Mapping and identification of candidate loci responsible for *Peromyscus* hybrid overgrowth

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Abstract

Crosses between two recently diverged rodent species of the genus *Peromyscus* result in dramatic parent-of-origin effects on growth and development. *P. maniculatus* females crossed with *P. polionotus* males yield growth-retarded conceptuses, whereas the reciprocal cross results in overgrowth and lethality. These hybrid effects are particularly pronounced in the placenta. We previously detected linkage to two regions of the genome involved in the overgrowth effects. One locus, termed *Peal*, is a paternally expressed autosomal locus mapping to a domain whose house mouse equivalent contains several clusters of imprinted genes. The other locus, termed *Mexl*, maps to a gene-poor region of the X chromosome. Here we use an advanced intercross line to verify and narrow the regions of linkage and identify candidate genes for *Mexl* and *Peal*. While we have previously shown that *Mexl* affects both pre- and postnatal growth, we show here that *Peal* affects only prenatal growth. Utilizing criteria such as mutant phenotypes and allelic expression, we identify the loci encoding the homeobox protein *Esx1* and the zinc-finger protein *Pw1/Peg3* as candidates. Both loci exhibit expression changes in the hybrids.

Introduction

Rodents of the genus *Peromyscus* are the most common indigenous North American mammals. *Peromyscus maniculatus*, the deer mouse, is the most widely distributed of the group, ranging from Alaska to Mexico City, from sea level to 14,000 feet elevation, and from the Pacific to Atlantic Oceans (Dewey and Dawson 2001). Despite near ubiquity in North America, *P. maniculatus* is largely excluded from the southeast United States. This region is inhabited by a recently diverged sister species, *P. polionotus* ("oldfield mouse").

Crosses between captive strains of *P. maniculatus* (BW) and *P. polionotus* (PO) yield striking parent-of-origin effects on growth (Dawson 1965; Rogers and Dawson 1970). Female BW animals crossed with PO males (bw × po) produce animals that exhibit prenatal growth retardation relative to the parental strains. The bw × po growth effects are particularly pronounced in the placenta, which average half the size of those of either parental genotype. The undergrowth persists through the life of the bw × po offspring, which are fertile and healthy.

Conversely, offspring of female PO animals crossed with BW males (PO × BW) exhibit placental and somatic overgrowth. A variable suite of phenotypes accompanies the PO × BW overgrowth; these range from conceptuses where there is no embryo proper (i.e., only placental and extra-embryonic tissues are present) to those that survive to late gestation. Typically the latter are deformed and oversized to the point that the mother dies at parturition. The very few PO × BW that have survived postnatally have been oversized and female (Dawson et al. 1993). These *Peromyscus* crosses represent a rare case in which the genetics of mammalian reproductive isolation may be examined.

The parent-of-origin effects seen in the F[1] crosses suggested that genes whose expression is dependent on parental origin might underlie the hybrid dys-

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genesis. Such loci include mitochondrial, sex chromosome-linked, and genes subject to genomic imprinting. Mitochondrial-nuclear genotype interactions have been ruled out as a source of the effects (Dawson et al. 1993). Imprinted genes are autosomal genes that show preferential expression of one parental allele over the other. Many imprinted genes are involved in growth pathways and are expressed in the placenta (Reik et al. 2003).

The X chromosome also shows parental gene expression bias. For example, the paternal copy is preferentially silenced in house mice (*Mus musculus*) and bovine extra-embryonic tissues (Takagi and Sasaki 1975; Xue et al. 2002). Moreover, the X chromosome is known to contain a disproportionately large number of placental genes (Khil et al. 2004; Moore et al. 2005).

To investigate the genetics of the overgrowth, we initially performed two backcrosses. In the first backcross we mated F_1 females (bw × po) with BW males (F_1 × BW hereafter). The second cross series consisted of female PO animals crossed with F_1 males (PO × F_1). We expected both backcrosses to mimic the overgrowth phenotypes seen in the PO × BW F_1cross, because both could contribute PO alleles maternally and BW alleles paternally.

We mapped two loci associated with overgrowth through genotypic analysis of the offspring from the two crosses: one linked to the paternally expressed gene *Pw1/Peg3*, and one linked to the X chromosome (Vrana et al. 2000). We showed that X chromosome loci are imprinted (maternally expressed) in *Peromyscus* extra-embryonic tissues as in *Mus*. Thus, both regions of linkage were consistent with the parent-of-origin effects.

We have termed the X-linked locus *Mexl* (Maternal Expressed X-linked Locus) and the *Pw1/Peg3*-linked locus *Peal* (Paternal Expressed Autosomal Locus). Both backcrosses could contribute the PO allele of *Mexl* maternally and the BW allele of *Peal* paternally to produce overgrowth. The linkage to each locus, however, is revealed in only one of the two backcrosses, depending on whether the paternal or maternal genome is varied in that cross.

We also previously performed a cross between F_1 (bw × po) females × F_1 (bw × po) males (Vrana et al. 2000). As expected, this cross yielded small, normal, and oversized offspring. Furthermore, the F_1 × F_1 offspring genotypes suggested a negative interaction between the two mapped loci (*Mexl* and *Peal*). That is, when both parental genotypes varied, we saw a significant reduction in those that had a maternally derived PO *Mexl* allele and those that had a paternally derived BW *Peal* allele.

