Journal of Mammalogy, 88(1):5–23, 2007

GENOMIC IMPRINTING AS A MECHANISM OF REPRODUCTIVE ISOLATION IN MAMMALS

PAUL B. VRANA*

Department of Biological Chemistry, University of California Irvine, Irvine, CA 92799, USA

Many traits exhibit nonequivalent effects upon maternal versus paternal inheritance. Such ''parent-of-origin'' effects may be caused by several mechanisms including sex chromosomes and maternal inheritance of mitochondrial DNA. Recently, a class of mammalian autosomal genes has emerged that shows expression of only 1 parental allele. This phenomenon has been termed ''genomic imprinting.'' Genomic imprinting is an epigenetic effect resulting from chromosomal marks established during gametogenesis. Such imprinted genes result in non-Mendelian inheritance patterns despite being located on autosomes. The chorioallantoic placenta and brain are prominent places of imprinted gene expression. Correspondingly, most imprinted genes appear to be involved in growth or behavior. Interspecific variation in which genes display genomic imprinting suggests that the process is under selection. There is also evidence for intraspecific variation in degree of imprinting of certain genes. Here I briefly review the current understanding of imprinting mechanisms and arguments for selection. The leading argument for positive selection of genomic imprinting is an extension of the concept of kin selection. Although this hypothesis remains controversial, the involvement of imprinted genes in placentation suggests a role in reproductive isolation. Interspecific hybrids in the cricetid genus *Peromyscus* exhibit parent-of-origin effects involving placental and somatic growth dysplasias. Female *P. maniculatus* crossed with male *P. polionotus* produce neonates smaller than either parental strain, with placentas half the parental size. Female *P. polionotus* crossed with male *P. maniculatus* produce dysmorphic overgrown embryos whose placentas average >2.5 times the mass of the parental strains. Hybrid dysgenesis in *Peromyscus* is affected by both the imprinting process and interactions among imprinted genes. I hypothesize that imprinted genes underlie multiple cases of reproductive isolation in the *P. maniculatus* species complex. Further, I suggest that such interactions have played a significant role in generating mammalian diversity. Finally, I examine the role of the environment in regulating genomic imprinting and argue that studying natural populations in wild-type habitats will be critical to understanding this phenomenon.

Key words: epigenetics, genomic imprinting, growth, kinship selection, parent-of-origin effects, *Peromyscus*, placenta, reproductive isolation

MAMMALIAN GROWTH AND PARENT-OF-ORIGIN EFFECTS

Eutherian growth control and the role of the placenta.—Mammals exhibit tremendous variation in morphology, behavior, natural history, and growth. Although much of mammalian growth is postnatal, substantial variation also is seen in prenatal growth (Ulijaszek 1998). Control of mammalian growth must be fairly tightly regulated within a species; the deleterious effects of both over- and undergrowth within a species are well documented. Individuals that are too small are less likely to survive past weaning due to intralitter competition. For example, overgrowth in humans is associated with heart failure and increased cancer risk, among other maladies (Gracia Bouthelier and Lapunzina 2005). Overgrowth also risks being maladaptive in terms of the organism's ecological niche. For example, being overly large might make an individual more vulnerable to predation or unable to procure sufficient food.

Eutherian mammals are obligate maternal parasites during early growth and development. That is, fetal nutrients are directly obtained from maternal tissues. In eutherians, a unique set of tissues form to mediate this process. Zygotic cells literally bore into maternal tissue, extract nutrients, and manipulate maternal physiology via paracrine hormones (Rinkenberger and Werb 2000). The major organ that performs these tasks (nutrient or waste transport and endocrine function) is the chorioallantoic placenta (Cross et al. 1994). Although all

* *Correspondent: pvrana@uci.edu*

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placentas arise from fusion of the chorionic and allantoic membranes and serve the same essential functions (Cross et al. 2003), eutherian placentas are as varied in morphology as the organisms that produce them (Carter and Enders 2004).

All eutherian placentas produce many factors thought to be critical for fetal growth. Targeted mutations (gene ''knockouts'') in laboratory strains of *Mus* have further revealed the dependence of the fetus on molecules produced by the placenta (Rossant and Cross 2001). A striking example involves a mutation created in the insulinlike growth factor 2 (*Igf2*) gene that reduced placental expression by approximately 10% (Constancia et al. 2002). Despite the remainder of the placenta and the embryo proper producing prodigious amounts of *Igf2*, the partial knockout animals were nearly as small as those with an earlier induced mutation that removed all *Igf2* expression from both placenta and associated embryo (DeChiara et al. 1990). The unusual thing about both *Igf2* mutations is that the growth retardation only occurs when young inherit the null allele from their father.

Genetic phenomena where a particular phenotype is dependent on whether the father or mother contributed the allele are termed ''parent-of-origin'' effects. Suppose there are 2 distinguishable varieties of an organism, A and B. Mendelian genetics presumes that the hybrid AB phenotype will be equivalent regardless of whether a female type A mates with a male type B or vice versa. In contrast, parent-of-origin effects occur whenever the outcome of ♀A × ♂B ≠ ♀B × ♂A. Many of these parent-of-origin effects were compiled in the volume *Mammalian Hybrids* (Gray 1972). Some cases were well documented, others merely suggestive. For example, a female tiger (*Panthera tigris*) crossed with a male lion (*Panthera leo*) produces offspring termed ligers. Ligers are larger than either parent species, often exceeding 3 m in length and 350 kg in mass (Morison et al. 2001). The offspring of the reciprocal cross (female lion × male tiger) are called tigons, and are typically smaller than either parent species.

Nonequivalence of parental genomes.—Intrinsic differences between mammalian maternal and paternal genomes were only demonstrated in the mid-1980s (Barton et al. 1984; McGrath and Solter 1984). Two research groups employed pronuclear transfer techniques to artificially create embryos whose genomes were completely maternally or paternally derived (Fig. 1). Such experiments are feasible because the male and female pronuclei are morphologically distinguishable shortly after fertilization. By removing the male pronucleus from such a recently fertilized zygote, and replacing it with another female pronucleus, gynogenetic embryos are produced. Alternatively, oocytes may be induced to divide and double the amount of their own DNA to form similar parthenogenotes. The 2 classes (gynogenotes and parthenogenotes) appear to be functionally equivalent in most cases.

Both groups observed failure of gynogenetic or parthenogenetic zygotes during preimplantation development. In particular, the extraembryonic tissues are poorly developed. Zygotes altered to contain 2 paternal pronuclei also died. In contrast to the gynogenetic phenotype, these so-called androgenotes produced little embryonic tissue per se. Rather, the androgenetic phenotype is an excess of extraembryonic tissues with poor development of the embryo proper.

A relatively common human disease termed hydatidiform mole is similarly characterized by excessive trophoblast (placental) tissue and the absence of a fetus. Several years before the pronuclear transfer experiments, researchers determined that hydatidiform moles are typically characterized by either lack of a maternal genome or by a 2:1 paternal : maternal genome ratio due to fertilization by multiple sperm (Wake et al. 1978). Together, examination of these data demonstrated that mammalian maternal and paternal genomes are not equivalent, and that both are needed in a 1:1 ratio for normal development.

Chimeric embryo experiments suggest that this parental nonequivalence is not limited to placental versus fetal development. Cells from (marked) androgenetic and gynogenetic zygotes may be aggregated with those of normal biparental embryos. If the proportion of uniparental cells is not too high, live births are possible. Androgenetic chimeras are significantly larger than nonchimeric littermates, whereas gynogenetic chimeras are smaller than the wild-type (Mann et al. 1990).

Chimeric animals also have been assessed to determine whether androgenetic or gynogenetic cells can contribute to all tissues. The 2 cell types show different distribution patterns in the chimeric animals. Androgenetic cells populate skeletal muscle and mesodermally derived tissues in general (Barton et al. 1991). In contrast, gynogenetic cells are predominantly found in the kidney, spleen, and central nervous system of chimeras (Fundele et al. 1990). Although androgenetic cells show relatively lower contributions to the brain, they are consistently found in certain areas such as the hypothalamus, septum, and preoptic area. Gynogenetic cells show a different distribution, contributing to the cortex and striatum (Keverne et al. 1996). Although providing valuable clues, the studies of chimeric embryos do not reveal what genes or proportion of the genome are involved in these effects.

Beechy, Cattanach, and colleagues took advantage of chromosomal translocations and breakages in laboratory *Mus* strains to further study these phenomena (Beechey et al. 1990). By breeding such mice, one can obtain offspring that have received both copies of a given chromosome (or a portion thereof) from 1 parent. This phenomenon is termed uniparental disomy. By comparing such disomic animals to wild-type animals, one can assess whether the maternal–paternal nonequivalence extends across the genome. Examination of disomy data suggested that the parent-of-origin effects are limited to a number of discrete genomic regions on 8 of the 19 *Mus* autosomes. Similar effects have been seen in humans with chromosomal translocations. Generally, the regions of the human genome exhibiting parent-of-origin effects corresponded to those identified in the *Mus* uniparental disomy studies.

