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Studying The Expression Of Vital Genes In Blastocysts With Different Quality

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Abstract:
Background and aim: Single-cell transcriptomics provides information on heterogeneity and common gene expression at the level of individual cells. This enables a cellular landscape to define intracellular gene regulatory networks. The aim of this study was to investigate mRNA expression of cMYC, OCT4, NANOG, SMAD2, and SMAD4 genes in embryos that are in the blastocyst stage.
Materials and methods: We collected 150 embryos with high, medium and low-quality, and then were biopsied at the blastocyst stage. Extraction of total RNA from all embryos was performed and cDNA was synthesized. Expression of the target genes in mRNA levels were investigated by qReal-Time PCR technique.
Result: Our results demonstrated that mRNA expression pattern of cMYC, OCT4, NANOG, SMAD2, and SMAD4 genes in low quality embryos is significantly different as compared high and medium quality embryos.
Conclusion: In general, we suggested that mRNA expression pattern of developmental transcription factors may associated with embryos quality.

Keywords: Blastocyst, Gene expression, Single cell, qRT-PCR, Quality of embryo

Introduction

In multicellular organisms, gene regulation causes cell transformation and morphogenesis in the embryo, which ultimately causes the emergence of different cell types in these organisms that have different gene expression profiles from the same genome (1, 2). It represents how evolution occurs at the molecular level and the basis of evolutionary development in biology. For example, in humans, most tissues have the same genome, and yet a variety of tissues and processes are created due to the process of regulating gene expression (3).
Measuring gene expression is critical for studying tissue differentiation, attributing disease risk to gene expression, studying the genomic landscape of diseases, drugs, or other perturbations (3). The activity of the embryo's genome takes place during the stages of development, the expression of human embryo's genes begins between the two-cell and eight-cell stages (4). Maternal RNA transcripts stored in the egg are only necessary until the completion of the first mitotic division, after that, the continued development of the embryo depends on the expression of the embryo's own genes, which leads to the formation of the blastocyst and, finally, to successful implantation (5). Our understanding of genes important for embryo implantation is limited. Studies have been conducted on the expression of genes in pre-implantation embryos, from pre-fertilized eggs to embryos in the blastocyst stage, and even embryonic stem cells (6).
There have been studies that have proven the importance of the expression of genes involved in the transition from the expression of the maternal genome to the expression of the fetal genome, including genes involved in DNA repair, cell growth and trophectoderm formation (6, 7). The regulation of gene expression during the period between gametogenesis and embryo implantation is very important because it occurs in the early stages of embryonic development (8). Because the number of cells at this stage is limited, studies on gene expression have been conducted in recent years, but the studies conducted to prove the role of genes in the development of embryos formed by IVF method are limited and there is not a lot of information about the role of gene expression of embryonic at the blastocyst stage (9, 10).
We selected genes that are differentially expressed in human blastocyst stage embryos compared to cleavage stage embryos. It appears that genes differentially expressed in blastocyst-stage (compared to cleavage-stage) embryos are likely to be important in both blastocyst structure and

function. In this study, mRNA expression of cMYC, OCT4, NANOG, SMAD2, and SMAD4 genes in embryos that are in the blastocyst stage has been investigated.

Materials and methods

Sample collection

In this retrospective study, 50 embryos with good quality, 50 embryos with middle quality, and 50 embryos with poor quality at the blastocyst stage were collected from couples undergoing assisted reproductive technology treatments in Milad Fertility Clinic and Omid Fertility Clinic in Tabriz, Iran from August 2020 to July 2021. The embryos used in our study were specifically donated for research use by patients who had undergone IVF treatment. Embryos were formed using intracytoplasmic sperm injection insemination. All embryos (n = 150) were cultured using embryo culture media in an incubator with 5.5-6.0% CO2 and 37°C temperature. Embryos designated for study on the fifth day post fertilization (D5) were cultured for 96 hours. Approval from the University of Azad University-Arsanjan branch (IR.IAU. A.REC.1400.001) was obtained prior to the performance of all experiments.

Blastocyst biopsy

All embryos were biopsied on day 5, during blastocyst stages. For biopsy from blastocysts, embryos were immobilized with a holding pipette (left) and the zona was punctured using a laser (Hamilton Thorne Biosciences, Beverly, MA, USA). Using the right biopsy pipette, gentle suction of trophectoderm cells was performed through the zona pellucida hole. 5-6 cells were separated from trophectoderm. Sampled trophectoderm cells were washed with Dulbecco's phosphate- buffered saline (DPBS) and placed in 0.2 ml RNase-DNase-free polymerase chain reaction tubes for referral to Genetics Center.

Gene expression assay

To extract RNA, the Single Cell RNA Purification Kit (cat no 51800) from Norgen Company, was used according to the following protocol. For cDNA synthesis, TruScript™ First Strand cDNA Synthesis Kit (Norgren, Canada) was used. The StepOnePlus™ Real-Time PCR system device and RealQ Plus 2x Master Mix Green product of the Ampliqun commercial company were used to perform the genes expression assay. The used primers are presented in Table 1.