While both backcrosses (PO × F_1 and F_1 × BW) did in fact yield both normal and oversized offspring, the PO × F_1 cross better recapitulated the F_1 phenotypes. The offspring of the PO × BW cross exhibited extreme placental and somatic overgrowth, multiple developmental abnormalities, and loss of imprinting (LOI) of certain genes (Vrana et al. 1998). LOI is characterized by a switch from monoallelic to biallelic expression, indicating a perturbation in control of genomic imprinting. In contrast, the offspring of the F_1 × BW cross exhibited mild placental/somatic overgrowth, little embryonic lethality or morphologic abnormalities, and no discernible LOI.

We examined the disparity between these two crosses and have provided genetic evidence for a maternal effect involved in the LOI (Duselis et al. 2005). This maternal effect appears to require PO homozygosity in mothers of affected offspring and to be due to a small number of loci. The maternal effect appears to be a separate mechanism contributing to the hybrid overgrowth. Thus, we hypothesize that the combination of the maternal effect and the *Mexl*-*Peal* interaction are responsible for the full hybrid overgrowth and suite of associated characteristics.

To further explore the genetics of the hybrid overgrowth, we have confirmed and refined the areas of linkage through a combination of backcrosses and intercrosses. Because no *Peromyscus* genome is yet mapped, we developed additional genetic markers for these regions. We surveyed these areas for candidate loci and investigated several through sequence identity and gene expression. We focused particularly on *Pw1/Peg3* and *Esx1* because they were the first candidate genes identified for *Peal* and *Mexl* (respectively) and because they remained candidates through the genetic analyses.

Materials and methods

Animal breeding/husbandry. We purchased BW and PO animals from the *Peromyscus* Genetic Stock Center (http://stkctr.biol.sc.edu/). We bred BW females with PO males as the basis of the advanced intercross line. These hybrid animals were then interbred for 2 more generations in the case of the G_3 animals, 9 more generations in the case of the G_{10}s, and 12 more generations in the case of G_{13}s. Animals picked to breed for the next generation were chosen randomly. Animals were housed with food and water *ad libitum* on a 16:8 light:dark cycle.

We collected embryos and placentas from pregnant females, using morphologic criteria and (when applicable) time since birth of previous litters to ascertain developmental stage. Only placentas from pregnancies determined to be past 14.5 days gesta-

Table 1. Placental weights (in grams) of *Peromyscus* crosses

Cross	Range	Mean	SD
BW × BW	0.078–0.241	0.181	0.043
PO × PO	0.098–0.250	0.181	0.036
BW × PO	0.038–0.145	0.096	0.027
PO × BW	0.232–0.776	0.511	0.149
G10/13 × BW	0.127–0.673	0.277	0.091
PO × G3/8	0.102–0.612	0.257	0.114

Backcross data include all mature placentas from this study. Parental and F$_1$ data from Duselis et al. (2005).

tion (i.e., mature placental weight) were used for comparisons with results from other crosses. The upper weight limit for placental weights in the parental strains was 0.25 g (Table 1). To allow for any unseen parental variation, we considered any placenta ≥0.3 g overgrown.

Development of markers and genotyping. Genes used as markers were chosen based on their location in the linkage areas. *Mus* and *Homo* sequences were obtained from ENSMBL (http://www.ensembl.org) and aligned. Areas in the 5′ or 3′ untranslated regions (UTRs) or small exons and introns that had a high level of conservation were used to design primers. Sequence was obtained from the PO and BW strains by PCR and cloned into the pCR 2.1-TOPO vector (Invitrogen Life Technologies, Carlsbad, CA). Several clones of each allele were sequenced to obtain the most accurate sequence. Sequences were then examined for restriction fragment length polymorphisms (RFLPs) using Vector NTI or TACG (http://workbench.sdsc.edu).

We isolated genomic DNA from embryonic tissues using the Qiagen whole-tissue kit (Qiagen, Valencia, CA) and genotyped them by PCR RFLP analysis. In addition, some *Prdx4* genotypes were ascertained by direct sequencing of PCR products amplified with the primers listed below. Digested PCR products were electrophoresed on 7.5% acrylamide gels and scored as BW, PO, or heterozygotes. *Pw1/Peg3*, *Myod1*, and *Xist* genotyping assays were as described in Vrana et al. (2000). We used modified versions of the earlier *Plp1* and *Xist* assays, which are described below.

Primers used for each respective gene were as follows: CkmF: AAC ACC CAC AAC AAG TTC AAG CTG, CkmR: GTC TGG ATG ACA TCG TCY ASA GTG; EsxF6: GTT TGG TTT CAG AAC AGA AGG, EsxR6: GAT ATT TCC AAA TAA CCT CCA CA; Glra2F: TCA GGA AAA TGC TGC CTC GT, Glra2R: ACA CAC CAT CTC CAT AAA TAC TGT

AG; Gpi1F: GTG TGG ACC ACC AGA CAG G, Gpi1R: TTC CGT ATG GGG TGC TGG GTC; Irs4F: TAG GCT TGC TCC CGC TGG A, Irs4R: RAG CCC CGG CCA TTT CCT GAG C; Plp1F3: GGT CCC AGA GAG AAT TAG GCT AGA, Plp1R1: GTA CAC AGG CGC AGC AGA GCA; Prdx4F: TTY YGC GCT YGC KTG GTC ATG GA, Prdx4R: TCT TSG CTT TGC TTA GRT GCA G; XistF2: CTC CCC CTT TAA GAG GGA ACT TGA, XistR2: TAC CCG GAA GTG ACC ACA AT. Annealing temperatures and restriction enzymes used were as follows: *Ckm:* 57°C, *Bst*UI; *Esx1:* 52°C, *Mbo* I ; *Glra2:* 59°C, *Hpy*CH4IV; *Gpi1:* 62°C, *Aci* I; *Irs4:* 64°C, *Bme*1580I; *Plp1:* 58°C, *Mse*I; *Prdx4:* 52°C, *Fnu*4HI; *Xist:* 52°C, *Bst*NI.

