OCT-4 EXPRESSION IN BLASTOCYST FROM TWO SELECTED LINES

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ABSTRACT

Pre-implantation development is characterized by distinct biological steps including first cleavage division, activation of the embryonic genome, compaction, and blastocyst formation. These changes need the expression well orchestrated of genes both of the maternal and/or embryonic genome. Usually prenatal survival seems to be mainly determined by the female, whereas the embryo-fetus plays a secondary role. In this preliminary study we focused on a major developmental gene expressed mainly by the embryo. Oct-4 expression patterns, transcript of POU5F1 gene, have been studied in several mammalian species, but are not known in rabbit. In other species, this transcript was highly expressed in early stages. It is related with undifferentiated stages by up-regulation of other genes as IFN, Sox-2 or Ets-2. The Oct-4 is related with cellular differentiation in mammalian embryos. In fact, Oct-4 is expressed in embryonic cell but the expression is not equal in different stage of development. The objective of this study was to examine the Oct-4 expression in pre-implantatory blastocyst from two rabbit lines selected for litter size. Embryos were recovered at the fifth and sixth day post-mating from 15 females of two selected lines by litter size at weaning. Total RNA was extracted and retrotranscripted individually from 117 blastocysts. The quantitative PCR for the Oct-4 expression was realized and the β-actin was used for housekeeping gene. The relative expression ratio of Oct-4 gene was calculated with the ΔΔCt method normalized to β-actin gene (ACTB). The results showed that the Oct-4 expression is not related with ovulation rate and selected line, but the relationship between Oct-4 expression and age of embryos is statistically significant (the means of Oct-4 relative expression were 89.16 and 303.06 for days 5 and 6 respectively, $P<0.05$). The characterization of profile secretion of this transcript can be a valuable tool to accurate assessment of rabbit embryo viability in genetic and biotechnological studies as *in vitro* production or cryopreservation of embryos.

Key words: POU5F1 gene, Oct-4 quantitative PCR, Blastocyst development.

INTRODUCTION

The reproductive efficacy in the rabbits is marked by the prenatal mortality (Adams, 1960; Torres, 1982; Bolet and Theau-Clement, 1994; Mocé *et al*. 2002). Most of these losses are characterized by the asynchronization between the embryo and the uterus that causes problems in the process of implantation and/or placentation.

Preimplantation development is characterized by distinct biological steps including first cleavage division, activation of the embryonic genome, compaction, and blastocyst formation. These changes require the well-orchestrated expression of genes derived from the maternal and/or embryonic genome. One of them, it is the gene POU5F1, which belongs to the POU transcription factor family. This gene presents an Oct4 transcript. Oct-4 is the earliest expressed transcription factor that is known to be crucial in pre-implantation development (Nichols *et al*., 1998; Fair *et al*., 2004; Dode *et al.*, 2007) and it has been related with the cellular differentiation in different mammalian species (Pesce *et al*., 1998; Hansis *et al*., 2001). Oct-4, as transcription factor protein, is known to bind to DNA and activate or repress transcription of several of these genes expressed during early embryonic development (Smith *et al*., 2007). In murine, it is known to be crucial in murine pre-implantation

development The mRNA and protein of Oct-4 have been found in murine oocytes and in the nuclei of subsequent cleavage stage embryos while in the expanded murine blastocyst stage both mRNA and protein were predominantly found in the inner cell mass (ICM) (Fair *et al*., 2004; Fisher *et al*., 2006; Dode *et al*., 2007). Even in fully expanded bovine and porcine blastocysts both ICM and trophectoderm cells were found to be positive for Oct-4 protein. Studies on Oct-4 showed that regulatory effects on target genes related to the cytoskeleton, apoptosis, cell cycle and metabolic processes. The vast majority (85%) of the differentially expressed transcripts between the in vivo produced embryo and in vitro cultured blastocysts, which it lead to suggest that the primary reason why in vitro embryos are inferior developmental competence compared to *in vivo* embryos is because of a deficiency of the machinery associated with transcription and translation. So study Oct-4 transcript in embryos can be an excellent indicator of ability of development of embryos pre-implantation.

