scientific reports

Check for updates

OPEN

Oviductal extracellular matrix hydrogels enhance in vitro culture of rabbit embryos and reduce deficiencies during assisted reproductive technologies

Emilio Francés-Herrero[1,2], Laura Lorenzo-Rebenaque[3], Cristina Casto-Rebollo[3], José Salvador Vicente[3], Patricia Sebastian-Leon[2], Clara Bueno-Fernandez[1,2], Adolfo Rodríguez-Eguren[2], María Gómez-Álvarez[2], Amparo Faus[2], Patricia Diaz-Gimeno[2], Francisco Marco-Jiménez[3] & Irene Cervelló[2]✉

In vitro embryo culture often falls short of mimicking the physiological dynamism occurring in the reproductive tract, prompting developmental plasticity in mammalian embryos with consequential genotypic and phenotypic deviations. Recent research highlights the potential of biological derivatives in in vitro culture to mitigate these effects, being the extracellular matrix (ECM) one of the most important components in retaining structural and biological signals derived from the native source tissue. Current bioengineering techniques could provide ECM-based biomaterials mimicking the native environment and offering optimal embryonic development. Rabbit oviducts (n = 24) were decellularized and solubilized to create tissue-specific ECM (OviECM) hydrogels. Following physicochemical characterization, these hydrogels were applied as coatings for the in vitro culture of two-cell embryos over 48 h, along with embryos cultured under In vitro control conditions (n = 218/group), which were subsequently transferred to recipient females. A subset of embryos was recovered on day 6 for transcriptomic analysis (n = 75–80/group), while the remaining embryos were used to assess implantation and birth rates. Rabbit weights were monitored over 20 weeks post-delivery, with blood tests conducted at weeks 8 and 20. Bayesian inference methods were used for statistical analysis. Differences were considered relevant if $P \geq 0.8$ (80%). No differences in embryo development and morphology were detected between the OviECM coating and In vitro control conditions. However, embryos cultured on these coatings exhibited upregulation of pathways involved in antigen presentation and immune system activation, as well as, increased cellular response to external stimulus and intracellular protein transport. The implantation and live birth rates were significantly higher in the coating group than in the In vitro control group (30.8% vs. 26.1% and 21.2% vs. 18.1%, respectively). During the first 20 weeks of life, the animals from the coating group showed higher weights than the In vitro control group $P0 > 0.8$. The animals of both experimental groups showed normal blood parameters. Implementation of OviECM coatings allows for improving in vitro conditions and decreases postnatal phenotypic deviations after assisted reproductive technology (ART). This study could initiate a new embryo culture techniques era to guarantee that ART is utilized in the most efficient and safest possible practice.

Keywords Decellularization, ECM hydrogel, Oviducts, Embryo culture, Phenotype effect

Abbreviations

ALR	Additive log-ratio

[1]Department of Pediatrics, Obstetrics and Gynecology, School of Medicine, Universitat de València, 46010 Valencia, Valencia, Spain. [2]IVIRMA Global Research Alliance, IVI Foundation, Instituto de Investigación Sanitaria La Fe (IIS La Fe), 46026 Valencia, Valencia, Spain. [3]Institute for Animal Science and Technology (ICTA), Universitat Politècnica de València, 46022 Valencia, Valencia, Spain. ✉email: irene.cervello@ivirma.com

ART	Assisted reproductive technology
BER	Balanced error rate
BSA	Bovine serum albumin
DC	Decellularized
DEG	Differentially expressed gene
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ERCC	External RNA Control Consortium
FDR	False discovery rate
GAG	Glycosaminoglycan
GF	Growth factor
GO	Gene ontology
IVF	In vitro fertilization
KEGG	Kyoto Encyclopedia of Genes and Genomes
LRT	Likelihood ratio test
NC	Naturally conceived
PBS	Phosphate buffered saline
PLS-DA	Partial least squares-discriminant analysis
OviECM	Oviductal extracellular matrix
VIP	Variable importance prediction

Background

Infertility is a reproductive system disorder defined by the World Health Organization as "the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse". Currently, around 15% of couples worldwide are affected by this condition and, for a significant number of them, assisted reproductive technology (ART) provides a helpful solution. Indeed, the number of children born using these methods exceeds 10 million[1].

In this context, the outcome of in vitro embryo culture has improved over the last decades, with many chemical formulations and physical platforms being developed to support it. Today it is known that the biophysical and chemical cues of the microenvironments impart significant spatiotemporal effects on embryonic development[2]. However, current embryo culture techniques still have limitations, as they are unable to fully replicate the dynamic conditions of the reproductive tract in vitro. This suboptimal environment prompts mammalian embryos to display significant developmental reprogramming, resulting in distinct epigenetic and phenotypic variations at pre- and postnatal stages[3].

Recently, it has been demonstrated that by using in vivo-derived culture innovations based on specific complements and others, epigenetic and phenotypic deviations can be ameliorated[4]. In this sense, bioengineering provides advanced platforms for in vitro embryo culture on which biochemical and biophysical microenvironments such as media, mechanical forces, and biomaterials are regulated[5]. Several biomedical investigations have used interesting approaches by culturing embryos or embryonic stem cells on culture dishes coated with different biomaterials[6–9]. However, the biomaterials used are nonspecific hydrogels such as gelatin, laminin, or collagen, and few studies have investigated the effect of the culture conditions beyond the pre-implantation phase.

In this context, extracellular matrix (ECM) hydrogels offer unique advantages due to their fine structures and complex biochemical characteristics[10]. Decellularized (DC) ECM is primarily comprised of structural proteins such as collagen, elastin, and proteoglycans but can also contain nontrivial concentrations of endogenous bioactive agents, such as growth factors. ECM hydrogels have been successfully derived from virtually all tissues and organs, including kidneys[11], liver[12], endometrium[13], and, recently, oviducts[14]. These unique hydrogels have found successful applications in regenerative and other biomedical purposes because their dynamic composition and three-dimensional organization closely resemble that of their native origin[15]. Furthermore, the removal of all native cell material, including its epitopes, through decellularization, allows for the use of different allogeneic and xenogeneic sources.

Based on previous studies[14], we have hypothesized that, by mimicking an oviductal environment in vitro, the embryonic gene expression and the post-natal growth would more closely resemble those of naturally conceived (NC) animals. To create a culture environment to mimic the oviduct, we chose the rabbit as an animal model given its phylogenetic closeness to humans, the size of its oviducts, and its high reproductive performance[16]. The main objective of this study was to evaluate the effect of an ECM hydrogel from DC rabbit oviducts (OviECM) used as a substrate for in vitro culture of embryos, compared to standard culture conditions. We aimed to assess its value in maintaining early embryonic development and, ultimately, the potential epigenetic and phenotypic changes induced in the embryos and the offspring.

Methods

Unless otherwise stated, all chemicals were reagent-grade and purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain). All experiments were performed in accordance with relevant guidelines and regulations.

Animals and ethical statements

The animal management and experimental procedures of this study were conducted in accordance with the ARRIVE guidelines[17]. New Zealand rabbits belonging to the Universitat Politècnica de València were used throughout the study. The animal study protocol was reviewed and approved (code number 2018/VSC/PEA/0116) by the Universitat Politècnica de València ethical committee before the initiation of the study. All experiments were performed following the guidelines and regulations outlined in Directive 2010/63/EU EEC. Animal experiments were conducted at an accredited animal care facility (Code: ES462500001091).

