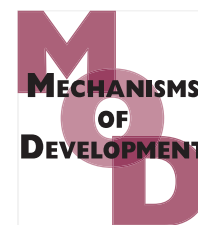


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Wnt signaling specifies and patterns intestinal endoderm

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ARTICLE INFO

Article history:

Received 15 February 2011

Received in revised form

11 July 2011

Accepted 28 July 2011

Available online 10 August 2011

Keywords:

Endoderm

Embryonic stem cell differentiation

Intestinal development

ABSTRACT

Wnt signaling has been implicated in many developmental processes, but its role in early endoderm development is not well understood. Wnt signaling is active in posterior endoderm as early as E7.5. Genetic and chemical activation show that the Wnt pathway acts directly on endoderm to induce the intestinal master regulator Cdx2, shifting global gene away from anterior endoderm and toward a posterior, intestinal program. In a mouse embryonic stem cell differentiation platform that yields pure populations of definitive endoderm, Wnt signaling induces intestinal gene expression in all cells. We have identified a set of genes specific to the anterior small intestine, posterior small intestine, and large intestine during early development, and show that Wnt, through Cdx2, activates large intestinal gene expression at high doses and small intestinal gene expression at lower doses. These findings shed light on the mechanism of embryonic intestinal induction and provide a method to manipulate intestinal development from embryonic stem cells.

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1. Introduction

There are two main reasons for studying the development of the intestine. First, the intestine is responsible for digestion and absorption of food. Second, colorectal cancers are the second leading cause of cancer-related mortality, and Inflammatory Bowel Syndromes afflict hundreds of thousands of people. Despite its essential functions and medical problems, rather little is known about how different cell types within the intestine arise during embryogenesis.

During mouse development, the endoderm forms between E6.5 and E7.5 as a flat sheet on the outside of the cup-shaped embryo. Endoderm begins to fold ventrally at the caudal end of the endoderm, at E8.25, forming a tube known as the

caudal intestinal portal (CIP). This nascent tube zips progressively anteriorly until it joins the anterior intestinal portal (AIP), forming an internal single-cell-thick gut tube by E8.75. The single-layered intestinal epithelial tube proliferates and begins to become stratified between E8.75 and E14.5, at which point the intestine begins to deform in the radial plan, eventually forming fingerlike protrusions into the lumen. The adult intestine is composed of these protrusions, known as villi, which contain mature intestinal absorptive, secretive, and endocrine cells, as well as crypts that contain stem cells that replenish the intestinal epithelium throughout adult life (van der Flier and Clevers, 2009).

While considerable insight has been gained about adult intestinal stem cell self-renewal, differentiation, and

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doi:10.1016/j.mod.2011.07.005

dysfunction in disease (Barker et al., 2008), relatively little attention has been given to the initial specification and morphogenesis of the intestine. Protohox genes of the Cdx family, the mammalian homologue of the *Drosophila* posterior determinant Caudal (Mlodzik et al., 1985), are required for posterior development in all germ layers (Chawengsaksophak et al., 2004; Subramanian et al., 1995; van den Akker et al., 2002). Cdx2 is the first known molecular marker for the future intestinal epithelium, first expressed in the posterior endoderm at E7.5 (Beck et al., 1995; Sherwood et al., 2009). Cdx2 has been shown to be necessary for intestinal specification in the endoderm (Gao et al., 2009), controlling a network of intestinal genes.

Tcf1/Tcf4 mutants have embryonic intestinal defects, implicating the Wnt pathway in intestinal development (Gregorieff et al., 2004) and inhibition of Wnt signaling appears necessary for proper differentiation of foregut endoderm derivatives (Heller et al., 2002; Kim et al., 2005; McLin et al., 2007; Okubo and Hogan, 2004). Numerous Wnt ligands are present in the early node and primitive streak, including Wnt3a (Takada et al., 1994), Wnt5a (Yamaguchi et al., 1999), and Wnt8a (Bouillet et al., 1996). The developing gut also expresses a multitude of secreted Wnt repressors including those of the Sfrp and Dkk families (Theodosiou and Tabin, 2003), so expression alone is insufficient to predict where Wnt signaling is occurring. Other signaling pathways including the Hedgehog and Bmp families (Roberts et al., 1998) and Notch (Stanger et al., 2005) have been implicated in intestinal development as well.

The small intestine is traditionally divided into three segments, the duodenum, jejunum and ileum, by morphological criteria, and the large intestine is similarly divided into the cecum, ascending, transverse, and descending colon and rectum. Villous architecture and Paneth cells at the base of crypts are present only in small intestine, while the large intestine is characterized by a flat epithelium with deep crypts. Many digestive enzymes are exclusive to the duodenum and pancreas (Whitcomb and Lowe, 2007). Enterocrine cells display segment-specific expression, with CCK primarily produced in anterior small intestine and PYY and GLP1 primarily in distal small intestine and large intestine (Ratineau et al., 2003). To what extent molecular distinctions exist among the small and large intestinal segments is unknown and no mechanism has been proposed for the acquisition of these differences.

Here we describe the role of Wnt signaling in intestinal specification and regionalization. We find that Wnt signaling activates Cdx2 in the early endoderm and is capable of inducing an intestinal gene program in anterior endoderm cells. Wnt signaling not only specifies intestinal fate from undifferentiated endoderm, it appears to have concentration-dependent effects on anterior–posterior intestinal segments. Consequently, a molecular heterogeneity appears before any morphological distinctions are apparent. Finally, we have transferred these findings to the *in vitro* differentiation of embryonic stem cells and show that Wnt signaling efficiently specifies intestinal fate in endoderm derived from mouse and human ES cells.

2. Materials and methods

2.1. Tissue manipulation and bead implantation

For all experiments, outbred ICR mice (Taconic, Germantown, NY) and transgenic strains were bred and maintained at the Harvard Biomedical Research Infrastructure. Embryos were considered to be E0.5 at noon of the day the plug was detected. Sox17-CreER mice were generated using a similar cloning strategy to that of Borowiak et al. (2009) and will be described in a manuscript in preparation.

Tissues were dissected in HBSS (Sigma, St. Louis, MO). AG 1-X8 beads, formate form, 200–400 μm diameter (Bio-Rad, Hercules, CA) were soaked in either DMSO (Sigma) or GSK3iXV (EMD Biosciences, Gibbstown, NJ) and implanted into the foregut cavity with forceps. Dissected tissues were cultured in DMEM:F12 (Invitrogen, Carlsbad, CA) with 10% FBS (Hyclone, Logan, UT) and penicillin/streptomycin (Invitrogen).

Electroporation experiments were performed according to published protocols (Pierreux et al., 2005). Embryos were bathed in 1 $\mu\text{g}/\mu\text{L}$ plasmid solution and electroporated using a T820 Electro Square Porator (BTX, Holliston, MA) using three 12-Volt pulses with 50 μs interval. Embryos were cultured in static culture for 24 h in 1:1 DMEM:F12 (Invitrogen): rat serum (Valley Biomedical, Winchester, VA) or dissected and cultured as dissected tissues as above.

2.2. Flow cytometry and microarray profiling

Tissues were dissociated in 0.25% Trypsin (Invitrogen) for 2–5 min at 37 degrees and, after neutralization and centrifugation, were stained on ice for 15 min in DMEM:F12 (Invitrogen) with 2% FBS (Hyclone) and 2 mM EDTA. Before flow cytometric sorting, cells were resuspended in staining buffer with calcein blue AM (Invitrogen) and sorted using a FACSAria (Becton Dickinson, San Jose, CA).

Sorted cell populations were collected in Trizol (Invitrogen), and RNA was isolated according to the manufacturer's instructions. RNA was amplified and biotinylated cRNA probe generated using the Ambion Illumina TotalPrep RNA Amplification Kit (Applied Biosystems/Ambion, Austin, TX). Biotinylated cRNA was hybridized onto Illumina MouseRef-8 v2 microarrays (Illumina, San Diego, CA) according to the manufacturer's instructions. Microarrays were scanned using BeadStation (Illumina), and data was analyzed using BeadStudio (Illumina). Two to three biological replicates were performed for each tissue analyzed.

