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Influence of *in vitro* manipulation on the stability of methylation patterns in the *Snurf/Snrpn*-imprinting region in mouse embryonic stem cells

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ABSTRACT

Recent work on embryonic stem (ES) cells showed that stem cell-derived tissues and embryos, cloned from ES cell nuclei, often fail to maintain epigenetic states of imprinted genes. This deregulation is frequently associated with *in vitro* manipulations and culture conditions which might affect the cells potential to develop into normal fetuses. Usually, epigenetic instability is reported in differentially methylated regions of mostly growth-related imprinted genes. However, little is known about the epigenetic stability of genes that function late in organogenesis. Hence, we set out to investigate the epigenetic stability of neuronal genes and analyzed DNA methylation patterns in the *Snurf/Snrpn* imprinted cluster in several cultured mouse ES cell lines. We also determined the effects of *in vitro* stress factors such as consecutive passaging, trypsination, mechanical handling, single cell cloning, centrifugation, staurosporine-induced neurogenesis and the insertion of viral (foreign) DNA into the host genome. Intriguingly, none of these *in vitro* manipulations interfered with the stability of the methylation patterns in the analyzed neuronal genes. These data imply that, in contrast to growth-related genes like *Igf2*, *H19*, *Igf2r* or *Grb10*, the methylation imprints of the analyzed neuronal genes in the *Snurf/Snrpn* cluster may be particularly stable in manipulated ES cells.

INTRODUCTION

Embryonic stem (ES) cells are regarded as a potential source of therapeutic material in transplantation medicine and for nuclear cloning experiments. However, ES cells are prone to differentiation and might easily be affected by culture conditions and *in vitro* manipulation, which could in turn result in a great variability in the methylation and transcription patterns of the ES cell genome. Indeed, recent work on ES cells has shown that stem cell-derived tissues and embryos often fail to maintain stable epigenetic states, especially in imprinted genes (1–3).

The correct inheritance of monoallelically expressed, imprinted genes requires germline-specific chromosomal modifications like DNA methylation and/or histone-acetylation. Loss of allele-specific methylation is associated with different forms of cancer, several neurobehavioral diseases and can be lethal during early stages of embryogenesis (4–7). The dysregulation of imprinted genes is probably the major cause for the abnormal phenotypes observed in animals produced through nuclear cloning (8–11). This cloning procedure is a very inefficient process as only a low percentage of all clones from different species survive after transfer of an ES cell nucleus (12–14). Abberant fetal growth is frequently observed in a large proportion of the animals resulting in increased placental weight, too low or too high birth weights, and skeletal and organ abnormalities (15–19). Further evidence for the effect of culture conditions on the epigenetic state of cultured pre-implantation mouse embryos came from the observation of the altered expression of the imprinted genes *Igf2* and *H19* in fetuses derived from cultured blastocysts (20,21). Depending on the nature of the environmental stress during the cultivation of cells, epigenetic changes can affect the organism's development long after the transfer of manipulated embryos into foster mothers (15,17–19).

Since the genome undergoes extensive changes in gene regulation at the blastocyst stages, the suspicion has been raised that *in vitro* manipulation might result in methylation abnormalities in ES cells. Therefore, we investigated the methylation stability of two well characterized maternally imprinted loci, *Ndn* and an imprinting control element (*Snrpn* Exon1) of the *Snurf/Snrpn* imprinting cluster on mouse chromosome 7C. We analyzed DNA methylation patterns of these genes in several cultured mouse ES cell lines and determined the effects of different *in vitro* stress factors. ES cells and single ES cell clones were cultivated in standard medium for over 30 consecutive passages to assess the influence of continuous cultivation. Furthermore, three mouse ES cell lines, IB10, RW4 and Bruce 4 were induced to enter neurogenesis in culture at low concentrations of Staurosporine (STS), a broad spectrum protein kinase inhibitor, that was previously shown to induce neurite outgrowth and neurosphere formation in ES cells (22). Finally, we addressed the

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question whether the insertion of a transgene affected the imprinting patterns of the *Snurf/Snrpn* imprinting cluster. Homologous recombination was used to integrate viral sequences from SV40 and adenovirus type 2 (Ad2) into a specific gene, B-lymphocyte kinase (BLK), in three C57BL/6 ES cell lines (23).

None of the imposed stress factors induced a significant loss or gain of DNA methylation in the imprinted genes analyzed. The same epigenetic stability of *Ndn* and *Snrpn* was found in ES cell-derived neuronal cells. However, both alleles of the *Ndn* gene were hypermethylated in two mouse tumors leading to a loss of imprinting (LOI).

These findings support the notion of a methylation maintenance function within the *Snurf/Snrpn* imprinting cluster that could actively maintain the allele-specific methylation of some imprinted genes during embryonal development.

MATERIALS AND METHODS

ES cell culture and differentiation

The mouse ES cell lines IB10 (129/ola), RW4 (129/sv), BL/6-III (C57BL/6) and Bruce 4 (C57BL/6) were grown on mitomycin C (10 μg/ml)-treated embryonic feeder layer cells in standard ES medium [DMEM; Invitrogen (IG), Karlsruhe, Germany] as described elsewhere (24). To prevent differentiation, the cells were kept at low densities in the presence of leukemia inhibitory factor (LIF). Cells were passaged every 2–3 days for up to 3 months without freezing the cells between passaging.

To achieve *in vitro* neurogenesis of ES cells, the cells were expanded without β-mercaptoethanol on gelatine-coated culture dishes to minimize the number of LIF-secreting feeder cells (25). Induction phase I: on day 0, ES cells were trypsinized and aggregated in hanging drops with 500–1000 cells/20 μl to form embryoid bodies. The cell aggregates were kept in culture for 3 days in the presence of 20 nM STS (Sigma, Steinheim, Germany), 20% FCS (Cytogen, Ober-Mörlen, Germany), 2 mM glutamine (IG), non-essential amino acids (stock solution 1:100; IG), 1 mM sodium pyruvate (IG); 100 IU/ml penicillin, 100 μg/ml streptomycin (IG), 5 μg/ml insulin (Sigma), 10 μg/ml transferrin (Sigma) and 10 μg/ml ascorbic acid (Sigma). Induction phase II: embryoid bodies were transferred to bacteriological dishes and grown as floating bodies in suspension for another 4 days. The medium was changed every 24 h with decreasing FCS concentrations (20, 10, 5 and 3%) to adapt the cells to low serum media. On day 7, cell aggregates were collected and plated in tissue culture dishes in neurobasal medium (IG) with the aforementioned supplements and 25 μM glutamic acid (Sigma), 1% FCS, B27 (IG) or G5 (IG). After several days, neuronal cells developed with high efficiency in the culture dishes [for details see Schumacher *et al.* (22)].

