From The Cover: Paternal and maternal genomes confer opposite effects on proliferation, cell-cycle length, senescence, and tumor formation

Lidia Hernandez, Serguei Kozlov, Graziella Piras, and Colin L. Stewart

PNAS 2003;100;13344-13349; originally published online Oct 27, 2003;
doi:10.1073/pnas.2234026100

Paternal and maternal genomes confer opposite effects on proliferation, cell-cycle length, senescence, and tumor formation

Lidia Hernandez, Serguei Kozlov, Graziella Piras*, and Colin L. Stewart†

Cancer and Developmental Biology Laboratory, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702

Edited by Rudolf Jaenisch, Whitehead Institute for Biomedical Research, Cambridge, MA, and approved September 4, 2003 (received for review June 28, 2003)

Loss of imprinting is the silencing of active imprinted genes or the activation of silent imprinted genes, and it is one of the most common epigenetic changes associated with the development of a wide variety of tumors. Here, we have analyzed the effects that global imprinted gene expression has on cell proliferation and transformation. Primary mouse embryonic fibroblasts (MEFs), whose entire genome is either exclusively paternal (androgenetic) or maternal (parthenogenetic), exhibit dramatically contrasting patterns of growth. In comparison with biparental MEFs, androgenetic proliferation is characterized by a shorter cell cycle, increased saturation density, spontaneous transformation, and formation of tumors at low passage number. Parthenogenetic MEFs reach a lower saturation density, senesce, and die. The maternally expressed imprinted genes *p57^{kip2}* and *M6P/Igf2r* retard proliferation and reduce the long-term growth of MEFs. In contrast, the paternally expressed growth factor *Igf2* is essential for the long-term proliferation of all genotypes. Increased *Igf2* expression in primary MEFs not only stimulates proliferation, but also results in their rapid conversion to malignancy with tumor formation of short latency. Our results reveal that paternally expressed imprinted genes, in the absence of maternal imprinted genes, predispose fibroblasts to rapid transformation. A potent factor in their transformation is IGF2, which on increased expression results in the rapid conversion of primary cells to malignancy. These results reveal a route by which malignant choriocarcinoma may arise from molar pregnancies. They also suggest that the derivation of stem cells from parthenogenetic embryos, for the purposes of therapeutic cloning, may be ineffective.

genomic imprinting | uniparental | *Igf2* | *p57^{kip2}* | cloning

One of the axioms in cancer is that tumor development arises as a consequence of cells undergoing a sequence of genetic mutations in the course of transformation. Recent data, however, have revealed that epigenetic changes, inherited traits in DNA other than an alteration to nucleotide sequence, can affect the expression of key regulatory cancer genes, such as the silencing of tumor suppressors (1). One means of epigenetic modification in neoplasia is an alteration to the pattern of imprinted gene expression. In mammals, imprinted genes are a subset of genes differentially expressed because of their parental origin (2). Mammalian development depends on the presence of both parental genomes (3, 4). Mouse embryos, whose entire genome is paternal in origin (androgenotes), die by early midgestation, with some exhibiting increased growth of the extraembryonic membranes (5). Similarly, in human pregnancy, an embryo with only a paternally derived genome does not develop, although its trophoblast forms and can transform into highly malignant choriocarcinoma (6). In contrast, an embryo with an entirely maternal genome (parthenote) shows retarded development, in particular, in the fetal membranes, with no embryo surviving beyond midgestation (7). In women, the spontaneous development of unfertilized eggs gives rise to usually benign ovarian teratomas (8).

In the formation of many tumors, alterations in the expression of imprinted genes or loss of imprinting is one of the most common alterations in cancer development (9, 10). Loss of imprinting is when a silenced imprinted allele is activated, or an active allele is silenced. For example, insulin-like growth factor 2 (*Igf2*) is one of the most frequently up-regulated genes in human tumor progression (11, 12), whereas expression of the *Igf2* receptor, a putative tumor suppressor regulating *Igf2* availability, is often lost in tumors (13, 14).

The cellular basis for the differences in growth associated with loss of imprinting is not yet known. To further investigate this, we examined the growth characteristics of primary androgenetic and parthenogenetic embryonic fibroblasts and determined the influence of three imprinted genes on cell proliferation. Our results reveal that each parental genome is associated with an intrinsic, but unique set of growth characteristics, with the paternal genome exhibiting a consistent tendency toward spontaneous transformation. In contrast, the maternal genome results in an early cessation of growth and subsequent death. Analysis of individual imprinted genes revealed that *Igf2* regulates transformation and functions as a potent oncogene, converting primary fibroblasts into forming rapidly growing tumors. Other maternally expressed imprinted genes may primarily act to moderate the action of this growth factor. Our results reveal that imprinted genes may have a significant role in the development of certain tumors, and their function in regulating cell proliferation may complicate the use of cells, derived from parthenogenetic embryos, for therapeutic purposes.

