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Differential effects of culture and nuclear transfer on relative transcript levels of genes with key roles during preimplantation

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Summary

It is well known that the preimplantation culture environment to which embryos are exposed influences the expression of developmentally important genes. Recently, it has been reported that MEMα, a culture medium commonly used for somatic cells, allows high rates of preimplantation development and development to term of mouse somatic cell nuclear transfer (SCNT) embryos. The objective of this study was to compare the differential effects of this medium and of the nuclear transfer procedure on the relative mRNA abundance of several genes with key roles during preimplantation. The relative mRNA levels of nine genes (Glut 1, Glut 5, G6PDH, Bax, Survivin, Gpx 1, Oct4, mTert and IGF2bp1) were quantified at blastocyst stage on cumulus cell cloned embryos cultured in MEMα, as well as on *in vivo* cultured and MEMα cultured controls. Only three of the nine transcripts analysed (Glut 5, Gpx 1 and Igf2bp1) were significantly down-regulated at blastocyst stage in *in vitro* produced controls. However, most genes analysed in our MEMα cultured cloned embryos showed altered transcription levels. Interestingly, between cloned and *in vitro* produced controls only the transcription levels measured for Glut 1 were significantly different. This result suggests that Glut 1 may be a good marker for embryo quality after cumulus cell nuclear transfer.

Keywords: Culture medium, mRNA transcription, Nuclear transfer

Introduction

Cloning by nuclear transplantation involves the transfer of nuclei from undifferentiated or differentiated cells into enucleated oocytes. Reconstructed embryos are chemically activated, cultured in a synthetic medium (usually until morula/blastocyst stage), and then transferred into the uterus of a pseudopregnant synchronized recipient (Campbell *et al.*, 1996). To date, and independently of the species, the overall efficiency of this cloning procedure does not exceed the 1–5% range of nuclear transfer (NT) embryos developed into live offspring (Wilmut *et al.*, 1997; Kato *et al.*, 1998; Wakayama *et al.*, 1998; Baguisi *et al.*, 1999; Onishi *et al.*, 2000; Polejaeva *et al.*, 2000), and of those reconstructed embryos that survive to term, a large proportion show abnormal phenotypic effects (Ogura *et al.*, 2002). Evidence from NT experiments indicates that the reprogramming of donor nuclei in reconstructed embryos is incomplete, resulting in altered gene expression, epigenetic defects and, consequently, poor developmental capability (Dean *et al.*, 2003; Shi *et al.*, 2003). To improve the cloning method it is necessary to achieve a better and more thorough understanding of the biological processes occurring after NT. It is essential that the overall physiology and metabolism of NT embryos operate efficiently in order to maintain viability.

There is also a large amount of data demonstrating that the post-fertilization embryo culture environment can have a dramatic effect on the gene expression pattern of the preimplantation embryo, and that this in turn can have serious implications for the normality of the implanting blastocyst (Khosla *et al.*, 2001; Natale *et al.*, 2001; Wrenzycki *et al.*, 2001; Rizos *et al.*, 2002, 2003; Lonergan *et al.*, 2003). *In vitro* culture and NT have

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been associated with the Large Offspring Syndrome (Young *et al.*, 1998; Wakayama, 1998, 1999). As yet, there is no definitive explanation of the mechanism through which the syndrome occurs, although an altered gene expression pattern and a suboptimal culture system are likely candidates. Gross phenotypic alterations such as this, demonstrate that manipulation of the embryonic environment can profoundly alter subsequent placental, fetal, peri- and postnatal development (Fernandez-Gonzalez *et al.*, 2004). Somatic cell nuclear transfer (SCNT) embryos are usually cultured in embryonic media of simple salt composition, based on the common belief that they have to assume the biological patterns of a normal embryo. However, recent data have raised some doubts about this requirement, under the hypothesis that the ongoing nuclear reprogramming and gene expression remodelling after NT generates an embryo with different physiological and metabolic demands (Chung *et al.*, 2002). Recently, there have been reports of high rates of preimplantation development (~60% blastocyst formation) and subsequent development to term (2% of activated embryos) of somatic cell clones after *in vitro* culture in MEMα, a culture medium commonly used for somatic cells (Gao & Latham, 2004). However, until now no data have been published regarding the effect of a somatic culture medium on the preimplantation mRNA levels of normal and SCNT embryos. The objective of this study was to perform this analysis on both embryo types after *in vitro* culture in MEMα, by measuring the relative transcript abundance at blastocyst stage of several genes with key roles during preimplantation. *In vivo* produced controls were also analysed. By comparing the different transcription profiles and associated preimplantation development, we hoped to better understand the physiology of cloned embryos and to identify genetic markers of embryo quality after SCNT.

