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Comparison of Gene Expression between Bovine Embryos Produced *in vitro* and Cloned Arabian Oryx Embryo from Bovine Ova

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ABSTRACT

Background: Embryonic biotechnology is an application used in animal production to expand the number of livestock animal species, provide animal products for global demand and preserve endangered species to improve reproduction, maintain biodiversity and genetic diversity and reduce the interval between generations. This animal production will supply goods and services for the human population's well-being leading to economic growth. This biotechnology encompasses the use of assisted reproductive techniques (ARTs) like *in vitro* fertilization and nuclear transfer using somatic cells. This study compared the relative expression of some genes between the embryos that were fertilized *in vitro* (IVF) and cloned embryos by nuclear transfer of bovine (SCNT-B) and Arabian Oryx (SCNT-O).

Methods: The oocytes were matured, then fertilized and cultured for the IVF technique, while for the SCNT technique, the matured oocytes were enucleated to recipe the donor cells for bovine and Arabian Oryx then cultured, the embryos for both techniques were used for the gene expression of the studied genes.

Result: The expression of each studied gene (OCT4, DNMT1, BAX, GLUT1 and HSP70) was assessed in the early and late cleavage stages of the embryos produced by IVF in bovine, SCNT in bovine and SCNT in Arabian Oryx; this gene expression was compared between the three types of embryos. There was a non-significant difference in the relative expression between the embryos produced by the techniques mentioned above.

Key words: Arabian Oryx, Bovine, Cloning, Gene expression, *In vitro* fertilization.

INTRODUCTION

Reproductive technology advancements like nuclear transfer, *in vitro* fertilization, artificial insemination and embryo transfer have significantly increased animal reproduction efficiency and are important tools for domestic and wild animal maintenance, especially for species that are endangered (Engdawork *et al*., 2024; Wolf *et al*., 2001).

In vitro fertilization (IVF) and somatic cell nuclear transfer (SCNT) or embryo cloning are two technologies with significant promise for producing a large number of domestic animal embryos for research purposes. Ova from slaughterhouses has supplied an essential source of embryos for several scientific objectives (Alofi and AlHimaidi, 2004).

In vitro fertilization technique from immature ovarian oocytes involves three essential stages maturation of the oocytes *in vitro* (IVM), *in vitro* fertilization (IVF) and *in vitro* culture of embryos (IVC) (Herrick *et al*., 2004; Singh *et al*., 2020). The reprogramming for genes starts after fertilization by degrading oocyte-specific transcripts (maternal) that are not later expressed and replacing these maternal transcripts with embryonic transcripts, then promoting a significant reprogramming of the gene expression pattern in conjunction with the production of new transcripts that are not expressed in the oocyte (Schultz, 2002; Wrenzycki *et al*., 2005).

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The nuclear transfer technique has several uses like breeding and clonal therapy. Although remarkable successes, the efficacy of SCNT remains low. Fibroblasts from various origins are frequently employed as donor cells for SCNT in domestic animals like sheep and cattle (100). SCNT provides an accurate genetic match by transferring the donor's nucleus to the recipient oocyte enucleated (Keefer, 2015; Tian *et al*., 2003; Yun *et al*., 2008), after the nucleus has been transferred, the cloned embryo that results must grow to produce a genomic copy or clone of the individual whose nucleus was used, as well as reprogram the donor nucleus' epigenetic markers to restore

the proper gene expression patterns necessary for embryonic development to term. However, gene expression in cloned embryos is not always properly restored during reprogramming, resulting in incomplete donor nucleus reprogramming and abnormal expression of development-related genes (Park *et al*., 2003; Rodriguez-alvarez and Castro, 2010; EM *et al*., 2014). Remodeling and reprogramming are two distinct processes. Remodeling involves transforming the donor nucleus into one that resembles a zygote after being transported into the cytoplasm of a mature enucleated egg. Whereas reprogramming involves altering the chromatin structure (Whitworth and Prather, 2010). This nuclear transfer is dependent on the transplanted somatic cell nucleus becoming able to complete development from differentiated to totipotent stages (Matoba and Zhang, 2018).

During embryo development in mammals, two main reprogramming processes are known as epigenetic alteration. The first occurs during germ cell formation, while the second occurs during the preimplantation embryo development stages. Maternal to zygotic transition (MZT) is the designation for the second reprogramming process that occurs at the 8- to 16-cell stage in bovine which is important for the development of fertilized oocytes (Vigneault *et al*., 2004).

