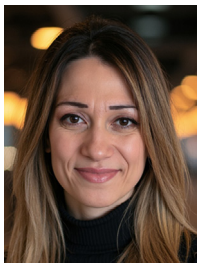
RBMO

ELSEVIER

ARTICLE

Sperm insulin-like growth factor 2 protein levels: implications for early embryo development



BIOGRAPHY

Dr Rossella Cannarella is a clinical andrologist and endocrinologist specializing in male infertility, human reproduction and sperm epigenetics. With extensive international training, including at the Cleveland Clinic and University of Massachusetts, she has published over 240 peer-reviewed articles. Her research focuses on spermatogenesis, testicular function and endocrine disruptors.

Rossella Cannarella[a,b,*], Oliver J. Rando[c], Sandrine Chamayou[d], Simona Romano[d], Antonino Guglielmino[d], Qiangzong Yin[c], Francesca Mancuso[e], Iva Arato[e], Cinzia Lilli[e], Stefano Brancorsini[e], Sabrina Farinacci[e], Giovanni Luca[e], Loredana Leggio[f], Nunzio Iraci[f], Scott D. Lundy[b], Sandro La Vignera[a], Aldo E. Calogero[a]

KEY MESSAGE

Sperm insulin-like growth factor 2 (IGF2) protein plays a critical role in early embryo development by influencing pronuclei formation during fertilization. Its effect on later stages of embryo development and assisted reproductive technology outcomes remains limited. This study highlights sperm-delivered IGF2's potential in early embryogenesis and its diagnostic value in male infertility.

ABSTRACT

Research question: What is the role of sperm-derived insulin-like growth factor 2 (IGF2) protein in early embryo development, and what is its potential influence on outcomes in assisted reproductive technology (ART)?

Design: In 27 infertile couples undergoing ART, IGF2 protein levels in spermatozoa were quantified using enzyme-linked immunosorbent assay. Their correlation with embryo morphokinetics was assessed via time-lapse imaging. The analysis was adjusted for female partner's age, body mass index and serum anti-Müllerian hormone levels. To explore underlying mechanisms, recombinant IGF2 was added to murine parthenote cultures. Transcriptome analysis was conducted at the blastocyst stage.

Results: Sperm IGF2 levels were significantly associated with the number of post-gradient sperm recovered ($r = 0.65$; $P < 0.001$) and with the timing of pronuclear formation ($r = -0.22$; $P = 0.04$), even after adjusting for confounding factors, indicating a role in the earliest stages of embryogenesis. No significant correlation was observed with later stages of embryo development, possibly owing to a developmental transition from paternal to embryonic IGF2 transcript expression. Furthermore, IGF2 levels did not differ significantly among ART outcome groups (biochemical pregnancy, clinical pregnancy and live birth). Transcriptome analysis of IGF2-treated parthenotes revealed enrichment in pathways related to protein binding, which is critical for pronuclear formation.

[a] Department of Clinical and Experimental Medicine, University of Catania, Catania, Italy.
[b] Glickman Urological and Kidney Institute, Cleveland Clinic, Cleveland, OH, USA.
[c] Department of Biochemistry and Molecular Biotechnology, University of Massachusetts Chan Medical School, Worcester, MA, USA.
[d] Centro HERA – Unità di Medicina della Riproduzione, Catania, Italy.
[e] Department of Medicine and Surgery, University of Perugia, Perugia, Italy.
[f] Department of Biomedical and Biotechnological Sciences (BIOMETEC), University of Catania, Catania, Italy.

KEYWORDS
sperm IGF2
IGF2
embryo kinetics
blastocyst
embryo development

*Corresponding author. E-mail address: rossella.cannarella@unict.it (R. Cannarella). https://doi.org/10.1016/j.rbmo.2025.105202 1472-6483/
Declaration: The authors report no financial or commercial conflicts of interest.

Conclusion: A potential involvement of sperm-delivered IGF2 in early post-fertilization events is suggested, with a limited apparent effect on later embryonic development or ART outcomes. Further investigation into the diagnostic and therapeutic relevance of IGF2 in the context of male-factor infertility is needed.

INTRODUCTION

Infertility, defined by the inability to conceive after 1 year of unprotected intercourse, is nowadays a major global health challenge, affecting couples on emotional, social and financial levels. Recent data from the World Health Organization estimate that infertility affects about 17.6% of couples, or one in six, attempting to conceive (https://www.who.int/news/). Despite extensive research, the cause of infertility remains undetermined in up to 30% of cases, leaving many couples without a definitive diagnosis (*Gelbaya et al., 2014*). This highlights the pressing need for further advanced research to uncover novel causes and develop effective treatment strategies, particularly for male infertility. Advancements in molecular biology techniques have provided valuable insights into previously underexplored physiological mechanisms, with significant implications for clinical practice (*Calogero et al., 2023*). Recent findings challenge the conventional perspective of spermatozoa as merely a vehicle for paternal DNA. It is now well established that mature spermatozoa contain a wide array of transcripts (*Santiago et al., 2021*) and proteins (*Castillo et al., 2018*) within their cytoplasm. Studies in animal models have shown that these molecules play a crucial role for various key processes, including sperm maturation, fertilization and early embryo development (*Castillo et al., 2018*; *Santiago et al., 2021*; *Vallet-Buisan et al., 2023*). Intriguingly, emerging evidence suggests that these sperm-derived components may also influence fetal health, potentially affecting outcomes beyond fertilization (*Ashapkin et al., 2023*). Identifying diagnostic and therapeutic markers among the diverse array of molecules present in spermatozoa is a significant challenge. One promising approach involves focusing on factors associated with paternally expressed imprinted genes, which are of particular interest owing to their role in supporting embryo growth. These genes are uniquely expressed from paternal chromosomes, with no maternal counterpart (*Barton et al., 1991*). Consequently, it can be hypothesized that such factors are present in spermatozoa but not in oocytes, positioning male gametes as a critical source of molecules essential for initiating the earliest stages of embryo development. This hypothesis was recently explored in our pilot study, in which we demonstrated that the levels of insulin-like growth factor 2 (*IGF2*) transcript delivered by spermatozoa to the oocyte positively influenced embryo development and morphology in humans (*Cannarella et al., 2024*). These findings were further corroborated by experiments in mice, in which transcriptome analysis of parthenotes injected with *Igf2* mRNA resulted in the enrichment in pathways associated with early embryo growth and development (*Cannarella et al., 2024*). This research opens a fascinating avenue for further investigation, requiring validation in larger cohorts and necessitating a deeper search of the molecular factors involved in this pathway.

Mature spermatozoa contain the *IGF2* transcript (*Cannarella et al., 2020*) and the IGF2 protein within their cytoplasm (*Cannarella et al., 2022*). Although its precise role remains largely undefined, IGF2 protein may regulate spermatogenesis by modulating the expression of key factors such as glial cell line-derived neurotrophic factor (*GDNF*), stem cell factor (*SCF*) and fibroblast growth factor 2 (*FGF2*) in Sertoli cells (*Cannarella et al., 2022*). Recently, IGF2 protein has been identified in seminal fluid, with its levels correlating with sperm quality; in a cohort of 320 infertile patients, lower IGF2 protein levels were associated with poor sperm quality, increased inflammation and DNA damage, suggesting IGF2 as a promising target for future fertility treatments (*Wu et al., 2024*). These findings emphasize the multifaceted roles of IGF2 and its potential as a biomarker and a therapeutic target for male fertility. It remains unclear, however, whether the levels of IGF2 protein in the sperm cytoplasm influences embryo development and subsequent outcomes.

To investigate this aspect, it is crucial to first understand the kinetics of gene expression onset in the embryo, a process known as zygotic genome activation (ZGA). This does not occur immediately after fertilization, as the maternal and paternal DNA strands must first recombine and organize. The regulation of ZGA is complex and varies slightly between species, particularly between mice and humans. In mice, ZGA occurs in two phases: the minor ZGA at the intermediate one-cell stage and the major ZGA at the late two-cell stage (*Tadros and Lipshitz, 2009*). In contrast, ZGA in humans begins at the eight-cell stage, typically around day 3 of development, after maternal RNA degradation (*Lee et al., 2014*; *Schulz and Harrison, 2019*). The sperm-carried *IGF2* gene transcript seems to influence embryo growth starting from day 2 after fertilization (*Cannarella et al., 2024*), when the transcript is translated into protein. From day 3 onwards, embryo growth is driven by the transcriptional activity of the *IGF2* gene within the embryo itself. Therefore, the hypothesis in the present study was that sperm-delivered IGF2 protein influences early events occurring on day 1 after fertilization, before the translation of sperm-derived transcripts and before the onset of ZGA.

MATERIALS AND METHODS

Human experimental protocol

Study protocol

This prospective, observational study focused on infertile couples undergoing treatment at an ART centre. Data were collected between March and September 2023, from women, including information on the cause of infertility, age, body mass index (BMI) and anti-Müllerian hormone (AMH), as well as the type of ART stimulation protocol used. For men, data included age, BMI and sperm parameters. All couples were only included once.

The study prospectively collected data on sperm concentration in semen samples, before and after sperm recovery, through density gradient centrifugation, as well as the ART techniques used (intracytoplasmic sperm injection [ICSI] and intracytoplasmic morphologically selected sperm injection [IMSI]). Additional information was gathered on embryo kinetics, along with the timing of embryo transfer and post-transfer beta-HCG serum levels. Data on pregnancy outcomes, including biochemical pregnancy (defined as a biochemical loss with serum HCG ≥5 IU/l and no gestational sac on ultrasound), extrauterine

pregnancy and clinical pregnancy (confirmed by ultrasound by 5–6 weeks of gestation), as well as miscarriage rate (pregnancy loss before 20 weeks following a documented clinical pregnancy) and live birth rate were also recorded.

