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RESEARCH

Protein levels of p-mTOR and p-RPS6 in cumulus cells serve as non-invasive biomarkers for embryo quality and pregnancy outcome in IVF

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Abstract

Purpose To investigate whether the levels of mTOR signaling and ribosome biogenesis proteins in cumulus cells (CCs) can serve as non-invasive biomarkers for predicting embryo quality and pregnancy outcomes in women undergoing IVF.

Methods In this prospective study, discarded CCs were collected from 83 IVF patients. The protein levels of mTOR, phosphorylated mTOR (p-mTOR), ribosomal protein S6 (RPS6), and phosphorylated S6 (p-RPS6) were quantified by Western blot and normalized to β-actin. These molecular data were correlated with clinical parameters, including ovarian reserve, embryonic development, and pregnancy outcomes. Statistical analyses were performed to determine optimal predictive thresholds and to evaluate single and combined protein models.

Results Reduced levels of p-mTOR, p-RPS6, and RPS6 in CCs were robustly associated with superior IVF outcomes. Specific cutoff values were identified (e.g., p-mTOR < 0.45, p-RPS6 < 0.80) for predicting enhanced blastocyst formation and higher clinical pregnancy rates. Combining these biomarkers into multi-protein models significantly improved predictive accuracy for both embryonic development and pregnancy success compared to any single protein alone.

Conclusion The assessment of p-mTOR, p-RPS6, and RPS6 in cumulus cells provides a powerful, non-invasive strategy for prognostic assessment in IVF. A molecular profile characterized by lower levels of these proteins is indicative of high oocyte developmental competence and a greater likelihood of successful pregnancy, offering a valuable tool for clinical decision-making prior to embryo transfer.

Keywords p-mTOR · p-RPS6 · Biomarker · Embryo quality · Pregnancy

Introduction

A significant challenge in reproductive medicine is improving the success rates of IVF, which is influenced by numerous factors, including oocyte quality and embryo

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development. The identification of key biomarkers capable of predicting critical parameters in the IVF process would substantially improve clinical decision-making and increase the likelihood of achieving a successful pregnancy.

Oocyte morphological assessment is noninvasive and routinely performed in assisted reproductive technology [1]. Oocytes with abnormal meiotic spindles and polar bodies and chromosome misalignment can contribute to failed fertilization, arrested embryo development and further reduce the capacity for pregnancy [2–6]. Notably, the telomere length of oocytes can also predict embryonic development and pregnancy, and short telomeres in oocytes lead to fragmented, aneuploid embryos and poor reproductive outcomes [7–10]. Oocyte-cumulus cell communication is mediated by transzonal projections, which plays critical roles in promoting the oocyte growth and quality [11, 12]. Thus, cumulus cells can serve as indirect and noninvasive assessment factors of oocyte and embryo competence [13]. A multitude of studies have demonstrated an association between biomarkers in cumulus–oocyte complexes and successful IVF outcomes, and these biomarkers are involved in DNA damage, apoptosis, telomere length, mitochondrial DNA content, microRNAs and some key genes expressed in female cumulus cells [14–21]. PROK1 expression in follicular fluid is also correlated with follicular size and subsequent oocyte competence [22]. Successful fertilization and high-quality embryos are crucial for pregnancy. In addition, several invasive or noninvasive methods are available for embryo selection, including conventional morphology assessment, morphokinetics, preimplantation genetic testing, and metabolomics [23–27].

Single-cell analysis of human ovaries revealed that mechanistic target of rapamycin (mTOR) signaling is implicated in ovary aging and rapamycin alleviates granulosa cell senescence and ovarian aging [28, 29], which is consistent with a recent study that rapamycin delays aging by targeting mTOR and ribosome biogenesis, thereby improving oocyte quality and embryo development, and ultimately enabling successful pregnancy and live birth [30]. mTOR is known to be a key factor in integrating nutrients, growth factors, and energy and stress inputs, regulating many fundamental cell processes, including protein synthesis, autophagy, cell growth and metabolism. Deregulating mTOR signaling is associated with cancer, aging and diabetes [31]. mTOR contains two catalytic subunits, mTOR complexes 1 (mTORC1) and 2 (mTORC2), and mTORC1 promotes protein synthesis largely through the phosphorylation of p70S6 kinase 1 (S6K1) and eIF4E binding protein (4EBP), and S6K1 subsequently phosphorylates eukaryotic initiation factor 4B (eIF4B), eukaryotic elongation factor 2 kinase (eEF2K) and RPS6 [32–34]. Accordingly, inhibiting mTOR can reduce translation. Many studies have shown that inhibiting mTOR can extend the lifespan of different species [35–37]. Decreased S6K1 reduces protein synthesis and extends lifespan [38, 39]. Furthermore, mTOR inhibition improves ovarian and endocrine function in reproductive aging mice, and the maintenance of primordial follicles is regulated by mTOR signaling [40–42]. Additionally, mTOR activation is conserved in mouse and human embryos, and its inhibition induces embryonic diapause (a diapause-like dormant state) [43]. Notably, mTOR may act as a early checkpoint for developmental progression and successful implantation [44].

Given that a noninvasive and accurate indicator is necessary during the IVF procedure, we further explored mTOR and key ribosome translation components as potential biomarkers in cumulus cells of women undergoing IVF to accurately and effectively assess pregnancy outcomes.

Materials and methods

Human subjects

A total of 83 female patients were included in this study (average age: 31.8 ± 3.7 years). The cumulus cells were randomly collected on the day of oocyte retrieval and used for western blotting. Patients underwent IVF treatment at the Children's Hospital of Shanxi and Women Health Center of Shanxi (Shanxi, China), and oocyte retrieval was completed between October and November 2023. The inclusion criteria for donors were IVF/ICSI with their own oocytes, and donors were excluded from the study if they had endometriosis, cancer, chronic infections, or autoimmune or genetic diseases.

IVF-ET procedures

All patients received standardized controlled ovarian stimulation, oocyte retrieval, fertilization, and planned embryo transfer. In brief, in the mid-luteal phase of the previous menstrual cycle, 0.1 mg of triptorelin acetate (triptorelin) was used for downregulation for 14–16 days. On the 2nd to 5th days of the menstrual cycle, the serum hormone levels (FSH, LH, and E2) and ultrasonography results were monitored. Gonadotropin (Gn) was used after the downregulation standard was reached (FSH ≤ 5 mIU/mL, LH ≤ 5 mIU/mL, E2 ≤ 50 pg/mL). Gonadotropin (Gn) was given at 75–300 IU. The Gn dose was adjusted according to the growth of the follicle and the hormone levels. When more than one follicle with a diameter greater than 18 mm appeared, intramuscular injection of human chorionic gonadotropin (hCG) 6000–10000 IU was given. Oocyte retrieval was performed 36 h later.

