Balancing on Sox

Involvement of Sox2 in determination and maintenance of organ identity of the gastrointestinal tract

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Voor Papa en Mama

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CHAPTER 1

General introduction and scope

Histology of the mature gastro-intestinal tract

The gastro-intestinal tract is organized in four distinct layers, which are consistent from mouth to anus. Surrounding the gastro-intestinal lumen is the mucosa, followed by the submucosa, tunica muscularis and serosa. The inner layer of any epithelial-lined hollow organ (e.g., mouth, gut, trachea, lung, etc) is called the mucosa, which itself consists of the epithelium, lamina propria and the muscularis mucosa, which forms the boundary between mucosa and submucosa. The submucosa is a connective tissue layer supporting the mucosa and allows flexibility during peristalsis. It contains a vascular plexus, which gives rise to the capillary bed of the mucosa and also a delicate nerve network, called Meissner's plexus. The tunica muscularis consists of an inner circular layer and an outer longitudinal layer. Between the two muscle layers lies the Auerbach's plexus. Coordinated contraction of these layers is responsible for rhythmic peristalsis. The adventitia or the serosa is the outermost layer of the gastrointestinal tract and has a supportive function.

The mucosa is the most characteristic layer of the gastrointestinal wall. Tissue specialization and surface shape are correlated with functional differentiation along the tract. Each region contains certain cell types which are adapted to carry out the characteristic functions of secretion and absorption.

The stomach is responsible for the formation and processing of the ingested food into a thick acidic fluid known as chyme. Anatomically four regions can be distinguished in the stomach: cardia, fundus, corpus and pylorus (Fig. 1). The cardia is a narrow region at the gastroesophageal junction. While the fundus is frequently filled with gas, it is the corpus that is mainly involved in the formation of the chyme. The pylorus separates the stomach and its acidic environment from the small intestine. Histologically, the fundus and corpus of the stomach are identical and consist of simple columnar epithelium with glands. The gastric mucosa is organized into numerous mucosal invaginations called gastric units, in which the stem cells and the various differentiated progeny are localized. The gastric units comprise the pit, which are formed by invaginations of the epithelial layer into the mucosa and the flask-shaped gland, which can be further subdivided into isthmus, neck, and base regions (Fig. 1). The four major functional cell types present are the mucus producing neck cells, the acid and intrinsic factor producing parietal (oxyntic) cells, the chief (zymogenic) cells, which secrete pepsin, and several types of endocrine cells secreting hormones such as gastrin and somatostatin[1-2] (Fig.1).

The small intestine digests and absorbs nutrients, which are the end products of the digestive process. The small intestine can be subdivided in three

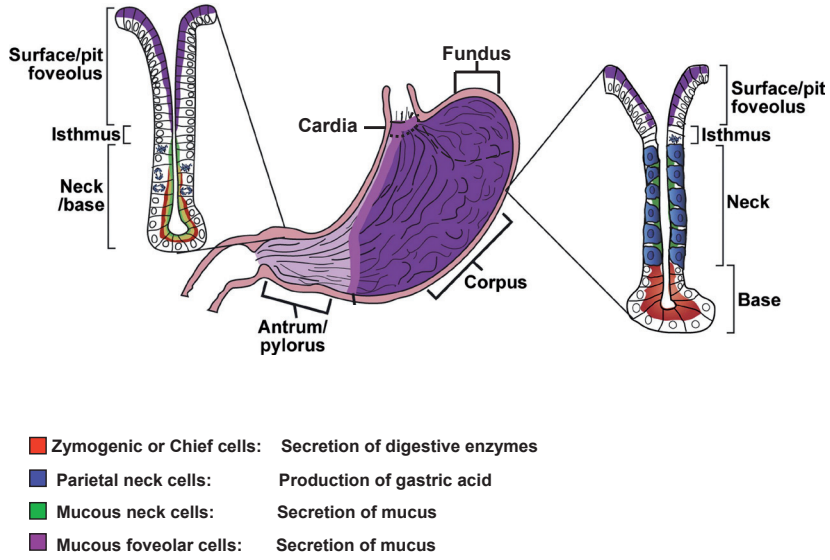

Figure 1: Schematic representation of the stomach and its main cell types.

Typical anatomy and histology of a mammalian stomach. There are a number of variations in mammalian gastric anatomy, however the most prominent regions in most mammals are the corpus and a distal antrum or pylorus. The gastric epithelium is organized into repeating gastric units that are invaginations from the surface and contain 4 main gastric cell types. The chief cells (red), the acid producing parietal cells (blue) and the mucous producing neck (green) and foveolar (purple) cells. Figure adapted from Mills et al, 2011[2].

segments, the duodenum, jejunum and ileum. The duodenum receives enzymes from the pancreas and bile from the liver, and contains Brunner's glands, which produce bicarbonate-rich fluid to neutralize the acidic contents from the stomach. The mucosa of the small intestine is specialized for absorption of nutrients and consists of a layer of simple columnar epithelium. To increase the intestinal absorptive area, the mucosa is formed into fingerlike structures, the villi. In between, at the base of the villi, the intestinal crypts of Lieberkühn reside.

Lastly, the large intestine comprises of the caecum, colon, rectum and anus and its function is to absorb water, electrolytes and gasses from the chyme, and subsequently to compact the residues into feces for eventual excretion. Villi are excluded from the large intestine, but instead the crypts of Lieberkuhn are richly present

The intestinal tract is characterized by four main intestinal cell lineages, which are classified into absorptive and secretory groups. The first group is represented by columnar cells, which are called enterocytes and colonocytes in the small and large intestine respectively, and are the most abundant cells present[3-4]. To increase the absorptive area, these cells harbor an apically located

brush border. The secretory group is represented by the goblet cells, entero-endocrine cells and the Paneth cells. The goblet cells produce and secrete mucins onto the intestinal surface, in order to lubricate and protect the mucosa[4-5]. The entero-endocrine cells produce peptide hormones, such as cholecystokinin, secretin and gastric inhibitory peptide[4, 6]. Lastly, the Paneth cells, which contain large secretory granules and express a number of proteins with antibacterial actions, such as lysozyme[4, 7]. Besides ensuring a sterile crypt environment, these cells have an important role in regulating the stem cell compartment in the crypts of Lieberkühn[8-9] (Fig.2).

Intestinal stem cells and the epithelial renewal process

The intestinal epithelium is a rapidly self-renewing tissue. The turnover time of the murine small intestinal epithelium is approximately 5 days. The epithelial renewal process takes place along the so called crypt-villus axis. This renewal process involves proliferation of multipotent progenitor cells or stem cells, which reside at the base of the crypts. Stem cells divide and give rise to other stem cells with self renewal capacity, and to a highly proliferative population of transit amplifying daughter cells. Transit amplifying cells are proliferative uncommitted progenitors, which undergo rapid cell division for 4-5 rounds[10]. Proliferation ceases in the upper third of the crypt .Transit amplifying cells then move out of the crypt compartment and subsequently differentiate into the four main cell types of the intestinal tract. The enterocytes, goblet and entero-endocrine cells form the epithelial lining of the villus and move up the villus, where they die after reaching the tip after 2-3 days. The Paneth cells on the other hand are the only terminally differentiated cell type, which reside within the crypt stem cell zone of only the small intestine, where they live for 6-8 weeks.

The primary driving force behind the proliferation of epithelial cells in the intestinal crypts is the Wnt/β-catenin signalling pathway, which is highly conserved throughout the animal kingdom (Fig.3)[11-13]. The central player in the canonical Wnt pathway is β-catenin. In the absence of a Wnt signal, β-catenin is targeted for proteasomal degradation through phosphorylations at N-terminal Serine residues. The degradation complex consists of the tumor suppressors axin and adenomatous polyposis coli (APC) and the kinases glycogen synthase kinase 3β and casein kinase I. When Wnt ligands signal through their Frizzled and low-density lipoprotein receptor–related protein (LRP) receptors, the destruction complex is inactivated, leading to accumulation of β-catenin because it is no longer targeted for degradation. Accumulated β-catenin can be transported into the nucleus and binds to transcription factors of the T cell factor/lymphocyte

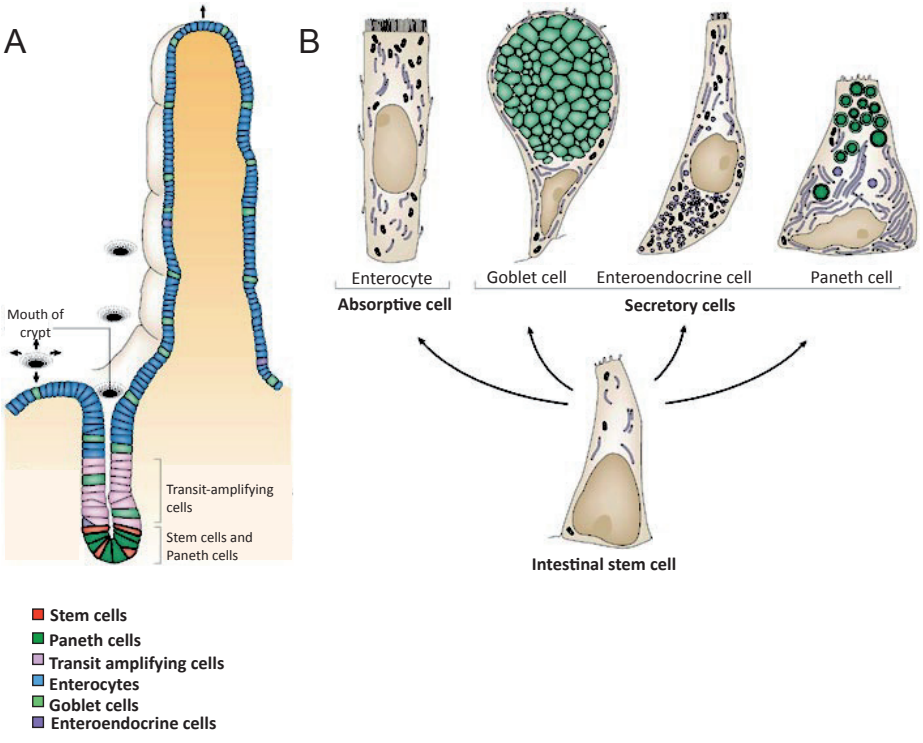

Figure 2: Schematic representation of the intestinal epithelium and its main cell types.

The intestinal epithelium has a highly structured organization. To increase the intestinal absorptive area, the mucosa is formed into fingerlike structures, called the villi. At the base of the villi the intestinal crypts of Lieberkuhn reside (A). The intestinal epithelial process takes place along the so-called crypt-villus axis. This process involves proliferation of multipotent stem cells (red), which reside at the base of the crypts, with subsequent migration and differentiation of the transit amplifying (TA) cells (pink) along the villi. The four main cell types which are formed are the enterocytes (blue), the enteroendocrine cells (purple), the Paneth cells (dark green) and the mucus producing goblet cells (light green)(B). Figure adapted from Crosnier et al, 2006[8].

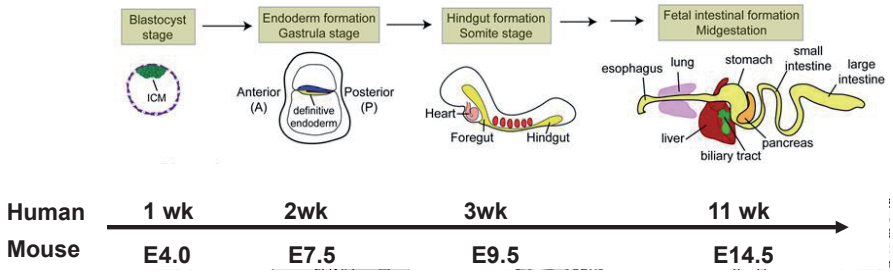

Figure 3: Timeline of human and mouse intestinal development.

Schematic representation of the blastocyst stage at E4.0 in mice or the first week post coitum in human, which is followed by the process of gastrulation resulting in the formation the endodermal layer at E7.5 in mice and second week post coitum in human. At E9.5 in mice / third week in human the regionalization of the primitive gut takes place and the foregut, midgut and hindgut can be discriminated. From E14.5 (week 11 human) onwards cytodifferentiation and villus formation in the intestinal tract takes place.

enhancer factor (TCF/LEF) family, thereby forming an active transcriptional complex that activates target genes. A large variety of β-catenin target genes, have been described since the discovery that this pathway represents the dominant force behind the proliferative activity of the healthy intestinal epithelium.

Whereas the involvement of the canonical Wnt/β-catenin signalling pathway in intestinal homeostasis is well described, the role of the non-canonical Wnt pathway is far less understood. Non-canonical Wnt signalling is described as the signalling routes that act independent of β-catenin. The most extensively studied non-canonical ligand Wnt5a gained attention over the last years, as it has been implicated in various human diseases including cancer, inflammatory bowel diseases and metabolic disorders[14-16].

Development of the gastro-intestinal tract

The gastro-intestinal tract develops through a complex mechanism of patterning, expansion and differentiation of the primitive gut. Early in vertebrate embryogenesis, gastrulation results in the three germ layers, the ectoderm, endoderm and mesoderm[17-19]. At the end of gastrulation, the morphologically homogeneous endoderm has already anterior (top) and posterior (bottom) characteristics, mainly by the expression of specific anterior or posterior markers[20-21]. Immediately after gastrulation, the endoderm is not yet committed to specific organ domains[18]. When explanted mouse E7.5 anterior endoderm is placed in contact with posterior mesoderm, posterior endodermal genes can be activated and vice versa. During development these anterior and posterior domains become more regionalized and by E9.5 in mice the foregut, midgut and hindgut regions can be discriminated. The foregut eventually gives rise to the esophagus, stomach and proximal part of the duodenum; the midgut develops into the caudal part of the duodenum, jejunum, ileum, caecum and proximal part of the colon; and the hindgut forms the caudal part of the colon and rectum.

Before the placental connections are established, during development of the embryo, the omphalomesenteric duct (also referred to as vitelline duct) connects the midgut to the yolk sac. Normally, the omphalomesenteric duct involutes completely between the fifth to ninth week of human gestation (E9.5-E10 of mouse development). However, if this resorption does not occur properly, the complete omphalomesenteric duct, or a part of it, may persist, which leads to various congenital anomalies of the intestine, such as Meckel's diverticulum, which accounts for the most prevalent congenital disorder of the gastro-intestinal tract and occurs approximately in 2% of the population.

In mice, from E9.5-E13.5 the gut tube increases in length and the circumference increases due to the expansion of the lumen, epithelium and mesenchyme. At E11.5 the stomach epithelium is pseudo-stratified with little difference between presumptive forestomach and glandular stomach. This differentiation of the epithelium is first noted on E12.5. The epithelium of the forestomach begins to stratify on E13.5 and keratinisation is first noted on E16.5. In the glandular stomach the epithelium begins to stratify on E12.5 and primitive glands are formed on E16.5[22].

As the gut tube grows, the epithelium of the prospective small and large intestine reorganizes around E14.5 from a pseudostratified epithelium, to a columnar form[23]. At the same time point in development the emergence of villi in the small intestine and the process of cytodifferentiation into the characteristic cell types found in the various regions, takes place. Although the precise mechanisms that initiate and control epithelial reorganisation and villus morphogenesis is still not fully understood, it has been shown that interaction between the gut epithelium and mesenchyme provides both instructive and permissive cues to allow normal intestinal development[24]. The intestinal development completes postnatally, when the definitive crypts of Lieberkühn are formed. The crypts emerge from the intervillus epithelium. The development of the gastro-intestinal tract described here with embryonic time points for the mouse, is basically identical in all mammals although with different timelines (Fig.4).

The role of Sox2 and Cdx2 in the development of the primitive gut endoderm

Various signalling families, including BMP[25-29], Hedgehog[30-31], FGF[32] and Notch[33] , have been implicated to play a role in gut development. However, until now very little is known about one of the most basic facets of organogenesis, i.e. the dynamics of the expression of key transcription factors, which are the ultimate targets of signalling pathways. Transcription factors are proteins that play a key role in this regulation, as they act as molecular switches that determine fundamental decisions regarding proliferation, differentiation or cell fate of distinct cell populations[34]. It has now abundantly been demonstrated that sets of transcription factors from various families act in concert to control cell fate decisions and differentiation into each lineage. These factors include basic-helix-loop-helix, POU domain, Runt domain, forkhead, HMG-box domain and homeodomain proteins. In this thesis the focus will lie on two specific members of the two latter super families of transcription factors, Sox2 and Cdx2 respectively.

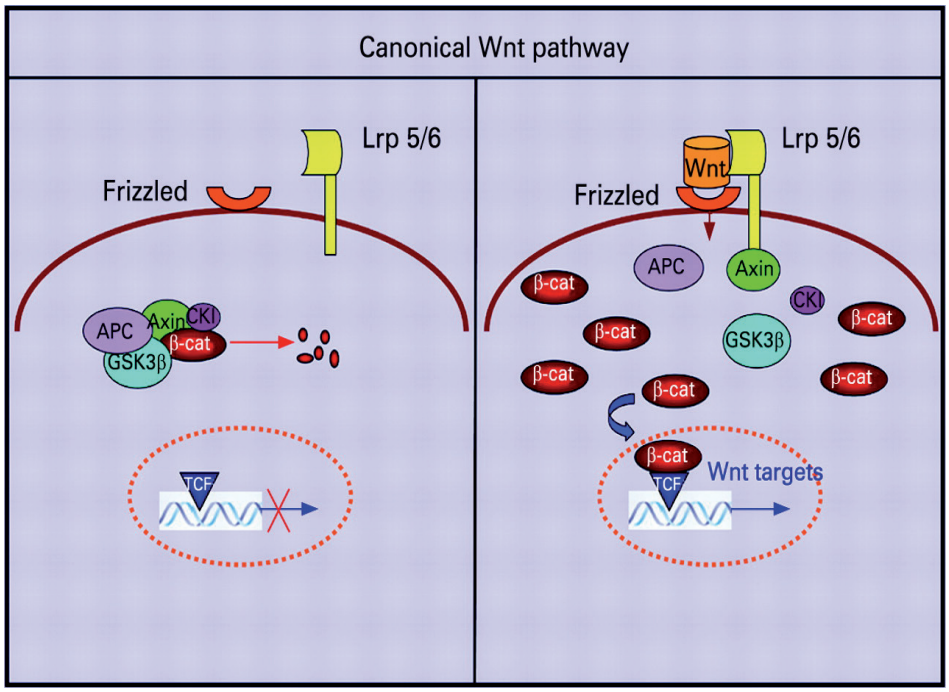

Figure 4; schematic representation of the canonical wnt/β-catenin signaling pathway.

In the absence of Wnt ligand, a "destruction complex" phosphorylates the free cytoplasmic pool of β-catenin which then undergoes proteasomal degradation. When Wnt ligands engage the frizzled receptors, formation of the "destruction complex" is inhibited, leading to accumulation of free β-catenin in the nucleus where it serves as a coactivator for the Tcf/Lef family of transcription factors[13].

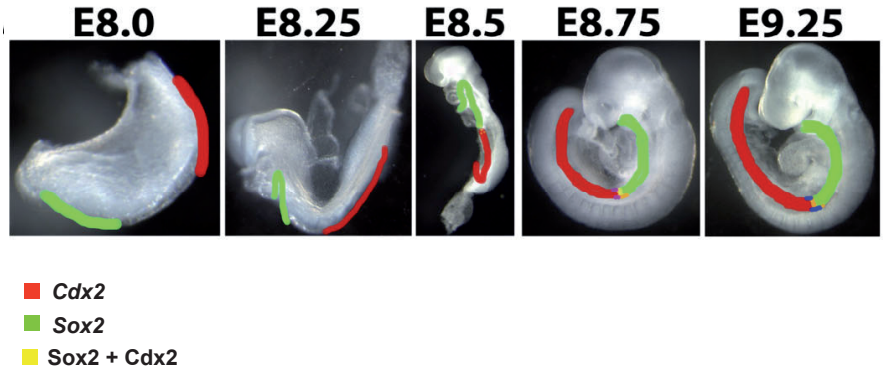

Figure 5: Sox2 and Cdx2 expression in the developing gut endoderm.

Schematic representation of the developing gut endoderm, from which the expression domains of Sox2 and Cdx2 become clear. Sox2 (green) starts to express at the anterior end of the endoderm, whereas Cdx2 starts at the posterior end. These domains will expand towards each other. From E9.25 the boundaries between the presumptive stomach and intestine are established and these domains will not overlap. Within the mature gastro-intestinal tract, Sox2 is expressed in the stomach and esophagus, whereas expression of Cdx2 is restricted to the intestinal epithelium. Figure adapted from Sherwood et al, 2009[35].

One of the earliest steps in patterning of the primitive gut is the regionalization into an anterior and posterior domain, which correlates with the expression of Sox2 and Cdx2 respectively[35]. In mice, expression of Sox2 and Cdx2 can be detected at E7.75 in the most anterior and posterior endodermal cells[35]. Between E8.0 and E8.5 the expression domains of Sox2 and Cdx2 move towards each other, and eventually at E9.5 a reciprocal expression pattern is seen for both transcription factors (Fig.5)[35]. Later in development Cdx2 becomes restricted to the intestinal epithelium, with a sharp boundary, marking the transition from stomach to duodenum, while Sox2 is expressed in the stomach and esophagus[35-38]. Although Sox2 and Cdx2 are reciprocally expressed, it remains unclear whether Sox2 or Cdx2 has a dominant role in determining the fate of the developing gut endoderm.

Sox genes are widespread throughout the animal kingdom and at present up to 20 Sox genes have been characterized in mouse and human[39-40]. They play an important role in different aspects of embryonic development and are also found to be expressed in a number of adult tissues[41-42]. Proteins are grouped into this family if they contain an HMG domain with strong similarity (>50% of amino acids) to the HMG domain of SRY, which is also known as SRY-box[43]. This box gave the Sox family its name: SRY related HMG box. The HMG- box domain is a 79 amino acid protein motif, through which proteins interact with DNA[44]. Although they share common DNA binding properties, the specificity of individual Sox proteins is the result of their expression pattern and their ability to associate with different partners[41, 45-46]. Sox2 plays an important role during various phases of vertebrate embryogenesis and is also expressed in a number of adult tissues. In mice, expression of Sox2 starts from the 4-8 cell stage of embryo development and is initially expressed in the inner cell mass (ICM) and extraembryonic ectoderm of blastocysts[47]. Sox2 is essential in both the establishment and the maintenance of pluripotent stem cells, which is supported by data showing that Sox2 null mutant mouse embryos die before gastrulation as a result of failure of epiblast development after implantation, and by the observation that deletion of Sox2 in embryonic stem cells results in inappropriate differentiation into trophoectoderm-like cells[48]. Furthermore, forced expression of Sox2, in combination with Oct4, c-Myc and Klf4 endows somatic cells with pluripotency, giving rise to induced pluripotent stem (iPS) cells[49-50]. Recent studies have also established Sox2 as a wide spread marker of pluripotent and adult stem/progenitor cell types[49-51].

At later phases of normal embryogenesis, Sox2 expression is essential for the formation of several endodermal and ectodermal tissues, including lens epithelium[52], sensory cells of the taste bud[53], the nervous system[54-56], the inner

ear[57], and the retina[58-59]. In addition, it is essential in the formation of the foregut, from which the esophagus, stomach and duodenum originates[60-63]. Modulating expression in mice showed the importance of Sox2 in the differentiation of the esophagus and stomach. Reduced Sox2 levels in mouse embryo models are associated with esophageal atresia and other developmental esophageal abnormalities including the presence of luminal mucus-producing cells, and ectopic expression of genes normally expressed in the glandular stomach[63]. Furthermore, in human, several congenital malformations, for instance anophthalmia, microphthalmia and the AEG (anophthalmia-esophageal-genital) syndrome[64], have been associated with heterozygote mutations in SOX2, confirming the importance of Sox2 in development and differentiation processes.

A second family of transcription factors which can be discriminated is the family of homeobox domain proteins. Homeobox genes are characterized by a 60 amino acid homeodomain[65]. Within this superfamily many subgroups can be distinguished, one of which is the group of Cdx (Caudal-type homeobox) genes. In mice and human there are three Cdx genes known, Cdx1, Cdx2, and Cdx4. These Cdx members play an essential role in the anterior-posterior (A-P) patterning in both vertebrate and invertebrate embryos[38, 66-71]. In mice, Cdx2 is expressed very early during embryogenesis, and is already observed at E3.5 in the trophoectoderm. Underscoring the importance of Cdx2 for normal development, homozygous Cdx2 -/- embryos fail to implant properly, resulting from a block in differentiation of the trophoblast[72]. At E8.5, expression shifts towards the posterior gut, neural tube and tail bud. At E12.5, the expression of Cdx2 is restricted to the primitive gut endoderm and in the mature digestive tract Cdx2 is exclusively expressed within the intestinal epithelium[20, 37, 72-73]. Cdx2 is an essential transcription factor for establishing intestinal cell fate. This was first shown in heterozygous Cdx2 knock-out mice, which developed polyps consisting of normally organized stomach mucosa in the paracaecal region of the intestine [74]. Importantly, Sox2 was ectopically expressed in the overlying endoderm[75]. It was suggested that local sporadic haploinsufficiency of Cdx2 caused an anterior homeotic shift in which undifferentiated intestinal endoderm defaulted to a gastric phenotype. Chimaeric studies using Cdx2-/- cells in wild-type hosts confirmed these observations and also studies done by Cdx2 ablation specifically in the embryonic endoderm showed an activation of the foregut transcriptional program, causing a forestomach phenotype[76-77]. In all cases, ectopic expression of Sox2 was noted. Table 1 shows an overview of the experiments described above and their results.

