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Demonstration of all-or-none loss of imprinting in mRNA expression in single cells

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ABSTRACT

Loss of imprinting (LOI) is the reactivation of the silenced allele of an imprinted gene, leading to perturbation of monoallelic expression. We tested the hypothesis that LOI of *PLAGL1*, a representative maternally imprinted gene, occurs through an all-or-none process leading to a mixture of fully imprinted and nonimprinted cells. Herein using a quantitative RT-PCR-based experimental approach, we measured LOI at the single cell level in human trophoblasts and demonstrated a broad distribution of LOI among cells exhibiting LOI, with the mean centered at ~100% LOI. There was a significant ($P < 0.01$) increase in expression after 2 days of 5-aza-2′-deoxycytidine (AZA) treatment and a significant ($P < 0.01$) increase in LOI after both 1 and 2 days of AZA treatment, while the distribution remained broad and centered at ~100% LOI. We propose a transcriptional pulsing model to show that the broadness of the distribution reflects the stochastic nature of expression between the two alleles in each cell. The mean of the distribution of LOI in the cells is consistent with our hypothesis that LOI occurs by an all-or-none process. All-or-none LOI could lead to a second distinct cell population that may have a selective advantage, leading to variation of LOI in normal tissues, such as the placenta, or in neoplastic cells.

INTRODUCTION

Genomic imprinting is the silencing of one parental allele in the zygotes of gametes leading to monoallelic expression of the gene in the offspring (1). Several epigenetic processes such as DNA methylation and histone modification regulate this sex-dependent pattern of gene expression (1). Most of the imprinted genes in mammals control tissue growth (2). The most predominant hypothesis to explain such conservation is the 'parental conflict hypothesis' (3). This hypothesis proposes that the purpose of the imprinting is to assure appropriate allocation of limited maternal resources to each conceptus. Perturbations of genomic imprinting, i.e. loss of imprinting (LOI), have been implicated in multiple human diseases, including reproductive abnormalities and cancer (4–7). In previous work, we have demonstrated variation of LOI for many paternally or maternally expressed genes among human placentas (8). In this study, we examined the mechanism of LOI by measuring cell-to-cell variation in imprinting status.

PLAGL1 encodes a zinc finger protein that is thought to function as a transcription factor, inducing apoptosis and cell cycle arrest at G_1 phase (9). *PLAGL1* is a paternally expressed (maternally imprinted) gene that belongs to an imprinting cluster located on chromosome 6q24 (10). It is polymorphically imprinted in different tissues; monoallelic expression has been shown in various human tissues (placenta, muscle, lung), while it is biallelically expressed in peripheral blood leukocytes (11,12). Dysregulation of *PLAGL1* has been observed in ovarian and breast cancer cells, while paternal uniparental disomy of 6q24 has been implicated in transient neonatal diabetes mellitus (13–15). We selected *PLAGL1* as our reference gene to study the mechanism of LOI, because *PLAGL1* was among the most highly expressed imprinted genes that we had assayed in our previous work and our cell line was heterozygous for the readout polymorphism, a prerequisite for the LOI measurement.

PLAGL1 has two promoters, but only one is active in human placentas (11). The inactive promoter is neither imprinted nor methylated. The active promoter is

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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silenced from the maternal allele by differential methylation in primary human cells at all or the majority of 51 CpG sites compared with lack of methylation at all or the majority of the sites in the paternal allele (16). The same type of pattern is seen in cell lines, but with more variation in methylation between individual subclones (14). We chose a readout polymorphism (rs9373409) in the 5′-UTR which is represented in all splice variants (16) and has a minor allele frequency >22% in all populations.

Stochasticity in transcription has been observed for many genes in both prokaryotic and eukaryotic cells (17–19). In previous work, we have shown that stochastic transcription of biallelically expressed genes in human cells can lead to cell-to-cell variation in mRNA copy number by as much as 1000-fold (20), and to imbalanced transcription between two alleles within single cell (21). Gene expression noise has a significant effect on many biological processes, contributing to phenotypic variability of genetically identical organisms and determining cellular fate following viral infection (22–26). To be noted, the measurements of LOI in *PLAGL1* at the single cell level take place in the context of significant transcriptional noise.

