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Sensitive Flow Cytometric Analysis Reveals a Novel Type of Parent-of-Origin Effect in the Mouse Genome

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Summary

The discovery of classic parental imprinting came, at least in part, from the analysis of transgene expression in mice [1]. It was noticed that some transgenes were only expressed following paternal transmission [2–4] and that others sometimes showed differential patterns of methylation depending on the parent of origin [5, 6]. Here, we present evidence of a novel and more subtle form of parental imprinting by taking advantage of the highly sensitive detection of murine transgene expression afforded by flow cytometry. We have produced nine lines of transgenic mice carrying a GFP reporter linked to the human α-globin promoter and enhancer elements, which direct expression to erythroid cells. A high proportion of transgenic lines, four of the nine, display significantly lower levels of expression following maternal transmission. Both the percentage of expressing cells and the mean fluorescence in expressing cells are between 10% and 30% lower following maternal transmission. These effects are reversible upon passage through the opposite germline. This finding raises the possibility that differences in the epigenetic state of the maternal and paternal chromosomes in adult somatic cells are more widespread than was previously thought.

Results and Discussion

The erythroid-specific transgene consists of a GFP reporter linked to the human α-globin promoter and enhancer. This enables the use of flow cytometry to measure GFP expression on a cell-by-cell basis in the peripheral blood of adult mice. The transgenic lines were produced and maintained in the inbred FVB/N strain; thus, all individuals within one transgenic line are isogenic. Some lines, e.g., GFP7, showed clear variegation, similar to that previously reported by us for erythroid-specific transgenes [7–9]. In contrast, other lines, e.g., GFP3, expressed the transgene in the majority of cells (Figure 1A). Each line displayed a unique fluorescence profile that was identical among individuals (of either sex) within a line (Figure 1A) but differed between lines (Figures 1A and 1B). We observed parent-of-origin effects in four of the nine lines tested: GFP3, GFP5, GFP6, and GFP9 all showed a small, but highly significant, drop in expression following maternal transmission (Figures 1B and 2; Table 1). Statistical analysis of the data showed that parent-of-origin effects were present in both the percentage of cells expressing the transgene (GFP^+ cells) and in the mean fluorescence intensity of the expressing cells (Figure 2 and Table 1). The percentage of expressing cells was between 10% and 20% lower following maternal transmission, and the mean fluorescence of expressing cells was between 10% and 30% lower following maternal transmission (Figure 2 and Table 1). Any line that showed significant differences in the percentage of expressing cells also showed differences in the mean fluorescence intensity and vice versa. Analysis of transgene expression in midgestation embryos, when erythroid-specific transgene expression is generally more robust [10, 11], revealed lower expression following maternal transmission in GFP3, GFP5, GFP6, GFP9, and, in an additional line, GFP8 (data not shown).

These parent-of-origin effects were found to be reversible upon passage through the opposite germline and are stable over at least three generations (Figure 3). When crossed to wild-type mice, male mice homozygous for the transgene (G4) produced offspring (paternal transmission, G5) (Figure 3B) that expressed the transgene at levels equivalent to those seen following paternal transmission at G3 (Figure 3A). When crossed to wild-type mice, female homozygotes (G4) produced offspring (maternal transmission, G5) (Figure 3B) that expressed the transgene at levels equivalent to those seen following maternal transmission at G2 (Figure 3A). This shows that the parent-of-origin effects were stable over a number of generations.

Furthermore, the percentage of expressing cells, which reflects the probability of expression from each allele, observed in homozygous individuals obeys the equation for independent events as calculated below. The probability that a red blood cell expresses the transgene after male transmission was 85%, and the probability of expression after female transmission was 68% (values taken from Figure 2). If the two alleles behave independently in homozygous offspring, one would expect the following, according to the equations of two independent events:

$$P(A \cup B) = P(A) + P(B) - P(A \cap B),$$

$$\text{where } P(A) = 0.85 \text{ and } P(B) = 0.68$$

$$P(A \cap B) = P(A) \times P(B) = 0.85 \times 0.68 = 0.58$$

$$P(A \cup B) = 0.85 + 0.68 - 0.58 = 0.95.$$

This is consistent with the empirically determined probability of 95% established by using genetically confirmed homozygous offspring (Figure 3). From this we can conclude that the epigenetic states at the maternal and paternal alleles behave independently.

