Oxidative damage-induced hyperactive ribosome biogenesis participates in tumorigenesis of offspring by cross-interacting with the Wnt and TGF-β1 pathways in IVF embryos

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ARTICLE

In vitro fertilization (IVF) increases the risk of tumorigenesis in offspring. The increased oxidative damage during IVF may be involved in tumor formation. However, the molecular mechanisms underlying this phenomenon remain largely unclear. Using a well-established model of oxidatively damaged IVF mouse embryos, we applied the iTRAQ method to identify proteins differentially expressed between control and oxidatively damaged zygotes and explored the possible tumorigenic mechanisms, especially with regard to the effects of oxidative damage on ribosome biogenesis closely related to tumorigenesis. The iTRAQ results revealed that ribosomal proteins were upregulated by oxidative stress through the Nucleolin/β-Catenin/n-Myc pathway, which stimulated ribosomes to synthesize an abundance of repair proteins to correct the damaged DNA/chromosomes in IVF-derived embryos. However, the increased percentages of γH2AX-positive cells and apoptotic cells in the blastocyst suggested that DNA repair was insufficient, resulting in aberrant ribosome biogenesis. Overexpression of ribosomal proteins, particularly Rpl15, which gradually increased from the 1-cell to 8-cell stages, indicated persistent hyperactivation of ribosome biogenesis, which promoted tumorigenesis in offspring derived from oxidatively damaged IVF embryos by selectively enhancing the translation of β-Catenin and TGF-β1. The antioxidant epigallocatechin-3-gallate (EGCG) was added to the in vitro culture medium to protect embryos from oxidative damage, and the expression of ribosome-/tumor-related proteins returned to normal after EGCG treatment. This study suggests that regulation of ribosome biogenesis by EGCG may be a means of preventing tumor formation in human IVF-derived offspring, providing a scientific basis for optimizing in vitro culture conditions and improving human-assisted reproductive technology.

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INTRODUCTION

In vitro fertilization (IVF) involves the manipulation of early embryos at a time when they may be particularly vulnerable to external disturbances. Environmental influences during embryonic development in vitro affect an individual’s susceptibility to epigenetic alterations and diseases such as cardiovascular disease, raising concerns about the potential consequences of IVF on the long-term health of offspring. The influence of IVF on tumor formation in offspring has been a topic of great concern for many researchers. There is increasing evidence that children who are born after IVF treatment have an increased risk of cancer1,2. A large registry-based study found a 42% higher risk of cancer in children conceived through IVF3. A 10-year follow-up study demonstrated that the total incidence of neoplasms was higher among IVF-conceived children (1.5/1000 person-years) than among naturally conceived children (0.59/1000 person-years; P < 0.001), and the association between IVF and total pediatric neoplasms remained significant after controlling for confounders (HR 2.48, 95% CI 1.71–3.50)4. However, the molecular mechanisms underlying this phenomenon remain largely unknown.

In vitro culture conditions, such as the culture medium, temperature, light, and oxygen concentration, which are unable to fully simulate the in vivo development environment, can lead to oxidative stress due to the production of excessive reactive oxygen species (ROS), causing developmental arrest in IVF-derived embryos. We have been researching the impacts of oxidative stress on IVF-derived embryos. In our previous studies, we treated mouse zygotes with different doses of hydrogen peroxide (H2O2) and found that 0.03 mM H2O2 was the minimum effective concentration able to reduce the blastocyst formation rate (without reducing the rates of 2-, 4-, and 8-cell embryo formation) by inducing DNA damage and chromosome aneuploidy, producing a model that strongly resembles the clinical phenomenon of oxidative damage in embryos during IVF5–7. DNA damage and chromosome aneuploidy are closely associated with tumorigenesis8,9. Cells from precancerous lesions and even hyperplastic lesions accumulate DNA alterations, confirming that the gradual accumulation of DNA damage leads to cancerous transformation8. Oxidative damage may be one of the most important reasons why IVF-conceived children have an
increased risk of pediatric neoplasms. To date, there is a lack of animal experimental research to reveal the effects and molecular mechanisms of oxidative damage on tumorigenesis in IVF-derived offspring. Therefore, we used an H$_2$O$_2$-induced model of oxidatively damaged IVF mouse embryos to study the tumorigenic mechanisms. It is also the first time that animal experiments have been applied to elucidate the possible pathogenesis of tumors in offspring derived from oxidatively damaged IVF embryos. Oxidative damage participates in tumorigenesis of offspring, possibly by altering the ribosome function of IVF-derived embryos.