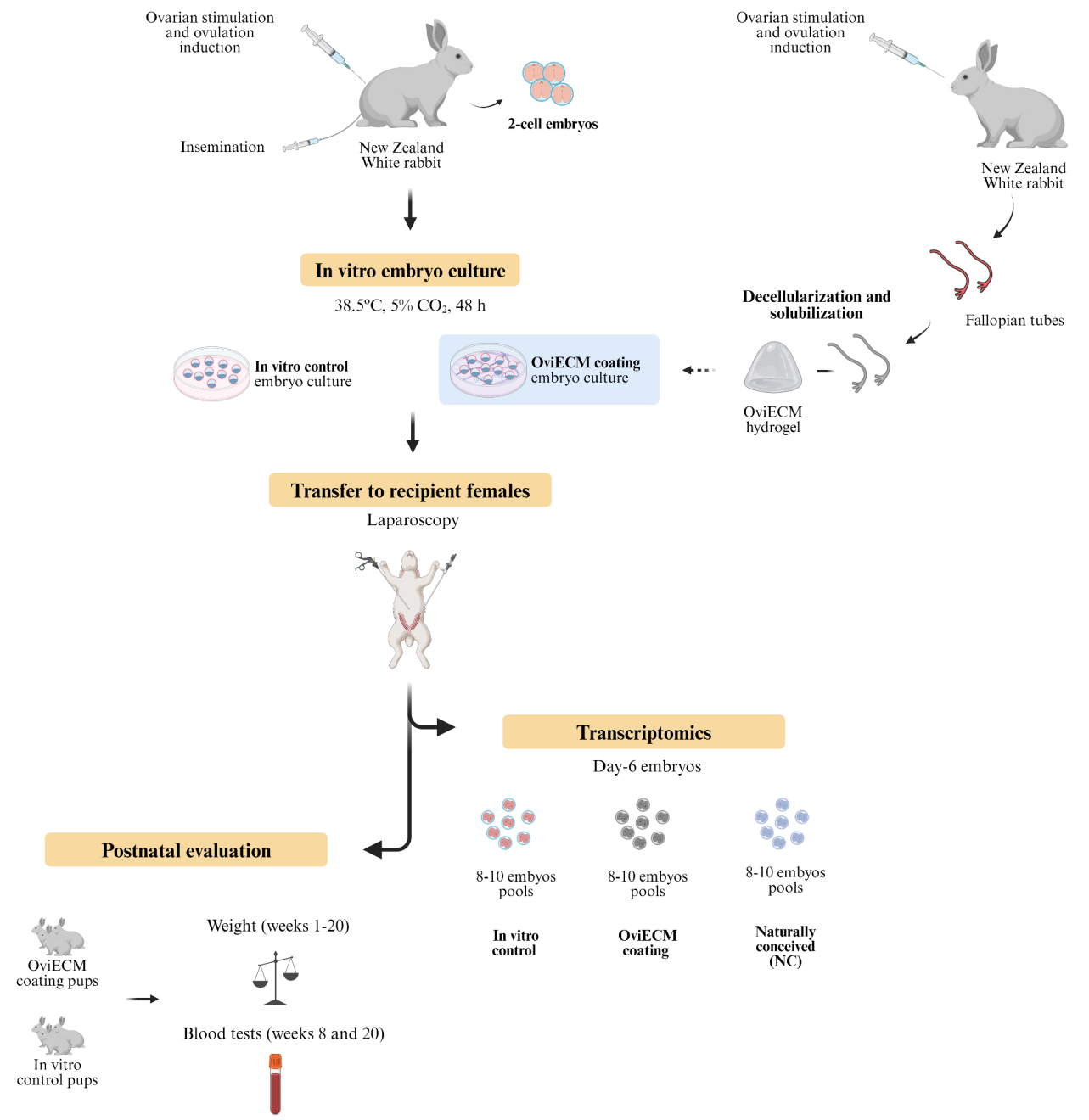

Fig. 1. Experimental design. This figure presents a comprehensive flowchart detailing the processes performed in the study. The methodology utilizes New Zealand White rabbits as the model organism, encompassing ovarian stimulation, ovulation induction, insemination, and two-cell embryo obtention. The embryos are cultured in vitro at 38.5 °C with 5% CO_2 for 48 h, using both In vitro control and OviECM coating culture conditions. Post-culture, embryos are transferred to foster mothers and, while some of them undergo transcriptomics analysis 72 h later, the rest are left to obtain offspring. Postnatal evaluation includes weight monitoring from weeks 1 to 20 and blood tests at weeks 8 and 20 to assess the health and development of the pups.

Experimental design

Figure 1 illustrates the experimental design conceived to elucidate the influence of the OviECM during in vitro rabbit embryo development. Donor females were stimulated and inseminated to produce 2-cell embryos. Embryos were pooled and cultured in vitro for 48 h (i) in standard (In vitro control) culture conditions or (ii) on OviECM coatings. Morulae were transferred into foster mothers via laparoscopy ($n=12–14$ embryos/horn), and a subset of them was recovered 72 h later for transcriptomic analysis. NC day-6 embryos were recovered to serve as a reference group (without any ART manipulation) in gene expression comparisons. Implantation and birth rates, as well as offspring's phenotype (weight and blood parameters) derived from the rest of in vitro cultured embryos were compared between In vitro control and OviECM coating conditions.

Oviduct decellularization

Ovulation was induced in six rabbits using hormonal treatment consisting of 1 µg of Buserelin acetate (Hoechst Marion Roussel S.A., Madrid, Spain). After 72 h, the animals were euthanized by intravenous administration of an overdose of T-61 (0.3 mL/kg, MSD Animal Health, New Jersey, USA) and the oviducts were isolated. Before decellularization, surrounding fat was removed and the oviducts were washed with PBS, cut into 5-mm pieces, and separated into 2 pools ($n=6$ oviducts/pool). Decellularization was performed through a 2-day SDS-based protocol and agitation with a magnetic stirrer at 300 RPM (Francés-Herrero et al., 2021). Briefly, oviduct pools were subjected to chemical detergents (SDS, Triton X-100), an enzymatic solution [DNAse 1 (D4513-1VL), diluted in 1.3 mM $MgSO_4$ and 2 mM $CaCl_2$] and PBS washes (Thermo Fisher Scientific).

Preparation of OviECM hydrogels

DC oviduct fragments were frozen in liquid nitrogen, ground in a mortar, and stored at −80 °C. The resulting powder was lyophilized (LyoQuest −85, Telstar) for 48 h at 20 Pa and −80 °C and finally stored at −20 °C. Acellular tissue hydrogels were created using a modified protocol[39] that conserves the ECM. Lyophilized powder was suspended at 1% (w/v) in 0.01 M HCl with 0.1% (w/v) pepsin and digested for 48 h at room temperature with constant agitation using a sterilized magnetic stirrer. The solution was iced, and digestion was stopped by neutralization to physiological pH with 10% (v/v) 0.1 M NaOH and 10% (v/v) 10× PBS. The resulting pre-gel solution was aliquoted and stored at −80 °C; this solution forms a stable hydrogel at 37 °C.

Collection of 2-cell embryos and in vitro culture

Ten nulliparous New Zealand white females were superstimulated with a combination of FSH (Corifollitropin alfa, 3 µg, Elonva, Merck Sharp & Dohme S.A.) and hCG (7.5 UI)[18]. Seventy-two hours after superstimulation, females were inseminated with pooled semen from New Zealand bucks of proven fertility. Ovulation was induced with 1 µg buserelin acetate (Suprefact; Hoechst Marion Roussel, S.A., Madrid, Spain). Females were euthanized 24 h after artificial insemination, and the reproductive tract was immediately removed. Embryos were recovered by flushing each uterine horn with 10 mL Dulbecco´s phosphate-buffered saline containing 0.2% (wt/vol) bovine serum albumin (BSA). Two-cells embryos were counted, evaluated according to IETS standards (symmetrical and spherical mass, comprising 2 blastomeres that were uniform in size, color, and density) and pooled to randomize the donor effect. Pools of 24–25 embryos were cultured in 4-well Nunc plates (Thermo Fisher Scientific) under a humidified atmosphere of 5% CO_2 at 38.5 °C over 48 h. Two different conditions were tested: (i) SAGE 1-Step™ HSA medium (CooperSurgical) as In vitro control group ($n=218$), and (ii) OviECM hydrogel coating with SAGE 1-Step™ medium, as OviECM coating group ($n=218$). To coat the wells, 250 µl of OviECM pre-gel solution was added and left overnight at 4 °C to allow nonspecific adherence of proteins. The coating solution was then aspirated, and the wells were rinsed once with PBS before adding the culture medium.

Embryo transfer procedure

After 48 h of in vitro culture, compact morulae that were uniform in size, color, and density were laparoscopically transferred into the oviduct of synchronous foster mothers, following the protocol described by Besenfelder and Brem[19]. Only receptive females (determined by the vulva color) were induced to ovulate by injection of 1 µg of Buserelin acetate (Hoechst Marion Roussel S.A., Madrid, Spain) 72 h before transfer[20]. Briefly, twenty-four foster mothers were anesthetized with xylazine (5 mg/kg; Rompun; Bayern AG, Leverkusen, Germany) intramuscularly and ketamine hydrochloride (35 mg/kg; Imalgene 1000; Merial S.A, Lyon, France) intravenously, and placed in Trendelenburg's position. Then, embryos were loaded in a 16G epidural catheter, which was inserted through a 17G epidural needle into the inguinal region. Finally, the process was monitored by single-port laparoscopy. The catheter was introduced in the oviduct through the infundibulum to release the embryos ($n=12–14$ embryos/side). The transfer procedure was described in detail in our recent report[21]. Embryo transfer was carried out in 2 batches.