A New Zealand–based group has continued to update the parent-of-origin effects list and maintains an Internet database (Appendix I; Morison et al. 2001). Many of the parent-of-origin effects listed involve growth and behavioral effects. Domesticated species of mammals predominate in these lists,

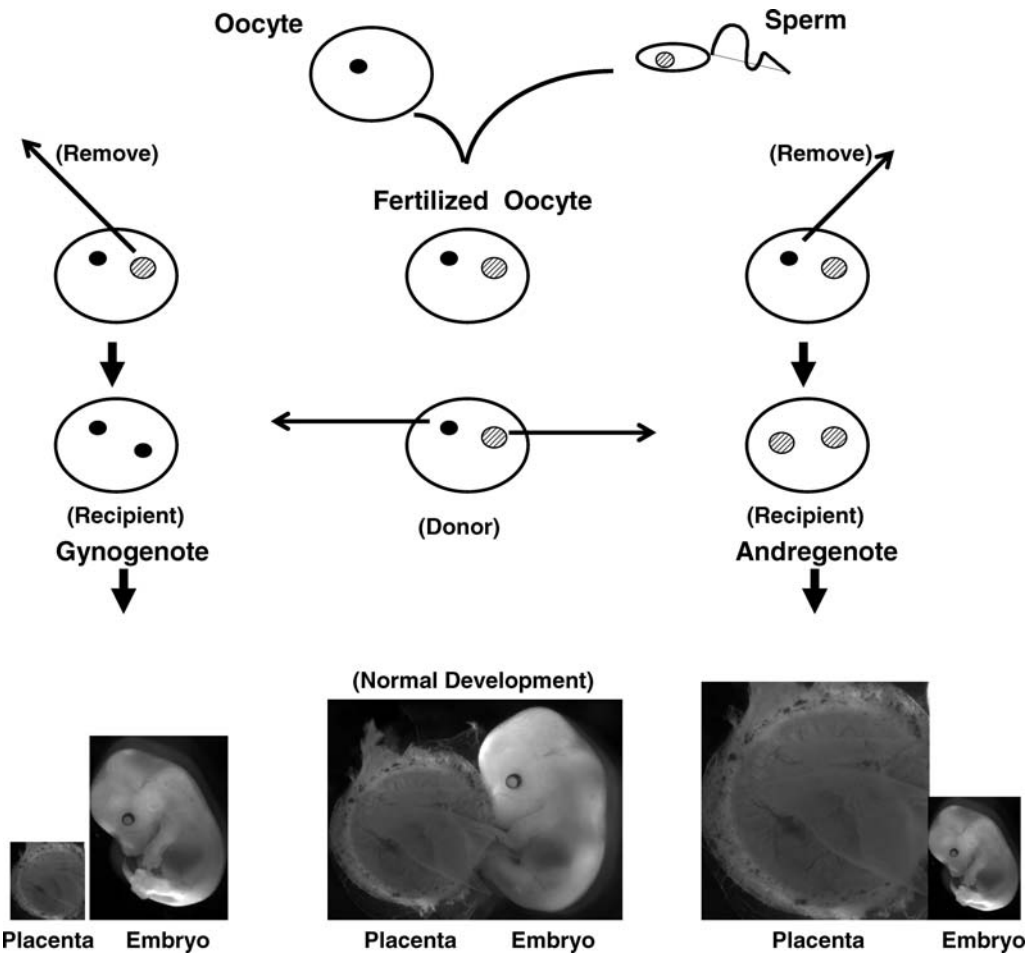

Fig. 1.—Schematic diagram of pronuclear transfer experiments. Filled circle = maternal pronucleus. Cross-hatched circle = paternal pronucleus. Photographs of placenta and embryo at bottom illustrate outcome of experiments in terms of relative growth (e.g., andregenotes exhibit placental overgrowth with little or no embryonic tissue). Actual morphologies vary. These experiments demonstrated the nonequivalence of the oocyte and sperm genomes, and suggested the existence of genes whose expression is dependent on parent of origin. They further suggested that these ''imprinted'' genes affect cell fate and growth of both the embryo and extraembryonic tissues. Photos courtesy of the author.

probably because of our greater knowledge of their genetics. For example, a domestic pig (*Sus scrofa*) variant that produces greater body mass upon paternal inheritance maps to the *Igf2* gene (Van Laere et al. 2003). Domestic sheep (*Ovis aries*) have a similar allele that shows increased muscle mass only in the hindquarters. The sheep variant, termed callipyge (*Cp*; Greek for ''beautiful buttocks''), also maps to a genomic locale known to exhibit parent-of-origin effects (Cockett et al. 1996).

Causes of Parent-of-Origin Effects

A number of mechanisms can cause parent-of-origin effects. It may be difficult to distinguish between these effects without detailed pedigree or molecular analyses (Fig. 2). One potential underlying cause of parent-of-origin effects is variation in mitochondrial DNA (mtDNA). The vast majority of zygotic mtDNA is derived from the oocyte (Hutchison et al. 1974). Thus, any phenotypes caused by mtDNA variation will show a matrilineal inheritance pattern; males will not pass the trait.

Another class of genes, termed ''maternal effect'' genes, are those in which offspring phenotype is dependent on maternal genotype. Mature oocytes, in contrast to sperm, contain many gene products critical for development. Because of the haploid nature of germ cells, alleles that would otherwise be recessive may exhibit phenotypes when they are expressed in oocytes. Thus, a female could be either heterozygous or homozygous for the allele for her offspring to show the effect. The offspring genotype at the gene in question, heterozygous or homozygous, does not affect the phenotype in this case.

Maternal effect loci in mammals may even produce phenotypes in offspring that have no copies of the causative allele. Genes that affect uterine environment, lactation, or postnatal care all have this potential. That is, allelic variation in these genes could affect the offspring without being passed on to them (e.g., if the allele is dominant and the mother is heterozygous; Fig. 2B).

Sex chromosome–linked genes may also underlie parent-of-origin effects. Most mammalian species have a Y chromosome found only in males. Although there are some known

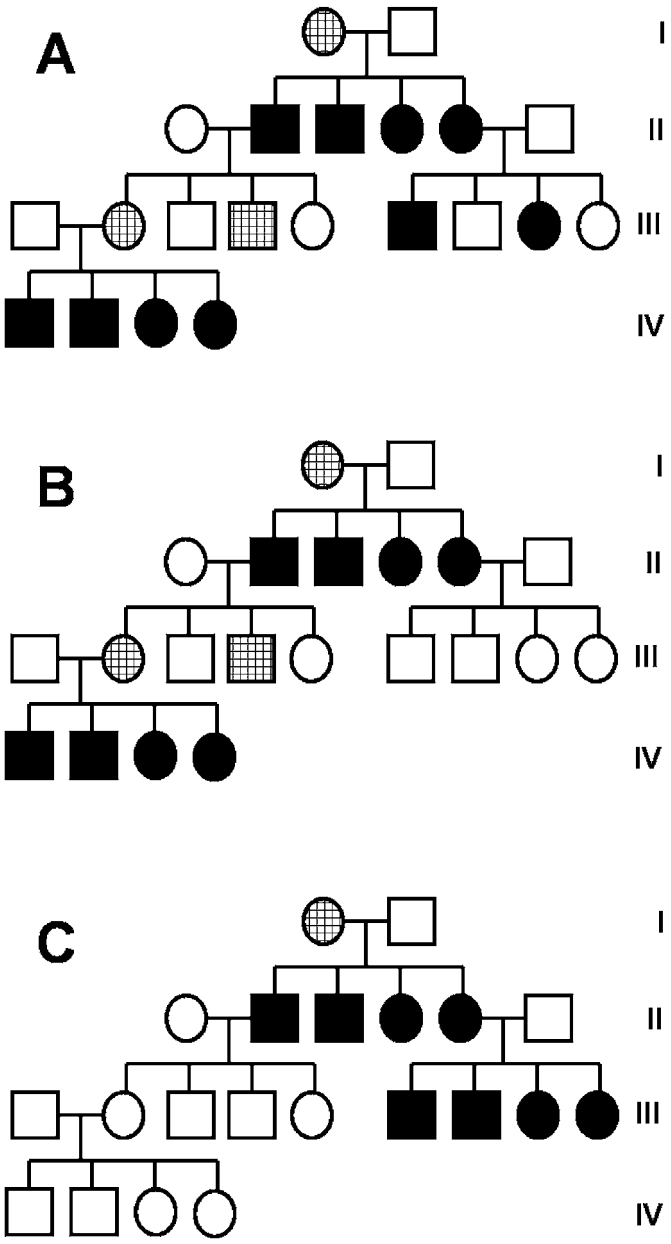

Fig. 2.—Pedigrees associated with 3 kinds of genes that exhibit parent-of-origin effects. Roman numerals refer to generation number. Squares = males; circles = females. Clear circle or square = does not exhibit new phenotype. Filled circle or square = exhibits phenotype associated with new allele. Cross-hatched = carriers of the allele that do not exhibit the phenotype. For all 3 pedigrees, a de novo mutation arises in the generation I female. Assume this female only passes the new allele and the new allele produces a selectively neutral phenotype. A) An imprinted, maternally expressed gene. B) A dominant maternal effect gene. C) A mitochondrial gene. In all 3 cases, the new allele arises in the germline of the female in the 1st generation—hence, she is not affected. In A and B, the generation I female is heterozygous for the gene in question. In C, the female passes on only 1 mitochondrial DNA (mtDNA) haplotype. Note that in A, the new allele is silent when passed through a male, but regains activity when passed through a female in subsequent generations. In B, individuals may be affected without themselves containing the allele in question. See the affected female and her offspring in generations II and III, for example. In the mtDNA pedigree (C), there are no carriers (i.e., individuals possessing the allele who do not exhibit the phenotype).

exceptions (e.g., the XY females of the transcaucasian mole vole [*Ellobius lutescens*]), most Y-linked genes will affect only males and be passed only to other males (Just et al. 2002). The parental effects on mammalian X chromosomes are more subtle. The best known are those in which recessive X-linked phenotypes are disproportionately seen in males. This sex disparity in X chromosome phenotypes seems obvious given the single copy in males versus 2 in females. However, females silence 1 of their 2 X chromosomes such that gene expression is roughly equivalent between the sexes (Lyon 1961).

Female mammals are typically thought to randomly inactivate 1 X chromosome. For some time, however, it has been known that *Mus* undergo preferential inactivation of the paternal X in the placenta and other extraembryonic tissues (Takagi and Sasaki 1975). Paternal X inactivation also has been observed in the placentas of deer mice (*Peromyscus maniculatus*; Vrana et al. 2000) and domestic cows (*Bos taurus*; Xue et al. 2002). Female marsupials of several genera have been shown to inactivate the paternal X chromosome in both extraembryonic and somatic tissues (Graves 1987). Examination of metatherian data suggests that marking of the paternal chromosome, rather than random choice, was the ancestral therian mechanism of X inactivation.

This hypothesis has been strengthened by recent developmental studies in *Mus* suggesting that the paternal X is initially marked for silencing in embryonic tissues as well (Sado and Ferguson-Smith 2005). Early in development, however, the paternal X is ''reset'' in cells destined to form the embryo itself, and the inactivation then becomes random. That is, the paternal X is inactivated in 50% of cells and the maternal X is silenced in the other 50%. This mosaicism of X inactivation is thought to protect against deleterious recessive mutations: a female that is heterozygous for such a mutation will still have a proportion of cells that have a functional copy of the gene.

Once one of the X chromosomes is inactivated, it stays silent in daughter cells produced by cellular division. The continuing silence of the same X allele in descendant cells is an example of epigenetics. Epigenetic effects are those in which heritable changes in gene activity are produced without a change in DNA sequence. Although epigenetic effects are typically thought of as occurring within an organism, they also may involve transmission to offspring. Such Lamarckian effects have been demonstrated in plants (Adams and Wendel 2005; Takeda and Paszkowski 2005).