Herein, we test the hypothesis that LOI is an all-or-none phenomenon at the single cell level, wherein partial LOI in tissue would reflect the fraction of cells with complete LOI. We quantify expression of the paternal and maternal alleles in single cells from a human placental trophoblast cell line heterozygous for a readout polymorphism in *PLAGL1* mRNA (8). The *PLAGL1* gene is known to be regulated by DNA methylation and histone modification (14,27). By treating the cell line with 5-aza-2′-deoxycytidine (AZA) or Trichostatin A (TSA), we were able to examine the mechanism of LOI at the single cell level under different perturbations.

MATERIALS AND METHODS

Cell culture

Primary cytotrophoblasts were extracted from human term placentas as described earlier (28). Primary cytotrophoblasts and the placental trophoblast cell line HTR-8/SV neo (HTR8) (29) were cultured at 37°C in DMEM and RPMI 1640 media (Gibco), respectively, supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen).

Cell treatment

HTR8 cells were treated separately with 0.5 μM AZA or 20 nM TSA. Fresh cell media and antibiotics were added every 24 h and treated cells were collected after 24 and 48 h.

Cell synchronization

HTR8 cells were synchronized at the G_1/S border using double treatment of thymidine as described earlier (30). Briefly, 2 mM of thymidine was added in growing cells for 12 h to accumulate the majority of the cells at G_1/S. Thymidine was replaced by fresh media for 12 h and was added back to the media for 12 h. Finally, the thymidine was removed and cells were collected every 2 h for up to 8 h. Cell synchronization was validated by FACS using a LSRII system (BD Bioscience) to separate treated cells stained by Vybrant®DyeCycle™ violet (Invitrogen) following standard protocols.

Nucleic acid extraction and cDNA synthesis

Nucleic acids extraction, DNase treatment and conversion of total RNA to single-stranded cDNA were performed as described earlier (8).

Quantitative PCR of total RNAs

cDNAs were amplified with gene-specific primers. The primers are listed in Supplementary Table S1. The expression levels of *PLAGL1*, *ZNF331* and *ACTB* genes were determined by quantitative PCR (qPCR) using the LightCycler480™ (Roche). All qPCR assays were carried out in triplicate in a reaction containing: buffer (50 mM Tris–OH + HCl, pH 7.5; 50 mM KOAc; 2% glycerol, 0.1 mg/ml BSA); 4 mM $Mg(OAc)_2$; 0.2 mM each dNTPs (dUTP replacing dTTP); 0.2 μM primers; 0.25× SYBR Green (Invitrogen); 5 U/μl AmpliTaq Gold (Applied Biosystems); 20 ng single-stranded cDNA template; final volume 20 μl. Cycling conditions for all genes were: 95.0°C for 10 min, followed by 50 cycles of 95.0°C for 30 s, 65.0°C for 30 s and 72.0°C for 30 s. The crossing point, Cp, was automatically calculated from the amplification curve without human intervention.

Single cell sorting

HTR8 cells and primary cytotrophoblasts were sorted directly into 384-well PCR plates as described earlier (21). Briefly, single cells were sorted into 384-well PCR plates (Roche) using the MoFlo high-speed cell sorter. Each well contained 5 μl cell lysis buffer [4 mM magnesium acetate (Sigma), 0.05% NP40 (Sigma), 0.8 U/μl Protector RNAse Inhibitor (Roche Applied Sciences)]. After sorting, the plates were immediately placed on dry ice and stored at −70°C for future use.

LOI assay and measurements on total RNA and single cells

LOI is a measurement of expression of the silenced allele, which may be calculated as:

$$LOI = 2^{-|\Delta Cp|}$$

where the $|\Delta Cp|$ refers to the absolute difference between the allele-specific Cp values on cDNA level corrected for the specificity of the allele-specific PCR (8).

The conditions for the measurement of the LOI levels in total RNA level have been described elsewhere (8). The sequences of the primers used for the amplification of the area bracketing the readout polymorphism and the quantitative allele-specific PCR (qASPCR) are listed in Supplementary Table S1. The SNP reference numbers

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for *PLAGL1* and *ZNF331* are rs9373409 and rs8109631, respectively.