For each cycle included in the study, data on embryo transfer and clinical outcomes were collected exclusively for the fresh transfers undertaken. After gradient separation and oocyte injection, an aliquot of spermatozoa used in ART was analysed for IGF2 protein levels via enzyme-linked immunosorbent assay (ELISA), with results compared with data from five donors with normozoospermia serving as controls. These donors were not undergoing ART but were recruited from a seminology laboratory based on their normozoospermic semen parameters, and their samples were processed using the same protocol applied to patient samples. Time-lapse imaging was used to monitor embryo morphokinetics, and the correlation between IGF2 levels and embryo development was assessed, adjusting for potential confounders. Additionally, IGF2 levels were also analysed in relation to the clinical outcomes of ART.

Patient selection

Couples eligible for homologous ART were required to have a clinical history of infertility for at least 12 months, with male factor infertility or idiopathic infertility as the primary indications. Couples with a history of repeated implantation failure, recurrent miscarriage or those undergoing heterologous ART were excluded from participation.

Male participants were required to be at least 18 years of age, free from major comorbidities or organ failure, e.g. heart, kidney or liver failure, and without history of alcohol abuse, drug use or smoking. Patients with cryptozoospermia or azoospermia were excluded owing to the inability to assess IGF2 protein levels in their semen samples.

Eligibility criteria for female participants included being at least 18 years of age, with no history of significant comorbidities (diabetes mellitus, tumours, thyroid diseases or hyperprolactinemia) or organ failure, e.g. heart, kidney or liver failure, and no current or past history of alcohol abuse, drug use or smoking. Additionally, participants were required to have regular menstrual cycles (25–35 days) and a serum AMH level greater than 0.5–1.1 ng/ml (*Ferraretti et al., 2011*). Female infertility caused by endometriosis was an exclusion criterion as well as the retrieval of fewer than five mature oocytes during ovarian stimulation, as suggestive of poor ovarian response (*Abusheikha et al., 2001*). Female infertility resulting from tubal dysfunction, occlusion or anovulation, however, was not considered grounds for exclusion.

Oocyte collection and morphology

Ovarian stimulation was carried out using long or short agonist protocols. Specifically, luteal-phase gonadotrophin releasing hormone (GnRH) agonist (Suprefact) (Hoechst Marion Roussel Deutschland GmbH, Frankfurt, Germany) was administered, followed by recombinant FSH (Gonal-F) (Merck-Serono, London, UK) or Puregon (MSD, Franklin Lakes, USA) starting on the third day of the menstrual cycle. Oocytes were retrieved 35 h after administration of 10,000 IU of HCG (Gonasi) (IBSA, Lodi, Italy), and oocyte–cumulus complexes were retrieved using transvaginal ultrasound-guided aspiration.

After retrieval, cumulus cells were removed and oocytes were handled and maintained in pre-equilibrated culture media, e.g. G-MOPS Plus and G-1/G-2 series (Vitrolife, Gothenburg, Sweden) during denudation and morphological assessment. Oocytes were then evaluated based on their morphology. Oocyte morphology was classified into four categories according to the appearance of the first polar body, the size of the perivitelline space and the presence of cytoplasmic inclusions, as described in previous studies (*Xia et al., 1997*). The oocytes were subsequently graded into two categories: grades 1–2 (poor quality) or grades 3–4 (good quality).

Sperm collection

Semen samples were collected through masturbation into a sterile container after a 3–4-day period of sexual abstinence. Sperm quality was assessed according to the World Health Organization guidelines (*WHO, 2010*). After liquefaction, spermatozoa were isolated using density gradient centrifugation, following the manufacturer's protocol (SpermGrad) (Vitrolife, Englewood, CO, USA) (*Ali et al., 2022*). Only motile spermatozoa were selected for use in ART procedures. Any remaining sample, after its use in ART, was preserved at −80°C for future protein extraction.

Measurement of sperm insulin-like growth factor 2 protein

Semen samples were thawed on ice. Total protein extracts were prepared by lysing semen samples in 100 μl of radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), as previously described (*Cannarella et al., 2022*). The lysates were centrifuged at 1000 x g (Eppendorf, Hamburg, Germany) for 10 min, and the supernatant was collected. Total protein content was measured using the Bradford method (*Bradford, 1976*). Sample aliquots were stored at −20°C for assessment of mature IGF2 via an enzyme-linked immunosorbent assay (ELISA) (Human IGF-II/IGF2 Immunoassay, Quantikine ELISA DG200) (R&D Systems, Inc., Minneapolis, MN, USA), following the manufacturer's instructions. Concentrations of IGF2, expressed as pg/ml, were normalized to the total protein content in the extracts, to provide an absolute value independent of sperm count and sample volume.

Embryo kinetics and morphology

The time of ICSI/IMSI was designated as time zero (t0) for each patient. The appearance of both pronuclei, referred to as tPN appear, was recorded as the time of pronuclear formation. This marks the onset of the pronuclear stage. The last observation of the two pronuclei was noted as tPN fading. Subsequent time points, such as t2, t3, t4, tn, corresponded to the development of the embryo at the two-cell, three-cell, four-cell and subsequent stages. The morula stage was defined as tM, and the emergence of a crescent-shaped area from the morula was labelled tB. The time at which the blastocyst cavity expanded and increased in volume was recorded as the time of the expanded blastocyst (tEB). All time points were documented in hours and fractions of an hour (*Ciray et al., 2014*).

Early embryos were cultured in pre-equilibrated sequential media (G-1/G-2 series) (Vitrolife, Gothenburg, Sweden) under standard incubator conditions (37° C, 5% CO_2, 5% O_2), and graded using a score ranging from 1 to 4 (1–2 indicated a good-quality embryo and 3–4 a poor-quality embryo), following previously established criteria.

The time points were calculated using the EmbryoScope time-lapse imaging system (Vitrolife, Gothenburg, Sweden), which continuously monitored embryo

development. The recorded images were analysed with the EmbryoViewer® software (Vitrolife, Gothenburg, Sweden), specifically designed for precise annotation of morphokinetic events. All annotations were carried out by trained embryologists according to standardized protocols. Regular inter-observer calibration sessions were held to ensure consistency and minimize discrepancies. Ambiguous or borderline cases were reviewed jointly to reach consensus decisions, thus enhancing the reliability of the timing assessments.

Statistical analysis

Data are presented as mean ± SD for variables with a normal distribution, and as median with interquartile range for non-normally distributed continuous variables. The Shapiro–Wilks test was used to assess data distribution. To compare sperm IGF2 protein levels between patients who had undergone ART and healthy donors with normozoospermia, a Student's t-test was conducted on log10-transformed data to normalize the distribution and stabilize variance, as IGF2 levels were positively skewed.

A linear regression (followed by Pearson's correlation coefficient to test the strength of the correlation) was used to examine the relationship between sperm IGF2 levels and the number of spermatozoa recovered after gradient centrifugation, which served as an indicator of sample quality, with post-gradient sperm count as the dependent variable and IGF2 levels as the independent one. Additionally, a linear regression analysis followed by Pearson's correlation analysis were used to evaluate the relationship between IGF2 levels and specific morphokinetic time points, as well as the strength of their correlation.

A stepwise multiple linear regression model was constructed to evaluate the relationship between sperm IGF2 levels and embryo morphokinetic parameters, while adjusting for potential confounders. In model 1, the confounding factors included the female partner's age, serum AMH levels, and the number of injected oocytes; in model 2, BMI was also included. Multiple embryos per woman were included in the analysis, introducing potential dependency. Because of software limitations, clustering was not modelled; instead, the number of injected oocytes was included as a covariate. This approach is consistent with previously published studies of this type (*Cannarella et al., 2024*).

A correlation analysis was used to evaluate the relationship between sperm IGF2 protein levels and the 2PN rate and embryo grade 1 on day 3. The 2PN rate was defined as the proportion of fertilized oocytes with two pronuclei 18–20 h after ICSI/IMSI. Good-quality embryo was defined according to standard morphological scoring, with uniform blastomeres and 10% or less fragmentation.

To assess whether sperm IGF2 levels could predict embryo developmental potential, a receiver operating characteristic (ROC) curve was constructed. The predictor variable was sperm IGF2 protein level, and the binary outcome was defined per patient: 'positive' if at least one embryo from the patient reached the blastocyst stage on day 5, and 'negative' if none did. By aggregating the data in this way, each patient contributed only one observation to the analysis, avoiding the issue of non-independent multiple embryos per patient. The area under the ROC curve (AUC) was used to evaluate the predictive value of sperm IGF2 levels for blastocyst formation. Embryo development to the blastocyst stage was evaluated using time-lapse imaging and morphological assessment according to standard criteria, including blastocoel expansion, zona thinning and cell number. Only embryos that met these criteria on the fifth day of culture were considered blastocysts for the analysis, and only fresh embryos selected for transfer were included in this evaluation. Patients were subsequently categorized based on beta-HCG results (positive versus negative), clinical pregnancy status (yes/no) and live birth outcome (yes/no). IGF2 levels were compared across these groups using the appropriate statistical tests (Student's t-test or Mann–Whitney U test for normally or non-normally distributed data). A sub-analysis was conducted by stratifying patients according to female partner age (≤35 years or >35 years), and a two-way analysis of variance with Bonferroni correction for multiple comparisons was used.

