Developmental Biology 374 (2013) 164–173

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Wnt3 function in the epiblast is required for the maintenance but not the initiation of gastrulation in mice

Giovane G. Tortelote, J. Manuel Hernández-Hernández, Alexandre J.C. Quaresma, Jeffrey A. Nickerson, Anthony N. Imbalzano, Jaime A. Rivera-Pérez *

Department of Cell and Developmental Biology, University of Massachusetts Medical School, 55 Lake Ave. North S7-228, Worcester, MA 01655, United States

ARTICLE INFO

Article history:
Received 24 July 2012
Received in revised form
8 October 2012
Accepted 9 October 2012
Available online 16 October 2012

Keywords:
Mouse
Embryo
Gastrulation
Epiblast
Primitive streak
Wnt3

ABSTRACT

The formation of the anteroposterior axis in mice requires a *Wnt3*-dependent symmetry-breaking event that leads to the formation of the primitive streak and gastrulation. *Wnt3* is expressed sequentially in two distinct areas of the mouse embryo before the appearance of the primitive streak; first in the posterior visceral endoderm and soon after in the adjacent posterior epiblast. Hence, although an axial requirement for *Wnt3* is well established, its temporal and tissue specific requirements remain an open question. Here, we report the conditional inactivation of *Wnt3* in the epiblast of developing mouse embryos. Contrary to previous studies, our data shows that embryos lacking *Wnt3* specifically in the epiblast are able to initiate gastrulation and advance to late primitive streak stages but fail to thrive and are resorbed by E9.5. At the molecular level, we provide evidence that *Wnt3* regulates its own expression and that of other primitive streak markers via activation of the canonical Wnt signaling pathway.

Introduction

In mice, the formation of the primitive streak on the posterior side of the embryo at embryonic day 6.5 (E6.5), marks the morphological initiation of gastrulation (Arnold and Robertson, 2009). Tissue recombination experiments have hinted that a signaling event emanating from the posterior visceral endoderm is responsible for the formation of the primitive streak and gastrulation (Belaoussoff et al., 1998). However, the identity of the signaling molecule and the molecular mechanisms by which the epiblast receives and reacts to this signal remain elusive.

Genetic studies indicate that the Wnt signaling pathway is at the core of the molecular events that control gastrulation in mouse embryos. Ablation of *Wnt3*, leads to complete absence of the primitive streak and gastrulation failure (Liu et al., 1999). Simultaneous inactivation of the Wnt co-receptors *Lrp5* and *Lrp6* or absence of their chaperone, *Mesd,* phenocopies the *Wnt3* null phenotype (Hsieh et al., 2003; Kelly et al., 2004). Conversely, ablation of *Axin*, a negative regulator of the canonical Wnt signaling pathway, or ectopic expression of the chick *Wnt8c* gene in mouse embryos lead to duplication of the primitive streak (Popperl et al., 1997; Zeng et al., 1997).

Wnt3 is a Wnt signaling molecule expressed in the early post-implantation mouse embryo in a sequential manner: first, it is observed in the posterior visceral endoderm, an extra-embryonic tissue, at around E5.5, and six hours later, at ~E5.75, *Wnt3* expression is evident in the epiblast, the region of the conceptus that forms the embryo (Rivera-Perez and Magnuson, 2005). The expression in the epiblast is restricted to a region directly abutting the posterior visceral endoderm (Rivera-Perez and Magnuson, 2005). Because of its dual expression pattern, the role of *Wnt3* in anteroposterior axis formation cannot be assigned to either tissue in standard knockout experiments. Moreover, the molecular events downstream of *Wnt3* signaling in the developing embryo are not understood.

In order to determine the function of *Wnt3* in the epiblast we conducted a tissue specific knockout of *Wnt3* using an epiblast-specific Cre line, and a conditional allele of *Wnt3*. In addition, we investigated the intracellular events that control *Wnt3* expression at the onset of gastrulation. Our results reveal that embryos lacking *Wnt3* specifically in the epiblast are capable of specifying the anteroposterior axis and initiate the process of gastrulation. However, they are not able to complete gastrulation and are resorbed at approximately E9.5. These results run contrary to previous studies that suggested that *Wnt3* is essential in the epiblast for establishment of a primitive streak and gastrulation (Barrow et al., 2007). We also provide evidence that, at the promoter level, *Wnt3* controls not only the expression of primitive streak markers but also its own expression through activation of

* Corresponding author. Fax: +1 508 856 1033.
E-mail address: jaime.rivera@umassmed.edu (J.A. Rivera-Pérez).

