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Sfrp1 and *Sfrp2* are required for normal male sexual development in mice

Nick Warr [a,1], Pam Siggers [a,1], Debora Bogani [a], Rachel Brixey [a], Laura Pastorelli [a], Laura Yates [a], Charlotte H. Dean [a], Sara Wells [b], Wataru Satoh [c], Akihiko Shimono [c,2], Andy Greenfield [a,*]

[a] *Mammalian Genetics Unit, MRC Harwell, Harwell Science and Innovation Campus, Oxfordshire OX11 0RD, UK*
[b] *The Mary Lyon Centre, MRC Harwell, Harwell Science and Innovation Campus, Oxfordshire OX11 0RD, UK*
[c] *Vertebrate Body Plan, Center for Developmental Biology, RIKEN Kobe, Minatojima-Minami, Chuou-Ku, Kobe 650-0047, Japan*

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ABSTRACT

Secreted frizzled-related proteins (Sfrps) are antagonists of WNT signalling implicated in a variety of biological processes. However, there are no reports of a direct role for Sfrps in embryonic organogenesis in mammals. Using *in vivo* loss-of-function studies we report here for the first time a redundant role for *Sfrp1* and *Sfrp2* in embryonic sexual development of the mouse. At 16.5 dpc, male embryos lacking both genes exhibit multiple defects in gonad morphology, reproductive tract maturation and gonad positioning. Abnormal positioning of the testis appears to be due to failed gubernaculum development and an unusually close association between the cranial end of the reproductive tract and the kidney. The testes of double homozygotes are smaller than controls, contain fewer cords from the earliest stages, but still express *Insl3*, which encodes the hormone required for gubernacular masculinisation. *Lgr8*, which encodes the Insl3 receptor, is also expressed in the mutant gubernaculum, suggesting that Sfrp1/Sfrp2 signalling is not required for expression of the ligand or receptor that controls transabdominal testicular descent. Similarities between the abnormalities of embryonic sexual development in $Sfrp1^{-/-}$ $Sfrp2^{-/-}$ embryos with those exhibited by the Looptail and Wnt5a mutants suggest that disrupted non-canonical Wnt signalling may cause these defects.

Introduction

Secreted frizzled-related proteins (Sfrps) are a family of secreted glycoproteins containing a cysteine-rich domain (CRD) at their N-terminus that is homologous to the CRD of the Wnt receptor, Frizzled (Fz) (Jones and Jomary, 2002; Kawano and Kypta, 2003). Several reports describe evidence of Sfrps antagonizing Wnt-mediated signalling by direct competitive interaction with Wnt ligands via the CRD (Wang et al., 1997; Leyns et al., 1997; Dann et al., 2001) or by formation of non-signalling complexes with Frizzled proteins (Bafico et al., 1999), with different Sfrps exhibiting differential specificities with respect to their inhibitory potential (Galli et al., 2006). However, despite this growing body of knowledge concerning molecular interactions of Sfrps, there is still a relative paucity of data describing their specific physiological roles.

In the mouse, five members of the *Sfrp* gene family have been identified using a variety of approaches (Rattner et al., 1997; Shirozu et al., 1996; Finch et al., 1997; Melkonyan et al., 1997). Phylogenetic analysis using protein sequence comparisons indicates that Sfrp1, Sfrp2 and Sfrp5 are the most closely related members of the family. We have previously analysed expression of *Sfrp2* and *Sfrp5* in the developing mouse reproductive organs and utilised ENU mutagenesis to generate point mutations in these genes that are predicted to severely disrupt function (Quwailid et al., 2004; Cox et al., 2006). Both *Sfrp2* and *Sfrp5* are expressed from early stages in the developing mesonephros, which contains the primordia of the male and female reproductive tracts. Both exhibit sexually dimorphic patterns of expression in the Müllerian duct, the female reproductive tract primordium. Although these sex-specific profiles suggest a possible role in the sexually dimorphic fate of the mesonephros, homozygosity for these ENU-induced point mutations in *Sfrp2* and *Sfrp5* does not cause any overt abnormalities in development or reduction in viability (Cox et al., 2006). This is consistent with reports of other recent genetic studies utilising targeted null alleles (Leaf et al., 2006; Satoh et al., 2008).

