF-1 in guinea pig *in vitro* oocyte maturation……… 134

5.4.1. Cortical granules migration and mitochondrial distribution patterns in the guinea pig………………………………………………………….. 134

5.4.2. Effect of EGF supplementation on the maturation medium………… 135

5.4.3. Effect of IGF-1 supplementation on the maturation medium………… 136

5.4.4. Effect of EGF and IGF-1 supplementation on the maturation medium…………………………………………………………………….. 137

6. CONCLUSIONS……………………………………………………………… 145

7. CONCLUSIONES…………………………………………………….............. 151

8. REFERENCES……………………………………………………….............. 157

LIST OF FIGURES

Figure 1. Anatomical representation of guinea pig female reproductive tract. 3

Figure 2. Estrous cycle of guinea pig………………………………………….… 4

Figure 3. Follicular stages and their endocrine and paracrine control in guinea pig…………………………………………………………..... 10

Figure 4. ErbB receptor family: the different endogenous ligands are shown and the members of each family……………………………………… 13

Figure 5. Influence of IGF-1 on the development of primordial to preovulatory follicles in mammalian species……………………………………... 16

Figure 6. Key components of the IGF-1R pathway …………………………… 17

Figure 7. Meiotic division………………………………………………………. 25

Figure 8. Schematic overview of the nuclear maturation………………………. 27

Figure 9. Schematic overview of the distribution of cytoplasmic organelles during maturation and fertilization…………………………………….. 31

Figure 10. Preimplantation embryonic development in guinea pig compared to mouse, and human.………………………………………………… 34

Figure 11. Adult guinea pig of Peruvian breed…………………………………... 53

Figure 12. A) Measurement in mm of guinea pig ovary, B) Guinea pig ovaries with follicles of different sizes visualized under stereoscopic microscope.…………………………………………………………….. 54

Figure 13. Follicles in guinea pig ovaries.. 61

Figure 14. Cytological assessment of vaginal smear of guinea pig................... 63

Figure 15. Cumulus- oocyte complexes (COCs) suitable for IVM.................... 66

Figure 16. Experimental design of EGF for IVM…………………………………. 66

Figure 17. Experimental design of IGF-1 for IVM……………………………….. 67

Figure 18. Experimental design of EGF and IGF-1 combination for IVM………. 67

Figure 19. Cumulus cell apoptosis measured by TUNEL assay in guinea pig COCs after *in vitro* maturation.. 69

Figure 20. Representative electrophoresis in a 2% agarose gel showing the expression. A) EGF, B) EGF-R, C) β-actin in guinea pig……………. 75

Figure 21. EGF and EGF-R mRNA expression in ovarian antral and preovulatory follicles, and corpus luteum. Densitometry readings of A) EGF, B) EGF-R (are presented as ratio of readings of corresponding β-actin samples in guinea pig ovarian structures studied………………………………………………………………...... 76

Figure 22. EGF and EGF-R mRNA expression in oocytes and their corresponding cumulus cells. Densitometry readings of A) EGF, B) EGF-R (are presented as ratio of readings of corresponding β-actin samples in guinea pig……………………………………………….. 76

Figure 23. Representative electrophoresis in a 2% agarose gel showing the expression of A) IGF-1, B) IGF-1R, C) β-actin in guinea pig ovary.. 77

Figure 24. IGF-1 and IGF-1R mRNA expression in ovarian antral and preovulatory follicles, and *corpus luteum.* Densitometry readings of A) IGF-1, B) IGF-1R (are presented as ratio of readings of corresponding β-actin samples in guinea pig ovarian structures studied…………………………………………………..……………… 78

Figure 25. IGF-1 and IGF-1R mRNA expression in oocytes and their corresponding cumulus cells. Densitometry readings of A) IGF-1, B) IGF-1R (are presented as ratio of readings of corresponding β-actin samples in guinea pig……………………………………………. 78

Figure 26. Representative electrophoresis in a 2% agarose gel showing the expression of A) IGFBP-1, B) IGFBP-2, C) IGFBP-3, D) IGFBP-4, E) IGFBP-5, F) IGFBP-6, G) β-actin in guinea pig…………………... 79

Figure 27.	IGFBPs 1-6 mRNA expression. Densitometric readings of IGFBPs 1-6 are presented as ratio of readings of corresponding β-actin samples in guinea pig ovarian……………………..……………….	80
Figure 28.	IGFBPs 1-6 mRNA expression in COCs. Densitometric readings of IGFBPs 1-6 are presented as ratio of readings of corresponding β-actin samples in guinea pig ovarian…………………..………………	81
Figure 29.	Immunolocalization of EGF-R in guinea pig ovary…………………...	82
Figure 30.	General view of Immunolocalization of EGF-R in guinea pig ovary	83
Figure 31.	Immunolocalization of IGF-1R in guinea pig ovary…………………..	85
Figure 32.	Immunolocalization of IGFBP-1 in guinea pig ovary ………………...	87
Figure 33.	Immunolocalization of IGFBP-2 in guinea pig ovary…………………	89
Figure 34.	Immunolocalization of IGFBP-3 in guinea pig ovary…………………	90
Figure 35.	Immunolocalization of IGFBP-4 in guinea pig ovary…………………	91
Figure 36.	Immunolocalization of IGFBP-5 in guinea pig ovary…………………	92
Figure 37.	Immunolocalization of IGFBP-6 in guinea pig ovary………………..	93
Figure 38.	Localization by immunocytochemistry of EGF-R in immature and matured Oo and CCs of guinea pig…………………………………...	94
Figure 39.	Localization of EGF-R in guinea pig preimplantation embryos by immunofluorescence..………………………..…………………….......	95
Figure 40.	Localization by immunocytochemistry of IGF-1R in immature and matured Oo and CCs of guinea pig ………………………..............	96
Figure 41.	Localization of IGF-1R in guinea pig preimplantation embryos by immunofluorescence………………………………….………………..	97
Figure 42.	Localization of IGFBP-1, 2 and 3 in the CCs and the oocytes guinea pig by immunofluorescence……………………………………………	98

Figure 43. Localization of IGFBP-4, 5 and 6 in the CCs and the oocytes guinea pig by immunofluorescence…………………………………………… 99

Figure 44. IGFBPs 1-6 quantification of the immunofluorescence intensity is presented as values measured in pixels……………..……………… 100

Figure 45. Cortical granule (CG) distribution in guinea pig oocytes after IVM period visualized under confocal laser microscopy………..……….. 102

Figure 46. Mitochondrial distribution in guinea pig oocytes after IVM period visualized under confocal laser microscopy……..………………….. 103

Figure 47. Steroidogenic production of COCs after IVM using different concentrations of EGF…………..…………………………………..... 104

Figure 48. Steroidogenic production of COCs after IVM using different concentrations of IGF-1………………………………………………... 106

Figure 49. Steroidogenic production of COCs after IVM using combinations of EGF and IGF-1…………………..………………………………….. 108

LIST OF TABLES

Table 1. Morphological classification of COCs………………………………….. 37

Table 2. Oligonucleotide primers used for RT-PCR analysis of different samples of guinea pig…………………………………………………… 58

Table 3. The nucleotide sequences of the β-Actin primer used as target gene in RT-PCR……………………………………………………………… 59

Table 4. Immunolocalization of EGF-R in the guinea pig ovary………………... 84

Table 5. Immunolocalization of IGF-1R in the guinea pig ovary……………….. 86

Table 6. Immunolocalization of IGFBPs 1 - 6 in the guinea pig ovary……….. 88

Table 7. Nuclear and cytoplasmic maturation in guinea pig oocytes and apoptosis in cumulus cells with different concentrations EGF in IVM medium………………………………………………………………. 105

Table 8. Nuclear and cytoplasmic maturation in guinea pig oocytes and apoptosis in cumulus cells with different concentrations of IGF-1 in IVM medium…………………………………………………………… 107

Table 9. Nuclear and cytoplasmic maturation in guinea pig oocytes with EGF and IGF -1 combined in IVM medium…………………………………... 109

LIST OF ABBREVIATIONS

× g	Centrifugal force
°C	Degrees celsius
µg	Micrograms
µL	Microliters
µm	Micrometers
µM	Micromolar
AF	Antral follicle
Akt	Protein kinase B
ANOVA	Analysis of variance
APC/C	Anaphase promoting complex
AREG	Amphiregulin
ARTs	Assisted reproductive techniques
ATP	Adenosine tri-phosphate
BCB	Brillant cresyl blue
BCL-2	*B-cell lymphoma 2*
BMP	Bone morphogenetic protein
bp	Base pair
BSA	Bovine serum albumin
BTC	β-cellulin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CCs	Cumulus cells
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
CL	*Corpus luteum*
cm	Centimeters
CO_2	Carbon dioxide
COCs	Cumulus-oocyte complexes
CSF	Cytostatic factor
C-terminal	Carboxi-terminal or COOH-terminal
Cx	Connexin
CYP	Cytochrome
DEPC	Diethyl pyrocarbonate
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide
dT	Deoxy-thymidine nucleotides
DTT	Dithiothreitol
E_2	Estradiol
eCG	Equine chorionic gonadotropin
ECM	*Extracellular matrix*
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor

EGF-R	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Extracellular region
EREG	Epiregulin
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FF	Follicular fluid
FGF	Fibroblast growth factor
FIGLA	Folliculogenesis Specific BHLH Transcription Factor
FOXL2	Forkhead box protein L2
FOXO	Forkhead box O
FSH	Follicle-stimulating hormone
g	Grams
GC	Granulosa cells
GDF-9	Growth differentiation factor
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
GPR3	G protein related receptor
GTP	Guanosine triphosphate
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
h	Hours
HBD	Heparin-binding domain
HCl	Hydrochloric acid
HEPES	4-(2-Hydroxyethyl)-1-piperazine-ethanesulphonic acid
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IETS	International Embryo Technology Society
IFN	Interferon
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 Receptor
IGFBPs	IGF- Binding Proteins 1-6
IP3	Phosphatidylinositol 3,4,5-triphosphate
IU	International unit
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
JM	Juxtamembrane region
kDa	Kilo daltons
KITL	Kernel Independent Transport Layer
KL	Kit ligand
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LIF	Lleukemia inhibitory factor
LRP	Lipoprotein receptor-related protein
MAPK	Mitogen-activated protein kinases
MEK	Mitogen-activated protein kinase kinase
mg	Milligrams
MI	Meiosis I

MII	Meiosis II
M-II	Metaphase II
min	Minutes
miRNA	Molecule microRNAs
mL	Milliliter
Mm	Molecular marker
mM	Milimolar
mm	Millimeters
MMPs	Matrix metalloproteinase
MPF	Meiosis Promoting Factor
mRNA	Messenger ribonucleic acid
mTORC	Mammalian target of rapamycin complex
n	Number
NC	Negative control
ng	Nanograms
NGF	Nerve growth factor
nm	Nanometers
NOBOX	Newborn ovary homeobox-encoding gene
N-terminal	Amino-terminal or NH2-terminal
NTRK2	Neurotrophic Receptor Tyrosine Kinase
O_2	Oxygen
Oct	Octamer-binding transcription factor
Oo	Oocyte
OV	Ovary
p.c.	*Post-coitum*
p.p.	*Post-partum*
P_4	Progesterone
PAPP-A	Pregnancy-associated plasma protein-A
PBS	Phosphate-buffered saline
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGF	Platelet-derived growth factor
PF	Preovulatory Follicle
PG	Prostaglandins
$PGF_{2\alpha}$	Prostaglandin F_2 alpha
pH	potential hydrogen
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PRL	Prolactin
PTEN	Phosphatase and tensin homolog
PVA	Polyvinyl alcohol
PVP	Polyvinyl pyrrolidone
Raf/Ras	Protein superfamily
RNA	Ribonucleic acid
rt	Room temperature
RT-	Reverse transcriptase negative
RTKs	Receptor tyrosine kinases

RT-PCR	Reverse Transcription and Polymerase Chain Reaction
s.e.m.	Standard error of the mean
SCC	Side chain cleavage
SCF	Stem cell factor.
SDF-1	Stromal derive factor-1
SDF-1	Stromal derive factor-1
SMAD	Intracellular proteins
StAR	Steroidogenic acute regulatory protein
STAT	Signal transducers and activators of transcription
TACE/ADAM	Tumor necrosis factor-alpha converting enzyme
TCM-199	Tissue culture medium 199
TE	Trophectoderm
TGF	Transforming growth factor
TM	Transmembrane domain
TNFR	Tumour necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing Ligand
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TZP	Transzonal projection
UK	United Kingdom
v/v	Volume/volume
VEGF	Vascular endothelial growth factor
wt/vol	Weight/volume
ZP	Zona pellucida

SUMMARY

Among non-primate species, guinea pig (*Cavia porcellus*) is used as a laboratory animal since the late 18th century and remains an essential species in many areas of research. It is an excellent animal model for the study of reproduction in humans and domestic animals because of spontaneous ovulation and active corpora lutea, long gestation period and type of placenta. As in woman, guinea pigs have small to medium-sized litters, low ovulation rate and only few preimplantation embryos can be obtained from one female. Similarly, the immature resting oocytes in the guinea pig ovary are on the true diplotene stage as in women and they are more sensitive to ionic radiation than mice oocytes. Therefore, some studies have suggested that guinea pig may represent an alternative animal model than mice for investigations on ovarian infertility in women that show premature ovarian failure as a consequence of radiotherapy. In this sense, it is widely known that the insulin like growth factor (IGF-1) and epidermal growth factor (EGF) are implied in the regulation of many ovarian processes throgouth their receptors. EGF and its receptor (EGF-R) are involved in the ovulation process, oocyte maturation, steroidogenesis of follicle and luteinization of *corpus luteum* in humans and animal species. In addition, Insulin-like growth factor-1 (IGF-1) system plays an important role in the regulation of ovarian folliculogenesis, steroidogenesis and apoptosis prevention and is luteotropic in *corpus luteum* and promotes early embryo development. Firthermore, the gene expression of IGFs and their binding proteins (IGFBPs) differ from women with normal ovaries and those with polycystic ovary syndrome (PCOS).

Nowadays, animal models for the study of ovarian physiology in relation with these growth factors and this syndrome are lacking but there is clear evidence that the role of the EGF and IGF system in relation to follicular health needs to be further investigated. Both systems are important for the normal function of the ovary in animals and human. However, no studies in guinea pig model exist in relation with the effect of both EGF and IGF-1 systems in the ovary and the quality of the oocytes. For these reasons, the characterization of EGF and IGF-1 system in the guinea pig ovary could be an approach to establish a suitable model for the study of mechanisms of ovarian health that can help to elucidate ovarian failure process derived of imbalance in these growth factors.

In this context, the development of *in vitro* follicle culture and *in vitro* oocyte maturation systems in the guinea pig seems to be necessary to provide an *in vitro* model to study the effect of EGF and IGF system in the ovarian physiology and to improve IVM systems in human Assisted Reproductive Tecnologies (ARTs) and in the guinea pig. By this way,

we can also considerably reduce the number of animals required in the lab and subjected to hormonal treatments to obtain Metaphase- II (M-II)-oocytes *in vivo* by replacing them with *in vitro* alternatives and avoiding the application of hormonal treatments. Besides, IVM system could be a important biothecnology for conservation of genetic pool of valuable guinea pig strains for their socioeconomic value in some Andean countries due to the poor results in superstimulation protocols in this species. Although some investigations of follicular development have been accomplished, IVM protocols are rarely developed in the guinea pig model, and cellular mechanisms that occur during oocyte maturation are unknown in this specie.

Therefore, main goals of the present PhD work were: 1) to characterize the EGF and the IGF-1 systems in different structures of the guinea pig ovary and; 2) to study the effect of both growth factors (EGF and IGF-1) supplemented *in vitro* on oocyte maturation. All these objectives would lead to the stablishment of a useful animal model for the study of both systems in ovarian physiology and the setting up of an *in vitro* defined maturation system based in the supplementation with EGF and IGF-1 useful in this specie as a souce of M-II- oocytes for ARTs improvement.

The **first experiment** include two studies carried out to investigate mRNA (messenger ribonucleic acid) expression of candidate genes ((EGF, EGF-R, IGF-1, IGF-1R (Insulin-like growth factor-1 Receptor), IGFBPs)) in different ovarian structures, and after that in cumulus-oocyte complexes (COCs) in order to describe the differential expression pattern of mRNA encoding for such genes. For this objective, a total of 26 ovaries from adult animals were used. Antral and preovulatory follicles as well as *corpus luteum* were mechanically isolated to study the gene expression of candidate genes in each structure per separate (**Experiment 1.a**) by RT-PCR (Reverse Transcription and Polymerase Chain Reaction). The gene expression of a total of 73 oocytes and their corresponding cumulus cells (CCs) separately were also evaluated (**Experiment 1.b**) by the same technique.

The **Experiment 1.a**. clearly demonstrated that mRNA for all candidate genes were expressed in the main ovarian structures involved in the final oocyte growth and maturation, such as antral and preovulatory follicles, and in the early embryo development, such as *corpus luteum*. Therefore, this Thesis demonstrates that the guinea pig ovary differentially expressesall the constituents of EGF and IGF-1 system included the six IGFBPs. Then, in the ovary the highest expression levels of EGF, EGF-R and IGF-1 and IGF-1R was showed in antral follicle compared to preovulatory follicle.

The relative mRNA expression of the IGFBP-1, 2 and 4 was significantly increased in the preovulatory follicles whereas IGFBP-3 and 5 were higher in antral follicle. The mRNA transcripts for IGFBP-6 showed similar gene expression among them. These results indicate that IGFBPs may be modulating the IGF-1 function in antral follicle and preovulatory follicle modulating IGF-1 bioavaibility. *Corpus luteum* showed diferentially expression being higher for EGF-R, IGF-1R and IGFBP-1 and 2 compared with the gene expression in preovulatory follicle. In current work, there were found mRNA transcripts for all IGFBPs but gene expression was found greater for IGFBP-1 and 2, whereas the rest of IGFBPs, showed lower mRNA transcripts compared with the follicles. These findings evidenced that, EGF and IGF-1 system modulate the antral follicle and preovulatory follicle development and the *corpus luteum* function by an autocrine and paracrine mechanism of action in the guinea pig ovary, since all structures studied have the ability to express both growth factors, their receptors and the IGFBPs in different levels. The highest gene expression of EGF and EGF-R, and IGF-1 and IGF-1R in antral follicle could be showing that this system can be particularly involved in the selection of the dominant follicle and adquisition of oocyte competence in the guinea pig model.

In the COCs (**Experiment 1.b**) the results showed that mRNA transcripts of all candidate genes were significantly higher in the oocytes than in CCs, except for IGF-1R and IGFBP- 5.The expression of IGFBP-6 was found similar in oocytes and CCs. This may suggest an important bidirectional communication between the oocyte and their CCs throgout this system and a key regulatory role of the IGFBPs in the IGF-1 availability. Our results suggest that cumulus cells respond to IGF-1 more than the oocyte since CCs showed a higher IGF-1R gene expression and lower mRNA transcripts for the majority of the IGFBPs. In fact, low levels of IGFBPs in CCs can be explained by the interaction of IGF-1 with follicle-stimulating hormone (FSH) and their role in steroidogenic production by CCs. Our results indicate that both isolated oocytes and their CCs can directly respond to EGF and IGF-1 throughout their receptors and that the IGF-1 action can be greatly modulated by IGFBPs in the COCs, since they can express mRNA transcripts for all IGFBPs. Therefore, these finfdings corroborate the implication of both systems in oocyte maturation in the guinea pig.

Once gene expression study was performed, the aim of the **second experiment** was to localize in the ovary and in the COCs the receptor of EGF and IGF-1 to confirm if all structures studied respond to both growth factors. Besides, we immunolocalized all IGFBPs described above to verify the possible implication of those IGFBPs in the modulation of IGF-1 function during foliculogenesis, early luteal function, oocyte

maturation and early embryo development.

Therefore, in **Experiment 2.a.** a total of 18 ovaries were used and 20 sections was analyzed for EGF-R and IGF-1R and a total of 12 ovaries and 288 sections for the IGFBPs. The ovarian follicles were classified as primordial, primary, secondary and antral follicle according to the morphology of the follicular cells. The intensity of immunohistochemical staining was graded as follows: (–) no immunostaining; (+) weak staining; (++) moderate staining and (+++) strong staining. The immunohistochemistry results in the ovary confirmed that EGF-R, IGF-1R and IGFBPs 1-6 were widely and differentially immunolocalized in primordial, primary, secondary and antral follicle, and in *corpus luteum* in guinea pig.

EGF-R and IGF-1R were immunolocalized with stronger intensity in granulosa cells and oocytes of antral follicle that confirms the findings of the gene expression study for both receptors. IGF-1R showed stronger immunostaining also in early follicles, which could be indicating that although both growth factors are implicated in the follicular development and oogenesis, probably IGF-1 is exerting a key role also in the earlier stages of folliculogenesis. However, further studies are needed to corroborate this hypothesis. In this sense, follicles at all stages of development also exhibited immunoreaction of each IGFBPs studied. All IGFBPs were distributed in all follicular compartments (granulosa and theca cells and oocyte) and in *corpus luteum,* although diferential intensity was evidenced. In general lines, all the IGFBPs except IGFBP-1 and 2 showed moderate to weak signal in all follicular structures (oocytes, granulosa cells, theca cells) and in all follicular stages included antral follicle. In contrast, IGFBP- 2 showed high immunostaining in the granulose cells of all follicular stages, whereas the IGFBP-1 signal decreased when the follicles achieved the antral stages. These findings, suggests the influence of EGF and IGF-1 in the follicular development in the guinea pig. It can confirm that the important role noted for IGF-1 in the earlier stages of follicle development can be differently modulated by the IGFBPs, and especially by the IGFBP-2, since it showed the most intense signal in the granulose cells in all stages of the follicular development.

In *corpus luteum* stronger intensity was found for EGF-R and IGF-1R as well as for IGFBP- 2 and 3, whereas signal was moderate and weak for the rest of IGFBPs studied. These findings indicate the key role in luteal function and angiogenesis for IGF-1 and EGF probably by enhancing progesterone (P_4) production on luteal cells of guinea pig as has been demonstrated by in other mammals.

In COCs indirect immunofluorescence assays carried out in **Experiment 2.b**. showed that EGF-R and IGF-1R were present in immature and *in vitro* matured oocytes and CCs. Both of them were detected in the cellular membrane but signal was significantly higher after maturation. These findings match with the mRNA results obtained in the first experiment of this Thesis, and confirm that the COCs of guinea pig can respond to EGF and IGF-1 throughout their receptors in a paracrine and autocrine manner during oocyte maturation process. Fluorescence signal was also found for all the IGFBPs in the cell membrane of both oocytes and CCs. Oocytes showed lower intensity for IGFBP- 3 and 6 compared with their CCs whereas in CCs IGFBP-1, 4 and 5 fluorescence was higher. Therefore, these results indicate that IGFBPs seems to be implicated in the modulation of the action of IGF-1 between the oocyte and CCs since all IGBBPs proteins were localized in both of them. However, are needed more investigations to study the differences found between the gene expression and immunofluorescence study of IGFBP-2, 3, 5 and 6 in the COCs of guinea pig.

In this experiment, and taking account that the EGF and IGF-1 system can be acting on the steroidogenic function of the *corpus luteum*, and therefore indirectly affect the early embryo development, we also decided to check if the EGF and IGF-1 system could also be involved directly on the early embryos. Therefore, the inmunolocalization of both receptors was studied in early embryos of guinea pig. A total of 23 females were used. Ovulation was confirmed by the presence of a mucous plug in the vagina and by means of vaginal smears. Embryos were recovered at day 2, 3 and 4 after the formation of mucous plug by flushing of the reproductive tract and classified as: 2 to 4-cells embryos, 8 to 16-cells embryos and morula. EGF-R and IGF-1R immunofluorescence was observed in the cell membrane of the blastomeres in all embryo stages. The intensity of the signal was temporally increased through preimplantation embryo development. These findings imply that both growth factors could be directly regulating the early embryo development throgouth their receptors in guinea pig.

On the other hand, set up an IVM protocol as an alternative method in this specie to the unsuccessful gonadotropin treatments for superovulation will be helpful; besides, it will be a procedure to obtain large numbers of M-II-oocytes *in vitro* for biomedical research applications in ARTs. However, find an IVM medium results a complex process given that every laboratory uses different methods and additives. Gonadotropins are the primary regulators of oocyte maturation, but taking account the results of the first and second experiments of this Thesis, in absence of hormones, EGF and IGF-1 could be

good candidates to be used in free-serum IVM systems in the guinea pig. Use of defined medium is highly desirable in ARTs as well. As a consequence, in **Experiment 3** we studied the direct effect of the EGF and IGF-1 on oocyte *in vitro* maturation in the guinea pig. Nevertheless, due to the lack of knowledge about the processes involved in the cytoplasmic maturation in guinea pig oocytes, fistly we characterized some cytoplasmic markers to stablish the cellular patterns that occur in guinea pigs during their maturation and to set the basis that improves IVM protocols in this specie.

In this sense, migration of cortical granules and redistribution of mitochondria are necessary events for the oocyte to acquire the capability to support fertilization and early embryonic development. Hence, in the **Experiment 3.a.** we characterized, by the first time in the guinea pig, the compatible cortical granules and mitochondrial migration patterns related with the oocyte cytoplasmic maturation. After that, we studied a possible defined- non hormonal IVM medium using EGF and/or IGF-1 (**Experiment 3.b., 3.c., and 3.d.**) assessing: M-II rate, cytoplasmic oocyte maturation, in terms of cortical granules migration and mitochondrial relocation, apoptosis rate and; estradiol (E_2) and P_4 production by the COCs as a possible indirect way to determine the quality of COCs after IVM. The viability of CCs as well as their steroidogenic ability is essential in the acquisition of the oocyte competence during maturation.

For the study of the cortical granules and mitochondrial patterns we used immature and *in vitro* matured oocytes. The results showed that cortical granules migrated to the periphery were the main pattern observed in the oocytes after IVM whereas in immature oocytes homogeneous distribution of cortical granules was prevalent. It has sense because cortical granules positioned under the plasma membrane of the mature oocyte prevent polyspermy; also is related to oocyte competence and to its capacity to be fertilized and to maintain viable early embryo development. Then, the cortical granules patterns defined for the guinea pig were: 1) non-migrated, in which the cortical granules appeared distributed throughout the cytoplasm (considered non- cytoplasmically matured); 2) partially migrated in which most of the cortical granules spread throughout the cortical area and; 3) migrated, in which cortical granules appeared adjacent to the plasma membrane (considered cytoplasmically matured). Regarding with the mitochondrial oocyte patterns characterized, our findings showed that mitochondria migrated to the periphery in the most of the oocytes after IVM, whereas in immature oocytes homogeneous distribution was prevalent. Therefore, the mitochondrial patterns defined for the guinea pig were: 1) non-migrated, where the mitochondria were homogeneously distributed throughout the cytoplasm (considered non-cytoplasmically

matured); 2) partially migrated, where the mitochondria were heterogeneously distributed with granular aggregations and; 3) migrated, when the mitochondria were relocated to the nuclear pole (considered cytoplasmically matured).

After that and to assess the possible effect of EGF and IGF-1 on the oocyte maturation in the guinea pig, a dose response experiments were performed as follows. A total of 1746 COCs were used in three experiments and each experiment was repeated four times.

In **Experiment 3.b**., COCs were IVM with 0, 10, 50, 100 ng/mL (nanograms/milliliter) EGF or 10% Fetal Calf Serum (FCS). The results showed that supplementation with 50 ng/mL of EGF in maturation media improved M-II rate and cytoplasmic maturation, both in terms of CG migration and mitochondrial redistribution; besides the quality of CCs seemed to be enhanced, since lower apoptotic rate and higher E_2 and P_4 secretion by COCs were found. In **Experiment 3.c.,** COCs were IVM with 0, 50, 100, 200 ng/mL IGF-1 or 10% FCS. Higher rates of M-II and CGs periphericlly located in oocytes, greater amounts of E_2 and P_4 released by COCs and lower index of apoptosis in CCs were found when 100 ng/mL IGF-1 were added to the maturation media. Both experiments corroborate that COCs respond to EGF and IGF-1 directly throughout their corresponding receptors and that both growth factors improve the oocyte maturation, steroidogenic response and the viability of the CCs. Therefore, these results suggest that the use of both growth factors appear to be as a suitable option to use in the IVM medium in the guinea pig specie.

In **Experiment 3.d**., 500 COCs were supplemented with the concentrations of EGF and IGF-1 that showed the best maturation results in experiments 3.b., and 3.c., (50 ng/mL EGF and 100 ng/mL IGF-1), with or without FCS as follows: group 0 (non-supplemented); group EI (EGF + IGF-1); group EI-FCS (EGF + IGF-1 + 10% FCS) and group FCS (10% FCS). As we expected, the addition of both growth factors together with FCS improved nuclear maturation, CG migration rate in oocytes, steroid-level secretion of COCs, and reduced apoptotic index in CCs compared to those COCs *in vitro-* matured without growth factors. The interaction between growth factors and CCs can modify steroid production, which indirectly affects oocyte maturation and proliferation of CCs. Surprisingly, both growth factors together did not exert a synergic effect on oocyte maturation compared when they were added alone. Moreover, no significant improvements were found in mitochondria patterns as well as occurred in experiment 3.c. in which only IGF-1 was added. The cellular and molecular mechanisms by which

IGF-1 alone or together with EGF influences on mitochondrial migration are unknown for any specie. The apoptotic rate of COCs in the group supplemented with both growth factors together with FCS was lower compared to the rate obtained in COCs cultured with 50 ng/mL EGF or 100 ng/mL IGF-1 alone but no significant differences were found among groups.

Therefore, based on our results, the use of 50 ng/mL EGF appears to be the better option to use in the IVM medium in the guinea pig specie. However, 100ng/mL IGF-1 could be also a suitable defined medium, since the percentage of mitochondrial migrated was quite well. Therefore, in the guinea pig model serum free *in vitro* maturation systems with EGF or IGF-1 alone could provide similar oocyte maturation rates than those that used FCS.

In conclusion, this Thesis has characterized the EGF and IGF-1 sytems in guinea pig ovary, COCs and early embryos establishing the posible role of EGF and IGF-1 system in the follicle development, oocyte maturation, luteogenesis and early embryo development in an autocrine a paracine manner. These findings provide the first evidence that both growth factors could be involved in the regulation of ovarian physiology in the guinea pig model. In this line, we evidencied the beneficial effect of supplementation of both growth factors *in vitro* on oocyte maturation, thus stablishing a valuable system for oocyte maturation in a defined medium for the guinea pig.

RESUMEN

Entre las especies no primates, la cobaya (*Cavia porcellus*) se utiliza como animal de laboratorio desde finales del siglo XVIII en muchas áreas de investigación. Además, es un buen modelo animal para el estudio de la reproducción en humanos y animales domésticos debido a su ovulación espontánea, cuerpo lúteo activo, largo período de gestación y tipo de placenta. De manera similar a la mujer, la cobaya tiene un tamaño de camada pequeño y baja tasa de ovulación, de tal forma que solo unos pocos embriones preimplantacionales pueden ser obtenidos de una hembra. Así mismo, los oocitos inmaduros en reposo en el ovario de la cobaya se encuentran en la etapa de Diplotene como en la mujer, y son más sensibles a la radiación iónica que los oocitos de ratones. Por lo tanto, algunos estudios han sugerido que la cobaya puede representar un modelo animal alternativo para las investigaciones sobre infertilidad ovárica como consecuencia de la radioterapia. En este sentido, el factor de crecimiento similar a la insulina (IGF-1) y también el factor de crecimiento epidérmico (EGF) están implicados en la regulación de muchos procesos fisiológicos ováricos a través de la unión con sus receptores. Así, el EGF y su receptor (EGF-R) regulan la ovulación, la maduración del oocito, la esteroidogénesis del folículo y la luteinización del cuerpo lúteo en humanos y en distintas especies animales. Además, el factor de crecimiento similar a la insulina tipo 1 (IGF-1) juega un papel importante en la regulación de la foliculogénesis ovárica, en la esteroidogénesis, en la prevención de la apoptosis; es luteotrópico en el cuerpo lúteo y promueve el desarrollo embrionario temprano. Además, la expresión génica del EGF, IGF-1 y sus proteínas de unión (IGFBPs) difieren de las mujeres con ovarios normales y de aquellas que tienen el síndrome de ovario poliquístico (PCOS, Polycystic Ovarian Syndrome).

Actualmente, los modelos animales para el estudio de la fisiología ovárica son escasos, pero hay evidencias claras de que el papel del sistema EGF e IGF en relación con la salud folicular necesita ser investigado más a fondo ya que ambos sistemas son importantes para la función normal del ovario en animales y humanos. Sin embargo, no existen estudios en el modelo de la cobaya en relación con el efecto de ambos sistemas EGF e IGF-1 en el ovario y la calidad de los oocitos. Por estas razones, la caracterización del sistema del EGF y del IGF-1 en el ovario de cobaya podría ser un enfoque para establecer un modelo adecuado para el estudio de la fisiología ovárica, que pueda ayudar a elucidar el proceso de fallo ovárico derivado del desequilibrio en estos factores de crecimiento.

En este contexto, el cultivo folicular *in vitro* y el desarrollo de sistemas de maduración *in vitro* (IVM, *In vitro* maturation) de oocitos en la cobaya son biotecnologías importantes a

desarrollar en estas especies para estudiar el efecto del sistema del EGF y del IGF-1 en la fisiología ovárica y para mejorar los sistemas IVM en las tecnologías de reproducción asistida (ARTs, Assisted Reproductive Techniques) en humana y en la cobaya. De esta manera, también podemos reducir considerablemente el número de animales requeridos en el laboratorio y sometidos a tratamientos hormonales *in vivo* para obtener oocitos en metafase II (M-II), reemplazándolos con alternativas *in vitro* y evitando la aplicación de tratamientos hormonales. Además, la IVM podría ser una biotecnología importante para la conservación de la reserva genética de valiosas cepas de cobayas, por su valor socioeconómico en algunos países andinos, debido a los pobres resultados en los protocolos de súper-estimulación que se obtienen en esta especie. Sin embargo, los protocolos de IVM raramente se han desarrollado en la cobaya y los mecanismos celulares que ocurren durante la maduración del oocito son desconocidos en esta especie.

Por lo tanto, los principales objetivos del presente trabajo doctoral fueron: 1) caracterizar el sistema del EGF y del IGF-1 en diferentes estructuras del ovario de la cobaya y; 2) estudiar el efecto de la suplementación de ambos factores de crecimiento (EGF e IGF-1) en la MIV de oocitos. Estos objetivos pretenden caracterizar el sistema EGF e IGF-1 y sentar las bases para utilizar la cobaya como modelo animal útil para el estudio de ambos sistemas en la fisiología ovárica, así como establecer un posible sistema de IVM basado en la suplementación con EGF e IGF-1 que resulte útil en esta especie.

El **primer experimento** incluye dos estudios llevados a cabo para investigar la expresión de mRNA (messenger Ribonucleic Acid) de EGF, EGF-R, IGF-1, IGF-1R, e IGFBPs 1-6 en diferentes estructuras ováricas y en los complejos cúmulo-oocito (COCs, cumulus-oocyte complexes) con el fin de describir el patrón de expresión de dichos genes. Para este objetivo se utilizaron un total de 26 ovarios de animales adultos. Los folículos antrales y preovulatorios y el cuerpo lúteo se aislaron mecánicamente para estudiar la expresión génica en cada estructura por separado (**Experimento 1.a**) mediante una reacción en cadena de polimerasa precedida de una retrotranscripción (RT-PCR). También se evaluó la expresión génica de un total de 73 oocitos y sus correspondientes células del cúmulo (CCs) (**Experimento 1.b**) utilizando la misma técnica.

El **experimento 1.a**. demostró claramente que el mRNA de todos los genes candidatos se expresaba los folículos antrales y preovulatorios, principales estructuras ováricas implicadas en el crecimiento y la maduración final del oocito y, en el cuerpo lúteo, cuya

producción esteroidogénica esta indirectamente relacionada con el desarrollo embrionario temprano. Por lo tanto, esta Tesis Doctoral demuestra que el ovario de la cobaya expresa diferencialmente el sistema del EGF e IGF-1, así como las IGFBPs 1-6. En el ovario, los niveles de expresión más altos de EGF, EGF-R, IGF-1 e IGF-1R se evidenciaron en los folículos antrales en comparación con los folículos preovulatorios. La expresión relativa de mRNA de las IGFBP-1, 2 y 4 fue superior en los folículos preovulatorios, mientras que las IGFBP-3 y 5 fueron mayores en los folículos antrales. Los transcritos de mRNA para la IGFBP-6 mostraron una expresión génica similar en ambos tipos. Estos resultados indican que las IGFBPs podrían regular la biodisponibilidad de IGF-1 en los folículos antrales y folículos preovulatorios. En el cuerpo lúteo la expresión génica del EGF-R, el IGF-1R y las IGFBP-1 y 2 fue superior en comparación con la expresión génica de los folículos preovulatorios. Se encontraron transcritos de mRNA para todas las IGFBPs aunque la expresión génica fue mayor para las IGFBP-1 y 2, mientras que para el resto de las IGFBPs se encontraron menores cantidades de mRNA en comparación con los folículos. Estos hallazgos evidenciaron que los sistemas EGF y del IGF-1 pueden estar implicados en la regulación del desarrollo folicular en los estadios antral y preovulatorio y en la función del cuerpo lúteo por un mecanismo de acción autocrino y paracrino, ya que todas las estructuras estudiadas tienen la capacidad de expresar ambos factores de crecimiento, sus receptores y las IGFBP 1-6 en diferentes niveles. El incremento en la expresión génica del EGF, IGF-1 y de sus receptores en los folículos antrales podría demostrar que este sistema está particularmente involucrado en la selección del folículo dominante y en la adquisición de la competencia de oocitos en la cobaya.

En los COCs (**Experimento 1.b**) los resultados mostraron que los transcritos de mRNA de todos los genes candidatos eran significativamente más altos en los oocitos que en las células del cúmulo (CCs, Cumulus cells), excepto para IGF-1R e IGFBP-5. La expresión de IGFBP-6 fue similar en oocitos y CCs. Esto puede sugerir que existe una importante comunicación bidireccional entre el oocito y sus CCs, a través de estos factores de crecimiento y sus receptores, y un papel regulador clave de las IGFBPs en la biodisponibilidad del IGF-1 y su efecto en el oocito. Las CCs podrían ser más receptivas a la acción del IGF-1 que el oocito ya que muestran una mayor expresión génica de su receptor y pero menor para la mayoría de la IGFBPs. De hecho, los menores niveles de mRNA de las IGFBPs en las CCs pueden estar relacionados con la interacción de IGF-1 con hormona folículo estimulante (FSH, Follicle-Stimulating hormone) y su papel en la producción de esteroides por las CCs. Por lo tanto, nuestros resultados indican que tanto los oocitos aislados como sus CCs pueden responder

directamente al EGF y al IGF-1 a través de sus receptores y que la acción de IGF-1 puede ser modulada en gran medida por IGFBPs en los COCs, ya que pueden expresar transcritos de mRNA para todas las IGFBPs. Por lo tanto, estos resultados corroboran la implicación de ambos sistemas en la maduración de los oocitos de cobaya.

Una vez realizado el estudio de expresión génica, el objetivo del **segundo experimento** fue localizar en el ovario y en los COCs el receptor del EGF y del IGF-1 para confirmar que todas las estructuras estudiadas respondían a ambos factores de crecimiento. Además, se immunolocalizaron todas las IGFBPs descritas anteriormente para verificar la posible implicación de las IGFBPs en la modulación de la función del IGF-1 durante la foliculogénesis, la función lútea temprana, la maduración de los oocitos y el desarrollo temprano del embrión.

Por lo tanto, en el **Experimento 2.a.** Se utilizaron un total de 18 ovarios y se analizaron 20 secciones para cada receptor de los factores de crecimiento (EGF-R e IGF-1R) y un total de 12 ovarios y 288 secciones para las IGFBPs 1-6. Los folículos ováricos se clasificaron como folículos primordiales, primarios, secundarios y antrales según la morfología de las células foliculares. La intensidad de la tinción inmunohistoquímica se clasificó de la siguiente manera: (-) no inmunoticción; (+) tinción débil; (++) tinción moderada y (+++) tinción fuerte. Los resultados de la inmunohistoquímica en el ovario confirmaron que el EGF-R, el IGF-1R y las IGFBPs 1-6 estaban amplia y diferencialmente inmunolocalizados en folículos primordiales, primarios, secundarios y antrales, y en cuerpo lúteo de los ovarios de cobaya.

El EGF-R y el IGF-1R fueron inmunolocalizados con una intensidad elevada en las células de granulosa y en los oocitos de los folículos antrales, de acuerdo con los hallazgos del estudio de expresión génica para ambos receptores. El IGF-1R también mostró una inmunotinción más fuerte en los folículos preantrales, lo que podría indicar que, aunque ambos factores de crecimiento están implicados en el desarrollo folicular y la oogénesis, probablemente el IGF-1 está también desempeñando un papel clave en las etapas tempranas de la foliculogénesis. Sin embargo, se necesitan más estudios para corroborar esta hipótesis. En este sentido, los folículos, en todas las etapas del desarrollo, también mostraron inmunorreacción para cada IGFBPs estudiadas. Todas las IGFBPs se inmunolocalizaron en todos los compartimentos foliculares (oocito, células de la granulosa y de la teca) y en el cuerpo lúteo, aunque se evidenciaron diferentes intensidades. En líneas generales, excepto la IGFBP-1 y 2, todas las IGFBPs mostraron una señal de moderada a débil en todas las estructuras foliculares y en todos

los estadios foliculares incluidos los folículos antrales. Por el contrario, la IGFBP-2 mostró una inmunotinción fuerte en principalmente en las células de la granulosa en todos los estadios del desarrollo folicular, mientras que la señal de la IGFBP-1 se redujo en las células de la granulosa conforme los folículos aumentaron de tamaño, es decir en los antrales. Estos resultados indican la posible influencia del EGF y del IGF-1 en el desarrollo folicular. La acción del IGF-1 desde las etapas tempranas de la foliculogénesis parece estar regulada de manera diferencial por las IGFBP 1-6, especialmente por la IGFBP-2, ya que es la que muestra la señal más intensa en las células de la granulosa de todas las etapas.

En el cuerpo lúteo se encontró mayor intensidad para EGF-R e IGF-1R, así como para las IGFBP-2 y 3, mientras que la señal para el resto de las IGFBPs fue moderada o débil. Estos hallazgos indican el papel clave del IGF-1 y del EGF en la función lútea y en la angiogénesis, probablemente aumentando la producción de progesterona en células luteales de la cobaya como ha sido demostrado en otros mamíferos.

Los ensayos de inmunofluorescencia indirecta llevados a cabo en el **Experimento 2.b.** demostraron que el EGF-R y el IGF-1R estaban presentes en los oocitos y en las CCs de COCs inmaduros y madurados *in vitro*. Ambos receptores fueron detectados en la membrana celular, pero la señal fue significativamente mayor después de la maduración. Estos hallazgos coinciden con los resultados del mRNA obtenidos en el primer experimento de esta Tesis Doctoral y confirman que los COCs de cobaya pueden responder directamente al EGF e IGF-1 a través de sus receptores de una manera paracrina y autocrina durante el proceso de maduración de los oocitos. La señal de fluorescencia para todas las IGFBPs también se encontró en la membrana celular de los oocitos y de las CCs. Los oocitos mostraron menor intensidad para la IGFBP-3 y 6 en comparación con sus CCs, mientras que en las CCs la fluorescencia de las IGFBP-1, 4 y 5 fueron mayores. Por lo tanto, estos resultados indican que las IGFBPs parecen estar implicadas en la modulación de la acción del IGF-1 entre el oocito y las CCs de la cobaya ya que todas las proteínas IGFBPs se localizaron en ambas estructuras. Sin embargo, se necesitan más estudios para ahondar sobre las diferencias encontradas entre los resultados de la expresión génica y de inmunofluorescencia de las IGFBP 2,3,5 y 6 en los COCs.

Teniendo en cuenta que los sistemas EGF y del IGF-1 pueden actuar sobre la función esteroidogénica del cuerpo lúteo y por lo tanto, afectar indirectamente al desarrollo embrionario temprano, en este experimento decidimos comprobar si tanto el EGF como

el IGF-1 podrían estar involucrados directamente en el desarrollo embrionario temprano. Por lo tanto, la inmunolocalización de ambos receptores fue estudiada en embriones tempranos de la cobaya. Se utilizaron un total de 23 hembras para este estudio. La ovulación fue confirmada por la presencia de un tapón mucoso en la vagina y por medio de frotis vaginales. Los embriones fueron recuperados al día 2, 3 y 4 después de la formación del tapón mucoso mediante el lavado del tracto reproductor y fueron clasificados como: embriones de 2 a 4 células, embriones de 8 a 16 células y mórulas. Se observó inmunofluorescencia para el EGF-R y del IGF-1R en la membrana celular de los blastómeros en todos los estadios embrionarios estudiados. La intensidad de la señal aumento temporalmente a lo largo del desarrollo embrionario preimplantacional. Estos hallazgos implican que ambos factores de crecimiento pueden estar involucrados en el desarrollo embrionario temprano de la cobaya a través de sus receptores.

Teniendo en cuenta estos resultados, sería útil instaurar un protocolo de IVM basado en la adición de los factores de crecimiento estudiados. De esta manera, la IVM se podría utilizar como un método alternativo en esta especie a los tratamientos de superovulación con gonadotropinas ya que no ofrecen resultados satisfactorios. Además, sería un procedimiento para obtener *in vitro* un gran número de oocitos en M-II para aplicaciones en la investigación biomédica en las ARTs. Sin embargo, encontrar un medio de IVM resulta un proceso complejo dado que cada laboratorio utiliza diferentes métodos y aditivos. Las gonadotropinas son los principales reguladores de la maduración de los oocitos, pero teniendo en cuenta los resultados del primer y segundo experimento de esta tesis, en ausencia de hormonas, el EGF y el IGF-1 podrían ser buenos candidatos para ser utilizados en sistemas de IVM libres de suero en la cobaya. El uso de un medio definido es también altamente deseable en las ARTs. Como consecuencia, en el **Experimento 3** se estudió el efecto de la suplementación del EGF y del IGF-1 sobre la maduración *in vitro* de oocitos de cobaya. Sin embargo, y dada la falta de conocimientos sobre los procesos celulares involucrados en la maduración citoplasmática del oocito de la cobaya, consideramos que el primer paso era caracterizar algunos de esos marcadores en el oocito para establecer los mecanismos celulares que ocurren en la cobaya durante su maduración y como base sobre la cual estudiar los protocolos de IVM en esta especie.

En este sentido, la migración de los gránulos corticales y la redistribución de las mitocondrias son eventos necesarios para que el oocito adquiera la capacidad para apoyar la fertilización y el desarrollo embrionario temprano. Por lo tanto, en el **Experimento 3.a**. caracterizamos por primera vez en la cobaya, los patrones

compatibles con la migración de los gránulos corticales y la redistribución de las mitocondrias altamente relacionados con la maduración citoplasmática del oocito. Después, ensayamos un posible medio de IVM definido y no hormonal usando EGF y/o IGF-1 exclusivamente **(Experimento 3.b., 3.c., y 3.d.**) evaluando: la tasa de M-II y la maduración citoplasmática del oocito en términos de migración de los gránulos corticales y redistribución mitocondrial. También se estudió la tasa de apoptosis en las CCs y la producción de estradiol (E_2) y progesterona (P_4) por los COCs como una posible vía indirecta para determinar la calidad de los COCs después de la IVM, ya que la viabilidad de las CCs y su capacidad esteroidogénica son factores esenciales en la adquisición de la competencia del oocito durante la maduración.

Para el estudio de los patrones de gránulos corticales y mitocondrias se utilizaron oocitos inmaduros y madurados *in vitro*. Los resultados demostraron que los gránulos corticales migrados a la periferia fueron el patrón principal observado en los oocitos después de la IVM, mientras que en los oocitos inmaduros se observó una distribución homogénea de los gránulos corticales. Estos resultados tienen cierto sentido fisiológico ya los gránulos corticales colocados debajo de la membrana plasmática del oocito maduro evitan la polispermia, por lo tanto, están relacionados con la competencia del oocito y con su capacidad para ser fecundado y para mantener un desarrollo embrionario temprano viable. Los patrones de gránulos corticales definidos para la cobaya fueron: 1) patrón no migrado, en el que los gránulos corticales aparecieron distribuidos en la totalidad del citoplasma y cuyos oocitos fueron considerados no madurado citoplasmáticamente; 2) patrón parcialmente migrado, donde la mayor parte de los gránulos corticales se extendieron a través del área cortical y; 3) patrón migrado, en el que los gránulos corticales aparecieron adyacentes a la membrana plasmática. Estos oocitos fueron considerados citoplasmáticamente maduros.

Respecto a la caracterización de los patrones mitocondriales de los oocitos, nuestros hallazgos demostraron que las mitocondrias migraron a la periferia en la mayoría de los oocitos después de la IVM, mientras que en los oocitos inmaduros fue frecuente encontrar una distribución homogénea de las mismas. Por lo tanto, los patrones mitocondriales definidos para la cobaya fueron: 1) patrón no migrado, donde las mitocondrias aparecieron distribuidas homogéneamente a lo largo del citoplasma y cuyos oocitos fueron considerados no maduros citoplasmáticamente); 2) patrón parcialmente migrado, donde las mitocondrias se encontraron distribuidas heterogéneamente con agregaciones granulares por el citoplasma y; 3) patrón migrado,

cuando las mitocondrias fueron redistribuidas en el polo nuclear. Estos oocitos fueron considerados citoplasmáticamente maduros.

Después de realizar esta caracterización y para evaluar el posible efecto del EGF e IGF-1 sobre la maduración de los oocitos en la cobaya, se realizaron experimentos de dosis-respuesta como se describe a continuación. Un total de 1746 COCs fueron utilizados en tres experimentos y cada experimento fue repetido cuatro veces.

En el **Experimento 3.b**., los COCs fueron MIV con 0, 10, 50, 100 ng/mL de EGF o 10% de suero fetal bovino (FCS, Fetal Calf Serum). Los resultados demostraron que la suplementación con 50 ng/mL de EGF en el medio de maduración mejoró la tasa de M-II y la maduración citoplasmática, tanto en términos de migración de los GC y redistribución mitocondrial; además la calidad de las CCs parecía mejorar, ya que mostraron una tasa de apoptosis menor y se incrementó la secreción de E_2 y P_4. En el **Experimento 3.c**., los COCs fueron IVM con 0, 50, 100, 200 ng/mL de IGF-1 o 10% de FCS. El medio suplementado con 100 ng/mL de IGF-1 produjo las tasas más altas de M-II, incrementó el porcentaje de oocitos con granulos corticales localizados en la periferia, redujo la tasa de apoptosis en las CCs significativamente y mejoró la secreción steroidogénica de los COCs. Ambos experimentos corroboran que los COCs responden al EGF e IGF-1 directamente a través de sus receptores correspondientes y que ambos factores de crecimiento mejoran la maduración de los oocitos, la respuesta esteroidogénica y la viabilidad de las CCs. Por lo tanto, estos resultados sugieren que el uso de ambos factores de crecimiento parece ser una opción adecuada para usar en el medio de MIV en la cobaya.

En el **Experimento 3.d**., 500 COCs fueron madurados *in vitro* utilizando las concentraciones de EGF e IGF-1 que mostraron los mejores resultados de maduración en los experimentos 3.b. y 3.c. (50 ng/mL de EGF y 100 ng/mL del IGF-1), con o sin FCS, como se detalla a continuación: grupo 0 (no suplementado); grupo EI (EGF + IGF-1); grupo EI-FCS (EGF + IGF-1 + 10% FCS) y grupo FCS (10% al FCS). Como se esperaba, la adición de ambos factores de crecimiento junto con FCS mejoró la maduración nuclear, la tasa de migración de los granulos corticales en los oocitos, la secreción de esteroides de los COCs y redujeron el índice apoptótico en las CCs en comparación con los COCs madurados *in vitro* sin factores de crecimiento. La interacción entre ambos factores de crecimiento y las CCs puede modificar la producción de esteroides, lo que afecta indirectamente a la maduración del oocito y a la proliferación de las CCs. Sorprendentemente, ambos factores de crecimiento juntos no tuvieron un

efecto sinérgico sobre la maduración de los oocitos cuando los resultados fueron comparados con los que se obtuvieron cuando se añadieron por separado. Además, no se encontraron mejoras significativas en los patrones mitocondriales entre los grupos experimentales, así como ocurrió en el experimento 3.c. en el que sólo se añadió IGF-1. Los mecanismos celulares y moleculares por los cuales el IGF-1 solo o junto con el EGF influyen en la migración mitocondrial son desconocidos para cualquier especie. La tasa apoptótica de los COCs en el grupo suplementado con ambos factores de crecimiento y con el FCS fue inferior comparado con la tasa obtenida en los COCs cultivados solo con 50 ng/mL de EGF o 100 ng/mL de IGF-1, aunque no se encontraron diferencias significativas entre ellos.

Basándose en nuestros resultados, y en nuestras condiciones experimentales, el uso de 50 ng/mL de EGF parece ser la mejor opción para el medio IVM en la cobaya. Sin embargo, la utilización de 100 ng/mL de IGF-1 no se descarta completamente, ya que, aunque no alcanzaron diferencias significativas, el porcentaje de migración mitocondrial alcanzado fue bastante bueno. Por lo tanto, nuestros resultados demuestran que con los sistemas de maduración *in vitro* suplementados con EGF o IGF-1, se podrían alcanzar tasas de maduración similares a las que se obtienen cuando se usa FCS.

En conclusión, en esta Tesis Doctoral se ha caracterizado el sistema EGF e IGF-1 en el ovario de la cobaya, es los COCs y en embriones tempranos, estableciendo el posible papel del sistema del EGF y del IGF-1 en el desarrollo folicular, la maduración del oocito, la luteogénesis y el desarrollo embrionario temprano de manera autocrina o paracrina. Estos hallazgos proporcionan la primera evidencia de que ambos factores de crecimiento podrían estar implicados en la regulación de la fisiología ovárica en la cobaya. En esta línea, se demostró el efecto beneficioso de la suplementación de ambos factores de crecimiento *in vitro* sobre la maduración del oocito, estableciendo así un sistema valioso para la maduración de oocitos en un medio definido sin suero para la cobaya.

1. INTRODUCTION

1.1 Guinea pig as an experimental model

The Guinea pig (*Cavia porcellus*) belongs to Caviidae family (Pritt, 2012). It is a hystricomorph rodent originating from South America (Quesenberry, 2004). They were first domesticated by the Andean Indians of Peru because they were used as a food source and as a sacrificial offering to the Incan Gods (Morales, 1995). Peru and Ecuador are the main countries in the Andean region where guinea pig were distributed. Nowadays this animal is a good low-cost protein source, especially for the poorest population in these regions (Avilés *et al.,* 2014). Also, the guinea pig is involved in folk traditions and traditional medicine (Morales, 1995). Therefore, genetic diversity conservation of this species is indispensable for their socio economical importance.

Since the late 18th century, guinea pig has been frequently used as a laboratory animal (Clemons and Seeman, 2011). Availability of commercial strains and their peaceful nature make them a useful research model that has been extensively and historical used. Then 'guinea pig' has become asynonym of 'experimental subject' (Anderson *et al.,* 2015). They are useful in research of anaphylaxis, asthma, immunology, infectious and nutritional diseases, and otology among others (Craig, 1995; Hauser *et al.,* 2005; Yu *et al.,* 2017). Despite its important role in laboratory animal science, guinea pig utilization has declined dramatically in recent decades. For example, this species was used in about 27,000 scientific experiments in the UK (United Kingdom) in 2004, that means<1% of total animal research (Dorsch *et al.,* 2008), mainly because mice and rats have replaced them. However, in some very specific research the guinea pig continues to be a reference model as for example in immunology due to the antigen-macrophage interaction similar to humans (Hoff *et al.,* 2011; Larrouy-Maumus *et al.,* 2017).

From a genetic point of view, it is a more suitable model than the mouse or rat since the primordial oocytes of guinea pig present at birth a typical diplotene configuration like the equivalent stage in women while primordial oocytes from mouse or rat have a dyctiate appearance. The pool of primordial oocytes is the most menaced, since they are present throughout life and genetic damage from radiation may accumulate for considerable periods before entering maturation and being ovulated. Studies have confirmed that guinea pig oocyte has high radio-resistance and are suitable for more detailed studies in relation to genetic risks (Jacquet *et al.,* 2001), representing one of the best models for the genetic hazard of radiation in human (Jacquet *et al.,*1995).

In addition, this species remains essential in reproductive research areas such as Artificial Reproductive Technics (ARTs). In this sense, it is the only laboratory rodent with a complete estrous cycle, consisting of both follicular and luteal phases that resemble those of cows, ewes and pigs and the menstrual cycle in humans. In contrast, most laboratory rodents like mice, rats and hamsters exhibit endocrinologically different incomplete estrous cycles which lack of functional luteal phases (Suzuki *et al.,* 2003). Additionaly to easily defined ovarian cycle and active *corpus luteum,* the guinea pig is also a suitable animal model for the study of reproduction in humans because this species presents also long gestation period and spontaneous ovulation (Suzuki *et al.,* 2003; Carter *et al.,* 2006, Carter, 2007). Pups exhibit mature central system at birth that makes them a very pertinent model for studies of teratology, for example in deleterious genes that act at different stages of gestation or in congenital diseases (Cardin *et al.,* 2016). Finally, it has been suggested that *in vitro* culture of guinea-pig blastocysts can serve as a good model for the study of implantation in humans, since the interstitial type of implantation and trophoblast formation observed in guinea pigs and women share similarities (Verkeste *et al.,* 1998; Mess, 2007).

1.2. Anatomical description of the guinea pig reproductive system

The reproductive system of guinea pig is constituted by a pair of ovaries and oviducts, a bicorne uterus, cervix and vagina (Figure 1).

i. **Ovary:** the ovaries have an ovoid shape and are located in the abdominal cavity, near the kidneys between the third and fourth lumbar vertebra. The averaged measurements are 7 mm (millimeters) long and 4 mm width. The ovaries surface is generally smooth except for the projecting mature follicles during ovulation cycles (Grégoire*,* 2012).

ii. **Oviduct**: the oviduct collects and transports the mature oocyte (in Methaphase II) and the early embryo to the uterine horn and the fertilization process take place in there. In the guinea pig the oviducts measuring about 57 mm long and 1.5 mm width (Grégoire*,* 2012).

iii. **Uterus**: the uterus is bicorne, Y-shaped and consists of two horns which form a V. Measuring approximately 37 mm long and 6 mm width (Grégoire *et al.,*2012). Dorsal side is in contact to the rectum and the ventral side is adjoining to the intestines and the dorsal side of the urinary bladder.

iv. **Cervix:** the cervix is a portion of the uterus with Y-shape. It´s connected at the vagina and consists of a series of small mucosal thickenings extending over the side walls to converge ventrally in the center (Grégoire, 2012). Its consistency is hard (Popesko *et al.,*1998).

v. **Vagina**: is a fibroelastic muscular tube, measuring 3 cm long by 1 cm width approximately. It is located in the pelvic cavity related in its dorsal side with rectum on its ventral face and body with the neck of the bladder. Features that distinguish other rodents and the guinea pig is the presence of an epithelial membrane covering the vaginal orifice except in the estrus phase and parturition (Grégoire, 2012).

vi. **Vulva**: is an external opening which has inverted Y-shaped. It has an outer diameter smaller than that of the cervix and has a thin wall. It joins in the pelvis floor and bends ventrally to the area of the ischial arch where it opens to the outside through the vaginal opening (Grégoire, 2012). Vulvar hole has recess forming two small lips at the bottom of the meatus having a similar appearance to the anal sphincter (Popesko *et al.,* 1998).

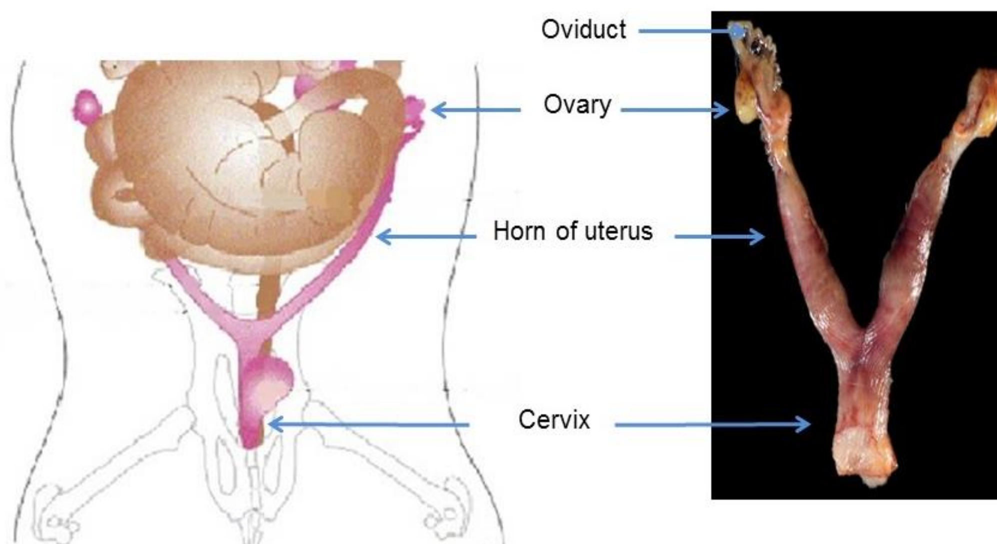

Figure 1. Anatomical representation of guinea pig female reproductive tract. Drawing adapted from Silverman, 2005.

1.3. Ovarian physiology

1.3.1. Estrous cycle: follicular and luteal phase

Guinea pigs are polyestrous animals and ovulate spontaneously (Sisk, 1976). The female is sexually mature at about three months old (Stockard and Papanicolaou, 1917). The first estrus occurs around 68 days of age (range 33-134 days) (Mills and Reeds, 1971) and begins the day after ovulation. Estrus have a mean length of 17.5 ± 2.1 days (range 15–21 days) (Myers *et al.,* 1936; Hutz *et al.,* 1990; Rocca and Wehner, 2009) (Figure 2). The follicular development begins the day after ovulation and consists in two waves. The largest follicles of the first wave reach their maximal diameter (500-750 µm) at Days 10–11 of the cycle but become atretic because progesterone (P_4) are elevated and the negative feedback provokes suppression of luteinizing hormone (LH) (Garris and Foreman, 1984). The demise of the *corpus luteum* allows the development of the second follicular cohort until ovulation. This wave takes about 6 -7 days; there is a recruitment of small follicles (Bland, 1980; Hutz *et al.,* 1990) and a selection of the 3 to 5 largest follicles at Day 10. Then P_4 concentrations decrease rapidly after Day 12 and it is a clear increase in LH levels between 13 and 15 Days (Wisel *et al.,* 1991), which will stimulate ovulation. Finally, ovulation occurs at the end of the second wave (Day 17 of the cycle) (Logothetopoulos *et al.,* 1995).

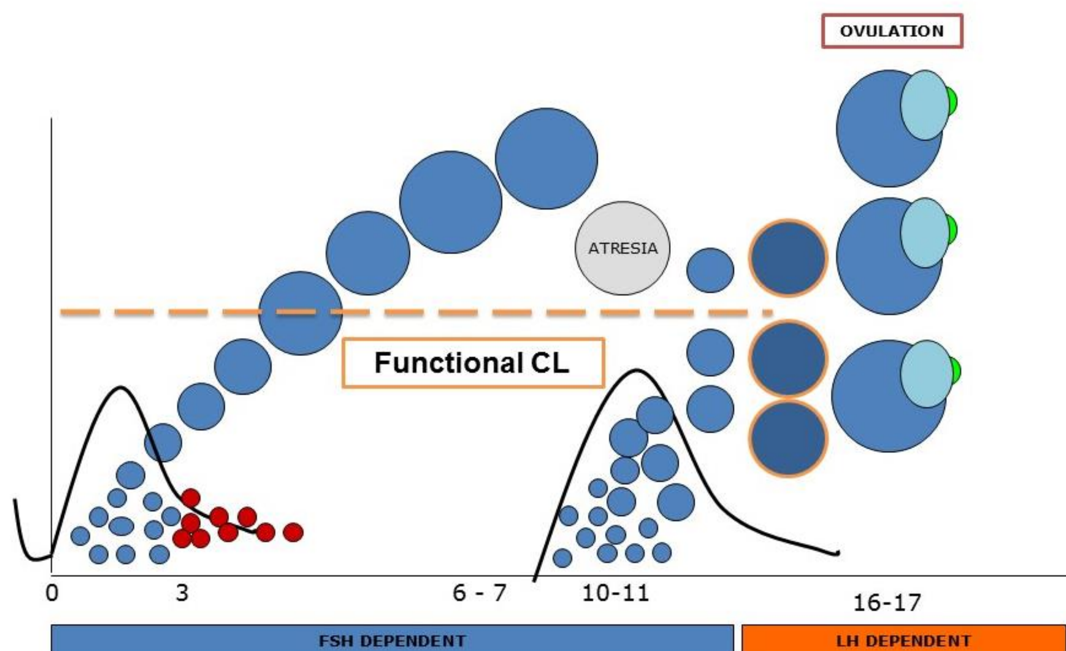

Figure 2. Estrous cycle of guinea pig. CL: *Corpus luteum*; FSH: Follicle-stimulating hormone; LH: luteinizing hormone.

The initial study of the estrous cycle in the guinea pig was done by Stockard and Papanicolaou (1917) and Ishii (1920). They identified different phases in the estrous cycle that are describes below. Phases can be easily identified by cytological study to assess the stage of the cycle:

i. **Proestrus:** This period maintains for 1-1.5 days. The external genital organs become swollen. Rupture of the vaginal membrane and a serous secretion is produced (in some instances turbid or viscous), but in the majority of cases is more or less transparent. This is followed by the estrus period. Excess of viscous transparent liquid secretion during the last 4 or 5 hours (h) of proestrus.

ii. **Estrus:** Secretion similar than final stage of proestrus continues for 6 or 7 h from the estrus period. The vaginal membrane continues open during estrus (2-3 days), covering the begining of period sexual behavior and the period of ovulation. Estrus lasts 8–11 h. Females exhibit lordosis, with rear quarters elevated and present swollen congested vulva and a vaginal membrane perforate (Harper, 1968; Phoenix, 1970). To assess the estrous cycle stage, also vaginal impedance measurements can be used in this species (Lilley *et al.,* 1997).

iii. **Metaestrus**: Lasts 3 days and might be considered as the period of recovery of the estrus changes.

iv. **Diestrus**: Lasts 11–12 days and complete the estrous cycle. It is characterized by the presence of active *corpus luteum* that produces P_4. Other studies have shown that there is an elevation of plasma P_4 and estradiol (E_2) coincident with this phase (Challis *et al.,* 1971; Garris and Foreman, 1984; Hutz *et al.,* 1990).

Some interesting data from this species is that a fertile postpartum estrus occurs 2 to 10 h post- parturition (Rowlands, 1949; Sisk, 1976). There is not demonstrated evidence of cycle synchronization in grouped females (Donovan and Lockhart, 1972; Harned and Casida, 1972).

1.3.2. Oogenesis and follicular development

The ovary is a highly organized organ constituted of germ cells (oocytes) and somatic cells (granulosa, theca and stromal cells). Main functions comprise endocrine action

(steroid biosynthesis) and reproductive function (foliculogenesis and oocyte production). Their functional subunits are the follicles that grow from primordial follicle to antral one. Oogenesis is the formation, development and maturation of female gametes, that begin in the germ cells of fetal ovary (Findlay *et al.,* 2015; Grive and Freiman, 2015). Following Deoxyribonucleic acid (DNA) replication, two consecutive rounds ofmeiotic division, called Meiosis I (MI) and Meiosis II (MII), begins and reduce chromosomes to generate haploid gametes (Cohen and Holloway, 2015).

In guinea pig once the gonad has been formed, the ovogonias undergo a phase of interstitial mitotic multiplication and surround themselves with layers of germ cells from embryonic stages to sexual maturity. The number of germ cells per ovary increases from about 27.000 at the half of gestation period ((30 days *post-coitum* (p.c.)) up to a maximum of 105.000 at 41 days p.c. Further, the initial stock of germ cells undergoes a heavy depletion to reach the number of 13.000 to 12-month *post-partum* (p.p.). This decrease is due to the continuous atresia process that affects germinal cells from the earliest stages of development and that will be referred below. The ovogenesis process ends around the 50th day p.c. There are a large number of oocytes in the leptotene stage at 35 days p.c. with zygotene at about 40 days p.c. and pachytene at 50 days p.c. Oocytes are in the diplotene stage at birth. This phase of meiosis is blocked until puberty. In guinea pig, the period of gonadal division and entry into meiosis takes place before the parturition, phenomenon comparable to the woman. The number of oocytes in the adult animal ovary is highly dependent on the initial recruitment of the pool of primordial follicles (McGee and Hsueh, 2000).

Folliculogenesis is the process of follicular development. Somatic cell types in the follicle interact by differents mechanisms promoting follicular growth, oocyte maturation and subsequent *corpus luteum* formation (Richards and Pangas, 2010; El-Hayek and Clarke, 2016). Below we will describe the most important characteristics of the different follicles present in the ovary:

The primordial follicles are consisting of one oocyte enclosed by a single layer of squamous granulosa cells and a basal lamina. They are continually recruited during reproductive life into the growth phase to produce fertilizable oocytes. Studies demonstrated that survival of primordial follicles is maintained through the PI3K (Phosphatidylinositol 3-kinase) pathway (Reddy *et al.,* 2005; Liu *et al.,* 2006). They lack vascular supply, so nutrition and paracrine signaling occur by diffusion from GC which create a close microenvironment. In early stage oocytes and follicles in which follicle-

stimulating hormone (FSH) receptors are not expressed yet, follicular growth is dependent on Kit-Kit ligand (KL) signaling (Albertini and Barret, 2003). Kit exists on the surface of oocytes, and KL is produced in the granulosa cells. KL activates the PI3K pathway in primordial oocytes, followed by phosphorylation of Akt (protein kinase B) and Foxo3a (forkhead box O3A) (John *et al.,* 2008). The FOXO family is a main target of Akt and it suppresses the activation of primordial follicles and maintains dormancy by promoting pro-apoptotic genes (Reddy *et al.,* 2005). Other studies, also have demonstrate phosphatase and tensin homolog (PTEN) plays an inhibitory role in the primordial oocyte activation (John *et al.,* 2008; Reddy *et al.,* 2005), and have reported successful *in vitro* activation of mammalian primordial follicles with synthetic PTEN inhibitors (Li *et al.,* 2010; Adhikari *et al.,* 2012). By last, granulosa cells -derived anti-Müllerian hormone mediated dialogue also inhibits follicle activation (Carlsson *et al.,* 2006). On the other hand, primordial follicle activation is initiated by molecules in primordial follicle granulosa cells (Zhang *et al.,* 2014), activated by mTORC1 complex (mammalian target of rapamycin complex) (Cheng *et al.,* 2015; Sun *et al.,* 2015; Zhang and Liu, 2015) that lead to diferenciation and proliferation of granulosa cells of recruited follicles. Transcription of some genes into germ cells such as Folliculogenesis Specific BHLH Transcription Factor (FIGLA), Newborn ovary homeobox-enconding gene (NOBOX), KL and neurotrophic Receptor Tyrosine Kinase 2 (NTRK2), as well as forkhead box protein L2 (FOXL2), kernel independent transport layer (KITL) and nerve growth factor (NGF) have also been observed, which are expressed in somatic cells and play a critical role during early folliculogenesis (Choi and Rajkovic, 2006). Many growth factors play a role as upstream regulators of primordial follicle activation, including leukemia inhibitory factor (LIF) (Nilsson *et al.,* 2002), basic fibroblast growth factor (β-FGF) (Nilsson *et al.,* 2001), platelet-derived growth factor (PDGF) (Nilsson *et al.,* 2006), stem cell factor (SCF), stromal derive factor-1 (SDF-1), and bone morphogenetic protein 4 (BMP-4) (Nilsson and Skinner, 2003; Williams and Erickson, 2014). This growth factor network is supported by surrounding stromal cell derived mediators, such a keratinocyte growth factor, BMP-4 and BMP-7 (Nilsson and Skinner, 2003). However, mTORC provokes KITL secretion in granulosa cells which binds kit in the oocyte so PI3K pathway activates, awaking the quiescent oocyte and starting their development.

