Inactivation of Imprinted Genes Induced by Cellular Stress and Tumorigenesis

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Abstract

Cellular proliferation under stressful conditions may result in permanent genetic and epigenetic changes. Using primary mouse embryonic fibroblasts, we have completed a screening test to identify gene expression changes triggered when cells proliferate under stress. In this manner, we have discovered a novel phenomenon that consists of the rapid and coordinated silencing of genes subject to imprinting, including *Cdkn1c*, *Igf2*, *H19*, *Ndn1*, *Grb10*, and *Meg3*. This generalized silencing of imprinted genes is independent of the stress-responsive tumor suppressors p53, $p19^{Arf}$, and $p16^{Ink4a}$, and it is also independent of the oxidative culture conditions and the stress response known as "mouse embryonic fibroblast senescence". In the case of *Cdkn1c* and *H19*, their silencing is associated with unscheduled *de novo* methylation of the normally expressed allele at their corresponding CpG island promoters, thus resulting in biallelic methylation. Finally, we provide evidence for frequent *de novo* methylation of *Cdkn1c* in a variety of murine cancer types. Altogether, our data support the concept that silencing of imprinted genes, including methylation of *Cdkn1c*, constitutes an epigenetic signature of cellular stress and tumorigenesis. (Cancer Res 2005; 65(1): 26-33)

Introduction

Many of the properties that characterize cancer cells can be regarded as adaptations that confer them the ability to proliferate under stressful conditions, such as hypoxia, oxidative stress, DNA damage, abnormal combinations of growth and differentiation factors, inappropriate extracellular matrix, and improper cell-to-cell contacts (1). Interestingly, *in vitro* cultivation of cells may entail nonphysiologic conditions that are reminiscent of the stresses present during tumorigenesis. In this context, it is not surprising that cultivation of normal cells *in vitro* frequently results in the activation of tumor suppressor pathways (2).

Under standard *in vitro* conditions, most types of primary mammalian cells proliferate only for a short number of cell doublings, typically less than 15, before entering into a stable proliferative arrest, variously known as "culture shock", "crisis", "mortality stage 0", or "mouse embryonic fibroblast (MEF) senescence" for the particular case of mouse embryo fibroblasts (3). The nature of the stresses underlying culture shock is incompletely understood, probably being the result of a complex combination of individual types of stress. Some stress-inducing motifs have emerged recently that contribute to culture shock, such as the high oxygen concentrations typically used for *in vitro* cultivation (4) or, in the case of epithelial cells, the absence of an appropriate extracellular matrix (5). Mechanistically, proliferation under *in vitro* stress results in the progressive derepression of the *Ink4a/Arf* tumor suppressor locus by epigenetic mechanisms that are in part controlled by Polycomb proteins, such as Bmi1 (4, 6, 7). The concomitant activation of the $p19^{Arf}$/p53 and $p16^{Ink4a}$/Rb pathways may lead to the formation of characteristic heterochromatic foci known as senescence-associated heterochromatic foci that contribute to the long-term permanence of the senescence-associated proliferative arrest (8). On another note, it must be emphasized that the cultivation of cells under *in vitro* stress has yielded a wealth of information about molecular pathways deregulated in cancer, which further reinforces the idea that *in vitro* stresses bear resemblance to the stresses present during tumorigenesis (2).

Another area where *in vitro* culture stress has been analyzed in some detail is the stability of imprinting. Imprinted genes are characterized by being monoallelically expressed from either the maternal or the paternal allele. Monoallelic expression reflects allele-specific epigenetic marks that are largely associated with DNA methylation (9). In the case of embryonic stem cells, cultivation *in vitro* results in a high degree of internal heterogeneity and instability in the expression levels of imprinted genes (10, 11). Also, cultivation of preimplantation embryos may result in the altered expression of imprinted genes (12, 13). Together, these data suggest that *in vitro* culture stress may impinge on the expression of imprinted genes.

In the present work, we have used primary MEFs to explore the gene expression profile of cells proliferating under *in vitro* stress conditions. In this manner, we have found that the transcriptional profile of cells is largely stable with the notable exception of imprinted genes. Our results indicate that exposure of cells to nonphysiologic stresses, including tumorigenesis, results in generalized silencing of imprinted genes.

Materials and Methods

Cell Culture. Primary MEFs were derived from day 13.5 embryos obtained from the corresponding colonies of wild-type, $p53^{-/-}$ (14), and $Ink4a/Arf^{-/-}$ (15) mice maintained at the Spanish National Center of Biotechnology, Madrid, in a mixed genetic background C57BL6; 129Sv. MEFs were prepared as previously described (16). Briefly, each embryo was dispersed and trypsinized in a 10-cm diameter dish and the resulting cells were incubated for 1 or 2 days until confluent. Attached cells were then transferred to a 15-cm diameter dish and incubated for 2 days. These cells were frozen in aliquots and considered passage 0 (P0). Unless otherwise stated, cells were maintained in DMEM supplemented with 10% fetal bovine serum and a commercial cocktail of antibiotics and antimycotics, in an incubator with 5% CO_2, at 37°C, and in the presence of 20% O_2. In the

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particular case of cells cultivated under hypoxic conditions, MEFs at P0 (thawed under normoxia, i.e., 20% O_2) were transferred to an incubator with control of the oxygen tension and were serially passaged according to the 3T3 protocol (see below) under 3% O_2 and 5% CO_2. The sex of the embryos used to derive MEFs was determined by PCR using the following pair of primers for the *Sry* gene, which is specific of the Y chromosome: SRY-F (5′-AGAGATCAGCCAGCAGCTGG-3′) and SRY-R (5′-TCTTGCCTGTATGTGATGGC-3′; ref. 17). For the serial 3T3 cultivation protocol, we seeded 1 $\times 10^6$ cells in a 10-cm diameter dish, cells were counted 3 days later, and 10^6 cells were replated. This procedure was repeated for 25 to 30 passages. The increase in population doubling level (PDL) was calculated according to the formula PDL = $\log(n_f/n_0)$ / log2, where n_0 is the initial number of cells and n_f is the final number of cells.