Oct-4 is produced in embryo stem cells, epiblast and primordial germ line cells or it has been detected in multipotent adult progenitor cells (Rosner *et al*., 1990; Schöler *et al*., 1990; Jiang *et al*., 2002). Oct-4 is a key determinant of the properties of embryo stem cells because it activates or represses target genes (Schöler HR *et al*., 1991; Ambrosetti *et al*., 1997; Shushan *et al*., 1998; Liu and Roberts, 1996). Oct-4 is known to bind to DNA and activate or repress transcription of several of these genes expressed during early embryonic development (Smith *et al*., 2007). Large-scale transcriptional analysis of mouse blastocysts using annealing control primer (ACP) technique after down-regulation of Oct-4 transcript by RNAi approach showed that eight transcripts (Atp6ap2, GK003, Ddb1, hRscp, Dppa1, Dpp3,Sap18, and Rent1) were down-regulated and two (Rps14 and Etif2b) were up-regulated in Oct-4 dsRNA-injected blastocysts. A similar study inhuman ES cells showed that the knockdown of Oct-4 has resulted in differential regulation of pluripotency markers (Nanog, Sox2). In the same study, down-regulation of Oct-4 transcript showed to affect the key components of WNT, TGF-b, FGF, MAPK, NOTCH, Hedgehog, JAK/STAT and ECM signaling pathways as well as regulators of the cytoskeleton, apoptosis, cell cycle and metabolic processes.

The objective of this preliminary study was to quantify the Oct4 expression in morphologically normal blastocysts from two selected rabbit lines.

MATERIALS AND METHODS

Animals and experimental design

Fifteen multiparous non lactating females were used. They belonged to the two different maternal lines from Animal Science Department at the Polytechnic University of Valencia (Spain). Eight females belonged to the line A (New Zealand White line, selected since 1980 for litter size at weaning with a family index) and 7 females belonged to the line V (synthetic maternal line, selected since 1982 on litter size at weaning with a BLUP under a repeatability animal model, Estany *et al*., 1989). All females were mated by fertile buck belonging to same selected line. Embryos were recovered from uterine horn by flushing 10 ml of PBS+BSA (2 g/l) at 5 and 6th day after mating. From each female, the number of corpora lutea as an estimate of ovulation rate and number of normal embryos were recorded. Blastocysts were used individually to realize the expression studies. A total of 117 blastocysts were recovered, 52 at 5 days (23 V and 29 A) and 65 at 6 days (28 V and 37 A).

RNA extraction

Total RNA was isolated from individual embryos by using Trizol® (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Samples were treated with DNAse as described by the manufacturer. Total RNA was collected in elution tubes in a volume of 10 µl. RNA quality and quantity was determinate electrophoretically by OD_{260}/OD_{280} nm absorption ratio >1.9. Reverse transcription reaction was performed by Quantitec Kit (Qiagen). Before addition of RT enzyme and random primers, samples and buffer were incubated at 42ºC for 2 min. The RT and random primers

were added, reactions were incubated at 42ºC for 15 min. The reaction was inactivated by incubation at 95ºC for 3 min. Embryos cDNA were stored at -20ºC.

Real-time PCR Oct-4

PCR reactions were run as duplicate in a total volume of 25 µl which consisted of SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 25 µM each of sequence-specific primers (Table 1) and 1 ng of cDNA. The PCR protocol included an initial step of 50ºC (2 min) and second step for denaturation of 95ºC (15 sec), followed by 45 cycles of 95ºC (30 sec), 60ºC (60 sec) and 72ºC (60 sec). At the end of the amplification cycles, a melting curve analysis was performed to verify specific amplification. Melting curve data were collected between 60º and 95ºC with a ramp time of 20 minutes. Relative quantification is based on the comparison of Ct at a constant level of fluorescence. The amount of transcript present is inversely related to the observed Ct. The relative expression ratio is calculated with the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001; Livak and Schmittgen, 2001). That is, to determinate a normalized arbitrary value for each gene, every data point was normalized to the reference gene ACTB (housekeeping), as well as to their respective control. The real time quantitative PCR was evaluated according to efficiency by melting curve (Pfaffl, 2001) and a coefficient of determination with serial dilutions for the cDNA of samples recovered pools.

Table 1. Oligonucleotide primers used for gene expression analysis

Gene	Primer	Sequence (5'-3')	Fragment size (bp)	Annealing Temperature (ºC)	Gene bank accession No.
B-actin (ACTB)	Forward	CACACGGTGCCCATCTACG	203	59	AF000313
	Reverse	GCCATCTCCTGCTCGAAGTC			
Oct-4	Forward	CATGAGCAGCAAGGGAAAAC	231	58	NM001099957
	Reverse	GGGCGATGAACCATACCG			

Statistical Analysis

The model used for Oct-4 expression was:

$$y_{ijklm} = \mu + b*TO + L_i + D_j + h_k + e_{ijklm},$$

where y_{ijklm} is Oct-4 expression of embryo, µ is the general mean, TO es the covariate ovulation rate, L_i is the fixed effect of line with two levels (A,V), D_j is the fixed effect of embryo age with two leves (5 or 6 days), h_k is the random effect of the female in which the embryos are collected, and e_{ijklm} is the residual. The real-time data of mRNA expression related with ovulation rate, female, selected line and recovered embryos days were analyzed across line using the General Linear Models procedure of Statgraphics PLUS. For the statistical analysis, interaction day-line has been analyzed, including the ovulation rate as covariate and the female as random factor.

