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ASSESSMENT OF "YAMANAKA" FACTOR GENE EXPRESSION IN EMBRYOS OBTAINED FROM GONADOTROPIN-STIMULATED RABBITS

APRECIEREA EXPRESIEI GENICE A FACTORILOR „YAMANAKA" LA EMBRIONII OBȚINUȚI DE LA IEPUROAICE STIMULATE CU GONADOTROPINE

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ABSTRACT | REZUMAT

„Yamanaka"factors are known for their ability to reprogram somatic cells into pluripotent stem cells, i.e., cells with the potential to differentiate into multiple cell types. In this study, we evaluated the gene expression of „Yamanaka" factors in embryos obtained from gonadotropin-stimulated rabbits. To induce ovulation and obtain embryos at various stages of early development, the rabbits were stimulated with extrapituitary gonadotropins. We extracted total RNA from these embryos and analysed the expression level of „Yamanaka" factors such as Oct4, Sox2, Klf4, and c-Mycusing molecular techniques such as RT-PCR or qPCR. Preliminary results indicate a detectable presence of „Yamanaka" factors in rabbit embryos, suggesting that these factors may also be expressed in this species. Following the experimental study, we obtained the following levels of gene expression: in the case of groups 3, 4, and 6, the expression of the four genes was expressed most intensively, while for groups 1 and 5, the gene expression was weakly expressed. Thanks to these results, it can be demonstrated that the gene expression of „Yamanaka" factors is best expressed in the morula and blastocyst stages.

Keywords: rabbit embryos, gene expression, gonadotropines, qR-PCR

Factorii „Yamanaka" și anume, factorii de transcripție Oct4, Sox2, Klf4 și c-Myc sunt cunoscuți pentru capacitatea lor de a reprograma celulele somatice în celule stem pluripotente, adică celule cu potențial de diferențiere în mai multe tipuri de celule. În acest studiu, am evaluat expresia genică a factorilor „Yamanaka" la embrionii obținuți de la iepuroaice stimulate cu gonadotropine. Pentru a induce ovulația și a obține embrioni în diferite stadii de dezvoltare timpurie, iepuroaicele au fost stimulate cu gonadotropine extrahipofizare (hCG, PMS G). S-a extras ARN-ul total din acești embrioni și s-a analizat nivelul de expresie al factorilor „Yamanaka", precum Oct4, Sox2, Klf4 și c-Myc, folosind tehnici moleculare precum RT-PCR sau qPCR. Rezultatele preliminare indică o prezență detectabilă a factorilor „Yamanaka" în embrionii de iepure, sugerând că acești factori pot fi exprimați și la această specie. În urma studiului experimental s-au obținut următoarele niveluri ale expresiei genice: în cazul loturilor 3,4 și 6 expresia celor patru gene a fost exprimată cel mai intens, în timp ce pentru loturile 1 și 5 expresia genică a fost slab exprimată. Datorită acestor rezultate se poate demonstra faptul că expresia genică a factorilor „Yamanaka" este cel mai bine exprimată în stadiile de morulă și blastocist.

Cuvinte cheie: embrioni de iepure, expresie genică, gonadotropine, qR-PCR

The rabbit (*Oryctolagus cuniculus*) is suitable for experimental testing, particularly in the fields of regenerative medicine and medical clinics. For these species, ovulation is triggered by mating, allowing for the precise determination of embryonic age (measured in hours post-coitum). Sexual maturity is reached at around 4-5 months of age, and the gestation period lasts for 31 days (1). Throughout the morula stage (up to the 16-cell stage), the embryo is a compact sphere of cells between which strong intercellular connections are first established. Shortly after, the blastocyst stage is reached, in which two areas are differentiated: the inner cell mass (ICM) made up of the pluripotent epiblast (EPI) and the hypoblast cells (HP) in the gastrula stage, as well as the trophectoderm (TE), which will later form the placenta (3). In rabbits, the blastocyst is developed in 3-6 days, and the time interval between the onset of cavity establishment (day 3 of embryonic development) and implantation (day 7) is considerably extended. At the time of implantation, the embryo reaches approximately 5 mm in diameter, consisting of an average of 5000 cells. During this period, embryonic development progresses through the processes of trophoblast differentiation, hypoblast migration and the onset of gastrulation on day 6, shortly before implantation (10). Yamanaka factors, also called cell re-