The primary objective of the study was to evaluate the correlation between sperm IGF2 levels and embryo morphokinetics. A power analysis based on correlation coefficients was conducted to determine the required sample size. Assuming a correlation coefficient of 0.4, a type I error (alpha) of 0.05, a type II error (beta, 1-Power) of 0.20 and a null hypothesis value of 0.0, the required sample size was calculated to be 46 blastocysts. On the basis of an estimated blastocyst formation rate of about 50% (Thomas et al., 2010), a minimum of 92 zygotes were included in the study.

Statistical analyses were conducted using MedCalc Software Ltd. (Version 19.6 – 64 bit) (MedCalc Software Ltd., Ostend, Belgium), with $P \leq 0.05$ considered statistically significant.

Ethical approval

The study was carried out at the Division of Endocrinology, Metabolic Diseases, and Nutrition at the University-Teaching Hospital Policlinico 'G. Rodolico-San Marco' in Catania, Italy, in collaboration with the ART centre 'HERA – Unità di Medicina della Riproduzione' also based in Catania. The research protocol was approved by the Ethics Committee 'Catania 1' at the University-Teaching Hospital Policlinico 'G Rodolico-San Marco' (approval number 18189, granted on 27 March 2023). All participants provided informed consent after receiving detailed information about the study objectives and procedures. The study was conducted in accordance with the ethical standards set forth in the Declaration of Helsinki.

ANIMAL EXPERIMENTAL PROTOCOL

Study protocol

Recombinant mouse IGF2 (rmIGF2) (50 μM) or a vehicle control was added to the oocyte culture immediately after oocyte retrieval, and the oocytes were placed in a parthenogenic medium, at a 1:1 concentration. After 96 h, a total of 60 blastocysts per group (divided into four pools of 15 embryos each) that reached the blastocyst stage were collected for subsequent transcriptome analysis (FIGURE 1).

Mouse strain

The experiments involved 12-week-old FVB/NJ mice, which were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and served as oocyte donors. All animal care and experimental procedures were conducted in compliance with the ethical standards established by the University of Massachusetts Medical School Institutional Animal Care and Use Committee, under protocol number 201200029. Mice were housed under a

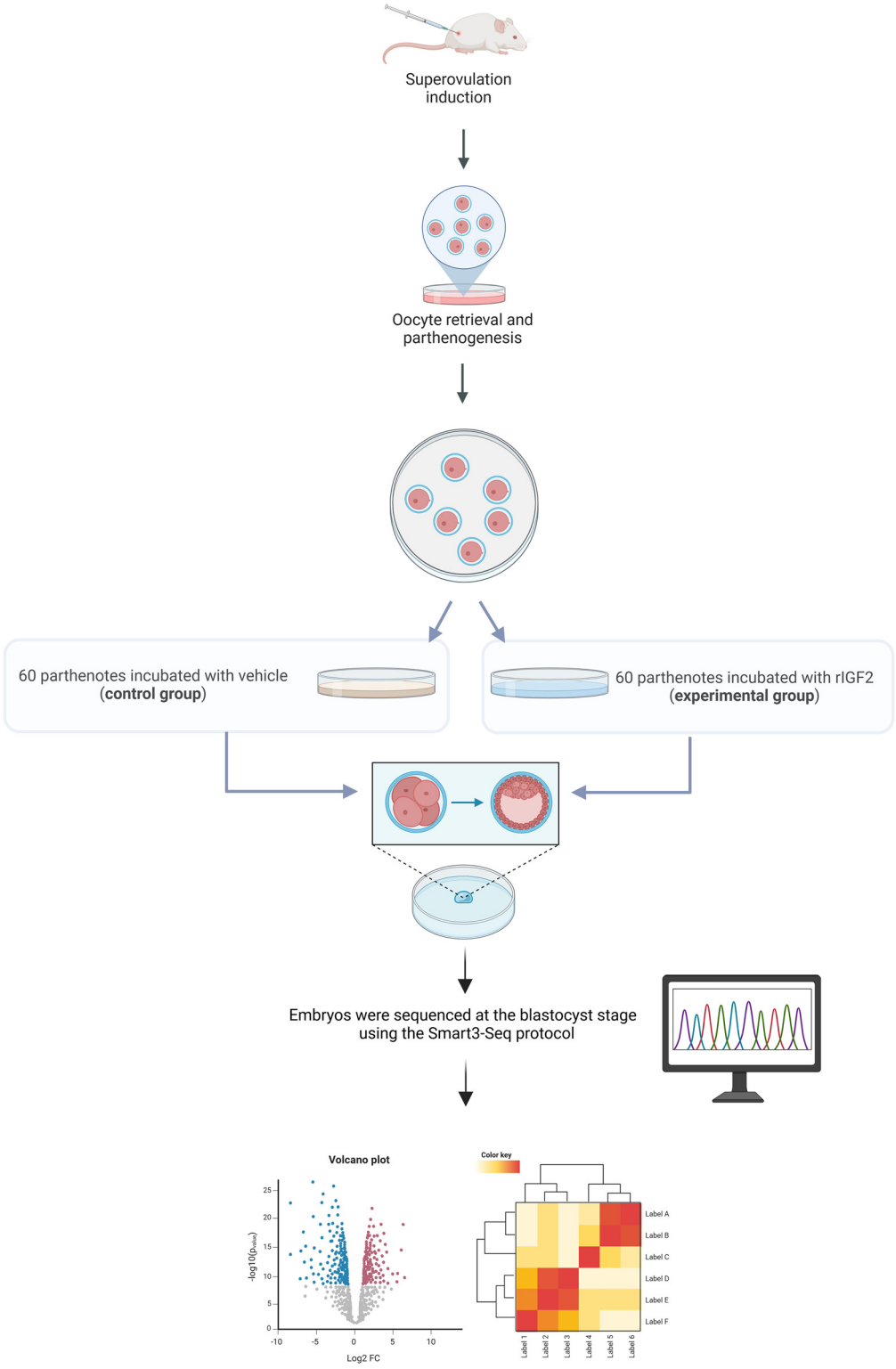


FIGURE 1 Animal experimental protocol. Oocytes were retrieved from 8–12-week-old superovulated FVB/NJ mice, separated from the cumulus cells, and incubated in a parthenogenic medium. Three hours after incubation, parthenotes were incubated with either vehicle (control group, $n = 60$) or recombinant mouse insulin-like growth factor 2 (rIGF2, 50 μM) (experimental group, $n = 60$). After 96 h, when the embryos reached the blastocyst stage, they were harvested and subsequently sequenced. Created in BioRender (Calogero A, 2025 [https://BioRender.com/dqefg7k]).

12-h light–dark cycle and provided with ad-libitum access to food and water.

Oocyte retrieval and parthenotes generation

Superovulation in female mice was induced by an intraperitoneal injection of pregnant mare's serum gonadotropin (PMSG, 5 IU) (Sigma-Aldrich, St Louis, MO, USA), followed by a second intraperitoneal injection of HCG (5 IU) (Sigma-Aldrich, St Louis, MO, USA) 48 h later. Oocytes were retrieved from the oviducts by dissecting the ampulla and placing it into potassium-supplemented simplex optimized medium (KSOM) (Millipore, Burlington, MA, USA) supplemented with 0.1% hyaluronidase (Sigma-Aldrich, St Louis, MO, USA) to facilitate the removal of cumulus cells. After a 5-min incubation with hyaluronidase, the oocytes were washed four to five times in fresh KSOM. They were then incubated for 60 min in an activation medium under controlled conditions (37°C, 5% CO_2, 5% O_2 and 95% relative humidity). The activation medium consisted of 5 μg/ml cytochalasin B (Sigma-Aldrich, St Louis, MO, USA), 4 mM EGTA (Sigma-Aldrich, St Louis, MO, USA) and 10 mM $SrCl_2$ (Sigma-Aldrich, St Louis, MO, USA). After activation, the oocytes were transferred to KSOM and incubated at 37°C until further use.

Parthenote incubation

A total of 365 parthenotes were treated with rmIGF2 (R&D Systems, Biotechne, Minneapolis, MN, USA) at a final concentration of 50 μM, following previously established protocols (*Muhammad et al., 2020*), whereas 404 parthenotes were exposed to a vehicle control consisting of KSOM medium alone. All parthenotes were cultured simultaneously under identical conditions. After 96 h of incubation, 60 blastocysts from each group were collected and placed into TCL buffer containing 1% beta-mercaptoethanol. The samples were then stored at −80°C until further processing for RNA sequencing library from individual embryos.

Smart-Seq3

For each experimental condition, four pools of 15 embryos were analysed. RNA-sequencing libraries were generated using the Smart-seq3 protocol with several modifications (*Hagemann-Jensen et al., 2020*). Briefly, individual blastocysts were collected in a 1x TCL buffer (catalogue number 1070498) (Qiagen, Hilden, Germany) containing 1% beta-mercaptoethanol (catalogue number M6250) (Sigma, Kanagawa, Japan). Total RNA was purified using 2.2 x SPRI beads (B23318) (Beckman Coulter, Brea, CA, USA), and the beads were resuspended in 6 μl of lysis buffer containing recombinant RNase inhibitor (2313B) (Takara, Shiga, Japan), Triton X-100, dNTPs, Smart-seq3 oligo-dT primer (5′-biotin-ACGAGCATCAGCAGCATACGAT 30VN-3′; IDT) and PEG 8000.

