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A set of imprinted genes required for normal body growth also promotes growth of rhabdomyosarcoma cells

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INTRODUCTION: In many normal tissues, proliferation rates decline postnatally, causing somatic growth to slow. Previous evidence suggests that this decline is due, in part, to decline in the expression of growth-promoting imprinted genes including *Mest*, *Plagl1*, *Peg3*, *Dlk1*, and *Igf2*. Embryonal cancers are composed of cells that maintain embryonic characteristics and proliferate rapidly in childhood. We hypothesized that the abnormal persistent rapid proliferation in embryonal cancers occurs in part because of abnormal persistent high expression of growth-promoting imprinted genes.

RESULTS: Analysis of microarray data showed elevated expression of *MEST*, *PLAGL1*, *PEG3*, *DLK1*, and *IGF2* in various embryonal cancers, especially rhabdomyosarcoma, as compared to nonembryonal cancers and normal tissues. Similarly, mRNA expression, assessed by real-time PCR, of *MEST*, *PEG3*, and *IGF2* in rhabdomyosarcoma cell lines was increased as compared to nonembryonal cancer cell lines. Furthermore, siRNA-mediated knockdown of *MEST*, *PLAGL1*, *PEG3*, and *IGF2* expression inhibited proliferation in Rh30 rhabdomyosarcoma cells.

DISCUSSION: These findings suggest that the normal postnatal downregulation of growth-promoting imprinted genes fails to occur in some embryonal cancers, particularly rhabdomyosarcoma, and contributes to the persistent rapid proliferation of rhabdomyosarcoma cells and, more generally, that failure of the mechanisms responsible for normal somatic growth deceleration can promote tumorigenesis.

In mammals, somatic growth is rapid in embryonic and early postnatal life but decelerates with age. This decline in growth rate is due, in large part, to a decrease in the rate of cell proliferation(1–4). We recently showed evidence that the decline in proliferation is driven by an extensive genetic program that occurs coordinately in multiple tissues during early postnatal life in mice (5,6). This complex program includes the down regulation of multiple growth-promoting imprinted genes, including *Igf2*, *Plagl1*, *Mest*, *Peg3*, *Dlk1*, *Gtl2*, and *Slc38a4* (7). Targeted ablation of these imprinted genes in mice causes decreased body size at birth, indicating that they are required for rapid embryonic growth (8–11). However, their expression subsequently declines markedly, probably contributing to normal postnatal growth deceleration (5,6).

Although *Mest*, *Peg3*, *Plagl1*, and *Igf2* are all required for normal somatic growth in mice, the molecular functions of the gene products are quite disparate. *Igf2* encodes insulin-like growth factor-II, a secreted protein that interacts with the type I insulin-like growth factor receptor to promote growth in a wide variety of cell types. Knockout of *Igf2* causes mice to be small in length and weight (8). *Plagl1*, pleiomorphic adenoma gene-like 1, encodes a C2H2 zinc-finger transcription factor. Unlike the related genes *Plag1* and *Plagl2*, which act as proto-oncogenes, *Plagl1* is considered to be a candidate tumor suppressor because reduced expression has been observed in tumor cells and because overexpression in tumor cells can induce apoptosis and cell-cycle arrest (12). However, knockout of *Plagl1* in mice causes intrauterine growth restriction as well as postnatal pulmonary dysfunction (13). *Mest* has sequence similarity to α/β fold hydrolases. Knockout of *Mest* in mice causes growth retardation of embryonic and extra-embryonic structures and abnormal maternal behavior (9). *Peg3* encodes a protein with 12 Kruppel-type zinc finger domains and two proline-rich periodic repeat domains. There is evidence that *Peg3* promotes apoptosis and has a variety of molecular actions, including effects on Bax/p53, TNF-α, and Wnt-related pathways (14). Mice deficient in *Peg3* are viable but smaller than wild-type animals, and female mutants show impaired maternal behavior including abnormal nest-building and gathering of pups (10). *MEST*, *PEG3*, *PLAGL1*, and *IGF2* all exhibit imprinting with silencing of the maternal allele and expression of the paternal allele. It has previously been observed that a disproportionate number of imprinted genes are involved in growth regulation and particularly that paternally expressed genes tend to promote body growth (15).

Embryonal cancers are composed of cells that maintain embryonic characteristics and show abnormally rapid proliferation and other malignant features. These tumors typically present in childhood and include rhabdomyosarcoma, Wilms' tumor, neuroblastoma, and retinoblastoma, which resemble embryonic muscle, renal, neural crest, and retinal tissue, respectively. Embryonal cancers are among the most common solid extra-cranial tumors of childhood, accounting for 8–10% of childhood malignancies (16).