Materials and Methods

Generation of Androgenetic and Parthenogenetic Mouse Chimeras. Mice were from our existing colony, and care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (15). Embryonic stem (ES) cells used were the androgenetic ES lines LB1, LB4, and LB10, wild-type biparental ES lines W9.5 and Bruce-4, which reproducibly form germ-line chimeras, and the parthenogenetic ES lines LG1 and 5 (16, 17). All lines were derived from 129S1 mice, except for Bruce-4, which was derived from C57BL/J mice. Heterozygous parthenogenetic embryos were produced by ethanol activation of eggs, with suppression of second polar body formation. The embryos were aggregated at the eight-cell stage with wild-type embryos. Homozygous parthenotes were similarly activated, except that second polar body formation was not suppressed. Once the second polar body was formed, the haploid

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: MEF, mouse embryonic fibroblast; BP-MEF, biparental MEF; P-MEF, parthenogenetic MEF; A-MEF, androgenetic MEF; *Igf2*, insulin-like growth factor 2; *Igf2r*, insulin-like growth factor 2 receptor; ES, embryonic stem.

*Present address: Celera, 45 West Gude Drive, Rockville, MD 20850.

†To whom correspondence should be addressed at: National Cancer Institute, Division of Basic Science, NCI-Frederick, Building 539/Room 121, Frederick, MD 21702. E-mail: stewartc@ncifcrf.gov.

www.pnas.org/cgi/doi/10.1073/pnas.2234026100

eggs were cultured overnight in 2 μg/ml cytochalasin D (Sigma) to inhibit the first cleavage division and diplodize the embryos. The resulting four- to eight-cell embryos were aggregated with wild-type embryos 2 days later. The parthenogenetic eggs were derived from mouse lines that constitutively expressed the *Neo*^{R} gene (18). The Igf2r null mice were as described (19). The *Igf2*$^{-/-}$ mice were a line carrying a deletion in the coding region (exon 5) of the *Igf2* gene (C.L.S., unpublished data).

Derivation of Primary Embryo Fibroblast Cell Lines with Different Parental Genotypes. Fibroblasts were established as described (20) and immediately cultured in selection medium (DMEM/10% FBS/500 μg/ml Geneticin). The purity of the androgenetic, parthenogenetic, and biparental fibroblast cultures was determined by both the glucose phosphate isomerase 1 assay (21) and by imprinted gene expression (22).

Growth and Continuous-Passage Assays. Cells were plated at concentrations of 10^4 or 10^5 per well and counted daily in triplicate. Continuous-passage assays were performed according to the 3T3 schedule (23), modified to allow for the lower cell numbers available from parthenogenetic primary cultures. Cells were plated at a density of 1.25×10^5 cells per well in quadruplicate, harvested 3 days later, pooled, and counted. From this suspension, 1.25×10^5 cells were replated into four new wells and assigned the next passage number. The cells were maintained for up to 40 passages.

Cell-Cycle Analysis. Cells (5×10^6 per dish) were plated in 150-mm tissue-culture dishes and harvested 24, 48, or 72 h after plating. For cell-cycle length determination (24) cells were pulse-labeled with BrdUrd (Roche Molecular Biochemicals) and counterstained with propidium iodide according to manufacturer's instructions. Cell sampling and data collection were performed by using a fluorescence-activated cell sorter (FACScan, BD Biosciences Immunocytometry Systems). Data acquisition was performed with ANALYSIS MODFIT LT 2.0 software (Verity Software, Topsham, ME), and cell-cycle time determinations were as described (25).

Tumor Potential of Primary Cell Lines. To determine the tumorigenicity of primary cell lines, 1×10^7 cells were s.c. injected into the flank of weanling nude mice. Control animals received culture medium only. Tumor sizes were recorded according to the National Cancer Institute *in vivo* cancer model procedures (26). Tumor tissue obtained at the time of sacrifice for histological analysis was fixed in 10% buffered formalin, embedded, sectioned, and stained with hematoxylin/eosin (Fisher).

Imprinted Gene Expression Analysis. Total cellular RNA was isolated, and gene expression was determined by using primer pairs as described (22).

Retroviral Vector Construction. Expression vectors for retroviral gene transfer into primary cells were derived from the pLXIN plasmid (Clontech) and contained either mouse *Igf2* cDNA or pLEGFP-C1 (Clontech). E1A- and Ras-containing vectors were a kind gift from K. Ryan (Cancer Research UK, Beatson Institute, Glasgow, U.K.). The mouse *Igf2* ORF (GenBank accession no. NM-010514) was amplified by RT-PCR from embryonic day 15 embryo total RNA with the following primers: mmIgf2-for, 5′-AGGAATTCTTGTGGTACCAATGGGGATC-3′ and mmIgf2-rev, 5′-TCTGAATTCGATTCACTGATGGTTGCTGGA-3′ (*Eco*RI sites used to clone the insert into pLXIN vector are underlined). The PCR product containing the full-length mouse *Igf2* was confirmed by DNA sequencing. To produce infectious supernatants, Phoenix-Eco virus-packaging cells were transfected by calcium phosphate precipitation with the expression constructs according to recommended protocols (27). Cells at 40–50% confluency were infected with three subsequent changes of virus-containing media supplemented with 8 μg/ml polybrene for the total of 36 h. Cells were allowed to recover for an additional 48 h in complete growth medium (DMEM plus 10% FBS) before analysis.

