Known unknowns for allele-specific expression and genomic imprinting effects
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Abstract
Recent studies have provided evidence for non-canonical imprinting effects that are associated with allele-specific expression biases at the tissue level in mice. These imprinting effects have features that are distinct from canonical imprinting effects that involve allele silencing. Here, I discuss some of the evidence for non-canonical imprinting effects in the context of random X-inactivation and epigenetic allele-specific expression effects on the autosomes. I propose several mechanisms that may underlie non-canonical imprinting effects and outline future directions and approaches to study these effects at the cellular level in vivo. The growing evidence for complex allele-specific expression effects that are cell- and developmental stage-specific has opened a new frontier for study. Currently, the function of these effects and the underlying regulatory mechanisms are largely unknown.

Introduction
Allele-specific expression (ASE) effects that arise due to epigenetic regulatory factors have previously been thought to be relatively rare in the genome. For example, the ASE effects associated with clusters of imprinted genes, olfactory receptors, protocadherins, and random X-inactivation have been considered exceptions to the rules of Mendelian inheritance. However, as I review below, new genomics-based approaches suggest that complex and tissue-specific ASE effects are prevalent in the genomes of mice and humans. Currently, these phenomena are both functionally and mechanistically poorly understood. Some of these ASE effects have been classified as random, whereas others have been classified as imprinting effects. Here, I discuss recent findings that challenge the concept of randomness in some cases and that suggest that complex parent-of-origin effects can influence ASE in a manner that has features distinct from those of canonical genomic imprinting. These new studies suggest that there are complex ASE effects that differ according to cell type and developmental stage and that exhibit variance between different individuals. In some cases, these effects appear to be influenced by the parental origin of the allele. I highlight new findings that are changing our understanding of allele-specific gene expression effects, and present a potential opportunity for the development of new therapeutic strategies that activate healthy alleles or silence mutated alleles at specific loci [1].

Defining allele-specific gene expression effects
I define ASE effects to include any phenomenon that results in differences in the relative expression of a maternal versus paternal allele (Figure 1). These effects potentially encompass (a) allele-specific silencing effects, in which one allele is completely silent; (b) ASE biases, in which a significantly higher level of expression arises from one allele versus the other; and (c) allele-specific isoforms, in which differences exist in the relative expression of specific transcripts from one allele versus the other. Each of these ASE effects can be further subcategorized depending upon whether the effects vary by cell type and developmental stage and between individuals. Furthermore, in each of these contexts, a parent-of-origin effect arises whenever a significant bias to
Figure 1. Subtypes of allele-specific expression effects

 Allele-specific expression effects could potentially take on four basic forms that involve (1) equal expression of the maternal (red) and paternal (blue) alleles (biallelic), (2) complete silencing of one allele (monoallelic), (3) a bias to express either the maternal or paternal allele at a higher level (allele-specific bias), or (4) the expression of different isoforms from the maternal versus the paternal allele (allele-specific isoforms).

express the maternal or paternal allele exists across a population of individuals. These categories provide testable models to explore the concept of randomness in ASE effects, as I discuss below.

Parent-of-origin effects and random X-inactivation

X-inactivation in female somatic tissues is considered by many to be a classic example of a random monoallelic effect because either the maternally (Xm) or paternally (Xp) inherited X chromosome is randomly inactivated in each cell [2]. The decision to express the Xm or Xp is typically thought to be a stochastic process that occurs in precursor cells, giving rise to a variegated pattern of Xm- and Xp-expressing cells in mature female somatic tissues. However, if X-inactivation is random in all cases, a problem appears to arise for X-linked imprinted genes. X-linked imprinting effects that act at the gene level and that result in the differential expression of specific genes on the Xm versus the Xp have been defined in mice. These effects have been shown to be both developmental stage- and tissue-specific. It is unclear how such effects could be functional and selected for if X-inactivation is highly variable between individuals. Even within litters, extreme variation was detected such that some littermates express primarily the Xp, whereas others express primarily the Xm. In their analysis, they found entire regions of the brain or other tissues that were populated primarily by cells that chose one X over the other. This extremely variegated pattern seems at odds with functional roles for X-linked imprinting would be similar between different individuals. However, an elegant new study by Wu and colleagues [7] demonstrates that this is not the case. The authors used an X-linked reporter system in combination with cell type-specific Cre drivers to systematically analyze X-chromosome inactivation patterns in different tissues and cell types. They conclude that X-inactivation is patchy and random between individuals.

