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REVIEW

Nuclear Cloning and Epigenetic Reprogramming of the Genome

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Cloning of mammals by nuclear transfer (NT) results in gestational or neonatal failure with at most a few percent of manipulated embryos resulting in live births. Many of those that survive to term succumb to a variety of abnormalities that are likely due to inappropriate epigenetic reprogramming. Cloned embryos derived from donors, such as embryonic stem cells, that may require little or no reprogramming of early developmental genes develop substantially better beyond implantation than NT clones derived from somatic cells. Although recent experiments have demonstrated normal reprogramming of telomere length and X chromosome inactivation, epigenetic information established during gametogenesis, such as gametic imprints, cannot be restored after nuclear transfer. Survival of cloned animals to birth and beyond, despite substantial transcriptional dysregulation, is consistent with mammalian development being rather tolerant to epigenetic abnormalities, with lethality resulting only beyond a threshold of faulty gene reprogramming encompassing multiple loci.

Epigenetic modification of the genome ensures proper gene activation during development and involves (i) genomic methylation changes, (ii) the assembly of histones and histone variants into nucleosomes, and (iii) remodeling of other chromatin-associated proteins such as linker histones, polycomb group, nuclear scaffold proteins, and transcription factors (*1*). The two parental genomes are formatted during gametogenesis to respond to the oocyte environment and proceed through development (Fig. 1A). The zygote biochemically remodels the paternal genome shortly after fertilization and before embryonic genome activation (EGA) occurs. To successfully recapitulate these processes, the somatic nuclei transferred into an oocyte must be quickly reprogrammed to express genes required for early development.

Epigenetic reprogramming after fertilization and nuclear transfer has been studied in *Xenopus* and mammals (*1*). Here we will concentrate on aspects of epigenetic gene regulation that are pertinent to our understanding of the reprogramming process after mammalian somatic cell nuclear transfer including chromatin structure, DNA methylation, imprinting, telomere length adjustment, and X chromosome inactivation with a focus on experimental data from the mouse. Also, we will compare and contrast the outcome of cloning experiments when either somatic or embryonic stem (ES) cells are used for nuclear transfer.

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Epigenetic Programming in Normal Development

The programming of the genome that occurs as primordial germ cells (PGCs) differentiate into mature gametes establishes the markedly different chromatin configurations of sperm and oocyte. As demonstrated by normal preimplantation development of uniparental embryos, both parental genomes share the ability to independently direct cleavage (early development to the blastocyst stage) despite profound differences in their epigenetic organization (*2*, *3*). In spermatogenesis, chromatin is sequentially remodeled, silenced, and ultimately compacted with protamines (*4*), processes crucial for normal fertilization (*5*). However, completion of these events is not strictly required for development as normal pregnancies can result from intracytoplasmic sperm injection with round spermatids or secondary spermatocytes (*6*, *7*).

In contrast, the genome of the oocyte is organized in a structure more like that of a somatic cell, with chromatin whose nucleosomes contain an oocyte-specific linker histone, H1oo (*8*). In comparison with the male pronucleus, the female pronucleus is more transcriptionally repressive (*9*–*11*), contains relatively deacetylated histone H4 (*12*), and is deficient in generalized transcription factors (e.g., Sp1) (*13*). This repressive chromatin structure may protect the oocyte genome against the extensive epigenetic modifications imposed on the paternal genome after fertilization.

In addition to differences in chromatin structure between the mature gametes, they also vary in their DNA methylation levels, which are then further modified after fertilization. Overall levels of DNA methylation are low in PGCs and rise during gametogenesis with the sperm becoming more methylated relative to the oocyte (*14*). Repetitive sequences (LINES, SINES, and satellite sequences) that are highly methylated in somatic tissues are not uniformly methylated in the gametes, with some elements methylated more in sperm (*15*, *16*) and others in oocytes (*17*). Methylation differences between the genomes of the gametes are also seen in tissue-specific genes, but most disappear during cleavage (*18*–*20*). Within hours after fertilization, the paternal genome is actively demethylated in contrast to the maternal genome, which appears to be passively demethylated during cleavage (*21*). By the blastocyst stage, the embryo's genome is hypomethylated and subsequently undergoes global de novo methylation, resulting in an apparently uniform pattern of methylation on both parental alleles by gastrulation (*14*, *22*). Recent data show some abnormal methylation at repetitive sequences (*23*) and frequent failure to reactivate Fgf4, Fgf2r, and IL6 (*24*) in nuclear transfer (NT) bovine preimplantation embryos. An unresolved question is to what extent the epigenetic modification of chromatin structure and DNA methylation, which occurs in normal development, needs to be mimicked for nuclear cloning to succeed.