The obtained oocytes were inseminated approximately 4 to 6 h by a conventional method or intracytoplasmic sperm injection according to the sperm quality, and a fertilization

check was then performed 16 to 18 h after insemination. The embryos were scored according to the morphological criteria [23]. The quality of the blastocysts was assessed according to the criteria of Gardner and Schoolcraft [24]. High-quality day-3 embryos or day-5-6 blastocysts were selected for fresh transfer or cryopreservation via vitrification for subsequent embryo transfer. Luteal-phase support was administered before embryo transfer and continued until 10 weeks of gestation. Biochemical pregnancy was defined as a serum level of human chorionic gonadotropin of more than 10 mIU per milliliter. Clinical pregnancy was defined as the presence of a gestational sac on ultrasonography. Ultrasonography was performed at 11 weeks of gestation to confirm ongoing pregnancy.

Cumulus cells

The cumulus−oocyte complex was retrieved through ultrasound-guided vaginal puncture, and small amounts of cumulus cells were mechanically stripped from MII oocytes of the same patients under stereomicroscopy (oocytes were used for clinical fertilization). Then, the isolated cumulus cells were dispersed into single cells with 0.03% hyaluronidase (H6254–500 MG; Sigma−Aldrich), washed twice in phosphate-buffered saline (PBS), and freshly collected cumulus cells were used immediately for Western blot analysis.

Western blot

Fresh cumulus cells were lysed in cell lysis buffer on ice for 30 min. After centrifugation at 10,000 × g for 10 min at 4 °C, the supernatant was transferred into new tubes. The concentration of each protein sample was measured via a bicinchoninic acid (BCA) assay kit according to the instructions, and the protein samples were boiled in SDS sample buffer at 95 °C for 5 min and stored at − 80 °C for one week. The protein in each cell extract was resolved via a 4−20% precast Tris−glycine gel (DG101−02−V2, TRANS) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membrane was blocked with 5% skim milk in TBST at room temperature for 2 h and then incubated with primary antibodies overnight at 4 °C. β-actin served as a loading control. The immunoreactive bands were then probed for 2 h at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. The protein bands were detected with a chemiluminescent HRP substrate (WBKLS0500, Millipore). The antibodies used for western blotting were as follows: p-mTOR (5536 T, Cell Signaling Technology), mTOR (2983S, Cell Signaling Technology), p-RPS6 (4858S, Cell Signaling Technology), RPS6 (2217S, Cell Signaling Technology), and β-actin (AC026, ABclonal). In each batch of experiments, the protein band density was quantified via ImageJ software with consistent parameters and normalized to that of β-actin [45]. The protein band density and relative protein levels are shown in Supplementary Table S1.

RNA-seq analysis

RNA-seq data for mouse oocytes and cumulus cells were downloaded from the previous publication (GSE184637). The trimmed clean reads were aligned to the UCSC mouse mm10 reference genome via HISAT2 with the default settings [46]. The read counts of each gene were calculated via featureCounts with default parameters [47]. GSEA was performed to identify enrichments associated with ribosomal subunits in oocytes and cumulus cells, and only gene sets with an FDR < 0.05 were considered significantly enriched [48]. Gene sets related to large and small ribosomal subunits were downloaded from Gene Ontology resources (GO:0015934 and GO:0015935).

Sample size calculation

A *priori* power analysis was performed to determine the sample size required to detect statistically significant changes associated with the clinical pregnancy rate. The study was designed to have a power of 0.85 at a two-sided significance level (α) of 0.05 to detect a 35% absolute difference in the clinical pregnancy rate between the two groups (from 35 to 70%). At least 35 patients per study group were needed, and we increased this number to 40 per study group (80 in total) to allow for a dropout rate of 10%. To account for potential experimental attrition (e.g., insufficient cellular material for protein extraction), the total sample size was increased to 82–84 (3%–5% failure rate).

Statistical analysis

Statistical analyses were performed via PRISM software (GraphPad 8 Software) and SPSS Statistics (version 26) software. Categorical data are presented as frequencies and percentages, and between-group comparisons were conducted with the chi-square test. Continuous data are expressed as the means (± SEMs), with a Mann−Whitney U test for between-group differences. *, **, *** and **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively. NS indicates not significant.

Results

Relative protein levels of p-mTOR, mTOR, p-RPS6 and RPS6 in human cumulus cells discarded from the IVF clinic

The transcription of ribosome genes is a prerequisite for protein translation, which is also downstream of mTOR [32, 49]. Ribosome genes are notably upregulated in oocytes and cumulus cells with increasing age and are associated with reduced fertility [30]. Additionally, genes related to large and small ribosomal subunits were aberrantly upregulated with age in mouse oocytes and cumulus cells (Fig. 1A–F), suggesting conserved ribosome dysregulation in aging oocytes and cumulus cells. The specific pattern of gene dysregulation identified the mTOR pathway as a potential candidate underlying the aging phenotype in oocytes and cumulus cells, which may affect subsequent embryo development.

To validate the effects of mTOR and related downstream proteins on embryonic development and pregnancy outcomes, we extensively measured protein levels in trace cumulus cells obtained from a large cohort of women and performed seven repeats of Western blot (at least 10 patients per batch) while ensuring consistent experimental conditions to reduce batch variation, and corresponding IVF clinical data, including indicators of ovarian reserve, embryo quality, and pregnancy outcomes, were also collected for each patient (Fig. 1G). A total of 83 cumulus cell samples were collected from women undergoing IVF treatment. The protein levels of p-mTOR, mTOR, p-RPS6, RPS6 and β-actin were subsequently detected in each cumulus cell sample under identical experimental conditions (Fig. 2 and Supplementary Fig. 1).

Next, the five proteins were quantified via ImageJ software and then divided by β-actin to calculate the relative levels of these four proteins in each cumulus cell sample (Fig. 3A). The results revealed that the relative protein levels of p-mTOR, mTOR, p- RPS6 and RPS6 as well as p-RPS6/RPS6 and p-mTOR/mTOR varied in the cumulus cells of each patient, indicating heterogeneity, but overall, the relative protein levels of p-mTOR, mTOR, p-RPS6 and RPS6 were consistent (Fig. 3A). In addition, most of the relative levels of these six proteins were between 0 and 2, the median value of each factor was basically between 0.6 and 0.8, and the frequency was high; that is, there were more people in this range (Fig. 3B). In summary, the levels of p-mTOR, mTOR, p- RPS6 and RPS6 in cumulus cells were heterogeneous, but the overall trends were consistent. However, whether this feature is related to embryonic development and pregnancy outcomes requires further detailed analysis.