Results

Derivation of Primary Fibroblasts of Different Parental Origin. A comparison between the cell cycles of biparental, androgenetic, and parthenogenetic ES lines revealed no differences in the duration of the different stages or in overall length of the cycle and were similar to those reported for other biparental ES lines (28) (data not shown). We therefore analyzed their differentiated derivatives, because many imprinted genes are first expressed upon ES cell differentiation. The differentiation of ES cell lines *in vitro* can be highly variable and inconsistent, often resulting in a heterogeneous mixture of cell types that do not retain imprinted gene expression (17). To circumvent this variability and to establish a uniform differentiated population, we produced androgenetic (A-MEFs) and parthenogenetic fibroblasts (P-MEFs) from chimeras explanted *in vitro* in which the biparental host fibroblasts (BP-MEFs) were eliminated by selection. Chimeric embryos at days 12–13 of gestation were used, because both androgenetic and parthenogenetic cells contribute extensively to the majority of tissues at these stages (16, 29). A-MEFs uniparental for the paternal genome were established from previously characterized androgenetic *Neo*^{R} ES lines, which have a diploid karyotype and whose development closely resembled that of androgenetic embryos (16). However, the derivatives of our parthenogenetic ES cells neither retained correct imprinted gene expression nor resembled parthenogenetic embryo chimeras during development (17). As an alternative, parthenogenetic four- to eight-cell stage embryos, constitutively expressing the *Neo*^{R} gene, were aggregated with wild-type embryos to produce P-MEFs uniparental for the maternal genome. Normal biparental, BP-MEFS were derived either from chimeras made from two biparental ES lines that reproducibly form germ-line chimeras by using the same procedure or from regular embryonic day 13 embryos (Fig. 1*A*). The genotypes of the primary fibroblast cultures were confirmed by *Gpi1* analysis and by expression of known imprinted genes (Fig. 1*B*), demonstrating that these lines stably retained appropriate imprinted gene expression over many generations. Expression of the fibroblast-specific protein 1 (FSP1) marker gene established that fibroblasts were the cell type derived from all three genotypes (data not shown) (30).

Genome-Specific Differences in Proliferation and Tumor Formation. The proliferation of the BP-MEFs, A-MEFs, and P-MEFs was determined by using cells at passage 3. A-MEFs reproducibly attained a higher saturation density and proliferated at a faster rate than the BP-MEFs, having a cell-cycle duration of ≈11 h compared with ≈22 h in the BP-MEFs (Fig. 1 *C* and *D*). The G_1 and G_2 phases were both present in the A-MEFs but reduced in length. In contrast, the cell-cycle duration in P-MEFS was similar to that of BP-MEFs (Fig. 1*D*), although they consistently reached a lower saturation density.

Primary mouse fibroblast lines undergo characteristic and reproducible changes to their rates of proliferation when passaged continuously. To assess these changes for the different parental genotypes, multiple, independently isolated lines of all three genotypes were cultured by using a continuous passage procedure (23). BP-MEFs, derived from both normal embryos and wild-type ES chimeras, underwent the typical pattern of growth for primary mouse fibroblasts. For the first four to five passages, the cells proliferated vigorously. Thereafter, they

