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A Wisp3 Cre-knockin Allele Produces Efficient Recombination in Spermatocytes during Early Prophase of Meiosis I

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Abstract

Individuals with the autosomal recessive skeletal disorder Progressive Pseudorheumatoid Dysplasia have loss-of-function mutations in WISP3, and aberrant WISP3 expression has been detected in tumors from patients with colon and breast cancer. In mice however, neither absence nor over-expression of WISP3 was found to cause a phenotype, and endogenous Wisp3 expression has been difficult to detect. To confirm that Wisp3 knockout mice have no phenotype and to identify potential sites of endogenous Wisp3 expression, we generated mice with a knockin allele (Wisp3<sub>GFP-Cre</sub>) designed to express Green Fluorescent Protein (GFP) and Cre-recombinase instead of WISP3. Heterozygous and homozygous knockin mice were fertile and indistinguishable from their wild-type littermates, confirming that mice lacking Wisp3 have no phenotype. We could not detect GFP-expression from the knockin allele, but we could detect Cre-expression after crossing mice with the knockin allele to Cre-reporter mice; the double heterozygous offspring had evidence of Cre-mediated recombination in several tissues. The only tissue that had high levels of Cre-mediated recombination was the testis, where recombination in spermatocytes occurred by early prophase of meiosis I. As a consequence, males that were double heterozygous for a Wisp3<sub>GFP-Cre</sub> and a floxed allele only contributed a recombined allele to their offspring. We detected no evidence of Cre-mediated recombination in the female ovary, although when double heterozygous females contributed the reporter allele to their offspring it had recombined ~7% of the time. Wisp3<sub>GFP-Cre</sub> expression therefore occurs less frequently and most likely at a later stage of oocyte development in female mice compared to male mice. We conclude that although WISP3 is dispensable in mice, male mice with a Wisp3<sub>GFP-Cre</sub> allele (Jackson Laboratory stock # 017685) will be useful for studying early prophase of meiosis I and for efficiently recombining floxed alleles that are passed to offspring.

Introduction

Wnt1 inducible signaling pathway protein 3 (WISP3/CCN6) is a member of the connective tissue growth factor family (CCN) of proteins [1]. The 6 CCN family members share a common structure comprised of an N-terminus signal peptide and four domains bearing homology to insulin-like growth factor binding protein, von Willebrand factor, thrombospondin 1, and cysteine-knot containing proteins [2,3]. CCN proteins are key signaling/regulatory factors involved in a wide variety of cellular processes including, cell adhesion, extracellular matrix remodeling, skeletal development, chondrogenesis, angiogenesis, wound repair, cell proliferation and tumourigenesis (reviewed in 1,4). Such a diversity of interactions is facilitated by the modular architecture of CCN proteins, whereby the 4 domains can either act individually or in multiple combinations to confer specificity [5].

WISP3 was first identified by DNA sequence homology and subsequently found to have altered expression levels in colon cancers [3]. Further studies showed that somatic WISP3 frameshift mutations occur frequently in mismatch repair deficient colorectal carcinomas [6,7] and WISP3 expression is frequently reduced or lost in inflammatory breast cancers [8,9]. Inherited loss-of-function mutations in WISP3 cause the autosomal recessive, skeletal disorder Progressive Pseudorheumatoid Dysplasia (PPD) [10–13].
Investigations into the in vivo role of WISP3 in PPD and, more generally, in cartilage formation/maintenance have met with little success [14,15]. Although morpholino-mediated knockdown of Wisp3 expression in zebrafish produces a craniofacial cartilage phenotype [16], neither over-expression [15] nor mutation of Wisp3 [14] produced any discernable phenotype in mice. Wisp3 and WISP3 mRNAs were detected by RT-PCR in several mouse [14] and human [3] tissues, respectively, but not by in situ hybridization. Immunohistochemistry has been unable to detect endogenous WISP3 protein.