Eukaryotic cytoplasmic ribosomes are large ribonucleoprotein complexes made up of small 40S and large 60S subunits. The 40S subunit consists of 18S ribosomal RNA (rRNA) and 33 different ribosomal proteins (Rps), whereas the 60S subunit consists of 25S, 5.8S, and 5S rRNA together with 47 ribosomal proteins (Rpl). Ribosome biogenesis involves the production and correct assembly of four rRNAs and 80 ribosomal proteins. Ribosomes are the molecular machines that produce all cellular proteins during a process called translation. Precise regulation of ribosome biogenesis is fundamental for maintaining normal cell growth and proliferation. Increasing evidence has underscored that multifaceted relations link abnormal ribosome biogenesis to cancer. Ribosome biogenesis is abnormally regulated in tumors under strong growth pressure and is mainly upregulated to increase the rate of protein synthesis. More importantly, most ribosomal proteins can influence proliferation and metastasis by performing extraribosomal regulatory functions involving binding to select critical target mRNAs. Hyperactive ribosome biogenesis can promote tumorigenesis via extraribosomal functions, including regulation of oncogene activation and tumor suppressor gene silencing and contribution to the activation of tumor-related signaling pathways. According to the literature, ribosomal proteins can be chemically modified by ROS, which may alter ribosome functions. However, no reports have been published on the alterations in ribosome functions induced by oxidative damage in IVF-derived embryos or on the role of abnormal ribosome function in tumorigenesis in offspring.

In this study, using a well-established model of oxidatively damaged IVF mouse embryos, we aimed to explore the relevant mechanisms of tumorigenesis in IVF-derived offspring, especially the effects of ROS on ribosome biogenesis closely related to tumorigenesis. In addition, the antioxidant (-)-epigallocatechin gallate (EGCG) has been shown to exert not just a promoting effect on embryo development but also a cancer chemopreventive effect by modulating various cell signaling pathways, such as inducing apoptosis, reducing proliferation, and regulating angiogenesis. Therefore, our further experiments focused on whether EGCG can antagonize ROS-induced alterations in ribosome biogenesis, which may be a means of preventing tumor formation in human offspring from IVF-derived embryos.

MATERIALS AND METHODS

Animals

Adult Kun-Ming mice (male: 3–6 months old; female: 4–8 weeks old) were obtained from the animal center of Shantou University Medical College. All work was carried out in accordance with the International Guiding Principles for Biomedical Research Involving Animals (2012 version) issued by the Council for the International Organizations of Medical Sciences. All experimental protocols were approved by the Laboratory Animal Ethics Committee of Shantou University Medical College (SUMP2014-014).

Reagents

Detailed information on the materials used in our experiment is listed in Supplementary Table 1.

TUNEL assay

A TUNEL assay was performed to analyze blastocyst apoptosis using the RiboAPO™ One-Step TUNEL Apoptosis Detection Kit (red) in accordance with the manufacturer’s instructions.

Karyotype analysis

Zygotes were treated with hypotonic solution (0.9% sodium citrate in distilled water containing 3% distilled water) at 37 °C for 40 min and then transferred into fixative I (methanol: acetic acid: H$_2$O = 5: 1: 2.5) for 5 min. When the color of the cell changed from brown to white and became translucent, the cell and a small amount of fixative II (methanol: acetic acid = 3: 1) were aspirated and released into a slide and immediately covered by a gentle flow of fixative II. The slide was placed into a Coplin jar filled with fixative II at room temperature overnight and then dipped into...
LC–MS/MS analysis. The labeled samples were mixed together in equal amounts, and 200 µg of the mixture was then fractionated with a high pH RP-HPLC column. The sample was loaded onto the column in buffer A (98% ddH2O and 2% acetonitrile, pH 10) and eluted with the following gradient: an initial increase to 5% buffer B (98% acetonitrile and 2% ddH2O, pH 10), a subsequent 64 min linear gradient from 5% buffer B and successive ramps to 18%, 32%, and 95% buffer B, with a flow rate of 0.7 mL/min. The sample was analyzed by a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), resuspended in solution C (99.9% ddH2O and 0.1% formic acid) and eluted with a gradient starting with 4% solvent D (99.9% acetonitrile and 0.1% formic acid) and ending at 95% solvent D with a flow rate of 350 nL/min for 70 min.

Database search. The LC/MS/MS spectra were searched against the UniProt protein database of mice using Proteome Discoverer software (version 1.3; Thermo Fisher Scientific). The proteins with a fold change (the ratio of intensity of protein expression in H2O2-treated zygotes to control zygotes, 119/118) >1.5 were considered significantly differentially expressed proteins. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to analyze the functional and pathway enrichment of differential genes.

Western blot analysis

Lysates of 300 zygotes (the control and H2O2-treated groups) were obtained using RIPA lysis buffer containing protease and phosphatase inhibitors. Protein samples were separated by SDS–PAGE, transferred onto PVDF membranes, and probed with primary antibody at 4 °C overnight, followed by HRP-conjugated secondary antibody at room temperature for 1 h. Protein bands were visualized by a chemiluminescence kit. Optical densities of bands were determined with BandScan 5.0 software (Glyko, Novato, CA, USA). The antibodies were optimized as described in Supplementary Table 1.