Analysis of Microarrayed Filters. MEFs *Ink4a/Arf*$^{-/-}$ at passage 1 (P1) and passage 20 (P20) of the 3T3 serial cultivation protocol were serum-starved (0.1% fetal bovine serum) for 4 days. Cell cycle arrest at G_0 was confirmed by fluorescence-activated cell sorting. Synchronization of cultures at G_0 was done with the aim of eliminating possible differences in the proliferative rate of the cultures. Total RNA was extracted from cells at different passages and polyadenylated RNA was prepared using Oligotex columns (Qiagen, Valencia, CA). Two microarrayed filters were purchased from Genome Systems, St. Louis, MO (Mouse Gene Discovery Array, version 1.1), each filter containing the same set of 19,315 mouse expressed-sequence tags (EST; each EST arrayed by duplicate in each filter). Synthesis of ^{33}P-labeled cDNA and hybridization of the filters were done following the instructions of the supplier. Hybridization signals were analyzed using a Molecular Dynamics Phosphorimager and ImageQuant software for quantification, with external and internal normalization controls (details on the quantification method used are provided elsewhere; ref. 18).

RNA Hybridization. For Northern analysis, 10 μg of total RNA were electrophoresed, blotted and hybridized according to standard procedures. The following ESTs (Genbank accession numbers) were used as probes: for *H19*, AL747191; for *Cd81*, BF783079; for *Ndn1*, AW742322; for *Grb10*, BF784469; and for *Meg3*, W54280. Other probes used were: for *Arf* exon 1β, we used a PCR fragment of 230 bp produced by primers mE1β-F (5′-GTCACAGTGAGGCCGCTG-3′) and mE1β-R (5′-TGGTCCAGGATTCCGGTG-3′); for *Ink4a* exon 1α, we used a PCR fragment of 204 bp produced by primers mE1α-F (5′-ACACGACTGGGCGATTGG-3′) and mE1α-R (5′-CTGAATCGGGGTACGACC-3′); and for Igf2 exon 4, we used a PCR fragment of 240 bp produced by primers mIgf2-F (5′-AGTCGATGTTGGTGCTTCTCA-3′) and mIgf2-R (5′-GGGTGGCACAGTATGTCTCC-3′). The cDNA of *Cdkn1c* was kindly provided by Dr. Mariano Barbacid (CNIO, Madrid), and the cDNA of γ-actin was kindly provided by Dr. Ignacio Palmero (IIB, Madrid).

Carcinogenesis. These experiments were done at the specific pathogen-free animal facility of the Spanish National Center of Biotechnology, Madrid, using wild-type mice (C57BL6 genetic background) of 3 to 5 months of age. For the induction of fibrosarcomas, mice received a single i.m. injection of a 40 μL solution containing 3-methyl-cholanthrene (Aldrich, Milwaukee, WI) in one of the rear legs, at a concentration of 25 μg/μL, and dissolved in sesame oil (Sigma, St. Louis, MO), as previously described (19). Mice were observed on a daily basis until tumors of more than 1.5 cm diameter developed on the injected leg, at which point, the animals were sacrificed and the tumors were extracted for further analysis. For the induction of urinary bladder carcinomas, mice of 3 to 5 months of age were exposed to *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (TCI, Japan) permanently present in the drinking water at a concentration of 0.025%, for 20 weeks, as previously described (20, 21). Mice were observed on a daily basis and were sacrificed when they manifested signs of morbidity. On necropsy, tumors in the urinary bladder were evident and were extracted for further analysis. For γ-radiation tumorigenesis, 1-month-old mice were irradiated weekly for 4 weeks with a 1.75 gray dose (22), from a ^{137}Cs source (MARK 1-30 Irradiator; Shepherd & Associates), at a rate of 1.14 gray/minute.

Analysis of DNA Methylation. Five to ten micrograms of genomic DNA from MEFs, adult liver, or fresh tumors were processed following the bisulfite genomic sequencing protocol, using 10 mmol hydroquinone and 3.6 mol/L sodium bisulfite, as described (23). Following chemical transformation, samples were dialyzed using desalting columns. After NaOH treatment, samples were resuspended in 50 μL of Tris EDTA [10, 0.1 mmol, (pH 8.0)] as described in the same protocol. One microliter of bisulfite-treated DNA was used as a template in a PCR reaction using primers specific for one of the two resulting noncomplementary DNA strands that do not include any CpG in their sequence to prevent any bias in the amplification from methylated or unmethylated molecules. PCR conditions were 10 cycles (1 minute at 94°C, 1 minute at the specific annealing temperature, and 3 minutes at 68°C) followed by 25 cycles (1 minute at 94°C, 30 seconds at the specific annealing temperature, and 3 minutes at 68°C with an increase of 20 seconds per cycle in the extension time). PCR amplification of bisulfite-treated DNA is sometimes inefficient and, for this reason, 5 μL of the first PCR reaction were used as a template for a second reaction using one of the first primers and another one internal to the first amplified fragment. Resulting products were recovered from the gel, cloned into pGEM-T (Promega, Madison, WI) and sequenced. The sequences of the primers used are available on request.

