RESEARCH ARTICLE

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Global Gene Expression Analysis of Bovine Somatic Cell Nuclear Transfer Blastocysts and Cotyledons

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SUMMARY

Low developmental competence of bovine somatic cell nuclear transfer (SCNT) embryos is a universal problem. Abnormal placentation has been commonly reported in SCNT pregnancies from a number of species. The present study employed Affymetrix bovine expression microarrays to examine global gene expression patterns of SCNT and in vivo produced (AI) blastocysts as well as cotyledons from day-70 SCNT and AI pregnancies. SCNT and AI embryos and cotyledons were analyzed for differential expression. Also in an attempt to establish a link between abnormal gene expression patterns in early embryos and cotyledons, differentially expressed genes were compared between the two studies. Microarray analysis yielded a list of 28 genes differentially expressed between SCNT and AI blastocysts and 19 differentially expressed cotyledon genes. None of the differentially expressed genes were common to both groups, although major histocompatibility complex I (MHCI) was significant in the embryo data and approached significance in the cotyledon data. This is the first study to report global gene expression patterns in bovine AI and SCNT cotyledons. The embryonic gene expression data reported here adds to a growing body of data that indicates the common occurrence of aberrant gene expression in early SCNT embryos.

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INTRODUCTION

The inefficiency associated with bovine SCNT has greatly limited its utility in a number of applications including production agriculture, conservation biology, and bio-pharmaceutical research. While it is difficult to ascertain the overall efficiencies due to differences in protocols, embryo transfer criteria, and data presentation, the overall efficiency of SCNT across species based on the number of embryos produced is less than 5% (Campbell et al., 2005). In cattle under the best conditions approximately 10–15% of SCNT embryos transferred develop to term (Oback and Wells, 2007).

A growing amount of data indicates the inefficiencies associated with SCNT largely result from deficiencies in nuclear reprogramming of the somatic nucleus following NT. Following the transfer of a differentiated cell or nucleus into an enucleated oocyte, the DNA must be reprogrammed from a cell-type-specific gene expression pattern to a totipotent embryonic-cell state. Modifications to the epigenetic order of the DNA are required in order for this to occur.

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The oocyte is well equipped to direct the nuclear reprogramming following normal fertilization, but less efficient at reprogramming somatic cells following SCNT. At fertilization, sperm chromatin is actively demethylated, and the maternal genome is demethylated in a passive, replication-dependent manner. This global demethylation is subsequently followed by de novo methylation of the genome starting at the 8–16-cell stage in bovine embryos and the blastocyst stage in the murine resulting in differentiation of cell lineages during development (Reik et al., 2001). Histone modifications are altered in a similar fashion following fertilization (Dean et al., 2003). A recent study analyzed the involvement of 24 chromatin factors (CFs) including transcription factors and nuclear binding proteins in reprogramming following fertilization in the mouse. Shortly after fertilization nearly all CFs were removed from chromatin, and shortly after pronuclear formation CFs are re-established on the chromatin in what is described as an ''erase-and-rebuild strategy'' (Sun et al., 2007).

A growing body of evidence supports the idea that SCNT inefficiency is a result of incomplete nuclear reprogramming. Differences in gene expression of embryos (Daniels et al., 2000; Han et al., 2003; Santos et al., 2003; Li et al., 2006) and fetuses (Hill et al., 2002; Schrader et al., 2003), as well as aberrant DNA methylation (Kang et al., 2001, 2002, 2003; Shi and Haaf, 2002; Mann et al., 2003; Young and Beaujean, 2004) and histone acetylation (Enright et al., 2003, 2005; Santos et al., 2003) in embryos and fetuses have all been reported previously. A follow-up study on the involvement of CFs on nuclear reprogramming evaluated the dynamics of the same CFs following SCNT and found similar patterns of CF removal and re-establishment in the somatic nucleus, but with some differences associated with timing and efficiency (Gao et al., 2007). In the case of control embryos, early development was characterized by a nearly wholesale removal of CFs from the DNA and export from the nucleus followed by sequential re-establishment of the CFs. In SCNT embryos, even after removal of the majority of CFs, some remained associated with DNA throughout early development, an indication of incomplete reprogramming (Gao et al., 2007). Epigenetic changes associated with differentiation of somatic cells likely make them more difficult to reprogram following SCNT.

A common phenotypic problem with bovine SCNT pregnancies is abnormal placentation. SCNT pregnancies are often noted to have larger and fewer placentomes than controls (Hill et al., 1999; Heyman et al., 2002; Hoffert et al., 2005; Constant et al., 2006; Oishi et al., 2006). Deficiencies in fetal-maternal nutrient and waste exchange contribute to the high rates of pregnancy failure and postpartum loss (Hill et al., 1999, 2000; Heyman et al., 2002; Constant et al., 2006).

Based on the growing amount of data implicating deficient nuclear reprogramming in many of the problems associated with SCNT, along with the apparent involvement of abnormal placentation in SCNT pregnancy loss, we conducted a series of experiments to evaluate global gene expression patterns in SCNT and AI blastocysts and cotyledons, the fetal contribution to the placentome and to determine whether aberrant gene expression in the early embryo can be linked with mid-gestational placental gene expression differences.

RESULTS

At time of collection SCNT fetuses appeared morphologically normal, and crown-rump lengths were not different from controls. No gross abnormalities were observed in SCNT placental tissues, although placentomes were slightly larger than controls as has been reported by other groups (Hill et al., 1999; Heyman et al., 2002; Hoffert et al., 2005; Constant et al., 2006; Oishi et al., 2006).

Using a probeset-level model to fit the embryo microarray data, 28 probes representing 28 different genes were identified as being differentially expressed ($q < 0.05$) with the FDR controlled at 0.05 (Table 1). By applying the probe-level model to the cotyledon data only 93 probes had a q-value of less than 1. Of those 93, 22 probes representing 19 different genes were identified as significantly differentially expressed ($q < 0.05$), controlling the FDR at 0.05 (Table 2).

One aim of the study was to determine whether commonly misexpressed genes could be detected in both SCNT embryos and cotyledons. Comparison of the lists of differentially expressed genes showed no genes that were common to both data sets, however two of the genes that were differentially expressed in embryos were among the list of 93 genes whose differential expression approached significance in the cotyledon data. In addition differential expression of MHCI approached significance in both microarray data sets. Expression of MHCI was analyzed in embryos and cotyledons because it approached significance in both groups and based on previous reports of its over-expression in cloned bovine placenta (Hill et al., 2002; Davies et al., 2004). In all, 22 genes were analyzed by qRT-PCR in cotyledons, and 29 genes were analyzed in embryos. Variability of gene expression levels between biological replicates was considerably higher among cotyledons (Fig. 1).