2.3. Antibodies and immunofluorescence

For immunofluorescence analysis, wholemount tissues were fixed for 15 min in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and washed in PBS with 1% Tween-20 (Sigma). Tissues were blocked by 1 h incubation at 4° in PBS with 20% donkey serum (Jackson ImmunoResearch, West Grove, PA) and 1% Tween-20. Primary and secondary antibody staining were performed by rocking

overnight at 4° in PBS with 5% donkey serum, and after primary and secondary antibody staining, washing was performed for >5 h in PBS with 1% Tween-20.

After staining, wholemount embryos were transferred to 1:1 glycerol:PBS with 1% Tween and placed between two coverslips. Confocal imaging was performed using an LSM 510Meta confocal microscope (Carl Zeiss Inc., Germany).

The following primary antibodies were used: rabbit anti- β -galactosidase, chicken anti-GFP (Millipore, Billerica, MA); G8.8 (anti-EpCAM; Developmental Studies Hybridoma Bank, Iowa City, IA); goat anti-Foxa2 (M-20), goat anti-Sox2 (Y-17; Santa Cruz Biotechnology, Santa Cruz, CA); biotinylated DBA (Vector Labs, Burlingame, CA); mouse anti-Cdx2 (Biogenex, San Ramon, CA); biotin anti-Ly-6A (D7; ebioscience, San Diego, CA) and FITC-conjugated rat anti-CD26 (BD Biosciences, San Jose, CA). Alexa Fluor 488, 594, 647 and Pacific Blue secondary antibody conjugates (Invitrogen) as well as PE and APC conjugates (Jackson ImmunoResearch) were used for secondary detection.

2.4. In situ hybridization

In situ hybridization was performed according to (Wilkinson and Nieto, 1993). cDNA was obtained from commercial clone libraries (Open Biosystems, Huntsville, AL) and digoxigenin-labeled probes were generated. Tissues were fixed overnight in 4% paraformaldehyde, dehydrated in methanol, bleached in hydrogen peroxide and treated with proteinase K, followed by re-fixation. Probe was added at 1 μ g/mL overnight at 70°. Embryos were washed and labeled overnight with anti-digoxigenin-AP antibody (Roche Applied Science, Indianapolis, IN). Embryos were developed in BM Purple (Roche Applied Science).

2.5. Embryonic stem cell culture and differentiation

Undifferentiated 129P2/OlaHsd mouse ES cells were maintained on gelatin-coated plates with mouse embryonic fibroblast (MEF) feeders in DMEM (Invitrogen) supplemented with 15% defined fetal bovine serum (FBS) (HyClone), 0.1 mM non-essential amino acids (Invitrogen), Glutamax (Invitrogen), penicillin/streptomycin (Invitrogen), 0.55 mM 2-mercaptoethanol (Sigma), and 1X ESGRO LIF (Chemicon, Temecula, CA).

Prior to differentiation, ES cells were passaged onto gelatin-coated plates for 30 min to deplete MEFs. MEF-depleted ES cells were then seeded at 1×10^4 cells/cm² onto gelatin-coated dishes in Advanced DMEM (Invitrogen) supplemented with N-2 (Invitrogen), B27 Supplement without vitamin A (Invitrogen), 0.05% Albumax II (Invitrogen), Glutamax, and 2.5 μ M Y-27632 (Tocris Bioscience, Ellisville, MO). After 40–44 h, media was changed to Advanced DMEM with 2% FBS, Glutamax, 5 nM GSK-3 inhibitor XV and 50 ng/mL E. coli-derived Activin A (Peprotech, Rocky Hill, NJ). After 1 day, cells were fed with Advanced DMEM with 2% FBS, Glutamax, 50 ng/mL Activin A and 1 μ M Dorsomorphin (Sigma). After 5 days of differentiation, cells were either stained for endodermal genes or passaged using 0.25% Trypsin onto plates coated with 804G cell conditioned media and treated with DMSO or GSK3iXV at the concentrations indicated in

Advanced DMEM with B-27 supplement without vitamin A and Glutamax.

ES cells with a doxycycline-inducible Cdx2 allele in the HPRT locus were created as described (Iacovino et al., 2009) and maintained and differentiated as above.

HUES8 human embryonic stem cells were maintained and differentiated toward endoderm as described (Chen et al., 2009). After endoderm induction, cells were treated with 0.05% Trypsin and re-plated onto plates coated with conditioned media from the 804G cell line in RPMI (Invitrogen) with 2% FBS, Glutamax and penicillin/streptomycin (Invitrogen). Cells were cultured for 48 h in the same media with 125 nM GSK3iXV when indicated prior to fixation and immunostaining.

3. Results

3.1. Wnt signaling is active in posterior endoderm between E7.5–E8.5

To determine whether Wnt signaling is active in early definitive endoderm, wholemount confocal analysis was performed on E7.5–E9.5 BAT-gal mouse embryos. This mouse strain reports canonical Wnt activity through expression of nuclear β -galactosidase (Maretto et al., 2003), and Wnt is known to be expressed during gastrulation and neurulation in the posterior region of the embryo. Cells co-expressing the pan-endodermal transcription Foxa2 and β -galactosidase can be seen in the posterior of the embryo starting at E7.5 (Fig. 1A–D). Many of these cells also express the posterior endodermal and future intestinal transcriptional regulator Cdx2. β -gal-expressing cells can also be found more anteriorly in the endoderm than Cdx2⁺ cells (Fig. 1D). At E8.25, β -gal⁺Foxa2⁺ cells occur as a swath of cells spanning the anterior border of Cdx2 expression in the midgut with the anterior border of β -gal expression occurring a few cell diameters more anteriorly than the border of Cdx2 expression (Fig. 1E–H). By E8.75, β -gal expression in the posterior endoderm can no longer be detected (Fig. 1I). Additionally, the Wnt target gene Axin2 (Jho et al., 2002) is expressed highly in E7.5–E8.5 endoderm but is downregulated by E9.5, and its expression is significantly weaker in anterior endoderm than in posterior endoderm at E8.5 (Supplemental Fig. 1). Thus, in the E7.5–E8.5 endoderm, a correlation between the anterior border of Cdx2 expression and Wnt activity is apparent.

3.2. Activation of Wnt signaling induces Cdx2 expression and posterior migration of definitive endoderm

To determine the effect of Wnt signaling on endodermal organ fate specification, a whole embryo electroporation strategy was used to introduce DNA into the E8.25 foregut endoderm (Pierreux et al., 2005). After electroporation of the foregut with a control GFP-expressing vector and 24 h of subsequent embryo culture, electroporated cells can be detected specifically in the foregut endoderm and, as expected, do not express Cdx2 (0/10 embryos have GFP⁺Cdx2⁺ cells, Fig. 2A–C). When a vector expressing constitutively active beta catenin

