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To assess the extent of abnormal gene expression in clones, we assessed global gene expression by microarray analysis on RNA from the placentas and livers of neonatal cloned mice derived by nuclear transfer (NT) from both cultured embryonic stem cells and freshly isolated cumulus cells. Direct comparison of gene expression profiles of more than 10,000 genes showed that for both donor cell types ≈4% of the expressed genes in the NT placentas differed dramatically in expression levels 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 set of genes differed between the embryonic stem 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 most expression abnormalities appear common to the NT procedure whereas others appear to reflect the particular donor nucleus.

The majority of cloned mammals derived by nuclear transfer (NT) die during gestation, display neonatal phenotypes resembling large offspring syndrome (1, 2), often with respiratory and metabolic abnormalities, and have enlarged and dysfunctional placentas (3–5). For a donor nucleus 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. Because few clones survive to birth, the question remains whether survivors are normal or merely the least severely affected animals, making it to adulthood despite harboring subtle abnormalities originating from inadequate nuclear reprogramming (6).

Given the long generational time of most animal species cloned, the long-term consequences of cloning on health have been difficult to assess. Evidence that cloned animals retain abnormalities capable of causing severe health consequences has been obtained for 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 (7). Additionally, mice cloned from cumulus cell donor nuclei were obese with increased body fat and size (8). Because obesity was not passed on to the offspring of the clones it is unlikely to reflect any genetic changes in the clones but instead to reflect epigenetic abnormalities arising from inadequate nuclear reprogramming. Examination of adult clones in other species has been described only for younger animals and limited to physical examinations and blood and urine chemistry (9).

Development of clones derived from embryonic stem (ES) cell nuclei to the blastocyst stage is less efficient than that of clones derived from somatic donor nuclei because the majority of ES cells are in S phase (6), a stage of the cell cycle that is incompatible with survival of clones (10). However, survival to birth or adulthood of blastocysts derived from ES cell donor nuclei is about 10–20 times more efficient than that of clones derived from somatic donor nuclei (11, 12). 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. Despite this enhanced developmental rate, it has been argued that epigenetic instability described in ES cells during *in vitro* culturing (13, 14) makes them a poor choice for NT donors (15). However, this argument is based largely on the expression of imprinted genes known to be particularly affected in ES cells. Nevertheless, common phenotypes, including dramatically overgrown placentas, have been described when using either ES cell or somatic cell donor nuclei for NT (3, 12).

Examination of gene expression in cloned animals has largely been limited to preimplantation embryos for a small number of genes important for early embryogenesis (16–18). In clones surviving to birth, the expression of a limited number of imprinted genes has been described, and several are expressed at abnormal levels (14, 15) with some changes reflecting epigenetic, in addition to chromosomal, abnormalities (19) arising in donor cells, in particular during the *in vitro* culture of ES cell donors. However, apart from about a dozen examined genes, it is not clear to what extent other imprinted gene expression or global gene expression may be abnormal in neonatal clones. Faulty imprinting has been proposed as a candidate for some cloning phenotypes because imprinted genes are frequently involved in fetal and placental growth (20) and are likely resistant to reprogramming because their imprints are established in the germ line and specifically maintained in the embryo (21). Furthermore, *in vitro* culturing of embryos can lead to a loss of imprinting and large offspring syndrome (22, 23). Because cloned embryos also display phenotypes resembling large offspring syndrome it is possible that some of these phenotypes result from imprinting abnormalities.

We report here the expression profiles of more than 10,000 genes in placentas and livers of neonatal clones from both ES cell and cumulus cell donor nuclei. Our results suggest that many expression abnormalities are common to the NT procedure whereas some reflect the particular donor nucleus. These results further emphasize the severity of placental dysfunction and illustrate abnormalities in clones surviving to birth.

Materials and Methods

RNA Preparation and Array Hybridization. Cloned mouse neonates were produced by NT 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 1.3 g and 0.09 g for normally fertilized embryos, 2.1 g and 0.32 g for ES cell NT mice (12), and 2.2 g (n = 12) and 0.33 g (n = 14) for cumulus cell NT mice. The distribution of increased placental and birth weights was similar to that seen in our previous study (14). RNA was isolated from a total of 24 placentas and 20 livers, and expression analysis was performed by using Affymetrix (Santa Clara, CA) gene arrays. Two sets of experiments

Abbreviations: NT, nuclear transfer; ES, embryonic stem.