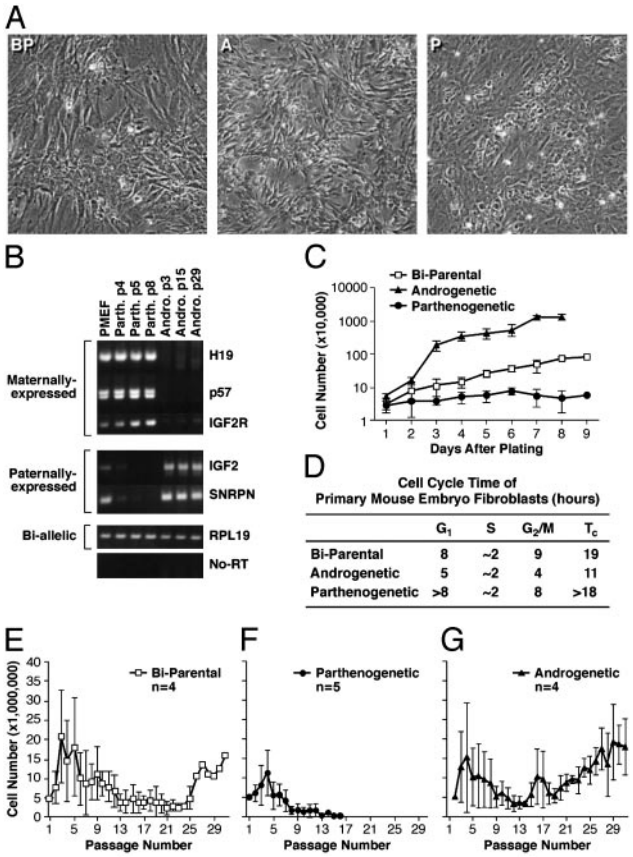

Fig. 1. Establishment and proliferation of A-MEFs, P-MEFs, and BP-MEFs. (*A*) BP-MEFs, A-MEFs, and P-MEFs in culture at low passage showed a typical fibroblastic morphology. (*B*) A-MEFs and P-MEFs appropriately and stably express known imprinted genes over many passages. Parth., parthenogenetic; Andro., androgenetic. A more extensive analysis of other imprinted gene expression in these lines is described in ref. 22. (*C*) Growth curves of the A-MEFs, P-MEFs, and BP-MEFs. (*D*) Duration of the different phases of the cycle in MEFs of all three genotypes. (*E–G*) Continuous-passage growth curves for the MEFs from each genotype: BP-MEFs (*E*), P-MEFs (*F*), and A-MEFs (*G*).

entered a senescent phase, and only at passage 20 did immortalized derivatives begin to appear (Fig. 1*E*). Senescence was characterized by the cells becoming larger, flatter, and senescence-associated β-galactosidase-positive (31). The P-MEFS (a total of seven lines) proliferated at a rate similar to the rate of BP-MEFs until passage 5–6, when they also became senescent. However, by passage 6–7, they started dying, and they were all dead by passage 13 (Fig. 1*F*). The A-MEFs (total of four independently derived lines) also vigorously proliferated and entered a brief phase of senescence; however, rapidly proliferating derivatives consistently emerged around passage 8–13, some 7–9 passages before the BP-MEFs (Fig. 1*G*).

Androgenetic MEFs Undergo Malignant Conversion at Low Passage. The BP-MEFs that emerge from senescence are immortalized and do not form tumors when injected into recipient mice. The conversion of immortalized fibroblasts to malignancy requires a second transforming event, such as the introduction of a mutant *Ras* gene (32). The A-MEFs, which appeared after a brief senescent phase, were not only immortalized but transformed because they exhibited anchorage-independent growth and reproducibly gave rise to rapidly growing fibrosarcomas with a latency of 1–2 weeks when injected into nude mice (Table 1). No tumors were produced from equivalent-passage BP-MEFS derived from embryos or from fibroblasts derived from biparental ES cells, although tumors (undifferentiated sarcomas and with a longer latency of 4 weeks) eventually did arise from BP-MEFs at a much later passage number (p44).

To determine the molecular basis underlying these patterns of proliferation, we analyzed the expression of the principal (nonimprinted) cell-cycle regulatory factors. Expression of the cyclins, cyclin-dependent kinases, telomerase activity, and cyclin-dependent kinase inhibitors (p21, p27, $p16^{INK4A}$, and $p19^{ARF}$) were all analyzed in low-passage MEFs of all three genotypes. All lines expressed cyclin D1 (Fig. 2*A*) and, after release from mitotic arrest (induced by serum starvation for 72 h), the levels of cyclin D1 were highest in the A-MEFs. All MEFs expressed cyclins E and A as they progressed through S phase (data not shown). Similarly, expression of the cyclin-dependent kinase inhibitors p21, p27, $p16^{INK4A}$, and $p19^{ARF}$ were all up-regulated (Fig. 2*B*) as the cells of all three genotypes entered senescence (p4). The response of all low-passage MEFs to DNA damage (by UV exposure), as measured by growth arrest and increased p53 expression, was induced in cells of all three parental genotypes, with the highest levels expressed by the A-MEFS (Fig. 4*C*). Also, P-MEFS established from $p16^{INK4A}/p19^{ARF}$ null embryos did not senesce and continued to vigorously proliferate as described for BP-MEFs (33) (data not shown). These results revealed that the principal growth-regulatory and checkpoint mechanisms were functional in the fibroblasts of all three genotypes.