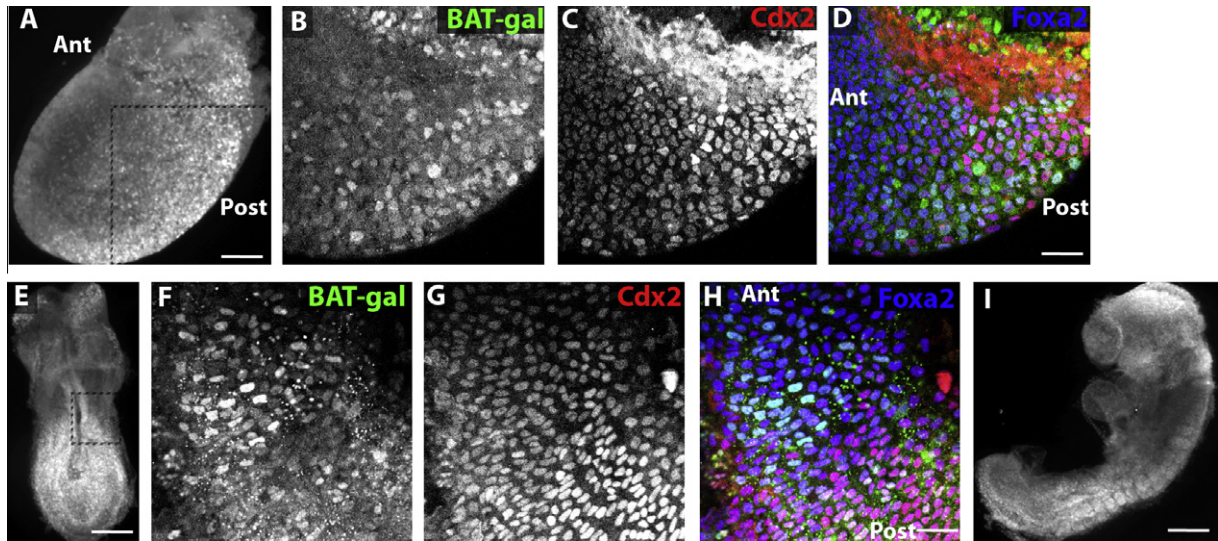


Fig. 1 – Wnt signaling is active at the anterior border of $Cdx2^+$ endoderm between E7.5–E8.5. (A) Wholemount immunofluorescence image of E7.5 BAT-Gal embryo stained for β -gal. Boxed area represents embryonic region imaged in higher detail in B–D. (B–D) Wholemount confocal immunofluorescence images of E7.5 BAT-Gal embryo stained for β -gal (B, green in D), $Cdx2$ (C, red in D), and $Foxa2$ (blue in D). (E) Wholemount immunofluorescence image of E8.25 BAT-Gal embryo stained for β -gal. Boxed area represents embryonic region imaged in higher detail in F–H. (F–H) Wholemount confocal immunofluorescence images of E8.5 BAT-Gal embryo stained for β -gal (F, green in H), $Cdx2$ (G, red in H), and $Foxa2$ (blue in H). (I) Wholemount immunofluorescence image of E8.75 BAT-Gal embryo stained for β -gal. Scale bar equals 500 μ m in A, E, and I and 50 μ m in D and H.

(CA β -catenin), which activates the Wnt signaling pathway (Toyofuku et al., 2000), fused to GFP was electroporated, a majority of electroporated cells express $Cdx2$ (10/10 embryos have GFP^+Cdx2^+ cells, Fig. 2D–F). Cells transfected with CA β -catenin tend to cluster and can be found within or nearby the endogenous $Cdx2^+$ posterior endodermal domain even though the electroporated domain in control GFP electroporations is around 20 cell diameters from the endogenous $Cdx2^+$ domain.

In the CA β -catenin electroporations, GFP is first detected within 4 h without significant $Cdx2$ co-expression (Supplemental Fig. 2). By 8 h, about half of the GFP-expressing cells co-express $Cdx2$ (Supplemental Fig. 2), and by 12 h, most GFP^+ cells co-express $Cdx2$ (Supplemental Fig. 2). In the first 8 h, less than 5% of control GFP-electroporated and 25% of CA β -catenin-electroporated cells can be found within 5 cell layers of the endogenous hindgut $Cdx2^+$ domain, whereas at and after 12 h, 90% of CA β -catenin-electroporated cells are found within 5 cell layers of the endogenous hindgut $Cdx2^+$ domain, which may be the result of selective survival or posterior migration (Supplemental Fig. 2), and explant culture of the nascent gut tube for an additional 48 h following the 24 h embryo culture reveals a persistence of electroporated cells selectively in the $Cdx2^+$ intestinal epithelium (Supplemental Fig. 2).

To determine whether Wnt is capable of inducing $Cdx2$ in the absence of endogenous $Cdx2$ -expressing endoderm, the anterior half of the embryo was explanted after foregut electroporation of CA β -catenin. In the absence of CA β -catenin electroporation, the foregut is devoid of $Cdx2$ expressing cells

and uniformly expresses $Sox2$ (data not shown; (Sherwood et al., 2009)). After 24 h *in vitro*, GFP^+ cells express $Cdx2$ and the vast majority display markedly reduced expression of the anterior endoderm marker $Sox2$ (Fig. 2G–M), although rare co-expression of $Cdx2$ and $Sox2$ can be detected (Fig. 2J–M). Electroporation of CA β -catenin into E7.75 anterior endoderm yields significant co-expression of GFP and $Cdx2$ (Supplemental Fig. 2); however, electroporation of CA β -catenin into E9.5 stomach does not yield any induction of $Cdx2$ (Supplemental Fig. 2), suggesting that CA β -catenin (Wnt signaling) is able to induce endodermal $Cdx2$ expression in endoderm from E7.5 to about E8.5.

3.3. *In vivo genetic and in vitro chemical activation of Wnt signaling induce $Cdx2$ expression in definitive endoderm*

To confirm that Wnt signals can induce $Cdx2$ expression in definitive endoderm, *in vivo genetic* and *in vitro chemical* activation of Wnt signaling were employed. Mice engineered to express CreER from the $Sox17$ locus were crossed with mice containing loxP sites flanking exon 3 of β -catenin, which when excised creates a constitutively active form. Tamoxifen was administered at E6.5, a time point found to cause mosaic, endoderm-exclusive recombination in all anterior-posterior regions (manuscript in preparation). At E9.5, no gross abnormalities can be detected, yet upon wholemount immunofluorescence analysis, clusters of $Cdx2$ -expressing cells are found throughout the anterior endoderm in half of the embryos (12/24), while no $Cdx2$ -expressing cells are detected in the foregut of the other half of the embryos (12/24) (Fig. 3A–C).

