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Imprinted Genes Are Up-Regulated by Growth Arrest in Embryonic Fibroblasts[1]

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It was previously reported that the imprinted insulin-like growth factor II and H19 genes are up-regulated in embryonic fibroblasts upon growth arrest. We have examined six other imprinted genes and found that all were up-regulated upon confluency and at least four of them by serum starvation. The significance of the results was confirmed by demonstrating that only one-third of randomly selected genes are up-regulated upon confluency. Our findings suggest that the cell cycle should be carefully controlled when imprinted genes are studied in cell cultures. Moreover, the unique property could have significance in the mechanistic or the evolutionary aspect of imprinting or both.

Key words: embryonic fibroblast, genomic imprinting, growth arrest.

Genomic or parental imprinting is relevant to a variety of biological phenomena but its evolutionary significance and molecular mechanisms are not fully understood. Identification of characteristics common to many imprinted genes should help us answer these questions. Recent papers have described unusual properties of the imprinted genes, *i.e.*, association with tandem repeats embedded in CpG-rich sequences (*1*), possession of few and small introns (*2*), unusual sex-specific recombination rates (*3*), allele-specific replication timing (*4*), and inter-allelic physical association in late S phase (*5*). Regarding the function of the imprinted genes, it has been noted that the paternally expressed genes tend to promote cell growth while the maternally expressed genes are often growth-inhibiting (*2*). This functional difference between the paternally and the maternally expressed genes might explain the evolution of imprinting in mammals (*6, 7*). We report here a new and peculiar behavior of the imprinted genes, which is of immediate practical importance and could have bearing on the evolution and mechanisms of imprinting.

We previously reported that two imprinted mouse genes, insulin-like growth factor II (*Igf2*) and *H19*, are coordinately up-regulated upon growth arrest in several embryonic fibroblast cell lines (*8, 9*). These two genes are closely linked on distal chromosome 7 and share at least two tissue-specific enhancers (*10*). To ask whether the unique regulation is also seen for other imprinted genes, we have examined six other genes, all of which were expected to be transcribed in fibroblasts. The genes included U2 auxiliary factor 1-related sequence 1 (*U2af1-rs1*) on chromosome 11, insulin-like growth factor II/mannose-6-phosphate receptor (*Igf2/Mpr*) on chromosome 17, small nuclear ribonucleoprotein-associated polypeptide N (*Snrpn*) on proximal chromosome 7, $p57^{KIP2}$ on distal chromosome 7, paternally expressed gene 1/mesoderm-specific transcript (*Peg1/Mest*) on chromosome 6 and paternally expressed gene 3 (*Peg3*) on proximal chromosome 7 (*11* and references therein). No other imprinted genes were known to be transcribed in fibroblasts at the time of this study.

A mouse embryonic fibroblast line called Normal Cl 1 (*8, 9*) was seeded at 0.4×10^6 cells/100-mm dish and, after 3 days, total RNA was isolated from cells in the logarithmic phase by the method of Chomczynski and Sacchi (*12*). Confluent phase RNA was isolated from cells on duplicate dishes 7 days after seeding. The total RNA templates were reverse-transcribed and the products subjected to semi-quantitative PCR as described (*8, 9*). Precise PCR conditions and the cycling number were determined for each primer set according to the amplification curve obtained in pilot experiments. The primer sequences and the detailed PCR conditions are available upon request. Surprisingly, all six genes tested were up-regulated upon confluency in Normal Cl 1 cells, although the degree of induction was different from gene to gene (Fig. 1). Almost identical results were obtained with newly derived embryonic fibroblasts after only four passages of mass culture (data not shown), indicating that the up-regulation by contact inhibition was not restricted to this particular cell line.

To test whether growth factor depletion could also induce these genes, Normal Cl 1 cells were seeded as described above and, three days after seeding, the medium containing

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Abbreviations: *Igf2*, the insulin-like growth factor II gene; *Igf2/Mpr*, the insulin-like growth factor II/mannose-6-phosphate receptor gene; *Peg1/Mest*, paternally expressed gene 1/mesoderm-specific transcript; *Peg3*, paternally expressed gene 3; *Snrpn*, the small nuclear ribonucleoprotein-associated polypeptide N gene; *U2af1-rs1*, U2 auxiliary factor 1-related sequence 1.