Genomic imprinting is an epigenetic phenomenon similar to X inactivation, but occurs on autosomes. Genomic imprinting may be the most common cause of parent-of-origin effects. So-called imprinted genes are expressed from only 1 parental allele (Tilghman 1999). That is, there are genes that are only expressed from the maternally inherited copy, and those that are only expressed from the paternally inherited copy (Fig. 3A).

A deleterious or maladaptive mutation in an imprinted gene will be exposed if it is on the expressed parental chromosome, despite the presence of a wild-type copy. Imprinted genes are reset every generation, such that a paternal allele passed from father to daughter becomes a maternal allele in her offspring

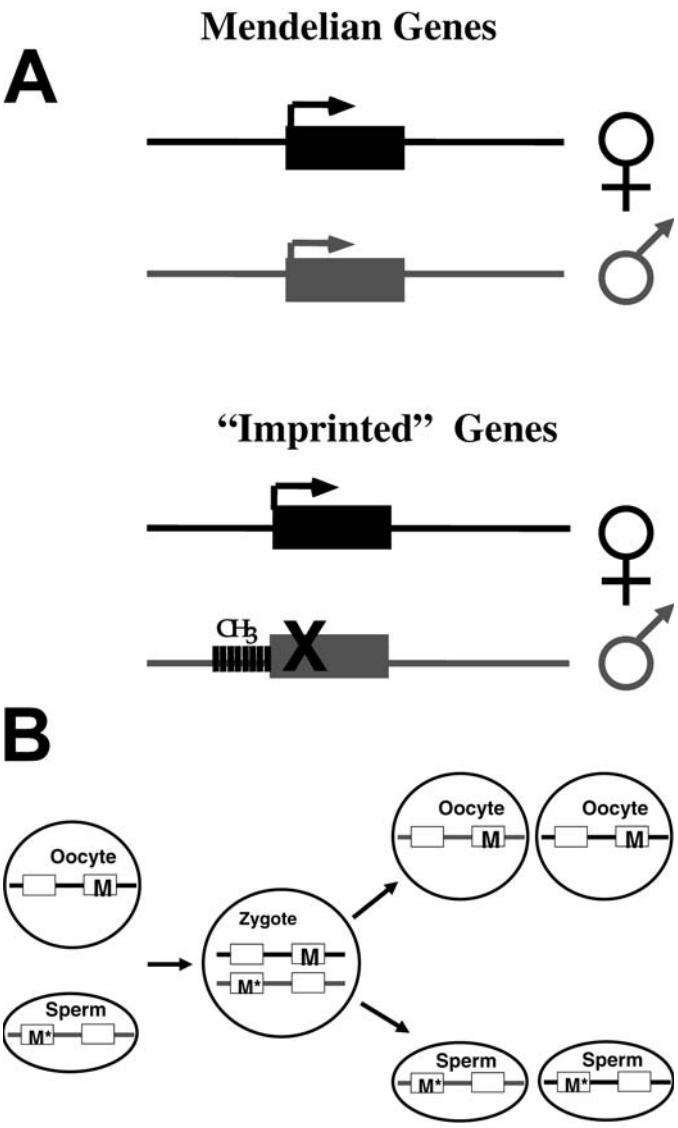

Fig. 3.—Schematic diagrams of genomic imprinting. A) Single imprinted gene that is only expressed from the maternal allele. Box on line represents gene. Male and female symbols refer to the parental origin of that allele. CH_3 refers to DNA cytosine methylation. Arrow branching from box indicates expression and X indicates lack of expression (silenced allele). B) Model of imprint resetting in germ lines. Gray and black lines represent 2 alleles of an imprinted region. Boxes represent regions of DNA where gametic marks (''imprints'') are applied. M* represents a paternal mark, and M represents a maternal mark. Note that the gray allele begins with a paternal mark (left), but may have a maternal mark in the next generation if it is inherited by a female. Reciprocally, the black allele begins with a maternal mark, but may be either switched to a paternal mark in the next generation, or reset with a maternal mark.

(Fig. 3B). Thus, the hallmark of imprinted inheritance is dependence of the phenotype on which parent contributed the allele, rather than which sex inherits it. The *Igf2* mutations noted only cause the growth retardation phenotypes in young whose father passed the mutation; this is because the *Igf2* gene is exclusively expressed from the paternal allele. Growth-retarded females cannot produce growth-retarded offspring if mated to a wild-type male. In contrast, grand-offspring produced from sons of this female may exhibit the growth phenotype (Fig. 2A).

The gene *Igf2* was the 1st gene reported to be imprinted (DeChiara et al. 1990), because of the unexpected inheritance pattern of the mutant phenotype. Almost simultaneously, a report came that the *Igf2* receptor (*Igf2r*) was expressed only from the maternal allele (Barlow et al. 1991). What is intriguing is that the *Igf2r* protein actually acts as a sink for *Igf2* by targeting it for degradation. *Igf2r* has the curious property of being expressed from the opposite parental allele as that of *Igf2*, and its product acting antagonistically to the *Igf2* gene product. The 3rd imprinted gene discovered also proved to be maternally expressed, and also to be involved in *Igf2* function, albeit indirectly (Bartolomei et al. 1991). This gene, termed *H19*, does not encode a protein; rather only an RNA is produced (Brannan et al. 1990). Whether this RNA itself is functional is open to debate; however, the DNA sequence itself is well conserved. Other imprinted genes were discovered slowly through the 1990s both by serendipity and designed screens. Sequencing of the *Mus* genome allowed more rapid discovery through identification and testing of genes located near known imprinted loci. Today the number of imprinted transcripts stands at greater than 80 (Morison et al. 2005), although an exact count is difficult.

Regardless of the total, a number of generalizations can be made regarding imprinted genes:

1. Most imprinted genes particularly affect prenatal and neonatal growth.
2. Most imprinted genes are expressed in extraembryonic tissues.
3. Imprinted genes are found in clusters.
   A. Imprinted domains have at least 1 region where the 2 parental alleles differ in the amount of DNA methylation.
   B. Clusters of imprinted genes tend to have both maternally and paternally expressed genes.
4. Maternally and paternally expressed genes tend to have antagonistic effects.
5. Many imprinted genes apparently do not encode proteins.
6. There are several common complications to genomic imprinting:
   A. Genes may be imprinted only in certain tissues.
   B. Genes may be imprinted only at certain times in development.
   C. Genes may have incomplete imprinting. That is, allelic expression may be biased against, but not completely absent from the ''silent'' allele.

One annoying facet of the genomic imprinting literature has been the inconsistency of terms used to describe a gene's parental expression pattern. Generally, the silenced allele is the one referred to as ''imprinted.'' An ongoing dilemma is whether to refer to a gene by its silenced (imprinted) or active (expressed) allele. For example, *Igf2* may be termed

a ''paternally expressed gene'' or a ''maternally imprinted gene.'' Proponents of the former point out that it is the silencing that is atypical and hence being studied. For those interested in allelic variation or population biology, however, it is more logical to refer to the active allele.

Mammalian genes subject to genomic imprinting encode products that fall into many families—secreted ligands, receptors, transcription factors, splicing factors, intracellular signaling molecules, diverse enzyme groups, and noncoding RNAs. Despite this diverse array of gene products, involvement in both pre- and postnatal growth, metabolism, or behavior emerge as common themes (Reik et al. 2003). Strengthening the case made by transgenic mouse models, a number of human diseases have been identified in which altered expression of imprinted genes is the primary defect. Many of these imprinting diseases are characterized by abnormal growth, including Beckwith–Wiedemann syndrome, Silver–Russell syndrome, and Prader–Willi syndrome. Prader–Willi syndrome is notable in that afflicted individuals are obese because of abnormalities of the central nervous system that lead to overeating (Cassidy 1984). Recent evidence also has strengthened the case for imprinted genes playing a role in 2 common maternal–fetal conditions, pre-eclampsia and gestational diabetes (Oudejans et al. 2004).

Some imprinted genes appear to directly function in both growth and behavior; an interesting example is that of the paternally expressed gene *Pw1*/*Peg3*. *Pw1*/*Peg3* was 1st identified in a screen for genes involved in muscle cell determination and later found to be imprinted (Relaix et al. 1998). The *Pw1*/*Peg3* gene encodes a large (~1,500 amino acid) protein with 12 zinc finger domains, and is expressed in numerous cell types, particularly in central nervous system and myogenic lineages. Mice that inherit an induced *Pw1*/*Peg3* mutation from their father are growth retarded, similar to those produced by the *Igf2* mutants (Li et al. 1999). These young also suckle poorly and show a later onset of puberty. Female young that inherit the mutation are fertile, but do not eat sufficiently during pregnancy and exhibit poor lactation and reduced maternal care (Curley et al. 2004). The young of mutant females also show growth retardation, even if they received a functional allele from their father, showing that *Pw1*/*Peg3* also has a maternal effect phenotype (e.g., Fig. 2B).

Molecular Mechanisms of Genomic Imprinting

Although imprinted domains tend to be relatively discreet, it is not entirely clear what defines them. All imprinted chromosomal regions examined contain a relatively small region of DNA where one parent's allele exhibits greater amounts of DNA methylation than that derived from the other parent. DNA methylation in mammals is primarily found on cytosine residues in the sequence CG (Bird 1986). Regions of the genome rich in DNA methylation tend to have little or no gene transcription activity. The enzymes that add these methyl groups, the DNA methyltransferases (*Dnmts*), are active in the germlines of both sexes (Mertineit et al. 1998). Indeed, mature sperm and oocytes exhibit very different patterns of DNA methylation. These gametic DNA methylation marks are typically thought to be the actual imprint or mark that specifies 1 allele be silenced.

The DNA methylation levels are not the only marks distinguishing the expressed and silenced alleles of imprinted genes. Methylation and other modifications of the histone proteins that act as chromosome scaffolds also are found in imprinted and other silenced regions. For example, a methylated version of histone 3 is associated with inactive genes. Reciprocally, an acetylated form of histone 3 is typically associated with active regions of chromatin, whereas removal of those acetyl groups signals silencing (Jones et al. 1998). Collectively, the state of modifications of these proteins has been termed the ''histone code.'' The silenced allele of an imprinted gene thus has a different code than the active allele (Hu et al. 2000).

Although the histone code clearly plays a role in genomic imprinting, the general thought is that it cannot be the primary mark or imprint: during mammalian spermatogenesis, histones are replaced by a class of structural proteins called protamines (Govin et al. 2004). Protamines are more efficient at packing DNA into the sperm nucleus. A small number of protamines may persist for a time after fertilization, and thus could play a role in conveying paternal marks (Meistrich et al. 2003).

