Incomplete reactivation of *Oct4*-related genes in mouse embryos cloned from somatic nuclei

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SUMMARY

The majority of cloned animals derived by nuclear transfer from somatic cell nuclei develop to the blastocyst stage but die after implantation. Mouse embryos that lack an *Oct4* gene, which plays an essential role in control of developmental pluripotency, develop to the blastocyst stage and also die after implantation, because they lack pluripotent embryonic cells. Based on this similarity, we posited that cloned embryos derived from differentiated cell nuclei fail to establish a population of truly pluripotent embryonic cells because of faulty reactivation of key embryonic genes such as *Oct4*. To explore this hypothesis, we used an in silico approach to identify a set of *Oct4*-related genes whose developmental expression pattern is similar to that of *Oct4*. When expression of *Oct4* and 10 *Oct4*-related genes was analyzed in individual cumulus cell-derived cloned blastocysts, only 62% correctly expressed all tested genes. In contrast to this incomplete reactivation of *Oct4*-related genes in somatic clones, ES cell-derived cloned blastocysts and normal control embryos expressed these genes normally. Notably, the contrast between expression patterns of the *Oct4*-related genes correlated with efficiency of embryonic development of somatic and ES cell-derived cloned blastocysts to term. These observations suggest that failure to reactivate the full spectrum of these *Oct4*-related genes may contribute to embryonic lethality in somatic-cell clones.

Key words: Nuclear cloning, Gene expression, Developmental pluripotency, *Oct4*, Embryogenesis

INTRODUCTION

After historic experiments by Briggs and King (Briggs and King, 1957; Briggs and King, 1952), animal cloning has emerged as a key approach to explore functional changes in the nuclear genome during differentiation (Gurdon, 1974; Gurdon, 1999; Kikyo and Wolffe, 2000; Rideout et al., 2001; Solter, 2000). Studies in amphibians have revealed that the potential of the nucleus to direct normal embryonic development decreases progressively with the developmental stage of the donor cell (Briggs and King, 1960; Gurdon, 1962; Gurdon et al., 1975). Similarly, in mammals, a direct relationship has been observed between the state of differentiation of a donor nucleus and its ability to generate cloned animals upon transfer into the oocyte (Campbell et al., 1996; Solter, 2000; Tsunoda et al., 1987; Willadsen, 1986). This was established most clearly in mouse cloning experiments using donor nuclei from somatic and pluripotent embryonic stem (ES) cells (Eggan et al., 2001; Rideout et al., 2000). Somatic and ES cell clones that develop to the blastocyst stage in vitro develop very differently

in utero after their transfer to recipient animals. As in all other mammals where nuclear cloning has been successful, only a small fraction (1-3%) of mouse blastocysts derived from somatic donor nuclei develop to term, and even fewer reach adulthood (Wakayama et al., 1998; Wakayama and Yanagimachi, 1999; Wakayama and Yanagimachi, 2001). By contrast, up to a third of blastocysts from mouse clones made by nuclear transfer of ES cell donor nuclei develop to birth (Eggan et al., 2001; Rideout et al., 2000). These observations reinforce the notion that a progressive decrease or loss of nuclear potency during cell differentiation is a general characteristic of vertebrate development.

Although as many as 70% of somatic clones can develop to the blastocyst stage in vitro after nuclear transfer, most arrest soon after implantation (Wakayama and Yanagimachi, 1999). Homozygous mutant embryos lacking *Oct4* (*Pou5f1* – Mouse Genome Informatics), a gene that encodes a POU-domain transcription factor that has an essential role in the control of developmental pluripotency, form morphologically normal but developmentally deficient blastocysts (Nichols et al., 1998;

Niwa et al., 2000). In particular, the cells of *Oct4*-deficient embryos fail to form the inner cell mass (ICM) that gives rise to all adult tissues (Nichols et al., 1998). The superficially normal development of *Oct4*-deficient embryos to the blastocyst stage suggests that the combined activity of maternal, zygotic and housekeeping genes might be sufficient to produce a phenocopy of normal preimplantation development. The subsequent failure of embryonic cells in the blastocyst to form the epiblast leads to the manifestation of developmental defects observed in *Oct4*-deficient embryos. The similar developmental phenotypes observed in *Oct4*-deficient and cloned blastocysts suggest that developmental failure in both cases results from the absence of a pluripotent ICM.

