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Extra-embryonic *Wnt3* regulates the establishment of the primitive streak in mice

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ABSTRACT

The establishment of the head to tail axis at early stages of development is a fundamental aspect of vertebrate embryogenesis. In mice, experimental embryology, genetics and expression studies have suggested that the visceral endoderm, an extra-embryonic tissue, plays an important role in anteroposterior axial development. Here we show that absence of *Wnt3* in the posterior visceral endoderm leads to delayed formation of the primitive streak and that interplay between anterior and posterior visceral endoderm restricts the position of the primitive streak. Embryos lacking *Wnt3* in the visceral endoderm, however, appear normal by E9.5. Our results suggest a model for axial development in which multiple signals are required for anteroposterior axial development in mammals.

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Introduction

The establishment of the head to tail axis is a fundamental step in generating the basic body plan of vertebrates (Stern et al., 2006). In amniote embryos, like those of birds and mammals, this axis is marked at its caudal end by the primitive streak, a region of epithelial to mesenchymal transition that serves as a conduit for the generation of mesoderm and endoderm during gastrulation (Stern, 2004). In mammals, understanding how the primitive streak forms has special significance because deciphering why the primitive streak forms where it does can explain how the anteroposterior axis is established during embryogenesis (Beddington and Robertson, 1998).

In mice, two components of the early post-implantation embryo, the extra-embryonic ectoderm and the posterior visceral endoderm, have been proposed to induce the formation of the primitive streak (Bachvarova, 1996; Beddington and Robertson, 1999; Conlon and Beddington, 1995). The extra-embryonic ectoderm is a trophectoderm derivative that abuts the epiblast, the tissue that gives rise to the primitive streak. Several lines of evidence suggest that Bmp4 emanating from the extra-embryonic ectoderm signals to the adjacent epiblast to activate primitive streak markers (Arnold and Robertson, 2009; Beddington and Robertson, 1999; Ben-Haim et al., 2006). The induced epiblast then undergoes an epithelial to mesenchymal transition to become the primitive streak, allowing gastrulation and the subsequent generation of mesoderm and endoderm.

A less studied potential signaling center is the posterior visceral endoderm, an extra-embryonic component derived from primitive endoderm located adjacent to the region of epiblast that becomes the primitive streak. Tissue recombination experiments have shown that the posterior visceral endoderm has the capacity to re-specify anterior ectoderm into a posterior mesodermal fate and that this reprograming is effected by a diffusible signal (Belaoussoff et al., 1998). A candidate molecule is *Wnt3*; *Wnt3* knockout embryos lack a primitive streak and fail to gastrulate (Liu et al., 1999). In addition, *Wnt3* expression is first observed in the posterior visceral endoderm of embryos dissected at embryonic day 5.5 and expands to the adjacent epiblast tissue a few hours later (Rivera-Perez and Magnuson, 2005). In support of the hypothesis that *Wnt3* derived from visceral endoderm has an inductive role in primitive streak formation, embryos in which *Wnt3* was conditionally inactivated in the epiblast were able to establish the primitive streak and initiate

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gastrulation although they failed to thrive soon afterwards (Tortelote et al., 2013). Intriguingly, in Wnt3-epiblast mutant embryos, *Wnt3* mRNA is present transiently in the posterior visceral endoderm suggesting that *Wnt3* signaling from the posterior visceral endoderm is responsible for the initiation of gastrulation (Tortelote et al., 2013).

In order to determine if *Wnt3* function in the posterior visceral endoderm is required for the formation of the primitive streak, we inactivated *Wnt3* in the visceral endoderm using *Transthyretin-Cre* mice (Ttr^{Cre}). Our results show that embryos lacking *Wnt3* solely in the visceral endoderm specify the primitive streak later than control littermates. This effect is exacerbated if a copy of *Wnt3* is additionally inactivated in the epiblast. Despite these defects, *Wnt3* visceral endoderm mutant embryos are able to gastrulate and develop normally. From these results, we conclude that *Wnt3* function in the posterior visceral endoderm regulates the formation of the primitive streak and gastrulation but its function is dispensable for embryo development. Our results also indicate that the anterior visceral endoderm plays a role in preventing the expansion of *Wnt3* signaling to the anterior side of the embryo restricting the formation of the primitive streak to the opposite side of the epiblast. We propose a model in which a series of coordinated events involving the visceral endoderm, extra-embryonic ectoderm and epiblast control the development of the primitive streak in mammals.

Materials and methods

Embryo staging and mice

Embryos were staged based on morphological landmarks as previously described (Downs and Davies, 1993; Rivera-Perez et al., 2010) or described in terms of dissection time. Noon of the day that a mating plug was observed was considered embryonic day 0.5 (E0.5) of gestation. Ttr^{Cre} mice were previously described (Kwon and Hadjantonakis, 2009). $Wnt3^{c}$ mice were obtained from Jeff (Barrow et al., 2003). *Cripto* mutant mice ($Cripto^{lacZ}$) were provided by Michael (Ding et al., 1998). Hex^{GFP} mice were provided by Tristan (Rodriguez et al., 2001). All *Wnt3* mutant alleles, Ttr^{Cre}, $Cripto^{lacZ}$ and Hex^{GFP} mice were maintained on a CD-1 outbred genetic background.