To measure the levels of LOI at the single cell level, a two-step hemi-nested RT-PCR protocol was carried out. For the first RT-PCR step, an aliquot of 5 µl of 2xAccuRT PCR reaction mix [2xAccuRT buffer, 4 mM magnesium acetate, 0.2 µM primer sets, control oligonucleotides (2000 copies/well for *PLAGL1* or 200 copies/well for *ZNF331*; the sequences of the control oligonucleotides are given in Supplementary Table S1), 0.2 mM each dNTP (dUTP) and 0.375 U/µl AccuRT with aptamer (an aptamer-based hot-start, magnesium-activated thermostable DNA polymerase kindly provided by Dr Tom Myers of Roche Molecular Systems)] was added into each well of a cell-sorted PCR plate. The cycling conditions were: 65.0°C for 30 min (reverse transcription), followed by 15 cycles of 95.0°C for 15 s and 60.0°C for 50 s. For the second PCR step, 2 µl of the PCR products from the first PCR were mixed with 8 µl of PCR reaction mix [2 × Lightcycler Probe Master Mix, 0.2 µM final concentrations of allele-specific primer sets and 0.1 µM final concentration of Roche LNA probe]. All the reactions were run in duplicate. The sequences of the primers used for the qASPCR are listed in Supplementary Table S1. We used the Roche LNA probes #74 and #50 for *PLAGL1* and *ZNF331* genes, respectively. The cycling conditions were: 95°C for 10 min, followed by 50 cycles of 95°C for 10 s, 65°C for 20 s and 72°C for 10 s.

Accuracy of the LOI measurements

The error of our LOI measurements was determined by performing the qASPCR assay using serial dilutions of a genomic DNA mixture from two homozygous individuals for the *PLAGL1* SNP (rs9373409, A/G). The DNA mix contained G:A copies in a 9:1 ratio (10% LOI). Genomic DNA was diluted from 6000 copies to 6 copies per reaction. All the reactions were repeated six times.

Statistical analysis

The statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). The Fisher exact test was used to determine the significance of the percentage of AZA-treated cells exhibiting LOI compared with the untreated control. The chi-square test was utilized to compare the LOI percentage and the expression of *PLAGL1* in RNA from treated versus untreated cells. The mean and variance of LOI for cells exhibiting LOI was obtained by bootstrapping using R version 2.90. The simulation of the distributions of LOI in the all-or-none LOI and gradient LOI models was computed in R version 2.90. The Kolmogorov–Smirnov test for comparison of the experimental and simulated distributions was also performed in R version 2.90.

Model

The model proposes that each allele of a gene at a specific time point can be either in an active state, during which transcription is very efficient, or an inactive state, in which transcription is hindered. The synthesized mRNA is proposed to follow an exponential decay. The amount of total mRNA from each allele (denoted as A1 or A2) at a certain time t_i is given by

$$A1 = \left[\sum_{t_1=0}^{t_1=t_i} p(t_1,\lambda_1)e^{\left[\frac{t_1-t_i}{k}\right]}\rho_e\right]\rho_m$$

$$A2 = \left[\sum_{t_2=0}^{t_2=t_i} p(t_2,\lambda_2)e^{\left[\frac{t_2-t_i}{k}\right]}\rho_e\right]\rho_m$$

where $p(t, \lambda)$ is a hypergeometric random function determining the transcription state at time t: 1 = active or 0 = inactive with the pulsing frequency λ; ρ_e is a Gaussian random variable from 0% to 100% for the transcription efficiency of each pulse; k is the parameter to control the mRNA exponential decay rate; ρ_m is a Gaussian random variable to estimate the measurement error. We set $\Delta Cp = 0.5$ as the SD of ρ_m.

The simplest simulation assumed that every pulse gave 100% transcription efficiency so that $\rho_e = 1$. For the all-or-none LOI model, 10% of the cells were set to follow transcriptional pulsing on both alleles with pulsing frequency $\lambda_1 = \lambda_2 = 0.2$ and 90% of the cells to exhibit pulsing only for the major allele with pulsing frequency $\lambda_1 = 0.2$. For the partial LOI model, we set the minor allele pulsing frequency to be $\lambda_2 = 0.02$, 10-fold less than the pulsing frequency of the major allele. Using the equations above, we carried out simulations of 200 pulsing steps in 2000 cells.

Furthermore, additional simulations were carried out with consideration of transcription efficiency ρ_e as a Gaussian random variable at the single cell level (Supplementary Figure S1A) or with consideration of only the cells in the top 25% of total mRNA copies (Supplementary Figure S1B). Both simulations showed similar distributions to that seen for the simple model.