An analysis of *Sfrp1* and *Sfrp2* during mouse embryogenesis reveals widespread and overlapping expression patterns (Leimeister et al., 1998). Such expression profiles raise the possibility of functional redundancy between closely related family members. Genetic proof of such redundancy was obtained recently when the genes encoding Sfrp1 and Sfrp2 were inactivated in the mouse by gene targeting (Satoh et al., 2006). Mice lacking *Sfrp1* appear to be viable and fertile, and the great majority of those lacking *Sfrp2* are also normal apart from very occasional syndactyly. Mice of the genotype $Sfrp1^{-/-}$ $Sfrp2^{+/-}$ also appeared viable and fertile, but embryos homozygous for both targeted mutations ($Sfrp1^{-/-}$ $Sfrp2^{-/-}$) die after 16.5 days *post coitum*

* Corresponding author. Fax: +44 01235 841200.
E-mail address: a.greenfield@har.mrc.ac.uk (A. Greenfield).
[1] These authors contributed equally to this work.
[2] Current address: Oncology Research Institute, National University of Singapore, Centre of Life Sciences, 28 Medical Drive, #05-06, Singapore 117456.

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(dpc) and exhibit occasional oedema, craniofacial defects and extra digits. The anteroposterior body axis is also shortened, primarily in the thoracic region, and this is attributed to defects in cell migration in the paraxial mesoderm. Aberrant somitogenesis also contributes to this dysmorphology and is correlated with perturbed Notch function.

Wnt signalling is known to play an important role in various aspects of embryonic sexual development (Carroll et al., 2005; Jeays-Ward et al., 2004; Kim et al., 2006), but there have been no previous mutational studies describing a direct role for Sfrps in embryonic organogenesis or embryonic sexual development. In order to address the function of *Sfrp* genes in mouse sexual development we have adopted a loss-of-function approach, using targeted null alleles, and analysed embryos lacking both *Sfrp1* and *Sfrp2* at later stages of gestation. This is the unique pairwise combination of *Sfrp* null alleles known to lead to phenotypic abnormalities in the embryo (Satoh et al., 2006, 2008). Here we report abnormalities in male reproductive organ development, most notably in the morphology of the developing testis and its failure to undergo the first transabdominal phase of descent, and attribute this to a failure of the reproductive tract and associated gubernaculum to masculinise appropriately and an unusually close physical association between the developing reproductive tract and kidney. A comparison is made between these abnormalities and those found in embryos lacking key non-canonical Wnt signalling molecules, Vangl2 and Wnt5a. Strong similarities between *Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ and *Wnt5a*$^{-/-}$ embryos suggest that Sfrp1 and Sfrp2 regulate non-canonical Wnt signalling during sexual development. We discuss these comparative data and the possible molecular mechanisms by which Sfrps modulate Wnt signalling during development of the reproductive organs.

Materials and methods

Generation of mutant embryos and expression analyses

Sfrp1$^{-/-}$ *Sfrp2*$^{-/-}$ embryos were generated by timed matings of *Sfrp1*$^{-/-}$ *Sfrp2*$^{+/-}$ females and males bred on a mixed 129J/C57BL/6J background (Satoh et al., 2006). Noon on the day of the copulatory plug was counted as 0.5 dpc. Embryos were staged accurately based on the number of tail somites or limb and gonad morphology. Dead or dying embryos late in gestation (after 16.5 dpc) were discarded. Wholemount *in situ* hybridization (WMISH) analysis of embryonic tissues was performed as previously described (Cox et al., 2006; Grimmond et al., 2000). Probes for *Sox9* (Wright et al., 1995), *Lim1* (Kobayashi et al., 2004), *Oct4* and 3β*HSD* (Siggers et al., 2002) have been previously described. An *Lgr8* probe was generated from IMAGE clone 40129664. Probes for *Insl3*, *Jag1* and *AR* were generated by reverse transcription polymerase chain reaction (RT-PCR) from embryonic gonad RNA samples (13.5 dpc) using the following primers: 5′-AGCTGCTGCAGTGGCTAGA-3′ and 5′-GGGACACAGGGAGGAGGT-3′ (*Insl3*); 5′-AATGGTGATGGCAGCCTTAG-3′ and 5′-GCCTGCCTGTCTCTTTTCAA-3′ (*Jag1*); 5′-CACCTTGTTCCCTTTCCAGA-3′ and 5′-TGGGGTCAACCTGCTCTTTA-3′ (*AR*).