To elucidate the expression pattern of Wisp3 in mice, we generated knockin mice in which a Green Fluorescent Protein (GFP) - internal ribosomal entry site - Cre recombinase (Cre) expression cassette was inserted in-frame into the first exon of Wisp3, three amino acids downstream of the endogenous Wisp3 translation initiation codon. This knockin allele, Wisp3<sup>GFP-Cre</sup>, was designed to express GFP and Cre in place of WISP3. Here we describe the expression pattern of the Wisp3<sup>GFP-Cre</sup> allele.

Materials and Methods

All animal work was performed as approved by the Institutional Animal Care and Use Committee at Boston Children’s Hospital.

Construction of the GFP-Cre Targeting Vector and generation of Wisp3<sup>GFP-Cre</sup> knockin mice

The following DNA elements were PCR amplified, subcloned, and ligated to produce a GFP-ires-Cre-Neo targeting vector (Figure 1).

1. FRT-PGK-EM7-Neo-FRT selection cassette from pL451 [17]
2. 5’ and 3’ Wisp3 targeting arms from BAC RP24_541B4
3. eGFP, IRES, and Cre from pIgCN21 [18]
4. Thymidine kinase selection cassette from pL253 [17]

The full-length targeting vector was linearized and used to perform homologous recombination in V6.5 (C57BL/6 x 129S4/SvJae)F1 ES cells using positive (G418) and negative (GFP) - internal ribosomal entry site - Cre recombinase (Cre). Correct targeting of ES cells was confirmed by long-range PCR (P<sub>1</sub> aacgcccaggtcctactccattcac and P<sub>2</sub> tgcctcagtggttggcg for 5’ targeting and P<sub>3</sub> gggagattggaagcaat and P<sub>4</sub> ccatgaaggaagccctatga for 3’ targeting), and one correctly targeted ES cell clone was generated knockin mice in which a Green Fluorescent Protein (GFP) - internal ribosomal entry site - Cre recombinase (Cre) expression cassette was inserted in-frame into the first exon of Wisp3, three amino acids downstream of the endogenous Wisp3 translation initiation codon. This knockin allele, Wisp3<sup>GFP-Cre</sup>, was designed to express GFP and Cre in place of WISP3. Here we describe the expression pattern of the Wisp3<sup>GFP-Cre</sup> allele.

Genotyping alleles at the Wisp3 and ROSA26 loci

Total DNA was extracted from mouse tail tips using the HotSHOT method [19]. Genotyping of mice with the ROSA<sup>26<sup>Tm</sup>g</sup> allele ((ROSA) 26So<sup>tm4(FLP)Dym</sup> (ACTB-tdTomato-EGFP)Luo<sup>+/−</sup>) Jackson Laboratory stock # 007576) was performed as recommended by the Jackson Laboratory for this strain. Genotyping of the Wisp3<sup>GFP-Cre</sup> allele was performed with a three-primer reaction (F ctaggctcttttgcac; R<sub>1</sub> gaaacctcaggtcaggtgc; and R<sub>2</sub> tgcgaagaagctcctagtg) designed to generate a 256 bp amplifier for the wild-type Wisp3 allele and a 180 bp amplifier for the Wisp3<sup>GFP-Cre</sup> knockin allele. PCR conditions were: 94°C for 2 min followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec, followed by a 7 minute 68°C polish.

Detecting Cre-recombination of the ROSA26<sup>Tm</sup>g allele

Total DNA was extracted from individual tissues using the DNeasy blood and tissue extraction kit (Qiagen). One primer pair (mT-F gcaacagtctgtgtcagttg and mT-R tgtgacccactctcctatg) was designed to generate a 200 bp amplifier from the non-recombined ROSA26<sup>Tm</sup>g allele and another primer pair (mg-F gtggcctctgctgtt and mg-R tgtgacccactctatg) was designed to generate a 376 bp amplifier from the Cre-recombined ROSA26<sup>Tm</sup>g allele. PCR conditions for both reactions were: 95°C for 1 min followed by 35 cycles of 94°C for 15 sec, 62°C for 30 sec, and 72°C for 1 min, followed by a 2.5 minute 72°C polish. The sensitivity of the latter primer pair for detecting Cre-recombination of the ROSA26<sup>Tm</sup>g allele was determined by performing PCR using template genomic DNA from non-recombined mice that contained serial diluted genomic DNA from mice that had completely recombined alleles. The unmixed genomic DNAs were used as negative and positive controls for each primer pair, and the recombination assay was performed using 200 ng of template DNA.