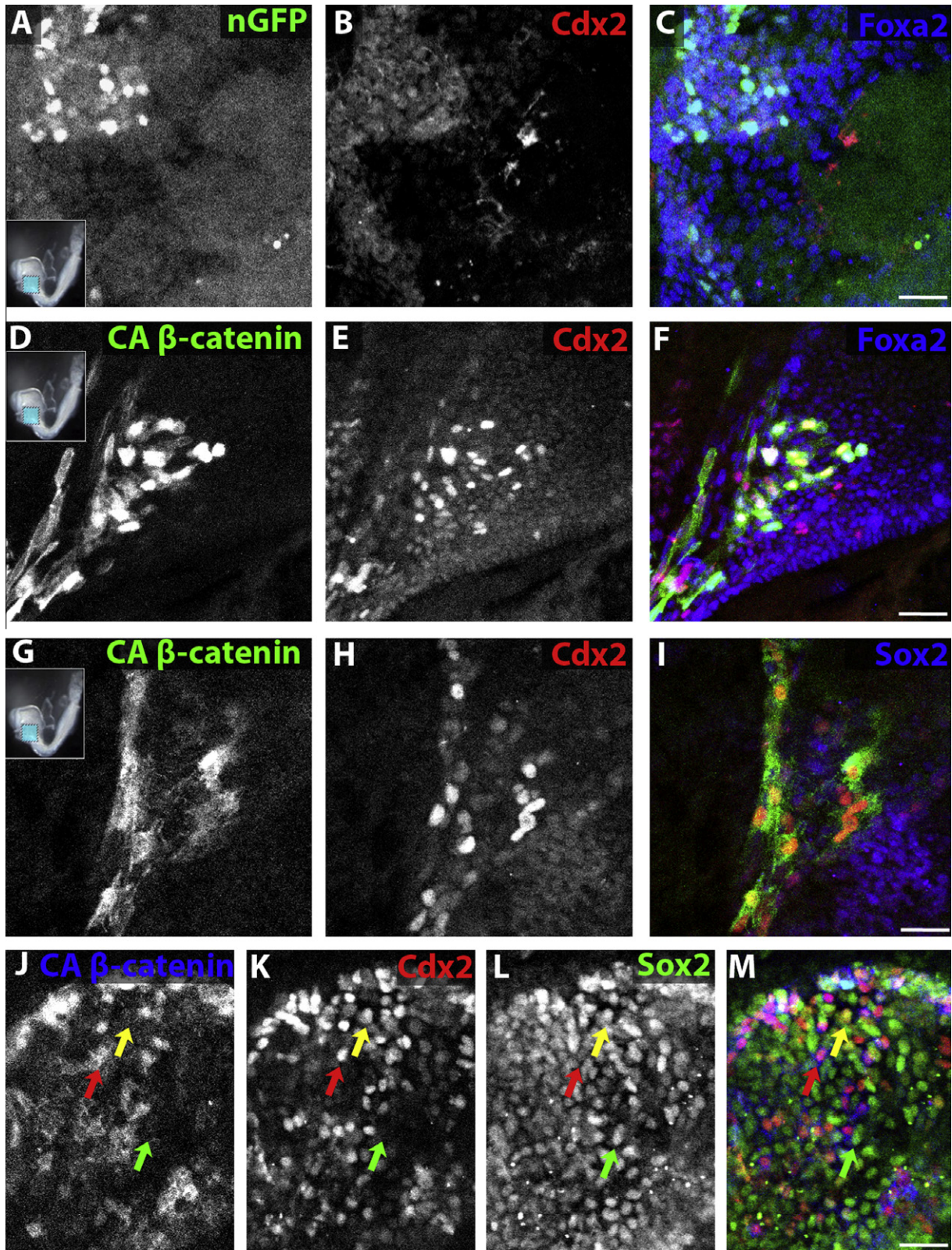


Fig. 2 – Electroporation of activated β -catenin induces *Cdx2* and represses *Sox2* in foregut endoderm. (A–K) Wholemount confocal immunofluorescence images of E8.25 foregutelectroporated with nuclear GFP (A–C) or activated β -catenin (D–M) and cultured for 24 h in whole embryo culture (A–F) or foregut explant culture (G–M). Electroporated embryos were all stained for GFP (A, D, G, J) and *Cdx2* (B, E, H, K) and some also for *Foxa2* (C, F) or *Sox2* (I, L). (J–M) Red arrow refers to a $Cdx2^+Sox2^-$ cell, green arrow to a $Sox2^+Cdx2^-$ cell and yellow arrow to a rare $Cdx2^+Sox2^+$ cell. Scale bar equals 100 μ m in C and F and 50 μ m in I and M.

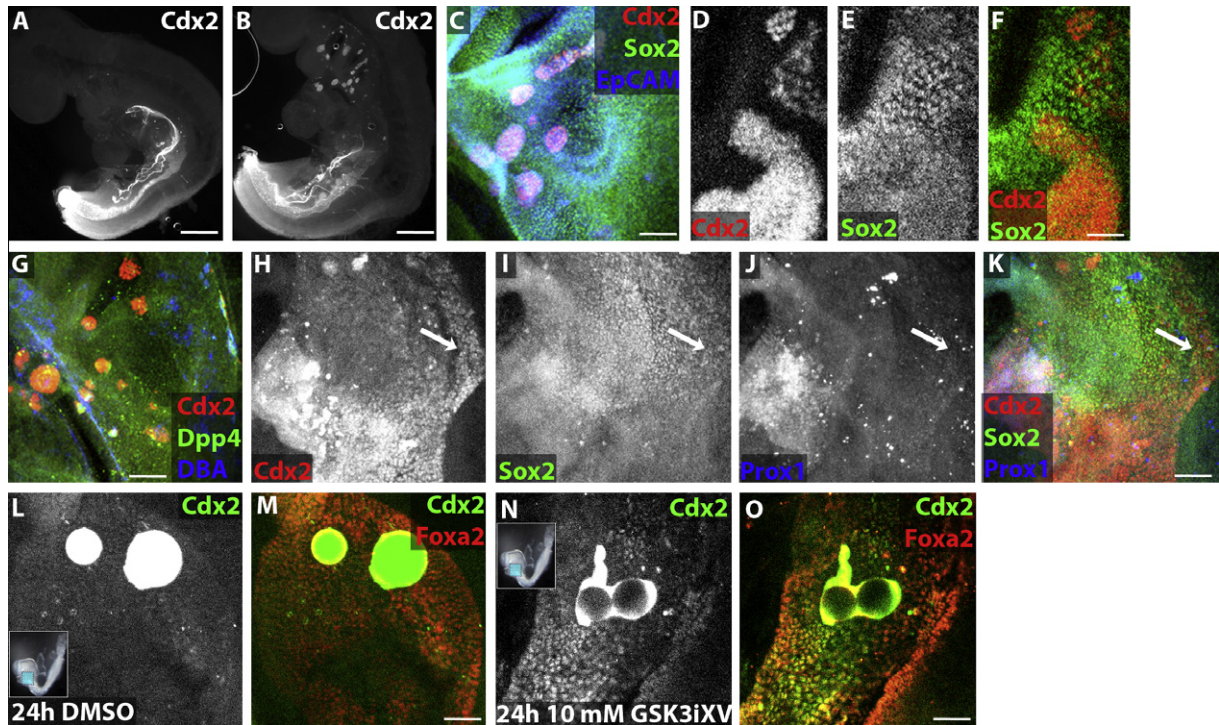


Fig. 3 – Genetic and chemical activation of Wnt signaling leads to intestine induction in the foregut. (A and B) Wholemount immunofluorescence images of E9.5 *Sox17^{CreER} X β -catenin^{+/+}* wildtype littermate (A) and *Sox17^{CreER} X β -catenin^{Ex3/+}* (B) embryos stained for Cdx2. (C–E) Wholemount confocal immunofluorescence images of E9.5 *Sox17^{CreER} X β -catenin^{Ex3/+}* foregut (C–G) or stomach–intestine border (H–K) stained for Cdx2 (C, D, G, H), Sox2 (C, E, I), EpCAM (C), Dpp4 (G), DBA (G), and Prox1 (J). Arrow in H–K indicates missing dorsal pancreatic domain replaced by Cdx2 expression. (L–O) Wholemount confocal immunofluorescence images of E8.25 explanted foreguts cultured for 24 h before fixation and stained for Cdx2 (L, N) and Foxa2 (M, O). Foreguts were implanted with beads soaked in DMSO (L–M) or 10 mM GSK3iXV (N–O). Scale bar equals 500 μ m in A–B, 200 μ m in C, G, and K, and 100 μ m in F, M, and O.

Some *Cdx2*⁺ cells within these clusters downregulate the anterior endoderm marker Sox2 while others retain Sox2 expression (Fig. 3D–F). A subset of these ectopic *Cdx2*⁺ cells express the intestinal markers Dpp4 and DBA (Fig. 3G). While most embryos segment their endodermal organs normally, one of 12 mutant embryos examined had a missing dorsal pancreatic domain replaced by *Cdx2*-expressing cells (Fig. 3H–K), a phenomenon that may be explained by variable recombination frequency and location. A similar phenotype of ectopic clusters of *Cdx2*-expressing cells is detected at E10.5 (data not shown), but mutant embryos cannot be recovered at later timepoints, presumably due to Tamoxifen toxicity at doses sufficient to induce recombination (Danielian et al., 1998), precluding analysis of intestinal maturation in the ectopic clusters.

To establish a method to allow for controlled manipulation of Wnt signaling in embryos, beads soaked in a highly selective chemical GSK3 inhibitor [GSK3 inhibitor XV (GSK3iXV)] (Atilla-Gokcumen et al., 2006), which activates Wnt signaling (Siegfried et al., 1994), were placed in the foregut cavity of E8.25 embryos. Immediately after bead implantation, the anterior half of the embryo was explanted and cultured for 24 h. *Cdx2*⁺ cells are never detected in the foregut in the absence of beads or surrounding DMSO-soaked

beads ($n = 10$, Fig. 3F–G), while large numbers of *Cdx2*⁺*Foxa2*⁺ endodermal cells can be found surrounding GSK3iXV-soaked beads in all embryos examined ($n = 20$, Fig. 3H–I). *Cdx2* protein can be detected between 6 and 8 h after treatment with GSK3iXV beads (data not shown). Consistent with the electroporation results, GSK3iXV soaked beads induce *Cdx2* expression when placed in the nascent foregut of E8.0 embryos for 24 h but are unable to induce *Cdx2* expression in the E9.5 oral cavity or stomach after 24 or 48 h (data not shown).

3.4. Characterization of intestinal induction of Wnt pathway

While *Cdx2* is a key regulator of intestinal formation, we sought to determine whether activation of Wnt signaling more broadly can induce intestinal fates. To obtain a global view of gene expression changes induced by the Wnt pathway, microarray analysis was performed on anterior explants with either DMSO- or GSK3iXV-soaked beads placed in the foregut cavity. At two time points, after 6 and 24 h *in vitro*, the pooled anterior explants were dissociated, and definitive endoderm was isolated by expression of the pan-endodermal cell surface protein EpCAM and minimal expression of the

Table 1 – Transcription factors and select other genes significantly affected by GSK3iXV treatment in E8.25 foregut endoderm. Expression of these genes is changed >2-fold for 6 h timepoint and >2.5-fold for 24 h timepoint in EpCAM + endoderm by treatment of E8.25 foregut endoderm with 10 mM GSK3iXV beads.

