Gene Expression and Imprinting in Mice Cloned by Nuclear Transfer

by

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B.S. Biology

Harvey Mudd College, 1994

Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Biology

at the

Massachusetts Institute of Technology

June 2002

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Acknowledgements

I'd like to thank all of the members of the Jaenisch lab whom I've worked in the company of through the years in the lab as well as many others in the MIT/Whitehead community who have made this experience a rewarding one. In particular, I'd like to thank Laurie Jackson-Grusby and Jessie Dausman who helped train me as first I endeavored to set up experiments in the lab. Later, Britt Rideout, Kevin Eggan, and Konrad Hochedlinger were all instrumental to my success though discussions and were extremely generous with reagents and moral support. I'd also like to thank Doug Menke for advice on other projects in my time here. Additonally, Kevin Eggan and Hidenori Akutsu toiled to make many clones which I was fortunately able to examine. Without their efforts I would have been unable to even begin most of the work that comprises this thesis. I'd also like to thank David Page and David Housman for guidance while on my thesis committee. Finally, I'd like to thank Rudolf Jaenisch for conveying a constant enthusiasm and passion for science and for his support, patience, and guidance through the years.

Table of contents

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ABSTRACT

Most cloned mammals derived by nuclear transfer (NT) die during gestation, and those surviving to birth frequently show increased birth weights, enlarged dysfunctional placentas, and neonatal mortality associated with respiratory distress and metabolic abnormalities. These abnormalities may reflect inadequate reprogramming of the donor nucleus to a state fully compatible with normal development. As inadequate reprogramming should lead to abnormal gene regulation in the clones, we have examined gene expression in clones to determine the extent of dysregulation and whether particular genes are commonly affected in clones.

Abnormal imprinted gene expression has been proposed as a likely cause for some phenotypes observed in clones. Imprinted genes frequently affect fetal growth and some phenotypes similar to those seen in cloning are found upon *in vitro* embryo culturing, a process known to affect the expression of some imprinted genes. Because imprinting is normally established in the gametes and maintained in the early embryo, imprinted genes are likely to be resistant to reprogramming after nuclear transfer. We have examined imprinted gene expression in neonatal cloned mice derived from embryonic stem (ES) cells. In ES cell NT mice and their placentas, we found that many imprinted genes had abnormal gene expression levels. Similar expression abnormalities were observed upon *in vitro* differentiation of the ES cell donor populations from which the NT mice were derived. Additionally, mice cloned from the same ES cell subclone showed significantly varying imprinted gene expression suggesting that the epigenetic state of the ES cell

genome was unstable. However, the abnormal expression of any given imprinted gene did not correlate with abnormal expression at any other loci or with the various cloning phenotypes. Thus, imprinted gene expression appeared stochastically affected in the clones and some abnormalities in gene expression could be traced to the donor ES cell populations.

To determine whether abnormal gene expression extended beyond the examined imprinted genes, we assessed global gene expression by microarray analysis on RNA from the placentas and livers of neonatal cloned mice. This analysis allowed us to determine whether differences in gene expression existed between different types of donor cells. Direct comparison of gene expression profiles of over 10,000 genes in ES cell and cumulus cells NT mice showed that in clones derived from both donor cell types approximately 4% of the expressed genes in the placentas differed dramatically from those in controls and that the majority of abnormally expressed genes were common to both types of clones. Importantly, however, the expression of a smaller subset of genes differed between the ES cell and cumulus cell derived clones. The livers of the cloned mice also showed abnormal gene expression although to a lesser extent, and with a different set of affected genes, than seen in the placentas. Our results demonstrate frequent abnormal gene expression in clones, in which many expression abnormalities appear common to the nuclear transfer procedure while others appear to reflect the particular donor nucleus. Surprisingly, many clones survived neonatally despite widespread gene dysregulation, indicating that mammalian development can tolerate many epigenetic abnormalities.

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Chapter 1.

Introduction

Nuclear Cloning

Nuclear cloning can be defined as the production of a new adult organism with the same nuclear genome as a donor organism. To generate a clone, the genome of a donor cell must be transferred to an oocyte which has had its own genome removed, and then proceed through development. In order for a donor nucleus of a differentiated cell to support development in a clone, it must be "reprogrammed" to a state compatible with embryonic development. The transferred nucleus must properly activate genes important for early embryonic development and also adequately suppress differentiation-associated genes that had been transcribed in the original donor cell. An understanding of the extent to which a donor nucleus can be reprogrammed following nuclear transfer has become a central issue for nuclear cloning. In this chapter, I will first review the advances in nuclear cloning and the phenotypes of cloned animals and then discuss the genetic and epigenetic modifications observed in cloned animals compared to normally developing animals.

Historical work in amphibian and mammalian cloning

Early nuclear transplantation experiments were designed to test whether nuclei become irreversibly changed in the course of development. To test this "nuclear

differentiation" theory, early work sought to transfer the nucleus of an irreversibly differentiated cell into an enucleated oocyte. In 1952, Briggs and King found that transfer of undifferentiated blastula nuclei into enucleated frog oocytes could support development to normal larva (Briggs and King, 1952) demonstrating the feasibility of nuclear transplantation.

Following their initial cloning experiment, Briggs and King subsequently used endodermal donor nuclei from progressively later stages of development: late gastrula, mid-neurula, and post neurula and found that nuclei from more advanced stages had a decreased potential to support clones reaching cleavage and blastula stages (Briggs and King, 1957). In 1966, Gurdon reported that fertile adult clones had been generated by nuclear transfer of intestinal epithelium cells of tadpoles (Gurdon and Uehlinger, 1966). Because the cells of the intestine were heterogeneous and presumably included stem cells it could not be concluded whether the live clones developed from nuclei of terminally differentiated cells. Later experiments attempted to clarify the identity of nuclear donors by using cells with known morphologies or that expressed markers characteristic of terminal differentiation. For example, nuclear transfer was performed with adult skin cells expressing keratin(Gurdon et al., 1975), lymphocytes expressing immunoglobulins(Du Pasquier and Wabl, 1977; Wabl et al., 1975) and erythrocytes(Brun, 1978). However, none of these experiments yielded embryos developing beyond the larval stage and only a small percentage (at most 6%) of the embryos developed that far. While clones derived from adult erythroblasts yielded larva, those derived from erythrocytes did not; however, erythrocyte donors from juvenile frogs did support development to the feeding tadpole stage(DiBerardino et al., 1986). While

these experiments indicate that nuclei of differentiated cell types could support extensive development of clones, in no case was it shown that adult frogs could develop from a transplanted adult nucleus.

One approach for improving cloning efficiencies has been to expose the donor nucleus to oocyte cytoplasm for a longer period of time following nuclear transfer, for example, by performing a second round of nuclear transfer from cloned one-cell embryos or from blastomeres of clones into a new recipient enucleated egg or zygote. These procedures ensure that the original donor nucleus has more time to be reprogrammed within the context of a very early oocyte or embryo, before being transferred to the ultimate oocyte. In 1962, Gurdon observed that by transferring nuclei of abnormal cloned embryos to new recipient oocytes, the subsequent clones developed further(Gurdon, 1962). A similar developmental enhancement was observed by first transferring erythrocyte nuclei to metaphase I oocytes, allowing development to metaphase II, activating the eggs, removing the original oocyte nucleus, and allowing development for another day before transferring the nuclei again (DiBerardino and Hoffner, 1983).

The conclusion from these amphibian cloning experiments was that nuclear developmental potential is restricted during development. Nevertheless, adult clones could be derived from larval stage nuclei. However, the differentiation status of the nuclei giving rise to adult clones was ambiguous. When donor nuclei were known to be from terminally differentiated cells or adult cells, development of clones to adults was not observed. The serial transfer experiments indicated that the developmental potential of

donor nuclei could be improved by an additional round of nuclear transfer, possibly by allowing more time for the nuclei to be reprogrammed in the oocyte and early embryo.

Some of the earliest nuclear transplantation studies in mammals used rabbits, with the nuclear donor being incorporated into oocytes with an intact nucleus, resulting in early tetraploid embryos after either micro-injection of morula nuclei or fusion with blastomeres (Bromhall, 1975). The first report of cloned mice that survived to term described nuclear transfer (NT) of nuclei from inner cell mass (ICM) cells into enucleated zygotes (Illmensee and Hoppe, 1981); however, attempts to repeat these experiments have been unsuccessful(McGrath and Solter, 1984b; Wakayama et al., 2000b). Subsequently, NT of donor nuclei from 8- to 16-cell sheep embryos into enucleated oocytes, arrested at metaphase II, produced live clones (Willadsen, 1986). In 1994, the first live clones were produced from donor cells of a cultured cell line (Sims and First, 1994) (derived from bovine ICM) and later from an established sheep cell line(Campbell et al., 1996).

More recently, mammalian serial nuclear transfer has been described. These experiments have been performed in the mouse for blastomere, embryonic fibroblast, and ES cell donors (Tsunoda and Kato, 1997) (Ono et al., 2001a; Ono et al., 2001b). In these experiments, improvements in cloning efficiencies seemed modest. Multiple rounds of nuclear transfer from early embryos up to the fifth transfer have generated cloned offspring in the goat(Yong and Yuqiang, 1998); however, there is no compelling evidence that these multiple rounds of nuclear transfer have improved cloning efficiency in mammals(Peura et al., 2001), possibly because they add more technically challenging steps.

Mammalian cloning successes from adult somatic cell donors

The first evidence that the nucleus of an adult cell could support development of a cloned animal to birth was the creation of the lamb Dolly in 1997 from the nucleus of a cell population derived from an adult mammary gland (Wilmut et al., 1997). Since then, an additional six mammalian species have been cloned from adult cells: cattle (Kato et al., 1998), goats (Baguisi et al., 1999), pigs (Onishi et al., 2000) (Polejaeva et al., 2000), mice (Wakayama et al., 1998), a cat(Shin et al., 2002), and rabbits (Chesne et al., 2002). In all of these examples, only a small percentage of the manipulated embryos survived until birth and some clones died perinatally, frequently as a consequence of cardiovascular and placental abnormalities. Cloned animals surviving through the first several days after birth have generally developed to puberty at a high frequency(Cibelli et al., 2002). However, recent reports suggest that clones surviving until later in adulthood are still abnormal(Ogonuki et al., 2002; Tamashiro et al., 2002). Thus, while it is possible for adult clones to be derived from adult donor cells, reprogramming rarely appears sufficient to support development to birth and may not ever be complete.

Embryonic versus somatic donors in mammals

As previously mentioned, cloning in amphibians had suggested a decreased developmental potential of clones derived from the nuclei of more differentiated cells(Briggs and King, 1957). Sufficient numbers of cloned mice have been generated with ES cell and somatic cell donors to make a comparison of their developmental rates(Wakayama et al., 1998; Wakayama and Yanagimachi, 1999) (Eggan et al., 2001;

Rideout et al., 2000; Wakayama et al., 1999). The cloning efficiency when using ES cell donor nuclei in comparison to somatic cell donor nuclei was about ten times higher when comparing development from the blastocyst stage to adults, 15% vs 1-2% (see Table 1). This striking increase in development rate suggests that less reprogramming is needed for nuclei of embryonically derived cells and that reprogramming is important for postimplantation development. However, the rate of development of ES cell derived clones to the blastocyst stage was actually lower (10-20% for ES cell donors versus 70% for cumulus cell donors). This is not thought to be a consequence of reprogramming. Instead, it is believed to reflect the high percentage of ES cell donors in S phase, a state that appears incompatible with cloning due to premature chromatin condensation caused by high levels of maturation(or M phase)-promoting factor (MPF) present in the recipient oocyte. MPF is a cyclin B-cdc2 kinase complex that induces nuclear envelope breakdown and chromatin condensation(Newport and Kirschner, 1984).

Given the low efficiency of cloning from somatic cells it is possible that the small number of surviving clones arise from a particular subset of cells, such as somatic stem cells present in adult tissues, that might be more amenable to reprogramming following nuclear transfer. The exact identity of the nuclei giving rise to the surviving clones has generally been ambiguous. However, it has been demonstrated that differentiated cell types, specifically B and T cells, are capable of being reprogrammed to give rise to an entire adult animal (Hochedlinger and Jaenisch, 2002). Consistent with the idea that a truly differentiated cell nucleus might be more difficult to reprogram, the cloning efficiencies in this experiment were lower than for previously reported somatic cloning

attempts and only by using a cloned ES cell intermediate in tetraploid embryo complementation were live clones generated(Hochedlinger and Jaenisch, 2002).

	Developmental rate	
Donor cell nucleus	Activated oocyte to blastocyst	Blastocyst to adult
ES cell	10-20%	15%
Cumulus cell	70%	1-2%

Table 1. Approximate developmental rates of ES cell and cumulus cell nuclear transfer embryos through development. A substantially greater percentage of ES cell NT animals than cumulus cell NT animals survive after embryo transfer. The reduced efficiency of ES cell nuclei in supporting development to the blastocyst stage is likely due to a greater percentage of ES cells in S-phase (Eggan et al., 2001; Rideout et al., 2000; Wakayama et al., 1998; Wakayama et al., 1999). Adapted from (Rideout et al., 2001).

Technical considerations

Before discussing other biological limitations in cloning, it is worth noting that cloning inefficiencies may to a certain extent reflect technical difficulties. In order to enucleate the recipient oocyte, the zona pellucida must be pierced and the chromosome-spindle complex aspirated completely without damaging the remaining oocyte cytoplasm. Then, the donor nucleus must be transferred alternatively by either i) direct injection of a nucleus, which had been mechanically separated from the donor cell, or ii) by placing the donor cell adjacent to the enucleated oocyte and fusing them via an electric pulse or an

inactivated Sendai virus. The manipulated egg must also be chemically or electrically activated to trigger further development. Furthermore, *in vitro* culturing of the oocytes, manipulated embryos, and the donor cells themselves may affect cloning efficiencies.

Status of donor nucleus

Besides the differentiation status of the donor nucleus, the effects of a number of other donor cell characteristics have also been examined. It had been posited that successful cloning requires the donor cell be in a quiescent G_0 state(Wilmut et al., 1997). However, since the creation of Dolly, clones have also been generated from non-quiescent donor cells (Cibelli et al., 1998) in a number of species, including cells arrested at metaphase with inhibitors of tubulin polymerization, such as nocodozole or colcemid (Wakayama et al., 1999) (Ono et al., 2001b) (Zhou et al., 2001).

The genetic background of the donor cell has been shown to play an important role in cloning efficiency, in particular for neonatal survival. Attempts to produce live cloned mice from inbred donor ES cell lines have almost invariably failed, in contrast to donor F1 ES cell lines (Eggan et al., 2001). Inbred cumulus cell donors have been successfully used as nuclear donors; however, the percentage of surviving clones is consistently reduced when using inbred rather than F1 somatic donor nuclei(Wakayama and Yanagimachi, 2001).

Mitochondrial contribution

Since reproductive nuclear cloning is perceived of as a means to create a genetically identical copy of an individual, it is worth noting that since eukaryotic cells

contain a cytoplasmically transmitted mitochondrial genome in addition to their nuclear DNA, the mitochondrial genome of a clone will likely be heteroplasmic and primarily be inherited from the recipient oocyte. The mitochondrial genome encodes 13 proteins, 22 tRNAs, and two rRNAs, which are important to oxidative phosphorylation and electron transfer(Attardi et al., 1986) and mutations in the mitochondrial genome are known to cause human diseases(Brown and Wallace, 1994). Even in the case of nuclear transfer by cell fusion, as opposed to the direct nuclear injection, it appears that the mitochondria of clones are nearly completely derived from the oocyte rather than the donor cell(Evans et al., 1999; Meirelles et al., 2001; Steinborn et al., 2000) except in cases of blastomere donors, likely as a consequence of their large cytoplasms (Steinborn et al., 1998) (Hiendleder et al., 1999). Thus, heteroplasmy is unlikely to account for the abnormal phenotypes in clones.

Overgrowth in cloned animals

Cloned animals frequently display phenotypes reminiscent of "large offspring syndrome" (LOS) , a term given to the frequently elevated birth weights and other characteristics following development of embryos in unusual environments, including: *in vitro* embryo culture, asynchronous embryo transfer, and a maternal diet high in urea(Young et al., 1998). In addition to increased birth weight, LOS is also characterized by increased gestation time, breathing difficulties, reluctance to suckle, perinatal death, altered metabolism, and gross abnormalities of several organs(Young et al., 1998).

One of the most frequently observed phenotypes in cloned mice is placental overgrowth. Placentas of cloned mice have been reported to have an average weight of

two to three times that of control mice when using either somatic or ES cell donor nuclei (Eggan et al., 2001; Wakayama and Yanagimachi, 1999). Histological examination and transcriptional expression patterns in term mouse placentas suggest that cloned placentas have an enlarged spongiotrophoblast layer and an increase in the number of glycogen producing cells(Tanaka et al., 2001). Moreover, there was disorganization between the spongiotrophoblast layer and labyrinth layer, and red blood cells frequently accumulated in placentas of clones. A range of severe placental abnormalities have also been observed in cloned bovine (Hill et al., 2000; Hill et al., 1999) and sheep(De Sousa et al., 2001) fetuses. The placental phenotypes seen in earlier stages of gestation are consistent with many cloned embryos dying throughout gestation and only a minority of clones developing functional placentas.