DNA extraction and Southern blotting

Prior to the isolation of genomic DNA, ES cells were grown on gelatine-coated culture dishes to minimize DNA contaminations by fibroblasts. ES cells were then trypsinized, transferred to 1.5 ml tubes and washed twice with MT-PBS (4 mM NaH_2PO_4, 16 mM Na_2HPO_4, 150 mM NaCl, pH 7.3) to prevent gelatine clogging in the tubes. For each culture dish with ~5×10^6 cells, the ES cells were resuspended in 500 μl of lysis buffer (100 mM Tris–HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 μg/ml Proteinase K) and incubated at 37°C for several hours. An equal volume of isopropanol was added at room temperature until a DNA precipitate was visible. Recovery of DNA was performed by lifting the DNA out of the isopropanol with a drawn out Pasteur pipet. The DNA was washed in EtOH, air dried and resuspended in TE. Preparation of genomic DNA from tumors, restriction cleavage, gel electrophoresis, membrane transfer and methylation quantitation were performed as described previously (25,26). Mouse tumor A was derived from an area between the eyes and frontal cortex of a 129/sv mouse, tumor B from the cerebral area of a DBA/2 mouse.

Insertion of foreign DNA

Homologous recombination was used to integrate Ad2 or SV40 promoter containing constructs with the luciferase gene as indicator into BL/6-III (C57BL/6) mice (23). The Ad2-construct (see Fig. 5a) had integrated into the BLK gene located on mouse chromosome 14. The SV40 construct (see Fig. 5b) had integrated randomly into the ES cell genome. Two of the resulting ES cell lines were analyzed: ES-A08, which contained the plasmid with the adenovirus type 2 E2AL promoter upstream of a luciferase reporter gene, and a second ES cell line (ES-S07), which carried the early SV40 promoter and a luciferase reporter gene.

RESULTS

Since different mouse strains exhibit strain-specific differences in DNA methylation (25), we selected ES cells from different genetic backgrounds, 129/sv, 129/ola and C57BL/6 and analyzed two imprinted loci on mouse chromosome 7c (Fig. 1). First, the differentially methylated region 1 (DMR1) of the *Snurf/Snrpn* bicistronic gene, and secondly, the brain-specific *Ndn* gene.

The *Snurf/Snrpn* DMR1 is located in the 5′ portion of the gene, spanning Exon 1 (Fig. 1a) and might play an essential role in the imprinting process of this chromosomal region. This imprinting center (IC) element contains two DNase I hypersensitive sites (27) on the unmethylated paternal chromosome, but not on the methylated maternal chromosome. The DMR1 functions as a classical silencer in transgenic *Drosophila* (28), suggesting that this element might have a specific chromatin structure and possibly interacts with non-histone proteins (29). The *Ndn* gene, a member of the MAGE gene family, is an intronless gene, maps ~1 Mb proximal to the IC (Fig. 1b) and encodes the neuronal protein Necdin. The *Ndn* gene displays several characteristics of imprinted genes in the *Snurf/Snrpn* region, including parental specific methylation and asynchronous DNA replication (reviewed in 7).

Imprint stability during ongoing ES cell passages

Upon prolonged culturing, the developmental potential of many ES cell lines becomes impaired resulting in ES-derived fetuses with growth abnormalities (30,31). Some of the high passage stem cells show quantitative changes in DNA methylation in some imprinted genes, i.e. in *Igf2*, *H19* or *U2af1-rs1* (1,32). Therefore, we analyzed the stability of DNA

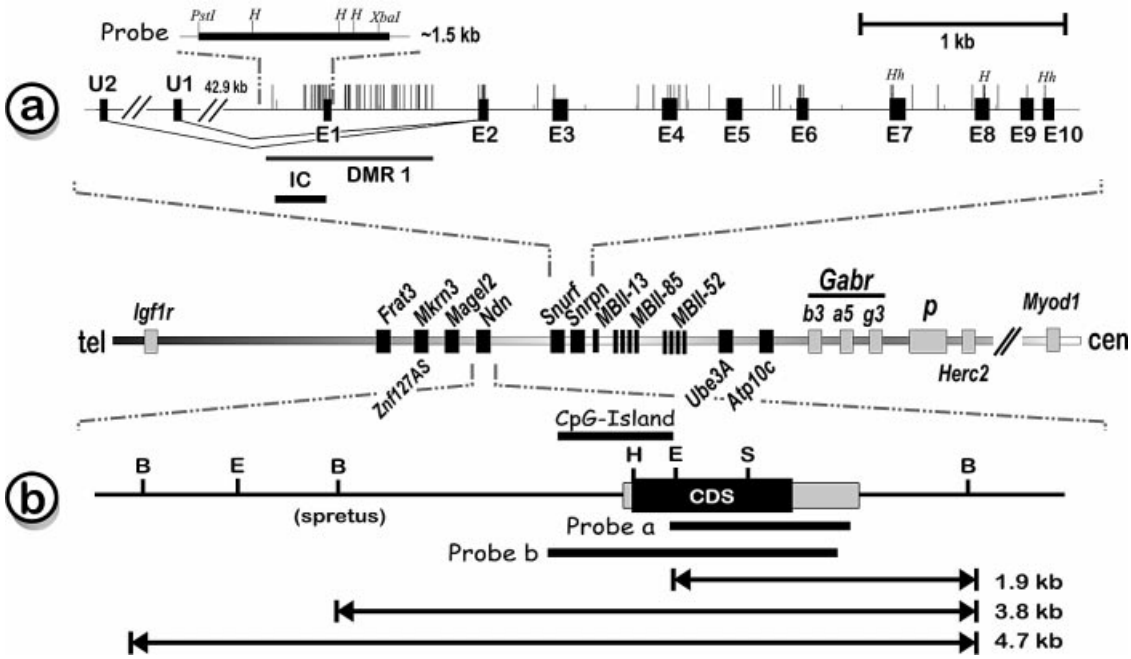

Figure 1. (**a**) Structure of the bicistronic *Snurf/Snrpn* gene within the imprinted neuronal gene cluster on mouse chromosome 7C. Deletion, uniparental disomy (UPD) or inappropriate imprinting of the homologous chromosomal region in humans results in the neurogenetic PWS. Deletion of the DMR1 in mice, which spans the promoter and exon1 of *Snurf/Snrpn*, results in the disability to maintain the correct paternal methylation imprint during embryonic development (reviewed in 7). The bicistronic structure of this gene leads to two different protein products, SNURF and SmN. Vertical lines represent CpG dinucleotides. (**b**) Genomic structure of the intronless *Ndn* gene about 1 Mb telomeric to the IC. *Ndn* has DNA binding capacity and may play a role as a growth suppressor that interacts with the transcription factors E2F1 and p53 in virtually all post-mitotic neurons in the brain. H, HpaII/MspI; E, EagI; S, SacII; B, BglII; B_S, polymorphic BglII site to identify the parental origin of an allele in mice with mixed genetic background.