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Received 9 March 2011; accepted 17 July 2011. doi:10.1038/pr.2011.6

We hypothesized that failure of the genetic program responsible for normal growth deceleration may contribute to the rapid proliferation and immature characteristics observed in embryonal cancers. Because this program appears to suppress the rapid cell proliferation that normally occurs in embryonic tissues, we reasoned that failure of the program in a clone of cells would allow persistent rapid proliferation in postnatal life, thus contributing to tumorigenesis. As a first test of this hypothesis, we focused on the growth-promoting imprinted genes that are normally down regulated postnatally, asking whether these genes show persistently elevated expression in embryonal cancers. Then, to determine whether these genes are required for the rapid growth of rhabdomyosarcoma, we knocked down expression of these genes in cultured rhabdomyosarcoma cells and examined the effect on proliferation rate.

RESULTS

To determine whether imprinted genes that have been implicated in physiological growth deceleration (6) are overexpressed in embryonal cancers, we first evaluated microarray data available from an NCI database (http://home.ccr.cancer.gov/oncology/oncogenomics/) (17) that includes embryonal tumor samples, xenografts, and cell lines. Rhabdomyosarcoma samples showed elevated expression of *MEST*, *PEG3*, *PLAGL1*, *IGF2*, and *DLK1* as compared to the reference RNA sample derived from seven nonembryonal human cancer cell lines and compared to nonembryonal cancers and normal tissues (including muscle and kidney) in this database (**Figure 1**). Wilms' tumor samples showed elevated expression of *MEST* and *IGF2* (**Figure 1**). Neuroblastoma samples showed elevated expression of only *DLK1* (**Figure 1**). Ependymoma samples showed elevated expression of *IGF2* (**Figure 1**). *SLC38A4* did not appear to be overexpressed consistently by any tumor type (data not shown), and *GTL2* expression could not be assessed because it was not represented in the database.

Because the microarray analysis showed elevated expression of multiple growth-promoting imprinted genes in rhabdomyosarcoma tumor samples, xenografts, and cell lines, we decided to focus subsequent studies on this embryonal cancer. To confirm the microarray findings, we first analyzed mRNA expression of *MEST*, *PEG3*, *PLAGL*, and *IGF2* in rhabdomyosarcoma cell lines (n = 5) using real-time PCR. *MEST*, *PEG3*, and *IGF2*