The emerging picture indicates that the histone, DNA, and possibly other chromatin modifications act in a combinatorial fashion to insure that a gene is either silenced or activated. For example, the *Dnmt* enzymes recruit histone deacetylases—both these states (methylated DNA and histones lacking acetyl groups) are associated with silenced genes. These modifications are similar to those seen in silent regions of the genome (heterochromatin) and the silenced X chromosome.

Multiple modifications are likely necessary to maintain silencing of a region. For example, evidence to date suggests that metatherian mammals have overall lower levels of DNA methylation than eutherian mammals (Loebel and Johnston 1996). Correspondingly, the silenced paternal X chromosome still shows some expression in female marsupials (Graves 1987). Similarly, eutherian placentas exhibit lower levels of DNA methylation than the embryo proper as well as a greater tendency toward abnormal expression of imprinted genes. For example, a subset of imprinted genes located near the *Lit1* gene on *Mus* chromosome 7 appear to use only a trimethylated form of histone 3 to maintain silencing. That is, the germline DNA methylation in this region has been lost. Strikingly, all these genes are primarily or exclusively expressed in the placenta (Lewis et al. 2004).

When imprinted gene transcription is perturbed such that there is a switch from monoallelic to biallelic expression, loss of imprinting is said to have occurred. Loss of imprinting can be induced by a number of factors, including genetic variation as well as induced mutations. For example, deletion of any one of a number of the *Dnmt* genes, whose products regulate DNA methylation, induces loss of imprinting. Many of these induced deletions of the *Dnmt*s have maternal effects on imprinting (Bourchis et al. 2001; Howell et al. 2001; Kaneda et al. 2004).

That is, females that initially inherit the mutation are normal, but their offspring exhibit loss of imprinting when mated to a wild-type male. The *Dnmt* maternal effects are specific to the oocyte, as demonstrated by embryo transfer experiments in which *Dnmt* mutant fertilized oocytes are moved to the uterus of a wild-type female, and vice versa.

As suggested by these data, *Dnmt* gene products have been found in mature oocytes before fertilization. Gene products in the DNA methyltransferase family have several functions. Maintenance *Dnmt*s methylate newly synthesized partner strands of already methylated regions (e.g., *Dnmt1*—Li et al. 1993), de novo methyltransferases add methylated cytosines to a region based on other cues (e.g., *Dnmt3a* and *Dnmt 3b*—Bestor 2000; Bestor and Verdine 1994), and *Dnmt3L* lacks the ability to actually methylate DNA, but appears to interact with other *Dnmt*s to target specific regions of the genome, particularly imprinted domains (Aapola et al. 2000). *Dnmt3L* also appears to directly activate histone modifying enzymes (Deplus et al. 2002).

The differentially methylated regions (DMRs) produced by *Dnmt* activity are thought to be key in regulating the entire cluster of imprinted genes in which they lie. One type of DMR (primary) originates in the germline of a parent and may mark an allele from the beginning of development. Other DMRs (secondary) are not present initially, but arise during development. Deletion or mutation of primary DMRs typically result in loss of imprinting of most genes within the associated cluster (Verona et al. 2003). In such cases, these DMRs also are referred to as imprint control regions or elements. Surprisingly, the actual DNA sequence at these regions is often very divergent between mammalian species. One common feature is that most primary DMRs have repeated sequences that are not found elsewhere in the genome. Interestingly, plants have a mechanism that senses repeated sequences and silences them via DNA methylation; this system is thought to be a viral defense mechanism. Whether the same repeat-induced silencing mechanism also exists in mammals is unclear. However, forceful arguments have been made that mammalian DNA methylation was originally selected for as a retroviral defense (McDonald et al. 2005).

Surprisingly little is known about mechanisms of DNA demethylation in mammals. Profiles of DNA methylation levels in house mice show a rapid drop during preimplantation development. The demethylation of paternal DNA appears to be an active process, whereas maternal DNA methylation is lost through lack of maintenance (i.e., newly synthesized strands are not methylated—Oswald et al. 2000). Most cytosine methylation across the genome is lost; however, a small amount at primary DMRs survives. The genome then begins a remethylation process, with germ cells, somatic tissues, and extraembryonic tissues differing in final density of methylated cytosines (Santos et al. 2002).

A recent study of domestic sheep challenges the assumption that DNA methylation dynamics are similar across mammals (Beaujean et al. 2004). Fertilized sheep oocytes do not actively demethylate the paternal genome. A sheep paternal pronucleus transplanted to a mouse oocyte is demethylated, suggesting no intrinsic protection against demethylation. The reciprocal experiment yielded more surprising results: sheep oocytes were able to demethylate transplanted mouse paternal pronuclei, despite being unable to demethylate conspecific male DNA.

The differences between genes, cluster regulation, species, and nomenclature underscore confusion in the field: no one model of imprinting control or gene organization fits all the known clusters of imprinted genes, let alone those yet to be discovered. Further, most of the data are from either laboratory *Mus* or humans. The former do not represent natural populations, the latter are not tractable for many experiments.

Imprinted domains usually contain at least 1 of each type of DMR, typically 1 primary and multiple secondary DMRs. The relationship of the imprinted genes to these DMRs and to each other varies greatly (Fig. 4). The heavily methylated regions overlie the areas where gene transcription begins in some cases, such as for *Pw1*/*Peg3* and neighboring *Usp29* gene (Fig. 4A; Li et al. 2000). In many cases noncoding RNAs (ncRNAs) are found in imprinted clusters. In particular, the silenced X chromosome specifically expresses a large ncRNA termed *Xist* (inactive X chromosome–specific transcript). The *Xist* locus is found in the region genetically known to mediate this process, the X-inactivation center (Brockdorff et al. 1991). The *Xist* RNA spreads from the X-inactivation center to coat the entire inactivated chromosome (Clemson et al. 1996). Whether the spreading *Xist* RNA directly or indirectly (i.e., through recruiting other factors) silences transcription is not understood.

Noncoding RNAs may play a direct role in autosomal imprinted gene regulation in certain clusters. The enormous (50,000+ bases) *Lit1* RNA is the only paternally expressed transcript in its cluster of imprinted genes (Mitsuya et al. 1999). The spreading *Lit1* RNA is speculated to interfere with transcription of other genes on the paternal allele (Fig. 4B; Mancini-Dinardo et al. 2006). *Lit1* is an example of a so-called antisense transcript, as are a number of ncRNAs. That is, these transcripts arise from the noncoding strand of DNA directly opposite a strand encoding a gene. The *Lit1* transcript begins in an intron of the *Kcnq1* potassium channel–encoding gene and crosses several others. Such overlapping transcripts render both counts of imprinted genes and elucidating the role of ncRNAs in regulation difficult.

The gene pair for which imprinted gene regulation has been best characterized is the *Igf2–H19* pair. The primary DMR lies between the 2 genes in this pair (Fig. 4C; Tremblay et al. 1995). The unmethylated maternally derived DNA sequence at the *Igf2–H19* primary DMR specifically binds the CTCF (CCCTC-binding factor) transcription factor (Hark et al. 2000). The bound CTCF protein on the maternal allele is thought to prevent the *Igf2* gene from interacting with a set of transcriptional-enhancing elements that lie on the other side (i.e., away from *Igf2*) of *H19* (Fig. 4C). In contrast, the CTCF protein is unable to bind to the methylated paternal allele. The absence of CTCF binding allows the *Igf2* gene to access the enhancer elements on the paternal allele. The paternal DNA methylation at the CTCF binding region extends into the *H19* gene, insuring its silence on this allele.

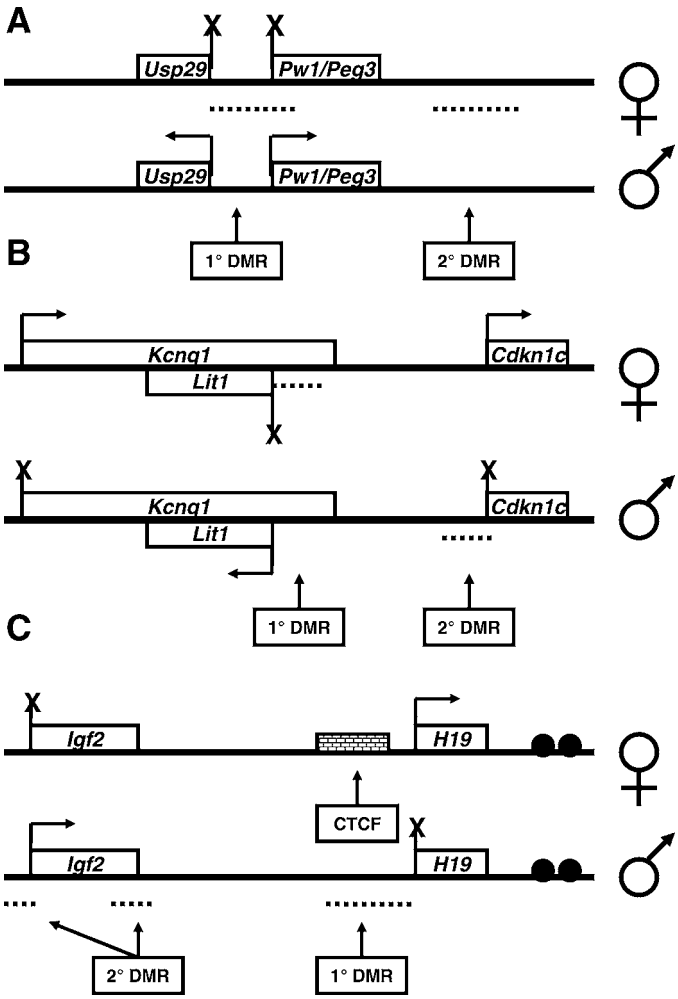

Fig. 4.—Organization of 3 imprinted domains. Clear boxes on lines represent individual genes; gene name is indicated within box. Branched arrows from genes indicate expressed allele. "X" indicates silenced allele. 1° DMR = primary differentially methylated region (germline origin). 2° DMR = secondary DMR (arises postfertilization). Dashed line delineates the DMR and underlies the methylated allele. Male and female symbols refer to the parental origin of that allele. A) *Pw1/Peg3* domain. Note that the 1° DMR lies over the promoters and transcription start sites of both genes. B) *Lit1* domain. 1° DMR overlies the *Lit1* promoter. *Lit1* transcript silences the *Kcnq1* on the paternal allele. C) *Igf2* domain. Filled circles represent enhancer elements. Shaded box indicates the CTCF transcription factor protein binding. The bound CTCF protein forms a barrier on the maternal allele that prevents *Igf2* from using the enhancers. On the paternal allele, the DNA methylation prevents CTCF from binding and spreads to cover the *H19* start site.