methylation patterns in non-growth-related imprinted genes during the consecutive culturing of two 129 ES cell lines, RW4 (129/sv) and IB10 (129/ola), which were cultured for up to 35 passages in standard ES cell medium (see Materials and Methods). The cells were propagated on tissue culture plastic dishes and were passaged every 2–3 days to minimize differentiation of the cells. ES cell aliquots from several passage numbers were collected to isolate genomic DNA for Southern blot analyses, as exemplified for the imprinted *Ndn* gene (Fig. 2). All genomic DNA samples were cleaved with BglII in addition to the methylation-sensitive restriction endonuclease EagI.

Both ES cell lines revealed low methylation levels of the *Ndn* alleles in the second passage (P2; 16.5% in IB10 cells and 15.5% in RW4 cells, respectively) compared with the fully imprinted 50% methylation in adult brain (Fig. 5). In concordance with an earlier report (33), analyses with cells of mixed BDF_1 genetic background indicated that the remaining methylated CpG dinucleotides originated from the maternal allele. The hypomethylated pattern of the *Ndn* gene observed in passage 2 was stably transmitted during further passages (Fig. 2). Significant changes in DNA methylation could not be observed in the two ES cell lines even after 2 months in culture (>P30–P35). Similar results were obtained for *Ndn* and *Snrpn DMR1* in C57BL/6 ES cells (data not shown). These data indicate that the methylation patterns of some imprinted genes can be stably maintained in ES cells even if the ES cells are cultivated for a prolonged time period. In contrast to these neuronal genes, growth-related genes, like the chromosomal neighbors *H19* and *Igf2*, might be more susceptible to alterations of DNA methylation during growth in culture (see Discussion).

Imprint stability in clonally derived ES cells

Previous reports on the expression patterns of imprinted mouse genes in ES cell-derived clones revealed that *Snrpn* might exhibit less epigenetic variation when compared with other imprinted genes (2) (see also Table 1). Therefore, cloning procedures should not significantly influence the epigenetic state of this gene locus in cloned animals or *in vitro* cell lines. To test this hypothesis, we isolated single C57BL/6 ES cells (Bruce 4) and grew them into clonally derived new cell populations. Ten of the resulting cell lines (K1–K10) were subjected to detailed methylation analyses of their *Ndn* gene and their DMR1 loci of the *Snurf/Snrpn* gene (Fig. 3a and b).

The 10 cell lines revealed only small variations in methylation patterns for the analyzed CpG sites as shown for the *Ndn* gene (Fig. 3a). All clones and the original cell line (Bruce 4-P17) exhibited methylation levels of ~15%. Southern analyses for the DMR1 locus revealed even lower variability in methylation patterns (Fig. 3b). Densitometric scans of the Southern blots with a phosphoimager did not detect any changes in methylation levels above 5% among the different cell lines. Hence, although located close to the epigenetically unstable *H19/Igf2* region on chromosome 7C, the *Snurf/Snrpn* region seems rather resistant to changes in DNA methylation during *in vitro* manipulation. Since we did not investigate the epigenetic stability of the *H19/Igf2* cluster in our study, it is unknown if these genes would exhibit a methylation instability in our experimental set-up. However, numerous publications

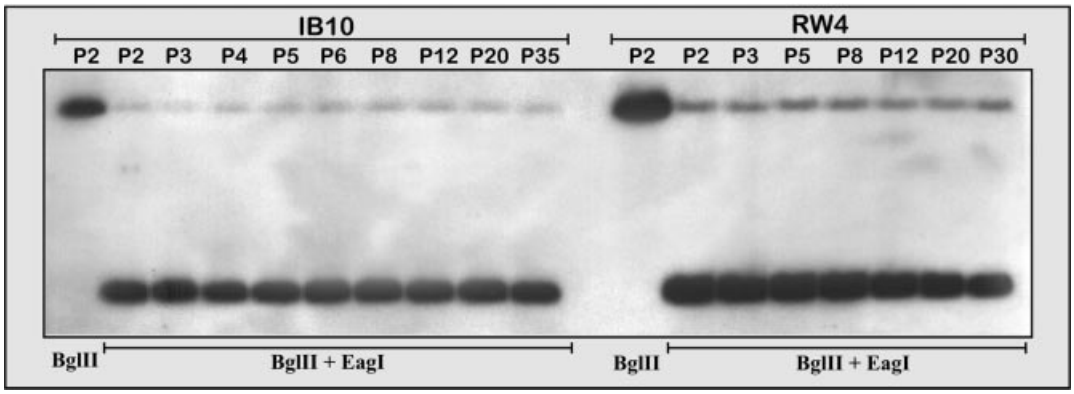

Figure 2. DNA methylation stability in mouse ES cells with ongoing passages (Px; Ndn probe a). (Left) DNA from IB10 ES cells (129/ola) were cleaved with BglII and with the methylation-sensitive restriction endonuclease EagI to study *Ndn* methylation. The ES cells were passaged in standard medium for 35 passages (~90 days) without significant changes in methylation levels (~15%). Methylation levels were assessed by relative hybridization intensities, as determined by scanning densitometry. (Right) Methylation analysis of RW4 ES cells (129/sv), which were cultured for 30 passages (~80 days). The methylation patterns were stably transmitted and approximately the same as in the IB10 cell line, with ~15% CpG methylation on the maternal allele (band on top of lanes).