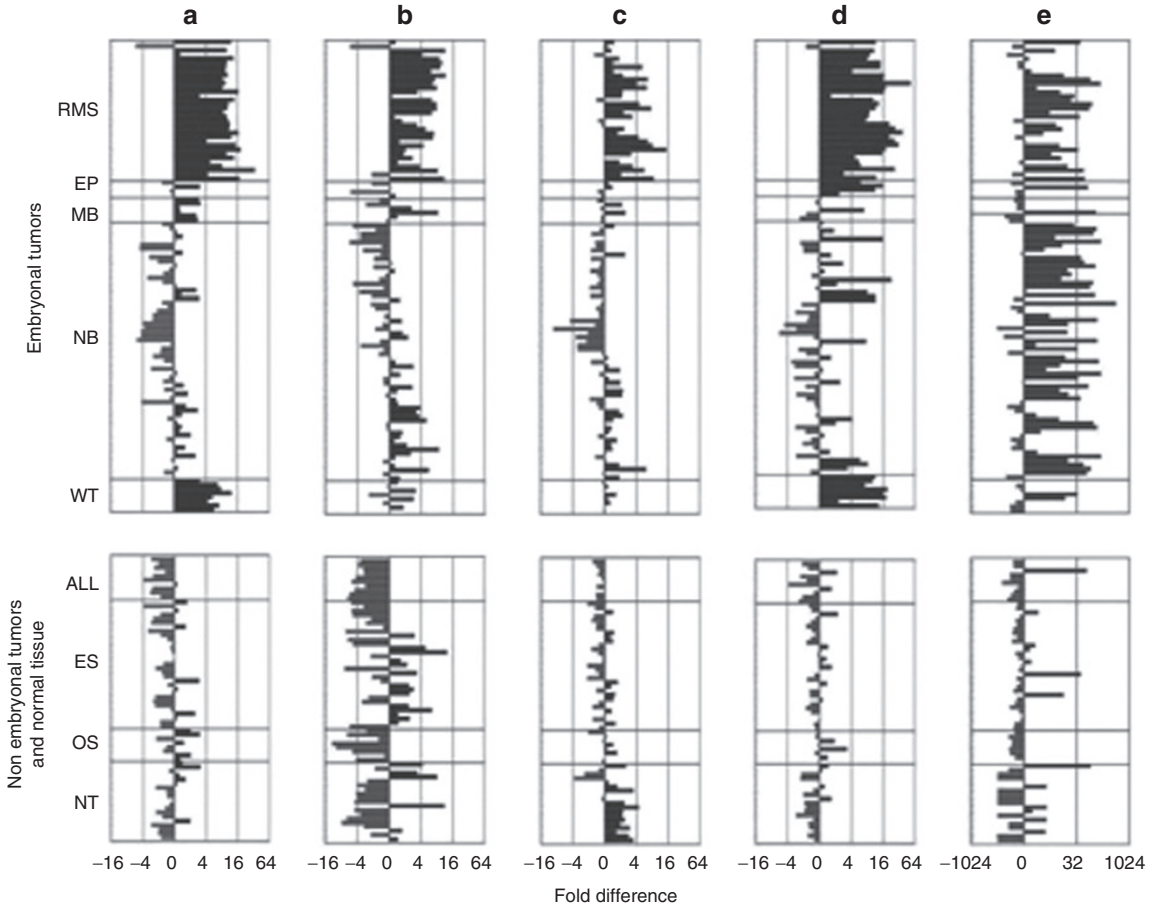

Figure 1. Expression of (**a**) *MEST*, (**b**) *PEG3*, (**c**) *PLAGL1*, (**d**) *IGF2*, and (**e**) *DLK1* in embryonal tumors, nonembryonal tumors and normal tissues. Data are from a National Cancer Institute expression microarray database. Each bar represents a single primary tumor, xenograft, or cell line or a normal tissue sample. The plotted value is the fold difference in mRNA expression of *MEST*, *PEG3*, *PLAGL1*, *IGF2*, or *DLK1* normalized to a reference RNA mixture from seven adult, nonembryonal tumor cell lines. ALL, acute lymphoblastic leukemia; EP, ependymoma; ES, Ewing's sarcoma; MB, medulloblastoma; NB, neuroblastoma; NT, normal tissue; OS, osteosarcoma; RMS, rhabdomyosarcoma; WT, Wilms' tumor.

mRNA levels were in general higher in rhabdomyosarcoma cell lines than in nonembryonal cancers ($n = 11$) of adult and pediatric origin (**Figure 2**). The corresponding difference for *PLAGL1* did not reach statistical significance.

To determine whether the high expression levels of these genes contribute to the rapid proliferation of rhabdomyosarcoma cells, we used siRNA interference to suppress the expression of *MEST*, *PLAGL1*, *PEG3*, and *IGF2* in the Rh30 rhabdomyosarcoma cell line. Each gene was targeted independently with two siRNAs. Transfection of all siRNAs decreased the target mRNAs by at least 50% as compared to values from a negative control siRNA, as measured by real-time PCR (**Figure 3**). siRNA targeting of *MEST*, *PEG3*, *PLAGL1*, and *IGF2* decreased proliferation as compared to values from a negative control siRNA, as assessed by ^{3}H-thymidine incorporation (**Figure 4**). This effect was seen for each of the two siRNAs targeting each gene, suggesting that the effect on proliferation was not due to off-target effects of the siRNA on other genes.

Next, we analyzed the microarray data to determine whether *MEST*, *PEG3*, *PLAGL1*, and *IGF2* are regulated coordinately in rhabdomyosarcomas. In rhabdomyosarcoma tumor samples, cell lines, and xenografts, there was a positive correlation between *IGF2* expression and expression *MEST*, *PEG3*, and *PLAGL1*. Thus, samples that showed higher levels of *IGF2* expression also tended to show higher expression levels of *MEST*, *PEG3*, and *PLAGL1* (**Figure 5**).

Because previous evidence suggests that *PLAGL1* may regulate expression of a large network of imprinted genes (13), we analyzed the effect of siRNA-mediated knockdown of *PLAGL1* on the expression of *MEST*, *PEG3*, and *IGF2* in Rh30 cells. *PLAGL1* knockdown did not have consistent effects on mRNA levels of any of these genes (**Figure 6**). Similarly, knockdown of *MEST*, *PEG3*, and *IGF2* had little effect on mRNA expression of any other gene studied, except for a possible reduction in *PLAGL1* expression after knockdown of *PEG3* (**Figure 6**).

