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LETTER

Sfrp5 Is Not Essential for Axis Formation in the Mouse

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Summary: *Secreted frizzled related protein* (*Sfrp*) genes encode extracellular factors that can modulate Wnt signaling. During early post-implantation mouse development *Sfrp5* is expressed in the anterior visceral endoderm (AVE) and the ventral foregut endoderm. The AVE is important in anterior–posterior axis formation and the ventral foregut endoderm contributes to multiple gut tissues. Here to determine the essential role of *Sfrp5* in early mouse development we generated *Sfrp5*-deficient mice by gene targeting. We report that *Sfrp5*-deficient mice are viable and fertile. To determine whether the absence of an axis phenotype might be due to genetic redundancy with *Dkk1* in the AVE we generated *Sfrp5;Dkk1* double mutant mice. AVE development and primitive streak formation appeared normal in *Sfrp5*$^{-/-}$;*Dkk1*$^{-/-}$ embryos. These results indicate that *Sfrp5* is not essential for axis formation or foregut morphogenesis in the mouse and also imply that *Sfrp5* and *Dkk1* together are not essential for AVE development. genesis 44:573–578, 2006. **Published 2006 Wiley-Liss, Inc.**†

Key words: *Sfrp5*; axis formation; foregut; visceral endoderm

Secreted frizzled related protein (*Sfrp*) genes encode extracellular molecules that contain a cysteine-rich domain with homology to the Wnt binding domain of Frizzled receptors (Rattner et al., 1997). Coimmunoprecipitation experiments as well as cell labeling experiments using anchored forms of Sfrps and Wnts indicate that Sfrps can bind Wnts (Leyns et al., 1997; Rattner et al., 1997; Wang et al., 1997). It has been hypothesized that Sfrps may function to modulate the stability or the diffusion of Wnt proteins or act as antagonists by binding Wnts extracellularly and preventing them from interacting with Frizzled receptors (Rattner et al., 1997). Five Sfrp genes have been identified in the mouse and they are expressed in various adult and embryonic tissues (Leimeister et al., 1998; Leyns et al., 1997; Rattner et al., 1997). We have previously described the expression of *Sfrp5* in the anterior visceral endoderm (AVE) and the ventral foregut endoderm of early mouse embryos (Finley et al., 2003). The AVE is a source of secreted factors that can suppress posteriorizing signals in the epiblast and the ventral foregut endoderm gives rise to anterior gut tissues, including epithelial cells of the liver bud (Kimura et al., 2000; Tremblay and Zaret, 2005). Here, to determine the essential role of *Sfrp5* in early mouse development we generated *Sfrp5*-deficient mice by gene targeting.

We devised a targeting strategy to delete nearly the entire coding region of *Sfrp5* (Fig. 1a). Two correctly targeted ES cell clones were identified by Southern hybridization (Fig. 1b). One clone, 1C7, gave rise to chimeric mice able to transmit the targeted mutation through the germ line. Mice heterozygous for the targeted mutation were maintained on a CD-1 outbred genetic background and appeared normal. Mice heterozygous for the targeted mutation were then intercrossed to generate *Sfrp5*$^{-/-}$ mice. Southern blot analysis using a *Sfrp5* cDNA fragment confirmed that the expected *Sfrp5* coding sequences were deleted (Fig. 1c).

Mice resulting from heterozygous intercrosses were genotyped at weaning by Southern analysis (Table 1). We found that approximately one quarter of the mice had the genotype *Sfrp5*$^{-/-}$, indicating that *Sfrp5* is not required for embryonic or postnatal survival. As *Sfrp5* is also expressed in the Müllerian duct at embryonic day (E) 14 (Akio Kobayashi and Richard Behringer, personal communication; Cox et al., 2006), we crossed *Sfrp5*$^{-/-}$ females to *Sfrp5*$^{+/-}$ and *Sfrp5*$^{-/-}$ male mice to test functionally whether reproductive tract development was altered. Both female and male *Sfrp5*$^{-/-}$ mice were fertile implying that loss of *Sfrp5* function does not alter Müllerian duct development. As the expression of the mouse *Sfrp5* gene in various adult tissues has not previously been described, we hybridized a *Sfrp5* cDNA

