Simultaneous gene quantitation of multiple genes in individual bovine nuclear transfer blastocysts

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Abstract

During somatic cell nuclear transfer (NT), the transcriptional status of the donor cell has to be reprogrammed to reflect that of an embryo. We analysed the accuracy of this process by comparing transcript levels of four developmentally important genes (Oct4, Otx2, Ifitm3, GATA6), a gene involved in epigenetic regulation (Dnmt3a) and three housekeeping genes (β-actin, β-tubulin and GAPDH) in 21 NT blastocysts with that in genetically half-identical in vitro produced (IVP, n = 19) and in vivo (n = 15) bovine embryos. We have optimised an RNA-isolation and SYBR-green-based real-time RT-PCR procedure allowing the reproducible absolute quantification of multiple genes from a single blastocyst. Our data indicated that transcript levels did not differ significantly between stage and grade-matched zona-free NT and IVP embryos except for Ifitm3/Fragilis, which was expressed at twofold higher levels in NT blastocystst. Ifitm3 expression is confined to the inner cell mass at day 7 blastocysts and to the epiblast in day 14 embryos. No ectopic expression in the trophectoderm was seen in NT embryos. Gene expression in NT and IVP embryos increased between two- and threefold for all eight genes from early to late blastocyst stages. This increase exceeded the increase in cell number over this time period indicating an increase in transcript number per cell. Embryo quality (morphological grading) was correlated to cell number for NT and IVP embryos with grade 3 blastocysts containing 30% fewer cells. However, only NT embryos displayed a significant reduction in gene expression (50%) with loss of quality. Variability in gene expression levels was not significantly different in NT, IVP or in vivo embryos but differed among genes, suggesting that the stringency of regulation is intrinsic to a gene and not affected by culture or nuclear transfer. Oct4 levels exhibited the lowest variability. Analysing the total variability of all eight genes for individual embryos revealed that in vivo embryos resembled each other much more than did NT and IVP blastocysts. Furthermore, in vivo embryos, consisting of 1.5-fold more cells, generally contained two- to fourfold more transcripts for the eight genes than did their cultured counterparts. Thus, culture conditions (in vivo versus in vitro) have greater effects on gene expression than does nuclear transfer when minimising genetic heterogeneity.

Introduction

During mammalian reproduction, fertilisation of the oocyte results in the fusion of two haploid genomes and marks the beginning of a cascade of events leading to the activation of the embryonic gene transcription program. Remarkably, fertilisation can be completely bypassed and live offspring obtained by replacing the maternal genome with a diploid genome derived from a multitude of host cells in a process termed nuclear transfer (NT) or cloning (for reviews, see Hochdelinger & Jaenisch 2002, Wilmot et al. 2002). This indicates that cytoplasmic factors present in the matured oocyte are able to reprogram the transcriptional state of the donor cell genome. The relatively low viability of NT embryos, with only a few percent of reconstructs reaching adulthood and a range of malformations observable (Wilmot et al. 2002), suggests that this reprogramming is inefficient. Errors in reprogramming cannot be ascribed to genetic causes as offspring of cloned animals displaying defects are normal (Shimozawa et al. 2002, Tamashiro et al. 2002, Wells et al. 2004). Thus, it is likely that epigenetic causes (‘stable alterations in gene expression potential’; Jaenisch & Bird 2003) underlie reprogramming defects. Epigenetic information is encoded by DNA methylation patterns and chromatin configuration, the latter including the types and modifications of histones as well as non-histone chromatin proteins including the polycomb/trithorax factors (Li 2002). That such epigenetic information differs between nuclear transfer and normal embryos has been well documented (Bourc’his et al. 2001, Dean et al. 2001, Kang et al. 2001, Santos et al. 2003).

As the expression of any given gene is a function of its epigenetic state (Li 2002, Jaenisch & Bird 2003) and the available repertoire of transcription factors and
modulators which impinge on its regulatory region, errors in epigenetic reprogramming would be predicted to affect the gene expression profile of the embryo. Epigenetic reprogramming during NT needs to result in the abolition of donor cell transcriptional status and its replacement with that of the embryo. However, expression of donor-specific genes has recently been observed in clones, showing that epigenetic reprogramming is incomplete (Gao et al. 2003, Ng & Gurdon 2005). Such epigenetic memory can lead to some loss of viability as demonstrated for mouse cloning, where nuclear transfer embryos developed more efficiently in donor cell culture medium than in embryo culture medium (Chung et al. 2002, Gao et al. 2003). However, reprogramming errors leading to incorrect activation/regulation of embryonic genes are likely to have more serious consequences on embryo viability. Numerous loss-of-function studies in the mouse have demonstrated that the absence of single genes will lead to embryonic lethality (see www.informatics.jax.org). For the inner cell mass (ICM)-specific gene Oct4, even a relatively small twofold change in transcript levels can lead to dramatic lineage fate changes (Niwa et al. 2000). Thus, the quantitative analysis of embryonic gene transcription in NT embryos is fundamental to understanding decreased viability.

