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Foxh1 and Foxa2 are not required for formation of the midgut and hindgut definitive endoderm

Kristen D. McKnight [a,b,1,2], Juan Hou [a,2], Pamela A. Hoodless [a,c,*]

[a] Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, BC, Canada
[b] Genetics Graduate Program, University of British Columbia, Vancouver, BC, Canada
[c] Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada

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ABSTRACT

The definitive endoderm forms during gastrulation and is rapidly transformed into the gut tube which is divided along the anterior–posterior axis into the foregut, midgut, and hindgut. Lineage tracing and genetic analysis have examined the origin of the definitive endoderm during gastrulation and demonstrated that the majority of definitive endoderm arises at the anterior end of the primitive streak (APS). Foxh1 and Foxa2 have been shown to play a role in specification of the APS and definitive endoderm. However, prior studies have focused on the role of these factors in specification of foregut definitive endoderm, while their role in the specification of midgut and hindgut definitive endoderm is less understood. Furthermore, previous analyses of these mutants have utilized definitive endoderm markers that are restricted to the anterior endoderm, expressed in extraembryonic endoderm, or present in other germ layers. Here, we characterized the expression of several novel definitive and visceral endoderm markers in *Foxh1* and *Foxa2* null embryos. In accordance with previous studies, we observed a deficiency of foregut definitive endoderm resulting in incorporation of visceral endoderm into the foregut. Interestingly, this analysis revealed that formation of midgut and hindgut definitive endoderm is unaffected by loss of *Foxh1* or *Foxa2*. This finding represents a significant insight into specification and regionalization of mouse definitive endoderm.

Introduction

The definitive endoderm, one of the three germ layers formed during gastrulation, gives rise to the lining of the respiratory and gastrointestinal tract and associated organs such as the liver and pancreas (reviewed in Wells and Melton, 1999). Before gastrulation, the embryonic portion of the mouse embryo consists of two cell layers—the inner epiblast and the outer extraembryonic visceral endoderm. Gastrulation begins at embryonic day (E) 6.5 and is visualized by the formation of the primitive streak on the posterior side of the embryo (reviewed in Tam and Behringer, 1997). During gastrulation, cells of the epiblast move through the primitive streak and emerge as mesoderm or definitive endoderm. As the cells of the definitive endoderm form and exit the streak, they intercalate into the pre-existing visceral endoderm layer and subsequently expand to form a single cell layer encompassing the entire embryonic region (Kwon et al., 2008; Lawson et al., 1986; Lawson and Pedersen, 1987; Tam and Beddington, 1992). Following gastrulation, the epithelial sheet of definitive endoderm transforms into a tube, beginning at the anterior with the foregut invagination and followed by the hindgut invagination at the posterior with the midgut remaining open until E9.0 when the embryo turns from the lordotic to fetal position. As the gut tube is forming, the derivative organs of the definitive endoderm begin to develop, beginning as small buds in the epithelial layer which then further differentiate as embryogenesis proceeds.

Fate mapping of the definitive endoderm has examined both the spatial and temporal origin of different regions of the definitive endoderm. These studies revealed that approximately 95% of definitive endoderm arises from the anterior end of the primitive streak (APS) during gastrulation (Lawson et al., 1986; Lawson and Pedersen, 1987). The APS is marked by the expression of *Foxa2* and *goosecoid* (*Gsc*) and is the location of the mouse embryonic organizer (Camus and Tam, 1999; Robb and Tam, 2004). In addition to the definitive endoderm, the APS also contains progenitors for the node, prechordal plate, and notochord (Kinder et al., 2001). The Nodal signaling pathway and the forkhead transcription factors Foxh1 and Foxa2 have

* Corresponding author. Terry Fox Laboratory, British Columbia Cancer Agency, 675 West 10th Avenue, Vancouver, British Columbia, Canada V5Z 1L3. Fax: +1 604 877 0712.
E-mail address: hoodless@bccrc.ca (P.A. Hoodless).
[1] Present address: Department of Developmental Biology, Stanford University School of Medicine, Beckman Center, 279 Campus Drive, Stanford, CA 94305-5329, USA.
[2] These authors contributed equally to this work.

