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The transcriptomic architecture of mouse Sertoli cell clone embryos reveals temporal–spatial-specific reprogramming

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Abstract

Somatic cell nuclear transfer, a technique used to generate clone embryos by transferring the nucleus of a somatic cell into an enucleated oocyte, is an excellent approach to study the reprogramming of the nuclei of differentiated cells. Here, we conducted a transcriptomic study by performing microarray analysis on single Sertoli cell nuclear transfer (SeCNT) embryos throughout preimplantation development. The extensive data collected from the oocyte to the blastocyst stage helped to identify specific genes that were incorrectly reprogrammed at each stage, thereby providing a novel perspective for understanding reprogramming progression in SeCNT embryos. This attempt provided an opportunity to discuss the possibility that ectopic gene expression could be involved in the developmental failure of SeCNT embryos. Network analysis at each stage suggested that in total, 127 networks were involved in developmental and functional disorders in SeCNT embryos. Furthermore, chromosome mapping using our time-lapse expression data highlighted temporal–spatial changes of the abnormal expression, showing the characteristic distribution of the genes on each chromosome. Thus, the present study revealed that the preimplantation development of SeCNT embryos appears normal; however, the progression of incorrect reprogramming is concealed throughout development.

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Introduction

With progression of mammalian development, which starts at fertilization, the totipotency of embryonic cells is rapidly lost and cell fate is progressively determined. Somatic cell nuclear transfer (SCNT) is an excellent approach for understanding the nuclear reprogramming of somatic cells, because it is the only way that somatic cells are turned into a totipotent stage, from which all types of cells, including cells of extra-embryonic tissues, can be differentiated (Wilmut *et al*. 1997, Wakayama *et al*. 1998, Gurdon & Wilmut 2011). In many species, embryo-cloning studies have demonstrated that the differentiated cell status is not irreversible and can be switched by various regulatory molecules (Thuan *et al*. 2010). However, only a limited proportion of SCNT embryos are allowed to develop into normal individuals and the majority die at different stages of development (Ono *et al*. 2001, Ogonuki *et al*. 2002).

To date, a number of studies have been conducted to understand how donor nuclei acquire totipotency and/or why the majority of SCNT fails to develop to term (Rideout *et al*. 2001, Hochedlinger & Jaenisch 2006, Peat & Reik 2012). Reprogramming of donor cells begins shortly after the transplantation of a donor cell nucleus into an enucleated oocyte (Gurdon *et al*. 1976), and Q-PCR studies have revealed that many genes were abnormally expressed in SCNT embryos (Bortvin *et al*. 2003, Inoue *et al*. 2006, Jincho *et al*. 2008). Transcriptome studies using microarray analysis are often an effective approach in studying the reprogramming, which occurs in SCNT embryos (Inoue *et al*. 2002, 2010, Pfister-Genskow *et al*. 2005, Smith *et al*. 2005, Somers *et al*. 2006, Wakayama *et al*. 2006, Beyhan *et al*. 2007, Vassena *et al*. 2007, Fukuda *et al*. 2010). Such studies have shown that a number of genes avoid being reprogrammed during the first and second cell cycles (Vassena *et al*. 2007), and a considerable number of genes were abnormally expressed at blastocysts (Inoue *et al*. 2002, Wakayama *et al*. 2006, Fukuda *et al*. 2010). Meantime, these results suggested that some part of the genes were corrected during development to the blastocyst stage. Furthermore, Inoue *et al*. (2010) obtained very interesting observations that inappropriate reprogramming of X chromosome-related genes could be responsible for developmental failure in SCNTs. Their gene expression profiles showed that 80% of the downregulated genes in Sertoli cell nuclear transfer (SeCNT) embryos recovered when *Xist*-knockout donor cells were used. In a previous study, we conducted

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further comprehensive transcriptome analysis of mouse SCNT embryos at the blastocyst stage by using a microarray designed for single embryos so as to gain better understanding on the rapid embryo loss around implantation (Fukuda *et al*. 2010). The results revealed that each type of SCNT embryos had a unique gene expression profile that strictly depended on three donor cells: Sertoli cells, ES cells, and cumulus cells. Furthermore, the analysis identified 35 genes that were inappropriately reprogrammed in most of the SeCNT blastocysts. Transcriptome studies in bovine have also shown global gene expression profiles in nuclear-transferred embryos at the blastocyst stage, and suggested that restrictive reprogramming errors might be magnified in the subsequent developmental stages in SCNT embryos (Pfister-Genskow *et al*. 2005, Smith *et al*. 2005, Somers *et al*. 2006, Beyhan *et al*. 2007).

Studies on reprogramming need to show the changing aspect of the gene expression profile of SCNT embryos throughout the preimplantation stages. For example, Tong *et al*. (2006) reported aberrant gene expression in cumulus-cloned embryos detected at the 2-cell, 4-cell, and blastocyst stages and found candidate genes related to cell structure, protein modification and cell signaling, and transcription factors (TFs). Since time-lapse gene expression profiles certainly provide beneficial information for understanding reprogramming in SCNT embryos, further precise and comprehensive transcriptome analysis should be performed in mice. In the present study, we used a transcriptomic approach to analyze the progression of reprogramming in SeCNT embryos throughout preimplantation development by using microarray analysis of single embryos. This novel research angle provided a comprehensive data set, which provided a whole picture of the reprogramming process in SeCNT embryos.