A number of investigations have examined gene expression in IVP (in vitro produced) and NT embryos using RT-PCR (Daniels et al. 2000, 2001b, Bortvin et al. 2003, Donnison & Pfeffer 2004, Hall et al. 2005), reporter transgenes (Boiani 2002) and microarrays (Pfister-Genskow et al. 2005, Smith et al. 2005, Somers et al. 2006). To date, no clear picture has emerged of embryonic genes that are consistently misexpressed (Wrenzycki et al. 2005). This may not be surprising, considering that aspects of the nuclear transfer procedure including donor cell type, recipient cytoplast, activation protocols and culture methods differ among studies and some of these have been demonstrated to have an effect on gene expression (Daniels et al. 2001, Wrenzycki et al. 2001a). However, a further problem inherent to studies examining gene expression in preimplantation embryos concerns the technical difficulty of procuring accurate quantitative data when working with such small quantities of material. This has been partially solved by examining pools of embryos (Donnison & Pfeffer 2004), performing semi-quantitative analyses (Daniels et al. 2000, 2001, Wrenzycki et al. 2001a, Hall et al. 2005), or recently, by using linear amplification before PCR (Camargo et al. 2005). However, as reprogramming may well result in small changes in transcript levels, it has become imperative to be able to distinguish subtle variations in individual embryos from variations due to technical noise.

We have sought here to develop a reproducible accurate assay allowing the quantification of multiple genes from a single blastocyst-stage embryo. Using this procedure, we examined the expression of a collection of developmentally important genes in individual NT as well as IVP and in vivo derived embryos. Our method, accurate enough to discern subtle gene expression differences between early, mid and late blastocyst stage embryos, yields insight into transcript level variation of multiple genes in single embryos and the effect of nuclear transfer, in vitro culture and embryo grading on embryonic gene transcription.

Materials and Methods

Generation of in vivo, IVP and NT embryos

Abattoir recovered ovaries were aspirated with oocytes matured in vitro and used to generate either zona-free IVP or nuclear transfer (NT) blastocysts. Generation of IVP embryos by in vitro fertilisation (using sperm from bull AESF 1) is as previously described (Thompson et al. 2000) except for zona removal after fertilisation and single embryo culture. Bovine NT, using cultured skin fibroblasts recovered from bull AESF 1 and embryo culturing of both the IVP and NT embryos using a synthetic oviduct fluid system, with embryos cultured singly was performed as described elsewhere (Oback et al. 2003). Grading and staging of development according to published guidelines (Robertson & Nelson 1998) was performed by only one of us (D N W). Briefly, zona-free blastocysts were classified as ‘early’ when a small blastocoel was visible, ‘mid’ when the blastocoel constituted approximately half the embryo and ‘late’ when the blastocyst had expanded. Grade 1 embryos were symmetrical with well-defined and uniform blastomeres. Grade 2 embryos had moderate irregularities in the shape or size of the inner cell mass or similar irregularities in the size, colour or density of the individual blastomeres. Grade 3 embryos had major irregularities in the size or overall shape of the ICM or the size, colour or density of the blastomeres. For in vivo embryos, eight Friesian cows were superovulated by i.m. injection of follicle-stimulating hormone (FSH) over 4 days with prostaglandin F2α administered on the morning and evening of the first and third day of FSH treatment according to standard protocols. The cows were artificially inseminated 12 and 24 h after oestrus (day 0) with semen from bull AESF 1. Blastocysts were recovered by non-surgical uterine flushing on day 7. Embryos were classified as ‘early’, ‘mid’ and ‘late’ according to the guidelines above. This work was performed under animal ethics approval Ruakura-AE10051.