In primary follicle the oocyte increase in size and the single layer of granulosa cells change to cuboidal shape and proliferate mitotically enclosing and surrounding the oocyte (Knight *et al.,* 2012; Orsi *et al.,* 2014). The growing oocyte secretes a glycoprotein called the zona pellucida (ZP) that assembles into an extracellular layer and physically separates the oocyte from the adjacent granulosa cells (El-Hayek and Clarke, 2016).

Although follicle growth is gonadotrophin independent in this stage, upregulation of FSH receptor expression by the action of granulosa cells is described (Oktay *et al.,* 1997; Findlay and Drummond, 1999) and is required for primary follicle development to the preantral stage (Oktay *et al.,* 1997). Then, stimulators of FSH receptor expression such activin, cyclic adenosine monophosphate (cAMP), and transforming growth factor-beta (TGFβ) family, mainly BMP-15 and growth derived factor 9 (GDF 9) are secreted (Findlay and Drummond, 1999). In primary follicles, granulosa cells continue to secrete KL, the oocyte is transcriptionally active and must generate sufficient proteins and mRNA (messenger ribonucleic acid) transcripts to support its own growth as well as oocyte maturation, fertilization and early embryo development (Williams and Erickson, 2014). The oocyte and granulosa cells come into contact through by intercellular connections denominated transzonal projection (TZP), that traverse the ZP and form adherents junction between oocyte surface and gap junctions from granulosa cells (Albertini *et al.,* 2001). Finally, gap junctions, ((formed by connexins (Cx)), established in this area to give cell-to-cell contact, facilitate the diffusion of metabolites, ions and signaling molecules (Bruzzone *et al.,* 1996). Between the oocyte and GC, gap junctions are form by Cx37 synthesized by the oocyte and Cx43 synthesized by the GC (Simon *et al.,* 1997; Grazul-Bilska *et al.,* 1997), so in granulosa cells gap junctions are only formed by Cx43.

The next stage of development is the secondary follicle. This is usually situated deeper in the ovarian cortex and its development begins with the acquisition of the second layer of granulosa cells. Active granulosa cells changes from a simple cuboidal epithelium to a stratified or pseudostratified columnar epithelium surrounding the oocyte (Williams and Erickson, 2014; Orsi *et al.,* 2014). This granulosa cells makes possible the change of nutrients and their transport to the oocyte (Herlands and Schultz, 1984). In addition, theca development layer occurs in this phase differentiating into two layers: internal theca comprising rounded interstitial cells and external theca, which differentiates into smooth muscle cells (Erickson *et al.,* 1985). Theca external development is accompanied of the perifollicular vascular development through angiogenesis (Orsi *et al.,* 2014; Williams and Erickson, 2014). Secondary follicle can be described by an active autocrine/paracrine system which depends of oocyte-derived cytokines (Orsi *et al.,* 2014). Studies in animal models suggest a crucial role of Growth differentiation factor-9 (GDF-9) and BMP-15. In its absence follicle growth and development stop at the primary stage (Chang *et al.,* 2002). The oocyte not only controls the follicular growth but also its own growth (Eppig, 2001; Matzuk *et al.,* 2002). In this phase, the gap junctions continue to develop between the granulosa cells for promoting the cellular communication. Studies in mice demonstrated that folliculogenesis arrests at the primary/secondary

transition in Cx43-deficient animals, so conexins play an indispensable role in secondary follicle formation (Juneja *et al.,* 1999). Angiogenesis is also crucial in this phase and is promoted by the FGF-β and vascular endothelial growth factor (VEGF) (Brown *et al.,* 1997). The blood now circulates around the follicle, bringing nutrients, gonadotropin, waste and secretory products from, the developing follicle. At the completion this phase of folliculogenesis, the secondary follicle is formed by interacting structural units: a fully grown oocyte surrounded by a zona pellucida, several layers of granulosa cells, a basal lamina, and internal and external theca with a capillary net (Williams and Erickson, 2014).

The antral follicle is formed when a full-filled cavity with follicular fluid (FF) are developed inside follicle. FF is a plasma exudates and protein concentration is generally lower than in plasma. It is conditioned by secretory products from the oocyte and granulosa cells (Hutt and Albertini, 2007) and creates a microenvironment where regulatory molecules must pass to and from (Williams and Erickson, 2014). Once the antrum expands, it separates the granulosa cells into two populations: the mural granulosa cells that line the inside of the follicular wall (with sterodogenic action) and the Cumulus Cells (CCs) that surround the oocyte. Now, follicle is dependent of gonadotropins it is regulated by FSH, LH, and an extensive array of growth factors (Orsi *et al.,* 2014). FSH receptors are located on the granulosa cells and is essential for the maturation of ovarian follicles and induces granulosa cells proliferation and antrum formation. Gonadotropins can increase intracellular cAMP; studies showed, that the oocyte is able to synthesize cAMP through, the activity of a G protein related receptor, (GPR3) (Mehlmann *et al.,* 2002, 2004). Antral follicle are heterogeneous in size, as their size is determined by FF volume in the antrum (Erickson and Shimasaki, 2000). As an antral follicle develops, GDF-9 and BMP-15 function as gradient signals for the generation of distinct classes of granulosa cells. These differences become important as the follicular cells and the oocyte are prepared for formation of preovulatory follicle (Graafian follicle) and its ovulation (Williams and Erickson, 2014). When the theca and granulosa cells proliferate and at the same time grows the antrum, the antral follicle becomes a preovulatory follicle. Also, some factors implied in angiogenesis as Insulin-like growth factor-1 (IGF-1) and VEGF intervene in the improvement of vascularization in selected follicles, which lead to more FSH support in these follicles and more LH receptors development. In addition, as estrogens level decreased in the recruited follicles, TGF-β factors modulate FSH secretion (Aerts and Bols, 2010).

As mentioned, in the preovulatory follicles the granulosa cells are of different types and according to their position have different responses to the stimulus of FSH. For example,

the granulosa cells membrane express P450 and LH receptor whereas CCs only have FSH receptors. The way in which granulosa cells differentiate may be controlled by the oocyte (Erickson and Shimasaki, 2000; Hutt and Albertini, 2007). The theca external is innervated by autonomic nerves, concentrically organized (Erickson and Shimasaki, 2000). The physiological significance of the theca external is unknown but it has been suggested that they may play a role in subsequent ovulation (Orsi *et al.,* 2014). The theca internal is formed by large epithelioid cells whose cytoplasm is filled with lipid droplets, smooth endoplasmic reticulum, and mitochondria (Young and McNeilly, 2010). These cells possess receptors for LH and insulin (e.g. produce androgens such androstenedione) (Orisaka *et al.,* 2006). In this follicle, FSH plays a vital role in LH receptor induction in the granulosa cells necessary to respond to LH surge and undergoes ovulation process. The expression of LH receptors remain suppressed until late in the follicular phase of the cycle (Erickson and Shimasaki, 2000; Eppig, 2001) and is completely dependent of FSH as mentioned. LH surge causes resumption of meiosis of the oocyte at the end of the follicular development in the preovulatory follicle (Figure 3).

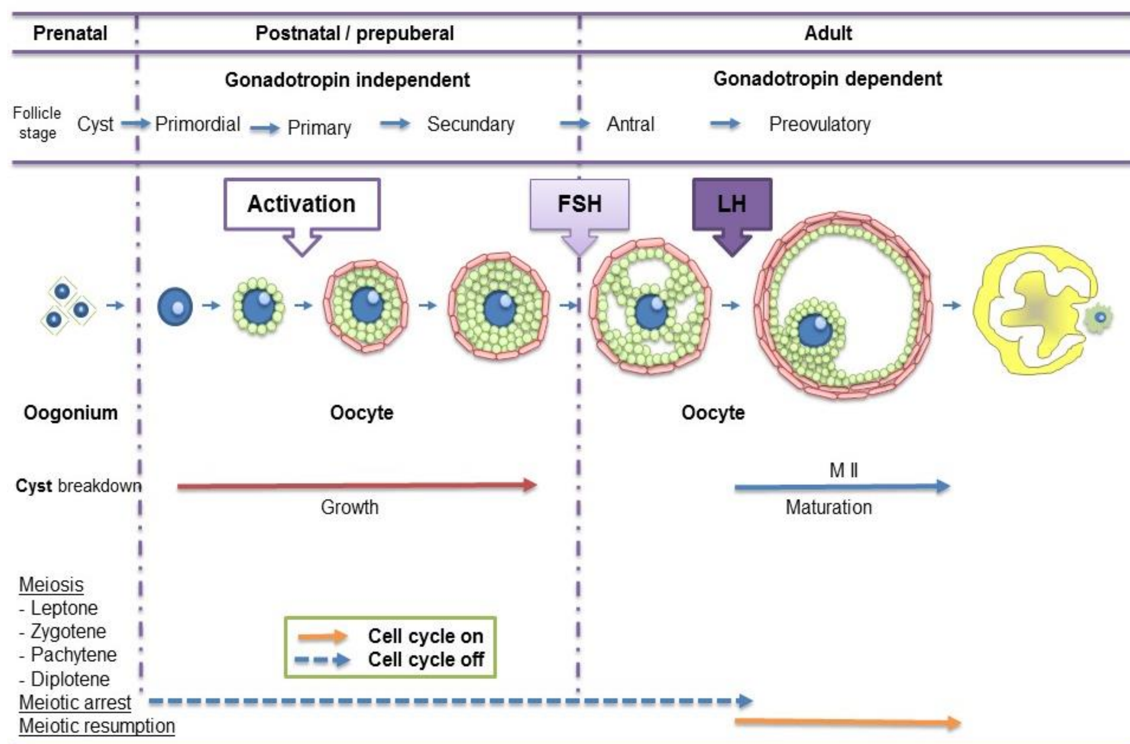

Figure 3. Follicular stages and their endocrine and paracrine control in guinea pig. Adapted from Pangas and Rajkovic, 2015.

1.3.2.1. Endocrine, paracrine and autocrine control of folliculogenesis

The follicular development is regulated by the hypothalamic-pituitary-ovarian axis, where the specialized hypothalamic neurons secrete pulses of gonadotropin-releasing hormone (GnRH) into the blood vessels regulated by the kisspeptin system (Messager *et al.,* 2005; Okamura *et al.,* 2013; Kanasaki *et al.,* 2017). The anterior pituitary secretes gonadotropins FSH and LH and the ovary itself produce steroid (e.g., androgens, estrogen and P_4) and peptide hormones (e.g., inhibin). The frequency of GnRH pulsatility determines the FSH secretion (slower pulses) or LH secretion (rapid pulses) that explains their differential predominance in the follicular phase and luteal, respectively (Mermillod *et al.,* 2008). Steroidogenesis in the ovary, occur via the two cell/two gonadotropin model describing cooperation between the granulosa and theca cells as follows: the androgens are synthesized from cholesterol in LH-stimulated theca cells, followed by conversion to estrogens by FSH in granulosa cells (Richard *et al.,* 2002). The cholesterol is imported by steroidogenic acute regulatory protein (StAR) (Stocco, 2001) and cleaved by cytochrome P450 (CYP) (Young and McNeilly, 2010). Both are mitochondrial enzymes. LH receptors and the enzyme CYP17, which converts pregnenolone and P_4 to dehydroepiandrosterone (DHEA) and androstenedione, respectively, are expressed primarily in theca cells, while FSH receptors and aromatase (CYP19), which converts androgens to estrogens, are expressed mainly in granulosa cells (Peter and Dubuis, 2000). Androgens diffused to granulosa cells where they play a dual role: are substrates for enzyme aromatase and also increase the activity of this enzyme, thereby increasing estrogen production (Taubøll *et al.,* 2003). Regulation of both GnRH pulsatility and gonadotropin production is due of the feedback to the pituitary. P_4 decrease GnRH pulse frequency and therefore LH secretion, while E_2 has positive feedback for the production of proges P_4 (Orsi *et al.,* 2014). On the other hand, as has been mentionated granulosa and theca cells are capable of making pregnenolone and/or P_4 from cholesterol. In the follicular phase, the relatively avascularized granulosa cells have limited oxygen (O_2) or cholesterol, thus low amounts of these steroids are produced. In contrast, after exposure to gonadotropins, the granulosa cells become "luteinized," and are then able to synthesize large amounts of pregnenolone and P_4 from cholesterol substrate (Wood and Strauss, 2002; Havelock *et al.,* 2004).

Although gonadotrophins are important in regulating folliculogenesis, to obtain competent oocytes is necessary to have a perfect interaction between follicular somatic cells and the oocyte, which is potentially regulated by paracrine factors produced in the ovary and autocrine produce by both granulosa cells and oocyte. One of the more

amazing event discovered in last decade is that local produced oocyte factors can drive follicle to success or atresia (Hutt and Albertini, 2007; Mermillod *et al.;* 2008). Oocyte plays an active role in the follicle growth by regulation of proliferation, differentiation of granulosa cells and by the secretion of extracellular matrix components, ensuring an adequate environment for follicular development (Eppig *et al.,* 2002; Hussein *et al.,* 2005). Then the oocyte has the ability to regulate LH receptors, steroids and hyaluronic acid secretion stimulating follicle growth and survival as well as cumulus expansion; also inhibits luteinization by suppressing P_4 production until it is released at ovulation (Hutt and Albertini, 2007). All these processes are supported by TGF-β superfamily factors, mainly GDF-9 and BMP-15 (Young and McNeilly, 2010). Evidence suggests that both oocytes and CCs produce and secreted proteins and cell surface receptors that are involved in an oocyte granulosa cell regulatory loop (Taft *et al.,* 2002).

In addition, other paracrine and autocrine factors are responsible for ensuring the success of follicular and oocyte developmentsuch as growth factors (Erickson *et al.,*1985). The intrinsic growth factors interact with the endocrine system to evoke the physiologic control of folliculogenesis including recruitment, preantral follicle growth, selection, stereiodogenesis, atresia and ovulation. Among these factors, we highlight the Epidermal Growth Factor (EGF) and IGF family ((IGF-1 and their IGF- Binding Proteins 1-6 (IGFBPs)) that describe below, for their important participation in follicular development, maturation and early embryo development. And also by their implication insome pathologies as polycystic ovarian syndrome (PCOS). Some authors have signaling growth factors EGF and IGF systems as one of the major factors that causes the multietiology syndrome PCOS, since development of follicles are stopped and also impaired steroidogenesis and hyperinsulinemia has been described (Guidice, 1995; Homburg, 1998).

i. EGF system

EGF is member of a large family of closely related proteins that includes amphiregulin (AREG), epiregulin (EREG), β-cellulin (BTC) and transforming growth factor-α (TGFα). All of these EGF-like ligands act by binding with high affinity to the cell surface receptor, EGF-R (Goodsell, 2003; Herbst, 2004; Citri and Yarden, 2006). EGF is an important regulator of physiological ovarian function since it acts via endocrine, autocrine and paracrine manner as a potent mitogenic factor but also can exert inhibitory actions. In general, EGF and EGF-like ligands are associated with cell growth, proliferation and differentiation. Notably, EGF cannot directly trigger maturation of denuded oocytes,

suggesting that secondary messengers induced by EGF-R signaling in the CCs are required for meiotic progression (Jamnongjit *et al.,* 2005). So, EGF has the ability to stimulate the proliferation of granulosa cells (Gospodarowicz and Birdwell, 1977; May *et al.,*1987) germinal vesicle breakdown (GVBD), polar body formation (Das *et al.,*1991), and cleavage of oocytes (Coskun *et al.,*1991). LH triggers secretion of EGF molecules, that in a paracrine signal through the EGF-R stimulates cumulus-cell expansion and oocyte maturation. In CCs, EGF promotes calcium (Ca^{2+}) efflux and improves their expansion during maturation (Hill *et al.,* 1999; O'Donnell *et al.,* 2004).

EGF-R is a growth factor dependent of tyrosine kinase activity and is considered to be the primary mechanism for the generation of intracellular signals that culminates in meiosis re-activation and maturation of the oocyte. It belongs to family of transmembrane receptor tyrosine kinases (RTKs) consists of the EGF-R (ERBB1), HER2 (ERBB2), HER3 (ERBB3), and HER4 (ERBB4). Each ErbB receptor comprises a large extracellular region (ER) a single spanning transmembrane domain (TM) an intracellular juxtamembrane region (JM), a tyrosine kinase domain and Carboxi-terminal (C-terminal) regulatory region (Ferguson *et al.,* 2003; Lafky *et al.,* 2008) (Figure 4). The dimerization of two ErbB receptors results in the auto-transphosphorylation of tyrosine residues (Ferguson *et al.,* 2003; Citri and Yarden, 2006).

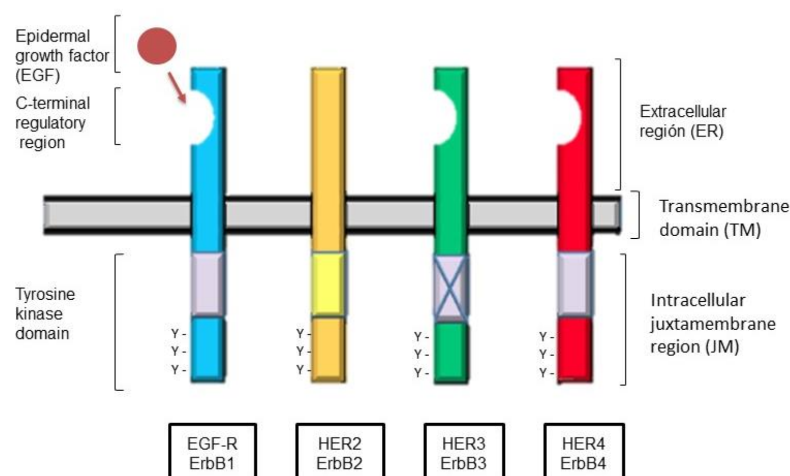

Figure 4. ErbB receptor family: the different endogenous ligands are shown and the members of each family.

The interaction between EGF and their receptor activates several systems of signal transduction implicated in important mechanism for the follicle development and oocyte maturation:

i. Raf/Ras/mitogen-activated protein kinase pathway: regulates cell proliferation and

survival. Raf/Ras (Protein superfamily) activation ((Ras-GTP (Guanosine triphosphate)) activates Raf-1 that can phosphorylate to activate the dual specificity protein kinases MEK1 (Mitogen-activated protein kinase kinase-1) and MEK2 (Mitogen-activated protein kinase kinase-2), which in turn phosphorylate to activate the serine/threonine specific protein kinases, Extracellular signal-regulated kinase-1 (ERK1) and Extracellular signal-regulated kinase-2 (ERK2) (Liebmann, 2001). Activated MAPKs (Mitogen-activated protein kinases) are imported into the nucleus where they phosphorylate specific transcription factors involved in cell proliferation (Gaeste, 2006; Citri and Yarden, 2006) and it is essential for granulosa cells division. It is also involved in processes such as oocyte maturation and embryo development (Walker *et al.,* 1998).

ii. PI3K/Akt pathway: this pathway is involved in cell growth, apoptosis resistance, invasion and migration. PI3K is a dimeric enzyme responsible of the anchorage to ErbB receptor-specific docking sites and generation of the second messenger phosphatidylinositol 3,4,5-triphosphate (IP3) that is responsible for phosphorylation and activation of the Akt (Vivanco and Sawyers, 2002).

iii. Signal transducers and activators of transcription (STAT): proteins interact with phosphotyrosine residues via their Src homology 2 domains and dimerization, translocate to the nucleus and drive the expression of specific target genes (Bromberg, 2002).

iv. Src kinase pathways: Src plays a critical role in the regulation of cell proliferation, migration, adhesion, angiogenesis, and immune function (Summy and Gallick, 2006). Src serves as a signal transducer and an enhancer of EGF-R activation (Leu and Maa, 2003).

In addition, the mural granulosa cells and cumulus cells both express EGF receptors. This suggests that LH could act by transactivating EGF-R signaling. This provides a pathway by which the LH signal received by the mural granulosa cells could be relayed to the cumulus cells and ultimately the oocyte. EGF is able to induce maturation of follicle-enclosed oocytes and expansion of the cumulus layer in the absence of LH (Ashkenazi *et al.,* 2005; Downs and Chen, 2008). EGF-like ligands released by the granulosa cells in response to LH, as well as EGF, trigger the decrease in follicular cyclic guanosine monophosphate (cGMP) independently of LH (Hsieh *et al.,* 2011; Norris *et al.,* 2010). In ovarian follicles EGF activates StAR post-translationally through

phosphorylation. Since activation of the EGF and LH receptors similarly increase StAR activity and steroidogenesis, then perhaps the EGF receptor is signaling downstream of the LH receptor (Jamnongjit *et al.,* 2005).

On the other hand, EGF can have a role in pathological situations because excess of activity in both EGF and TGFα can prevent aromatase function and disminish apoptosis of follicles (Homburg, 1998).

ii. IGF System

The IGF system is denominated the "IGF autocrine/paracrine system" which is composed of different elements (Jones and Clemmons, 1995).Two ligands: IGF-1 and IGF-2 (Humbel, 1990), two receptors, the type I receptor that mediates most of the somatomedin-like actions of both IGF-1 and-2 (Roth and Kiess, 1994) and six IGF binding proteins (IGFBPs 1–6) circulatingin addition to proteases that modulate ligand availability (Hwa *et al.,* 1999; Paveli´c *et al.,* 2007; Mctavish *et al.,* 2009; Zha and Lackner, 2010).

IGF-1 or somatomedin-C is a 70 amino acid hormone that is structurally similar to pro-insulin and is synthesized in the liver and fibroblasts (Hwa *et al.,* 1999; Laviola *et al.,* 2007) but also in the ovary (Adashi, 1998). This peptide of low molecular weight promotes cellular mitosis and differentiation in a variety of systems (Giudice, 1992) and IGF system is considered as intraovarian regulator of folliculogenesis by endocrine, autocrine and paracrine pathways (Kooijiman, 2006). It plays an important role in the regulation of cell proliferation, differentiation and protein synthesisas well as in the protection of multiple tissues from apoptosis (Laviola *et al.,* 2007). In the ovary, IGF-1 is important in the follicular development process by enhancement of cell proliferation and differentiation of granulosa cells (Hernández *et al.,* 1988), acts as a biological amplifier by the action of FSH in the ovary (Hsu and Hammond, 1987) by increasing the aromatase activity and biosynthesis of estrogen (Young and McNeilly, 2010), induces antrum formation (Baker *et al.,* 1996; Zhou *et al.,* 1997; Adams *et al.,* 2000; Martins *et al.,* 2010; Sharma *et al.,* 2010), increases P_4 level and keep resistance to apoptosis events (Ewton *et al.,* 1994; Spicer and Echternkamp, 1995; Gutierrez *et al.,* 1997; Mani *et al.,* 2010). Figure 5 show the main effects of IGF-1 in the ovary of different mammalian speciesat different stages of follicular development.

Follicular stage	IGF - 1
Primordial	- Initiation of growth (primate)
Primary	- Growth (bovine)
Secondary	- Follicle survival (caprine) - Follicle growth (caprine, bovine) - GC proliferation (caprine, ovine) - GC differentiation (murine) - Estradiol production by GC (bovine) - Induce FSH expression in GC (murine) - Theca cell survival (murine) - Cortical granules formation in oocytes (murine)
Antral Preovulatory	- GC proliferation (ovine, bovine, swine) - Progesterone production by GC (ovine) - Estradiol production by GC (ovine) - Increase follicle sensitivity to gonadotrophin (bovine,murine) - Oocyte viability (bovine) - Increases LH-R in GC and theca (murine) - Follicle dominance and multiple ovulation (bovine, murine) - Oocyte maturation (bovine, murine) - Secretion of inhibin-A, activin-A, follistatin by GC (bovine)

Figure 5. Influence of IGF-1 on the development of primordial to preovulatory follicles in mammalian species. By Silva *et al.* (2009). GC (Granulosa cells)

The biological actions of IGF-1 are mediated by the Insulin-like growth factor-1 Receptor (IGF-1R) (Laviola *et al.,* 2007). It is a transmembrane tyrosine kinase with high homology to the insulin receptor (Izadyar *et al.,* 1998) and consists of two extracellular α-subunits and two membrane-spanning β-subunits (Figure 6). The α-subunit is extracellular and contains the ligand binding domain for IGF-1, IGF-2, and insulin. The β-subunit has a hydrophobic domain that traverses the cell membrane, contains an Adenosine tri-phosphate (ATP) binding site and ligand-activated tyrosine kinase activity, as well as sites for autophosphorylation (Liu *et al.,* 1993; Gennigens *et al.,* 2006; Laviola *et al.,* 2007; Yin *et al.,* 2009). Additional tyrosine residues in the C-terminal domains of the β-subunit are also important in signal transduction by the IGF-1 receptor (Yuen and Macaulay, 2008).

Signalling pathway involve the activation of the PI3K (Le Roith *et al.,* 2001) which cause induction of antiapoptotic proteins (Meinbach and Lokeshwar, 2006) and activation of the recruitment of Akt (protein kinase B) to the membrane. Activated Akt then mediates a host of cell signaling events, including disinhibition of the mTORC1 complex and increased protein synthesis and cell growth. Also activated IGF-1R results in stimulation of the RAS/MAP kinase pathway, which also leads to increased cell proliferation (Zha and Lackner, 2010) (Figure 6). Finally, MAPK/ERK kinase signaling pathways is activated (Le Roith *et al.,* 2001). The ligands IGF-1 are both capable of binding and stimulating the catalytic activity of the IGF-1R.

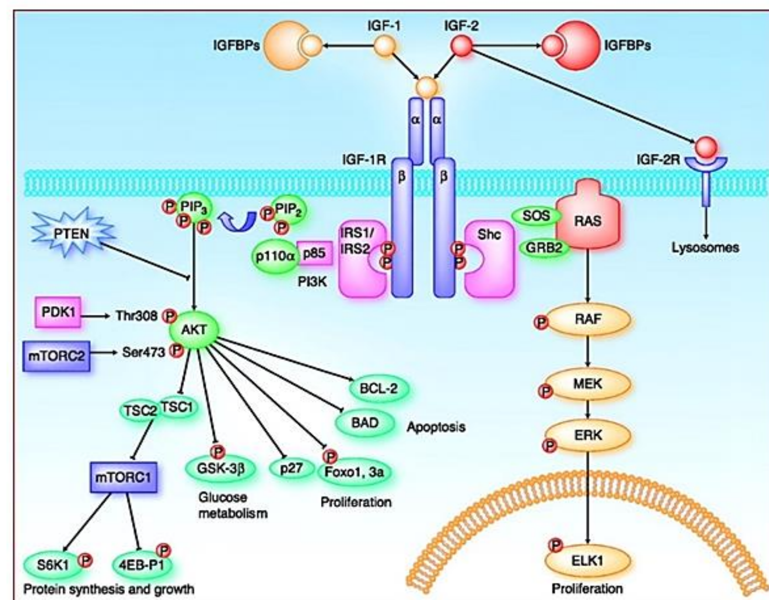

Figure 6. Key components of the IGF-1R pathway. (By Zha and Lackner, 2010).

The IGFs interact on a 1:1 molar ratio with six specific, high-affinity IGFBPs (Rechler, 1993; Jones and Clemmons, 1995). They are often divided into two classes: the pituitary growth hormone (GH)-dependent main serum IGFBPs (IGFBP-3) and the low molecular weight, GH-independent IGFBPs 1,2,4,5 and 6 (Binoux *et al.,* 1988; Hardouin *et al.,* 1987). Less than 5% of IGF is free in circulation whereas almost 90% circulates as a complex with IGFBPs. The ligand affinities of the IGFBPs are comparable to or greater than the affinity of the cellular IGF receptors but the affinity of IGFBPs for IGFs can be reduced when IGFBPs are bound to extracellular matrix or when they undergo proteolysis (Spicer, 2004; Clemmons, 2016). Thus, IGFBPs modulate IGF bioactivities both in the circulation and in the extracellular environment (Hwa *et al.,* 1999), regulating the ability of the IGFs to interact with their receptors. IGFBPs 1-6 can both inhibit and potentiate IGF action at the target cells, depending on particular post transductional

changes, including phosphorylation, proteolytic degradation, and binding to the extracellular matrix (Rajaram *et al.,* 1997). All of them are known to inhibit IGF actions (Yu and Rohan, 2000; Puche and Castilla-Cortázar, 2012). Also, IGFBP-1, IGFBP-3 and -5 can potentiate IGF action (Conover, 1992; Baxter, 2000; Firth and Baxter, 2002; Kiepe *et al.,* 2002). In general, the IGFBPs have four essential functions in the regulation of IGF activities: (1) they act as transport proteins in plasma; (2) they leng then the half-lives of IGFs by regulating their metabolic clearance; (3) they provide a means of tissue and cell type-specific targeting and (4) they directly modulate interaction of the IGFs with their receptors and thereby indirectly control their bioreactivity (Silva *et al.,* 2009). However, there is increasing evidence that the IGFBPs possess IGF-1 independent effects (Guler *et al.,* 1989; Zapf *et al.,* 1995; Firth and Baxter, 2002; Clemmons, 2016). In this sense, IGFBP-3 and -5 can induce apoptosis or modulate cell survival in the absence of the ligand (Schedlich *et al.,* 2000; Torng *et al.,* 2009; Beauchamp *et al.,* 2010; Kim *et al.,* 2011; Gullu *et al.,* 2012; Baxter, 2013, 2015), possibly by sequestering extracellular IGF and thereby limiting peptide access to specific cell surface receptors.

IGFBPs for IGF are expressed in different species in specific ovarian cell types. Their expression appears to be independently regulated during the reproductive cycle (Spicer, 2004; Chen *et al.,* 2010). The amount of mRNA for all six IGFBPs in follicles has distinct patterns and these IGFBPs have distinct functions in cattle (Schams *et al.,* 2002; Rodríguez *et al.,* 2013) and anoestrous sheep (Hastie *et al.,* 2004), but they participate as the rest of IGF system the important biological function in follicles such as cellular growth, proliferation, differentiation, survival against apoptosis and migration (Khandwala *et al.,* 2000).The IGFBPs are inhibitory to gonadotrophin-induced follicular growth and differentiation and inhibit the actions of IGF at the level of target cells (Spicer and Echternkamp, 1995). Thus changes in intrafollicular IGFBPs lead to changes in IGF bioavailability and the up or down regulation of gonadotropin actions on follicular cells (Spicer, 2004).

- **IGFBP-1**

IGFBP-1 was the first member of a IGFBPs family to be characterized. This family is involved in modulating the effects of the IGF-1 and 2 whit roles in growth and apoptosis, metabolism and development (Lee *et al.,* 1993). The IGFBP-1 primary amino acid sequence contains 12 N- terminal and 6 C- terminal cysteine residues which are conserved in other mammalian; these cysteine-rich areas are essential for optimal IGFBPs. Availability of IGF-1 in relation to levels of IGFBP-1 (ratio between IGF-1 and

IGFBP-1) may be useful as a marker for biological response of the IGF-1 system in various treatments or also as marker for mature oocytes (i.e. alterations in the levels of IGFBPs may generate the low IGF-1 levels preventing the protein from binding to its receptor and altering the normal development of function) (Kawano *et al.,* 1997; Donaghy, *et al.,*1995). The post-translational modifications can be modificate the phosphorylation of IGFBP-1, for example during fetal stresses (hypoxemia). IGFBP-1 can be increased in a sixfold of its afiniity for IGF-1 resulting in fetal growth restriction (Abu *et al.,* 2014). Serum IGFBP-1 levels seems to be lower in PCOS (Cataldo, 1997).

❖ **IGFBP-2**

The IGFBP-2 was isolated originally from rat liver (Lyons and Smith 1986; Mottola *et al.,* 1986) and their N- terminal amino acid sequence was reported and isolated from adult rat serum (Binkert *et al.,* 1989; Shimonaka *et al.,* 1989) ovine plasm (Walton *et al.,* 1990) and pig ovarian FF (Shimasaki *et al.,* 1990). The IGFBP-2 precursor is 36- kilo daltons (kDa) (328 amino acid residues) in size and consists of three distinct regions: The N-terminal (Amino-terminal) region (amino acid residues 43-136); the C-terminal region (amino acid residues 229-309) (Bunn *et al.,* 2003; Mark *et al.,* 2005). The N-terminal and C-terminal domains are cysteine-rich. The cysteine distribution in IGFBP-2 is highly similar to that of IGFBP-1. Studies have indicated that IGFBP-2 may be important during early stages of follicular development (Giudice, 1992), supporting the role for the IGF system in oocyte maturation. Also IGFBP-2 is expressed in fetal tissues that are highly proliferative as the apical ectodermal ridge and after birth in the glial cells (Fuller *et al.,* 1999; Fukushima and Kataoka, 2007). The expression of IGFBP-2 decreases significantly after birth, but gradually increases again and found in seminal plasma, milk and cerebrospinal fluid (Blum *et al.,* 1993; Schwander and Mary, 1993; van den Beld *et al.,* 2003). Other studies have also shown that IGFBP-2 bind to glycosaminoglycans on the cell surface and extracellular matrix through its heparin-binding domain (HBD) (Russo *et al.,* 2005). IGFBP-2 down regulates phosphatase and PTEN expression, but has not yet been determined the receptor that mediated this process and the signaling elements (Perks *et al.,* 2007).

❖ **IGFBP-3**

IGFBP-3, is the most abundant of the IGFBPs in the circulation in humanand the main IGF transports protein in the blood stream, where it carries the growth factors

predominantly in stable complexes. The IGFBP-3 primary amino acid sequence contains 12 N- terminal and 6 C- terminal cysteine residues. The IGF-1/IGFBP-3 ratio has sometimes been used as an index of IGF bioavailability in the circulation IGFBP-3 is also predominant in FF although, its role in FF has not been clearly demonstrated (Thierry van Dessel *et al.,* 1996). It can modulate the actions of insulin-like growth factors (IGF-1 and -2) also exerts IGF-1 independent actions as suppression of cell proliferation and stimulation of apoptosis in a variety of cell lines (Valentinis *et al.,* 1996; Oh *et al.,* 1995; Lalou *et al.,* 1996; Butt *et al.,* 1999).

IGFBP-3 also interacts with cell-surface proteins, affecting cell signaling from outside the cell or after internallization, and also enters the cell nucleus where it binds to nuclear hormone receptors and other ligands (Martin and Baxter, 1986). Studies had demonstrated that IGFBP-3 also exerts antiproliferative effects in many cell types by blocking the ability of IGF-1 to activate the IGF-1R (which stimulates cell proliferation) (Takaoka *et al.,* 2006). A subsequent study showed that IGFBP-3 can activate the TGF-β1and-β2 receptors, and inhibit the signaling of SMAD (Intracellular proteins) in the TGFβ pathway (Fanayan *et al.,* 2002). Other studies showed that IGFBP-3 could stimulate sphingosine kinase. IGFBP-3 functions through this mechanism to transactivate either the EGF or the IGF receptor, but this mechanism is still unknown. IGFBP-3 also binds to caspase 3 and 8 linked to the cell death (Ingermann *et al.,* 2010).

- **IGFBP-4**

The IGFBP-4 is the smallest among the six IGFBPs and it exists in two forms; non-glycosylated (24 kDa) and N-glycosylated forms (28 kDa) (Durai *et al.,* 2006). IGFBP-4 has two extra cysteine residues (Qin *et al.,* 1998) and three domains: N-terminal, C-terminal and the central domain, of which the N-terminal sequence is important for the binding of IGF (Fernandez-Tornero *et al.,* 2005). IGFBP-4 acts as a transport protein for IGF-1 and IGF-2 and inhibits IGF-1 induced cellular growth both *in vitro* and *in vivo*. IGFBP-4 level and expression in various tissues are influenced by IGFBPs protease, growth factors and hormones (Durai *et al.,* 2006). Other studies have demonstrated that IGFBP-4 has an inhibitory effect on both granulosa and theca cell steroidogenesis (Seppälä *et al.,* 1984). Intrafollicular concentrations of IGFBP-4 strongly decrease during the last steps of folliculogenesis (Monget *et al.,* 1996; Stewart *et al.,* 1996). These changes are mainly due to an increase in proteolytic degradation of IGFBP-4. However, the follicular atresia is associated with the appearance of IGFBP-4 transcripts in rat

granulosa cells (Erickson *et al.,* 1992) and increases in IGFBP-2, -4, and -5 in sheep AF (Besnard *et al.,* 1996).

- **IGFBP-5**

IGFBP-5 is formed by 18 cysteine residues: 12 in the N-terminal and 6 in the C-terminal regions of the protein. Initially was report that IGFBP-5 bound to the TGFβ receptor, later was determined to be a low-density lipoprotein receptor-related protein 1 (LRP-1) (Kalus *et al.,* 1998; Beattie *et al.,* 2006). Studies have demonstrated that IGFBP-5 interacts with IGF-1 and IGF-2. It was initially demonstrated that inhibit IGF function in cell growth, apoptosis, survival, and differentiation (Lee *et al.,* 2016), by blocking the interaction between IGF and the IGF-1R in rat granulosa cells (Kalus *et al.,* 1998). Studies also, demonstrated that IGFBP-5 stimulates markers of bone formation and in the humans, IGFBP-5 mRNAs were detected in liver, kidney and brain tissues and an osteosarcoma (Shimasaki and Ling, 1991).

- **IGFBP-6**

IGFBP-6 consists of 3 domains: The N- and C-terminal domains, which contain internal disulfide bonds, 5 in the N-terminal IGFBPs domain and 3 in the C-terminal domain (Neumann and Bach, 1999; Chandrashekaran *et al.,* 2007). IGFBP-6 expression is regulated by cAMP, IGF, p53, β-catenin, and TGF-β (Bach *et al.,* 2013). Studies hace demonstrated that IGFBP-6 may enter the nucleus and modulate cell survival and differentiation (Bach, 2015). A principal role for IGFBP-6 is inhibition of IGF-2 actions, angiogenesis and promotion of cancer cell migration. This type of IGFBPs has a binding preference for IGF-2 over IGF-1. It is antiproliferative and proapoptotic in a number of cell lines *in vitro* (Bach, 2005; Bach *et al.,* 2013). It has also been reported to have IGF-1 independent actions, such as angiogenesis (Zhang *et al.,* 2011). It has been reported in humans to be a tumor suppressor through regulation of EGR-1 expression (Kuo *et al.,* 2010). IGFBP-6 has been isolated from FF or ovarian tissue. Studies indicate that IGFBPs expression and production in the developing follicle is dependent on both cell type and follicle size and is regulated by IGF-1 and gonadotropins. Singh et al. (2015) describe in buffalo that the expression of IGFBP-6 is greater in preovulatory follicles that other binding proteins.

Regarding, the role of IGF system in pathogenesis of PCOS IGFBPs concentrations are decreased, thus it will be more active IGF-1 that induces LH receptors and activation of

P450 which leads to hyperandrogenism due to the excess of androgen produced in the theca cells (Guidice, 1995; Homburg, 1998).

1.3.2.2. Follicular atresia

Apoptosis or programmed cell death is cell self-destruction under physiological control (Ameisen *et al.,* 2002). It is a complex process that involves a variety of different signaling pathways and regulatory networks caused by extrinsic and intrinsic factors (Quirk *et al.,* 2006; Sirotkin *et al.,* 2010) and give as results multitude of morphological, histological and biochemical changes in the dying cell. The cell undergoes shrinkage, chromatin margination, nuclear condensation, segmentation, and division into apoptotic bodies that may be phagocytosed (Hussein, 2004; Matsuda-Minehata *et al.,* 2006). This event plays an important role in the normal function of all tissues including the follicle (Amsterdam *et al.,* 2003). In mammalian and human follicular development, apoptosis can be initiated in different follicular stages and cell compartments, i.e., theca and granulosa cells (Sharma, 2003; Sharma and Bhardwaj, 2007, 2009), CCs, the oocyte itself (Hussein, 2004) and *corpus luteum* (Sharma and Batra, 2005). However, mainly apoptosis signals in the follicle are seen in granulosa cells.

Many studies have shown that apoptosis in granulosa and germ cells of diverse species is precisely regulated by a cohort of intracellular signals and gene products conserved through evolution (Tilly, 1996; Tilly *et al.,* 1997; Quirck *et al.,* 2006). The complex range of molecular mechanisms (Hussein, 2004; Manabe *et al.,* 2004; Inoue *et al.,* 2011) and signaling pathways are mainly regulated bya type of non-coding RNA (Ribonucleic Acid) molecule microRNAs (miRNA) (Liu *et al.,* 2014; Nie *et al.,* 2015; Xiong *et al.,*2016; Worku *et al.,* 2017). Some miRNAs regulating apoptosis in granulosa cells are miR-22, let-7 family (lethal-7), miR-26b. Also, apoptosis is activated by the binding of the death receptors (Fas, tumour necrosis factor receptor (TNFR), interferon (IFN) and TNF-related apoptosis-inducing Ligand (TRAIL) receptors) to their ligands. The expression of these proteins is closely linked with gonadotrophin levels. Low levels of gonadotrophin are associated with disappearance of FasL/Fas and p53 (in the granulosa cells) (Hu *et al.,* 2001). The FasL-Fas signaling pathway in the granulosa cells can be suppressed by IGF-1 or EGF mediated by the PI3K/Akt pathway (Quirk *et al.,* 2000; Hu *et al.,* 2001). It also modulates the apoptotic role of miR-23a and -27a (Nie *et al.,* 2015). Similarly, the TGF-β1 signaling pathway has been found to be involved in granulosa cells apoptosis and follicular atresia by down regulating miR-26b, in which case SMAD4 is the target gene (Liu *et al.,* 2014). Finally, several intracellular molecules as: *B-cell lymphoma 2*

(Bcl-2), Bax and caspases (Kim and Tilly, 2004) can regulate this process. Bcl-2 (pro-survival factor) is found in the developing follicles while Bax (pro-apoptotic) is seen in the atretic follicles (Hussein, 2004). This expression is related to gonadotrophin levels again; higher levels of gonadotrophins increase the expression of Bcl-2 and decrease the expression of Bax (Sugino *et al.,* 2000). Caspases are the main effector molecules in ovarian apoptosis. They are activated by cell surface receptors or members of the Bcl-2 family of proteins. In the ovary, caspase- 3 is expressed in luteal and thecal cells of healthy *corpus luteum* as well as in the granulosa cells of follicles. This expression is regulated by gonadotrophin and may be altered as part of the apoptotic process in the granulosa cells (Hussein, 2004).

In guinea pig, various factors can influence follicular atresia as age, nutrition, X-irradiation (Jacquet *et al.,* 2001) and phases of the ovarian cycle. During the second half of the ovarian cycle, the atresia can occur in the early stages of development of follicles as a process of degeneration of the granulosa cells and phenotypic transformation of theca cells (Logothetopoulos *et al.,* 1995; Sun *et al.,* 2014).

1.3.3. Oocyte maturation

The events that arise during oocyte maturation depend not only on the correct dynamics of chromosome separation during nuclear maturation (known as nuclear maturation) but also to the redistribution of cytoplasmic organelles and the previous storage of mRNA proteins, and transcription factors during oogenesis (known as citoplasmic maturation). Therefore, nuclear maturation events are accompained by cytoplasmic maturation processes. A complete nuclear maturation does not guarantee the complete cytoplasmic maturation. Then, when oocyte has not completed cytoplasmic maturation it shows poor quality and is unable to successfully complete normal developmental progression (Krisher, 2004). Therefore, both processes are essential to obtain a competent oocyte. Changes to nuclear and cytoplasmic level are also dependent on maternal genetic background and may be responsible for maternal effects on de-novo methylation, gene expression and congenital aberrations (Picard *et al.,* 2001). Selection of good quality oocytes is particularly important for the improvement of assisted reproduction techniques (Marteil *et al.,* 2009).

Estrogen act at the oocyte surface by producing changes in reactivity of Ca^{2+} that play an important role in intracellular signaling. It is essential for oocyte activation during fertilization including the block to polyspermy and the completion of meiosis, cortical

granule exocytosis and the transition into the embryonic mitotic cell cycle (Machaca, 2007; Ullah *et al.,* 2008; Ferreira *et al.,* 2009). In immature oocytes Ca^{2+} release is activated by protein kinase C (Tesarik and Mendoza, 1995). Only when the oocyte grows, it becomes competent and need to release cortical granules the oocyte first generates spontaneous Ca^{2+} transients (Carroll and Swann, 1992; Carroll *et al.,* 1994) and the ability to generate Ca^{2+}, is acquired in the oocyte at metaphase II (M-II), since it is possible that in this phase that Ca^{2+} channels are regulated by mitotic cell cycle control proteins or by factors released from the nucleus that control Ca^{2+} release during mitosis (Kono *et al.,* 1995; Carroll *et al.,* 1996; Ullah *et al.,* 2008).

1.3.3.1. Nuclear maturation

The MI which consists of prophase I, metaphase I, anaphase I and telophase I are denominate “reductional” because it results in daughter cells that contain only half of the number of chromosomes that existed in the originating parent cell. In this process, homologous chromosomes pair during prophase I are separated at metaphase I, resulting in the formation of two daughter cells each containing just one homologous chromosome. Homologous chromosomes come together and pair in a process known as synapsis and then exchange genetic information through crossing over by recombination (Tankimanova *et al.,* 2004). Synapsis and recombination events are necessary to keep homologous chromosomes tightly associated with each other to be equally segregated to daughter cells. Daughter cells now consist of paired chromatids (Storlazzi *et al.,* 2003) and must then enter MII that consisting of prophase II, M-II, anaphase II, and telophase II (Figure 7) (Cohen and Holloway, 2015). This division is called “equational” because overall number of chromosomes in the daughter cells is the same as in the parental cell at the end of MI (Cohen and Holloway, 2015).

Regarding to metaphase I, the first substage of prophase I is known as leptonema. This occurs immediately after DNA replication and involves the initial assembly of a proteinic structure, termed the synaptonemal complex. This complex begins to form a scaffold along each homologous chromosome. The second substage of prophase I, termed zygonema, consists of pairing of homologous chromosomes chromatids; then occurs the synapse (Liu and Keefe, 2008). Simultaneously, the DNA begins to condense, making the chromosomes more visible cytologically. Once the homologs have been completed the synapsis, the cell enters the third substage of prophase I, known as pachynema. Now the chromosomes are fully condensed and have a short, flat cytological appearance. After pachynema, the central constituent of the synaptonemal complex

break down, and the chromosomes keep away each other (Kouznetsova *et al.,* 2011). This fourth substage is known as diplonema, when the two homologs become clearly visible again. The homologs remain joined at the location of meiotic crossover, called chiasmata even if the central element was broken down and the synaptonemal complex disassembled. The fifth and final substage of prophase I is diakinesis which means “moving through”. At this point, the homologs are connected only by the remnants of the synaptonemal complex of the centromeres and at the crossover sites (Cohen and Holloway, 2015).

During MI, the chiasmata form the tension necessary to ensure stable the microtubules which maintain homolog interactions. In following stage, Anaphase I, chromosomes containing a pair of chromatids joined solely by cohesin at the centromeres (Liu and Keefe, 2008), are separated to the opposite poles. Then in telophase I, a clearly asymmetric division is produced and a rudimentary structure called the first polar body, which contains the excess of chromosomes, is formed and extruded to the periphery. There is no intervening S-phase before entry into MII, and oocytes progress to M-II rapidly, only to arrest again under the influence of cytostatic factor (CSF). It induces stabilization and reaccumulation of meiosis promoting factor (MPF). (Rieder *et al.,* 1994; Lara-Gonzalez *et al.,* 2012). Inhibition of anaphase promoting complex (APC/C) action avoid CSF binding to MPF (Hunt *et al.,* 2012) resulting in the second meiotic arrest at M-II. MII resumption only occurs upon fertilization by the influx of Ca^{2+} ions triggered by sperm penetration (Madgwick and Jones, 2007).

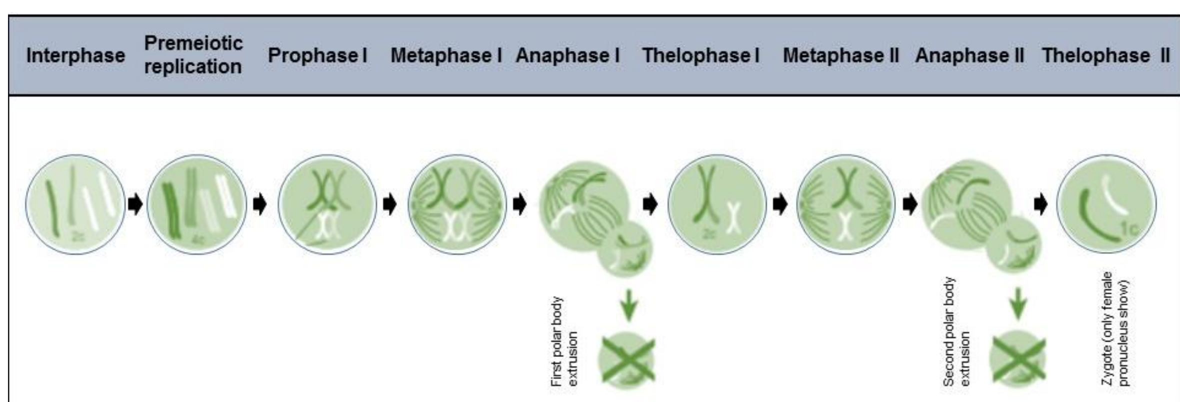

Figure 7. Meiotic division. Adapted from Cohen and Holloway, 2015

Nuclear maturation covers chromatin changes during oocyte maturation from GVBD throughout MI to MII. Morphologically the nuclear stages displayed during oocyte meiotic maturation (Marteil *et al.,* 2009; Li and Albertini, 2013) are:

i. **GV (germinal vesicle):** the nucleus of the oocyte is arrested in prophase of meiosis with a nucleus surrounded by a nuclear membrane wherein the nucleus is characterized by the presence of chromatin filaments.

ii. **GVBD**: In this stage the nuclear envelope of the GV will break down in a process known as GVBD, when the oocyte resumes meiosis in response to the LH surge that occurs prior to ovulation.

iii. **MI (Meiosis I):** The first meiotic division leads thanks to crossing-over to the formation of daughter cells with distinct genetical compositions.

❖ **Prophase I**: Chromosomes start to condense, crossing-over between pairs of homologous chromosomesand form chiasmata.

❖ **Metaphase I:** Homologous pairs of chromosomes (bivalents) arranged as a double row along the metaphase plate.

❖ **Anaphase I:** The homologous chromosomes in each bivalent pair are separated and move to the opposite poles of the cell.

❖ **Telophase I**: The chromosomes become diffuse and the nuclear membrane reforms.

iv. **MII (Meiosis II):** An haploid number of chromosomes condense again. The nuclear membrane and nuclei break up while the spindle network appears. The chromosomes move again to the equatorial plane. The sister chromatids are pulled apart to opposite poles by the spindle and begin moving to opposite ends (poles) of the cell. A nuclear envelope is formed around each nucleus. Distinct nuclei form at the opposite poles and cytokinesis occurs. At the end of M-II, each cell has one half the numbers of chromosomes (Marteil *et al.,* 2009).

The resumption of meiosis is marked by the breakage of the nuclear membrane VG, the disappearance of nucleolus, formation of meiotic spindle and chromatin condensation in homologous chromosomes reaching metaphase I, then the pairs of chromosomes are separated homologues and progresses to metaphase of the second meiotic division (M-II) accompanied by extrusion of the first polar body. The oocyte remains M-II stage in the oviduct until fertilization. At that time occurring meiosis complete extrusion of the second polar body (Figure 8) which is located between oocyte cell membrane and perivitelline space.

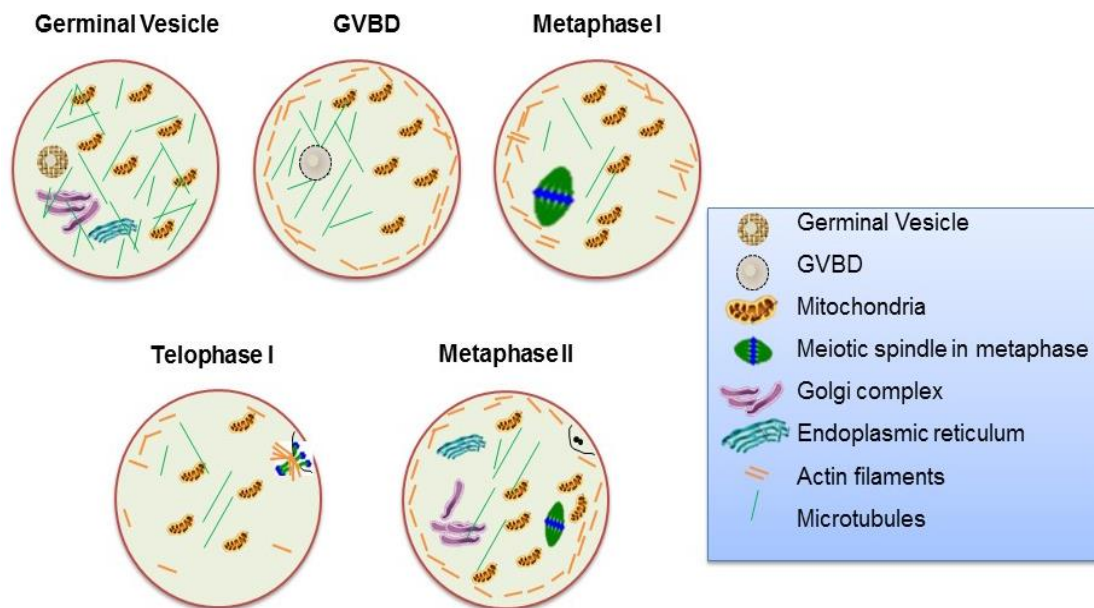

Figure 8. Schematic overview of the nuclear maturation. Redrawed figure adapted from Ferreira *et al.* (2009)

As it has been indicated before, the beginning of oocyte maturation is determined by influence of LH in the granulosa cells (Eppig *et al.,* 2004; Norris *et al.,* 2010). The presence of gap junctions between granulosa cells and oocyte provide the cGMP necesary to keep the meiotic arrest after entering the oocyte (Norris *et al.,* 2009). The process by which LH activates the pathways that reduce cGMP is not known. cAMP is synthesized by follicular somatic cells and is transported into the oocyte also through gap junctions (Adhikari and Liu, 2014). Then, LH increase provokes activation of cAMP and cGMP lowering in the COCs. Oocytes arrest under the influence of CSF. Activity of CSF induces stabilization and reaccumulation of MPF, which keeps the prophase I arrest in the oocyte. MPF is a complex of a catalytic kinase, consisting of the regulatory subunit

cyclin B1 and thecatalytic subunit p34cdc2 (cyclin dependent kinase 1 (CDK1) or Cdc2) and CDK1 in the nucleus. It regulates the passage of prophase to-metaphase restarting the meiosis in immature oocytes (Brunet and Maro, 2005; Santamaria *et al.,* 2007; Oh *et al.,* 2010; Adhikari *et al.,* 2012).

1.3.3.2. Cytoplasmic maturation

During cytoplasmic maturation fully grown oocytes undergo structural and functional modifications that allow them to be fertilized and to continue embryo development (Duranthon and Renard, 2001; Sirard *et al.,*2006). Some of these changes include the redistribution of cell organelles like cortical granules, mitochondria, golgi apparatus and endoplasmic reticulum and the ability to undergo cortical granules exocytosis (Ducibella *et al.,*1993; Yoshida *et al.,* 1993). Trafficking of cytoplasmic organelles during maturation occurs through the actions of cytoskeletal microfilaments and microtubules and repositioning of the organelles depends on the needs of the cell during each stage of development (Ferreira *et al.,* 2009). The microtubules of cytoskeleton play an important role in the separation of chromosomes, extrusion of the first and second polar body and migration of cortical granules (Adams and Hertig, 1966; Adona *et al.,* 2008). Although migration of cortical granules and mitochondria has been assessed as an indicator of cytoplasmic maturation, the ultimate sign of cytoplasmic maturation is the ability of the oocyte to undergo fertilization (Ducibella *et al.,* 1988; Ferreira *et al.,* 2009). Other morphological changes include cumulus cell expansion, expulsion of the polar body and the increase in the perivitelline space (Motlik *et al.,* 1990; Kastrop *et al.,* 1991).

In addition, mRNA transcription (Kastrop *et al.,* 1991; Farin and Yang, 1992), protein translation (Sirard *et al.,* 1989) and post-translational modification of proteins (Levesque and Sirard, 1995; Chian *et al.,* 2004) take place.

i. Cortical granules migration

Cortical granules are organelles exclusively found in oocytes and their composition includes a diverse population of proteins, structural molecules, enzymes and glycosaminoglycans. Cortical granules are derived from the Golgi complex (Wessel *et al.,* 2002) and endoplasmic reticulum (Lucidi *et al.,* 2003) in a continuous process until

ovulation. These are considered vesicles that once released its contents after the cortical reaction, they do not re-synthesized.

They are considered the most important organelles in the cytoplasm to prevent polispermia. They increase during early stages of maturation synchronously with the increased size of the oocyte. In nuclearly mature oocytes cortical granules migrate to the periphery (Figure 9), through its binding to anchoring proteins to the cytoskeleton and are located in the arranged cortical cytoplasm monolayer just below the oolema (Wessel *et al.,* 2002), close to their site of action (the ZP) but their translocation is necessary for fertilization. All these modifications establish an extracellular blocking to polyspermy (Hosoe and Shioya, 1997). A failure during oocyte maturation causes the impossibility of correct fertilization and inactivating specific receptors that recognize and bind to spermatozoa (Ducibella *et al.,* 1994). In many vertebrate and invertebrate species, exocytosis of cortical granules after spermatozoa penetration into the oocyte triggers a rapid modification of extracellular matrix of oocytes because cortical granules are not produced at the site of their final localization (Ducibella *et al.,* 1994; Wessel *et al.,* 2002).

Cortical granules distribution have been used as a reliable indicator for the cytoplasmic maturation of oocytes (Damiani *et al.,* 1995). For oocytes in the GV stage, cortical granules are distributed throughout the cytoplasm (Hosoe and Shioya, 1997). Partially matured oocytes show cortical granules localized in the ooplasm and distributed as individual particles as well as small aggregates (Bevers and Izadyar, 2002). At the end of the maturation period (M-II) the granules are distributed more or less dispersed in the cortical ooplasm aligning the oolemma (Conner *et al.,* 1997; Wang *et al.,* 1997). During oocyte maturation a reorganization of cortical granules is observed in various species. In immature mouse and hamsters oocytes, cortical granules are found in the whole cytoplasm (the cortex cytoplasm and the inner cytoplasm) (Ducibella *et al.,* 1988). In matured porcine and bovine oocytes showed asymmetric distribution of cortical granules (Hosoe and Shioya, 1997, Wang *et al.,* 1997).

ii. Mitochondrial distribution

Mitochondria and their distribution play an extremely important role since they are a key component of the metabolic machinery responsible for the supply of energy required for the maturation process, chromosomal condensation and segregation (Calarco, 1995; Liu *et al.,* 2001; Brevini *et al.,* 2005; Nagai *et al.,* 2006; Shoubridge and Wai, 2007; Yu *et al.,*

2010), spindle formation and first polar body extrusion (Krisher and Bavister, 1998; Stojkovic *et al.,* 2001) in the oocyte as has observed in several species as: mouse (Nagai *et al.,* 2006; Yu *et al.,* 2010), pig (Sun *et al.,*2001; Torner *et al.,* 2004; Brevini *et al.,* 2005), cow (Krisher and Bavister, 1998; Stojkovic *et al.,* 2001; Ferreira *et al.,* 2009), dog (Valentini *et al.,*2010; De los Reyes *et al.,* 2011), hamster (Barnett *et al.,* 1996) and human (Van Blerkom, 2004; Dell´Aquila *et al.,* 2009; Liu *et al.,* 2010). Mitochondria produce most of the ATP by oxidative phosphorylation (Stojkovic *et al.,* 2001) and may serve to supply energy directly to the nucleus. During oocyte maturation, breakdown of the nuclear membrane requires elevated ATP levels (Yu *et al.,* 2010). Also, the mitochondria around the nuclei might serve to reduce the O_2 concentration around the nucleus, thus playing a role in protection from O_2 radicals (Dumollard *et al.,* 2007). For these reason, mitochondria density increases at specific stages of oocyte maturation accompanied by concomitant focal bursts of ATP production (Yu *et al.,* 2010). Number of mitochondria presents in the cytoplasm of mammalian oocytes varies according to the stage of development of the cell (Krisher and Bavister, 1998). During the pre-migratory stage of germ cells, the number of mitochondria increasing in the oogonium stage. The number of mitochondria in primary oocytes increases during oocyte maturation (Cummins, 2001; Shoubridge and Wai, 2007).

On the other hand, mithocondria is reallocated to supply the high demand for ATP and Ca^{2+} needed during cytoplasmic maturation (Van Blerkom and Runner, 1984; Calarco, 1995). A translocation of mitochondria to the perinuclear region is observed in the GVBD in mouse (Van Blerkom and Runner, 1984; Calarco, 1995; Nishi *et al.,*2003), bovine (Stojkovic *et al.,* 2001) and pig (Sun *et al.,* 2001). The aggregation of mitochondria around the nucleus is correlated with the acquisition of maturation and developmental competences (Nishi *et al.,* 2003; Nagai *et al.,* 2006). The abnormal distribution of mitochondria is related to the inappropriate formation of the cytoplasmic microtubule network, which can lead to the retardation or arrest of oocyte development due to the abnormal ATP distribution (Nagai *et al.,* 2006).

In addition, mithocondria are important in regulating apoptosis (Torner *et al.,* 2004), Ca^{2+} signaling (Krisher, 2004; Dumollard *et al.,* 2007), reactive oxygen species, and production of intermediary metabolites (Liu and Keefe, 2008, Liu *et al.,* 2000; Dumollard *et al.,* 2003).

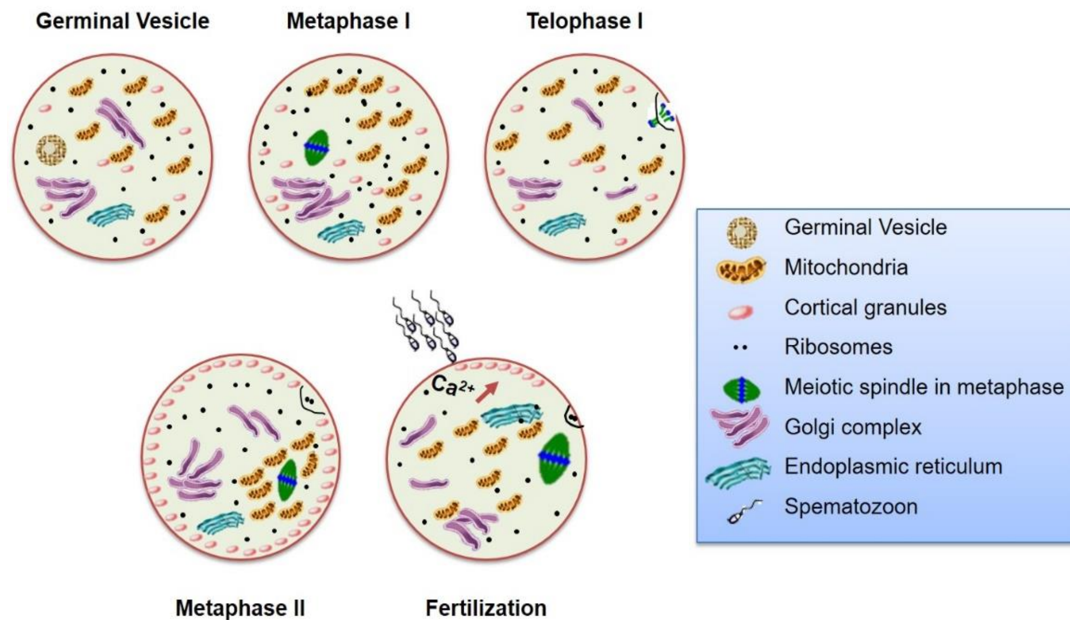

Figure 9. Schematic overview of the distribution of cytoplasmic organelles during maturation and fertilization. Redrawed figure adapted from Ferreira *et al.* (2009).

1.3.4. Ovulation and *corpus luteum* formation

Ovulation is a biological process that is regulated by intraovaric connections between theca, granulosa, CCs and the oocyte. It depends upon specific cellular and molecular events that occur sequentially during follicular development and is a slow extrusion process that occurs by degradation of the follicle wall (Richards, 2007; Richards *et al.,* 2015). Before the ovulation there is an increase of gonadotropins that promove production of hyaluronic acid that binds CCs and expands spaces between the cells (Eppig, 2001). In addition, a normal ovulation requires prostaglandins (PG) production y GDF9 (Elvin *et al.,* 1999) and is induced by the LH. After the ovulation, the CCs continue associated with the oocyte, facilitating the capture of the COCs by the ciliated cells of the infundibulum and its transport along the oviduct (Lam *et al.,* 2000; Tanghe *et al.,* 2002; Kôlle *et al.,* 2009). On the ovarian surface, the ovulation involves the dissolution of collagen fibers in theca layer; the PG and cytokines permit the release of the expanded COCs. The COCs are then picked up by the ciliated cells of the fimbria and transported to the oviduct, where fertilization occurs. If the oocyte is not released before to luteinization it will be trapped in the *corpus luteum* (Richards *et al.,* 2015).

Luteinization begins prior to ovulation and continues until the fully *corpus luteum* are formed. It involves the proliferation and differentiation cellular (Murphy, 2004). Some morphological events must occur: breakdown of the basement membrane separating the granulosa and theca layers, invasion of microvascular cells into the granulosa layer and theca cells are transported toward the center of the follicle cavity (Redmer *et al.,* 2001). In many species, including rodents theca-derived luteal cells appear to disperse throughout the parenchyma of the *corpus luteum* (Stocco *et al.,* 2007). However, the fate of theca cells may depend upon their differentiated state or proximity to luteinizing of granulosa cells at ovulation (Stouffer and Hennebold, 2014). In addition, a volume of granulosa cells increases of up to 10-fold by hypertrophy (Smith *et al.,* 1994), gap junctions between granulosa and theca cells disappear and changes in the cytoskeleton play an important role in optimizing cell size (Stouffer and Hennebold, 2014). Also changes in the extracellular matrix (ECM) and integrins and serine proteases ((e.g. matrix metalloproteinases (MMPs)) (Liu *et al.,* 1997), appear to be critical in the *corpus luteum* development. The granulosa and theca cells which initially produced estradiol-17β, are differentiate into luteal cells that produce P_4 after the LH surge (Reynolds *et al.,* 2000). The principal function of *corpus luteum* is to synthesize and secrete steroid and P_4 to permit implantation of the embryo in the endometrium and maintenance of pregnancy in mammals (Reynolds and Redmer, 1999). In some species the luteinization may occur in response to pregnancy and the angiogenic process in the *corpus luteum* is remarkable (Murphy, 2004; Stouffer and Hennebold, 2014).

Corpus luteum function is controlled by several hormones secreted by the pituitary, decidua and placenta (e.g. prolactin (PRL) and GH have crucial role for *corpus luteum* development (Risk and Gibori, 2001; Bachelot and Binart, 2005 a, b). However, *corpus luteum* has some level of autonomy by its own P_4 production and the PG, oxytocin and noradrenaline play a role in regulation of P_4 synthesis (Devoto *et al.,* 2009) that protect these cells from apoptosis (Kliem *et al.,* 2009). miRNAs may be regulators of ovarian function, including the follicular-luteal transition (Donadeu *et al.,* 2012; McBride *et al.,* 2012) and also have demonstrated an interaction between the Notch signalling pathway and P_4 that maintains the functionality of the *corpus luteum* (Sugino *et al.,* 2005). *In vitro* Notch induces P_4 production through the activation of CYP P450 cholesterol side chain cleavage enzyme (P450scc). Also the P_4 might be interacting with the Notch/PI3K/Akt signalling pathway for the survival of luteal cells (Stouffer and Hennebold, 2014).

In absence of uterine and embryonic signals, P_4 production GnRH and VEGF (molecules that promote endothelial cell survival and blood vessels are diminished) decreases (Fraser *et al.,* 2005), Prostaglandin F_2 alpha ($PGF_{2\alpha}$) induces a decrease in secretion of P_4, blood flow and induces DNA fragmentation and apoptosis by effects of the protein kinase (PKC) system (Davis and Rueda, 2002; Stouffer and Hennebold, 2014). PGF2a decreases luteal concentrations of mRNA encoding receptor for LH, low-density lipoprotein (LDL) and StAR. Finally, lipid peroxidation (induces membrane damage) and the loss of gonadotropin receptors contributing *corpus luteum* regression and the decrease of steroidogenic capacity.

Guinea pig show spontaneous ovulation that occurs late in the period of estrus or shortly after its end (Blandau and Young, 1939). Rowlands in 1957 shows that ovulation in the guinea pig occurs about 12 h after the onset of mating behavior. Ovulation usually takes place after the opening of the vaginal membrane for more than 1 day and always before the membrane is regenerated. On average, only 3 or 4 matured follicles ovulate. If fertilization does not occur, 13 days after ovulation the level of P_4 decreases and FSH secretion by theca cells increase (Reed and Hounslow, 1971). The formation of a functional *corpus luteum* is the most outstanding feature that differentiates the guinea pig estrous cycle from that of the rat or hamster. Its luteal cells develop from the follicular cells surrounding the ovarian follicle after the ovulation process. The theca cells luteinize into small luteal cells (thecal-lutein cells) and granulosa cells luteinize into large luteal cells (granulosa-lutein cells). In guinea pigs, luteal cells secrete P_4 that begins around day 10 post ovulation and reaches a peak on day 15 or 16, in the proestrus period (Joshi *et al.,* 1973; Sadeu *et al.,* 2007; Gregoire *et al.,* 2012). In addition, in other studies has been found that the P_4 concentrations increase in the ovaries, placenta and uterus, becoming an essential factor to maintain the early embryo development and gestation (Heap and Deanesly, 1966; Csapo *et al.,* 1981; Sun *et al.,* 2014). Several studies have proposed that FSH, estrogen and prostaglandin F25 can be an essential component of luteotropic complex in the guinea pig (Choudary and Greenwald, 1969; Blatchley *et al.,* 1971; Grégoire *et al.,* 2012).

1.3.5. Preimplantation embryo development

Preimplantation embryonic development is a complex series of steps that begins just after fertilization when the zygote is formed and finalizes in the blastocyst stage, before

implantation. This period takes place in the guinea pig 6 to 7 days as it showed in the Figure 10.