Imprinted Gene Effects on Proliferation. We then investigated what effect individual imprinted genes might have on proliferation. Mutation of the maternal alleles of two genes that are maternally expressed, the cyclin-dependent kinase inhibitor $p57^{kip2}$ (CDKN1C) and the type Igf2 receptor (*Igf2r*), results in abnormal fetal growth and lethality (19). To determine their roles in

Table 1. Androgenetic MEFs and Igf2-transduced primary cells transform and form tumors at low passage

MEF	Cell line (passage no.)	Tumors, *n*	Tumor type
Biparental	MEF (p20)	0/8	
	MEF (p44)	8/8	Sarcoma
	MEF (p16) + Igf2 Rv	7/7	Fibrosarcoma
	MEF (p8) $M6Pr^{-/-}$ + Igf2 Rv	4/4	Fibrosarcoma
	MEF (p16) $Igf2^{-/-}$ + Igf2 Rv	3/3	Fibrosarcoma
Androgenetic	LB1.10 (p10)	0/8	
	LB1.10 (p25)	8/8	Chondrosarcoma, sarcoma
	LB1.10 (p34)	8/8	Sarcoma
	LB Poly2 (p20)	8/8	Sarcoma
Parthenogenetic	+Igf2 Rv (p16)	3/3	Fibrosarcoma

Igf2 Rv, Igf2 retroviral vector.

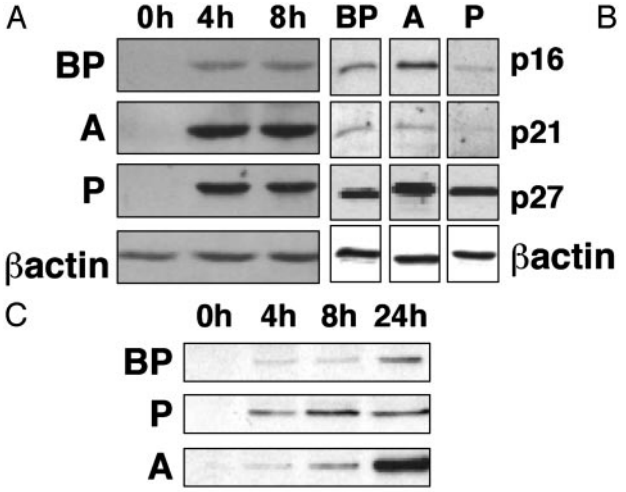

Fig. 2. (*A*) MEFs of all three genotypes show an increase in cyclin D levels after release from serum starvation. (*B*) MEFS of all three genotypes express cyclin-dependent kinase inhibitors p21, p27, and $p16^{ink4}$ as they start to senesce. (*C*) After UV treatment MEFs of all genotypes induce p53, with an ≈10-fold increase in the A-MEFs compared with the other genotypes. A, androgenetic; P, parthenogenetic; BP, biparental.

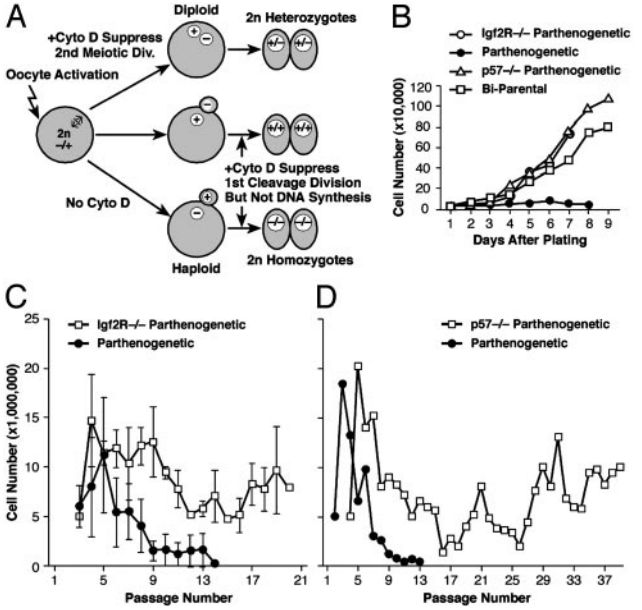

Fig. 3. (*A*) Protocol for producing parthenogenetic embryos homozygous or wild type for the mutant $p57^{kip2}$ or *Igf2r*. (*B*) Loss of either $p57^{kip2}$ or *Igf2r* in P-MEFs enhances their rates of proliferation, and loss of *Igf2r*, but not $p57^{kip2}$ loss, affects BP-MEF proliferation (data not shown). (*C* and *D*) $Igf2r^{-/-}$ or $p57^{kip2-/-}$ P-MEFs show enhanced long-term proliferation and do not die.

proliferation, P-MEF lines deficient in expression of these genes were established. We derived these lines by modifying the procedure by which parthenogenetic embryos (from females heterozygous for each mutated gene) were produced before making chimeras.

At ovulation mouse oocytes are arrested at the second meiotic division. The usual route for making parthenotes is to activate the eggs and suppress the formation of the second polar body with the resulting embryos remaining diploid. The majority of the genome of these diploid parthenotes would remain heterozygous for any mutated allele when derived from a heterozygous mother, with the possible exception of regions close to centromeres, which may be homozygous after the first meiotic division. However, if after activation, second polar body formation is allowed to proceed, then the activated egg is haploid and will either be null or wild-type for the allele in question. Suppressing the first cleavage division will cause the haploid embryo to become diploid and homozygous at all loci. As a result, two types of parthenogenetic embryos will be produced, those homozygous for the mutated allele and those wild-type for the functional allele (34) (Fig. 3*A*).