Materials and methods

Animals

B6D2F1 mice (Harlan Iberica SL, Barcelona, Spain) were used as donors of oocytes and sperm for all experiments. Females were 6–8 weeks old at the time of the experiments, and males at least 3 months old. Mice were fed *ad libitum* with a standard diet and maintained in a temperature- and light-controlled room (23 °C; 14 h light, 10 h dark). All animal experiments were performed in accordance with Institutional Animal Care and USE Committee guidelines and in compliance with guidelines established in the *Guide for Care and Use of Laboratory Animals* as adopted and promulgated by the Society for the Study of Reproduction.

Oocyte and embryo collection

Metaphase II (MII) oocytes and fertilized pronuclear stage embryos were collected from superovulated female mice, 14 and 20 h post-human chorionic gonadotrophin (hphCG) injection, respectively. Cumulus cells were dispersed with 300 IU/ml hyaluronidase. Oocytes were cultured in CZB until use, and embryos in alpha-Modified Eagle's Medium (MEMα; Sigma, Madrid, Spain) supplemented with 1 mM glutamine and 5 mg/ml bovine serum albumin (BSA) at 37 °C in an atmosphere of 5% CO_2 in air. *In vivo* cultured preimplanting blastocyst stage embryos were collected from superovulated females on day 4 post-mating and immediately processed for gene expression analysis.

Nuclear transplantation and oocyte activation

NT with cumulus cell donor nuclei was done as described previously (Wakayama *et al.*, 1998; Moreira *et al.*, 2003) with minor alterations. Cells obtained from four to eight cumulus–oocyte complexes were washed in MEMα, resuspended in 100 µl of this medium and cultured at 37 °C in an atmosphere of 5% CO_2 in air until use. MII oocyte enucleation (~15 hphCG) was carried out by aspiration of a translucent cytoplasmic area containing the metaphase plate in CZB medium containing 5 µg/ml cytochalasin B. Enucleated oocytes were washed and returned to culture. Two hours later, oocyte reconstitution was performed. Cumulus cells were transferred to CZB/10% polyvinylpyrrolidone, lysed in a pipette attached to a piezo unit, and a single nucleus injected into an enucleated oocyte. Oocytes were cultured in CZB for at least 1 h before artificial activation. A PMM-150 FU piezo-impact unit (Prime Tech, Japan) and Eppendorf micromanipulators (Hamburg, Germany) were used.

Recipient oocytes were activated for 6 h with 10 mM $SrCl_2$ in Ca^{2+}-free CZB medium containing 5 µg/ml cytochalasin B in order to prevent polar body extrusion and maintain diploidy. At the end of the treatment, reconstructed embryos were washed and cultured in MEMα. As a control for oocyte quality, parthenogenetic activation of MII oocytes was carried out as for NT embryos.