We have examined the hypothesis that the limited developmental potency of cloned embryos is a result of incomplete reactivation of genes that, like *Oct4*, function specifically in pluripotent embryonic cells. To test this hypothesis, we have identified 10 candidate genes with an expression pattern similar to *Oct4* (designated as *Oct4*-related genes in this paper) and have compared expression of these genes in normal preimplantation embryos to embryos cloned from somatic cumulus cells and pluripotent ES cells.

MATERIALS AND METHODS

UniGene analysis

Oct4-related genes were identified via electronic analysis of the mouse dataset of UniGene, a regularly updated database of electronically partitioned clusters of expressed sequence tags (ESTs) sequenced from a variety of organisms and their tissues (Schuler, 1997). Previously, electronic analysis of UniGene was used to identify genes whose expression is enriched in the germ cell lineage (Rajkovic et al., 2001). The UniGene mouse dataset can be found at ftp://ncbi.nlm.nih.gov/repository/UniGene. Each individual UniGene cluster is a collection of expressed sequence tags (ESTs) that are grouped together based on sequence similarities. Each EST record is linked to information about the cDNA library from which it was sequenced and its biological source (available at http://www.ncbi.nlm.nih.gov/UniGene/lbrowse.cgi?ORG=Mm). We examined biological source descriptions of all cDNA libraries that contribute to the UniGene mouse dataset and identified 105 cDNA libraries of interest prepared from pre-implantation embryos, gastrulating embryos, embryonic and adult gonads, posterior regions of early and late gestation embryos that also contained developing gonads and germ cells, and ES and embryonic carcinoma (EC) cells. Few cDNA libraries lacking sufficient biological source descriptions were excluded from our analysis.

The UniGene mouse dataset partitions 2.5 million ESTs sequenced from over 500 cDNA libraries into about 88,000 EST clusters. The 105 cDNA libraries of interest to us contributed over 350,000 ESTs to the dataset. Taking these parameters into account, we estimated that a UniGene cluster of six or more ESTs derived from cDNA libraries of interest has a statistically significant chance of identifying a gene specific to tissues containing pluripotent cells. We further restricted electronic selection criteria by requiring that contributing ESTs be derived from at least two distinct stages of development, including one from the preimplantation embryo. This latter criterion was essential to improve selection for genes with a continuous *Oct4*-like expression pattern and to avoid selecting highly expressed genes specific to a particular cell-type or developmental stage, such as genes specifically involved in spermatogenesis or meiosis.

We then used a custom Perl script to electronically identify 102

UniGene clusters that satisfied the selection criteria. EST contigs were assembled using Sequencher 4.1.2 (Gene Codes Corporation). Subsequent sequence analysis and expression analysis by RT-PCR narrowed the number of clusters of interest to 66. We then performed a more detailed RT-PCR-based expression analysis on embryonic and germ cell samples to identify genes with developmental expression profiles closely matching that of *Oct4*. Finally, we assessed the reproducibility of detection of candidate genes in single preimplantation embryos and chose 10 *Oct4*-related genes for further analysis.

Embryonic RNA purification, cDNA synthesis and PCR analysis

Embryos were collected in 100 µl of TRIzol reagent (GibcoBRL) in non-stick, RNAse-free microtubes (Ambion), extracted twice with 20 µl of chloroform using Phase Lock Gel Heavy 0.5 ml tubes (Eppendorf) spun at 5000 g. Total RNA was precipitated with an equal volume of isopropanol in the presence of 1.5 µl of GlycoBlue (Ambion). Residual genomic DNA was removed using DNA-free reagent (Ambion). RNA was re-precipitated, washed in 70% ethanol and dissolved in 2 µl of RNAse-free water (Ambion). First strand cDNA synthesis and universal amplification (four cycles) were performed using the SMART PCR cDNA synthesis kit (Clontech). cDNAs was purified using the QIAquick PCR purification kit (Qiagen), eluted in 100 µl of water. PCR products were resolved in 2% agarose gels.

Primordial germ cell and embryonic tissue isolation

PGCs purified from day 10 (E10) of embryonic development of *Oct4-GFP* transgenic mouse line (A.B. and D.C.P., unpublished) were collected directly into TRIzol reagent using a Becton Dickinson cell sorter. Epiblasts of early gastrulation embryos were dissected cleanly from extra-embryonic tissues. Embryonic gonads were collected from E12 embryos of both sexes by carefully dissecting them from mesonephroi. RNA was prepared from collected tissues using TRIzol reagent and processed for cDNA synthesis as described above.