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been shown to play critical roles in specification of the APS and its derivative tissues, including the definitive endoderm (Ang and Rossant, 1994; Hoodless et al., 2001; Schier, 2003; Shen, 2007; Weinstein et al., 1994; Yamamoto et al., 2001). Nodal, a member of the transforming growth factor beta (TGF-β) family of ligands, regulates gene expression via Smads 2, 3, and 4, along with sequence-specific DNA-binding factors, such as Foxh1. Genetic manipulation of components of the Nodal pathway has revealed a graded response to Nodal signaling in the mouse embryo, where specification of the anterior definitive endoderm and prechordal plate require the highest levels of signaling, intermediate levels of signaling specify the node and notochord (axial mesoderm), and lower levels of signaling are involved in specification of paraxial and lateral mesoderm (Vincent et al., 2003). In addition, Smad2 has been demonstrated to play a role in endoderm specification, as *Smad2* null ES cells have also been shown to be unable to contribute to the definitive endoderm lineage (Tremblay et al., 2000).

Foxh1 is a Smad cofactor involved in regulating gene expression downstream of both Nodal and Activin (Chen et al., 1996; Labbe et al., 1998; Liu et al., 1999; Weisberg et al., 1998). Loss of *Foxh1* in the mouse embryo results in a spectrum of phenotypes ranging from a failure to establish the anterior–posterior axis in the most severe phenotypic class to a failure to specify the APS its midline derivatives, such as the node and notochord, in the majority of embryos (Hoodless et al., 2001; Yamamoto et al., 2001). Chimera studies revealed that *Foxh1* null ES cells are largely excluded from the definitive endoderm, with a small number of mutant cells remaining in the hindgut region, suggesting that *Foxh1* plays a critical role in the specification of this tissue but may not be required in the posterior endoderm (Hoodless et al., 2001). However, chimera studies primarily test the ability of a mutant cell population to effectively compete with wild-type cells to contribute to a tissue lineage, but not their differentiation potential based on marker analysis.

In accordance with its loss of expression in *Foxh1* null embryos, *Foxa2* null embryos also fail to specify the APS and have a phenotype that is slightly more severe than *Foxh1* null embryos (Ang and Rossant, 1994; Weinstein et al., 1994) due to a severe constriction between the extraembryonic and embryonic regions which is thought to sterically hinder embryonic development. *Foxa2* tetraploid chimeras generated using *Foxa2* null ES cells (such that the extraembryonic tissues are wild-type and embryonic tissues are mutant) rescues the constrictions and results in a phenotype more similar to *Foxh1* null embryos (Dufort et al., 1998). Histological analysis of chimeras revealed that loss of *Foxa2* affected specification of the foregut definitive endoderm while *Shh* expression suggested that the hindgut definitive endoderm was still present (Dufort et al., 1998). Recently, it was shown that *Foxa2* null cells are unable to properly integrate into the definitive endoderm during gastrulation due to aberrant establishment of cell polarity and epithelialization of the mutant cells, demonstrating that Foxa2 plays multiple roles in endoderm formation in the mouse (Burtscher and Lickert, 2009).

Study of the definitive endoderm, and in particular the midgut and hindgut, has lagged behind that of the other two germ layers, primarily due to the lack of both pan-endodermal and region-specific genetic markers of this tissue. Furthermore, while a role for Nodal, Foxh1, and Foxa2 in foregut specification is well established, the function of these factors in midgut and hindgut formation remains less understood. We recently performed gene expression profiling of the mouse definitive endoderm and identified a panel of novel definitive and visceral endoderm markers (Hou et al., 2007; McKnight et al., 2007). Here, we analyze these markers in *Foxh1* and *Foxa2* null embryos to directly address the role of these transcription factors in midgut and hindgut formation. Supporting previous findings, we show that formation of the foregut definitive endoderm is significantly reduced in these mutants, resulting in the incorporation of visceral endoderm into the foregut invagination. Furthermore, we demonstrate for the first time that formation of the midgut and hindgut definitive endoderm does not require Foxh1 or Foxa2.

