UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

TESIS DOCTORAL

Parámetros moleculares de competencia ovocitaria en las células del cúmulo humanas y bovinas

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Álvaro Martínez Moro

DIRECTOR

Pablo Bermejo Álvarez

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UNIVERSIDAD COMPLUTENSE DE MADRID

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Departamento de Fisiología Animal

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Madrid, 2023

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Tesis doctoral

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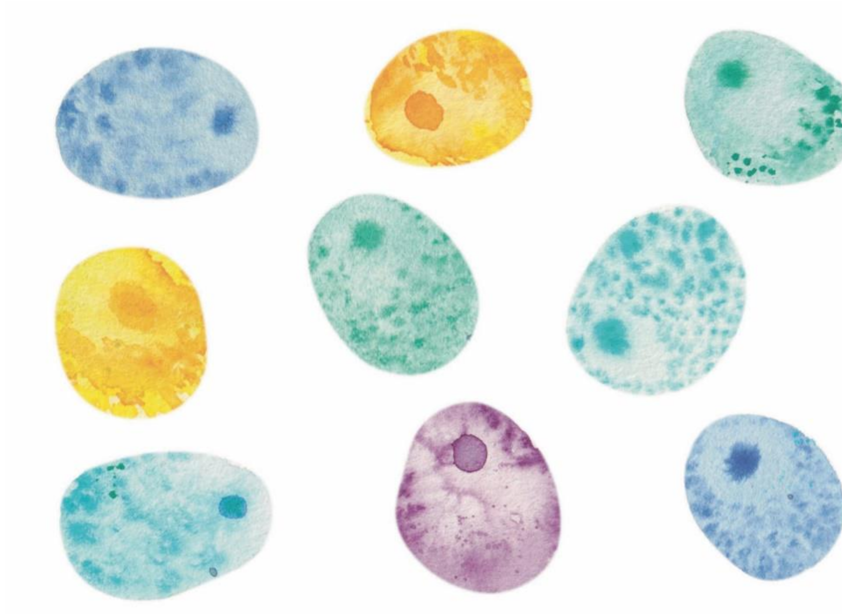

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Miguel de Cervantes (1605).

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Abstract

Artificial Reproductive Techniques based on *in vitro* embryo production from oocytes grant very relevant applications in farming and human medicine. In cattle, *ovum* pick-up is employed to increase greatly the offspring of high-genetic merit females by obtaining cumulus-oocyte complexes from these females that will be fertilized *in vitro* and transferred to recipient females of a lower genetic merit. In humans, which display a mono-ovulatory follicular development similar to that of cattle, *in vitro* fertilization is routinely employed in assisted reproduction clinics to treat several causes of female and male infertility. In both cases the low efficiency of the technique in terms of development to blastocysts and pregnancy success constitutes the main limitation. Embryo selection based on morphological criteria constitute the main way to improve pregnancy rates, but molecular analyses could improve the predictive value of morphological selection. In this context, molecular analyses in cumulus cells –which are intimately related with the oocyte- provide a non-invasive way to obtain molecular data whose values could be correlated with oocyte competence. The identification of such molecular markers of oocyte competence would serve 1) to use them as a criteria to select oocytes/embryos based on their chances to establish pregnancy following embryo transfer, and 2) to elucidate molecular aspects of oocyte competence to improve *in vitro* maturation protocols.

In this thesis, we have carried out analyses in human and bovine cumulus cells surrounding oocytes with different developmental potential. For bovine experiments, three groups of cumulus oocyte complexes diverging in their developmental competence were compared: 1) oocytes unable to generate cleaved embryos (Cl-), 2) oocytes that generate cleaved embryos that are unable to develop to blastocysts (Bl-), and 3) oocytes that generate embryos able to develop to the blastocyst stage (Bl+). For human experiments, other three groups were compared: 1) oocytes unable to generate blastocysts (Bl-), 2) oocytes that produce blastocysts that do not establish pregnancy following embryo transfer (P-), and 3) oocytes able to generate embryos that establish pregnancy (P+).

Chapter 1 analyzed the relative mitochondrial DNA (mtDNA) amount in human and bovine cumulus cell samples obtained from individual cumulus-oocyte complexes of known developmental potential. The rationale behind this analysis is based on the positive correlation observed between oocyte´s mtDNA amount and its developmental competence, which could be conserved in cumulus cells. In both species, mtDNA content

in cumulus cells –analyzed by quantitative PCR- was not associated with oocyte developmental potential. In human samples, no correlation was observed between mtDNA amount and donor age, body mass index (BMI) or smoking status. Furthermore, an experiment on bovine samples observed no correlation between mtDNA amount in cumulus cells and that of the oocyte, clearly proving that mtDNA amount in cumulus cells is not associated to oocyte developmental potential.

Chapters 2 and 3 report transcriptomics analyses carried out by Deep sequencing (RNA-seq) in bovine and human cumulus cells, respectively. In both cases, the analysis was performed in samples composed by cumulus cells from several oocytes sharing the same developmental ability (10 cumulus-oocyte complexes in bovine and 5 in humans). The analysis of bovine samples (chapter 2) identified 19335 genes expressed in cumulus cells from which only 49, 50 and 18 were differentially expressed (p adjusted value <0.05 and fold-change >1.5-fold) for the comparisons Bl+ vs. Bl-, Bl+ vs. Cl- y Bl- vs. Cl, respectively. Between the genes potentially related with developmental competence to reach the blastocyst stage (Bl+), 10 were differentially expressed on both comparisons (Bl`vs. Bl- and Bl+ vs. Cl-): 4 were upregulated in Bl+ group (*GSTA1*, *PSMB8*, *FMOD* y *SFRP4*) and 6 downregulated in that group (*HBE1*, *ITGA1*, *PAPPA*, *AKAP12*, *ITGA5* y *SLC1A4*). These genes are involved in functions such as integrin-mediated cellular adhesion, oxygen availability, IGF and Wnt signaling and protein kinase A activity, and suggest a more advanced follicular development of the more competent oocytes. The analysis of human samples (Chapter 3) identified 17469 expressed genes, from which only 10, 11 and 5 were differentially expressed (p adjusted value <0.05 and fold-change >1.5 fold) in the comparisons P+ vs. P-, P+ vs. Bl- y P- vs. Bl-, respectively. Among them *CHAC1* was upregulated in the groups able to reach the blastocyst stage (P+ and P- vs. Bl-), while *CENPE*, *CD93*, *PECAM1* and *HSPA1B* showed the opposite pattern. Only *EPN3* was related with pregnancy potential, being downregulated in P+ group compared with P- or Bl-, but data dispersion impeded the establishment of a clear cut-off required to be used as a selection marker. These results evidence that global cumulus cells transcriptomic is largely unrelated with oocyte developmental potential in both species, although the transcriptional level of a small subset of genes varied significantly between groups.

Chapter 4 reports a metabolomics analysis on human cumulus cells obtained from individual cumulus-oocyte complexes. The analysis quantified 98 compounds of diverse

categories, including lipids, carbohydrates, amino acid related compounds, vitamins and co-factors. Between them 5 were differentially abundant between groups: asparagine, proline and malonate abundance was lower in group P- compared with Bl-, malonate and 5-oxyproline abundance was higher in group P+ compared with P-, and erithronate was more abundant in group P+ compared with Bl-. Besides, two unexpected compounds were identified in cumulus cells: the neurotransmitter N-acetyl-aspartyl-glutamate (NAG), the pseudodipeptide β-citrylglutamate, which may play signaling and chromatin remodeling roles, respectively.

Resumen

La generación de embriones *in vitro* a partir de ovocitos permite aplicaciones muy relevantes en ganadería y medicina humana. En el ganado vacuno, la aspiración ovárica permite aumentar la descendencia de hembras de alto mérito genético mediante la obtención de complejos cúmulo-ovocito que serán fecundados *in vitro* y transferidos a hembras receptoras. En humanos, donde el desarrollo folicular monoovulatorio es similar al bovino, la fecundación *in vitro* se emplea para tratar distintas causas de infertilidad femenina y masculina. En ambos casos, su limitación principal es la baja eficiencia en términos de desarrollo a blastocisto y éxito de gestación. La selección morfológica de embriones constituye la principal vía para la mejora de las tasas de gestación, pero distintos análisis moleculares podrían mejorar su valor predictivo. Los análisis moleculares de las células del cúmulo –íntimamente relacionadas con el ovocito- permiten obtener datos moleculares de forma no invasiva cuyos valores podrían estar correlacionados con la competencia del ovocito. La identificación de estos marcadores moleculares de competencia del ovocito permitiría 1) emplearlos para seleccionar ovocitos/embriones en base a sus probabilidades de gestación post-transferencia y 2) entender aspectos moleculares de la competencia del ovocito para mejorar los protocolos de maduración *in vitro*.

En esta tesis, hemos llevado a cabo análisis en células del cúmulo humanas y bovinas procedentes de ovocitos con distinta capacidad para el desarrollo. En la especie bovina se establecieron tres grupos de complejos cúmulo-ovocito en función de su capacidad para el desarrollo 1) ovocitos que no dan lugar a embriones divididos (Cl-), 2) ovocitos que generan embriones que se dividen, pero no se desarrollan hasta blastocisto (Bl-) y 3) ovocitos que generan embriones capaces de desarrollarse a blastocisto (Bl+). En la especie humana, se establecieron otros tres grupos: 1) ovocitos que no dan lugar a blastocistos (Bl-), 2) ovocitos que dan lugar a blastocistos, pero no a gestaciones tras la transferencia (P-) y 3) ovocitos que generan embriones capaces de establecer embarazo (P+).

En el capítulo 1 se llevó a cabo el análisis de la cantidad relativa de ADN mitocondrial (ADNmt) en muestras de células del cúmulo humanas y bovinas correspondientes a ovocitos individuales con capacidad de competencia probada. La lógica detrás de este análisis se basa en la correlación positiva entre la cantidad de ADNmt del ovocito y su competencia, que podría mantenerse en las células del cúmulo. El contenido de ADNmt en las células del cúmulo en ambas especies –analizado mediante PCR cuantitativa- no

mostró diferencias significativas en función de la competencia del ovocito correspondiente. En las muestras humanas tampoco se observó una correlación entre la cantidad de ADNmt y la edad, índice de masa corporal (IMC) o consumo de tabaco de la donante. Tampoco se observó una correlación entre la cantidad de ADNmt de las células del cúmulo bovinas y la cantidad de ADNmt de su ovocito, demostrando claramente la falta de correlación entre la cantidad de ADNmt de las células del cúmulo y la competencia del ovocito.

En los capítulos 2 y 3 se realizaron análisis transcriptómicos mediante secuenciación masiva de transcritos (*RNA-seq*) en muestras de células del cúmulo bovinas y humanas. En ambos casos se analizaron muestras formadas por células del cúmulo procedentes de varios ovocitos con la misma capacidad de competencia (10 complejos cúmulo-ovocito/muestra en bovino y 5 en humanos). En el caso de la especie bovina (capítulo 2), se identificaron 19335 genes expresados de los cuales únicamente 49, 50 y 18 mostraron diferencias estadísticamente significativas (valor p ajustado <0,05 y cambio de expresión >1,5 veces) en las comparativas Bl+ vs. Bl-, Bl+ vs. Cl- y Bl- vs. Cl-, respectivamente. Entre los genes potencialmente relacionados con competencia para desarrollo a blastocisto (Bl+), 10 mostraron diferencias significativas en ambas comparativas (Bl+ vs. Bl- y Bl+ vs. Cl-): 4 transcritos fueron más abundantes en el grupo Bl+ (*GSTA1*, *PSMB8*, *FMOD* y *SFRP4*) y 6 menos abundantes en dicho grupo (*HBE1*, *ITGA1*, *PAPPA*, *AKAP12*, *ITGA5* y *SLC1A4*). Estos genes están implicados en funciones como la adhesión celular mediada por integrinas, la disponibilidad de oxígeno, la señalización IGF y Wnt y la actividad de proteína kinasa A, y sugieren un mayor desarrollo folicular de los ovocitos más competentes. En el análisis de muestras humanas (capítulo 3), se identificaron 17469 genes expresados, de los cuales únicamente 10, 11 y 5 mostraron diferencias estadísticamente significativas (valor p ajustado <0,05 y cambio de expresión >1,5 veces) en las comparativas P+ vs. P-, P+ vs. Bl- y P- vs. Bl-, respectivamente. *CHAC1* mostró una abundancia mayor en los grupos capaces de alcanzar el estadio de blastocisto (P+ y P- vs. Bl-), mientras que *CENPE*, *CD93*, *PECAM1* y *HSPA1B* mostraron el patrón opuesto. Únicamente *EPN3* se relacionó con competencia para establecimiento de gestación, siendo menos abundante en el grupo P+ comparado con los grupos P-o Bl-, pero la dispersión de datos impide establecer un límite claro para su empleo como marcador de selección. Estos resultados evidencian que el transcriptoma global de las células del cúmulo muestra una baja correlación con la capacidad de

competencia del ovocito en ambas especies, aunque existe un pequeño número de genes cuyos niveles transcripcionales variaron de forma significativa entre los distintos grupos.

Por último, en el capítulo 4 se realizó un estudio metabolómico en muestras de células del cúmulo humanos procedentes de ovocitos individuales. El análisis cuantificó 98 compuestos de diversas categorías, incluyendo lípidos, carbohidratos, compuestos relacionados con aminoácidos, vitaminas y cofactores. Entre ellos 5 mostraron diferencias entre grupos: la asparragina, la prolina y el malonato mostraron abundancias menores en el grupo P- comparado con Bl-, el malonato y la 5-oxiprolina mostraron abundancias mayores en el grupo P+ comparado con el P-, y el eritronato fue más abundante en el grupo P+ comparado con el Bl-. Además, se identificaron dos compuestos inesperados en células del cúmulo: el neurotransmisor N-acetil-aspartil-glutamato (NAG) y el pseudodipéptido β-citrilglutamato, que pueden ejercer funciones de señalización y remodelación de la cromatina, respectivamente.

Introducción

Historia de la reproducción asistida humana

La fertilidad fue, es y será una cuestión tan importante como desconocida. Desde un punto de vista histórico, el principal símbolo de la fertilidad ha sido la mujer. Muestra de ello son las pinturas rupestres, así como las representaciones de diosas de la fertilidad (Afrodita, Venus o Taueret), tratando de atraer prosperidad y fertilidad. Por el contrario, la infertilidad es uno de los problemas sociales –y médicos- más importantes en la historia del ser humano, siendo considerado como una maldición enviada por los dioses que solo sufrían las mujeres, puesto que suponía una amenaza para la supervivencia de la propia estirpe y el mantenimiento del estatus en la sociedad (Morice *et al*., 1995).

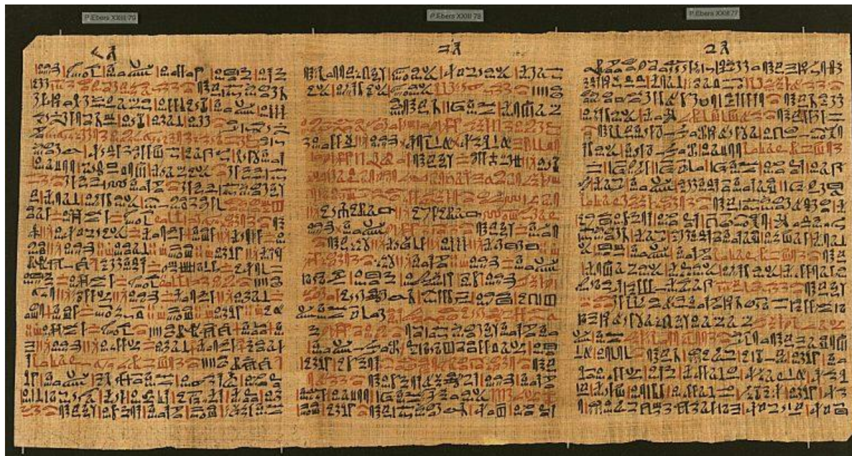

Figura 1. Fragmento del papiro de Ebers. Redactado en el Antiguo Egipto durante el reinado de Amenhotep I (1500 a.C.), es considerado uno de los primeros escritos de medicina, donde se describen patologías de distintas ramas como la oftalmología, la gastroenterología, la cirugía y la ginecología. Actualmente se conserva en la biblioteca de la Universidad de Leipzig.

Civilizaciones como la egipcia consideraban la infertilidad no como un castigo divino, sino como una patología que debía ser diagnosticada y tratada. El conocimiento de los galenos egipcios era muy amplio, abarcando la anatomía reproductora, la ginecología y la fertilidad, llegando a desarrollar el papiro de Ebers, considerado como el primer tratado ginecológico de la historia. En cuestiones de fertilidad, fueron capaces de establecer metodologías para el diagnóstico de infertilidad basándose en los órganos genitales y el tracto digestivo de la mujer (Lefebvre, 1952). Este diagnóstico estaba especialmente enfocado a la infertilidad femenina y, pese a no conllevar tratamiento alguno, este tipo de evaluación en la fertilidad fue recogido por otras civilizaciones posteriores (Ghalioungui & Ammar, 1963). Aunque no existían métodos diagnósticos para evaluar la fertilidad masculina, la medicina egipcia daba a entender que la problemática para engendrar un

hijo no podía caer únicamente sobre la responsabilidad de la mujer, aludiendo la posible existencia de la infertilidad masculina.

La medicina griega trajo consigo nuevos avances medicina y tratamiento de la infertilidad, sentando las bases de la medicina occidental. La principal figura de la medicina helena fue Hipócrates, quien partió de los conocimientos de la medicina egipcia para elaborar sus tratados. Según Hipócrates, la infertilidad podría tener diferentes orígenes: posición del cérvix, afectación de la cavidad uterina o prolapso uterino entre otros diagnósticos (Morice *et al*., 1995). La medicina griega no se limitó al diagnóstico de la infertilidad, sino que desarrolló tratamientos de diferente índole como la apertura del cérvix mediante un ungüento formado por comino, resina y miel, aunque algunos de estos tratamientos podrían considerarse como un riesgo *per se* para la propia fertilidad (Hipocrates, 1988). La importancia en los avances del conocimiento sobre la infertilidad en las civilizaciones egipcia y griega, sentaron las bases de la medicina reproductiva ejercida siglos después, ya que en los siglos sucesivos tuvieron mayor influencia las creencias religiosas que la evidencia. Figuras como Sorano de Efeso, Avicena o Arnau de Villanova trataron de aportar conocimiento y mejorar el diagnóstico, pero su trabajo se vio desplazado por influencia divina (Drabkin, 1951).

El renacimiento es considerado como uno de los periodos más importantes desde un punto de vista científico. Los estudios sobre anatomía llevados a cabo desde Italia por Vesalio o Leonardo da Vinci sentaron las bases de esta ciencia por medio de la observación y la disección (Morice *et al*., 1995). Estos avances fueron refrendados en el siglo XVII, cuando Gabriel Falopio describió las trompas, el clítoris, la vagina y la placenta (Falloppio, 1561) . En 1651, William Harvey desarrollo la teoría de la epigénesis la cual resumió como *"ex ovo omnia"* (Todo ser vivo procede del huevo) refutando la existencia del homúnculo dentro del gameto del varón (Harvey, 1651). En 1672, Reignier De Graaf, describió el ovario y el ciclo folicular, suponiendo un cambio en la comprensión del funcionamiento reproductivo femenino (De Graaf, 1672). En 1677 se produjo uno de los mayores descubrimientos técnicos que cambió el estudio y la metodología diagnóstica de muchas patologías: un sistema de lentes precursor de los actuales microscopios. Su descubridor, Anthony Van Leeuwenhoek se convirtió también en uno de los primeros en observar los espermatozoides (Lewenhoeck, 1677). Durante los siglos XVIII y XIX se sucedieron diversos avances relacionados con el diagnóstico de la infertilidad, como la identificación de distintas patologías que pueden causar infertilidad como obstrucción

tubárica, leucorrea, agenesia folicular, anormalidades en útero o vagina o situaciones de dismenorrea (Morice *et al*., 1995). También en el siglo XIX se publica el libro *“The microscope as an aid in the diagnosis and treatment of sterility”*, donde se defiende la importancia del microscopio en la valoración de la calidad espermática para comprender la infertilidad (Sims, 1868).

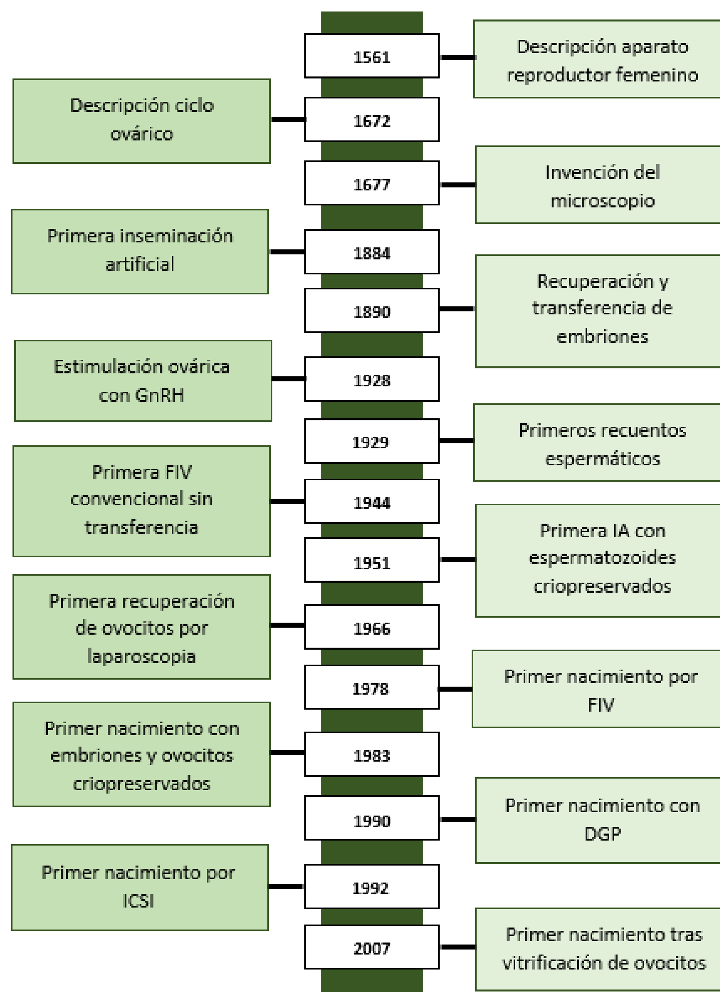

Figura 2. Infografía cronológica de la evolución histórica de la reproducción asistida humana.

A finales de siglo XIX, en 1884, se desarrolla en Filadelfia (EEUU) la técnica de inseminación artificial con muestra seminal de donante. Esta primera inseminación, de dudosa ética profesional, fue llevada a cabo por Pancoast, quien había asumido la infertilidad de la pareja como femenina. Tras explorar a la mujer y no encontrar afectación alguna, observó que la pareja tenía gonorrea, lo cual afectaba a la calidad espermática. Esta información, que no fue revelada a la pareja, determinó el diagnóstico de la misma, requiriendo a la paciente para una segunda exploración. Tras haber sido dormida y delante de seis estudiantes de medicina, inseminó mediante una jeringuilla de goma a la paciente,

empleando la muestra seminal de uno de los estudiantes (Hard, 1909). De esta forma, se estableció una de las primeras técnicas de reproducción asistida.

El siglo XX supone el empuje definitivo en el desarrollo de la reproducción asistida. Los avances en endocrinología reproductiva en las primeras décadas del siglo, permitieron realizar estimulaciones ováricas que fueron optimizadas posteriormente con la administración de gonadotropinas humanas que contenían hormona folículo estimulante (FSH) y hormona luteinizante (LH) (Aschheim & Zondek, 1928; Gemzell *et al*., 1958). Otra metodología de estimulación ovárica controlada que se desarrolló empleaba el citrato de clomifeno (Greenblatt *et al*., 1962). Las estimulaciones ováricas precisan de un desencadenante de la ovulación, empleado por primera vez en 1978 (Wu, 1978). De forma paralela, los avances científicos facilitaron la monitorización en la estimulación ovárica, sentando las bases de los actuales protocolos de estimulación. En 1881, Jacques y Pierre Curie publicaron los resultados obtenidos al aplicar un campo eléctrico sobre algunos minerales, los cuales producían ondas sonoras de alta frecuencia, suponiendo el fundamento físico de la piezoelectricidad, sobre el que se basan los aparatos ultrasónicos. En 1951, Douglas Horwry desarrolló un equipo capaz de mostrar imágenes bidimensionales (Serrano, 2000) y que fue posteriormente desarrollado e implementado hasta poder llevar a cabo los procedimientos de reproducción asistida como eran la punción ovárica o la transferencia embrionaria (Russell *et al*., 1987) sin necesidad de laparoscopias (Edwards *et al*., 1966).

Durante las primeras décadas del siglo XX también se desarrollaron técnicas de recuento espermático (Macomber & Sanders, 1929) y en la década de los 50 se produjeron los primeros embarazos generados mediante inseminaciones con espermatozoides criopreservados (Chang, 1951a). Estos avances en el conocimiento y tratamiento de los espermatozoides desembocaron con el desarrollo exitoso de la primera fecundación *in vitro* de embriones humanos en 1944, que no fueron transferidos (Austin, 1951; Chen & Wallach, 1994; Rock & Hertig, 1944). Años más tarde, en 1966, tuvo lugar la primera recuperación de ovocitos empleando la técnica laparoscópica (Edwards *et al*., 1966), la cual ha evolucionado hasta la punción ovárica actual (Russell *et al*., 1987).

El acontecimiento histórico de mayor importancia en el desarrollo de la reproducción asistida tuvo lugar el 25 de Julio de 1978 con el nacimiento de Louise Brown, el primer bebé engendrado por fecundación *in vitro* (Steptoe & Edwards, 1978). Tras el nacimiento de Louise Brown se sucedieron las diferentes técnicas tal y como las conocemos en la

actualidad, aunque con metodologías ligeramente diferentes, consiguiéndose hitos como el primer embarazo con ovocitos donados (Buster *et al.*, 1983), tras haber congelado y descongelado el embrión (Trounson & Trounson, 1983) o empleando ovocitos donados mediante fecundación *in vitro* y transferencia embrionaria (Lutjen *et al.*, 1984). Las técnicas ginecológicas fueron variando conforme el desarrollo científico-técnico progresaba. La evolución tecnológica facilitó la progresión de los protocolos de recuperación ovocitaria y transferencia, tanto de gametos como de cigotos a la trompa de Falopio mediante laparoscopia (Asch *et al.*, 1986; Devroey *et al.*, 1986), hasta la realización de estos procedimientos gracias a la ecografía transvaginal (Russell *et al.*, 1987).

BRITAIN'S BIGGEST EVENING SALE

Evening News

LONDON: THURSDAY JULY 27 1978

8p

LATE SPECIAL CITY PRICES

Meet Louise, the world's first test-tube arrival

SUPERBABE

Wide-eyed Louise Brown pictured in hospital 18 hours after she was born. Today she's doing well. See Page Three

Figura 3. Portada de la edición especial del periódico *"Evening News"* de 27 de julio de 1978.

El avance en las técnicas de micromanipulación de gametos se produjo fundamentalmente en las dos últimas décadas del siglo XX. En el año 1988, Ng y colaboradores informan del primer embarazo empleando una novedosa técnica en la que se inyectaban uno o varios espermatozoides en el espacio perivitelino (*SUZI, Subzonal Sperm Insertion*) (Ng *et al.*, 1988). De manera similar, en 1989 se realiza un pequeño agujero en la zona pelúcida para facilitar la entrada del espermatozoide (*PZD, Partial Zonal Dissection*)(Malter & Cohen, 1989). Finalmente, en el año 1992 tiene lugar el desarrollo de la técnica más empleada en la actualidad en reproducción asistida humana, microinyección intracitoplasmática de espermatozoides (*ICSI*, por sus siglas en inglés). Esta técnica consiste en la introducción de un espermatozoide en el interior del citoplasma del ovocito, facilitando las posibilidades reproductivas de varones con diferentes patologías que impiden o dificultan la fecundación *in vitro* (Palermo *et al.*, 1992).

Por último, otras técnicas empleadas en reproducción asistida han surgido gracias al avance en otros campos del conocimiento como la genética o la tecnología óptica y computacional. En particular, los avances en genética han sido claves para el desarrollo del diagnóstico genético preimplantacional, que permite seleccionar aquellos embriones que no presentan mutaciones o cromosomopatías (Handyside *et al.*, 1990). Por otro lado, los actuales sistemas de incubación *time-lapse* permiten monitorizar el desarrollo del

embrión a tiempo real para, en base a unos criterios de selección de embriones, mejorar las tasas de éxito en los centros de reproducción asistida.

En España, la cronología científica de los diferentes hitos se sucedía con un breve retraso. El primer nacimiento por técnicas de fecundación *in vitro* tuvo lugar el 12 de julio de 1984, fecha en la que Victoria Anna nació en el Instituto Dexeus (Carol, 1984; Veiga Lluch, 2014). El primer diagnóstico genético tuvo lugar en 1994 (Veiga *et al*., 1994), 4 años después de haberse conseguido a nivel mundial. El éxito en los lavados seminales de pacientes seropositivos tuvo lugar en 1998, con la consecución del embarazo sin afectación de VIH a la pareja o al recién nacido (Marina *et al*., 2006; Marina *et al*., 1998; Marina *et al*., 1998). La criopreservación fue una de las técnicas de mayor importancia en España. El procedimiento inicial, conocido como congelación y descongelación lenta, permitió que en el año 2002 tuviera lugar el nacimiento de los primeros bebés procedentes de la congelación y descongelación de ovocitos (Marina & Marina, 2003). La congelación lenta sentó las bases de la congelación ultrarrápida o vitrificación, consiguiéndose en 2008 el nacimiento del primer bebé mediante vitrificación de ovocitos (Cobo *et al*., 2008). Los avances mencionados han permitido no solo el desarrollo de la medicina y biología de la reproducción a nivel de conocimiento, sino también organizativo al convertirse paulatinamente en un campo multidisciplinar en el que profesionales de diferentes ámbitos trabajan para mejorar las técnicas de reproducción asistida al tiempo que se facilita el deseo de paternidad y maternidad de pacientes con patologías de diferente índole que afectan a su fertilidad.

Historia de la reproducción asistida animal

El manejo reproductivo de los animales ha jugado un papel fundamental en la mejora genética de los animales de granja y en el suministro de alimentos a la población, además de permitir la preservación de especies y razas en peligro de extinción. Por otra parte, el empleo de técnicas de reproducción asistida en animales ha sido clave no sólo para generar conocimiento aplicable a la reproducción humana sino para generar todo tipo de biomodelos de enfermedades o procesos mediante modificaciones genéticas.

Los inicios de la reproducción animal asistida son inciertos e incluyen mitos y leyendas, como la que sitúa el origen de la inseminación artificial en animales en el año 1322, cuando un jefe árabe supuestamente utilizó clandestinamente el semen de un potro pura

sangre árabe perteneciente al ejercito enemigo para inseminar una yegua. La supuesta metodología empleada, difícilmente creíble desde los conocimientos actuales, se basó en la recogida del semen a través de una esponja situada en la vagina de una yegua maniquí y en su transporte en leche de camella hasta la yegua receptora. La primera inseminación artificial registrada tuvo lugar en 1777, cuando Spallanzani trató de desarrollar esta técnica en reptiles. Años más tarde, en 1780, tras haber conseguido realizar de forma exitosa la técnica en anfibios, trató de replicarlo en especies superiores, consiguiendo finalmente realizar la primera inseminación artificial en mamíferos.

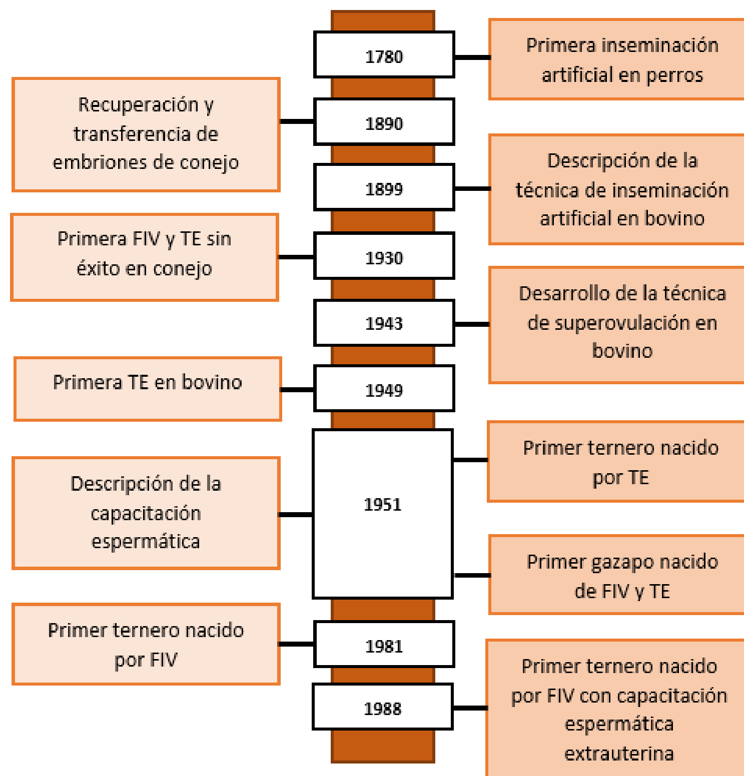

Figura 4. Cronología de los hitos históricos más relevantes de la reproducción asistida animal.

Esta primera inseminación se realizó en una perra de raza Spaniel empleando semen de un macho recogida por eyaculación espontánea, logrando el nacimiento de tres cachorros. Posteriormente, en 1803, dató el descenso en la motilidad en los espermatozoides al ser enfriados con nieve y su recuperación tras aplicarles calor, lo que supone un ligero esbozo del cómo afecta la criopreservación a los espermatozoides (Orland, 2017). Casi un siglo después, en 1899, Ivanoff estandarizó métodos de inseminación artificial enfocados principalmente a animales de granja (caballos, vacas y ovejas) y otros animales domésticos como el perro, aunque su detallada descripción de la técnica sentó las bases para su utilización en la medicina reproductiva humana (Ivanoff, 1922). Unos años más tarde se desarrollaron crioprotectores para poder preservar las muestras seminales

utilizando como base la yema de huevo (Phillips, 1939; Phillips & Spitzer, 1946), un avance esencial para permitir la inseminación de hembras en tiempos y lugares distintos al de la recogida del semen. El trabajo de Ivanoff fue secundado por Milovanov, científico ruso que estableció las bases para el desarrollo de los criaderos de ganado bovino (Milovanov, 1964). Rusia continuó, junto con China, desarrollando programas de inseminación artificial, especialmente en animales de granja, lo cual permitió la mejora sustancial tanto de la técnica, como de la colecta y manipulación de la muestra seminal.

El desarrollo de la inseminación artificial permitió expandir la descendencia que tendría de forma natural un macho de alto mérito genético y, por tanto, acelerar la selección genética en una determinada raza. Sin embargo, la selección genética de las hembras seguía limitada al número de crías que podía generar cada animal de forma natural a lo largo de su vida, ya que no era posible transferir embriones de una hembra de alto mérito genético a otras hembras receptoras. La primera transferencia de embriones en mamíferos fue llevada a cabo por Walter Heape en conejos en el año 1890, utilizando embriones obtenidos por lavado oviductal y consiguiendo el nacimiento de gazapos sanos (Heape, 1890). La transferencia embrionaria en bovino, la especie de interés ganadero en la que más se aplica la técnica, fue desarrollado por Umbaugh en 1949, cuyo grupo en primera instancia no consiguió el recién nacido sano, posiblemente debido a un brote de brucelosis (Umbaugh, 1951). El primer ternero nacido mediante transferencia de embriones tuvo lugar en 1951, apenas dos años después del desarrollo de la técnica por Umbaugh (Willet *et al*., 1951).

El desarrollo en la transferencia de embriones en bovino evolucionó gracias al descubrimiento de la técnica de superovulación, que permitía la obtención de múltiples embriones en cada colecta. La superovulación consigue un aumento de la producción ovocitaria mediante la estimulación folicular producida mediante la inyección de gonadotropinas. Las gonadotropinas empleadas inicialmente eran de caballo, ya que tenía una vida media larga y permitían realizar protocolos con un menor número de inyecciones (Casida *et al*., 1943; Rowson, 1951). Posteriormente, los estudios de superovulación empleando FSH equina y porcina fueron utilizados como base en los programas de transferencia embrionaria (Dziuk *et al*., 1954; Elsden *et al*., 1978; Hasler *et al*., 1983).

El desarrollo de las técnicas de reproducción asistida *in vitro* en animales comenzó en el siglo XIX. Uno de los primeros científicos en intentar la fecundación *in vitro* fue Schenk,

quien realizó fecundaciones de óvulos procedentes de conejas y cobayas sin demasiado éxito (Schenk, 1874, 1880). La primera fecundación *in vitro* exitosa se realizó en conejos usando semen capacitado en el útero de una coneja (Chang, 1959). Por otra parte, el desarrollo de la transferencia de embriones (Heape, 1890) generó un gran interés por el cultivo *in vitro*, que empleó inicialmente sustancias biológicas como plasma sanguíneo o suero para su desarrollo. De esta forma, se desarrollaron distintos medios de cultivo que permitieron el desarrollo embrionario (Brinster & Troike, 1979; Whitten, 1971). Whitten propuso una nueva composición de los medios de cultivo utilizados en embriología, tanto para la recolección como para el cultivo de embriones. Estos medios de cultivo se basaban en el tampón Krebs-Ringer suplementado con albúmina de suero bovino y fueron capaces de promover el desarrollo de un embrión de ratón hasta el estadio de blastocisto (Whitten, 1971). Brinster y Troike basaron su investigación en determinar las necesidades nutricionales de un embrión en cultivos *in vitro* en base a su actividad metabólica, empleando microgotas de medio de cultivo, que se han mantenido hasta la actualidad para el cultivo de embriones humanos y animales (Brinster & Troike, 1979). Estos avances permitieron la consecución de la primera gestación a término mediante producción *in vitro* completa, es decir, llevando a cabo maduración, fecundación y cultivo *in vitro* (Goto *et al*., 1988). Este hito permitió progresar significativamente en la compresión de las necesidades metabólicas de embriones y gametos, reformulándose la composición de los medios de cultivo en la búsqueda de las mayores tasas de éxito hasta llegar a los medios que empleamos en la actualidad (Holm *et al*., 1999).

El descubrimiento de las técnicas de fecundación y cultivo *in vitro* abrió la posibilidad a la producción de embriones completamente *in vitro*, una técnica con grandes aplicaciones para la investigación, ya que permite la observación del desarrollo embrionario y la manipulación de los embriones, y para la ganadería, ya que permite la obtención de un mayor número de embriones de hembras de alto mérito genético mediante aspiración ovárica. El principal escollo inicial para la producción de embriones *in vitro* en especies ganaderas como el ganado bovino fue la necesidad de desarrollar métodos de capacitación espermática. La capacitación espermática consiste en una serie de cambios que experimenta el espermatozoide que son necesarios para la fecundación del ovocito (Austin, 1951; Chang, 1951b). Como se ha comentado anteriormente, la primera fecundación *in vitro* se llevó a cabo con espermatozoides capacitados en el útero de una coneja (Chang, 1959) pero, a día de hoy, sigue sin existir un método reproducible para

capacitar espermatozoides de conejo *in vitro*. Por este motivo, la primera fecundación exitosa de ovocitos bovinos madurados *in vitro* tuvo lugar en 1977 empleando semen capacitado en el oviducto de vacas o en el útero de conejos (Iritani & Niwa, 1977). A finales de la década de 1970 se había demostrado también la posibilidad de fecundar en el oviducto de la hembra ovocitos madurados *in vitro*, consiguiéndose el nacimiento de terneros vivos sanos (Newcomb *et al*., 1978). Gracias a estos estudios, en 1981 se produjo el nacimiento del primer ternero vivo procedente de fecundación *in vitro* en la Universidad de Pennsylvania (Brackett *et al*., 1982). Este hito supuso el desencadenante de nuevos nacimientos de terneros con distintas metodologías como el cultivo de embriones en oviductos de ovino o conejo, o capacitando del semen con ionóforo de calcio (Hanada *et al*., 1986; Leibfried-Rutledge *et al*., 1987). La capacitación del semen, especialmente en muestras criopreservadas, siguió siendo el talón de Aquiles en el desarrollo de la producción de embriones hasta que Parrish y colaboradores en 1988, demostraron que la heparina desencadenaba la capacitación en espermatozoides bovinos, permitiendo llevar a cabo el proceso completo de producción de embriones (maduración, fecundación y cultivo) *in vitro* (Parrish *et al*., 1988).

Técnicas de reproducción asistida humana

El desarrollo científico a lo largo de la historia ha permitido una evolución en el tratamiento de enfermedades desde un punto de vista diagnóstico, técnico y resolutivo. Como hemos visto anteriormente, la reproducción asistida no es un excepción y han sido numerosas las técnicas desarrolladas en cuarenta años (Niederberger *et al*., 2018) desde que tuvo lugar el primer recién nacido vivo mediante la técnica de fecundación *in vitro* (Steptoe & Edwards, 1978). Este desarrollo ha llevado a un avance en el conocimiento de las causas de infertilidad que ha ido incrementando su incidencia en la población durante las últimas décadas (Borght & Wyns, 2018).

La selección de la técnica de reproducción asistida de elección depende del diagnóstico de infertilidad de la mujer o pareja que acuden a la unidad especializada (Ferring *et al*., 2000; Remohí *et al*., 2017). El diagnóstico es el proceso deductivo mediante el cual se trata de identificar la causa u origen que provoca un cuadro de esterilidad en la/los paciente/s. El proceso diagnóstico debe realizarse simultáneamente a ambos miembros de la pareja si los hubiere puesto que el foco de la patología está muy diversificado, encontrando un 30% de los casos debidos a una afectación femenina, un 30% a factores

masculinos, un 25% producida por alteraciones en ambos miembros de la pareja, y un 15% tienen causas de origen desconocido o incierto (Hull *et al*., 1985).

Las pruebas diagnósticas empleadas en el estudio de la mujer o pareja estéril se han ido simplificando debido a la imperfección de algunas metodologías que demoran el tratamiento y encarecen el proceso, por lo que en muchos casos son los propios tratamientos los que aportan la información para establecer un diagnóstico de infertilidad (Ferring *et al*., 2000; Remohí *et al*., 2017). Para ello, es fundamental poder analizar cuatro pilares diagnósticos:

1. Anamnesis y exploración física
2. Existencia de ovulación
3. Integridad de los canales genitales
4. Análisis de los espermatozoides mediante seminograma

Los objetivos que las pruebas diagnósticas realizadas aportan en un estudio de infertilidad son similares a los analizados en otras patologías:

1. Descubrir el origen de la infertilidad para poder establecer la pauta más adecuada.
2. Proporcionar a los pacientes información actualizada, veraz y basada en estudios científicos.
3. Eliminar prejuicios asociados con la responsabilidad de la situación infértil de los pacientes.
4. Establecer las posibles vías capaces de solucionar la situación particular que se muestre en cada caso.

Inseminación Artificial

La inseminación artificial es la técnica reproductiva en la que se depositan, con ayuda del medio instrumental adecuado, los espermatozoides en el aparato genital de la mujer. Se trata de una técnica sencilla y de las menos intrusivas de las existentes en reproducción asistida. La elección de la inseminación artificial depende del diagnóstico previo, debido a que deben darse unas condiciones reproductivas específicas: la mujer debe tener capacidad para ovular, ya sea de forma natural o mediante tratamiento, y al menos una de las trompas de Falopio, encargadas de recoger al ovocito a través de las fimbrias, debe ser permeable para permitir el encuentro entre los gametos (Aboulghar *et al*., 2009; Duran *et al*., 2002; Starosta *et al*., 2020).