DISCUSSION

We have previously shown evidence that the normal postnatal deceleration in mammalian body growth is driven by a genetic program that involves downregulation of many growth-promoting genes (5–7). Some of these downregulated growth-promoting genes are imprinted, including *Igf2*, *Plagl1*, *Mest*, *Peg3*, *Dlk1*,

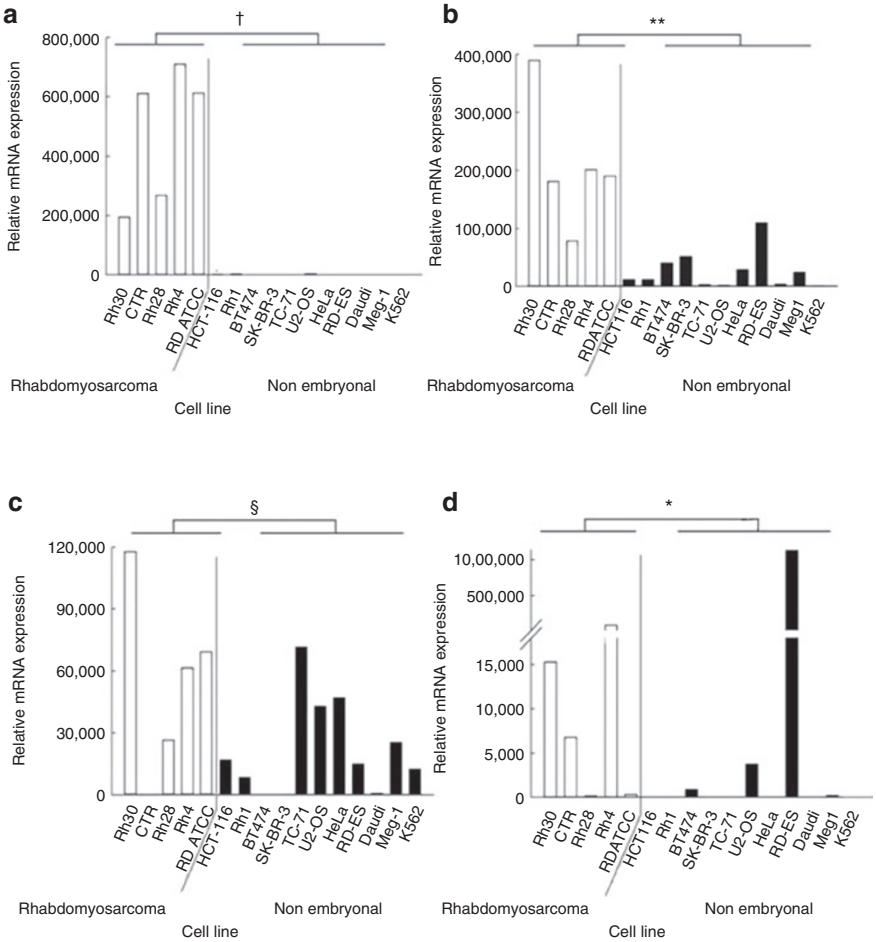

Figure 2. Relative mRNA expression of (**a**) *IGF2*, (**b**) *MEST*, (**c**) *PLAGL1*, and (**d**) *PEG3* in rhabdomyosarcoma cell lines and nonembryonal cancer cell lines, measured by real-time PCR. Levels of *IGF2*, *MEST*, and *PEG3* mRNA were significantly higher in the five rhabdomyosarcoma cell lines than in 11 nonembryonal cancer cell lines. *PLAGL1* did not reach significance. White bars, relative mRNA expression in rhabdomyosarcoma cell lines; black bars, relative mRNA expression in nonembryonal cancer cell lines. *$P = 0.05$; **$P < 0.005$; †$P < 0.001$; §nonsignificant.

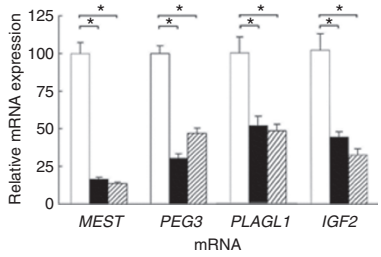

Figure 3. Effect of *IGF2*, *MEST*, *PEG3*, and *PLAGL1* siRNA transfection on target mRNA levels in Rh30 cells. mRNA levels were measured by real-time reverse transcription PCR 48 h after siRNA transfection and compared to mRNA levels in cells transfected with a negative control siRNA. Two independent siRNAs were used for each gene. All siRNAs significantly reduced expression of the target gene. White bars, transfection with negative control siRNA; black bars, transfection with siRNA no. 1; hatched bars, transfection with siRNA no. 2. *$P < 0.001$.