Results

Changes in Gene Expression During *In vitro* Cultivation of Mouse Embryonic Fibroblasts. To identify changes in gene expression during *in vitro* culture of primary MEFs, we compared the global transcription profiles of cell populations after 1 (P1) and 20 (P20) passages *in vitro* (Fig. 1*A*). It is well established that wild-type MEFs enter into a permanent and stable proliferative arrest known as MEF senescence on proliferation *in vitro* for about 10 to 15 cell doublings, equivalent to four to five passages (Fig. 1*A*, *white circles*; ref. 3). For this reason, and to prevent the identification of changes associated with the establishment of MEF senescence, we used MEFs derived from mice deficient in the tumor suppressor locus *Ink4a/Arf*, which have the ability to proliferate indefinitely *in vitro* without showing any signs of senescence (Fig. 1*A*, *black circles*; ref. 15). Total RNA from passages P1 and P20 were used to hybridize a commercial microarray filter containing about 19,000 ESTs (see MATERIALS AND METHODS). Analysis of the filters showed that about 2,800 ESTs gave a measurable hybridization signal with RNA from P1 MEFs. One EST was identified as significantly up-regulated between P1 and P20, and three ESTs were identified as significantly down-regulated (more than 3-fold up-regulation or down-regulation; see Fig. 1*B* for an example). These four cases were subject to further validation by Northern blotting, obtaining confirmation for only one EST, which corresponded to the *Cdkn1c* gene, encoding the cell-cycle inhibitor p57^{Kip2}. Thus, a first general conclusion from our screening is that MEFs do not undergo dramatic variations in gene expression under standard *in vitro* stress conditions. Also, the stability of the expression pattern after a period of 20 passages argues against the emergence of cell subpopulations. The behavior of *Cdkn1c* seems to reflect a selective phenomenon likely restricted to a small fraction of genes. The down-regulation of *Cdkn1c* was observed not only in *Ink4a/Arf*-null MEFs but also in *p53*-null MEFs (which share with the *Ink4a/Arf*-null MEFs the characteristic of not undergoing MEF senescence), and in wild-type MEFs (Fig. 2*A*). Therefore, proliferation under *in vitro* stress conditions results in

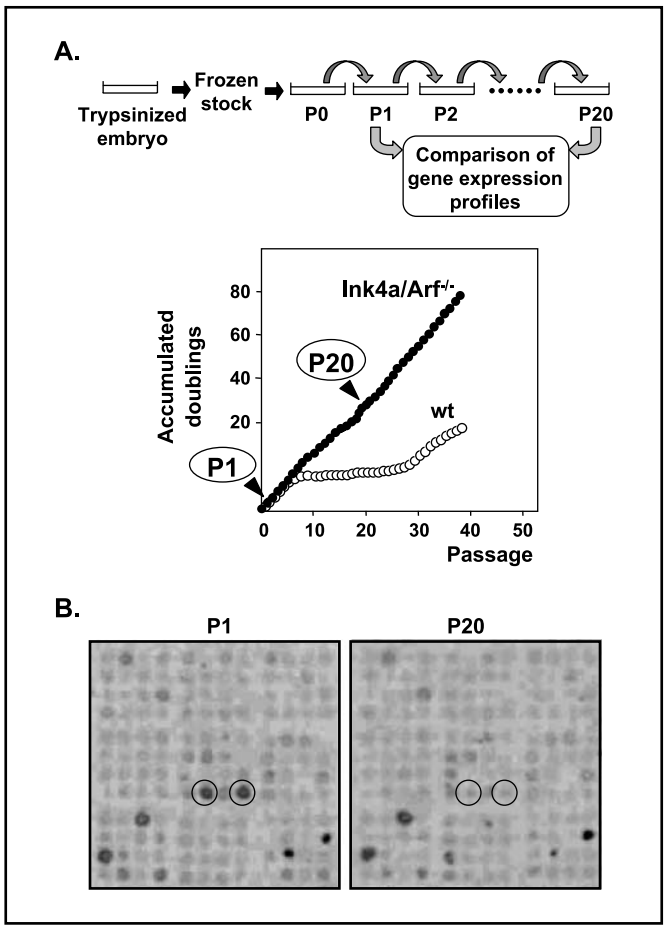

Figure 1. Changes in gene expression during *in vitro* cultivation of MEFs. *A,* (*top*) scheme of the serial passage protocol of primary MEFs. *Bottom,* accumulation of cell doublings (*y*-axis) along serial 3T3 passages (*x*-axis) for *Ink4a*/*Arf*-null MEFs (•) and wild-type MEFs (○). *Large ovals containing P1 and P20,* corresponding passages used for the microarray screening. *B,* magnification of a small region of two microarray filters hybridized, respectively, with total RNA of *Ink4a*/*Arf*-null MEFs at passages 1 (P1) and 20 (P20). All the ESTs are found in pairs in each filter. The *Cdkn1c* signal was clearly detectable at P1 and reduced to background levels at P20 (*circles,* duplicate spots of the *Cdkn1c* EST in each filter).

the down-regulation of *Cdkn1c* independently of the functional status of the tumor suppressors $p16^{Ink4a}$, $p19^{Arf}$, or p53, and of the establishment of MEF senescence.