Gene expression. RT-PCR was performed on 1.0 μg of DNase I-treated RNA using TaqMan® RT-PCR reagents (Applied Biosystems, Foster City, CA). TaqMan assays for *Pw1/ Peg3*, *Esx1*, and an endogenous control, *Rpl32*, were obtained through the Assays-by-Design service offered by Applied Biosystems after submitting *Peromyscus* sequence for these genes. In each reaction approximately 75 ng of cDNA was used along with 2.5 μl of 20 × assay mix and TaqMan Universal Mastermix-No UNG (Applied Biosystems) for a total reaction volume of 50 μl. Reactions were done in triplicate in 96-well plates and run on an ABI 7900 real-time PCR instrument. Two or more samples were analyzed for each parental strain and each reciprocal hybrid. Data were analyzed and expression levels calculated as per the manufacturer's recommendations. Northern analysis was performed by standard methods. Quantitation and visualization was conducted on an Typhoon 9400 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Results

***Crosses used to localize* Mexl.** To refine the area of linkage on the X chromosome, we first bred (bw × po) F$_1$ animals together for multiple generations to create an advanced intercross line (AIL) (Darvasi and Soller 1995). Advanced intercross females were then bred to BW males. We reasoned that such "advanced backcrosses" would enable us to localize the area of the X chromosome responsible for the overgrowth without potential confounding effects of allelic variation at interacting paternal loci.

Our prior analyses were based largely on weight of mature placentas as the result of several factors. First, some PO × BW, PO × AIL, and AIL × BW matings resulted in conceptuses that consisted of only placentas and associated extra-embryonic tis-

Table 2. Maternal genotypes of the 20 largest AIL × BW placentas

Sex	Plac Wt	Xist	Plp1	Esx1	Irs4	Prdx4	Glra2
M	0.327	PO	BW	PO	BW	PO	BW
F	0.328	PO	PO	PO	PO	PO	BW
M	0.333	PO	PO	PO	PO	PO	BW
F	0.334	PO	PO	PO	PO	PO	PO
F	0.338	PO	PO	PO	PO	PO	BW
M	0.338	PO	PO	PO	PO	PO	BW
F	0.347	PO	PO	PO	PO	PO	BW
F	0.357	PO	PO	PO	BW	PO	PO
M	0.358	PO	PO	PO	PO	PO	BW
F	0.359	PO	PO	PO	PO	PO	BW
M	0.362	BW	PO	PO	BW	BW	BW
F	0.372	PO	PO	PO	PO	PO	PO
M	0.397	PO	PO	PO	PO	PO	BW
M	0.419	PO	PO	PO	PO	PO	BW
M	0.43	PO	PO	PO	PO	PO	BW
M	0.453	PO	PO	PO	PO	PO	BW
F	0.456	PO	PO	PO	PO	PO	BW
M	0.476	PO	PO	PO	PO	BW	PO
M	0.59	PO	PO	PO	BW	PO	BW
M	0.673	PO	PO	PO	PO	PO	BW

Note that only *Esx1* genotypes completely cosegregate with the overgrowth.

sues. Second, those crosses that produced severe overgrowth frequently resulted in maternal lethality. Third, it was very difficult to ensure exact time of embryonic development and thus compare weights of the embryo proper. In contrast, maximum placental weight is reached in *Peromyscus* at the equivalent stage as in *Mus*, at approximately 14.5 days of gestation (E14.5).

We had previously undertaken two crosses in which maternal genotype varied. We established X linkage for both adult body weight and placental weight in an F_1 female × BW male cross. We then bred a third generation AIL (G_3) female × male BW cross resulting in 87 offspring. Placental overgrowth in these embryos had cosegregated most closely with a marker for the gene *Plp1* (*Mus* position = 56 cM). The next closest marker in the initial analysis was that for the *Xist* gene (*Mus* position = 42 cM) (Vrana et al. 2000).

Fluorescence *in situ* hybridization (FISH) analysis has confirmed that the whole *Mus* X chromosome is syntenic with the *Peromyscus* X chromosome (Dr. Rachel O'Neill, University of Connecticut, personal. communication) Furthermore, gene order on the X chromosome appears to be generally conserved between the two species (Vrana et al. 2000). We first developed additional markers corresponding to genes on the distal portion of the X chromosome, where we believed *Mexl* to lie. These markers included genotyping assays for the genes *Esx1* (*Mus* position 57.0 cM), *Irs4* (58.0 cM), *Prdx4* (67.7 cM), and *Glra2* (72.0 cM).

