The Classical Mouse Mutant Postaxial Hemimelia
Results from a Mutation in the Wnt 7a Gene

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The study of spontaneous mutations has aided the understanding of developmental processes. A large collection of spontaneous or “classical” mouse mutations has been accumulated over many decades. One of the mutations causes the postaxial hemimelia (px) phenotype, which consists of limb patterning defects accompanied by Müllerian duct-associated sterility in both sexes. We were intrigued that both the limb and the Müllerian duct px phenotypes are similar to those caused by mutations in the gene encoding the Wnt 7a signaling molecule. In this paper, we investigate the nature of the px mutation. Morphological analysis and breeding experiments demonstrate that the px phenotype indeed results from a mutation in the Wnt 7a gene. Molecular analysis demonstrates that px results from a 515-bp deletion in the Wnt 7a gene. This generates an abnormal splicing event, which ultimately produces a truncated Wnt 7a protein of half the normal size. Thus, the px mutation is predicted to be a likely null allele of the Wnt 7a gene. Our results provide another interesting example of a classical mutation that disrupts an important patterning gene in development. © 1998 Academic Press

Key Words: Wnt 7a; limb/reproductive patterning; px mutation.

INTRODUCTION

Functional studies of development have been facilitated by the isolation of mutations affecting a variety of developmental processes. In mice, a substantial collection of spontaneous mutations has been supplemented by a growing number of targeted mutations. Previously characterized classical mouse mutations produce a wide range of phenotypes. For instance, the Brachyury mutation, resulting from a disruption of the T transcription factor, causes defects in gastrulation and a subsequent loss of posterior structures in the embryo (Herrmann et al., 1990). The S1 and W gene products, corresponding to the stem cell factor and its c-kit receptor, are required for normal neural crest cell migration, and their absence results in albinism, anemia, and sterility (Geissler et al., 1988; Zsebo et al., 1990). The Reeler mutation, resulting from mutations in the extracellular molecule reelin, disrupts formation of the cerebral cortex laminae (D’Arcangelo et al., 1995).

There have been a number of particularly interesting spontaneous mouse mutations that disrupt normal patterning of the limb bud. For example, syndactylism results from a disruption of the Serrate2 transmembrane ligand and generates an apical ectodermal ridge (AER) hyperplasia and subsequent fusion of skeletal elements (Sidor et al., 1997). The TGF-β family member GDF5 is disrupted in brachyphalangism and regulates chondrogenesis of the limb skeleton (Storm et al., 1994). Limb deformity (formin) mutants exhibit an improper differentiation of the AER and subsequent shortening of the anteroposterior axis and oligodactyly and syndactyly (Woychik et al., 1985, 1990).

The postaxial hemimelia (px) mutation also exhibits interesting defects in limb bud patterning (Russell, 1957; Searle, 1964). px(px) homozygotes have limb deformities with ectopic dorsal footpads and sesamoid bones accompanied by the frequent loss of posterior bones. In addition, px mutants are sterile, as their Müllerian ducts fail to differentiate normally. Interestingly, both the limb and the urogenital px phenotypes are similar to those observed in Wnt 7a mutants.

The Wnt 7a gene encodes a secreted signaling molecule...
that appears to play several important roles in vertebrate development. Wnt 7a is expressed in the limbs, central nervous system, and urogenital tract (Parr et al., 1993; Dealy et al., 1993; Parr and McMahon, 1998). Functional studies have demonstrated that Wnt 7a is needed for normal patterning of the limb buds (Parr and McMahon, 1995; Yang and Niswander, 1995). We have generated a targeted mutation in the mouse Wnt 7a gene and demonstrated that it functions as a dorsalizing factor in the limb bud and is necessary for proper Müllerian duct development (Parr and McMahon, 1995, 1998). The similarity of px/px and Wnt 7a mutant phenotypes suggested that px might be a Wnt 7a allele. In this report, we show that the px phenotype indeed results from a mutation in the Wnt 7a gene.

**MATERIALS AND METHODS**

**Wnt 7a and Px Mice**

The production of mice with a targeted mutation in the Wnt 7a gene (Wnt7a<sup>tm1Amc</sup>; this allele is called simply Wnt7a for the remainder of the paper) has been described previously (Parr and McMahon, 1995). Px mice were obtained from The Jackson Laboratories.

**Preparation and Photography of Limbs and Urogenital Systems**

To examine the limb skeleton, animals were skinned, eviscerated, and fixed in ethanol overnight. The skeletons were incubated in acetic acid for 24 h and then stained in 70% ethanol, 5% glacial acetic acid, 0.015% alcian blue, 0.005% alizarin red for 1–3 days. They were cleared in 1% KOH for 1 day and then passed through a glycerol series (20, 50, 80, and 100% glycerol) prior to being photographed in 100% glycerol.

The limb footpads and handpads and the urogenital systems were photographed as whole mounts. All photographs were taken on an Olympus SZ10 photomicroscope. Slides were scanned into a Power Macintosh computer and figures compiled in Adobe Photoshop.

Whole-mount in situ hybridizations were performed as previously described (Cygan et al., 1997).