Expression of imprinted genes is controlled by parent-of-origin–specific methylation marks that are established during late stages of gametogenesis (*25*, *26*) and when lost cannot be reset except by passage through the germ line (*27*). In normal embryos, regions of imprinted allele-specific methylation are strictly maintained in the preimplantation embryo and are crucial for later development of the embryo (*25*, *26*). Eventually, imprints are removed from the paternal and maternal genomes in PGCs, probably before mitotic arrest in the testis and meiotic arrest in the ovaries (*28*). Genome-wide imbalance in or disruption of imprinted gene expression results in postimplantation lethality, as demonstrated in uniparental embryos (*2*, *3*) and nuclear transfer embryos derived from male PGCs (*29*) or nongrowing oocytes (*30*).

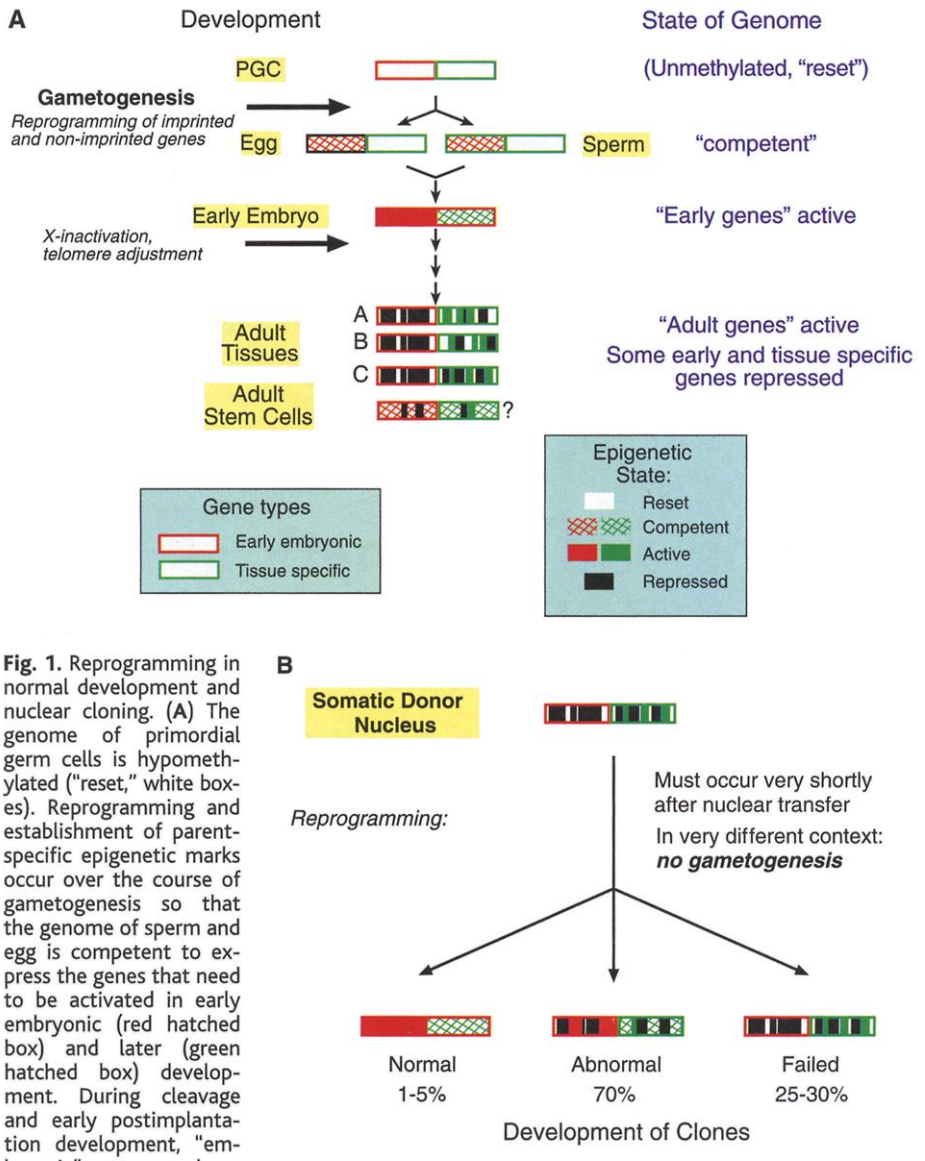

Fig. 1. Reprogramming in normal development and nuclear cloning. (**A**) The genome of primordial germ cells is hypomethylated ("reset," white boxes). Reprogramming and establishment of parent-specific epigenetic marks occur over the course of gametogenesis so that the genome of sperm and egg is competent to express the genes that need to be activated in early embryonic (red hatched box) and later (green hatched box) development. During cleavage and early postimplantation development, "embryonic" genes, such as Oct 3/4, become activated (solid red box) and are repressed at later stages (black boxes) when tissue-specific genes (green boxes) are activated in adult tissues (labeled A, B, and C). Adult stem cells are thought to be less differentiated and may be more effective NT donors because they may require less reprogramming (see text). Epigenetic reprogramming of imprinted and nonimprinted genes occurs during gametogenesis in contrast to X inactivation and the readjustment of telomere length, which take place postzygotically. (**B**) Reprogramming of a somatic nucleus after nuclear transfer may result in (i) no activation of "embryonic" genes and early lethality, (ii) faulty activation of embryonic genes and an abnormal phenotype, or (iii) faithful activation of "embryonic" and "adult" genes and normal development of the clone. The latter outcome is the exception, and the percentage in each category is estimated from data on cumulus cell NT animals (*52*).

