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 blastocysts. *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.

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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 [Hochedlinger & Jaenisch 2002](#), [Wilmut 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 ([Wilmut 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, 2001, Wrenzycki *et al.* 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 cytoplasm, 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

Abbatoir 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 (DNW). 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 F_{2α} 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 1 U/µl RNase-free DNase1 (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 $(1.94/1.90)^{30} = 1.87$ -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.

Table 1 Primers for PCR.

Gene	Primer sequence, 5' to 3', forward, reverse	Size (bp)	Introns	PCR Effic	Ta	Reference
Rabbit α -globin	GCAGCCACGGTGGCGAGTAT, GTGGGACAGGAGCTTGAAAT	256	2	1.89	56	Davis <i>et al.</i> (1996)
Housekeeping						
β -actin	CAGAAGGACTCGTACGTGGG, TTGGCCTTAGGGTTCAGGG	200	0	1.98	56	Coussens & Nobis (2002)
β -tubulin	CAGAGCAAGAACAGCAGCTACTT, GTGAACTCCATCTCGTCCATGCCCTC	228	1	1.91	56	
GAPDH	CTCCAACGTGTCTGTTGTG, TGAGCTTGACAAAGTGGTCG	222	2	1.97	56	Coussens & Nobis (2002)
Inner cell mass						
Oct4	CCAGGACATCAAAGCTCTTCA, AAAACCACACTCGGACCA	428	3	1.98	56	Donnison & Pfeffer (2004)
Ifm3	ACGACCACGGTGATCAACATC, TAGTGGCCTCCGTAGTTCTGC	317	1	1.92	56	
Trophectoderm						
cdx2	GGCTCTCAGAGAGGCAGGTAA, AGCCTTGACAGGAAGACA	194	0	1.93	56	
IFNtau	GCTATCTCTGTCTCCATGAGATG, AGTGAGTTCAGATCTCCACCCATC	359	0	1.87	56	Wrenzycki <i>et al.</i> (2001a)
Primitive endoderm						
Gata6	ACCGGCCACTACCTTTGCA, GCTGCAGTCATCTGCGTTAGAAG	367	2	1.90	56	
HNF4 α	TGATGAGTTGGTGCTGCCCT, TGAGGGCTCGGACCCTGAA	503	3	1.84	60	
Reprogramming						
Dnmt3a	CTGGTGCTGAAGGACTTGGGC, CAGAAGAAGGGGCGGTATC	317	1	1.83	56	Golding & Westhusin (2003)
HDAC1	ACTACTACGACGGGGATGTTG, GCCAAGACGATATCATTGACG	428	1	1.88	56	McGraw <i>et al.</i> (2003)
HDAC2	GACATATGAGACTGCAGTTGC, ACCTCCTTCTCCTTCATCCTC	355	3	1.80	56	McGraw <i>et al.</i> (2003)
HAT1	CAGATATATAAGGCTGACATGAC, GCTGTAATATCAAGAACTGTAGG	345	3	1.77	56	McGraw <i>et al.</i> (2003)
GCN5	CAGAAATGTCTTTTCCACCAG, GGATTGAGCTCACACTCCATC	401	2	1.85	56	McGraw <i>et al.</i> (2003)
Trithorax	CCCCTTCCACCACTCCATT, GCTTTGGGGACTCCTGGAAT	405	0		56	
Signalling						
BMP4	CTCAGGGCAGAGCCATGAGCT, CGTCTCTGGGATGCTGCTGA	317	1	1.81	56	
Wnt3a	AGGCCATCGCCAGTCACATG, TCCTGGCAGCTGACGTAGCA	445	1		58	
Metabolism						
Glut1	AGGAGCTGTTCCACCCCTGGGAGCTGACT, TGTGGGTGAAGGAGACTCTGGCTGATAAAA	327	0	1.90		Wrenzycki <i>et al.</i> (1999)
Connexin 44	CGGAAGGAGCGTGAGGAGGAGC, GGAGGCAGAGGGCGCGTAGT	519	0		64	
HSP70.1	AAGGTGCTGGACAAGTGCCAGGAGGTGATT, ACTTGAAGTAACAGAAACGGGTGAAAAA	488	0	1.85	60	Wrenzycki <i>et al.</i> (1999)
Imprinted						
Nnat	TTTTCGAAATCCTCCAGGGACAC, TTGTCGGTGCTGCTTCTCCA	309	1		60	Ruddock <i>et al.</i> (2004)
Other						
Otx2	CAATGCAGTCACCAGCCATCTCAA, TGCCTCCTGGGACATTGATCA	335	1	1.95	56	
gp130	GGCCGTGTACCTGTTCTTA, CCAGGTATGTTCCCTTCCA	257	1		55	
IL-6	TGCACTCTTCAAACGAGTGCG, ACATTCAAGCCACATAGCCA	377	0		56	
LIFR	CGCAGATGATGGACATT, CCGCTTTTCAATGCAAACTT	265	1	1.94	56	Coussens & Nobis (2002)
Traube	TGGTAGATCTCCAGGAAGAGTTG, CTCAGGAAGGATCTCCGGCT	464	2	1.77	56	