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were run by using two different array versions. RNA was isolated from mouse placentas after C-section at term as described (14). The cloning efficiencies for the ES cell NT mice have been reported (12, 14), and the survival rates of the cumulus clones were similar to previous reports (24). Preparation of targets, hybridization, washing, and scanning were carried out with slight modifications to those protocols described (25). 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 ($F_{1.2–3}$ 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 (three female, two 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 nonfunctional probe sets of the 12,654 on the array.

Twenty liver samples were analyzed, 10 samples on each array version. The first comparison of version 1 arrays was made by using the following 10 samples: two 129 normally developing mice, three tetraploid embryo complementation mice using a V6.5 ES cell subclone 89 (14), three ES cell NT mice from V6.5 subclone 89, and two ES cell NT mice from a J1 line. In the second set of experiments using array version 2, we used another 10 samples: three normally developing mice (two B6/DBA2 F_1, one B6/129 F_1), 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 F_1 cumulus cell NT mice. In most cases the livers were from different conceptuses than the placentas.

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 by using both a multiple testing under dependency false discovery rate (ref. 36; www.math.tau.ac.il/~ybenja/depApr27.pdf) and a Westfall–Young stepdown algorithm family-wise error rate (see ref. 37) 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, or coefficient of variation, for each gene was calculated by using the eight control samples, including the *in vitro* cultured control. Only those genes with a coefficient of variation of less than 0.25 were included in the final candidate list. This process eliminated sex-specific genes such as *Xist* and other genes that proved by Northern analysis to have highly variable expression among isogenic controls. The only ES cell NT data used to generate Table 3 were for the animals derived by NT from the V6.5 ES cell subclones; the J1 line data were excluded. In generating predictors, both weighted voting and k-nearest neighbor algorithms were used.

Northern Analysis. Northern analysis was performed as described (14) with 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.

Results

Abnormal Gene Expression Profiles in Placentas of Clones Derived from ES Cell and Cumulus Cell Donors. Each of the two types of NT placentas was first compared with the controls. Genes showing a 2-fold or more expression difference between the means of the controls and either of the ES cell NT or cumulus cell NT placentas were determined for 15 samples on the more recent array version. Fig. 1*A* 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 2-fold in cumulus clones as compared with normally fertilized controls and to differ significantly by a Student's *t* test ($P < 0.05$). Similarly, dysregulation of 221 genes was detected in ES cell-derived clones. Because 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 chance. A schematic comparison of genes abnormally expressed in either type of experimental placenta is given in Fig. 1*B*. Many of the abnormally regulated genes were common to the two types of clones, whereas some were dysregulated either only in cumulus or 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% of the genes showing a 2-fold change in ES cell NT placentas, either up or down relative to controls, also showed a consistent, more than 2-fold change in the placentas of cumulus cell clones. An additional 17% (93% in total) showed a more 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. The variability between clones appeared higher than between controls but the differences were not statistically significant.