Production of cloned embryos from cumulus and ES cell nuclei

Nuclear transfer of ES cell and cumulus cell nuclei into enucleated metaphase II oocytes was carried out as previously described (Eggan et al., 2000; Wakayama et al., 1998; Wakayama and Yanagimachi, 2001). Three hours after nuclear transfer, oocytes were activated for 5 hours with 10 mM Sr^{2+} in Ca^{2+}-free CZB media in the presence of 5 µg/ml Cytochalasin B. Embryos were cultured in vitro for 96 hours, preimplantation development was assessed by Hoffman contrast microscopy, and individual embryos were isolated for gene expression studies.

Embryo culture

All embryo culture was carried out in microdrops on bacterial petri dishes (Falcon) under mineral oil (Squibb). HEPES-buffered CZB was used for room temperature operations, while long-term culture was carried out in bicarbonate buffered KSOM at 37°C under an atmosphere of 5% CO_2 in air.

GenBank Accession Numbers

cDNA sequences for examined genes: *Pramel4*, AF490340; *Pramel5*, AF490341; *Pramel6*, AF490342; *Pramel7*, AF490343; *Ndp52l1*, AF490344; *Dppa1*, AF490345; *Dppa2*, AF490346; *Dppa3*, AF490347; *Dppa4*, AF490348; *Dppa5*, AF490349.

Primer sequences and RT-PCR conditions for mouse genes: *Pramel4*, G73527; *Pramel5*, G73528; *Pramel6*, G73529; *Pramel7*, G73530; *Ndp52l1*, G73531; *Dppa1*, G73532; *Dppa2*, G73533; *Dppa3*, G73534; *Dppa4*, G73535; *Dppa5*, G73536; *Ptgs2*, G73537; *Hsh*, G73538; *Tnfsg6*, G73539; *Oct4*, G73540; *Gapd*, G73541.
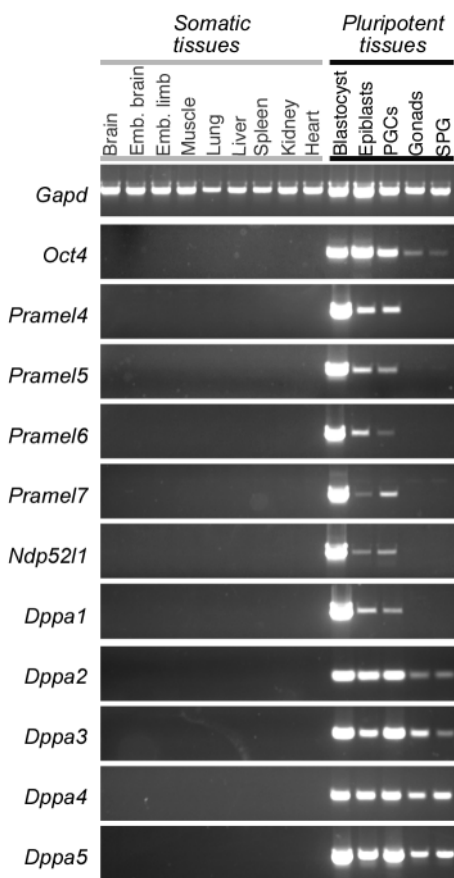
Fig. 1. Expression of *Oct4*-related genes in somatic and embryonic tissues assayed by RT-PCR. Total cellular RNA samples were prepared from (left to right): adult brain, embryonic brain, embryonic limbs, adult skeletal muscles, lung, liver, spleen, kidney, heart, blastocysts (embryonic day E3.5), epiblasts (E7.0), purified primordial germ cells (E10), embryonic gonads (E12) and purified primitive type A spermatogonia (postnatal day 6). *Gapd* served as a ubiquitously expressed control. *Oct4* is expressed in embryonic and germline tissues as described previously (Rosner et al., 1990; Yeom et al., 1996). Control reactions lacking reverse transcriptase or input cDNA showed no amplification products (data not shown). The ability of DNA-free reagent (Ambion) to eliminate residual genomic DNA in embryonic RNA samples was tested extensively in control experiments.