There are physiological changes that happen during the maternal-to-zygotic transition such as DNA methylation and chromatin structural remodeling and the new gene expression starts at MZT which means that the gene expression of the maternal turns off in the embryos produced by the IVF technique (Camargo *et al*., 2006), the expression of the somatic donor cell genes turns off in the embryos that produced by the SCNT technique throughout the transition from gametes into a pluripotent embryo and to the early differentiation of cell stages embryo (Al-Malahi *et al*., 2022; EM *et al*., 2014).

The genes selected for this study OCT4 gene (Octamer-binding transcription factor-4), DNMT1 gene (DNA methylation), BAX gene (apoptotic gene), GLUT1 gene (Glucose transporter) and HSP70 gene (cytoprotective factor) have a significant role in the development of good-quality embryos according to the reprogramming and remodeling (EM *et al*., 2014).

MATERIALS AND METHODS

Chemicals, reagents, media and tools for embryo production and gene analysis

All of them were from Sigma-Aldrich (St. Louis, MO, USA) unless defined otherwise.

Collection of ovaries and oocytes for maturation

The ovaries were collected from the bovine that was slaughtered in the slaughterhouse of Riyadh city to apply the *in vitro* fertilization and embryo cloning techniques, then the oocytes from these ovaries were collected and matured for 22-24 h in TCM-199 Earle's salt maturation medium for 21-24 h at 39°C and 5% CO_2 in the air with humidity as reported in (Al-Malahi *et al*., 2022; Ammari *et al*., 2022; Amran *et al*., 2024) for IVF and cloning techniques.

In vitro fertilization technique

The matured oocytes were fertilized in IVF-BO medium at 39°C and 5% CO_2 for 24 h incubation in the air with humidity by the cryopreserved semen of the bull that was capacitated previously depending on (Al-Malahi *et al*., 2022; Amran *et al*., 2023; Cánepa *et al*., 2014).

Nuclear transfer technique (cloning)

The matured oocytes were denuded by using hyaluronidase enzyme and the oocytes with the extruded first polar body were chosen for enucleation, the zona pellucida of the oocytes was dissected by pierced and then the 1st polar body was pushed out with the surrounding cytoplasm by using a glass needle under micromanipulator. Adult Arabian oryx and bovine fibroblast somatic cells were used as donor cell sources after being cultured in (Dulbecco's modified minimum essential medium DMEM) to the recipient enucleated oocyte, the nuclear fibroblast somatic cells were fused with the enucleated oocyte by two direct current pulses, a single AC pulse of 0.2 kV/cm for one second followed by a single DC pulse of 2.5 kV/cm for 50 µs in the cell fusion medium Zimmerman and then were activated for 5 min with 5 µM ionomycin at room temperature and followed by incubation in SOF medium for 6 hours at 38.5°C and 5% CO_2, as detailed in (Al-Ghadi *et al*., 2020; Ammari, *et al*., 2022; Dinnyés *et al*., 2000).

Culture embryos

The fertilized and fused oocytes were cultured in IVC-SOF medium at 39°C in a 5% O_2, 5% CO_2 and 90% N_2 for 7-9 days of incubation in the air with humidity (Alotaibi *et al*., 2022; Cánepa *et al*., 2014).

Embryo collection and freezing

Embryos produced by both techniques mentioned were collected during the 7-9 days of culture in different cleavage stages from the early stages (2-4 and 8 cell stages) to the late stages (morula and blastocyst). These embryos were frozen in 0.1% PVA-PBS at -80°C until RNA extraction (Al-Malahi *et al*., 2022; Long *et al*., 2007).

Extraction of RNA and gene expression

The embryos' RNA was extracted and gene expression for the genes HSP70 (heat shock protein), OCT4 (pluripotency gene), GLUT1 (glucose transporter), BAX (an apoptosis gene) and DNMT1 (DNA methyltransferase) were determined as detailed in our study (Al-Malahi *et al*., 2022). Table 1 displays the forward and reverse primers for the studied genes.

The comparative CT method was used to calculate the quantification of the expression levels gene concerning the GAPDH as a reference gene by the $2^{-\Delta\Delta Ct}$ formula (Amarnath *et al*., 2007).