The level of p-mTOR protein is associated with improved blastocyst development and pregnancy outcomes

Clinical data were collected from 83 patients, 73 of whom had completed embryo transfer and follow-up; 9 patients had not completed embryo transfer for their own reasons (time schedule), and 1 patient had no embryos for transfer (Fig. 3C, pie chart on the left). Among the 73 patients who had completed embryo transfer, forty-five patients were confirmed to be in clinical pregnancy, and the rest were not pregnant. (Fig. 3C, Pie chart in the middle). Among the follow-up results of the ongoing pregnancies of these 45 patients, forty-three patients were confirmed to have ongoing pregnancies (34 singletons and 9 twins), and 2 patients were lost to follow-up (Fig. 3C, Pie chart on the right).

Similar to the clinical use of AMH as a biochemical marker of the ovarian reserve [50], a clear threshold is needed for these proteins to predict clinical indicators. Depending on the relative protein level distribution, the median value of each protein distribution was used as the threshold for group analysis. First, we used the median p-mTOR protein level of 0.6 (Fig. 3B) as the grouping threshold and divided the data into two groups, ≥ 0.6 and < 0.6, and group analysis revealed that there was no significant difference in ovarian reserve-related indicators between the two groups (Supplementary Fig. 2 A). However, the number of blastocysts was significantly reduced when p-mTOR was ≥ 0.6 (Supplementary Fig. 2B). Next, we analyzed pregnancy outcomes, including biochemical pregnancy, indicated by hCG levels, clinical pregnancy and ongoing pregnancy, and there was no significant difference in these three indicators between the two groups. Notably, when the grouping threshold was narrowed to 0.45, patients' rates of clinical pregnancy and ongoing pregnancy significantly decreased in the group with p-mTOR ≥ 0.45 (Supplementary Fig. 2 C; Supplementary Table S2). When the median mTOR protein level of 0.7 was used as the grouping threshold, there was no significant difference in the ovarian reserve or embryo development-related indicators between the groups (Supplementary Fig. 3 A and B), but the clinical pregnancy rate and ongoing pregnancy rate decreased when the mTOR level was ≥ 0.7 (Supplementary Fig. 3 C; Supplementary Table S2). In summary, p-mTOR protein in cumulus cells could be used as a key factor in clinically predicting female embryo quality and pregnancy outcomes.

Levels of p-RPS6 and RPS6 proteins are linked to ovarian reserve, embryonic development, and pregnancy outcomes

mTOR phosphorylates and activates the ribosomal subunit S6 kinase, which further phosphorylates RPS6[33].

