Postaxial hemimelia Jackson, a new spontaneous mouse mutation in *Wnt7a*

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Mutation (allele) symbol: *Wnt7a*$^{px\text{-}J}$
Mutation (allele) name: postaxial hemimelia Jackson
Gene symbol: *Wnt7a*
Strain of origin: C57BL/6J
Current strain name: C57BL/6J- *Wnt7a*$^{px\text{-}J}$/GrsrJ
Stock #016098 (jaxmice.jax.org)
Phenotype categories: Skeletal

Abstract
A spontaneous, recessively-inherited mutation causing deformed limbs has been identified in wingless-related MMTV integration site 7A (*Wnt7a*). This new allele includes a 23kb intragenic deletion in *Wnt7a* that encompasses part of intron 3 and a portion of exon 3. A single nucleotide variant from G to A at 91,344,546 of Chromosome 6 (NCBI37/mm9) predicted to result in the missense mutation R143W in Wnt7a was also detected. Map position, phenotype, and whole exome sequencing indicate that this is an allele of Wnt7a. This mutation has been designated postaxial hemimelia Jackson *Wnt7a*$^{px\text{-}J}$.

Origin and Description
In 2007 a spontaneous, recessive mutation causing abnormal limbs was identified in a C57BL/6J mouse at The Jackson Laboratory. Mutants have malformed, shortened forelimbs that resemble flippers and all exhibit missing or shortened digits of the front paws. Infrequently mutants also have missing digits of the hind paws. Mutant gait is not noticeably abnormal although animals exhibit a stance that is lower in the front.

Although mutants have a normal life span they fail to breed naturally. Consistent with this observation, a visual assessment by microscopy revealed that mutant sperm, although viable, appeared sluggish. This viability has since been confirmed with successful *in vitro* fertilization from cryopreserved mutant sperm. Additionally, the ovaries from mutants have been used successfully for ovarian transplants. The colony is maintained by breeding the hosts of homozygous ovarian transplants with C57BL/6J males or unaffected male siblings of the donor. Obligate heterozygous progeny from these matings are then intercrossed to produce mutant offspring.

Genetic Analysis
The mode of inheritance of the new mutation was established by intercrossing non-mutant siblings of a mutant. Normal and mutant progeny were recovered from this

mating indicating that the mutation was inherited in a recessive manner, and that the non-mutant siblings that produced mutant offspring were heterozygous carriers.

Using our standard mapping procedures[1], an intercross was set up to map this mutation. Females carrying transplanted ovaries from homozygous mutants were mated to CAST/EiJ males. The unaffected F1 progeny produced from these matings were intercrossed to produce affected F2 animals. DNA isolated from affected F2 offspring was used for linkage analysis. The mutation was mapped to an 18 Mb Chromosome 6 segment located between *D6Mit188* (75.3 Mb) and *D6Mit102* (93.4 Mb).

Whole exome sequencing along with commercial alignment and SNP/INDEL (insertions/deletions) calling tools were then used to identify candidate coding mutations in the mapped region. A second alignment tool (BWA) and SNP caller (SAM Tools) were used for additional single nucleotide variant (SNV) and INDEL analysis. A SNV from G to A at 91,344,546 was found on Chromosome 6 (NCBI37/mm9) in exon 3 of *Wnt7a*. This is a missense mutation, resulting in the substitution of arginine to tryptophan at amino acid residue 143. Primers were generated that produce a 264 base pair product spanning the predicted mutation: Wnt7a-1F (GTTCAGCTTTGCTGAGGGTC) and Wnt7a-1R (ACCAGAGTGGAGCAGAAGGA). Sanger sequence analysis of genomic DNA samples from two wild type animals, two heterozygotes, and three homozygous mutants revealed amplification of the wild type allele in wild type and heterozygous samples, but surprisingly no amplification in the mutant samples.

