

Gene expression profiling of individual bovine nuclear transfer blastocysts

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Abstract

During somatic cell nuclear transfer the gene expression profile of the donor cell has to be changed or reprogrammed extensively to reflect that of a normal embryo. In this study we focused on the switching on of embryonic genes by screening with a microarray consisting of 5000 independent cDNA isolates derived from a bovine blastocyst library which we constructed for this purpose. Expression profiling was performed using linearly amplified RNA from individual day 7 nuclear transfer (NT) and genetically half-identical *in vitro* produced (IVP) blastocysts. We identified 92 genes expressed at lower levels in NT embryos whereas transcripts of 43 genes were more abundant in NT embryos ($P \leq 0.05$, ≥ 1.5 -fold change). A range of functional categories was represented among the identified genes, with a preponderance of constitutively expressed genes required for the maintenance of basal cellular function. Using a stringent quantitative SYBR-green real time RT-PCR based approach we found, when comparing the means of the expression levels of a larger set of individual embryos, that differences were small (< 2 -fold) and only significant for two of the seven analysed genes (*KRT18*, *SLC16A1*). Notably, examination of transcript levels of a single gene in individual embryos could not distinguish an NT from a control embryo. This unpredictability of individual gene expression on a global background of multiple gene expression changes argues for a predominantly stochastic nature of reprogramming errors.

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Introduction

Mammalian nuclear transfer (NT) cloning has produced live, viable offspring in many species, yet cloning efficiencies are always very low, with generally less than 4% of reconstructs reaching term (Wilmot *et al.* 2002). Early gestational losses of NT embryos are often associated with aberrant placental development in livestock and mice (Hill *et al.* 2000, De Sousa *et al.* 2001, Ogura *et al.* 2002). Anomalies in foetal and perinatal stage cloned calves and lambs are heterogeneous, including excessive birth weight (large offspring syndrome), defects in the gastrointestinal, cardiopulmonary, hepatic and renal systems as well as skeletal malformations (Wells *et al.* 1997, Zakhartchenko *et al.* 1999, 2001, Lanza *et al.* 2001, Gibbons *et al.* 2002, Pace *et al.* 2002, Rhind *et al.* 2003). Furthermore, survival beyond term is also affected with annual mortalities of 8% reported in cattle (Wells *et al.* 2004) and early death in mice (Ogonuki *et al.* 2002).

The primary cause of defects in clones is postulated to be epigenetic, based on the observation that

abnormalities of clones are not transmitted to subsequent generations (Shimozawa *et al.* 2002, Tamashiro *et al.* 2002, Wells *et al.* 2004). For the survival of a NT reconstructed embryo, the epigenetic memory of the donor cell nucleus has to be erased and the chromatin remodeled into an embryo-equivalent state in a reprogramming process that leads to the correct initiation of the embryonic gene expression programme. Such chromatin remodelling in clones is often incomplete as demonstrated by aberrant DNA methylation patterns in bovine NT embryos (Bourc'his *et al.* 2001, Dean *et al.* 2001, Kang *et al.* 2001, 2002). Likewise, histone lysine methylation and acetylation changes in bovine clones showed disparity relative to control embryos (Santos *et al.* 2003). Observations that NT mouse embryos, in contrast to control embryos, developed more efficiently in donor cell culture medium than in embryo culture medium, implies that donor cell transcription may not have ceased after transfer (Gao *et al.* 2003). Improved viability was observed when using embryonic stem (ES) as opposed to somatic cells

(Hochedlinger & Jaenisch, 2002). ES cells more closely resemble embryos in terms of their gene expression profile than do differentiated cells and hence less nuclear reprogramming may be required upon using ES cells for NT. Thus the superior NT efficiencies when using ES cells suggests that correct embryonic genome activation constitutes a major hurdle during somatic nuclear transfer.

Gene expression studies in nuclear transfer embryos have been published for several species. In mice, pluripotency genes involved in early development have been reported to be misregulated in cloned embryos (Boiani *et al.* 2002, Bortvin *et al.* 2003). The expression of cattle NT blastocysts has been previously studied in an attempt to identify marker genes that would predict clone developmental competence (Daniels *et al.* 2000, Donnison & Pfeffer 2004). Many of the previous studies measured the expression of only a small number of genes by semi-quantitative RT-PCR (Daniels *et al.* 2000, 2001, Wrenzycki *et al.* 2001, Park *et al.* 2003). Detailed quantitative analyses of individual blastocysts has revealed a large degree of embryo to embryo variation and only subtle changes in expression levels (Camargo *et al.* 2005). These studies, however, offer only a limited picture of the gene transcription changes occurring after nuclear transfer. In order to obtain a more complete picture of the accuracy and extent of nuclear reprogramming, a global method is required. One group has recently used such an approach, creating a microarray from cDNA derived from cultured genital ridge cells, and identified 18 genes as significantly differentially expressed between NT and *in vitro* produced (IVP) embryos (Pfister-Genskow *et al.* 2005).

We have here addressed this issue further, focusing on gene *activation* in NT embryos. To this end we have constructed a novel bovine blastocyst cDNA library and synthesised a 5000 feature blastocyst-stage microarray, allowing us to compare embryonic gene expression in individual NT blastocysts with that of genetically half-identical IVP blastocysts. Genes identified from the microarray as differentially expressed were analysed further by quantitative real time RT-PCR. We interpret our results in terms of a stochastic model for reprogramming.

Materials and Methods

IVP and NT embryo production

Abattoir recovered ovaries were aspirated, with oocytes matured *in vitro* and used to generate either zona-free IVP or NT blastocysts. Generation of IVP embryos by *in vitro* fertilisation (IVF; using sperm from bull AESF 1) was as previously described (Thompson *et al.* 2000) with the exceptions of zona removal after IVF 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 has been previously described (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, 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. Single embryos were washed in PBS and transferred in a minimal volume into individual tubes. These were immediately flash frozen in liquid nitrogen and kept at -80°C until RNA extraction. A reference standard was created from a pool of 200 zona-intact day 7 IVP blastocysts.