The *Igf2* secondary DMRs are involved in formation of DNA loops that facilitate the interaction with the enhancer elements (Murrell et al. 2004). On the paternal allele, the primary DMR associates with the secondary DMR farthest from the start site of *Igf2* transcription. This arrangement brings the enhancer elements in close proximity to the start site of *Igf2*. On the maternal allele, the primary DMR interacts with a secondary DMR very close to the *Igf2* start site; this arrangement isolates the *H19* gene and enhancer elements.

At least 1 other pair of imprinted genes resembles the *Igf2–H19* configuration, the *Dlk1–Gtl2* pair: *Dlk1* is a protein coding gene involved in growth pathways, whereas *Gtl2* produces ncRNA transcripts of various lengths (Schmidt et al. 2000). A primary DMR lies between the 2 genes. A mutation in this regulatory domain, rather than the genes themselves, appears to be responsible for the hypertrophic buttock musculature of the domestic sheep callipyge phenotype (Charlier et al. 2001). However, the emerging picture is that imprinted domains and local methods of regulation are quite heterogeneous. Knockouts of several potential genome-wide imprinting regulation genes have had effects only on a subset of imprinted genes and clusters. Even responses to widespread changes in histone modification and DNA methylation are not uniform among imprinted genes. Although there are commonalities among these regions, the variation suggests that different genes and clusters have become imprinted at different points in history.

Phylogenetic Distribution of Genomic Imprinting in Mammals

The similar mechanistic and phylogenetic distribution between autosomal genomic imprinting and X chromosome inactivation has strengthened the hypothesis that the 2 phenomena share an evolutionary origin in mammals. The lack of evidence for either autosomal or X imprinting in monotremes suggests that both processes evolved after the divergence of this lineage from therian mammals (Fig. 5). Imprinting variation among eutherian mammals suggests that there have been multiple steps in the evolution of this phenomenon, and that it is ongoing.

Comparative genomic studies suggest that the clusters of genes found to be imprinted in *Mus* are generally well conserved across eutherians (complete mammalians genomes, available at http://www.ensembl.org). However, general gene content conservation does not prove identical imprinting status of those genes or conservation of the boundaries of the domain. Directly assessing the imprinting status of a gene requires the ability to distinguish the 2 parental alleles (Vrana 2006). One must identify heterozygous individuals as well as ascertaining 1 or preferably both parental genotypes. Genera of mammals for which genomic imprinting has been directly demonstrated are listed in Table 1.

The presence of DMRs has been used as a proxy for imprinting status: if a gene has a DMR present, then it is thought to be imprinted. Typically the differential allelic DNA methylation patterns are present even in nonexpressing tissues. A counterexample to this inference is the *Igf2r* gene. The gene is not imprinted in humans despite the presence of a DMR (Kalscheuer et al. 1993; Riesewijk et al. 1996; Smrzka et al. 1995). *Igf2r* also is not imprinted in prosimian ring-tailed lemurs (*Lemur catta*), common tree shrews (*Tupaia glis*; order Scandentia), and Philippine flying lemurs (*Cynocephalus volans*; order Dermoptera—Killian et al. 2001). *Igf2r* imprinting has been demonstrated in the metatherian genus *Didelphis*, the rodent genera *Peromyscus* and *Rattus*, as well as in 3 artiodactyl species (domestic sheep, pigs, and cows). The in-

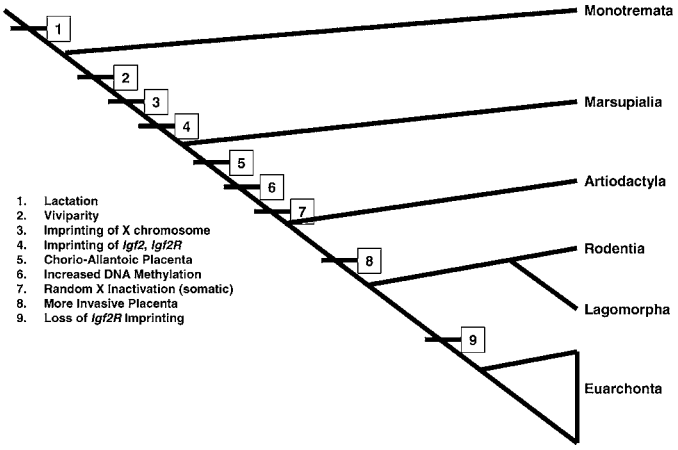

FIG. 5.—Phylogenetic tree depicting mammalian groups investigated for genomic imprinting of specific loci. Origins of key events related to genomic imprinting are depicted by numbered boxes on the tree. Horizontal bar next to Euarchonta indicates multiple orders (Dermoptera, Primates, and Scandentia). Only groups that have been investigated with regard to genomic imprinting are shown. Partly adapted from Reik and Lewis (2005). Note that no imprinting has been demonstrated in Monotremes, and that it has not yet been demonstrated that mammalian X-chromosome inactivation preceded autosomal genomic imprinting.

terpretation of these data is that *Igf2r* imprinting was present in the common ancestor of therian mammals, but has been lost in Primates, Dermoptera, and Scandentia. These 3 orders have been grouped together in the superorder Euarchonta, suggesting a single loss of imprinting status in this gene (Fig. 5).

Interspecific discrepancies in imprinting status have now been observed in a number of other genes (approximately 32 between humans and house mice as of this writing [Appendix I]). For example, Vrana et al. (2001) discovered a placental lactogen gene in *Peromyscus* showing paternal expression. Muroid placental lactogen genes are duplications of the prolactin gene, encoding the well-known pregnancy hormone. In contrast, primates have no duplications of prolactin; the analogous loci termed placental lactogens in Primates are instead duplications of the growth hormone gene (Soares 2004). Human and house mouse orthologs are reciprocally imprinted (i.e., expressed from the opposite parental allele) in at least 2 cases (Morison et al. 2005).

Humans are the only species that has been examined for intraspecific imprinting variation. One study revealed variation in the degree of parental bias in imprinted multiple loci within the relatively homogenous Japanese population (Sakatani et al. 2001). A number of inherited diseases also show significant phenotypic differences dependent on which parent contributed the allele.

Recently, a class of human hydatidiform moles was uncovered that has a normal 1:1 ratio of parental genomes rather than an excess of paternal DNA (Judson et al. 2002). An allele that apparently predisposes women to these placental overgrowths has been mapped to the imprinted domain containing the *Pw1*/*Peg3* gene. These biparental hydatidiform moles are associated with changes in DNA methylation at imprinted domains. An emerging view is that the majority of cases in which this syndrome recurs are due to such susceptibility alleles; it will be particularly interesting to determine if these alleles are deleterious in all genetic backgrounds or environments. The changes in DNA methylation at DMRs suggest that changes in imprinted gene expression are directly responsible for the hydatidiform mole phenotypes, and reinforce the notion that placental tissues are particularly sensitive to these changes.

TABLE 1.—Mammalian genera with demonstrated genomic imprinting. Auto. = 1 or more autosomal genes demonstrated to be imprinted. X chr. = X chromosome demonstrated to be imprinted in extraembryonic or other tissues. Y = yes, imprinting demonstrated. N = no, imprinting not demonstrated. ND = not determined. Lagomorph data from Okamura et al. (2005); see text for other sources.

Order	Genus	Auto.	X Chr.
Artiodactyla	*Bos*	Y	Y
	Ovis	Y	ND
	Sus	Y	ND
Lagomorpha	*Oryctolagus*	Y	ND
Marsupialia	*Monodelphis*	Y	ND
	Didelphis	Y	ND
	Macropus	Y	Y
Primates	*Macaca*	Y	ND
	Homo	Y	N
Rodentia	*Mus*	Y	Y
	Rattus	Y	ND
	Peromyscus	Y	Y

RATIONALE FOR GENOMIC IMPRINTING

Understanding the biological role of genomic imprinting is particularly interesting in that it is a counterintuitive process: Why give up the advantages of diploidy at a locus by silencing 1 allele and potentially exposing deleterious recessive mutations? What selective pressures have given rise to this seemingly bizarre form of gene transcriptional regulation? Are these the same forces that maintain the process?

Chromatin modifications such as DNA methylation and histone modifications that underlie monoallelic expression already were present before the origin of mammalian imprinting (Reik and Lewis 2005). The raw material for genomic imprinting must be provided by differences in such modifications that arise during oogenesis and spermatogenesis. Imprinting selection hypotheses must explain why only certain genes retain evidence of these gametic differences.

One clear consequence of imprinting is that it precludes parthenogenesis; this was 1st noted when the reciprocal pronuclear transplantations were performed. This hypothesis assumes that new allelic combinations generated by sexual reproduction are so important that the processes cannot be obviated. The antiparthenogenesis hypothesis predicts that imprinting will not be found in groups where parthenogenesis exists. However, this prediction has not yet been tested,

although the opportunity exits within amniotes (e.g., a proportion of wild turkeys [*Meleagris gallopavo*] are parthenogenetic, as are several populations of the lizard genus *Cnemidophorus*—Harada and Buss 1981; Kearney and Shine 2004; Olsen and Marsden 1954).

Genomic imprinting also could function as a means of preventing inappropriate development of oocytes still in the ovary (Varmuza and Mann 1994). Varmuza and Mann (1994) noted that invasive trophoblast tissue was one of the major tissues impaired in parthenogenetic embryos, thus rendering most such growths benign. In contrast, risk of metastasizing tumorigenic trophoblast is much greater in hydatidiform mole pregnancies. This ''ovarian time bomb'' hypothesis described a consequence of imprinting, but does not explain maternally expressed genes, nor predict that they should also be involved in the same growth pathways as paternally expressed genes. The hypothesis does predict that there should be no imprinting outside of eutherian mammals, that is, before the innovation of the trophoblast lineage. The finding that *Igf2* (Killian et al. 2000, 2001; O'Neill et al. 2000) is imprinted in didelphid marsupials and the small macropodid *Macropus eugenii* (tammar wallaby) argues strongly against this hypothesis.