After the previously published work, we next genotyped the G3 × BW offspring with the additional distal X chromosome markers in an attempt to refine the area of phenotypic linkage. In this cross, however, *Esx1* genotypes were identical to those of *Plp1* for all placentas larger than the parental range. We therefore bred AIL females of greater generation numbers to BW males in the expectation that their offspring would provide greater numbers of recombinant offspring.

Specifically, we utilized both G_{10} and G_{13} females in backcrosses to BW males. The two crosses yielded 86 conceptuses of age E14.5 or later. These conceptuses yielded placentas with a spectrum of weights from those within the parental weight range to those exhibiting extreme overgrowth (Table 1). Note that the parental strains show the same mean weight of approximately 0.18 g. We also included eight younger conceptuses whose placental weight already exceeded the maximum weight seen in both parental strains. Thus, we genotyped 94 total offspring of these crosses with the distal X chromosome markers and compared the results with placental weights.

Esx1 maternal genotypes showed the greatest correlation with the largest placentas (Table 2), suggesting this is the closest marker to the *Mexl* locus. The distribution of maternal *Esx1* genotypes by placental weight over all the offspring AIL female × BW male cross is shown in Fig. 1. These data reveal that the PO *Esx1* genotypes are also found in normal-sized placentas and that they constitute
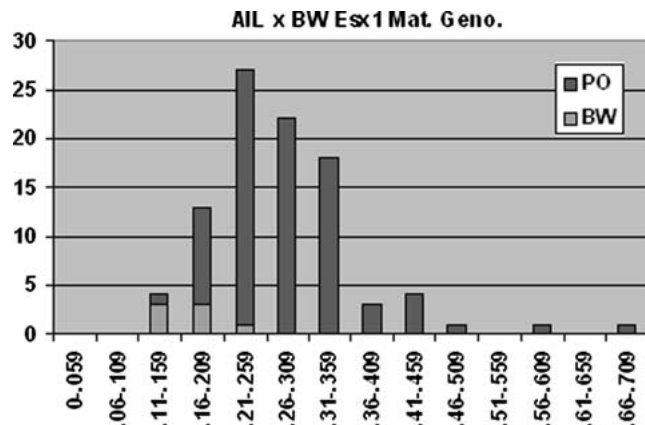
Fig. 1. Distribution of the maternal *Esx1* alleles in the AIL × BW crosses. Vertical axis represents number of animals, horizontal axis indicates placental weight in grams.

greater than 92% (87/94) of the total maternal genotypes. We had previously found substantial evidence for allele fixation at multiple autosomal loci in the AIL (Duselis et al. 2005): seven of nine loci tested yielded chi-squared values corresponding to p values of 0.05 or lower. In this case, approximately 31 of the 94 maternal *Esx1* alleles are predicted to be of the PO genotype (one third of the original intercross X alleles were a PO genotype). Analysis of this disparity results in a chi-squared score of 101 ($p < 0.0005$), suggesting that the *Esx1* genotypes in the AIL are extremely skewed.

Gene order in this distal portion of the X chromosome appears to be different than that in *Mus*, where the gene order is (proximal-distal) *Xist*, *Plp1*, *Esx1*, *Irs4*, *Prdx4*, *Glra2*. An analysis of the data in Table 2 using the Map Manager software (Manly and Olson 1999) suggests the gene order in *Peromyscus* is *Prdx4*, *Xist*, *Esx1*, *Plp1*, *Irs4*, with *Glra2* either proximal or distal to the other loci.

***Crosses used to localize* Peal.** To localize the paternally expressed gene affecting hybrid growth (*Peal*), we undertook crosses in which the paternal genome was varied but the maternal genome remained constant. These crosses therefore were PO females crossed with AIL males. Crosses in which the paternal genome is varied but the maternal genome is PO are more difficult to undertake than the reciprocal because of the maternal effect on genomic imprinting control (Duselis et al. 2005). These PO × AIL crosses always result in offspring with some degree of imprinting perturbation and also often in maternal lethality. Resorptions are also common in these crosses, resulting in our harvesting embryos from approximately one quarter of matings. We obtained 16 litters from PO × AIL (G_3 and G_{13}) crosses, resulting in 96 mature placentas for

Table 3. Paternal genotypes of the 20 largest PO × AIL placentas

Plac Wt	Sex	Ckm	Pw1	Gpi1	Myod1
0.344	M	BW	BW	BW	BW
0.346	F	BW	BW	BW	BW
0.351	M	BW	BW	BW	BW
0.352	F	BW	BW	BW	BW
0.354	M	BW	BW	BW	BW
0.358	F	BW	BW	BW	BW
0.362	F	BW	BW	BW	BW
0.385	F	BW	BW	BW	BW
0.391	F	BW	BW	BW	BW
0.419	F	BW	BW	BW	PO
0.421	M	BW	BW	BW	PO
0.428	M	BW	BW	BW	PO
0.447	M	BW	BW	BW	BW
0.451	M	BW	BW	BW	BW
0.452	F	BW	BW	BW	BW
0.523	F	BW	BW	BW	BW
0.561	F	BW	BW	BW	BW
0.568	F	BW	BW	BW	BW
0.610	M	BW	BW	BW	BW
0.612	M	PO	BW	PO	PO

Note that only *Pw1/Peg* genotypes completely cosegregate with the overgrowth.

genetic analysis. As in the PO × F$_1$ cross, these offspring displayed mild to severe placental and somatic overgrowth with no obvious sexual dimorphism (Table 1).