Silencing of Imprinted Genes. It is well-established that the *Cdkn1c* gene is subject to imprinting (24), being located within a gene cluster that includes other imprinted genes, such as *Igf2* and *H19* (25). Genetic and epigenetic aberrations in this cluster are responsible for the Beckwith-Wiedemann syndrome (BWS; ref. 25). In the light of the specific inactivation of *Cdkn1c*, we asked whether *in vitro* stress affected the expression of other imprinted genes. Except for *Cdkn1c*, no other gene from the BWS locus was either present or gave a detectable signal in the microarray. Thus, using Northern blotting, we tested the expression of imprinted genes from this locus. As shown in Fig. 2*A*, *Igf2* and *H19* were strongly expressed at the earliest passage (P0) in primary MEFs, and were subsequently down-regulated paralleling the behavior of *Cdkn1c*. Also, as observed for *Cdkn1c*, the rapid and severe down-regulation of *Igf2* and *H19* occurred independently of the status of *Ink4a/Arf* and *p53* (Fig. 2*A*). In sharp contrast with the behavior of imprinted genes, the expression of *Arf*, which is one of the two transcripts synthesized from the *Ink4a/Arf* locus, was significantly up-regulated in wild-type MEFs until cells reached MEF senescence (around P6). Loss of *Arf* expression occurs in 25% to 50% of spontaneously immortalized MEF cultures (26, 27), and this was the case in the particular example shown in Fig. 2*A* (note the loss of *Arf* expression at P16 and P30). These results indicated that repression of *Cdkn1c* on *in vitro* stress is shared with other imprinted genes present at the BWS cluster. Of note, this silencing was independent of the parental origin of the imprinting because *Cdkn1c* and *H19* are expressed from the maternal allele, whereas *Igf2* is expressed from the paternal allele (25).

The BWS cluster also comprises genes that are not subject to imprinting, as well as others that are imprinted only temporarily (28). We asked whether the down-regulation observed for *Cdkn1c*, *Igf2*, and *H19*, was extensive to other genes located in the BWS cluster but not subject to imprinting. A particularly suitable gene to address this question was *Cd81* (also known as *Tapa1*), which is a gene centrally located in the BWS cluster,

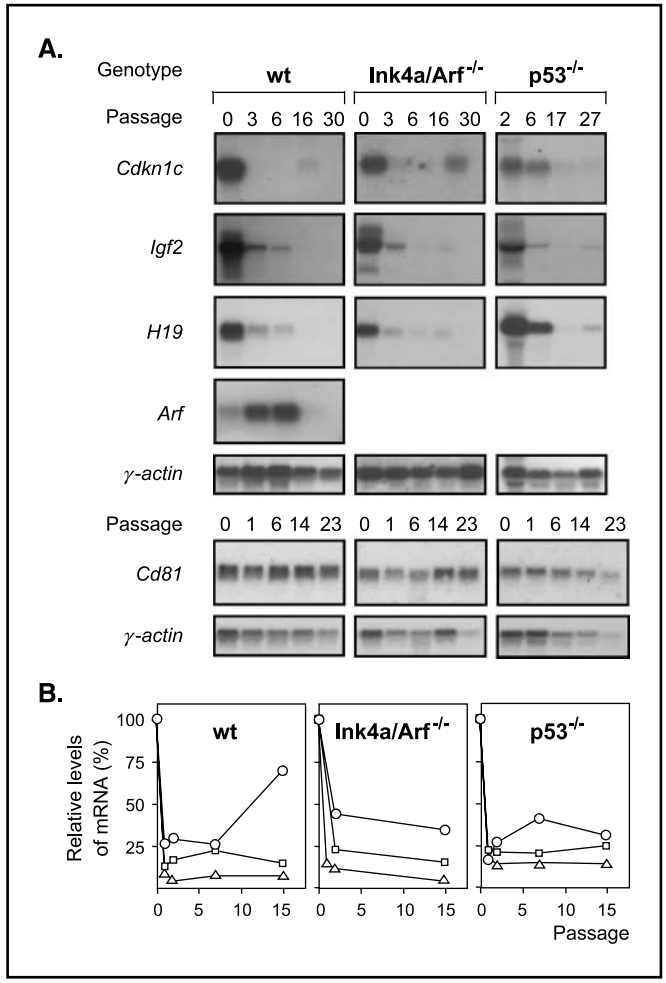

Figure 2. Coordinated down-regulation of imprinted genes on proliferation of MEFs *in vitro*. *A,* Northern blots of serially passaged cultures of the indicated genotypes were hybridized using probes for *Cdkn1c*, *Igf2*, *H19*, *Arf*, and *Cd81*. γ-*Actin* was included as a loading control. Data for *Cdkn1c* and *Igf2* were repeated at least thrice for each of the three genotypes using independent MEF cultures, and obtaining in all cases the same results as those shown here. *B,* quantification of *Grb10* (○), *Ndn1* (□), and *Meg3* (△) mRNA levels in MEFs of the indicated genotype.

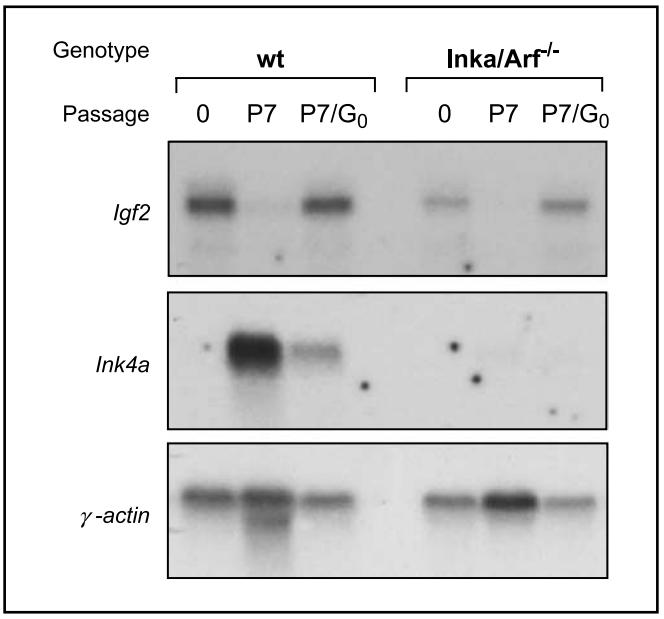

Figure 3. Down-regulation of *Igf2* by *in vitro* stress requires cell proliferation. Primary MEFs of the indicated genotypes were plated from the frozen stocks (P0), serially passaged seven times (P7), or maintained in culture for an equivalent time but without passaging the cells ($P7/G_0$). Under the latter conditions, cultures are quiescent for essentially the entire duration of the experiment (21 days corresponding to seven passages). Total RNA was extracted from the different cultures and Northern blots were done to detect *Igf2* and *Ink4a*. The same results were obtained in at least three additional experiments with independent cultures (data not shown).