The severity of placental abnormalities compared to embryonic defects may reflect development of the trophectoderm (TE), which gives rise to the placenta, and as the first functionally specified lineage offers the least time for reprogramming. Placental phenotypes may also contribute directly to embryonic phenotypes of clones. These cloning phenotypes also implicate growth regulating genes as being inadequately reprogrammed. For this reason and others discussed later in the chapter, imprinted genes represent likely candidates for abnormal gene expression.

Health of aging clones

Since so few clones survive to birth, the question remains whether the survivors are normal or merely the least severely affected animals, making it to adulthood despite harboring subtle abnormalities. Given the long generational time of most animal species

cloned to date, the long-term effects of cloning have been difficult to assess. Only recent reports in mice have begun to adequately address the health of adult clones. The first evidence that cloned animals still retain abnormalities capable of causing severe health consequences came from an examination of mice cloned from Sertoli cells that, in comparison to normally developing controls of the same sex and background, had reduced lifespans and frequent pneumonia and hepatic failure(Ogonuki et al., 2002). Additionally, mice cloned from cumulus cell donor nuclei were obese with increased body fat and size(Tamashiro et al., 2002). As this phenotype was not passed on to the offspring of the clones, obesity is unlikely to reflect any genetic change in the clones but instead to reflect epigenetic abnormalities arising from either inadequate nuclear reprogramming or the cloning procedure itself.

Bovine clones have been reported to reach puberty later and at greater weights than controls(Enright et al., 2002). However, the largest set of sexually mature cloned cattle examined to date revealed no gross abnormalities by physical examination or by blood and urine chemistry (Lanza et al., 2001).

In contrast to the murine studies, the bovine studies cannot yet assess clones at later stages of their respective lifespans. Because the mouse clones derived from Sertoli cells, unlike those derived from cumulus cells, did not display obesity it raises the possibility that some reprogramming errors may be specific to the nuclei of given donor cells. The studies in mice provide strong evidence for the notion that all clones are abnormal and illustrate the potential dangers of human cloning.

Genomic Reprogramming

Normal embryonic development of clones is presumably dependent on both silencing of tissue specific gene expression of donor cells as well as proper activation of early embryonic genes. Here, I will review the reprogramming events that occur in normal development during gametogenesis and after fertilization. It is important to note that pre- and post-zygotic reprogramming occurs and that cloned nuclei are only exposed to post-zygotic reprogramming machinery. I will also describe experiments examining reprogramming in cloned animals.

Epigenetic status and maturation of gametes

A zygote is the product of an egg fertilization by a sperm, each bearing a genome in a very specific epigenetic state. A large number of epigenetic modifications are made in the sperm and oocyte during gametogenesis and any given donor nucleus that is transferred during cloning would thus likely contrast in many functionally important ways with the epigenetic states normally observed in a zygote.

Early in germ cell development, primordial germ cells (PGCs) reactivate the X-chromosome (in females) (Monk and McLaren, 1981; Nesterova et al., 2002), demethylate their genome(Monk et al., 1987), and erase allele specific imprints(Szabo and Mann, 1995). The genome then becomes largely re-methylated in both germ lines by embryonic day (E)18.5 (Kafri et al., 1992). The chromatin state of the sperm is sequentially modified throughout spermatogenesis, with somatic histones being first replaced by testis specific subtypes, then by transition proteins and eventually by protamines in elongating spermatids (Steger, 1999). Several days before the completion of spermatogenesis transcriptional silencing occurs and the mature sperm genome

becomes extremely compacted(Steger, 1999). The final genomic modifications in spermatogenesis are not essential for development since intracytoplasmic sperm injections from round spermatids and secondary spermatocytes have been successful(Kimura and Yanagimachi, 1995a; Kimura and Yanagimachi, 1995b).

Female germ cells arrest in the diplotene stage of the first meiotic prophase around E13. In mice, oocytes are about 15-20 μm in diameter at this timepoint but will grow to about 75-80 μm when fully mature(Bao et al., 2000). The highly diffuse bivalent chromosomes of the oocyte condense (Chouinard, 1975) as the oocyte reaches about 60 μm in diameter. Only fully mature oocytes are capable of supporting development to term; however, the nuclei of oocytes near the time of this chromosomal condensation can be transferred to fully mature oocytes and still proceed through development following fertilization (Bao et al., 2000). Methylation differences are also apparent between the egg and sperm. For example, *L1*, *IAP*, and *MUP* sequences have been shown to be hypometylated in oocytes in comparison to sperm(Sanford et al., 1984; Sanford et al., 1987).

Many of the gametic modifications to the genome occur over an extended period of time and may be important for embryonic development. It is unlikely that some of these changes can be reprogrammed in the oocyte following nuclear transfer. For example, differential modifications of the parental genomes important for genomic imprinting, as discussed later in the introduction, are established in the respective germlines and preserved in during embryogenesis.

Embryonic gene activation

After fertilization, maternally inherited RNAs and proteins are responsible for directing initial development. The breakdown of these maternal components necessitates that embryonic gene activation (EGA) take place to continue successful embryonic development. The timing of this transition takes place by the 2-cell stage in mice but not until the 8- to 16-cell stage in the cow and rabbit (Telford et al., 1990). EGA is not a discrete event; it begins with a limited activation of certain genes followed by a more global gene activation(Latham, 1999). Throughout this transition the general degree of transcriptional repression decreases, the chromatin structure changes, including changes in histone acetylation, and new transcription factors appear(Wang and Latham, 1997). While many of the events of EGA appear to be conserved between the various mammalian species cloned so far, the differences in the time allowed for reprogramming the donor nucleus prior to its transcriptional activation could account for small differences in cloning efficiencies observed between species.

DNA methylation

DNA methylation is the most thoroughly described epigenetic modification in animals and has been implicated in a number of processes including: genomic imprinting, X-inactivation, cellular differentiation, tissue-specific gene expression, regulation of chromatin structure, aging, carcinogenesis, and silencing of endogenous retroviruses(Reik et al., 2001). Methylation occurs at positon 5 in the pyrimidine ring of cytosines and occurs almost exclusively at CpG dinucleotides(Clark et al., 1995).

The effects of methylation upon chromatin structure and gene expression are likely mediated in many ways by a family of genes that share a highly conserved methyl

CpG-binding domain (MBD) (Wade, 2001). The ability of two of these family members, *MeCP2* and *MBD1*, to repress transcription has been shown(Nan et al., 1997) (Fujita et al., 2000; Fujita et al., 1999). Furthermore, both MeCP2 and MBD3 have been shown to associate in complexes with histone deactylases and other proteins important to chromatin structure (Jones et al., 1998b; Nan et al., 1998) (Humphrey et al., 2001; Wade et al., 1999; Zhang et al., 1999).

In eutherian mammals, there are many dynamic changes to methylation levels in the embryo (see Figure 1). Within four hours after fertilization, a rapid demethylation of the paternal genome occurs in the mouse, followed by further demethylation of the genome during cleavage stage divisions (Dean et al., 2001; Santos et al., 2002). By the morula stage little methylation remains and subsequent *de novo* methylation begins within the ICM of the blastocyst(Santos et al., 2002). The embryonic and extraembryonic lineages are then progressively and independently methylated to different final extents(Monk et al., 1987). Maintenance of methylation on hemi-methylated CpG dinucleotides following replication is accomplished via a DNA methyltransferase encoded by *Dnmt1*(Bestor, 1992; Li et al., 1992). *De novo* methylation appears to be largely accomplished by *Dnmt3a* and *Dnmt3b*(Lyko et al., 1999; Okano et al., 1999). Targeted disruption of *Dnmt1* in mice results in a demethylated genome and early embryonic lethality around E9-E10(Lei et al., 1996; Li et al., 1992). *Dnmt3a*$^{-/-}$mice frequently become runted and die in their first postnatal month while *Dnmt3b*$^{-/-}$ mice die embryonically; *Dnmt3a/Dnmt3b* compound mutant mice die before E11.5 with abnormalities similar to *Dnmt1*$^{-/-}$ mice. (Okano et al., 1999).

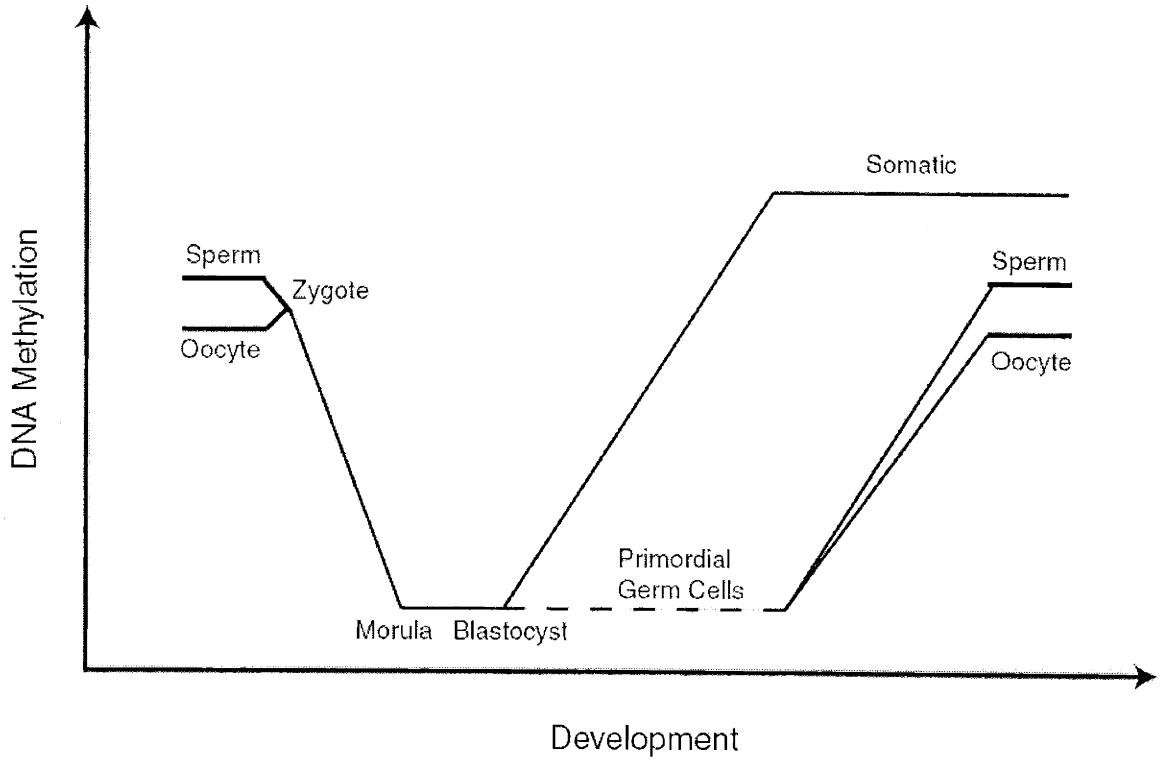

Figure 1. Developmental changes in DNA methylation level during development. Following fertilization there is a rapid demethylation primarily affecting the paternal genome, followed by demethylation of both parental genomes that is complete by the morula stage. *De novo* methylation of the genome begins in the ICM of the blastocyst and methylation levels continue to increase in the developing somatic lineages. Early in their development the primordial germ cells have demethylated their genome. The male and female germline subsequently re-establish methylation to different levels.

DNA methylation in clones

Recapitulating proper timing and specificity of DNA methylation events are likely very important to the future development of clones. Two separate groups have analyzed methylation in cloned bovine embryos from fibroblast donor cells utilizing an antibody against 5-methyl cytosine. While early demethylation of the genome was observed at interphase (Dean et al., 2001), this rapid demethylation was not detected by the other group in metaphase spreads (Bourc'his et al., 2001). In both studies, the cloned embryos

did not become normally demethylated and instead often showed precocious *de novo* methylation (Dean et al., 2001) with euchromatin abnormally hypomethylated and centromeric heterochromatin abnormally hypermethylated (Bourc'his et al., 2001).

Methylation at satellite sequences has been examined by bisulfite sequencing in cloned cow and pig preimplantation embryos. While satellite sequences of the cloned pigs became demethylated like controls in progressing to the blastocyst stage (Kang et al., 2001b), cloned bovine embryos retained methylation levels similar to the donor cells(Kang et al., 2001a). Finally, the methylation status of two single copy gene promoters in cloned bovine blastocysts suggests that single copy sequences are normally demethylated (Kang et al., 2002). Single copy sequences were demethylated in both the ICM and TE. In contrast, the satellite sequences had more methylation in the TE than the ICM of cloned blastocysts, but were equivalently methylated in these two lineages in *in vitro* fertilized controls(Kang et al., 2002). Since the placenta arises from the TE lineage, this abnormal methylation might be related to the severity of placental phenotypes.

Using restriction landmark genome scanning (RLGS), methylation patterns of two neonatal cloned mice have been examined in the placenta, skin and kidney (Ohgane et al., 2001). Methylation at several sites in each clone differed from those in the controls and the other clone, including sites which showed tissue specific methylation.

The results of these experiments indicate that the timing and extent of methylation in cloned animals can be abnormal, with possible species specific differences. Further experimentation is needed to clarify some of the inconsistencies and to further describe methylation in clones at both single copy genes and repetitive elements, especially in

post-implantation development where it is not clear that many methylation differences persist.

Telomere length

The ends of mammalian chromosomes have long tandem arrays of hexameric repeats, known as telomeres, that typically shorten during DNA replication because the DNA polymerases fail to replicate the 5' ends of chromosomes(Levy et al., 1992). Telomeres are important to the stability, replication, and segregation of chromosomes during mitosis(Blackburn, 1991) and once the telomeres shorten to a critical level they enter cycle cycle arrest, apparently by activating a DNA damage response pathway(Saretzki et al., 1999). Telomerase is a multisubunit reverse-transcriptase complex capable of adding repeats onto the ends of chromosomes, but its activity appears limited primarily to the germ line, early embryo, immortalized cell lines, and tumor cells(Counter et al., 1998; Wright et al., 1996).

Telomere length in clones

When cloning from somatic cells, it seems possible that the replicative lifespan of cells would be reduced in clones unless telomere length is restored following NT. It was concluded from an analysis of the sheep Dolly and embryonic tissues of sheep clones that telomere lengths were shorter in NT sheep than in controls(Shiels et al., 1999). In contrast, telomere lengths have been demonstrated to be restored in mouse(Wakayama et al., 2000a) and bovine (Betts et al., 2001; Lanza et al., 2000) clones. Telomerase activity,

which is also present in normally fertilized embryos, thus generally appears capable of restoring telomeres to normal lengths in cloned embryos.

The ability of clones to be generated from other cloned animals in reiterations for up to six generations (Wakayama et al., 2000a) suggests that, at least in the mouse, acquired changes to the genome such as telomere length or mutations are not rapidly accumulated in a way that hinders cloning efficiency; however, the cloning efficiencies were somewhat reduced with successive generations.

X-chromosome inactivation

In eutherian mammals, dosage compensation for the X-chromosome is achieved by silencing one of the two X-chromosomes in a process mediated by a noncoding RNA, known as *Xist*, which coats the inactive X and is necessary for the initiation and propagation but not maintenance of silencing on the X chromosome(Csankovszki et al., 1999; Marahrens et al., 1997; Penny et al., 1996). The inactive X-chromosome is also characterized by: condensed chromatin(Barr and Carr, 1962), late replication timing(Priest et al., 1967), hypoacetylated histone H4(Jeppesen and Turner, 1993), enriched histone macroH2A1(Costanzi and Pehrson, 1998), and hypermethylated CpG islands(Norris et al., 1991). X-inactivation occurs first at the time of implantation in the cells of the TE, in which the paternal X-chromosome is preferentially inactivated, and then randomly in the embryonic lineages just before gastrulation (McMahon et al., 1983).

X-chromosome inactivation in clones

Following nuclear transfer, both X-chromosomes become active during preimplantation and random X-inactivation is recapitulated in the embryo proper (Eggan et al., 2000). There is non-random X-inactivation in the TE; the silenced allele being the same X-chromosome originally silenced in the somatic donor cell. When ES cell donor nuclei, which show biallelic *Xist* expression, are used in NT, random X-inactivation in the TE occurs. Thus, the epigenetic marks responsible for X-inactivation in the embryo can be removed and reestablished during the cloning process.

Genomic imprinting and fetal growth

Genomic imprinting is a process whereby the two parental genomes retain functional differences. Imprinted genes are differentially expressed between the parental alleles with a given imprinted gene showing expression either only maternally or only paternally. This differential expression of a large number of genes throughout the genome has only been described for eutherian mammals and its role in mammals may reflect the continuing dependence of developing embryos upon maternal resources, a scenario in which the parental genomes have a "conflict of interest" and are thus engaged in a "tug of war" (Moore and Haig, 1991). Consistent with this hypothesis, many of the identified imprinted genes have an effect upon fetal and placental growth (Tilghman, 1999).