Real-time quantitative PCR analysis

Approximately 150 zygotes were prepared, and total RNA was extracted with an RNNaprep pure Micro Kit. RNA samples were reverse-transcribed into cDNA using FastKing gDNA Dispelling RT SuperMix. Real-time quantitative PCR (RT–qPCR) analysis was performed by Talent qPCR PreMix (SYBR Green) on the CFX Connect Real-Time PCR Detection System (BIO-RAD, CA, USA). The primers were described in Supplementary Table 2. RT–qPCR was conducted as described previously.19 Relative mRNA expression normalized to GAPDH was calculated using the 2−ΔΔCt method.

Statistical analysis

Data were collected from at least three independent experiments. Data expressed as the means ± standard deviations (SDs) were compared with Student’s t test. Data shown as percentages were analyzed using Pearson’s χ2 test. P < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS software 19.0 (IBM, Armonk, NY, USA).

RESULTS

Oxidative stress-induced developmental arrest, DNA damage, and chromosome aneuploidy in IVF mouse embryos

Oxidative stress. The iTRAQ technique can qualitatively and quantitatively analyze the dynamic changes of proteins in cells under different conditions, producing truly comprehensive information on cell function and process mechanisms.20 In addition, due to its advantage of high sensitivity, the iTRAQ technique is more suitable than other techniques for gaining insights into the changes in proteomic profiles from samples comprising a very small number of cells, such as IVF-derived embryos. Therefore, we used iTRAQ labeling to identify proteins differentially expressed between the control and H2O2-treated zygotes and successfully found 93 upregulated and 147 downregulated proteins among 1464 total identified proteins, providing a powerful platform for us to study the possible mechanisms of tumorigenesis in IVF-derived offspring. GO and KEGG analyses indicated that H2O2 treatment resulted in decreased activity of glutathione peroxidase (Gpx4 and Gpx6) and glutathione transferase (Gstm1, Gstm5, Gstm7, and Gsto2), contributing to the accumulation of ROS in IVF-derived embryos (Fig. 1a).

The yields of ROS between different groups were compared using DCF fluorescence intensity in zygotes (7.5 hpi) (Fig. 1b, c). The mean DCF fluorescence intensity in zygotes of the H2O2-treated group was approximately twofold higher than that in zygotes of the control group (P < 0.001), which indicated that the production of ROS was augmented. EGCG (20 µg/mL) treatment significantly reduced ROS generation in the H2O2-treated zygotes to a level nearly three-quarters that of the control group (P < 0.001).

Developmental arrest

The cleavage rate and blastocyst formation rate were monitored to explore the effects of ROS and EGCG on embryo development in vitro (Fig. 2a, b). Compared to the control group, exposure of zygotes to 0.03 mM H2O2 did not clearly reduce the rates of 2- and 4-cell embryo formation (P > 0.05) but led to a significant reduction in the blastocyst formation rate (P < 0.05), which was similar to the findings observed clinically. Conversely, the addition of EGCG to H2O2-treated zygotes effectively promoted blastocyst formation, causing blastocyst formation to return to the normal level (P < 0.05 vs. H2O2 treatment). In addition, the developmental potential of IVF-derived blastocysts with respect to the total cell number and allocation to inner cell mass (ICM) and trophoblast-derm was evaluated (Fig. 2c and Table 1). The average number of ICM cells and the ratio of ICM to total cells in blastocysts in the H2O2-treated group were decreased to ~50% of the control values (P < 0.05), suggesting that oxidative stress could lead to embryo developmental arrest. In the blastocysts that developed from H2O2/EGCG-treated zygotes, EGCG treatment not only increased the ICM cell number, which was not significantly different from that of the control group (P < 0.05 vs. H2O2 treatment), but also significantly increased the blastocyst total cell number, which was nearly 1.5-fold higher than that of the control group (P < 0.05 vs. both H2O2 treatment and control). These results indicate that EGCG improves the development and quality of in vitro embryos.

DNA damage. DNA damage was determined by measuring phosphorylated H2AX (γH2AX) using immunofluorescence staining, and apoptosis was detected by TUNEL assay (Fig. 3a–f). While there were few γH2AX foci and little TUNEL-positive staining in the control group, H2O2 treatment led to a remarkable increase in the proportion of γH2AX-positive embryos at the 1-cell, 2-cell, and 4-cell stages as well as in the percentages of γH2AX-positive cells and TUNEL-positive apoptotic cells in blastocysts (P < 0.05). These findings indicated that ROS caused DNA damage in IVF-derived embryos that was repaired by activation of the G2/M cell cycle checkpoint, but the associated DNA repair was incomplete until the blastocyst stage, and thus, the transferred blastocysts appeared to consist of oxidatively damaged and apoptotic cells. As expected, no γH2AX foci or TUNEL staining was observed in the majorities of H2O2/EGCG-treated embryos (P < 0.05 vs. H2O2 treatment). EGCG protected embryos from ROS-induced DNA
damage and increased the undamaged cells in preimplantation blastocysts.