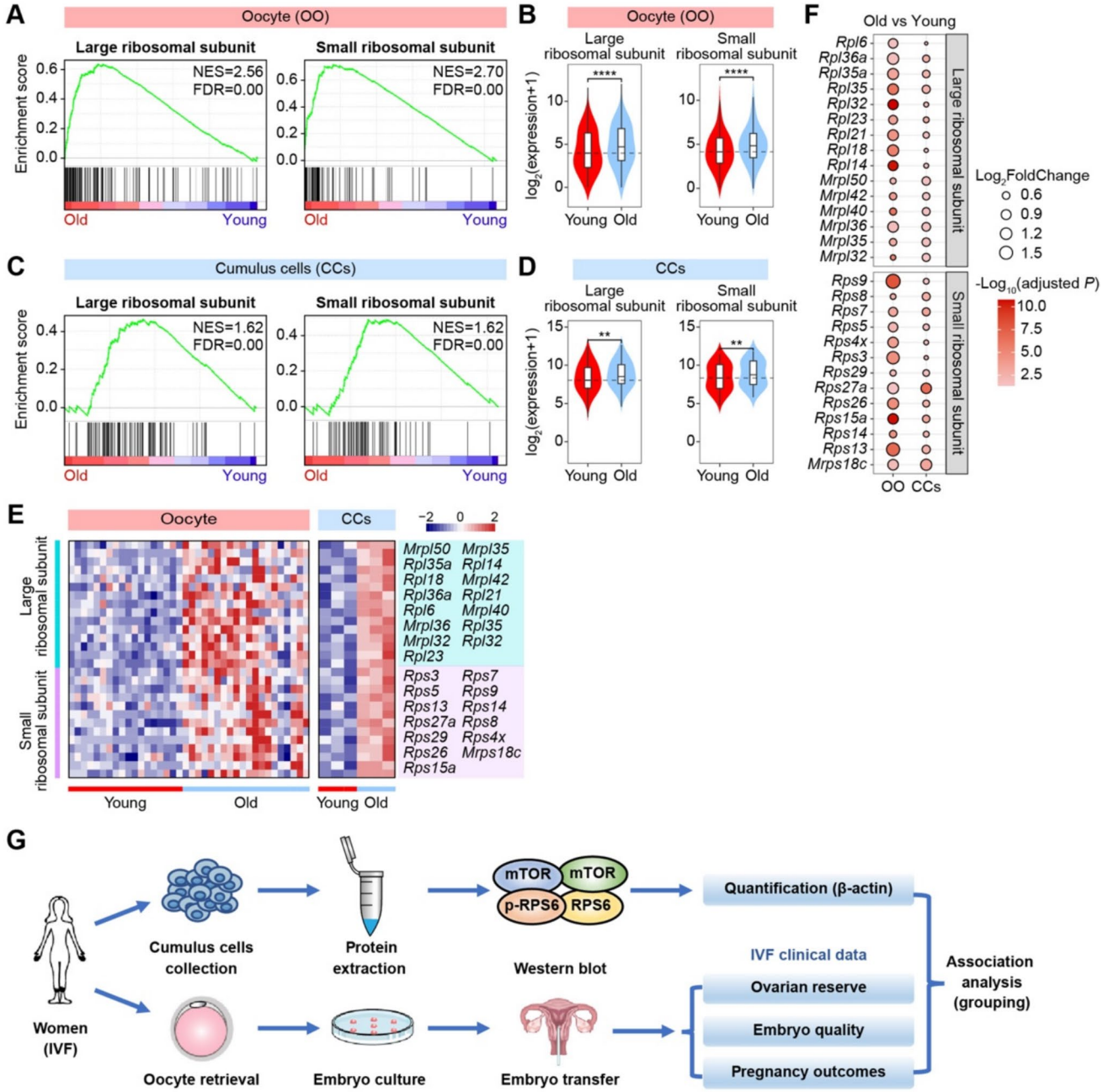

Fig. 1 Increased expression of ribosomal subunit-related genes during the aging of mouse oocytes and cumulus cells and workflow. (**A**) Gene set enrichment analysis (GSEA) indicating that upregulated genes in aging oocytes were highly enriched in large and small ribosomal subunit-related gene sets. Red, upregulated genes in old group. NES, normalized enrichment score; FDR, false discovery rate. (**B**) Violin plot showing the comparison of upregulated genes associated with large (left) and small (right) ribosomal subunits in young and old oocytes. $^{****}P < 0.0001$, two-tailed unpaired *t* test. (**C**) Gene set enrichment analysis (GSEA) indicating that upregulated genes in aging cumulus cells were highly enriched in large and small ribosomal subunit-related gene sets. (**D**) Violin plot showing the comparison of upregulated genes associated with large (left) and small (right) ribosomal subunits in young and old cumulus cells. $^{**}P < 0.01$, two-tailed unpaired *t* test. (**E–F**) Heatmaps showing the expression levels of ribosomal subunit-related genes whose expression is upregulated in the oocytes and cumulus cells of old mice (E). Bubble plot showing the fold changes (log2fold change) and significance (adjusted *P* value) between the young and old groups (F). (**G**) A Workflow integrating protein biomarkers in cumulus cell and clinical IVF data. The diagram illustrates the study pipeline, from the collection of trace cumulus cells through rigorous, batched Western blot analysis with internal controls, to the systematic compilation of patient-specific clinical parameters including ovarian reserve, embryo quality and pregnancy outcomes

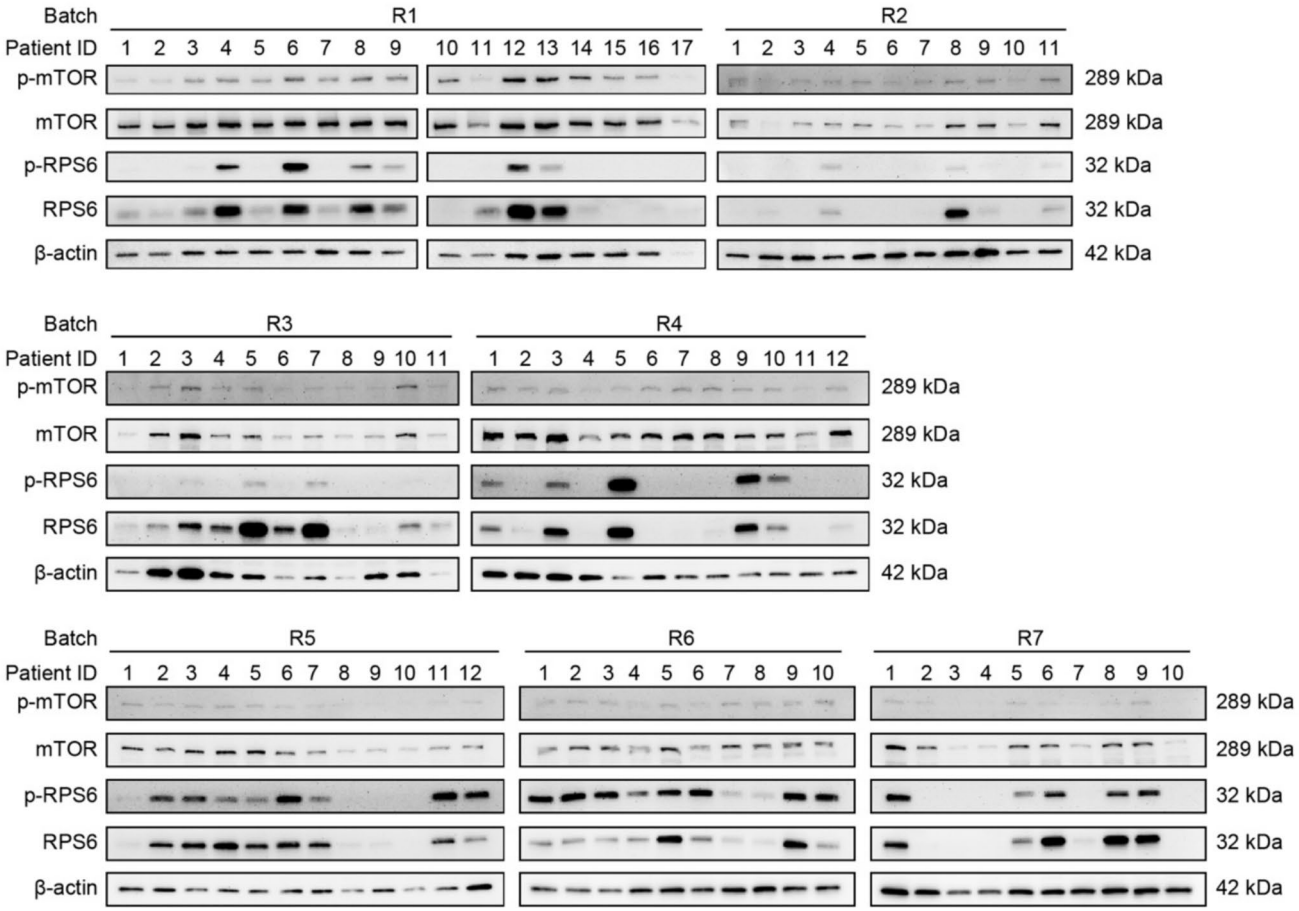

Fig. 2 Western blot analysis of the protein levels of p-mTOR, mTOR, p-RPS6 and RPS6 in female cumulus cells from the same patient. β-actin served as the loading control. Cumulus cells (CCs) were collected from women undergoing IVF on the day of oocyte retrieval. R1– R7 represent seven batches of CCs collected, with samples within each batch being biological replicates (R) harvested on the same day. The numbers 1–12 represent the patient identification numbers (Patient ID) corresponding to the CC samples from each collected batch; $n = 83$ donors

Therefore, we focused on whether the protein levels of p-RPS6 and RPS6 could also predict pregnancy outcomes. Similarly, we divided the levels of p-RPS6 into two groups with a median value of 0.8 as the grouping threshold and found that the AMH level, AFC, and number of oocytes retrieved and MII oocytes were significantly lower in the p-RPS6 ≥ 0.8 group (Supplementary Fig. 4 A). Importantly, AMH and the AFC are key indicators for clinically evaluating women's ovarian reserve [51]. In terms of embryonic development indicators, the numbers of blastocysts and A-grade blastocysts in the p-RPS6 ≥ 0.8 group were significantly lower than those in the p-RPS6 < 0.8 group (Supplementary Fig. 4B). We further analyzed the three pregnancy-related indicators and found that 0.8, as the grouping threshold of p-RPS6, can effectively indicate the pregnancy outcome of women. Specifically, when p-RPS6 was ≥ 0.8, hCG levels decreased significantly, followed by a significant decrease in the clinical pregnancy rate and ongoing pregnancy rate (Supplementary Fig. 4 C; Supplementary Table S2).