RESULTS

Identification of the *Oct4*-related genes

Oct4 is the best-studied gene essential for the maintenance of a pluripotent state in early embryonic and ES cells (Pesce and Scholer, 2001). *Oct4* encodes a POU-domain transcription factor and is expressed in pluripotent cells of the early embryo and in the embryonic and adult germ cells. We were interested in identifying genes whose expression patterns are like that of *Oct4* and that might be involved in establishing or maintaining developmental pluripotency. We used the following criteria for electronic selection: (1) expression in cleavage-stage embryos, blastocysts and in the embryonic germline, and (2) the genes should not be expressed in somatic cells.

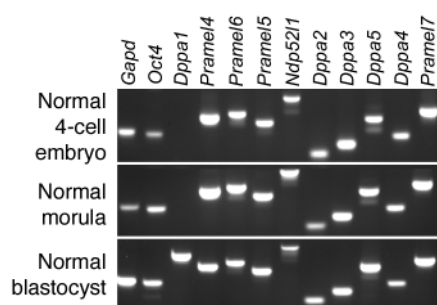An electronic analysis of the UniGene mouse dataset

Fig. 2. Gene expression during normal preimplantation development. Embryos of indicated developmental stages were harvested from pregnant females. Gene expression was assayed by RT-PCR in individual embryos. *Gapd* served as a ubiquitously expressed control. Expression of all test genes is readily detectable from the four-cell stage with the exception of *Dppa1*. *Dppa1* expression is upregulated at the morula-to-blastocyst transition. Control reactions omitting cDNA or reverse transcriptase showed no amplification products (data not shown).

generated a list of 66 candidate genes, ten of which were selected for this study based on their *Oct4*-like temporal expression pattern and reproducibility of detection in single preimplantation embryos (for details see Materials and Methods). Fig. 1 shows that these 10 candidate genes were expressed during cleavage in early epiblasts and germ cell-containing samples, but they were not expressed in any of the somatic tissues tested. Expression analysis of normal four-cell, morula- and blastocyst-stage embryos established that all but one of these *Oct4*-related genes were detectable, beginning with the four-cell stage of development (Fig. 2). Strong expression of *Dppa1* was readily seen at the morula-to-blastocyst transition.

Comparison of predicted ORFs for the 10 genes revealed sequence similarities to previously characterized human and mouse proteins (summarized in Table 1). Of particular interest are four *Oct4*-related genes that encode proteins similar to the PRAME antigen detected in a wide range of human tumors, including melanomas, leukemias, sarcomas and renal carcinomas (van Baren et al., 1998). Two additional mouse Pramel genes, *Pramel1* and *Pramel3*, have previously been shown to exhibit male germ stem cell-specific expression (Wang et al., 2001). Products of three other genes, *Dppa2*, *Dppa3* and *Dppa4*, share a SAP putative DNA-binding motif often found in proteins implicated in chromosomal organization, as well as in various aspects of RNA biology (Aravind and Koonin, 2000). *Dppa3* was described recently as a primordial germ cell (PGCs)-specific gene *PGC7* (Sato et al., 2002), as was *Stella* (Saitou et al., 2002). *Stella* has been implicated in the initiation of the germ cell lineage, and its expression marks the emerging primordial germ cells in the epiblast (Saitou et al., 2002).

The results of expression analysis summarized in Figs 1 and 2 indicate that in silico selection identified a class of genes that were transcribed in pluripotent embryonic but not in differentiated somatic cells. The homologies, expression pattern and limited functional data suggest that these genes may play important roles in early development. We compared the expression of these genes in normal blastocysts to blastocysts cloned from different donor nuclei.

Table 1. *Oct4*-related genes

Gene	Gene name	UniGene ID	Protein features
Pramel4	PRAME-like 4	Mm.21737	Similar to human tumor antigen PRAME (van Baren et al., 1998)
Pramel5	PRAME-like 5	Mm.26222	Similar to human tumor antigen PRAME (van Baren et al., 1998)
Pramel6	PRAME-like 6	Mm.21815	Similar to human tumor antigen PRAME (van Baren et al., 1998)
Pramel7	PRAME-like 7	Mm.6018	Similar to human tumor antigen PRAME (van Baren et al., 1998)
Ndp52l1	Nuclear domain 10 protein 52-like 1	Mm.27779	Coiled coil protein, similar to nuclear domain 10 protein 52 (Korioth et al., 1995)
Dppa1	Developmental pluripotency associated 1	Mm.18119	Signal peptide, single transmembrane domain, similarity to Kim-1 (Ichimura et al., 1998)
Dppa2	Developmental pluripotency associated 2	Mm.27857	Putative DNA-binding motif SAP (Aravind and Koonin, 2000); weak similarity to Dppa3 and Dppa4
Dppa3	Developmental pluripotency associated 3	Mm.27982	Putative DNA-binding motif SAP (Aravind and Koonin, 2000); weak similarity to Dppa2 and Dppa4; also known as Stella (Saitou et al., 2002) and PGC7 (Sato et al., 2002)
Dppa4	Developmental pluripotency associated 4	Mm.35597	Putative DNA-binding motif SAP (Aravind and Koonin, 2000); weak similarity to Dppa2 and Dppa3
Dppa5	Developmental pluripotency associated 5	Mm.139314	Similarity to KH RNA-binding motif (Siomi et al., 1994); also known as retinoic acid downregulated protein Ph34 (Astigiano et al., 1991)