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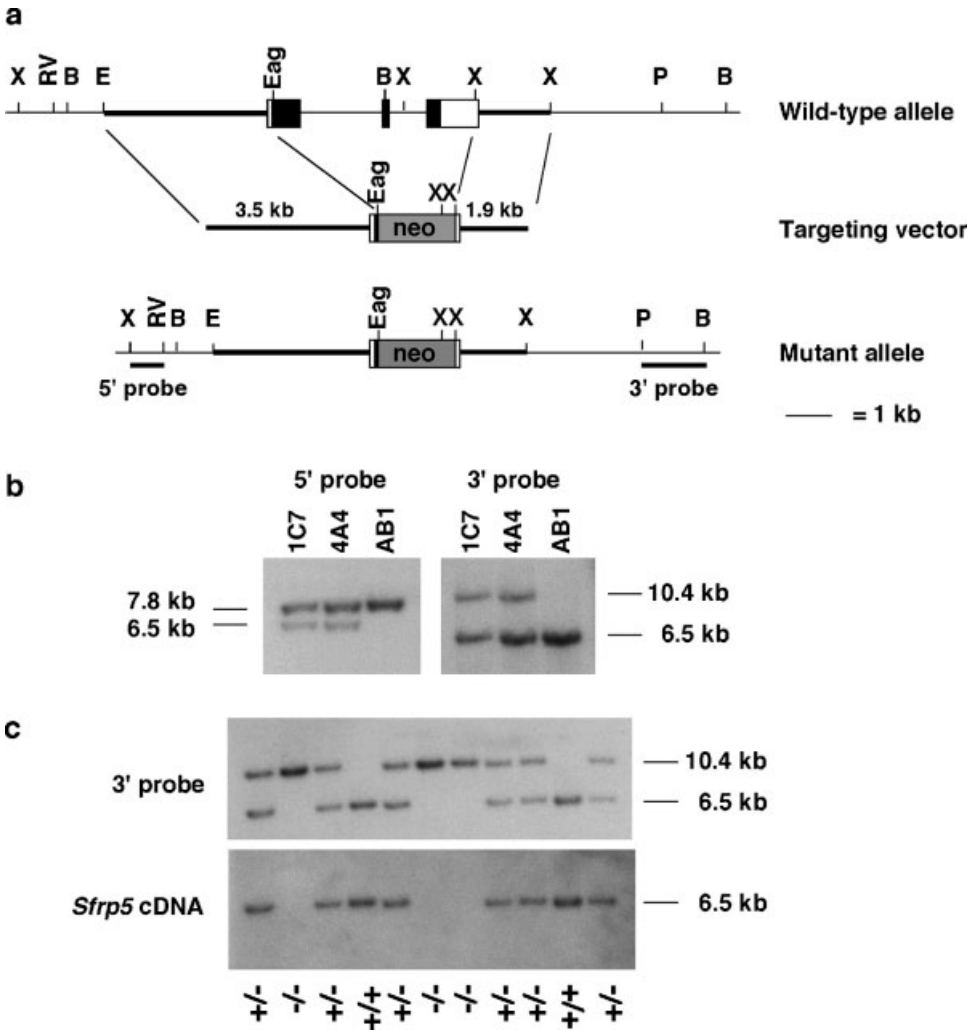

