

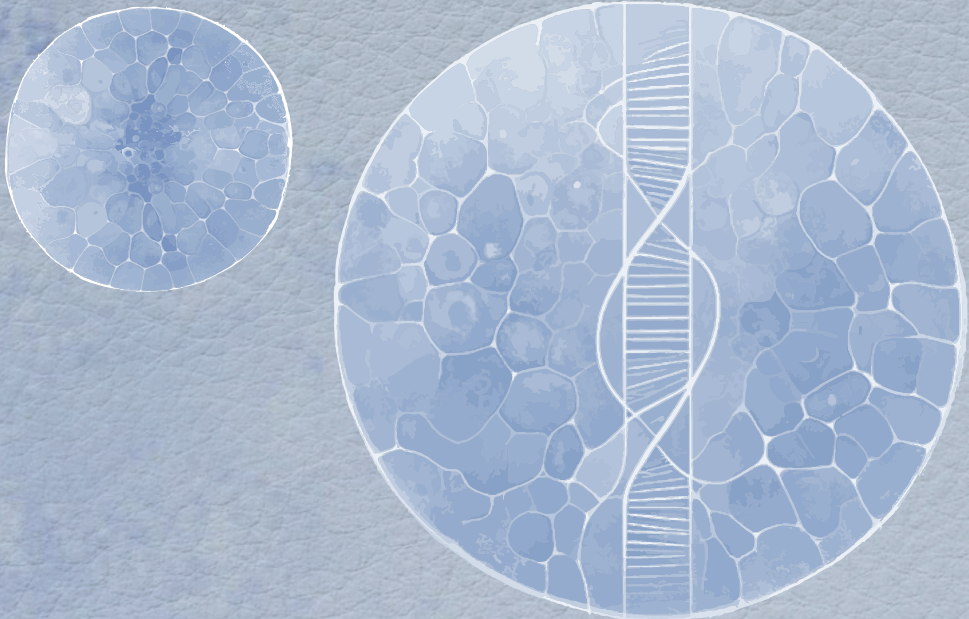
VNIVERSITAT  VALÈNCIA

Facultad de Medicina y Odontología
Departamento de Obstetricia, Ginecología y Medicina Regenerativa
Programa de Doctorado en Medicina (3139)

ESTUDIO DEL MOSAICISMO GENÉTICO PREEMBRIÓNARIO E IMPACTO EN LA APOPTOSIS Y EN LAS VARIABLES MORFOCINÉTICAS

TESIS DOCTORAL

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Dirigida por

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Prof. José Alejandro Remohí Giménez

Valencia, marzo 2024



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**Estudio del mosaicismo genético preembrionario
e impacto en la apoptosis
y en las variables morfocinéticas**

Autor

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Trabajo presentado para optar al grado de

Doctor por la Universidad de Valencia

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CERTIFICAN:

Que el presente trabajo de investigación, titulado: **“Estudio del mosaicismo genético preembrionario e impacto en la apoptosis y en las variables morfocinéticas”** ha sido realizado íntegramente por Ángel Martín Bastida bajo su dirección. Dicha memoria está concluida y reúne todos los requisitos para su presentación y defensa como TESIS DOCTORAL ante un tribunal.

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"La perfección es una pulida colección de errores".

Mario Benedetti

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GLOSARIO DE ABREVIATURAS

GLOSARIO DE ABREVIATURAS

ADN	Ácido desoxirribonucleico
ARN	Ácido ribonucleico
ASEBIR	Asociación para el Estudio de la Biología de la Reproducción
ASRM	Sociedad Americana de Medicina Reproductiva, del inglés <i>American Society for Reproductive Medicine</i>
BAF	Frecuencia de alelos B, del inglés <i>b-allele frequency</i>
BRCA	del inglés <i>Breast cancer</i>
cc1	Duración del primer ciclo celular blastomérico
cc2a	Duración del segundo ciclo celular de la primera blastómera en dividirse
cc2b	Duración del segundo ciclo celular de la segunda blastómera en dividirse
cc2m	cc2 promedio
cc3a	Duración del tercer ciclo celular de la primera blastómera en dividirse
cc3b	Duración del tercer ciclo celular de la segunda blastómera en dividirse
cc3c	Duración del tercer ciclo celular de la tercera blastómera en dividirse
cc3d	Duración del tercer ciclo celular de la cuarta blastómera en dividirse
cc3m	cc3 promedio
CCN	Número de copias cromosómicas, del inglés <i>chromosomal copy number</i>
CCS	Screening cromosómico completo, del inglés <i>comprehensive chromosome screening</i>
cfDNA	ADN libre de células, del inglés <i>cell-free DNA</i>
CGH	Hibridación genómica comparativa, del inglés <i>comparative genomic hybridization</i>
CMA	Microarrays cromosómicos, del inglés <i>chromosomal microarray analysis</i>

GLOSARIO DE ABREVIATURAS

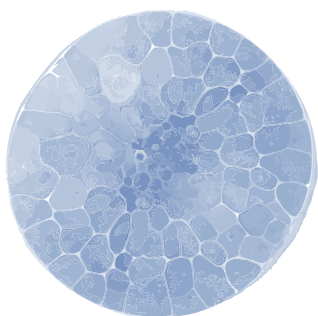
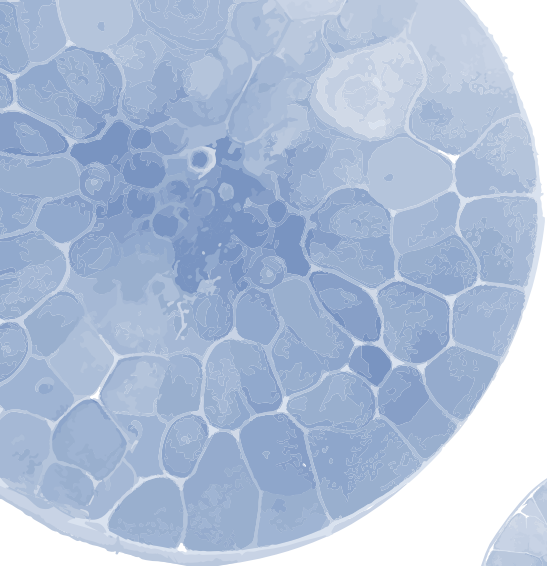
COGEN	Congreso sobre Controversias en Preconcepción, Preimplantación y Diagnóstico Genético Prenatal, del inglés <i>Congress on Controversies in Preconception, Preimplantation and Prenatal Genetic Diagnosis</i>
CVS	Biopsia de vellosidades coriónicas, del inglés <i>chorionic villus sampling</i>
D+1	Primer día del desarrollo embrionario
D+2	Segundo día del desarrollo embrionario
D+3	Tercer día del desarrollo embrionario
D+4	Cuarto día del desarrollo embrionario
D+5	Quinto día del desarrollo embrionario
D+6	Sexto día del desarrollo embrionario
D+7	Séptimo día del desarrollo embrionario
DAPI	del inglés <i>4',6-diamidino-2-phenylindole</i>
DEGs	Genes diferencialmente expresados, del inglés <i>differentially expressed genes</i>
dpf	Días post-fecundación
DSBs	Roturas de doble cadena, del inglés <i>double-strand breaks</i>
ECC1	Duración del primer ciclo celular embrionario
ECC2	Duración del segundo ciclo celular embrionario
ECC3	Duración del tercer ciclo celular embrionario
ESHRE	Sociedad Europea de Reproducción Humana y Embriología, del inglés <i>European Society of Human Reproduction and Embryology</i>
FISH	Hibridación in situ fluorescente, del inglés <i>fluorescence in situ hybridization</i>
FIV	Fecundación in vitro
HLA	Antígeno leucocitario humano, del inglés <i>human leukocyte antigen</i>
hpf	Horas post-fecundación
hpi	Horas post-inseminación
IA	Inteligencia Artificial

GLOSARIO DE ABREVIATURAS

ICCs	Coeficientes de correlación intraclase, del inglés <i>intraclass correlation coefficients</i>
ICCm	ICC promedio
ICSI	Inyección intracitoplasmática de espermatozoides, del inglés <i>intracytoplasmic sperm injection</i>
KEGG	Enciclopedia Kyoto de Genes y Genomas, del inglés <i>Kyoto Encyclopedia of Genes and Genomes</i>
MAPD	Desviación media porcentual absoluta, del inglés <i>mean absolute percentage deviation</i>
MCI	Masa celular interna
miPGT-A	PGT-A mínimamente invasivo, del inglés <i>minimally-invasive PGT-A</i>
NGS	Secuenciación de nueva generación, del inglés <i>next-generation sequencing</i>
niPGT-A	PGT-A no invasivo, del inglés <i>non-invasive PGT-A</i>
NS	Estadísticamente no significativo, del inglés <i>not statistically significant</i>
PCR	Reacción en cadena de la polimerasa, del inglés <i>polymerase chain reaction</i>
PGDIS	Sociedad Internacional de Diagnóstico Genético Preimplantacional, del inglés <i>Preimplantation Genetic Diagnosis International Society</i>
PGT	Test Genético Preimplantacional, del inglés <i>Preimplantation genetic testing</i>
PGT-A	PGT para la detección de aneuploidías, del inglés <i>PGT for aneuploidy</i>
PGT-M	PGT para la detección de enfermedades monogénicas, del inglés <i>PGT for monogenic disorders</i>
PGT-SR	PGT para la detección de alteraciones estructurales, del inglés <i>PGT for structural rearrangements</i>
POCs	Restos abortivos, del inglés <i>products of conception</i>
qPCR	PCR cuantitativa, del inglés <i>quantitative PCR</i>
RHA	Reproducción humana asistida

GLOSARIO DE ABREVIATURAS

RNA-seq	Secuenciación de ARN, del inglés <i>RNA sequencing</i>
s2	Sincronía del segundo ciclo celular embrionario
SART	Sociedad de Tecnología de Reproducción Asistida, del inglés <i>Society for Assisted Reproductive Technology</i>
SNPs	Polimorfismos de nucleótido único, del inglés <i>single nucleotide polymorphisms</i>
t2	Tiempo de división a 2 células
t3	Tiempo de división a 3 células
t4	Tiempo de división a 4 células
t5	Tiempo de división a 5 células
t6	Tiempo de división a 6 células
t7	Tiempo de división a 7 células
t8	Tiempo de división a 8 células
t9	Tiempo de división a 9 células
tB	Tiempo de formación del blastocisto
tDC	Duración de la compactación
TE	Trofoectodermo
tM	Tiempo de formación de la mórula
tPB2	Tiempo de extrusión del segundo corpúsculo polar
tPNa	Tiempo de aparición de los pronúcleos
tPNf	Tiempo de desaparición de los pronúcleos
TRA	Técnicas de reproducción asistida
tSB	Tiempo de inicio de la blastulación
tSC	Tiempo de inicio de la compactación
TUNEL	del inglés <i>Terminal deoxynucleotidyl transferase dUTP nick end labeling</i>
μm	Micrómetros



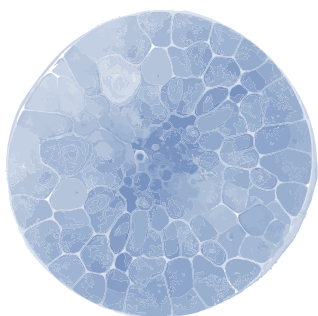
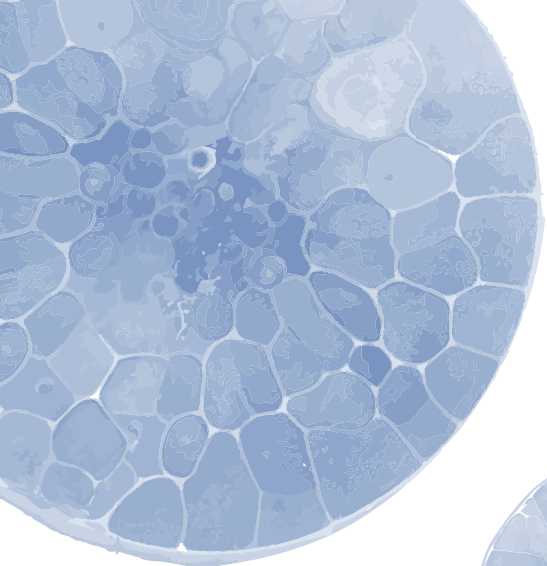
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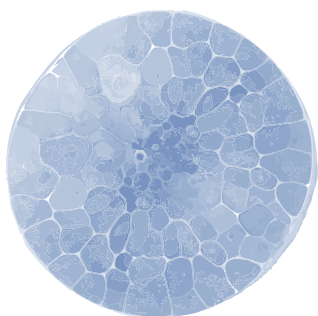
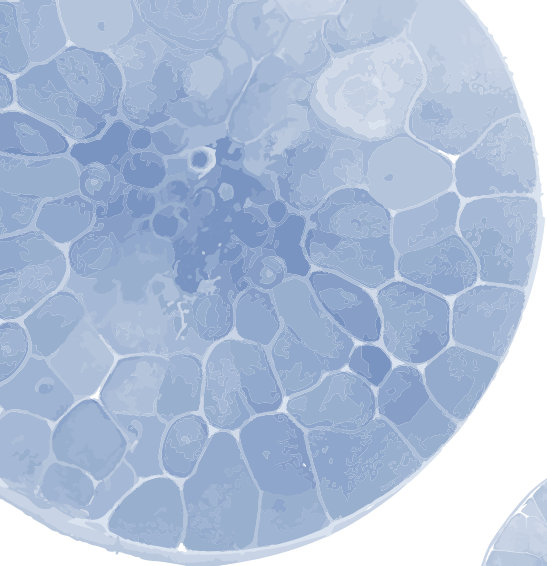
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RESUMEN

RESUMEN

El mosaicismo embrionario es la presencia de líneas celulares con cariotipos diferentes en un mismo embrión, y surge como consecuencia de errores mitóticos durante las divisiones celulares post-cigóticas. En los últimos años, el diagnóstico de mosaicismo en el Test Genético Preimplantacional para la detección de aneuploidías (PGT-A) se ha basado principalmente en la identificación de valores intermedios en el número de copias cromosómicas en los perfiles de secuenciación de nueva generación (NGS). Los embriones diagnosticados como mosaicos de células euploides y aneuploides han demostrado tener la capacidad de dar lugar a bebés sanos. Sin embargo, las razones de su éxito reproductivo se desconocen.

Por un lado, se ha propuesto que los embriones mosaicos poseen cierta capacidad intrínseca para autocorregirse, lo cual podría ocurrir a través de la apoptosis selectiva y la reducción de la proliferación de las células aneuploides. No obstante, actualmente no existe evidencia directa de estos mecanismos en embriones humanos. Por otro lado, la interpretación de los datos procedentes de los estudios que involucran la transferencia de embriones mosaicos se ha visto sesgada por el uso de métodos de análisis retrospectivos, la adopción de estrategias de priorización de embriones euploides y una alta incidencia de diagnósticos falsos positivos derivados del empleo de plataformas de PGT-A poco específicas.

En este contexto, el objetivo principal de la presente tesis doctoral es generar nuevos conocimientos en el campo de la embriología clínica sobre la dinámica de desarrollo y las características moleculares de los embriones diagnosticados como mosaicos tras PGT-A, con el fin de comprender las causas que determinan su potencial reproductivo. Para ello, se plantearon los siguientes objetivos específicos:

1) Determinar la incidencia, tipología y distribución cromosómica del mosaicismo en el estadio de blastocisto; **2)** Evaluar el comportamiento morfocinético y las características morfológicas de los blastocistos mosaicos; **3)** Caracterizar el perfil de expresión génica de los blastocistos mosaicos; y **4)** Examinar la incidencia y colocalización de eventos apoptóticos en los blastocistos mosaicos.

La hipótesis formulada propone que los blastocistos diagnosticados como mosaicos de células euploides y aneuploides constituyen una población minoritaria de embriones en el programa de PGT-A que presenta características específicas en términos morfológicos, cromosómicos, morfocinéticos y de expresión génica, en comparación con los blastocistos uniformemente euploides y aneuploides.

Este trabajo de investigación de tesis doctoral se estructura como un compendio de tres publicaciones que abordan los objetivos propuestos:

I. The morphokinetic signature of mosaic embryos: evidence in support of their own genetic identity. Martín Á, Rodrigo L, Beltrán D, Meseguer M, Rubio C, Mercader A, de Los Santos MJ. Fertil Steril. 2021 Jul;116(1):165-173. doi: 10.1016/j.fertnstert.2020.12.031. Epub 2021 Mar 22. PMID: 33766460. 5-year impact factor: 7.5.

En este artículo, se describe la incidencia, tipología y distribución cromosómica del mosaicismo embrionario en el estadio de blastocisto, y se utiliza la tecnología *time-lapse* para describir sus implicaciones morfocinéticas. Los resultados obtenidos demuestran que los blastocistos diagnosticados como mosaicos mediante NGS constituyen una población minoritaria de embriones que presenta características morfológicas y cromosómicas específicas, así como perfiles morfocinéticos solapantes con respecto a los blastocistos euploides y aneuploides.

RESUMEN

II. Mosaic results after preimplantation genetic testing for aneuploidy may be accompanied by changes in global gene expression. Martin A, Mercader A, Dominguez F, Quiñonero A, Perez M, Gonzalez-Martin R, Delgado A, Mifsud A, Pellicer A, De Los Santos MJ. Front Mol Biosci. 2023 Apr 14;10:1180689. doi: 10.3389/fmolb.2023.1180689. PMID: 37122560. 5-year impact factor: 5.2.

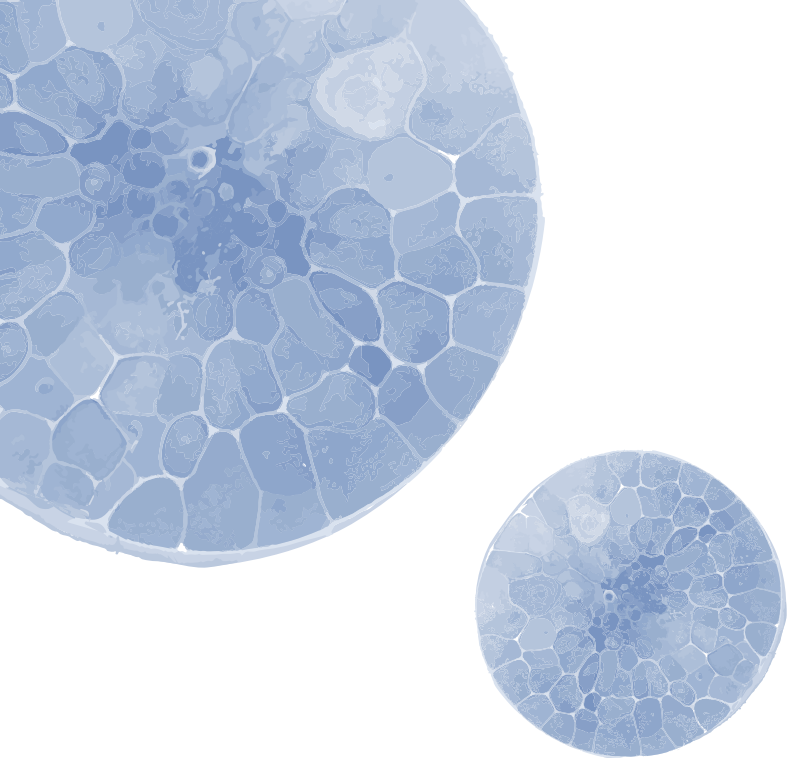
En este artículo, se emplea la tecnología de secuenciación de ARN para evaluar el impacto de diferentes grados de mosaicismo embrionario en el perfil de expresión génica de las células de la masa celular interna (MCI) y el trofoectodermo (TE) del blastocisto humano. Los resultados obtenidos demuestran que, cuando el diagnóstico de mosaicismo mediante NGS se apoya en el análisis de la expresión génica y en la identificación de retrasos en la duración de los ciclos celulares mediante el seguimiento individualizado de las divisiones blastoméricas, el grado de mosaicismo se asocia a una desregulación progresiva del transcriptoma embrionario con respecto a los perfiles euploides.

III. Trophectoderm cells of human mosaic embryos display increased apoptotic levels and impaired differentiation capacity: a molecular clue regarding their reproductive fate? Martín Á, Mercader A, Beltrán D, Mifsud A, Nohales M, Pardiñas ML, Ortega-Jaén D, de Los Santos MJ. Hum Reprod. 2024 Feb 2;deae009. doi: 10.1093/humrep/deae009. PMID: 38308811. 5-year impact factor: 7.1.

En este artículo, se realiza inmunofluorescencia y microscopía confocal para analizar la incidencia de eventos apoptóticos en los blastocistos mosaicos durante la segregación de los linajes celulares de MCI y TE. Los resultados obtenidos demuestran que los blastocistos diagnosticados como mosaicos mediante NGS y genotipado de polimorfismos de nucleótido único, en comparación con los blastocistos euploides, presentan mayores

niveles de apoptosis y una capacidad de diferenciación alterada en las células del TE, y que la magnitud de ambos efectos se correlaciona con el número y la tipología de los cromosomas aneuploides.

En conjunto, los resultados obtenidos en la presente tesis doctoral sugieren la existencia de mecanismos que conducen a la depleción clonal de las células aneuploides en los blastocistos mosaicos. El conocimiento generado a partir de esta investigación enriquece nuestra comprensión sobre la naturaleza biológica de los blastocistos mosaicos y ofrece nuevas perspectivas respecto a los factores moleculares que determinan su potencial reproductivo.



SUMMARY

SUMMARY

SUMMARY

Embryonic mosaicism is the presence of cell lines with different karyotypes in the same embryo, and it arises as a consequence of mitotic errors during post-zygotic cell divisions. In recent years, the diagnosis of mosaicism after Preimplantation Genetic Testing for Aneuploidy (PGT-A) has been primarily based on the identification of intermediate chromosomal copy number values in next-generation sequencing (NGS) profiles. Embryos diagnosed as mosaics of euploid and aneuploid cells have shown the capacity to give rise to healthy babies. However, understanding the reasons behind their reproductive potential requires further investigation.

On the one hand, it has been proposed that mosaic embryos possess some intrinsic capacity for self-correction, potentially through selective apoptosis and reduced proliferation of aneuploid cells. However, there is currently no direct evidence of these mechanisms in human embryos. On the other hand, the interpretation of data from studies involving mosaic embryo transfers has been hampered by the use of retrospective analysis methods, the adoption of euploid embryo prioritization strategies, and the high incidence of false-positive mosaic diagnoses stemming from the use of poorly specific PGT-A platforms.

In this context, the main objective of this doctoral thesis is to generate new knowledge in the field of clinical embryology on the developmental dynamics and molecular characteristics of embryos diagnosed as mosaic after PGT-A, aiming to elucidate the factors that influence their reproductive potential. To this end, the following specific objectives were proposed:

1) To determine the incidence, typology, and chromosomal distribution of mosaicism at the blastocyst stage; **2)** To evaluate the morphokinetic behavior and morphological characteristics of mosaic blastocysts; **3)** To

characterize the gene expression profile of mosaic blastocysts; and **4)** To examine the incidence and colocalization of apoptotic events in mosaic blastocysts.

The formulated hypothesis proposes that blastocysts diagnosed as mosaics of euploid and aneuploid cells constitute a minority population of embryos within the PGT-A program that exhibits specific characteristics in terms of morphology, chromosomal composition, morphokinetics, and gene expression when compared to uniformly euploid and aneuploid blastocysts.

This doctoral thesis is structured as a compendium of three publications that address the proposed objectives:

I. The morphokinetic signature of mosaic embryos: evidence in support of their own genetic identity. Martín Á, Rodrigo L, Beltrán D, Meseguer M, Rubio C, Mercader A, de Los Santos MJ. Fertil Steril. 2021 Jul;116(1):165-173. doi: 10.1016/j.fertnstert.2020.12.031. Epub 2021 Mar 22. PMID: 33766460. 5-year impact factor: 7.5.

In this article, the incidence, typology, and chromosomal distribution of embryonic mosaicism at the blastocyst stage are described, and time-lapse imaging technology is used to discuss its morphokinetic implications. The findings demonstrate that blastocysts diagnosed as mosaic through NGS constitute a minority population that exhibit specific morphological and chromosomal characteristics, as well as overlapping morphokinetic profiles with respect to their euploid and aneuploid counterparts.

II. Mosaic results after preimplantation genetic testing for aneuploidy may be accompanied by changes in global gene expression. Martin A, Mercader A, Dominguez F, Quiñonero A, Perez M, Gonzalez-Martin R, Delgado A, Mifsud A, Pellicer A, De Los Santos MJ. Front Mol Biosci. 2023

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Apr 14;10:1180689. doi: 10.3389/fmolb.2023.1180689. PMID: 37122560.
5-year impact factor: 5.2.

In this article, RNA sequencing technology is employed to assess the impact of different degrees of embryonic mosaicism on the gene expression profile of inner cell mass (ICM) and trophectoderm (TE) cells of the human blastocyst. The findings demonstrate that, when the diagnosis of mosaicism through NGS is supported by gene expression analysis and the identification of delays in cell cycle duration through individualized tracking of blastomere divisions, the degree of mosaicism is associated with a progressive deregulation of the embryonic transcriptome in comparison to the euploid profiles.

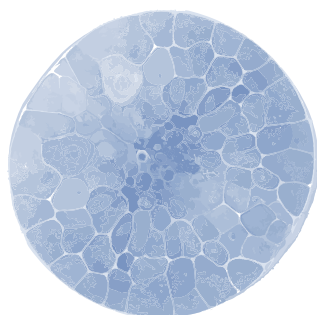
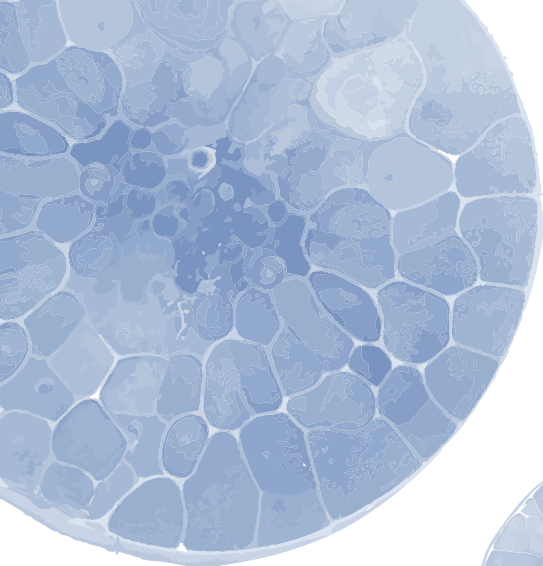
III. Trophectoderm cells of human mosaic embryos display increased apoptotic levels and impaired differentiation capacity: a molecular clue regarding their reproductive fate? Martín Á, Mercader A, Beltrán D, Mifsud A, Nohales M, Pardiñas ML, Ortega-Jaén D, de Los Santos MJ. Hum Reprod. 2024 Feb 2;deae009. doi: 10.1093/humrep/deae009. Epub ahead of print. PMID: 38308811. 5-year impact factor: 7.1.

In this article, immunofluorescence and confocal microscopy are used to analyze the incidence of apoptotic events in mosaic blastocysts during the segregation of ICM and TE cell lineages. The findings demonstrate that blastocysts diagnosed as mosaic through NGS and single nucleotide polymorphism genotyping, compared to euploid blastocysts, show higher levels of apoptosis and an altered differentiation capacity in TE cells, and that the magnitude of both effects correlates with the number and typology of aneuploid chromosomes.

Taken together, the results obtained in this doctoral thesis support the existence of mechanisms leading to the clonal depletion of aneuploid cells in mosaic blastocysts. The knowledge generated from this research improves

SUMMARY

our understanding of the biological nature of mosaic blastocysts and offers new insights regarding the molecular factors that determine their reproductive potential.



COMPENDIO DE ARTÍCULOS QUE AVALAN LA TESIS

Compendio de artículos que avalan la tesis

COMPENDIO DE ARTÍCULOS QUE AVALAN LA TESIS

La presente tesis doctoral se basa en tres publicaciones en forma de compendio en las cuales el candidato es el primer autor. El texto completo de las publicaciones se muestra a continuación.

Martín Á, Rodrigo L, Beltrán D, Meseguer M, Rubio C, Mercader A, de Los Santos MJ. The morphokinetic signature of mosaic embryos: evidence in support of their own genetic identity. *Fertil Steril*. 2021 Jul;116(1):165-173. doi: 10.1016/j.fertnstert.2020.12.031. PMID: 33766460. 5-year impact factor: 7.5.

Martín A, Mercader A, Dominguez F, Quiñonero A, Perez M, Gonzalez-Martin R, Delgado A, Mifsud A, Pellicer A, De Los Santos MJ. Mosaic results after preimplantation genetic testing for aneuploidy may be accompanied by changes in global gene expression. *Front Mol Biosci*. 2023 Apr 14;10:1180689. doi: 10.3389/fmolb.2023.1180689. PMID: 37122560. 5-year impact factor: 5.2.

Martín Á, Mercader A, Beltrán D, Mifsud A, Nohales M, Pardiñas ML, Ortega-Jaén D, de Los Santos MJ. Trophectoderm cells of human mosaic embryos display increased apoptotic levels and impaired differentiation capacity: a molecular clue regarding their reproductive fate? *Hum Reprod*. 2024 Feb 2;deae009. doi: 10.1093/humrep/deae009. PMID: 38308811. 5-year impact factor: 7.1.

The morphokinetic signature of mosaic embryos: evidence in support of their own genetic identity.

Martín Á, Rodrigo L, Beltrán D, Meseguer M, Rubio C, Mercader A, de Los Santos MJ.

Fertil Steril. 2021 Jul;116(1):165-173. doi: 10.1016/j.fertnstert.2020.12.031. PMID: 33766460.

Factor de impacto (2022): 6,7.

Factor de impacto 5 años: 7,5.



The morphokinetic signature of mosaic embryos: evidence in support of their own genetic identity

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Objective: To provide full morphokinetic characterization of embryos ranked with different degrees of chromosomal mosaicism.

Design: Retrospective cohort study.

Setting: University-affiliated private in vitro fertilization clinic.

Patient(s): We analyzed 1,511 embryos from 424 intracytoplasmic sperm injection cycles by culturing embryos in a time-lapse imaging system and performing next-generation sequencing. We assessed 106 mosaic embryos.

Intervention(s): None.

Main Outcome Measure(s): Comparison of chromosomal, morphological, and morphokinetic characteristics of blastocysts classified as euploid, aneuploid, low-degree mosaic (30% to <50% aneuploid cells in trophectoderm biopsy), and high-degree mosaic (50% to <70% aneuploid cells in trophectoderm biopsy). Statistical analysis was performed using χ^2 , Kruskal-Wallis, or analysis of variance tests according to data type and distribution. A two-way random effects model was used to calculate interoperator correlation of annotations, and a logistic mixed effects model was performed to evaluate the effect of confounders on morphokinetic timing.

Result(s): The mosaicism rate was ~7% regardless of parental age. Mosaicism and uniform aneuploidies were not evenly distributed across chromosomes. The percentage of high-quality blastocysts significantly decreased from euploid (66.9%) to mosaic (52.8%) and aneuploid (47.7%). Aneuploid blastocysts significantly delayed development compared with euploid blastocysts in start of compaction (median, 84.72 hours postmicroinjection [hpm], interquartile range [IQR], 13.2; vs. median, 82.10 hpm, IQR, 11.5), start of blastulation (median, 101 hpm, IQR, 11.7; vs. median, 98.29 hpm, IQR, 10.5), and timing of blastocyst (median, 108.04 hpm, IQR, 11.50; vs. median, 104.71 hpm, IQR, 11.35). However, embryo morphokinetics were not correlated to the degree of mosaicism or to a mosaicism configuration that was apt for embryo transfer.

Conclusion(s): Morphokinetic timing of mosaic embryos overlaps with that of euploid and aneuploid embryos, which may reflect their unique genetic and developmental identity. Although this suggests mosaic embryos are not simply a misdiagnosis by-product, further studies are needed to reveal the true identity of this particular type of embryo. (Fertil Steril® 2021;116:165–73. ©2020 by American Society for Reproductive Medicine.)

El resumen está disponible en Español al final del artículo.

Keywords: Mosaicism, morphokinetics, preimplantation genetic testing for aneuploidy, next-generation sequencing

Discuss: You can discuss this article with its authors and other readers at <https://www.fertstertdialog.com/posts/30819>

Aneuploidy in preimplantation embryos is a major cause of infertility and in vitro fertilization (IVF) failure (1). Aneuploid cells originating from meiotic errors are widespread in the embryo, while those

of mitotic origin coexist with euploid cells and lead to mosaicism (2).

Meiotic aneuploidies have been associated with age-related decline of maternal fertility (3). Preimplantation genetic testing for aneuploidy (PGT-A)

by next-generation sequencing (NGS) is currently used to infer the chromosomal status of the embryo from a trophectoderm (TE) fraction, which is supported by disaggregation studies showing high karyotype concordance (>95%) for uniform whole chromosome aneuploidies between the TE and inner cell mass (ICM) (4). Although the clinical usefulness of PGT-A has been discussed at length (5), several studies suggest that transfer of embryos with euploid TE biopsies can significantly increase pregnancy and live birth rates per patient and reduce the risk of miscarriage and multiple pregnancy as well as the time to pregnancy (6–8).

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ORIGINAL ARTICLE: GENETICS

Unlike uniform aneuploidy, the impact of embryonic mosaicism on human fecundity remains unclear (9). Development of new NGS protocols has increased the sensitivity of mosaicism detection, providing more accurate quantification of copy number changes present in TE samples (10, 11). Consequently, several clinics have reported healthy live births after transfer of blastocysts diagnosed as mosaics. However, these studies suggest that mosaic blastocysts implant less and miscarry more than their euploid counterparts (12). Due to limitations surrounding diagnosis of mosaicism (12, 13), it is unknown to what extent the outcomes from replacing putative mosaic embryos are due to technical artefacts or to their inherent developmental potential.

Use of time-lapse imaging (TLI) in IVF practice has favored the emergence of studies that combine PGT-A and morphokinetics to find significant kinetic markers or to build algorithms predicting ploidy (14). These algorithms can be used to help identify embryos at increased risk of harboring complex chromosomal abnormalities (15), which may be of special interest when PGT-A is not feasible due to biological, technical, social, or legal-ethical constraints.

Mitotic aneuploidies have been associated with morphokinetic alterations in the timing of several cell cycle parameters, such as the interval from the first to second mitosis (t3–t2) and synchrony between the third and fourth cell appearance (t4–t3) (16). More recently, morphokinetic delays have been reported in NGS-tested high-degree mosaic embryos (17). Most mitotic errors causing mosaicism arise from chromosome nondisjunction or anaphase lag during early cleavage (2), which suggests that kinetic measurements and algorithms could be used to detect mosaicism based on the pace of embryo development.

Here we used TLI to describe the morphokinetic implications of clinical mosaicism detected at the blastocyst stage. Identification of parameters associated with mosaicism may shed new light on the accuracy of a mosaicism diagnosis and may confirm the effects of mitotic aneuploidy on embryo development dynamics.

MATERIAL AND METHODS

Study Design and Population

The study was approved by the National Commission of Human Reproduction, the general director of research, innovation, technology, and quality, and by our institutional review board, the ethics committee of Clinical Research IVI RMA Valencia, which complies with Spanish law on assisted reproductive technologies (14/2006) and biomedical research (14/2007).

This retrospective cohort study included 424 consecutive intracytoplasmic sperm injection (ICSI) cycles conducted from April 2018 to April 2019 at IVI RMA Valencia. Embryos ($n = 1,511$) were monitored from the zygote to blastocyst stage by means of TLI. Blastocysts were analyzed by NGS after a single TE biopsy, and embryo development was assessed.

Egg donation cycles represented 9.2% of the total (39/424). Main clinical indications for PGT-A included female age (≥ 35 years; 52.1% = 221/424), implantation failure (11.8% = 50/424), and male factor (8.7% = 37/424). Multiple

indications accounted for 17.7% of cycles (75/424). The mean age and body mass index (BMI) of patients providing oocytes were 36.8 ± 5.4 years and 22.9 ± 3.5 kg/m², respectively. The mean age of patients providing sperm was 39 ± 7.5 years.

In Vitro Fertilization

Controlled ovarian stimulation was performed using GnRH antagonist or long agonist protocols, as described elsewhere (18, 19). Recombinant hCG or GnRH agonist (Ovitrelle, Merck Serono) was administered to trigger ovulation, and transvaginal oocyte retrieval was performed 36 hours after recombinant hCG or GnRH agonist administration. Oocyte retrieval, denudation, and ICSI were performed according to standard clinical practice at Instituto Valenciano de Infertilidad (20).

Time-Lapse Culture

Immediately after ICSI, zygotes were incubated in preequilibrated dishes with Gems culture medium (Genea Biomedx; see [Supplemental Methods](#)) in a TLI system (Geri, Genea Biomedx; or Embryoscope, Vitrolife) at 37°C, 6% CO₂, and 5% O₂ (balanced with N₂). Embryo development was recorded up to the blastocyst stage.

Embryo Grading

Embryo quality was assessed according to guidelines from the Spanish Association for the Study of Biology of Reproduction, with slight modifications (21) (see [Supplemental Methods](#)).

Embryo Biopsy

A TE biopsy was performed using the pulling method (see [Supplemental Methods](#)). Only embryos that developed beyond a full blastocyst (graded A, B, or C) were considered for biopsy (21). Aspirated cells were collected into DNase-free polymerase chain reaction tubes, immediately stored at –20°C, and kept in a freezer until they were shipped to a genetics laboratory (Igenomix). Blastocysts were moved back to a tri-gas incubator and vitrified following standard procedures (22, 23).

NGS and Mosaicism Classification

Library construction was performed at Igenomix using the Ion ReproSeq PGS kit and Ion Chef System (Thermo-Fisher Scientific). DNA sequencing was conducted using the Ion S5 System instrument (Thermo-Fisher Scientific). For bioinformatic analysis, readings were aligned to the reference human genome using Ion Reporter software (Thermo-Fisher Scientific), which provided copy number counts for each chromosome pair. Aneuploidy patterns were termed “whole chromosome” if they involved gain or loss of one or more complete chromosomes or “partial/segmental” if they included subchromosomal gains or losses ≥ 10 MB in at least one of the 23 chromosome pairs. The presence of diploid and aneuploid cells in a TE biopsy represented a mosaic result. An internal algorithm validated by Igenomix was used for mosaicism calling (11). Briefly, the detection of mosaicism was

validated using mixes of genomic DNA with different percentages of euploid and aneuploid chromosomes. The established algorithm was evaluated in samples prepared to have increasing percentages of mosaicism using two cell lines with trisomy in chromosome 8 or 9. Finally, the algorithm was retrospectively applied to 14,108 TE biopsies analyzed in 10 different diagnostic laboratories. Based on the thresholds established, TE biopsies were classified as [1] euploid, < 30% of aneuploid cells; [2] low-degree mosaic, 30% to <50% of aneuploid cells; [3] high-degree mosaic, 50% to <70% of aneuploid cells; and [4] aneuploid, $\geq 70\%$ of aneuploid cells. Blastocysts displaying chromosome imbalances in two to five chromosomes were classified as complex aneuploid, and those with imbalances in six or more chromosomes as chaotic aneuploid. Blastocysts with segmental and/or sex chromosome mosaicism were not part of the study.

In addition, according to the transfer policy of IVI Valencia (24), mosaic blastocysts were classified as nontransferable if harboring aneuploidies in chromosomes 2, 7, 11, 13, 14, 15, 16, 18, 21, or 22; or as potentially transferrable if harboring aneuploidies in chromosomes 1, 3, 4, 5, 6, 8, 9, 10, 12, 17, 19, or 20.

TLI Assessment

All annotations were performed by two embryologists specifically trained in morphokinetics. The exact timing of 19 variables was annotated as described elsewhere (25): extrusion of the second polar body (tPB2); pronuclear appearance (tPNA); pronuclear fading (tPNF); division to 2, 3, 4, 5, 6, 7, 8, and 9 cells (t2, t3, t4, t5, t6, t7, t8, and t9); start of compaction (tSC); morula (tM); duration of compaction (tDC = tM - tSC); start of blastulation (tSB); blastocyst (tB); duration of the first (ECC1 = t2 - tPNF), second (ECC2 = t4 - t2), and third (ECC3 = t5 - t3) embryo cell cycles; and synchrony of ECC2 (s2 = t4 - t3). All timings were expressed in hours postmicroinjection (hpm).

Statistical Analysis

To ensure minimal interoperator variability between the annotator embryologists, an internal quality control was run on a random subset of 20 embryos, and intraclass correlation coefficients (ICCs) were calculated using a two-way random effects model with absolute agreement (26). The mean ICC was 0.968, indicating "almost perfect agreement" between the two embryologists. The lowest agreement was for t8, although its value still indicated "very strong agreement."

Distribution characteristics of variables were visualized in histograms and analyzed using Kolmogorov-Smirnov and Levene's tests. Morphokinetic timings were compared using Kruskal-Wallis (nonparametric) or one-way analysis of variance (parametric) tests. Continuous variables were expressed as mean $\pm$ standard deviation or as median and interquartile range (IQR), as appropriate. Pairwise comparisons of groups' proportions were performed by means of the χ^2 test.

A logistic mixed effects model was performed with incubator type as the explanatory variable and morphokinetic

timings as response variables. The mixed effect was included to reduce bias per patient and protocol. Female age, female BMI, dose of follicle-stimulating hormone, and type of incubator were considered.

Data were analyzed using 95% confidence levels ($P < .05$ was considered statistically significant) with R (v4.0.2; R Foundation for Statistical Computing) and SPSS (v26; IBM Corporation). Figures were made in GraphPad Prism (v8.4.0; GraphPad Software).

RESULTS

Mosaicism Rate and Correlation with Parental Age

Next-generation sequencing revealed euploidy in 45.8% of blastocysts (692/1,511), low-degree mosaicism in 4.5% of blastocysts (68/1,511), high-degree mosaicism in 2.5% of blastocysts (38/1,511), and aneuploidy in 47.2% of blastocysts (713/1,511). The mosaicism rate of our PGT-A program was 7% (106/1,511; Fig. 1A). The incidence of uniform aneuploidy strongly increased with female age ($P = 2.6365e-25$), but mosaicism did not depend on female or male age ($P > .05$; Fig. 1B).

Frequency of Mosaicism and Aneuploidy per Chromosome

Aneuploidy distribution across chromosomes was investigated. A total of 132 mosaic aneuploidies were identified. Mosaicism was more frequent ($>5\%$) in chromosomes 19 (12.9% = 17/132), 18 (7.6% = 10/132), 13 (7.6% = 10/132), 20 (6.1% = 8/132), 22 (5.3% = 7/132), 21 (5.3% = 7/132), and 14 (5.3% = 7/132). Mosaicism was less frequent in chromosomes 17 (1.5% = 2/132), 1 (1.5% = 2/132), and 15 (0.8% = 1/132; Fig. 2A).

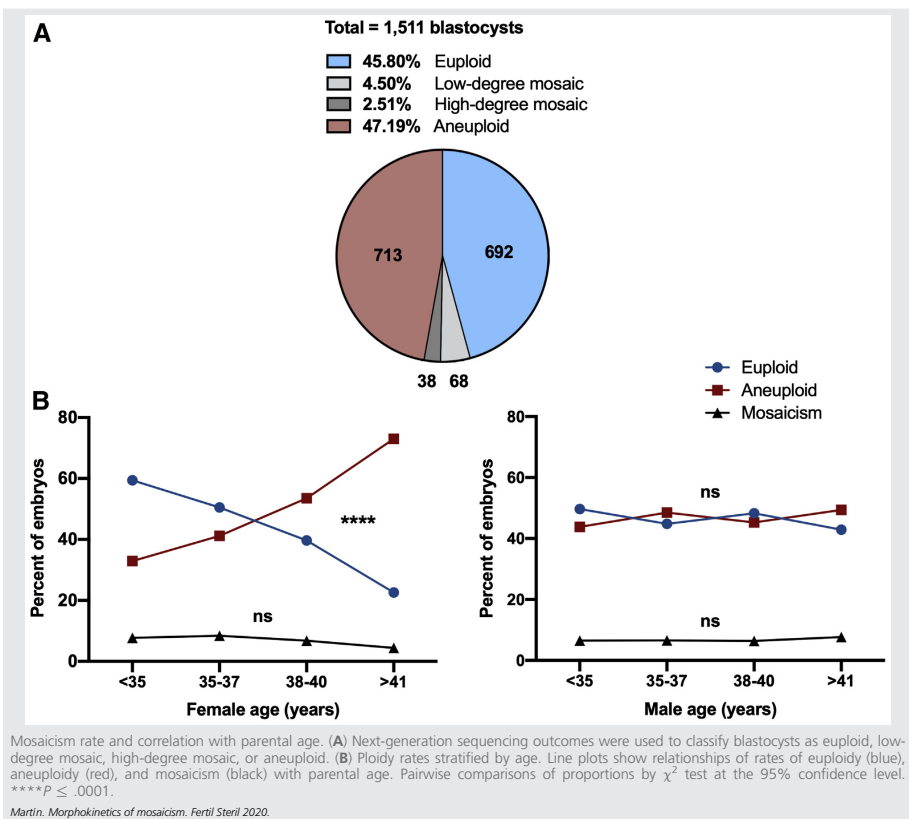
A total of 1,065 uniform aneuploidies were identified. Aneuploidy was more frequent ($>5\%$) in chromosomes 22 (12.6% = 134/1,065), 21 (12.2% = 130/1,065), 16 (10.4% = 111/1,065), 15 (9% = 96/1,065), and 18 (5.5% = 59/1,065). Chromosomes less affected by aneuploidy ($\leq 2\%$) were chromosomes 1 (1.4% = 15/1,065), 6 (1.9% = 20/1,065), and 17 (2% = 21/1,065; Fig. 2B). The frequency of mosaicism and aneuploidy was negatively correlated with chromosome length ($P = .026$ and $P = .0025$, respectively; Fig. 2C, 2D).

Distribution of Aneuploid Embryos per Type of Abnormality

From the 713 aneuploid embryos, 94.5% (674/713) were only affected by autosomal aneuploidy. Whole chromosome autosomal imbalances were detected as the sole abnormality in 81% (578/713) of embryos; 44% (314/713) had single aneuploidies, 33.1% (236/713) had complex aneuploidies, and 3.9% (28/713) had chaotic aneuploidy. Segmental autosomal aneuploidy was present as the sole abnormality in 8.6% of embryos (61/713), and 4.9% (35/713) had both whole chromosome and segmental aneuploidies in their autosomes.

Further, 3% of embryos (21/713) were solely affected by sex chromosome aneuploidy: 1.3% (9/713) were monosomic for chromosome X (X0), 0.3% (2/713) were trisomic for the

FIGURE 1



same chromosome (XXX), 0.8% (6/713) had an XXY karyotype, and 0.6% (4/713) were affected by segmental aneuploidy. Finally, 2.5% (18/713) were simultaneously affected by both autosomal and sex chromosome aneuploidy (Supplemental Table 1).

Mosaicism and Embryo Quality

A significantly higher proportion of euploid blastocysts were ranked as better-quality blastocysts (A or B; 66.9% = 463/692) in comparison with mosaic (considering both low- and high-degree mosaicism; 52.8% = 56/106; $P = .014$) and aneuploid blastocysts (47.7% = 340/713; $P = .336$ –.13). Therefore, the morphological quality of blastocysts increased gradually from aneuploid embryos (lowest rank) to mosaic

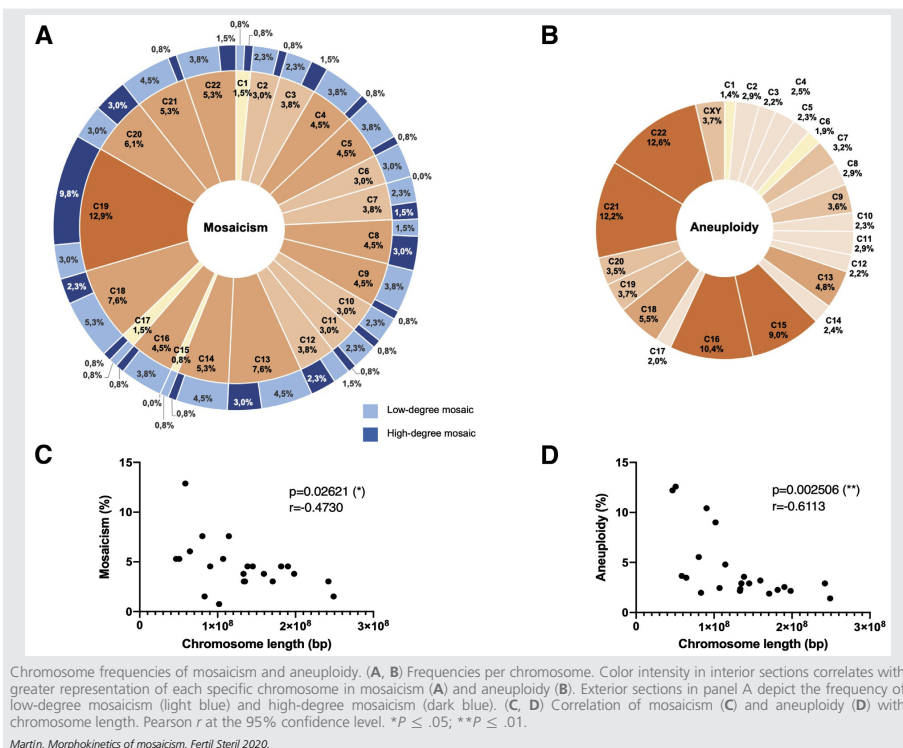
(intermediate rank) and euploid embryos (highest rank; Fig. 3).

Morphokinetic Analysis of Mosaicism

No variables included in the logistic mixed effects model (i.e., female age, female BMI, follicle-stimulating hormone dose, and type of incubator) had a significant effect on morphokinetics ($P > .05$). The logistic mixed effects model for incubator comparison is shown in Supplemental Table 2, and morphokinetic timings by incubator are shown in Supplemental Figure 1. The complete model is available on request from the authors.

The morphokinetic variable tM met the parametricity criteria ($pKS > .05$; $pLev > .05$), and the remaining variables

FIGURE 2



had nonparametric distributions. Variables were analyzed accordingly (Supplemental Table 3). Aneuploid embryos were delayed compared with euploid in tSC (median, 84.72 hpm, IQR, 13.2; vs. median, 82.10 hpm, IQR, 11.5, respectively; $P = .016$), tSB (median, 101 hpm, IQR, 11.7; vs. median, 98.29 hpm, IQR, 10.5, respectively; $P = 2.89 \times 10^{-7}$), and tB (median, 108.04 hpm, IQR, 11.50; vs. median, 104.71 hpm, IQR, 11.35, respectively; $P = 5.77 \times 10^{-8}$; Fig. 4). However, morphokinetic comparisons in low- and high-degree mosaic embryos revealed no significant differences between euploid and aneuploid embryos ($P > .05$). Potentially transferrable and nontransferrable mosaics were also similar in morphokinetics ($P > .05$; Supplemental Figure 2).

DISCUSSION

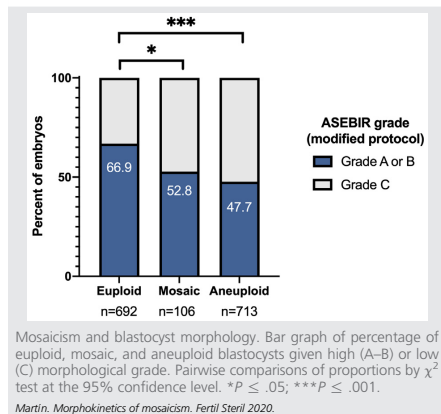
Cellular mechanisms leading to mosaicism in preimplantation embryos have been studied in detail (2). However, their ori-

gins and consequences on embryo development deserve further attention.

Several laboratory factors, including culture conditions (e.g., temperature, pH, or media composition), biopsy, and even insemination method may influence embryo proneness to mitotic error. Moreover, mosaicism rates can vary greatly across laboratories due to differences in NGS protocols and reporting practices (12). This highlights the importance of rigorous validation on the diagnostic test, first by measuring its analytical accuracy and then by performing nonselection studies and randomized clinical trials (27). Acknowledging this variability, recent prevalence estimates of mosaicism are in the range of 5%–10% after NGS (28), which is in line with our data.

Mosaicism is largely independent of age (29, 30), which was confirmed in our cohort. However, it may decrease in older women at the expense of generating fewer euploid embryos. Indeed, in a recent study comprising more than 15,000

FIGURE 3



NGS cycles, mosaicism rates significantly decreased in women age ≥ 40 years (5.1%) versus <40 years (6.6%) (31). Although we observed the same trend, it did not reach statistical significance.

We show that mosaicism and aneuploidy are more frequent in shorter chromosomes, which indicates differential proneness and/or tolerance to error for certain chromosomes (29, 32). In our study, mosaicism and aneuploidy were more frequent in chromosomes 18, 21, and 22, while chromosomes 1 and 17 were relatively unaffected. Mosaicism was less frequent than aneuploidy in chromosomes 15 and 16 and more frequent in chromosomes 13, 19, 14, and 20. Interestingly, the frequencies of aneuploidy per chromosome are similar across studies, but those of mosaicism do vary (29, 32–34). In fact, mosaicism in chromosome 1 was highly prevalent in a previous study (29). It is possible that tolerance to mitotic error relies on the degree of mosaicism (35–37) or that differences are due to methodological aspects related to NGS.

We also report a significant association between blastocyst morphology and chromosomal content, similar to previous studies (38, 39), which indicates why transfer of blastocysts with good TE morphology results in higher implantation, clinical pregnancy, and live birth rates (40, 41), also considering mosaicism.