Epigenetic Reprogramming in Nuclear Cloning

The epigenetic conformation of any somatic nucleus is markedly different from that of the

mature gametes, and it is remarkable that the oocyte can reverse the epigenetic modifications imposed on the genome during differentiation to recreate a state of totipotency. Little is known about the initial molecular events that accomplish reprogramming in the mammalian oocyte. Transplanted *Xenopus* nuclei lose more than 85% of their protein and simultaneously incorporate a substantial amount of protein from the cytoplasm (*31*). The nucleosomal adenosine triphosphatase ISWI (a member of the SWI2/SNF2 family) appears to play a crucial role in this process by stimulating the release of TATA-binding proteins from the donor nucleus (*32*). In mammals, initial reprogramming events appear to occur uniformly, with transplanted nuclei becoming transcriptionally silent (*33–35*) and near normal transcriptional activity reappearing by the two-cell stage (*33*). These observations suggest that the initial transcriptional activity of the donor nucleus is controlled predominantly by the egg cytoplasm, consistent with active and appropriate chromatin remodeling after NT. However, all information concerning these initial events after nuclear transfer is based on the analysis of pools of embryos. Because cloned embryos derived by NT seem to fail because of stochastic reprogramming errors, the extent of these errors may only become apparent by analyzing gene expression in individual embryos. Emerging experimental evidence is consistent with faulty or incomplete reprogramming causing abnormal development of cloned embryos (see later).

In principle, the poor survival of nuclear clones could be due to genetic or epigenetic abnormalities. Genetic abnormalities have been shown to accumulate during organismal aging and during in vitro cultivation of cells (*36*). However, the high-frequency and cross-species similarities in abnormalities inherent to cloned animals and the normalcy of their natural offspring argue that epigenetic rather than genetic changes are responsible for these developmental problems. Thus, the most likely explanation for the developmental failure of NT embryos is the inability to "reprogram" the epigenetic profile of the somatic donor nucleus to that of a fertilized zygote (*37*).