Table 1. Survey of methylation stability of imprinted genes

Imprinted genes	Growth-related genes							Neuronal genes		
	Igf2	*H19*	*Meg1/Grb10*	*U2af1-rs1*[a]	*Igf2r*	*Peg1/Mest*	*P57*^{Kip2}	*Snrpn*	*Ndn*	*Peg3*
Imposed *in vitro* stress factors										
Passaging/culturing	+[5,8,11,13]	+[5,6,7,8,13]	+[5]	+[8]	+[8]	*[5]		*[1,15]	*[1]	
NT	+[3]	+[3]+[16]	+[3]		*[3]+[16]	+[3]		*[3]		*[3]+[16]
Transgenes					+[4]			*[1]	*[1]	
ES differentiation		+[3]						*[1]	*[1]	
ES cell clones		+[3]				+[3]		*[1]	*[1]	
Spermatid injection		+[12]								
Epigenetic instability after treatment with factors that influence the imprint machinery										
TSA treatment	+[9]			+[10]			+[9]	*[9,10]		*[9]
AcaC/TSA treatment		+[9]	*[9]	+[9]		*[9]	+[9]	*[9]		+[9]
Dnmt1 over-expression	+[2]	+[2]			*[2]			*[2]		*[2]
Epigenetic instability and carcinogenesis										
Tumors	+[x]	+[x]						+[14]	+[1]	

Some imprinted genes may be particularly susceptible to epigenetic changes, which occur during pre-implantation development. Reported epigenetic instability (+) and epigenetic stability (*) of cultured mammalian pre-implantation stage embryos, fibroblasts, primary T-lymphocyte clones, spermatids, ES cells and ES cell-derived clones. TSA inhibits histone-deacetylases. [1]Schumacher *et al.* (22), this study; [2]Cox *et al.* (64); [3]Humpherys *et al.* (2); [4]Mueller *et al.* (38); [5]Khosla *et al.* (21); [6]Doherty *et al.* (66); [7]Sasaki *et al.* (20); [8]Dean *et al.* (1); [9]Kharroubi *et al.* (67); [10]Gregory *et al.* (68); [11]Blondin *et al.* (69); [12]Shamanski *et al.* (70); [13]Ungaro *et al*. (71); [14]Miura *et al.* (72); [15]LaSalle *et al*. (73); [16]Mann *et al.* (3); x, several reports.
[a]The exact influence of the splice-factor *U2af1-rs1* on embryonal growth remains to be elucidated, however, mice with maternal and paternal disomy for chromosome 11 show abnormal growth phenotypes (74).

on the epigenetic stability of these extensively studied genes are available (Table 1) that support the view of the *H19/Igf2* region as a rather epigenetically unstable imprinting cluster.

Imprint stability in ES cells during induced neurogenesis

The main reason for some imprinted genes, like *H19* or *Igf2*, to be more susceptible to methylation changes during ES cell culture than others, may be related to their function and activity within this cell type. ES cells mimic the behavior of cells in the early embryo, where the *Igf2* and *H19* genes might participate in growth processes (34,35). However, *Ndn* and the bicistronic *Snurf/Snrpn* genes are expressed predominantly in the developing and in the adult brain (reviewed in 7). Therefore, we analyzed the methylation patterns of the *Snurf/Snrpn* imprinting cluster in the three mouse ES cell lines, IB10, RW4 and Bruce 4, which were induced to enter neurogenesis in culture by low concentrations of STS [Schumacher *et al.* (22)]. These cells differentiated with high efficiency into epidermal growth factor-responsive neural precursor cells, formed neurospheres, and developed further to neurons and astrocytes. Fourteen days after STS induction, when first neuron-like cells were visible in the culture dishes, DNA was extracted and analyzed with methylation-sensitive restriction enzymes.

As exemplified for cell line Bruce 4, the methylation patterns of the imprinted genes were essentially unaffected during neurogenesis (Fig. 4). The allele specifically methylated DMR1 sequence revealed no measurable differences in methylated 5′-CCGG-3′ sites (HpaII recognition sequence) in ES cells grown in STS medium as compared with ES cells