Unlike meiotic aneuploidies, the origins of mosaicism trace back to a stage that can be monitored in the laboratory. Identification of mosaicism at the blastocyst stage is not an easy task, since it relies on the premise that aneuploid cells are distributed equally in the biopsied fraction compared with the remaining embryo. Indeed, reanalysis of disaggregated putative mosaics has shown low levels of karyotype concordance across fractions (4). Time-lapse imaging allows

visualization of embryo development and correlation of cell division timings with parameters related to developmental potential (e.g., ploidy).

To the best of our knowledge, this study is the second attempt to describe morphokinetic features of putative mosaicism. In a previous study, tB was delayed in aneuploid compared with euploid blastocysts (17), which is in line with our data and reinforces the idea that uniform aneuploidies can significantly impact the pace of preimplantation development (14). In addition, that previous study reported delays in t5, t8, and ECC3 in high-degree mosaic compared with euploid blastocysts (17), whereas we did not find any significant parameter in low- and high-degree mosaic blastocysts.

Compared with published work, our study has some important discrepancies. One major difference is that mosaicism rate was nearly 40% in the previous study (17). It is likely that a proportion of these mosaic embryos would have been categorized differently in our study, affecting morphokinetic distributions. In addition, only embryos from women age ≤ 37 years with at least five mature oocytes were included in that study, which does not represent our PGT-A population. Embryos inseminated by conventional IVF also were included in that study, which may influence morphokinetic timing (42). Finally, that previous study performed statistical comparisons using the Mann-Whitney U test, although the number of groups was four, which may have underestimated P values.

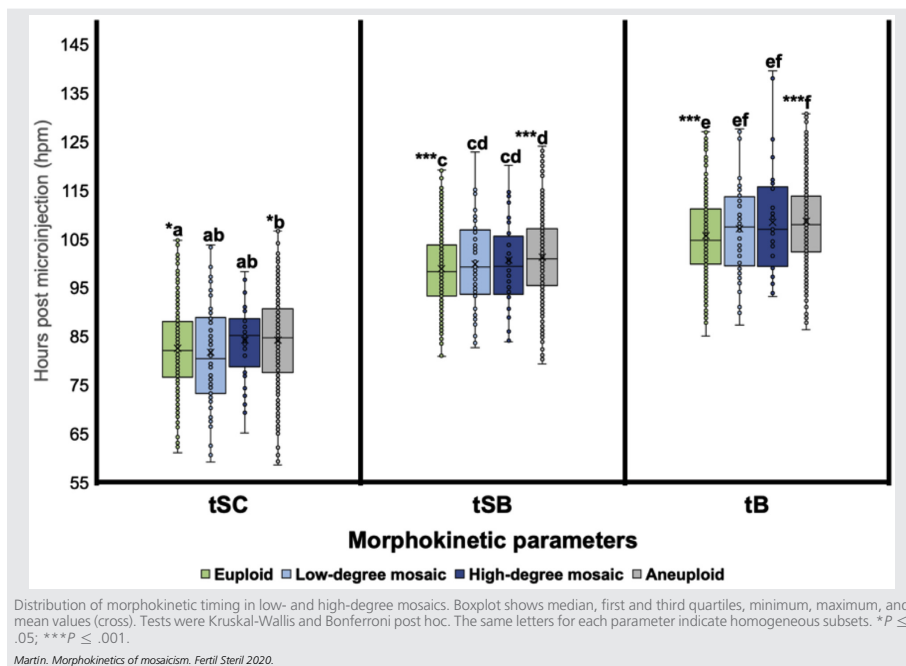
Limitations in our study should be also noted. First, given its retrospective nature, a selection bias may exist due to lack of randomization. Second, this was a single-center study with a limited sample size, so generalization of our findings to larger populations should be made with caution. Third, although internal quality control was performed, interoperator variability in morphokinetic annotations cannot be ruled out. Fourth, discrimination between low- and high-degree mosaicism with current PGT-A procedures may be subject to both technical and biological limitations.

Assuming the lack of clinical utility of the morphokinetic approach when evaluating mosaicism for embryo selection purposes, our main interest was centered on knowing more about the biology of early human development by characterizing the cleavage activity of a group of human embryos that, with the current validated genetic platforms, have been diagnosed as aneuploid/euploid mosaic.

These categories of embryos find themselves in a sort of limbo: some of them will be able to give rise to live births, while others will not. Apart from possible technical artefacts (at least 15% of rebiopsied embryos have shown no concordance within different zones of the TE) (43), there is also the possibility that mosaic embryos may have their own identity and, as has been shown in the mouse, be able to implant once aneuploid cells have been eliminated by different mechanisms (37).

In conclusion, we demonstrate that putative mosaic embryos fit neither euploid nor aneuploid morphokinetic categories. Mosaic embryo morphokinetics may overlap with euploid and aneuploid categories due to some of these mosaic embryos being false-positive diagnoses. However, our results

FIGURE 4



also leave the door open to the hypothesis that mosaicism has been correctly diagnosed and that mosaic embryos swim between the euploid and aneuploid waterfalls, as both molecular and clinical outcomes indicate (35). This may support the idea of their own genetic and developmental identity, pushing them aside from being considered by-products of misdiagnosis in PGT-A with current NGS platforms. However, further studies are needed to increase our current understanding of embryonic mosaicism and the impact of mitotic aneuploidies on preimplantation dynamics.

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Firma morfocinética de embriones mosaico: evidencia que apoya su propia identidad genética.

Objetivo: proveer de una caracterización morfocinética completa de embriones clasificados con grados de mosaicismo cromosómico diferente.

Diseño: Estudio retrospectivo de cohorte.

Lugar: Clínica privada de fecundación in vitro afiliada a Universidad

Pacientes: Analizamos 1,511 embriones de 424 ciclos de inyección intracitoplasmática de esperma mediante cultivo de embriones en sistema de imagen time lapse y secuenciación de nueva generación. Se evaluaron 106 embriones mosaico.

Intervenciones: Ninguna.

Medidas principales: Comparación de las características cromosómicas, morfológicas y morfocinéticas de blastocistos clasificados como euploides, aneuploides, mosaico de bajo grado (30% a <50% de células aneuploides en la biopsia de trofotodermo) y mosaico de alto grado (50% a <70% de células aneuploides en biopsia de trofotodermo). El análisis estadístico se realizó utilizando χ^2 , Kruskal-Wallis, o análisis de pruebas de varianza según el tipo y distribución de los datos. Se utilizó un modelo bidireccional de efectos aleatorios para calcular la correlación inter-operador de anotaciones, y se realizó un modelo logístico de efectos mixtos para evaluar el efecto de los factores de confusión sobre el tiempo morfocinético.

Resultados: La tasa de mosaicismo fue ~7% independientemente de la edad de los padres. El mosaicismo y las aneuploidías uniformes no se distribuyeron uniformemente entre los cromosomas. El porcentaje de blastocistos de alta calidad disminuyó significativamente de euploide (66,9%) a mosaico (52,8%) y aneuploide (47,7%). Los blastocistos aneuploides retrasaron significativamente el desarrollo en comparación con los blastocistos euploides en el inicio de la compactación (mediana, 84,72 horas después de la microinyección [hpm], rango intercuartílico [IQR], 13,2; frente a mediana, 82,10 hpm, IQR, 11,5), inicio de blastulación (mediana, 101 hpm; IQR, 11,7; frente a mediana, 98,29 hpm, IQR, 10,5) y momento de aparición de blastocisto (mediana, 108,04 hpm, IQR, 11,50; frente a mediana, 104,71 hpm, IQR, 11,35). Sin embargo, la morfocinética del embrión no se correlacionó con el grado de mosaicismo o con una configuración de mosaicismo que fuera apta para la transferencia de embriones.

Conclusiones: El tiempo morfocinético de los embriones mosaico se superpone con el de los embriones euploides y aneuploides, lo que puede reflejar su identidad genética y de desarrollo única. Aunque esto sugiere que los embriones en mosaico no son simplemente un subproducto de un diagnóstico erróneo, se necesitan más estudios para revelar la verdadera identidad de este tipo particular de embrión.

Supplemental Material

*The morphokinetic signature of mosaic embryos:
evidence in support of their own genetic identity*

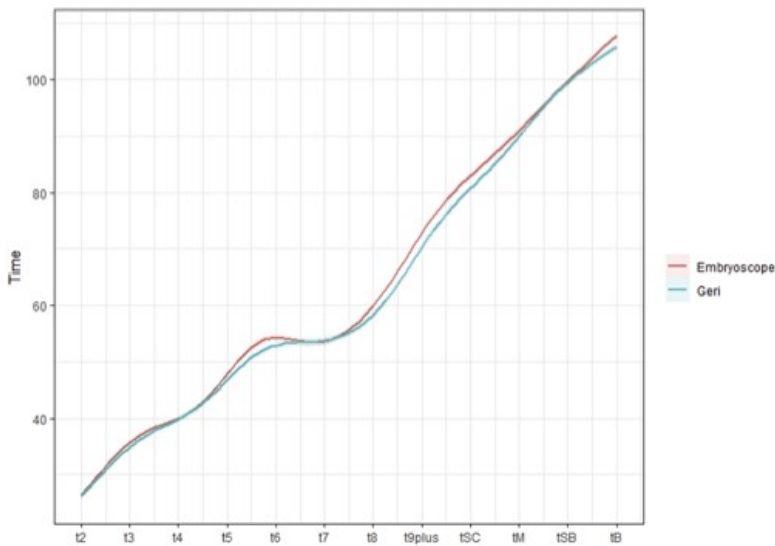
Ángel Martín, M.Sc., Lorena Rodrigo, Ph.D., Diana Beltrán, M.Sc., Marcos Meseguer, Ph.D., Carmen Rubio, Ph.D.,
Amparo Mercader, Ph.D., Maria Jose de los Santos, Ph.D.

Fertility and Sterility

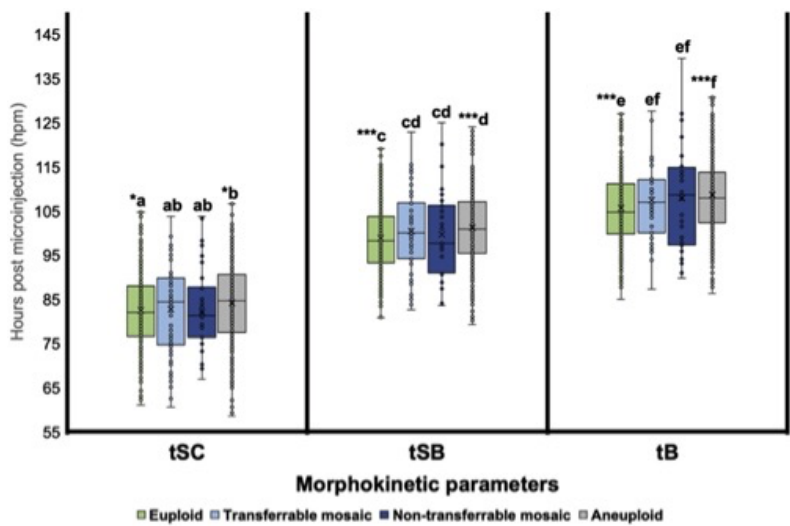
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Supplemental Figures



Supplemental Figure 1 Kinetic embryo development by incubator. The x-axis indicates morphokinetic variables. The y-axis indicates time expressed in hours postmicroinjection (hpm).



Supplemental Figure 2 Distribution of morphokinetic timings in potentially transferrable and non-transferrable mosaics. Boxplot shows median, first and third quartiles, minimum, maximum, and mean values (cross). Tests were Kruskal-Wallis and Bonferroni post hoc. The same letters for each parameter indicate homogeneous subsets. * $P \leq .05$; *** $P \leq .001$.

Supplemental Methods

Time-lapse culture

Geri and Embryoscope incubators were programmed to acquire images of each embryo every 5 or 15 minutes through 7 or 11 different focal planes, respectively. Embryos were cultured to the blastocyst stage in Geri dishes (Genea Biomedx, Australia) or EmbryoSlides (Vitrolife, Sweden). Geri dishes were filled with 80 μ L of Gems medium under a 4-mL overlay of mineral oil. EmbryoSlides were filled with 28 μ L of Gems medium under a 1.5-mL overlay of mineral oil. TLI slides were incubated overnight to enable pre-equilibration to incubator conditions.

Embryo grading

Blastocysts were graded according to the extent of blastocoel expansion, cellularity and organization of the TE, and size, shape, and compaction degree of the ICM. Blastocysts graded “A” had a prominent ICM with many compacted cells, accompanied by a smooth and cohesive TE epithelium. Blastocysts graded “B” had fewer TE cells and a discernible ICM. Blastocysts graded “C” either displayed very few TE cells or had not yet commenced expansion. Grade “D” was given to either blastocysts showing signs of degeneration or embryos that had not yet commenced blastulation.

Embryo biopsy

Briefly, all embryos underwent assisted hatching on day 3 using laser technology (Fertilase, Octax). Hatched embryos were placed in fresh medium and cultured until day 5 or 6 of development. After viewing under an inverted microscope, blastocysts were held firmly with a holding pipette on the opposite site where TE cells were herniating. TE cells were carefully drawn into the biopsy pipette by applying gentle suction while applying short laser pulses. Detachment of TE cells was achieved after 4–5 laser pulses.

Supplemental Tables

Aneuploidy type	n	%
Autosomal aneuploidy	674	94.53%
Whole chromosome aneuploidy	578	81.07%
1 chromosome (single)	314	44.04%
2–5 chromosomes (complex)	236	33.10%
≥6 chromosomes (chaotic)	28	3.93%
Segmental aneuploidy (≥10 MB)	61	8.56%
Whole chromosome + segmental aneuploidy	35	4.91%
Sex chromosome aneuploidy	21	2.95%
Whole chromosome aneuploidy	17	2.38%
X0	9	1.26%
XXX	2	0.28%
XXY	6	0.84%
Segmental aneuploidy (≥10 MB)	4	0.56%
Autosomal + sex chromosome aneuploidy	18	2.52%
Total	713	100%

Supplemental Table 1. Classification of aneuploid embryos per type of abnormality.

Effect	Term	Estimate	SE	Statistic	P	Conf.low	Conf.high
Fixed	(Intercept)	0.0252	0.2439	-0.3801	0.7038	0.0000	4,42E+11
Fixed	t2	10.580	0.3740	0.1596	0.8732	0.5292	2,12E+05
Fixed	t3	0.8086	0.2430	-0.7068	0.4797	0.4486	1,46E+05
Fixed	t5	10.478	0.1930	0.2536	0.7998	0.7303	1,50E+05
Fixed	t6	10.209	0.2494	0.0846	0.9326	0.6325	1,65E+05
Fixed	t7	10.517	0.1827	0.2902	0.7716	0.7482	1,48E+05
Fixed	t8	10.407	0.1071	0.3879	0.6981	0.8506	1,27E+05
Fixed	t9+	0.9532	0.1234	-0.3704	0.7111	0.7395	1,23E+05
Fixed	tM	0.9897	0.1516	-0.0675	0.9462	0.7330	1,34E+05
Fixed	tSC	0.9130	0.1398	-0.5948	0.5520	0.6763	1,23E+05
Fixed	tSB	11.824	0.2589	0.7654	0.4440	0.7699	1,82E+05
Fixed	tB	0.9117	0.1837	-0.4588	0.6464	0.6143	1,35E+05

Supplemental Table 2. Logistic mixed model for incubator comparison. Estimate indicates odds ratio effect over the probability to belong to Embryoscope incubator. Conf.low and conf.high indicate odds ratio 95% confidence interval. A significant *P*-value rejected the null hypothesis OR=1. SE, standard error; t2–t9+, timing of division to 2–9+ cells; tM, timing of morula; tSC, timing of start of compaction; tSB, timing of start of blastulation; tB, timing of blastocyst

	pKS	pLev	pKW	pANV
tPB2	1.2e-5	0.949	0.465	-
tPNa	3.3e-23	0.190	0.042	-
tPNf	8.7e-15	0.113	0.268	-
t2	4.43e-19	0.084	0.467	-
t3	4.05e-7	0.507	0.331	-
t4	5.7e-21	0.044	0.121	-
t5	5.0e-6	0.428	0.598	-
t6	1.03e-16	0.110	0.478	-
t7	9.87e-28	0.062	0.345	-
t8	3.82e-49	0.495	0.108	-
t9	8.00E-06	0.116	0.920	-
tSC*	0.200	0.010	0.016	-
tM*	0.200	0.067	-	0.060
tDC	5.63e-30	0.668	0.579	-
tSB***	0.007	0.014	2.89e-7	-
tB***	1.33e-9	0.018	5.77e-8	-
ECC1=t2-tPNf	4.1e-186	0.217	0.670	-
ECC2=t4-t2	9.49e-90	0.479	0.137	-
ECC3=t5-t3	2.07e-92	0.023	0.743	-
s2=t4-t3	1.87e-291	0.116	0.107	-

Supplemental Table 3. Distribution and statistical analysis of morphokinetic timing. Comparison of euploid, low-degree mosaic, high-degree mosaic, and aneuploid embryos. *Parametric distribution. * $P \leq 0.05$; *** $P \leq 0.001$. pKS, P -value Kolmogorov-Smirnov test; pLev, P -value Levene's test; pKW, P -value Kruskal-Wallis test; pANV, P -value one-way ANOVA test; tPB2, timing of extrusion of the second polar body; tPNa, timing of pronuclear appearance; tPNf, timing of pronuclear fading; t2-t9, timing of division to 2-9 cells; tSC, timing of start of compaction; tM, timing of morula; tDC, timing of duration of compaction (tDC=tM-tSC); tSB, timing of start of blastulation; tB, timing of blastocyst; ECC1-ECC3, duration of first-third embryo cell cycles; s2, synchrony of ECC2.

Mosaic results after preimplantation genetic testing for aneuploidy may be accompanied by changes in global gene expression.

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Mosaic results after preimplantation genetic testing for aneuploidy may be accompanied by changes in global gene expression

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Aneuploidy in preimplantation embryos is a major cause of human reproductive failure. Unlike uniformly aneuploid embryos, embryos diagnosed as diploid-aneuploid mosaics after preimplantation genetic testing for aneuploidy (PGT-A) can develop into healthy infants. However, the reason why these embryos achieve full reproductive competence needs further research. Current RNA sequencing techniques allow for the investigation of the human preimplantation transcriptome, providing new insights into the molecular mechanisms of embryo development. In this prospective study, using euploid embryo gene expression as a control, we compared the transcriptome profiles of inner cell mass and trophectoderm samples from blastocysts with different levels of chromosomal mosaicism. A total of 25 samples were analyzed from 14 blastocysts with previous PGT-A diagnosis, including five low-level mosaic embryos and four high-level mosaic embryos. Global gene expression profiles visualized in cluster heatmaps were correlated with the original PGT-A diagnosis. In addition, gene expression distance based on the number of differentially expressed genes increased with the mosaic level, compared to euploid controls. Pathways involving apoptosis, mitosis, protein degradation, metabolism, and mitochondrial energy production were among the most deregulated within mosaic embryos. Retrospective analysis of the duration of blastomere cell cycles in mosaic embryos revealed several mitotic delays compared to euploid controls, providing additional evidence of the mosaic status. Overall, these findings suggest that embryos with mosaic results are not simply a misdiagnosis by-product, but may also have a genuine molecular identity that is compatible with their reproductive potential.

KEYWORDS

embryonic mosaicism, RNA sequencing, preimplantation genetic testing for aneuploidy, next-generation sequencing, blastomere cell cycle

1 Introduction

Preimplantation genetic testing for aneuploidy (PGT-A) enables the identification of embryos carrying chromosomal alterations after *in vitro* fertilization (IVF). Embryos with uniform aneuploidies, resulting from meiotic errors during gametogenesis, are deselected for transfer due to the high risk of adverse clinical outcomes (Capalbo et al., 2022). Intermediate chromosome copy-number values in trophoctoderm (TE) biopsies are often interpreted as evidence of chromosomal mosaicism (Paulson and Treff, 2020). Embryos with putative diploid-aneuploid mosaicism, which originates from mitotic errors after fertilization, have the potential to develop into healthy offspring (McCoy, R. C., 2017; Treff and Marin, 2021; Greco et al., 2023). However, the clinical management of these embryos remains the subject of an intense debate.

Retrospective cohort studies involving large data sets of embryo transfers have suggested that mosaic embryos have a reduced developmental potential compared to their euploid peers, and that the level and type of mosaicism can determine reproductive outcomes (Abhari and Kawwass, 2021). However, prospective non-selection studies have shown that mosaic embryos with less than 50% of aneuploid cells in the TE biopsy have equivalent developmental potential compared to fully euploid ones (Capalbo et al., 2021; Tiegs et al., 2021; Wang et al., 2021).

This view is supported by several systematic reviews and meta-analyses that have used the Newcastle-Ottawa scale to assess the quality of non-randomized studies (Mourad et al., 2021; Ma et al., 2022). Importantly, these studies agree that transferring a mosaic embryo entails a low risk of confirming the abnormal karyotype in the ensuing pregnancy. In fact, reports on this matter are limited (Kahraman et al., 2020; Schlade-Bartusiak et al., 2022; Greco et al., 2023).

In line with the debate, a recent position statement issued by the PGDIS on the management of embryonic mosaicism indicates that the decision to transfer a mosaic embryo can be prioritized either based on the level of mosaicism or the type of mosaicism (Leigh et al., 2022). Conversely, the ESHRE Working Group on Chromosomal Mosaicism avoids formulating specific recommendations to the transfer of high-level mosaic embryos due to the absence of robust clinical data (De Rycke et al., 2022).

Two major hypotheses have been proposed to explain why mosaic embryo transfers are compatible with positive pregnancy outcomes and the delivery of healthy offspring.

The first hypothesis considers that mosaicism is overestimated by false positive diagnoses. This notion is primarily supported by studies involving embryo disaggregation, which have demonstrated low concordance rates of the mosaic result among the diagnostic TE biopsy, the inner cell mass (ICM) and/or the remaining TE cells (Popovic et al., 2020; Capalbo et al., 2021; Marin et al., 2021). Furthermore, even diagnostic platforms validated for detecting mosaicism may not fully account for the technical noise introduced by TE biopsies, which can potentially be misinterpreted as a biological signal, leading to false positive diagnoses of mosaicism (Treff and Marin, 2021).

The second hypothesis supports the ability of mosaic embryos to self-correct. Reversion of aneuploidy appears to be a rare phenomenon in human embryos, given the low frequency of

uniparental disomy at the blastocyst stage (Gueye et al., 2014; Taylor et al., 2014). Rather, a more-supported view of self-correction may be the negative selection of aneuploid cells during embryonic development through mechanisms of cell competition, such as apoptosis or differential cell proliferation (Bolton et al., 2016; Baker, 2020; Singla et al., 2020; Coticchio et al., 2021).

Against this background, there is an overriding need to study the biological implications of mosaic findings on the specific functions of the preimplantation embryo. RNA-sequencing (RNA-seq) techniques can be used to measure relative changes in gene expression across multiple experimental conditions (Ura et al., 2022). Conveniently, the optimization of library preparation protocols for use with low input samples has paved the way for the study of the human preimplantation transcriptome (Tang, F. et al., 2009; Islam et al., 2011; Ramskold et al., 2012; Picelli et al., 2014; Hwang et al., 2018).

In view of recent findings suggesting that moderate levels of mosaicism in the TE biopsy are not detrimental for embryo development (Capalbo et al., 2022), it is paramount to investigate whether such mosaic findings are accompanied by changes in global gene expression, compared to embryos having fully euploid karyotypes. Here, we used RNA-seq to compare the transcriptome profiles of ICM and TE samples from embryos diagnosed as euploid, low-level mosaic and high-level mosaic after PGT-A. The identification of pathways specifically deregulated in embryos with different levels of putative mosaicism may shed new light on the mechanisms leading to their reproductive competence.

2 Material and methods

2.1 Ethical approval

The study was approved by the National Commission of Human Reproduction (CNRHA), the general direction of research, innovation, technology, and quality and by our institutional review board, the ethics committee of Clinical Research IVI-RMA Valencia (#1710-VLC-102-MD), which complies with Spanish law on assisted reproductive technologies (14/2006) and biomedical research (14/2007).

2.2 Study design and population

This prospective cohort study was conducted on a total of 14 blastocysts donated to research by 12 couples who underwent IVF treatment with intracytoplasmic sperm injection (ICSI) and PGT-A at IVI-RMA (Valencia, Spain). The study cohort included blastocysts classified as euploid ($n = 5$), low-level euploid-aneuploid mosaic ($n = 5$) and high-level euploid-aneuploid mosaic ($n = 4$) after PGT-A. All participants provided written informed consent.

The mean age of patients providing oocytes and sperm was 35.8 ± 5.3 and 36.5 ± 6.2 years, respectively. Indications for PGT-A included advanced female age (≥ 35 years) ($41.7\% = 5/12$), implantation failure ($25\% = 3/12$), male factor ($8.3\% = 1/12$), previous chromosomopathy ($8.3\% = 1/12$) and multiple indications ($16.7\% = 2/12$).

2.3 Ovarian stimulation and *in vitro* fertilization

Controlled ovarian stimulation was performed using GnRH antagonist protocol, as described elsewhere (Labarta et al., 2018). Recombinant hCG (rhCG) (Ovitrelle, Merck Serono, Germany) or GnRH agonist (Decapeptyl, Ipsen Pharma, France) was administered to trigger ovulation, and transvaginal oocyte retrieval was performed 36 h after rhCG or GnRH agonist administration. Oocyte retrieval, denudation, and ICSI were performed according to standard clinical practice at Instituto Valenciano de Infertilidad (Alegre et al., 2019). Embryos were incubated in Gens culture medium (Genea Biomedx, Australia) under mineral oil at 37°C, 6% CO₂, and 5% O₂ (balanced with N₂) up to the blastocyst stage.

2.4 Preimplantation genetic testing for aneuploidy

All embryos underwent assisted hatching on day 3 using laser technology (Fertilase®, Octax, Sweden). On days 5 or 6, embryo quality was assessed according to guidelines from the Spanish Association for the Study of Biology of Reproduction, with slight modifications (Arday et al., 2008). Embryos that developed beyond a full blastocyst (graded A, B, or C) were considered for biopsy, which was performed by the pulling method (Aoyama and Kato, 2020). After biopsy, blastocysts were vitrified by the Kitazato method (Kuwayama, 2007; Cobo et al., 2009).

PGT-A was performed by next-generation sequencing (NGS) (Igenomix, Spain). Library construction, DNA sequencing and bioinformatic analysis were performed using the Ion ReproSeq PGS kit, Ion Chef Instrument, Ion S5 System and Ion Reporter software (Thermo Fisher Scientific, United States). An internal algorithm validated by Igenomix was used for mosaicism calling (Garcia-Pascual et al., 2020). TE biopsies were classified as i) Euploid: <30% of aneuploid cells; ii) Low-level mosaic: 1–2 chromosomes aneuploid in 30%–<50% cells; and iii) High-level mosaic: 1–2 chromosomes aneuploid in 50%–<70% cells. Segmental and/or sex chromosomal mosaicism were not detected.

2.5 Collection of inner cell mass and trophectoderm samples

Blastocyst warming was performed by the Kitazato method (Kuwayama, 2007; Cobo et al., 2009). Following re-expansion, blastocysts were placed under an inverted microscope and held firmly with a holding pipette (SHP-120B-35 Sunlight Medical, United States). The ICM was identified as a group of cohesive, oval-shaped cells located inside the embryo. An ICM biopsy was performed by the pulling method, whereby cells were carefully drawn into a biopsy pipette (SBB-30Z-30 Sunlight Medical, United States) by applying gentle suction and 4–5 laser pulses of 3.1 milliseconds (Aoyama and Kato, 2020). The ICM biopsy and the remaining TE were separately collected from 14 blastocysts into RNase-free PCR tubes containing 2 µL of 10X reaction buffer (SMART-Seq v4 Ultra-Low Input RNA kit for Sequencing,

Takara Bio, United States), which were immediately stored at -80°C until processing (Figure 1A). Table 1 summarizes the main characteristics of the embryos included in the study.

2.6 Library preparation and RNA sequencing

Total RNA was extracted from 28 samples using a SMART-Seq V4 Ultra-Low Input RNA Sequencing kit (Takara Bio, United States) that uses oligo (dT) priming. cDNA was synthesized with 3'SMART-Seq CDS primer II (Takara Bio, United States) and PCR-amplified (17 cycles) from 10 pg of RNA following manufacturer's instructions. Amplified cDNA was purified using AMPure XP magnetic beads (Illumina, United States). After confirming cDNA integrity on a 2,100 Bioanalyzer (Agilent Technologies, United States), 1 ng of cDNA per sample were fragmented, and libraries were constructed using NexteraXT DNA sample preparation (Illumina, United States) according to manufacturer's instructions. Samples were quantified using Qubit dsDNA Quantitation Assay (Thermo Fisher Scientific, United States), and three samples were excluded due to poor cDNA quality. An RNA pool was generated with 25 samples using equal concentration (5 nM) of RNA per sample. Barcodes and adapters were included in the library to sequence and identify all samples. Sequencing was performed in duplicate in a single run using an Illumina NovaSeq 6000 S1 platform (Illumina, United States) with a 200-nucleotide read length in a paired-end design (100-bp fragments). FastQC was used for checking the quality of the raw sequence data. Fragments that did not meet quality requirements were trimmed using Trimmomatic (Bolger et al., 2014). Alignment and quantification were performed using the Salmon algorithm (reference genome GRCh38) (Patro et al., 2017). An average of 54, 238, 594 reads were sequenced per sample, and 83.8% were successfully mapped (Figure 1B). Raw counts were directly used for differential gene expression analysis.

2.7 Bioinformatic analysis

Correlation studies, principal component analysis (PCA) and differential gene expression analysis were performed with the DESeq2 package (Love et al., 2014). Venn diagrams were generated with the software Venn (Chen et al., 2021). Volcano plots were generated with the software VolcanoR (Goedhart and Luijsterburg, 2020). The fgsea algorithm was used for functional enrichment analysis on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) terms; i.e., Biological Process (GO-BP), Molecular Function (GO-MF) and Cellular Component (GO-CC) (Korotkevich et al., 2019). Enrichment scores (ES) were calculated as the degree to which each gene set was overrepresented at the top or bottom of the ranked list of genes in the expression dataset. Normalized enrichment scores (NES) were obtained after normalization across the analyzed gene sets. Gene set enrichment results were analyzed using the tool EnrichmentMap in Cytoscape (Shannon et al., 2003).

Six different comparisons were analyzed: (C-1) TE of euploid blastocysts (*n* = 4) vs. TE of low-level mosaic blastocysts (*n* = 5); (C-2) TE of euploid blastocysts (*n* = 4) vs. TE of high-level mosaic

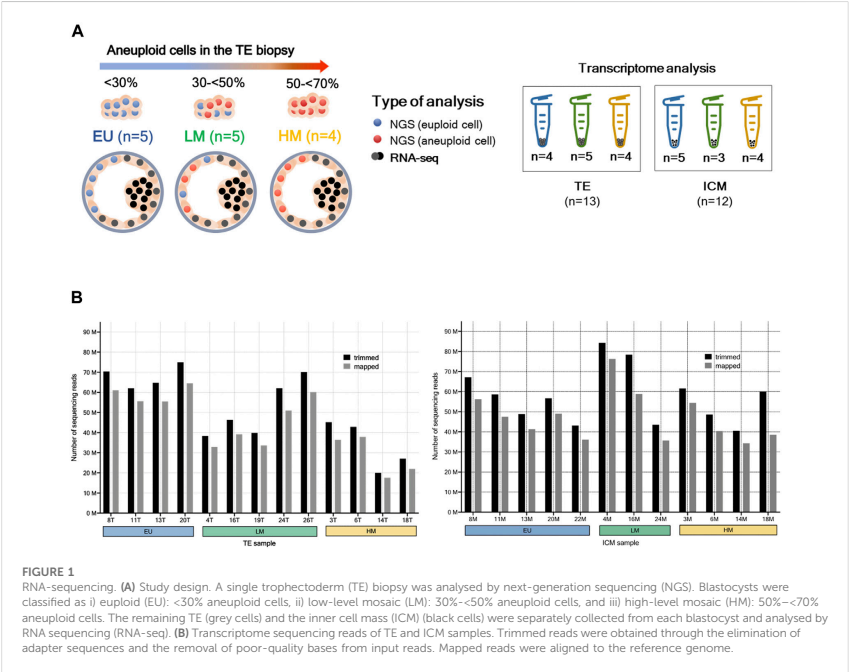


TABLE 1 Study population and embryo characteristics. PGT-A was performed by next-generation sequencing (mosaic range defined between 30%–70% aneuploid cells). ^a Days post-fertilization. ^b Embryo quality assessed according to guidelines from the Spanish Association for the Study of Biology of Reproduction (ASEBIR).

Embryo number	Day of biopsy ^a	Grade ^b	NGS (% of aneuploid cells in the TE biopsy)	PGT-A diagnosis
EU1 (08)	5	B	<30%	Euploid
EU2 (11)	6	B	<30%	Euploid
EU3 (13)	5	C	<30%	Euploid
EU4 (20)	5	B	<30%	Euploid
EU5 (22)	5	B	<30%	Euploid
LM1 (04)	5	B	+6 (30%–50%), –7 (30%–50%)	Low-level mosaic
LM2 (16)	6	C	–22 (30%–50%)	Low-level mosaic
LM3 (19)	5	B	+4 (30%–50%)	Low-level mosaic
LM4 (24)	5	C	+8 (30%–50%), –22 (30%–50%)	Low-level mosaic
LM5 (26)	5	B	+12 (30%–50%), +20 (30%–50%)	Low-level mosaic
HM1 (03)	6	C	+4 (50%–70%)	High-level mosaic
HM2 (06)	5	B	+8 (50%–70%)	High-level mosaic
HM3 (14)	5	B	+19 (50%–70%)	High-level mosaic
HM4 (18)	5	C	–20 (50%–70%)	High-level mosaic

blastocysts ($n = 4$); (C-3) TE of low-level mosaic blastocysts ($n = 5$) vs. TE of high-level mosaic blastocysts ($n = 4$); (C-4) ICM of euploid blastocysts ($n = 5$) vs. ICM of low-level mosaic blastocysts ($n = 3$); (C-5) ICM of euploid blastocysts ($n = 5$) vs. ICM of high-level mosaic blastocysts ($n = 4$); and (C-6) ICM of low-level mosaic blastocysts ($n = 3$) vs. ICM of high-level mosaic blastocysts ($n = 4$).

2.8 Validation of RNA-seq by quantitative PCR

To corroborate gene expression data from RNA-seq, cDNA samples from each comparison group were analyzed by quantitative real-time PCR (qRT-PCR) on a StepOnePlus system (Applied Biosystems, United States) using Power-Up SYBR green (Thermo Fisher Scientific, United States). Duplicate cDNA amplifications were performed on the same cDNA samples used in RNA-seq (primer descriptions provided in [Supplementary Table S1](#)).

Six genes were randomly selected amongst the top differentially-expressed genes, based on their fold change, statistical significance and inclusion within significantly-enriched functions in the context of embryo development:

- i) Rho GTPase Activating Protein 36 (*ARHGAP36*): FC = 8.1; padj = 1.5E-04 (C-1).
- ii) Neural EGFL Like 1 (*NELL1*): FC = -7.9; padj = 1.8E-03 (C-1) and FC = -8.6; padj = 1.2E-06 (C-2).
- iii) Neurotrophic Receptor Tyrosine Kinase 3 (*NTRK3*): FC = 5.5; padj = 2.9E-04 (C-4).
- iv) Cadherin 5 (*CDH5*): FC = 8.2; padj = 6.6E-03 (C-5) and FC = 8.3; padj = 8.5E-03 (C-6).
- v) Vimentin (*VIM*): FC = -8.8; padj = 2.1E-04 (C-5).
- vi) Laminin Subunit Gamma 3 (*LAMC3*): FC = -4.5; padj = 1.2E-05 (C-6).

Selected genes are highlighted in [Supplementary Figure S1](#). These genes were found in several GO annotation classes in order to avoid bias towards a particular function, and were related to embryonic cell growth, differentiation and survival (*ARHGAP36*; *NELL1*; *NTRK3*; *VIM*), as well as to cell migration, adhesion and organization of cells into tissues during embryogenesis (*CDH5*; *LAMC3*).

Gene expression levels were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and quantified by the $\Delta\Delta CT$ method. Fold change was calculated as the normalized gene expression ($2^{\Delta\Delta CT}$) in each sample divided by the normalized gene expression ($2^{\Delta\Delta CT}$) in a random reference sample.

2.9 Analysis of blastomere cell cycles by time lapse imaging

The development of low-level mosaic embryos ($n = 4$) and high-level mosaic embryos ($n = 4$) was retrospectively analyzed from the 1- to 8- cell stage by means of time lapse imaging (TLI). The timings of 8 direct morphokinetic variables (expressed in hours after ICSI) were annotated: extrusion of the second polar body (tPB2); division

to 2, 3, 4, 5, 6, 7, and 8 cells (t2, t3, t4, t5, t6, t7, and t8). Duration of blastomere cell cycles were calculated as indirect morphokinetic variables (expressed in hours) as described by ([Grau et al., 2015](#)): duration of the first blastomere cell cycle (cc1 = t2-tPB2); duration of the second cell cycle of the first blastomere to cleave from the 2- to the 3-cell stages (cc2a = t3-t2); duration of the second cell cycle of the second blastomere to cleave from the 2- to the 4-cell stages (cc2b = t4-t2); mean cc2 (cc2m) as the mean of cc2a and cc2b; duration of the third cell cycle of the first (cc3a), second (cc3b), third (cc3c), and fourth (cc3d) blastomeres to cleave to the 5-, 6-, 7-, and 8-cell stages, respectively. Importantly, tracking blastomere origin was necessary to determine cc3a, cc3b, cc3c, and cc3d. Mean cc3 (cc3m) was calculated as the mean of cc3a, cc3b, cc3c, and cc3d. Since TLI videos were not available for the same euploid embryos used in the RNA-seq study, morphokinetic timings of putative mosaic embryos were compared to those from a random subset of 25 embryos of similar morphological quality which had been diagnosed as euploid with the same NGS protocol and analyzed in a previous study ([Martin et al., 2021](#)).

2.10 Statistical analysis

Differential gene expression analysis was performed using the parametric Wald test with Benjamini-Hochberg adjustment (padj). Genes with padj < 0.05 and a log2FoldChange (FC) of ± 2 were considered significantly differentially expressed (DEGs). GO terms and KEGG pathways with padj < 0.05 were considered significantly deregulated. Validation analyses were performed by using a two-tailed *t*-test for independent samples, where $p < 0.05$ was considered statistically significant. Data analysis and figure preparation were performed in R and GraphPad Prism 9 (GraphPad Software, United States). Morphokinetic timings were compared by one-way ANOVA and Bonferroni *post hoc*, and variables were expressed as mean $\pm$ standard deviation.

3 Results

3.1 Comparison of global gene expression profiles

We first analyzed the differences in global gene expression across the six comparisons using PCA and cluster heatmaps. In the PCA, we observed that the dominant expression patterns captured by the first two principal components were not related to the original PGT-A result. Samples were only minimally separated by the PGT-A diagnosis when comparing euploid embryos to high-level mosaic embryos, both in TE comparisons and in ICM comparisons ([Supplementary Figure S2](#)). In contrast, in the cluster heatmaps, samples were grouped according to the PGT-A diagnosis in all comparisons, and expression vectors for samples within the same cluster were much more similar than those for samples from different clusters ([Supplementary Figure S3](#)). Only one TE sample collected from a low-level mosaic embryo was clustered in a different group ([Supplementary Figure S3A](#)). Notably, the TE samples of low-level mosaic embryos and high-

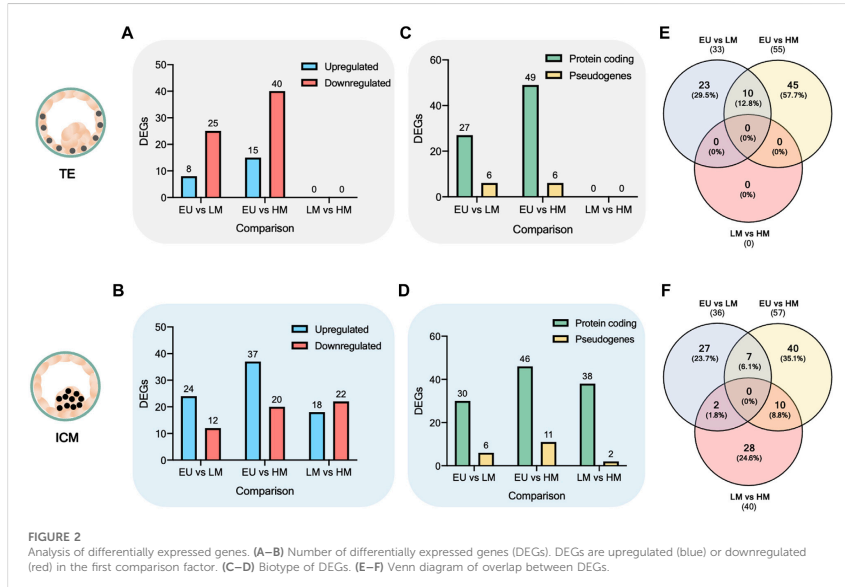


FIGURE 2

Analysis of differentially expressed genes. (A–B) Number of differentially expressed genes (DEGs). DEGs are upregulated (blue) or downregulated (red) in the first comparison factor. (C–D) Biotype of DEGs. (E–F) Venn diagram of overlap between DEGs.

level mosaic embryos showed the greatest degree of similarity (Supplementary Figure S3C).

3.2 Analysis of differentially expressed genes

Next, we identified the statistically significant DEGs across the six comparisons. We observed that the number of DEGs increased in both the TE (Figure 2A) and the ICM (Figure 2B) when comparing euploid embryos to low-level mosaic embryos (33 DEGs in the TE and 36 DEGs in the ICM) and high-level mosaic embryos (55 DEGs in the TE and 57 DEGs in the ICM). However, no statistically significant DEGs were found between the TE of low-level mosaic embryos and high-level mosaic embryos, indicating that the differences observed between both groups were not statistically significant. In contrast, 40 DEGs were found between the ICM samples of low-level and high-level mosaic embryos. A volcano plot was used to represent the differential expression of genes between groups (Supplementary Figure S1).

Most of the DEGs found were protein coding, accounting for 76 out of 88 (86.4%) DEGs in TE comparisons (Figure 2C) and 114 out of 133 (85.7%) DEGs in ICM comparisons (Figure 2D), while the rest were categorized as pseudogenes. The full list of DEGs is provided in Supplementary Table S2.

Next, we used the Venn diagram to identify overlapping and unique statistically significant DEGs. A total of 17 DEGs were found

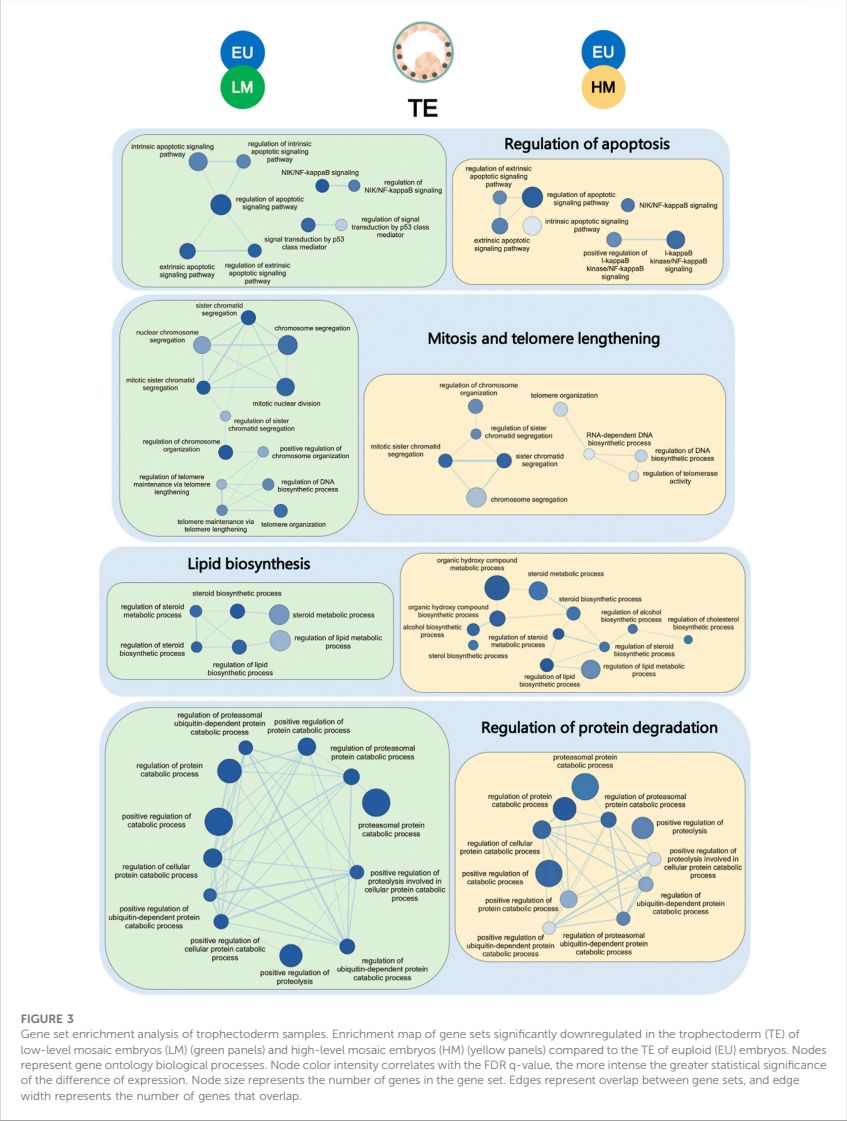
commonly significantly deregulated (upregulated or downregulated) when comparing euploid embryos to both low-level mosaic embryos and high-level mosaic embryos, respectively (Supplementary Table S3). 10 out of the 17 DEGs were deregulated in the TE (Figure 2E), and 7 DEGs were deregulated in the ICM (Figure 2F).

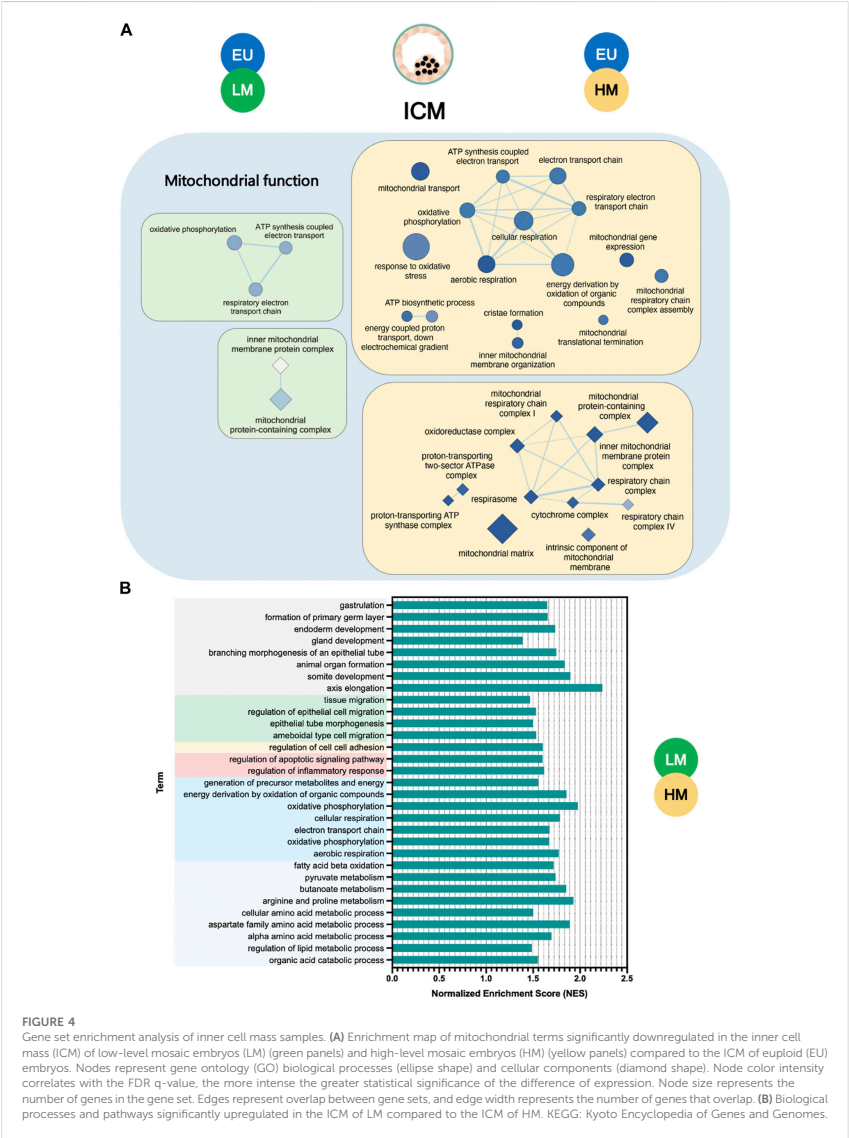
In addition, 10 DEGs were found commonly significantly deregulated in the ICM of high-level mosaic embryos, compared to the ICM of both euploid and low-level mosaic embryos (Supplementary Table S4). Finally, a total of 40 DEGs were found exclusively significantly deregulated in the ICM of high-level mosaic embryos compared to euploid embryos (Supplementary Table S5).

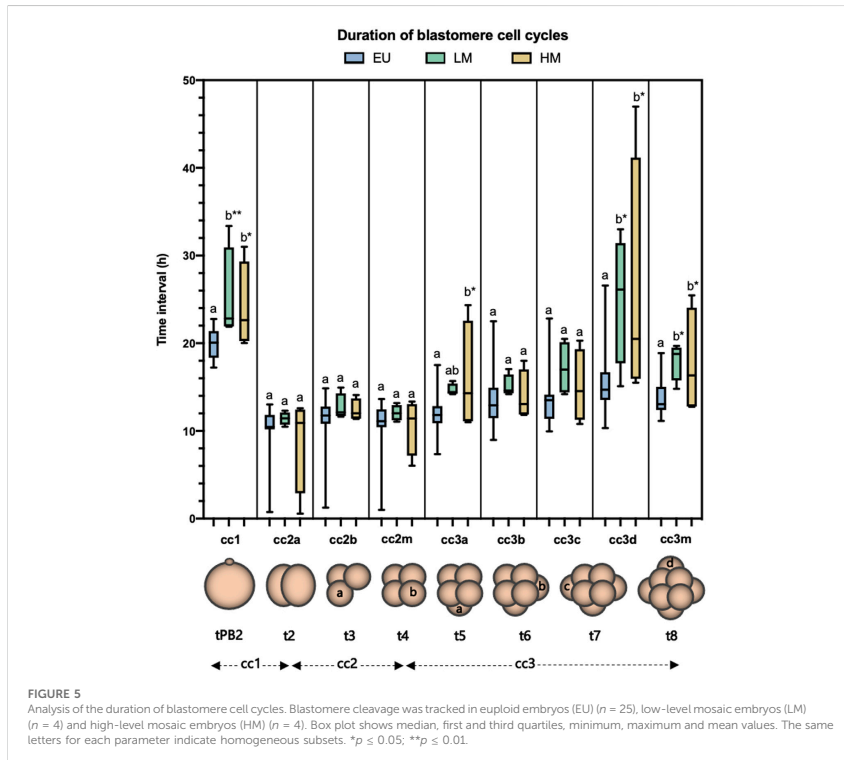
3.3 Functional enrichment analysis

3.3.1 Trophoblast cells

Since the TE cells of low-level mosaic embryos and high-level mosaic embryos had similar gene expression profiles (0 DEGs), we expected to find commonly deregulated pathways compared to euploid embryos. Indeed, gene set enrichment analysis revealed 124 terms that were commonly significantly deregulated in the TE of both low-level mosaic embryos and high-level mosaic embryos, compared to euploid controls. 85 out of the 124 (68.6%) deregulated terms were GO-Biological Process (GO-BP), 27 (21.8%) were GO-Cellular Component (GO-CC), 7 (5.6%) were







GO-Molecular Function (GO-MF), and 5 (4%) were KEGG pathways. These included chromosome segregation, ERAD pathway, regulation of apoptotic signaling pathway, and steroid biosynthesis (Supplementary Table S6).

Indeed, the enrichment map of terms significantly deregulated in the TE samples of mosaic embryos revealed a global disruption of pathways involved in the regulation of apoptosis, mitosis, telomere maintenance, biosynthesis of lipids and degradation of proteins, when compared to euploid controls (Figure 3). Other disrupted pathways included those involved in WNT intercellular signaling and in the generation of precursor metabolites and energy through the TCA cycle.

3.3.2 Inner cell mass cells

Unlike the response observed in TE cells, we found only 12 common terms, mainly involved in mitochondrial function, to be significantly deregulated in the ICM of low-level and high-level embryos, compared to euploid controls. The extent of disruption of mitochondrial pathways, as measured by the

number and statistical significance of downregulated terms, was greater in high-level mosaic embryos than in low-level mosaic embryos when compared to euploid controls (Figure 4A).

In fact, gene set enrichment analysis revealed key significant transcriptome differences (134 terms) between the ICM of low-level and high-level mosaic embryos (Supplementary Table S7). Out of the 134 terms, 130 (97%) were upregulated in low-level mosaic embryos and were related to energy production, cellular respiration, embryonic development, cell migration and adhesion, metabolism of organic compounds, regulation of apoptosis, and inflammatory response (Figure 4B).

3.4 Validation of RNA-seq results by quantitative PCR

qRT-PCR was performed on a selection of DEGs (*ARHGAP36*, *NELL1*, *NTRK3*, *VIM*, *CDH5*, and *LAMC3*) to validate the findings

of the RNA-seq experiment (Supplementary Figure S4). The results of the qRT-PCR analysis were consistent with those of RNA-seq, demonstrating significant differences in the expression of two of the six genes evaluated (*ARHGAP36* and *NELL1*), and similar trends in the remaining four genes (*NTRK3*, *VIM*, *CDHS*, and *LAMC3*). The lack of statistical significance in these genes may be due to natural variations in gene expression between individual samples.