Day-6 embryo differential gene expression analysis

Three days after transfer, 6 foster mothers by group were euthanized and embryos were recovered by flushing each uterine horn with 10 mL Dulbecco´s phosphate-buffered saline containing 0.2% (wt/vol) BSA. In addition, a control group ($n=4$) defined as naturally conceived (NC) was generated, in which the females were superstimulated and inseminated as previously described. Recovered embryos ($n=72$, 80, and 128 for the OviECM coating, in vitro control, and NC groups, respectively) were pooled and randomly divided into groups, generating a total of 35 pooled samples ($n=9$, 10, and 16 for the OviECM coating, In vitro control, and NC groups, respectively). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. mRNA libraries were prepared from total RNA using the TruSeq stranded mRNA Library Preparation kit (20020594, Illumina). The libraries generated were quantified by fluorimetry and verified for size using the Agilent 2100 Bioanalyzer. An equimolecular pool of all samples was prepared

and sequenced on the NextSeq 550 NGS platform (Illumina) by 2×75 bp paired-end sequencing (High Output Reagent Cartridge 150 cycles, 400 M reads).

Embryo survival rate

In the remaining 6 foster mothers by group, a laparoscopy was performed to record the number of implanted embryos 10 days after embryo transfer. Briefly, the females were anesthetized following the procedure described in the embryo transfer procedure section. The implantation rate was calculated as the successful implantation of the total number of embryos transferred per recipient. In addition, at birth, the total number of pups and the individual weight of each pup were recorded. Fetal losses were calculated as the difference between the total number of pups born and the number of implanted embryos. The litter birth rate was defined as the number of kits at birth concerning the number of embryos transferred.

Growth performance during postnatal development

A total of 56 animals (31 from OviECM coating and 25 from In vitro control group) were weighed weekly from 1 to 20 weeks of age.

Evaluation of hematological parameters

A total of 16 individual blood samples (8 from OviECM coating and 8 from In vitro control group) were recovered from the central ear artery. Animals were selected randomly, keeping 1–2 animals of each litter (parity) within each experimental group. Blood samples were dispensed into an EDTA-coated tube (Deltalab S.L., Barcelona, Spain). Blood count was performed from EDTA tubes at most 10 min after the collection using an automated veterinary hematology analyzer MS 4e automated cell counter (MeletSchloesing Laboratories, Osny, France) according to the manufacturer's instructions. The blood parameters recorded were as follows: white blood cells (WBC), lymphocytes (LIN%), monocytes (MON%), granulocytes (GRA%), red blood cells (RBC), and hematocrit (HCT). All samples were processed in duplicate.

Bioinformatics and statistical data analysis

Bioinformatic analyses to obtain the transcriptomic profile of each sample were performed according to Overbey et al., 2021[22]. Transcriptomic raw data quality was evaluated using FastQC v0.11.9 program[23] and a summary statistic report was generated using MultiQC vXX tool[24]. Low-quality bases and sequencing adapters were removed using TrimGalore software (Krueger 2019)[25]. After trimming, a further quality check was performed to ensure a good quality of the remaining reads. A splice-aware aligner, STAR v2.7.3a[26], was employed for processing the trimmed reads. First, an indexed genome file was generated for the *Oryctolagus Cuniculus* reference genome (OryCun2.0) using its FASTA and GTF files. The trimmed reads were then mapped to the indexed reference genome and transcriptome in STAR's "two-pass mode" to improve the detection and quantification of the reads (Dobin et al., 2013)[27]. Quantification of reads was performed using RSEM[28] in two phases: (i) creation of an index for the reference genome, and (ii) calculation of gene and isoform expression. A total of 29,587 genes were identified.

Normalized gene counts for transcript per million were used in the analyses for the detection of differentially expressed genes (DEGs). Genes having zero counts in all measured samples were removed and low counts genes (10 counts) were filtered. After that, 17,070 genes from the 29,587 remained in the dataset. In this study, two different analyses were used for the detection of differentially expressed genes: the DESEq2 analysis[29] and the Partial Least Square Discriminant analysis (PLS-DA). The former was based on the DESEq2 R-Bioconductor package (version 3.18)[30], with ERCC (External RNA Control Consortium) normalization. Then, LRT (likelihood ratio test), included in the DEseq2 R-package, was applied to the normalized data to generate the F statistic p-value. Adjusted p-values were obtained by computing the False Discovery Rate (FDR) following the Benjamini and Hochberg method[31]. Relevant DEGs were those with an FDR less than or equal to 0.05, and log2 fold-change values greater than 1 or less than −1.

Partial least square discriminant analysis (PLS-DA) was performed to identify the expressed genes more important for the prediction and classification among the groups. Variables (expressed genes) with more than 20% of zeros among all the groups were considered outliers and removed from the dataset. In total 14,641 genes were considered in this analysis. Additive log-ratio (ALR) was used to transform the data and consider that is a subcomposition of the real data. Thus, a reference variable was selected to use as a denominator in all ALR according to[32]. After ALR transformation, data was normalized to mean zero and standard deviation of one. PLS-DA of pairwise comparations were performed to identify DEGs between the In vitro control, OviECM coating, and NC groups. For that, the "mixOmics" R package was used[33]. The number of components for achieving optimal classification performance was determined based on the model's lowest balanced error rate (BER). This rate was calculated through 4-fold cross-validation after 100 iterations. The variables with a variable importance prediction (VIP) higher than 1 were defined as important variables for the classification and prediction of the groups. The predictive capability of each model in every pairwise comparison was assessed using the final model—specifically, the one featuring the selected variables (VIP > 1) and the optimal number of components. For that, a confusion matrix with the percentage of true and false positives was calculated after 1000 iterations. The predictive capability was additionally determined by permuting the groups, confirming the relevance of the selected genes to discriminate each group.

All genes identified by DESEq2 and PLS-DA were considered candidate genes with differences in their expression among the pairwise comparison: NC – In vitro control, NC – OviECM coating, In vitro control – OviECM coating. Specifically, candidate genes were those overlapping between methodologies, with a VIP and a log2 fold-change higher than 1, and a FDR lower than 0.05. The lists of candidate genes for the different comparisons were analyzed using ShinyGO 0.77 (http://bioinformatics.sdstate.edu/go/) for network

development and gene ontologies (GO) enrichment analysis KEGG. The GO were extracted with biomaRt available from Bioconductor to R[34], using the OryCun2.0 of Ensembl[35] such as reference database.

Statistical analyses

The relevance of the differences between groups for proportional data (morula in vitro development, implantation rate, offspring rate at birth, and fetal losses) or continuous data (growth performance and hematological parameters) was estimated using Bayesian inference. Binomial data for morula in vitro development, implantation rate, offspring rate at birth, and fetal losses were assigned a value of 1 if positive development was achieved, or 0 if it was not.

Bayesian inference is based on probabilities, providing great flexibility to construct all kinds of confidence intervals with a chosen probability. In all cases, the progeny origin was included as a treatment with two levels (OviECM coating and In vitro control). Bounded flat priors were used for all unknowns and the marginal posterior distributions were estimated by Gibbs sampling. After some exploratory analyses, results were based on Markov chain Monte Carlo chains consisting of 60,000 iterations, with a burn-in period of 10,000, and a log of 10. Summary statistics from the marginal posterior distributions were calculated directly from the samples saved. Convergence was tested using the Geweke Z criterion and Monte Carlo sampling errors were computed using time-series procedures. In all cases, the Monte Carlo Standard Error was small, and lack of convergence was not detected by the Geweke test. The statistics obtained from the marginal posterior distributions of the phenotypic differences between experimental groups were the mean of the difference ($D_{C-OviECM}$; computed as In vitro control-OviECM coating), the probability of the difference being greater than 0 when $D_{C-OviECM} > 0$ or lower than 0 when $D_{C-OviECM} < 0$ (P_0), and the highest posterior density interval at 95% of probability (HPD95%). $D_{C-OviECM}$ estimated the mean of the differences between C and OviECM traits, P_0 estimated the probability of $D_{C-OviECM} \neq 0$, and HPD95% estimated the accuracy. Statistical differences were considered if $|D_{C-OviECM}|$ surpassed the relevant value (R; proposed as one-third of the SD of the trait) and $P_0 > 0.8$ (80%). Statistics analyses were computed with the Rabbit R package[36] developed by the Institute for Animal Science and Technology (Valencia, Spain). A more detailed description of these features can be found in a previous review[37].

Results

Differential gene expression in pre-term embryos and predicted biomarkers

A genome-wide expression analysis of pre-implantation day 6 embryos under In vitro control conditions, or exposed to OviECM coatings or NC, was conducted to evaluate the effects of the culture conditions at the molecular level. In vitro (control and OviECM) vs. NC embryo development was the most conditioning variable for embryo gene expression profiles, regardless of culture conditions (Fig. 2a). However, the main component analysis was able to differentiate, and separate samples grown under In vitro control condition vs. OviECM coating (Fig. 2a). Specifically, 78 differentially expressed genes (DEGs) were found between both in vitro culture conditions via DESEq2 analysis. In both cases, more than 2000 DEGs were found when compared against NC embryos (Supplementary Fig. 1).