RNA extraction and reverse transcription

Poly(A) RNA was prepared from four or five groups of pools of 10 embryos, following the manufacturer's instructions using a Dynabeads mRNA Direct KIT (DYNAL). Briefly, samples were lysed in 50 µl DYNAL lysis/binding buffer. After vortexing and a brief centrifugation, the samples were incubated at room temperature for 10 min. Ten microlitres of prewashed Dynabeads oligo (dT)25 were added to each sample. After 5 min of hybridization, the beads were separated

Table 1 Details of primers used for RT-PCR

Gene	Primer	Sequence (5′–3′)	Fragment size (bp)	Annealing temperature (°C)	GenBank accession no.
β-actin	5′	GAGAAGCTCTGCTACGTCG	255	59	K00622.1
	3′	CCAGACAGCACCGTGTTGG			
Glut 1	5′	TTCTCGGTGGGAGGCATGATTGG	240	56	M60448.1
	3′	GAAGCTCCGTGGGGGATACCTCC			
Glut 5	5′	CATCTCCATCATCGTCCTCA	531	56	AF308830
	3′	GTAGATGGTGGTGAGGAGAC			
G6PDH	5′	TTGCAGCAGCTGTCCTCTATGTG	220	59	NM 008062
	3′	GCCAGGCTTCTTGGTCATCATC			
Bax	5′	AAGCTGAGCGAGTGTCTCCGGCG	361	59	MN 07527
	3′	GCCACAAAGATGGTCACTGTCTGCC			
Survivin	5′	CATTCATCCGGTTGTGCTTTCC	175	59	AY606044
	3′	GCTGCTCAATGGCACAGCGGAC			
GPX	5′	TCGCCAAGGCAGATGTGAGAGC	216	56	L10325.1
	3′	TTCATGCCAGTGGGTCAGGGTG			
Oct 4	5′	GGCGTTCTCTTTGGAAAGGTGTTC	312	60	NM 13633.1
	3′	CTCGAACCACATCCTTCTCT			
mTert	5′	TGCGGCCCATTGTGAACATGAG	209	59	NM_009354
	3′	AGCACGCACACGCAGCACAAAG			
Igf2bp1	5′	AATGGGCGTCGTGGAGGCTTTG	400	56	NM_009951
	3′	TCCGCCCTTCCTTGCCAATGAG			

from the binding buffer using the DYNAL magnetic separator. Subsequently, the beads were washed in DYNAL buffer A and B and the poly(A) RNA was eluted from them by adding 11 µl of DEPC-treated water. The eluted poly(A) mRNA was used in the reverse transcription–polymerase chain reaction (RT-PCR) in a total volume of 20 µl using 2.5 µM random hexamer primer, 1× RT buffer, 20 IU RNase inhibitor, 50 IU AMV reverse transcriptase enzyme (Promega, Madrid, Spain), 5 mM $MgCl_2$ and 1 mM of each dNTP. Tubes were heated to 70 °C for 5 min to denature secondary RNA structures before the addition of the reverse transcriptase enzyme. The reaction was then incubated at room temperature for 10 min and then at 42 °C for 60 min to allow reverse transcription of RNA, followed by 93 °C for 1 min to denature the reverse transcriptase enzyme.

Quantitative real-time PCR

PCR was performed using a Rotorgene 2000 Real Time CyclerTM (Corbett Research, Sydney, Australia) and SYBR Green (Molecular Probes, Eugene, OR) as a double-stranded DNA-specific fluorescent dye. The PCR reaction mixture (25 µl) contained 2.5 µl 10× buffer, 3 mM $MgCl_2$, 2 IU Taq Express (MWGAG Biotech, Ebersberg, Germany), 100 µM of each dNTP, and 0.2 µM of each primer. In addition, the double-stranded DNA dye, SYBR Green I, (1:3000 of 10 000× stock solution) was included in each reaction. The PCR protocol included an initial step at 94 °C (2 min), followed by 40 cycles at 94 °C (15 s), 56–59 °C (30 s) and 72 °C (30 s). Fluorescence data were acquired at 85 °C. The melting protocol consisted of 60 s at 40 °C and 5 s per degree (during which fluorescence was monitored) while raising the temperature from 50 to 94 °C. Product identity was confirmed by ethidium-bromide-stained 2% agarose gel electrophoresis. As negative controls, real-time PCR-reaction tubes were prepared without RNA or reverse transcriptase. The comparative CT method was used for quantification of expression levels. Primer sequences, annealing temperature, approximate sizes of amplified fragments, as well as GenBank accession numbers, are shown in Table 1.