One potential outcome from random X-inactivation could be that female tissues are composed of an equal number of Xm- and Xp-expressing cells that are relatively evenly distributed (Figure 2A). In this case, each individual would have a substantial Xm and Xp contribution to a given structure, and thus the effects of Xlr imprinting would be similar between different individuals. However, some studies have proposed a potential mechanism for X-linked parent-of-origin effects on behavior [5]. Imprinting for the Xlr genes is specific to brain region, tissue, and the developmental stage [6]. For example, Xlr3b exhibits biallelic expression (equal expression from the Xm and Xp) in the midbrain and hindbrain at E14.5 but is paternally repressed in these regions after birth. In the liver, the paternal Xlr3b allele is strongly repressed, but the paternal Xlr4b allele exhibits weak repression (a maternal bias). In the brain, the paternal allele for both genes is strongly repressed and expression arises exclusively from the Xm. Thus, X-linked imprinting effects at the gene level can be highly tissue- and developmental stage-specific. How can these effects be functionally meaningful and selected for if the pattern of X-inactivation is patchy and random between individuals?
Figure 2. The relationship between X-linked imprinted genes and random X-inactivation patterns

(A) If random X-inactivation in female tissues resulted in an equal number of Xm and Xp expressing cells, then X-linked imprinted genes like Xlr3b would have a consistent effect on different individuals. However, this pattern does not occur. (B) The study by Wu and colleagues [7] uncovered highly variable X-inactivation patterns in different tissues and individuals. This random and variegated pattern results in some brain regions or tissues being primarily populated by Xm+ cells in one individual and Xp+ cells in another. This pattern would introduce extreme variance for the effects of X-linked imprinted genes, like Xlr3b, in a population of individuals. (C) A parent-of-origin effect that biases X-inactivation toward the Xm could potentially buffer the variance associated with random X-inactivation, such that a minimum contribution of Xm+ cells exists in tissues where Xlr3b imprinting functions, such as the brain. In this scenario, pronounced Xm biases would still arise, perhaps increasing the impact of Xlr3b imprinting effects (individual #1), but the Xm inactivation bias would buffer against extreme Xp biases that could negate the effects of Xlr3b imprinting (individual #2).
parent-of-origin effects that bias X-inactivation toward the expression of the Xm in the brain, nor did they find any bias in any of the other cell types or tissues they analyzed. However, others have reported these effects, as described below.

Genomic imprinting effects that influence X-inactivation are known to occur in mice in the zygote, when the Xp is specifically silenced because of a paternally inherited imprint. This imprinting effect is maintained in extra-embryonic tissues. In contrast, at the epiblast stage in the embryo proper, the Xp is reactivated and random X-inactivation occurs [8]. However, using an RNA-Seq-based approach in F1 hybrid mice, we and others detected an overall bias to express the Xm over the Xp in the adult female mouse brain [9,10]. These independent studies used different mouse strains, suggesting that the effect is reproducible in multiple genetic backgrounds. Our study found further support for the Xm bias in the brain by using an X-linked enhanced green fluorescent protein (EGFP) transgenic reporter line [9]. These results support earlier work that first uncovered parent-of-origin effects influencing X-inactivation in the embryo [11-14], and the results were recently replicated [15].

Interestingly, parent-of-origin effects that bias X-inactivation patterns can be highly cell type-specific. A recent study of mouse mammary tissues revealed that the Xp is preferentially expressed over the Xm in mouse alveolar cells [16]. The authors found that a paternally inherited deletion of the X-linked gene Rnf12, a really interesting new gene (RING) finger LIM domain-interacting protein, inhibits alveolar differentiation and milk production in pregnant female offspring. No effect was observed when the mutated allele was maternally inherited. Using the same X-linked EGFP reporter line indicated above as well as an analysis of X-linked ASE patterns in hybrid mice, the authors were able to demonstrate that the Xp is preferentially expressed in alveolar cells, thus explaining the parent-of-origin effect. In summary, parent-of-origin effects that bias random X-inactivation in mice have been identified by multiple studies, but it is not yet clear how to reconcile these observations with the highly variegated and random X-inactivation patterns described by Wu and colleagues. I propose that an important study of different alleles of the X-inactivation control element (Xce) in mice provides some possible insights [15].