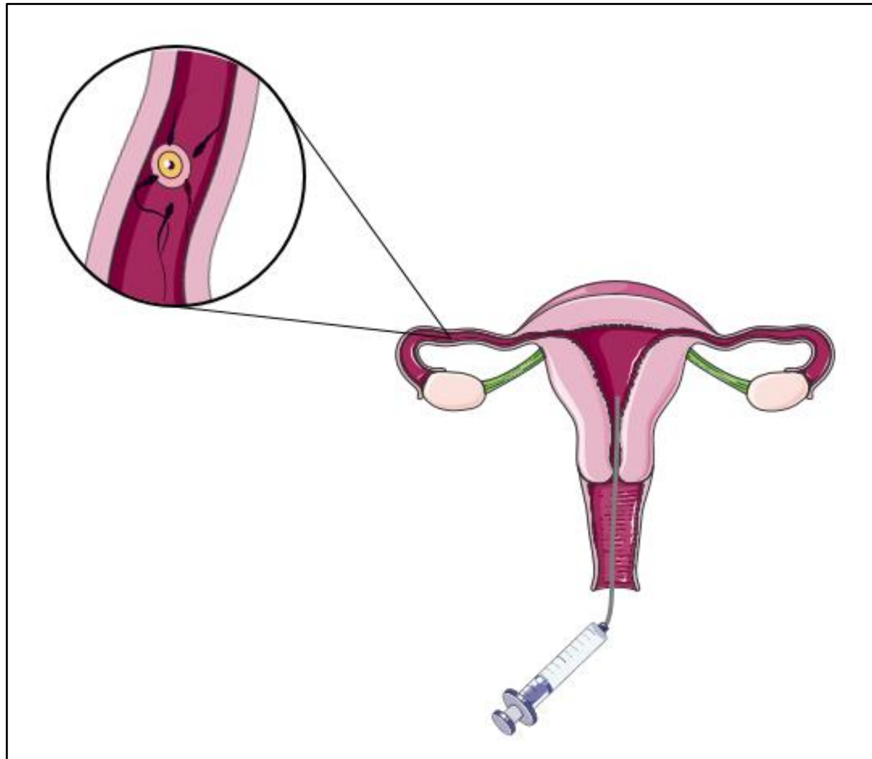

Figura 5. Representación gráfica de la inseminación artificial. Para la realización de esta técnica se emplea un catéter que permite que los espermatozoides sean depositados en el interior de la cavidad uterina, cerca de la trompa de Falopio (*Imagen modificada de Servier Medical Art: https://smart.servier.com/*)

La inseminación artificial es una técnica que puede realizarse sin tratamiento hormonal, en lo que se conoce como ciclo natural, realizándose la inseminación en el momento en el que vaya a tener lugar la ovulación (Kalu *et al.*, 2007). Sin embargo, la mayoría de las inseminaciones se hacen tras llevar a cabo una estimulación ovárica controlando la respuesta mediante ecografías periódicas que permitan conocer el momento óptimo en el que desencadenar la ovulación y realizar la inseminación propiamente dicha (Nandi & El-Toukhy, 2018; Wild, 2018). El proceso de inseminación es sencillo, breve y debiera ser completamente indoloro. Consiste en introducir un catéter fino a través del cuello para depositar en el útero un volumen que contiene los espermatozoides previamente capacitados (Huang *et al.*, 2004; Miller *et al.*, 2005; Ramón *et al.*, 2009).

Los riesgos asociados a la inseminación artificial son relativamente leves, ya que la técnica no presenta complejidad, y se centran en los riesgos asociados a la estimulación hormonal –escasos por ser una estimulación leve-, y en la posibilidad de gestaciones ectópicas o múltiples, que conllevan un mayor riesgo de complicaciones obstétricas y perinatales. En el caso de los abortos, causa y consecuencia de muchos tratamientos de reproducción asistida, la probabilidad de aborto tras gestación es la misma al realizar una

inseminación artificial que dicha probabilidad existente en la población general fértil (Evans *et al*., 2020; Khalil *et al*., 2001; Nuojua-Huttunen *et al*., 1999).

Existen dos tipos de inseminación artificial, en función del origen del gameto masculino que se emplee: homóloga (o de pareja) o heteróloga (o de donante). La inseminación artificial homóloga puede ser útil ante defectos en la calidad del moco uterino, situaciones de esterilidad de origen desconocido o situaciones de alteración reproductiva leve (ciertos trastornos de ovulación, endometriosis no severas, alteraciones leves de las trompas que no implique la obstrucción completa de la trompa, etc.) (Helmerhorst *et al*., 2006; R. K. K. Lee *et al*., 2002; Veltman-Verhulst *et al*., 2016). Esta técnica puede emplearse ante afectaciones leves de la calidad espermática, siempre que el recuento de espermatozoides móviles tras procesar la muestra seminal no sea inferior a 5 millones de espermatozoides por mililitro. La probabilidad de gestación en la inseminación artificial con semen de pareja es del 10%-15% por ciclo, el cual tiende a repetirse hasta aproximadamente los cuatro ciclos, ya que pasado el cuarto ciclo ese porcentaje disminuye. En cualquier caso, la probabilidad de éxito depende de las condiciones específicas de cada diagnóstico (Ejzenberg *et al*., 2019; Matorras, 2002; Osuna *et al*., 2004; Tiegs *et al*., 2020).

La inseminación artificial heteróloga o con muestra seminal de donante, se emplea en situaciones de esterilidad por factor masculino severo, afectaciones genéticas del varón no resueltas mediante diagnóstico genético preimplantacional, sensibilización grave de la mujer frente al factor Rh o en situaciones de esterilidad reproductiva de carácter social (mujeres sin pareja o lesbianas) que precisan recurrir a muestras seminales de donante. La probabilidad de gestación en las inseminaciones artificiales heterólogas es del 20%-25%, dependiendo el diagnóstico previo (Carpinello *et al*., 2021; Chen *et al*., 2018; Kop *et al*., 2018; Linara-Demakakou *et al*., 2020; Wolf *et al*., 2001).

Obtención de ovocitos

La inseminación artificial es, junto con los coitos dirigidos, la única técnica que no requiere la extracción de los ovocitos para su fecundación e implantación posterior. En el caso de la FIV o la ICSI, es siempre necesario obtener los ovocitos del ovario para su posterior fecundación en el laboratorio. En humanos es habitual realizar la punción ovárica como método de obtención ovocitaria, obteniendo ovocitos madurados *in vivo.* La maduración folicular previa a la punción es controlada mediante la administración de

hormonas. Como se explicará más adelante, esto constituye una diferencia con respecto a los protocolos empleados en la especie bovina, donde los ovocitos se recuperan de folículos en desarrollo y por tanto se lleva a cabo maduración *in vitro*.

Los tratamientos farmacológicos previos a la aspiración de los ovocitos maduros persiguen una estimulación ovárica controlada y una inducción de la ovulación. La estimulación ovárica pretende:

1. Obtener una respuesta suprafisiológica, incrementando el número de ovocitos obtenidos y, por tanto, el número de embriones disponibles a transferir.
2. Evitar grandes fluctuaciones hormonales, especialmente picos espontáneos de LH que puedan comprometer la obtención de los ovocitos por una ovulación prematura.
3. Inducir la maduración folicular, mediante la adición de distintos fármacos que faciliten tanto el reclutamiento ovocitario como la maduración necesaria para el desarrollo posterior.
4. Disminuir los riesgos asociados a los tratamientos de estimulación ovárica.

Los protocolos más empleados de estimulación ovárica se inician en ausencia de actividad folicular, confirmada mediante ecografía transvaginal y valores de estradiol en sangre inferiores a 60 pg/ml. Existen dos esquemas básicos de estimulación: de dosis fija o sostenida y *step down.* En el esquema dosis fija o sostenida la pauta de gonadotropinas se mantiene hasta la aplicación del inductor de la ovulación, mientras que en el protocolo *step down,* la cantidad administrada disminuye cuando los folículos llegan a un diámetro superior a 12 mm, imitando el perfil de concentraciones de gonadotropinas durante las diferentes fases del ciclo.

Uno de los riesgos de los protocolos de estimulación ovárica es la elevación de los niveles de LH (pico espontáneo de LH) debido a los niveles de estradiol, lo cual desencadenaría la ovulación espontánea. El riesgo de ovulación espontánea de los protocolos iniciales se disminuyó gracias al empleo de los agonistas de la GnRH inicialmente, y posteriormente de los antagonistas de la GnRH, que permiten un mejor control de la estimulación ovárica (Timmons *et al.*, 2019).

Los agonistas de la GnRH son fármacos que inicialmente se unen a los receptores hipofisarios, induciendo la liberación de gonadotropinas, agotando la reserva hipofisaria

de estas hormonas y provocando el efecto *flare-up*. Pasada una semana del efecto *flare-up*, se observa una supresión sobre los receptores hipofisarios, provocando un estado hipogonadotrófico y evitando el riesgo del pico de LH. Los protocolos de estimulación empleando agonistas de la GnRH varían de acuerdo al inicio respecto al ciclo menstrual, aprovechándose o no el efecto *flare-up* (Conn & Crowley, 1994).

1. El protocolo largo se puede iniciar en fase folicular o fase lútea del ciclo previo al de estimulación. A partir de los 10 días de estimulación con el agonista se evalúa la supresión ovárica por medio de ecografía y análisis de estradiol. Confirmada la supresión ovárica, se disminuye la dosis de análogo y se inicia la administración de gonadotropinas hasta alcanzar los criterios de inducción de la ovulación (Thi Minh Le *et al*., 2019; Wu *et al*., 2021).
2. El protocolo corto comienza durante la fase folicular. El efecto del agonista junto con el de las gonadotropinas endógenas propias de la fase folicular permite un aumento en el reclutamiento folicular. La administración del agonista continúa hasta que se provoque la inducción de la ovulación (Kumar & Sharma, 2014;Wu *et al*., 2021).

Los antagonistas de la GnRH son fármacos que compiten por el mismo receptor que la GnRH pero sin desencadenar respuesta, por lo que se produce una inhibición de las gonadotropinas sin producirse el efecto *flare up* de los agonistas. Además, la acción de los antagonistas puede revertirse por la administración de análogos de la GnRH. Existen dos tipos de protocolos en función de las dosis utilizadas:

1. Protocolos de dosis múltiples, en los que se administra el antagonista durante varios días. Los protocolos de dosis múltiples pueden ser rígidos, que se inician el día 5-6 de la estimulación, o flexibles, que se inician si se alcanza un tamaño folicular de 14 mm o niveles de estradiol superiores a 150 pg/ml durante 48h (Thi Minh Le *et al*., 2019; Yang *et al*., 2021).
2. Protocolos de dosis única, en los que se administra una dosis única de 3 mg al alcanzarse los criterios de aplicación, repitiéndose si la estimulación se prolonga más de 3 días desde su administración (Thi Minh Le *et al*., 2019; Yang *et al*., 2021).

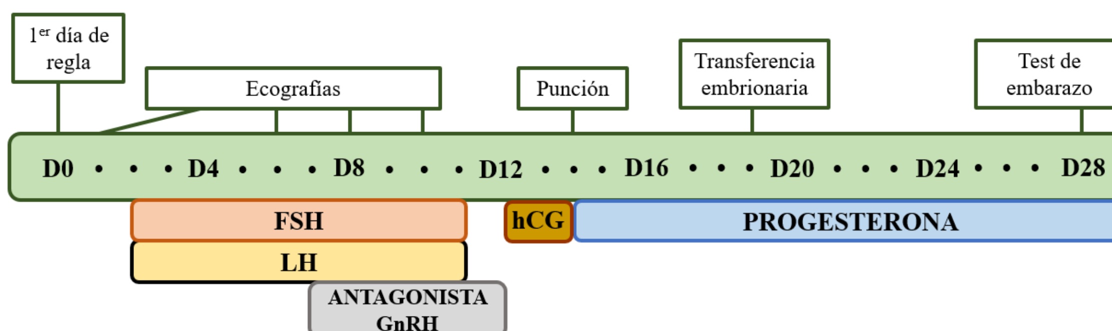

Figura 6. Infografía correspondiente a la estimulación ovárica empleando un protocolo de dosis múltiples con antagonistas. La estimulación comienza después del sangrado menstrual seguido de una ecografía para comprobar la actividad ovárica. Posteriormente se inicia la estimulación ovárica con FSH, pudiendo emplearse LH en el caso de que fuera necesario. 7 días después del sangrado menstrual, se administra el agonista de la GnRH durante varios días. Tras la observación de al menos tres folículos de un diámetro superior a 17 mm y con niveles de estradiol en sangre entre 500 y 3000 pg/ml, se administra el desencadenante de la ovulación 36 h antes de la punción. En los ciclos con ovocitos propios y transferencia en el mismo ciclo, el día de la punción puede ser necesario apoyar el mantenimiento de la fase lútea progesterona hasta la prueba de embarazo. En el caso de las donantes, después de la punción se llevan a cabo controles ecográficos y hormonales valorando la recuperación de la fisiología basal.

La inducción de la ovulación se lleva a cabo mediante la administración de gonadotropina coriónica humana (hCG). El momento óptimo de aplicación de la hCG se controla mediante observación ecográfica o análisis de estradiol, administrándose cuando se observen 3 o más folículos de un tamaño de 17 mm o superior, o con niveles de estradiol entre 500 y 3000 pg/ml. En caso de obtener valores de estradiol superiores o equivalentes a 3000 pg/ml, existe riesgo de hiperestimulación ovárica, que puede controlarse mediante administración de un análogo de la GnRH, aunque su efectividad está limitada a protocolos de estimulación ovárica con antagonistas (Herbemont *et al*., 2019; Maged *et al*., 2021). La administración de la hCG debe programarse entre 34 y 36 horas antes de la aspiración folicular o punción ovárica (Ding *et al*., 2017).

La aspiración folicular es una intervención sencilla, de corta duración y que se realiza bajo anestesia. Una vez realizada la sedación, el ginecólogo procede a la aspiración folicular guiado por un ecógrafo transvaginal equipado con una aguja de aspiración encargada de acceder a los folículos. Los ovocitos considerados maduros suelen estar asociados a folículos cuyo diámetro sea de 17 mm, y son recogidos mediante la aguja de aspiración hacia un tubo calefactado a 37º C que es trasladado al laboratorio de embriología para recuperar los complejos cúmulo ovocito.

Donación de ovocitos

Las técnicas de obtención de ovocitos posibilitan la donación de ovocitos, mediante la cual el gameto femenino es aportado por una mujer distinta a la que recibirá este o el embrión resultante (Lutjen *et al*., 1984; Trounson *et al*., 1983). La donación de ovocitos se emplea fundamentalmente para contrarrestar la baja competencia ovocitaria en pacientes de edad avanzada (Yeh *et al*., 2014), en parejas lesbianas en las que una mujer gesta el embrión generado a partir de un ovocito de su pareja, y en diversas situaciones clínicas como el fallo ovárico precoz, menopausia prematura o quirúrgica, afectaciones genéticas o fallos en los tratamientos de reproducción asistida con gametos propios que no han podido ser resueltos mediante diagnóstico genético, como fallos de fecundación, implantación o abortos de repetición (Blanchette, 1993; Lydic *et al*., 1996; Shah & Nagarajan, 2014). La tasa de gestación clínica en las donaciones de ovocitos es superior al 50 % independientemente de la indicación por la que se recurra a esta técnica (Remohí *et al*., 2017; Remohí *et al*., 1997). El proceso de donación de ovocitos suele iniciarse con la sincronización entre la donante y la receptora. La sincronización permite que el endometrio de la receptora sea receptivo al embrión de la donante y pueda transferirse directamente sin necesidad de criopreservación. Una vez extraídos los ovocitos de la donante, se realiza la fecundación (homóloga o heteróloga), el cultivo *in vitro* y la transferencia del embrión con mejor pronóstico de implantación (Melnick & Rosenwaks, 2018). La receptora debe recibir un tratamiento que estimula el crecimiento del endometrio, región uterina donde se producirá la recepción e implantación del embrión y que será descrito en la sección de transferencia de embriones (Glujovsky *et al*., 2020; Grzegorczyk-Martin *et al*., 2012; Madero *et al*., 2016).

El marco legal entorno a la donación de ovocitos en España se ha ido fraguando desde 1998, siendo en la actualidad regulada por la Ley 14/2006, de 26 de mayo, sobre las técnicas de reproducción humana asistida y los Reales Decretos 9/2014, de 4 de julio, y 412/1996, de 1 de marzo. La donación de ovocitos debe que cumplir aspectos legales y sanitarios, La donante debe tener entre 18 y 35 años, además de presentar buena condición de salud, tanto física como psicológica, por lo que previo a su aceptación como donante han de realizarse pruebas que permitan evaluar su estado de salud, tanto desde un punto de vista reproductivo (buena reserva ovárica, test genéticos recomendados normales etc.), no ser adoptada (debido a que se desconocerían antecedentes familiares) y no tener más de seis hijos nacidos en España. La donación de ovocitos debe ser voluntaria, altruista y

totalmente anónima, salvo en aquellas situaciones donde pueda ponerse en peligro la salud del hijo/a o situaciones de procesamiento penal. Es por ello que, pese a la situación económico-legal, la donante de ovocitos debe firmar un consentimiento informado previo a la realización de la donación justificando de este modo que lo hace de forma voluntaria y libre de coacción. Sin embargo, pese a que las condiciones legales y sanitarias de la donante cumplan con lo establecido, no se puede asegurar la fertilidad de dicha donante, que sólo se considera probada cuando la propia donante o alguna de sus donaciones haya concluido con un recién nacido vivo sano.

En España, según los datos del Registro de la Sociedad Española de Fertilidad del año 2018 se realizaron 32.746 tratamientos de recepción de ovocitos donados incluyendo pacientes de origen nacional e internacional. Estos tratamientos de donación de ovocitos suponen entre un 20-30% de todos los tratamientos iniciados en nuestro país. La principal razón de que estas cifras vayan en aumento es el mayor porcentaje de éxito comparado con tratamientos con ovocitos propios, ya que superan el 50% de éxito en términos de embarazo por ciclo comparado con el 36% en tratamientos con ovocitos propios (Chen & Wallach, 1994; Remohí *et al*., 1997; Sociedad Española de Fertilidad, 2019).

Maduración in vitro de ovocitos humanos

Aunque la recuperación de ovocitos maduros es la técnica más empleada para la obtención de ovocitos en humanos, la estimulación ovárica asociada puede suponer un riesgo médico en ciertos casos, en los cuales se puede recurrir a la obtención de ovocitos inmaduros, una técnica que no requiere estimulación ovárica (Hart & Walls, 2018; Sauerbrun-Cutler *et al*., 2015; Smitz *et al*., 2011; Walls & Hart, 2018). La principal aplicación de la maduración *in vitro* en humanos consiste en su empleo en pacientes con síndrome de ovario poliquístico, en los que existe un elevado riesgo de síndrome de hiperestimulación ovárica al realizar el tratamiento hormonal necesario para obtener ovocitos maduros. En este sentido, la maduración *in vitro* puede considerarse como una alternativa segura y sencilla a la obtención de ovocitos maduros (Hart & Walls, 2018; Sauerbrun-Cutler *et al*., 2015; Smitz *et al*., 2011). Además de su aplicación en pacientes con trastornos hormonales con alto riesgo de hiperestimulación ovárica, también es aplicable a mujeres que padezcan distintos tipos de cáncer (De Vos *et al*., 2014; Noyes *et al*., 2011; Smitz *et al*., 2011). En el caso de niñas y mujeres en edad reproductiva los tratamientos de cáncer ponen en riesgo el deseo de una futura maternidad. En estos casos,

las pacientes oncológicas pueden recurrir a procesos de preservación de fertilidad si desean tener descendencia genéticamente propia (De Vos *et al*., 2014; Donnez & Dolmans, 2017; Taylan & Oktay, 2019) y la estimulación ovárica necesaria para la recuperación de ovocitos maduros puede retrasar el tratamiento oncológico. Este retraso en el tratamiento se evita al realizar la obtención de ovocitos inmaduros, que además evita el empleo de hormonas que podría estimular a las células de determinados tipos de tumores. En este sentido, la maduración *in vitro* permite preservar ovocitos procedentes de pacientes oncológicas en edad fértil, mientras que en niñas prepúberes puede recurrirse a la preservación de tejido ovárico (Donnez & Dolmans, 2017; Taylan & Oktay, 2019).

La técnica de maduración *in vitro* precedió a la obtención de folículos maduros, siendo la elegida para lograr el primer nacimiento vivo sano en la especie humana (Yul Cha *et al*., 1991). Este hito supuso un primer paso para la utilización de la maduración *in vitro* en reproducción asistida, lográndose el nacimiento de miles de bebés empleando esta técnica madurativa en el laboratorio (Hart & Walls, 2018; Sauerbrun-Cutler *et al*., 2015). Sin embargo, la principal limitación de la técnica es que las tasas de éxito son más bajas, siendo del 10%-15% por embrión transferido, muy por debajo de los obtenidos mediante FIV convencional o ICSI, cercanos al 45%. Además, aunque se han logrado múltiples nacimientos de niños tras la maduración *in vitro* y transferencia en fresco, existen pocos casos de éxito tras la criopreservación de ovocitos madurados *in vitro* (Child *et al*., 2002).

Fecundación in vitro y microinyección intracitoplásmica del espermatozoide.

Las técnicas de reproducción asistida más empleadas son la fecundación *in vitro* (FIV) y la microinyección intracitoplásmica del espermatozoide (ICSI por sus siglas en inglés *Intra Cytoplasmic Sperm Injection*). Estos procedimientos ponen en contacto los gametos masculinos y los femeninos, logrando así un desarrollo embrionario fuera del aparato reproductor femenino (Palermo *et al*., 1992; Steptoe & Edwards, 1978).

La fecundación *in vitro* convencional o FIV, originalmente fue ideada para salvar un obstáculo tubárico bilateral, aunque terminó extendiéndose a otras patologías reproductivas como los fallos de inseminación artificial, disfunción ovárica, endometriosis, etc. Esta técnica consiste en simular la fecundación del ovocito en condiciones de cultivo *in vitro*, tras obtener y preparar los gametos, para posteriormente realizar la transferencia embrionaria (de Ziegler *et al*., 2019; Jankowska, 2017; Steptoe

& Edwards, 1978). La elección de la FIV convencional depende de los parámetros obtenidos en el seminograma, que debe presentar unos valores mínimos después de haber procesado la muestra: buena movilidad (superior al 50 %), concentración superior a 1 millón de espermatozoides/ml y un máximo de 3 % de espermatozoides con formas normales, datos correspondientes en muchos casos a fallos en tratamientos con inseminación artificial (Ruiz *et al*., 1997).

El primer paso de la FIV convencional consiste en la obtención los ovocitos mediante punción ovárica tras estimulación hormonal de la paciente, como ya se ha explicado (Fatemi & Garcia-Velasco, 2015; Frydman & Nargund, 2014; Mansour *et al*., 1994; Youssef *et al*., 2014). Una vez recuperados los ovocitos se valorará el grado de inmadurez a través del análisis en la expansión de los complejo cúmulo-ovocito (Ng *et al*., 1999), aunque esta expansión no siempre se correlaciona con el estado de maduración ovocitario (Gardner *et al*., 2013; Laufer *et al*., 1984). Al mismo tiempo que se analizan los complejo cúmulo-ovocito se efectúa un seminograma siguiendo las recomendaciones de la Organización Mundial de la Salud. Seguidamente se capacitará la muestra y se realizará el conteo de espermatozoides para ajustar la concentración de espermatozoides en la gota donde se realizará la fecundación *in vitro* a aproximadamente 10^5 espermatozoides/ml (Remohí *et al*., 2017). La FIV debe realizarse entre cuatro y seis horas después de la punción folicular.

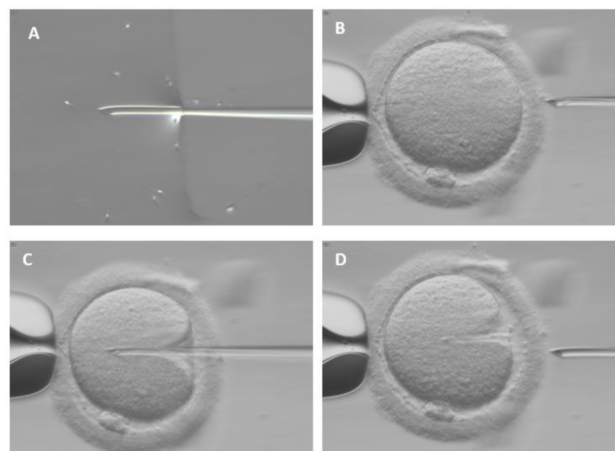

Figura 7. Secuencia del proceso de realización de una microinyección espermática. La imagen ***A*** corresponde con la selección del espermatozoide a mediante la pipeta de ICSI. En la imagen ***B***, el ovocito ha sido fijado gracias a la pipeta *"holding"* situando el corpúsculo polar en posición perpendicular a la recta imaginaria establecida por las dos pipetas. La imagen ***C***, muestra la introducción de la pipeta de ICSI y absorción posterior del oolema para provocar su ruptura. Finalmente, en la imagen ***D*** se observa la deposición del espermatozoide en el interior del ovocito (Ross, 2013). *(Imágenes cedidas por la clínica IVF Spain Madrid).*

Pasadas 17-20 horas deberá valorarse si la fecundación ha sido exitosa, retirando previamente las células del cúmulo que pudieran quedar adheridas mediante pipeteo con una pipeta de diámetro variable (Huang *et al.*, 2013; Zhang *et al.*, 2013). Transcurridos los 5 o 6 días del desarrollo embrionario se realizará la transferencia embrionaria mediante la selección del mejor embrión según criterios morfológicos, aunque también pueden emplearse otros criterios como la cinética embrionaria (Gardner *et al.*, 2013; Glujovsky *et al.*, 2016).

La microinyección intracitoplasmática de espermatozoides (ICSI) consiste en la introducción de un espermatozoide en el interior de un ovocito que previamente ha sido decumulado (Palermo *et al.*, 1992). Actualmente la mayor parte de los tratamientos se orientan hacia el empleo de ICSI por diversos motivos, asociados a la calidad de la muestra seminal y al diagnóstico de la mujer o el historial reproductivo de la pareja. Una de las principales ventajas de ICSI es el poder realizar un tratamiento de reproducción asistida en casos donde el factor masculino presenta una alteración de moderada a grave (azoospermia, espermatozoides procedentes de biopsias testiculares, recuperación seminal baja, oligoastenoteratozoospermia, etc.). En otras variantes de los tratamientos de reproducción asistida como el diagnóstico genético preimplantacional indicado en casos de fallos de implantación, abortos o alteraciones genéticas- se ha recomendado realizar ICSI para evitar cualquier tipo de contaminación, ya sea de células de la granulosa o de espermatozoides, que pudiera alterar el resultado del diagnóstico genético (Remohí *et al.*, 2017). La microinyección espermática también es la técnica de elección en tratamientos donde los ovocitos han sido previamente criopreservados, madurados *in vitro*, o cuando otros tipos de tratamientos como la inseminación artificial o la fecundación *in vitro* convencional han fracasado (Crosignani *et al.*, 2007; Remohí *et al.*, 2017; Stern & Nangia, 2020).

El procedimiento de la microinyección espermática es similar al de la FIV convencional, con algunas particularidades. Los complejos cúmulo-ovocitos han de ser denudados para comprobar el correcto estadio de madurez (metafase II). Trascurrido un tiempo después de la denudación, tiene lugar la microinyección en un microscopio invertido, donde serán necesarias una placa, una pipeta de sujeción y una pipeta de microinyección. Una vez realizada la preparación previa, se seleccionarán los espermatozoides para su posterior microinyección en el citoplasma del ovocito (Esteves *et al.*, 2018; Karabulut *et al.*, 2019; Palermo *et al.*, 1992; Rubino *et al.*, 2016).

La selección de FIV o ICSI depende, en la mayoría de los casos, del diagnóstico de cada paciente. La elección de cada técnica no viene preestablecida por el éxito que tengan, ya que diversos estudios mostraron que, ante diagnósticos similares, los resultados no presentan diferencias estadísticamente significativas. En la mayoría de los centros de reproducción asistida, la elección de la técnica está asociada a reducir el riesgo de producirse un fallo de fecundación, por lo que se opta por asegurarse la introducción del espermatozoide en el interior del ovocito (Crosignani *et al*., 2007; Drakopoulos *et al*., 2019; Gozlan *et al*., 2007; Haas *et al*., 2021; Ruiz *et al*., 1997; Yang *et al*., 1996).

Transferencia de embriones

La transferencia embrionaria representa la acción mediante la cual el/los embrión/es se deposita en el interior de la cavidad endometrial, suponiendo el último obstáculo para la consecución de la gestación en los procesos de reproducción asistida. Existen numerosos factores que pueden afectar al éxito del proceso de transferencia embrionaria como son el número y la calidad de los embriones transferidos (Crosignani *et al*., 2000; Cuevas Saiz *et al*., 2018; Pons *et al*., 2014), la presencia de sangre o moco en el catéter de transferencia (Alvero *et al*., 2003; Goudas *et al*., 1998), fallos de implantación precios o la contractilidad uterina entre otros (Awonuga *et al*., 1998; Phillips *et al*., 2013). Además, ciertos aspectos instrumentales o metodológicos como serían el tipo de cánula (Buckett, 2006; Van Weering *et al*., 2002), la monitorización ecográfica, la propia dificultad del proceso o la experiencia del especialista pueden tener cierta repercusión en la obtención de resultados satisfactorios (Phillips *et al*., 2013; Schoolcraft *et al*., 2001).

La exitosa realización de la transferencia de embriones depende de la calidad embrionaria y también de la receptividad endometrial, además de la pericia de los profesionales que la realizan. La preparación endometrial resulta de gran importancia, especialmente en los tratamientos con ovocitos donados donde, además de la sincronización donante-receptora, se administran inicialmente estrógenos y posteriormente progesterona para mantener el espesor endometrial adecuado y apoyar la fase lútea. La aplicación inicial de estradiol persigue obtener un espesor endometrial de 6 mm durante los primeros 7 días desde el inicio de la estimulación. Si se obtiene dicho espesor se continúa la pauta de administración, en el caso de que sea menor se incrementa la dosis de estradiol. El soporte de la fase lútea con progesterona se inicia la noche de la punción ovárica de la donante,

administrándose progesterona hasta la prueba de embarazo (Lawrenz *et al*., 2019; van der Linden *et al*., 2015).

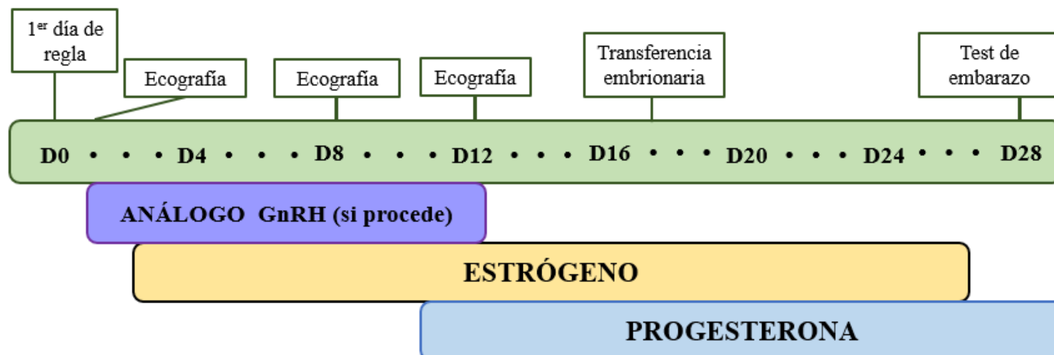

Figura 8. Esquema correspondiente a la preparación endometrial previa al proceso de transferencias de embriones criogenizados y de receptoras de ovocitos. La preparación se inicia después del sangrado menstrual con una ecografía para comprobar la actividad ovárica. Posteriormente se inicia la estimulación del endometrio con estrógenos, y análogos de la GnRH si fuera necesario. Durante el crecimiento endometrial se llevará a cabo diferentes controles ecográficos para comprobar el espesor del endometrio. Una vez que el endometrio ha alcanzado un espesor óptimo se inicia la ingesta de la progesterona, 5 días antes de la transferencia embrionaria, manteniéndose hasta la prueba de embarazo.

La transferencia embrionaria es un procedimiento considerado invasor, por lo que requiere que las condiciones sean lo más asépticas posible. El objetivo principal es realizar la transferencia de embriones con la menor afectación posible, precisamente para evitar contracciones uterinas que expulsen al embrión fuera de la cavidad, así como minimizar el riesgo de afectación por agentes externos a los embriones. El proceso se inicia con la colocación del espéculo y limpiando el exceso de progesterona y moco cervical existente, para posteriormente iniciar la ecografía abdominal sobre la paciente. Una vez localizada la línea endometrial, se introduce el catéter guía a través del orificio cervical externo hasta haber alcanzado el orificio cervical interno. Una vez alcanzado este punto con el catéter guía se procede a cargar los embriones en el catéter de transferencia, el cual pasará a través del catéter guía llevando la punta a dos centímetros del fondo uterino. Una vez la localización uterina se considera la adecuada, se procede a la descarga de los embriones, extrayendo ambos catéteres y comprobando que en ningún caso se ha quedado el embrión retenido en la cánula (Schoolcraft *et al*., 2001).

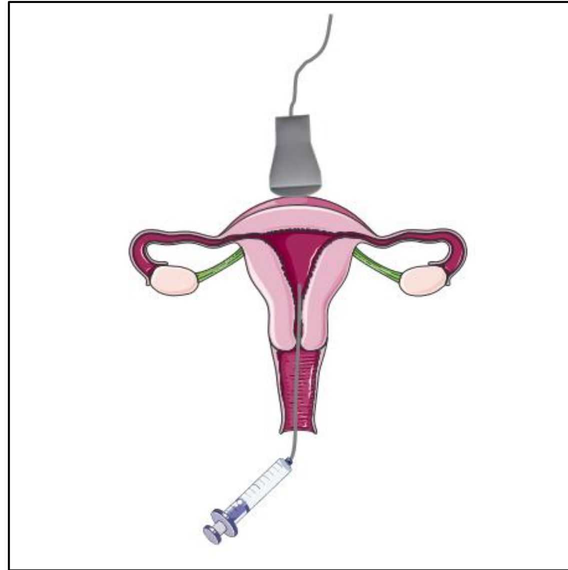

Figura 9. Infografía correspondiente a la ecografía y el proceso de transferencia. Se trata de un procedimiento clave en reproducción asistida en el que gracias a la ecografía se deposita el embrión en la cavidad endometrial por medio de varios catéteres. (*Imagen modificada de Servier Medical Art: https://smart.servier.com/*).

Extracción espermática

En reproducción asistida, algunos varones presentan alteraciones de diferente índole que hacen necesaria la extracción de los espermatozoides directamente del aparato reproductor masculino, ya sea del tejido testicular, del epidídimo o de otro segmento de la vía seminal, para su criopreservación y posterior utilización en tratamientos de reproducción asistida (Schoysman *et al*., 1993).

La extracción espermática se realiza con una doble finalidad: reproductiva y diagnóstica. La mayoría de estos procedimientos para conseguir espermatozoides tienen como objetivo realizar una fecundación, pero las biopsias testiculares también son útiles para realizar estudios histológicos y citogenéticos, con la finalidad de aportar un diagnóstico y/o información complementaria sobre la pareja infértil (Amer *et al*., 1999; Ben-Yosef *et al*., 1999). A nivel reproductivo, esta técnica se realiza principalmente para obtener espermatozoides de pacientes con ausencia u obstrucción de los conductos deferentes, fibrosis quística, infecciones, vasectomías, así como fallos de implantación y/o abortos de repetición debidos a índices de fragmentación del ADN espermático alterados (Dohle *et al*., 2012; Lopes & Esteves, 2019).

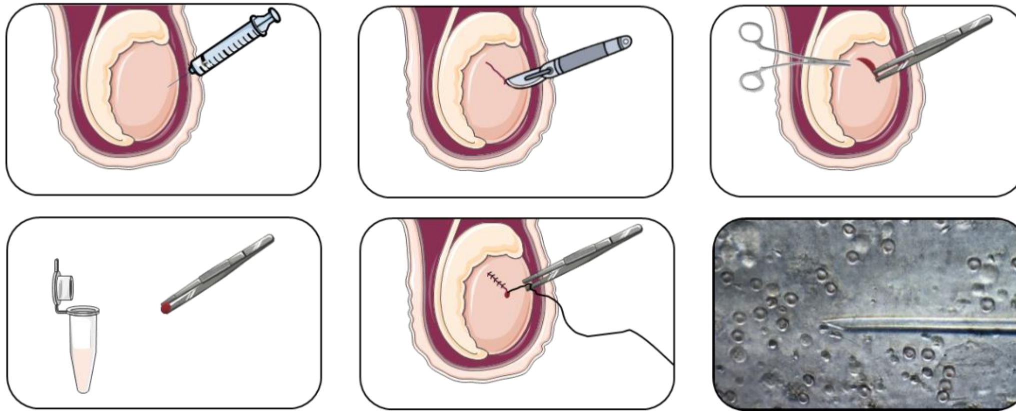

Figura 10. Secuencia gráfica de una extracción espermática testicular o biopsia testicular (TESE). El procedimiento se inicia con la anestesia local para insensibilizar la zona. Seguidamente se efectúa una incisión a través de la piel y se extrae una pequeña muestra de tejido testicular y se ponen los puntos de sutura correspondientes. La muestra extraída es observada al microscopio comprobando la existencia de espermatozoides antes de realizar la técnica que corresponda (*Imagen modificada de Servier Medical Art: https://smart.servier.com/. Imagen de biopsia testicular cedida por IVF Spain*).

Las dos modalidades más utilizadas son:

- Extracción espermática testicular (*TESE Testicular Sperm Extraction*) o biopsia testicular. Es una intervención quirúrgica superficial basada en la extracción de una o varias porciones de tejido mediante incisiones en los testículos. A continuación, estas porciones son disgregadas y observadas en el microscopio para detectar la presencia o ausencia de espermatozoides, previo paso al procesamiento para su uso reproductivo o su criopreservación (Schoysman *et al*., 1993).
- Microdisección o microtese (*Microdissection Testicular Sperm Extraction*). Se trata de una técnica similar al TESE donde se utiliza el microscopio quirúrgico en el campo para identificar y seleccionar de manera individualizada los túbulos seminíferos que tengan más probabilidad de presentar una espermatogénesis activa (Schlegel, 1999).
- Aspiración espermática testicular (*TESA Testicular Sperm Aspiration*). Consiste en la punción con aguja en varias zonas del testículo. Esta técnica permite obtener espermatozoides del epidídimo por lo que su exitosa realización evita tener que recurrir a TESE y MicroTESE, las cuales son más complejas, costosas y suponen mayores molestias para el paciente (Lewin *et al*., 1996).

La extracción de espermatozoides ha demostrado tener unas tasas de éxito muy similares a las obtenidas empleando espermatozoides de eyaculado, especialmente cuando se trata

de azoospermias obstructivas (Mercan *et al*., 2000). Sin embargo, patologías relacionadas con la producción espermática del testículo pueden tener un porcentaje más bajo debido a que la calidad celular suele estar disminuida (Donoso *et al*., 2007).

El carácter invasivo de la extracción de espermatozoides, en cualquiera de sus vertientes, supone riesgos asociados a las complicaciones de las técnicas, como puede ser la inflamación del escroto, aparición de hematomas, complicaciones hemorrágicas o infecciosas. Además, algunas patologías genéticas que concurren en azoospermias de distinta índole tienen el riesgo congénito asociado en su transmisión a la descendencia, por lo que es necesario realizar estudios urológicos y genéticos previos a este tipo de intervenciones (Dohle *et al*., 2012; McLachlan *et al*., 2007).

Preservación de la fertilidad

La preservación de la fertilidad comprende un conjunto de tratamientos en los que se pretende congelar gametos para su utilización posterior. Las técnicas de preservación de la fertilidad surgieron en un inicio como metodología para permitir mantener la capacidad fértil en un nicho de pacientes concreto: pacientes que pudieran sufrir la pérdida parcial o total reproductiva debido a procesos quirúrgicos o tratamientos agresivos (pacientes oncológicos) (Chen, 1986; Noyes *et al*., 2011). De este modo, se permite preservar un conjunto de gametos que, si bien no asegura la consecución de un embarazo, facilita lograrlo sin tener que recurrir a los gametos donados (Argyle *et al*., 2016; Fabbri *et al*., 1986).

Existen distintos tratamientos de preservación de fertilidad:

- Congelación de ovocitos: Este proceso generalmente consiste en la realización de un tratamiento de estimulación ovárica para conseguir recuperar un número de ovocitos madurados óptimo que serán criopreservados mediante la vitrificación. Esta técnica requiere de una cierta madurez ovárica y por ello se emplea en mujeres jóvenes que se enfrentan a tratamientos oncológicos (Hipp *et al*., 2019; Noyes *et al*., 2011) , aunque como ya se ha comentado también se puede realizar con ovocitos madurados *in vitro* (De Vos *et al*., 2014; Hart & Walls, 2018; Sauerbrun-Cutler *et al*., 2015; Smitz *et al*., 2011; Walls & Hart, 2018). Este procedimiento ha ido optimizándose con el tiempo, llegando a desarrollarse protocolos como el *dual stim* que consiste en la estimulación doble, obteniendo

ovocitos durante la fase lútea (Luo *et al*., 2020; Tsampras *et al*., 2017). En los últimos años se ha producido un incremento de pacientes sin patologías cuyo objetivo es posponer la maternidad, conociendo las dificultades que puede acarrear el ser madre pasados los 35 años (Cobo *et al*., 2016; Cobo & García-Velasco, 2016). También se puede realizar este tipo de tratamientos en personas que presentan disforia de género y tengan deseo reproductivo, por lo que se recomendaría realizar esta preservación antes de que se pudieran iniciar tratamientos hormonales y/o quirúrgicos que puedan alterar su fertilidad (Ainsworth *et al*., 2020; Chen *et al*., 2018).

- Congelación de tejido ovárico: El desarrollo de esta técnica se inició con niñas que debían hacer frente a tratamientos oncológicos que pudieran afectar a la capacidad reproductiva, pero debido a su dificultad sigue sin ser considerada como una metodología de rutina. La congelación del tejido ovárico consiste en extraer mediante cirugía parte del tejido ovárico que será congelado y para ser reimplantado en la paciente después del tratamiento oncológico (Arav & Patrizio, 2019; Kristensen & Andersen, 2018; Rivas Leonel *et al*., 2019).
- Congelación de semen: Al igual que los procesos anteriores, surgió como método de preservación para pacientes con patologías que disminuían la fertilidad de los varones (McBride & Lipshultz, 2018). Otras situaciones pueden ser la preservación por tratamiento hormonal y/o quirúrgico en casos de disforia de género y por la disminución en la calidad seminal (Moravek, 2019). Este procedimiento consiste en la obtención de espermatozoides, ya sea por masturbación, electroeyaculación, aspirado del epidídimo o biopsia testicular, para su posterior tratamiento con crioprotectores y congelación (Di Santo *et al*., 2012; Grin *et al*., 2021).

Técnicas de reproducción asistida en ganado bovino

La especie bovina constituye el animal de granja en el que se aplican de forma más extendida una mayor variedad de técnicas de reproducción asistida. La elevada difusión de estas técnicas en la ganadería bovina es consecuencia del gran interés en la mejora genética del ganado bovino -principalmente en el ganado lechero, pero también en razas cárnicas-, del elevado precio del recién nacido comparado con otras especies de menor tamaño –que permite compensar el coste de determinadas técnicas que no tienen sentido

en otro tipo de producción- y en la capacidad económica del sector, con grandes empresas de genética operando a nivel global.