Statistical analysis

For statistical analysis and choosing the appropriate test for data analysis, the obtained results were first entered into SPSS 26 software, and after checking the normality of the distribution of variables using the Smirnov Kolmogorov sample one test method, the data were analyzed with REST software.

Results

In the present study, 50 embryos with excellent quality, 50 embryos with medium quality, and 50 embryos with low quality were analyzed in terms of cMYC, OCT4, NANOG, SMAD2, and SMAD4 genes mRNA expression. Our results demonstrated that with decrease in quality of the embryos, the expression of the target genes also decreases (Figure 1). We found no significant difference in expression of target genes between high quality and medium quality embryos. However, expression level of target genes in low quality embryos is lower than high quality embryos (Table 2).

Discussion

Embryo development requires coordinated events that are finely regulated at the molecular and cellular level. With advances in genetic technologies such as single-cell RNA analysis, we can now assess embryo gene expression with greater precision and gain new insights into complex processes that were difficult to unravel in the not-so-distant past. Several genes and regulatory pathways have been identified for each stage of embryonic development. For example, genes are highly expressed in early stages and are involved in cell adhesion, cell cycle, and transcriptional regulation. In embryology, there are many technical, ethical and scientific limitations that significantly make the use of non-human embryos more feasible for extensive studies in life sciences (11, 12).

In this study, we report on preimplantation development with a focus on genes whose defects lead to developmental arrest. According to the results of our study, the expression levels of cMYC, OCT4, NANOG, SMAD2, and SMAD4 in grade low quality embryos were lower than high and medium quality embryos.

Transcription factor Oct4 plays an important role in the early stages of embryonic development and in maintaining the inner cell mass's multiplicity and the stability of mouse embryonic stem cells. Sanna et al investigated the expression of Oct4, Nanog, Sox2 and Stat3 genes in sheep blastocysts in vitro. According to their report, Oct4 and Nanog were expressed in the inner cell mass, trophoblast and embryonic stem cells, and their expression was significantly higher in the inner cell mass than in the trophoblast, and this indicates the role of Oct4 and Nanog in

the development of the produced embryos (12). In a study, Niwa et al showed that the transcription factors Nanog, Sox2 and other molecules of the Stat3 signaling pathway along with Oct4 have prominent roles in the growth of the bovine embryo, the maintenance of pluripotency and the self- renewal of stem cells (13). Xiao-li et al. proved the importance of Oct4 and Nanog transcription factors for embryonic stem cells as well as for preimplantation embryos. Their results showed that maternal Oct4 and Nanog transcripts were maintained until cleavage stage and then slowly decreased. Then the expression of their genes gradually increases in morula and blastocyst (14). In a study conducted by Kirchhof et al and his colleagues, they showed the expression of Oct4 in all stages of pig and cow embryo development (15). The results of these studies are consistent with the results of our study and show the importance of Oct4 and Nanog gene expression in how the embryo grows and develops. In another study, He et al. reported that Oct4 protein was not detected in all trophectoderms of in vitro embryos of different species, which they suggest that Oct4 protein expression may be different in embryos of different species (16). Keefer et al and his colleagues reported that the expression pattern of Nanog protein is similar to Oct4 and both were detected in the cell nucleus from the 8-cell stage to the blastocyst stage, both in the ICM and in the trophectoderm. They proved that Oct4 and Nanog control the stages. Primary mammals play an important role (17). In another study by Hambiliki on goat embryos, they observed that the
expression of Oct4 and Nanog in trophectoderm is reduced compared to ICM cells, which is contrary to the results of mouse and human embryos (18). Wu and colleagues showed that the maternal Oct4 gene is critical until the cleavage stage, and after that stage both Oct4 and Nanog proteins are expressed in ICM cells (19). Suzuki et al. analyzed the expression pattern of cMyc during oogenesis and embryo growth and development before implantation and concluded that cMyc is reduced during oocyte development and up to the morula stage in embryos in blastocysts. Intranuclear analysis showed that cMyc was concentrated in small granules. After fertilization in particular, their number and size decrease and increase during the development of the embryo. In this way, the granules disappeared in the 1-cell stage and then reappeared in the 2-cell stage. Therefore, changes in cMyc expression during pre-implantation embryonic development indicate the importance of its function in these stages (20). In another study, Rajput et al investigated the expression of cMyc in oocytes and two-cell, four-cell, morulae and blastocyst embryos. According to their report, cMyc expression plays a vital role in embryo development in the pre-implantation stages, and its expression changes in each stage of embryo development (21). According to the results of our study, the expression of cMyc in low-quality embryos has a decreasing trend, which can affect the abnormality of the number of cells and the process of cell divisions.
In a study, Singh et al observed a gradual increase in the expression of Oct4 and Nanog transcripts from the 2-cell stage to the blastocyst. Also, in another study, they studied the expression of Oct4, cMyc, and Nanog genes in different stages of buffalo embryo development, and according to their report, the relative frequency of Nanog transcripts. Oct4 was significantly higher in high-quality embryos as compared high and medium-quality embryos. The relative abundance of cMyc transcript in grade A was significantly higher compared to grades C and grades D, but it was not significantly different from the values of grade B, and in buffalo, Nanog, Oct4 and cMyc were highly expressed in blastocysts compared to other embryo stages (22). In a study, Zhang et al showed that in most mammalian species, including cattle, Smad2, Smad3, and Smad4 are required for early bovine embryo development, and follistatin-specific embryonic actions are lost early in development when Smad2/3 signaling is disrupted. the river in total, their results showed that the TGF-b superfamily of cognate signaling pathway components, (Smad2/3/4), play an essential role in the early stages of embryonic development, from the 8 to 16-cell and blastocyst stages, depending on the function of Smad2/3 and Smad4 genes (23). Nomura et al. showed that the level of Smad2 protein expression is low in cow and mouse embryos. Embryo mortality is observed
between 5-6 days, which emphasizes the functional requirement of this protein for post- implantation development (24). In summary, the studies presented here, by comparing the expression of Smad2 and Smad4 genes in embryos of different developmental quality, show that both Smad2 and Smad4 are key genes that influence early human embryogenesis, with changes in expression. They include the smallest morphological changes.