It is estimated that as much as 0.1% of the genome is imprinted and over 40 imprinted genes have been identified(Morison et al., 2001). Disruptions of the imprinted genes *Igf2*, *Igf2r*, and *H19* have been shown to affect fetal growth by affecting the IGF signaling pathway (DeChiara et al., 1990; Filson et al., 1993; Lau et al., 1994; Leighton

et al., 1995a; Wang et al., 1994). Additionally, the imprinted genes *Insulin2*(Duvillie et al., 1997), $p57^{Kip2}$ (Zhang et al., 1997), *Gnas*(Weinstein et al., 2000), and *Rasgrf1*(Itier et al., 1998) have also been shown to affect fetal development and/or the cell cycle. The imprinted gene *Mash-2* is essential for extraembyronic development; placentas of null mice lack spongiotrophoblast cells and their precursors, and the chorionic ectoderm is reduced (Guillemot et al., 1994). Mice with uniparental inheritance of chromosome 12 exhibit placentomegaly with multiple defects at the maternal-fetal interface(Georgiades et al., 2001). Additonally, placental overgrowth in crosses between two rodent species of *Peromyscus* appear to be related to changes in the expression of multiple imprinted genes (Vrana et al., 2000; Vrana et al., 1998).

Erasure, establishment and mechanisms of genomic imprinting

Imprinted genes appear to share several differential epigenetic features between the parental alleles including DNA methylation(Brandeis et al., 1993) and chromatin structure(Greally et al., 1999). Like many other mono-allelically expressed genes, imprinted genes also show asynchronous replication timing with the paternal allele replicating early for the genes examined (Kitsberg et al., 1993). Many imprinted genes have defined regulatory sequences in which one or the other of the parental alleles is significantly methylated. These differentially methylated sequences (DMRs) are established in the germ line and can be maintained in the embryo and adult(Brandeis et al., 1993), even during the phase of global genomic demethylation taking place in pre-implantation. While methylation is typically associated with gene repression, approximately one-third of the identified imprinted gene DMRs are hypermethylated on

the expressed allele (Reik and Walter, 2001). Additionally, the vast majority of methylation imprints appear to be established maternally. Differential chromatin compaction(Greally et al., 1999) and histone H3 and H4 acetylation(Gregory et al., 2001) have also been described for several DMRs.

Imprinted genes are often clustered together and many imprinted genes produce non-coding RNAs. These non-coding RNAs are often transcribed in an overlapping anti-sense direction to the coding RNAs (Lee et al., 2000; Rougeulle et al., 1998; Smilinich et al., 1999; Wroe et al., 2000; Wutz et al., 1997). A role for some of these genes and their non-coding RNAs(Sleutels et al., 2002) in regulating the expression of adjacent genes has been shown. For example, *H19* is a non-coding RNA located adjacent to the imprinted gene *Igf2*. The replacement of its transcriptional unit does not disturb imprinting of *Igf2*(Jones et al., 1998a), while larger deletions of the *H19* gene lead to biallelic *Igf2* expression(Leighton et al., 1995a; Ripoche et al., 1997). The DMR upstream of the H19 gene has enhancer blocking activity in which the binding of CTCF, a zinc finger protein implicated in vertebrate boundary function(Bell et al., 1999), is abolished when the region is methylated (Hark et al., 2000). It appears that this boundary element and an enhancer downstream of *H19*(Leighton et al., 1995b) account for functional imprinting in the *H19/Igf2* locus; when the enhancer is blocked from activating *Igf2* on the unmethylated maternal allele *H19* is instead activated, while on the methylated paternal allele activation of *Igf2* is not blocked.

Normal methylation levels are required for the parental specific expression of imprinted genes as evidenced by either biallelic or silenced expression of imprinted genes in mice lacking *Dnmt1*(Li et al., 1993). ES cells lacking *Dnmt1* expression are

dramatically hypomethylated; re-expression of *Dnmt1* in null ES cells restores methylation to the bulk genome but not at imprinted DMRs (Biniszkiewicz et al., 2002; Tucker et al., 1996), with some exceptions, including the *H19* DMR which becomes methylated if *Dnmt1* is abundantly expressed (Biniszkiewicz et al., 2002). However, these rescued cells can contribute substantially to chimeras, unlike *Dnmt1* null ES cells, but only with transmission through the germline does normal imprinting become re-established, suggesting that imprints are only established during gametogenesis(Tucker et al., 1996).

While the epigenetic states of the parental genomes are quite distinct prior to fertilization, each is capable in the context of uniparental embryos to support normal pre-implantation development. Embryos derived from parthogenetic (PG) and gynogenetic mouse embryos, composed exclusively of maternal genomes via either oocyte activation or pronuclear transfer respectively, typically survive until embryonic day 10 with a poorly developed extraembryonic lineage(Surani and Barton, 1983). The survival of gynogenetic embryos has been increased to E13 by transfering non-growing (ng) oocyte (diplotene stage of first meiosis) chromatin into mature fully grown (fg) oocytes (Kono et al., 1996). This enhanced survivability is consistent with many developmentally important imprints being established subsequent to the ng oocyte stage, a notion further supported by NT experiments using growing oocytes(Obata and Kono, 2002).

The embryonic development of androgenetic (AG) embryos (derived entirely from a paternal genome by pronuclear transfer) is characterized by poor development of the embryonic lineage(McGrath and Solter, 1984a). The different developmental potential of these embryos compared to PG embryos is also evident in chimeric embryos

made between AG and PG embryos. The PG cells were confined to the embryo but the trophoblast consisted almost entirely of AG cells (Surani et al., 1987). In the brains of chimeras generated with normal embryos, AG cells contributed substantially to the hypothalamic structures but not the cortex structures, while PG and gynogenetic cells contributed substantially to the cortex, striatum and hippocampus but not to the hypothalamic structures(Keverne et al., 1996). A role for imprinting in brain function is further indicated by behavioral abnormalities, including poor maternal care, that have been observed upon disruption of the imprinted genes *Peg1/Mest*(Lefebvre et al., 1998) and *Peg3*(Li et al., 1999).

Abnormal genomic imprinting during ES cell culture and in ES cell derived mice

It has been demonstrated that imprints can be lost during the *in vitro* culturing of ES cells(Dean et al., 1998). One way to characterize these changes and to examine the *in vivo* consequences of these imprinting changes in ES cells is to generate mice derived entirely from ES cells. This is possible by a technique termed tetraploid embryo complementation (see Figure 2); ES cells are either injected into tetraploid blastocysts or aggregated with early tetraploid embryos. This process yields mice that are exclusively derived from the ES cells because the tetraploid host is only capable of contributing to the extra-embryonic lineages(Nagy et al., 1990). Mice generated by tetraploid embryo complementation with ES cells that have been cultured for prolonged periods, showed increased birth weights, swollen edemic skin and perinatal death (Nagy et al., 1993). In these ES cells and in tetraploid-ES cell derived fetuses, the methylation of *H19, Igf2, Igf2r, U2af1-rs1* was abnormal and the expression of these imprinted genes in the fetuses

revealed frequent faulty imprinting for all of the examined genes (Dean et al., 1998). A study using F1 ES cell lines at earlier passages generated entirely ES cell derived mice by tetraploid embryo complementation and by NT. Tetraploid-ES cell derived mice did not show elevated birth weights(Eggan et al., 2001). These results suggest that imprinting abnormalities arise in culture and may affect the development of ES cell derived animals.

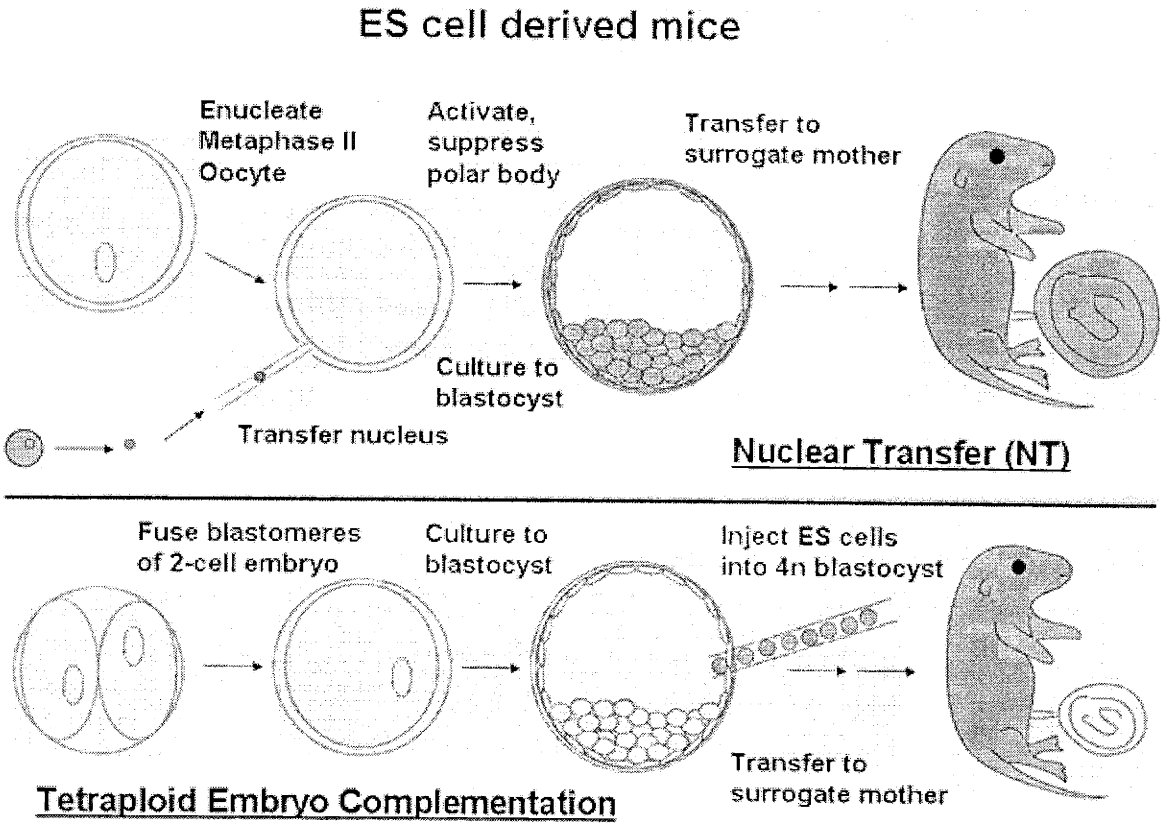

Figure 2. Comparison of two techniques for generating ES cell derived mice: nuclear transfer (NT) and tetraploid embryo complementation. In NT, the nucleus of a donor cell is transferred into an enucleated oocyte. Both the embryonic and extra-embryonic lineages are derived from the ES cell nucleus. In tetraploid embryo complementation, the blastomeres of a 2-cell embryo are fused, giving rise to a one-cell tetraploid embryo. ES cells are then injected into a tetraploid blastocyst. The injected ES cells give rise to the entirety of the neonate since the tetraploid cells are unable to contribute to the embryonic lineages. However, the extra-embryonic lineages, including the placenta, are largely derived from the cells of the tetraploid host blastocyst.

Genomic imprinting in clones

Thus imprinted genes are likely to cause some of the phenotypes in clones since they are frequently involved in fetal and placental growth and are likely resistant to reprogramming since their imprints are established in the germline and specifically maintained in the embryo. Furthermore, *in vitro* culturing of embryos can lead to a loss of imprinting and large offspring syndrome(Khosla et al., 2001). Since cloned embryos also display phenotypes resembling LOS it is possible that some aspects of LOS result from imprinting abnormalities.

Consistent with imprinting playing a role in cloning phenotypes, in chapter 2 we show that imprinted gene expression in ES cell NT neonates and their placentas is frequently abnormal. Reduced expression of *H19* was always associated with hypermethylation of the *H19* DMR. Since similar abnormal gene expression could be recapitulated in donor ES cells upon both *in vitro* differentiation and *in vivo* in mice generated by tetraploid embryo complementation, the loss of imprinting could be explained by a perpetuation of abnormalities present in the donor ES cells prior to NT that could not be reprogrammed. Abnormal expression of any given imprinted gene did not correlate with birth or placental weights, neonatal survival, or with the expression of other imprinted loci. These results suggest that many stochastic expression differences for imprinted genes can be tolerated in the clones, and that cloning phenotypes may reflect the cumulative effect of these and possibly other non-imprinted gene expression changes.

Recently several imprinted genes have been shown to be expressed from the appropriate parental allele in developing clones derived from cumulus cell nuclei (Inoue et al., 2002). The expression of *H19* and *Igf2* were found to be expressed at normal levels in these clones and placentas, in contrast to ES cell derived clones as presented in Chapter 2, but the expression of other imprinted genes and several non-imprinted genes were reduced in the placentas of the cumulus clones (Inoue et al., 2002). In Chapter 3, we confirm these expression changes for the imprinted genes in cumulus cell NT clones and placentas. These data are consistent with *H19/Igf2* being particularly susceptible to abnormalities that arise as a consequence of the environment (Doherty et al., 2000) (Khosla et al., 2001), such as the *in vitro* culture of ES cells. Furthermore, these results remain consistent with abnormal expression levels of a number of imprinted genes affecting the growth of cloned animals

Gene expression analysis in clones

To date, an examination of gene expression in cloned animals has largely been limited to preimplantation embryos and primarily to a small number of genes known to be important for early embryogenesis. In bovine morulae and blastocyst stage NT embryos cloned from somatic and embryonic donor cells, FGF4 expression was frequently absent, in contrast to *in vitro* fertilized controls. In a similar analysis using granulosa cell donors, IL6 and FGFr2 expression levels were also frequently changed (Daniels et al., 2000) (Daniels et al., 2001). Gene expression was examined in cloned bovine blastocysts following both delayed and immediate activation of reconstituted embryos and using donor cells at different passage numbers and points in the cell

cycle(Wrenzycki et al., 2001). Changing each of these parameters, as well as varying *in vitro* culturing media, all appeared to have some affect on the expression pattern of at least one of the eight transcripts monitored. In early amphibian clones derived from intestinal epithelial cells, silencing of the only tissue specific gene examined was effective whereas activation of early embryonic genes was sporadic and to greater expression levels than typically seen in controls(Byrne et al., 2002).

Since many clones surviving past the first few days of birth appear superficially normal, it is important to define in a rigorous way whether these clones resemble normal animals. One way to assess this is through a comprehensive analysis of gene expression in clones for a number of tissues. In chapter 3 we describe the first genome-wide analysis of gene expression in both clones and their placentas. The expression of about 4% of expressed genes was significantly altered in the placentas of the clones. Both cumulus and ES cell NT placentas showed striking similarities with respect to the genes that were affected. However, there were also a number of genes that were expressed differentially between the placentas of the ES cell NT and cumulus cell NT mice. The livers of the clones also showed significant expression differences but these were more subtle than those observed in the placentas. Our results suggest that many expression abnormalities are common to the nuclear transfer procedure while some reflect the particular donor nucleus. These results further emphasize the severity of placental dysfunction and illustrate abnormalities in clones surviving to birth.

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Chapter 2.

Epigenetic Instability in ES Cells and Cloned Mice

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Respective contributions: Kevin Eggan and Hidenori Akutsu generated all cloned mice examined. Kevin Eggan also generated mice by tetraploid embryo complementation for this study. Konrad Hochedlinger, William M. Rideout III, and Detlev Biniszkiewicz performed related assays and provided technical advice. Some of the cloned mice were generated in the laboratory of Ryuzo Yanagimachi. Rudolf Jaenisch assisted in the experimental design and writing of the paper.

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ABSTRACT

Cloning by nuclear transfer (NT) is an inefficient process in which most clones die before birth and survivors often display growth abnormalities. In an effort to correlate gene expression with survival and fetal overgrowth, we have examined imprinted gene expression in both mice cloned by nuclear transfer and in the embryonic stem (ES) cell donor populations from which they were derived. The epigenetic state of the ES cell genome was found to be extremely unstable. Similarly, variation in imprinted gene expression was observed in most cloned mice, even in those derived from ES cells of the same subclone. Many of the animals survived to adulthood despite widespread gene dysregulation, indicating that mammalian development may be rather tolerant to epigenetic aberrations of the genome. These data imply that even apparently normal cloned animals may have subtle abnormalities in gene expression.

Nuclear transfer technology has been used to derive live clones in several species including sheep (1), cattle (2), goats (3), pigs (4, 5), and mice (6), but only a few percent of nuclear transfer embryos develop to term. Even those clones that survive to term frequently die of respiratory and circulatory problems and show increased placental (7-9) and birth (10) weights, often referred to as "large offspring syndrome" (11). Abnormal

regulation of imprinted genes has been shown to affect fetal growth (12, 13), and it has been proposed that improper expression of these genes may contribute to the abnormalities observed in cloned offspring (14, 15).

With one notable exception, poor survival of NT embryos has so far been independent of the donor tissue; a significantly higher fraction of blastocysts cloned from ES cell nuclei than from any somatic cell type survive to adulthood (10, 16, 17). This result is consistent with the idea that the nucleus from an undifferentiated embryonic cell might be more amenable to or require less reprogramming than the nucleus from a differentiated somatic cell. In an effort to correlate changes in gene expression with the survival and fetal overgrowth of cloned animals, we have examined imprinted gene expression in mice cloned by nuclear transfer and in the donor ES cell populations from which they were derived.