FIG. 1. Targeted deletion of the *Sfrp5* gene. (**a**) Schematic representation of targeting. The coding region of the *Sfrp5* gene (in black) was deleted with the exception of sequences in the first exon encoding the first eight amino acids, which correspond to part of the signal sequence. The targeting vector contained a PGKneobpA-loxP cassette flanked by 3.5 kb of 5′ *Sfrp5* homology and 1.9 kb of 3′ *Sfrp5* homology (thicker black lines). (**b**) Southern analysis of targeted ES clones using 5′ and 3′ *Sfrp5* probes. AB1 ES cell DNA was included as a control. (**c**) Southern analysis of DNA isolated from the yolk sacs of E9.5 embryos derived from a *Sfrp5* heterozygous intercross hybridized with 3′ *Sfrp5* probe (upper panel) and a *Sfrp5* cDNA probe corresponding to the 3′ region of *Sfrp5* (lower panel). B, *Bgl*II; E, *Eco*RI; Eag, *Eag*I; RV, *Eco*RV; P, *Pst*I; X, *Xba*I.

Table 1
Sfrp5$^{+/-}$ × *Sfrp5*$^{+/-}$ *Intercrosses*

Genetic background	Litters	Pups	Avg. litter size	+/+	+/−	−/−
CD-1	7	83	11.9	19	42	22
129S6/SvEv[a]	6	29	4.8	7	16	6
C57BL/6[a]	3	21	7	3	12	6

[a]Backcrossed for 5 generations.

probe to a commercial RNA dot blot containing 22 different mouse tissues. We found abundant expression of *Sfrp5* at E7 but weak or no expression of *Sfrp5* at other embryonic time points or in adult tissues (see Fig. 2). Although a control hybridization was not performed, our results correspond reasonably well to the expression profile of *Sfrp5* suggested by analysis of mouse EST clones (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist = Mm.103731). Necropsies of several *Sfrp5*$^{-/-}$ mice at 6 weeks of age were unremarkable (data not shown). We did not observe any overt abnormalities in *Sfrp5*$^{-/-}$ mice that were followed for ∼1 year. To determine whether placing the *Sfrp5* mutation on a different genetic background might reveal a phenotype, we backcrossed *Sfrp5*$^{+/-}$ mice to C57BL/6 and 129S6/SvEv mice for 5 generations. On predominantly C57BL/6 or 129S6/SvEv genetic backgrounds we obtained viable and fertile *Sfrp5*$^{-/-}$ mice at the expected frequency (Table 1).

Although *Sfrp5*$^{-/-}$ mice are viable there could be subtle defects in *Sfrp5*$^{-/-}$ embryos that are compen-

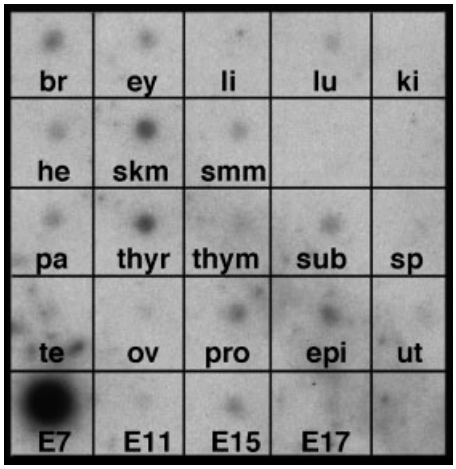

FIG. 2. Expression of *Sfrp5* in adult tissues and embryonic time points revealed by RNA dot blot hybridization. br, brain; ey, eye; li, liver; lu, lung; ki, kidney; he, heart; skm, skeletal muscle; smm, smooth muscle; pa, pancreas; thyr, thyroid; thym, thymus; sub, submaxillary gland; sp, spleen; te, testis; ov, ovary; pro, prostate; epi, epididymis; ut, uterus. Embryonic day (E) 7, E11, E15, and E17. The blot was exposed overnight.