The samples were incubated at 72°C for 10 min and then placed on ice. Reverse transcription was conducted by adding 2 μl of reverse transcription mix, which contained 25 mM Tris-HCL, pH 8.3 (T6791) (Sigma, Kanagawa, Japan) 30 mM NaCl (AM9759) (Ambion, Thermo Fisher Scientific, Waltham, MA, USA), 1 mM GTP (R0461) (Thermo Fisher Scientific, Waltham, MA, USA), 2.5 mM $MgCl_2$ (AM9530G) (Ambion Thermo Fisher Scientific, Waltham, MA, USA), 8 mM DTT (R0861) (Thermo Fisher Scientific, Waltham, MA, USA), 0.5 U/μl RNase inhibitor (2313B) (Takara, Shiga, Japan), 2 μM of various Smart-seq3 TSOs (5′-biotin AGAGACAGATTGCGCAA TGNNNNNNNNrGrGrG-3′; IDT) and 2 U/μl of Maxima H-minus reverse transcriptase enzyme (EP0751) (Thermo Fisher Scientific, Waltham, MA, USA).

Reverse transcription was carried out at 42°C for 90 min, followed by 10 cycles of 50°C for 2 min, 42°C for 2 min and 85°C for 5 min. Polymerase chain reaction (PCR) preamplification was carried out by adding 12 μl of PCR mix containing 1x KAPA HiFi master mix, 0.1 μM Smart-seq3 forward PCR primer (5′-TCGTCGGCAGCGTC AGATGTGTATAAGAGACAGA TTGCGCAATG-3′; IDT), and 0.1 μM Smart-seq3 reverse PCR primer (5′-ACGAGCATCAGCAGCATACGA-3′; IDT). Polymerase chain reaction was carried out under the following conditions: 3 min at 98°C, followed by 19 cycles of 20 s at 98°C, 30 s at 65°C, 6 min at 72°C and a final 5 min at 72°C. The PCR product was purified with 0.8 x SPRI beads and eluted in nuclease-free water (AM9932) (Ambion, Thermo Fisher Scientific, Waltham, MA, USA).

Purified cDNA was assessed for quality using a bioanalyser and quantified using the Qubit 1X dsDNA HS Assay Kit (Q33231) (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were prepared for sequencing with the Illumina Nextera XT DNA Library Prep Kit (FC-131-1096) (Illumina, San Diego, CA, USA). Sequencing was conducted on an Illumina NextSeq500 instrument, using paired-end reads (45 bp for read 1 and 30 bp for read 2).

The length of the reverse-transcribed cDNA fragments and the quality of the libraries were assessed by measuring the fragment sizes. Only the samples that passed quality control were subsequently sequenced (Appendix 1 and Appendix 2).

Analysis of smart-seq3 sequencing data

The raw FASTQ files were processed by first distinguishing between 5′ end reads containing unique molecular identifiers (UMI) and internal reads, based on the 11-bp tag sequence (ATTGCGCAATG). The UMI sequences from the 5′ end reads were extracted using UMI-tools (*Smith et al., 2017*), followed by transcriptome alignment with STAR (*Dobin et al., 2013*). Only uniquely mapped reads were retained, and PCR duplicates were removed using UMI tools. For the internal reads, STAR was used for alignment, and Picard was used for deduplication (http://broadinstitute.github.io/picard). The BAM files generated from both 5′ end and internal reads were then combined. Read counts were quantified using FeatureCounts (*Liao et al., 2014*), and batch effects were corrected using Combat-seq (*Zhang et al., 2020*). Differential gene expression analysis was conducted with DESeq2 (*Love et al., 2014*), applying a false discovery rate threshold of 0.05 and selecting genes with a fold change greater than 2. Raw data are available at: https://doi.org/10.5281/zenodo.14559305.

Pathway enrichment analysis

For functional enrichment analysis, the pathway enrichment analysis tools g:Profiler g:GOSt (*Reimand et al., 2007*; *2011*; *2016*) and EnrichR (*Chen et al., 2013*; *Kuleshov et al., 2016*) were used, applying the modified Fisher's exact test and the Fisher's exact test variant, respectively.

The analysis was conducted using the modified Fisher's exact test. The g:Profiler g:GOSt tool (version e109_eg56_p17_1d3191d) was applied to the gene list, using the g:SCS method for multiple testing correction, with a significance threshold of an adjusted $P < 0.05$. For EnrichR, an unadjusted $P < 0.005$ was considered as significant. The findings were cross validated through an

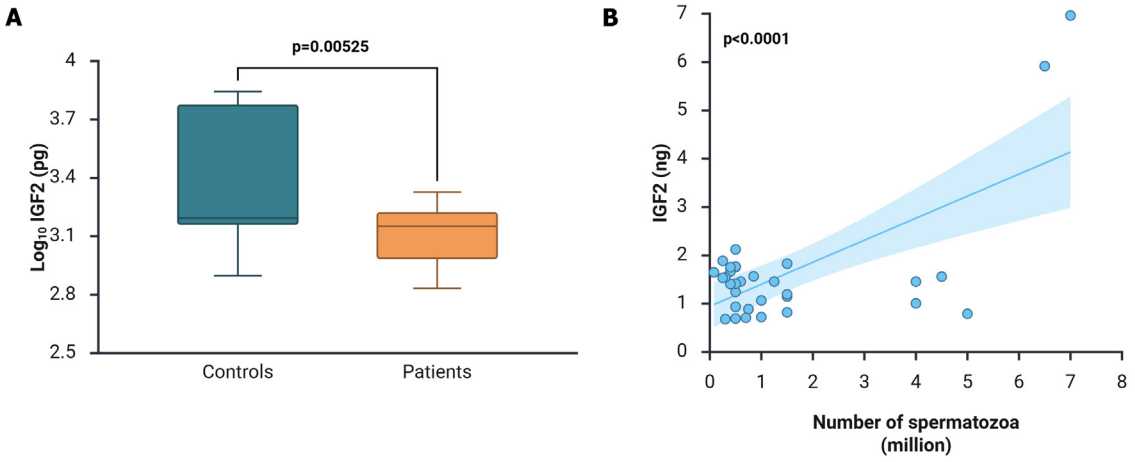


FIGURE 2 Sperm insulin-like growth factor 2 (IGF2) levels: Intergroup comparison and correlation analysis. (A) Intergroup comparison of IGF2 levels. Levels were quantified using enzyme-linked immunosorbent assay and normalized to the total protein extracted from spermatozoa obtained after gradient centrifugation. Patients: 26 men with oligozoospermia; controls: five donors with normozoospermia. The mean IGF2 levels of non-log-transformed data were 1.33 ± 0.42 ng in patients and 3.33 ± 2.87 ng in controls. To address data skewness, a logarithmic transformation was applied. For logarithmic transformation, data in pg were used. Boxplot shows median with interquartile range and range. Data distribution was assessed using the Shapiro–Wilks test, and intergroup comparison was conducted using the Student's t-test for independent samples. Statistical significance was set at $P \leq 0.05$; (B) scatter plot showing the relationship between absolute IGF2 levels and the number of spermatozoa retrieved after gradient centrifugation. IGF2 levels normalized to the total protein extracted from spermatozoa obtained after gradient centrifugation. The line of best fit was obtained through linear regression, whereas the strength of the association was evaluated using Pearson's correlation coefficient. The surrounding band denotes the 95% confidence interval of the regression estimate ($n = 31$).

extensive literature review (*Chicco and Agapito, 2022*). Specifically, to investigate the molecular basis of sperm IGF2 influence on early embryo development, gene ontology terms were corroborated by examining studies linking them to early embryogenesis.

RESULTS

Human study

Descriptive analysis

A total of 27 white couples met the inclusion criteria and were included in the study. All couples sought treatment at the ART centre for male factor infertility, with each male partner presenting with oligozoospermia. None of the enrolled couples presented with female-factor infertility. The median sperm concentration for the male partners was 4.5 million/ml, with 11 of the 27 cases (40.7%) having concentrations between 1 and 5 million/ml, and five of the 27 cases (18.5%) having concentrations below 1 million/ml.

The median age of female partners was 36.7 (interquartile range: 31.8–38.1) years, and two of the 27 women (7.4%) were aged over 40 years. The ovaries were stimulated with a long-acting GnRH agonist in 16 couples (59.3%) and a short-acting GnRH agonist in 11 (40.7%). Twenty-four couples underwent ICSI, whereas three couples received IMSI. A summary of the couples' clinical characteristics and hormonal values is presented in Supplementary Table 1.

A total of 192 embryos were generated through ART, and their development was monitored using time-lapse technology (Supplementary Table 2). On average, 7.1 ± 2.6 embryos per couple were included in the analysis, resulting in the transfer of two blastocysts in 20 couples, one blastocyst in six couples, and no transfer in one couple. Among the 26 couples who underwent embryo transfer, implantation occurred in 16 patients (61.5%), as shown by positive beta-HCG results. Clinical pregnancy was confirmed in 12 women (46.2%) through visualization of the gestational sac and detection of the fetal heartbeat. Of the remaining patients, three (11.5%) had only a biochemical pregnancy, two (7.7%) had a miscarriage and one (3.8%) had an ectopic pregnancy. Among those who achieved a clinical pregnancy, 11 (42.3%) delivered live births, including two sets of twins.