RESULTS AND DISCUSSION

The results of PCR Real Time efficiency indicated a high reproducibility of real-time PCR specific for Oct-4 expression. Linear regression of calibration curves provided a mean slope of 3.27, suggesting a mean slope efficiency of 94%, with a coefficient of determination (R^2) of 0.995. The results of dissociation stage of two genes are showed in Figure 1. No differences were observed between lines for Oct-4 expression. However, the recovery day seems to have an effect on the expression of this gene (Table 2). The Oct-4 expression showed statistical differences between embryos from fifth and sixth days (89.16, 303.06 for 5^{th} and 6^{th} days respectively, $P<0.05$, Figure 2). The covariate ovulation rate was not significant.

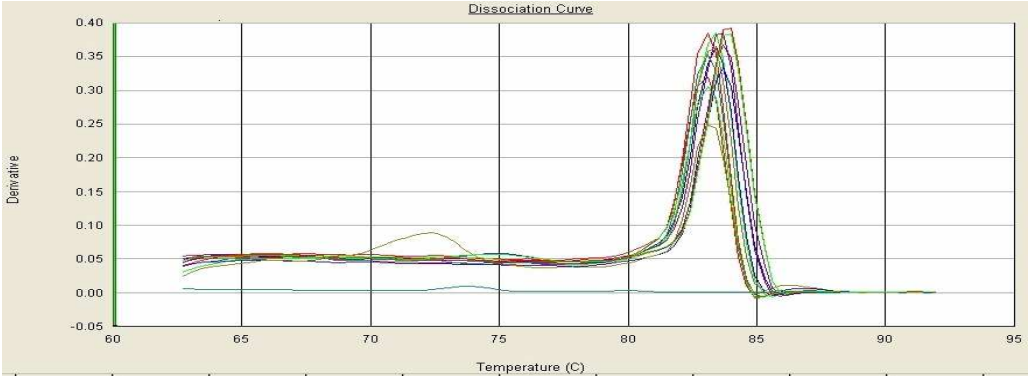

Figure 1: Dissociation curve of Oct-4

Table 2: Results of statistical analysis

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Line	11,5234	1	11,5234	1,68	0,1969
Day	55,2667	2	27,6334	4,03	0,0199*
Line*Day	7,46421	2	3,7321	0,54	0,5812
Residual	897,144	131	6,84843		

*P<0.05, statistical significance. Durbin-Watson statistic 1, 6175 (P=0.0123)

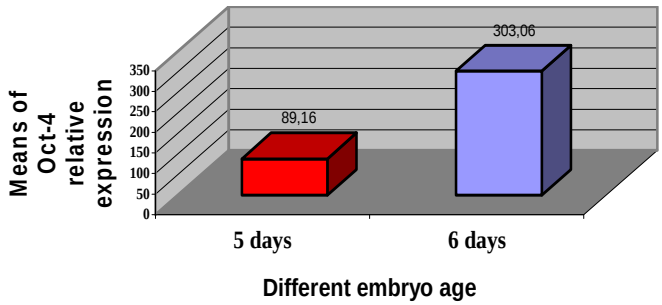

Figure 2: Means of Oct-4 relative expression at different embryo age

Similar results have been observed in other species, Oct-4 expression pattern differing according to the stage of embryonic development. In mouse Oct-4 expression is higher in early stage that is known to be crucial in murine preimplantation development (Okamoto *et al*., 1990; Rosner *et al*., 1990; Schöler *et al*., 1990). In bovine embryos, the Oct-4 transcript was quantified through the preimplantation developmental stages. This transcript was found to be highly abundant at early developmental stages (Nganvongpanit *et al*., 2006). The Oct-4 gene and transcript has been related with undifferentiated and maintenance stages (Ovitt and Schöler, 1998). The expression Oct-4 gene is up-regulated in early stage and lower in advanced stages of embryo development. The Oct-4 protein can be a repressor as well as a transactivator of other genes during embryonic development (Ezashi *et al*., 2001).

CONCLUSIONS

In conclusion, our results show that the Oct-4 expression in rabbit is not different from other species, being higher in blastocyst of 6th after mating. The characterization of profile secretion of this transcript can be a valuable tool to accurate assessment of embryo viability in genetic and biotechnological studies as *in vitro* production or cryopreservation embryos.