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programming factors, are a group of four transcription factors that can reprogram specialised cells into pluripotent stem cells. The factor *Oct4* (Octamer-binding transcription factor 4), encoded by the *POU5F1* gene, is an essential transcription protein for maintaining the pluripotency of embryonic stem cells. During blastocyst formation, the expression of *Oct4* remains high in the inner cell mass (ICM) and is not expressed in the trophoectoderm. After embryo implantation, the transient regulation of *Oct4* in a group of inner cell mass cells initiates their differentiation into primary endoderm cells (hypoblasts). Subsequently, Oct4 expression is reduced in these cells (11). During gastrulation, *Oct4* decreases and becomes limited to the primordial germ cells (9). Sox2 (the SRY-box containing gene 2) is also a key transcriptional protein for maintaining stem cell pluripotency and regulating cell differentiation. This gene acts in parallel with *POU5F1*by regulating the expression of target genes involved in the maintenance of pluripotency. Also, the gene is expressed both in the extraembryonic ectoderm, in the outer layer of the blastocyst (trophoblast), and in neural stem cells. Forced expression of the *POU5F1* gene can compensate for the loss of the *Sox2* gene in ESCs, suggesting that the main role of the *Sox2* gene in pluripotent cells is to control the expression of the *POU 5F1*gene. However, iPSCs (induced pluripotent stem cells) cannot be generated without *Sox2* gene expression (18). The Klf4 factor (Kruppel-like factor 4) is a transcription protein that controls the expression of genes involved in cell proliferation and the regulation of cell differentiation. The fact that the Klf4 factor is required for the efficient generation of iPSCs could be explained by its role in maintaining the pluripotency of embryonic cells. *Klf4* gene expression was observed to be stimulated by leukaemia inhibitory factor (LIF), which maintains pluripotency in mouse ESCs. In addition, Klf4 enables the maintenance of self-renewal capacity in iPS cells through its cooperation with the ***c-Myc***gene. In iPS cells, there is a balance between these two factors, in which Klf4 suppresses **c-Myc-**induced apoptosisand **c-Myc** neutralises the cytostatic effect of the *Klf4* gene, thus maintaining the immortality of iPS cells (20). The **c-Myc**(Myc proto-oncogene) oncoprotein regulates the transcription of genes that are associated with cell growth, proliferation, and apoptosis. **c-Myc**is involved in a wide range of cellular processes, including cell cycle control, metabolism, signal transduction, self-renewal, maintenance of pluripotency, and control of cell fate decisions. c-Myc transcription factors also have clear roles in cell reprogramming and the establishment of the pluripotent state (17). The objective of the study was to analyse the expression of **Yamanaka"** factors (the *Sox2*, *Oct 4*, *Klf4, c-Myc* genes) in several developmental stages (morula and blastocyst stages) of the rabbit's species.

MATERIALS AND METHODS

Animals

For this study, we used New Zealand female rabbits. At the time of the experiments, the females were 4 months old and weighed 3–3.3kg.All females were housed in individual boxes with natural light and a temperature of 15–25°C. They were given water and commercial food (pellets) ad libitum.

In this study, we used rabbit embryos in different embryonic stages. Females were divided into four groups, with eight in each group. The biological material from Group A was divided into two subgroups, one of which was stimulated with the male (A1-A3), and the second was unstimulated and designated as the control group (A5-A8). Group B received hormonal superovulation therapy based on PMSG and HCG (B5-B8), and some rabbits were also stimulated with the male (B1-B4) (Table 1).