flanked by *Cdkn1c* at one side, and *Igf2* and *H19* at the other side (25). Interestingly, *Cd81* is expressed monoallelically before day E8.5 and biallelically at later stages (29). This implies that in MEFs, which are obtained from day E13.5 embryos, *Cd81* is biallelically expressed. Northern analysis showed that, unlike the transcriptional repression of the other examined genes at the BWS cluster, *Cd81* expression remained constant at all passages (Fig. 2*A*). This result suggested that the relevant property common to the down-regulated genes at the BWS cluster was the fact that they were subject to imprinting at the time of cell exposure to *in vitro* culture stress.

A relevant question at this stage was whether other imprinted genes located outside the BWS cluster undergo repression on cell proliferation *in vitro*. We tested seven additional imprinted genes, but only three of them, *Meg3*, *Ndn1*, and *Grb10*, were detectably expressed by Northern blot in MEFs at passage P0 (the remaining tested genes, *Ube3a*, *Sgce*, *Zac1*, and *U2af1-rs1*, were below detection level). Remarkably, the expression of *Meg3*, *Ndn1*, and *Grb10* followed a decline similar to that of the imprinted genes examined at the BWS cluster (Fig. 2*B*). Taking into account the global stability of expression on proliferation *in vitro*, the observed down-regulation of all the imprinted genes tested (*Cdkn1c*, *Igf2*, *H19*, *Meg3*, *Ndn1*, and *Grb10*) seems to be highly specific for this class of genes.

We asked next whether exposure to *in vitro* culture conditions was sufficient for the observed silencing or if cell proliferation was also required. We addressed this point by splitting cultures of primary MEFs into two experimental groups, one was subjected to routine 3T3 passages (with plate transfer and dilution every 3 days), and the other one was maintained under the same culture conditions but without passaging (i.e., with medium changes every 3 days). These last cultures reached saturation density in 2 to 3 days and stayed confluent for the remaining 21 days, (equivalent to seven passages, abbreviated as $P7/G_0$). We chose to analyze the expression of *Igf2* because of its involvement in regulating cell proliferation. Interestingly, *Igf2* expression remained unchanged in the $P7/G_0$ cultures, thus indicating that its silencing requires not only the exposure to stress but also cell proliferation (Fig. 3). We also examined the behavior of the *Ink4a/Arf* locus by analyzing the expression of one of the two transcripts encoded by this locus, which in this occasion was the *Ink4a* transcript, and we observed that the induction of this gene by culture stress also requires cellular proliferation (Fig. 3). Thus, *in vitro* culture stress requires concomitant cell proliferation to repress *Igf2* and to induce *Ink4a*.

The oxidative stress produced by normoxic conditions (20% O_2), which are usually employed to cultivate cells, is one of the main factors that contribute to *in vitro* culture stress. Indeed, cultivation of primary MEFs under hypoxia (3% O_2), which is closer to the *in vivo* physiologic conditions, prevents the stress response known as MEF senescence (4). To determine if the oxidative stress associated with normoxia was at the basis of the stress that triggers the silencing of imprinted genes, we serially cultivated wild-type MEFs under 3% and 20% O_2, respectively. As reported (4), MEFs under hypoxia did not undergo proliferative arrest up to at least passage 9 (P9), whereas MEFs under normoxia underwent MEF senescence between P4 and P6 (results not shown). We observed that the silencing of *Igf2* occurred when cells were grown both under hypoxia and normoxia, thus ruling out the idea that silencing of *Igf2* is related to oxidative stress (Fig. 4). In comparison, *Arf* was induced when cells were grown under normoxia, but was marginally induced when cells were grown under hypoxia (Fig. 4). Experiments in which cells were transferred for two passages from

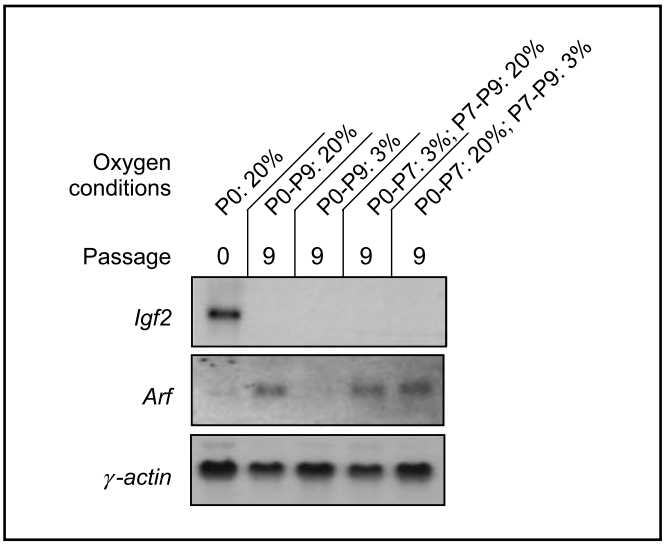

Figure 4. Down-regulation of *Igf2* by *in vitro* stress is independent of the oxidative stress associated to normoxia. Primary wild-type MEFs were serially passaged according to the 3T3 protocol until passage 9 (P9) either under normoxia (20% O_2) or under hypoxia (3% O_2). At passage 7 (P7), subcultures were also initiated in which the oxygen conditions were changed. Total RNA was extracted from the different cultures and Northern blots were done to detect *Igf2* and *Arf*. The same results were obtained with two additional MEF cultures from independent embryos (data not shown).

hypoxia to normoxia, clearly indicated that oxidative stress was at the basis of the induction of *Arf* (Fig. 4).