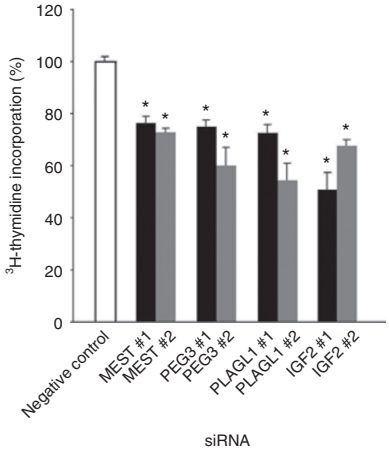

Figure 4. Effects of siRNA transfection on proliferation of Rh30 rhabdomyosarcoma cells. Cells transfected with siRNA targeting *MEST*, *PEG3*, *PLAGL1*, and *IGF2* caused significant reduction in the rate of proliferation as compared to negative control siRNA, as measured by tritiated thymidine incorporation. This effect was confirmed with a second siRNA sequence for each gene target. White bars, transfection with negative control siRNA; black bars, transfection with siRNA no. 1; gray bars, transfection with siRNA no. 2. *$P < 0.001$.

and *Slc38a4*. In this study, our analysis of available microarray data suggested that some of these imprinted genes, particularly *IGF2*, *PLAGL1*, *MEST*, and *PEG3*, are overexpressed in some embryonal cancers, especially rhabdomyosarcoma, relative to nonembryonal cancers and to normal tissues. Relative overexpression in rhabdomyosarcoma cell lines as compared to nonembryonal cancer cell lines was confirmed by real-time PCR for *MEST*, *PEG3*, and *IGF2*. For the analysis, tumors had to be classified as either embryonal or nonembryonal in origin. We classified Ewing's sarcoma as a nonembryonal tumor because recent molecular studies suggest that these tumors are derived from the mesenchymal stem cell (18), which is an adult stem cell present in marrow, rather than from an embryonal cell. Previous studies have focused primarily on *IGF2*, which has been shown to be overexpressed in some rhabdomyosarcomas (19–22), Wilms' tumors (23,24), and hepatoblastomas (25). Our findings also confirm and extend a previous report that *MEST*, *PEG3*, and

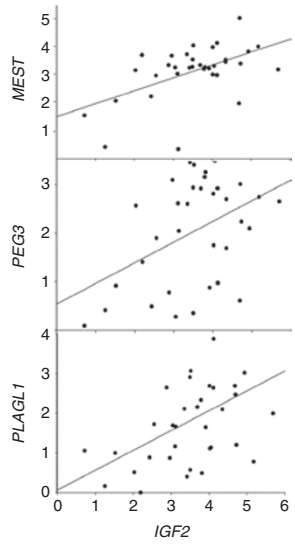

Figure 5. Correlation of expression of *IGF2* with expression of *MEST*, *PEG3*, and *PLAGL1* in rhabdomyosarcoma primary tumors, cell lines, and xenografts. Plotted values are derived from a National Cancer Institute expression microarray database and represent the fold difference in expression of the mRNA as compared to a reference RNA. Samples that expressed *IGF2* mRNA at high levels also expressed higher levels of *MEST*, *PEG3*, and *PLAGL1*, suggesting that these genes are regulated coordinately in rhabdomyosarcomas. *MEST*: $P < 0.005$, $r = 0.497$; *PEG3*: $P < 0.001$, $r = 0.617$; *PLAGL1*: $P < 0.001$, $r = 0.386$.