RNA isolation and amplification

For the amplification procedure, RNA was isolated from individual embryos and the reference standard pool using the RNAqueous micro kit (Ambion, Austin, TX). The manufacturer's protocol was followed with the following modifications. To each thawed sample 200 ng of poly-deoxy-inosinic-deoxy-cytidylic acid (poly[d(I-C)]; Roche, Mannheim, Germany) was added. The elution volume of 40 μl was reduced to 10 μl with a SVC100H speedvac concentrator and the DNase I treatment step was omitted. Non-stick microfuge tubes were used (Neptune #3435.S3, Raylab, Auckland, New Zealand).

Individual blastocyst samples were subjected to two rounds of linear amplification using the Arcturus RiboAmp RNA amplification kit (Arcturus, Gene Works, Auckland, New Zealand) using a modified version A. The modifications included using the centrifuge conditions described in version C of the manufacturer's protocol and a first round *in vitro* transcription length of 5 h at 42°C and 4°C overnight. For the second round of *in vitro* transcription, reagent volumes were doubled and samples incubated for 6 h at 42°C then 4°C overnight. Sample yields and the integrity of the amplified antisense RNA (aRNA) was examined spectrophotometrically and by gel electrophoresis. Yields were as follows (in μg): IVP embryos—35, 35, 53 and 71; NT embryos—67, 81, 33, 34 and 25. aRNA was stored at -80°C .

For the reference standard, the first round of linear amplification was performed with the Arcturus kit and the yield determined by spectrophotometer. Aliquots (280 ng) were made to which 200 ng poly[d(I-C)] was added. For the second linear amplifications a modified version of the protocol by Wang and colleagues was used (Wang *et al.* 2000). Modifications included the use of Microcon YM-100 columns (Millipore, North Ryde, Australia) for double-stranded DNA clean-up, an incubation period of 5 h at 37°C and 4°C overnight

for the *in vitro* transcription reaction and the use of the RNaqueous micro kit (Ambion) for aRNA clean-up. All second round amplifications of the standard reference were pooled and the yield quantified with a spectrophotometer. The standard reference aRNA was stored at -80°C .

cDNA blastocyst library construction

Total RNA was obtained from 640 day 7 and 8 bovine IVP blastocysts using the Trizol procedure (Invitrogen, Auckland, New Zealand). PolyA⁺ RNA was isolated using the MicroPolyA Purist kit (Ambion). PolyA⁺ RNA (160 ng) was reverse-transcribed following the SMART cDNA protocol (PT3000-1, 2001; Clontech, BD Biosciences, Auckland, New Zealand), but using SuperscriptII (Invitrogen) for reverse transcription. Protocol PT3000-1 was followed using 24 cycles for LD-PCR amplification. cDNA ligated to λ TriplEx2 vector was packaged using MaxPlax λ Packaging Extract (Epicentre, Madison, WI) and titered using *E. coli* XL-1 blue cells according to standard protocols.

Microarray generation

The primary λ pTriplEx2 library was plated at low density on 15 cm plates containing IPTG and X-Gal to allow for individual recombinant white plaques to be picked. Five thousand plaques were randomly selected and each added to 50 μl λ -dilution buffer (100 mM NaCl, 10 mM MgSO₄, 35 mM Tris-HCl pH 7.5, 0.01% gelatine) and 2 μl of chloroform in 96-well plates, before storage at 4 $^{\circ}\text{C}$. The insert of each phage was amplified in a 50 μl PCR reaction containing 2 μl phage, 0.25 μl each of primers λ TriplEx5' (5'-CTCGGGAAGCGCGCCATTGTGTTGGT-3') and 3' (5'-ATACGACTCACTATAGGGCGAATTGGCC-3'; Clontech), 5 μl 10 \times PCR buffer (Roche), 1 μl 10 mM dNTP and 0.2 μl 5 Units/ μl Taq DNA polymerase (Roche). Cycling conditions were 94 $^{\circ}\text{C}$ for 5 min, followed by 40 cycles of 94 $^{\circ}\text{C}$ for 15 s, 58 $^{\circ}\text{C}$ for 30 s and 72 $^{\circ}\text{C}$ for 2 min, with a final extension of 72 $^{\circ}\text{C}$ for 7 min. Two microlitres were used for gel electrophoresis to examine the amplified inserts.

Preparation of microarray slides, preparation of PCR-amplified DNA for microarray printing and microarray printing was according to published protocol (Diez-Tascón *et al.* 2005). The microarrays were printed with an ESI array robot (Toronto, Canada) using a 32 pin head. Each pin head printed a block of 180 spots. We included 243 blank spots (no DNA) per array. Array details can be accessed at the ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress/>) under A-MEXP-312.

cDNA labelling and microarray hybridisation

Two micrograms of amplified aRNA from the individual blastocyst samples and aliquots of the reference sample

were converted into cDNA and aminoallyl-labelled using the Invitrogen Superscript cDNA Labelling System according to the manufacturer's protocol. Single embryo samples were labelled with Cyanin (Cy) 5 dye and the standard reference sample with Cy3 (Amersham, Auckland, New Zealand). After the final elution the two samples to be compared on each microarray were combined, 10 μl 3 M (pH 5.3) sodium acetate and 2 μl 20 mg/ml glycogen (Roche) added before ethanol precipitation and resuspension in 10 μl DEPC-water. Microarray pre-hybridisation, hybridisation and post-hybridisation conditions were as previously described (Diez-Tascón *et al.* 2005). Dye-reversal hybridisation where the standard reference was labelled with either Cy3 or Cy5 and the two samples hybridised against one slide gave a correlation coefficient of 0.995.

Microarray analysis

Microarray slides were scanned at a resolution of 10 μm by a GenePix 4000A microarray scanner (Axon Molecular Devices, Sunnyvale CA). Cy 5 and Cy 3 fluorescence was measured at a 16 bit pixel resolution. Tagged image files were converted into data files using GenePix Pro 4.1 software (Axon). GenePix analysis included automatic as well as manual flagging of bad spots. Microarray data was manipulated and displayed using Data Desk 6.1 (Data Description, Ithaca, NY) and deposited in the public ArrayExpress database under accession number E-MEXP-556. Graphical displays were also generated using S-PLUS 6.1 (Insightful Corporation, Seattle, WA) and Microsoft Excel. Statistical analysis was conducted using a spatial mixed model (Baird *et al.* 2004) with GenStat software (VSN International, Oxford, UK). Differential expression between the mean of the IVP and NT blastocyst samples was determined using a t-test, with an arbitrary cut-off of 1.5-fold change and a 5% significance level. Reproducibility in hybridisation was monitored by including 384 features spotted in duplicate at different positions within each microarray slide. Comparison of the log intensity ratios across this data set revealed an average correlation ratio of 0.83.