By producing parthenotes during this procedure, P-MEFs were established from the Neo^R chimeras that carried no functional allele of the gene. A total of three P-MEF lines were derived that were homozygous nulls for the *Igf2r* and one line null for $p57^{kip2}$. Their proliferation and viability were assessed by using standard growth and continuous-passage assays. Both the $Igf2r^{-/-}$ and $p57^{kip2-/-}$ P-MEFs showed increased proliferation rates compared with P-MEFs (Fig. 3*B*). The $Igf2r^{-/-}$ P-MEFs also showed little indication of senescence and continued to proliferate for multiple passages (Fig. 3 *C* and *D*). These results suggest that both genes independently contributed to senescence and eventual death of the P-MEFs. Similar results were also obtained from BP-MEFS derived from embryos that had maternally inherited the mutant allele, with loss of the *Igf2r* resulting in an increased rate of proliferation. Loss of $p57^{kip2}$ had no effect on BP-MEF proliferation (data not shown). Attempts were made to determine whether complete loss of either protein would affect parthenogenetic growth and viability *in vivo*. No $p57^{kip2-/-}$ postimplantation embryos were recovered and only one parthenogenetic $Igf2r^{-/-}$ null embryo (of 12 transferred) was recovered at embryonic day 10, which although viable, was retarded and had a small placenta.

Another imprinted gene affecting embryonic growth is the paternally expressed insulin-like growth factor 2 (*Igf2*). P-MEFs do not express *Igf2*, whereas A-MEFs express twice the levels of mRNA and protein found in BP-MEFs (35). BP-MEFs made from $Igf2^{-/-}$ embryos senesced, and, similar to the P-MEFs, they consistently failed to immortalize and all died (Fig. 4*A*). In androgenetic embryos *Igf2* is an important contributor to their abnormal growth, because chimeras made from androgenetic $Igf2^{-/-}$ ES cells do not exhibit the characteristic overgrowth or edema (36). We produced chimeras by using two independently derived $Igf2^{-/-}$ androgenetic ES lines. The ES derivatives contributed extensively and uniformly to tissues in midgestation embryos and were overtly normal as reported (data not shown). We were unable to establish any $Igf2^{-/-}$ A-MEF lines. The fibroblasts proliferated for the first two to three passages but then rapidly senesced and died (Fig. 4*A*). This rapid senescence was not due to failure to express the Neo^R, because the ES cells from which they were derived were viable in the concentration of G418 used for selection. Attempts to increase their viability by supplementing the culture medium with recombinant IGF2 were unsuccessful, revealing that either the cells need to produce their own IGF2 and/or some additional factor(s) are expressed in BP-MEFs that complements IGF2 and are essential to their viability.

Primary Cell Transformation by IGF2. In a converse set of experiments IGF2 expression was increased in the MEF lines by introducing a murine *Igf2* cDNA by using a retroviral vector (Fig. 4*B*). More than 90% of low-passage (p 2–3) P-MEFs and BP-MEFs (all $Igf2^{-/-}$) were infected, as assessed by Southern blot analysis. Within 1 week, the cells were expressing elevated levels of IGF2 (Fig. 4*B*). Within 2–3 weeks, the cells assumed a spindle-shaped, refractile morphology, and their rate of proliferation had increased significantly (Fig. 4 *C* and *D*). To determine the percentage of cells transformed by *Igf2*, we performed a colony-forming assay, because primary or low-passage MEFs

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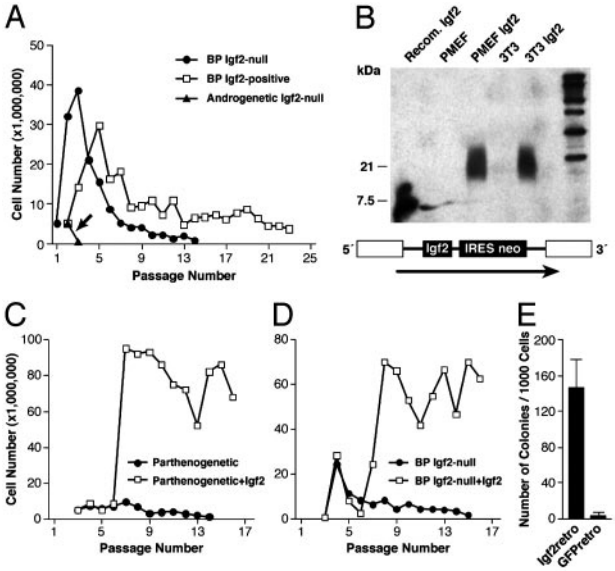