Statistical analysis

Data on embryo development and mRNA expression were analysed using the SigmaStat (Jandel Scientific, San Rafael, CA) software package. The chi-square test was used for analysis of embryo development and one-way repeated-measures ANOVA (followed by multiple pair-wise comparisons using the Student–Newman–Keuls method) were used for the analysis of differences in mRNA expression assayed by quantitative real-time PCR. Differences of $p < 0.05$, were considered significant.

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Table 2 *In vitro* development of NT and *in vivo* fertilized embryos cultured in MEMα

Culture medium/ manipulation	Cultured oocytes/ zygotes	2-cell embryos (%)[1]	4-cell embryos (%)[1]	Blastocysts (%)*
MEMα/NT	87	52[a] (60)	36[a] (41)	29[a] (33)
MEMα/*in vivo* fertilized	60	60[b] (100)	60[b] (100)	57[b] (95)

*Percentages from cultured oocytes.
[a,b]Values with different superscripts are significantly different ($p < 0.01$).

Results

In this study, the effect of a preimplantation exposure to a somatic culture medium (MEMα) was assessed on *in vivo* fertilized and NT embryos by characterizing and comparing their *in vitro* development and relative transcription level profiles at blastocyst stage. *In vitro* development outcomes in MEMα are shown in Table 2. As expected from non-manipulated zygotes, the developmental rates of *in vivo* fertilized embryos were much higher than that of cloned embryos. Such developmental difference was evident at all developmental stages monitored, reaching a maximum at blastocyst stage. Fifty-seven (95%) of 60 *in vivo* fertilized embryos developed to blastocyst stage, compared with only 29 (33%) of 87 NT embryos. It is important to highlight that the preimplantation exposure to this somatic medium did not affect the developmental potential of the *in vitro* produced blastocysts obtained from fertilized embryos. Of 36 *in vivo* fertilized *in vitro* produced blastocysts transferred to recipient females, 34 (94%) implanted (data not shown). These implantation results, as well as those concerning development to term, were repeatable and comparable to the ones currently obtained in our laboratory using the traditional embryonic culture medium, KSOM (Potassium Simplex Optimized Media).

When the transcript levels of Glut 1, Glut 5, G6PDH, Bax, Survivin, Gpx 1, Oct4, mTert and IGF2bp1 were assessed by quantitative real-time PCR on developing blastocysts, non-manipulated *in vivo* cultured controls were also analysed (Fig. 1). The results of our analysis indicated that, when culture of fertilized embryos was done in MEMα, the normal transcript profile of three (Glut 5, Gpx 1 and IGF2bp1) of the nine analysed genes was not maintained (ANOVA, $p < 0.05$). Moreover, the relative transcript level of eight genes was modified in cloned blastocysts ($p < 0.05$), suggesting, as previously reported for other culture media, that altered gene expression probably accounts for the poorer developmental capability of these embryos. Interestingly, the mRNA level profiles detected in cloned and MEMα cultured controls was not significantly different. Between these two experimental groups, only for Glut 1 were significant relative transcript differences detected. Possible implications of this finding will be discussed further.

Discussion

Mouse cloning by SCNT is inefficient, and most cloned embryos suffer developmental arrest before or after implantation, indicating defects in early essential events. It is possible that transplanted donor nuclei impose nutrient demands on reconstructed embryos that are not satisfied by tradition embryo culture media. In supporting of this hypothesis, high rates of preimplantation development and subsequent development to term of somatic cell clones have recently been reported after *in vitro* culture in MEMα, a medium usually used for culture of somatic cells (Gao *et al*., 2003; Gao & Latham, 2004). However, despite the growing acceptance of the use of this medium for *in vitro* culture of SCNT embryos, a characterization of the induced embryonic transcription level profiles has not been done. The important comparison with *in vitro* produced controls has also never been reported. Our study tried to achieve both these objectives. The outcomes of our experiments indicated that the high rates of preimplantation development of SCNT embryos *in vitro* cultured in MEMα were not associated with normal embryonic patterns of transcription (Fig. 1). Interestingly, however, the overall transcription pattern of these embryos was very similar to that seen in *in vivo* fertilized MEMα cultured controls. This finding indicates that *in vitro* culture in MEMα can partially overcome the dysfunctional state of SCNT embryos. However, *in vitro* culture in MEMα does not completely solve the developmental potential differences between non-manipulated *in vivo* fertilized controls and SCNT embryos. This becomes evident when implantation rates are compared; the ones obtained after SCNT are much poorer.