Conclusion

The results of our study provide valuable insights into how the transcriptome is controlled and utilized during real human embryonic development. Therefore, new biomarkers of embryo quality can be adopted to improve the diagnosis and treatment of infertility. Embryos with different quality in terms of the expression of cMYC, OCT4, NANOG, SMAD2, and SMAD4 genes have different trends. it can indicate the possibility of improving the regulation of gene expression after embryo implantation. Therefore, it is recommended to investigate effect of environmental factors on these genes expression in embryos. It will be of great help in the science of infertility and preventing the wastage of low-quality embryos in infertility centers by applying special conditions in the growth and development of embryos.

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Table 1. Sequences of the used primers.

Gene	Primer sequences	Product size
cMYC	Forward: 5'-AGCGACTCTGAGGAGGAAC-3' Reverse: 5'-CTGCGTAGTTGTGCTGATG-3'	144 bp
OCT4	Forward: 5'-TGGATGTCAGGGCTCTTTGT-3'	124 bp
	Reverse: 5'-AGTGCTTTGTGTGTACTTACTCA-3'	
NANOG	Forward: 5'-CTGGTTGCCTCATGTTATTATGC-3' Reverse: 5'-CTGGAAGATGTTAGAGAAATAGGAC-3'	138 bp
SMAD2	Forward: 5'-CCGACACACCGAGATCCTAAC-3' Reverse: 5'-AGGAGGTGGCGTTTCTGGAAT-3'	136 bp
SMAD4	Forward: 5'-CACTGCCAACTTTCCCAACA-3' Reverse: 5'-ATCCATTCTGCTGCTGTCCT-3'	134 bp
GAPDH	Forward: 5'-CAAGATCATCAGCAATGCCT-3' Reverse: 5'-GCCATCACGCCAGTTTCC-3'	127 bp

Table 2. The target genes mRNA expression in embryos with different quality.

Genes	High/medium quality	High/low quality
NANOG	0.217	<0.001
OCT4	0.781	<0.001
cMYC	0.499	<0.001
SMAD2	0.104	<0.001
SMAD4	0.655	<0.001

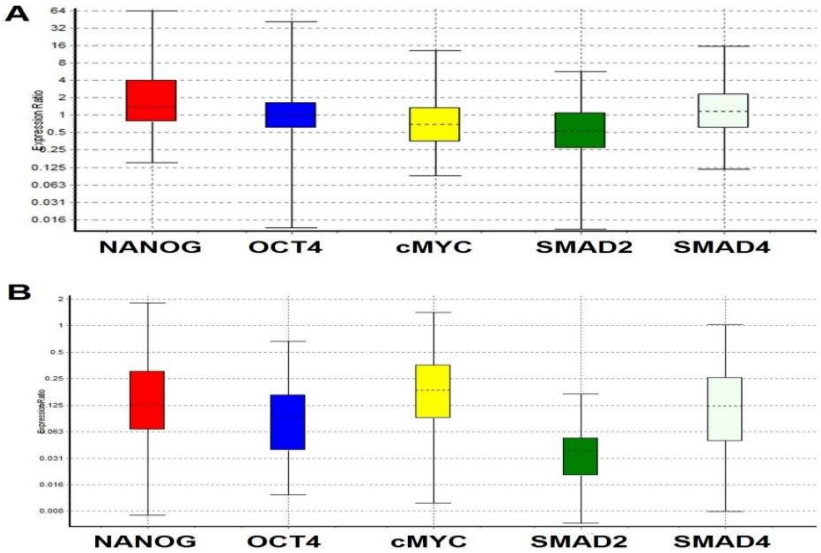

Figure 1. Relative expression of the target genes in high/medium-quality embryos (A) high/low- quality embryos (B).