Sequencing of significantly differentially expressed probes

Inserts of interest were PCR amplified as described above and sequenced (Eck *et al.* 2004) from the 5' direction using the TriplEx5'LD primer (#9107-1; Clontech) before separation on a Prism 3100 DNA sequencer (Applied Biosystems). Sequence information was analysed using BLASTN searches of public databases. An expected value of e^{-10} or less was considered to be a significant match.

Total RNA isolation, DNase treatment and reverse transcription

Single day 7 zona-free IVP or NT blastocysts were placed in 100 µl of Trizol (Invitrogen, Auckland, New Zealand) to which 5 pg rabbit α -globin mRNA (Sigma, Sydney, Australia) and 800 ng of MS2 RNA (Roche, Auckland, New Zealand) were added. Samples were extracted with 20 µl of chloroform followed by the addition of 10 µg linear acrylamide (Ambion, Austin, Texas) and 65 µl of cold isopropanol. After 10 min at room temperature, samples were centrifuged at 14 000 rpm (16 000 g) for 30 min, washed with 150 µl 70% ethanol and air-dried. After resuspension in 7 µl DEPC-treated water, 2 µl 1 U/µl RNase-free DNase1 (Invitrogen, Auckland, NZ) and 1 µl of 10×DNase I buffer was added and samples incubated for 1 h at 37 °C. Samples were precipitated with 1.5 µl 3M sodium acetate (pH 5.5) and 45 µl 100% ethanol, washed in 70% ethanol and resuspended in 12 µl DEPC-treated water.

To each sample 1 µl 10 mM dNTP and 1 µl 10 mM oligo dT₁₄VN anchored primer (Invitrogen) were added 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 reverse transcription 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 of TE buffer in non-stick 0.65 ml tubes (Neptune #3435.S3, Raylab, Auckland, New Zealand).

Real time PCR analysis

Seven of the genes identified by the microarray as differentially expressed—cytochrome c oxidase I; keratin 18 (*KRT18*); myosin, light peptide 6, alkali, smooth muscle and non-muscle (*MYL6*); ribosomal protein L21 (*RPL21*); solute carrier family 16, member A1 (*SLC16A1*); β -tubulin isoform 5 (*TUBB*); and tyrosine 5-monooxygenase/tryptophan 5-monooxygenase activation protein, theta isoform (*YWHAQ*)—were selected for quantitative expression analysis by SYBR-green real time PCR. In addition, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and keratin 8 (*KRT8*) were examined. Primers (Table 1) were designed using GCG's Prime (Accelrys, San Diego, CA) and, where possible, were selected to cover putative intron sequences as determined by comparison to the homologous human gene loci.

Real-time PCR was performed on a Roche LightCycler 2 instrument using 10 µl reactions containing 2 µl of

Table 1 Primers used for quantitative real time PCR analysis.

Gene	Primer sequence (5' to 3'; forward, reverse)	Size (bp)	Introns	PCR effic ^a
<i>GAPDH</i>	CTGTTGAAGTCGCAGGAGAC, TATCATCCCTGCTTCTACTG	247	0	1.9
<i>MYL6</i>	AAGAACAAGGACCAGGGCA, GCAGACATCATCCAAGAAAGAG	391	3	1.9
<i>RPL21</i>	GATGACCAACACAAAGGGAA, GGGTTAAAGGAGGCAATACAG	480	0	1.8
<i>KRT18</i>	TGATAATGC CCGTCTTGCT, GTGCTCTCCTCAATCTGCT	375	3	1.9
<i>TUBB</i>	GAGGCGAGCAAAAAGATTAGA, CAAGATAGCAGCAGGGACA	229	2	1.9
<i>YWHAQ</i>	TGTCCAACGAAGAGCGCAA, CCGATCATCACCACAAGCAA	308	1	1.9
<i>SLC16A1</i>	ACCAGTTTTAGGTCGTCTCA, GGCTTCTCAGCAACATCTACA	207	1	1.9
<i>Cyt c ox^b</i>	CGGAGACGACCAATCTACAA, GGAAACTCCTGCTAAGTGTAAA	325	0	1.8
<i>KRT8</i>	GCTACATTAACAACCTCCGTC, TCATCAGTCAGCCCTTCCA	235	2	1.9

^aPCR reaction efficiency.

^bCytochrome c oxidase I.

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 programme 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 base pairs), ramp speed 20 °C/s. The fluorescence readings were recorded after each 72 °C step. Dissociation curves were performed after each PCR run to ensure that a single PCR product had been amplified. Products were also analysed by gel electrophoresis and sequencing on first primer pair usage to ensure that the correct gene fragment was amplified. Each blastocyst sample was measured in duplicate per run by using an undiluted and 1:2 diluted aliquot. A no template control of 2 µl TE buffer, an RT⁻ control and a standard dilution series were included in each real-time run.

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) and fluorometrically with PicoGreen (Invitrogen) using the LightCycler. Standards consisted of a 10-fold dilution series containing 10⁵ to 10 copies/µl. Sample concentrations calculated from the standard curves were converted into an estimate of copy number per blastocyst after correcting for recovery and reverse transcription losses using values obtained for α -globin recovery. The average measurement for each blastocyst sample (at least two sample values) was used in statistical analysis of real time PCR results by an unbalanced ANOVA using GenStat (VSN).

Results

Experimental strategy

We here endeavoured to obtain a global picture of the effect of NT on the expression of genes transcribed at the blastocyst stage. To this end we made a novel bovine blastocyst cDNA library allowing the synthesis of a blastocyst-specific microarray. Our bovine day 7 blastocyst cDNA library had a complexity of 1.1 million. The use of oligo-dT mediated reverse transcription ensured inclusion of the 3' trailers of cDNA isolates, a necessary requisite when hybridising to linearly amplified cDNA which exhibits a similar 3' bias. Blue/white selection on X-Gal plates showed over 90% of phages to contain inserts. Amplification of inserts by PCR using vector specific primers yielded an insert size ranging from 0.1 to 5 kbp with a median of 0.7 kbp. For the generation of our blastocyst-specific microarray, 5000 insert-containing clones were randomly selected from the primary non-amplified library, PCR amplified and spotted.