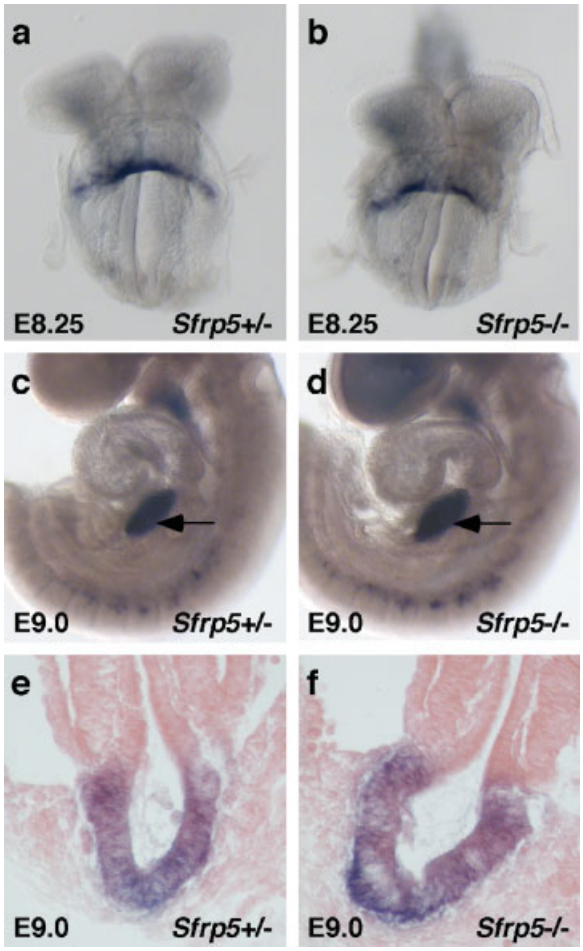

FIG. 3. Foregut morphogenesis in *Sfrp5*-deficient embryos. (**a**,**b**) Expression of *Hex* in the ventral foregut endoderm of E8.25 $Sfrp5^{+/-}$ and $Sfrp5^{-/-}$ embryos with 4–5 somites. (**c**,**d**) Expression of *Hex* in the presumptive liver (arrow) of E9.0 $Sfrp5^{+/-}$ and $Sfrp5^{-/-}$ embryos with 16–18 somites. Embryo in (d) is slightly more advanced developmentally than (c). $Sfrp5^{+/-}$ and $Sfrp5^{-/-}$ embryo pairs for each stage are shown at the same magnification. (**e**,**f**) Histological sections of embryos in (c) and (d). Magnification is ×200.

sated for later in development. To determine whether there might be subtle defects in foregut morphogenesis in $Sfrp5^{-/-}$ embryos, we examined *Hex* expression in E8.25 embryos (4–5 somites) by whole mount in situ hybridization. *Hex* is expressed in the ventral foregut endoderm and experiments in *Xenopus* suggest that inhibition of Wnt signaling is necessary for *Hex* expression in the endoderm (Foley and Mercola, 2005; Thomas et al., 1998). In $Sfrp5^{-/-}$ embryos ($n = 4$), *Hex* was expressed in manner similar to $Sfrp5^{+/-}$ embryos (Fig. 3a,b). In addition we observed no obvious differences in the shape of the foregut pocket or in the morphogenesis of the overlying heart in $Sfrp5^{-/-}$ embryos. As the liver derives from the ventral foregut endoderm, we also investigated whether liver bud development might be altered or delayed in $Sfrp5^{-/-}$ embryos. To examine liver bud development we analyzed the expression of *Hex* in E9.0 embryos (16–18 somites). We found that *Hex* was expressed in the liver bud of $Sfrp5^{-/-}$ embryos ($n = 6$) in a manner similar to that in $Sfrp^{+/-}$ embryos and that growth of the liver bud was not retarded (Fig. 3c–f). These findings suggest that foregut morphogenesis occurs normally in *Sfrp5*-deficient mice.