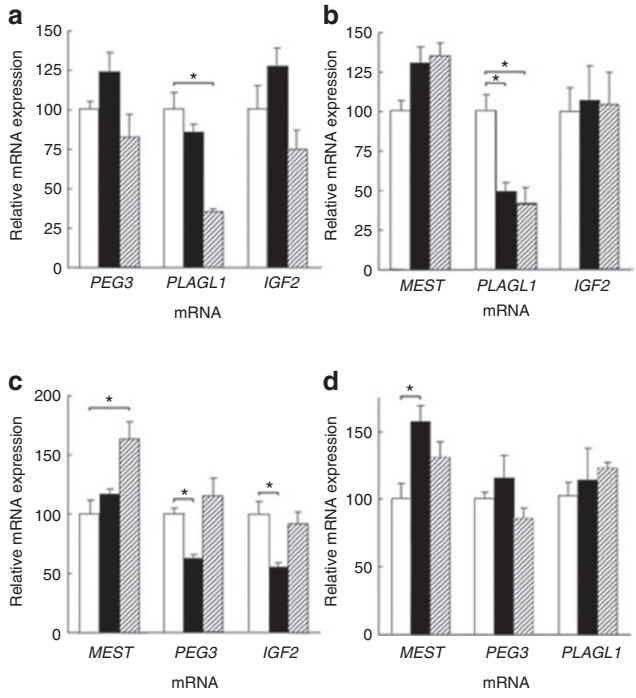

Figure 6. Effect of siRNA-mediated knockdown of (**a**) *MEST*, (**b**) *PEG3*, (**c**) *PLAGL1*, and (**d**) *IGF2* expression on expression of other imprinted genes of interest. Rh30 rhabdomyosarcoma cells were transfected with siRNA targeting *MEST*, *PEG3*, *PLAGL1*, or *IGF2*, and the resulting effect on mRNA levels was assessed by real-time reverse transcription PCR. For each target gene, two different siRNA sequences were used independently. The only consistent effect observed was that *PEG3* knockdown decreased *PLAGL1* mRNA levels. White bars, transfection with negative control siRNA; black bars, transfection with siRNA no. 1; hatched bars, transfection with siRNA no. 2. *$P < 0.05$.

DLK1 are overexpressed in Wilms' tumors, indicating that this overexpression occurs in other embryonal cancers as well (23).

We also found that inhibiting the expression of *MEST*, *PEG3*, *PLAGL1*, and *IGF2* slowed the proliferation of rhabdomyosarcoma cells in culture. In this experiment, we used siRNA to knock down gene expression and observed significant decreases in cell proliferation, as assessed by tritiated thymidine incorporation. Two lines of evidence suggest that the decreases in proliferation were actually mediated by decreased expression of the target gene. First, using real-time reverse transcription PCR (RT-PCR), we demonstrated that siRNA transfection did knock down the target mRNA levels by ~50–80%. Second, for each gene, we used two independent siRNAs and observed that both inhibited proliferation. It would be unlikely that these two siRNAs, which have different sequences, would both have off-target effects causing decreased proliferation. The results of the siRNA knockdown experiment indicate that *MEST*, *PEG3*, *PLAGL1*, and *IGF2* are all required for the rapid proliferation observed in these cells and suggest that the observed overexpression of these genes in rhabdomyosarcomas contributes to their rapid neoplastic proliferation.

Prior studies suggest that *MEST*, *PEG3*, *PLAGL1*, and *IGF2* are normally expressed at high levels in embryonic and early postnatal life, promoting the normal rapid body growth that occurs at this time, but are subsequently downregulated in multiple tissues simultaneously, contributing to normal somatic growth deceleration (6,7). The current findings suggest that this downregulation fails to occur in some embryonal cancers and that this failure contributes to the persistent rapid proliferation of rhabdomyosarcoma cells and possibly their persistent embryonal characteristics.

Previous evidence also suggests that *MEST*, *PEG3*, *PLAGL1*, and *IGF2* are members of an imprinted coregulated gene network (13). Consistent with this proposal, we found that *MEST*, *PEG3*, and *PLAGL1* expression correlated with *IGF2* expression among rhabdomyosarcoma samples. Therefore, the data suggest that these genes are regulated coordinately by a common mechanism. Varrault *et al.* have proposed that *PLAGL1* may be a master regulator of this imprinted gene network (13). However, we found that siRNA-mediated knockdown of *PLAGL1* expression in a rhabdomyosarcoma cell line did not consistently alter the expression of *MEST*, *PEG3*, or *IGF2*. Therefore, it is unlikely that this program of declining gene expression is coordinated by *PLAGL1* in rhabdomyosarcoma cells. We also tested for other possible interactions among *MEST*, *PEG3*, *PLAGL1*, and *IGF2* expression levels and found only that knockdown of *PEG3* decreased expression of *PLAGL1*. Therefore, the findings suggest that the concordant regulation is not coordinated by any of these genes.