Expression of *Oct4*-related genes in mouse cumulus cell blastocysts cloned from cumulus donor nuclei

For expression analyses, total RNA was isolated from individual control and cloned embryos as well as from cumulus donor cells. Expression of *Oct4* and related genes was not detected in cumulus cells, although sensitive RT-PCR analysis occasionally revealed small amounts of mRNA contamination that were probably the result of oocyte lysis during enzymatic and mechanical disruption of oocyte-cumulus-cell complexes (Fig. 3). Parthenogenetic and in vitro fertilized embryos served as controls for the nuclear transfer procedure and culture conditions. All tested *Oct4*-related genes were expressed in all normal control, in vitro fertilized and parthenogenetic blastocysts (Fig. 2, Table 2 and data not shown).

We examined expression of the *Oct4* and 10 *Oct4*-related genes in a total of 48 individual cumulus cloned preimplantation embryos that developed to phenotypically normal blastocysts, after transfer of a donor cumulus cell nucleus into enucleated oocytes. While each of 15 control embryos derived from fertilized zygotes expressed all 11 genes, 18 of 48 cloned blastocysts failed to correctly reactivate expression of at least one of the 11 test genes (Figs 3, 4). Of the tested genes, *Oct4* was incorrectly expressed more than any other, and 25% of all examined cloned blastocysts failed to express detectable levels of *Oct4*. However, other *Oct4*-related genes also failed to be activated in multiple cloned embryos. For example, *Pramel4* was not expressed in 17% of cloned blastocysts, while *Dppa1* expression was undetectable in 13% of all cloned blastocysts. Overall, 11 out of the 18 (or 62%) clones with incomplete gene reactivation failed to express one gene, while the remaining cumulus clones failed to activate between 2 and 7 of the test genes. Two of the genes, *Pramel6* and *Pramel7*, are transcribed in opposite directions from their respective promoters, which are positioned within 16 kb from each other on chromosome 2 (data not shown). Despite this close chromosomal proximity, we conclude that reactivation of one of the two genes occurred independently of the other, as several blastocysts expressed one but not the other of the two genes (Fig. 4).

Clones that arrested in development before reaching the blastocyst stage were also analyzed. As shown in Fig. 3 and summarized in Fig. 4 and Table 2, only 25% of clones arrested at the morula stage and none of those arrested at the eight-cell stage expressed all 11 tested *Oct4*-related genes.

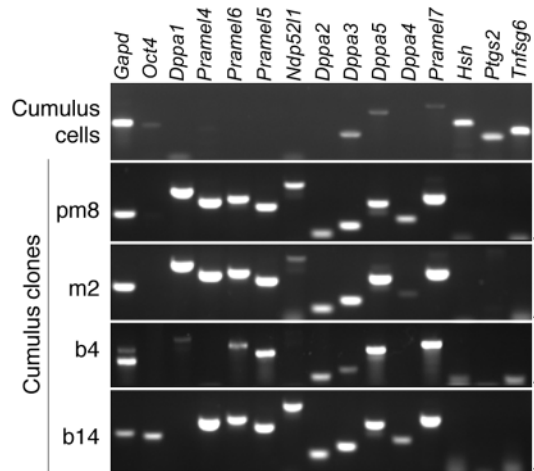We also examined the expression of three genes that are