Inseminación artificial

La inseminación artificial (IA) es la técnica reproductiva más empleada para la mejora y conservación de recursos zoogenéticos. Esta técnica se basa en la obtención de un semen procedente de un macho concreto y genéticamente valioso para su posterior deposición (generalmente tras su conservación mediante congelación en el ganado bovino) en el aparato reproductor de la hembra con el objetivo de lograr una gestación. La principal ventaja de la IA es la aceleración de la mejora genética mediante la obtención de un mayor número de animales procedentes de sementales de alto mérito genético (Funk, 2006; Vishwanath, 2003), para lo cual es necesario obtener un alto número de dosis seminales de cada macho en un tiempo determinado y ser capaz de conservarlas para poder inseminar vacas en otros momentos y lugares (Colenbrander *et al*., 1993; Jewgenow *et al*., 2017). El desarrollo de las técnicas de criopreservación permitió el almacenamiento y conservación de muestras seminales durante un periodo de tiempo indefinido, así como el transporte del material genético con mayor facilidad, reduciendo drásticamente el número de sementales necesarios y dando lugar a grandes avances en la mejora genética (Bertolini & Bertolini, 2009; Colenbrander *et al*., 1993; Funk, 2006; Vishwanath, 2003). La IA en ganado vacuno se realiza de forma transcervical, depositando la dosis seminal en el cuerpo del útero. Para ello se introduce una cánula (pistola de inseminación o inyector) a través de la vagina atravesando el cérvix gracias a la manipulación del mismo a través del recto (Diskin, 2018; Thomas *et al*., 2021). Una vez atravesado el cérvix se depositan los espermatozoides en el cuerpo del útero de modo que puedan avanzar por ambos cuernos, aumentando las posibilidades de fecundar ovocitos procedentes de uno u otro ovario (Moore & Hasler, 2017; Stevenson & Britt, 2017).

La IA debe tener lugar en un periodo entre las nueve horas después de iniciarse el celo hasta seis horas después de su finalización, siendo el periodo más adecuado entre las 12 y 18 h de la aparición del celo (Diskin, 2018; Thomas *et al*., 2021). Para ello, la IA en bovino puede realizarse de forma natural, detectando el celo de las hembras para poder realizar la inseminación en el momento con mayor probabilidad de éxito; o bien mediante IA a tiempo fijo, empleando un tratamiento hormonal para controlar el momento de ovulación y permitir la inseminación de un mayor número de animales en un breve

espacio de tiempo. En el caso de la IA a celo natural, la detección del celo es de gran importancia. El celo de las vacas dura aproximadamente 18 horas, siendo por ello necesario realizar la detección de celos al menos dos veces al día. Teniendo en cuenta el periodo óptimo de inseminación, y dado que la detección de celos se suele realizar por la mañana y por la tarde la IA se realiza a las 12 h de la detección del celo, inseminando por la tarde a las vacas que entran en celo por la mañana y por la mañana a las que entran en celo por la tarde. La inseminación artificial a tiempo fijo consiste en intervenir el ciclo estral de la hembra bovina mediante hormonas, lográndose la ovulación de los animales en un mismo periodo. Este control se lleva a cabo bloqueando el ciclo estral durante 7-9 días mediante la administración de progesterona o progestágenos, generalmente combinada con control ecográfico o rotura de cuerpos lúteos mediante administración de análogos de prostaglandina F2α, tras la cual se reanuda el ciclo y tiene lugar la ovulación (Bó & Baruselli, 2014; Lamb *et al.*, 2006; Clifford Lamb & Mercadante, 2016). La IA a tiempo fijo se lleva a cabo entre las 52 y las 56 horas después de haberse retirado los dispositivos de liberadores de progesterona/progestágeno (Lindley *et al.*, 2021; Jeffrey S. Stevenson, 2016).

La técnica de inseminación artificial presenta ventajas y desventajas que deben tenerse en cuenta durante su implementación. Entre las ventajas existentes, reside no solo la aceleración de la mejora genética, sino también la eliminación del riesgo de transmisión de enfermedades relevantes como la tricomoniasis y la posibilidad de utilizar con semen sexado (Bermejo-Álvarez *et al.*, 2008; Johnson *et al.*, 1989; Seidel, 2014). Las principales desventajas están relacionadas con la necesidad de pericia técnica, de detección o sincronización de celos y de relativa docilidad del semental y la hembra para la recolección de semen e inseminación (Hamid *et al.*, 2021; Ibrahim *et al.*, 2014).

Superovulación (MOET)

Del mismo modo que la IA permite la obtención de un gran número de descendientes de un toro de alto mérito genético, la obtención de embriones de una hembra de alto mérito genético para su transferencia a hembras de menor mérito genético permite ampliar la descendencia que dicha hembra tendría de forma natural, sirviendo para acelerar la mejora genética. Para este fin, el procedimiento más empleado conlleva la superovulación de la hembra donante, de la cual se recogen los embriones mediante lavado uterino y se

transfieren en fresco o congelados a otras hembras. Este procedimiento se denomina MOET por sus siglas en inglés *Multiple Ovulation Embryo Transfer*.

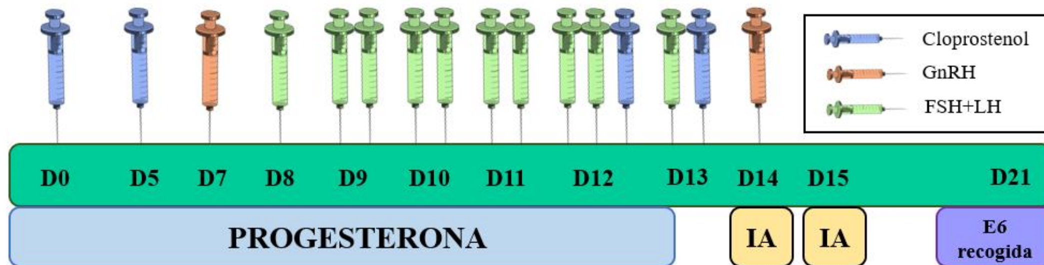

Figura 11. Esquema de un protocolo convencional de superovulación bovina. El empleo de un análogo de GnRH (cloprostenol) elimina los posibles cuerpos lúteos, mientras que la progesterona impide el crecimiento folicular endógeno. La estimulación ovárica se realiza con 10 inyecciones en dosis decrecientes y se utiliza un análogo de GnRH para asegurar la ovulación. *(Imagen adaptada de* (Ramos-Ibeas *et al*., 2020)*)*.

La superovulación consiste en el aumento fisiológico de las ovulaciones propias de la especie debido a la administración de fármacos (Mogollón Waltero & Burla Dias, 2013) con el objeto de obtener un mayor número de embriones de una hembra de interés (Daly *et al*., 2020; Palma, 2013). El procedimiento consiste en estimular el reclutamiento folicular mediante hormonas como la gonadotropina coriónica equina (eCG o PMSG) y la hormona folículo estimulante (FSH), que son aplicadas entre los días 8 y 12 del ciclo estral en la especie bovina (Baruselli *et al*., 2019; Fallis, 2013). La utilización de la eCG permite una respuesta superovulatoria con una dosis, pero posee un periodo de vida largo, teniendo implicaciones en el crecimiento dispersos de los folículos y los niveles de estrógenos (Kimura *et al*., 2007; Takedomi *et al*., 1995). Esta situación estrogénica, además de poder inducir la formación de quistes en el ovario, puede comprometer los primeros estadios embrionarios debido a una disminución de la calidad ovocitaria y, por ende, embrionaria (Goulding *et al*., 1996). Por este motivo, generalmente se emplean preparaciones de FSH, aunque requieren un mayor número de inyecciones. Los protocolos convencionales administran dos dosis diarias en cantidades decrecientes durante cuatro o seis días sumando un total de 700 Unidades Internacionales de FSH por animal (Figura 11). Aunque estos protocolos son los más empleados y los recomendados por los suministradores de hormonas, distintos estudios sugieren que el empleo de tres dosis o incluso una única dosis puede ser eficaz en determinadas condiciones (Demoustier *et al*., 1988; Fallis, 2013; Takedomi *et al*., 1995). En cualquier caso, la eficacia de los protocolos de superovulación bovina basados en FSH es variable en función de las

variaciones de FSH-LH de las preparaciones entre distintas marcas comerciales y lotes dentro del mismo proveedor (Bó & Mapletoft, 2014; Deguettes *et al.*, 2020; Mikkola *et al.*, 2019).

Obtención de embriones in vivo

Los primeros protocolos de obtención de embriones bovinos tras el tratamiento de superovulación y la inseminación eran quirúrgicos e implicaban posibles riesgos como la formación de adherencias o infecciones (Givens & Marley, 2008; Hasler, 2014). Por ello, se desarrollaron métodos de lavado uterino no quirúrgicos, basados en la introducción de una sonda Foley la cavidad uterina por vía transcervical. Dicha sonda está acoplada a un sistema mediante el cual se introduce medio de lavado en el útero y se recoge en un filtro que retiene a los embriones. Para evitar el reflujo del líquido introducido en el útero hacia la vagina y la posible pérdida de embriones, la sonda lleva asociado un globo que bloquea la salida de líquido a través del cérvix. Las manipulaciones del cérvix para la introducción de la sonda y del útero para controlar su llenado y vaciado se realizan mediante palpación rectal, empleándose anestesia epidural para evitar molestias al animal (Baruselli *et al.*, 2010; Bó *et al.*, 2012; Phillips & Jahnke, 2016). La recogida de embriones *in vivo* se realiza en el estadio de mórula o blastocisto temprano, hacia el sexto día de desarrollo embrionario (séptimo desde la aparición del celo). La recogida en este estadio permite la obtención de embriones con la zona pelúcida intacta, un aspecto crítico para cumplir con los requisitos sanitarios de comercialización de embriones establecidos por la asociación internacional de transferencia de embriones (IETS) (Stringfellow, 2010).

Aspiración ovárica

La aspiración ovárica u OPU (*Ovum pick-up*) puede ser utilizada como alternativa a los protocolos MOET. Al igual que ocurre en humanos, la obtención de ovocitos inmaduros mediante punción sin estimulación ovárica requiere de maduración *in vitro*, estando intrínsecamente ligada a la producción de embriones *in vitro* descrita a continuación. Las motivaciones de realizar esta técnica en ganadería son lógicamente diferentes a las de su aplicación en humana. En bovino, la aspiración ovárica se emplea para obtener embriones de hembras de alto mérito genético (Daly *et al.*, 2020; Ferré *et al.*, 2020; Seneda *et al.*, 2020). Para este fin OPU tiene como ventaja frente a MOET que puede realizarse sin emplear hormonas, puede utilizarse en animales gestantes, y puede repetirse en intervalos

cortos, lo que facilita obtener un mayor número de embriones por hembra/año que MOET, siendo esta su principal ventaja (Baruselli *et al.*, 2012; Palma, 2001; Van Wagtendonk-De Leeuw, 2006). En concreto, se puede realizar hasta dos sesiones de OPU por semana, obteniéndose un promedio de 4-5 ovocitos por vaca y sesión (Palma, 2013; Seneda *et al.*, 2020; Van Wagtendonk-De Leeuw, 2006). Además, OPU se puede realizar en hembras jóvenes, acortando el intervalo inter-generacional y acelerando la mejora genética (Van Wagtendonk-De Leeuw, 2006).

El procedimiento de obtención de ovocitos es similar al humano, aunque obteniendo ovocitos inmaduros para aumentar el número de ovocitos obtenidos por sesión y la frecuencia de aspiraciones. La aspiración se realiza bajo anestesia epidural introduciendo el transductor con la aguja a través de la vagina orientados hacia el ovario a puncionar. Los ovocitos procedentes de la aspiración de folículos se recogen en un tubo calefactado y son madurados *in vitro* para producir embriones *in vitro* (Nagano, 2019; Seneda *et al.*, 2020).

Producción de embriones in vitro

El proceso de producción de embriones *in vitro* (PEIV) permite obtener embriones a partir de ovocitos obtenidos mediante aspiración ovárica (OPU) o mediante la aspiración de ovarios recogidos en matadero de hembras sacrificadas para consumo. Como se ha descrito anteriormente, la combinación de OPU-PEIV permite obtener un mayor número de embriones por hembra y año que la obtenida mediante MOET. El empleo de ovocitos aspirados de hembras sacrificadas en matadero lógicamente no sirve para obtener un gran número de embriones de la hembra sacrificada, pero tiene aplicaciones interesantes en investigación y ganadería. En investigación proporciona una fuente de embriones sin necesidad de recurrir al empleo de animales de experimentación, permitiendo el estudio de fallos reproductivos en la especie bovina y su uso como modelo para la embriología humana (Pérez-Gómez *et al.*, 2021). A nivel de ganadería, la PEIV a partir de ovarios procedentes de matadero permite la generación de embriones bovinos a un coste bajo, que pueden emplearse para realizar transferencias de embriones con el objeto de mejorar las tasas de fertilidad en condiciones de estrés térmico en los que la tasa de preñez es mayor realizando transferencia de embriones que realizando IA (Demetrio *et al.*, 2007; Pereira *et al.*, 2016), o para conseguir terneros de razas cárnicas preñando vacas lecheras. Ésta última aplicación posibilita generar preñeces en vacas lecheras de bajo mérito

genético (necesarias para la producción de leche) aumentando el valor del ternero generado, dado que el valor de los terneros de razas cárnicas de alto valor, como la raza Angus, es mucho más elevado que el de razas lecheras cuando se emplean para la producción de carne. Para esta aplicación, el semental empleado para la fecundación *in vitro* puede ser seleccionado de forma individual y, aunque la genética de la hembra no se puede seleccionar de forma individual, sí puede seleccionarse su raza.

La PEIV es un proceso que consta de tres etapas: la maduración de ovocitos, la fecundación y el cultivo embrionario. Los ovocitos inmaduros obtenidos mediante OPU o aspiración de ovarios recogidos en el matadero se obtienen de folículos de 2 a 8 mm de diámetro, que son receptivos a la acción de gonadotropinas. Los complejos cúmulo-ovocito recogidos se seleccionan en base a criterios morfológicos basados en la presencia y compactación de las células del cúmulo y en la apariencia del ovoplasma, siendo los de mejor calidad aquellos rodeados por más de 4 capas de células de la granulosa y mostrando un ovoplasma homogéneo (Hawk & Wall, 1994). La maduración *in vitro* tiene lugar durante 24 horas en medios específicos de maduración (Lonergan *et al*., 1996). Pasado este tiempo, se prepara la muestra de semen para la fecundación, añadiéndose una concentración de 10^6 espermatozoides/ml e incubándose en medio de fecundación capacitante durante unas 20 horas (Parrish *et al*., 1986). Transcurrida la fecundación, las células del cúmulo se eliminan, generalmente mediante agitación en vórtex, y los presuntos cigotos se incuban en medio de cultivo embrionario durante 7-9 días (Holm *et al*., 1999).

La aplicación de PEIV al manejo reproductivo bovino está limitada por tres condicionantes: 1) la necesidad de personal y equipamiento especializado para llevarla a cabo, 2) las bajas tasas de desarrollo embrionario y 3) la reducida supervivencia embrionaria post-transferencia de los embriones producidos *in vitro* con respecto a los producidos *in vivo.* La eficiencia del proceso de producción de embriones entendida como la proporción entre el número de blastocistos obtenidos y el número de ovocitos recogidos es normalmente inferior al 40 % (Ismael Lamas-Toranzo *et al*., 2018). Aunque más del 90% de los ovocitos inmaduros son capaces de reiniciar la meiosis y llegar a la metafase II *in vitro* y más del 80 % son capaces de ser fecundados *in vitro* y llevar a cabo la primera división mitótica; más de la mitad de los embriones fecundados no alcanzan el estadio de blastocisto *in vitro*. Sin embargo, aunque el mayor descenso en la proporción de embriones útiles tiene lugar durante el cultivo embrionario posterior a la fecundación, el

origen de este descenso parece tener lugar durante la maduración *in vitro* ya que la tasa de blastocistos obtenida realizando únicamente fecundación y cultivo *in vitro* duplica (~80 vs. ~35 %) la obtenida realizando todo el procedimiento (Rizos *et al*., 2002). Estos resultados evidencian que las condiciones subóptimas de maduración *in vitro* son las principales responsables de las bajas tasas de desarrollo embrionario hasta blastocisto obtenidas en PEIV (Dieleman *et al*., 2002; Rizos *et al*., 2002; Tesfaye *et al*., 2007). Por otra parte, los blastocistos producidos *in vitro* son considerados de menor calidad que los generados *in vivo* (Lonergan, 2007) mostrando menores tasas de gestación postransferencia (Lonergan & Fair, 2008). La reducida supervivencia post-transferencia de los embriones *in vitro* parece ser consecuencia de las condiciones subóptimas en las que se desarrollan (Hansen *et al*., 2010).

Pese a sus limitaciones, la aplicación de PEIV se encuentra en un claro ascenso que se refleja en los datos de las estadísticas de transferencias de embriones bovinos, recogidas por la Sociedad Internacional de Transferencia Embrionaria (IETS International Embryo Transfer Society). En 2019 se transfirieron un total de 1.116.551 embriones bovinos en los países que informan de su actividad a la IETS, siendo 797.190 (~71 %) producidos *in vitro*. Esta cifra supone un incremento del 5,1% con respecto al año anterior.

Sincronización de la receptora y transferencia embrionaria

La transferencia de embriones en animales puede realizarse empleando embriones recuperados del útero de la hembra (MOET) o generados mediante PEIV asociado a OPU o a aspiración de ovarios post-mortem. La receptora debe tener un tamaño adecuado, buena salud, estado de carnes y capacidad de producción lechera para poder llevar la gestación a término y la cría del ternero (Lindley *et al*., 2021; Palma, 2001; Seneda *et al*., 2020). La alimentación de la receptora supone un punto importante durante todo el proceso, tanto en el éxito final de la transferencia, como en la gestación y lactación de los terneros, de mayor valor genético de los que hubiera producido (Armstrong *et al*., 2003). Además, las receptoras deben estar libres de enfermedades transmisibles (Baruselli *et al*., 2018; Bó *et al*., 2012; Palma, 2001).

Existen distintos protocolos de sincronización de receptoras, que tiene por objeto lograr un estado endometrial adecuado para la recepción del embrión transferido. Para este fin, el desfase de la sincronización entre la vaca receptora y el embrión a transferir no debe

superar +/- 1 día, de modo que las transferencias se realizan generalmente entorno al día 7 después de la aparición de celo en la receptora (Bó *et al*., 2012; Lindley *et al*., 2021). La estimulación de las receptoras se realiza con la finalidad de inducir la formación de cuerpos lúteos de tamaño óptimo, los cuales producirán progesterona y detendrán la actividad ovárica. En primer lugar, para favorecer la sincronización del celo, se coloca un dispositivo intravaginal con progesterona o progestágeno, el cual se mantendrá entre 7 y 9 días (Macmillan & Thatcher, 1991). Al retirar la progesterona o progestágeno, se administra PGF2α y se estimula el desarrollo folicular con ECG, produciendo la estimulación folicular de cara a la formación de los cuerpos lúteos necesarios para la producción de la progesterona (Hubner *et al*., 2020; Oosthuizen *et al*., 2018; Troxel *et al*., 1983).

La selección de embriones obtenidos *in vivo* o producidos *in vitro* antes de la transferencia permite aumentar las tasas de éxito. Dicha selección se lleva a cabo mediante morfología (Lonergan *et al*., 2003, 2006), existiendo distintas clasificaciones, con pequeñas variaciones en los criterios morfológicos aplicados (Aguilar *et al*., 2002; Bo & Mapletoft, 2013; Lindner & Wright, 1983). Los embriones seleccionados se cargan en pajuelas de plástico que se emplean para la transferencia en fresco o criopreservación. La criopreservación permite el transporte y almacenamiento de embriones, posibilitando una mayor difusión de la mejora genética (Do *et al*., 2019; Leibo & Sztein, 2019). El proceso de la transferencia, tanto en fresco como tras la descongelación, debe realizarse rápidamente para minimizar la pérdida de viabilidad del embrión (Galli *et al*., 2003; Phillips & Jahnke, 2016). La transferencia se lleva a cabo de forma no quirúrgica, introduciendo un catéter de inseminación en el útero a través del cérvix y bajo anestesia epidural. Para realizar esta técnica, es necesario tener cierta experiencia y precaución para evitar provocar contracciones uterinas y sangrado cervical o endometrial que podría afectar a la receptividad del endometrio o a la viabilidad del embrión (Dochi, 2019; Givens & Marley, 2008; Phillips & Jahnke, 2016).

Métodos de selección embrionaria

Las distintas técnicas de reproducción asistida basadas en generación de embriones *in vitro* en humanos y animales de granja comparten una limitación principal: la baja eficiencia. En humanos, la eficiencia de los tratamientos se analiza como tasa de recién nacido vivo en base al número de ciclos realizados (Goodman *et al*., 2020; Kushnir *et al*.,

2017). En la especie bovina, el rendimiento se determina en base al porcentaje de blastocistos con buena morfología obtenidos por sesión de aspiración ovárica o el porcentaje de blastocistos obtenidos por complejo cúmulo-ovocito puesto a madurar, y en las tasas de preñez obtenidas tras la transferencia de embriones, que continúa siendo relativamente baja, alcanzando el 45% cuando los blastocistos son considerados de buena calidad morfológica (Alkan *et al.*, 2020; Ferré *et al.*, 2020; Moore & Hasler, 2017).

Durante las primeras décadas de la reproducción asistida humana, la principal estrategia para mejorar el éxito de las técnicas de reproducción asistida se basaba en la transferencia múltiple de embriones (Gleicher & Barad, 2009). Como contrapartida, la transferencia de dos o más embriones provocó un aumento en las gestaciones múltiples y con ello aumentaron los riesgos y complicaciones asociadas, tanto gestacionales como perinatales (Declercq *et al.*, 2015; Hasler, 2003, 2014; Lewi *et al.*, 2006; Moore & Hasler, 2017; Nakhuda & Sauer, 2005; Norwitz *et al.*, 2005). Esta problemática desencadenó que se iniciara una política de transferencia embrionaria orientada a la transferencia de un único embrión. La transferencia de un embrión conllevaba un descenso en las tasas de éxito, por lo que la investigación se centró en mejoras metodológicas, recurriendo a nuevos criterios de selección embrionaria que permitieran la selección del embrión con mayor potencial de éxito, tanto de implantación como de desarrollo (Gardner *et al.*, 1998, 2000; Giorgetti *et al.*, 1995). Estas técnicas para seleccionar el embrión con mayor potencial de éxito pueden ser de dos tipos: invasivas y no invasivas:

- Las técnicas invasivas son aquellas en las que se precisa obtener una parte del embrión en la que se pueden realizar análisis genéticos o de otra índole, en base a los cuales se selecciona el embrión con mejor pronóstico de desarrollo.
- Las técnicas no invasivas consisten en establecer una relación entre diferentes parámetros relacionados con el embrión de forma directa o indirecta para poder inferir la información que permita seleccionar el embrión a transferir.

Técnicas invasivas

La técnica de selección embrionaria invasiva más empleada en la actualidad es el diagnóstico genético preimplantacional, que sirve para identificar embriones portadores de patologías de origen genético, cromosómico o mitocondrial. Con independencia de la afectación que se pretenda evitar, se debe realizar la biopsia para la obtención del ADN

sobre el cual realizar los análisis genéticos. Estas biopsias pueden realizarse sobre el ovocito –corpúsculo polar- o bien sobre el embrión –blastómeras en día 3, día 5 o día 6 de desarrollo embrionario o bien sobre el blastocele- requiriendo en ambos casos la intrusión sobre el ovocito o embrión.

Las patologías genéticas en humanos, tanto a nivel cromosómico como a nivel monogénico, han centrado la mayor parte de los análisis genéticos realizados para mejorar la selección embrionaria (Munné & Cohen, 1998; Papas & Kutteh, 2021; Rubio *et al.*, 2007).

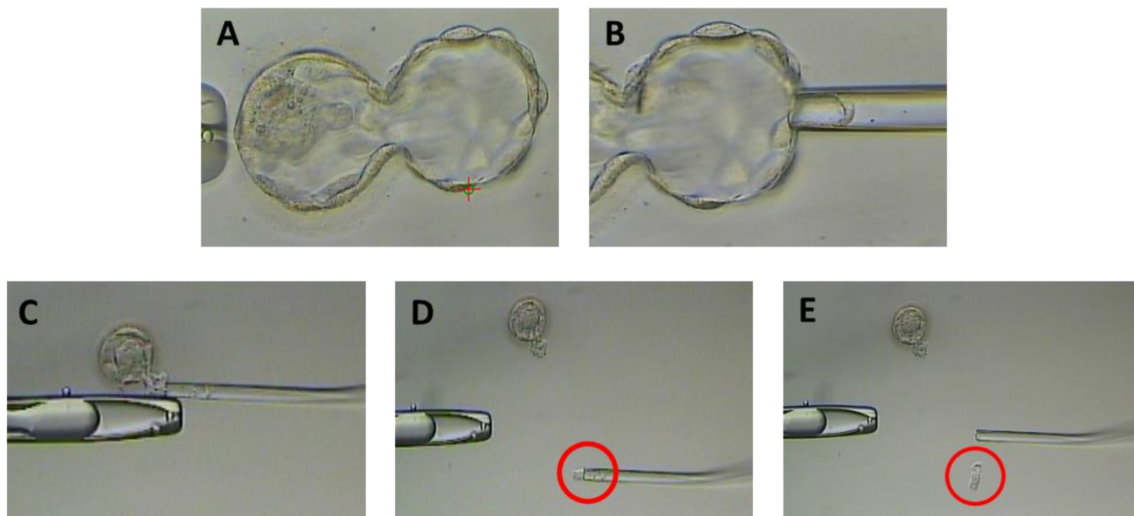

Figura 12. Secuencia de imágenes sobre la metodología de la biopsia embrionaria *flicking*. En las imágenes se puede observar un blastocisto sujeto por la pipeta *holding* (A). El trofoectodermo es absorbido por la pipeta de biopsia ligeramente (B). Tras fijar con la pipeta de biopsia el fragmento que queremos obtener aplicarán pulsos de láser aislados para debilitar las uniones intercelulares. Seguidamente se soltará el embrión de la pipeta *holding*, manteniéndolo sujeto por la pipeta de biopsia (C). Las pipetas *holding* y de biopsia se sitúan en el mismo plano, para posteriormente conseguir obtener las células mediante un proceso mecánico y rápido en el que las pipetas han de cruzarse sesgándose dichas células (Círculo rojo de la imagen D). Finalmente se consigue obtener el blastocisto y por otro lado la muestra de biopsia (Círculo rojo de la imagen E). *(Imágenes cedidas por IVF Spain Madrid).*

Los embriones humanos portadores de trastornos genéticos de distinta índole son relativamente frecuentes (Angell *et al.*, 1983; Boiso *et al.*, 2002; Munné & Cohen, 1998) y la incidencia de estas cromosomopatías tiene una mayor prevalencia en embriones de pacientes con edades reproductivas elevadas, diagnóstico de hibridación in situ fluorescente (FISH) en espermatozoides alterado o fragmentación del ADN espermático elevado entre otras causas. Mediante el diagnóstico genético preimplantacional se consigue reducir los riesgos de aneuploidías, disminuyendo la tasa de aborto espontáneo (Boiso *et al.*, 2002; Gleicher & Barad, 2009; Ribas-Maynou *et al.*, 2012; Rubio *et al.*, 2007) y también permite aumentar la tasa de implantación y reducir el tiempo para lograr

un embarazo, las tasas de aborto espontáneo y de recién nacido vivo afecto (Ajduk & Zernicka-Goetz, 2013; Iews *et al*., 2018; Rubio *et al*., 2007).

El diagnóstico genético también se ha empleado para seleccionar embriones empleando como marcador la cantidad de ADN mitocondrial (ADNmt) presente en la biopsia embrionaria. El análisis del contenido del ADNmt puede tener un valor predictivo, habiéndose descrito una correlación negativa entre cantidad de ADNmt y capacidad de desarrollo post-transferencia (Diez-Juan *et al*., 2015; Fragouli & Wells, 2015) aunque otros estudios no han logrado demostrar tal correlación (Klimczak *et al*., 2018; Scott et al., 2020; Treff *et al*., 2017; Victor *et al*., 2017). Como explicación a la relación inversa entre ADNmt en biopsias embrionarias y pronóstico de gestación se ha sugerido que las concentraciones elevadas del ADNmt podrían indicar un incremento anormal en el número de mitocondrias debido a una reserva mitocondrial insuficiente o defectuosa al final de la ovogénesis. Dicho aumento anormal podría desencadenar un desarrollo embrionario en condiciones de estrés oxidativo (Fragouli *et al*., 2013; May-Panloup *et al*., 2005; May-Panloup *et al*., 2016; Seli, 2016). En este sentido, dado que la posibilidad de replicación del ADN mitocondrial antes de la implantación es controvertida (Pikó & Taylor, 1987), también se ha sugerido que un aumento en los valores relativos de ADNmt (proporción ADNmt:ADN genómico) en embriones de peor calidad puede ser consecuencia de una degradación del ADN genómico en dichos embriones (Ho *et al*., 2018). Los niveles alterados de ADNmt en biopsias embrionarias también se han correlacionado con la edad de la paciente (Fragouli & Wells, 2015; Murakoshi *et al*., 2013) y con aneuploidías embrionarias (Diez-Juan *et al*., 2015; Fragouli *et al*., 2015; Seli, 2016).

En la especie bovina, se ha tratado de relacionar la expresión génica del embrión con el futuro desarrollo embrionario, comparando la expresión génica de biopsias de embriones bovinos con la distintas capacidades para establecer la gestación y el desarrollo a término (El-Sayed *et al*., 2006; Salilew-Wondim *et al*., 2010, 2021). Aunque estos estudios son ciertamente interesantes a nivel científico para entender los mecanismos moleculares implicados en el desarrollo embrionario temprano (Pérez-Gómez *et al*., 2021), su aplicación en ganadería es limitada, debido a las dificultades técnicas, logísticas y económicas que implican la realización de una biopsia y el análisis transcriptómico.

Los inconvenientes de las técnicas invasivas basadas en biopsia embrionaria no solo se ciñen al potencial daño causado por su carácter invasivo. El tiempo adicional que supone la espera de resultados hasta la transferencia embrionaria o el elevado coste de dicha técnica son también factores a valorar de cara a su aplicación (Lamb et al., 2004). Además, existe una cierta controversia sobre los beneficios del diagnóstico genético preimplantacional según qué tratamientos. Centrándonos en estudios recientes, Doyle y colaboradores observaron que las tasas de recién nacido vivo por transferencia y la tasa de recién nacido vivo acumulada por ciclo en los tratamientos con ovocitos donados eran similares con o sin diagnóstico genético. (Doyle *et al*., 2020). Otro estudio tampoco observó una mejora de la tasa de implantación, embarazo clínico y recién nacido vivo asociada al diagnóstico genético en embriones procedentes de ovocitos de pacientes o donantes (Schaeffer *et al*., 2020). En la misma línea, otro estudio tampoco observó una mejora de la tasa de nacidos vivos o tasa acumulada por ciclo empleando embriones procedentes de mujeres jóvenes con o sin diagnóstico genético (Zhou *et al*., 2021). El empleo de la técnica parece, por tanto, no estar indicado para la mayoría de los pacientes (Facadio Antero *et al*., 2021), aunque sigue estando indicada en pacientes portadores de enfermedades de base genética, particularmente de herencia dominante, en casos de fallo repetido en los tratamientos de reproducción asistida o para evitar el incremento de las aneuploidías en pacientes de edad avanzada (Carvalho *et al*., 2020; De Rycke & Berckmoes, 2020; Murphy *et al*., 2019; Sato *et al*., 2019).

Técnicas no invasivas basadas en el embrión

La selección morfológica embrionaria es la metodología de selección embrionaria más utilizada en los laboratorios de reproducción asistida. Esta técnica metodológica se basa en la observación puntual de distintos parámetros morfológicos objetivos del embrión relacionados con la capacidad de desarrollo posterior de este, tanto en términos de implantación como de recién nacido vivo (Cuevas Saiz *et al*., 2018; Gardner *et al*., 2000; Hurtado de Mendoza *et al*., 2015; Pons *et al*., 2014). La selección morfológica embrionaria presenta algunos inconvenientes: la visualización puntual hace que algunos de los eventos que ocurren durante el desarrollo embrionario no sean recogidos y la elección del mejor embrión para transferir puede estar sesgada por la pérdida de eventos tales como las divisiones tricotómicas o divisiones reversas (Milewski & Adjuk, 2017). Por otro lado, este tipo de valoración embrionaria hace necesaria la extracción de los

embriones del incubador a lo largo del desarrollo embrionario, afectando a la estabilidad de las condiciones de cultivo (Kirkegaard *et al*., 2015; Meseguer *et al*., 2012).

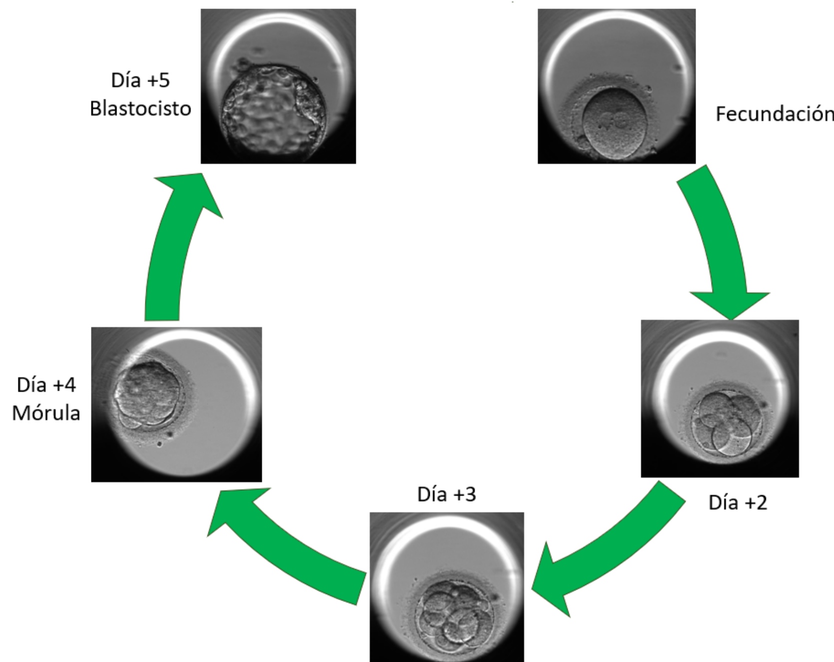

Figura 13. Esquema correspondiente al desarrollo embrionario humano procedente de sistemas *time-lapse*. El desarrollo embrionario se inicia con la unión de ambos gametos en el día +0. El día +1 o fecundación se comprobará la consecución del cigoto, observándose los pronúcleos, los corpúsculos y el halo citoplásmico. Durante los días +2 y +3 tienen lugar las divisiones mitóticas llegando a tener al menos 8 células antes de formar una mórula durante el día +4, en el cual se produce la compactación celular. Finalmente, en el día +5 tiene lugar la reestructuración del embrión, dando lugar al blastocisto.

Los métodos de selección clásicos basados en morfología embrionaria se han ido combinando con otros parámetros como los ritmos de división de los embriones durante su desarrollo. Uno de estos parámetros analizados desde un punto de vista cinético es la división temprana, que puede emplearse como criterio único de selección embrionario (Sakkas *et al*., 2001; Salumets *et al*., 2003; Shoukir *et al*., 1997) o en combinación con otros valores de puntuación (Lechniak *et al*., 2008; Lundqvist *et al*., 2001; Terriou *et al*., 2007), ya que tiene un alto valor predictivo en la tasa de embarazo y el potencial de implantación (Lundin *et al*., 2001; Salumets *et al*., 2003; Shoukir *et al*., 1997). La división temprana no ha sido el único marcador cinético empleado para la selección de embriones; los tiempos en los que se producen las divisiones celulares hasta llegar a cinco o nueve células (Meseguer *et al*., 2011; Motato *et al*., 2016), o la formación de la mórula han sido empleados como criterios de selección de embriones (Harada *et al*., 2020; Motato *et al*., 2016).

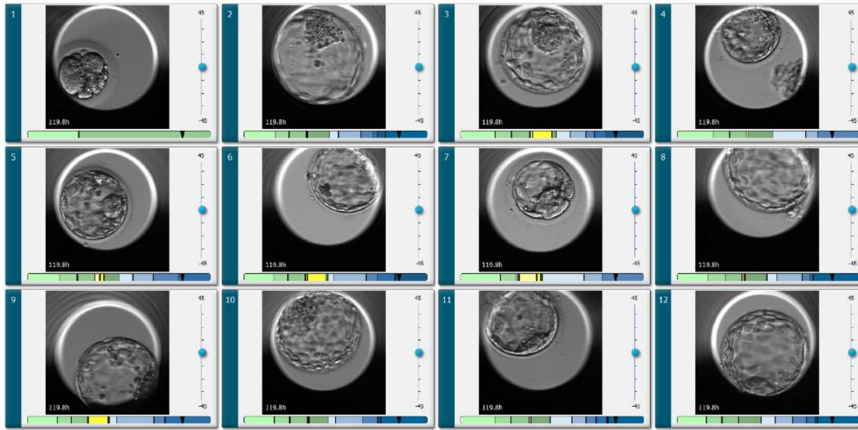

Figura 14. Imágenes correspondientes al *viewer* del sistema time-lapse Embryoscope® de un embrión humano en el quinto día de desarrollo. El eje derecho permite visualizar diferentes planos focales del embrión con el objetivo de analizar posibles eventos que determinen la selección embrionaria. El eje izquierdo corresponde con el eje temporal desde la unión de los gametos. Este software permite analizar individualmente lo embriones maximizando la imagen de cada uno de ellos. (Imagen cedida por IVF Spain Madrid-IVF Life)

La observación continua del desarrollo embrionario en el incubador, conocida como tecnología *time-lapse*, supone un complemento en la rutina de las unidades de reproducción asistida. La selección embrionaria mediante *time-lapse* consiste en valorar los embriones gracias a las fotografías tomadas cada 5-15 minutos que aportan una secuencia continua del desarrollo embrionario, analizando de forma sencilla la cinética embrionaria. Los sistemas *time-lapse* fueron utilizados por primera vez por Payne *et al*., en 1997 con el objetivo de poder valorar la fecundación de los cigotos humanos (Payne *et al*., 1997). Los sistemas de visualización continua han evolucionado desde entonces, publicándose diversos estudios que han tratado de relacionar la cinética de desarrollo embrionario con la capacidad predictiva de implantación (Basile *et al*., 2015; Dal Canto *et al*., 2012; Meseguer *et al*., 2011; Wong *et al*., 2010), confirmándose dicha asociación en un ensayo clínico controlado aleatorizado (Rubio *et al*., 2014). Pese a los resultados obtenidos, la última revisión Cochrane concluye que no hay una evidencia científica sólida que demuestre que el empleo incubadores con sistemas *time-lapse* frente a incubadores convencionales mejore las tasas de embarazo clínico o recién nacido vivo (Armstrong *et al*., 2019). Encontrar un consenso entre distintos ensayos clínicos controlados aleatorizados es complicado debido a la disparidad de diseños experimentales entre los mismos y a los múltiples factores que intervienen en el proceso de implantación (Kovacs & Lieman, 2019).

Los sistemas *time-lapse* además de ser un sistema predictor de la capacidad de desarrollo del embrión, han sido utilizados como método sustitutivo no invasivo del diagnóstico genético preimplantacional. Para esta aplicación, algunos autores han sugerido que los algoritmos y cinética asociadas al desarrollo embrionario podrían estar relacionados con la euploidia de los mismos (Basile *et al*., 2014; Campbell *et al*., 2013). Sin embargo, estos resultados han generado cierta controversia, ya que otros estudios no han observado una asociación clara entre la tasa de euploidia y la morfocinética embrionaria durante su desarrollo hasta el estadio de blastocisto (Rienzi *et al*., 2015; Yang *et al*., 2014).

Además de la valoración morfológica del embrión o la evaluación de su cinética de desarrollo, existen otras metodologías analíticas no invasivas que pueden ser de utilidad en la selección embrionaria. Estas técnicas no invasivas se basan en el análisis de sustancias (metabolitos, ARN o ADN) en el medio de cultivo sobre el que se ha desarrollado el embrión. El diagnóstico genético preimplantacional no invasivo consiste en el análisis del ADN libre potencialmente presente en el medio de cultivo en el que se ha desarrollado el embrión. El ADN obtenido del medio de cultivo es amplificado y posteriormente analizado mediante secuenciación masiva. De este modo, se podrían potencialmente identificar embriones que presenten cromosomopatías (Barroso Villa *et al*., 2020; Capalbo *et al*., 2018; Rubio-Lluesa, 2019; Rubio *et al*., 2021). El análisis de los metabolitos o metabolómica es una técnica de estudio en desarrollo, que aún no se ha implementado en los laboratorios de reproducción asistida. El análisis de los metabolitos secretados al medio de cultivo podrían servir como metodología de selección embrionaria, existiendo una correlación entre el desarrollo embrionario y el propio metabolismo del embrión (Nel-Themaat & Nagy, 2011; Singh & Sinclair, 2007; Uyar & Seli, 2014; Vergouw *et al*., 2008). Por último, el análisis transcriptómico no invasivo consiste en analizar el contenido de ARNm libre supuestamente presente en el medio de cultivo. Esta técnica presenta ciertas limitaciones como la degradación del ARN, la necesidad de amplificar el material genético aislado, la esterilidad que debe acompañar a todo el proceso, así como el elevado coste motivado por la necesidad de equipos y personal especializados (Ajduk & Zernicka-Goetz, 2013). Debido a estas limitaciones y a lo controvertido de la técnica, resulta difícil la implementación de este tipo de análisis en la rutina clínica.

Técnicas no invasivas basadas en análisis de células del cúmulo

La calidad del ovocito es un factor clave en las tasas de éxito en reproducción asistida, como queda patente en base a las tasas de desarrollo obtenidas con ovocitos bovinos madurados *in vivo* frente a aquellos madurados *in vitro* (Dieleman *et al*., 2002; Rizos *et al*., 2002; van Leemput *et al*., 1999) y en las menores tasas de éxito de la maduración *in vitro* vs. *in vivo* en humanos (Hart & Walls, 2018; Sauerbrun-Cutler *et al*., 2015; Walls & Hart, 2018). Comprender la regulación molecular que determina la competencia ovocitaria entendida como la capacidad de dicho ovocito para generar un embrión viable es, por tanto, clave para mejorar las tasas de éxito en tratamientos de reproducción asistida humana y animal.

Los complejos cúmulo-ovocito están constituidos por el ovocito y unas células denominadas células del cúmulo que son esenciales para el crecimiento y maduración del ovocito. La interacción entre el ovocito y las células del cúmulo determina el metabolismo de carbohidratos, lípidos y proteínas esenciales durante las primeras etapas del desarrollo temprano, además de regular funciones paracrinas y autocrinas (Banwell & Thompson, 2008; Roelen, 2019; Sánchez & Smitz, 2012). Las células del cúmulo proporcionan un soporte continuo de metabolitos y nutrientes esenciales durante desarrollo del ovocito hasta el estadio de metafase II. Las uniones estrechas entre las células son necesarias para mantener el flujo de sustancias, la coordinación de vías metabólicas, y el establecimiento de señales paracrinas claves en el metabolismo del ovocito y su maduración (Downs, 2015; Rohen & Lütjen-Drecoll, 2008). Una vez madurado el ovocito estas uniones se pierden dando lugar a la expansión de las células del cúmulo, uno de los indicadores morfológicos de la correcta maduración del ovocito.

La expansión de las células del cúmulo en el momento en el que dejan de ser necesarias es el primer paso para su eliminación (Banwell & Thompson, 2008; Fair, 2010). Este proceso de eliminación también ocurre al aplicar técnicas de reproducción asistida. Durante la ICSI las células del cúmulo deben ser eliminadas para permitir la correcta visualización del ovoplasma y el caso de la FIV también son desechadas antes de la puesta en cultivo (Remohí *et al*., 2017). Siendo un material biológico eliminado de forma rutinaria y que ha estado en contacto estrecho con el ovocito durante la foliculogénesis y la maduración, estas células pueden constituir un material biológico interesante sobre el

que realizar distintos análisis no invasivos encaminados a obtener un valor predictivo de la futura capacidad de desarrollo del embrión generado.