Next, we also divided the RPS6 protein levels into two groups using the median value of 0.8 as the grouping threshold. We found that when RPS6 was ≥ 0.8, the AMH and AFC decreased significantly, and the number of oocytes obtained and MII oocytes also decreased significantly (Supplementary Fig. 5 A), accompanied by a significant reduction in the number of zygotes and blastocysts (Supplementary Fig. 5B). However, the two groups (RPS6 < 0.8 vs. RPS6 ≥ 0.8) showed no significant differences in the three pregnancy outcomes. Notably, when the threshold was adjusted to 1.10, both the clinical and ongoing pregnancy rates were significantly lower in the RPS6 ≥ 1.10 group (Supplementary Fig. 5 C; Supplementary Table S2). In summary, the relative levels of p-RPS6 and RPS6 proteins in cumulus cells could also be used as key factors in the clinical evaluation of the female ovarian reserve, embryonic development, and

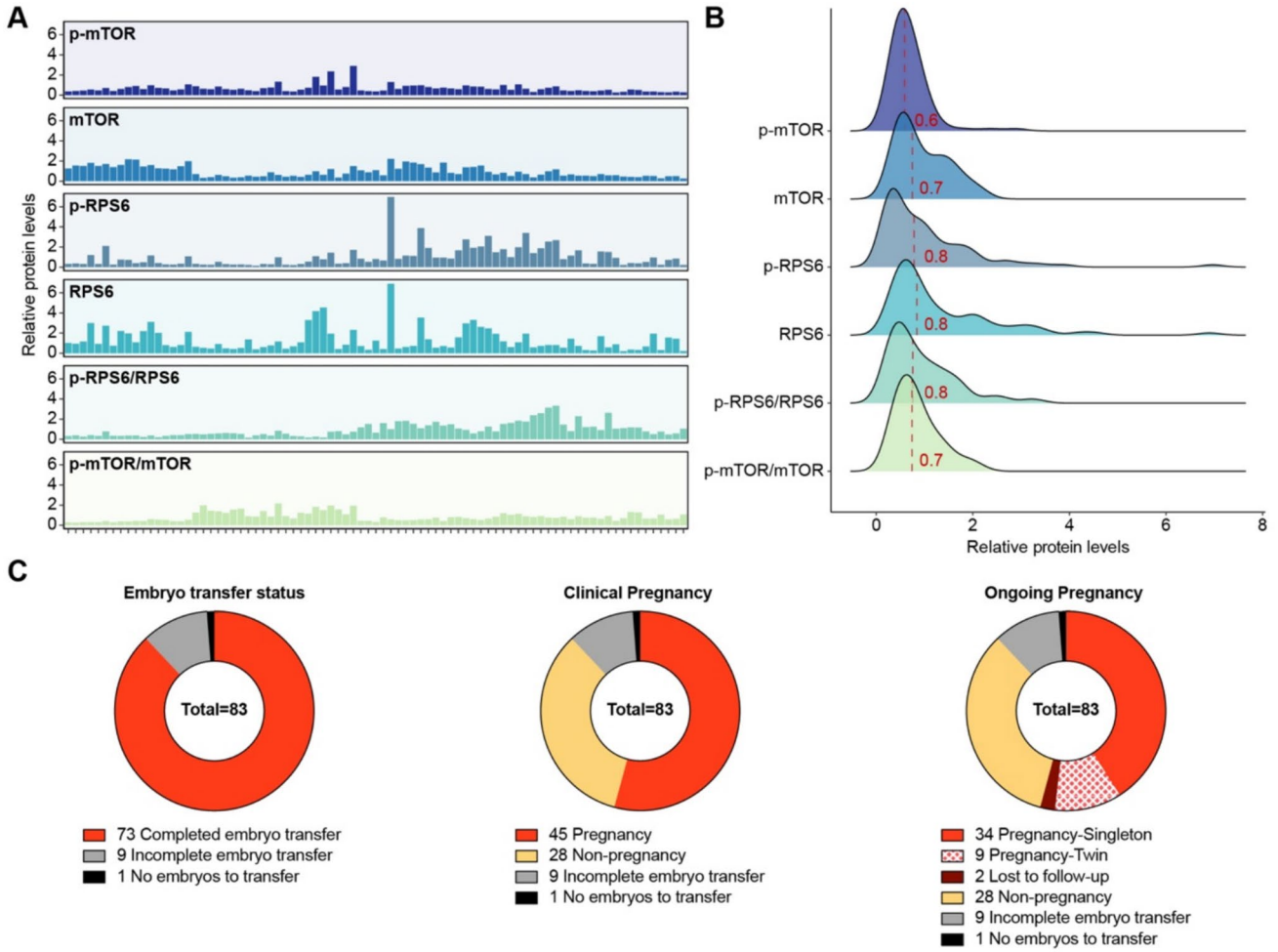

Fig. 3 Relative protein levels of p-mTOR, mTOR, p-RPS6 and RPS6 in cumulus cells from 83 women undergoing IVF. (**A**) The relative protein levels of p-mTOR, mTOR, p-RPS6 and RPS6 in CCs from 83 women are shown as ratios based on normalization to the β-actin band from the same patient. (**B**) Density ridgeline plots showing the distribution of p-mTOR, mTOR, p-RPS6 and RPS6 protein levels. The red dotted line represents the median value of the distribution of each protein. (**C**) Pie chart showing the embryo transfer status and pregnancy status of 83 women. Pregnancy outcome was evaluated by three indicators: biochemical, clinical and ongoing pregnancy. Biochemical pregnancy was defined as a human chorionic gonadotropin level of more than 10 mIU per milliliter, as measured 10 days after embryo transfer. Clinical pregnancy was defined as the presence of a gestational sac in the uterine cavity 30 days after embryo transfer, as detected by ultrasonography. Ultrasound scans were performed at 11 weeks gestation to confirm ongoing pregnancy

pregnancy outcomes. Moreover, compared with p-mTOR, p-RPS6 and RPS6 have been more broadly evaluated in these three aspects.