3.5 Analysis of blastomere cell cycles by time lapse imaging

The duration of cc1 and cc3m was significantly longer in both low-level mosaic embryos ($n = 4$) (cc1 = 25.23 ± 5.49 h, $p = 0.004$; cc3m = 18.02 ± 2.19 h, $p = 0.02$) and high-level mosaic embryos ($n = 4$) (cc1 = 24.07 ± 4.97 h, $p = 0.027$; cc3m = 17.72 ± 6.13 h, $p = 0.03$), compared to euploid embryos ($n = 25$) (cc1 = 19.89 ± 1.68 h; cc3m = 13.68 ± 2.05 h). No significant morphokinetic differences were found between low-level mosaic embryos and high-level mosaic embryos (Figure 5).

4 Discussion

Most RNA-seq studies of the human preimplantation transcriptome have focused on investigating the transcriptional networks that govern the delineation of early cell lineages (Xue et al., 2013; Yan et al., 2013; Blakeley et al., 2015; Petropoulos et al., 2016; Boroviak et al., 2018; Stirparo et al., 2018; Meistermann et al., 2021). Other studies have attempted to identify molecular markers of embryonic competence by correlating transcriptome profiles with clinical variables related to human embryonic development, such as ploidy status or live birth potential (Kirkegaard et al., 2015; Licciardi et al., 2018; Groff et al., 2019; Ntostis et al., 2019; Sanchez-Ribas et al., 2019; Starostik et al., 2020). Furthermore, some groups have developed protocols for digital karyotyping based on gene expression data (Macaulay et al., 2015; Weissbein et al., 2016; Fan et al., 2018), some of which have been optimized for the detection of mosaic abnormalities (Griffiths et al., 2017; Starostik et al., 2020). These approaches are fundamental to understanding the molecular mechanisms of embryonic development and implantation capacity.

Transcriptome analysis of human preimplantation embryos has revealed a link between aneuploidy and different manifestations of cellular stress. Stress responses involving the deregulation of pathways related to cell proliferation, DNA damage, apoptosis, protein degradation, and mitochondrial energy metabolism have been reported in different types of samples, including whole embryos, TE biopsies, and even single cells (Regin et al., 2022). These findings underscore the complex interplay between aneuploidy and cellular stress, and suggest that the molecular mechanisms underlying this relationship are multifaceted. However, while the stress caused by the presence of aneuploid cells may compromise early development, it does not necessarily induce embryonic arrest, as both meiotic and mitotic errors can persist to the blastocyst stage (McCoy et al., 2015).

To the best of our knowledge, only one previous study has assessed the transcriptome profile of blastocysts diagnosed as mosaic after NGS-based PGT-A (Maxwell et al., 2022). In such study, global gene expression was progressively disrupted from euploid embryos (used as a control for comparisons) to mosaic embryos (with chromosomal copy numbers ranging from 20% to 80% in the TE biopsy) and aneuploid embryos (which shared the same aneuploid chromosomes as mosaic embryos).

Our aim was to investigate the impact of different levels of putative mosaicism on the gene expression profile of human blastocysts. In contrast to the previous study, all embryos were subjected to the same NGS protocol and were separated into ICM and TE fractions before RNA-seq analysis. We believe this approach to be more informative considering the critically different roles played by the ICM and the TE in promoting embryogenesis and the initiation and maintenance of implantation, respectively. Additionally, in line with recent observations regarding the reproductive capabilities of mosaic embryos (Capalbo et al., 2022), we used a threshold of 50% to differentiate between low-level and high-level mosaic embryo populations.

One important difference between our study and the previous work (Maxwell et al., 2022) is that we were unable to obtain low-level and high-level mosaic embryos with the same aneuploidies. This can be attributed to the low incidence of mosaicism at the blastocyst stage and the uncertainty surrounding the management of mosaic embryos, which led us to contact only those patients with supernumerary embryos and favorable clinical outcomes to participate in our research. Although three out of the four aneuploidies of high-level mosaic embryos were also present in low-level mosaic embryos, our data may be biased by chromosome-specific gene expression. To minimize this confounding effect, we performed gene set enrichment maps and identified global transcriptome responses to multiple aneuploidies, rather than analyzing the effects of each aneuploidy separately.

In our cohort, we found no significant association between PCAs and PGT-A categories. However, cluster heatmaps revealed a clear separation of samples according to the original diagnosis in all comparisons. The day of embryo biopsy, as well as the morphological grade of embryos, were similarly distributed across the experimental groups. This suggests that, despite the potential effect of natural variations in gene expression of individual samples or factors with undefined roles in embryo development, the original mosaic diagnoses were predictive of the remaining embryonic fractions.

These findings are supported by the identification of morphokinetic delays in several cell cycle timings within the same mosaic embryos analyzed by RNA-seq. It has been demonstrated that mitotic errors during early cell divisions can result in slower cleavage and longer cell cycles (Chavez et al., 2012), potentially due to the spindle assembly checkpoint stalling mitosis caused by incorrect chromatid alignments (Regin et al., 2022). Interestingly, the duration of cell cycles in embryos with both low-level and high-level mosaic results have been studied in detail, and timings have been found to overlap with those of euploid and aneuploid embryos (Martin et al., 2021). However, a more effective strategy for identifying cell cycle delays by TLI and providing clues about the presence of

mosaicism is to track blastomere divisions individually (Grau et al., 2015).

Compared to euploid embryos, which were used as control, the gene expression distance based on the number of DEGs increased with level of mosaicism. This increase was statistically significant only in ICM samples, whereas the TE samples from low-level and high-level mosaic embryos displayed virtually equivalent gene expression profiles. Several genes related to embryo development were commonly found to be significantly deregulated in mosaic embryos, compared to euploid controls. These included ICM-related genes, such as *TRIM36* (which has been linked to chromosomal instability when overexpressed) (Miyajima et al., 2009) and *SPATA20* (which is impaired in infertile patients) (Omolaoye et al., 2022); as well as TE-related genes such as *NELL1* (which plays a role in protein modification and cellular metabolism in human preimplantation embryos) (Sun et al., 2022).

Accordingly, gene set enrichment analysis revealed a common set of pathways that were significantly deregulated in mosaic embryos compared to euploid controls. These pathways included those deregulated in TE cells such as apoptosis, cell proliferation, metabolism and protein degradation, as well as pathways deregulated in ICM cells, which were primarily related to mitochondrial function. These pathways were similar to those found in the previous study (Maxwell et al., 2022). Additionally, our findings align with previous observations demonstrating that, although the impact of mosaicism on gene expression is largely dependent on the affected chromosomes (Licciardi et al., 2018; Ntostis et al., 2019; Sanchez-Ribas et al., 2019), some reproducible effects may exist regardless of the specific chromosomal aberration (Sheltzer, 2013; Zhu et al., 2018).

The deregulation of pathways involved in apoptosis and cell proliferation observed in our study supports previous immunofluorescence studies performed in the mouse, which indicate that mosaic embryos undergo apoptosis and anti-proliferative mechanisms during the blastocyst stage (Bolton et al., 2016; Singla et al., 2020). Interestingly, in human embryos, these mechanisms appear to occur more frequently in the TE than in the ICM, as opposed to mouse embryos (Victor et al., 2019), which is also consistent with our findings. It is indeed possible that the incidence of apoptosis in human TE cells has been underestimated by the use of a single pluripotency marker (Victor et al., 2019), since such cells can retain the expression of pluripotency genes even after blastocyst expansion (Gerri et al., 2020). Furthermore, the deregulation of *p53* may serve as a specific indicator of chromosome segregation errors in apoptotic pathways (Tang, Y. et al., 2011).

Moreover, the perturbation of metabolic processes observed in the TE of mosaic embryos may be attributed to the altered stoichiometry of enzymes and their regulators caused by aneuploidy (Zhu et al., 2018; Chunduri and Storchová, 2019; Groff et al., 2019). The disruption of pathways involved in protein degradation by the ubiquitin-proteasome system in the TE of mosaic blastocysts suggests proteotoxic stress resulting from the accumulation of unfolded proteins, which is a well-known consequence of transcriptome deregulation (Walter and Ron, 2011). Additionally, deregulation of telomere elongation pathways in the TE might reflect potential differences in telomere maintenance dynamics across cell lineages, as reported in other studies (Iqbal et al., 2011; Varela et al., 2011; Kalmbach et al., 2014).

At the ICM level, pathways related to mitochondria were found to be commonly deregulated in both low-level and high-level mosaic embryos, when compared to euploid controls. The presence of aneuploid cells in the embryo could lead to mitochondrial dysfunction through various mechanisms. For instance, trisomy 21 can alter the expression of genes involved in mitochondrial biogenesis and function, which in turn can result in mitochondrial defects (Izzo et al., 2018). Additionally, chromosomal alterations can lead to the accumulation of reactive oxygen species, which can disrupt mitochondrial membrane potential, reduce ATP production, and impair the electron transport chain, ultimately leading to cellular stress (Zhu et al., 2018).

Unlike what was observed in TE cells, the gene expression profiles of low-level mosaic embryos and high-level mosaic embryos differed significantly at the ICM level. This difference in gene expression involved the significant upregulation of the genes *ALDH2*, *GPX2*, and *CDH5* in low-level mosaic embryos. These genes are key for embryo implantation and pregnancy (Zhou et al., 1997; Roland-Zejly et al., 2011; Sata et al., 2012; Kinnear et al., 2019). Indeed, they belong to the "embryo implantation universe" proposed by (Sanchez-Ribas et al., 2019). Intriguingly, these genes were also significantly upregulated in euploid embryos compared to high-level mosaic embryos, but their expression levels were comparable between euploid embryos and low-level mosaic embryos. A similar expression pattern was observed in the hypoblast marker *SOX17*, supporting the notion that aneuploidy can alter cell fate decisions during early embryonic development and lead to defects in cell-lineage formation (Shahbazi et al., 2020).

Consistently, pathways involving the formation of the primary germ layer, endoderm development, energy production, metabolism of organic compounds, and regulation of apoptosis were significantly upregulated in the ICM of low-level mosaic embryos compared to high-level mosaic embryos.

Taken together, these findings challenge the notion that embryos with mosaic results are simply a by-product of misdiagnosis due to false positive mosaic calls. While this might be the most common scenario, the reproductive potential of these embryos may also be explained by the complex interplay of molecular mechanisms which become deregulated in the presence of aneuploid cells. Our findings also suggest that within mosaic embryos, those with low-level mosaicism may exhibit transcriptome profiles more closely resembling those of euploid embryos, while high-level mosaic embryos represent a distinct biological category characterized by molecular features indicative of a greater extent and burden of the cellular stress arising from aneuploidy. These findings align well with current perspectives on the reproductive potential of mosaic embryos, and contribute to the growing body of evidence suggesting that low and high levels of mosaicism after PGT-A deserve distinct clinical management.

Some limitations of our study should be acknowledged. First, given the difficulty of obtaining embryos with the highest morphological quality for research purposes, our analysis was restricted to embryos graded B or C. In addition, global gene expression may exhibit high heterogeneity even among high-quality embryos at the same developmental stage (Shaw et al., 2013; Ntostis et al., 2019). This means that our results may not be generalizable to other embryo populations, especially those with

higher morphological scores. Furthermore, beyond the potential effect of chromosome-specific gene expression, the impact of mosaic aneuploidies on global transcriptome profiles may be confounded by specific adaptive mechanisms, particularly considering the extraordinary plasticity of early human development (Coticchio et al., 2021). Finally, while transcriptomics can serve as a sensitive indicator of cellular stress, it provides little to no information on protein activity. Therefore, our results should be interpreted with caution, and further studies focusing on protein are necessary to confirm these findings.

In conclusion, our study demonstrates that mosaic results following PGT-A may be accompanied by significant alterations in gene expression across blastocyst compartments as well as by significant delays in cell cycle timings during the first mitotic divisions. The level of mosaicism was associated with the extent of transcriptome deregulation, and pathways involving apoptosis, mitosis, protein degradation, metabolism, and mitochondrial energy production were among the most deregulated within mosaic embryos. We believe that these findings contribute towards the molecular characterization of mosaic embryos and offer new insights into the factors that determine their developmental potential.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: Gene Expression Omnibus (GEO); GSE194306 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE194306>].

Ethics statement

The studies involving human participants were reviewed and approved by the Clinical Research Ethics Committee of IVI-RMA Valencia. IVI Foundation, Health Research Institute La Fe, Avenida Fernando Abril Martorell, 106-Biopol, Torre A, 1st floor. 1.03.46026 Valencia. Spain. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AMA and MD conceived the study and designed experiments. AP and MD identified and recruited patients. AMA, AMe, AQ, and

MP performed experiments. AMA, AQ, MP, AD, and AMi collected the data. AMA, FD, RG-M, and MD analyzed and interpreted the data. AMA and MD wrote and edited the manuscript. AP contributed to the final version of the manuscript. MD supervised the project and secured funding and ethical approvals. All authors approved the manuscript and provided critical feedback and discussion.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmolb.2023.1180689/full#supplementary-material>

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Supplemental Material

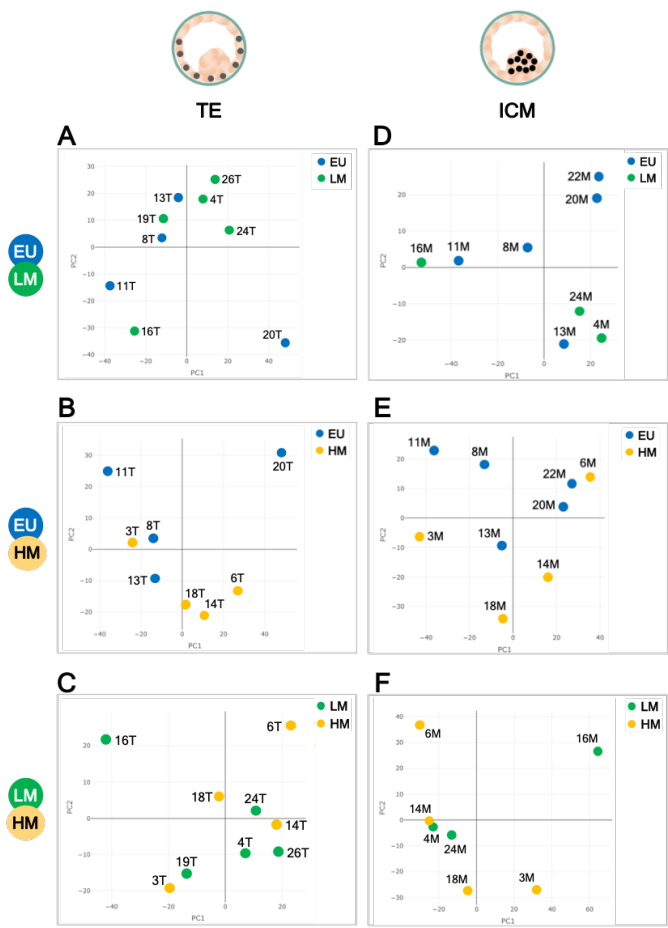
Mosaic results after preimplantation genetic testing for aneuploidy may be accompanied by changes in global gene expression

A. Martin, A. Mercader, F. Dominguez, A. Quiñonero, M. Perez, R. Gonzalez-Martin, A. Delgado, A. Mifsud, A. Pellicer, M.J. De los Santos*

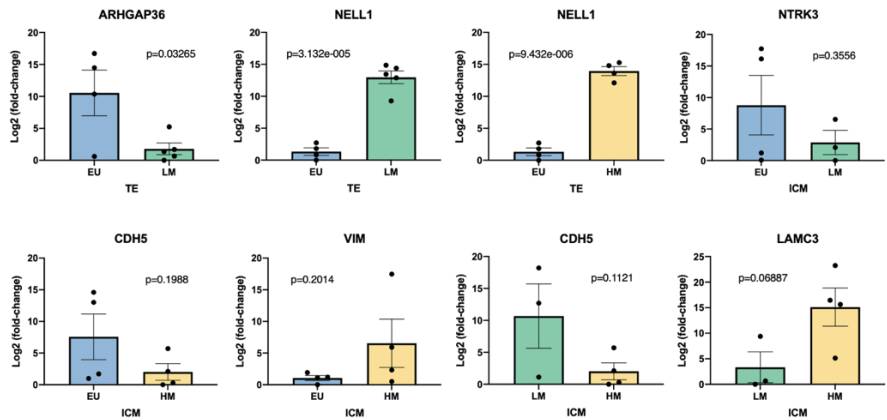
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Supplementary Figure 2. Principal Component Analysis. (A-C) Trophoctoderm comparisons (TE). (D-F) Inner cell mass comparisons (ICM). EU: euploid. LM: low-level mosaic. HM: high-level mosaic.



Supplementary Figure 4: Validation of RNA-seq by quantitative PCR. The expression levels of the selected genes ARHGAP36, NELL1, NTRK3, CDH5, VIM and LAMC3 were validated by quantitative PCR, quantified by the $\Delta\Delta CT$ method and expressed as fold regulation. Statistical analysis was performed by using a t-test for independent samples (two-tailed). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ were considered statistically significant. ICM: inner cell mass; TE: trophectoderm; EU: euploid; LM: low-level mosaic; HM: high-level mosaic.



1.2 Supplementary Tables

Supplementary Table 1. List of primer sequences used for the validation of the RNA-seq results by quantitative PCR. FW, forward primer; RV, reverse primer.

Name	Primer sequence (5'->3')
NTRK3 FW	GTGAGTCCCACACCTCCTATC
NTRK3 RV	AAGCAGCAAGTCCAAGTCTA
CDH5 FW	CCAGCCCAAAGTGTGTGAGA
CDH5 RV	TGGTATGCTCCCGGTCAAAC
VIM FW	GTGGACCAGCTAACCAACGA
VIM RV	GCCAGAGACGCATTGTCAAC
LAMC3 FW	AGCAATGTCCGTCCTGCTAC
LAMC3 RV	CCCCTCCGTCAAAGTCAGTC
ARHGAP36 FW	ACTTGGCCTTGGTGTGTTGGA
ARHGAP36 RV	ATCATGGCACGGACCACATT
NELL1 FW	CTTGGGATGGACCCTGACC
NELL1 RV	TCACATGAGGAGCTGCATGG

Supplementary Table 2. List of all differentially expressed genes found across the six comparisons analyzed. Genes with a Benjamini-Hochberg adjusted p-value (padj) < 0.05 and a log2foldchange of ± 2 was considered significantly differentially expressed. log2FoldChange > 0 indicates significant upregulation in the first comparison factor. EU: euploid. LM: low-level mosaic. HM: high-level mosaic. TE: trophectoderm. ICM: inner cell mass.

Fraction	Comparison	Gene symbol	Gene biotype	log2FoldChange	padj
TE	(C-1) EU-LM	ARHGAP36	protein coding	8,06	1,50E-04
TE	(C-1) EU-LM	KLF4P1	pseudogene	2,27	1,90E-02
TE	(C-1) EU-LM	PAGE5	protein coding	5,1	1,90E-02
TE	(C-1) EU-LM	MTND6P4	pseudogene	4,15	2,60E-02
TE	(C-1) EU-LM	RPP21	protein coding	3,47	2,70E-02
TE	(C-1) EU-LM	GUSBP4	pseudogene	2,08	3,00E-02
TE	(C-1) EU-LM	DGKK	protein coding	4,86	3,30E-02
TE	(C-1) EU-LM	CXorf49	protein coding	4,08	4,10E-02
TE	(C-1) EU-LM	RBMV1J	protein coding	-10,55	5,30E-06
TE	(C-1) EU-LM	RPL7P16	pseudogene	-7,11	1,00E-04
TE	(C-1) EU-LM	RBMV1F	protein coding	-9,13	1,50E-04
TE	(C-1) EU-LM	RBMV1B	protein coding	-9,54	1,50E-04
TE	(C-1) EU-LM	CNR1	protein coding	-3,25	2,20E-04
TE	(C-1) EU-LM	TXLNGY	pseudogene	-11,76	3,60E-04
TE	(C-1) EU-LM	AC004556.3	protein coding	-6,12	4,20E-04
TE	(C-1) EU-LM	LYRM9	protein coding	-4,53	5,80E-04
TE	(C-1) EU-LM	KLHL3	protein coding	-5,42	6,30E-04
TE	(C-1) EU-LM	TSPY10	protein coding	-8,86	6,30E-04
TE	(C-1) EU-LM	NELL1	protein coding	-7,86	1,80E-03
TE	(C-1) EU-LM	LRRC17	protein coding	-6,73	4,80E-03
TE	(C-1) EU-LM	NLRP13	protein coding	-4,58	8,20E-03
TE	(C-1) EU-LM	TSPY1	protein coding	-8,59	8,20E-03
TE	(C-1) EU-LM	RBMV2FP	pseudogene	-7,62	1,00E-02
TE	(C-1) EU-LM	PKDCC	protein coding	-4,04	1,20E-02
TE	(C-1) EU-LM	HAL	protein coding	-7,41	1,70E-02
TE	(C-1) EU-LM	COL24A1	protein coding	-6,56	1,80E-02
TE	(C-1) EU-LM	RBMV1E	protein coding	-6,73	1,80E-02
TE	(C-1) EU-LM	CPNE4	protein coding	-4,27	1,90E-02
TE	(C-1) EU-LM	KBTD3	protein coding	-2,18	2,10E-02
TE	(C-1) EU-LM	MAGEA8	protein coding	-6,44	2,60E-02
TE	(C-1) EU-LM	THBS4	protein coding	-4,12	2,70E-02
TE	(C-1) EU-LM	IGKC	protein coding	-2,08	4,00E-02
TE	(C-1) EU-LM	NCR1	protein coding	-7,34	4,50E-02
TE	(C-2) EU-HM	SKA1	protein coding	22,93	1,20E-07

TE	(C-2) EU-HM	SLC6A9	protein coding	3,73	9,40E-05
TE	(C-2) EU-HM	IL32	protein coding	4,68	2,50E-04
TE	(C-2) EU-HM	MTND4P12	pseudogene	2,93	7,00E-04
TE	(C-2) EU-HM	ASS1	protein coding	2,9	1,20E-03
TE	(C-2) EU-HM	TIMP3	protein coding	5,19	1,20E-03
TE	(C-2) EU-HM	DGKK	protein coding	4,79	6,70E-03
TE	(C-2) EU-HM	TRPV2	protein coding	5,64	1,60E-02
TE	(C-2) EU-HM	TMEM176B	protein coding	5,69	2,00E-02
TE	(C-2) EU-HM	RASGRP1	protein coding	3,07	2,00E-02
TE	(C-2) EU-HM	C4orf33	protein coding	2,42	2,90E-02
TE	(C-2) EU-HM	ITPRIP	protein coding	3,26	3,10E-02
TE	(C-2) EU-HM	CYP19A1	protein coding	4,61	3,50E-02
TE	(C-2) EU-HM	POGLUT1	protein coding	2,42	3,50E-02
TE	(C-2) EU-HM	LOXL4	protein coding	7,78	3,80E-02
TE	(C-2) EU-HM	CHST5	protein coding	-3,95	1,70E-09
TE	(C-2) EU-HM	ATP8A1	protein coding	-3,77	5,30E-07
TE	(C-2) EU-HM	NELL1	protein coding	-8,61	1,20E-06
TE	(C-2) EU-HM	ABCA1	protein coding	-4,29	2,40E-05
TE	(C-2) EU-HM	CCN2	protein coding	-2,26	4,50E-04
TE	(C-2) EU-HM	TSPY8	protein coding	-9,53	7,90E-04
TE	(C-2) EU-HM	RSPO2	protein coding	-4,47	9,40E-04
TE	(C-2) EU-HM	TSPY1	protein coding	-9,24	1,20E-03
TE	(C-2) EU-HM	AC013268.1	pseudogene	-7,4	1,20E-03
TE	(C-2) EU-HM	PKHD1L1	protein coding	-4,53	1,90E-03
TE	(C-2) EU-HM	GABRA3	protein coding	-6,16	2,50E-03
TE	(C-2) EU-HM	ADCY5	protein coding	-5,08	2,50E-03
TE	(C-2) EU-HM	CPNE4	protein coding	-5,57	2,80E-03
TE	(C-2) EU-HM	ZP4	protein coding	-3,85	1,10E-02
TE	(C-2) EU-HM	GYG2P1	pseudogene	-4,05	1,10E-02
TE	(C-2) EU-HM	TSPY9P	protein coding	-8,46	1,40E-02
TE	(C-2) EU-HM	RPL7P16	pseudogene	-7,35	1,40E-02
TE	(C-2) EU-HM	TEX101	protein coding	-5,48	1,60E-02
TE	(C-2) EU-HM	MAGEA8	protein coding	-8,25	2,00E-02
TE	(C-2) EU-HM	TXLNGY	pseudogene	-12,2	2,00E-02
TE	(C-2) EU-HM	SLC30A8	protein coding	-3,93	2,00E-02
TE	(C-2) EU-HM	AC008758.1	protein coding	-8,11	2,00E-02
TE	(C-2) EU-HM	GRIN2B	protein coding	-5,98	2,70E-02
TE	(C-2) EU-HM	LEF1	protein coding	-3,94	2,90E-02
TE	(C-2) EU-HM	CD37	protein coding	-3,19	2,90E-02

TE	(C-2) EU-HM	HBZ	protein coding	-7,22	2,90E-02
TE	(C-2) EU-HM	SHISAL2B	protein coding	-6,65	2,90E-02
TE	(C-2) EU-HM	TSPY3	protein coding	-8,1	2,90E-02
TE	(C-2) EU-HM	MARCHF2	protein coding	-2,05	3,10E-02
TE	(C-2) EU-HM	TNFRSF10C	protein coding	-6,97	3,10E-02
TE	(C-2) EU-HM	GAGE2E	protein coding	-8,04	3,10E-02
TE	(C-2) EU-HM	EBF1	protein coding	-5,3	3,30E-02
TE	(C-2) EU-HM	PIMREG	protein coding	-4,33	3,30E-02
TE	(C-2) EU-HM	PHACTR3	protein coding	-3,52	3,80E-02
TE	(C-2) EU-HM	SRRM3	protein coding	-5,72	3,80E-02
TE	(C-2) EU-HM	NLRP13	protein coding	-4,2	3,80E-02
TE	(C-2) EU-HM	PDGFRL	protein coding	-3,48	4,10E-02
TE	(C-2) EU-HM	RBMV2FP	pseudogene	-7,86	4,10E-02
TE	(C-2) EU-HM	TDRD6	protein coding	-2,36	4,10E-02
TE	(C-2) EU-HM	RBMV1J	protein coding	-10,76	4,30E-02
ICM	(C-4) EU-LM	GJB4	protein coding	22,53	3,70E-09
ICM	(C-4) EU-LM	PPP1R11	protein coding	25,41	3,70E-09
ICM	(C-4) EU-LM	ADGRD1	protein coding	3,01	1,40E-06
ICM	(C-4) EU-LM	HTR2C	protein coding	8,54	7,10E-06
ICM	(C-4) EU-LM	MTND4P12	pseudogene	2,58	2,00E-04
ICM	(C-4) EU-LM	NTRK3	protein coding	5,54	2,90E-04
ICM	(C-4) EU-LM	WWC1	protein coding	2,16	1,10E-03
ICM	(C-4) EU-LM	SVEP1	protein coding	5,64	1,10E-03
ICM	(C-4) EU-LM	PORCN	protein coding	2,16	4,70E-03
ICM	(C-4) EU-LM	CHMP1B2P	pseudogene	4,21	4,80E-03
ICM	(C-4) EU-LM	MLYCD	protein coding	2,34	4,90E-03
ICM	(C-4) EU-LM	C10orf90	protein coding	8,38	5,30E-03
ICM	(C-4) EU-LM	MTND1P32	pseudogene	7,45	5,30E-03
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ICM	(C-4) EU-LM	NOTCH2NLB	protein coding	2,02	6,40E-03
ICM	(C-4) EU-LM	SLC12A7	protein coding	2,58	1,30E-02
ICM	(C-4) EU-LM	LRRC6	protein coding	2,32	1,40E-02
ICM	(C-4) EU-LM	AC080023.2	pseudogene	8,52	2,10E-02
ICM	(C-4) EU-LM	BX664615.2	protein coding	8,42	2,60E-02
ICM	(C-4) EU-LM	ANK1	protein coding	3,93	3,50E-02
ICM	(C-4) EU-LM	SPATA20	protein coding	8,33	3,80E-02
ICM	(C-4) EU-LM	ANKK1	protein coding	9,57	3,80E-02
ICM	(C-4) EU-LM	RNF14P1	pseudogene	7,77	4,00E-02
ICM	(C-4) EU-LM	RPL10P9	pseudogene	6,35	4,80E-02

ICM	(C-4) EU-LM	AC004556.3	protein coding	-6,54	3,10E-71
ICM	(C-4) EU-LM	BOC	protein coding	-8,02	7,00E-05
ICM	(C-4) EU-LM	TRIM36	protein coding	-7,32	1,40E-03
ICM	(C-4) EU-LM	FCGRT	protein coding	-5,36	1,80E-03
ICM	(C-4) EU-LM	AHNAK2	protein coding	-7,49	2,00E-02
ICM	(C-4) EU-LM	H2BC15	protein coding	-2,1	2,70E-02
ICM	(C-4) EU-LM	PCDH9	protein coding	-3,01	3,50E-02
ICM	(C-4) EU-LM	PIK3R2	protein coding	-2,38	3,70E-02
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ICM	(C-4) EU-LM	PMAIP1	protein coding	-2,05	4,00E-02
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ICM	(C-5) EU-HM	PLAU	protein coding	22,33	2,50E-11
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ICM	(C-5) EU-HM	GAGE12C	protein coding	24,64	9,60E-11
ICM	(C-5) EU-HM	LRRC20	protein coding	21,61	1,70E-09
ICM	(C-5) EU-HM	DRD2	protein coding	21,42	4,70E-09
ICM	(C-5) EU-HM	ANXA1	protein coding	22,62	5,80E-09
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ICM	(C-5) EU-HM	KLK13	protein coding	5,44	1,40E-03
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ICM	(C-5) EU-HM	MSTO2P	pseudogene	7,59	4,00E-03
ICM	(C-5) EU-HM	ALDH2	protein coding	2,22	6,60E-03
ICM	(C-5) EU-HM	CDH5	protein coding	8,18	6,60E-03
ICM	(C-5) EU-HM	C10orf90	protein coding	7,01	6,60E-03
ICM	(C-5) EU-HM	SEPTIN6	protein coding	6,61	6,70E-03
ICM	(C-5) EU-HM	HNRNPA1P16	pseudogene	6,8	9,60E-03
ICM	(C-5) EU-HM	GPR1	protein coding	2,89	1,00E-02

ICM	(C-5) EU-HM	AC093809.1	pseudogene	7,58	1,00E-02
ICM	(C-5) EU-HM	SLC6A9	protein coding	3,81	1,10E-02
ICM	(C-5) EU-HM	SPATA20	protein coding	7,24	2,80E-02
ICM	(C-5) EU-HM	CAMKV	protein coding	7,53	2,80E-02
ICM	(C-5) EU-HM	SOX17	protein coding	6,08	3,10E-02
ICM	(C-5) EU-HM	MT1H	protein coding	3,07	3,30E-02
ICM	(C-5) EU-HM	APOBEC3C	protein coding	5,48	3,30E-02
ICM	(C-5) EU-HM	PRDM11	protein coding	5,78	3,40E-02
ICM	(C-5) EU-HM	PAPOLB	protein coding	8,01	3,90E-02
ICM	(C-5) EU-HM	GBA3	pseudogene	7,34	4,10E-02
ICM	(C-5) EU-HM	VCY1B	protein coding	-27,01	4,80E-13
ICM	(C-5) EU-HM	DLK2	protein coding	-3,38	3,10E-05
ICM	(C-5) EU-HM	CCK	protein coding	-8,54	4,10E-05
ICM	(C-5) EU-HM	TXLNGY	pseudogene	-11,89	4,70E-05
ICM	(C-5) EU-HM	ACVR1C	protein coding	-4,83	7,30E-05
ICM	(C-5) EU-HM	VIM	protein coding	-8,8	2,10E-04
ICM	(C-5) EU-HM	FCGRT	protein coding	-5,31	2,00E-03
ICM	(C-5) EU-HM	JAG1	protein coding	-2,79	4,50E-03
ICM	(C-5) EU-HM	ITIH6	protein coding	-8,88	4,50E-03
ICM	(C-5) EU-HM	MORC1	protein coding	-8,12	5,30E-03
ICM	(C-5) EU-HM	TSPY1	protein coding	-8,77	5,30E-03
ICM	(C-5) EU-HM	CNNM2	protein coding	-2,37	6,60E-03
ICM	(C-5) EU-HM	ARHGEF28	protein coding	-6,27	8,30E-03
ICM	(C-5) EU-HM	TRIM36	protein coding	-6,53	1,40E-02
ICM	(C-5) EU-HM	H2BC20P	pseudogene	-4,64	1,50E-02
ICM	(C-5) EU-HM	CXCL16	protein coding	-2,78	2,10E-02
ICM	(C-5) EU-HM	NELL1	protein coding	-5,25	3,30E-02
ICM	(C-5) EU-HM	NPHP3-ACAD11	protein coding	-7,2	4,00E-02
ICM	(C-5) EU-HM	PON1	protein coding	-4,46	4,10E-02
ICM	(C-5) EU-HM	RBMY1J	protein coding	-10,68	4,40E-02
ICM	(C-6) LM-HM	PLAU	protein coding	9,75	8,40E-05
ICM	(C-6) LM-HM	PIMREG	protein coding	6,69	8,40E-05
ICM	(C-6) LM-HM	BOC	protein coding	8,19	3,20E-04
ICM	(C-6) LM-HM	GPX2	protein coding	5,39	5,70E-04
ICM	(C-6) LM-HM	SMLR1	protein coding	9,29	8,50E-04
ICM	(C-6) LM-HM	PIK3R5	protein coding	8,21	1,70E-03
ICM	(C-6) LM-HM	LGALS2	protein coding	6,97	6,70E-03
ICM	(C-6) LM-HM	CDH5	protein coding	8,31	8,50E-03
ICM	(C-6) LM-HM	LMLN2	protein coding	7,86	1,00E-02

ICM	(C-6) LM-HM	PK1A	protein coding	8,38	1,10E-02
ICM	(C-6) LM-HM	ALDH2	protein coding	2,92	1,40E-02
ICM	(C-6) LM-HM	MTMR11	protein coding	9,44	1,50E-02
ICM	(C-6) LM-HM	TRIM17	protein coding	5,16	1,50E-02
ICM	(C-6) LM-HM	GYPC	protein coding	6,34	1,70E-02
ICM	(C-6) LM-HM	FGA	protein coding	8,35	3,60E-02
ICM	(C-6) LM-HM	SOX17	protein coding	6,02	3,90E-02
ICM	(C-6) LM-HM	PYCR1	protein coding	2,17	4,40E-02
ICM	(C-6) LM-HM	TMEM107	protein coding	2,18	4,50E-02
ICM	(C-6) LM-HM	DLK2	protein coding	-4,65	1,80E-10
ICM	(C-6) LM-HM	GAGE2E	protein coding	-23,77	9,80E-07
ICM	(C-6) LM-HM	GAGE12F	protein coding	-4,03	4,80E-06
ICM	(C-6) LM-HM	LAMC3	protein coding	-4,49	1,20E-05
ICM	(C-6) LM-HM	PKHD1L1	protein coding	-5,98	1,20E-05
ICM	(C-6) LM-HM	HTR2C	protein coding	-9,28	2,00E-04
ICM	(C-6) LM-HM	SULT4A1	protein coding	-8,73	2,20E-04
ICM	(C-6) LM-HM	BX842568.1	pseudogene	-8,44	3,20E-04
ICM	(C-6) LM-HM	CCK	protein coding	-8,84	8,50E-04
ICM	(C-6) LM-HM	APOC4	protein coding	-9,17	3,10E-03
ICM	(C-6) LM-HM	RNF144B	protein coding	-6,28	3,20E-03
ICM	(C-6) LM-HM	IL22RA1	protein coding	-5,79	3,90E-03
ICM	(C-6) LM-HM	PON1	protein coding	-5,1	9,50E-03
ICM	(C-6) LM-HM	GAGE12J	protein coding	-2,94	1,50E-02
ICM	(C-6) LM-HM	ITIH6	protein coding	-9,19	1,70E-02
ICM	(C-6) LM-HM	KDM4E	protein coding	-4,39	1,70E-02
ICM	(C-6) LM-HM	PGGHG	protein coding	-8,11	1,80E-02
ICM	(C-6) LM-HM	C1QTNF9	protein coding	-6,49	2,20E-02
ICM	(C-6) LM-HM	DNASE1L1	protein coding	-5,43	3,10E-02
ICM	(C-6) LM-HM	AC016582.1	pseudogene	-5,22	3,30E-02
ICM	(C-6) LM-HM	ZNF19	protein coding	-3,02	3,80E-02
ICM	(C-6) LM-HM	CCL15-CCL14	protein coding	-8,76	3,90E-02

Supplementary Table 3. List of commonly differentially expressed genes found between euploid embryos (EU) and both low-level mosaic embryos (LM) and high-level mosaic embryos (HM). Genes with a Benjamini-Hochberg adjusted p-value (padj) < 0.05 and a log2foldchange of ± 2 was considered significantly differentially expressed. log2FoldChange > 0 indicates significant upregulation in the first comparison factor.

Fraction	Category	Gene Set Enrichment Term	NES		padj	
			(C1)	(C2)	(C1)	(C2)
			EU-LM	EU-HM	EU-LM	EU-HM
TE	BP	GO:0090630-activation of gtpase activity	1,67	1,8	2,99E-02	1,46E-02
TE	BP	GO:0019882-antigen processing and presentation	1,5	1,58	1,90E-02	2,59E-02
TE	BP	GO:0042590-antigen processing and presentation of exogenous peptide antigen via mhc class i	1,62	1,73	4,03E-02	4,53E-02
TE	BP	GO:0198738-cell cell signaling by wnt	1,58	1,35	3,36E-04	3,62E-02
TE	BP	GO:0044839-cell cycle g2 m phase transition	1,8	1,57	8,91E-05	1,32E-02
TE	BP	GO:0006884-cell volume homeostasis	1,79	1,89	2,38E-02	4,37E-02
TE	BP	GO:0006520-cellular amino acid metabolic process	1,52	1,95	1,28E-02	1,66E-05
TE	BP	GO:0042180-cellular ketone metabolic process	1,48	1,64	4,63E-02	2,10E-02
TE	BP	GO:0022412-cellular process involved in reproduction in multicellular organism	-1,53	-1,51	6,40E-03	2,64E-02
TE	BP	GO:0071453-cellular response to oxygen levels	1,88	1,57	6,32E-05	1,18E-02
TE	BP	GO:0035967-cellular response to topologically incorrect protein	1,78	1,85	1,78E-03	3,58E-03
TE	BP	GO:1902476-chloride transmembrane transport	-1,64	-1,78	3,23E-02	3,71E-02
TE	BP	GO:0007059-chromosome segregation	1,5	1,43	6,75E-03	3,54E-02
TE	BP	GO:0016569-covalent chromatin modification	1,81	1,42	2,93E-07	2,13E-02
TE	BP	GO:0050906-detection of stimulus involved in sensory perception	-1,61	-1,72	6,62E-03	2,51E-03
TE	BP	GO:0010256-endomembrane system organization	1,59	1,73	5,53E-04	4,86E-05
TE	BP	GO:0030968-endoplasmic reticulum unfolded protein response	1,78	1,89	3,96E-03	4,94E-03
TE	BP	GO:0036503-erad pathway	1,66	1,74	2,16E-02	3,62E-02
TE	BP	GO:0097191-extrinsic apoptotic signaling pathway	1,65	1,63	1,93E-03	1,33E-02
TE	BP	GO:0009566-fertilization	-1,55	-1,9	4,95E-02	5,01E-04
TE	BP	GO:0048144-fibroblast proliferation	1,75	1,66	1,30E-02	4,53E-02

TE	BP	GO:0006091-generation of precursor metabolites and energy	1,4	1,65	1,54E-02	1,26E-04
TE	BP	GO:0007281-germ cell development	-1,56	-1,54	1,30E-02	3,62E-02
TE	BP	GO:0007030-golgi organization	1,68	1,64	9,88E-03	4,07E-02
TE	BP	GO:0007249-i kappa b kinase nf kappa b signaling	1,57	1,69	1,06E-02	5,77E-03
TE	BP	GO:0097193-intrinsic apoptotic signaling pathway	1,52	1,45	1,38E-02	4,83E-02
TE	BP	GO:0048232-male gamete generation	-1,74	-1,72	8,16E-06	1,95E-05
TE	BP	GO:0033619-membrane protein proteolysis	1,84	1,87	1,53E-02	3,35E-02
TE	BP	GO:0000070-mitotic sister chromatid segregation	1,83	1,79	6,59E-04	4,79E-03
TE	BP	GO:0034660-ncrna metabolic process	2,3	1,36	1,26E-07	4,53E-02
TE	BP	GO:1902532-negative regulation of intracellular signal transduction	1,77	1,51	1,36E-06	4,43E-03
TE	BP	GO:0043409-negative regulation of mapk cascade	1,9	1,56	2,29E-04	4,83E-02
TE	BP	GO:0042326-negative regulation of phosphorylation	1,66	1,38	2,22E-04	3,54E-02
TE	BP	GO:0038061-nik nf kappa b signaling	1,92	1,77	3,30E-04	9,49E-03
TE	BP	GO:0036499-perk mediated unfolded protein response	1,86	2,04	4,99E-02	2,81E-02
TE	BP	GO:0009896-positive regulation of catabolic process	1,8	1,54	3,81E-06	4,79E-03
TE	BP	GO:0043123-positive regulation of i kappa b kinase nf kappa b signaling	1,68	1,66	3,60E-03	1,70E-02
TE	BP	GO:0045732-positive regulation of protein catabolic process	1,92	1,58	2,22E-05	2,87E-02
TE	BP	GO:0045862-positive regulation of proteolysis	1,78	1,51	1,34E-05	1,36E-02
TE	BP	GO:1903052-positive regulation of proteolysis involved in cellular protein catabolic process	1,81	1,61	2,23E-03	4,48E-02
TE	BP	GO:1901522-positive regulation of transcription from rna polymerase ii promoter involved in cellular response to chemical stimulus	1,95	1,94	2,83E-02	4,76E-02
TE	BP	GO:2000060-positive regulation of ubiquitin dependent protein catabolic process	1,86	1,66	2,78E-03	4,53E-02
TE	BP	GO:0043687-post translational protein modification	1,55	1,44	2,84E-03	3,00E-02
TE	BP	GO:0010498-proteasomal protein catabolic process	1,87	1,78	1,26E-07	5,28E-06
TE	BP	GO:0034502-protein localization to chromosome	2,11	1,81	2,22E-04	2,11E-02

TE	BP	GO:0070646-protein modification by small protein removal	1,7	1,44	4,62E-04	4,31E-02
TE	BP	GO:0007265-ras protein signal transduction	1,42	1,47	1,38E-02	1,54E-02
TE	BP	GO:2001233-regulation of apoptotic signaling pathway	1,79	1,61	1,13E-05	1,59E-03
TE	BP	GO:1902749-regulation of cell cycle g2 m phase transition	1,86	1,64	1,35E-04	1,16E-02
TE	BP	GO:1903362-regulation of cellular protein catabolic process	1,85	1,68	3,39E-05	4,43E-03
TE	BP	GO:0045540-regulation of cholesterol biosynthetic process	1,97	2,41	1,11E-02	1,06E-04
TE	BP	GO:0033044-regulation of chromosome organization	1,7	1,5	3,03E-04	1,72E-02
TE	BP	GO:0051090-regulation of dna binding transcription factor activity	1,44	1,61	1,30E-02	7,21E-04
TE	BP	GO:2000278-regulation of dna biosynthetic process	1,68	1,66	1,59E-02	4,12E-02
TE	BP	GO:2001236-regulation of extrinsic apoptotic signaling pathway	1,75	1,73	2,59E-03	1,61E-02
TE	BP	GO:0046890-regulation of lipid biosynthetic process	1,73	1,94	3,13E-03	4,65E-04
TE	BP	GO:0019216-regulation of lipid metabolic process	1,41	1,49	3,13E-02	1,70E-02
TE	BP	GO:0019219-regulation of nucleobase containing compound metabolic process	1,69	1,36	9,17E-06	3,62E-02
TE	BP	GO:0061136-regulation of proteasomal protein catabolic process	1,91	1,75	1,32E-04	4,42E-03
TE	BP	GO:0032434-regulation of proteasomal ubiquitin dependent protein catabolic process	1,94	1,74	3,70E-04	1,39E-02
TE	BP	GO:0042176-regulation of protein catabolic process	1,92	1,68	1,31E-07	5,26E-04
TE	BP	GO:0071900-regulation of protein serine threonine kinase activity	1,32	1,35	4,95E-02	4,53E-02
TE	BP	GO:0031647-regulation of protein stability	1,47	1,59	3,15E-02	6,83E-03
TE	BP	GO:0010469-regulation of signaling receptor activity	-1,51	-1,6	5,18E-03	7,26E-04
TE	BP	GO:0033045-regulation of sister chromatid segregation	1,64	1,8	3,15E-02	1,63E-02
TE	BP	GO:0062012-regulation of small molecule metabolic process	1,72	1,77	1,16E-04	1,26E-04
TE	BP	GO:0050810-regulation of steroid biosynthetic process	1,9	2,27	3,60E-03	4,12E-05
TE	BP	GO:0019218-regulation of steroid metabolic process	1,72	2,12	5,32E-03	2,57E-04
TE	BP	GO:2000058-regulation of ubiquitin dependent protein catabolic process	1,89	1,69	2,84E-04	2,59E-02
TE	BP	GO:0030111-regulation of wnt signaling pathway	1,62	1,41	8,07E-04	4,83E-02

TE	BP	GO:0034976-response to endoplasmic reticulum stress	1,68	1,96	9,68E-04	5,28E-06
TE	BP	GO:0009408-response to heat	1,69	1,62	3,76E-03	3,62E-02
TE	BP	GO:0009314-response to radiation	1,47	1,54	6,74E-03	2,51E-03
TE	BP	GO:0035966-response to topologically incorrect protein	1,87	2,06	3,27E-04	7,64E-06
TE	BP	GO:0034612-response to tumor necrosis factor	1,44	1,43	3,56E-02	4,92E-02
TE	BP	GO:0007606-sensory perception of chemical stimulus	-1,59	-1,76	5,05E-03	1,08E-03
TE	BP	GO:0007608-sensory perception of smell	-1,54	-1,63	3,14E-02	1,49E-02
TE	BP	GO:0000819-sister chromatid segregation	1,84	1,78	3,08E-04	3,60E-03
TE	BP	GO:0006694-steroid biosynthetic process	1,85	2,32	6,02E-04	3,83E-07
TE	BP	GO:0008202-steroid metabolic process	1,46	1,92	2,12E-02	5,28E-06
TE	BP	GO:0016126-sterol biosynthetic process	2,11	2,55	3,36E-04	9,98E-07
TE	BP	GO:0032200-telomere organization	1,71	1,61	6,21E-03	4,24E-02
TE	BP	GO:0006099-tricarboxylic acid cycle	2,16	2,09	1,42E-03	6,76E-03
TE	BP	GO:0030433-ubiquitin dependent erad pathway	1,68	1,78	4,95E-02	2,87E-02
TE	BP	GO:0019058-viral life cycle	1,69	1,5	3,18E-04	1,63E-02
TE	CC	GO:0015629-actin cytoskeleton	1,26	1,41	3,92E-02	1,27E-02
TE	CC	GO:0101031-chaperone complex	2,12	1,89	3,63E-03	4,93E-02
TE	CC	GO:0034707-chloride channel complex	-1,78	-1,77	1,60E-02	4,45E-02
TE	CC	GO:0098687-chromosomal region	1,73	1,62	6,83E-05	4,48E-03
TE	CC	GO:0000775-chromosome centromeric region	1,42	1,51	4,60E-02	3,21E-02
TE	CC	GO:0097014-ciliary plasm	-1,7	-1,64	9,60E-03	3,98E-02
TE	CC	GO:0031461-cullin ring ubiquitin ligase complex	1,8	1,66	8,13E-04	1,51E-02
TE	CC	GO:0031045-dense core granule	-1,99	-2,09	5,76E-03	8,01E-03
TE	CC	GO:1905369-endorpeptidase complex	1,6	1,85	2,54E-02	1,76E-02
TE	CC	GO:0044440-endosomal part	1,72	1,5	1,78E-06	4,48E-03
TE	CC	GO:0101002-ficolin 1 rich granule	1,69	1,92	7,00E-03	2,91E-03
TE	CC	GO:0031300-intrinsic component of organelle membrane	1,43	1,56	1,79E-02	4,21E-03

TE	CC	GO:0000776-kinetochore	1,43	1,53	4,40E-02	4,33E-02
TE	CC	GO:0005770-late endosome	1,61	1,64	3,70E-03	4,72E-03
TE	CC	GO:0031902-late endosome membrane	1,66	1,57	9,95E-03	3,48E-02
TE	CC	GO:0043202-lysosomal lumen	1,54	1,85	3,29E-02	8,56E-03
TE	CC	GO:0005759-mitochondrial matrix	1,77	1,85	1,20E-06	1,31E-06
TE	CC	GO:0005635-nuclear envelope	1,59	1,46	9,24E-05	1,20E-02
TE	CC	GO:0034399-nuclear periphery	2,04	1,55	2,21E-05	4,33E-02
TE	CC	GO:0016607-nuclear speck	1,7	1,37	4,45E-05	3,98E-02
TE	CC	GO:0055037-recycling endosome	1,5	1,6	2,66E-02	1,76E-02
TE	CC	GO:0005819-spindle	1,51	1,46	4,24E-03	1,76E-02
TE	CC	GO:1990351-transporter complex	-1,47	-1,5	1,55E-02	1,81E-02
TE	CC	GO:0000151-ubiquitin ligase complex	1,75	1,56	6,41E-05	9,71E-03
TE	CC	GO:0005775-vacuolar lumen	1,48	1,53	2,33E-02	4,53E-02
TE	CC	GO:0005774-vacuolar membrane	1,79	1,53	3,05E-06	4,48E-03
TE	CC	GO:0031983-vesicle lumen	1,41	1,61	1,88E-02	4,48E-03
TE	MF	GO:0005254-chloride channel activity	-1,64	-1,75	2,64E-02	3,19E-02
TE	MF	GO:0005230-extracellular ligand gated ion channel activity	-1,76	-1,82	1,18E-02	1,62E-02
TE	MF	GO:0030594-neurotransmitter receptor activity	-1,74	-1,66	7,44E-03	3,89E-02
TE	MF	GO:0004984-olfactory receptor activity	-1,6	-1,54	1,16E-02	2,61E-02
TE	MF	GO:0022803-passive transmembrane transporter activity	-1,33	-1,45	4,51E-02	2,43E-02
TE	MF	GO:0030545-receptor regulator activity	-1,53	-1,6	2,58E-03	1,25E-03
TE	MF	GO:0022835-transmitter gated channel activity	-2,03	-2,1	1,59E-03	1,25E-03
TE	KEGG	00020_KEGG-citrate cycle tca cycle	2,39	2,26	1,85E-05	5,74E-04
TE	KEGG	11266_KEGG-lysosome	1,93	1,81	5,32E-04	4,98E-03
TE	KEGG	13380_KEGG-neuroactive ligand receptor interaction	-1,66	-1,53	2,39E-03	1,80E-02
TE	KEGG	5872_KEGG-steroid biosynthesis	2,02	2,32	1,28E-02	4,47E-04
TE	KEGG	19428_KEGG-wnt signaling pathway	1,59	1,49	2,12E-02	4,34E-02

Supplementary Table 4. List of commonly differentially expressed genes found in inner cell mass (ICM) samples between high-level mosaic embryos (HM) and both low-level mosaic embryos (LM) and euploid embryos (EU). Genes with a Benjamini-Hochberg adjusted p-value (padj) < 0.05 and a log2foldchange of ± 2 was considered significantly differentially expressed. log2FoldChange > 0 indicates significant upregulation in the first comparison factor.

Fraction	Gene symbol	Gene biotype	log2FoldChange		padj	
			(C-5)	(C-6)	(C-5)	(C-6)
			EU-HM	LM-HM	EU-HM	LM-HM
ICM	PLAU	protein coding	22,33	9,75	2,46E-11	8,37E-05
ICM	DLK2	protein coding	-3,38	-4,65	3,09E-05	1,76E-10
ICM	CCK	protein coding	-8,54	-8,84	4,12E-05	8,47E-04
ICM	SMLR1	protein coding	9,37	9,29	1,03E-04	8,47E-04
ICM	GPX2	protein coding	4,15	5,39	7,44E-04	5,74E-04
ICM	ITIH6	protein coding	-8,88	-9,19	4,52E-03	1,65E-02
ICM	ALDH2	protein coding	2,22	2,92	6,61E-03	1,40E-02
ICM	CDH5	protein coding	8,18	8,31	6,61E-03	8,49E-03
ICM	SOX17	protein coding	6,08	6,02	3,13E-02	3,90E-02
ICM	PON1	protein coding	-4,46	-5,1	4,08E-02	9,51E-03

Supplementary Table 5. List of exclusively differentially expressed genes found in inner cell mass (ICM) samples between euploid embryos (EU) and high-level mosaic embryos (HM). Genes with a Benjamini-Hochberg adjusted p-value (padj) < 0.05 and a log2foldchange of ± 2 was considered significantly differentially expressed. log2FoldChange > 0 indicates significant upregulation in the first comparison factor.