0012-1606/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2012.10.013

the canonical Wnt pathway. In summary, our results show that *Wnt3* function in the epiblast is essential for the maintenance of gastrulation but not its initiation and provide mechanistic evidence for how *Wnt3* regulates its own expression in order to orchestrate gastrulation in mice.

Materials and methods

Embryo staging and mouse strains

Embryos were staged using morphological landmarks as previously described (Downs and Davies, 1993; Rivera-Perez et al., 2010) or described as days of development. Noon of the day that a mating plug was observed was considered embryonic day 0.5 (E0.5) of development. CD-1 mice were obtained from Charles River Laboratories. *Sox2*^{cre} mice were obtained from the Jackson laboratory (Stock no. 004783). *Wnt3*^{c} mice were obtained from Dr. Jeff Barrow (Barrow et al., 2003). *Wnt3*^{lacZ} mice were provided by Dr. Richard Behringer. These mice carry an *IRES-lacZ* cassette that was inserted in the unique ClaI site of exon 4 of the *Wnt3* locus creating a null allele (MGI:5439832). All animals were maintained as CD-1 outbred stocks.

Whole-mount RNA in situ hybridization

We performed whole-mount *in situ* hybridization as described (Rivera-Perez and Magnuson, 2005). Briefly, embryos were dissected using forceps and fixed overnight at 4 °C in 4% paraformaldehyde prepared in PBS. After fixation, the embryos were dehydrated in methanol series and stored in 100% methanol at −20 °C. Hybridization was conducted at 70 °C. The probes were: *Wnt3*, a 0.8 kb cDNA fragment containing exons 3–5 (Liu et al., 1999). *Wnt3* 3′F, a 1050 bp fragment of exon 5 containing a piece of the 3′UTR. *Brachyury*, full length cDNA probe of 1308 bp (Herrmann, 1991). *Fgf8*, full length cDNA, 1100 bp. *Axin2*, 2420 bp cDNA piece containing part of exon 2, exons 3–9 and a portion of exon 10. *Sp5,* full length cDNA, 1540 bp. *Hesx1*, cDNA piece containing part of exon1, exon2 and a portion of exon 3, 394 bp (Thomas and Beddington, 1996b). *Hex,* 527 bp fragment from 281–818 bp of cDNA sequence (Thomas et al., 1998) and *Dkk1,* full cDNA sequence, 1235 bp (Miura et al., 2010). All riboprobes were prepared using a digoxigenin RNA labeling kit (Roche Cat. no. 1175025).

Preparation of mouse embryonic fibroblasts

MEFs were prepared using E13.5 CD-1 embryos as previously described (Nagy et al., 2003).

Preparation of Wnt3-conditioned media

To obtain *Wnt3*-conditioned media, we transfected HeLa cells with pCMV-*Wnt3*-2a-eGFP. This plasmid produces a bicistronic message that contains the self-cleaving 2A peptide (Szymczak and Vignali, 2005), inserted between *Wnt3* and eGFP. To generate pCMV-*Wnt3*-2a-eGFP, a 1.2 kb fragment containing the *Wnt3* coding region was PCR-amplified from a *Wnt3* cDNA plasmid obtained from Open Biosystems (Cat. no. 40039305). The amplified fragment was digested with XhoI and BspEI and subcloned into the pCAG-SH-RG plasmid (Stewart et al., 2009). Next the *Wnt3*-2a-EGFP cassette was PCR-amplified, digested with NheI and BglII and subcloned into pCMV-GFP (Clontech, Cat. no. 6082-1) digested with NheI and BamHI. All transfections were carried out using FUGENE HD reagent (Promega Cat. no. E2311). The transfection success was evaluated by the presence of eGFP, using fluorescence microscopy. The efficiency of the transfection, using HeLa cells was 60%–70%. Twenty-four hours after transfection, the media was replaced by MEF media composed of DMEM (Gibco, Cat. no. 12100-046), 10% FBS, 1 × Glutamax (Invitrogen, Cat. no. 35050-061), Penicillin (100 U/ml), Streptomycin (100 μg/ml) (Invitrogen, Cat. no. 15140-122). Media was collected every 24 h for three days, stored at 4 °C and used within 4 days. Before use *Wnt3*-conditioned media was cleared by centrifugation (5 min, 1000 g) and filtered through a 0.45 μm filter. Control conditioned media (GFP-conditioned media), was generated by transfecting HeLa cells with a pCMV-eGFP construct.