Following qRT-PCR analysis of embryo cDNA, expression levels were found to follow the same trends as microarray data for 25 of the 28 genes, and 9 were significantly different based on qRT-PCR results (Fig. 2). In the case of the cotyledons, 19 of the 22 cotyledon genes followed the same trends as microarray data, however only two were significantly different (Fig. 3). By qRT-PCR analysis expression of MHCI was determined to be higher in SCNT blastocysts and cotyledons, but the difference was only significant in the blastocysts. While microarray-based expression studies are a powerful means of generating lists of differentially expressed candidate genes, qRT-PCR is widely accepted as a more robust test of differential expression. For this reason qRT-PCR validation is requisite for validation of microarray results.

DISCUSSION

The present study employed microarray and qRT-PCR analysis to evaluate gene expression patterns in SCNT and control blastocysts and cotyledons. The goal of the study was to identify differentially expressed genes that might be

TABLE 1. Results of Microarray Experiments Comparing Gene Expression of Control and SCNT Blastocysts

Gene symbol	Gene name	Fold change	Q-value	NCBI ID
Over-expressed in SCNT blastocysts				
LOC540552	Hypothetical LOC540552	19.16	0.018	CB534828
*S-NID-2	Similar to NID-2	12.04	0.018	CK770586
PAH	Phenylalanine hydroxylase	8.77	0.028	CK849069
LOC533044	Similar to phosphoserine aminotransferase 1	7.59	0.049	CB166901
TL21877	Transcribed locus	5.54	0.035	BF707348
MHCI JSp. 1	MHC Class I JSp. 1	5.12	0.122	M21044.1
LOC614726	Similar to adaptor protein Lnk	4.85	0.037	AW670030
LOC785058	Hypothetical protein LOC785058	4.84	0.037	BM433653
LOC507982	Similar to WDSUB1 protein	4.63	0.018	AW307635
ANXA4	Annexin A4	4.19	0.018	M22248.1
ANXA1	Annexin A1	3.74	0.037	NM_175784.2
*S-N33	Strong similarity to protein sp:Q13454 (H.sapiens) N33_HUMAN N33 protein	3.65	0.037	CB534173
MGC152029	Similar to source of immunodominant MHC-associated peptides	3.51	0.037	CK849836
*S-Laminin	Similar to Laminin beta-1 chain precursor	3.39	0.037	CK849175
DR1	Down-regulator of transcription 1, TBP-binding (negative cofactor 2)	3.36	0.049	AW356106
LOC511508	Similar to KIAA0438	3.30	0.045	BI536262
TL12963	Transcribed locus	3.24	0.037	CF930841
LOC785489	Similar to *O*-acyltransferase (membrane bound) domain containing 2	3.04	0.037	AW658325
MGC143403	Similar to coronin, actin binding protein, 1C	3.03	0.037	CB428145
LOC539967	Hypothetical LOC539967	2.88	0.037	BM480824
MGC29463	Hyp protein	2.88	0.028	AU276541
LOC539627	Similar to KIAA0551 protein	2.41	0.037	CB221260
LOC514267	Hypothetical LOC514267	2.23	0.039	CK780156
LOC786956	Hypothetical protein LOC786956	2.20	0.038	CK947614
LOC510084	Similar to ankyrin repeat domain 10	2.05	0.049	CK770463
TL24300	Transcribed locus	2.01	0.038	BP100594
Reduced expression in SCNT blastocysts				
LOC513234	Similar to ovary-specific acidic protein	3.24	0.038	CK778634
LOC616217	Hypothetical LOC616217	4.38	0.037	BF045590
IER3	Immediate early response 3	4.26	0.037	CK775895

TABLE 2. Results of Microarray Experiments Comparing Gene Expression of Cotyledons from Control and SCNT Pregnancies

Gene symbol	Gene name	Fold change	Q-value	NCBI ID
Over-expressed in SCNT cotyledons				
PAG10	Pregnancy-associated glycoprotein 10	3.90	0.001	NM_176621.2
TKDP3	Trophoblast Kunitz domain protein 3	3.45	0.023	BE682514
TKDP5	Trophoblast Kunitz domain protein 5	3.03	0.002	BP108664
MGC139527	Similar to Sorting nexin-10	2.68	<0.001	CK847894
IL6	Interleukin 6	2.32	0.007	NM_173923.2
MGC139339	Similar to cell death-inducing DFFA-like effector a	2.24	0.004	CK849502
B4GALT1	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1	2.13	0.001	NM_177512.2
MHCI JSp. 1	MHC Class I JSp. 1	1.93	0.253	M21044.1
LOC540923	Similar to plasma glutamate carboxypeptidase	1.89	0.046	BF652540
TSPAN1	Tetraspanin 1	1.86	0.004	CK847262
LOC540552	Hypothetical LOC540552	1.57	0.219	CB534828
Reduced expression in SCNT cotyledons				
LOC782061	Similar to AKR1C1 protein	4.77	0.001	AY135401.1
LOC613334	Similar to Fragile X mental retardation 1 neighbor	2.68	0.001	CK847504
RBP1	Retinol binding protein 1, cellular	2.20	0.012	CK957614
LOC528380	Hypothetical LOC528380	2.15	0.001	CK771895
MGC142541	Similar to membrane-associated RING-CH protein III	1.99	0.015	BI849604
LOC515356	Similar to Cytochrome b5 domain containing 2	1.82	0.019	CK770131
MGC142636	Similar to carbonyl reductase 3	1.75	0.021	CK778163
MGC139085	Similar to paraoxonase 3	1.74	0.009	CK959273
KRT10	Keratin 10	1.69	0.050	NM_174377.1
LOC514936	Hypothetical LOC514936	1.66	0.006	BM088453
LOC533044	Similar to Phosphoserine aminotransferase 1	1.52	0.434	CB166901