A microarray approach necessitated an amplification step to generate sufficient cDNA to hybridise to the arrays, in particular as we wished to examine the expression profile of individual blastocysts, in line with observations that each NT reconstructed embryo exhibits a unique expression profile (CS, DNW, PLP, unpublished observations). We used linear mRNA amplification to achieve this. To assess the degree of bias introduced by this technique, total RNA isolated from two pooled IVP blastocysts was divided equally and both samples concurrently subjected to two rounds of amplification. Comparison of the amplified cDNA after hybridisation to our microarray (Fig. 1A) revealed a strong correlation ($r=0.99$) between the separate amplifications (Fig. 1B). We conclude that minimal bias was introduced by the linear amplification procedure, yet a small number of false positives are unavoidable.

Genetic effects were minimised by comparing NT embryos to IVP 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. The choice to compare NT not to *in vivo* embryos but rather to IVP embryos cultured in parallel, focused this work on effects specific to the NT procedure as opposed to gene expression differences arising due to embryo culture (Wrenzycki *et al.* 2005). We attempted to eliminate effects of embryo culture by comparing NT and IVP embryos grown under identical conditions in the same experimental run. Furthermore, all embryos were graded by only one person (DNW) ensuring consistency. For these analyses we used only the top two grades of expanded day 7 blastocysts, representing the grades used for transfers into recipient cows. Such NT embryos derived from fibroblast cells of this particular bull yield blastocyst development rates and implantation/attachment rates equal to IVP embryos,

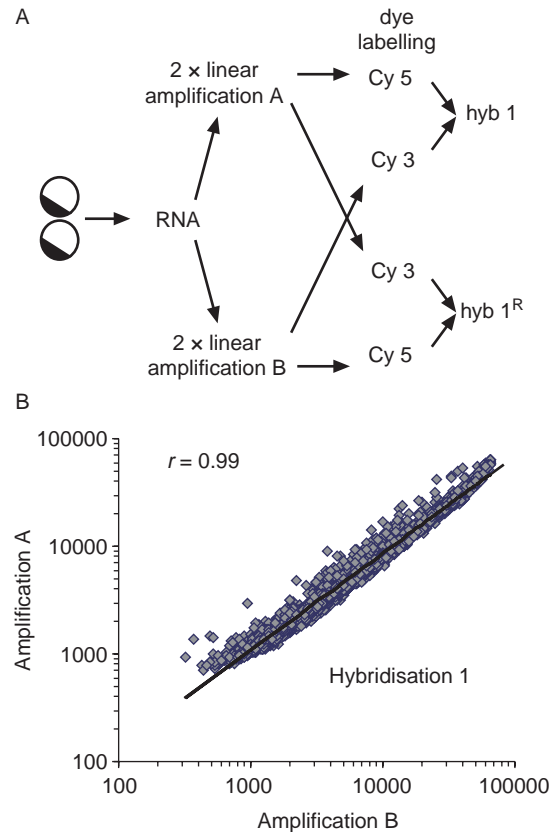


Figure 1 Testing bias resulting from linear amplification. (A) Experimental design for linear amplification bias control testing. RNA derived from 2 blastocysts was split equally and linearly amplified independently (A and B). (B) Plot of the fluorescence intensity generated by hybridising Cy5 labelled amplification A and Cy3 labelled amplification B (hyb 1) target to the 5000 features of the blastocyst-specific microarray. Note the dye-reversal microarray comparison (Hybridisation 1^R) also displayed a correlation coefficient of $r = 0.99$ (not shown).

but, unlike their IVP counterparts, show high mortality rates thereafter (data not shown). Of the six IVP and NT embryos used for linear amplification we discarded two IVP and one NT embryos based on both inadequate amplification efficiency (below 200 000 \times) and aberrant gel electrophoretic pattern (strong bias for short fragments). The remaining five NT and four IVP blastocysts were individually hybridised with the reference standard against the microarray. The use of blastocyst cDNA as a reference standard ensured signal generation from all features of our blastocyst-specific microarray.

Microarray results

Statistical analysis using a spatial mixed model (Baird *et al.* 2004) identified 164 microarray features (spots) as demonstrating significant differential expression between the means of the NT and IVP blastocysts ($P \leq 0.05$, ≥ 1.5 -fold change). Thus of the 5000 features

examined, 3% were consistently differentially expressed in the NT blastocysts. Of these features, 121 demonstrated lower expression and 43 higher expression in NT relative to IVP blastocysts. A graphical representation of the mean-adjusted relative expression intensities of each individual blastocyst across the 164 selected features highlights feature variation in individual embryos but also reveals the consistency of differences between the groups of IVP and NT embryos (Fig. 2). In particular, genes normally expressed at the blastocyst stage tend to be expressed at lower levels in NT embryos.

Sequence identification revealed some redundancy among the identified features. *Mitochondrial 16S rRNA* was detected seven times, a myosin isoform (*MYL6*) five times, *cytochrome C oxidase I* four times, keratin 18 (*KRT18*) and *mitochondrial 12S rRNA* thrice whereas 12 genes were detected twice (Table 2). The consistent behaviour of these independent duplicates (cDNA isolated from separate phages and thus spotted at different concentrations on the microarray) indicates the reproducibility of the microarray hybridisation procedure. The 121 features expressed at lower levels in NT embryos comprised 92 genes of which 8 (7%; 9 features as one gene was detected twice) could not be assigned to any known gene homologue. All of the 43 features found at higher levels in NT embryos corresponded to separate genes, though 26 (60%) were unknown.