We had previously detected linkage of the overgrowth phenotype to the *Pw1/Peg3* locus using a PO female × F$_1$ male cross. *Pw1/Peg3* is located on *Mus* Chromosome 7 at 6.5 cM. *Mus* whole Chromosome 7 FISH probes hybridize entirely to *Peromyscus* Chromosome 1 (Dawson et al. 1999), suggesting that gene content in this region is conserved. Therefore, we designed genotyping assays for other loci located on *Mus* proximal Chromosome 7. The new assays include those for *Ckm* (*Mus* location = cM 4.5) and *Gpi1* (cM 11.0). We genotyped the PO × AIL offspring with these as markers as well as those for *Pw1/Peg3*, *Myod1* (cM 23.5), and *H19* (cM 69.0). The latter, however, did not show any genotype-phenotype association (data not shown).

Pw1/Peg3 genotypes exhibited the tightest correlation with placental overgrowth; all placentas above 0.35 g had a BW paternal *Pw1/Peg3* genotype. In contrast, at least one of these hyperplastic placentas had a PO genotype at *Ckm*, *Gpi1*, and *Myod* (Table 3). A complete distribution of paternal *Pw1/Peg3* genotype over the placental weight range reveals the roughly normal distribution of PO alleles around the parental mean, in contrast to the skewed distribution of the BW alleles (Fig. 2). The allelic content of the cross, however, was not skewed: 57 of the 96 genotyped animals had a BW paternal allele.
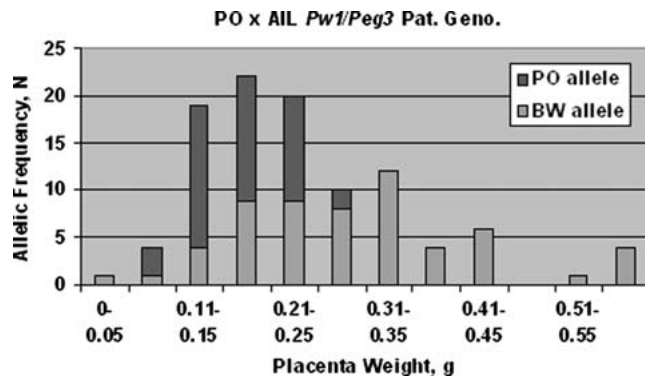
Fig. 2. Distribution of the paternal *Pw1/Peg3* alleles in the PO × AIL crosses. Vertical axis represents number of animals, horizontal axis indicates placental weight in grams.
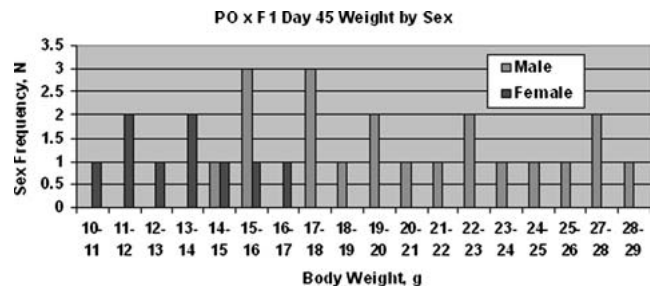

Fig. 3. Distribution of sex and weight in the PO × F1 cross at postnatal day 45. Vertical axis represents number of animals, horizontal axis indicates body weight in grams. Parental mean body weights (± one standard deviation) are as follows : PO♀ = 15.3 ± 1.2, PO♂ = 12.4 ± 0.4, BW♀ = 15.5 ± 1.6, BW♂ = 17.4 ± 1.4.

To estimate how great an increase in resolution these crosses provided, we compared recombination rates between the F_1 and AIL backcrosses. The PO × F_1 cross produced four recombinants between *Pw1/Peg3* and *Myod1* in 109 animals for a distance of 3.7 cM. In contrast, there were 13 recombinants between these two loci in the 103 PO × AIL offspring (12.6 cM). As expected, the majority of the recombinants were found in the later generation AIL crosses. Recombination frequencies between all gene pairs used in mapping *Peal* are shown in Table 4.

The recombination frequencies confirmed the linkage of these loci on *Peromyscus* Chromosome 1 as on *Mus* Chromosome 7. The *Mus* recombination map, however, differs from the physical map in the relative positions of *Ckm* and *Pw1/Peg3*. Our data suggest that the gene order determined for *Peromyscus* (*Pw1/Peg3*, *Ckm*, *Gpi1*, *Myod1*) is the same as that for the *Mus* (as well as *Rattus*) physical map (http://www.ncbi.nlm.nih.gov/mapview/).

We also tested whether *Pw1/Peg3* genotype was also associated with postnatal overgrowth. The PO × F_1 backcross produced a limited number of survivors, which were weighed at 45 days after birth. Unlike the conceptuses, the postnatal survivors displayed considerable sexual dimorphism (Fig. 3), with only a subset of males exhibiting overgrowth.

Note that all males from this cross have the same sex chromosome genotypes (X^{PO} Y^{PO}). *Pw1/Peg3* genotypes, however, showed no correlation with body weight (Table 5). In contrast, earlier studies had already demonstrated that *Mexl* is associated with both prenatal and postnatal growth (Vrana et al. 2000). These data suggest that other autosomal loci may be involved in the overgrowth phenotypes.