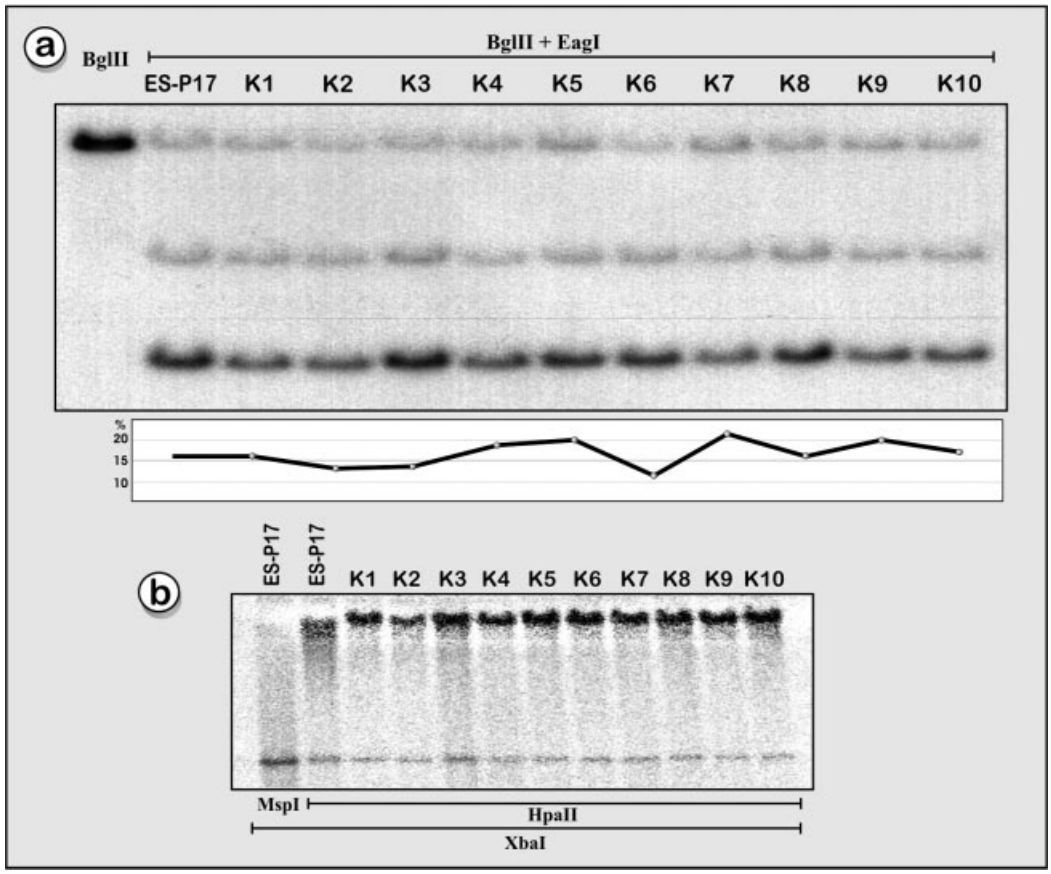

Figure 3. Stability of DNA methylation in imprinted neuronal genes in single cell-derived clonal ES cell lines. (**a**) Methylation-sensitive Southern blot analyses with restriction enzymes BglII and EagI revealed only small variations in methylation patterns of the *Ndn* gene in 10 Bruce 4 (C57BL/6) ES cell clones (*Ndn* probe a). (Bottom) The relative methylation levels in all ES cell subclones (K1–K10) varied around 15% (±5%) and were nearly identical to the methylation level observed in the original cell line (ES-P17). (**b**) Phosphoimager scan of a Southern blot with genomic DNA of 10 Bruce 4 subclones (5 μg/lane), cleaved with XbaI and the methylation-sensitive HpaII. The DNA on the membrane was hybridized with a mouse *Snurf/Snrpn* DMR1 probe. The first lane represents a control with the HpaII-isoschizomer MspI, which cleaves DNA also at methylated 5′-CCGG-3′ sites. The *Snurf/Snrpn* DMR1 was hypermethylated (65 ± 5%) in the Bruce 4 cell line ES-P17 and in all 10 subclones derived from these ES cells.

grown in standard medium (Fig. 4a). In contrast to the DMR1 element, *Ndn* is known to be heavily demethylated during early embryogenesis (36) and remains in a hypomethylated state at least until the blastocyst state, when ES cells are prepared (A. Schumacher and W. Doerfler, unpublished results). Indeed, the HpaII-recognition sequence within the *Ndn* gene was unmethylated in ES cells and remained unmethylated in the neuronal cells (Fig. 4b). This finding indicated that epigenetic mechanisms were active on the *Ndn* gene only late in organogenesis. However, the telomeric *Ndn* gene harbors another CpG site that was partly methylated (8–17%) on the maternal allele. Nevertheless, the remaining CpG methylation was constant during *in vitro* neurogenesis, with a low demethylation activity of <5% in some samples.

Imprint stability in SV40- and adenovirus-transfected ES cells

Integration of foreign DNA into an established host genome can lead to changes in methylation in both the inserted DNA and in host sequences (reviewed in 37). Therefore, we analyzed two transgenic C57BL/6 cell lines, ES-A08, which contains a plasmid with the Ad2 E2AL promoter upstream of a luciferase reporter gene (see Fig. 5a), and a second ES cell line, ES-S07, which carries the early SV40 promoter (23). The transgenic fragment in ES-A08 was integrated via homologous recombination in intron 3 of the BLK on mouse chromosome 14, whereas the transgenic construct with the SV40 promoter had integrated randomly into the ES cell genome in cell line ES-S07. Although the Ad2 transgene itself was heavily *de novo* methylated (23), integration did not affect the methylation pattern of the imprinted *Ndn* gene (Fig. 5b, lane 3). *Ndn* was allele specifically methylated in both transgenic cell lines and in the original non-transgenic ES-C57BL/6 cell line. The paternal allele seemed to maintain the low methylation level of the gametes (Fig. 5b, lane 1). Comparison of the EagI restriction patterns from the DNA of mice with a mixed $(BxD)F_1$ background carrying a maternal C57BL/6 and paternal DBA/2 allele indicated that the methylation resided on the maternal allele (Fig. 5b, lane 6). Although the insertion of foreign DNA was previously found to influence the DNA methylation of an imprinted rodent gene (*Igf2r*) (38), the integration of the hypomethylated SV40 transgenic construct did not interfere with the imprinted methylation pattern of *Ndn* in the ES cells (Fig. 5, lane 4).

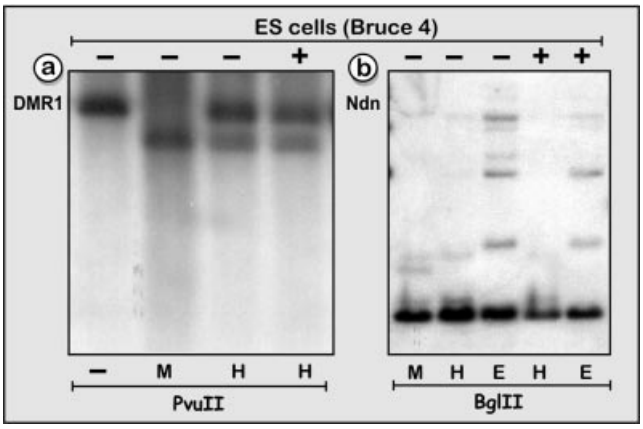

Figure 4. The DNAs from differentiated ES cells show no significant changes in methylation levels of imprinted neuronal genes. For Southern blot analyses, 10 µg of genomic DNA from STS-induced ES cells (+) with neuronal morphology was cleaved with PvuII (for the *Snurf/Snrpn* DMR1 region) or with BglII (for the *Ndn* region) and then either with HpaII (H lanes), MspI (M lanes) or EagI (E lanes). (**a**) The *Snurf/Snrpn* DMR1 remained hypermethylated during STS-induced neurogenesis. (**b**) The hypomethylated EagI site within the *Ndn* gene (*Ndn* probe b) exhibited no significant methylation variation during *in vitro* neurogenesis. The HpaII restriction site remained completely unmethylated.

Epigenetic dysregulation in tumor tissues

Methylation alterations in promoter regions are often the earliest and most frequent events known to occur in human cancers and are associated with loss of gene function (39,40). Some imprinted genes, notably *IGF2* and *H19*, have been recognized to harbor dense methylation in normally unmethylated CpG islands (5). Therefore, we analyzed two mouse tumors for the stability of DNA methylation in the mouse *Ndn* gene, which is thought to control the permanent arrest of cell growth and differentiation during nervous system development (41).

Ndn was highly hypermethylated in both tumors, as compared with the brain tissue controls (Fig. 6). To assess whether paternal or maternal *Ndn* loci were affected, a restriction fragment length polymorphism between C57BL/6 and DBA/2 DNA was used to distinguish parental alleles in F_1 progeny DNA. The methylation patterns of tumor A (mouse strain 129; Fig. 6, left) indicated that the paternal allele had adopted the hypermethylated pattern of the maternal allele. Densitometric scans revealed an overall *Ndn* methylation level of 90% in tumor A and 100% in tumor B (mouse strain DBA/2, Fig. 6, right). This result indicates that the paternal allele in the DBA/2 tumor has become heavily *de novo* methylated. Thus, cellular dysregulations, possibly imposed through *in vitro* manipulation or environmental factors, might

