Epigenetic Instability in ES Cells and Cloned Mice

David Humpherys,[1,2]* Kevin Eggan,[1,2]* Hidenori Akutsu,[3]* Konrad Hochedlinger,[1] William M. Rideout III,[1] Detlev Biniszkiewicz,[1] Ryuzo Yanagimachi,[3] Rudolf Jaenisch[1,2]†

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 (Leukemia Inhibitory Factor) in media containing 10^{-7}M 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

[1]Whitehead Institute for Biomedical Research, [2]Department of Biology, Massachusetts Institute of Technology, 9 Cambridge Center, Cambridge MA 02142, USA. [3]Department of Anatomy and Reproductive Biology, John A. Burns School of Medicine, University of Hawaii, Honolulu, HI 96822, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: jaenisch@wi.mit.edu

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.

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 lineag-

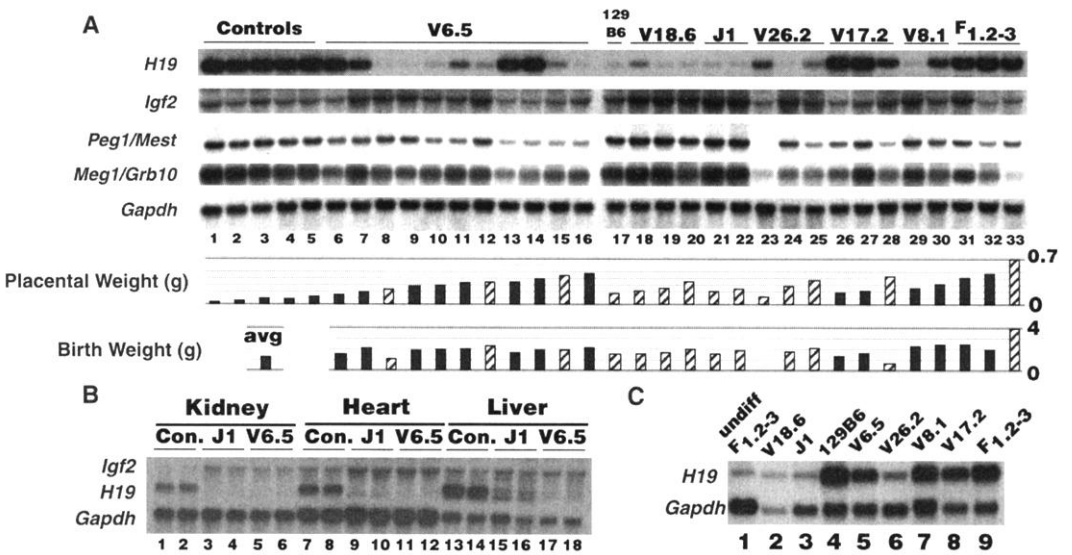

Fig. 1. Perinatal silencing of H19 in placentas and tissues of mice cloned from ES cells. (**A**). Northern blot analysis of *H19*, *Igf2*, *Peg1/Mest*, *Meg1/Grb10*, and *Gapdh* in placentas after c-section at 19.5 dpc. The ES cell line used as a nuclear donor is indicated above the lanes. Genetic backgrounds 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.

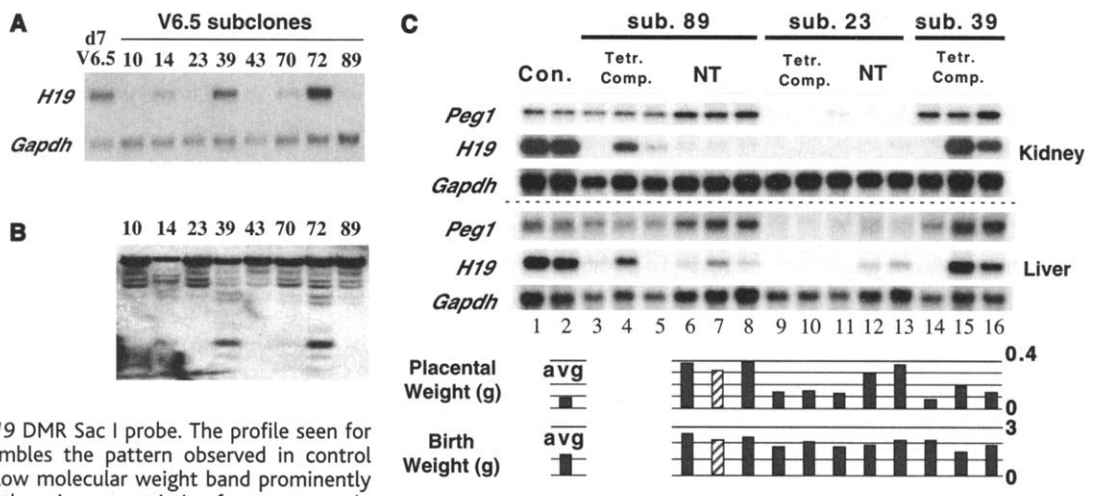

Fig. 2. H19 methylation and expression in subclones of the V6.5 ES cell line and expression in mice cloned from these subclones. (**A**) Northern analysis of V6.5 subclones after 6 days RA treatment. The original ES line from which the subclones were derived after 7 days RA treatment is shown for comparison (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.

es, 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).

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.

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%)

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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′-CCAAAAG-CTCCTCAAAGACG-3′ and 5′-TCTGTTAGAAGCAGCC-GATG-3′, followed by nested primers 5′-CTTTCCCCC-ATCCTCACC-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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24. Subclones of the V6.5 ES cell line were obtained as part of the targeting of a tet 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.).

6 April 2001; accepted 4 June 2001