Preliminary PLS-DA analyses revealed that the number of genes relevant for classification and prediction of the "NC" vs. "In vitro control", "NC" vs. "OviECM coating" and "In vitro control" vs. "OviECM coating" classes were 5501, 5321, and 4296 genes (Fig. 2b), respectively, with total classification errors of 0, 0 and 0.2852. Subsequent PLS-DA from candidate genes were carried out to identify the most important genes that could be used as biomarkers for prediction between the two classes. For the classification and prediction of the "NC" and "In vitro control" classes, a model was obtained with 2 genes, the ENSOCUG00000004865 gene (ARMCX3) and the ENSOCUG00000006768 gene (FAM78A), with a predictive capacity of 100% for both classes. Similarly, for the "NC" and "OviECM coating" classes, the ENSOCUG00000004699 (PRUNE1) and ENSOCUG00000027246 (novel gene) genes constituted a model with a prediction error of 0. Finally, for the 'In vitro control' and 'OviECM coating' groups, a model with 814 was needed to achieve a predictive error of 0.056, indicating that these conditions are the most similar (Fig. 2c).

All genes identified by DESEq2 and PLS-DA were considered candidate genes with differences in their expression among the pairwise comparison. Specifically, 1297, 1541, and 53 candidate RNA (match in both analyses) were identified for the comparisons NC vs. In vitro control, NC vs. OviECM coating, and In vitro control vs. OviECM coating, respectively (Fig. 2b).

Analysis of the altered functions among the samples from the different conditions revealed that embryos cultured in both in vitro conditions exhibited downregulation in lipid metabolism pathways compared to NC embryos, while they showed upregulation in pathways involved in cell differentiation, regulation of developmental processes, regulation of response to stimulus, or intracellular signal transduction (Supplementary Data). Embryos cultured in vitro on OviECM coatings showed upregulation of pathways involved in antigen presentation and immune system activation, beneficial for survival and correct embryonic development, compared to those cultured on standard medium (In vitro control) (Fig. 3a). In addition, increased cellular response to external stimulus was observed, indicating adaptation to stimulus and physiological signals. Among the most relevant genes are IL1RN, MFSD2A, TGFBR2, or ZEB2 (Fig. 3b).

Assisted reproductive technology outcomes

Embryos cultured under In vitro control condition and on OviECM coatings were transferred to foster mothers to assess their implantation potential (ten days after transfer) and live birth rate. No differences were observed in the percentage of embryos that reached the morula stage between the In vitro control and OviECM coating groups (96% and 98%, respectively). Images of embryos before and after 48 h of in vitro culture are shown in Supplementary Fig. 2. Nevertheless, the use of OviECM coatings during in vitro embryo culture induced a significant increase (4.7%, $P = 0.8$) in implantation rates in comparison to the standard In vitro control condition.

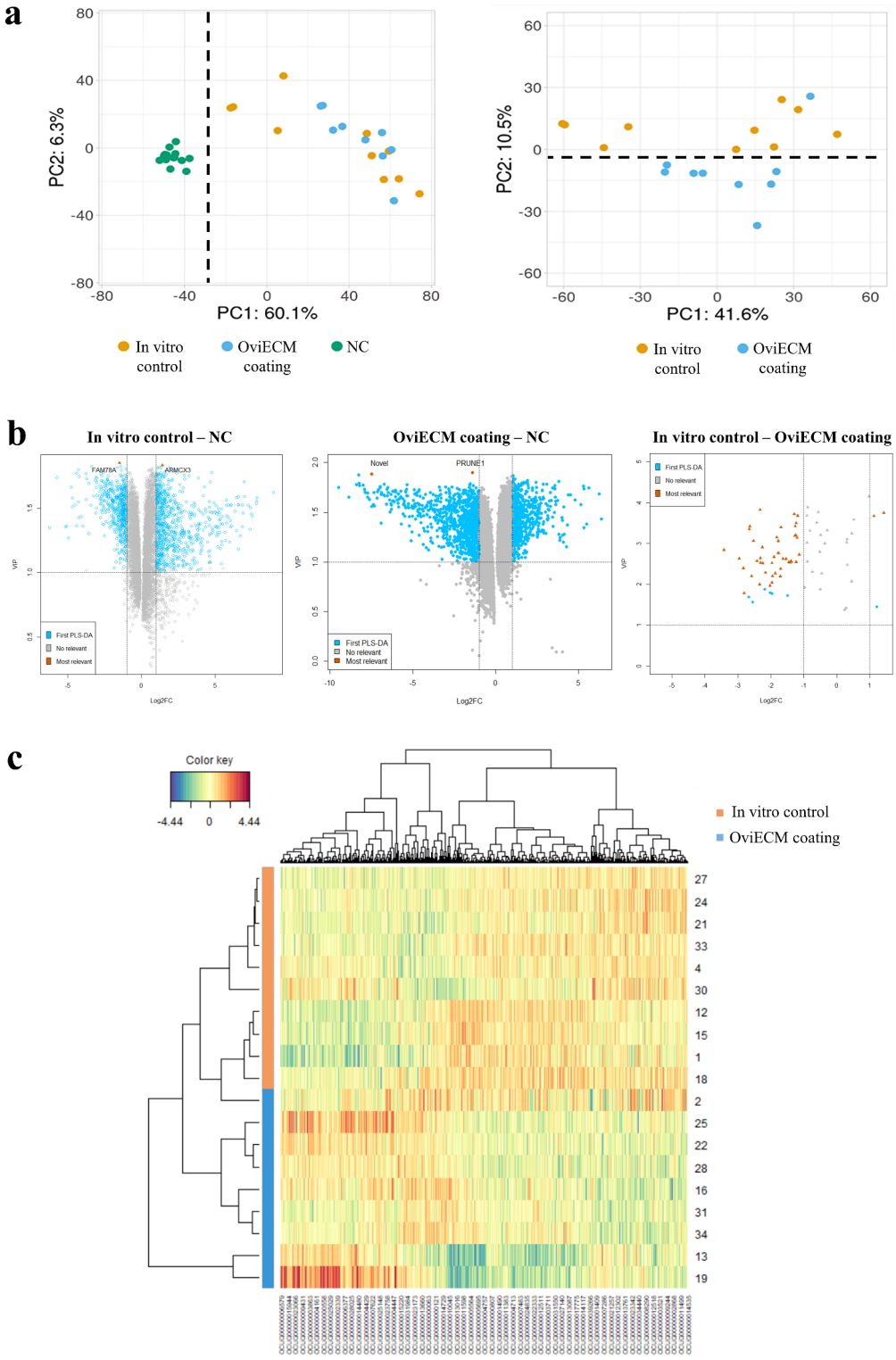

Fig. 2. Comparative analysis of transcriptomic datasets. (**a**) PCA plots showing gene expression variance between embryos developed NC, under In vitro control condition, or exposed to OviECM coatings. (**b**) Volcano plots highlighting DEGs identified between NC vs. In vitro control, NC vs. OviECM coating, and In vitro control vs. OviECM coating conditions, using both PLS-DA and DESEq2 analyses. (**c**) Heatmap elaborated from the 814 genes with the highest predictive capacity for the classification of the In vitro control and OviECM coating classes.