Fraction	Comparison	Gene symbol	Gene biotype	log2FoldChange	padj
ICM	(C-5) EU-HM	VCY1B	protein coding	-27,01	4,80E-13
ICM	(C-5) EU-HM	TRIM43	protein coding	22,19	3,55E-11
ICM	(C-5) EU-HM	GAGE12C	protein coding	24,64	9,64E-11
ICM	(C-5) EU-HM	LRRC20	protein coding	21,61	1,75E-09
ICM	(C-5) EU-HM	DRD2	protein coding	21,42	4,71E-09
ICM	(C-5) EU-HM	ANXA1	protein coding	22,62	5,80E-09
ICM	(C-5) EU-HM	TRIM49D1	protein coding	8,63	5,01E-07
ICM	(C-5) EU-HM	IFITM1	protein coding	3,13	1,46E-05
ICM	(C-5) EU-HM	TXLNGY	pseudogene	-11,89	4,67E-05
ICM	(C-5) EU-HM	ACVR1C	protein coding	-4,83	7,32E-05
ICM	(C-5) EU-HM	VIM	protein coding	-8,8	2,11E-04
ICM	(C-5) EU-HM	CPXM2	protein coding	6,91	3,26E-04
ICM	(C-5) EU-HM	SLC28A1	protein coding	7,45	3,26E-04
ICM	(C-5) EU-HM	SLPI	protein coding	5,58	9,06E-04

ICM	(C-5) EU-HM	KLK13	protein coding	5,44	1,36E-03
ICM	(C-5) EU-HM	GVINP1	pseudogene	3,67	1,36E-03
ICM	(C-5) EU-HM	GLRX	protein coding	3,94	1,69E-03
ICM	(C-5) EU-HM	HNRNPA1P21	pseudogene	6,1	2,04E-03
ICM	(C-5) EU-HM	MTND6P4	pseudogene	4,89	3,68E-03
ICM	(C-5) EU-HM	AP000356.3	pseudogene	7,74	3,75E-03
ICM	(C-5) EU-HM	MSTO2P	pseudogene	7,59	3,95E-03
ICM	(C-5) EU-HM	JAG1	protein coding	-2,79	4,52E-03
ICM	(C-5) EU-HM	MORC1	protein coding	-8,12	5,35E-03
ICM	(C-5) EU-HM	TSPY1	protein coding	-8,77	5,35E-03
ICM	(C-5) EU-HM	SEPTIN6	protein coding	6,61	6,69E-03
ICM	(C-5) EU-HM	ARHGEF28	protein coding	-6,27	8,34E-03
ICM	(C-5) EU-HM	HNRNPA1P16	pseudogene	6,8	9,60E-03
ICM	(C-5) EU-HM	GPR1	protein coding	2,89	1,01E-02
ICM	(C-5) EU-HM	AC093809.1	pseudogene	7,58	1,01E-02
ICM	(C-5) EU-HM	SLC6A9	protein coding	3,81	1,09E-02
ICM	(C-5) EU-HM	H2BC20P	pseudogene	-4,64	1,55E-02
ICM	(C-5) EU-HM	CXCL16	protein coding	-2,78	2,15E-02
ICM	(C-5) EU-HM	CAMKV	protein coding	7,53	2,83E-02
ICM	(C-5) EU-HM	NELL1	protein coding	-5,25	3,32E-02
ICM	(C-5) EU-HM	MT1H	protein coding	3,07	3,32E-02
ICM	(C-5) EU-HM	APOBEC3C	protein coding	5,48	3,32E-02
ICM	(C-5) EU-HM	PRDM11	protein coding	5,78	3,35E-02
ICM	(C-5) EU-HM	PAPOLB	protein coding	8,01	3,94E-02
ICM	(C-5) EU-HM	NPHP3-ACAD11	protein coding	-7,2	4,05E-02
ICM	(C-5) EU-HM	GBA3	pseudogene	7,34	4,08E-02

Supplementary Table 6. List of commonly significantly deregulated terms from functional enrichment analysis found in trophoctoderm (TE) samples between euploid embryos (EU) and both low-level mosaic embryos (LM) and high-level mosaic embryos (HM). padj: Benjamini-Hochberg adjusted p-value. KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: gene ontology; BP: biological process; CC: cellular component; MF: molecular function. Normalized enrichment score (NES) > 0 indicates overrepresentation of the term in the first comparison factor.

Fraction	Category	Gene Set Enrichment Term	NES		padj	
			(C1)	(C2)	(C1)	(C2)
			EU-LM	EU-HM	EU-LM	EU-HM
TE	BP	GO:0090630-activation of gtpase activity	1,67	1,8	2,99E-02	1,46E-02
TE	BP	GO:0019882-antigen processing and presentation	1,5	1,58	1,90E-02	2,59E-02
TE	BP	GO:0042590-antigen processing and presentation of exogenous peptide antigen via mhc class i	1,62	1,73	4,03E-02	4,53E-02
TE	BP	GO:0198738-cell cell signaling by wnt	1,58	1,35	3,36E-04	3,62E-02
TE	BP	GO:0044839-cell cycle g2 m phase transition	1,8	1,57	8,91E-05	1,32E-02
TE	BP	GO:0006884-cell volume homeostasis	1,79	1,89	2,38E-02	4,37E-02

TE	BP	GO:0006520-cellular amino acid metabolic process	1,52	1,95	1,28E-02	1,66E-05
TE	BP	GO:0042180-cellular ketone metabolic process	1,48	1,64	4,63E-02	2,10E-02
TE	BP	GO:0022412-cellular process involved in reproduction in multicellular organism	-1,53	-1,51	6,40E-03	2,64E-02
TE	BP	GO:0071453-cellular response to oxygen levels	1,88	1,57	6,32E-05	1,18E-02
TE	BP	GO:0035967-cellular response to topologically incorrect protein	1,78	1,85	1,78E-03	3,58E-03
TE	BP	GO:1902476-chloride transmembrane transport	-1,64	-1,78	3,23E-02	3,71E-02
TE	BP	GO:0007059-chromosome segregation	1,5	1,43	6,75E-03	3,54E-02
TE	BP	GO:0016569-covalent chromatin modification	1,81	1,42	2,93E-07	2,13E-02
TE	BP	GO:0050906-detection of stimulus involved in sensory perception	-1,61	-1,72	6,62E-03	2,51E-03
TE	BP	GO:0010256-endomembrane system organization	1,59	1,73	5,53E-04	4,86E-05
TE	BP	GO:0030968-endoplasmic reticulum unfolded protein response	1,78	1,89	3,96E-03	4,94E-03
TE	BP	GO:0036503-erad pathway	1,66	1,74	2,16E-02	3,62E-02
TE	BP	GO:0097191-extrinsic apoptotic signaling pathway	1,65	1,63	1,93E-03	1,33E-02
TE	BP	GO:0009566-fertilization	-1,55	-1,9	4,95E-02	5,01E-04
TE	BP	GO:0048144-fibroblast proliferation	1,75	1,66	1,30E-02	4,53E-02
TE	BP	GO:0006091-generation of precursor metabolites and energy	1,4	1,65	1,54E-02	1,26E-04
TE	BP	GO:0007281-germ cell development	-1,56	-1,54	1,30E-02	3,62E-02
TE	BP	GO:0007030-golgi organization	1,68	1,64	9,88E-03	4,07E-02
TE	BP	GO:0007249-i kappa b kinase nf kappa b signaling	1,57	1,69	1,06E-02	5,77E-03
TE	BP	GO:0097193-intrinsic apoptotic signaling pathway	1,52	1,45	1,38E-02	4,83E-02
TE	BP	GO:0048232-male gamete generation	-1,74	-1,72	8,16E-06	1,95E-05

TE	BP	GO:0033619-membrane protein proteolysis	1,84	1,87	1,53E-02	3,35E-02
TE	BP	GO:0000070-mitotic sister chromatid segregation	1,83	1,79	6,59E-04	4,79E-03
TE	BP	GO:0034660-ncrna metabolic process	2,3	1,36	1,26E-07	4,53E-02
TE	BP	GO:1902532-negative regulation of intracellular signal transduction	1,77	1,51	1,36E-06	4,43E-03
TE	BP	GO:0043409-negative regulation of mapk cascade	1,9	1,56	2,29E-04	4,83E-02
TE	BP	GO:0042326-negative regulation of phosphorylation	1,66	1,38	2,22E-04	3,54E-02
TE	BP	GO:0038061-nik nf kappab signaling	1,92	1,77	3,30E-04	9,49E-03
TE	BP	GO:0036499-perk mediated unfolded protein response	1,86	2,04	4,99E-02	2,81E-02
TE	BP	GO:0009896-positive regulation of catabolic process	1,8	1,54	3,81E-06	4,79E-03
TE	BP	GO:0043123-positive regulation of i kappab kinase nf kappab signaling	1,68	1,66	3,60E-03	1,70E-02
TE	BP	GO:0045732-positive regulation of protein catabolic process	1,92	1,58	2,22E-05	2,87E-02
TE	BP	GO:0045862-positive regulation of proteolysis	1,78	1,51	1,34E-05	1,36E-02
TE	BP	GO:1903052-positive regulation of proteolysis involved in cellular protein catabolic process	1,81	1,61	2,23E-03	4,48E-02
TE	BP	GO:1901522-positive regulation of transcription from rna polymerase ii promoter involved in cellular response to chemical stimulus	1,95	1,94	2,83E-02	4,76E-02
TE	BP	GO:2000060-positive regulation of ubiquitin dependent protein catabolic process	1,86	1,66	2,78E-03	4,53E-02
TE	BP	GO:0043687-post translational protein modification	1,55	1,44	2,84E-03	3,00E-02
TE	BP	GO:0010498-proteasomal protein catabolic process	1,87	1,78	1,26E-07	5,28E-06

TE	BP	GO:0034502-protein localization to chromosome	2,11	1,81	2,22E-04	2,11E-02
TE	BP	GO:0070646-protein modification by small protein removal	1,7	1,44	4,62E-04	4,31E-02
TE	BP	GO:0007265-ras protein signal transduction	1,42	1,47	1,38E-02	1,54E-02
TE	BP	GO:2001233-regulation of apoptotic signaling pathway	1,79	1,61	1,13E-05	1,59E-03
TE	BP	GO:1902749-regulation of cell cycle g2 m phase transition	1,86	1,64	1,35E-04	1,16E-02
TE	BP	GO:1903362-regulation of cellular protein catabolic process	1,85	1,68	3,39E-05	4,43E-03
TE	BP	GO:0045540-regulation of cholesterol biosynthetic process	1,97	2,41	1,11E-02	1,06E-04
TE	BP	GO:0033044-regulation of chromosome organization	1,7	1,5	3,03E-04	1,72E-02
TE	BP	GO:0051090-regulation of dna binding transcription factor activity	1,44	1,61	1,30E-02	7,21E-04
TE	BP	GO:2000278-regulation of dna biosynthetic process	1,68	1,66	1,59E-02	4,12E-02
TE	BP	GO:2001236-regulation of extrinsic apoptotic signaling pathway	1,75	1,73	2,59E-03	1,61E-02
TE	BP	GO:0046890-regulation of lipid biosynthetic process	1,73	1,94	3,13E-03	4,65E-04
TE	BP	GO:0019216-regulation of lipid metabolic process	1,41	1,49	3,13E-02	1,70E-02
TE	BP	GO:0019219-regulation of nucleobase containing compound metabolic process	1,69	1,36	9,17E-06	3,62E-02
TE	BP	GO:0061136-regulation of proteasomal protein catabolic process	1,91	1,75	1,32E-04	4,42E-03
TE	BP	GO:0032434-regulation of proteasomal ubiquitin dependent protein catabolic process	1,94	1,74	3,70E-04	1,39E-02
TE	BP	GO:0042176-regulation of protein catabolic process	1,92	1,68	1,31E-07	5,26E-04
TE	BP	GO:0071900-regulation of protein serine threonine kinase activity	1,32	1,35	4,95E-02	4,53E-02

TE	BP	GO:0031647-regulation of protein stability	1,47	1,59	3,15E-02	6,83E-03
TE	BP	GO:0010469-regulation of signaling receptor activity	-1,51	-1,6	5,18E-03	7,26E-04
TE	BP	GO:0033045-regulation of sister chromatid segregation	1,64	1,8	3,15E-02	1,63E-02
TE	BP	GO:0062012-regulation of small molecule metabolic process	1,72	1,77	1,16E-04	1,26E-04
TE	BP	GO:0050810-regulation of steroid biosynthetic process	1,9	2,27	3,60E-03	4,12E-05
TE	BP	GO:0019218-regulation of steroid metabolic process	1,72	2,12	5,32E-03	2,57E-04
TE	BP	GO:2000058-regulation of ubiquitin dependent protein catabolic process	1,89	1,69	2,84E-04	2,59E-02
TE	BP	GO:0030111-regulation of wnt signaling pathway	1,62	1,41	8,07E-04	4,83E-02
TE	BP	GO:0034976-response to endoplasmic reticulum stress	1,68	1,96	9,68E-04	5,28E-06
TE	BP	GO:0009408-response to heat	1,69	1,62	3,76E-03	3,62E-02
TE	BP	GO:0009314-response to radiation	1,47	1,54	6,74E-03	2,51E-03
TE	BP	GO:0035966-response to topologically incorrect protein	1,87	2,06	3,27E-04	7,64E-06
TE	BP	GO:0034612-response to tumor necrosis factor	1,44	1,43	3,56E-02	4,92E-02
TE	BP	GO:0007606-sensory perception of chemical stimulus	-1,59	-1,76	5,05E-03	1,08E-03
TE	BP	GO:0007608-sensory perception of smell	-1,54	-1,63	3,14E-02	1,49E-02
TE	BP	GO:0000819-sister chromatid segregation	1,84	1,78	3,08E-04	3,60E-03
TE	BP	GO:0006694-steroid biosynthetic process	1,85	2,32	6,02E-04	3,83E-07
TE	BP	GO:0008202-steroid metabolic process	1,46	1,92	2,12E-02	5,28E-06
TE	BP	GO:0016126-sterol biosynthetic process	2,11	2,55	3,36E-04	9,98E-07
TE	BP	GO:0032200-telomere organization	1,71	1,61	6,21E-03	4,24E-02
TE	BP	GO:0006099-tricarboxylic acid cycle	2,16	2,09	1,42E-03	6,76E-03
TE	BP	GO:0030433-ubiquitin dependent erad pathway	1,68	1,78	4,95E-02	2,87E-02
TE	BP	GO:0019058-viral life cycle	1,69	1,5	3,18E-04	1,63E-02

TE	CC	GO:0015629-actin cytoskeleton	1,26	1,41	3,92E-02	1,27E-02
TE	CC	GO:0101031-chaperone complex	2,12	1,89	3,63E-03	4,93E-02
TE	CC	GO:0034707-chloride channel complex	-1,78	-1,77	1,60E-02	4,45E-02
TE	CC	GO:0098687-chromosomal region	1,73	1,62	6,83E-05	4,48E-03
TE	CC	GO:0000775-chromosome centromeric region	1,42	1,51	4,60E-02	3,21E-02
TE	CC	GO:0097014-ciliary plasm	-1,7	-1,64	9,60E-03	3,98E-02
TE	CC	GO:0031461-cullin ring ubiquitin ligase complex	1,8	1,66	8,13E-04	1,51E-02
TE	CC	GO:0031045-dense core granule	-1,99	-2,09	5,76E-03	8,01E-03
TE	CC	GO:1905369-endoropeptidase complex	1,6	1,85	2,54E-02	1,76E-02
TE	CC	GO:0044440-endosomal part	1,72	1,5	1,78E-06	4,48E-03
TE	CC	GO:0101002-ficolin 1 rich granule	1,69	1,92	7,00E-03	2,91E-03
TE	CC	GO:0031300-intrinsic component of organelle membrane	1,43	1,56	1,79E-02	4,21E-03
TE	CC	GO:0000776-kinetochore	1,43	1,53	4,40E-02	4,33E-02
TE	CC	GO:0005770-late endosome	1,61	1,64	3,70E-03	4,72E-03
TE	CC	GO:0031902-late endosome membrane	1,66	1,57	9,95E-03	3,48E-02
TE	CC	GO:0043202-lysosomal lumen	1,54	1,85	3,29E-02	8,56E-03
TE	CC	GO:0005759-mitochondrial matrix	1,77	1,85	1,20E-06	1,31E-06
TE	CC	GO:0005635-nuclear envelope	1,59	1,46	9,24E-05	1,20E-02
TE	CC	GO:0034399-nuclear periphery	2,04	1,55	2,21E-05	4,33E-02
TE	CC	GO:0016607-nuclear speck	1,7	1,37	4,45E-05	3,98E-02
TE	CC	GO:0055037-recycling endosome	1,5	1,6	2,66E-02	1,76E-02
TE	CC	GO:0005819-spindle	1,51	1,46	4,24E-03	1,76E-02
TE	CC	GO:1990351-transporter complex	-1,47	-1,5	1,55E-02	1,81E-02
TE	CC	GO:0000151-ubiquitin ligase complex	1,75	1,56	6,41E-05	9,71E-03
TE	CC	GO:0005775-vacuolar lumen	1,48	1,53	2,33E-02	4,53E-02
TE	CC	GO:0005774-vacuolar membrane	1,79	1,53	3,05E-06	4,48E-03
TE	CC	GO:0031983-vesicle lumen	1,41	1,61	1,88E-02	4,48E-03

TE	MF	GO:0005254-chloride channel activity	-1,64	-1,75	2,64E-02	3,19E-02
TE	MF	GO:0005230-extracellular ligand gated ion channel activity	-1,76	-1,82	1,18E-02	1,62E-02
TE	MF	GO:0030594-neurotransmitter receptor activity	-1,74	-1,66	7,44E-03	3,89E-02
TE	MF	GO:0004984-olfactory receptor activity	-1,6	-1,54	1,16E-02	2,61E-02
TE	MF	GO:0022803-passive transmembrane transporter activity	-1,33	-1,45	4,51E-02	2,43E-02
TE	MF	GO:0030545-receptor regulator activity	-1,53	-1,6	2,58E-03	1,25E-03
TE	MF	GO:0022835-transmitter gated channel activity	-2,03	-2,1	1,59E-03	1,25E-03
TE	KEGG	00020_KEGG-citrate cycle tca cycle	2,39	2,26	1,85E-05	5,74E-04
TE	KEGG	11266_KEGG-lysosome	1,93	1,81	5,32E-04	4,98E-03
TE	KEGG	13380_KEGG-neuroactive ligand receptor interaction	-1,66	-1,53	2,39E-03	1,80E-02
TE	KEGG	5872_KEGG-steroid biosynthesis	2,02	2,32	1,28E-02	4,47E-04
TE	KEGG	19428_KEGG-wnt signaling pathway	1,59	1,49	2,12E-02	4,34E-02

Supplementary Table 7. List of significantly deregulated terms from functional enrichment analysis found in inner cell mass (ICM) samples between low-level mosaic embryos (LM) and high-level mosaic embryos (HM). padj: Benjamini-Hochberg adjusted p-value. KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: gene ontology; BP: biological process; CC: cellular component; MF: molecular function. Normalized enrichment score (NES) > 0 indicates overrepresentation of the term in the first comparison factor.

Fraction	Comparison	Category	Gene Set Enrichment Term	NES	padj
ICM	C6 (LM-HM)	BP	GO:0006613-cotranslational protein targeting to membrane	2,73	1,28E-07
ICM	C6 (LM-HM)	BP	GO:0072599-establishment of protein localization to endoplasmic reticulum	2,6	1,28E-07
ICM	C6 (LM-HM)	BP	GO:0006612-protein targeting to membrane	2,34	1,28E-07
ICM	C6 (LM-HM)	BP	GO:0000184-nuclear transcribed mrna catabolic process nonsense mediated decay	2,34	3,12E-07
ICM	C6 (LM-HM)	BP	GO:0070972-protein localization to endoplasmic reticulum	2,31	3,12E-07
ICM	C6 (LM-HM)	BP	GO:0006605-protein targeting	1,86	5,90E-07
ICM	C6 (LM-HM)	BP	GO:0090150-establishment of protein localization to membrane	1,96	7,66E-07
ICM	C6 (LM-HM)	BP	GO:0015980-energy derivation by oxidation of organic compounds	1,86	1,03E-04

ICM	C6 (LM-HM)	BP	GO:0001655-urogenital system development	1,81	1,03E-04
ICM	C6 (LM-HM)	BP	GO:0006091-generation of precursor metabolites and energy	1,55	7,86E-04
ICM	C6 (LM-HM)	BP	GO:0022409-positive regulation of cell cell adhesion	1,8	7,86E-04
ICM	C6 (LM-HM)	BP	GO:0060602-branch elongation of an epithelium	2,3	1,02E-03
ICM	C6 (LM-HM)	BP	GO:0045333-cellular respiration	1,79	1,92E-03
ICM	C6 (LM-HM)	BP	GO:0072001-renal system development	1,7	1,92E-03
ICM	C6 (LM-HM)	BP	GO:0006413-translational initiation	1,79	1,92E-03
ICM	C6 (LM-HM)	BP	GO:2001233-regulation of apoptotic signaling pathway	1,6	2,25E-03
ICM	C6 (LM-HM)	BP	GO:0019080-viral gene expression	1,77	2,29E-03
ICM	C6 (LM-HM)	BP	GO:0022407-regulation of cell cell adhesion	1,6	3,34E-03
ICM	C6 (LM-HM)	BP	GO:0003401-axis elongation	2,24	3,92E-03
ICM	C6 (LM-HM)	BP	GO:0051186-cofactor metabolic process	1,52	4,63E-03
ICM	C6 (LM-HM)	BP	GO:0061326-renal tubule development	1,97	5,33E-03
ICM	C6 (LM-HM)	BP	GO:0000956-nuclear transcribed mrna catabolic process	1,71	6,28E-03
ICM	C6 (LM-HM)	BP	GO:0001667-ameboidal type cell migration	1,53	6,96E-03
ICM	C6 (LM-HM)	BP	GO:0050727-regulation of inflammatory response	1,62	6,96E-03
ICM	C6 (LM-HM)	BP	GO:0001763-morphogenesis of a branching structure	1,72	7,09E-03
ICM	C6 (LM-HM)	BP	GO:0050821-protein stabilization	1,72	8,19E-03
ICM	C6 (LM-HM)	BP	GO:1901605-alpha amino acid metabolic process	1,7	1,04E-02
ICM	C6 (LM-HM)	BP	GO:0050673-epithelial cell proliferation	1,52	1,21E-02
ICM	C6 (LM-HM)	BP	GO:0045785-positive regulation of cell adhesion	1,52	1,21E-02
ICM	C6 (LM-HM)	BP	GO:0052547-regulation of peptidase activity	1,5	1,37E-02
ICM	C6 (LM-HM)	BP	GO:0072028-nephron morphogenesis	1,89	1,38E-02
ICM	C6 (LM-HM)	BP	GO:0048754-branching morphogenesis of an epithelial tube	1,75	1,54E-02
ICM	C6 (LM-HM)	BP	GO:0022900-electron transport chain	1,67	1,54E-02
ICM	C6 (LM-HM)	BP	GO:0007369-gastrulation	1,65	1,54E-02
ICM	C6 (LM-HM)	BP	GO:0090103-cochlea morphogenesis	2,08	1,65E-02
ICM	C6 (LM-HM)	BP	GO:0030850-prostate gland development	2,03	1,65E-02
ICM	C6 (LM-HM)	BP	GO:0019216-regulation of lipid metabolic process	1,49	1,78E-02
ICM	C6 (LM-HM)	BP	GO:0061053-somite development	1,9	1,78E-02

ICM	C6 (LM-HM)	BP	GO:0045834-positive regulation of lipid metabolic process	1,71	1,79E-02
ICM	C6 (LM-HM)	BP	GO:0072079-nephron tubule formation	2,05	1,89E-02
ICM	C6 (LM-HM)	BP	GO:0016054-organic acid catabolic process	1,55	1,89E-02
ICM	C6 (LM-HM)	BP	GO:0007156-homophilic cell adhesion via plasma membrane adhesion molecules	-1,75	1,90E-02
ICM	C6 (LM-HM)	BP	GO:0060192-negative regulation of lipase activity	2,04	2,06E-02
ICM	C6 (LM-HM)	BP	GO:0034340-response to type i interferon	1,83	2,28E-02
ICM	C6 (LM-HM)	BP	GO:0060512-prostate gland morphogenesis	2	2,96E-02
ICM	C6 (LM-HM)	BP	GO:0098754-detoxification	1,75	3,15E-02
ICM	C6 (LM-HM)	BP	GO:0072171-mesonephric tubule morphogenesis	1,87	3,30E-02
ICM	C6 (LM-HM)	BP	GO:0042060-wound healing	1,37	3,30E-02
ICM	C6 (LM-HM)	BP	GO:0043434-response to peptide hormone	1,43	3,34E-02
ICM	C6 (LM-HM)	BP	GO:0048546-digestive tract morphogenesis	1,91	3,34E-02
ICM	C6 (LM-HM)	BP	GO:0048732-gland development	1,39	3,34E-02
ICM	C6 (LM-HM)	BP	GO:1904035-regulation of epithelial cell apoptotic process	1,82	3,34E-02
ICM	C6 (LM-HM)	BP	GO:0030178-negative regulation of wnt signaling pathway	1,55	3,35E-02
ICM	C6 (LM-HM)	BP	GO:0006119-oxidative phosphorylation	1,67	3,35E-02
ICM	C6 (LM-HM)	BP	GO:0009617-response to bacterium	1,36	3,35E-02
ICM	C6 (LM-HM)	BP	GO:0035270-endocrine system development	1,7	3,35E-02
ICM	C6 (LM-HM)	BP	GO:0009636-response to toxic substance	1,38	3,35E-02
ICM	C6 (LM-HM)	BP	GO:0048645-animal organ formation	1,84	3,42E-02
ICM	C6 (LM-HM)	BP	GO:0070555-response to interleukin 1	1,55	3,42E-02
ICM	C6 (LM-HM)	BP	GO:0019731-antibacterial humoral response	1,98	3,51E-02
ICM	C6 (LM-HM)	BP	GO:0060914-heart formation	1,96	3,51E-02
ICM	C6 (LM-HM)	BP	GO:0002526-acute inflammatory response	1,78	3,52E-02
ICM	C6 (LM-HM)	BP	GO:0031016-pancreas development	1,77	3,52E-02
ICM	C6 (LM-HM)	BP	GO:1903039-positive regulation of leukocyte cell cell adhesion	1,56	3,52E-02
ICM	C6 (LM-HM)	BP	GO:0090102-cochlea development	1,87	3,62E-02
ICM	C6 (LM-HM)	BP	GO:0006401-rna catabolic process	1,43	3,62E-02
ICM	C6 (LM-HM)	BP	GO:0051604-protein maturation	1,52	3,65E-02
ICM	C6 (LM-HM)	BP	GO:0009066-aspartate family amino acid metabolic process	1,89	3,67E-02

ICM	C6 (LM-HM)	BP	GO:0031338-regulation of vesicle fusion	1,97	3,75E-02
ICM	C6 (LM-HM)	BP	GO:0035357-peroxisome proliferator activated receptor signaling pathway	1,95	3,78E-02
ICM	C6 (LM-HM)	BP	GO:0001704-formation of primary germ layer	1,65	3,80E-02
ICM	C6 (LM-HM)	BP	GO:0060562-epithelial tube morphogenesis	1,5	3,82E-02
ICM	C6 (LM-HM)	BP	GO:0009060-aerobic respiration	1,77	3,91E-02
ICM	C6 (LM-HM)	BP	GO:0043062-extracellular structure organization	1,41	3,94E-02
ICM	C6 (LM-HM)	BP	GO:0032355-response to estradiol	1,66	3,94E-02
ICM	C6 (LM-HM)	BP	GO:0031018-endocrine pancreas development	1,89	3,96E-02
ICM	C6 (LM-HM)	BP	GO:0007492-endoderm development	1,74	3,96E-02
ICM	C6 (LM-HM)	BP	GO:0071887-leukocyte apoptotic process	1,73	3,96E-02
ICM	C6 (LM-HM)	BP	GO:0031647-regulation of protein stability	1,5	4,11E-02
ICM	C6 (LM-HM)	BP	GO:0042773-atp synthesis coupled electron transport	1,69	4,12E-02
ICM	C6 (LM-HM)	BP	GO:0006520-cellular amino acid metabolic process	1,5	4,21E-02
ICM	C6 (LM-HM)	BP	GO:0071542-dopaminergic neuron differentiation	1,9	4,21E-02
ICM	C6 (LM-HM)	BP	GO:0051154-negative regulation of striated muscle cell differentiation	1,95	4,21E-02
ICM	C6 (LM-HM)	BP	GO:0051051-negative regulation of transport	1,39	4,21E-02
ICM	C6 (LM-HM)	BP	GO:0016485-protein processing	1,57	4,21E-02
ICM	C6 (LM-HM)	BP	GO:0010632-regulation of epithelial cell migration	1,53	4,21E-02
ICM	C6 (LM-HM)	BP	GO:0009069-serine family amino acid metabolic process	1,92	4,21E-02
ICM	C6 (LM-HM)	BP	GO:0042398-cellular modified amino acid biosynthetic process	1,9	4,57E-02
ICM	C6 (LM-HM)	BP	GO:0060993-kidney morphogenesis	1,75	4,57E-02
ICM	C6 (LM-HM)	BP	GO:0072009-nephron epithelium development	1,64	4,57E-02
ICM	C6 (LM-HM)	BP	GO:2000108-positive regulation of leukocyte apoptotic process	1,85	4,57E-02
ICM	C6 (LM-HM)	BP	GO:0043542-endothelial cell migration	1,48	4,60E-02
ICM	C6 (LM-HM)	BP	GO:0006635-fatty acid beta oxidation	1,72	4,60E-02
ICM	C6 (LM-HM)	BP	GO:0001823-mesonephros development	1,72	4,60E-02
ICM	C6 (LM-HM)	BP	GO:0090130-tissue migration	1,47	4,60E-02
ICM	C6 (LM-HM)	BP	GO:0034116-positive regulation of heterotypic cell cell adhesion	1,94	4,83E-02
ICM	C6 (LM-HM)	BP	GO:0009142-nucleoside triphosphate biosynthetic process	1,71	4,89E-02

ICM	C6 (LM-HM)	BP	GO:0032869-cellular response to insulin stimulus	1,53	4,90E-02
ICM	C6 (LM-HM)	CC	GO:0022626-cytosolic ribosome	2,53	2,15E-08
ICM	C6 (LM-HM)	CC	GO:0044391-ribosomal subunit	2,33	2,15E-08
ICM	C6 (LM-HM)	CC	GO:0005840-ribosome	2,13	2,15E-08
ICM	C6 (LM-HM)	CC	GO:0015934-large ribosomal subunit	2,18	5,94E-06
ICM	C6 (LM-HM)	CC	GO:0022625-cytosolic large ribosomal subunit	2,38	8,43E-06
ICM	C6 (LM-HM)	CC	GO:0044455-mitochondrial membrane part	1,72	5,61E-04
ICM	C6 (LM-HM)	CC	GO:0098798-mitochondrial protein complex	1,71	5,61E-04
ICM	C6 (LM-HM)	CC	GO:0044445-cytosolic part	1,67	2,18E-03
ICM	C6 (LM-HM)	CC	GO:0098800-inner mitochondrial membrane protein complex	1,78	2,22E-03
ICM	C6 (LM-HM)	CC	GO:0015935-small ribosomal subunit	2	2,22E-03
ICM	C6 (LM-HM)	CC	GO:0005788-endoplasmic reticulum lumen	1,58	3,06E-03
ICM	C6 (LM-HM)	CC	GO:0022627-cytosolic small ribosomal subunit	2,04	3,88E-03
ICM	C6 (LM-HM)	CC	GO:0031983-vesicle lumen	1,56	3,88E-03
ICM	C6 (LM-HM)	CC	GO:0043194-axon initial segment	-1,92	2,57E-02
ICM	C6 (LM-HM)	CC	GO:0043235-receptor complex	-1,51	2,57E-02
ICM	C6 (LM-HM)	CC	GO:0070469-respirasome	1,68	2,57E-02
ICM	C6 (LM-HM)	CC	GO:0098803-respiratory chain complex	1,71	3,60E-02
ICM	C6 (LM-HM)	CC	GO:0005759-mitochondrial matrix	1,36	4,25E-02
ICM	C6 (LM-HM)	CC	GO:0030867-rough endoplasmic reticulum membrane	1,84	4,54E-02
ICM	C6 (LM-HM)	MF	GO:0003735-structural constituent of ribosome	2,54	7,46E-08
ICM	C6 (LM-HM)	MF	GO:0051082-unfolded protein binding	1,89	8,46E-03
ICM	C6 (LM-HM)	MF	GO:0009055-electron transfer activity	1,84	2,32E-02
ICM	C6 (LM-HM)	KEGG	KEGG_03010-ribosome	2,66	1,74E-08
ICM	C6 (LM-HM)	KEGG	KEGG_00190-oxidative phosphorylation	1,98	1,09E-04
ICM	C6 (LM-HM)	KEGG	KEGG_05012-parkinsons disease	1,91	5,06E-04
ICM	C6 (LM-HM)	KEGG	KEGG_05016-huntingtons disease	1,71	5,24E-03
ICM	C6 (LM-HM)	KEGG	KEGG_05010-alzheimers disease	1,73	6,41E-03
ICM	C6 (LM-HM)	KEGG	KEGG_00330-arginine and proline metabolism	1,93	7,38E-03
ICM	C6 (LM-HM)	KEGG	KEGG_03320-ppar signaling pathway	1,75	1,93E-02
ICM	C6 (LM-HM)	KEGG	KEGG_05200-pathways in cancer	1,42	2,28E-02
ICM	C6 (LM-HM)	KEGG	KEGG_00310-lysine degradation	1,82	2,86E-02

ICM	C6 (LM-HM)	KEGG	KEGG_00650-butanoate metabolism	1,85	3,65E-02
ICM	C6 (LM-HM)	KEGG	KEGG_05215-prostate cancer	1,63	3,65E-02
ICM	C6 (LM-HM)	KEGG	KEGG_00620-pyruvate metabolism	1,74	3,94E-02
ICM	C6 (LM-HM)	KEGG	KEGG_00563-glycosylphosphatidylinositol gpi anchor biosynthesis	-1,79	4,54E-02
ICM	C6 (LM-HM)	KEGG	KEGG_00260-glycine serine and threonine metabolism	1,8	4,62E-02

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Embryology

Trophectoderm cells of human mosaic embryos display increased apoptotic levels and impaired differentiation capacity: a molecular clue regarding their reproductive fate?

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ABSTRACT

STUDY QUESTION: Are there cell lineage-related differences in the apoptotic rates and differentiation capacity of human blastocysts diagnosed as euploid, mosaic, and aneuploid after preimplantation genetic testing for aneuploidy (PGT-A) based on concurrent copy number and genotyping analysis?

SUMMARY ANSWER: Trophectoderm (TE) cells of mosaic and aneuploid blastocysts exhibit significantly higher levels of apoptosis and significantly reduced differentiation capacity compared to those of euploid blastocysts.

WHAT IS KNOWN ALREADY: Embryos diagnosed as mosaic after PGT-A can develop into healthy infants, yet understanding the reasons behind their reproductive potential requires further research. One hypothesis suggests that mosaicism can be normalized through selective apoptosis and reduced proliferation of aneuploid cells, but direct evidence of these mechanisms in human embryos is lacking. Additionally, data interpretation from studies involving mosaic embryos has been hampered by retrospective analysis methods and the high incidence of false-positive mosaic diagnoses stemming from the use of poorly specific PGT-A platforms.

STUDY DESIGN, SIZE, DURATION: Prospective cohort study performing colocalization of cell-lineage and apoptotic markers by immunofluorescence (IF). We included a total of 64 human blastocysts donated to research on Day 5 or 6 post-fertilization (dpf) by 43 couples who underwent *in vitro* fertilization treatment with PGT-A at IVI-RMA Valencia between September 2019 and October 2022. A total of 27 mosaic blastocysts were analyzed.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The study consisted of two phases: Phase I (caspase-3, $n = 53$ blastocysts): $n = 13$ euploid, $n = 22$ mosaic, $n = 18$ aneuploid. Phase II (terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), $n = 11$ blastocysts): $n = 2$ euploid, $n = 5$ mosaic, $n = 4$ aneuploid. Following donation for research, vitrified blastocysts were warmed, cultured until re-expansion, fixed, processed for IF, and imaged using confocal microscopy. For each blastocyst, the following cell counts were conducted: total cells (DAPI+), TE cells (GATA3+), inner cell mass (ICM) cells (GATA3–/NANOG+), and apoptotic cells (caspase-3+ or TUNEL+). The incidence of apoptosis was calculated for each blastocyst by dividing the number of caspase-3+ cells (Phase I) or TUNEL+ cells (Phase II) by the number of TE or ICM cells. Statistical analysis was performed according to data type and distribution ($P < 0.05$ was considered statistically significant).

MAIN RESULTS AND THE ROLE OF CHANCE: Phase I: Mosaic blastocysts displayed a similar number of total cells (49.6 ± 15 cells at 5 dpf; 58.8 ± 16.9 cells at 6 dpf), TE cells (38.8 ± 13.7 cells at 5 dpf; 49.2 ± 16.2 cells at 6 dpf), and ICM cells (10.9 ± 4.2 cells at 5 dpf; 9.7 ± 7.1 cells at 6 dpf) compared to euploid and aneuploid blastocysts ($P > 0.05$). The proportion of TE cells retaining NANOG expression increased gradually from euploid blastocysts (9.7% = 63/651 cells at 5 dpf; 0% = 0/157 cells at 6 dpf) to mosaic blastocysts (13.1% = 104/794 cells at 5 dpf; 3.4% = 12/353 cells at 6 dpf) and aneuploid blastocysts (27.9% = 149/534 cells at 5 dpf; 4.6% = 19/417 cells at 6 dpf) ($P < 0.05$). At the TE level, caspase-3+ cells were frequently observed (39% = 901/2310 cells). The proportion of caspase-3+ TE cells was significantly higher in mosaic blastocysts ($44.1\% \pm 19.6$ at 5 dpf; $43\% \pm 16.8$ at 6 dpf) and aneuploid blastocysts ($45.9\% \pm 16.1$ at 5 dpf; $49\% \pm 15.1$ at 6 dpf) compared to euploid blastocysts (26.6% $\pm$ 16.6 at 5 dpf; 17.5% $\pm$ 14.8 at 6 dpf) ($P < 0.05$). In contrast, at the ICM level, caspase-3+ cells were rarely observed (1.9% = 11/596 cells), and only detected in mosaic blastocysts (2.6% = 6/232 cells) and aneuploid blastocysts (2.5% = 5/197 cells) ($P > 0.05$). Phase II: Consistently, TUNEL+ cells were only observed in TE cells (32.4% = 124/383 cells). An increasing trend was identified toward a higher proportion of TUNEL+ cells in the TE of mosaic blastocysts (37.2% $\pm$ 21.9) and aneuploid blastocysts (39% $\pm$ 41.7), compared to euploid blastocysts (23% $\pm$ 32.5), although these differences did not reach statistical significance ($P > 0.05$).

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LIMITATIONS, REASONS FOR CAUTION: The observed effects on apoptosis and differentiation may not be exclusive to aneuploid cells. Additionally, variations in aneuploidies and unexplored factors related to blastocyst development and karyotype concordance may introduce potential biases and uncertainties in the results.

WIDER IMPLICATIONS OF THE FINDINGS: Our findings demonstrate a cell lineage-specific effect of aneuploidy on the apoptotic levels and differentiation capacity of human blastocysts. This contributes to unravelling the biological characteristics of mosaic blastocysts and supports the concept of clonal depletion of aneuploid cells in explaining their reproductive potential.

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TRIAL REGISTRATION NUMBER: N/A.

Keywords: embryonic mosaicism / aneuploidy / trophoctoderm / apoptosis / lineage segregation

Introduction

Despite significant advances in reproductive technology, assisted human reproduction remains remarkably inefficient. According to the latest report from the European Society of Human Reproduction and Embryology (ESHRE) on IVF monitoring, clinical pregnancy rates per transfer have plateaued at around 34% (European IVF Monitoring Consortium for ESHRE et al., 2023). One major cause of this inefficiency is embryonic aneuploidy, a condition of karyotype imbalance involving the loss or gain of one or more chromosomes. Meiotic aneuploidy is caused by errors in chromosome segregation during gametogenesis, leading to embryos composed entirely of chromosomally abnormal cells (Hassold and Hunt, 2001). In contrast, mitotic aneuploidy arises from errors during post-zygotic cell divisions, resulting in embryos with a mixture of euploid and aneuploid cells, a status known as mosaicism (Levy et al., 2021).

Preimplantation genetic testing for aneuploidy (PGT-A) enables the identification of embryos with chromosomal alterations after IVF. Embryos with uniform aneuploidies are deselected for transfer since they lead to implantation failure, miscarriage, and congenital anomalies (Capalbo et al., 2022). Conversely, embryos with mosaic aneuploidies rarely result in aneuploid pregnancies (Kahraman et al., 2020; Schlade-Bartusiak et al., 2022; Greco et al., 2023) but are rather associated with normal development and the birth of healthy babies (Abhari and Kawwass, 2021; Capalbo et al., 2021; Mourad et al., 2021; Treff and Marin, 2021; Viotti et al., 2021; Ma et al., 2022; Viotti et al., 2023). However, the reasons for the reproductive potential of mosaic embryos require further investigation.

Two main hypotheses are under consideration. On the one hand, it has been proposed that mosaic embryos have some intrinsic capacity for self-correction, which may occur through selective apoptosis and reduced proliferation of aneuploid cells (Baker, 2020; Coticchio et al., 2021; Regin et al., 2022). Although direct evidence of these mechanisms in human embryos is lacking, time-lapse imaging studies have shown that mitotic errors during early embryonic divisions lead to slower cleavage and longer cell cycles (Chavez et al., 2012; Lee et al., 2019; Martin et al., 2023). Consistently, transcriptome studies have demonstrated that embryonic aneuploidy leads to deregulation of genes and pathways associated with cell proliferation, apoptosis, and autophagy (Vera-Rodriguez et al., 2015; Licciardi et al., 2018; Sanchez-Ribas et al., 2019; Maxwell et al., 2022; Martin et al., 2023).

On the other hand, it has been suggested that mosaic results based solely on the identification of intermediate chromosomal copy number profiles in trophoctoderm (TE) biopsies lead to false positive diagnoses. Indeed, studies performing multifocal blastocyst biopsies have demonstrated that most mosaic results remain confined to a single TE sample (Popovic et al., 2020; Capalbo et al., 2021; Marin et al., 2021; Girardi et al., 2023).

In addition, mosaic results can be indistinguishable from technical noise superimposed on euploid or aneuploid profiles (Treff and Marin, 2021). Moreover, given the uncertainty surrounding these findings, embryos with mosaic results are typically deprioritized for transfer (Abhari and Kawwass, 2021; Cheng et al., 2022; Leigh et al., 2022). Therefore, compelling evidence indicates that the outcomes of mosaic embryo transfers have been confounded by the misdiagnosis of euploid and aneuploid embryos and a selection bias toward patients with poor reproductive prognosis (Treff and Marin, 2021).

Novel PGT-A methods integrate both copy number and single nucleotide polymorphism (SNP) genotyping data analysis, which has made it possible to distinguish between meiotic and mitotic aneuploidies in TE samples (Rana et al., 2023). These platforms have revealed that more than 30% of embryos diagnosed as mosaic based solely on copy number profiles display at least one error of meiotic origin, indicating the presence of uniform aneuploidies (Popa et al., 2023). Since the detection of mitotic-origin aneuploidy more accurately predicts mosaicism in the remaining embryo, these methods offer an unparalleled opportunity to investigate the molecular mechanisms responsible for the reproductive potential of mosaic embryos.

Here, we performed immunofluorescence (IF) and confocal imaging to compare the cell lineage-specific incidence of apoptosis among blastocysts diagnosed as euploid, mosaic, and aneuploid by a novel PGT-A platform that integrates copy number and SNP genotyping data analysis. The differential expression of apoptotic markers in mosaic embryos may shed new light on the impact of mitotic aneuploidy on embryo viability at the stage when the first lineage segregation occurs.

Materials and methods

Ethical approval

The study was approved by the National Commission of Human Reproduction (CNRHA), the general direction of research, innovation, technology, and quality, and by our institutional review board, the ethics committee of Clinical Research IVI-RMA Valencia (No. 1710-VLC-102-MD), which complies with Spanish law on assisted reproductive technologies (14/2006) and biomedical research (14/2007). All participants provided written informed consent prior to their inclusion in the study, and the research procedures were carried out in compliance with the Declaration of Helsinki.

Study population and design

This prospective cohort study was performed on a total of 64 human blastocysts with previous PGT-A diagnosis on Day 5 or 6 post-fertilization (dpf). Blastocysts were donated to research by 43 couples who underwent IVF treatment with ICSI at IVI-RMA (Valencia, Spain) between September 2019 and October 2022.

The mean age of patients providing oocytes and sperm was 34.8 ± 6.3 and 38.3 ± 7.4 years, respectively. Indications for PGT-A included advanced maternal age, recurrent miscarriage, recurrent implantation failure, and aneuploidy screening.

The study was divided into two phases (Fig. 1). In the first phase, we analyzed the expression of the apoptotic marker active/cleaved caspase-3 on a total of 53 blastocysts: $n = 13$ euploid ($n = 11$ at 5 dpf; $n = 2$ at 6 dpf), $n = 22$ mosaic ($n = 16$ at 5 dpf; $n = 6$ at 6 dpf), and $n = 18$ aneuploid ($n = 11$ at 5 dpf; $n = 7$ at 6 dpf) (Table 1). In the second phase, we analyzed the level of DNA fragmentation, a hallmark of late-stage apoptosis, by performing terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining on a total of 11 blastocysts: $n = 2$ euploid ($n = 1$ at 5 dpf; $n = 1$ at 6 dpf), $n = 5$ mosaic ($n = 4$ at 5 dpf; $n = 1$ at 6 dpf), and $n = 4$ aneuploid ($n = 4$ at 5 dpf) (Supplementary Table S1).

IVF-related procedures

Ovarian stimulation, oocyte retrieval, and ICSI were performed as described previously by Bori et al. (2022). Embryo culture was performed in Gems culture medium (Genea Biomedx, Australia)

under mineral oil at 37°C , 6% CO_2 , and 5% O_2 (balanced with N_2) from the zygote to the blastocyst stage. Embryo quality was assessed according to guidelines from the Association for the Study of Reproductive Biology (ASEBIR), with slight modifications (Arday et al., 2008).

Preimplantation genetic testing for aneuploidy

At 3 dpf, embryos underwent assisted hatching by laser technology (Octax NaviLase™, Sweden). The day of embryo biopsy depended exclusively on blastocyst quality and pace of development. Only embryos that developed beyond a full blastocyst with a defined inner cell mass (ICM) and a cohesive TE epithelium (grade A, B, or C in the ASEBIR scoring system) were considered eligible for biopsy. Blastocysts not reaching the guide mark at 5 dpf (116 ± 2 h post-fertilization (hpf)) were given one more day in culture and were biopsied at 6 dpf (140 ± 2 hpf) if meeting the established criteria.

A TE biopsy was performed by the pulling method (Aoyama and Kato, 2020). Approximately five to eight TE cells with visible nuclei opposite the ICM were drawn into a biopsy pipette by applying gentle suction and 4–5 intercellular laser pulses (intensity 1.8 ms, diameter $13 \mu\text{m}$). Aspirated TE cells were collected into DNase-free polymerase chain reaction tubes using UV-sterilized material and sterile gloves. Biopsy samples were labelled, stored at -20°C , and shipped to a reference genetics laboratory. Biopsied blastocysts were moved back to culture and vitrified following the Kitazato protocol (Kuwayama, 2007; Cobo et al., 2009).

The method used for PGT-A is known as PGTseq and is based on targeted next-generation sequencing (NGS). It employs multiplex targeted PCR to amplify regions on all 24 chromosomes, followed by massive parallel sequencing of the resulting amplicons. Targeted NGS specifically examines areas of the genome that can accurately assess whole chromosome aneuploidy without introducing artifacts. The test was validated, and its performance characteristics were determined by Juno Genetics (Tiegs et al., 2021).

Briefly, the genetic material of the biopsied cells was extracted, amplified, and sequenced. To determine the chromosomal constitution of the biopsied sample, the relative number of DNA fragments derived from each chromosome was considered. The PGTseq software generated a graph representing the chromosome copy numbers in the sample. The mosaic range was defined as intermediate copy number variations between 30% and 70%. In addition, a custom SNP genotyping assay was performed in parallel on every biopsy. The analysis of polymorphic markers scattered across the genome allowed for a second, independent confirmation of chromosomal copy numbers and assisted in detecting mosaicism and DNA contamination in the biopsy (Supplementary Fig. S1).

The PGTseq assay detected all forms of haploidy and triploidy (including XXX triploidy, which is missed by NGS alone), while balanced structural chromosome rearrangements and uniparental disomy were not detected.

Embryo fixation and immunofluorescence

Blastocysts donated to research were warmed using the Kitazato method (Kuwayama, 2007; Cobo et al., 2009) and cultured until re-expansion (2–4 h) in Gems culture medium (Genea Biomedx, Australia) under mineral oil at 37°C , 6% CO_2 , and 5% O_2 (balanced with N_2). Following re-expansion, blastocysts were washed for 2 min in PBS (D8537, Sigma, USA) and fixed in a freshly prepared PBS solution containing 4% paraformaldehyde (15710, EMS, USA) for 20 min at room temperature (RT). Fixed embryos were washed twice for 2 min in a PBS solution containing 0.1%

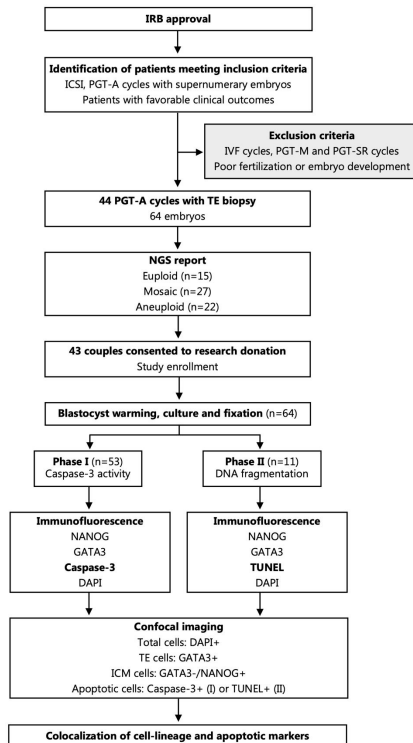


Figure 1. Study flowchart. IRB, Institutional Review Board; PGT-A, preimplantation genetic testing for aneuploidy; PGT-M, preimplantation genetic testing for monogenic diseases; PGT-SR, preimplantation genetic testing for structural rearrangements; NGS, next-generation sequencing; TE, trophectoderm; ICM, inner cell mass.

Table 1. Study population and characteristics of embryos analyzed by caspase-3 staining.

Embryo ID	Day of analysis ^a	Grade ASEBIR ^b	Grade TE	Grade ICM	Hatching	PGT-A diagnosis	NGS (% mosaicism)
EU01	5	B	B	B	Yes	Euploid	<30%
EU02	5	B	B	B	No	Euploid	<30%
EU03	5	B	B	B	Yes	Euploid	<30%
EU04	5	B	B	B	Yes	Euploid	<30%
EU05	5	B	B	B	Yes	Euploid	<30%
EU06	5	B	B	B	Yes	Euploid	<30%
EU07	5	C	C	B	No	Euploid	<30%
EU08	5	C	C	B	No	Euploid	<30%
EU09	5	C	C	B	Yes	Euploid	<30%
EU10	5	C	C	C	No	Euploid	<30%
EU11	5	C	C	B	No	Euploid	<30%
EU12	6	B	B	B	Yes	Euploid	<30%
EU13	6	C	C	C	Yes	Euploid	<30%
MO01	5	B	B	B	Yes	Mosaic	+3(30%), +4(30%), +12(40%), +17(30%), +21(30%)
MO02	5	B	B	B	No	Mosaic	+Y(30%), +7(40%), +10(40%)
MO03	5	B	B	B	Yes	Mosaic	-X(30%)
MO04	5	B	B	B	No	Mosaic	-8(40%)
MO05	5	B	B	B	No	Mosaic	-14(40%), -19(40%)
MO06	5	B	B	B	Yes	Mosaic	-14(40%)
MO07	5	B	B	B	Yes	Mosaic	-16(30%)
MO08	5	B	B	B	Yes	Mosaic	+6p(30%)
MO09	5	C	C	B	Yes	Mosaic	-2(30%), -7(30%)
MO10	5	C	C	B	No	Mosaic	-3(70%), -4(30%)
MO11	5	C	C	B	Yes	Mosaic	-22(40%)
MO12	5	C	C	B	No	Mosaic	-6(40%), +7(40%)
MO13	5	C	C	A	Yes	Mosaic	-12p(40%), +12q(40%)
MO14	5	C	C	B	Yes	Mosaic	-2(40%)
MO15	5	C	C	B	No	Mosaic	-2(70%)
MO16	5	C	C	B	Yes	Mosaic	-10(30%)
MO17	6	B	B	A	Yes	Mosaic	-X(50%), +3(40%), -1p(50%), -1q(40%)
MO18	6	B	B	B	No	Mosaic	-3(50%)
MO19	6	B	B	B	Yes	Mosaic	+8(60%)
MO20	6	C	C	C	No	Mosaic	-3(30%), -16(40%), -18(50%)
MO21	6	C	C	B	Yes	Mosaic	+4q(30%)
MO22	6	C	C	B	Yes	Mosaic	+9p(50%)
AN01	5	B	B	B	Yes	Aneuploid	+16(>70%)
AN02	5	B	B	B	No	Aneuploid	+1(>70%), +17(>70%)
AN03	5	B	B	B	No	Aneuploid	+22(>70%)
AN04	5	B	B	B	Yes	Aneuploid	-22(>70%)
AN05	5	B	B	B	No	Aneuploid	+15(>70%)
AN06	5	B	B	B	Yes	Aneuploid	-21(>70%)
AN07	5	B	B	A	No	Aneuploid	+2(>70%)
AN08	5	C	C	B	No	Aneuploid	+15(>70%)
AN09	5	C	C	B	No	Aneuploid	-4q(>70%)
AN10	5	C	C	B	Yes	Aneuploid	+10(>70%)
AN11	5	C	C	A	Yes	Aneuploid	+16(>70%)
AN12	6	B	B	B	Yes	Aneuploid	-16(>70%), +22(>70%)
AN13	6	B	B	B	No	Aneuploid	-8(>70%)
AN14	6	B	B	A	No	Aneuploid	-22(>70%)
AN15	6	B	B	B	No	Aneuploid	+15(>70%)
AN16	6	C	C	C	Yes	Aneuploid	-20(>70%)
AN17	6	C	C	B	Yes	Aneuploid	-Yp(>70%), -Yq(>70%)
AN18	6	C	C	B	No	Aneuploid	-7(>70%)

^a Days post-fertilization.^b Embryo quality assessed according to guidelines from the Spanish Association for the Study of Biology of Reproduction (ASEBIR). TE, trophoctoderm; ICM, inner cell mass; PGT-A, preimplantation genetic testing for aneuploidy; NGS, next-generation sequencing.