RESULTS

We tested the hypothesis that LOI was an all-or-none phenomenon at the single cell level using the maternally imprinted gene *PLAGL1*. Figure 1 illustrates the experimental design for studying the effect of treatment of single HTR8 trophoblasts with AZA. Because of cell-to-cell variability in gene expression, *PLAGL1* expression could only be measured in a subset of the cells (center panels). LOI in the *PLAGL1* gene in the expressing cells was measured by examining allele-specific expression in the presence and absence of AZA (right panels).

Genomic imprinting is regulated primarily by DNA methylation and histone modification. We treated the trophoblasts either with AZA, a DNMT1 inhibitor or TSA, an HDAC inhibitor, and looked at the impact of these drugs on the *PLAGL1* expression and LOI profile on total RNA. Table 1 shows the relative expression levels of *PLAGL1* and the percent LOI together with confidence limits for the allele-specific PCR triplicate measurements. There was a significant ($P < 0.01$) increase in expression after 2 days of AZA treatment and a significant ($P < 0.01$) increase in LOI after both 1 and 2 days of AZA treatment.

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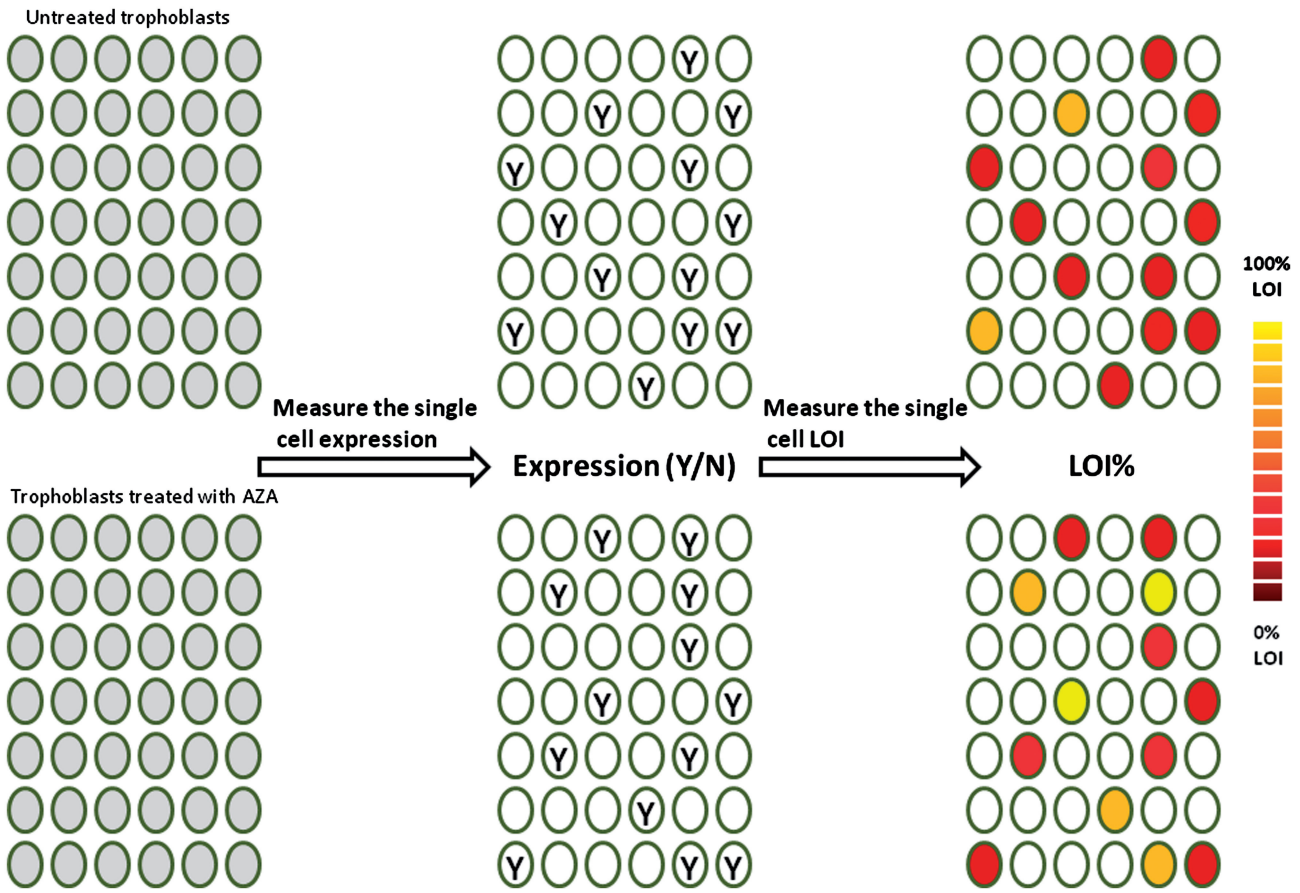