In addition to this single nucleotide variant, manual examination of the BWA alignment to NCBI37 (mm9) revealed a large deletion spanning an estimated 23 kb of genomic DNA within the *Wnt7a* gene and encompassing one of the SNV amplification primers described above thereby explaining its failure to amplify. A direct PCR assay for the deletion was designed by selecting a pair of PCR primers that flank the deleted nucleotides and a third primer within the deletion: Wnt7a-Ex3-1L (AGAAGCAAGGCCAGTACCAC), Wnt7a-Int3-1R (CAGAAATCATTCCATCTGTGC), and Wnt7a-1R (same as above). This assay clearly distinguishes among wild type (263 base pair product), heterozygous, and homozygous mutant (201 base pair product) genotypes. Sequence analysis of genomic DNA confirmed a 23,106 bp deletion, encompassing nucleotides 91,321,380-91,344,486 on Chromosome 6 (NCBI37/mm9). This deletion disrupts a portion of exon 3 (amino acids 163-190) and removes the 5-prime end of intron 3, including the splice donor site (NM 009527.3). Potential effects of this mutation include changes in splicing and in the structure of the *Wnt7a* transcript, which would lead to altered protein length and composition. Previously described deletions in this region of the genome have resulted in the use of a cryptic splice site within exon 3 resulting in a frameshift and subsequent early termination codon. This previously described cryptic splice site is retained in the segment of exon 3 present in the *px-J* allele, making it possible that the deletion described here will result in similar aberrant splicing.

Pathology

A routine pathological screen[2] was performed on one male and one female mutant at 45 days of age and on one female at 46 weeks of age. Other than a dilated uterus containing

a cyst in the 46-week old female, no noteworthy abnormalities were found in any other organ. The eyes of two mutants and two controls all at 4 weeks of age were examined by ophthalmoscopy and found to be normal. Hearing, assessed by auditory brainstem response testing (ABR) of 4 mutants and three +/? controls showed normal hearing at 2 months of age.

Discussion

Wnt7*a* regulates dorsal-ventral and anterior-posterior limb pattern formation, and previously studied *Wnt7a* mutants all exhibit anterior-posterior patterning defects restricted to limbs. The phenotypes observed in these mutants are similar to those seen in targeted (null) mutations in *Wnt7a*, as well as the chemically induced allele *gimpy* and spontaneous allele postaxial hemimelia (*px*). Both *gimpy* and *px* mutants exhibit limb and digit malformation, as well as sterility. *px* and null mutants contain Mullerian duct defects, and smaller uteri than wild type animals. These defects could explain the breeding difficulties observed in *px-J* mutants, but have not yet been measured.

The detection of the larger deletion in this mutant provides an interesting model for detecting structural variants in exome datasets. Manual examination of the mutant exome data aligned with the mouse reference sequence (NCBI37/mm9) revealed that the 3-prime end of exon 3 was not represented in the mutant dataset. Since this region is normally represented in control datasets, it seemed likely that mutants have a deletion that spans this region, and that this deletion was outside the detection limits of the standard DNA Seq pipeline (described above). Since this mutation has been validated using PCR and Sanger sequencing, we are now able to use these data as a guide to optimize our DNA Seq pipeline parameters for future datasets. This pipeline will detect larger duplications and deletions by implementing coverage based tools, as well as SNVs/INDELs in exome data.

Acknowledgements

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References

1 Standard Mapping Protocol used in the MMR

Linkage crosses

To map new mouse mutations we use CAST/Ei, an inbred strain of *Mus musculus castaneus*, as our standard linkage testing strain. In some cases, because of breeding difficulties or reduced phenotypic penetrance, we use other strains. An intercross of F1 hybrids is usually used to analyze linkage of recessive mutations and a backcross to analyze linkage of dominant mutations. Our goal is to produce enough informative mice from each mapping cross to test the recombinational products from at least 100 meioses.

DNA isolation

DNA is extracted from the frozen tail tips of mutant (homozygous) F2 mice or backcross progeny by a standard hot sodium hydroxide and Tris (Hot SHOT) procedure (Truett, et al., 2000) or from spleens using standard phenol extraction methods.