Results

Midgut and hindgut formation is unperturbed in Foxh1 and Foxa2 null embryos

To specifically study midgut and hindgut formation in *Foxh1* and *Foxa2* null embryos we analyzed the expression of the midgut marker *Nephrocan* (*Nepn*) and the hindgut marker *SRY box containing gene 17* (*Sox17*). In E8.5 wild-type embryos, *Nepn* is expressed specifically in the midgut, where the "midgut" is defined as the open region of the gut tube at this embryonic stage (Figs. 1A and B) (Hou et al., 2007). *Nepn* is not expressed in the midline cells of the notochordal plate (arrowhead in Fig. 1C). Interestingly, strong expression of *Nepn* in the midgut is observed in both *Foxh1* and *Foxa2* null embryos (Figs. 1D, E, G, and H). Consistent with a loss of the notochordal plate in these mutants, the bilateral expression domain of *Nepn* is fused at the midline (arrowheads in Figs. 1F and I). The domain of *Nepn* expression is reduced in *Foxa2* null embryos compared to *Foxh1* null embryos, consistent with the more severe phenotype and smaller size of these mutants.

Sox17 is expressed in the hindgut definitive endoderm (arrow in Fig. 1M, where the "hindgut" is defined as the closed region of the gut tube at the posterior of the embryo), as well as in the hemangioblasts (arrowheads in Fig. 1K) and visceral endoderm of E8.5 wild-type embryos (Kanai-Azuma et al., 2002). *Sox17* expression is observed in both the hindgut and foregut of *Foxh1* null embryos (Figs. 1O, P, arrow in Q). The bilateral hemangioblast expression domain is fused in the midline of *Foxh1* null embryos (arrowhead in Fig. 1O). In *Foxa2* null embryos, weak expression of *Sox17* in the hindgut and strong expression in the foregut invagination is observed (Figs. 1S, T, arrow in U). The expression of *Nepn* provides the first conclusive evidence that the midgut definitive endoderm forms in *Foxh1* and *Foxa2* null embryos. Furthermore, the expression of *Sox17* suggests that the hindgut definitive endoderm is also formed in these mutants.

Foregut definitive endoderm is significantly reduced in Foxh1 and Foxa2 null embryos

We observed expression of *Sox17* in the foregut region of *Foxh1* and *Foxa2* null embryos; however, as *Sox17* is expressed in both the visceral and definitive endoderm, it was unclear if cells expressing *Sox17* in the mutant embryos were visceral or definitive endoderm. To address this, we examined the expression of the definitive endoderm-specific foregut markers *thyrotropin-releasing hormone* (*Trh*) and *peptide YY* (*Pyy*) in *Foxh1* and *Foxa2* null embryos (where the "foregut" is defined as the closed region of the gut tube at the anterior of the embryo) (Hou et al., 2007; McKnight et al., 2007). In E8.5 wild-type embryos, *Trh* is expressed in the definitive endoderm, with expression enriched in the foregut compared to the hindgut (Figs. 2A–D) (Hou et al., 2007; McKnight et al., 2007). Expression is also observed in the posterior neural tube (arrowhead in Fig. 2D). Similar to *Nepn*, *Trh* is not expressed in the notochordal plate (arrowhead in Fig. 2C, inset). Expression of *Trh* is observed in the hindgut definitive endoderm of *Foxh1* null embryos, with a significantly lower level in the foregut (Figs. 2E–H). Consistent with the failure to specify the notochord in these mutants, *Trh* is expressed across the midline in *Foxh1* null embryos (arrowhead in Fig. 2G). In *Foxa2* null embryos, expression of *Trh* is also observed in the anterior region near the foregut (boxed region 1 in Fig. 2K and arrow in inset K1) and in the hindgut region (boxed region 2 in Fig. 2K and arrow in inset K2).

In wild-type embryos, *Pyy* is specifically expressed in the foregut invagination at E8.5 and is not expressed in the notochordal plate