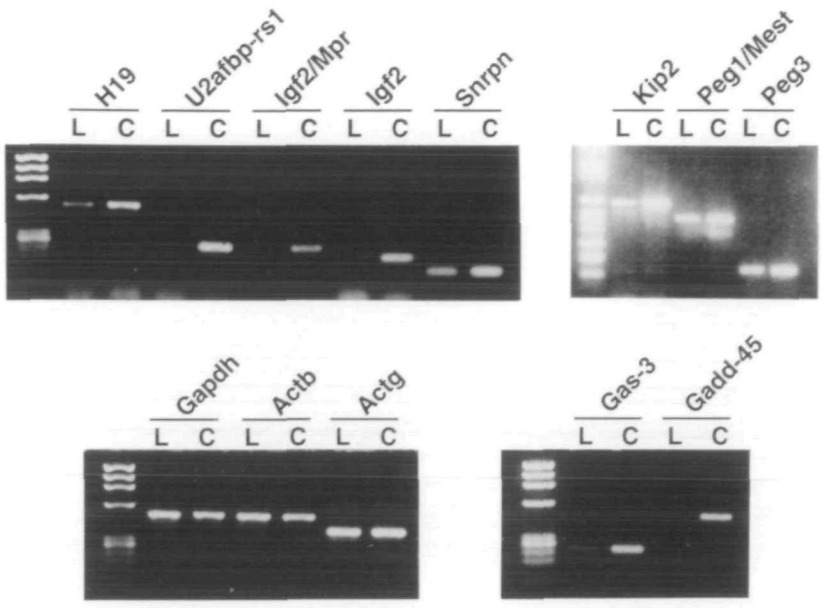

Fig. 1. **Up-regulation of eight imprinted mouse genes upon confluency in the embryonic fibroblast line Normal Cl 1.** Three housekeeping genes (*Gapdh, Actb,* and *Actg*) and two arrest-specific genes (*Gas-3* and *Gadd-45*) are also included as controls. Total RNA isolated from cells in the logarithmic (L) and confluent (C) phases were subject to semi-quantitative reverse transcriptase-PCR under non-saturating conditions. Gene symbols not appearing in the text are: *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Actb*, β-actin; *Actg*, γ-actin; *Gas-3*, growth arrest-specific gene 3; *Gadd-45*, growth arrest and DNA damage inducible gene 45.

10% serum was replaced by a 0.5% serum medium. RNA was isolated from cells 48 h after reducing the serum concentration. The low serum medium in duplicate dishes was then changed to that containing 20% serum and, after culturing for a further 48 h, RNA was isolated from the re-stimulated cells. The experiment showed that the imprinted genes in serum-starved cells were expressed at levels comparable to those in confluent cells (Fig. 2). When these cells were stimulated to re-initiate cell division by adding 20% serum, most genes were down-regulated with the exception of *Snrpn* and *Peg3*, which showed little or no decrease in expression (Fig. 2). These experiments established that all of the eight imprinted genes examined were up-regulated by contact inhibition and at least six of them by growth factor depletion.

To assess the significance of these findings, we next determined what proportion of all mRNA species is up-regulated upon growth arrest. Total RNA isolated from mass cultures of fibroblast cells in the logarithmic and confluent phases was reverse-transcribed and the products subjected to differential display analysis as described (*13*). Among the 665 bands detected by combinations of four sets of degenerate anchored oligo(dT) primers and three arbitrary decamer primers (OPA-2, OPA-6, and OPA-8 from Operon Technologies), 193 bands (29.0%) appeared at higher intensities in the confluent phase than in the logarithmic phase, while 329 bands (49.5%) remained unchanged. The remaining 143 bands (21.5%) appeared at higher intensities in the logarithmic samples. Assuming that the experiment represents random sampling of mRNA, the distribution of the number of up-regulated genes in a pool of eight genes is described as a binomial B (8, 0.29). If we assume that this applies to the imprinted genes, the probability of all eight genes being up-regulated is calculated as 0.00005 and that of six or more genes 0.0094 (<0.01). The binomial distribution can thus be rejected at a risk of less than 0.01 for the imprinted genes, which in turn suggests a significant association between imprinting and arrest-specific up-regulation.

Preliminary studies suggest that the arrest-specific

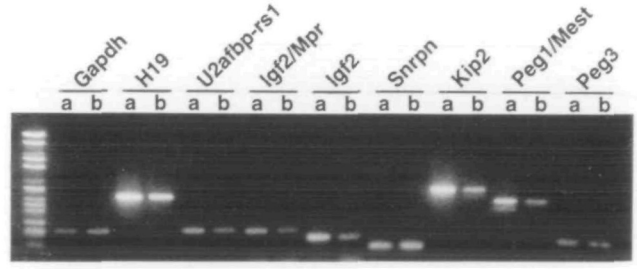

Fig. 2. **Up-regulation of six imprinted mouse genes by serum starvation in the embryonic fibroblast line Normal Cl 1.** Total RNA isolated from the serum-starved (a: 0.5% 48 h) and re-stimulated (b: 20% 48 h) cells were subject to semi-quantitative reverse transcriptase-PCR under non-saturating conditions. *Gapdh* is included as a control.

increase in expression may be diminished or even lost in tumor-derived cell lines and well-established lines maintained for many years. Thus the phenomenon may be restricted to primary cultures and newly derived cell lines. Also, it is unknown whether other imprinted genes, which were undetectable in embryonic fibroblasts, display a similar growth-dependent expression pattern. However, an immediate conclusion drawn from the above results is that one should control growth conditions carefully when examining the expression of imprinted genes in cultured cells. This information will be particularly important if new imprinted genes are to be identified based upon differences in the expression levels between two different cell lines (for example, parthenogenetic and androgenetic cell lines).

Besides this practical significance, it is potentially of more importance and interest that the unique property may provide a clue to understanding the evolutionary reasons and molecular mechanisms of imprinting. Since the growth-dependent regulation was observed independently of whether the gene is expressed maternally (*H19, Igf2/Mpr*, and *p57^{KIP2}*) or paternally (*Igf2, U2af1-rs1, Snrpn, Peg1/Mest*, and *Peg3*), or whether the gene influences fetal growth positively (*Igf2*) or negatively (*H19, Igf2/Mpr*, and

$p57^{KIP2}$), it is difficult to relate directly the unique property to the gene function or embryonic development. Nevertheless, it is possible that the imprinted genes might share common components in their activation pathways, which could play roles in their parental-origin-specific expression or repression. Alternatively, the arrest-specific up-regulation might be mechanistically related with the differentiation-associated activation of imprinted genes because, for example, *Igf2, Igf2/Mpr*, and *H19* are up-regulated upon differentiation of ES cells (although *Snrpn* is an exception, which displays down-regulation) (*14*). In any case, further studies on these genes may provide an understanding of how this regulation is related to the evolutionary and mechanistic aspects of imprinting.

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