The differentially expressed genes were classified by function using gene ontology (SOURCE, Stanford University, CA) (Fig. 3). Of the known genes over-expressed in NT blastocysts, those coding for transcription factors and proteins involved in signal transduction and metabolism predominated. Genes expressed at lower levels in NT blastocysts were associated with a large range of gene ontology terms (Fig. 3). Under-representation of transcripts in NT derived embryos encoded by the mitochondria (*12S* and *16S rRNA* and *cytochrome C oxidase I*) were detected multiple times. Other recurrent functions included cytoskeletal (*TUBB*, *TPX2*, *KRT18*, *TMSB4X*, *ACTB*, *ACTG1*, *MYL6*, *TPM4*, *DIAPH3*, *VIL2*, *PHACTR4*, *EPLIN*), protein biosynthesis (*EEF1A1*, *EFG2*, *BWZ1*, *EIF5A* and ribosomal proteins *L4*, *L5*, *L21*, *L23*, *LP0*, *S4X*, *S7*, *S20*), protein binding/folding (*GORASP2*, *HSPA9B*, *HSPA5*, *LGALS3*, *HAVCR1*, *CCT4*, *TCP1*) and metabolism/biosynthesis (*ADH5*, *PTGS2*, *SCP2*, *ACSL3*, *NDUFA1*, *MDH2*, *IDH3B*, *OAZ1*). The reduced levels of many genes required for the viability of every cell points to a general defect in the NT embryo's cellular machinery.

Real time PCR verification

Due to the small sample size, the expression levels of a further 17 NT and 16 IVP embryos were measured by the quantitative technique of SYBR-green based real time RT-PCR. As internal housekeeping genes typically used

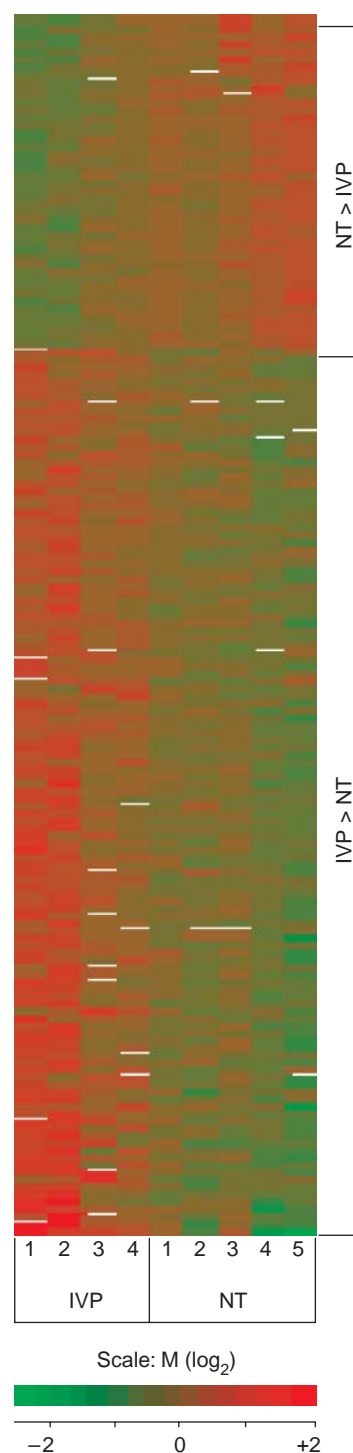


Figure 2 Heat map of the mean adjusted relative expression intensity ($\log_2[\text{IVP}/\text{NT}]$) of the microarray identified differentially expressed genes ($P \leq 0.05$, ≥ 1.5 -fold change). Columns represent individual blastocyst samples, rows represent individual genes. White lines represent absence of a microarray value.

for normalisation may be affected by the NT procedure, we used an exogenous spike of rabbit α -globin to adjust for differences in RNA recovery and reverse transcription efficiencies (Donnison & Pfeffer 2004). All PCR standard

Table 2 List of genes identified from the microarray analysis as significantly differentially expressed between NT and IVP blastocysts ($P \leq 0.05$, ≥ 1.5 -fold change)^a.