Experimental approach	Results
Sox2	
Sox2 null mutant mice	Failure of epiblast development after implantation.
Deletion of Sox2 in ES cells	Inappropriate differentiation into trophoectoderm-like cells.
Reduced levels of Sox2 in mouse foregut	Development of esophageal abnormalities (atresia, ectopic expression of genes normally expressed in the glandular stomach and presence of luminal mucous producing cells)
Forced expression of Sox2 in somatic mouse cells	Induction of pluripotent stem cells, in combination with forced expression of Oct4, c-Myc and Klf4
Cdx2	
Cdx2 null mutant mice	Block in differentiation of the trophoblast.
Heterozygote Cdx2 knock-out mice	Development of polyps in the paracaecal region, consisting of stomach mucosa.
Chimaeric studies with Cdx2 null cells in wild type host mice	Undifferentiated intestinal endoderm defaults to gastric phenotype.
Cdx2 ablation in embryonic mouse endoderm	Ectopic activation of the foregut program in the presumptive intestine

Table 1: Overview of experiments altering Sox2 and Cdx2 expression in mice

Homeostasis of the mature intestinal tract and human colorectal cancer

Recently, a number of studies have reported ectopic expression of Sox2 in a number of cancer types including lung, esophagus and breast cancer[78-79]. In these tumor types, aberrant Sox2 expression was associated with worse prognosis, attributed to the induction of a precursor-like state induced by Sox2. As such, the ectopic SOX2 expression previously observed in CRCs may likewise affect tumor progression[80].

Worldwide over 1,200,000 people are annually diagnosed with CRC, resulting in approximately 600,000 deaths every year. As such, CRC is the most frequent malignancy, and is the second most common cause of cancer-related mortality. CRC has been classified in different subtypes according to criteria such as their mutational status and histological appearance. Mucinous colorectal carcinoma is defined as a tumor with more than 50% mucinous differentiation on histologic examination, according to the WHO. The incidence of mucinous carcinoma among colorectal cancer patients is between 10-25%[81-82]. Most evidence suggests that mucinous CRC portend a worse clinical prognosis. The reason for the worse prognosis in mucinous CRC is not clear. Aberrant expression of SOX2 in a subset of CRCs with mucinous differentiation was reported previously[80]. Given the important role of SOX2 in determining cell fate the ectopic SOX2 expression

observed in mucinous tumors represented an attractive candidate to investigate in further detail. As aberrant expression of SOX2 has been linked to the induction of a precursor-like state in other tumor types, the ectopic SOX2 expression observed in CRCs may likewise affect tumor progression.

Scope of this thesis

In order to take up nutrients from food and liquid, which is needed for the growth and maintenance of the body, a highly specialized digestive tract is warranted. The organs of the digestive tract together have a function in the ingestion, digestion and absorption of nutrients as well as the elimination of indigestible components of the food. Both normal and abnormal development of these organs takes place under the control of various transcriptional regulators, such as Sox2, which plays an important role in stomach and esophageal development. Once formed, organ identity is strongly maintained and rarely converted into another tissue type. However, ectopic gastro-intestinal tissue arises outside its organ boundaries, in various situations including Barrett's esophagus in which the normal squamous esophageal epithelium is replaced by specialized mucosal epithelium, intestinal metaplasia of the stomach and gastric heteroplasia in the developing intestinal tract. The mechanisms underlying the formation of this ectopic tissue are not fully understood.

The overall aims of the research described in this thesis, are to investigate in more detail the role of Sox2 in establishing and maintaining organ identity. Furthermore, we examined the connection of ectopic Sox2 expression with various human diseases of the intestinal tract, such as colorectal cancer and various congenital anomalies.

In **chapter 2** we tested the Villin-rtTA inducer mouse line, by crossing it with a mouse line that expresses the histone H2B- green fluorescent protein (H2B-GFP) controlled by a tetracycline responsive regulatory element (TRE). We show that by administering different concentrations of doxycycline, the Villin-rtTA2-M2 system drives transgene expression in a dosage-dependent fashion specifically in the intestinal epithelium. Thus, we have generated a novel doxycycline-inducible mouse model, providing a valuable tool to study the effect of different gene dosages on intestinal physiology and pathology.

First, we used the mouse line described in Chapter 2 to specifically induce Sox2 expression in the presumptive intestinal tract during development. The results of this study are described in **Chapter 3** of this thesis. The findings provide strong evidence that expression of Sox2 in the developing gut drives

the activation of the foregut transcriptional program and leads to conversion from an intestinal into a premature gastric-like epithelium, despite simultaneous expression of Cdx2.

Next, we investigated the effect of ectopic expression of Sox2 in the mature intestinal tract using the same mouse model, of which the results are described in **chapter 4.** We found that ectopic expression severely alters the morphology of the villi and consequently alters intestinal homeostasis. However, in contrast to the developing gut, ectopic expression of Sox2 does not result in a conversion of intestinal cells into more gastric-like cells, indicating a loss of plasticity of the mature intestinal cells.

As shown in mice, a strict balance between the transcription factors Sox2 and Cdx2 is of great importance for normal intestinal development (Chapter 3) and imbalances in expression of either factor results in developmental defects in mice. However, in human little is known about the expression patterns of SOX2 and CDX2 in gut development. Therefore we examined the expression pattern of both transcription factors in intestinal duplications and vitelline duct anomalies, including Meckel's diverticulum, which are congenital anomalies of the intestine, in which ectopic gastric tissue can be present. We compared cases with gastric heteroplasia, with cases consisting solely of intestinal tissue. The results described in **chapter 5**, show that also in human gut development a strict balance between SOX2 and CDX2 appears necessary for proper development.

In **chapter 6**, we investigated whether we can detect aberrant SOX2 expression in colorectal cancer, and whether this can be correlated with specific tumor characteristics and prognosis. We did not observe a correlation with mucinous differentiation as was previously suggested. In addition, our data may point to a weak association with Sox2 expression and worse prognosis.

The overwhelming majority of CRC cases are initiated by activating mutations in the Wnt/β-catenin signaling pathway. Whereas the involvement of the canonical Wnt/β-catenin signalling pathway in intestinal homeostasis is well described, the role of the non-canonical Wnt pathway is far less understood. In **chapter 7**, we investigated the role of Wnt5a induction during different time frames of embryonic gut development and the mature intestinal tract. Our results indicate a role for Wnt5a during a restricted time-frame of embryonic development, but suggest no impact during homeostatic postnatal stages.

Lastly in the general discussion (**chapter 8**), we discuss our findings and indicate their clinical relevance.

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CHAPTER 2

Generation of a tightly regulated doxycycline-inducible model for studying mouse intestinal biology

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Abstract

In order to develop a sensitive and inducible system to study intestinal biology, we generated a transgenic mouse model expressing the reverse tetracycline transactivator rtTA2-M2 under control of the 12.4 kb murine Villin promoter. The newly generated Villin-rtTA2-M2 mice were then bred with the previously developed tetO-HIST1H2BJ/GFP model to assess inducibility and tissue-specificity. Expression of the histone H2B-GFP fusion protein was observed exclusively upon doxycycline induction and was uniformly distributed throughout the intestinal epithelium. The Villin-rtTA2-M2 was also found to drive transgene expression in the developing mouse intestine. Furthermore, we could detect transgene expression in the proximal tubules of the kidney and in a population of alleged gastric progenitor cells. By administering different concentrations of doxycycline, we show that the Villin-rtTA2-M2 system drives transgene expression in a dosage-dependent fashion. Thus, we have generated a novel doxycycline-inducible mouse model, providing a valuable tool to study the effect of different gene dosages on intestinal physiology and pathology.

Introduction

The intestinal epithelium consists of distinct crypt-villus units, which undergo renewal through a tightly regulated sequence of proliferation, differentiation, migration and cell death. Distinct gene expression levels are necessary to maintain tissue homeostasis. Alterations of these levels will lead to disruption of the equilibrium between proliferation and cell death, and loss of tissue architecture [1].

Many genes have been implicated in intestinal development and carcinogenesis. The temporal and spatial control over expression of specific genes at distinct dosage levels represents a powerful approach to evaluate their role during development as well as in tumor initiation and progression to malignancy. To address these questions, a model system is needed where tight regulation of intestine-specific gene expression at different dosages and at various time-points during development and adulthood is feasible.

Different promoters have been described which are able to drive intestine-specific expression. While activity of the Fabpl596 promoter is restricted to the small intestine and cecum, its derivative Fabpl4x promoter has been shown to drive expression in more distal intestinal segments including ileum, cecum and colon. Both promoters reveal patchy transgene expression patterns [2-3]. More recently, the Villin promoter has become the promoter of choice for intestine-specific gene expression. Using a 9 kb promoter fragment, Pinto et al. demonstrated transgene expression throughout the cephalocaudal axis of the gut [4]. Both this promoter construct and a 12.4 kb variant thereof have been used successfully to generate various transgenic models for intestinal specific expression [5-8] The Villin promoter is also active in the putative stem cells of the intestinal epithelium, as demonstrated by long-term labeling of crypt-villus units using a tamoxifen-dependent and Villin-driven Cre recombinase [8].

Inducible transgenic mouse models represent unique experimental tools to study transgene expression in a tightly regulated fashion. Ideally, such an inducible mouse model should drive homogeneous transgene expression in the tissue of interest exclusively upon induction and in a dosage-dependent manner. Furthermore, induction of gene expression should be reversible [9]. Such a model is currently not available for the intestinal tract. Therefore, we set out to generate a mouse model allowing robust induction of intestine-specific gene expression.

Materials and methods

Transgenic mice

The Villin-rtTA2-M2 construct was generated as follows. The rtTA2-M2 sequence was PCR-amplified from the pUHrT62-1 vector [10] (gift from W. Hillen, Erlangen) using the forward primer

5'-ATTCTCGAGGCCGCCACCATGTCTAGACTG-3' and the reverse primer 5'-TAGATCGATTATCCTGGGAGCATGTCAAGG-3'. The PCR product was inserted into the vector containing the 12.4 kb Villin promoter [5] (a gift from D. Gumucio, Michigan) by digesting PCR product and vector using restriction enzymes XhoI and ClaI, and ligating both fragments. The final vector was sequence verified using the sequencing primers 5'-CTTTTCGGCCTGGAACTAATC-3' and 5'-CTGTCCAGCATCTCGATTG-3'. The 14.7 kb expression cassette was cut out of the plasmid backbone by PmeI digest, gel-purified and prepared for injection into fertilized FVB/N oocytes. Founders were identified by PCR amplification of tail DNA using transgene specific primers (5'-CAAGACTTTCTGCGGAACAAC-3' and 5'- GTGTCTCTCTTTCCTCTTTTG-3').

Transgenic Villin-rtTA2-M2 animals were bred with tetO-HIST1H2BJ/GFP (H2BGFP) animals [11] (kindly provided by E. Fuchs, New York). Transgene expression was induced in compound heterozygous animals and their littermates by replacing normal drinking water with 5% sucrose water containing 2 mg/ml doxycycline (Sigma, D9891). Dox-treated water was changed every 2 days. After 7 days of doxycycline treatment, mice were sacrificed and tissues were analysed for H2B-GFP-expression.

Transgenic Villin-rtTA2-M2 animals were also bred with tetO-myc-Sox2 transgenic mice [12]. Doxycycline was administered to dams from gestational age 10.5 onwards in the drinking water (2 mg/ml doxycycline, 5% sucrose) and embryos were analysed at day 18.5.

Cryosectioning

The intestinal tract was removed, cut open and washed in PBS. Next, sections of ~10 cm were spread out flat on paper and fixed in 4% PFA for 60 min and washed in PBS for 30 min. Swiss-rolls were placed in KP-CryoBlock (Klinipath, Duiven, NL) and frozen on dry ice. The left kidney was dissected, washed in PBS and frozen on dry ice using KP-CryoBlock. Five µm sections were

cut and placed on SuperFrost Plus slides. The sections were stained with DAPI and analysed by fluorescence microscopy.

Immunohistochemistry

Dissected tissues were fixed in 4% PFA overnight, processed and embedded in paraffin. Immunohistochemistry for GFP and Myc-tag was done using citrate antigen retrieval followed by overnight incubation in 5% non-fat dry milk/PBS with, respectively, 2.5 µg/ml rabbit anti-GFP antibody (Molecular Probes) and 12.5 µg/ml anti-C-myc clone 9E10 (Roche). The Rabbit ChemMate™ EnVision™ kit (DakoCytomation) was employed as a secondary antibody, according to manufacturer's instructions.

FACS analysis

Duodenum, jejunum, ileum and colon were dissected, cut-open, washed in PBS and chopped into small pieces using a razor blade. Tissue pieces were left at 37˚C in digest medium (RPMI + p/s, 10%FCS, 5 ng/ml EGF, 0.03 ng/ml Hydrocortisone, 1x Insulin/Transferrin, 4 mg/ml Collagenase, 0.1 mg/ml Dispase, 50 µg/ml DNAse) for 2.5h until 90% were single cells. The cell suspension was passed through a 40µm cell strainer, washed by doubling the volume using 2% FCS/PBS, and spun down for 8 min at 1200 rpm at 4˚C. To exclude non-epithelial cells from analysis, $1x10^6$ cells were stained 30min in 100µl of lineage staining solution consisting of 2% FCS/PBS, 5 µg/ml Biotin anti-mouse CD31, 2.5 µg/ml Biotin anti-mouse CD45 and 5 µg/ml Biotin anti-mouse TER-119 (all BD Pharmingen) at 4˚C. After a washing step, staining with the secondary antibody (0.2 µg/ml Strepavidin-PerCP-Cy5.5; BD Pharmingen) was performed in the dark at 4˚C. Cells were then washed and re-suspended in 0.2 µg/ml Hoechst in 2%FCS/PBS in order to facilitate discrimination of live from dead cells. Fluorescent activated cell sorting analysis was performed on samples from each part of the intestine separately using a BD FACSAria.

Western Blotting

Intestinal tissue specimens (~2 mm) were snap-frozen in liquid nitrogen and subsequently lysed in 200 µl Laemmli sample buffer for 30 minutes using a thermoshaker set at 1000 rpm and 75°C. Twenty µl of each sample was loaded on a 10% SDS-PAGE gel. Western blotting was performed according to standard procedures. Immunoreactive bands were detected using 4 µg/ml

rabbit polyclonal anti-GFP primary antibody (Molecular Probes) in 5% non-fat dry milk/PBS, followed by HRP-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories), and ECL-based chemiluminescence. As loading control, tubulin expression was assessed using 0.1 ug/ml rabbit polyclonal anti-tubulin (Abcam).

Fluorescence in situ hybridisation (FISH)

Femur bone marrow cultures were treated with 100 ng/ml colcemid (GIBCO-BRL, KaryoMAX Colcemid solution) for 15 min to arrest cells at metaphase, and subsequently treated with 75 mM KCl, and fixed with methanol/acetic acid (3:1). FISH was carried out to determine the chromosomal site of transgene integration, basically as previously described [13]. As a specific probe a Biotin-16-dUTP-labeled Villin-rtTA construct was used. Slides were embedded in DABCO/Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI) as counterstain. Initial determination of chromosomal location of the wild-type and the transgene integration signal was established by inverted DAPI band analysis. To confirm the specific localization of the integration site, we performed dual-colour FISH using a Digoxigenin-11-dUTP-labeled probe specific for the telomeric region of mouse chromosome 2 (P1 clone 5309 [14]). Fluorescence signals were visualized with a Leica DM 6000B fluorescence microscope (Leica, Rijswijk, The Netherlands) using Mac Probe Software (version 4.3, Applied Imaging, Newcastle upon tyne, United Kingdom).

Southern blot analysis of transgene copy number

Transgene copy number for the Villin-rtTA2-M2 line was determined from the intensity of fragments obtained after hybridizing HincII digested liver DNA with a 225 bp probe prepared by PCR amplification of a region within intron 1 of the endogenous *Vil1* gene using specific primers: 5'-GAGGGAGGGGTATGTTTTAAG-3'and 5'-GTGGACGAGCCTAGAGGAG-3'. The probe was designed so that no repetitive sequences were present using repeatmasker software (http://www.repeatmasker.org/). This probe recognizes a 6.5 kb endogenous *Vil1* band and a 3.1 kb transgenic band. PhosphorImager analysis was performed using Image-Quant software (Molecular Dynamics, Sunnyvale, CA).

Results and discussion

Transgenic Villin-rtTA2-M2 founder lines were generated with a construct consisting of 12.4 kb murine Villin promoter sequence in front of the reverse tetracycline transactivator rtTA2-M2, previously described as an improved rtTA variant ('Tet-On') with strongly reduced background activity and a 10-fold increased sensitivity for doxycycline-driven induction [10]. Southern blot analysis identified two transgenic founders. Functionality of the transgene was tested by breeding the transgenic animals with tetO-HIST1H2BJ/GFP mice. These mice express histone H2B-green fluorescent protein (H2B-GFP) controlled by a tetracycline-responsive regulatory element [11]. Compound transgenic animals and wild type littermates were given drinking water supplemented with 5% sucrose, either with or without 2 mg/ml doxycycline. Induction of H2B-GFP gene expression was analyzed by fluorescence microscopy and immunohistochemistry. One transgenic line was identified that showed homogeneous and high H2B-GFP expression levels throughout the entire length of the intestinal tract, from the pyloric area to the distal colon and rectum (Fig. 1).

The H2B-GFP fusion protein was expressed in all epithelial cells of the small and large intestine, with slightly reduced expression levels in small intestinal crypts and the lower half of the colonic crypts (Fig. 1e,f,g,j,k). In agreement with previous reports [4-5], a very limited number of crypt-villus structures did not show H2B-GFP induction. No nuclear background fluorescence could be observed in the epithelial cells of all control animals, i.e. induced and un-induced wild type or single transgenic littermates, whereas a low level of auto-fluorescence was present in the cytoplasm, only detectable with long exposure times (Fig. 1a,c,h). Closer examination of the un-induced double-transgenic animals occasionally revealed a small number of positive epithelial nuclei barely detectable above background levels (data not shown). This very low level of leakiness in un-induced animals was not detectable by FACS (see below) or by western analysis for the H2B-GFP protein, whereas a strong induction of transgene expression was observed in the doxycycline-treated animals (Fig. 2).

Notably, transgene expression was also observed in two additional organs outside the intestinal tract, namely kidney and stomach (Fig. 3).

In line with what has been reported for the 9 kb Villin promoter [8], the 12.4 kb variant employed for the generation of the Villin-rtTA2-M2 transgenic model revealed expression in the proximal tubules of the kidney (Fig. 3c). The same was found to be true for a limited number of stomach cells located in the antrum, at or below the isthmus of the glands (Fig. 3d). Their position along the crypt axis

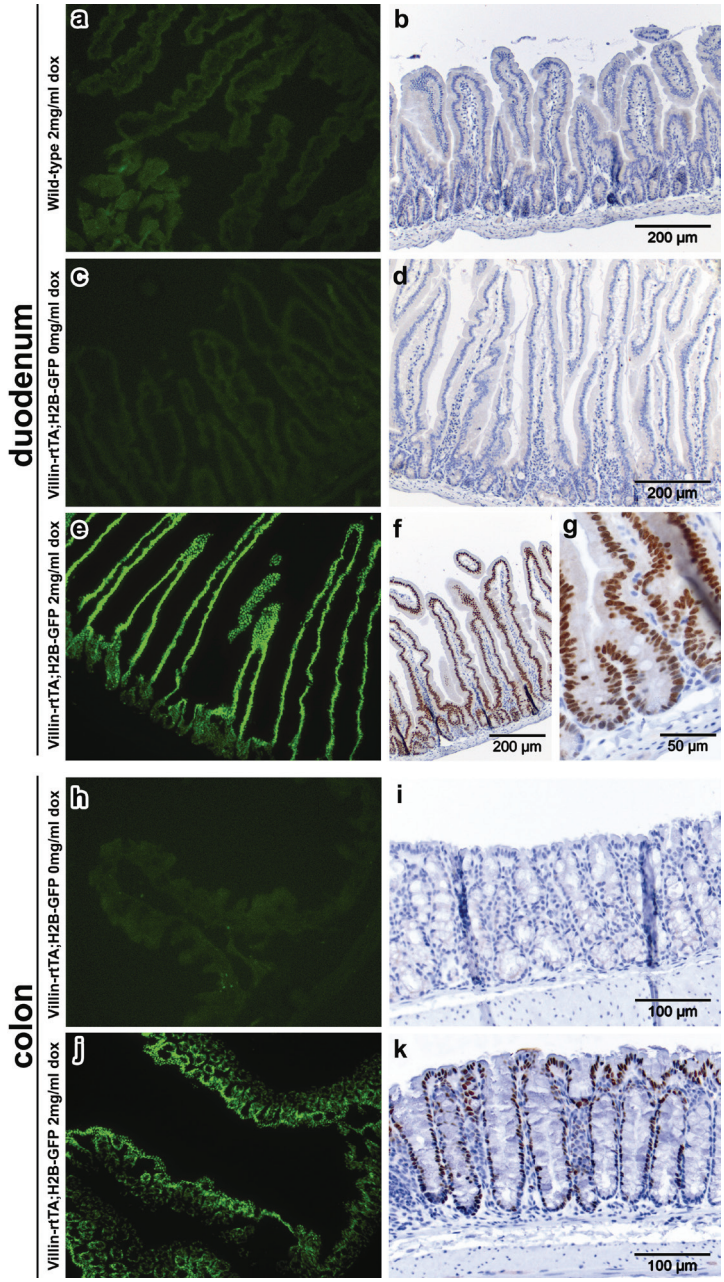

Figure 1. "Tet On" regulated expression of the H2B-GFP fusion protein in the adult mouse intestine.

Immunofluorescence (a, c, e, h, j; original magnification 100x) and immunohistochemistry (b, d, f, g, i, k) analysis for H2B-GFP expression. Intestines from doxycycline-induced wild-type mice (a, b) have a similar GFP-background as un-induced Villin-rtTA;H2B-GFP littermates (c, d). After 7 days of doxycycline treatment, double transgenic mice show strong expression of nuclear H2B-GFP throughout the epithelial cells of the intestine (e,f,g,j,k). Expression reaches higher levels in the small intestinal villi than in the crypts (e, f, g).

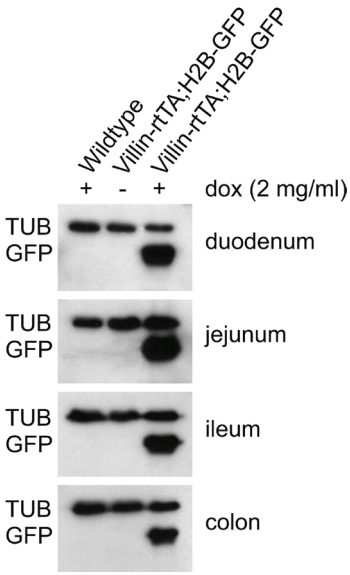

Figure 2. Induction of H2B-GFP expression after doxycycline treatment.

Western Blot analysis revealed no detectable levels of transgene expression in induced wild type animals and un-induced compound transgenic littermates. Doxycycline treatment of double transgenic animals resulted in high expression levels of H2B-GFP fusion protein throughout the intestinal tract. "TUB" is tubulin used as loading control; "GFP" is the doxycycline-induced H2B-GFP protein.

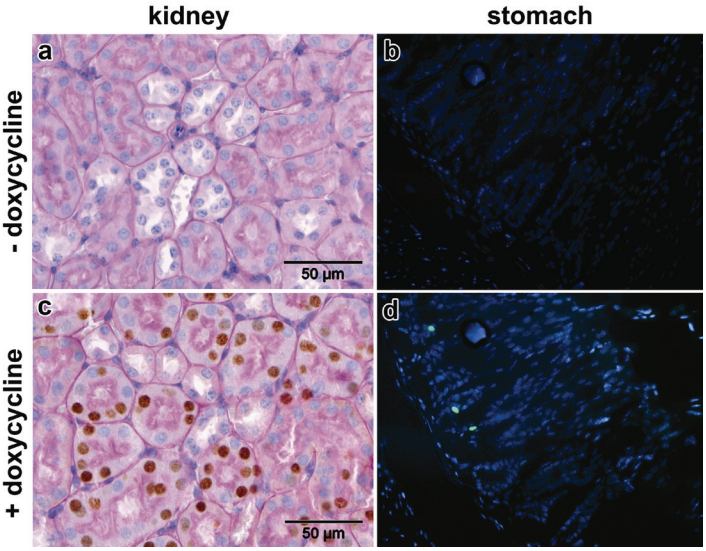

Figure 3. Transgene expression in kidney proximal tubules and stomach progenitor cells.

a,b: Kidney and stomach of un-induced double transgenic animals do not show detectable levels of H2B-GFP. **c:** In the kidney of doxycycline-induced double transgenic animals, nuclear GFP staining (brown) is clearly observed in the proximal tubules as identified by PAS staining of the brush border (pink). **d:** Using fluorescence analysis (original magnification 250x), doxycycline-induced H2B-GFP was detected in a small population of cells of the stomach.

is reminiscent of the gastric progenitor cells with multi-lineage potential recently reported to express a β-galactosidase reporter cassette driven by the same Villin 12.4 kb promoter employed here [15]. Hence, the Villin-rtTA2-M2 model fully recapitulates the expression pattern previously reported for other Villin transgenic models.

The Villin promoter has previously been shown to be expressed already from early phases of mouse embryonic development [8 16]. From 6.5 dpc onwards, the Villin promoter is active in the extra-embryonic visceral endoderm and from 9.5 dpc a clear activity has been shown in the hindgut. In the developing intestine, expression could be observed from 10.5 dpc onwards [8 16]. Furthermore, parts of the urogenital tissue and the developing auditory system show Villin promoter activity during embryonic development [16]. In order to demonstrate the utility of the Villin-rtTA2-M2 model for developmental studies, we bred transgenic animals with TetO-Myc-Sox2 mice [12] and analyzed the resulting embryos upon doxycycline induction. Administration of doxycycline to pregnant females from gestational age 10.5 days onwards in the drinking water resulted in intestine-specific expression of Myc-Sox2 in the embryos (Fig. 4).

This shows that the Villin-rtTA2-M2 mouse model is also able to drive transgene expression in the mouse embryo and can be used to study the effect of induced gene expression during development.