Tween-20 (P9416, Sigma, USA). Embryos were permeabilized in PBS containing 0.5% Triton X-100 (T8787, Sigma, USA) for 30 min at RT. Permeabilized embryos were washed three times for 2 min in PBS containing 0.1% Tween-20 and 2% BSA (A9647, Sigma, USA). Embryos were then incubated in blocking buffer (PBS containing 5% BSA) for 4 h at 4°C. Primary antibodies were incubated overnight at 4°C in blocking buffer: goat anti-NANOG 1:80 (AF1997, R&D-Systems, USA), mouse anti-GATA3 1:100 (MAB6330, R&D-Systems, USA), rabbit anti-caspase-3 (active/cleaved) 1:1000 (NB10056113, Novus Biologicals, USA). Secondary

antibodies were incubated for 1 h at RT in blocking buffer: Alexa Fluor 647 Donkey anti-goat 1:500 (ab150135, Abcam, UK), Alexa Fluor 568 Donkey anti-mouse 1:500 (ab175700, Abcam, UK), Alexa Fluor 488 Donkey anti-rabbit 1:500 (ab150061, Abcam, UK). In addition, embryos were stained with 165 µM DAPI (D8417, Merck, Germany) for 15 min at RT to reveal nuclei.

For TUNEL staining, blastocysts were incubated in 12 µl of TUNEL reaction mixture (In Situ Cell Death Detection Kit, Roche, Germany) for 1 h at 37°C in humid conditions after the incubation with secondary antibodies.

Confocal imaging

Stained blastocysts were placed in glass-bottom dishes (Nunc Glass Bottom Dish, Fisher Scientific, USA) in 15 μ l drops of PBS containing 2% BSA overlaid with mineral oil (FerticultTM, Fertipro, Belgium) and imaged with an FV1000 confocal microscope using the software FV10-ASW 4.2 b (Olympus, Japan).

Confocal imaging was accomplished by capturing a z-series stack of 3- μ m-thick optical sections encompassing the whole blastocyst. A virtual channel was set up to image all fluorescent signals simultaneously, and a Kalman filter was applied to reduce high-gain noise. A 40 $\times$ oil immersion lens was used for imaging.

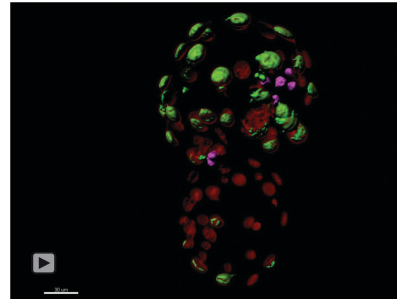
The 635-, 559-, and 488-nm laser wavelengths of a light-emitting diode (LED) were used to image Alexa Fluor 647, Alexa Fluor 568, and Alexa Fluor 488, respectively. The 405-nm laser wavelength of a LED was used to visualize DAPI staining. The 488-nm laser wavelength of a LED was used to visualize TUNEL+ nuclei. Laser acquisition parameters, including laser intensity (LI), high voltage (HV), and offset, were adjusted to compensate for the loss of fluorescent signal due to the presence of the zona pellucida, and two laser configurations were established: (i) non-hatching blastocysts: 635 nm (LI=43.6%, HV=685, offset=14), 559 nm (LI=56%, HV=700, offset=8), 488 nm (LI=40%, HV=585, offset=89), 405 nm (LI=47.8%, HV=950, offset=11); (ii) hatching/hatched blastocysts: 635 nm (LI=43.6%, HV=670, offset=34), 559 nm (LI=56%, HV=595, offset=8), 488 nm (LI=40%, HV=520, offset=89), 405 nm (LI=47.8%, HV=770, offset=25).

Colocalization of cell-lineage and apoptotic markers

Z-stack projection files in the OIF format were visualized with the software FV10-ASW 4.2 b (Olympus, Japan), and fluorescent channels were analyzed for each blastocyst. The analysis was conducted in a blinded fashion, as images were assigned a numerical code unrelated to blastocyst classification. For each blastocyst, the following cell counts were performed: number of total cells (DAPI+), number of TE cells (GATA3+), number of ICM cells (GATA3-/NANOG+), and number of apoptotic cells (caspase-3+). Individual focal planes were assessed to confirm the colocalization of cell markers. The incidence of apoptosis was calculated for each blastocyst by dividing the number of caspase-3+ cells by the number of TE or ICM cells. The level of DNA fragmentation was calculated for each blastocyst by dividing the number of TUNEL+ cells by the number of TE or ICM cells. Three-dimensional (3D) confocal z-stacks were reconstructed using Imaris software (Bitplane, Oxford Instrument Company, USA) (Video 1).

Statistical analysis

The sample size was not pre-defined, and embryos were homogeneously allocated into different experimental rounds according to the day of the biopsy, embryo quality, and PGT-A diagnosis. Qualitative data were analyzed using chi-square or Fisher exact tests depending on expected frequencies. The distribution characteristics of quantitative variables were visualized in histograms and analyzed using Kolmogorov-Smirnov and Levene's tests (Supplementary Table S2). Quantitative data that presented a normal distribution were analyzed using an ANOVA test. Quantitative data that did not present a normal distribution were analyzed using a Kruskal-Wallis test. Continuous variables were expressed as mean $\pm$ standard deviation. Data were analyzed using 95% confidence levels ($P < 0.05$ was considered statistically



Video 1. Three-dimensional confocal z-stack reconstruction of a human blastocyst with apoptotic trophoctoderm cells. Imaris software was used to colocalize cell-lineage and apoptotic markers. GATA3+ cells are represented in red, NANOG+ cells in pink, and Caspase-3+ cells in green.

significant) with SPSS (v26, IBM Corporation). Figures were made in GraphPad Prism (v9, GraphPad Software). The number of cells or embryos analyzed (n), statistical tests, and P-values are all stated in each figure or figure legend.

Results

Immunostaining for DAPI, NANOG, and GATA3 markers was performed on all 64 blastocysts (47 blastocysts at 5 dpf and 17 blastocysts at 6 dpf). We identified a total of 3387 DAPI+ cells (2369 cells at 5 dpf and 1018 cells at 6 dpf), 1138 NANOG+ cells (930 cells at 5 dpf and 208 cells at 6 dpf), and 2693 GATA3+ cells (1851 cells at 5 dpf and 842 cells at 6 dpf). Additionally, 53 out of the 64 blastocysts (38 blastocysts at 5 dpf and 15 blastocysts at 6 dpf) were stained for caspase-3, revealing a total of 912 caspase-3+ cells (604 cells at 5 dpf and 308 cells at 6 dpf). The remaining 11 blastocysts (9 blastocysts at 5 dpf and 2 blastocysts at 6 dpf) were stained for TUNEL, uncovering a total of 124 TUNEL+ cells (99 cells at 5 dpf and 25 cells at 6 dpf).

Phase I: caspase-3 staining

Total cell counts

We first determined the mean total cell counts by analyzing DAPI staining. Blastocysts analyzed at 5 dpf and 6 dpf exhibited similar total cell counts (51.9 $\pm$ 16 cells and 60.5 $\pm$ 21.9 cells, respectively) ($P > 0.05$). Furthermore, at 5 dpf, total cell counts were comparable among euploid blastocysts (59.2 $\pm$ 20.6 cells), mosaic blastocysts (49.6 $\pm$ 15 cells), and aneuploid blastocysts (48.5 $\pm$ 14.2 cells) ($P > 0.05$). Similarly, at 6 dpf, no significant differences in total cell counts were found among euploid blastocysts (78.5 $\pm$ 0.7 cells), mosaic blastocysts (58.8 $\pm$ 16.9 cells), and aneuploid blastocysts (59.6 $\pm$ 23.5 cells) ($P > 0.05$) (Supplementary Fig. S2A).

Analysis of NANOG and GATA3 expression

Next, we examined the expression of the cell-lineage markers NANOG and GATA3. On the one hand, the proportion of NANOG+ cells decreased significantly from 38.2% (755/1979 cells) at 5 dpf to 20.3% (188/927 cells) at 6 dpf ($P < 0.0001$). On the other hand, the proportion of GATA3+ cells increased significantly from 77.8% (1540/1979 cells) at 5 dpf to 83.1% (770/927 cells) at 6 dpf ($P < 0.01$). Consistently, the proportion of GATA3+/

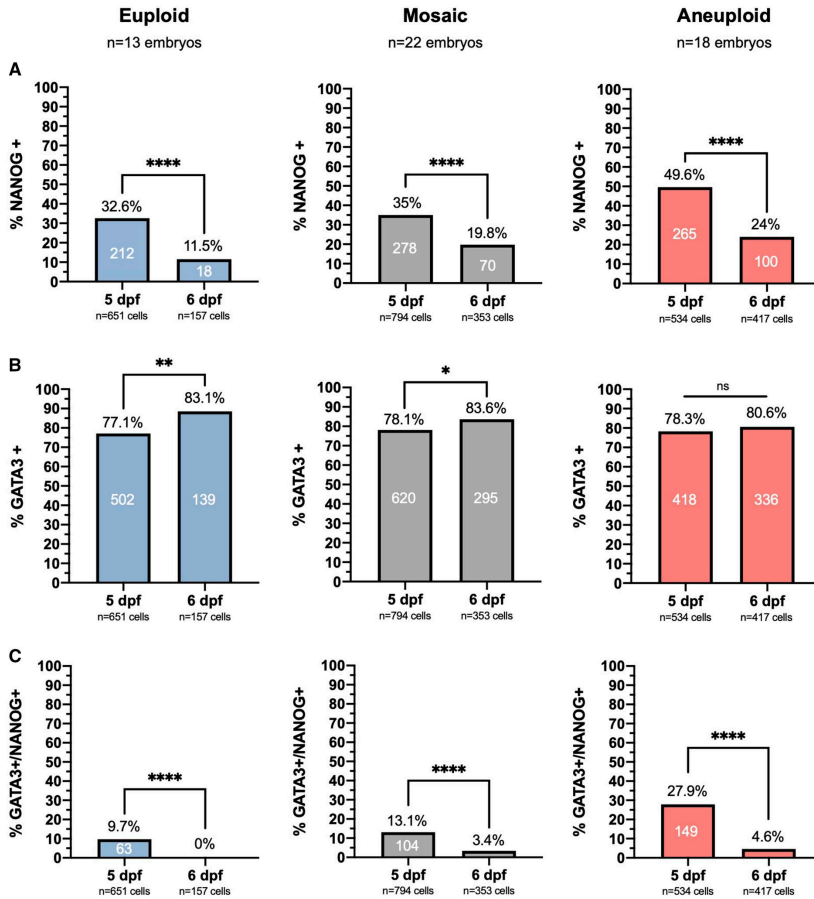


Figure 2. Analysis of NANOG and GATA3 expression throughout the blastocyst stage. (A) Percent of NANOG+ cells. (B) Percent of GATA3+ cells. (C) Percent of GATA3+/NANOG+ cells. Blue, grey, and red bars show euploid, mosaic, and aneuploid embryos, respectively. The values below each bar (n) indicate the number of cells analyzed. The values displayed within each bar indicate the number of positive cells. The values displayed above each bar indicate the percent of positive cells. 5 dpf, Day 5 post-fertilization; 6 dpf, Day 6 post-fertilization. Data were analyzed using chi-square or Fisher exact tests at the 95% confidence level. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$; **** $P < 0.0001$; ns, not significant.

NANOG+ cells decreased significantly from 16% (316/1979 cells) at 5 dpf to 3.3% (31/927 cells) at 6 dpf ($P < 0.0001$).

Interestingly, when stratifying by genetic status, only in aneuploid blastocysts did the proportion of GATA3+ cells fail to increase significantly from 5 dpf to 6 dpf ($P > 0.05$) (Fig. 2). Furthermore, at 5 dpf, aneuploid blastocysts displayed a significantly higher proportion of GATA3+/NANOG+ cells (27.9% = 149/534 cells) compared to mosaic blastocysts (13.1% = 104/794 cells) and euploid blastocysts (9.7% = 63/651 cells) ($P < 0.0001$). Similarly, at 6 dpf, the proportion of GATA3+/NANOG+ cells was significantly higher in aneuploid blastocysts (4.6% = 19/417 cells)

and mosaic blastocysts (3.4% = 12/353 cells) compared to euploid blastocysts (0% = 0/157 cells) ($P < 0.05$) (Fig. 3).

Remarkably, a similar effect was observed when blastocysts were further stratified based on their expected viability according to the type of aneuploid chromosome(s). The highest proportion of GATA3+/NANOG+ cells was found in aneuploid blastocysts containing only non-viable aneuploidies (17.7% = 168/951 cells), while the lowest proportions of GATA3+/NANOG+ cells were observed in euploid blastocysts (7.8% = 63/808 cells) and mosaic blastocysts containing only viable aneuploidies (2.5% = 2/81 cells) ($P < 0.01$). Additionally, the proportion of GATA3+/NANOG+ cells

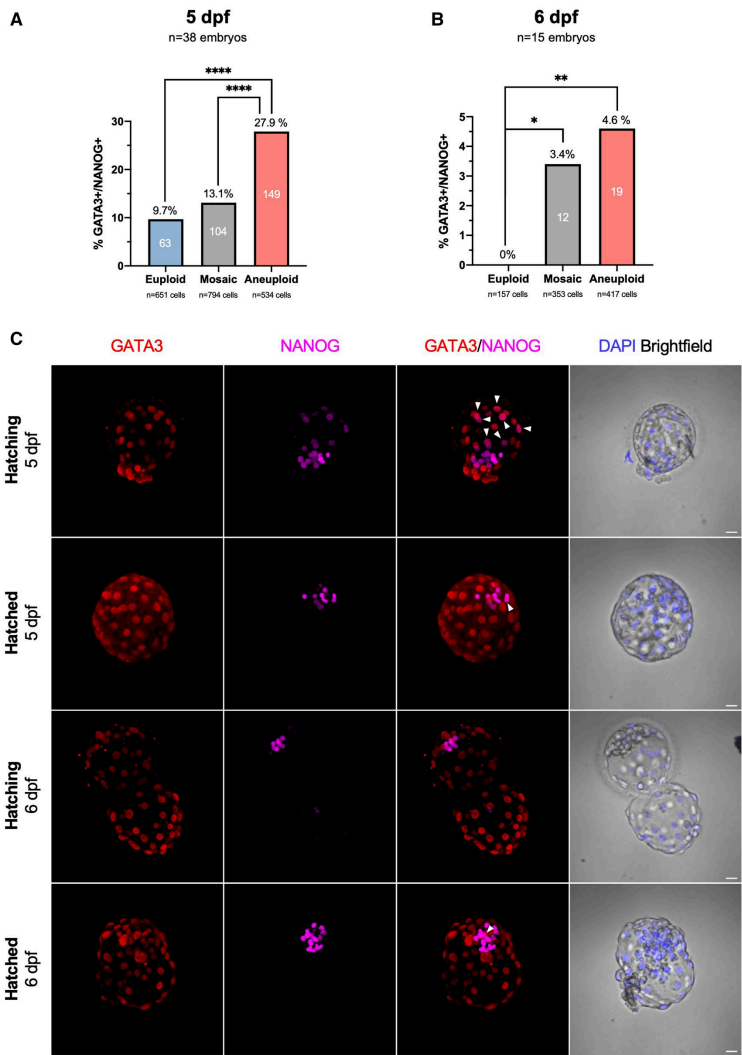


Figure 3. Persistence of NANOG expression in trophoderm cells. (A) Day 5 post-fertilization (5 dpf). **(B)** Day 6 post-fertilization (6 dpf). Blue, grey, and red bars show euploid, mosaic, and aneuploid embryos, respectively. The values below each bar (n) indicate the number of cells analyzed. The values displayed within each bar indicate the number of positive cells. The values displayed above each bar indicate the percent of positive cells. Pairwise comparisons of proportions by chi-square test at the 95% confidence level. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$. **(C)** Representative images of human embryos analyzed at different developmental stages and immunostained for NANOG and GATA3. Arrowheads indicate cells coexpressing GATA3 and NANOG. Scale bars, 20 μ m.

was significantly lower in mosaic blastocysts containing only non-viable aneuploidies (9.3% = 80/856 cells) compared to their aneuploid counterparts (17.7% = 168/951 cells) ($P < 0.001$). Finally,

mosaic blastocysts containing a mixture of viable and non-viable aneuploidies displayed a similar proportion of GATA3+/NANOG+ cells (16.2% = 34/210 cells) compared to aneuploid

blastocysts containing only non-viable aneuploidies (17.7% = 168/951 cells) ($P > 0.05$), which can be attributed to the higher number of chromosomes affected in the mosaic group (Supplementary Fig. S3A) (Table 1).

Quantification of TE and ICM cells

Then, we quantified TE cells as GATA3+ cells and ICM cells as GATA3-/NANOG+ cells. Blastocysts analyzed at 6 dpf exhibited a significantly higher number of GATA3+ cells (51.3 ± 18.7 TE cells) compared to those analyzed at 5 dpf (40.5 ± 14.5 TE cells) ($P < 0.05$). In contrast, the numbers of GATA3-/NANOG+ cells remained virtually unchanged (11.6 ± 4.6 ICM cells at 5 dpf; 10.5 ± 5.1 ICM cells at 6 dpf) ($P > 0.05$).

When considering the chromosomal constitution, we observed that, at 5 dpf, euploid blastocysts displayed similar numbers of GATA3+ cells and GATA3-/NANOG+ cells (45.6 ± 17.6 TE cells; 13.5 ± 4 ICM cells) compared to mosaic blastocysts (38.8 ± 13.7 TE cells; 10.9 ± 4.2 ICM cells) and aneuploid blastocysts (38 ± 11.8 TE cells; 10.5 ± 5.1 ICM cells) ($P > 0.05$). Similarly, at 6 dpf, no significant differences were found in the number of GATA3+ cells and GATA3-/NANOG+ cells among euploid blastocysts (69.5 ± 2.1 TE cells; 9 ± 2.8 ICM cells), mosaic blastocysts (49.2 ± 16.2 TE cells; 9.7 ± 1.1 ICM cells), and aneuploid blastocysts (48 ± 21.6 TE cells; 11.6 ± 3.2 ICM cells) ($P > 0.05$) (Supplementary Fig. S2B and C).

Analysis of caspase-3 expression in TE cells

Next, to identify TE cells undergoing early apoptotic events, we analyzed the expression of the apoptotic marker caspase-3 in these cells. Overall, apoptosis was a frequent phenomenon in TE cells. In fact, out of the 2310 TE cells analyzed, 901 (39%) were caspase-3+. Remarkably, 192 (21.3%) of these 901 apoptotic TE cells were GATA3+/NANOG+, while the remaining 709 cells (78.7%) were GATA3-/NANOG-.

The incidence of apoptosis in TE cells was significantly associated with the PGT-A diagnosis. At 5 dpf, a significantly higher proportion of caspase-3+ TE cells was observed in mosaic blastocysts (44.1% $\pm$ 19.6) and aneuploid blastocysts (45.9% $\pm$ 16.1) compared to euploid blastocysts (26.3% $\pm$ 17) ($P < 0.05$) (Fig. 4A) (Video 1). At 6 dpf, a similar increasing trend was observed when comparing the proportion of caspase-3+ TE cells among euploid blastocysts (17.5% $\pm$ 14.8), mosaic blastocysts (43% $\pm$ 16.8), and aneuploid blastocysts (49% $\pm$ 15.1). However, these differences did not reach statistical significance ($P > 0.05$) (Fig. 4B).

Interestingly, the incidence of apoptosis in TE cells was also correlated with the type of aneuploid chromosome(s). Although the proportion of caspase-3+ TE cells showed significant differences only between euploid blastocysts (24.9% $\pm$ 16.4) and aneuploid blastocysts containing only non-viable aneuploidies (47.1% $\pm$ 15.3) ($P < 0.05$), an increasing trend was observed across all intermediate categories. Notably, this trend almost reached statistical significance when comparing euploid blastocysts to mosaic blastocysts containing only non-viable aneuploidies (43.9% $\pm$ 18.2) ($P = 0.06$) (Supplementary Fig. S3B).

Analysis of caspase-3 expression in ICM cells

Subsequently, we applied a similar analysis to identify ICM cells experiencing early apoptotic events by assessing their caspase-3 expression. In contrast to what was observed in TE cells, apoptosis was an exceedingly rare event in ICM cells. Indeed, only 11 (1.9%) out of the 596 ICM cells analyzed were caspase-3+ ($P < 0.0001$).

Although the incidence of apoptosis in ICM cells was not significantly associated with the PGT-A diagnosis ($P > 0.05$), euploid blastocysts were the only group lacking any caspase-3+ ICM cells (0/149 cells at 5 dpf; 0/18 cells at 6 dpf). In contrast, six caspase-

3+ ICM cells were found in mosaic blastocysts (1.7% = 3/174 cells at 5 dpf; 5.2% = 3/58 cells at 6 dpf), and five caspase-3+ ICM cells were found in aneuploid blastocysts (0.9% = 1/116 cells at 5 dpf; 4.9% = 4/81 cells at 6 dpf), respectively (Fig. 5).

Phase II: TUNEL assay

Due to the limited number of blastocysts included in this study phase, especially after scrutiny by ploidy status and dpf, the analysis of the immunostaining markers DAPI, NANOG, and GATA3 was conducted solely to confirm the differential occurrence of apoptosis between human TE and ICM cells. For this purpose, we performed the TUNEL assay to assess the level of DNA fragmentation (a marker of late-stage apoptosis) on an additional cohort of 11 blastocysts.

Consistently, out of the 98 ICM cells analyzed, none (0%) were found to be TUNEL+, whereas 124 out of the 383 TE cells analyzed (32.4%) were TUNEL+ ($P < 0.0001$) (Supplementary Fig. S4A). Despite the small sample size, a non-significant increasing trend was observed toward higher levels of DNA fragmentation in the TE of mosaic blastocysts (37.2% $\pm$ 21.9) and aneuploid blastocysts (39% $\pm$ 41.7), compared to euploid blastocysts (23% $\pm$ 32.5) ($P > 0.05$) (Supplementary Fig. S4B).

Discussion

Advances in sequencing technologies have revolutionized our understanding of the complexity and plasticity of the human embryonic genome (Coticchio et al., 2021). Successful embryonic development relies on the faithful transmission of genetic material. However, chromosome segregation errors are frequent during early post-zygotic cell divisions, leading to widespread mosaicism in pre-implantation embryos (McCoy, 2017). While the detrimental effect of aneuploidy on cellular viability and clinical outcomes has been extensively demonstrated (Williams et al., 2008; Zhu et al., 2018; Shahbazi et al., 2020; Capalbo et al., 2022; Tosh et al., 2022), relatively little is known about the mechanisms responsible for the survival of embryos containing a mixture of euploid and aneuploid cells. This knowledge is critical for guiding the implementation of genetic screening approaches and broadening our understanding of human reproductive capacity.

A preliminary strategy for investigating the impact of mosaicism on embryonic competence is to compare its prevalence across different developmental time points. These studies have shown that mosaicism declines over embryo development, affecting more than 70% of embryos at cleavage stages (Vanneste et al., 2009; van Echten-Arends et al., 2011; Mertzaniidou et al., 2013), 2–50% of embryos at the blastocyst stage (Viotti, 2020; Levy et al., 2021; Rana et al., 2023), and <2% of established pregnancies, of which 90% represent confined placental mosaicism (Malvestiti et al., 2015). In product of conceptions, mosaicism is typically detected in less than 1% of cases (Menasha et al., 2005; Hardy et al., 2016).

Several models, which are not mutually exclusive, have been proposed to account for this reduction (McCoy, 2017). First, the embryonic mortality model indicates that mosaic embryos are negatively selected due to the detrimental impact of aneuploid cells on embryonic fitness. This has been evidenced by studies showing a high prevalence of mosaic abnormalities in arrested embryos (Ruangvutitert et al., 2000; Bielanska et al., 2002; Rubio et al., 2007; Santos et al., 2010).

Second, the aneuploidy rescue model suggests that mosaicism can be corrected through the reversion of aneuploidy into euploidy. While this has been theorized, evidence supporting this mechanism in humans is scarce (Coorens et al., 2021;

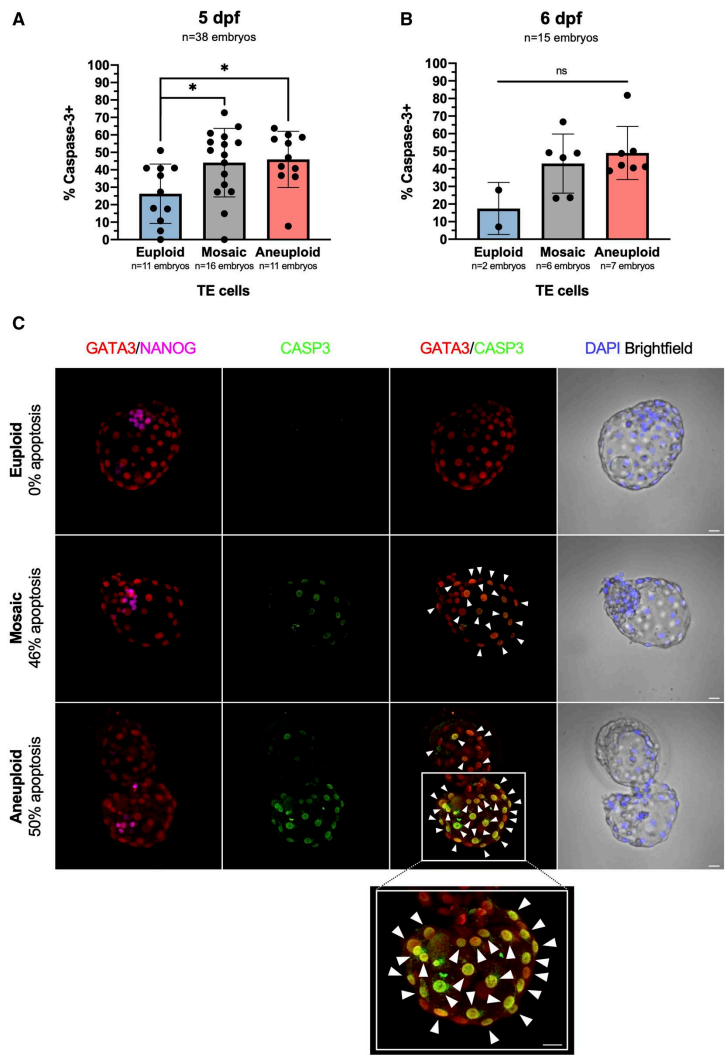


Figure 4. Analysis of caspase-3 expression in trophoblast (TE) cells. (A) Day 5 post-fertilization (5 dpf). (B) Day 6 post-fertilization (6 dpf). Blue, grey, and red bars show euploid, mosaic, and aneuploid embryos, respectively. The values below each bar (n) indicate the number of embryos analyzed. Each dot represents one analyzed embryo. The apoptotic rate was calculated for each embryo by dividing the number of caspase-3+ cells by the total number of TE cells. The error bars represent the standard deviation. Apoptotic rates were compared using Kruskal-Wallis or one-way analysis of variance (ANOVA) tests with Bonferroni post hoc at the 95% confidence level. * $P < 0.05$; ns, not significant. (C) Representative images of human embryos immunostained for NANOG, GATA3, and caspase-3. Arrowheads indicate apoptotic TE cells. A magnified section is included for a better appreciation of the apoptotic TE cells. Scale bars, 20 μ m.

Schlade-Bartusiak et al., 2022), and it seems to play a minor role based on the low incidence of uniparental disomy in blastocysts (Gueye et al., 2014; Taylor et al., 2014).

Third, the preferential allocation model involves the selective distribution of aneuploid cells into extraembryonic lineages. Although there is contradictory evidence regarding this

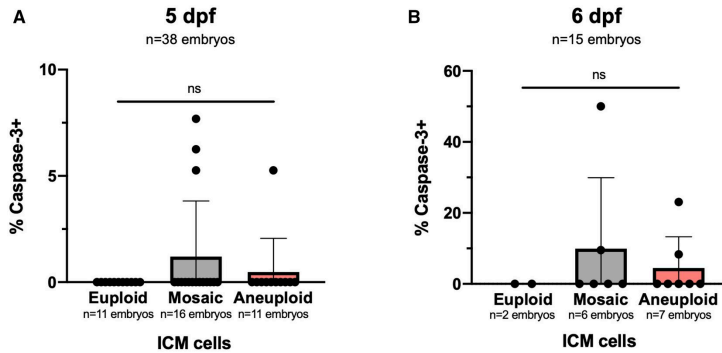


Figure 5. Analysis of caspase-3 expression in inner cell mass (ICM) cells. (A) Day 5 post-fertilization (5 dpf). (B) Day 6 post-fertilization (6 dpf). Blue, grey, and red bars show euploid, mosaic, and aneuploid embryos, respectively. The values below each bar (n) indicate the number of embryos analyzed. Each dot represents one analyzed embryo. The apoptotic rate was calculated for each embryo by dividing the number of caspase-3+ cells by the total number of ICM cells. The error bars represent the standard deviation. Apoptotic rates were compared using Kruskal-Wallis or ANOVA tests at the 95% confidence level. ns, not significant.

mechanism (Capalbo et al., 2013; Popovic et al., 2018; Starostik et al., 2020), recent studies suggest that aneuploid cells may primarily segregate to the blastocoel fluid, to peripheral cells located distantly from the ICM, and, to a lesser extent, to the TE cell lineage (Yang et al., 2021; Griffin et al., 2023). These findings are consistent with the identification of physiological bottlenecks that confine aneuploid cells arising within the first few cell divisions to placental lineages (Coorens et al., 2021).

Finally, the clonal depletion model implies that aneuploid cells are either eliminated by apoptosis or fail to propagate efficiently within mosaic embryos, thereby reducing their detrimental impact. Although direct evidence of these mechanisms in human embryos is lacking, studies performed in artificially generated mouse and human chimeras suggest that embryo viability can be restored through targeted apoptosis of aneuploid cells and competitive growth of euploid cells (Bolton et al., 2016; Singla et al., 2020; Yang et al., 2021). In addition, the removal of aneuploid cells may occur either through their exclusion via partial compaction (Zhan et al., 2016; Lagalla et al., 2017, 2020; Hur et al., 2023) or through their extrusion in the form of expelled fragments or debris (Lagalla et al., 2020; Orvieto et al., 2020).

In this scenario, to gain new insights into the clonal depletion mechanism, we studied the impact of mosaic and uniform aneuploidies on the apoptotic rates of human blastocysts during the first lineage segregation event to TE and ICM cells.

Predictably, overall changes in the expression of cell-lineage markers between 5 dpf and 6 dpf evidenced the segregation of ICM and TE cell types. On the one hand, the proportion of NANOG+ cells decreased significantly from 5 dpf to 6 dpf, which is consistent with the restriction of pluripotency maintenance genes such as SOX2, OCT4, and NANOG to ICM cells during the first lineage segregation process (Gerri et al., 2020). On the other hand, the proportion of GATA3+ cells increased significantly from 5 dpf to 6 dpf, indicating the progression of TE differentiation driving the expression of a multitude of trophoblast-related genes, including TEAD1, YAP1, CDX2, and GATA3, the latter being the key player in the initiation of the placental progenitor program (Regin et al., 2023).

Consistently, the number of TE cells increased significantly from 5 dpf to 6 dpf. In contrast, the number of ICM cells

remained unchanged, resulting in comparable total cell counts between 5 dpf and 6 dpf. Overall, cell counts were in line with previous reports, although the range was considerable across studies (Hardy et al., 1989; Niakan and Eggan, 2013; Iwasawa et al., 2019). This variability can be ascribed to differences in laboratory factors, including culture conditions (e.g. oxygen tension, temperature, pH, or media composition), fertilization technique, or biopsy procedure. Additionally, variations in cell numbers may be attributed to embryo-related factors, such as embryo quality or chromosomal sex (ESHRE Working Group on Time-Lapse Technology et al., 2020), as well as to the methodology used for cell counting.

In our cohort, cell counts were not significantly affected by the chromosomal status of blastocysts. However, we observed a trend toward a lower number of TE cells in mosaic and aneuploid blastocysts compared to euploid blastocysts, both at 5 dpf and 6 dpf. Interestingly, these blastocysts displayed a significantly higher proportion of caspase-3+ TE cells at 5 dpf, and a similar, albeit non-significant trend was observed in those analyzed at 6 dpf. Taken together, these data point to increased apoptosis as the cause of the decreasing trend in TE cell numbers. In contrast, in ICM cells, apoptosis was remarkably rare in mosaic and aneuploid blastocysts and undetectable in euploid blastocysts.

These results fit well with classic studies showing that apoptosis is a common feature of human preimplantation development, even in normally developing blastocysts of good morphology (Hardy et al., 1989; Hardy, 1997). Furthermore, our findings are consistent with more recent research on mouse embryos (Bolton et al., 2016; Singla et al., 2020) and human gastruloids (Yang et al., 2021), which demonstrate that the impact of aneuploidy on apoptosis is cell-type dependent. However, these studies indicate that aneuploid cells are preferentially eliminated from the ICM, with the TE displaying greater resistance to apoptosis and instead exhibiting proliferative defects. In contrast, research on human embryos has revealed a higher incidence of apoptosis in TE cells at both the mRNA and protein expression levels (Victor et al., 2019; Martin et al., 2023). Importantly, this incidence correlates with the presence of mosaic and uniform aneuploidies, which is reinforced by our data.

Furthermore, we report concurrent findings by using the TUNEL assay. While active caspase-3 staining enables the visualization of cells initiating the apoptotic pathway, the TUNEL assay allows for the detection of DNA fragmentation, a downstream consequence of caspase-3 activation, and thus a key marker of late-stage apoptosis (Kari *et al.*, 2022). Therefore, the fact that both methods yielded consistent results suggests that the differential susceptibility to apoptosis observed in human ICM and TE cells is not an artifact of the technique but a genuine biological response.

In fact, it is well established that pluripotent cells exhibit distinct biological characteristics compared to differentiated cells. These include atypical cell cycles with truncated gap phases, resulting in unusually high rates of proliferation (Padgett and Santos, 2020), highly decondensed chromatin (Delgado-Olguin and Recillas-Targa, 2011), as well as a glycolytic-based metabolism with increased proteasome activity (Milagre *et al.*, 2023). These intrinsic differences may not only affect the fidelity of mitotic divisions but also the way these cells tolerate aneuploidy (Taapken *et al.*, 2011; Rebuzzini *et al.*, 2015). In fact, while aneuploidy typically results in the death of differentiated cells, pluripotent cells are more prone to continue proliferating despite the aneuploid state, and frequently acquire and expand p53-dominant negative mutations (Merkle *et al.*, 2017). In this regard, the reduced frequency of apoptosis observed in ICM cells may be due to an uncoupling between apoptosis and aneuploidy-induced stress signals, as shown in human embryonic stem cells (Mantel *et al.*, 2007). However, whether human blastocysts possess additional mechanisms to cope with aneuploidy in ICM cells or if apoptosis is activated later in development remains to be elucidated.

In addition, we report that a considerable proportion of TE cells retain nuclear NANOG expression at 5 dpf, which declined significantly by 6 dpf. This observation aligns with previous studies demonstrating that in humans, the nuclear expression of NANOG becomes restricted to the ICM only after the first lineage segregation at the late blastocyst stage (Gerri *et al.*, 2020; Tocci, 2020). Furthermore, NANOG has been found to co-localize with OCT4 in human blastocysts (Hambiliki *et al.*, 2012). Therefore, given that TE cells can retain expression of stemness genes common to ICM cells, it is possible that the incidence of apoptosis in ICM cells has been overestimated in the study of Victor *et al.* (2019) due to the use of OCT4 as the sole cell-lineage marker.

Interestingly, the persistence of NANOG expression in TE cells was associated with the ploidy status of the blastocysts, as evidenced by the significantly higher proportion of GATA3+/NANOG+ cells found in mosaic and aneuploid blastocysts compared to fully euploid ones. In fact, complete TE differentiation, meaning the absence of any GATA3+/NANOG+ cells, was only observed in euploid blastocysts at 6 dpf. Consistently, only in aneuploid blastocysts did the proportion of TE cells fail to increase significantly from 5 dpf to 6 dpf.

These findings align well with several prior studies on human embryonic and trophoblast stem cells that have reported changes in cellular differentiation capacity in response to aneuploidy (Maclean *et al.*, 2012; Bosman *et al.*, 2015; Shahbazi *et al.*, 2020; Haim-Abadi *et al.*, 2023). Intriguingly, in mouse embryonic stem cells, aneuploidy has been shown to impact differentiation by delaying the exit from pluripotency through increased retention of NANOG and OCT4 expression, thereby reinforcing our findings (Zhang *et al.*, 2016).

Moreover, the expression of NANOG in TE cells declined more rapidly in mosaic blastocysts than in euploid blastocysts. This observation is also consistent with the findings of Zhang *et al.*

(2016) who reported that the differentiation defects of aneuploid embryonic stem cells can be mitigated by the presence of neighboring euploid cells, which express critical differentiation factors that operate in a paracrine manner. Given that cell fate is driven by intricate transcription factor networks that depend on genetic and epigenetic responses, it appears intuitive that increasing aneuploid cell loads would progressively affect differentiation.

Interestingly, both the differentiation and survival capacity of TE cells were also influenced by the number and type of aneuploid chromosome(s). This was evidenced by the significantly higher proportions of GATA3+/NANOG+ cells and caspase-3+ TE cells found in blastocysts harboring uniform, multiple and/or non-viable aneuploidies compared to those harboring mosaic, single and/or viable aneuploidies. This observation offers novel molecular insights into the notion that the deleterious effect of aneuploidy in human embryos depends not only on the aneuploid cell load but also on the extent and characteristics of the specific chromosomes involved (Frasniasak *et al.*, 2014; Shahbazi *et al.*, 2020). While these data suggest a potential correlation between the differentiation defect, aberrant chromosomal status, and increased apoptotic susceptibility of human TE cells, the intricacies of this relationship remain elusive.

From a clinical perspective, to the best of our knowledge only one previous study has investigated the reproductive potential of embryos diagnosed as mosaic using concurrent copy number and SNP genotyping analysis (Babariya *et al.*, 2023). This study showed that embryos with less than 50% mosaicism have equivalent sustained implantation rates than fully euploid ones, while those exceeding the threshold resulted in significantly poorer outcomes. These findings complement our own and support the clonal depletion model, suggesting that, although the detrimental impact of aneuploidy at the cellular level may vary depending on the affected chromosome(s), mosaic embryos can progress to normal pregnancies provided they contain sufficient euploid cells after the removal or hindered growth of aneuploid cells. We propose that this could occur as a result of cellular stress stemming from the aberrant genetic state of aneuploid cells, as demonstrated in transcriptome studies (Vera-Rodriguez *et al.*, 2015; Licciardi *et al.*, 2018; Sanchez-Ribas *et al.*, 2019; Maxwell *et al.*, 2022; Martin *et al.*, 2023), rather than as a protective mechanism specifically aimed at preserving embryonic competence.

Our study has several strengths. Firstly, mosaic diagnoses were conducted using a novel PGT-A platform that integrated both copy number and SNP genotyping analysis, thereby reducing the risk of false positives (Popa *et al.*, 2023; Rana *et al.*, 2023). Secondly, embryos were distributed uniformly across experimental rounds based on the day of the biopsy, embryo quality, and PGT-A diagnosis. This approach allowed us to minimize the confounding effect of experimental artifacts, particularly fluctuations in laser acquisition settings due to temperature changes, electrical noise, or vibrations. Thirdly, we used TUNEL staining as a secondary apoptotic marker in a separate cohort of blastocysts, which reinforced the results obtained with caspase-3 staining.

However, some limitations of the study should be also noted. Firstly, given the difficulty of obtaining embryos with the highest morphological grade for research, our analysis was limited to embryos categorized as grade B or C. This means that our results may not apply to embryo populations with superior morphological quality. Secondly, while we report increased apoptosis and impaired differentiation in mosaic blastocysts, it remains unknown whether these processes selectively occur in aneuploid cells. Thirdly, although the observed effects were stratified based

on the type of affected chromosome(s), mosaic and aneuploid blastocysts harbored different aneuploidies. Thus, our results may be biased by the chromosome-specific effect of aneuploidy on apoptosis and cell differentiation. Fourthly, 6 dpf blastocysts exhibited lower cleavage capacity than 5 dpf blastocysts. Therefore, we do not know what would be the molecular and cell lineage differentiation status of 5 dpf blastocysts purportedly left in culture until 6 dpf. Finally, we did not assess karyotype concordance between TE and ICM cells. As a result, the possibility of false positives cannot be ruled out.

In conclusion, our study provides compelling evidence of distinct types of alterations of cellular function associated with the presence of mosaic and uniform aneuploidies in human blastocysts. These findings might help explain why most uniformly aneuploid blastocysts fail to progress in development and suggest that analogous mechanisms could lead to removal of aneuploid cells in mosaic blastocysts. We believe that these data contribute to unveiling the biological identity of mosaic blastocysts and offer valuable insights into the factors that determine their reproductive potential.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

Most of the data supporting this article are available within the article itself and in its online [Supplementary Material](#). The totality of the data underlying this article will be shared on reasonable request to the corresponding author.

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Authors' roles

A.Mar. and M.J.d.I.S. conceived the study and designed experiments. A.Mar., A.Mer., D.B., A.Mif., M.N., and M.J.d.I.S. identified and recruited patients. A.Mar., M.L.P., and D.O.-J. performed experiments and collected the data. A.Mar., M.L.P., and D.O.-J. analyzed and interpreted the data. A.Mar. and M.J.d.I.S. wrote and edited the article. A.Mer., D.B., A.Mif., and M.N. provided critical feedback and discussion. M.J.d.I.S. supervised the project and secured funding and ethical approvals. All authors approved the final version of the article.

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Conflict of interest

None of the authors has any conflict of interest to declare.

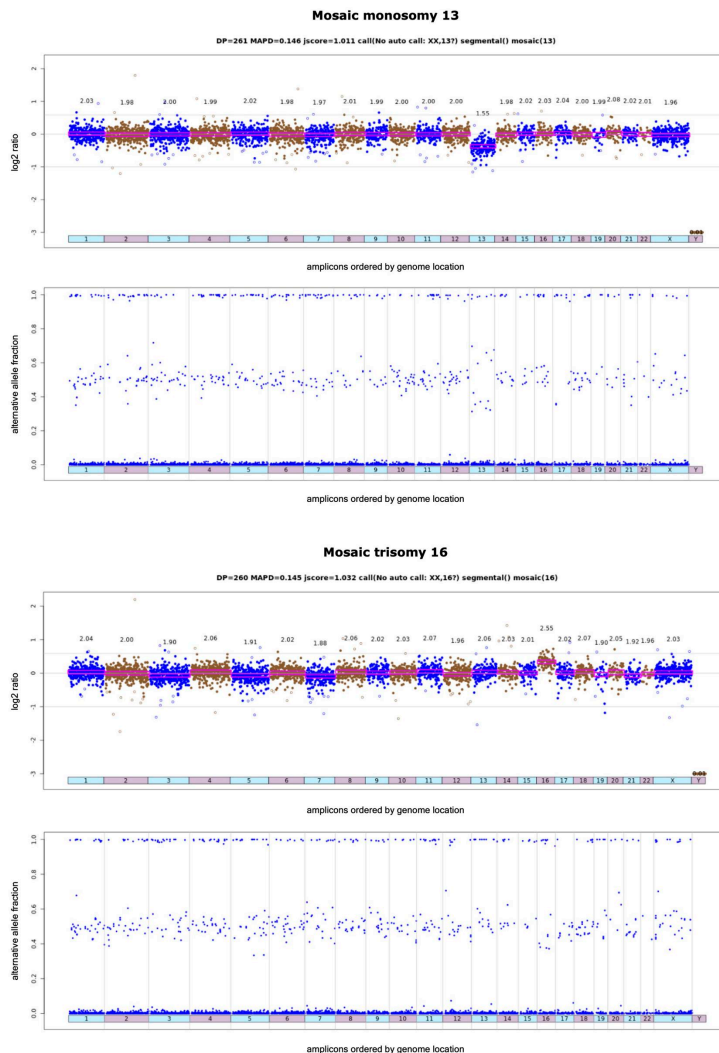
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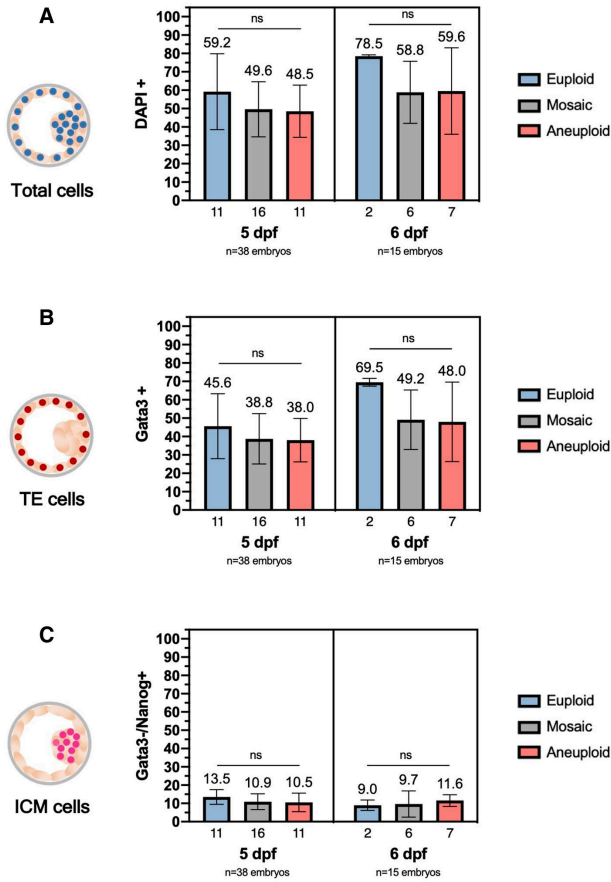
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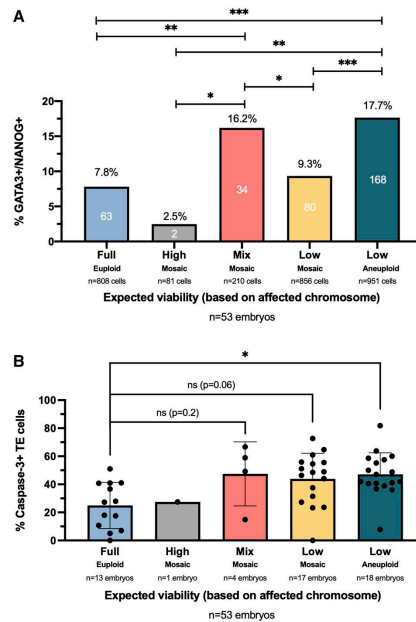
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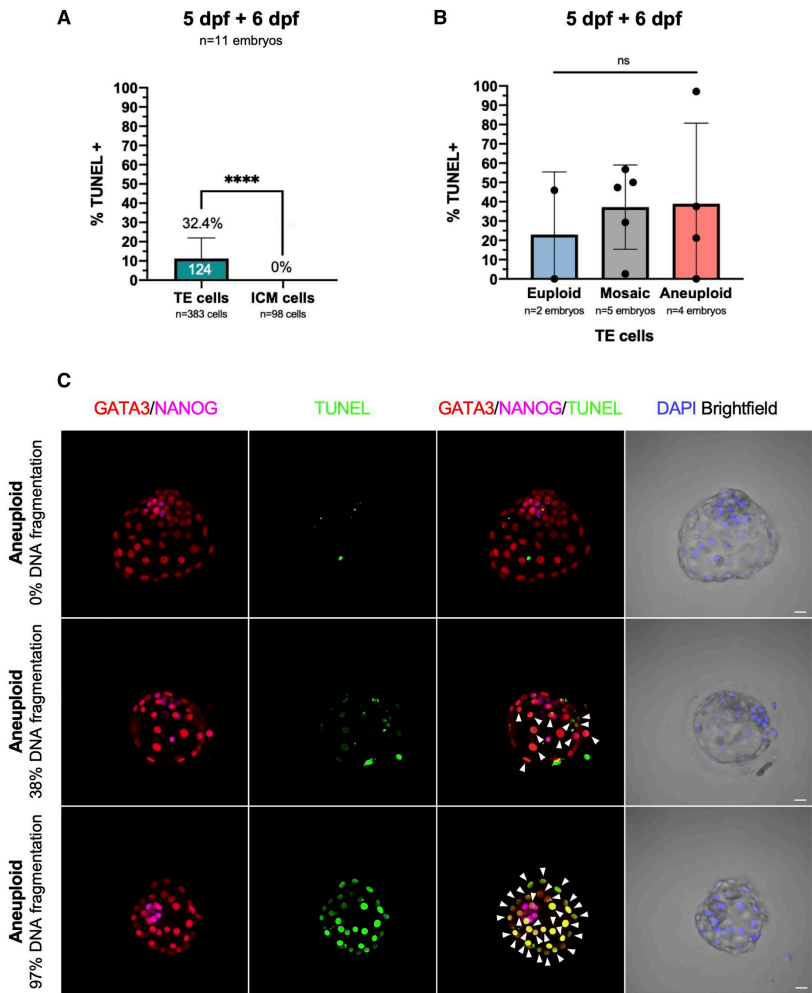
Supplementary Figure S1. PGTseq diagrams of mosaicism diagnoses. For each sample, the PGTseq software generates a chromosome copy number (CCN) profile and a B-allele frequency (BAF) plot using single nucleotide polymorphisms (SNPs) data. The BAF plots consist of two types of SNPs: homozygous and heterozygous. Homozygous AA SNPs are located at the top of the BAF plot, heterozygous AB SNPs are in the middle, and homozygous BB SNPs are at the bottom. A CCN of 2 is represented by an AB SNP profile not deviating from the middle (0.5). A trisomy or the presence of a mosaic chromosome abnormality is represented by a split of the AB SNPs for the affected chromosome, with some shifting upwards and others shifting downwards. Monosomy is represented by a complete loss of the middle AB SNPs for the affected chromosome. Triploidy is represented by a split of the AB SNPs for all chromosomes. The presence of contamination is indicated by a highly noisy profile, where it is impossible to distinguish between the AA, AB, and BB SNPs. The mean absolute percent deviation (MAPD) score describes the "noise" level of a specific chromosome profile and measures the spread of amplicons for that chromosome from the reference line (0). Samples with a MAPD > 0.3 exhibit "noisy" profiles (less compact across the reference line) and can be challenging to interpret.



Supplementary Figure S2. Cell counts comparisons. (A) Total cell counts. (B) Trophectoderm (TE) cell counts. (C) Inner cell mass (ICM) cell counts. The values displayed below each bar indicate the number of embryos analyzed. The values above each bar indicate the mean cell count and the error bars represent the standard deviation. Cell counts were compared using Kruskal–Wallis or one-way ANOVA tests at the 95% confidence level. 5 dpf, Day 5 post-fertilization; 6 dpf, Day 6 post-fertilization; ns, not significant.



Supplementary Figure S3. Persistence of NANOG expression (A) and caspase-3 expression (B) in trophoblast (TE) cells stratified by blastocyst's expected viability based on the type of aneuploid chromosome(s). Each dot represents one analyzed embryo. Blastocyst's expected viability was classified as full (euploid blastocysts), high (blastocysts containing only viable aneuploidies, including +13, +18, +21, +X, +Y, and -X), mix (blastocysts containing a mixture of viable and non-viable aneuploidies), or low (blastocysts containing only non-viable aneuploidies). Mosaic autosomal monosomies were considered equivalent to mosaic autosomal trisomies to account for potential post-zygotic non-disjunction events leading to reciprocal aneuploidies. Pairwise comparisons of proportions were conducted by chi-square test at the 95% confidence level. Apoptotic rates were compared using the Kruskal–Wallis test with Bonferroni post hoc at the 95% confidence level. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ns, not significant.



Supplementary Figure S4. TUNEL assay. (A) Comparison of DNA fragmentation levels between trophectoderm (TE) and inner cell mass (ICM) cells. The values below each bar (n) indicate the number of cells analyzed. The values displayed within each bar indicate the number of positive cells. The values displayed above each bar indicate the percent of positive cells. **** $P < 0.0001$. (B) Analysis of DNA fragmentation in TE cells. The values below each bar (n) indicate the number of embryos analyzed. Each dot represents one analyzed embryo. The level of DNA fragmentation was calculated for each embryo by dividing the number of TUNEL+ cells by the total number of TE cells. The error bars represent the standard deviation. The levels of DNA fragmentation were compared using Kruskal–Wallis or one-way analysis of variance (ANOVA) tests at the 95% confidence level. ns, not significant; 5 dpf, Day 5 post-fertilization; 6 dpf, Day 6 post-fertilization. (C) Representative images of human embryos immunostained for NANOG, GATA3, and TUNEL. Arrowheads indicate TUNEL+ cells. Scale bars, 20 μ m.