Figure 1. Illustration of heterogeneity in the expression and LOI of the *PLAGL1* gene. Individual trophoblasts (shown as ovals), either nontreated (upper panel) or treated with AZA (lower panel) were tested for the expression level of the *PLAGL1* gene. We detected *PLAGL1* expression only in a subset of the cells (labeled with Y). The cells expressing *PLAGL1* showed different LOI levels [shown as a color gradient from dark red (0% LOI) to light yellow (100% LOI), with a numerical bias toward LOI].

Table 1. LOI% and expression of *PLAGL1* on the total RNA level

	LOI% (low95%, up95%)	Relative expression[a] (× 1000) (low95%, up95%)
Without treatment		
Primary cytotrophoblasts/AG	3.4 (2.8, 4.1)	
HTR8/AG	2.3 (0.4, 4.2)	2.1 (1.6, 2.7)
AZA treatment of HTR8/AG		
1 day	8.4 (5.4, 12.9)**	3.4 (2.8, 4.2)*
2 days	12.7 (9.8, 16.4)**	6.0 (4.9, 7.3)**
TSA treatment of HTR8/AG		
1 day	2.5 (2.0, 3.0)	3.8 (2.4, 6.0)
2 days	4.4 (3.9, 4.9)*	3.2 (2.5, 4.0)

[a]Normalized against *ACTB*.
Treated versus untreated (chi-square): $^{**}P < 0.01$; $^{*}P < 0.1$.

TSA treatment resulted in no significant changes in expression or LOI.

Single cell measurements are presented in Figure 2. Figure 2A and B present measurement controls for primary cytotrophoblasts from individuals homozygous for the two alleles of the *PLAGL1* readout polymorphism. Because the LOI measurement method cannot detect LOI in readout polymorphism homozygotes, measured LOI must reflect allele-specific PCR measurement error. All their calculated LOI values were between 0% and 35%. To exclude all contributions from monoallelic expressing cells, we present the distribution of heterozygous cells exhibiting LOI within the range of 35–100%. The means and the variances for the distributions were computed by a bootstrapping method. We found that the mean *PLAGL1* LOI measurements of the AZA treated cells at 0, 1 and 2 days were 87%, 97.2% and 92.3%, respectively, while the SDs were 7.4%, 7.3% and 5.8%, respectively. To explore possible bias in the 35% cutoff, we repeated the same analyses using cutoffs of 10 and 20% (Supplementary Table S2). For all the AZA-treated samples, the mean LOI with each cutoff was centered at ~100% with SDs of 5–9%.

Figure 2C depicts the analysis of LOI for *ZNF331*, which is not imprinted in HTR8 cells (31), and whose expression was between 2- and 4-fold greater than that of *PLAGL1*. The mean LOI and standard deviation of the mean for the nonimprinted gene *ZNF331* were 98.6% and 2.2%, respectively. The distributions of LOI measured for both genes in cells within the selected range were centered at ~100% LOI.

The PCR reaction for *PLAGL1* was reproducibly able to detect six copies of duplex DNA template