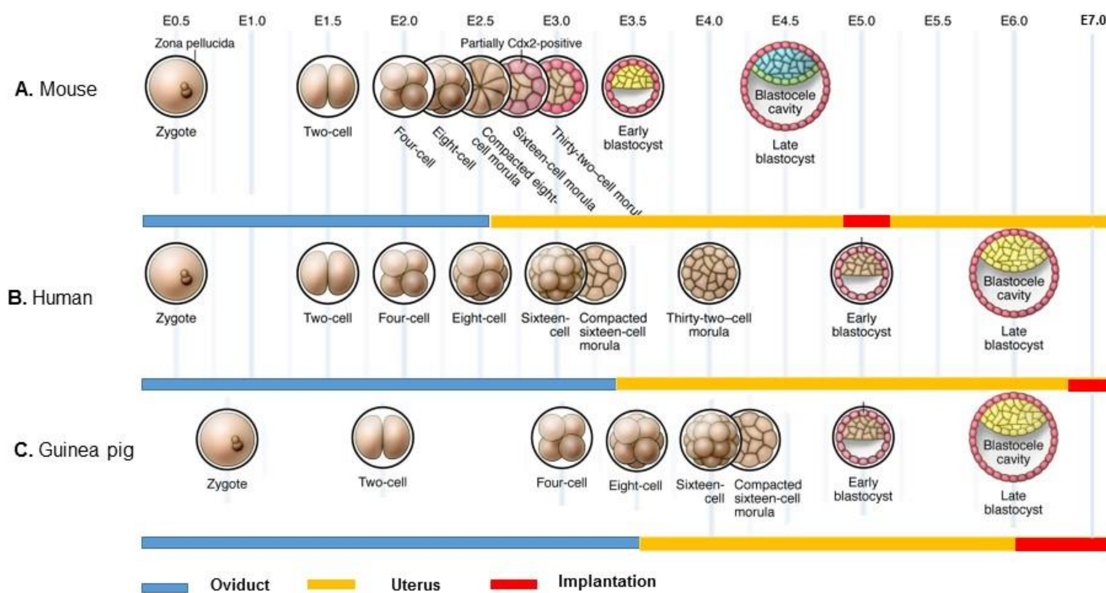

Figure 10. Preimplantation embryonic development in guinea pig compared to mouse, and human. Day 0.5 to 1: zygote; Day 1.5 to 2: 2 cell embryo; Day 3 to 3.5: 4 to 8-cell embryos; Day 4: 16 to-32-cells embryo and compact morula; Day 5: early blastocyst; Day 6: late blastocysts. Figured adapted from Cockburm and Rossante (2010) with guinea pig data recovered from several authors (Blandau, 1949; Hunter *et al.,* 1969; Dorsch *et al.,* 2008).

The fertilized oocyte is called zygote and the male and female pronucleus are identified at the pole of the egg opposite the place of expulsion of the polar bodies. Blastomeres start to divide evenly and after cleavage division, embryos reach 16-cells stage where an increase in intercellular adhesion (compactation) takes place. This event is associated with the formation of gap junctions; E-cadherin becomes localized in regions of cell-cell contact (Cockburn and Rossant, 2010), as well as β-catenin and Rho family GTPases (Natale and Watson, 2002). From the 32-cell stage, cells on the outside of the embryo form the trophectoderm (TE), while inner-cells contribute to the formation of inner cell mass (ICM). In the TE the polarity and transcription factor caudal type homeobox2 (Cdx2) (Cdx2mRNA) are important for determining cell fate (Jedrusik *et al.,* 2008), whilethe pluripotency markers Oct4 (Octamer-binding transcription factor), Nanog and SRYbox (Sox2) are involved inestablishing the ICM fate. When the TE is formed a fluid-filled cavity known as the blastocele also begins to form. The blastocele is essential for

development of the ICM. During blastocele formation water movement is facilitated by aquaporins, which are present in the TE (Watson and Barcroft, 2001). Finally, with the formation of the blastocele the embryo is considered a blastocyst. In rodents normal embryonic development to the blastocyst stage requires the ovarian estrogen and P_4 (Tazuke and Giudice, 1996; Dey *et al.,* 2004).

In guinea pig, blastocyst reaches the uterine cavity and attaches to the endometrium at Day 6 -7 p.c. Penetration of the zona pellucida is performed by projections of the embryonic trophoblastic cells is one of the characteristic of implanting guinea-pig embryos (Hunter *et al.,* 1969; Suzuki *et al.,* 1993). In order to complete this process, there is an embryonic-maternal dialogue, in which the embryo and the endometrium induce changes in each other to promote embryonic development and endometrial receptivity (Tazuke and Guidice, 1996). Studies in rodents have demonstrated that this process is controlled by the action of hormones (estrogen and P_4), cytokines and growth factors as EGF and IGF-1, glycoproteins and carbohydrate ligands (that are expressed in the uterine luminal epithelium and blastocyst cell surfaces), primary adhesion molecules (selectins, heparan sulfate, proteoglycans, Muc-1, integrins and cadherins) (Kimber and Spanswick, 2000; Dey *et al.,* 2004; Cockburn and Rossant, 2017) These factors mediate interactions between trophoblast with the ECM (Dey *et al.,* 2004; Eftekhari-Yazdi *et al.,* 2006; Ramathal *et al.,* 2014), allow the initial attachment phase of implantation, bind to specific cell surface receptors, resulting in cellular mitosis or differentiation by autocrine, paracrine, juxtacrine, or endocrine mechanisms. All these effector molecules participate in the attachment and invasion phase (Tazuke and Giudice, 1996) that activate some signaling pathways as COX-2, Hippo and TGFβ superfamily (TGFβ/activin/nodal) among other, that are essential for controlling celll fate during the embryo development and implantation. Finally, early stages of placental and yolk sac development involve some mechanisms specific to caviomorph rodents (e.g. yolk sac involution, development of a subplacenta) (Kaufmann and Davidoff, 1977; Uhlendorf and Kaufmann, 1979).

1.4. SYSTEMS OF OOCYTE MATURATION *IN VITRO*

1.4.1. Definition and importance of *in vitro* maturation (IVM) in ARTs

In vitro maturation (IVM) of mammalian oocytes is an efficient method to produce II-mature oocytes for their use in the ARTs (Mahmoudi *et al.,* 2005). It consists in maturing oocytes from ovarian follicles in culture. Although this technique was described in the

early past century (Pincus and Enzman, 1934), efficiency is satisfactory only in some species but not in others. Indeed, in guinea pig scarce studies in this area has been developed.

IVM has been proposed as an alternative ARTs and it is the most important step before successful monospermic fertilization and proper embryo growth. In contrast to standard techniques usually employed in human as *in vitro* fertilization (IVF) or intracytoplasmatic inyection of sperm (ICSI), the IVM is a technique that avoids hormonal stimulation because immature oocytes will be matured *in vitro* after collection (Strowitzki, 2013). In animals, use of ARTs allows accelerate genetic improvement toward type of production and permit maximizing the benefits of outstanding individuals with reproductive problems (Hafez and Hafez, 2013). In laboratory animals reduce the number of animals required in the lab and subjected to hormonal treatments to obtain MII-oocytes *in vivo* by replacing them with *in vitro* alternatives. Then, IVM is a useful tool in ARTs in both human and animals so studies in animal models like guinea pig could be a remarkable approach to improve IVM system. Thus, the guinea pig is a valuable laboratory animal model for reproductive studies; however, female produces 2 to 4 oocytes in each ovarian cycle (Logothetopoulos *et al.,* 1995; Harkness *et al.,* 2016) and shows little ovarian response to exogenous FSH and equine chorionic gonadotrophin (eCG) (Reed and Houstaw, 1971). Superovulation was scarcely studied in this species (Shi *et al.,* 2000; Dorsh *et al.,* 2008) so an IVM system could be an alternative method to the use of superovulation protocols in assisted ARTs.

1.4.2. IVM system

1.4.2.1. Ovary collection and oocyte selection

IVM consist of several steps. The first one is the collection of ovaries and selection of oocytes. The availability of enough number of oocytes is the pre-requisite to any investigation for the development and optimization of reproduction techniques. Ovaries of the slaughtered animals are the cheapest and the most abundant source of primary oocytes for large scale production of embryos through IVM and IVF (Agrawal *et al.,* 1995). Transport may be performed in physiological solution or phosphate-buffered saline (PBS) with or without antibiotics at anuniformily temperature above 30°C. The shelf-life of the collected ovaries should not exceed 5 h as it could diminish the developmental capacity and viability of the oocytes. Moreover, an important aim of an oocyte recovery method is to maximize the total number of oocytes and the yield of high-

quality oocytes recovered per ovary, which can be used for *in vitro* technologies (Shirazi *et al.,* 2005). However, no enough reports evaluating the relative efficacy of the methods have been published in the guinea pig. The development of an efficient oocyte collection procedure is, therefore, very significant.

The oocyte collection methods actuality used are slicing, puncture and aspiration. In the slicing method, the whole ovarian is chopped into small pieces with a surgical blade. In the puncture method, the whole ovarian surface is punctured by needle. In aspiration methods, the oocytes are collected by aspirating from the visible follicles using diameters different needle attached to a syringe or using a constant negative pressure with a vacuum pump (Wang *et al.,* 2007) depending each species. In any case, a close association between CCs and the oocyte is crucial in the production of developmentally competent IVM mammalian oocytes (Zuelke and Brackett, 1990; Saeki *et al.,*1991; Kidder and Mhawi, 2002; Gittens *et al.,* 2003).

On a routine basis, oocyte quality is evaluated immediately after recovery by non-invasive visual assessment of the morphology of COCs. Homogeneity of the ooplasm and number of compact granulosa layers surrounding the oocyte (so-called CCs) are assessed, enabling the elimination of oocytes in which maturation has initiated *in vivo* or those that have any signs of atresia in either the oocyte cytoplasm or CCs (Wasielak and Bagacki, 2007). For the IVM process COCs are classified in different categories based on the following criteria according to De Loos *et al.,* (1989) (Table1):

Table 1. Morphological classification of COCs. By: De Loos *et al.* (1989)

Category	COCs
1	- Compact multilayered cumulus investment - Homogeneous ooplasm - Total COCs light and transparent
2	- Compact multilayered cumulus investment - Homogeneous ooplasm but with a coarse appearance, and a darker zone at the periphery of the oocyte - Total COCs slightly darker and less transparent
3	- Less compact cumulus investment - Ooplasm irregular with dark clusters - Total COCs darker than categories 1 and 2
4	- Expanded cumulus investment - Cumulus cells scattered in dark clumps in a jelly matrix - Ooplasm irregular with dark clusters - Total COCs dark and irregular

1.4.2.2. Maturation media

Choosing a specific medium for IVM of immature oocytes was derived by adapting methods developed from culturing other cell types and mimicking the composition of ovarian microenvironment of the enclosed oocyte in the follicle. Complex culture media such as tissue culture medium 199 (TCM-199), Ham's-F10 and Whitten's medium buffered with bicarbonate or 4-(2-Hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) among others has been used in rodent IVM culture. Numerous supplements can be added (Bevers *et al.,* 1997; Trounson *et al.,* 1998) to provide the necessary nutrients for the process of oocytes maturation.

The biophysical parameters must be controlled in the culture media as pH (potential hydrogen), carbon dioxide (CO_2) and osmolarity. Most *in vitro* culture media develops a neutral or slightly alkaline pH, being the best results between 7.2 and 7.6. For the CO_2, the gas phase used consists of 5% CO_2 in air to mimick the follicle and oviduct of mammalian females or 20% of CO_2 as in air. Osmolarity ranges between 275 and 290 mOsm.
Despite many supplements can add to the maturation media, normally energy substrates and protein source, as well as different hormones and growth factors are added. Different energy substrates are provided due to the influence on oocyte meiotic and cytoplasmic maturation (Rose-Hellekant *et al.,* 1998; Chung *et al.,* 2007; Garner and Lane, 2013). This energy source used in most culture media is generally lactate, pyruvate and glucose because they are the main substrates for energy metabolism of oocytes (Gardner and Lane, 2013). Oocyte utilization of pyruvate is dependent upon CCs that can convert glucose or lactate into pyruvate to be used by oocytes (Pool, 2004). Furthermore, glutamine can serve as an energy substrate to improve *in vitro* nuclear maturation of oocytes. On the other hand, protein source are essential and non-essential amino acids that would be used by protein synthesis, but also can be utilized as an energy source and intracellular buffer. Essential and non-essential amino acids are added to culture media serum-supplemented or serum-free culture media (Gardner and Lane, 1998; 2014; Rezaei *et al.,* 2003). Also Fetal calf serum (FCS) and bovine serum albumin (BSA) are the most used protein sources for *in vitro* culture (Carolan *et al.,* 1995; Lazzari *et al.,* 2000). FCS contains a complex array of protein components that are required by many cells to grow; it provides a wide variety of macromolecular proteins, low molecular weight nutrients, carrier proteins for water-insoluble components and other compounds necessary for *in vitro* growth of cells, such as hormones, growth factors, vitamins and attachment factors (Chung *et al.,* 2007; Chian *et al.,* 2004). However, large offspring

syndrome (LOS) has been described mainly in ruminants with the adition of serum (Farin *et al.,* 2001). Efforts to avoid FCS and BSA as indeterminant compounds in the medium are done in the last decade. Synthetic compounds as polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP) can be substitute as well as BSA.

As gonadotrophins (FSH and LH) play an important role in the physiological development and function of follicles *in vivo* and oocyte maturation as we explained before, some medium includes these supplements. LH probably acts to induce GVBD by an indirect action mediated by CCs because it is believed that there are no LH receptors on oocytes (Yadav *et al.,* 2010; Sha *et al.,* 2010). Besides, E_2 may be important in regulating oocyte maturation (Lonergan, 2011) and also is used in some laboratories.

Finally, growth factors are usually present in the culture media. The most used are EGF, IGF-1, TGFα and TGF-β.

EGF has been shown to stimulate *in vitro* oocyte maturation thus resulting in an increased developmental competence of oocytes in different species (mouse: Das *et al.,* 1991; Smitz *et al.,* 1998; Merriman *et al.,* 1998; De La Fuente *et al.,* 1999; sheep: Guler *et al.,* 2000; cattle: Lonergan *et al.,* 1996; Sakaguchi *et al.,* 2002; pig: Reed *et al.,* 1993; Coskun and Lin, 1995; Singh *et al.,* 1997; Prochazka *et al.,* 2000, 2003; rat: Dekel and Sherizly, 1985; rabbit: Lorenzo *et al.,* 1996; buffalo: Kumar and Purohit, 2004 and human: Gómez *et al.,* 1993; Goud *et al.,* 1998). It was reported that EGF stimulates granulosa cells proliferation *in vitro* (pig: May *et al.,* 1992) and estrogen production through aromatase activation (human: Misajon *et al.,* 1999; goat: Behl and Pandey, 2001); modulates steroidogenesis and estrogen production (Hsueh *et al.,*1981; human: Misajon *et al.,* 1999). Also EGF is particularly important in controlling oestrogen receptor function (Ignar-Trowbridge *et al.,* 1996) and alone or associated with gonadotropins induces cumulus expansion and promotes nuclear and cytoplasmic maturation of immature oocytes during culture *in vitro* (Lorenzo *et al.,* 1994; Wang and Niwa, 1995; De La Fuente *et al.,* 1999).

In vitro studies have also shown that EGF maintains follicular survival (Wandji *et al.,* 1996), promotes follicular growth (bovine: Wandji *et al.,* 1996; caprine: Kajarajan *et al.,* 2006; Silva *et al.,* 2013; hamster: Roy, 1993; mouse: Boland and Gosden, 1994 and human: Roy and Kole, 1998), proliferation of granulosa cells obtained from preantral follicles Morbeck *et al.,* 1993) and stimulates antrum formation (Silva *et al.,* 2013).

IGF-1 promoted nuclear maturation of oocytes, stimulated proliferation and inhibited apoptosis in the surrounding CCs, increased developmental competence of oocytes (Lorenzo *et al.,* 1996; Patiño *et al.,*2001; Sirotkin *et al.,* 2003; Hunter *et al.,* 2004; Velazquez *et al.,* 2009). Likewise, IGF-1 is known to stimulate protein synthesis when added to medium for mouse embryos *in vitro* (Simmen *et al.,* 1993) and known to increase E_2 production by the theca granulosa cells in serum free culture (Shores *et al.,* 2000).

In some studies, it have been shown that EGF and IGF-1 in combination act synergistically and accelerate the cumulus expansion and the progression of meiosis (Lorenzo *et al.,* 1996; Sakaguchi *et al.,* 2000; Purohit, 2001; Sakaguchi *et al.,* 2002; Isobe and Terada, 2001). In bovine oocyte activation is known to be associated with Ca^{2+} dependent electrical events (Tosti *et al.,* 2002). A combination of EGF and IGF-1 under conditions *in vitro* stimulates a cascade of events including protein synthesis which generate positive signals for mitotic resumption (Spicer and Chamberlain, 2000). On the other hand, the addiction the protein sources how FCS or BSA could neutralize the accelerating effect of EGF and IGF-1 of the meiosis of *in vitro* (Sakaguchi *et al.,* 2000) stimulating cytoplasmic oocyte maturation.

2. OBJETIVES AND THESIS OUTLINE

OBJETIVE AND THESIS OUTLINE

Guinea pig (*Cavia porcellus*) has been widely used as a laboratory animal and due to its reproductive characteristics can be considered an excellent animal model for the study of ovarian physiology in woman. However, IVM protocols are rarely developed in this specie, and cellular mechanisms that occur during oocyte maturation are unknown. Replacement of animal models with *in vitro* alternatives, such as IVM to obtain M-II-oocytes, involves a reduction of animals used in the lab, avoid the application of hormonal treatments and give us a tool to develop a useful IVM system to study the oocyte maturation processes in these species for biomedical research in the ARTs and for conservation of valuable genetic pool.

Growth factors, such as EGF and IGF-1 systems are implicated in a wide range of events in the ovary. The EGF throgouth the EGF-R is involved in the ovulation process, oocyte maturation, granulosa cell proliferation, apoptosis and steroidogenesis of follicle and, luteinization of *corpus luteum* in human and several animal species. On the other hand, IGF-1 regulates luteal steroidogenesis by an increase in E_2 and P_4 production and acts as anti-apoptotic factor during oocyte maturation. Moreover, IGF-1 system is implied in the development of PCOS in woman, and the gene expression of IGFs and IGFBPs differ from women with normal ovaries and those with PCOS.

In this context, there is a clear evidence that the role of the components of EGF and IGF-1 system in relation to follicular health needs to be investigated in animal models, to increase our understanding of the relationship between the constituents and the processes involved in the ovarian health or failure; as well as to develop useful IVM defined system for this species in order to obtain competent oocytes for ARTs and for genetic conservation of resources.

MAIN OBJETIVE

The main objective of this Thesis was to characterizethe EGF and the IGF-1 systems in different structures of the guinea pig ovary, to use the guinea pig as a possible animal model for the study of ovarian physiology. Furthermore, we studied the effect of both growth factors (EGF and IGF-1) alone or in combination on *in vitro* oocyte maturation as a possible way to set up an *in vitro* defined maturation system in this specie. To achieve this main objective, a total of three specific objectives have been proposed and developed in different experiments:

EXPERIMENT 1. Characterization of gene expression patterns of EGF (EGF and EGF-R) and IGF-1 system (IGF-1, IGF-1R and IGFBPs 1-6) in ovarian follicles, *corpus luteum* and cumulus-oocyte complexes (oocyte and cumulus cells) in the guinea pig model

The objective of this experiment was to describe the gene expression patterns of EGF and IGF-1 systems in the ovary of the guinea pig as a possible animal model for human. The basis of the physiological mechanism of action of both growth factors during the final oocyte growth, oocyte maturation and early embryo development was elucidated. For this purpose, in current experiment was analyzed:

Experiment 1.a. The gene expression patterns of EGF, IGF-1, their receptors (EGF-R and IGF-1R), and the IGFBPs 1-6 in: 1) the preovulatory follicle and, 2) the ovarian structure mainly involved in the early embryo development, such as the *corpus luteum*.

Experiment 1.b. The mRNA transcripts for EGF and IGF-1 system in cumulus-oocyte complexes to reveal the possible direct implication of both growth factors during oocyte maturation in the guinea pig model and their autocrine and/ or paracrine response.

EXPERIMENT 2. Immunolocalization of EGF-R and IGF-1R and their ligands (IGFBPs 1-6) in follicles, *corpus luteum*, cumulus-oocyte complexes and early embryos in the guinea pig model

In line with the expression study, the objective of this experiment was to characterize the possible pattern of distribution for the EGF-R and IGF-1R and IGFBPs 1-6 proteins to establish the possible respond of the follicles, *corpus luteum*, cumulus-oocyte complexes and early embryos to both growth factors and the role of IGFBPs. For this purpose, in current experiment was analyzed:

Experiment 2.a. The diferential localization by immunohistochemistry of EGF-R, IGF-1R and their ligands (IGFBPs 1-6) in guinea pig ovaries.

Experiment 2.b. The differential and temporal distribution by immunocytochemistry of EGF-R and IGF-1R and their ligands (IGFBPs 1-6) in cumulus-oocyte complexes and in the early embryos of guinea pig.

EXPERIMENT 3. Study of the effect of EGF and IGF-1 on nuclear and cytoplasmic *in vitro* oocyte maturation, apoptosis and steroidogenic production by the cumulus-oocyte complexes in the guinea pig model.

The specific objective of this experiment after the immunolocalization of the EGF-R and IGF-1R in the oocytes and CCs was to investigate the direct role of different concentrations of EGF and IGF-1 during the *in vitro* oocyte maturation process in the guinea pig, to set up a possible defined medium useful in this specie. Furthermore, the description of patterns of cortical granules and mitochondrial distribution was done, due to their importance in the cytoplasmic maturation of the oocytes and the acquisition of their developmental competence. For these purpose in current experiment was performed:

Experiment 3.a. Characterization of cortical granules and mitochondrial distribution patterns during *in vitro* oocyte maturation in the guinea pig.

Experiment 3.b. Dose response study by supplementation of EGF on *in vitro* oocyte maturation system of guinea pig by assessment of nuclear and cytoplasmic oocyte maturation, apoptotic rate in CCs and steroidogenic response of cumulus-oocytes complexes.

Experiment 3.c. Dose response study by supplementation of IGF-1 on *in vitro* oocyte maturation medium of guinea pig evaluating of nuclear and cytoplasmic oocyte maturation, apoptotic rate in CCs and steroidogenic response of cumulus-oocytes complexes.

Experiment 3.d. Study of the effect of combination of EGF and IGF-1 on *in vitro* oocyte maturation of guinea pig by the study of nuclear and cytoplasmic oocyte maturation, apoptotic rate in CCs and steroidogenic response of cumulus- oocytes complexes.

3. MATERIALS AND METHODS

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company (Alcobendas, Madrid, Spain).

3.1. Animals and facilities

All the animals used in the experiments were adult (3-4 months) female guinea pigs (*Cavia porcellus*), sexually mature with body weights ranged 450-550 g (Figure 11).

Figure 11. Adult guinea pig of Peruvian breed

Ovaries and reproductive tract were obtained from animals that were purchased from a breeding farm and maintained in the Agroecological station of the Department of Biological Sciences of the Universidad Técnica Particular de Loja (UTPL) or from a commercial farm (Sivisapa Farm, Chuquiribamba-Loja, Ecuador). In both facilities the animals were kept under the following environmental conditions: temperature (18-22 °C) and humidity (60 ± 5%); photoperiod constant with 12-h light/12 h darkness. They were caged in groups of eight to ten animals and were fed guinea pig pellets (vitamin C enriched) (Pronaca, Quito, Ecuador) and watered *ad libitum*. The experimental procedures described were approved by the Ethics Committee for Animal experimentation of the UTPL. All the animals were sacrificed according to ethical guidelines on Animal care (Close *et al.,* 1997).

3.2. Ovarian collection and transport

The ovaries (Figure 12) were obtained from slaughtered animals by ventral midline laparotomy and transported to the laboratory in a flask desk with PBS + antibiotic (100 IU (International Unit), 100 IU/mL) (A5955, antibiotic antimicotic, lyophilized ϒ- sterilized by irradiation, cell culture tested) at 37 °C.

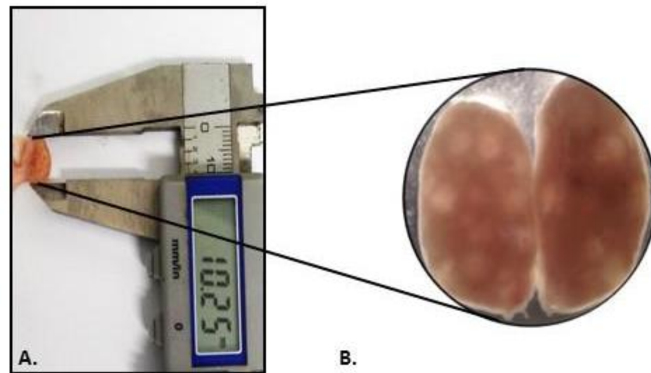

Figure 12. A) Measurement in mm of guinea pig ovary, B) Guinea pig ovaries with follicles of different sizes visualized under stereoscopic microscope.

3.3. Gene expression of EGF, IGF-1 and their receptors (EGF-R and IGF-1R), and IGFBPs 1-6 in ovaries, follicles and *corpus luteum*, and COCs in the guinea pig model

A total of 19 non-synchronized females were used to collect ovaries and their structures for the study of the gene expression of EGF, IGF-1 and their receptors (EGF-R and IGF-1R), and IGFBPs 1-6 by RT-PCR according with the procedures described below.

3.3.1. Collection of ovaries, follicles and corpus luteum

A sum of 13 ovaries was used to obtain total RNA from the ovary, follicles and *corpus luteum*. Ovaries (n=8) were collected and rinsed in PBS + 0.1% BSA (A9647; Bovine Serum Albumin, Fraction V, ≥ 96%) and embedded in RNAlater (Life Technologies, Gaithersburg, MD, USA) where they were maintained at 4 ºC overnight. Then the supernatant was removed and the ovaries were stored at -80 °C until RNA extraction. The rest of the ovaries (n=18) were used to obtain the follicles and *corpus luteum*. Then, ovaries were dissected into two halves and follicles were isolated in sterile conditions in laminar flood cabin by microdissection under a stereoscopic microscope. First, the connective tissue around the follicles was removed using a 26-gauge needle. Isolated follicles were categorized in antral (500 - 700 µm) (n=180) and preovulatory follicles (>700 µm) (n=90) and were stored at –80 ºC until used. Also all *corpus luteum* (n=10) found in the ovaries were processed by the same technique.

3.3.2. Collection and selection of cumulus-oocyte complexes

Cumulus oocyte complexes (COCs) (n= 73) were collected from the rest of the ovaries (n=12) by follicular aspiration of the visible antral follicle (≥700 μm) in the ovarian surface with a 25 G needle connected to a syringe 2 mL using a stereomicroscope. COCs collected were washed several times in PBS+ 0.1% BSA, and CCs were mechanically removed by gentle repeated aspiration with a fine-bore pipette to get denuded oocytes and their corresponding CCs per separate. Groups of approximately 15 denuded oocytes were allocated in eppendorf (n=73) and then snap frozen in liquid nitrogen. The remaining CCs from those oocytes was centrifuged at 2000 × g for 5 min and the pellet was snap frozen in liquid nitrogen. Both oocytes and CCs were stored at -80 °C until RNA extraction process.

3.3.3. Extraction of total RNA, mRNA purification and Reverse Transcription

3.3.3.1. RNA extraction in ovaries, follicles and corpus luteum

Total RNA was extracted using the PureLink RNA Mini kit (Ambion - Life Technologies, Gaithersburg, MD, USA) following the manufacturer's instructions. A total of 750 μLTRIzol® Reagent (Ambion - Life Technologies, Gaithersburg, MD, USA) was added to all samples of ovaries, antral follicles, preovulatory follicles and *corpus luteum* to maintain RNA integrity with inhibition of RNase activity. Mechanic manually homogenization with a plastic pestle was performed at room temperature (rt). Then, they were incubated for 5 min at rt to allow the complete dissociation of nucleoprotein complexes. After that, it was added 150 μL of chloroform (Panreac, Barcelona, Spain) to separate the aqueous (RNA-containing) layer from the organic layer (containing DNA and proteins), and the tube was vigorously shook by hand for 15 seconds. The samples were incubated for 3 min at rt and centrifuged at 12000 × g for 15 minutes (min) at 4 ºC. Then, the aqueous phase of the sample was removed by angling the tube at 45 ºC and pipetting the solution out to place into a new tube. A volume of 70 % ethanol (Panreac, Barcelona, Spain) for DNA precipitation, equal to the sample volume was added and 700 μL of each sample were transferred to the commercial spin cartridge (with the collection tube) supplied in the kit, and centrifuged at 12000 × g for 15 seconds at rt. After that, 700 μL of wash buffer was added to the spin cartridge and centrifuged for 15 seconds at rt. When the supernatant was discarded, the spin cartridge was placed into a new collection tube and 500 μL of another wash buffer with ethanol was added. Again, another

centrifugation at 12000 × g for 15 seconds at rt and then after throw away the supernatant, the empty membrane was dried with bound RNA by a 12000 × g for 2 min centrifugation. Finally, 100 µL of RNase-free water was added to the spin cartridge, incubated at rt for 1 minute and centrifuged for 2 min at 12000 × g to elute the total RNA from the membrane into the recovery tube. At that moment, the purified RNA was quantified by Biophotometer plus (Eppendorf, Hamburg, Germany) and stored at -80 ºC.

3.3.3.2. RNA extraction in oocytes and cumulus cells

Total RNA isolation in oocytes and CCs was similar to procedure described for tissues with some modifications. For these samples 300 µL of lysis buffer of the PureLink RNA Mini kit (Ambion - Life Technologies, Gaithersburg, MD, USA) and 3 µL of 2-mercaptoethanol was added. The lysate was homogenized through a syringe (1 mL) with 20-gauge needle; then it was added 300 µL of 70% ethanol (Panreac, Barcelona, Spain) and the total volume of the sample was transferred to a spin cartridge (with the collection tube) following then the same procedure that described above. The isolated total RNA was also quantified and stored at -80 ºC.

3.3.3.3. mRNA purification in ovaries, antral and preovulatory follicles, corpus luteum, oocytes and cumulus cells

Specific extraction of mRNA was performed by incubation of the samples (ovaries, antral follicles, preovulatory follicles, *corpus luteum,* oocytes and CCs) with super-paramagnetic polystyrene beads coated with oligo ((deoxy-thymidine nucleotides (dT)) sequence, and processed according to the manufacturer's protocol for Dynabeads® mRNA Purification kit (Ambion - Life Technologies, Gaithersburg, MD, USA) with minor modifications. Briefly; 100 µL of the total RNA extracted of each type of sample was added to the Dynabeads/Binding Buffer suspension (100 µL). The solution tube was mixed thoroughly for 3-5 min at rt to allow mRNA to anneal to the oligo (dT) on the beads and it was placed on the magnet until solution was clear; afterward the supernatant was removed. The tubes were removed from the magnet and the mRNA-bead complex washed twice with 200 µL of Washing Buffer provided by the kit. All the supernatant was removed between each washing step with the help of the magnet. Finally, 20 µL of 10 milimolar (mM)Tris- Hydrochloric acid (HCl) was added and the samples were maintained in heat to 75 °C for 2 min in a thermoblock. Finally, the tube was placed immediately on the magnet and the eluted mRNA transferred to a new RNase-free tube (Ambion - Life Technologies, Gaithersburg, MD, USA).

3.3.3.4. Reverse transcription

Reverse transcription reaction was performed using SuperScript™ First-Strand Synthesis System for RT-PCR, to synthesize complementary DNA (cDNA) from mRNA, using poly(A) RNA, Oligo (dT), random hexamers and SuperScript II™ RT (reverse transcriptase Moloney murine leukaemia virus enzyme (MMLV) genetically engineered with reduced RNase H activity) (Invitrogen - Life Technologies, Gaithersburg, MD, USA).

First, to get a total volume of 10 µL of mRNA for each reaction the following components was combined in a sterile tube: 2 µL of mRNA sample, 1 µL of 10 mM deoxynucleotide (dNTP) mix, 2 µL Random hexamers (50 ng/mL), 1 µL Oligo dT primers (0.5 µg/µL), 4 µL DEPC-treated (Diethyl pyrocarbonate) water. This mixture was heated at 65 ºC for 5 min in a thermoblock to denature the secondary mRNA structure and then place on ice for 1 min. In a separate tube, it was prepared the 2X reaction mix, adding each component in the following order: 2 µL of 10X RT buffer, 4 µL 25 mM $MgCl_2$, 2 µL 0.1 M Dithiothreitol (DTT) and 1 µL RNase OUT™ (40 U/µL). Then, 9 µL of the 2X reaction mix was added to each mRNA mixture tube and the control sample tube. After that, it was mixed gently and collected by brief centrifugation. The reactions were incubated at 42 ºC for 2 min. After added 1 µL of SuperScript™ II RT (1U/ µL) to each tube, they were incubated at 42 °C for 50 min in a Thermo-Shaker for microtubes (TS-100, BioSan, Latvia) to allow retrotranscription of mRNA, followed by incubation at 70 ºC for 15 min to denature the RT enzyme and finally cooled on ice. At the end, a brief spin was performed and 1 µL of RNase H (2 U/µL) (Invitrogen - Life Technologies, Gaithersburg, MD, USA) was added to each tube. After, incubation for 20 min at 37 °C to get a greater full-length cDNA synthesis, the volumen total of cDNA samples (720 µL) were stored at -20 ° C until use. Reverse transcriptase negative (RT-) controls containing all the RT-PCR reagents, except SuperScript™ II Reverse Transcriptase (RT) were used to check genomic DNA contamination.

3.3.4. Polymerase chain reaction

PCR (Polymerase Chain Reaction) reactions were performed with an equal amount of cDNA (2 µL) in triplicate in a 0.5-mL thin-walled PCR tube eppendorf following the manufacturer's instructions of Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA). First, the PCR master mix was prepared to obtain a total volume of 25 µL adding the following components to each tube: 2.5 µL of 10 X PCR Buffer (Invitrogen, Carlsbad, CA), 0.75 µL of 50 µM $MgCl_2$ (Invitrogen, Carlsbad, CA), 0.5 µL of 10 mM dNTP mix

(Invitrogen, Carlsbad, CA), 0.5 µL of each forward and reverse primers, 1 µL of cDNA,0.1 µLTaq DNA polymerase (Invitrogen, Carlsbad, CA) and 19.15 µL of DEPC-treated water to complete a total volume of 25 µL. In the negative control the cDNA was replaced by DEPC-treated water. Specific primers were used at a concentration of 10 µM/µL in each reaction and are summarized in Table 2. Intron-spanning primers were designed using the Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primersblast/) and validated using OligoAnalyser program. Each sample was analyzed in triplicate using eppendorf for PCR (Eppendorf, Hamburg, Germany) and template controls (water in place of cDNA) were used in each reaction. The RT-PCR (for ovaries, follicles, *corpus luteum* and COCs) was performed in a Mastercycler (Eppendorf, Hamburg, Germany) with the following conditions: an initial denaturation and activation of the polymerase for 2 min at 94 °C, followed by 40 cycles (94 °C for 30 seconds; specific temperature (see Table 2 for each gene) for 30 seconds and 72 ºC for 40 seconds). The final extension at 72 ºC incubation was continued for a further 7 min to increase PCR sensitivity.

Table 2. Oligonucleotide primers for amplification of genes of interest in RT-PCR analysis of different samples of guinea pig.

Target gene	Primer sequence (5´to 3´)	Sense	Annealing temperature (◦C)	Fragment size (pb)
EGF	CAGACAGTGGGAAGTCTGT	sense	53	89
	TCGTGGGCGAGAGATATTTT	antisense		
EGF-R	CCTTCCTGAAGACCATCCAG	sense	55	130
	GCCAGAGCATAGGTGTTCTCA	antisense		
IGF-1	GACGCAGAAGGAAGTACATT	sense	56	104
	TGACGTGTCGTTCTTCACC	antisense		
IGF-1R	TGTCCACAGAGACCTTGCTG	sense	53	122
	CTTTCCCTCCTTTCCGGTAG	antisense		
IGFBP-1	AAAGTGGAAGCCGTGCCA	sense	58	11
	CCATTCTTGTTGCAGTTAGG	antisense		
IGFBP-2	GCAGGTTGCAGACAATGGTGA	sense	55	139
	GACAGCCAGTTCCTTCATGC	antisense		
IGFBP-3	CCTCCGCTCCAGGAAATG	sense	55	60
	GGGTCTCCACGCTGCTAGT	antisense		
IGFBP-4	AGCCCGCCGACAAGGATG	sense	58	141
	CCCCGACGACCTTCATCT	antisense		
IGFBP-5	GCCAAGATCGAGAAAGACTC	sense	56	71
	GCGTAGGTCTCTTCGGTCA	antisense		
IGFBP-6	AAGCGGCAGTGTCGATCTTCA	sense	58	112
	GGCAGTGTGAGCTTCTGTCA	antisense		

Gene expression of studied genes was normalized to β -actin expression according to Dubey *et al.* (2015) and Ritter *et al.* (2015). Normalization is the result of the relative expression of each studied genes respect to actin (Table 3).

Table 3. The nucleotide sequences of the β-Actin primer used as target gene in RT-PCR**.**

Target gene	Primer sequence (5´to 3´)	Sense	Annealing temperature (◦C)	Fragment size (pb)
β -ACTIN	AAGATGACCCAGATCATGTTCG	sense	53-58	158
	GCGTAGCCCTCGTAGATGG	antisense		

PCR products of follicle, *corpus luteum* and COCs, were separated by electrophoresis on 2 % agarose gel and visualized by Real safe nucleic acid staining solution (Durviz, Valencia, España). The molecular marker used was 100 bp ladder (Biotools, Madrid, Spain). The image of all gels was recorded using a Universal Hood II system equipped with Gel Doc XR camera with MZL (BIO-RAD Laboratories, Milan, Italy).

Semi-quantitative RT-PCR was chosen to estimate the transcript of EGF, EGF-R, IGF1, IGF-1R and IGFBPs. Gene β-actin (housekeeping) was examined in all samples (follicles, CCs, oocytes and *corpus luteum*) to confirm the integrity of the mRNA according to Dubey *et al.* (2015). To normalize different experimental samples, band density of β-actin was also calculated, along with band density of targeted genes, using densitometry by means of Image J 1.47v software (NIH, Bethesda, Maryland (http://imagej.nih.gov/ij)). Relative expression was determined in arbitrary units, defined as the ratio of mRNA level to the corresponding β-actin mRNA level. The results were expressed as the difference of the densitometric readings for the genes analyzed on β-actin; therefore, values indicate relative changes in mRNA levels. Mean values of three measurements for each gene in each sample band were taken for the analysis.

3.4. Immunolocalization of EGF-R and IGF-1R receptors and IGFBPs 1-6 in the guinea pig ovary

3.4.1. Ovary fixation

Ovary samples were fixed in PBS containing 4% wt/vol (weigth/volume) buffered neutral paraformaldehyde solution (pH 7.2–7.4) and embedded in paraffin blocks which were

cut in 5 µm sections and mounted on glass slides in the Histology Facilities of the National Center for Biotechnology (CNB- CSIC, Madrid, Spain).

3.4.2. Immunohistochemistry of EGF-R and IGF-1R in guinea pig ovaries

Immunohistochemical study for EGF and IGF-1 receptors (EGF-R and IGF-1R, respectively) was performed in 10 sections of 9 ovaries for each receptor (n=18). Immunohistochemical staining was performed by avidin–biotin immunoperoxidase system according to a previously described protocol by Arias-Álvarez *et al.* (2010) with some modifications. Sections were deparaffinized in xylene (Panreac, Barcelona, Spain) and alcohol (Panreac, Barcelona, Spain) series (100º, 96º and 70º). The endogenous peroxidase activity was blocked by incubating the sections with 0.3% v/v (volume/volume) hydrogen peroxide (Panreac, Barcelona, Spain) in methanol solution (Panreac, Barcelona, Spain) for 30 min, and nonspecific protein-binding sites was blocked with normal goat serum (1:10, sc-2043, Santa Cruz Biotechnology, CA, USA) in PBS at rt for 30 min. Then, the sections were incubated with EGF-R rabbit polyclonal IgG (1:100, sc-03, Santa Cruz Biotechnology, CA, USA) or IGF-1R rabbit polyclonal antibody (1:100, sc-7952, Santa Cruz Biotechnology) overnight at 4 °C in a humidified chamber. Subsequently, the sections were incubated with a secondary biotinylated anti-rabbit IgG antibody (1:200, Vector Laboratories, Burlingame, CA, USA) for 30 min at rt and then incubated with Avidin-Biotin-Complex (ABC Vector Elite kit, Vector Laboratories Burlingame, CA, USA). After the incubation with chromogen (Vector Nova RED substrate Kit for Peroxidase, Vector Laboratories Burlingame, CA, USA) for 3 min, the sections were counterstained with hematoxylin, dehydrated and mounted in Depex medium. Finally, they were observed under a light microscope equipped with a digital camera (F550; Leica, Wetzlar, Germany). The intensity of immunohistochemical staining was graded as follows: (–) no immunostaining; (+) weak staining; (++) moderate staining and (+++) strong staining.

Some of the sections were stained with hematoxylin – eosin and the ovarian follicles were previously categorized as primordial, primary, secondary and antral follice according to the morphology of the follicular cells described by Sadeu *et al.* (2007) (Figure 13).

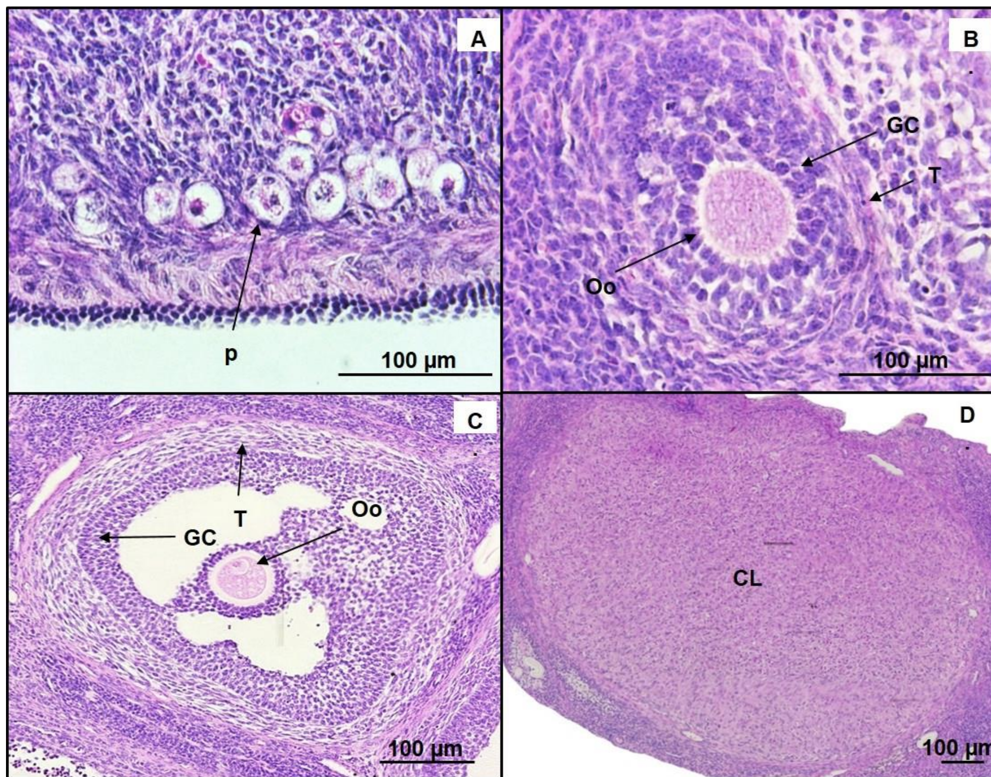

Figure 13. Follicles in guinea pig ovaries. A) Primary follicle, B) Secondary follicle, C) antral follicle and, D) *corpus luteum.* GC (Granulosa Cells), Oo (Oocyte), T (Theca cells), CL (*Corpus luteum*), p (primordial follicle). Scale is 100 µm.

3.4.3. Immmunohistochemistry of IGFBPs 1-6 in guinea pig ovaries

A total of 12 ovaries from 6 animals were used for the immunolocalization of IGFBPs. Each IGFBPs (1, 2, 3, 4, 5 and 6) were analyzed in 4 sections per ovary, therefore a total of 288 sections were studied (48 sections per each IGFBPs).

Samples for IGFBPs immunolocalization were processed as described above for the immunohistochemical study of EGF-R and IGF-1R with some modifications. The sections were incubated with blocking horse serum at a concentration of 1:10 (sc-2483, Santa Cruz Biotechnology, CA, USA) in PBS for 30 min. Primary goat polyclonal antibody IGFBP-1 (1:200, sc-6072), IGFBP-2 (1:200, sc-6001), IGFBP-3 (1:50, sc-6003), IGFBP-4 (1:50, sc-6005), IGFBP-5 (1:50, sc-6006) and IGFBP-6 (1:50, sc-6007) (Santa Cruz Biotechnology, CA, USA) were diluted in the blocking serum and incubated at 4 °C overnight. According to data sheet of products, each specific antibody had shown no cross-reactivity with other IGFBPs family. Secondary biotinylated anti-goat IgG antibody (Vector Laboratories, Burlingame, CA, USA) was used at a concentration of 1:200 in PBS. In all the negative controls, the primary antibody was replaced with blocking serum.

Sections were counterstained with hematoxylin, photographed and assessed under a light microscope equipped with a digital camera (F550; Leica, Wetzlar, Germany). The intensity of immunohistochemical staining was graded as described before in: (–) no immunostaining; (+) weak staining; (++) moderate staining and (+++) strong staining.

3.5. Immunolocalization of EGF-R and IGF-1R and IGFBPs 1-6 in cumulus-oocyte complexes and early embryos

3.5.1. Collection and selection of cumulus-oocyte complexes

COCs were collected by aspiration of antral follicles (>700 μm diameter) on the ovary surface under a stereoscopic microscope (Sadeu *et al.,* 2007), with a 25 G needle connected to a syringe 2 mL. Then COCs were washed in 35 mm Petri dishes (NunclonTM Surface, NuncTM, Roskilde, Denmark) with PBS (pH 7.4) + 0.1% BSA at 37 °C and selected for *in vitro* maturation based on morphological criteria of homogeneity in the cumulus and cytoplasm, as described by Jacquet *et al. (*1995).

For the immunocytochemistry a total of 160 COCs were used immediately after follicular aspiration and 48 COCs after *in vitro* maturation. Among them, a total of 80 immature COCs and 24 *in vitro* matured oocytes were denuded mechanically by repeated aspiration with a fine-bore pipette, as described above. Oocytes were treated with 0.5% pronase (Protease type XIV, from *Streptomyces griseous®*) in PBS for a few min to remove the ZP. All the oocytes (denuded or with CCs) before and after *in vitro* maturation were fixed in PBS containing 4% wt/vol buffered neutral paraformaldehyde solution (pH 7.2–7.4) for 30 min and stored in PBS at 4 ºC until processed.

3.5.2. Collection and selection of early embryos

A total of 23 females were used for the immunocytochemistry of early embryos. First, the exact day of ovulation and copulation was determined so each female was checked twice a day (at 6:00 am and 18:00 pm) to detect the opening of the vaginal membrane. If half of the vaginal membrane was perforated, the female was separated and placed with a sexually tested male in the cage; subsequently, the vaginal smears continued once every 12 h for every female. Vaginal smears to evaluate estrus were performed with sterile swab, which was previously moistened with saline solution and gently inserted into the vagina at an angle of approximately 45 ºC. After 1 cm of the swab was inserted, the angle of insertion varied to 90 ºC and continued inserting it until completing 2 cm lenght.

When the swab was fully inserted, the end was rotated a 2 to 3 revolutions, allowing the cotton tip to pick up an adequate load of cells. Then the swab was removed gently. The smears were prepared immediately after with drawal of the swab by rolling the cotton tip along the length of a glass slide. For fixation, slides were dipped 10 min in a container of methanol (Panreac, Barcelona, Spain). Then, the slides were then allowed to dry completely. After fixation slides were stained with Giemsa 10% (G48900) for 15 min and rinsed in distilled water. Examination was done under an optical microscope (Olympus, CX31R, Tokyo, Japan). Ovulation (Day 0) was confirmed by leukocyte influx in the vaginal smear on the day following the observation of a cornified cell-rich vaginal smear (Young, 1937; Suzuki *et al.,* 2003; Grégoire *et al.,* 2012) (Figure 14).

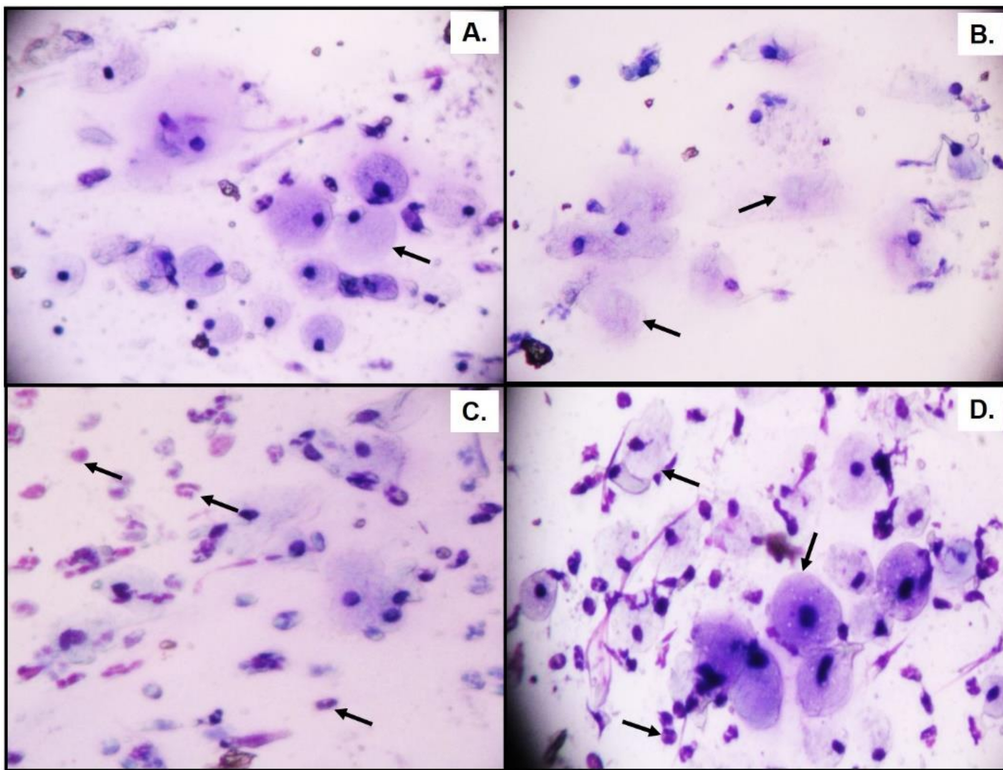

Figure 14. Cytological assessment of vaginal smear of guinea pig. A) Proestrus, round nucleated epithelial cells predominated), B) Estrus (the predominant cells are cornified squamous epithelial cells), C) Metaestrus (cornified squamous epithelial cells and small stained leukocytes), D) Diestrus (nucleated epithelial cells, cornified squamous epithelial cells and leukocytes are present). In all the pictures, the representative cell is indicated by the arrow. Photographs by the author.

Finally, the presence of a mucous plug in the vagina and vaginal smear analized confirmed the copulation. Once the plug was checked, the females were separated from

the male. At day 2, 3 and 4 post-ovulation the females were sacrificed and the reproductive tract was collected and flushed with PBS supplemented with 0.1% BSA at 37 °C from the oviduct to the uterus. We recovered embryos in different developmental stages that were classificated as: 2 to 4-cells embryos (n=22, recovered at 2 days after having detected the mucous plug), 8 to 16-cells embryos (n=21, recovered at 3 days after having detected the mucous plug) and morulae (n=16, recovered at 4 days after having detected the mucous plug). After several washes in PBS-BSA (0.1 %) selected embryos of grade 1 and 2 ((according to the International Embryo Technology Society (IETS) guidelines)) were fixed in PBS containing 4% wt/vol buffered neutral paraformaldehyde solution (pH 7.2–7.4) during 30 min and then stored at 4 ºC in PBS until their use.

3.5.3. Immunocytochemistry of EGF-R and IGF-1R in cumulus-oocyte complexes and early embryos

Immunocytochemistry for the detection of EGF-R and IGF-1R were carried out in immature COCs (n=40), of which were obtained denuded oocytes (n=20) and CCs (n=20). Also, *in vitro* matured COCs (n=24), *in vitro* matured oocytes (n=24) and early embryos (n=59) were used. Process was performed according to a previously described protocol by Agirregoitia *et al.* (2012), with some modifications. After fixing in PBS containing 4% wt/vol buffered neutral paraformaldehyde solution for 30 min, samples were placed on a coverslip covered with poly-L-lysine and then permeabilized with 1% Triton X-100 for 10 min at rt. The next step was an incubation for 45 min in PBS and 10% v/v goat serum at rt. For indirect immunofluorescence staining, the coverslips were incubated with EGF-R or IGF-1R rabbit polyclonal IgG (1:100, sc-03 and sc-7952, respectively, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. In the negative controls the primary antibody incubation was replaced with goat serum after the slides were washed three times with PBS. All the samples were incubated with Alexa Fluor 488 goat anti rabbit secondary antibody (1:1000 A-11034, Life Technologies, USA) for 1 h at rt in the dark; later, they were washed three times with PBS (during the second washed the nuclei was stained with Hoechst 33342) and finally they were washed with distilled water. For assembly of the oocytes, a drop of Prolong (Gold antifade reagent, Invitrogen, Oregon, USA) was placed on a slide. A drop of paraffin was placed in each corner of the coverslip to preserve the shape of oocytes and COCs intact. The samples were examined by confocal scanning microscopy (Leica, SP2, Wetzlar, Germany) in the Interdepartmental Investigation Service (SIdI) of the Universidad Autónoma de Madrid

(Madrid, Spain), using using 488 nanometers (nm) excitation laser. The format, laser gain and offset were kept constant for every sample.

3.5.4. Immunocytochemistry of IGFBPs 1-6 in oocytes and cumulus-oocyte complexes

Immunocytochemistry for the detection of IGFBPs 1-6 were carried out in a total of 60 COCs and 60 denuded oocytes. For the IGFBPs localization, the protocol and primary antibodies used was performed as described above in section (3.4.3) with some modifications. Blocking serum employed was donkey serum diluted to 10% v/v (sc-2044, Santa Cruz Biotechnology, CA, USA) and Alexa Fluor 488 of donkey anti goat was used as secondary antibody (Ref: 705-545-147, Jackson ImmunoResearch, PA, USA). For assembly of the oocytes procedure was the same that described before in section (3.5.3.) as well as the visualization.

3.5.5. Quantification of the immunofluorescence intensity

For quantification of the immunofluorescence intensity a total of 40 oocytes and their corresponding CCs were used before (n=32) and after (n=8) *in vitro* maturation to asses EGF-R and IGF-1R and IGFBP 1-6. In the 2-4 cell embryos (n=6), 8-16 cell embryos (n=6) and morula (n=6) the EGF-R and IGF-1R were localized as well. All the images were acquired under the same intensity parameters and the Image J program was used for quantification. A First, an analysis area was selected for each receptor and the ROI tool (region of interest) was used to provide an average fluorescence intensity. An identical ROI (4 equidistant intensity values measured in pixels) were taken for each sample. Measure the display area in pixels was analyzed statistically (Papadopulos *et al.,* 2007; Jensen, 2013; Dunning *et al.,* 2014).

3.6. Role of EGF and IGF-1 on nuclear and cytoplasmic *in vitro* oocyte maturation

An IVM dose response study using EGF or IGF-1 and their combination were carried out. Assessment of nuclear maturation (M-II rate), cytoplasmic oocyte maturation, in terms of cortical granule migration and mitochondria relocation, apoptosis rate in CCs and steroidogenic production by the COCs in the spent maturation medium were performed.

3.6.1. Recovery, selection of cumulus-oocyte complexes and in vitro oocyte maturation systems

Ovaries (n=220) were transported from the abattoir to the laboratory at 37 °C in PBS supplemented with antibiotic (100 IU, 100 IU/mL) (A5955, antibiotic antimicotic, lyophilized Ƴ- sterilized by irradiation, cell culture tested).

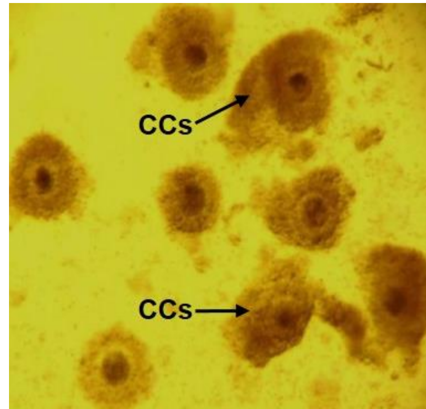

Figure 15. Cumulus- oocyte complexes (COCs) suitable for IVM. (CCs) cumulus cells.

Ovaries were transported and COCs (Figure 15) were obtained as described before (see section 3.2). The maturation medium consisted of TCM-199 (M4530) enriched with 2 mM/mL glutamine (G3126, L Glutamine, cell culture tested, irradiated®Ƴ), 0.1 mg/mL sodium pyruvate (P2256) and 0.003% wt/vol BSA. Stock solutions of EGF from mouse submandibulary glands (E4127) and IGF-1 from human recombinant growth factors (I3769) were diluted and added to TCM-199 as described above. Groups of 30-32 COCs with intact and unexpanded CCs were cultured at 37 °C in 5% CO_2 in air and maximum humidity for 17 h in an incubator (Forma™ Series II 3110 Water-Jacketed CO2 Incubator, USA).

A total of 1746 COCs were used in three experiments and each experiment was repeated four times (Figure 16, 17 and 18). In ***Experiment 1*****,** 622 COCs were IVM with different concentration of EGF and 10% FCS.

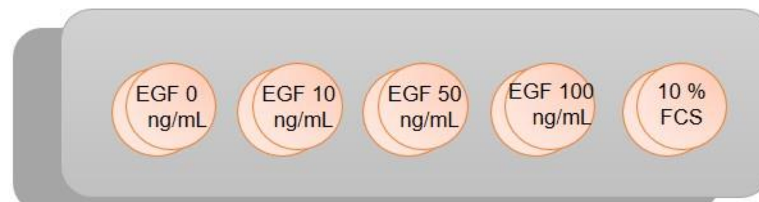

Figure 16. Experimental design of EGF for IVM.

In ***Experiment 2*****,** 624 COCs were IVM with different of concentration IGF-1 and 10% FCS.

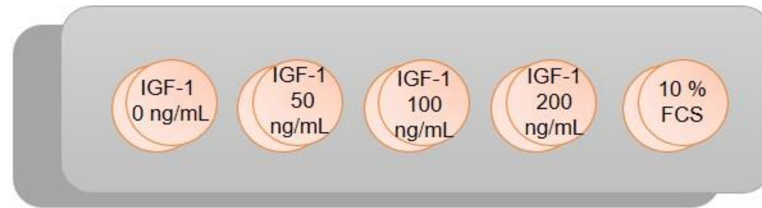

Figure 17. Experimental design of IGF-1 for IVM.

In ***Experiment 3*****,** 500 COCs were supplemented with the concentrations of EGF and IGF-1 that showed the best maturation results in experiments 1 and 2 (50 ng/mL EGF and 100 ng/mL IGF-1), with or without FCS as follows: group 0 (non- supplemented); group EI (EGF + IGF-1); group EI-FCS (EGF + IGF-1 + 10% (v/v) FCS) and group FCS (10% (v/v) FCS)

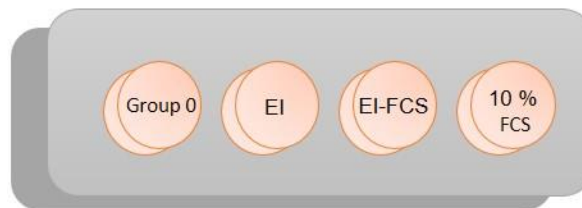

Figure 18. Experimental design of EGF and IGF-1 combination for IVM

3.6.2. Assessment of nuclear and cytoplasmic maturation by confocal microscopy

3.6.2.1. Nuclear oocyte maturation

After IVM, oocytes (n=1588) were mechanically denuded by repeated aspiration with a fine-bore pipette and fixed in 4% wt/vol buffered neutral paraformaldehyde solution. Chromosomes were stained with Hoechst 33342 (10 µg/mL) and nuclear maturation was measured as M-II under fluorescent microscope (F550; Leica, Germany). Among these oocytes a total of 594 oocytes were also stained to visualize cortical granules (n=382) and mitochondria (n=212).

3.6.2.2. Cortical granule and mitochondrial distribution

Visualization of cortical granules was performed according to Arias-Álvarez *et al*. (2009) with minor modifications; oocytes were treated with 0.5% wt/vol pronase after CCs

removal, fixed in 4% v/v paraformaldehyde, and stored in PBS at 4 ºC. Then, oocytes were treated with 0.02% v/v Tritón X-100 and 7.5% wt/vol BSA in PBS. Cortical granules were stained with FITC of *Lens culinaris* (100 mg/mL). For mitochondrial visualization, oocytes were treated with 0.5% wt/vol pronase and stained with 180 nMMitoTracker Red CMX Ros (Molecular Probes Inc., USA) following manufacturer's instructions. The fluorescent dye only was accumulated in active mitochondria. Oocytes were examined under a confocal laser-scanning microscope (Leica, TCS SP2, Germany) at 488 and 546 nm to visualize cortical granules and mitochondria, respectively. The format, laser, gain and offset were kept constant for every sample. Sections of each 5 µm were made for each oocyte and a maximum projection was accomplished for each one.

Furthermore, a total of 40 immature guinea pig oocytes were also used for visualize cortical granules (n=20) and mitochondrial migration patterns (n=20) before maturation. These migration patterns were described based in the adaptation from previous studies in other species for cortical granules (Damiani *et al.,* 1995; Arias-Alvarez *et al.,* 2009) and for mitochondria (Li and Fan, 1997; Leoni *et al.,* 2015).

3.6.3. Cumulus cell apoptosis

Apoptosis were analyzed as previously described by Arias-Alvarez *et al.* (2010) using the In Situ Cell Death Detection Kit, POD (Roche Diagnostics S.L., Spain) following the manufacturer's instructions. COCs were washed three times in PBS supplemented with 0.1% polyvinylpyrrolidone (PVP) (P0930, powder, BioXtra) and then fixed in 4% paraformaldehyde solution for 1 h at rt. The COCs were pretreated with Triton X-100 0.5% diluted in PBS for 1 h 20 min in a humid chamber. Incubation with the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) reaction was made under the same conditions (In Situ Cell Death Detection Kit, POD, Roche Diagnostics SL, Applied Science, Barcelona, Spain). Positive control sections were treated with deoxyribonuclease (DNase I) (Roche Diagnostics SL, Applied Science) for 10 min at rt in a humidified chamber prior to incubation with the TUNEL reagent. For negative controls, the samples were incubated only with the label solution TUNEL reaction mixture without the enzyme solution. In order to avoid RNA interferences, all COCs were treated with RNAses before staining. COCs were counterstained with Hoechst 33342 for 15 min, mounted between a coverslip and a glass slide in a mounting solution (ProLong® Gold antifade reagent, Invitrogen, USA) and observed under a fluorescent microscope (F550; Leica, Germany) (Figure 19). Samples were analyzed using IMAGE J/FIJI 1.46 software (Ferreira and Rasband, 2012). The apoptosis index was calculated as the relation

between green area and blue area ×100, and was log10-transformed before statistical analysis to achieve normal distribution.

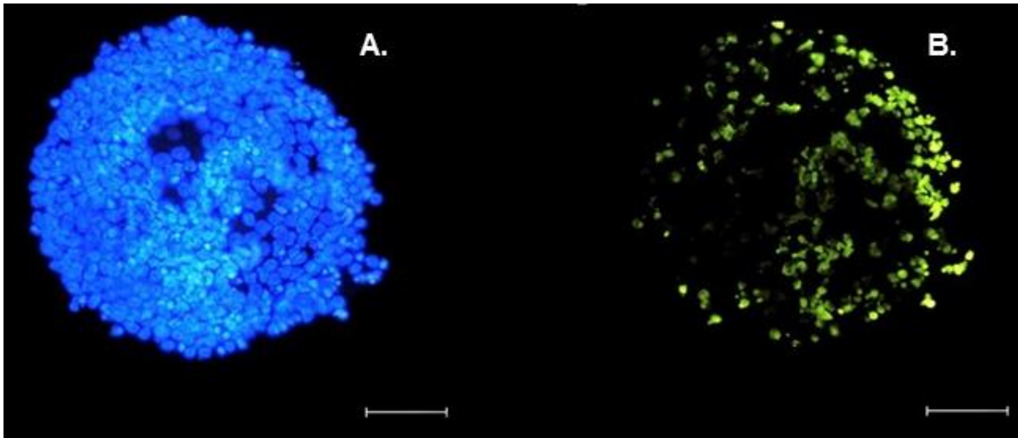

Figure 19. Cumulus cell apoptosis measured by TUNEL assay in guinea pig COCs after *in vitro* maturation. A) Showing all nucleus of CCs stained with Hoechst, B) showing TUNEL staining labelled cells considered as apoptotic. Scale bar 30 µm.

3.6.4. Steroidogenic production of cumulus-oocyte complexes

Steroid concentration in the spent maturation medium of each well were collected before and after oocyte maturation and stored at -20 ºC until processing. Then, E_2 and P_4 were measured in duplicate by Enzyme-Linked ImmunoSorbent Assay (ELISA) assay, based on the principle of competitive binding using a specific kit for E_2 and P_4 (DEMEDITEC Diagnostics GmbH, Kile, Germany) (Arias-Alvarez *et al.,* 2010). The absorbance of the samples was analyzed with a spectrophotometer (Benchmark Plus Microplate Spectrophotometer, BIO-RAD, Hercules, California USA). Inter and intra-assay coefficients of variation were 6.71% and 5.60% for P_4, and 5.43% and 5.47% for E_2, respectively. The range of the assay was between 0-40 ng/mL (P_4) and 0-200 pg/mL (E_2). Results are expressed as the average E_2 and P_4 levels produced by each COCs after IVM.

3.7. Statistical analysis

Data were analyzed using the SPSS version 19 (IBM SPSS Statistics).

The mRNA transcript expression between all genes of interest studied in antral follicle, preovulatory follicle and *corpus luteum* were analyzed by a one-way ANOVA test using Tukey post- hoc test or Kruskall-Wallis test for non-parametrical samples. To compare

gene expression between oocytes and their corresponding CCs a t-student test was used. The Shapiro' Wilks test was previously used to assess normality and homogeneity of variance.

The quantification of the immunofluorescence intensity for EGF-R, IGF-1R in the early embryos stages and of IGFBPs in immature CCs and oocytes was analyzed by a one-way ANOVA test using the Tukey post-hoc test or the Kruskall- Wallis for non-parametric samples. A t-student test was used to compare the immunofluorescence intensity between mature and immature CCs and oocytes and for comparing diferential immunofluorescence between oocytes and CCs for each IGFBP. The Shapiro 'Wilks test was previously used to assess the normality and homogeneity of the variance.

Mean values were subjected to Chi-square test to compare nuclear maturation, cortical granules and mitochondria migration rates of IVM oocytes among experimental groups.

Apoptotic index and steroidogenesis production by the COCs after IVM were analyzed by a one-way ANOVA test using Tukey post- hoc test as well. The Shapiro' Wilks test was previously used to assess normality and homogeneity of variance.

Data were expressed as the mean ± s.e.m. (standard error of the mean) and the differences were considered significant when $P<0.05$.

4. RESULTS

4.1. EXPERIMENT 1. Expression of mRNA encoding EGF, IGF-1 and their receptors (EGF-R and IGF-1R), and the IGFBPs 1-6 in the preantral and antral follicles, *corpus luteum* and cumulus-oocyte complexes in the guinea pig model.

We assessed the gene expression of EGF, EGF-R, IGF-1, IGF-1R and the IGFBPs 1-6 in the guinea pig ovary. Gene expression of all genes mentioned were localized in the ovarian structures involved in the final oocyte growth and maturation, such as antral follicles and preovulatory follicles, and in the early embryo development, such as *corpus luteum*. Oocytes and their corresponding CCs, also diferentially express all candidate genes. Therefore, this Thesis demonstrates that the guinea pig ovary expresses the EGF and IGF-1 system, included all IGFBPs. For each sample the mean expression of total RNA was determined: whole ovary: 814.3 ng/μL (nanograms/microlitter), preovulatory follicles: 549 ng/μL, antral follicles: 485.3 ng/μL, oocyte: 352.6 ng/μL, CCs: 248.6 ng/μL and *corpus luteum*: 575.3 ng/μL.

4.1.1. Expression of mRNA encoding EGF and EGF-R in guinea pig ovaries, antral follicles, preovulatory follicles, corpus luteum, denuded oocytes and their cumulus cells

Expression of mRNA showed that both EGF and EGF-R were expressed in the guinea pig ovary and in all structures studied in the current work (antral follicles, preovulatory follicles, denuded oocytes and CCs). The amplified cDNA fragments were identified with 89 bp for EGF, 130 bp for EGF-R and 158bp for β-actin (Figure 20).

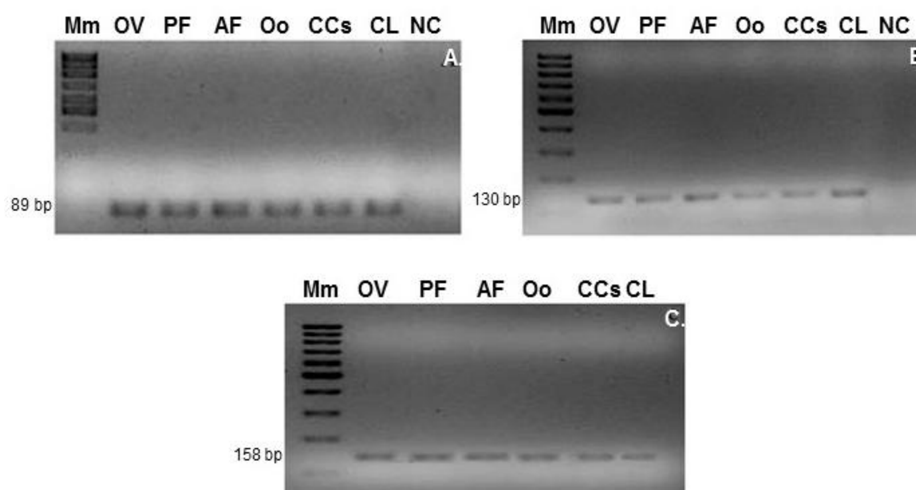

Figure 20. Representative electrophoresis in a 2% agarose gel showing the expression. A) EGF, B) EGF-R, C) β-actin in guinea pig. OV (Whole ovary), PF (Preovulatory Follicle), AF (Antral Follicles), Oo (Oocytes), CCs (Cumulus Cells), CL (*Corpus luteum*), NC (Negative Control) and Mm (100 bp DNA marker).

As it is shown in Figure 21, expression level of EGF and EGF-R was significantly higher in antral follicles compared to the preovulatory follicles (P<0.05). *Corpus luteum* showed lower mRNA transcripts of EGF than those observed in antral follicles and preovulatory follicles (P<0.05) but showed similar values of EGF-R than those found in the antral follicles. In oocytes mRNA transcripts of both EGF and EGF-R were significantly higher than in CCs (Figure 22).

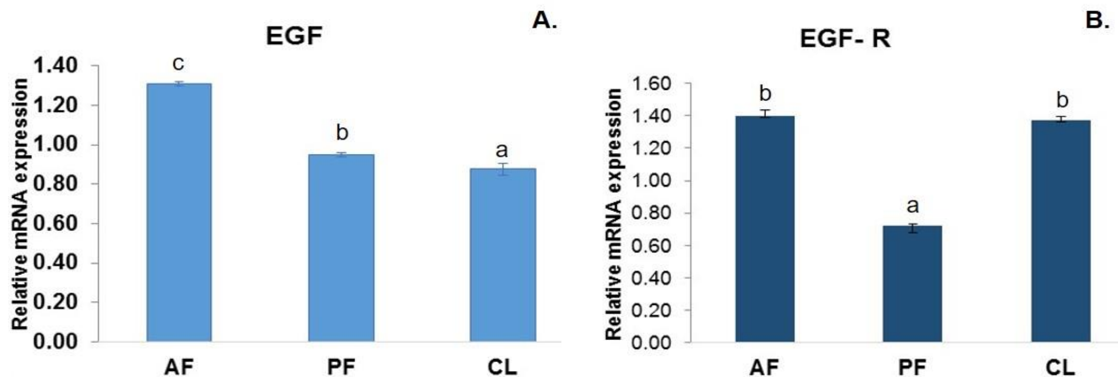

Figure 21. EGF and EGF-R mRNA expression in ovarian antral and preovulatory follicles, and *corpus luteum.* Densitometry readings of A) EGF, B) EGF-R (are presented as ratio of readings of corresponding β-actin samples in guinea pig ovarian structures studied. AF (Antral Follicles), PF (Preovulatory Follicle), and *CL (Corpus luteum).* Bar diagram represent the mean of the normalized integral density for each mRNA band (mean ± s.e.m). Different letters indicate significant differences (P< 0.05).