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REFERENCES

Ambrosetti D.C., Basilico C., Dailey L. 1997. Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites. *Mol. Cell. Biol., 17, 6321– 6329.*

Adams C.E. 1960. Studies on prenatal mortality in the rabbit, *Oryctolagus cuniculus:* the amount and distribution of loss before and after implantation. *J. Endocrin., 19, 325-344.*

Ben-Shushan E., Thompson J.R., Gudas L.J. 1998. Rex-1, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1, binding to an adjacent site. *Mol. Cell. Biol., 18, 1866–1878.*

Bolet G., Theau-Clement M. 1994. Fertilization rate and preimplantation embryonic development in two rabbit strains of different fecundity, in purebreeding and crossbreeding. *Anim. Reprod. Sci., 36, 153-162*.

Dode MAN, Dufort I, Massicotte L, Sirard M.A. 2006. Quantitative expression of candidate genes for developmental competence in bovine two-cell embryos. *Mol. Reprod. Dev., 73, 288–97.*

Estany J., Baselga M., Blasco A., Camacho J. 1989. Mixed model methodology for estimation of genetic response to selection in litter size in rabbits. *Livest. Prod. Sci., 21, 67–75.*

Fair T., Murphy M., Rizos D., Moss C., Martin F., Boland M.P. 2004. Analysis of differential maternal mRNA expression in developmentally competent and incompetent bovine two-cell embryos. *Mol. Reprod. Dev., 67, 136–44*.

Fischer Russell D., Baqir S., Bordignon J., Betts D.H. 2006. The impact of oocyte maturation media on early bovine embryonic development. *Mol. Reprod. Dev., 73, 1255–70*.

Ezashi T., Ghosh D., Roberts R.M. 2001. Repression of Ets-2-induced transactivation of the tau interferon promoter by Oct-4. *Mol. Cell. Biol., 21, 7883–7891*.

Hansis C., Tang Y., Grifo J., Krey L. 2001. Analysis of Oct4 expression and ploidy in individual human blastomeres. *Mol. Human Repr., 7, 155-61*.

Jiang Y., Jahagirdar B.N., Reinhardt R.L. 2002. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature, 418, 41–49.*

Livak K.J., Schmittgen T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Method, 25, 402-408.*

Liu L., Roberts R.M. 1996. Silencing of the gene for the beta subunit of human chorionic gonadotropin by the embryonic transcription factor Oct-3/4. *J. Biol. Chem., 271, 16683–16689.*

Mocé L., Santacreu M.A., Climent A. 2002. Effect of divergent selection for uterine capacity on progesterone, estradiol and cholesterol levels around implantation time in rabbits. *World Rabbit Science, 10, 89-97*.

Nganvongpanit K., Müller H., Rings F., Hoelker M., Jenner D., Tholen E., Havlicek V., Besenfelder U., Schellander K., Tesfaye D. 2006. Selective degradation of maternal and embryonic transcripts in in vitro produced bovine oocytes and embryos using sequence specific double-stranded RNA. *Reproduction, 131, 861-874.*

Nichols J., Zevnik B., Anastassiadis K. 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell, 95, 379–391.*

Okamoto K., Okazawa H., Okuda A., Sakai M., Muramatsu M., Hamada H. 1990. A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. *Cell, 60, 461–472.*

Ovitt C.E., Schöler H.R1. 1998. The molecular biology of Oct-4 in the early mouse embryo. *Molecular Human Reproduction, 4, 1021–1031*.

Pesce M., Wang X., Wolgemuth D., Schöler H. 1998. Differential expression of the Oct4 transcription factor during mouse germ cell differentiation. *Mech. Dev., 71, 89-98.*

Pfaffl M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucl. Acids Res., 29, 2002-7.*

Roberts R.M., Ezashi T., Das P. 2004. Trophoblast gene expression: Transcription factors in the specification of early trophoblast. *Repr. Biol. Endocrin., 2, 47.*

Rosner M.H., Vigano M.A., Ozato K. 1990. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature, 345, 686–692*.

Schöler H., Ruppert S., Suzuki N., Chowdhury K., Gruss P. 1990. New type of POU domain in germ line-specific protein Oct4. *Nature, 344, 435-9.*

Schöler H. 1991. Octamania: the POU factors in murine development. *Trends Genet., 7, 323-9.*

Schöler H.R., Dressler G.R., Balling R., Rohdewohld H., Gruss P. 1990. Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. *EMBO Journal, 9, 2185–2195.*

Schöler H.R., Ciesiolka T., Gruss P. 1991. A nexus between Oct-4 and E1A: Implications for gene regulation in embryonic stem cells. *Cell, 66, 291–304.*

Smith C., Berg D., Beaumont S., Standley N.T., Wells D.N., Pfeffer P.L. 2007. Simultaneous gene quantitation of multiple genes in individual bovine nuclear transfer blastocysts. *Reproduction, 133, 231–242*.

Torres S. 1982. Etude de la mortalité embryonnaire chez la lapine. *In: Proc. III Journées de la Recherche Cunicole, Paris France, Communication nº15*.