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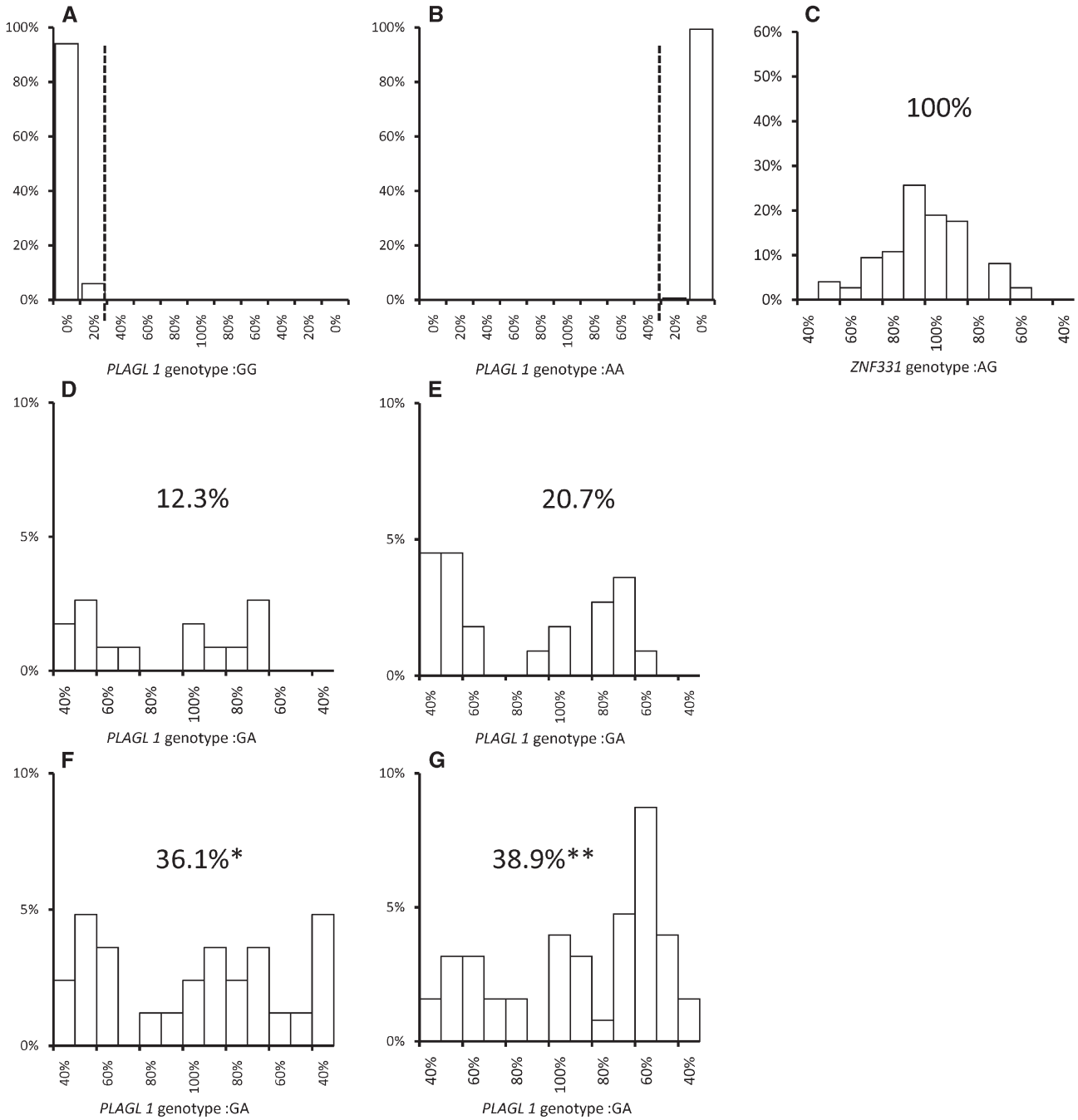

Figure 2. Single cell LOI distributions of *PLAGL1* and *ZNF331* in human trophoblasts. Histograms show the percentage of cells exhibiting a given LOI. In order to present data with positive and negative values of ΔCp based on a single allele and limit LOI to 0–100%, data with negative ΔCp were calculated using the alternative allele. (**A–B**) LOI for primary *PLAGL1* homozygous trophoblast controls. (**C**) LOI for ZNF331 heterozygotes (scale limited to 35–100% LOI). (**D–G**) LOI of *PLAGL1* heterozygotes with significant LOI (35–100%). (**D**) Primary cytotrophoblasts; (**E**) Untreated HTR8 cells. (**F**) HTR8 cells treated 1 day with AZA. (**G**) HTR8 cells treated 2 days with AZA. The Fisher's exact test was used to determine significance of the percentage of cells exhibiting LOI compared with the untreated control. (*$P < 0.05$; **$P < 0.01$).