Tissue Collection/Preparation

Mice were euthanized immediately prior to tissue collection. For histologic analyses, dissected tissues were washed thrice in 1x phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (USB) in PBS at 4°C overnight. Tissues were then washed once with 1x PBS and cryoprotected in 30% sucrose in PBS and placed directly in Allprotect Tissue Reagent (Qiagen) or RNAlater (Invitrogen) at 4°C for a minimum of 24 hrs. DNA and RNA were extracted using either DNeasy Blood & Tissue Kit (Qiagen) or Trizol (Invitrogen) following the manufacturers’ recommendations.
Figure 1. Generation of a knockin GFP-CRE allele at the Wisp3 locus. (Upper panel) Schematic diagram of the Wisp3 locus, the targeting vector, and the Wisp3lac22Δex3-5 and Wisp3GFP-Cre alleles (not drawn to scale). Exons 1-5 are indicated as rectangles, with the 5′ untranslated region (UTR) of exon 1 a thinner rectangle. The Wisp3GFP-Cre targeting construct contains a thymidine kinase cassette (TK) for negative selection, an internal ribosomal entry site (IRES), a Cre-recombinase (Cre) cassette, a polyA addition signal (rGpA), and a FRT (diamonds) flanked Neomycin resistance cassette (NeoR), all inserted into Wisp3 exon 1. DNA sequences from the correctly targeted Wisp3GFP-Cre allele are shown. The two potential translation initiation codons of endogenous Wisp3 are underlined, as is the translation initiation codon in the GFP expression cassette. NotI and SalI restriction sites are in italics. FRT sequence 3′ of the NeoR cassette, the remainder of Wisp3 exon 1, and beginning of Wisp3 intron 1 are indicated. Arrows indicate the locations of the PCR primers used to generate the amplifiers shown in the lower panels. The P1 and P4 primers lie outside of the targeting arms (Lower panels). A) PCR amplifiers demonstrating correctly targeted alleles in Wisp3GFP-Cre/GFP-Cre mice and their absence in wild-type mice. B) PCR amplimers used to genotype wild-type, heterozygous, and homozygous knockin littermates and the Wisp3 locus in C57BL6/J mice (control). Sizes of the molecular weight standards (MS Std) in kb or bp are indicated.

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RT-PCR of Wisp3
Mouse RNA were bought from Ambion (AM5730G), Zyagen (MR-108, MR-109) and BioChain (R4334566). Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare) with 2 μg RNA and OligoDT(20) primers (Invitrogen).
PCR was performed using Taq-pro Complete (Denville scientific) with Wisp3 specific, exon/intron spanning, primers (ExF ttgctcggtagtttacatt and ExR ccccttgaaattccatt). Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare) with 2 µg RNA and OligoDT(20) primers (Invitrogen). PCR conditions were: 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec, followed by a 7 minute 68°C polish.

Results and Discussion
Creation of Wisp3<sup>GFP-Cre</sup> knockin mice
Figure 1 depicts the targeting strategy used to generate the Wisp3<sup>GFP-Cre</sup> allele. Mouse Wisp3 has 2 potential translation initiation codons in exon 1. We used a NotI restriction site to add three alanine residues between the second translation initiation codon of Wisp3 and the translation initiation codon of GFP (Figure 1). Depending on which of the 3 potential translation initiation codons was used by the knockin allele, the GFP protein product would contain 14, 4, or 0 additional amino acid residues at its amino terminus. We confirmed correct targeting by long-range PCR (Figure 1) and sequencing (data not shown), and excised the Neo- cassette with a Flippase expressing mouse. After confirming the Neo-cassette had been excised, we intercrossed Wisp3<sup>GFP-Cre</sup> mice and produced Wisp3<sup>−/−</sup>, Wisp3<sup>GFP-Cre</sup>, and Wisp3<sup>GFP-Cre/GFP-Cre</sup> offspring (Figure 1) at the expected Mendelian frequencies. Wisp3<sup>GFP-Cre</sup> and Wisp3<sup>GFP-Cre/GFP-Cre</sup> mice were indistinguishable from their wild-type littermates when followed to >12 months-of-age, and they were fertile. Seventeen matings between Wisp3<sup>GFP-Cre/GFP-Cre</sup> mice produced an average of ~9 pups/litter and a male: female pup ratio of 1.07.