Cloned embryos were produced by transfer of low passage (p8 to p12) ES cell nuclei into enucleated oocytes, which were then transferred into surrogate mothers and delivered by caesarian section (c-section) at 19.5 days post coitum (dpc) as previously described (10). Total RNA was isolated from placentas and organs of newborn mice, and Northern hybridization analysis (18) was used to quantify expression of *H19* and *Igf2* in normal and cloned neonates. *H19* and *Igf2* RNA levels were similar in placentas of normal pups (Fig. 1A, lanes 1 through 3) and in placentas derived from normal zygotes that had been cultured in vitro to the blastocyst stage before transfer to surrogate mothers (lanes 4 and 5). In contrast to controls, expression of both genes varied widely between the placentas of cloned embryos. Some clones expressed both genes at levels similar to control placentas (lanes 6, 13, 14, 26, 27, 32, 33), whereas most clones showed aberrant

levels of *H19* and/or *Igf2* transcripts. In many clones, *H19* was silenced and *Igf2* was expressed at higher levels than normal (lanes 8, 9, 18 through 22, 24, 29), as one would predict from the usual reciprocal expression pattern of these two genes (19). However, the reciprocal expression of *H19* and *Igf2* was not observed in a few clones (lanes 15 through 17).

Similarly, Northern analysis of the imprinted genes *Peg1/Mest* and *Meg1/Grb10* revealed variability in the RNA levels amongst the clones, although the extent of variation was less than that observed for *H19* (Fig. 1A). Expression levels of these genes were noticeably lower than for controls in many clones (*Peg1*, lanes 13 through 16, 23, 28 and *Meg1*, lanes 23, 25, 28, 32, 33). When the expression of *Peg3* and *Snrpn* was examined, there was less variation than for the other genes (data not shown). A comparison of expression levels among several genes in a given placenta revealed that the abnormal expression of one imprinted gene did not correlate with abnormal expression at other imprinted gene loci (Fig. 1A).

It has been established that the differentially methylated region (DMR) upstream of the *H19* gene affects its expression with the expressed maternal allele being hypomethylated and the silent paternal allele being methylated (20). To determine whether the abnormalities in gene expression were associated with epigenetic alterations in the imprinted domain upstream of *H19*, we analyzed the methylation pattern of the *H19* DMR as previously described (21). As predicted, the DMR was highly methylated in those placentas that had silenced the *H19* gene and was partially unmethylated in those that showed *H19* expression (22). In contrast, we did not observe a difference in DNA methylation levels between control and cloned offspring at the *Igf2r* DMR 2 (data not

shown), again indicating that epigenetic alterations at one imprinted locus did not necessarily predict changes at other loci.

To determine whether similar alterations in *H19* and *Igf2* expression were present in the organs of newborn clones, we examined RNA isolated from the kidney, heart, and liver of NT mice. *H19* expression was silenced in the heart and kidney and reduced in the liver of all four clones examined (Fig. 1B). *Igf2* expression was increased in kidney and heart but was comparable to controls in the liver (Fig. 1B, lanes 15 through 18). The less dramatic elevation in hepatic *Igf2* expression compared with mesodermal expression changes is consistent with previous observations (19).

Increased placental and birth weights are common phenotypes of cloned animals (7, 10). We investigated, therefore, whether the altered expression of imprinted genes observed in cloned animals correlated with fetal overgrowth and neonatal mortality. The results summarized in Fig. 1A show that the majority of NT pups, regardless of their postnatal survival, exhibited increased birth and placental weights and displayed stochastic changes in the expression of a number of imprinted genes. However, no significant correlation between any of these parameters was established.

The results described so far indicate that expression and methylation of imprinted genes varied widely in placentas and tissues of neonatal ES-cell NT mice. Because these clones were derived from ES cells that had been cultured in vitro, we investigated whether faulty reprogramming during nuclear cloning caused improper imprinted gene expression or whether these errors were the result of preexisting losses of imprinting in the donor cell population, as has been previously observed (23). The ES cell lines used in the nuclear transfer experiments were differentiated in the absence of feeders or LIF

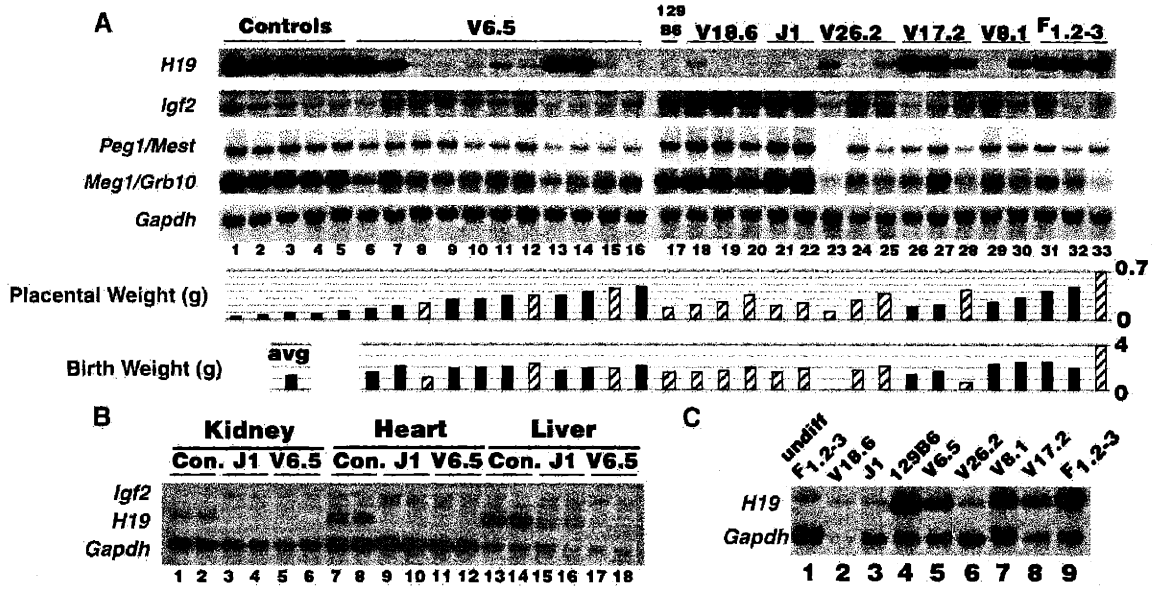


Figure 1. Perinatal silencing of H19 in placentas and tissues of mice cloned from ES cells. (**A**). Northern blot analysis of *H19, Igf2, Peg1/Mest, Meg1/Grb10*, and *Gapdh* in placentas after c-section at 19.5 dpc. The ES cell line used as a nuclear donor is indicated above the lanes. Genetic backgrounds of the ES cell lines are given in (10). V18.6, J1, and V26.2 are inbred lines, whereas the remainder are F_1 lines. Samples in lanes 1 through 3 were from normal embryos and those in lanes 4 and 5 were from embryos that were in vitro cultured to the blastocyst stage. The placentas are ordered by weight from smallest to largest among clones from a given cell line. *Igf2* and *Gapdh* are shown for the same blot, whereas the blots shown for *H19, Peg1/Mest*, and *Meg1/Grb10* are from equivalently loaded blots showing similar *Gapdh* expression. Placental and birth weights for the mice examined are indicated below the respective lanes [solid bar indicates that the mouse initiated normal breathing, hatched bar indicates death soon after delivery; compare results with (10)]. For controls, an average birth weight is indicated for normal mice (10). (**B**) Northern analysis of newborn kidney, heart, and liver from wild-type 129 mice (lanes 1, 2, 7, 8, 13, and 14) and mice cloned from the J1 and V6.5 ES cell lines. The blot was sequentially probed, without stripping, for *H19*, *Igf2*, and *Gapdh*. *Igf2* transcripts from multiple promoters are present, including one just smaller than the prominent *H19* transcript (see, for example, lanes 9, 10, 15, and 16). (**C**) Northern analysis of ES lines for *H19* and *Gapdh* after 7 days differentiation in retinoic acid (RA). Undifferentiated $F_{1.2\text{-}3}$ ES cell RNA (lane 1) is included for comparison.

(Leukemia Inhibitory Factor) in media containing 10^7M all-trans retinoic acid. *H19* expression was then determined by Northern analysis. Wide variation in expression was observed in different ES cell lines; the J1 and V26.2 lines showed the lowest *H19* expression levels (Fig. 1C, lanes 3 and 6) and exhibited the highest degree of *H19* DMR methylation, as determined by Southern analysis (data not shown).

Given the variability of *H19* gene expression in the placentas of cloned mice even when derived from the same cell line, we assessed the extent of heterogeneity in *H19* expression and methylation among subclones derived from individual colonies of the V6.5 ES cell line (24). As judged by Northern analysis, ES-cell subclones differed widely in *H19* expression after differentiation in retinoic acid (Fig. 2A). Subclones that expressed *H19* (subclones 39 and 72) showed a methylation profile consistent with one hypomethylated *H19* allele (Fig. 2B), whereas subclones that silenced *H19* (subclones 10, 23, 43, 89) were hypermethylated in both alleles (Fig. 2, A and B). Methylation differences between subclones were often more dramatic than the differences observed between the various ES cell lines.

To assess the epigenetic heterogeneity among individual clonally related cells, we investigated whether gene expression would be similar in animals derived from cells of the same subclone. We attempted to generate mice from each of four subclones that expressed both *Peg1* and *H19* (subclones 39 and 72), *Peg1* but not *H19* (subclone 89), or neither *Peg1* nor *H19* (subclone 23, *Peg1* expression not shown). Mice derived entirely from donor ES cells were generated by two different methods: (i) tetraploid embryo complementation and (ii) nuclear cloning.

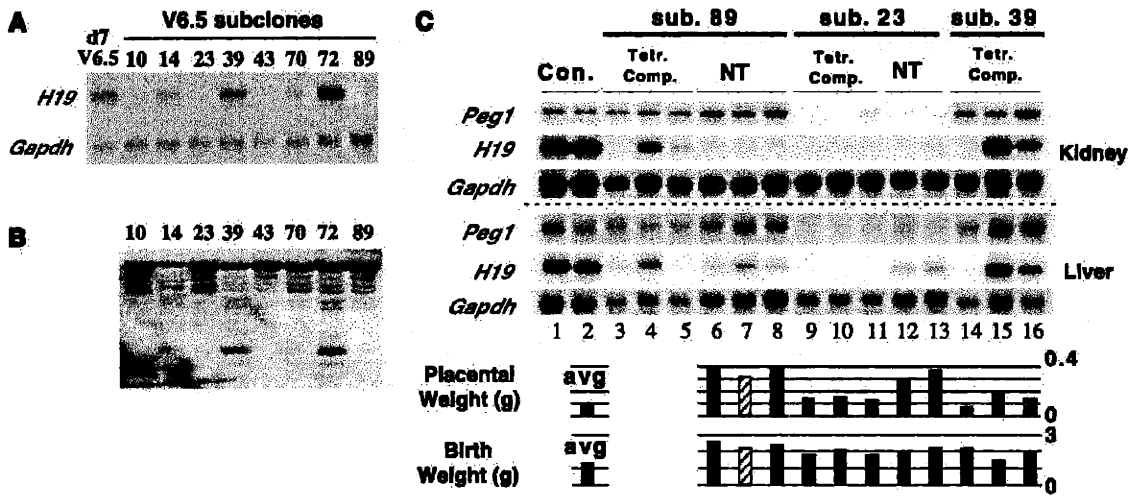


Figure 2. H19 methylation and expression in subclones of the V6.5 ES cell line and expression in mice cloned from these subclones. (**A**) Northern analysis of V6.5 subclones after 6 days RA treatment. The original ES line from which the subclones were derived after 7 days RA treatment is shown for comparison (lane 1). Subclones have been given numerical designations. (**B**) DNA from indicated undifferentiated subclones digested with Sac I and Hha I. The blot was probed with an *H19* DMR Sac I probe. The profile seen for subclone 72 most closely resembles the pattern observed in control placentas at term. The intense low molecular weight band prominently visible in subclones 39 and 72 is the primary restriction fragment seen in *Dnmt1*$^{-/-}$ ES cells (21). (**C**) Comparison of *H19* and *Peg1* expression by Northern analysis in neonatal kidney and liver from mice generated via tetraploid embryo complementation and nuclear transfer from three (89, 23, 39) of the different V6.5 (B6/129) subclones characterized in (A) and (B). Neonatal RNA (lanes 1 and 2) from 129 inbred mice is shown for comparison. *Peg1* expression was analyzed because subclone 23 had failed to express *Peg1* upon RA differentiation. Placental and birth weights and postnatal survival are indicated as in Fig. 1.

Tetraploid embryo complementation is technically easier than nuclear transfer and involves production of composite embryos by injecting ES cells into a tetraploid blastocyst (10, 25, 26). Because the host tetraploid blastocyst cannot contribute to embryonic lineages, the composite embryos give rise to mice that are entirely derived from descendants of the injected ES cells. We successfully generated tetraploid ES-cell pups from three of the four subclones (Table 1). Most pups had increased birth weights relative to normal but not in vitro cultured controls, and they initiated normal breathing after delivery by c-section. Fig. 2C shows the great variability observed among pups derived from both different and identical ES-cell subclones. For example, two pups derived from subclone 89, which had silenced *H19* in the donor cells, did not express *H19* in kidney and liver (lanes 3 and 5), but one did (lane 4). Consistent with the expression pattern found in the donor cell population, none of the pups derived from subclone 23 expressed *H19*. Two of the pups generated from subclone 39 showed high *H19* expression levels (lanes 15 and 16), which is consistent with the donor cells; however, a third pup had silenced *H19* (lane 14). Normal Peg 1 expression was seen in all pups derived from ES cell subclones 39 and 89 but not in pups derived from subclone 23.

Roughly 10 donor ES cells were injected into each blastocyst for tetraploid embryo complementation. To analyze gene expression in animals derived from a single nucleus, we used nuclear transfer to generate animals from different ES-cell subclones. A total of 613 enucleated oocytes were reconstituted with nuclei from the four ES-cell subclones, and five viable pups were obtained: two from subclone 23 and three from subclone 89. We were unable to obtain mice from subclones 39 and 72 (Table 1). Low but variable levels of *H19* were present in the kidney and liver of all five clones (Fig. 2C,

lanes 6 through 8, 12, and 13), whereas *Peg1* expression levels were similar to expression levels observed in the donor subclones. *Peg1* was not expressed in the kidney and was barely detectable in the liver of two NT pups derived from subclone 23 (lanes 12 and 13) but was present at levels comparable to controls in NT pups derived from subclone 89 (lanes 6 through 8).

Table 1. Summary of pups derived by nuclear transfer (NT) and tetraploid (Tetra.) embryo complementation for V6.5 subclones. Percentage of live embryos at each stage are indicated. No., number; surv., surviving manipulation; activ., activated with psuedo pronuclei; trans., transferred to recipient.

Subclone	No. oocytes		No. embryos	No. at 19.5 dpc		
	Surv.	Activ. (% surv.)	Trans. (% activ.)	Dead	Live	Breathing (% trans.)
NT 23	94	75 (80%)	28 (37%)	0	2	2 (7%)
NT 39	156	105 (67%)	24 (23%)	0	0	0
NT 72	348	321 (92%)	65 (20%)	0	0	0
NT 89	156	112 (72%)	32 (29%)	1	3	3 (9%)
Tetra. 23			74	0	3	2 (3%)
Tetra. 39			110	0	3	3 (3%)
Tetra. 72			124	0	0	0
Tetra. 89			76	0	3	3 (4%)

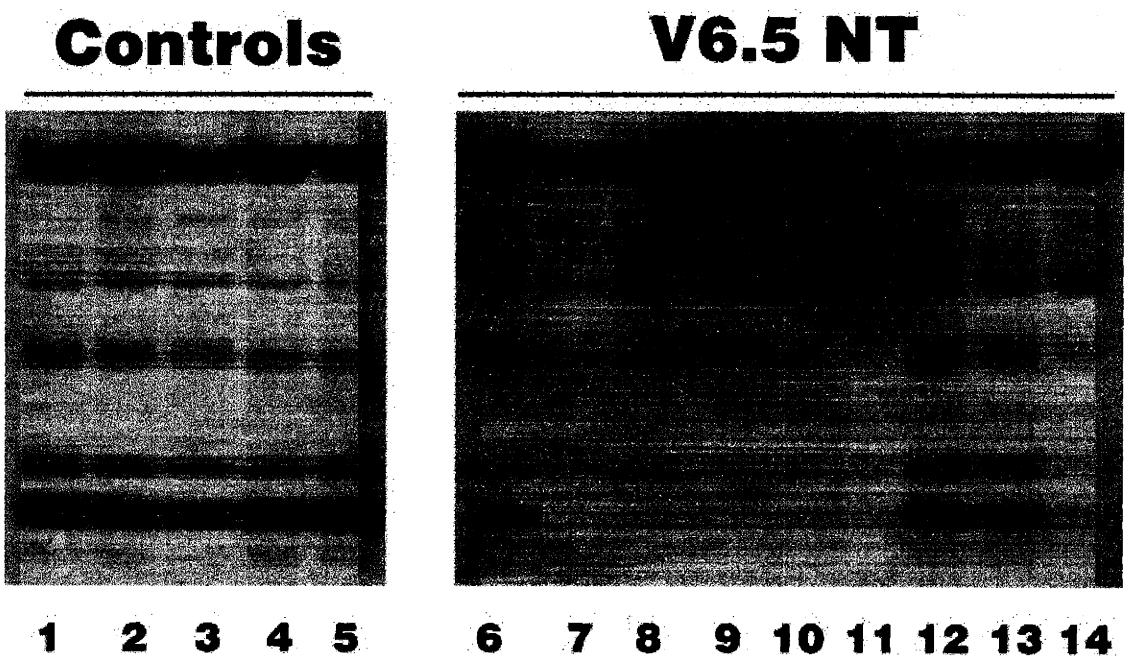
Unlike NT placentas, which are derived exclusively from oocytes reconstituted with ES cell nuclei, the embryonic component of placentas generated by tetraploid complementation was largely derived from the tetraploid host blastocyst and to a lesser extent from the injected ES cells (27). Consistently, *H19* expression was normal in the placentas of pups generated by tetraploid complementation but frequently abnormal in those from nuclear transfer animals (22)

We conclude that expression of *H19*, *Peg1*, and presumably other imprinted genes varies widely between individual ES-cell subclones. Surprisingly, expression was significantly different among mice derived from cells of the same ES-cell subclone, whether originating from several cells after tetraploid complementation or from a single cell after nuclear transfer. The variability of gene expression in these mice likely reflects epigenetic changes that occurred during in vitro culture among sister cells derived from a single cell, consistent with the notion that the epigenetic state of ES cells is extremely unstable. The level of *H19* expression was not a useful criterion to predict whether given donor cell populations could be used to generate viable mice by either of the two methods. Because ES cells are a potential in vitro source of many cell types for transplantation medicine, it will be important to assess whether the epigenetic state of human ES cells is as unstable as that of murine ES cells. It should be emphasized, however, that epigenetic instability of murine ES cells does not impair the routine generation of normal chimeric mice.