Identification of* Mexl *and* Peal *candidate loci. We examined the Mouse Genome Informatics Database (http://www.informatics.jax.org/) for known genes between 50 and 80 cM of the *Mus* X chromosome. Our criteria for candidate gene identification were (1) loci in the region that show mutant phenotypes similar to those of the *Peromyscus* hybrids; (2) genes that exhibit great divergence between mouse and human (suggesting rapid evolution that might lead to the interspecific negative interaction); (3) genes thought to be directly involved in growth pathways (e.g., by cell culture assays or paralogy); and (4) genes highly expressed in the placenta.

Another potential criterion for *Mexl* candidate genes is allelic expression; the genetic analyses suggest it is a maternally expressed gene. Prior work

Table 4. Gene order on *Peromyscus* Chr 1/*Mus* Chr 7

Gene pair	Mus Mb	Pero cM
Ckm − Pw1/Peg3	10.3	3.8
Pw1/Peg3 − Gpi1	23.4	4.9
Gpi1 − Myod1	10.8	8.7
Ckm − Gpi1	13.1	1.9
Ckm − Myod1	23.9	10.7
Pw1/Peg3 − Myod1	34.2	12.6

Distances between gene pairs are shown. House mouse (*Mus*) is in megabases (Mb) and *Peromyscus* (Pero) is in centimorgans (cM).

Table 5. *Pw1/Peg3* paternal genotypes of the ten largest PO × F_1 45-day animals

Body Wt	Sex	Pw1
20.2	M	BW
21.9	M	PO
22.1	M	PO
22.5	M	PO
23.2	M	BW
24	M	BW
25.9	M	PO
27.3	M	PO
27.9	M	BW
28	M	BW

Table 6. Potential candidate genes for *Mexl*

Gene	Function	Map	Criteria
Esx1	Placental Dev	57.0	Plac. Exp., Mutant Growth Pheno.
Fgd1	Nucleotide Exchange	64.0	Growth, Plac Exp.
Figf	C-fos Induced GF	70.0	Growth, Plac. Exp.
Irs4	Insulin Signaling	58.0	Mutant Growth Pheno.
Magea1-8	Unknown	66.1	Plac. Exp., altered in tumors
Ott	Unknown	62.5	Plac. Exp.
Phex	Proteolysis	65.4	Mutant Growth Pheno.

Map = cM position on *Mus* X chromosome. Growth = implicated in growth pathways; Plac. Exp. = placental expression; *Magea1-8* refers to multiple family members with the corresponding names.

has demonstrated, however, that the paternal X chromosome is preferentially inactivated in *Peromyscus* extra-embryonic tissues, as it is in *Mus* (Vrana et al. 2000). That is, the majority of the X chromosome is expressed from the maternal allele in the placenta. Genes in this region fitting one or more of the above criteria are listed in Table 6. Of the 86 genes in this interval, eight fit one and four fit at least two of these criteria. *Esx1* alone fulfills three of these criteria (i.e., 1, 2, and 4).

In contrast to the distal X chromosome, proximal *Mus* Chromosome 7 is gene dense. For example, there are at least 40 distinct genes within 1 cM of *Pw1/Peg3*. An additional criterion that may be used to identify candidate genes in this region is their allelic expression. That is, our genetic analyses strongly suggest that *Peal* is normally expressed exclusively from the paternal allele (Vrana et al. 2000). Like other autosomal imprinted domains, only a subset of genes in this region exhibit mono-allelic paternal expression. The known paternally expressed genes in this domain include *Usp29*, *Zfp264*, and *Pw1/Peg3* (http://www.mgu.har.mrc.ac.uk/research/imprinting/).

Comparative genomic analyses, however, suggest that this imprinted domain is rapidly evolving: Domestic cows, humans, and mice show differences in both gene content and imprinting status. For example, *Zim2*, like *Pw1/Peg3*, is a paternally expressed zinc-finger gene. The *Zim2* gene overlaps the *Pw1/Peg3* gene (sharing several small 5′ 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*, which lies between *Zim2* and *Pw1/Peg3*. Both the human and the domestic cow genomes lack *Zim1*; the latter has an unrelated, nonimprinted gene termed *Ast1* in the equivalent position (Kim et al. 2004).

The only imprinted gene in this region to be functionally characterized is *Pw1/Peg3*. This gene was originally discovered in a screen for novel myogenic regulatory factors. Further biochemical and cell-culture assays have revealed that the gene product acts in the tumor necrosis factor (TNF)/NFkappaB signal transduction pathway and has a key role in p53-mediated apoptosis (Relaix et al. 1998, 2000). The gene-targeting reduction in *Pw1/Peg3* product resulted in animals with both placental and somatic growth retardation as well as behavioral defects (Curley et al. 2004; Li et al. 1999). Thus, one might predict that overexpression of the gene resulting from the LOI seen in the PO × BW hybrids would lead to overgrowth.

Another locus that maps to the equivalent region in humans, 19q13.4, also affects placental growth (Panichkul et al. 2005). This locus influences susceptibility to biparental complete hydatidiform moles (BiCHM), which are characterized by the presence of hyperplastic trophoblast villi and lack of a fetus. Hydatidiform moles typically have either no maternal genome or have two paternal genomes (e.g., due to fertilization by two sperm) (Li et al. 2002; Szulman 1987). Recent studies, however, have confirmed the existence of hydatidiform moles that have normal biparental genomic contributions (BiCHM). The BiCHM tissues exhibit altered DNA methylation suggestive of LOI (Judson et al. 2002).