Chromosomal aneuploidy. We used DAPI staining to observe lagging chromosomes/micronuclei and multinuclei of IVF-derived embryos caused by chromosome mis-segregation during first mitosis (Fig. 4a–d). The rates of lagging chromosomes/micronuclei and multinuclei were found to increase with H2O2 treatment (P < 0.05). However, the rates in the H2O2/EGCG-treated group were significantly lower than those in the H2O2-treated group (P < 0.05) and were close to those in the control group (P > 0.05).

Historically, α-tubulin, the main component of spindle microtubules, has been known to govern spindle microtubule dynamics during chromosome segregation. We next examined the localization of α-tubulin to assess abnormal spindle formation, which is closely associated with chromosome mis-segregation (Fig. 4e–g). In normal zygotes, newly nucleated microtubules self-organized into bipolar spindles during prometaphase; metaphase nuclei were predominantly condensed into tight bars aligned on the metaphase plate with two tight triangular bi-oriented microtubule arrays; chromosomes arrived at the cell poles, and spindle microtubules disappeared during telophase. In contrast, 25.45% (14/55) of the H2O2-treated zygotes exhibited features of aberrant microtubule formation, including loss of spindle bipolarity, microtubule breakage, monopolar spindles, and elongated spindles accompanied by chromosome misalignment at metaphase, which may act as a trigger for micronuclei formation (vs. control 2.08% [1/48], P = 0.002). It is worth mentioning that two
bipolar spindles were observed in a few H$_2$O$_2$-treated metaphase zygotes; however, the two spindles failed to align and come into close apposition to form a compound barrel-shaped structure, producing 2-cell embryos with one or two binucleated blastomeres, which provides a potential rationale for the multinucleated blastomere formation observed in human IVF-derived embryos. In contrast, the H$_2$O$_2$/EGCG-treated zygotes presented no obvious spindle abnormalities, and only one cell with chromosome breakage was observed (1.96 [1/51] vs. H$_2$O$_2$ treatment, $P = 0.001$).

Micronuclei and multinuclei are considered biomarkers of aneuploidy, so we examined the karyotypes of 2-cell embryos to identify the effects of ROS and EGCG on aneuploidy (Fig. 4h, i). The aneuploidy rate in the control group was lower than that in the H$_2$O$_2$-treated group ($P < 0.05$), revealing that ROS produced by the in vitro culture conditions may be one of the most important reasons for the high incidence of aneuploidy in IVF-derived embryos. This effect of ROS on aneuploidy was reversed by EGCG supplementation ($P < 0.05$), indicating that EGCG protects embryos in vitro from ROS-induced chromosome aneuploidy.

Identification of differentially expressed proteins in response to oxidative damage by iTRAQ labeling

DNA damage and aneuploidy formation induced by ROS during IVF may contribute to tumorigenesis in offspring. However, the molecular mechanisms underlying this phenomenon remain largely unclear. To determine possible mechanisms, we used iTRAQ labeling to identify proteins that were differentially expressed between the control and H$_2$O$_2$-treated zygotes. The MS/MS spectra used for the identification of H2A and Mat2a are shown in Fig. 5a, b. To validate the data obtained from the iTRAQ method, Mat2a protein levels were measured by western blotting and found to be suppressed by ROS in IVF-derived embryos ($P < 0.05$), consistent with the iTRAQ findings (Fig. 5c).

All of the identified and differentially expressed proteins were analyzed according to the GO database and classified into "cellular component", "molecular function", and "biological process" subcategories (a given gene product may exhibit one or more functional annotations).

Cellular component. Both the total identified proteins and the differentially expressed proteins were mainly enriched for the GO terms associated with cell part, intracellular part, and organelle. The cytoplasmic compartment was the most represented by the identified proteins (78.21%, 1145/1464), followed by the membrane (60.45%, 885/1464) and nuclear (22.81%, 334/1464) compartments. H$_2$O$_2$ treatment increased the proportion of nuclear proteins (45.42%, 109/240; $P < 0.001$) and decreased the proportion of cytoplasmic proteins (68.33%, 164/240; $P = 0.001$). The increased expression of Ranbp2, Lmna, Ipo5, and Lrrc59, which play positive roles in regulating protein import into the nucleus, suggested that oxidative stress triggered nuclear-cytoplasmic transport participating in DNA/chromosome repair in IVF-derived embryos. Furthermore, the upregulated proteins had a special relationship with the ribonucleoprotein complex (ribosome), whereas the downregulated proteins were more related to the cilium (Fig. 6a–c).