Selected genes upregulated within 6 hours	Selected genes upregulated between 6 and 24 h	Selected genes downregulated within 6 h	Selected genes downregulated between 6 and 24 h
Defcr-rs2	Hoxd8	Hhex	Rarb
Dkk4	Hoxd9	Wnt8a	Foxel
Evxl	Hoxc9	Hesxl	Hoxb1
Cdx2	Hoxa7	Gsc	Tbx1
Sox17	Fzd10	Sox2	Foxal
Axin2	Sct		Pax1
Wnt6	Sp7		
Cdx1	T		
Tcf7			
Klf5			
Lef1			
Sp6			
Tbx3			

extraembryonic endoderm-specific lectin DBA (Sherwood et al., 2007).

A small number of genes are differentially expressed at 6 h, and a much larger set of genes becomes differentially expressed at 24 h (selected genes highlighted in Table 1). As expected, Cdx2 mRNA is highly upregulated by GSK3iXV beads within 6 h (9.5-fold at 6 h, 21.8-fold at 24 h). Additionally, several positive and negative regulators of the Wnt pathway are strongly induced, including Dkk1, 3 and 4, Axin2, Fzd1 and 10, Wnt6 and 10a. Crucially, GSK3iXV beads downregulate expression of transcription factors involved in anterior endoderm development, starting with a subset of factors including Hhex, Otx2 and Sox2 at 6 h and progressing to most known anterior endodermal transcription factors by 24 h (Table 1). To obtain a more comprehensive view of the intestinal induction of GSK3iXV, a set of 50 genes unique to the intestine within the E11.5 endoderm (Sherwood et al., 2009) were selected. Intestinal gene expression after treatment with GSK3iXV versus control beads was relatively unchanged after 6 h (mean foldchange 1.27, median foldchange 0.89, standard deviation 1.81) but dramatically induced by 24 h (mean 3.25, median 1.24, standard deviation 10.3). This upregulation of intestinal genes, combined with the downregulation of genes involved in anterior endodermal development, provides strong evidence that Wnt pathway activation induces an intestinal gene expression program.

3.5. Wnt signaling induces intestinal differentiation in mouse embryonic stem cell-derived endoderm

As our experiments demonstrated a role for Wnt signaling in embryonic intestinal specification from endoderm, we sought to determine whether Wnt signaling could be used to direct a similar differentiation in ES cell derivatives. To this end, we modified existing protocols to improve the efficiency of mouse ES cell differentiation into definitive endoderm. TGF β ligands, in particular Activin A, have been widely used in endoderm differentiation of ES cells (Kubo et al., 2004; Tada et al., 2005; Gadue et al., 2006; Hansson et al., 2009); however, addition of Activin A to ES cells for 5 days yields a heteroge-

neous population with only about half of the cells expressing the endodermal markers Sox17 and Foxa2 (Fig. 4A–B).

By evaluating candidate signaling pathways to enhance or inhibit Activin A-induced endodermal differentiation, an optimized endodermal induction protocol was developed such that a near-uniform population of definitive endoderm can be derived from ES cells within 5 days. Culturing embryonic stem cells in serum-free media without growth factors for two days prior to addition of Activin A improves endodermal yield (Supplemental Fig. 3). From day 2–3, activating Wnt signaling, achieved by low-dose GSK3iXV, increases endodermal efficiency and antagonizing Wnt signaling impairs endoderm formation (Supplemental Fig. 3). From day 3–5, inhibiting Bmp signaling with the small molecule Dorsomorphin increases endodermal differentiation whereas Bmp4 strikingly inhibits endoderm differentiation (Supplemental Fig. 3), as has been found previously (Hansson et al., 2009). By combining all of these components, a near-uniform population of endoderm can be obtained after 5 days of differentiation (Fig. 4C–D).

The effect of Wnt induction on ES cell-derived endoderm was then analyzed. Treatment of ES cell-derived endoderm with GSK3iXV induces a striking induction of Cdx2 (Fig. 4E–H). In the absence of GSK3iXV, fewer than 1% of Foxa2-expressing endoderm cells express Cdx2 (Fig. 4E–F, Supplemental Fig. 3), whereas 24 h after addition of 100 nM GSK3iXV, over 99% of Foxa2-expressing endoderm cells express Cdx2 (Fig. 4G–H, Supplemental Fig. 3). Cdx2 induction occurred within a range of 20 nM–500 nM GSK3iXV (data not shown). Treatment with Wnt3a is also able to induce Cdx2 expression, although only in around 40% of endoderm, likely due to low bioactivity of Wnt3a (Fig. 4I–J, Supplemental Fig. 3).

Microarray analysis was performed on ES-derived endoderm treated with DMSO or GSK3iXV for 24 h. As expected, GSK3iXV treatment induces transcripts of intestinal marker genes such as Cdx2 and Dpp4 and downregulates anterior endodermal genes such as Hhex and Hesx1 (selected genes highlighted in Table 2 with a complete list in Supplemental Table 2). GSK3iXV treatment of ES-derived endoderm produces strikingly similar gene expression changes to E8.25