While the familial BiCHM susceptibility locus has been localized to the *Pw1/Peg3* domain, it does not correlate with coding region polymorphisms of the gene. The gene *NALP7*, known to be involved in apoptotic and inflammatory pathways, has emerged as the primary candidate for those BiCHM whose susceptibility locus maps to Chromosome 19 (Murdoch et al. 2006). The *Mus* genome, however, apparently lacks an ortholog in this region.

While we do not yet know the structure of this domain in *Peromyscus*, Northern analysis suggests that there are no genes sharing exons with *Pw1/Peg3*. We hybridized placental and embryonic RNA with multiple probes derived from regions equivalent to the *Mus* upstream exons and the large (~5 kb) final exon. All tests revealed only the approximately 9-kb transcript also observed in *Mus* (data not shown).
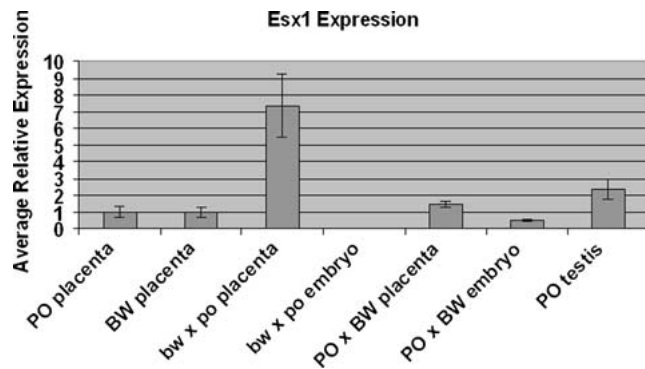
Fig. 4. Quantitative PCR analysis of *Esx1* expression. Each indicated genotype-tissue type combination represents multiple samples, with the exception of testis. Standard error bars are indicated.

Fig. 5. Quantitative PCR analysis of *Pw1/Peg3* expression. Each indicated genotype-tissue type combination represents multiple samples, with the exception of testis. Standard error bars are indicated.

Expression analysis of candidate loci. Upon identifying *Esx1* and *Pw1/Peg3* as candidate genes for *Mexl* and *Peal* (respectively), we asked whether expression levels varied between the two species and if perturbations occurred in the hybrid offspring. We tested expression levels in both genes via Northern analysis and quantitative PCR analysis.

Esx1 expression has been shown to be confined to the placenta and testis in *Mus*; Northern analysis of PO and BW mid-late gestation embryos suggests that this is also true in *Peromyscus*. However, we included hybrid embryos in the quantitative PCR assays in the event spatial expression was perturbed. *Esx1* did not exhibit discernible expression differences between the two parental strains nor between the oversized hybrids and the parental strains (Fig. 4). In contrast, the growth-retarded bw × po placentas exhibit substantial upregulation of the *Esx1* transcript. Note that the complete loss of *Esx1* results in an enlarged placenta (Li and Behringer 1998).

It is unclear whether *Esx1* is a viable candidate gene for the genetic incompatibilities underlying the hybrid growth retardation. While preliminary evidence suggests the involvement of the X chromosome in the genetics of the bw × po hybrid phenotypes, it is not yet clear whether the same region is involved. *Esx1* is predominantly expressed in the labyrinth region of the placenta. The other major layer of the muroid placenta, the spongiotrophoblast or junctional zone, is greatly reduced in the bw × po placentas (Rogers and Dawson 1970; Vrana et al. 2001). Therefore, the increased *Esx1* expression in bw × po may be a consequence of a relative overabundance of the expressing cells.

The *H19* gene is also predominantly transcribed in the labyrinthine portion of the *Peromyscus* placenta (Vrana et al. 2001). However, total *H19* expression levels in the bw × po placenta are no
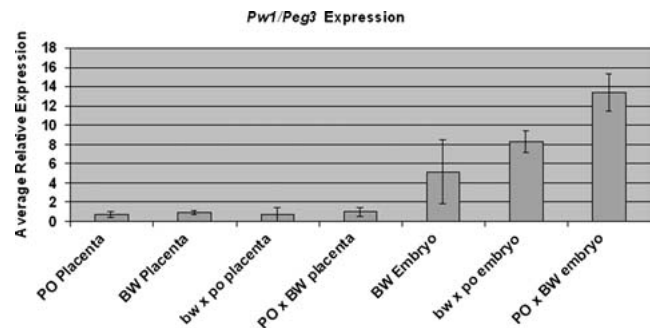
higher than that seen in either parental strain (Vrana et al. 1998). Thus, it is possible that the increased *Esx1* expression plays a causal role in the growth-retarded hybrid placentas.

We also tested *Esx1* allelic usage in the *Peromyscus* hybrids. Like other X-linked genes, *Esx1* was expressed exclusively from the maternal allele in extra-embryonic tissues (data not shown). In contrast, *Pw1/Peg3* has been shown to lose imprinting in the PO × BW cross (Duselis et al. 2005; Vrana et al. 1998). That is, the gene is expressed from both alleles in the oversized hybrids. Corresponding to this biallelic expression, we observed increased expression levels in PO × BW conceptuses, particularly in the embryo proper (Fig. 5). Surprisingly, *Pw1/Peg3* also showed increased expression in the bw × po embryos, despite maintaining strict paternal expression.