Table 1. Comparison of ICM cell number and ICM/total cell ratio in IVF embryos with different treatments.

| Treatment                        | ICM cell number | Total cell number | ICM/total cell ratio |
|----------------------------------|-----------------|-------------------|---------------------|
| Control group                    | 14.33 ± 1.15    | 47.33 ± 4.51      | 30.06 ± 5.07        |
| H$_2$O$_2$-treated group         | 7 ± 1*          | 41 ± 3            | 17.19 ± 3.12*       |
| EGCG-treated group               | 17.9 ± 1.3      | 70.51 ± 3.5*      | 25.39 ± 1.75        |
| H$_2$O$_2$ + EGCG-treated group  | 17.33 ± 2.08*   | 69.33 ± 5.51*     | 24.95 ± 1.36*       |

* $P < 0.05$ vs. control, *$P < 0.05$ vs. H$_2$O$_2$ treatment.
H$_2$O$_2$ treatment principally caused significant increases in the expression of ribosomal proteins, including small (Rps3a1, Rps7, Rps11, Rps14, Rps18, and Rps20) and large (Rpl6, Rpl8, Rpl10a, Rpl11, Rpl15, Rpl23, Rpl27, Rpl34, Rpl36a, and Rpl37a) ribosomal subunits and heterogeneous nuclear ribonucleoproteins (hnRNP A3, hnRNP F, hnRNP K, and hnRNP U).
Molecular function. Through analysis of molecular functions, we found that most of the total identified proteins were enriched for the binding function term (70.8%, 1037/1464), followed by catalytic activity (45.7%, 669/1464). A total of 82.8% (77/93) of the upregulated proteins were assigned to a binding function; in particular, H2O2 treatment increased the expression of proteins acting as structural constituents of the ribosome and involved in rRNA binding. In addition, 61.2% (90/147) of the downregulated proteins were assigned to catalytic activity; in particular, H2O2 treatment decreased the expression of proteins involved in
antioxidant activity, mainly glutathione peroxidase activity and glutathione transferase activity, resulting in the accumulation of ROS in IVF-derived embryos (Fig. 6d–f).

Biological process. The GO biological process analysis indicated that the top three biological process terms of all identified proteins were cell process, single-organism process, and metabolic process (Fig. 7a). While the downregulated proteins generally participated in the biological process of production, the 93 upregulated proteins were largely associated with gene expression and macromolecule metabolic processes such as DNA repair and replication and protein synthesis and degradation (Fig. 7b).

To better understand the involved pathways, these differentially expressed proteins were further analyzed by KEGG pathway enrichment analysis. According to the KEGG pathway maps, the major upregulated proteins were annotated into the ribosomal pathway, while the downregulated proteins were abundant in the glutathione metabolism pathway (Fig. 1a). Thus, the GO and KEGG functional enrichment analyses consistently revealed that suppression of glutathione peroxidase and glutathione transferase activity was responsible for the overproduction of ROS and subsequent responses to oxidative stress, which plays critical roles in the promotion of ribosomal protein expression (ribosome biogenesis) and ribosome function in IVF-derived embryos.

The ribosome is a molecular machine in charge of protein synthesis. It can be seen from the above functional analysis that ribosomal proteins were upregulated by ROS, which may have enhanced ribosome function to enable synthesis of an abundance of repair proteins to correct the damaged DNA/chromosomes in IVF-derived embryos. Three main molecular mechanisms were implicated in the repair of oxidative DNA damage in IVF-derived embryos. First, the upregulation of Psme3, hnRNP K, and H2BC3 suggests that the ATM/P53-dependent DNA damage checkpoint is fully activated and that G2/M cell cycle arrest is maintained for several hours until repair is effected and cells reenter the cell cycle. Our previous results confirmed that ROS caused G2/M cell cycle arrest via the ATM-Chk1-Cdc25-Cdc2 pathway, providing more time for DNA repair in IVF mouse zygotes. Second, the increase in Prkar2b expression and the corresponding decrease in Akap4 expression lead to dysfunction of cAMP-dependent PKA signaling, which engages in DNA repair by phosphorylating DNA damage response elements, such as ATR/ATM. Third, the elevated expression of Ranbp2, Dek, and H2BC3 promotes DNA double-strand break repair via nonhomologous end-joining, but not homologous recombination, during G2/M cell cycle arrest.

Possible mechanisms involved in tumorigenesis in offspring derived from oxidatively damaged IVF embryos

From the abovementioned 240 differentially expressed proteins, we enriched 58 tumor-related proteins, including 45 upregulated and 13 downregulated proteins, and analyzed tumor-related signaling pathways (Supplementary Tables 3 and 4). In addition to oncogene activation and tumor suppressor gene inactivation, several other possible signaling pathways involved in tumorigenesis in offspring derived from oxidatively damaged embryos were identified. The first identified pathway was the ribosome signaling pathway. The upregulation of ribosomal proteins (large 60S subunit, small 40S subunit, and hnRNPs) suggested that oxidative damage stimulated ribosome biogenesis in IVF-derived embryos, and persistent hyperactivation of ribosome biogenesis conferred many favorable advantages to cancer cells. Moreover, Nucleolin, n-Myc, Ddx17, and Syn2 participate in cancer progression when overexpressed by altering ribosome biogenesis. The second potential pathway identified was the Wnt signaling pathway. The upregulated expression of Nucleolin, Prksch, Prkar2b, and Zbed3 and the downregulated expression of Nfatc4 promoted cancer cell proliferation, migration, and invasion by activating the Wnt/β-Catenin signaling pathway. The third identified pathway was the TGF-β signaling pathway. Oxidative stress results in increased expression of calreticulin, Psme3, and vimentin, which regulate TGF-β1-induced proliferation and epithelial-mesenchymal transition (EMT) by modulating Smad signaling. Besides, Srsf5, Rdx, Trz2b, and NuMA1 function as novel oncogenes and are upregulated by ROS to regulate the cell cycle and apoptosis in IVF-derived embryos.