The relative transcript level analysis done in this study tried to characterize a particular set of genes involved in important physiological processes: glucose (Glut 1) and fructose (Glut 5) transport and metabolism; oxidative stress (G6PDH); apoptosis (Bax, Survivin, Gpx 1); and cell proliferation and differentiation (Oct4, mTert, Igf2bp1). The results of our analysis showed higher expression of Glut 1 in NT embryos. This may be a reminiscent feature of the cumulus cells used in this study as nuclear donors, which are specialized in metabolizing glucose and passing the resulting metabolites to developing oocytes at high rates (Downs & Utecht, 1999). Because the nuclear reprogramming in SCNT embryos is usually incomplete, the propensity

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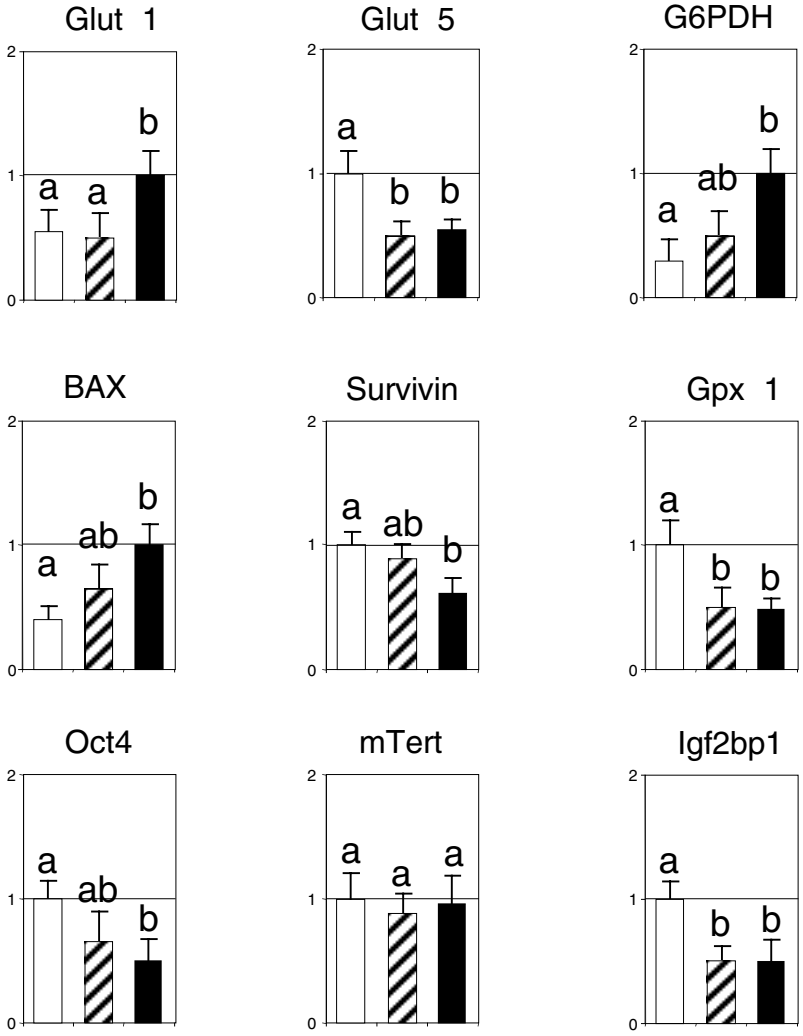