The combination of multiple proteins could better predict clinical indicators during IVF

With respect to the prediction of ovarian reserve, embryonic development, and pregnancy outcome, p-mTOR, p-RPS6, and RPS6 displayed significant directional trends that effectively predicted these three aspects, but they are not sufficient for evaluating the entire IVF procedure individually. A combined analysis of these proteins as a group could achieve this goal, providing a more holistic evaluation. Thus, we combined these proteins in different combinations and then performed group analysis. The combined results of p-RPS6 and RPS6 showed that when the protein levels of p- RPS6 and RPS6 were both greater than 0.8, AMH, the AFC, the number of oocytes obtained, and MII oocytes significantly decreased, along with a significant reduction in the number of zygotes, embryos and good-quality embryos on day 3, blastocysts and A-grade blastocysts on days 5–6 (Fig. 4A). However, other dual-protein combinations have not achieved such comprehensive evaluation effects (Supplementary Fig. 6). Consistently, the multiple combinations of "p-mTOR&p-RPS6&RPS6" or "p-mTOR&mTOR&p-RPS6&RPS6" can also comprehensively and effectively evaluate indicators related to ovarian reserve and embryonic

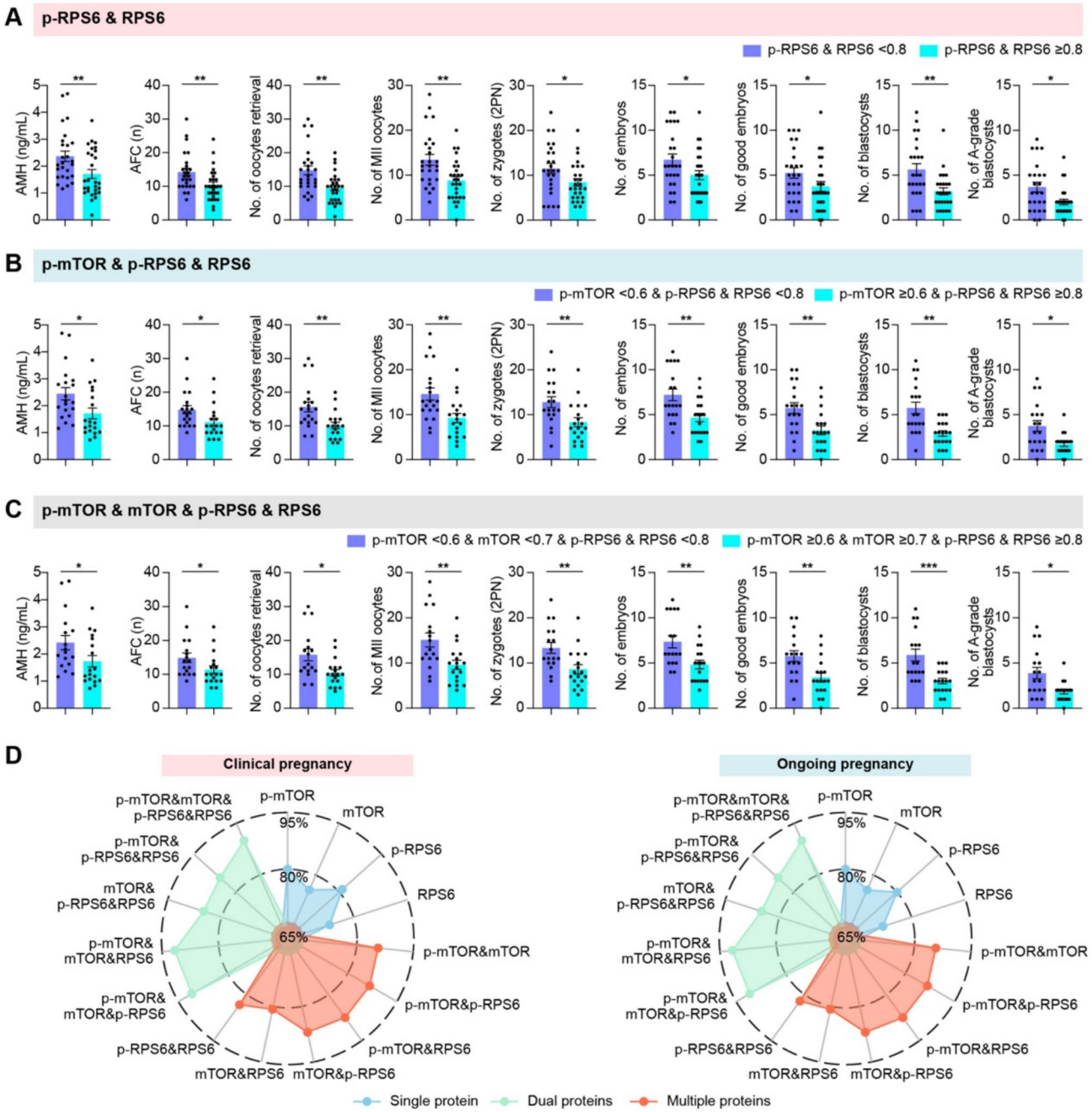

Fig. 4 Protein combinations used to assess ovarian reserve, embryo development and pregnancy outcomes. (**A**) Comparison of indicators associated with ovarian reserve and embryo development in the two groups, with p-RPS6 and RPS6 protein levels of 0.8 as the grouping threshold. $^{*}P<0.05$, $^{**}P<0.01$, Mann−Whitney U test. (**B**) Comparison of indicators associated with ovarian reserve and embryo development in the two groups, with p-RPS6 and RPS6 protein levels of 0.8 and a p-mTOR protein level of 0.6 as the grouping threshold. $^{*}P<0.05$, $^{**}P<0.01$, Mann−Whitney U test. (**C**) The protein levels of p-mTOR, mTOR, p-RPS6 and RPS6 were combined as the basis for grouping, and the changes in the ovarian reserve and embryo development-related indicators between the two groups were compared. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, Mann−Whitney U test. (**D**) The radar chart represents the percentages of clinical pregnancies (left) and ongoing pregnancies (right) associated with the four proteins under various conditions. Three colors represent three conditions, including a single protein (four), a combination of two proteins (six), and a combination of more than three proteins (five). For detailed comparisons and statistics between groups, see Supplementary Tables S2–S4

development (Fig. 4B and C), but the remaining combinations of the three proteins cannot be comprehensively evaluated (Supplementary Fig. 7). To summarize the above results, different combinations of p-mTOR, mTOR, p-RPS6 and RPS6 proteins could be used to better evaluate women's ovarian reserve and embryonic development.