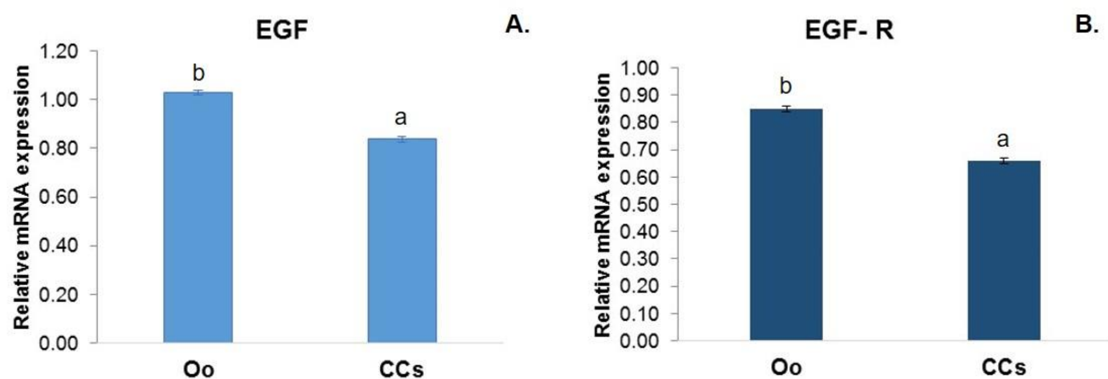

Figure 22. EGF and EGF-R mRNA expression in oocytes and their corresponding cumulus cells. Densitometry readings of A) EGF, B) EGF-R (are presented as ratio of readings of corresponding β-actin samples in guinea pig. Oo (Oocyte) and CCs (Cumulus Cells). Bar diagram represent the mean of the normalized integral density for each mRNA band (mean ± s.e.m). Different letters indicate significant differences (P< 0.05).

4.1.2. Expression of mRNA encoding IGF-1 and IGF-1R in guinea pig antral follicles, preovulatory follicles, corpus luteum, denuded oocytes and their cumulus cells

Expression of IGF-1 and IGF-1R mRNA could be detected in the whole ovary and specifically in antral and preovulatory follicles, *corpus luteum*, denuded oocytes and their CCs. The amplified cDNA fragments were 104 and 122 bp, for IGF-1 and IGF-1R, respectively (Figure 23).

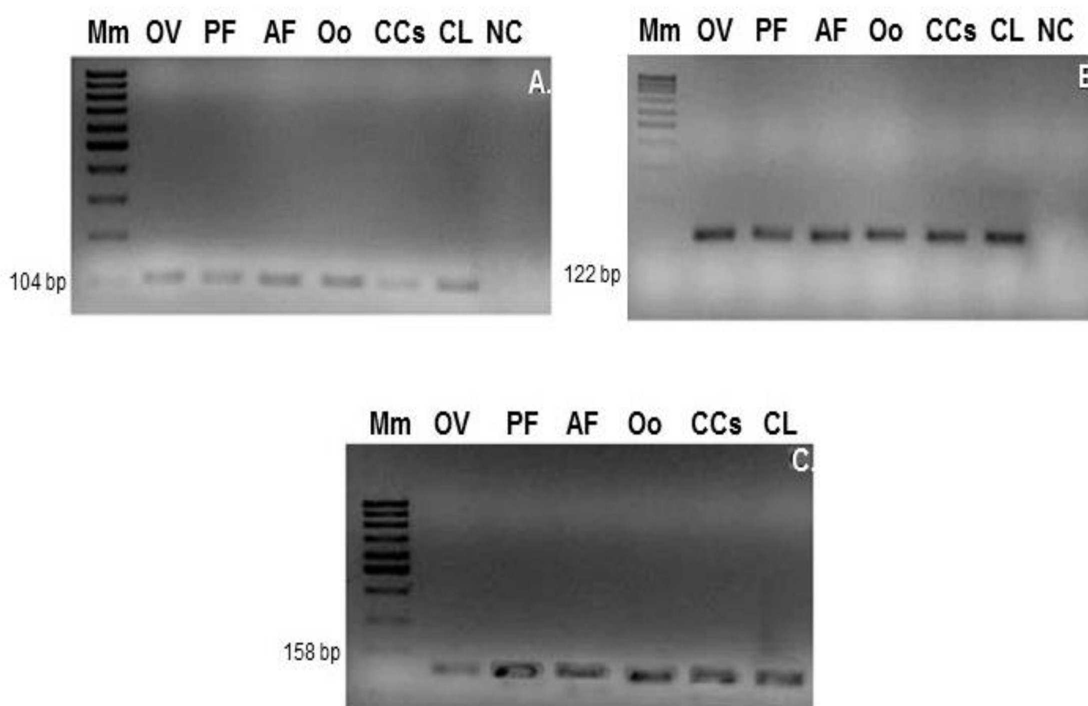

Figure 23. Representative electrophoresis in a 2% agarose gel showing the expression of A) IGF-1, B) IGF-1R, C) β-actin in guinea pig ovary. OV (Whole ovary), PF (Preovulatory Follicle), AF (Antral Follicles), Oo (Oocytes), CCs (Cumulus Cells), CL (*Corpus luteum*), NC (Negative Control) and Mm (100 bp DNA marker).

As depicted in Figure 24 expression level of IGF-1 and IGF-1R was significantly higher in antral follicles compared to the preovulatory follicles ($P<0.05$). *Corpus luteum* showed the highest mRNA transcripts of IGF-1R ($P<0.05$) but showed intermediate values of IGF-1 compared with those found in antral and preovulatory follicles. Oocytes showed significantly higher IGF-1 mRNA transcripts ($P<0.05$) but lower IGF-1R ($P<0.05$) than their corresponding cumulus cells.

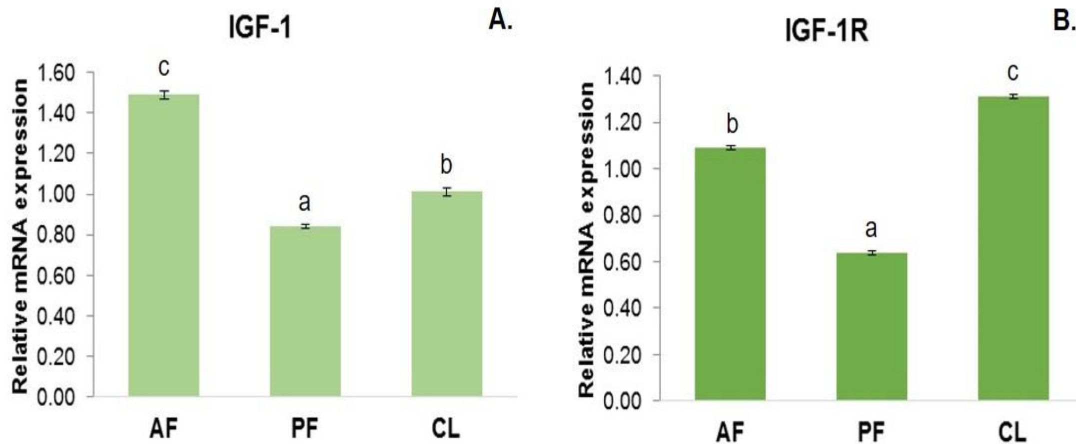

Figure 24. IGF-1 and IGF-1R mRNA expression in ovarian antral and preovulatory follicles, and corpus luteum. Densitometry readings of A) IGF-1, B) IGF-1R (are presented as ratio of readings of corresponding β-actin samples in guinea pig ovarian structures studied. AF (Antral Follicles), PF (Preovulatory Follicle), and *CL (Corpus luteum).* Bar diagram represent the mean of the normalized integral density for each mRNA band (mean ± s.e.m). Different letters indicate significant differences (P< 0.05).

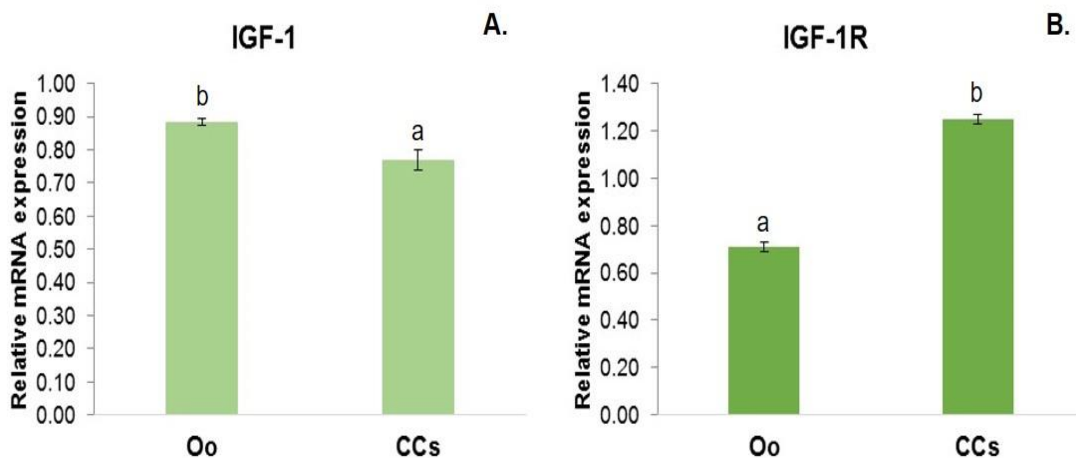

Figure 25. IGF-1 and IGF-1R mRNA expression in oocytes and their corresponding cumulus cells. Densitometry readings of A) IGF-1, B) IGF-1R (are presented as ratio of readings of corresponding β-actin samples in guinea pig. Oo (Oocyte) and CCs (Cumulus Cells). Bar diagram represent the mean of the normalized integral density for each mRNA band (mean ± s.e.m). Different letters indicate significant differences (P< 0.05).

4.1.3. Expression of mRNA encoding IGFBPs 1-6 in guinea pig ovaries, antral follicles, preovulatory follicles, corpus luteum, denuded oocytes and their cumulus cells

The mRNA expression of the IGFBPs was found in all structures studied by RT-PCR and is shown in Figure 26. The amplified cDNA fragments were 117, 139, 60, 141, 71 and 112 bp, for IGFBP-1, 2, 3, 4, 5 and 6, respectively.

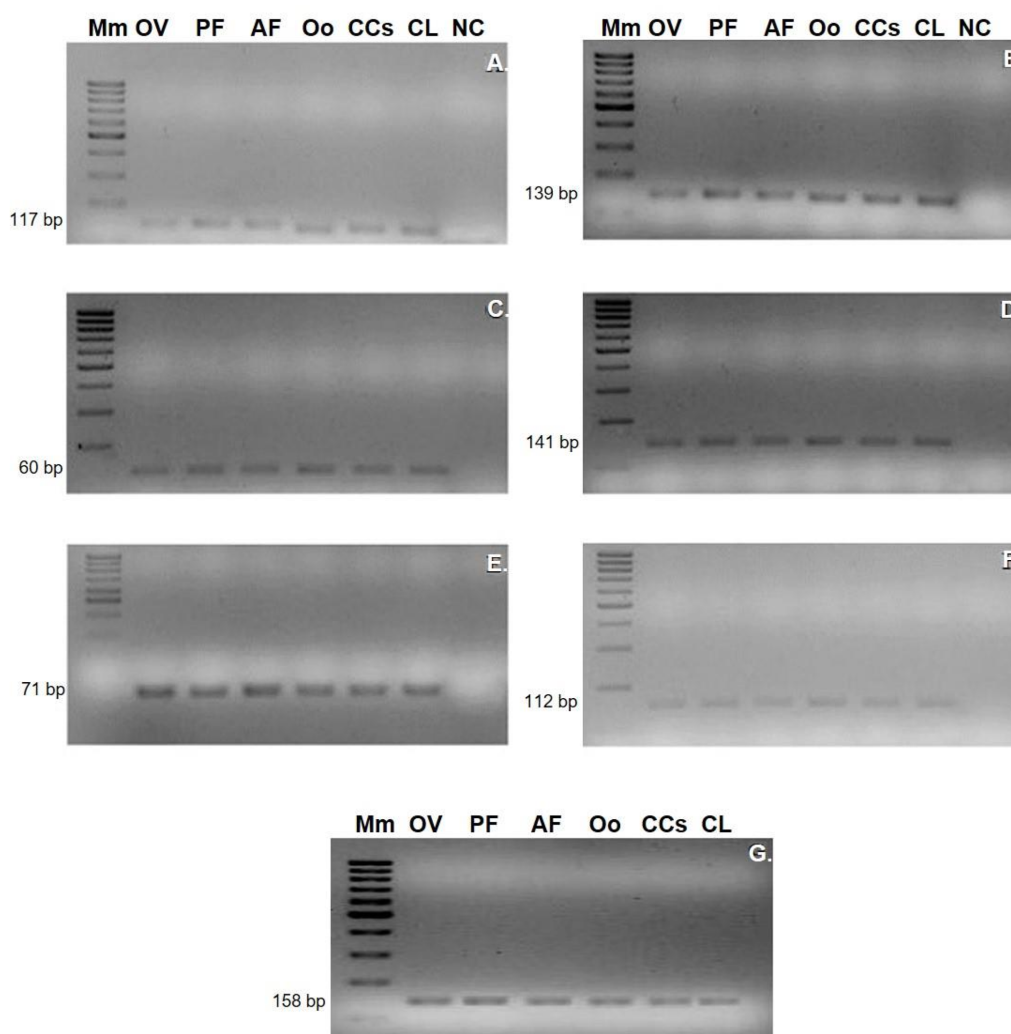

Figure 26. Representative electrophoresis in a 2% agarose gel showing the expression of A) IGFBP-1, B) IGFBP-2, C) IGFBP-3, D) IGFBP-4, E) IGFBP-5, F) IGFBP-6, G) β-actin in guinea pig. OV (Whole ovary), PF (Preovulatory Follicle), AF (Antral Follicles), Oo (Oocytes), CCs (Cumulus Cells), CL (*Corpus luteum*), NC (Negative Control) and Mm (100 bp DNA marker).

In the ovary, the relative mRNA expression of the IGFBPs in different stages of follicles and *corpus luteum* is shown in Figure 27. IGFBP 1, 2 and 4 showed similar expression pattern in follicles. mRNA transcripts were significantly down-regulated in antral follicles

compared to preovulatory follicles (P<0.05). *Corpus luteum* showed the highest values (P<0.05) for IGFBP-1 and 2 compared to follicles whereas IGFBP-4 showed intermediate results. IGFBP 3 and 5 showed similar pattern of gene expression in the structures studied. IGFBP-3 and 5 were significantly up-regulated in antral follicles compared to the gene expression displayed in preovulatory follicles. *Corpus luteum* exhibited the lowest expression pattern of IGFBP- 3 and 5. mRNA transcript of IGFBP-6 was similar in both follicular stages studied whereas *Corpus luteum* showed the lowest levels of gene expression of IGFBP-6.

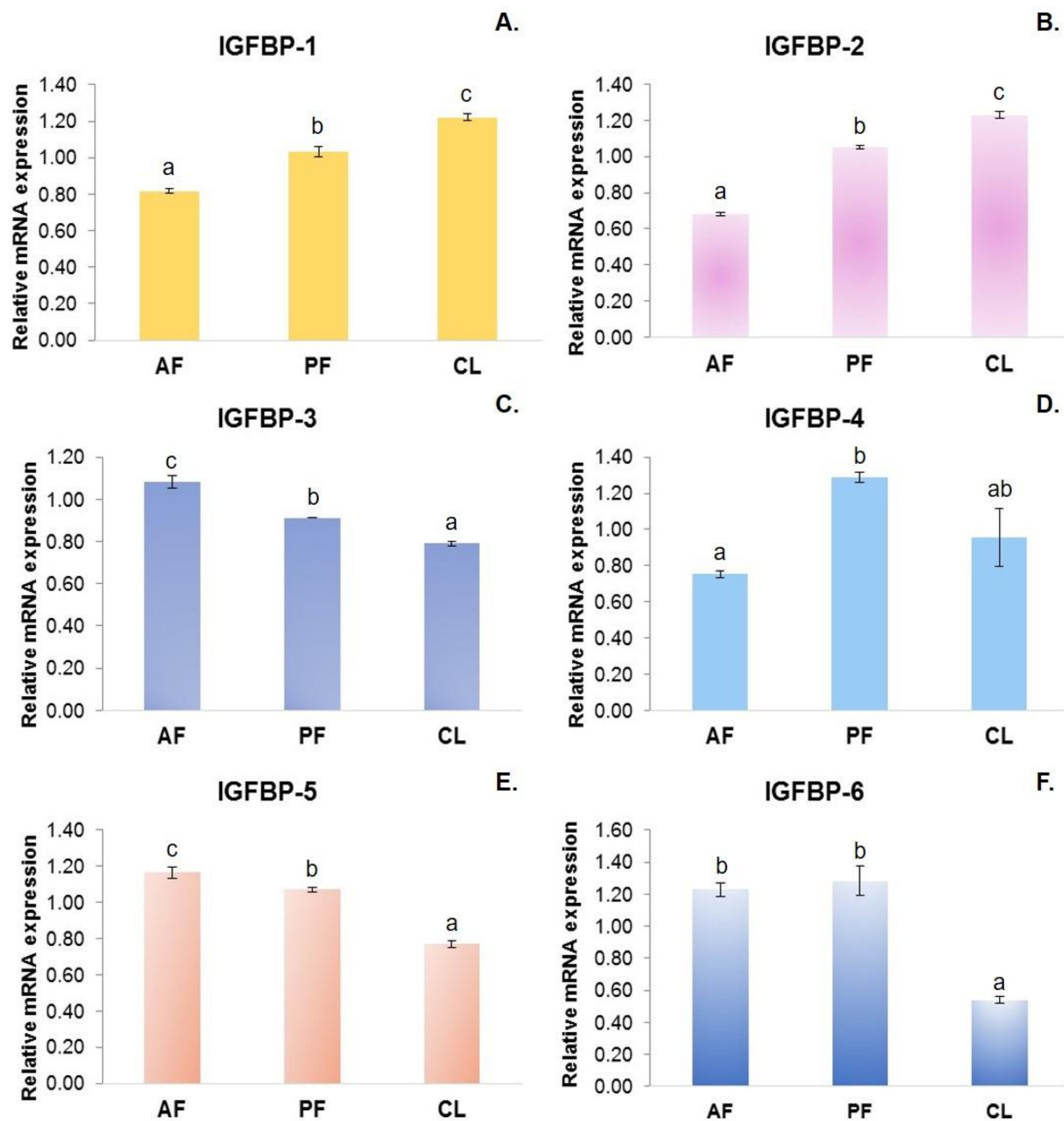

Figure 27. IGFBPs 1-6 mRNA expression. Densitometric readings of IGFBPs 1-6 are presented as ratio of readings of corresponding β-actin samples in guinea pig ovarian. AF (Antral Follicles), PF (Preovulatory Follicle), and *CL (Corpus luteum).* Bar diagram represent the mean of the normalized integral density for each mRNA band (mean ± s.e.m). Different letters indicate significant differences (P< 0.05).

The relative mRNA expression of the IGFBPs was different in oocytes and their corresponding CCs as it is showed in Figure 28. IGFBP-1, 2, 3 and 4 mRNA expression was found higher in oocytes than in their corresponding CCs ($P<0.05$) and contrarily to IGFBP 5. The expression of IGFBP-6 was found similar in oocytes and CCs.

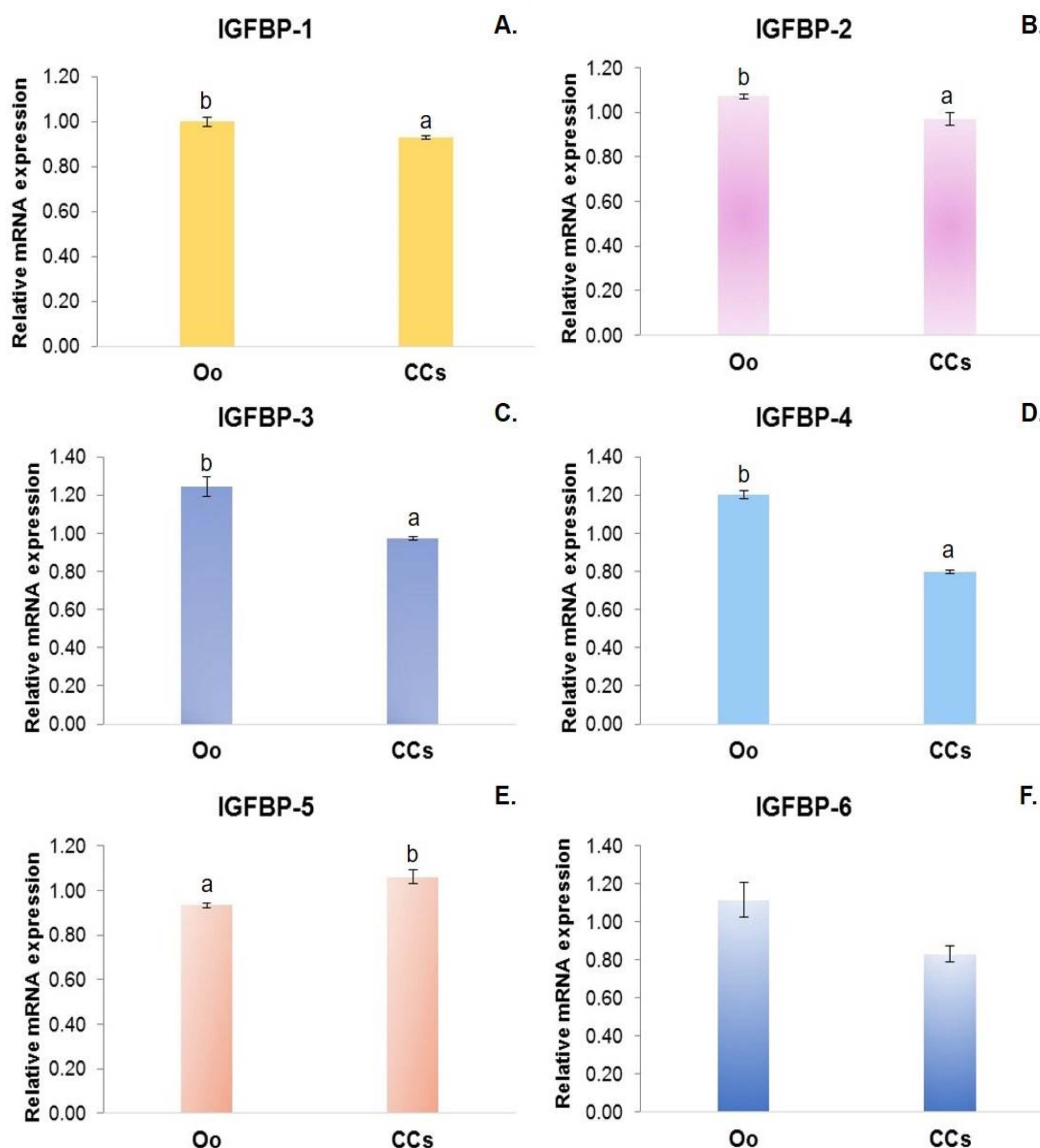

Figure 28. IGFBPs 1-6 mRNA expression in COCs. Densitometric readings of IGFBPs 1-6 are presented as ratio of readings of corresponding β-actin samples in guinea pig ovarian. Oo (Oocyte) and CCs (Cumulus Cells). Bar diagram represent the mean of the normalized integral density for each mRNA band (mean ± s.e.m). Different letters indicate significant differences ($P< 0.05$).

4.2. EXPERIMENT 2. Immunolocalization of EGF-R and IGF-1R and their ligands (IGFBPs 1-6) in ovaries, follicles, *corpus luteum*, cumulus-oocyte complexes and early embryos in the guinea pig model

4.2.1. Experiment 2.a. Immunolocalization of EGF-R and IGF-1R and their ligands (IGFBPs 1-6) by immunohistochemistry in ovaries of guinea pig.

4.2.1.1. Immunolocalization of EGF-R in guinea pig ovaries

The immunolocalization of EGF-R was found in *Corpus luteum* and follicles of guinea pig in all stages of development as is illustrated in Figure 29. In follicles, EGF-R was localized in the granulosa and theca cells and in oocytes (Figure 30). Non-specific immunoreaction was observed when staining was performed in the negative control. In the secondary and AF, EGF-R immunostaining was distributed in the oocyte, granulosa and theca cells. Luteal cells in the CL showed EGF-R immunostaining as well.

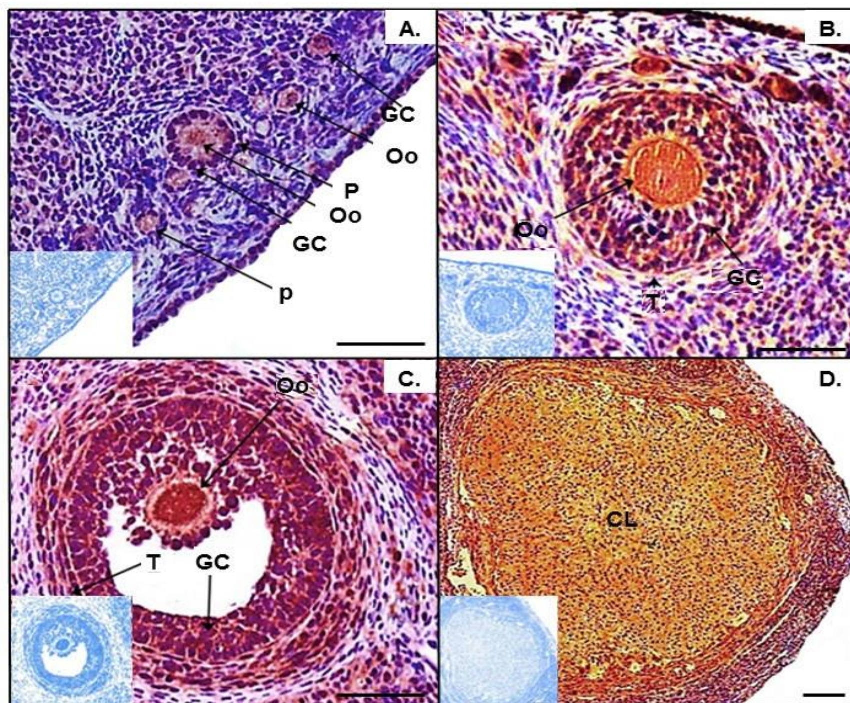

Figure 29. Immunolocalization of EGF-R in guinea pig ovary. Red staining denotes the localization of the receptor. A) Primordial and primary follicles (GC, Oo: ++), B) Secondary follicles (GC, Oo and T: ++), C) Antral follicles (GC, Oo and T: +++), D) *Corpus luteum* (CL: +++). Gc (Granulose Cells), Oo (Oocyte), T (Theca cells), CL (*Corpus luteum*), p (Primordial follicle), P (Primary follicle). Representative photomicrographs are shown. Non-specific immunoreaction was observed in the negative control. Scale is 200 µm.

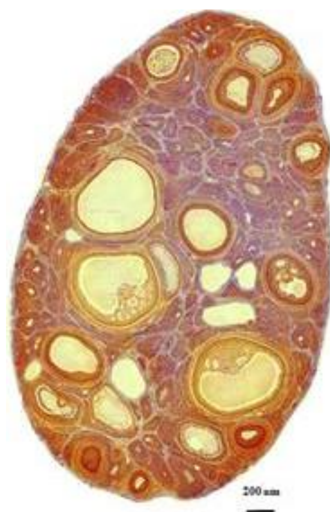

Figure 30. General view of Immunolocalization of EGF-R in guinea pig ovary

As is shown in Table 4, the most of primordial and primary follicles showed moderate staining (++) in the granulosa cells and oocyte. In the secondary follicles a stronger EGF-R immunoreaction (+++) was showed in the granulosa cells and oocyte. Most of the theca cells showed moderate immunoreaction (++). In antral follicle, a stronger EGF-R immunostaining was localized in both the oocyte, granulosa and theca cells (+++) in the majority of follicles. Furthermore, a stronger EGF-R immunoreactivity (+++) was observed in the *corpus luteum* (Figure 30).

4.2.1.2. Immunolocalization of IGF-1R in guinea pig ovaries

IGF-1R immunoreaction was detected in oocytes, granulosa cells and theca cells in ovarian follicles of guinea pig in all stages of development as well as in *corpus luteum* (Figure 31). IGF-1R immunostaining was showed in the granulosa and theca cells as well as in the luteal cells. Non- specific immunoreaction was observed when staining was performed in the negative control.

Differences in the intensity of staining in the stromal cells, granulosa and theca cells, oocytes and *corpus luteum* were shown in Table 5. The primordial and primary follicles showed strong staining (+++) in oocytes and granulosa cells. In the secondary follicles, strong immunoreaction (+++) was observed in granulosa cells and oocytes whereas the most of the theca cells showed weak immunoreaction (+). In antral follicle, a stronger (+++) IGF-1R immunostaining was also detected in the oocyte, granulosa and theca cells. Furthermore, *corpus luteum* presented a stronger IGF-1R staining (+++) too.

Table 4. Immunolocalization of EGF-R in the guinea pig ovary

Follicles	Granulosa cells			Theca cells			Oocytes			Luteal cells		
	+	++	+++	+	++	+++	+	++	+++	+	++	+++
Primordial n (%)	39 (27.0)	61 (42.7)	44 (30.5)	-	-	-	12 (27.9)	21 (48.8)	10 (23.3)	-	-	-
Primary n (%)	9 (11.5)	48 (61.5)	21 (26.9)	-	-	-	10 (33.3)	17 (56.7)	3 (10.0)	-	-	-
Secondary n (%)	3 (12.5)	8 (33.3)	13 (54.2)	4 (16.8)	11 (45.8)	9 (37.5)	2 (12.5)	2 (12.5)	12 (75.0)	-	-	-
Antral n (%)	2 (3.8)	7 (13.2)	44 (83.0)	3 (5.7)	13 (24.5)	37 (69.8)	3 (11.1)	3 (11.1)	21 (77.8)	-	-	-
Corpus luteum n (%)	-	-	-	-	-	-	-	-	-	2 (20.0)	2 (20.0)	6 (60.0)

(-) No staining (+) weak staining; (++) moderate staining and (+++) strong staining.

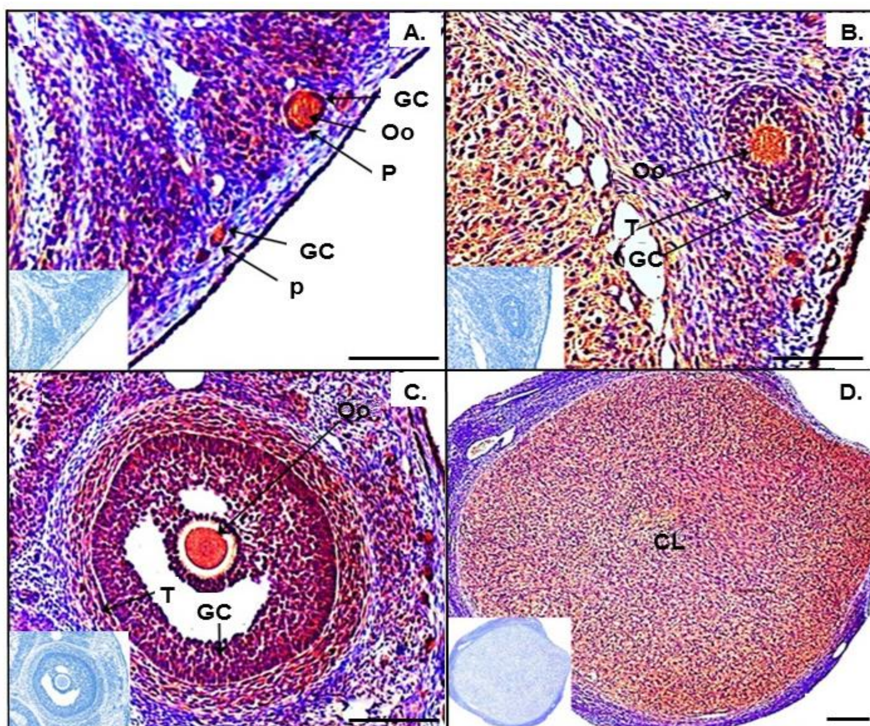

Figure 31. Immunolocalization of IGF-1R in guinea pig ovary. Red staining denotes the localization of the receptor. A) Primordial and primary follicles (GC, Oo: ++), B) Secondary follicles (GC, Oo: +++; T: +), C) Antral follicles (GC, Oo and T: +++), D) *Corpus luteum* (CL: +++). GC (Granulosa Cells); Oo (Oocyte); T (Theca cells); CL *(Corpus luteum*); p (primordial follicle); P (primary follicle). Representative photomicrographs are shown. Non-specific immunoreaction was observed in the negative control. Scale is 200 µm.

4.2.1.3. Immunolocalization of IGFBPs 1-6 in guinea pig ovaries

The IGFBPs 1, 2, 3, 4, 5 and 6 proteins were inmunolocalized in ovaries of guinea pig. Follicles at all stages of development exhibited immunoreaction to each IGFBP and the signal was distributed in all follicular compartments (granulosa and theca cells and oocyte) and in *corpus luteum* although with different intensity (Table 6). Non- specific immunostaining was observed in the negative controls.

Table 5. Immunolocalization of IGF-1R in the guinea pig ovary

Follicles	Granulosa cells			Theca cells			Oocytes			Luteal cells		
	+	++	+++	+	++	+++	+	++	+++	+	++	+++
Primordial n (%)	55 (14.7)	113 (30.2)	206 (55.1)	-	-	-	27 (36.9)	36 (49.31)	10 (13.7)	-	-	-
Primary n (%)	39 (16.0)	92 (37.7)	113 (46.3)	-	-	-	12 (10.6)	21 (18.6)	80 (70.8)	-	-	-
Secondary n (%)	5 (13.5)	12 (32.4)	20 (54.1)	21 (56.8)	7 (18.9)	9 (24.3)	2 (7.1)	5 (17.9)	21 (75.0)	-	-	-
Antral n (%)	2 (4.4)	9 (19.6)	35 (76.0)	6 (13.0)	16 (34.8)	24 (52.2)	2 (10.0)	2 (10.0)	16 (80.0)	-	-	-
Corpus luteum n (%)	-	-	-	-	-	-	-	-	-	2 (20.0)	2 (20.0)	6 (60.0)

(-) No staining (+) weak staining; (++) moderate staining and (+++) strong staining.

i. IGFBP-1

As depicted Figure 32 and Table 6, primordial and primary follicles showed strong staining (+++) in granulosa cells for IGFBP-1. In the secondary follicles a stronger immunoreaction (+++) was observed in the theca cells whereas oocyte and granulosa cells showed moderate staining (++). In antral follicle, oocyte exhibited a moderate staining (++) and granulosa and theca cells showed a weak staining (+). Also a moderate IGFBP-1 immunoreactivity (++) was observed in the *corpus luteum*.

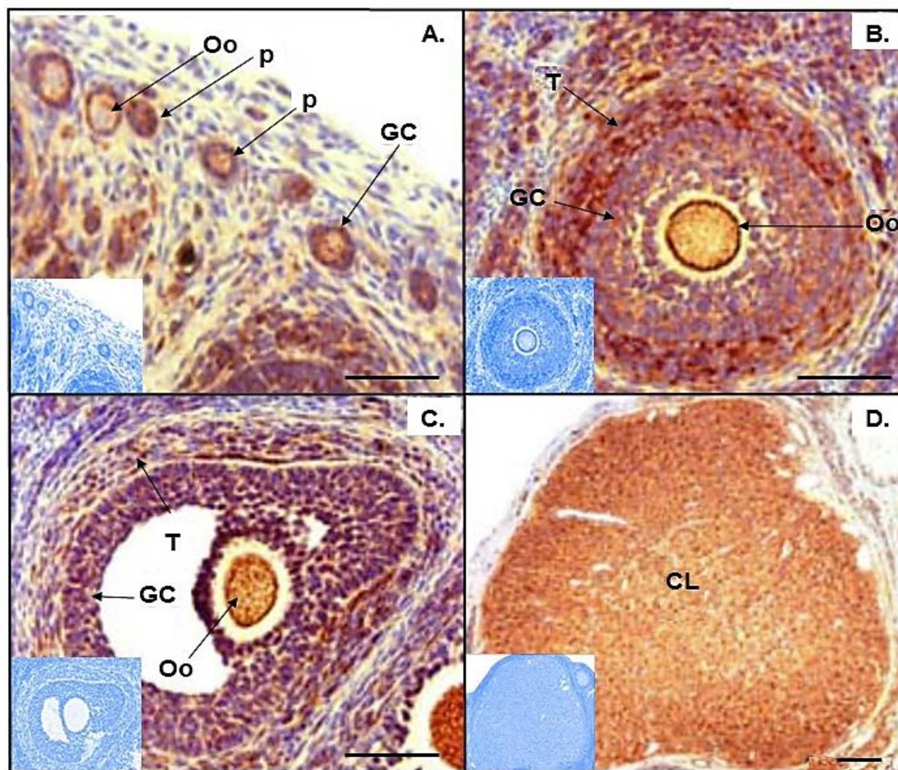

Figure 32. Immunolocalization of IGFBP-1 in guinea pig ovary. Red staining denotes the localization of the receptor. A) Primordial follicles (GC: +++), B) Secondary (T: +++; GC:++), C) Antral follicle (GC and T: +; Oo:++), D) *Corpus luteum* (CL: ++/+++). GC (Granulosa Cells); Oo (Oocyte); T (Theca cells); CL (*Corpus luteum*); p (primordial follicle); P (primary follicle). Representative photomicrographs are shown. Non-specific immunoreaction was observed in the negative control. Scale is 200 µm.

Table 6. Immunolocalization of IGFBPs 1-6 in the guinea pig ovary

	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
Primordial Follicle						
Oocyte	+	+	+	+	+	+
(n)	(774)	(698)	(693)	(503)	(689)	(673)
Granulosa cells	+++	+++	+	+	+	+
(n)	(774)	(698)	(693)	(503)	(689)	(673)
Primary Follicle						
Oocyte	+	+	+	+	+	+
(n)	(679)	(624)	(597)	(657)	(672)	(646)
Granulosa cells	+++	+++	+	+	+	+
(n)	(679)	(624)	(597)	(657)	(672)	(646)
Secondary follicle						
Oocyte	++	+	+	+	+	+
(n)	(103)	(128)	(114)	(119)	(123)	(118)
Granulosa cells	++	+++	+	+	+	+
(n)	(103)	(128)	(114)	(119)	(123)	(118)
Theca cells	+++	+	+	+	+	+
(n)	(103)	(128)	(114)	(119)	(123)	(118)
Antral						
Oocyte	++	+	+	+	+	+
(n)	(94)	(91)	(93)	(95)	(90)	(93)
Granulosa cells	+	+++	+	+	++	+
(n)	(94)	(91)	(93)	(95)	(90)	(93)
Theca cells	+	++	+	+	+	+
(n)	(94)	(91)	(93)	(95)	(90)	(93)
Corpus luteum						
Luteal cells	++	+++	+++	+	+	+
(n)	(13)	(13)	(13)	(13)	(13)	(13)

n: represent number of structures analyzed; (+) weak staining; (++) moderate staining and (+++) strong staining.

ii. IGFBP-2

Immunoreaction of IGFBP-2 in the granulosa cell of primordial, primary secondary and antral follicle was intense (+++) (Figure 33 and Table 6). Oocyte was considerate weak stained (+) to IGFBP-2 in all stages of follicular development. Inmunostaining was increased in the theca cells from weak staining (+) in secondary to moderate staining (++) in antral follicle. In the *corpus luteum* a strong staining (+++) was observed (Table 6). There was no staining in the negative controls.

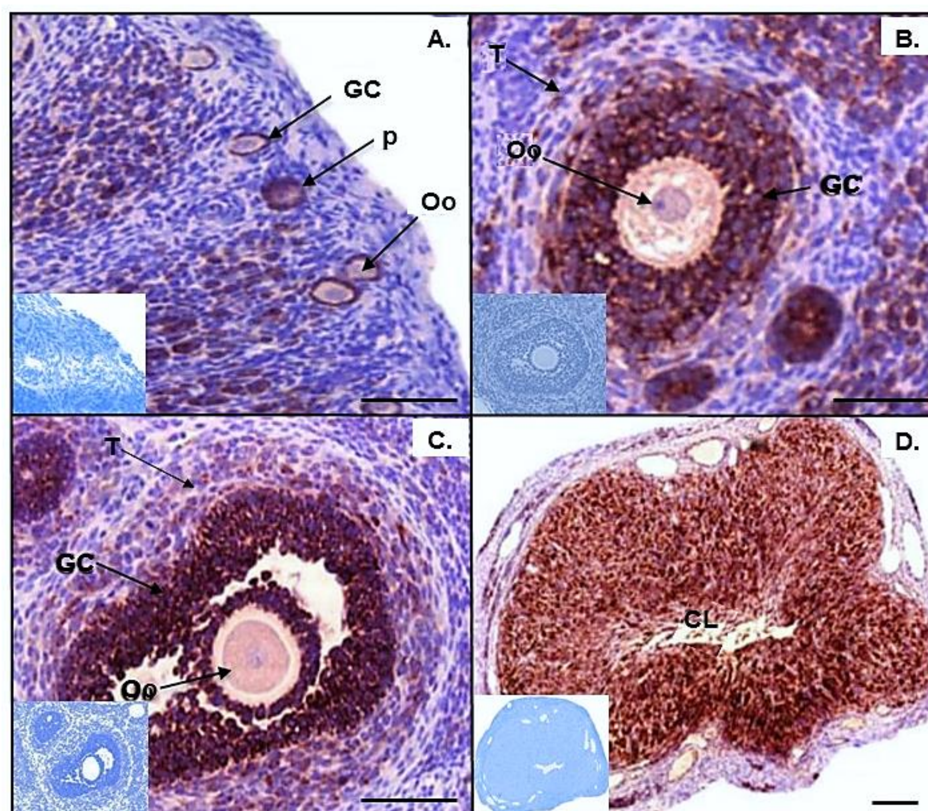

Figure 33. Immunolocalization of IGFBP-2 in guinea pig ovary. Red staining denotes the localization of the receptor. A) Primordial follicles (Oo: +; GC: +++), B) Secondary (Oo: +; GC: +++; T:+), C) Antral follicle (Oo: +; GC: +++; T: ++), D) *Corpus luteum* (CL: +++). GC (Granulosa Cells); Oo (Oocyte); T (Theca cells); CL (*Corpus luteum*); p (primordial follicle). Representative photomicrographs are shown. Non-specific immunoreaction was observed in the negative control. Scale is 200 µm.

iii. IGFBP-3

As it is shown in Figure 34 and Table 6, oocyte, granulosa and theca cells of all follicles from primordial to antral showed weak staining (+), whereas *Corpus luteum* presented the strongest signal (+++). There was no staining in the negative controls.

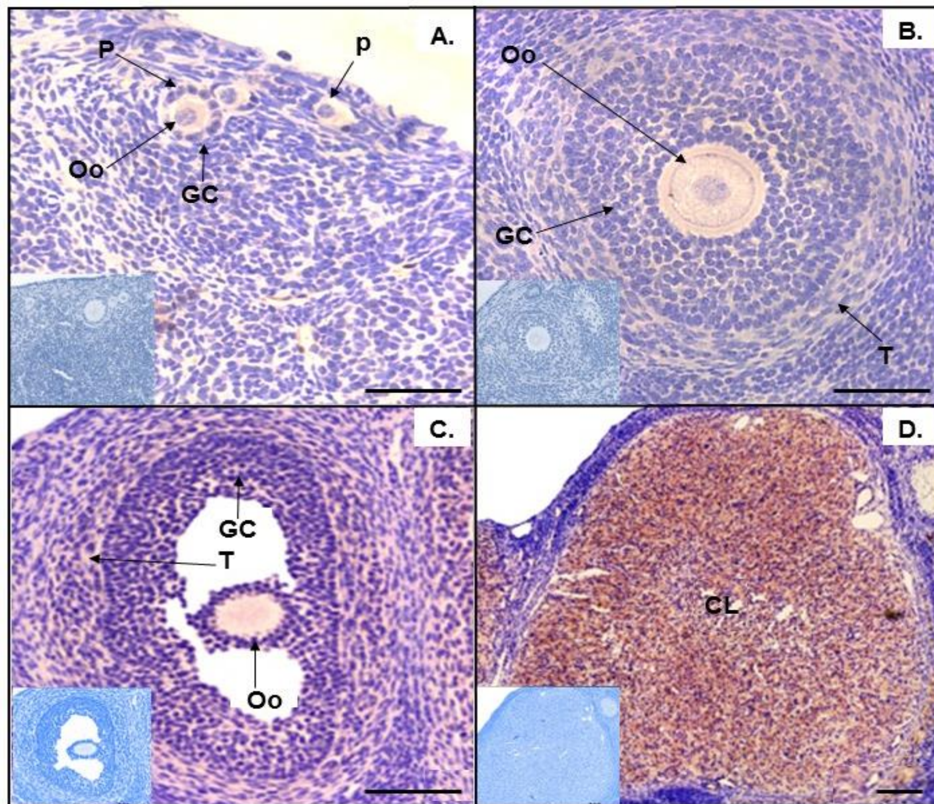

Figure 34. Immunolocalization of IGFBP-3 in guinea pig ovary. Red staining denotes the localization of the receptor. A) Primary and primordial follicles (Oo: +; GC: +), B) Secondary (Oo, GC and T:+), C) Antral follicle follicle (Oo, GC, and T: +), D) *Corpus luteum* (CL: +++). GC (Granulosa Cells); Oo (Oocyte); T (Theca cells); CL *(Corpus luteum*); p (primordial follicle), P (Primary). Representative photomicrographs are shown. Non-specific immunoreaction was observed in the negative control. Scale is 200 µm.

iv. IGFBP-4

The localization of IGFBP-4 (Figure 35) was similar for all the compartments in the ovary, which exhibited weak staining (+) in oocytes, granulosa cells and theca cells in every stage of follicular development (Table 6). *Corpus luteum* show weak staining for IGFBP-4 as well. Negative controls didn´t show any immunoreaction signal.

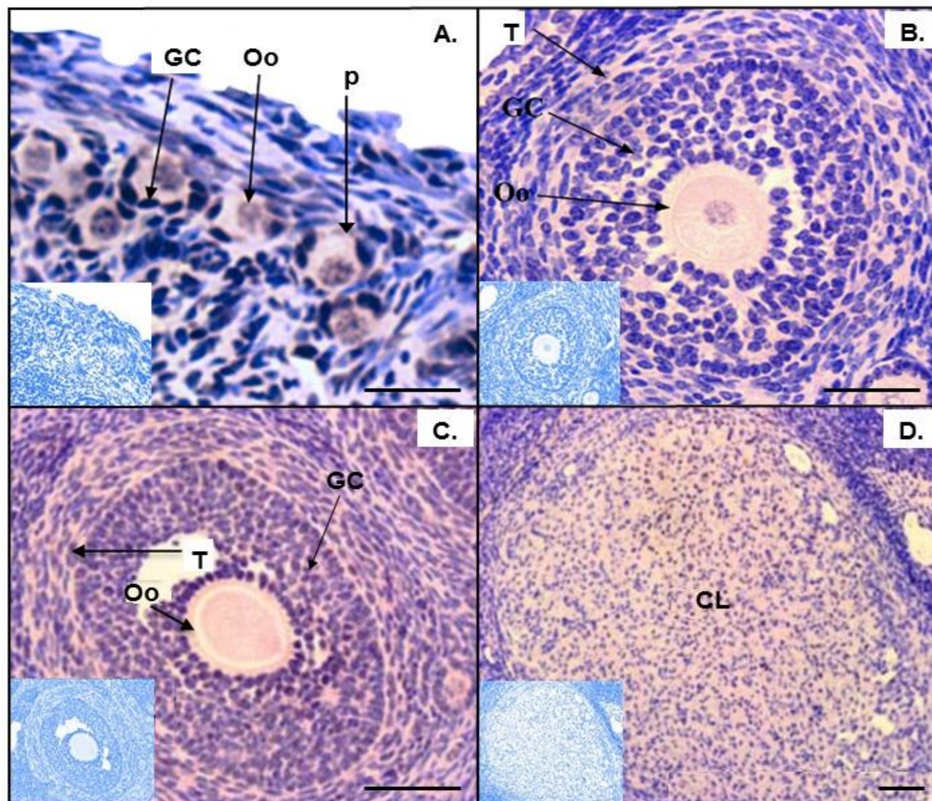

Figure 35. Immunolocalization of IGFBP-4 in guinea pig ovary. Red staining denotes the localization of the receptor. A) Primordial follicles (Oo and CG:+), B) Secondary follicle (Oo, GC and T: +), C) Antral follicle (Oo, GC and T: +), D) *Corpus luteum* (CL: +). GC (Granulosa Cells); Oo (Oocyte); T (Theca cells); CL (*Corpus luteum*); p (primordial follicle), P (Primary). Representative photomicrographs are shown. Non-specific immunoreaction was observed in the negative control. Scale is 200 µm.

v. IGFBP-5

As it is shown in Figure 36 and Table 6 the intensity of IGFBP-5 signal was weak (+) in oocytes, granulosa cells and theca cells in primordial, primary, secondary and antral follicle of guinea pig. Oocytes in antral follicle showed weak/moderate staining (+/++) In the *corpus luteum* a weak staining (+) was also evidenced. There was no staining in the negative controls.

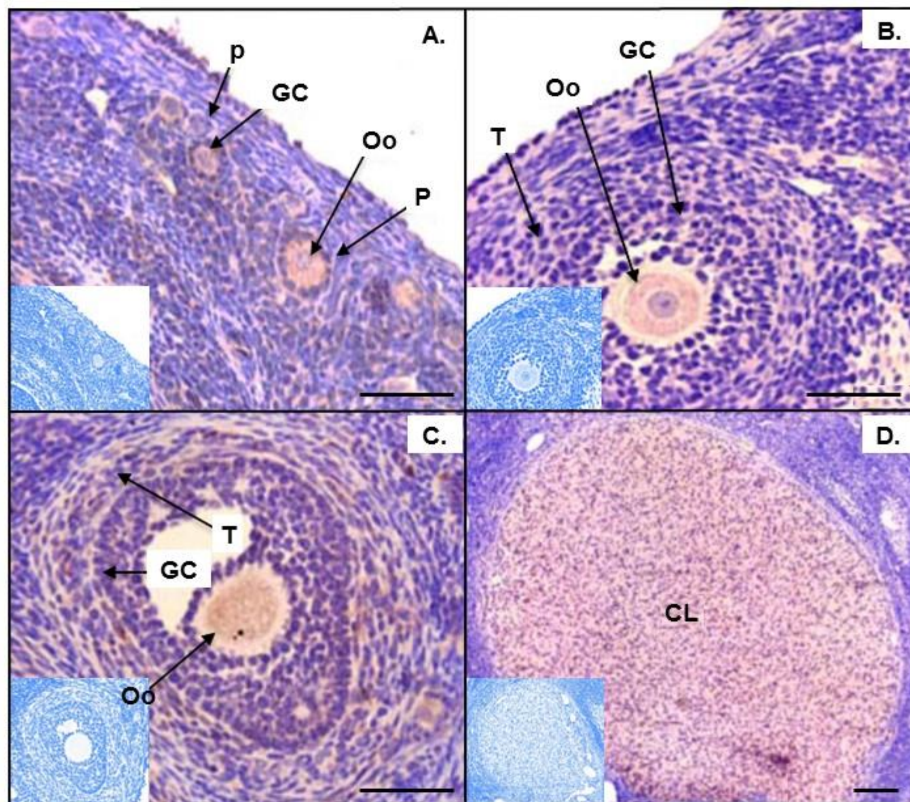

Figure 36. Immunolocalization of IGFBP-5 in guinea pig ovary. Red staining denotes the localization of the receptor. A) Primordial and primary follicle (GC and Oo:+), B) Secondary follicle (GC, Oo and T: +), C) Antral follicle (Oo: ++ GC and T: +), D) *Corpus luteum* (CL: +). GC (Granulosa Cells); Oo (Oocyte); T (Theca cells); CL (*Corpus luteum*); p (primordial follicle), P (Primary). Representative photomicrographs are shown. Non-specific immunoreaction was observed in the negative control. Scale is 200 µm.

vi. IGFBP-6

It was found a weak (+) presence of IGFBP-6 in granulosa cells regardless of follicle type as well as in the *corpus luteum* (Figure 37 and Table 6). Signal in oocyte was weak (+) for all follicular stages. IGFBP-6 intensity in theca cells showed a weak (+) staining in secondary follicles and antral follicle. No specific signal was observed in the negative controls

.

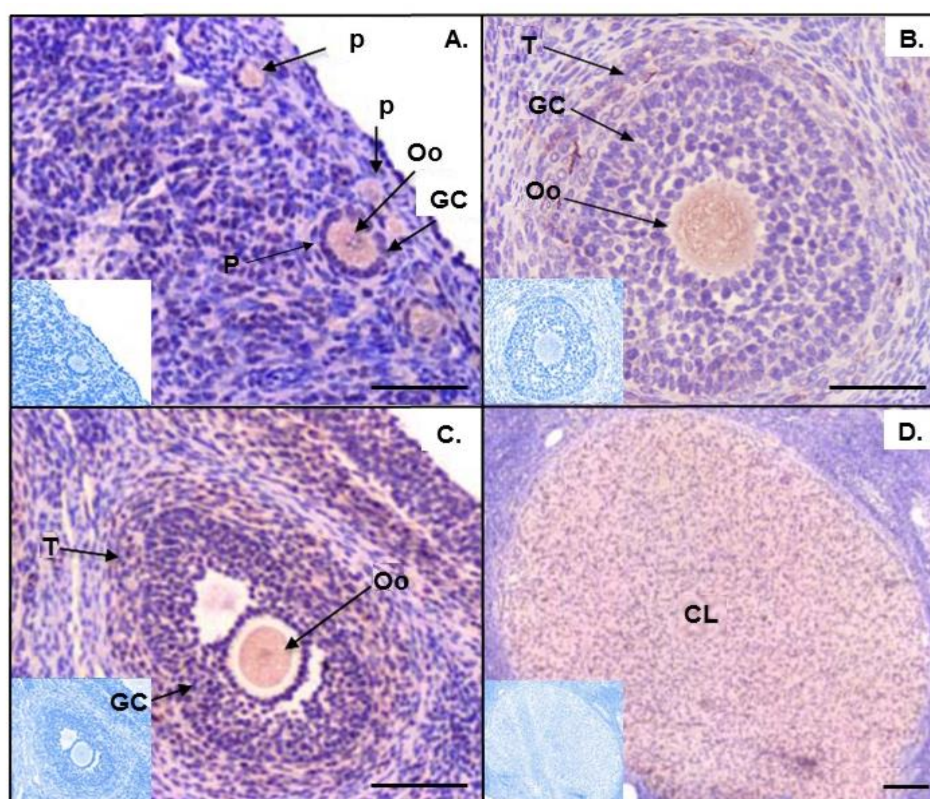

Figure 37. Immunolocalization of IGFBP-6 in guinea pig ovary. Red staining denotes the localization of the receptor. A) Primordial and primary follicles (Oo and GC: +), B) Secondary follicle (Oo, GC and T: +), C) Antral follicle (Oo; GC and T: +), D) *Corpus luteum* (CL: +). GC (Granulosa Cells); Oo (Oocyte); T (Theca cells); CL (*Corpus luteum*); p (primordial follicle), P (Primary). Representative photomicrographs are shown. Non-specific immunoreaction was observed in the negative control. Scale is 200 µm.

4.2.2. Experiment 2.b. Immunolocalization of EGF-R and IGF-1R and their ligands (IGFBPs 1-6) by immunocytochemistry in cumulus-oocyte complexes and in the early embryos of guinea pig.

4.2.2.1. Immunolocalization of EGF-R in cumulus-oocyte complexes and early embryos

Indirect immunofluorescence assays showed that EGF-R was present in immature- and *in vitro* matured oocytes and their corresponding CCs (Figure 38). EGF-R was detected in the cellular membrane of the cells for both oocytes and CCs regardless if they were matured or immature. Significant differences were found in the immunofluorescence staining pattern between immature and matured COCs (P<0.05), being intensity higher in both oocyte and CCs after *in vitro* maturation (P<0.05). There was no staining in the negative controls.

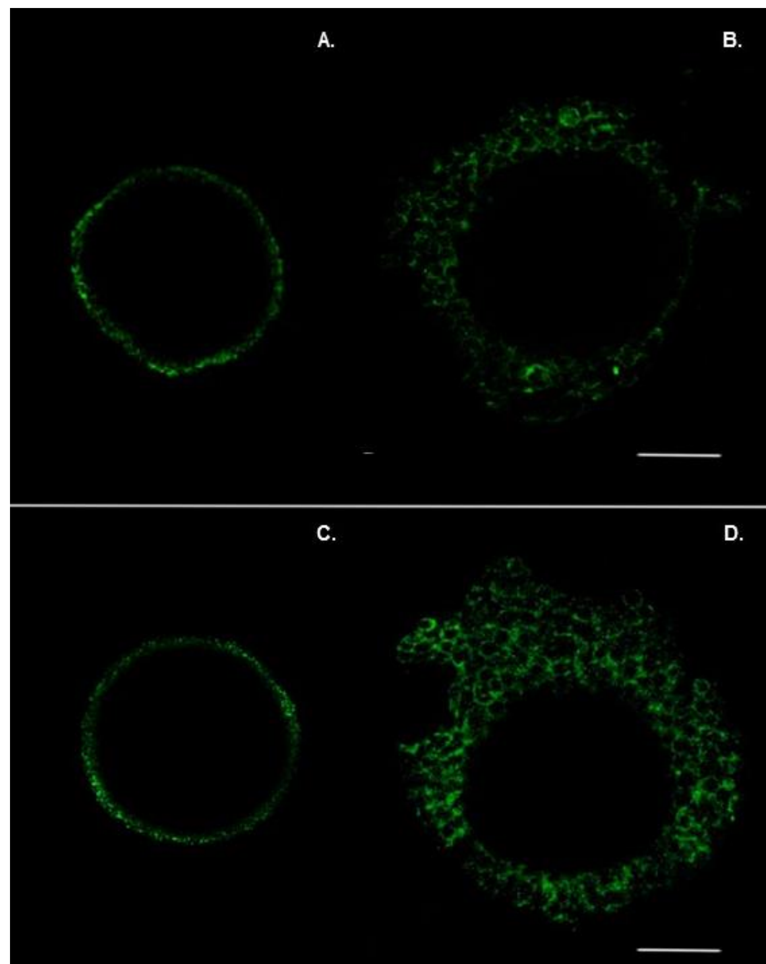

Figure 38. Localization by immunocytochemistry of EGF-R in immature and matured Oo and CCs of guinea pig. A) Immature Oo, B) Immature CCs, C) Oo matured, D) CCs after *in vitro* maturation. The distribution of EGF-R is shown in green fluorescence. Oo (Oocyte) and CCs (Cumulus Cells). Scale 30 µm. Representative photomicrographs are shown.

In the embryos, EGF-R immunoreactivity was observed also in the cell membrane of the blastomeres (Figure 39). EGF-R was expressed at increasing levels on preimplantation embryos to morula stage. Four cell embryos exhibited significantly lower inmunostaining compared to morulas ($P<0.05$) whereas in 8-16 cell embryo displayed intermediate levels.

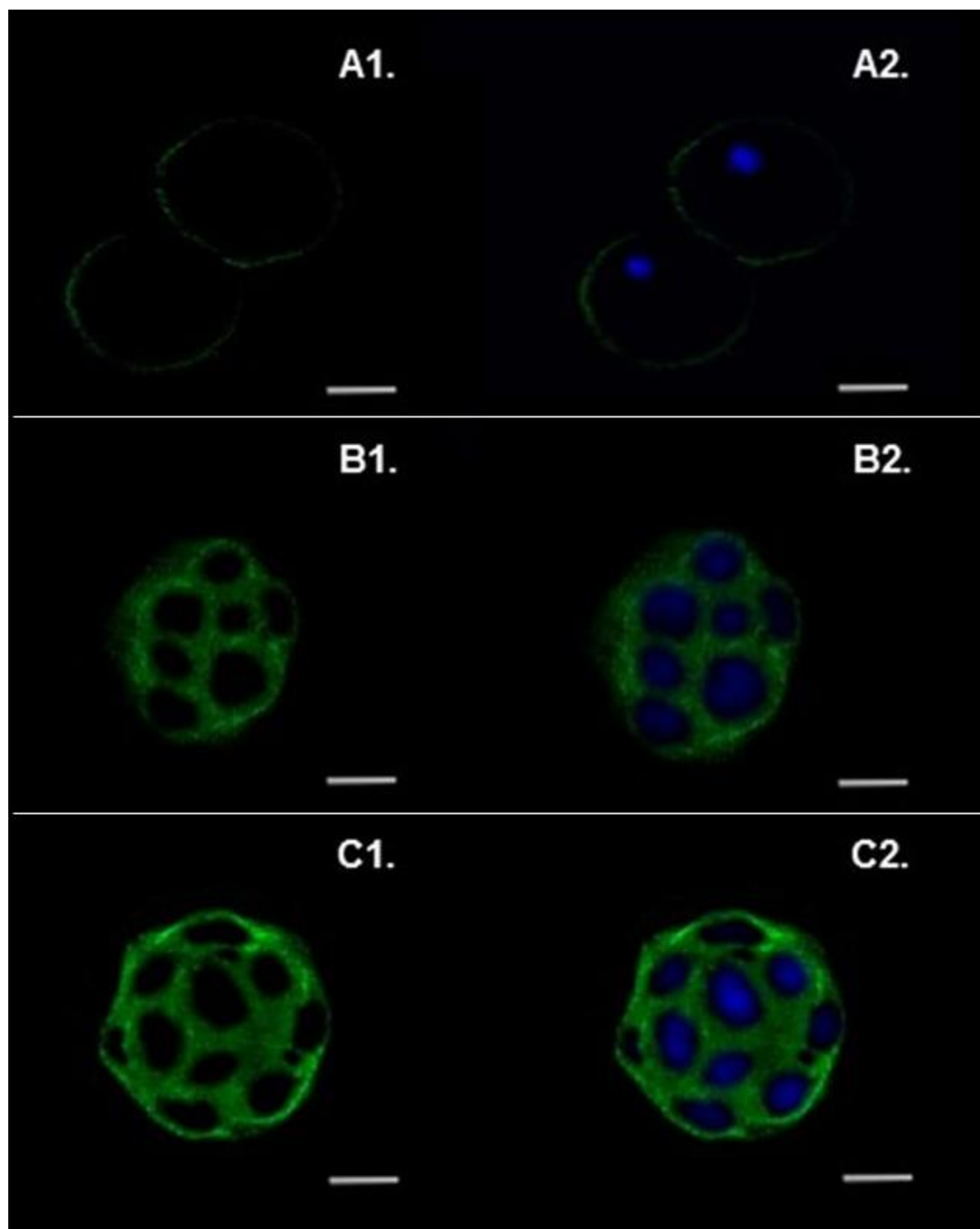

Figure 39. Localization of EGF-R in guinea pig preimplantation embryos by immunofluorescence. A1), A2) two-cell embryo, B1), B2) 8-16 cells embryo, C1), C2) Morula. The right panel represents the merge from the blue staining that denotes the nucleus and the green the positive staining). Representative photomicrographs are shown. Scale 30 μm.

4.2.2.2. Immunocytochemical localization of IGF-1R in cumulus-oocyte complexes and early embryos

IGF-1R staining was detected in CCs and in oocytes. Distribution was shown in the cell membrane of the cells for both oocytes and CCs (Figure 40). We observed a diferential change in the localization of the receptor between matured and immature oocytes; the intensity of the signal was significantly higher in oocytes and CCs after *in vitro* maturation. There was no staining in the negative controls.

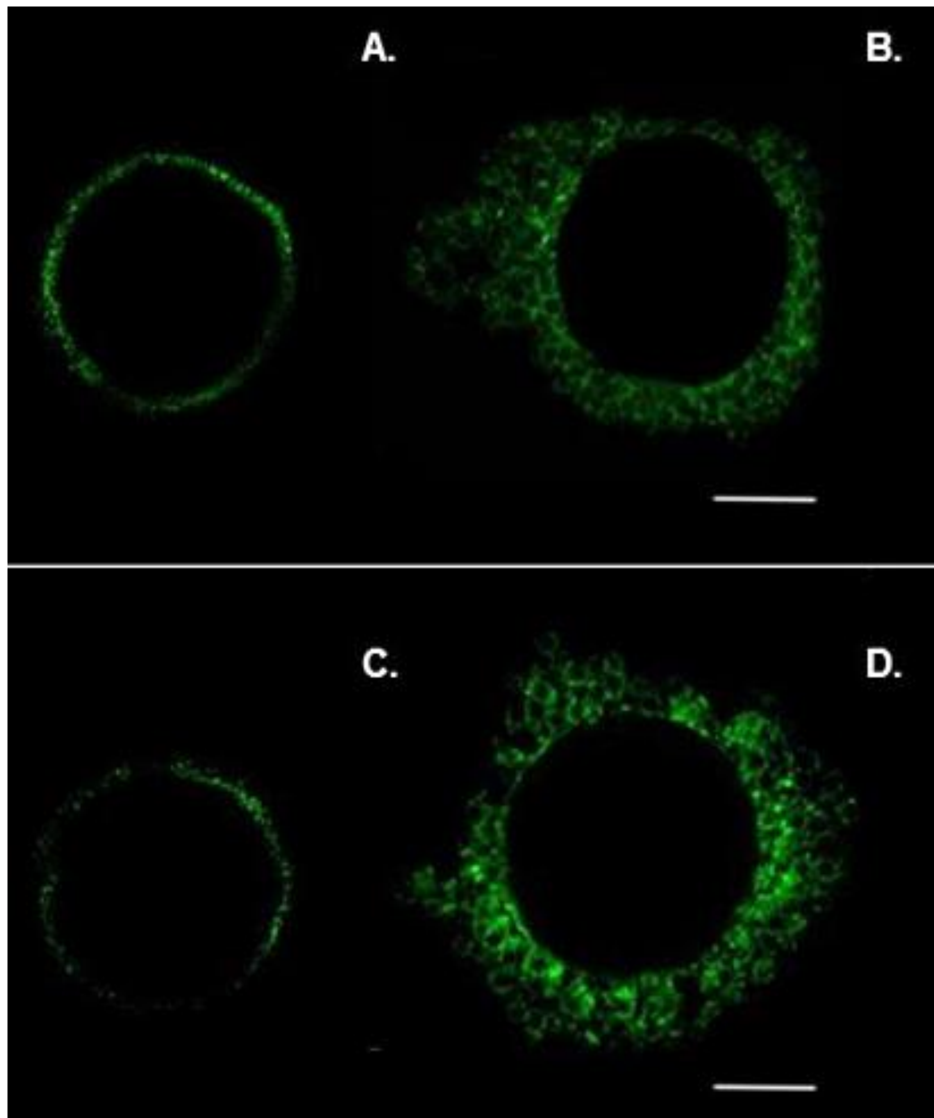

Figure 40. Localization by immunocytochemistry of IGF-1R in immature and matured Oo and CCs of guinea pig. A) Immature oocyte, B) Immature COCs, C) Oocyte maturated, D) CCs after *in vitro* maturation. Oo (Oocyte) and CCs (Cumulus Cells). Green fluorescence denotes the distribution of IGF-1R. Representative photomicrographs are shown. Scale 30 µm.

In embryos, the IGF-1R was uniformly immunolocalized in the cell membrane of the blastomeres in all developmental stages studied. Developing embryos increased the intensity of the signal during the early development. Four cell stage embryos exhibited the lowest immunostaining compared to morula stage that show the highest signal ($P<0.05$). Eight to 16 cell stage show intermediate fluorescence value (Figure 41).

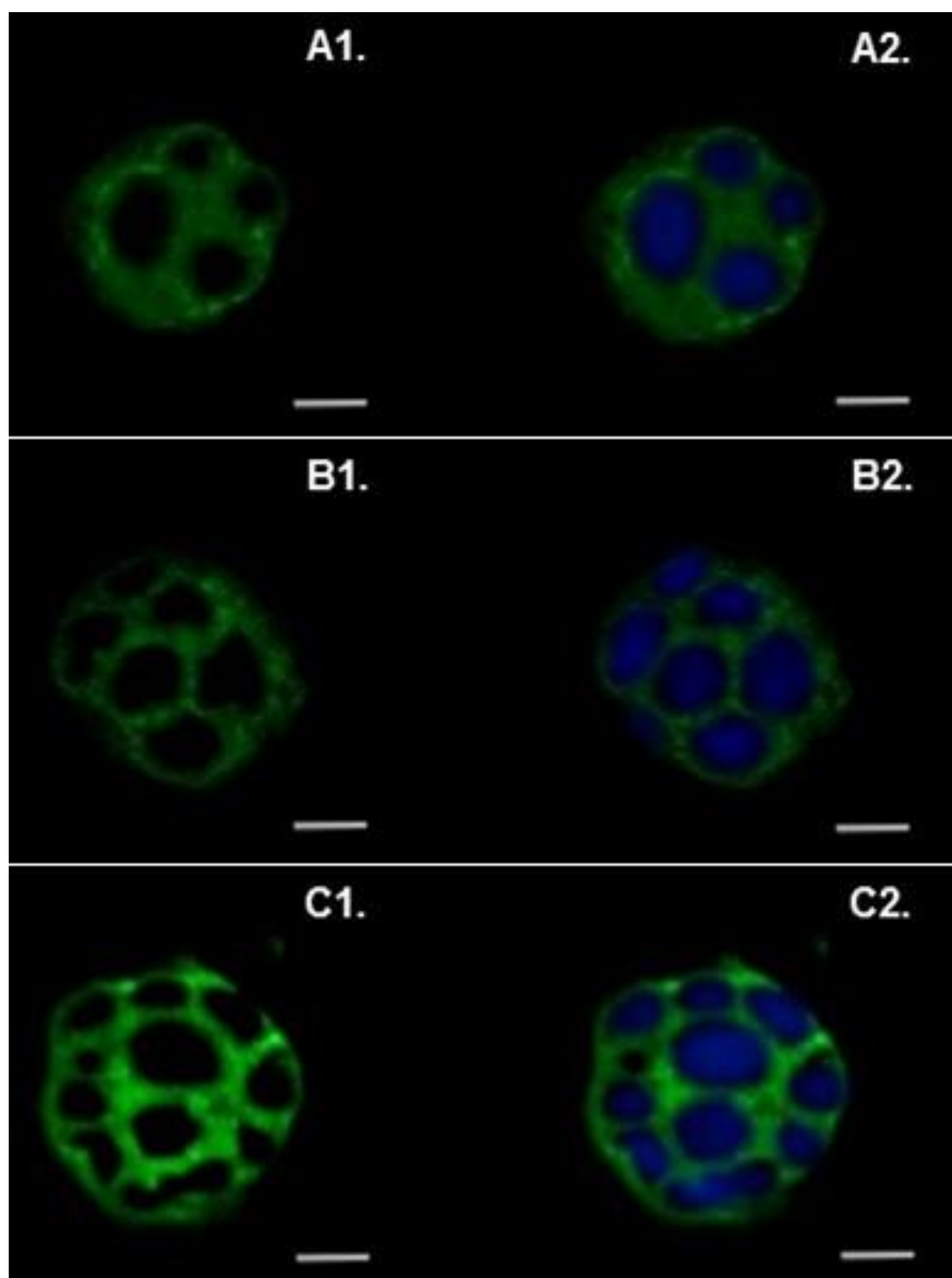

Figure 41. Localization of IGF-1R in guinea pig preimplantation embryos by immunofluorescence. A1), A2) Four-cell embryo, B1), B2) 8-16 cells embryo, C1), C2) Morula. The right panel represents the merge from the blue staining that denotes the nucleus and the green the positive staining). Representative photomicrographs are shown. Scale 30 µm.