Fig. 3. Gene expression in cumulus cells and in individual embryos cloned from cumulus cell nuclei. Cumulus cells were enzymatically separated from cumulus cell-oocyte complexes. Cumulus cells exhibit abundant expression of *Hsh*, *Ptgs2* and *Tnfsg6* genes but expression of these genes is not detectable in all cumulus clones regardless of their extent of development in vitro (Fulop et al., 1997a; Fulop et al., 1997b; Joyce et al., 2001). Ghost bands (marked with an asterisk) do not correspond to expected products and are presumed to be PCR artifacts. A small amount of *Oct4*, *Dppa3*, *Dppa5* and *Pramel7* expression detected in cumulus cell sample is likely to be the result of contamination by mRNAs from oocytes damaged during the isolation procedure. *Oct4* expression is clearly limited to the oocyte in the follicle (Pesce et al., 1998). Pre-morula *pm8*, morula *m2* and blastocysts *b4* and *b14* are examples of incompletely reprogrammed cumulus clones. A full summary of gene expression is shown in Fig. 4.
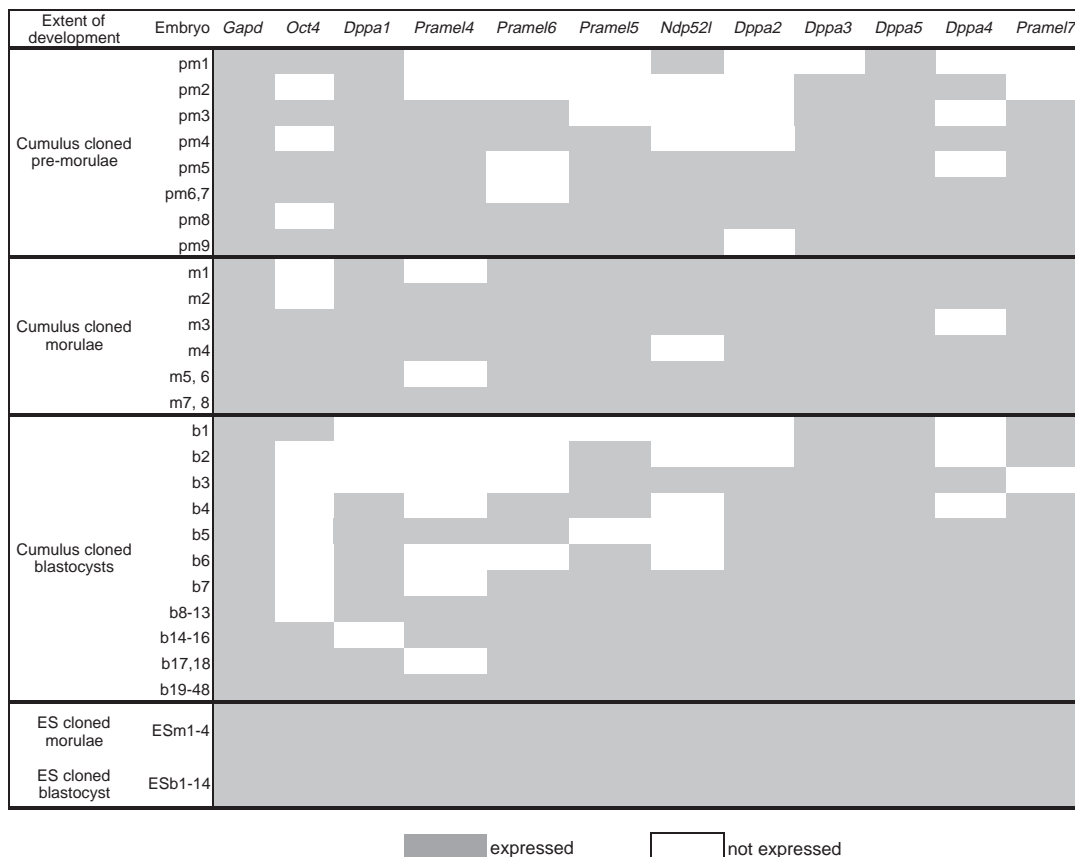
Fig. 4. Summary of embryonic gene expression in all studied clones. Cloned embryos are named according to the developmental stage to which they progressed during 4 days of culture following nuclear transfer (*pm*, pre-morula embryos; *m*, morula; *b*, blastocyst). Investigated genes are shown in columns with gray squares indicating expression and white squares lack of expression of a given marker gene. Embryos within each developmental group are arranged such that the most transcriptionally defective are shown above those with fewer defects. Embryos with identical expression profiles are combined in a single row. Some of the primary data are shown in Figs 3 and 5.

active in the cumulus donor cells but not in normal blastocysts. These genes are prostaglandin-endoperoxide synthase 2 (*Ptgs2*) (Joyce et al., 2001), hyaluronan synthase homologue (*Hsh*) (Fulop et al., 1997b) and TNF-stimulated gene 6 (*Tnfsg6*) (Fulop et al., 1997a). In contrast to incomplete reactivation of the *Oct4*-related genes, all cumulus cell-derived

clones uniformly lacked expression of the three 'somatic' genes regardless of the extent of development in vitro (Fig. 3).