For clones to complete development, genes normally expressed during embryogenesis, but silent in the somatic donor cell, must be reactivated. During gametogenesis in normal development, a complex process of epigenetic remodeling assures that the genome of the two gametes, when combined at fertilization, can faithfully activate early embryonic gene expression (Fig. 1A). In a cloned embryo, reprogramming has to occur in a cellular context radically different from gametogenesis and within the short interval between transfer of the donor nucleus into the egg and the time when zygotic transcription becomes necessary for further development. We envisage a spectrum of different outcomes to the reprogramming process from (i) no reprogramming of the genome, resulting in immediate death of the NT embryo, through (ii) partial reprogramming, allowing initial survival of the clones but resulting in an abnormal phenotype and/or lethality at various stages of development, to (iii) faithful reprogramming producing normal animals (Fig. 1B). The phenotypes observed in nuclear clones suggest that complete reprogramming is the exception.

For the following discussion, it is useful to distinguish epigenetic changes that normally occur before formation of the zygote from those that are established in the developing embryo after fertilization. For example, the DNA methylation pattern of the genome, including that of imprinted and nonimprinted genes is substantially modified during gametogenesis. In contrast, X chromosome inactivation and the readjustment of telomere length are events that take place postzygotically.

X chromosome inactivation and telomere length. Dosage compensation in mammals is achieved by extinguishing gene expression from one X chromosome in female somatic cells, a process known as X inactivation [for review, see (*38*)]. Both X chromosomes are active during preimplantation cleavage of the female embryo and the choice of which X chromosome to inactivate is random in the embryonic (epiblast) lineage of the embryo. In contrast, an unidentified parental imprint causes preferential inactivation of the paternal X in the trophectoderm (TE), a tissue that contributes to the placenta of the embryo. Using somatic nuclei containing genetically marked X chromosomes as NT donors, X inactivation was random in the epiblast lineage of cloned mice but nonrandom in the TE (*39*). Thus, the epigenetic marks that distinguish the active X from the inactive X in somatic cells are removed and reestablished on either X in the embryonic lineage after NT. In contrast, the TE lineage of the clone showed a nonrandom inactivation pattern dictated by the state of X inactivation in the donor cell and was similar to normal imprinted X inactivation in the TE. Thus, the process of X inactivation is faithfully recapitulated in cloned female embryos.

Telomere length is also adjusted postzygotically in embryos. Once somatic differentiation begins, telomeres progressively shorten in most cells, which raises the question of whether cloned animals inherit the shortened telomeres of their nuclear predecessors, leading to premature aging. Shortened telomeres were seen in the first cloned sheep (*40*) but not in cloned calves (*41*, *42*). When nearly senescent embryonic bovine cells were used as nuclear donors, telomere length and cellular proliferative lifespan were restored and even enhanced by the cloning process (*43*). Telomerase activity is reactivated in cloned embryos to a level similar to that of controls (*41*) and is consistent with restored telomere length seen in cloned calves (*41*, *42*) and mice (*44*). Given the observation that expression of telomerase in senescent somatic cells restores telomere length (*45*), it is likely that enzyme activity in the early embryo readjusts the shortened telomeres of the donor genome. In summary, it appears that postzygotic reprogramming including X inactivation and telomere length adjustment is faithfully accomplished after nuclear transfer and, therefore, would not be expected to impair survival of cloned animals.

Phenotypes of nuclear clones. In all mammalian species where cloning has been successful, at best a few percent of nuclear transfer embryos develop to term, and of those, many die shortly after birth. Abnormalities observed in cloned animals include respiratory distress and circulatory problems, which are thought to be the most common causes of neonatal death (*46–48*). Even apparently healthy survivors may suffer from immune dysfunction or kidney or brain malformation, perhaps contributing to their death at later stages (*43*, *49*). Most frequently cloned animals that have survived to term are overgrown, a condition referred to as "large offspring syndrome" (*48*). In particular, the placenta is often oversized and dysfunctional, a pathology that may contribute to fetal overgrowth (*50–52*) (see Fig. 2). Although a substantially higher fraction of ES cell NT blastocysts survive to term than clones derived from somatic donor cells (*53–55*) (see section on "ES cell versus somatic donor nuclei: developmental potency"), similar phenotypic abnormalities are seen in both types of clones. Because ES cells can be cultured indefinitely and both genetically and epigenetically manipulated, they provide an excellent tool to study the relative roles of epigenetic and genetic errors in the development and survival of clones.