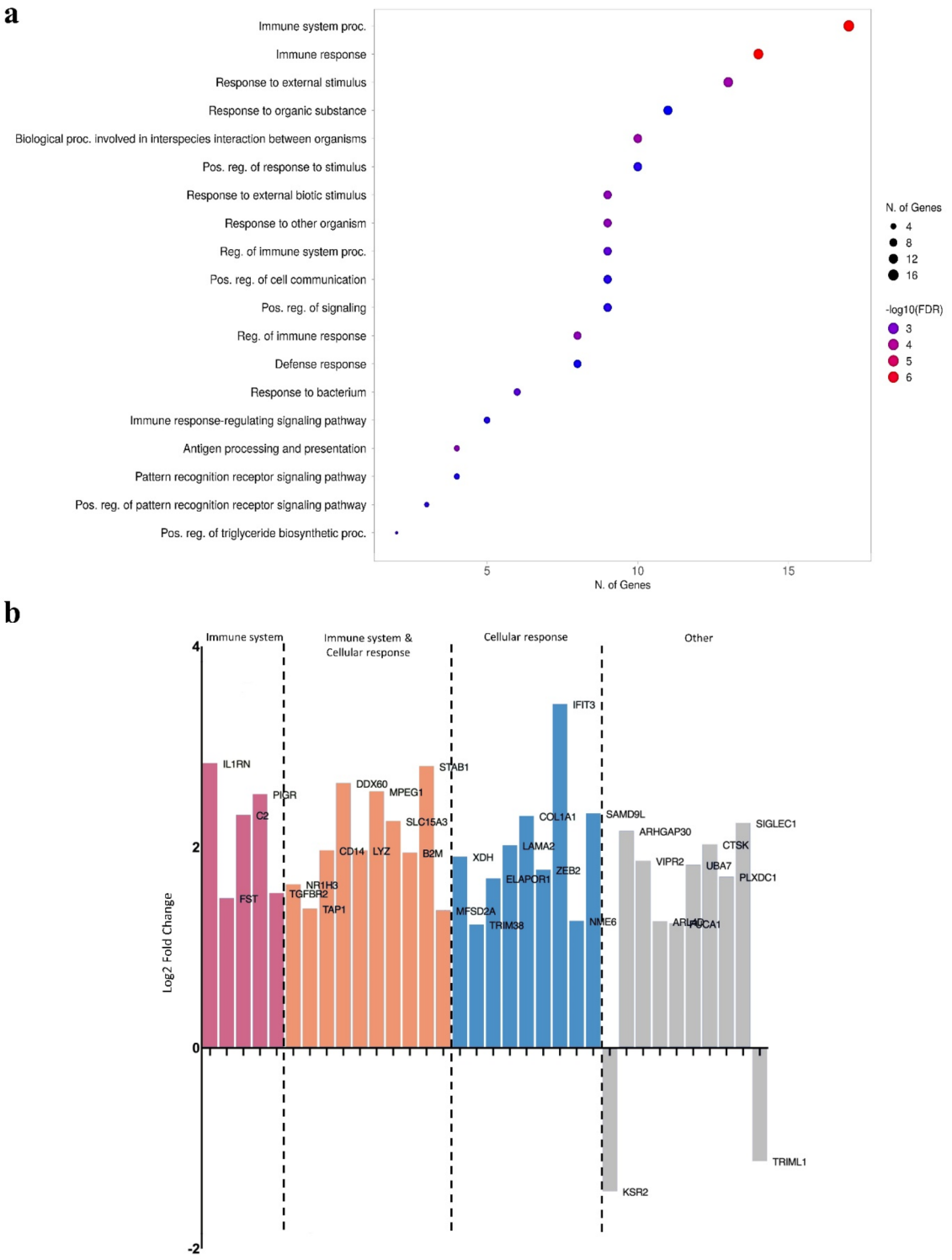

Fig. 3. Functional enrichment of DEGs between Control and OviECM in vitro conditions. (**a**) Scatter plot of GO enrichment analysis, highlighting biological processes impacted by genes overexpressed in OviECM coating compared to In vitro control condition. (**b**) Bar graph showing the most relevant DEGs, predominantly overexpressed in OviECM coating condition, mainly involving immune system and cellular response functions.

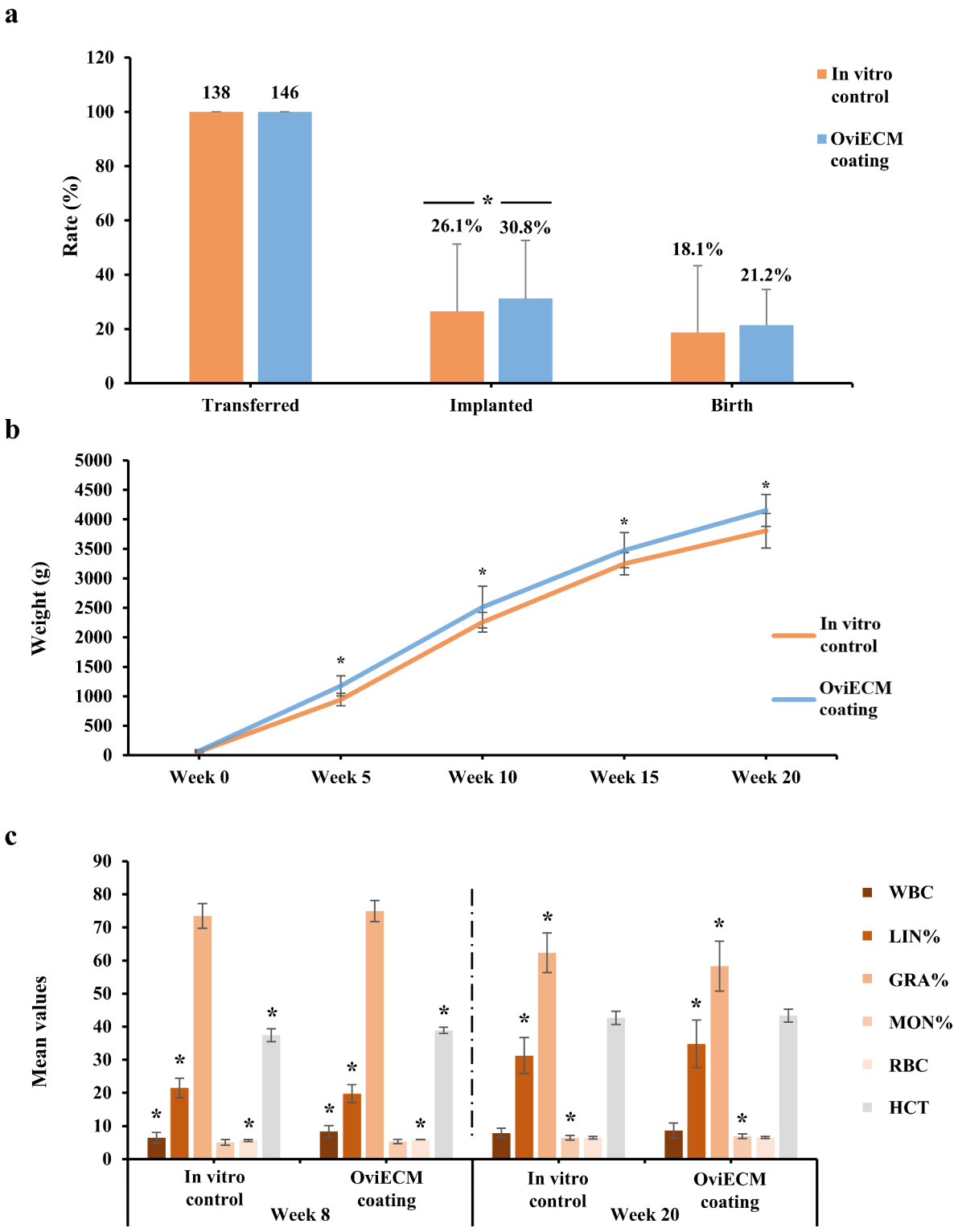

Fig. 4. In vitro embryo culture: ART outcomes and phenotypic parameters. (**a**) Number of transferred embryos in both in vitro conditions (In vitro control and OviECM coating), implantation, and birth rates. (**b**) Weight of offspring until adulthood (20 weeks) for In vitro control and OviECM coating groups. (**c**) Blood parameters of the offspring at 8 and 20 weeks of life for In vitro control and OviECM coating groups. WBC: white blood cells (10^9/L); LIN%: %lymphocytes; GRA%: %granulocytes; MON%: %monocytes; RBC: red blood cells (10^12/L); HCT: haematocrit (%). Data are shown as mean ± SD. *Asterisks denote significant differences between In vitro control and OviECM coating groups ($|D_{C\text{-}OviECM}|$ surpass R-value and its $P_0 > 0.80$).

Litter birth rate was similar in both groups (3.1%, $P=0.73$) (Fig. 4a). Supplementary Table 1 provides statistical details for all the variables analyzed in the study.

Phenotypic evaluation during postnatal development: growth and health parameters

Ultimately, we wanted to test whether there could be any lasting and visible effect on the phenotype and health of the rabbit offspring obtained from the embryos cultured in vitro on OviECM coatings and transferred to recipient females, in comparison to gold standard techniques (In vitro control condition). From birth to weaning age (week 4), no differences in body weight or hematological values were observed. However, from week 5 to week 20 (reproductive age), animals born from embryos exposed to OviECM coating showed significantly higher body weight than those in the In vitro control group ($P0>0.8$) (Fig. 4b). Regarding hematological values, non-remarkable differences were found between groups, with all parameters within the physiological ranges of the species (Fig. 4c).

Discussion

In vitro embryo culture methodology has significantly improved over the last few decades, with numerous chemical formulations and physical platforms being developed[38]. However, in vitro conditions fail to mimic the physiological dynamics and specific microenvironment within the reproductive tract. The female reproductive tract has complex hydrated surfaces coated with macromolecules, hydroxylated compounds, GFs, and different components of the ECM. This stands in stark contrast to the inert culture surfaces currently used in ART, e.g. glass, polystyrene, and polydimethylsiloxane[39]. This could be one of the contributing factors to the developmental gaps exhibited in in vitro cultured mammalian embryos, reducing ART success rates[5,40,41].