Gene name	GenBank accession#	Fold change	Gene name	GenBank accession#	Fold change
Expression reduced in clones					
<i>TUBB</i> (Beta-tubulin)	XM_600385	2.60	<i>MYL6</i> (Myosin) (5x)	NM_175780	1.65
Mitochondrial 12S rRNA (3x)	AY337545	2.41	<i>RPL4</i> (Ribosomal L4) (2x)	NM_001014894	1.65
<i>PTP4A1</i> (Tyrosine phosphatase)	XM_538984	2.35	<i>SNX2</i> (Sorting nexin 2) (2x)	XM_612796	1.65
			<i>BWZ1</i> (Leucine zipper)	NM_014038	1.64
				BC01779446	
<i>KRT18</i> (Cytokeratin 18) (3x)	XM_582930	2.28	<i>HSPA5</i> (GRP78)	NM_005347	1.62
<i>HSPA9B</i>	XM_595707	2.26	<i>DIAPH3</i> (Diaphanous homologue)	NM_019670	1.61
<i>ADH5</i> (Alcohol dehydrogenase)	XM_581812	2.25	<i>ATP5B</i> (ATP synthase) (2x)	NM_175796	1.59
<i>EPLIN</i>	XM_612416	2.20	<i>RPL21</i> (Ribosomal L21)	XM_588172	1.59
<i>UBE2V2</i> (Ubiquitin conjugating)	NM_003350	2.19	<i>EIF5A</i> (Translation initiation)	XM_580919	1.59
<i>EFG2</i> (Elongation factor)	NM_170691	2.16	<i>IER3IP1</i>	NM_016097	1.59
<i>SLC7A3</i> (Transporter)	XM_581045	2.14	<i>RPS20</i> (Ribosomal S20)	XM_590875	1.58
<i>SLC16A1</i> (Transporter)	XM_582117	2.11	<i>CKB</i> (Creatine kinase)	NM_001015613	1.58
<i>TMSB4X</i> (Thymosin, beta)	NM_001002885	2.10	<i>ACTB</i> (Beta actin)	XM_612548	1.58
<i>PTGS2</i> (Prostaglandin synthase)	NM_174445	2.08	<i>RPL23</i> (Ribosomal L23)	XM_581066	1.57
<i>RALY</i> (RNA binding)	NM_001014847	2.07	<i>YWHAH</i>	NM_174794	1.57
<i>GSP11</i> (G1 to S phase transition)	NM_146066	2.00	<i>SLC39A1</i> (Transporter)	NM_001859	1.57
<i>TRNT1</i>	XM_585851	1.98	<i>RCN1</i> (Reticulocalbin 1)	XM_580691	1.57
<i>ABCB6</i> (ABC transporter)	XM_584946	1.97	<i>GORASP2</i> (Golgi stacking)	NM_015530	1.56
<i>AP2M1</i> (Adaptor-related complex)	XM_595615	1.96	<i>SLC20A1</i> (Transporter)	XM_592075	1.55
<i>YWHAQ</i> (2x)	XM_582688	1.94	Cytochrome c oxidase I (4x)	AF493542	1.55
<i>CDC42</i>	NM_044472	1.94	<i>RPL5</i> (Ribosomal L5)	XM_614883	1.55
<i>EEF1A1</i> (Elongation factor) (2x)	XM_600690	1.91	<i>GDI2</i> (GDP dissociation)	XM_586879	1.55
<i>SCP2</i> (Sterol carrier)	XM_589307	1.90	<i>H3F3A</i> (H3 histone, family 3a)	NM_001014389	1.54
<i>HAVCR1</i> (Cellular receptor) (2x)	XM_585668	1.90	<i>IDH3B</i> (Isocitrate dehydrogenase)	NM_174856	1.54
<i>PHACTR4</i>	AL840643	1.89	<i>TIA-2</i> (Membrane glycoprotein)	XM_586758	1.54
<i>LGALS3</i> (Glectin 3)	XM_588340	1.89	<i>PSMD2</i> (Proteasome)	XM_589975	1.54
<i>ACSL3</i> (Acyl-CoA synthetase)	NM_203372	1.89	<i>RPLP0</i> (Ribosome phosphoprotein)	NM_001012682	1.53
<i>TEBP</i> (Progesterone receptor) (2x)	NM_001007806	1.87	<i>C10orf119</i>	XM_592775	1.53
<i>MLP</i> (MARCKS-like protein)	XM_588062	1.86	<i>VIL2</i> (Villin 2)	NM_174217	1.52
<i>ATRX</i>	XM_592333	1.85	<i>TPM4</i> (Tropomyosin 4)	NM_001001491	1.52
<i>RPS7</i> (Ribosome S7)	XM_581800	1.84	<i>CUL3</i> (Cullin 3)	NM_003590	1.51
<i>NPM1</i> (Nucleophosmin) (2x)	XM_587109	1.84	<i>NAP1L1</i> (Nucleosome assembly)	XM_613876	1.50
<i>TPX2</i> (Microtubule associated)	XM_594789	1.84	Unknown (9x)		
<i>PABPC1</i> (Poly(A) binding) (2x)	NM_174568	1.83	Expression elevated in clones		
<i>OAZ1</i> (Ornithine antizyme)	XM_583507	1.83	<i>CALM2</i> (Calmodulin 2)	NM_001009759	1.52
<i>CD22</i> (Antigen)	XM_592455	1.82	<i>PCNA</i>	XM_593532	1.52
<i>TCPI1</i> (t-complex chaperone)	XM_589481	1.82	<i>CT120</i>	XM_585198	1.52
<i>BASP1</i>	NM_174780	1.82	<i>PIP5K2B</i> (Kinase)	XM_592455	1.52
Mitochondrial 16S rRNA (7x)	AB099133	1.80	<i>SNX14</i> (Sorting nexin 14)	XM_532222	1.52
<i>PAI-RBP1</i> (mRNA binding)	XM_584549	1.80	<i>AP3D1</i> (Adaptor related complex)	NM_173998	1.53
<i>TEGT</i> (Bax inhibitor 1)	NM_003217	1.79	<i>RNP24</i> (Coated vesicle)	XM_588281	1.54
<i>SET</i>	XM_580367	1.78	<i>PLAG1</i>	NM_002655	1.54
<i>NDUFA1</i> (NADH dehydrogenase)	NM_175794	1.78	<i>GNG5</i> (G protein)	NM_174811	1.56
<i>RRBP1</i> (Ribosome binding)	NM_001003179	1.77	<i>OAZIN</i>	XM_583507	1.59
<i>IL23A</i> (Interleukin)	XM_588269	1.76	<i>NDUFA4</i> (NADH dehydrogenase)	NM_175820	1.59
<i>HNRPA1</i> (Ribonucleoprotein) (2x)	XM_614145	1.75	<i>DDX24</i> (DEAD box)	XM_537542	1.59
<i>PEX19</i> (Peroxisome)	NM_002857	1.74	<i>SERTAD1</i> (SERTA domain)	XM_612612	1.59
<i>CCT4</i> (Chaperonin)	NM_006430	1.74	<i>IPLA2(GAMMA)</i> (Phospholipase)	XM_613745	1.61
<i>ACTG1</i> (Gamma actin)	XM_612548	1.71	<i>ARHE</i> (Ras homolog)	XM_592424	1.67
<i>MAPRE1</i> (Microtubule associated)	NM_012325	1.68	<i>JJAZ1</i> (joined to JAZF1)	XM_582605	1.72
<i>MDH2</i> (2x)	XM_590742	1.67	<i>KIAA1970</i> (UBPH)	NM_019116	1.79
<i>RTLFL</i> (Reverse transcriptase-like)	XM_585472	1.67	Unknown (26x)		
<i>RPS4X</i> (Ribosomal S4)	XM_614302	1.66			

^aSome genes were detected more than once and are indicated in bold within parenthesis. Fold change refers to difference in the mean expression of the NT blastocysts relative to the IVP. There are 34 genes that are unknown—2 of the underexpressed unknowns are the same.

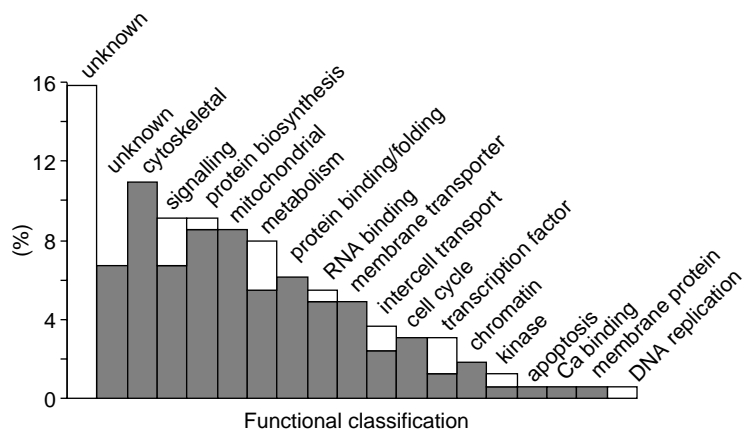


Figure 3 Classification of microarray identified significantly differentially expressed genes into functional categories, with each category expressed as a percentage of the total. Within each category, the fraction of genes which are underexpressed in NT embryos are shaded, those overexpressed are not.