Figure 1 Relative mRNA abundance of Glut 1, Glut 5, G6PDH, Bax, Survivin, Gpx 1, Oct4, mTert and IGF2bp1 in mouse blastocysts obtained from: oocytes fertilized and cultured *in vivo* (white bars); *in vivo* fertilized embryos *in vitro* cultured in MEMα (dashed bars); and nuclear transfer embryos *in vitro* cultured in MEMα (black bars). [a,b]Different superscripts indicate significantly different abundances ($p < 0.05$).

for increased glucose uptake and oxidative glucose metabolism may continue during preimplantation embryo development, leading to altered physiology and metabolism at blastocyst stage. The reduced mRNA level of Glut 5, an important transporter of fructose, essential for nucleotide synthesis after zygotic transcription, in MEMα cultured controls and NT embryos suggests that this medium does not allow a correct metabolic shift from the pentose phosphate pathway (PPP) towards fructose uptake via Glut 5 production of ribose-5-phosphate. In NT embryos this was concomitant with higher transcript levels of G6PDH, which catalyses the first irreversible step of the PPP. The fact that G6PDH is also involved in maintenance of intracellular redox states by NADPH production, alternatively suggests higher oxidative stress levels in these embryos.

In this study, we have also analysed the expression of genes (Bax, Survivin and Gpx 1) governing cell death pathways involved in the regulation of preimplantation embryo survival (Jurisicova & Acton, 2004). It is known that the majority of NT embryos suffer from various cellular defects, including reduced cell number, abnormal nuclear ploidy, altered genomic imprinting and altered mRNA expression, and are thus likely to be a poor reflection of molecular pathways found in healthy embryos. In our experiment, a higher transcript level of Bax, known to be a caspase-dependent pro-apoptotic regulator (Cory *et al.*, 2003), was observed in our MEMα cultured NT embryos. Concomitantly, we also detected lower Survivin mRNA expression in these embryos. Survivin is implicated both in the regulation of cell cycle progression and in the inhibition of apoptosis (Altieri, 2003), and it has been

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shown that its disruption leads to embryonic lethality between morula and blastocyst stage (Kawamura *et al.*, 2003). Relative to Gpx1 (glutathione peroxidase1), which plays an important role in protecting cells from oxidative damage, its transcription levels were found to be reduced in NT and MEMα cultured controls, most likely reflecting a culture medium effect. When combined, these results suggest that preimplantation development in a somatic culture environment does not completely solve increased apoptosis and oxidative damage in somatic cell clones, and that Survivin misregulation may be involved in the poor morula–blastocyst transition commonly seen in NT embryos.

Our analysis of Oct4, a redox-modulated transcription factor required to maintain totipotency (Guo *et al.*, 2004), detected low transcript levels exclusively in NT embryos, indicating that preimplantation culture in MEMα is not sufficient to correct this feature of somatic cell clones. Our results on mTert transcription (a marker of the telomerase activity) did not indicate significant differences between the *in vivo* fertilized controls and NT embryos. These results were not surprising since they have previously been reported in cattle as well (Xu & Yang, 2001). Finally, when the transcript levels of IGF2bp1 (insulin growth factor 2 binding protein 1) were measured, the outcomes suggested compromised transcription in NT embryos and *in vivo* fertilized controls cultured in MEMα, suggesting, as for Gpx 1, a culture medium effect.

The high blastocyst formation rates obtained from SCNT embryos *in vitro* cultured in MEMα, demonstrates that for the mouse, media commonly used for culture of somatic cells can efficiently support the *in vitro* development of these artificially produced somatic–embryonic hybrids. It is important to highlight that although the pattern of gene expression is significantly different from that of *in vivo* produced controls, the relative transcription level profiles that *in vitro* culture in MEMα generates in mouse SCNT embryos and *in vivo* fertilized controls are similar. The alterations in physiology and metabolism that MEMα may induce seem to be beneficial for mouse SCNT development. A finding of this study that we wish to stress is the observation that after preimplantation exposure to MEMα only the relative abundance of Glut 1 is significantly different in SCNT embryos and *in vivo* fertilized controls, suggesting that Glut 1 can be used as a marker of embryo quality after SCNT.

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