Fig. 4. (*A*) In continuous passage, loss of *Igf2* in BP-MEFs results in their senescence and eventual death. *Igf2*$^{-/-}$ A-MEFs have a short lifespan in culture and cannot be propagated (arrow). (*B*) Transduction of MEFs with an *Igf2*-expressing retroviral vector results in a significant production of IGF2. Total cell protein (50 μg) was added per lane. The marker lane was loaded with 1 μg of recombinant Igf2 and the gel hybridized with a mouse-specific Igf2 antibody (Amano). (*C*) Infection of P-MEFs with the *Igf2* vector enhances their growth rate and viability compared with control (*Gfp*-infected) cells. (*D*) Transduced Igf2 also had a similar enhancing effect on proliferation on the *Igf2*-deficient BP-MEFs. (*E*) BP-MEFs (passage 3) infected with the *Igf2* vector and plated at clonal density (1,000 cells per 150-mm dish) show an ≈150-fold increase in colonies compared with control infections. This assay was repeated five times.

do not proliferate when plated as single cells at clonal density. Immediately after infection, the BP-MEFs were plated at a density of 1,000 cells per 150-mm dish, and within 2–3 weeks the number of colonies formed were counted. Infection with the *Igf2* vector resulted in a >100-fold increase in colony formation (Fig. 4*E*). Controls using the empty vector or expressing *Gfp* were also efficiently infected, but cellular proliferation and colony formation were not increased. When injected into mice, all the *Igf2*-infected primary lines efficiently formed fibrosarcomas with a short latency of ≈1 week (Table 1). Control infections of BP-MEFs with a retroviral vector expressing E1A resulted in the immortalization of the BP-MEFs, but these did not form tumors as reported (32).

Discussion

Here we describe the effects that the paternal and maternal genome, and specific imprinted genes, have on cell proliferation and tumor formation. ES cells of paternal, maternal, or biparental genotypes showed no differences in rates of proliferation or cell-cycle parameters and were essentially identical with those described (37). On differentiation, proliferation and cell-cycle parameters diverged according to their genotype. Parthenogenetic fibroblasts had a cell-cycle length similar to that of biparental fibroblasts, but they were growth-retarded and eventually died.

The effects of maternally expressed imprinted genes *Igf2R* and *p57*^{kip2} on the proliferation of P-MEFs was analyzed by deriving P-MEFs lines deficient in expression of either gene. As a result, the proliferation of the *Igf2r*$^{-/-}$ or *p57*$^{kip2-/-}$ P-MEFs had growth rates similar to BP-MEFs and attained a similar saturation density. Furthermore, both lines in continuous passage proliferated for multiple passages. The IGF2R acts as a receptor for a variety of ligands affecting cell proliferation or survival, including IGF2, transforming growth factor β, and leukemia inhibitory factor (38). Removal of IGF2R conceivably would increase the availability of IGF2 that binds to and stimulates the IGF1R and reduce the proteolytic activation of transforming growth factor β (a potential inhibitor), which can act as a growth inhibitor or increase the availability of leukemia inhibitory factor that functions as a survival factor for MEFs (C.L.S., unpublished observations). *p57*^{kip2} is a cyclin-dependent kinase inhibitor regulating the transition of cells from G_1 to S. Its loss may enhance progression through the cell cycle, because its overexpression causes complete cell-cycle arrest (39). p57^{kip2} levels are reduced by elevated levels of IGF2 (40), and loss of the IGF2R also reduces p57^{kip2} levels, presumably by increasing the availability of IGF2 from the serum in which the cells are grown, which in turn may act through the p53-related protein, p73β on p57^{kip2} (41). Clearly, loss of either p57^{kip2} or IGF2R is sufficient to rescue the growth inhibition and death of P-MEFs *in vitro*, but it does not significantly improve parthenogenetic development *in vivo*.

In contrast to the retarded growth of the P-MEFs, the androgenetic MEFs exhibited enhanced proliferation. A-MEFs have a shorter cell-cycle length (≈11 h vs. 20–22 h) and attain a higher saturation density. In a continuous passage assay they consistently emerged from a brief senescent phase some seven to eight passages before the BP-MEFs. Presenescent A-MEFS were not transformed and could be arrested by the introduction of *Ras*, indicating that *Ink4a-Arf* checkpoint controls were intact at this stage of *in vitro* growth. However, A-MEF postsenescent derivatives were transformed, as they exhibited anchorage-independent growth and readily formed tumors when injected into adult nude mice.