Possible explanations for the abnormal phenotypes of clones include reprogramming errors, epigenetic damage incurred during in vitro cultivation of embryos before their transfer into the uterus, and undefined parameters of the nuclear transfer procedure itself that could somehow affect development of the clone. To distinguish between these possibilities, Eggan *et al.* produced mice composed exclusively from ES cells by tetraploid complementation and compared with them NT embryos (*55*). Embryos made by tetraploid complementation with ES cells develop into conceptuses where the embryo proper (epiblast) is composed entirely of the injected ES cells and the TE lineage arises from the tetraploid host cells (*56*). Comparing embryos derived by both methods from the same ES cell line is informative as to the mechanisms of abnormal phenotypes: Any shared phenotypes are likely due to inherent genetic or epigenetic deficiencies in the donor cells, whereas differences would suggest problems

arising as a consequence of nuclear transfer. Indeed, a significant increase in birth weight was seen in nuclear clones relative to newborns derived by tetraploid complementation (*55*). This phenotypic difference may be caused by the nuclear transfer procedure or may be due in part to the abnormal placenta specific to NT pups. In contrast, long-term survival of both clones and mice derived by tetraploid embryo complementation was strongly determined by the genetic background of the donor ES cells: When the ES donors cells were derived from several inbred strains, all mice died at birth because of respiratory distress. In contrast, F_1 ES cells of various genetic backgrounds generated adult mice by either technique (*55*). Thus, animals derived exclusively from ES cells, either from a single nucleus by NT or from several cells as in tetraploid complementation, may suffer from similar phenotypic abnormalities. In contrast, standard diploid chimeras do not show these abnormalities because the presence of cells from the fertilized embryo ensures a normal phenotype.

ES cell versus somatic donor nuclei: Developmental potency. Nuclear cloning of both ES and somatic cells is inefficient. However, the timing of developmental arrest is influenced by the origin of the donor nucleus. Between 60 and 70% of embryos derived from the transfer of somatic nuclei into the oocyte survive to the blastocyst stage. In contrast, only 10 to 20% survive after transfer of an ES cell nucleus (*51, 52, 55*) (Table 1). This difference in initial survival of clones is likely due to differences in the cell cycle stage of the donor cells, as only G_0 or G_1 nuclei appear to be efficient in promoting development of NT embryos (*57, 58*). In contrast, because about 60% of ES cell populations are in S phase (*59*), most NT embryos derived from randomly picked ES cells arrest. Developmental failure likely arises as the result of an S-phase nucleus being exposed to high levels of MPF (maturation promoting factor) activity in the MII oocyte that induces nuclear envelope breakdown and premature chromatin condensation. After activation and formation of the pseudopronucleus, rereplication of the DNA may occur resulting in abnormal ploidy (*60*). However, ES cell NT embryos that reach the blastocyst stage, presumably originating from G_1 cells, develop to term at a 10- to 20-fold higher efficiency than those from any somatic donor cells (Table 1). This observation suggests that the nucleus of an undifferentiated embryonic cell might be more amenable to, or require less reprogramming, than the nucleus of a differentiated somatic cell. The epigenetic state of the genome in an ES cell may more closely resemble that of the early embryo, which enables ES cells to serve as more effective nuclear donors.

Direct comparison of cloning efficiency between embryonic and somatic cells has been done only in the mouse. However, species-specific differences in somatic NT clone survival are seen, with 5 to 10% of transferred embryos giving viable offspring in cows (*61*, *62*) and goats (*63*). The timing of EGA (2-cell in mice compared with 8-16 cell in ungulates) (*1*) may permit more extensive "reprogramming" of the somatic donor nucleus before the developing embryo requires zygotic gene transcription. Surviving cloned goats and pigs appear to lack the placental abnormalities seen in other species (*63*, *64*) [but see (*65*)], suggesting either a more stringent requirement for normal placental development in utero or species-specific differences. Nonetheless, most of the somatic NT clones from all species die between the blastocyst stage and birth.