Dual-color FISH (fluorescent *in situ* hybridization) analysis of metaphase chromosome spreads was performed with chromosome-specific probes to determine the chromosomal integration site of the Villin-rtTA2-M2 transgene [13]. As shown in Figure 5a, use of a chromosome 2 specific telomeric probe revealed

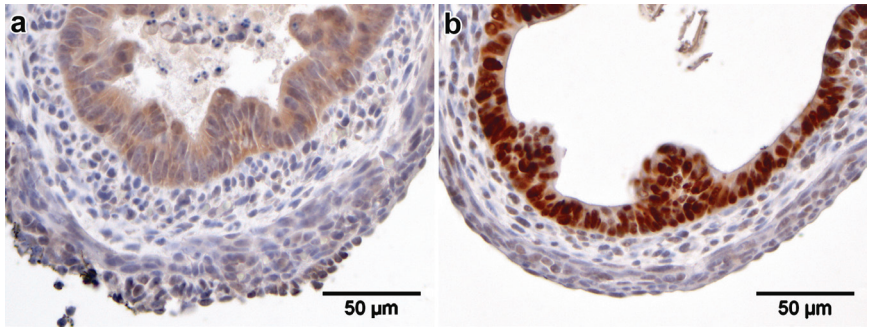

Figure 4. Transgene expression in the developing intestine.

Cross section through embryonal intestine at 18 dpc after 8 days of doxycycline induction. Immunostaining with an antibody against the myc epitope present within the Sox2 transgene reveals strong positive nuclear staining in intestinal epithelial cells (b). In wild type or single transgenic littermates only background staining is observed, mainly in the cytoplasm (a).

that the Villin transgene has a single integration site at chromosomal position 2A5. An additional, though considerably weaker signal was observed for the endogenous *Vil1* gene on chromosome 1. The strong intensity of the transgenic signal indicates that multiple copies are likely to be integrated. Accordingly, Southern analysis revealed that approximately 35 copies of the transgene are present (Fig. 5b).

The inducible Villin-rtTA2-M2 system allows the titration of levels of transgene expression. Therewith it is possible to investigate the impact of gene dosages on differentiation processes in the intestine. To this aim, double transgenic Villin-rtTA2-M2/H2B-GFP mice were administered different doxycycline dosages (between 2 ng/ml and 2 mg/ml) via their drinking water for 7 days before being sacrificed. Frozen sections of these mice were analyzed and revealed that not only the expression level of H2B-GFP fusion protein but also that the relative number of crypt-villus units actively expressing the transgene decreased with lower doxycycline dosages. The overall pattern became patchier, clearly visible at a doxycycline concentration of 4 µg/ml (data not shown). H2B-GFP expression of duodenum, jejunum, ileum and colon of these animals were assessed separately using flow cytometry. To that aim, mouse intestinal specimens were digested and dissociated into single cell suspensions. Moreover, single cell gating was

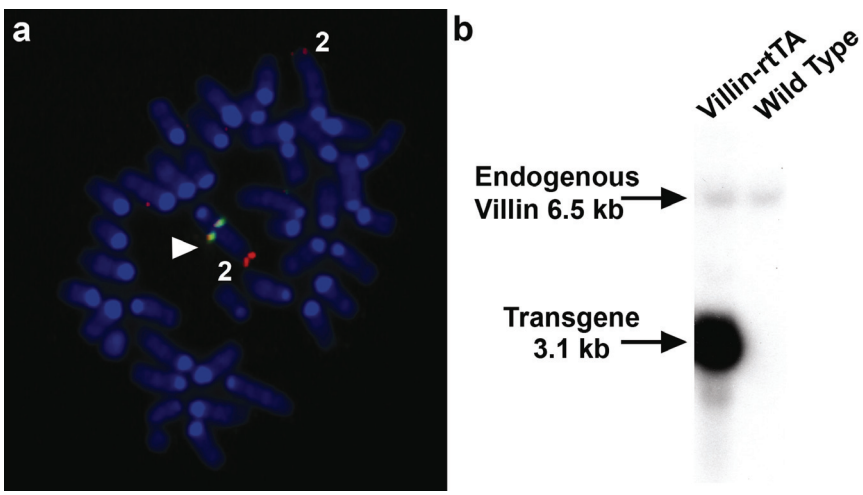

Figure 5. Assessment of transgene integration site and copy number.

a: FISH analysis indicates that the Villin-rtTA2-M2 transgene integration maps on chromosome 2. The probe for the Villin-rtTA2-M2 transgene is labelled in green; the telomeric probe for chromosome 2 is labelled in red. **b:** Southern blot analysis of HincII digested DNA from a Villin-rtTA2-M2 transgenic and wild-type animal. The endogenous Villin band (6.5 kb) was used as a reference in PhosphorImager analysis to estimate the number of transgenic integrations to approximately 35.

applied to exclude doublets. Voltages of the FACS were also adjusted so that the background levels are around 10^2 relative units. Background GFP (FITC) levels of epithelial cells from un-induced Villin-rtTA2-M2/H2B-GFP mice are indistinguishable from those derived from wild type controls (Fig. 6). In contrast, among intestinal cells from doxycycline-induced compound transgenic mice, high GFP levels were observed, compatible with up to an approximately 1000-fold induction. Stepwise reduction of doxycycline dosages led to a similar reduction of the average signal intensity per cell (Fig. 6).

Thus, treating transgenic animals with lower dosages of doxycycline can help to circumvent potential toxicity associated with the over-expression of certain transgenes.

In conclusion, we have generated an inducible mouse model that upon doxycycline administration shows homogeneous transgene expression throughout

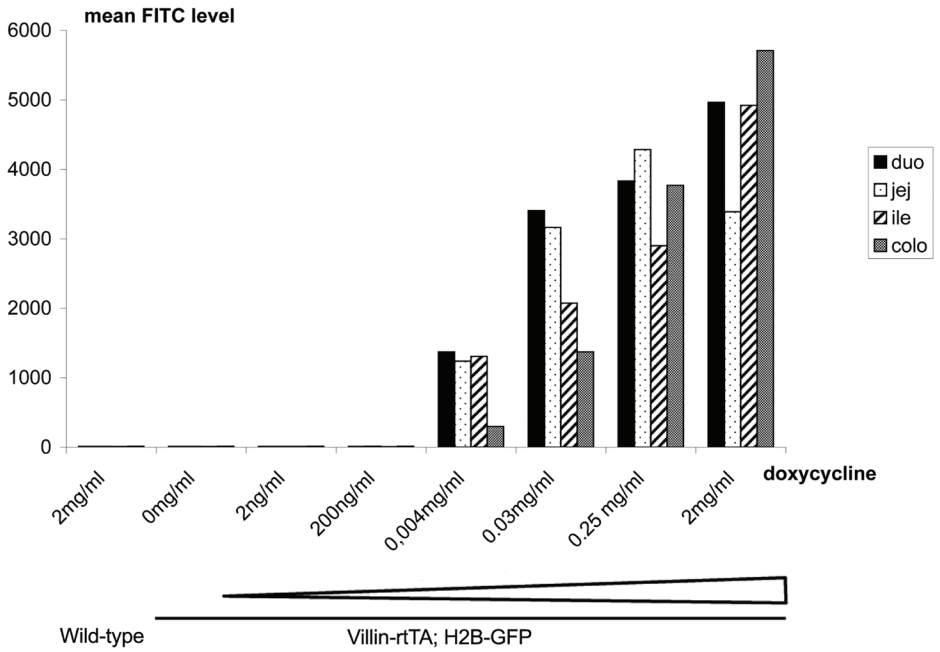

Figure 6. The inducible Villin-rtTA2-M2 system allows titration of levels of transgene expression.

Transgenic Villin-rtTA2-M2/H2B-GFP mice were administered different dosages of doxycycline between 2 ng/ml and 2 mg/ml in the drinking water for 7 days. Intestinal tissue specimens of duodenum, jejunum, ileum and colon were then analyzed by flow cytometry. Background GFP (FITC) levels of intestinal cells from un-induced Villin-rtTA2-M2/H2B-GFP double transgenic mice are indistinguishable from wild type controls. In contrast, we detected doxycycline-dosage dependent levels of GFP with a maximum induction of approximately 1000-fold in intestinal cells from doxycycline-induced double-transgenic mice.

the intestinal tract. Notably, expression levels can be tightly regulated without any significant leakiness. Therewith, this mouse model offers new opportunities for studying the impact of distinct gene dosages on intestinal homeostasis in the adult as well as during development of the mouse intestine, and will be a valuable tool for studying intestinal biology and pathology in vivo. This mouse model can furthermore be used for the identification of putative intestinal stem cells using a chase for several weeks after a pulse of doxycycline-induced H2B-GFP expression. These experiments have been performed and have resulted in the identification of a low number of H2B-GFP-label retaining cells at the bottom of the crypt (Roth et al., manuscript in preparation).

Acknowledgements

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CHAPTER 3

Sox2 redirects the developmental fate of the intestinal epithelium towards a premature gastric phenotype

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Abstract

various factors play an essential role in patterning the digestive tract. During development, Sox2 and Cdx2 are exclusively expressed in the anterior and posterior part of the primitive gut, respectively. However, it is unclear if these transcription factors influence each other in determining specification of the naïve gut endoderm. We therefore investigated whether Sox2 redirects the fate of the prospective intestinal part of the primitive gut. Ectopic expression of Sox2 in the posterior region of the primitive gut caused anteriorization of the gut towards a gastric-like phenotype. Sox2 activated the foregut transcriptional program, in spite of sustained co-expression of endogenous Cdx2. However, binding of Cdx2 to its genomic targets and thus its transcriptional activity was strongly reduced. Recent findings indicate that endodermal Cdx2 is required to initiate the intestinal program and to suppress anterior cell fate. Our findings suggest that reduced Cdx2 expression by itself is not sufficient to cause anteriorization, but that Sox2 expression is also required. Moreover, it indicates that the balance between Sox2 and Cdx2 function is essential for proper specification of the primitive gut and that Sox2 may overrule the initial patterning of the primitive gut, emphasizing the plasticity of the primitive gut.

Introduction

The vertebrate digestive tract develops through a complex mechanism of patterning, expansion and differentiation of the primitive gut, which itself is formed after gastrulation as a result of folding of the definitive endodermal layer [1-3]. One of the earliest steps in patterning of the primitive gut is the regionalization into an anterior and posterior domain, which correlates with the expression of two transcription factors, Sox2 and Cdx2 respectively [4]. During development these domains become more subdivided and by E9.5 the foregut, midgut and hindgut regions can be discriminated. The foregut eventually gives rise to the esophagus, stomach and proximal part of the duodenum; the midgut develops into the caudal part of the duodenum, jejunum, ileum, caecum and proximal part of the colon; and the hindgut forms the caudal part of the colon and rectum. Once regionalization has occurred, the gastro-intestinal tract becomes specified and cells differentiate into the characteristic types found in the various regions.

Sox2 is a member of a highly conserved family of transcription factors founded by the sex-determining gene Sry. Although individual Sox proteins share common DNA binding properties, their specificity is the result of their expression pattern and their ability to associate with different partners [5-6]. Sox proteins are recognized as key players in the regulation of embryonic development and determination of cell fate and we recently described two novel partners for Sox2 [7]. Sox2 plays an important role during early vertebrate embryogenesis and is required later in development in the brain, neural tube, germ cells and in the foregut and its derivatives [8-13].

Expression of Sox2 starts at the 4-8 cell stage of embryonic development and Sox2 gene inactivation results in early peri-implantation lethality [8]. Modulating expression in mice showed the importance of Sox2 in the differentiation of the esophageal epithelium and morphogenesis of the esophagus and trachea [10 12 14]. Several congenital malformations in humans, for instance anophthalmia, microphthalmia and the AEG (anophthalmia -esophageal-genital) syndrome [15], have been associated with heterozygote mutations in SOX2. In addition, Sox2 is one of the key players in the induction of pluripotent stem cells from somatic cells [16], confirming the importance of Sox2 in development and differentiation processes.

Cdx2 is a homeobox transcription factor involved in the establishment of the anterior-posterior polarity of the gut endoderm and is expressed from E8.5 in the posterior part of the gut [17]. Later in development Cdx2 becomes restricted to the intestinal epithelium, with a sharp boundary, marking the transition from stomach to duodenum [4 18-20].

Conditional ablation of Cdx2 from the developing gut endoderm results in severe malformation of the intestinal tract and anteriorization of the posterior gut, evidenced by changes in the expression pattern of various transcriptional regulators [21]. Interestingly, quantitative RNA expression analysis of Cdx2 mutant intestinal samples demonstrated ectopic activation of Sox2 expression in the posterior region. The aberrant expression of Sox2 after Cdx2 ablation coincided with an early anteriorization event of the gut. The apparent reciprocal expression pattern of Sox2 and Cdx2 suggests that these factors are able to influence each other. However, it remains unclear whether Sox2 or Cdx2 has a dominant role in determining the fate of the developing gut endoderm. In addition, aberrant SOX2 expression may underlie the formation of gastric metaplasia associated with intestinal inflammation[22], and the formation of various human congenital anomalies of the gut, such as Meckel's diverticulum in which ectopic gastric tissue can be present in the small intestine [23]. Furthermore, our group and others have observed aberrant expression of SOX2 in human colorectal cancers, suggesting a role for SOX2 in colorectal tumor initiation or progression (*manuscript in preparation*)[24].

Therefore, we ectopically expressed Sox2 in epithelial cells of the prospective intestinal part of the primitive gut to investigate whether Sox2 is able to change the differentiation of the developing gastro-intestinal tract. Our data show that expression of Sox2 in the embryonic mouse intestinal epithelium leads to a fluid-filled, translucent swollen intestine with aberrant villi. The proliferation of epithelial cells in the Sox2 expressing intestines are no longer confined to the crypts, but rather distributed randomly throughout the intestinal epithelium. Moreover, Sox2 induced the expression of esophageal and stomach specific markers, resulting in an anteriorization of the intestinal epithelium. Although the expression level of Cdx2 is unaffected by ectopic Sox2 expression, we further show that induction of Cdx2 target gene expression is strongly reduced, because Sox2 leads to a reduced binding of Cdx2 to its target genes.

These findings provide strong evidence that expression of Sox2 in the developing gut drives the activation of the foregut transcriptional program and leads to conversion from an intestinal into a premature gastric epithelium, despite simultaneous expression of Cdx2. This indicates that the balance between Sox2 and Cdx2 function is essential for proper specification of the primitive gut and that Sox2 can exert a dominant effect over Cdx2 to alter intestinal cell fate by redirecting the intestinal transcriptional program, emphasizing the plasticity of the primitive gut.

Materials and methods

Transgenic animals

The myc-Sox2 and Villin-rtTA transgenic mouse lines were previously described.[10 25] Administration of doxycycline to dams from E8.5 or E14.5 onward in the drinking water (2mg/ml doxycycline, 5% sucrose) induced expression of myc-Sox2 in intestinal epithelium of double transgenic embryos. Each experiment was performed on at least three independent litters containing double transgenic, single transgenic and wild type pups. All double transgenic animals receiving doxycycline expressed nuclear Sox2 in the intestinal epithelium, throughout the entire intestinal tract and showed described phenotype.

Histology

Embryos were obtained by caesarean section at E18.5. The gastro-intestinal tract was isolated and stretched on pieces of filter paper, followed by fixation in 4% PBS-buffered paraformaldehyde (PFA) overnight at 4°C. Intestines were cut into 4-5 pieces and pre-embedded in 5% bacto-agar (BD) in PBS, before being embedded in paraffin according to routine protocols.

Immunohistochemistry (IHC), Periodic Acid Schiff staining (PAS) and electron microscopy were performed as described previously.[37,38] Antibodies are listed in Supplemental Table I. Differentiated and proliferative cells in the epithelium were quantified by counting at least 3 microscopic fields relative to the total number of epithelial cells.

Microarray analysis

The intestinal tracts of three control and two double transgenic embryos, which received doxycycline from E8.5 onwards, were isolated at E18.5 and individually used for transcriptome analysis using Affymetrix Mouse Genome 430 2.0 microarrays as previously described.[10] Subsequent analysis of the data was performed as described in detail in the supplementary data.

Chromatin immunoprecipitation (ChIP)

Small intestines of, 10 double transgenic and 10 control E18.5 embryos were pooled and mechanically disrupted. Subsequently, the material

was crosslinked, sonicated and used to immunoprecipitate either Cdx2 or Sox2 complexes. The precipitated material was de-crosslinked and the co-immunoprecipitated DNA fragments were used as template in qPCR reactions to amplify specific target sequences (primers are listed in supplementary table II). Details of the protocol are provided in the supplementary data.

Quantitative-PCR

RNA isolation, cDNA synthesis and subsequent qPCR analysis was performed as previously described.[39] Data were analyzed using the $2^{-\Delta\Delta Ct}$-method.[40] The gene-specific primers used are listed in Supplemental Table III.

Results

Ectopic Sox2 expression in the caudal part of the developing gut endoderm

Sox2 expression in the developing gut is restricted to the anterior part, which forms the stomach and other foregut derivatives. The posterior part is devoid of Sox2 expression, but expresses the homeobox protein Cdx2. Expression of Sox2 and Cdx2 is thought to be mutually exclusive, but it is unclear whether one has a dominant effect on the determination of the fate of the developing gut endoderm. Therefore we investigated the role of Sox2 in the patterning of the primitive gut and its influence on Cdx2 expression.

Conditional expression of Sox2 in the intestinal epithelium was achieved by crossing mice carrying a myc-tagged Sox2 gene under the control of a tet-inducible promoter with the Villin-rtTA transgenic mouse line, which drives expression of the rtTA gene in all intestinal epithelial cells [10 25]. The rtTA protein drives expression of the Sox2 transgene in the intestinal epithelium in a doxycycline dependent manner. Induction of Sox2 in embryos was achieved by administration of doxycycline to the mother from E8.5 onwards. Double transgenic pups that received doxycycline through their mother were born at Mendelian ratio, but failed to thrive. Therefore, pups were isolated at E18.5 by caesarean section in order to investigate the intestinal abnormalities.

Externally, the littermates showed no significant morphological abnormalities and all embryos were equally well developed. Intestinal tracts isolated from pups carrying only one of the two transgenes, or non-induced myc-Sox2/Villin-rtTA double transgenic animals, appeared indistinguishable from non-transgenic control animals and showed no macroscopic or microscopic

abnormalities. However, isolation of the gastro-intestinal tract of double transgenic embryos that received doxycycline immediately revealed major differences compared to single or non-transgenic animals (Fig. 1A).

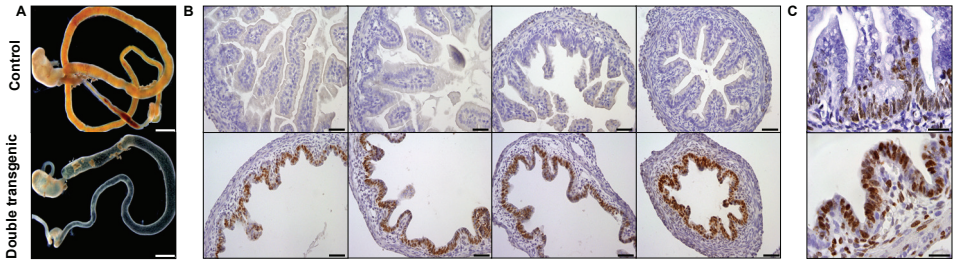

Figure 1: Ectopic expression of Sox2 severely affects the intestinal tract

(A) Macroscopic appearances of the digestive tracts from stomach until rectum isolated at E18.5 of a non-transgenic embryo (top) and double transgenic embryo treated with doxycycline (bottom), showing that Sox2 induction leads to dilated and fluid-filled intestines. (B) IHC using an antibody against Sox2 on cross-sections of the duodenum, jejunum, ileum, and colon reveals specific nuclear staining in the epithelium of double transgenic animals throughout the intestinal tract, whereas Sox2 is absent in the control. (C) IHC using an antibody against Ki67 shows an increased number of cycling cells in the double transgenic embryos, compared to control intestine. Moreover, proliferating cells were randomly distributed throughout the intestinal epithelium of the double transgenic animals, whereas proliferation is restricted to the prospective crypt compartment at the base of villi in control intestines. Scale bars: 2 mm (A), 50 μm (B), 20 μm (C).

In all double transgenic pups ectopic expression of Sox2 in the intestinal tract resulted in markedly abnormal intestines that were translucent and fluid-filled. Immunohistochemical analyses using an antibody against Sox2 (Fig. 1B), or an antibody against the N-terminal myc-epitope present in the transgenic Sox2 protein (data not shown), revealed that nuclear Sox2 was expressed throughout the entire intestinal epithelium of the double transgenic animals.

Assessment of Sox2 expression at specific time points of development, i.e. E.10.5, E12.5, E14.5 and E16.5, revealed that robust Sox2 expression could only be detected in the double transgenic intestinal epithelium from E14.5 onwards (Fig. S1A), coinciding with the phase of cell specification in the embryonic gut. The level of ectopic Sox2 expression was compared with endogenous Sox2 levels in the stomach by qPCR analysis, showing a 6-fold increase on RNA level (Fig. S1B). As Sox2 expression in the E18.5 embryonic stomach is restricted to the basal cells of the forestomach, the qPCR analysis will likely overestimate transgenic Sox2 expression. Therefore, we compared expression levels by IHC at limiting antibody concentrations, showing that on a cell-to-cell basis transgenic

Sox2 is only expressed at slightly higher levels than endogenous stomach Sox2 (Fig S1C).

Ectopic Sox2 expression causes alterations in proliferation and malformation of the developing intestinal tract

Cross-sections of the intestines ectopically expressing the Sox2 transgene showed a two-fold increase in luminal space (Fig. S2). In addition, the small intestines did not form true, morphological villi. Even the epithelial folds that resembled villi were markedly reduced per equivalent surface area (Fig. 1B).

One possible explanation for the dilated intestinal tract could be a disturbed balance between proliferation and apoptosis. Using Ki67 staining to mark cycling cells (Fig. 1C and S3A), we observed that proliferation in the cells of the double transgenic animals was no longer confined to the intervillous, primitive crypt regions as in the controls, but distributed randomly throughout the epithelium of Sox2 expressing embryos. In addition, the total number of Ki67-positive epithelial cells increased from approximately 35-40% in controls to 85-90% in the Sox2 expressing embryos (Fig S3A). Staining for activated caspase 3 revealed no differences in apoptosis (data not shown).

Thus, ectopic expression of Sox2 impairs villus formation and greatly alters the appearance of normal intestinal epithelium. These changes are accompanied by an increase and mislocalization of cycling cells.

Ectopic Sox2 expression in the intestine results in loss of intestinal identity

Next, we analyzed the differentiation potential of the embryonic intestinal epithelium to understand the phenotypic abnormalities observed in the Sox2 expressing intestines. Both mucin2 (Muc2) immunohistochemistry and qPCR showed a dramatic loss in the number of intestinal mucin producing goblet cells in the double transgenic animals (Fig. 2A/C and S3B).

Similar results were obtained for enterocytes using qPCR for *Lactase (Lct)* (Fig. 2C). We also observed reduced numbers of synaptophysin (Syp) positive cells and reduced expression of *Chromogranin A (ChgA),* both markers for enteroendocrine cells though not exclusive for the intestinal epithelium (Fig. 2B/C and S3C). In addition, Periodic Acid Schiff (PAS) staining demonstrated that the intestinal brush border layer was entirely absent in the double transgenic animals, while clearly present in the controls (Fig. 2D). As the brush border

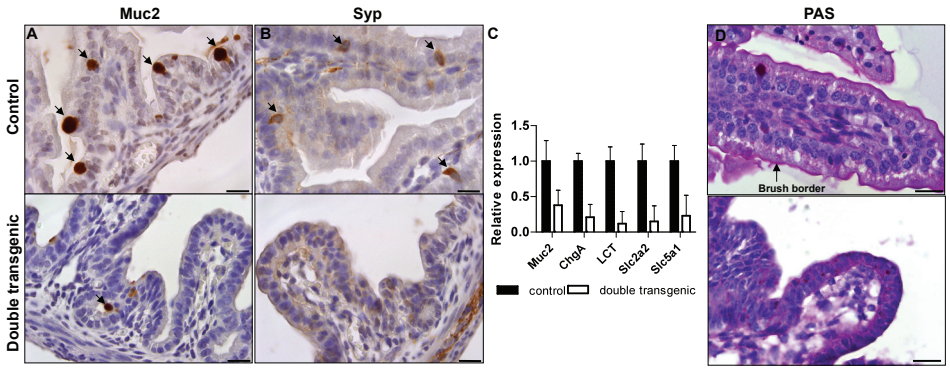

Figure 2: Sox2 affects the normal differentiation of intestinal epithelium

Cross-sections of the duodenum at E18.5 of controls and double transgenic embryos, which received doxycycline. IHC using antibodies against Mucin2 (A) and synaptophysin (B), showed a reduced number of goblet cells and enteroendocrine cells, respectively, in the double transgenic animals. (C) Analysis of the expression level of marker genes of goblet cells (*Muc2*), enteroendocrine cells (*ChgA*) and enterocytes (*Lct*) by qPCR showed a significant reduction of expression in the small intestine of double transgenic embryos at E18.5. Additionally, qPCR was used to determine the expression levels of two genes specific for the intestinal brush border, i.e. members of the solute carrier family (Slc), *Slc2a2* and *Slc5a1*, which are involved in glucose transport. Both are down-regulated in double transgenic embryos. (D) Periodic Acid Schiff staining (PAS) revealed the absence of the intestinal brush border in Sox2 induced animals compared to the controls. Scale bars: 20 μm (A, B), 25 μm (D).

contains several transporters for micronutrients, lack of absorption across this border may explain both the dilatation of the small intestine and the fluid retention we observed. We tested the expression level of several transporters expressed in the intestinal epithelium by qPCR. Analysis of two members of the solute carrier family, *Slc2a2* and *Slc5a1*, which are responsible for active and passive glucose transport, respectively [26], showed that these genes were downregulated in the double transgenic animals (Fig. 2C). In addition, the expression levels of several members of the Aquaporin family, which are of major importance for water transport, were downregulated, as shown by qPCR (Fig. S4).

Overall, ectopic expression of Sox2 in the intestinal epithelium results in loss of intestinal identity which is accompanied by reduced expression of various transporters.

Sox2 expression in the intestine results in cell fate conversion

In order to better understand the underlying changes induced by expression of Sox2 in the intestinal epithelium, we performed microarray analysis using intestines of E18.5 pups of three control and two double transgenic animals, which received doxycycline from E8.5 onwards. Hierarchical clustering

of differentially expressed genes revealed large differences between controls and double transgenic intestines (Fig. 3A).