Results and discussion

Gene expression profile during preimplantation development

To gain a better understanding of the molecular mechanisms that underlie the reprogramming of SeCNT embryos, we performed transcriptomic profiling using single preimplantation-stage embryos from all preimplantation stages. Here, we analyzed SeCNTs because of their relatively high and stable development to term compared with other SCNTs (Ogura *et al*. 2000, Meissner & Jaenisch 2006). Of the activated SeCNT embryos, more than 60% developed to the blastocyst stage, and nine pups (10.1%) were obtained after the

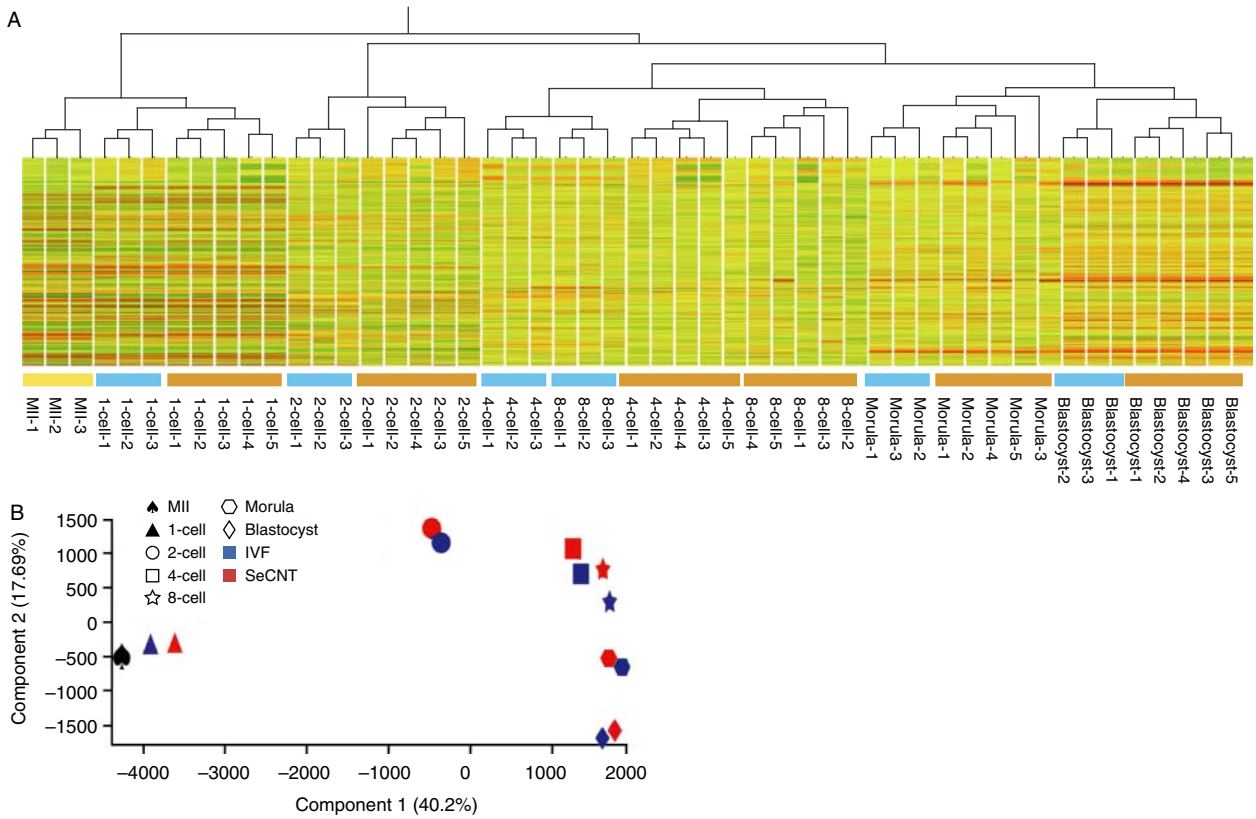


Figure 1 Hierarchical clustering dendrogram and principal component analysis (PCA). (A) Hierarchical clustering dendrogram of 51 samples. In total, 17 822 probe sets passed the cut-off RAW value of <100 and were used for hierarchical clustering. Yellow, metaphase II oocytes; blue, IVF samples; orange, SeCNT samples. (B) PCA results in a 2D display. Blue symbols, IVF embryos; red symbols, SeCNT embryos.

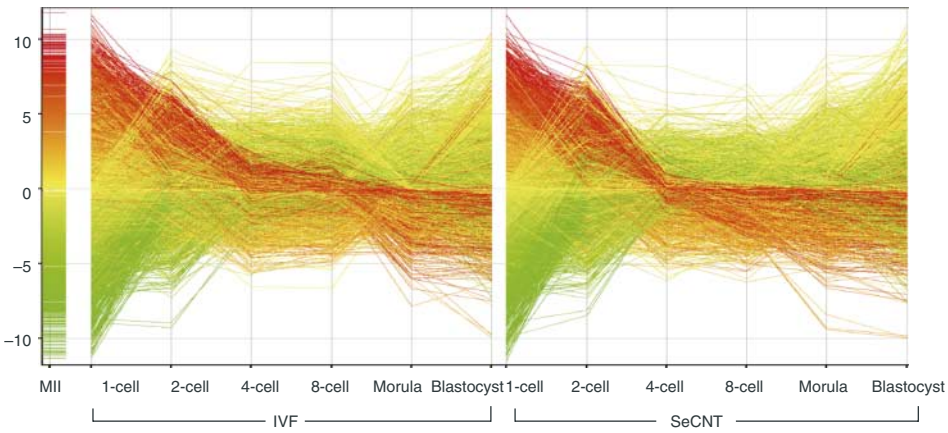
embryos were transferred to recipients in the present study (Supplementary Table 1, see section on supplementary data given at the end of this article). However, the majority of the embryos were lethal. At present, we are unable to select viable embryos for further analysis, but this might be possible in the near future by using a real-time imaging system (Mizutani *et al*. 2012).

IVF B6D2F2 (B6D2F1/B6D2F1) embryos were used as controls, because 95% or more of the IVF eggs develop to the blastocyst stage in our laboratory. Moreover, inappropriate gene expression caused by *in vitro* culturing can be eliminated among differentially expressed genes in SeCNTs. We analyzed IVF embryos (B6D2F1/B6D2F1) ($n=3$ at each stage), SeCNT embryos ($n=5$ at each stage), and MII oocytes (B6D2F1, $n=3$). The genetic background of donor Sertoli cells (B6CBF1) differed from that of the IVF controls (B6D2F2). Therefore, the genetic background might have affected the differences in gene expression profiles. However, the differences were perhaps not significant because a comparison of the gene expression profiles between the B6D2F2 and B6CBF1 embryos revealed that the correlation coefficient was rather high (0.97). The CEL file data that were obtained from Mouse GeneChip 430 2.0 were entered into GeneSpring GX 12.0 for the analysis. A dendrogram was constructed by performing a hierarchical clustering analysis (Fig. 1A), and the principal component analysis (PCA) results are shown in a 2D display (Fig. 1B).