The most compelling and controversial hypothesis to explain the existence of genomic imprinting is based on kin selection (Hamilton 1964). Trivers (1974) noted that parents and their offspring might have differing genetic interests. Because only 50% of an embryo's alleles are necessarily shared with its mother, nonshared alleles may be selected to extract more maternal resources than would be advantageous for the mother's other current or future offspring.

Haig (1996) particularly explored potential ''parental antagonism'' in mammalian pregnancies, positing the placenta as a major ''battleground.'' This kinship–parental antagonism hypothesis (Haig 1996; Moore and Haig 1991) proposes that in any nonmonogamous, outbred population, parental interests will differ: males benefit most when their offspring extract the greatest amount of maternal resources possible during gestation and postnatal care. This selection on paternally transmitted alleles is particularly strong if a female's other offspring are likely to be sired by other males that are not closely related to the 1st male.

This antagonism can be depicted as being realized through different parental optima for expression levels of a given gene. For example, paternal interests may benefit from higher expression levels of a prenatal growth factor such as *Igf2*. A small increase in *Igf2* levels would result in greater birth weight of the offspring, and hence a greater rate of survival. These larger neonates then impose a greater cost on the mother. Total expression level of such a gene is predicted to gravitate toward the higher of 2 parental optima: once 1 parent's allele has been completely silenced, it can no longer affect overall levels of gene expression. Wilkins and Haig (2003) termed this phenomenon the ''loudest voice prevails'' effect.

The other parent may counter such a strategy by modulating expression levels of a gene with antagonistic effects. The maternal expression of *Igf2r*, which lowers *Igf2* protein levels, is an example of this effect. A striking example of reciprocally imprinted antagonistic genes lies within the *Gnas* locus. The *Gnas* gene complex encodes ''G protein'' subunits, which are key components of intracellular signaling from hormone receptors (Peters et al. 1999). Deletion of a shared exon between 2 variants of the protein results in reciprocal metabolic phenotypes depending on which parent contributes the mutant allele; inheriting a maternal deletion results in obese offspring, whereas inheriting a paternal deletion yields very lean offspring (Chen et al. 2005).

The kin selection hypothesis predicts that imprinted genes should affect growth control and behavior; different genes may acquire imprinting at different times, resulting in somewhat heterogeneous mechanisms; no selection for imprinting should occur in oviparous taxa that also lack parental care; and selection should be reduced in monogamous or highly inbred taxa. Many, but not all, imprinted genes have met a simple interpretation of the hypothesis: that paternally expressed genes should promote growth (maternal allele silent, as per the ''loudest voice prevails'' effect), and maternally expressed genes should inhibit growth (Haig 2004).

Zeh and Zeh (2000) proposed that parental antagonism plays a major role in differing speciation patterns across taxa. They noted that mammals appear to have much more rapid reproductive isolation than nonviviparous vertebrate groups. The role of viviparity in rapid reproductive isolation is supported by the great diversity in eutherian placentation, including morphology, degree of maternal invasion, and gene expression (Carter and Enders 2004). Placental abnormalities are common in both interspecific hybrids and embryo transfer experiments (Allen et al. 1993). The numerous associations between altered imprinted gene expression and placental phenotypes suggest a role for imprinted genes in mammalian reproductive isolation.

Further study of monotremes and marsupials should help clarify the role of selection on mammalian imprinting. Both groups would appear to be subject to parental antagonism because of lactation. However, monotremes are oviparous. If the viviparity hypothesis is correct, genomic imprinting should play a more significant role in marsupials than in monotremes. As noted, studies have confirmed both autosomal and X chromosome imprinting in the former, whereas there is no evidence for genomic imprinting in monotremes. The number of imprinted genes or severity of parental bias in expression also should be less in metatherian mammals than that observed in eutherian mammals. Relatively weaker selection for imprinting of genes during prenatal development is predicted in marsupials because of their typically shorter gestation times, simpler and more transitory placenta, and the greater amount of yolk in metatherian than eutherian ova.

Hurst (1997) and others have criticized the kin selection hypothesis of genomic imprinting on grounds that it is not rigorously testable or sufficiently quantitative, and that it is too accommodating of seemingly contradictory data. For example, the kin selection hypothesis predicts rapid evolution of imprinted genes (McVean and Hurst 1998), but the gene regions examined to refute this hypothesis were limited to

expressed portions. Given that genomic imprinting is essentially a phenomenon of gene transcription, regulatory changes seem to be more likely.

Burt and Trivers (1998) noted that genes expected to be under selection in parental antagonism involving genomic imprinting could include the imprinted genes themselves, genes that encode products that affect imprinting status of 1 or more imprinted genes, and genes that themselves or whose products are affected by imprinted genes. This scenario obviously presents a large and complex set of targets to examine for selection.

The prevalence of mammalian imprinted genes involved in growth pathways is such that they cannot be ignored by any hypothesis that seeks to explain the phenomenon. A straightforward alternative to the kin selection hypothesis is that mammalian growth requires very tight regulation, and silencing 1 allele helps accomplish this. That mammalian physiology requires such regulation is not obvious, except that unlike many other vertebrate groups, mammals show determinate growth. The higher metabolic rates associated with homeothermy also might result in greater free-radical production and hence DNA damage, presumably responsible for the high rate of mammalian cancer. *IGF2* is one of the most common transcripts found in human tumors (Kim et al. 1998; Rainier et al. 1993). Perhaps without the epigenetic regulation of imprinting, growth control genes would show greater amounts of ectopic expression, even during normal development. Mammalian growth requires a control mechanism such that it is tightly regulated, but can evolve rapidly enough to allow for the huge variation observed between species. Differential imprinting of growth-related genes could provide this sort of flexibility. This mammalian growth sensitivity model does not predict that paternally expressed loci should be growth promoting and maternally expressed loci growth retarding. If this trend among imprinted genes holds, then the parental antagonism hypothesis has greater explanatory power.

The monoallelic expression of imprinted genes suggests a role in reproductive isolation under the Dobzhanksy–Muller speciation model (Muller 1942; Orr 1993). This model posits 2 or more interacting loci in which an ancestral population is homogenous (AABB). Population isolation leads to derived alleles: population 1 = AAbb and population 2 = aaBB. Both A–b and a–B combinations are either selectively advantageous or neutral relative to the ancestral A–B interaction. However, a–b interactions may produce a deleterious phenotype and thus limit hybridization between the 2 populations. Imprinting of 1 or more of the loci involved in this model may increase the chances of hybrid dysgenesis. If the new alleles are recessive, heterozygous hybrids (AaBb, Aabb, and aaBb) would not necessarily be at a disadvantage. Should allele A or B be silenced, however, the chances of an a–b (deleterious) interaction increase. A similar model involving X-linked genes has been invoked by several authors to explain Haldane's rule, the disproportionate effects of hybridization on the heterogametic sex (Haldane 1922). Our work on hybrid dysgenesis in the genus *Peromyscus* is consistent with such a model: we have implicated an X-linked locus and another locus mapping to an imprinted domain in the effects (described below). At the very least it is clear that placentation is a major barrier to mammalian interspecies hybridization. Imprinted genes clearly play a significant role in this process.

Genomic Imprinting in the *Peromyscus maniculatus* Species Complex

One of the best documented cases of reproductive isolation involving parent-of-origin effects involves the cricetid rodent genus *Peromyscus* (Steppan et al. 2004). The deer mouse (*P. maniculatus*) is the most widespread of this most common group of North American mammals. *P. maniculatus* extends from Alaska to Mexico City, from Pacific to Atlantic coasts, and from sea level to approximately 14,000 feet (Dewey and Dawson 2001). Notably absent from this range is the southeastern United States, a gap filled by a recently diverged species, the oldfield mouse (*P. polionotus*). Ongoing colonies of randomly bred individuals from a single locale were established in 1948 for *P. maniculatus* (descended from 40 animals of the subspecies *bairdii* from Washtenaw County, Michigan) and in 1952 for *P. polionotus* (from 21 animals of the subspecies *subgriseus* from Ocala National Forest, Florida). These colonies have been used for genetic, developmental, physiological and behavioral studies (Dewey and Dawson 2001).

Early studies suggested that *P. maniculatus* × *P. polionotus* hybrids could occasionally be produced. Watson (1942) 1st demonstrated that hybrids were more readily obtained when crossing female *P. maniculatus* with male *P. polionotus* than the reciprocal combination. Liu (1953) demonstrated that this asymmetry was due to fetal mortality in the latter. Dawson (1965) 1st quantified the hybrid phenotypes and genetics, showing that female *P. maniculatus* (strain BW, subspecies *P. m. bairdii*, from Washtenaw County, Michigan) mated with male *P. polionotus* (strain PO, subspecies *P. p. subgriseus*, from Ocala National Forest, Florida) resulted in offspring significantly smaller than either parental strain. These BW × PO hybrids were not only growth retarded at birth, but remained smaller throughout life (Fig. 6C). Maintaining this undersized phenotype is atypical, because most mammals that are growth retarded at birth exhibit faster or prolonged growth, presumably to reach a set selected size. Both sexes of the BW × PO hybrids are fertile. The reciprocal cross, female PO crossed with male BW, results in disproportionately large offspring, few of which survive past birth. The rare postnatal survivors were almost entirely female, fertile, and remained larger than either parental strain (Dawson et al. 1993).

Rogers and Dawson (1970) showed that the hybrid phenotypes are particularly pronounced in the placenta: PO × BW placentas average 5–6 times greater in mass than those of the BW × PO cross. The latter hybrids show reduced amounts of the spongiotrophoblast layer of the muroid placenta (Fig. 6A). The spongiotrophoblast contains endocrine cells as well as gives rise to cell lineages that invade the maternal uterine wall (Cross et al. 2003). Additional studies by multiple groups have demonstrated the repeatability of these phenotypes (Duselis et al. 2005; Maddock and Chang 1979).