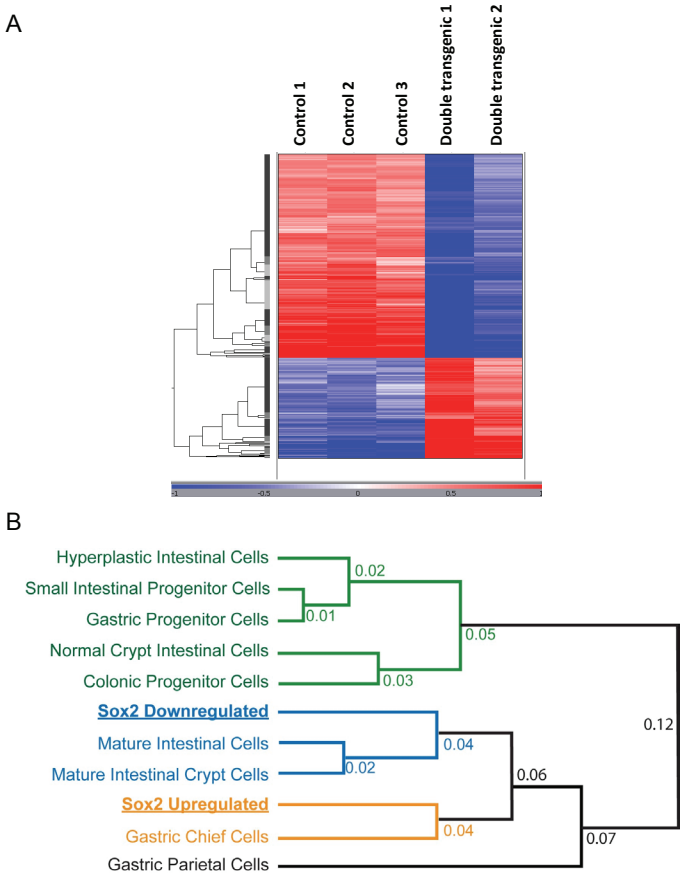

Figure 3: Transcriptome analysis reveals upregulation of gastric cell-specific transcripts by Sox2

(A) OmniViz Treescape showing the hierarchical clustering of Affymetrix probe sets that matched the selection query. Gene expression levels compared with the geometric mean are indicated in red for up-regulated genes and in blue for down-regulated genes. The color intensity correlates with the degree of change. (B) Sox2-upregulated and Sox2-downregulated gene lists were compared based on their associated GO term fractional representations with previously described GO term profiles of several other gastrointestinal cells and tissues [41]. "Mature intestinal crypt cells" refers to an expression profile of genes derived from β-catenin deleted mice [42], which causes crypt cells to mature. "Hyperplastic intestinal cells" refers to PTEN-deficient intestinal cells, which become hyperplastic [43].

Differentially expressed genes in the Sox2 induced animals are involved in development and signal transduction, and are associated with a more immature and proliferative phenotype (Fig S5A-C). In addition, close examination showed

that the downregulated genes in the Sox2 overexpressing animals are involved in intestinal transport and lipid handling, typical processes for the absorptive function of the intestine (Fig S5D).

We next used the pattern of GO term enrichment to integrate our Sox2 profiles with a database of gastric and intestinal cells and tissues of varying degrees of differentiation (Fig. 3B). That analysis showed that genes whose expression was induced by ectopic Sox2 most resembled the gene expression profile of gastric zymogenic chief cells, whereas genes whose expression was decreased by Sox2 most resembled genes expressed in mature intestinal cells. Thus, by GO term enrichment, Sox2 induced genes expressed by gastric cells and reduced expression of normal intestinal genes. Ectopic Sox2 had mixed effects on parietal cell genes, potentially due to overlap in the types of genes parietal cells express and those that intestinal enterocytes express. For example, genes regulating lipid metabolism are characteristic of both enterocytes and parietal cells [27].

We followed up the global patterns of gene expression by confirming expression changes in individual markers with defined rostral-caudal patterns of expression in the gastro-intestinal tract. We analyzed markers identifying the presence of the esophagus and non-glandular gastric basal cells, the acid producing glandular gastric parietal cells, and gastric mucous neck cells. This showed that unlike the controls, the double transgenic animals had focal expression of p63-positive basal cells (Fig. 4A and S3D), characteristic of basal cells of forestomach and esophagus.

Sox2-expressing intestines also showed abundant H+/K+ ATPase4ß positive cells of the gastric parietal lineage (Fig. 4B and S3E) and GSII lectin positive mucous neck cells (Fig. 4C) within the intestinal epithelium. Mist1, a marker for the zymogenic chief cell lineage in adult stomach [28], did not reveal a specific staining pattern (data not shown), which is in accordance with the lack of mature chief cells in the embryonic stomach. In addition, ectopic expression of the stomach specific *p63*, *H+/K+ ATPase4α*, *Mucin5ac (Muc5ac*, marks gastric surface pit cells*)* and *Keratin13 (Ker13*, marks suprabasal cells) genes was detected using qPCR in the double transgenic intestine (Fig. 4D). Ectopic expression of Sox2 in the intestinal epithelium did not clearly influence the underlying mesenchyme, as indicated by the unchanged low expression of the stromally expressed stomach-specific gene *Barx1* (Fig. 4D) [29]. Checking the expression of *Gastrin* revealed a reduced expression (Fig. 4D), which is in line with Gastrin producing cells being present embryonically in the intestine and pancreas, while emerging in the

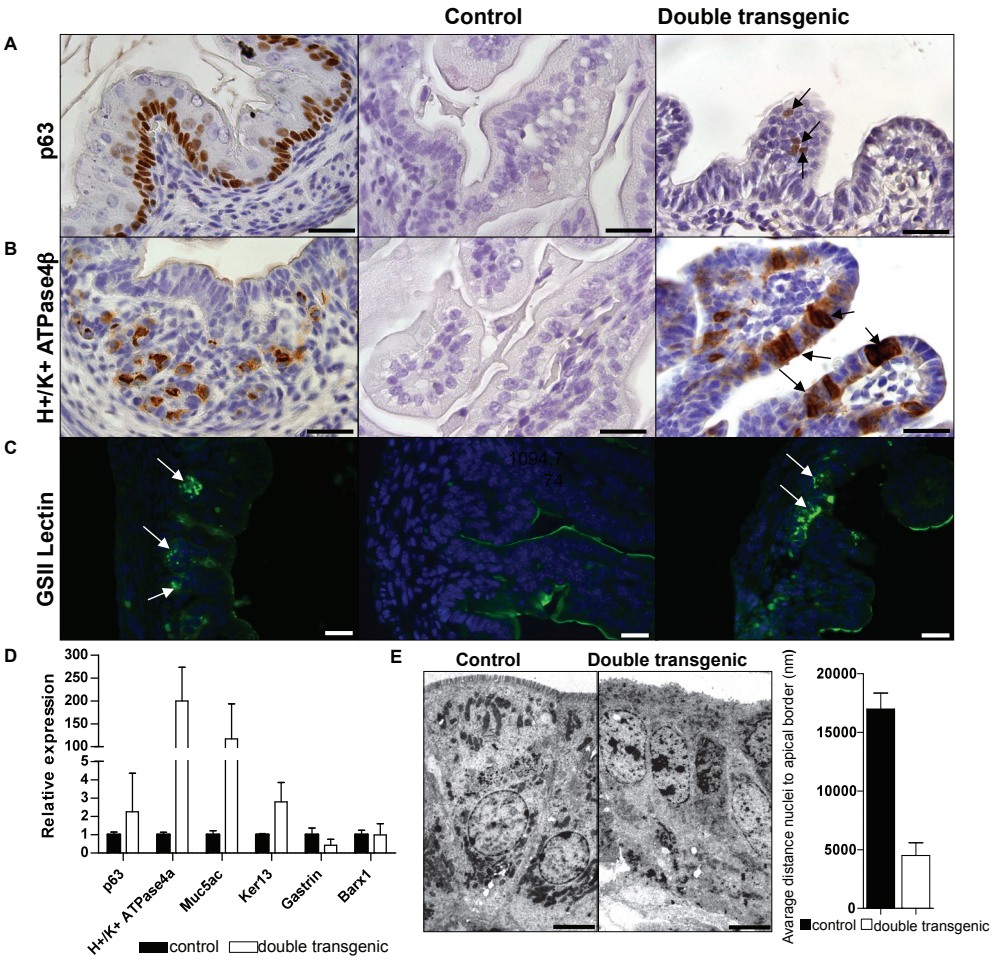

Figure 4: Sox2 induces stomach-like cells in the intestinal environment

Cross-sections of the stomach and duodenum at E18.5 of controls and double transgenic embryos, which received doxycycline. IHC using markers for basal cells (p63) (A) and parietal cells (H+/K+ATPase4ß) (B) showed positive staining in the stomach. Ectopically expressed Sox2 induced the appearance of basal cells and parietal cells in duodenum of double transgenic animals (arrows), whereas control duodenum is devoid of staining. Immunofluorescence for GSII lectin (C), a marker for the mucous neck cells in the stomach, showed positive cells in the double transgenic intestine (arrows), while no expression was found in the control. (D) Analysis of the expression of marker genes for basal cells (*p63*), suprabasal cells (*Ker13*), parietal cells (*H+/K+ATPase4α*) and gastric pit/surface-cell mucin (*Muc5ac*) by qPCR showed an increase in the small intestine of double transgenic animals. No significant change in expression level of the stomach-specific mesenchymal marker *Barx1* was detected. Expression of the Gastrin hormone was reduced in double transgenic animals. (E) Nuclei of normal intestinal epithelium are oriented towards the basal membrane, whereas the nuclei of the double transgenic intestinal epithelial cells are positioned more apically, shown by EM. Scale bars: 20 μm (A-C), 6 μm (E).

stomach only at birth [30]. Next, electron microscopy confirmed the lack of microvilli on the double transgenic intestinal epithelial cells (Fig 4E and S6). Moreover, it showed that ectopic Sox2 expression induced a more apical localisation of the nuclei, in contrast to the basal orientation in controls (Fig 4E).

Collectively, our data show that Sox2 expression in the intestinal epithelium causes anteriorization of the intestine with intestinal epithelial cells adopting the morphological and gene expression profile of immature gastric cells.

Ectopic Sox2 expression in the intestine alters the functionality of Cdx2, but not its expression level

Considering all the changes caused by Sox2 expression, we examined whether Cdx2 expression was also decreased, thereby fitting the suggestion that Sox2 and Cdx2 are expressed in a mutually exclusive manner. qPCR showed that expression of the *Cdx2* mRNA was not altered (data not shown). Surprisingly, IHC for Sox2 (Fig. 5A) and Cdx2 (Fig. 5B) revealed that both transcription factors were expressed throughout the entire intestinal epithelium of the Sox2 induced animals, whereas only Cdx2 was expressed in the control. Moreover, nuclear co-

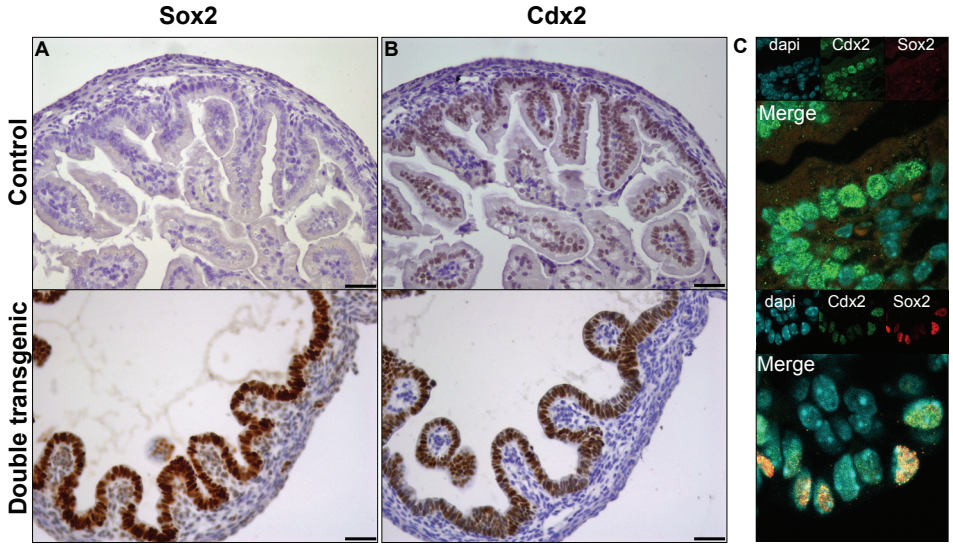

Figure 5: Sox2 and Cdx2 are co-expressed

IHC on sequential sections of E18.5 duodenum showed Sox2 expression only in double transgenic intestines (A), and Cdx2 expression throughout the epithelium in both control and double transgenic embryos (B). Confocal microscopy (C) of control and double transgenic animals showed that Cdx2 and Sox2 are co-expressed in the same intestinal epithelial cells in Sox2 expression embryos. Individual images of DAPI (blue), Cdx2 (green) and Sox2 (red) stainings are shown as inserts. Scale bars: 50 μm

localization of Cdx2 and Sox2 was shown by immunofluorescence using confocal microscopy (Fig. 5C).

Thus, although Sox2 induced cellular changes leading to the occurrence of anterior gastro-intestinal cell types normally devoid of Cdx2, Cdx2 remained co-expressed in the Sox2 double transgenic intestines. However, the microarray analysis revealed several known Cdx2 target genes to be clearly downregulated in these Cdx2/Sox2 positive cells, including the well-established target gene *Cdh17* [31]. These reduced expression levels were confirmed by qPCR for the target genes, *Hnf4α*, *Heph*, *Cdx1* and *Mep1α* (Fig. 6A) [21 32]. Using IHC we confirmed that the number of Hnf4α positive cells was strongly decreased in the double transgenic animals (Fig. 6B).

These results suggest that ectopic expression of Sox2 does not directly affect the expression of Cdx2 itself, but interferes with the subsequent activation of Cdx2 target genes. Therefore, we analyzed the binding of Cdx2 to the promoter regions of two known target genes by chromatin immunoprecipitation (ChIP) using embryonic intestines from control and double transgenic animals (Fig. 6C and S7). We show that ectopic expression of Sox2 in the intestine leads to a strong decrease in binding of Cdx2 to its targets Cdh17 and Hnf4α, whereas Sox2 does not bind to these sites. Specificity of the Sox2 ChIP was confirmed by the detection of increased binding of Sox2 to its target Sox21 in the double transgenic embryos (Fig. S7). Thus, ectopic Sox2 expression in the intestinal tract does not affect the expression of Cdx2, but inhibits its function as a transcriptional regulator by interfering with the binding of Cdx2 to its gene target sites, which in turn prevents the activation of the intestinal transcriptome.

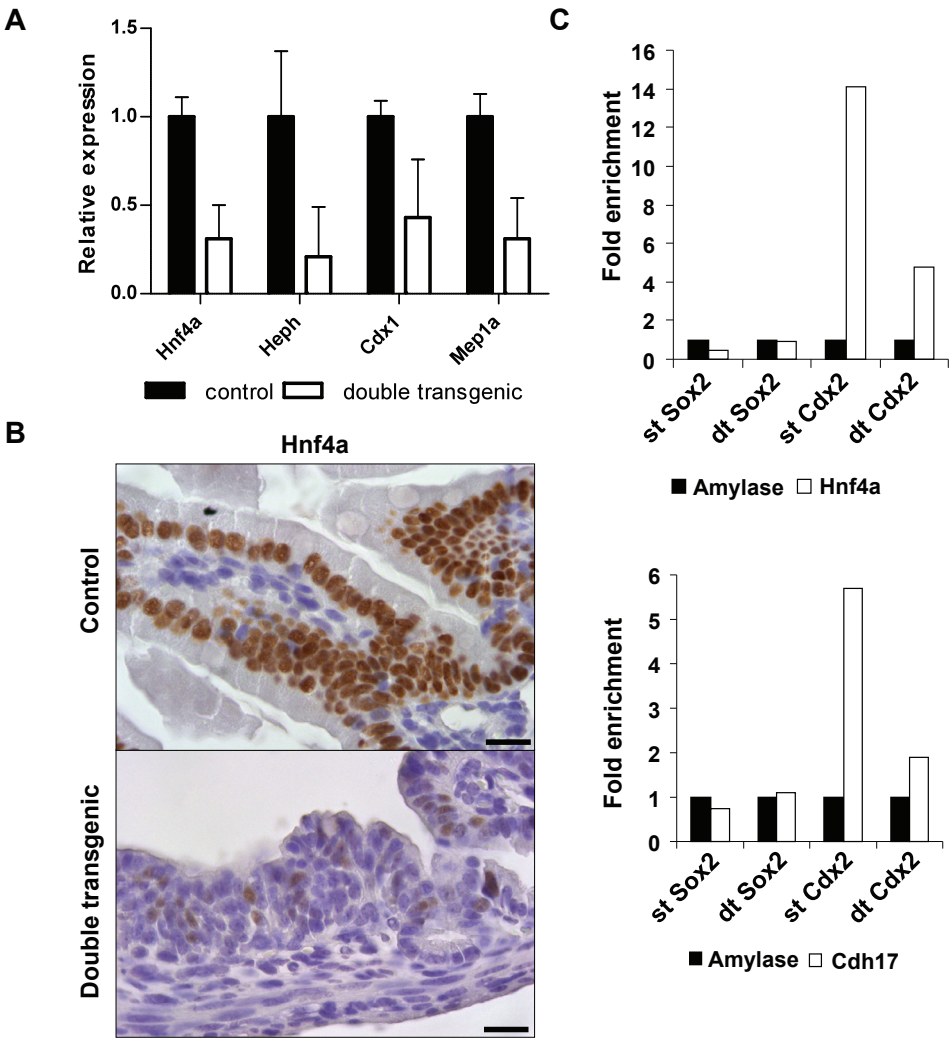

Figure 6: Sox2 affects the expression of Cdx2 target genes by inhibiting Cdx2 binding to target genes

(A) The expression levels of the Cdx2 target genes *Hnf4α, Heph, Cdx1* and *Mep1α* are significantly down-regulated in the small intestine of double transgenic animals at E18.5. (B) IHC with an antibody against Hnf4α on cross-sections of duodenum at E18.5 of control and Sox2 overexpressing animals showed a dramatic loss of staining in the double transgenic animals. (C) ChIP assay showed a dramatic loss of binding of Cdx2 to *Cdh17* and *Hnf4a* in the double transgenic animals. Amylase served as a negative control. Scale bars: 20 μm (B)

Discussion

During development of the gastro-intestinal tract, Sox2 is expressed in the anterior part of the primitive gut, while the homeobox gene Cdx2 is expressed in the posterior part [4]. Various studies have suggested that the expression of Sox2 and Cdx2 is mutually exclusive, but it remains elusive whether one of the two is dominant over the other. Therefore, we ectopically expressed Sox2 in the prospective intestinal part of the primitive gut to investigate whether Sox2 has a dominant role over Cdx2 in the specification of the developing endoderm. We show that ectopic expression of Sox2 in the posterior region of the primitive gut caused aberrant activation of the foregut transcriptional program, leading to anteriorization of the gut with features resembling those of an immature embryonic stomach. Sox2 induction did not alter Cdx2 expression, but interfered with its function as a transcriptional regulator, providing evidence that Sox2 can exert a dominant effect over Cdx2 on cell fate.

Ectopic expression of Sox2 in the embryonic intestinal tract leads to a translucent and dilated intestine, which had a reduced number of villus-like outfoldings per surface area. These villi were poorly developed and showed increased numbers of cycling cells that were no longer confined to the intervillous regions, but were localized randomly throughout the intestinal epithelium. The differentiation towards goblet cells, enteroendocrine cells and enterocytes was dramatically affected. Instead, the Sox2 expressing epithelium differentiated into gastric-like cell types, such as parietal cells and basal cells, demonstrated by IHC, qPCR and GO term analysis. GO term comparative expression profile analysis also revealed that the genes upregulated by Sox2 were closer to cells of mature gastric zymogenic chief cell differentiation than to any of a variety of normal intestinal tissue gene expression profiles. These dramatic changes were accomplished in the short time-frame that ectopic Sox2 becomes expressed, i.e. E14.5-E18.5, demonstrating the dominant influence of Sox2 in the developing gut. Apparently, Sox2 is able to convert the cell fate of already committed embryonic intestinal cells into stomach-like cells. The prominent effect of Sox2 in determining cell fate was also highlighted by our previous study, in which we have shown that ectopic expression of Sox2 in the lung also results in dramatic changes in differentiation [10].

The fluid filled, swollen appearance of intestines of the Sox2 induced animals is most likely caused by the dramatic decreased number of enterocytes combined with the disruption of the intestinal brush border. As a consequence of the decreased absorptive area, fluid can not be absorbed by the intestine, resulting

in fluid retention. Our studies with ectopic Sox2 expression in intestines of mouse embryos complement a recent study reporting the effect of Cdx2 ablation in mouse embryos [21]. Cdx2 ablation severely affects the normal intestinal development, since mutant embryos fail to form the colon and rectum properly. Furthermore, the duodenum of the Cdx2 mutant animals was distended and became translucent. Also the disturbed proliferative pattern strongly resembles our observations. In our study the total number of epithelial cells was not significantly altered (data not shown), which is in accordance with the equal number of cells in the mitotic phase of the cell cycle as revealed by phospho-Histone H3 staining (20-25% for both genotypes, data not shown). These results indicate that although Sox2 expression strongly affects the number and position of cycling cells, it most likely also leads to an increased duration of the cell cycle, effectively resulting in an equal production of epithelial cells.

Interestingly, Gao *et al.* also showed ectopic activation of Sox2 in the Cdx2 mutant intestine, which implied that Sox2 may have been responsible for inducing the phenotypes that were observed [21]. Our results strongly support this concept, as we demonstrated that Sox2 induces the differentiation toward a premature stomach phenotype, in spite of sustained Cdx2 expression. We conclude that loss of Cdx2 by itself is not sufficient to drive the activation of the foregut transcriptional program, but requires expression of Sox2 to dominantly induce anteriorization.

Inactivation of Cdx2 in adult intestines induced the expression of gastric markers, but did not lead to major morphological changes [29]. Although these authors showed that the gut stem cells in the crypts showed some plasticity to express gastric genes, the plasticity of the cells in the developing gut is much greater since we and Gao *et al* showed complete induction of gastric genes and cell types [21]. This indicates that the adult intestinal epithelial cells have partly lost their potential to change their fate upon Cdx2 ablation.

More recently, Cdx2 was shown to also regulate epithelial cell polarity [33]. Depletion of Cdx2 in the developing mouse embryo from E15.5 onwards, i.e. equivalent to the time point that we induce Sox2, led to the formation of irregular packed epithelial cells containing large subapical vacuoles and a disorganized pattern of several apical and basolateral markers. In our case, staining for ZO-1 (apical), E-Cadherin (basolateral), and Laminin-1 (basement membrane) revealed that epithelial polarity was not clearly affected (data not shown). We also did not observe the vacuoles and irregular packing of epithelial cells. These differences in phenotype are possibly explained by the incomplete functional downregulation of Cdx2 induced by Sox2 expression.

Ectopic expression of Sox2 did not affect the expression of the intestinal specific transcription factor Cdx2. This is in apparent contrast with work published by Benahmed and co-workers, who investigated the regulation of *Cdx2* expression [34]. Using cellular approaches with a 13 kb genomic fragment covering part of the *Cdx2* locus, they showed that Sox2 had a profound inhibitory effect on *Cdx2* transcriptional activity. As our study shows that the endogenous locus is not affected by Sox2, this either implies that the proposed Sox2 binding site in the 13 kb fragment is not easily accessible in the endogenous setting or that for proper *Cdx2* regulation additional domains are required, e.g. the 3' located enhancer. Previous studies have provided limited data to suggest that Cdx2 regulates Sox2 expression, but no direct binding of Cdx2 to the Sox2 promoter region was shown [4 21 35]. Nishiyama et al recently described a genome wide analysis of CDX2 target sites using ChIP technology and clearly demonstrated that CDX2 did not bind to the SOX2 locus [36]. Verzi et al analyzed the Caco-2 intestinal carcinoma cell line for putative CDX2 binding sites and identified a ChIP peak in an intergenic region in proximity to the SOX2 locus [32]. However, it remains unclear whether the binding site had functional relevance to the SOX2 gene.

Although Cdx2 was not affected at the transcriptional level, its function is severely affected as deduced from the strong downregulation of several Cdx2 target genes. We show that Sox2 interferes with the binding of Cdx2 to its genomic target sites, thereby preventing its transcriptional activity. At present, the precise molecular mechanism to explain the inhibitory effect on Cdx2 DNA binding is unclear. Possibly Sox2 competes with co-factors that are needed by Cdx2 for its binding activity [32], or Sox2 may induce differentiation into cell types that lack the expression of factors required by Cdx2 to bind efficiently to its target sequences.

In conclusion, we show that ectopic expression of Sox2 leads to impairment of villus formation and strongly alters the appearance of the normal intestinal epithelium. We show anteriorization of the primitive gut, demonstrated by cell fate conversion from intestinal epithelial into immature stomach-like cells. This demonstrates the dominant influence of Sox2 in the developing gut, since Sox2 is able to convert the cell fate of already committed intestinal cells into stomach-like cells. Sox2 induction does not alter Cdx2 expression, but inhibits its function as transcriptional activator by interfering with its DNA binding capacity. This supports the idea that a balance between Sox2 and Cdx2 may dictate proper patterning of the foregut endoderm and that Sox2 may exert a dominant effect on intestinal cell fate by altering the differentiation program, emphasizing the plasticity of the primitive gut.

Our work improves the understanding of the mechanisms underlying the formation of the gastrointestinal tract and provides insight into the possible origin of various human congenital anomalies of the gut, such as Meckel's diverticulum, in which ectopic gastric tissue can be present in the small intestine. In addition, it may clarify the functional consequences of the ectopic SOX2 expression that we and others observed in human colorectal cancer (*manuscript in preparation*)[24].