Collectively, we conclude from the above observations that proliferation *in vitro* under stressful conditions results in generalized silencing of imprinted genes. Further analyses have allowed us to exclude the involvement of the stress-responsive tumor suppressors *p53*, *Arf*, and *Ink4a*, as well as other processes related to MEF senescence, including oxidative stress.

Silencing of *Cdkn1c* and *H19* is Associated with *De novo* Methylation. The promoters of some imprinted genes are included in CpG islands that are nonmethylated in the expressed allele and methylated in the nonexpressed allele. This is the case of *Cdkn1c* and *H19*, whose promoters are contained in CpG islands that are methylated only in the paternal and maternal alleles, respectively. The coexistence of methylated and nonmethylated alleles is in contrast with the general situation of CpG islands associated with nonimprinted genes, which under normal conditions, are free of methylation regardless of their transcriptional status (30, 31). As a working hypothesis, we considered the possibility that the stress-induced silencing of *Cdkn1c* and *H19* could be associated with *de novo* methylation of their CpG island promoters, thus resulting in biallelic methylation. It has been previously established that the promoters of genes associated with CpG islands are located between the transcription initiation site and the 5′ boundary of the CpG-rich region (32). Following this criterion, we tested the promoter regions of *Cdkn1c* and *H19* for methylation at various passages (Fig. 5*A*). To measure methylation, we used the bisulfite modification method, which allows to determine the methylation status of every CpG in a given genomic region (23). As this method provides information for individual molecules, several clones were sequenced for every promoter. All sequenced clones fell into two categories, either "nonmethylated" (with essentially none of the CpG dinucleotides methylated) or "methylated" (with more than 40% of the CpG dinucleotides methylated; see Fig. 5*B* for examples corresponding to *Cdkn1c*). Interestingly, at passage P1, and even more clearly at passages P7 or P31, the percentage of methylated *Cdkn1c* and *H19* promoters increased to more than 80%, reaching 100% in several instances (Table 1). In addition, in the case of methylated alleles, their methylation density per individual clone increased from 40% to 75% in P0 MEFs, to essentially 100% in some P1 and P7 clones and in most of the P31 clones (Fig. 5*B*). Remarkably, those clones that remained nonmethylated were completely devoid of methylation at all their CpG dinucleotides (Fig. 5*B*, for quantifications see Table 1). The coexistence in the same cell population of promoters that are fully methylated and promoters that are completely free of methylation suggests that, at the level of individual promoters, the methylation process is highly processive, resulting in very high densities of methylation (close to 100%) per promoter. Together, these data show that proliferation under *in vitro* stress triggers a rapid process of unscheduled *de novo* methylation that results in biallelic methylation of *Cdkn1c* and *H19*.

Monoallelic methylation is not exclusive of imprinted genes, but also affects genes present at the X chromosome in the particular

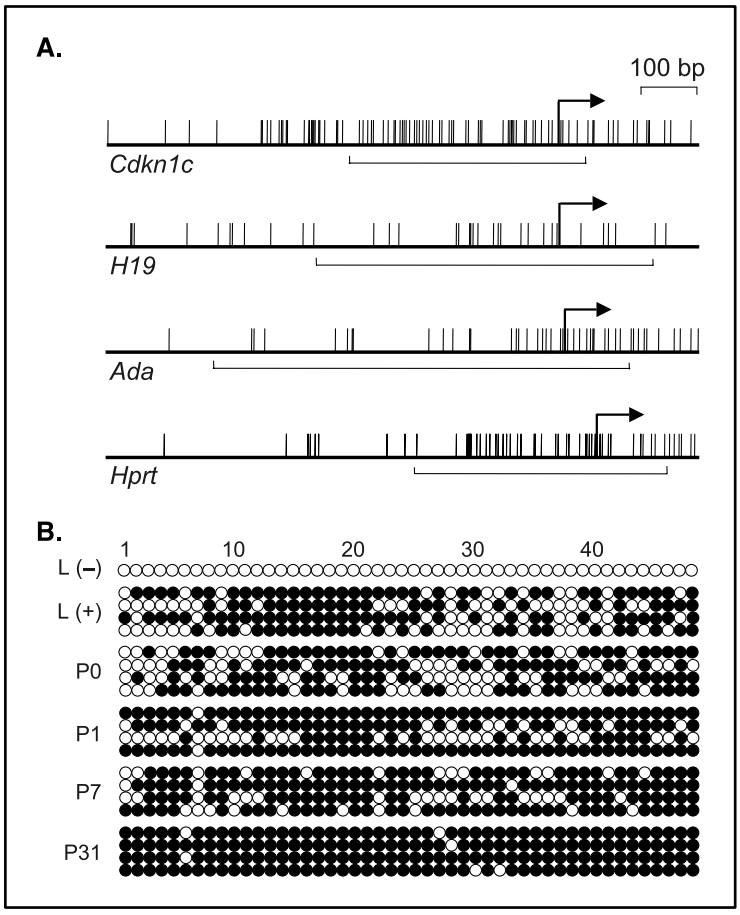

Figure 5. *De novo* methylation of CpG island promoters. *A,* CpG distribution across the promoter regions of the murine *Cdkn1c*, *H19*, *Ada*, and *Hprt* genes. *Vertical lines,* CpG dinucleotides; *arrows,* transcription initiation site; *brackets,* encompass the regions analyzed for methylation. *B,* circles represent the 48 CpG sites included in the region tested for methylation in the *Cdkn1c* gene promoter. As an example, one nonmethylated L (−) and four methylated L (+) clones from the liver, as well as four clones from MEFs at each of the indicated passages. Only examples of methylated clones are shown in P0 and P1 to illustrate the clonal differences among them. All nonmethylated clones in each case are identical to L (−). Complete methylation data for all the promoters analyzed by bisulfite analysis are indicated in Tables 1 and 2.