A good ovarian reserve and good embryonic development are likely to increase the pregnancy rate of women. Therefore, we further explored whether protein combinations can be used to evaluate a woman's pregnancy outcomes more effectively. Interestingly, different combinations can effectively assess hCG levels, including "p-mTOR&p-RPS6", "mTOR&p-RPS6", "p-RPS6&RPS6", "p-mTOR&mTOR&p- RPS6", "p-mTOR&p-RPS6&RPS6" and "p-mTOR&mTOR&p-RPS6&RPS6" (Supplementary Fig. 8). Moreover, the clinical and ongoing pregnancy rates of these combinations increased significantly when the level of each protein was less than the corresponding threshold. (Fig. 4D; Supplementary Table S3 and S4). Furthermore, multiple protein combinations may be more effective in assessing pregnancy rates than single proteins (Fig. 4D), such as p-mTOR (absolute differences: 25.3% clinical pregnancy and 27.1% ongoing pregnancy) and "p-mTOR&mTOR&p-RPS6&RPS6" (absolute differences: 56.7% clinical pregnancy and 56.7% ongoing pregnancy) (Supplementary Table S2 and S4).

In brief, the levels of p-mTOR, mTOR, p-RPS6 and RPS6 proteins can be used to independently evaluate women's ovarian reserve, embryonic development, and pregnancy outcomes, but they do not cover all three indicators. Notably, when these proteins are combined, a variety of clinical indicators can be comprehensively assessed (Fig. 5). When the levels of these four proteins were below the corresponding thresholds, the number of high-quality day-3 or day-5-6 embryos was significantly increased. This approach also substantially improved pregnancy outcomes, demonstrating the critical role of these biomarkers in predicting IVF success.

Discussion

Using western blotting, we confirmed the heterogeneity of p-mTOR, mTOR, p-RPS6, and RPS6 protein levels in the cumulus cells of women undergoing IVF, which was associated with IVF clinical outcomes. Specifically, p-mTOR, p-RPS6, and RPS6 were inversely associated with indicators related to female ovarian reserve, embryonic development, and pregnancy outcomes during the IVF procedure. Moreover, elevated levels of p-mTOR, mTOR, p- RPS6, and RPS6 proteins in cumulus cells, either individually or in combination, were associated with poor pregnancy outcomes in women undergoing IVF (Fig. 5). In future, these biomarkers also could benefit the patients who are expected to respond precisely to rapamycin treatment.

By analyzing relative protein levels in cumulus cells and clinical IVF indicators, we identified more accurate and extensive indicators for evaluating IVF outcomes. Successful IVF outcomes resulted from high-quality embryo selection, but what were the selection criteria? This is a key issue that ART has faced. The current methods can be divided into invasive and noninvasive methods, and many methods exist, but most of them focus on oocyte and embryo competence. The morphological evaluation of oocytes and embryos is performed via a routine IVF procedure, which is still dependent on ocular evaluation, leading to different interpretations of embryo quality [52]. Morphokinetic assessment by time-lapse microscopy combined with standard morphological assessment can improve embryo selection and subsequent reproductive outcomes; however, this technology requires diligent and meticulous analysis by experienced TLM embryologists [25]. Many studies have focused on cumulus cells and mural granulosa cells, and these two types of cells are essential for oocyte development. A series of studies confirmed that several key genes in cumulus cells were correlated with good-quality oocytes and embryos, the implantation rate and successful pregnancy via different methods [21]. For example, lower CITED2 was linked to higher fertilization and implantation rates, whereas higher *VCAN* and *PTGS2* were associated with successful term birth [53, 54]. *HAS2*, *GREM1*, *GDF9* and *BMP15* expression influences oocyte maturation, fertilization, embryo quality and pregnancy outcome [19, 55].

Additionally, the telomere length of cumulus cells was also a good indicator. The relative telomere length in cumulus cells surrounding oocytes that developed into poor embryos was significantly shorter than that in those that produced good-quality embryos [16], and notably, the maximum telomere length in spare eggs was significantly inversely related to cytoplasmic fragmentation in embryos [7]. Significantly increased mtDNA copy numbers in cumulus cells are associated with good-quality embryos or implanted embryos, but the quantification of mtDNA is affected by BMI and smoking [17, 56]. Fluorescence lifetime imaging microscopy of NAD(P)H and FAD^+ could be used to characterize the metabolic state of cumulus cells, oocytes and embryos in a noninvasive manner, which could aid in embryo selection for successful pregnancy; moreover, the safety of this approach is of concern [57]. Although the above indicators can be used to evaluate IVF-related indicators, the detection of some indicators might be affected by other patient factors, and the safety of some methods still needs to be explored; alternatively, some clinical indicators might be evaluated. Therefore, there is a greater need for more accurate, safe, and comprehensive evaluation indicators in IVF procedures. Compared with previous clinical

A

Biomarker (Single protein)	Embryo quality						Pregnancy			
	Threshold	Zygotes	Embryos (Day3)	Good embryos	Blastocysts (Day5-6)	A-grade blastocysts	Threshold	Biochemical	Clinical	Ongoing
p-mTOR/β-actin	<0.60	NP	NP	NP	High	NP	<0.45	NP	+	+
	≥0.60				Low		≥0.45		−	−
mTOR/β-actin	<0.70	NP	NP	NP	NP	NP	<0.70	NP	+	+
	≥0.70						≥0.70		−	−
p-RPS6/β-actin	<0.80	NP	NP	NP	High	High	<0.80	+	+	+
	≥0.80				Low	Low	≥0.80	−	−	−
RPS6/β-actin	<0.80	High	NP	NP	High	NP	<1.10	NP	+	+
	≥0.80	Low			Low		≥1.10		−	−

B

Biomarker (Dual proteins)	Embryo quality						Pregnancy			
	Threshold	Zygotes	Embryos (Day3)	Good embryos	Blastocysts (Day5-6)	A-grade blastocysts	Threshold	Biochemical	Clinical	Ongoing
p-mTOR&mTOR	<0.60&0.70	NP	NP	NP	NP	NP	<0.45&0.70	NP	+	+
	≥0.60&0.70						≥0.45&0.70		−	−
p-mTOR&p-RPS6	<0.60&0.80	NP	NP	High	High	High	<0.45&0.80	+	+	+
	≥0.60&0.80			Low	Low	Low	≥0.45&0.80	−	−	−
p-mTOR&RPS6	<0.60&0.80	High	NP	NP	High	High	<0.45&1.10	NP	+	+
	≥0.60&0.80	Low			Low	Low	≥0.45&1.10		−	−
mTOR&p-RPS6	<0.70&0.80	NP	NP	NP	High	High	<0.70&0.80	+	+	+
	≥0.70&0.80				Low	Low	≥0.70&0.80	−	−	−
mTOR&RPS6	<0.70&0.80	NP	NP	NP	NP	NP	<0.70&1.10	NP	+	+
	≥0.70&0.80						≥0.70&1.10		−	−
p-RPS6&RPS6	<0.80&0.80	High	High	High	High	High	<0.80&1.10	+	+	+
	≥0.80&0.80	Low	Low	Low	Low	Low	≥0.80&1.10	−	−	−