In conclusion, our findings provide evidence that, in addition to *IGF2*, other imprinted genes, including *MEST*, *PEG3*, and *PLAGL1*, are overexpressed in embryonal tumors including rhabdomyosarcomas and that this overexpression promotes growth of rhabdomyosarcoma cells. Our previous studies suggest that these genes are also highly expressed in embryonic cells and that the subsequent decline in expression contributes to normal growth deceleration. Therefore, taken together, the findings suggest that the postnatal program of gene expression that normally limits proliferation in juvenile tissues fails to occur in some embryonal cancers and contributes to the persistent rapid proliferation and perhaps embryonic characteristics of at least one embryonal cancer, rhabdomyosarcoma. Thus, *MEST*, *PEG3*, *PLAGL1*, *IGF2*, and *DLK1* may provide a link between the genetic mechanisms responsible for normal growth deceleration and the pathologic proliferation of cells in embryonal tumors. Because the imprinted genes that we studied appear to be part of a larger genetic program responsible for growth deceleration, our findings raise the possibility that failure of other components of this program may also contribute to the growth of rhabdomyosarcoma, and possibly other embryonal cancers.

METHODS

Microarray

We analyzed an expression microarray database from the laboratory of Javed Khan (17), which is based on 163 cancer samples (75 xenograft, 70 primary tumors, and 18 cell lines), including embryonal and nonembryonal pediatric cancers. The embryonal tumor samples include rhabdomyosarcoma (n = 34), neuroblastoma (n = 62), Wilms' tumor (n = 8), medulloblastoma (n = 6), and ependymoma (n = 4). Cancers of nonembryonal origin include acute lymphoblastic leukemia (n = 10), Ewing's sarcoma (n = 31), and osteosarcoma (n = 8). Of the 163 samples analyzed in the microarray, 18 were cell lines (12 neuroblastoma, 3 rhabdomyosarcoma, and 3 Ewing's sarcoma), 70 were primary tumor samples (30 neuroblastoma, 21 rhabdomyosarcoma, and 19 Ewing's sarcoma), and the remainder were xenografts. Some of the cell lines, xenografts, and primary tumor samples might have been derived from a common origin. Normal pediatric tissue samples (n = 19) include gastrointestinal tissue, kidneys, ureters, muscle, uterus, testes, lung, heart, brain, and adrenal glands. Expression of mRNA was normalized to a combined sample of total RNA from seven human cancer cell lines (CHP212, RD, HeLa, A204, K562, RD-ES, and CA46). For this prior study, all samples required appropriate institutional review board and material transfer agreement approval from the donating institution (17).

Cell Culture

Rh30, Rh4, Rh28, CTR, RD-ATCC, Rh1, Daudi, K562, MEG-1, and HCT116 cell lines were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA), and HeLa, BT474, SKBR3, TC-71, RD-ES, and U-2 OS cell lines were cultured in Dulbecco's modified essential medium (Invitrogen). Both culture media were supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 U/ml streptomycin (Invitrogen), and 300 μg/ml L-glutamine (Invitrogen). Cells were cultured at 37 °C in 5% CO_2 and split every 3–4 days (at ~75% confluence for adherent cell lines) using 0.25% trypsin with 0.038% EDTA (Invitrogen) for 1 min. Cell lines were provided by Javed Khan, National Cancer Institute, National Institutes of Health (Duadi, K562, MEG-1, HCT116, HeLa, BT474, SKBR3, TC-71, RD-ES, U-2 OS), and Lee Helman, National Cancer Institute, National Institutes of Health (Rh30, Rh4, Rh28, CTR, RD-ATCC, Rh1). Rh30 (26–28), Rh4 (28), Rh28 (28), CTR (28), and RD-ATCC (28–30) are rhabdomyosarcoma cell lines, whereas the remaining 13 cell lines are derived from cancers of nonembryonal origin: HeLa, cervical cancer (31); HCT116, colon cancer (32); BT474 (33,34) and SKBR3 (34), breast cancer; TC-71 (35), RD-ES (36), and Rh1 (26,37), Ewing's sarcoma; U-2 OS, osteosarcoma (38–40); Daudi (41), Burkitt's lymphoma; and K562 (42) and MEG-1 (43), chronic myeloid leukemia.