In the dendrogram (Fig. 1A), the samples were clearly divided into five groups according to the embryonic development stages in which SeCNT embryos were distinguished from IVF embryos at each stage. The gene expression profiles changed depending on the developmental stage rather than the difference between the IVF and SeCNT embryos. However, in the IVF groups, the gene expression profiles of 4-cell- and 8-cell-stage embryos were very similar and formed one cluster, and were definitely different from the SeCNT embryos. These results indicate that SeCNT embryos expressed transcriptomes that resembled that of IVF embryos. However, particular gene clusters were differentially expressed between the two groups (Kohda *et al*. 2005). The results from the PCA (Fig. 1B) supported the results of the dendrogram and showed three significant changes in the profiles between 1- and 2-cell stages, 2- and 4-cell stages, and the morula and blastocyst stages. These three changing phases reflected major events : firstly, the initial phase of reprogramming; secondly, zygotic gene activation; and finally, first cell differentiation into inner-cell-mass cells and trophoblastic cells (Chastant *et al*. 1996, Kawasumi *et al*. 2007, Sawai 2009, Egli *et al*. 2011).

Genes were differentially expressed in SeCNT embryos at each stage

In an attempt to identify particular genes that needed to be reprogrammed correctly, we compared the gene expression profiles of SeCNT and control IVF embryos at each stage. The probe sets passed the filter of cut-off RAW values of <100 and subjected to a two-way ANOVA ($P<0.001$, 4793 probe sets). The number of differentially expressed genes is shown in Fig. 2, and genes that showed twofold or greater changes are listed



Number of genes differentially expressed at preimplantation stages

	1-cell	2-cell	4-cell	8-cell	Morula	Blastocyst
Foldchange>2	751	1308	1420	2239	695	368
up/down	278/473	272/1036	343/1077	400/1839	215/480	162/206
Foldchange>3	253	409	478	958	250	130
up/down	123/130	107/302	120/358	137/821	88/162	70/60
Foldchange>5	62	91	112	268	83	54
up/down	44/18	28/63	30/82	36/232	30/53	33/21

Figure 2 Time-lapse gene expression profile of the differentially expressed genes in SeCNT embryos during the preimplantation stages. In total, 4793 probe sets passed the two-way ANOVA and were used for fold-change selection at each stage. The number of genes differentially expressed at each stage is shown in the table by 2-, 3-, and 5-fold changes. Up/down means the probe sets were up/downregulated in SeCNT embryos. Each line represents one probe set: the color of the line represents the gene expression in MII; the line in red represents a higher expression in MII; and the line in green represents a lower expression.

in Supplementary Table 2, see section on supplementary data given at the end of this article.

Generally, the transcriptomic profiles of SeCNT embryos resembled those of IVF embryos (Fig. 2). A considerably large number of genes were differentially expressed at threefold or greater levels at each stage: a minimum of 130 genes at the blastocyst stage and a maximum of 958 genes at the 8-cell stage. However, genes showing fivefold or greater changes were relatively limited. Interestingly, of the differentially expressed genes, the number of downregulated genes in SeCNT embryos was higher than that of the upregulated genes from the 2-cell (107/302) to the morula (88/162) stage. The number was also six times higher at the 8-cell stage (137/821) but not at the blastocyst stage. Key genes, which would have affected the development of SeCNT embryos, were incorrectly reprogrammed throughout preimplantation development. Thus, the cloned embryos feigned seemingly proper embryonic development so that the progression of incorrect reprogramming was concealed throughout development. Therefore, SeCNT embryos rapidly lost their developmental capability from the 8-cell stage (Latham 2005), when the number of genes that were inappropriately expressed was greatest. Our previous study analyzed SCNT embryos that were constructed from three types of donor cells and showed that the abnormal gene expression profiles of SeCNT embryos were influenced by donor cell-specific gene expression (Fukuda *et al*. 2010). Furthermore, a gene ontology analysis suggested that the level of gene expression in the donor cells was responsible for disorders pertaining to specific biological functions in SeCNT embryos.

Because TFs always have a significant influence on the regulation of gene expression, their reprogramming failure directly affects wide-scale gene expression and the development of SeCNT embryos. We found that TFs, which represented 10–25% of the differentially expressed genes, were abnormally expressed at several stages of development, and, in particular, a large number of genes were inappropriately expressed with threefold or greater changes at the 2-cell (up: 18, down: 89), 4-cell (up: 19, down: 97), and 8-cell (up: 33, down: 154) stages (Supplementary Table 3, see section on supplementary data given at the end of this article). Therefore, the reprogramming failure of TFs might have been partly responsible for the significant abnormality in the transcriptome in SeCNT embryos. Notably, TFs such as *Yy1* (Donohoe *et al*. 1999) and *Tial1* (Beck *et al*. 1998), which have been reported via knockout studies to be involved in embryonic lethality, were completely repressed in SeCNT embryos. Furthermore, as we discuss below, many TFs function upstream to regulate the network of differentially expressed genes.

To gain a better understanding of the functions of the differentially expressed genes, we carried out a gene annotation analysis by IPA using genes that were

Table 1 Top ten functions of differentially expressed genes at each embryonic stage by IPA.