Bioengineering approaches based on the utilization and applications of tissue-specific ECM may help to solve this problem. The ECM plays crucial roles in many organ systems, and DC tissues that harness the intrinsic qualities of the ECM have many possible purposes[11,42–46]. We hypothesized that, in terms of improving current ART outcomes, DC tissue could be used to improve embryo culture systems, given that the biophysical and chemical cues of embryonic microenvironments exert significant spatiotemporal effects on development[2,47]. DC ECM primarily consists of structural proteins, such as collagen, elastin laminin, and GAGs, but it can also contain nontrivial concentrations of endogenous GFs, secretions, and matrix-bound nanovesicles[10,14,48,49]. Such ECM components can influence embryo development by regulating the activity of signaling pathways and supplying specific biomolecules and cytokines to the medium[50]. Thus, using tissue-specific ECM hydrogels as in vitro coatings[40] is a promising way to achieve reliable embryo culture[11].

In this study, oviducts were selected as the source tissue as these structures are essential for successful spontaneous conception in humans and facilitate early embryo development, enabling embryos to reach the uterine cavity and implant in a receptive endometrium[51]. Our goal was to create an OviECM hydrogel from DC rabbit oviducts rich in bioactive components capable of mimicking the natural microenvironment in which preimplantation embryos grow. Specifically, we wanted to evaluate whether OviECM hydrogel coatings used in in vitro conditions could influence embryo gene expression, ART outcomes such as implantation and birth rates, and offspring phenotype.

In that sense, the gene expression analysis revealed how the effect of manipulating and culturing embryos in vitro, compared to natural embryo development (in vivo), had a greater impact on the transcriptomic profile of the embryos than that induced by adding an OviECM coating to the standard in vitro culture. By assessing the expression levels of just two genes, we can discern whether an embryo has been cultured in vitro or has developed naturally. These two genes differ depending on whether one is talking about In vitro control conditions or OviECM coatings. In the first case, these genes are ARMCX3, which is overexpressed in the NC group, and FAM78A, which is overexpressed in the In vitro control group. ARMCX3 encodes a member of the ALEX family of proteins, which may play a role in tumor suppression, and other proteins containing the arm repeat are involved in development, maintenance of tissue integrity, and tumorigenesis[52]. In contrast, overexpression of FAM family genes is associated with a worse prognosis in several types of cancer, leading to increased proliferation, invasion, metastasis, and resistance to treatment[53–55]. In the second case, the predictive genes are PRUNE1 and ENSOCUG00000027246, both of which were found to be overexpressed in the NC group. Although PRUNE1 is expressed in adult tissues, it is highly expressed in early embryonic and fetal stages and is suggested to play an important role in the developing human brain[56]. Further, it has a crucial role in microtubule polymerization, cell migration, cell differentiation, and proliferation[57,58].

The differences found between the in vitro and in vivo groups are consistent with results reported in similar studies[59] and highlight the gap that ART can still bridge through improvements in culture platforms. One of the most remarkable observations in our work was a downregulation of lipid metabolism in embryos cultured in vitro compared to those developed in natural conditions. Lipids have the potential to serve as an energy reserve for the initial stages of embryonic growth, before the activation of the embryonic genome. Moreover, they play a crucial role in synthesizing the plasma membrane. Reduced lipid metabolism could lead to a heightened accumulation of lipids in embryos produced through in vitro methods, which has been described as a direct consequence of the stress experienced during the developmental process conducted outside the natural environment[60].

Embryos cultured on OviECM coatings showed an upregulation of genes involved in antigen presentation and immune system activation, compared to those cultured on standard medium. This activation of immune functions, necessary and beneficial for survival and correct early embryonic development[61,62], could be due to the presence of epitopes in the OviECM coating (from the native oviductal tissue). For example, the gene IL1RN is responsible for the transcription of the protein known as Interleukin-1 Receptor Antagonist (IL-1Ra). This protein functions as an inhibitor of Interleukin-1 (IL-1), a cytokine with proinflammatory properties, by competing for the same receptor binding sites. In the specific context of embryonic development, particularly during the preimplantation stages, the modulation of inflammatory responses could be of critical importance.

The process of embryo implantation within the endometrial lining of the uterus involves intricate interactions between the embryo and maternal tissue. These interactions encompass both immune and inflammatory responses, highlighting the potential significance of IL-1Ra in this context[63–65].

The embryos cultured in vitro on OviECM coating also presented an overexpression of pathways related to cellular response compared to embryos cultured in In vitro control conditions. This could be advantageous for maintaining cellular homeostasis by responding to physiological signals[66]. For instance, MFSD2A (Major Facilitator Superfamily Domain Containing 2 A) is known for its role in the transport of long-chain polyunsaturated fatty acids across the blood-brain barrier, which is crucial for proper brain development and function. The TGFBR2 gene encodes the type II receptor for transforming growth factor beta (TGF-β). TGF-β and its receptors participate in various cellular processes, including growth, differentiation, and development, suggesting its potential role in the early stages of embryonic development[67]. Another example is ZEB2 (Zinc Finger E-Box Binding Homeobox 2), which encodes a transcription factor involved in developmental processes, such as cell differentiation and migration. It is known for its role in the epithelial-mesenchymal transition (EMT) process, which is crucial in embryonic development, as well as in cancer progression. In embryos, ZEB2 plays a role in the formation of structures such as the neural tube and neural crest. Given its significance in embryogenesis, it is plausible that the ZEB2 gene is actively expressed at various stages of development, including preimplantational stages[68].

Although no differences in embryo development were observed between the two in vitro conditions, when the OviECM coating was used, higher and statistically significant implantation rates were obtained. In addition to the gene expression changes described in this work, the increase in fertility rates observed when using OviECM coatings could be due to metabolic adaptations experienced by the embryos, previously reported by our group[14]. Although a multitude of bioengineered microenvironments have been proposed for early embryo culture[50], few studies have moved beyond the in vitro stage. In this respect, our study is pioneering and opens the horizon for further research.

It has been shown in several systematic reviews and meta-analyses that singletons born after ART are at increased risk of adverse perinatal outcomes when compared with naturally conceived children concerning preterm birth, lower birthweight, perinatal mortality, and congenital abnormalities[69–74]. ART children have also shown alterations in DNA methylation and transcript level of several genes such as those controlling growth (insulin-like growth factor (IGF)2/H19 and its receptor, IGF2R) in cord blood and placentae[75,76]. In this context, the effect of specific in vitro culture factors (manipulation, culture medium, supplementation) on epigenetic and phenotypic traits of offspring has been reported in numerous studies[3,4,77,78]. Zandstra et al.. reported in 2018 that the use of different commercial in vitro culture media for human embryos is associated with differences in body weight, BMI, truncal adiposity, waist circumference, and waist/hip ratio at the age of 9[3].

In the rabbit species, ART offspring often exhibit lower birth weights than those conceived naturally[79]. In our study, the pups derived from embryos cultured on OviECM coatings presented significantly higher weights than those derived from embryos exposed to In vitro control culture conditions. These findings show how the presence of signaling molecules and oviduct-specific ECM biochemical factors allows improving in vitro conditions and decreases postnatal phenotypic deviations after ART. Concerning the general state of health of the offspring, as measured by haemograms, physiological values were found for all experimental groups and at all times of analysis. This suggests that, although ART is associated with measurable differences in terms of gene expression or phenotype, these do not necessarily imply health disorders in the offspring.

Conclusions

Using tissue-specific ECM hydrogels from DC rabbit oviducts (OviECM) in in vitro embryo culture influences ART outcomes, decreasing postnatal phenotypic deviations. Specifically, a different gene expression profile of embryos and offspring with a higher body weight were observed. This study has been carried out in an animal model and, although the offspring generated is healthy (based on hemograms studies), the scope of this technique is still unknown. So, the next step would be to study embryonic reprogramming under OviECM coating in vitro culture and to continue working towards increasing the complexity of embryo culture platforms, bringing them progressively closer to the reality of the in vivo maternal environment. This study could initiate a new embryo culture techniques era to guarantee that ART is utilized in the most efficient and safest possible practice.

Data availability

Sequence data that support the findings of this study have been deposited in the BioProject database with the primary accession code PRJNA1138426.