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Supplementary Materials and Methods

Microarray analysis

The intestinal tract of three control and three double transgenic embryos, which received doxycycline from E8.5 onwards, were isolated at E18.5 and individually used for total RNA isolation with Trizol reagent (Invitrogen life technologies, Carlsbad, CA, USA). RNA was purified using RNeasy MinElute Cleanup kit (Qiagen, Valencia, CA, USA) and cDNA was synthesized using the GeneChip Expression 3'-Amplification Reagents One-Cycle cDNA Synthesis kit (Affymetrix, Santa Clara, CA, USA). Biotin-labelled cRNA synthesis, purification and fragmentation were performed according to standard conditions. Fragmented biotinylated cRNA was subsequently hybridised onto Affymetrix Mouse Genome 430 2.0 microarray chips.

To examine the quality of the various arrays, several R packages (including affyQCreport) were run starting from the CEL files. All created plots, including the percentage of present calls, noise, background, and ratio of GAPDH 39 to 59 (1.4) indicated a high quality of all samples and an overall comparability, except for one sample, which was excluded from further analysis. Raw intensity values of all samples were normalized by RMA normalization (Robust Multichip Analysis), background correction and quantile normalization using Partek version 6.4 (Partek Inc., St. Louis, MO).

The normalized datafile was transposed and imported into OmniViz version 6.0.1 (BioWisdom Ltd., Cambridge, UK) for further analysis. For each probe set, the geometric mean of the hybridization intensity of all samples was calculated. The level of expression of each probe set was determined relative to this geometric mean and 2log-transformed. The geometric mean of the hybridization signal of all samples was used to ascribe equal weight to gene expression levels with similar relative distances to the geometric mean. Differentially expressed genes were identified using statistical analysis of microarrays (SAM). Cutoff values for significantly expressed genes were the number of falsely called less than 1 (FDR of 0.0006 or less) and a fold change of 2. Functional annotation of the statistical analysis of microarrays results was done using Ingenuity Pathway Analysis (Ingenuity, Mountain View, CA) and DAVID (http://david.abcc.ncifcrf.gov). DAVID calculates significant overrepresentation of gene ontology (GO)-classified biological processes. The results are shown for biological processes, which are significantly (P <0.05) enriched after multiple testing.

To analyze higher-order differentiation patterns defined by the 449 genes induced and 906 downregulated significantly by Sox2, each gene list was re-expressed in terms of the fractional representation of GO terms associated with its member genes using the GOurmet software suite.[1] The distribution of GO term fractional representations were then used as a metric to classify the Sox2 expression profiles to a database of expression profiles of mature, proliferating, and hyperplastic gastrointestinal tissues, as described before.[2]

Chromatin immunoprecipitation (ChIP)

The small intestines of, respectively, 10 double transgenic and 10 control E18.5 embryos were pooled and mechanically disrupted, after incubating for 20 minutes in ColagenaseII at 37°C. Next we filtered the cell suspension using a 70μm cell strainer. Formaldehyde was added to a final concentration of 1% for 10 min on RT to crosslink the samples and this process was stopped by adding 0.15 M glycine. Cells were washed with PBS and lysed for 10 minutes on ice in cell lysis buffer (10mM Tris pH 8.0, 10 mM Nacl, 0.2% NP-40 and complete protease inhibitors). After centrifugation the nuclei were resuspended in nuclei lysis buffer (50mM Tris pH 8.0, 10mM EDTA, 1% SDS and complete protease inhibitors) and the chromosomal DNA was fragmented by sonication (20 cycles of 15 seconds, 45 seconds in between cycles) to yield DNA fragments with an average size of 500bp.

Equal amounts of sample were diluted 1:10 with ChIP dilution Buffer (0.01% SDS, 1.1% Tx-100, 1.2 mM EDTA, 16.7mM Tris-HCL pH 8.1 and 167 mM Nacl) and from each sample 50 μl was removed to serve as input control. The samples were pre-cleared with 80μl Prot A/G agarose beads for 1 hour. Next, these samples were incubated O/N with pre-formed complexes of 10 μg antibody specific for Sox2 or Cdx2, or control IgG (goat and mouse) with 150 μl Prot A/G agarose beads.

Beads were washed with Low salt immune complex buffer (0.1% SDS, 1% Tx-100, 2mM EDTA, 20mM Tris-HCL pH 8.0 and 50 mM Nacl), High salt immune complex buffer (0.1% SDS, 1% Tx-100, 2mM EDTA, 20mM Tris-HCL pH 8.0 and 150 mM Nacl), LiCl immune complex buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA and 10mM Tris-HCL pH 8.0) and twice with TE (10mM Tris-HCL pH 8.0, 1mM EDTA pH 8.0). The DNA was eluted by adding twice 250 μl elution buffer (1% SDS and 0.1m NaHCO3). Next the samples and the input were incubated at 65°C O/N using 20 μl 5M NaCl to de-crosslink the DNA and proteins. The eluted material was phenol-extracted and ethanol-precipitated. The

DNA was resuspended in 26 μl of water and qPCRs were performed to analyze the enrichment of Cdx2 for binding to *Hnf4a* and *Cdh17*, and Sox2 for binding to *Sox21*, using Amylase as control.

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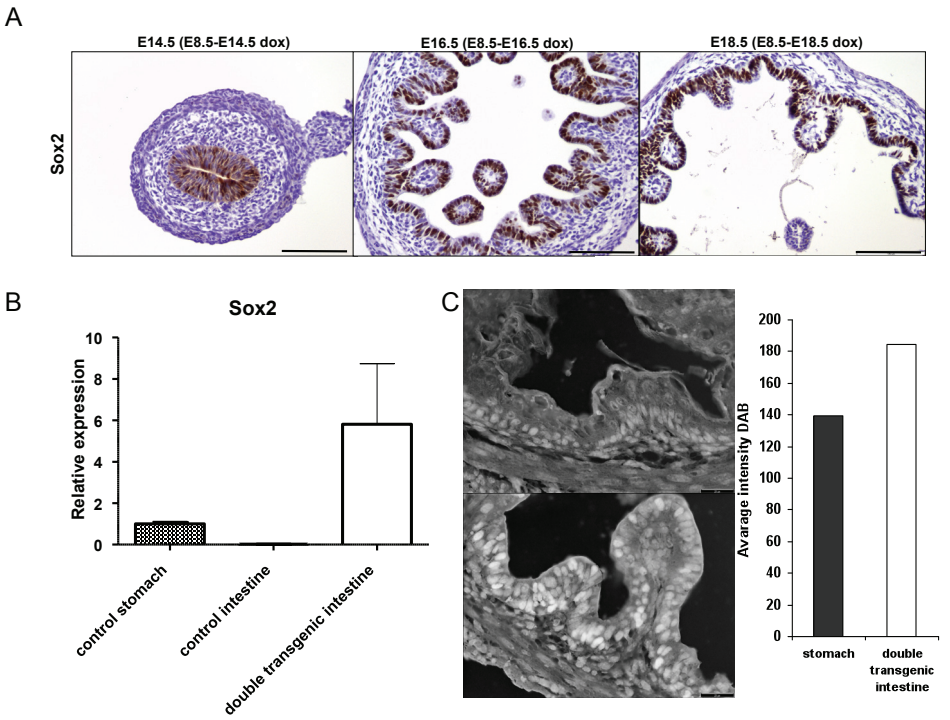


Figure S1: Robust expression of induced Sox2 at E16.5

IHC using an antibody against Sox2 on intestinal cross-sections at E14.5, E16.5 and E18.5 of double transgenic animals shows that robust Sox2 expression is observed from E14.5 onwards (A). In order to evaluate the expression level of induced Sox2, we compared Sox2 mRNA expression levels of Dox-induced double transgenic embryos with the stomach and intestine of control animals. We show an approximate 6-fold increase of expression of induced Sox2 compared to the endogenous level in the stomach. As expected Sox2 expression in the control intestine was negligible (B). Additionally, we analysed the unaffected stomach and the intestinal tract of the induced double transgenic animals with immunohistochemistry using limiting amounts of Sox2 antibody. Because the titration of the antibody showed diminishing staining, we could perfectly compare the intensity of the staining in de stomach and intestine and quantify the expression level of the ectopically expressed Sox2 in the intestinal epithelium using ImageJ. This revealed an average increase in intensity of 32% in the intestine, compared to endogenous Sox2 expression in the stomach (C). Scale bars: 50 μm.

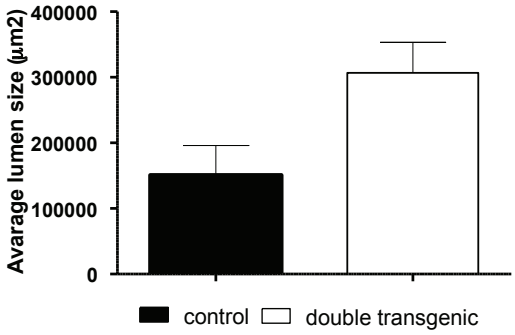


Figure S2: Ectopic Sox2 induces significant enlargement of the intestine

The average intestinal lumen size was measured in at least three controls and double transgenic animals. The double transgenic animals show a two-fold increase in lumen size.

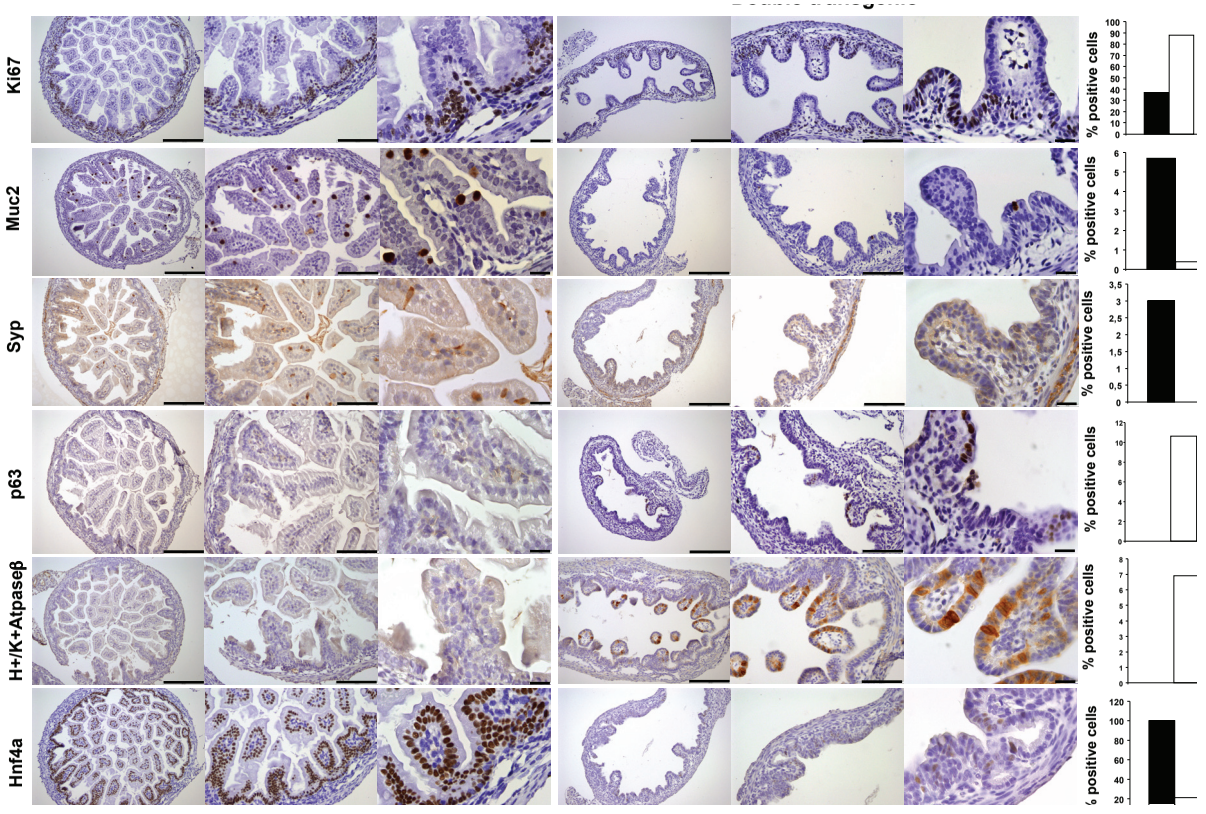


Figure S3: Overview of IHC on control and double transgenic intestines

Overview of IHC results with antibodies against Ki67 (A), Muc2 (B), Syp2 (C), p63 (D), H+/K+ Atpase4β (E) and Hnf4a (F) on cross-sections of duodenum of control (left panels) and double transgenic (center panels) animals at E18.5. Each panel consists of three images, representing 100, 200 and 400 times magnification (scale bars: 200, 100 and 20 μm, respectively). Quantification is represented as the number of positive cells per total number of epithelial cells in at least 3 microscopic fields (right panels). Black bars represent the control and white bars the double transgenic animals.

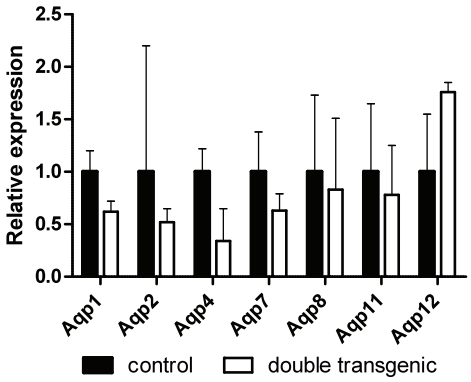


Figure S4: Quantification of the expression of aquaporin family members

Analysis of the expression of Aquaporin family members Aqp1, Aqp2, Aqp4, Aqp7, Aqp8, Aqp11 showed a downregulation in the double transgenic animals, whereas Aqp 12 was upregulated compared to the controls.

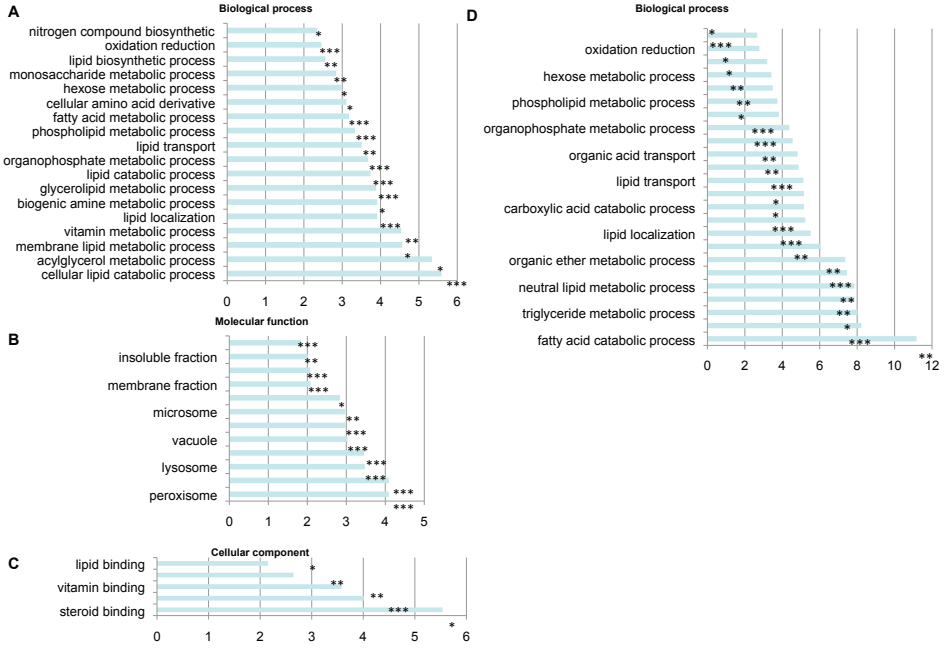


Figure S5: GO enrichment analysis of control and double transgenic intestines

Enriched GO terms within the set of 1,354-regulated probesets (both up- and downregulated genes) for GO biological processes (A), GO molecular function (B) and GO cellular component (C). Enriched categories are those identified as significantly enriched (P<0.05) after multiple testing. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. (C) Enriched GO terms within the set of 906-downregulated probesets for GO biological processes (D). Enriched categories are those identified as significantly enriched (P<0.05) after multiple testing. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

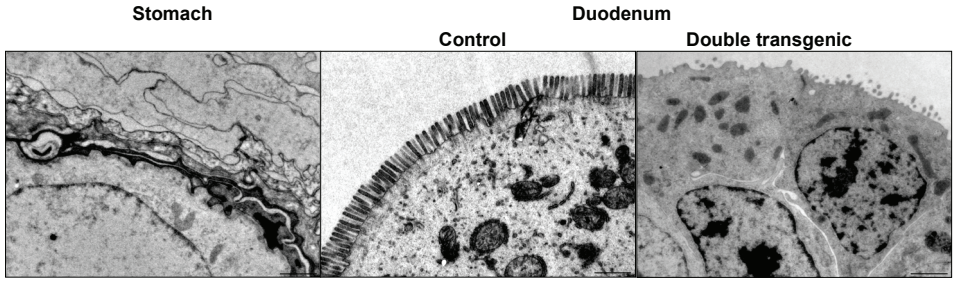
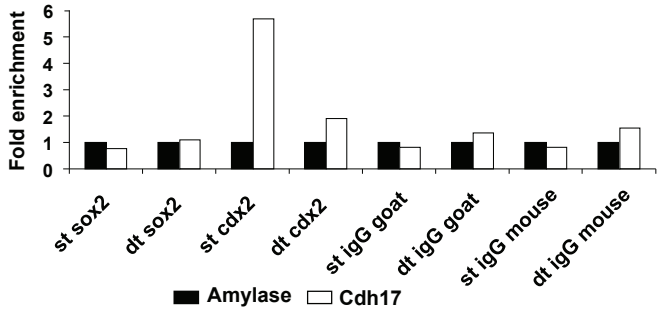
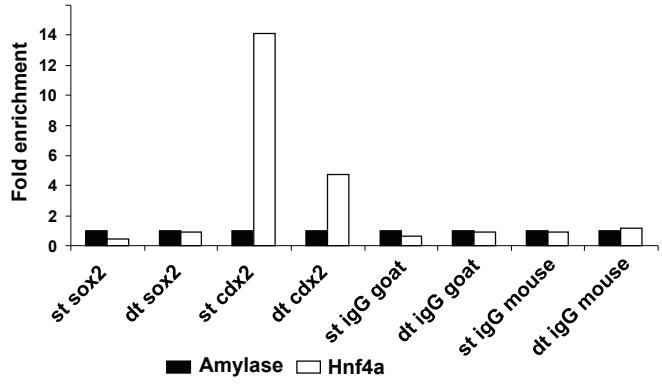


Figure S6: Sox2 affects the intestinal brush border

Close up electron microscopy images of the intestinal brush border of control and double transgenic E18.5 pups, and of the embryonic stomach, which does not have a brush border. Scale bars: 1.4μm





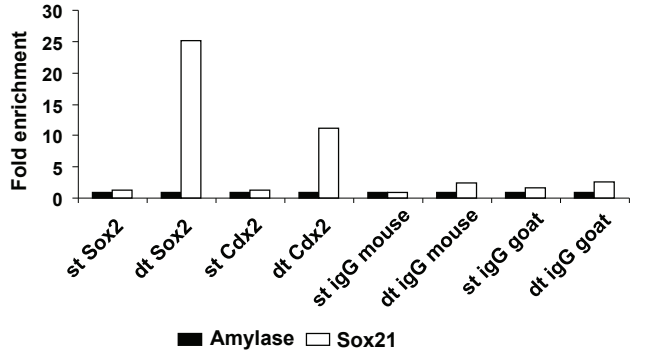


Figure S7:Sox2 interferes with Cdx2 binding to target genes

ChiP assay for Cdx2 shows loss of binding to Cdh17 (top panel) and Hnf4a (middle panel) in the double transgenic animals, compared to the control. Sox2 does not bind to Cdh17 or Hnf4a, but does bind to its downstream target Sox21 (bottom). Mouse IgG or goat IgG serve as negative antibody controls Cdx2 and Sox2, respectively. Amylase served as negative control for the qPCR.

Antigen	Clone	Target	Source	Concentration	Dilution	Amplification-Kit
Myc-epitope	9E10	Myc-epitope	Roche	5mg/ml	1:800	None
Sox2	401196	Sox2	Immune systems	1mg/ml	1:500	ABC-kit *
Cdx2	Cdx2-88	Cdx2	Biogenex	10-15mg/ml	1:20	ABC-kit*
Phospho-histone H3	32219	mitosis	Upstate, cell sinaling solutions	1mg/ml	1:800	ABC-kit*
Ki67	TEC-3	proliferation	Dakocytomation		1:50	ABC-kit*
Cleaved Caspase 3	5A1E	apoptosis	Cell Signaling	100µg/ml	1:100	ABC-kit*
Mucin 2	H-300 Sc-15334	goblet cells	Santa Cruz Biotechnology, INC	1mg/ml	1:400	ABC-kit*
Synaptophysin	A0010	entero-endocrine cells	DakoCytomation	300µg/ml	1:250	Envision**
p63	4A4 Sc-8431	basal cells	Santa Cruz Biotechnology, INC	200µg/ml	1:200	ABC-kit*
H/K+ ATPase 4B	2G11	parietal cells	thermo scientific	10µg/ml	1:2000	ABC-kit*
HNF4	K9218	HNF4	Abcam	1mg/ml	1:100	ABC-kit*
GSII Lectin	L21415	mucous neck cells	Molecular probes	1mg/ml	1:2000	None
Mist1		zymogenic chief cells	Gift of Jason Mills		1:500	None
ZO-1	61-7300	Apical membranes	Invitrogen	250µg /ml	1:50	None
Laminin	L9393	Basement membranes	Sigma		1:400	None
E-cadherin	24E10	Lateral membranes	Cell SignalingTechnology		1:20	ABC-kit*

* StreptABCcomplex/HRP (Dako)
** Envision+systems (Dako)

Genomic region	Sequence (forward)	Sequence (reverse)
Sox21	GCAGGCGCATAAATAAATAA	ATATCCATTCAAAGGGCATT
Cdh17	TTAAAACAACACCACCACCAC	CCCCAGTCAAACATTAACCAC
Hnf4a	AGGCTGAGGCTATGAGAAC	AACTCTCCCCTGACTCCTTGC
Amylase	CTCCTTGTACGGGTTGGT	AATGATGTGCACAGCTGAA

Gene	Sequence (forward)	Sequence (reverse)
Aqp4	CTGTGATTCCAAACGAACTG	GGCTCCAGTATAATTGATTGCA
Aqp8	CTACTGGGACTTCCATTGGA	CCGATGAGGAGCCTAATGAG
H+/K+Atpase4a	GACCACTGATGATAATCTGTACCT	GATATTTGTGCTCTTGAACTCCTG
Cdx2	GTATGTCTGTGTTGTAAATGCC	AAACAATTCCGGTCTTCTTCAG
ChgA	CAGAAGTGTTTGAGAACCAGAG	TTCTCTTCTCCATAGTGTCCC
Krt13	CTGACTCTGGCTAAGACTGAC	AATTCCTTCATCTCCTCTTCGT
Muc2	TGCAACAACTTAACTGCTCTG	TCAGTATGGTAATAGCCAGCC
Muc5ac	CATGACCTGTTATAGCTCCGA	CTCAGTAACAACACAGCCTC
p63	CATTGTCAGTTTCTTAGCAAGG	CTCAATCTGATAGATGGTGGT
Slc2a2	CAGAAGACAAGATCACCGGA	GCATTGATCACACCGATGTC
Slc5a1	ATTGAAATAGACACAGAAGCCC	GTCATCTTTGGTCCTTTATCCT
Lct	GCTTCCTATCAGGTTGAAGGT	GTCGTCATTCCCAATCTTCAG
Hnf4a	CTTTGATCCAGATGCCAAGG	GGTCGTTGATGTAATCCTCCA
Mep1a	CATCTTCAGCTATAAATGGCTC	CTTCTGAAACAATCACAGTCCT
Heph	GCAGAAGAGATAGAGTGGGA	ATAGCTGTCTTTCTCAGATGTG
Cdx1	AAAGGAGTTTCACTACAGCC	GAACCAGATCTTTACCTGCC
Aqp1	CTCCCTAGTCGACAATTCAC	CCAATGATCTCAATGCCAG
Aqp2	ACCTCCTTGGGATCTATTTCAC	ATCATCAAACTTGCCAGTGAC
Aqp7	GAGCTACAGTTCAGTTGCAG	ATGAAGTAGGTTCTCTGAAGTG
Aqp11	TCACAGGAGCATTGTTTAACC	ATCAGCACACCTACAGAAGG
Aqp12	CACAGCCTTCTTGTCTACAG	GGATTGAAGAAGGCAGATGTG

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CHAPTER 4

Ectopic expression of Sox2 in the mature intestinal tract induces aberrant proliferation, rather than changes in the intestinal differentiation program

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Abstract

Increasing evidence is obtained that a balance between the transcription factors Sox2 and Cdx2 is essential for proper regionalization of the primitive gut, from which the stomach, intestine and other endodermal tissues arise. In the digestive tract Sox2 is normally expressed in the rostral part up to the stomach, while Cdx2 is expressed in the intestine. Conditional knockout of Cdx2 in the developing gut was shown to lead to an anteriorization of the intestine towards a more gastric-like phenotype, and caused ectopic expression of Sox2 in the intestine. Recently, we showed that ectopic expression of Sox2 in the posterior region resulted in a similar conversion of the intestine towards a gastric like phenotype, in spite of sustained Cdx2 expression. These data suggested that reduced expression of Cdx2 by itself is not sufficient to cause anteriorization, but requires simultaneous expression of Sox2, and indicate that Sox2 might have a dominant role over Cdx2. While our understanding of the roles of Sox2 and Cdx2 in the developing gastrointestinal tract increases, far less is known about their roles in the mature intestine. Therefore, we ectopically induced Sox2 in the mature intestinal epithelium and examined whether Sox2 expression alters the normal homeostasis. We show that ectopic expression has a great impact on the morphology of the villi, resulting in the death of the animals within a week. Sox2 expanded the proliferative compartment throughout the crypt-villus structures, indicating that the intestinal cells were converted to a progenitor-like phenotype. Immunohistochemistry showed that the main intestinal cell types are still present and that no conversion toward a gastric cell type occurred. Moreover, expression of Cdx2 was not clearly altered. Our data indicate that ectopic expression of Sox2 in the mature intestinal epithelium does not alter the intestinal differentiation program, suggesting that the mature intestinal epithelium harbors a limited degree of plasticity. Additionally, ectopic expression of Sox2 drives cells into a progenitor phenotype.