**Molecular Biology**

Genomic DNA for Southern blot analysis and PCR amplification was isolated by proteinase K digestion of mouse tail samples. Southern blots were performed by standard methods (Sambrook et al., 1989).

RNA from the brains of neonatal wild type and px/px mice was isolated by the Trizol method (Life Technologies). M-MLV reverse transcriptase (Life Technologies) was used to synthesize cDNA from 10 μg of total RNA in a 20-μl reaction primed by random oligonucleotide primers. One microliter of the cDNA was subsequently used in PCR.

PCR to amplify genomic DNA used the following conditions: 1× (94°C for 1.5 min) and 34× (94°C for 30 s, 60°C for 30 s, 65°C for 3 min). Amplification of cDNA used the following conditions: 1× (94°C for 2 min), 30× (94°C for 30 s, 60°C for 30 s, 72°C for 1 min).

The following oligonucleotide primers were used to amplify px exons: Exon 1, 5′-CTGGCGGGATCAGCACAGCC and 5′-CGTCGACGCAGCGGAAAGAAC; exon 2, 5′-GGTGTCTAGAACTGTTGCTCTGTGAGAGGT and 5′-AGACGAATTCGCTCTTATAGCT; exon 3, 5′-CCCCGGCCAACCCTGAGCGAC

FIG. 1. Wnt 7a and px mutants have identical ventralized limb phenotypes. (A–D) Dorsal view of adult limbs shows ectopic footpads (fp) on Wnt 7a/Wnt 7a (B), Wnt 7a/px (C), and px/px (D) limbs. (E–H) Skeletal preparations of adult digits show ectopic dorsal sesamoid bones (ds) in addition to the normal ventral sesamoids (vs). (I–J) Neonatal limbs demonstrate the loss of posterior skeletal elements, digit 5, and the ulna (u), in mutant limbs. r, radius; h, humerus; s, scapula.
FIG. 2. Wnt 7a and px mutants show the same Müllerian duct defects. (A–C) Wild-type and mutant urogenital tracts from neonatal males have the same basic structures. (D–F) However, a higher magnification view of the vas deferens (v) from adult males demonstrates the nonregressed Müllerian duct (md) in Wnt 7a (E) and px (F) mice. (G–I) All the structures of the female urogenital tract are present in mutants at neonatal stages. The mutant uteri (u; K and L) clearly are smaller and less well developed than wild type (J) as adults. k, kidney; t, testis; o, ovary.
and 5'-CTCTCGAGAGCTCCTCAGT; exon 4, 5'-GGTGTTGAAGGGCCAGGCTTGCACTTGGC and 5'-AGACGAATTCGCAAGTCGGC.

Oligos used to amplify cDNA were exon 3, 5'-CAGGGCCGTTCCTGC and 5'-AGACGAATTCTGCACTTGGC and 5'-AGACGAATTCGC-ATCCAGGCAGTGGCCTGC.

PCR products were gel purified and subcloned into the pGem T vector (Promega) for sequencing. Sequencing reactions were performed with Sequenase T7 DNA polymerase (Amersham) and run on a 6% polyacrylamide/8 M urea gel. The location of the 3' end of the deletion was confirmed by comparison to wild-type sequence of intron 3 obtained from automated fluorescent cycle sequencing.

RESULTS AND DISCUSSION

We previously generated a likely null mutation in the mouse Wnt 7a gene by gene targeting (Parr and McMahon, 1995). Homozygous mutant mice exhibit limb patterning defects; the distal limb is ventralized and posterior bones are frequently absent. Moreover, both sexes are sterile as a consequence of improper Müllerian duct development (Parr and McMahon, 1998).

Wnt 7a has been mapped to the middle of mouse chromosome 6 (Adamson et al., 1994; B. Gavin, N. Copeland, N. Jenkins, and A. McMahon, unpublished observations). We noticed that the spontaneous recessive viable mouse mutation postaxial hemimelia (px; Russell, 1957; Searle, 1964) also maps to the same region. Interestingly, px homozygotes were reported to exhibit a set of defects similar to those observed in Wnt 7a mutants. Both sexes are sterile due to Müllerian duct abnormalities, and px limbs are ventralized and missing posterior digits. The similar phenotypes and chromosomal proximity suggested that px may encode a Wnt 7a allele. We undertook a series of experiments to test this possibility and determine the molecular nature of the px mutation.

Breeding Results and Phenotypes

To directly test whether px is allelic to Wnt 7a, we crossed Wnt 7a heterozygotes with px heterozygotes. If Wnt 7a and px are allelic, one-quarter of the offspring from this mating (the trans-heterozygotes) should exhibit the same phenotype as Wnt 7a homozygotes and px homozygotes. In fact, 23% (10/44) of the offspring from the Wnt 7a/+ × px/+ matings showed the same limb and Müllerian duct deformities as Wnt 7a and px homozygotes. Thus, the breeding
results demonstrate that px is indeed an allele of Wnt7a and should be designated Wnt7apx.