We previously generated a mutant allele of Wisp3, Wisp3<sup>323aza103S</sup>, by replacing exons 3 -5 with a lacZ reporter and a Neomycin selection cassette (Figure 1) [14]. This mutant allele was designed to produce a mutant allele of mouse Wisp3 that was comparable to several homozygous deletion and nonsense WISP3 mutations that cause Progressive Pseudorheumatoid Dysplasia in humans [10,12]. These homozygous Wisp3-mutant mice also appeared normal and were fertile on two different genetic backgrounds (129Sv/Ev and C57BL/6) [14]. Thus, the inability of two different Wisp3 loss-of-function alleles to recapitulate the phenotype of Progressive Pseudorheumatoid Dysplasia on different genetic backgrounds argues strongly against WISP3 function in the skeleton being conserved between humans and mice. We cannot preclude the possibility that another murine CCN family member is compensating for WISP3 deficiency. To date, only Wisp1/ Wisp3 double knockout mice have been studied; they are indistinguishable from Wisp1 knockouts alone (personal communication from Professor Karen Lyons, UCLA, Los Angeles, CA).

Comparing sites of endogenous Wisp3 expression to sites of GFP and Cre expression from the Wisp3<sup>GFP-Cre</sup> allele
Wisp3 expression is poorly represented in expressed sequence tags (EST) and RNA sequencing (RNA-seq) data sets, and has not been reliably detected by in situ hybridization [14]. Therefore, to determine which mouse tissues express Wisp3 mRNA, we performed RT-PCR using commercially available total RNA from multiple mouse tissues. We were able to amplify spliced Wisp3 RNA from several tissues, including cartilage, kidney, testis, heart, liver, and brain (Figure 2). To determine whether the Wisp3<sup>GFP-Cre</sup> allele expressed GFP in these tissues, we examined frozen sections from Wisp3<sup>GFP-Cre/GFP-Cre</sup> mice by fluorescence microscopy. We were unable to detect GFP expression from the Wisp3<sup>GFP-Cre</sup> allele in any tissue (Figure 3 and data not shown). We next determined whether Cre was expressed from the Wisp3<sup>GFP-Cre</sup> allele by crossing Wisp3<sup>GFP-Cre</sup> mice with ROSA26<sup>tmGtmG</sup> mice and looking for evidence of Cre-mediated recombination at the ROSA26 locus in multiple tissues by PCR and by fluorescence microscopy. We first determined that our PCR protocol could detect ~0.1% recombination of the ROSA26<sup>tmGtmG</sup> allele by mixing genomic DNA from mice in which the ROSA26<sup>tmGtmG</sup> allele was intact or fully recombined (data not shown). We then used this assay to identify which tissues in Wisp3<sup>GFP-Cre; ROSA26<sup>tmGtmG</sup></sup> double heterozygous mice had evidence of Cre-mediated recombination, and found evidence of recombination in testis, heart, and brain. Because the PCR assay was not quantitative, we also examined these same tissues for recombination using fluorescence microscopy; cells with a ROSA26<sup>tmGtmG</sup> allele that had not recombined have membranes that fluoresce red, while cells with a Cre-recombined ROSA26<sup>tmGtmG</sup> allele have membranes that fluoresce green. Testis was the only tissue in which fluorescence microscopy revealed Cre-mediated recombination of the ROSA26<sup>tmGtmG</sup> allele (Figure 3 and data not shown).