Group B received 120 IU/female PMSG analogue (Folligon, MSD) intramuscularly, followed by 180 IU/female HCG (Chorulon, MSD) 48 hours later, but 6 hours before mating. The embryos were harvested by washing the oviducts and uterine horns with PBS (137 mM Sodium chloride, 2.7 mM Potassium Chloride, and

Table 1

The embryonic stages of the groups analysed

Experimental group	ID molecular analysis	Total number of embryos collected	Embryonic stage	Stimulated/Non-Stimulated
A1-A2	ID 1	19	Morula + blastocyst	Stimulated with male
A5-A8	ID 2	26	Morula + blastocyst	Non stimulated
B1-B4	ID 3	9	Blastocyst	Stimulated with male + hormones
B1-B4	ID 4	24	Morula quality 1	Stimulated with male + hormones
B1-B4	ID 5	23	Morula degraded	Stimulated with male + hormones
B5-B8	ID 6	19	Blastocyst	Stimulated with hormones

Table 2

Primers corresponding to the expression markers

Study type	Expression marker	Primer sense sequence 5'-3'	Primer antisense sequence 3'-5'
Embryonic cell pluripotency	Oct4	5'AAGCGGGGACCCTCGTGCAG	5'TCTGGCGCCGGTTACA
	Sox2	5'AGCCCCAAGATGCACAACTC	5'CTCCGGGAAGCGTGTACTTA
	Klf4	5'AGCGGGAAGGGAGAAGACAC	5'AGGACGAGGAAGAGGCAGAC
	c-Myc	5'TCGGACTCTCTGCTCTCCTC	5'CTTGTCGTTCTCCTCGGTGT
Reference gene	GAPDH	5'CAAGTTCCACGGCACGGTCA	5'CTCGGCACCAGCATCACCC

10 mM Phosphate Buffer/100 mL, VWR Life Science) after the ovariohysterectomy operation. The embryos were harvested using 10 ml of Phosphate Buffer Solution (PBS) supplemented with 0.2% Bovine Serum Albumin (BSA) for each oviduct/uterine horn. The harvest fluid was collected in Petri dishes and examined under a stereo loupe. The identified embryos were transferred to Petri dishes and noted in the laboratory register. To analyse the efficiency of the treatments to which the experimental groups were subjected, the Anova One-Way test was used at a significance threshold of $p<0.05$.

Quantitative studies of gene expression using the reaction qR-PCR

Protein sequences were transcribed into corresponding mRNA sequences. The obtained mRNA sequences were then transcribed into cDNA sequences. The sequences of the specific primers for the indicator genes of the pluripotency process were taken from the specialised literature. Oligonucleotides were synthesised in Eurogentec laboratories in Belgium and purchased for use in this study. The sequences of the primers used throughout this study are listed in Table 2.

RNA extraction and determination of the quality and quantity of nucleic acids

The extraction and purification of the total RNA from the samples were carried out with the Total RNA Isolation System kit (Promega, USA).The quantity and quality of the extracted RNA were verified by the spectrophotometric method with the UV-VIS spectrophotometer Nanodrop 8000 (Thermo Scientific).

Complementary DNA synthesis

cDNA was synthesised using the Hight-capacity cDNA Reverse Transcription kit (Thermo Scientific, Lithuania).The solution thus prepared is transferred to ice, 10µl of RNA is added, mixed gently, and then transferred to the thermocycler. The synthesis and amplification programme consisted of maintaining the samples at 25°C for 10 minutes, then at 37°C for 120 min. Reverse transcriptase activity was stopped by keeping the samples at 85°C for 5 minutes (Table 3). The quantity and quality of cDNA were verified by the spectrophotometric method using the UV-VIS spectrophotometer Nanodrop 8000 (Thermo Scientific) (Table 4).

Table 3

The composition of the reverse transcription reaction

Buffer solution X10	2µl
dNTP Mix25(100mM)	0.8 µl
Primer 10X RT	2 µl
Reverse transcriptase MultiScribe	1 µl
RNase inhibitor	1 µl
Nuclease-free water	3.2 µl
Total reagent volume	10 µl

qPCR reactions for assessing gene expression

The kit used for the qPCR reactions was the GoTaq qPCR Master Mix Kit (Promega USA). It is a ready-to-use solution optimised for the quantitative PCR reaction. It includes a DNA polymerase (GoTaq DNA Polymerase) and dNTPs (nucleotide triphosphate) in a bu-