Expression of *Oct4*-related genes in mouse ES cells and ES cell-derived cloned embryos

The hypothesis that incomplete reactivation of the *Oct4*-related

Table 2. Expression of *Oct4*-related genes in cloned and control embryos

Embryo type	Developmental stage after 4 days of culture	Number of embryos studied	Percent of embryos with all 11 tested genes activated
Cumulus clones	Pre-morula	9	0%
	Morula	8	25%
	Blastocyst	48	62%
ES cell clones	Pre-morula*	N/A	N/A
	Morula	4	100%
	Blastocyst	14	100%
Control embryos	Four-cell embryo[†]	6	100%
	Morula[†]	6	100%
	Blastocyst[†]	7	100%
	Parthenogenetic blastocysts	22	100%
	In vitro fertilized blastocysts	8	100%

*Up to 85% of ES clones arrest as one to two-cell embryos probably because of cell cycle incompatibility with the oocyte. The remaining 15% of ES cell clones progress to morula and blastocyst stages of development. Therefore a pre-morula class of four- to 16-cell embryos is missing among the ES cell-derived clones.

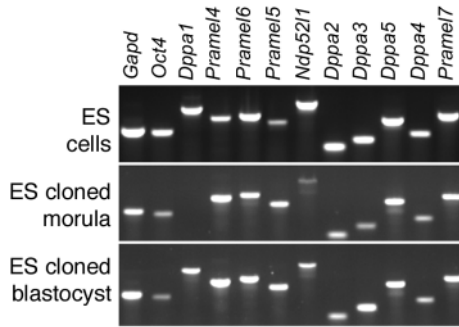[†]Control embryos collected at indicated stages of development.

Fig. 5. Gene expression in ES cells and in individual cloned embryos from ES cell nuclei. *Oct4* and *Oct4*-related genes are abundantly expressed in the donor ES cells and in ES cell nuclei-derived clones. Note that although *Dppa1* gene is strongly expressed in ES cells, it is initially downregulated in ES cell cloned morulae before re-activation in the blastocyst stage ES clones. This pattern of *Dppa1* expression is reminiscent of its expression during normal embryogenesis (see Fig. 2). A full summary of gene expression is shown in Fig. 4.

genes may contribute to the early lethality of somatic clones predicts that these genes should be expressed faithfully in blastocysts derived from nuclei of donor cells that are expressing these genes. To test this possibility, we examined expression of the 11 test genes in donor ES cells and in individual ES cell clones. As expected from the electronic selection criteria, all tested genes were expressed in undifferentiated ES cells (Fig. 5). In contrast to cumulus donor cell-derived clones, all ES cell nuclei-derived clones expressed all eleven tested genes (Figs 4 and 5, Table 2 and data not shown). In addition, unlike somatic clones, ES cell clones correctly recapitulated temporal regulation of the *Dppa1* gene. Like normal embryos in which *Dppa1* expression is upregulated at the morula-to-blastocyst transition (Fig. 2), *Dppa1* was expressed in blastocyst-stage ES clones but not in morula-stage clones (Fig. 5). Thus, the ES cell-derived clones appear to have faithfully recapitulated expression of the *Oct4*-related genes with a similar kinetics to the normal embryos.

DISCUSSION

In this study, we explored the biological causes of early embryonic lethality observed in somatic cell clones. Based on the similarities between the previously described phenotypes exhibited by *Oct4*-deficient embryos and somatic cloned blastocysts, we posited that somatic clones fail because of incomplete reactivation of genes functioning in the pluripotent cells of the preimplantation embryo. The goal of this study was to identify genes that can serve as suitable molecular markers for developmental pluripotency, and to examine their expression in both normal and cloned embryos derived from differentiated somatic and undifferentiated cells.