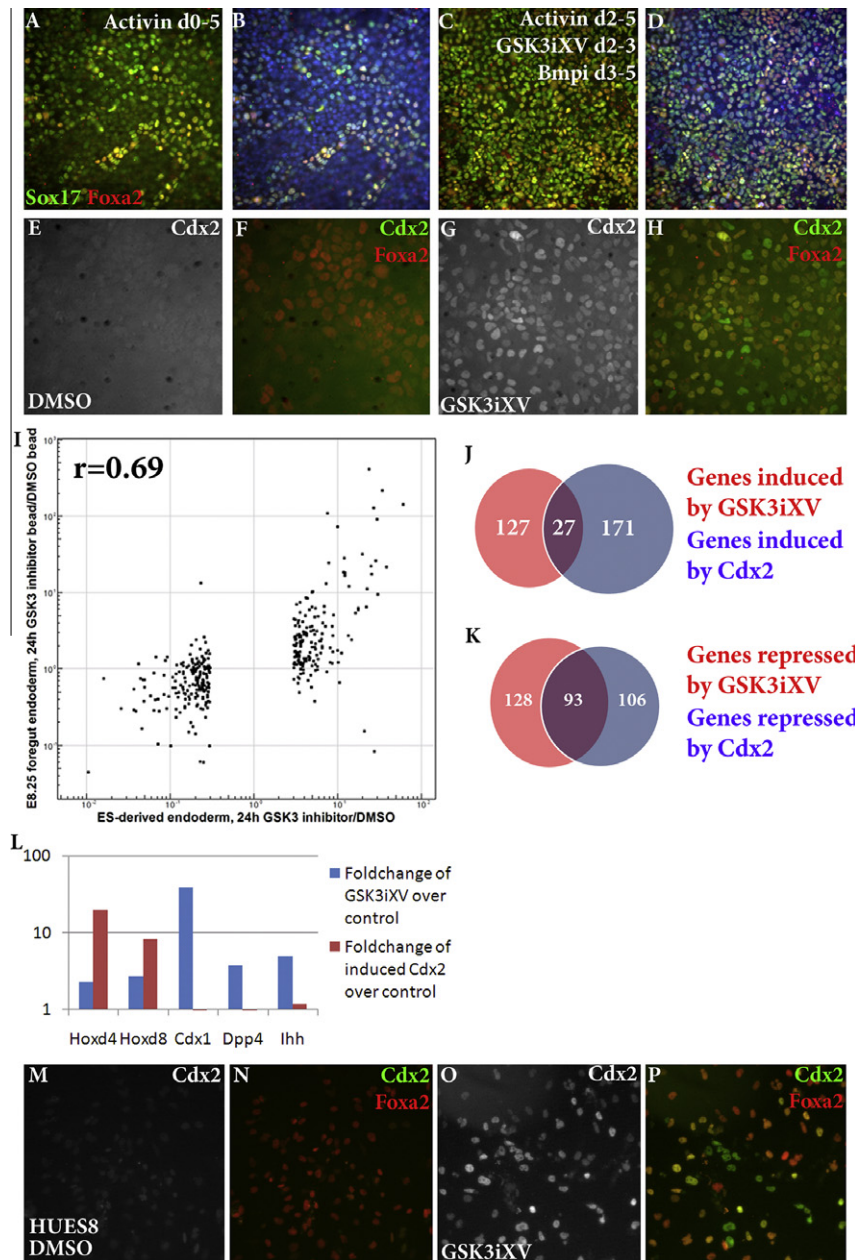


Fig. 4 – Wnt induces intestinal differentiation from mouse and human ES cell-derived endoderm. (A–D) Immunofluorescence analysis of mouse ES cells treated with Activin A from days 0–5 (A–B) or Activin A from days 2–5, GSK3iXV from day 2–3 and Dorsomorphin from days 3–5 (C–D) before fixation and stained for Sox17 (green) and Foxa2 (red). Nuclei are stained with Hoechst 33342 (B, D). (E–J) Immunofluorescence analysis of mouse ES-derived endoderm treated with DMSO (E–F) or 50 nM GSK3iXV (G–H) or 1 μ g/mL Wnt3a (I–J) for 24 h before fixation and stained for Cdx2 (white in E, G, I, green in F, H, J) and Foxa2 (red in F, H, J). (K) Graph comparing the microarray foldchange induced by 24 h of GSK3iXV treatment in ES-derived endoderm (X-axis) and E8.25 endoderm (Y-axis). Only genes significantly affected by GSK3iXV in E8.25 endoderm are displayed, and the correlation coefficient (r) is shown. (L–M) Venn diagrams displaying the fraction of genes induced (L) or downregulated (M) by GSK3iXV treatment (red) and Cdx2 induction (blue) of ES-derived endoderm as determined by microarray analysis. (N) Graph displaying the microarray foldchange in certain genes induced by 24 h of GSK3iXV treatment (blue) or 24 h of Cdx2 induction (red). (O–R) Immunofluorescence analysis of HUES8 human ES-derived endoderm treated with DMSO (O–P) or 125 nM GSK3iXV (Q–R) for 24 h before fixation and stained for Cdx2 (white in O, Q, green in P, R) and Foxa2 (red in P, R). Scale bar equals 100 μ m in B and D, 50 μ m in F, H, J, P, and R.

GSK3iXV bead implantation. Using the set of genes significantly upregulated or downregulated by E8.25 GSK3iXV bead

as compared to DMSO bead implantation (3-fold cutoff), fold-change in this bead implantation setting was plotted versus

Table 2 – Transcription factors and select other genes significantly affected by GSK3iXV treatment in ES cell-derived endoderm. Expression of these genes is changed >3-fold by treatment of ES cell-derived endoderm with 50 nM GSK3iXV.

Selected genes upregulated in GSK3iXV-treated ES-derived Endoderm	Selected genes downregulated in GSK3iXV-treated ES-derived endoderm
Axin2	Foxd4
Cdx1	Gata3
Cdx2	Gsc
Defcr-rs2	Hes1
Dkk4	Hhex
Dpp4	Isl1
Evx1	Nkx2–3
Gbx2	Otx2
Sp8	Tbx1
Tcf2	
Wnt6	

foldchange of ES-derived endoderm treated with GSK3iXV as compared to DMSO (Fig. 4I). The correlation between gene expression changes in E8.25 embryonic endoderm and ES-derived endoderm is highly significant ($r = 0.69$), suggesting that Wnt signaling plays a similar role in embryo-derived and ES-derived endoderm.

3.6. Comparison of Wnt intestinal induction and Cdx2 induction

Cdx2 is known to be a key regulator of intestinal fate (Gao et al., 2009), so we investigated whether induction of Cdx2 alone in ES-derived endoderm would be sufficient to induce an intestinal gene program. An ES cell line that allows doxycycline-inducible expression of genes (Iacovino et al., 2009) was used to affect uniform induction of Cdx2 after ES cell endodermal differentiation. Microarray analysis was performed on ES cell-derived endoderm treated for 24 h with either GSK3iXV alone, Doxycycline-inducible Cdx2 alone, or both. Only 27 of the 154 (18%) of the genes induced by GSK3iXV are also induced by Doxycycline-induced expression of Cdx2 (Fig. 4J, full list in Supplemental Table 4). A larger degree of overlap exists between genes downregulated by GSK3iXV and Cdx2 (93/221, 42%, Fig. 4K, full list in Supplemental Table 4). The lack of overlap between induced genes can be partially explained by the abundance of Wnt pathway feedback genes induced by GSK3iXV. To refine understanding of the role of Cdx2-independent Wnt induction in intestinal gene expression, the set of genes uniquely expressed in E11.5 intestinal endoderm, long after Wnt-mediated induction has ceased according to BAT-GAL reporter expression, was analyzed. While a large majority of E11.5 intestinal genes are induced by both GSK3iXV and Cdx2, including the intestinal Hox genes (Fig. 4L), such as Cdx1, Ihh and Dpp4 are induced by Wnt signaling and not Cdx2 (Fig. 4L). Together, this analysis indicates that the role of Wnt in intestinal induction is not simply the induction of Cdx2.

3.7. Wnt induces Cdx2 in human ES-derived endoderm

To address whether Wnt induction of intestinal fate is species-specific, the role of Wnt signaling was also examined in human ES-derived endoderm. The human ES cell line HUES8 was differentiated into definitive endoderm (Chen et al., 2009) with ~50–70% efficiency (Supplemental Fig. 3). In the absence of GSK3iXV, <5% of Foxa2⁺ cells express Cdx2 (Fig. 4M–N), whereas treatment of HUES8 ES-derived endoderm with GSK3iXV for 48 h induces Cdx2 in >80% of Foxa2⁺ endoderm (Fig. 4O–P). Thus, Wnt signaling induces intestinal fate in both mouse and human ES-derived endoderm.

3.8. Wnt signaling affects anterior-posterior intestinal domain specification

The intestine becomes subdivided into anterior-posterior domains, each with unique morphogenetic, enzymatic and absorptive properties. By E12.5, the cecum is clearly discernable morphologically, but there is no clear visual distinction among the segments of the small intestine or large intestine until late gestation. As there is a paucity of known molecular markers for these intestinal regions during midgestation, we first sought molecular markers of distinct intestinal regions. At E14.5, the large intestine posterior to and including the cecum, the anterior half of the small intestine, the posterior half of the small intestine, and the stomach were dissected (Fig. 5A), dissociated, and endoderm was isolated on the basis of EpCAM expression. Microarray analysis was performed on these four endodermal populations, and a set of differentially expressed genes was determined (selected genes highlighted in Table 3 with a complete list in Supplemental Table 3). Several genes that displayed preferential expression in one E14.5 intestinal segment were further analyzed by in situ hybridization or immunofluorescence analysis. Genes confirmed to be enriched in anterior small intestine, in addition to the previously documented Pdx1 (Ahlgren et al., 1996; Offield et al., 1996) and Onecut2 (Jacquemin et al., 2003), include Anpep and Tm4sf4 (Fig. 5D and Supplemental Fig. 3). Cib2, Fzd10, and Osr2 showed preferential expression in posterior small intestinal endoderm from E10.5 through E14.5 (Fig. 5B, E and Supplemental Fig. 4), and Hoxd10, Ly6a (Sca-1), and Xpnpep2 displayed preferential expression in the large intestine (Fig. 5C, F and Supplemental Fig. 4).

To determine whether Wnt signaling specifies a particular intestinal domain, we utilized the genes found by microarray to be specific to particular domains. Comparative expression in anterior endoderm treated for 24 h with GSK3iXV beads versus with DMSO beads was calculated for each domain-specific gene set. As predicted, genes expressed in the stomach are on average downregulated by Wnt pathway activity (mean foldchange 0.90, median foldchange 0.76). Genes expressed in anterior small intestine are unaffected by Wnt signaling (mean 0.97, median 0.95), whereas genes expressed in posterior small intestine (mean 4.45, median 1.31) and large intestine (mean 3.24, median 1.34) are significantly upregulated by Wnt signaling.