To determine the identity of genes most likely to be dysregulated

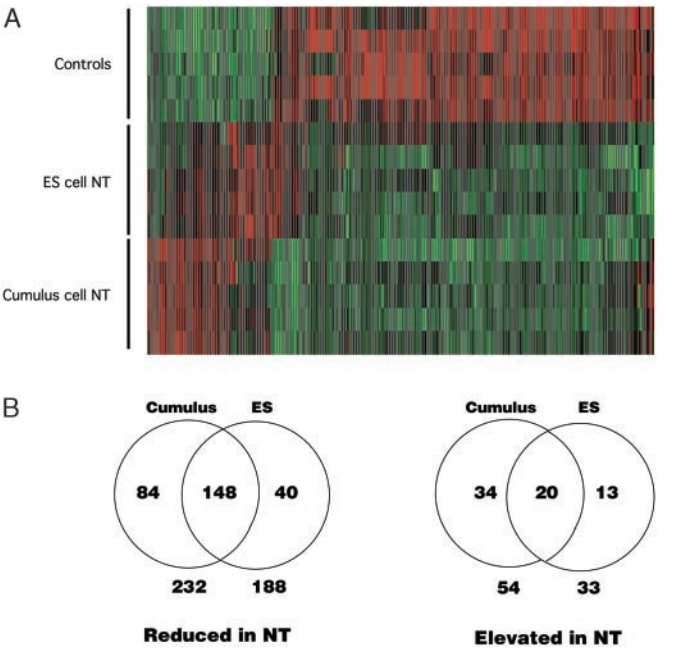

Fig. 1. (*A*) Representation of the expression levels of all genes showing a 2-fold difference in mean expression between any two sample types. Placentas are along the vertical axis and genes along the horizontal axis. Average expression across the 15 samples is indicated by black. Expression more than the average level is indicated by increasing red intensity, and 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 2-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 2-fold in both types of clones is indicated in the overlapping circles.

Table 1. Twenty-five genes that were most significantly (*t* test, $P < 0.05$) up- or down-regulated between the indicated groups of placentas

ES cell list	ES/Con	Cumulus list	Cum/Con
Elevated in clones			
Ada adenosine deaminase	3.34∧	**Prlpi prolactin-like protein I**	3.34∧
Ncam neural cell adhesion	3.20∧	**Ada adenosine deaminase**	2.43∧
Car2 carbonic anhydrase 2	2.82∧	**Pthr parathyroid hormone r**	2.16
Ada adenosine deaminase	2.67∧	**Ada adenosine deaminase**	2.14∧
Chemokine (C-C) receptor	2.58∧	Jak1 Janus kinase 1	2.11∧
Prlpg prolactin-like protein	2.47	**Car2 carbonic anhydrase 2**	2.05
Onzin	2.26∧	**Cd83 CD83 antigen**	2.03∧
Mmp15 matrix metalloprot	2.23∧	**Mmp15 matrix metalloprot**	2.02∧
Gpc1 glypican 1	2.15∧	Sparc secreted glycoprot	2.01
2610042L04Rik RIKEN cDNA	2.14∧	**Fbln1 fibulin 1**	1.94∧
Lysozyme M	2.11	**Prlpg prolactin-like protein**	1.94∧
Prkcl protein kinase C	2.07	Tyrosine phosphatase LAR	1.92∧
Prlpi prolactin-like protein	2.05∧	Cbx4 chromobox homolog	1.88∧
Oxidized LDL receptor	2.04	1200008D14Rik RIKEN cDNA	1.86
Pl1 placental lactogen 1	2.04∧	Hdac6 histone deacetylase	1.85∧
Fbln1 fibulin 1	2.01∧	**Chemokine (C-C) receptor 1**	1.82∧
Cd83 CD83 antigen	2.00∧	**Ctss cathepsin S**	1.80∧
Col15a1 Procollagen,XV	1.96	**Ncam neural cell adhesion**	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	1.86∧	**Lysozyme M**	1.75
Reduced in clones			
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 dehydrogenase	0.46∧	**Hsd11b1 hydroxysteroid 11**	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**	0.44∧
Ccr4 carbon catabolite hom	0.48∧	**Folr1 folate receptor 1**	0.44∧
Na dep vitamine transport	0.48∧	Carbonic anhydrase IV	0.44∧
Eng endoglin	0.48	Col18a1 procollagen XVIII	0.47∧
AA589632 EST	0.49∧	**AI785303 EST**	0.47∧
2610019F03Rik RIKEN cDNA	0.51∧	**Ephrin B1**	0.47∧
Grb10 growth factor rbp	0.52∧	**Grb10 growth factor rbp**	0.48∧
AI785303 EST	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 prot 2	0.51∧
1810004P07Rik RIKEN cDNA	0.53	**2610019F03Rik RIKEN cDNA**	0.52∧
MHC class III region	0.55∧	Vegf	0.52∧
Carbonic anhydrase IV	0.55∧	Perp-pending, Pmp22related	0.52
Mpp1 membrane protein	0.55∧	**MHC class III region**	0.53∧

All genes shown have a coefficient of variation of less than 25% for controls. Gene names correspond to their Unigene identification where possible, and otherwise their Affymetrix identifier. Genes are displayed from the greatest to least fold-change and the specified NT/control expression ratio is indicated. Genes that changed in both donor cell data sets appear in bold. Genes showing a consistent expression pattern when data from the different array versions were assessed independently are indicated as follows. Genes with a 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. The complete list of changed genes is in Table 5.