This recent study of the Xce defined the relative strength and identity of six different Xce alleles (Xce°, Xceb, Xced, Xce, and Xced) through genetic crosses conducted with 10 different mouse strains. The authors found an Xm expression bias in most crosses and propose that the parent-of-origin effects that influence X-inactivation are encoded in an undefined region outside of the Xce. However, the authors also discovered that parent-of-origin effects on X-inactivation do not occur in crosses involving the Xce° and Xceb alleles. The weak Xce° allele is present in the 129/Sv mouse strain, and the Xceb allele is present in C57BL/6J mice. Importantly, 129/Sv and C57BL/6J hybrids are commonly used for gene targeting and the generation of transgenic mice. Indeed, the background for the targeted transgenic reporter lines generated by Wu and colleagues to map X-inactivation patterns is reported as a 129/Sv x C57BL/6J mixed background. Thus, the lack of parent-of-origin effects on X-inactivation in these lines may be related to the genetic background of the mice.

Further studies focusing on the relationship between the genetic background and parent-of-origin effects influencing X-inactivation are warranted. It is of interest to determine whether Mus subspecies that lack parent-of-origin influences on X-inactivation also lack imprinting effects for genes, such as the Xlr genes. If a relationship exists between these two phenomena, then the problem of how X-linked imprinted genes evolved in the face of seemingly stochastic and highly variegated inactivation patterns might be resolved by the existence of a parental bias that buffers random X-inactivation and provides a stable background for X-linked imprinted genes, such as the Xlr genes (Figure 2C). A major point that emerges from the studies presented above is that parent-of-origin effects influence random X-inactivation in mice—the prototypical example of a random allelic effect—and I propose a potential functional role for this effect. Next, I discuss the potential for parent-of-origin effects to influence random monoallelic expression effects on the autosomes, which are now known to be widespread in mice and humans.

**Autosomal random monoallelic expression effects**

Widespread random monoallelic expression (RMAE) effects influence approximately 20% of genes in the genome and were first described by Gimelbrant and colleagues [17] in clonally derived human lymphoblastoid cell lines. Subsequently, numerous studies have provided supporting evidence for the prevalence of RMAE effects in cell lines derived from mice and humans [18–23]. Furthermore, Nag and colleagues [24] defined an epigenetic signature for genes that exhibit random monoallelic effects, which consists of the simultaneous appearance of chromatin marks for active transcription (H3K36me3) and transcriptional silencing (H3K27me3) in a gene body. This signature is present for 20% of ubiquitously expressed genes and 30% of tissues-specific genes in humans. RMAE effects are stable over multiple cell divisions and distinct from other forms of ASE effects. These pioneering studies have fundamentally changed our understanding of gene
expression. However, little is known about the nature of these effects in vivo or the potential for parent-of-origin effects to influence random ASE.

Typically, studies of RMAE effects involve deriving five to eight clonal cell lines that are propagated in vitro. Genes exhibiting RMAE effects have been defined as those that exhibit variance between different clones in terms of the number of alleles and identity of the allele that is expressed for a given gene [25]. The apparently stochastic nature of this outcome in clones from the same cell type propagated under similar culture conditions presents a challenge in terms of defining the functional significance of the effects and the underlying mechanisms. Two recent studies of RMAE effects in embryonic stem cells (ESCs) that were differentiated into neural precursor cells (NPCs) revealed that the number of genes exhibiting RMAE effects increases by over fivefold as a consequence of differentiation [20,22]. Thus, changes to ASE effects are associated with cellular lineage commitment, and RMAE effects appear to be highly tissue- and cell type-specific. Interestingly, in addition to the analysis of cell lines, the study by Gendrel and colleagues [22] analyzed allele expression effects in vivo by using in situ probes targeting nascent RNAs. The authors report that all RMAE candidate genes analyzed exhibit monoallelic expression in at least a subpopulation of cells in vivo. Of note, many biallelic control genes also exhibited monoallelic expression in a substantial subpopulation of cells in vivo (approximately 20% to 40% of cells). The relative proportions of monoallelic and biallelic cells varied in different tissues for different genes. For example, Eya1 exhibits monoallelic expression in essentially all expressing cells in the adult lens but in only 50% of expressing cells in the developing kidney. Overall, these early studies demonstrate that ASE effects are common in vitro and in vivo.