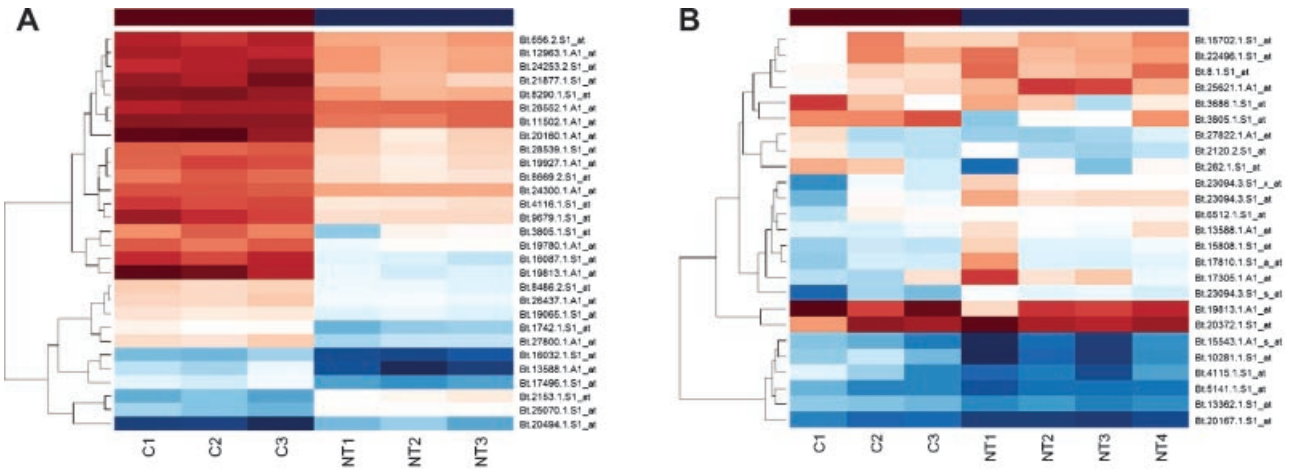

Figure 1. Heatmaps for embryo and cotyledon data indicating differences in specific gene expression profiles between control and SCNT cells. **A**: Twenty-eight significant probe-sets from embryo array data plus MHCI probe-set and (**B**) 22 significant probe-sets from cotyledon array data (representing 19 different genes) plus MHCI and two probe-sets that approached significance and were common to embryo data set. Control embryos and cotyledons are labeled C, and SCNT embryos and cotyledons are labeled NT. Dark red represents low expression, and dark blue represents high expression.

involved in the low efficiencies and frequent fetal abnormalities associated with bovine SCNT.

For the embryo experiments, 28 genes were determined to be differentially expressed between SCNT and AI blastocysts. qRT-PCR analysis of those 28 genes verified 25 (89%) followed the same trend as predicted in the microarray data. Statistical analysis of qRT-PCR data indicated significant differences for nine of those genes plus MHCI ($P < 0.05$). This relatively low concordance between qRT-PCR and microarray data is due in part to the fact that only six chips were run in the experiment, due to the limited numbers of available samples coupled with the expense of a microarray project. A relatively high degree of variability between groups was observed in the qRT-PCR results despite using pooled embryo samples. The other factor that might account for the partial discordance is that the RNA for the microarray and qRT-PCR experiments was derived from two separate embryo collections. This design lends additional credibility to the study in that ten of the same genes were found to be differentially expressed in both embryo collections.

Several previous studies have evaluated global gene expression differences between bovine SNCT and control blastocysts (Pfister-Genskow et al., 2005; Smith et al., 2005; Somers et al., 2006; Beyhan et al., 2007; Kato et al., 2007; Zhou et al., 2008). These reports as well as the study reported here are similar in that they all report a relatively small subset of genes that are differentially expressed between SCNT and control blastocysts, indicating the majority of genes are reprogrammed to express the appropriate genes at the appropriate levels by the blastocyst stage. An early study evaluated global gene expression patterns in bovine SCNT, IVF and AI blastocysts as well as expression patterns in the donor cells (Smith et al., 2005). As expected, the donor cell gene expression patterns were far divergent from the expression patterns in any of the blastocysts. Surprisingly SCNT embryo expression profiles were more similar to AI embryos than IVF compared to AI. (Smith et al., 2005). Pfister-Genskow et al. (2005) used a robust statistical design to evaluate gene expression differences between IVF and SCNT blastocysts and found 18 differentially expressed genes using a custom printed array. Another study evaluating global gene expression differences between bovine SCNT and IVF embryos reported 164 differentially expressed genes (Somers et al., 2006).

More recent studies have published similar results. One group reported 20 differentially expressed genes between NT and IVF blastocysts (Beyhan et al., 2007), and another evaluated expression profiles of day 15 IVF and NT embryos and reported 36 differentially expressed genes (Kato et al., 2007).

In the present study 28 genes were found to be differentially expressed between SCNT and AI blastocysts. Varying levels of discordance between qRT-PCR and microarray data was reported in each of the studies. The first study selected six genes for validation by qRT-PCR, and five were reported to validate microarray results, however statistical treatment is not discussed (Smith et al., 2005). Pfister-Genskow et al. (2005) evaluated six genes by qRT-PCR and had high validation rates with 5/6 validated using amplified RNA and 6/6 validated using un-amplified RNA. In the study by Somers seven genes were evaluated by qRT-PCR, and of the seven only two were found to be significantly different (Somers et al., 2006). Beyhan evaluated expression of six of the 20 differentially expressed genes by qRT-PCR, and was able to validate five of them (Beyhan et al., 2007). In the study by Kato et al., where 36 genes were identified as differentially expressed in day 15 embryos, the authors evaluated ten genes by qRT-PCR in 8-cell and blastocyst stage IVF and SCNT embryos with only moderate concordance with the microarray data. day 15 embryos were not evaluated by qRT-PCR (Kato et al., 2007). The disparity between microarray and qRT-PCR results reported