Discussion

These *Peromyscus* species and their hybrids offer a novel system for studying the role of natural genetic variation on epigenetic regulation, reproductive isolation, growth, and development. Here we have refined the areas of the genome containing two loci (*Mexl* and *Peal*) contributing to the hybrid parent-of-origin effects. *Peal* remains unbounded on one side. The combination of tight linkage to *Pw1/Peg3* in combination with requirement for paternal expression, however, will likely result in few other *Peal* candidate loci. As most X-linked loci are maternally expressed in rodent placentas, it would be difficult to identify *Mexl* candidates on the basis of allelic expression. *Mexl*, however, appears to be bounded by *Plp1* and *Esx1* (Table 2). These two loci lie only about 300 kb apart in *Mus* (http://www.ensembl.org/Mus_musculus). Thus, a comprehensive survey of this region in *Peromyscus* should not be difficult.

We have identified candidate genes for both loci, *Esx1* for *Mexl* and *Pw1/Peg3* for *Peal*. Genotypes of both candidate loci are completely concordant with placental hypertrophy in their respective backcrosses. While both genes show equivalent expression levels in mature placentas of the parental strains, this does not rule out interspecific regulatory changes. For example, the genes may show different temporal and/or spatial expression patterns.

Despite the apparent requirement of these alleles for hypertrophy, those particular genotypes (PO maternal allele of *Esx1*, BW paternal allele of *Pw1/Peg3*) did not always result in overgrowth. Our approach of crossing AIL animals to a parental strain rules out incomplete penetrance because of the necessity of genotypes at interacting loci inherited from the other parent.

It seems likely, however, that other alleles from the varying parental genome may act as modifiers to the phenotypes. For example, the lack of correlation of *Pw1/Peg3* genotypes with postnatal overgrowth in the PO × F_1 cross suggests the involvement of other loci contributing to the hybrid overgrowth. Furthermore, the PO × F_1 placental analysis revealed a class of mildly overgrown placentas that were also not dependent on *Pw1/Peg3* genotype (Vrana et al. 2000). Another unidentified locus affecting postnatal overgrowth in the F_1 female × BW backcross has been linked to the autosomal platinum coat-color allele, which does not exhibit linkage to *Pw1/Peg3* (W. Dawson, J. Rice, J. Weston, C. Ramsdell, and M. Dewey; submitted).

The *Pw1/Peg3* region is gene dense, potentially rendering further genetic tests of *Peal* difficult. A first approach would be to characterize the genes in the region and their allelic expression, i.e., nonpaternally expressed genes could be ruled out. A systematic study of this domain has not yet been performed in any species. Furthermore, comparative studies to date suggest one cannot assume that imprinting status in this region necessarily applies to other taxa (Morison et al. 2005).

In contrast, the paucity of genes on the distal X chromosome of muroid rodents examined to date makes potential testing of candidate loci more feasible. While *Esx1* looks promising as a candidate, it is unclear how its gene product might regulate postnatal growth, given its lack of embryonic and postnatal expression (save testis) (Li and Behringer 1998; Li et al. 1997).

Crosses between the mixed genetic background *Mus* strain C57BL/6 (B6) and *Mus spretus* also yield reciprocal parent-of-origin effects on placental size. The *Mus* crosses, however, do not yield significant reciprocal effects on embryo or postnatal growth.

Similar to the *Peromyscus* crosses, the *Mus* hybrid placental growth effects show genetic linkage to the X chromosome and altered *Esx1* expression (Zechner et al. 2002). No genetic role for any autosomal locus has yet been found in the *Mus* hybrids (Zechner et al. 1997).

The greater inquiries underlying this work are whether these allelic variants of imprinted genes and imprinting regulatory genes have undergone positive selection, whether similar changes underlie other cases of reproductive isolation and human disease, and what the commonalities are in the biological roles of imprinted genes.

It is clear that a major role of imprinted gene products is to modulate prenatal growth. The placenta, a key endocrine organ, expresses many of these loci (Reik et al. 2003). Eutherian placentas, however, vary tremendously in overall morphology, cell types, and gene expression (Carter and Enders 2004; Cross et al. 2003). Numerous associations between altered imprinted gene expression and placental phenotypes suggest at least a role for imprinted genes in this aspect of mammalian diversity. Evidence suggests roles for imprinted genes in a number of human growth and placental disorders (Fisher and Hodges 2003; Reik and Walter 2001). Included in the latter group are the two most common, preeclampsia (Oudejans et al. 2004) and gestational diabetes (Reik et al. 2003). Thus, it seems likely that natural variation at imprinted loci can contribute to mammalian speciation. This work represents a significant advance in isolating such variation.

An opportunity exists to generally test whether *Mexl* and *Peal* play a role in reproductive isolation in other populations. The *Peromyscus maniculatus* species complex contains a number of partially isolated populations. Furthermore, preliminary studies suggest parent-of-origin effects in hybrids between several of these populations (Dice 1949; Liu 1954). Because very little is known about genetic and epigenetic variation at imprinted loci in any mammalian species (Vrana 2006), the present work provides a preliminary framework to test hypotheses about this aspect of mammalian biology.

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