Cell stage (number of genes included)	***P* value**
1-Cell stage	
Post-translational modification (16)	6.36×10^{-6}
DNA replication, recombination, and repair (15)	3.91×10^{-5}
Cell-to-cell signaling and interaction (14)	2.92×10^{-4}
Cellular development (16)	5.81×10^{-4}
Gene expression (38)	8.29×10^{-4}
Cell cycle (11)	9.62×10^{-4}
Cell morphology (18)	9.62×10^{-4}
Cellular function and maintenance (19)	9.62×10^{-4}
Cellular assembly and organization (18)	1.74×10^{-3}
Cellular compromise (8)	5.09×10^{-3}
2-Cell stage	
Free radical scavenging (5)	2.81×10^{-4}
Cellular growth and proliferation (88)	3.01×10^{-4}
Nucleic acid metabolism (4)	3.01×10^{-4}
Small-molecule biochemistry (29)	3.01×10^{-4}
Protein synthesis (33)	5.18×10^{-4}
Post-translational modification (21)	5.93×10^{-4}
Cellular compromise (12)	8.92×10^{-4}
Cellular movement (19)	1.02×10^{-3}
Cell cycle (59)	1.50×10^{-3}
Cellular morphology (23)	1.64×10^{-3}
4-Cell stage	
DNA replication, recombination, and repair (41)	4.69×10^{-7}
Gene expression (72)	4.69×10^{-7}
Cellular assembly and organization (29)	8.04×10^{-5}
Cellular development (35)	2.04×10^{-4}
Cell cycle (53)	3.24×10^{-4}
Cellular growth and proliferation (91)	3.38×10^{-4}
Amino acid metabolism (5)	9.15×10^{-4}
Small-molecule biochemistry (26)	9.15×10^{-4}
Post-translational modification (18)	2.08×10^{-3}
Lipid metabolism (9)	3.03×10^{-3}
8-Cell stage	
Gene expression (146)	2.14×10^{-6}
Cell cycle (108)	4.60×10^{-6}
Cellular assembly and organization (103)	4.60×10^{-6}
DNA replication, recombination, and repair (71)	4.60×10^{-6}
Post-translational modification (36)	1.33×10^{-5}
Cell-to-cell signaling and interaction (15)	1.45×10^{-4}
Drug metabolism (9)	1.45×10^{-4}
Small-molecule biochemistry (72)	1.45×10^{-4}
Cell death (184)	5.19×10^{-4}
Amino acid metabolism (22)	6.20×10^{-4}
Morula stage	
Cell death (60)	1.58×10^{-4}
Cellular movement (19)	1.95×10^{-4}
Post-translational modification (6)	7.01×10^{-4}
Protein folding (5)	7.01×10^{-4}
Cellular growth and proliferation (31)	1.47×10^{-3}
Cellular assembly and organization (19)	1.57×10^{-3}
Cellular compromise (5)	2.18×10^{-3}
DNA replication, recombination, and repair (7)	2.22×10^{-3}
Gene expression (40)	2.22×10^{-3}
Cell-to-cell signaling and interaction (21)	2.89×10^{-3}
Blastocyst stage	
Lipid metabolism (19)	3.23×10^{-6}
Small-molecule biochemistry (28)	3.23×10^{-6}
Vitamin and mineral metabolism (15)	3.23×10^{-6}
Cellular assembly and organization (10)	5.88×10^{-5}
Cellular growth and proliferation (40)	9.10×10^{-5}
DNA replication, recombination, and repair (5)	2.17×10^{-4}
Gene expression (21)	2.17×10^{-4}
Carbohydrate metabolism (12)	2.76×10^{-4}
Cellular movement (21)	3.08×10^{-4}
Cell-to-cell signaling and interaction (16)	9.54×10^{-4}

Table 2 Number of zygotic activated genes differentially expressed in SeCNT embryos.

	1-cell	2-cell	4-cell	8-cell	Morula	Blastocyst
Zygotic activated in IVF	659 (76)[a]	1301 (120)	331 (4)	31 (6)	201 (14)	281 (–)[b]
Downregulated in SeCNTs	76 (7)	156 (19)	37 (0)	4 (1)	6 (3)	4 (–)
Repressed in SeCNTs	11	30	16	0	0	0
KO data[c]	20	53	19	2	2	0
Related to lethality	14	35	13	0	1	0

[a]Values in parentheses indicate the number of stage-specific expressed genes. [b]Stage-specific genes were identified as only having gene expression at a particular stage. The blastocyst stage was lacking at a later stage for comparison. (–) indicates no data. [c]Knockout data obtained from MGI (http://www.informatics.jax.org).

differentially expressed at levels threefold or greater. The top ten functions of the genes at each stage are listed in Table 1. Several gene functions were frequently listed in the top ten list throughout the preimplantation stages, such as post-translational modification, gene expression, DNA replication/recombination/repair, and cell cycling. The results show that characteristic disorders occurred at each stage. Interestingly, 'cell death' was listed as the top function in the morula stage and was also included in the list of the top ten functions at the 8-cell stage. This coincides well with SeCNT embryo development, because these embryos were significantly retarded and arrested around these stages (Tveden-Nyborg *et al.* 2005). It is thought that differentiation of the embryonic cells into cell lineages of trophoblasts or inner cell mass is determined after the morula stage, but in the cloned embryos, some specific errors such as those detected here might lead to functional disorders in cell death and cell cycling (Park *et al.* 2004). Furthermore, genes that are regarded as having 'free radical scavenging' functions are listed in the top ten list at the 2-cell stage. This suggests that SeCNT embryos are exposed to oxidative stress, by which the embryos may suffer short- and long-term disorders (Ohashi *et al.* 2006). Furthermore, these results suggest that genes whose functions are related to metabolic activity (e.g. lipid metabolism, vitamin and mineral metabolism, and carbohydrate metabolism) were insufficiently expressed, especially at the blastocyst stage.

Zygotic gene activation during embryo development

The zygotic activation of genes involves *de novo* transcription from the embryonic genome, which mainly occurs from the late 1-cell stage after fertilization (Bouniol *et al.* 1995), and this transition is an essential program for normal development (Minami *et al.* 2007). First, by using the transcriptome data of IVF embryos, we attempted to identify the genes with greater than threefold expression changes during specific stages (Table 2 and Supplementary Table 4, see section on supplementary data given at the end of this article) and the number of genes at each stage: 1-cell 659, 2-cell 1301, 4-cell 331, 8-cell 31, morula 201, and blastocyst 281. Here, for convenience, these genes are named zygotic activated genes. A minority of these genes was transiently expressed only at specific stages. In addition, more than 50% of the zygotic activated genes expressed from the 2-cell stage corresponded to those that were identified by an α-amanitin inhibition test (Zeng & Schultz 2005). As well, these genes were activated mainly in the 2-cell stage; their levels decreased once by the 8-cell stage and then increased slightly at the morula and blastocyst stages. This finding suggests that dynamic changes in gene expression profiles occurred at the morula and blastocyst stages.