La importancia del poder predictivo de las células del cúmulo residiría en la estrecha relación que mantiene con el ovocito, lo cual puede permitir inferir la competencia de los ovocitos que rodean, facilitando la selección embrionaria en la optimización de los tratamientos reproductivos (Assidi *et al*., 2008; Melanie Hamel *et al*., 2008; McKenzie *et al*., 2004). Durante el crecimiento folicular, tanto en las células del cúmulo como en el ovocito, se produce la síntesis de numerosos transcritos. En el caso del ovocito la transcripción se detiene en las últimas fases del desarrollo folicular, correspondiendo con el proceso madurativo del ovocito, mientras que las células del cúmulo se mantienen transcriptómicamente activas. En esta línea, resulta comprensible que el valor predictivo del análisis transcriptómico de las células del cúmulo sobre la calidad del ovocito haya sido estudiado tanto en bovino (Assidi *et al*., 2008; Bessa *et al*., 2013; Bunel *et al*., 2014, 2015; Dawit Tesfaye *et al*., 2009) como en humano (Assou *et al*., 2008; Demiray *et al*., 2019; Fortin *et al*., 2019; Kranc *et al*., 2019; McKenzie *et al*., 2004) en la búsqueda de marcadores sobre el desarrollo embrionario posterior.

Los resultados obtenidos de los estudios sobre la transcriptómica en las células del cúmulo presentan resultados controvertidos. Dicha controversia surge de la necesidad de establecer genes o familias de genes candidatos para ser analizados y que relacionaban genes implicados en procesos apotóticos, gluconeogénesis, factores de transcripción (Ahmed *et al*., 2020; Assou *et al*., 2008; Gasca *et al*., 2007), genes relacionados con la adhesión, proliferación y diferenciación celular (Kranc *et al*., 2019), con la respuesta a la estimulación ovárica (P. Feuerstein *et al*., 2007), funciones paracrinas (Cillo *et al*., 2007) o implicados en la maduración ovocitaria (P. Feuerstein *et al*., 2007; McKenzie *et al*., 2004; van Montfoort *et al*., 2008). Las dificultades en la repetitividad de los experimentos y la correlación con genes presentes en los diferentes estudios posiblemente estuvieran relacionados con las limitaciones asociadas a las técnicas de análisis como la PCR cuantitativa (qPCR) (Boucret *et al*., 2015; Fortin *et al*., 2019; Kranc *et al*., 2019) o los *microarrays* (Said Assou *et al*., 2006; Demiray *et al*., 2019; Gasca *et al*., 2007; van Montfoort *et al*., 2008), los cuales precisaban de genes o familias de genes candidatos. El desarrollo de la secuenciación masiva y más concretamente orientada al ARN (RNAseq) (Andrei *et al*., 2018; Barone *et al*., 2020; Xiong *et al*., 2019) ha permitido la apertura de una nueva línea de investigación sin las limitaciones de analizar un conjunto de genes en

concreto, facilitando la repetitividad de los experimentos. Desafortunadamente, los resultados obtenidos en estos y otros estudios de expresión génica en las células del cúmulo son muy dispares, mostrando escasas coincidencias en las listas de genes candidatos entre distintos artículos (R. Kordus & LaVoie, 2017). En el caso de estudios humanos, se ha asociado esta la falta de coincidencia entre los genes a numerosas covariables como estimulación ovárica, origen de la infertilidad o la edad de la paciente (Armstrong *et al.*, 2003; Walsh *et al.*, 2012; Sharma *et al.*, 2020).

En la especie bovina también se ha intentado relacionar la competencia ovocitaria con la abundancia relativa de transcritos específicos en las células del cúmulo mediante análisis de qPCR o *microarray*. Estos estudios difieren en los criterios empleados para determinar la competencia del ovocito, siendo generalmente criterios indirectos como el tamaño del folículo (Melo *et al.*, 2017), la metodología de maduración *in vitro* (Assidi *et al.*, 2008), el origen *in vitro* o *in vivo* de los ovocitos (Salhab *et al.*, 2013; Tesfaye *et al.*, 2009), la edad de las hembras empleadas (Bettegowda *et al.*, 2008), o el momento de recolección durante el ciclo estral y/o control hormonal (Assidi *et al.*, 2010; Bunel *et al.*, 2014). Desafortunadamente, los genes potencialmente asociados con competencia embrionaria no coinciden entre los distintos estudios. Además, estos genes supuestamente implicados en calidad del ovocito tampoco coinciden los genes candidatos identificados en otros estudios en los que se determinó la competencia de cada ovocito de forma directa, mediante el empleo de cultivo embrionario individual y se analizaron las diferencias transcriptómicas mediante *microarray* (Bunel *et al.*, 2015) o mediante qPCR para transcritos específicos (Kussano *et al.*, 2016). La disparidad de resultados puede deberse a que los criterios indirectos de competencia ovocitaria empleados involucran a distintos procesos biológicos (crecimiento folicular, respuesta hormonal, edad…) que pueden tener un efecto transcriptómico propio que enmascare a los marcadores reales de competencia ovocitaria directa. Sin embargo, además de las posibles razones biológicas, existen limitaciones técnicas del análisis transcriptómico asociadas al número de genes analizados, muy limitado en estudios de qPCR y limitado y sesgado en el caso de *microarrays*, y al limitado rango dinámico de los *microarrays*. En este sentido, el ARN-seq no parte de una selección previa de genes y ofrece un mayor rango dinámico, aumentando la precisión de los resultados.

La cantidad relativa de ADN mitocondrial en células del cúmulo es otro parámetro molecular que podría emplearse como marcador de selección para ovocitos. La cantidad

de ADNmt del ovocito se ha correlacionado positivamente con su calidad mediante observaciones indirectas, ya que la determinación de este parámetro implica la destrucción del ovocito. En concreto, se ha observado que la cantidad de ADNmt es mayor en cohortes de ovocitos en los que hay fallo de fecundación comparados con otros en los que el porcentaje de fecundación es normal (Reynier *et al*., 2001) o en ovocitos fecundados comparados con ovocitos sin fecundar (Santos *et al*., 2006). También se ha observado una reducción de la cantidad de ADNmt en pacientes con insuficiencia ovárica (May-Panloup *et al*., 2005) o de edad avanzada (Chan *et al*., 2006; Murakoshi *et al*., 2013) y estudios en modelos animales también han correlacionado positivamente cantidad de ADNmt del ovocito con su competencia (El Shourbagy *et al*., 2006; Ismael Lamas-Toranzo *et al*., 2018). En esta línea la determinación del ADNmt en células del cúmulo podría permitir la selección de ovocitos con alta cantidad de ADNmt (más viables) si el contenido en ADNmt en las células se correlaciona con el del ovocito y existen publicaciones que han observado una correlación entre cantidad de ADNmt de las células del cúmulo y calidad embrionaria basada en morfología (Desquiret-Dumas *et al*., 2017; Ogino *et al*., 2016) o en probabilidades de implantación (Diez-Juan *et al*., 2015; Taugourdeau *et al*., 2019).

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Objetivos

El objetivo principal de este proyecto es analizar parámetros de competencia ovocitaria en biopsias de células del cúmulo con el objeto de seleccionar aquellos embriones con mayor probabilidad de llevar a cabo una gestación a término.

Los objetivos específicos se derivan de los distintos parámetros a analizar: contenido de ADN mitocondrial, análisis transcriptómico y análisis metabolómico. La conjunción de estos parámetros en muestras clínicas y en el biomodelo bovino da lugar a los siguientes objetivos específicos:

1. Analizar la cantidad de ADN mitocondrial en muestras de células del cúmulo procedentes de complejos cúmulo-ovocito bovinos con diferente competencia para el desarrollo *in vitro*.
2. Analizar la cantidad de ADN mitocondrial en muestras de células del cúmulo procedentes de complejos cúmulo-ovocito humanos con diferente competencia para el desarrollo *in vitro* y post-transferencia.
3. Evaluar las posibles diferencias transcripcionales entre muestras de células del cúmulo procedentes de complejos cúmulo-ovocito bovinos con diferente competencia para el desarrollo *in vitro*.
4. Evaluar las posibles diferencias transcripcionales entre muestras de células del cúmulo procedentes de complejos cúmulo-ovocito humanos con diferente competencia para el desarrollo *in vitro* y post-transferencia.
5. Analizar la cantidad de distintos compuestos bioquímicos presentes en muestras de células del cúmulo procedentes de complejos cúmulo-ovocito humanos con diferente competencia para el desarrollo *in vitro* y post-transferencia.

Capítulo 1

mtDNA content in cumulus cells does not predict development to blastocyst or implantation

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Álvaro Martínez-Moro, Ismael Lamas-Toranzo, Leopoldo González-Brusi, Alba Pérez-Gómez, Ester Padilla-Ruiz, Javier García-Blanco, Pablo Bermejo-Álvarez

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Abstract

STUDY QUESTION: Is relative mitochondrial DNA (mtDNA) content in cumulus cells (CCs) related to embryo developmental competence in humans and/or the bovine model?
SUMMARY ANSWER: mtDNA content in CCs provides a poor predictive value of oocyte developmental potential, both *in vitro* and following embryo transfer.
WHAT IS KNOWN ALREADY: CCs are closely connected to the oocyte through transzonal projections, serving essential metabolic functions during folliculogenesis. These oocyte-supporting cells are removed and discarded prior to ICSI, thereby providing interesting biological material on which to perform molecular analyses designed to identify markers that predict oocyte developmental competence. Previous studies have positively associated oocyte mtDNA content with developmental potential in animal models and women. However, it remains debatable whether mtDNA content in CCs could be used as a proxy to infer oocyte developmental potential
STUDY DESIGN, SIZE, DURATION: mtDNA content was analyzed in CCs obtained from 109 human oocytes unable to develop to blastocyst, able to develop to blastocyst but failing to establish pregnancy or able to develop to blastocyst and to establish pregnancy. mtDNA analysis was also performed on bovine cumulus samples collected from 120 oocytes unable to cleave, oocytes developing into cleaved embryos but arresting development prior to the blastocyst stage or oocytes developing to blastocysts.
PARTICIPANTS/MATERIALS, SETTING, METHODS: Human CCs samples were obtained from women undergoing IVF. Only unfrozen oocytes and embryos not submitted to preimplantation genetic testing were included in the analysis. Bovine samples were obtained from slaughtered cattle and individually matured, fertilized and cultured *in vitro*. Relative mtDNA was assessed by quantitative PCR analysis.

MAIN RESULTS AND THE ROLE OF CHANCE: mtDNA content in human and bovine CCs did not differ according to the developmental potential of their enclosed oocyte. Moreover, mtDNA content in bovine oocytes did not correlate with that of their corresponding CCs.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: The lack of correlation found between mtDNA content in human CCs and oocytes was also assessed in bovine samples. Although bovine folliculogenesis, mono-ovulatory ovulation and early embryo development exhibit considerable similarities with that of humans, they may not be fully comparable.

WIDER IMPLICATIONS OF THE FINDINGS: The use of molecular markers for oocyte developmental potential in CCs could be used to enhance success rates following single embryo transfer. However, our data indicate that mtDNA in CCs is not a good proxy for oocyte quality.

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ORIGINAL ARTICLE

mtDNA content in cumulus cells does not predict development to blastocyst or implantation

Álvaro Martínez-Moro[1,2], Ismael Lamas-Toranzo[1], Leopoldo González-Brusi[1], Alba Pérez-Gómez[1], Ester Padilla-Ruiz[2], Javier García-Blanco[2], and Pablo Bermejo-Álvarez [1,*]

[1]Animal Reproduction Department, INIA, CSIC, Madrid, Spain [2]IVF Spain, Madrid, Spain

*Correspondence address. Animal Reproduction Department, INIA, CSIC, Avda. Puerta de Hierro 18, 28040 Madrid, Spain. E-mail: bermejo.pablo@inia.csic.es https://orcid.org/0000-0001-9907-2626

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SUMMARY ANSWER: mtDNA content in CCs provides a poor predictive value of oocyte developmental potential, both *in vitro* and following embryo transfer.

WHAT IS KNOWN ALREADY: CCs are closely connected to the oocyte through transzonal projections, serving essential metabolic functions during folliculogenesis. These oocyte-supporting cells are removed and discarded prior to ICSI, thereby providing interesting biological material on which to perform molecular analyses designed to identify markers that predict oocyte developmental competence. Previous studies have positively associated oocyte mtDNA content with developmental potential in animal models and women. However, it remains debatable whether mtDNA content in CCs could be used as a proxy to infer oocyte developmental potential

STUDY DESIGN, SIZE, DURATION: mtDNA content was analyzed in CCs obtained from 109 human oocytes unable to develop to blastocyst, able to develop to blastocyst but failing to establish pregnancy or able to develop to blastocyst and to establish pregnancy. mtDNA analysis was also performed on bovine cumulus samples collected from 120 oocytes unable to cleave, oocytes developing into cleaved embryos but arresting development prior to the blastocyst stage or oocytes developing to blastocysts.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Human CCs samples were obtained from women undergoing IVF. Only unfrozen oocytes and embryos not submitted to preimplantation genetic testing were included in the analysis. Bovine samples were obtained from slaughtered cattle and individually matured, fertilized and cultured *in vitro*. Relative mtDNA was assessed by quantitative PCR analysis.

MAIN RESULTS AND THE ROLE OF CHANCE: mtDNA content in human and bovine CCs did not differ according to the developmental potential of their enclosed oocyte. Moreover, mtDNA content in bovine oocytes did not correlate with that of their corresponding CCs.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: The lack of correlation found between mtDNA content in human CCs and oocytes was also assessed in bovine samples. Although bovine folliculogenesis, mono-ovulatory ovulation and early embryo development exhibit considerable similarities with that of humans, they may not be fully comparable.

WIDER IMPLICATIONS OF THE FINDINGS: The use of molecular markers for oocyte developmental potential in CCs could be used to enhance success rates following single embryo transfer. However, our data indicate that mtDNA in CCs is not a good proxy for oocyte quality.

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Key words: mitochondria / mtDNA / oocyte quality / cumulus cells / granulosa cells / embryo development / developmental competence / developmental proxy / implantation

WHAT DOES THIS MEAN FOR PATIENTS?

Embryo viability (i.e. development and survival) plays a major role in successful reproduction and therefore improving the methods used for embryo selection is crucial to improve pregnancy rates following artificial reproductive techniques (e.g. IVF). Cumulus cells (CCs) completely surround an oocyte (egg) and provide the oocyte with essential nourishment but they are often removed prior to fertilization. Being a 'by product' of the IVF process, CCs can be used in analyses aimed to infer the odds for embryo survival following transfer to the uterus. In this study, we have tested whether the amount of mitochondrial DNA (mtDNA) in CCs differs between eggs that are unable to develop outside the body (*in vitro*), are able to develop *in vitro* but fail to establish pregnancy or those that are able to establish a pregnancy. Our results showed no differences in mtDNA content between the three groups, suggesting that mtDNA in CCs is of poor predictive value for the developmental potential of an egg.

Introduction

Embryo viability plays a major role in reproductive success and, thus, the implementation of methods for embryo selection is crucial to enhance pregnancy rates following ART. Morphological evaluation is the most widely used method for embryo selection, constituting an excellent predictor of pregnancy success. However, clinical pregnancy rates remain around 35% (Wyns *et al.*, 2020), suggesting that there is still room for improvement. Time-lapse screening of embryo development (Armstrong *et al.*, 2019) or diverse molecular analyses performed on embryo biopsies or in the culture medium of the embryos (Mastenbroek *et al.*, 2011) have been proposed as complementary methods to assist embryo selection. For this purpose, cumulus cells (CCs) provide an interesting biological material on which to perform molecular analyses, as they are closely connected to the oocyte throughout folliculogenesis and oocyte maturation and are discarded prior to fertilization (Kordus and LaVoie, 2017).

Molecular analyses on CCs aiming to predict pregnancy success have been majorly based on transcriptomics analyses, leading to the identification of diverse putative markers for oocyte quality and subsequent embryo development (Iager *et al.*, 2013; Wathlet *et al.*, 2013; Borup *et al.*, 2016; Parks *et al.*, 2016; Artini *et al.*, 2017; Martínez-Moro *et al.*, 2022). However, the different transcriptional markers identified vary greatly between studies, and others have failed to find a correlation between pregnancy success and gene expression in CCs, either for genes previously suggested as potential markers (Burnik Papler *et al.*, 2015) or in global transcriptomics analyses (Burnik Papler *et al.*, 2015; Green *et al.*, 2018). Compared to transcriptomics analyses, the evaluation of relative mitochondrial DNA (mtDNA) amount in CCs constitutes a faster, easier and less expensive molecular analysis, and it provides an easier-to-compare unique data value per sample, which makes it appealing for its implementation in routine IVF.

Several lines of evidence suggest that mtDNA content in CCs may be a good predictor for oocyte quality. During oocyte growth, mtDNA undergoes massive proliferation (Cao *et al.*, 2007; Cree, *et al.*, 2008) and oocyte mtDNA content has been consistently correlated to oocyte maturity during folliculogenesis and subsequently to fertilization outcome in animal models (El Shourbagy *et al.*, 2006; Lamas-Toranzo *et al.*, 2018). In humans, oocyte mtDNA content was found to be lower in cohorts of oocytes undergoing fertilization failure compared to cohorts displaying normal fertilization rates (Reynier *et al.*, 2001). Similarly, mtDNA content was reported to be higher in human zygotes compared to unfertilized oocytes (Santos *et al.*, 2006) and in *in vivo* matured human oocytes compared to *in vitro* matured oocytes (Zhao *et al.*, 2016), whereas a reduction in oocyte mtDNA has been associated with ovarian insufficiency (May-Panloup *et al.*, 2005) and aging (Chan *et al.*, 2005; Murakoshi *et al.*, 2013).

Given the essential roles of CCs during oocyte growth and maturation, a similar positive correlation between mtDNA content in CCs and oocyte quality may occur. Oocyte and CC mtDNA content have been positively correlated in human samples (Boucret *et al.*, 2015), and mtDNA content in human CCs has been reported to correlate positively with embryo quality, as assessed by morphology on Days 3 and 5 (Ogino *et al.*, 2016) or 2 (Desquiret-Dumas *et al.*, 2017). Furthermore, going beyond conventional embryo selection based on morphology, another article associated mtDNA content in human CCs with the odds of implantation (Taugourdeau *et al.*, 2019). In view of these findings, the objective of this study was to determine if mtDNA content in CCs is correlated to the developmental potential of its enclosed oocyte. To achieve this, we have analyzed mtDNA in human samples collected from IVF cycles showing diverse developmental competence, and in bovine oocytes, an animal model with some similarities to the human reproductive process.

Materials and methods

Collection of human samples

CCs were obtained at IVF Spain Madrid from IVF cycles between June 2018 and June 2020. The study was approved by the Ethical Committee from La Princesa University Hospital (Madrid) and all patients and donors were informed and agreed to participate in the study. The study was conducted on both autologous and heterologous (oocyte donation) embryo transfers meeting the following inclusion criteria: absence of uterine abnormalities; donor age ≤37 years; recipient age ≤50 years; and sperm count in ejaculate >2 million spermatozoa/ml. Only unfrozen oocytes and embryos not submitted to preimplantation genetic testing were included in the analysis. Characteristics of the patients included in the study are presented in Table I.

Table I Patient characteristics in a study of mitochondrial DNA content of cumulus cells.

	Pregnant	Non-pregnant
Patient age (years)	36.77 ± 0.85	37.79 ± 0.78
Patient BMI (kg/m^2)	24.37 ± 0.85	23.83 ± 0.88
Endometrial thickness (mm)	9.07 ± 0.35	9.35 ± 0.26
Donor age (years)	31.77 ± 1	32.97 ± 1.09
Donor BMI (kg/m^2)	24.9 ± 0.9	23.34 ± 0.91

Data are shown as mean ± SEM, no significant differences were found between groups (Student's *t*-test $P > 0.05$).

Cumulus–oocyte complexes (COCs) were obtained from both patients and donors stimulated by FSH treatment starting on the second menstruation day. Ovulation was induced by GnRH analog in the presence of three or more follicles larger than 17 mm (Melo *et al.*, 2009). Oocyte retrieval was performed 36 h after hCG injection by transvaginal ultrasound guidance. Prior to embryo transfer, endometrium was stimulated by the administration of 6 mg/day of estradiol (Meriestra®). Embryo transfer was performed only if the endometrium measured 7–13 mm (Prapas *et al.*, 1998). Luteal support started on the night of oocyte retrieval and involved the administration of 400 mg of progesterone every 12 h until a pregnancy test (Edwards *et al.*, 1984).

COCs were retrieved by follicular aspiration, washed and individually cultured in G-IVF Plus Media (Vitrolife, Göteborg, Sweden) (6.3% CO_2, 5% O_2, 89% N_2 at 37°C). Denudation was performed individually in a hyaluronidase solution (Irvine) 2 h after COCs recovery. CCs detached from oocytes were collected from denudation medium, pelleted by centrifugation at 1500*g* for 10 min, snap frozen in liquid nitrogen and stored at −80°C until analysis. Oocytes were fertilized by ICSI in G-MOPS Plus medium (Vitrolife, Göteborg, Sweden). Spermatozoa were introduced in 7% polyvinylpyrrolidone solution to slow their movement. Following ICSI, the presumptive zygotes were cultured individually in Continuous Single Culture SAGE 1-Step™ medium (Origio Malov, Denmark) for 6 days, up to the blastocyst stage. Morphological score was used to select blastocysts for embryo transfer (Gardner *et al.*, 2015). Morphological score was similar for both groups transferred (P− and P+, see below). Pregnancy was assessed at the fourth or fifth week post-fertilization by fetal heart rate detection by ultrasound echography. Once embryo development was known, the previously stored CCs were allocated into three groups according to oocyte developmental potential: oocytes not developing to blastocyst (Bl−); oocytes developing to blastocyst but failing to establish pregnancy following embryo transfer (P−); or oocytes developing to blastocyst and able to establish pregnancy (P+).

Collection of bovine samples

Bovine embryo production was performed following conventional protocols with minor modifications (Lamas-Toranzo *et al.*, 2020). Two independent experiments were conducted in bovine samples. The first experiment aimed to collect CC samples from oocytes exhibiting different developmental potential. Bovine ovaries were collected at slaughterhouse and transported at 35–37°C to the laboratory. COCs were obtained by aspiration of 2–8 mm follicles and selected using conventional morphological criteria (Hawk and Wall, 1994). COCs were matured individually in 40 µl drops of TCM-199 supplemented with 10% fetal calf serum and 10 ng/ml epidermal growth factor, and covered under mineral oil at 39°C and 5% CO_2 in air with a humidified atmosphere for 24 h. Following maturation, CCs were removed individually by pipetting in medium supplemented with 0.1% hyaluronidase. CCs were collected from the media by centrifugation at 750 g for 5 min, snap frozen in liquid nitrogen and stored at −80°C until analysis. Denuded oocytes were individually inseminated with 10^6 frozen-thawed bull spermatozoa/ml and incubated in 40 µl drops of Tyrode's albumin lactate pyruvate medium (Parrish *et al.*, 1986) covered under mineral oil for ∼20 h in the same atmosphere conditions as above. Following IVF, presumptive zygotes were cultured individually in 10 µl drops of SOF (Synthetic Oviduct Fluid) medium (Holm *et al.*, 1999) at 39°C and in a 5% CO_2 and 5% O_2 humidity-saturated atmosphere. Cleavage and blastocyst rates were assessed at 48 h and 9 days post-insemination, respectively, in five independent replicates. Once the embryo development was known, the previously stored CCs were allocated to one of three groups according to oocyte developmental potential: oocytes not cleaving following IVF (Cl−); oocytes cleaving but not developing to blastocysts (Bl−); and oocytes developing to blastocyst (Bl+).

A second experiment aimed to determine if relative mtDNA amount in CCs correlated with that of the enclosed oocyte. CCs and oocytes samples were collected from *in vitro* matured bovine oocytes. CCs samples were collected as described above. For oocyte samples, the zona pellucida was removed by brief incubation in PBS pH 2 and zona-free oocytes were snap frozen individually and stored at −80°C until analysis.

mtDNA analysis

CCs and oocyte samples were digested using a Picopure DNA Extraction kit (Applied Biosystems, San Francisco, CA, USA) following the manufacturer's recommendation, using 20 µl and 10 µl of reagent per CCs and oocyte sample, respectively. In CCs, relative mtDNA quantification was determined as previously described (Lamas-Toranzo *et al.*, 2018). Relative quantification is required to compensate for the differences between samples in the number of CCs. Relative quantification was performed by using PCR primers that amplify a mitochondrial sequence (*MT-ND2* (mitochondrially encoded NADH dehydrogenase 2) and *COX1* (cytochrome c oxidase subunit 1) for human and bovine samples, respectively) and others to amplify a genomic sequence (*PPIA*, peptidylprolyl isomerase A) to adjust for the total amount of DNA present in the sample. Primer sequences are shown in Table II. Samples were run in duplicate, using 4 µl of digested sample in a 20 µl quantitative PCR (qPCR) reaction (Gotaq qPCR, Promega, Madison, WI, USA) in a MIC thermocycler (BioMolecular Systems, Upper Coomera, Australia). PCR conditions were optimized to achieve efficiencies close to 1 and then the comparative cycle threshold method was used to obtain relative values following $2^{-\Delta\Delta Cq}$ calculation, as described (Schmittgen and Livak, 2008). Absolute quantification was performed for oocytes following a similar method (Bermejo-Alvarez *et al.*, 2008), using the primers designed for mtDNA sequences and 3 µl of digested sample in a 20 µl PCR reaction. To compare groups, oocyte data were analyzed by the $2^{-\Delta Cq}$ method (Schmittgen and Livak, 2008).

Table II Sequences for the PCR primers used to quantify mitochondrial DNA in human and bovine samples.

Gene	Species	Primer sequences (5′–3′)	Fragment size (bp)	GenBank accession no.
MT-ND2	Human	CAGCACCACGACCCTACTAC GGAGGGTGATGGTGGCTATG	194	NC_012920.1
PPIA	Human	TCTGCACTGCCAAGACTGAG CCCAGCTAAGGGCCAAGTTT	230	NC_0000007.14
COX1	Bovine	TTTGATGCTTGGGCCGGTAT GGAATGAGGGAGGGAGGAGT	265	NC_006853.1
PPIA	Bovine	CGGGATTTATGTGCCAGGGT CCAAAGTACCACGTGCTTGC	218	NC_037331.1

MT-ND2, mitochondrially encoded NADH dehydrogenase 2; *PPIA*, peptidylprolyl isomerase A; *COX1*, cytochrome c oxidase subunit 1.

The number of human samples analyzed was 109 (37, 38 and 34 for BI−, P− and P+ groups, respectively). Experiments in the bovine model analyzed 120 samples in the first experiment (40/group) and 170 in the second (85 oocytes and their corresponding CCs). Statistical differences were analyzed by *t*-test or ANOVA using SigmaStat (Jandel Scientific, Leighton Buzzard, UK). *P*-values above 0.05 were not considered statistically significant.

Results

The analysis of relative mtDNA amount in 109 samples of human CCs revealed that this parameter was not associated with donor BMI or age (Fig. 1A and B). No differences in CCs mtDNA content were found between donors who were non-smokers or smokers (Fig. 1C) or between samples recovered from autologous or heterologous (oocyte donation) cycles (Fig. 1D). Recipient age, BMI and endometrial thickness were also similar between pregnant and non-pregnant groups (Table I). Thereafter, mtDNA content in human CCs was tested as a proxy for the developmental competence of their enclosed oocyte. mtDNA content in human CCs was remarkably similar between samples collected from oocytes not developing to blastocysts versus oocytes developing to blastocysts, irrespective of the pregnancy outcome (Fig. 1E), suggesting that this parameter provides a poor predictive value of oocyte developmental potential for *in vitro* embryo development. Similarly, relative mtDNA abundance did not vary between samples collected from oocytes ultimately establishing a pregnancy versus those developing to blastocyst but failing to establish pregnancy (Fig. 1D).

Aiming to reduce the effects of possible confounders inherently associated with human samples, mtDNA analysis in CCs was also performed in the bovine model. Cattle share with humans a mono-ovulatory folliculogenesis and similar timing of embryo development, which together with the possibility of using ovaries collected from the slaughterhouse makes it an excellent model for experimentation in human IVF (Lamas-Toranzo et al., 2018). As observed for human samples, relative mtDNA abundance in bovine CCs did not vary according to their developmental potential, being similar in non-cleaving oocytes, oocytes developing into cleaved embryos but arresting their development prior to blastocyst stage and oocytes developing into blastocysts (Fig. 2A). The use of an animal model also allows us to compare mtDNA content in oocytes and CCs, as mtDNA analysis in oocytes involves destroying the oocyte. mtDNA content in CCs was unrelated to that of their enclosed oocyte (Fig. 2B).

Discussion

Previous analyses of oocyte mtDNA in cohorts of oocytes showing different developmental potential or in unfertilized oocytes versus zygotes have identified a positive correlation between mtDNA amount and oocyte quality in humans (Reynier et al., 2001; Santos et al., 2006; Zhao et al., 2016) and experimental animal models (El Shourbagy et al., 2006; Lamas-Toranzo et al., 2018). Unfortunately, the analysis of mtDNA in oocytes is not compatible with subsequent development, while CCs are readily available following ICSI. In this sense, mtDNA analysis in CCs provides an alluring alternative method to select embryos based on their developmental potential. mtDNA analysis is less complex and faster than other molecular analyses, such as transcriptomics, yielding a result in a matter of hours, well before embryo transfer is performed, and thereby being readily applicable to IVF cycles without the need for embryo freezing. However, in contrast to previous observations (Ogino et al., 2016; Desquiret-Dumas et al., 2017; Taugourdeau et al., 2019), we have not found a correlation between mtDNA content in CCs and the developmental potential of the enclosed oocyte in either human or bovine samples. The reason for such a discrepancy is unknown, but it may be caused by differences in mtDNA analysis or by the different criteria used to assess embryo developmental potential.

mtDNA content in human CCs has been associated with embryo developmental potential, inferred indirectly using morphokinetical parameters (Ogino et al., 2016; Desquiret-Dumas et al., 2017; Yang et al., 2021). While this association is certainly interesting from a biological perspective, it does not provide any more information for embryo selection than conventional morphological selection. In other words, for a practical use, mtDNA analysis should provide additional predictive value of implantation rate to that already available by morphokinetical parameters. A previous study reported a positive correlation between mtDNA content in CCs and implantation potential (Taugourdeau et al., 2019), but the dispersion of the reported data (215 ± 375 versus 59 ± 72 for implanting versus non-implanting, respectively) may make this possible correlation not readily applicable for embryo selection, as it would be difficult to establish a given threshold to predict implantation potential. As uterine receptivity plays

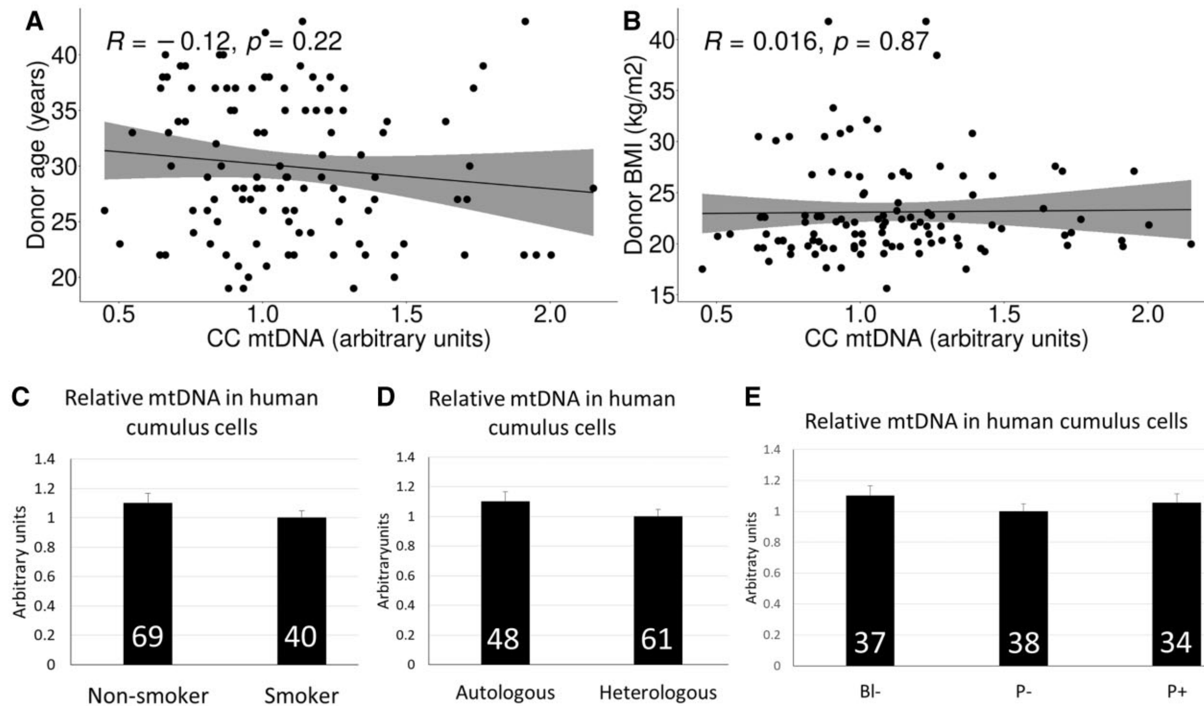

Figure 1. Analyses of mitochondrial DNA (mtDNA) content in 109 samples of human cumulus cells (CCs). (**A**) Donor age plotted against mtDNA content in human CCs. No correlation was found between both parameters. (**B**) Donor BMI plotted against mtDNA content in human CCs. No correlation was found between both parameters. (**C**) Relative mtDNA abundance in human CCs from donors who were non-smokers or smokers. No significant differences were found by Student's *t*-test ($P > 0.05$). The number of samples analyzed per group is indicated within each column. (**D**) Relative mtDNA abundance in human CCs from autologous and heterologous (oocyte donation) cycles. No significant differences were found by *t*-test ($P > 0.05$). The number of samples analyzed per group is indicated within each column. (**E**) Relative mtDNA abundance in human CCs obtained from oocytes not developing to blastocysts (Bl−), developing to blastocyst but failing to establish pregnancy (P−) or establishing pregnancy (P+). Data are presented as mean ± SEM. No significant differences were found by ANOVA ($P > 0.05$). The number of samples analyzed per group is indicated within each column.

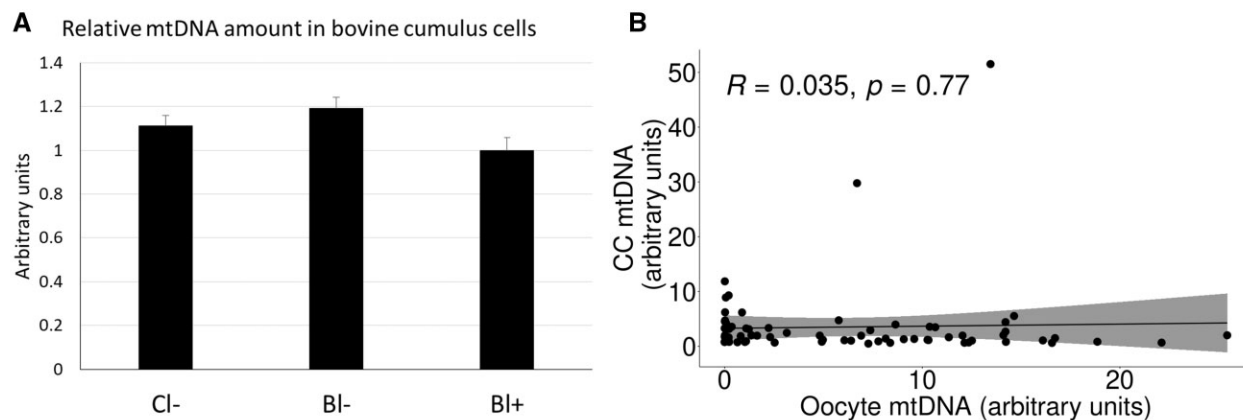

Figure 2. Relative mitochondrial DNA (mtDNA) abundance in bovine cumulus cells does not vary according to oocyte developmental potential. (**A**) Relative mtDNA abundance in bovine cumulus cells obtained from oocytes not cleaving (Cl−), cleaving but not developing to blastocyst (Bl−) or developing to blastocyst (Bl+). Data are presented as mean ± SEM. No significant differences among groups were found by ANOVA ($P > 0.05$). Forty samples were analyzed per group. (**B**) mtDNA content in bovine cumulus cells plotted against mtDNA content of their corresponding oocytes (85 samples). No correlation was found between both parameters.

a major role in implantation potential (Craciunas et al., 2019), our experimental design aimed to identify a putative gradual increase in CCs mtDNA between oocytes not developing to blastocyst versus oocytes developing to blastocyst (unaffected by recipient parameters) and between oocytes developing to blastocyst failing to implant versus able to implant. Despite obtaining a small intra-group variability (<7% SEM), mtDNA content in human CCs was unrelated to oocyte developmental potential. In agreement with our data, two recent articles found no correlation between mtDNA in human CCs and morphological parameters on Day 3 or subsequent implantation potential (Liu et al., 2021) or oocyte maturity, fertizability, embryo quality and pregnancy rates (Kumar et al., 2021). To further test a possible relation between mtDNA amount in CCs and embryo development, experiments were conducted on the bovine model, where experimental conditions are tightly controlled, as no hormonal treatments are used prior to oocyte collection and all oocytes are fertilized by the same fertile male. Data obtained here from the bovine model also failed to link mtDNA content in CCs with subsequent embryo development and found no correlation between mtDNA amount in CCs and oocytes, further refuting the use of this parameter for embryo selection.

While CC mtDNA content was not found to be a good predictor of implantation potential and qPCR-based oocyte mtDNA analysis is not compatible with subsequent development, the analysis of mtDNA content in embryo biopsies may provide good predictive value. Elevated mtDNA in trophectoderm biopsies has been associated with poor blastocyst implantation potential (Diez-Juan et al., 2015; Fragouli et al., 2015), although others have failed to find such a correlation (Treff et al., 2017; Victor et al., 2017; Klimczak et al., 2018; Scott et al., 2020). The possible negative correlation between implantation potential and mtDNA content in embryo biopsies has been suggested to be caused by a compensatory mechanism for an insufficient or faulty mitochondrial pool at the end of oogenesis (May-Panloup et al., 2016). However, embryonic mtDNA replication before implantation still remains a matter of controversy (Piko and Taylor, 1987) and a higher relative mtDNA in poor-quality embryos has also been suggested to be a consequence of degradation of genomic DNA (Ho et al., 2018). In any case, the positive correlations reported for mtDNA in oocytes and different developmental parameters (Reynier et al., 2001; Chan et al., 2005; May-Panloup et al., 2005; El Shourbagy et al., 2006; Santos et al., 2006; Murakoshi et al., 2013; Zhao et al., 2016; Lamas-Toranzo et al., 2018) suggest that different strategies aimed at increasing or correcting the mitochondrial pool in the oocyte may help to improve ART outcomes (Craven et al., 2017).

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Authors’ roles

Human samples were collected by A.M.-M., E.P.-R. and J.G.-B. Bovine embryo production and samples collection, and initial optimization of mtDNA analysis for human and bovine samples were performed by A.M.-M., I.L.-T., L.G.-B., A.P.-G. and P.B.-A. A.M.-M. performed final mtDNA analysis. Data analysis was performed b A.M.-M., L.G.-B. and P.B.-A. P.B.-A. designed experiments and managed funding. A.M.-M. and P.B.-A. wrote the manuscript, being supervised by all authors.

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Conflict of interest

The authors declare no conflict of interest.

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Capítulo 2

RNA-sequencing reveals genes linked with oocyte developmental potential in bovine cumulus cells

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Álvaro Martínez-Moro, Leopoldo González-Brusi, Ismael Lamas-Toranzo, Elena O'Callaghan, Anna Esteve-Codina, Pat Lonergan, Pablo Bermejo-Álvarez

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Abstract

Cumulus cells provide an interesting biological material to perform analyses to understand the molecular clues determining oocyte competence. The objective of this study was to analyze the transcriptional differences between cumulus cells from oocytes exhibiting different developmental potentials following individual *in vitro* embryo production by RNA-seq. Cumulus cells were allocated into three groups according to the developmental potential of the oocyte following fertilization: (1) oocytes developing to blastocysts (Bl+), (2) oocytes cleaving but arresting development before the blastocyst stage (Bl−), and (3) oocytes not cleaving (Cl−). RNAseq was performed on 4 (Cl−) or 5 samples (Bl+ and Bl−) of cumulus cells pooled from 10 cumulus-oocyte complexes per group. A total of 49, 50, and 18 differentially expressed genes (DEGs) were detected in the comparisons Bl+ versus Bl−, Bl+ versus Cl− and Bl- versus Cl−, respectively, showing a fold change greater than 1.5 at an adjusted p value <0.05. Focussing on DEGs in cumulus cells from Bl+ group, 10 DEGs were common to both comparisons (10/49 from Bl+ vs. Bl−, 10/50 from Bl+ vs. Cl−). These DEGs correspond to 6 upregulated genes (HBE1, ITGA1, PAPPA, AKAP12, ITGA5, and SLC1A4), and 4 downregulated genes (GSTA1, PSMB8, FMOD, and SFRP4) in Bl+ compared to the other groups, from which 7 were validated by quantitative PCR (HBE1, ITGA1, PAPPA, AKAP12, ITGA5, PSMB8 and SFRP4). These genes are involved in critical biological functions such as integrin-mediated cell adhesion, oxygen availability, IGF and Wnt signaling or PKA pathway, highlighting specific biological processes altered in incompetent *in vitro* maturation oocytes.

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RESEARCH ARTICLE

RNA-sequencing reveals genes linked with oocyte developmental potential in bovine cumulus cells

Álvaro Martínez-Moro[1,2] | **Leopoldo González-Brusi**[1] | **Ismael Lamas-Toranzo**[1] | **Elena O'Callaghan**[3] | **Anna Esteve-Codina**[4] | **Pat Lonergan**[3] | **Pablo Bermejo-Álvarez**[1]

[1]Department of Animal Reproduction, INIA, CSIC, Madrid, Spain

[2]Embryology, IVF Spain, Madrid, Spain

[3]Agriculture and Food Science, School of Agriculture and Food Science, University College Dublin, Dublin, Ireland

[4]Functional Genomics, CNAG-CRG, Centre for Genomic Regulation, Barcelona Institute of Science and Technology, Barcelona, Spain

Correspondence
Pablo Bermejo-Álvarez, Department of Animal Reproduction, INIA-CSIC, 28040 Madrid, Spain.
Email: bermejo.pablo@inia.es

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Abstract

Cumulus cells provide an interesting biological material to perform analyses to understand the molecular clues determining oocyte competence. The objective of this study was to analyze the transcriptional differences between cumulus cells from oocytes exhibiting different developmental potentials following individual in vitro embryo production by RNA-seq. Cumulus cells were allocated into three groups according to the developmental potential of the oocyte following fertilization: (1) oocytes developing to blastocysts (Bl+), (2) oocytes cleaving but arresting development before the blastocyst stage (Bl−), and (3) oocytes not cleaving (Cl−). RNAseq was performed on 4 (Cl−) or 5 samples (Bl+ and Bl−) of cumulus cells pooled from 10 cumulus-oocyte complexes per group. A total of 49, 50, and 18 differentially expressed genes (DEGs) were detected in the comparisons Bl+ versus Bl−, Bl+ versus Cl− and Bl- versus Cl−, respectively, showing a fold change greater than 1.5 at an adjusted p value <0.05. Focussing on DEGs in cumulus cells from Bl+ group, 10 DEGs were common to both comparisons (10/49 from Bl+ vs. Bl−, 10/50 from Bl+ vs. Cl−). These DEGs correspond to 6 upregulated genes (*HBE1*, *ITGA1*, *PAPPA*, *AKAP12*, *ITGA5*, and *SLC1A4*), and 4 downregulated genes (*GSTA1*, *PSMB8*, *FMOD*, and *SFRP4*) in Bl+ compared to the other groups, from which 7 were validated by quantitative PCR (*HBE1*, *ITGA1*, *PAPPA*, *AKAP12*, *ITGA5*, *PSMB8* and *SFRP4*). These genes are involved in critical biological functions such as integrin-mediated cell adhesion, oxygen availability, IGF and Wnt signaling or PKA pathway, highlighting specific biological processes altered in incompetent in vitro maturation oocytes.