(Supplementary Table S3). When examining *PLAGL1* at the single cell level, mRNA expression could only be detected in 40% of the cells. To test whether expression of *PLAGL1* was dependent on the cell cycle phase, we compared the *PLAGL1* expression levels between cells with no synchronization and synchronized to G_1/S phase. The synchronization was confirmed by FACS analysis. We found that there was no significant difference at the expression levels at any time points ($P = 0.51$, 0.41, 0.77, 0.54 for 2, 4, 6 and 8 h, respectively) after synchronization. Thus, the results in Figure 2D–G were limited to cells expressing mRNA above the limit of detection. Figure 2D depicts a LOI histogram for primary cytotrophoblasts. Although the distribution of cells exhibiting

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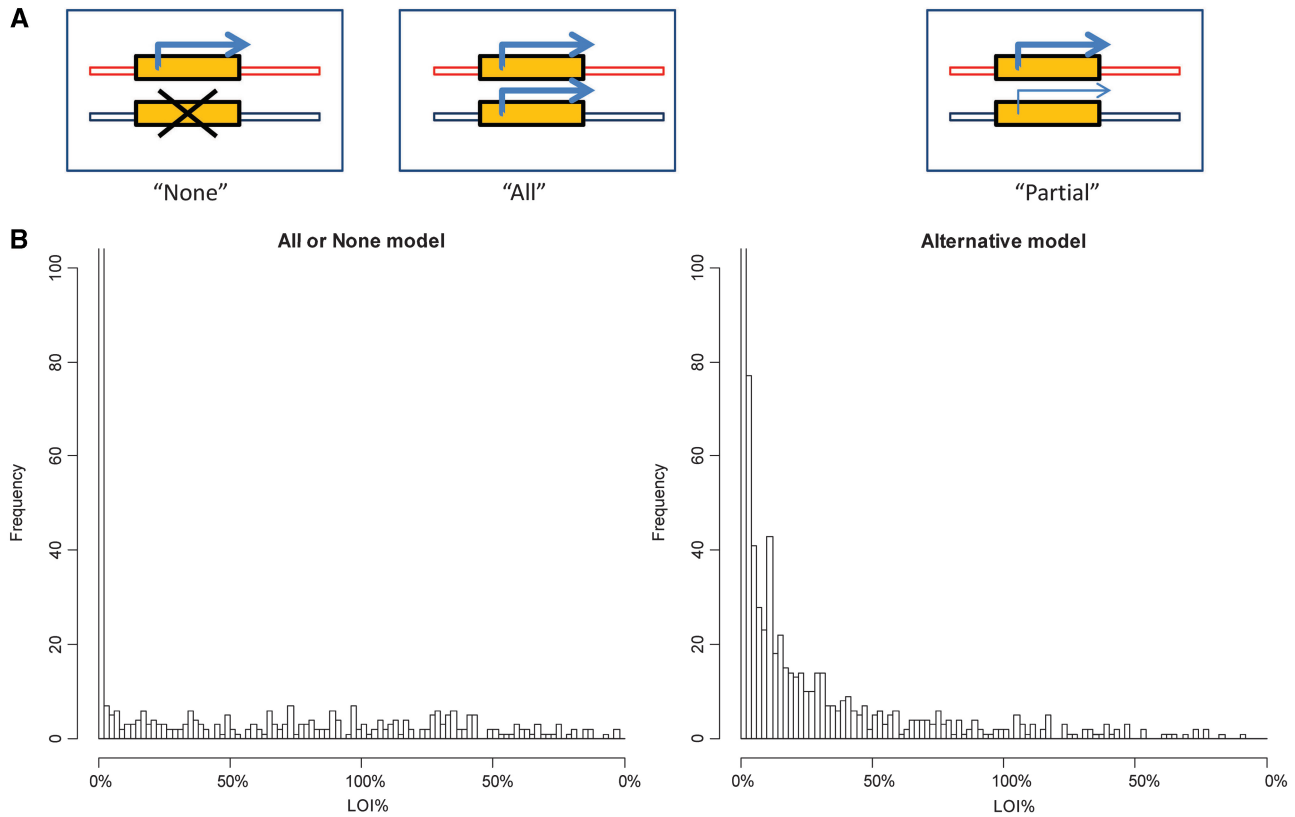

Figure 3. Models for the distributions of LOI in single cells. Simulations of single cell LOI distributions in 2000 cells were presented as histograms of cell counts with different percentage of LOI. The format for data presentation is the same as in Figure 2. (**A**) In the all-or-none model, we defined two distinct populations of cells: fully imprinted or having completely lost imprinting in the minor allele. In the partial LOI model, the transcription of the minor allele is less efficient than the major allele in each cell. (**B**) Simulated LOI distributions in single cells for each model (left side: all-or-none model; right side: partial LOI model).

LOI was wider than the distribution seen in Figure 2C, the results still suggested a distribution centered at ~100% LOI.