Notably, some of the zygotic activated genes were incompletely activated and showed reduced expression in the 1- to 4-cell stages of SeCNT embryos: 1-cell 76, 2-cell 168, and 4-cell 47. Interestingly, after the 8-cell stage, only some of the genes were incorrectly expressed, although the number of zygotic activated genes was significantly increased at the morula and blastocyst stages. Furthermore, we detected that some of the zygotic activated genes were completely repressed (<100 of the RAW value) at the 1-cell ($n=11$), 2-cell ($n=30$), and 4-cell ($n=16$) stages of SeCNT embryos. This observation showed that a number of zygotic activated genes could not be reprogrammed correctly in SeCNT embryos, which suggests that these genes are involved in prenatal and postnatal developmental disorders.

Table 3 Number of networks constructed by the differentially expressed genes (fold change >3).

	1-cell	2-cell	4-cell	8-cell	Morula	Blastocyst
No. of networks	12	21	24	45	15	10
No. of overlapped networks	1	2	1	1	1	2
No. of networks in overlapped networks	9	13, 2	15	40	7	8, 2

Gene network analysis using differentially expressed genes

To understand the interactions of the differentially expressed genes, we performed a gene network analysis at each stage by using IPA: in total, 127 networks were formed (Table 3 and Supplementary Table 5, see section on supplementary data given at the end of this article). The frequency of network descriptions is shown in Supplementary Table 6: the top ten were hereditary disorder ($n=19$), cancer ($n=18$), embryonic development ($n=15$), cell cycle ($n=13$), cellular assembly and organization ($n=13$), molecular transport ($n=13$), developmental disorder ($n=12$), neurological disease ($n=11$), and small-molecule biochemistry ($n=11$). Thus, the IPA network analysis strongly suggested that many genes that are critical to growth and survival were dysfunctional in SeCNT embryos, which resulted in embryo death.

The four networks with extremely high score values of >50 are shown in Fig. 3 and these functions are as follows: 1-cell stage: post-translational modification, organismal injury and abnormalities, and digestive system development and function; 2-cell stage: free radical scavenging, cellular compromise, DNA replication, DNA recombination, and DNA repair; 4-cell stage: post-translational modification, protein degradation, and protein synthesis; and 8-cell stage: cell-to-cell signaling and interaction, tissue development, and cardiovascular system development and function.

Notably, several genes that were integrated into the networks were completely repressed. For example, eight genes, including zygotic activated genes and genes that encode TFs, were completely repressed in the top network at the 2-cell stage (Fig. 3B). Interestingly, it has been reported that the deletion of some of these repressed genes, *Tial1* (Beck *et al*. 1998), *Ilk* (Lange *et al*. 2009), *Hdac2* (Trivedi *et al*. 2007), *Sod2* (Li *et al*. 1995), and *Foxa1* (Kaestner *et al*. 1999), results in prenatal or postnatal death, which suggests that this network might be involved in developmental disorders of SeCNT embryos at the 2-cell stage. Furthermore, these