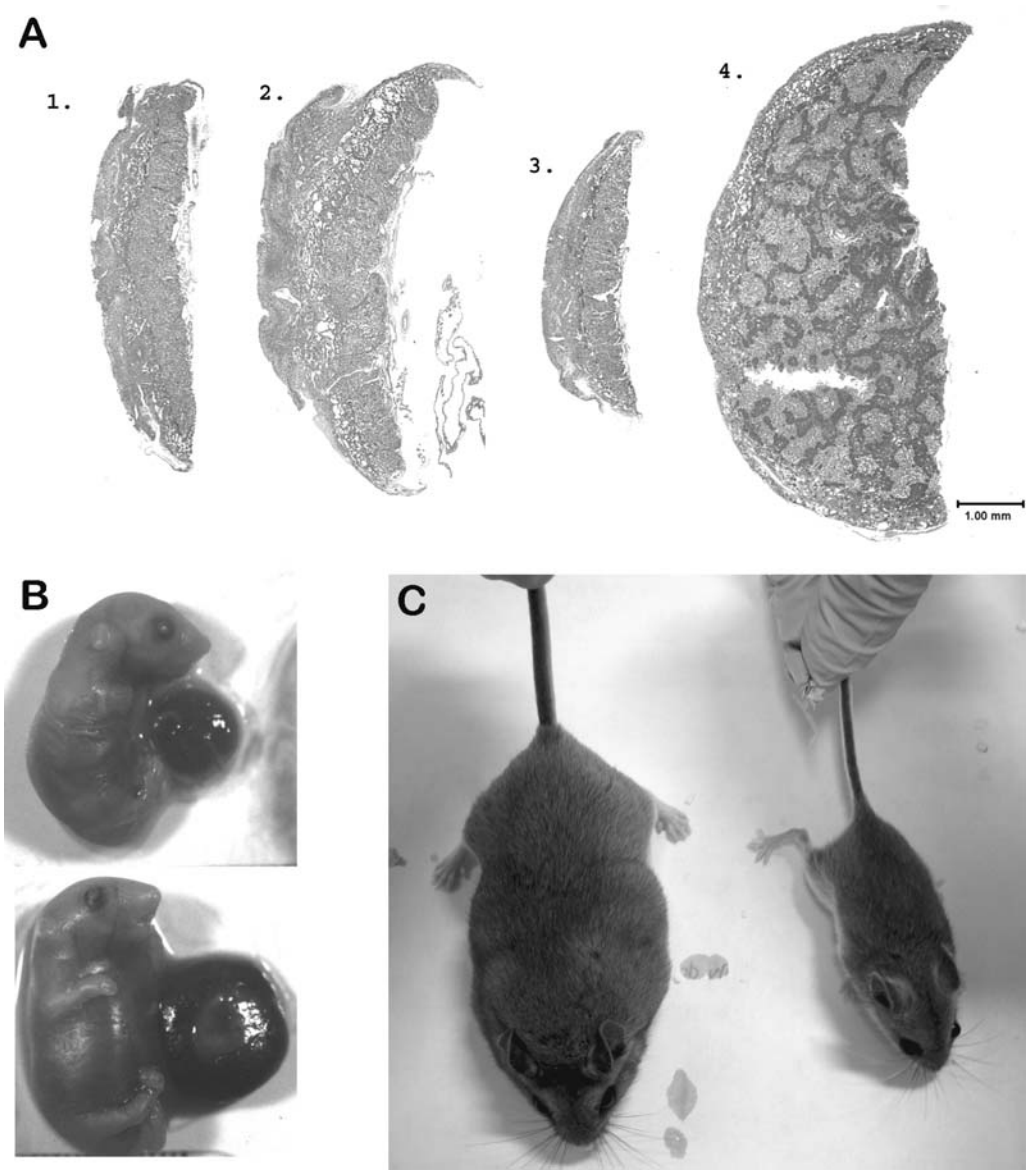

Fig. 6.—*Peromyscus* interspecfic growth phenotyopes. A) Stained cross sections of mature placentas from parental strains and reciprocal hybrids. PO = *Peromyscus* strain PO, subspecies *P. polionotus subgriseus.* BW = *Peromyscus* strain BW, subspecies *P. maniculatus bairdii*. 1. PO. 2. BW. 3. BW × PO. 4. PO × BW. Maternal interface is to the left. Note that the BW section has more of the maternal deciduas than the PO section. B) Normal (upper) and overgrown (lower) embryo with associated placentas. C) Adult growth phenotypes; both animals are mature males. Left: PO × F_1 backcross, right: BW × PO F_1 hybrid. All photos courtesy of the author.

One potential explanation of such reciprocal hybrid growth phenotypes is interactions between species-specific mitochondrial and nuclear genes. Dawson et al. (1993) tested this hypothesis using selective backcrosses to obtain animals whose genomic content was 1 strain, while their mtDNA was derived from the other. Contrary to the mtDNA hypothesis, these crosses resulted in lessened growth effects, suggesting that interactions between nuclear genes with parent-of-origin effects are responsible for the hybrid dysgenesis. As an alternative hypothesis, they proposed that mispairing of imprinted chromosomal domains might be the cause of the phenotypes.

My colleagues and I began our investigations of the *Peromyscus* growth phenotypes by verifying the phenotypes. Consistent with earlier reports, we found growth phenotypes that were particularly pronounced in the placenta. Indeed, in the female PO × male BW cross, we frequently observed conceptuses consisting only of a large, dysmorphic placental mass, reminiscent of hydatidiform mole disease. Further, we noted that when embryos were present in the PO × BW cross, they were not only larger, but always exhibited abnormal morphologies. Many of these phenotypes are similar to diseases in humans and transgenic *Mus* caused by altered expression of imprinted genes. As suggested by earlier studies, our current research shows that fewer than 50% of pregnancies from PO × BW crosses survive past midgestation (A. Duselis and P. Vrana, in litt.).

We then tested whether the phenotypes might be a consequence of *P. polionotus* having lost genomic imprinting. *P. polionotus*, unlike *P. maniculatus*, has been described as largely monogamous (Foltz 1981). Reciprocal growth observations could be explained if imprinting does not occur, or control is weakened by lack of selection in the monogamous species, as suggested by the kin selection hypothesis. In contrast, we found that imprinting has been maintained in *P. polionotus* at all 3 loci tested (Vrana et al. 1998). However, because, the 2 species are thought to have diverged in the Pleistocene (Blair 1950), there likely has been insufficient time for a complete loss of genomic imprinting.

We next tested the parental expression status of known imprinted genes in these hybrids. We found only minor perturbations of imprinting in the undersized hybrids, but the more lethal PO × BW oversized hybrids showed complete loss of imprinting of a number of unlinked loci. For at least 2 genes (*H19* and *Pw1*/*Peg3*), this loss of imprinting was accompanied by reduced DNA methylation on the silenced allele. We do not yet know if the alterations in DNA methylation are limited to imprinted regions; however, analysis with methylation-sensitive restriction enzymes suggest the changes are not genome-wide. Examination of these data strongly suggests that at least some aspects of imprinting control can evolve rapidly.

Our efforts to elucidate the genetics of the *Peromyscus* hybrid dysgenesis have primarily focused on the overgrowth. We have shown that 2 distinct genetic mechanisms contribute to the PO × BW phenotypes. First, we identified 2 regions of the genome that contribute to the overgrowth. We mapped these loci by performing 2 backcrosses with the undersized hybrids: 1 in which the maternal genome was varied (female F_1 × male BW), and 1 in which the paternal genome is varied (female PO × male F_1). The former revealed an association of overgrowth with the distal PO X chromosome, whereas the latter cross showed linkage to the BW *Pw1*/*Peg3* gene (Vrana et al. 2000). Analysis of intercross animals (F_1 × F_1) suggested that the PO X locus must be inherited maternally, and the BW *Pw1*/*Peg3* linked locus inherited paternally to manifest the overgrowth (Vrana et al. 2000). Another unmapped locus associated with the platinum coat-color mutation also affects growth in backcross animals and shows dependence on parental origin (W. D. Dawson et al., in litt.)

Our current refining of the areas of linkage has revealed candidate loci in both domains: within this region of the X chromosome is the homeobox containing gene *Esx1* (Loschiavo et al., in press). Based on a gene knockout, *Esx1* directly impacts placental structure and growth and indirectly affects embryo growth (Li and Behringer 1998). Surprisingly, the gene shows little mouse–human conservation–there is only 65% amino acid identity in the DNA binding homeodomain (Fohn and Behringer 2001). Outside that key motif, the 2 sequences are essentially not alignable.

In contrast to the genomic region surrounding *Esx1*, the *Pw1*/*Peg3* imprinted domain is very gene dense. The *Pw1*/*Peg3* gene knockout suggests a role in placental and embryonic growth as well as behavior. Further, we have found a number of amino acid substitutions between the PO and BW alleles.

Table 2.—Summary of selected *Peromyscus* crosses. LOI = loss of imprinting. BW = strain of *P. maniculatus*, PO = strain of *P. polionotus*, F_1 = offspring of female BW crossed with male PO, G_3 = result of intercrossing the F_1 animals for 2 generations. Female genotype shown 1st in all crosses. * = mean substantially reduced due to dead or resorbing conceptuses. † = although the BW × PO conceptuses exhibited a small amount of LOI, here LOI will refer to the genes observed to lose imprinting in the PO × BW hybrids. Lethality refers to observed lethality in the cross offspring.

Cross	Placental weight (mg)		LOI	Lethality
	Range	Mean		
BW × BW	0.078–0.241	0.181	N	N
PO × PO	0.098–0.250	0.181	N	N
BW × PO	0.038–0.145	0.096	N†	N
PO × BW	0.232–0.776	0.511*	Y	Y
F_1 × BW	0.105–0.439	0.21	N	N
G_3 × BW	0.104–0.730	0.293	Y	Y
F_1 × F_1	0.030–0.344	0.138	N	N
G_3 × G_3	0.033–0.538	0.205	Y	Y
PO × F_1	0.096–1.088	0.315*	Y	Y

However, comparative genomic analyses suggest that the entire domain is rapidly evolving: domestic cows, humans, and mice show differences in both gene content and imprinting status. For example, *Zim2* is another paternally expressed zinc-finger gene that overlaps *Pw1*/*Peg3* (sharing some exons) in humans (Kim et al. 2000). *Zim2* is present in *Mus*, but does not share exons with *Pw1*/*Peg3*, and is maternally expressed. The *Mus* genome has another maternally expressed zinc-finger gene, *Zim1*, that lies between *Zim2* and *Pw1*/*Peg3*. Both the human and domestic cow genomes lack *Zim1*; the latter has an unrelated, nonimprinted gene termed *Ast1* in the equivalent position (Kim et al. 2004).

However, these 2 mapped loci do not explain all the PO × BW phenotypes. Both backcrosses can contribute PO alleles maternally and BW alleles paternally; both yielded overgrowth. However, the female F_1 × male BW offspring did not exhibit the loss of imprinting, aberrant morphologies, or the degree of placental overgrowth observed in the PO × BW F_1 cross (Table 2). In contrast, the female PO × male F_1 cross produced litters that exhibited all the attributes of the PO × BW cross, including maternal lethality due to inability to pass term fetuses through the birth canal.