Total RNA Isolation, DNase treatment and RT

cDNA generation from blastocysts was optimised as follows. Single day 7 blastocysts were placed in 100 μl of Trizol (Invitrogen) to which 5 pg rabbit α-globin mRNA (Sigma) and 800 ng MS2 RNA (Roche) were added. The use of non-stick tubes (Neptune #3435.S3, Raylab, Auckland, New Zealand) marginally enhanced
recoveries. Samples were extracted with 20 μl chloroform followed by the addition of 10 μg linear acrylamide (Ambion, Austin, Texas, USA) and 65 μl cold isopropanol. After 10 min at room temperature, samples were centrifuged at 14 000 r.p.m. (16 000 g) for 30 min, washed with 150 μl 70% ethanol and air-dried. The use of phage MS2 RNA in addition to linear acrylamide as a carrier improved recoveries by up to fivefold. After resuspension in 7 μl diethylpyrocarbonate (DEPC)-treated water, 2 μl 1U/μl RNase-free DNase I (Invitrogen) and 1 μl 10× DNase I buffer was added and samples incubated for 1 h at 37 °C. The samples were precipitated with 1.5 μl 3 M sodium acetate (pH 5.5) and 45 μl 100% ethanol, washed in 70% ethanol and resuspended in 12 μl DEPC-treated water.

For reverse transcription (RT), 1 μl water, 1 μl 10 mM dNTP and 1 μl 10 mM oligo dT14VN anchored primer (Invitrogen) were added to 11 μl of each sample before incubation at 65 °C for 5 min. Four microlitres 5× first strand buffer (Invitrogen), 1 μl 40 U/μl Protector RNase inhibitor (Roche), 1 μl 200 U/μl Superscript III (Invitrogen) were added and the samples incubated at 50 °C for 60 min, then 70 °C for 15 min. A RT negative (RT–) control was included. This was followed by the addition of 0.5 μl 2 U/μl RNase H (Invitrogen) for 30 min at 37 °C. After the addition of 2 μl sodium acetate (pH 5.5), samples were passed through Qiaquick mini elute columns (Qiagen, Auckland, New Zealand) and resuspended in 40 μl T0.1E buffer in 0.65 ml non-stick tubes. In the RT reactions, omission of dithiothreitol and the use of anchored oligo-dT primers increased the consistency, though not the yield, of cDNA as measured using α-globin mRNA. Importantly, inclusion of an RNaseH step after RT to digest RNA still bound to cDNA increased copy numbers fourfold. In this regard, an RNaseA digestion step reduced yields, presumably by digesting away the MS2 RNA carrier (data not shown).

Real-time PCR

We used SYBR-green based real-time RT-PCR for quantification using a Roche LightCycler 2 instrument with 10 μl reactions containing 2 μl FastStart DNA Master Sybr Green I reaction and enzyme mix (Roche), 4 nmol of each primer, 80 ng MS2 RNA and 2 μl template. The thermal program included a 10 min incubation at 95 °C to activate the FastStart Taq polymerase followed by 45 cycles of 95 °C for 10 s, annealing temperature (see Table 1) for 5 s and 72 °C for 10–20 s (this varied with amplicon size – 1 s for every 25 bps) with ramp speed 20 °C/s. The fluorescence readings were recorded after each 72 °C step. A no-template control, an RT – control and a standard dilution series were included in each real-time run. Dissociation curves were performed after each PCR run to ensure that a single PCR product had been amplified. The products were also analysed by gel electrophoresis and sequencing on first primer pair usage to ensure that the correct gene fragment was amplified.

We determined the sensitivity of detection and amplification efficiency for each gene-specific primer pair by preparing standard curves. The standard curves were obtained using PCR fragments that were excised from a 1% agarose gel, purified using a Roche Gel Extraction Kit, resuspended in TE and quantified with both a NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA) and fluorometrically with PicoGreen (Invitrogen) using the LightCycler. Standards consisted of a tenfold dilution series containing 105 to 101 copies/μl. PCR results from the LightCycler were compared with predicted values using an equation that relates reaction efficiency, Cp value (cycle number corresponding to the apex of second derivative of fluorescence intensity curve) and copy number (Wilkening & Bader 2004). Only primer pairs that gave an amplification efficiency (the increase in product per PCR cycle) within 0.2 of the theoretical maximum of 2.0 were used for quantification (Table 1). We found amplification efficiencies to be highly reproducible between PCR experiments (within 0.04), but nevertheless included a standard curve for each run, as even a slight increase of 0.04 at an amplification efficiency of 1.9 would, after 30 cycles, cause an error in copy number estimation of (1.94/1.90)10 = 1.87-fold or 87%. Using this strategy, we observed typical variation in copy numbers of PCRs performed on different days of between 2 (α-globin) and 4% (Oct4).