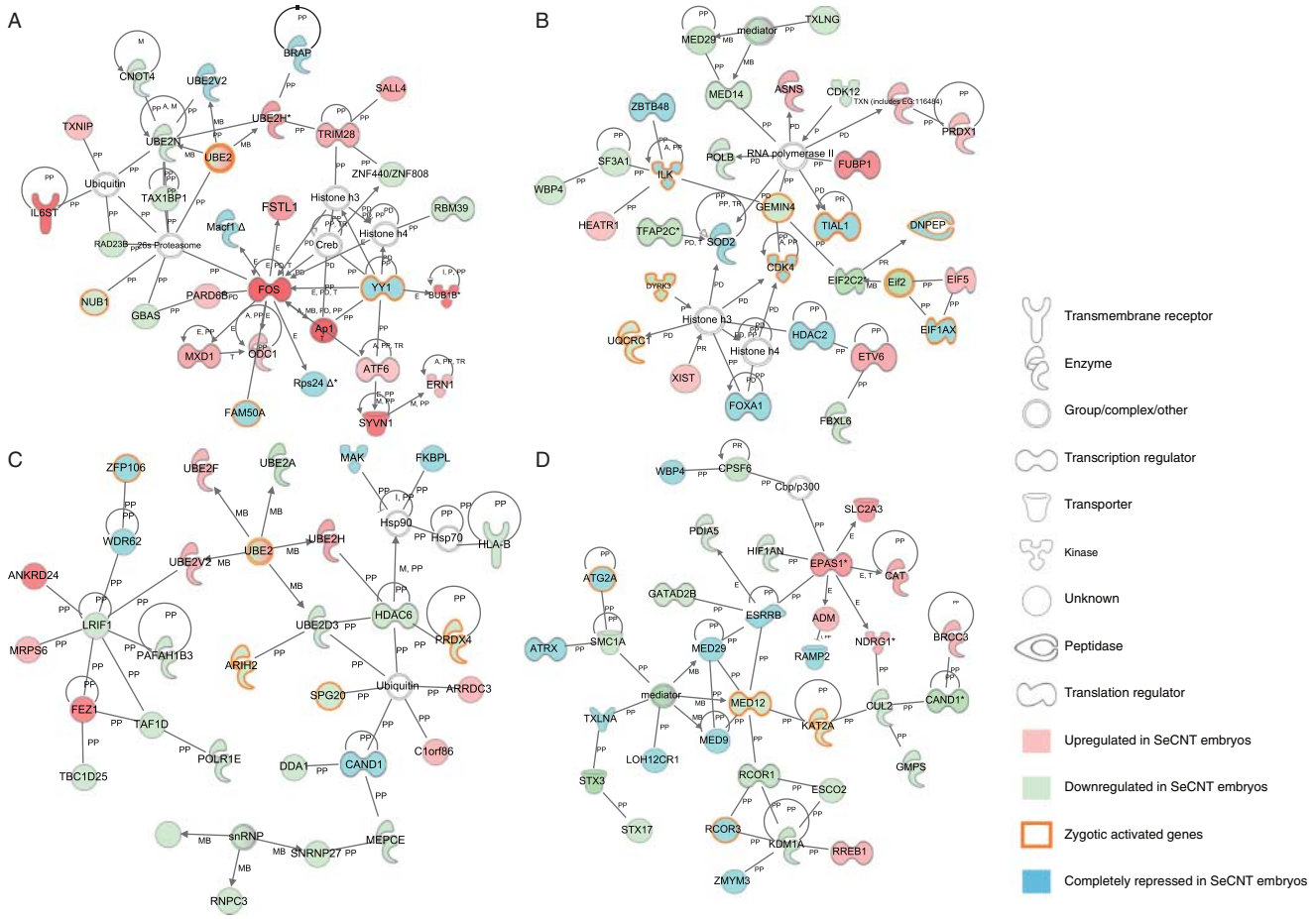


Figure 3 Top gene networks of the differentially expressed genes in SeCNT embryos. The networks were constructed by IPA using the differentially expressed gene list in Supplementary Table 2, fold change >3. Top network of the 1-cell stage (A), 2-cell stage (B), 4-cell stage (C), and 8-cell stage (D) that had a score >50. The score was given by IPA, which is based on the hypergeometric distribution and calculated with right-tailed Fisher's exact test. Green, genes downregulated in SeCNT embryos; blue, genes completely repressed in SeCNT embryos; red, genes upregulated in SeCNT embryos. Orange frames represent the zygotic activated genes.

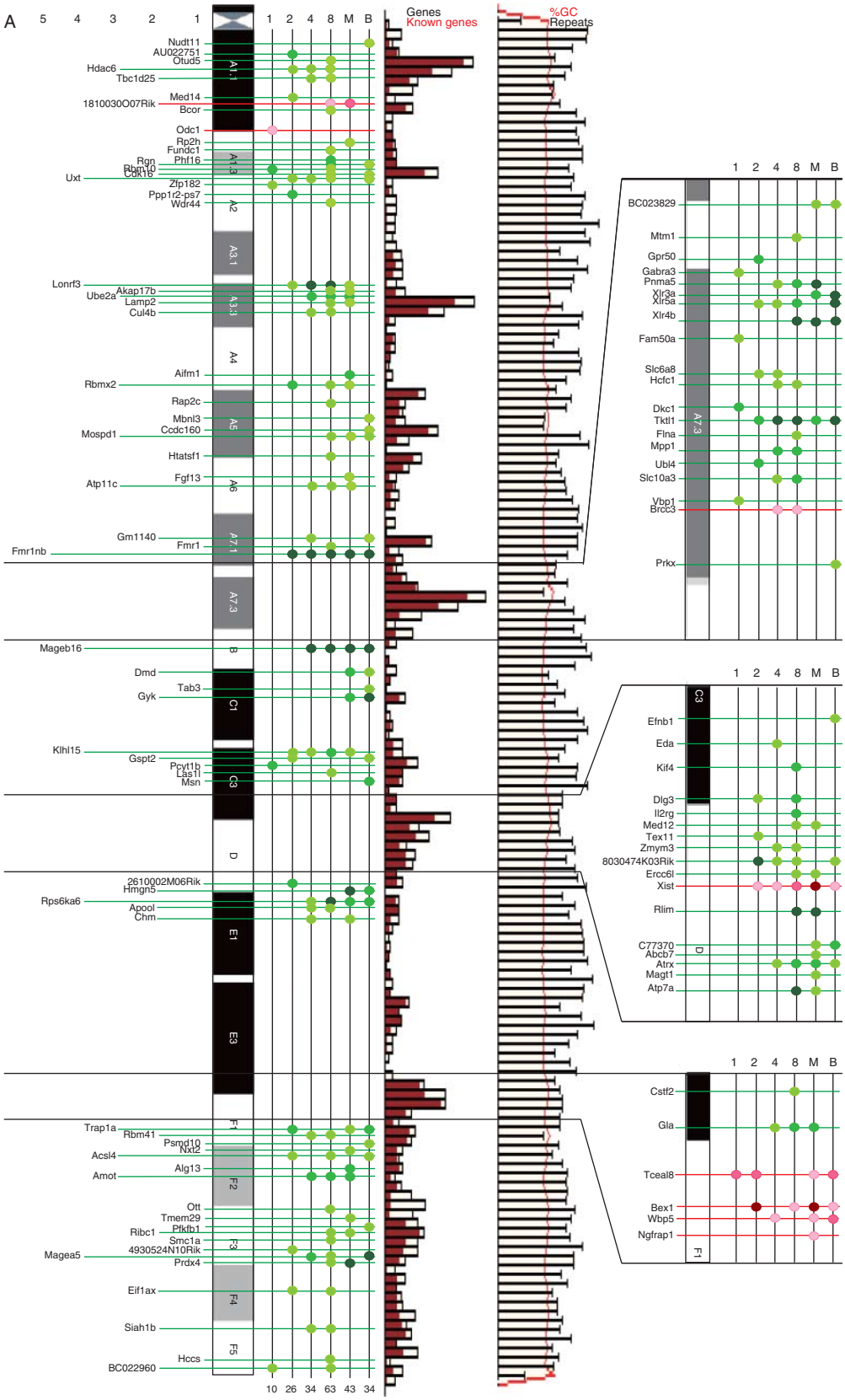
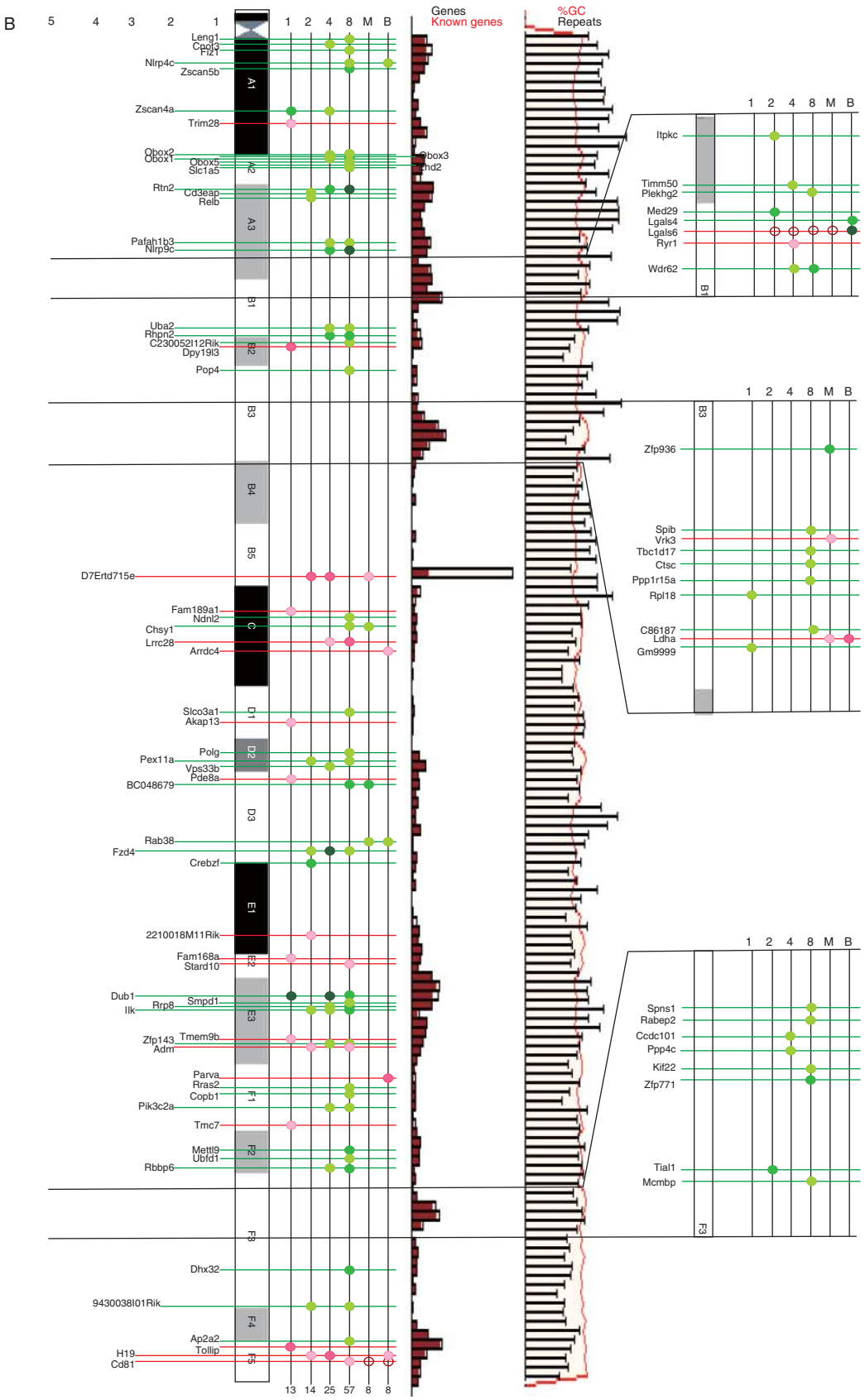