Given the small number of clones examined and the approaches used in these studies, it has been difficult to determine whether any RMAE genes exhibit a significant bias favoring the paternal versus the maternal allele when an allele-specific silencing event occurs. In vivo, the parental bias could arise in a highly specific cell type, similar to the Xp bias that occurs in mouse alveolar cells (see above). In a recent genome-wide study of RMAE effects in pre-B lymphoblastoid cell lines from hybrid mice, the authors provided evidence for allele “skewing” effects, in which the maternal and paternal alleles are unequal in terms of their probability of being silenced when a monoallelic expression event occurs [18]. The study examined RMAE genes that had more than one clone in which an allele-specific silencing event occurred and counted the number of times the same allele was selected. The study did not examine sufficient clones to statistically define skewing effects at the gene level; however, the authors demonstrated that skewing effects in their data are consistent with one of two models. Either 40% of random monoallelic genes exhibit a strict preference for one allele, or 80% of random monoallelic genes prefer one parental allele 85% of the time. Similarly, in their study of RMAE effects in NPCs, Gendrel and colleagues [22] noted that for many genes exhibiting monoallelic effects, one allele was preferentially expressed. Neither study was able to determine whether the skewing effects involve a parent-of-origin effect due to the absence of a reciprocal cross and the limited number of clones examined.

In summary, we know little about whether parent-of-origin effects can influence RMAE effects on the autosomes. Rather than advocating for more in vitro studies, I believe that the potential for parent-of-origin influences to bias ASE effects is better studied in vivo (at least in mice). Parent-of-origin effects that bias or silence the expression of one parental allele over the other in a subpopulation of cells only could emerge as allele-specific expression biases at the tissue level. Interestingly, some studies of genomic imprinting biases have detected these biases, as I detail below.

**Non-canonical autosomal imprinting effects**

Canonical genomic imprinting involves allele-specific silencing effects that do not vary between healthy individuals in mice [26,27]. Most imprinted genes reside in clusters, of which there are a total of 16 in the mouse. Canonical imprinting is mechanistically defined by the effects of differentially methylated regions (DMRs) in the genome that are located at specific sites, called imprinting control regions (ICRs) [26,27]. ICRs are cis-acting elements that regulate imprinting at nearby genes, resulting in the formation of imprinted gene clusters in most cases. In total, 55 DMRs have been identified in the mouse genome [28]. Maternally methylated DMRs are located at promoters and can regulate the expression of long non-coding RNAs that regulate imprinting effects for neighboring genes. Paternally methylated DMRs are located in intergenic regions and have been shown to regulate distinct promoter-enhancer interactions on maternal and paternal chromosomes. Several proteins that play essential roles in the establishment of DMRs in the parental gametes and the maintenance of these marks in the embryo have been defined and recently reviewed [26,27]. Importantly, all of the genes that exhibit the canonical imprinting effects I have detailed here have been identified in the mouse genome. However, although canonical imprinted genes exhibit allele silencing, a growing list of genes in the genome...
have been shown to exhibit biases to express either the maternal or paternal allele and much less is known about the frequency and nature of these effects.