Quantitative Real-Time RT-PCR

Total RNA was isolated from cultured cells using the RNeasy kit (Qiagen, Valencia, CA) and then reverse transcribed using Superscript

Table 1. siRNA sequences used for transfection experiments

siRNA	Sense sequence (5′→3′)	Antisense sequence (5′→3′)	Ambion ID no.	Concentration (nmol)
MEST no. 1	GGUACAAGCAGAAUCGAUCtt	GAUCGAUUCUGCUUGUACCtg	106797	75
MEST no. 2	GGGAUUGUUGUGUAGUCAtt	UUGACUACACAACAAUCCCtt	106798	100
PEG3 no. 1	GCACCAGUCGAGGUCUAAAtt	UUUAGACCUCGACUGGUGCtt	144245	100
PEG3 no. 2	CGUAACUGUUCAUAAGAAUtt	AUUCUUAUGAACAGUUACGtg	144246	100
PLAGL1 no. 1	GGCUUUUCCGUAAACAUAUtt	AUAUGUUUACGGAAAAGSStc	108090	25
PLAGL1 no. 2	CGCGUGUUCUGUAAUCAAtt	UUUGAUUACAGAACACGCGtt	115818	100
IGF2 no. 1	CAAUUGUGGAACCCACAUUtt	AAUGUGGGUUCCACAAUUGtg	254540	50
IGF2 no. 2	AAUUACCUGCCCAUUCGUCtt	GACGAAUGGGCAGGUAAUUtg	238102	75

III Reverse Transcriptase (Invitrogen), both according to the manufacturers' instructions. Quantitative real-time PCR was performed using the following assays containing primers and specific intron-spanning FAM-labeled TaqMan probes (Applied Biosystems, Foster City, CA): *MEST*, Hs083380_91; *PEG3*, ZIHs00377844_m1; *PLAGL1*, Hs00414677; and *IGF2*, Hs00171254_m1. An assay for 18S rRNA using a VIC/TAMRA-labeled TaqMan probe (Applied Biosystems) was used for normalization. Reactions were performed in triplicate using cDNA, TaqMan universal PCR Master Mix (Applied Biosystems), and the ABI 7900HT Sequence Detection System (Applied Biosystems), according to the manufacturer's instructions with the following thermal cycling conditions: 1 cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The quantity of each mRNA was calculated using the formula: relative expression$_i$ = $(2)^{CTr}/(2)^{CTi}$ where r represents 18S rRNA (for internal normalization), i represents the gene of interest, and CT represents the threshold cycle. For convenience, relative expression values were multiplied by 10^6.

siRNA Transfection

One day prior to transfection, Rh30 cells were plated in 6-well plates at a density of 200,000 cells per well to reach ~50% confluence at the time of transfection. Cells were transfected with siRNA (Silencer siRNA; Ambion, Austin, TX) targeting *MEST*, *PEG3*, *PLAGL1*, and *IGF2* (**Table 1**), using 5 µl LipofectAMINE 2000 (Invitrogen) in 2.25 ml total volume per well in 6-well plates. Final concentrations of siRNA required to achieve maximal knockdown were optimized. A scrambled sequence siRNA of similar length (cat. no. AM4635; Ambion) was used as a negative control at a concentration of 25 nmol. Cells were cultured at 37 °C in 5% CO_2 for an additional 48 h after transfection, then used to isolate RNA for real-time RT-PCR as described previously or treated with ^{3}H-thymidine as described in the following.

Tritiated Thymidine Incorporation

Cell proliferation was assessed by tritiated thymidine incorporation. Forty-eight hours after transfection with siRNA, tritiated thymidine ([Methyl-^{3}H] thymidine in aqueous solution, 25 Ci/mmol, cat. no.TRK120; Amersham, Piscataway, NJ) was added to the culture medium at a concentration of 1 µCi/ml. Cells were incubated for an additional 4 h at 37 °C in 5% CO_2, after which cells were detached with trypsin, and incorporation of tritiated thymidine was measured by liquid scintillation counting. For each experiment, three replicate wells were used per siRNA. The experiment was repeated, and results were combined.

Statistical Analysis

Data are presented as mean ± SEM. Specific mRNA levels in rhabdomyosarcoma as compared to nonembryonal cancers, and effects of knockdown by siRNA on mRNA levels were evaluated by one-way ANOVA. Effects of siRNA on tritiated thymidine incorporation were normalized to results from the negative control scrambled siRNA and then evaluated by two-way ANOVA to account for effects of multiple experiments. Correlation of gene expression was evaluated by linear regression using SigmaStat (Systat Software, San Jose, CA).

ACKNOWLEDGMENTS

The authors thank Javed Khan and Lee Helman of the Pediatric Oncology Branch, National Cancer Institute, for helpful advice on the experimental approach and for providing cell lines.

STATEMENT OF FINANCIAL SUPPORT

This work was supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health.

Disclosure: The authors have nothing to disclose.

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