Intergroup comparison

Sperm IGF2 levels measured in the post-gradient aliquot discarded after oocyte injection in 27 patients were compared with those in the post-gradient fraction from five donors with normozoospermia, who served as controls. Sperm IGF2 levels in patients followed a normal distribution ($P = 0.20$), with a mean of 1.33 ± 0.42 ng (range: 0.68–2.12). Donor IGF2 values were positively skewed (3.33 ± 2.87 ng); therefore, statistical comparisons were made on log10-transformed data. After normalizing for total protein concentration, IGF2 levels were significantly lower in patients than in controls (log-transformed $P = 0.005$). The non-logarithmic mean values are presented here to provide clinically interpretable data, whereas log-transformed values can be assessed in FIGURE 2, panel A. A positive correlation was observed between the absolute IGF2 levels and the number of spermatozoa recovered after gradient centrifugation ($r = 0.65$; $P < 0.001$) (FIGURE 2, panel B).

Sperm insulin-like growth factor 2 protein levels and embryo kinetics

At tPNappear, sperm IGF2 protein levels were negatively correlated with this time point ($r = -0.22$, $P = 0.04$) (FIGURE 3 and TABLE 1), a result that was confirmed in the adjusted analyses ($P \leq 0.05$) (TABLE 2). Interestingly, the direction of the correlation reversed at PNfading and t2. This resulted in a positive association between sperm IGF2 and these time points, both in unadjusted and adjusted analyses. Specifically, using the adjusted

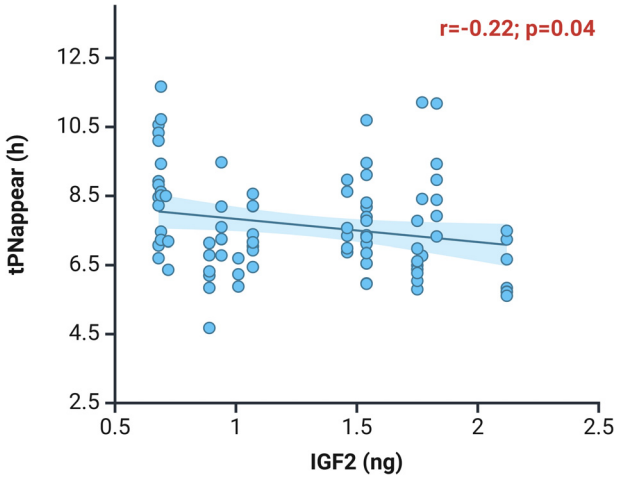


FIGURE 3 Sperm insulin-like growth factor 2 (IGF2) levels and time of pronuclei appearance (tPN_{appear}): Correlation analysis. A scatter plot showing the relationship between absolute IGF2 levels (normalized to the total protein extracted from spermatozoa obtained after gradient centrifugation) and the tPN_{appear}. The line of best fit was obtained through linear regression, whereas the strength of the association was evaluated using Pearson's correlation coefficient. The surrounding band denotes the 95% confidence interval of the regression estimate ($n = 88$).

model 2, sperm IGF2 levels were positively associated with early embryo morphokinetic parameters from tPNfading to t5 ($P \leq 0.05$), indicating that higher IGF2 levels were linked to faster progression through these early developmental stages. No significant associations were observed for later time points, from t7 to tEB. Correlation coefficients for significant time points ranged from 0.29 to 0.44, reflecting moderate associations after adjustment for female age, BMI, serum AMH and number of injected oocytes (model 2) (TABLE 1 and TABLE 2). No significant correlation was found between sperm IGF2 protein levels and the 2PN rate ($P = 0.68$) either. Concerning early embryo morphology, sperm IGF2 levels did not seem to influence good-quality embryo rate in day 3 ($P = 0.16$) (data not shown).

Predictive value of embryo development

To evaluate whether sperm IGF2 protein levels could predict embryo development, a ROC curve was constructed. As expected, the analysis revealed that sperm IGF2 protein levels were not predictive of blastocyst formation. The threshold of 0.82 ng yielded the highest area under the curve for predicting blastocyst development on day 5, as assessed by standard morphological criteria (blastocoel expansion, cell number and zona thinning), with a sensitivity of 86.7% and a specificity of 30.6% (AUC 0.521; $P = 0.667$) (FIGURE 4).

TABLE 1 CORRELATION ANALYSIS BETWEEN SPERM INSULIN-LIKE GROWTH FACTOR 2 PROTEIN LEVELS AND EMBRYO MORPHOKINETICS

Parameters	Unadjusted	
	r	*P*-value
tPN_{appear}	−0.22	0.04[a]
tPN_{fading}	0.21	0.01[a]
t2	0.25	0.003[a]
t3	0.15	0.08
t4	0.13	0.155
t5	0.28	0.004[a]
t6	0.16	0.11
t7	0.19	0.06
t8	0.15	0.16
t9	0.17	0.12
tM	0.04	0.71
tB	0.05	0.69
tEB	0.16	0.30

Pearson's coefficient (r) was used for correlation analysis.

[a] Statistical significance was set at $P \leq 0.05$.

PN, pronuclei; t, time; tB, time when a crescent-shaped area begins to emerge from the morula; tEB, time when an increase in volume and expansion of the blastocoel cavity becomes visible; tM, time of morula.

TABLE 2 MULTIPLE REGRESSION ANALYSIS BETWEEN SPERM INSULIN-LIKE GROWTH FACTOR 2 PROTEIN LEVELS AND EMBRYO MORPHOKINETICS.

Parameters	Adjusted (model 1)			Adjusted (model 2)		
	β	$r_{partial}$	*P*-value	β	$r_{partial}$	*P*-value
tPN_{appear}	0.001	−0.35	0.02[a]	0.001	−0.31	0.05[a]
tPN_{fading}	0.003	0.29	0.006[a]	0.004	0.36	0.001[a]
t2	0.005	0.38	0.0004[a]	0.005	0.44	0.0001[a]
t3	0.004	0.26	0.03[a]	0.005	0.33	0.006[a]
t4	0.003	0.21	0.07	0.004	0.29	0.02[a]
t5	0.005	0.24	0.08	0.009	0.36	0.009[a]
t6	0.003	0.16	0.27	0.007	0.29	0.04[a]
t7	0.004	0.18	0.23	0.005	0.22	0.14
t8	0.004	0.16	0.27	0.007	0.26	0.08
t9	0.005	0.19	0.23	0.008	0.28	0.08
tM	−0.000	−0.01	0.93	−0.000	−0.008	0.96
tB	−0.001	−0.03	0.87	−0.001	−0.04	0.85
tEB	−0.004	−0.22	0.33	−0.003	−0.25	0.27

Adjusted stepwise multi-regression models included the following confounding factors: the female partner's age, serum anti-Müllerian hormone levels and number of injected oocytes in model 1; and all variables included in model 1 plus the body mass index in model 2.

[a] Statistical significance was set at $P \leq 0.05$

PN, pronuclei; t, time; tB, time when a crescent-shaped area begins to emerge from the morula; tEB, time when an increase in volume and expansion of the blastocoel cavity becomes visible tM, time of morula.

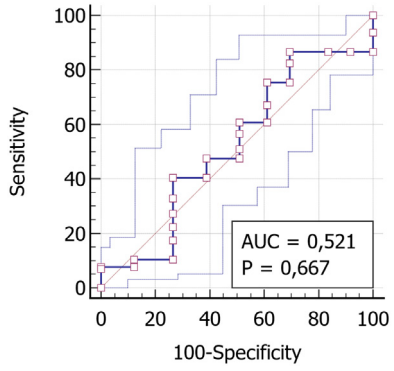


FIGURE 4 Predictive analysis of sperm insulin-like growth factor 2 (IGF2) protein levels for embryo development to the blastocyst stage on day 5. Receiver operating curve analysis included a sample size of 192 embryos. Sperm IGF2 protein levels were quantified using enzyme-linked immunosorbent assay, with the ratio of IGF2 concentration to total protein concentration in the sample taken into account. The central solid line represents the empirical receiver operating characteristic (ROC) curve. The two flanking lines indicate the 95% confidence band obtained via bootstrap resampling (1000 iterations). The area under the curve (AUC) and its 95% confidence interval were calculated using DeLong's method. Reported AUC values refer to the central ROC estimate: AUC = 0.521 (95% CI 0.448 to 0.594, $P = 0.667$).

Outcomes of assisted reproductive technology

Patients were categorized based on ART outcomes. The mean IGF2 protein levels did not differ significantly between groups with positive versus negative beta-HCG levels (Supplementary Figure 1, panel A), those who achieved clinical pregnancy compared with those who did not (Supplementary Figure 1, panel B) or those who delivered a live baby versus those who did not (Supplementary Figure 1, panel C). Further sub-group analysis based on the age of the female partners (≤ or > than 35 years) revealed no significant difference (Supplementary Table 3).

Animal study

To further explore the potential role of IGF2 in early embryonic development, how exposure to the protein affects gene expression in preimplantation embryos was investigated. Parthenogenetically activated mouse oocytes were incubated either with rIGF2 or in control medium until they reached the blastocyst stage. RNA sequencing was then conducted to assess transcriptional changes. In rIGF2-treated parthenotes, 46 genes were upregulated and 63 genes were downregulated compared with controls (FIGURE 5, Supplementary Figure 2 and Supplementary Table 4). g:Profiler g:GOSt, one gene ontology molecular function term, one gene ontology biological process term and two gene ontology cellular component terms were identified. The most strongly associated terms were 'protein binding' (GO:0005515), with $P = 0.003075$, and 'positive regulation of biological process' (GO:0048518), with $P = 0.00615$ (FIGURE 6). The genes contributing to the enrichment of these pathways are presented in Supplementary Figure 3 and Supplementary Table 5.