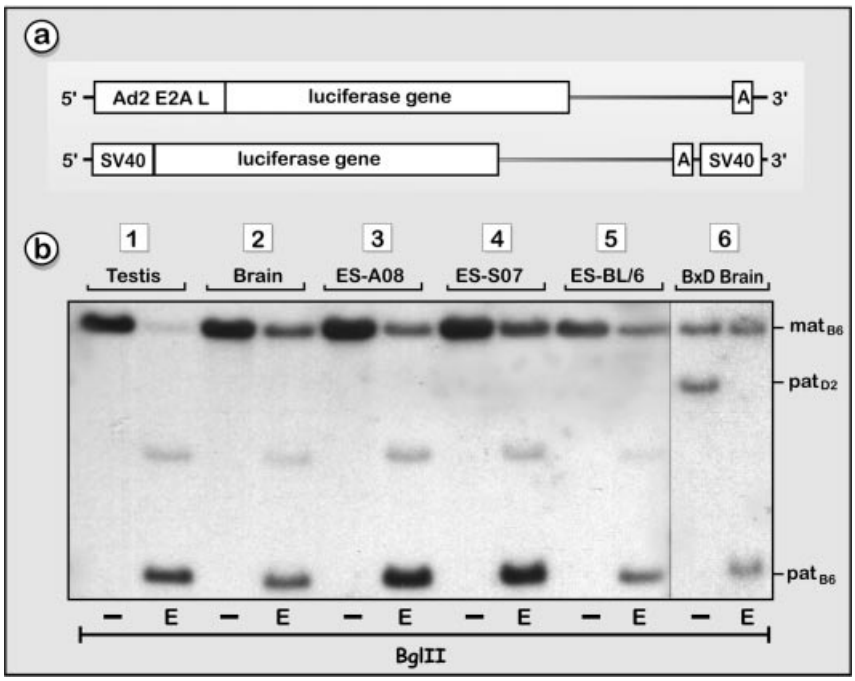

Figure 5. Southern blot analyses of the DNAs from ES clones transgenic for the Ad2-Luc and SV40-Luc constructs. (**a**) Map of the transgenes used in this study. Ad2-Luc contains the Ad2 E2A late promoter upstream of the luciferase reporter gene with the SV40 poly(A) signal downstream (23). SV40-Luc has the SV40 promoter upstream of the reporter gene and the SV40 enhancer 3′ of the poly(A) signal. (**b**) For Southern blot analyses, 10 µg of genomic DNA from non-transgenic controls and from transgenic ES cells was cleaved with BglII and then with EagI. The DNA on the membrane was hybridized with mouse *Ndn* probe b (see Fig. 1). Lane 1, testis DNA of control mice of the same genetic 129/ola background revealed a hypomethylated *Ndn* gene, indicating a maternal origin for the methylated allele in ES cells. Analysis with control brain DNA from a mixed genetic background [BxD(C57BL/6 × DBA/2)F1; lane 6] confirmed the maternally derived methylation imprint. Lane 2, the *Ndn* gene was imprinted in brain from control animals. Lanes 3 and 4, both transgenic constructs exhibited an allele-specific methylation pattern for the EagI site. However, the surrounding HpaII and SacII sites (see Fig. 1b) were not fully methylated on the maternal allele (data not shown). The methylation patterns were not significantly different compared with the non-transgenic ES cells (lane 5). The adenovirus construct in ES-A08 integrated via homologous recombination in intron 3 of the BLK gene on mouse chromosome 14, whereas the SV40 transgenic construct integrated randomly into the genome of ES cell line ES-S07. B6, C57BL/6 allele; D2, DBA/2 allele.

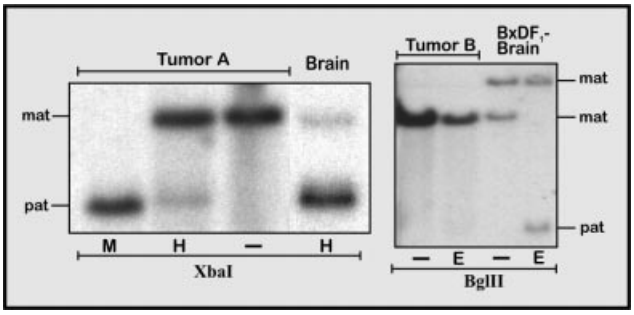

Figure 6. Epigenetic instability of the neuronal *Ndn* gene in mouse tumors. For Southern blot analyses, 5 µg of genomic DNA from tumor tissues was cleaved with XbaI for tumor A and BglII for tumor B and then with HpaII, MspI or EagI, respectively. (Left) The HpaII site of *Ndn* in DNA from control brain is partly methylated on the maternal allele and fully demethylated on the paternal allele. Southern blot analyses of tumor DNA, derived from a 129 mouse (tumor A), revealed *de novo* hypermethylation of the paternal allele (~90% methylation). (Right) Tumor B, derived from the cerebral area of a DBA/2 mouse, exhibited fully *de novo* methylated alleles (100% methylation). The EagI site of *Ndn* in control DNA of $BxDF_1$ mice was allele specifically methylated, with a hypermethylated maternal allele and fully demethylated paternal allele. It is noteworthy that not all CpG dinucleotides within the *Ndn* gene are equally methylated in adult brain. Whereas the EagI and and SmaI (data not shown) sites show a fully developed imprinting pattern, the HpaII site displays only an incomplete methylation of the maternal allele, indicating that the methylation pattern for this CpG dinucleotide does not change much during development. H, HpaII; M, MspI; E, EagI.

preferentially affect allele specifically methylated genes, which in turn leads to a LOI.

DISCUSSION

With the development of technologies like *in vitro* fertilization and intra-cytoplasmatic sperm injection (ICSI), reports have accumulated which show that the *in vitro* cultivation and manipulation of ES cells can dramatically impair the development of the early embryos, fetal growth and affect post-natal development (2,15,18,19,21). The improper regulation of several imprinted genes may also contribute to the growth abnormalities observed in cloned animals (9,10). Moreover, abnormal regulation of imprinted genes has been shown to affect fetal growth (21,42,43).

One of the best understood imprinting clusters in the mammalian genome is positioned on mouse chromosome 7 and plays an essential role in growth regulation. This cluster contains the imprinted genes *Igf2* and *H19*, whose human homologs are involved in the human fetal overgrowth syndrome, the Beckwith-Wiedemann syndrome (reviewed in 35,44). Several studies have documented abnormal epigenetic patterns in these growth-related genes and in cultured and manipulated cells of many species (see Table 1). These findings have led to the hypothesis that presumably most or all imprinted genes in ES cells are epigenetically unstable (2). However, here we have shown that imprinted neuronal genes, which are thought to be active later in organogenesis, have not been significantly affected by *in vitro* manipulations (Table 1).