Introduction

The vertebrate digestive tract develops through a complex mechanism of patterning, expansion and differentiation of the primitive gut, which itself is formed after gastrulation as a result of folding of the definitive endodermal layer [1-3]. In order to meet the changing digestive and absorptive requirements, the structure and physiology of the intestinal tract mature during postnatal growth[4-5]. To increase the absorptive area and the surface area of the small intestinal wall, the mucosa is formed into finger-like structures, the villi. In between, at the base of the villi, the intestinal crypts of Lieberkuhn reside. The large intestine lacks villi, but instead the crypts of Lieberkuhn are richly present. The intestinal epithelium gradually develops into a highly structured layer and is eventually composed of four main differentiated cell types, the absorptive enterocytes and colonocytes in the small and large intestine respectively, the mucin producing goblet cells, entero-endocrine cells and the Paneth cells that are specific for the small intestine. These cells are derived from the stem cells which are localized near or at the base of the crypts and give rise to the highly proliferating transit amplifying (TA) cells.

Early establishment of the anterior-posterior polarity (A-P) in the developing gut is to a large extent determined by two crucial transcription factors, i.e. the HMG-box protein Sox2 and the homeodomain containing Cdx2. Sox2 is a member of a highly conserved family of transcription factors founded by the sex-determining gene Sry. Although individual Sox proteins share common DNA binding properties, their specificity is the result of their expression pattern and their ability to associate with different partners [6-8]. Sox proteins are recognized as key players in the regulation of embryonic development and determination of cell fate. Sox2 plays an important role during early vertebrate embryogenesis and is required later in development in the brain, neural tube, germ cells and in the foregut and its derivatives [9-14]. In the digestive tract Sox2 starts to be expressed at E8.5 in the future foregut and is expressed in the rostral part of the gastro-intestinal tract up to the stomach. Sox2 is also one of the key players in the induction of pluripotent stem cells from somatic cells[15],underscoring its involvement in establishing progenitor identity.

Cdx2 is a member of the homeodomain transcription factors, which play crucial roles in diverse developmental processes as well in the adult organism, with alterations in their function being linked to a number of disorders including metabolic diseases and cancer[16]. Cdx2 is expressed from E8.5 in the posterior part of the gut[17], and later in development becomes restricted to the intestinal

epithelium. The expression domains of Sox2 and Cdx2 have a sharp boundary, marking the transition from stomach to duodenum [18-21].

Homozygous Cdx2 -/- embryos fail to implant properly, resulting from a block in differentiation of the trophoblast[22]. Cdx2 is also an essential transcription factor for establishing intestinal cell fate. This was first shown in heterozygous Cdx2 knock-out mice, which developed polyps consisting of normally organized stomach mucosa in the paracaecal region of the intestine[23]. Importantly, Sox2 was ectopically expressed in the overlying endoderm[24]. It was suggested that local sporadic haploinsufficiency of Cdx2 caused an anterior homeotic shift in which undifferentiated intestinal endoderm defaulted to a gastric phenotype. Chimaeric studies using Cdx2-/- cells in wild-type hosts confirmed these observations and also studies done by Cdx2 ablation specifically in the embryonic endoderm showed an activation of the foregut transcriptional program, causing a forestomach phenotype[25-26]. In all cases, ectopic expression of Sox2 was noted. This latter observation suggested that Sox2 may have been responsible for inducing the phenotypes that were observed. To test this hypothesis, we recently showed that ectopic expression of Sox2 in the developing embryonic gut drives the activation of the foregut transcriptional program and led to a rapid conversion from an intestinal into a premature gastric epithelium, despite simultaneous expression of Cdx2[27]. However, binding of Cdx2 to its genomic targets and thus its transcriptional activity was strongly reduced. This suggests that the balance between Sox2 and Cdx2 function is essential for proper specification of the primitive gut and that Sox2 can exert a dominant effect over Cdx2 to alter intestinal cell fate by redirecting the intestinal transcriptional program, emphasizing the plasticity of the primitive gut.

While our understanding of the roles of Sox2 and Cdx2 in the developing gastrointestinal tract has increased over the last years, far less is known about their roles in the mature gastro-intestinal tract. Studies done in order to elucidate the consequences of Cdx2 ablation in the mature intestinal tract resulted in various phenotypes. Stringer et al reported that Cdx2 knockout in the mature intestinal tract despite the expression of various gastric-associated genes retained a normal crypt-villus architecture, showing that Cdx2 loss only resulted in a partial conversion towards gastric epithelium[28]. Interestingly, in contrast to embryonic ablation of Cdx2, no induction of Sox2 expression was observed. Furthermore, they demonstrated that in absence of Cdx2, the intestinal stem cells were not able to differentiate into enterocytes, goblet cells or Paneth cells. In apparent contrast Hryniuk et al show that acute Cdx2 loss in the entire mature intestinal epithelium caused a rapid decline in health and death of the animals within a

week, which was accompanied by disruption of normal villus morphology and a decrease in transit amplifying and stem cell compartments[29]. However, long term loss of Cdx2, achieved by suboptimal dosing to evoke partial ablation compatible with long term survival, resulted in dysplastic regions in the small intestine, which were devoid from Cdx2 and showed some gastric characteristics. Thus, Cdx2 is necessary and sufficient to direct gut epithelial cells to an intestinal nature in adults. Nevertheless, the adult intestine showed a reduced plasticity towards gastric conversion. In the embryonic experiments that we and others performed, loss of Cdx2 functionality was in all cases accompanied with increased Sox2 expression, suggesting that both are required to influence the plasticity of the intestinal cells. Since in the mature intestinal tract no ectopic Sox2 expression was observed, we wondered whether the lack of Sox2 expression underlies the limited plasticity of the adult epithelium.

In the present study we used an inducible mouse model that upon doxycyline administration expresses transgenic Sox2 specifically in the mature intestinal tract. We show that ectopic Sox2 expression does not alter the differentiation process into the main intestinal cell types, but does have a great impact on villus morphology, proliferation and crypt formation. Our results indicate that the mature intestinal epithelium retains a limited degree of plasticity compared with the developing gut. Moreover, ectopic expression of Sox2 drives cells into a more progenitor-like phenotype, in concordance with its association with establishing progenitor identity.

Materials and methods

Transgenic animals

The myc-Sox2 and Villin-rtTA transgenic mouse lines were described previously[11 30]. The administration of doxycycline to the mice in their drinking water (2mg/ml doxycycline, 5% sucrose) for 1,3 and 5 days induced expression of myc-Sox2 in intestinal epithelium of double transgenic animals. Each experiment was performed on at least three control and three double transgenic mice, which received doxycycline for the indicated time points.

Histology

After the mice were sacrificed, the intestinal tract was removed, cut open and washed in PBS. Next, sections of approximately 10 cm were spread out on filter paper and fixed in 4% phosphate buffered paraformaldehyde overnight, processed and embedded in paraffin. Immunohistochemistry (IHC), Periodic Acid Schiff staining (PAS) and Hematoxylin and Eosin staining was performed as described previously[31]. Antibodies are listed in Supplemental Table I. Differentiated and proliferative cells in the epithelium were quantified by counting at least 3 microscopic fields relative to the total number of epithelial cells.

Results

Ectopic Sox2 expression affects the morphology of the mature intestinal tract

In the normal digestive tract, Sox2 is expressed in the esophagus and stomach, while it is completely excluded from the intestinal tract [32]. In contrast, Cdx2 is exclusively expressed in the intestine and not in the esophagus and stomach[33]. Previously, we showed that ectopic expression of Sox2 in the developing embryonic gut resulted in cell fate conversion of primitive intestinal cells into more gastric-like cells, in spite of sustained Cdx2 expression. These data suggested that Sox2 is able to convert already committed cell types into other ones. In order to investigate whether Sox2 is able to alter the homeostasis of the mature intestinal tract and whether it is also able to convert fully differentiated intestinal cells towards a more gastric like phenotype, we ectopically expressed Sox2 specifically in the intestinal epithelium of adult animals. Therefore, we crossed transgenic mice carrying a doxycycline-inducible, myc-tagged Sox2 gene with the Villin-rtTA transgenic mouse line, which drives expression of the rtTA protein in all intestinal epithelial cells [11 30].

First we noted that treatment with doxycycline for more than 3 days led to a decrease in general fitness of the double transgenic animals, all showing signs of diarrhea and reduced fur shininess, symptoms which were not observed in control animals. After 5 days of treatment this effect became even more striking and double transgenic mice actually started to die from this day onward, most likely as a result of dehydration, while control animals appeared healthy, independent of the number of days of doxycycline treatment.

We studied the expression pattern of the ectopic Sox2 protein, by performing IHC using an antibody against the myc-epitope (data not shown) and Sox2. In the control animals, Sox2 was absent in the intestinal epithelium (Fig.1A), while in the double transgenic animals nuclear Sox2 was detectable in the intestinal epithelium throughout the entire intestinal tract. At day 1 of induction, ectopic Sox2 was clearly expressed in both the villus and crypt compartment (Fig.1B). At later time points expression of Sox2 became more restricted to the villus, in addition to few scattered Sox2-positive cells in the crypt compartment (Fig. 1C,D).

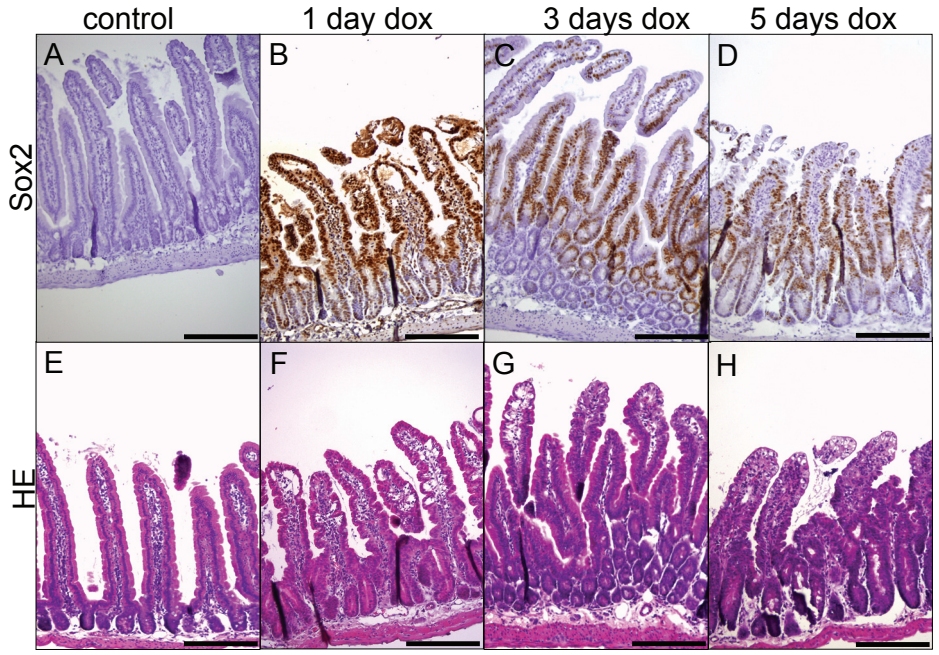

Figure 1: Ectopic expression of Sox2 in the mature intestinal epithelium causes morphological changes.

Nuclear Sox2 expression is present in the intestinal epithelium of double transgenic animals after 1(B), 3(C) and 5(D) days of doxycycline treatment, while in controls Sox2 is absent (A). After 3 days of inductionSox2 becomes restricted to the villus.HE staining shows that the villi in the double transgenic animals gradually shorten (F, G, H), upon Sox2 induction, compared to the controls (E). Scale bars: 200μm (A-H).

The loss of crypt expression may be the result of silencing of the transgenic Villin-promoter, which we also observed in Apc-driven tumor formation (unpublished observation).

Histological analysis revealed that the villi of the small intestine gradually shortened after prolonged induction of Sox2 expression (Fig. 1F,G,H), compared to the controls (Fig.1E). In addition, the epithelial layer of intestines of the double transgenic animals was disorganized and became multilayered (Fig.2E), whereas in the control animals a single layer of intestinal cells were well positioned and aligned (Fig.2A). Furthermore, we detected in the double transgenic animals the formation of vacuoles in the tip of the villi (Fig.2F). Additionally, the highly structured crypt-villus units progressively lost their organization and showed an aberrant morphology. The crypts were elongated, most apparent after 3 days of treatment (Fig.2G), compared to the controls (Fig.2C). The expression of Sox2 also induced anomalous crypts, which were present not only at the base

of the villus, but also seemed to be positioned within villus structures (Fig. 2H). Thus, ectopic expression of Sox2 affects the morphology of the intestinal villi and causes the formation of aberrant crypt structures.

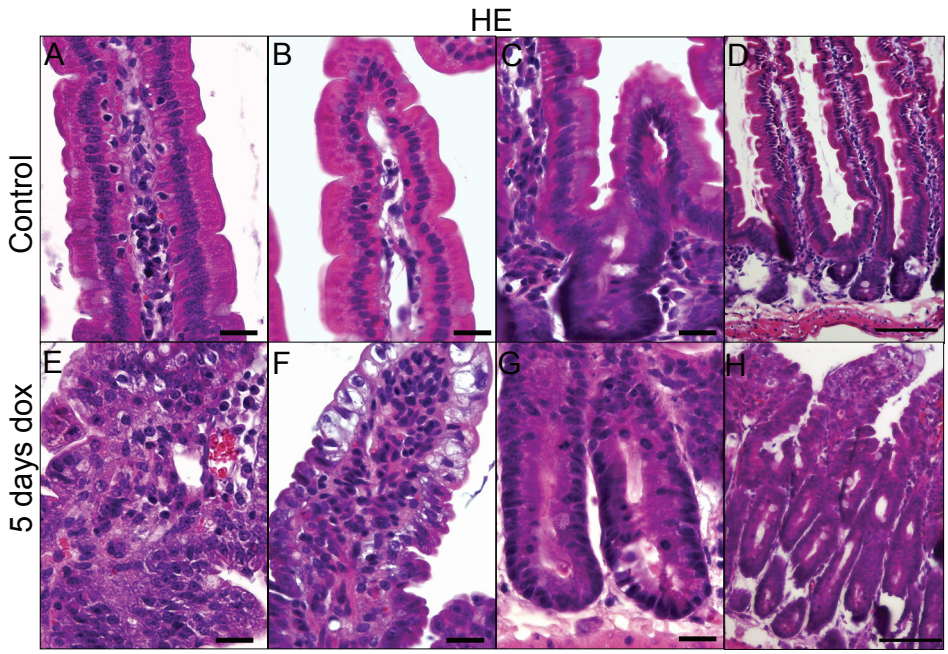

Figure 2: Ectopic expression of Sox2 in the mature intestinal epithelium causes disorganization of the intestinal epithelium and results in aberrant crypt-villus morphology.

Ectopic expression of Sox2 in the mature intestinal epithelium leads to a disorganized, multi-layered epithelial layer (E), compared to the controls (A).A representative double transgenic animal is shown, which was treated with doxyxyclin for 5 days. Furthermore vacuoles were observed in the tip of the villi specifically in the double transgenic animals (F). Additionally the crypt villus axis was altered, since crypt became elongated over time (G) and cryp-like structures were observed within the villus, most apparent in after 3 days of Sox2 induction (H). Scale bars: 20μm (A-C and E-G) and 100μm (D,H).

Ectopic expression of Sox2 causes aberrant positioning of proliferating cells

Since we observed structural changes in the villi, we examined the proliferative capacity of the epithelial cells. Therefore, we used the general proliferation marker Ki-67 (data not shown), and stained for phosho-Histone H3, which marks mitotic cells. Proliferation is normally restricted to the crypt compartment (Fig.3A), but in the double transgenic animals the proliferative

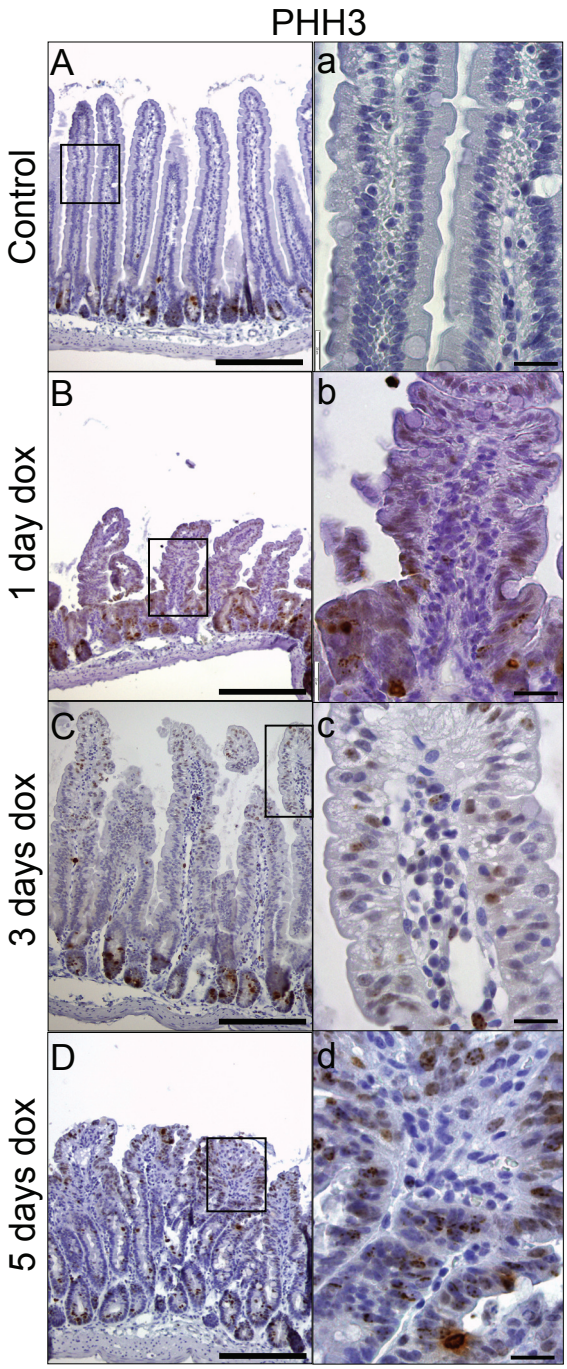

Figure 3: Ectopic expression of Sox2 in the mature intestinal epithelium affects proliferation.

IHC using phosho-Histone H3 (PHH3) shows that in the control animals proliferation is restricted to the crypt compartment (A), In the double transgenic animals dividing cells are also observed in the villus and in the aberrantly positioned crypts (B,C,D). More detailed images of the villi in control and double transgenic animals (a-d). Scale bars: 200μm (A-D) and 20μm (a-d).

zone of cells is expanded from the crypts to the villus structure (Fig.3B-D and b-d). These data indicate that ectopic expression of Sox2 in the mature intestinal epithelium causes epithelial cells to convert to a more progenitor-like phenotype.

Intestinal differentiation is not altered in mice ectopically expressing Sox2

Animals with Sox2 induction detoriated progressively with signs of diarrhea and overall reduced health. This indicated a general defect in the

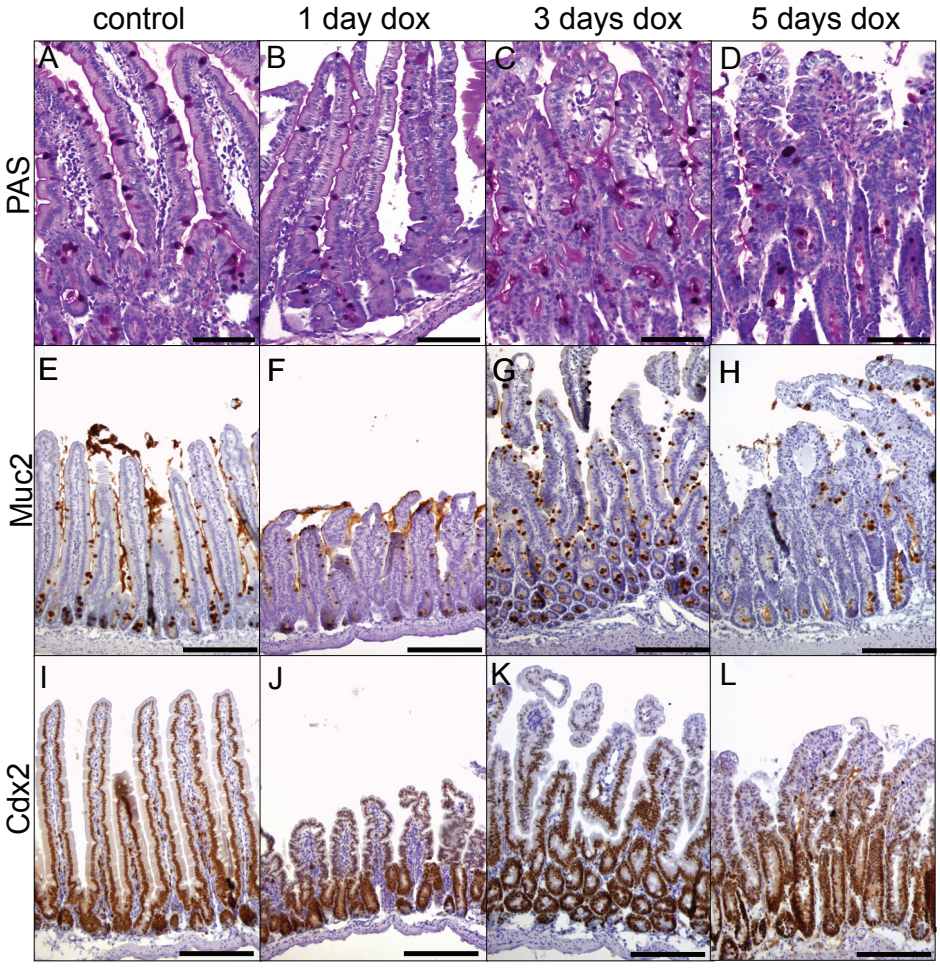

Figure 4: Ectopic expression of Sox2 in the mature intestinal tract does not alter the differentiation of the intestinal cells.

PAS staining marks the intestinal brush border as a clear pink lining in the contols (A). Until three days of Sox2 induction the brush border of the double transgenic animals are intact (B, C), however after induction for longer period the brush border appears to vanish (D). IHC for Muc2, which is a marker for the goblet cells, shows that goblet cells are present in both the control (E) and the double transgenic animals (F-H), although in the latter the localisation is chaotic, because of the dramatic morphological alterations. IHC for Cdx2 shows that it is expressed in the intestinal epithelium in both the control (I) and the double transgenic animals (J-L). After three days of induction Cdx2 expression is decreased at the tip of the villi, whereas is robustly expressed in the crypts (K, L).).Scale bars: 100μm (A-D) and 200μm (E-L).

absorption capacity of the intestinal epithelium. Previously, we showed that ectopic expression of Sox2 during intestinal development hampered the formation of the brush border, which is essential for intestinal absorptive capacity[27]. Therefore, we performed a PAS staining, which marks the intestinal brush border, visible as a thin pink apical lining (Fig. 4A). In the double transgenic animals the brush border appeared mostly intact until 3 days of induction (Fig. 4B,C). After 5 days of induction, the intestinal brush border is locally vanished (Fig. 4D). Although the effects on the brush border are not as dramatic as previously described for the embryonic gut, it may partially explain the observed effects on general health.

Since ectopic expression of Sox2 caused alterations in the intestinal architecture and resulted in ectopically located proliferating cells, we wondered whether the intestinal identity was affected. Therefore, we performed IHC using an antibody against Mucin2, which is a marker for the mucin producing goblet cells. We show the presence of goblet cells both in the control (Fig.4E) animals as well as in the double transgenic animals which were treated with doxycycline for various days (Fig. 4F,G,H). However, the localization of the goblet cells was not clearly defined due to the dramatic alterations in the morphology of the intestinal epithelium.

The presence of Paneth was analyzed using a specific staining for lysozyme (data not shown). We observed that in both the control and double transgenic animals Paneth cells are present, but the localization of the Paneth cells was not always restricted to the crypt compartment in the Sox2 expressing animals.

Ectopically expressed Sox2 does not induce gastric cell types in adult mice

Previously, we observed that ectopic expression of Sox2 in the embryonic intestinal tract, led to the rapid formation of premature gastric-like epithelium. Therefore, we analyzed the appearance of the gastric acid producing parietal cells with an antibody against N^+/K^+-ATPase. While we found parietal cells in the stomach, we could not detect positive cells in the control or double transgenic animals (data not shown). Also Mist1-positive zymogenic chief cells, GSII lectin positive mucus neck cells and gastric Muc5ac, were not detectable in the Sox2 induced intestines, while the stomach was positive (data not shown).

In conclusion, it appears that the main intestinal cell types are present in both control and double transgenic animals, although their localization is

affected due to the disruption of villus morphology. However, the intestinal brush border appeared to gradually decrease and was locally vanished after 5 days of Sox2 induction, most likely accounting for the symptoms observed in the double transgenic animals. In contrast to the phenotypic changes observed in the intestine when Sox2 was expressed during embryonic development, ectopic Sox2 expression does not induce the conversion of intestinal cells into gastric cell types when expressed in the mature gastrointestinal tract, but rather appears to induce a more progenitor phenotype of the cells.