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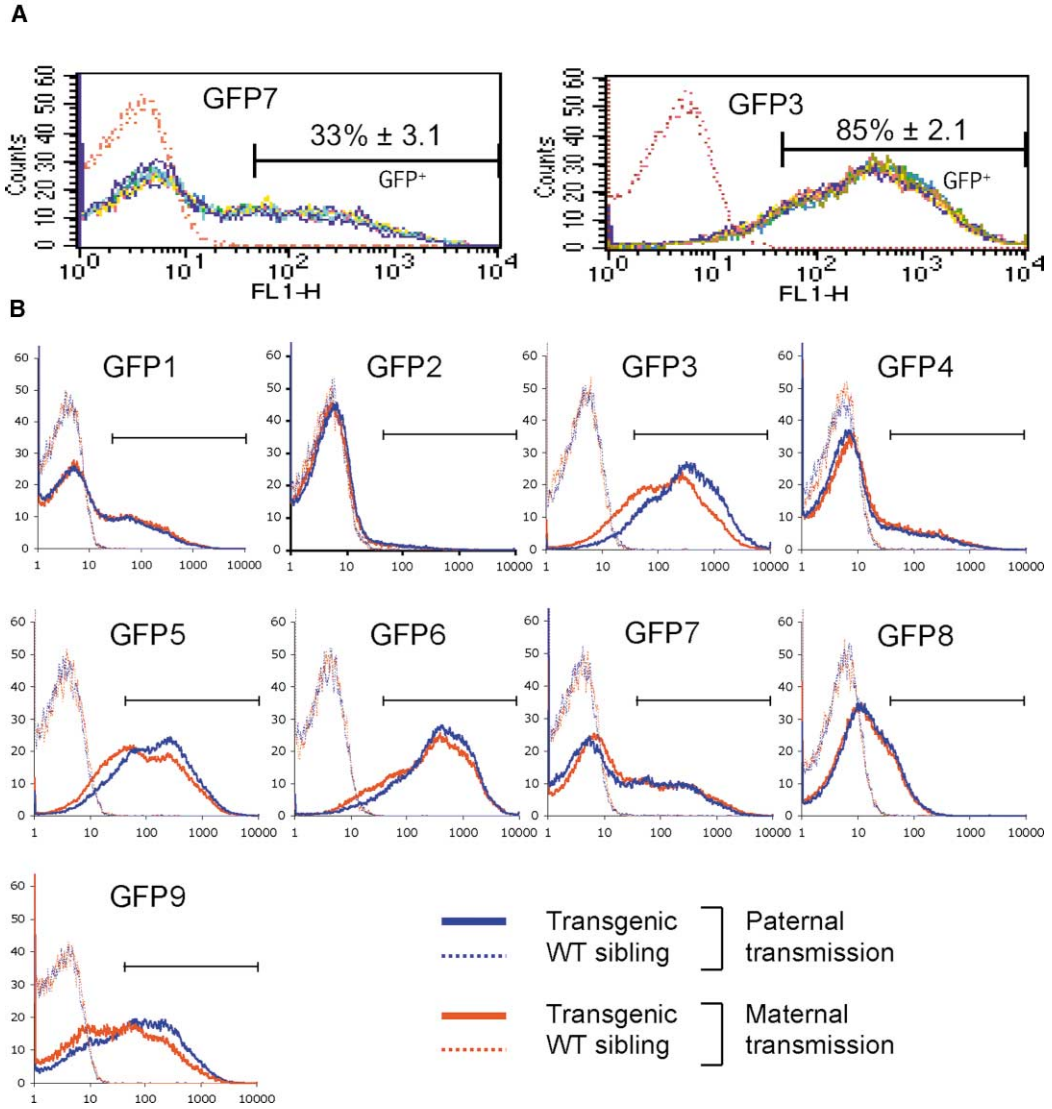

Figure 1. Subtle Parental Effects in the Expression Profile of GFP Transgenic Lines

(A) Flow cytometric analysis of erythrocytes from 3-week-old littermates of lines GFP7 and GFP3 is representative of expressing lines. Erythrocytes from each of the nine established transgenic mouse lines show a unique fluorescence intensity distribution when flow cytometry is used. For each line, blood from ten 3-week-old mice, produced from more than one litter (paternal transmission), was analyzed, and the graphs were overlayed. In both cases, two nontransgenic littermates are included (dotted lines). Line GFP7 has a clear peak of nonfluorescing cells, while line GFP3 contains few nonfluorescing cells. The fluorescence profile within a line is conserved in all transgenic offspring (independent of sex) from that line. The gate GFP$^+$ was used to quantify the percentage of expressing cells and the mean fluorescence of expressing cells and was set to exclude 99.9% of wild-type (nontransgenic) cells.