We applied MetaCore software to analyze the protein–protein interaction (PPI) networks of the differentially expressed proteins and identified a total of 59 biological networks. Within the networks analyzed, 23 pathways were associated with cellular metabolic processes, especially the cellular macromolecule biosynthetic processes, which produces DNA repair products but also provides sufficient protein for tumor cells to grow and proliferate (Supplementary Fig. 1). Ten pathways were associated with the cellular response to oxidative stress. Ten tumor-related pathways were primarily enriched for the regulation of cell apoptotic process (4 pathways), cell proliferation (2), cell cycle (1), cell differentiation (1), mesenchyme migration (1), and other terms, such as DNA conformation change (1), suggesting that the ribosome, Wnt, and TGF-β1 signaling pathways may promote tumorigenesis by regulating apoptosis (Supplementary Figs. 2 and 3).

mRNA levels of the key ribosome-/tumor-related differentially expressed proteins Nucleolin, n-Myc, Rpl15, Rpl36a, hnRNP K, β-Catenin, and TGF-β1

As we have noted previously, Nucleolin and n-Myc play positive roles in regulating the expression of nearly all ribosomal proteins, including Rpl15, Rpl36a, and hnRNP K, which may participate in tumorigenesis in offspring derived from oxidatively damaged embryos by cross-interacting with the Wnt and TGF-β1 pathways. Therefore, RT-qPCR was used to determine the mRNA expression levels of these key tumor-related differentially expressed proteins in 1-cell (M phase) and 8-cell embryos from different groups (Fig. 8a–g). The results showed high mRNA expression of Nucleolin, n-Myc, Rpl15, Rpl36a, hnRNP K, β-Catenin, and TGF-β1 in the 1-cell and 8-cell embryos of the H2O2-treated group compared with the control group (P < 0.05), which was consistent with the iTRAQ findings.

However, EGCG, due to its strong antioxidant activity, prevented the oxidative damage-induced dysfunction of ribosome biogenesis.
The mRNA expression of Nucleolin, n-Myc, Rpl15, Rpl36a, hnRNP K, β-Catenin, and TGF-β1 was significantly lower in the 1-cell and 8-cell embryos of the H2O2/EGCG-treated group than in those of the H2O2-treated group (P < 0.05), suggesting that EGCG treatment regulated the abnormal expression of ribosome-/tumor-related genes in oxidatively damaged IVF embryos. Furthermore, the levels of most ribosome-/tumor-related genes tended to decrease with the development of oxidatively damaged embryos but were still significantly higher than those in the control group (P < 0.05). Only Rpl15 expression increased remarkably from the 1-cell to 8-cell stages, suggesting that Rpl15 may be the most critical ribosomal protein in carcinogenesis in IVF-derived offspring. The Rpl15 level was restored to the normal level by EGCG supplementation under oxidative stress.

DISCUSSION
A growing number of scientists are beginning to realize that IVF-conceived children are at an increased risk of pediatric neoplasms. To date, research has focused mostly on the prevalence of neoplasms and the factors that induce them (e.g., causes of infertility, use of gonadotropins for controlled ovarian stimulation, and application of IVF or intracytoplasmic sperm injection). There is a lack of animal experimental research to reveal the molecular and genetic mechanisms of tumorigenesis in IVF-derived offspring. In this study, the in vitro culture conditions led to excessive production of ROS, which caused DNA damage and aneuploidy formation in IVF-derived embryos. The association of DNA damage/chromosome aneuploidy and tumorigenesis has long been recognized. Oxidative damage may be one of the most important reasons for tumorigenesis in IVF-conceived children. Therefore, we used this model of oxidatively damaged IVF mouse embryos to study the tumorigenic mechanisms. It is also the first time that animal experiments have been used to elucidate the possible pathogenesis of tumors in offspring derived from oxidatively damaged IVF embryos. We concluded that ROS-induced damage to DNA and chromosomes promotes tumor progression mainly via alterations in ribosome biogenesis as well as via activation of the Wnt/β-Catenin and TGF-β1/Smad signaling pathways.