Our standard curves indicated a loss of linearity between 20 and 200 copies, thus setting a limit to quantitation. As we were using only 1/20th of a blastocyst per PCR to allow for duplicates and the analysis of more than one gene per embryo, we introduced a stringent sensitivity criterion by performing duplicates at a one in two dilution. Only if the copy number lay well within the linear range, the dilution would yield the expected copy number. This criterion resulted in the rejection of many genes whose transcripts, though readily detectable by real-time or standard RT-PCR, were below the reliability/sensitivity threshold of quantification.

Sample concentrations calculated from the standard curves were converted into an estimate of copy number per blastocyst after correcting for recovery and RT losses using values obtained for α-globin recovery.

PCR primers

PCR primers were designed using Vector NTI (Invitrogen) or obtained from the literature (Table 1). Where possible, primers were designed to cover putative introns as determined by comparison with genomic bovine sequences or the homologous mouse loci. For genes without a GenBank bovine sequence, the mouse sequence was blasted against the TIGR bovine database, and primers were designed from the TIGR sequence.

www.reproduction-online.org
<table>
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<tr>
<th>Gene</th>
<th>Primer sequence, 5’ to 3’, forward, reverse</th>
<th>Size (bp)</th>
<th>Introns</th>
<th>PCR Effic</th>
<th>Ta</th>
<th>Reference</th>
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<td>56</td>
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<td>464</td>
<td>2</td>
<td>1.77</td>
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Ta, annealing temperature; Effic., efficiency of PCR reaction.
Cell counting of blastocyst embryos

The number of cells in day 7 blastocysts was determined by either: (1) counting the Giemsa stained nuclei of fixed embryos spread onto glass slides; or (2) by combining the differential cell counts of the trophectoderm and ICM using procedures as previously described (Iwasaki et al. 1990). In vivo embryos were obtained as described above using a total of 20 animals in two separate experiments.

Whole mount in situ hybridisation

Zona-free IVP or NT blastocysts (from bull AESF 1) were fixed for 30 min at RT in 4% paraformaldehyde in PBS containing 0.1% Tween-20, then dehydrated in a methanol series and kept at least overnight at −20 °C. Day 14 embryos were recovered by flushing 7 days after day 7 IVP embryos were bulk transferred to synchronised recipient cows (McMillan et al. 2001). Day 14 embryos were slit open and fixed for 1 h at RT. Whole mount in situ hybridisation was performed as described (Nagy et al. 2003), but washing and hybridisation steps were performed in four-well plates (#176740; Nunc, Auckland, New Zealand) with embryos transferred during steps rather than replacing solutions. The post-rehydration Protease K (Roche) digestion contained 10 μg/ml enzyme and was done for 30 s for zona-free embryos. The embryos were photographed with a Nikon Coolpix digital camera mounted on an inverted microscope.

A bovine Ilitm3/Fragilis PCR fragment (primers in Table 1) was cloned into pGEM-T easy in the SP6 orientation and antisense digoxigenin-labelled RNA produced by T7 RNA polymerase transcription (Roche) after plasmid linearisation by Spel digestion according to standard protocols.

Statistics

The log of the expression for each gene was analysed by treatment (NT/IVP/AI), stage, quality (grades 1 and 2 were usually combined) and their interactions to give means and standard error of differences of means using the linear model routine in GenStat (VSN International, Oxford, UK) that accommodated unequal numbers of blastocysts in each group. Variances, calculated separately for each treatment/stage/quality combination as the S.D. of the log of the mean expression level, were compared using Bartlett’s test.

Results

Expression levels at the blastocyst stage of a set of 26 bovine genes

We wished to examine the expression of genes which would either be important for early development or be involved in reprogramming (Table 1). Of the 26 genes examined, six could not be detected in our quantitative assay using 1/20th of a blastocyst as input. Of these, Wnt3a, Trithorax, Connexin44, IL-6, Gp130 and the putatively imprinted gene Neuronatin fell within the non-linear range of detection when using a full blastocyst-equivalent amount of cDNA, indicating that these genes are expressed at less than 200 copies per embryo.

Of the remaining 20 genes, 12 could be detected in 1/20th of a blastocyst but fell within the non-quantifiable region. Using full-blastocyst equivalents of cDNA, we confirmed that the transcript levels of these genes lay in the 200–2000 copies per blastocyst range. All of the histone modification enzymes (HDAC1 and 2, HAT1 and GCN5) as well as Cdx2, BMP4, HNF4a, LIF receptor, Traube, Interferon-γ, Hsp70 and Glut1 were expressed at these levels.