curve reaction efficiencies were within 10% of the ideal amplification efficiency of 2.0 (Table 1). Furthermore, duplicates of all blastocyst samples were measured using a 1:2 dilution to ensure that readings were within the linear range. Quantitative results of 7 downregulated genes are presented (Fig. 4). These included three genes coding for cytoskeletal proteins, namely β -tubulin 5 (*TUBB*; highest fold difference in the microarray analysis), *KRT18* and *MYL6* (detected several times). We also analysed the mitochondrial gene *Cytochrome C oxidase I*, one of the eight genes coding for ribosomal subunit proteins (*RPL21*), one of the four genes coding for transporter proteins, (*SLC16A1*) and one of the signal transduction mediators (*YWHAQ*). We measured *GAPDH* transcript levels as an internal reference standard allowing comparison with previous quantitative results (CS, DNW and PLP, unpublished observation).

We observed moderate embryo to embryo variation in the expression levels of individual genes in both IVP and NT embryos (Fig. 4). When comparing the mean expression levels between IVP and NT embryos, only two of the seven microarray candidate genes as well as *GAPDH* exhibited a significant ($P \leq 0.05$) decrease in transcript levels in NT blastocysts (Table 3). These two genes, *KRT18* and the *SLC16A1*, showed subtle decreases in transcript levels of 1.4- and 1.5-fold respectively. We conclude from these analyses that only a subset of the genes identified as underexpressed in NT embryos by microarray analysis are consistently underexpressed when analysing a larger set of embryos.

We next examined the expression levels of keratin 8 (*KRT8*), the cytoplasmic partner of *KRT18* (Waseem *et al.* 1990). Interestingly, *KRT8* was recently reported as being expressed at lower levels in NT embryos in a microarray study similar to ours (Pfister-Genskow *et al.* 2005). However, real time quantitation of transcript levels of *KRT8* in a set of 16 embryos revealed no significant

difference between NT embryos and their half siblings ($P = 1.00$) (Fig. 4, Table 3). It should be noted that both *keratin 18* and *8* transcripts are extremely abundant in blastocysts, reaching levels of several hundred thousand copies (Table 3).

Discussion

The stochastic nature of the gene expression profile differences in individual NT embryos

During somatic cell nuclear transfer the gene expression profile of the donor cell has to be changed or reprogrammed extensively to reflect that of a normal embryo. Incomplete reprogramming has been proposed to underlie the loss of viability that is known to occur in NT-derived embryos. In our hands and using skin fibroblast donor cells, development to term and beyond of transferred NT embryos is less than 10% compared to around 40% for IVP blastocysts (Kruip & den Daas 1997, Oback *et al.* 2003, Smeaton *et al.* 2003, Wells *et al.* 2003, 2004).

An interesting question regarding incomplete reprogramming concerns the issue of whether particular gene loci are preferentially affected (reprogramming error hotspots) or whether defects are of a stochastic/random global nature. Consistent gene expression changes at a defined set of loci would support the hotspot scenario whereas unpredictability of expression profiles would suggest a stochastic model of reprogramming. Expression profile studies using pools of embryos (Wrenzycki *et al.* 2001, Donnison & Pfeffer 2004) are unsuitable for answering this question as stochastic effects are averaged out. Studies of individual NT-generated fetuses or newborns (Humpherys *et al.* 2001) are severely biased as these animals had already survived many critical phases of embryogenesis during which a large fraction of NT embryos succumb. We thus compared the gene expression of individual NT and

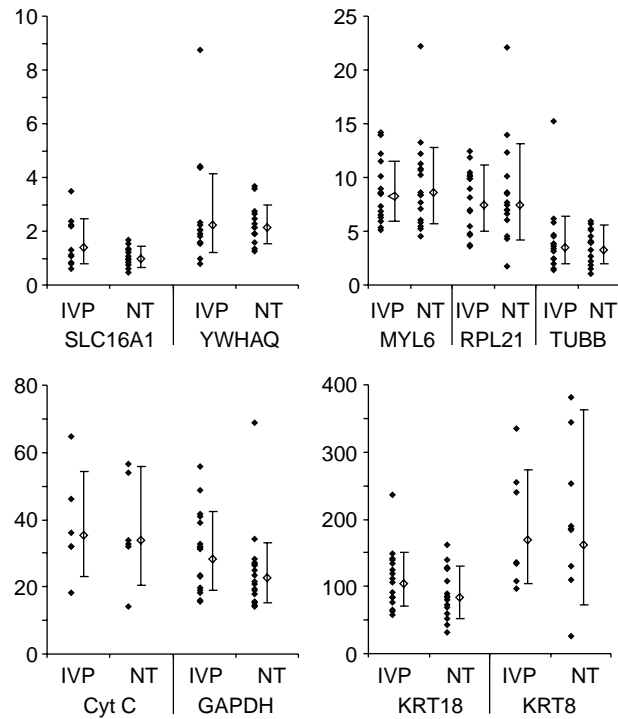


Figure 4 Individual and average gene expression levels (in thousands of transcripts per embryo) in IVP and NT day 7 blastocysts as determined by quantitative real time RT-PCR. Expression in individual blastocysts is represented by diamonds (IVP, $n = 16$; NT, $n = 17$) with the mean and standard deviation (back transformed from log scale) depicted adjacent to each data set. Each individual blastocyst sample was measured twice, once at a 1:2 dilution. Cyt C; cytochrome C oxidase I; KRT, cytokeratin.

genetically half-identical IVP embryos at an early stage (blastocyst) using a global microarray approach. We concentrated on the switching on of embryonic genes by screening a microarray consisting of cDNA derived from a blastocyst library which we constructed for this purpose. Genetic and developmental variation as well as embryo culture effects were minimised in this study.

We identified 92 genes expressed at lower and 43 genes expressed at higher levels in NT embryos ($P < 0.05$, ≥ 1.5 -fold change). The preponderance of underexpressed genes in NT embryos when examining

a set of cDNA isolates known to be expressed at the blastocyst stage suggests an impaired capacity of NT embryos for activation of embryo-specific genes. However, a range of functional categories was represented among the identified genes, with a high proportion involved in protein biosynthesis, signalling, cytoskeleton, mitochondrial, protein binding/folding and metabolism/biosynthesis. Most of these genes are not embryo-specific but would be expected to be constitutively active in every cell as they are required for the maintenance of the basal cellular machinery.