The gene ontology term 'positive regulation of biological process' is defined as 'any process that activates or increases the frequency, rate or extent of a biological process. Biological processes are regulated by various mechanisms, including the control of gene expression, protein modification, or interaction with a protein or substrate molecule' (see gene ontology:0048518, definition). According to this definition, this term is closely interconnected with the gene ontology term 'protein binding'. Indeed, a significant overlap was observed in the genes enriching both pathways (Supplementary Table 5). 'miR127 In Mesendoderm Differentiation' and 'Mechanisms Associated With Pluripotency' were the top associated terms from gene ontoloogy ($P = 1.03 \times 10^{-3}$ and $P = 1.06 \times 10^{-3}$, respectively) with EnrichR (Supplementary Figure 4), both of which are associated with early embryogenesis.

DISCUSSION

Mature IGF2 protein has been identified in both the sperm cytoplasm (*Cannarella et al., 2022*) and seminal fluid, with the latter correlating with sperm quality (*Wu et al., 2024*). In the present study, we aimed to investigate whether sperm IGF2 protein levels influence embryo morphokinetics and ART outcomes. Given the timeline of IGF2 activity, we hypothesized that sperm-delivered IGF2 protein could affect events occurring on the first day after fertilization, as sperm-carried IGF2 mRNA is translated (and influences embryo development) on day 2 (*Cannarella et al., 2024*), whereas embryo *IGF2* gene transcription begins (and exerts its effects) on day 3 (*Pringle*

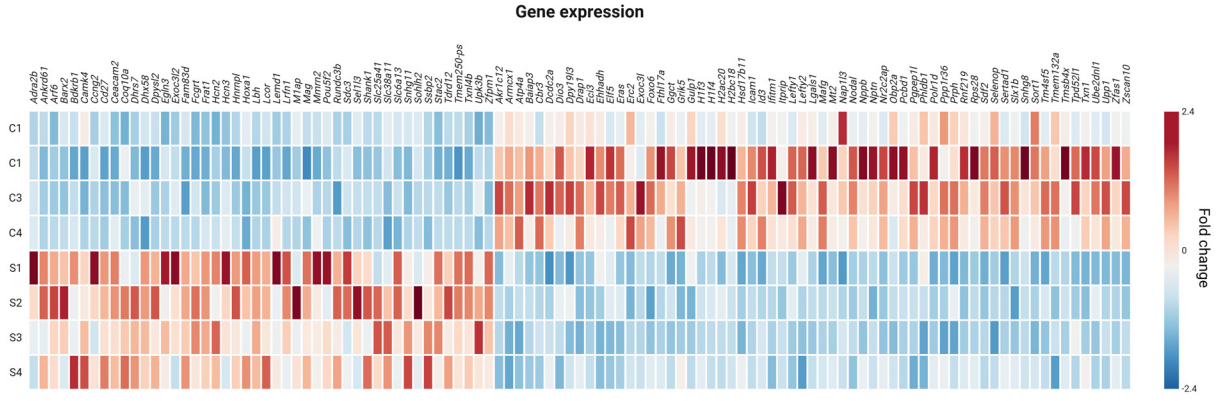
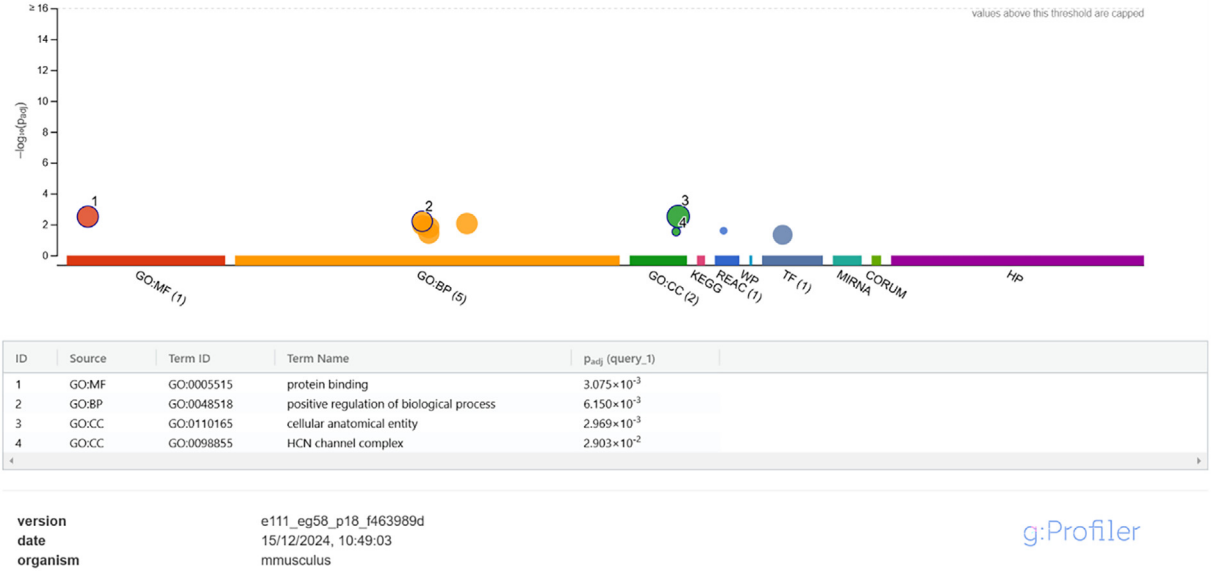


FIGURE 5 Heatmap showing differently expressed genes in murine parthenotes incubated with recombinant mouse insulin-like growth factor 2 (rIGF2). Parthenogenetically activated FVB/NJ-strain oocytes were treated for 3 days with 50 μM rmIGF2 ($n = 60$) compared with vehicle controls ($n = 60$). Differential gene expression was considered statistically significant with an adjusted $P < 0.05$ and a more than twofold change. A total of 46 genes were found upregulated and 63 were downregulated. The analysis included four replicates (15 embryos each) for both vehicle-incubated (controls, C1-4) and rIGF2-incubated samples (S1-4), with several animals contributing oocytes for each replicate.



ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:MF	GO:0005515	protein binding	3.075×10^{-3}
2	GO:BP	GO:0048518	positive regulation of biological process	6.150×10^{-3}
3	GO:CC	GO:0110165	cellular anatomical entity	2.969×10^{-3}
4	GO:CC	GO:0098855	HCN channel complex	2.903×10^{-2}

version	e111_eg58_p18_f463989d
date	15/12/2024, 10:49:03
organism	mmusculus

g:Profiler

FIGURE 6 Overview of enrichment analysis results obtained with g:Profiler. Upper panel: the Manhattan interactive plot shows the results of the enrichment analysis. The x-axis represents functional terms, grouped and colour-coded according to data sources: molecular function (MF) from gene ontology (red), biological process (BP) from gene ontology (orange), cellular component (CC) from gene ontology (GO) (green), Kyoto Encyclopedia of Genes and Genomes from biological pathways (fuchsia), Reactome (REAC) from biological pathways (electric blue), Wiki Pathways (WP) from biological pathways (sky blue), TRANSFAC (TF) from regulatory motifs in DNA (blue), mirTarBase (MIRNA) from regulatory motifs in DNA (water green), CORUM from protein databases (green), human phenotype (HP) from human phenotype ontology (violet). The y-axis shows the adjusted *P*-values of enrichment, displayed on a negative log10 scale. Light circles represent non-significant terms (if present). Bottom panel: source, term identifier, term name and adjusted *P*-values of significant terms are shown. The query can be found at: https://biit.cs.ut.ee/gplink/l/aknyiXTPrSr.

and Roberts, 2007; *Lee et al., 2014*; *Schulz and Harrison, 2019*). Supporting this hypothesis, regression analyses revealed that sperm IGF2 protein levels positively associated with the timing of pronuclei formation, a critical event on day 1. This finding is further supported by our animal experiments, in which rIGF2 incubation enriched pathways related to protein binding, a key process for pronuclei formation mediated by microtubule interactions (*Scheffler et al., 2021*), potentially providing mechanistic insight into a causal link. Particularly, the pathway enrichment analysis results align with the hypothesis that incubation with rIGF2 protein may have activated a pathway that enhances the frequency of protein modification and interaction, including microtubule interaction, which plays a crucial role in pronuclear formation (*Scheffler et al., 2021*). This mechanistic pathway offers a substrate-level explanation for the findings observed in humans.

For the animal experiments, we carefully considered whether to deliver IGF2 via direct microinjection or by supplementing the culture medium. Although microinjection offers precise delivery, it imposes significant physical stress on the embryo that can independently alter gene expression and introduce confounding effects. Therefore, we chose incubation with IGF2 in the culture medium as a more physiological and less invasive approach. This method reduces technical bias in transcriptomic analyses and better mimics the natural conditions surrounding fertilization and early embryonic development. Our choice of IGF2 dosage was informed by a previous in-vitro study using incubation methods. Notably, this demonstrated that supplementing culture media with IGF2 significantly enhances oocyte maturation by reducing reactive oxygen species production and minimizing spindle and chromosome abnormalities, resulting in a higher proportion of embryos reaching the blastocyst stage (*Muhammad et al., 2020*). These findings underscore the crucial role of IGF2 availability in maintaining oocyte quality and supporting early embryo development.