4.2.2.3. Immunolocalization of IGFBPs 1-6 in cumulus-oocyte complexes

Indirect immunofluorescence assay was used to detect the localization of IGFBPs 1 to 6 in oocytes and CCs of guinea pig (Figure 42, 43 and 44). In both, all the IGFBPs (Figure 40 and 41) was localized in the cellular membrane of oocyte and CCs.

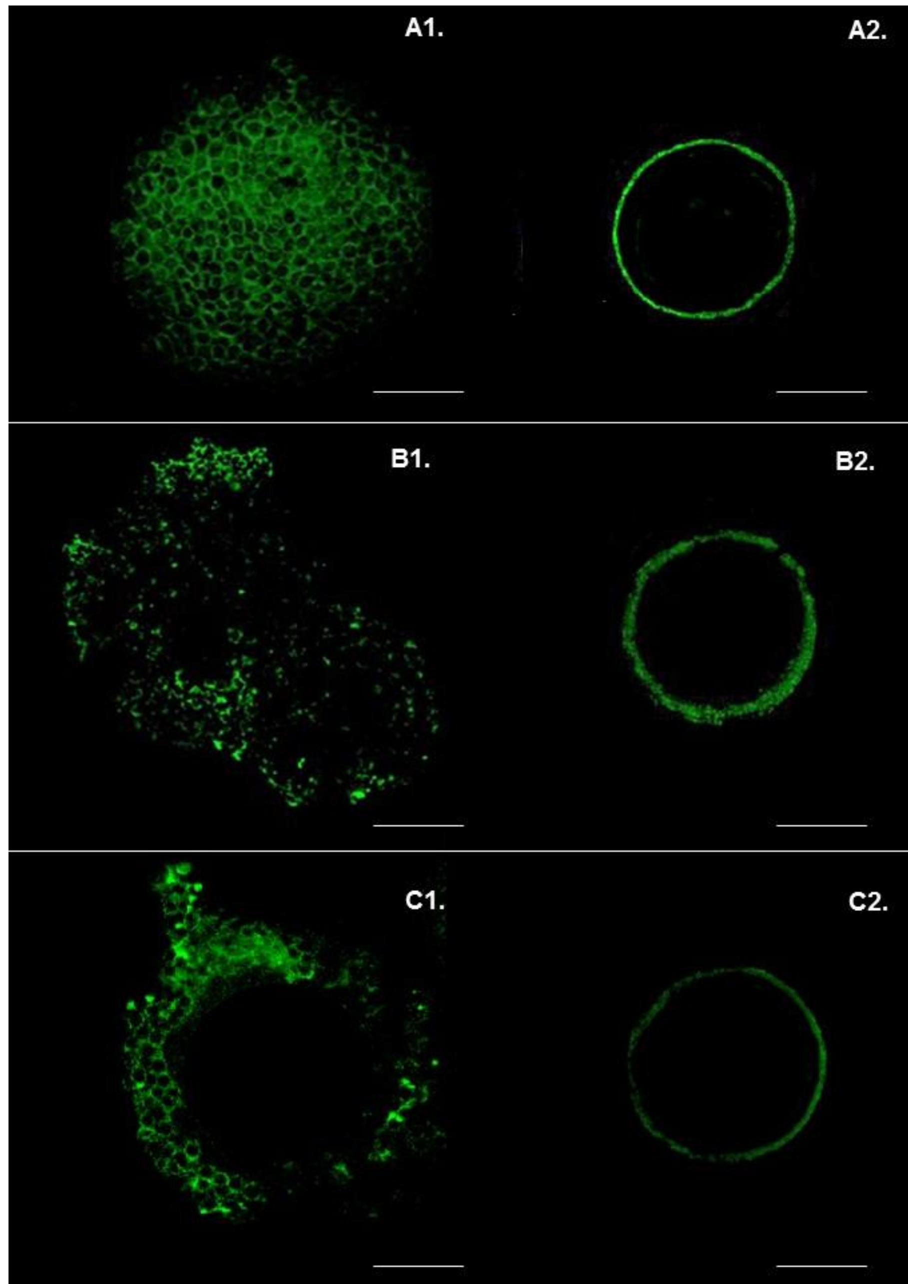

Figure 42. Localization of IGFBP-1, 2 and 3 in the CCs and the oocytes guinea pig by immunofluorescence. A1), A2) IGFBP- 1, B1), B2) IGFBP- 2, C1, C2) IGFBP- 3. The left panel represents the CCs and right the panel represents the oocytes. Green immunofluorescence indicates the localization of protein. CCs (Cumulus Cells). Representative photomicrographs are shown. Scale 30 µm.

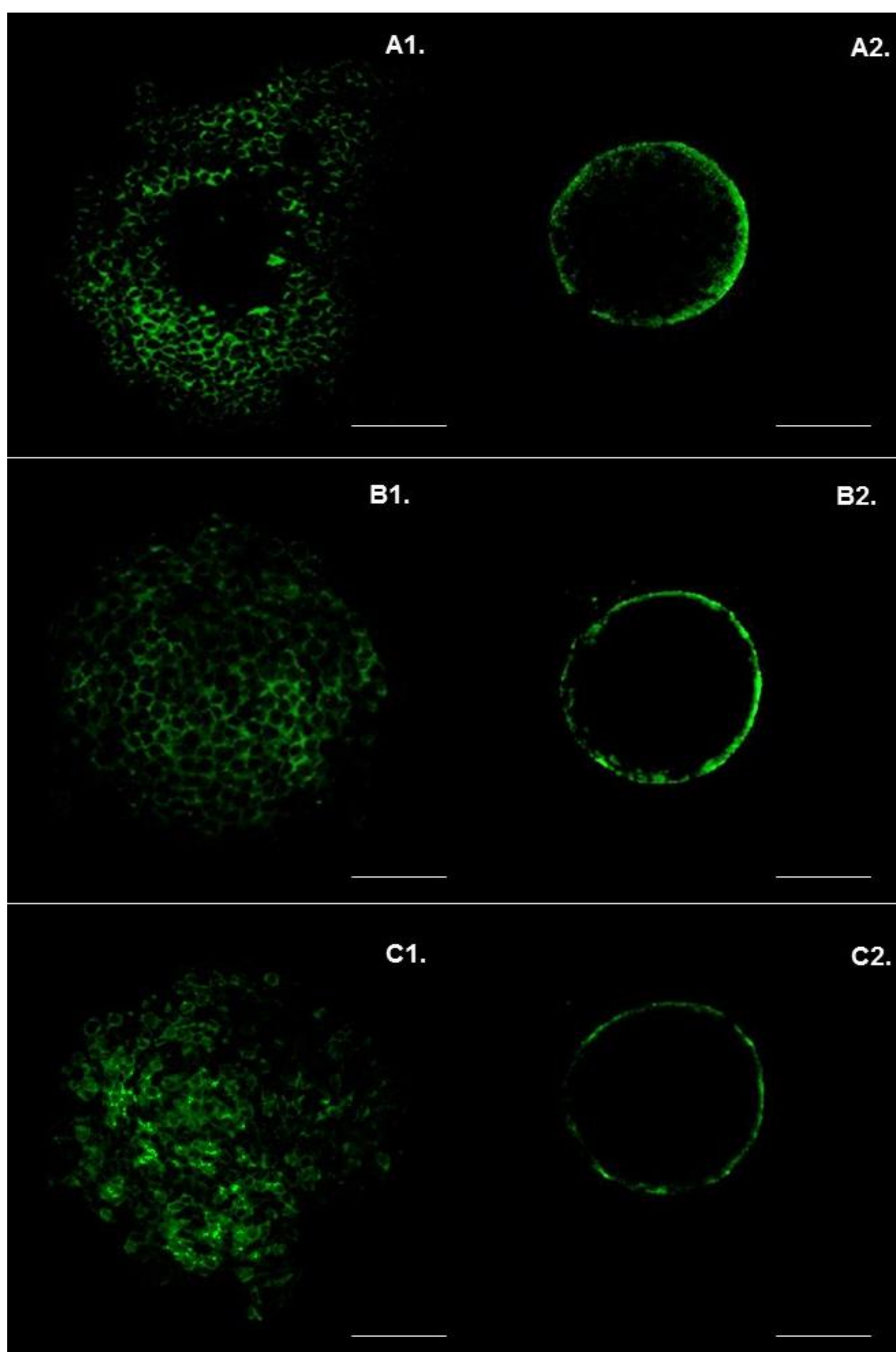

Figure 43. Localization of IGFBP-4, 5 and 6 in the CCs and the oocytes guinea pig by immunofluorescence. A1), A2) IGFBP- 4, B1), B2) IGFBP- 5, C1, C2) IGFBP- 5. The left panel represents the CCs and right the panel represents the oocytes. Green immunofluorescence indicates the localization of protein. CCs (Cumulus Cells). Representative photomicrographs are shown. Scale 30 μm.

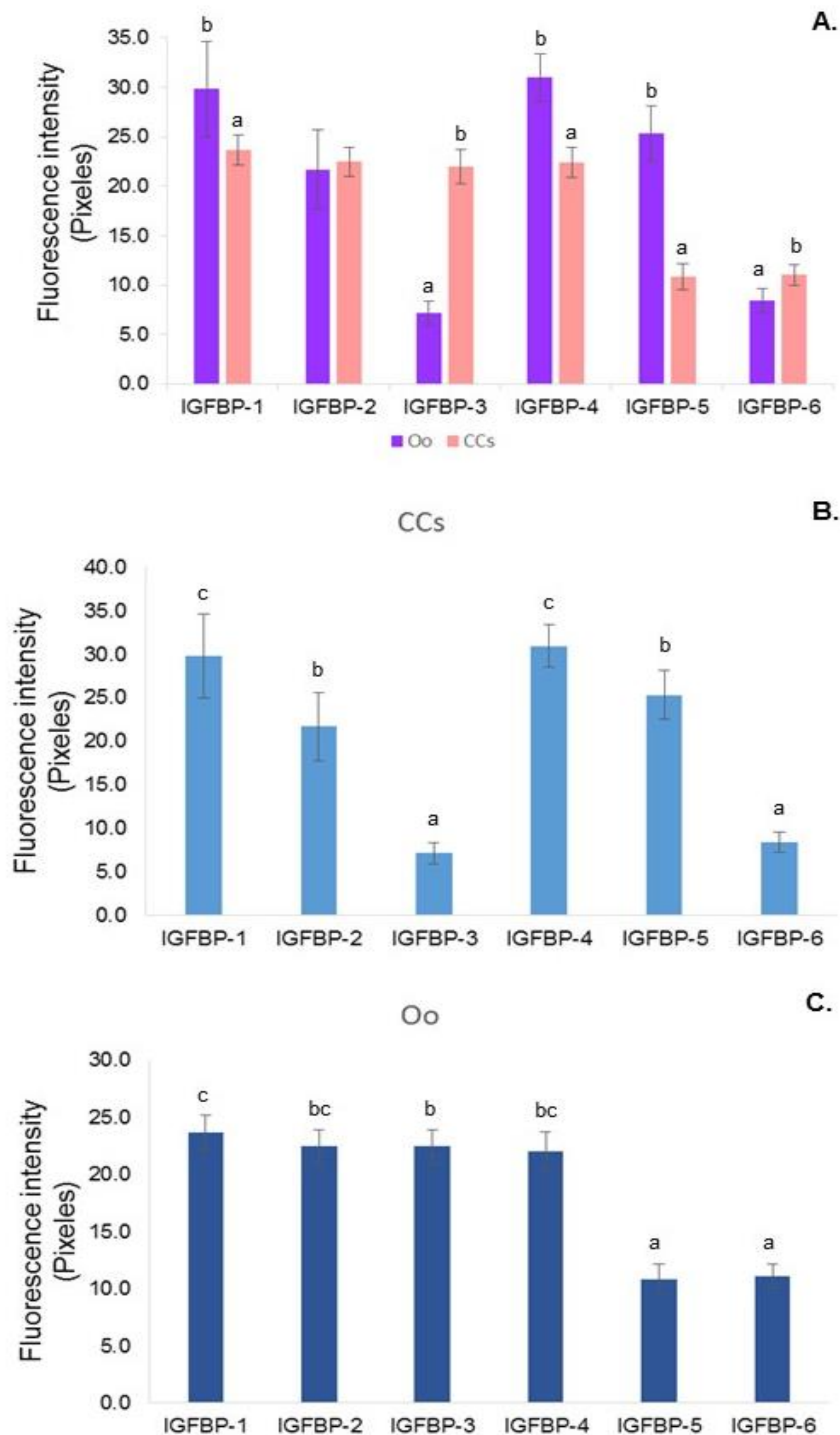

Figure 44. IGFBPs 1-6 quantification of the immunofluorescence intensity is presented as values measured in pixels. A) Represents the comparison between Oo and CCs for each IGFBPs, B) Represents the fluorescence intensity of each IGFBPs for the CCs, C) Represents the fluorescence intensity of each IGFBPs for the Oo. Oo (Oocytes), CCs (Cumulus cells). Different letters indicate significant differences (P<0.05).

The intensity of the signal for IGFBP-1, 4 and 5 was significantly higher in oocytes than in CCs (P<0.05) (Figure 42, 43 and 44). In contrast, fluorescence for IGFBP-3 and 6 was significantly lower (P<0.05), (Figure 42, 43 and 44) in oocytes compared to the surrounding cumulus cells. IGFBP-2 did not show significant differential staining between CCs and oocyte (Figure 42). Among IGFBPs in CCs, IGFBP3 and 6 showed the lowest intensity of staining whereas IGFBP-1 and 4 exhibited the higher signal (P<0.05, Figure 43). In oocytes IGFBP-5 and 6 showed the lower signal compared to IGFBP-1 which presented the higher intensity (P<0.05). The rest of IGFBPs showed intermediate results.

4.3. EXPERIMENT 3. Study the effect of EGF and IGF-1 on nuclear and cytoplasmic *in vitro* oocyte maturation, apoptosis and steroidogenic production by the cumulus-oocyte complexes in the guinea pig model.

4.3.1. Experiment 3a: Characterization of cortical granules and mitochondrial distribution patterns during *in vitro* oocyte maturation in the guinea pig

4.3.1.1. Cortical granules patterns

Cortical granules patterns observed (Figure 45) before and after oocyte maturation were defined as: non-migrated A1), cortical granules distributed throughout the cytoplasm (considered non- cytoplasmically matured); partially migrated B1), most of the cortical granules spread throughout the cortical area and migrated C1), cortical granules adjacent to the plasma membrane and peripheral distribution (considered cytoplasmically matured). Cortical granules migrated to the periphery were the main patterns observed in the oocytes after IVM culture (71.9 - 76.9%, see above) whereas in immature oocytes homogeneous distribution of cortical granules was prevalent (70%).

4.3.1.2. Mitochondrial patterns

The mitochondrial patterns (Figure 46) were classified as: non-migrated (A1) when the mitochondrias were homogeneously distributed throughout the cytoplasm (considered non-cytoplasmically matured); partially migrated (B1), when the mitochondria were heterogeneously distributed with granular aggregations and migrated (C1), when the mitochondria were relocated to the nuclear pole (considered cytoplasmically matured). Peripheral distribution of mitochondria is the most abundant pattern in mature oocytes

(66.7- 80.0%). However, mitochondria distributed throughout the cytoplasm were found mainly in immature oocytes (60%).

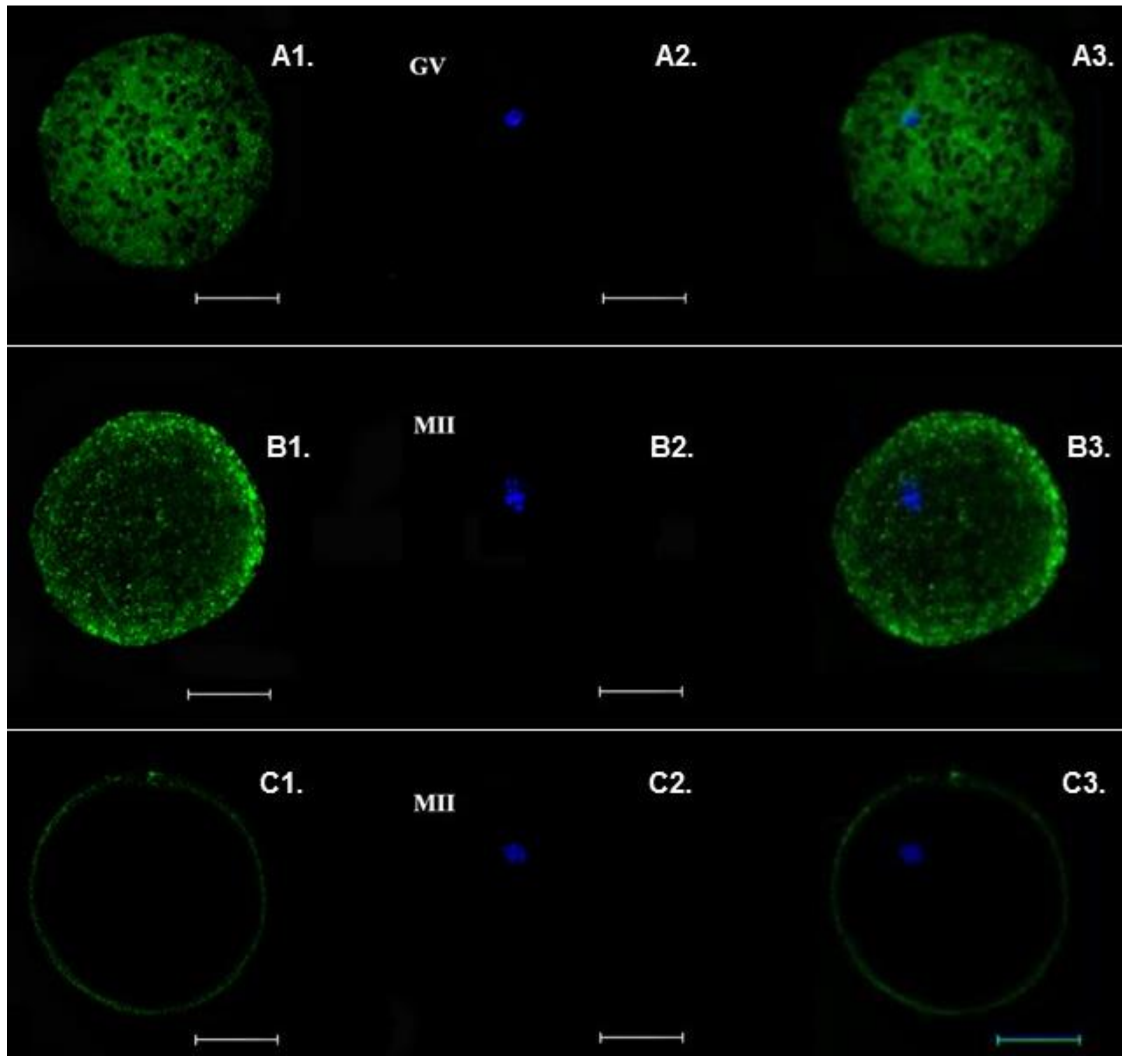

Figure 45. Cortical granule (CG) distribution in guinea pig oocytes after IVM period visualized under confocal laser microscopy. Left panel shown CG migration patterns: A1) Homogeneous CG distribution; B1) Cortical CG distribution; C1) Peripheral CG distribution. Middle panel shown nuclear chromatin of oocytes stained with Hoechst 33342: A2) Germinal vesicle; B2 and C2) Metaphase II. Right panel shown merges the both nucleus and CG.

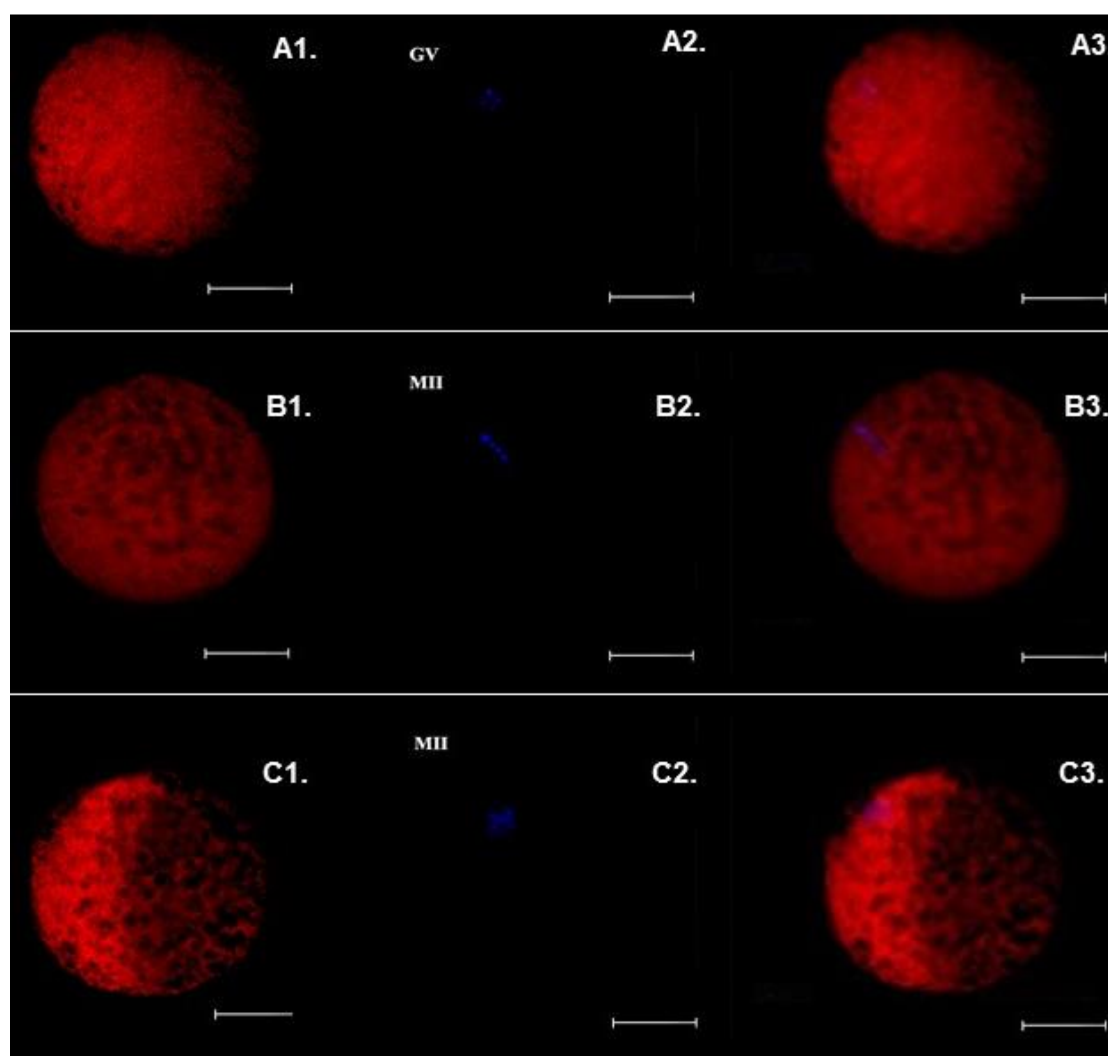

Figure 46. Mitochondrial distribution in guinea pig oocytes after IVM period visualized under confocal laser microscopy. Left panel shown CG migration patterns: A1) Non-migrated; B1) partially migrated; C1) migrated. Middle panel shown nuclear chromatin of oocytes stained with Hoechst 33342: A2) Germinal vesicle; B2 and C2) Metaphase II. Right panel show merges the both nucleus and mitochondria.

4.3.2. Experiment 3.b. Role of EGF on *in vitro* oocyte maturation system of guinea pig by the study of nuclear and cytoplasmic oocyte maturation, apoptotic rate in cumulus cells and steroidogenic response of cumulus-oocyte complexes.

A higher M-II rate was found when the oocytes were IVM with 50 ng/mL EGF compared to the rest of the experimental groups (P<0.05) (see Table 7). Also, this group showed higher percentage of oocytes with peripheral migration pattern of cortical granules and migrated mitochondrial distribution compared to group without EGF (negative control

group) (P<0.05). There were no significant differences among groups in the other cortical granules and mitochondrial migration patterns studied.

Apoptosis rate was significantly lower for groups with 50 ng/mL EGF or 10% FCS compared with those cultured without EGF (P<0.05)

Before IVM, E_2 and P_4 were detected in the control spent maturation medium at a concentration of 0.16±0.007 ng/mL and 0.02±0.005 pg/mL, respectively. After IVM a significant increase of E_2 and P_4 production by COCs was found when maturation media was supplemented with 50 ng/mL EGF (Figure 45).

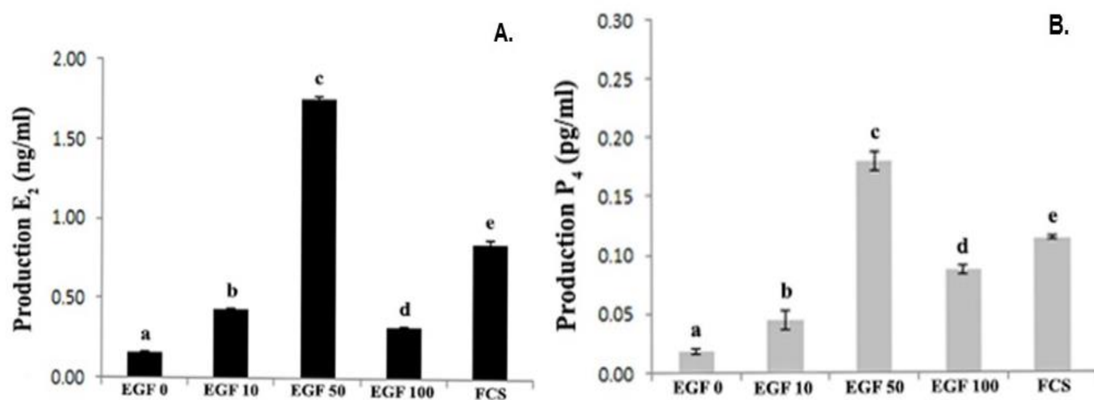

Figure 47. Steroidogenic production of COCs after IVM using different concentrations of EGF. Bars represent mean concentrations of A) E_2, B) P_4 produced per each COCs under different experimental conditions. Means in bars with different letters in each chart indicate significant differences (P<0.05).

Table 7. Nuclear and cytoplasmic maturation in guinea pig oocytes and apoptosis in cumulus cells with different concentrations of EGF in IVM medium.

	0 ng/mL EGF	10 ng/mL EGF	50 ng/mL EGF	100 ng/mL EGF	10% FCS
Nuclear maturation					
M-II rate n (%)	115 (43.5)[a]	114 (51.8)[a]	116 (75.9)[b]	108 (53.7)[a]	111 (50.5)[a]
Cytoplasmic maturation					
Cortical granules distribution patterns					
Migrated n (%)	10 (32.4)[a*]	14 (45.2)[ab]	23 (71.9)[b]	12 (50.0)[ab]	16 (64.0)[ab*]
Partially migrated n (%)	15 (48.4)	11 (35.5)	7 (21.9)	10 (41.7)	7 (28.0)
Non- migrated n (%)	6 (19.4)	6 (19.4)	2 (6.3)	2 (8.3)	2 (8.0)
Mitochondrial distribution patterns					
Migrated n (%)	5 (27.8)[a]	8 (57.1)[ab]	12 (80.0)[b]	5 (31.3)[a]	10 (66.7)[ab]
Partially migrated n (%)	4 (22.2)	3 (21.4)	1 (6.7)	6 (37.5)	1 (6.7)
Non-migrated n (%)	9 (50.0)	3 (21.4)	2 (13.3)	5 (31.3)	4 (26.7)
Apoptosis of cumulus cells n(%)	13 (28.7±1.4)[a]	11 (21.1±1.2)[ab]	11 (17.2 ± 0.9)[b]	13 (19.7±1.2)[ab]	10 (16.0±1.2)[b]

Means in rows with different letters differ: a,b $P<0.05$; *$P<0.1$

4.3.3. Experiment 3.c. Role of IGF-1 on *in vitro* oocyte maturation of guinea pig by the study of nuclear and cytoplasmic oocyte maturation, apoptotic rate in cumulus cells and steroidogenic response of cumulu-oocyte complexes.

As shown in Table 8, supplementation with 100 ng/mL IGF-1 significantly stimulated nuclear oocyte maturation in guinea pig oocytes (P<0.05). This group showed higher percentage of cortical granules peripheral pattern compared with the group without IGF-1 or with 50 ng/mL IGF-1 (P< 0.05). The lowest rates of non-migrated oocytes were found for groups supplemented with 100 and 200 ng/mL IGF-1 compared to non-supplemented group (P<0.05). The percentage of partially migrated and non-migrated was similar for all groups. No significant differences were found in mitochondrial patterns among experimental groups.

Apoptotic index of the CCs was significantly lower in groups with 50 or 100 ng/mL IGF-1 than in group without IGF-1 (P<0.05) (Table 8). The COCs supplemented with 10% FCS group showed intermediate values of apoptosis in CCs.

P_4 and E_2 production by CCs were found significantly higher in the group supplemented with 100 ng/mL of IGF-1 compared to the other studied groups (P<0.05) (Table 8 and Figure 46).

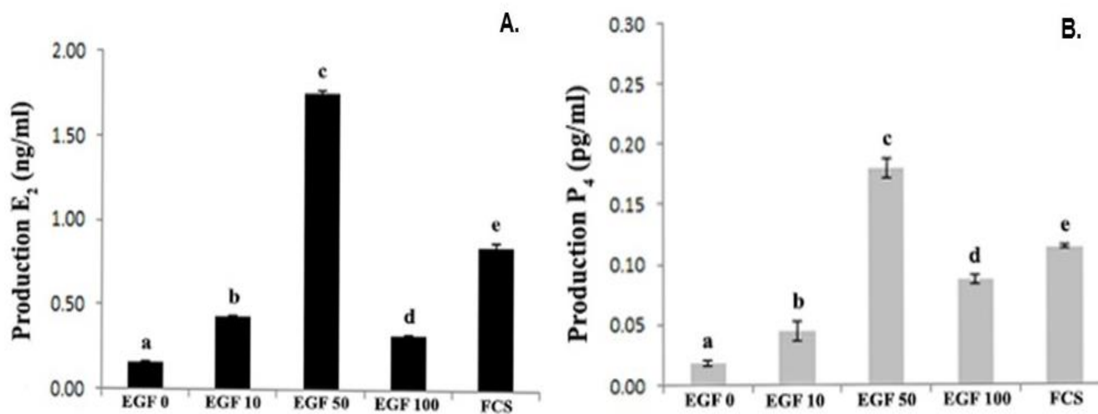

Figure 48. Steroidogenic production of COCs after IVM using different concentrations of IGF-1. Bars represent mean concentrations of A) E_2 , B) P_4 produced per each COCs under different experimental conditions. Means in bars with different letters in each chart indicate significant differences (P<0.05).

Table 8. Nuclear and cytoplasmic maturation in guinea pig oocytes and apoptosis in cumulus cells with different concentrations of IGF-1 in IVM medium.

	0 ng/mL IGF-1	50 ng/mL IGF-1	100 ng/mL IGF-1	200 ng/mL IGF-1	10% FCS
Nuclear maturation					
M-II rate n (%)	113 (38.9)^{a}	114 (48.2)^{a}	112 (72.3)^{b}	112 (54.5)^{a}	117 (50.4)^{a}
Cytoplasmic maturation					
Cortical granules distribution patterns					
Migrated n (%)	9 (31.0)^{a}	13 (43.3)^{a}	23 (76.7)^{b}	16 (55.2)^{ab}	15 (53.6)^{ab}
Partially migrated n (%)	10 (34.5)	15 (50.0)*	6 (20.0)*	12 (41.4)	10 (35.7)
Non- migrated n (%)	10 (34.5)^{a}	2 (6.7)^{b}	1 (3.4)^{b}	1 (3.4)^{b}	3 (10.7)^{a}
Mitochondrial distribution patterns					
Migrated n (%)	6 (40.0)	8 (53.3)	11 (73.3)	7 (46.7)	9 (60.0)
Partially migrated n (%)	5 (33.3)	3 (20.0)	4 (26.7)	4 (26.7)	3 (20.0)
Non-migrated n (%)	4 (26.7)	4 (26.7)	0 (0.0)	4 (26.7)	3 (20.0)
Apoptosis of cumulus cells n (%)	11 (34.6±1.5)^{a}	12 (14.5±1.1)^{bc}	11 (12.8± 0.9)^{b}	11 (21.7±1.2)^{ac}	11 (17.5±1.2)^{abc}

Means in rows with different letters differ: a,b P<0.05; *P<0.1

4.3.4. Experiment 3.d. Study of the effect of EGF and IGF-1 on *in vitro* oocyte maturation of guinea pig by the study of nuclear and cytoplasmic oocyte maturation, apoptotic rate in cumulus cells and steroidogenic response of cumulus-oocyte complexes

M-II rate significantly increased in oocytes IVM with the combination of both growth factors alone or with 10% FCS compared to the group without growth factors (negative control) and the FCS group (positive control) (P<0.05) (Table 9). The highest percentage of oocytes with migrated cortical granules distribution was shown in the EI-FCS group (P<0.05) whereas group 0 showed the highest rate of oocytes with non-migrated pattern of cortical granules (P<0.05). There were not found significant differences in the mitochondrial distributions studied among groups.

The percentage of apoptosis in CCs of EI-FCS group was significantly lower compared to the rest of experimental groups (P<0.05).

The combination of EGF+IGF-1 and FCS showed higher P_4 and E_2 production by COCs (P<0.05) (Figure 49).

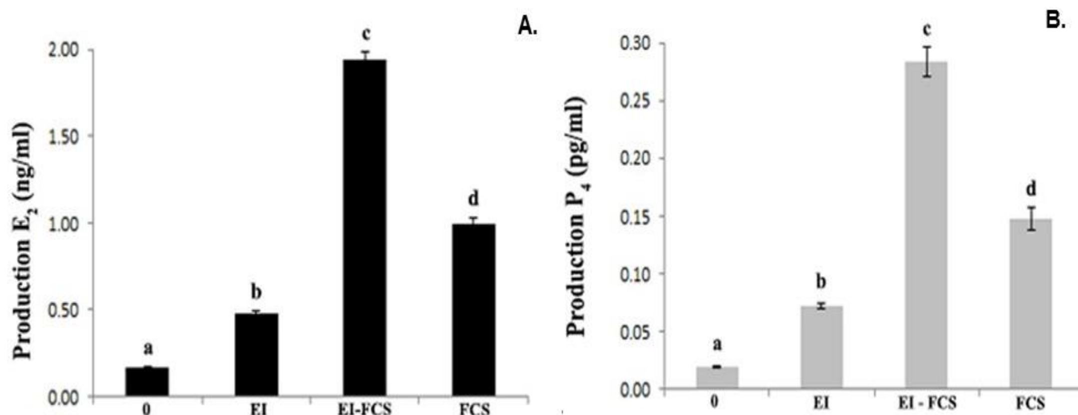

Figure 49. Steroidogenic production of COCs after IVM using combinations of EGF and IGF-1. Bars represent mean concentrations of A) E_2, B) P_4 produced per each COCs under different experimental conditions. Means in bars with different letters in each chart indicate significant differences (P<0.05).

Table 9. Nuclear and cytoplasmic maturation in guinea pig oocytes with EGF and IGF -1 combined in IVM medium. Means in rows with different letters differ: [a, b,c] P<0.05; [*] P<0.1.

	0	EI	EI-FCS	FCS
Nuclear maturation				
M-II rate n (%)	112 (38.4)[a]	115 (78.3)[b]	116 (83.7)[b]	113 (55.8)[c]
Cytoplasmic maturation				
Cortical granules distribution patterns				
Migrated n (%)	5 (23.8)[a*]	13 (59.1)[ab*]	20 (76.9)[b]	12 (50.0)[ab]
Partially migrated n (%)	6 (28.6)	7 (31.8)	5 (19.2)	11 (45.8)
Non- migrated n (%)	10 (47.6)[a]	2 (9.1)[b]	1 (3.8)[b]	1 (4.2)[b]
Mitochondrial distribution patterns				
Migrated n (%)	5 (35.7)	8 (53.3)	10 (66.7)	8 (53.3)
Partially migrated n (%)	4 (28.6)	2 (13.3)	2 (13.3)	4 (26.7)
Non-migrated n (%)	5 (35.7)	5 (33.3)	3 (20.0)	3 (20.0)
Apoptosis of cumulus cells n (%)	11 (25.8±1.4)[a]	11 (22.7±1.3)[a]	11 (6.6±0.7)[b]	11 (22.8±1.3)[a]

Group 0: not supplemented with EGF and IGF-1; EI group: supplemented with 50 ng/mL EGF+100 ng/mL IGF-1; EI-FCS group, supplemented with 50 ng/mL EGF + 100 ng/mL IGF-1 + 10% FCS; and FCS group: supplemented with 10% (v:v) FCS

5. DISCUSSION

The follicular development is regulated by several factors which stimulate the differentiation and proliferation of somatic cells and the development and growth of the oocytes enclosed. The intraovarian system of EGF and IGF-1 plays a key role in those events, since they are involved in steroidogenesis, granulosa cell proliferation, apoptosis, oocyte maturation outcome and *corpus luteum* formation and function. The biologic effects of both factors are mediated throughout their corresponding cell membrane receptors (EGF-R and IGF-1R) and in IGF system also for the IGFBPs 1-6, which can potentiate or inhibit the IGF action at the target cells. Therefore, the investigation of the expression patterns and localization of these developmentally important genes in animal models is necessary for the study of the ovarian physiology in animals and human.

In this context, oocyte IVM has emerged as a promising procedure for infertility treatment in woman since it has the potential to substitute standard IVF and superovulation protocols. IVM may be the method of choice, not only for infertile couples but also for obtaining meiotically competent oocytes for preservation of fertility in cancer patients (Lim *et al.,* 2013). Although advances in IVM protocols have led to satisfactory pregnancy rates, the technique yielded limited success and further improvements are needed. Furthermore, comparison of multiple media appears very difficult in a human IVM context, in which material donated for research is extremely rare. Therefore, the use of animal models is needed as well. The guinea pig is a valuable laboratory animal model for reproductive studies since it shares similar characteristics related to the ovarian physiology, but the IVM systems are rarely developed in this specie.

For these reasons, in this Thesis it has been characterized the EGF and its receptor; the IGF-1 and its receptor, and the IGFBPs 1-6, described in woman´s ovary, to use the guinea pig as a possible animal model for the study of ovarian physiology in human. Finally, we studied the effect of both growth factors (EGF and IGF-1) on *in vitro* oocyte maturation as a possible way to set up an *in vitro* defined maturation system in this specie; it would be valuable as source of M-II oocytes needed for ARTs (used in human experiments and guinea pig conservation of genetic pool).

5.1. Gene expression and immunolocalization of EGF and EGF-R

5.1.1. Characterization of EGF and EGF-R system in the guinea pig ovary

The mRNA expression of EGF and EGF-R showed a similar pattern in the antral and preovulatory follicles in the guinea pig in which significantly higher expression was shown for both genes in antral follicles than in preovulatory ones. Both protein and mRNA for EGF-R have been found in antral follicles in other species, but also in pre- antral and earlier follicles (pig: Singh *et al.,* 1995; cat: Goritz *et al.,* 1996; human: Maruo *et al.,* 1993; Bennett *et al.,* 1996; Qu *et al.,* 2000; hamster: Roy and Greenwald, 1990; Singh *et al.,* 1995; Garnett *et al.,* 2002). The simultaneous expression of EGF and EGF-R in guinea pig suggests that an autocrine and paracrine mode of EGF action may exist to regulate final follicular growth, including the selection and regression process and oocyte maturation in the ovary. However, the presence of EGF in human ovarian tissue is still in debate. EGF gene expression was not observed in thecal or granulosa cells of rats, bovines, and humans (Skinner and Coffey, 1988; Yeh *et al.,* 1993; Tamura *et al.,* 1995). The presence of EGF in antral follicles in porcine and human has been reported contradictorily by several laboratories (Reeka *et al.,* 1998; Hsu *et al.,* 1987, Westergaard and Andersen, 1989; Volpe *et al.,* 1991).

Concomitantly, we also have observed EGF-R immunolocalization in all developing follicles of the ovary in both granulosa and theca cells, oocytes and luteal cells. EGF-R signal dynamically changes in oocytes and granulosa cells throughout the follicular stages; from moderate immunoreactivity in primordial follicles to strong signal in antral follicles, as also was appreciated in theca cells. EGF-R inmunolocalized with strong intensity in antral follicles and *corpus luteum* confirms the findings of the gene expression study for this receptor. Previous studies have localized EGF-R in granulosa, theca and luteal cells in human (Maruo *et al.,* 1993; Reeka *et al.,* 1998; Tamura *et al.,* 1995; Qu *et al.,* 2000) and in other mammalian species such as, primate (de Prada *et al.,* 2009), bovine (Vlodavsky *et al.,* 1978), goat (Gall *et al.,* 2004; Silva *et al.,* 2006; Pereira *et al.,* 2012); sheep (Santos *et al.,* 2014), cat (Goritz *et al.,* 1996), porcine (Fujinaja *et al.,* 1992), equine (Lorenzo *et al.,* 2002) and hamster (Garnett *et al.,* 2002).

All these observations suggest that EGF may regulate the growth of follicles through binding to EGF-R in ovarian tissues. In human the results are controversial, since some authors was not found inmunostaining for EGF or EGF-R in primordial follicles (Maruo *et al.,* 1993) but other have reported positive reaction in all stages of the follicular

development in granulosa cells, oocytes and theca (Qu *et al.,* 2000). EGF throughout it receptor promotes granulosa cell proliferation, increases the follicular daily growth rate in rat follicles (Dekel and Sherizly, 1985; Das *et al.,* 1994), stimulate the growth of primary and secondary follicles of goat (Silva *et al.,* 2004; Celestino *et al.,* 2011) and, in bovine antral follicles, improves antrum formation *in vitro* (Santos *et al.,* 2014).

Similarly, with our results, the EGF-R expression related to follicle size increased in oocytes and in granulosa cells in human (Maruo *et al.,* 1993). In this sense, several authors also have shown strong inmunostaining in granulosa cells of antral follicles of several species (rat: St-Arnaud *et al.,* 1983, Ergin *et al.,* 2008; pig: Fuginaga *et al.,* 1992; hamster: Roy and Greenwald, 1990; human: Maruo *et al.,* 1993, Qu *et al.,* 2000; rabbit: Lorenzo *et al.,* 2000; goat: Silva *et al.,* 2006 and bovine: Caixeta *et al.,* 2009). The increasing staining in theca cells when the follicle growth showed in the present study may be related with the involvement of EGF on the growth of granulosa cells as has been pointed out. Thecal cells are important site of synthesis of growth factors involved in cell-to-cell interactions since they secreted androgens, which indeed stimulate the growth of granulosa cells (Skinner *et al.,* 1987).

It is known that the growth of follicles depends on gonadotropins after preantral stage (Scaramuzzi *et al.,* 2011). Prior to the LH surge, FSH significantly stimulated LH and EGF-R expression in the granulosa cell (Erickson *et al.,* 1989; El-Hayek *et al.,* 2016). Indeed, the EGF inhibited FSH-induced aromatization of androgen to estrogen and the formation of LH receptors in human granulosa cells (Peters *et al.,* 1994). In this sense, Volpe *et al.* (1991) found that EGF levels in FF were significantly higher in patients with polycystic ovary disease than those of normally ovulating infertile women in an IVF-ET program. Oocytes acquire meiotic competence at an early antral stage, and meiosis resumes in PF as a result of the LH surge. EGF-R is a decisive mediator of LH signaling (Jaffe and Egbert, 2016). LH binds to its receptors on mural granulosa cells and stimulates the expression of EGF-like peptides *in vivo* (Park *et al.,* 2004). These peptides then act directly on CCs, bind to the EGF-R and consequently stimulate the resumption of meiosis and expansion of the CCs in a variety of species (cattle: Lonergan *et al.,* 1996; sheep: Guler *et al.,* 2000; dog: Hatoya *et al.,* 2009). The increased expression of mRNA EGF and EGF-R in the antral follicles accompanied with the EGF-R strong inmunoreactivity found in present study support EGF participation in the developmental competence of the oocyte in the guinea pig.

In current work, abundance of EGF and EGF-R mRNA was found in *corpus luteum* being EGF-R gene expression increased. The inmunolocalization of EGF-R found in antral follicles persisted in *corpus luteum*, with high intensity as well. EGF-R expression was described in *corpus luteum* in human (Maruo, 1993) and other species too (rat: Tekpetey *et al.,* 1995; pig: Kennedy *et al.,* 1993, Singh *et al.,* 1995). These findings, support EGF affects the function of *corpus luteum* during the early luteal phase in the guinea pig by autocrine and paracrine ways. *Corpus luteum* secretion of P_4 is essential for the establishment and maintenance of pregnancy. Inadequate P_4 production is a major cause of infertility and embryonic loss in several species (Equine: Bergfelt *et al.,* 1992; Bovine: Wiltbank *et al.,* 2014; goat: Thammasiri *et al.,* 2016). EGF augments P_4 biosynthesis in luteinized human granulosa cells in culture and in rat luteal cells (Tekpetey *et al.,* 1995) in a dose-dependent manner (Jones *et al.,*1982). Furthermore, growth of the *corpus luteum* depends on the development of new blood vessels. Apart from their steroidogenic properties, EGF play an important role in dynamic luteogenesis (Assarsson *et al.,* 1995) by stimulating angiogenesis as well (Chouhan *et al.,* 2014). Besides, EGF endowed with well-established anti-apoptotic properties during luteal development (Ptak *et al.,* 2004; Yang and Rajamahendran, 2000; Parborell *et al.,* 2002; Okuda *et al.,* 2009) possibly through stimulating expression of survival factors and suppressing that of pro-apoptotic factors as has been evidenced in granulosa cells (Babitha *et al.,* 2014).

5.1.2. Characterization of EGF and EGF-R system in the guinea pig cumulus-oocyte complexes

In this context, we also observed that EGF and EGF-R gene expression was localized in both oocytes and CCs. These findings confirm that both oocytes and CCs, can directly respond to EGF by an autocrine and paracrine way. The presence of EGF and its receptor indicates that this system may exert a key role in the resumption of meiosis, cAMP production and CCs expansion in guinea pig as has been demonstrated in human (Yerushalmi *et al.,* 2014) and other species (mouse: Su *et al.,* 2010; goat: Gall *et al.,* 2004; pig: Sugimura *et al.,* 2015). Also, we have showed the EGF-R inmunolocalization in the cell membrane of both immature and matured oocytes and their corresponding CCs. Localization pattern of EGF-R revealed higher signal in *in vitro* matured COCs compared with the immature ones. This is according with the results of Bhardwaj *et al.* (2016) in which brilliant cresyl blue (BCB) positive oocytes showed significantly higher expression of EGF-R transcripts than BCB negative oocytes. In mouse CCs, their

expression increased dramatically after 4 h of culture in FSH-enriched media (Shimada *et al.,* 2006). This increase is also reported in bovine (Assidi *et al.,* 2008). Over expression of *EGF-R* in CCs can enhance the proliferation of CCs and oocyte meiosis resumption and maturation through the activation of *MAPK3/1* in mice (Yang *et al.,* 2016). Therfore EGF-R could be used as molecular marker of oocyte competence. Recently has been described proteolytic enzyme of EGF-like factors (TACE/ADAM17) which activity increased in CCs that expressed in FSH/LH-stimulated GC and CCs together with activation of the EGFR-MAPK3/1 pathway. When Tumor necrosis factor-alpha-converting enzyme (TACE/ADAM) activity is suppressed granulosa cell luteinization, cumulus expansion and oocyte maturation were inhibited in an *in vitro* culture (Yamashita and Shimada, 2012).

In other species it has been evidenced that EGF-R are only located in CCs, but not in the oocyte (murine: Downs *et al.,* 1989; bovine: Yoshida *et al.,* 1998; mouse: Park *et al.*, 2004, Panigone *et al.,* 2008; Su *et al.,* 2010). The hypothesis is that EGF major site of action when regulating oocyte maturation is the CCs in human (Qu *et al.,* 2000; Tamura *et al.,* 1995) and rat (Dekel and Sherizly, 1985). As has been mentioned previously, the mechanisms of action involve EGF-like peptides which act directly on CCs, bind to the EGF-R and consequently stimulate the resumption of meiosis and expansion of the CCs. However, previous studies have reported that in the oocytes EGF-R associated tyrosine kinase activation triggers meiosis even if they are denuded (Park *et al.,* 2004; Li *et al.,* 2008). In fact, EGF-R has been localized in human (Maruo *et al.,* 1993; Bennett *et al.,* 1996; Qu *et al.,* 2000), hamster (Garnett *et al.,* 2002), pig (Singh *et al.,* 1995; Uhm *et al.,* 1999; Sugimura *et al.,* 2015), macaque (de Prada *et al.,* 2009), mouse (Das *et al.,* 1991; Hill *et al.,* 1999) and goat (Gall *et al.,* 2004) oocytes. Although, some works have demonstrated that EGF acts on denuded oocytes or COCs (mouse: Das *et al.,* 1991; cattle: Lonergan *et al.,* 1996), the presence of a functional receptor for EGF in oocyte is still controversial in mammalian species (Gall *et al.,* 2004). Our findings imply that EGF may act on CCs and directly on the oocyte throughout its receptor. This suggests that the effect of EGF could be in part independent of the CCs and that the oocyte could be a direct target for EGF via its receptor (Gall *et al.,* 2004; Pereira *et al.,* 2012) through an autocrine/paracrine pathway (Jamnongjit *et al.,* 2005; Park *et al.,* 2004). Furthermore, Jamnongjit *et al.* (2005) demonstrated that EGF signaling through the EGF-R is essential for steroidogenesis in mouse COCs. The EGF-R-mediated steroidogenesis is regulated by StAR pathway and increased the signaling by posttranslational modifications or phosphorylation (Jammongit *et al.,* 2005). The EGFR-induced steroidogenesis (P_4 and E_2) indirectly promoted the maturation where oocytes are held in meiotic arrest.

5.1.3. Characterization of EGF and EGF-R system in the guinea pig early embryos

Finally, in the guinea pig embryos EGF-R was temporally inmunolocalized in the cell membrane throughout the preimplantation developmental stages studied. Increased EGF-R signal in the periphery of blastomeres as development progressed from 2 cell embryo to morula stage has been found. Our findings indicate that the EGF could be involved in the early embryo development in the guinea pig by two ways: 1) by stimulating the survival and P_4 production in the *corpus luteum*, but also; 2) acting directly by its cell surface receptor in the early embryos. However, further studies are needed to corroborate this hypothesis. These findings are according toThongkittidilok *et al.* (2015), which found that cat early embryos expressed EGF-R. Besides, EGF-R is expressed in the 2-cell to blastocyst stage in the mouse and from the 8-cell stage to the blastocyst stage in humans (Paria *et al.,* 1993; Kane *et al.,* 1997) thus transcription after maternal transition has been suggested. EGF-R was detected in porcine morula and blastocyst as well (Kim *et al.,* 1999; Wei *et al.,* 2001). EGF binds with its receptor and stimulates MAP/ERK and PI3K/Akt pathways that, in turn, enhance cell proliferation (Fujihara *et al.,* 2014). By this mechanism, EGF can directly promotes cleavage and enhances blastocyst formation in the mouse, cow, buffalo, sheep and cat (Paria *et al.,* 1990; Lee *et al.,* 1995; Lim *et al.,* 1996; O´Neil*,* 1997; Hardy and Spanos, 2002; Sirisathien *et al.,* 2003; Neira *et al.,* 2010; Shabankareh *et al.,* 2010; Song *et al.,* 2011; Chandra *et al.,* 2012). It is responsible of increased protein synthesis in embryos (Wood and Kaye, 1989) and it is involved in the implantation of the embryo (Wiley *et al.,* 1992; Das *et al.* 1994) and regulates the development of the peri-implantation uterus by the ERK1/2 MAPK pathway (Jeong *et al.,* 2016). In fact, embryos from knockout EGF-R mothers fail to implant (Threadgill *et al.,* 1995).

5.2. Expression and localization of IGF-1 and IGF-1R

5.2.1. Characterization of IGF-1 and IGF-1R system in the guinea pig ovary

The IGF-1 system consisting of peptide hormone (IGF-1), cell surface receptor (IGF-1R) and six circulating binding proteins (IGFBPs 1–6) (Paveli´c *et al.,* 2007). The intrinsic tyrosine kinase pathway gets activated and facilitates the essential biological role of IGF-1. All the components act together and regulate a number of crucial biological outcomes such as cellular growth, proliferation, differentiation, survival against apoptosis, and migration (Khandwala *et al.,* 2000; Pollak *et al.,* 2008). Besides according to the results

of earlier studies, the IGF system differ between womans with PCOS and normal ovaries (Homburg, 1998; Suikkari *et al.,* 1989; Vendola *et al.,* 1999; Stubbs *et al.,* 2013)

In the present study, relative mRNA expression of IGF-1 and IGF-1R was higher in antral follicles than in preantral ones. Both mRNA transcripts and protein have been identified in human antral follicles (El-Roeiy *et al.,* 1993; Voutilainen *et al.,* 1996), and preantral follicles (Samoto *et al.,* 1993; Qu *et al.,* 2000). IGF-1 gene expression have been also identified in rat (Murphy *et al.,* 1987; Oliver *et al.,* 1989), porcine (Hammond *et al.,* 1988), bovine (Armstrong *et al.,* 2000) and mice (Demeestere *et al.,* 2004) ovary. Besides, IGF-1R transcripts were described in rat (Ergin *et al.,* 2008), buffalo (Zhou and Bondy *et al.,* 1993; Zhao *et al.,* 2001; Uniyal *et al.,* 2015), and rat (Oliver *et al.,* 1989) ovary as well. These results support the notion that the selection of a future dominant follicle could be affected when the intrafollicular IGF system is altered (Fortune *et al.,* 2004.) Althouugth the pathogenesis of PCOS is still unclear, it is demonstrated that in PCOS women, concentrations of IGF-1 in the follicular fluid are higher than in healthy women (Abd *et al.,* 2005). The increased secretion of IGF-1 and androgen by theca cells is attributable to this alteration.

According to these findings, the immunohistochemical analysis revealed that most of the follicles in all stages of development studied in guinea pig exhibited moderate expression for IGF-1R in oocytes and granulosa cells in preantral follicles but the staining was stronger in the most of antral follicles. Theca cells presented differential expression since IGF-1R staining changed from mainly weak in secondary follicles to strong signal in the most of antral follicles. These findings are according to the literature in other species, where IGF-1R was immunolocalized in the follicles (human: Qu *et al.,* 2000b; rat: Zhao *et al.,* 2002), GC (buffalo: Singh *et al.,* 2015; bovine: Gong *et al.,* 1994) and oocytes (human: El-Roeiy *et al.,*1993, 1994; bovine: Wang *et al.,* 2009a; primate: Zhou and Bondy, 1993; Vendola *et al.,* 1999; buffalo: Singh *et al.,* 2015). Conversely, immunoreactive IGF-1 has been localized in several studies in the theca-interstitial compartment (bovine: Schams *et al.,* 2002) but not in others (human: Poljicanín *et al.,* 2015). IGF-1 acts throughout its receptor in the follicle cells and oocyte (Murphy *et al.,* 1987; Yu and Ge, 2007; Zhou *et al.,* 2016) and is mainly produced in granulosa cells in the ovary. Then, observations here described was expected as its key function is granulosa cell proliferation and differentiation and steroidogenesis stimulation of different follicular stages via PI3K-Akt activation (Louhio *et al.,* 2000; Fortune, 2003; Monget *et al.,* 2002; Cara and Rosenfield, 1988).

The moderate inmunostaining in granulosa cells and oocytes of IGF-1R in all preantral follicles suggests physiological functions before recruitment but, the increase in inmunostaining of IGF-1R and the mRNA gene expression of IGF-1 and IGF-1R in antral follicles stages advise a key role in the later stages of the follicular development in an autocrine and paracrine manner as well. According to our results, in several species, IGF-1 mRNA was localized in follicles of all sizes (human: Qu *et al.,* 2000b; Samoto *et al.,* 1993; pig: Zhou *et al.,* 1996; cow: Schams *et al.,* 2002). IGF-1 increased progression from the primordial to the primary stage in *in vitro* cultured human ovarian cortex but in absence of IGF-1 the follicles also growth (Louhio *et al.,* 2000; Stubbs *et al.,* 2013). As occur with EGF, the intraovarian IGF-1 system plays a key role in regulating proliferation and differentiation of granulosa and theca cells, steroidogenesis and apoptosis either alone or in synergy with gonadotropins (Baker *et al.,* 1996). Increase in IGF-1R expression is concomitant with increase in FSH and LH receptors as follicle enlarges size in cattle (Wandji *et al.,* 1992). IGF-1 contributes to improve the final maturation of follicle events (Monget and Bondy, 2000) and the amplification of FSH action by increasing the aromatase activity (Baker *et al.,* 1996; Zhou *et al.,* 1997), production of estrogens and LH receptors (Magoffin and Weitsman, 1994; Tsuchiya *et al.,* 1999; Huang *et al.,* 2001), and antrum formation (Monget and Bondy, 2000). Therefore, it is suggested that IGF-1 has a permissive rather than a decisive role on follicular cell proliferation and survival and was not essential for the recruitment of primordial follicles and growing of preantral ones (Baker *et al.,* 1996), where an endocrine mechanism for the IGF-1 regulation of antral follicles has been suggested (Armstrong*,* 2000). Besides, IGF-1 seems to play a key role in increasing the sensitivity of non- gonadotropin dependent follicles (small antral follicles) to gonadotropin action and to their transition to the dependence of gonadotropin (Monget *et al.,* 2002). In fact, previous studies have shown that in woman IGF are possible regulators of beginning of follicular growth, and accelerated preantral follicular growth on PCOS may be due to increased activity of endogenous IGF (Stubbs *et al.,* 2013).

On the other hand, IGF-1 and IGF-1R genes were also expressed in the *corpus luteum.* IGF-1R mRNA transcripts were higher than IGF-1 ones. Data was confirmed by the high expression for IGF-1R in the luteal cells by immunohistochemistry. mRNA expression of IGF-1, and IGF-1R was also observed during the early luteal phase (Days 1–4) in bovine *corpus luteum* (Vandehaar *et al.*, 1995; Kirby *et al.,* 1996; Perks *et al.,* 1999; Woad *et al.,* 2000). mRNA expression of IGF-1R and localization in large luteal cells was also observed in pig *corpus luteum* (Ge *et al.,* 2000) but not was detected in rats (Oliver *et al.,* 1989). IGF-1 acts by stimulating the uptake of cholesterol, modifying its cytoskeleton,

and actively stimulating the secretion of P_4 (Schams *et al.,* 1980; McArdle and Holtorf, 1989; Einspanier *et al.,* 1990) in rats (Chouhan *et al.,* 2014), sheep (Khan-Dawood *et al.,* 1994), pigs (Pescador *et al.,* 1999; Miller *et al.,* 2003; Ptak *et al.,* 2004), and cattle (McArdle and Holtorf, 1989; Sauerwein *et al.,* 1992). As well as EGF, IGF-1 maintains luteal size (Parmer *et al.,* 1991) promotes proliferation, decreases of DNA fragmentation, and prevents the spontaneous onset of apoptosis by stimulating P_4 production or by regulating the expression of survival gene and pro-apoptotic gene (Ptak *et al.,* 2004; Yang *et al.,* 2000; Parborell, *et al.,* 2002; Sugino, *et al.,* 2000). However, a less potent role than EGF in angiogenesis has been elucidated (Chouhan *et al.,* 2014). Therefore, the IGF-1 seems to play an important role in the process of early *corpus luteum* function and therefore in the endometrium development, embryo survival and early embryonic losses (Webb *et al.,* 2002). However, these functions are modulated by the IGFBPs.

5.2.2. Characterization of IGF-1 and IGF-1R system in the guinea pig cumulus-oocyte complexes

IGF-1 system expression has been scarcely studied in the COCs. It is known that the oocyte produces paracrine signals which act together with surrounding CCs to regulate gonadotropin and IGF-1 stimulated steroidogenesis (Li *et al.,* 2000). In guinea pig, we evidenced IGF-1 and IGF-1R gene expression suggesting an autocrine and paracrine role of IGF-1 in the bidirectional communication of oocytes and their corresponding CCs. Higher mRNA transcripts of IGF-1R were found in CCs than in oocytes which can be related with higher response of CCs to IGF-1 action than oocytes. According to our results, IGF-1R was detected in human CCs but the mRNA levels were different if the follicles were obtained from woman with PCOS or not (Kwon *et al.,* 2010). IGF-1R was also localized in bovine (Nuttinck *et al.,* 2004; Wang *et al.,* 2009) and ovine (Pissani *et al.,* 2008) CCs and oocytes. But controversially, it is not completely clear if IGF-1 is expressed in the oocytes of all mammals. According with our results, some authors detected IGF-1 in immature bovine (Yoshida *et al.,* 1998; Satrapa *et al.,* 2013) and sheep (Pissani et al; 2008) oocytes but others have reported no expression in the oocytes being mRNA restricted exclusively to CCs (Nuttinck *et al.,* 2004; Wang *et al.,* 2009). The intensity of the inmunofluorescence for IGF-1R increased in both oocytes and CCs during *in vitro* maturation in the guinea pig. It disagrees to previous studies where no modifications in the localization of IGF-1R within the oocyte were detected after oocyte maturation (Yoshida *et al.,* 1998; Nuttinck *et al.,* 2004). In contrast other reports have shown that IGF-1R increase after oocyte maturation depends on the type of IVM medium used (Warzych *et al.,* 2007). Higher gene expression of IGF-1R after oocyte maturation

is related to higher blastocyst rate and lowere apoptosis in embryos deloped form those oocytes (Warzych *et al.,* 2007). Other studies also have detected mRNA for IGF- in immature and matured oocytes (bovine: Wang *et al.,* 2009, Farin *et al.,* 2010) and buffalo (Vikash *et al.,* 2011) suggesting that supplementation of IGF-1 may be useful for *in vitro* oocyte maturation.

5.2.3. Characterization of IGF-1 and IGF-1R system in the guinea pig early embryos

IGF-1 is an important growth factor during preimplantation embryo development in mammals (bovine: Schultz *et al.,* 1992; Matsui *et al.,* 1995, Palma *et al.,* 1997, Yoshida *et al.,* 1998; Lonergan *et al.,* 2000, Yaseen *et al.,* 2001, Moore *et al.,* 2007; Warzych *et al.,* 2007; buffalo: Pawshe *et al.,* 1998; murine: Schultz *et al.,* 1992) since can enhance embryo cell proliferation and mitogenesis (Adashi *et al.,* 1985; Kamada *et al.,* 1992) via Akt phosphorylation (Green and Day, 2014). IGF-1R is regulated during embryogenesis and IGF-1 has effects on glucose, metabolism and cell differentiation (Bassas *et al.,* 1985; Heyner *et al.,* 1989, 1993; Rappolee *et al.,* 1992; Adamson, 1993; Schultz *et al.,* 1993). In current work, we demonstrated the distribution of the IGF-1R in guinea pig embryos in the cell membrane of the totality of embryo blastomers from 4 cell embryos to morula stage showing the temporal localization of IGF-1R protein. Bedzhov *et al.* (2012) also localized IGF-1R throughout the embryo membrane, but they discovered that co-expression with E-cadherin in cell contact sites in the embryo was essential for appropriate activation of the receptor and to avoid apoptotic signaling. Some authors have also found IGF-1R in all the preimplantation stages (cattle: Watson *et al.,* 1992; Rappolee *et al.,* 1992; Yoshida *et al.,* 1998; Qu *et al.,* 2017) while others only detected it after maternal transition: 8-cell stage in mouse (Smith *et al.,* 1993; Schultz et al; 1993) and pig (Xia *et al.,* 1994) embryos and morulae and blastocysts in rabbit (Santos *et al.,* 2008). High gene expressions of IGF-2, IGF-1 receptor, IGF-2 receptor, and insulin receptor are associated with high growth potential in day 3– day 6 human embryos (shortly before implantation) (Liu *et al.,* 1997). These results suggest that human embryonic development at the preimplantation stage is regulated by IGF from both embryos. Probably, IGF-1 and insulin are provided both by the mother and the embryo (Kane *et al.,* 1997). The insulin is needed to increase the protein synthesis and development of blastocyst (Harvey and Kaye, 1988, 1990, 1992; Rappolee *et al.,* 1992). The IGF-1 and IGF-1R are highly expressed in TE (precursor to placenta) to regulate embryonic growth (Rappolee *et al.,* 1992) highlighting the differential role of IGF-1 in the embryos.

In addition, as we mentioned previously an increasing immunoreaction pattern as development progressed in the guinea pig embryo is shown. It could be related to the regulation of the metabolic processes that may modulate cell proliferation rates when the embryo genome is activated in which IGF system is involved (Senior *et al*., 1990). Our results match with the increased expression from the 3- to 4-cell stage up to the blastocyst stage found in human embryos (Lee *et al.,* 1993). In cattle, greatest increase occurred after hatching (Yaseen *et al.,* 2001) although IGF-1R is expressed in all the preimplantation stages (Yoshida *et a*l., 1998; Schams *et al.,* 2002; Wang *et al.,* 2009; Vikash *et al.,* 2011) suggesting that supplementation of IGF-1 may be useful for *in vitro* embryo development. In fact, many studies point out the importance of IGF-1 and IGF-1R in the paracrine dialog between preimplantation embryos and uterine tissues (Murphy and Barron, 1993; Chandra *et al.,* 2012) since higher gene expression of IGF-1R has been related to higher blastocyst rate and survival as well (Warzych *et al.,* 2007).

All the described findings in guinea pig confirm the important role of IGF-1 and IGF-1R in regulation mainly of final maturation stages of folliculogenesis, oocyte maturation, *corpus luteum* formation and function and, embryo development. However, there are differences in the spatial and temporal location of the IGF-1 system among mammals suggesting different mechanisms of action (Yuan *et al.,* 1998; Gutierrez *et al.,* 1997; Schams *et al.,* 1999; Armstrong *et al.,* 2000; Mao *et al.,* 2002; Sharma *et al.,* 2010; Magalhaes-Padilhaa *et al.,* 2012). Besides, the bioactivity of IGF during follicular development and *corpus luteum* formation is mediated by the cell membrane receptors and the family of specific IGFBPs 1-6. Hence, changes in any component of this system could potentially disturb follicular development and related events.

5.3. Expression and localization of IGFBPs 1 to 6

5.3.1. Expression and localization of IGFBPs 1 to 6 in guinea pig ovary

Insulin-like growth factor binding proteins present within the ovary bind with IGF, modulating the concentrations of bioactive IGF. The proteins, IGFBPs 1 to 6, share a common domain organization consisting of cysteine-rich N and C terminal domains connected by a flexible linker region. IGF binding sites are located on both the N and C domains and both are required for wild-type affinity (Hwa *et al.,* 1999; Carrick *et al.,* 2001).

IGFBPs are present within the ovary where they are mainly produced, secreted and degraded (Besnard *et al.,* 1996; Stewart *et al.,* 1996; Zhou *et al.,* 1996). As described in the results, we clearly demonstrate that the IGFBPs 1–6 are located within ovary in all follicular stages, *corpus luteum* and oocyte and CCs of guinea pig, but different expression pattern is showed for each IGFBPs indicating a regulatory role in the IGF-1 bioavailability during the developmental stages of follicular development. In general, the IGFBPs, by sequestering IGF away from the IGF receptor, show inhibitory effects on IGF action (Firth and Baxter, 2002). In the ovary IGFBPs inhibits follicular growth and differentiation induced by gonadotrophins; because their shift lead to changes in IGF-1 bioavailability (Spicer and Echternkamp, 1995), making it non-available to its receptors (Armstrong, 1997). In contrast, *in vitro* studies showed stimulatory activity (Schams *et al.,* 1999; Dubey *et al.,* 2015) and it is also described IGF-1-independent actions (Wright *et al.,* 2002), such as regulation of glucose homeostasis by IGFBP-1 and IGFBP-3 (Murphy, 2003). The proteolytic degradation of IGFBPs in healthy follicles has an important role reversing inhibition action of the IGFBPs and increasing availability of IGF or generating IGFBPs fragments with novel bioactivity (Firth and Baxter, 2002). Then, IGF-1 action can be inhibited or potentiated by IGFBPs 1-6 (Jones and Clemmons, 1995; Rajaram *et al.,* 1997) and these actions depends on cell surface association, ECM association and phosphorylation or proteolysis, which alter the affinity of IGFBPs for the ligands (Schams *et al.,* 2002; Singh *et al.,* 2015).

The existence of IGFBPs 1-6 mRNA transcripts in the guinea pig ovary is according to findings in other species (cattle: Schams *et al.,* 2002; sheep: Hasti *et al.,* 2004, buffalo: Singh *et al.,* 2015, macaque: Brogan *et al.,* 2010, rat and mouse: Goubau *et al.,* 1996; human: El-Roeiy *et al.,* 1994; Voutilainen *et al.,* 1996).

Gene expression of IGFBP-1 was detected in antral and preovulatory follicles, *corpus luteum*, and in COCs in the guinea pig. This agrees with the fact that IGFBP-1 mRNA has been detected in granulosa cells of dominant follicles in human (El-Roeiy *et al.,* 1994), luteinizing follicles (Thierry *et al.,* 1996) and follicles containing mature oocytes (Kawano *et al.,* 1997). In our work, relative mRNA expression of IGFBP-1 was higher in preovulatory follicles than in antral follicles whereas the IGF-1 mRNA transcrips followed the inverse relationship. Higher inmunofluorescent signal for IGFBP 1 and 3, in oocytes and CCs obtained from preovulatory follicles as also found in guinea pig, as described below. High IGFBP-1 and low IGF-1 in the ovary may play an important role in follicular growth and maturation (Wang *et al.,* 2006). IGFBP-1, -2 and -3 inhibit IGF-1 action on the ovary. Therefore, IGFBP-1 and -3 are observed in dominant follicles. IGFBP-1

modulates the mitogenic activity of IGF-1 in granulosa and CCs in the later stages of follicular development contributing to the proliferation of cells, probably enhancing gonadotropin action (Singh *et al.,* 2015), and to the processes related to the stablishment of follicular dominance and oocyte developmental competence. IGFBP-1 is believed to inhibit IGF-1-induced androgen production by theca cells and in turn inhibit/prevent follicular atresia. In fact, IGFBP-1 is observed in granulosa cells of dominant follicles but not in those atetric (El-Roeiy *et al.,* 1994). An increase in IGF-1 expression and a decrease in IGFBP-1 and IGF-2 in theca and stroma cells isolated from PCOS suggests a negative effect of IGF-1 on folliculogenesis and ovulation. In these woman and excess of androgen and insulin levels in follicles, affects granulosa cells by increasing IGFBP-2 and -4 levels and reducing IGFBP-1 levels (Greisen *et al.,* 2002; Druckmann and Rohr *et al.,* 2002; Balen, 2004). However, in the immunohystochemical study the expression for IGFBP-1 has been described higher signal in preantal follicular stages but the intensity decreased in granulosa cells and theca of antral follicles in the guinea pig. These results may be indicating that the relatively amount of mRNA for the IGFBPs does not necessarily indicate that there is a constant inhibition of the action of the IGF (Besnard *et al.,* 1996, 1997) and other IGFBPs could be modulating the bioavaiability of IGF-1 during follicular development.

In *corpus luteum*, IGFBP-1 gene expression was higher than in follicles of both sizes although moderate immunoreaction was observed in luteal cells. In agreement with our results, IGFBP-1 expression was also localized in luteinized granulosa cells and *corpus luteum* for several species (Buyalo *et al.,* 1994; Baxter 2000; Bondy, 1993; Sayre *et al.,* 2000; Neuvians *et al.,* 2003) and human (Hartshorne and Lee, 1990) whereas, in others, IGFBP-1 mRNA could not be detected (rat: Nakatani *et al.,* 1991, sheep: Hastie and Haresign, 2006). Therefore, controversial results about the luteal function of IGFBP-1 are reported. Some studies attributed a stimulatory role of IGFBP-1 on luteal function since they found a significantly high expression of IGFBP-1, -3, -5, and -6 either in the early or in the mid-luteal phase (Uniyal *et al.,* 2015). However, other authors have reported an increase of IGFBP-1 mRNA transcripts during luteolysis (Sayre *et al.,* 2000) which may inhibit the steroidogenic effects of IGF-1.