Figure 4 (*figure continued*)



(*legend continued*)

observations provide an opportunity to discuss the possibility that these ectopic gene expressions could be involved in later embryo development. In addition, in total, eight overlapped networks were constructed (Supplementary Figure 1, see section on supplementary data given at the end of this article) and a hugely overlapped network was obtained for the 8-cell stage that consisted of 40 gene networks. This suggests that various functional disorders occur at the 8-cell stage in SeCNT embryos, which leads to a significant proportion of developmental arrest at this stage.

Chromosome maps of differentially expressed genes

To obtain an overview of the global transcriptomic changes in SeCNT embryos throughout preimplantation development, we constructed a map of each chromosome by using the data on the differentially expressed genes (Fig. 4A and B). This analysis showed temporal–spatial changes in the abnormal expressions of the X-linked genes after nuclear transfer. The mapping data of the X chromosome showed that the abnormally reprogrammed genes were concentrated in specific regions, such as the A7.3 and D bands. However, the reason for this is still unclear. Interestingly, *Tceal8*, *Bex1*, *Wbp5*, and *Ngfrap1*, which are located on the pseudoautosomal region in the F1 region of chromosome (Chr) X (Jeppesen & Turner 1993), were upregulated. Matoba *et al*. (2011) reported that the abnormal repression of X-linked genes could be corrected by either gene knockout or siRNA inhibition of *Xist*, which leads to a significant improvement in the survival rate of SeCNT embryos. However, we detected neither a positive nor a negative correlation between these genes and *Xist*. This finding implies that the expression errors that occurred in Chr X genes were not under the control of *Xist* alone and that other factors could have been involved.

Generally, the male Chr X is protected from inactivation and remains active (Barakat & Gribnau 2012). Recent studies have shown that abnormal reprogramming of Chr X inactivation, which is regulated by a non-coding RNA encoded on Chr X called *Xist*, is the main cause of the developmental failure of SCNT embryos (Inoue *et al*. 2010). Interestingly, *Xist* expression was first detectable at the 2-cell stage and lasted until the blastocyst stage in SeCNT embryos (Supplementary Figure 2, see section on supplementary data given at the end of this article). However, its expression was not detected in IVF male embryos throughout development. Because of this abnormal activation of *Xist* in SeCNT embryos, a number of X-linked genes were abnormally downregulated.

The chromosome maps of Chr 7 and others are shown in Fig. 4B and Supplementary Figure 3 respectively. In addition to Chr X, the differentially expressed genes formed many clusters on every autosome. Some chromosomal regions contained large regions that did not show any differentially expressed genes, for example the particularly large regions in Chr 7 (B4–B5, 35 Mb; Fig. 4B), Chr 1 (E1.1–E3.1, 36 Mb), and Chr 14 (D3–E4, 38 Mb) (Supplementary Figure 3). On further examination, we found that some of these regions showed low gene density and a high repeat sequence frequency. Furthermore, other regions, such as the B1–B2.1 region of Chr 6, did not contain any differentially expressed genes despite their high gene density. Therefore, some factors other than gene density were possibly involved in the distribution of the differentially expressed genes. Figure 4B highlights the interesting fact that *Ilk* and *Tial1* on Chr 7, whose knockouts are lethal (Beck *et al*. 1998, Lange *et al*. 2009), were repressed from the 2-cell stage to the blastocyst stage. This suggests that these genes are involved in developmental disorder in SeCNT embryos during the preimplantation stage.