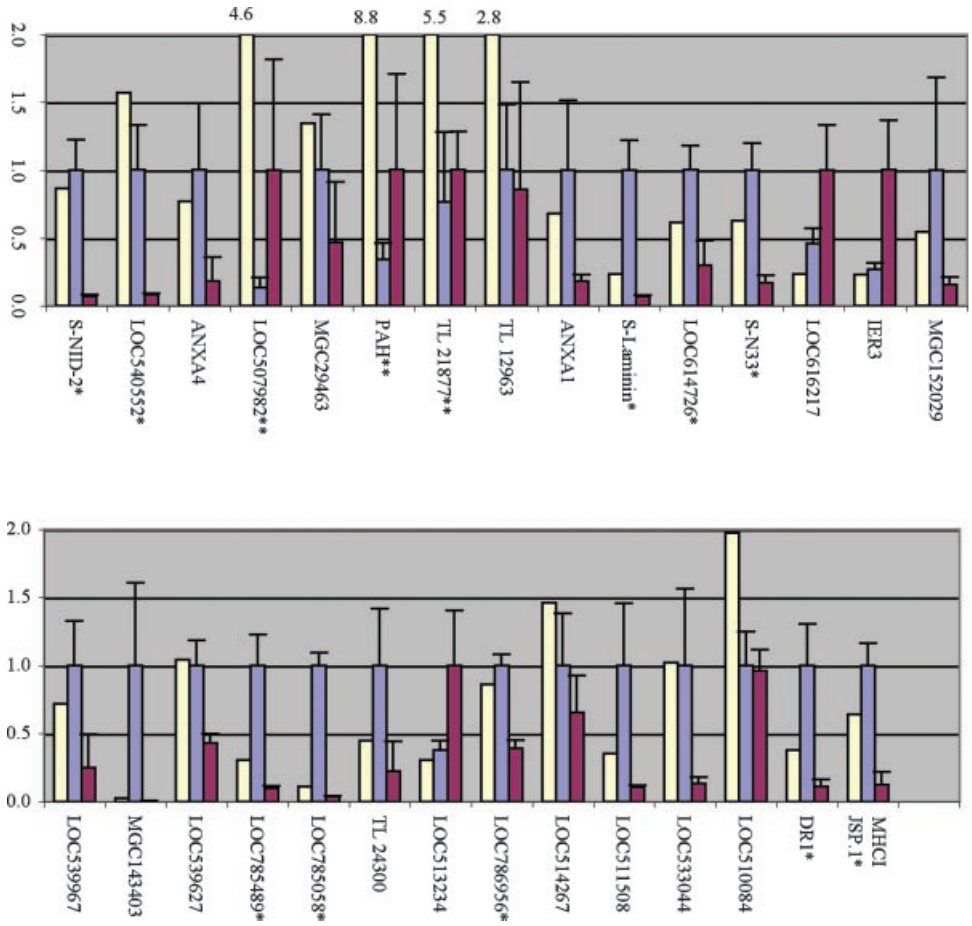

Figure 2. Gene expression profiles of control and SCNT blastocysts using qRT-PCR analysis. The yellow bars represent predicted SCNT expression relative to AI expression based on microarray data, blue and maroon bars represent actual expression in SCNT and AI blastocysts respectively as determined by qRT-PCR. Genes are ordered by significance based on microarray data from most-to-least. The y-axis represents scaled expression values for purposes of comparison of SCNT, AI, and microarray-predicted SCNT expression levels. Error bars represent SEM. Note: for scaling purposes, some bars are truncated. Actual values are noted above truncated bars. * AI and SCNT gene expression differs significantly based on qRT-PCR ($P < 0.05$). ** qRT-PCR results are opposite microarray predictions.

in several of these papers illustrates the importance of comprehensive qRT-PCR validation of microarray experiments. Accordingly, we applied qRT-PCR analysis to every gene determined by microarray analysis to be differentially expressed.

Interestingly, there is very little overlap in differentially expressed genes between these studies arguing for a stochastic (Smith et al., 2005) or cell line-dependent (Beyhan et al., 2007) reprogramming event following SCNT. Likewise, there does not seem to be much consensus between other gene expression studies evaluating expression differences between SCNT and control bovine blastocysts using other methodologies (Daniels et al., 2000; Niemann et al., 2002; Wrenzycki et al., 2004; Li et al., 2006; Oishi et al., 2006). The lack of concensus between studies does not indicate any study is flawed; rather it emphasizes the need for continued research to better understand the factors that affect gene expression following SCNT. The impact of differences in nuclear transfer protocols, culture conditions, and donor cell lines on gene expression likely explains the lack of consensus between experiments (Wrenzycki et al., 2001; Beyhan et al., 2007).

Ten genes were verified by qRT-PCR to be significantly different in SCNT blastocysts. The majority of those genes are not well annotated, but several were identified as being similar to genes in other species. Genes similar to osteonidogen (Nid-2), Laminin beta-1 (Lamb1), adaptor protein Lnk, tumor suppressor candidate 3 (N33), and *O*-acyltransferase domain containing 2 (Oact2) were all identified as differentially expressed as well as three hypothetical proteins—LOC540552, LOC786956, and LOC785058. In addition, down-regulator of transcription 1 (Dr1) and MHCI were identified by qRT-PCR as significantly different. In every case, these genes were over-expressed in SCNT embryos.

NID-2 and LAMB1 have been shown to be important in development of the basement membranes, a process critical for normal embryo development (Kohfeldt et al., 1998). Nid-2 was reported as being over-expressed in SCNT embryos