The housekeeping genes β-actin, β-tubulin and GAPDH as well as one gene involved in reprogramming (the DNA methyltransferase Dnmt3a) and the lineage restricted genes Oct4, Otx2, Ilitm3 and GATA6 could be reliably quantitated in 1/20th of a blastocyst and we focussed on these genes for our subsequent analyses. Average expression levels of these eight genes were 4000–100 000 copies per blastocyst embryo (Fig. 1).

Comparison of gene expression in individual NT, IVP and in vivo blastocysts

We measured gene expression levels of eight genes in individual staged and graded day 7 blastocysts derived by NT (n=21), IVP (n=19) or in vivo embryos (n=15). Zona-free NT and IVP embryos were cultured in parallel. In vivo and IVP embryos were fertilised with sperm from the same animal that was used to generate the NT embryos, thus ensuring a large degree of genetic identity between the different categories of embryos. An overall comparison of expression levels in transferable grade 1 and 2 embryos (Fig. 1) revealed a highly significant effect of embryo type (in vivo/IVP/NT) on transcript levels for all genes (actin, P=0.03; Oct4, P<0.01; others, P<0.001). This effect is predominantly due to the higher transcript levels of the in vivo embryos (Fig. 1). Refining our analysis to stage matched groups revealed that in vivo embryos contained two- to fourfold more mRNA than both IVP and NT blastocysts for all genes at most stages (exceptions where P>0.05 were actin at earlier stages, Oct4 at late blastocyst stage, Ilitm3 compared with mid- and late blastocyst NT embryos and Dnmt3a compared with mid-blastocyst IVP embryos). We observed 1.5-fold higher cell numbers in in vivo embryos (Fig. 3). Interestingly, stage-matched NT and IVP embryos exhibited remarkably similar gene expression levels with consistent differences in expression levels at all stages seen only for the gene Ilitm3/Fragilis (P<0.01 across all stages and P=0.02 at early and P=0.03 at late blastocyst stage). However, the increase in expression was small with levels 2.5-, 1.5- and 1.6-fold higher in early, mid and late NT than in IVP blastocysts (Fig. 1).
Temporal effects on gene expression

We noted a highly significant embryo-stage effect on gene transcription in transferable grade embryos ($P<0.01$ for all genes). IVP and NT gene expression increased between two- and threefold for all eight genes from early to late stages (Fig. 1, $P<0.05$). Counting cell numbers in 120 NT and 69 IVP zona-free embryos revealed no significant difference between these two groups (Fig. 2). However, grade 1 and 2 late blastocysts contained 1.5- to 1.3-fold (NT and IVP respectively; $P<0.05$) more cells than early blastocysts, with early- and mid-blastocyst cell numbers not being significantly different from each other. Thus, whereas cell number is correlated to gene expression levels, both increasing with developmental age, the increase in gene expression exceeds the increase in cell numbers over this period, suggesting that the number of transcripts per cell is also increasing.

It should be pointed out that the temporal increases in gene expression were more subtle or not seen in in vivo embryos (Fig. 1), even though average cell numbers increased from 130 (S.E.M. ±7; n=12) to 160 (S.E.M. ±8; n=13) between mid- and late-stage blastocysts (Fig. 2).

**The effect of nuclear transfer and in vitro culture on gene expression variability**

We compared gene expression variability of transferable grade NT, IVP and in vivo embryos grouped according to developmental stage. Figure 3A depicts the variability for each of the genes at late blastocyst stages where we could compare the largest number of transferable grade embryos (n=6 for each type of embryo). None of the differences in variability was significant (Bartlett’s test, $P>0.1$ for all eight genes). Similar results were seen at earlier stages with only one exception (Dnmt3a at mid blastocyst; $P=0.03$). We conclude that individual gene expression variance is equal for in vivo, IVP and NT embryos.
As this study measured eight different genes from a single embryo, we could ask the question as to how different genes behaved within a single embryo. To this end, we summed the residuals (deviation from the log of the average expression level) for all genes of each late-stage transferable grade embryo (Fig. 3B). As expected from the previous analysis, the average sum of residuals (diamonds in Fig. 3B) is nearly identical for all the three types of embryos. However, individual in vivo embryos deviated much less from this average than did the NT or IVP embryos (see range and S.E.M. bars in Fig. 3B). This implies that in vivo embryos are more alike to each other when including gene expression data from many genes than are the cultured embryos.