Ribosomes are RNA-protein complexes responsible for protein synthesis. This study found that both rRNAs and ribosomal proteins were significantly downregulated in the 1-cell and 8-cell embryos of the H2O2/EGCG-treated group compared to the H2O2-treated group. The expression of ribosome- and tumor-related genes was restored to normal levels by EGCG supplementation under oxidative stress.
proteins can be chemically modified by ROS, which prompts ribosomes to synthesize an abundance of DNA repair proteins to correct damaged DNA in IVF-derived embryos at an early developmental stage. However, the increased percentages of γH2AX- and TUNEL-positive cells in the blastocyst suggested that DNA repair was insufficient, possibly resulting in persistent hyperactivation of ribosome biogenesis. Increasing evidence shows that persistent hyperactive ribosome biogenesis confers competitive advantages to cancer cells. In one study, RNA sequencing of freshly isolated circulating breast cancer cells revealed a subset with strong ribosome and protein synthesis signatures that were correlated with poor clinical outcomes. The essential role of increased ribosome biogenesis and protein synthesis in sustaining tumor cell growth and proliferation is well established. In addition, the execution of EMT, a migratory cellular program associated with tumor development and metastasis, is fueled by upregulation of ribosomal proteins. We also found that several ribosome-/tumor-related genes were particularly actively expressed even in 8-cell preimplantation embryos, and persistent hyperactivation of ribosome biogenesis could play essential roles in the initiation and progression of cancers in IVF-derived offspring.

Rpl15 was identified as the most significantly upregulated ribosomal protein in our iTRAQ experiment, and RT-qPCR analysis revealed stepwise upregulation of Rpl15 from the 1-cell to 8-cell stages in response to oxidative damage. It has been found that
Rpl15 can promote metastatic growth in multiple organs. The number of nucleoli and the expression of nucleolar proteins increased when Rpl15 was overexpressed, and abnormal increases in nucleolar size and number caused by dysregulation of ribosome biogenesis have emerged as hallmarks of the majority of spontaneous cancers. Mechanistically, overexpression of Rpl15 selectively enhances translation of other ribosomal proteins. Furthermore, Rpl15 siRNA-mediated downregulation induces cell cycle arrest by inhibiting cyclin-dependent kinases and results in a significant increase in the percentage of apoptosis in cancer cells. Therefore, Rpl15 is the most critical ribosomal protein in carcinogenesis, increasing the risk of tumor formation in IVF-derived offspring.

The mechanisms of hyperactive ribosome biogenesis in response to ROS in IVF-derived embryos are associated with several features. ROS upregulate the expression of Nucleolin, n-Myc, Ddx17, and Syne2, which are most widely known for their positive participation in steps throughout ribosome biogenesis, including synthesis and processing of rRNAs, assembly of ribosomal proteins, transport to the cytoplasm and association of ribosomal subunits. On the other hand, the Wnt/β-Catenin/c-Myc signaling pathway is the major pathway that works in concert with each of the three RNA polymerases (RNA Pol I, II, and III) to regulate ribosome biogenesis. Decreases in ribosomal proteins are accompanied by decreased expression and activity of β-Catenin. In our oxidatively damaged IVF embryos,
the upregulation of Nucleolin promoted Wnt/β-Catenin signaling, which globally affects multiple steps in ribosome biogenesis and enhances the expression of nearly all ribosomal proteins via a Myc-dependent pathway. Myc (both c-Myc and n-Myc) has been shown to serve as a direct positive regulator of ribosome biogenesis. ROS caused an increase in n-Myc (not c-Myc), suggesting that n-Myc may be the critical molecule by which Wnt/β-Catenin signaling regulates ribosome biogenesis in preimplantation embryos.