Supplementary Table S1. Study population and characteristics of embryos analyzed by TUNEL staining.

Embryo ID	Day of analysis ^a	Grade ASEBIR ^b	Grade TE	Grade ICM	Hatching	PGT-A diagnosis	NGS (% mosaicism)
EU14	5	B	B	B	Yes	Euploid	<30%
EU15	6	B	B	B	Yes	Euploid	<30%
MO23	5	B	B	C	No	Mosaic	–16(50%)
MO24	5	B	B	B	No	Mosaic	–14(40%)
MO25	5	C	C	C	No	Mosaic	–10(70%), –14(40%)
MO26	5	C	C	C	No	Mosaic	–3(30%), –13(20%)
MO27	6	B	B	B	Yes	Mosaic	+19(70%)
AN19	5	B	B	B	Yes	Aneuploid	+8(>70%)
AN20	5	B	B	B	Yes	Aneuploid	–2(>70%), +3(>70%), –7(>70%), –9(>70%), +11(>70%), +1(>70%)
AN21	5	B	B	B	Yes	Aneuploid	–17(>70%)
AN22	5	C	C	C	No	Aneuploid	+1(>70%), +9(>70%), +13(>70%), +16(>70%)

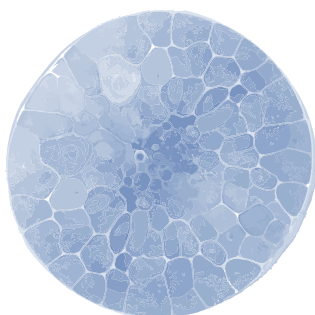
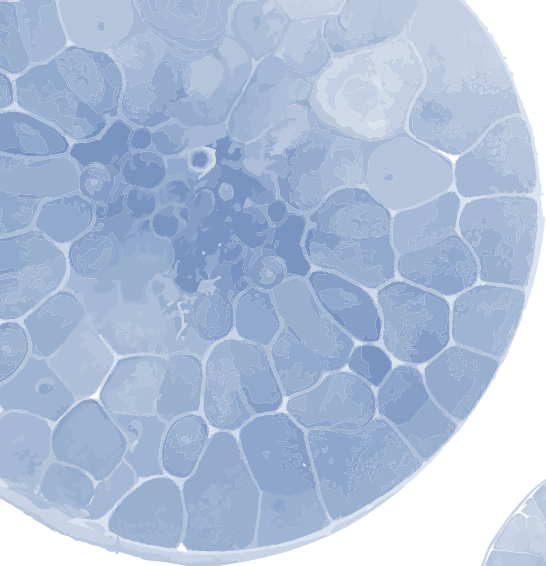
^a Days post-fertilization.
^b Embryo quality assessed according to guidelines from the Spanish Association for the Study of Biology of Reproduction (ASEBIR). TE, trophoctoderm; ICM, inner cell mass; PGT-A, preimplantation genetic testing for aneuploidy; NGS, next-generation sequencing.



Supplementary Table S2. Distribution and statistical analysis of quantitative variables.

Variable	pKS	pLev	pKW	pANV	pBNF (pairwise comparisons)		
					Euploid	Euploid	Mosaic
					Mosaic	Aneuploid	Aneuploid
Phase I: caspase-3 (n = 53 blastocysts)							
5 dpf (n = 38 blastocysts)							
Total cells	0.053	0.349	–	0.254	–	–	–
TE cells	0.037	0.423	0.475	–	–	–	–
ICM cells	0.200	0.692	–	0.216	–	–	–
% caspase-3+ cells in TE	0.200	0.628	–	0.023	0.047	0.044	1.000
% caspase-3+ cells in ICM	1.3E–30	0.002	0.286	–	–	–	–
6 dpf (n = 15 blastocysts)							
Total cells	0.200	0.038	0.309	–	–	–	–
TE cells	0.200	0.101	–	0.359	–	–	–
ICM cells	0.200	0.093	–	0.741	–	–	–
% caspase-3+ cells in TE	0.095	0.813	–	0.169	–	–	–
% caspase-3+ cells in ICM	3.1E–07	0.220	0.645	–	–	–	–
Phase II: TUNEL (n = 11 blastocysts)							
5 dpf + 6 dpf (n = 11 blastocysts)							
% TUNEL+ cells in TE	0.200	0.589	–	0.836	–	–	–

pKS, P-value Kolmogorov–Smirnov test; pLev, P-value Levene’s test; pKW, P-value Kruskal–Wallis test; pANV, P-value one-way ANOVA test; pBNF, Bonferroni adjusted P-value; Dpf, days post-fertilization; TE, trophectoderm; ICM, inner cell mass.



ARTÍCULOS EN COAUTORÍA NO INCLUIDOS EN LA TESIS

Artículos en coautoría no incluidos en la tesis

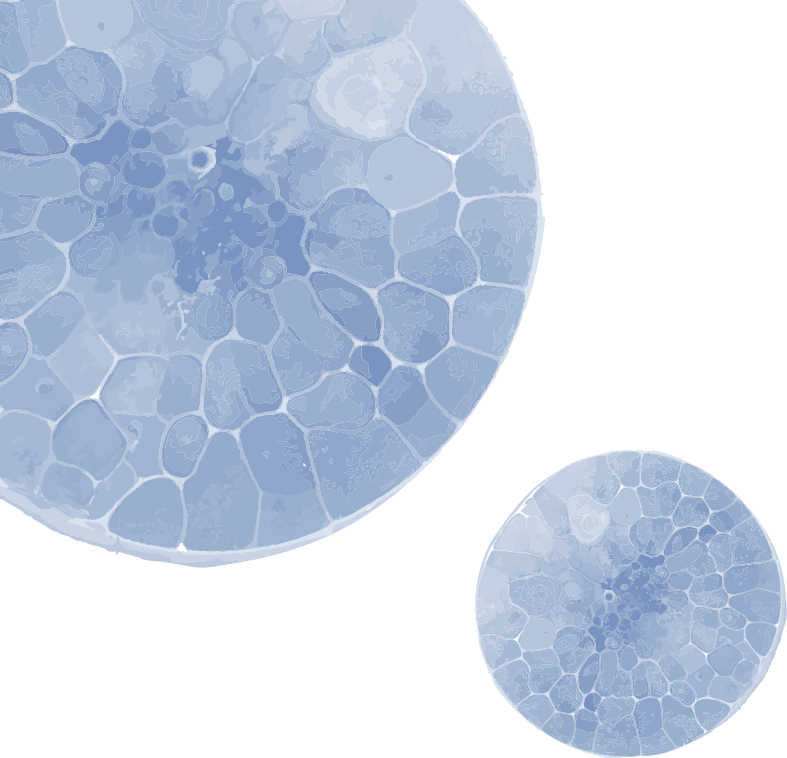
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1.1. Infertilidad y Reproducción Asistida

La infertilidad es una enfermedad del sistema reproductivo que se define por la incapacidad de lograr un embarazo tras 12 meses o más de relaciones sexuales regulares sin protección. Según datos de la Organización Mundial de la Salud, entre 48 millones de parejas y 186 millones de personas sufren de infertilidad en todo el mundo, lo que equivale a una prevalencia del 17,5% en la población adulta, afectando aproximadamente a 1 de cada 6 personas a lo largo de su vida (World Health Organization, 2023).

El índice de fecundidad, definido como el número promedio de nacimientos por mujer, varía considerablemente entre las distintas regiones del mundo. Mientras que las regiones menos desarrolladas experimentan un crecimiento más rápido de la población y niveles más altos de natalidad, la disminución de la población, el envejecimiento y las bajas tasas de natalidad son motivo de preocupación en las regiones más desarrolladas. No obstante, en las últimas décadas, estos indicadores han disminuido a nivel global y se prevé que sigan descendiendo (**Figura 1**).

A pesar del declive en el número de nacimientos totales, se ha observado un aumento drástico en el número de tratamientos de Reproducción Humana Asistida (RHA), especialmente en las regiones más desarrolladas. Desde 2005 hasta 2019, el número de tratamientos de RHA aumentó 2,6 veces en Europa (de 418.111 a 1.077.813) (European IVF Monitoring Consortium for the European Society of Human Reproduction and Embryology, 2009-2023), 3 veces en Estados Unidos (de 109.812 a 330.773) (Centers for Disease Control and Prevention, 2007-2021) y 2 veces

en Australia y Nueva Zelanda (de 43.726 a 88.929) (Australia and New Zealand Assisted Reproduction Database, 2007-2021).

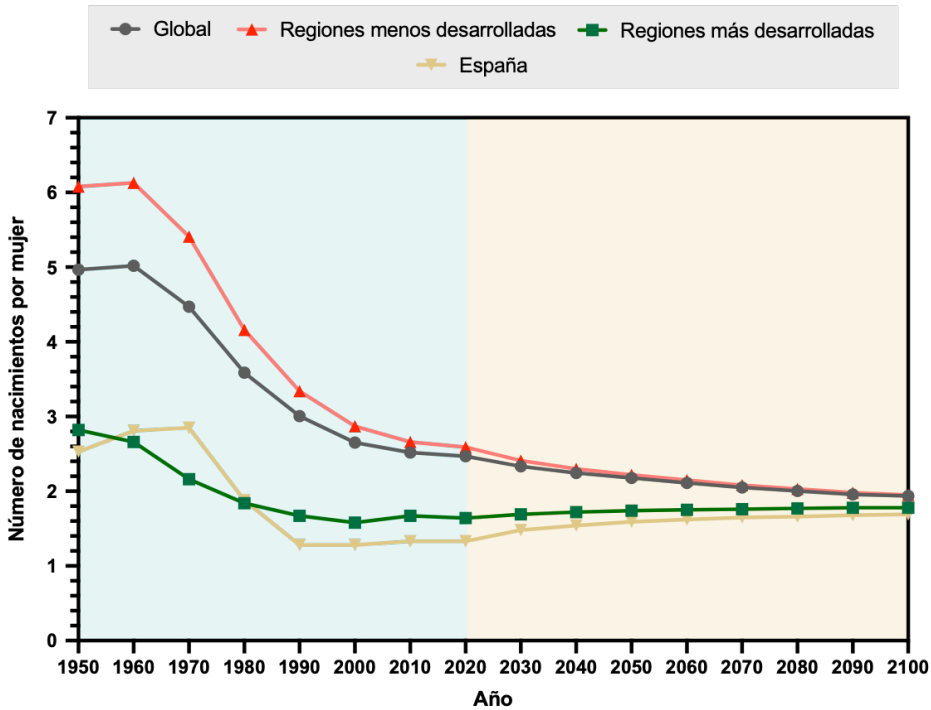


Figura 1 Estimaciones (1950-2020) y proyecciones (2020-2050) del número promedio de nacimientos por mujer en diferentes regiones del mundo. Las regiones menos desarrolladas incluyen todas las regiones de África, Asia (excepto Japón), América Latina y el Caribe, así como Melanesia, Micronesia y Polinesia. Las regiones más desarrolladas incluyen Europa, América del Norte, Australia/Nueva Zelanda y Japón. Fuente: United Nations, 2020. World Population Prospects 2019. Volume II: Demographic Profiles (ST/ESA/SER.A/427).

De manera similar, en España, el número de tratamientos de RHA aumentó 2,6 veces en 15 años, pasando de 69.077 en 2005 a 180.713 en 2019. Notablemente, aunque el número total de niños nacidos en España disminuyó 1,3 veces en el mismo período, de 466.371 en 2005 a 360.617 en 2019, el número de niños concebidos mediante tratamientos de RHA aumentó 3,6 veces, pasando de 10.295 en 2005 (2,2% del total de

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nacimientos) a 37.428 en 2019 (10,4% del total de nacimientos) (Sociedad Española de Fertilidad, 2008-2021) (**Figura 2**).

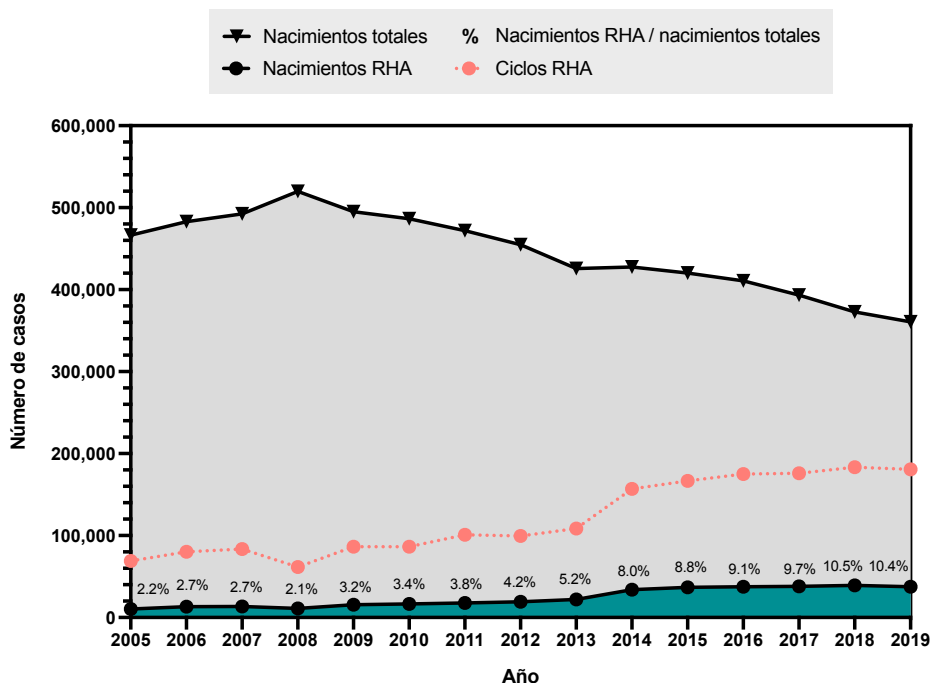


Figura 2 Número de ciclos de Reproducción Humana Asistida (RHA) y nacimientos registrados en España desde 2005 hasta 2019. Los porcentajes indican la contribución de los nacimientos por RHA (área verde) al total de nacimientos (área gris) por año. Fuente: Sociedad Española de Fertilidad, 2008-2021. Registro Nacional de Actividad 2005-2019.

El aumento en el uso de las Técnicas de Reproducción Asistida (TRA) ha ido acompañado de importantes avances tecnológicos en el campo. Estos avances incluyen el desarrollo de nuevas plataformas de cultivo embrionario, gracias a la integración de la microscopía de lapso de tiempo (*time-lapse*) en los sistemas de incubación y la formulación de nuevos medios de cultivo. Además, se han mejorado técnicas del laboratorio de fecundación in vitro (FIV), como la vitrificación para la criopreservación de óvulos y embriones, la inyección intracitoplasmática de espermatozoides (ICSI) para el

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tratamiento de pacientes con infertilidad masculina severa, y la biopsia embrionaria junto con las pruebas genéticas preimplantacionales. Asimismo, los avances tecnológicos han tenido un impacto positivo en el ámbito clínico de las TRA, con la implementación de nuevos sistemas ecográficos, mejoras en la producción de gonadotropinas y el desarrollo de protocolos personalizados de estimulación hormonal (Niederberger *et al.*, 2018).

Sin embargo, a pesar de tales avances, en la actualidad, las tasas de gestación clínica y parto por transferencia parecen haber alcanzado un pico alrededor del 33% y el 24%, respectivamente, según datos de la Sociedad Europea de Reproducción Humana y Embriología (ESHRE) (**Figura 3**).

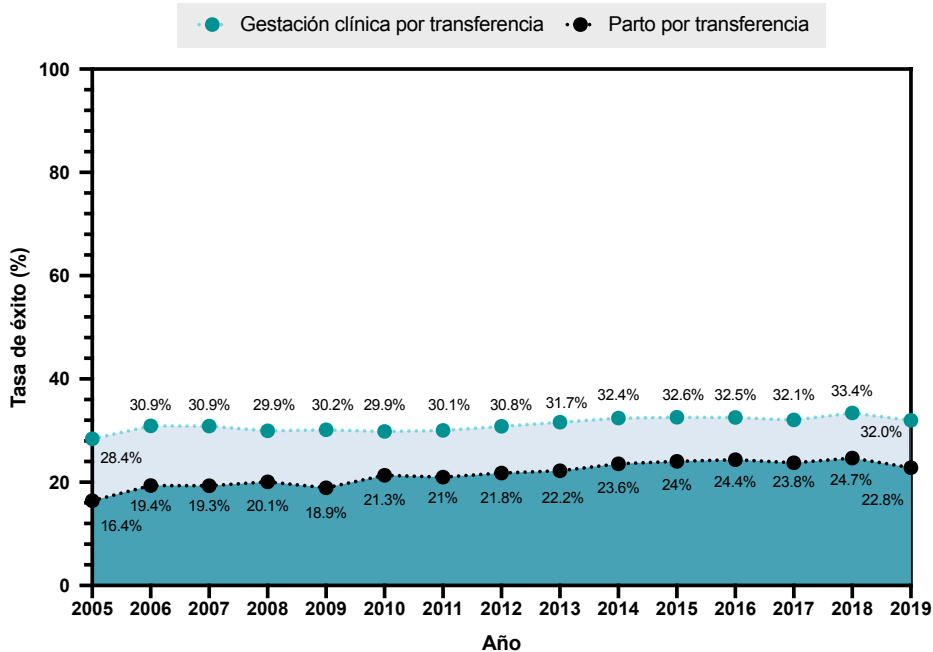


Figura 3 Tasa de gestación clínica y tasa de parto por transferencia embrionaria en Europa desde 2005 hasta 2019. Los datos representados incluyen ciclos sin donación de gametos, tanto en fresco como con embriones congelados. Fuente: European IVF Monitoring Consortium for the European Society of Human Reproduction and Embryology, 2009-2023.

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En este contexto, es evidente la necesidad de explorar nuevas vías para mejorar el éxito reproductivo de las personas con problemas de infertilidad.

1.2. Herramientas de selección embrionaria

En términos generales, el éxito reproductivo requiere un endometrio receptivo, un embrión funcional en el estadio de blastocisto y una comunicación sincronizada entre los tejidos maternos y el embrión (Governini *et al.*, 2021). En este sentido, uno de los enfoques dirigidos a mejorar las tasas de éxito de los tratamientos de RHA se centra en la identificación y selección de los embriones con capacidad reproductiva tras la FIV. Esto cobra particular relevancia en el contexto actual, donde la tendencia es transferir un único embrión en el estadio de blastocisto para minimizar los riesgos asociados a los embarazos múltiples (Cutting, 2018; ESHRE Guideline Group on the Number of Embryos to Transfer, 2024).

Los métodos actuales de selección embrionaria pueden implicar procedimientos invasivos, como la biopsia embrionaria; o procedimientos no invasivos, como la evaluación morfológica o morfocinética de los embriones, o el análisis del medio de cultivo. Estos métodos se basan en características biofísicas, bioquímicas y fisicoquímicas de los embriones, y permiten clasificarlos en función de parámetros asociados a su potencial reproductivo, tales como la morfología, cinética, genética, fisiología y metabolismo.

1.2.1. Valoración morfológica convencional

Desde los inicios de la FIV (Edwards *et al.*, 1969), la evaluación morfológica ha sido el método más extendido y eficiente para valorar la calidad embrionaria. Dicha evaluación consiste en el análisis microscópico

de parámetros morfológicos en diferentes estadios del desarrollo embrionario.

Gracias a la integración y ponderación de distintos parámetros morfológicos, se han elaborado sistemas para clasificar los embriones según el potencial de implantación esperado (Abeyta and Behr, 2014; Machtinger and Racowsky, 2013). En consecuencia, varias sociedades han desarrollado esquemas de consenso con el propósito de estandarizar la clasificación entre laboratorios. En Estados Unidos, la Sociedad de Tecnología de Reproducción Asistida (SART) desarrolló el esquema de clasificación SART (Racowsky *et al.*, 2010). En Europa, el Grupo Ejecutivo Alpha y el Grupo de Interés Especial de Embriología de la ESHRE desarrollaron el consenso de Estambul (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011), que definió los estándares mínimos para la evaluación morfológica de ovocitos y embriones. En España, la Asociación para el Estudio de la Biología de la Reproducción (ASEBIR) desarrolló los Criterios ASEBIR para la Evaluación Morfológica de Ovocitos, Embriones Tempranos y Blastocistos Humanos (Cuevas Saiz *et al.*, 2018).

El sistema de clasificación ASEBIR permite distinguir entre cuatro categorías de embriones: A (embrión de óptima calidad con máxima capacidad de implantación), B (embrión de buena calidad con elevada capacidad de implantación), C (embrión regular con probabilidad de implantación media) y D (embrión de mala calidad con probabilidad de implantación baja o nula). En el primer día del desarrollo embrionario (D+1), correspondiente al estadio de cigoto, la fecundación se evalúa mediante la observación del número y tamaño de los pronúcleos y corpúsculos polares entre las 16-18 horas tras la inseminación (hpi). En el segundo día (D+2) y el tercer día (D+3) del desarrollo embrionario, correspondientes al estadio de escisión, se evalúan, entre las 43-45 hpi (D+2) y las 67-69 hpi (D+3), el número, tamaño y simetría de las blastómeras, así como la presencia de

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fragmentación, multinucleación, vacuolas y alteraciones en la zona pelúcida o el citoplasma. En el cuarto día (D+4) del desarrollo embrionario, correspondiente al estadio de mórula, se evalúa la compactación entre las 90-94 hpi. Por último, en el quinto día (D+5) y el sexto día (D+6) del desarrollo embrionario, correspondientes al estadio de blastocisto, se evalúan, entre las 114-118 hpi (D+5) y las 136-140 hpi (D+6), el grado de expansión del blastocelo, el grosor de la zona pelúcida, el tamaño y la compactación de la masa celular interna (MCI), y el número de células, cohesión y homogeneidad del trofoectodermo (TE) (Cuevas Saiz *et al.*, 2018).

Sin embargo, la evaluación estática de la morfología embrionaria en intervalos temporales predefinidos presenta varias limitaciones. En primer lugar, las evaluaciones son subjetivas, lo que puede resultar en una alta variabilidad inter- e intraobservador (Arce *et al.*, 2006; Paternot *et al.*, 2011; Storr *et al.*, 2017). En segundo lugar, al extraer los embriones del incubador para realizar las valoraciones, se alteran las condiciones de cultivo, lo que implica observaciones limitadas en tiempo y, por lo tanto, la omisión de eventos críticos potencialmente relacionados con la viabilidad embrionaria (Montag *et al.*, 2011; Zhang *et al.*, 2010).

1.2.2. Análisis morfocinético

La integración de la microscopía *time-lapse* en los sistemas de incubación ha permitido monitorizar de forma ininterrumpida el desarrollo de los embriones obtenidos mediante FIV. Actualmente, existen múltiples sistemas que incorporan esta tecnología, cuyas características principales se resumen en el documento de buenas prácticas elaborado por la ESHRE (ESHRE Working group on Time-lapse technology, 2020). Frente a la evaluación estática de la morfología embrionaria, el uso de la tecnología *time-lapse* presenta una serie de ventajas.

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En primer lugar, los embriones no necesitan ser extraídos del incubador durante el cultivo *in vitro*, evitando así su exposición a las condiciones ambientales y asemejándose más al entorno *in vivo* (Swain, 2014). En segundo lugar, el uso de incubadores con tecnología *time-lapse* simplifica la logística del laboratorio, ya que permite la evaluación en cualquier momento durante el flujo de trabajo diario. En tercer lugar, la monitorización continua del cultivo *in vitro* permite la evaluación de parámetros morfocinéticos relacionados con la dinámica del desarrollo embrionario. Estos parámetros pueden ser cualitativos, como la formación e internalización de fragmentos, patrones anormales de división, expansión y colapso del blastocelo, exclusión y extrusión de células, y multinucleación; o cuantitativos, como el tiempo en el que se producen eventos durante el desarrollo, como las divisiones celulares (Del Gallego *et al.*, 2019).

Desde la comercialización del primer incubador con tecnología *time-lapse* para su uso en RHA en 2008, se han recopilado grandes cantidades de datos morfocinéticos generados mediante anotaciones manuales. Estos datos han sido utilizados para identificar parámetros morfocinéticos predictivos de la calidad embrionaria, los cuales, en combinación con parámetros morfológicos, se han utilizado para elaborar algoritmos de selección embrionaria (ESHRE Working group on Time-lapse technology, 2020; Del Gallego *et al.*, 2019; Garcia-Belda *et al.*, 2024; Gimenez *et al.*, 2023). Estos algoritmos se han implementado en las clínicas de RHA como herramientas de predicción de los resultados clínicos, como la formación del blastocisto, el riesgo de aneuploidías, el potencial de implantación e incluso la probabilidad de obtener un recién nacido vivo.

Sin embargo, los algoritmos de selección embrionaria basados en anotaciones manuales de parámetros morfocinéticos presentan una serie de limitaciones. Por un lado, no eliminan la variabilidad inter- e intraobservador, la cual, además, se ve exacerbada por la definición inexacta del momento

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de inseminación como punto temporal de referencia (Barrie *et al.*, 2017; Sundvall *et al.*, 2013). En este sentido, se ha sugerido la utilización de puntos de referencia alternativos o variables indirectas (como la duración entre eventos) para elaborar algoritmos morfocinéticos (Cetinkaya *et al.*, 2015; Kirkegaard *et al.*, 2013; Liu, Chapple, *et al.*, 2015; Liu, Copeland, *et al.*, 2015). No obstante, el rendimiento global de dichos algoritmos se ha visto limitado por la influencia de variables que difieren entre laboratorios y que afectan al cultivo embrionario, tales como las características de los pacientes, del tratamiento y del entorno (Barrie *et al.*, 2017).

Por estas razones, aunque la tecnología *time-lapse* ha demostrado ser segura para cultivar embriones humanos (Cruz *et al.*, 2011; Insua *et al.*, 2017; Nakahara *et al.*, 2010), y múltiples estudios han mostrado mejores resultados clínicos tras la selección embrionaria basada en criterios morfocinéticos frente a la evaluación estándar de la morfología (Meseguer *et al.*, 2012; Pribenszky *et al.*, 2017; Siristatidis *et al.*, 2015), la implementación de estos algoritmos se ha visto limitada por su baja reproducibilidad entre clínicas (Armstrong *et al.*, 2019; Freour *et al.*, 2015; Kieslinger *et al.*, 2016, 2023; Liu, Copeland, *et al.*, 2015; Rienzi *et al.*, 2015).

1.2.3. Evaluación del desarrollo embrionario mediante inteligencia artificial

La inteligencia artificial (IA) es la capacidad de las máquinas para llevar a cabo tareas que normalmente requieren de la inteligencia humana, tales como el aprendizaje, la toma de decisiones y la resolución autónoma de problemas. En los últimos años, la aplicación de la IA en los laboratorios de FIV ha permitido optimizar los sistemas de selección embrionaria (Glatstein *et al.*, 2023).

Por un lado, la aplicación de técnicas de IA basadas en el procesamiento de datos, como los clasificadores bayesianos (Morales *et al.*, 2008), los árboles de decisión (Romero-Ruiz *et al.*, 2019), y más recientemente, las técnicas de machine learning y deep learning basadas en redes neuronales artificiales (Curchoe and Bormann, 2019; Tran *et al.*, 2019), han permitido desarrollar algoritmos de selección embrionaria capaces de reconocer patrones en los datos de manera autónoma y aprender de ellos para hacer predicciones. Además, estos algoritmos se han beneficiado de la aplicación de técnicas de IA basadas en el procesamiento de imágenes, las cuales permiten detectar estructuras o eventos en el embrión mediante la conversión de las imágenes en matrices de píxeles, representadas por valores de intensidad (Arsalan *et al.*, 2022; Louis *et al.*, 2021). Esto ha permitido automatizar las anotaciones morfocinéticas, reduciendo la subjetividad en las observaciones (Conaghan *et al.*, 2013; Manna *et al.*, 2013).

Actualmente, existen múltiples algoritmos de selección embrionaria basados en IA, como Eeva™ (Merck, Darmstadt, Alemania) (Wong *et al.*, 2010), KIDScore D5™ (Vitrolife, Copenhagen, Dinamarca) y más recientemente, iDAScore™ (Vitrolife, Copenhagen, Dinamarca) (Berntsen *et al.*, 2022; Theilgaard Lassen *et al.*, 2023). Estos algoritmos han sido entrenados para predecir la probabilidad de implantación, gestación clínica, o gestación evolutiva antes de la transferencia embrionaria (Adamson *et al.*, 2016; Aparicio-Ruiz *et al.*, 2016; Bori *et al.*, 2022; Cimadomo, Chiappetta, *et al.*, 2023; Gazzo *et al.*, 2020; Reignier *et al.*, 2019; Theilgaard Lassen *et al.*, 2023; VerMilyea *et al.*, 2014; Zhu *et al.*, 2023).

La evidencia disponible hasta la fecha sugiere que los algoritmos de selección embrionaria basados en IA, cuando se utilizan como herramienta de apoyo en la toma de decisiones, tienen el potencial de mejorar la selección embrionaria en comparación con la evaluación realizada

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únicamente por los embriólogos. En este sentido, los modelos más eficaces se han logrado al combinar imágenes de embriones con parámetros clínicos (Salih *et al.*, 2023).

Este enfoque adquiere especial relevancia al considerar el impacto del factor uterino en los resultados clínicos (Galliano *et al.*, 2015). Así, la inclusión de este y otros factores de confusión en futuros modelos, tales como la constitución genética de los embriones (Jiang and Bormann, 2023), contribuirá a optimizar los algoritmos y mejorar las tasas de éxito en los tratamientos de RHA, pasando de predecir la probabilidad de implantación a predecir la probabilidad de obtener un recién nacido vivo y sano.

1.2.4. Análisis genético

El Test Genético Preimplantacional (PGT) es una prueba que permite detectar alteraciones genéticas y cromosómicas en los embriones obtenidos por FIV. Mediante la selección de los embriones no afectados, es posible evitar la transmisión de enfermedades genéticas a la descendencia, así como la transferencia de embriones con alteraciones que puedan comprometer su viabilidad o dar lugar a recién nacidos con cromosomopatías (ESHRE PGT Consortium Steering Committee, 2020).

En términos generales, el PGT es una prueba invasiva, ya que conlleva el análisis del ADN presente en una biopsia embrionaria, usualmente tras la apertura de un orificio en la zona pelúcida mediante eclosión asistida. Existen diferentes variantes metodológicas del PGT, las cuales difieren en función de múltiples factores, como el estadio de desarrollo en el que se realizan la eclosión asistida y la biopsia embrionaria, las técnicas empleadas en estos procedimientos, el tipo de alteración que se pretende detectar y las técnicas de análisis genético utilizadas para tal fin.

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Así, por un lado, la biopsia embrionaria puede realizarse en el estadio de escisión, mediante la extracción de 1-2 blastómeras en D+3. Entre las técnicas de biopsia descritas en este estadio destacan la aspiración (extracción celular mediante succión directa) y la extrusión por desplazamiento (extracción celular mediante introducción de líquido en el espacio perivitelino o mediante opresión de la zona pelúcida con la micropipeta de biopsia) (Tarin and Handyside, 1993). Por otro lado, la biopsia embrionaria puede realizarse en el estadio de blastocisto, mediante la extracción de ~5-10 células de TE en D+5, D+6 o D+7, utilizando las técnicas de pulling (extracción celular mediante aplicación de fuerza de tracción con micropipeta de biopsia y micropulsos de láser) o flicking (extracción celular mediante aplicación de micropulsos de láser y fuerza de fricción entre las micropipetas de sujeción y biopsia) (Aoyama and Kato, 2020).

Asimismo, la eclosión asistida puede llevarse a cabo durante la biopsia embrionaria o como un procedimiento independiente previo a ésta, mediante métodos mecánicos (disección parcial de la zona pelúcida con una microaguja), químicos (perforación de la zona pelúcida con ácido Tyrode) o físicos (fotoablación de la zona pelúcida mediante micropulsos de láser) (De Vos and Van Steirteghem, 2001).

Respecto a las técnicas moleculares de análisis genético, las primeras que se aplicaron en el contexto del PGT fueron la reacción en cadena de la polimerasa (PCR) y la hibridación in situ fluorescente (FISH). Inicialmente, estas técnicas fueron empleadas para prevenir la transmisión de enfermedades ligadas a los cromosomas sexuales (Griffin *et al.*, 1993; Handyside *et al.*, 1990; Munne, Weier, *et al.*, 1993). Posteriormente, se utilizaron para detectar aneuploidías, translocaciones y enfermedades monogénicas autosómicas (Handyside *et al.*, 1992; Liu *et al.*, 1994; Munne, Lee, *et al.*, 1993). Más adelante, surgieron las técnicas de Screening

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Cromosómico Completo (CCS), las cuales, en lugar de analizar regiones cromosómicas concretas, permitieron el análisis integral de toda la dotación cromosómica. Estas técnicas incluyen la hibridación genómica comparativa (CGH) (Voullaire *et al.*, 1999; Wells *et al.*, 1999), los arrays de CGH (aCGH) (Gutierrez-Mateo *et al.*, 2011; Wells *et al.*, 2008), los arrays de polimorfismos de nucleótido único (arrays de SNPs) (Treff *et al.*, 2010), la determinación de haplotipos mediante arrays de SNPs (Karyomapping) (Handyside *et al.*, 2010), la PCR cuantitativa (qPCR) (Treff *et al.*, 2012), y, más recientemente, la secuenciación de nueva generación (NGS) (Treff *et al.*, 2013; Yin *et al.*, 2013).

El desarrollo de nuevas tecnologías de análisis genético, sumado a las mejoras en las técnicas de biopsia (Mao *et al.*, 2024), cultivo embrionario (Gardner *et al.*, 2000; Swain *et al.*, 2016), criopreservación (Cobo *et al.*, 2009; Kuwayama, 2007) y preparación endometrial (Zhang *et al.*, 2023), han permitido optimizar los protocolos de PGT, y como resultado, su uso se ha incrementado exponencialmente en los últimos años. De hecho, en Europa, el número de ciclos de PGT aumentó 11 veces en 15 años, pasando de 5.846 en 2005 a 64.089 en 2019. De manera similar, en España, el número de ciclos de PGT aumentó 7,3 veces, pasando de 1.937 en 2005 a 14.189 en 2019 (**Figura 4**).

A pesar de las múltiples variantes descritas, actualmente la metodología más extendida para realizar PGT consiste en el análisis, mediante NGS, de una biopsia de TE obtenida en el estadio de blastocisto. Además, con frecuencia, la biopsia se lleva a cabo tras realizar la eclosión asistida por láser. Asimismo, las plataformas más recientes de PGT incorporan un genotipado adicional basado en el análisis de SNPs, el cual permite confirmar las alteraciones identificadas mediante NGS, detectar otras que la NGS no identifica por sí sola (como la haploidía y la triploidía), determinar

el origen parental de las anomalías y confirmar la presencia de mosaicismo y/o contaminación (Huang *et al.*, 2024; Rana *et al.*, 2023).

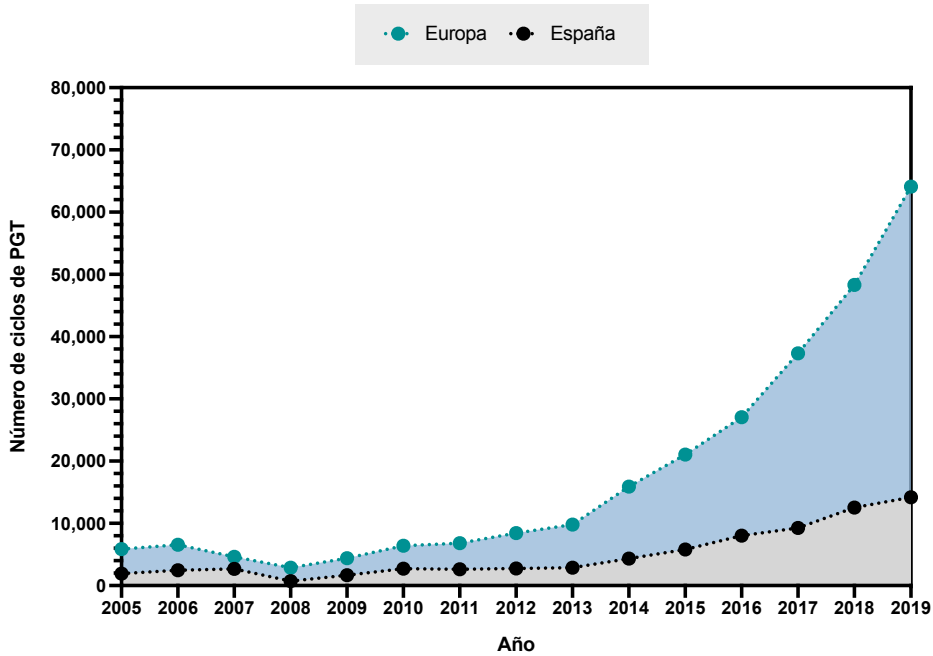


Figura 4 Número de ciclos de Test Genético Preimplantacional (PGT) en Europa y en España desde 2005 hasta 2019. Fuentes: European IVF Monitoring Consortium for the European Society of Human Reproduction and Embryology, 2009-2023; Sociedad Española de Fertilidad, 2008-2021. Registro Nacional de Actividad 2005-2019.

Por último, en función del tipo de alteración que se pretende detectar, se distinguen varias modalidades del PGT: PGT para la detección de aneuploidías (PGT-A), PGT para la detección de alteraciones estructurales (PGT-SR) y PGT para la detección de enfermedades monogénicas (PGT-M) (Harris *et al.*, 2021).

1.2.4.1. PGT para la detección de aneuploidías (PGT-A)

El PGT-A permite detectar alteraciones cromosómicas numéricas y, por lo tanto, seleccionar embriones euploides para la transferencia, es decir, aquellos con una dotación normal de cromosomas en sus células. En seres humanos, esto implica tener un total de 46 cromosomas completos, conformados por 22 pares de autosomas y un par de cromosomas sexuales, lo que equivale a una dotación diploide.

El fundamento del PGT-A, en comparación con otros métodos de selección embrionaria, radica en el hecho de que existen embriones que, aun presentando la máxima calidad morfológica y un comportamiento morfocinético óptimo, carecen de potencial reproductivo debido a su composición cromosómica aberrante (Capalbo *et al.*, 2014; Minasi *et al.*, 2016). Mediante la selección de los embriones euploides, el PGT-A en el estadio de blastocisto permite aumentar las tasas de implantación, embarazo y recién nacido vivo por transferencia, reduciendo, al mismo tiempo, el riesgo de aborto y cromosomopatías congénitas, así como el riesgo asociado a los embarazos múltiples, el tiempo necesario para lograr un embarazo y el estrés emocional relacionado con resultados adversos repetidos (Kasaven *et al.*, 2023). Sin embargo, a pesar de su amplio uso y de los múltiples estudios y ensayos clínicos aleatorizados que han demostrado su utilidad clínica, diversos autores han cuestionado su utilización basándose en otros trabajos en los que no se ha demostrado un beneficio (Morales, 2024).

A pesar del debate generado en torno a ambas posturas (Rosenwaks *et al.*, 2018), una de las visiones más aceptadas actualmente es que el PGT-A podría mejorar significativamente los resultados clínicos en mujeres de edad

avanzada, debido al mayor riesgo de aneuploidías embrionarias en esta población de pacientes (ESHRE PGT Consortium Steering Committee, 2020; Mao *et al.*, 2024; Simopoulou *et al.*, 2021).

Asimismo, en los últimos años se han desarrollado nuevas estrategias para determinar el estatus cromosómico de los embriones sin necesidad de biopsiarlos. Estas estrategias conllevan el análisis del ADN libre de células (cfDNA) presente en el medio de cultivo en el que se ha desarrollado (niPGT-A) (Stigliani *et al.*, 2013) o bien el análisis del cfDNA presente en el fluido del blastocele tras su aspiración por blastocentesis (miPGT-A) (Palini *et al.*, 2013).

A pesar de que múltiples estudios han demostrado el potencial de estas técnicas, otros han destacado los desafíos que actualmente limitan su aplicación clínica (Del Collado *et al.*, 2023). En primer lugar, la concordancia entre el cfDNA y la biopsia de TE es insuficiente (Rubio *et al.*, 2020) y puede variar ampliamente entre estudios (Cinnioglu *et al.*, 2023). De hecho, se desconoce en qué medida el cfDNA refleja el ADN del embrión, o si es ADN aberrante que ha sido eliminado mediante procesos de apoptosis o necrosis (Domingo-Muelas *et al.*, 2023; Handayani *et al.*, 2023; Tomic *et al.*, 2022). Además, el cfDNA está fragmentado y suele presentar una baja concentración, lo cual puede afectar al éxito de su amplificación y posterior análisis (Kuznyetsov *et al.*, 2020). Por último, la optimización de las técnicas de niPGT-A o miPGT-A requiere la modificación de los protocolos estándar de laboratorio, lo cual puede conllevar cambios en la estrategia y la duración del cultivo embrionario, un aumento en el número de lavados necesarios para evitar la contaminación con ADN materno, o cambios en el manejo de los embriones para evitar la contaminación cruzada (Del Collado *et al.*, 2023).

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En la actualidad, el test basado en niPGT-A comercializado bajo el nombre EMBRACE™ (Embryo Analysis of Culture Environment) (Rubio *et al.*, 2020) se utiliza como herramienta de priorización no invasiva para mejorar la selección embrionaria frente a la selección basada únicamente en criterios morfológicos y morfocinéticos. No obstante, el gold standard para realizar PGT-A continúa siendo el método invasivo.

1.2.4.2. PGT para la detección de alteraciones estructurales (PGT-SR)

El PGT-SR permite detectar alteraciones cromosómicas estructurales en los embriones, tales como translocaciones, deleciones, duplicaciones, inserciones, anillaciones o inversiones. Estas alteraciones se originan como consecuencia de la ruptura o unión incorrecta de varios segmentos cromosómicos, lo cual puede ocurrir cuando uno o ambos progenitores son portadores de alteraciones cromosómicas estructurales balanceadas, como translocaciones recíprocas, Robertsonianas o inversiones.

El PGT-SR se considera una estrategia efectiva en parejas portadoras de dichas alteraciones, especialmente en aquellas con resultados previos desfavorables, ya que podría reducir significativamente el riesgo de aborto y cromosomopatías congénitas, así como aumentar las tasas de recién nacido vivo (Franssen *et al.*, 2006; Griffin and Ogur, 2023; Huang *et al.*, 2019; Munne *et al.*, 2000). Asimismo, se ha planteado la posibilidad de un efecto intercromosómico durante la meiosis en parejas portadoras de alteraciones balanceadas, lo cual podría aumentar la incidencia de alteraciones cromosómicas numéricas en los embriones. Por tanto, dado que actualmente el PGT-SR y el PGT-A comparten la misma tecnología, ambas modalidades suelen aplicarse de manera simultánea en esta población de pacientes (Boynukalin *et al.*, 2021; Mateu-Brull *et al.*, 2019).

1.2.4.3. PGT para la detección de enfermedades monogénicas (PGT-M)

El PGT-M permite detectar mutaciones causantes de enfermedades monogénicas o mendelianas, y evitar su transmisión a la descendencia mediante la selección de los embriones no afectados.

Inicialmente, el PGT-M fue empleado en la detección de enfermedades debilitantes de inicio en la infancia, como fibrosis quística, atrofia muscular espinal y distrofia muscular de Duchenne (Liu *et al.*, 1996). Con el tiempo, su aplicación se ha ampliado para incluir enfermedades de inicio en la adultez, como la enfermedad de Huntington y la enfermedad de Alzheimer de inicio temprano (Ethics Committee of the American Society for Reproductive Medicine, 2018), así como síndromes de predisposición al cáncer, como las mutaciones BRCA y el síndrome de Lynch (Dallagiovanna *et al.*, 2023; Wang and Hui, 2009). Además, el PGT-M ha demostrado ser útil en la detección de algunas condiciones no mortales pero graves que se pueden identificar desde el nacimiento (Child *et al.*, 2016; Lavery *et al.*, 2013; Sciorio *et al.*, 2021).

Técnicamente, el PGT-M permite detectar prácticamente cualquier enfermedad monogénica siempre que se conozca la mutación causante. En la práctica, la lista de indicaciones para las cuales se permite el PGT-M puede variar considerablemente de un país a otro, dependiendo de la regulación correspondiente (De Rycke and Berckmoes, 2020). Por otro lado, el PGT-M permite realizar el análisis de los antígenos leucocitarios humanos (tipaje HLA) para seleccionar embriones histocompatibles con un familiar de primer grado, generalmente un hermano, que padece una enfermedad hematopoyética grave, congénita o adquirida, cuyo único tratamiento es el

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trasplante de células de cordón umbilical o médula ósea (Caligara *et al.*, 2023; De Rycke *et al.*, 2020).

1.2.5. Evaluación molecular de la fisiología embrionaria

Una buena morfología embrionaria, un comportamiento morfocinético óptimo y una constitución cromosómica euploide no identifican necesariamente un blastocisto viable, ya que no proporcionan información sobre su estado fisiológico (Cimadomo, Rienzi, *et al.*, 2023). En este sentido, la identificación de biomarcadores moleculares de viabilidad embrionaria, que aporten información sobre las funciones y mecanismos compatibles con un correcto desarrollo, continúa siendo uno de los principales retos a los que se enfrenta la tecnología en RHA para mejorar la selección embrionaria (Gardner *et al.*, 2015).

En los últimos años, los avances producidos en el campo de las denominadas ciencias ómicas han permitido el estudio de los perfiles moleculares embrionarios, ofreciendo una visión global de la fisiología del embrión gracias al análisis simultáneo de miles de moléculas en una sola muestra biológica (Horgan *et al.*, 2009). Aunque los métodos actuales permiten el análisis de biopsias embrionarias e incluso células individuales (Heumos *et al.*, 2023), existe una tendencia hacia el desarrollo de enfoques ómicos no invasivos utilizando muestras descartadas en la práctica clínica, como el medio de cultivo embrionario (Sanchez *et al.*, 2017).

Con el objetivo de identificar nuevos biomarcadores de viabilidad embrionaria, actualmente se están explorando diferentes disciplinas en el ámbito de las ciencias ómicas, las cuales se clasifican en función del tipo de molécula analizada. Por un lado, se han llevado a cabo estudios

transcriptómicos, proteómicos y metabolómicos, dirigidos a caracterizar, respectivamente, la composición, abundancia y variabilidad de transcritos, proteínas y metabolitos embrionarios en función de diferentes variables clínicas, como el estadio de desarrollo, la composición cromosómica o la capacidad de implantación (Hernandez-Vargas *et al.*, 2020). Además, se están explorando otros enfoques, como el estudio del exoma (regiones codificantes del genoma) y del secretoma (proteínas secretadas por el embrión), así como la caracterización de los perfiles epigenómicos (conjunto de modificaciones epigenéticas del ADN) y lipidómicos (análisis de todas las especies de lípidos) embrionarios (Hernandez-Vargas *et al.*, 2020). Por último, el desarrollo de nuevos enfoques multiómicos, basados en la aplicación de la biología de sistemas, permite integrar información obtenida a partir de diferentes tecnologías ómicas, facilitando la obtención de firmas moleculares globales más predictivas de las funciones y los estados fisiológicos embrionarios (Baysoy *et al.*, 2023).

Sin embargo, a pesar de la gran cantidad de información proporcionada por estos estudios, hasta la fecha no se ha identificado un perfil molecular capaz de predecir la competencia embrionaria. Es importante destacar que la asociación de la regulación diferencial de una o más moléculas con el resultado clínico no es suficiente para su uso como herramienta de selección embrionaria. En este sentido, se necesitan desarrollar nuevos modelos computacionales capaces de elaborar firmas moleculares a partir de grandes cantidades de datos ómicos. Además, los biomarcadores candidatos deben ser validados mediante ensayos clínicos en amplias cohortes de pacientes, y deben ser medibles con una sensibilidad, especificidad y precisión significativas sin comprometer la viabilidad embrionaria.

1.3. Mosaicismo embrionario

El PGT-A permite identificar los embriones con aneuploidías dentro de una cohorte; es decir, aquellos que presentan cualquier desviación del número diploide normal de cromosomas en sus células. Las aneuploidías pueden ser de origen meiótico, si surgen por errores en la segregación de los cromosomas durante la gametogénesis, o de origen mitótico, si surgen por errores en las divisiones celulares tras la fecundación. Las aneuploidías meióticas se conocen como aneuploidías uniformes, ya que suelen afectar a todas las células del embrión, mientras que las aneuploidías mitóticas dan lugar a mosaicismo (Hassold and Hunt, 2001; Levy *et al.*, 2021).

1.3.1. Definición y tipos

El mosaicismo embrionario es la presencia de líneas celulares con cariotipos diferentes en un mismo embrión (Delhanty *et al.*, 1993). En función de los cariotipos coexistentes, se distinguen varios tipos de mosaicismo. Por un lado, el mosaicismo diploide-aneuploide implica la coexistencia de una línea celular diploide y una o más líneas celulares aneuploides. Por otro lado, el mosaicismo aneuploide se refiere a la presencia de líneas celulares con distintas aneuploidías. El mosaicismo de ploidía o mixoploide implica cualquier combinación de líneas celulares haploides, diploides y poliploides en el mismo embrión (McCoy, 2017).

Según el número de cromosomas implicados en la aneuploidía, también es posible distinguir entre el mosaicismo simple, cuando implica un solo cromosoma, y mosaicismo complejo, cuando afecta a más de uno, aunque existen discrepancias entre autores a la hora de establecer esta distinción (Coonen *et al.*, 2004; Ma *et al.*, 2022; Viotti, McCoy, *et al.*, 2021). Por otro lado, el mosaicismo caótico es un tipo de mosaicismo caracterizado por

patrones severos de irregularidad cromosómica con múltiples líneas celulares aneuploides (McCoy, 2017).

Además, en función del tamaño del fragmento de ADN implicado en la aneuploidía, podemos distinguir entre mosaicismo genético, si la aneuploidía implica cambios a nivel de pequeñas secuencias de ADN o pequeñas variaciones en el número de copias, o de mosaicismo citogenético, si la aneuploidía implica cambios a nivel de cromosomas. A su vez, dentro del mosaicismo citogenético podemos distinguir entre mosaicismo segmentario (también conocido como segmental o parcial), cuando ocurren cambios a nivel de fragmentos de cromosomas, y mosaicismo cromosómico, cuando los cambios afectan a cromosomas completos (Viotti, 2020).

Por otro lado, según el estadio de desarrollo en el que se origina, el mosaicismo embrionario puede tener distintas configuraciones. Hablamos de mosaicismo generalizado cuando está presente tanto en el linaje embrionario como en el extraembrionario, ya que deriva de un error mitótico anterior a la segregación de estos linajes. En cambio, hablamos de mosaicismo confinado cuando se restringe a un único linaje celular, debido a un error mitótico que ocurre tras la diferenciación celular. De manera similar, cuando las células aneuploides quedan confinadas a un determinado linaje celular, también surge un patrón de mosaicismo en relación con el resto de células. Finalmente, el nivel o grado de mosaicismo generalmente se refiere al porcentaje de células aneuploides en un embrión con mosaicismo diploide-aneuploide (Vera-Rodriguez and Rubio, 2017) **(Figura 5).**

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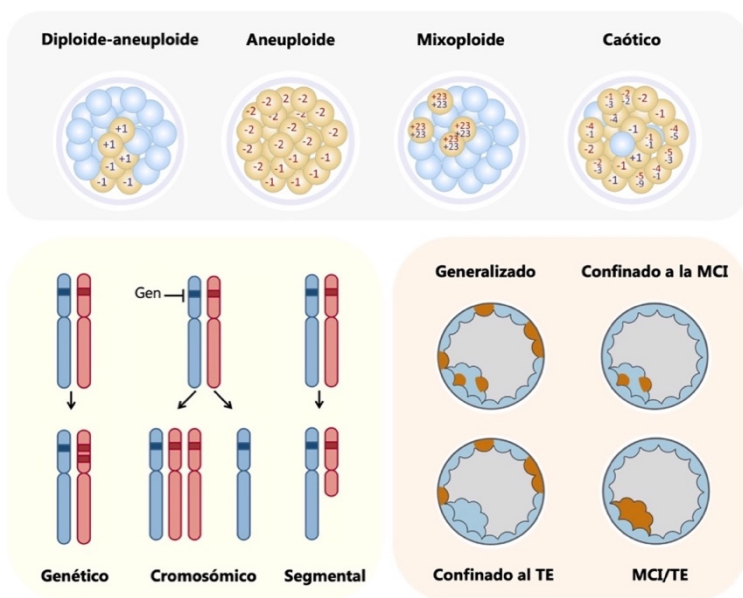


Figura 5 Tipos de mosaicismo embrionario según los cariotipos coexistentes (panel gris), el tamaño del fragmento de ADN implicado en la aneuploidía (panel amarillo) y su localización (panel naranja). Las células aneuploides se muestran con el número de cromosomas sobrantes (+) o ausentes (-), o en color rojo. (Adaptado de McCoy, 2017; Vera-Rodriguez and Rubio, 2017).

1.3.2. Origen molecular

1.3.2.1. Mosaicismo cromosómico

Los errores mitóticos que causan el mosaicismo cromosómico ocurren por fallos en la segregación de los cromosomas durante las divisiones celulares post-cigóticas. Existen múltiples mecanismos moleculares de error mitótico (Taylor *et al.*, 2014). El más frecuente es el retraso en la anafase, en el cual una de las cromátidas hermanas no migra correctamente al polo de la célula debido a fallos en la formación del huso mitótico, quedando como consecuencia excluida del núcleo de la célula hija. Esto resulta en la

formación de una línea celular disómica y otra monosómica para el cromosoma implicado. Otro mecanismo es la no disyunción cromosómica, que ocurre cuando las cromátidas hermanas no se separan correctamente, y una de las células hijas hereda ambas. El resultado es una línea celular trisómica y otra monosómica para el cromosoma implicado. Otros mecanismos menos frecuentes incluyen la formación de micronúcleos y la endoreduplicación, que ocurre cuando un cromosoma se replica pero no se completa la división celular, debido a un fallo en la citocinesis o a una salida prematura del ciclo celular (Kort *et al.*, 2016) **(Figura 6)**.