Polymerase chain reaction

PCR primer pairs (MapPairs, from Research Genetics, Huntsville, Ala., or from Integrated DNA Technologies, Coralville, Ia.) are used to type MIT microsatellite markers positioned throughout the genome. PCR reactions contain 20 ng genomic DNA in 10 ul containing 50 mM KCL, 10 mM Tris-HCL

(pH 9.0 at 250C), and 0.01% Triton-X-100, 2.25 mM MgCl2, 100 nM of each primer (forward and reverse), 100 uM of each of four deoxyribonucleoside triphosphates, and 0.5 Units of Taq DNA polymerase (Amplitaq from Applied Biosystems #N808-0145). Amplification consists of one cycle of denaturation at 940C for 3 minutes followed by 30 cycles, each consisting of 940C for 15 sec. denaturation, 550C for 2 minutes of annealing, and 720C for 2 minutes of extension. After the 30 cycles, the final product is extended for 7 minutes at 720C. The PCR products are run on 2.5% Metaphor agarose gels or 6% polyacrylamide (non-denaturing) gels. The gels are then stained with ethidium bromide, destained with distilled water, visualized on a UV light table, and photographed.

Pooled DNA Method (Taylor et al 1994).

In the case of an intercross, a pool of DNA prepared from 25-30 mutant F2 mice is compared with DNA from F1 hybrids. In the case of a backcross, a DNA pool from 25-30 mutant N2 mice is compared with a pool from 25-30 unaffected N2 mice. These DNA samples are typed by PCR for MIT markers located throughout the genome. For linked markers, the mutant strain allele will predominate in the DNA pool from mutant mice compared with controls. When a particular marker indicates linkage by analysis of the pooled sample, individual DNA samples are typed with that marker and additional markers in the same region to confirm linkage. Once a linkage is confirmed, additional DNAs from individual mice are typed to obtain a finer map position.

Linkage analysis

Gene order and recombination frequencies are calculated with the Map Manager computer program (Manley1993, 2001), a MacIntosh program for storage and analysis of genotyping data.

References for Mapping Protocol:

Manley KF (1993) A MacIntosh program for storage and analysis of experimental mapping data. Mamm Genome 4: 303-313.

Manly KF, Cudmore RH Jr, and Meer JM (2001) Map Manager QTX, cross-platform software for genetic mapping. Mamm Genome12: 930-932.

Taylor BA, Navin A, and Phillips SJ (1994) PCR-amplification of simple sequence repeat variants from pooled DNA samples for rapidly mapping new mutations of the mouse. Genomics 21: 626-32.

Truett GE, Heeger P, Mynatt RL, Truett AA, Walker JA, and Warman ML(2000) Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and Tris (HotSHOT). Biotechniques 29:52-54

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2 Standard Histology Protocol used in the Mouse Mutant Resource

For fixation of tissues, mice were deeply anesthetized with tribromoethanol (avertin) until they no longer displayed a withdrawal reflex in the hind limbs and then perfused intracardially with Bouin's fixative following a flush of the vasculature with saline solution. After soaking in Bouin's for one week to demineralize bones, tissues were dissected. Six segments of spine with axial muscles and spinal cord in situ, representing cervical, thoracic and lumbar spinal segments, were dissected. The brain was removed and sliced into 6 cross sectional pieces at the levels of olfactory lobes, frontal cortex, striatum, thalamus, midbrain, rostral and caudal medulla with cerebellum. Midsagittal slices of hind leg through the knees were prepared. Slices of basal skull through the pituitary and inner ears were taken. Both eyes, salivary glands and submandibular lymph node, trachea plus thyroid and sometimes parathyroid were removed and cassetted. A longitudinal slice of skin from the back was removed. The thymus, slices of lung, and a longitudinal slice of heart were cassetted. Similarly slices of liver through gall bladder, kidney with adrenal attached, pancreas and spleen were prepared. The stomach was sliced longitudinally to include both squamous and glandular portions. Loops of small intestine from 3 levels and slices of large intestine and cecum were removed, as were slices of urinary bladder. The whole uterus, with ovaries attached, was taken. In males testes were sliced longitudinally. The accessory male organs including seminal vesicles, coagulating gland and prostate were removed en block. Altogether in most cases all tissue fit into a total of 10 cassettes. The cassettes were processed in an automatic tissue processor to dehydrate tissues which were then embedded in paraffin. Six micron sections were cut and stained with hematoxylin and eosin (H&E). Sections of brain and spinal cord in vertebral bones also were stained with luxol fast blue (LFB) for myelin and cresylecht violet (CV) for cellular detail.