The difference between the 2 crosses was suggested by another cross: we 1st bred the small hybrid animals together in nonsibling matings for 2 generations to create a 3rd generation (G_3) intercross. We then performed a series of crosses in which G_3 females were mated with BW males. We expected this cross to yield the same phenotypic range as the F_1 × BW cross: in both cases the maternal genome is an equivalent mix of the 2 strains, whereas the paternal genome is BW. The 3rd generation intercross animals differ in 1 important aspect from F_1 animals: whereas both types would contain roughly 50% BW and 50% PO alleles, all F_1 mice would be uniformly heterozygous at all autosomal loci (Fig. 7). In contrast, individual G_3 animals would either be heterozygous, or

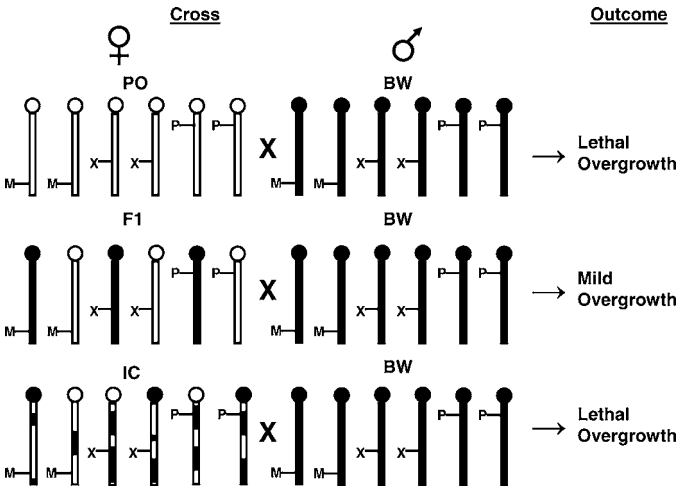

FIG. 7.—Genetic model of overgrowth in *Peromyscus* crosses. Three pairs of chromosomes are represented for males and females in each cross. One gene on each chromosome is represented: M = maternal effect gene, X = X chromosome linked gene, P = *Pw1*/*Peg3* linked paternally expressed gene. PO = strain of *P. polionotus*, BW = strain of *P. maniculatus*. Unfilled areas of chromosomes represent PO genotype; filled areas of chromosomes represent BW genotype. F_1 in cross refers to the small BW × PO hybrid females; IC refers to intercross females derived from BW × PO animals interbred for multiple generations (e.g., G_3 = 3rd-generation intercross). At top the PO × BW hybrid cross is illustrated. Next is an female F_1 crossed with a male BW. Note that this cross results in mild overgrowth due to the interaction of the PO X locus, and the BW P locus. Because the female F_1 animal is heterozygous for the M locus, however, the extreme (lethal) overgrowth and associated loss of imprinting are not present. In contrast, IC females may be homozygous for the PO allele of the M gene, resulting in lethal overgrowth. IC females also may be heterozygous (or homozygous BW) for the M locus (last cross). Note that the only difference between the 2 female IC × male BW crosses is homozygosity at the M locus.

homozygous for the BW or the PO allele of each gene. The female G_3 × male BW crosses yielded a number of litters that exhibited more extreme overgrowth, developmental abnormalities, and loss of imprinting. The differences between the F_1 × BW and G_3 × BW crosses suggested that maternal homozygosity of PO alleles might underlie the loss of imprinting and associated defects (Fig. 7).

Comparing an F_1 × F_1 (using the small hybrids) cross with a G_3 × G_3 cross revealed a similar effect. Again, both females are an equal mix of PO and BW alleles, as are the males. Both crosses produce a spectrum of sizes from undergrowth to overgrowth. However, more severe overgrowth appears in the G_3 × G_3 cross, but not the F_1 × F_1 cross (Table 2). Examination of a number of crosses has supported our maternal effect hypothesis (Duselis et al. 2005). The maternal effect appears to be due to a small number of loci, and not to be associated with the *Dnmt* genes. The PO maternal effect gene product(s) are predicted to be essential to normal imprinting, but cannot recognize or maintain imprints in the presence of incoming foreign BW paternal DNA or other sperm factor.

Our preliminary analysis of the undergrowth phenotypes also indicated involvement of an X chromosome locus. The *Esx1* gene appears to be proportionately overexpressed in the BW × PO placentas. However, we have detected no linkage to *Pw1*/*Peg3* or nearby imprinted genes. Ongoing analysis of the BW × PO animals suggests some changes in imprinted gene expression levels without formal loss of imprinting.

One major question is whether the *Peromyscus* hybrid effects are limited to the populations from which the BW and PO strains were derived. We have tested crosses with BW and the *P. polionotus* subspecies *leucocephalus* (strain LS), originating from individuals on Santa Rosa Island, Florida. We have not detected any differences between PO × BW hybrids and LS × BW hybrids. Further, PO × LS hybrids do not exhibit any abnormal prenatal growth phenotypes, or any loss of imprinting.

Several subspecies of *P. maniculatus* limited to the Pacific Northwest have been designated a separate species, *P. keeni* (Chirhart et al. 2005). Several preliminary studies have suggested that *P. keeni* crosses with neighboring subspecies of *P. maniculatus* yield nonviable offspring and exhibit parent-of-origin effects (Dice 1949; Liu 1954). Several other former subspecies of *P. maniculatus* also have been proposed to have species-level status (Chirhart et al. 2005). It would be particularly interesting to assess gene flow in these *Peromyscus* populations at the X-linked and imprinted regions we have already implicated in the hybrid dysgenesis. I predict that these same regions will stand out in showing disjunct allele flow between populations. It may also be informative to assess patterns of DNA methylation at specific differentially methylated regions across the *P. maniculatus* complex. Very few data are available on population-level epigenetic variation in any species.

To further assess the generality of the *P. maniculatus bairdii* × *P. polionotus subgriseus* hybrid effects, several experiments appear paramount. First would be to assess imprinted gene status and growth effects in hybrid fetuses. An easier assay is to test whether DNA methylation or other epigenetic marks are altered in hybrid animals. Mating patterns also should be assessed to determine if they differ between the 2 populations.

ENVIRONMENTAL EFFECTS ON EPIGENETIC GENE REGULATION

Epigenetics is a still-emerging field that has relevance for many areas of biology. One of the most important but least understood aspects of epigenetic gene regulation is the role of the environment. Examination of data to date suggests that environmental effects on epigenetics are significant. Domestic cow embryos that have been produced by in vitro fertilization often exhibit overgrowth and corresponding misexpression of imprinted genes (Allen and Reardon 2005; Blondin et al. 2000). Human children derived from in vitro fertilization exhibit a much higher incidence of Beckwith–Wiedemann and other syndromes associated with misregulation of imprinted genes (Allen and Reardon 2005; Niemitz and Feinberg 2004). Studies with *Mus* embryos have shown that even small changes in culture media may affect epigenetic marks and the expression levels of imprinted genes (Doherty et al. 2000). Again, extraembryonic tissues seem particularly sensitive to these effects (Mann et al. 2004).

Although the cultured embryo studies are suggestive, they involve unnatural environments. The ''Fetal Programming of Adult Disease'' hypothesis posits that even small alterations in fetal growth due to changes in maternal diet or environment result in increased susceptibility to cardiovascular disease, certain cancers, and other ailments decades later (Barker 1992, 1993, 1994). Although we do not know how these signals are transduced, perhaps maternal signals influence fetal epigenetic marks. One example of such an effect involves the hypoxia-inducible factor proteins in the developing placenta. The hypoxia-inducible factor proteins sense O_2 levels, in this case those in the maternal uterine blood vessels. Interestingly, a class of enzymes that modulate epigenetic marks, histone deacetylases, have been shown to be reliant on hypoxia-inducible factor activity in developing placental cells (Maltepe et al. 2005). In turn, histone deacetylase activity affects the proportions of cell types, presumably via gene expression. Hypoxia-inducible factor activity has also been implicated in activating *Igf2*, further linking imprinted genes and pathways in these critical events (Feldser et al. 1999).

More striking is a recent study of human monozygotic twins that strongly suggests that environmental effects on epigenetic marks are inevitable during postnatal life (Fraga et al. 2005). Global patterns of DNA methylation and histone acetylation are nearly identical in most 3-year-old monozygotic twins. In contrast, 50-year-old twins inevitably exhibited significant differences in these profiles. Further, differences in level of gene expression were correlated with the degree of variation in epigenetic marks.

Nearly entirely unexplored are potential situations where altered gene expression via epigenetic change is an adaptive response to an environmental change. Clearly, the potential benefits of such changes are enormous. Haig (2004) has noted that under the kin selection hypothesis, it would be advantageous to modulate genic imprinting status based on such factors as interrelatedness and potential length of relationship. Such changes could potentially be mediated by pheromones. A recent study shows that male and female *Mus* avoid urinary odors of their maternal, but not paternal, origin (Isles et al. 2002). Thus imprinted gene–environmental interactions also may play a role in dispersal patterns. I suggest that epigenetic changes underlie the complex interplay between genetics and environment.

Imprinted genes can be regulated by these same epigenetic changes, and have been shown to be involved in mammalian growth, metabolism, and behavior. Differential growth underlies not only overall size differences, but within specific tissues may underlie other morphological changes as well. I predict that changes in control of imprinted gene expression or imprinted gene interactions will be shown to be a major force underlying the spectacular diversity seen in mammalian size, morphology, and behavior.

Acknowledgments

I am grateful for many discussions with S. Tilghman, W. Dawson, M. O'Neill, J. Fossella, R. Ingram, and M. Dewey in developing this research program. Awards from the March of Dimes and American Cancer Society made this research possible. I thank J. Weston, S. Lipkin, and A. Duselis for discussion and commentary on this manuscript.

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Special Features Editor was Edward J. Heske.

APPENDIX I

Internet resource sites.—Maps of imprinted domains and imprinted gene function: http://www.mgu.har.mrc.ac.uk/imprinting/imprinting.html; parent-of-origin effect listing and interspecific comparisons of imprinting status: http://igc.otago.ac.nz/home.html; listings of genomic imprinting meetings and key papers: http://www.geneimprint.com; comparative placentation: http://medicine.ucsd.edu/cpa/placenta.html; *Peromyscus* Genetic Stock Center: http://stkctr.biol.sc.edu/; Riken candidate imprinted transcript map: http://fantom2.gsc.riken.go.jp/imprinting/; complete mammalian genomes: http://www.ensembl.org/index.html. All Web site URLs were valid as of August 2006.