Received: 30 July 2024; Accepted: 23 October 2024
Published online: 11 November 2024

References

1. Pinborg, A., Wennerholm, U-B. & Bergh, C. Long-term outcomes for children conceived by assisted reproductive technology. *Fertil. Steril.***120**, 449–456 (2023).
2. Vianello, S. & Lutolf, M. P. Understanding the mechanobiology of early mammalian development through Bioengineered models. *Dev. Cell.***48**, 751–763 (2019).
3. Zandstra, H. et al. Association of culture medium with growth, weight and cardiovascular development of IVF children at the age of 9 years. *Hum. Reprod.***33**, 1645–1656 (2018).
4. Canovas, S., Ross, P. J., Kelsey, G. & Coy, P. DNA methylation in embryo development: epigenetic impact of ART (assisted Reproductive technologies). *BioEssays News Rev. Mol. Cell. Dev. Biol.* ;**39**. (2017).
5. Simopoulou, M. et al. Considerations regarding embryo culture conditions: from media to Epigenetics. *Vivo Athens Greece.* **32**, 451–460 (2018).

6. Fuchs, C. et al. Self-organization phenomena in embryonic stem cell-derived embryoid bodies: axis formation and breaking of symmetry during cardiomyogenesis. *Cells Tissues Organs.***195**, 377–391 (2012).
7. Kolahi, K. S. et al. Effect of substrate stiffness on early mouse embryo development. *PloS One*. **7**, e41717 (2012).
8. Morris, S. A. et al. Dynamics of anterior-posterior axis formation in the developing mouse embryo. *Nat. Commun.***3**, 673 (2012).
9. Warmflash, A., Sorre, B., Etoc, F., Siggia, E. D. & Brivanlou, A. H. A method to recapitulate early embryonic spatial patterning in human embryonic stem cells. *Nat. Methods*. **11**, 847–854 (2014).
10. Cramer, M. C. & Badylak, S. F. Extracellular matrix-based biomaterials and their influence upon cell behavior. *Ann. Biomed. Eng.***48**, 2132–2153 (2020).
11. Su, J., Satchell, S. C., Shah, R. N. & Wertheim, J. A. Kidney decellularized Extracellular Matrix hydrogels: Rheological characterization and human glomerular endothelial cell response to Encapsulation. *J. Biomed. Mater. Res. A*. **106**, 2448–2462 (2018).
12. Willemse, J. et al. Hydrogels derived from decellularized liver tissue support the growth and differentiation of cholangiocyte organoids. *Biomaterials*. **284**, 121473 (2022).
13. Campo, H. et al. Tissue-specific decellularized endometrial substratum mimicking different physiological conditions influences in vitro embryo development in a rabbit model. *Acta Biomater.***89**, 126–138 (2019).
14. Francés-Herrero, E. et al. Development of Decellularized Oviductal Hydrogels as a support for rabbit embryo culture. *Reprod. Sci. Thousand Oaks Calif.***28**, 1644–1658 (2021).
15. Catoira, M. C., Fusaro, L., Di Francesco, D., Ramella, M. & Boccafoschi, F. Overview of natural hydrogels for regenerative medicine applications. *J. Mater. Sci. Mater. Med.***30**, 115 (2019).
16. Fischer, B., Chavatte-Palmer, P., Viebahn, C., Navarrete Santos, A. & Duranthon, V. Rabbit as a reproductive model for human health. *Reprod. Camb. Engl.***144**, 1–10 (2012).
17. Sert, N. P. et al. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *PLOS Biol.***18**, e3000410 (2020).
18. Viudes-de-Castro, M. P. et al. A single injection of corifollitropin alfa supplemented with human chorionic gonadotropin increases follicular recruitment and transferable embryos in the rabbit. *Reprod. Domest. Anim. Zuchthyg.* **54**, 696–701 (2019).
19. Besenfelder, U., Strouhal, C. & Brem, G. A method for endoscopic embryo collection and transfer in the rabbit. *Zentralbl Veterinarmed A*. **45**, 577–579 (1998).
20. Marco-Jiménez, F., Lavara, R., Jiménez-Trigos, E. & Vicente, J. S. In vivo development of vitrified rabbit embryos: effects of vitrification device, recipient genotype, and asynchrony. *Theriogenology*. **79**, 1124–1129 (2013).
21. Garcia-Dominguez, X., Marco-Jimenez, F., Viudes-de-Castro, M. P. & Vicente, J. S. Minimally invasive embryo transfer and embryo vitrification at the optimal embryo stage in rabbit model. *J. Vis. Exp. JoVE* ; (2019).
22. Overbey, E. G. et al. NASA GeneLab RNA-seq consensus pipeline: standardized processing of short-read RNA-seq data. *iScience*. **24**, 102361 (2021).
23. Andrews, S. et al. FastQC: a quality control tool for high throughput sequence data [Internet]. Babraham Institute; (2010). http://www.bioinformatics.babraham.ac.uk/projects/fastqc
24. Ewels, P., Magnusson, M., Lundin, S. & Käller, M. MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*. **32**, 3047–3048 (2016).
25. Krueger, F. Trim Galore: A wrapper tool around Cutadapt and FastQC to consistently apply quality and adapter trimming to FastQ files. Available at: https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/ (2019).
26. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. **29**, 15–21 (2013).
27. Dobin, A.. et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics.***29**, 15–21 (2013).
28. Li, B. & Dewey, C. N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinform.***12**, 323 (2011).
29. Love, M. I., Huber, W. & Anders, S. Moderated estimation of Fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.***15**, 550 (2014).
30. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing. (2021). https://www.R-project.org/
31. Benjamini, Y. & Hochberg, Y. Controlling the false Discovery rate: a practical and powerful Approach to multiple testing. *J. R Stat. Soc. Ser. B Methodol.***57**, 289–300 (1995).
32. Greenacre, M., Martínez-Álvaro, M. & Blasco, A. Compositional Data Analysis of Microbiome and Any-Omics Datasets: A Validation of the Additive Logratio Transformation. Front Microbiol [Internet]. [cited 2024 Feb 6];12. https://www.frontiersin.org/journals/microbiology/articles/ (2021). https://doi.org/10.3389/fmicb.2021.727398
33. Rohart, F., Gautier, B., Singh, A., Lê Cao, K-A. & mixOmics An R package for 'omics feature selection and multiple data integration. *PLoS Comput. Biol.***13**, e1005752 (2017).
34. Durinck, S., Spellman, P. T., Birney, E. & Huber, W. Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. *Nat. Protoc.***4**, 1184–1191 (2009).
35. Zerbino, D. R. et al. Ensembl 2018. *Nucleic Acids Res.***46**, D754–D761 (2018).
36. Martínez-Álvaro, M., Ibáñez-Escriche, N. & Casto-Rebollo, C. *Innovación en Al Aprendizaje estadístico: Inferencia Bayesiana Amigable en El lenguaje de programación R* (Universidad de Zaragoza, Servicio de Publicaciones, 2023).
37. Blasco, A. The use of bayesian statistics in meat quality analyses: a review. *Meat Sci.***69**, 115–122 (2005).
38. Swain, J. E. & Smith, G. D. Advances in embryo culture platforms: novel approaches to improve preimplantation embryo development through modifications of the microenvironment. *Hum. Reprod. Update*. **17**, 541–557 (2011).
39. Carrascosa, J. P., Horcajadas, J. A. & Moreno-Moya, J. M. Chapter 15 - The Molecular Signature of the Endometrial Receptivity: Research and Clinical Application. In: Horcajadas JA, Gosálvez J, editors. Reproductomics [Internet]. Academic Press; [cited 2023 Jul 16]. pp. 279–301. (2018). https://www.sciencedirect.com/science/article/pii/B9780128125717000162
40. Mantikou, E. et al. Embryo culture media and IVF/ICSI success rates: a systematic review. *Hum. Reprod. Update*. **19**, 210–220 (2013).
41. Vajta, G., Rienzi, L., Cobo, A. & Yovich, J. Embryo culture: can we perform better than nature? *Reprod. Biomed. Online*. **20**, 453–469 (2010).
42. DeQuach, J. A. et al. Simple and high yielding method for preparing tissue specific extracellular matrix coatings for cell culture. *PloS One*. **5**, e13039 (2010).
43. French, K. M. et al. A naturally derived cardiac extracellular matrix enhances cardiac progenitor cell behavior in vitro. *Acta Biomater.***8**, 4357–4364 (2012).
44. Sackett, S. D. et al. Extracellular matrix scaffold and hydrogel derived from decellularized and delipidized human pancreas. *Sci. Rep.***8**, 10452 (2018).
45. Young, D. A., Choi, Y. S., Engler, A. J. & Christman, K. L. Stimulation of adipogenesis of adult adipose-derived stem cells using substrates that mimic the stiffness of adipose tissue. *Biomaterials*. **34**, 8581–8588 (2013).
46. Zhang, X. & Dong, J. Direct comparison of different coating matrix on the hepatic differentiation from adipose-derived stem cells. *Biochem. Biophys. Res. Commun.***456**, 938–944 (2015).
47. Amorim, C. A. Special issue devoted to a New Field of Regenerative Medicine: Reproductive tissue Engineering. *Ann. Biomed. Eng.***45**, 1589–1591 (2017).
48. Huleihel, L. et al. Matrix-bound nanovesicles within ECM bioscaffolds. *Sci. Adv.***2**, e1600502 (2016).
49. Hussey, G. S. et al. Lipidomics and RNA sequencing reveal a novel subpopulation of nanovesicle within extracellular matrix biomaterials. *Sci. Adv.***6**, eaay4361 (2020).