**Relationship of grading to gene expression**

We next examined gene expression in grade 3 embryos which are normally not transferred to recipients. Comparing late stage grade 1 with grade 3 IVP embryos revealed a slight reduction in transcription levels for five out of the eight genes, but this reduction was not significant apart for Gata6 (Fig. 4A). In marked contrast, gene expression levels in grade 3 NT embryos were only 42–62% of that of grade 1 NT blastocysts. Most of these differences were significant below the 5% level (Fig. 4B). This difference in gene expression between grade 3 IVP and NT embryos can not be attributed to differences in cell numbers as both IVP and NT grade 3 embryos contained very similar cell numbers (Fig. 2). At the late blastocyst stage, grade 3 IVP embryos contained 65% as many cells as transferable grades, whereas for NT embryos the value was 75%. Thus, although grade 3 embryos show a highly significant reduction in cell number per embryo, gene expression is only concomitantly significantly reduced in NT embryos. The differential behaviour of grade 3 IVP and NT embryos suggests that such embryos, though morphologically similar, are different at a molecular level.

**Spatial pattern of Ifitm3 expression**

Ifitm3 was the only gene significantly misexpressed when comparing NT and IVP embryos. Whole mount in situ hybridisation revealed that Ifitm3 expression is restricted to the ICM in day 7 bovine blastocysts (Fig. 5A). We next asked whether the significant upregulation of this gene in NT embryos could be attributed to ectopic expression in the trophectoderm. However, in all embryos examined (n=11), Ifitm3 expression was confined to the ICM (Fig. 5B). Ifitm3 expression remains confined to the epiblast at later stages in IVP (Fig. 5D) as well as NT embryos (data not shown).
Discussion

Effects of somatic cell nuclear transfer

The primary focus of this work was to examine the effect of nuclear transfer on gene expression in individual blastocysts. Our approach centred on the ability to accurately quantify transcript levels of multiple genes in a single blastocyst. We thus developed a robust cDNA isolation scheme and have introduced criteria aimed at eliminating technical variation in our data (described in detail in the Materials and Methods section). We found three criteria to be crucial for accurate quantification. First, the addition of an exogenous spike – rabbit α-globin mRNA, (Temeles et al. 1994, Donnison & Pfeffer 2004) – was essential as housekeeping genes (internal standards) are themselves subject to reprogramming effects. Second, the inclusion of a standard curve with each experimental run eliminated significant errors arising through small changes in amplification efficiencies. The use of ΔΔCt should be avoided except in instances where the amplification efficiencies of the two genes compared is demonstrated to be equal. Third, performing duplicates at a higher dilution ensured that samples were within the linear range, where quantification is meaningful.

In this study, we minimized genetic effects by comparing NT embryos with IVP and in vivo embryos sharing 50% genetic identity with the NT embryos by virtue of being generated using sperm from the bull from which the skin fibroblast donor cells were derived. Furthermore, by culturing NT embryos in parallel to IVP embryos under identical (zona-free) conditions, effects specific to the NT procedure as opposed to gene expression differences arising due to embryo culture (Wrenzycki et al. 2005) can be addressed. On the other hand, the inclusion of in vivo embryos allows comparisons with a more physiological state. Does nuclear transfer affect gene expression at the blastocyst stage? Based on the simultaneous expression data for eight different genes, transferable grade NT embryos are remarkably similar to their genetically half-identical IVP embryos. This result is in line with other studies (Camargo et al. 2005, Somers et al. 2006). For seven of the genes, expression levels did not differ significantly at blastocyst stages. As several of the analysed genes (Oct4, Otx2, Gata6, Dnmt3a) are not expressed in skin fibroblasts (Smith et al. 2005), this suggests that reprogramming of these genes has occurred correctly. Secondly, with two exceptions (Dnmt3a and Gapdh at mid blastocyst), the time course of gene activation is highly similar in NT and IVP embryos, increasing at similar rates in a developmental series based on the morphological criteria of blastocyst formation and expansion. Importantly, the increase in gene expression (two- to threefold) exceeded the increase in cell numbers (1.3- to 1.5-fold) over this developmental period, suggesting that the number of transcripts per cell was also increasing.