A strength of our animal experiments was the use of parthenogenetic embryos, which, by lacking a paternal genome, helped us in the attempt to identify the effects of sperm-delivered IGF2 without interference from embryo *IGF2* gene transcription.

Regarding the human study, consistent with our hypothesis, correlations between IGF2 levels and embryo morphokinetics changed direction on tPNfading and on day 2 (possibly owing to the influence of sperm-carried IGF2 mRNA as a confounder) and were no longer significant at later preimplantation stages. Adjusting for IGF2 mRNA would help isolate the effect of IGF2 protein without interference. This is not, however, feasible, as the post-gradient aliquot is entirely used for protein evaluation, making it impossible to assess IGF2 mRNA in the same fraction. This limitation is a result of low cytoplasmic content of sperm cells and, in infertile patients, the typically limited number of spermatozoa in post-gradient samples. In addition, reflecting its biological action being primarily restricted to day 1 after fertilization, sperm IGF2 protein levels showed limited predictive value for blastocyst development by day 5, in contrast to the transcript (*Cannarella et al., 2024*).

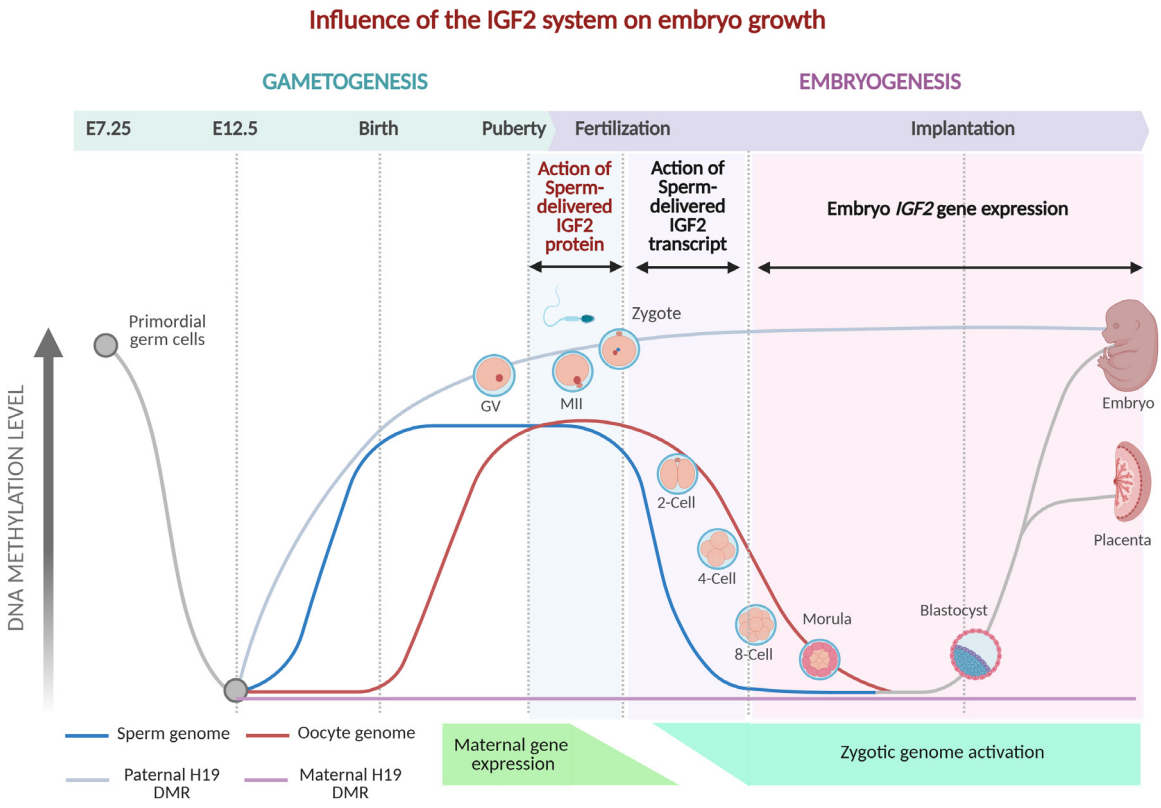


FIGURE 7 Influence of the Insulin-like growth factor 2IGF2 system on embryo growth. The IGF2 protein delivered by spermatozoa to the oocyte plays a critical role during the first day post-fertilization, before translation of the sperm-derived transcript and before zygotic genome activation. *IGF2* is an imprinted gene expressed exclusively from the paternal chromosome; thus, the spermatozoon serves as the source of IGF2 for the oocyte. Specifically, spermatozoa deliver both IGF2 protein and transcript. The protein is believed to act rapidly after fertilization by stimulating pronuclear formation in the zygote. The transcript, translated into protein after the first 24 hours after fertilization, supports embryo growth up to day 3. Thereafter, the embryo begins transcribing its own IGF2. Expression levels are regulated by the methylation status of the H19 differentially methylated region (DMR), inherited from the paternal chromosome. Created in BioRender. Calogero, A. (2025) https://BioRender.com/zq6ijlb.

An additional finding was the significant difference in sperm IGF2 levels between patients with oligozoospermia and donors with normozoospermia. Normalized protein analysis confirmed higher IGF2 expression in spermatozoa from patients with oligozoospermia, which may be associated with differences in *H19* DMR methylation patterns that regulate *IGF2* expression (*Cannarella et al., 2023*). Furthermore, sperm IGF2 protein levels were positively correlated with the number of spermatozoa retrieved after post-gradient centrifugation. As post-gradient sperm retrieval is influenced by both total sperm count and sperm progressive motility, our finding may suggest that higher sperm quality is associated with elevated IGF2 levels. This finding aligns with a previous study linking low seminal fluid IGF2 to poor sperm quality, increased inflammation and DNA damage (*Wu et al., 2024*). Although no significant differences in sperm IGF2 levels were observed between couples grouped by ART outcome, the sample size for this analysis was insufficient, and therefore, no definitive conclusions can be drawn.

This study represents an exploratory attempt to dissect the physiopathological role of individual sperm-borne growth factors, specifically IGF2 protein, in early embryonic development. Although no association was found between IGF2 protein levels and clinical outcomes, our findings contribute to a broader understanding of how sperm-derived molecules may influence the oocyte at fertilization (FIGURE 7). It is plausible that these factors act synergistically, with their combined action triggering key molecular events in the zygote. Clarifying the relative contribution of the IGF2 protein versus its mRNA (investigated in our previous study) may help prioritize future investigations, especially considering the technical challenges of analysing both. Early post-fertilization events are increasingly recognized as key determinants of embryonic competence (*Coticchio et al., 2023*). In particular, a cross-sectional study involving 905 patients and 1466 blastocysts reported variations in the timing of paternal pronucleus appearance (tpPNappear) across blastocysts developing on days 4 to 7, with delayed timing observed in day 7 compared with day-5 blastocysts (*Fujiwara et al., 2025*). These findings suggest that identifying the molecular signals influencing these early stages may be crucial to improving our understanding of embryo development.

These considerations should be interpreted with caution owing to several limitations. The presence of some weak *P*-values further suggests that certain associations should be validated in larger cohorts. Notably, the cohort mirrors real-world clinical scenarios, with two of the 27 couples having female partners over 40 years old, consistent with current epidemiological trends. Although this may be viewed as a limitation, it also

strengthens the generalizability of the findings to typical ART patients, rather than a specific clinical subset. Furthermore, although multivariate regression analysis accounted for several maternal confounders, including age, BMI and serum AMH levels, other potential confounders such as lifestyle factors, environmental exposures and genetic background were not addressed, which could influence the observed associations. Additionally, the study included a control group of healthy donors, but this group was limited in size and may not fully represent the broader fertile population.

Another important limitation lies in the translational aspect of our findings. The human clinical data and animal experimental models differ significantly in design and biological context, which complicates direct comparisons and extrapolations between species. Such differences should be carefully considered when interpreting the results. Finally, although measuring sperm *IGF2* transcript levels and adjusting the analysis accordingly would have allowed for a more precise understanding of the protein's role, this was not feasible owing to the low sperm concentrations obtained after post-gradient centrifugation in patients with oligozoospermia. Consequently, protein and transcript levels could not be assessed from the same sample, limiting the ability to fully disentangle their respective contributions.

Despite these limitations, the present study uncovers previously unrecognized mechanisms and provides novel insights into fundamental aspects of reproductive medicine. Future research should aim to validate these findings in larger, well-controlled and possibly multicentre cohorts, with comprehensive consideration of potential confounders and standardized methodologies.

In conclusion, these preliminary findings indicate a possible role for sperm-delivered IGF2 in early post-fertilization events, particularly pronuclei formation through modulation of microtubule interactions. Although our results suggest that healthy spermatozoa may contribute to the earliest stages of embryo development, these observations should be interpreted with caution owing to the exploratory nature of the study and its inherent limitations. Further research is necessary to validate these findings and to elucidate the potential implications for reproductive biology and infertility. Nonetheless, this work opens the possibility of uncovering novel molecular factors involved in early embryo development, which may ultimately inform future diagnostic or therapeutic strategies for idiopathic infertility.