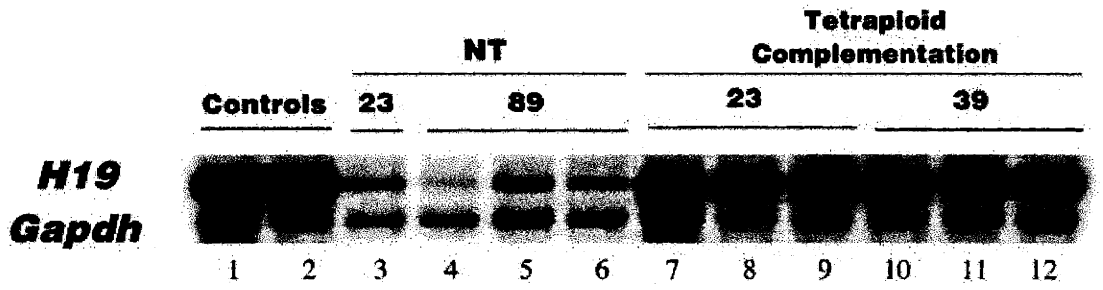
No significant correlation between the anomalous fetal growth of cloned mice and abnormal expression of any single imprinted gene was seen (see weights in Fig. 1A and Fig. 2C). It is possible that the disturbance of placental and fetal growth is due to the cumulative action of many abnormally expressed genes which may have opposing influences on fetal growth (12, 13) and that the effect of a single imprinted gene is insufficient to produce a significant correlation with the overgrowth.

The widespread dysregulation of genes in cloned animals suggests that, contrary to previous conclusions (23), mammalian development may be rather tolerant to epigenetic abnormalities and that lethality may only result from the cumulative effects of

a stochastic loss of normal gene regulation at multiple loci. Our results indicate that even apparently healthy cloned animals can have gene expression abnormalities that are not severe enough to impede development to birth but that may cause subtle physiological abnormalities which could be difficult to detect.



Supplemental Figure 1. Southern analysis after digestion of placental DNA with Sac I and the methylation-sensitive restriction enzyme, Hha I. Blot was probed with *H19* DMR Sac I fragment. Lanes contain DNA corresponding to samples in Fig. 1A, where lanes 1 through 5 are the same controls and lanes 6 through 14 match lanes 7 through 15, respectively.



Supplemental Figure 2. Northern analysis comparison of H19 expression in placentas derived by the NT and tetraploid complementation techniques. Placentas from embryos that were in vitro cultured to the blastocyst stage (lanes 1 and 2) are shown for comparison.

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18. Total RNA was isolated using TriReagent (Molecular Research Center, Cincinnati, OH). Five to ten micrograms were resolved in 1% agarose-formaldehyde gels and transferred to Zetabind (Cuno, Meridian, CT) or Genescreen (NEN, Boston, MA) filters, which were then hybridized with probe in Church buffer overnight. The probes were generated by random hexamer labeling of the following sources: *H19* generated from a Bam HI-Stu I 754-base pair (bp) genomic fragment, *Peg1/Mest* was amplified from cDNA with primers 5'-CCAAAAGCTCCTCAAAGACG-3' and 5'-TCTGTTAGAAGCAGCCGATG-3', followed by nested primers 5'-CTTTCCCCCATCCTCACC-3' and 5'-CAAGCTCCTCCCTCACTCTG-3'; *Meg1/Grb10* was generated from IMAGE clone #746564, and *Igf2* was generated from a 1.2-kb cDNA fragment.
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transactivator vector into the ROSA26 locus (16). Individual targeted subclones were derived by picking and expanding drug resistant colonies. Derivation, culture, and targeted mutagenesis of ES cells were carried out as previously described (16).

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28. We thank A. Wutz for providing subclones of the V6.5 cell line. Supported by the Victoria and Bradley Geist Foundation, the Kosasa Family Foundation, and the Harold Castle Foundation (R.Y.) and by NIH grants 5-R35-CA44339 and RO1-CA84198 (R.J.).

Chapter 3.

Abnormal gene expression in cloned mice derived from ES cell and cumulus cell nuclei

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Respective Contributions: Kevin Eggan and Hidenori Akutsu generated all cloned mice. Konrad Hochedlinger, Kevin Eggan , and Adam Freidman assisted in generating control mice. Adam Friedman also assisted in processing data files. Cumulus cell derived clones were generated in the laboratory of Ryuzo Yanagimachi. Eric S. Lander, Todd Golub, and Rudolf Jaenisch assisted in the writing of the paper.

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The majority of cloned mammals derived by nuclear transfer (NT) die during gestation, display a phenotype resembling "Large Offspring Syndrome"[1,2], and have an enlarged and dysfunctional placenta[3,4]. Previous examinations of a limited number of imprinted genes in the placentas of clones have shown that several are expressed at abnormal levels [5,6] and that these changes can reflect epigenetic abnormalities arising in donor cells, in particular during the *in vitro* culture of ES cell donors. However, it is not clear beyond the few examined genes to what extent imprinted gene expression or global gene expression differences, if any, may depend on a given type of donor cell nuclei. To address these issues we have performed a direct comparison of gene expression profiles for over 10,000 genes in the placentas and livers of neonatal cloned mice derived from both cultured ES cells and freshly isolated cumulus cells. In clones derived from both donor cell types, the expression patterns in the placentas differed dramatically from those in controls and the majority of genes abnormally expressed were common to both types of clones. Importantly, however, the expression of a subset of genes differed between the cumulus and ES cell derived clones. Expression profiling of the livers from cloned mice also revealed abnormal gene expression, although to a lesser extent than in the placentas. Our results suggest that abnormal gene expression in clones is influenced by both the nuclear transfer procedure as well as the type of donor nucleus.

Cloned mouse neonates were produced by nuclear transfer from ES and cumulus cell nuclei. Most clones derived from both donor cell types exhibited fetal overgrowth and an enlarged placenta. The average birth and placental weights, respectively, were 1.3g and 0.09g for normally fertilized embryos, 2.1g and 0.32g for ES cell NT mice[7], and 2.2g (N=12) and 0.33g (N=14) for cumulus cell NT mice. RNA was isolated from a total of 24 placentas and 20 livers and expression analysis was performed using Affymetrix gene arrays. Two sets of experiments were run using two different array versions.

Each of the two types of NT placentas was first compared to the controls. Genes showing a two-fold or greater expression difference between the means of the controls and either of the two types of NT placentas were determined for 15 samples on the more recent array version. Fig. 1a gives a visual comparison of gene expression differences between the three groups of animals. The expression of 286 genes was found to be changed at least two-fold in cumulus clones as compared to normally fertilized controls and to differ significantly by a student t-test ($p<0.05$). Similarly, dysregulation of 221 genes was detected in ES cell derived clones. Since many genes might be expected to show significant differences by chance when thousands of genes are examined, we calculated adjusted p-values for this data set to account for the large number of genes. Fifty six percent of the genes in this subset were calculated to have an adjusted p-value of less than or equal to 0.05 for a false discovery rate. Also, 101 genes in total had an adjusted p-value meeting a threshold of 0.05 for a family-wise error rate (probability of at least one error in the group), showing that the differences we observed did not arise by

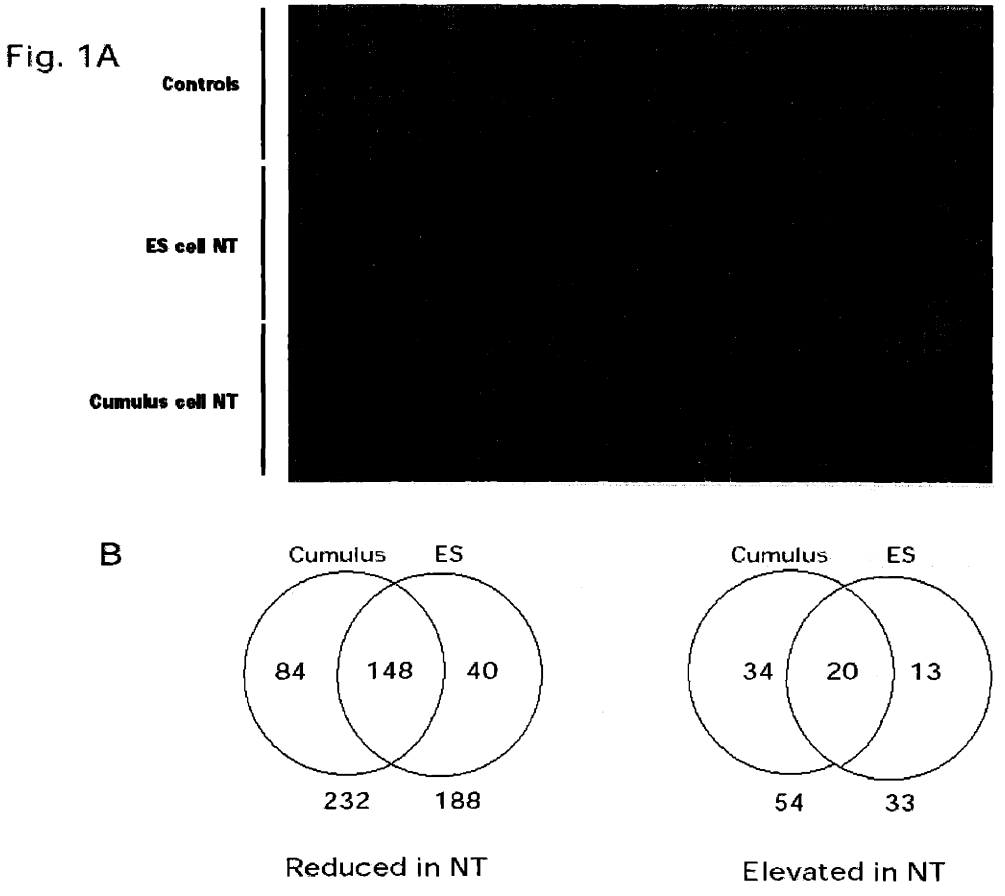

Figure 1. (A) Representation of the expression levels of all genes showing a two-fold difference in mean expression between any two sample types. Placentas are along the vertical axis and genes along the horizontal axis. Average expression across the fifteen samples is indicated by black. Expression greater than the average level is indicated by increasing red intensity, while green indicates reduced expression. Genes with similar expression profiles have been clustered and displayed with GeneCluster and TreeView. (B) The number of genes that show a two-fold difference in average expression and differing significantly from controls by a t-test ($p<0.05$) are indicated for each of the donor cell types beneath the diagrams. There are two separated diagrams for genes either reduced or elevated in the NT placentas. The number of genes altered two-fold in both types of clones is indicated in the overlapping circles.

chance. A schematic comparison of genes abnormally expressed in either type of experimental placenta is given in Fig 1b. Many of the abnormally regulated genes were common to the two types of clones, while some were dysregulated either only in cumulus or in ES cell donor derived clones but not in both (Fig. 1). Of the 188 genes with reduced expression in the ES cell NT placentas, 148 (79%) were also reduced in the cumulus cell NT placentas. In total, 76 percent of the genes showing a two-fold change in ES cell NT placentas, either up or down relative to controls, also showed a consistent, greater than two-fold change in the placentas of cumulus cell clones. An additional 17 percent (93 percent in total) showed a greater than 1.5-fold change. These results suggest that the majority of expression abnormalities were generally common to all cloned mice rather than specific to those derived from one particular cell type.

In order to determine the identity of genes most likely to be dysregulated in NT placentas, both data sets were incorporated into the analysis. These data included control RNAs from placentas of both sexes, from placentas of *in vitro* cultured embryos and from both interstrain (*M. musculus* x *M. musculus*) and interspecific (*M. musculus* x *M. castaneus*) F1 samples. Genes showing high variation in the controls were excluded from the candidate list to eliminate genes with altered expression as a result of background variation. Table 1a lists the most altered candidate genes following this selection. These candidates were also analyzed with the data from each array version assessed independently. The majority of the genes shown exhibited a consistently altered expression in both sets of experiments.

To demonstrate that the overall expression patterns were substantially different between the clones and controls, we generated predictors to classify our samples

Table 1A Genes with greatest fold-change between clones and controls

Elevated in Clones

ES List	ES/Con		Cumulus List	Cum/Con	
Ada Adenosine deaminase	3.34	^	Pripl Prolactin-like protein I	3.34	^
Ncam Neural cell adhesion mo	3.20	^	Ada Adenosine deaminase	2.49	^
Car2 Carbonic anhydrase 2	2.82	^	Pthr Parathyroid hormone rec	2.16	
Ada Adenosine deaminase	2.67	^	Ada Adenosine deaminase	2.14	^
Chemokine (C-C) receptor 1,-l	2.58	^	Jak1 Janus kinase 1	2.11	^
Pripg Prolactin-like protein G	2.47		Car2 Carbonic anhydrase 2	2.05	
onzin	2.26	^	Cd83 CD83 antigen	2.03	^
Mmp15 Matrix metalloproteins	2.23	^	Mmp15 Matrix metalloproteins	2.02	^
Gpc1 Glypican 1	2.15	^	Sparc Secreted acidic cystein	2.01	
2610042L04Rik RIKEN cDNA 2	2.14	^	Fbln1 Fibulin 1	1.94	^
lysozyme M	2.11		Pripg Prolactin-like protein G	1.94	^
Prkcl Protein kinase C, lamda	2.07		tyrosine phosphatase LAR (Pt	1.92	^
Pripl Prolactin-like protein I	2.05	^	Cbx4 Chromobox homolog 4 (	1.88	^
oxidized LDL receptor (Lox-1)	2.04		1200008D14Rik RIKEN cDNA	1.86	
Pl1 Placental lactogen 1	2.04	^	Hdac6 Histone deacetylase 6	1.85	^
Fbln1 Fibulin 1	2.01	^	chemokine (C-C) receptor 1,-l	1.82	^
Cd83 CD83 antigen	2.00	^	Ctss Cathepsin S	1.80	^
Col15a1 Procollagen, type XV	1.96		Ncam Neural cell adhesion mo	1.80	^
Ctss Cathepsin S	1.90		Pl1 Placental lactogen 1	1.79	
Glk Galactokinase	1.86	^	Adcy7 Adenylate cyclase 7	1.78	^
Dtx1 Deltex 1 homolog (Droso	1.86	^	lysozyme M	1.75	
Rora RAR-related orphan rece	1.85		AW228162 Expressed sequen	1.72	
EST: AW122677	1.85	^	Madh2 MAD homolog 2 (Droso	1.72	
Cd83 CD83 antigen	1.85		Anxa11 Annexin A11	1.71	
Plac1 Placental specific prote	1.83	^	P4ha1 Procollagen-proline, 2-	1.70	
Vr1 Vanilloid receptor-like pr	1.82	^	Mmp12 Matrix metalloproteins	1.69	^
Adcy7 Adenylate cyclase 7	1.80		Gpc1 Glypican 1	1.69	^
Gpr97 G protein-coupled rece	1.77		P4ha2 Procollagen-proline, 2-	1.67	^
Pthr Parathyroid hormone rec	1.77	^	1500011E07Rik RIKEN cDNA	1.67	
Il1r1 Interleukin 1 receptor, ty	1.76		Uchrp Ubiquintin c-terminal h	1.66	
Pdcd4 Programmed cell death	1.74	^	Pdcd4 Programmed cell death	1.65	
Hspg2 Perlecan (heparan sulf	1.69		Dtx1 Deltex 1 homolog (Droso	1.63	
P4ha1 Procollagen-proline, 2-	1.68		Lgals9 Lectin, galactose bindi	1.63	^
Lgals9 Lectin, galactose bindi	1.67		Trap1a Tumor rejection antige	1.62	^
AI848508 Expressed sequenc	1.67		Hs3st1 Heparan sulfate (gluco	1.61	^
Gjb5 Gap junction membrane	1.66	^	Nrk Nik related kinase	1.61	
1700012A18Rik RIKEN cDNA	1.65		Erp29 Endoplasmic reticulum	1.61	