Table 1. Methylation of *Cdkn1c*, *H19*, and *Hprt* gene promoters in MEFs at different passages

Gene	Samples			
	P0	P1	P7	P31
Cdkn1c	8/12 (67%)	9/13 (69%)	11/11 (100%)*	10/10 (100%)*
H19	13/21 (62%)	18/19 (95%)*	12/15 (80%)*	
Hprt (female)		4/7 (57%)	6/12 (50%)	
Hprt (male)		0/12 (0%)		0/12 (0%)

NOTE: Fractions indicate the number of methylated clones relative to the total number of clones analyzed. Percentages are indicated in parentheses. Clones were considered methylated when more than 40% of their CpG dinucleotides were methylated; nonmethylated clones were essentially free of methylation. The data for each passage was obtained from independent cultures derived from different embryos.
*Ratios that were significantly different (Fisher exact test, $P < 0,05$) from the expected ratio of methylated clones, which in the case of *Cdkn1c*, *H19*, and *Hprt* (in females) is 50% of the total number of analyzed clones.

case of female-derived (XX) cells (33, 34). For this reason, we considered the possibility that the abovementioned phenomenon of aberrant *de novo* methylation could also affect genes in the X chromosome. To explore this hypothesis, we analyzed the stability of methylation in the promoter region of the *Hprt* gene, which is located in the X chromosome. We monitored the methylation status of *Hprt* both in female (XX) and male (XY) MEFs at the same stages as we did for *Cdkn1c* and *H19*. In contrast with the behavior of *Cdkn1c* and *H19*, female-derived cells maintained a ratio of *Hprt* methylated promoters at P1 and P7 that was not significantly different (Fisher exact test, $P > 0.05$) from the expected ratio of 50%. In the case of male (XY) MEFs, their single copy of *Hprt* remained free of methylation even at P31 (Table 1). These results indicate that, at least in the case of *Hprt*, monoallelic methylation is not sufficient to render the nonmethylated allele susceptible to aberrant *de novo* methylation on *in vitro* stress and further emphasizes the differential susceptibility of imprinted genes *Cdkn1c* and *H19* to *de novo* methylation.

***De novo* Methylation of *Cdkn1c* During Tumorigenesis.** As mentioned in the INTRODUCTION, proliferation under *in vitro* stress recapitulates aspects of the stress associated with tumorigenesis. It is also well known that gene inactivation by *de novo* methylation of CpG island promoters is a common mechanism of epigenetic gene inactivation in tumors (35). For this reason, we asked whether *de novo* methylation of *Cdkn1c* occurred frequently during the development of tumors in mice. We monitored the methylation of the *Cdkn1c* promoter in three types of experimentally induced mouse tumors (see MATERIALS AND METHODS), i.e., bladder carcinomas (epithelial origin), thymic lymphomas (hematopoietic origin) and fibrosarcomas (mesenchymal origin). To classify an individual tumor as "aberrantly methylated" at the *Cdkn1c* locus, we chose a threshold of 80% of its promoters methylated because, considering our promoter sampling size, this percentage corresponds to a significant variation (Fisher exact test, $P < 0.05$) relative to the expected ratio of 50%. Table 2 shows that the promoter region of *Cdkn1c* was aberrantly methylated in seven out of the eight (88%) tumors analyzed. As previously done with MEFs, we analyzed the methylation status of the *Hprt* gene. In total, four out of eight (50%) of the tumors showed aberrant methylation of *Hprt* in male-derived and in female-derived tumors (Table 2), although this percentage is an underestimation if we consider that in males the normal status of *Hprt* is nonmethylated. Finally, we measured the methylation status of the promoter-associated CpG island of a nonimprinted autosomal gene, such as

Table 2. Methylation of *Cdkn1c*, *Hprt*, and *Ada* gene promoters in tumors

Gene	Tumor type (ID and sex)								Aberrant methylation in all tumors†
	Carcinomas*		Lymphomas		Sarcomas				
	#1 Female	#2 Male	#1 Female	#2 Male	#1 Female	#2 Female	#3 Male	#4 Male	
Cdkn1c	15/15 (100%)	15/15 (100%)	13/13 (100%)	10/10 (100%)	11/18 (61%)	12/12 (100%)	14/16 (87%)	13/15 (87%)	7/8 (88%)
Hprt	8/13 (61%)	12/13 (92%)	11/14 (78%)	5/9 (55%)	16/18 (89%)	14/15 (93%)	8/13 (61%)	10/12 (83%)	4/8 (50%)
Ada	0/8 (0%)	0/6 (0%)			0/14 (0%)			0/17 (0%)	0/4 (0%)

*Fractions indicate the number of methylated clones relative to the total number of clones analyzed. Percentages are indicated in parentheses. Clones were considered "methylated" when more than 40% of their CpG dinucleotides were methylated; nonmethylated clones were essentially free of methylation.
† Fractions indicate the number of aberrantly methylated tumors relative to the total number of tumors analyzed. Tumors were considered as aberrantly methylated for a particular promoter when the percentage of methylated promoters was higher than 80% (which, in the case of genes with an expected normal ratio of 50%, corresponds to a Fisher exact test of $P < 0.05$). Percentages of methylated tumors are indicated in parentheses.

Ada. In contrast to the situation with *Cdkn1c*, the four tumors analyzed remained completely free of methylation at the *Ada* promoter. Together, these observations indicate that during tumorigenesis, as well as during proliferation of MEFs under stress, aberrant *de novo* methylation of the imprinted gene *Cdkn1c* is a highly frequent event.