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 inter-strain (*Mus musculus* × *M. musculus*) and interspecific (*M. musculus* × *Mus castaneus*) F_1 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 1 is a shortened list of those genes with most altered expression levels. The complete list is available as Table 5, which is published as supporting information on the PNAS web site, www.pnas.org. 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 as has been done to assign tumors to known classes (26). Using classifiers based on 6–10 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-derived 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 with controls. A list of genes showing the greatest fold changes between the two types

Table 2. Genes differing in their expression levels between the two types of NT placentas are ordered from greatest to least average expression difference for each comparison

Gene	ES/Cum	ES	Cum
Expression higher in ES			
Sod3 superoxide dismut	2.08	–	<
Mus musculus oxi LDL r	1.91	>	–
5930418K15RikRIKENcDNA	1.69	–	<
Rgs16 regulator of G protein	1.66	>	<
2610042L04Rik RIKEN cDNA	1.62	>	–
Rpo1-1 RNA polymerase 1-1	1.62	–	<
Ncam neural cell adhesion	1.62	≫	>
Vnn1 vanin 1	1.53	<	≪
Perp-pending P53 effector	1.52	<	≪
Prkcl protein kinase C, lamda	1.50	≫	>
Htr4 5 hydroxytryptamine r	1.48	–	<
Eif4ebp1 initiation factor 4E	1.48	–	<
F2rl1 coagulation factor II r	1.44	<	≪
Sox13 SRY-box	1.40	–	<
Srp9 signal recognition particle	1.40	–	<
Il11ra2 Interleukin 11 receptor	1.39	–	<
Vegf	1.38	<	≪
H2-L histocompatibility 2	1.36	–	<
1200003F12Rik RIKEN cDNA	1.35	>	<
Csf2ra CSFr	1.35	–	–
Rora RAR-related orphan r	1.35	≫	>
Egfr epidermal growth factor r	1.35	–	<
Col18a1 procollagen, type XVIII	1.34	<	≪
2810021G24Rik RIKEN cDNA	1.33	<	≪
Gjb3 Gap junction channel	1.33	–	<
Il1r1 Interleukin 1 receptor	1.32	≫	>
Sdf1 stromal cell-derived factor 1	1.31	>	–
Putative G-protein coupled r	1.31	–	<
Expression lower in ES			
Xlr3b X-linked lymph-reg	0.53	<	>
Prlpi prolactin-like protein I	0.58	>	≫
DXImx46e DNA segment	0.62	–	>
Mlycd malonyl-CoA decarb	0.66	–	–
R74626 EST	0.67	–	>
Pim-1 protein kinase	0.68	–	>
Hdac6 histone deacetylase 6	0.69	>	≫
U2af1-rs1 U2 (U2AF), 35 kDa	0.70	–	>

The ES cell NT/cumulus cell NT expression ratio is indicated. Beneath each donor type is shown the relative expression of the gene compared to controls, where symbols indicate the following: – = not significantly different from controls, > = significantly elevated above controls, < = significantly reduced below controls. When expression was affected in both NT types, two symbols are used to indicate which type of clone was more severely affected.

of clones but little variation in controls are shown in Table 2 and Table 6, which is published as supporting information on the PNAS web site.

Gene Expression in Somatic Lineages of Neonatal Clones. To determine the status of gene expression in the somatic lineages of cloned mice we analyzed neonatal livers by 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 3). 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 because of insufficient sample size.

We also generated entirely ES cell-derived mice by tetraploid embryo complementation as a comparison for the ES cell NT mice. Because these mice are not generated by NT and do not exhibit overgrown placentas (12), 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 NT 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 3, 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.