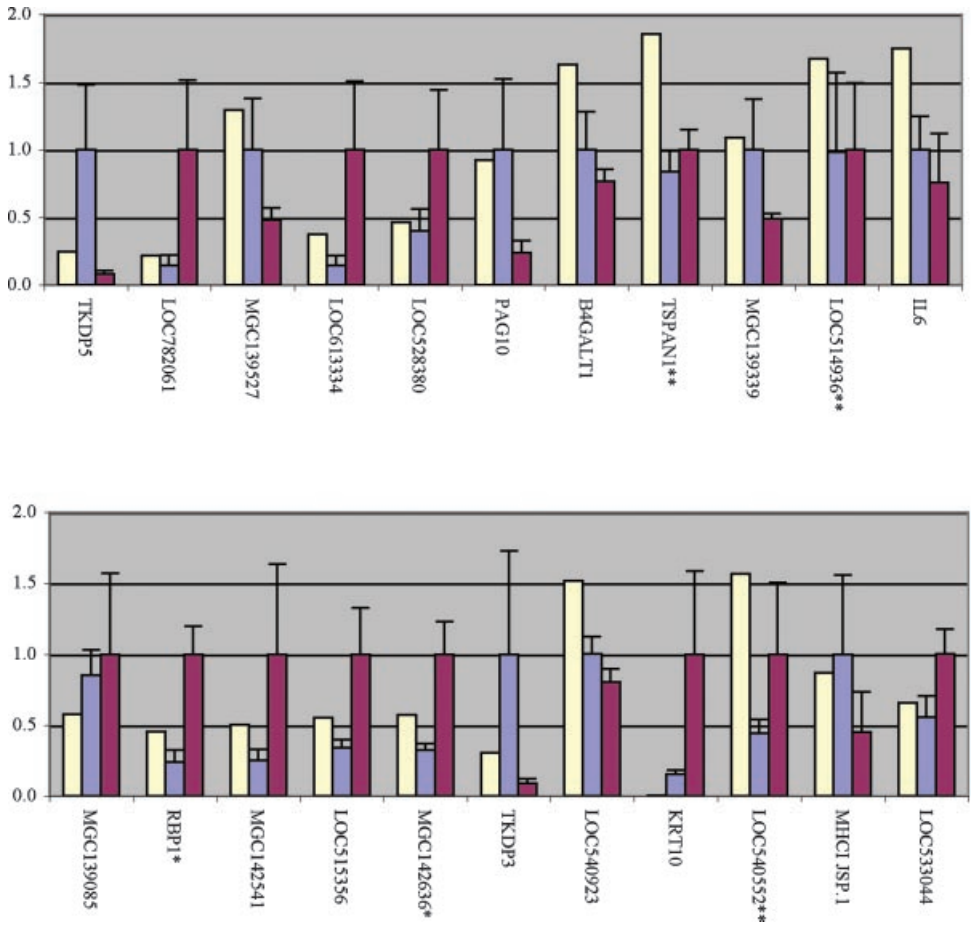

Figure 3. Gene expression profiles of control and SCNT cotyledons using qRT-PCR analysis. The yellow bars represent predicted SCNT expression relative to AI expression based on microarray data, blue and maroon bars represent actual expression in SCNT and AI cotyledons respectively as determined by qRT-PCR. Genes are ordered by significance based on microarray data from most-to-least. The y-axis represents scaled expression values for purposes of comparison of SCNT, AI, and microarray-predicted SCNT expression levels. Error bars represent SEM. * AI and SCNT gene expression differs significantly based on qRT-PCR ($P < 0.05$). ** qRT-PCR results are opposite microarray predictions.

in another recent study (Pfister-Genskow et al., 2005). Interruption in the association between NID-2 and the gamma chain of laminin resulted in lethal lung and renal developmental impairment (Willem et al., 2002). The effect of over-expression of either of these genes is unknown. Adaptor protein Lnk is a broad inhibitor of a number of growth factor and cytokine signaling pathways critical for early development (Buza-Vidas et al., 2006). Surprisingly, over-expression of Lnk in the mouse had little affect on phenotype (Takaki et al., 1997). N33 is a putative tumor suppressor involved in regulation of cell proliferation (Sun et al., 2004); however, little is known about its role in early development. The functions of Oact2, LOC540552, LOC786956, and LOC785058 are unknown. DR1 binds to the TATA binding protein (TBP) and blocks the binding of RNA polymerases II and III. In this way, DR1 can act as a potent transcriptional regulator (White et al., 1994). If Dr1 is commonly over-expressed in SCNT embryos it could have a profound impact on transcriptional regulation in early embryos. MHCI molecules are important for antigen presentation associated with cell mediated immunity. The over-expression of MHCI in the bovine SCNT placenta has been reported previously and is proposed as a factor in the frequent losses in bovine SCNT pregnancies (Hill et al., 2002). MHCI was reported to be over-expressed in SCNT blastocysts in one study (Pfister-Genskow et al., 2005) in agreement with our data, but based on array data was under-expressed and based on qRT-PCR was over-expressed in SCNT blastocysts in another paper (Beyhan et al., 2007). Evaluation of MHCI expression in cotyledons in the present study also found expression to be higher in SCNT cotyledons compared with AI but not significantly.

Gene expression was recently evaluated in IVF, AI, and SCNT placentomes between 174 days and term to investigate potential associations with fetal hydrops (Everts et al., 2008). In this study the authors found a large number of differentially expressed genes between groups (Everts et al., 2008). With an FDR of 0.3 they found 162 and 276 differentially expressed genes with greater than twofold difference for IVF versus SCNT and AI versus SCNT re-

spectively with no apparent overlap with our gene list (Everts et al., 2008). This disparity between gene lists likely results in part from the different methodologies between studies, that is, analysis of whole placentomes versus cotyledons. It is also likely as with the various embryo microarray studies that different donor cell lines and lab-to-lab variability has a significant impact on gene expression.

Results of our microarray experiments comparing AI and SCNT cotyledons revealed 19 genes to be significantly differentially expressed when controlling the FDR at 0.05. Of the 19 genes predicted to be different between SCNT and AI cotyledons, 16 were found to follow the same trends in expression by qRT-PCR analysis; however only two were verified to be significantly different. This again reflects a large degree of variability between samples. In fact, greater variability was observed between cotyledon expression patterns than between embryo expression patterns. This is likely due to the fact that multiple embryos were pooled to reduce variability in the microarray and qRT-PCR experiments, and cotyledons were analyzed individually. Future experiments of this nature might benefit from pooling cotyledons from each pregnancy in order to reduce variability. In addition, the blastocysts were all subjected to essentially the same environment—in vitro culture conditions for SCNT embryos and pre-implantation uterine environment for AI embryos. Cotyledons were collected from different dams, and factors such as condition of the dam or nutrient demands of the fetus could easily impact gene expression in cotyledons. The probe-level model did not prove to be a highly effective means of detecting differentially expressed genes in the cotyledon experiments. Nevertheless, two genes were validated by qRT-PCR to be significantly different, and these genes might be of physiological importance.

A gene similar to carbonyl reductase 3 (Cbr3) was found to be under-expressed, and retinol binding protein 1 (Rbp1) was over-expressed in Day-70 SCNT cotyledons. CBR3 is a cytosolic enzyme that catalyzes the reduction of prostaglandins, steroids and other carbonyls (Forrest and Gonzalez, 2000). The function of CBR3 has not yet been characterized in the placenta. RBP1 serves as the carrier protein for retinol (vitamin A), a vitamin critical for normal embryonic development. Either an excess or a deficiency in vitamin A can result in embryonic defects (Cohlan, 1953; Ross et al., 2000).

The physiological relevance of the genes determined by microarray analysis and qRT-PCR to be differentially expressed in SCNT blastocysts and cotyledons has not yet been elucidated nor have the properties of these genes that make them less amenable to reprogramming. Continued research evaluating the role of these genes in development is required, however several of the genes merit further research based on function; in particular Dr1, MHCI, and Rbp1.