Failure to maintain their epigenetic state in manipulated cells may not be a general characteristic of all imprinted genes. Although the prolonged culture of mouse ES cells can affect the epigenetic state of Igf2, H19, Igf2r and U2af-rs1 (1), we have not observed aberrant methylation patterns in the *Snurf/Snrpn* imprinting cluster in passaged ES cells. Even after 35 passages or 3 months in culture, changes of methylation levels in the analyzed genes were not apparent. Although it was suggested that freezing of ES cells and early embryos could damage methylation patterns, our ES cell lines, which had been subjected to two to five additional cycles of freezing and thawing, did not reveal alterations in DNA methylation patterns either (data not shown). Nevertheless, it seems likely that other stress factors in ES cell or embryo cultures are involved in epigenetic dysregulation. Epigenetic abnormalities were observed most frequently when serum supplemented the culture medium (21; reviewed in 45). However, the ES cell lines studied here tolerated serum concentrations between >15 and 20% FCS without interference with their methylation imprint in the Ndn or Snrpn DMR1 loci and other imprinted regions in the *Snurf/Snrpn* cluster. In low passage ES cells (P2–P8), we observed no variation in methylation patterns of the neighboring imprinted loci Ipw, Mkrn3 and Magel2 (data not shown).

All methylation analyses in this study were performed using methylation-sensitive restriction enzymes, which covered several different restriction sites within the *Snurf/Snrpn* imprinted cluster. It could be argued that bisulfite sequencing would provide a more detailed methylation profile. However, we also employed the bisulfite protocol extensively for other regions of the *Snurf/Snrpn* region in mouse. Compared with these results we believe that an overview of the status of DNA methylation in the regions investigated will provide sufficiently detailed information.

The methylation imprints in the *Ndn* and *Snrpn* regions are stable among individual ES cell subclones

Recently, it was demonstrated that expression patterns of the growth-related genes *H19* and *Peg1* vary widely among individual ES cell subclones (2) (see Table 1). The neuronal imprinting cluster around *Snurf/Snrpn* appears to differ in this respect. Our Southern analyses revealed only stably propagated methylation patterns in the *Ndn* and *Snurf/Snrpn* regions of individual ES cell subclones, although the cells had been exposed to several stress factors like mechanical manipulation, high serum concentration, disrupted cell to cell contacts, trypsinization and centrifugation. The reasons for the differences in epigenetic stability of the imprinted neuronal- and growth-related genes observed in ES cells may depend on their transcriptional activity. Many imprinted genes, in particular genes that are part of the insulin and insulin-like growth factor systems and the associated signal-transduction pathways (46), are active during early embryogenesis. Indeed, several of these genes, like *Ig2f* or *Igf2r*, display high epigenetic instability in ES cells (see Table 1; *Grb10* might also be involved in the insulin pathway). Although the *Snrpn* gene is also expressed throughout mouse pre-implantation development (32), most of the neuronal genes are thought to perform their function only very late in brain development. For example, in the early embryo the *Ndn* gene is still inactive and becomes expressed only when the mouse brain is close to full development (47). To further examine this notion, we also analyzed several ES cell lines, which had been induced to differentiate into neuronal cells by using the neurotrophic factor STS. After 2 weeks following STS exposure, we

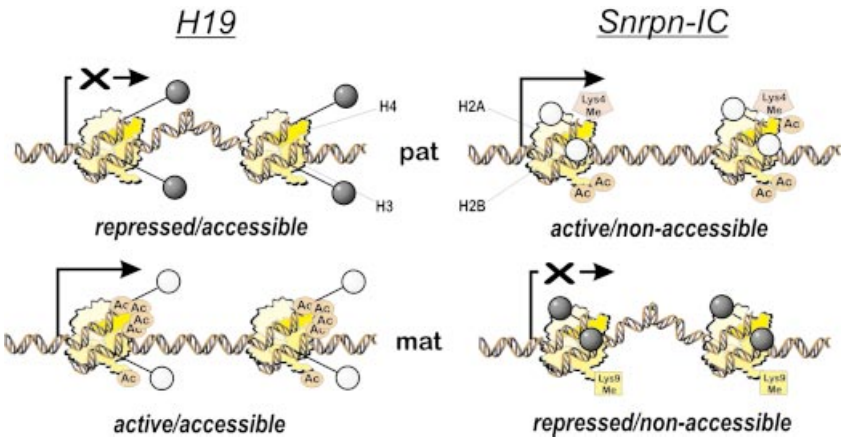

Figure 7. The *Snrpn* IC and the *H19/Igf2* imprinting region may be differentially accessible to modifying enzymes. It seems possible that the overall chromatin structure in these regions may contain several dynamic levels of regulation. For example, a DNA sequence could be either in a methylated repressed state (heterochromatin) or in an active unmethylated conformation (euchromatin). Independent of these conformations, modifying enzymes could have different access to these chromosomal regions. Since the *Snrpn* IC and *H19/Igf2* region are active in ES cells, they could both have an open chromatin structure on their active alleles, but may expose their CpG dinucleotides differently due to a different nucleosomal structure. The *H19* region was reported to be accessible to methyltransferases in ES cells (50), which could lead to an increased susceptibility to epigenetic changes during *in vitro* culture. In contrast, the *Snrpn* IC could be already in an epigenetic maintenance mode, independent of its transcriptional activity. Both genomic regions have complex patterns of known epigenetic modifications (49,51,75–77). Histone H3 on the paternal allele has lysine 4 methylation and is acetylated, accompanied with a hypomethylation of the CpG dinucleotides. On the other hand, on the maternally inherited allele, chromatin is marked by hypermethylation on lysine 9 of H3 and hypermethylation of the DNA (75). Dark lollipops, CpG methylated; white lollipops, CpGs are unmethylated; Ac, histone-acetylation; Me, histone methylation; Lys, lysine residues.

assessed the methylation patterns of the *Snurf/Snrpn DMR1* and *Ndn* genes in these cells. At this point, the cells had largely been differentiated to neuronal cells but had not formed structured tissues. Both loci were epigenetically stable during this differentiation process. In contrast, it was shown that *H19* expression varied dramatically in ES cells that were differentiated in media containing retinoic acid (2). Some of the differentiated cells exhibited a very low expression of *H19*, which was associated with a high degree of *H19* methylation.