Thirdly, NT and IVP embryos resembled each other in that the level of embryo-to-embryo variation for all genes did not differ significantly, suggesting that the NT procedure does not cause any of the analysed genes to be consistently expressed in a less controlled manner than in the IVP counterparts. Lastly, by virtue of having measured multiple genes in single embryos, we could obtain a measure of the total gene expression variability of individual embryos. Bearing in mind that this measure represents only a small sample of the more than 10 000 genes expressed at blastocyst stages, our results indicated wide variation among single embryos ranging

Figure 5 Spatial pattern of Ifitm3 expression in bovine embryos. Whole mount in situ hybridisation of zona-free day 7 IVP (panel A), NT (panel B) and day 14 bovine embryos (panel D). At blastocyst stages, Ifitm3 expression is confined to the inner cell mass (ICM) with no expression seen in the trophectoderm (TE). By day 14, expression is still confined to the epiblast (epi) which is derived from the ICM. Panels C and E are embryos hybridised to a control (sense) probe, scale bar is 20 μm.
from some closely resembling ‘normality’ – as characterised by approaching the average expression level of most genes – to distinctly aberrant embryos. However, as compared to IVP embryos, the mean and distribution of total gene expression variability of NT embryos did not differ. It is intriguing to speculate whether such ‘abnormal’ NT and IVP embryos are developmentally compromised. Considering the lower viability of NT embryos, one might have expected a larger fraction of such abnormal embryos in this treatment group. This question will only be resolved by future studies in which development of biopsied embryos is followed.

A recent report claimed that NT embryos are more similar in gene expression to in vivo than to IVP embryos (Smith et al. 2005). In our hands, it is in vivo embryos that differ the most in several aspects from their in vitro grown half-siblings and from genetically related NT embryos. For all eight genes and at most morphological stages, transferable grade in vivo embryos contain the most transcripts, generally between two- and fourfold more than the cultured zona-free blastocysts. Only part of this increase can be explained by the 1.5-fold higher cell counts of in vivo embryos. A trend for higher relative gene transcript levels in in vivo blastocysts has been previously reported (Tesfaye et al. 2004, Camargo et al. 2005), though lower levels of gene transcripts have also frequently been seen (for recent review see Wrenzycki et al. 2005 and references therein). Furthermore, zona removal, a necessary requirement for our NT procedure, potentially may influence gene expression (Ribas et al. 2006). The pertinent point here is that culture per se (inclusive of zona removal) may have a stronger influence on gene expression levels than does the nuclear transfer treatment.

Intriguingly, in vivo gene expression levels do not differ as much between the morphologically early and late blastocyst stages as in cultured embryos. Thus, the changes in gene expression levels between in vivo and cultured embryos are most pronounced at early stages. A likely explanation accounting for this observation is that morphological staging of in vivo zona intact and cultured zona-free embryos does not relate equally to number of cell division and thus developmental stage. It has been observed that in vivo embryos commence blastulation (our morphological landmark) one cell cycle later than cultured embryos and complete this process much more rapidly (Van Soom et al. 1997). Thus, morphologically early in vivo blastocysts are more equivalent in developmental stage and cell number to in vitro mid blastocysts.

In contrast to overall expression levels, variability of expression for any of the eight genes analysed is not significantly different between the two types of cultured and the in vivo blastocysts. The variability in levels appears to be intrinsic to genes, as different genes show different average s.d. of the log of the average expression with more highly expressed genes generally, but not always, displaying less variation in their levels. Notably, Oct4 shows the least variability in expression (1.25-fold; compared with Otx2 variation of 1.5-fold) in line with reports that twofold variation in this gene’s expression levels can induce lineage changes in mouse ES cells (Niwa et al. 2000). Interestingly, when comparing gene expression profiles on an individual embryo basis, in vivo embryos show much lower total variability, when comparing many genes than their NT or IVP counterparts. Thus, in vivo embryos are more normal/like to each other in terms of their gene expression levels. This question has not been addressed before using a quantitative assay. In one microarray experiment, bovine NT blastocysts were seen to show the lowest embryo-to-embryo variation, followed by in vivo, then IVP, embryos (Smith et al. 2005). IVP embryos in this study were not related to NT or in vivo embryos and were genetically heterogeneous, whereas NT embryos were genetically identical to each other. The in vivo embryos were derived from two sets of siblings. This might suggest that genetic homogeneity reduces gene expression variability. Though the results are not directly comparable, our results clearly demonstrate albeit for a small set of genes that in genetically equivalent embryos (in vivo and IVP – all one sire) culture per se is sufficient to induce increased total gene expression heterogeneity. The genetic homogeneity of NT embryos is not sufficient to reduce this heterogeneity caused by in vitro culture, possibly because of increased epigenetic variation through incomplete reprogramming.