Imprinted genes. The most common phenotypes observed in animals cloned from either somatic or ES cell nuclei are fetal growth abnormalities such as increased placental and birth weight. Because similar phenotypes have been observed in human patients and in mice as a consequence of both naturally occurring and targeted mutagenesis of imprinted genes, these apparent similarities suggested that aberrant expression of imprinted genes might cause some of the abnormalities seen in clones (*66*). When imprinted gene expression was analyzed in clones derived from ES cell nuclei, considerable differences were apparent and few if any of the analyzed clones showed normal expression of all tested genes (*67*). Altered expression of a particular imprinted locus did not correlate with the expression at any other imprinted locus, suggesting that dysregulation of imprinted gene expression was due to a stochastic process. Importantly, in a given cloned mouse, no substantial correlation was seen between the abnormal expression of any single imprinted gene and the degree of anomalous fetal overgrowth. However, the observed overgrowth in most cloned fetuses and placentas may result from the cumulative dysregulation of several imprinted genes, which can have opposing influences on fetal growth (*25*, *26*). Therefore, it might be expected that the effect of a single imprinted gene could be insufficient to produce a substantial correlation with abnormal growth.

That normal imprinting can be disturbed in animals by in vitro cultivation of preimplantation embryos emphasizes the vulnera-

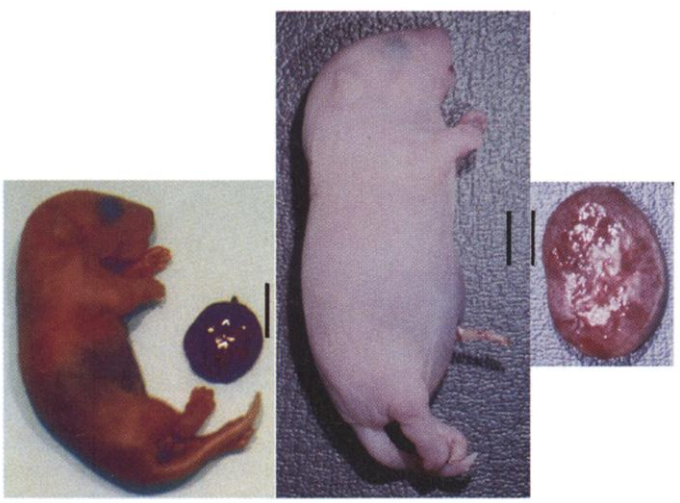

Fig. 2. Photographs of ES cell–derived mice at term. The pup and placenta on the left are derived from tetraploid embryo complementation and appear grossly normal. In contrast, the pup on the right, derived by nuclear transfer of the same ES cell line, shows a dramatic example of the commonly observed overgrowth phenotype seen in cloned mice. Extensive fetal and placental overgrowth are observed accompanied by edema, and this animal did not survive. Scale bars, 1 cm. [from Eggan *et al.* (*55*); copyright 2001 National Academy of Sciences, U.S.A.]

Table 1. Development of ES cell and somatic cell NT embryos. A substantially lower proportion of ES cell–derived NT embryos reach the blastocyst stage than do embryos derived from somatic cells. About 60% of the cells in actively growing ES cell cultures are in S phase of the cell cycle; in contrast, cumulus cells or starved fibroblasts are in phase G_0 or G_1. Because NT embryos derived from S phase donor nuclei are expected to arrest during early cleavage because of DNA replication errors, only those ES cell NT embryos derived from G_1 cells are expected to cleave successfully, whereas most somatic cell NT clones reach the blastocyst stage. However, ES cell NT embryos that reach the blastocyst stage complete development to term at a 10- to 20-fold higher efficiency than blastocyts derived from any somatic donor cells.

Donor cell nucleus	Development to			References
	Blastocyst	Newborn*	Adult*	
ES cell				(*53–55*)
Inbred	10–20%	5–21%	0 (inbred)	
F_1			15% (F_1)	
Cumulus cell	70%	2–3%	1–2%	(*52*)
Fibroblast	58%	0.5–1%	0.5%	(*51*)

*Percentage of transferred blastocysts.

bility of imprinting marks to environmental influences (*68–70*). Importantly, the frequent misexpression of imprinted genes in surviving clones illustrates that development can tolerate substantial errors in imprinting. This conclusion is supported by preliminary evidence from expression profiling approaches suggesting that expression of imprinted as well as nonimprinted genes varies widely between individual somatic and ES cell NT clones (*71*). It will be important to investigate whether phenotypic abnormalities in NT animals can be traced to specific genes or whether errors in reprogramming lead to a stochastic dysregulation of many genes.