Our results imply a key role for IGFBP-1 in the development of the dominant follicle and in *corpus luteum* regulation in the guinea pig. However, its implication in the stablisment of the follicular dominance and function of *corpus luteum* need further investigations.

For IGFBP-2 significantly higher mRNA transcripts have been showed in preovulatory follicles than in antral follicles. mRNA IGFBP-2 was detected in mouse granulosa cells in which is highly expressed (Seppälä *et al.,* 1984, Nakatani *et al.,* 1991, Adashi *et al.,* 1997, Wandji *et al.,* 1998). However, in the rat ovary IGFBP-2 mRNA is specifically localized in thecal cells (Nakatani *et al.,* 1991). In situ hybridization studies have shown that, in human dominant follicles, IGFBP-2 and IGFBP-3 mRNA are localized in both granulosa cells and theca cells (Zhou and Bondy*,* 1993; El-Roeiy *et al.,* 1993). It is proposed that lower levels of mRNA for IGFBP-2 in antral follicle can be associated with increased sensitivity to FSH (Armstrong *et al.,* 1996; 1998). FSH may increase production of proteases and decreases IGFBP-2 (Schams *et al.,* 1999) resulting in a greater expression of the IGF-1 in the granulosa cells and theca cells (Singh *et al.,* 2015). Atretic follicles present predominantly as IGFBPs, IGFBP-2 and IGFBP-4 which are selectively eliminated in healthy follicles (Wright *et al.,* 2002). However, Giudice (1992) indicated that IGFBP-2 may be also important during early stages of follicular development. In fact, mRNA IGFBP-2 was detected in granulosa cells of primordial, primary, and small antral follicle in the rhesus monkey (Arraztoa *et al.,* 2002). In our immunohistological study, we also showed that IGFBP-2 showed higher sinal in granulosa cells in all follicular stages, including the primordial follicles, whereas theca cells and oocyte presented fewer inmunostaining. These findings suggest a possible regulatory role of IGFBP-2 in the follicular development according to Sharms *et al.,* (2002) and Ge *et al.,* (2003).

In *corpus luteum*, IGFBP-2 was highly expressed as well as has been demonstrated in the early luteal stage in buffalo (Uniyal *et al.,* 2015) but, in mid-, late, and regressing luteal phases in equine (Watson *et al.,* 2005). In sheep gene expression of IGFBP-2 was relatively constant during the luteal phase of the cycle, and decreased 20-40 h after $PGF_{2\alpha}$ induced luteolysis, indicating that they may not play an active role in luteolysis (Hastie and Haresign, 2006). Although in present study the expression of IGFBP-2 was found high, it has been suggested that the inhibitory effects might have been masked by higher expression of other stimulatory factors (Uniyal *et al.,* 2015). Also a role for IGFBP-2 in the developing *corpus luteum* has been elucidated in bovine (Gregson *et al.,* 2016).

In later stages of follicular development, differential gene expression of IGFBP-3 was described since relative mRNA expression was significantly higher in the antral follicles than in preovulatory follicle. Higher expression of IGFBP-3 in antral follicle correlates with higher gene expression of IGF-1 and IGF-1R in those follicles compared to the preovulatory follicle. IGFBP-3 is present in granulosa cells of different type of follicles as

has been reported in buffalo (Dubey *et al.,* 2015; Singh *et al.,* 2015), cattle (Yuan *et al.,* 1998; Armstrong, 2002), and pig (Samaras *et al.,* 1993). Besides, IGFBP-3 was expressed in oocytes of bovine preantral follicle (Armstrong *et al.,* 2002) and in oocytes and stroma in rhesus monkey ovaries (Arraztoa *et al.,* 2002). It is also expressed in luteinized granulosa of porcine (Wandji *et al.,* 2000) and human follicles (Adashi *et al.,* 1990). In current work, the immunohistochemical study showed slight expression of IGFBP-3 in all follicular stages in oocytes, granulosa and theca cells. Previous authors also have detected IGFBP-3 mRNA are in both human granulosa and theca cells (Zhou and Bondy, 1993; el-Roeiy *et al.,* 1994) and no differences were demostrated in mRNA levels of IGFBP-3 betwenn granulosa cells from inmature- and mature follicles (Kwon *et al.,* 2010). It is in contrast with previous findings in which demonstrated that IGFBP-3 presence increases with the enlargement in follicular size in bovine (Rebouças *et al.,* 2014). Besides higher concentrations of IGFBP-3 in estrogen dominant follicles have found compared with androgen dominant ones (Thierry Van Dessel *et al.,* 1996; Ge *et al.,* 2003), but this results are contradictory (San Roman and Magoffin *et al.,* 1993). Although, in humans, IGFBP-3 is the most important and abundant binding protein that intervenes in the maintenance of the circulating IGF-1 level (Batista *et al.,* 2012), the IGFBP-3 exerts dual functions in modulating IGF action *in vivo*. Soluble IGFBP-3 inhibits IGF-1 action by sequestering and preventing IGF-1 receptor binding, whereas surface-associated IGFBP-3 enhances the growth-promoting effects of IGF-1 in bovine fibroblasts (Conover *et al.,*1990).

Increasing levels of IGFBP-3 has been observed in the early (Schams *et al.,* 2002; Brogan *et al.,* 2010) and mid-luteal phase (Uniyal *et al.,* 2015), which correlates with the highest expression for IGF-1, IGF-2 and IGF-1R and it is attributed to the stimulatory effect of IGF-1 on the production of IGFBP-3 as studied in cultured porcine GCs (Grimes and Hammond, 1992) and bovine fibroblast cells (Bale *et al.,* 1992). In contrast, gene expression of IGFBP-3 was highest throughout the luteal phase but lower in regressing *corpus luteum* in sheep (Hastie and Haresign, 2006). In primate *corpus luteum* IGFBP-3 plays a regulatory role in angiogenesis and luteal function as well (Fraser *et al.,* 1998). In current work, *corpus luteum* showed lower mRNA transcripts than in the those found in follicles and moderate inmunostaining. In some cases the glycosylation in the biological actions of IGFBP-3 may affect the detection of protein and the interaction with IGF-1 (Giudice, 1992; Schams *et al.,* 1999; Masnikosa *et al.,* 2010; Rodriguez *et al.,* 2011).

IGFBP-4 mRNA was expressed in antral and preovulatory follicles and *corpus luteum*, in guinea pig with a similar pattern than IGFBP-1 and 2 although *corpus luteum* showed intermediate values. IGF binding to IGFBP-4 may be mediated through the activation/production of specific IGFBP-4 proteases present in the granulosa cells (Besnard *et al.,* 1996) and in porcine, equine and bovine preovulatory follicles (Mazerbourg *et al.,* 2000). IGFBP-4 specific protease is stimulated in human granulosa cells by FSH (Iwashita *et al.,* 1998) being in the dominant follicle increased. The higher mRNA levels of IGFBP-4 in preovulatory follicles in guinea pig are according with previous findings in which found high protease PAPP-A (Pregnancy-associated plasma protein-A) and low IGFBP-4 during the selection of dominant follicles (Conover *et al.,* 1999,1990; San Roman and Magoffin, 1993; Hourvitz *et al.,* 2000). This situation shift to low PAPP-A and high IGFBP-4 in the late and more mature follicles shortly before oocyte maturation and ovulation (Wang *et al.,* 2006). Therefore, decreased levels of IGFBP-4 and increased levels of its protease PAPP-A (Conover *et al.,* 1999) have been associated with the selection of dominant follicles (San Roman and Magoffin, 1993; Hourvitz *et al.,* 2000; Mazerbourg *et al.,* 2001). In contrast, an excess of androgen and insulin levels in follicles of woman with PCOS, increases IGFBP-2 and -4 levels and reducing IGFBP-1 levels (Greisen *et al.,* 2002; Druckmann and Rohr *et al.,* 2002; Balen 2004).

Schams *et al.* (2002) confirm the IGFBP-4 expression and regulation in pre-ovulatory follicles in bovine species. It is described that it is essentially expressed in thecal cells from healthy and atretic follicles (human: Zhou and Bondy *et al.,* 1993; sheep: Besnard *et al.,* 1996; Armstrong *et al.,* 1996), but it has also been localized in granulosa cells in pig (Grimes *et al.,* 1994) and buffalo (Singh *et al.,* 2015) and in atretic follicles in the rat (Nakatani *et al.,* 1991; Erickson *et al.,* 1992). IGFBP-4, a potent inhibitor of FSH-induced estradiol secretion by granulosa cells, is expressed in atresic follicles in the rat (Nakatani *et al.,* 1991; Erickson *et al.,* 1992). In current work, we didn´t observe any difference in the immunolocalization between cellular compartments according to human (El-Roeiy *et al.,* 1994), where IGFBP-4 is expressed in both granulosa and thecal cells. It has been demonstrated that IGFBP-4 inhibits steroid production in granulosa and theca cells in woman (Mason *et al.,* 1998). In our study, the signal was weak for this protein according to Mason et al. (1998).

In *corpus luteum* gene expression of IGFBP-4 showed intermediate values compared to follicles, being signal found by immunohistochemistry weak. Uniyal et al. (2015) and Hastie and Haresign, (2006) found maximum IGFBP-4 expression in the early luteal

phase, and afterwards decreased. In current work, IGFBP-2 showed higher gene expression and inmunostaining than IGFBP-4. In buffalo, it has been reported that IGFBP-2 is more important inhibitory binding protein than IGFBP-4 (Uniyal *et al.,* 2015). Hossner *et al.,* (1997) also showed that IGFBP-4 exhibits only inhibitory effects in pig

IGFBP-5 is considered to have a key role in the IGF axis (Beatti *et al.,* 2006) and shows a relative constant expression in all cycle (Schams *et al.,* 2002). It was detected in all studied tissues and immunolocalized with a weak reaction in all the cells from primordial to antral follicles, and in *corpus luteum* in guinea pig. mRNA expression pattern was similar to IGFBP-3, in which antral follicle showed the maximum relative expression.

In humans, IGFBP-3 is the most important and abundant binding protein that intervenes in the maintenance of the circulating IGF-1 level (Batista *et al.,* 2012) and second one is IGFBP-5 (Firth and Baxter, 2002). In human, higher expression of IGFBP-5 in granulosa cells was observed in woman with PCOS suggesting that this increase is related with inhibition of oocyte maturation by decreasing IGF-1 availability (Kwon *et al.,* 2010). The IGFBP-5 mRNA was mainly present in theca cells from healthy follicles and in granulosa cells from atretic follicles (Zhou and Bondy *et al.,* 1993). However, expression in granulosa cells appears to be species-dependent. In the rodent, it is concentrated in a subpopulation of small follicles probably destined for selection (mouse: Wandji *et al.,* 1998) or atresia (rat: Erickson *et al.,* 1992). However, in the porcine, IGFBP-5 is not detected in the granulosa of any follicles, small or large (Wandji *et al.,* 2000). In the sheep, cow, and goat as well as in the rat, IGFBP-5 mRNA is detected in the granulosa of atretic follicles (Besnard *et al.,* 1996, Erickson *et al.,* 1992) since follicular atresia, is characterized by a decrease in intrafollicular IGFBP-4 and -5 proteolytic activity (Besnard *et al.,* 1996). Also the IGFBP-5 was isolated in the porcine FF (Shimasaki *et al.,* 1990*;* Shimasaki and Ling,1991).

In current work gene expression and inmunolocalization of IGFBP-5 in *corpus luteum* were low. IGFBP-5 has been localized in *corpus luteum* in previous studies (Erickson *et al.,* 1992). In sheep IGFBP-5 mRNA gene expression increased in early regressing *corpus luteum* 20 h after prostaglandin-induced luteolysis (Hastie and Haresign, 2006), which was also evident in a previous study in cattle (Neuvians *et al.,* 2003). E_2 has been suggested to participate in the regulation of luteal steroidogenesis and luteal cell morphology. In this sense, a recent study has suggested an inhibitory role for E_2 in the regulation of luteal steroidogenesis, morphology and proliferation. IGFBP-5 was one the

E_2 responsive genes with important role in the mediation of this hormone actions such as E_2-induced phosphorylation of PI3K/Akt kinase pathway in rats (Tripathy *et al.,* 2016).

We evidenced the expression of IGFBP–6 in the guinea pig ovary. mRNA abundance was comparable between antral and preovulatory follicles being both of them significantly higher than in *corpus luteum*, as occurred for IGFBP-3 and 5 too. Also, protein distribution was weak in all the compartments of the ovary except for the oocyte, which signal increased from secondary to antral follicle. During final growth of bovine follicles, IGFBP–6 has been detected in the theca-interstitial cell and granulosa cell compartment (Schams *et al.,* 1999). Moreover, studies have demonstrated that this binding protein has lower binding affinity by IGF-1 (Bach, 2015). Recent evidences suggested that IGFBP-6 induced apoptosis appeared to be IGF-dependent (Hale *et al.,* 2000) or independent (Iosef *et al*., 2010; Sueoka *et al.,* 2000) in different cell lines and may be inhibit oocyte maturation (Kwon *et al.,* 2010). In this line, IGFBP-6 mRNA levels were lower in mature follicles than in those inmatue (Kwon *et al.,* 2010).

The gene expression and immunolocalization of IGFBP-6 in *corpus luteum* was low as occur with IGFBP-5. In bovine IGFBP-6 is poor expressed during estrus cycle, but a significant up-regulation for IGFBP-6 mRNA at 64 h after induced luteolysis occur (Neuvians *et al.,* 2003). In contrast, a reduction of IGFBP-6 gene expression was observed in sheep *corpus luteum* after $PGF_{2\alpha}$-induced luteolysis, indicating that all of them may not play an active role in luteolysis in sheep (Hastie and Haresign, 2006).

5.3.2. Expression and localization of IGFBPs 1 to 6 in guinea pig cumulus-oocyte complexes

Current work it is demonstrated that oocytes and CCs expressed all IGFBPs genes. The inmunolocalization of IGFBPs family was confirmed by the expression of mRNA transcripts in both. In other especies IGFBP-1 (Kamangar *et al.,* 2006), 2 (Nuttinck *et al.,* 2004), 3 (Ingman *et al.,* 2000), 4 (Giudice, 2001) 5 (Ingman *et al.,* 2000) and 6 (Kamangar *et al.,* 2006) are also located in oocytes. IGFBP-1 (Kwon *et al.,* 2010; Kim *et al.,* 2013), 2 (Nuttinck *et al.,* 2004; Kwon *et al.,* 2010; Kim *et al.,* 2013), 3 (Kwon *et al.,* 2010; Ingman *et al.,* 2000), 4 (Nuttinck *et al.,* 2004; Kwon *et al.,* 2010) 5 (Ingman *et al.,* 2000, Kwon *et al.,* 2010) and 6 (Kwon *et al.,* 2010) are also located in CCs.

Our findings indicate that IGFBPs seems to be implicated in the oocyte maturation by the modulation of the action of IGF-1 between the oocyte and CCs since all IGBBPs

proteins were localized in both of them. Wang *et al.* (2006) have shonw that he combination of high IGF-2, high IGFBP-3, high IGFBP-4, and low PAPP-A levels in FF at the time of oocyte retrieval correlates with better oocyte maturation and early embryo development. In addition, high IGFBP-1, high IGFBP-4, and low IGF-1 levels may be favorable factors in later embryo development after oocyte retrieval. According with these results, the highest signal was found for IGFBP-1 in both oocytes and CCs and for IGFBP-4 in CCs of guinea pig. Preovulatory follicles also showed higher gene expression levels for IGFBP-1 and 4 and lower levels for IGF-1 and IGF-1R compared to antral follicles. However, the lowest fluorescence intensity was found for IGFBP- 6 in oocytes and CCs and for IGFBP-3 in CCs, whereas the rest of IGFBPs (2 and 5) showed intermediate results in CCs. Previous results have showed a decrease of IGFBP-6 in mature-follicles in both normal woman and woman with PCOS suggesting that IGFBP-6 may inhibit oocyte maturation (Kwon *et al.,* 2010). Other authors have reported that granulosa cell production of IGFBP-2 and -5 plays an inhibitory role in intrafollicular IGF action, and IGFBP-2 is prominent in atretic follicles in primates (Liu *et al.,* 1993; Spicer *et al.,* 1997; Spicer and Chamberlain, 1999; Davidson *et al.,* 2002).

Comparing the results between the oocytes and their corresponding CCs, the present study showed that mRNA transcripts for the majority of IGFBPs are up-regulated in oocytes compared to their gene expression in CCs, except for IGFBP-5. Together with the results found for IGF-1 and IGF-1R in ooctes and CCs, in the experiment 1b we can hipothesize that IGF-1 can be acting mainly on CCs in the guinea pig throgouth its receptor. Elevation of IGF-1 in luteinized CCs of matured follicles implies that IGF-1 plays an important role in steroid synthesis at the time preceding ovulation (Kwon *et al.,* 2010b). According with the results in gene expression IGFBP-1 and 4 fluorescence was also higher in oocytes. However, CCs showed higher intensity for IGFBP- 3 and 6 compared to oocytes. While the mRNA expression of IGFBP-1 to 6 is diverse and consistent with complex regulation, there is a generalized increase in mRNA levels of IGFBP-3, 5, and 6 during the first 12 h following hCG, with a concomittent increase in protein for IGFBP-3 and 5 in the FF (Brogan *et al.,* 2010). Increase in IGFBP-3 and 5 can induce a reduction in IGF-1 bioavailability helping to final maturation events as the earliest steps in granulosa cell luteinization (Brogran *et al.,* 2010).

We have shown for the first time the patterns of mRNA expression and protein inmunolocation for all IGFBPs in the guinea pig COCs. More investigations are needed to study the differences found between the gene expression and immunofluorescence study for IGFBP-2, 3, 5 and 6 in the COCs of guinea pig and their implication in the

oocyte maturation in these species. A deedply research about the changes of those IGFBPs throgouth oocyte maturation are required to understand the role of the IGFBPs in the maturation of the guinea pig oocyte.

5.4. Role of EGF and IGF-1 in guinea pig *in vitro* oocyte maturation

5.4.1. Cortical granules migration and mitochondrial distribution patterns in the guinea pig

In this Thesis we established by the first time the cortical granules and mitochondrial distribution patterns in guinea pig. Cortical migration is related to density decrease of cortical granules in the central area of oocyte compared to the periphery as maturation proceeds (Adona *et al.,* 2008). It has been used as a reliable indicator for the cytoplasmic maturation of oocytes in cattle (Damiani *et al.,* 1995), porcine (Wang *et al.,* 1997), horse (Carneiro *et al.,* 2001), mouse (Li and Albertini, *2013*) and rabbit (Arias-Alvarez *et al.,* 2009). However, different patterns are shown among species. From our best knowledge, no references described cortical granules migration pattern in guinea pig oocytes. Our findings showed that cortical granules migrated to the periphery in most of the oocytes after IVM whereas in immature oocytes homogeneous distribution of cortical granules was prevalent. These findings are similar to that found in calf (Damiani *et al.,* 1995), rabbit (Arias–Alvarez *et al.,* 2009), bovine (Hosoe and Shioya, 1997), and equine (Willis *et al.,* 1994) oocytes. In human mature oocytes also form one to three discontinuous layers of cortical granules beneath the oolemma (Sathananthan *et al.,* 1985; Ghetler *et al.,* 2006; Shahedi *et al.,* 2013). Conversely, mice oocytes showed asymmetric distribution of cortical granules (Ducibella *et al.,* 1988). This difference in the distribution of cortical granules in mouse and the similarity of the distribution to other species, including humans, emphasizes the importance of using the guinea pig as a potential biomodel for IVM studies.

On the other hand, reorganization of mitochondria is also an essential process for oocyte maturation. The capacity of mitochondria to produce ATP by oxidative phosphorylation and their distribution could vary among species, perhaps due to site specific requirements within the ooplasm in the maturing oocyte (Van Blerkom, 2004). Schon *et al.* (2000) and Barnett *et al,* (1996) suggest that changes in the location of mitochondria can be correlated with the ability of hamster and cattle embryos to develop *in vitro*. Regarding mitochondrial patterns, we found that there wasa relationship between oocyte maturation and mitochondrial distribution. We determined that great number of the

oocytes after IVM showed peripheral distribution to the nuclear pole. This is according to mitochondrial migration pattern described in horse (Torner *et al.,* 2007), mouse (Nishi *et al.,* 2003; Calarco, 1995) and human oocytes (Eichenlaub–Ritter *et al.,* 2004) in which a clustering of polarized mitochondrial in the hemisphere containing the second metaphase plate was described (Van Blerkom, 2011; Eichenlaub–Ritter *et al.,* 2004). However, mitochondria homogeneously distributed throughout the cytoplasm was found mainly in immature oocytes of guinea pig according to previously described in other species such as swine (Cran, 1985; Sun *et al.,* 2001), cattle (Krisher and Bavister, 1998), mouse (Li and Fan, 1997; Wang *et al.,* 2009b), hamster (Barnett *et al.,* 1996), cat (González *et al.,* 2012), dog (De Los Reyes *et al.,* 2011) and human (Wilding *et al.,* 2001).

5.4.2. Effect of EGF supplementation on the maturation medium

As we have extensively reported in this work, EGF intervene on several events related to the oocyte growth, maturation process and the resumption of meiosis (Gall *et al.,* 2005) as well as on embryonic development (Cai *et al.,* 2003). EGF is associated to cumulus expansion (Downs, 1989; Tsafriri *et al.,* 2005) and EGF-like ligands induce the activity of MAPK and stimulate the occurrence of GVBD in the immature follicles (Pang and Ge, 2002). Studies have reported that EGF promotes Ca^{2+} efflux in the CCs (O'Donnell *et al.,* 2004) and stimulate the estrogen production by aromatase activation in the GC (Misajon *et al.,* 1999; Behl and Pandey, 2001). In addition, EGF can inhibit LH receptor or inhibin secretion (Luciano *et al.,* 1994; Hattori *et al.,* 1995). When EGF is supplemented in the maturation medium *in vitro*, it was described the stimulation of ovarian granulosa cell proliferation (May *et al.,* 1987;1992; Morbeck *et al.,* 1993), steroidogenesis (Hsueh *et al.,* 1981; Misajon *et al.,* 1999), and the improvementof nuclear maturation by inducing the resumption of meiosis in several species (bovine: Lorenzo *et al.,* 1994; Lonergan *et al.,* 1996; rats: Dekel and Sherizly 1985; Hsu *et al.,* 1987; mice: Downs 1989; Das *et al.,* 1991; dog: Hatoya *et al.,* 2009; rabbit: Lorenzo *et al.,* 1996; pigs: Singh *et al.,* 1997, Prochazka *et al.,* 2000; goat: Cognié *et al.,* 2002; Silva *et al.,* 2004; sheep: Guler *et al.,* 2000 and human: Westergaard and Andersen, 1989; Das *et al.,* 1991). Also, cytoplasmic maturation of mouse (Das *et al.,* 1991), bovine (Kobayashi *et al.,* 1994), porcine (Ding and Foxcroft, 1994) and human (Goud *et al.,* 1998) oocytes is enhanced with EGF addition in the IVM medium.

Our results of IVM showed that supplementation with 50 ng/mL of EGF enhances M-II rate and cytoplasmic maturation, both in terms of cortical granules migration and

mitochondrial redistribution in guinea pig oocytes; besides the quality of CCs seemed to be enhanced, since lower apoptotic rate and higher E_2 and P_4 secretion by COCs were found. Lower (10 ng/mL) and higher (100 ng/mL) concentrations showed intermediate values in cortical granules and mitochondria migration rates, in CCs apoptosis and steroidogenesis production but nuclear maturation was not improved in neither of them. These results agree with those of previous authors reporting that EGF at 50 ng/mL concentration promotes nuclear and cytoplasmic maturation (Farin *et al.,* 2007), improves oocyte developmental competence and mitochondrial activity (Richani *et al.,* 2014), stimulates granulosa cell proliferation (May *et al.,* 1987) and steroidogenesis (Hsueh *et al.,* 1981), acting as a mitogen and apoptotic survival factor during early embryo development (Byrne *et al.,* 1999).

5.4.3. Effect of IGF-1 supplementation on the maturation medium

In the ovary, IGF-1 is involved in the ovarian function (Adashi *et al.,* 1985; Adashi and Rohan, 1992) because it stimulates the granulosa cells proliferation and E_2 secretion (Gutierrez *et al.,* 2000; Zhao *et al.,* 2001; McCaffery *et al.,* 2000) and also it has been reported as anti-apoptotic factor during oocyte maturation in human (Van Blerkom and Davis, 1998), porcine (Guthrie *et al.,* 1998) and bovine oocytes (Wasielak and Bogacki, 2007).These studies confirm that IGF-1 is an important factor in the maturation medium. Indeed, when IGF-1 is added into the maturation medium, studies have demonstrated substantial improvements in nuclear (Lorenzo *et al.,* 1994, 1996) and cytoplasmic maturation, in terms of cortical granules migration rates (Wang, 1997; Liu and Min, 2011). Ergin *et al.* 2008 showed that IGF-1 regulates rat luteal steroidogenesis by an increase in P_4 and E_2 production. However, other authors failed to detect any stimulatory effect of IGF-1 on murine (Downs, 1989; Demeestere *et al.,* 2004) and sheep oocytes (Guler *et al.,* 2000). In guinea pig, the addition of 100 ng/mL IGF-1 to the maturation medium significantly increases rates of M-II and cortical granules migration in oocytes, produces higher amounts of E_2 and P_4 released by COCs and lower index of apoptosis in CCs. These findings match with that described in other species, as it is reported to enhance the proportion of oocytes that reach M-II of oocytes in bovine (Lorenzo *et al.,* 1994; Herrler *et al.,* 1992), horse (Carneiro *et al.,* 2001), cat (Yıldırım *et al.,* 2014), human (Gómez *et al.,* 1993), pig (Xia *et al.,* 1994) and rabbit (Lorenzo *et al.,* 1996); and the cytoplasmic maturation of oocytes (Liu *et al.,* 2003) also in terms of cortical granules migration in horse (Carneiro *et al.,* 2001), calf (Damiani *et al.,* 1995) and pig (Wang *et al.,* 1997).However, in the present study no differences were found in mitochondria patterns with the different concentrations of IGF-1 tested in IVM, but the percentages are

high. No previous works in the literature were found about the effect of IGF-1 on mitochondrial distribution in any specie. Further studies are needed to deduce intracellular mechanisms that link the effect of IGF-1 and mitochondrial function in this specie. Maybe, it was necessary the addition of gonadotrophins to the IVM medium to accomplish the complete cytoplasmic maturation since IGF-1R expression is associated with increase in FSH and LH receptors presence, which amplificate FSH effect (Baker *et al.,* 1996). For the rest of IGF concentrations, we did not find nuclear maturation enhancement whereas migrated cortical granules increased when medium is supplemented with 200 ng/mL but apoptotic index is also increased with this concentration, reaching better results with 50 ng/mL. In any case, steroidogenic secretion is lower than when we use 100 ng/mL.

5.4.4. Effect of EGF and IGF-1 supplementation on the maturation medium

As we expected, the addition of both growth factors together with FCS improved nuclear maturation, cortical granules migration rate and steroid-level secretion of COCs and reduced apoptotic index compared to COCs *in vitro*- matured without growth factors. The stimulatory effects of EGF and IGF-1 on aromatase activity of CCs had also been described in other species (Lorenzo *et al.,* 1997). The interaction between growth factors and CCs can modify steroid production, which indirectly affects oocyte maturation and proliferation of CCs (Gutierrez *et al.,* 1997). Also, it has been demonstrated that IGF and EGF interaction can promote the oocyte maturation and activation of MAPK pathway (Yoshimura *et al.,* 1996; Pozios *et al.,* 2001; Adams *et al.,* 2004; Laviola *et al.,* 2007; Paul *et al.,* 2009; Su *et al.,* 2010; Nelson and Van Der Kraak, 2010; Yao *et al.,* 2014). The concentrations at which both growth factors improve nuclear oocyte maturation in guinea pig oocytes are similar to that found in other species (rabbit: Lorenzo *et al.,* 1996; cattle: Lorenzo *et al.,* 1994; Sakaguchi *et al.,* 2000; buffalo: Kumar and Purohit, 2004; Jainudeen *et al.,* 1993; Totey *et al.,* 1992; pig: Isobe and Terada, 2001).Surprisingly, both growth factors together without FCS did not exert a synergic effect on oocyte maturation compared when they were added alone as has been demonstrated in rabbit (Lorenzo *et al.,* 1997). Moreover, no significant improvements were found in mitochondria patterns as well as occurred with only IGF-1 supplementation, but the percentages achieved were quite similar. The cellular and molecular mechanisms by which IGF-1 alone or together with EGF influences on mitochondrial migration are unknown for any specie but combination of EGF and IGF-1 under *in vitro* conditions probably stimulate a cascade of events, including protein synthesis which eventually generate positive signals for resumption of meiosis in oocytes (Purohit *et al.,* 2005; Gehm

et al., 2000; Sakaguchi *et al.,* 2000), as has demonstrated in the present work. The apoptotic rate of COCs in the group IVM with both growth factors together with FCS was lower compared to the rate obtained in COCs cultured with 50 ng/mL EGF or 100 ng/mL IGF-1 but no significant differences were found. IGF-1 E_2 released by COCs in the maturation medium supplemented with 100 ng/mL IGF-1 were similar to the combined medium with FCS whereas P_4 showed intermediate values. IGF-1 increased E_2 production by bovine granulosa cells cultured under serum-free conditions irrespective of the size of follicle (Gong *et al.,* 1994). IGF-1 and EGF stimulate cell number and E_2 production, even in the absence of FSH, demonstrating the gonadotropic action of these growth factors (Adashi and Rohan, 1992). Other studies also demonstrated that P_4 secretion by CCs may be modulated by E_2 because the cells are able to secrete P_4 when maturation is performed in the presence of low concentrations of E_2 (Jamnongjit *et al.,* 2005).

The majority of IVM protocols are based on serum-enriched culture media (Otoi *et al.,* 1999; Luvoni *et al.,* 2005) since FCS is considered crucial for animal oocyte maturation. However, the exact beneficial effects of serum are unknown and in most cases unpredictable (Bolamba *et al.,* 2002; Lee *et al.,* 2007) due to the adverse long term effects of FCS during ARTs protocols on the health and behavior of the progeny (Fernández- Gonzalez *et al.,* 2004). Therefore, establishment of serum free and chemically defined media are preferred for future IVM protocols used in ARTs in both animals and humans. In our experimental conditions and based in our results, a defined IVM medium supplemented only with 50 ng/mL EGF or 100ng/mL IGF-1 successfully support nuclear and cytoplasmic maturation, and improves COCs quality because it stimulates steroidogenic capacity and reduces the apoptotic rate. The addition of both growth factors in the combined medium with or without FCS did not substantially improve the oocyte maturation parameters measured, except for the apoptotic percentage found that was lower when FCS was added. FCS can be contained gonadotropins that may be acting as a survival factors in the CCs as has been previously mentioned. However, to check this possibility is preferred to include FSH and/or LH in the IVM medium instead of FCS. In any case, the medium proposed in this Thesis reached good results of nuclear and cytoplasmic maturation, avoiding FCS addition to elude possible negative long- term effects in the fetal development and health of progeny. However, further studies are needed to assess the long term consequences of these media.

In summary, in this Thesis it has been characterized the EGF and IGF-1 system included IGFBPs in the ovary of the guinea pig, oocytes and their corresponding CCs and in early

embryos for the first time. The data indicate that both systems are play a key regulatory role in follicular development, oocyte maturation, luteogenesis and *corpus luteum* function, and early embryogenesis by autocrine and paracrine pathway. mRNA transcripts and protein inmunolocalization of EGF and IGF-1, EGF-R, IGF-1R and IGFBPs 1-6 are differentially expressed and widely distributed in the ovary, oocytes and embryos. Although this does not necessarily involve that transcripts are translated in protein or that the receptors are functionally involved in signal transduction, the presence of transcripts and the proteins implies a physiological role in ovarian function in guinea pig, especially in antral follicles, early *corpus luteum*, cumulus-oocyte complexes and early embryo development. Therefore, EGF and IGF-1R could be exerting a pivotal role on the acquisition of oocyte developmental competence and function of early *corpus luteum*, and indeed, in oocyte maturation and early embryo development. Part of this hypothesis is confirmed with the *in vitro* maturation experiment. This Thesis present the first study that describe in detail the cortical granules and mitochondrial patterns as a indicators of cytoplasmic maturation in guinea pig as well as the effect of EGF and IGF-1 on the oocyte *in vitro* maturation. Our results suggest that both EGF and IGF-1 improves nuclear and cytoplasmic maturation, increases steroidogenic response and reduces apoptotic rate in CCs, improving the guinea pig COCs quality *in vitro*. In our experimental conditions, a defined IVM medium with 50 ng/mL of EGF or 100 ng/mL of IGF-1 and without FCS is a suitable IVM medium for guinea pig. These findings may serve as a base to future investigations of the mechanisms and agents involved in the generation of physiological signals regulating oocyte maturation for the improvement of IVM systems in the guinea pig animal model useful for ARTs.

6. CONCLUSIONS

1. The mRNA transcripts of EGF and IGF-1, their receptors and the IGFBPs genes in antral and preovulatory follicles, in *corpus luteum* and in cumulus-oocyte complexes implies that both systems are directly involved in the acquisition of oocyte developmental competence during oocyte maturation and in the luteal function; and therefore indirectly involved in the early embryo development in the guinea pig.

2. The differential immunolocalization of EGF and IGF-1 receptors and the IGFBPs in all stages of follicular development and early embryos demonstrates the responsiveness of such structures to both growth factors throughout their receptor and the influence of these systems on follicular development, oogenesis and early embryo development in the guinea pig model. Both growth factors acting in a paracrine and autocrine manner having IGFBPs 1-6 an important regulatory role on IGF-1 function.

3. Cortical granules and mitochondrial distribution patterns are defined by the first time in guinea pig. Oocytes are considered cytoplasmically matured when they show cortical granules adjacent to the plasma membrane and when the mitochondria are relocated to the nuclear pole. In contrast, those oocytes that show cortical granules and mitochondria homogeneously distributed throughout the cytoplasm are considered non-cytoplasmically matured. The cytoplasmic patterns characterized contribute to improve the knowledge of oocyte maturation in the guinea pig model.

4. In our experimental conditions, a defined IVM medium supplemented with 50 ng/mL EGF appears to be the best option to use in the IVM medium in the guinea pig since successfully supports nuclear and cytoplasmic maturation, and improves COCs quality because it stimulates steroidogenic capacity and reduces the apoptotic rate. However, we cannot discard the supplementation with 100 ng/ mL IGF-1 as a suitable option, since the percentage of oocytes with mitochondrias migrated is quite well. The addition of both growth factors with FCS does not substantially improve the oocyte maturation parameters.

7. CONCLUSIONES

1. La presencia de transcritos y la inmunolocalización de las proteínas del EGF, IGF-1, sus receptores y las IGFBPs, en los folículos antrales y preovulatorios, en el cuerpo lúteo y en los complejos cúmulo oocito implica que ambos sistemas pueden estar directamente involucrados en la adquisición de la competencia del oocito durante la maduración, y en la función luteal, y por lo tanto, indirectamente relacionados con el desarrollo embrionario temprano en la cobaya.

2. La inmunolocalización de los receptores del EGF, del IGF-1 y de las IGFBPs en todas las etapas del desarrollo folicular y en los embriones tempranos de cobaya demuestra el posible papel regulador de estos factores de crecimiento sobre todos los estadios del desarrollo folicular, la oogenesis y sobre el desarrollo temprano del embrión de manera directa y a través de sus receptores. Ambos factores de crecimiento actuarían mediante un mecanismo paracrino y autocrino teniendo las IGFBPs 1-6 un papel regulador importante en la función del IGF-1.

3. Los patrones de distribución de los gránulos corticales y las mitocondrias se definen por primera vez en la cobaya. Los oocitos se consideran citoplásmicamente maduros cuando muestran gránulos corticales adyacentes a la membrana plasmática y cuando las mitocondrias son redistribuidas al polo nuclear. Por el contrario, los oocitos que muestran gránulos corticales y mitocondrias homogéneamente distribuidos en el citoplasma se consideran inmaduros citoplasmáticamente. Por lo tanto, los patrones citoplasmáticos caracterizados contribuyen a mejorar el conocimiento sobre los procesos que acontecen durante la maduración de oocitos en la cobaya.

4. En nuestras condiciones experimentales, el medio de maduración *in vitro* suplementado con 50 ng/mL de EGF parece ser la mejor opción para estimular la maduración de los oocitos de cobaya *in vitro*, ya que proporciona los mejores resultados de maduración nuclear y citoplasmática y mejora la calidad de los COCs ya que estimula la capacidad esteroidogénica y reduce la tasa de apoptosis en las células del cúmulo. Sin embargo, no podemos descartar la suplementación con 100 ng/mL de IGF-1 como una opción también adecuada, ya que el porcentaje de oocitos con mitocondrias migradas es satisfactorio. La adición de ambos factores de crecimiento con suero fetal no mejora sustancialmente los parámetros de maduración de los oocitos. Por lo tanto, el medio propuesto podría ser válido como una herramienta para mejorar las técnicas de reproducción asistida in the cobaya.

8. REFERENCES

1. Abd El Aal, DE., Mohamed, SA., Amine, AF. (2005). Vascular endothelial growth factor and insulin-like growth factor-1 in polycystic ovary syndrome and their relation to ovarian blood flow. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *118*(2), 219-224.
2. Abu Shehab, M., Damerill, I., Shen, T., Rosario, F. J., Nijland, M., Nathanielsz, P. W., Gupta, M. B. (2014). Liver mTOR controls IGF-I bioavailability by regulation of protein kinase CK2 and IGFBP-1 phosphorylation in fetal growth restriction. *Endocrinology*, *155*(4), 1327-1339.
3. Adams, E. C., Hertig, A. T., Foster, S. (1966). Studies on guinea pig oocytes. II. Histochemical observations on some phosphatases and lipid in developing and in atretic oocytes and follicles. *Developmental Dynamics*, *119*(2), 303-339.
4. Adams, T. E., Epa, V. C., Garrett, T. P. J., Ward, C. W. (2000). Structure and function of the type 1 insulin-like growth factor receptor. *Cellular and Molecular Life Sciences*, *57*(7), 1050.
5. Adams, T. E., McKern, N. M., Ward, C. W. (2004). Mini Review: Signalling by the Type 1 Insulin-like Growth Factor Receptor: Interplay with the Epidermal Growth Factor Receptor. *Growth Factors*, *22*(2), 89-95.
6. Adamson, E. D. (1993). Activities of growth factors in preimplantation embryos. *Journal of cellular biochemistry*, *53*(4), 280-287.
7. Adashi, E. Y. (1998). The IGF family and folliculogenesis. *Journal of reproductive immunology*, *39*(1), 13-19.
8. Adashi, E.Y., Resnick, C.E., D'Ercole, A.J., Svoboda, M.E., Van Wyk, J.J., (1985). Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. EndocrineReviews 6, 400–420.
9. Adashi, E.Y., Resnick, C.E., Payne, D.W., Rosenfeld, R.G., Matsumoto, T., Hunter, M.K., Gargosky, S.E., Zhou, J., Bondy, C.A., (1997). The mouse intraovarian insulin-like growth factor I system: departures from the rat paradigm. Endocrinology 138, 3881–3890.
10. Adashi, E.Y., Resnick, C.E., Rosenfeld, R.G., (1990). Insulin-Like Growth Factor-I (IGF-I) and IGF-II Hormonal Action in Cultured Rat Granulosa Cells: Mediation via Type I but not Type II IGF Receptors*. Endocrinology 126, 216–222.
11. Adashi, E.Y., Rohan, R.M., (1992). Intraovarian regulation: peptidergic signaling systems. Trends Endocrinol. Metab. 3, 243–248.
12. Adhikari, D., Liu, K., (2014). The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes. Mol. Cell. Endocrinol. 382, 480–487.

13. Adhikari, D., Zheng, W., Shen, Y., Gorre, N., Ning, Y., Halet, G., Kaldis, P., Liu, K., (2012). Cdk1, but not Cdk2, is the sole Cdk that is essential and sufficient to drive resumption of meiosis in mouse oocytes. Hum. Mol. Genet. 21, 2476–2484.
14. Adona, P.R., Pires, P.R.L., Quetglas, M.D., Schwarz, K.R.L., Leal, C.L.V., (2008). Prematuration of bovine oocytes with butyrolactone I: effects on meiosis progression, cytoskeleton, organelle distribution and embryo development. Anim. Reprod. Sci. 108, 49–65.
15. Aerts, J., Bols, J. (2010). Ovarian Follicular Dynamics: A Review with Emphasis on the Bovine Species. Part I: Folliculogenesis and Pre-antral Follicle Development. *Reproduction in domestic animals*, *45*(1), 171-179.
16. Agirregoitia, E., Peralta, L., Mendoza, R., Expósito, A., Ereño, E.D., Matorras, R., Agirregoitia, N., (2012). Expression and localization of opioid receptors during the maturation of human oocytes. Reprod. Biomed. Online 24, 550–557.
17. Agrawal K, Sharma T, Sexana C and Sharma N, (1995). Chronology of first meiotic events of caprine oocytes matured *in vitro*. Indian J. Anim. Sci. 285–288.
18. Albertini, D. F., Combelles, C. M., Benecchi, E., Carabatsos, M. J. (2001). Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction*, *121*(5), 647-653.
19. Albertini, D.F., Barrett, S.L., (2003). Oocyte-somatic cell communication. Reprod. Suppl. 61, 49–54.
20. Ameisen, J.C., (2002). On the origin, evolution, and nature of programmed cell death: a timeline of four billion years. Cell Death Differ. 9, 367–393.
21. Amsterdam, A., Sasson, R., Keren-Tal, I., Aharoni, D., Dantes, A., Rimon, E., Land, A., Cohen, T., Dor, Y., Hirsh, L., (2003). Alternative pathways of ovarian apoptosis: death for life. Biochem. Pharmacol. 66, 1355–1362.
22. Anderson, L.C., Otto, G., Pritchett-Corning, K.R., Whary, M.T. (Eds), (2015). Laboratory Animal Medicine. Elsevier.
23. Arias-Álvarez, M., García-García, R.M., Rebollar, P.G., Nicodemus, N., Millán, P., Revuelta, L., Lorenzo, P.L., (2010). Follicular, oocyte and embryo features related to metabolic status in primiparous lactating does fed with high-fibre rearing diets. Reprod. Domest. Anim. 45, e91–e100.
24. Arias-Álvarez, M., García-García, R.M., Rebollar, P.G., Nicodemus, N., Revuelta, L., Millán, P., Lorenzo, P.L., (2009). Effects of a lignin-rich fibre diet on productive, reproductive and endocrine parameters in nulliparous rabbit does. Livest. Sci. 123, 107–115.
25. Armstrong, D., (1997). Ovarian follicular dominance: the role of intraovarian growth factors and novel proteins. Rev. Reprod. 2, 139–146.

26. Armstrong, D., (2000). Expression of mRNA encoding IGF-I, IGF-II and type 1 IGF receptor in bovine ovarian follicles. J. Endocrinol. 165, 101–113.
27. Armstrong, D., (2002). Insulin-like growth factor (IGF) system in the oocyte and somatic cells of bovine preantral follicles. Reproduction 123, 789–797.
28. Armstrong, D.G., Baxter, G., Gutierrez, C.G., Hogg, C.O., Glazyrin, A.L., Campbell, B.K., Bramley, T.A., Webb, R., (1998). Insulin-like growth factor binding protein -2 and -4 messenger ribonucleic acid expression in bovine ovarian follicles: effect of gonadotropins and developmental status. Endocrinology 139, 2146–2154.
29. Armstrong, D.T., Xia, P., de Gannes, G., Tekpetey, F.R., Khamsi, F., (1996). Differential effects of insulin-like growth factor-I and follicle-stimulating hormone on proliferation and differentiation of bovine cumulus cells and granulosa cells. Biol. Reprod. 54, 331–338.
30. Arraztoa, J. A., Monget, P., Bondy, C., Zhou, J. (2002). Expression patterns of insulin-like growth factor-binding proteins 1, 2, 3, 5, and 6 in the mid-cycle monkey ovary. *The Journal of Clinical Endocrinology & Metabolism*, *87*(11), 5220-5228.
31. Ashkenazi, H., Cao, X., Motola, S., Popliker, M., Conti, M., Tsafriri, A. (2005). Epidermal growth factor family members: endogenous mediators of the ovulatory response. Endocrinology 146, 77–84.
32. Assarsson, B., Nilsson, I., Selstam, G. (1995). Ovarian levels of epidermal growth factor receptor mRNA in the rat-a postovulatory decrease. *Acta Physiologica*, *154*(2), 177-183.
33. Assidi, M., Dufort, I., Ali, A., Hamel, M., Algriany, O., Dielemann, S., Sirard, M. A. (2008). Identification of potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-stimulating hormone and/or phorbol myristate acetate *in vitro*. *Biology of reproduction*, *79*(2), 209-222.
34. Avilés, D. F., Martínez, A. M., Landi, V., Delgado, J. V. (2014). El cuy (Cavia porcellus): un recurso andino de interés agroalimentario The guinea pig (Cavia porcellus): An Andean resource of interest as an agricultural food source. *Animal Genetic Resources/Ressources génétiques animales/Recursos genéticos animales*, *55*, 87-91.
35. Babitha, V., Yadav, V. P., Chouhan, V. S., Hyder, I., Dangi, S. S., Gupta, M., Sarkar, M. (2014). Luteinizing hormone, insulin like growth factor-1, and epidermal growth factor stimulate vascular endothelial growth factor production in cultured bubaline granulosa cells. *General and comparative endocrinology*, *198*, 1-12.
36. Bach, L. A. (2005). IGFBP-6 five years on; not so 'forgotten'?. *Growth hormone & IGF research*, *15*(3), 185-192.
37. Bach, L. A. (2015). Recent insights into the actions of IGFBP-6. *Journal of cell*

communication and signaling, *9*(2), 189-200.

38. Bach, L. A., Fu, P., & Yang, Z. (2013). Insulin-like growth factor-binding protein-6 and cancer. *Clinical Science*, *124*(4), 215-229.
39. Bachelot, A., Binart, N., (2005a). Corpus luteum development: lessons from genetic models in mice. Curr. Top. Dev. Biol. 68, 49–84.
40. Bachelot, A., Binart, N., (2005b). Corpus Luteum Development: Lessons from Genetic Models in Mice, in: Current Topics in Developmental Biology. pp. 49–84.
41. Baker, J., Hardy, M.P., Zhou, J., Bondy, C., Lupu, F., Bellvé, A.R., Efstratiadis, A., (1996). Effects of an Igf1 gene null mutation on mouse reproduction. Mol. Endocrinol. 10, 903–918.
42. Bale, L. K., Conover, C. A. (1992). Regulation of insulin-like growth factor binding protein-3 messenger ribonucleic acid expression by insulin-like growth factor I. *Endocrinology*, *131*(2), 608-614.
43. Balen A (2004). The pathophysiology of polycystic ovary syndrome: trying to understand PCOS and its endocrinology. Best Pract. Res. Clin. Obstet. Gynaecol.18(5), 685–706.
44. Barnett, D.K., Kimura, J., Bavister, B.D., (1996). Translocation of active mitochondria during hamster preimplantation embryo development studied by confocal laser scanning microscopy. Dev. Dyn. 205, 64–72.
45. Bassas L, De Pablo F, Lesniak MA, Roth J (1985). Ontogeny of receptors for insulin-like peptides in chick embryo tissues: early dominance of insulin-like growth factor over insulin receptors in brain. Endocrinology 117:2321-29, 1985.
46. Batista, J. G., Soares-Jr, J. M., Maganhin, C. C., Simões, R. S., Tomaz, G., Baracat, E. C. (2012). Assessing the benefits of rosiglitazone in women with polycystic ovary syndrome through its effects on insulin-like growth factor 1, insulin-like growth factor-binding protein-3 and insulin resistance: a pilot study. *Clinics*, *67*(3), 283-287.
47. Baxter, R.C., (2000). Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. Am. J. Physiol. Endocrinol. Metab. 278, E967–76.
48. Baxter, R.C., (2013). Insulin-like growth factor binding protein-3 (IGFBP-3): Novel ligands mediate unexpected functions. J. Cell Commun. Signal. 7, 179–189.
49. Baxter, R.C., (2015). Nuclear actions of insulin-like growth factor binding protein-3. Gene 569, 7–13.
50. Beattie, J., Allan, G.J., Lochrie, J.D., Flint, D.J., (2006). Insulin-like growth factor-binding protein-5 (IGFBP-5): a critical member of the IGF axis. Biochem. J 395, 1–19.
51. Beauchamp, M.-C., Yasmeen, A., Knafo, A., Gotlieb, W.H., (2010). Targeting insulin

and insulin-like growth factor pathways in epithelial ovarian cancer. J. Oncol. 2010, 257058.

52. Bedzhov, I., Liszewska, E., Kanzler, B., Stemmler, M. P. (2012). Igf1r signaling is indispensable for preimplantation development and is activated via a novel function of E-cadherin. *PLoS Genet*, *8*(3), e1002609.
53. Behl, R. Pandey, R.S. (2001). Effect of epidermal growth factor on steroidogenesis by caprine granulosa cells in culture: interaction with FSH. *Small Rum. Res.* 40, 57–62.
54. Bennett, R.A., Osathanondh, R., Yeh, J., (1996). Immunohistochemical localization of transforming growth factor-alpha, epidermal growth factor (EGF), and EGF receptor in the human fetal ovary. J. Clin. Endocrinol. Metab. 81, 3073–3076.
55. Bergfelt, D. R., Woods, J. A., Ginther, O. J. (1992). Role of the embryonic vesicle and progesterone in embryonic loss in mares. *Journal of reproduction and fertility*, *95*(2), 339-347
56. Besnard, N., Pisselet, C., Monniaux, D., Locatelli, A., Benne, F. (1996). Expression of Messenger Ribonucleic Acids of Insulin-Like Growth Factor Binding Proteins-2, -4 , and -5 in the Ovine Ovary : Localization and Changes during Growth and Atresia of Antral Follicles ', *1367*, 1356–1367.
57. Besnard, N., Pisselet, C., Monniaux, D., Monget, P., (1997). Proteolytic Activity Degrading Insulin-like Growth Factor-Binding Protein−2, −3, −4, and −5 in Healthy Growing and Atretic Follicles in the Pig Ovary1. Biol. Reprod. 56, 1050–1058.
58. Bevers, M.M., Dieleman, S.J., van den Hurk, R., Izadyar, F., (1997). Regulation and modulation of oocyte maturation in the bovine. Theriogenology 47, 13–22.
59. Bevers, M.M., Izadyar, F., (2002). Role of growth hormone and growth hormone receptor in oocyte maturation. Mol. Cell. Endocrinol. 197, 173–178.
60. Bhardwaj, R., Ansari, M. M., Pandey, S., Parmar, M. S., Chandra, V., Kumar, G. S., Sharma, G. T. (2016). GREM1, EGFR, and HAS2; the oocyte competence markers for improved buffalo embryo production *in vitro*. *Theriogenology*, *86*(8), 2004-2011.
61. Binkert, C., Landwehr, J., Mary, J. L., Schwander, J., Heinrich, G. (1989). Cloning, sequence analysis and expression of a cDNA encoding a novel insulin-like growth factor binding protein (IGFBP-2). *The EMBO journal*, *8*(9), 2497.
62. Binoux, M., Hossenlopp, P., (1988). Insulin-like growth factor (IGF) and IGF-binding proteins: comparison of human serum and lymph. J. Clin. Endocrinol. Metab. 67, 509–514.
63. Bland, K.P., (1980). Biphasic follicular growth in the guinea-pig oestrous cycle. J. Reprod. Fertil. 60, 73–76.
64. Blandau, R.J., (1949). Observations on implantation of the guinea pig ovum. Anat.

Rec. 103, 19–47.

65. Blandau, R.J., Young, W.C., (1939). The effects of delayed fertilization on the development of the guinea pig ovum. Am. J. Anat. 64, 303–329.
66. Blatchley, F.R., Donovan, B.T., Poyser, N.L., Horton, E.W., Thompson, C.J., Los, M., (1971). Identification of Prostaglandin F2α in the Utero-ovarian Blood of Guinea-pig after Treatment with Oestrogen. Nature 230, 243–244.
67. Blum, W.F., Jenne, E.W., Reppin, F., Kietzmann, K., Ranke, M.B., Bierich, J.R., (1989). Insulin-Like Growth Factor I (IGF-I)-Binding Protein Complex Is a Better Mitogen than Free IGF-I*. Endocrinology 125, 766–772.
68. Bolamba, D., Russ, K.D., Olson, M.A., Sandler, J.L., Durrant, B.S., (2002). *In vitro* maturation of bitch oocytes from advanced preantral follicles in synthetic oviduct fluid medium: serum is not essential. Theriogenology 58, 1689–1703.
69. Boland, N.I., Gosden, R.G., (1994). Effects of epidermal growth factor on the growth and differentiation of cultured mouse ovarian follicles. Reproduction 101, 369–374.
70. Bondy, C.A., Lee, W.H., (1993). Patterns of insulin-like growth factor and IGF receptor gene expression in the brain. Functional implications. Ann. N. Y. Acad. Sci. 692, 33–43.
71. Brevini, T.A.L., Vassena, R., Francisci, C., Gandolfi, F., (2005). Role of adenosine triphosphate, active mitochondria, and microtubules in the acquisition of developmental competence of parthenogenetically activated pig oocytes. Biol. Reprod. 72, 1218–1223.
72. Brogan, R. S., Ph, D., Mix, S., Puttabyatappa, M., Vandevoort, C. A., Ph, D., Ph, D. (2010). Expression of the insulin-like growth factor and insulin systems in the luteinizing macaque ovarian follicle. *Fertility and Sterility*, *93*(5), 1421–1429.
73. Bromberg, J., (2002). Stat proteins and oncogenesis. J. Clin. Invest. 109, 1139–1142.
74. Brown, L.F., Detmar, M., Claffey, K., Nagy, J.A., Feng, D., Dvorak, A.M., Dvorak, H.F., (1997). Vascular permeability factor/vascular endothelial growth factor: A multifunctional angiogenic cytokine, in: Experientia Supplementum. pp. 233–269.
75. Brunet, S., Maro, B., (2005). Cytoskeleton and cell cycle control during meiotic maturation of the mouse oocyte: integrating time and space. Reproduction 130, 801–811.
76. Bruzzone, R., White, T.W., Paul, D.L., (1996). Connections with connexins: the molecular basis of direct intercellular signaling. Eur. J. Biochem. 238, 1–27.
77. Bunn, R.C., John L. Fowlkes, J.L. (2003). Insulin-like growth factor-binding protein proteolysis. Trends in Endocrinology and Metabolism, Vol.14, No.4, pp. 174-181, ISSN 1043-2760

78. Butt, A.J., Firth, S.M., Baxter, R.C., (1999). The IGF axis and programmed cell death. Immunol. Cell Biol. 77, 256–262.
79. Byrne, A.T., Southgate, J., Brison, D.R., Leese, H.J., (1999). Analysis of apoptosis in the preimplantation bovine embryo using TUNEL. J. Reprod. Fertil. 117, 97–105.
80. Cai, L., Zhang, J., Duan, E. (2003). Dynamic distribution of epidermal growth factor during mouse embryo peri-implantation. Cytokine, 23(6), 170-178.
81. Caixeta E.S., Ripamonte P., Franco M.M., Junior J.B., Dode M. (2009). Effect of follicle size on mRNA expression in CCsand oocytes of Bos indicus: an approach to identify marker genes for developmental competence. Reprod Fertil Dev, 21, pp. 655–664
82. Calarco, P. G. (1995). Polarization of mitochondria in the unfertilized mouse oocyte. Developmental genetics, 16(1), 36-43.
83. Cara, J.F., Rosenfield, R.L., (1988). Insulin-like growth factor I and insulin potentiate luteinizing hormone-induced androgen synthesis by rat ovarian thecal-interstitial cells. Endocrinology 123, 733–739.
84. Cardin, R.D., Bravo, F.J., Pullum, D.A., Orlinger, K., Watson, E.M., Aspoeck, A., Fuhrmann, G., Guirakhoo, F., Monath, T., Bernstein, D.I., (2016). Replication-defective lymphocytic choriomeningitis virus vectors expressing guinea pig cytomegalovirus gB and pp65 homologs are protective against congenital guinea pig cytomegalovirus infection. Vaccine 34, 1993–1999.
85. Carlsson, I.B., Scott, J.E., Visser, J.A., Ritvos, O., Themmen, A.P.N., Hovatta, O., (2006). Anti-Müllerian hormone inhibits initiation of growth of human primordial ovarian follicles in vitro. Hum. Reprod. 21, 2223–2227.
86. Carneiro, G., Lorenzo, P., Pimentel, C., Pegoraro, L., Bertolini, M., Ball, B., Liu, I. (2001). Influence of insulin-like growth factor-I and its interaction with gonadotropins, estradiol, and fetal calf serum on *in vitro* maturation and parthenogenic development in equine oocytes. *Biology of Reproduction*, *65*(3), 899-905.
87. Carolan, C., Lonergan, P., Van Langendonckt, A., Mermillod, P., (1995). Factors affecting bovine embryo development in synthetic oviduct fluid following oocyte maturation and fertilization *in vitro*. Theriogenology 43, 1115–1128.
88. Carrick, F. E., Wallace, J. C., Forbes, B. E. (2001). The interaction of insulin-like growth factors (IGFs) with insulin-like growth factor binding proteins (IGFBPs): a review. *Letters in Peptide Science*, *8*(3), 147-153.
89. Carroll, J., Jones, K. T., Whittingham, D. G. (1996). Ca2+ release and the development of Ca2+ release mechanisms during oocyte maturation: a prelude to fertilization. Reviews of reproduction, 1(3), 137-143.

90. Carroll, J., Swann, K., Whittingham, D., Whitaker, M. (1994). Spatiotemporal dynamics of intracellular [Ca2+] i oscillations during the growth and meiotic maturation of mouse oocytes. Development, 120(12), 3507-3517.
91. Carter, A., Enders, A., Jones, C., Mess, A., Pfarrer, C., Pijnenborg, R., Soma, H., (2006). Comparative Placentation and Animal Models: Patterns of Trophoblast Invasion – A Workshop Report. Placenta 27, 30–33.
92. Carter, A.M., (2007). Animal Models of Human Placentation – A Review. Placenta 28, S41–S47.
93. Cataldo, N. A. (1997). Insulin-like growth factor binding proteins: do they play a role in polycystic ovary syndrome?. In Seminars in reproductive endocrinology (Vol. 15, No. 02, pp. 123-136). Copyright© 1997 by Thieme Medical Publishers, Inc..
94. Celestino, J. J. H., Bruno, J. B., Saraiva, M. V. A., Rocha, R. M., Brito, I. R., Duarte, A. B. G., Silva, J. R. V. (2011). Steady-state level of epidermal growth factor (EGF) mRNA and effect of EGF on in vitro culture of caprine preantral follicles. *Cell and tissue research*, *344*(3), 539
95. Challis, J.R., Heap, R.B., Illingworth, D.V., (1971). Concentrations of oestrogen and progesterone in the plasma of non-pregnant, pregnant and lactating guinea-pigs. J. Endocrinol. 51, 333–345.
96. Chandra V, Mishra A, and Taru Sharma G, (2012). Effect of growth factors (epidermal growth factor, platelet derived growth factor, and insulin-like growth factor-1) on buffalo (Bubalus bubalis) embryos produced *in vitro*. Indian J. Anim. Sci. 82, 1510.
97. Chandrashekaran, I.R., Yao, S., Wang, C.C., Bansal, P.S., Alewood, P.F., Forbes, B.E., Wallace, J.C., Bach, L.A., Norton, R.S., (2007). The N-Terminal Subdomain of Insulin-like Growth Factor (IGF) Binding Protein 6. Structure and Interaction with IGFs. Biochemistry 46, 3065–3074.
98. Chang, H., Brown, C.W., Matzuk, M.M., (2002). Genetic Analysis of the Mammalian Transforming Growth Factor-β Superfamily. Endocr. Rev. 23, 787–823.
99. Chen, W., Wang, Y., Li, W., Lin, H., (2010). Insulin-like growth factor binding protein-2 (IGFBP-2) in orange-spotted grouper, Epinephelus coioides: molecular characterization, expression profiles and regulation by 17β-estradiol in ovary. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 157, 336–342.
100. Cheng, Y., Kim, J., Li, X.X., Hsueh, A.J., (2015). Promotion of ovarian follicle growth following mTOR activation: synergistic effects of AKT stimulators. PLoS One 10, e0117769.
101. Chian, R.-C., Buckett, W.M., Tan, S. L., (2004). In-vitro maturation of human oocytes. Reprod. Biomed. Online 8, 148–166.

102.Choi, Y., Rajkovic, A. (2006). Genetics of early mammalian folliculogenesis. Cellular and molecular life sciences, 63(5), 579-590.

103.Choudary, J.B., Greenwald, G.S., (1969). Reversal by gonadotrophins of the luteolytic effect of oestrogen in the cyclic guinea-pig. J. Reprod. Fertil. 19, 503–510.

104.Chouhan, V. S., Dangi, S. S., Vazhoor, B., Yadav, V. P., Gupta, M., Pathak, M. C., Singh, G. (2014). Stimulatory effect of luteinizing hormone, insulin-like growth factor-1, and epidermal growth factor on progesterone secretion and viability of cultured bubaline luteal cells. Theriogenology, 82(9), 1212-1223.

105.Chung, J.-T., Tosca, L., Huang, T.-H., Xu, L., Niwa, K., Chian, R.C., (2007). Effect of polyvinylpyrrolidone on bovine oocyte maturation in vitro and subsequent fertilization and embryonic development. Reprod. Biomed. Online 15, 198–207.

106.Citri, A., Yarden, Y., (2006). EGF–ERBB signalling: towards the systems level. Nat. Rev. Mol. Cell Biol. 7, 505–516.

107.Clemmons, D.R., (2016). Role of IGF Binding Proteins in Regulating Metabolism. Trends Endocrinol. Metab. 27, 375–391.

108.Clemons, D. J., and Seeman, J. L. (2011). *The laboratory guinea pig*. CRC Press.

109.Close, B., Banister, K., Baumans, V., Bernoth, E. M., Bromage, N., Bunyan, J., Morton, D. (1997). Recommendations for euthanasia of experimental animals: Part 2. *Laboratory animals*, *31*(1), 1-32.

110.Cockburn, K., Rossant, J., (2010). Making the blastocyst: lessons from the mouse. J. Clin. Invest. 120, 995–1003.

111.Cognié Y, P.N.A.M.P., (2002). Cysteamine improves *in vitro* goat oocyte maturation in defined medium. AETE.

112.Cohen, P.E., Kim Holloway, J., (2015). Mammalian Meiosis, in: Knobil and Neill's Physiology of Reproduction. pp. 5–57.

113.Conner, S., Leaf, D., Wessel, G., (1997). Members of the SNARE hypothesis are associated with cortical granule exocytosis in the sea urchin egg. Mol. Reprod. Dev. 48, 106–118.

114.Conover, C. A., Oxvig, C., Overgaard, M. T., Christiansen, M., Giudice, L. C. (1999). Evidence that the insulin-like growth factor binding protein-4 protease in human ovarian follicular fluid is pregnancy associated plasma protein-A. *The Journal of Clinical Endocrinology & Metabolism*, *84*(12), 4742-4745.

115.Conover, C. A., Ronk, M., Lombana, F., Powell, D. R. (1990). Structural and biological characterization of bovine insulin-like growth factor binding protein-3. *Endocrinology*, *127*(6), 2795-2803

116.Conover, C.A., (1992). Potentiation of insulin-like growth factor (IGF) action by IGF-binding protein-3: studies of underlying mechanism. Endocrinology 130, 3191–3199.

117.Coskun, S., Lin, Y.C., (1995). Mechanism of action of epidermal growth factor-induced porcine oocyte maturation. Mol. Reprod. Dev. 42, 311–317.

118.Coskun, S., Sanbuissho, A., Lin, Y.C., Rikihisa, Y., (1991). Fertilizability and subsequent developmental ability of bovine oocytes matured in medium containing epidermal growth factor (EGF). Theriogenology 36, 485–494.

119.Craig, S.J., Conboy, G.A., Hanna, P.E., (1995). Baylisascaris sp. infection in a guinea pig. Lab. Anim. Sci. 45, 312–314.

120.Cran D.G. (1985). Qualitative and quantitative structural changes during pig oocyte maturation. J. Reprod. Fertil. 74237-245.

121.Csapo, A.I., Eskola, J., Tarro, S., (1981). Gestational changes in the progesterone and prostaglandin F levels of the guinea-pig. Prostaglandins 21, 53–64.

122.Cummins, J.M., (2001). Epigenetic and experimental modifications in early mammalian development: Part I: Mitochondria: potential roles in embryogenesis and nucleocytoplasmic transfer. Hum. Reprod. Update 7, 217–228.

123.Damiani, P., Fissore, R.A., Cibelli, J.B., Long, C.R., Balise, J.J., Robl, J.M., Duby, R.T., (1995). Evaluation of developmental competence, nuclear and ooplasmic maturation of calf oocytes. Mol. Reprod. Dev. 45, 521–534.

124.Das K, Stout LE, Hensleigh HC, Tagatz GE, Phipps WR, Leung BS, (1991): Direct positive effect of epidermal growth factor on the cytoplasmic maturation of mouse and human oocytes. FertilSteril 55, 1000–1004.

125.Das, S. K., Tsukamura, H., Paria, B. C., Andrews, G. K., Dey, S. K. (1994). Differential expression of epidermal growth factor receptor (EGF-R) gene and regulation of EGF-R bioactivity by progesterone and estrogen in the adult mouse uterus. *Endocrinology*, *134*(2), 971-981.

126.Davidson, T. R., Chamberlain, C. S., Bridges, T. S., Spicer, L. J. (2002). Effect of follicle size on *in vitro* production of steroids and insulin-like growth factor (IGF)-I, IGF-II, and the IGF-binding proteins by equine ovarian granulosa cells. *Biology of reproduction*, *66*(6), 1640-1648.

127.Davis, J. S., Rueda, B. R. (2002). The corpus luteum: an ovarian structure with maternal instincts and suicidal tendencies. *Front Biosci*, *7*, d1949-d1978.

128.De La Fuente, R., O'Brien, M.J. Eppig, J.J. (1999). Epidermal growth factor enhances preimplantation developmental competence of maturing mouse oocytes. *Hum. Reprod.* 14, 3060–8.

129.De Loos, F., van Vliet, C., van Maurik, P., Kruip, T.A., (1989). Morphology of immature bovine oocytes. Gamete Res. 24, 197–204.

130.De los Reyes, M., Palomino, J., Parraguez, V.H., Hidalgo, M., Saffie, P., (2011). Mitochondrial distribution and meiotic progression in canine oocytes during *in vivo*

and *in vitro* maturation. Theriogenology 75, 346–353.

131. De Prada, J. K. N., Lee, Y. S., Latham, K. E., Chaffin, C. L., VandeVoort, C. A. (2009). Role for cumulus cell-produced EGF-like ligands during primate oocyte maturation *in vitro*. American Journal of Physiology-Endocrinology and Metabolism, 296(5), E1049-E1058.
132. Dekel, N., Sherizly, I., (1985). Epidermal Growth Factor Induces Maturation of Rat Follicle-Enclosed Oocytes*. Endocrinology 116, 406–409.
133. Dell'Aquila, M.E., Ambruosi, B., De Santis, T., Cho, Y.S., (2009). Mitochondrial distribution and activity in human mature oocytes: gonadotropin-releasing hormone agonist versus antagonist for pituitary down-regulation. Fertil. Steril. 91, 249–255.
134. Demeestere, I., Gervy, C., Centner, J., Devreker, F., Englert, Y., Delbaere, A., (2004). Effect of Insulin-Like Growth Factor-I During Preantral Follicular Culture on Steroidogenesis, *In vitro* Oocyte Maturation, and Embryo Development in Mice1. Biol. Reprod. 70, 1664–1669.
135. Devoto, L., Fuentes, A., Kohen, P., Céspedes, P., Palomino, A., Pommer, R., Strauss, J. F. (2009). The human corpus luteum: life cycle and function in natural cycles. *Fertility and sterility*, *92*(3), 1067-1079.
136. Dey, S.K., Lim, H., Das, S.K., Reese, J., Paria, B.C., Daikoku, T., Wang, H., (2004). Molecular Cues to Implantation. Endocr. Rev. 25, 341–373.
137. Ding, J., Foxcroft, G. R. (1994). Epidermal growth factor enhances oocyte maturation in pigs. *Molecular reproduction and development*, *39*(1), 30-40.
138. Donadeu, F.X., Schauer, S.N., Sontakke, S.D., (2012). Involvement of miRNAs in ovarian follicular and luteal development. J. Endocrinol. 215, 323–334.
139. Donaghy, A., Ross, R., Gimson, A., Hughes, S. C., Holly, J., Williams, R. (1995). Growth hormone, insulinlike growth factor-1, and insulinlike growth factor binding proteins 1 and 3 in chronic liver disease. Hepatology, 21(3), 680-688.
140. Donovan, B.T., Lockhart, A.N., (1972). Growth and regression of the corpora lutea formed in guinea-pigs in response to treatment with exogenous gonadotrophin. J. Endocrinol. 54, 327–332.
141. Dorsch, M.M., Glage, S., Hedrich, H.J., (2008). Collection and cryopreservation of preimplantation embryos of Cavia porcellus. Lab. Anim. 42, 489–494.
142. Downs SM. 1989. Specificity of epidermal growth factor action on maturation of the murine oocyte and cumulus oophorus *in vitro*. Biol Reprod 41:371–379
143. Downs, S.M., Chen, J., (2008). EGF-like peptides mediate FSH-induced maturation of cumulus cell-enclosed mouse oocytes. Mol. Reprod. Dev. 75, 105–114.
144. Downs, S.M., Daniel, S.A.J., Eppig, J.J., (1988). Induction of maturation in cumulus cell-enclosed mouse oocytes by follicle-stimulating hormone and epidermal growth

factor: Evidence for a positive stimulus of somatic cell origin. J. Exp. Zool. 245, 86–96.