We used an in silico selection method to identify a class of embryonic *Oct4*-related genes whose expression is limited to the pluripotent cells of the early embryo and the germline. Although the biological functions of these genes have yet to be elucidated, we have found that they serve as informative markers for pluripotent cell types and thus can be validly used

in the analysis of cloned embryos. The electronic selection that led to the identification of this set of genes was modeled after the properties and expression pattern of the *Oct4* gene, which has been shown to have an essential role in early development. Recently, our observations have been corroborated by the results of an expression profiling study that identified *Dppa2*, *Dppa4*, *Dppa5* and *Oct4* among genes found at high levels in mouse ES cells (Ramalho-Santos et al., 2002). In addition, expression of the *Oct4*-related genes is downregulated during ES cells differentiation (data not shown) in strong agreement with a prior description of *Dppa5* as a cDNA downregulated during EC cell differentiation (Astigiano et al., 1991). Finally, one of the *Oct4*-related genes, *Dppa3*/*Stella*/*PGC7*, has been implicated in the earliest steps of germline initiation, further strengthening the argument that these genes might represent factors with specific roles in early development (Saitou et al., 2002).

Analysis of expression of the *Oct4*-related genes in somatic and ES cell-derived clones revealed that successful reactivation of the full set correlates with development of cloned embryos to term. Mouse clones derived from nuclei of ES cells, where the genes are already active, recapitulate their expression pattern with the same kinetics as observed during normal embryogenesis. In contrast to ES clones, almost 40% of the cumulus cell-derived blastocysts, though morphologically normal, failed to reactivate these genes faithfully. Activation of these genes in embryos that were arrested at the pre-blastocyst stage was even less efficient. The significance of this result is difficult to assess as impaired viability and earlier developmental arrest of these embryos might be have been caused by physical damage after nuclear transfer, leading to RNA degradation.

Incomplete reactivation of Dppa genes in cumulus clones strongly agrees with a recent independent study of *Oct4* expression in somatic clones (Boiani et al., 2002). In that study, 82% of clones reactivated *Oct4*. In situ mRNA analysis also revealed that some of these *Oct4*-positive clones exhibited aberrant *Oct4* expression in the trophoectoderm. These findings suggest that somatic clones capable of reactivation of *Oct4* may also fail to reproduce its normal spatial pattern of expression. Our work confirms these prior findings and attributes incomplete recovery of pluripotency in somatic clones to a larger set of specific genes.

Our observations could plausibly reflect a more profound transcriptional dysregulation in cumulus cell-derived cloned embryos. Future experiments using single-embryo expression profiling on microarrays will examine the entirety of mouse transcriptome and reveal which genes consistently fail to be expressed in somatic cell-derived cloned embryos. We emphasize, however, that all examined cumulus cell-derived cloned blastocysts expressed *Gapd*, while the expression of three genes, *Ptgs2*, *Hsh* and *Tnfsg6*, which are normally abundantly expressed in donor cumulus cells, was undetectable in cloned embryos (Fig. 3). These observations indicate that mechanisms controlling gene expression in cumulus cell-derived clones selectively affect whether housekeeping and 'somatic' genes are expressed. By contrast, they can only achieve incomplete reactivation of expression of *Oct4* and 10 *Oct4*-related genes.

Why somatic clones fail to reactivate the *Oct4*-related genes remains to be determined. It is now generally accepted that

epigenetic mechanisms play important roles in the control of gene expression during embryogenesis (Reik and Walter, 2001; Renard, 1998; Rideout et al., 2001; Surani, 2001). However, little is known about the molecular mechanism of epigenetic reprogramming in the context of normal development and somatic cloning. Recently, initial studies of bulk genomic DNA methylation in normal and cloned embryos have been reported (Barton et al., 2001; Bourc'his et al., 2001; Dean et al., 2001; Fairburn et al., 2002; Kang et al., 2001; Mann and Bartolomei, 2002; Santos et al., 2002). These studies have revealed significant differences in the pattern and dynamics of DNA methylation between normal and cloned embryos. However, current approaches for analysis of epigenetic modifications in single pre-implantation embryos have significant limitations. Importantly, they lack the resolution to allow straightforward identification of individual genes whose expression and epigenetic make-up could be meaningfully correlated with the development of clones. In this regard, the *Oct4*-related genes identified in our work represent a set of loci whose epigenetic control should be examined in the future. Such analysis might provide specific examples of the role of epigenetic control of gene expression in the development of cloned and normal embryos.

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