The epigenetic stability of the *Snurf/Snrpn* cluster might be accounted for by the structural features of this chromosomal region. *Ndn* expression is not only dependent on the methylation patterns within the promoter region. Although the Ndn gene is hypomethylated in ES cells and blastocysts, it is not expressed (36,47,48). Thus, CpG methylation cannot be the sole determinant in the somatic maintenance of genomic imprints. It has been suggested that non-histone protein binding and alterations in nucleosome and chromatin structures facilitate the somatic maintenance of epigenetic signals at DMRs (29,43). These modifications are likely to affect the epigenetic stability of some imprinted chromosomal regions but not of others. For example, it was shown that the DMRs of the mouse *Snurf/Snrpn* and *U2af1-rs1* genes have allele-specific patterns of acetylation on histones H3 and H4 (49). However, both DMRs behave differently when treated with trichostatin A (TSA), which induces global hyperacetylation of H3 and H4. In ES cells only, TSA led to a selective increase in maternal acetylation at *U2af1-rs1* without interfering with its methylation patterns (49) (Table 1). In contrast, no changes in acetylation were observed in TSA-treated ES cells at the DMR locus in the *Snurf/Snrpn* region.

Another factor, which could influence the epigenetic stability of a chromosomal region, is the ability of the maintenance methyltransferase DNMT1 to methylate nucleosomal sites dependent on the DNA substrate. Indeed, CpG sites, which are packed into nucleosomes reconstituted onto DNA derived from the epigenetically unstable H19 locus, are efficiently methylated, whereas nucleosomes assembled in other chromosomal regions block DNA methylation (50).

One key player in maintaining DNA methylation patterns could be the H3 Lys9 methylation within the Prader–Willi syndrome (PWS) IC. The maintenance of CpG methylation of the PWS IC in mouse ES cells requires the function of histone methyltransferase G9a (51). However, H3 Lys9 methylation is not sufficient for the *de novo* CpG methylation of the PWS IC, which may require molecules not present in ES cells (51,52). These results demonstrate a role for histone modifications in the maintenance of parent-specific CpG methylation and in regulating accessibility to methyltransferases of imprinted regulatory regions (see Fig. 7). Other known histone modifications which could affect the epigenetic stability, are histone ubiquitinilation, phosphorylation or sumoylation, which regulates transcriptional repression (reviewed in 53). Histone-lysine is a key substrate for multiple modifying enzymes, e.g. acetylation and methylation can occur at the same lysine residues. In contrast to histone acetylation, lysine methylation on histones appears to be quite stable and may provide a 'memory' mark (54). Fuks *et al.* (55) provided evidence that the methyl-CpG-binding protein MeCP2 reinforces a repressive chromatin state by acting as a bridge between two global epigenetic modifications, DNA methylation and histone methylation.

Insertion of foreign DNA and epigenetic stability

The fate of foreign DNA in mammalian cells is of considerable interest. Transgenic organisms are generated in increasing numbers and human somatic gene therapy relies almost exclusively on the transfer, fixation and stable expression of

the DNA constructs. The manipulation of ES cells by transgene insertion could compromise the epigenetic state of imprinted genes. Previously, our laboratory has demonstrated that in one mouse line transgenic for bacteriophage λ DNA, hypermethylation was observed in the imprinted *Igf2r* gene (38) (Table 1). Two other mouse lines, transgenic for an adenovirus promoter-indicator gene construct, showed hypomethylation in the *Igf2r* locus. In contrast to *Igf2r*, we could not identify methylation changes within the *Snurf/Snrpn* imprinting cluster in several transgenic ES cell lines. This report demonstrates that the methylation patterns of imprinted genes can be transmitted in cultured ES cells despite the presence of viral sequences in the genome. Of course, it is conceivable that the chromosomal regions affected by the insertion event are dependent on the site(s) of foreign DNA insertion.

The epigenetic stability of the *Snurf/Snrpn* imprinting cluster might be maintained by an active control element within DMR1

During evolution, several imprinting clusters developed different mechanisms for regulating their imprint. The reciprocally imprinted *Igf2/H19* locus is regulated by a methylation-sensitive insulator region that binds the factor CTCF on the unmethylated allele (reviewed in 56), whereas the *Snurf/Snrpn* imprinting cluster is controlled by a bipartite, *cis*-acting IC (reviewed in 7,57). Three reports on deletions covering the mouse and human ICs indicate that the *PWS-SRO* (DMR1) is primarily essential for maintaining the paternal unmethylated state during embryonic development rather than a control element for a germline methylation switch (7,58–60). Hence, there is the potential that small epimutations within the *SNURF/SNRPN* imprinting cluster are repaired throughout embryonic development, resulting in an epigenetically stable chromosomal region.

However, our results on mouse tumor tissues demonstrate that under certain circumstances, genes within the *Snurf/Srpn* imprinting cluster can also be epigenetically dysregulated. In both mouse tumors we observed a significant *de novo* methylation of the *Ndn* gene on the paternal allele. Cancer tissues often exhibit aberrantly methylated promoter regions, which are associated with loss of gene function (61,62). Imprinted genes, especially *IGf2* and *H19*, are commonly associated with tumorigenesis (5). However, methylation data obtained from tumors should be regarded with caution as not only imprinted genes are affected in cancer tissues, overall DNA methylation patterns in tumor cells are generally prone to significant changes (40,63).

Perspectives and open questions

Although the methylation patterns in ES cells may be stably transmitted during *in vitro* manipulation, it is possible that some imprinted neuronal genes are affected in their epigenetic stability during later developmental stages. Two recent reports revealed abnormal methylation patterns at the *SNRPN* locus in patients who were conceived by ICSI and who developed Angelman syndrome (64,65). This observation raises questions about the reliability of these *in vitro* manipulations. Hypomethylation of the *SNRPN* locus in the two affected children indicated that they had a sporadic imprinting defect on the maternal chromosome. The lack of an IC mutation and the detection of mosaic methylation patterns in one of the patients make an inherited defect unlikely and point to a post-zygotic epigenetic defect. However, it is not known whether the imprint abnormality is causally related to the technique of ICSI.

The influence of imprinted genes on brain development is not restricted to the *Snurf/Snrpn* region; other imprinted genes also regulate parental behavior in mammals. Deletions of *Mest*, *Peg3* or *Gfr1* in mice give rise to abnormal, mostly sex-specific behavior (reviewed in 7). In summary, there are several indications that imprinted genes are differentially affected by *in vitro* manipulation depending on their somatic function and time course of expression. However, although the *Snurf/Snrpn* region analyzed in our study was not significantly affected by *in vitro* manipulation, these results may not be representative of all imprinted genes expressed in the mammalian brain. As assisted reproductive technologies are widely used, it will be important to investigate further exactly how environmental factors influence the epigenome.

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