An essay by Khatib first drew attention to the fact that some imprinted genes exhibit allele-specific expression biases rather than allele-silencing [29]. However, the phenomenon has largely been overlooked. Previously, we and others performed genome-wide screens for imprinting effects in brain [9,30–32] or placental tissues [33] by using RNA-Seq analyses of the transcriptome of reciprocal F1 hybrid mice. Single-nucleotide variants in the RNA-Seq data were used to distinguish expression from the maternally versus paternally inherited allele for each expressed gene. The novel “imprinted genes” uncovered in these studies typically involved significant maternal or paternal allele-specific expression biases rather than the canonical genomic imprinting effects defined above. For example, Casd1, Pde4d, Bcl2l1, Cobl, Ddc, Mrpl48, and Il18 were found to exhibit imprinting effects in the brain, and in all cases an allele-specific expression bias rather than allele silencing was found [9,30,31]. Here, I refer to these effects as non-canonical imprinting effects since the phenomenon involves allele expression biases rather than the allele silencing that is typically associated with canonical imprinting. In the case of Ddc, canonical imprinting occurs in the heart involving the expression of the paternal allele [34], whereas a non-canonical imprinting effect was found in the brain involving a bias to express the maternal allele [30]. The number of genes that exhibit non-canonical imprinting effects in the genome is currently debated. Some RNA-Seq-based surveys of imprinting have found very little evidence for novel imprinting effects [31,32], whereas others have suggested that the effects are more widespread [30,33]. The studies by Gregg and colleagues [9,30] in which many novel imprinting effects were uncovered have been shown to have a high false discovery rate, and the conclusion that many non-canonical imprinting effects exist in the genome has been challenged [35]. However, it is unlikely that published screens for non-canonical imprinting effects have reached saturation, particularly for effects that are similar in magnitude to the parent-of-origin effects that influence X-inactivation (Huang et al., unpublished data). The detection of the Xm bias in the female brain by RNA-Seq was achieved by pooling data across all X-linked genes [9,10]. These studies were not sufficiently powered to statistically detect the maternal bias for individual X-linked genes. Indeed, little attention has been paid to type II statistical error rates in published surveys of imprinting effects. Emerging studies that more effectively manage type I and type II statistical errors will provide important insights into the nature and prevalence of non-canonical imprinting effects. A new study of genomic imprinting effects in the mouse placenta, for example, suggests that non-canonical imprinting effects may influence the expression of many genes in this tissue [36].

In the above study by Finn and colleagues [36], the authors performed RNA-Seq to analyze ASE in F1 hybrid mice derived from reciprocal matings of Cast x C57BL/6J mice. Their study used large numbers of biological replicates (n = 11 to 12 for each cross) and an RNA-Seq-based approach called 3SEQ, which specifically targets the 3’ end of transcripts. The study revealed 78 novel imprinted genes in the placenta, some of which were independently validated by using pyrosequencing. All of these genes exhibit an allele-specific expression bias rather than canonical imprinting. A maternal bias was detected for 75 out of the 78 novel genes and beyond these high confidence candidates, the authors provide further evidence that hundreds of transcripts exhibit maternal expression biases in the E11.5 placenta. A concern is that these biases arise due to maternal decidual contamination; however, the authors performed multiple controls that suggest that this contamination is unlikely to explain their results. The widespread maternal effect was developmental stage- and tissue-specific, as it was not present in the yolk sac or the E17.5 placenta. Finally, the authors analyzed individual offspring from multiple litters in their study, which allowed them to discover that the maternal non-canonical imprinting effects in their data can vary according to litter. These results suggest that non-canonical imprinting effects are more widespread and dynamic in the placenta than was previously appreciated. These effects contrast sharply with the relatively stable and robust allele-silencing effects associated with canonical imprinted genes in somatic tissues.

The nature of non-canonical imprinting effects at the cellular level in vivo is unknown, and multiple models might explain the effects. One testable hypothesis is that some non-canonical imprinting effects reflect allele-specific silencing events in a subpopulation of cells. Under this model, a tissue is composed of a mosaic of cells in which some cells express both alleles for a given non-canonical imprinted gene and some express one specific parental allele (Figure 3A), or all cells exhibit monoallelic expression, but the decision to select the maternal or paternal allele is skewed such that a parental bias emerges at the level of a cell population (Figure 3B). The rationale for these models emerges from both the RMAE field and the imprinting field. Cell type-specific imprinting effects are known to occur. For example, Dlk1 is imprinted in most cells that express the gene in the
Figure 3. Potential parent-of-origin effects on allele-specific expression at the cellular level

(A) An allele-specific expression bias could emerge because of silencing of one parental allele in a subpopulation of cells. (B) A bias could emerge because of random monoallelic expression effects that are skewed toward the selection of one parental allele over the other. (C) A bias to express one parental allele over the other could exist in all or most cells expressing the non-canonically imprinted gene. (D) The non-canonical imprinting effect could arise at the gene level because of the expression of distinct and overlapping isoforms from the maternal versus the paternal allele, with one isoform expressed at a higher level.