Preexisting errors versus faulty reprogramming. The abnormal phenotype of cloned animals could be due to faulty epigenetic reprogramming or to preexisting epigenetic errors in the donor nucleus. Errors in the donor nucleus would be expected to increase with the age of the donor animal and/or the length of in vitro cultivation of the donor cells, whereas faulty reprogramming may depend on the cell type of the donor nucleus. Conflicting evidence on the age dependency of nuclear cloning efficiency has been published. In bovine, no obvious difference was seen between fibroblasts from young and old donors or between low- and high-passage fibroblasts (*72*, *73*). In contrast, cloning problems in the sheep appear to increase with the passage number of the donor cells (*46*, *49*, *74*). However, because the overall efficiency of nuclear cloning was low in these experiments, it is difficult to ascertain the importance if any between the different experimental conditions.

The length of in vitro culture of ES cells has shown a direct correlation with the frequency of errors in imprinting (*75*), and notable alterations in imprinted gene expression were seen among different ES cell lines (*67*). Although ES NT mice had roughly similar imprinted gene expression as the donor cell line, substantial variation of imprinting was seen between mice derived from nuclei of sister cells from a given ES cell subclone (*67*). This indicates that the epigenetic state of the ES cell genome is highly unstable and that preexisting epigenetic aberrations present in ES cell populations can cause gene dysregulation of imprinting in ES cell NT embryos.

Given the extreme epigenetic instability of ES cells, it raises the possibility that in vitro cultivation of any cell type may result in a similar loss of normal imprinting. It will be important, therefore, to compare imprinted gene expression in clones derived directly from primary cells and from other cultured donor cells such as tail-tip and embryonic fibroblast. Indeed, preliminary results are consistent with imprinted gene expression being more faithfully recapitulated in cumulus cell as compared with ES cell NT pups (*71*) (see below), suggesting that imprinting errors are infrequent in vivo and are primarily enhanced by in vitro cultivation.

In vitro culture-induced epigenetic instability is also relevant to the potential applications of human ES cell technologies in the clinic. ES cells can differentiate into many different cell types, which someday might be used in transplantation medicine (*76*). Therefore, 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 their capacity to in vitro differentiate to many different cell types nor does it impede their potential to generate normal chimeric mice as is routinely done in many laboratories. Also, it is worth noting that expression of imprinted genes is important for embryonic development to proceed normally but is of little or no relevance to proper function of differentiated cells in the postnatal animal. Therefore, proper function of differentiated cells derived in vitro from ES cells may not be impeded by dysregulation of imprinted genes.

ES cells, somatic cells, and epigenetic reprogramming. Because widespread dysregulation of imprinting was seen in ES cell but not in cumulus cell–derived clones (*67*, *71*), cumulus cell nuclei might be thought of as more faithfully reprogrammed after transfer into the oocyte. If this were so, why would postimplantation development of ES cell NT embryos be much more efficient than that of somatic cell NT embryos? Comparing the phenotype and gene expression pattern of cumulus and ES cell nuclear clones may offer a potential solution to this puzzle. It is known that ES cells express genes such as Oct3/4 that are normally active in the blastocyst and are known to be crucial for early postimplantation development (*77*). These embryonic genes are silent in somatic cells such as fibroblasts and cumulus cells. This predicts that for somatic donor nucleus-derived NT clones to survive beyond implantation, "Oct3/4 like" genes need to be reactivated. Therefore, the early death of most somatic clones may be due to faulty or failed reprogramming of these early embryonic genes. In contrast, ES cell nuclear clones may survive the immediate postimplantation period because crucial "embryonic" genes, such as Oct3/4, are already active in the donor nucleus and need not be reactivated. The relatively efficient development of somatic cell NT embryos up to the blastocyst stage could be due to use of maternal resources that direct cleavage divisions.

Although ES cell NT embryos commonly survive the early stages of postimplantation development, they often show phenotypes at later stages, including fetal overgrowth (Fig. 2), which may be due to stochastic, epigenetic abnormalities affecting expression of imprinted and nonimprinted genes. Clearly, genome-wide expression analyses of individual cloned embryos and animals will be required to validate these hypotheses.