One potential explanation for why axis formation occurs normally in *Sfrp5*-deficient mice is that *Sfrp5* is redundant with *Dkk1* in the visceral endoderm. *Dkk1* encodes a secreted factor that binds the Wnt co-receptor LRP5/6, triggering its internalization and thereby preventing Wnt signaling from occurring (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001). Thus Dkk1 acts mechanistically different from Sfrps, which bind Wnt proteins directly (Leyns et al. 1997; Rattner et al. 1997; Wang et al., 1997). We have previously reported that the expression of *Sfrp5* and *Dkk1* overlap in the AVE region at the mid-streak stage, with *Sfrp5* expressed more broadly than *Dkk1* (Finley et al., 2003). *Dkk1*-deficient mice have anterior head defects but chimera experiments indicate that *Dkk1* function is not required in extraembryonic tissues (Mukhopadhyay et al., 2001). To determine whether *Sfrp5* and *Dkk1* act redundantly in the visceral endoderm we generated compound mutant mice. Mice heterozygous for both *Sfrp5* and *Dkk1* had no readily apparent phenotype. As both *Sfrp5* and *Dkk1* are located on chromosome 19, we used meiotic recombination to place the *Sfrp5* and *Dkk1* targeted mutations on the same chromosome. The *Sfrp5* and *Dkk1* mutations were then maintained in a cis configuration by crossing a *Sfrp5*;*Dkk1* cis heterozygous female mouse to a $Sfrp5^{-/-}$ male. Five of the 8 resulting

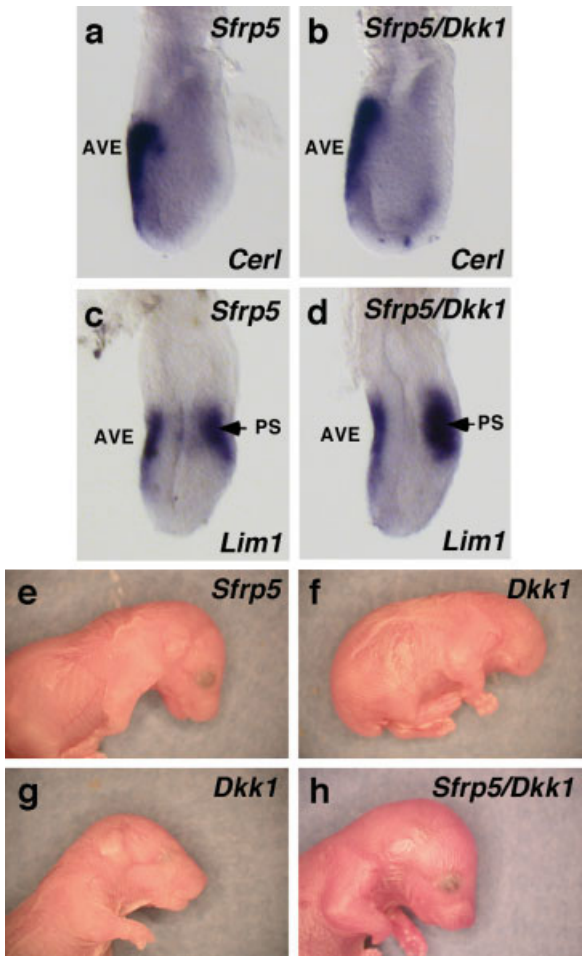

FIG. 4. Analysis of *Sfrp5*;*Dkk1* double mutant mice. (**a**,**b**) *Cerl* expression in the AVE of E6.75 $Sfrp5^{-/-}$ and $Sfrp5^{-/-}$;$Dkk1^{-/-}$ mid-streak stage embryos. (**c**,**d**) *Lim1* expression in the AVE and primitive streak of E6.5 $Sfrp5^{-/-}$ and $Sfrp5^{-/-}$;$Dkk1^{-/-}$ early streak stage embryos. Embryo pairs for each marker gene are presented at the same magnification. Embryo in (d) is slightly more developmentally advanced than (c). (**e**) E17.5 $Sfrp5^{-/-}$ embryo with normal head. (**f**) E17.5 headless $Dkk1^{-/-}$ embryo. (**g**) E17.5 $Dkk1^{-/-}$ embryo with anterior head structures. (**h**) E17.5 $Sfrp5^{-/-}$;$Dkk1^{-/-}$ embryo with anterior head structures. The magnifications for (e–h) are not equivalent. AVE, anterior visceral endoderm; PS, primitive streak.