50. Gu, Z., Guo, J., Wang, H., Wen, Y. & Gu, Q. Bioengineered microenvironment to culture early embryos. *Cell. Prolif.***53**, e12754 (2020).
51. Ezzati, M., Djahanbakhch, O., Arian, S. & Carr, B. R. Tubal transport of gametes and embryos: a review of physiology and pathophysiology. *J. Assist. Reprod. Genet.***31**, 1337–1347 (2014).
52. Kurochkin, I. V., Yonemitsu, N., Funahashi, S. I. & Nomura, H. ALEX1, a novel human armadillo repeat protein that is expressed differentially in normal tissues and carcinomas. *Biochem. Biophys. Res. Commun.***280**, 340–347 (2001).
53. Lee, S-Y. et al. FAM83A confers EGFR-TKI resistance in breast cancer cells and in mice. *J. Clin. Invest.***122**, 3211–3220 (2012).
54. Liu, C. et al. Positive feedback loop of FAM83A/PI3K/AKT/c-Jun induces migration, invasion and metastasis in hepatocellular carcinoma. *Biomed. Pharmacother Biomedecine Pharmacother*. **123**, 109780 (2020).
55. Zheng, Y-W. et al. FAM83A promotes Lung Cancer Progression by regulating the wnt and Hippo Signaling Pathways and indicates poor prognosis. *Front. Oncol.***10**, 180 (2020).
56. Karaca, E. et al. Genes that affect brain structure and function identified by rare variant analyses of mendelian neurologic disease. *Neuron*. **88**, 499–513 (2015).
57. Zollo, M. et al. PRUNE is crucial for normal brain development and mutated in microcephaly with neurodevelopmental impairment. *Brain J. Neurol.***140**, 940–952 (2017).
58. Kobayashi, T. et al. Glycogen synthase kinase 3 and h-prune regulate Cell Migration by modulating focal adhesions. *Mol. Cell. Biol.***26**, 898–911 (2006).
59. Ramos-Ibeas, P. et al. Embryo responses to stress induced by assisted reproductive technologies. *Mol. Reprod. Dev.***86**, 1292–1306 (2019).
60. de Andrade Melo-Sterza, F. & Poehland, R. Lipid metabolism in bovine oocytes and early embryos under in vivo, in Vitro, and stress conditions. *Int. J. Mol. Sci.***22**, 3421 (2021).
61. Hoijman, E. et al. Cooperative epithelial phagocytosis enables error correction in the early embryo. *Nature*. **590**, 618–623 (2021).
62. Schilling, M. A. et al. Transcriptional Innate Immune Response of the Developing Chicken Embryo to Newcastle Disease Virus Infection. Front Genet [Internet]. 2018 [cited 2023 Jul 19];9. https://www.frontiersin.org/articles/https://doi.org/10.3389/fgene.2018.00061
63. Kruessel, J. S. et al. Different pattern of interleukin-1 beta-(IL-1 beta), interleukin-1 receptor antagonist- (IL-1ra) and interleukin-1 receptor type I- (IL-1R tI) mRNA-expression in single preimplantation mouse embryos at various developmental stages. *J. Reprod. Immunol.***34**, 103–120 (1997).
64. Montazeri, M. et al. Interleukin-1 receptor antagonist mediates toll-like receptor 3-induced inhibition of trophoblast adhesion to endometrial cells in vitro. *Hum. Reprod. Oxf. Engl.***31**, 2098–2107 (2016).
65. Spandorfer, S. D., Neuer, A., Liu, H. C., Rosenwaks, Z. & Witkin, S. S. Involvement of interleukin-1 and the interleukin-1 receptor antagonist in in vitro embryo development among women undergoing in vitro fertilization-embryo transfer. *J. Assist. Reprod. Genet.***20**, 502–505 (2003).
66. Barlan, K. & Gelfand, V. I. Microtubule-based transport and the distribution, Tethering, and Organization of Organelles. *Cold Spring Harb Perspect. Biol.***9**, a025817 (2017).
67. Jia, S. & Meng, A. TGFβ family signaling and development. *Dev. Camb. Engl.***148**, dev188490 (2021).
68. Yang, S. et al. A Zeb2-miR-200c loop controls midbrain dopaminergic neuron neurogenesis and migration. *Commun. Biol.***1**, 1–11 (2018).
69. Declercq, E. et al. Perinatal outcomes associated with assisted reproductive technology: the Massachusetts Outcomes Study of Assisted Reproductive Technologies (MOSART). *Fertil. Steril.***103**, 888–895 (2015).
70. Messerlian, C., Maclagan, L. & Basso, O. Infertility and the risk of adverse pregnancy outcomes: a systematic review and meta-analysis. *Hum. Reprod. Oxf. Engl.***28**, 125–137 (2013).
71. Raatikainen, K., Kuivasaari-Pirinen, P., Hippeläinen, M. & Heinonen, S. Comparison of the pregnancy outcomes of subfertile women after infertility treatment and in naturally conceived pregnancies. *Hum. Reprod. Oxf. Engl.***27**, 1162–1169 (2012).
72. Romundstad, L. B. et al. Effects of technology or maternal factors on perinatal outcome after assisted fertilisation: a population-based cohort study. *Lancet Lond. Engl.***372**, 737–743 (2008).
73. Wise, L. A. et al. Prospective study of time to pregnancy and adverse birth outcomes. *Fertil. Steril.***103**, 1065–1073e2 (2015).
74. Pinborg, A., Henningsen, A-K-A., Malchau, S. S. & Loft, A. Congenital anomalies after assisted reproductive technology. *Fertil. Steril.***99**, 327–332 (2013).
75. Katari, S. et al. DNA methylation and gene expression differences in children conceived in vitro or in vivo. *Hum. Mol. Genet.***18**, 3769–3778 (2009).
76. Song, S. et al. DNA methylation differences between in vitro- and in vivo-conceived children are associated with ART procedures rather than infertility. *Clin. Epigenetics*. **7**, 41 (2015).
77. Khosla, S., Dean, W., Reik, W. & Feil, R. Culture of preimplantation embryos and its long-term effects on gene expression and phenotype. *Hum. Reprod. Update*. **7**, 419–427 (2001).
78. Sunde, A. et al. Time to take human embryo culture seriously. *Hum. Reprod. Oxf. Engl.***31**, 2174–2182 (2016).
79. Vicente, J. S., Viudes-de-Castro, M. P. & Marco-Jiménez, F. Vivo rabbit embryo production and cryopreservation review. Application to ex situ conservation and rederivation. *World Rabbit Sci.***31**, 65–75 (2023).

Acknowledgements

The authors express their sincere thanks to the staff of the experimental farm of the Universitat Politècnica de València and to the Multigenic Analysis Service from the Univesity of Valencia, especially to Úrsula Estada.

Author contributions

E.F.-H., F.M.-J., and I.C. originated the study concept. F.M.-J. and I.C. supervised the project. E.F.-H., F.M.-J, J.S.V., and A.P. carried out the experiments. E.F.-H., L.L-R., C.C., P.S.-L., C.B.-F., A.R.-E., M.G.-A., and P.D.-G. analyzed the data. E.F.-H. and F.M.-J. wrote the manuscript with input from all authors. All authors contributed to data analyses, and data interpretation, reviewed and approved the manuscript before submission.

Funding

This work was supported by the Instituto de Salud Carlos III (ISCIII) and co-funded by the European Union (Fondo Social Europeo «El FSE invierte en tu futuro») [PI17/01039, PI21/00305, CP19/00149 (I.C.)]; Spanish Ministry of Science, Innovation, and Universities [FPU18/06327 (E.F.-H.), MS21-142 [C.B.-F.], FPU19/04850 (A.R.-E.), FPU20/00251 (M.G.-Á.)]; and Generalitat Valenciana [PROMETEO/2018/137, CIPROM/2021/058 (I.C., and A.P.)].

Declarations

Ethics approval and consent to participate
This study was approved by the Ethical Committee for Experimentation with Animals of the Polytechnic University of Valencia, Spain.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-77583-4.

Correspondence and requests for materials should be addressed to I.C.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.