Los errores de segregación cromosómica se atribuyen a diferentes causas moleculares, y ocurren con mayor frecuencia durante las primeras divisiones embrionarias (Munné *et al.*, 1994). Una de las principales causas moleculares de error mitótico es el funcionamiento defectuoso de los mecanismos de control del ciclo celular. Debido a que hasta el estadio de 4-8 células el genoma embrionario humano se encuentra prácticamente inactivo, los mecanismos de control del ciclo celular deben llevarse a cabo a expensas de factores acumulados en el citoplasma ovocitario. En consecuencia, dichos mecanismos, que conllevan la oscilación de ciclinas y sus quinasas asociadas, operan de forma atenuada durante las primeras divisiones embrionarias. La atenuación de los puntos de control del ciclo celular permite el acortamiento de los ciclos celulares, en detrimento de la fidelidad y la temporalización de la transmisión de la información genética (McCoy, 2017).

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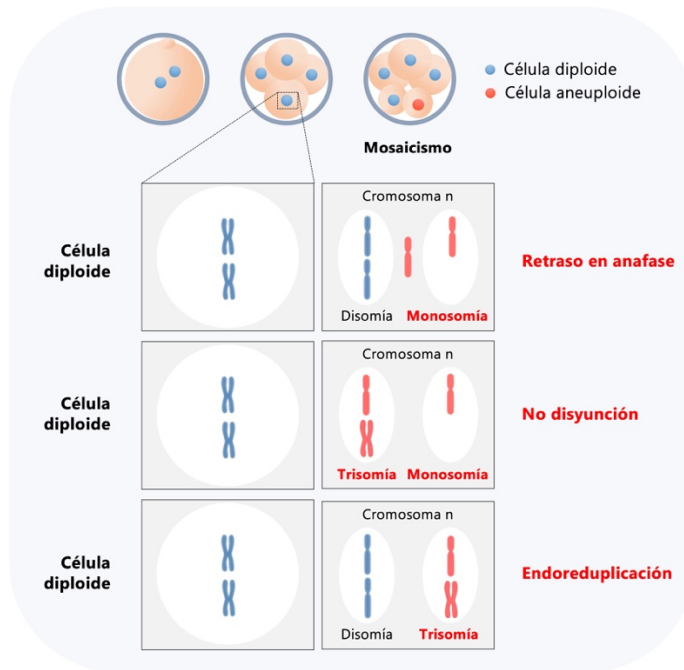


Figura 6 Mecanismos moleculares de error mitótico que generan mosaicismo cromosómico en embriones humanos.

Otra causa molecular de error mitótico son las alteraciones en el centrosoma espermático, el cual actúa como centro organizador de microtúbulos durante las primeras divisiones embrionarias, así como los defectos en la funcionalidad del huso mitótico (McCoy *et al.*, 2015). La presencia extra de centriolos, debido a fallos en su duplicación, predispone a los cromosomas a establecer uniones aberrantes con el huso mitótico (monotelia, sintelia y merotelia) y, en consecuencia, a sufrir retraso en anafase (Ganem *et al.*, 2009).

Por último, los errores en la segregación cromosómica pueden originarse por defectos en la cohesión de los cromosomas, debidos a fallos en la funcionalidad de las cohesinas embrionarias y/o en sus proteínas reguladoras, como las securinas y las separasas. Estas proteínas son

responsables de mantener unidas las cromátidas hermanas y facilitar su separación en la anafase. Por tanto, un funcionamiento defectuoso de estas puede resultar en eventos de no disyunción o, alternativamente, en una separación prematura de las cromátidas (Mantikou *et al.*, 2012).

1.3.2.2. Mosaicismo segmentario

El mosaicismo segmentario tiene su origen en correcciones defectuosas de roturas en el ADN, por lo que su etiología es distinta a la del mosaicismo cromosómico (Viotti, 2020). Las roturas de doble cadena (DSBs) se producen cuando las horquillas replicativas se detienen y colapsan durante la síntesis del ADN, por razones como alteraciones en su integridad, ausencia de constituyentes de su síntesis o tensión asociada a su estructura secundaria (Babariya *et al.*, 2017; Mehta and Haber, 2014). Normalmente, las DSBs inducen la activación de mecanismos de reparación del ADN, y el fallo en su ejecución suele activar el proceso apoptótico (Aguilera and Garcia-Muse, 2013). Sin embargo, dado que los mecanismos de reparación y apoptosis están comprometidos durante las primeras divisiones embrionarias, cuando una célula repara incorrectamente una DSB, puede producirse la duplicación o delección del segmento que contiene la rotura (Escriba *et al.*, 2019).

1.3.3. Factores de riesgo

En cuanto a los factores que incrementan la probabilidad de ocurrencia de los eventos moleculares que generan mosaicismo, es posible distinguir entre factores extrínsecos, si están relacionados con el tratamiento de RHA; y factores intrínsecos, si son características propias de los pacientes o de los embriones generados por estos. Como factores de riesgo extrínsecos se han propuesto el protocolo de estimulación ovárica, las condiciones del

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laboratorio o del cultivo *in vitro*, y el método de fecundación, entre otros (Morbeck *et al.*, 2017; Palmerola *et al.*, 2019; Swain, 2019; Viotti, 2020). Por otro lado, entre los factores de riesgo intrínsecos propuestos destacan la presencia de variantes genéticas de riesgo (McCoy *et al.*, 2015) y la edad paterna avanzada (Coll *et al.*, 2021), la cual podría estar relacionada con un incremento en el riesgo de aparición de mutaciones *de novo* (Kong *et al.*, 2012; Silber *et al.*, 2003). No obstante, hasta la fecha, los estudios publicados al respecto son limitados y a menudo presentan resultados contradictorios, lo que dificulta el establecimiento de causalidades y mecanismos de acción.

1.3.4. Diagnóstico

1.3.4.1. Estadio preimplantacional

El diagnóstico de mosaicismo embrionario en PGT-A se basa en la identificación de valores intermedios en el número de copias cromosómicas en los perfiles de NGS tras la biopsia de TE (Munne and Wells, 2017; Paulson and Treff, 2020) (**Figura 7**).

En cada protocolo diagnóstico, es necesario realizar experimentos de validación preliminares para determinar el rango de mosaicismo. Estos experimentos se basan en el uso de mezclas artificiales de líneas celulares con proporciones crecientes de células aneuploides (Goodrich *et al.*, 2016), o bien en la utilización del ADN contenido en dichas células (Garcia-Pascual *et al.*, 2020). Estos procedimientos son fundamentales para determinar la sensibilidad de la plataforma, es decir, el porcentaje de verdaderos positivos (biopsias con mosaicismo identificadas correctamente), y su especificidad, es decir, el porcentaje de verdaderos negativos (biopsias sin mosaicismo identificadas correctamente).

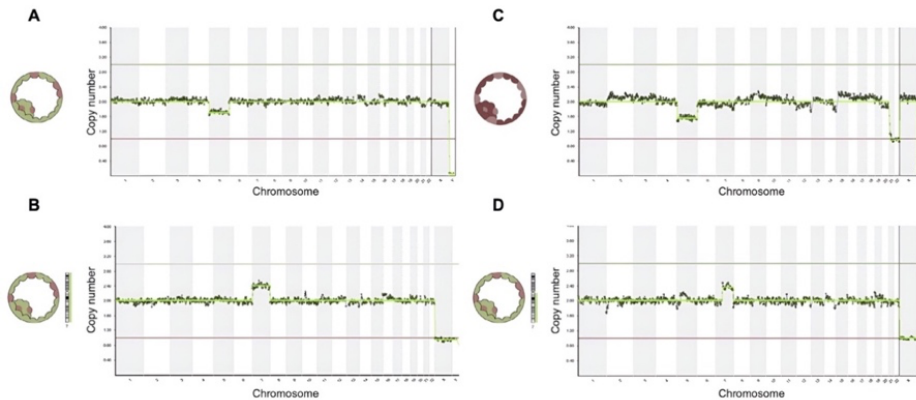


Figura 7 Diagnóstico de mosaicismo en diagramas NGS de variación en el número de copias. El eje x indica el cromosoma, y el eje y indica el número de copias de cada cromosoma. **(A)** Mosaico diploide-aneuploide (monosomía en mosaico del cromosoma 5). **(B)** Mosaico aneuploide (monosomía en mosaico del cromosoma 5 con aneuploidías uniformes adicionales). **(C)** Mosaico cromosómico diploide-aneuploide (trisomía en mosaico del cromosoma 7). **(D)** Mosaico segmentario diploide-aneuploide (trisomía en mosaico del brazo largo del cromosoma 7) (Adaptado de Coll *et al.*, 2021).

Los rangos más comúnmente utilizados para el diagnóstico de mosaicismo son 20%-80% y 30%-70%, refiriéndose al porcentaje estimado de células aneuploides en la biopsia. Asimismo, es habitual establecer subclasificaciones para diferenciar entre mosaicismo de bajo y alto grado. Los rangos diagnósticos menos restrictivos aumentan el riesgo de falsos positivos, mientras que los más restrictivos aumentan el riesgo de falsos negativos (Girardi *et al.*, 2023).

Por otro lado, gracias a la integración del genotipado de SNPs en la secuenciación NGS, las plataformas más recientes de PGT-A permiten determinar el origen parental de las aneuploidías, es decir, si estas han sido heredadas del óvulo o del espermatozoide, y, en consecuencia, confirmar la presencia del mosaicismo detectado por NGS (Rana *et al.*, 2023) **(Figura 8)**.

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Figura 8 Diagnóstico de mosaicismo en diagramas NGS de variación en el número de copias y análisis de SNPs. Monosomía en mosaico del cromosoma 13. La plataforma de análisis genera un perfil de número de copias cromosómicas (CCN) y un gráfico de frecuencia de alelos B (BAF) utilizando datos de SNPs. Los gráficos BAF consisten en dos tipos de SNPs: homocigotos y heterocigotos. Los SNPs homocigotos AA se ubican en la parte superior del gráfico BAF, los SNPs heterocigotos AB están en el medio, y los SNPs homocigotos BB están en la parte inferior. Un CCN de 2 se representa por un perfil de SNP AB que no se desvía del medio (0,5). Una trisomía o la presencia de una anomalía cromosómica en mosaico se representa por una división de los SNPs AB para el cromosoma afectado, con algunos desplazándose hacia arriba y otros hacia abajo. La monosomía se representa por una pérdida completa de los SNPs AB medios para el cromosoma afectado. La triploidía se representa por una división de los SNPs AB para todos los cromosomas. La presencia de contaminación se indica por un perfil altamente ruidoso, donde es imposible distinguir entre los SNPs AA, AB y BB. La desviación media porcentual absoluta (MAPD) describe el nivel de "ruido" de un perfil cromosómico específico y mide la dispersión de amplicones para ese cromosoma a partir de la línea de referencia (0). Las muestras con un MAPD > 0,3 exhiben perfiles "ruidosos" (menos compactos a lo largo de la línea de referencia) y pueden ser difíciles de interpretar (Martin *et al.*, 2024).

1.3.4.2. Estadio prenatal

Una vez ocurrida la gestación, el mosaicismo embrionario puede detectarse en la biopsia de vellosidades coriónicas (CVS) (entre las semanas 10 y 13 de embarazo) o mediante amniocentesis (entre las semanas 15 y 20 de embarazo) (Jelin *et al.*, 2019). Las técnicas de análisis más utilizadas son el cariotipo por bandejo G, el FISH y los microarrays cromosómicos (CMA). Las técnicas FISH y CMA se realizan mediante cultivo corto (directo o semidirecto), mientras que el análisis del cariotipo fetal requiere del cultivo largo de las células a analizar (Li *et al.*, 2022).

En la CVS se analizan, previa punción placentaria, células citotrofoblásticas (representativas de la placenta) mediante cultivo corto, y células mesenquimales del mesodermo extraembrionario (representativas del feto) mediante cultivo largo (Bianchi *et al.*, 1993). En cambio, en la amniocentesis se analizan células del líquido amniótico (amniocitos), las cuales se desprenden de la capa interna del amnion y provienen de varios órganos anatómicos fetales, como el tracto urogenital, el aparato respiratorio o las superficies epiteliales (Cremer *et al.*, 1981; Morris and Bennett, 1974; Tyden *et al.*, 1981).

1.3.5. Prevalencia

1.3.5.1. Estadio preimplantacional

Se estima que el mosaicismo está presente en más del 70% de los embriones en estadios de escisión (van Echten-Arends *et al.*, 2011; Mertzaniidou *et al.*, 2013), lo que concuerda con la alta incidencia de errores mitóticos durante las primeras divisiones embrionarias. De hecho, se puede afirmar que el genoma del embrión en sus primeras etapas exhibe

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inestabilidad cromosómica (McCoy, 2017; Vanneste *et al.*, 2009), una característica distintiva de muchos tumores marcada por frecuentes ganancias y pérdidas cromosómicas (Gordon *et al.*, 2012).

En el estadio de blastocisto, las tasas de mosaicismo reportadas tras NGS varían ampliamente entre clínicas, desde un 2% hasta un 50% (Levy *et al.*, 2021; Rana *et al.*, 2023; Viotti, 2020). Esta variabilidad puede atribuirse tanto al impacto diferencial de factores de riesgo de mosaicismo como a aspectos relacionados con el diagnóstico genético, tales como las técnicas de eclosión asistida y la biopsia embrionaria. Además, la metodología utilizada para procesar las muestras y los criterios empleados para interpretar los resultados genómicos también podrían contribuir a dicha variabilidad, así como a la detección del mosaicismo como artefacto técnico de laboratorio (Fragouli *et al.*, 2019; Munne and Wells, 2017). En este sentido, una alta incidencia de mosaicismo en el programa de PGT-A de una clínica o centro diagnóstico durante un tiempo prolongado puede ser indicativa de prácticas de laboratorio inadecuadas (Leigh *et al.*, 2022).

1.3.5.2. Estadio prenatal

La incidencia de mosaicismo reportada tras CVS es de un 1-2% en la población general. De esta, aproximadamente un 87% corresponde a mosaicismo confinado a la placenta y el 13% restante es mosaicismo verdadero fetal (Association of Clinical Cytogeneticists, 1994; Ledbetter *et al.*, 1992; Malvestiti *et al.*, 2015; Medical Research Council, 1991; Vejerslev and Mikkelsen, 1989). En amniocentesis, la incidencia de mosaicismo es de aproximadamente un 0,5% (Li *et al.*, 2022), mientras que en restos abortivos (POCs) y recién nacidos, el mosaicismo está presente en el 0,2-1% de los casos (Hansteen *et al.*, 1982; Hardy *et al.*, 2016; Menasha *et al.*, 2005).

1.3.6. Manejo clínico

Aunque la existencia del mosaicismo en embriones humanos ha estado documentada desde hace más de 30 años (Evsikov and Verlinsky, 1998; Griffin *et al.*, 1991; Munne, Lee, *et al.*, 1993), la cuestión de su manejo clínico solo ha cobrado relevancia recientemente, tras la introducción de plataformas de PGT-A con suficiente sensibilidad para detectarlo en biopsias de TE. Los embriones aneuploides tras PGT-A son descartados para la transferencia, ya que dan lugar a fallos de implantación, abortos y anomalías congénitas (Capalbo *et al.*, 2022). Sin embargo, desde que se reportó el nacimiento de bebés sanos tras la transferencia de embriones diagnosticados como mosaicos diploide-aneuploide tras PGT-A (Greco *et al.*, 2015), un número creciente de clínicas ha comenzado a considerar la transferencia de este tipo de embriones en casos donde no se disponen de embriones euploides; y en consecuencia, han surgido estudios en los que se evalúa su potencial reproductivo.

Los estudios de cohorte retrospectivos sugieren que los embriones mosaicos tienen capacidad de generar descendencia sana, aunque poseen un potencial reproductivo inferior al de los embriones euploides, concretamente, una menor tasa de implantación y de recién nacido vivo y una mayor tasa de aborto. Asimismo, estos estudios sugieren que ciertas características del mosaicismo, como el grado de mosaicismo, el tipo de aneuploidía, y el número de cromosomas afectados, podrían condicionar los resultados clínicos (Abhari and Kawwass, 2021; Ma *et al.*, 2022; Mourad *et al.*, 2021; Treff and Marin, 2021; Viotti *et al.*, 2023; Viotti, McCoy, *et al.*, 2021). Sin embargo, en el primer estudio prospectivo aleatorizado de transferencias de embriones mosaicos, se reportaron tasas similares de recién nacido vivo y aborto clínico entre embriones euploides y aquellos con hasta un 50% de mosaicismo (Capalbo *et al.*, 2021).

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Posteriormente, estos resultados fueron respaldados por dos revisiones sistemáticas y metaanálisis que evaluaron la calidad de los estudios no aleatorizados, sugiriendo que los peores resultados clínicos observados tras la transferencia de embriones con niveles moderados de mosaicismo podrían deberse a un sesgo de selección hacia pacientes de peor pronóstico (Capalbo *et al.*, 2021; Ma *et al.*, 2022; Mourad *et al.*, 2021) **(Tabla 1)**.

Es importante destacar que estos estudios coinciden en que, al menos cuando el mosaicismo se detecta en niveles moderados, la transferencia de embriones mosaicos conlleva un riesgo añadido relativamente bajo de resultados clínicos adversos en comparación con la transferencia de embriones euploides, incluyendo complicaciones en el embarazo, cariotipos prenatales y postnatales alterados, y anomalías congénitas (Capalbo *et al.*, 2021; Lee *et al.*, 2020; Treff and Marin, 2021; Viotti *et al.*, 2023; Yakovlev *et al.*, 2022) **(Tabla 2)**.

De hecho, las últimas recomendaciones de la ESHRE indican que no es aceptable descartar los embriones con mosaicismo de bajo grado, y que la selección entre éstos y los embriones euploides debe hacerse en base a criterios morfológicos. Por el contrario, la ESHRE evita formular recomendaciones para la transferencia de embriones con mosaicismo de alto grado, debido a la ausencia de datos clínicos sólidos (ESHRE Working Group on Chromosomal Mosaicism, 2022) **(Tabla 3)**.

Estudio	Diseño	Embriones transferidos (n)		Implantación (%)		Recién nacido vivo (%)		Aborto clínico (%)	
		Mosaicos	Euploides	Mosaicos Euploides	P	Mosaicos Euploides	P	Mosaicos Euploides	P
<i>Viotti et al. (2023)</i>	Retrospectivo	2.031	14.673	45,0	59,1 ****	25,0	53,8 ****	22,2	8,9 ****
<i>Yakovlev et al. (2022)</i>	Retrospectivo	118	513	39,0	47,0 **	28,8	40,7 **	26,1	12,0 *
<i>Capalbo et al. (2021)</i>	Prospectivo	413	484	55,4	55,8 ns	42,5	43,4 ns	11,9	12,0 ns
<i>Viotti et al. (2021)</i>	Retrospectivo	1.000	5.561	41,8	57,2 ****	31,3	52,3 ****	25,0	8,6 ****
<i>Zhang et al. (2020)</i>	Prospectivo	137	476	40,1	59,0 ***	27,1	47,0 ***	33,3	20,5 *
<i>Lee et al. (2020)</i>	Retrospectivo	83	216	51,8	65,7 *			5,1	12,9 ns
<i>Munné et al. (2020)</i>	Retrospectivo	253	2.654	49,0	83,0 ***	37,5	63,0 ***	25,0	7,0 ***
<i>Victor et al. (2019)</i>	Prospectivo	100	478	38,0	49,6 *				
<i>Zhang et al. (2018)</i>	Retrospectivo	102	268			46,6	59,1 *	20,3	12,7 ns
<i>Zore et al. (2019)</i>	Retrospectivo	20	357			30,0	53,8 *	40,0	18,2 *
<i>Spinella et al. (2018)</i>	Prospectivo	78	251	38,5	54,6 *	30,8	46,6 *	7,8	8,0 ns
<i>Fragouli et al. (2017)</i>	Retrospectivo	44	51	30,1	55,8 *	27,8	47,0 *	55,6	17,2 *
<i>Lledó et al. (2017)</i>	Retrospectivo	52	382	26,9	37,2 ns			7,1	18,1 ns
<i>Munne et al. (2017)</i>	Retrospectivo	143	1.045	53,0	71,0 *			24,0	10,0 *

Tabla 1 Resumen de estudios comparativos de transferencias de embriones mosaicos y sus resultados reproductivos. ns: P>0.05; *P≤0.05; **P≤0.01; ***P≤0.001; ****P≤0.0001.

Estudio	Resultado PGT-A	Resultado clínico	Prueba diagnóstica		
			CVS	Amniocentesis	Test postnatal
<i>Viotti et al. (2023)</i>	mos (-2) [22%]	Aborto espontáneo			mos (+2,+14,+X)
<i>Greco et al. (2023)</i>	mos (+1q,-7,-8,+9,-19,-20,+21) [40%]	Gestación interrumpida	mos (+21)	mos (+21)	
<i>Greco et al. (2023)</i>	mos (-1p) [40%]	Gestación interrumpida		mos (-1p)	mos (-1p)
<i>Kahaman et al. (2020)</i>	mos (-2) [35%]	Nacimiento sin defectos congénitos manifiestos		mos (+2)	mos (-2)
<i>Viotti et al. (2023)</i>	mos (+4q,-Xq) [40%]	Nacimiento sin defectos congénitos manifiestos	mos (+4q)		

Tabla 2 Gestaciones con confirmación del mosaicismo en el test prenatal, postnatal o de restos abortivos (POC) tras la transferencia de embriones con diagnóstico de mosaicismo en PGT-A mediante NGS. CVS: biopsia de vellosidades coriónicas.

Recomendación	Monosomía > trisomía	Grado de mosaicismo	Tipo de mosaicismo	Cromosoma implicado	Diagnóstico prenatal	Nuevo ciclo
PGDIS (2016)	Sí	Sí Umbral no definido	Un cromosoma	Sí	Sí Amniocentesis (precoc > 14 semanas)	Sí
				Prioridad 1: 1, 3, 4, 5, 6, 8, 9, 10, 11, 12, 17, 19, 20, 22		
				Prioridad 2: 14, 15 (UPD)		
COGEN (2017)	No	Sí Bajo grado (20-40%) > alto grado (40-70%)	Sí Desestimar mosaicos complejos	Prioridad 3: 2, 7, 16 (IUGR) y 13, 18, 21 (aneuploidias viables)	Sí Amniocentesis > CVS	Sí
				Prioridad más alta: 1, 3, 4, 5, 6, 8, 9, 10, 11, 12, 17, 19, 20		
				Prioridad baja: 14, 15 (UPD) y 2, 7, 16 (IUGR)		
Grati et al. (2018)	No	NR	NR	Prioridad más baja: 13, 18, 21, 22 (aneuploidias viables)	Sí Amniocentesis	NR
				Sí		
				Puntuación 0: 1, 3, 10, 12, 19 Puntuación 1: 4, 5, 47, XY Puntuación 2: 2, 7, 11, 17, 22 Puntuación 3: 6, 9, 15 Puntuación 4-5: 8, 20, 47, XXX, 47, XXY Transferencia no recomendada: 13, 14, 16, 18, 21, 45, X		
PGDIS (2019)	No	Sí Umbral no definido	NR	Sí Criterios descritos en Grati et al. (2018)	Sí Amniocentesis (precoc > 14 semanas)	Sí
ASRM (2020)	No	Falta de evidencia	Falta de evidencia	Falta de evidencia	Discutir riesgo-beneficio	Sí
Votri et al. (2021)	No	Sí Bajo grado > alto grado (umbral 50%)	Sí Segmentario > 1 cromosoma > 2 cromosomas > complejos	No	NR	NR
PGDIS (2021)	No	Sí Umbral no definido	Sí Segmentario > Cromosoma completo	No	Sí Sin distinción (euploides y mosaicos)	Sí
ESHRE (2022)	No	Sí Sin recomendaciones (alto grado)	NR	No	No (bajo grado)	No (bajo grado)
ASRM (2023)	No	Falta de evidencia	Falta de evidencia	Falta de evidencia	Discutir riesgo-beneficio	NR

Tabla 3 Resumen de las recomendaciones emitidas por diferentes sociedades científicas con respecto a la priorización y el manejo prenatal de los embriones mosaicos (Adaptado de Muñoz et al., 2024).

1.3.7. Hipótesis que explican el potencial reproductivo de los embriones mosaicos

Dos hipótesis se han propuesto para explicar el hecho de que una alta proporción de embriones mosaicos tengan pleno potencial de desarrollo.

1.3.7.1. Capacidad de autocorrección

Por un lado, en base al descenso observado en la prevalencia del mosaicismo a lo largo del desarrollo embrionario, se ha propuesto que los embriones mosaicos poseen una cierta capacidad intrínseca de autocorrección. Para explicar esta reducción, se han propuesto a su vez varios modelos que no se excluyen mutuamente (McCoy, 2017).

En primer lugar, el modelo de mortalidad embrionaria sugiere que los embriones mosaicos son seleccionados negativamente durante el desarrollo debido al impacto perjudicial de las células aneuploides sobre la viabilidad embrionaria. Esto ha sido evidenciado por estudios que muestran una alta prevalencia de aneuploidías en mosaico en embriones bloqueados (Bielanska *et al.*, 2002; Ruangvutilert *et al.*, 2000; Rubio *et al.*, 2007; Santos *et al.*, 2010).

En segundo lugar, el modelo de rescate de la aneuploidía propone que el mosaicismo puede corregirse mediante la reversión de la aneuploidía a euploidía. Aunque este mecanismo ha sido teorizado, las pruebas que lo apoyan en humanos son escasas (Coorens *et al.*, 2021; Schlade-Bartusiak *et al.*, 2022), y parece desempeñar un papel menor en base a la baja incidencia de disomía uniparental tanto en blastocistos (0,06%) (Gueye *et al.*, 2014; Taylor *et al.*, 2014), como en amniocitos, recién nacidos y POCs (<0,001%) (Benn, 2021).

En tercer lugar, el modelo de asignación preferencial implica la distribución selectiva de las células aneuploides a los linajes extraembrionarios. Aunque existen pruebas contradictorias respecto a este mecanismo (Capalbo *et al.*, 2013; Popovic *et al.*, 2018; Starostik *et al.*, 2020), estudios recientes sugieren que las células aneuploides pueden segregarse preferentemente al fluido del blastocelo, a células periféricas distantes de la MCI, y en menor medida, a las células del TE (Griffin *et al.*, 2023; Yang *et al.*, 2021). Estos hallazgos son coherentes con la identificación de cuellos de botella fisiológicos que confinan las células aneuploides surgidas en las primeras divisiones celulares a los linajes placentarios (Coorens *et al.*, 2021).

Por último, el modelo de depleción clonal plantea que las células aneuploides son eliminadas por apoptosis o no se propagan eficientemente en los embriones mosaicos, reduciendo así su impacto perjudicial (Baker, 2020; Coticchio *et al.*, 2021; Regin, Spits, *et al.*, 2022). Aunque faltan evidencias directas de estos mecanismos en humanos, estudios en quimeras humanas y de ratón generadas artificialmente sugieren que la viabilidad del embrión puede restablecerse mediante la apoptosis selectiva de las células aneuploides y la proliferación competitiva de las células euploides (Bolton *et al.*, 2016; Singla *et al.*, 2020; Yang *et al.*, 2021). Asimismo, estudios que utilizan la tecnología *time-lapse* han demostrado que los errores mitóticos durante las divisiones embrionarias tempranas se asocian con ciclos celulares más largos (Chavez *et al.*, 2012; Lee *et al.*, 2019; Martin *et al.*, 2023). Dichos estudios también han revelado que la eliminación de células aneuploides puede ocurrir mediante su exclusión a través de una compactación parcial (Hur *et al.*, 2023; Lagalla *et al.*, 2017, 2020; Zhan *et al.*, 2016) o su extrusión en forma de fragmentos o debris celular (Lagalla *et al.*, 2020; Orvieto *et al.*, 2020). De manera congruente, estudios del transcriptoma embrionario humano han demostrado que las aneuploidías generan estrés proteotóxico y conducen a la desregulación de genes y vías

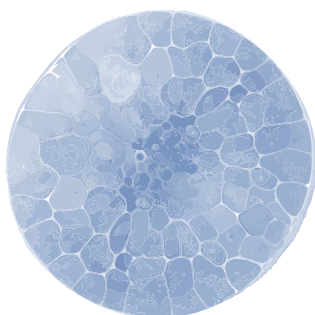
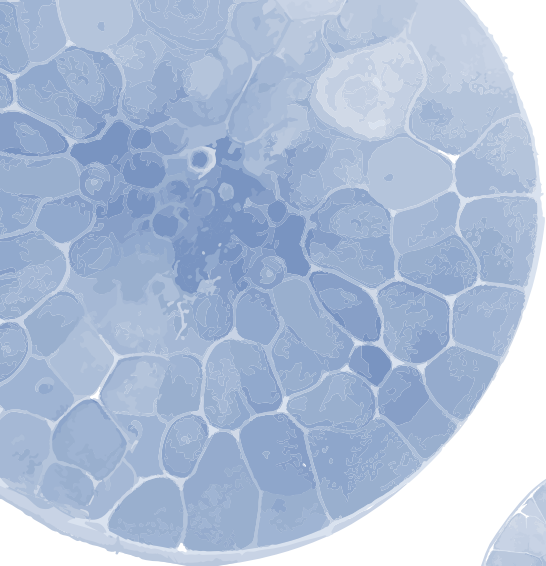
INTRODUCCIÓN

moleculares asociadas con la proliferación celular, la apoptosis y la autofagia (Licciardi *et al.*, 2018; Martin *et al.*, 2023; Maxwell *et al.*, 2022; Regin, Lei, *et al.*, 2022; Sanchez-Ribas *et al.*, 2019; Vera-Rodriguez *et al.*, 2015).

1.3.7.2. Falsos positivos

Por otro lado, se ha demostrado que el diagnóstico de mosaicismo en PGT-A basado únicamente en la identificación de valores intermedios en el número de copias cromosómicas en los perfiles de NGS conduce a falsos positivos. Por tanto, esta hipótesis sugiere que los resultados de las transferencias de embriones mosaicos se han visto sesgados por el diagnóstico erróneo de embriones euploides y aneuploides (Treff and Marin, 2021).

De hecho, estudios de desagregación embrionaria, en los que se realizan biopsias multifocales, han demostrado que la mayoría de los resultados de mosaicismo en blastocistos se limitan a una única muestra de TE (Capalbo *et al.*, 2021; Girardi *et al.*, 2023; Marin *et al.*, 2021; Popovic *et al.*, 2020). En este sentido, la introducción de nuevas plataformas de PGT-A, que incorporan el genotipado de SNPs al análisis NGS, ha revelado que más del 30% de los embriones diagnosticados como mosaicos basándose únicamente en los resultados de NGS, muestran al menos un error de origen meiótico tras el análisis de SNPs, indicando la presencia de aneuploidías uniformes (Cascante *et al.*, 2023; Popa *et al.*, 2023). Por otro lado, los resultados de mosaicismo basados en los perfiles de NGS pueden ser indistinguibles del ruido técnico superpuesto a perfiles euploides o aneuploides (Treff and Marin, 2021). En este documento, se emplea el término 'embrión mosaico', dado su uso habitual entre los profesionales de RHA, con el fin de simplificar la lectura del texto.



MOTIVACIÓN, OBJETIVOS E HIPÓTESIS

2| MOTIVACIÓN, OBJETIVOS E HIPÓTESIS

2.1. Motivación

Los avances en las tecnologías de secuenciación masiva han revolucionado nuestra comprensión sobre la complejidad y la plasticidad del genoma del embrión humano (Coticchio *et al.*, 2021). Aunque el efecto perjudicial de las aneuploidías embrionarias sobre la viabilidad celular y los resultados clínicos ha sido ampliamente demostrado (Capalbo *et al.*, 2022; Shahbazi *et al.*, 2020; Tosh *et al.*, 2022; Williams *et al.*, 2008; Zhu *et al.*, 2018), se sabe relativamente poco sobre los factores que subyacen a la competencia reproductiva de los embriones mosaicos. Este conocimiento es crítico para orientar la implementación de estrategias de cribado genético y mejorar nuestra comprensión sobre la capacidad reproductiva humana.

En los últimos años, la introducción de sistemas de cultivo embrionario con tecnología *time-lapse* en los laboratorios de FIV ha favorecido la aparición de estudios diseñados para identificar marcadores morfocinéticos relacionados con variables de relevancia clínica, así como para construir algoritmos predictores del éxito o fracaso reproductivo (Armstrong *et al.*, 2019). Algunos de estos algoritmos han sido utilizados como herramienta de selección embrionaria en base a la predicción del estatus cromosómico (Del Carmen Nogales *et al.*, 2017; Reignier *et al.*, 2018). En este contexto, dado que los errores mitóticos que causan el mosaicismo implican eventos de no disyunción o retrasos en la anafase (McCoy, 2017), el análisis morfocinético podría contribuir a la identificación del mosaicismo mediante el estudio del ritmo de las divisiones celulares embrionarias. De hecho, se ha establecido una correlación entre la presencia de aneuploidías de origen mitótico y ciertas alteraciones morfocinéticas en embriones humanos, como

el intervalo entre la primera y segunda división mitótica y la sincronía entre la segunda y tercera división mitótica (Chavez *et al.*, 2012). Asimismo, se han observado retrasos morfocinéticos en embriones diagnosticados como mosaicos mediante PGT-A (Lee *et al.*, 2019).

Por otro lado, el desarrollo de plataformas de secuenciación de ARN (RNA-seq) altamente sensibles ha favorecido la aparición de estudios del transcriptoma del embrión humano (Hwang *et al.*, 2018; Islam *et al.*, 2011; Picelli *et al.*, 2014; Tang *et al.*, 2009). La mayoría de estos estudios se han centrado en investigar las redes transcripcionales que dirigen la segregación de los linajes celulares (Blakeley *et al.*, 2015; Boroviak *et al.*, 2018; Meistermann *et al.*, 2021; Petropoulos *et al.*, 2016; Stirparo *et al.*, 2018; Xue *et al.*, 2013; Yan *et al.*, 2013). No obstante, otros estudios han tratado de identificar marcadores moleculares de competencia embrionaria, mediante la correlación de los perfiles transcriptómicos con variables de relevancia clínica, como el estatus cromosómico o el resultado de la transferencia (Groff *et al.*, 2019; Kirkegaard *et al.*, 2015; Licciardi *et al.*, 2018; Ntostis *et al.*, 2019; Sanchez-Ribas *et al.*, 2019; Starostik *et al.*, 2020). Además, se han desarrollado estrategias para inferir el estatus cromosómico de los embriones a partir de los datos de RNA-seq (Fan *et al.*, 2018; Macaulay *et al.*, 2015; Weissbein *et al.*, 2016), algunas de las cuales se han optimizado para detectar mosaicismo (Griffiths *et al.*, 2017; Starostik *et al.*, 2020). Asimismo, mediante análisis transcriptómico, se ha establecido una correlación entre la presencia de aneuploidías y la desregulación de múltiples vías moleculares relacionadas con la proliferación celular, daño en el ADN, apoptosis, degradación de proteínas y metabolismo energético mitocondrial en diferentes tipos de muestras, incluyendo embriones, biopsias de TE e incluso células individuales (Regin, Spits, *et al.*, 2022).

En este escenario, existe la necesidad de estudiar el impacto del mosaicismo en la dinámica de desarrollo y las funciones moleculares de los

MOTIVACIÓN, OBJETIVOS E HIPÓTESIS

embriones humanos, para poder profundizar en la comprensión de los mecanismos que les confieren capacidad reproductiva. Teniendo en cuenta que niveles moderados de mosaicismo parecen ser inocuos para el desarrollo embrionario, es crucial determinar si el diagnóstico de niveles crecientes de mosaicismo va acompañado de la adquisición de características morfocinéticas y moleculares indicativas de una desregulación progresiva de la proliferación celular y una pérdida de la viabilidad celular; o si, por el contrario, los embriones mosaicos constituyen una entidad biológica compatible con la normalidad cromosómica, y los resultados reproductivos observados tras su transferencia son producto de artefactos diagnósticos.

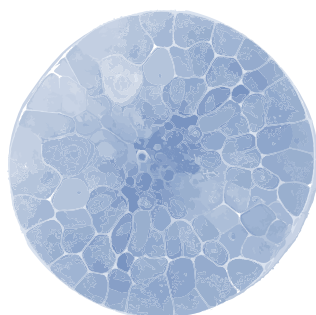
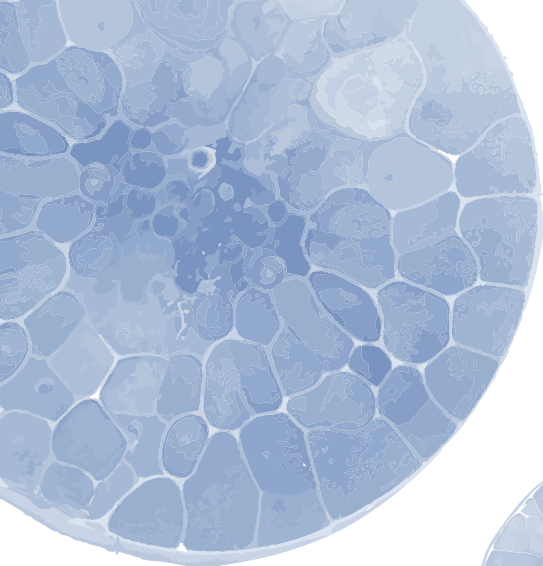
2.2. Objetivos

El objetivo principal de la presente tesis doctoral es generar nuevos conocimientos en el campo de la embriología clínica sobre la dinámica de desarrollo y las características moleculares de los embriones diagnosticados como mosaicos tras PGT-A, con el fin de comprender las causas que determinan su potencial reproductivo. Para ello, se plantearon los siguientes objetivos específicos:

1. Determinar la incidencia, tipología y distribución cromosómica del mosaicismo en el estadio de blastocisto.
2. Evaluar el comportamiento morfocinético y las características morfológicas de los blastocistos mosaicos.
3. Caracterizar el perfil de expresión génica de los blastocistos mosaicos.
4. Examinar la incidencia y colocalización de eventos apoptóticos en los blastocistos mosaicos.

2.3. Hipótesis

Los blastocistos diagnosticados como mosaicos de células euploides y aneuploides constituyen una población minoritaria de embriones en el programa de PGT-A que presenta características específicas en términos morfológicos, cromosómicos, morfocinéticos y de expresión génica en comparación con los blastocistos uniformemente euploides y aneuploides.



JUSTIFICACIÓN DE COMPENDIO DE ARTÍCULOS

3 | JUSTIFICACIÓN DE COMPENDIO DE ARTÍCULOS

La presente tesis doctoral se presenta como un compendio de tres publicaciones que proporcionan nuevos conocimientos sobre la dinámica de desarrollo y las propiedades moleculares de los embriones mosaicos.

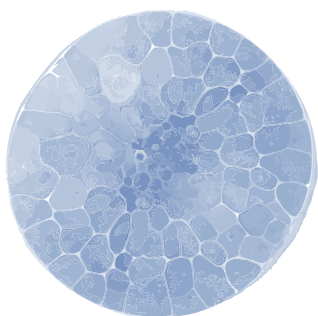
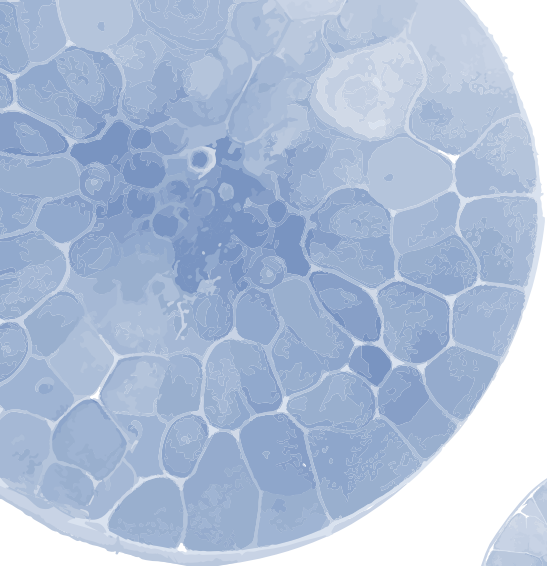
En estos trabajos, la caracterización del embrión mosaico se lleva a cabo a través de varios enfoques complementarios: por un lado, mediante el análisis de su perfil morfocinético; y por otro, mediante el análisis de su perfil de expresión génica tanto a nivel transcripcional como de proteína, con especial énfasis en la expresión de marcadores de apoptosis y de segregación de los linajes celulares. Esta aproximación multidimensional ofrece una perspectiva integral de la identidad biológica del embrión mosaico, acercándonos a la comprensión de las causas que subyacen a su potencial reproductivo.

En el artículo I, **“The morphokinetic signature of mosaic embryos: evidence in support of their own genetic identity”**, publicado en la revista *Fertility and Sterility*, se describe la incidencia, tipología y distribución cromosómica del mosaicismo embrionario en el estadio de blastocisto, y se utiliza la tecnología *time-lapse* para describir sus implicaciones morfocinéticas.

En el artículo II, **“Mosaic results after preimplantation genetic testing for aneuploidy may be accompanied by changes in global gene expression”**, publicado en la revista *Frontiers in Molecular Biosciences*, se emplea la tecnología de RNA-seq para evaluar el impacto de diferentes grados de mosaicismo embrionario en el perfil de expresión génica de las células de la MCI y el TE del blastocisto.

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En el artículo III, **“Trophectoderm cells of human mosaic embryos display increased apoptotic levels and impaired differentiation capacity: a molecular clue regarding their reproductive fate?”**, publicado en la revista *Human Reproduction*, se realiza inmunofluorescencia y microscopía confocal para analizar la incidencia de eventos apoptóticos en los blastocistos mosaicos durante la segregación de los linajes celulares de MCI y TE.



RESULTADOS

4| RESULTADOS

4.1. Ritmo de desarrollo del embrión mosaico: análisis morfocinético

En el artículo I, se llevó a cabo un estudio retrospectivo de cohortes comparando características cromosómicas, morfológicas y morfocinéticas entre blastocistos euploides (<30% mosaicismo) (n=692), mosaicos de bajo grado (30%-<50% mosaicismo) (n=68), mosaicos de alto grado (50%-<70% mosaicismo) (n=38) y aneuploides (>70% mosaicismo) (n=713) tras PGT-A mediante NGS. Se analizaron un total de 1.511 blastocistos cultivados en incubadores con tecnología *time-lapse*. Los resultados obtenidos se resumen a continuación:

En primer lugar, se analizó la incidencia de mosaicismo y su correlación con la edad de los progenitores. A diferencia de la incidencia de aneuploidías uniformes (47,2%=713/1.511), la cual se correlacionó positivamente con la edad materna ($P<0,0001$), la incidencia de mosaicismo (7%=106/1.511) no mostró correlación con la edad de los progenitores ($P>0,05$) (**Figura 9**).

A continuación, se describió la distribución del mosaicismo en función de los cromosomas implicados y la tipología de las anomalías cromosómicas. En los 106 embriones mosaicos analizados, se identificaron un total de 132 aneuploidías. El 75,5% de los embriones (80/106) presentaron aneuploidías simples y el 24,5% (26/106) presentaron aneuploidías dobles. Los cromosomas con mayor prevalencia de mosaicismo fueron el 19 (12,9%), 18 (7,6%), 13 (7,6%), 20 (6%), 22 (5,3%) 21 (5,3%) y 14 (5,3%); y los cromosomas con menor prevalencia el 17

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(1,5%), 1 (1,5%) y 15 (0,8%). No se encontró correlación entre el grado de mosaicismo y el riesgo de presentar aneuploidía simple o doble ($P>0,05$).

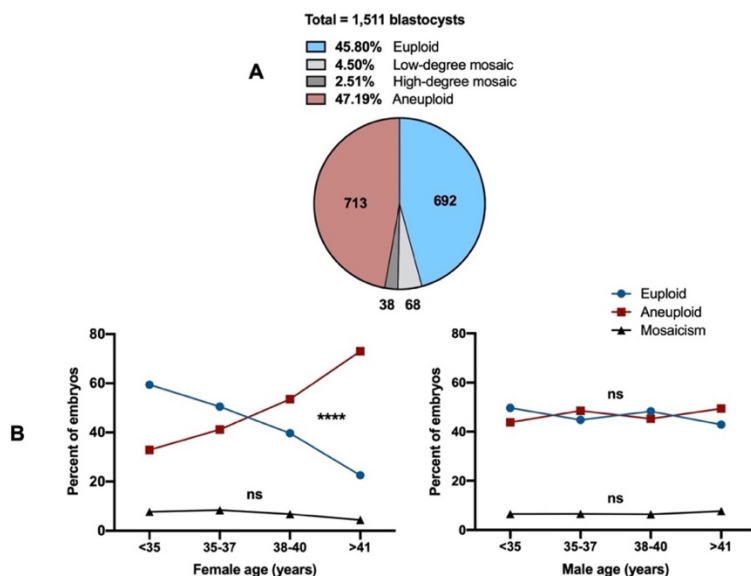
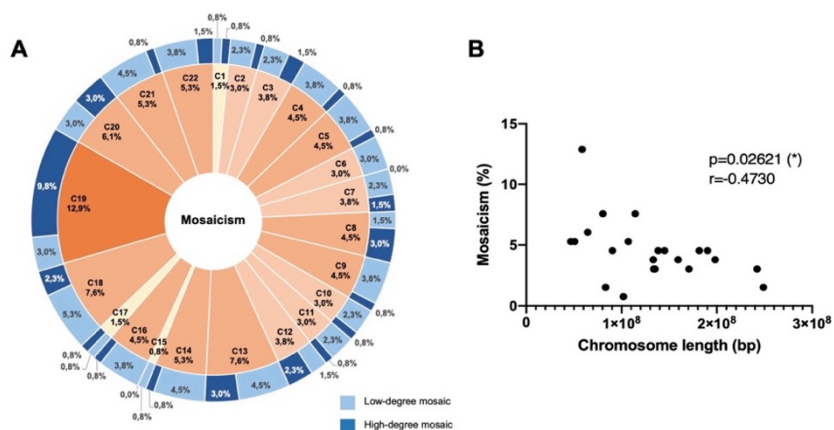


Figura 9 (A) Incidencia de euploidía, mosaicismo y aneuploidía. **(B)** Estratificación según la edad de los progenitores. Las comparaciones de proporciones entre las categorías se realizaron mediante la prueba de Chi cuadrado, con un nivel de confianza del 95%. **** $P\leq 0,0001$.

Sin embargo, la frecuencia de mosaicismo se correlacionó negativamente con la longitud cromosómica ($P=0,026$) (**Figura 10**).



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Figura 10 (A) Distribución de la incidencia de mosaicismo por cromosoma. La intensidad del color en las secciones internas indica una mayor incidencia de mosaicismo en ese cromosoma. Las secciones externas muestran la incidencia de mosaicismo de bajo grado (azul claro) y de alto grado (azul oscuro) por cromosoma. **(B)** Correlación entre la incidencia de mosaicismo y la longitud cromosómica medida en pares de bases (bp). p : coeficiente de correlación de Pearson con un nivel de confianza del 95%. * $P \leq 0,05$; ** $P \leq 0,01$.

Posteriormente, se evaluó la correlación entre la calidad morfológica y la constitución cromosómica de los blastocistos. El porcentaje de blastocistos de buena calidad morfológica (A o B según el criterio ASEBIR) fue significativamente mayor en blastocistos euploides (66,9%=463/692), en comparación con blastocistos mosaicos (52,8%=56/106) ($P=0,014$) y blastocistos aneuploides (47,7%=340/713) ($P<0,001$) (**Figura 11**).

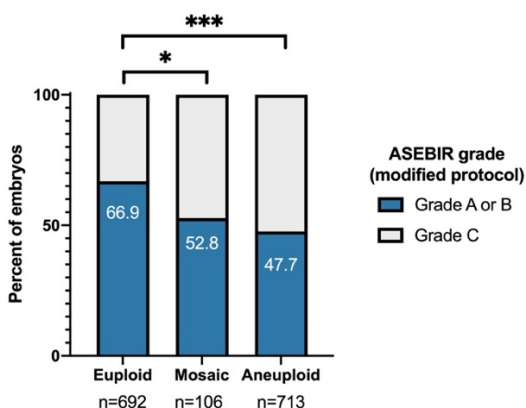


Figura 11 Porcentaje de blastocistos euploides, mosaicos y aneuploides según su calidad morfológica. Las comparaciones de proporciones entre las categorías se realizaron mediante la prueba de Chi cuadrado, con un nivel de confianza del 95%. * $P \leq 0,05$; *** $P \leq 0,001$.

Por último, se compararon los tiempos de 19 variables morfocinéticas entre los grupos de estudio: extrusión del segundo corpúsculo polar (tPB2); aparición de pronúcleos (tPNa); desaparición de pronúcleos (tPNf); división a 2, 3, 4, 5, 6, 7, 8 y 9 células (t2, t3, t4, t5, t6, t7, t8 y t9); inicio de la compactación (tSC); formación de mórula (tM); duración de la

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compactación ($t_{DC}=t_M-t_{SC}$); inicio de la blastulación (t_{SB}); formación del blastocisto (t_B); duración del primer ciclo celular embrionario ($ECC1=t_2-t_{PNf}$), duración del segundo ciclo celular embrionario ($ECC2=t_4-t_2$) y tercer ciclo celular embrionario ($ECC3=t_5-t_3$); y sincronía del segundo ciclo celular embrionario ($s_2=t_4-t_3$).

Para reducir el sesgo por paciente y protocolo, y considerar el impacto de posibles variables de confusión en los tiempos morfocinéticos (edad materna, índice de masa corporal materno, dosis de hormona folículo estimulante y tipo de incubador), se incluyó en el análisis un modelo de regresión logística de efectos mixtos (**Figura 12**). Además, con el fin de tener en cuenta la variabilidad entre anotadores, se calcularon los coeficientes de correlación intraclase (ICCs) en una muestra aleatoria de 20 embriones, alcanzando un acuerdo casi absoluto entre operadores ($ICC_m=0,968$).

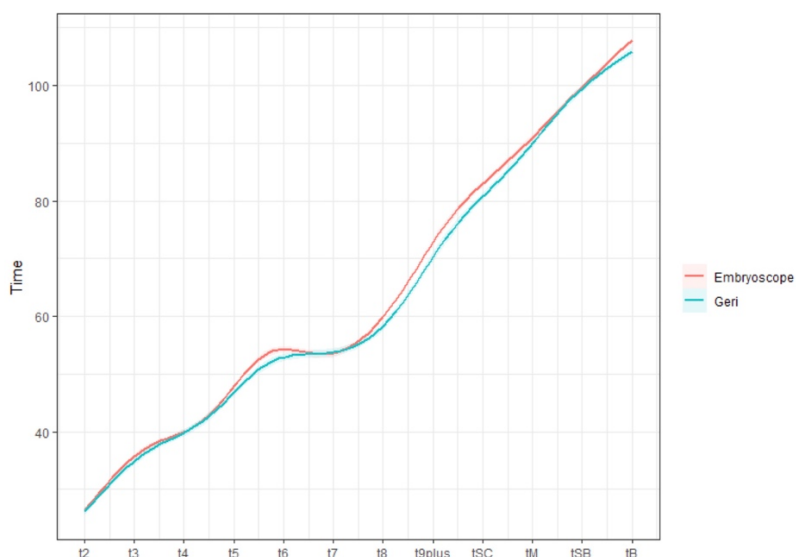


Figura 12 Perfil morfocinético embrionario según el tipo de incubador utilizado. En el eje x se representan las variables morfocinéticas directas. En el eje y se muestra el tiempo expresado en horas post-fecundación (hpf).

RESULTADOS

Al comparar los tiempos morfocinéticos entre los grupos diagnósticos, se observaron diferencias significativas entre embriones euploides y aneuploides en las variables tSC, tSB y tB ($P < 0,05$). Sin embargo, la morfocinética embrionaria no se correlacionó ni con el estatus de mosaicismo, ni con el grado de mosaicismo (bajo o alto) ni con la presencia de un patrón de mosaicismo compatible con la transferencia embrionaria ($P > 0,05$) (**Figura 13**).

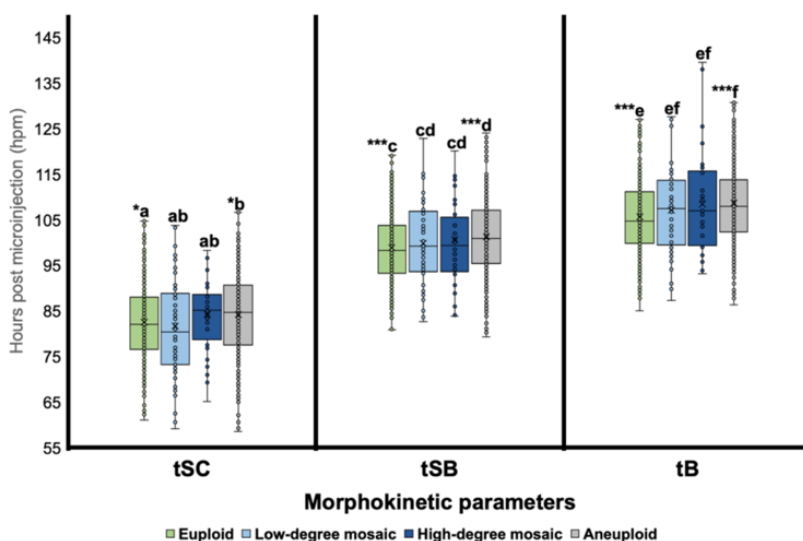


Figura 13 Comparación de variables morfocinéticas entre grupos diagnósticos. El diagrama de caja y bigotes muestra la mediana, la media, los cuartiles primero y tercero, y los valores mínimo y máximo. Las medianas se compararon utilizando la prueba de Kruskal-Wallis seguida del análisis post hoc de Bonferroni. Las mismas letras indican subconjuntos homogéneos para cada parámetro. tSC: inicio de la compactación; tSB: inicio de la blastulación; tB: formación del blastocisto. * $P \leq 0,05$; *** $P \leq 0,001$.