Reduced in Clones

ES List	ES/Con		Cumulus List	Cum/Con	
Eln Elastin	0.36	^	Eln Elastin	0.30	^
1600025H15Rik RIKEN cDNA	0.38	^	1600025H15Rik RIKEN cDNA	0.34	^
5033414D02Rik RIKEN cDNA	0.43	^	Vnn1 Vanin 1	0.37	^
EST: AW121826	0.45	^	EST: AW121826	0.39	^
Hsd11b1 Hydroxysteroid 11-b	0.46	^	Hsd11b1 Hydroxysteroid 11-b	0.42	^
Slc1a4 Solute carrier family 1	0.47	^	5033414D02Rik RIKEN cDNA	0.42	^
Folr1 Folate receptor 1	0.47	^	1200011C15Rik RIKEN cDNA	0.44	^
1200011C15Rik RIKEN cDNA	0.48	^	F2rl1 Coagulation factor II (thr	0.44	^
Ccr4 Carbon catabolite repres	0.48	^	Folr1 Folate receptor 1	0.44	^
SODIUM-DEPENDENT MULTI	0.48	^	carbonic anhydrase IV	0.44	^
Eng Endoglin	0.48		Col18a1 Procollagen, type XV	0.47	^
AA589632 Expressed sequenc	0.49	^	AI785303 Expressed sequenc	0.47	^
2610019F03Rik RIKEN cDNA 2	0.51	^	Ephrin B1	0.47	^
Grb10 Growth factor receptor	0.52	^	Grb10 Growth factor receptor	0.48	^
AI785303 Expressed sequenc	0.53	^	Slc1a4 Solute carrier family 1	0.50	^
Vnn1 Vanin 1	0.53	^	ESTs, Highly similar to SL56_	0.50	^
Trfr Transferrin receptor	0.53	^	1-Cys peroxiredoxin protein 2	0.51	^
1810004P07Rik RIKEN cDNA	0.53		2610019F03Rik RIKEN cDNA 2	0.52	^
major histocompatibility locus	0.55	^	Vegf Vascular endothelial grot	0.52	^
carbonic anhydrase IV	0.55	^	Perp-pending P53 apoptosis e	0.52	
Mpp1 Membrane protein, palm	0.55	^	major histocompatibility locus	0.53	^
Idb3 Inhibitor of DNA binding	0.55	^	Grb10 Growth factor receptor	0.53	^
2500002L14Rik RIKEN cDNA 2	0.56		Ccr4 Carbon catabolite repres	0.53	^
Vamp8 Vesicle-associated me	0.56		HSB2 RAT HEAT-SHOCK PRO	0.54	^
Fabp3 Fatty acid binding prot	0.56	^	SODIUM-DEPENDENT CHOLI	0.54	^
Capn2 Calpain 2	0.57		AA409659 Expressed sequenc	0.54	^
S100a10 Calcium binding prot	0.57	^	Cd81 CD 81 antigen	0.55	^
0610039C21Rik RIKEN cDNA	0.58	^	Eng Endoglin	0.55	^
Gsn Gelsolin	0.58	^	Ddah2 Dimethylarginine dimet	0.55	
F2rl1 Coagulation factor II (thr	0.58	^	Ly64 Lymphocyte antigen 64	0.55	^
Grb10 Growth factor receptor	0.58	^	1810004P07Rik RIKEN cDNA	0.56	^
Cd81 CD 81 antigen	0.58		p53 regulated PA26-T2 nuclea	0.56	
Laptm4b Lysosomal-associate	0.58	^	Srb1 Scavenger receptor class	0.56	
2410026J11Rik RIKEN cDNA 2	0.59		Gsn Gelsolin	0.57	
Ephrin B1	0.59		C80731 Expressed sequence	0.57	^
Aqp8 Aquaporin 8	0.59	^	Tgfbr3 Transforming growth f	0.57	^
AA409659 Expressed sequenc	0.60	^	Tmprss2 Transmembrane pro	0.58	^

1B Genes with greatest fold-change in expression between the two types of clones

Genes expression higher in ES NT	ES/Cum	ES	Cum
Sod3 Superoxide dismutase 3, extrac	2.08	-	▼
Mus musculus oxidized LDL receptor	1.91	▲	-
5930418K15Rik RIKEN cDNA 5930418	1.69	-	▼
Rgs16 Regulator of G-protein signalir	1.66	▲	▼
2610042L04Rik RIKEN cDNA 2610042	1.62	▲	-
Rpo1-1 RNA polymerase 1-1 (40 kDa s	1.62	-	▼
Ncam Neural cell adhesion molecule	1.62	▲▲	▲
Vnn1 Vanin 1	1.53	▼	▼▼
Perp-pending P53 apoptosis effector	1.52	▼	▼▼
Prkcl Protein kinase C, lamda	1.50	▲▲	▲
Htr4 5 hydroxytryptamine (serotonin)	1.48	-	▼
Eif4ebp1 Eukaryotic translation initiat	1.48	-	▼
F2rl1 Coagulation factor II (thrombin)	1.44	▼	▼▼
Sox13 SRY-box containing gene 13	1.40	-	▼
Srp9 Signal recognition particle 9 kDa	1.40	-	▼
Il11ra2 Interleukin 11 receptor, alpha	1.39	-	▼
Vegf Vascular endothelial growth fact	1.38	▼	▼▼
H2-L Histocompatibility 2, L region	1.36	-	▼
1200003F12Rik RIKEN cDNA 1200003	1.35	▲	▼
Csf2ra Colony stimulating factor 2 rec	1.35	-	-
Rora RAR-related orphan receptor alp	1.35	▲▲	▲
Egfr Epidermal growth factor recepto	1.35	-	▼
Col18a1 Procollagen, type XVIII, alpha	1.34	▼	▼▼
2810021G24Rik RIKEN cDNA 2810021	1.33	▼	▼▼
Gjb3 Gap junction membrane channe	1.33	-	▼
Il1r1 Interleukin 1 receptor, type I	1.32	▲▲	▲
Sdf1 Stromal cell derived factor 1	1.31	▲	-
putative G-protein coupled receptor (	1.31	-	▼

Genes expression lower in ES NT	ES/Cum	ES	Cum
Xlr3b X-linked lymphocyte-regulated	0.53	▼	▲
Pripl Prolactin-like protein I	0.58	▲	▲▲
DXImx46e DNA segment, Chr X, Immu	0.62	-	▲
Mlycd Malonyl-CoA decarboxylase	0.66	-	-
R74626 Expressed sequence R74626	0.67	-	▲
pim-1 protein kinase, Proviral integra	0.68	-	▲
Hdac6 Histone deacetylase 6	0.69	▲	▲▲
U2af1-rs1 U2 small nuclear ribonucle	0.70	-	▲

▲= significantly higher expression in given NT type than in controls

▼= significantly lower expression in given NT type than in controls

Double arrows indicate which clone type shows more dramatic affect

Table 1. Genes that were significantly (t-test, $p<0.05$) up- or down-regulated between the indicated groups of placentas. All genes shown have a coefficient of variation (CV) of less than 25% for controls. Gene names correspond to their Unigene identification where possible, and otherwise their Affymetrix identifier. (A) Genes with the most altered expression levels between clones and controls are shown. Genes showing the greatest fold-change are listed at the top and the specified NT/control expression ratio is indicated. Genes that appear in both donor lists have been shaded. Furthermore, genes which showed a consistent expression pattern when data from the different array versions were assessed independently are indicated; genes with an NT/control expression ratio of greater than 1.5 or less than 0.65 for both sets of experiments, for elevated and reduced expression respectively, are indicated by an "^" next to the expression ratio. (B) Genes differing in their expression levels between the two types of NT placentas are ordered with the greatest average expression difference at the top for each comparison. The ES cell NT/ cumulus cell NT expression ratio is indicated. Beneath each donor type is listed the expression of the genes compared to controls, where symbols indicate the following: "-" = not significantly different, "▲" = significantly elevated above controls, "▼" = significantly reduced below controls. When expression was affected in both NT types, two arrows are used to indicate which type of clone was more severely affected.

as has been done to assign tumors to known classes[8]. Using classifiers based on six to ten genes, we were able to correctly remove data corresponding to a given sample, one at a time, and then correctly reassign each of the 24 placental samples to either the control or the clone group based on the remaining samples. These results demonstrate that NT and control placentas have expression profiles that can be readily distinguished.

In addition to comparing the placentas of clones with those of controls, we compared the placentas of the two NT types with each other. We found significant differences between ES cell and cumulus cell derived clones. However, the number of abnormally expressed genes was about 10 fold less than when the two types of clones were combined and compared to controls. A list of genes showing the greatest fold-changes between the two types of clones but little variation in controls are shown in Table 1B.

To determine the status of gene expression in the somatic lineages of cloned mice we analyzed neonatal livers using two sets of array data. We analyzed gene expression in the livers of five normal mice, seven mice derived by ES cell NT, and two mice derived by NT from cumulus cells. This analysis revealed differences in gene expression between tissues of control and cloned pups derived from ES cell donor nuclei. However, these differences were less pronounced and the affected genes were generally distinct from those affected in the placentas (Table 2). Livers of the clones derived from cumulus cell donor nuclei also showed abnormalities in gene expression but we were not able to assess the statistical significance of these differences due to insufficient sample size.

Table 2: Altered gene expression in livers of clones

Expression reduced in ES cell NT Liver

Gene	ES/Con	Cum/Con	Tetra/Con
Consistent with being caused by ES cell donor			
Rasgrp2 RAS, guanyl releasing protein 2	0.28	0.71	0.27
H19	0.32	1.06	0.12
Amy2 Amylase 2, pancreatic	0.60	1.99	0.64
Aanat Arylalkylamine N-acetyltransferase	0.62	0.92	0.49
I kappa B alpha	0.63	1.17	0.74
Prkcc Protein kinase C, gamma	0.63	0.95	0.54
Yes Yamaguchi sarcoma viral (v-yes) oncogene homolog	0.63	1.53	0.67
Pcdha13 Protocadherin alpha 13	0.64	0.89	0.64
AW554572 Expressed sequence AW554572	0.67	0.89	0.61
AI850305 Expressed sequence AI850305	0.67	1.07	0.75
Consistent with being caused by NT			
Cyp2a4 Cytochrome P450, 2a4	0.48	0.59	0.90
Cyp3a16 Cytochrome P450, 3a16	0.60	0.36	0.88
Klf3 Kruppel-like factor 3 (basic)	0.65	0.65	0.98
Cpt1a Carnitine palmitoyltransferase 1, liver	0.65	0.68	1.54
Tpst1 Protein-tyrosine sulfotransferase 1	0.67	0.69	0.82

Expression elevated in ES cell NT Liver

Gene	ES/Con	Cum/Con	Tetra/Con
Consistent with being caused by ES cell donor			
Bcap31 B-cell receptor-associated protein 31	2.07	0.95	1.77
Cetn3 Centrin 3	1.99	1.26	2.06
S100a10 S100 calcium binding protein A10 (calpactin)	1.63	1.13	1.62
Psg-ps1 Pregnancy specific glycoprotein pseudogene 1	1.58	1.12	1.44
Sc5d Sterol-C5-desaturase homolog (probe 1)	1.56	1.00	1.64
Es31 Esterase 31	1.52	0.87	1.42
Sc5d Sterol-C5-desaturase homolog (probe 2)	1.52	0.95	1.30
Consistent with being caused by NT			
Slfn4 schlafen4	1.71	1.88	1.25
Abca2 ATP-binding cassette, sub-family A (ABC1), member 2	1.55	1.34	1.00
2610007K22Rik RIKEN cDNA 2610007K22 gene	1.54	1.54	1.05
Rpn1 Ribophorin I	1.52	1.38	1.08

Table 2. Subset of genes with expression levels varying most between ES cell NT livers and controls. The first ratio indicates the average expression in the ES cell NT livers compared to controls. The ratio of expression in livers between the cumulus cell NT mice and controls and the tetraploid embryo complementation mice and controls are also included. Genes are separated into two groups based on genes with expression profiles consistent with abnormal expression due to the ES cell donors (listed first in each group of changes) or due to the NT process. These genes represent approximately 50% of the most affected genes in ES cell NT livers

We also generated entirely ES cell derived mice by tetraploid embryo complementation as a comparison for the ES cell NT mice. Since these mice are not generated by nuclear transfer and do not exhibit overgrown placentas[7], we can use them to further define which expression changes in ES cell NT mice are likely to reflect either the ES cell donor or the nuclear transfer procedure itself, including possible secondary effects of dysfunctional NT placentas. Six mice derived by tetraploid embryo complementation, from the same ES cell subclones used to generate the NT embryos, were analyzed with arrays. Among the genes affected severely in ES cell NT mice, we screened for gene expression changes in the livers most consistent with being caused either by NT (common changes in just both NT types) or by characteristics of the ES cell donor (common changes in just ES cell derived livers). As summarized in Table 2, we found genes with expression patterns falling into both of these expression classes, including H19 which was affected specifically in the ES cell derived animals.

We next focused on the expression profiles of imprinted genes in both the placentas and livers of clones. While H19 was among the most variable genes in ES cell derived animals, its expression showed no significant variability in either the livers or placentas of cumulus cell derived clones, confirming previous results[5,6]. In contrast, the expression levels of 3 other imprinted genes (*Dlk*, *Meg1/Grb10*, and *Peg1/Mest*) in the placentas were similar for both types of clones and were significantly different from controls for both donor cell types (Table 3). In liver, expression of *Peg1/Mest* and *Meg1/Grb10* were not significantly reduced in the clones, except in pups derived from ES donor cell line subclone #23[5] which had previously been shown to lack *Peg1/Mest* expression upon *in vitro* differentiation. Conversely, *Cdkn1c (p57)* appeared to be

Table 3: Imprinted gene expression

Placenta			Liver	
Imprinted Gene	ES/Con	Cum/Con	ES/Con	Cum/Con
Nnat	0.51	0.55 *	N/A	N/A
Meg1/Grb10 (probe1)	0.52 **	0.48 **	1.18	0.72
Meg1/Grb10 (probe2)	0.58 **	0.53 **	1.06	0.86
Peg1/MEST	0.65 *	0.59 **	0.66	1.30
Dlk1	0.70 **	0.67 **	1.21	1.17
H19	0.72	1.00	0.33 **	1.06
Slc22a1l	0.80	0.65 *	1.08	0.95
Insulin I	0.87	1.13	0.84	0.85
Igf2r	0.90	0.96	0.99	1.01
Necdin	0.93	0.95	1.00	1.00
Nesp	0.98	1.04	1.13	1.18
Cdkn1c (P57)	0.99	0.97	1.63 *	1.53
Igf2	1.03	0.96	1.15 *	0.80
U2af1-rs1	1.12	1.58 **	N/A	N/A
Sgce	1.16	1.36 **	1.17 *	0.81
Peg3	1.24	0.92	1.05	1.16

Table 3. Ratio of average expression levels for imprinted genes in both types of NT placentas and livers as compared to controls. Genes not expressed above floored expression level are indicated with N/A. Genes showing a significant change relative to controls are indicated: *=$p<0.05$, **=$p<0.01$ by t-test.

elevated in liver but not placenta of cloned pups. When expression of *H19*, *Igf2*, *Igf2r*, and *SNRPN* in placentas and embryos of both cumulus and ES cell NT mice was tested using allele-specific assays of F1 *Mus/Cast* clones, we failed to detect inappropriate activation of the normally silent allele (data not shown) confirming data published by others[6]. This is consistent with our quantitative analyses and argues that the decreased expression of those imprinted genes is caused by reduced expression of the normally active allele and may not involve the other normally inactive allele. However, our results do not support the claim[6] that abnormal expression of imprinted genes is generally more pronounced in ES cell donor derived clones than in cumulus cell donor derived clones. Instead it appears that *H19* and *Igf2* are exceptional genes whose expression and methylation levels have been shown to be highly sensitive to environmental influences[9-11] such as *in vitro* cultivation.

To confirm the expression levels of several genes analyzed on the arrays, Northern blot analysis was performed with both placental samples used in the array analysis and with additional controls and clones (Fig. 2). Consistent with previous results[5,6] and our array analysis, H19 expression varied dramatically in placentas of ES cell donor derived clones (lanes 19–37) but not in cumulus cell donor derived clones (lanes 9–18) and controls (lanes 1-7). Expression of *Meg1/Grb10* and *Peg1/Mest*, however, were reduced in both cumulus cell and ES cell derived clones. We also probed for two non-imprinted genes whose expression levels appeared significantly altered by array analysis. Consistent with the array analyses, Northern hybridization demonstrated that *Carbonic anhydrase 2* was upregulated, with expression levels higher in the ES cell derived clones than in cumulus cell derived clones (compare Table 1a). Northern analysis

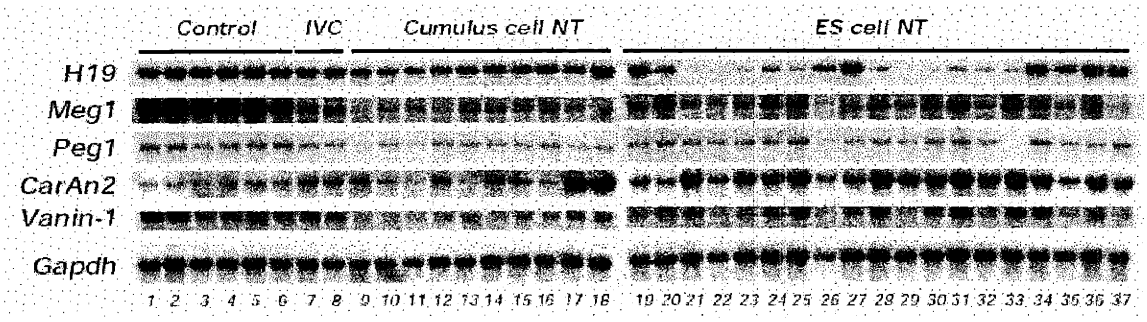

Figure 2. Northern analysis of several genes dysregulated in NT placentas at term. Lanes 1-6 contain RNA from naturally derived B6/129 controls, while the RNAs in lanes 7-8 are derived from the placentas from normal B6/129 zygotes that had been cultured *in vitro* before transfer to a surrogate mother. RNAs in lanes 9-18 are from cumulus cell NT placentas of the indicated genetic backgrounds: 9-13 DBA/Cast, 14-15 129/Cast, 16 AJ/Cast, 17-18 B6/DBA. RNAs in lanes 19-37 are from placentas of ES cell NT mice: lanes 19-29 are derived from the V6.5 (B6/129) line, lanes 30-33 are from targeted subclones[10] of the V6.5 line (lanes 30-32: subclone #89, lane 33: subclone #23), lane 34 is from the V17.2 (Balb/129) line and lanes 35-37 are from the $F_{1\text{-}2.3}$ (129/Cast) line.

of *Vanin-1* RNA showed reduced expression in clones which was more pronounced in cumulus than in ES cell derived clones, also in agreement with the array analyses. Of the genes showing expression changes by Northern analysis, only *H19* and *Igf2* appeared to be similarly affected in both neonatal tissues and the placentas, relative to controls (data not shown).