Table 3 Mean IVP and NT expression (copy number) \pm S.E.M. for the genes analysed by real time PCR^a.

Genes	Copy number per blastocyst		<i>P</i> value	Fold change (IVP/NT)
	IVP Mean \pm S.E.M.	NT Mean \pm S.E.M.		
<i>GAPDH</i>	28 300 \pm 2120	21 800 \pm 1630	0.02	1.3
<i>MYL6</i>	8310 \pm 694	8260 \pm 689	0.97	1.0
<i>RPL21</i>	7460 \pm 854	7120 \pm 815	0.74	1.0
<i>KRT18</i>	104 000 \pm 11200	73 900 \pm 7970	0.03	1.4
<i>TUBB</i>	3620 \pm 667	3040 \pm 561	0.52	1.2
<i>YWHAQ</i>	2200 \pm 312	2190 \pm 309	0.86	1.0
<i>SLC16A1</i>	1240 \pm 179	844 \pm 122	0.04	1.5
Cyt c oxi ^b	28 000 \pm 5449	30 000 \pm 5840	0.87	0.9
<i>KRT8</i>	168 000 \pm 55400	189 000 \pm 62500	1.00	0.9

^aThe *P* value represents the statistical difference between the IVP and NT means and the fold change represents the difference of the NT mean relative to the IVP mean.

^bCytochrome c oxidase I.

Misregulation of these genes in NT embryos therefore points to errors that may not be directly attributable to the donor cell's transcription profile.

It should be pointed out that our results are derived from examining a subset of blastocyst-specific genes. Currently it is believed that about 10 000 genes are transcribed in the mammalian blastocyst (Zeng *et al.* 2004). Based on frequency distributions of expressed sequence tags (ESTs) in non-normalised cDNA libraries (in-house data), we estimate our 5000 randomly chosen microarray features to represent around 1700 different genes. Of these genes we observed 8% to be consistently ($P < 0.05$) over- or underexpressed by at least 1.5-fold (none of sample deviated by more than 2.6-fold). Based on this random sample of around 1700 genes the 95% confidence interval for misexpression of genes after nuclear transfer lies in the range of 6.7–9.4%.

Interestingly, our quantitative data indicated that only a proportion of genes found to be misexpressed in the microarray data set are consistently misregulated when examining a larger set of embryos. On comparing the means of the expression levels of these misregulated genes, the changes seen are small (< 2 -fold). Considering the range of variation detected in the IVP samples, examination of transcript levels of a single gene in individual embryos cannot distinguish an NT from a control embryo. This unpredictability of individual gene expression on a global background of multiple gene expression changes argues for a predominantly stochastic nature of reprogramming errors.

Studies in the mouse can also be interpreted in light of stochastic reprogramming. Thus the expression of *Oct4* was found to be independent to that of a transgenic *Oct4*-enhancer driven reporter gene in individual NT derived blastocysts (Boiani *et al.* 2002). In a non-quantitative study of 10 putative pluripotency genes in mouse NT embryos, the expression profile was found to differ from embryo to embryo (Bortvin *et al.* 2003). In the bovine, detailed measurements of transcript levels of a small set of genes in individual embryos has yielded no (Camargo *et al.* 2005) or only one (CS, DNW, PLP, unpublished data) candidate that is significantly different in a set of IVP and NT embryos. Yet for that candidate, as for *GAPDH*, *KRT18* and *SLC16A1* identified in this study, changes in levels are small and, as in the mouse, seen only for a subset of NT embryos. Lastly, the stochastic reprogramming error model would easily account for the observed heterogeneity in the timing and cause of nuclear transfer associated defects: the manifestation of reprogramming errors would be dependent on the particular functions of the random set of genes affected.

Genes expressed at lower levels in NT embryos

The genes we found here to be more frequently misexpressed in NT embryos were *KRT18*, *SLC16A1*

and *GAPDH*. *GAPDH* may represent genes whose regulation is influenced by multiple regulatory pathways as it codes for an enzyme implicated in a wide range of cellular processes including glycolysis, tRNA binding, cytoskeleton interaction by the binding of tubulin and microtubules, apoptosis, vesicular transport and telomere length regulation (Sirover 1999, Tisdale 2002, Sundararaj *et al.* 2004). *GAPDH* is often used for gene expression normalisation (Bustin 2000, Pfister-Genskow *et al.* 2005). Our finding illustrates the need for caution in the choice of genes used for normalisation. Exogenous standards circumvent this problem.

The membrane transporter *SLC16A1* regulates the uptake of monocarboxylates including pyruvate (Garcia *et al.* 1994). Pyruvate uptake increases twofold after the expanded blastocyst stage in IVP embryos, suggesting a metabolic requirement for pyruvate at this stage (Rieger *et al.* 1992). Thus a reduced expression of *SLC16A1* in NT embryos may affect their viability. Interestingly our microarray identified reduced mRNA levels for three additional membrane solute carrier transporters, suggesting an altered metabolism in NT blastocysts.

The cytoskeletal keratin proteins *KRT18* and *KRT8* have been previously detected at early embryonic stages (Chisholm & Houlston 1987). In the only other global study examining gene expression in individual NT preimplantation embryos using microarrays, *KRT8* was found to be underexpressed in clones (Pfister-Genskow *et al.* 2005). We examined *KRT8* expression in our NT embryos using stringent criteria for quantification but found no significant difference in the mean expression levels between our NT and genetically half-identical IVP blastocysts. Though this discrepancy may have arisen from the use of different protocols for NT production and gene quantification in the two studies, it may also simply reflect the stochastic nature of reprogramming errors.

The biological relevance of the multiple small changes in expression is not clear, although it has previously been shown that an increase or decrease of only 50% in *Oct4* expression induced alternative differentiation pathways in mouse embryonic stem cells (Niwa *et al.* 2000). As every NT embryo appears to be quite unique in terms of its transcript levels, future studies will have to attempt to correlate gene expression profiles of individual embryos with their subsequent development, a technically daunting task.

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