This result leaves open the possibility that Wnt signaling specifically induces posterior small intestine and large intestine fate or that Wnt signaling can specify multiple intestinal

domains at different concentrations or times. To investigate the role of Wnt in intestinal subtype specification in more detail, E8.25 anterior explants were cultured for 48 h with either 1 μ M or 10 nM GSK3iXV in the media. While both concentrations efficiently induced Cdx2 in the endoderm, 1 μ M but not 10 nM GSK3iXV induced large intestine-specific Ly6a (Fig. 5G–J), suggesting that higher levels of Wnt signaling preferentially induce more posterior intestinal genes.

The ability of Wnt dosage to affect intestinal patterning in ES-derived endoderm was also investigated. Neither high nor low doses of GSK3iXV were able to turn on expression of Ly6a (Fig. 5K–L). However, Doxycycline-mediated induction of Cdx2 alone or in the context of GSK3iXV treatment yielded extensive expression of Ly6a (Fig. 5M–N). Microarray profiling confirmed that Doxycycline-induced Cdx2 or the combination of GSK3iXV and ectopic Cdx2 induced an extensive set of large intestine-specific genes not induced by GSK3iXV alone,

including *Hoxa7*, *Lpl*, and *Xpnp2* (Supplemental Fig. 5). While we have as yet been unable to induce robust large intestine gene expression from ES cells using exogenous factors, Cdx2 dosage appears to be crucial in this process.

4. Discussion

While genetic evidence has previously suggested a role for Wnt signaling in intestinal development, this report establishes a clear, direct and multifaceted role for Wnt signaling in intestinal specification and patterning. Wnt signaling acts directly on definitive endoderm to induce Cdx2, and application of Wnt to the early foregut endoderm is sufficient to downregulate the anterior endoderm program and to induce a gene expression program that resembles early embryonic intestine. As further proof of its role in intestinal specification, Wnt signaling is sufficient to induce ES cell-derived endoderm to an intestinal fate. Wnt signaling is involved not only in specification of the intestine but in its anterior–posterior patterning, as high-level Wnt and Cdx2 appear to be necessary for large intestine specification.

The effect of Wnt signaling on endoderm is transient. While Wnt possesses the ability to induce Cdx2 between E7.5 and E8.5, electroporation and bead experiments both demonstrate that Wnt signaling after E8.5 is no longer capable of inducing Cdx2. This loss of competence correlates with the time at which BAT-gal mice cease displaying midgut endodermal activity (Fig. 11), and this loss of active embryonic Wnt signaling is seen at a similar embryonic stage in *Xenopus* (Schohl and Fagotto, 2002). E8.5 is also the stage at which Cdx2 and Sox2 expression domains meet at the stomach-intestine border and at which hepatic and pancreatic genes begin to be expressed at this border (Sherwood et al., 2009). Thus, at E8.5, posterior foregut ceases to re-

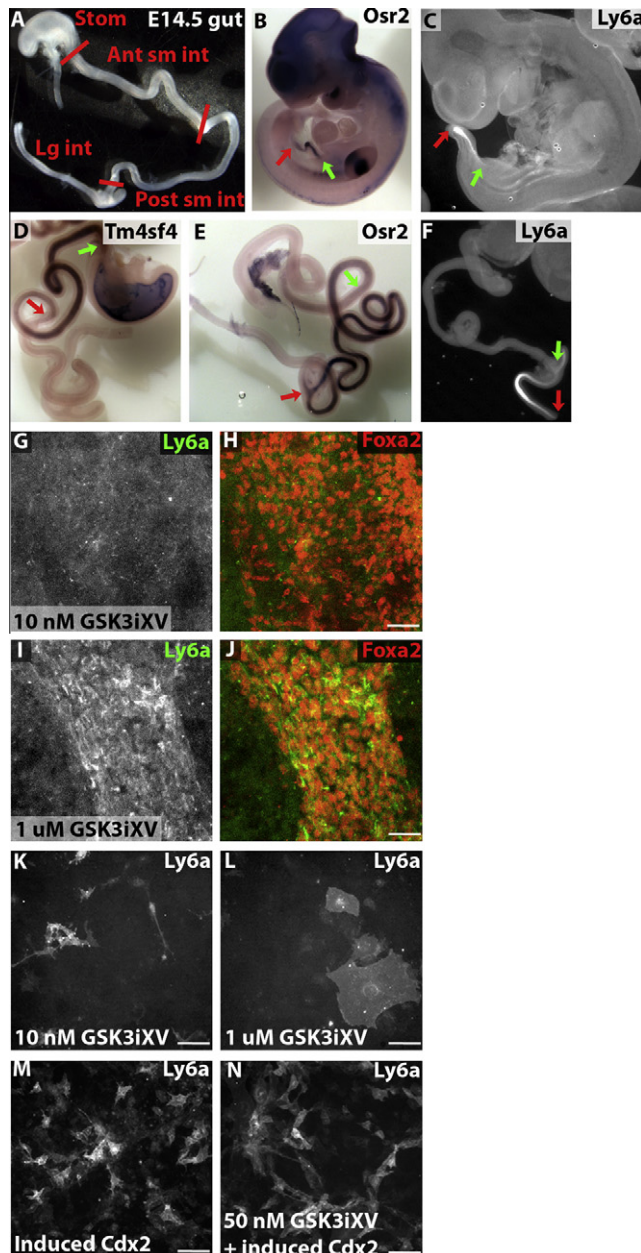


Fig. 5 – Intestinal molecular regionalization occurs by E10.5 and can be affected by Wnt and Cdx2 concentration. (A) Wholemount image of E14.5 gut with the different anterior–posterior divisions dissected for microarray analysis labeled. Distinction between anterior and posterior small intestine was chosen as the midpoint of the small intestine. **(B–F)** Wholemount in situ hybridization **(B, D, E)** or immunofluorescence **(C, F)** images of E10.5 embryos **(B–C)** or E14.5 gut **(D–F)** showing expression of posterior small intestine-specific *Osr2* **(B, E)**, large intestine-specific *Ly6a* **(C, F)**, and anterior small intestine-specific *Tm4sf4* **(D)**. Green arrows refer to the anterior border of the intestinal staining and red arrows refer to the posterior border of the intestinal staining. **(G–J)** Wholemount confocal immunofluorescence images of E8.25 foreguts cultured for 24 h before fixation and stained for *Ly6a* (white in G, I, green in H, J) and *Foxa2* (red in H, J). Foreguts were treated with soluble GSK3iXV at 10 nM **(G–H)** or 1 μ M **(I–J)**. **(K–M)** Immunofluorescence images of inducible Cdx2 ES-derived endoderm treated with 10 nM GSK3iXV **(K)**, 1 μ M GSK3iXV **(L)**, 1 μ g/mL Doxycycline **(M)**, or 50 nM GSK3iXV and 1 μ g/mL Doxycycline **(N)** for 24 h before fixation and stained for *Ly6a*. Scale bar equals 50 μ m in H, J, K–N.

Table 3 – Selected genes preferentially expressed in specific E14.5 gut segments. These genes have expression >3-fold higher in one E14.5 gut segment as compared to all other segments.

Stomach	Anterior small intestine	Posterior small intestine	Large intestine
Bace2	Anpep	Cib2	Evx1
Corin	Dkl1	Fzd10	Gnal4
Evil	Gpx3	Guca2a	Hoxa7
Gsta4	Edg7	Hoxd4	Hoxb7
Idb4	Ipfl	Olfml1	Hoxd10
Ly6h	Onecut2	Osr2	Ly6a
Sox21	Slc40a1	Rnase4	Slc5a8
Vsig2	Tm4sf4	Sdhl	Xpnp2

ceive Wnt signals or inhibitor activity outweighs ligand presence, and the endoderm loses competence to activate intestinal genes in response to such signaling. It has recently been shown that Wnt signaling in the E9.5 endoderm is vital for lung specification (Goss et al., 2009), and Wnt also plays an important role in the development of the pharyngeal pouches (Balcunaite et al., 2002), liver and pancreas (Verzi and Shivdasani, 2008). As the Wnt-Cdx2 axis is dominant over anterior fates, the loss of competence to activate Cdx2 in response to Wnt signaling is a crucial step in endodermal development.