Table 4

Checking the quality and quantity of DNA in samples

0	ng/ul	0	0	NaN	NaN	50	260	0	0	Blank	DNA-50
114.1	ng/ul	2.282	1.297	1.76	1.98	50	260	2.282	-0.03	Measure	DNA-50
103.7	ng/ul	2.074	1.177	1.76	2	50	260	2.074	-0.087	Measure	DNA-50
113.2	ng/ul	2.265	1.277	1.77	2	50	260	2.265	0.092	Measure	DNA-50
113.4	ng/ul	2.268	1.277	1.78	1.95	50	260	2.268	0.062	Measure	DNA-50
125.2	ng/ul	2.504	1.407	1.78	1.96	50	260	2.504	0.075	Measure	DNA-50
127.2	ng/ul	2.543	1.452	1.75	1.94	50	260	2.543	0.032	Measure	DNA-50
98.7	ng/ul	1.974	1.118	1.77	2.11	50	260	1.974	0.038	Measure	DNA-50

ffer optimised for PCR.It contains a dye,SYBR GREEN, and is supplemented with the passive reference dye, ROX. Each sample was analysed in two repetitions. In order to have a control for each individual primer, a sample without a cDNA template was analysed. The gene expression ratio was normalised with the expression value of the β-actin gene, a gene with a constitutive expression value. For all samples, the number of threshold cycles (Ct) was determined. The Δ (ΔCt) method was used for relative quantification.

RESULTS AND DISCUSSIONS

Standardising an effective superovulation stimulation protocol will lead to maximum production of oocytes, zygotes, and ultimately embryos. Ovulation in the rabbit does not occur spontaneously but is induced after mating by the secretion and increase in the level of luteinizing hormone (LH). Ovulation has been found to occur 10–12 hours after the LH surge (14). In addition to other hormonal protocols, purified menopausal serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) can be used to enhance fertility in rabbits (5).

Analysis of the expression results for the *Sox2* gene

During the embryogenesis process in the rabbit, the gene expression of the *Sox2* gene shows a specific pattern of regulation. It is observed that the gene expression level of the *Sox2* gene is lower in all developmental stages except the eight-cell and blastocyst stages. At the eight-cell stage, which occurs after repeated division of the initial zygote, an increase in *Sox2* gene expression is observed. This may be associated with its important role in maintaining cell pluripotency and early embryonic development. *Sox2* is a key transcription factor in the differentiation and maintenance of pluripotent stem cells (2).

There was a gene overexpression of group 4 in the morula stage, group that was stimulated with the male and hormones, compared to the control group, which was not stimulated (Fig.1).

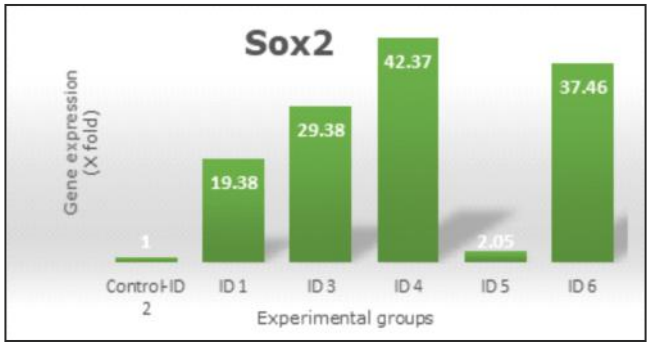

Fig.1. Graphical analysis of *Sox2* gene expression values

Treatment with LH and FSH can further contribute to the growth and development of the embryo. Although FSH alone can induce follicular growth, small amounts of LH are required to support follicular development. LH exerts a direct effect on the stimulation and modulation of folliculogenesis. LH also regulates itself directly by activating LH receptors. Activation of this receptor results in upregulation of cyclooxygenases 1, 2, and 5, oviductal glycoprotein (OGP), endothelin 1, and endothelin receptors type A and B. All of these have important roles in various oviductal functions. Thus, the oviduct contains LH receptors, and their activation results in an increased synthesis of OGP that binds to the embryo and contributes to its growth and development (4, 21). Pronounced gene expression is also highlighted in the case of group 6, which was stimulated only with the male, and the analysis was carried out for the blastocyst stage. At the blastocyst stage, which is the later stage of early embryonic development, there is a significant increase in *Sox2* gene expression.