offspring had the genotype $Sfrp5^{-/-}$;$Dkk1^{+/-}$. These mice were viable and fertile. The compound mutant mice were maintained by $Sfrp5^{-/-}$;$Dkk1^{+/-}$ intercrosses. No viable $Sfrp5^{-/-}$;$Dkk1^{-/-}$ mice were identified.

To determine if *Sfrp5* and *Dkk1* act redundantly in the AVE we intercrossed $Sfrp5^{-/-}$;$Dkk1^{+/-}$ mice. Mid-streak stage embryos were recovered at E6.75 and whole mount in situ hybridization was performed using *Cerl* as a marker for the AVE (Shawlot et al., 1998). We found that *Cerl* was expressed in the AVE of $Sfrp5^{-/-}$;$Dkk1^{-/-}$ mutant embryos ($n = 3$) and that the AVE was properly localized to the proximal region of the embryo (Fig. 4a,b). The AVE plays an important role in blocking posteriorizing signals and positioning the primitive streak (Kimura et al., 2000; Perea-Gomez et al., 2002). To determine whether primitive streak development was altered in *Sfrp5*;*Dkk1* embryos, we examined E6.5 early streak stage embryos using the molecular marker *Lim1*, which is expressed in both the AVE and the primitive streak (Shawlot and Behringer, 1995; Perea-Gomez et al., 1999). If Wnt signaling is increased in $Sfrp5^{-/-}$;$Dkk1^{-/-}$ embryos we might expect to see an enlarged or ectopic primitive streak. We found, however, that the primitive streak was located opposite the AVE and that the expression of *Lim1* in the primitive streak was not abnormally expanded in $Sfrp5^{-/-}$;$Dkk1^{-/-}$ embryos ($n = 3$) relative to $Sfrp5^{-/-}$ embryos (Fig. 4c,d). Together these results suggest that AVE differentiation and AVE function are not compromised in $Sfrp5^{-/-}$;$Dkk1^{-/-}$ embryos.

To determine whether *Sfrp5* and *Dkk1* synergize to regulate head development we examined E17.5 mice. Although loss of *Dkk1* leads to a severe truncation of anterior head structures (Fig. 4e,f), which could make determining whether *Sfrp5* and *Dkk1* synergize difficult, we took advantage of a fortuitous finding that substantial head development can occur in $Dkk1^{-/-}$ mice on a CD-1 genetic background. We found that 3/10 E17.5 $Dkk1^{-/-}$ mice examined on CD-1 genetic background had anterior head structures (Fig. 4g). If *Sfrp5* and *Dkk1* synergize to regulate head development, we might expect few or no $Sfrp5^{-/-}$;$Dkk1^{-/-}$ mice on a CD-1 background to have anterior head structures. We found, however, that 9/18 $Sfrp5^{-/-}$;$Dkk1^{-/-}$ double mutant mice had anterior head structures (Fig. 4h). This suggests that *Sfrp5* and *Dkk1* do not synergize to regulate head development. We also performed necropsies and skeletal staining of $Sfrp5^{-/-}$;$Dkk1^{-/-}$ mice but we did not detect any new overt phenotypes relative to $Dkk1^{-/-}$ mice.