KEYWORDS
granulosa cell, integrin, in vitro maturation, oocyte quality, transcription

1 | INTRODUCTION

In vitro embryo production (IVP) enables relevant applications for cattle reproductive management, including alleviating the negative effects of heat stress (Baruselli et al., 2020) and accelerating genetic improvement, especially when combined with sexed semen and embryo genomic selection (Ferre et al., 2020). Nevertheless, the general efficiency of the IVP process remains relatively low, as only between 30% and 40% of in vitro matured oocytes reach the blastocyst stage following fertilization and culture. Reduced oocyte

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competence clearly stands out as a major causative factor for the reduced developmental rates in IVP, as in vivo matured oocytes exhibit significantly higher developmental rates following in vitro fertilization (IVF) than those matured in vitro (Dieleman et al., 2002; Rizos et al., 2002; van de Leemput et al., 1999). In this context, understanding the underlying molecular regulation of oocyte competence is critical to improve IVP, but molecular analyses in oocytes are typically invasive, involve destroying the oocyte and are thus incompatible with subsequent embryo development. One solution is to use the surrounding cumulus cells as proxies of oocyte quality as they constitute an attractive matrix on which to perform molecular analyses, and are closely connected to the oocyte during growth and final maturation, serving essential metabolic and signaling functions.

Previous attempts to discover genes whose transcript abundance in cumulus cells may serve as predictor for bovine oocyte competence have been focused on comparing cumulus cells from groups of oocytes whose developmental competence was indirectly inferred by follicle size (Melo et al., 2017), the use of different in vitro maturation (IVM) media (Assidi et al., 2008), or their origin: in vivo versus in vitro (Salhab et al., 2013; Tesfaye et al., 2009), prepubertal versus adult (Bettegowda et al., 2008), or collected before versus after LH surge (Assidi et al., 2010) or at different times following FSH withdrawal (Bunel et al., 2014). Unfortunately, the candidate genes identified on the microarray-based experiments mentioned above show very poor correlation between studies. Besides, these candidates genes were not coincident with those identified on a microarray-based experiment where oocyte developmental competence was directly assessed by performing individual IVP (Bunel et al., 2015). Another study analyzing the expression of candidate genes by quantitative PCR (qPCR) in cumulus cells from oocytes showing different developmental competence also showed results discordant with microarray data (Kussano et al., 2016), indicating that the molecular signature of the developmentally competent oocyte still remains elusive.

The reasons for the lack of agreement between studies may have a biological basis, as the different classification criteria used to indirectly infer oocyte competence dealt with diverse biological processes such as follicle growth or hormonal response which may have a transcriptional effect on their own different from the sought-after transcriptional signature of developmental competence. Another possible source of inconsistency may be technical, as microarray based experiments rely on a finite number of probes that vary depending on the manufacturer. In contrast to microarray, RNA-seq provides an unbiased search for candidate transcripts and yield a higher dynamic range, ultimately leading to higher accuracy. The objective of this study was to apply RNA-seq to uncover the transcriptional differences between cumulus cells enclosing oocytes that exhibit different developmental competence. To that aim, individual IVP was performed to infer directly the developmental potential of each oocyte. Once the developmental potential of each cumulus-oocyte complexe (COC) was known, the stored cumulus cells were allocated to one of three groups according to the oocyte's developmental potential: (1) Oocytes not cleaving following IVF (Cl−), (2) oocytes cleaving but not developing to blastocysts (Bl−) and (3) oocytes developing to blastocyst (Bl+).

2 | RESULTS

To correlate cumulus cell transcription with the developmental competence of the enclosed oocyte, COCs were individually matured. Cumulus cell samples were collected from 396 individual COCs in 7 replicates. Developmental potential of each individual oocyte was assessed at 48 h postinsemination (cleavage rate 52.3%, 207 embryos cleaved) and at day 8 postinsemination (blastocyst rate 13.4%, 53 blastocysts). Once the developmental potential of each COC was known, the stored cumulus cells were allocated to one of three groups according to the oocyte's developmental potential: (1) Oocytes not cleaving following IVF (Cl−), (2) oocytes cleaving but not developing to blastocysts (Bl−) and (3) oocytes developing to blastocyst (Bl+).

RNA-seq detected the expression of 19,335 genes in bovine cumulus cells samples. Using a raw p value <0.05, inappropriate for RNA-seq data as described below, the analysis identified 1609, 1466, and 1420 differentially expressed genes (DEGs) for the comparisons Bl+ versus Bl−, Bl+ versus Cl−, and Bl− versus Cl−, respectively (Figure 1). These DEGs were narrowed down to 77, 80, and 32 DEGs for the comparisons Bl+ versus Bl−, Bl+ versus Cl−, and Bl− versus Cl−, respectively, when an adjusted p value < 0.05 was used. Such adjusted p value takes into account the data overdispersion inherently associated with RNA-seq data, yielding a more reliable result. From these subsets of DEGs obtained at an adjusted p value <0.05, 49, 50, and 15 DEGs, for the comparisons Bl+ versus Bl−, Bl+ versus Cl−, and Bl− versus Cl−, respectively, exhibited a fold change greater than 1.5 (Figure 1 and Table 1).

Enrichment analysis failed to find enriched terms in the 15 DEGs in Bl− versus Cl− comparison, but four common enrichment terms (FDR < 0.05) were found in the comparisons Bl+ versus Bl− and Bl+ versus Cl−: “integrin domain superfamily,” “cell surface receptor signaling pathway,” “response to organic substance,” and “response to chemical.” There were also terms exclusive to Bl+ versus Cl− and Bl+ versus Bl− comparisons: “Extracellular region” and “proteosomal complex” were exclusive to the Bl+ versus Cl− comparison and “anatomical structure morphogenesis,” “positive regulation of cell communication,” or “cell communication” were exclusive to Bl+ versus Bl− comparison.

Interaction networks allow to determine if the proteins encoded by DEGs interact directly (physical) or indirectly (functional) with each other, aiming to uncover network properties associated to developmental potential. The interaction network from DEGs in the Bl− versus Cl− comparison did not show a significant connectivity, as the observed interactions (5) were close to the expected random observations (3). In contrast, there were statistically significant relationships for DEGs in Bl+ versus Bl− and Bl+ versus Cl− comparisons. In the case of the 50 DEGs in the Bl+ versus Cl− comparison, there were 59 observed interactions versus 33 expected (Figure 2). Several clusters of genes in the interaction network coded for proteins that were related to enriched terms were selected. such as (1) extracellular matrix organization, including *PXDN*, *TNC*, *ITGA1*, *ITGA8*, and *FMOD*), (2) cytokine signaling in

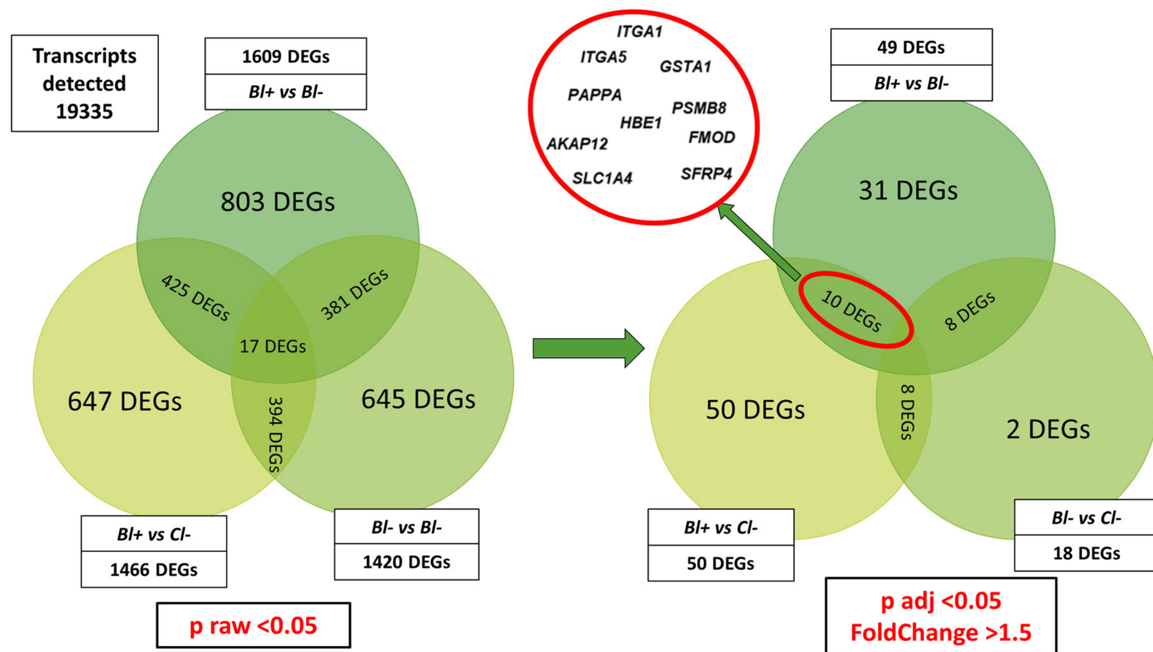


FIGURE 1 Venn diagram of differentially expressed genes (DEG) for the comparisons of the three groups exhibiting different developmental ability following IVF (Bl+, Bl−, and Cl−). Image on left shows DEG at a p raw <0.05. These lists of DEG are reduced by applying an adjusted $p < 0.05$ and a fold change greater than 1.5 (right image). 10 DEGs were common to Bl+ versus Bl− and Bl+ versus Cl− comparisons (red circle on right image)

immune system including *PSME2*, *SAMHD1*, *IFI6*, *GBP4*, *PSMB9*, *PSMB8*, *LGALS9*, *IFITM3*, *IFI35*, and *IFI27*, and (3) G alpha (q) signaling events, including *NTS*, *F2RL2*, and *HCTRC1*. Interaction analysis of the 49 DEGs in the Bl+ versus Bl− comparison observed 69 interactions versus 23 expected (Figure 2). Although the connectivity found Bl+ versus Bl− was weaker than for Bl+ versus Cl−, several nodes including *NTRK2*, *CDH17*, and *ACTN1* or *HMCN2* displayed a high number of connections.

A more stringent selection of DEGs was obtained through the three-group experimental design. Such a design constitutes a double check for DEGs potentially associated with oocyte developmental potential as both Bl+ versus Bl− and Bl+ versus Cl− comparisons contrast COCs exhibiting good developmental potential (Bl+) to COCs resulting in developmental arrest (Bl− and Cl−) (Figure 1). At an adjusted p value > 0.05 and fold change >1.5, 10 DEGs were common to both comparisons (10/49 from Bl+ vs. Bl−, 10/50 from Bl+ vs. Bl−). These DEGs correspond to 4 genes upregulated (*GSTA1*, *PSMB8*, *FMOD*, and *SFRP4*) and 6 genes downregulated (*HBE1*, *ITGA1*, *PAPPA*, *AKAP12*, *ITGA5*, and *SLC1A4*) in Bl+ compared to the other groups and none exhibited statistically significant differences in the Bl− versus Cl− comparison. Nine of those genes were selected for RNA-seq validation by qPCR, as we were unable to amplify *SLC1A4* by qPCR from cumulus cell complementary DNA (cDNA) samples at an efficiency compatible with reliable quantification. qPCR was performed using the same RNA samples than those employed for RNA-seq, so although this provides a technical validation (using different retrotranscription and technique) no validation in independent biological samples was tested. qPCR confirmed the significant differences observed by RNA-seq in both comparisons for *HBE1*, *ITGA1*, *PAPPA*, *ITGA5*, and *SFRP4*. In the case of *AKAP12* and *PSMB8* only the Bl+ versus Cl− comparison remained significant, whereas no significant difference between groups were observed for *GSTA1* and *FMOD* (Figure 3).

3 | DISCUSSION

The transcriptional analysis of individual COCs requires two critical modifications to conventional bovine IVP procedures: (1) individual IVP (iIVP) and (2) cumulus cell removal before fertilization. As a consequence of these modifications, blastocyst rates are halved compared to conventional IVP, impairing a direct application for commercial IVP. Given that we have previously observed that cumulus cell removal before IVF does not cause a significant reduction in blastocyst rate when IVP is conducted in groups (Lamas-Toranzo et al., 2019), we believe that the major cause of the reduced developmental rate is iIVP, as previously reported by others (Bunel et al., 2015). In this sense, while current methods allow performing iIVP to elucidate molecular clues of developmental competence (i.e., this experiment) further advances on iIVP are needed to be able to apply oocyte selection markers for commercial IVP purposes.

The transcriptional analysis of cumulus cells obtained from oocytes of known developmental potential has identified different biological pathways that are likely involved in oocyte quality. Among the different DEGs obtained in this analysis, integrins may be a key

TABLE 1 Differentially expressed genes at a p adjusted value <0.05 and fold change >1.5 at the three comparisons

	baseMean	log2FoldChange	shrunkenlfc	lfcSE	stat	filter	p value	padj	Fold change	Shrunken Fold change
ENSBTAG00000037815, HBE1, protein_coding	26.4787138	−2.12882978	−0.77751908	0.3854938	−5.5223451	1	3.35E-08	0.00012695	−4.37362577	−1.71418056
ENSBTAG00000012519, XDH, protein_coding	34.9303151	−1.70602939	−0.61150107	0.39614703	−4.30655603	1	1.66E-05	0.00980409	−3.26261644	−1.52784805
ENSBTAG00000002319, HMCN2, protein_coding	60.1771415	−1.64057208	−0.50575982	0.4298763	−3.81638177	0	0.00013542	0.0356707	−3.11789443	−1.41987096
ENSBTAG00000015409, STK32B, protein_coding	15.6495314	−1.55173881	−0.53294504	0.4260127	−3.64247075	0	0.00027003	0.04909611	−2.9317027	−1.44687977
ENSBTAG00000035005, protein_coding	17.6713968	−1.52954229	−0.57825979	0.3999492	−3.82434143	0	0.00013112	0.03554952	−2.88694232	−1.49304721
ENSBTAG00000000817, SYNJ2, protein_coding	48.4180788	−1.12361283	−0.55634793	0.29865507	−3.7622426	0	0.0001684	0.03880699	−2.1789194	−1.47054194
ENSBTAG00000017733, CA2, protein_coding	1232.68419	−1.09137678	−0.5943611	0.27937775	−3.90645558	1	9.37E-05	0.03011709	−2.13077282	−1.50980382
ENSBTAG00000010273, EREG, protein_coding	63.657073	−1.04827932	−0.56793004	0.28411068	−3.6896864	0	0.00022453	0.0463371	−2.06806183	−1.48239512
ENSBTAG00000016525, ITGA1, protein_coding	100.3518	−1.02332528	−0.62989844	0.23029216	−4.44359582	1	8.85E-06	0.00654253	−2.03259851	−1.54745606
ENSBTAG00000010647, NTRK2, protein_coding	44.2282193	−0.96405071	−0.5662427	0.25350133	−3.80294139	0	0.00014299	0.03649014	−1.9507795	−1.48066237
ENSBTAG00000020895, LOXL4, protein_coding	9013.3992	−0.9606973	−0.5492939	0.2537134	−3.78654545	0	0.00015276	0.03705543	−1.94625035	−1.46336931
ENSBTAG00000008814, ADGRA2, protein_coding	544.473729	−0.94162586	−0.6037932	0.2221158	−4.23934655	1	2.24E-05	0.01084811	−1.92069157	−1.519707
ENSBTAG00000002888, TMTC2, protein_coding	1371.22778	−0.92371015	−0.56201345	0.24029339	−3.84409312	0	0.000121	0.03485529	−1.89698747	−1.47632817
ENSBTAG00000018255, ACTN1, protein_coding	1406.51659	−0.91290287	−0.646985	0.19274936	−4.73621729	1	2.18E-06	0.00301075	−1.88283017	−1.56589231
ENSBTAG00000052736, protein_coding	70.0576043	−0.91195359	−0.56554114	0.23423242	−3.89337057	0	9.89E-05	0.03080768	−1.88159169	−1.47994251
ENSBTAG00000004010, PAPPA, protein_coding	705.782085	−0.89773068	−0.58690686	0.21375835	−4.19974556	1	2.67E-05	0.01231594	−1.86313302	−1.50202295
ENSBTAG00000005714, ACTC1, protein_coding	1295.44064	−0.8687399	−0.54698365	0.2328306	−3.73121023	0	0.00019056	0.0420548	−1.82606725	−1.46102782
ENSBTAG00000004375, ESRP2, pseudogene	66.9836549	−0.85983042	−0.61682149	0.19224247	−4.47263519	1	7.73E-06	0.00628409	−1.81482497	−1.5334929
ENSBTAG00000017086, GRB10, protein_coding	468.565501	−0.82402184	−0.53702793	0.21830105	−3.77470396	0	0.0001602	0.03767073	−1.77033432	−1.4509803
ENSBTAG00000014270, UNC5B, protein_coding	1451.4862	−0.80782213	−0.57019158	0.19370083	−4.17046284	0	3.04E-05	0.01313486	−1.75056682	−1.48472071
ENSBTAG00000014705, HES4, protein_coding	401.094545	−0.80463733	−0.57900276	0.18921065	−4.25260065	0	2.11E-05	0.01084811	−1.74670664	−1.49381631
ENSBTAG00000014788, AKAP12, protein_coding	3125.78806	−0.80402001	−0.56642493	0.19181549	−4.19163227	0	2.77E-05	0.01235307	−1.74595939	−1.4808494
ENSBTAG00000013745, ITGA5, protein_coding	8536.22896	−0.80039934	−0.67011624	0.13183965	−6.0710062	1	1.27E-09	1.76E-05	−1.74158314	−1.59120117
ENSBTAG00000003955, MYO7A, protein_coding	295.849762	−0.79463621	−0.54856033	0.20508284	−3.87470846	0	0.00010675	0.03208839	−1.73463991	−1.46262541

TABLE 1 (Continued)

	baseMean	log2FoldChange	shrunkenlfc	lfcSE	stat	filter	*p* value	padj	Fold change	Shrunken Fold change
ENSBTAG00000007763, SLC1A4, protein_coding	731.552183	−0.76493065	−0.51011009	0.20801395	−3.67730452	0	0.00023571	0.0471329	−1.69928831	−1.42415887
ENSBTAG00000007156, AGAP2, protein_coding	502.509705	−0.75188674	−0.53930729	0.18867277	−3.98513651	0	6.74E-05	0.02453977	−1.68399369	−1.45327456
ENSBTAG00000016685, FMO4, protein_coding	281.168645	−0.74909699	−0.5273272	0.19717086	−3.79922766	0	0.00014515	0.03649014	−1.68074049	−1.44125659
ENSBTAG00000003100, SMTN, protein_coding	1828.20159	−0.73778949	−0.60298346	0.14279815	−5.16665997	1	2.38E-07	0.0005492	−1.66761874	−1.51885428
ENSBTAG00000010531, CYP1B1, protein_coding	302.952778	−0.73459353	−0.57562419	0.15782087	−4.65460328	0	3.25E-06	0.00374026	−1.6639286	−1.49032212
ENSBTAG00000051704, TNFRSF10D, protein_coding	2153.66879	−0.71567807	−0.57329256	0.14921157	−4.79639795	0	1.62E-06	0.00258255	−1.6422549	−1.48791547
ENSBTAG00000046476, IGSF1, protein_coding	53.4421741	−0.71226507	−0.50483777	0.19486683	−3.65513751	0	0.00025704	0.04802905	−1.63837439	−1.41896379
ENSBTAG00000001042, MXD1, protein_coding	412.83263	−0.64709909	−0.49469463	0.16738173	−3.866008	0	0.00011063	0.03254679	−1.56601615	−1.40902249
ENSBTAG00000020650, ADCYAP1, protein_coding	7138.37598	−0.63633302	−0.48586002	0.168616	−3.77385919	0	0.00016074	0.03767073	−1.5543733	−1.40042043
ENSBTAG00000001511, BCL6, protein_coding	5546.65961	−0.58499101	−0.49417875	0.12946284	−4.51860152	0	6.22E-06	0.00537952	−1.50002964	−1.40851874
ENSBTAG00000021516, GSTA1, protein_coding	5460.78137	0.61970182	0.54315932	0.11335477	5.46692312	0	4.58E-08	0.00012695	1.53655757	1.45716002
ENSBTAG00000024493, DHRS3, protein_coding	2369.08918	0.65928401	0.50356721	0.16823569	3.9188118	0	8.90E-05	0.03001018	1.57929864	1.41771467
ENSBTAG00000015086, HSD11B1, protein_coding	1492.89658	0.67415399	0.48931148	0.18314371	3.68101086	0	0.00023231	0.0471329	1.59566079	1.40377477
ENSBTAG00000003039, PSMB8, protein_coding	709.1219	0.68317143	0.52216713	0.16951175	4.03023058	0	5.57E-05	0.02082352	1.60566556	1.43611087
ENSBTAG00000021964, CDH17, protein_coding	1535.98143	0.68771286	0.58492491	0.12580542	5.46648021	0	4.59E-08	0.00012695	1.61072797	1.49996092
ENSBTAG00000014912, FMOD, protein_coding	739.722034	0.73701708	0.6215327	0.13030585	5.65605543	1	1.55E-08	0.00010708	1.66672615	1.5385088
ENSBTAG00000002333, HOPX, protein_coding	1016.20725	0.74721593	0.6000701	0.14865524	5.02650252	1	5.00E-07	0.00098667	1.67855049	1.51579021
ENSBTAG00000014291, WNT2B, protein_coding	319.903568	0.93282217	0.57235467	0.23656942	3.9431224	0	8.04E-05	0.0278018	1.9090067	1.48694849
ENSBTAG00000021880, COQ8A, protein_coding	191.22917	0.96592022	0.63707829	0.21754298	4.44013509	1	8.99E-06	0.00654253	1.95330904	1.55517646
ENSBTAG00000012543, EDA, protein_coding	19.6165426	1.2074367	0.57659555	0.31657913	3.81401229	0	0.00013673	0.0356707	2.30926974	1.49132589
ENSBTAG00000015366, SFRP4, protein_coding	886.750538	1.25803286	0.68623077	0.28545786	4.40707038	1	1.05E-05	0.00689889	2.39169408	1.60907411
ENSBTAG00000003668, RADX, protein_coding	28.5049993	1.32787195	0.59111845	0.32947259	4.0302957	1	5.57E-05	0.02082352	2.51032116	1.50641414
ENSBTAG00000022759, PAG11, protein_coding	70.901648	1.83737746	0.43593861	0.49388336	3.720266	0	0.00019901	0.04233467	3.57359827	1.35279066

(Continues)

TABLE 1 (Continued)

	baseMean	log2FoldChange	shrunkenlfc	lfcSE	stat	filter	*p* value	padj	Fold change	Shrunken Fold change
ENSBTAG00000016030, HOXD10, protein_coding	9.66888124	4.89511024	0.34181762	1.14396661	4.27906743	0	1.88E-05	0.01038009	29.7560319	1.2673523
ENSBTAG00000033330, HOXD11, protein_coding	22.7369796	5.40528012	0.2810518	1.18585902	4.5581136	0	5.16E-06	0.00475788	42.3790727	1.21508042

	baseMean	log2FoldChange	shrunkenlfc	lfcSE	stat	filter	*p* value	padj	Fold change	Shrunken fold change
ENSBTAG00000037815, HBE1, protein_coding	26.4787138	−1.91848857	−0.64987859	0.40607055	−4.72452032	1	2.31E-06	0.00297571	−3.78026813	−1.56903615
ENSBTAG00000005404, MSC, protein_coding	9.47009056	−1.68620844	−0.53638082	0.45222608	−3.72868466	0	0.00019248	0.03691229	−3.2180984	−1.45032961
ENSBTAG00000021846, CELSR3, protein_coding	28.7980484	−1.33691093	−0.70503025	0.28588494	−4.67639503	1	2.92E-06	0.00312092	−2.52609855	−1.63017884
ENSBTAG00000050986, PXDN, protein_coding	759.097847	−1.28697186	−0.63829334	0.30510584	−4.21811607	1	2.46E-05	0.01152678	−2.44015343	−1.55648679
ENSBTAG00000008111, ESYT3, protein_coding	35.4964486	−1.28597795	−0.62360821	0.31108815	−4.1338056	1	3.57E-05	0.01301056	−2.43847291	−1.54072375
ENSBTAG00000016525, ITGA1, protein_coding	100.3518	−1.22846434	−0.74425043	0.24213649	−5.07343748	1	3.91E-07	0.00135643	−2.3431744	−1.67510373
ENSBTAG00000000575, TNC, protein_coding	1942.73478	−1.18594336	−0.65829251	0.27295316	−4.34486042	1	1.39E-05	0.00974389	−2.27512113	−1.57821363
ENSBTAG00000013081, PSPH, protein_coding	529.96833	−1.04225433	−0.57189496	0.2726827	−3.82222385	0	0.00013225	0.02976424	−2.0594432	−1.48647475
ENSBTAG00000004010, PAPPA, protein_coding	705.782085	−1.03006758	−0.65821181	0.22648823	−4.54799598	1	5.42E-06	0.00480358	−2.04211991	−1.57812536
ENSBTAG00000000898,F2RL2, protein_coding	391.727257	−1.01147449	−0.57030922	0.2651496	−3.81473136	0	0.00013633	0.02976424	−2.01597045	−1.48484179
ENSBTAG00000011171, PIEZO2, protein_coding	266.685041	−0.95741882	−0.5973885	0.23464523	−4.08028251	1	4.50E-05	0.01484478	−1.94183258	−1.51297537
ENSBTAG00000005305, NTS, protein_coding	800.137496	−0.94308085	−0.56728208	0.24642762	−3.82700958	0	0.00012971	0.02976424	−1.9226296	−1.48172948
ENSBTAG00000007763, SLC1A4, protein_coding	731.552183	−0.93784076	−0.61181017	0.2203764	−4.25563146	1	2.08E-05	0.01152678	−1.91565898	−1.52817543
ENSBTAG00000049806, protein_coding	45.5574402	−0.86036732	−0.55532883	0.22431935	−3.83545739	0	0.00012533	0.02976424	−1.81550049	−1.46950354
ENSBTAG00000014788, AKAP12, protein_coding	3125.78806	−0.84079252	−0.57935931	0.20341816	−4.13332085	0	3.58E-05	0.01301056	−1.79103374	−1.49418554
ENSBTAG00000012004, TGFB3, protein_coding	492.916789	−0.82260429	−0.52851344	0.22533801	−3.65053501	0	0.00026169	0.04700897	−1.76859571	−1.44244213
ENSBTAG00000006731, SLC7A5, protein_coding	1615.61458	−0.80300952	−0.5712345	0.19264121	−4.16842025	0	3.07E-05	0.0124073	−1.74473693	−1.48579441
ENSBTAG00000013745, ITGA5, protein_coding	8536.22896	−0.69648492	−0.57619276	0.1398384	−4.98064151	0	6.34E-07	0.0014989	−1.62055156	−1.49090957
ENSBTAG00000010366, HCRTR1, protein_coding	92.8984086	−0.67396878	−0.50576093	0.1736652	−3.88085101	0	0.00010409	0.02645027	−1.59545595	−1.41987205
ENSBTAG00000006770, MTBP, protein_coding	197.974827	−0.66753843	−0.52635602	0.15623066	−4.27277469	0	1.93E-05	0.01141521	−1.58836054	−1.4402867
ENSBTAG00000022007, SAMHD1, protein_coding	507.068088	−0.65998868	−0.49619588	0.17198734	−3.83742589	0	0.00012433	0.02976424	−1.58007022	−1.41048946

TABLE 1 (Continued)

	baseMean	log2FoldChange	shrunkenlfc	lfcSE	stat	filter	*p* value	padj	Fold change	Shrunken fold change
ENSBTAG00000000446, ATP11A, protein_coding	817.397518	−0.63853304	−0.5112286	0.15082022	−4.23373624	0	2.30E-05	0.01152678	−1.55674543	−1.42526344
ENSBTAG00000021516, GSTA1, protein_coding	5460.78137	0.58634961	0.5090243	0.12023851	4.87655405	0	1.08E-06	0.00166247	1.5014429	1.42308743
ENSBTAG00000005814, PSME2, protein_coding	1461.85184	0.59167211	0.46356199	0.15837816	3.73581881	0	0.00018711	0.03637277	1.50699237	1.3789422
ENSBTAG00000037988, ZSCAN31, protein_coding	394.510381	0.59644896	0.46825745	0.15731561	3.79141629	0	0.00014979	0.03166985	1.51199038	1.38343748
ENSBTAG00000014912, FMOD, protein_coding	739.722034	0.59878705	0.50092357	0.1380527	4.33738015	0	1.44E-05	0.00974389	1.51444276	1.41511919
ENSBTAG00000003458, CDCA7, protein_coding	128.32517	0.6126092	0.47091108	0.16214237	3.7782179	0	0.00015795	0.0322565	1.52902204	1.38598445
ENSBTAG00000017810, EFHC1, protein_coding	255.040583	0.63268455	0.51540401	0.14234854	4.44461552	0	8.80E-06	0.00657635	1.55044737	1.42939437
ENSBTAG00000014878, COX7A1, protein_coding	448.645919	0.63496081	0.48829927	0.16612315	3.8222294	0	0.00013225	0.02976424	1.55289556	1.40279021
ENSBTAG00000008954, PSMB9, protein_coding	322.288285	0.64130912	0.5126677	0.15221499	4.2131797	0	2.52E-05	0.01152678	1.55974385	1.42668586
ENSBTAG00000010365, SQOR, protein_coding	297.827243	0.64827557	0.5409818	0.13217637	4.90462525	0	9.36E-07	0.00166247	1.56729371	1.45496233
ENSBTAG00000019015, IFITM3, protein_coding	17098.1904	0.66380895	0.52294932	0.15690873	4.23054199	0	2.33E-05	0.01152678	1.58425981	1.4368897
ENSBTAG00000021378,S100A13, protein_coding	410.889638	0.737533	0.54657618	0.17711502	4.16414715	0	3.13E-05	0.0124073	1.66732229	1.46061523
ENSBTAG00000007389, IFI35, protein_coding	476.508804	0.79118103	0.56433895	0.19007248	4.16252268	0	3.15E-05	0.0124073	1.7304905	1.4787098
ENSBTAG00000005182, BoLA, protein_coding	1270.66579	0.79322265	0.53057632	0.21296559	3.72465179	0	0.00019559	0.03700729	1.73294114	1.44450613
ENSBTAG00000007602, ITGA8, protein_coding	123.960196	0.82296642	0.54099383	0.22217648	3.70411138	0	0.00021213	0.03909584	1.76903969	1.45497446
ENSBTAG00000052055, PRSS35, protein_coding	926.617163	0.87588891	0.57257477	0.22005409	3.98033467	0	6.88E-05	0.02170224	1.83513846	1.48717536
ENSBTAG00000003039, PSMB8, protein_coding	709.1219	0.87604555	0.64973839	0.18024151	4.86039847	1	1.17E-06	0.00166247	1.8353377	1.56888367
ENSBTAG00000012208, protein_coding	641.996258	0.92782533	0.52508522	0.25480351	3.64133656	0	0.00027123	0.04811215	1.90240621	1.43901858
ENSBTAG00000003152, IFI27, protein_coding	12519.18	1.00530287	0.63919636	0.22516773	4.46468453	1	8.02E-06	0.00651819	2.00736487	1.55746135
ENSBTAG00000017741, HACD4, protein_coding	96.8920071	1.01382425	0.57832167	0.25558096	3.96674396	0	7.29E-05	0.02199943	2.01925659	1.49311126
ENSBTAG00000007554, IFI6, protein_coding	9514.43329	1.01384156	0.60995997	0.24571896	4.12602094	1	3.69E-05	0.01309452	2.01928083	1.52621686
ENSBTAG00000037533,C4A, protein_coding	1687.1508	1.04743914	0.62189015	0.24716276	4.23785181	1	2.26E-05	0.01152678	2.06685781	1.53889004
ENSBTAG00000004155, SPATA20, protein_coding	143.31563	1.15486135	0.81924597	0.18903435	6.10926728	1	1.00E-09	7.55E-06	2.22662923	1.76448354
ENSBTAG00000006864, protein_coding	533.66699	1.20916202	0.54071426	0.32344862	3.73834343	0	0.00018524	0.03637277	2.31203306	1.45469254
ENSBTAG00000015366, SFRP4, protein_coding	886.750538	1.57569053	0.79740628	0.30320757	5.19673877	1	2.03E-07	0.00095938	2.98078131	1.73797374

(Continues)

TABLE 1 (Continued)

	baseMean	log2FoldChange	shrunkenlfc	lfcSE	stat	filter	*p* value	padj	Fold change	Shrunken fold change
ENSBTAG00000006846, LGALS9, protein_coding	189.319423	1.57652444	0.53710214	0.41616931	3.78818044	0	0.00015175	0.03166985	2.98250475	1.45105493
ENSBTAG00000020203, TMEM151A, protein_coding	44.2503514	1.8064424	0.61919315	0.41960817	4.30506965	1	1.67E-05	0.0103706	3.4977869	1.5360159
ENSBTAG00000040244, APOL3, protein_coding	47.3460324	2.10452986	0.5057058	0.53922484	3.90288007	0	9.51E-05	0.02549885	4.30057588	1.4198178
ENSBTAG00000014529, GBP4, protein_coding	57.7785598	2.58915172	0.41020341	0.62389815	4.14995896	0	3.33E-05	0.01275406	6.01744781	1.32887316
	baseMean	**log2FoldChange**	**shrunkenlfc**	**lfcSE**	**stat**	**filter**	***p* value**	**padj**	**Fold change**	**Shrunken fold change**
ENSBTAG00000011538, KIF1A, protein_coding	70.20266	−2.23957319	−0.64752587	0.47159611	−4.74892212	1	2.05E-06	0.00688155	−4.72257331	−1.56647948
ENSBTAG00000005679, TMEM130, protein_coding	21.2912193	−2.0531094	−0.58276117	0.4826633	−4.25370933	0	2.10E-05	0.03312846	−4.14999442	−1.49771298
ENSBTAG00000033726, GRIP1, protein_coding	42.6648451	−1.04563585	−0.59317774	0.26370095	−3.96523354	1	7.33E-05	0.04934713	−2.06427597	−1.50856592
ENSBTAG00000007698, TMEM59L, protein_coding	445.22969	−0.83954751	−0.55161438	0.21807519	−3.8498076	0	0.00011821	0.04972235	−1.7894888	−1.46572493
ENSBTAG00000012012, CYB5A, protein_coding	912.792784	−0.75546844	−0.57766426	0.16723207	−4.51748539	0	6.26E-06	0.01684609	−1.68817965	−1.49243103
ENSBTAG00000047706, ING2, protein_coding	1370.59626	−0.73789537	−0.54506528	0.17903156	−4.12159382	0	3.76E-05	0.03376308	−1.66774113	−1.45908636
ENSBTAG00000002699, KIT, protein_coding	3268.693	−0.65390318	−0.52709465	0.14709654	−4.4454016	0	8.77E-06	0.01968028	−1.57341929	−1.44102428
ENSBTAG00000015248, PLA2G16, protein_coding	1726.79268	−0.63501221	−0.48962246	0.16425182	−3.86608937	0	0.00011059	0.04972235	−1.5529509	−1.40407739
ENSBTAG00000003081, RWDD4, protein_coding	1842.91844	−0.62907124	−0.49540946	0.15522398	−4.05266796	0	5.06E-05	0.03786511	−1.54656904	−1.4097208
ENSBTAG00000004375, ESRP2, pseudogene	66.9836549	0.79182826	0.55735351	0.20436503	3.87457796	0	0.00010681	0.04972235	1.73126703	1.47156729
ENSBTAG00000004155, SPATA20, protein_coding	143.31563	0.79989979	0.54641291	0.19081576	4.19200067	0	2.77E-05	0.03312846	1.74098019	1.46044995
ENSBTAG00000011131, NMUR2, protein_coding	302.704748	0.84025472	0.54728294	0.21787477	3.85659483	0	0.00011498	0.04972235	1.79036621	1.46133095
ENSBTAG00000003955, MYO7A, protein_coding	295.849762	0.90394908	0.60207688	0.21824976	4.14181022	1	3.45E-05	0.03312846	1.87118095	1.51790014
ENSBTAG00000054774, processed_pseudogene	325.949244	1.18039991	0.5864884	0.28826976	4.09477533	1	4.23E-05	0.03554937	2.26639593	1.50158734
ENSBTAG00000049042,, protein_coding	22.5892647	1.79384363	0.56725143	0.45930221	3.90558458	0	9.40E-05	0.04972235	3.46737442	1.48169801

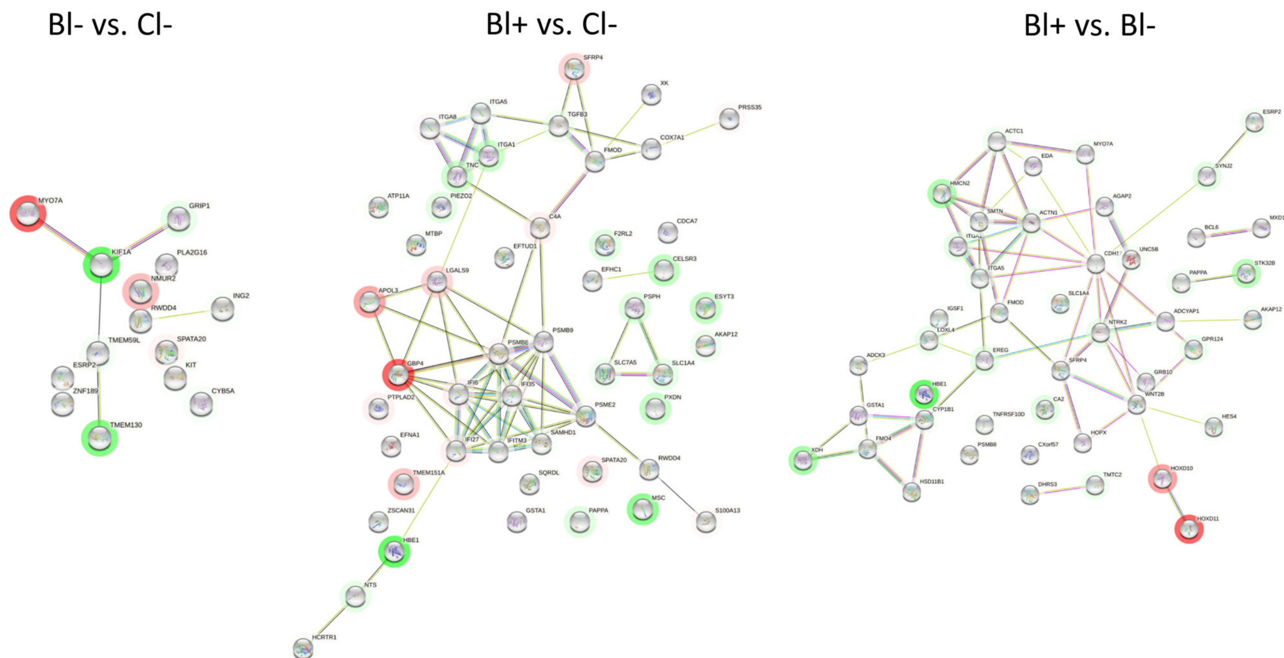
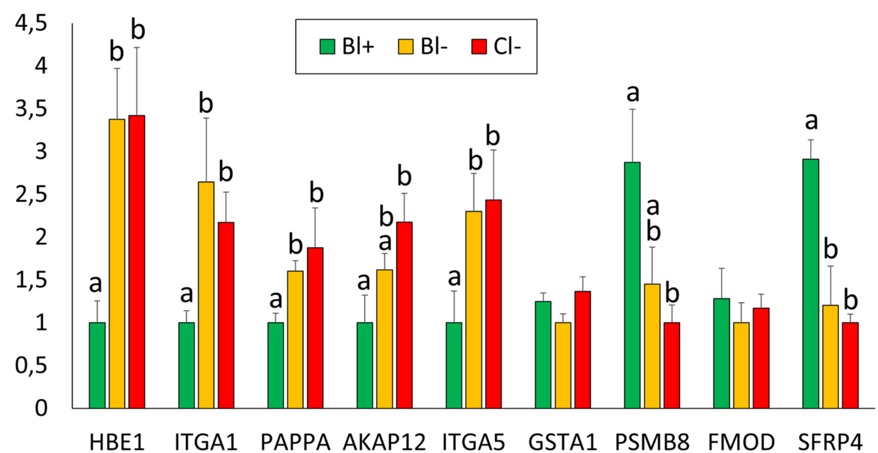


FIGURE 2 Interaction networks obtained from the DEGs at a *p* adjusted value <0.05 and fold change >1.5 at the three comparisons (from left to right Bl− vs. Cl−, Bl+ vs. Cl− and Bl+ vs. Bl−) using STRING. The color of edges and lines represents different types of evidence for protein-to-protein interaction: red for fusion evidence, green for neighborhood evidence, blue for co-occurrence evidence, purple for experimental evidence, yellow for textmining evidence, light blue for database evidence and black for coexpression evidence. DEG, differentially expressed genes.

FIGURE 3 Relative mRNA abundance of nine genes common to the comparisons Bl+ versus Bl− and Bl+ versus Cl− determined by qPCR. Mean ± standard error of the mean. Different letters indicate significant differences based on ANOVA ($p < 0.05$). ANOVA, analysis of variance; mRNA, messenger RNA.



player involved in oocyte competence, as two of the genes validated by qPCR (Integrin Subunit Alpha 1 and 5, *ITGA1* and *ITGA5*) were downregulated in cumulus cells from oocytes developing to blastocysts (Bl+) compared to other groups, and enrichment analysis of biological annotations also highlighted "integrin domain superfamily" in both Bl+ versus Bl− and Bl+ versus Cl− comparisons. Integrin-mediated cell adhesions provide dynamic links between the extracellular matrix and the cytoskeleton and, in the context of oocyte maturation, they control both cumulus cell expansion and luteinization, increasing their expression during cumulus expansion (Kitasaka et al., 2018) and ovulation (Wissing et al., 2014). In this sense, the negative correlation between integrin expression in cumulus cells and oocyte quality may indicate that competent IVM oocytes may achieve cumulus cell expansion earlier or to a greater extend, thereby resuming the expression of the integrins required for expansion before the end of IVM. In agreement with this hypothesis, cumulus expansion intensity is positively linked to embryo development (Qian et al., 2003), and *ITGA5* was found to be upregulated in cumulus cells from rhesus monkey oocytes matured in vitro compared to those matured in vivo (Lee et al., 2011).

Other genes downregulated in cumulus cells from competent oocytes include *HBE1*, *PAPPA*, and *AKAP12*. *HBE1* encodes for Epsilon 1 subunit of hemoglobin. Hemoglobin has been reported to be expressed by mouse preimplantation embryos where it was

TABLE 2 Details of primers used for qPCR

Gene	Primer sequence (5′–3′)	Fragment size (bp)	GenBank accession no.
PPIA	CGGGATTTATGTGCCAGGGT	218	NM_178320.2
	CCAAAGTACCACGTGCTTGC		
HBE1	CTGAGTGAGCTGCACTGTGA	219	NM_001110507.1
	AGGCACTGGGGACACAAAAT		
ITGA1	AGGGCAGAACTTCAGAGTGA	100	XM_024981466.1
	TGCCTGGTAGCCCATCTTTG		
PAPPA	CAAGGAGGGCAAGTGGAACA	234	XM_024996354.1
	AGGCACATGAGCTCACACAG		
AKAP12	AAAACCCGAACCCACGGAAT	154	XM_024997063.1
	TGAGCAGTTGACACGTCTGT		
ITGA5	AGTGGATCAAGGCAGAAGGC	197	NM_001166500.1
	GAGGAATCAGGCATCGGAGG		
GSTA1	GTGCCCACCTGCTGAAAAAG	202	NM_001078149.1
	GAAGTTGGCCAAAAGGCTGG		
PSMB8	TGGCCTTCAAGTTCCAGCAT	210	NM_001040480.1
	TACGCTCCCCATTCCTCAGA		
FMOD	GGCCTGGCCTCAAATACCTT	153	NM_174058.2
	GCAGAAGCTGCTGATGGAGA		
SFRP4	CCACACATCCTGCCTCATCA	180	NM_001075764.1
	TGCTGTTCGCTTCTTGTCCT		

suggested to play a role on embryonic oxygen regulation (Lim et al., 2019). As oxidative stress exerts a negative impact on oocyte competence (Bennemann et al., 2018; Bermejo-Alvarez et al., 2010), the lower expression of *HBE1* in cumulus cells from competent oocytes may indicate a reduced exposure to oxygen during folliculogenesis, before IVM, or a higher ability to deal with oxidative stress during IVM. Pregnancy-associated plasma protein-A (PAPPA) regulates ovarian follicle dominance by degrading IGFBP-4 in preovulatory follicles of several species, including cattle (Mazerbourg et al., 2001; Rivera & Fortune, 2001). Similarly to integrins, *PAPPA* expression increases during bovine folliculogenesis (Mazerbourg et al., 2001) and thereby the lower expression in cumulus cells from more competent oocytes may indicate that they have attained full developmental competence by the end of IVM. Finally, A-Kinase Anchoring Protein 12 (*AKAP12*) overexpression in the cumulus cells from mice lacking estrogen receptor beta has been suggested to contribute to the sequestration of PKA regulatory units, leading to reduced cAMP levels (Binder et al., 2013). In this perspective, the higher expression of *AKAP12* in cumulus cells from incompetent bovine oocytes may be linked to reduced cAMP, diminishing the odds to progress to the blastocyst stage (Luciano et al., 1999).