Although *Sox2* gene expression is initially detected at the morula stage, in the later stages of development it becomes more specifically localised in the inner cell mass of the blastocyst. Thus, embryos at this stage show a greater potential for development, and the significant increase in the expression of the *Sox2* gene can be related to the essential role of the gene in the formation of the inner cell layer of the blastocyst that will later give rise to the embryo.This might be the reason why we observed an overexpression of the *Sox2* gene at this stage of embryonic development (8, 15).

Considering group 5, which was stimulated with the male and hormones, gene expression is reduced due to the fact that it was in the degraded morula embryonic stage. A poor-quality morula shows several cells and fragments excluded from the main mass of compacted cells. Thus, if the morula was of good quality, *Sox2* gene expression would have been present in adjacent cells.

Analysis of the expression results for the *Oct4* gene

The expression level of the *Oct4* gene increases at the oocyte stage, indicating an important role of this gene in early embryo development. With oocyte fertilization and zygote formation, *Oct4* continues to be expressed and maintained throughout all embryonic stages. This persistent expression of the *Oct4* gene suggests that it plays an essential role in the maintenance of cell pluripotency and embryonic development (9).The expression level of the *Oct4* gene increases in the oocyte stage, indicating an important role for this gene in early embryo development. With oocyte fertilisation and zygote formation, *Oct4* continues to be expressed and maintained throughout all embryonic sta-

ges. This persistent expression of the *Oct4* gene suggests that it plays an essential role in the maintenance of cell pluripotency and embryo development (13). In our experiment, Fig. 2 highlights the gene overexpression recorded in group 4 (morula stage), which was stimulated with the male and hormones (Fig.2).

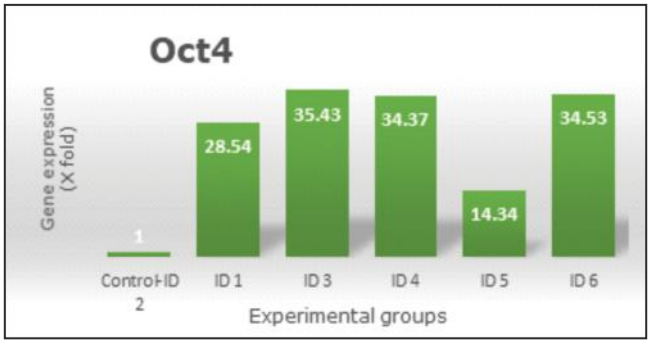

Fig. 2. Graphical analysis of *Oct4* gene expression values

A gene overexpression is evident both in group 3, which was male and hormonally stimulated and is in the blastocyst stage, and in group 6, which is also in the blastocyst stage but without hormonal stimulation. Functionally, *Oct4* is essential for early embryonic development.Throughout the morula stage,*Oct4* gene expression is abundant and uniform in all embryonic cells. However, as the outer cells of the embryo differentiate into the trophectoderm, gene expression becomes restricted to cells in the inner cell mass of the blastocyst. When primitive endoderm cells differentiate and migrate away from the ectoderm, their *Oct4* protein levels transiently increase.Subsequently, *Oct4* gene expression is inhibited in the primitive endoderm and maintained in the epiblast, concurrent with embryo implantation and gastrulation (12).Finally, *Oct4* gene expression becomes restricted to primordial germ cells (PGCs), which are first specified in the extraembryonic mesoderm at the base of the allantois bud during gastrulation. PGCs give rise to gametes that, after fertilisation will develop into a new organism in the next generation (6).Thus, considering the *in vivo* experimental model, it was highlighted that the *Oct4* gene was clearly expressed in all samples taken, but at a higher level in group 3, followed by group 6, group 4, group 1, and experimental group 5 compared with control group cells.

Analysis of expression results for the *Klf4* gene

The gene expression of the *Klf4* gene during embryonic development in the rabbit shows a similarity with the expression of the *Oct4* gene. The expression level of the *Klf4* gene increases from the oocyte stage, as with the *Oct4* gene and is maintained throughout all stages of embryonic development (7).This increase in *Klf4* gene expression from the oocyte stage suggests an important role forthis gene in early embryonic development. As the embryonic develops, *Klf4* gene expression remains constant and is maintained throughout the various stages of embryonic development. No significant differences in *Klf4* gene expression can be observed in the analysed groups, with the exception of group 5, due to the fact that it was in the degraded morula stage. The well-exemplified gene expression is justified by the fact that the *Klf4* gene, according to the specialized literature, is maintained during all stages of embryonic development(Fig. 3).