Our findings indicate that *Sfrp5* is not required for axis formation. It has recently been reported that mice homozygous for an ENU-induced point mutation in *Sfrp5* that likely creates a null allele are viable and do not have reproductive tract defects (Cox et al., 2006). Here we saw no defects in axis formation in *Sfrp5*-deficient mice and no defects in AVE differentiation or primitive streak formation in *Sfrp*;*Dkk1* double mutant mice. It is possible that *Sfrp5* does not antagonize Wnt signaling but rather promotes Wnt signaling by stabilizing Wnts or facilitating the binding of Wnts to Frizzled receptors. Supporting this idea it has been shown that at low concentrations Sfrp1 can increase Wnt signaling in cell culture experiments (Üren et al., 2000). Alternatively, multiple redundant Wnt antagonists may be present in the AVE. Consistent with this possibility it has been recently shown that *Sfrp1* is also expressed in the AVE (Kemp et al., 2005). It will be necessary to generate *Sfrp1*;*Sfrp5* double mutant mice and *Sfrp1*;*Sfrp5*;*Dkk1* triple mutant mice to determine whether these genes act redundantly to influence visceral endoderm development and axis formation in the early mouse embryo.

METHODS

Generation of *Sfrp5*-Deficient Mice

DNA from a 129/SvEv mouse genomic library was used to construct the targeting vector. The linearized targeting vector was electroporated into 1×10^7 AB1 ES cells that were then selected for resistance to G418. G418-resistant clones were screened for correct targeting by Southern analysis. Two targeted clones were injected into C57BL/6 blastocysts to generate chimeric mice. Selection for G418 resistance, Southern analysis, and blastocyst injection were as described by Ramirez-Solis et al. (1993). Chimeras were mated to C57BL/6 mice and CD-1 mice.

Genotyping of Mice at the *Sfrp5* and *Dkk1* Locus

Southern analysis at the *Sfrp5* locus was performed by digestion of mouse genomic DNA with *Xba*I and hybridization with a 0.65 kb *Eco*RV–*Not*I 5′ *Sfrp5* probe. This strategy identified a 7.8 kb wild-type *Sfrp5* allele and a 6.5 kb targeted allele. Digestion of mouse genomic DNA with *Bgl*II and hybridization with a 1.1 kb *Pst*I 3′ *Sfrp5* probe identified a 6.5 kb wild-type allele and a 10.4 kb targeted allele. Genotyping at the *Dkk1* locus was performed as described by Mukhopadhyay et al. (2001). E6.5 *Sfrp5*$^{-/-}$;*Dkk1* embryos were genotyped by PCR of embryo fragments using the *Dkk1* and *neo* PCR primers described by Mukhopadhyay et al. (2001). E8.25 and E9.0 *Sfrp5* embryos were genotyped by Southern hybridization using DNA isolated from yolk sacs.

Whole Mount in Situ Hybridization, Histology, and Dot Blot Hybridization

Digoxigenin-labeled antisense RNA probes were generated using the DIG RNA Labeling Kit (Roche). Whole mount in situ hybridization was performed as described by Wilkinson (1992) with the exception that BM Blocking Reagent (Roche) was used instead of sheep serum for antibody blocking and preabsorption of the anti-digoxigeninin–alkaline phosphatase antibody to mouse embryo powder was omitted. Embryos were obtained from mice maintained on an outbred CD-1 genetic background.

For histological analysis E9.0 embryos were post-fixed in 4% paraformaldehyde for 2 h. Embryos were then dehydrated in 70% ethanol for 5 min, 96% ethanol for 10 min, and twice in 100% ethanol for 10 min. Embryos were then placed in xylene 2 × 15 min and then in 1:1 mix of paraffin and xylene for 1 h at 60°C. Embryos were then placed in paraffin overnight at 60°C. The next day the paraffin was changed twice over a period of 4–6 h before the embryos were oriented for sectioning. Embryos were sectioned at 8 μm and sections were counterstained with eosin for 5 min.

Expression of *Sfrp5* in adult mouse tissues was determined by hybridization of a ^{32}P-labeled *Sfrp5* cDNA probe to a commercial RNA dot blot (Clontech) containing polyA$^+$ RNA according to the manufacturer's protocol.

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