The experiment steps are viewed in (Fig 1). This study took 1 year and 5 months, it was done in the Zoology Department, Science College, KSU, Riyad, Saudi Arabia.

Statistical analysis

The SPSS 20 software was used to analyze all data. The non-parametric tests (Kruskal-Wallis test) and (Mann-Whitney test) were used for comparison between the gene's relative abundance of the (IVF) bovine embryos and both (SCNT) cloned embryos, *P*-values of less than 0.05 were considered significant.

RESULTS AND DISCUSSION

The expression of each studied gene (OCT4, DNMT1, BAX, GLUT1 and HSP70) was assessed in the early cleavage stages of the three types of embryos; this gene expression was compared between the three types of embryos. There was an increase in the gene expression for the genes OCT4, DNMT1, GLUT1 and HSP70 in the SCNT-B embryo compared to the other embryos (Fig 2), while the expression of the BAX gene, was high in both types of embryos IVF and SCNT-O compared to SCNT-B embryo (Fig 2), but these differences non-significant (*P*>0.05). The expression of each studied gene (OCT4, DNMT1, BAX, GLUT1 and HSP70) was assessed in the late cleavage stage of the three types of embryos, this gene expression was compared between the three types of embryos. There was an increase in the gene expression for all genes OCT4, DNMT1, BAX, GLUT1 and HSP70 in the SCNT-B embryo compared to the other embryos, but these differences were non-significant (*P*>0.05) (Fig 3).

The study's focus genes were chosen because they have been demonstrated to become transcriptionally active during embryonic genome activation (EGA) and participate

Table 1: Primers of the selected genes that were used to amplify by real-time polymerase chain reaction.

Gene	Forward primer (F)	Reverse primer (R)	Accession no.
GAPDH	GGTTGTCTCCTGCGACTTCAA	AATGCCAGCCCCAGCAT	NM_001034034.1 (Cánepa *et al.*, 2014)
HSP70	AGCAAAGAACCAAGTCGCAATG	AAGGTAGGCTTCTGCGATTTCC	BC105182 (Rodríguez-Alvarez *et al.*, 2010)
OCT4	GGTTCTCTTTGGAAAGGTGTTC	ACACTCGGACCACGTCTTTC	NM174580 (Rodríguez-Alvarez *et al.*, 2010)
GLUT1	CTGATCCTGGGTCGCTTCA	GGATACCTCCCCCACGTACA	M60448 (Amarnath *et al.*, 2007)
BAX	GCGCATCGGAGATGAATTG	CCAGTTGAAGTTGCCGTCAGA	U92569 (Amarnath *et al.*, 2007)
DNMT1	TTCGGAACTTCGTCTCCTTCA	GCCAAAGGTGCACTGGTACC	AY173048 (Amarnath *et al.*, 2007)