Although the Cre activity we observed for the Wisp3<sup>GFP-Cre</sup> allele in testis was consistent with the endogenous Wisp3 expression we observed in this tissue by RT-PCR, and other investigators observed in human testis [3], there was no correlation between Cre activity and Wisp3 expression in other tissues. For example, cartilage, liver, and kidney had detectable Wisp3 expression by RT-PCR but no evidence of Cre activity in the reporter mice. One explanation for why Wisp3 expression may have been detected by RT-PCR, while Cre-mediated recombination was not, is that only a tiny fraction (<0.1%) of cells in these tissues express Wisp3 but at very high levels. These few high-expressing cells could make transcript detectable by RT-PCR in a total tissue RNA pool, but since only a very small fraction of cells actually express Cre from the Wisp3<sup>GFP-Cre</sup> allele, Cre-mediated recombination occurred too infrequently to be detected with our recombination assay. The alternative and more likely explanation is that Cre-expression from the Wisp3<sup>GFP-Cre</sup> allele does not truly mirror endogenous Wisp3 expression. That we could not find cells, other than spermatocytes and their descendents in the testis, expressing membrane bound GFP in the Wisp3<sup>GFP-Cre</sup>.
Figure 2. Endogenous Wisp3 expression and Wisp3\textsuperscript{GFP-Cre} expression are not identical. (Upper panel) RT-PCR amplimers indicating the presence of Wisp3 transcript in total RNA from a several different mouse tissues. A schematic of Wisp3 mRNA is indicated (not drawn to scale) along with the locations of the intron-spanning PCR primers and the expected amplimer size for correctly splice mRNA (Lower panel). A schematic of the ROSA26\textsuperscript{mTmG} allele before and after Cre-recombination (not drawn to scale) along with the locations of the PCR primers and the expected amplimer sizes for the non-recombined and recombinated alleles. PCR amplimers indicating non-recombined ROSA26\textsuperscript{mTmG} DNA (upper gel) in all tissues and Cre-mediated recombination (lower gel arrowheads) in testis, heart, and brain recovered from Wisp3\textsuperscript{GFP-Cre};ROSA26\textsuperscript{mTmG} mice (floxed and recombined template DNA serves as controls for the two primer pairs). Note that there is poor correlation between endogenous Wisp3 expression (upper panel) and Wisp3\textsuperscript{GFP-Cre} activity (lower panel). doi: 10.1371/journal.pone.0075116.g002
The strong Cre-activity we observed in the testis of Wisp3\textsuperscript{+/-}GFP-Cre\textsuperscript{mTmG} males led us to determine the efficiency of this allele in producing germline recombination. We bred two Wisp3\textsuperscript{+/-}GFP-Cre\textsuperscript{mTmG} males to wild-type females. Of 186 offspring generated from these crosses, 85 mice inherited the ROSA26\textsuperscript{mTmG} allele and this allele had recombined in all (100%). To identify the earliest stage during spermatogenesis at which Cre activity became detectable, we collected testes from male Wisp3\textsuperscript{+/-}GFP-Cre\textsuperscript{mTmG} mice at different postnatal ages and examined them for evidence of Cre-activity by PCR and fluorescence microscopy (Figure 4). By fluorescence microscopy, Cre-recombination of the ROSA26\textsuperscript{mTmG} allele was apparent in the seminiferous tubules of 15-day-old (P15) mice. Our PCR-based assay was able to detect evidence of Cre-mediated recombination in testis as early as P10 (data not shown). This timeframe coincides with the onset of early prophase of meiosis I during mouse spermatogenesis [20].

We performed the converse experiment by breeding wild-type males and three Wisp3\textsuperscript{+/-}GFP-Cre\textsuperscript{mTmG} females. Of 157 offspring, 72 inherited the ROSA26\textsuperscript{mTmG} allele and this allele had recombined in 5 of the 72 mice (~7%). All three females had pups with a recombinated allele. Recombination appears to have occurred during female gametogenesis, since none of the 5 pups had evidence of somatic mosaicism for the non-recombined and recombinated alleles by PCR (data not shown). We examined the ovaries of the Wisp3\textsuperscript{+/-}GFP-Cre\textsuperscript{mTmG} dams by fluorescence microscopy to look for evidence of Cre-mediated recombination in oocytes but found none (data not shown). Most oocytes in fertile female mice are arrested at the diplotene stage of prophase I [21,22]. Since that stage of oocyte arrest is later than the stage in meiosis in which Wisp3\textsuperscript{+/-}GFP-Cre\textsuperscript{mTmG}-mediated recombination had already occurred in males, it appears as though Wisp3\textsuperscript{+/-}GFP-Cre expression commences later and less often during female meiosis compared to male meiosis.