The data presented here as well as in a number of other studies evaluating gene expression differences between clones and controls all lend support to the idea that incomplete epigenetic reprogramming lies at the heart of poor SCNT efficiency. Unfortunately a large degree of variability is observed in every aspect of SCNT, from rates of in vitro development to differences in pregnancy establishment and maintenance rates. The variability even extends to phenotypes of genetically identical cloned offspring (Lee et al., 2004). It can arise from differences in manipulation or culture conditions (Wrenzycki et al., 2001), donor cell type (Powell et al., 2004; Batchelder et al., 2005), oocyte source (Miyoshi et al., 2003; Aston et al., 2006), and a host of other factors. Continued global gene expression studies under a variety of conditions will shed light on some of the factors most important in the nuclear reprogramming process as well as offer insights into the complex and poorly characterized field of epigenetic reprogramming. Until the factors affecting reprogramming efficiency are better characterized or methods for augmentation of epigenetic reprogramming are developed, it is unlikely SCNT efficiency will improve to any great degree.

MATERIALS AND METHODS

Donor Cell Culture

Primary bovine fibroblast cultures were established from lung tissue, as we have found lung tissue to culture well and have achieved relatively good SCNT tissues utilizing this tissue source (Aston et al., 2006). Tissues were washed thoroughly and minced, suspended in DMEM/Ham's F12 (1:1) (HyClone Laboratories, Logan, UT) supplemented with 15% fetal bovine serum (FBS; HyClone Laboratories) and 100 U/ml penicillin/100 μg/ml streptomycin (HyClone Laboratories), seeded in 25 cm^2 tissue culture flasks, and cultured at 39°C in a humidified atmosphere of 5% CO_2 in air for several days. Cells between passages one and four were then harvested and re-suspended in tissue culture medium containing 10% DMSO, frozen, and stored in liquid N_2 until use in SCNT. Prior to SCNT, cells were thawed and grown to 80–100% confluence. Cells were treated with trypsin (0.25%) and resuspended in manipulation medium for use in SCNT.

Oocyte Maturation

Maturation of bovine oocytes was performed as described previously (Li et al., 2004a,b). Briefly, cumulus oocyte complexes (COCs) were aspirated from 3 to 8 mm follicles using an 18-gauge needle from ovaries collected from a local abattoir. Only those oocytes with uniform cytoplasm and intact layers of cumulus cells were selected and matured in TCM 199 containing 10% FBS, 0.5 μg/ml FSH (Sioux Biochemicals, Sioux City, IA), 5 μg/ml LH (Sioux Biochemicals), and 100 U/ml penicillin/100 μg/ml streptomycin for 18–22 hr.

SCNT Embryo Production

Following maturation, cumulus cells were removed from oocytes by vortexing COC in PB1 (calcium and magnesium containing phosphate buffered saline [HyClone Laboratories], 0.32 mM sodium pyruvate, 5.55 mM glucose, 3 mg/ml BSA) medium containing 10 mg/ml hyaluronidase. Oocytes with a first polar body were used as recipient cytoplasts. Enucleation was employed to remove the first polar body and metaphase plate, and single cells were subsequently transferred to the perivitelline space of recipient cytoplasts. Fusions of NT couplets were performed in mannitol fusion medium (Wells et al., 1999) by two electric DC pulses of 2.2 kV/cm for 25 μs. Following fusion, embryos were held in CR2 medium supplemented with 3% FBS for 1–2 hr prior to activation (Rosenkrans and First, 1994). Fused embryos were activated between 23 and 25 hr after the onset of maturation by exposure to 5 μM ionomycin for 5 min followed by 5 hr incubation in 10 μg/ml cycloheximide. For the purposes of the microarray experiments we produced three groups

of 10 grade 1–2 blastocysts from a single cell line. For real-time PCR (qRT-PCR) validation, an additional three groups of five embryos were produced. Embryos were placed in RNAlater RNA stabilization reagent (Ambion Inc., Austin, TX) and stored at −20°C until RNA extraction.

AI Embryo Production

Control embryos for microarray studies were collected from super-ovulated cows using established protocols. Donor cows were synchronized using the EAZI-BREED™ CIDR® (CIDR; Pfizer, New York, NY) vaginal progesterone implant. On Day 0, a CIDR was inserted and 2 cm^3 of estradiol was injected intramuscularly (IM) into donor animals. A 2.34 mg dose of follicle stimulating hormone (FSH) was injected IM the morning and evening of Day 5, followed by 2.16 mg of FSH injected the morning and evening of Day 6. On the morning of Day 7, donor animals received 25 mg of Lutalyse (IM) and a subsequent 25 mg dose of Lutalyse in the evening. Estrus was observed the morning of day 9 and animals were artificially inseminated three times each at 12-hr intervals with the initial insemination being in the morning of Day 9. Seven days after the initial breeding, embryos were collected from donor animals by intra-uterine flush using embryo filters. Following collection, embryos were rinsed in flush medium, placed in RNAlater (Ambion Inc.) and stored at −20°C until RNA extraction. Three groups of ten grade 1 and 2 blastocysts were collected for the microarray studies, and an additional three groups of five embryos were collected for qRT-PCR validation.

Cotyledon Collection

Control pregnancies were established by artificial insemination of CIDR-synchronized cows, and SCNT pregnancies were established by nonsurgical embryo transfer of Days 7–8 SCNT blastocysts. Pregnancies were verified by ultrasound at approximately embryonic Day-30 and again at Day-60. On Days 69–70 post-insemination/activation, recipient animals were slaughtered at a local abattoir. Cotyledonary tissue was collected within 30 min of slaughter, snap frozen and stored in cryovials in liquid N_2 until RNA extraction. Cotyledons of similar size and location were collected by manual separation of placentomes from three AI pregnancies and four SCNT pregnancies. While the presence of maternal cells in manually separated fetal cotyledons is likely and indeed has been documented (Bridger et al., 2007), the goal for analysis was to enrich for the fetal contribution to gene expression and minimize the maternal contribution. Placental tissue was collected at day 70 in an effort to analyze tissues from NT fetuses that were normal and healthy and had a high probability of maintaining pregnancy. All of the pregnancies collected were verified by ultrasound on day 60 to have a fetal heartbeat.