In this study, we discovered a notable effect when extending our analyses to grade 3 NT and IVP embryos. Only grade 3 NT embryos revealed significant reductions in expression levels for nearly all examined genes. This suggests that grade 3 NT embryos, which can be morphologically recognised, are also abnormal in terms of their gene expression profile. This effect is not simply due to a loss in cell numbers as grade 3 IVP embryos show a similar cell number reduction compared to transferable grade embryos as do NT blastocysts yet gene transcription is minimally affected. Furthermore, the loss in NT grade 3 cell number (around 30%) is less than the loss in gene expression (around 50%). As we generally do not transfer grade 3 zona-free NT embryos to recipients, we presently do not know if the observed changes in gene expression specific to grade 3 NT embryos reflect differences in viability.

**Ifitm3 and embryonic development**

The only gene differentially expressed between NT and IVP embryos was Ifitm3. Levels of this gene were elevated in NT embryos by about twofold at all blastocyst stages. Ifitm3 (interferon-induced transmembrane protein 3), also known as 1-8U/Mil1/Fragilis, is highly interferon-inducible (Lewin et al. 1991). Members of this family have been implicated in homotypic adhesion and
in mediating an interferon-dependent anti-proliferative effect (Evans et al. 1990, 1993, Brem et al. 2003). Specifically, Ifitm3 overexpression reduces proliferation rates in human cell lines (Brem et al. 2003). Ifitm3 expression marks the onset of germ cell competence in the epiblast of pregerm stage mouse embryos, and its expression at later stages remains associated with primordial germ cells (Saitou et al. 2002, Tanaka & Matsui 2002). It has been proposed that the function of Ifitm3 in this system is twofold: first, that it is involved, through homotypic adhesion, in the segregation of prospective germ cells from surrounding somatic cells and second, that it mediates the observed increase in cell cycle time in nascent germ cells from 6 to 16 h (Saitou et al. 2002).

Previous to this report Ifitm3 expression could not be detected at the blastocyst stage in mice (Tanaka & Matsui 2002). Our results indicate levels of around 10,000 transcripts of Ifitm3 in the bovine blastocyst, with levels rising from early to expanded blastocyst stages. Interestingly, we found Ifitm3 expression to be restricted to the inner cell mass in line with transcripts being detected in mouse ES cells (Saitou et al. 2002). This expression is maintained in the ICM/epiblast until at least day 14. In the mouse, it appears that the transforming growth factor-β factor BMP4 lies upstream of Ifitm3 (Saitou et al. 2002). Transcripts for Bmp4 (200–2000 copies/embryo) were detected in our analyses and our unpublished data suggests that this gene’s expression is restricted to the ICM, raising the possibility that it may be involved in Ifitm3 transcriptional activation.

Why are Ifitm3 transcript levels elevated in NT embryos? Our whole mount in situ hybridisation results exclude the possibility of ectopic expression in the TE. The observation that offspring of defective animals created by nuclear transfer are normal strongly points to epigenetic changes causing defects. Such epigenetic changes are most likely due to the requirement for extensive chromatin remodelling of the donor nucleus. Hence, those genes most dependent on chromatin-remodelling activities for their expression would be predicted to be most affected by nuclear transfer. In this regard, it is relevant to note that the Ifitm3 promoter is highly sensitive to the chromatin-remodelling activity of the SWI/SNF-like ATPase dependent BAF complex (Liu et al. 2002).

Would elevated expression of Ifitm3 be expected to affect embryo viability? Increased Ifitm3 mRNA levels in the ICM of NT embryos could be expected to reduce the proliferation rate of ICM cells eventually biasing the ICM:TE ratio to TE (trophectoderm). However, it should be noted that when comparing late-blastocyst NT Ifitm3 levels to in vivo embryos, there is no significant difference, suggesting that the observed increase in Ifitm3 expression relative to IVP embryos should not have adverse consequences on NT embryo viability.

In conclusion, this study represents the first report of the simultaneous absolute quantification of several genes in individual blastocysts, allowing new insights into transcript fluctuations and the effects on transcription levels resulting from somatic cell nuclear transfer as well as in vitro embryo culture. We have discovered the upregulation in NT embryos of a gene not hitherto explicitly associated with blastocyst specific expression.

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