Discussion

The aim of this work was to identify gene expression changes triggered when cells proliferate *in vitro*. The analysis of the expression of approximately 2,800 ESTs in MEFs indicated that the large majority of genes are stably expressed when proliferating under stress. Imprinted genes constitute a remarkable exception. We have tested a total of six imprinted genes (*Cdkn1c*, *Igf2*, *H19*, *Meg3*, *Ndn1*, and *Grb10*) and all of them became severely and coordinately down-regulated during the first cell duplications. This occurred in the face of the general transcriptional stability observed for nonimprinted genes, and despite the fact that the six imprinted genes analyzed are functionally unrelated and are located at various genomic locations. The silencing of *Igf2* in association with *in vitro* culture of primary fibroblasts has been observed in two previous instances (36, 37). Our results confirm these previous reports and suggest that this phenomenon could apply to the great majority of imprinted genes.

Exploring the mechanistic basis for the generalized silencing of imprinted genes, we have found that, in the case of *Cdkn1c* and *H19*, it is associated with *de novo* methylation of their corresponding promoter regions. Methylation analysis of individual molecules indicated that *de novo* methylation is already noticeable after just one passage, which represents approximately two cell divisions, and eventually affects virtually every CpG across a particular promoter. Specifically, cell populations that initially had about 50% of their *Cdkn1c* and *H19* alleles completely free of methylation underwent, in a few passages, *de novo* methylation resulting in almost 100% of their *Cdkn1c* and *H19* alleles being fully methylated (see Table 1). These features suggest the existence of an active mechanism of *de novo* methylation that is triggered by proliferation under stressful conditions, and which selectively affects the promoter regions of imprinted genes. The fact that the silencing affects the bulk of the cell populations in the span of a few cell divisions excludes other possibilities such as genetic mutations followed by a selection process. Histone modification precedes DNA methylation during the inactivation of the X chromosome in mammals (38) and during transgene silencing (39), and it is possible that a similar process could occur prior to *de novo* methylation of *Cdkn1c* and *H19*.

As the process of tumorigenesis entails proliferation under stressful conditions, we reasoned that cancer cells directly extracted from primary tumors could represent a good model to test if aberrant methylation of *Cdkn1c* also occurs *in vivo*. We have experimentally induced three different types of tumors in mice and found that the *Cdkn1c* promoter was *de novo* methylated in 88% of the tumors analyzed, a level comparable to that observed in MEFs. This observation is in agreement with similar ones by other investigators reporting inactivation of *Cdkn1c* by *de novo* methylation in a large proportion of Wilms tumors, as well as, in hematopoietic, gastric and colorectal cancers (40–43). Moreover, other imprinted genes are also frequently silenced in a variety of human tumors, as in the case of *Igf2* in about 50% of human liver cancers (44), *Peg3* in gliomas (45), and *Arh1* in breast cancer (46). Also, the process of organismal aging is thought to involve the accumulation of various forms of cellular damage, and this could be the basis of the progressive loss of expression of *Igf2* in association with human aging (37). Our results unify these observations and allow us to propose that imprinting is an extraordinarily unstable epigenetic situation that can be perturbed by a variety of stressors such as *in vitro* proliferation, tumorigenesis, and aging, generally resulting in the permanent silencing of imprinted genes.

Previously, other experimental systems have been reported in which *de novo* methylation occurs in a rapid and predictable manner in *in vitro* cultured cells. In particular, cancer cell lines with aberrant methylation at a specific gene can undergo transient demethylation when treated with 5-aza-deoxycytidine, which is followed by rapid remethylation in a process that requires proliferation (47). Also, introduction of ectopic minigenes carrying an imprinting control region into a cancer cell line results in *de novo* methylation of the imprinting control region (48). Finally, culture stress of primary preparations of smooth muscle cells results in the selective *de novo* methylation of the estrogen receptor α gene (49). In the case of the present report, the silencing and methylation of *Cdkn1c* and *H19* in MEFs cultivated *in vitro* constitutes an attractive experimental system amenable to mechanistic dissection using genetically modified cells. An additional advantage of this model is that *de novo* methylation of the CpG islands associated with the imprinted genes tested also occurs during development of a variety of tumors in the animal (Table 2).

An important question derived from our observations is why imprinted genes are more sensitive to epigenetic inactivation than the remaining genes in the genome. A characteristic feature of imprinted genes is that, in general, their promoters or regulatory regions coexist in the diploid genome in a dual state, i.e. fully methylated and nonmethylated. Conceivably, the presence of a methylated copy of a gene could render it more susceptible to aberrant *de novo* methylation. In this regard, it is interesting to note that, in the fungus *Ascobolus*, different methylation status can be transferred from one allele to its diploid partner by a mechanism involving homologous recombination (50). A similar recombination-based mechanism could explain the "transfer" of the methylation status from the methylated allele of an imprinted gene to the nonmethylated allele. Although to be consistent with our observations, this putative mechanism should not operate in the case of the X chromosome in female-derived cells. Other alternatives can be envisaged based on the known involvement of noncoding RNAs in the establishment and maintenance of imprinting (51) and in the recent discovery that small interference RNAs can trigger heterochromatinization and DNA methylation in mammalian cells (52, 53).

Finally, the generalized silencing of imprinted genes during *in vitro* cultivation and the methylation of at least *Cdkn1c* and *H19* can have a detrimental impact on the success of animal cloning by nuclear transfer. It has been consistently observed that animal cloning is very inefficient, and also that successfully developed cloned mammals suffer serious disorders and shortened life span (54, 55). The main reason underlying aberrant cloning is thought to be the incomplete or incorrect reprogramming of the epigenetic information present in the donor somatic nuclei (54). In the light of our present report, we speculate that cultivation of donor cells *in vitro* may result in the stable silencing of imprinted genes, thus making successful reprogramming even less efficient and further contributing to aberrant animal cloning.

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