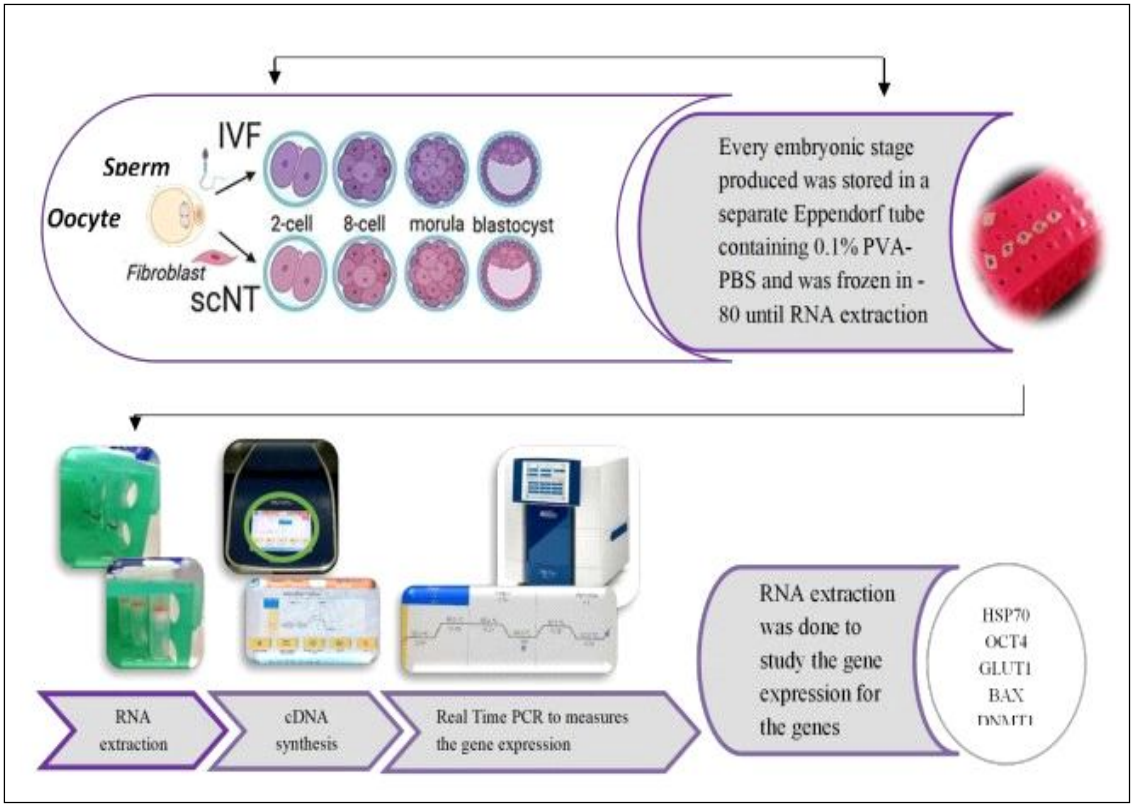


Fig 1: General view of the experiment steps that started from the collection of the immature oocytes to the studying of the gene expression.

in important biological processes during pre-implantation development (Ross *et al*., 2010). It could be feasible to obtain a further understanding of the reprogramming process and the gene expression patterns of preimplantation embryos by studying the gene expression before and after the maternal to-embryonic transition (Camargo *et al*., 2006), but the studies differed from each other in their results around the differences in the gene expression between the groups of embryos produced by the IVF or SCNT techniques and within the groups of SCNT.

The correct reprogramming which includes epigenetic modifications (DNA methylation and Histone modifications) is necessary for proper embryonic development and differentiation of cells (Cheng *et al*., 2019; Noh *et al*., 2016), suppressing sequences not intended to be expressed in the cell also constructs the fundamental gene expression infrastructure of the entire organism, such as various harmful parasitic and viral sequences and non-tissue-relevant genes found in the genome (Hall, 2013; Jeltsch *et al*., 2018), this regulation happen by the gene DNMT1. This results in the formation of various cell and tissue types through the control of certain gene expression patterns, which results in the states of gene expression being stable (Canovas *et al*., 2017; Dang and Zhang, 2019; Dor and Cedar, 2018). These epigenetic modifications are switches for the gene expression regulation (Hall, 2013; Mariño-Ramírez *et al*., 2005; Van and Santos, 2018), so the abnormalities in gene expression return to the impaired transcriptional regulation mechanisms (Kishigami *et al*., 2006; Salehi *et al*., 2019).

Therefore, there may be an opposite interaction between the transporter glucose gene for energy that is important for embryo development GLUT1 and the apoptosis gene Bax, that means if the level expression of the GLUT1 gene is low causally related to the high incidence of apoptosis, from the other hand also there is an opposite