Sox2 expression in the intestinal tract partially affects Cdx2 expression

In the developing gut, a strict balance between Sox2 and Cdx2 turned out to be essential for proper regionalization and organ specificity. Increasing evidence is provided that Cdx2 is essential for the maintenance of intestinal identity in adult mice. It was recently shown that conditional knock-out of Cdx2 specifically in the mature intestinal epithelium also resulted in morphologically altered villi and aberrant crypt villus structures[29]. Additionally, it was shown that conditional knock-out of Cdx2 in adult intestinal epithelium, resulted in ectopic expression of various gastric genes[28]. However it did not lead to actual conversion into gastric tissue. Interestingly, Cdx2 knock-out did not lead to upregulation of Sox2. Therefore, we performed IHC for Cdx2 in order to examine whether there were changes in the Cdx2 expression pattern. In concordance with our study in which we examined the effect of ectopically expressed Sox2 on embryonic gut development[27], we show that Cdx2 expression is clearly present in both the control (Fig. 4I) and double transgenic animals (Fig.4 J,K,L). However, from 3 days of doxycycline treatment onwards the expression of Cdx2 seems to decrease in the tip of the villi coinciding with the area in which the vacuoles are observed, whereas expression downward to the crypts is clearly present. Thus, ectopic expression of Sox2 in the mature intestinal does not hamper the expression of Cdx2 dramatically, but results in a slight decrease in Cdx2 expression in the tip of the villi.

Discussion

In the developing gut, a balance between the transcription factors Sox2 and Cdx2 is essential for normal development. In mice embryos, it was shown independently that both loss of intestinal Cdx2 as well as induced Sox2 expression, led to the activation of the foregut transcriptional program, resulting in a gastric-like phenotype[26-27]. This plasticity observed during development suggested that intestinal cells have a capacity greater than just forming intestinal epithelium. Since the adult intestinal epithelium has a high turnover, we wondered whether the epithelial cells in the adult intestine have the same plasticity as the embryonic cells. Therefore, we ectopically expressed Sox2 in the intestinal epithelium of adult mice.

Starting at three days of induction the animals became progressively ill and suffered from diarrhea and dehydration. Examination of the intestines revealed dramatic changes in the villi, such as progressive shortening and disorganization of the intestinal epithelium. The epithelium changed from a clear single layer of columnar cells into a multilayered population of cells. Also the intestinal brush border vanished locally after 5 days of doxycycline treatment, which may account for the progressive illness of the induced animals, most likely due to problems with intestinal absorption

Recently, the effect of conditional knock-out of Cdx2 in the mature intestine has been described by independent groups. Complete loss of Cdx2 throughout the whole intestinal epithelium, caused a rapid decline in health and ultimately death of the animals within a week, which was accompanied by disruption of normal villus morphology and a decrease in transit-amplifying and stem cell compartments[29]. Loss of Cdx2 only in a subset of the crypt-villus units, allowed long-term survival of the animals resulting in loss of intestinal identity, in addition to the acquisition of some gastric characteristics of the Cdx2-negative epithelium[28-29]. Importantly, no induction of ectopic Sox2 expression was observed[28]. The latter is in clear contrast to the embryonic situation in which Cdx2 loss in all cases was accompanied with ectopic Sox2 expression[25-26].

Our current results show that induced Sox2 expression in the mature intestinal tract has a very different outcome compared with loss of Cdx2. The main intestinal differentiation program was not affected as shown by the presence of goblet and Paneth cells, and no clear evidence of gastric conversion was noted. This might be due to the fact that Cdx2 itself is still expressed throughout the intestinal epithelium and retains sufficient transcriptional functionality to induce an intestinal program.

The most profound effect induced by Sox2 is the induction of a proliferative phenotype throughout the entire crypt-villus axis, which likely also underlies the formation of the aberrant crypts and the multilayered epithelium. This phenotype is in accordance with the induction of pluripotency and progenitor identity that has frequently been attributed to Sox2[14-15 34-39]. It is however in stark contrast to the Cdx2 knock-out phenotype characterized by reduced proliferation that remains restricted to the crypt zone[28-29].

In conclusion, our results suggest that Sox2 expression by itself is not sufficient to aberrantly induce gastric epithelium within the adult intestinal tract, and most likely requires the simultaneous loss of Cdx2 functionality. On the other hand, although Cdx2 loss results in some gastric characteristics in the mature intestine, it shows a clearly reduced efficiency when compared with the embryonic gut. In the latter the aberrant Sox2 expression accompanying Cdx2 loss, may facilitate a full gastric conversion by inducing more progenitor-like cells allowing for an easier reprogramming of the cells into another differentiated phenotype. Our results also confirm the limited degree of plasticity of the adult intestinal epithelium compared to its embryonic counterpart.

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CHAPTER 5

Expression pattern of SOX2 and CDX2 in human vitelline duct anomalies and intestinal duplications

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Submitted

Abstract

Congenital gastric type heteroplasia is common in intestinal duplications and anomalies, which find their origin in incomplete resorption of the omphalomesenteric duct. During development, two transcription factors play a determining role in the anterior-posterior specification of the gastrointestinal tract. SOX2 is expressed exclusively in the anterior part of the primitive gut, while CDX2 is expressed solely in the posterior part. In the absence of CDX2 during gut differentiation, ectopic tissue, mostly gastric tissue may arise. However, we recently showed that Sox2 leads to aberrant formation of gastric-like cells in the prospective intestine of transgenic mice, even in the presence of Cdx2, suggesting that Sox2 may have a dominant role in determining intestinal cell fate. Therefore we investigated the expression pattern of SOX2 and CDX2 in three congenital intestinal anomalies in which ectopic gastric tissue may be present, Meckel's diverticulum (N=8), persistent ductus omphalomesentericus (N=14) and intestinal duplications (N=8). CDX2 was detected in intestinal epithelial cells in tissue lacking gastric heteroplasia, where SOX2 was absent. In gastric-type heteroplasia, a reciprocal expression pattern existed between SOX2 and CDX2 in the gastric and intestinal tissue, respectively. Interestingly, patches of CDX2 positive cells were present within the gastric mucosa in a subset of Meckel's diverticula and intestinal duplications, suggesting that it is not the absence of CDX2 leading to gastric tissue in the prospective intestinal tissue, but rather the ectopic expression of SOX2 in the intestine. This is in concordance with our previous mouse studies. Collectively, this indicates that also in humans a fine balance between SOX2 and CDX2 expression in de gastrointestinal tract is essential for proper development and that ectopic expression of SOX2 may lead to malformations of the gut.

Introduction

Intestinal gastric-type heteroplasia, which refers to the presence of ectopic gastric tissue in the intestine, is a rare pathological abnormality detected in several congenital anomalies[1]. The most commonly observed intestinal anomalies are persistent omphalomesenteric ducts, Meckel's diverticula and intestinal duplications (Fig. 1). The latter arise after gastrulation when the crescent-shaped endodermal germ layer folds to form the primitive gut[2]. During the first trimester of embryonic development, the intestinal epithelium proliferates leading to a temporary block of the lumen, followed by a recanalisation in week 9 of gestation. Incomplete recanalisation is believed to underlie the formation of intestinal duplications (Fig. 1A).

Meckel's diverticula arise from the omphalomesenteric duct (also referred to as vitelline duct) that connects the midgut with the yolk sac before placental connections are established. Normally, the omphalomesenteric duct involutes completely between the fifth to ninth week of gestation. However, if this resorption does not occur properly, the complete omphalomesenteric duct, or part of it, may persist, which leads to various congenital anomalies[3]. Persistence of the complete omphalomesenteric duct within the abdominal wall may cause a patent ductus omphalomesentericus. The lumen of the ductus omphalomesentericus remains open from ileum to the umbilicus resulting in faecal contents discharged from the umbilicus (Fig. 1B). The umbilicus may also be closed by the skin, leaving a blunt-ended umbilicus (Fig. 1C), or a fibrous cord may remain, connecting the small intestine to the umbilicus through a so called umbilico-ileal fistula (Fig. 1D). Failure of the most proximal portion of the duct to obliterate, results in a small protrusion from the ileum, typically at the antimesenteric site, the so-called Meckel's diverticulum, which accounts for the most prevalent congenital disorder of the gastro-intestinal tract occurring in approximately 2% of the population (Fig.1E). In many cases Meckel's diverticulum does not result into clinical symptoms throughout life.

The diverticula as well as other vitelline duct anomalies contain all layers of the intestinal wall. In addition, ectopic tissue, mostly gastric, is commonly observed in both intestinal duplications and omphalomesenteric duct anomalies, accounting for most of the complications of these malformations. The ectopic gastric tissue often causes internal bleedings as a result of ulceration of the adjacent ileal mucosa inflicted by the acid produced by the gastric mucosa[1],

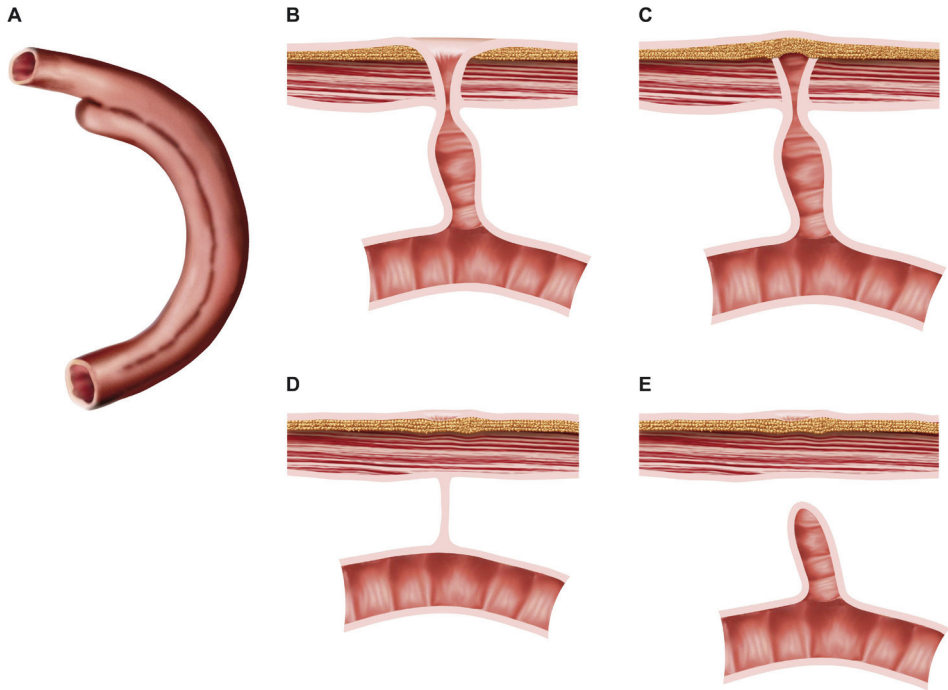

Figure 1: Schematic representation of congenital intestinal anomalies.
(A) Intestinal duplication. (B) Open persistent ductus omphalomesentericus. (C) Persistent ductus omphalomesentericius. (D) Umbilico-ileal fistula. (E) Meckel's diverticulum.

leading to anal blood loss or blood containing stool or with slow onset ferriprive anemia[3].

The mechanisms responsible for the determination of organ identity in the gastrointestinal tract in humans are poorly understood and are mainly based on the extrapolation from animal studies. One of the earliest steps in patterning of the primitive gut is the regionalization into an anterior and posterior domain, which correlates with the mutually exclusive expression of two transcription factors, Sox2 and Cdx2, respectively[4]. Sox2 is a member of a highly conserved family of transcription factors founded by the sex-determining gene Sry. Sox proteins are key players in the regulation of embryonic development and determination of cell fate[5-6]. Sox2 plays an important role during early vertebrate embryogenesis and is required for proper development of the brain, neural tube, trachea and germ cells[7-12]. In addition, it is one of the main factors involved in the differentiation of the gastric epithelium[11 13]. Several congenital malformations in humans, such as anophthalmia, microphthalmia and the AEG (anophthalmia-esophageal-genital) syndrome[14], have been associated with heterozygote mutations in SOX2.

Cdx2 is a homeobox transcription factor involved in the establishment of the anterior-posterior polarity of the gut endoderm and is expressed from E8.5 in the posterior part of the gut[15]. Later in development, Cdx2 becomes restricted to the intestinal epithelium, with a sharp boundary, marking the transition from stomach to duodenum[4 16-18]. Cdx2 plays a key role in determining intestinal identity, as it was shown that loss of Cdx2 function in mouse embryos resulted in colonic atresia and in the conversion of intestinal cell types to a gastric-like phenotype, concomitant with ectopic activation of Sox2 expression[19-20]. We recently showed the emergence of a comparable phenotype by ectopic expression of transgenic Sox2 in the prospective gut endoderm. Exogenous Sox2 did not affect Cdx2 expression itself, but prevented the binding of Cdx2 to its genomic targets and thus its transcriptional activity. Moreover, we showed that ectopic expression of Sox2 in the developing embryonic gut epithelium was capable of driving cells towards a gastric fate, despite the fact that intestinal organ identity had already been established[21]. Taken together, these results show that a strict balance between Sox2 and Cdx2 function is required for proper specification of the primitive gut.

In human gastrointestinal congenital diseases very little is known about the expression pattern of these transcription factors. It has been proposed that at sites of gastric heteroplasia the expression of CDX2 was reduced during gut formation[22]. However, the expression of SOX2 has not been investigated in these congenital anomalies. Therefore, we analyzed the expression pattern of SOX2 and CDX2 in various congenital anomalies of the gastrointestinal tract with gastric-type heteroplasia.

Materials and methods

Paraffin embedded tissue from 8 patients with intestinal duplications, 14 with persistent ductus omphaloentericus and 8 with Meckel's diverticulum were obtained from the department of Pathology of the Erasmus Medical Centre, Rotterdam. Patient details are described in Table 1. Sections of 5 μm were used for H&E staining or immunohistochemistry and were evaluated by at least one pathologist. For immunohistochemistry, sections were deparaffinized and rehydrated, followed by antigen retrieval with microwave treatment in Tris-EGTA pH 9.0. Sections were blocked with 5% nonfat dry milk in PBS for 10 min and incubated with primary antibody diluted in 5% nonfat dry milk in PBS overnight at 4°C. Primary antibodies that were used are Sox2 (Immune systems), CDX2 (Biogenex), and MUC5AC (45M1, Abcam). Secondary antibodies against the correct IgG species were conjugated with peroxidase (StreptABC complex/HRP, DAKO) followed by DAB colorimetric detection.

Table 1: Patient details: sex, age at surgery, localization of resected tissue and clinical diagnosis

	Sex	Age at surgery	Localisation	Diagnosis
1	V	7 days	terminal colon	intestinal duplication
2	M	13 days	terminal colon	intestinal duplication
3	M	3 days	small intestine*	intestinal duplication
4	V	170 days	ileocaecal border	intestinal duplication
5	M	155 days	distal ileum	intestinal duplication
6	M	97 days	small intestine	intestinal duplication with ectopic gastric mucosa
7	V	55 days	terminal ileum	intestinal duplication with ectopic gastric mucosa
8	V	147 days	ileocaecal border	intestinal duplication with ectopic gastric mucosa
9	V	9 days	small intestine*	persistent ductus omphalomesentericus
10	M	16 days	small intestine*	persistent ductus omphalomesentericus
11	M	12 days	small intestine*	persistent ductus omphalomesentericus
12	M	1 day	small intestine*	persistent ductus omphalomesentericus
13	M	5 days	small intestine*	persistent ductus omphalomesentericus
14	M	1 day	small intestine*	persistent ductus omphalomesentericus
15	M	38 days	small intestine*	persistent ductus omphalomesentericus
16	M	1 day	small intestine*	persistent ductus omphalomesentericus
17	M	294 days	umbilicus	persistent ductus omphalomesentericus
18	M	165 days	umbilicus	persistent ductus omphalomesentericus
19	M	1,1 years	small intestine*	persistent ductus omphalomesentericus with ectopic gastric mucosa
20	M	212 days	small intestine*	persistent ductus omphalomesentericus with ectopic gastric mucosa
21	M	58 days	umbilicus	persistent ductus omphalomesentericus with ectopic gastric mucosa
22	V	2 days	umbilicus	persistent ductus omphalomesentericus with ectopic gastric mucosa
23	V	1,7 years	small intestine*	Meckel's diverticulum
24	M	64,9 years	small intestine*	Meckel's diverticulum
25	V	92 days	terminal ileum	Meckel's diverticulum
26	M	74,3 years	small intestine*	Meckel's diverticulum with ectopic gastric mucosa
27	V	6,4 years	small intestine*	Meckel's diverticulum with ectopic gastric mucosa
28	M	12 years	Ileocaecal border	Meckel's diverticulum with ectopic gastric mucosa
29	M	41,2 years	Ileoceacal border	Meckel's diverticulum with ectopic gastric mucosa
30	V	40,3 years	terminal ileum	Meckel's diverticulum with ectopic gastric mucosa

* precise localisation unknown

Results

Given the importance of SOX2 and CDX2 in the formation of the gastro-intestinal tract, we analyzed the expression pattern of both transcription factors in intestinal anomalies with gastric-type heteroplasia, including intestinal duplications, persistent omphalomesenteric ducts and Meckel's diverticula.

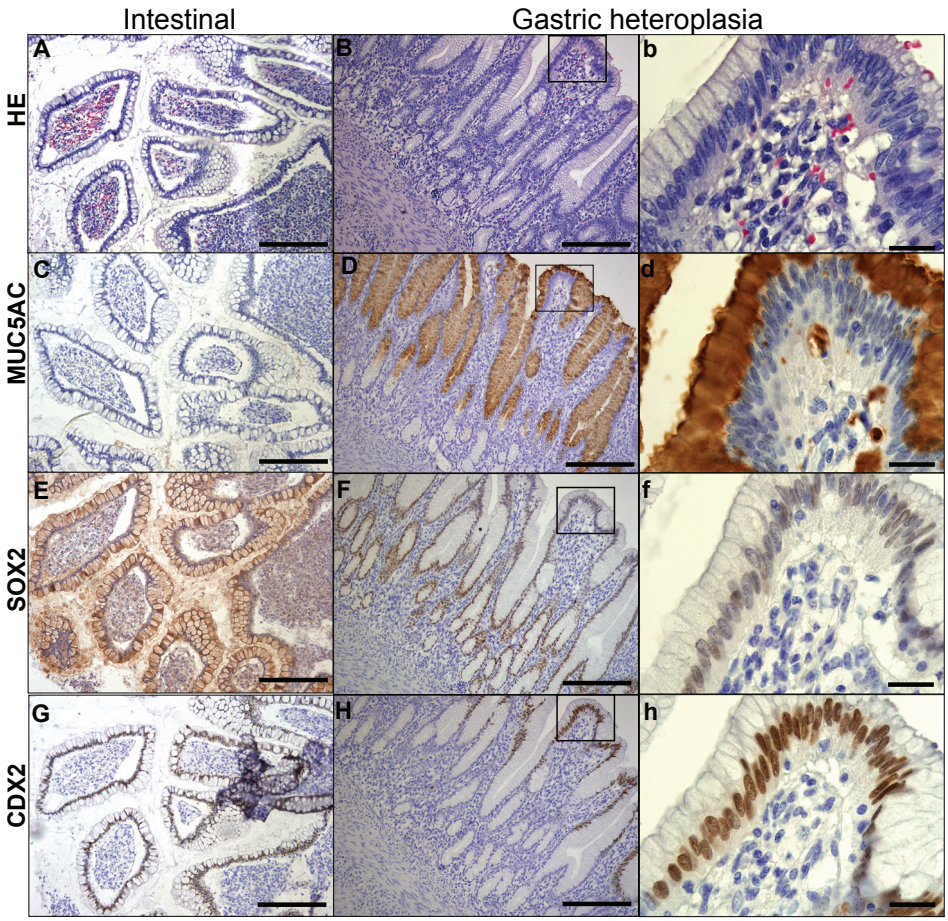

Figure 2: Histological analysis of intestinal duplications.

HE staining, of a representative case of intestinal duplication with gastric heteroplasia, showing intestinal epithelium (A) and ectopic gastric mucosa in the intestinal duplication (B). IHC for MUC5AC shows specific expression in the gastric heteroplasia (D), while no staining is observed in the intestinal epithelium (C). IHC for SOX2 shows expression of SOX2 in the ectopic gastric mucosa (F), but not in the intestinal epithelium (E). IHC for CDX2 shows exclusively nuclear expression in intestinal epithelium (G), however also ectopic expression of CDX2 in parts of the gastric heteroplasia is present (H). Higher magnifications of B,D,F and H are shown in b,d,f and h respectively. Scale bars: A-H 200μm and b,d,f,h 20 μm

We examined 8 cases of intestinal duplications of which 3 showed the presence of gastric tissue as determined by histological evaluation (representative case shown in Fig. 2). The expression of MUC5AC confirmed the presence of gastric tissue in all three cases (Fig. 2D, d), whereas the intestinal mucosa was completely devoid of MUC5AC (Fig. 2C), which was comparable with the 5 cases in which no gastric heteroplasia was present (data not shown). In these latter cases no specific nuclear SOX2 expression was observed, except in the neuronal plexi (data not shown), which we used as an internal control for SOX2

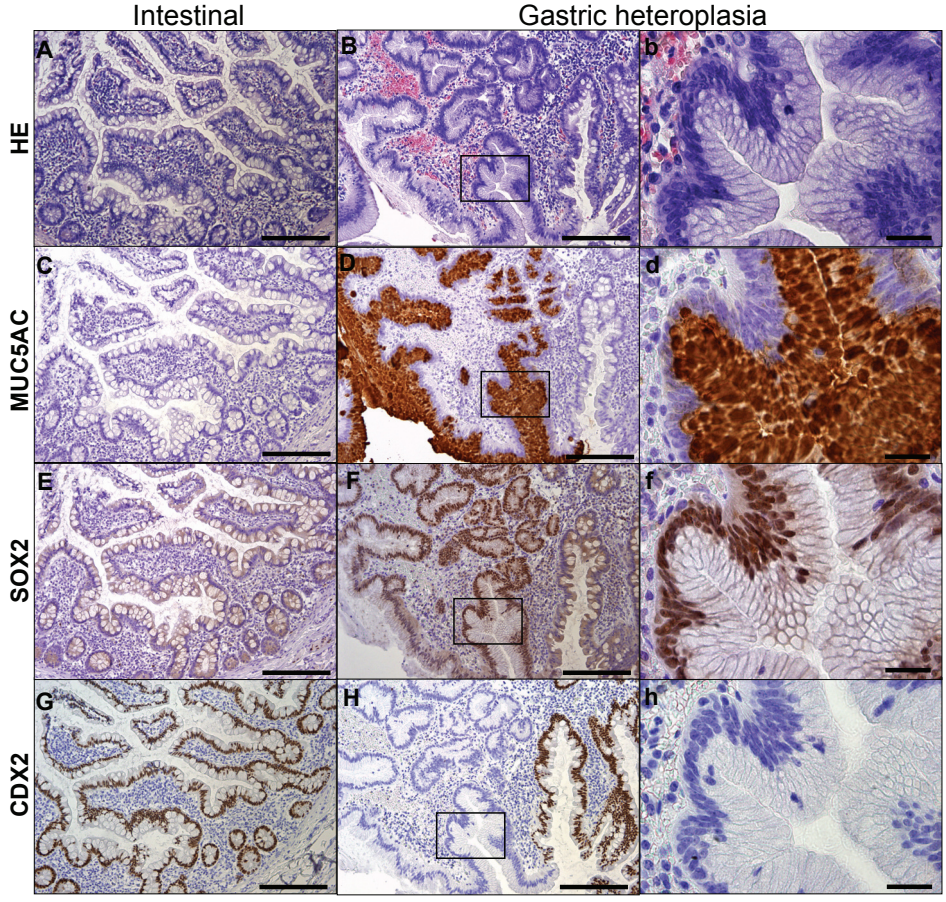

Figure 3: Histological analysis of persistent ductus omphalomesentericus.

HE staining, showing intestinal epithelium (A) and ectopic gastric mucosa (B) in a representative case of persistent ductus omphalomesentericus with gastric heteroplasia. IHC for MUC5AC shows specific expression in the gastric heteroplasia (D), while no staining is observed in the intestinal epithelium (C). IHC for SOX2 shows expression of SOX2 in the ectopic gastric mucosa (F), but not in the intestinal epithelium (E). IHC for CDX2 shows \ nuclear expression in intestinal epithelium (G), while no specific expression of CDX2 is present in parts of the gastric heteroplasia (H). Higher magnifications of B,D,F and H are shown in b,d,f and h respectively. Scale bars: A-H 200μm and b,d,f,h 20 μm

expression[23]. In the duplications in which gastric tissue was present, specific nuclear SOX2 could be detected in the gastric mucosa (Fig.2F, f), while in the surrounding intestinal epithelium specific nuclear expression of SOX2 was absent (Fig. 2E). Next we analyzed the distribution of CDX2 expression, in order to determine the presence of the intestinal epithelium. We detected CDX2 uniformly in the intestinal epithelium of all samples (Fig.2G). Interestingly, we also observed CDX2 expression in 2 of the 3 duplications with gastric-type heteroplasia within the parts which were histologically determined to be gastric tissue (Fig. 2H,h). Moreover, these CDX2 positive cells also showed a reduced, but not entirely absent, expression of SOX2 (compare Fig. 2f and 2h).

We subjected the omphalomesenteric duct anomalies to an identical analysis. First we analyzed the expression pattern of SOX2 and CDX2 in the persistent omphalomesenteric ducts. A total of 14 cases were examined, of which 4 samples contained ectopic gastric tissue based on histological evaluation (representative case shown in Fig.3) and IHC for MUC5AC, which was specifically expressed at these sites (Fig. 3D,d). The intestinal mucosa was devoid of MUC5AC (Fig.3C), as also was seen in the 10 samples consisting exclusively of intestinal tissue (data not shown). In the 4 samples with ectopic gastric tissue, nuclear staining was observed for SOX2 in the gastric mucosa (Fig. 3F, f), whereas in the surrounding intestinal epithelium specific SOX2 expression was absent (Fig. 3E). CDX2 staining was exclusively detected in the intestinal epithelium (Fig.3G), whereas it was not expressed in the gastric tissue (Fig. 3H, h).