145. Druckmann, R., Rohr, U. D., (2002). IGF-1 in gynaecology and obstetrics: update 2002. *Maturitas*, *41*, 65-83.
146. Dubey, P. K., Nath, A., Chandra, V., Sarkar, M., Saikumar, G., Sharma, G. T. (2015). Expression of mRNA encoding IGF-I, IGF-II, type-I, and II IGF-receptors and IGF-binding proteins-1-4 during ovarian follicular development in buffalo (Bubalus bubalis). *Animal biotechnology*, *26*(2), 81-91.
147. Ducibella, T., Anderson, E., Albertini, D.F., Aalberg, J., Rangarajan, S., (1988). Quantitative studies of changes in cortical granule number and distribution in the mouse oocyte during meiotic maturation. Dev. Biol. 130, 184–197.
148. Ducibella, T., Duffy, P., Buetow, J., (1994). Quantification and Localization of Cortical Granules during Oogenesis in the Mouse1. Biol. Reprod. 50, 467–473.
149. Ducibella, T., Kurasawa, S., Duffy, P., Kopf, G.S., Schultz, R.M., (1993). Regulation of the Polyspermy Block in the Mouse Egg: Maturation-Dependent Differences in Cortical Granule Exocytosis and Zona Pellucida Modifications Induced by Inositol 1,4,5-Trisphosphate and an Activator of Protein Kinase C1. Biol. Reprod. 48, 1251–1257.
150. Dumollard, R., (2003). Mitochondrial respiration and Ca2 waves are linked during fertilization and meiosis completion. Development 130, 683–692.
151. Dumollard, R., Duchen, M., Carroll, J., (2007). The role of mitochondrial function in the oocyte and embryo. Curr. Top. Dev. Biol. 77, 21–49.
152. Dunning, K. R., Anastasi, M. R., Zhang, V. J., Russell, D. L., Robker, R. L. (2014). Regulation of fatty acid oxidation in mouse cumulus-oocyte complexes during maturation and modulation by PPAR agonists. PLoS One, 9(2), e87327.
153. Durai, R., Davies, M., Yang, W., Yang, S., Seifalian, A., Goldspink, G., Winslet, M., (2006). Biology of insulin-like growth factor binding protein-4 and its role in cancer (review). Int. J. Oncol. doi:10.3892/ijo.28.6.1317
154. Duranthon, V., Renard, J.P., (2001). The developmental competence of mammalian oocytes: a convenient but biologically fuzzy concept. Theriogenology 55, 1277–1289.
155. Eftekhari-Yazdi, P., Valojerdi, M.R., Ashtiani, S.K., Eslaminejad, M.B., Karimian, L., (2006). Effect of fragment removal on blastocyst formation and quality of human embryos. Reprod. Biomed. Online 13, 823–832.
156. Eichenlaub-Ritter, U., Vogt, E., Yin, H., Gosden, R., (2004). Spindles, mitochondria and redox potential in ageing oocytes. Reproductive biomedicine online, 8(1), 45-58.

157.Einspanier, R., Miyamoto, A., Schams, D., Müller, M., Brem, G. (1990). Tissue concentration, mRNA expression and stimulation of IGF-I in luteal tissue during the oestrous cycle and pregnancy of cows. Journal of reproduction and fertility, 90(2), 439-445.

158.El-Hayek, S., Clarke, H.J., (2016). Control of Oocyte Growth and Development by Intercellular Communication Within the Follicular Niche. Results Probl. Cell Differ. 58, 191–224.

159.El-Roeiy, A., Chen, X., Roberts, V. J., Leroith, D., Roberts Jr, C. T., Yen, S. S. C., (1993). Expression of insulin-like growth factor-I (IGF-I) and IGF-II and the IGF-I, IGF-II, and insulin receptor genes and localization of the gene products in the human ovary. *The Journal of Clinical Endocrinology & Metabolism*, *77*(5), 1411-1418.

160.El-Roeiy, A., Chen, X., Roberts, V. J., Shimasakai, S., Ling, N., Leroith, D., Yen, S. S., (1994). Expression of the genes encoding the insulin-like growth factors (IGF-I and II), the IGF and insulin receptors, and IGF-binding proteins-1-6 and the localization of their gene products in normal and polycystic ovary syndrome ovaries. *The Journal of Clinical Endocrinology & Metabolism*, *78*(6), 1488-1496.

161.Elvin, J.A., Yan, C., Wang, P., Nishimori, K., Matzuk, M.M., (1999). Molecular Characterization of the Follicle Defects in the Growth Differentiation Factor 9-Deficient Ovary. Mol. Endocrinol. 13, 1018–1034.

162.Eppig, J., 2001. Oocyte control of ovarian follicular development and function in mammals. Reproduction 122, 829–838.

163.Eppig, J., Wigglesworth, K., Pendola, L. (2002). The mammalian oocyte orchestrates the rate of ovarian follicular development. Proceedings of the National Academy of Sciences, 99(5), 2890-2894.

164.Eppig, J.J., Viveiros, M.M., Bivens, C.M., de La Fuente, R., (2004). Regulation of Mammalian Oocyte Maturation, in: The Ovary. pp. 113–129.

165.Ergin, K., Gürsoy, E., Koca, Y.B., Başaloğlu, H., Seyrek, K., (2008). Immunohistochemical detection of insulin-like growth factor-I, transforming growth factor-β2, basic fibroblast growth factor and epidermal growth factor-receptor expression in developing rat ovary. Cytokine 43, 209–214.

166.Erickson, G.F., (1992). Localization of insulin-like growth factor-binding protein-5 messenger ribonucleic acid in rat ovaries during the estrous cycle. Endocrinology 130, 1867–1878.

167.Erickson, G.F., Gabriel Garzo, V., Magoffin, D.A., (1989). Insulin-Like Growth Factor-I Regulates Aromatase Activity in Human Granulosa and Granulosa Luteal Cells*. J. Clin. Endocrinol. Metab. 69, 716–724.

168.Erickson, G.F., Magoffin, D.A., Dyer, C.A., Hofeditz, C., (1985). The Ovarian

Androgen Producing Cells: A Review of Structure/Function Relationships1,2. Endocr. Rev. 6, 371–399.

169. Erickson, G.F., Nakatani, A., Ling, N., Shimasaki, S., (1992). Cyclic changes in insulin-like growth factor-binding protein-4 messenger ribonucleic acid in the rat ovary. Endocrinology 130, 625–636.

170. Erickson, G.F., Shimasaki, S., (2000). The role of the oocyte in folliculogenesis. Trends Endocrinol. Metab. 11, 193–198.

171. Ewton, D.Z., Roof, S.L., Magri, K.A., McWade, F.J., Florini, J.R., (1994). IGF-II is more active than IGF-I in stimulating L6A1 myogenesis: Greater mitogenic actions of IGF-I delay differentiation. J. Cell. Physiol. 161, 277–284.

172. Fanayan, S., Firth, S.M., Baxter, R.C., (2001). Signaling through the Smad Pathway by Insulin-like Growth Factor-binding Protein-3 in Breast Cancer Cells: relationship to transforming growth factor- 1 signaling. J. Biol. Chem. 277, 7255–7261.

173. Farin, C. E., Alexander, J. E., & Farin, P. W., (2010). Expression of messenger RNAs for insulin-like growth factors and their receptors in bovine fetuses at early gestation from embryos produced *in vivo* or *in vitro*. *Theriogenology*, *74*(7), 1288-1295.

174. Farin, C.E., Rodriguez, K.F., Alexander, J.E., Hockney, J.E., Herrick, J.R., Kennedy-Stoskopf, S., (2007). The role of transcription in EGF- and FSH-mediated oocyte maturation *in vitro*. Anim. Reprod. Sci. 98, 97–112.

175. Farin, C.E., Yang, L., (1992). Inhibition of germinal vesicle breakdown by 5,6-dichlorobenzimidazole riboside in bovine oocytes matured *in vitro*. Theriogenology 37, 208.

176. Farin, P. W., Crosier, A. E., Farin, C. E., (2001). Influence of *in vitro* systems on embryo survival and fetal development in cattle. *Theriogenology*, *55*(1), 151-170.

177. Ferguson, K. M., Berger, M. B., Mendrola, J. M., Cho, H. S., Leahy, D. J., Lemmon, M. A. (2003). EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. Molecular cell, 11(2), 507-517.

178. Fernandez-Gonzalez, R., Moreira, P., Bilbao, A., Jimenez, A., Perez-Crespo, M., Ramirez, M.A., De Fonseca, F.R., Pintado, B., Gutierrez-Adan, A., (2004). Long-term effect of *in vitro* culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. Proceedings of the National Academy of Sciences 101, 5880–5885.

179. Fernández-Tornero, C., Lozano, R.M., Rivas, G., Jiménez, M.A., Ständker, L., Díaz-Gonzalez, D., Forssmann, W.-G., Cuevas, P., Romero, A., Giménez-Gallego, G., (2005). Synthesis of the blood circulating C-terminal fragment of insulin-like growth factor (IGF)-binding protein-4 in its native conformation. Crystallization, heparin and IGF binding, and osteogenic activity. J. Biol. Chem. 280, 18899–18907.

180.Ferreira, E.M., Vireque, A.A., Adona, P.R., Meirelles, F.V., Ferriani, R.A., Navarro, P.A.A.S., (2009). Cytoplasmic maturation of bovine oocytes: Structural and biochemical modifications and acquisition of developmental competence. Theriogenology 71, 836–848.

181.Ferreira, T., Rasband, W., (2012). ImageJ User Guide

182.Findlay, J.K., Drummond, A.E., (1999). Regulation of the FSH Receptor in the Ovary. Trends Endocrinol. Metab. 10, 183–188.

183.Findlay, J.K., Hutt, K.J., Hickey, M., Anderson, R.A., (2015). How Is the Number of Primordial Follicles in the Ovarian Reserve Established? Biol. Reprod. 93, 111.

184.Firth, S.M., Baxter, R.C., (2002). Cellular Actions of the Insulin-Like Growth Factor Binding Proteins. Endocr. Rev. 23, 824–854.

185.Fortune, J. E., Rivera, G. M., Yang, M. Y., (2004). Follicular development: the role of the follicular microenvironment in selection of the dominant follicle. *Animal Reproduction Science*, *82*, 109-126.

186.Fortune, J.E., (2003). The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. Anim. Reprod. Sci. 78, 135–163.

187.Fraser, H. M., Lunn, S. F., Kim, H., Erickson, G. F., (1998). Insulin-like growth factor binding protein-3 mRNA expression in endothelial cells of the primate corpus luteum. *Human Reproduction*, *13*(8), 2180-2185.

188.Fraser, H.M., Bell, J., Wilson, H., Taylor, P.D., Morgan, K., Anderson, R.A., Duncan, W.C., (2005). Localization and quantification of cyclic changes in the expression of endocrine gland vascular endothelial growth factor in the human corpus luteum. J. Clin. Endocrinol. Metab. 90, 427–434.

189.Fujihara, M., Comizzoli, P., Keefer, C. L., Wildt, D. E., Songsasen, N. (2014). Epidermal Growth Factor (EGF) Sustains In Vitro Primordial Follicle Viability by Enhancing Stromal Cell Proliferation via MAPK and PI3K Pathways in the Prepubertal, but Not Adult, Cat Ovary 1. Biology of reproduction, 90(4), Article-86.

190.Fujinaga, H., Yamoto, M., Nakano, R., Shima, K., (1992). Epidermal Growth Factor Binding Sites in Porcine Granulosa Cells and their Regulation by Follicle-Stimulating Hormone. Biol. Reprod. 46, 705–709.

191.Fukushima, T., Kataoka, H. (2007). Roles of insulin-like growth factor binding protein-2 (IGFBP-2) in glioblastoma. Anticancer research, 27(6A), 3685-3692.

192.Fuller, G. N., Rhee, C. H., Hess, K. R., Caskey, L. S., Wang, R., Bruner, J. M., Zhang, W. (1999). Reactivation of insulin-like growth factor binding protein 2 expression in glioblastoma multiforme. Cancer research, 59(17), 4228-4232.

193.Gaestel, M. (2006). MAPKAP kinases—MKs—two's company, three's a crowd. Nature reviews Molecular cell biology, 7(2), 120-130.

194.Gall L, Boulesteix C, Ruffini S, Germain G, (2005): EGF-induced EGF-receptor and MAP kinase phosphorylation in goat cumulus cells during *in vitro* maturation. MolReprod Dev 71, 489–494.

195.Gall, L., Chene, N., Dahirel, M., Ruffini, S., Boulesteix, C., (2004). Expression of epidermal growth factor receptor in the goat cumulus-oocyte complex. Mol. Reprod. Dev. 67, 439–445

196.Gardner, D.K., Lane, M., (1998). Culture of viable human blastocysts in defined sequential serum-free media. Hum. Reprod. 13 Suppl 3, 148–59; discussion 160.

197.Gardner, D.K., Lane, M., (2013). Mammalian Preimplantation Embryo Culture, in: Methods in Molecular Biology. pp. 167–182.

198.Garnett K, Wang J, Roy SK. (2002). Spatiotemporal expression of epidermal growth factor receptor messenger RNA and protein in the hamster ovary: Follicle stage-specific differential modulation by follicle-stimulating hormones, luteinizing hormone, estradiol, and progesterone. BiolReprod 67:1593–1604.

199.Garris, D.R., Foreman, D., (1984). Follicular Growth and Atresia during the Last Half of the Luteal Phase of the Guinea Pig Estrous Cycle: Relation to Serum Progesterone and Estradiol Levels and Utero-Ovarian Blood Flow*. Endocrinology 115, 73–77.

200.Ge, Z., Miller, E., Nicholson, W., Hedgpeth, V., Gadsby, J. (2003). Insulin-like growth factor (IGF)-I and IGF binding proteins-2,-3,-4,-5 in porcine corpora lutea during the estrous cycle; evidence for inhibitory actions of IGFBP-3. *Domestic animal endocrinology*, *25*(2), 183-197.

201.Ge, Z., Nicholson, W., Plotner, D., Farin, C., Gadsby, J., (2000). Insulin-like growth factor I receptor mRNA and protein expression in pig corpora lutea. Reproduction 120, 109–114.

202.Gehm, B.D., McAndrews, J.M., Jordan, V.C., Jameson, J.L., (2000). EGF activates highly selective estrogen-responsive reporter plasmids by an ER-independent pathway. Mol. Cell. Endocrinol. 159, 53–62.

203.Gennigens, C., Menetrier-Caux, C., Droz, J.P., (2006). Insulin-Like Growth Factor (IGF) family and prostate cancer. Crit. Rev. Oncol. Hematol. 58, 124–145.

204.Ghetler, Y., Skutelsky, E., Ben Nun, I., Ben Dor, L., Amihai, D., Shalgi, R., (2006). Human oocyte cryopreservation and the fate of cortical granules. Fertil. Steril. 86, 210–216.

205.Gittens, J. E., Mhawi, A. A., Lidington, D., Ouellette, Y., Kidder, G. M. (2003). Functional analysis of gap junctions in ovarian granulosa cells: distinct role for connexin43 in early stages of folliculogenesis. *American Journal of Physiology-Cell Physiology*, *284*(4), C880-C887.

206.Giudice, L. C. (1995). The insulin-like growth factor system in normal and abnormal human ovarian follicle development. The American journal of medicine, 98(1), S48-S54.

207.Giudice, L. C. (2001). Insulin-like growth factor family in Graafian follicle development and function. Journal of the Society for Gynecologic Investigation, 8(1 suppl), S26-S29.

208.Giudice, L.C., 1992. Insulin-Like Growth Factors and Ovarian Follicular Development*. Endocr. Rev. 13, 641–669.

209. Gómez, E., Tarín, J. J., Pellicer, A. (1993). Oocyte maturation in humans: the role of gonadotropins and growth factors. *Fertility and sterility*, *60*(1), 40-46.

210.Gong, J.G., McBride, D., Bramley, T.A., Webb, R., (1994). Effects of recombinant bovine somatotrophin, insulin-like growth factor-I and insulin on bovine granulosa cell steroidogenesis *in vitro*. J. Endocrinol. 143, 157–164.

211.González, R., Gómez, M. C., Pope, C. E., Brandt, Y. C. B. (2012). Characterization of Mitochondrial and Actin Patterns in Cat Oocytes and Blastocysts. Reproduction in Domestic Animals, 47(s6), 118-120.

212.Goodsell, D.S., (2003). The Molecular Perspective: Epidermal Growth Factor. Stem Cells 21, 702–703.

213.Goritz, F., Jewgenow, K., Meyer, H.H.D., (1996). Epidermal growth factor and epidermal growth factor receptor in the ovary of the domestic cat (Felis catus). Reproduction 106, 117–124.

214.Gospodarowicz, D., III, C.R., Birdwell, C.R., (1977). Effects of Fibroblast and Epidermal Growth Factors on Ovarian Cell Proliferation*in vitro*. I. Characterization of the Response of Granulosa Cells to FGF and EGF. Endocrinology 100, 1108–1120.

215.Goubau, S., Murphy, B. D., Han, V. K., Schultz, G. A., (1996). Insulin-like growth factor-I (IGF-I) colocalizes with IGF-binding proteins (IGFBPs) in mouse and rat ovary. *Endocrine*, *4*(3), 213-221.

216.Goud, P.T., Goud, A.P., Qian, C., Laverge, H., Van der Elst, J., De Sutter, P. Dhont, M., (1998). *In vitro* maturation of human germinal vesicle stage oocytes: role of cumulus cells and epidermal growth factor in the culture medium. *Hum. Reprod.* 13, 1638–44.

217.Goud, P.T., Goud, A.P., Qian, C., Laverge, H., Van der Elst, J., De Sutter, P., Dhont, M., (1998). In-vitro maturation of human germinal vesicle stage oocytes: role of cumulus cells and epidermal growth factor in the culture medium. Hum. Reprod. 13, 1638–1644.

218.Grazul-Bilska, A.T., Reynolds, L.P., Redmer, D.A., (1997). Gap Junctions in the

Ovaries1. Biol. Reprod. 57, 947–957.

219. Green, C. J., Day, M. L., (2014). Insulin-like growth factor 1 acts as an autocrine factor to improve early embryogenesis *in vitro*. *International Journal of Developmental Biology*, *57*(11-12), 837-844.

220. Gregoire Anne. Cryoconservation des ressources génétiques chez le cochon d'Inde (Cavia porcellus): production et congélation des embryons. Sciences agricoles. Université Claude Bernard. Lyon I, (2012). Francais.

221. Grégoire, A., Allard, A., Huamán, E., León, S., Silva, R.M., Buff, S., Berard, M., Joly, T., (2012). Control of the estrous cycle in guinea-pig (Cavia porcellus). Theriogenology 78, 842–847.

222. Gregson, E., Webb, R., Sheldrick, E. L., Campbell, B. K., Mann, G. E., Liddell, S., Sinclair, K. D., (2016). Molecular determinants of a competent bovine corpus luteum: first-vs final-wave dominant follicles. *Reproduction*, *151*(6), 563-575.

223. Gregson, E., Webb, R., Sheldrick, E. L., Campbell, B. K., Mann, G. E., Liddell, S., Sinclair, K. D., (2016). Molecular determinants of a competent bovine corpus luteum: first-vs final-wave dominant follicles. *Reproduction*, *151*(6), 563-575.

224. Greisen, S., Flyvbjerg, A., Ledet, T., Ovesen, P., (2002). Regulation of insulin-like growth factor binding protein secretion by human granulosa luteal cells in a polycystic ovary-like environment. *Fertility and sterility*, *78*(1), 162-168.

225. Greisen, S., Flyvbjerg, A., Ledet, T., Ovesen, P., (2002). Regulation of insulin-like growth factor binding protein secretion by human granulosa luteal cells in a polycystic ovary-like environment. *Fertility and sterility*, *78*(1), 162-168.

226. Grimes, R. W., Barber, J. A., Shimasaki, S., Ling, N., Hammond, J. M., (1994). Porcine ovarian granulosa cells secrete insulin-like growth factor-binding proteins-4 and-5 and express their messenger ribonucleic acids: regulation by follicle-stimulating hormone and insulin-like growth factor-1. *Biology of reproduction*, *50*(3), 695-701.

227. Grimes, R. W., Hammond, J. M., (1992). Insulin and insulin-like growth factors (IGFs) stimulate production of IGF-binding proteins by ovarian granulosa cells. *Endocrinology*, *131*(2), 553-558.

228. Grive, K.J., Freiman, R.N., (2015). The developmental origins of the mammalian ovarian reserve. Development 142, 2554–2563.

229. Guler, A., Poulin, N., Mermillod, P., Terqui, M., Cognie, Y., (2000). Effect of growth factors, EGF and IGF-I, and estradiol on *in vitro* maturation of sheep oocytes. *Theriogenology*, *54*(2), 209-218.

230. Guler, H.P., Zapf, J., Schmid, C., Froesch, E.R., (1989). Insulin-like growth factors I and II in healthy man. Estimations of half-lives and production rates. Acta

Endocrinol. 121, 753–758.

231. Gullu, G., Karabulut, S., Akkiprik, M., (2012). Functional roles and clinical values of insulin-like growth factor-binding protein-5 in different types of cancers. Chin. J. Cancer 31, 266–280.
232. Guthrie, H.D., Garrett, W.M., Cooper, B.S., (1998). Follicle-Stimulating Hormone and Insulin-Like Growth Factor-I Attenuate Apoptosis in Cultured Porcine Granulosa Cells1. Biol. Reprod. 58, 390–396.
233. Gutierrez, C. G., Campbell, B. K., Armstrong, D. G., Webb, R. (1997). Insulin-like growth factor-I (IGF-I) production by bovine granulosa cells *in vitro* and peripheral IGF-I measurement in cattle serum: an evaluation of IGF-binding protein extraction protocols. *Journal of endocrinology*, *153*(2), 231-240.
234. Gutierrez, C. G., Ralph, J. H., Telfer, E. E., Wilmut, I., Webb, R., (2000). Growth and Antrum Formation of Bovine Preantral Follicles in Long-Term Culture *In vitro* 1. *Biology of Reproduction*, *62*(5), 1322-1328.
235. Hafez, E.S.E., Hafez, B., (2013). Reproduction in Farm Animals. Wiley-Blackwell.
236. Hale K, Murray AW., Cosgrove LJ., Bach LA., Hartfield PJ., (2000) Prevention of apoptosis by insulin-like growth factor (IGF)-I and IGF-II is differentially attenuated by IGF-binding proteins in PC12 cells. Neurosci Res Commun 27:75–83
237. Hammond, J.M., Hsu, C.J., Klindt, J., Tsang, B.K., Downey, B.R., (1988). Gonadotropins increase concentrations of immunoreactive insulin-like growth factor-I in porcine follicular fluid *in vivo.* Biology of Reproduction 38, 304–308.
238. Hardouin, S., Hossenlopp, P., Segovia, B., Seurin, D., Portolan, G., Lassarre, C., Binoux, M., (1987). Heterogeneity of insulin-like growth factor binding proteins and relationships between structure and affinity. 1. Circulating forms in man. Eur. J. Biochem. 170, 121–132.
239. Hardy, K., Spanos, S., (2002). Growth factor expression and function in the human and mouse preimplantation embryo. *Journal of Endocrinology*, *172*(2), 221-236.
240. Harkness, J.E., Murray, K.A., Wagner, J.E., (2016). Biology and Diseases of Guinea Pigs, in: Laboratory Animal Medicine. pp. 203–246.
241. Harned, M. A., Casida, L. E., (1972). Failure to obtain group synchrony of estrus in the guinea pig. *Journal of mammalogy*, *53*(1), 223-225.
242. Harper, L. V. (1968). The effects of isolation from birth on the social behaviour of guinea pigs in adulthood. *Animal behaviour*, *16*(1), 58-64.
243. Harvey M, Kaye PL., (1992): Insulin-like growth factor-I stimulates growth of mouse preimplantation embryos *in vitro.* Mol ReprodDev 31:195–199.
244. Harvey M., Kaye PL., (1990): Insulin increases the number of the inner cell mass and stimulates morphological development of the mouse blastocyst *in vitro.*

Development 110:963–967.

245.Harvey MB, Kaye PL., (1988): Insulin stimulates protein synthesis in compacted mouse embryos. Endocrinology 122:1182–1184.

246.Hastie, P. M., and Haresign, W., (2006). Expression of mRNAs encoding insulin-like growth factor (IGF) ligands, IGF receptors and IGF binding proteins during follicular growth and atresia in the ovine ovary throughout the oestrous cycle. Animal reproduction science, 92(3), 284-299.

247.Hastie, P.M., Onagbesan, O.M., Haresign, W., (2004). Co-expression of messenger ribonucleic acids encoding IGF-I, IGF-II, type I and II IGF receptors and IGF-binding proteins (IGFBP-1 to -6) during follicular development in the ovary of seasonally anoestrous ewes. Anim. Reprod. Sci. 84, 93–105.

248.Hatoya, S., Sugiyama, Y., Nishida, H., Okuno, T., Torii, R., Sugiura, K., Kida, K., Kawate, N., Tamada, H., Inaba, T., (2009). Canine oocyte maturation in culture: Significance of estrogen and EGF receptor gene expression in cumulus cells. Theriogenology 71, 560–567.

249.Hattori, M.A., Yoshino, E., Shinohara, Y., Horiuchi, R, Kojima, I. (1995). A novel action of epidermal growth factor in rat granulosa cells: its potentiation of gonadotrophin action. *J. Mol. Endocrinol.* 15, 283–91.

250.Hauser, D.S., Stade, M., Schmidt, A., Hanauer, G., (2005). Cardiovascular parameters in anaesthetized guinea pigs: A safety pharmacology screening model. J. Pharmacol. Toxicol. Methods 52, 106–114.

251.Havelock, J.C., Rainey, W.E., Carr, B.R., (2004). Ovarian granulosa cell lines. Mol. Cell. Endocrinol. 228, 67–78.

252. Heap, R. B., Deanesly, R., (1966). Progesterone in systemic blood and placentae of intact and ovariectomized pregnant guinea-pigs. *Journal of Endocrinology*, *34*(4), 417-423.

253.Herbst, R. S. (2004). Review of epidermal growth factor receptor biology. International Journal of Radiation Oncology* Biology* Physics, 59(2), S21-S26.

254.Herlands, R.L., Schultz, R.M., (1984). Regulation of mouse oocyte growth: Probable nutritional role for intercellular communication between follicle cells and oocytes in oocyte growth. J. Exp. Zool. 229, 317–325.

255.Hernandez, E.R., Resnick, C.E., Svoboda, M.E., Van Wyk, J.J., Payne, D.W., Adashi, E.Y., (1988). Somatomedin-C/Insulin-Like Growth Factor I as an Enhancer of Androgen Biosynthesis by Cultured Rat Ovarian Cells*. Endocrinology 122, 1603–1612.

256.Herrler, A., Lucas-Hahn, A., Niemann, H., (1992). Effects of insulin-like growth factor-I on in-vitro production of bovine embryos. Theriogenology 37, 1213–1224.

257.Heyner S, Shi C, Garside WT, Smith RM., (1993): Functions of the IGFs in early mammalian development. Mol ReprodDev 35:421–426.

258.Heyner S, Smith RM, Schultz GA., (1989): Temporally regulated expression of insulin and insulin-like growth factors and their receptors in early mammalian development. BioEssays 11(6):171– 177.

259.Hill JL, Hammar K, Smith PJS, Gross DJ., (1999). Stage-dependent effects of epidermal growth factor on Ca 2+ efflux in mouse oocytes. MolReprod Dev 53:244–253.

260.Hoff, D.R., Ryan, G.J., Driver, E.R., Ssemakulu, C.C., De Groote, M.A., Basaraba, R.J., Lenaerts, A.J., (2011). Location of intra- and extracellular M. tuberculosis populations in lungs of mice and guinea pigs during disease progression and after drug treatment. PLoS One 6, e17550.

261.Homburg, R., (1998). Involvement of growth factors in the pathophysiology of polycystic ovary syndrome. *Gynecological Endocrinology*, *12*(6), 391-397.

262.Homburg, R., and Amsterdam, A., (1998). Polycystic ovary syndrome—loss of the apoptotic mechanism in the ovarian follicles?. *Journal of endocrinological investigation*, *21*(9), 552-557.

263.Hosoe, M., Shioya, Y., (1997). Distribution of cortical granules in bovine oocytes classified by cumulus complex. Zygote 5, 371–376.

264.Hossner KL, McCusker RH, Dodson MV., (1997) Insulin-like growth factors and their binding proteins in domestic animals. Anim Sci ;64:1–15.

265.Hourvitz A, Widger AE, Filho FL, Chang RJ, Adashi EY, Erickson GF., (2000). Pregnancy-associated plasma protein-A gene expression in human ovaries is restricted to healthy follicles and corpora lutea. J Clin Endocrinol Metab;85:4916 – 20

266.Hsieh, M., Thao, K., Conti, M., (2011). Genetic dissection of epidermal growth factor receptor signaling during luteinizing hormone-induced oocyte maturation. PLoS One 6, e21574.

267.Hsu, C.-J., Hammond, J.M., (1987). Gonadotropins and Estradiol Stimulate Immunoreactive Insulin-Like Growth Factor-I Production by Porcine Granulosa Cells *in vitro*. Endocrinology 120, 198–207.

268.Hsueh, A.J.W., Welsh, T.H., Jones, P.B.C., (1981). Inhibition of ovarian and testicular steroiodogenesis by epidermal growth factor. Endocrinology 108, 2002–2004.

269.Hu, C.-L., Cowan, R.G., Harman, R.M., Porter, D.A., Quirk, S.M., (2001). Apoptosis of Bovine Granulosa Cells After Serum Withdrawal Is Mediated by Fas Antigen (CD95) and Fas Ligand1. Biol. Reprod. 64, 518–526.

270.Huang, C., Weitsman, S., Dykes, B., Magoffin, D., (2001). Stem cell factor and insulin-like growth factor I stimulate luteinizing hormone-dependent differentiation of rat ovarian theca cells. Biol. Reprod. 64, 451–6.

271.Humbel, R.E., (1990). Insulin-like growth factors I and II. Eur. J. Biochem. 190, 445–462.

272.Hunt, P.A., Lawson, C., Gieske, M., Murdoch, B., Smith, H., Marre, A., Hassold, T., VandeVoort, C.A., (2012). Bisphenol A alters early oogenesis and follicle formation in the fetal ovary of the rhesus monkey. Proc. Natl. Acad. Sci. U. S. A. 109, 17525–17530.

273.Hunter, M.G., Robinson, R.S., Mann, G.E., Webb, R., (2004). Endocrine and paracrine control of follicular development and ovulation rate in farm species. Anim. Reprod. Sci. 82-83, 461–477.

274.Hunter, R.H.F., Hunt, D.M., Chang, M.C., (1969). Temporal and cytological aspects of fertilization and early development in the guinea pig, Cavia porcellus. Anat. Rec. 165, 411–429.

275.Hussein, M.R., (2004). Apoptosis in the ovary: molecular mechanisms. Hum. Reprod. Update 11, 162–178.

276.Hussein, T.S., Froiland, D.A., Amato, F., Thompson, J.G., Gilchrist, R.B., (2005). Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. J. Cell Sci. 118, 5257–5268.

277.Hutt, K. J., Albertini, D. F. (2007). An oocentric view of folliculogenesis and embryogenesis. Reproductive biomedicine online, 14(6), 758-764.

278.Hutz, R.J., Bejvan, S.M., Durning, M., Dierschke, D.J., Fischer, C.L., Zachow, R.J., (1990). Changes in Follicular Populations, in Serum Estrogen and Progesterone, and in Ovarian Steroid Secretion *in vitro* during the Guinea Pig Estrous Cycle12. Biol. Reprod. 42, 266–272.

279.Hwa, V., Oh, Y., Rosenfeld, R. G., (1999). The insulin-like growth factor-binding protein (IGFBP) superfamily 1. *Endocrine reviews*, *20*(6), 761-787.

280. Ignar-Trowbridge, D.M., Pimentel, M., Parker, M.G., McLachlan, J.A., Korach, K.S., (1996). Peptide growth factor cross-talk with the estrogen receptor requires the A/B domain and occurs independently of protein kinase C or estradiol. Endocrinology 137, 1735–1744.

281.Ingermann, A.R., Yang, Y.-F., Han, J., Mikami, A., Garza, A.E., Mohanraj, L., Fan, L., Idowu, M., Ware, J.L., Kim, H.-S., Lee, D.-Y., Oh, Y., (2010). Identification of a Novel Cell Death Receptor Mediating IGFBP-3-induced Anti-tumor Effects in Breast and Prostate Cancer. J. Biol. Chem. 285, 30233–30246.

282.Ingman, W. V., Owens, P. C., Armstrong, D. T., (2000). Differential regulation by

FSH and IGF-I of extracellular matrix IGFBP-5 in bovine granulosa cells: effect of association with the oocyte. *Molecular and cellular endocrinology*, *164*(1), 53-58.

283. Inoue, N., Matsuda, F., Goto, Y., Manabe, N., (2011). Role of cell-death ligand-receptor system of granulosa cells in selective follicular atresia in porcine ovary. J. Reprod. Dev. 57, 169–175.

284. Iosef, C., Vilk, G., Gkourasas, T., Lee, K. J., Chen, B. P., Fu, P., Han, V. K., (2010). Insulin-like growth factor binding protein-6 (IGFBP-6) interacts with DNA-end binding protein Ku80 to regulate cell fate. *Cellular signalling*, *22*(7), 1033-1043.

285. Ishii, O., (1920). Observations on the sexual cycle of the guinea pig. Biol. Bull. 38, 237–250.

286. Isobe, N., Terada, T., (2001). Effect of the factor inhibiting germinal vesicle breakdown on the disruption of gap junctions and cumulus expansion of pig cumulus-oocyte complexes cultured *in vitro*. Reproduction 121, 249–257.

287. Iwashita, M., Kudo, Y., Takeda, Y., (1998). Effect of follicle stimulating hormone and insulin-like growth factors on proteolysis of insulin-like growth factor binding protein-4 in human granulosa cells. *Molecular human reproduction*, *4*(4), 401-405.

288. Izadyar, F., Zeinstra, E., Bevers, M.M., (1998). Follicle-stimulating hormone and growth hormone act differently on nuclear maturation while both enhance developmental competence of *in vitro* matured bovine oocytes. Mol. Reprod. Dev. 51, 339–345.

289. Jacquet, P., Buset, J., Vankerkom, J., Baatout, S., de Saint-Georges, L., Baugnet-Mahieu, L., Desaintes, C., (2001). Radiation-induced chromosome aberrations in guinea-pig growing oocytes, and their relation to follicular atresia. Mutat. Res./Fundam. Mol. Mech. Mutag. 473, 249–254.

290. Jacquet, P., de Saint-Georges, L., Vankerkom, J., Baugnet-Mahieu, L., (1995). A method for chromosome preparation of guinea-pig oocytes. Mutation Research/Environmental Mutagenesis and Related Subjects 334, 309–316.

291. Jaffe, L., Egbert, J. R., (2016). Regulation of Mammalian Oocyte Meiosis by Intercellular Communication Within the Ovarian Follicle. *Annual Review of Physiology,* (0).

292. Jainudeen, M.R., Takahashi, Y., Nihayah, M., Kanagawa, H., (1993). *In vitro* maturation and fertilization of swamp buffalo (Bubalus bubalis) oocytes. Anim. Reprod. Sci. 31, 205–212.

293. Jamnongjit, M., Gill, A., Hammes, S.R., (2005). Epidermal growth factor receptor signaling is required for normal ovarian steroidogenesis and oocyte maturation. Proceedings of the National Academy of Sciences 102, 16257–16262.

294. Jedrusik, A., Parfitt, D.-E., Guo, G., Skamagki, M., Grabarek, J.B., Johnson, M.H.,

Robson, P., Zernicka-Goetz, M., (2008). Role of Cdx2 and cell polarity in cell allocation and specification of trophectoderm and inner cell mass in the mouse embryo. Genes Dev. 22, 2692–2706.

295.Jensen, E. C., (2013). Quantitative analysis of histological staining and fluorescence using ImageJ. The Anatomical Record, 296(3), 378-381.

296.Jeong, W., Jung, S., Bazer, F. W., Song, G., Kim, J. (2016). Epidermal growth factor: Porcine uterine luminal epithelial cell migratory signal during the peri-implantation period of pregnancy. *Molecular and cellular endocrinology*, *420*, 66-74.

297. John, G. B., Gallardo, T. D., Shirley, L. J.,Castrillon, D. H., (2008). Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth. *Developmental biology*, *321*(1), 197-204.

298. Jones, J. I., Clemmons, D. R., (1995). Insulin-like growth factors and their binding proteins: biological actions. *Endocrine reviews*, *16*(1), 3-34.

299.Jones, P. B., Welsh, T. H., Hsueh, A. J., (1982). Regulation of ovarian progestin production by epidermal growth factor in cultured rat granulosa cells. *Journal of Biological Chemistry*, *257*(19), 11268-11273.

300.Joshi, H.S., Watson, D.J., Labhsetwar, A.P., (1973). Ovarian secretion of oestradiol, oestrone, 20-dihydroprogesterone and progesterone during the oestrous cycle of the guinea-pig. J. Reprod. Fertil. 35, 177–181.

301.Juneja, S.C., Barr, K.J., Enders, G.C., Kidder, G.M., (1999). Defects in the Germ Line and Gonads of Mice Lacking Connexin431. Biol. Reprod. 60, 1263–1270.

302.Kajarajan K, Rao BS, Vagdevi R, Tamilmani G, Arunakumari G, Sreenu M, Amarnath D, Naik BR, Rao VH, (2006): Effect of various growth factors on the *in vitro* development of goat preantral follicles. Small Rum Res 63, 2014–2212

303.Kalus, W., (1998). Structure of the IGF-binding domain of the insulin-like growth factor-binding protein-5 (IGFBP-5): implications for IGF and IGF-I receptor interactions. EMBO J. 17, 6558–6572.

304.Kamada, S., Kubota, T., Taguchi, M., Wen-Rong, H., Sakamoto, S., Aso, T., (1992). Effects of Insulin-Like Growth Factor-II on Proliferation and Differentiation of Ovarian Granulosa Cells. Horm. Res. 37, 141–149

305.Kamangar, B. B., Gabillard, J. C., Bobe, J. (2006). Insulin-like growth factor-binding protein (IGFBP)-1,-2,-3,-4,-5, and-6 and IGFBP-related protein 1 during rainbow trout postvitellogenesis and oocyte maturation: molecular characterization, expression profiles, and hormonal regulation. *Endocrinology*, *147*(5), 2399-2410.

306.Kanasaki, H., Oride, A., Mijiddorj, T., Sukhbaatar, U., Kyo, S. (2017). How is GnRH regulated in GnRH-producing neurons? Studies using GT1-7 cells as a GnRH-producing cell model. General and Comparative Endocrinology.

307.Kane, M. T., Morgan, P. M., Coonan, C., (1997). Peptide growth factors and preimplantation development. *Human Reproduction Update*, *3*(2), 137-157.

308.Kastrop, P.M.M., Hulshof, S.C.J., Bevers, M.M., Destrée, O.H.J., Kruip, T.A.M., (1991). The effects of α-amanitin and cycloheximide on nuclear progression, protein synthesis, and phosphorylation during bovine oocyte maturation *in vitro*. Mol. Reprod. Dev. 28, 249–254.

309.Kaufmann, P., Davidoff, M., (1977). Possibilities of Comparison with the Human Placenta, in: The Guinea-Pig Placenta. pp. 81–83.

310.Kawano, Y., Narahara, H., Matsui, N., Nasu, K., Miyamura, K., Miyakawa, I., (1997). Insulin-Like Growth Factor-Binding Protein-1 in Human Follicular Fluid: A Marker for Oocyte Maturation. Gynecol. Obstet. Invest. 44, 145–148.

311.Kennedy, T.G., Brown, K.D. Vaughan, T.J. (1993). Expression of the genes for the epidermal growth factor receptor and its ligands in porcine corpora lutea. Endocrinology 132, 1857–9.

312.Khan-Dawood, F. S., Gargiulo, A. R., Dawood, M. Y., (1994). *In vitro* microdialysis of the ovine corpus luteum of pregnancy: effects of insulin-like growth factor on progesterone secretion. *Biology of reproduction*, *51*(6), 1299-1306.

313.Khandwala, H.M., (2000). The Effects of Insulin-Like Growth Factors on Tumorigenesis and Neoplastic Growth. Endocr. Rev. 21, 215–244.

314.Kidder, G.M., Mhawi, A.A., (2002). Gap junctions and ovarian folliculogenesis. Reproduction 123, 613–620.

315.Kiepe, D., Ulinski, T., Powell, D.R., Durham, S.K., Mehls, O., Tönshoff, B., (2002). Differential effects of insulin-like growth factor binding proteins-1, -2, -3, and -6 on cultured growth plate chondrocytes. Kidney Int. 62, 1591–1600.

316.Kim, C. H., Chae, H. D., Cheon, Y. P., Kang, B. M., Chang, Y. S., Mok, J. E. (1999). The effect of epidermal growth factor on the preimplantation development, implantation and its receptor expression in mouse embryos. *Journal of Obstetrics and Gynaecology Research*, *25*(2), 87-93.

317.Kim, J.-H., Choi, D.S., Lee, O.-H., Oh, S.-H., Lippman, S.M., Lee, H.-Y., (2011). Antiangiogenic antitumor activities of IGFBP-3 are mediated by IGF-independent suppression of Erk1/2 activation and Egr-1-mediated transcriptional events. Blood 118, 2622–2631.

318.Kim, M.-R., Tilly, J.L., (2004). Current concepts in Bcl-2 family member regulation of female germ cell development and survival. Biochim. Biophys. Acta 1644, 205–210.

319.Kim, Y. J., Ku, S. Y., Kim, Y. Y., Liu, H. C., Chi, S. W., Kim, S. H., Moon, S. Y., (2013). MicroRNAs transfected into granulosa cells may regulate oocyte meiotic competence during *in vitro* maturation of mouse follicles. *Human*

Reproduction, *28*(11), 3050-3061.

320.Kimber, S.J., Spanswick, C., (2000). Blastocyst implantation: the adhesion cascade. Semin. Cell Dev. Biol. 11, 77–92.

321.Kirby, C. J., Thatcher, W. W., Collier, R. J., Simmen, F. A., Lucy, M. C., (1996). Effects of growth hormone and pregnancy on expression of growth hormone receptor, insulin-like growth factor-I, and insulin-like growth factor binding protein-2 and-3 genes in bovine uterus, ovary, and oviduct. *Biology of reproduction*, *55*(5), 996-1002.

322.Kliem, H., Berisha, B., Meyer, H.H.D., Schams, D., (2009). Regulatory changes of apoptotic factors in the bovine corpus luteum after induced luteolysis. Mol. Reprod. Dev. 76, 220–230.

323.Knight, P.G., Satchell, L., Glister, C., (2012). Intra-ovarian roles of activins and inhibins. Mol. Cell. Endocrinol. 359, 53–65.

324.Kobayashi, K., Yamashita, S., Hoshi, H., (1994). Influence of epidermal growth factor and transforming growth factor-α on *in vitro* maturation of cumulus cell-enclosed bovine oocytes in a defined medium. *Journal of reproduction and fertility*, *100*(2), 439-446.

325.Kölle, S., Dubielzig, S., Reese, S., Wehrend, A., König, P., Kummer, W., (2009). Ciliary transport, gamete interaction, and effects of the early embryo in the oviduct: ex vivo analyses using a new digital videomicroscopic system in the cow. Biol. Reprod. 81, 267–274.

326.Kono, T., Carroll, J., Swann, K., Whittingham, D. G. (1995). Nuclei from fertilized mouse embryos have calcium-releasing activity. Development, 121(4), 1123-1128.

327.Kooijman, R., (2006). Regulation of apoptosis by insulin-like growth factor (IGF)-I. Cytokine Growth Factor Rev. 17, 305–323.

328.Kouznetsova, A., Benavente, R., Pastink, A., Höög, C., (2011). Meiosis in Mice without a Synaptonemal Complex. PLoS One 6, e28255.

329.Krisher R, Bavister, (1998). Correlation of mitochondrial organization with developmental competence in bovine oocytes matured *in vitro.* Biology of reproduction 56.

330.Krisher, R.L., (2004). The effect of oocyte quality on development. J. Anim. Sci. 82 E-Suppl, E14–23.

331.Krisher, R.L., Bavister, B.D., (1998). Responses of oocytes and embryos to the culture environment. Theriogenology 49, 103–114.

332.Kumar, D., Purohit, G.N., (2004). Effect of epidermal and insulin like growth factor-1 on cumulus expansion, nuclear maturation and fertilization of buffalo cumulus oocyte complexes in simple serum free media DMEM and Ham's F-10.

VeterinarskiArhiv. 74, 13–25.

333.Kuo, Y.-S., Tang, Y.-B., Lu, T.-Y., Wu, H.-C., Lin, C.-T., (2010). IGFBP-6 plays a role as an oncosuppressor gene in NPC pathogenesis through regulating EGR-1 expression. J. Pathol. 222, 299–309.

334.Kwon, H., Choi, D. H., Bae, J. H., Kim, J. H., Kim, Y. S., (2010). mRNA expression pattern of insulin-like growth factor components of granulosa cells and cumulus cells in women with and without polycystic ovary syndrome according to oocyte maturity. *Fertility and sterility*, *94*(6), 2417-2420.

335.Lafky, J.M., Wilken, J.A., Baron, A.T., Maihle, N.J., (2008). Clinical implications of the ErbB/epidermal growth factor (EGF) receptor family and its ligands in ovarian cancer. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer 1785, 232–265.

336.Lalou, C., Lassarre, C., Binoux, M., (1996). A proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 that fails to bind IGFs inhibits the mitogenic effects of IGF-I and insulin. Endocrinology 137, 3206–3212.

337.Lam, X., Gieseke, C., Knoll, M., Talbot, P., (2000). Assay and Importance of Adhesive Interaction Between Hamster (Mesocricetus auratus) Oocyte-Cumulus Complexes and the Oviductal Epithelium1. Biol. Reprod. 62, 579–588.

338.Lara-Gonzalez, P., Westhorpe, F.G., Taylor, S.S., (2012). The spindle assembly checkpoint. Curr. Biol. 22, R966–80.

339.Larrouy-Maumus, G., Layre, E., Clark, S., Prandi, J., Rayner, E., Lepore, M., de Libero, G., Williams, A., Puzo, G., Gilleron, M., (2017). Protective efficacy of a lipid antigen vaccine in a guinea pig model of tuberculosis. Vaccine 35, 1395–1402.

340.Laviola, L., Natalicchio, A., Giorgino, F., (2007). The IGF-1 signaling pathway. Curr. Pharm. Des. 13, 663e669.

341.Lazzari G, Duchi R, Crotti G, Turini P, Notari C, Galli C, (2000). Effect of *in vitro* culture in SOF-BSA versus SOF-serum on the development of IVM-IVF bovine embryos up to day 12. AETE.

342.Le Roith, D., Bondy, C., Yakar, S., Liu, J.-L., Butler, A., (2001). The Somatomedin Hypothesis: 2001. Endocr. Rev. 22, 53–74.

343.Lee, C.-T., Risom, T., Strauss, W.M., (2007). Evolutionary conservation of microRNA regulatory circuits: an examination of microRNA gene complexity and conserved microRNA-target interactions through metazoan phylogeny. DNA Cell Biol. 26, 209–218.

344.Lee, E. S., Fukui, Y., (1995). Effect of various growth factors in a defined culture medium on *in vitro* development of bovine embryos matured and fertilized *in vitro*. *Theriogenology*, *44*(1), 71-83.

345.Lee, P.D.K., Conover, C.A., Powell, D.R., (1993). Regulation and Function of Insulin-

Like Growth Factor-Binding Protein-1. Exp. Biol. Med. 204, 4–29.

346. Lee, W., Hwang, R., Cho, J., Lee, Y., Choi, H., Bae, S., Kim, G. (2016). IGFBP5-derived peptide as a novel angiogenesis inhibitor for treatment of ovarian cancer.
347. Leoni, G. G., Palmerini, M. G., Satta, V., Succu, S., Pasciu, V., Zinellu, A., Berlinguer, F., (2015). Differences in the kinetic of the first meiotic division and in active mitochondrial distribution between prepubertal and adult oocytes mirror differences in their developmental competence in a sheep model. *PloS one*, *10*(4), e0124911.
348. Leu, T.-H Maa., (2003). Functional implication of the interaction between EGF receptor and C-SRC. Front. Biosci. 8, s28–38.
349. Levesque, J.T., Sirard, M.A., (1995). Effects of different kinases and phosphatases on nuclear and cytoplasmic maturation of bovine oocytes. Mol. Reprod. Dev. 42, 114–121.
350. Li, C.J., Fan, B.Q., (1997). Changes in the 3-dimensional distribution of mitochondria during meiotic divisions of mouse oocytes. Theriogenology 48, 33–41.
351. Li, J., Kawamura, K., Cheng, Y., Liu, S., Klein, C., Liu, S., Duan, E.-K., Hsueh, A.J.W., (2010). Activation of dormant ovarian follicles to generate mature eggs. Proc. Natl. Acad. Sci. U. S. A. 107, 10280–10284.
352. Li, M., Liang, C. G., Xiong, B., Xu, B. Z., Lin, S. L., Hou, Y., Sun, Q. Y. (2008). PI3-kinase and mitogen-activated protein kinase in cumulus cells mediate EGF-induced meiotic resumption of porcine oocyte. *Domestic animal endocrinology*, *34*(4), 360-371.
353. Li, R., Albertini, D.F., (2013). The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte. Nat. Rev. Mol. Cell Biol. 14, 141–152.
354. Li, R., Norman, R.J., Armstrong, D.T., Gilchrist, R.B., (2000). Oocyte-Secreted Factor(s) Determine Functional Differences Between Bovine Mural Granulosa Cells and Cumulus Cells1. Biol. Reprod. 63, 839–845.
355. Liebmann, C., (2001). Regulation of MAP kinase activity by peptide receptor signalling pathway: Paradigms of multiplicity. Cell. Signal. 13, 777–785.
356. Lilley, K. G., Epping, R. J., Hafner, L. M., (1997). The guinea pig estrous cycle: correlation of vaginal impedance measurements with vaginal cytologic findings. *Comparative Medicine*, *47*(6), 632-637.
357. Lim, J. M., Hansel, W., (1996). Roles of growth factors in the development of bovine embryos fertilized *in vitro* and cultured singly in a defined medium. *Reproduction, fertility and development*, *8*(8), 1199-1205.

358.Lim, S., Chae, J., Choo, W., Ku, H., Lee, J., Hur, Y., .Lee, D. (2013). In vitro maturation: Clinical applications. Clinical and experimental reproductive medicine, 40(4), 143-147.

359.Liu XJ, Malkowski M, Guo Y, Erickson GF, Shimasaki S, Ling N.,
(1993). Development of specific antibodies to rat insulin-like growth factor-binding proteins (IGFBP-2 to -6): analysis of IGFBP production by rat granulosa cells. Endocrinology;132:1176–1183

360.Liu, J., Yao, W., Yao, Y., Du, X., Zhou, J., Ma, B., Liu, H., Li, Q., Pan, Z., (2014). MiR-92a inhibits porcine ovarian granulosa cell apoptosis by targeting Smad7 gene. FEBS Lett. 588, 4497–4503.

361.Liu, K., Liu, Y.X., Hu, Z.Y., Zou, R.Y., Chen, Y.J., Mu, X.M., Ny, T., (1997). Temporal expression of urokinase type plasminogen activator, tissue type plasminogen activator, plasminogen activator inhibitor type 1 in rhesus monkey corpus luteum during the luteal maintenance and regression. Mol. Cell. Endocrinol. 133, 109–116.

362.Liu, K., Rajareddy, S., Liu, L., Jagarlamudi, K., Boman, K., Selstam, G., Reddy, P., (2006). Control of mammalian oocyte growth and early follicular development by the oocyte PI3 kinase pathway: new roles for an old timer. Dev. Biol. 299, 1–11.

363.Liu, L., Hammar, K., Smith, P.J.S., Inoue, S., Keefe, D.L., (2001). Mitochondrial modulation of calcium signaling at the initiation of development. Cell Calcium 30, 423–433.

364.Liu, L., Keefe, D.L., (2000). Cytoplasm Mediates Both Development and Oxidation-Induced Apoptotic Cell Death in Mouse Zygotes1. Biol. Reprod. 62, 1828–1834.

365.Liu, L., Keefe, D.L., (2008). Defective cohesin is associated with age-dependent misaligned chromosomes in oocytes. Reprod. Biomed. Online 16, 103–112.

366.Liu, L., Trimarchi, J.R., Keefe, D.L., (2000). Involvement of Mitochondria in Oxidative Stress-Induced Cell Death in Mouse Zygotes1. Biol. Reprod. 62, 1745–1753.

367.Liu, M., (2011). The biology and dynamics of mammalian cortical granules. *Reproductive Biology and Endocrinology*, *9*(1), 149.

368.Liu, M., Sims, D., Calarco, P., Talbot, P., (2003). Biochemical heterogeneity, migration, and pre-fertilization release of mouse oocyte cortical granules. *Reproductive Biology and Endocrinology*, *1*(1), 77.

369.Liu, S., Li, Y., Gao, X., Yan, J.-H., Chen, Z.J., (2010). Changes in the distribution of mitochondria before and after *in vitro* maturation of human oocytes and the effect of *in vitro* maturation on mitochondria distribution. Fertil. Steril. 93, 1550–1555.

370.Liu, X.J., Malkowski, M., Guo, Y., Erickson, G.F., Shimasaki, S., Ling, N., (1993). Development of specific antibodies to rat insulin-like growth factor-binding proteins (IGFBP-2 to -6): analysis of IGFBP production by rat granulosa cells. Endocrinology

132, 1176–1183.

371. Logothetopoulos, J., Dorrington, J., Bailey, D., Stratis, M., (1995). Dynamics of follicular growth and atresia of large follicles during the ovarian cycle of the guinea pig: Fate of the degenerating follicles, a quantitative study. Anat. Rec. 243, 37–48.

372. Lonergan P, Carolan C, Van Langendonckt A, Donnay I, Khatir H, Mermillod P. (1996). Role of epidermal growth factor in bovine oocyte maturation and preimplantation embryo development *in vitro*. Biol Reprod 54:1420–1429.

373. Lonergan, P., (2011). Influence of progesterone on oocyte quality and embryo development in cows. Theriogenology 76, 1594–1601.

374. Lonergan, P., Gutiérrez-Adán, A., Pintado, B., Fair, T., Ward, F., Fuente, J.D., Boland, M., (2000). Relationship between time of first cleavage and the expression of IGF-1 growth factor, its receptor, and two housekeeping genes in bovine two-cell embryos and blastocysts produced *in vitro*. Mol. Reprod. Dev. 57, 146–152.

375. Lorenzo, P. L., Liu, I. K. M., Carneiro, G. F., Conley, A. J., Enders, A. C. (2002). Equine oocyte maturation with epidermal growth factor. Equine veterinary journal, 34(4), 378-382.

376. Lorenzo, P. L., Liu, I. K. M., Enders, A. C., (2000). Oocyte Maturation-nuclear maturation of equine oocyte with epidermal growth factor: inhibitory effect by tyrphostin a-47 and localization of follicular egf receptor. Theriogenology 53, 458–458.

377. Lorenzo, P.L., Illera, J.C., Silván, G., Munro, C.J., Illera, M.J., Illera, M., (1997). Steroid-level response to insulin-like growth factor-1 in oocytes matured *in vitro*. J. Reprod. Immunol. 35, 11–29.

378. Lorenzo, P.L., Illera, M.J., Illera, J.C., Illera, M., (1994). Enhancement of cumulus expansion and nuclear maturation during bovine oocyte maturation *in vitro* by the addition of epidermal growth factor and insulin-like growth factor I. Reproduction 101, 697–701.

379. Lorenzo, P.L., Rebollar, P.G., Illera, M.J., Illera, J.C., Illera, M., Alvarino, J.M.R., (1996). Stimulatory effect of insulin-like growth factor I and epidermal growth factor on the maturation of rabbit oocytes *in vitro*. Reproduction 107, 109–117.

380. Louhio, H., Hovatta, O., Sjoberg, J., Tuuri, T., (2000). The effects of insulin, and insulin-like growth factors I and II on human ovarian follicles in long-term culture. Mol. Hum. Reprod. 6, 694–698.

381. Luciano, A.M., Lucian, A.M., Pappalardo, A., Ray, C., Peluso, J.J., (1994). Epidermal Growth Factor Inhibits Large Granulosa Cell Apoptosis by Stimulating Progesterone Synthesis and Regulating the Distribution of Intracellular Free Calcium1. Biol. Reprod. 51, 646–654.

382.Lucidi, P., Bernabò, N., Turriani, M., Barboni, B., Mattioli, M., (2003). Reprod. Biol. Endocrinol. 1, 45.
383.Luvoni, G.C., Chigioni, S., Allievi, E., Macis, D., (2005). Factors involved (*in vivo* and *in vitro* maturation of canine oocytes. Theriogenology 63, 41–59.
384.Lyons, R.M., Smith, G.L., (1986). Characterization of Multiplication-Stimulating Activity (MSA) carrier protein. Mol. Cell. Endocrinol. 45, 263–270.
385.Machaca, K., (2007). Ca2+ signaling differentiation during oocyte maturation. J. Cell. Physiol. 213, 331–340.
386.Madgwick, S., Jones, K.T., (2007). How eggs arrest at metaphase II: MPF stabilisation plus APC/C inhibition equals Cytostatic Factor. Cell Div. 2, 4.
387.Magalhães-Padilha, D. M., Duarte, A. B. G., Araújo, V. R., Saraiva, M. V. A., Almeida, A. P., Rodrigues, G. Q., Gastal, E. L., (2012). Steady-state level of insulin-like growth factor-I (IGF-I) receptor mRNA and the effect of IGF-I on the *in vitro* culture of caprine preantral follicles. *Theriogenology*, *77*(1), 206-213.
388.Magoffin, D.A., Weitsman, S.R., (1994). Insulin-Like Growth Factor-I Regulation of Luteinizing Hormone (LH) Receptor Messenger Ribonucleic Acid Expression and LH-Stimulated Signal Transduction in Rat Ovarian Theca-Interstitial Cells1. Biol. Reprod. 51, 766–775.
389.Mahmoudi, R., Subhani, A., Etesam, F., Subhani, A., Pasbakhsh, P., Abolhasani, F., (2005). The Effects of cumulus cells on *in vitro* maturation of mouse germinal vesicle stage oocytes. *International Journal of Reproductive BioMedicine*, *3*(2), 74-78.
390.Manabe, N., Goto, Y., Matsuda-Minehata, F., Inoue, N., Maeda, A., Sakamaki, K., Miyano, T., (2004). Regulation mechanism of selective atresia in porcine follicles: regulation of granulosa cell apoptosis during atresia. J. Reprod. Dev. 50, 493–514.
391.Mani, A.M., Fenwick, M.A., Cheng, Z., Sharma, M.K., Singh, D., Wathes, D.C., (2010). IGF1 induces up-regulation of steroidogenic and apoptotic regulatory genes via activation of phosphatidylinositol-dependent kinase/AKT in bovine granulosa cells. Reproduction 139, 139–151.
392.Mao, J., Wu, G., Smith, M. F., McCauley, T. C., Cantley, T. C., Prather, R. S., Day, B. N., (2002). Effects of Culture Medium, Serum Type, and Various Concentrations of Follicle-Stimulating Hormone on Porcine Preantral Follicular Development and Antrum Formation *In vitro* 1. *Biology of reproduction*, *67*(4), 1197-1203.
393.Mark, S., Kübler, B., Höning, S., Oesterreicher, S., John, H., Braulke, T., Ständker, L., (2005). Diversity of human insulin-like growth factor (IGF) binding protein-2 fragments in plasma: primary structure, IGF-binding properties, and disulfide bonding pattern. *Biochemistry*, *44*(9), 3644-3652.

394.Marteil, G., Richard-Parpaillon, L., Kubiak, J.Z., (2009). Role of oocyte quality in meiotic maturation and embryonic development. Reprod. Biol. 9, 203–224.

395.Martin, J. L., Baxter, R. C. (1986). Insulin-like growth factor-binding protein from human plasma. Purification and characterization. Journal of Biological Chemistry, 261(19), 8754-8760.

396.Martins, F.S., Celestino, J.J.H., Saraiva, M.V.A., Chaves, R.N., Rossetto, R., Silva, C.M.G., Lima-Verde, I.B., Lopes, C.A.P., Campello, C.C., Figueiredo, J.R., (2010). Interaction between growth differentiation factor 9, insulin-like growth factor I and growth hormone on the *in vitro* development and survival of goat preantral follicles. Braz. J. Med. Biol. Res. 43, 728–736.

397.Maruo T, Ladines-Llave CA, Samoto T, Matsuo H, Manalo AS, Ito H, Mochizuki M., (1993). Expression of epidermal growth factor and its receptor in the human ovary during follicular growth and regression. Endocrinology 132:924–931.

398.Maruo, T., (1993). Expression of epidermal growth factor and its receptor in the human ovary during follicular growth and regression. Endocrinology 132, 924–931.

399.Masnikosa, R., Baričević, I., Lagundžin, D., and Nedić, O., (2010). Characterisation of N-glycans bound to IGFBP-3 in sera from healthy adults. Biochimie, 92(1), 97-101.

400.Mason, H. D., Cwyfan-Hughes, S., Holly, J. M., Franks., (1998). Potent inhibition of human ovarian steroidogenesis by insulin-like growth factor binding protein-4 (IGFBP-4). *The Journal of Clinical Endocrinology & Metabolism*, *83*(1), 284-287.

401.Matsuda-Minehata, F., Inoue, N., Goto, Y., Manabe, N., (2006). The regulation of ovarian granulosa cell death by pro- and anti-apoptotic molecules. J. Reprod. Dev. 52, 695–705.

402.Matsui, M., Takahashi, Y., Hishinuma, M., Kanagawa, H., (1995). Insulin and Insulin-Like Growth Factor-I(IGF-I) Stimulate the Development of Bovine Embryos Fertilized *In vitro*. J. Vet. Med. Sci. 57, 1109–1111.

403.Matzuk, M.M., Burns, K.H., Viveiros, M.M., Eppig, J.J., (2002). Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 296, 2178–2180.

404.May, J.V., Bridge, A.J., Gotcher, E.D. Gangrade, B.K., (1992). The regulation of porcine theca cell proliferation *in vitro*: synergistic actions of epidermal growth factor and platelet derived growth factor. *Endocrinology*131, 689–97.

405.May, J.V., Buck, P.A., Schomberg, D.W., (1987). Epidermal Growth Factor Enhances [125I]Iodo-Follicle-Stimulating Hormone Binding by Cultured Porcine Granulosa Cells*. Endocrinology 120, 2413–2420.

406.Mazerbourg, S., Overgaard, M. T., Oxvig, C., Christiansen, M., Conover, C. A.,

Laurendeau, I., Monget, P., (2001). Pregnancy-associated plasma protein-A (PAPP-A) in ovine, bovine, porcine, and equine ovarian follicles: involvement in IGF binding protein-4 proteolytic degradation and mRNA expression during follicular development. *Endocrinology*, *142*(12), 5243-5253.

407. Mazerbourg, S., Zapf, J., Bar, R. S., Brigstock, D. R., Monget, P., (2000). Insulin-Like Growth Factor (IGF)-Binding Protein-4 Proteolytic Degradation in Bovine, Equine, and Porcine Preovulatory Follicles: Regulation by IGFs and Heparin-Binding Domain-Containing Peptides 1. *Biology of reproduction*, *63*(2), 390-400.
408. McArdle, C. A., & Holtorf, A. P., (1989). Oxytocin and progesterone release from bovine corpus luteal cells in culture: effects of insulin-like growth factor I, insulin, and prostaglandins. *Endocrinology*, *124*(3), 1278-1286.
409. McBride, D., Carre, W., Sontakke, S.D., Hogg, C.O., Law, A., Donadeu, F.X., Clinton, M., (2012). Identification of miRNAs associated with the follicular-luteal transition in the ruminant ovary. Reproduction 144, 221–233.
410. McCaffery, F. H., Leask, R., Riley, S. C., Telfer, E. E., (2000). Culture of bovine preantral follicles in a serum-free system: markers for assessment of growth and development. *Biology of Reproduction*, *63*(1), 267-273.
411. McGee, E.A., Hsueh, A.J.W., (2000). Initial and Cyclic Recruitment of Ovarian Follicles1. Endocr. Rev. 21, 200–214.
412. McTavish, H., Griffin, R.J., Terai, K., Dudek, A.Z., (2009). Novel insulin-like growth factor-methotrexate covalent conjugate inhibits tumor growth *in vivo* at lower dosage than methotrexate alone. Transl. Res. 153, 275–282.
413. Mehlmann, L.M., (2002). Meiotic Arrest in the Mouse Follicle Maintained by a Gs Protein in the Oocyte. Science 297, 1343–1345.
414. Mehlmann, L.M., Saeki, Y., Tanaka, S., Brennan, T.J., Evsikov, A.V., Pendola, F.L., Knowles, B.B., Eppig, J.J., Jaffe, L.A., (2004). The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. Science 306, 1947–1950.
415. Meinbach, D.S., Lokeshwar, B.L., (2006). Insulin-like growth factors and their binding proteins in prostate cancer: Cause or consequence?. Urologic Oncology: Seminars and Original Investigations 24, 294–306.
416. Mermillod, P., Dalbiès-Tran, R., Uzbekova, S., Thélie, A., Traverso, J.-M., Perreau, C., Papillier, P., Monget, P., (2008). Factors affecting oocyte quality: who is driving the follicle? Reprod. Domest. Anim. 43 Suppl 2, 393–400.
417. Merriman, J.A., Whittingham, D.G. Carroll, J., (1998). The effect of follicle stimulating hormone and epidermal growth factor on the developmental capacity of *in vitro* matured mouse oocytes. *Hum. Reprod.* 13, 690–5.
418. Mess, A., (2007). The Guinea pig placenta: model of placental growth dynamics.

Placenta 28, 12–815.

419.Messager, S., Chatzidaki, E. E., Ma, D., Hendrick, A. G., Zahn, D., Dixon, J., Colledge, W. H., (2005). Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(5), 1761-1766.

420.Miller, E. A., Ge, Z., Hedgpeth, V., Gadsby, J. E., (2003). Steroidogenic responses of pig corpora lutea to insulin-like growth factor I (IGF-I) throughout the oestrous cycle. *Reproduction*, *125*(2), 241-249.

421.Mills, P. G., and Reed, M., (1971). The onset of first oestrus in the guinea-pig and the effects of gonadotrophins and oestradiol in the immature animal. *Journal of Endocrinology*, *50*(2), 329-337.

422.Misajon, A., Hutchinson, P., Lolatgis, N., Trounson, A.O. Almahbobi, G., (1999). The mechanism of action of epidermal growth factor and transforming growth factor alpha on aromatase activity in granulosa cells from polycystic ovaries. *Mol. Hum. Reprod.* 5, 96–103.

423.Monget, P., Besnard, N., Huet, C., Pisselet, C., Monniaux, D., (1996). Insulin-Like Growth Factor-Binding Proteins and Ovarian Folliculogenesis. Horm. Res. 45, 211–217.

424.Monget, P., Bondy, C., (2000). Importance of the IGF system in early folliculogenesis. Molecular and cellular endocrinology, 163(1), 89-93.

425.Monget, P., Fabre, S., Mulsant, P., Lecerf, F., Elsen, J. M., Mazerbourg, S., Monniaux, D., (2002). Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. *Domestic Animal Endocrinology*, *23*(1), 139-154.

426.Monget, P., Fabre, S., Mulsant, P., Lecerf, F., Elsen, J.-M., Mazerbourg, S., Pisselet, C., Monniaux, D., (2002). Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. Domest. Anim. Endocrinol. 23, 139–154.

427.Moore, K., Kramer, J.M., Rodriguez-Sallaberry, C.J., Yelich, J.V., Drost, M., (2007). Insulin-like growth factor (IGF) family genes are aberrantly expressed in bovine conceptuses produced *in vitro* or by nuclear transfer. Theriogenology 68, 717–727.

428.Morales, E., (1995). The Guinea Pig: Healing, Food, & Ritual in the Andes. University Arizona Press, Tucson, AZ.

429.Morbeck, D.E., Flowers, W.L. Britt, J.H. (1993). Response of porcine granulosa cells isolated from primary and secondary follicles to FSH, 8-bromo-cAMP and epidermal growth factor *in vitro*. *J. Reprod. Fertil.* 99, 577–84.

430.Motlík, J., Lie, B., Shioya, Y., (1990). Two Sensitivity Levels of Cattle Oocytes to Puromycin. Biol. Reprod. 43, 994–998.

431.Mottola, C., MacDonald, R. G., Brackett, J. L., Mole, J. E., Anderson, J. K., Czech,

M. P. (1986). Purification and amino-terminal sequence of an insulin-like growth factor-binding protein secreted by rat liver BRL-3A cells. Journal of Biological Chemistry, 261(24), 11180-11188.

432.Murphy, B.D. (2004). Luteinization. The Ovary. pp. 185–199.

433.Murphy, L. J., (2003). The role of the insulin-like growth factors and their binding proteins in glucose homeostasis. *Journal of Diabetes Research*, *4*(4), 213-224.

434.Murphy, L.J., Barron, D.J., (1993). The IGFs and their binding proteins in murine development. Mol. Reprod. Dev. 35, 376–381.

435.Murphy, L.J., Bell, G.I., Friesen, H.G., (1987). Tissue distribution of insulin-like growth factor I and II messenger ribonucleic acid in the adult rat. Endocrinology 120, 1279–1282.

436.Myers, H.I., Young, W.C., Dempsey, E.W., (1936). Graafian follicle development throughout the reproductive cycle in the guinea pig, with especial reference to changes during oestrus (sexual receptivity). Anat. Rec. 65, 381–401.

437.Nagai, S., Mabuchi, T., Hirata, S., Shoda, T., Kasai, T., Yokota, S., Shitara, H., Yonekawa, H., Hoshi, K., (2006). Correlation of Abnormal Mitochondrial Distribution in Mouse Oocytes with Reduced Developmental Competence. Tohoku J. Exp. Med. 210, 137–144.

438.Nakatani, A., Shimasaki, S., Erickson, G.F., Ling, N., (1991). Tissue-Specific Expression of Four Insulin-Like Growth Factor-Binding Proteins (1, 2, 3, and 4) in the Rat Ovary*. Endocrinology 129, 1521–1529.

439.Natale, D.R., Watson, A.J., (2002). Rac-1 and IQGAP are potential regulators of E-cadherin–catenin interactions during murine preimplantation development. Mech. Dev. 119, S21–S26.

440.Neira, J. A., Tainturier, D., Pena, M. A., Martal, J., (2010). Effect of the association of IGF-I, IGF-II, bFGF, TGF-β1, GM-CSF, and LIF on the development of bovine embryos produced *in vitro*. *Theriogenology*, *73*(5), 595-604.

441.Nelson, S.N., Van Der Kraak, G., (2010). The role of the insulin-like growth factor (IGF) system in zebrafish (Danio rerio) ovarian development. Gen. Comp. Endocrinol. 168, 103e110.

442.Neumann, G. M., Bach, L. A. (1999). The N-terminal disulfide linkages of human insulin-like growth factor-binding protein-6 (hIGFBP-6) and hIGFBP-1 are different as determined by mass spectrometry. Journal of Biological Chemistry, 274(21), 14587-14594.

443.Neuvians, T. P., Pfaffl, M. W., Berisha, B., Schams, D., (2003). The mRNA expression of the members of the IGF-system in bovine corpus luteum during induced luteolysis. *Domestic animal endocrinology*, *25*(4), 359-372.

444.Nie, M., Yu, S., Peng, S., Fang, Y., Wang, H., Yang, X., (2015). miR-23a and miR-27a promote human granulosa cell apoptosis by targeting SMAD5. Biol. Reprod. 93, 98.

445.Nilsson, E., Detzel, C., Skinner, K., (2006). Platelet-derived growth factor modulates the primordial to primary follicle transition. Reproduction 131, 1007–1015.

446.Nilsson, E., Kezele, P., Skinner, K. (2002). Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries. Molecular and cellular endocrinology, 188(1), 65-73.

447.Nilsson, E., Parrott, J.A., Skinner, M.K., (2001). Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. Mol. Cell. Endocrinol. 175, 123–130.

448.Nilsson, E., Skinner, K., (2003). Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. Biol. Reprod. 69, 1265–1272.

449.Nishi, Y., Takeshita, T., Sato, K., Araki, T., (2003). Change of the Mitochondrial Distribution in Mouse OoplasmDuring *In vitro* Maturation. J. Nippon Med. Sch. 70, 408–415.

450.Norris, R.P., Freudzon, M., Nikolaev, V.O., Jaffe, L.A., (2010). Epidermal growth factor receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to LH. Reproduction 140, 655–662.

451.Norris, R.P., Ratzan, W.J., Freudzon, M., Mehlmann, L.M., Krall, J., Movsesian, M.A., Wang, H., Ke, H., Nikolaev, V.O., Jaffe, L.A., (2009). Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. Development 136, 1869–1878.

452.Nuttinck, F., Charpigny, G., Mermillod, P., Loosfelt, H., Meduri, G., Freret, S., Heyman, Y., (2004). Expression of components of the insulin-like growth factor system and gonadotropin receptors in bovine cumulus–oocyte complexes during oocyte maturation. *Domestic animal endocrinology*, *27*(2), 179-195.