Imprinted Gene Expression in Clones. We next focused on the expression profiles of imprinted genes in both the placentas and livers of clones. Although 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 (14, 15). In contrast, the expression levels of three 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 4). In liver, expression of *Peg1/Mest* and *Meg1/Grb10* was not significantly reduced in the clones, except in pups derived from ES donor cell line subclone 23, which had previously been shown to lack *Peg1/Mest* expression upon *in vitro* differentiation (14). Conversely, *Cdkn1c* (*p57*) appeared to be 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 by using allele-specific assays of F_1 *Mus/Cast* clones, we failed to detect inappropriate activation of the normally silent allele (data not shown), confirming data published by others (15). This finding 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 (15) 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 (22, 27, 28) such as *in vitro* cultivation.

Northern Analysis of Abnormally Expressed Genes. 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 (14, 15) and our array analysis, H19 expression varied dramatically in placentas of ES cell donor-derived clones (Fig. 2, lanes 19–37) but not in cumulus cell donor-derived clones (Fig. 2, lanes 9–18) and controls (Fig. 2, 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 nonimprinted genes whose expression levels appeared significantly altered by array analysis. Consistent with the array analyses, Northern hybridization demonstrated that *Carbonic anhydrase 2* was up-regulated, with expression levels higher in the ES cell-derived clones than in cumulus cell-derived clones (compare with Tables 1 and 5). Northern analysis of *Vanin-1* RNA showed reduced expression in clones that 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).

Table 3. Altered gene expression in livers of clones

Expression in ES cell NT liver for each gene	Mice		
	ES/Con	Cum/Con	Tetra/Con
Reduced			
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
Elevated			
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

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 caused by the ES cell donors (listed first in each group of changes) or caused by the NT process. These genes represent approximately 50% of the most affected genes in ES cell NT livers.

Discussion

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 (29). Thus, some of the changes in placental gene expression may reflect changes in 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 after NT and epigenetic errors inherited from the specific type of donor nucleus. These results are consistent with NT experiments in amphibians, in which the differentiation status of the donor cell has been shown to affect the developmental potential of cloned animals (30–34). The data presented here indicate that highly variable gene expression, observed previously for a limited number of genes in both amphibian (34) and mammalian clones (14, 15, 35), 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 (13, 14). When used as donors for NT, this instability contributes to widespread dysregulation of imprinted genes in the cloned mice. Cumulus cells are not

Table 4. Imprinted gene expression

Imprinted gene	Placenta		Liver	
	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

Ratio of average expression levels for imprinted genes in both types of NT placentas 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.

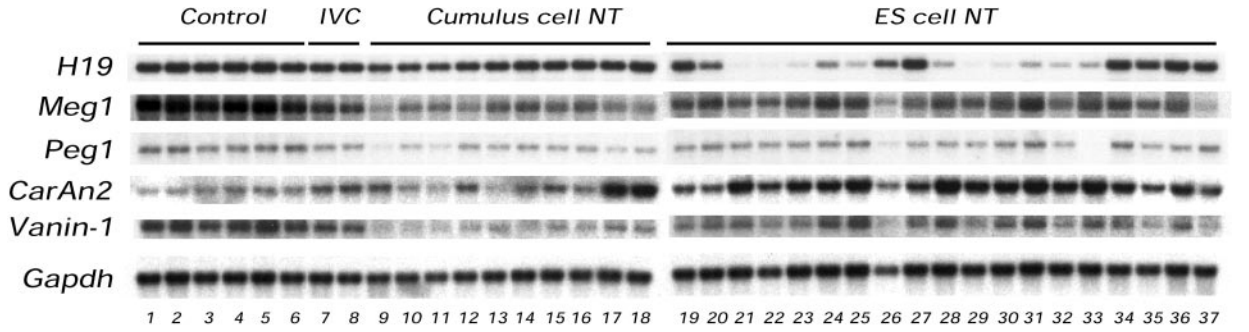

Fig. 2. Northern analysis of several genes dysregulated in NT placentas at term. Lanes 1–6 contain RNA from naturally derived B6/129 controls, whereas the RNAs in lanes 7 and 8 are derived from the placentas from normal B6/129 zygotes that had been cultured *in vitro* before transfer to a surrogate mother. RNAs in lanes 9–18 are from cumulus cell NT placentas of the indicated genetic backgrounds: lanes 9–13, DBA/Cast; lanes 14 and 15, 129/Cast; lane 16, AJ/Cast, and lanes 17 and 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 (14) 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 $F_{1-2.3}$ (129/Cast) line.