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 Proteinase K (Roche) digestion contained 10 $\mu\text{g}/\text{ml}$ 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 *Ifitm3/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 *SpeI* digestion according to standard protocols.

Statistics

The log of the expression for each gene was analysed by treatment (NT/IVP/Al), 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- τ* , *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*, *Ifitm3* 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 embryos 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, *Ifitm3* 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 *Ifitm3/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).

Figure 1 Average gene expression levels in NT, IVP and *in vivo* day 7 transferable grade blastocysts as determined by real-time RT-PCR. Solid squares represent NT (grade 1 and 2, $n=13$) solid triangles IVP ($n=11$) and open triangles *in vivo* ($n=13$) embryos. Error bars represent the S.E.M. The Y-axis shows transcript numbers in thousands. EB, early blastocyst; MB, mid blastocyst; LB, late blastocyst stage.

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.

Figure 2 Cell numbers per blastocyst in *in vivo* and zona-free NT and IVP day 7 blastocysts. Solid symbols show transferable grade 1 and 2 embryos, open symbols grade 3. NT embryos ($n=120$) are depicted by squares, IVP ($n=69$) by triangles, *in vivo* ($n=25$) by diamonds. ANOVA analysis for the zona-free embryos indicated no treatment effect (NT versus IVP), but significant grading (grades 1 and 2 vs 3) and a staging effect whereby early (EB) and mid (MB) blastocysts contained significantly fewer cells than late blastocysts (LB) ($P<0.05$). Error bars represent S.E.M.

Figure 3 Gene expression and embryo variability in NT, IVP and *in vivo* late blastocyst stage transferable grade embryos. All values depicted here are based on a set of six embryos of each type. (A) Average variability representing the s.d. from the log of the mean expression level of each gene is depicted for the three types of embryo analysed. Genes are arranged on the X-axis in order of increasing variability. (B) The total variability for each embryo across all eight genes analysed was calculated by summing the absolute value of the deviation of that embryo from the mean expression level (residual) for each gene. The mean total variability and S.E.M. there of is depicted next to each group of embryos by a filled diamond and error bars.

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

Figure 4 Embryo grade is correlated with gene expression levels in NT but not IVP embryos. Transcript levels in grade 3 blastocysts (black bars) are expressed as a percentage of levels in grade 1 blastocysts for IVP (Panel A) and NT (Panel B) embryos. Significance levels of the differences between grade 1 and 3 embryos are depicted where relevant. Only late blastocyst stage embryos are shown. Error bars are the S.E.M.

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).

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 trophoblast (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 μ m.

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 $\Delta\Delta C_t$ 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

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/alike 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 pregastrula 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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