Figure 1 shows the identical limb deformities observed in Wnt7a/Wnt7a, px/px, and Wnt7a/px animals. In wild-type mice, footpads and handpads are present only on the ventral surface of the limbs. In contrast, the limbs of Wnt7a homozygotes, px homozygotes, and Wnt7a/px compound heterozygotes exhibit ectopic pads on the dorsal limb surface (Figs. 1A–1D). Similarly, although a series of small sesamoid bones is normally present on the ventral part of wild-type digits, the sesamoids are duplicated dorsally in Wnt7a/Wnt7a, px/px, and Wnt7a/px limbs (Figs. 1E–1H). Finally, the frequent loss of posterior digits is also seen in all three classes of mutant limbs (Figs. 1I–1L).

Urogenital abnormalities are also identical in Wnt7a and px mutants (Fig. 2). The Müllerian duct does not regress properly in either px or Wnt7a males (Figs. 2A–2F), a phenotype similar to that observed in Müllerian inhibitory substance (MIS) mutants (Behringer et al., 1994). Indeed, recent studies have demonstrated that Wnt7a signaling is required for expression of the gene encoding the type II component of the MIS receptor in the Müllerian duct mesenchyme (Parr and McMahon, 1998). Consequently, Wnt7a and px mutants are unable to respond to MIS produced by the testis. In px and Wnt7a females, although the Müllerian ducts are present, they fail to differentiate normally (Figs. 2G–2L; see Parr and McMahon, 1998). The walls of the uterus are smaller and less muscular than those of wild type, and coiled oviducts are not observed.

The similarity of the Wnt7a and px phenotypes not only confirms the status of px as a Wnt7a allele, but indicates that both px and the targeted mutation are probably null alleles.
Molecular Nature of the px Mutation

We next examined the molecular basis of the px mutation. A Southern blot of px/+ and px/px DNA probed with a full-length Wnt 7a cDNA detected no gross deletions or rearrangements in the Wnt 7a locus (not shown). This result suggested that the px mutation could be a point mutation, a very small deletion/rearrangement, or a regulatory mutation which may lie well outside of the coding region.

In situ hybridization of a Wnt 7a probe to embryonic day 11.5 px/px embryos demonstrated that Wnt 7a transcripts are still present in their normal location in the dorsal limb bud ectoderm (Figs. 3A and 3B). Thus, a regulatory mutation affecting the temporal/spatial expression of Wnt 7a is unlikely to be the cause of the px mutation. Interestingly, expression of the Lmx-1b gene in the dorsal mesenchyme of the limb bud is reduced to a similar extent in px mutants and in the targeted Wnt 7a allele (Figs. 3C and 3D; Cygan et al., 1997).

Since a point mutation or small deletion in or near the coding region seemed the most likely cause of the px mutation, we used PCR to amplify portions of the Wnt 7a gene from px/px genomic DNA and px/px brain cDNA. The amplified regions were subsequently subcloned, and the entire coding region was sequenced. We detected two possible point mutations within the exons, both in exon 4 (not shown). One was a silent mutation in the third position of a codon. The other created an amino acid substitution from leucine (wild type) to lysine (px). Theoretically this might create a severe alteration in the Wnt 7a protein, but other Wnt proteins, such as Wnt 1 and Wnt 7b, possess a lysine residue at this position. Therefore, we believe that this substitution reflects a normal polymorphism within the mouse population. As a consequence, there are no alterations in px codons that could account for the mutant phenotype.

The sequence of the px cDNA revealed that the middle of the third exon was joined to the beginning of the fourth exon, with the 3’ end of the third exon missing (Fig. 4). Inspection of the cDNA sequence demonstrated that a cryptic splice site in the middle of the third exon was utilized in the px cDNA. These alterations in the px cDNA create a frameshift in the reading frame and a stop codon is encountered at the beginning of the fourth exon. Therefore, the px protein would be approximately half the size of the normal Wnt 7a protein and almost certainly would be nonfunctional. To probe possible splicing abnormalities in px, we used PCR to amplify the splice junctions from px/px genomic DNA. The splice sites matched consensus sequences for exons 1, 2, and 4. However, we discovered a 515-bp deletion at the end of exon 3. The deletion removes the last 106 bp of exon 3 and part of the adjoining intron (see Fig. 4). Thus, the normal 3’ splice site is missing in px, which activates the cryptic splice site and generates an aberrant cDNA.

Our results offer convincing proof that px encodes a mutant allele of Wnt 7a. The similarity of Wnt 7a and px phenotypes, the comparable decreases in Lmx-1b expression, the breeding experiments demonstrating that px is allelic to Wnt 7a, and the discovery of a deletion in the Wnt 7a gene in px DNA combine to establish the molecular nature of the px mutation.

px almost certainly represents a null allele of Wnt 7a. The truncated protein encoded by the px allele would lack the C-terminal half of the wild-type Wnt 7a protein. Since Wnt proteins with a single amino acid substitution in the fourth exon apparently lack normal function (McMahon and Moon, 1989), the absence of half the protein should yield a nonfunctional gene product. The identical phenotype of px and Wnt 7a knockout alleles is a further indication that the knockout allele is also a null allele.

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