453.O'Donnell, J.B., Hill, J.L., Gross, D.J., (2004). Epidermal growth factor activates cytosolic [Ca2] elevations and subsequent membrane permeabilization in mouse cumulus-oocyte complexes. Reproduction 127, 207–220.

454.O'Neill C. (1997) Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos in vitro. Biol Reprod ; 56: 229–237.

455.Oh, J.S., Han, S.J., Conti, M., (2010). Wee1B, Myt1, and Cdc25 function in distinct compartments of the mouse oocyte to control meiotic resumption. J. Cell Biol. 188, 199–207.

456.Oh, Y., Gucev, Z., Ng, L., Müller, H.L., Rosenfeld, R.G., (1995). Antiproliferative

actions of insulin-like growth factor binding protein (IGFBP)-3 in human breast cancer cells. Prog. Growth Factor Res. 6, 503–512.

457.Okamura, H., Yamamura, T., Wakabayashi, Y. (2013). Kisspeptin as a master player in the central control of reproduction in mammals: an overview of kisspeptin research in domestic animals. Animal Science Journal, 84(5), 369-381.

458.Oktay, K., (1997). Ontogeny of Follicle-Stimulating Hormone Receptor Gene Expression in Isolated Human Ovarian Follicles. J. Clin. Endocrinol. Metab. 82, 3748–3751.

459.Okuda, K., Bowolaksono, A., Acosta, T. J., (2009). Anti-Apoptotic Roles of Luteinizing Hormone in Bovine Luteal Cells. *Biology of Reproduction*, *81*(Suppl 1), 511-511.

460.Oliver, J. E., Aitman, T. J., Powell, J. F., Wilson, C. A., Clayton, R. N., (1989). Insulin-like growth factor I gene expression in the rat ovary is confined to the granulosa cells of developing follicles. Endocrinology, 124(6), 2671-2679.

461.Orisaka, M., Tajima, K., Mizutani, T., Miyamoto, K., Tsang, B.K., Fukuda, S., Yoshida, Y., Kotsuji, F., (2006). Granulosa cells promote differentiation of cortical stromal cells into theca cells in the bovine ovary. Biol. Reprod. 75, 734–740.

462.Orsi, N.M., Ellissa Baskind, N., Cummings, M., (2014). Anatomy, Development, Histology, and Normal Function of the Ovary, in: Pathology of the Ovary, Fallopian Tube and Peritoneum. pp. 1–32.

463.Otoi, T., Fujii, M., Ooka, A., Tanaka, M., Suzuki, T., (1999). Effect of serum on the *in vitro* maturation of canine oocytes. Reprod. Fertil. Dev. 11, 387.

464.Palma, G.A., Muller, M., Brem, G., (1997). Effect of insulin-like growth factor I (IGF-I) at high concentrations on blastocyst development of bovine embryos produced *in vitro*. Reproduction 110, 347–353.

465.Pang, Y., Ge, W. (2002). Epidermal growth factor and TGFα promote zebrafish oocyte maturation *in vitro*: potential role of the ovarian activin regulatory system. *Endocrinology*, *143*(1), 47-54.

466.Pangas, Stephanie A., and Aleksandar Rajkovic. (2015). “Follicular Development.” In Knobil and Neill’s Physiology of Reproduction, 947–95. Elsevier.

467.Panigone, S., Hsieh, M., Fu, M., Persani, L., Conti, M., (2008). Luteinizing Hormone Signaling in Preovulatory Follicles Involves Early Activation of the Epidermal Growth Factor Receptor Pathway. Mol. Endocrinol. 22, 924–936.

468.Papadopulos, F., Spinelli, M., Valente, S., Foroni, L., Orrico, C., Alviano, F., Pasquinelli, G., (2007). Common tasks in microscopic and ultrastructural image analysis using ImageJ. Ultrastructural pathology, 31(6), 401-407.

469.Parborell, F., Pecci, A., Gonzalez, O., Vitale, A., Tesone, M., (2002). Effects of a Gonadotropin-Releasing Hormone Agonist on Rat Ovarian Follicle Apoptosis: Regulation by Epidermal Growth Factor and the Expression of Bcl-2-Related Genes 1. *Biology of reproduction*, *67*(2), 481-486.

470.Paria, B.C., Das, S.K., Andrews, G.K., Dey, S.K., (1993). Expression of the epidermal growth factor receptor gene is regulated in mouse blastocysts during delayed implantation. Proceedings of the National Academy of Sciences 90, 55–59.

471.Paria, B.C., Dey, S.K., (1990). Preimplantation embryo development *in vitro*: cooperative interactions among embryos and role of growth factors. Proceedings of the National Academy of Sciences 87, 4756–4760.

472.Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M., (2004). EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science 303: 682–684.

473.Parks, W.C., Wilson, C.L., López-Boado, Y.S., (2004). Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat. Rev. Immunol. 4, 617–629.

474.Parmer, T. G., Roberts, C. T., LeRoith, D., Adashi, E. Y., Khan, 2., Solan, N., Gibori, G., (1991). Expression, action, and steroidal regulation of insulin-like growth factor-I (IGF-I) and IGF-I receptor in the rat corpus luteum: their differential role in the two cell populations forming the corpus luteum. *Endocrinology*, *129*(6), 2924-2932.

475.Patiño, R., Yoshizaki, G., Thomas, P., Kagawa, H., (2001). Gonadotropic control of ovarian follicle maturation: the two-stage concept and its mechanisms. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129, 427–439.

476.Paul, S., Pramanick, K., Kundu, S., Bandyopadhyay, A., Mukherjee, D., (2009). Involvement of PI3 kinase and MAP kinase in IGF-1- and insulin-induced oocyte maturation in Cyprinuscarpio. Mol. Cell Endocrinol. 309, 93e100.

477.Paveli ´ CJ, Matijevi ´ CT, Knezevi ´ CJ, (2007). Biological and physiological aspects of action of insulin-like growth factor peptide family. Indian J. Med. Res. 125, 511–522.

478.Pawshe, C.H., Rao, K.B.C.A., Totey, S.M., (1998). Effect of insulin-like growth factor I and its interaction with gonadotropins on *in vitro* maturation and embryonic development, cell proliferation, and biosynthetic activity of cumulus-oocyte complexes and granulosa cells in buffalo. Mol. Reprod. Dev. 49, 277–285.

479.Pereira, A. F., Alcântara Neto, A.S., Albuquerque, E. S., Luciano, M. C. S., Teixeira, D. I. A., Freitas, V. J. F., Melo, L. M., (2012). Goat Oocyte Production by Standard or One-shot FSH Treatments and Quantitative Analysis of Transcripts for EGF Ligands and its Receptor after *In vitro* Maturation. Reproduction in domestic animals, 47(2), 244-251.

480.Perks, C. M., Peters, A. R., Wathes, D. C. (1999). Follicular and luteal expression of

insulin-like growth factors I and II and the type 1 IGF receptor in the bovine ovary. *Journal of reproduction and fertility*, *116*(1), 157-165.

481. Perks, C.M., Vernon, E.G., Rosendahl, A.H., Tonge, D., Holly, J.M.P., (2007). IGF-II and IGFBP-2 differentially regulate PTEN in human breast cancer cells. Oncogene 26, 5966–5972.
482. Pescador, N., Stocco, D.M., Murphy, B.D. (1999) Growth factor modulation of steroidogenic acute regulatory protein and luteinization in the pig ovary**.** *Biol Reprod.*; 60:1453–1461.
483. Peter, M., Dubuis, J.M., (2000). Transcription factors as regulators of steroidogenic P-450 enzymes. Eur. J. Clin. Invest. 30 Suppl 3, 14–20.
484. Peters, K. E., Bergfeld, E. G., Cupp, A. S., Kojima, F. N., Mariscal, V., Sanchez, T., Kittok, R. J. (1994). Luteinizing hormone has a role in development of fully functional corpora lutea (CL) but is not required to maintain CL function in heifers. *Biology of reproduction*, *51*(6), 1248-1254.
485. Phoenix CH., (1970) Guinea pigs. In: Hafez ESE (ed) Reproduction and breeding techniques for laboratory animals. Lea and Febiger, Philadelphia, p 244
486. Pickard, B., Dean, W., Engemann, S., Bergmann, K., Fuermann, M., Jung, M., Reis, A., Allen, N., Reik, W., Walter, J., (2001). Epigenetic targeting in the mouse zygote marks DNA for later methylation: a mechanism for maternal effects in development. Mech. Dev. 103, 35–47.
487. Pincus, G., Enzmann, E.V., (1934). Can Mammalian Eggs Undergo Normal Development *in vitro*? Proc. Natl. Acad. Sci. U. S. A. 20, 121–122.
488. Pisani, F., Antonini, S., Pocar, P., Ferrari, S., Brevini, A., Rhind, M., Gandolfi, F. (2008). Effects of pre-mating nutrition on mRNA levels of developmentally relevant genes in sheep oocytes and granulosa cells. Reproduction, 136(3), 303-312.
489. Poljicanin, A., Filipovic, N., Pusic, T. V., Soljic, V., Caric, A., Saraga-Babic, M., Vukojevic, K., (2015). Expression pattern of RAGE and IGF-1 in the human fetal ovary and ovarian serous carcinoma. *Acta histochemica*, *117*(4), 468-476.
490. Pollak, M., (2008). Insulin and insulin-like growth factor signaling in neoplasia. Nat. Rev. Cancer 8, 915–928.
491. Pool, T.B., (2004). Development of culture media for human assisted reproductive technology. Fertil. Steril. 81, 287–289.
492. Popesko, P., (1998). Atlas de anatomía topográfica de los animales domésticos.
493. Pozios, K.C., Ding, J., Degger, B., Upton, Z., Duan, C., (2001). IGFs stimulate zebrafish cell proliferation by activating MAP kinase and PI3-kinase-signaling pathways. Am. J. Physiol. Regul. Integr. Comp. Physiol. 280, R1230eR1239.

494.Pritt, S., (2012). Taxonomy and history. In: Suckow, M.A., Stevens, K.W., Wilson, R.P. (Eds.), The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents. Academic Press, Waltham, MA, pp. 563–574.

495.Prochazka, R., Kalab, P., Nagyova, E., (2003). Epidermal Growth Factor-Receptor Tyrosine Kinase Activity Regulates Expansion of Porcine Oocyte-Cumulus Cell Complexes In Vitro1. Biol. Reprod. 68, 797–803.

496.Prochazka, R., Srsen, V., Nagyova, E., Miyano, T. Flechon, J.E., (2000). Developmental regulation of effect of epidermal growth factor on porcine oocyte-cumulus cell complexes: nuclear maturation, expansion, and F-actin remodeling. *Mol. Reprod. Dev.* 56, 63–73.

497.Ptak, A., Kajta, M., Gregoraszczuk, E. L., (2004). Effect of growth hormone and insulin-like growth factor-I on spontaneous apoptosis in cultured luteal cells collected from early, mature, and regressing porcine corpora lutea. *Animal reproduction science*, *80*(3), 267-279.

498.Puche, J. E., Castilla-Cortázar, I. (2012). Human conditions of insulin-like growth factor-I (IGF-I) deficiency. Journal of translational medicine, 10(1), 224.

499.Purohit, G., (2001). *Effect of serum free media and growth factors on IVM, IVF and Intravaginal culture of buffalo oocytes* (Doctoral dissertation, Ph. D. Thesis, Rajasthan Agricultural University, Bikaner).

500.Purohit, G.N., Brady, M.S., Sharma, S.S., (2005). Influence of epidermal growth factor and insulin-like growth factor 1 on nuclear maturation and fertilization of buffalo cumulus oocyte complexes in serum free media and their subsequent development *in vitro*. Anim. Reprod. Sci. 87, 229–239.

501.Qin, X., Strong, D.D., Baylink, D.J., Mohan, S., (1998). Structure-function analysis of the human insulin-like growth factor binding protein-4. J. Biol. Chem. 273, 23509–23516.

502.Qu, J., Godin, P. A., Nisolle, M., Donnez, J., (2000). Distribution and epidermal growth factor receptor expression of primordial follicles in human ovarian tissue before and after cryopreservation. Human Reproduction, 15(2), 302-310.

503.Qu, J., Godin, P. A., Nisolle, M., Donnez, J., (2000). Expression of receptors for insulin-like growth factor-I and transforming growth factor-β in human follicles. *Molecular human reproduction*, *6*(2), 137-145.

504.Qu, P., Qing, S., Liu, R., Qin, H., Wang, W., Qiao, F., Wang, Y., (2017). Effects of embryo-derived exosomes on the development of bovine cloned embryos. *PloS one*, *12*(3), e0174535.

505.Quesenberry, K.E., Donnelly, T.M., Hillyer, E.V., (2004). Biology, husbandry, and clinical techniques of guinea pigs and chinchillas. In: Ferrets, Rabbits and Rodents:

Clinical Medicine and Surgery, 2nd ed., eds. K.E. Quesenberry, J.W. Carpenter. St. Louis, MO: Saunders Publishing, pp. 232–244, 437–444.

506. Quirk, S.M., Cowan, R.G., Harman, R.M., (2006). The susceptibility of granulosa cells to apoptosis is influenced by oestradiol and the cell cycle. J. Endocrinol. 189, 441–453.

507. Quirk, S.M., Harman, R.M., Cowan, R.G., (2000). Regulation of Fas antigen (Fas, CD95)-mediated apoptosis of bovine granulosa cells by serum and growth factors. Biol. Reprod. 63, 1278–1284.

508. Rajaram, S., Baylink, D.J., Mohan, S., (1997). Insulin-Like Growth Factor-Binding Proteins in Serum and Other Biological Fluids: Regulation and Functions1. Endocr. Rev. 18, 801–831.

509. Ramathal, C., Durruthy-Durruthy, J., Sukhwani, M., Arakaki, J. E., Turek, P. J., Orwig, K. E., Pera, R. A. R., (2014). Fate of iPSCs derived from azoospermic and fertile men following xenotransplantation to murine seminiferous tubules. *Cell reports*, *7*(4), 1284-1297.

510. Rappolee DA, Sturm KS, Behrendtsen O, Schultz GA, Pedersen RA, Werb Z., (1992): Insulin-like growth factor II acts through an endogenous growth pathway regulated by imprinting in early mouse embryos. Genes Dev 6(6):939–952

511. Rebouças, E. L., Costa, J. J., Passos, M. J., Silva, A. W., Rossi, R. O., van den Hurk, R., Silva, J. R., (2014). Expression levels of mRNA for insulin-like growth factors 1 and 2, IGF receptors and IGF binding proteins in *in vivo* and *in vitro* grown bovine follicles. *Zygote*, *22*(04), 521-532.

512. Rechler, M.M., (1993). Insulin-like Growth Factor Binding Proteins, in: Vitamins & Hormones. pp. 1–114.

513. Reddy, P., Shen, L., Ren, C., Boman, K., Lundin, E., Ottander, U., Lindgren, P., Liu, Y.-X., Sun, Q.-Y., Liu, K., (2005). Activation of Akt (PKB) and suppression of FKHRL1 in mouse and rat oocytes by stem cell factor during follicular activation and development. Dev. Biol. 281, 160–170.

514. Redmer, D.A., Doraiswamy, V., Bortnem, B.J., Fisher, K., Jablonka-Shariff, A., Grazul Bilska, A.T., Reynolds, L.P., (2001). Evidence for a role of capillary pericytes in vascular growth of the developing ovine corpus luteum. Biol. Reprod. 65, 879–889.

515. Reed, M., Hounslow, W.F., (1971). Induction of ovulation in the guinea-pig. J. Endocrinol. 49, 203–211.

516. Reed, M.L., Estrada, J.L., Illera, M.J., Petters, R.M., (1993). Effects of epidermal growth factor, insulin-like growth factor-I, and dialyzed porcine follicular fluid on porcine oocyte maturation *in vitro*. J. Exp. Zool. 266, 74–78.

517.Reeka, N., Berg, F. D., Brucker, C., (1998). Presence of transforming growth factor alpha and epidermal growth factor in human ovarian tissue and follicular fluid. *Human Reproduction*, *13*(8), 2199-2205.

518.Reynolds, L. P., Redmer, D. A., (1999). Growth and development of the corpus luteum. J. Reprod. Fertil. Supplement, 181–191.

519.Reynolds, L.P., Grazul-Bilska, A.T., Redmer, D.A., (2000). Angiogenesis in the Corpus Luteum. Endocrine 12, 1–10.

520.Rezaei, N., Ak, A.-J., (2003). Role of essential and non-essential amino acids contained in maturation medium on bovine oocyte maturation and subsequent fertilization and early embryonic development *in vitro*. Theriogenology 59, 497.

521.Richani, D., Sutton-McDowall, M.L., Frank, L.A., Gilchrist, R.B., Thompson, J.G., (2014). Effect of Epidermal Growth Factor-Like Peptides on the Metabolism of *In vitro*- Matured Mouse Oocytes and Cumulus Cells1. Biol. Reprod. 90.

522. Richards, J., (2007). Genetics of Ovulation. Semin. Reprod. Med. 25, 235–242.

523.Richards, J.S., Liu, Z., Shimada, M., (2015). Ovulation, in: Knobil and Neill's Physiology of Reproduction. pp. 997–1021.

524.Richards, J.S., Pangas, S.A., (2010). The ovary: basic biology and clinical implications. J. Clin. Invest. 120, 963–972.

525.Richards, J.S., Russell, D.L., Ochsner, S., Hsieh, M., Doyle, K.H., Falender, A.E., Lo, Y.K., Sharma, S.C., (2002). Novel signaling pathways that control ovarian follicular development, ovulation, and luteinization. Recent Prog. Horm. Res. 57, 195–220.

526.Rieder, C.L., Schultz, A., Cole, R., Sluder, G., (1994). Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. J. Cell Biol. 127, 1301–1310.

527.Risk, M., Gibori, G., (2001). Mechanisms of Luteal Cell Regulation by Prolactin, in: Endocrine Updates. pp. 265–295.

528.Ritter, L. J., Sugimura, S., Gilchrist, R. B. (2015). Oocyte induction of EGF responsiveness in somatic cells is associated with the acquisition of porcine oocyte developmental competence. *Endocrinology*, *156*(6), 2299-2312.

529.Rocca, M.S., Wehner, N.G., (2009). The guinea pig as an animal model for developmental and reproductive toxicology studies. Birth Defects Res. B Dev. Reprod. Toxicol. 86, 92–97.

530.Rodríguez, F.M., Salvetti, N.R., Colombero, M., Stangaferro, M.L., Barbeito, C.G., Ortega, H.H., Rey, F., (2013). Interaction between IGF1 and IGFBPs in bovine cystic ovarian disease. Anim. Reprod. Sci. 140, 14–25.

531.Rodríguez, F.M., Salvetti, N.R., Panzani, C.G., Barbeito, C.G., Ortega, H.H., Rey,

F., (2011). Influence of insulin-like growth factor-binding proteins-2 and -3 in the pathogenesis of cystic ovarian disease in cattle. Anim. Reprod. Sci. 128, 1–10.

532. Rose-Hellekant, T. A., Libersky-Williamson, E. A., Bavister, B. D. (1998). Energy substrates and amino acids provided during *in vitro* maturation of bovine oocytes alter acquisition of developmental competence. *Zygote*, *6*(04), 285-294.
533. Roth, R. A., Kiess, W. (1994). Insulin-like growth factor receptors: recent developments and new methodologies. Growth regulation, 4, 31-38.
534. Rowlands, I. W. (1949). Post-partum breeding in the guinea-pig. *Journal of Hygiene*, *47*(03), 281-287.
535. Rowlands, I.W., (1957). Insemination of the guinea-pig by intraperitoneal injection. J. Endocrinol. 16, 98–106.
536. Roy, S. K., Greenwald, G. S. (1990). Immunohistochemical localization of epidermal growth factor-like activity in the hamster ovary with a polyclonal antibody. Endocrinology, 126(3), 1309-1317.
537. Roy, S., Kole (1998). Ovarian transforming growth factor-beta (TGF-beta) receptors: in-vitro effects of follicle stimulating hormone, epidermal growth factor and TGF-beta on receptor expression in human preantral follicles. Mol. Hum. Reprod. 4, 207–214.
538. Roy, S.K., (1993). Epidermal Growth Factor and Transforming Growth Factor-β Modulation of Follicle-Stimulating Hormone-Induced Deoxyribonucleic Acid Synthesis in Hamster Preantral and Early Antral Follicles1. Biol. Reprod. 48, 552–557.
539. Roy, S.K., Greenwald, G.S., (1990). Immunohistochemical Localization of Epidermal Growth Factor-Like Activity in the Hamster Ovary with a Polyclonal Antibody*. Endocrinology 126, 1309–1317.
540. Russo, V.C., Schütt, B.S., Andaloro, E., Ymer, S.I., Hoeflich, A., Ranke, M.B., Bach, L.A., Werther, G.A., (2005). Insulin-Like Growth Factor Binding Protein-2 Binding to Extracellular Matrix Plays a Critical Role in Neuroblastoma Cell Proliferation, Migration, and Invasion. Endocrinology 146, 4445–4455.
541. Sadeu, J.C., Adriaens, I., Cortvrindt, R., Smitz, J., (2007). Study of folliculogenesis *in vivo* in guinea pig. Theriogenology 68, 1228–1239.
542. Saeki, K., Hoshi, M., Leibfried-Rutledge, M.L., First, N.L., (1991). *In vitro* Fertilization and Development of Bovine Oocytes Matured in Serum-Free Medium1. Biol. Reprod. 44, 256–260.
543. Sakaguchi, M., Dominko, T., Leibfried Rutledge, M.L., Nagai, T., First, N.L., (2000). A combination of EGF and IGF-1 accelerates the progression of meiosis in bovine follicular oocytes *in vitro* and fetal calf serum eutralizes the acceleration effect. Theriogenology 54 (8), 1327–1342.

544.Sakaguchi, M., Dominko, T., Yamauchi, N., Leibfried Rutledge, M.L., Nagai, T. First, N.L., (2002). Possible mechanism for acceleration of meiotic progression of bovine follicular oocytes by growth factors *in vitro. Reproduction*123, 135–42

545.Samaras, S. E., Guthrie, H. D., Barber, J. A., Hammond, J. M., (1993). Expression of the mRNAs for the insulin-like growth factors and their binding proteins during development of porcine ovarian follicles. *Endocrinology*, *133*(5), 2395-2398.

546.Samoto T, Maruo T, Matsuo H, Katayama K, Barnea ER, Mochizuki M., (1993). Altered expression of insulin and insulin-like growth factor-I receptors in follicular and stromal compartments of polycystic ovaries. *Endocrine journal*, *40*(4), 413-424.

547.San Roman, G. A., Magoffin, D. A., (1993). Insulin-like growth factor-binding proteins in healthy and atretic follicles during natural menstrual cycles. *The Journal of Clinical Endocrinology & Metabolism*, *76*(3), 625-632.

548.Santamaría, D., Barrière, C., Cerqueira, A., Hunt, S., Tardy, C., Newton, K., Cáceres, J.F., Dubus, P., Malumbres, M., Barbacid, M., (2007). Cdk1 is sufficient to drive the mammalian cell cycle. Nature 448, 811–815.

549.Santos Navarrete, A., Ramin, N., Tonack, S., Fischer, B., (2008). Cell lineage-specific signaling of insulin and insulin-like growth factor I in rabbit blastocysts. *Endocrinology*, *149*(2), 515-524.

550.Santos, L.P., Barros, V., Cavalcante, A., Menezes, V.G., Macedo, T., Santos, J., Araújo, V.R., Queiroz, M., Matos, M., (2014). Protein Localization of Epidermal Growth Factor in Sheep Ovaries and Improvement of Follicle Survival and Antrum Formation *In vitro*. Reprod. Domest. Anim. 49, 783–789.

551.Sathananthan, A.H., Ng, S.C., Chia, C.M., Law, H.Y., Edirisinghe, W.R., Ratnam, S.S., (1985). The Origin and Distribution of Cortical Granules in Human Oocytes with Reference to Golgi, Nucleolar, and Microfilament Activity. Ann. N. Y. Acad. Sci. 442, 251–264.

552.Satrapa, R. A., Castilho, A. S., Razza, E. M., Pegorer, M. F., Puelker, R., & Barros, C. M., (2013). Differential expression of members of the IGF system in OPU-derived oocytes from Nelore (Bos indicus) and Holstein (Bos taurus) cows. *Animal reproduction science*, *138*(3), 155-158.

553.Sauerwein, H., Miyamoto, A., Günther, J., Meyer, H. H. D., Schams, D., (1992). Binding and action of insulin-like growth factors and insulin in bovine luteal tissue during the oestrous cycle. *Journal of reproduction and fertility*, *96*(1), 103-115.

554.Sayre, B. L., Taft, R., Inskeep, E. K., Killefer, J., (2000). Increased Expression of Insulin-Like Growth Factor Binding Protein-1 During Induced Regression of Bovine Corpora Lutea 1. *Biology of reproduction*, *63*(1), 21-29.

555.Scaramuzzi, R. J., Baird, D. T., Campbell, B. K., Driancourt, M. A., Dupont, J., Fortune, J. E., Monget, P., (2011). Regulation of folliculogenesis and the determination of ovulation rate in ruminants. *Reproduction, Fertility and Development*, *23*(3), 444-467.

556.Schams, D., Berisha, B., Kosmann, M., Amselgruber, W.M., (2002). Expression and localization of IGF family members in bovine antral follicles during final growth and in luteal tissue during different stages of estrous cycle and pregnancy. Domest. Anim. Endocrinol. 22, 51–72.

557.Schams, D., Berisha, B., Kosmann, M., Einspanier, R., Amselgruber, W.M., (1999). Possible role of growth hormone, IGFs, and IGF-binding proteins in the regulation of ovarian function in large farm animals. Domest. Anim. Endocrinol. 17, 279–285.

558.Schams, D., Koll, R., Li, C.H., (1988). Insulin-like growth factor-I stimulates oxytocin and progesterone production by bovine granulosa cells in culture. J. Endocrinol. 116, 97–100.

559.Schedlich, L.J., Le Page, S.L., Firth, S.M., Briggs, L.J., Jans, D.A., Baxter, R.C., (2000). Nuclear import of insulin-like growth factor-binding protein-3 and -5 is mediated by the importin beta subunit. J. Biol. Chem. 275, 23462–23470.

560.Schon, E.A., Kim, S.H., Ferreira, J.C., Magalhaes, P., Grace, M., Warburton, D., Gross, S.J., (2000). Chromosomal non-disjunction in human oocytes: is there a mitochondrial connection? Hum. Reprod. 15, 160–172.

561.Schultz, G.A., Hahnel, A., Arcellana-Panlilio, M., Wang, L., Goubau, S., Watson, A., Harvey, M., (1993). Expression of IGF ligand and receptor genes during preimplantation mammalian development. Mol. Reprod. Dev. 35, 414–420.

562.Schultz, G.A., Hogan, A., Watson, A.J., Smith, R.M., Heyner, S., (1992). Insulin, insulin-like growth factors and glucose transporters: temporal patterns of gene expression in early murine and bovine embryos. Reprod. Fertil. Dev. 4, 361.

563.Senior, P.V., Byrne, S., Brammar, W.J., Beck, F., (1990). Expression of the IGF-II/mannose-6-phosphate receptor mRNA and protein in the developing rat. Development 109, 67–73.

564.Seppälä, M., Wahlsträm, T., Koskimies, A.I., Tenhunen, A., Rutanen, E.-M., Koistinen, R., Huhtaniemi, I., Bohn, H., Stenman, U.-H., (1984). Human Preovulatory Follicular Fluid, Luteinized Cells of Hyperstimulated Preovulatory Follicles, and Corpus Luteum Contain Placental Protein 12*. J. Clin. Endocrinol. Metab. 58, 505–510.

565.Sha, W., Xu, B.-Z., Li, M., Liu, D., Feng, H.L., Sun, Q.Y., (2010). Effect of gonadotropins on oocyte maturation *in vitro*: an animal model. Fertil. Steril. 93, 1650–1661.

566.Shabankareh, H. K., Zandi, M., (2010). Developmental potential of sheep oocytes cultured in different maturation media: effects of epidermal growth factor, insulin-like growth factor I, and cysteamine. *Fertility and sterility*, *94*(1), 335-340.

567.Shahedi, A., Khalili, M.A., Soleimani, M., Morshedizad, S., (2013). Ultrastructure of *in vitro* Matured Human Oocytes. Iran. Red Crescent Med. J. 15, e7379.

568.Sharma K and Batra, S., (2005). Ultrastructure of the regressing corpus luteum in the goat ovary. Indian J. Anim. Sci. 75, 936–937.

569.Sharma RK, A. Bhardwaj .J.K., (2007). Granulosa cell apoptosis in situ in caprine ovary. J. Cell Tissue Res 7, 1111–1114.

570.Sharma, G. T., Dubey, P. K., Kumar, G. S., (2010). Effects of IGF-1, TGF-α plus TGF-β 1 and bFGF on *in vitro* survival, growth and apoptosis in FSH-stimulated buffalo (Bubalis bubalus) preantral follicles. *Growth Hormone & IGF Research*, *20*(4), 319-325.

571.Sharma, K., Bhardwaj, K. (2009). Ultrastructural characterization of apoptotic granulosa cells in caprine ovary. Journal of microscopy, 236(3), 236-242.

572.Sharma, R.K., (2003). Structural analysis of cumulus and corona cells of goat antral follicles: possible functional significance. Indian J. Anim. Sci. 73, 28–32.

573.Shi, F., Ozawa, M., Komura, H., Watanabe, G., Tsonis, C., Suzuki, A., Taya, K., (2000). Induction of superovulation by inhibin vaccine in cyclic guinea-pigs. Reproduction 118, 1–7.

574.Shimada, M., Hernandez-Gonzalez, I., Gonzalez-Robanya, I., Richards, J. S., (2006). Induced expression of pattern recognition receptors in cumulus oocyte complexes: novel evidence for innate immune-like functions during ovulation. *Molecular Endocrinology*, *20*(12), 3228-3239.

575.Shimasaki, S., and Ling, N., (1991). Identification and molecular characterization of insulin-like growth factor binding proteins (IGFBP-1,-2,-3,-4,-5 and-6). *Progress in growth factor research*, *3*(4), 243-266.

576.Shimasaki, S., Shimonaka, M., Ui, M., Inouye, S., Shibata, F., Ling, N., (1990). Structural characterization of a follicle-stimulating hormone action inhibitor in porcine ovarian follicular fluid. Its identification as the insulin-like growth factor-binding protein. J. Biol. Chem. 265, 2198–2202.

577.Shimonaka, M., Schroeder, R., Shimasaki, S., Ling, N., (1989). Identification of a novel binding protein for insulin-like growth factors in adult rat serum. Biochem. Biophys. Res. Commun. 165, 189–195.

578.Shirazi, A., Shams-Esfandabadi, N., Hosseini, S.M., (2005). A comparison of two recovery methods of ovine oocytes for *in vitro* maturation. Small Rumin. Res. 58, 283–286.

579.Shores, E., Picton, H., Hunter, M., (2000). Differential regulation of pig theca cell steroidogenesis by LH, insulin-like growth factor I and granulosa cells in serum-free culture. Reproduction 118, 211–219.

580.Shoubridge, E.A., Wai, T., (2007). Mitochondrial DNA and the Mammalian Oocyte, in: Current Topics in Developmental Biology. pp. 87–111.

581.Silva, C.M.G., Castro, S.V., Faustino, L.R., Rodrigues, G.Q., Brito, I.R., Rossetto, R., Saraiva, M.V.A., Campello, C.C., Lobo, C.H., Souza, C.E.A., Moura, A.A.A., Donato, M.A.M., Peixoto, C.A., Figueiredo, J.R., (2013). The effects of epidermal growth factor (EGF) on the in vitro development of isolated goat secondary follicles and the relative mRNA expression of EGF, EGF-R, FSH-R and P450 aromatase in cultured follicles. Res. Vet. Sci. 94, 453–461.

582.Silva, J.R.V., Figueiredo, J.R., van den Hurk, R., (2009). Involvement of growth hormone (GH) and insulin-like growth factor (IGF) system in ovarian folliculogenesis. Theriogenology 71, 1193–1208.

583.Silva, J.R.V., van den Hurk, R., Figueiredo, J.R., (2006). Expression of mRNA and protein localization of epidermal growth factor and its receptor in goat ovaries. Zygote 14, 107–117.

584.Silva, J.R.V., Van den Hurk, R., Matos, M.H.T., Santos, R.R., Pessoa, C., Moraes, M.O. Figueiredo, J.R., (2004). Influences of FSH and EGF on primordial follicles during *in vitro* culture of caprine ovarian cortical tissue. *Theriogenology*61, 1691–704.

585.Silverman, S., Tell, L.A., Nugent-Deal, J., Palmer-Holtry, K., (2005). Domestic Guinea Pig (Cavia porcellus), in: Radiology of Rodents, Rabbits, and Ferrets. pp. 105–157.

586.Simmen, R.C.M., Ko, Y., Simmen, F.A., (1993). Insulin-like growth factors and blastocyst development. Theriogenology 39, 163–175.

587.Simon, A.M., Goodenough, D.A., Li, E., Paul, D.L., (1997). Female infertility in mice lacking connexin 37. Nature 385, 525–529.

588.Singh, B., Meng, L., Rutledge, J.M. Armstrong, D.T., (1997). Effects of epidermal growth factor and follicle-stimulating hormone during *in vitro* maturation on cytoplasmic maturation of porcine oocytes. *Mol. Reprod. Dev.* 46, 401–7.

589.Singh, B., Rutledge, J.M., Armstrong, D.T., (1995). Epidermal growth factor and its receptor gene expression and peptide localization in porcine ovarian follicles. Mol. Reprod. Dev. 40, 391–399.

590.Singh, J., Paul, A., Thakur, N., Yadav, V.P., Panda, R.P., Bhure, S.K., Sarkar, M., (2015). Localization of IGF proteins in various stages of ovarian follicular development and modulatory role of IGF-I on granulosa cell steroid production in

water buffalo (Bubalus bubalis). Anim. Reprod. Sci. 158, 31–52.

591.Sirard, M. A., Florman, H. M., Leibfried-Rutledge, M. L., Barnes, F. L., Sims, M. L., First, N. L. (1989). Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. Biology of reproduction, 40(6), 1257-1263.

592.Sirard, M.-A., Richard, F., Blondin, P., Robert, C., (2006). Contribution of the oocyte to embryo quality. Theriogenology 65, 126–136.

593.Sirisathien, S., Hernandez-Fonseca, H. J., Brackett, B. G., (2003). Influences of epidermal growth factor and insulin-like growth factor-I on bovine blastocyst development *in vitro*. *Animal reproduction science*, *77*(1), 21-32.

594.Sirotkin AV, Makarevich AV, Grossmann R, Kotwica J, Schaeffer H-J, Marnet P-G, Kwon H, Sanislo P, Florkovicova I, Petrak J, Rafal J, Pivko J, Hetenyi L., (2003). Regulation and evaluation of ovarian function and embryogenesis in normal and transgenic animals *in vitro* and *in vivo*. Research Institute of Animal Production, Nitra, Slovakia.

595.Sirotkin, A.V., Lauková, M., Ovcharenko, D., Brenaut, P., Mlyncek, M., (2010). Identification of microRNAs controlling human ovarian cell proliferation and apoptosis. J. Cell. Physiol. 223, 49–56.

596.Sisk DB., (1976). Physiology. In: The Biology of the Guinea Pig. Eds. JE Wagner, PJ Manning, New York: Academic Press, pp. 63-98.

597.Skinner, M. K., Coffey JR, R. J., (1988). Regulation of ovarian cell growth through the local production of transforming growth factor-α by theca cells. *Endocrinology*, *123*(6), 2632-2638.

598.Skinner, M.K., Lobb, D., Dorrington, J.H., (1987). Ovarian Thecal/Interstitial Cells Produce an Epidermal Growth Factor-Like Substance*. Endocrinology 121, 1892–1899.

599.Smith, M.F., McIntush, E.W., Smith, G.W., (1994). Mechanisms associated with corpus luteum development. J. Anim. Sci. 72, 1857–1872.

600.Smith, R. M., Garside, W. T., Aghayan, M., Shi, C. Z., Shah, N., Jarett, L., Heyner, S., (1993). Mouse preimplantation embryos exhibit receptor-mediated binding and transcytosis of maternal insulin-like growth factor I. *Biology of reproduction*, *49*(1), 1-12.

601.Smitz, J., Cortvrindt, R., Hu, Y., 1998. Epidermal growth factor combined with recombinant human chorionic gonadotrophin improves meiotic progression in mouse follicle-enclosed oocyte culture. Hum. Reprod. 13, 664–669.

602.Song, H. J., Kang, E. J., Maeng, G. H., Ock, S. A., Lee, S. L., Yoo, J. G., Rho, G. J., (2011). Influence of epidermal growth factor supplementation during *in vitro*

maturation on nuclear status and gene expression of canine oocytes. *Research in veterinary science*, *91*(3), 439-445.

603. Spicer, L. J., Chamberlain, C. S., (1999). Insulin-like growth factor binding protein-3: its biological effect on bovine granulosa cells. *Domestic animal endocrinology*, *16*(1), 19-29.
604. Spicer, L. J., Stewart, R. E., Alvarez, P., Francisco, C. C., Keefer, B. E., (1997). Insulin-like growth factor-binding protein-2 and-3: their biological effects in bovine thecal cells. *Biology of reproduction*, *56*(6), 1458-1465.
605. Spicer, L.J., (2004). Proteolytic degradation of insulin-like growth factor binding proteins by ovarian follicles: a control mechanism for selection of dominant follicles. Biol. Reprod. 70, 1223–1230.
606. Spicer, L.J., Chamberlain, C.S., (2000). Production of insulin-like growth factor-I by granulosa cells but not thecal cells is hormonally responsive in cattle. J. Anim. Sci. 78, 2919.
607. Spicer, L.J., Echternkamp, S.E., (1995). The ovarian insulin and insulin-like growth factor system with an emphasis on domestic animals. Domest. Anim. Endocrinol. 12, 223–245.
608. St-Arnaud, R., Walker, P., Kelly, P.A., Labrie, F., (1983). Rat ovarian epidermal growth factor receptors: characterization and hormonal regulation. Mol. Cell. Endocrinol. 31, 43–52.
609. Stewart, R.E., Spicer, L.J., Hamilton, T.D., Keefer, B.E., Dawson, L.J., Morgan, G.L., Echternkamp, S.E., (1996). Levels of insulin-like growth factor (IGF) binding proteins, luteinizing hormone and IGF-I receptors, and steroids in dominant follicles during the first follicular wave in cattle exhibiting regular estrous cycles. Endocrinology 137, 2842–2850.
610. Stocco, C., Telleria, C., Gibori, G., (2007). The Molecular Control of Corpus Luteum Formation, Function, and Regression. Endocr. Rev. 28, 117–149.
611. Stocco, D.M., (2001). Tracking the Role of a StAR in the Sky of the New Millennium. Mol. Endocrinol. 15, 1245–1254.
612. Stockard, C.R., Papanicolaou, G.N., (1917). The existence of a typical oestrous cycle in the guinea-pig with a study of its histological and physiological changes. Am. J. Anat. 22, 225–283.
613. Stojkovic, M., Machado, S.A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Gonçalves, P.B., Wolf, E., (2001). Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after *in vitro* maturation: correlation with morphological criteria and developmental capacity after *in vitro* fertilization and culture. Biol. Reprod. 64, 904–909.

614.Storlazzi, A., (2003). Meiotic double-strand breaks at the interface of chromosome movement, chromosome remodeling, and reductional division. Genes Dev. 17, 2675–2687.

615.Stouffer RL, Hennebold JD., (2014). Structure, function and regulation of the corpus luteum. In: Plant TM, Zeleznik AJ, editors. Knobil and Neill's physiology of reproduction, 3rd edn. San Diego: Elsevier Academic Press, p. 1703–26.

616.Strowitzki, T., (2013). *In vitro* Maturation (IVM) of human oocytes. Arch. Gynecol. Obstet. 288, 971–975.

617.Stubbs, S. A., Webber, L. J., Stark, J., Rice, S., Margara, R., Lavery, S., Franks, S., (2013). Role of Insulin-like growth factors in initiation of follicle growth in normal and polycystic human ovaries. *The Journal of Clinical Endocrinology & Metabolism*, *98*(8), 3298-3305.

618.Su, Y. Q., Sugiura, K., Li, Q., Wigglesworth, K., Matzuk, M. M., Eppig, J. J., (2010). Mouse oocytes enable LH-induced maturation of the cumulus-oocyte complex via promoting EGF receptor-dependent signaling. Molecular Endocrinology, 24(6), 1230-1239.

619.Sueoka N, Lee HY, Wiehle S, Cristiano RJ, Fang B, Ji L, Roth JA, Hong WK, Cohen P, Kurie JM. (2000). Insulin-like growth factor binding protein-6 activates programmed cell death in non-small cell lung cancer cells. Oncogene 19:4432–4436

620.Sugimura, S., Ritter, L.J., Rose, R.D., Thompson, J.G., Smitz, J., Mottershead, D.G., Gilchrist, R.B., (2015). Promotion of EGF receptor signaling improves the quality of low developmental competence oocytes. Dev. Biol. 403, 139–149.

621.Sugino, N., Suzuki, T., Kashida, S., Karube, A., Takiguchi, S., Kato, H., (2000). Expression of Bcl-2 and Bax in the Human Corpus Luteum during the Menstrual Cycle and in Early Pregnancy: Regulation by Human Chorionic Gonadotropin1. J. Clin. Endocrinol. Metab. 85, 4379–4386.

622.Sugino, N., Suzuki, T., Sakata, A., Miwa, I., Asada, H., Taketani, T., Yamagata, Y., Tamura, H., (2005). Angiogenesis in the Human Corpus Luteum: Changes in Expression of Angiopoietins in the Corpus Luteum throughout the Menstrual Cycle and in Early Pregnancy. J. Clin. Endocrinol. Metab. 90, 6141–6148.

623.Suikkari, A. M., Ruutiainen, K., Erkkola, R., Sepälä, M., (1989). Low levels of low molecular weight insulin-like growth factor-binding protein in patients with polycystic ovarian disease. *Human Reproduction*, *4*(2), 136-139.

624.Summy, J.M., Gallick, G.E., (2006). Treatment for advanced tumors: SRC reclaims center stage. Clin. Cancer Res. 12, 1398–1401.

625.Sun, Q.Y., Wu, G.M., Lai, L., Park, K.W., Cabot, R., Cheong, H.T., Day, B.N., Prather, R.S., Schatten, H., (2001). Translocation of active mitochondria during pig

oocyte maturation, fertilization and early embryo development *in vitro*. Reproduction 122, 155–163.

626. Sun, S.Y., Zhang, W., Han, X., Huang, R.H., Shi, F.X., (2014). Cell proliferation and apoptosis in the fetal and neonatal ovary of guinea pigs. Genet. Mol. Res. 13, 1570–1578.

627. Sun, X., Su, Y., He, Y., Zhang, J., Liu, W., Zhang, H., Hou, Z., Liu, J., Li, J., (2015). New strategy for *in vitro* activation of primordial follicles with mTOR and PI3K stimulators. Cell Cycle 14, 721–731.

628. Suzuki, O., Koura, M., Noguchi, Y., Takano, K., Yamamoto, Y., Matsuda, J., (2003). Optimization of superovulation induction by human menopausal gonadotropin in guinea pigs based on follicular waves and FSH-receptor homologies. *Molecular reproduction and development*, *64*(2), 219-225.

629. Suzuki, O., Ogura, A., Asano, T., Noguchi, Y., Yamamoto, Y., Oike, M., (1993). Development of preimplantation guinea-pig embryos in serum-free media. *Reproduction, fertility and development*, *5*(4), 425-432.

630. Taft, R.A., Denegre, J.M., Pendola, F.L., Eppig, J.J., (2002). Identification of genes encoding mouse oocyte secretory and transmembrane proteins by a signal sequence trap. Biol. Reprod. 67, 953–960.

631. Takaoka, M., Smith, C.E., Mashiba, M.K., Okawa, T., Andl, C.D., El-Deiry, W.S., Nakagawa, H., (2006). EGF-mediated regulation of IGFBP-3 determines esophageal epithelial cellular response to IGF-I. Am. J. Physiol. Gastrointest. Liver Physiol. 290, G404–16.

632. Tamura, M., Sasano, H., Suzuki, T., Fukaya, T., Funayama, Y., Takayama, K., Takaya, R., Yajima, A., (1995). Expression of epidermal growth factors and epidermal growth factor receptor in normal cycling human ovaries. Hum. Reprod. 10, 1891–1896.

633. Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M., de Kruif, A., (2002). Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Mol. Reprod. Dev. 61, 414–424.

634. Tankimanova, M., Hultén, M.A., Tease, C., (2004). The initiation of homologous chromosome synapsis in mouse fetal oocytes is not directly driven by centromere and telomere clustering in the bouquet. Cytogenet. Genome Res. 105, 172–181.

635. Tauboll, E., Gregoraszczuk, E.L., Kołodziej, A., Kajta, M., Ropstad, E., (2003). Valproate inhibits the conversion of testosterone to estradiol and acts as an apoptotic agent in growing porcine ovarian follicular cells. Epilepsia 44, 1014–1021.

636. Tazuke, S., Giudice, L., (1996). Growth Factors and Cytokines in Endometrium, Embryonic Development, and Maternal: Embryonic Interactions. Semin. Reprod.

Med. 14, 231–245.

637.Tekpetey, F.R., Singh, B., Barbe, G. Armstrong, D.T., (1995). Localisation of epidermal growth factor (EGF) receptor in the rat corpus luteum, and EGF and transforming growth factor-alpha stimulation of luteal cell steroidogenesis *in vitro*. Mol. Cell. Endocrinol. 110, 95–102

638.Tesarik, J., Mendoza, C. (1995). Nongenomic effects of 17 beta-estradiol on maturing human oocytes: relationship to oocyte developmental potential. *The Journal of Clinical Endocrinology & Metabolism*, *80*(4), 1438-1443.

639.Thammasiri, J., Navanukraw, C., Jaikan, W., Moonmanee, T., Khanthusaeng, V., Boonkong, S., Uopasai, S., (2016). Corpora lutea growth and development in thai-native goats throughout the estrous cycle. *Chiang mai university journal*, *11*(S01

640.Thierry van Dessel, H.J., Chandrasekher, Y., Yap, O.W., Lee, P.D., Hintz, R.L., Faessen, G.H., Braat, D.D., Fauser, B.C., Giudice, L.C., (1996). Serum and follicular fluid levels of insulin-like growth factor I (IGF-I), IGF-II, and IGF-binding protein-1 and -3 during the normal menstrual cycle. J. Clin. Endocrinol. Metab. 81, 1224–1231.

641.Thongkittidilok, C., Tharasanit, T., Songsasen, N., Sananmuang, T., Buarpung, S., Techakumphu, M., (2015). Epidermal growth factor improves developmental competence and embryonic quality of singly cultured domestic cat embryos. *Journal of Reproduction and Development*, *61*(4), 269-276.

642.Threadgill DW, Dlugosz AA, Hansen LA, Tennenbaum T, Lichti U, Yee D, LaMantia C, Mourton T, Herrup K, Harris RC., (1995) Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science 269 230–234

643.Tilly, J.L., (1996). Apoptosis and ovarian function. Rev. Reprod. 1, 162–172.

644.Tilly, J.L., Tilly, K.I., Perez, G.I., (1997). The genes of cell death and cellular susceptibility to apoptosis in the ovary: a hypothesis. Cell Death Differ. 4, 180–187.

645.Torner, H., Alm, H., Kanitz, W., Goellnitz, K., Becker, F., Poehland, R., Bruessow, K.-P., Tuchscherer, A., (2007). Effect of initial cumulus morphology on meiotic dynamic and status of mitochondria in horse oocytes during IVM. Reprod. Domest. Anim. 42, 176–183.

646.Torner, H., Brüssow, K.-P., Alm, H., Ratky, J., Pöhland, R., Tuchscherer, A., Kanitz, W., (2004). Mitochondrial aggregation patterns and activity in porcine oocytes and apoptosis in surrounding cumulus cells depends on the stage of pre-ovulatory maturation. Theriogenology 61, 1675–1689.

647.Torng, P.-L., Lin, C.-W., Chan, M.W.Y., Yang, H.-W., Huang, S.-C., Lin, C.-T., (2009). Promoter methylation of IGFBP-3 and p53 expression in ovarian endometrioid carcinoma. Mol. Cancer 8, 120.

648.Tosti, E., Boni, R., Cuomo, A., (2002). Fertilization and activation currents in bovine oocytes. Reproduction 124, 835–846.

649.Totey, S.M., Singh, G., Taneja, M., Pawshe, C.H., Talwar, G.P., (1992). *In vitro* maturation, fertilization and development of follicular oocytes from buffalo (Bubalus bubalis). J. Reprod. Fertil. 95, 597–607.

650.Tripathy, S., Asaithambi, K., Jayaram, P., Medhamurthy, R., (2016). Analysis of 17β-estradiol (E 2) role in the regulation of corpus luteum function in pregnant rats: Involvement of IGFBP5 in the E 2-mediated actions. *Reproductive Biology and Endocrinology*, *14*(1), 19.

651.Trounson, A., Anderiesz, C., Jones, G.M., Kausche, A., Lolatgis, N., Wood, C., (1998). Oocyte maturation. Hum. Reprod. 13, 52–62.

652.Tsafriri, A., Cao, X., Ashkenazi, H., Motola, S., Popliker, M., Pomerantz, S. H., (2005). Resumption of oocyte meiosis in mammals: on models, meiosis activating sterols, steroids and EGF-like factors. Molecular and cellularendocrinology, 234(1), 37-45.

653.Tsuchiya, M., Menegishi, T., Kishi, H., Tano, M., Kameda, T., Hirakawa, T., Ibuki, Y., Mizutani, T. Miyamoto, K., (1999). Control of the expression of leuteinizing hormone receptor by local factors in rat granulosa cells. Arch. Biochem. Biophys. 367, 185–92.

654.Uhlendorf, B., Kaufmann, P., (1979). Development of the placental stalk in the guinea pig. Anat. Histol. Embryol. 8, 233–237.

655.Uhm, S.J., Chung, H.M., Kim, S.W., Kim, N.-H., Lee, H.T., Chung, K.S., (1999). Expression of epidermal growth factor receptor in porcine oocytes and preimplantation embryos. Theriogenology 51, 258.

656.Ullah, G., Jung, P., Machaca, K., (2008). Modeling Ca2 signaling differentiation during oocyte maturation. Cell Calcium 42, 556–564.

657.Uniyal, S., Panda, R.P., Chouhan, V.S., Yadav, V.P., Hyder, I., Dangi, S.S., Gupta, M., Khan, F.A., Sharma, G.T., Bag, S., Sarkar, M., (2015). Expression and localization of insulin-like growth factor system in corpus luteum during different stages of estrous cycle in water buffaloes (Bubalus bubalis) and the effect of insulin-like growth factor I on production of vascular endothelial growth factor and progesterone in luteal cells cultured *in vitro*. Theriogenology 83, 58–77.

658.Valentini, L., Iorga, A.I., De Santis, T., Ambruosi, B., Reynaud, K., Chastant-Maillard, S., Guaricci, A.C., Caira, M., Dell'Aquila, M.E., (2010). Mitochondrial distribution patterns in canine oocytes as related to the reproductive cycle stage. Anim. Reprod. Sci. 117, 166–177.

659.Valentinis, B., Bhala, A., DeAngelis, T., Baserga, R., Cohen, P., (1995). The human

insulin-like growth factor (IGF) binding protein-3 inhibits the growth of fibroblasts with a targeted disruption of the IGF-I receptor gene. Mol. Endocrinol. 9, 361–367.

660. Van Blerkom, J., (2004). Mitochondria in human oogenesis and preimplantation embryogenesis: engines of metabolism, ionic regulation and developmental competence. Reproduction 128, 269–280.

661. Van Blerkom, J., (2011). Mitochondrial function in the human oocyte and embryo and their role in developmental competence. Mitochondrion 11, 797–813.

662. Van Blerkom, J., Davis, P.W., (1998). DNA strand breaks and phosphatidylserine redistribution in newly ovulated and cultured mouse and human oocytes: occurrence and relationship to apoptosis. Hum. Reprod. 13, 1317–1324.

663. Van Blerkom, J., Runner, M.N., (1984). Mitochondrial reorganization during resumption of arrested meiosis in the mouse oocyte. Am. J. Anat. 171, 335–355.

664. Van den Beld, A. W., Bots, M. L., Janssen, J. A. M. L. L., Pols, H. A. P., Lamberts, S. W. J., Grobbee, D. E. (2003). Endogenous hormones and carotid atherosclerosis in elderly men. American journal of epidemiology, 157(1), 25-31.

665. Vandehaar, M. J., Sharma, B. K., Fogwell, R. L. (1995). Effect of Dietary Energy Restriction on the Expression of Insulin-like Growth Factor-I in Liver and Corpus Luteum of Heifers1. *Journal of dairy science*, *78*(4), 832-841.

666. Velazquez, M.A., Zaraza, J., Oropeza, A., Webb, R., Niemann, H., (2009). The role of IGF1 in the *in vivo* production of bovine embryos from superovulated donors. Reproduction 137, 161–180.

667. Vendola, K., Zhou, J., Wang, J., Famuyiwa, O.A., Bievre, M., Bondy, C.A., (1999). Androgens Promote Oocyte Insulin-Like Growth Factor I Expression and Initiation of Follicle Development in the Primate Ovary1. Biol. Reprod. 61, 353–357.

668. Verkeste, C.M., Slangen, B.F., Daemen, M., van Straaten, H., Kohnen, G., Kaufmann, P., Peeters, L.L., (1998). The extent of trophoblast invasion in the preplacental vasculature of the guinea-pig. Placenta 19, 49–54.

669. Vikash C, Saikumar G, Sharma GT., (2011) Temporal expression pattern of insulin-like growth factors (IGF-1 and IGF-2) ligands and their receptors (IGF-1R and IGF-2R) in buffalo (Bubalus bubalis) embryos produced *in vitro*. Livestock Sci 2011; 135:225–230.

670. Vivanco, I., Sawyers, C.L., (2002). The phosphatidylinositol 3-Kinase–AKT pathway in human cancer. Nat. Rev. Cancer 2, 489–501.

671. Vlodavsky, I., Brown, K. D., Gospodarowicz, D., (1978). A comparison of the binding of epidermal growth factor to cultured granulosa and luteal cells. Journal of BiologicalChemistry, 253(10), 3744-3750.

672. Volpe, A., Coukos, G., D'Ambrogio, G., Artini, P. G., Genazzani, A. R., (1991).

Follicular fluid steroid and epidermal growth factor content, and *in vitro* estrogen release by granulosa-luteal cells from patients with polycystic ovaries in an IVF/ET program. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *42*(3), 195-199.

673.Voutilainen, R., Franks, S., Mason, H. D., Martikainen, H., (1996). Expression of insulin-like growth factor (IGF), IGF-binding protein, and IGF receptor messenger ribonucleic acids in normal and polycystic ovaries. *The Journal of Clinical Endocrinology & Metabolism*, *81*(3), 1003-1008.

674.Walker, F., Kato, A., Jorge Gonez, L., Hibbs, M.L., Pouliot, N., Levitzki, A., Burgess, A.W., (1998). Activation of the Ras/Mitogen-Activated Protein Kinase Pathway by Kinase-Defective Epidermal Growth Factor Receptors Results in Cell Survival but Not Proliferation. Mol. Cell. Biol. 18, 7192–7204.

675.Walton, E., Grant, P., Owens, A., Wallace, C., Ballard, J. (1990). Isolation and characterization of ovine plasma IGF binding proteins 1, 2 and a 24 kDa species. 72nd Annu. Mtg. of the Endocrine Society, 69, 180.

676.Wandji, S. A., Pelletier, G., Sirard, M. A., (1992). Ontogeny and cellular localization of 125I-labeled insulin-like growth factor-I, 125I-labeled follicle stimulating hormone, and 125I-labeled human chorionic gonadotropin binding sites in ovaries from bovine fetuses and neonatal calves. Biology of reproduction, 47(5), 814 822.

677.Wandji, S.A., (1998). Expression of Mouse Ovarian Insulin Growth Factor System Components During Follicular Development and Atresia. Endocrinology 139, 5205–5214.

678.Wandji, S.A., Eppig, J.J., Fortune, J.E., (1996). FSH and growth factors affect the growth and endocrine function *in vitro* of granulosa cells of bovine preantral follicles. Theriogenology 45, 817–832.

679.Wandji, S.A., Gadsby, J.E., Simmen, F.A., Barber, J.A., Hammond, J.M., (2000). Porcine ovarian cells express messenger ribonucleic acids for the acid-labile subunit and insulin-like growth factor binding protein-3 during follicular and luteal phases of the estrous cycle. Endocrinology 141, 2638–2647.

680.Wandji, S.A., Pelletier, G., Sirard, M.A., (1992). Ontogeny and cellular localization of 125I-labeled insulin-like growth factor-I, 125I-labeled follicle-stimulating hormone, and 125I-labeled human chorionic gonadotropin binding sites in ovaries from bovine fetuses and neonatal calves. Biol. Reprod. 47, 814–822.

681.Wang, L. M., Feng, H. L., Ma, Y. Z., Cang, M., Li, H. J., Yan, Z., Liu, D. J., (2009). Expression of IGF receptors and its ligands in bovine oocytes and preimplantation embryos. *Animal reproduction science*, *114*(1), 99-108.

682.Wang, L. Y., Wang, D. H., Zou, X. Y., Xu, C. M., (2009). Mitochondrial functions on

oocytes and preimplantation embryos. Journal of Zhejiang University Science B, 10(7), 483-492.

683.Wang, T. H., Chang, C. L., Wu, H. M., Chiu, Y. M., Chen, C. K., Wang, H. S., (2006). Insulin-like growth factor-II (IGF-II), IGF-binding protein-3 (IGFBP-3), and IGFBP-4 in follicular fluid are associated with oocyte maturation and embryo development. *Fertility and sterility*, *86*(5), 1392-1401

684.Wang, W.H., Sun, Q.Y., Hosoe, M., Shioya, Y., Day, B.N., (1997). Quantified analysis of cortical granule distribution and exocytosis of porcine oocytes during meiotic maturation and activation. Biol. Reprod. 56, 1376–1382.

685.Wang, Z.-G., Yu, S.-D., Xu, Z.-R., (2007). Effects of Collection Methods on Recovery Efficiency, Maturation Rate and Subsequent Embryonic Developmental Competence of Oocytes in Holstein Cow. Asian-australas. J. Anim. Sci. 20, 496–500.

686.Wanga, W., Niwa, K., (1995). Synergetic effects of epidermal growth factor and gonadotropins on the cytoplasmic maturation of pig oocytes in a serum–free medium. Zygote 3, 345–350.

687.Warzych, E., Wrenzycki, C., Peippo, J., Lechniak, D., (2007). Maturation medium supplements affect transcript level of apoptosis and cell survival related genes in bovine blastocysts produced *in vitro*. Mol. Reprod. Dev. 74, 280–289.

688.Wasielak, M., Bogacki, M., (2007). Apoptosis inhibition by insulin-like growth factor (IGF)-I during *in vitro* maturation of bovine oocytes. J. Reprod. Dev. 53, 419–426.

689.Watson AJ, Hogan A, Hahnel A, Wiemer KE, Schultz GA., (1992). Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. MolReprod Dev; 31: 87-95.

690.Watson ED, Bae SE, Al-Zi'abi MO, Hogg CO, Armstrong DG., (2005). Expression of mRNA encoding insulin-like growth factor binding protein-2 (IGFBP-2) during induced and natural regression of equine corpora lutea. Theriogenology; 64:1371–80

691.Watson, A.J., Barcroft LC., (2001). Regulation of blastocyst formation. Front. Biosci. 6, d708–730.

692.Webb R, Woad KJ, Armstrong DG. CL., (2002). (CL) function: local control mechanisms. Domest Anim Endocrinol; 23:277–85.

693.Wei, Z., Park, K. W., Day, B. N., Prather, R. S. (2001). Effect of epidermal growth factor on preimplantation development and its receptor expression in porcine embryos. Molecular reproduction and development, 60(4), 457-462.

694.Wessel, G.M., Conner, S.D., Berg, L., (2002). Cortical granule translocation is microfilament mediated and linked to meiotic maturation in the sea urchin oocyte.

Development 129, 4315–4325.

695.Westergaard, L.G., Anhdersen, C.Y., (1989). Epidermal growth factor (EGF) in human preovulatory follicles. Hum. Reprod. 4, 257–260.

696.Wilding, M., Dale, B., Marino, M., di Matteo, L., Alviggi, C., Pisaturo, M.L., Lombardi, L., De Placido, G., (2001). Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. Hum. Reprod. 16, 909–917.

697.Wiley LM, Wu JX, Harari I, Adamson ED., (1992). Epidermal growth factor receptor mRNA and protein increase after the four-cell preimplantation stage in murine development. DevBiol 149:247–260

698.Williams, C.J., Erickson, G.F., (2014). Morphology and Physiology of the Ovary, in: De Groot, L.J., Chrousos, G., Dungan, K., Feingold, K.R., Grossman, A., Hershman, J.M., Koch, C., Korbonits, M., McLachlan, R., New, M., Purnell, J., Rebar, R., Singer, F., Vinik, A. (Eds.), Endotext. MDText.com, Inc., South Dartmouth (MA).

699.Willis, P., Caudle, A.B., Fayrer-Hosken, R.A., (1994). Fine structure of equine oocytes matured *in vitro* for 15 hours. Mol. Reprod. Dev. 37, 87–92.

700.Wiltbank, M. C., Souza, A. H., Carvalho, P. D., Cunha, A. P., Giordano, J. O., Fricke, P. M., Diskin, M. G., (2014). Physiological and practical effects of progesterone on reproduction in dairy cattle. *Animal*, *8*(s1), 70-81

701.Wisel, M.S., Datta, J.K., Saxena, R.N., (1991). Changes in the levels of protein and steroid hormones in the plasma and steroid hormone receptors in the uterus of normal cycling guinea pigs. Steroids 56, 148–153.

702.Woad KJ, Baxter G, Hogg CO, Bramley TA, Webb R, Armstrong DG., (2000). Expression of mRNA encoding insulin like growth factors-I and -II and the type 1 IGF receptor in the bovine corpus luteum at defined stages of the oestrous cycle. J Reprod Fertil;120:293–302.

703.Wood, J.R., Strauss, J.F., III, (2002). Rev. Endocr. Metab. Disord. 3, 33–46.

704.Wood, S.A., Kaye, P.L., (1989). Effects of epidermal growth factor on preimplantation mouse embryos. Reproduction 85, 575–582.

705.Worku, T., Rehman, Z.U., Talpur, H.S., Bhattarai, D., Ullah, F., Malobi, N., Kebede, T., Yang, L., (2017). MicroRNAs: New Insight in Modulating Follicular Atresia: A Review. Int. J. Mol. Sci. 18. doi:10.3390/ijms18020333

706.Wright RJ, Holly JM, Galea R, Brincat M, Mason HD., (2002). Insulin-like growth factor (IGF)-independent effects of IGF binding protein-4 on human granulosa cell steroidogenesis. Biol Reprod; 67:776–781

707.Xia, P., Tekpetey, F.R., Armstrong, D.T., (1994). Effect of IGF-I on pig oocyte maturation, fertilization, and early embryonic development *in vitro*, and on granulosa and cumulus cell biosynthetic activity. Mol. Reprod. Dev. 38, 373–379.

708.Xiong, F., Hu, L., Zhang, Y., Xiao, X., Xiao, J., (2016). miR-22 inhibits mouse ovarian granulosa cell apoptosis by targeting SIRT1. Biol. Open 5, 367–371.

709.Yadav, P., Kharche, S.D., Goel, A.K., Jindal, S.K., Sharma, M.C., (2010). 361 Effect of hormones, egf and β-mercaptoethanol on*in vitro*maturation of caprine oocytes. Reprod. Fertil. Dev. 22, 337.

710.Yamashita, Y., Shimada, M., (2012). The release of EGF domain from EGF-like factors by a specific cleavage enzyme activates the EGFR-MAPK3/1 pathway in both granulosa cells and cumulus cells during the ovulation process. *Journal of Reproduction and Development*, *58*(5), 510-514.

711.Yang, L., Wei, Q., Li, W., Xi, Q., Zhao, X., Ma, B., (2016). NPR2 is involved in FSH-mediated mouse oocyte meiotic resumption. *Journal of ovarian research*, *9*(1), 6.

712.Yang, M.Y., Rajamahendran, R., (2000). Morphological and biochemical identification of apoptosis in small, medium, and large bovine follicles and the effects of follicle-stimulating hormone and insulin-like growth factor-I on spontaneous apoptosis in cultured bovine granulosa cells**.** *Biol Reprod*;62:1209–1217.

713.Yao, K., Lau, S.W., Ge, W., (2014). Differential regulation of kit ligand an expression in the ovary by IGF-1 via different pathways. Mol. Endocrinol. 28, 138e150.

714.Yaseen, M.A., Wrenzycki, C., Herrmann, D., Carnwath, J.W., Niemann, H., (2001). Changes in the relative abundance of mRNA transcripts for insulin-like growth factor (IGF-I and IGF-II) ligands and their receptors (IGF-IR/IGF-IIR) in preimplantation bovine embryos derived from different *in vitro* systems. Reproduction 122, 601–610.

715.Yeh, J., Lee, G. Y., Anderson, E., (1993). Presence of transforming growth factor-alpha messenger ribonucleic acid (mRNA) and absence of epidermal growth factor mRNA in rat ovarian granulosa cells, and the effects of these factors on steroidogenesis *in vitro*. *Biology of reproduction*, *48*(5), 1071-1081.

716.Yerushalmi, G. M., Salmon-Divon, M., Yung, Y., Maman, E., Kedem, A., Ophir, L., Fadini, R., (2014). Characterization of the human cumulus cell transcriptome during final follicular maturation and ovulation. *Molecular human reproduction*, *20*(8), 719-735

717.Yıldırım, K., Vural, M.R., Küplülü, S., Ozcan, Z., Polat, I.M., (2014). The effects of EGF and IGF-1 on FSH-mediated *in vitro* maturation of domestic cat oocytes derived from follicular and luteal stages. Reprod. Biol. 14, 122–127.

718.Yin, M., Guan, X., Liao, Z., Wei, Q., (2009). Insulin-like growth factor-1 receptor-targeted therapy for non-small cell lung cancer: a mini review. Am. J. Transl. Res. 1, 101–114.

719.Yoshida, M., Cran, D.G., Pursel, V.G., (1993). Confocal and fluorescence microscopic study using lectins of the distribution of cortical granules during the

maturation and fertilization of pig oocytes. Mol. Reprod. Dev. 36, 462–468.

720. Yoshida, Y., Miyamura, M., Hamano, S., Yoshida, M., (1998). Expression of growth factor ligand and their receptor mRNAs in bovine ova during *in vitro* maturation and after fertilization *in vitro. Journal of veterinary medical science*, *60*(5), 549-554.
721. Yoshimura, Y., Ando, M., Nagamatsu, S., Iwashita, M., Adachi, T., Sueoka, K., (1996). Effects of insulin-like growth factor-I on follicle growth, oocyte maturation, and ovarian steroidogenesis and plasminogen activator activity in the rabbit. Biol. Reprod. 55, 152e160.
722. Young, J.M., McNeilly, A.S., (2010). Theca: the forgotten cell of the ovarian follicle. Reproduction 140, 489–504.
723. Young, W. C., (1937). The vaginal smear picture, sexual receptivity and the time of ovulation in the guinea pig. *The Anatomical Record*, *67*(3), 305-325.
724. Yu, H., Rohan, T., (2000). Role of the insulin-like growth factor family in cancer development and progression. J. Natl. Cancer Inst. 92, 1472–1489.
725. Yu, S., Ge, W., (2007). The GH-IGF axis and its potential role in the ovary of zebrafish, Danio rerio. Biol. Reprod. 77.
726. Yu, S., Zhao, C., Che, N., Jing, L., Ge, R., (2017). Hydrogen-rich saline attenuates eosinophil activation in a guinea pig model of allergic rhinitis via reducing oxidative stress. J. Inflamm. 14. doi:10.1186/s12950-016-0148-x
727. Yu, Y., Dumollard, R., Rossbach, A., Lai, F.A., Swann, K., (2010). Redistribution of mitochondria leads to bursts of ATP production during spontaneous mouse oocyte maturation. J. Cell. Physiol. 224, 672–680.
728. Yuan W, Bao B, Garverick HA, Youngquist RS, Lucy MC., (1998). Follicular dominance in cattle is associated with divergent patterns of ovarian gene expression for insulin-like growth factor-I (IGF-1), IGF-1I and IGF-binding protein-2 in dominant and subordinate follicles. DomestAnimEndocrinol 1998; 15:55–63.
729. Yuen, J.S.P., Macaulay, V.M., (2008). Targeting the type 1 insulin-like growth factor receptor as a treatment for cancer. Expert Opin. Ther. Targets 12, 589–603.
730. Zapf, J., Hauri, C., Futo, E., Hussain, M., Rutishauser, J., Maack, C.A., Froesch, E.R., (1995). Intravenously injected insulin-like growth factor (IGF) I/IGF binding protein-3 complex exerts insulin-like effects in hypophysectomized, but not in normal rats. J. Clin. Invest. 95, 179–186.
731. Zha, J., Lackner, M.R., (2010). Targeting the Insulin-like Growth Factor Receptor-1R Pathway for Cancer Therapy. Clin. Cancer Res. 16, 2512–2517.
732. Zhang, C., Lu, L., Li, Y., Wang, X., Zhou, J., Liu, Y., Fu, P., Gallicchio, M.A., Bach, L.A., Duan, C., (2011). IGF binding protein-6 expression in vascular endothelial cells is induced by hypoxia and plays a negative role in tumor angiogenesis. International

Journal of Cancer 130, 2003–2012.

733. Zhang, H., Liu, K., (2015). Cellular and molecular regulation of the activation of mammalian primordial follicles: somatic cells initiate follicle activation in adulthood. Hum. Reprod. Update 21, 779–786.

734. Zhang, H., Risal, S., Gorre, N., Busayavalasa, K., Li, X., Shen, Y., Bosbach, B., Brännström, M., Liu, K., (2014). Somatic cells initiate primordial follicle activation and govern the development of dormant oocytes in mice. Curr. Biol. 24, 2501–2508.

735. Zhao, J., Taverne, M. A. M., Van der Weijden, G. C., Bevers, M. M., van Den Hurk, R., (2002). Immunohistochemicallocalisation of growth hormone (GH), GH receptor (GHR), insulin-like growth factor I (IGF-1) and type I IGF-1 receptor, and gene expression of GH and GHR in rat pre-antral follicles. *Zygote*, *10*(01), 85-94.

736. Zhao, J., Taverne, M.A.M., Van der Weijden, G.C., Bevers, M.M. Van den Hurk, R., (2001). Insulin-like growth factor-I (IGF-1) stimulates the development of cultured rat pre-antral follicles. Mol. Reprod. Dev. 58, 287–96.

737. Zho, J., Bondy, C., (1993). Anatomy of the Human Ovarian Insulin-Like Growth Factor System. Biol. Reprod. 48, 467–482.

738. Zhou, J., Adesanya, O.O., Vatzias, G., Hammond, J.M., Bondy, C.A., (1996). Selective expression of insulin-like growth factor system components during porcine ovary follicular selection. Endocrinology 137, 4893–4901.

739. Zhou, J., Rajendra Kumar, T., Matzuk, M.M., Bondy, C., (1997). Insulin-Like Growth Factor I Regulates Gonadotropin Responsiveness in the Murine Ovary. Mol. Endocrinol. 11, 1924–1933.

740. Zhou, R., Yu, S.M.Y., Ge, W., (2016). Expression and functional characterization of intrafollicular GH–IGF system in the zebrafish ovary. Gen. Comp. Endocrinol. 232, 32–42.

741. Zuelke, K.A., Brackett, B.G., (1990). Luteinizing hormone-enhanced *in vitro* maturation of bovine oocytes with and without protein supplementation. Biol. Reprod. 43, 784–787.