Finally, we focused on imprinted genes, which are expressed from either the paternal or the maternal allele, because aberrant expression and DNA methylation are considered to be the causes of abnormal development in SCNT embryos. To date, the number of imprinted genes that have been identified is over 100, excluding small transcripts. However, only five imprinted genes, *Phf17* (Chr 3), *H19* and *Cd81* (Chr 7), *Impact* (Chr 18), and *Xlr4b* (Chr X), are abnormally expressed during the preimplantation stage; the expression of all the other imprinted genes is established to be after implantation (Dean *et al*. 2001, Kang *et al*. 2001). This result implies that the abnormal expression of many imprinted genes in SCNT embryos is caused by the failure to reverse the methylation of the imprinted genes in DNA methylation regions after implantation. Further comparison between methylome (Kobayashi *et al*. 2012) and transcriptome data may provide insight into the molecular mechanism that underlies reprogramming in SCNT embryos.

Figure 4 Chromosome map views of the differentially expressed genes on Chr X and Chr 7. (A) Temporal–spatial changing aspects of the differentially expressed genes on Chr X. (B) Temporal–spatial changing aspects of the differentially expressed genes on Chr 7. Genes differentially expressed in SeCNT embryos with a greater than threefold change were matching to the chromosome map. Genes that were upregulated in SeCNT embryos are shown as red dots and lines; genes downregulated are shown as green dots and lines. All the location data were mapped by using the database Ensembl (http://asia.ensembl.org). The gradient color from light to dark means a fold change >3, 5, and 10. The bar graph in the middle panel shows the gene density of the chromosome region. Each bar represents $\sim$800 kb long. Black frame represents the number of genes in that region; the red bar represent the number of known genes. The line graph in the right panel shows the GC% and the number of repeat sequences. The red line represents GC% in the region and the black line represents the number of repeat sequences. The magnified panels were five times enlarged from the original chromosome region.

Conclusion

Our study is the first to provide a comprehensive gene expression profile of single SeCNT embryos from the 1-cell to the blastocyst stage, and it provides a temporal–spatial view of the reprogramming mechanisms in preimplantation SeCNT embryos (Supplementary Figure 4, see section on supplementary data given at the end of this article). The present results show that the structural features of a chromosome, which are due to the presence of specific epigenetic modifications, could be responsible for the distribution of the differentially expressed genes, i.e. the formation of high- and low-density domains on each chromosome. Furthermore, the differentially expressed genes may control the changes in epigenetic modifications. The data obtained here suggest that the genes expressed abnormally during the zygotic activation period (around the 1-cell and 2-cell stages) are responsible for early embryo loss both during and after implantation. Stage-specific gene expression regulates the activation of several zygotic genes, and incorrect reprogramming may be potentially responsible for embryo loss during the post-implantation period. Thus, the present study revealed that the SeCNT feign proper preimplantation development; however, the progression of incorrect reprogramming is concealed throughout development.

Materials and Methods

Embryos

Recipient MII oocytes were collected from mature B6D2F1 (C57BL/6NJcl×DBA/2JJcl) female mice after superovulation was induced in these mice. The donor SR cells were obtained from 3-day-old male B6CBF1 mice and harvested, as described previously (Ogura *et al*. 2000). Oocytes IVF with male B6D2F1 sperm were used as control wild types. MII oocytes were collected 14 h after hCG injection. For the 1-cell, late 2-cell, 4-cell, 8-cell, morula, and blastocyst stages, the embryos were cultured for 16, 32, 48, 62, 72, and 84 h after sperm addition or activation.

Nuclear transfer and culture

Sertoli cell-cloned embryos were produced by the injection of a donor nucleus into enucleated oocytes with a piezo-driven system (Prime Tech Ltd, Ibaraki, Japan), by using a previously described method (Ogura *et al*. 2000). The activated cloned embryos were cultured in potassium simplex optimization medium (KSOM) at 37 °C, under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 until use. Each embryo was lysed in 50 μl of buffer RLT containing 1% β-mercaptoethanol (Qiagen).

Microarray and data analysis

Samples of cRNA were amplified according to a previously described modified protocol (Fukuda *et al*. 2010). After the quality of the amplified product was verified by Experion capillary electrophoresis (Bio-Rad), 10 μg of fragmented cRNA were hybridized to a GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA), which contains 45 101 probe sets. Affymetrix data file CEL output by GeneChip Operating Software (GCOS) version 1.3 (Affymetrix) was then loaded into GeneSpring GX 12.0 (Agilent Technologies, Santa Clara, CA, USA) with default normalization: summarization algorithm with RMA (robust M-algorithm) and baseline to the median of all samples. The box plots of all signal values for each sample are shown in Supplementary Figure 5, see section on supplementary data given at the end of this article.

To determine which genes were differentially expressed during each stage, we compared the two sets (IVF vs SeCNT) of data at each stage separately. PCA was employed to analyze the gene expression patterns of all the embryos. Hierarchical clustering was performed with Pearson's correlation analysis for the measurement of similarity and clustering algorithm with average linkage. The genes were investigated by gene ontology analysis using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc., Red Wood, CA, USA). All microarray data are compliant with the Minimum Information About a Microarray Experiment (MIAME). The raw CEL file data have been deposited in a MIAME-compliant database (GEO; http://www.ncbi.nlm.nih.gov/geoprofiles, GSE41358).

The chromosome map views of the differentially expressed genes were constructed by mapping the genes showing threefold or greater changes onto the chromosome maps obtained from the database Ensembl (http://asia.ensembl.org). The chromosome map views constructed were compared with gene density and GC% summaries obtained from the chromosome summary view of Ensembl.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-12-0435.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

F Cao and T Kono conceived the idea and designed the experimental strategy; A Fukuda and H Watanabe produced all embryos for microarray; F Cao and T Kono wrote the paper; and T Kono supervised the study.

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