C

Biomarker (Multiple proteins)	Embryo quality						Pregnancy			
	Threshold	Zygotes	Embryos (Day3)	Good embryos	Blastocysts (Day5-6)	A-grade blastocysts	Threshold	Biochemical	Clinical	Ongoing
p-mTOR&mTOR &p-RPS6	<0.60&0.70&0.80	NP	NP	High	High	High	<0.45&0.70&0.80	+	+	+
	≥0.60&0.70&0.80			Low	Low	Low	≥0.45&0.70&0.80	−	−	−
p-mTOR&mTOR &RPS6	<0.60&0.70&0.80	High	NP	NP	High	High	<0.45&0.70&1.10	NP	+	+
	≥0.60&0.70&0.80	Low			Low	Low	≥0.45&0.70&1.10		−	−
mTOR&p-RPS6 &RPS6	<0.70&0.80&0.80	NP	NP	High	High	High	<0.70&0.80&1.10	NP	+	+
	≥0.70&0.80&0.80			Low	Low	Low	≥0.70&0.80&1.10		−	−
p-mTOR&p-RPS6 &RPS6	<0.60&0.80&0.80	High	High	High	High	High	<0.45&0.80&1.10	+	+	+
	≥0.60&0.80&0.80	Low	Low	Low	Low	Low	≥0.45&0.80&1.10	−	−	−
p-mTOR&mTOR &p-RPS6&RPS6	<0.60&0.70&0.80&0.80	High	High	High	High	High	<0.45&0.70&0.80&1.10	+	+	+
	≥0.60&0.70&0.80&0.80	Low	Low	Low	Low	Low	≥0.45&0.70&0.80&1.10	−	−	−

Fig. 5 Summary of biomarkers from cumulus cells used to predict embryo quality and pregnancy. (**A**) Predictive effects of a single protein on embryo quality and pregnancy-related indicators during IVF-ET. Each protein in cumulus cells, which was obtained from the same patient and standardized to its own β-actin, was detected via WB. High-low represents the quality of the embryo, and "+" and "−" represent pregnancy and nonpregnancy, respectively. "NP" indicates nonpredictability. (**B**) Prediction effects of dual proteins on embryo quality and pregnancy-related indicators. (**C**) Effects of multiple proteins (≥three proteins) on predicting embryo quality and pregnancy-related indicators

evaluation indicators, the relative levels of the three proteins p-mTOR, p-RPS6 and RPS6, whether independently or in combination, could not only predict ovarian reserve and embryonic development but also pregnancy outcomes.

Studies of reproduction and beyond have evaluated the effects of various interventions on mTOR and downstream targets, with promising results. In animal models, mTORC1 is acutely sensitive to the drug rapamycin, and inhibition of the mTOR signaling pathway by rapamycin can extend the lifespan of aged mice [35], and reducing mTOR expression also increases the overall lifespan and reduces the number of aging tissue biomarkers [58]. Deletion of S6K1 in mice could increase the life span and protect against age-related diseases [38]. Notably, inhibition of mTOR by rapamycin also delays reproductive aging, which is characterized by fertility rebound and improved oocyte quality [40, 59]. Here, we showed that lower levels of p-mTOR, p-RPS6, and RPS6 in cumulus cells are associated with higher-quality embryos in women undergoing IVF, and these results were consistent with the studies described above. In terms of pregnancy

outcomes, our results further revealed that higher levels of p-mTOR, mTOR, p-RPS6, and RPS6 proteins were detrimental to pregnancy (biochemical, clinical, ongoing pregnancy) in women undergoing IVF. A clinical trial demonstrated that sirolimus, the most commonly used mTOR inhibitor in the clinic for allograft rejection, significantly improved pregnancy and live birth rates in RIF (recurrent implantation failure) patients [60]. Although our study confirmed that the p-mTOR, mTOR, p-RPS6, and RPS6 proteins, either alone or in combination, could be used to assess IVF-related clinical indicators, further studies are needed to integrate our biomarkers with established clinical indicators to explore whether they can truly improve female pregnancy rates.

This study should be interpreted within the context of its limitations. Despite the relatively large number of cumulus cell samples, owing to the scheduling issues of the participants, nine patients did not complete embryo transfer, so the sample size for evaluating subsequent pregnancy outcomes was still somewhat limited. Additionally, in the subsequent evaluation of pregnancy indicators, we tracked only three indicators: biochemical, clinical, and ongoing pregnancy. If indicators such as the live birth rate and birth weight could be analyzed, they might have more meaningful clinical value. Furthermore, the indicators we mined were valid and comprehensive, but whether they can be used clinically needs to be tested, so these exploratory findings should be validated in independent cohorts. While our study utilized Western blotting, we acknowledge its limitations regarding technical variability and labor intensity. A comparative analysis with non-invasive biomarkers from follicular fluid or embryo culture media, quantified via other methods such as ELISA, can help validate the depth and clinical relevance of our findings.

Conclusions

Relative protein levels of p-mTOR, mTOR, p-RPS6, and RPS6 could be used independently or in combination to evaluate indicators related to the female ovarian reserve, embryonic development, and pregnancy outcomes during the IVF procedure. Moreover, increased levels of p-mTOR, mTOR, p-RPS6, and RPS6 proteins portended poor pregnancy outcomes in women undergoing IVF. Although these data are insufficient to support specific clinical findings, these biomarkers can be used to analyze the reasons for IVF failure and provide ideas for subsequent treatment. Furthermore, if our assessment biomarkers can be combined with known clinical assessment indicators such as preimplantation genetic testing, it might ultimately help identify euploid embryos with greater developmental potential and improve the pregnancy rate of women undergoing IVF.

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Author contribution J.L. and P.Z. designed the experiments, conducted the major experiments, analyzed the data, and prepared the manuscript. H.W. C.L., G.F., and Y.Y. conducted part of the experiments and discussed the experiments and data analysis. X.W. provided clinical materials and relevant guidance for clinical diagnosis and revised the manuscript. L.L. conceived the project, designed the experiments, and revised the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Children's Hospital of Shanxi and Women Health Center of Shanxi (No. IRB-KYSB-2023–017).

Consent to participate Written informed consent was obtained from the donors.

Conflict of interest The authors declare no competing interests.

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