Ribosome biogenesis in cancer depends on multiple factors. In addition to protein synthesis function, most ribosomal proteins can influence cellular processes by performing extraribosomal regulatory functions involving binding to select critical target mRNAs. For example, hyperactive ribosome biogenesis can drive activation of oncogenes (e.g., En2 and n-Myc) and silencing of tumor suppressor genes (e.g., Per2 and Diras2) in oxidatively damaged IVF embryos. More importantly, hyperactive ribosome biogenesis can trigger the concomitant activation of tumor-related signaling pathways. Here, we show, for the first time, that oxidative damage establishes oncogenic cooperation among the ribosome, Wnt, and TGF-β1 signaling pathways in IVF-derived embryos. As mentioned above, oxidative damage activates the Wnt/β-Catenin signaling pathway, which stimulates the expression of ribosomal proteins. Conversely, knockdown of ribosomal
proteins downregulates β-Catenin expression and blocks Wnt signaling⁵⁵. In addition, ribosomal proteins, which are frequently amplified in many types of human cancers, mediate the TGF-β1/Smad signaling pathway. In line with our results, Rpl22l1 binds to intronic sequences of Smad2 premRNA and modulates splicing of the premRNA encoding Smad2, an essential transcriptional effector of TGF-β1 signaling; Smad2 phosphorylation and expression are reduced upon knockdown of Rpl22l1⁵⁶,⁵⁷. In contrast, EMT induction by TGF-β1 in cancer cells primarily mediates transcriptional downregulation of ribosomal proteins⁵⁸. Consequently, the persistent hyperactivation of ribosome biogenesis plays a tumor-promoting role in offspring derived from oxidatively damaged IVF embryos by cross-interacting with the Wnt/β-Catenin and TGF-β1/Smad pathways. Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a DNA-RNA-binding protein that regulates a wide range of biological processes and disease pathologies. Many studies have identified hnRNP K as an oncogene, as it is overexpressed in cancer tissues compared with nonneoplastic tissues, and its expression level is related to the prognoses of different types of malignancies⁵⁹. hnRNP K is a novel internal ribosomal entry site trans-acting factor⁶⁰; hnRNP K is also central to regulating activation of the Wnt/β-Catenin and TGF-β1/Smad signaling pathways⁶¹–⁶³. β-Catenin, which accumulates in the nucleus, activates Wnt signaling through complexation with hnRNP K⁶⁰,⁶⁴. hnRNP K promotes the TGF-β1-induced EMT process in lung cancer cells; the EMT phenotype of lung cancer cells can be increased via self-production of TGF-β1 and significantly decreased by silencing hnRNP K expression⁶⁵,⁶⁶. Therefore, through cross-talk among the β-Catenin, ribosome, and TGF-β1 pathways, hnRNP K may be involved in processes critical to ribosome biogenesis and cancer progression in oxidatively damaged IVF embryos. Due to its excellent antioxidative and antitumor properties, efforts have been made to use EGCG to produce healthy embryos and offspring. In vitro culture conditions stress mouse embryos and contribute to the reduced cell numbers of blastocysts. Blastocyst biopsy (in which a small number of cells are taken from the trophectoderm at the blastocyst stage) may not add additional risk of poor neonatal outcomes⁶⁷. Nevertheless, the blastocyst total cell number and the ICM-trophectoderm score are correlated with positive outcomes of clinical pregnancy rate and live birth rate⁶⁸. Mouse experiments have also revealed that offspring derived from blastocysts with fewer cells display decreased spleen weights, decreased values of several organ-to-body weight ratios (for the heart, lungs, spleen and liver), reduced crown-rump lengths, abnormal limb development and other features⁶⁹. However, there is no literature on the threshold number of blastocyst cells necessary to prevent negative impacts on clinical outcomes and offspring health. In this research, we treated mouse zygotes with a low concentration (0.03 mM) of H₂O₂ to create a model strongly recapitulating the clinically observed phenomenon of oxidative damage in IVF embryos and found that the ROS level in the H₂O₂/EGCG-treated group was close to that in the EGCG-treated group and that the total cell numbers of IVF blastocysts in both groups were nearly 1.5-fold higher than that in the control group. Thus, EGCG has a trophic pro-proliferative effect, increasing the blastocyst cell number and improving the clinical outcomes of IVF. In addition, we discovered that in vitro culture conditions contribute to the reduced cell number of blastocysts as well as to the increased numbers of yH2AX-positive cells and TUNEL-positive cells in blastocysts. These results indicate that preimplantation blastocysts may consist of oxidatively damaged and apoptotic cells that are harmful for IVF clinical outcomes. However, after EGCG treatment, embryo developmental competence was improved, as indicated by the increased ICM and total cell number, in association with the reductions in the numbers of damaged and apoptotic cells in blastocysts developed from H₂O₂/EGCG-treated zygotes. Furthermore, EGCG supplementation reversed the effects of ROS on the expression of ribosome-/tumor-related proteins in preimplantation embryos. Our data show, for the first time, that EGCG is beneficial for protecting IVF embryos from ROS-induced DNA/chromosome damage and maintaining ribosome biosynthesis at a level appropriate for the growth of embryonic cells, representing a new strategy for preventing offspring tumorigenesis. In conclusion, transient increases in ribosomal protein synthesis are beneficial for DNA repair. However, as a consequence of insufficient or inefficient DNA repair, persistent hyperactivation of ribosome biogenesis is one of the most important mechanisms of tumorigenesis in offspring derived from oxidatively damaged IVF embryos. Rpl15 plays a critical role in accelerating tumor progression by selectively enhancing the translation of other ribosomal proteins and cell cycle/apoptosis regulators. Oxidative damage establishes oncogenic cooperation among the β-Catenin, ribosome, and TGF-β1 signaling pathways. hnRNP K may be essential for cross-talk among the Wnt/β-Catenin, ribosome, and TGF-β1/Smad pathways. This study advances our current understanding of the molecular mechanisms of tumorigenesis in IVF-derived offspring. More importantly, our findings that EGCG increased the blastocyst formation rate/blastocyst undamaged cell number and inhibited hyperactivation of ribosome biogenesis provide a scientific basis for the use of EGCG to optimize the in vitro culture conditions of human embryos. Frankly speaking, the results of this paper were obtained from animal experimental models, which is the greatest limitation of this study. Given ethical factors and the scarcity of human samples, we chose not to conduct experiments directly on human embryos. To clarify whether similar results occur in human embryos, we will use discarded IVF embryos to observe the effects of oxidative damage on ribosome biogenesis and the carcinogenic effects of the interaction of hyperactive ribosome biogenesis and abnormal X chromosome inactivation.

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COMPETING INTERESTS
The authors declare no competing interests.

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