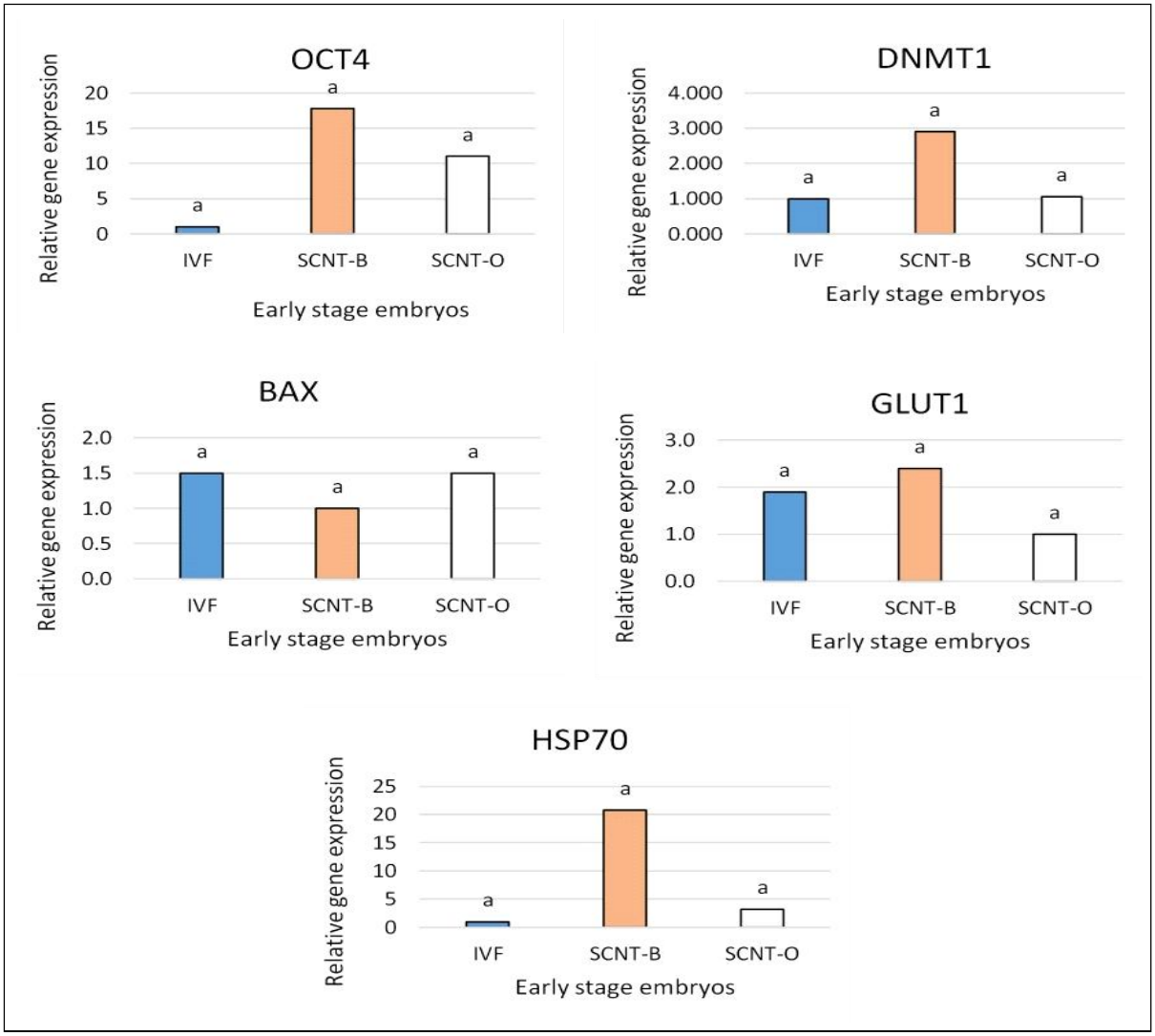


Fig 2: Comparison of expression of the studied genes in the early cleavage stage of the embryos produced by IVF, SCNT-B and SCNT-O.