In summary, oligonucleotide array expression analyses indicate a pronounced dysregulation of several hundred genes in the placentas of cloned mice, representing at least 4% of the expressed genes. These differences were pronounced allowing an easy distinction between clones and controls based solely on gene expression profiles. Histological analyses of the placentas of cloned pups have demonstrated a frequent overgrowth of the spongiotrophoblast layer and an increase in the number of glycogen producing cells[12]. Thus, some of the changes in placental gene expression may reflect changes in the relative abundance of certain cell types. However abnormal gene expression in the placentas did not correlate with placental size, indicating that these changes in cellular composition are unlikely to account for many of the observed expression changes. Our data show that many factors may contribute to altered gene expression including faulty reprogramming following nuclear transfer and epigenetic errors inherited from the specific type of donor nucleus. These results are consistent with nuclear transfer experiments in amphibians, in which the differentiation status of the donor cell has been shown to affect the developmental potential of cloned animals[13-16]. The data presented here indicate that highly variable gene expression, observed previously for a limited number of genes in both amphibian[16] and mammalian clones[5,6], affects much of the genome and further emphasizes that many changes are tolerated

during cellular differentiation and even in surviving clones. In addition, *in vitro* cultivated ES cells have been shown to be epigenetically unstable[5,17]. When used as donors for nuclear transfer, this instability contributes to widespread dysregulation of imprinted genes in the cloned mice. Cumulus cells are not cultured prior to nuclear transfer, yet clones derived from these cells also exhibit abnormal expression levels of many imprinted genes. Thus, *in vitro* culture can not be the sole cause of disrupted imprinted gene expression in cloned animals. Since the number of gene expression abnormalities were comparable in clones derived from cumulus cell and ES cell donor nuclei, our results are not consistent with the claim that clones from somatic donor nuclei are more "normal" than those derived from ES cell donors[6].

The altered expression of hundreds of genes in NT placentas may be related to the high mortality rate of cloned embryos during in utero development. Because of the atypical maternal-fetal environment during gestation of cloned embryos, even surviving clones may not be normal at birth and/or later in life. Gene expression changes in livers of cloned pups were less pronounced than in the placentas and affected a largely distinct set of genes. As the trophectoderm is the first lineage to be established in the embryo, eventually giving rise to the placenta, the reduced time period available for reprogramming in this lineage may contribute to the increased relative severity of placental phenotypes. The use of tetraploid embryo complementation to generate mice derived from ES cells as a comparison to those derived by nuclear transfer provides a means to begin distinguishing phenotypes that are attributable to specific aspects of cloning.

Our results are consistent with the hypothesis that most clones, independent of their cellular origin, may have gene expression abnormalities causing subtle phenotypes[18]. Recent studies showing premature death, pneumonia, hepatic failure[19] and obesity[20] in aging cloned mice could be a consequence of these gene expression abnormalities. Thus, the conclusion that cloned adult animals are "normal" should not be based on superficial clinical examinations[21] but rather on detailed molecular analyses of tissues from adult cloned animals.

Methods

RNA preparation and array hybridization. RNA was isolated from mouse placentas after C-section at term as previously described[5]. The cloning efficiencies for the ES cell NT mice have been reported [5,7] and the survival rates of the cumulus clones were similar to previous reports [22]. Preparation of targets, hybridization, washing, and scanning were carried out with slight modifications to protocols previously described[23]. A total of 24 placental samples were examined on Affymetrix arrays. In the first set of experiments, the following nine placental samples were hybridized to Murine genome U74A version 1 arrays: two 129/Cast normal controls, one B6/DBA2 placenta derived from normal zygotes cultured *in vitro* to the blastocyst stage before embryo transfer, two 129/Cast placentas derived by ES cell ($F_{1.2\text{-}3}$ line) NT, two 129/Cast placentas derived by cumulus cell NT, and two B6/DBA2 placentas derived by cumulus cell NT. An additional 15 samples were analyzed on Murine genome U74A version 2 arrays: five B6/129 normal control placentas (3 female, 2 male), five B6/129 placentas derived by ES cell (V6.5 line)

NT, and five DBA2/Cast placentas derived by cumulus cell NT. The version 1 arrays contained 2,608 non-functional probe sets out of the 12,654 on the array.

Twenty liver samples were analyzed; ten samples on each array version. The first comparison on version 1 arrays were made using the following ten samples: two 129 normally developing mice, three tetraploid embryo complementation mice using a V6.5 ES cell subclone #89[5], three ES cell NT mice from V6.5 subclone #89, and 2 ES cell NT mice from a J1 line. In the second set of experiments using array version 2, we used another ten samples: three normally developing mice (two B6/DBA2 F1, one B6/129 F1), three tetraploid embryo complementation mice using a V6.5 ES cell subclone #23, two ES cell NT mice from V6.5 subclone #23, and two B6/DBA2 F1 cumulus cell NT mice.

Analysis of array data. Intensity values on each array were scaled to the first control array such that plotting the data sets on two axes gave a slope of one and a y-intercept of zero. Genes with a calculated expression level below 50 units were set to a value of 50 units. A multiple hypothesis testing correction was performed using both a multiple testing under dependency false discovery rate (Benjamini, Y and Yekutieli, D. The control of the false discovery rate in multiple testing under dependency. Thesis. Tel Aviv University; see http://www.math.tau.ac.il/~ybenja/depApr27.pdf) and a Westfall-Young stepdown algorithm family-wise error rate (see Dudoit, *et. al.*, UC Berkeley technical report #578, (2000), http://www.stat.berkeley.edu/tech-reports/) when calculating adjusted p-values. The expression of many genes varied considerably in the controls. In analysis of the combined data sets, the standard deviation as a percentage of the average expression (CV) for each gene was calculated using the 8 control samples, including the

IVC control. Only those genes with a CV of less than 0.25 were included in the final candidate list. This eliminated sex specific genes such as *Xist* and other genes which proved by Northern analysis to have highly variable expression among isogenic controls. The only ES cell NT data used to generate Table 2 were for the animals derived by NT from the V6.5 ES cell subclones, the J1 line data was excluded. In generating predictors, both weighted voting and k-nearest neighbor algorithms were used.

Northern analysis. Northern analysis was performed as previously described[5] using the same probes for *H19*, *Peg1/Mest*, and *Meg1/Grb10*. Probes for *Vanin-1* and *Carbonic anhydrase 2* were generated from IMAGE clones 517746 and 1481304, respectively.

Acknowledgements

We thank Pablo Tamayo and Matt Tudor for assistance in data analysis. This work was supported by the Victoria and Bradley Geist Foundation, the Kosasa Family Foundation, and the Harold Castle Foundation to R.Y. and by NIH grants 5-R35-CA44339 and RO1-CA84198 to R.J. This work was supported in part by grants from Affymetrix, Inc. and Bristol-Myers Squibb Company.

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Chapter 4.

Perspectives

Few cloned animals survive to birth and those that do survive frequently show abnormalities. To determine the extent of gene dysregulation in neonatal clones, we have analyzed imprinted and global gene expression profiles in cloned mice derived from cumulus and ES cell donor nuclei. Many imprinted genes were expressed at abnormal levels in the term placentas of both ES cell and cumulus cell clones. The abnormal expression of a few imprinted genes was specific to the ES cell NT placentas and was likely due to epigenetic instability at those loci during *in vitro* culture of the ES cell donors. These particular imprinted genes were similarly affected in the tissues of ES cell NT mice; however, imprinted gene expression in the neonatal tissues otherwise appeared less affected than in the placenta. Similarly, global gene expression in the placentas was also dramatically affected. Most of the genes affected were commonly dysregulated in both the ES cell and cumulus cell NT placentas. Importantly, other genes showed significant expression differences between the two types of NT placentas. Significant differences in gene expression in the liver between the NT and controls neonates were also observed, but generally a different set of genes were affected than in the placentas. Our results indicate that widespread gene dysregulation occurs in clones, particularly in the placenta, and that some expression changes are specific to the donor cell.

To date, efforts have been made to describe gene expression in cloned preimplantation embryos and neonates. While changes in gene expression clearly exist, it remains to be established whether there are a few key genes responsible for the cloning phenotypes. Gene expression profiling at more timepoints in gestation may give insights into the gene expression changes leading to *in utero* death. Unfortunately, our results indicate that many genes are affected to varying levels and thus it may be exceedingly difficult to identify which genes are most responsible for mortality at various developmental stages. Further analysis may elucidate if instead of a few key genes being important, it is the cumulative effect of many abnormally regulated genes in embryogenesis that leads to death and other abnormalities. This type of analysis will likely contribute to an understanding of normal development, including identification of genes whose expression levels can be variable without causing death or other phenotypes.

The severity of placental phenotypes suggests that reprogramming may be particularly inadequate in cells giving rise to the placenta. This explanation is consistent with the fact that the placenta is derived from the trophectoderm which, as the first embryonic lineage specified, has the least time for reprogramming. One means of addressing this issue is to determine whether using extra-embryonic derived cells (e.g. trophectodermal cells) for NT affects placental phenotypes. From a practical standpoint, the placental phenotypes observed in cloned animals may be circumvented by using cloned ES cells in tetraploid embryo complementation, so that cloned cells only give rise to the embryonic lineages while the tetraploid host blastocyst give rise to the placenta. A comparison of such animals with NT clones, non-cloned ES cell-tetraploid animals, and controls should be insightful.

Since the nuclei of various donor cells appear to have different developmental potentials and our results indicate that some expression differences are specific to given donor cell types, one question is whether clones derived from different donor cell types give rise to specific phenotypes. Recently described abnormalities in adult mice suggest that this may be the case. For example, while NT mice derived from cumulus cells became obese(Tamashiro et al., 2002), NT mice derived from Sertoli cells in another study did not show altered weights, but died prematurely(Ogonuki et al., 2002).

Since the creation of Dolly just five years ago, the cloning field has rapidly advanced to the point where clones have been derived from a number of different cell types and mammalian species. However, cloning remains inefficient in all species and many clones show abnormalities. Despite this fact, several groups are proposing to do human reproductive cloning. It still remains a mystery how the oocyte is able to successfully reprogram the nucleus of a differentiated cell and whether rare surviving somatic clones are actually derived from the nuclei of fully differentiated cells. Many important developmental processes such as X-inactivation, genomic methylation, imprinting, and gene expression are now being described in cloned animals. Our results indicate extensive abnormal gene expression in neonatal mice and their placentas and thus support the notion that reprogramming of nuclei in clones is rarely, if ever, complete. These expression abnormalities and other phenotypic abnormalities now described throughout the entire developmental lifespan of cloned animals indicate that proposals to do human reproductive cloning are indefensible.

One of the most important unresolved questions in the cloning field is that of the initially stated question in the earliest nuclear transplantation studies. The question still

remains whether some differentiated cell nuclei acquire irreversible genetic changes that limit their developmental capacity. While many nuclear changes during development are largely reversible by reprogramming in the oocyte and early embryo, genetic changes should not be reversible. For example, it is known that lymphocytes undergo genomic rearrangements at their immunoglobulin and T cell receptor loci, in B and T cells, respectively. It has been shown recently that nuclei of both of these cell types can be reprogrammed to give rise to an entire mouse with these rearrangements intact(Hochedlinger and Jaenisch, 2002). This also illustrates that the rest of the genome remained capable of directing development. However, the possibility remains that the nuclei of other cells types may have other genomic rearrangements that would prohibit development following nuclear transfer. Genomic rearrangements might be expected and have been proposed in other developmental systems where cellular diversity is required, such as in the brain and olfactory epithelium. In the brain an abundantly expressed class of genes, protocadherins, has a genomic structure reminiscent of the immunoglobulin and T cell receptor loci (Wu and Maniatis, 2000). There is also data indicating that genes involved in genomic rearrangements in lymphocytes are expressed and required in the brain (Gu et al., 2000). To define how amenable various differentiated donor cell nuclei are to reprogramming such experiments will require the use of donor cells of a clearly defined differentiation status, with unambiguous molecular markers.

Nuclear transfer is therefore a useful tool for determining whether genomic modifications, occurring either developmentally or sporadically, are genetic or epigenetic. The nature of the changes in various nuclei and their ability to be reprogrammed can be tested. For example, transplantation of nuclei from tumorigenic

cells (attempted as early as 1965 in amphibians(King and DiBerardino, 1965)) could help decipher genomic changes in the donor cells because abnormalities resulting from epigenetic changes are likely to be reprogrammed while genetic changes cannot. Cloning can also be used to elucidate developmental processes that occur in a small number of non-replicating cells since it enables the propagation of nuclear information into a large, clonal population of cells that can be analyzed both *in vivo* and *in vitro* by derivation of NT ES cell lines.

A better understanding of early embryonic events and their molecular components may enable designs for improving reprogramming. For example, the genes responsible for early demethylation of the genome are unknown and yet likely important to the reprogramming process. Furthermore, elucidation of the factors that protect sequences from erasure of methylation, as in the case of imprinted genes, and the specificity involved in subsequent *de novo* methylation events in embryos should contribute to a better understanding of how reprogramming aberrations becomes propagated through development. Aside from DNA methylation, very little is known about epigenetic information in cloned animals; information pertaining to chromatin structure would also help assess reprogramming in the clones. It is also of interest to determine if inadequate reprogramming might occur over large genomic regions or rather in a gene-specific fashion.

The potential benefits of nuclear transfer for therapeutic purposes are enormous. In contrast to reproductive cloning, which involves *in utero* development of a clone in a host female, therapeutic cloning restricts the development of an NT embryo *in vitro* to the point where a source of pluripotent cells, ideally ES cells, can be isolated and

differentiated into a particular subset of genetically equivalent cells for treating a patient. Thus, while reproductive cloning necessitates that a nucleus become reprogrammed sufficiently to enable proper development of all lineages through all stages of development, therapeutic cloning merely requires that ES cells differentiate into a set of cells that are capable of functioning within an adult organism. By going through an ES cell intermediate during development, as is done in therapeutic cloning, the cloned nuclei may also benefit from the additional time permitted for reprogramming. An *in vitro* selection event may also take place such that cells in the ICM which had undergone more extensive reprogramming will be the ones that develop into the ES cell lines. Due to these differences, we do not foresee the abnormalities associated with reproductive cloning as being a significant factor in applications of cloning for therapeutic purposes.

Epigenetic abnormalities that arise during the *in vitro* culturing of ES cells may affect their suitability for therapeutic applications. However, the fact that ES cells can contribute extensively to apparently normal chimeras at high frequencies following diploid blastocyst injection shows that ES cells can function within the context of an otherwise normally developing organism. Furthermore, while imprinted genes are frequently affected in ES cells, the defined effects of imprinting are primarily for control of embryonic growth and not differentiation and should not pose a significant problem for therapies. Nevertheless, it will be useful to further define the effects of cell culturing on epigenetic regulation in ES cells, especially in species other than the mouse, and to determine whether they can be minimized. Recently, the efficacy of therapeutic cloning has been shown by the generation of pluripotent ES cell lines from cloned blastocysts(Hochedlinger and Jaenisch, 2002; Kawase et al., 2000; Wakayama et al.,

2001) and by therapeutic cloning as modeled in *Rag2* deficient mice(Rideout et al., 2002). At this time, the main challenges for therapeutic cloning are not in the generation of cloned pluripotent ES cell lines, but rather in learning how to differentiate those lines into the appropriate lineages and how to achieve engraftment of the differentiated cells (Rideout et al., 2002).

To better understand how a somatic nucleus becomes reprogrammed by an oocyte, it will be important to compare the ability of other pluripotent cell types, such as embryonic and somatic stem cells and germ cell derived lines, to reprogram somatic nuclei. Such a comparison may provide additional information about the machinery necessary for various aspects of reprogramming and what machinery is lost in these cell types. Ultimately, reprogramming may be improved upon in those cell types. If extracts or cell types other than oocytes can be used to reprogram a donor cell without going through an embryonic intermediate, the ethical issues associated with therapeutic cloning would be circumvented.