Similar to the primary cytotrophoblasts, untreated HTR8 cells showed a similar wide distribution of LOI (Figure 2E). To follow up on the LOI results seen in Table 1, HTR8 cells were treated with AZA for 1 or 2 days. The percentage of cells exhibiting LOI increased significantly ($P < 0.05$ and $P < 0.01$ at 1 and 2 days, respectively), while the distribution remained wide and centered at ~100% LOI (Figure 2F and G). This distribution is consistent with our hypothesis that LOI may occur by an all-or-none process.

We examined two possible models for the interpretation of the single cell data. The first is the all-or-none LOI model during which cells either are fully imprinted or have completely lost their imprinting; the second is the partial LOI model where the silenced allele exhibits incomplete activation (Figure 3A). In order to distinguish between the models, we developed a mathematical model based on transcriptional pulsing from the two alleles, which simulated the variations of the mRNA synthesis at the single cell level (19). Simulations for both models (Figure 3B) used the equations described in Materials and Methods section. The shapes of the computed distributions were independent of pulse size, threshold for detection or PCR error (Supplementary Figure S1). The distribution of LOI observed in our experiments (Figure 2D–G) fit the all-or-none LOI model (left side of Figure 2B). The Kolmogorov–Smirnov test showed a statistically significant difference between experiment and simulation based on the alternative model ($P < 0.05$ for each of 100 simulations), but no significant difference based on the all-or-none model ($P > 0.05$ for each of 100 simulations).

DISCUSSION

We observed a low but significant level of LOI in both primary cytotrophoblasts and the cell line HTR8 (Table 1). In order to examine the mechanism of LOI, we tested the effects of two drugs that have been shown to affect epigenetic silencing. TSA affects histone acetylation and was previously shown to increase *PLAGL1* in cancer cell lines (14). Our results indicated only a small effect on expression, suggesting that regulation of *PLAGL1* by histone acetylation is less important in placental trophoblasts. In contrast, treatment with the methylation inhibitor AZA substantially increased both expression and LOI.

If LOI were a function of the degree of methylation, this LOI could reflect heterogeneity in methylation among

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individual cells leading to cells with different degrees of LOI. We hypothesized, however, that LOI was an all-or-none phenomenon, with LOI reflecting only the fraction of cells expressing both alleles. Testing of this hypothesis requires a functional assay of single cell LOI based on transcriptional profiling.

We examined the effect of AZA treatment on expression and LOI at the single cell level. *PLAGL1* was expressed at low levels (~0.2% of *ACTB* expression levels, see Table 1), with expression unaffected by synchronization of the cells. Expression increased with AZA treatment. Our single cell measurements showed highly heterogeneous LOI distributions in both human primary cytotrophoblasts and HTR8 cells. The AZA treatment increased the number of cells exhibiting high LOI, while the heterogeneity among single cells remained the same. The median LOI remained close to 100%, consistent with our hypothesis that LOI was an all-or-none phenomenon. It should be noted that a process with many steps (e.g. loss of methylation at individual sites) would be consistent with all-or-none behavior if there is a one rate-determining step that governs the switch from imprinted to nonimprinted expression.

We examined the possibility that the PCR reaction contributed significantly to the wide distribution in LOI seen at the single cell level. However, the rise in the variance with serial dilution of template could be accounted for by the expected variability in pipetting small numbers of molecules. Thus, we proposed that the large variation in single cell LOI measurements reflected the stochastic nature in expression between the two alleles and among the single cells. *ZNF331* (Figure 2C), which is expressed at a 2- to 4-fold higher level in total RNA than *PLAGL1*, was detectable in all the cells yet showed significant cell-to-cell LOI variation. The fact that *PLAGL1* mRNA levels in 60% of the cells were below the detection limit suggested an even greater cell-to-cell variation in expression, possibly due to transcriptional pulsing (32). Herein, we proposed a transcription pulsing model to show that transcriptional pulsing could also contribute to chromosome to chromosome variation in expression which would be reflected in a wide distribution of LOI among cells that are expressing both alleles. Stochastic expression by transcriptional pulsing will not affect the observed mean LOI at 100%, which is the important parameter for supporting the all-or-none hypothesis for LOI for *PLAGL1* in trophoblasts. All-or-none LOI leads to a second distinct cell population which could have a selective advantage, leading to widespread LOI in normal tissues, such as the placenta (8) or in neoplastic cells (11).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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