cultured before NT, yet clones derived from these cells also exhibit abnormal expression levels of many imprinted genes. Thus, *in vitro* culture cannot be the sole cause of disrupted imprinted gene expression in cloned animals. Because the number of gene expression abnormalities was 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 (15).

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 NT 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 (6). Recent studies showing premature death, pneumonia, hepatic failure (7), and obesity (8) in aging cloned mice could be a consequence of these gene expression abnormalities. Interestingly, the cloned mice showing premature death did not show obesity, which could reflect the use of two different somatic donor cell types in these studies. Conclusions about the normalcy of surviving cloned animals therefore should not be based on superficial clinical examinations (9) but rather on detailed molecular analyses of tissues from adult cloned animals.

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1. Young, L. E., Sinclair, K. D. & Wilmut, I. (1998) *Rev. Reprod.* **3,** 155–163.
2. Chavatte-Palmer, P., Heyman, Y. & Renard, J. P. (2000) *Gynecol. Obstet. Fertil.* **28,** 633–642.
3. Wakayama, T. & Yanagimachi, R. (1999) *Nat. Genet.* **22,** 127–128.
4. Hill, J. R., Roussel, A. J., Cibelli, J. B., Edwards, J. F., Hooper, N. L., Miller, M. W., Thompson, J. A., Looney, C. R., Westhusin, M. E., Robl, J. M. & Stice, S. L. (1999) *Theriogenology* **51,** 1451–1465.
5. Hill, J. R., Burghardt, R. C., Jones, K., Long, C. R., Looney, C. R., Shin, T., Spencer, T. E., Thompson, J. A., Winger, Q. A. & Westhusin, M. E. (2000) *Biol. Reprod.* **63,** 1787–1794.
6. Rideout, W. M., III, Eggan, K. & Jaenisch, R. (2001) *Science* **293,** 1093–1098.
7. Ogonuki, N., Inoue, K., Yamamoto, Y., Noguchi, Y., Tanemura, K., Suzuki, O., Nakayama, H., Doi, K., Ohtomo, Y., Satoh, M., *et al*. (2002) *Nat. Genet.* **30,** 253–254.
8. Tamashiro, K. L., Wakayama, T., Akutsu, H., Yamazaki, Y., Lachey, J. L., Wortman, M. D., Seeley, R. J., D'Alessio, D. A., Woods, S. C., Yanagimachi, R. & Sakai, R. R. (2002) *Nat. Med.* **8,** 262–267.
9. Lanza, R. P., Cibelli, J. B., Faber, D., Sweeney, R. W., Henderson, B., Nevala, W., West, M. D. & Wettstein, P. J. (2001) *Science* **294,** 1893–1894.
10. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. & Campbell, K. H. (1997) *Nature (London)* **385,** 810–813.
11. Rideout, W. M., Wakayama, T., Wutz, A., Eggan, K., Jackson-Grusby, L., Dausman, J., Yanagimachi, R. & Jaenisch, R. (2000) *Nat. Genet.* **24,** 109–110.
12. Eggan, K., Akutsu, H., Loring, J., Jackson-Grusby, L., Klemm, M., Rideout, W. M., 3rd, Yanagimachi, R. & Jaenisch, R. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 6209–6214.
13. Dean, W., Bowden, L., Aitchison, A., Klose, J., Moore, T., Meneses, J. J., Reik, W. & Feil, R. (1998) *Development (Cambridge, U.K.)* **125,** 2273–2282.
14. Humpherys, D., Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W. M., 3rd, Biniszkiewicz, D., Yanagimachi, R. & Jaenisch, R. (2001) *Science* **293,** 95–97.