4.2. Perfil molecular del embrión mosaico: análisis transcriptómico

En el artículo II, se llevó a cabo un estudio prospectivo de cohortes comparando los perfiles transcriptómicos de fracciones de MCI y TE obtenidas a partir de blastocistos euploides (<30% mosaicismo) (n=5), mosaicos de bajo grado (30%-<50% mosaicismo) (n=5) y mosaicos de alto grado (50%-<70% mosaicismo) (n=4) tras PGT-A mediante NGS. Se analizaron un total de seis comparaciones (**Figura 14**):

(C-1): TE de euploides (n=4) vs. TE de mosaicos de bajo grado (n=5).

(C-2): TE de euploides (n=4) vs. TE de mosaicos de alto grado (n=4).

(C-3): TE de mosaicos de bajo grado (n=5) vs. TE de mosaico de alto grado (n=4).

(C-4): MCI de euploides (n=5) vs. MCI de mosaicos de bajo grado (n=3).

(C-5): MCI de euploides (n=5) vs. MCI de mosaicos de alto grado (n=4).

(C-6): MCI mosaicos de bajo grado (n=3) vs. MCI de mosaicos de alto grado (n=4).

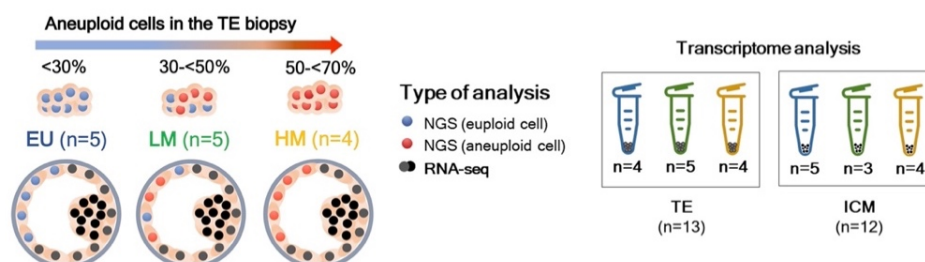


Figura 14 Diseño del estudio II. EU: euploide. LM: mosaico de bajo grado. HM: mosaico de alto grado. NGS: Secuenciación de nueva generación. TE: trofoectodermo. ICM: masa celular interna.

Los resultados obtenidos se resumen a continuación:

En primer lugar, se analizaron las diferencias en los perfiles globales de expresión génica en las seis comparaciones. El análisis de componentes principales reveló una falta de separación de las muestras según el resultado original del PGT-A. Las muestras solo se separaron mínimamente al

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comparar blastocistos euploides con mosaicos de alto grado, tanto en las comparaciones del TE como de la ICM. Por el contrario, en los mapas de calor, las muestras se clasificaron según el diagnóstico de PGT-A en todas las comparaciones. De hecho, los vectores de expresión de las muestras del mismo grupo fueron mucho más similares entre sí que aquellos de muestras pertenecientes a grupos diferentes. Solo una muestra de TE, obtenida de un embrión mosaico de bajo grado, fue clasificada en un grupo distinto, específicamente en el grupo euploide. Las muestras de TE de embriones mosaicos de bajo y alto grado mostraron los perfiles más similares (**Figura 15**).

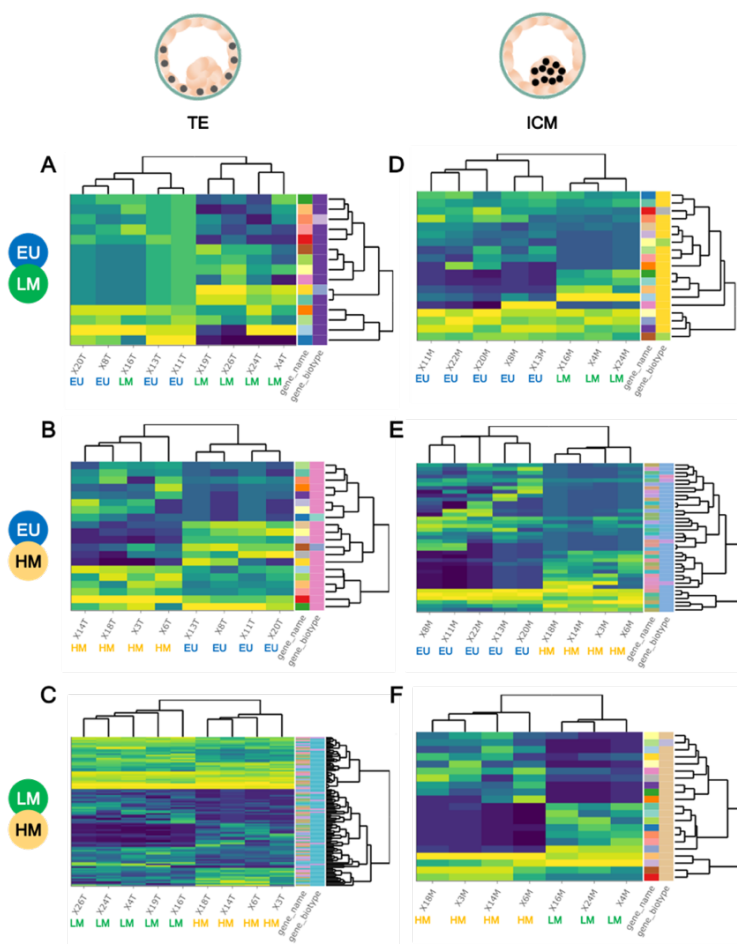
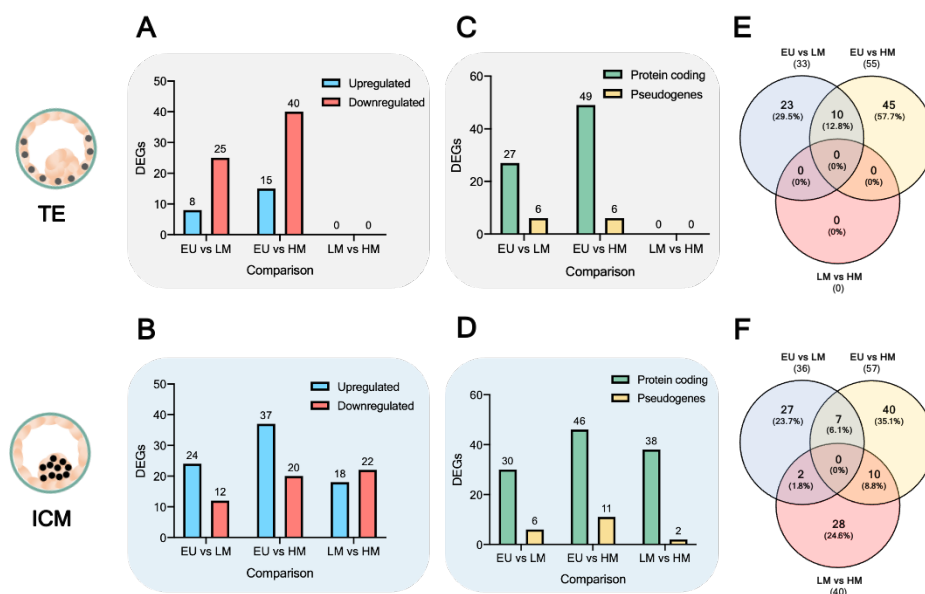


Figura 15 Mapas de calor y agrupamiento jerárquico. (A-C) Comparaciones de trofoectodermo. (D-F) Comparaciones de masa celular interna. Los conteos normalizados se obtuvieron dividiendo los conteos brutos de cada gen por el factor de tamaño, que se calculó mediante el método de la mediana. Las líneas amarillas representan mayor abundancia y las líneas azules representan menor abundancia frente a la otra condición. EU: euploide. LM: mosaico de bajo grado. HM: mosaico de alto grado.

A continuación, identificamos los genes diferencialmente expresados de forma significativa (DEGs) en las seis comparaciones. Por un lado, observamos que el número de DEGs aumentó tanto en el TE como en la MCI al comparar embriones euploides con embriones mosaicos de bajo grado (33 DEGs en el TE y 36 DEGs en la MCI) y con embriones mosaicos de alto grado (55 DEGs en el TE y 57 DEGs en la MCI). Sin embargo, no se encontraron DEGs entre el TE de embriones mosaicos de bajo grado y mosaicos de alto grado, lo que indica que las diferencias observadas entre ambos grupos no fueron estadísticamente significativas. Por el contrario, se identificaron 40 DEGs entre las muestras de MCI de embriones mosaicos de bajo grado y alto grado (**Figura 16 A-D**).



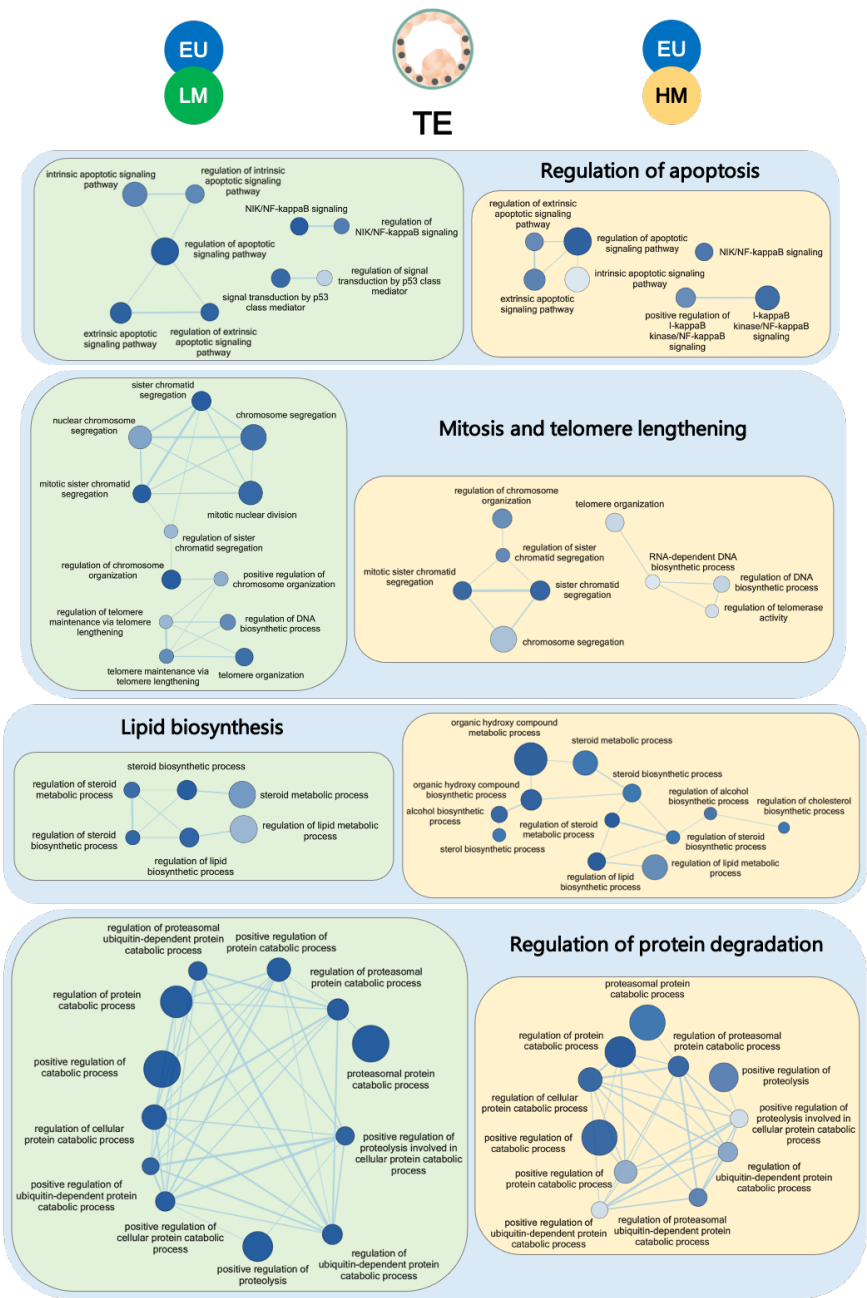
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Figura 16 Genes diferencialmente expresados de forma significativa (DEGs). (A-B) Número de DEGs sobreexpresados (azul) o infraexpresados (rojo) en el primer factor de comparación. **(C-D)** Biotipo de los DEGs. **(E-F)** Diagrama de Venn mostrando el solapamiento entre los DEGs. TE: trofoectodermo. ICM: masa celular interna.

Posteriormente, utilizamos el diagrama de Venn para identificar los DEGs solapantes y únicos. Se encontraron un total de 17 DEGs comúnmente desregulados (regulados al alza o a la baja) al comparar embriones euploides con embriones mosaicos de bajo grado y de alto grado, respectivamente. De estos 17 DEGs, 10 estaban desregulados en el TE y 7 en la MCI. Además, se identificaron 10 DEGs comúnmente desregulados en la MCI de embriones mosaicos de alto grado, en comparación con la MCI de embriones euploides y mosaicos de bajo grado. Finalmente, se encontraron un total de 40 DEGs exclusivamente desregulados en la MCI de embriones mosaicos de alto grado en comparación con embriones euploides (**Figura 16 E-F**).

Seguidamente, para comprender las implicaciones funcionales de las diferencias observadas a nivel de genes individuales, llevamos a cabo un análisis de enriquecimiento funcional. Dado que las células del TE de embriones mosaicos de bajo y alto grado mostraron perfiles de expresión génica similares (0 DEGs), esperábamos encontrar vías moleculares comúnmente desreguladas en comparación con los embriones euploides. De hecho, el análisis de enriquecimiento funcional reveló 124 términos desregulados significativamente en el TE de los embriones mosaicos de bajo y alto grado, en comparación con los controles euploides. El mapa de enriquecimiento funcional de las muestras de TE de los embriones mosaicos reveló una alteración global de las vías involucradas en la regulación de la apoptosis, la mitosis, el mantenimiento de telómeros, la biosíntesis de lípidos y la degradación de proteínas, en comparación con los controles euploides (**Figura 17**). Otras vías alteradas incluyeron aquellas involucradas en la

señalización intercelular WNT y en la generación de metabolitos precursores y energía a través del ciclo de Krebs.



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Figura 17 Mapa de términos funcionales comúnmente desregulados en el trofoectodermo (TE) de embriones mosaicos de bajo grado (LM) (paneles verdes) y embriones mosaicos de alto grado (HM) (paneles amarillos), en comparación con el TE de embriones euploides (EU). Los nodos representan procesos biológicos. La intensidad del color de los nodos se correlaciona con el valor q de la tasa de descubrimiento falso; cuanto más intenso es el color, mayor es la significancia estadística de la diferencia en la expresión. El tamaño del nodo representa la cantidad de genes en el conjunto. Las aristas representan la superposición entre los conjuntos de genes, y el ancho de las aristas indica la cantidad de genes solapados.

A continuación, aplicamos una estrategia de análisis similar en las células de la MCI. A diferencia de la respuesta observada en las células del TE, encontramos solo 12 términos, principalmente involucrados en la función mitocondrial, comúnmente desregulados de forma significativa en la MCI de embriones mosaicos de bajo y alto grado, en comparación con los controles euploides. La magnitud de la alteración de las vías mitocondriales, medida por el número y la significancia estadística de los términos desregulados, fue mayor en los embriones mosaicos de alto grado que en los de bajo grado al compararlos con los controles euploides. De hecho, el análisis de enriquecimiento funcional reveló importantes diferencias transcriptómicas (134 términos) entre la MCI de embriones mosaicos de bajo y alto grado. De los 134 términos, 130 (97%) estaban sobreexpresados en los embriones mosaicos de bajo grado, y se relacionaron con la producción de energía, la respiración celular, el desarrollo embrionario, la migración y adhesión celular, el metabolismo de compuestos orgánicos, la regulación de la apoptosis y la respuesta inflamatoria.

Por último, para aportar más pruebas de la presencia de mosaicismo en los embriones analizados, se calculó la duración de los ciclos celulares mediante el análisis retrospectivo de las divisiones blastoméricas hasta el estadio de 8 células, utilizando imágenes obtenidas mediante tecnología *time-lapse*. Es importante destacar que, para garantizar la precisión del análisis, la evaluación de los tiempos morfocinéticos implicó el seguimiento

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individualizado de las divisiones en todas las blastómeras. Dicho análisis reveló la existencia de retrasos significativos en la duración del primer (cc1) y tercer ciclo celular (cc3) tanto en los embriones mosaicos de bajo grado (n=4) (cc1=25,23±5,49 h; cc3=18,02±2,19 h) como en los embriones mosaicos de alto grado (n=4) (cc1=24,07±4,97 h; cc3=17,72±6,13 h), en comparación con los embriones euploides (n=25) (cc1=19,89±1,68 h; cc3=13,68±2,05 h) ($P<0,05$) (**Figura 18**).

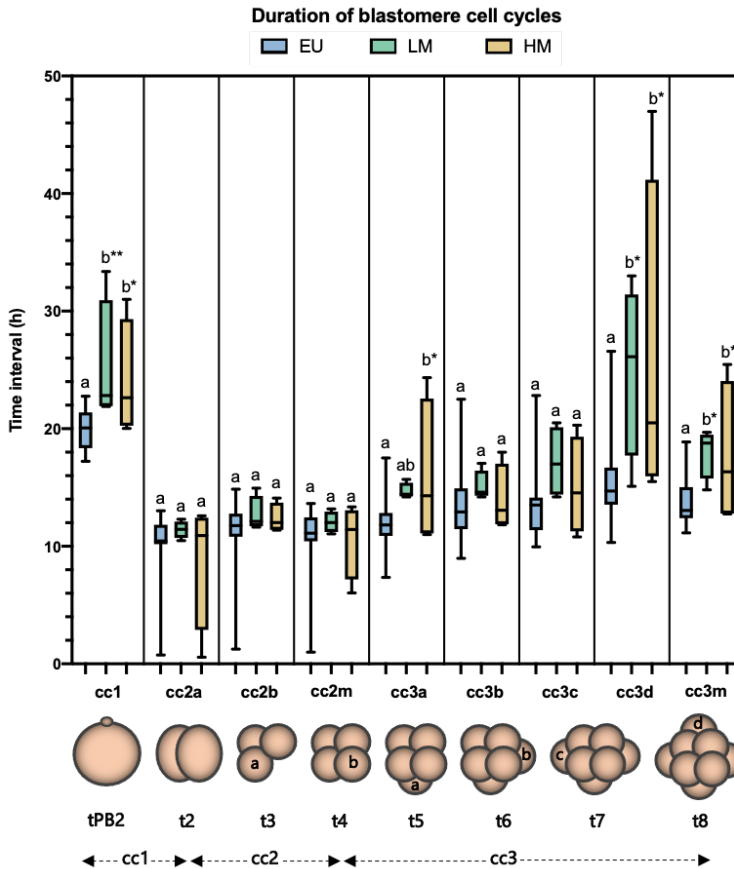


Figura 18 Duración de los ciclos celulares blastoméricos en embriones mosaicos de bajo grado (LM) y embriones mosaicos de alto grado (HM), en comparación con un subconjunto aleatorio de embriones euploides (EU). El diagrama de caja y bigotes muestra la mediana, la media, los cuartiles primero y tercero, y los valores mínimo y máximo. Las medias se compararon utilizando la prueba de ANOVA de un

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factor seguida del análisis post hoc de Bonferroni. Las mismas letras indican subconjuntos homogéneos para cada parámetro. * $P \leq 0.05$; ** $P \leq 0.01$.

4.3. Perfil molecular del embrión mosaico: análisis inmunohistoquímico

En el artículo III, se llevó a cabo un estudio prospectivo de cohortes comparando la incidencia de eventos apoptóticos y la capacidad de diferenciación celular de blastocistos euploides (<30% mosaicismo) (n=15), mosaicos (30%-<70% mosaicismo) (n=27) y aneuploides (>70% mosaicismo) (n=22) tras PGT-A mediante NGS y genotipado de SNPs.

El estudio se dividió en dos fases. En la primera fase, se analizó la expresión del marcador apoptótico caspasa-3 activa/escindida en un total de 53 blastocistos: n=13 euploides (n=11 en D+5; n=2 en D+6), n=22 mosaicos (n=16 en D+5; n=6 en D+6) y n=18 aneuploides (n=11 en D+5; n=7 en D+6). En la segunda fase, se evaluó el nivel de fragmentación del ADN, un indicador distintivo de la apoptosis en estadios avanzados, mediante la tinción TUNEL en un total de 11 blastocistos: n=2 euploides (n=1 en D+5; n=1 en D+6), n=5 mosaicos (n=4 en D+5; n=1 en D+6) y n=4 aneuploides (n=4 en D+5). En ambas fases, los marcadores apoptóticos se colocalizaron con el marcador nuclear DAPI y con los marcadores de linaje celular NANOG y GATA3 (**Figura 19**).

Los resultados obtenidos se resumen a continuación:

La inmunotinción con DAPI, NANOG y GATA3 se realizó en los 64 blastocistos (n=47 en D+5; n=17 en D+6), revelando un total de 3.387 células DAPI+ (2.369 células en D+5 y 1.018 células en D+6), 1.138 células NANOG+ (930 células en D+5 y 208 células en D+6) y 2.693 células GATA3+ (1.851 células en D+5 y 842 células en D+6). Adicionalmente, en

53 de los 64 blastocistos (38 blastocistos en D+5 y 15 blastocistos en D+6) se realizó inmunotinción con caspasa-3, revelando un total de 912 células caspasa-3+ (604 células en D+5 y 308 células en D+6). Los 11 blastocistos restantes (9 blastocistos en D+5 y 2 blastocistos en D+6) se tiñeron con TUNEL, identificándose un total de 124 células TUNEL+ (99 células en D+5 y 25 células en D+6).

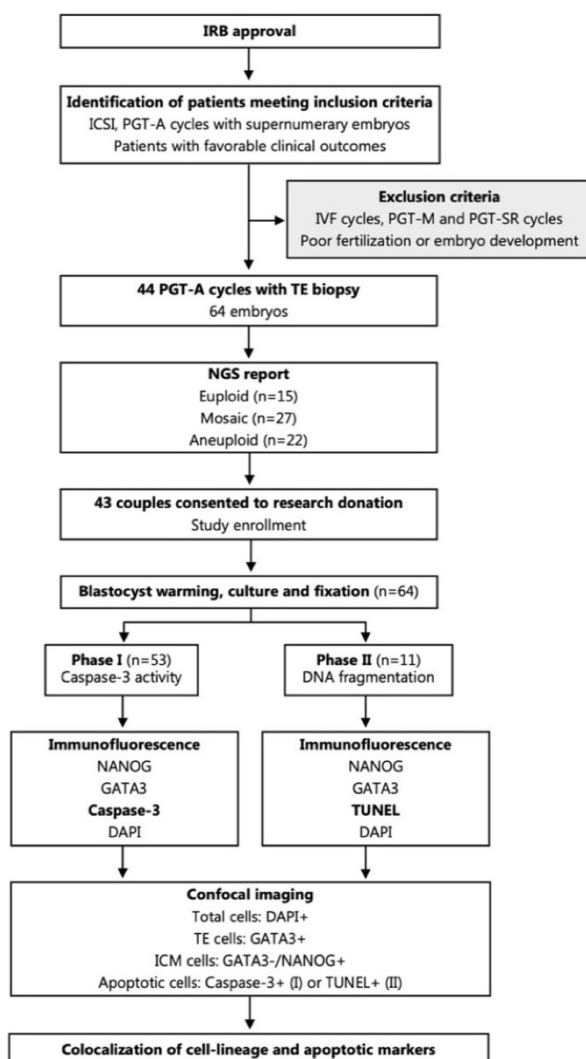


Figura 19 Diseño del estudio III.

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Fase I: tinción con caspasa-3

En primer lugar, se determinó el recuento total promedio de células analizando la tinción con DAPI. Los blastocistos analizados en D+5 y D+6 mostraron recuentos totales similares ($51,9 \pm 16$ células y $60,5 \pm 21,9$ células, respectivamente) ($P > 0,05$). Además, los recuentos totales fueron comparables entre blastocistos euploides ($59,2 \pm 20,6$ células en D+5; $78,5 \pm 0,7$ células en D+6), blastocistos mosaicos ($49,6 \pm 15$ células en D+5; $58,8 \pm 16,9$ células en D+6) y blastocistos aneuploides ($48,5 \pm 14,2$ células en D+5; $59,6 \pm 23,5$ células en D+6) ($P > 0,05$).

A continuación, se evaluó la expresión de los marcadores de linaje celular NANOG y GATA3. Por un lado, la proporción de células NANOG+ disminuyó significativamente del 38,2% (755/1.979 células) en D+5 al 20,3% (188/927 células) en D+6 ($P < 0,0001$). Por otro lado, la proporción de células GATA3+ aumentó significativamente del 77,8% (1.540/1.979 células) en D+5 al 83,1% (770/927 células) en D+6 ($P < 0,01$). De manera consistente, la proporción de células GATA3+/NANOG+ disminuyó significativamente del 16% (316/1.979 células) en D+5 al 3,3% (31/927 células) en D+6 ($P < 0,0001$).

Cabe destacar que, al estratificar por estatus cromosómico, tan solo en blastocistos aneuploides la proporción de células GATA3+ no aumentó significativamente entre D+5 y D+6 ($P > 0,05$). Además, la proporción de células GATA3+/NANOG+ fue significativamente mayor en blastocistos aneuploides (27,9%=149/534 células en D+5; 4,6%=19/417 células en D+6) y blastocistos mosaicos (13,1%=104/794 células en D+5; 3,4%=12/353 células en D+6), en comparación con blastocistos euploides (9,7%=63/651 células en D+5; 0%=0/157 células en D+6) ($P < 0,05$) (**Figura 20**).

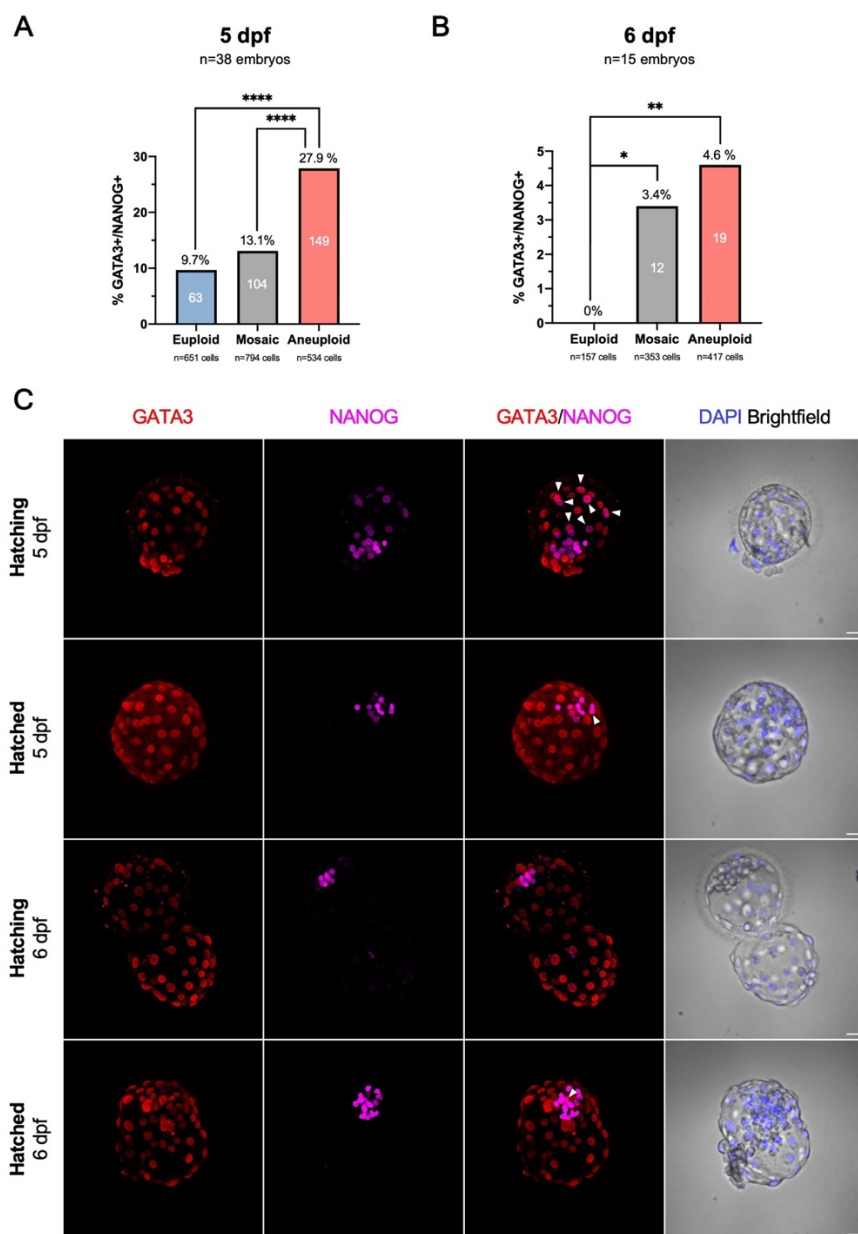


Figura 20 Persistencia de la expresión de NANOG en las células del trofoectodermo. (A) Día 5 post-fecundación (5 dpf). **(B)** Día 6 post-fecundación (6 dpf). Las barras azules, grises y rojas muestran embriones euploides, mosaicos y aneuploides respectivamente. Los valores debajo de cada barra (n) indican el número de células analizadas. Los valores mostrados dentro de cada barra indican el número

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de células positivas. Los valores mostrados encima de cada barra indican el porcentaje de células positivas. Las comparaciones de proporciones entre las categorías se realizaron mediante la prueba de Chi cuadrado, con un nivel de confianza del 95%. * $P \leq 0.05$; ** $P \leq 0.01$; **** $P \leq 0.0001$. **(C)** Imágenes representativas de embriones analizados en diferentes etapas de desarrollo. Las cabezas de flecha indican células coexpresando GATA3 y NANOG. Barras de escala, 20 μm .

De manera consistente, se observó un efecto similar al estratificar los blastocistos según su viabilidad esperada en base al cromosoma implicado en la aneuploidía. La mayor proporción de células GATA3+/NANOG+ se observó en blastocistos aneuploides con únicamente aneuploidías no viables (17,7%=168/951 células), mientras que las menores proporciones de células GATA3+/NANOG+ se observaron en blastocistos euploides (7,8%=63/808 células) y en blastocistos mosaicos con solo aneuploidías viables (2,5%=2/81 células) ($P < 0,01$). Además, la proporción de células GATA3+/NANOG+ fue significativamente menor en blastocistos mosaicos con únicamente aneuploidías no viables (9,3%=80/856 células) en comparación con sus homólogos aneuploides (17,7%=168/951 células) ($P < 0,001$). Finalmente, los blastocistos mosaicos con una mezcla de aneuploidías viables y no viables mostraron una proporción similar de células GATA3+/NANOG+ (16,2%=34/210 células) comparada con la de los blastocistos aneuploides con solo aneuploidías no viables (17,7%=168/951 células) ($P > 0,05$), lo que puede atribuirse al mayor número de cromosomas afectados en el grupo de mosaicos **(Figura 21)**.

Posteriormente, se cuantificaron las células del TE como células GATA3+ y las células de la MCI como células GATA3-/NANOG+. Los blastocistos analizados en D+6 mostraron un número significativamente mayor de células GATA3+ ($51,3 \pm 18,7$ células TE) en comparación con aquellos analizados en D+5 ($40,5 \pm 14,5$ células TE) ($P < 0,05$). En cambio, el número de células GATA3-/NANOG+ no mostró cambios significativos entre

ambos estadios ($11,6 \pm 4,6$ células MCI en D+5; $10,5 \pm 5$ células MCI en D+6) ($P > 0,05$).

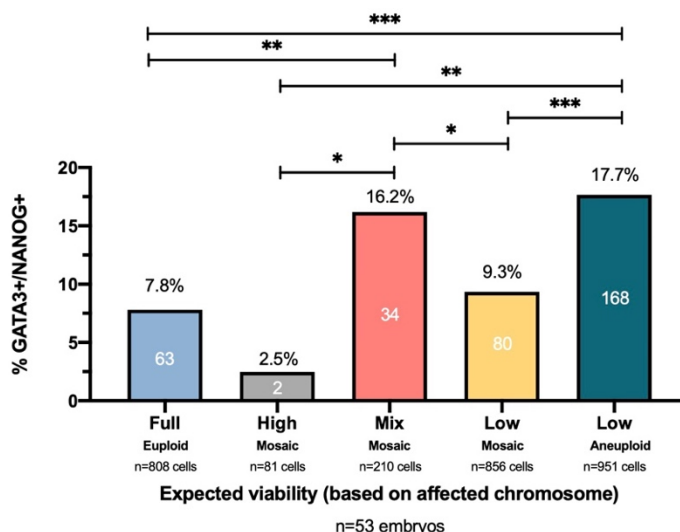


Figura 21 Persistencia de la expresión de NANOG en las células del trofoectodermo estratificada por la viabilidad esperada de los blastocistos en base al cromosoma implicado en la aneuploidía. Cada punto representa un embrión analizado. La viabilidad esperada se clasificó como completa (blastocistos euploides), alta (blastocistos con solo aneuploidías viables, incluyendo +13, +18, +21, +X, +Y y -X), mixta (blastocistos con una mezcla de aneuploidías viables y no viables), o baja (blastocistos con solo aneuploidías no viables); y las monosomías autosómicas en mosaico se consideraron equivalentes a las trisomías autosómicas en mosaico. Las comparaciones de proporciones entre las categorías se realizaron mediante la prueba de Chi cuadrado, con un nivel de confianza del 95%. * $P \leq 0,05$; ** $P \leq 0,01$; *** $P \leq 0,001$.

Al considerar la constitución cromosómica, no se observaron diferencias significativas en el número de células GATA3+ ni GATA3-/NANOG+ entre blastocistos euploides ($45,6 \pm 17,6$ células TE y $13,5 \pm 4$ células MCI en D+5; $69,5 \pm 2,1$ células TE y $9 \pm 2,8$ células MCI en D+6), blastocistos mosaicos ($38,8 \pm 13,7$ células TE y $10,9 \pm 4,2$ células MCI en D+5; $49,2 \pm 16,2$ células TE y $9,7 \pm 7,1$ células MCI en D+6) y blastocistos aneuploides ($38 \pm 11,8$ células

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TE y $10,5 \pm 5,1$ células MCI en D+5; $48 \pm 21,6$ células TE; $11,6 \pm 3,2$ células MCI en D+6) ($P > 0,05$).

A continuación, para identificar las células del TE en las fases iniciales de la apoptosis, se analizó la expresión de la proteína caspasa-3 en dichas células. En general, la apoptosis resultó ser un fenómeno frecuente en las células del TE. De hecho, de las 2.310 células de TE analizadas, 901 (39%) fueron caspasa-3+. Notablemente, 192 (21,3%) de estas 901 células de TE apoptóticas fueron GATA3+/NANOG+, mientras que las 709 células restantes (78,7%) fueron GATA3+/NANOG-.

La incidencia de apoptosis en las células del TE se asoció significativamente con el diagnóstico de PGT-A. En D+5, se observó una proporción significativamente mayor de células de TE caspasa-3+ en blastocistos mosaicos ($44,1\% \pm 19,6$) y blastocistos aneuploides ($45,9\% \pm 16,1$) en comparación con blastocistos euploides ($26,3\% \pm 17$) ($P < 0,05$) (**Figura 22A**). En D+6, se observó una tendencia creciente similar al comparar la proporción de células de TE caspasa-3+ entre blastocistos euploides ($17,5\% \pm 14,8$), blastocistos mosaicos ($43\% \pm 16,8$) y blastocistos aneuploides ($49\% \pm 15,1$). Sin embargo, estas diferencias no alcanzaron significación estadística ($P > 0,05$) (**Figura 22B**).

Notablemente, la incidencia de apoptosis en las células del TE también se correlacionó con el tipo de cromosoma implicado en la aneuploidía. Aunque la proporción de células de TE caspasa-3+ solo mostró diferencias significativas entre blastocistos euploides ($24,9\% \pm 16,4$) y blastocistos aneuploides con únicamente aneuploidías no viables ($47,1\% \pm 15,3$) ($P < 0,05$), se observó una tendencia creciente en todas las categorías intermedias. Es de resaltar que esta tendencia casi alcanzó significación estadística al comparar blastocistos euploides con blastocistos mosaicos con únicamente aneuploidías no viables ($43,9\% \pm 18,2$) ($P = 0,06$) (**Figura 23**).

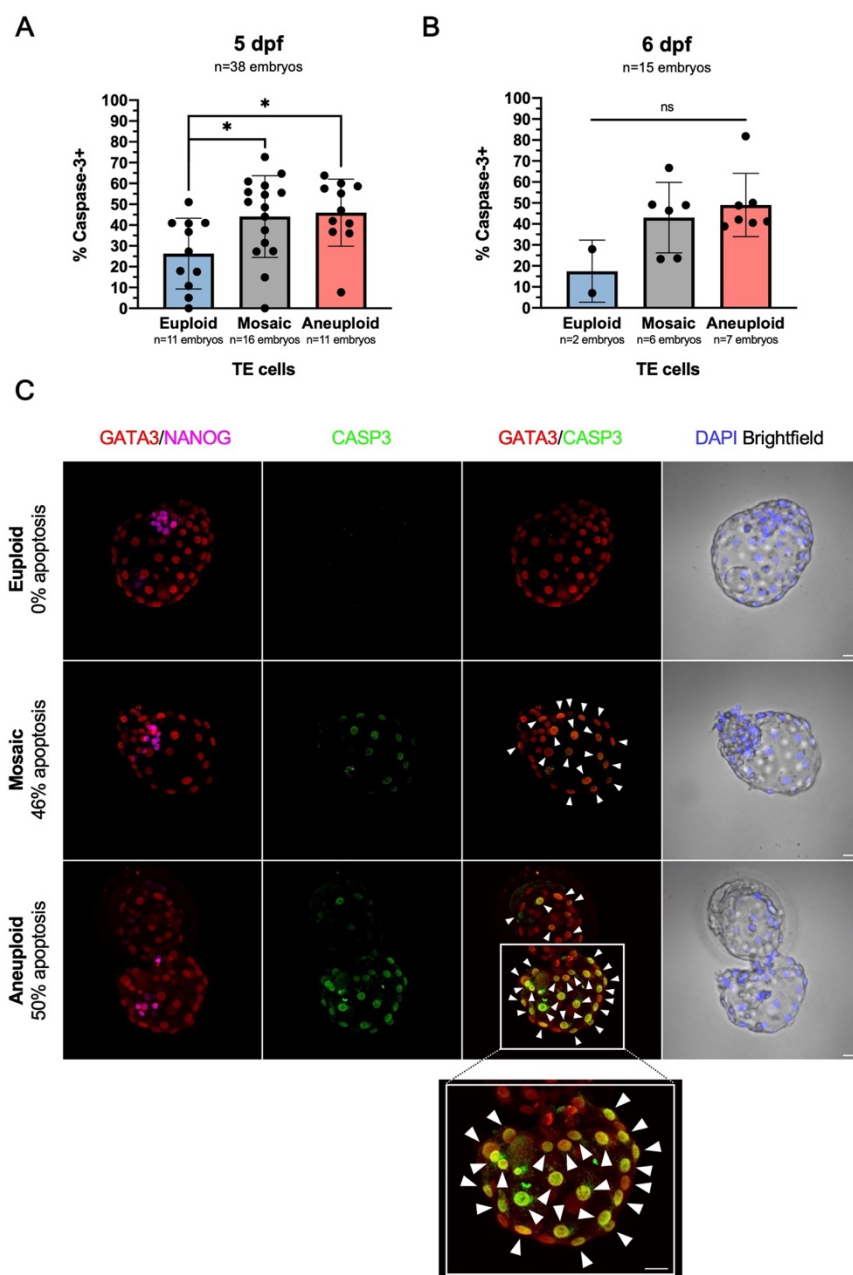


Figura 22 Expresión de caspasa-3 en las células del trofoectodermo (TE). **(A)** Día 5 post-fecundación (5 dpf). **(B)** Día 6 post-fecundación (6 dpf). Las barras azules, grises y rojas muestran embriones euploides, mosaicos y aneuploides respectivamente. Los

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valores bajo cada barra (n) indican el número de embriones analizados. Cada punto representa un embrión analizado. Las tasas de apoptosis se calcularon para cada embrión dividiendo el número de células caspasa-3+ por el número total de células de TE. Las barras de error representan la desviación estándar. Las tasas de apoptosis se compararon utilizando la prueba de Kruskal-Wallis o ANOVA seguida del análisis post hoc de Bonferroni, con un nivel de confianza del 95%. ns: $P>0,05$; $*P\leq 0,05$. **(C)** Imágenes representativas de embriones marcados con NANOG, GATA3 y caspasa-3. Las cabezas de flecha indican células del TE apoptóticas. Se incluye una sección ampliada para una mejor apreciación de las células del TE apoptóticas. Barras de escala, 20 μm .

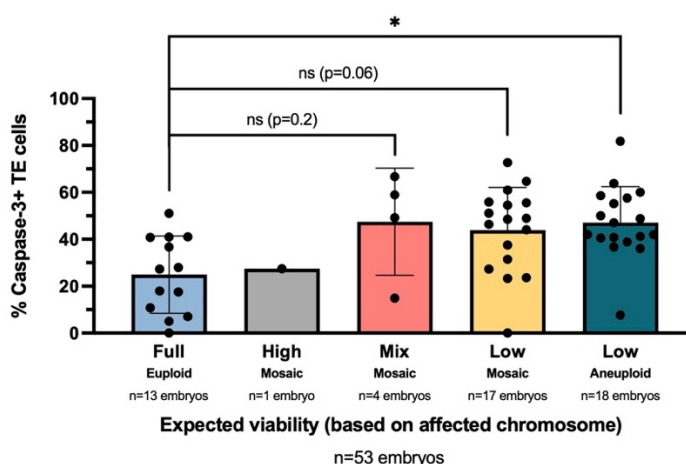


Figura 23 Expresión de caspasa-3 en las células del trofoectodermo estratificada por la viabilidad esperada de los blastocistos en base al cromosoma implicado en la aneuploidía. Cada punto representa un embrión analizado. La viabilidad esperada se clasificó como completa (blastocistos euploides), alta (blastocistos con solo aneuploidías viables, incluyendo +13, +18, +21, +X, +Y y -X), mixta (blastocistos con una mezcla de aneuploidías viables y no viables), o baja (blastocistos con solo aneuploidías no viables); y las monosomías autosómicas en mosaico se consideraron equivalentes a las trisomías autosómicas en mosaico. Las tasas de apoptosis se compararon utilizando la prueba de Kruskal-Wallis seguida del análisis post hoc de Bonferroni, con un nivel de confianza del 95%. ns: $P>0,05$; $*P\leq 0,05$.

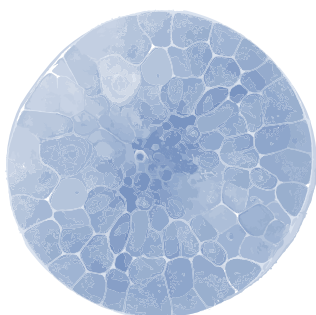
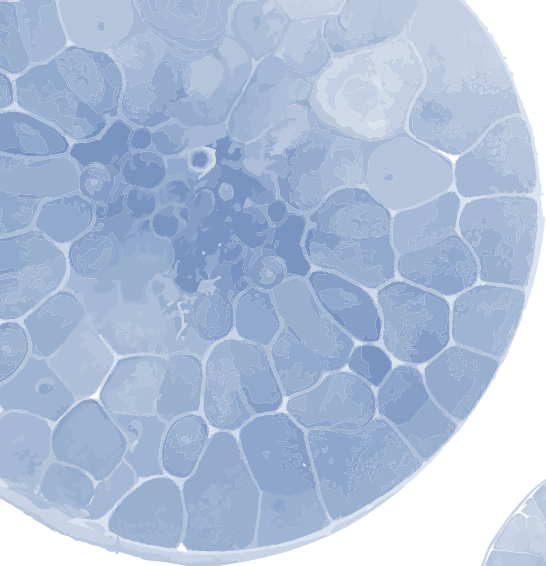
Posteriormente, aplicamos un análisis similar para identificar células de la MCI experimentando eventos apoptóticos tempranos. A diferencia de lo observado en las células del TE, la apoptosis fue un evento muy poco frecuente en las células de la MCI. De hecho, solo 11 (1,9%) de las 596

células de la MCI analizadas fueron caspasa-3+ ($P<0,0001$). Aunque la incidencia de apoptosis en las células de la MCI no se asoció significativamente con el diagnóstico de PGT-A ($P>0,05$), los blastocistos euploides fueron el único grupo sin células de la MCI caspasa-3+ (0/149 células en D+5; 0/18 células en D+6). Por el contrario, se identificaron 6 células de la MCI caspasa-3+ en blastocistos mosaicos ($1,7\%=3/174$ células en D+5; $5,2\%=3/58$ células en D+6), y 5 células de la MCI caspasa-3+ en blastocistos aneuploides ($0,9\%=1/116$ células en D+5; $4,9\%=4/81$ células en D+6).

Fase II: tinción con TUNEL

Debido al número limitado de blastocistos incluidos en esta fase del estudio, especialmente tras subclasificarlos por estatus cromosómico y día de desarrollo, el análisis de los marcadores DAPI, NANOG y GATA3 se realizó únicamente para confirmar la ocurrencia diferencial de la apoptosis entre las células del TE y de la MCI. Con este propósito, realizamos el ensayo TUNEL para evaluar el nivel de fragmentación del ADN en una cohorte adicional de 11 blastocistos.

De manera consistente, de las 98 células de la MCI analizadas, ninguna (0%) resultó ser TUNEL+, mientras que 124 de las 383 células del TE analizadas (32,4%) fueron TUNEL+ ($P<0,0001$). A pesar del pequeño tamaño de la muestra, se observó una tendencia no significativa hacia mayores niveles de fragmentación del ADN en el TE de blastocistos mosaicos ($37,2\%\pm 21,9$) y blastocistos aneuploides ($39\%\pm 41,7$), en comparación con blastocistos euploides ($23\%\pm 32,5$) ($P>0,05$).



CONCLUSIONES Y PERSPECTIVAS FUTURAS

5 | CONCLUSIONES Y PERSPECTIVAS FUTURAS

1. Los blastocistos diagnosticados como mosaicos de células euploides y aneuploides constituyen una población minoritaria de embriones en el programa de PGT-A, en comparación con aquellos que presentan configuraciones uniformemente euploides o aneuploides.

2. Los blastocistos diagnosticados como mosaicos de bajo y alto grado en PGT-A mediante NGS presentan características morfológicas y cromosómicas específicas, así como perfiles morfocinéticos solapantes con respecto a los blastocistos euploides y aneuploides. No obstante, cuando la evaluación de los tiempos morfocinéticos conlleva el seguimiento individualizado de las divisiones blastoméricas, el mosaicismo se asocia con retrasos morfocinéticos en la duración de los ciclos celulares.

3. Cuando el diagnóstico de mosaicismo mediante NGS se apoya en el análisis de la expresión génica y en la identificación de retrasos en la duración de los ciclos celulares blastoméricos, el grado de mosaicismo se asocia a una desregulación progresiva del transcriptoma embrionario con respecto a los perfiles euploides. Las principales funciones celulares desreguladas en los blastocistos mosaicos se relacionan con la apoptosis, las divisiones mitóticas, la degradación de proteínas, el metabolismo y la producción de energía mitocondrial.

4. Los blastocistos diagnosticados como mosaicos en PGT-A mediante NGS y genotipado de SNPs, en comparación con los blastocistos euploides, presentan mayores niveles de apoptosis y una capacidad de diferenciación alterada en las células del TE. Además, la magnitud de ambos efectos se correlaciona con el número y la tipología de los cromosomas aneuploides,

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siendo menor en blastocistos con aneuploidías en mosaico, únicas y/o viables que en blastocistos con aneuploidías uniformes, múltiples y/o no viables. Por el contrario, la apoptosis es un fenómeno muy poco frecuente en las células de la MCI.

5. Estos hallazgos podrían ayudar a explicar por qué la mayoría de los blastocistos con aneuploidías uniformes no progresan en el desarrollo, y sugieren que mecanismos similares podrían conducir a la depleción clonal de las células aneuploides en los blastocistos mosaicos. Estos datos contribuyen a desvelar la identidad biológica de los blastocistos mosaicos y ofrecen nuevas perspectivas sobre los factores moleculares que determinan su potencial reproductivo.

La investigación presentada sienta las bases para futuros estudios sobre los mecanismos que subyacen a la eliminación de las células aneuploides en los blastocistos humanos. En este sentido, las perspectivas futuras deben centrarse en varios ejes fundamentales con la intención de expandir nuestro conocimiento sobre las repercusiones clínicas del mosaicismo embrionario.

Por un lado, es necesario llevar a cabo estudios para determinar si la menor frecuencia de apoptosis observada en las células de la MCI, en comparación con las células del TE, se debe a un desacoplamiento molecular entre la apoptosis y las señales de estrés celular inducidas por las aneuploidías, o si la MCI posee mecanismos protectores compensatorios. Esto incluye la posibilidad de que la apoptosis se active en otras etapas del desarrollo, o que las células aneuploides sean segregadas preferentemente hacia los tejidos extraembrionarios.

Además, se requieren estudios para profundizar en nuestra comprensión de cómo otras respuestas de estrés celular inducidas por las aneuploidías, tales como los defectos en la diferenciación, el daño en el ADN,

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y el estrés mitótico, proteotóxico y metabólico, afectan a la capacidad de desarrollo y la viabilidad de los embriones mosaicos.

Es importante destacar que los estudios centrados en los mecanismos que rigen la supervivencia celular en embriones humanos son limitados debido a restricciones técnicas y éticas. Por ejemplo, no es posible llevar a cabo manipulaciones genéticas en seres humanos para inducir la expresión de proteínas fluorescentes, para luego generar embriones quiméricos y rastrear las células euploides y aneuploides, como en el modelo de ratón (Bolton *et al.*, 2016; Singla *et al.*, 2020).

No obstante, el reciente desarrollo de blastoides humanos, estructuras que emulan morfológica y transcripcionalmente a los blastocistos y que se generan a partir de células madre embrionarias con cariotipos conocidos, proporciona una plataforma única para investigar los mecanismos subyacentes a la eliminación de las células aneuploides y sus implicaciones clínicas en el contexto del mosaicismo embrionario (Kagawa *et al.*, 2022; Liu *et al.*, 2021; Rivron *et al.*, 2018; Yu *et al.*, 2021).

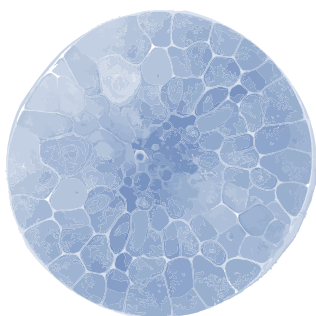
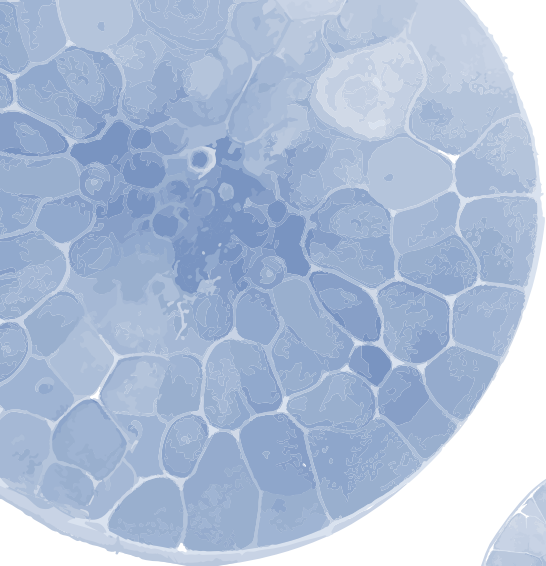
En particular, el uso de marcadores fluorescentes que se mantienen durante el desarrollo en poblaciones celulares específicas sin comprometer su viabilidad, combinado con sistemas de incubación con tecnología *time-lapse*, permitiría el seguimiento de la apoptosis de células aneuploides, la proliferación de células euploides y la segregación de ambos tipos celulares en los linajes embrionarios.

Por otra parte, la capacidad de generar blastoides con distintos niveles de mosaicismo y tipos de aneuploidías, para estudiar su potencial de desarrollo mediante modelos de implantación, permitiría una comprensión más detallada de cómo las aneuploidías específicas afectan a la viabilidad embrionaria (Deglincerti *et al.*, 2016; Rawlings *et al.*, 2021; Shahbazi *et al.*, 2016).

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Estos estudios deben complementarse con avances en las tecnologías de diagnóstico genético, poniendo un énfasis especial en el desarrollo de métodos no invasivos. Esto facilitaría un diagnóstico más preciso del mosaicismo embrionario, reduciendo los sesgos diagnósticos por falsos positivos y ampliando nuestro conocimiento sobre sus verdaderas implicaciones clínicas.

Por lo tanto, las perspectivas futuras en este campo no solo apuntan hacia una mayor comprensión del impacto de las aneuploidías en la biología del desarrollo del embrión humano, sino también hacia la aplicación práctica de estos conocimientos mediante su implementación en los sistemas de selección embrionaria. La integración de enfoques tanto experimentales como clínicos permitirá optimizar las herramientas de diagnóstico de la salud embrionaria, mejorando en última instancia las opciones reproductivas de las personas con problemas de infertilidad.



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