Other genes (*SFRP4*, *PSMB8*, *FMOD*, and *GSTA1*) were upregulated in cumulus cells from competent oocytes, although the differences in *FMOD* and *GSTA1* expression were not confirmed by qPCR. Secreted Frizzled Related Protein 4 (SFRP4) inhibits Wnt signaling, a pathway known to play multiple functions during folliculogenesis (Hernandez Gifford, 2015). The role of SFRP4 during folliculogenesis remain under debate, as its ablation in mice has been reported to increase (Zamberlam et al., 2019) or decrease (Christov et al., 2011) litter size, and whereas mRNA content in human cumulus cells was positively associated with in vivo meiotic progression (Devjak et al., 2012), protein content in human follicular fluid has been negatively associated with in vitro meiotic progression (Pla et al., 2021). The differences between studies may be linked to the different hormonal environment of in vitro and in vivo maturation, as *SFRP4* expression is modulated by LH in a species-specific manner (Maman et al., 2011). The agreement between our findings and those obtained in human IVM (Pla et al., 2021) suggest that, in contrast to rodents, bovine and humans may share a similar regulation of *SFRP4*, although dedicated experiments would be required to test this hypothesis. Proteasome 20 S subunit beta 8 (PSMB8) is a component of the proteasome, which modulates oocyte meiotic maturation (Huo et al., 2004). In agreement with the positive association between *PSMB8* expression and oocyte quality observed in bovine cumulus cells, the expression level of its antisense (PSMB8-AS1) was higher in cumulus cells from old versus young women (Bouckenheimer et al., 2018). Finally, although qPCR data did not detect significant differences between groups in glutathione S-Transferase Alpha 1

(*GSTA1*) expression, the positive association between *GSTA1* expression and oocyte quality observed by RNA-seq is consistent with previous findings. GSTA1 catalyzes the conjugation of glutathione to molecules such as prostaglandins A2 and J2 and it has been found to display steroid isomerase activity in bovine (Raffalli-Mathieu et al., 2007). *GSTA1* expression in bovine cumulus cells increases following FSH and/or phorbol myristate addition to IVM media, which resulted in improved rates of blastocyst development (Assidi et al., 2008). *GSTA1* transcript abundance is also higher in cumulus cells from in vivo matured bovine oocytes compared to IVM counterparts (Salhab et al., 2013) and in bovine oocytes selected by Brilliant Cresyl Blue staining (Janowski et al., 2012).

In conclusion, the transcriptome of bovine cumulus cells from COCs exhibiting different developmental competence following individual IVP differs in a small subset of genes involved in critical biological functions such as integrin-mediated cell adhesion, oxygen availability, IGF and Wnt signaling or PKA pathway. Such biological functions are required during folliculogenesis to attain full oocyte competence, thereby highlighting specific processes altered in incompetent IVM bovine oocytes.

4 | MATERIALS AND METHODS

4.1 | Individual IVP and collection of cumulus cells samples

Bovine embryos were produced from slaughterhouse ovaries following conventional protocols (Lamas-Toranzo et al., 2020), adapted to individual embryo production. Bovine ovaries were transported at 35–37°C from a local slaughterhouse to the laboratory. COCs were aspirated from surface visible antral follicles (2–8 mm) and selected using conventional morphological criteria (Hawk & Wall, 1994). Individual in vitro production (iIVP) was required to determine the developmental ability of each oocyte. COCs were matured individually in 40 µl drops of TCM-199 supplemented with 10% fetal calf serum (FCS) and 10 ng/ml epidermal growth factor covered under mineral oil at 39°C and 5% CO_2 in air with humidified atmosphere for 24 h. Following maturation, the cumulus cells from each individual COC were removed by pipetting in medium supplemented with 0.1% hyaluronidase. Cumulus cells were collected from the media by centrifugation at 1500 rpm for 5 min, snap frozen in liquid nitrogen and stored at −80°C until analysis. Denuded oocytes were individually inseminated in 40 µl drops of TALP medium covered under mineral oil and containing 1×10^6 frozen-thawed bull spermatozoa/ml. After 20 h of gamete coincubation, presumptive zygotes were cultured individually in 10 µl drops of synthetic oviduct fluid medium supplemented with 5% FCS at 39°C and in a 5% CO_2 and 5% O_2 water-saturated atmosphere. Embryo development was assessed for each individual oocyte at 48 h postinsemination (cleavage, at least 4-cells) and at day 8 postinsemination (blastocyst formation).

4.2 | Transcriptional analysis

RNA extraction was performed on 4 (CI−) or 5 (BI− and BI+) samples per group each of which was composed of cumulus cells obtained from 10 individual COCs. Individual samples (i.e., 40 CI−, 50 BI−, and 50 BI+) were collected from 7 independent replicates. The rationale behind analyzing three experimental groups was to double-check for the correlation between transcriptional change and developmental competence; for instance a "positive marker" for oocyte competence should be upregulated in BI+ versus BI− but also between BI+ and CI−. Total RNA was extracted using MagMAX *mir*Vana Total Isolation Kit (Applied Biosystems) following the manufacturer's instruction with minor modifications. Briefly 200 µl of Lysis Binding Mix were added to the sample, followed by gentle pipetting and 5 min incubation at room temperature. Then 20 µl of Binding Beads Mix were added and shaken gently for 5 min. Beads-mRNA complexes were washed once in each Wash Solution 1 and 2. Following the washing step, samples were treated with 50 µl of Turbo DNAse treatment, 50 µl of Rebinding Buffer and 100 µl of Isopropanol were added to the sample and mixed gently. Finally, following a double wash in Wash Solution 2, total RNA was eluted in 20 µl of Elution Buffer and stored at −80°C until analysis.

RNA samples were quantified by Qubit RNA BR Assay (Thermo Fisher Scientific) and RNA integrity was estimated by using RNA 6000 Nano Bioanalyzer 2100 Assay (Agilent). RNA amount oscillated between 0.285 and 0.676 µg and RIN values between 8 and 9.2. RNA-seq libraries were prepared with KAPA Stranded mRNA-Seq Illumina Platforms Kit (Roche) following the manufacturer's recommendations. Briefly, 50–100 ng of total RNA was used for the poly-A fraction enrichment with oligo-dT magnetic beads, following the mRNA fragmentation. The strand specificity was achieved during the second strand synthesis performed in the presence of dUTP instead of dTTP. The blunt-end double stranded cDNA was 3′ adenylated and Illumina platform compatible adaptors with unique dual indexes and unique molecular identifiers (Integrated DNA Technologies) were ligated. The ligation product was enriched with 15 PCR cycles and the final library was validated on an Agilent 2100 Bioanalyzer with the DNA 7500 assay.

The libraries were sequenced on a HiSeq. 4000 system (Illumina) with a read length of 2 × 76 bp + 8 bp + 8 bp using the HiSeq. 4000 SBS kit (Illumina) obtaining >30 M reads/sample. Image analysis, base calling and quality scoring of the run were processed using the manufacturer's software Real Time Analysis (RTA 2.7.7). Differential expression was analysed by DESeq. 2 software obtaining raw p values, adjusted *p* values, raw fold changes and shrunken fold changes for all genes detected. Enrichment in biological annotations and a network of biological interactions for each of the three comparisons were performed on differentially expressed genes (DEGs) with an adjusted *p* value <0.05 and fold change >1.5 using STRING (v11; Szklarczyk et al., 2019) through the package "STRINGdb" in R (v4.0.4). Only enriched terms with FDR < 0.05 calculated by the Benjamini-Hochberg procedure were selected.

RNA-seq validation was performed on independently retrotranscribed cDNA obtained from the RNA samples mentioned above using qScript cDNA Supermix (Quantabiosciences, containing a blend of random and oligo dT primers optimized to deliver unbiased representation of 5′ and 3′ sequences) in a 20 µl final volume. Relative mRNA abundance of 9 selected DEGs was analyzed in 4 (Cl−) or 5 (Bl− and Bl+) samples by qPCR as previously described (Lamas-Toranzo et al., 2018) using specific primers detailed in Table 2. Experiments were conducted to contrast the relative levels of each transcript and the housekeeping gene *PPIA* in each sample. cDNA was diluted to 55 µl in 10 mM Tris-HCl (pH 7.5) and qPCR was performed in duplicate by adding a 2 µl aliquot of diluted cDNA to the PCR mix containing the specific primers for each DEG. PCR efficiencies were optimized to achieve efficiencies close to 1 and then the comparative cycle threshold (Cq) method was used to quantify expression levels as described in (Schmittgen & Livak, 2008). As primers were designed using the same annealing parameters, all qPCRs were performed using the same cycling conditions: 40 cycles of 94°C 15 s, 56°C 30 s, and 72°C 20 s followed by a final melting assay to assess product identity. Cq value was taken in the log-linear phase of the reaction, and ΔCq value was determined by substracting the *PPIA* Cq value for each sample from each gene Cq value of the sample. Calculation of ΔΔCq involved using the highest sample ΔCq value (i.e., the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCq sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta Cq}$. qPCR data were analysed by one way analysis of variance using the statistical software Sigma Stat (Jandel Scientific).

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

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Capítulo 3

The human cumulus cell transcriptome provides poor predictive value for embryo transfer outcome

The human cumulus cell transcriptome provides poor predictive value for embryo transfer outcome

Álvaro Martínez-Moro, Leopoldo González-Brusi, Ismael Lamas-Toranzo, Pilar González-Dosal, Félix Rodríguez-Juárez, Pablo Bermejo-Álvarez

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Abstract

Research question: Is the transcriptome of cumulus cells a good predictor of the embryo's developmental competence?

Design: Cumulus cells were collected from donor oocytes and their transcriptome was analysed by RNA sequencing analysis at >30 x10^6 reads in samples grouped according to the developmental potential of their enclosed oocyte: not able to develop to the blastocyst stage (Bl-), able to develop to the blastocyst stage but failing to establish a pregnancy (P-), or able to develop to the blastocyst stage and to establish a clinical pregnancy (P+).

Results: The cumulus cell trancriptome was largely independent of the developmental potential as, using a false discovery rateadjusted *P-value* of <0.05, only 10, 11 and 5 genes were differentially expressed for the comparisons P+ versus P-, P+ versus Bl-, and P- versus Bl-, respectively, out of a total of 17,469 genes expressed. Between the differentially expressed genes, those showing little overlap between samples from different groups were CHAC1, up-regulated in the P- and P+ groups compared with the Bl- group, and CENPE, CD93, PECAM1 and HSPA1B, which showed the opposite expression pattern. Focusing on the pregnancy potential, only EPN3 was consistently downregulated in the P+ compared with the P- and Bl- groups.

Conclusions: The cumulus cell transcriptome is largely unrelated to the establishment of clinical pregnancy following embryo transfer, although the expression level of a subset of genes in cumulus cells may indicate the ability to develop to the blastocyst stage.

RBMO
ELSEVIER

The human cumulus cell transcriptome provides poor predictive value for embryo transfer outcome

BIOGRAPHY

Álvaro Martínez-Moro is a PhD Candidate at INIA, CSIC, Madrid Spain. He holds a degree in biology and MSc degrees in human reproduction, advanced biotechnology, genetics and health law and biomedicine. He is an Embryologist at IVF-Spain Madrid (former Procreatec) and is currently investigating molecular markers of oocyte competence and the kinetics of embryo development.

Álvaro Martínez-Moro[1,2], Leopoldo González-Brusi[1], Ismael Lamas-Toranzo[1], Pilar González-Dosal[2], Félix Rodríguez-Juárez[2], Pablo Bermejo-Álvarez[1,*]

KEY MESSAGE

The cumulus cell transcriptome of human oocytes exhibiting diverse developmental abilities was analysed and was found to be largely unrelated to development to the blastocyst stage or the establishment of clinical pregnancy.

ABSTRACT

Research question: Is the transcriptome of cumulus cells a good predictor of the embryo's developmental competence?

Design: Cumulus cells were collected from donor oocytes and their transcriptome was analysed by RNA sequencing analysis at $>30 \times 10^6$ reads in samples grouped according to the developmental potential of their enclosed oocyte: not able to develop to the blastocyst stage (Bl−), able to develop to the blastocyst stage but failing to establish a pregnancy (P−), or able to develop to the blastocyst stage and to establish a clinical pregnancy (P+).

Results: The cumulus cell trancriptome was largely independent of the developmental potential as, using a false dscovery rate-adjusted *P*-value of <0.05, only 10, 11 and 5 genes were differentially expressed for the comparisons P+ versus P−, P+ versus Bl−, and P− versus Bl−, respectively, out of a total of 17,469 genes expressed. Between the differentially expressed genes, those showing little overlap between samples from different groups were *CHAC1*, up-regulated in the P− and P+ groups compared with the Bl− group, and *CENPE*, *CD93*, *PECAM1* and *HSPA1B*, which showed the opposite expression pattern. Focusing on the pregnancy potential, only *EPN3* was consistently downregulated in the P+ compared with the P− and Bl− groups.

Conclusions: The cumulus cell transcriptome is largely unrelated to the establishment of clinical pregnancy following embryo transfer, although the expression level of a subset of genes in cumulus cells may indicate the ability to develop to the blastocyst stage.

INTRODUCTION

The overall change in embryo transfer policy to transfer only one embryo at a time (*Maheshwari et al., 2011*) to prevent multiple pregnancies (*Hazekamp et al., 2000*) emphasizes the need for embryo selection to maximize the odds of attaining a successful pregnancy. Embryo morphology has been the most commonly used parameter to infer its prospective developmental ability (*Gardner and Balaban, 2016*). However, although morphological grading arguably constitutes the best available predictor of pregnancy success, clinical pregnancy rates remain around 35% (*Wyns et al., 2020*), suggesting that there is still room for improvement. Time-lapse screening of embryo development (*Armstrong et al., 2019*) can be considered as an evolution of conventional morphological criteria, as it allows the assessment of embryo morphology as a continuum, but there is no current consensus on a favourable impact on pregnancy rates (*Chen et al.,*

[1] Animal Reproduction Department, INIA, CSIC, Madrid, Spain
[2] IVF Spain Madrid, Madrid, Spain

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*Corresponding author: E-mail address: bermejo.pablo@inia.csic.es (P. Bermejo-Álvarez). https://doi.org/10.1016/j.rbmo.2023.01.012 1472-6483/© 2023 The Author(s). Published by Elsevier Ltd on behalf of Reproductive Healthcare Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

KEYWORDS
Cumulus cells
Developmental competence
Implantation predictor
Oocyte
RNA-seq
Transcriptome

2017). From this perspective, molecular proxies may serve as complementary methods to assist embryonic selection based on morphological parameters.

Cumulus cells are somatic cells closely connected to the oocyte that play essential metabolic and signalling functions during folliculogenesis and oocyte maturation (Robert, 2021). As oocyte competence is acquired through bidirectional signalling between the oocyte and the surrounding cumulus cells (Richani et al., 2021), and these supporting cells are discarded prior to intracytoplasmic sperm injection (ICSI), they constitute an interesting biological material on which to perform molecular analyses to infer the developmental ability of their enclosed oocyte. To that end, different studies have searched for possible transcriptional differences in cumulus cell samples associated with oocytes ultimately establishing pregnancy versus those failing to do so.

Initial studies were conducted using quantitative PCR (qPCR) analysis for a select subset of genes suggesting specific transcripts putatively associated with implantation potential (Anderson et al., 2009; Artini et al., 2017; Gebhardt et al., 2011; Iager et al., 2013; Kordus et al., 2019; Krieg et al., 2018; Wathlet et al., 2011; Wathlet et al., 2012; Wathlet et al., 2013). Subsequent studies employed microarray technology that allowed the analysis of a larger subset of genes (Assidi et al., 2015; Feuerstein et al., 2012; Hamel et al., 2010). Some of these transcripts have been validated in independent sample sets and have even been used to improve clinical outcomes (Van Vaerenbergh et al., 2021), but overall the cumulus cell transcripts putatively associated with pregnancy potential are highly divergent between studies performed by independent research groups, and other studies based on the qPCR analysis of previously suggested genes (Burnik Papler et al., 2015b) or microarray analysis (Burnik Papler et al., 2015a) have failed to replicate those results.

In contrast to qPCR or microarray analysis, sequencing (RNA-seq) provides an unbiased selection of transcripts and displays a higher dynamic range, hence providing far more precise measurements of transcript levels (Wang et al., 2009). To date, only one study has investigated whether the transcriptional levels of genes expressed by cumulus cells are associated with pregnancy prognosis by RNA-seq, finding no correlation between the cumulus cell transcriptome and pregnancy outcomes (Green et al., 2018). The objective of the current study has been to employ RNA-seq technology to determine whether cumulus cell transcription can be used as a proxy to enhance the already available predictive value of morphological evaluation. To that aim, cumulus cells were collected from individual oocytes and, once the developmental potential of each oocyte was known, the stored samples were allocated to one of three groups according to the oocyte's developmental potential: (i) oocytes not developing to the blastocyst stage (Bl−); (ii) oocytes developing to the blastocyst stage but not establishing pregnancy (P−); or (iii) oocytes ultimately establishing a pregnancy (P+).

MATERIAL AND METHODS

Sample collection

Cumulus cells were obtained from donor oocyte cycles at IVF-Spain Madrid between June 2018 and June 2020. All the recipients and donors agreed to participate in the study, which was approved by the Ethical Committee of La Princesa University Hospital, Madrid on 8 March 2018 (reference: Acta CEIm 04/18, registration number: 3361). Inclusion criteria were the following: (i) an absence of uterine abnormalities; (ii) donor age ≤37 years; (iii) recipient age ≤50 years; and (iv) a normal male sperm count (>2 million spermatozoa/ml in the ejaculate). Age, body mass index, smoking status and endometrial thickness were similar between the experimental groups (TABLE 1). Only non-cryopreserved oocytes and embryos obtained from donors and not submitted to preimplantation genetic testing were included in the analysis, and only single-embryo transfers were conducted.

Donors were stimulated using FSH starting on the second day of menstruation, and ovulation was induced with a gonadotrophin-releasing hormone analogue or human chorionic gonadotrophin when three or more follicles larger than 17 mm were present (Melo et al., 2009). Ovum retrieval was performed 36 h after induction using transvaginal ultrasound guidance. Endometrium stimulation treatment in recipients consisted of 6 mg/day of estradiol (Meriestra, Sandoz, Spain), and embryo transfer was performed only if the endometrium measured 7–13 mm (Prapas et al., 1998). Luteal support was started on the night of the ovum retrieval day with 400 mg of progesterone every 12 h until pregnancy test was carried out (Edwards et al., 1984).

Cumulus–oocyte complexes (COC) were retrieved following follicular aspiration, washed and individually cultured in G-IVF Plus Media (Vitrolife, Sweden) (6.3% CO_2, 5%O_2, 89% N_2 at 37°C). Denudation was performed individually 2 h after COC recovery, using hyaluronidase solution (Irvine, Spain). Cumulus cells detached from oocytes were collected from the denudation medium, collected, pelleted at

TABLE 1 RECIPIENT AND DONOR CHARACTERISTICS ACCORDING TO OOCYTE DEVELOPMENTAL POTENTIAL

Characteristic	Bl−	P−	P+
Recipients	No transfer	19	22
Age (years)	–	41.28 ± 0.63	41.32 ± 0.60
BMI (kg/m^2)	–	21.78 ± 0.51	21.66 ± 0.56
Smoking	–	1	5
Endometrial thickness (mm)	–	8.60 ± 0.27	8.37 ± 0.24
Donors[a]	19	19	18
Age (years)	23.92 ± 0.83	24.28 ± 0.73	24.84 ± 0.75
BMI (kg/m^2)	23.06 ± 0.67	23.93 ± 0.74	22.97 ± 0.86
Smoking	16	15	14

Data are shown as mean ± SEM or *n*. No significant differences were found between groups (analysis of variance $P > 0.05$, Fisher test for smoking status).

[a] All donors in the Bl− group donated to either the P+ or P− group.

Bl−, oocyte not able to develop to a blastocyst; P−, oocyte able to develop to a blastocyst but not able to establish a pregnancy; P+, oocyte able to develop to a blastocyst and able to establish a pregnancy; BMI, body mass index.

1500*g* for 10 min, snap-frozen in liquid nitrogen and stored at −80°C until analysis. Oocytes were fertilized by ICSI using G-MOPS Plus (Vitrolife, Sweden), with spermatozoa placed in 7% polyvinylpyrrolidone solution to slow their movement. Following ICSI, the presumptive zygotes were individually cultured in Continuous Single Culture SAGE 1-Step (Irvine, Spain) for 6 days, up to the blastocyst stage. The morphological score was used to select blastocysts for embryo transfer (Gardner et al., 2015). Only day 5 blastocysts with scores of 4–5 were transferred (P− or P+ groups). Recipients were assessed for the occurrence of clinical pregnancy at the fourth or fifth week after fertilization, defining it by fetal heart rate detection on ultrasound echography.

Once the embryo development was known, the previously stored cumulus cells were allocated into three groups according to the oocyte's developmental potential: (i) oocytes not developing to the blastocyst stage (Bl−); (ii) oocytes developing to the blastocyst stage (score 4–5; Gardner et al., 2015) but failing to establish a clinical pregnancy following embryo transfer (P−); and (iii) oocytes developing to the blastocyst stage that were able to establish a pregnancy (P+). As only fresh embryo transfers were performed there was no common recipient between groups P+ and P−.

Transcriptional analysis

RNA extraction was performed on five pooled samples per group. The pooled samples were composed of cumulus cells obtained from five individual COC. These samples were randomly selected from those collected once the oocyte's developmental potential was known. Analysis in pooled samples allows a reduction of the number of assessments required to analyse a large number of biological samples, although it is acknowledged that the contribution of each COC to the pool is not even, given that human COC are diverse in size. The rationale behind analysing three experimental groups was to double-check for the relationship between transcriptional levels in cumulus cells and developmental competence in oocytes; for instance a 'positive marker' for oocyte competence should be up-regulated in P+ versus P− and also between P− and Bl+.

Total RNA was extracted using a MagMAX *mir*Vana Total Isolation Kit (Applied Biosystems, USA) following the manufacturer's instruction with minor modifications (Ramos-Ibeas et al., 2022). Briefly 200 μl of Lysis Binding Mix was added to the sample, followed by gentle pipetting and 5 min incubation at room temperature. Then 20 μl of Binding Beads Mix was added and the mixture was gently shaken for 5 min. The bead–mRNA complexes were washed once in each of Wash Solution 1 and 2. Following the washing step, samples were treated with 50 μl of Turbo DNase treatment, and 50 μl of Rebinding Buffer and 100 μl of isopropanol were added to the sample and gently mixed in. Finally, following a double wash in Wash Solution 2, total RNA was eluted in 20 μl of Elution Buffer and stored at −80°C until analysis.

The RNA samples were quantified by Qubit RNA BR Assay (Thermo Fisher Scientific, Spain) and the RNA integrity was estimated using an RNA 6000 Nano Bioanalyzer 2100 Assay (Agilent, USA), with a minimum RIN value of 8.4. RNA-seq libraries were prepared with a KAPA Stranded mRNA-seq Illumina Platforms Kit (Roche, Switzerland) following the manufacturer's recommendations. Briefly, 50–100 ng of total RNA was used for poly-A fraction enrichment with oligo-dT magnetic beads, after the mRNA fragmentation. The strand specificity was achieved during the second strand synthesis performed in the presence of dUTP instead of dTTP. The blunt-end double-stranded cDNA was 3′-adenylated, and Illumina platform-compatible adaptors with unique dual indexes and unique molecular identifiers (Integrated DNA Technologies, Spain) were ligated. The ligation product was enriched with 15 PCR cycles and the final library was validated on an Agilent 2100 Bioanalyzer with a DNA 7500 assay.

The libraries were sequenced on a HiSeq 4000 (Illumina, USA) with a read length of 2×76 bp+8 bp+8 bp using a HiSeq 4000 SBS kit (Illumina, USA) obtaining >30 M reads/sample. Image analysis, base calling and quality scoring of the run were processed using the manufacturer's Real Time Analysis (RTA 2.7.7) software. The number of raw reads (150 bp paired-end reads) ranged from 38 to 57 million per sample. Paired-end read fastq files were quality checked with FastQC (Andrews, 2010) and preprocessed with fastp (Chen et al., 2018). The resulting files were pseudoaligned and quantified using kallisto (Bray et al., 2016) against the reference transcriptome of *Homo sapiens* (cDNAs and ncRNAs from the genome assembly GCA_000001405.28). Differential expression was analysed using DESeq2 software (Love et al., 2014) obtaining the raw *P*-value, false discovery rate (FDR)-adjusted *P*-value and shrunken fold changes for all the genes detected. A test on biological annotation enrichment was performed with STRING (v11) https://string-db.org/.

RESULTS

The expression of 17,469 genes (including 15,593 protein-coding genes) was detected by RNA-seq in human cumulus cells. Principal component analysis showed little divergence between the 15 cumulus cell samples analysed, as the first two components explained only 37.4% of the variance and no distinction was observed between experimental groups (FIG. 1A). Sample clustering according to gene expression confirmed this observation, as clusters were not grouped according to their developmental potential (FIGURE 1B). The test on biological annotation enrichment using STRING (v11) found no enriched terms with the studied differentially expressed genes (DEG).

Using a raw *P*-value of <0.05, the analysis identified 920, 1354 and 1148 DEG for the comparisons P+ versus P−, P+ versus Bl−, and P− versus Bl+, respectively (Supplementary Table). These DEG were narrowed down to 5, 11 and 10 genes for the comparisons P− versus Bl−, P+ versus Bl−, and P+ versus P−, respectively, when an FDR-adjusted *P*-value of <0.05 was used (Figures 2–4 and TABLE 2). Such adjusted *P*-values take into account the data overdispersion inherently associated with RNA-seq data, yielding a more reliable result.

The box plots (Figures 2–4) showed that the expression of some DEG was similar in samples belonging to groups exhibiting different developmental abilities, thereby precluding the establishment of a given threshold to blindly discriminate samples based on the oocyte developmental potential. However, the expression levels of other DEG showed little overlap between groups and a consistent relationship with oocyte developmental potential when all three groups were considered. Between them, *CHAC1* was up-regulated in most samples obtained from oocytes able to reach the blastocyst stage (P− and P+ groups) compared with the samples obtained from oocytes unable to attain preimplantation development

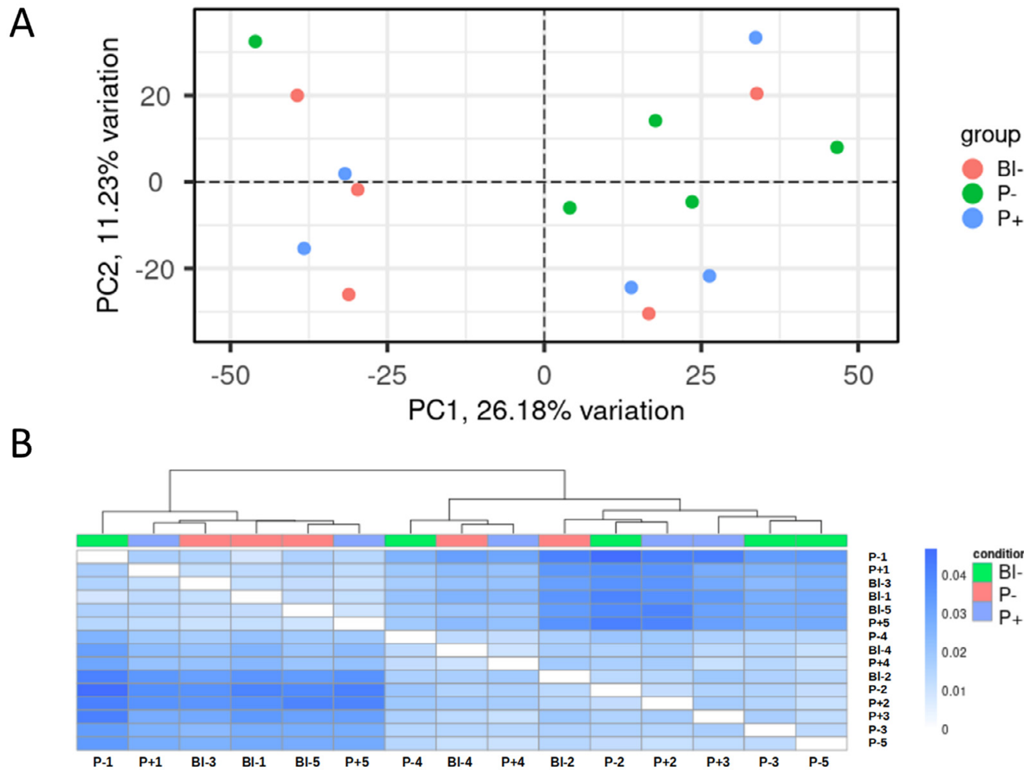

FIGURE 1 Transcriptional analysis of cumulus cells surrounding oocytes of different developmental ability: unable to develop to blastocyst stage (Bl–), able to develop to blastocyst stage but not implanting (P–), or able to develop to blastocyst stage and implanting (P+); five samples per group, and five cumulus–oocyte complexes per sample. (A) Principal component analysis. (B) Unsupervised hierarchical clustering.

(Bl– group), and *CENPE*, *CD93*, *PECAM1* and *HSPA1B* showed the opposite expression pattern (FIGURES 2 and 3). Focusing on predictors of pregnancy potential (FIGURES 3 and 4), only *EPN3* showed a consistent pattern, being downregulated in the P+ group compared with P– and Bl–. *RMPR* was up-regulated in the P+ versus Bl– group, but the association of *RMPR* expression and pregnancy outcome could be an artefact caused by the low abundance (0–40 counts/sample) of their transcripts.

DISCUSSION

The search for molecular markers of oocyte competence in cumulus cells constitutes an enticing way to improve the predictive value of conventional embryo selection. To raise the predictive value already available by embryo morphology assessment, routinely performed at a fixed time or by time-lapse equipment, such molecular markers must be able to distinguish between two cohorts of oocyte, both able to reach the blastocyst stage but differing in their ability to establish pregnancy. Unfortunately, RNA-seq analysis showed that the cumulus cell transcriptome provides a poor predictive value for embryo transfer outcome.

These results are in agreement with previous findings using qPCR (Burnik Papler et al., 2015b), microarray analysis (Burnik Papler et al., 2015a) and RNA-seq (Green et al., 2018), and with the lack of coherence between the lists of transcripts putatively associated with oocyte

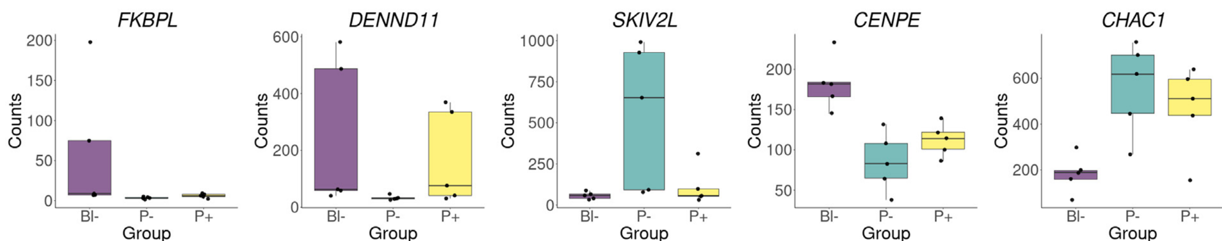

FIGURE 2 Differentially expressed genes (false delivery rate-adjusted *P*-value <0.05) between cumulus cells surrounding oocytes unable to develop to the blastocyst stage (Bl–) versus able to develop to the blastocyst stage but not establishing a pregnancy following embryo transfer (P–). The group able to establish a pregnancy following embryo transfer (P+) is also included. The dots on each box plot indicate the value (counts) for each individual sample, the median is represented by a bold line, the upper and lower borders of the boxes depict the interquartile range, and the limits of the bars represent the maximum and minimum values excluding outliers (five samples per group and five cumulus–oocyte complexes per sample).

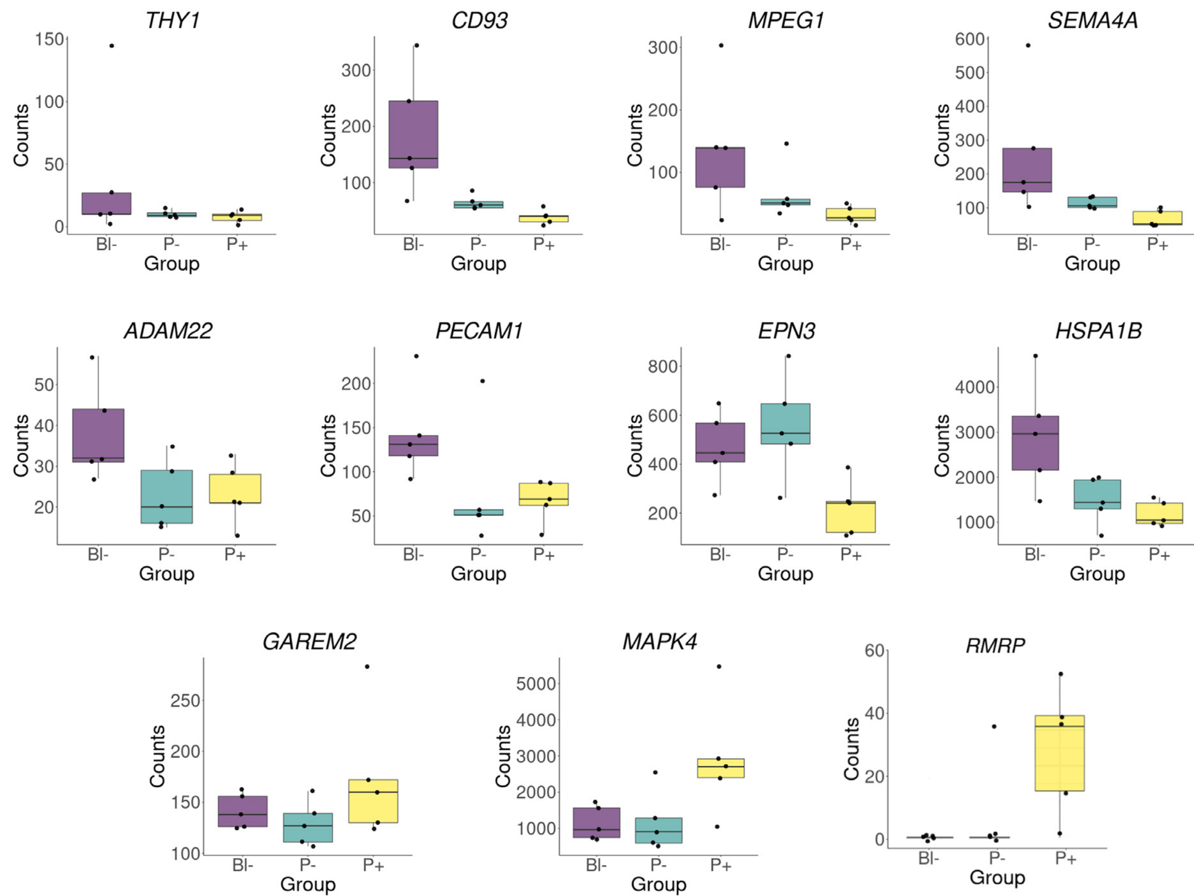

FIGURE 3 Differentially expressed genes (false delivery rate-adjusted *P*-value <0.05) between cumulus cells surrounding oocytes unable to develop to blastocyst stage (Bl−) versus able to develop to blastocyst stage and establish a pregnancy following embryo transfer (P+). The group unable to establish a pregnancy following embryo transfer (P−) is also included. The dots on each box plot indicate the value (counts) for each individual sample, the median is represented by a bold line, the upper and lower borders of the boxes depict the interquartile range, and the limits of the bars represent the maximum and minimum values excluding outliers (five samples per group and five cumulus−oocyte complexes per sample).

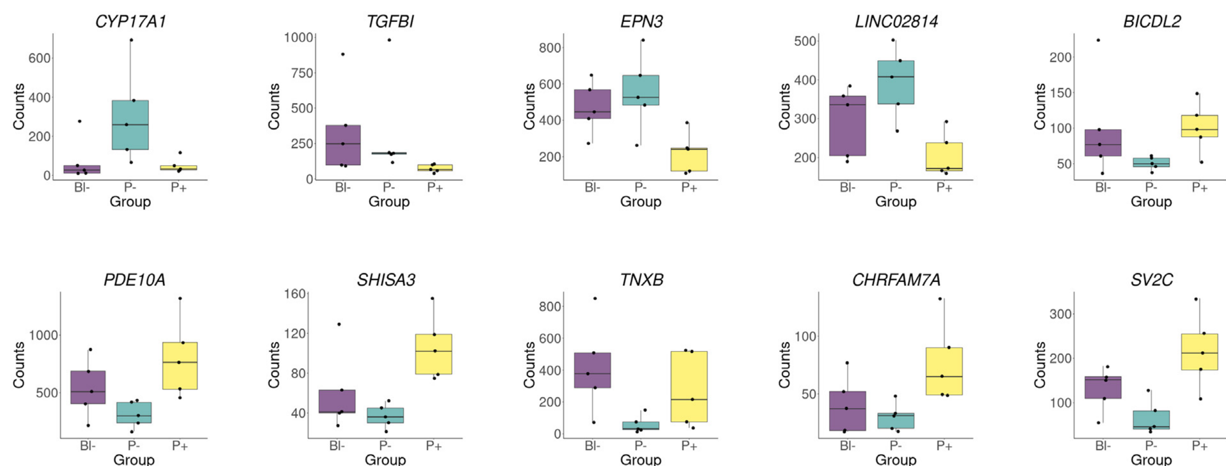

FIGURE 4 Differentially expressed genes (false delivery rate-adjusted *P*-value <0.05) between cumulus cells surrounding oocytes able to develop to blastocyst stage and establish a pregnancy (P+) or not (P−) following embryo transfer. The group unable to develop to blastocyst stage (Bl−) is also included. The dots on each box plot indicate the value (counts) for each individual sample, the median is represented by a bold line, the upper and lower borders of the boxes depict the interquartile range, and the limits of the bars represent the maximum and minimum values excluding outliers (five samples per group and five cumulus−oocyte complexes per sample).

TABLE 2 GENES DIFFERENTIALLY EXPRESSED IN CUMULUS CELLS SURROUNDING OOCYTES ACCORDING TO EMBRYO OUTCOME

Gene	Bl−	P−	P+	Fold change	FDR-adjusted *P*-value
					Bl− versus P−
FKBPL	59.2	3.2	6	−19	0.023
DENND11	246	33.2	170.4	−6.9	0.048
CENPE	182.2	85.2	112.4	−2.3	0.011
CHAC1	183	558	467.2	3.2	0.016
SKIV2L	56.8	548.6	110	3.5	0.01
					Bl− versus P+
THY1	38.8	36.8	7.8	−10	0.046
CD93	185	102.4	38.6	−4.9	0.0003
MPEG1	136.2	60.4	31.4	−4.3	0.044
SEMA4A	256.2	208.8	67.8	−3.8	0.011
ADAM22	38.2	30.4	23.2	−3.2	0.044
PECAM1	142.6	114	67	−2.4	0.022
EPN3	469	552	221	−2.3	0.022
HSPA1B	2927.2	1926.8	1184	−2.3	0.0053
GAREM2	141.6	132.4	173.8	2	0.018
MAPK4	1139	1216	2904.8	2.8	0.039
RMRP	0	6.2	24.6	130	0.0003
					P− versus P+
CYP17A1	74.8	306.6	49	−6.3	0.0073
TGFBI	339.4	327.8	73.6	−3.9	0.04
EPN3	469	552	221	−2.7	0.032
LINC02814	294.4	393	205.8	−2	0.012
BICDL2	99.4	50.6	101	2.2	0.044
PDE10A	536.4	308.4	801	2.7	0.022
SHISA3	60	36.8	106	2.8	0.0054
TNXB	419.2	58.8	273.8	2.8	0.012
CHRFAM7A	40.2	29.8	77.2	2.8	0.044
SV2C	131.8	66.8	217.2	3.4	0.017

Gene expression is reported as the mean counts (reads) on RNA sequencing.

Five samples per group, and five cumulus–oocyte complexes per sample.

Bl−, oocyte not able to develop to a blastocyst; P−, oocyte able to develop to a blastocyst but not able to establish a pregnancy; P+, oocyte able to develop to a blastocyst and able to establish a pregnancy; FDR, false discovery rate.

competence provided by different articles employing qPCR or microarray techniques (Anderson et al., 2009; Artini et al., 2017; Assidi et al., 2015; Feuerstein et al., 2012; Gebhardt et al., 2011; Hamel et al., 2010; Iager et al., 2013; Kordus et al., 2019; Krieg et al., 2018; Wathlet et al., 2011; Wathlet et al., 2012; Wathlet et al., 2013).

A plausible explanation for the lack of reliable markers of pregnancy potential in cumulus cells is that the success of embryo transfer depends on multiple variables, some of which, especially uterine receptivity (Craciunas et al., 2019), may outweigh the role of oocyte competence in the prospective establishment of pregnancy. In agreement with this, the search for other potential molecular markers of oocyte competence in cumulus cells, such as mitochondrial DNA abundance (Kumar et al., 2021, Liu et al., 2021, Martínez-Moro et al., 2022b), has also failed to predict the post-transfer developmental potential.

Another limitation in finding usable markers of oocyte competence in cumulus cells is the heterogeneous nature of the samples, as follicle size, COC size or the stimulation protocol used (Borgbo et al., 2013) exert an effect on cumulus cell transcription that may mask potential predictors of oocyte quality. The current analyses were performed in pools of individual samples, an approach that focuses on identifying markers of oocyte competence that are usable on most patients, i.e. not affected by confounding individual co-variates.

Sample pooling entails two limitations for the analysis: (i) the contribution of each COC to the pool is not equal, as the number of cells per COC varies between samples, and (ii) the weight of individual factors related to each patient or each specific COC for developmental outcome or cumulus cell transcription cannot be estimated. Morphological parameters of cumulus masses such as cell density or the degree of cell attachment have been correlated with oocyte developmental potential (Wang and Sun, 2006), and their effect on cumulus cell transcription may be obscured in pooled samples. However, if those morphological features of the COC are truly predictive of developmental outcomes – a topic requiring further investigation (Balaban et al. 1998) – they will be also associated with potential oocyte quality molecular markers. In this sense, 'universal' rather than 'feature-specific' markers of oocyte competence would be required for clinical applications, as tailoring gene expression data to the myriad of potentially confounding patient or COC co-variates will not be a realistic option.