Generation of $Wnt3^{lacZ}$ knock-in mice

To target the *Wnt3* locus, we designed a targeting vector containing a 6 kb NotI-BamHI genomic DNA fragment (129S6/SvEvBrd) that included exons 3 and 4. A *lacZ* cassette and a floxed PGK*neo*bpA cassette were inserted into the ClaI site in exon 4. The *IacZ* cassette contained an internal ribosomal entry site (IRES) and an SV40 polyA signal sequence. An HSV-*tk*pA cassette was added to the 5′-homologous arm. Gene targeting was conducted in AB1 ES cells as previously described (Mishina et al., 1995). A total of 192 G418; FIAU double-resistant ES cell colonies were screened for homologous recombination by Southern blotting using a *Wnt3* 3′ UTR probe (Liu et al., 1999). Twenty-seven lines were positive in the initial screen ing, and two lines gave rise to germ-line chimeras. We obtained $Wnt3^{lacZneo}$ heterozygous mice after crossing chimeras to C57BL/6 mice. $Wnt3^{lacZneo}$ heterozygous mice were crossed to $Sox2^{Cre}$ mice (Hayashi et al., 2002) to remove the *neo* cassette and generate $Wnt3^{lacZ}$ heterozygous mice. Heterozygous mice for both alleles were normal and fertile. To determine whether the $Wnt3^{lacZneo}$ and $Wnt3^{lacZ}$ alleles are null alleles, we generated $Wnt3^{lacZneo/lacZneo}$ and $Wnt3^{lacZ/lacZ}$ homozygous embryos or crossed $Wnt3^{lacZneo}$ and $Wnt3^{lacZ}$ heterozygous mice with mice carrying the $Wnt3^{\Delta 3,4}$ null allele (Barrow et al., 2003). All types of mutants phenocopy the previously reported phenotype of *Wnt3* null embryos (Barrow et al., 2007; Liu et al., 1999), that is, they lack a primitive streak, fail to elongate the anteroposterior axis and are resorbed by ~E9.5 indicating that they are null alleles. The $Wnt3^{lacZneo}$ strain has been registered with the Mouse Genome Informatics international database under the identifier MGI:5439832 (Wnt3tm1Bhr).

Germ layer isolation

To separate the epiblast from the visceral endoderm, we followed the germ layer separation protocol described previously (Nagy, 2003). Briefly, embryos were cut just below the epiblast – extra-embryonic ectoderm boundary, incubated in a solution containing a mixture of pancreatin and trypsin and pipetted up and down using a pulled Pasteur pipette. Germ layer separation was performed retrospectively after wholemount *in situ* hybridization was conducted.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was performed as previously described (Rivera-Perez and Magnuson, 2005) using the following probes: *Wnt3* (full length cDNA, 2084 bp). *Brachyury* (full length cDNA, 1784 bp) (Herrmann, 1991). All riboprobes were prepared using a digoxigenin RNA labeling kit (Roche Cat. no. 1175025).

β-Galactosidase staining protocol

We performed β-galactosidase assays as described by Sundararajan and co-workers (Sundararajan et al., 2012). After β-galactosidase staining, embryos were rinsed in wash solution, re-fixed in 4% paraformaldehyde at room temperature for 20 min, cleared in glycerol and imaged.

Genotyping

The genotype of the embryos was determined retrospectively after wholemount *in situ* hybridization or after β-galactosidase assays. Each litter was imaged before genotyping to allow unique identification of each embryo. Embryos were placed in 15–20 μl of PCR lysis buffer (50 mM KCl, 10 mM Tris–HCl pH8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% IGEPAL (Sigma, Cat. no. 18896) and 0.45% Tween 20) containing 100 mg/ml Proteinase K and incubated overnight at 56 °C. After lysis, the proteinase K was inactivated at 95 °C for 5 min and 1 μl of the lysate was used for the PCR reaction. Mice were genotyped at postnatal day 10 using a 2 mm tail tip piece.

PCR reactions were carried out using the following oligonucleotides: *Wnt3* wild-type allele, ptmw1 GAC TTC CTC AAG GAC AAG TAC G and ptmw2 GAA GAC GCA ATG GCA TTT CTC (309 bp); Wild-type and $Wnt3^{c}$ alleles, Wnt3F3 5′ TGG CTT CAG CAT CTG TTA CCT TC 3′ and Wnt3R6 5′ AAG ATC CCC ATA CTG CCA TCA C 3′ (389 bp and 546 bp, respectively); $Wnt3^{\Delta 3,4}$ allele, Wnt3F10 5′ GGG AGC CGC CCG TTG CTA 3′ and Wnt3R6 5′ AAG ATC CCC ATA CTG CCA TCA C 3′ (388 bp); $Wnt3^{lacZ}$ allele, LacZF 5′TGG CGT TAC CCA ACT TAA TCG 3′ and LacZR 5′ATG TGA GCG AGT AAC AAC CCG 3′ (324 bp); $Wnt3^{lacZneo}$ allele, NeoF2 TGG CTA CCC GTG ATA TTG CTG and ptmw2 GAA GAC GCA ATG GCA TTT CTC (~500 bp); Ttr^{Cre} transgene, qCreF1 5′ GAA CCT CAT GGA CAT GTT CAG G 3′ and qCreR1 5′ AGT GCG TTC GAA CGC TAG AGC CTG T 3′ (320 bp); *Cripto* wild type allele, Cripto5′UTRf CCT CCG AAG TCC TCA ATC AC and CriptoR3 TCC GAA GTG GCT ATC TCC AG (333 bp); and $Cripto^{lacZ}$ Cripto5′UTRf CCT CCG AAG TCC TCA ATC AC and CriptolacZ GAT TAA GTT GGG TAA CGC CAG (~300 bp).

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