Given that ES cells are more efficient donors for nuclear transfer than somatic cells, an interesting question is whether somatic stem cells, in some ways similar to ES cells, have an epigenetic make up that would permit easier reprogramming than that of fully differentiated cells (see Fig. 1A). If so, most, if not all, cloned animals may be derived from somatic stem cells present at a low frequency rather than from terminally differentiated cells constituting the majority of the population. As no cellular or genetic markers have been used to unambiguously identify, the actual somatic donor cell that has given rise to a particular clone, this possibility cannot be excluded.

How "normal" are cloned animals? Regardless of donor cell type, NT cloned animals die at various stages of development, either during gestation, as neonates, or postnatally. Only a few clones are long-term survivors. The rare nature of clones that do develop to an apparently healthy adulthood raises the question of whether these animals in fact have a normal pattern of gene expression. The dysregulation of gene expression in cloned animals is consistent with the notions that mammalian development may tolerate a substantial degree of epigenetic abnormality and that lethality may result only from the cumulative effect of stochastic losses in normal gene regulation at multiple loci. Embryos may die at various stages of pre- or postnatal development depending on a certain threshold for faulty expression of the particular genes affected in a given clone or because of the random dysregulation of a key gene(s) crucial for a specific developmental stage. Consequently, embryos developing to birth obviously have appropriate expression of genes crucial for early development but may still have epigenetic defects affecting expression of genes activated later in development or adulthood. These considerations raise the possibility that even apparently healthy cloned animals may have subtle gene expression abnormalities that were not severe enough to cause lethality or an obvious postnatal phenotype. On the basis of the available data, it seems almost certain that clones of all species, including humans, would be subject to these epigenetic abnormalities and their associated phenotypes (*78*).

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REVIEW

The Centromere Paradox: Stable Inheritance with Rapidly Evolving DNA

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Every eukaryotic chromosome has a centromere, the locus responsible for poleward movement at mitosis and meiosis. Although conventional loci are specified by their DNA sequences, current evidence favors a chromatin-based inheritance mechanism for centromeres. The chromosome segregation machinery is highly conserved across all eukaryotes, but the DNA and protein components specific to centromeric chromatin are evolving rapidly. Incompatibilities between rapidly evolving centromeric components may be responsible for both the organization of centromeric regions and the reproductive isolation of emerging species.

Inheritance of genetic information requires a faithful copying mechanism. DNA replication and repair provide high-fidelity inheritance over evolutionary periods, whereas epigenetic inheritance is less rigid. DNA methylation can mediate epigenetic inheritance during development and even between generations of complex organisms (*1*). Stable protein-based inheritance is well-established for prions (*2*), and chromatin-based mechanisms are thought to maintain developmental states (*3*). Over the past decade, several authors have argued that centromeres, the sites of spindle attachment at mitosis and meiosis, can also be maintained epigenetically (*4*–*8*). Here, we examine recent studies on the basis for centromeric inheritance. These point to a novel chromatin-based mechanism for the maintenance of centromere location during multiple rounds of cell division. This mechanism may be responsible for the enigmatic organization of centromeric DNA and for the rapid onset of reproductive isolation as species emerge.

DNA Sequences at Centromeres

Delimiting the precise boundaries of centromeres has proven to be a daunting task. In animals and plants, centromeres are contained within regions of highly repetitive satellite DNA, which confounds even the most powerful mapping methods. Chromosomes in *Saccharomyces cerevisiae* are exceptional in that they lack satellite sequences and their centromeres have been precisely localized. Each of these "point" centromeres specifies spindle attachment with only ~125 base pairs (bp) of DNA. However, this simplicity is evolutionarily derived, as centromeres from other fungal lineages include arrays of repeats (*9*, *10*), much like what is found in animals and plants.

The simplicity of *S. cerevisiae* centromeres on small chromosomes led to the idea that specific repeated sequence elements might specify centromere location for larger chromosomes when present in a sufficient number of copies (*11*). However, this hypothesis has fallen out of favor for reasons that have been extensively reviewed (*5*–*7*). Most compelling is the lack of any common repetitive elements in many "neocentromeres" that

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