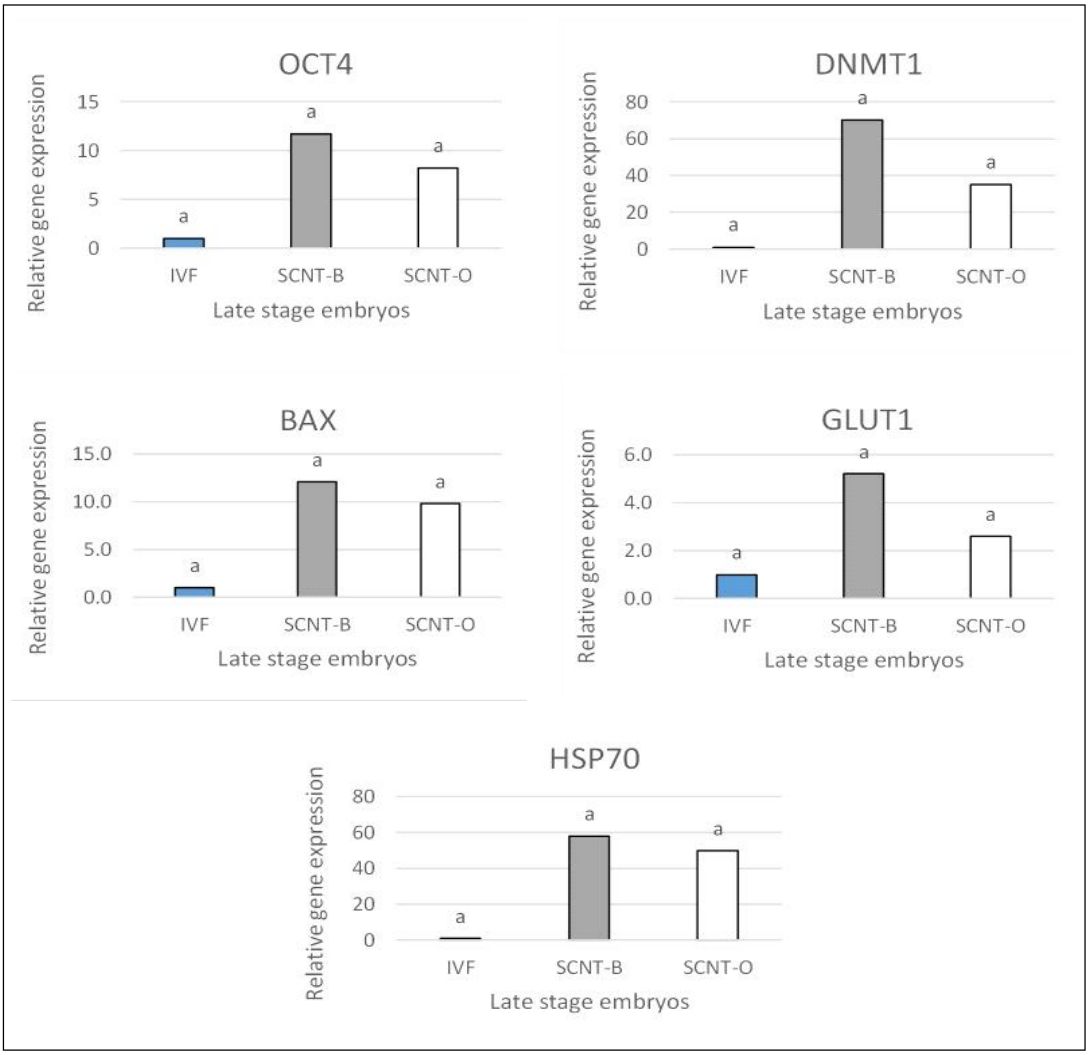


Fig 3: Comparison of expression of the studied genes in the late cleavage stage of the embryos produced by IVF, SCNT-B and SCNT-O.

interaction between the BAX gene of embryos and cytoprotective gene HSP70 causing increase the level expression of HSP70 gene to protects the embryos from the apoptosis caused by stress damage (Al-Malahi *et al*., 2022).

Our results in (Fig 2 and 3) cleared that there were non-significant differences when we compared the groups of embryos (IVF, SCNT-B and SCNT-O) before and after the maternal-to-zygotic transition for each of the studied genes separately (OCT4, DNMT1, BAX, GLUT1 and HSP70), these findings consistent with the results of (Amarnath *et al*., 2007) whose compared the gene expression of genes profile included some like our studied genes (OCT4, DNMT1 and BAX) between the IVF and SCNT buffalo embryos at the 8-cell stage and blastocyst and also agreed with EM *et al.* (2014) whose compared between the same embryos at the 8-cell stage for the genes (GLUT1, OCT4, DNMT1 and BAX). The insignificant differences between these types of embryos may be explained by the problems in the correct reprogramming of these genes or the quality of oocytes or embryos, the culture conditions, or the various modifications of the SCNT protocol like passage number of donor cells, source of donor cells, donor cell cycle stage and activation protocol, all of them can affect the gene expression embryonic patterns and the differences between the embryos produced by different reproductive techniques.

CONCLUSION

The transition from the maternal genome to the embryonic genome at the determined stage of development and the correct reprogramming and remodeling of the studied genes ensures balance in the genetic expression of these genes during the stages of embryonic development and this, in turn, ensures the continuation of high-quality embryonic development.

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Author contributions

All authors participate equally.

Institutional review board statement

Not applicable.

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Data availability statement

Not applicable.

Conflicts of interest

The authors declare no conflict of interest.

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