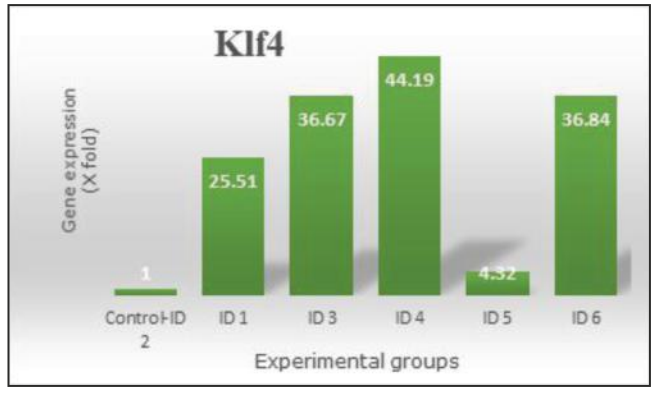

Fig. 3. Graphical analysis of *Klf4* gene expression values

Analysis of the expression results for the *c-Myc* gene

The *c-Myc* gene plays important roles in cellular processes such as DNA replication and cell cycle control. This means that the *c-Myc*gene contributes to the regulation of cell division and ensures correct DNA replication during the cell cycle (19).

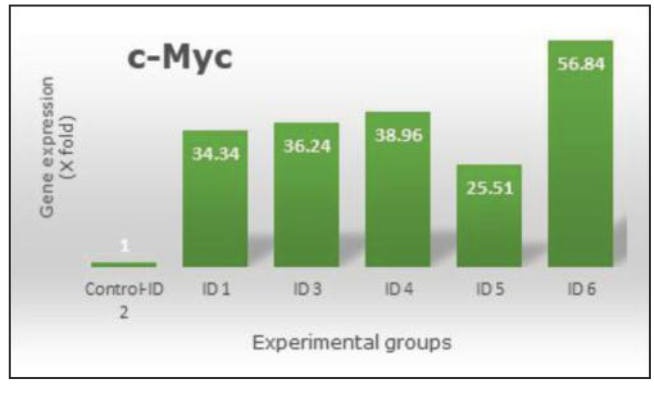

Fig. 4. Graphical analysis of *c-Myc* gene expression values

Another interesting aspect of *c-Myc*is how it affects apoptosis, the process by which cells programmed to die are removed from the body. *c-Myc*can modulate apoptosis, meaning it can influence cell survival or

death in various physiological and pathological contexts (16). In this study, the level of *c-Myc*gene expression can be detected at a low level in group 5, and higher levels can be detected in group 6, followed by groups 4, 3, and 1 (Fig. 4). This can be correlated with the fact that the gene has increased expression at the blastocyst stage present in group 6, and the degraded morula stage embryos in group 5 show reduced gene expression.

In this paper, some similarities were found between the analyses carried out for the four genes. For example, the overexpression of the four genes was recorded in groups 3, 4, and 6, these being in the morula and blastocyst stages. Also, the levels and expression patterns of these genes may vary depending on certain stimuli, such as the gonadotropins used in this study. In the cases of the groups to which the analogue hormones FSH and LH were administered, an overexpression of the *Oct4*, *Sox2*, *Klf4,* and *c-Myc* genes was found, with the exception of group 5, in which there was a degraded morula stage.

CONCLUSIONS

The overexpression of the four assessed genes was recorded in groups 3, 4, and 6, these being in the morula and blastocyst stages.Sox2 this gene was much more intensely expressed in the stage of morula quality I than in the blastocyst stage. In the cases of the groups to which the analogue hormones FSH and LH were administered, an overexpression of the *Oct4*, *Sox2*, *Klf4,* and *c-Myc* genes was found, with the exception of group 5, in which there was a degraded morula stage.

The gene expression of „Yamanaka" factors is best expressed in the morula and blastocyststages. Due to the obtained results, it can be demonstrated that the gene expression of „Yamanaka" factors is best expressed in the morula and blastocyst stages.

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