15. Inoue, K., Kohda, T., Lee, J., Ogonuki, N., Mochida, K., Noguchi, Y., Tanemura, K., Kaneko-Ishino, T., Ishino, F. & Ogura, A. (2002) *Science* **295,** 297.
16. Daniels, R., Hall, V. & Trounson, A. O. (2000) *Biol. Reprod.* **63,** 1034–1040.
17. Daniels, R., Hall, V. J., French, A. J., Korfiatis, N. A. & Trounson, A. O. (2001) *Mol. Reprod. Dev.* **60,** 281–288.
18. Wrenzycki, C., Wells, D., Herrmann, D., Miller, A., Oliver, J., Tervit, R. & Niemann, H. (2001) *Biol. Reprod.* **65,** 309–317.
19. Eggan, K., Rode, A., Jentsch, I., Samuel, C., Hennek, T., Tintrup, H., Zevnik, B., Erwin, J., Loring, J., Jackson-Grusby, L., *et al*. (2002) *Nat. Biotechnol.* **20,** 455–459.
20. Tilghman, S. M. (1999) *Cell* **96,** 185–193.
21. Brandeis, M., Kafri, T., Ariel, M., Chaillet, J. R., McCarrey, J., Razin, A. & Cedar, H. (1993) *EMBO J.* **12,** 3669–3677.
22. Khosla, S., Dean, W., Brown, D., Reik, W. & Feil, R. (2001) *Biol. Reprod.* **64,** 918–926.
23. Young, L. E., Fernandes, K., McEvoy, T. G., Butterwith, S. C., Gutierrez, C. G., Carolan, C., Broadbent, P. J., Robinson, J. J., Wilmut, I. & Sinclair, K. D. (2001) *Nat. Genet.* **27,** 153–154.
24. Wakayama, T., Perry, A. C., Zuccotti, M., Johnson, K. R. & Yanagimachi, R. (1998) *Nature (London)* **394,** 369–374.
25. Tamayo, P., Slonim, D., Mesirov, J., Zhu, Q., Kitareewan, S., Dmitrovsky, E., Lander, E. S. & Golub, T. R. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 2907–2912.
26. Golub, T. R., Slonim, D. K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J. P., Coller, H., Loh, M. L., Downing, J. R., Caligiuri, M. A., *et al*. (1999) *Science* **286,** 531–537.
27. Doherty, A. S., Mann, M. R., Tremblay, K. D., Bartolomei, M. S. & Schultz, R. M. (2000) *Biol. Reprod.* **62,** 1526–1535.
28. Biniszkiewicz, D., Gribnau, J., Ramsahoye, B., Gaudet, F., Eggan, K., Humpherys, D., Mastrangelo, M. A., Jun, Z., Walter, J. & Jaenisch, R. (2002) *Mol. Cell. Biol.* **22,** 2124–2135.
29. Tanaka, S., Oda, M., Toyoshima, Y., Wakayama, T., Tanaka, M., Yoshida, N., Hattori, N., Ohgane, J., Yanagimachi, R. & Shiota, K. (2001) *Biol. Reprod.* **65,** 1813–1821.
30. Briggs, R. & King, T. (1957) *J. Morphol.* **100,** 269–302.
31. Gurdon, J. B., Laskey, R. A. & Reeves, O. R. (1975) *J. Embryol. Exp. Morphol.* **34,** 93–112.
32. DiBerardino, M. A. (1980) *Differentiation* **17,** 17–30.
33. McKinnell, R. G. & Di Berardino, M. A. (1999) *BioScience* **49,** 875–885.
34. Byrne, J. A., Simonsson, S. & Gurdon, J. B. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 6059–6063.
35. De Sousa, P., Winger, Q., Hill, J. R., Jones, K., Watson, A. J. & Westhuisen, M. E. (1999) *Cloning* **1,** 63–69.
36. Benjamini, Y. & Yekutieli, D. (2001) Thesis (Tel Aviv University, Tel Aviv).
37. Dudoit, S., Yang, Y. H., Callow, M. J. & Speed, T. P. (2000) *University of California, Berkeley Technical Report 578*, e-Print Archive, http://www.stat.berkeley.edu/tech-reports/(Univ. of California, Berkeley).