In the search of a marker predicting the establishment of pregnancy, only *EPN3* was overexpressed in most samples from the P− and Bl− groups compared with P+. The role of EPN3 during cumulus cell development is unknown as *Epn3* knockout mice are fertile (Ko et al., 2010). However, as EPN3 seemingly mediates the epithelial-to-mesenchymal transition (Spradling et al., 2001), a key process experienced by cumulus cells during follicle development (Mora et al., 2012), it could be postulated that the reduced expression of *EPN3* in the most competent group (P+) may reflect an earlier stage of

follicular development in the less competent (Bl− and P−) groups. On the other hand, it has been suggested that instead of applying a given threshold of expression of a single gene to predict the odds of embryo survival, multivariate models involving the expression of a subset of genes could be employed (Wathlet et al., 2011). Unfortunately, unsupervised clustering and principal component analysis were unable to deliver an applicable predictive value, and whereas models biased towards a few selected genes could cluster samples according to group in the current data (Supplementary Figure 1), such a cherry-picking data-tailored procedure would not be repeatable using independent data.

The identification of DEG between COC differing in their ability to reach the blastocyst stage does not provide an additional predictive value of pregnancy potential over conventional embryo selection, but it may uncover interesting clues to understand the mechanisms underpinning oocyte developmental competence. Previous studies have reported that the expression of different genes in cumulus cells can predict the pre-hatching developmental potential, assessed by embryo morphology (Adriaenssens et al., 2010; Cillo et al., 2007; Huang et al., 2013; Liu et al., 2018; McKenzie et al., 2004; Zhang et al., 2005), time-lapse parameters (Parks et al., 2016) or development to the blastocyst stage (Feuerstein et al., 2007; Scarica et al., 2019). Unfortunately, these studies show a very poor correlation between them and could not be replicated by others employing microarray analysis (Feuerstein et al., 2012) or RNA-seq (Green et al., 2018, and the current analysis).

Studies in animal models, particularly in bovine models, have uncovered a small, yet larger than in humans, set of transcripts associated with development to the blastocyst stage (Bunel et al., 2015, Martinez-Moro et al., 2022a). The easier detection of oocyte competence markers in animals models may obey three fundamental differences compared with studies conducted in human samples: (i) animal samples are obtained from a more homogeneous and fertile population, which potentially reduces the confounding effects of co-variates; (ii) the interference of male factors is eliminated by using semen from a single male to fertilize all the oocytes in the study; and (iii) in contrast to humans, animal oocytes are obtained from immature follicles and matured *in vitro*, thereby constituting a more divergent population in terms of oocyte competence where differences in molecular markers are expected to be more evident.

A series of genes was identified as being potentially associated with oocyte competence to attain preimplantation development in the current analysis, but these results must be considered carefully, as no validation in an independent set of individual samples was conducted. Between them, *CHAC1* was positively associated with developmental competence. CHAC1 (ChaC glutathione specific gamma-glutamylcyclotransferase 1, previously known as BOTCH) is a protein involved in glutathione cleavage and a negative regulator of NOTCH signalling (Chi et al., 2012), a pathway involved in pre-granulosa cell recruitment (Vanorny et al., 2014). The up-regulation of CHAC1 – i.e. NOTCH inhibition – may indicate a more advanced developmental stage of cumulus cells in oocytes able to develop to the blastocyst stage, when cell recruitment is no longer needed. Also in agreement with a positive association with developmental competence, CHAC1 was found to be down-regulated in cumulus cells from cows suffering with metritis, a condition exerting detrimental effects on follicle development (Piersanti et al., 2019).

Other genes, namely *CENPE*, *CD93*, *PECAM1* and *HSPA1B*, were less abundant in cumulus cells surrounding oocytes able to develop to blastocysts. *CENPE* encodes for centromere-specific protein E and its higher abundance in less competent COC could be linked to a higher rate of cell replication probably associated with immature stages of follicle development, as cell replication is largely absent in fully matured oocytes. CD93 is a phagocytic receptor and its up-regulation in COC unable to develop to blastocysts could indicate a pro-phagocytosis state in less competent COC, as granulosa cells have recently been suggested to eliminate apoptotic oocytes by phagocytosis (Yefimova et al., 2020).

PECAM1 (platelet endothelial cell adhesion molecule-1) inhibits apoptosis by activating Akt and eNOS (Fleming et al., 2005; Limaye et al., 2005), and it has been reported to be down-regulated in primordial follicles following chemotherapy with cyclophosphamide (Titus et al., 2021). Its up-regulation in COC unable to develop to blastocyst stage may again reflect a lesser degree of follicular development compared with more competent COC, as at later stages of follicular development Akt can be activated by high oestradiol concentrations (Quirk et al., 2006) and thus may not require PECAM1-mediated apoptosis inhibition. A previous study reported that PECAM1 protein concentrations in follicular fluid were not associated with the establishment of pregnancy (Benifla et al., 2001), which agrees with the lack of differences between the P− and P+ groups in the current study.

Finally, HSPA1A (heat shock protein family A [hsp70] member 1A) belongs to the Hsp70 protein family, and is one of the major proteins induced by heat and other stress stimuli (Huang et al., 2013); therefore its up-regulation in COC unable to develop to blastocyst stage may be associated with prior exposure to stressful conditions.

In conclusion, the transcriptome analysis of human cumulus cells obtained from COC exhibiting diverse developmental competence revealed genes potentially associated with oocyte competence to reach the blastocyst stage, but was unable to identify useful markers to predict pregnancy potential.

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AU CONTRIBUTIONS

Human samples were collected by A.M.-M., PG-D and FR-J. Transcriptome analysis was performed by A.M.-M., L.G.-B., I.L.-T. and P.B.-A. L.G.-B. performed bioinformatics analysis. P.B.-A. designed the experiments and managed the funding. A.M.-M., L.G.-B. and P.B.-A. wrote the manuscript, which all authors approved.

DATA AVAILABILITY

Data will be made available on request.

DECLARATION OF COMPETING INTEREST

The authors report no financial or commercial conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.rbmo.2023.01.012.

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Capítulo 4

Metabolomics analysis of human cumulus cells obtained from cumulus-oocyte-complexes with different developmental potential

Metabolomics analysis of human cumulus cells obtained from cumulus-oocyte-complexes with different developmental potential

Álvaro Martínez-Moro, Leopoldo González-Brusi, Ana Querejeta-Fernández, Javier García-Blanco, Ester Padilla-Ruiz, Pablo Bermejo-Álvarez

Study question: Is the abundance of biochemical compounds in cumulus cells related to oocyte quality?

Summary answer: Malonate, 5-oxyproline and erythronate were positively associated with pregnancy potential.

What is known already: Cumulus cells are removed and discarded prior to ICSI, thereby constituting an interesting biological material on which to perform molecular analysis aimed to predict oocyte developmental competence. mtDNA content and transcriptional analyses in cumulus cells have shown to provide a poor predictive value of oocyte competence, but the untargeted analysis of biochemical compounds (metabolomics) has been unexplored.

Study design, size, duration: Cumulus cells obtained from cumulus oocyte complexes (COCs) of known developmental potential: 1) oocytes not developing to blastocyst following ICSI (Bl-), 2) oocytes developing to blastocyst but failing to establish pregnancy following embryo transfer (P-), and 3) oocytes developing to blastocyst able to establish a pregnancy (P+). Metabolomics analyses were performed on 12 samples per group, each composed by the cumulus cells recovered from one individual COC.

Participants/materials, setting, methods: Human cumulus cells samples were obtained from IVF treatments. Only unfrozen oocytes and embryos not submitted to preimplantation genetic testing were included in the analysis. Metabolomics analysis was performed by Ultra High Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS).

Main results and the role of chance: The analysis identified 98 compounds, from which 5 were differentially abundant between groups: asparagine, proline and malonate were less abundant in P- compared to Bl-, malonate and 5-oxoproline were less abundant in P- group compared to P+, and erythronate was less abundant in Bl- group compared to P+.

No significant association between the abundance of the compounds identified and donor age or body mass index was noted.

Limitations, reasons for caution: Data dispersion and the lack of coherence between developmental groups precludes the direct use of metabolic markers in clinical practice, where uterine environment plays a major role on pregnancy outcome. The abundance of other compounds not detected by the analysis may be associated to oocyte competence. As donors were lean (only 2 showing BMI>30) and young (<34 years-old), a possible effect of obesity or advanced age on cumulus cells metabolome could not be determined.

Wider implications of the findings: The abundance of Malonate, 5-oxyproline and erythronate in cumulus cells was significantly higher in COCs ultimately establishing pregnancy, providing clues on pathways required for oocyte competence. The untargeted analysis uncovered the presence of compounds that were not expected in cumulus cells, such as β-citrylglutamate and the neurotransmitter N-acetyl-aspartyl-glutamate (NAAG), which may play chromatin remodelling and signalling roles, respectively.

Study funding/competing interest(s): Research was supported by the Industrial Doctorate Project IND2017/BIO-7748 funded by Madrid Region Government. The authors declare no competing interest.

Keywords: Cumulus cells, oocyte quality, metabolomics, cumulus signaling, naag, β53 citrylglutamate.

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Original Article

Reproductive biology

Metabolomics analysis of human cumulus cells obtained from cumulus–oocyte complexes with different developmental potential

Álvaro Martínez-Moro[1,2], Leopoldo González-Brusi[1], Ana Querejeta-Fernández[3], Ester Padilla-Ruiz[2], Javier García-Blanco[2] and Pablo Bermejo-Álvarez [1,*]

[1]Animal Reproduction Department, INIA, CSIC, Madrid, Spain
[2]IVF Spain, Madrid, Spain
[3]Departamento de Química Inorgánica, Facultad de Ciencias Químicas, Universidad Complutense, Madrid, Spain

*Correspondence address. Departamento de Química Inorgánica, Facultad de Ciencias Químicas, Universidad Complutense, 28040 Madrid, Spain. E-mail: bermejo.pablo@inia.csic.es https://orcid.org/0000-0001-9907-2626

ABSTRACT

STUDY QUESTION: Is the abundance of certain biochemical compounds in human cumulus cells (CCs) related to oocyte quality?

SUMMARY ANSWER: Malonate, 5-oxyproline, and erythronate were positively associated with pregnancy potential.

WHAT IS KNOWN ALREADY: CCs are removed and discarded prior to ICSI, thereby constituting an interesting biological material on which to perform molecular analysis aimed to predict oocyte developmental competence. Mitochondrial DNA content and transcriptional analyses in CC have been shown to provide a poor predictive value of oocyte competence, but the untargeted analysis of biochemical compounds (metabolomics) has been unexplored.

STUDY DESIGN, SIZE, DURATION: CCs were obtained from three groups of cumulus–oocyte complexes (COCs) of known developmental potential: oocytes not developing to blastocyst following ICSI (Bl−); oocytes developing to blastocyst but failing to establish pregnancy following embryo transfer (P−); and oocytes developing to blastocyst able to establish a pregnancy (P+). Metabolomics analyses were performed on 12 samples per group, each sample comprising the CC recovered from a single COC.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Human CC samples were obtained from IVF treatments. Only unfrozen oocytes and embryos not submitted to preimplantation genetic testing were included in the analysis. Metabolomics analysis was performed by ultra-high performance liquid chromatography-tandem mass spectroscopy.

MAIN RESULTS AND THE ROLE OF CHANCE: The analysis identified 98 compounds, five of which were differentially abundant ($P < 0.05$) between groups: asparagine, proline, and malonate were less abundant in P− compared to Bl−, malonate and 5-oxoproline were less abundant in P− group compared to P+, and erythronate was less abundant in Bl− group compared to P+. No significant association between the abundance of the compounds identified and donor age or BMI was noted.

LIMITATIONS, REASONS FOR CAUTION: Data dispersion and the lack of coherence between developmental groups preclude the direct use of metabolic markers in clinical practice, where the uterine environment plays a major role in pregnancy outcome. The abundance of other compounds not detected by the analysis may be associated with oocyte competence. As donors were lean (only two with BMI $> 30\,kg/m^2$) and young (<34 years old), a possible effect of obesity or advanced age on the CC metabolome could not be determined.

WIDER IMPLICATIONS OF THE FINDINGS: The abundance of malonate, 5-oxyproline, and erythronate in CC was significantly higher in COCs ultimately establishing pregnancy, providing clues on the pathways required for oocyte competence. The untargeted analysis uncovered the presence of compounds that were not expected in CC, such as β-citrylglutamate and the neurotransmitter N-acetyl-aspartyl-glutamate, which may play roles in chromatin remodeling and signaling, respectively.

STUDY FUNDING/COMPETING INTEREST(S): Research was supported by the Industrial Doctorate Project IND2017/BIO-7748 funded by Madrid Region Government. The authors declare no competing interest.

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Keywords: cumulus cells / oocyte quality / metabolomics / metabolome / cumulus signaling / malonate / oxyproline / erythronate / NAAG / β-citrylglutamate

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Introduction

Oocyte growth and maturation occur alongside the adjacent somatic cells, called cumulus cells (CC), which communicate directly with the oocyte through transzonal projections that allow the transfer of regulatory factors and metabolites to the oocyte, forming an interconnected structure termed the cumulus–oocyte complex (COC). Within the COC, the oocyte and the surrounding CC are co-dependent in terms of both metabolism and signaling pathways: oocytes fail to progress in meiosis when they are devoid of CC (De La Fuente and Eppig, 2001; Fatehi et al., 2002), and CC fail to proliferate and expand (Vanderhyden et al., 1990), and show an altered metabolism when they are separated from the oocyte (Sugiura et al., 2005). Given that these supporting cells are discarded prior to ICSI, analysis of the biochemical compounds present in this easily accessible biological material may serve to shed light on the metabolic and signaling pathways used by the COC and to uncover molecular proxies to assist oocyte or embryo selection.

Current knowledge of COC metabolic and signaling pathways has been gathered from pioneer experiments that evaluated the developmental effects of the addition of specific metabolites or activators/inhibitors of specific metabolic or signaling routes to the media where COC develops (Biggers et al., 1967; Barbehenn et al., 1974; Herrick et al., 2006). This information, combined with the analysis of metabolites in the spent medium where COC developed, the expression of key metabolic enzymes, transporters or signaling receptors, and the unequivocal answers provided by gene ablation approaches (Cetica et al., 2002; Bermejo-Alvarez et al., 2010; Sutton-McDowall et al., 2010; Lamas-Toranzo et al., 2018) has served to uncover the details of COC metabolism and signaling pathways required for oocyte competence (Sobinoff et al., 2013; Richani et al., 2021). High-throughput metabolomics analysis provides an alternative, and largely unexplored, means to gain novel insights into metabolic and signaling pathways. Such analysis allows an untargeted search of biochemical compounds and has been applied to different reproductive fluids and tissues, such as human follicular fluid (de la Barca et al., 2017; Castiglione Morelli et al., 2019; Huang et al., 2022), but, to our knowledge, a global untargeted analysis of biochemical compounds has not been conducted in human CC.

Beyond the interest in providing basic knowledge on COC metabolism and signaling pathways, the analysis of the biochemical compounds present in CC could provide predictive markers of oocyte or embryo developmental competence. These markers could assist in embryo selection to maximize clinical pregnancy rate, which remains around 35% (European IVF-monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE), 2020). Unfortunately, although significant efforts have been devoted to uncover useful markers of developmental competence, embryo selection based on morphology still constitutes the only undisputed tool to improve pregnancy rate (Gardner and Balaban, 2016). Metabolomics analyses in the medium where the embryo develops (spent medium) have yielded promising preclinical markers to infer prospective pregnancy success, but the markers identified by independent groups vary and final clinical performance has been poor (reviewed by Hernandez-Vargas et al., 2020). Focusing on the search for molecular markers that could predict pregnancy potential in CC, global transcriptomics analysis (Green et al., 2018; Martinez-Moro et al., 2023) or the amount of mitochondrial DNA (Kumar et al., 2021; Liu et al., 2021; Martinez-Moro et al., 2022a,b) have been reported to provide a poor predictive value of pregnancy outcome. Compared to these analyses, metabolomics provides a downstream view of CC function, integrating possible differences between competent and non-competent COCs occurring at upstream levels, such as gene expression, post-transcriptional modification, or enzymatic activity.

The objective of this study was to investigate the biochemical compounds present in human CC and to determine if they could be used as predictors of subsequent embryo development. CCs were collected from individual COCs and, once the developmental outcome of each oocyte was known, the stored samples were allocated to one of three groups according to the oocyte's developmental potential: oocytes not developing to blastocyst (Bl−); oocytes developing to blastocyst but not establishing pregnancy (P−); or oocytes ultimately establishing a pregnancy (P+).

Materials and methods

Sample collection

CCs were obtained from donor cycles at IVF Spain Madrid between June 2018 and June 2020. All patients agreed to participate in the study (providing written informed consent), which was approved by the Ethical Committee from La Princesa University Hospital (Madrid). Inclusion criteria were the following: absence of uterine abnormalities; donor age ≤37 years; recipient age ≤50 years; and normal male sperm count (>2 millions spermatozoa/ml in ejaculate). BMI, endometrial thickness, and donor age were similar between experimental groups (Supplementary Table S1), although recipient age was higher in P+ group (Table 1). Only unfrozen oocytes and embryos not submitted to preimplantation genetic testing were included in the analysis.

Patients and donors were stimulated using recombinant FSH (corifollitropin alfa, Elonva®, MSD Biotech, Ireland) starting on the second menstruation day and ovulation was induced with GnRH analog (ganirelix, Orgalutran®, MSD Biotech, Ireland) analog when three or more follicles larger than 17 mm were present (Melo et al., 2009). Ovum pick-up was performed 36 h after hCG analog (triptorelin, Decapeptyl®, Tecnofarma, Chile) injection by transvaginal ultrasound guidance. Endometrium stimulation treatment in recipients consisted of 6 mg/day of estradiol (Meriestra®, Sandoz, Spain) and embryo transfer was performed only if the endometrium measured 7–13 mm (Prapas et al., 1998). Luteal support started on the night of oocyte retrieval with 400 mg of progesterone administered every 12 h until a pregnancy test (Edwards et al., 1984).

COCs were retrieved following follicular aspiration, washed, and individually cultured in G-IVF Plus Media (Vitrolife, Sweden) (6.3% CO_2, 5% O_2, 88.7% N_2 at 37 °C). Denudation was performed individually 2 h after COC recovery, using hyaluronidase solution (Irvine). CCs detached from oocytes were collected from denudation medium, pelleted by centrifugation at 1500×*g* for 10 min at

Table 1. Characteristics of patients and donors in a study of the metabolome of cumulus cells obtained from cumulus–oocyte complexes.

	Bl−	P−	P+
Age patients (years)	–	39.25 ± 1.95[a]	44.50 ± 0.75[b]
BMI patients (kg/m^2)	–	25.18 ± 1.83	21.92 ± 1.03
Smoker (patient)	–	3/12	2/12
Endometrial thickness (mm)	–	9.16 ± 0.42	8.80 ± 0.45
Age donors (years)	27.33 ± 1.17	25.92 ± 1.37	27.33 ± 1.16
BMI donors (kg/m^2)	22.59 ± 1.12	21.36 ± 0.89	22.64 ± 1.24
Smoker (donors)	6/12	4/12	5/12

Data are shown as mean ± SEM (n = 12/group), significant differences are indicated by different letters (ANOVA; $P < 0.05$).

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room temperature, snap frozen in liquid nitrogen, and stored at −80°C until analysis. Oocytes were fertilized by intracytoplasmic sperm injection (ICSI) using G-MOPS Plus (Vitrolife, Sweden) and spermatozoa were allocated in 7% polyvinylpyrrolidone solution to slow their movement. Following ICSI, the presumptive zygotes were individually cultured in Continuous Single Culture SAGE 1-Step^{TM} (Irvine, CA, USA) for 6 days, up to the blastocyst stage. Morphological score was used to select blastocysts for embryo transfer (Gardner *et al.*, 2015). Pregnancy was assessed at the fourth or fifth week post-fertilization by fetal heart rate detection by ultrasound echography. Once the embryo development was known, the previously stored CCs were allocated into three groups according to the oocyte's developmental potential: oocytes not developing to blastocyst (Bl−); oocytes developing to blastocyst but failing to establish pregnancy following embryo transfer (P−); or oocytes developing to blastocyst able to establish pregnancy (P+).

Metabolomics analysis

Metabolomics analyses were performed on 12 samples per group, which were shipped in dry ice to Metabolon Inc. (Durham, NC, USA). Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. Several recovery standards were added prior to the first step in the extraction process for quality control (QC) purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min followed by centrifugation at 1493×*g* for 10 min at RT. The resulting extract was divided into five fractions: two for analysis by two separate reversed-phase ultra-high performance liquid chromatography-mass spectrometry reverse phase methods [(RP)/UPLC-MS/MS] with positive ion model electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by hidrophilic interaction chromatography (HILIC)/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly in TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under passive nitrogen gas before preparation for analysis.

Several types of controls were analyzed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample (or alternatively, use of a pool of well-characterized human plasma) served as a technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of QC standards, carefully chosen not to interfere with the measurement of endogenous compounds, were spiked into every analyzed sample, that allowed instrument performance monitoring and aided chromatographic alignment. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e. non-instrument standards) present in 100% of the pooled matrix samples. Experimental samples were randomized across the platform run with QC samples spaced evenly among the injections.

Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS) was performed on a Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyser operated at 35 000 mass resolution (ThermoFisher, MS, USA). The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1 × 100 mm, 1.7 μm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions, but it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1 × 100 mm, 1.7 μm) using methanol, acetonitrile, water, 0.05% PFPA, and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using methanol and water with 6.5 mM ammonium bicarbonate at pH8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1 × 150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10 mM ammonium formate, pH 10.8. The MS analysis alternated between MS and data-dependent MS^n scan using dynamic exclusion. The scan range varied slighted between methods but covered 70–100 m/z.

Raw data were extracted, peak identified, and QC processed using Metabolon's hardware and software (Metabolon, NC, USA). Compounds were identified by comparison to library entries of purified standards. More than 3300 standard compounds are registered for analysis. Biochemical identifications are based on three criteria: retention index within a narrow retention time/index window of the proposed identification, accurate mass match to the library±10 parts per million, and the MS/MS forward and reverse scores between the experimental data and authentic standards. Peaks were quantified using area-under-the-curve. A matrix with the normalized peak intensities was processed in an in-house R script, making use of the metabolomics package *omu* (Tiffany and Baumler, 2019). Pairwise differential analysis was performed through a Welch test implemented within the *omu_summary* function applying the Benjamini–Hochberg correction. Principal component analysis (PCA) and boxplots were obtained from *ggplot2* package and heatmaps with hierarchical clustering were drawn by the package *ComplexHeatmap* (Gu *et al.*, 2016).

Results

Metabolomics analysis identified 98 compounds of known identity in human CC (excluding xenobiotics: Supplementary Table S2). The list of compounds comprised 30 amino acid-related compounds, including amino acids involved in protein synthesis and amino acids and related compounds involved in other processes, such as glutathione, taurine, creatine, spermidine, β-citrylglutamate, and N-acetyl-aspartyl-glutamate (NAAG). Nine carbohydrates were detected, including five involved in glycolysis (glucose, 3-phosphoglycerate, pyruvate, lactate, and glycerate), and others, such as 6-phosphogluconate (involved in pentose phosphate pathway), fructose, mannitol/sorbitol, and the amino-sugar erythronate. Compounds of the tricarboxylic acid (TCA) cycle (citrate, aconitate, alpha-ketoglutarate, and malate), phosphate (involved in oxidative phosphorylation), nucleotides (including AMP/cAMP), and vitamins and cofactors—such as threonate, nicotinamide, and alpha-tocopherol—were also identified in human CC. Lipid constituted the largest group of

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compounds detected. A large proportion of the 45 lipid-related compounds detected were phospholipids, including 20 glycerophospholipids and nine sphyngolipids. Other lipid-related compounds detected were steroid hormones and precursors, fatty acids, and myo-inositol.

No evident association was observed between the abundance of all biochemical compounds identified in CC and the developmental competence of the enclosed oocyte, as PCA and hierarchical clustering failed to group samples according to subsequent embryo development (Figs 1A and 2). There was also no association between the abundance of all biochemicals detected and donor weight, age, or smoking status (Figs 1B–D and 2). The abundance of five individual compounds showed statistically significant differences between the groups that differed in developmental ability (Fig. 3). In particular, asparagine, proline, and malonate were less abundant in P− compared to Bl−, malonate, and 5-oxoproline were less abundant in P− group compared to P+, and erythronate was less abundant in Bl− group compared to P+. Tendencies ($0.05 < P < 0.1$) were noted for another seven compounds: citrate, 2-hydroxybutyrate, serine, epiandrosterone sulfate, cysteine, N-acetylkynurenine, and alanine (Fig. 2). When data were analyzed depending on the ability to reach the blastocyst stage (i.e. Bl− versus Bl+, the latter composed by P− and P+ samples), no statistically significant differences were found, although trends (P 0.07–0.08) were found for erythronate—more abundant in Bl+ group- and N-acetylkynurenine—less abundant in Bl− group- (Supplementary Fig. S1), and Bl− samples roughly clustered together when all biochemicals detected were considered in an hierarchical clustering (Fig. 2).

Discussion

CCs constitute a readily available material on which to perform analyses aimed at inferring the developmental ability of the oocyte they nourished (Kordus and LaVoie, 2017). Mitochondrial DNA and transcriptomics analyses of CC have failed to provide reliable and clinically useful markers of the embryo potential to establish pregnancy (Green et al., 2018; Kumar et al., 2021; Liu et al., 2021; Martinez-Moro et al., 2022a,b, 2023), but metabolomics has remained largely unexplored, probably owing to the technical difficulties of performing such analysis of the limited amount of cells present in a COC. Herein, using an improved MS/MS protocol, more sensitive than nuclear magnetic resonance approaches (Emwas, 2015), we were able to quantify the relative abundance of 98 compounds.

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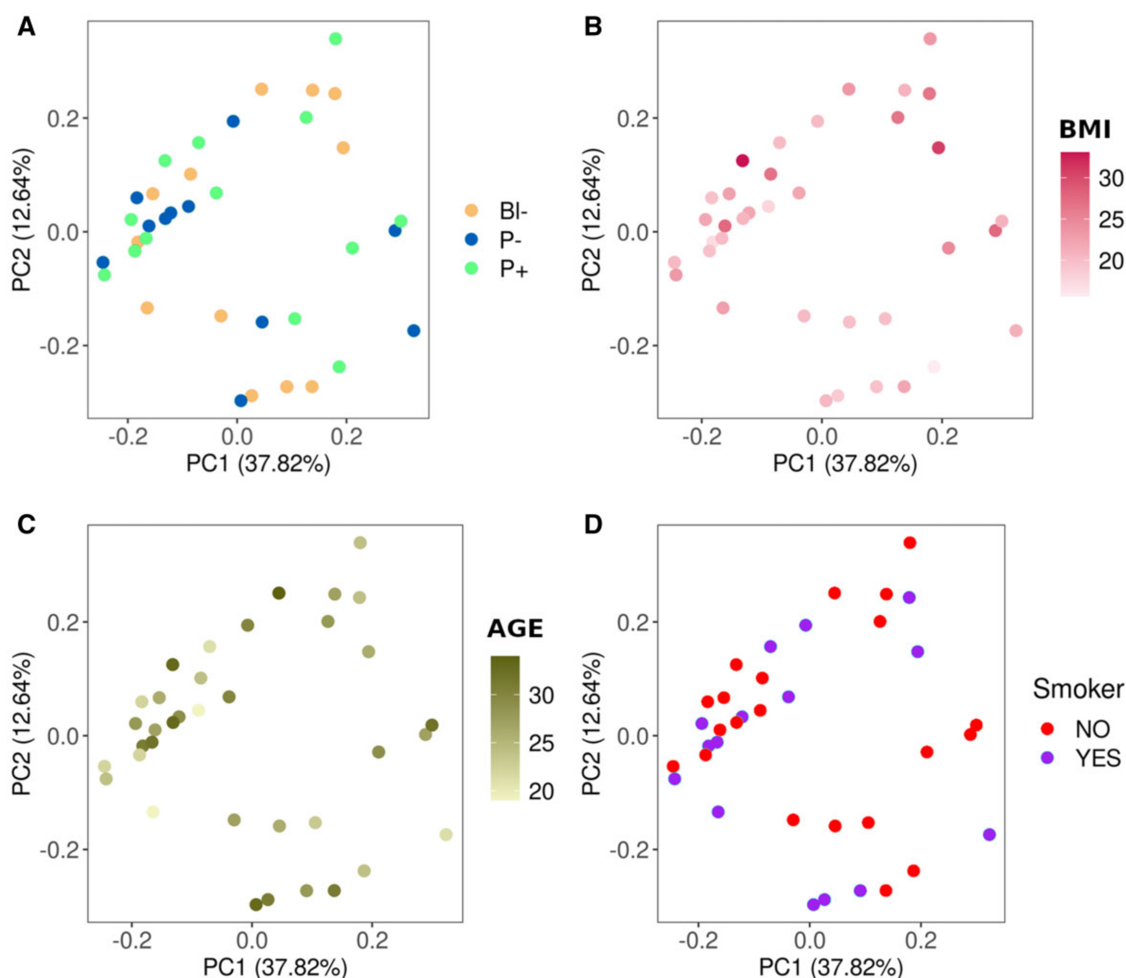

Figure 1. Principal component analysis of the metabolomics data obtained from the cumulus cells of 36 single human cumulus–oocyte complexes. The analyses are presented according to developmental ability (**A**), donor BMI (kg/m^2) (**B**), age (years) (**C**), or smoking status (**D**). (A) Cumulus cells were classified into three groups depending on the developmental ability of their corresponding oocyte: oocytes unable to develop to blastocysts (Bl−, orange), able to develop to blastocyst but not establishing pregnancy (P−, blue) or able to establish pregnancy (P+, green). (B and C) Color scales were employed for numerical variables: BMI (light to dark red) and age (light to dark green). (D) Smoking status is depicted as red for non-smokers and purple for smokers. PC, principal component.

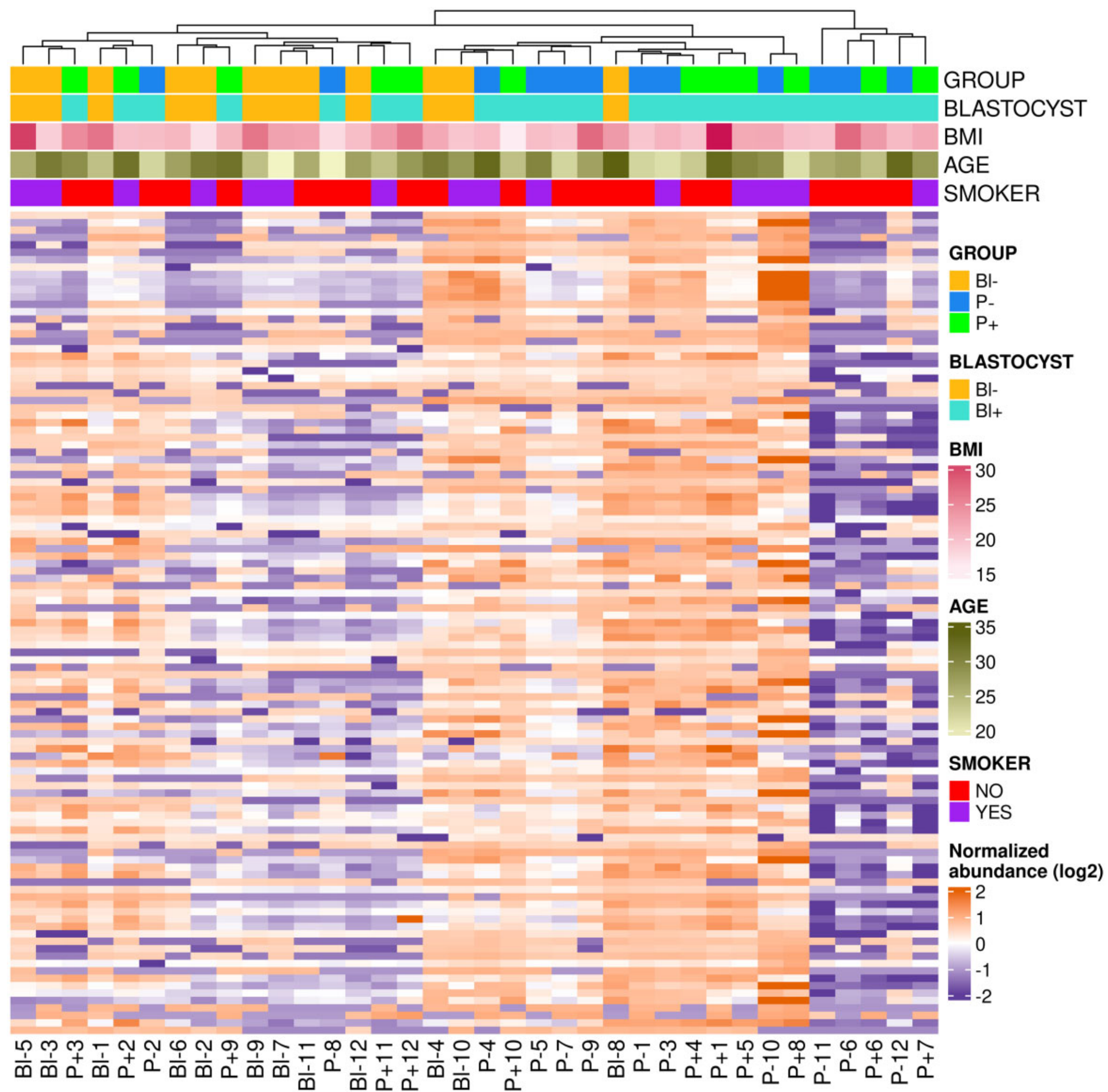

Figure 2. Hierarchical clustering of the metabolomics data obtained from the cumulus cells of 36 single human cumulus–oocyte complexes. Upper color bars indicate developmental ability and donor characteristics. The uppermost color bar indicates developmental ability: oocytes unable to develop to blastocysts (Bl−, orange), able to develop to blastocyst but not establishing pregnancy (P−, blue) or able to establish pregnancy (P+, green). The next bar down distinguishes oocytes unable to develop to blastocysts (Bl−, orange) and those able to develop to blastocyst (Bl+, turquoise). The next two color bars indicate donor BMI (light to dark red) and age (light to dark green). The lowest color bar indicates smoking status, red for non-smokers, and purple for smokers.

Despite the high number of compounds analyzed, the comparison between the three groups differing in their developmental ability failed to yield clear biochemical markers of oocyte competence. Although some metabolites showed statistically significant differences between groups, such differences did not follow a logical trend. A reliable marker to infer the oocyte's ability to reach the blastocyst stage should display differences in both Bl− versus P− and Bl− versus P+ comparisons (i.e. the group showing impaired preimplantation development compared to either group successfully developing to blastocysts). Conversely, a dependable proxy of the ability to establish pregnancy would be expected to display significant differences in both Bl− versus P+ and P− versus P+ comparisons (i.e. the group establishing pregnancy compared to the groups arresting their development earlier), although in this case the value of P+ versus Bl− is less informative. None of the five compounds showing statistically significant differences between groups fulfilled those stringent conditions and the Bl− versus Bl+ comparison also failed to yield significant differences. However, malonate and 5-oxyproline were more abundant in P+ compared to P, and erythronate was more abundant in P+ compared to Bl− and numerically higher in P+ versus P−.

Malonate inhibits succinate dehydrogenase (Pardee and Potter, 1949), a component of the TCA cycle and complex II of the mitochondrial electron transport chain. The increased malonate content in P+ group may indicate a diminished TCA activity in more competent COCs. Such reduction in TCA activity could reduce oxidative stress, shifting metabolism to anaerobic routes (Bermejo-Alvarez *et al.*, 2010). 5-Oxyproline (pyroglutamic acid) is a precursor of glutamate, an amino acid suggested to play signaling roles in oocytes and preimplantation embryos (Spirkova *et al.*, 2022). Glutamate is also one of the three amino acids forming glutathione, together with cysteine and glycine, all detected in CC by the analysis. Glutathione constitutes the major non-enzymatic anti-oxidant (Meister and Anderson, 1983) and has been linked to oocyte quality, as mice unable to synthesize

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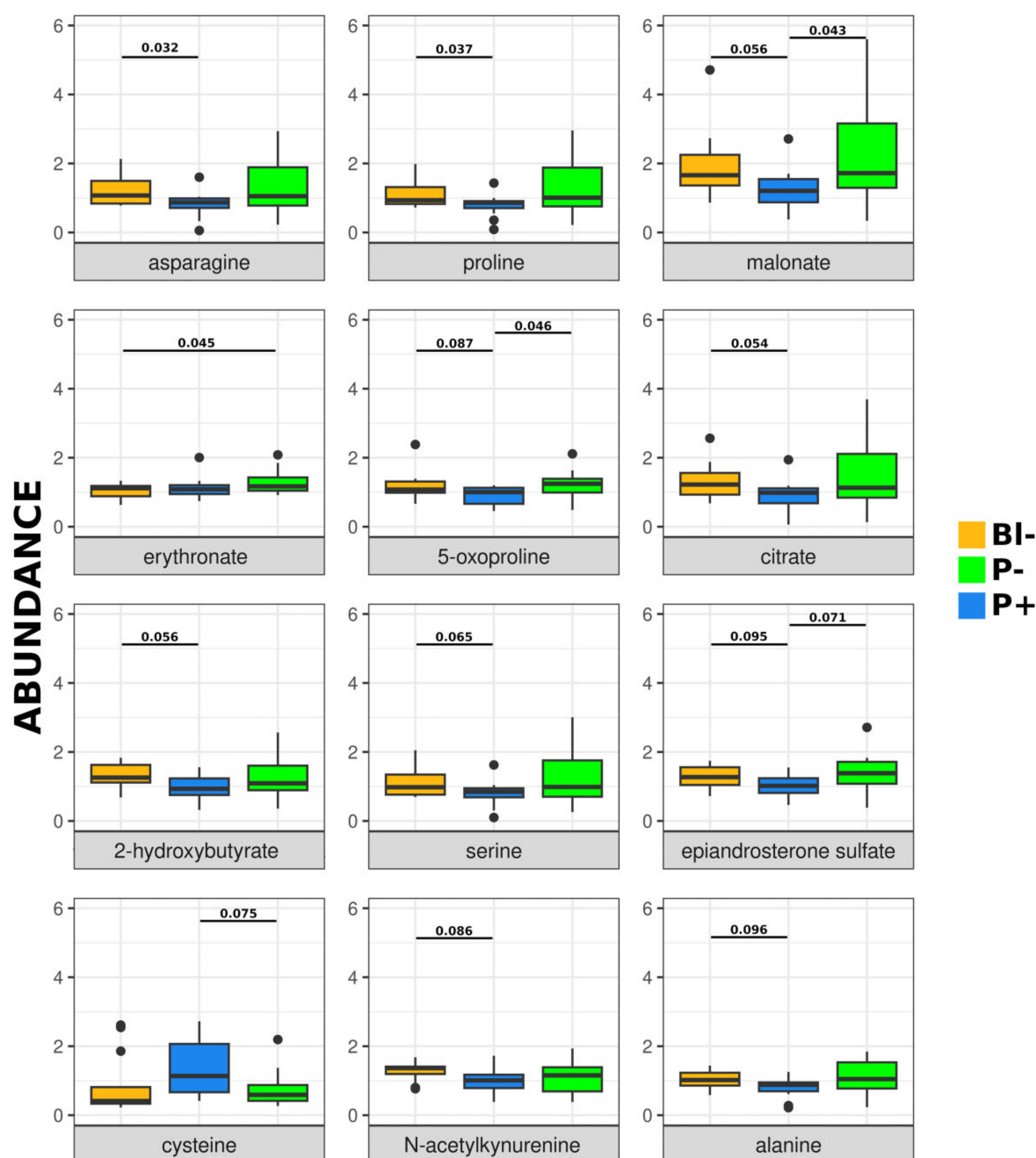

Figure 3. Relative abundance of biochemical compounds in cumulus cells surrounding human oocytes of different developmental ability. Oocytes unable to develop to blastocysts (Bl−, orange), able to develop to blastocyst but not establishing pregnancy (P−, blue) or able to establish pregnancy (P+, green). Compounds above the black line (asparagine, proline, malonate, erythronate, and 5-oxyproline) were differentially abundant between groups based on Welch test, P-values are indicated for each compound and statistically significant comparison. Individual points indicate samples diverging greatly from the group (included in the analysis). The seven compounds below the black line tended to differ ($0.05 < P < 0.01$) between groups.

glutathione (*Glmc* KO) show an accelerated age-related decline in female fertility that is associated with preimplantation embryonic mortality (Nakamura *et al.*, 2011), and glutathione content in the oocyte has been positively associated with oocyte competence (Perreault *et al.*, 1988). Paradoxically, 5-oxyproline content in CC was elevated in women with polycystic ovary syndrome, an increase suggested to be caused by a reduced capacity to synthesize glutathione (Turathum *et al.*, 2022). Despite the differences observed in 5-oxyproline levels, our analysis found no differences in glutamate or glutathione content in CC between groups exhibiting different developmental ability. From this perspective, the increased amount of 5-oxyproline in the P+ group might be indicative of an available surplus after the synthesis of glutamate and/or glutathione, which could have been transported from the CC into the oocyte before cumulus expansion. Finally, erythronic acid is formed by the oxidation of N-acetyl-D-glucosamine (GlcNAc), a constituent of hyaluronic acid (HA). HA is a linear glycosaminoglycan, consisting of alternating units of glucuronic acid and GlNAc that plays an essential role during CC expansion (Chen *et al.*, 1990; Salustri *et al.*, 1992). The increased content in erythronate may therefore be indicative of a greater or earlier CC expansion, which has been positively associated with oocyte quality in animal models (Qian *et al.*, 2003; Martinez-Moro *et al.*, 2022a,b).

Despite the large number of lipid compounds detected, their amounts were not associated with developmental ability. In contrast, a prior lipidomics analysis conducted on human CC comparing groups equivalent to P− and P+ here suggested a higher

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abundance of phosphatidylcholine in P+ and a higher abundance of phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol in P− (Montani et al., 2012). Unfortunately, these compounds were not among the 98 compounds identified by our analysis, although 11 compounds related to phosphatidylcholine and four related to phosphatidylethanolamine were detected and did not show differences between groups. A possible explanation for the discrepancies between studies may be the different methods used for lipid detection and characterization, but given that no relative amount data or fold-change was provided on the lipidomics study (Montani et al., 2012), data analysis constitutes a major difference between the studies. Donor BMI and age have been found to exert an effect on different molecular markers in CC (reviewed by Richani et al. (2021), Babayev and Duncan (2022)), but their effects on the CC metabolome were unexplored. Here, we did not observe a significant correlation between donor BMI and age and the CC metabolome but the population analyzed does not allow us to draw solid conclusions, as donors were mostly lean (only 2/30 with BMI > 30 kg/m^2) and young (the oldest being 34 years old).

Beyond the search for proxies of oocyte competence, the untargeted analysis of biochemical compounds present in CC provides a novel means to uncover signaling pathways. The metabolomics analysis identified amino acid-related compounds whose presence in CC was unexpected. Among them, β-citrylglutamate is a pseudodipeptide abundant in several neonatal tissues whose concentration declines rapidly after birth, with the exception of testes, where it increases with age coinciding with the development of late spermatocytes into early spermatids (Montani et al., 1982). The presence of this compound in CC was, therefore, unexpected and its role in follicular development is unknown. In mice, the β-citrylglutamate synthase Rimklb is expressed in Leydig cells and its ablation has been observed to result in male infertility (Maekura et al., 2021) or subfertility caused by impaired histone to protamine exchange and retention of transition protein 2 during spermiogenesis (Wang-Eckhardt et al., 2022). Despite the remarkable differences in chromatin compaction between male and female mammalian gametogenesis, β-citrylglutamate could intervene in chromatin condensation during oogenesis. As *Rimklb* knock-out (KO) mice do not show female infertility, β-citrylglutamate role may not be required for female gametogenesis, but it may be possible that KO COCs are not devoid of β-citrylglutamate, as another β-citrylglutamate synthase may be acting during oogenesis.