Several transgenic and knockin mice express Cre-recombinase in male spermatogonia, spermatocytes, and/or spermatids (reviewed in 23,24). Mice that express Cre-recombinase during spermatogenesis or oogenesis, and that are heterozygous for a floxed allele, have great utility in generating recombinated alleles in their progeny. Male Wisp3\textsuperscript{GFP-Cre} mice, which we have donated to The Jackson Laboratory (stock # 017685), should be particularly useful for this purpose since they are healthy, fertile, and highly efficient Cre-deleters in early spermatocytes. Also, since the Wisp3\textsuperscript{GFP-Cre} allele is a knockin, rather than a transgene, its chromosomal location is known (Chr10:39150971-39163794; in the GRCm38/mm10
(genome assembly) and it is unlikely to be subject to epigenetic silencing. In addition to transmitting recombined alleles to progeny, Wisp3\textsuperscript{GFP-Cre} mice can be used to specifically determine the consequence of altering gene expression in spermatocytes during meiosis I. The Wisp3\textsuperscript{GFP-Cre} allele produces minimal Cre-recombinase activity in cells/tissues other than spermatocytes, and thus Wisp3\textsuperscript{GFP-Cre} mice are unlikely to have significant off-target effects. This contrasts with transgenic strains containing Cre-drivers that are active during male meiosis I, such as Pgk2-Cre [25,26], Hspa2-Cre [27,28], and Synapsin-Cre [29]. These strains are efficient Cre-deleters during paternal transmission but they exhibit significant Cre-activity in multiple tissues during embryogenesis and postnatally, which can limit their usefulness in studying male spermatogenesis.

**Conclusion**

The Wisp3\textsuperscript{GFP-Cre} allele produces extremely efficient Cre-recombinase activity in male spermatocytes by early prophase of meiosis I, and minimal recombination in other tissues. Thus, males with a Wisp3\textsuperscript{GFP-Cre} allele and floxed alleles at other loci can be used to study the roles of these other loci during male meiosis I, without causing significant off-target effects. Importantly, by driving complete recombination in male gametes, the Wisp3\textsuperscript{GFP-Cre} allele should be very useful for converting a floxed allele to a recombined allele in offspring.

Wisp3\textsuperscript{GFP-Cre} mice have been donated to the Jackson Laboratory (stock #017685).

Three reporters (LacZ, GFP, and CRE) have been knocked into the Wisp3 locus, and none have mirrored the Wisp3 expression pattern suggested by RT-PCR. Consequently, the Wisp3\textsuperscript{GFP-Cre} allele may not inform us regarding the endogenous expression pattern of Wisp3. Furthermore, the lack of a phenotype in Wisp3\textsuperscript{GFP-Cre} mice indicates that the mice do not recapitulate the phenotype of WISP3 deficiency in humans and are unlikely to inform us regarding the pathogenesis of human Progressive Pseudorheumatoid Dysplasia. Despite their lack of utility in teaching us about PPD, male mice with Wisp3\textsuperscript{GFP-Cre} alleles are excellent "Cre- deleters" and male and female mice offer a new means for dissecting spermatogenesis and oogenesis. As with any genetically modified allele, it is possible that the Wisp3\textsuperscript{GFP-Cre} allele, alone, could produce a phenotype depending on the mouse's genetic background. Investigators should consider this possibility when using the Wisp3\textsuperscript{GFP-Cre} allele in their experiments.

**Author Contributions**

Conceived and designed the experiments: SH LK BNN MH MLW. Performed the experiments: SH LK BNN MH MLW. Analyzed the data: SH LK BNN MH MLW. Wrote the manuscript: SH LK BN MH MLW.
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