AUTHORS' ROLES

RC: conception and study design, data acquisition, human data analysis, animal superovulation, oocyte retrieval, rIGF2 incubation transcriptome analysis, writing of the first draft; OJR: design of the animal study, animal data analysis and interpretation; SC: design of the human study, human data analysis and interpretation; SR: collection of human samples, human data analysis and interpretation; AG: design of the human study, human data analysis and interpretation; QY: transcriptome and bioinformatic analysis, animal data analysis and interpretation; FM: measurement of sperm Igf2, human data analysis and interpretation; IA: measurement of sperm IGF2, human data analysis and interpretation; CL: measurement of sperm IGF2, human data analysis and interpretation; SB: recruitment of donors with normozoospermia; SF: recruitment of donors with normozoospermia; GL: measurement of sperm IGF2, human data analysis and interpretation; LL: human data analysis and interpretation; NI: human data analysis and interpretation; SDL: critical contribution to study design; SLV: design of the human study, human data analysis and interpretation; AEC: conception and design of the study, project administration, data interpretation. All the authors revised the article critically, approved the final version of the article, and are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

This study was supported by a grant from the American Society for Reproductive Medicine.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version at doi:10.1016/j.rbmo.2025.105202.

REFERENCES

Abusheikha, N., Lass, A., Burnley, A., Brinsden, P., 2001. In vitro fertilization cycles converted to intrauterine insemination because of poor follicular response have low success rates. Fertil Steril 75 (3), 634–635. https://doi.org/10.1016/s0015-0282(00)01739-8. PMID: 11239557.

Ali, A.H., Ajina, T., Ali, M.B., Mehdi, M., 2022. Efficacy of density gradient centrifugation technique (DGC) in enhancing sperm cell DNA quality for assisted reproductive technique. Middle East Fertil Soc J 27, 22.

Ashapkin, V., Suvorov, A., Pilsner, J.R., Krawetz, S.A., Sergeyev, O., 2023. Age-associated epigenetic changes in mammalian sperm: implications for offspring health and development. Hum Reprod Update 29 (1), 24–44.

Barton, S.C., AC, Ferguson-Smith, Fundele, R., Surani, MA., 1991. Influence of paternally imprinted genes on development. Development 113 (2), 679–687.

Bradford, MM., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248–254.

Calogero, A.E., Cannarella, R., Agarwal, A., Hamoda, T.A.A., Rambhatla, A., Saleh, R., et al., 2023. The Renaissance of Male Infertility Management in the Golden Age of Andrology. World J Mens Health 41 (2), 237–254.

Cannarella, R., Condorelli, R.A., La Vignera, S., Bellucci, C., Luca, G., Calafiore, R., et al., 2020. IGF2 and IGF1R mRNAs Are Detectable in Human Spermatozoa. World J Mens Health 38 (4), 545–551.

Cannarella, R., Crafa, A., Barbagallo, F., Lundy, S.D., La Vignera, S., Condorelli, R.A., et al., 2023. H19 Sperm Methylation in Male Infertility: A Systematic Review and Meta-Analysis. Int J Mol Sci 24 (8), 7224.

Cannarella, R., Mancuso, F., Arato, I., Lilli, C., Bellucci, C., Gargaro, M., et al., 2022. Sperm-carried IGF2 downregulated the expression of mitogens produced by Sertoli cells: A paracrine mechanism for regulating spermatogenesis? *Front Endocrinol* (*Lausanne*) 13, 1010796.

Cannarella, R., Rando, O.J., Condorelli, R.A., Chamayou, S., Romano, S., Guglielmino, A., et al., 2024. Sperm-carried IGF2: towards the discovery of a spark contributing to embryo growth and development. Mol Hum Reprod 30 (9), gaae034.

Castillo, J., Jodar, M., Oliva, R., 2018. The contribution of human sperm proteins to the development and epigenome of the preimplantation embryo. Hum Reprod Update 24 (5), 535–555.

Chen, E.Y., Tan, C.M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G.V., et al., 2013. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics 14 (1), 1–14.

Chicco, D., Agapito, G., 2022. Nine quick tips for pathway enrichment analysis. PLoS Comput Biol 18 (8), e1010348.

Ciray, H.N., Campbell, A., Agerholm, I.E., Aguilar, J., Chamayou, S., Esbert, M., et al., 2014. Time-Lapse User Group. Proposed guidelines on the nomenclature and annotation of dynamic human embryo monitoring by a time-lapse user group. Hum Reprod 29 (12), 2650–2660.

Coticchio, G., Ezoe, K., Lagalla, C., Zacà, C., Borini, A., Kato, K., 2023. The destinies of human embryos reaching blastocyst stage between Day 4 and Day 7 diverge as early as fertilization. Hum

Reprod. 38 (9), 1690–1699. https://doi.org/10.1093/humrep/dead136. PMID: 37381073.
Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al., 2013. STAR: Ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21.
Ferraretti, A.P., La Marca, A., Fauser, B.C., Tarlatzis, B., Nargund, G., Gianaroli, L., ESHRE working group on Poor Ovarian Response Definition. 2011. ESHRE consensus on the definition of 'poor response' to ovarian stimulation for in vitro fertilization: the Bologna criteria. Hum Reprod. 26(7):1616–1624. doi:10.1093/humrep/der092.
Fujiwara, N., Ezoe, K., Coticchio, G., Cimadomo, D., Rienzi, L., Kato, K., 2025. Diverse developmental velocities of blastocyst development emerge as early as a few hours after insemination: morphokinetic analysis of fertilisation in days 4-7 blastocysts. J Assist Reprod Genet. https://doi.org/10.1007/s10815-025-03476-8. Epub ahead of print. PMID: 40234312.
Gelbaya, T.A., Potdar, N., Jeve, Y.B., Nardo, LG., 2014. Definition and epidemiology of unexplained infertility. Obstet Gynecol Surv 69 (2), 109–115.
Hagemann-Jensen, M., Ziegenhain, C., Chen, P., Ramskold, D., Hendriks, G.J., Larsson, A.J.M., et al., 2020. Single-cell RNA counting at allele and isoform resolution using Smart-seq3. Nat Biotechnol 38, 708–714.
Kuleshov, M.V., Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., et al., 2016. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res 44 (W1), W90–W97.
Lee, M.T., Bonneau, A.R., Giraldez, AJ., 2014. Zygotic genome activation during the maternal-to-zygotic transition. Annu Rev Cell Dev Biol 30, 581–613.
Liao, Y., Smyth, G.K., featureCounts, Shi W., 2014. An efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923–930.
Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15, 550.
Muhammad, T., Li, M., Wang, J., Huang, T., Zhao, S., Zhao, H., et al., 2020. Roles of insulin-like growth factor II in regulating female reproductive physiology. Sci China Life Sci 63 (6), 849–865.
Pringle, K.G., Roberts, CT., 2007. New light on early post-implantation pregnancy in the mouse: roles for insulin-like growth factor-II (IGF-II)? Placenta 28 (4), 286–297.
Reimand, J., Arak, T., Adler, P., Kolberg, L., Reisberg, S., Peterson, H., et al., 2016. Profiler—a web server for functional interpretation of gene lists (2016 update). Nucleic Acids Res 44 (W1), W83–W89.
Reimand, J., Arak, T., Vilo, J.g, 2011. Profiler—a web server for functional interpretation of gene lists (2011 update). Nucleic Acids Res 39 (suppl 2), W307–W315.
Reimand, J., Kull, M., Peterson, H., Hansen, J., Vilo, J.g, 2007. Profiler—a web-based toolset for functional profiling of gene lists from large-scale experiments. Nucleic Acids Res 35 (suppl 2), W193–W200.
Santiago, J., Silva, J.V., Howl, J., Santos, M.A.S., Fardilha, M., 2021. All you need to know about sperm RNAs. Hum Reprod Update 28 (1), 67–91.
Scheffler, K., Uraji, J., Jentoft, I., Cavazza, T., Mönnich, E., Mogessie, B., et al., 2021. Two mechanisms drive pronuclear migration in mouse zygotes. Nat Commun 12 (1), 841.
Schulz, K.N., Harrison, MM., 2019. Mechanisms regulating zygotic genome activation. Nat Rev Genet 20 (4), 221–234.
Smith, T., Heger, A., Sudbery, I., 2017. UMI-tools: Modeling sequencing errors in Unique Molecular Identifiers to improve quantification accuracy. Genome Res 27, 491–499.
Tadros, W., Lipshitz, HD., 2009. The maternal-to-zygotic transition: a play in two acts. Development 136 (18), 3033–3042.
Vallet-Buisan, M., Mecca, R., Jones, C., Coward, K., Yeste, M., 2023. Contribution of semen to early embryo development: fertilization and beyond. Hum Reprod Update 29 (4), 395–433.
World Health Organization, 2010. WHO laboratory manual for the examination and processing of human semen, 5th edn. Cambridge University Press, Cambridge.
Wu, J.G., Zhou, C.P., Gui, W.W., Liang, Z.Y., Zhang, F.B., Fu, Y.G., et al., 2024. Correlation of IGF2 levels with sperm quality, inflammation, and DNA damage in infertile patients. Asian J Androl. https://doi.org/10.4103/aja202487 Epub ahead of print. PMID: 39468803.
Xia, P., 1997. Intracytoplasmic sperm injection: correlation of oocyte grade based on polar body, perivitelline space and cytoplasmic inclusions with fertilization rate and embryo quality. Hum Reprod 12 (8), 1750–1755.
Zhang, Y., Parmigiani, G., Johnson, WE., 2020. ComBat-seq: Batch effect adjustment for RNA-seq count data. NAR Genom. Bioinform 2, lqaa078.

Received 7 April 2025; received in revised form 21 June 2025; accepted 4 August 2025.