This schematic illustrates different models by which non-canonical imprinting effects, which involve biases to express either the maternal or paternal allele at the tissue level (bar plots; red, maternal allele expression; blue, paternal allele expression), could emerge at the cellular level. (A) An allele-specific expression bias could emerge because of silencing of one parental allele in a subpopulation of cells. (B) A bias could emerge because of random monoallelic expression effects that are skewed toward the selection of one parental allele over the other. (C) A bias to express one parental allele over the other could exist in all or most cells expressing the non-canonically imprinted gene. (D) The non-canonical imprinting effect could arise at the gene level because of the expression of distinct and overlapping isoforms from the maternal versus the paternal allele, with one isoform expressed at a higher level.

The fact that ASE effects change during cellular differentiation [20,22] and that some canonical and non-canonical imprinting effects appear to change during differentiation [37] or development [30,36] suggests the possibility that allele-specific regulatory mechanisms may function to coordinate specific gene expression programs and influence cell fate decisions. A role for differential allelic regulation in cell fate decisions was previously observed for Nanog [48]. Furthermore, studies of RMAE effects have recently provided evidence that monoallelic expression effects are associated with reduced expression and could function to influence gene expression levels [22]; only a few genes that compensate for the loss of one allele have been identified [20]. Finally, one mechanistic model for non-canonical imprinting involves DMRs that are functional in a specific subpopulation of cells. However, a recent survey of DMRs in the brain found few novel DMRs outside of known imprinted gene clusters [28]. Thus, this mechanism is unlikely unless a cell type-specific analysis of DMRs is required to detect them. Interestingly, other mechanisms that influence ASE effects may exist. Studies by Gendrel and colleagues [22] or Eckersley-Maslin and colleagues [20] (or both) are known imprinted genes, such as Cdh11c, Airn, Sgce, Mcts2, and H13. Thus, in some cases, genes that exhibit RMAE effects in vitro exhibit imprinting effects in vivo.
of RMAE effects suggest that allele silencing might not require DNA methylation and that some other mechanism for stable allele-specific silencing remains to be defined [20,22,48].

**Outlook**

Genetic studies in mice clearly indicate that parent-of-origin effects influence several complex traits [49,50]. However, many mechanisms and details remain to be understood, and the function of imprinting is debated. Here, I have discussed some observations in the ASE field that provide evidence for non-canonical imprinting effects involving maternal or paternal biases that influence random X-inactivation and the allelic expression of some autosomal genes. It is likely that published RNA-Seq-based screens for imprinting are not saturated for the detection of non-canonical imprinting effects, and in the light of the discovery that ASE effects are widespread and arise in subpopulations of cells in vivo, a deeper investigation into the nature and function of non-canonical imprinting effects in different tissues and cell types in vivo is warranted. Several models might explain the detection of these effects at the tissue level, including maternally or paternally biased RMAE effects, overlapping transcripts, and cell type-specific imprinting. The recent developments in the ASE field have exposed many questions and opened an exciting frontier that is likely to advance our understanding of the genetic and epigenetic factors that influence phenotypes and disease susceptibility. An important question that I have not touched on is the possibility that ASE effects can be influenced by physiological and environmental factors acting on parents or offspring or both. A challenge for the field involves the development of in vivo approaches to further analyze allele-specific expression effects accurately in specific cell types in vivo, such as allele-specific in situ hybridization approaches that can discern the parental allele being expressed [51,52] or RNA-Seq-based methods that permit the study of ASE effects in specific cell populations [19,53-55]. Most enticing is the fact that the functions and regulatory mechanisms for many of these effects are currently unknown.

**Abbreviations**

ASE, allele-specific expression; DMRs, differentially methylated regions; EGFP, enhanced green fluorescent protein; ICR, imprinting control region; NPC, neural precursor cell; RMAE, random monoallelic expression; Xce, X-inactivation control element.

**Disclosures**

The author declares that he has no disclosures.

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