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Appendix

Identifying imprinted genes by comparison of *Dnmt1* rescued cells with wildtype cells

Introduction

Imprinted genes are a class of mammalian genes whose expression is dependent on their parental origin such that a given gene will be expressed only from its maternally or paternally inherited copies. A few sites in imprinted genes retain their methylation status during the global demethylation and *de novo* methylation in the early embryo and may serve as a signal in the imprinting process (Brandeis et al., 1993). *Dnmt1* mutant mice are embryonic lethal (E8-E10) and have negligible methylation at all examined sequences (Li et al., 1992). Furthermore, monoallelic expression of imprinted genes is not maintained in these mutant embryos (Li et al., 1993). *Dnmt1*$^{-/-}$ ES cells are capable of proliferating but die upon differentiation and cannot contribute to embryonic lineages when injected in blastocysts.

Expression of the wild-type *Dnmt1* cDNA in *Dnmt1*$^{-/-}$ ES cells with a cDNA homologous insertion protocol (CHIP) increases global methylation but fails to restore methylation of certain imprinted genes (Tucker et al., 1996). These "rescued" ES cells can be *in vitro* differentiated although they show no expression of the imprinted genes *Igf2* and *Igf2r,* and *H19* overexpression. Rescued ES cells contribute to chimeras and their imprints are restored after the cells are transmitted through the germline(Tucker et

al., 1996). Since the hypomethylation associated with some of these imprinted genes in rescued cells correlates with their inactivation, we have compared rescued fibroblasts cultured from chimeras with isogenic controls in an attempt to identify new imprinted genes that have been silenced in these rescued cells. We have used cDNA subtractions to selectively amplify genes not expressed in the rescued cells by using cDNA from these cells as a driver and control cDNA as a tester. Although three known imprinted genes were dramatically enriched in our subtracted pool by two indepenedent subtraction techniques, no new imprinted genes were identified.

Methods

We have generated chimeras by injecting diploid Balb/c blastocysts with *Dnmt1*$^{-/-}$ CHIP rescued ES cells (Tucker et al., 1996). Primary mouse embryonic fibroblasts (mEFs) were generated from two embryos with pigmented eyes at E14.5. Rescued cells were selected out of this culture with puromycin (2ug/ml) for four days. The same puromycin selection on a normal mEFs was sufficient to kill all of the cells in the culture. Rescued cells were grown without selection for another 4 days prior to isolating RNA. Control primary mEFs were derived from strain and sex-matched embryos cultured without puromycin for an equivalent period of time. Immortalized lines were also generated from these cells with SV40 large T antigen as previously described(Jat et al., 1986).

Two independent cDNA subtractions between the rescued and control mEFs were performed. One subtraction was performed according to the commercially available PCR-Select protocol (Diatchenko et al., 1996) (Clontech). The second subtraction

technique employed was a modified (D. Menke, personal communication) SABRE protocol (Lavery et al., 1997). This protocol is illustrated schematically and described in Figure 1. This protocol uses two parallel subtractions. cDNAs were synthesized from the rescued mEF and control mEF RNAs. Each cDNA pool was divided and cut with *AluI* or *AluI* and *RsaI*. The indicated adaptors were then ligated to the digests. We used 129 control mEF cDNA as tester and rescued mEF cDNA as driver.

Results

Before proceeding with the subtraction we first confirmed by Northern analysis that both *Igf2* and *Igf2r* were silenced in the primary and immortalized cells used in this study(Figure 2). Two cDNA subtractive hybridizations were performed to identify candidate imprinted genes. With the first subtraction protocol (Clontech), involving a single round of subtraction, we were able to demonstrate a substantial enrichment in the subtracted product for cDNA fragments of two imprinted genes, *Igf2* and *Igf2r*, previously demonstrated to be downregulated in the rescued cells (Figure 3, lane CT_s vs CT_u). The second subtraction technique (modified SABRE), involving multiple rounds of subtraction, also dramatically enriched for cDNAs corresponding to these two genes(Figure 3, lanes F_4 and F_{4x} vs F_0). An initial sequencing of clones from the final subtracted pools revealed that another imprinted gene, *Meg1/Grb10*, was represented and we confirmed that it was also significantly enriched in each of the subtractions (Figure 3). We also determined by Norhtern analysis that *Meg1/Grb10* was silenced in the rescued cells (Figure 2). The clones of the subtracted products were first analyzed by probing Southern blots containing the clones with a cocktail of known imprinted genes. The

known imprinted genes comprised as much as 60% of the final subtracted pool in the final round of the SABRE protocol and only 10% of the subtracted product in the Clontech protocol.

The SABRE protocol also appeared more effective in eliminating transcripts common to both the tester and driver pools based on the reduction of *Gapdh* in the subtracted product (Figure 3). It was confirmed by sequencing and Southern analysis that other abundant sequences were eliminated more effectively in the SABRE protocol. Over 50% of the clones in the Clontech protocol were shown to corresponded to abundant transcripts while only about 10% corresponded to abundant transcripts in the modified SABRE protocol. Remaining clones were sequenced until genes represented by multiple independent fragments were identified. We attempted to screen for clones that were present in the initial tester but not the driver by using individual clones as probes on Southerns containing the tester and driver pools. Clones showing any indication of differential expression were used as probes for Northern analysis of the rescued and control RNAs. While clones corresponding to *Igf2*, *Igf2r*, and *Meg1/Grb10* met these criteria, no additional clones were shown to be silenced in the rescued cells by this approach.

Discussion

We were able to isolate by cDNA subtraction the two imprinted genes, *Igf2* and *Igf2r*, previously demonstrated to be silenced in the *Dnmt1*$^{-/-}$ rescued cells and one additional known imprinted gene, *Meg1/Grb10*. The ability to identify these imprinted genes validates both the experimental design as well as the subtraction method employed.

However, as we were not able to identify any other novel imprinted genes with this strategy, we can assume that there may not be many imprinted genes whose expression is abolished in the absence of methylation or whose expression in mEFs is normally high enough to be isolated by these techniques. Given the effectiveness of the subtraction itself, it might thus be possible to examine other differentiated cell types. In addition, the subtraction might be further improved by include cDNAs corresponding to known imprinted genes in the driver so these sequences do not rapidly take over the enriched population.

These strategies are designed only to detect imprinted genes where the normally active allele is silenced; however, in many cases the silent allele may be activated instead and detecting a two-fold expression difference is difficult. Using Affymetrix expression chips, we have also compared the rescued mEFS with controls. While *Igf2* showed a dramatic silencing in the rescued cells by this assay, few other genes showed large, reproducible changes. The most promising candidates based on these data failed to show significant differences by Northern.

Rather than using a cDNA comparison or subtraction, it may also be possible to identify genomic sequences corresponding to differential methylated regions (DMRs), using other methods. For example, we have previously performed a genomic subtraction that could ideally isolate any DMRs that remained biallelically hypomethylated in the rescued cells (unpublished data). This subtraction compared fragments from genomic pools of rescued and normal cells based on their ability to be bound by a column containing the methyl CpG binding domain (MBD) of MeCP2. DMRs of imprinted genes should be bound in wildtype but not rescued cells. A genomic subtraction was

used in which bound fractions from wildtype cells were used as a tester and bound fragments in rescued cells were used as a driver. While several DMRs were shown to elute in the expected fractions, enriching for these fragments via subtraction was unsuccessful. Many repetitive sequences were present in the final subtraction products and many of these repetitive sequences showed significant methylation differences between the *Dnmt1* rescued and control cells (data not shown). Because of the methylation differences still present at non-imprinted genes between the rescued and control cells, future subtractions would likely be better suited to rescued cells showing a higher DNA MTase levels than the CHIP allele, which has reduced protein levels compared to a normal allele(Biniszkiewicz et al., 2002).

Acknowledgements

I would like to thank Doug Menke for developing the details of the iterative subtraction protocol used in this analysis and for advice in proceeding with the subtraction.

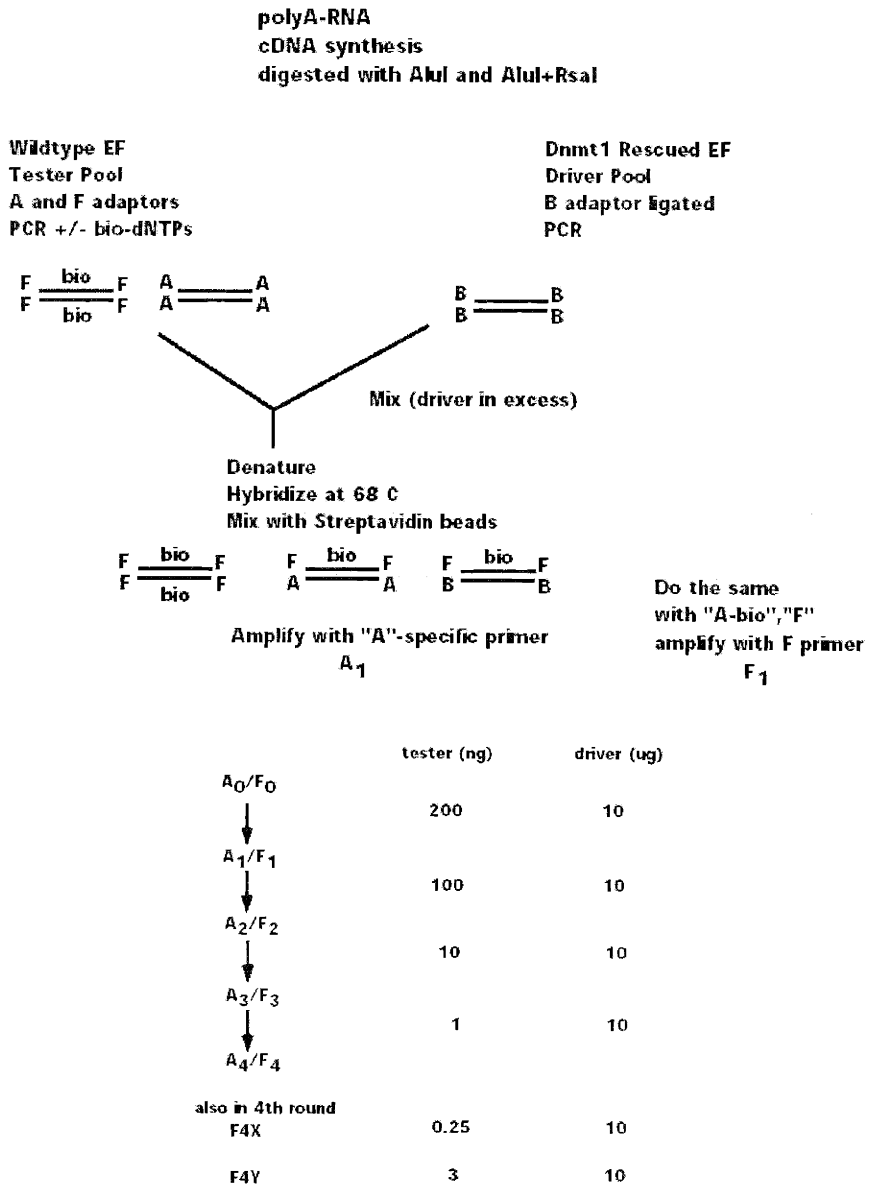

Figure 1. Schematic diagram of SABRE-modified subtraction. Adaptors A and F were ligated to the control mEF cDNA digests. Adaptor B was ligated to the rescued mEF cDNA digests. The tester cDNA were then amplified with primers corresponding to the adaptors using biotinylated and non-biotinylated dNTPs, and the driver was amplified with non-biotinylated dNTPS. Two subtraction are set up: 1) "F-bio" and "A" tester with "B" driver in excess, and 2) "A-bio" and "F" tester with B in excess. Samples were

denatured at 95°C for 15 min and ramped down to 68°C over 45 minutes. Hybridization was continued for 2-4 days with longer hybridizations in later rounds. Streptavidin beads were used to purify biotinylated cDNAs as described(Lavery et al., 1997). To selectively amplify the cDNAs common to the testers and not the driver, PCR amplification is done with the primer corresponding to the adaptor of the non-biotinylated tester of that hybridization. This generates the tester for the next round. The amounts of tester and driver are indicated for each round of subtraction. In the final round, three different pairs of subtractions were performed in parallel with the indicated ratios of tester:driver.

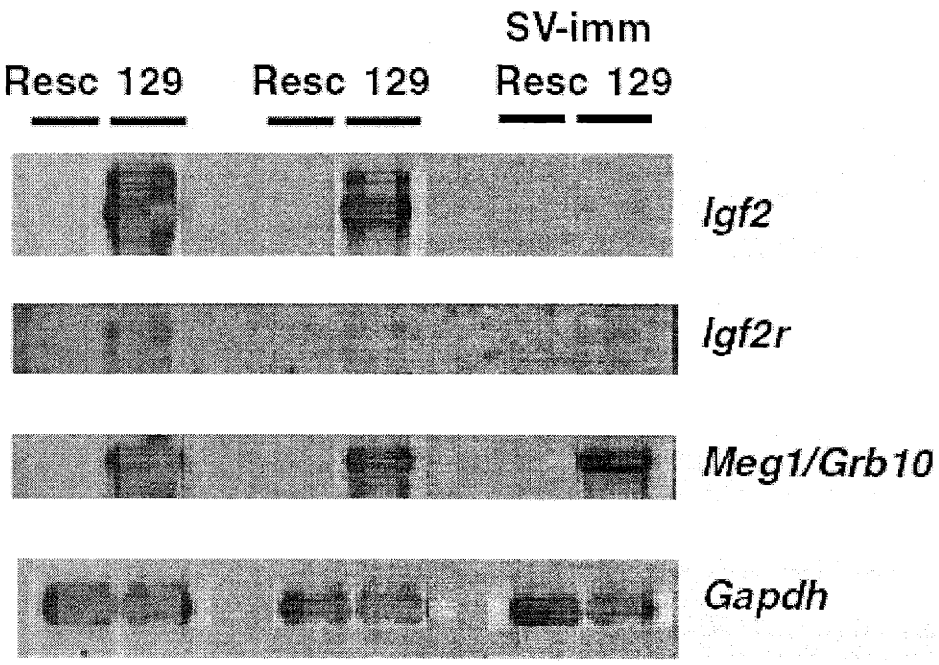

Figure 2 Northern analysis of *Dnmt1* rescued and 129 control mEFs each derived from independent mice. The last two lanes show expression following immortalization of these cells with SV40 large T antigen.

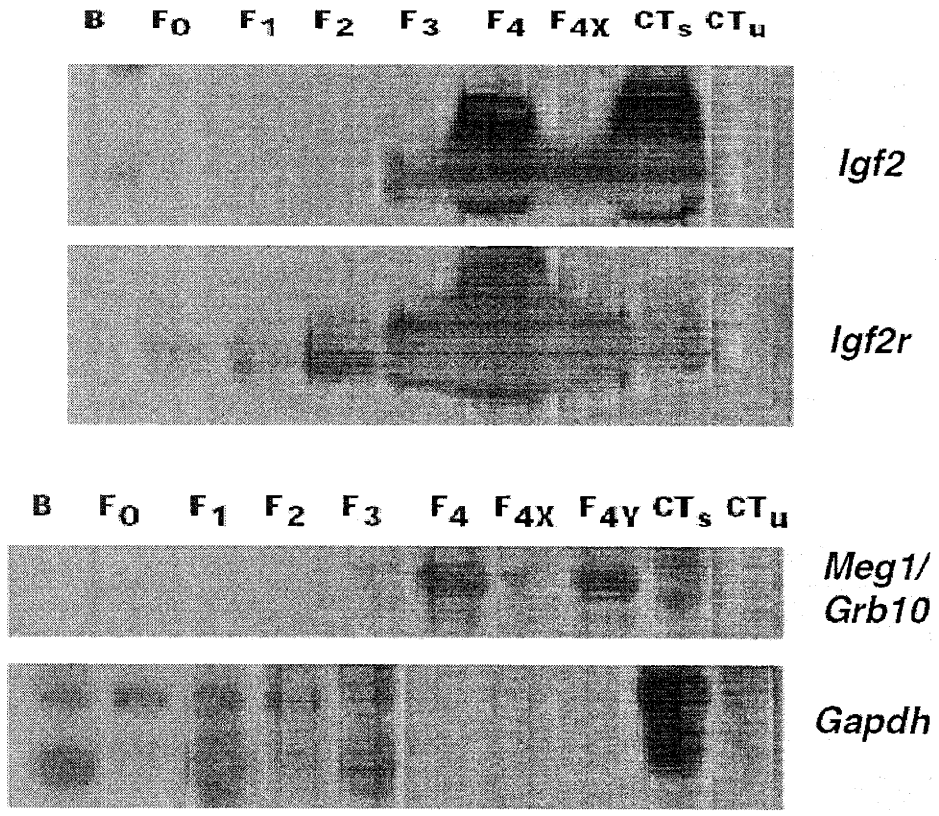

Figure 3 Southern analysis of equal quantities of amplified driver and tester probed with imprinted genes and *Gapdh*. B= driver, $F_{\#}$ = subtracted product at round # of iterative protocol (in the 4th round of subtraction, three different tester:driver ratios were tested, see Figure 1). CT_s= subtracted product after Clontech protocol, CT_u= unsubtracted pool in Clontech protocol.

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