The reduction in cell-cycle length was intriguing because it approximates to the cycle length of undifferentiated ES cells. Unlike ES cells, in which G_1 is significantly reduced, the difference in cell-cycle length of A-MEFs and P-MEFs, compared with the BP-MEFs was due to a shortening in the duration of G_1 and G_2 (28). Transformation to the malignant state requires the introduction of an additional factor, such as an activated *Ras* gene into the immortalized derivatives. The molecular basis for the transformation of the A-MEFs is unclear. However, it was noted that all the transformed A-MEF lines were aneuploid, which may be accounted for the by a failure in proper G_1/S checkpoint controls in these cells, because of increased IGF2 expression and/or lack of p57^{kip2}. The genetic and molecular analysis determining the effects specific imprinted genes had on proliferation of the different fibroblast lines support this. We made several attempts to introduce and to express p57^{kip2} in the MEFs. On all occasions when p57^{kip2} was expressed, growth arrest and death of the transfected/infected cells occurred. This suggests that the level of p57^{kip2} may be critical to its effectiveness at regulating G_1/S transition, with overexpression resulting in cell death.

Effects of IGF2 on Proliferation and Transformation. P-MEFs and BP-MEFs, deficient for *Igf2*, showed a decline in proliferative rates, with the cells eventually senescing and dying. A particularly striking observation was noted with the A-MEFs. Attempts to derive A-MEF lines from two independent androgenetic *Igf2*$^{-/-}$ ES-lines were unsuccessful. Despite a significant and uniform (≈50%) chimeric contribution of the *Igf2*$^{-/-}$ androgenetic derivatives to embryonic day 13 embryos, it proved to be impossible to establish any *Igf2*$^{-/-}$ A-MEFs lines from these chimeras. As reported, chimeras made between *Igf2*$^{-/-}$ androgenetic ES cells and normal embryos do not exhibit the size increase or edema associated with normal androgenetic chimeras. Why the *Igf2*$^{-/-}$ A-MEF derivatives, which survived for two passages, should die so rapidly remains unclear. One possibility is that the *Igf2*$^{-/-}$ androgenetic derivatives,

in fact, depend on some maternally encoded factor that, in the absence of IGF2, is essential for their survival and growth *in vitro*. The identification of such a factor(s) would clearly be of great interest as it may shed further light on the function of the Igf-signaling pathway and the role of the different parental genomes in regulating proliferation.

IGF2 Transforms Primary Cells. Initial attempts to enhance proliferation were made by increasing the exogenous concentration of IGF2 in the culture to a maximum of 100 ng and had little or no effect on the long-term proliferation of the P-MEFs. Subsequently, a retroviral vector was used to introduce an *Igf2* cDNA into the MEF lines. Introduction by this route not only resulted in the maintenance of proliferation in P-MEFs cells, but also transformed both BP- and P-MEFs into highly malignant cells. Increasing the concentration of exogenous IGF2, while increasing viability and proliferation in some instances, does not induce a transformed phenotype, even in immortalized cells, as platelet-derived growth factor β does (42). IGF2 affects the rate at which tumors grow (43), but it has not been shown to act as a primary transforming agent. Our results are particularly striking because the IGF2 retroviral vector transforms primary low-passage fibroblasts, making it a unique method for transformation. Previous studies indicated that mouse cells require at least two transforming events (44). Although about 2–3 weeks was required for IGF2-mediated transformation to be apparent, it appears unlikely that transformation depended on a secondary event. Plating the cells at clonal density revealed that nearly 100% of infected cells were transformed. The molecular basis to transformation is under investigation. One possibility for the tumorigenic effect of IGF2 is that some of the regulatory sequences responsible for regulating *Igf2*, located at the 3′ end of the gene, were deleted on expression of the cDNA. Deletion of these sequences results in elevated IGF2 expression and increased proliferation (45).

In summary, our results show that paternal and maternal genomes each establish a unique pattern of growth and proliferation that is intrinsic to the cells. These patterns are strongly influenced by imprinted genes, with *Igf2* having a particularly pronounced effect on proliferation, both at enhancing growth and at long-term viability. The transforming effect of *Igf2* supports the notion that maternally expressed imprinted genes may act to regulate the growth-stimulating effects of *Igf2* at many different levels. The results reveal that imprinted gene expression has a marked effect on proliferation and the susceptibility of cells to tumor formation. The effects of the androgenetic genome on tumor formation may explain the development of the gestational trophoblastic disease, choriocarcinoma, which develops from complete hydatidiform mole whose entire genome is of paternal origin. It would be interesting to determine what effect the paternal genome has on the growth characteristics of trophoblast lines established from androgenetic blastocysts. Furthermore, the growth retardation associated with the maternal genome may explain why ovarian teratomas are relatively benign, and in mice they are usually nontransplantable (46).

Our results also have implications for therapeutic cloning, where it has been suggested that the derivation of stem cell lines from parthenogenetic embryos could overcome some of the ethical objections to using normal fertilized embryos (47). Our results indicate that parthenogenetic cell lines would be of little use in therapies involving tissue reconstitution because such cells would be at a competitive growth disadvantage.

We thank John McLaughlin for the *Igf2* null androgenetic ES cells, Steve Elledge and Ron Depinho for the $p57^{kip2}$ and $p16/p19^{arf}$ null mice, Leslie Mounkes and the Center for Cancer Research Fellows Editorial Board for comments.

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