Wnt3 treatment and immunofluorescence

MEFs cells were grown on cover slips placed in 12-well plates containing MEF media. When the cells reached about 70% confluence the media was replaced either with MEF media, *Wnt3*-conditioned or GFP-conditioned media and incubated overnight. The cells were then fixed in 4% PFA for 40 min and washed in TBS-1 (10 mM Tris–HCl, pH7.7, 150 mM NaCl, 3 mM KCl, 1.5 mM $MgCl_2$, 0.05% Tween 20, 0.1% bovine serum albumin and 0.2% glycine). Cells were blocked in TBS-1 for 1 h and then incubated overnight at 4 °C with mouse anti-β-catenin antibody diluted 1:50 (BD Transduction lab, Cat. no. 610154) in TBS-1. The next day, cells were washed three times for 15 min with TBS-1 and incubated for one hour with goat anti-mouse Alexa 488 secondary antibody (Molecular probes, Cat. no. A10680) diluted 1:1500 at room temperature. Cells were then washed twice for 15 min in TBS-1 and incubated with DAPI (Sigma Cat. no. D9564) for 30 min. Cells were mounted on the glass slides using prolong gold mounting media (Molecular Probes, Cat. no. P36930) and visualized using fluorescence microscopy (Leica DMI 6000B). Quantification of cells with nuclear localization of β-catenin was performed by selecting five adjacent fields per treatment (Control media, GFP-conditioned media, *Wnt3*-conditioned media). The experiments were repeated three times and were scored by two investigators. One investigator scored the cells blindly. Each field averaged around 20 cells. Cells were assessed based on their nuclear staining (blue) and β-catenin staining (green). All cells with accumulation of β-catenin in their nuclei (blue) were counted and plotted as a percentage of the total number of cells. The scoring of β-catenin positive nuclei was validated by confocal microscopy (Leica SP1 laser scanning confocal microscopy). Cells that were scored as negative had fluorescence above or below the nucleus, but did not have nuclear fluorescence in an optical section through the nucleus. Cells scored as positive had a high level of nuclear fluorescence in optical sections through the nucleus.

RNA isolation and quantitative PCR

For quantitative PCR analysis, P3 MEF cells were grown in 10 cm tissue culture plates, until they reached 70% confluence. At this point, they were incubated overnight in control or *Wnt3*-conditioned media. The next day cells were harvested and the RNA was isolated using TRIzol (Invitrogen, Cat. no.15596-026). The RNA was digested with DNAse I for 30 min at 37 °C to yield DNA-free RNA samples and purified using a DNA-free RNA kit (Zymo Research, Cat. no. R1013). One microgram of total RNA was utilized for reverse transcription reactions to generate cDNA using the Superscript III First Strand kit (Invitrogen, Cat. no. 18080-051). Quantitative PCR was performed with Fast SYBR Green Master Mix (Applied Biosystem, Cat. no. 4385612). The primers used for qPCR were designed with at least one of the primers spanning an intronic region in order to prevent amplification of genomic DNA contaminants. The following primers were used: *Axin2*: 5′-CTCCTTGGAGGCAAGAGC-3′ and 5′-GGCCACG-CAGCACCGCTG-3′ (Jho et al., 2002). *Brachyury*: 5′-TACCCCAGCCCC-TATGCTCA-3′ and 5′-GGCACTCCGAGGCTAGACCA-3′; *Cyclin D1*: 5′-TCGTGGCCTCTAAGATGAAG-3′ and 5′-TTTTGGAGAGGAAGTGTTCG-3′ (Zhang et al., 2009); *Gapdh* (5′-AAGGTCATCCCAGAGCTGAA-3′ and