Detection of the *Sfrp1* KI *lacZ* reporter was performed using a protocol based on (Whiting et al., 1991). Embryos were dissected in PBS to expose the developing reproductive organs/tracts, fixed on ice (1% PFA, 0.2% glutaraldehyde, 2 mM $MgCl_2$, 5 mM EGTA, 0.02% NP-40 in PBS) and then washed in PBS/0.02% NP-40. Staining was carried out in the dark, at room temperature for 16 h or until blue colour fully developed in X-gal stain (PBS containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% deoxycholate, 0.02% NP-40, 1 mg/ml X-Gal). Samples were post-fixed in 4% PFA/PBS.

Mouse mutants utilised and genotyping

Genotyping for the targeted alleles of *Sfrp1* and *Sfrp2* was performed as previously described (Satoh et al., 2006). The identification and analysis of the *Sfrp2*^{HC50F} and *Sfrp2*^{I153N} mutations has been described previously (Quwailid et al., 2004; Cox et al., 2006). Mice heterozygous for these mutations were maintained on a C57BL/6J background. Genotyping for both alleles was performed by an allelic discrimination PCR (AD-PCR) performed using an ABI Prism 7000 Sequence Detection System according to manufacturer's guidelines as previously described (Cox et al., 2006).

Looptail mice (*Vangl2*$^{Lp/+}$) were maintained on the C3H/HeH background and identified by the presence of a prominent tail loop (Murdoch et al., 2001). Homozygous embryos were identified by the occurrence of severe neural tube defects characteristic of this mutant.

Mice expressing green fluorescent protein (GFP) ubiquitously (Tg(GFPU)5Nagy/J) were purchased from The Jackson Laboratory and carriers identified by neonatal fluorescence.

Embryos were sexed by a PCR assay that simultaneously amplifies the *Ube1y1* and *Ube1x* genes, using the following primer pair: 5′-TGGATGGTGTGGCCAATG-3′ and 5′-CACCTGCACGTTGCCCTT-3′. Y- and X-linked amplicons give products of 335 bp and 253 bp, respectively.

Testosterone assay

Whole embryos (*Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ and control littermates) were collected at 14.5 dpc and homogenised in PBS. Testosterone levels were measured using a previously validated ELISA kit from Neogen (# 402510) according to the manufacturer's instructions. Three embryos in each class were measured in quadruplicate. Given in the text are the mean values for each class of embryo.

Exogenous administration of testosterone

Androgens were administered to embryos using a protocol adapted from (Hammes et al., 2005). Pregnant mice were injected subcutaneously with a daily dose of 2 mg of dihydrotestosterone (DHT: 5α-androstan-17β-OH-ol-3-one, Sigma-Aldrich) in a 1:9 mix of ethanol/sunflower oil from days 10.5 to 17.5 dpc (or with just ethanol/oil as control) and embryos were harvested at 17.5/18.5 dpc.

Organ culture

Culturing of embryonic gonads and recombination experiments between sub-dissected gonads and marked mesonephroi were performed based on methodologies described in (Martineau et al., 1997). XY urogenital ridges (UGRs), consisting of gonad and attached mesonephros, were collected at 11.5 dpc and initially cultured to establish conditions under which testis cords formed reliably after 48 h culture. Samples were incubated on 1.5% agar blocks at 37 °C/5% CO_2 in Dulbecco's Minimal Eagle's Medium (DMEM)/10% fetal calf serum (FCS)/50 μg/ml ampicillin/200 mM L-glutamine. For recombination cultures, 11.5 dpc XY male UGRs from mutant embryos (*Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$) which also carried a ubiquitously expressed GFP transgene (Tg(GFPU)5Nagy/J)) and littermate controls were sub-dissected into component gonad and mesonephros in PBS. These were then reassembled in appropriate combinations and cultured for 48 h, as above. Migration from the marked mesonephros into the attached gonad was imaged using a Leica TCS SP5 confocal microscope. No migration was observed into control XX gonads during these experiments.

Immunohistochemistry

The following antibodies were utilised in this study: PECAM-1 (BD Bioscience, #553708); phospho-histone H3 (pHH3) (Upstate/Millipore, #06-570); anti-cleaved caspase 3 (Cell Signalling, #9661); active (non-phosphorylated) β-catenin (ABC) antibody (Upstate/Millipore, #05-665). Immunostaining was performed on sectioned material, apart from PECAM-1, which was performed on wholemounts and visualised using confocal microscopy. For quantitative analysis of cell proliferation in the gonad mesonephros, cryosections were