RNA Extraction

RNA extraction from embryos. Total RNA was extracted and DNA was digested with DNase I from AI and NT embryos using the RNAqueous micro kit (Ambion Inc.) according to manufacturer's recommendations with modifications. Prior to RNA extraction each sample was spiked with 50 μg yeast tRNA as a carrier. The RNA was eluted from the RNAqueous column using two 20 μl volumes of pre-warmed (75°C) elution solution. Following RNA purification microarray samples were reduced to 3–5 μl using speed vacuum centrifugation in order to yield a sufficient RNA concentration for amplification using the Affymetrix 2-round labeling kit. All of the RNA extracted from the first three groups of ten AI and SCNT embryos was utilized for the microarray experiments, and a second group of embryos was collected and RNA-extracted for qRT-PCR validation. In order to obtain sufficient RNA for qRT-PCR reactions, the RNA was amplified using the TargetAmp 2-Round Amplification Kit 2.0 (Epicentre, Madison, WI). Both the Affymetrix 2-round labeling and the TargetAmp2 RNA amplification kits employ the T7 linear amplification procedure which has been validated for fidelity and successfully employed in numerous microarray studies (Gomes et al., 2003; Jenson et al., 2003; McClintick et al., 2003) including several embryo microarray studies (Pfister-Genskow et al., 2005; Smith et al., 2005; Beyhan et al., 2007; Kato et al., 2007). Amplified RNA was reverse-transcribed and stored at −20°C until qRT-PCR analysis.

RNA extraction from cotyledons. Cotyledons were removed from liquid N_2, and approximately 30 mg of tissue was placed in RLT Buffer (Qiagen Inc., Valencia, CA) containing beta-mercapto ethanol (βME) and subsequently homogenized using a rotor stator homogenizer. The RNA extraction was performed using the RNeasy Mini RNA Extraction Kit (Qiagen) according to manufacturer's recommendations.

Microarray Expression Studies

Preliminary checks of the RNA were not performed on embryo samples as previous experience indicated RNA concentration- and quality-determination using the nanodrop and bioanalyzer are not reliable with RNA extracted from embryos. Blastocyst stage bovine embryos contain approximately 2 ng total RNA. In order to attain sufficient quantities of RNA for hybridization on Affymetrix GeneChips a two-round labeling protocol was used according to manufacturer's protocols. Following the two-round labeling procedure RNA quantity and integrity were assessed using an Agilent 2100 Bioanalyzer. Following quality assessment, labeled RNA was hybridized to the Affymetrix bovine microarray chip and subsequently scanned according to manufacturer's protocols. Microarray analysis of cotyledons was also performed according to manufacturer's protocols. Since sufficient RNA could be obtained from cotyledons, single-round labeling was used rather than the two-round labeling. Following microarray analysis, qRT-PCR of cotyledon RNA and amplified blastocyst RNA was used to validate microarray data.

Reverse Transcription and SYBR Green qRT-PCR

Reverse transcription was performed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) with random primers. The cDNA was stored at −20°C until use. SYBR Green real-time PCR (Abgene, Rochester, NY) was used to validate differential expression of genes in cotyledons and blastocysts that was determined, by microarray analysis, to be differentially expressed. Each real-time PCR reaction was performed in duplicate. qRT-PCR was performed in white thin-walled 96-well plates. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta actin were initially utilized as housekeeping reference genes. In agreement with previously published data GAPDH was found to be most consistently expressed in preimplantation bovine embryos and was therefore used as the internal control housekeeping gene (Robert et al., 2002). Primers for qRT-PCR analysis (Table 3) were designed using Primer3 primer-design software (Rozen and Skaletsky, 2000). All primers utilized were designed and optimized in order to ensure similar reaction efficiencies between target genes and GAPDH. In addition, cDNA was diluted such that threshold cycles were similar between the target and housekeeping genes. These measures were taken because a critical assumption for the application of the delta-delta C_t method ($\Delta\Delta C_t$) is that the efficiencies of target and housekeeping primers be very similar. Even small variances in primer efficiencies can result in large differences following 30–40 PCR cycles. A standard PCR protocol with a 15 μl reaction volume was used. The reactions consisted of Absolute™ QPCR SYBR® Green PCR Master Mix (Abgene) containing fluorescein reference dye, forward and reverse primers at 200–300 nM final concentration and 1 μl diluted template cDNA.

TABLE 3. Specific Primer Sequences Used in SYBR Green qRT-PCR Analyses to Validate Microarray Gene Expression Results

Primer name	Sequence: forward and reverse (5′–3′)	Size (bp)
Primers used in both experiments		
GAPDH	GATTGTCAGCAATGCCTCCT TTGAGCTCAGGGATGACCTT	240
MHCI JSp. 1	TCCTTGTCACTGGAGCTGTG ACAGACGCATTCAGATGCAG	240
LOC540552	TGTTGGAGTTGTTCCTTGCT ACAGCCACAAAAATGTCCTG	115
LOC533044	TTACGTTTTCAACGGCTGTG TCACTGGCCAAACACCATAA	235
Cotyledon primers		
LOC782061	CAAGCAGCTGGAGAAGATCC TCCAAGAGAAAGGGGAGGTT	201
MGC142541	TGTGGAAGTTTCTGCACTGG CATGTTTGGCAGCTTAGCAA	199
LOC613334	GCAGTGAAAATGCTGATGGA CTCTCAGAGGGGCAAAACAG	216
RBP1	CGACTTTACCGGGTACTGGA TCAAACTCCTTCCCAACCTG	207
MGC139085	CTCCCTGAACTGGCATTCTC GTTCTGAGGCCCTCTCTCCT	207
LOC540923	TGCCTGGAGCCAGTCTACTT AGAGGGGCTTCCTAAAGCTG	244
MGC142636	GACGCTCACAGAGGAAGACC GTCGGCTTTCCTCTTCTCCT	175
LOC528380	CCACTTTGCTGCTGACTTGA GCTGCATTTGACTCAGAAAGG	210
IL6	TGCAGTCTTCAAACGAGTGG TAAGTTGTGTGCCCAGTGGA	182
PAG10	GAATGGGACAGTGGTTGCTT AAGACAGCAGGAGGCAGTGT	177
B4GALT1	CAGTGATAGGCCCTCTCTGC GCTTTGATTCTTTGGGGTGA	185
LOC515356	TGTGAGCAGAGACTGGATCG AGCTTAGAGGGGGACAGAGC	211
KRT10	CAAAGCTGCCTCCATAGCTC ATCCCTCAGAATTTCGAGCA	200
TKDP5	ACGGTGGCTGTAATGGAAAG GGAAAGGAAAAGGCAGGTTC	230
TKDP3	TATCATCCGTGGTGTGGCTA GTGCCGTGACCTACCACTTT	246
TSPAN1	ACCACTGCTGCTGTCGTATG GGGCTCTGGAATAGGAGGAC	195
LOC514936	AAGCCACTTCAGCCACAGTT AGGAAGGACAAAGGGGAAGA	219
MGC139339	TCCTACGACATCCACTGCAC CCCCTACCCTCTCTTGATCC	192
MGC139527	GCCTTTTTGTTTTCTGTTTGG ATATTGCCAAGGAGCTGGTG	209
Embryo primers		
LOC511508	TGGCACACGTTGTTGATTTT GATGAAGCAAAGGGACCAAA	171
LOC510084	TTCTTGGGGTGTTCTGCTTT CGGAGGACACTGGTTTTGTT	237
LOC614726	TGGACCGTGTAGGAAAAAGG AGCACTCAGCCCACAAACTT	169
S-N33	TCGTGGCAGAGTCACACATT GGTAGCCGTGGTACTTGGAA	192
LOC616217	CAGTTTTTAATGCGCAAGCA AAGCTGTCTTTCTGGGCAAA	209
IER3	GCAAGCACCCAGAACTAAGC TTCCCCGAATCTTCACAGAC	154
TL21877	GGCATTGCTTCCATTTGATT CAATGAGAAACAGAGGAAAATCG	247
DR1	TGCTTAGGTTGCATTGGTTG TGCCATTTCAAAGGAAGCAT	207