(B) Erythrocytes from 3-week-old mice from nine lines were analyzed by flow cytometry. Data from individuals ($n \geq 8$) that inherited the transgene from their father (blue) or their mother (red) were averaged separately and were overlayed in each graph. The sex of the offspring did not affect the level of expression in any line (data not shown). In all cases, more than one parent of each sex was used. As an internal control for the consistency of the settings of the flow cytometer, a wild-type (WT) sibling was used from each litter (dotted line). Lines GFP1, GFP2, GFP4, GFP7, and GFP8 show identical profiles for paternal and maternal inheritance, whereas profiles from lines GFP3, GFP5, GFP6, and GFP9 showed both a lower percentage of expressing cells and a lower mean fluorescence intensity if the transgene was inherited from the mother. Transgene copy numbers for lines GFP1–GFP9 are: 11, 4, 30–35, 800–900, 25–35, 45–55, 35–40, 2, 20, respectively (data not shown).

Using flow cytometric analysis of transgene expression, a far more sensitive assay than is generally used to measure gene expression, we have found that four of nine transgenic lines at 3 weeks of age (and an additional line at midgestation) show a novel type of parent-of-origin effect. The transgenes were less active following maternal inheritance, and the increased silencing was associated with a decrease in the amount of GFP per cell as well as a decrease in the probability that a cell will express the transgene. Occasional reports of parent-of-origin effects at murine transgenes, where the causative factor appeared to be not the construct itself,

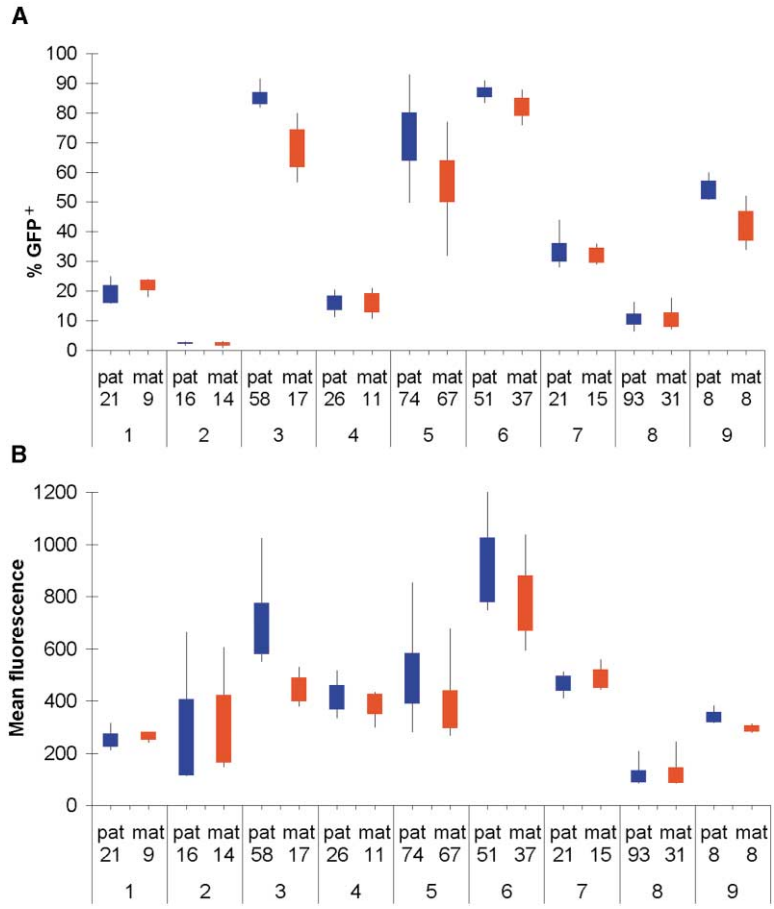

Figure 2. Box-Whisker Plots Showing Parent-of-Origin Effects on the Percentage and Mean Fluorescence of GFP$^+$ Cells

(A and B) The percentage of expressing cells and the mean fluorescence (determined by gate GFP$^+$) ± 1 standard deviation were determined for paternally (blue box) and maternally (red box) inherited transgenes (n is between 8 and 93 individual mice per data set). The data range is symbolized by black "whiskers." Lines GFP3, GFP5, GFP6, and GFP9 show a clear difference between paternal and maternal transmission of the transgene. In all four cases, the paternally inherited transgene is expressed in a higher percentage of cells and shows a higher mean fluorescence at a level of significance of $p < 0.01$ for each pair (using the two-tailed Student's t test).