Both the BAT-gal expression in the endoderm (Fig. 1) and the anterior creeping of endodermal Cdx2 (Sherwood et al., 2009) suggest a model in which a posterior source of Wnt diffuses anteriorly, and progressively more anterior cells reach a threshold to activate Cdx2. Our study suggests direct activation of Cdx2 by Wnt signaling, as Cdx2 mRNA is seen within 6 h of Wnt treatment in embryonic and ES-derived endoderm, and Tcf/Lef factors have been demonstrated to act directly on the promoters of Cdx1 and Cdx4 (Pilon et al., 2006; Prinos et al., 2001), so direct action on Cdx2 is a likely mechanism. The relationship between Wnt and Cdx2 in the nascent intestine may be more complex, however, as evidence from other developmental stages suggests that Cdx2 can induce Wnt signaling (Young et al., 2009), Cdx2 and the Wnt-responsive transcription factor Tcf4 co-occupy a large number of regulatory sequences (Verzi et al., 2010), and Cdx2 has been shown to inhibit Wnt responsiveness (Guo et al., 2010). This model of posterior Wnts patterning the intestinal endoderm provides a convenient explanation for the observation that only higher doses of Wnt in embryos and Cdx2 in ES-derived endoderm are able to induce large intestinal gene expression. However, the inability of high-dose Wnt alone to induce large intestinal gene expression in ES-derived endoderm suggests either that additional signaling mechanisms are required in concert with high-dose Wnt to induce high-level Cdx2 expression and large intestinal fates or that the protocol used for ES cell intestinal endoderm induction fails to provide the temporal competence window required for this induction.

Several experiments suggest that an additional aspect of intestinal organogenesis involves differential adhesion and possibly cell migration. After electroporation of CA β -catenin into foregut endoderm, electroporated cells are found at more posterior positions after longer periods of embryo culture, and after 24 h, a significant percentage of cells are found near to the endogenous hindgut Cdx2⁺ domain (Fig. 2D–F, Supplemental

Fig. 2). In the endoderm-specific genetic activation of β -catenin, ectopic Cdx2⁺ cells are found as clusters in the pharyngeal endoderm and as strands in the midgut region anterior to and connected to the endogenous Cdx2⁺ intestinal domain (Fig. 3). It is clear from the cluster formation in the genetic activation experiments that Wnt-induced Cdx2⁺ cells have differential adhesion that allows them to cluster and at times disengage from the pharyngeal endodermal epithelium as protrusions, an interesting finding in light of the known role of Cdx2 in controlling cell polarity and cytoskeletal arrangement (Gao and Kaestner, 2010). It is also tempting to speculate that the lack of ectopic clusters in the midgut occurs because Cdx2⁺ cells close enough to the endogenous intestinal domain migrate to and adhere to the endogenous intestinal domain, which could provide an explanation as well for the electroporation results. These experiments cannot rule out selective survival or proliferation as alternative explanations, so future timelapse imaging experiments will be informative to determine whether migration and differential adhesion play a role in the formation of a strict boundary for the nascent intestinal domain.

Induction of intestinal fate by Wnt seems to rely heavily on Cdx2. Cdx2 is one of the earliest genes activated in the endoderm by activation of Wnt signaling. Wnt signaling has been shown to directly induce Cdx1 in ES cells (Lickert et al., 2000), and our results strongly suggest a direct activation of Cdx2 in endoderm, as Cdx2 mRNA is induced within 6 h and Cdx2 protein is induced within 8 h. As has been recently documented in Cdx2-deficient endoderm (Gao et al., 2009), Cdx2 orchestrates a network of intestinal transcription, so it is unsurprising that 24 h after Wnt activation, gene expression has altered significantly toward that of native embryonic intestinal endoderm. Wnt signaling, however, has a more extensive role than simply activating Cdx2, as is demonstrated in experiments comparing genes induced by Wnt or by direct induction of Cdx2 in ES cell-derived endoderm. One potentially crucial difference is that Wnt induces Cdx1 along with Cdx2, and these two genes could have distinct targets during intestinal development. Additionally, Wnt signaling appears to induce Indian Hedgehog independently of Cdx2, and Hedgehog signaling is known to be important in the coordination of intestinal endoderm and mesoderm development (Roberts et al., 1998).

In the process of analyzing the role of Wnt in intestinal regional patterning, we have identified several genes differentially expressed in intestinal segments. This process has revealed two surprising results. The first is that, while the

small intestine is traditionally divided into three anterior-posterior segments by morphology, gene expression patterns suggest two molecularly distinct small intestinal regions. The duodenum has unique expression of genes such as *Ipf1*, *One-cut1* and *2*, *Anpep* and *Tm4sf4*, while *Osr2*, *Fzd10* and *Cib2* have graded anterior borders at the presumed duodenum-jejunum boundary and are expressed until their graded posterior boundaries slightly past the cecum (Fig. 5E, Supplemental Fig. 3). The large intestine-specific genes identified span the entire large intestine posterior to the cecum (Fig. 5F, Supplemental Fig. 3), although reports of specific roles for *Hox13* members in the anorectal endoderm (Warot et al., 1997) suggest that some genes may be expressed only in subregions of the large intestine. Second, these regional expression patterns are established at least as early as E10.5 and possibly earlier (Fig. 5B–C, Supplemental Fig. 3) even though morphological distinction among regions starts around E12.5 with the development of the cecum and does not become clear until villus formation after E14.5. The early establishment of regionalization is potentially a consequence of its reliance on differential Wnt levels, as Wnt acts in intestinal specification exclusively between E7.0 and E8.5.

Finally, we demonstrate that activation of Wnt signaling is sufficient to induce intestinal differentiation of ES cell-derived endoderm (Fig. 4). This induction is highly efficient, as greater than 95% of ES cell-derived endoderm cells begin to express *Cdx2* after treatment with GSK3iXV. While GSK3 inhibition has been found to affect pathways other than Wnt (Wu and Pan, 2010), the concordance of phenotypes using GSK3iXV, activated β -catenin, and Wnt3a in multiple assays lends confidence to the conclusion that GSK3 inhibition is exerting its intestinal inductive action primarily through activation of Wnt signaling. Recently, a protocol has been developed demonstrating three-dimensional intestinal organoid differentiation from human embryonic stem cell-derived endoderm by the addition of *Fgf4* and Wnt3a (Spence et al., 2011). In these experiments, *Fgf* and Wnt signals are required in conjunction to allow for *Cdx2* expression and intestinal differentiation; however, equivalent experiments in this work demonstrate that strong Wnt stimulation through GSK3 inhibition is sufficient to induce uniform *Cdx2* (Fig. 4), and the discrepancy is likely a result of the increased effectiveness of small molecule GSK3 inhibitors over Wnt3a (Fig. 4). In Spence et al.'s work (Spence et al., 2011), human ES-derived mesoderm is also present and becomes incorporated into the organoids, so *Fgf* signaling may be acting to pattern this mesenchyme. It will be interesting to replicate these experiments using ES cell-derived populations containing small and large intestinal marker expression to assess the differential morphological and transcriptional properties of these populations.

The gene expression changes induced by Wnt pathway activation are strikingly similar between embryo-derived endoderm and ES cell-derived endoderm. The few differences could result from differences in timing, as the embryonic experiments were performed in E8.25 foregut, which already expresses some specialized genes involved in anterior endoderm organogenesis whereas ES cell-derived endoderm appears by gene expression to resemble early embryonic endoderm, or from indirect effects of non-endodermal tissues

in the bead experiments. This finding is significant for the ES cell field, as it lends confidence to the fact that ES cells do respond equivalently to embryonic cells when exposed to extra-cellular signals.

It will be interesting to investigate ES cell-derived intestine further to determine whether it possesses the ability to differentiate down mature intestinal lineages or to adopt adult intestinal stem cell activity. Derivation of intestinal populations from ES cells will lead to greater understanding of intestinal diseases and will hopefully lead to *in vitro* models of intestinal diseases and tissue replacement therapy.

Acknowledgements

D.A.M. is an investigator of the Howard Hughes Medical Institute. R.I.S. was supported by a National Science Foundation Graduate Research Fellowship and the Sternlicht Fellowship. E.O.M. is the David and Sylvia Lieb Fellow of the Damon Runyon Cancer Research Foundation, DRG-1937-07. This work was supported by the N.I.H. The authors would like to thank Brian Tilton, Anastasie Kweudjeu, George Kenty, and the Molecular Genetics Core Facility at Children's Hospital Boston for technical assistance. The G8.8 antibody was obtained from the Developmental Studies Hybridoma Bank.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2011.07.005.

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