Table 3. (*Continued*)

Primer name	Sequence: forward and reverse (5′–3′)	Size (bp)
TL24300	TCCCTGGAAGTGTTTCCAAC TCCTACCCATCAAGAAGCTCA	202
LOC513234	GCGACAGTGGAGACAACAGA GAACCTGCACAGGCTTCTTC	206
LOC514267	TGCCTTCATGTTATGCGGTA GCCTTGTGAAAGCACCTCTC	201
MGC152029	TGCCTTTAGCTCATGTCGTG GGTTCTTTGGTGCGAATTGT	250
LOC539967	CAAGGAAGTCCTGCCTTCAG AAGGCTGGCTCCTGTGAATA	215
LOC539627	ACATGGACAAGGCACATTGA ACTGTACCCCAAATCCCACA	215
LOC785489	AAGGGGTCTGTGTCTGTTGG TGGGACACACAGCGTACATT	236
LOC785058	AGTTGCCCGAAGGTACTGTG TTCAGTCCAGCTTTCCCAAG	160
MGC29463	CAGTGAGGAGGGTGGGATAA GGGGTTTGGAGTTCAGCATA	231
S-Laminin	TCGGGAATCTCTTTGAGGAA GAACTTGTGGTGGAGGCATT	184
S-NID-2	CCCTTCTCCAAACTGCTCTG TCCCTTCTCCAGTCGGTATG	161
LOC786956	CAGAAGAGGTGCTCCCTCAC TGAACAGAATGCCAAGGACA	178
MGC143403	GGAGAAAGCACACGAAGGAG CCCCATTGCTAGTGTCCATT	167
TL12963	ATGCCACATTGCAAAAGATG TGCCCAAACATAGTCTCACA	258
ANXA1	AAGGCTTTGCTTTCTCTTGC GACGAGTTCCAATACCCTTCA	346
PAH	TGCTTGCTATGAGCACAACC GCAGTGGAAGACTCGGAAAG	193
ANXA4	AAATCCATGAAGGGCTTGG GGGAATCTTCCTGGGCTTT	221
LOC507982	CAAGCACCCTGACCAACATT CCTCCGTGCCCTTAGAGTTT	151

The same PCR protocol was used for all primers: 15 min at 95°C for activation of the hot start Thermo-Start® DNA Polymerase; 40 cycles of 95°C for 15 sec, 58°C for 30 sec, and 72°C for 15 sec (data collection step), then 95°C for 30 sec followed by an 80-cycle melt curve initiated by 30 sec at 55°C with a temperature increase of 0.5°C each cycle.

Statistical Analysis

Analysis of cotyledon microarray data. After RMA (Irizarry et al., 2003) preprocessing the limma/eBayes model (Smyth, 2004) was applied to the data to test for differential expression between controls and clones. As all of the clones were bulls, and two of the controls were heifers, a gender covariate was added to the model. The results of this preliminary probeset-level analysis did not yield any significant genes when controlling the false discovery rate at 0.05.

The inability of more traditional probeset-level models to detect significance in these data motivated a consideration of various probe-level models, which have performed favorably in previous applications (Bolstad, 2004). RMA background correction and quantile normalization was performed, and again the limma/eBayes model with the gender covariate was applied to the data. By analyzing the data in this manner, a number of genes were determined to be differentially expressed after controlling the false discovery rate (FDR) at 0.05 (adjusted *P*-value [q] < 0.05).

Analysis of embryo microarray data. Similar to the cotyledon data, after RMA (Irizarry et al., 2003) preprocessing the limma/eBayes model (Smyth, 2004) was fit to the data to test for differential expression between controls and clones. Unlike the cotyledon data, after controlling the FDR at 0.05 differentially expressed genes were found using the probeset-level data ($q < 0.05$). As a probeset model is a more conservative analysis, and differentially expressed genes were detected between embryos using this model, further analysis using a probe-level model was not necessary for the embryo data.

qRT-PCR analysis. The delta-delta C_t method was used for real-time PCR data evaluation (Livak and Schmittgen, 2001). Data was normalized for differing amounts of input cDNA using ΔC_t (C_t for the GAPDH housekeeping gene minus C_t for the gene of interest). Next, $\Delta\Delta C_t$ was calculated by subtracting the ΔC_t of each sample from the ΔC_t of a reference cDNA sample. The n-fold increase or decrease in expression levels of each gene at each embryonic stage was calculated using the formula $\mathbf{2}^{-\Delta\Delta C_t}$. Pair-wise comparisons between SCNT- and AI-$\Delta\Delta C_t$ values were performed for each gene using the Student's *t*-test. A probability of $P < 0.05$ was considered significant.

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