Table 1. Quantitative Analysis of Expression Profiles of GFP Transgenic Lines

		Paternal Transmission		Maternal Transmission		
GFP Line	Copy Number	% Expressing Cells (M2)	n	% Expressing Cells (M2)	n	p Value
GFP 1	11	19 ± 3	21	22 ± 2	9	NS
GFP 2	~4	2 ± 1	16	2 ± 1	14	NS
GFP 3	30–35	85 ± 2	58	68 ± 6	17	<0.0001
GFP 4	~900	16 ± 3	26	16 ± 3	11	NS
GFP 5	25–35	72 ± 8	74	57 ± 7	67	<0.0001
GFP 6	45–55	87 ± 2	51	82 ± 3	37	<0.0001
GFP 7	35–40	33 ± 3	21	32 ± 3	15	NS
GFP 8	~2	10 ± 2	93	10 ± 2	31	NS
GFP 9	20	54 ± 3	8	42 ± 5	8	<0.0001
		Mean Fluorescence (M2)		Mean Fluorescence (M2)		
GFP 1	11	249 ± 26	21	266 ± 15	9	NS
GFP 2	~4	260 ± 146	16	293 ± 129	14	NS
GFP 3	30–35	677 ± 98	58	443 ± 46	17	<0.0001
GFP 4	~900	413 ± 47	26	388 ± 39	11	NS
GFP 5	25–35	486 ± 97	74	367 ± 73	67	<0.0001
GFP 6	45–55	902 ± 124	51	775 ± 106	37	<0.0001
GFP 7	35–40	467 ± 30	21	485 ± 35	15	NS
GFP 8	~2	111 ± 23	93	115 ± 30	31	NS
GFP 9	20	338 ± 20	8	294 ± 12	8	<0.0001

Analysis of data presented in Figures 1A and 1B. The percentage of expressing cells was determined by using a GFP$^+$ gate, which was set to exclude 99.9% of wild-type cells.

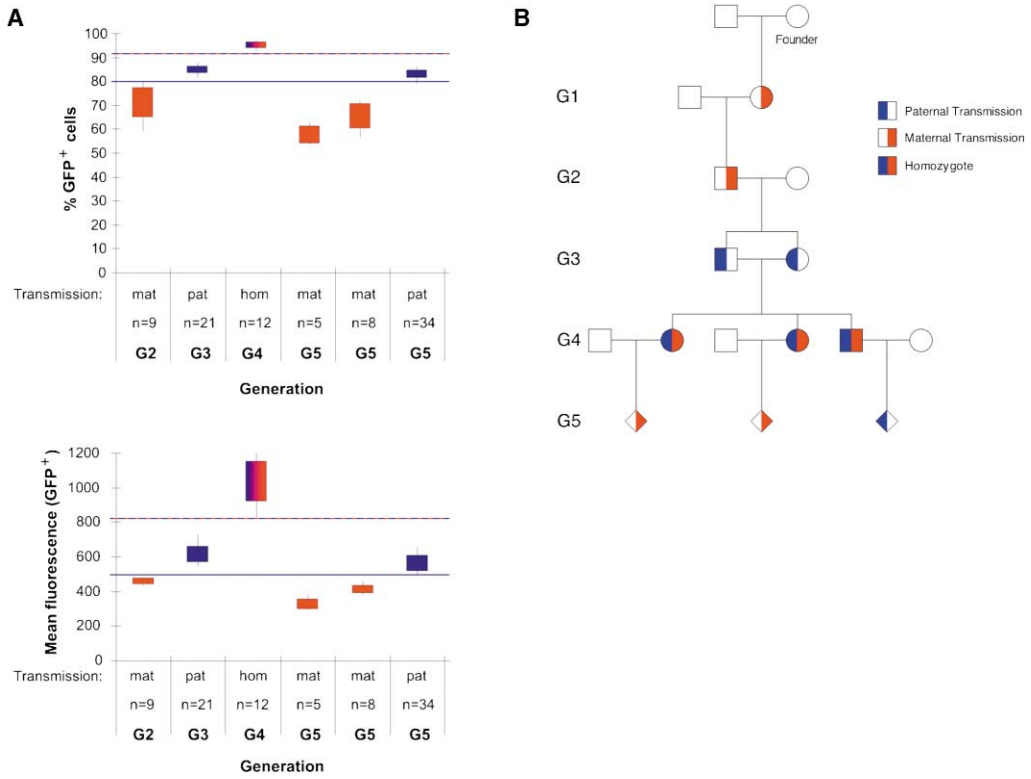

Figure 3. Expression Patterns Recorded over Five Generations

(A) The parent-of-origin effect is reversible upon passage through the opposite germline. Line GFP3 was used in this study. Individuals that received the transgene from their mother (red), whether at generation 2 (G2) or generation 5 (G5), express the transgene at lower levels than individuals that received the transgene from their father (blue), whether at G3 or G5. Individuals that are homozygous for the transgene (blue and red) express at a level consistent with the two alleles continuing to behave independently (see text). Homozygosity was verified by mating the mice to wild-type mice and finding that all offspring ($n > 10$) were positive for the transgene. The boxes represent the mean $\pm$ one standard deviation, and the whiskers extend to the highest and lowest value in each data set.