Another unexpected compound detected in human CC was NAAG, a dipeptide that consists of glutamate and N-acetylaspartate (both also detected in CCs by the analysis) coupled via a peptide bond. NAAG acts as neurotransmitter through activation of the presynaptic metabotropic receptor 3 (GRM3, also known as mGluR3) (Wroblewska et al., 1997), which is almost exclusively expressed in brain (Fagerberg et al., 2014) and is undetectable in CC from humans and close mammalian models (Martinez-Moro et al., 2022a,b, 2023). The presence of NAAG in CC was therefore unexpected and its role is unknown, but it may mediate signaling roles through NMDA (N-methyl-D-aspartate) receptors, as observed in neurons (Khacho et al., 2016), given that multiple NMDA receptors (GRINA, GRIN2A GRIN2C, GRIN2D, and GRIN3B) are expressed in human CC (Martinez-Moro et al., 2022a,b). Other amino acid-related compounds detected, such as taurine, creatine, and spermidine, have been previously observed in COCs or follicular fluid. Taurine has been previously detected in CC (Lobo et al., 2001), where it probably plays anti-oxidant and biological membrane stabilization roles (Goberdhan, 2010). Creatine has been suggested to act as a probable modulator of ATP concentration in the oocyte (Scantland et al., 2014), and spermidine is a polyamine involved in post-translational modifications (Pegg, 2016) that has been reported to be more abundant in follicular fluid from women with diminished ovarian reserve (Gokce et al., 2023).

In conclusion, the abundance of the 98 compounds identified in CC was largely unrelated to the developmental competence of the enclosed oocyte, although malonate, 5-oxyproline, and erythronate were positively associated with pregnancy potential. However, given data dispersion and the lack of coherence between the three groups analyzed, the predictive value in clinical practice is poor. Two unexpected amino acid-related compounds, β-citrylglutamate and NAAG, were detected in CC, where they could play chromatin remodeling and signaling roles, respectively.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Authors' roles

Human samples were collected by A.M.-M., J.G-B., and E.P.-R. L.G.-B. performed data analysis. Data interpretation was performed by A.M.-M., L.G.-B., A.Q.-F., and P.B.-A. A.M.-M. and P.B.-A. wrote the article, being supervised by all authors.

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Conflict of interest

The authors declare no conflict of interest.

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Discusión general

Contexto aplicativo de la investigación

Los tratamientos de reproducción asistida basados en técnicas de fecundación *in vitro* (FIV) o inseminación intracitoplásmica (ICSI) tienen distintas aplicaciones en humanos y animales de granja. En humanos, estos tratamientos se emplean para solucionar distintas causas de infertilidad masculina o femenina, mientras que en animales de granja constituyen una herramienta para obtener un mayor número de descendientes de hembras de alto mérito genético o para generar embriones a partir de ovarios procedentes de matadero a un coste inferior a los embriones *in vivo* que pueden emplearse en múltiples aplicaciones (Baruselli *et al*., 2020; Ferré *et al*., 2020). En ambos casos, existe la necesidad de mejorar la eficiencia del proceso en términos de desarrollo a blastocisto y éxito de embarazo/preñez (Gliozheni *et al*., 2020), bien sea para evitar la repetición de los tratamientos hormonales y quirúrgicos asociados a la técnica en mujeres, o para mejorar la eficacia reproductiva y aumentar el número de descendientes de elevado valor genético en animales. En esta tesis, hemos llevado a cabo experimentos para buscar marcadores de competencia ovocitaria en células del cúmulo humanas y bovinas. Dichos marcadores podrían emplearse para seleccionar los ovocitos con mayor competencia embrionaria, es decir, aquellos en los que las probabilidades de éxito post-transferencia sean más altas. Además, el estudio de estos marcadores sirve para conocer la base molecular de la adquisición de competencia ovocitaria durante la foliculogénesis y la maduración del ovocito, un conocimiento esencial para avanzar en las técnicas de maduración *in vitro*.

La especie bovina constituye la especie de mamífero no primate con un desarrollo y dinámica folicular más cercana a la humana, ya que en ambas especies se establece un único folículo dominante que ovula de forma espontánea y no estacional (Sirard, 2017). Por ello, la realización de experimentos en ambos modelos puede dar lugar a sinergias que ayuden a entender factores comunes que intervengan en la competencia del ovocito. Sin embargo, las diferencias en el propósito de la aplicación de las técnicas de FIV o ICSI en ganadería y reproducción asistida humana dan lugar a una diferencia fundamental en el procedimiento: La ICSI en humanos se realiza sobre ovocitos madurados *in viv*o mientras que en la especie bovina se realiza FIV sobre ovocitos madurados *in vitro*. En los tratamientos de reproducción asistida humana, el éxito del proceso consiste en la obtención de una gestación viable, de modo que se realiza una estimulación hormonal

para obtener ovocitos madurados en una única sesión (D'Angelo *et al.*, 2019). En ganadería, el enfoque es diferente, ya que lo que se pretende es obtener un gran número de embriones de una hembra de alto mérito genético que serán transferidos a otras hembras receptoras. Para este propósito es necesario poder realizar múltiples aspiraciones de ovocitos en la misma hembra y, en este contexto, la obtención de ovocitos inmaduros permite obtener un mayor número de ovocitos y reducir el lapso de tiempo entre sesiones de aspiración (Merton *et al.*, 2003; Petyim *et al.*, 2003). Además, la aspiración de ovocitos inmaduros puede realizarse sin tratamiento hormonal o con menores dosis totales que las necesarias para una superovulación, lo que supone una ventaja económica y logística, permite reducir el uso de hormonas en animales de granja y posibilita la obtención de ovocitos en hembras preñadas. La desventaja de la obtención de ovocitos inmaduros es que su competencia es significativamente menor a la de los madurados *in vivo* en términos de capacidad de desarrollo a blastocisto (Dieleman *et al.*, 2002; Rizos *et al.*, 2002; van Leemput *et al.*, 1999).

Las diferencias en el procedimiento humano y bovino han de tenerse en cuenta en la interpretación de los resultados de los experimentos y en el interés aplicativo de los mismos. En lo referente a la interpretación, los resultados humanos provienen de complejos cúmulo-ovocito madurados *in vivo* tras la aplicación de tratamientos hormonales y, por ello, están condicionados por la variabilidad a la respuesta hormonal entre donantes, que afecta a la funcionalidad de las células del cúmulo (Borgbo *et al.*, 2013). En contraposición, los resultados bovinos se obtienen de complejos cúmulo-ovocito obtenidos sin estimulación hormonal previa y madurados *in vitro* en condiciones experimentales controladas. Además del mayor control experimental de las muestras bovinas, que reduce la variabilidad no deseada (intra-grupo), se esperan mayores diferencias en competencia ovocitaria (inter-grupo) en comparación con las muestras humanas, debido a la mayor diversidad de desarrollo folicular asociada a la aspiración de ovocitos inmaduros, que desemboca en una menor competencia promedio para el desarrollo (Dieleman *et al.*, 2002; Rizos *et al.*, 2002; van Leemput *et al.*, 1999). A nivel aplicativo, los marcadores de competencia ovocitarios tendrían una aplicación directa en la selección de embriones previa a la transferencia en la especie humana. En el ganado bovino los márgenes de beneficio económico de la transferencia embrionaria son más estrechos, de modo que el balance entre el coste económico del análisis del marcador de competencia y el beneficio económico derivado de la mejora en la tasa de preñez no sería

positivo en el caso de emplear técnicas actualmente costosas como la transcriptómica o la metabolómica. Además, para realizar estos análisis es necesario realizar un cultivo individualizado y obtener las células del cúmulo antes de la FIV, ya que en caso contrario degeneran durante el proceso, dejando de ser útiles para el análisis de marcadores de competencia. Ambas premisas contrastan con los protocolos habituales de producción de embriones bovinos: la FIV se realiza con células del cúmulo y su eliminación antes del proceso reduce ligeramente las tasas de desarrollo (Lamas-Toranzo *et al.*, 2019), mientras que la maduración, fecundación y cultivo *in vitro* individualizados disminuyen notoriamente las tasas de desarrollo comparadas con el tratamiento en grupos (Bunel *et al.*, 2015). En este sentido, los análisis transcriptómicos y metabolómicos en células del cúmulo bovinas son más útiles como generadores del conocimiento necesario para mejorar las técnicas de maduración *in vitro* bovina que para la obtención de marcadores de competencia aplicables a la práctica veterinaria.

Discusión de los resultados

Las células del cúmulo constituyen un material biológico interesante sobre el que realizar análisis moleculares no invasivos para determinar la competencia ovocitaria (Kordus & LaVoie, 2017). Estas células se encuentran comunicadas directamente con el ovocito a través de proyecciones transzonales, formando una estructura interconectada denominada complejo cúmulo-ovocito. En esta estructura, las células del cúmulo cumplen funciones metabólicas y de señalización durante la ovogénesis y la maduración hasta la expansión del cúmulo (Richani *et al.*, 2021), momento en el que las proyecciones transzonales se pierden y las células del cúmulo dejan de ser esenciales, quedando por tanto disponibles para realizar análisis moleculares no invasivos. Sobre estas células se han realizado múltiples análisis moleculares, siendo el análisis de la cantidad de ADN mitocondrial (ADNmt) y el análisis de la expresión génica los más empleados en la bibliografía previa. En esta tesis hemos realizado análisis de la cantidad de ADNmt, análisis transcriptómicos y un novedoso análisis metabolomico.

El diseño experimental de todos los experimentos de la tesis está basado en la comparación de tres grupos experimentales que se diferencian en su capacidad para el desarrollo (Figura 15). En el caso de los experimentos con complejos cúmulo-ovocito bovinos, las muestras se agruparon en función de su capacidad de desarrollo *in vitro* en 1) ovocitos que no llegan a dividirse tras la fecundación *in vitro* (Cl-, de división en inglés

–cleavage-), 2) ovocitos que llegan a dividirse, pero no a formar blastocistos (Bl-), y 3) ovocitos que llegan a formar blastocistos (Bl+). En el caso de los experimentos con complejos cúmulo-ovocito humanos, las muestras se dividieron en función de su capacidad de desarrollo *in vitro* e *in vivo* en ovocitos que no llegan a formar blastocistos (Bl-) y en dos grupos de ovocitos capaces de formar blastocistos que fueron transferidos: en uno de los grupos los blastocistos no dieron lugar a gestación (P-, de gestación en inglés *–pregnancy*-) y en el otro sí (P+). Este diseño en tres grupos escalados de menor a mayor competencia embrionaria permite una evaluación más robusta y fehaciente de los parámetros de competencia embrionaria que el análisis de dos grupos. Por ejemplo, en el caso del bovino, un parámetro de competencia para la división debería aparecer en las comparativas Cl- vs Bl- y Cl- vs. Bl+, ya que los ovocitos de los grupos Bl- y Bl+ son capaces de dividirse tras la fecundación. De forma similar, en humanos, un parámetro de competencia para desarrollo a blastocisto debería aparecer en las comparativas Bl- vs. P- y Bl- vs. P+, ya que los ovocitos de los grupos P- y P+ son capaces de alcanzar el estadio de blastocisto tras la ICSI.

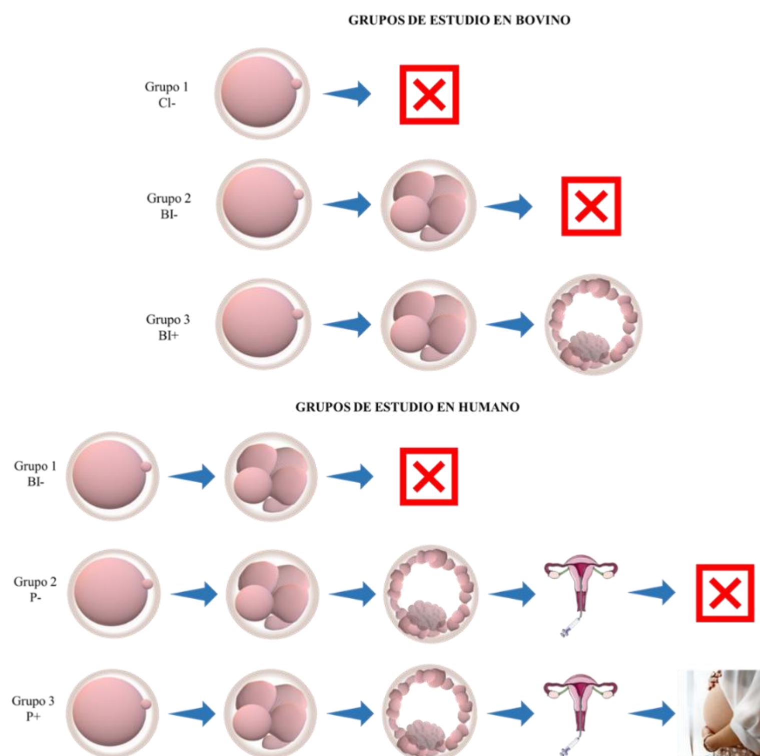

Figura 15. Esquema de los grupos de estudio a analizar en las dos especies.
(Imagen modificada de Servier Medical Art: https://smart.servier.com/ y http://albumarium.com/)

El análisis de la cantidad del ADNmt presenta una serie de ventajas para su aplicación en la práctica clínica con respecto a otros análisis más complejos como el análisis

transcriptómico o el metabolómico: 1) la cantidad de ADNmt puede estimarse de forma sencilla, económica y rápida, ya que sólo requiere extracción de ADN y PCR cuantitativa (qPCR) y 2) el análisis proporciona un único dato por muestra, permitiendo una comparación rápida y sencilla. La lógica detrás del estudio de la cantidad de ADN mitocondrial en células del cúmulo como posible marcador de competencia ovocitaria se basa en múltiples evidencias que correlacionan la cantidad de ADN mitocondrial del ovocito con su competencia. Durante el crecimiento del ovocito el ADN mitocondrial se multiplica exponencialmente (Cao *et al.*, 2007; Cree *et al.*, 2008) y, por tanto, es esperable que su cantidad sirva como un marcador de desarrollo folicular y, por tanto, de la competencia del ovocito. Aunque el análisis del ADNmt del ovocito implica la destrucción del mismo y, por ello, no es posible obtener una correlación directa entre la cantidad de ADNmt de un determinado ovocito y su competencia para el desarrollo, varios análisis que han comparado grupos de ovocitos con distinta competencia para el desarrollo han observado una correlación positiva entre ambos parámetros. En modelos animales, la técnica de tinción vital de azul brillante de cresilo (BCB por sus siglas en inglés Brilliant Cresyl Blue) permite diferenciar ovocitos en un estado más avanzado de crecimiento folicular (BCB+) más competentes para el desarrollo que los BCB- (Ericsson *et al.*, 1993). Empleando esta técnica combinada con el análisis de ADNmt se ha observado una mayor cantidad de ADNmt en ovocitos porcinos y bovinos BCB+ comparados con los BCB- (El Shourbagy *et al.*, 2006; Lamas-Toranzo *et al.*, 2018). En humanos se ha observado una mayor cantidad de ADNmt en grupos de ovocitos que promediaban una tasa de fecundación normal comparado con grupos de ovocitos que mostraban fallos en la fecundación (Reynier *et al.*, 2001), en cigotos comparados con ovocitos sin fecundar (Santos *et al.*, 2006) y en ovocitos madurados *in vivo* comparados con in vitro –menos competentes para el desarrollo que los *in vivo*- (Zhao *et al.*, 2016). Además, se ha observado una reducción de la cantidad de ADNmt del ovocito en casos de insuficiencia ovárica (May-Panloup *et al.*, 2005) y envejecimiento (Chan *et al.*, 2006; Murakoshi *et al.*, 2013).

El análisis del ADNmt de forma no invasiva (en células del cúmulo) no obtuvo el resultado deseado, no existiendo correlación entre el ADNmt y la competencia para el desarrollo de ovocitos humanos o bovinos. Estos resultados contrastan con estudios previos en los que se asoció el ADNmt con parámetros indirectos de competencia embrionaria (morfocinética) (Desquiret-Dumas *et al.*, 2017; Ogino *et al.*, 2016), una

asociación que, de ser cierta, podría tener cierto interés biológico pero que no tiene aplicación práctica, ya que no proporciona información adicional a la ya disponible mediante la observación del desarrollo embrionario in vitro. Otro estudio correlacionó la probabilidad de implantación con la cantidad del ADNmt del ovocito (Craciunas *et al*., 2019), sin embargo, la dispersión de los datos de dicho estudio (215±375 vs. 59±72 para ovocitos que dan lugar a implantación vs. ovocitos que no dan lugar a implantación, respectivamente) pone en duda la significación estadística de los mismos y hace inviable su aplicación práctica en embriología clínica, al no poderse establecer un valor límite a partir del cual rechazar o seleccionar como transferible a un embrión. Por último, realizamos un experimento para probar la hipótesis base sobre la que se asienta la lógica del análisis de la cantidad de ADNmt de las células del cúmulo como parámetro de competencia embrionaria: la supuesta correlación entre la cantidad de ADNmt en el ovocito (el parámetro de competencia ovocitaria contrastado indirectamente por múltiples estudios previos (Chan *et al*., 2006; El Shourbagy *et al*., 2006; Lamas-Toranzo *et al*., 2018; May-Panloup *et al*., 2005; Murakoshi *et al*., 2013; Reynier *et al*., 2001; Santos *et al*., 2006; Zhao *et al*., 2016) y la cantidad de ADNmt de las células del cúmulo. Para ello recurrimos al modelo bovino, observando que ambos parámetros no estaban relacionados, confirmando el nulo valor predictivo de la cantidad de ADNmt de las células del cúmulo sobre el futuro desarrollo del ovocito que rodean. Nuestros resultados concuerdan con estudios recientes que no observan correlación entre la cantidad de ADNmt de células del cúmulo humanas y la morfología embrionaria en día 3 o potencial de implantación (Liu *et al*., 2021), o estado de maduración del ovocito, probabilidad de fecundación, calidad embrionaria y tasas de gestación (Kumar *et al*., 2022).

La mayor parte de los análisis moleculares llevados a cabo en células del cúmulo para poder inferir la competencia del ovocito se han basado en la abundancia de transcritos. En el modelo bovino, se ha correlacionado el nivel de expresión de ciertos genes con distintos parámetros asociados indirectamente a la competencia del ovocito como el tamaño del folículo (Melo *et al*., 2017), el empleo de distintos medios de maduración *in vitro*, o su origen (*in vivo* vs. *in vitro* (Assidi *et al*., 2008), prepuberal vs. adulto (Bettegowda *et al*., 2008), recogidos antes vs. después del pico de LH (Assidi *et al*., 2010) o a distintos tiempos tras la administración de FSH (Bunel *et al*., 2014). Desafortunadamente, los genes identificados como potenciales marcadores de competencia ovocitaria no coinciden entre estudios, ni con un análisis de *microarray*

basado en competencia real para el desarrollo (Bunel *et al*., 2015), que tampoco ha podido ser replicado mediante qPCR (Kussano *et al*., 2016). De forma similar, los transcritos identificados como marcadores de competencia ovocitaria en células del cúmulo humanas, identificados mediante qPCR (Anderson *et al*., 2009; Artini *et al*., 2017; Gebhardt *et al*., 2011; Iager *et al*., 2013; Kordus *et al*., 2019; Krieg *et al*., 2018; Wathlet *et al*., 2011, 2012, 2013) o *microarray* (Assidi *et al*., 2015; Feuerstein *et al*., 2012; Hamel *et al*., 2010), también muestra poca coherencia entre estudios, y no han podido ser replicados por estudios independientes basados en qPCR (Burnik Papler, *et al*., 2015) o *microarray* (Burnik Papler, *et al*., 2015). La razón de esta discrepancia puede ser biológica, ya que el criterio para separar entre grupos experimentales se basa en muchos casos en una medida indirecta de la competencia del ovocito, pero también pueden existir razones técnicas, ya que tanto la técnica de *microarray* como, sobre todo, la técnica de qPCR están limitadas a un número de sondas (*microarray*) o genes candidatos (qPCR). En contraposición a estas limitaciones, la secuenciación masiva de ARN permite el análisis no sesgado de transcritos y proporciona una precisión mayor debido a su elevado rango dinámico, siendo por estas razones la técnica empleada en esta tesis.

El estudio transcriptómico de células del cúmulo bovinas identificó un número muy limitado de transcritos asociados a competencia del ovocito (49, 50 y 15 para las comparaciones Bl+ vs. Bl-, Bl+ vs. Cl- y Bl- vs. Cl- de un total de 19335 genes identificados), evidenciando que el transcriptoma de las células del cúmulo es muy similar entre complejos cúmulo-ovocito, independientemente de su capacidad para el desarrollo. Entre los genes diferencialmente expresados se identificaron dos integrinas (ITGA1 e ITGA5) cuyos transcritos eran menos abundantes en el grupo capaz de desarrollarse a blastocisto (Bl+) comparado con cualquiera de los otros grupos, es decir, cumpliendo los criterios estrictos del diseño de tres grupos escalonado descritos anteriormente. Las integrinas están implicadas en el control de la expansion de las células del cúmulo, aumentando su expresión en la fase de expansión (Kitasaka *et al*., 2018) y ovulación (Wissing *et al*., 2014). En esta línea, la menor expresión de integrinas en el grupo más competente podría deberse a que en este grupo la expansión de las células del cúmulo se alcance antes que en los menos competentes, no siendo necesaria la expresión de los genes implicados en la expansión a partir de ese momento. De acuerdo con esta posibilidad, la intensidad de la expansión de las células del cúmulo se ha relacionado con la capacidad para el desarrollo embrionario (Qian *et al*., 2003) y la expresión de *ITGA5* es más elevada

en células del cúmulo de macaco procedentes de complejos cúmulo-ovocito madurados *in vitro* comparado con los maduradas *in vivo* (Lee *et al*., 2011). De forma similar a *ITGA1* e *ITGA5*, el nivel de transcripción del gen codificante para la proteína plasmática A asociada a gestación (PAPPA) fue significativamente menor en el grupo más competente para el desarrollo. La proteína PAPPA aumenta su expresión durante la foliculogénesis bovina (Mazerbourg *et al*., 2001) y media la degradación de la proteína IGFBP-4 en los folículos preovulatorios para asegurar la dominancia folicular (Mazerbourg *et al*., 2001; Rivera & Fortune, 2001). El nivel de expresión más bajo en el grupo más competente podría indicar que han alcanzado un estadio de desarrollo más avanzado que los no competentes, en el que ya no es necesaria la expresión de *PAPPA*.

El nivel de los transcritos del gen codificante de una subunidad de la hemoglobina (HBE1) y de la proteína de anclaje a la kinasa A 12 (AKAP12) también fue menor en el grupo más competente. La expresión de subunidades de hemoglobina en embriones preimplantacionales ha sido descrita previamente y se ha sugerido que juegan un papel en la regulación del oxígeno celular (Lim *et al*., 2019). La correlación inversa entre la transcripción de *HBE1* y la competencia ovocitaria puede indicar una menor exposición al oxígeno en los complejos cúmulo-ovocito más competentes, dado que los niveles elevados de oxígeno y el estrés oxidativo reducen la competencia del ovocito (Bennemann *et al*., 2018; Bermejo-Álvarez *et al*., 2010). La sobre-expresión de *AKAP12* en las células del cúmulo de ovocitos incompetentes podría estar relacionada con una menor cantidad de cAMP en el ovocito –una molécula relacionada con competencia ovocitaria (Luciano *et al*., 1999)-, ya que la sobreexpresión de este gen se ha relacionado con el secuestro de unidades regulatorias de la proteína kinasa A que dan lugar a una reducción en los niveles de cAMP (Binder *et al*., 2013).

Otros dos genes, la proteína secretada relacionada con Frizzled 4 (SFRP4) y la subunidad beta 8 del proteasoma 20 S (PSMB8), mostraron un mayor nivel de expresión en células del cúmulo procedentes de ovocitos competentes. La proteína secretada relacionada con Frizzled 4 (SFRP4) inhibe la señalización WNT (Hernandez Gifford, 2015), que interviene en múltiples procesos durante la foliculogénesis. Su papel durante la foliculogénesis es desconocido ya que los estudios realizados para elucidarlo han dado lugar a resultados contradictorios. En ratones, su eliminación ha dado lugar a fenotipos reproductivos opuestos –aumento (Zamberlam *et al*., 2019) o disminución (Christov *et al*., 2011) del tamaño de camada- y mientras que su nivel de transcripción en células del

cúmulo humanas se ha correlacionado positivamente con progresión meiótica *in vivo* (Devjak *et al*., 2012), la cantidad de proteína en el fluido folicular humano correlaciona negativamente con progresión meiótica *in vitro* (Pla *et al*., 2021). Estas discrepancias pueden deberse a los distintos ambientes hormonales que regulan la maduración *in vivo* vs. *in vitro*, ya que la expresión de *SFRP4* está modulada por la hormona luteinizante (Maman *et al*., 2011). La similitud entre nuestros resultados y los obtenidos durante la maduración *in vitro* humana (Pla *et al*., 2021) sugieren que la regulación de *SFRP4* es similar en bovino y humano, al contrario a lo observado entre ratones y humanos (Maman *et al*., 2011), aunque –como se discutirá más adelante- *SFRP4* no es un buen marcador de competencia ovocitaria en complejos cúmulo-ovocito humanos madurados *in vivo*. PSMB8 interviene en la meiosis (Huo *et al*., 2004) y la expresión de su antisentido (*PSMB8-AS1*) se correlaciona positivamente con la edad de la mujer (Bouckenheimer *et al*., 2018), lo que concuerda con su posible papel de marcador positivo de competencia embrionaria. Por último, la expresión del mRNA codificante para s-transferasa de glutatión alfa 1 (GSTA1) también fue más elevada en el grupo más competente, aunque estos resultados no se pudieron corroborar mediante qPCR. *GSTA1* se ha identificado como un marcador positivo de competencia ovocitaria en estudios que compararon grupos de ovocitos con distinta competencia para el desarrollo, agrupados en base a la composición del medio de maduración (Assidi *et al*., 2008) o su origen: *in vivo* vs. *in vitro* (Salhab *et al*., 2013) o BCB+ vs. BCB- (Janowski *et al*., 2012).

El estudio transcriptómico de las células del cúmulo humanas procedentes de ovocitos con distinta competencia para el desarrollo pretendía identificar transcritos que permitiesen seleccionar el embrión con mayor probabilidad de gestación. La comparativa pertinente (P- vs. P+) identificó 10 genes diferencialmente expresados de 17469 genes detectados y entre ellos sólo la Epsina 3 (*EPN3*) también mostró diferencias significativas en la comparativa entre los grupos P+ y Bl-. El papel de este gen es desconocido, ya que su ablación en ratones no afecta a la reproducción (Ko *et al*., 2010), pero dado que interviene en la transición epitelio-mesénquima (Spradling *et al*., 2001), experimentada por las células del cúmulo durante la foliculogénesis (Mora *et al*., 2012), su menor expresión en el grupo más competente podría indicar un estadio de desarrollo folicular más avanzado en dicho grupo.

El análisis de transcritos relacionados con el desarrollo a blastocisto en células del cúmulo humanas no tiene una utilidad en la práctica clínica, ya que no aporta un valor adicional

a la observación directa del desarrollo embrionario, pero puede aportar información acerca de la base molecular de la competencia del ovocito. A diferencia de lo observado en el modelo bovino, no se identificó ningún gen que cumpliese con las estrictas condiciones del diseño experimental de tres grupos escalonados, es decir, que mostrase diferencias significativas en ambas comparativas (Bl- vs. P+ y Bl- vs. P-), aunque 5 genes mostraron diferencias significativas en una de las comparativas y tendencias coherentes en la otra. Entre ellos, la gamma-glutamylcyclotransferasa específica de glutatión 1 CHAC1 mostró un nivel de expresión mayor en la mayoría de las muestras de los grupos P+ o P- comparado con el grupo Bl-. CHAC1 interviene en la división del glutatión y es un regulador negativo de la señalización NOTCH (Chi *et al.*, 2012), que está implicada en el reclutamiento de células precursoras de la granulosa (Vanorny *et al.*, 2014). La sobreexpresión de *CHAC1* (inhibición de NOTCH) podría indicar un estado de desarrollo más avanzado en las células del cúmulo de los ovocitos capaces de alcanzar el estadio de blastocisto, que no necesitarían reclutar más células de la granulosa. De acuerdo con esta asociación positiva con la competencia para el desarrollo, se ha observado una redución en la expresión de *CHAC1* en células del cúmulo de vacas con metritis, una condición que afecta negativamente al desarrollo folicular (Piersanti *et al.*, 2019). Otros cuatro genes mostraron la tendencia opuesta (un mayor nivel de expresión en la mayoría de las muestras del grupo Bl- comparado con las muestras de los otros grupos): *CENPE*, *CD93*, *PECAM1* y *HSPA1A*. La sobreexpresión de transcritos de la proteína E específica de centrómero (*CENPE*) en el grupo Bl- podría indicar una mayor tasa de división celular probablemente asociada a estadios más inmaduros de desarrollo folicular, dado que la replicación celular no ocurre en ovocitos maduros. CD93 es un receptor de fagocitos y su sobreexpresión puede indicar un estado propenso a la fagocitosis en los complejos cúmulo-ovocito menos competentes, un proceso que parece estar implicado en la eliminación de ovocitos apoptóticos (Yefimova *et al.*, 2020). La molécula mediadora de adhesión celular plaqueta-endotelio (PECAM1) inhibe la apoptosis activando Akt y eNOS (Fleming *et al.*, 2005; Limaye *et al.*, 2016) y la expresión de su transcrito disminuye en folículos primordiales tras el tratamiento quimioterápico con ciclofosfamida (Titus *et al.*, 2021). Su sobreexpresión en el grupo Bl- podría indicar un menor grado de desarrollo folicular comparado con los grupos más competentes para el desarrollo, ya que en estadios tardíos de foliculogénesis los niveles elevados de estradiol pueden activar Akt (Quirk *et al.*, 2006), no siendo necesario por tanto la inhibición de la apoptosis mediada por PECAM1. La concentración de la proteína PECAM1 en el fluido

folicular no está correlacionada con probabilidad de gestación (Benifla *et al*., 2001), en concordancia con la ausencia de diferencias entre los grupos P+ y P- observada por nuestro estudio. Por último, la proteína de estrés térmico de la familia A (hsp10) 1A (HSPA1A) es una de las principales proteínas inducidas por calor y otros estímulos estresantes (Huang *et al*., 2001) y, por ello, su sobre-expresión en el grupo P- podría indicar una exposición previa a condiciones estresantes.

El menor número de transcritos relacionados con competencia para el desarrollo a blastocisto observado en el experimento con muestras humanas comparado con el experimento bovino puede deberse a varios factores. Como se ha descrito anteriormente, los experimentos con muestras bovinas y humanas parten de sistemas biológicos distintos (*in vitro* para las muestras bovinas vs. *in vivo* para las humanas), una diferencia que también podría estar detrás de la falta de coherencia entre los genes diferencialmente expresados entre ambos modelos. Además, los experimentos bovinos se realizan en condiciones experimentales controladas, eliminando el posible efecto de infertilidad o subfertilidad masculina mediante el empleo de semen de un único macho de fertilidad conocida.

Por último, se realizó un análisis metabolómico, capaz de identificar y cuantificar compuestos bioquímicos presentes en células del cúmulo de forma no dirigida. El análisis metabolómico proporciona una ruta alternativa e inexplorada para entender la base molecular de la competencia ovocitaria. En comparación con el análisis transcriptómico, la metabolómica proporciona una visión más finalista del funcionamiento celular, ya que integra posibles diferencias en niveles superiores (transcripción, traducción y actividad enzimática). El análisis metabolómico identificó 98 compuestos en células del cúmulo humanas, incluyendo 45 compuestos relacionados con lípidos, 30 relacionados con amino ácidos, compuestos relacionados con el ciclo de los ácidos tricarboxílicos, carbohidratos, fosfatos, nucleótidos, vitaminas y cofactores. Ninguno de estos compuestos mostró diferencias significativas en su abundancia en células del cúmulo que se ajustasen a la lógica del diseño experimental de tres grupos escalonados (no mostró diferencias significativas entre dos comparativas), sin embargo, la cantidad de 5 compuestos fue significativamente diferente en alguna comparativa. La cantidad de asparagina, prolina y el malonato fue más elevada en células del cúmulo del grupo Bl- comparado con P-, pero no se observaron diferencias significativas en la comparación Bl- vs. P+. Sin embargo, la cantidad de malonato fue más elevada en el grupo P+ comparado con el grupo P-, un

resultado que confunde su papel como predictor positivo o negativo de la competencia ovocitaria. El malonato inhibe a la enzima succinato deshidrogenasa (Potter & Pardee, 1948), que forma parte del ciclo de Krebs. La mayor cantidad de malonato detectada en el grupo P+ podría indicar una menor actividad del ciclo de Krebs que podría estar asociado a una preferencia por el metabolismo anaeróbico, beneficioso para la competencia del ovocito (Bermejo-Álvarez *et al*., 2010).

Otro de los cinco compuestos que mostró diferencias significativas en su abundancia fue la 5-oxiprolina, más abundante en el grupo P+ comparado con P-. Este compuesto es un precursor del glutamato, un aminoácido también detectado en el análisis que parece ejercer funciones de señalización en ovocitos y embriones preimplantacionales (Spirkova *et al*., 2022). El glutamato es también uno de los tres componentes del glutatión, el principal antioxidante no enzimático (Meister & Anderson, 1983). La cantidad de glutatión en el ovocito se ha correlacionado con su competencia (Perreault *et al*., 1988), y las ratonas incapaces de sintetizar glutatión (Glmc KO) muestran una reducción prematura de la fertilidad asociada a mortalidad embrionaria (Nakamura *et al*., 2015). Paradójicamente, un estudio observó que la cantidad de 5-oxiprolina en células del cúmulo era más elevada en mujeres con síndrome del ovario poliquístico, argumentando que el incremento podría deberse a una reducción en la capacidad de síntesis de glutatión (Turathum *et al*., 2022). Dado que el análisis no observó diferencias en la cantidad de glutamato o glutatión, el aumento en la cantidad de 5-oxiprolina en las células del cúmulo del grupo P+ podría indicar un mayor remanente disponible de este compuesto después del transporte de glutamato y/o glutatión al ovocito.

El eritronato fue el último de los cinco compuestos que mostró diferencias significativas en su cantidad, siendo significativamente más elevada en el grupo P+ comparado con el grupo Bl- y numéricamente más alta en el grupo P+ con respecto a P-. El eritronato es un compuesto formado por la oxidación de la N-acetil-D-glucosamina, un constituyente del ácido hialurónico. El ácido hialurónico juega un papel esencial en la expansión de las células del cúmulo (Lin Chen *et al*., 1990; Salustri *et al*., 1992), de modo que su mayor cantidad en el grupo más competente puede ser indicativo de una mayor o más temprana expansión de las células del cúmulo, que se ha asociado a calidad ovocitaria (Qian *et al*., 2003) y concuerda con las diferencias en la cantidad de transcritos de integrinas observadas en las células del cúmulo bovinas.

Además del estudio de potenciales marcadores de competencia ovocitaria, el análisis metabolómico permitió identificar dos compuestos no esperados en células del cúmulo: β-citrilglutamato y N-acetil-aspartil-glutamato. El β-citrilglutamato es un pseudodipéptido ausente en tejidos adultos salvo en testículo (Miyake *et al*., 1982), donde interviene en el intercambio entre histonas y protaminas (Wang-Eckhardt *et al*., 2022). El ratón nulo para la sintasa de β-citrilglutamato Rimklb muestra infertilidad o subfertilidad masculina (Maekura *et al*., 2021), pero no femenina, de modo que este compuesto podría no ser necesario para la ovogénesis. Sin embargo, también es posible que los complejos cúmulo-ovocito del ratón KO para Rimklb tengan β-citrilglutamato sintetizado por una enzima distinta a Rimklb. En este sentido, a pesar de las grandes diferencias en la remodelación de la cromatina entre la gametogénesis masculina y femenina, el β-citrilglutamato podría intervenir en la condensación de la cromatina durante la ovogénesis. El N-acetil-aspartil-glutamato (NAAG) es un dipéptido que actúa como neurotransmisor a través de la activación del receptor metabotrópico presináptico 3 (GRM3, también conocido como mGLuR3) (Wroblewska *et al*., 1997), expresado casi en exclusiva en cerebro y ausente en células del cúmulo humanas o bovinas según nuestros análisis transcriptómicos. El papel de NAAG en las células del cúmulo es incierto, pero podría mediar señalización a través de receptores de N-metil-D-aspartato (NMDA), como se ha observado en neuronas (Khacho *et al*., 2016), dada la expresión de múltiples receptores NMDA identificados por nuestro análisis transcriptómico (GRINA, GRIN2A, GRIN2C, GRIN2D y GRIN3B).

Perspectiva sobre la búsqueda de marcadores de selección de ovocitos

Los resultados generales de la tesis muestran una escasa correlación entre los distintos parámetros analizados en células del cúmulo y la probabilidad de gestación. La escasa correlación entre ambos parámetros puede deberse a múltiples factores, siendo los más relevantes la variabilidad asociada a factores individuales de donante y/o paciente y el hecho de que la receptividad uterina –un factor independiente de la calidad embrionaria u ovocitaria- juega un papel fundamental en la implantación (Craciunas *et al*., 2019). La selección de embriones basada en criterios morfológicos clásicos (Gardner & Balaban, 2016) continúa siendo el mejor parámetro predictor de la probabilidad de gestación y la búsqueda de parámetros que lo complementen continúa siendo un desafío que no han podido resolver nuestros análisis en células del cúmulo.

Como alternativa al análisis de células del cúmulo, los análisis del fluido folicular parten de una premisa similar a los de las células del cúmulo: el valor del parámetro analizado puede ser similar al del ovocito o estar relacionado con algún proceso que interviene en la calidad del ovocito. Como ocurre en el caso de las células del cúmulo, múltiples publicaciones han observado correlación entre distintos parámetros moleculares del fluido folicular y la competencia del ovocito, pero hay poca coherencia entre estudios actualmente tampoco se conoce ningún marcador fiable de competencia ovocitaria presente en el fluido folicular (Revelli *et al.*, 2009). Además, este material biológico presenta una serie de desventajas funcionales y logísticas con respecto a las células del cúmulo. A nivel funcional, el fluido folicular no está tan íntimamente relacionado con el ovocito como las células del cúmulo. A nivel logístico, la obtención de fluido folicular para la selección de ovocitos requiere la realización de punciones foliculares individuales (una por folículo), un procedimiento que aumenta el malestar de la donante y el riesgo de sangrado vaginal.

Los análisis moleculares en el ovocito permiten obtener una información directa no dependiente de la hipotética correlación entre el contenido del ovocito y el de las células del cúmulo o el fluido folicular. El principal problema de los análisis convencionales es que implican la destrucción del ovocito, pero se están desarrollando técnicas no invasivas que permitirían el análisis del ovocito manteniendo su viabilidad. En particular, se ha analizado la cantidad de determinados compuestos bioquímicos en ovocitos en base a su auto-fluorescencia mediante microscopía multi- o hiper-espectral (Campbell *et al.*, 2022; Gosnell *et al.*, 2016; Sutton-Mcdowall *et al.*, 2017) o FLIM (por sus siglas en inglés Fluorescence Lifetime Imaging Microscopy) (Sanchez *et al.*, 2019). Desafortunadamente, la inocuidad del método –el principal problema de otras aproximaciones probadas anteriormente como la microscopía Raman (Perevedentseva *et al.*, 2019)- y la eficacia como parámetro selectivo en la práctica real aún no han sido demostradas. En cualquier caso, la aplicación de éstas y otras técnicas biofísicas al estudio directo de la competencia ovocitaria constituye una aproximación novedosa y prometedora para ayudar a la selección de ovocitos y embriones.

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Conclusiones

CONCLUSIONES

1) La cantidad relativa de ADN mitocondrial en células del cúmulo bovinas es similar entre complejos cúmulo-ovocito con diferente competencia para el desarrollo *in vitro*, no estando correlacionada con la cantidad de ADN mitocondrial presente en el ovocito.

2) La cantidad relativa de ADN mitocondrial en células del cúmulo humanas es similar entre complejos cúmulo-ovocito con diferente competencia para el desarrollo *in vitro* y post-transferencia. Tampoco se encontró relación entre la cantidad relativa de ADN mitocondrial en células del cúmulo y la edad, índice de masa corporal o consumo de tabaco de la donante.

3) El transcriptoma de las células del cúmulo bovinas muestra un número muy limitado de genes cuya expresión correlaciona con la competencia del ovocito para el desarrollo *in vitro* hasta el estadio de blastocisto, siendo esta correlación positiva para los genes GSTA1, PSMB8, FMOD y SFRP4 y negativa para HBE1, ITGA1, PAPPA, AKAP12, ITGA5 y SLC2A4.

4) El transcriptoma de las células del cúmulo humanas está muy poco relacionado con la capacidad para el desarrollo *in vitro* y post-transferencia del ovocito. El gen EPN3 fue el único que mostró una variación de expresión compatible con un marcador negativo de competencia para el desarrollo post-transferencia, estando sobre-expresado en las células del cúmulo de ovocitos incapaces de completar el desarrollo *in vitro* o de generar una gestación comparado con las de ovocitos capaces de generar gestación.

5) La cantidad de 98 compuestos bioquímicos detectados en células del cúmulo humanas no está claramente relacionada con la competencia para el desarrollo *in vitro* o post-transferencia, aunque se observó una correlación positiva entre la cantidad de eritronato, malonato y 5-oxiprolina y probabilidad de gestación. El análisis identificó la presencia de β-citrilglutamato y N-acetil-aspartil-glutamato en células del cúmulo humanas.

Abreviaciones

ADNmt ADN mitocondrial

AKAP12 Proteína de anclaje a kinasa 12

Akt Proteína kinasa B

AMP Adenosina monofosfato

ARNm ARN mensajero

BCB Blue Creasly Brilliant

cAMP Adenosin monofosfato cíclico

CD93 *Cluster* de diferenciación 93

CENPE Proteína E específica del centrómero

CHAC1 *ChaC glutathione specific gamma-glutamylcyclotransferase 1*

DGP Diagnóstico genético preimplantacional

eCG Gonadotropina coriónica equina

eNOS Endotelial óxido nítrico sintasa

EPN3 Epsina 3

FIV Fecundación *in vitro*

FLIM *Fluorescence Lifetime Imaging Microscopy*

FSH Hormona folículo estimulante

GlmcKO Ratonas incapaces de sintetizar glutatión

GnRH Hormona liberadora de gonadotropinas

GRM3 Receptor metabotrópico presináptico 3

GRINA *Glutamate receptor, ionotropic, Nmethyl Daspartateassociated protein 1*

GRIN2A *Glutamate ionotropic receptor NMDA type subunit 2A*

GRIN2C *Glutamate ionotropic receptor NMDA type subunit 2C*

GRIN3B *Glutamate ionotropic receptor NMDA type subunit 3B*

GSTA1	s-transferasa de glutatión alfa 1
HBE1	Subunidad de la hemoglobina 1
hCG	Gonadotropina coriónica humana
HSPA1A	Proteína de estrés térmico de la familia A (hsp10) 1A
IA	Inseminación artificial
ICSI	Inyección intracitoplásmica del espermatozoide
IMC	Índice de masa corporal
IGFBP-4	Proteína de unión al crecimiento similar a la insulina 4
ITGA1	Integrina alfa 1
ITGA5	Integrina alfa 5
LH	Hormona Luteinizante
MOET	Multiple ovulation and embryo transfer
NAAG	N-metil-D-aspartato
NMDA	N-metil-D-aspartato
OPU	Ovum pick-up
PAPPA	Proteína plasmática A asociada a gestación
PCR	Reacción en cadena de la polimerasa
PECAM1	Molécula mediadora de adhesión celular plaqueta-endotelio 1
PEIV	Producción de embriones *in vitro*
PGF2α	Prostaglandina F2α
PSMB8	Subunidad beta 8 del proteasoma 20 S
qPCR	PCR cuantitativa a tiempo real
SFRP4	Proteína secretada relacionada con Frizzled 4
TE	Transferencia embrionaria

TESA Aspiración espermática testicular

TESE Extracción espermática testicular

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