(B) A simplified pedigree of the breeding strategy. Wild-type offspring at G1, G2, G3, and G4 are not shown. Heterozygous offspring at G4 are not shown. At each generation, the number of mice assayed and used as data points in (A) is always much higher than the number shown in this panel. Those shown here are only those used to generate subsequent generations.

but the site of integration, have been made by a number of groups [2, 3, 6, 12]. In all those cases, silencing was observed following maternal inheritance. The parent-of-origin effects reported here clearly do not behave in the same way as classic parental imprinting, since, in the latter case, the parent-of-origin effect is so strong that it results in monoallelic expression. Furthermore, the frequency of the effect seen here is much higher than would be expected based on the estimated number of classic parentally imprinted genes in the mouse. One could argue that such an effect is particular to transgenes and tells us nothing about the site of integration. However, the fact that each line has a characteristic expression profile that does not appear to correlate with transgene copy number and the fact that not all the lines display a parent-of-origin effect argues against this hypothesis. The effects we report are reminiscent of the unusual parent-of-origin effects reported at alleles such as *agouti viable yellow* (A^{vy}) and *axin fused* ($Axin^{Fu}$) in which expression is controlled by the epigenetic state of a neighboring retrotransposon [13]. At A^{vy}, the probability that the offspring will overexpress the allele is influenced by whether it is inherited from the sire or the dam. Overexpression of *agouti* is approximately 10% more likely following maternal inheritance of the allele. At $Axin^{Fu}$, another allele at which expression of a mutant transcript is controlled by the epigenetic state of a retrotransposon in intron 6, expression of the mutant transcript is approximately 30% more likely following paternal inheritance [14]. In many respects, the transcriptional activity of retrotransposons appears to be similar to that of transgenes. They are subject to stochastic epigenetic silencing that manifests as variegation and variable expressivity [13–16]. Two independent studies analyzing the activity of reporter genes at numerous independent P element insertions on the Y chromosome of *Drosophila melanogaster* found that most locations showed differential expression according to the parental source of the chromosome; this finding indicates that there is chromosome-wide parental imprinting [17]. It is interesting to note that in flies both imprinting and retrotransposons appear to be confined to heterochromatin of which the majority is contained within the Y chromosome.

The results presented here in mice, combined with those observed in *Drosophila*, are consistent with the idea that the insertion of DNA, by transgenesis or retrotransposition, renders the DNA susceptible to silencing;

this susceptibility differs to some degree depending on whether the allele has passed through the male or the female germline. It is likely that more sensitive assays of transgene expression from other transgene constructs expressed in other cell types will reveal similar effects. Such subtle parent-of-origin effects may in fact be fairly widespread since a significant proportion of the mammalian genome is derived from retroelements.

The widely accepted explanation for the evolution of classic parental imprinting is that the maternal and paternal alleles have conflicting interests with regard to the transfer of nutrients from mother to offspring. Recently, models have emerged that challenge this hypothesis. Both Sapienza and Lloyd suggest that classic parental imprinting is a by-product of the fact that the paternal and the maternal chromosomes are epigenetically different over large regions [18, 19]. Our results are consistent with these ideas and raise the interesting possibility that subtle parent-of-origin effects also occur at some endogenous genes. Indeed, it has been reported that the penetrance of some disease phenotypes in humans is affected by the parent-of-origin [20, 21]. Sensitive assays may reveal subtle differences in expression between the maternal and paternal alleles at these and other loci.

Experimental Procedures

Transgenic Mice

The transgene construct, αPEGFP, contains the human α-globin promoter from −570 to +37 linked to the *hGFP* reporter gene with the SV40 polyadenylation signal and a 4.1-kb fragment containing the human α-globin locus enhancer region (HS-40) [8, 22]. The transgenic lines were produced by microinjection of fertilized eggs in the FVB/N inbred strain. Homozygosity of GFP3 in some mice (see Figure 3) was confirmed genetically by testing that all offspring produced from mating these mice with wild-type mice were transgenic.

Flow Cytometry

Analysis was performed on a FACSCalibur (Becton Dickinson). The gate GFP^+ was used to quantify the percentage of expressing cells and the mean fluorescence of expressing cells and was set to exclude 99.9% of wild-type (nontransgenic) cells.

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