In addition, we examined 8 cases of Meckel's diverticulum of which 3 cases with exclusively intestinal tissue (data not shown) and 5 with histologically gastric type heteroplasia (representative case shown in Fig. 4). The appearance of gastric tissue was confirmed using MUC5AC (Fig. 4D, d), while in the intestinal mucosa no MUC5AC was present (Fig. 4C). As expected, no specific SOX2 expression was observed in the intestinal epithelium (Fig. 4E), whereas in the gastric tissue, SOX2 was expressed in the gastric epithelium (Fig. 4F, f). IHC for CDX2 showed clear expression in the intestinal epithelium (Fig. 4G) and no staining in the ectopic gastric tissue, except for 1 sample, in which we observed CDX2 expression in the ectopic gastric mucosa (Fig. 4H, h).

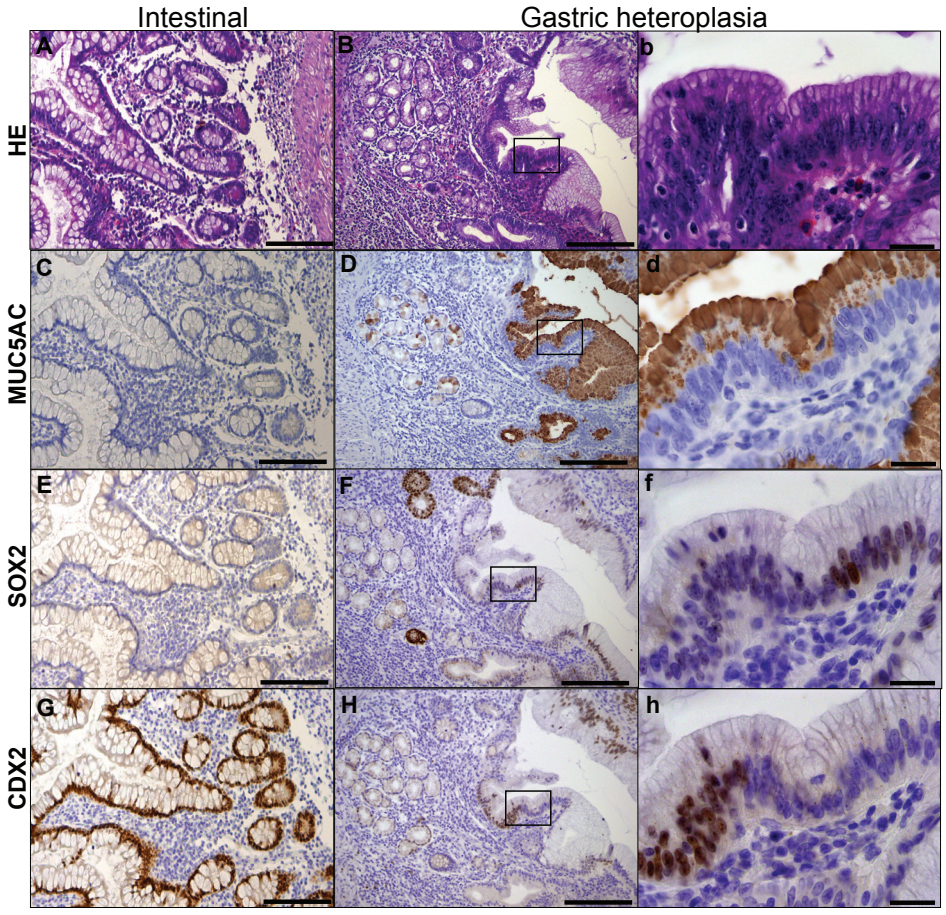

Figure 4: Histological analysis of Meckel's diverticulum.

HE staining, showing intestinal epithelium (A) and ectopic gastric mucosa (B) in a representative case of Meckel's diverticulum with gastric heteroplasia. IHC for MUC5AC shows specific expression in the gastric heteroplasia (D), while no staining is observed in the intestinal epithelium (C). IHC for SOX2 shows expression of SOX2 in the ectopic gastric mucosa (F), but not in the intestinal epithelium (E). IHC for CDX2 shows exclusively nuclear expression in intestinal epithelium (G), however also ectopic expression of CDX2 in parts of the gastric heteroplasia is present (H). Higher magnifications of B, D, F and H are shown in b,d,f and h respectively. Scale bars: A-H 200μm and b,d,f,h 20 μm

Discussion

Recent studies have addressed the role of Cdx2 and Sox2 in the development of the primitive gut into specific organ domains[11 20-21]. The transcription factor Sox2 is normally expressed in the anterior part of the presumptive gut, i.e., esophagus and stomach, while Cdx2 is predominantly expressed in the posterior part of the gut[2]. Specific gene ablation of *Cdx2* in the intestine of the mouse showed an anteriorization event of the intestinal tract, leading to a gastric-like phenotype associated with ectopic Sox2 expression[20]. Recently, we showed that this anteriorization event also occurred upon ectopic Sox2 expression in the embryonic intestinal epithelium, despite the fact that Cdx2 was still expressed in the intestine. Furthermore, we showed that Cdx2 did no longer bind to its endogenous target sequences in the presence of Sox2, indicating a functional loss of Cdx2 activity. This strongly indicated that it is not solely the lack of Cdx2 that causes the stomach-like phenotype, but rather requires the expression of Sox2 in the intestinal epithelium[21].

Although substantial insight has been gained about gut formation in mice, the mechanisms and the expression pattern of SOX2 and CDX2 during the development of the gastrointestinal tract and in developmental anomalies of the digestive tract in humans are largely unknown. Therefore, we investigated 3 types of congenital malformations of the intestine in humans, i.e. intestinal duplications, persistent omphalomesenteric ducts and Meckel's diverticulum, in which frequently ectopic gastric tissue has been observed. In general, a reciprocal expression pattern of CDX2 and SOX2 was observed in the intestine and gastric tissue, respectively. Interestingly, we observed 3 cases that expressed CDX2 in the ectopic gastric type heteroplasia. This would suggest that the primary reason for the appearance of gastric tissue is not just the lack of CDX2, as was proposed by Martin et al[22], but rather the ectopic expression of SOX2, as we showed in our study in mice[21].

Gastric heteroplasia is seen both in intestinal duplications as well as anomalies of the omphalomesenteric duct. Both types of malformation arise during the first trimester of gestation, though different causes underlie these anomalies. Duplicatures are thought to result from improper recanalization of the intestinal tube, whereas omphalomesenteric duct anomalies are caused by improper resorption of this duct. However, ectopic tissue, mainly gastric tissue, is still observed in both kinds of anomalies. A possible explanation could be that the intestinal epithelium has a certain degree of plasticity in the first trimester of embryonic development. In support of this, Sherwood et al pointed out a phase

at E8.75-E9.25 of mouse development when cells at the border of stomach and intestine co-express both transcription factors. At E9.5 the cellular border remains, but the cells exclusively express either Sox2 or Cdx2[4]. This phase of embryonic development coincides with the timing of involution of the omphalomesenteric duct. As such, the double-positive cells that we observe, might represent descendants from the population of cells described above, which co-express both factors during a short phase of intestinal development. Alternatively, after the intestinal epithelium is already formed it may still be influenced by ectopic expression of factors such as SOX2, resulting in the conversion of the intestinal epithelium into a gastric phenotype.

In conclusion, we showed the expression pattern of SOX2 and CDX2 in intestinal duplications, persistent omphalomesentericus and Meckel's diverticulum. Our data suggest, as also observed in mice, that ectopic expression of SOX2 in the intestinal epithelium might contribute to the cell fate conversion from intestinal tissue to gastric tissue, in spite of sustained CDX2 expression. This supports the general concept based on animal studies that a strict balance between SOX2 and CDX2 during early development is essential for normal development of the gastrointestinal tract, also in human.

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CHAPTER 6

Aberrant SOX2 expression in colorectal cancers shows no direct relation with the expression of the gastric mucin MUC5AC, but correlates with non-mucinous differentiation

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Submitted

Abstract

***Aim*:** Colorectal cancer (CRC) can be subdivided in mucinous and non-mucinous CRC. A recent study suggested a putative link between SOX2 expression observed selectively in mucinous CRC, which portend to have a worse clinical prognosis. In this study we aimed to further elucidate the expression behavior of SOX2, CDX2 and MUC5AC in both mucinous and non-mucinous CRC, and to examine whether SOX2 expression can be correlated with specific tumor characteristics and prognosis.

Methods and results: We performed immunohistochemical analysis on 34 cases of non-mucinous CRC, 12 cases of mucinous CRC and 15 case-matched normal intestinal mucosa. In contrast to the previously suggested link between SOX2 and mucinous CRC, we show ectopic expression of SOX2 predominantly in non-mucinous cancers. While normally SOX2 and CDX2 are expressed in a reciprocal manner, SOX2-positive tumor cells co-express CDX2. Furthermore, we show that MUC5AC was equally expressed in both classes of CRC independent of SOX2. This gastric mucin was also detected in morphological normal intestinal tissue neighboring tumor sites, while in control samples the normal intestinal mucosa MUC5AC was absent. Additionally, the SOX2 expressing non-mucinous CRC had a significantly worse survival than the SOX2-negative part ($p= 0.0224$) in the first patient group that we tested, however we did not observe this association in a second patient group, in which we tested 56 non-mucinous CRCs.

Conclusions: We observe ectopic SOX2 expression predominantly in non-mucinous CRC and show the appearance of cells that simultaneously express both SOX2 and CDX2, while normally these transcripts are expressed in a mutually exclusive manner. Furthermore, in contrast to previous suggestions that the aberrant expression of the gastric mucin MUC5AC can be attributed to SOX2, our results suggest that SOX2 is not absolutely required.

Introduction

Colorectal cancer (CRC) affects over 400,000 people annually in Europe, resulting in approximately 220,000 deaths every year. CRC accounts for the most frequent malignancy within the European Union, and is the second most common cause of cancer-related mortality. CRC has been classified in different subtypes according to criteria based on their histological appearance and mutational status [1]. Proper classification of CRC is becoming increasingly important in the clinic, as it will more often determine prognostic outcome and choice of therapeutic intervention.

Approximately 75-90% of the CRCs are non-mucinous, while the remaining 10-25% are mucinous or signet ring cell carcinomas[2-3]. Mucinous CRC is defined as a tumor with more than 50% mucinous differentiation on histological examination, according to the WHO. These tumors are more common in the proximal colon and often show a flat appearance, making their early identification more difficult. As a result, they present on average with a higher stage of tumor progression at first diagnosis. In addition to their delayed detection, mucinous differentiation by itself is associated with a modest increase in mortality compared with their non-mucinous counterparts, even when corrected for stage[3]. Elevated levels of mucin have also been associated with worse prognosis for various other tumor types, including pancreatic cancer[4]. Furthermore, in comparison to their non-mucinous counterparts, mucinous CRCs more often show ectopic expression of MUC5AC, a mucin whose expression is normally restricted to the upper gastrointestinal tract[2 5-7].

The etiology of this subset of cancers and the mechanism underlying the mucinous differentiation and worse prognosis are still poorly understood. Recently, Park and co-workers reported aberrant expression of SOX2 specifically in a subset of CRCs with mucinous differentiation[7]. They also reported concordant expression of SOX2 with MUC5AC in colorectal cancers or cell lines thereof, and the activation of a MUC5AC reporter construct by SOX2. In support of their results, overexpression of SOX2 in COS-7 cells induced the mRNA expression of endogenous MUC5AC, indicating that the MUC5AC expression observed in mucinous CRCs, may indeed result from the ectopic SOX2 expression[8]. The SOX2 gene encodes for a HMG-box containing transcription factor with a potent role in determining cell fate. In recent years, it has gained substantial attention as one of the factors required to obtain induced pluripotent stem (iPS) cells from adult somatic cells[9]. In the adult gastrointestinal tract, SOX2 expression is restricted to the gastric and esophageal epithelium, whereas it is completely

excluded from the intestine[10-11]. Additionally, we and others have shown that during embryonic development a strict balance between SOX2 and CDX2, which is a key transcription factor in determining intestinal identity, is essential for proper development of the intestinal tract [12-13].

Recently, a number of studies have reported ectopic expression of Sox2 in different kinds of cancers, including lung, esophagus and breast cancer[10 14]. In these tumor types, aberrant Sox2 expression was associated with worse prognosis, attributed to the induction of a precursor-like state induced by Sox2. As such, the ectopic SOX2 expression observed in CRCs may likewise affect tumor progression.

In this study we aimed to further elucidate the expression patterns of SOX2, CDX2 and MUC5AC in both mucinous and non-mucinous CRC, and to examine whether SOX2 expression can be correlated with specific tumor characteristics and prognosis.

Materials and methods

Human samples

Formalin-fixed, paraffin embedded tissue blocks were obtained from the department of Pathology, Erasmus Medical Centre Rotterdam. Material of patients from the first group were selected based on their tumor being either mucinous (N=12) or non-mucinous (N=34), and healthy intestinal tissue (N=15). Mucinous CRC was defined as a tumor with more than 50% mucinous differentiation on histological examination, according to the WHO 2010. The second group of patients were selected based on their tumor being non-mucinous and at least 2 years follow-up time (N=56). All H&E and immunohistochemically stained sections were evaluated by a pathologist.

Immunohistochemistry

Sequential 5 μm sections of the paraffin blocks were used for H&E staining and immunohistochemistry. For immunohistochemistry, sections were deparaffinized and rehydrated, followed by antigen retrieval with microwave treatment in Tris-EGTA pH 8.0. Sections were blocked with 5% non-fat dry milk in PBS for 10 minutes and incubated with primary antibody diluted in 5% non-fat dry milk in PBS overnight at 4°C. The following antibodies were used: 1:500 SOX2 (Immune systems), 1:200 MUC5AC (Abcam) and 1:20 CDX2 (Biogenex). Secondary antibodies against the correct IgG species were conjugated with peroxidase (Dako) using StreptABC complex/HRP (Dako) or HRP-DAB colorimetric detection. Immunohistochemistry for SOX2 and CDX2 were only scored as positive when a clear nuclear staining was observed.

Statistical analysis

Ordinal variables were tested for association using the Chi-square test or the Fisher's exact test when appropriate. Continuous variables were tested with the Wilcoxon ranksum test with study-set as grouping variable. Survival curves were visualized with Kaplan Meier curves. The logrank test or when appropriate the logrank test for trend was used to test differences between survival curves. Time to survival was analyzed using the Cox proportional hazards model. Overall survival was defined as time from diagnosis to death or last date of follow-up. Endpoint was death of any cause. A P-value < 0.05 was considered statistically significant. All P-values are two-sided. Computations were done with the STATA statistical package, release 11.2 (STATA Corp. College Station. TX).

Results

SOX2 is expressed in non-mucinous CRC, rather than mucinous CRC

SOX2 has been associated with various malignancies in humans, including lung, esophageal and breast cancer[10 14]. Since it was previously reported that SOX2 was expressed specifically in mucinous colorectal cancers [7], we initially performed immunohistochemistry on 34 non-mucinous CRC, 12 mucinous CRC and 15 samples of normal mucosa, to determine the expression pattern of SOX2 in human CRC. Specific nuclear expression of SOX2 was detected in 13 of the 34 (38%) non-mucinous CRCs (Fig. 1A,a), whereas only a single mucinous CRC showed SOX2 expression (8%). A representative case of a SOX2-negative mucinous tumor is shown in Fig.1B. In general, SOX2 was clearly detectable in patches of the tumor. Morphologically the ectopically SOX2 expressing cells were indistinguishable from the SOX2-negative cells in the same tumor. Furthermore, we did not observe SOX2 expression specifically at the invasive front of a tumor. As expected, SOX2 was absent in normal intestinal tissue (data not shown). As an internal positive control for SOX2 expression, we used the submucosal and myenteric plexuses present in the intestine, which are known to express SOX2 (Fig. 1B insert)[15].

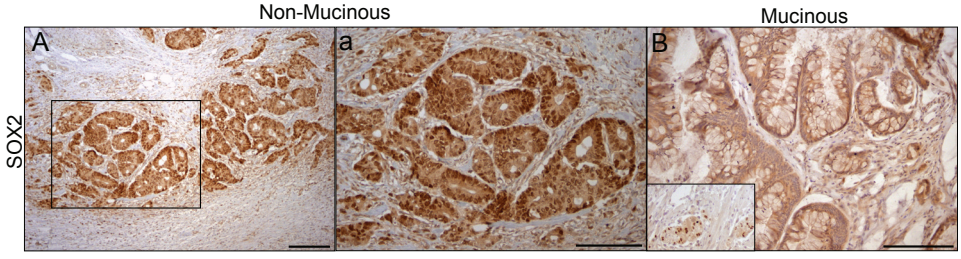

Figure 1: SOX2 expression is observed predominantly in non-mucinous CRC.

Immunohistochemistry for SOX2 in non-mucinous and mucinous CRCs. SOX2 expression was present in 13/34 non-mucinous CRCs (A, (overview) and (a) higher magnification of (A), whereas it was observed only in 1/12 mucinous CRCs. (B) Representative figure of a SOX2-negative mucinous CRC. Insert shows SOX2-positive neuronal plexus. In all cases, SOX2 IHC was only scored as positive when a clear nuclear staining was observed. The faint cytoplasmic staining was observed in most samples, and was considered as aspecific background staining. Scale bars: A, B and a 200μm

CDX2 is co-expressed in the SOX2 expressing CRC cells

In the mature gastrointestinal tract SOX2 is normally exclusively expressed in the rostral part of the digestive tract, i.e. in the esophagus and stomach epithelium, whereas it is completely excluded from the CDX2-positive intestine[11]. Since we detected ectopic SOX2 expression in intestinal tumors, we wondered whether there was simultaneous loss of CDX2, which would be suggestive for loss of intestinal identity. All samples stained uniformly for CDX2 (Fig 2B,D) including the SOX2-positive patches (Fig 2C), indicating that intestinal identity was retained. Thus, in contrast to the mutual exclusive expression pattern of SOX2 and CDX2 in the normal gastro-intestinal tract, we observed co-expression of both proteins in a subset of CRCs.

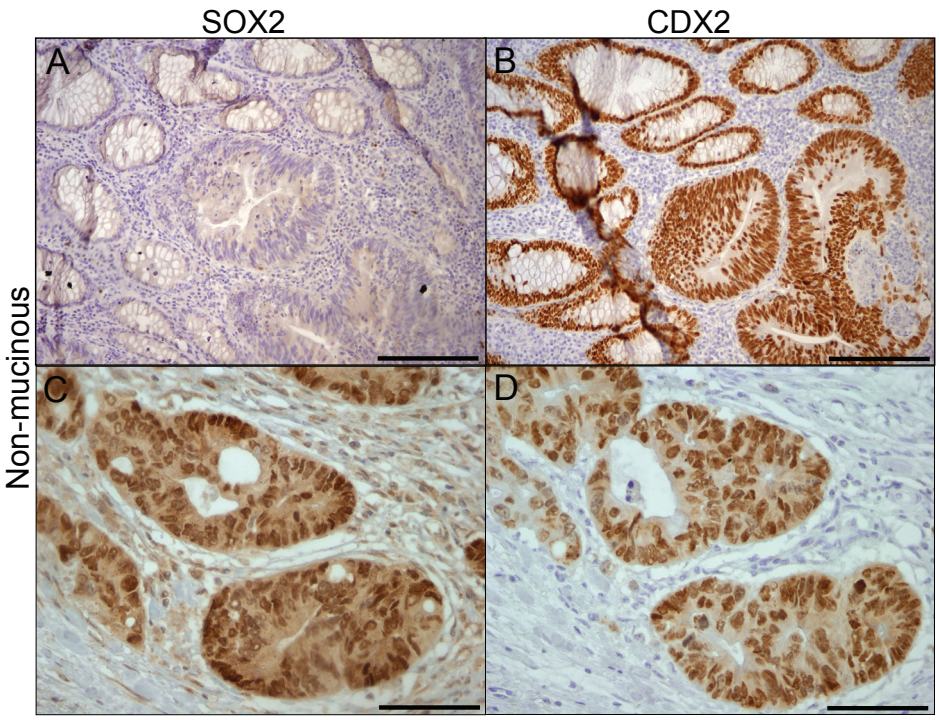

Figure 2: Co-expression of SOX2 and CDX2 in CRC.

Immunohistochemistry for (A,C) SOX2 and (B,D) CDX2 in two examples of non-mucinous CRCs. The upper tumor shows the reciprocal expression of SOX2 and CDX2, typically observed in the normal intestinal epithelium. Interestingly, SOX2 and CDX2 are clearly co-expressed in tumors with SOX2-positive patches. Scale bars: A, B 200μm, C,D 50μm

MUC5AC is expressed independently from SOX2 in both non-mucinous and mucinous CRC

Since it was previously shown that expression of SOX2 directly correlated with MUC5AC [7-8], we performed immunohistochemistry for this gastric foveolar mucin. Whereas MUC5AC expression was absent in the mucosa of normal

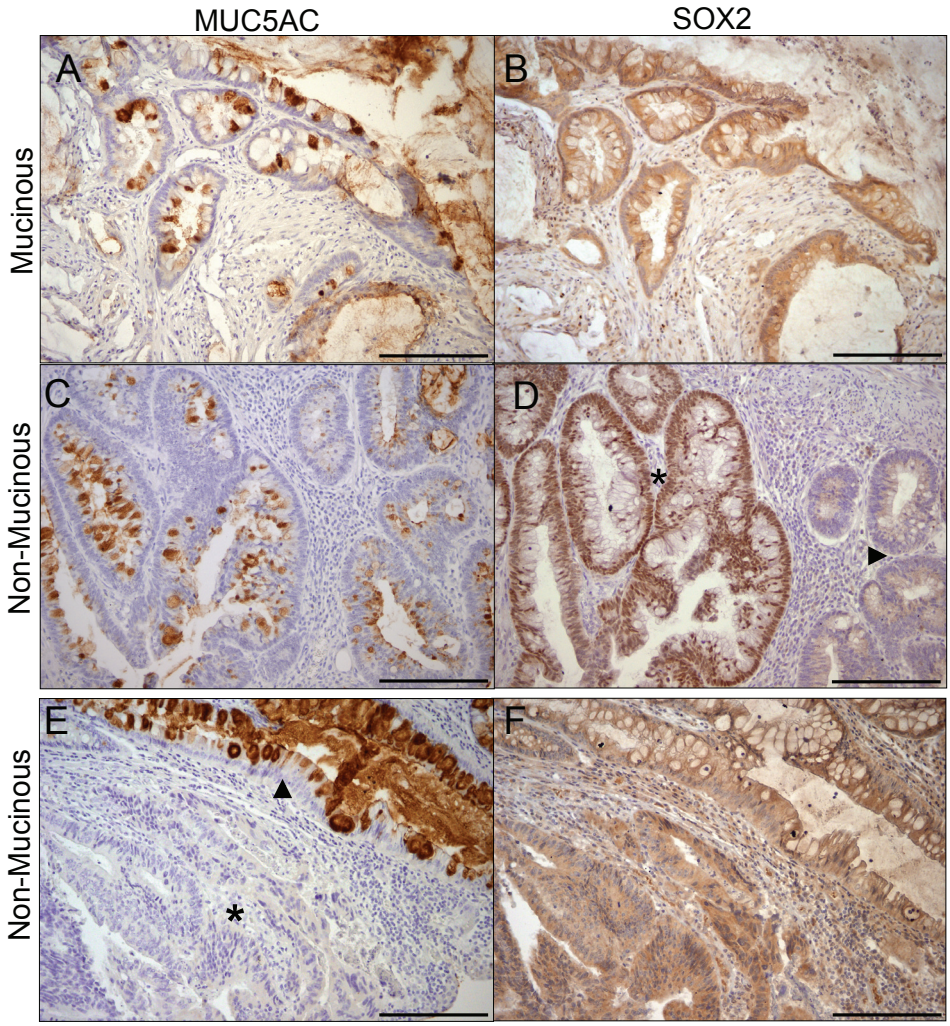

Figure 3: Aberrant expression of MUC5AC is independent of SOX2 expression.

Immunohistochemistry for MUC5AC (left) and SOX2 (right) on CRCs. (A,B) Example of a mucinous tumor showing positive MUC5AC staining, despite the complete absence of nuclear SOX2. (C,D) Example of a non-mucinous tumor, partially showing co-expression of MUC5AC and SOX2 (asterisk), and a MUC5AC-positive section devoid of SOX2 (arrowhead). (E,F) Ectopic MUC5AC expression was also observed in histologically normal SOX2-negative epithelium (E; arrowhead) adjacent to non-mucinous tumor tissue (E; asterisk). Scale bars: A-F 200μm

intestinal tissue, specific staining was observed in both the mucinous (Fig. 3A) and non-mucinous CRC (Fig. 3C) with similar frequency in both types. Ten out of 12 mucinous CRC expressed MUC5AC (83%), and 22 of the 34 non-mucinous CRC (65%). MUC5AC was detectable in mucinous CRC, but showed no correlation with SOX2 expression (Fig 3B). Moreover, MUC5AC was not exclusively expressed at sites with SOX2 expression in the non-mucinous CRC, as regions within the tumors displayed both cells that were only positive for MUC5AC and cells that co-expressed SOX2 and MUC5AC (Fig. 3C,D; asterisk and arrowhead, respectively. Furthermore, expression of MUC5AC was also detected in some areas of morphologically unaffected mucosa adjacent to tumor tissue (Fig. 3E), rather than in the actual carcinoma. These sites did not display SOX2 expression (Fig 3F). Thus, in our sample set we observed numerous examples of cells aberrantly expressing MUC5AC independent of SOX2.

Ectopic SOX2 expression and patient survival

Next, we analyzed the survival of patients with mucinous or non-mucinous CRC in relation with the expression of SOX2, to identify whether SOX2 expression can be correlated with survival. Comparing the overall survival of the total group of non-mucinous CRC with their mucinous counterpart did not show significant differences in survival (p=0.26) (Fig 4A). However, when we subdivided the group of non-mucinous CRC into SOX2-positive and negative groups, the patients with SOX2-positive CRC showed a statistically significant reduced survival compared to both the mucinous and the non-mucinous SOX2-negative group (p=0.02) (Fig 4B).

The finding that patients with a SOX2 expressing tumor showed a shorter survival than patients with SOX2-negative non-mucinous CRC, suggests that SOX2 might be useful as a prognostic marker. Therefore, we performed an explorative analysis on an additional set of 56 non-mucinous CRCs, which were analyzed for SOX2 expression. Out of the 56 tumors, 19 ectopicallly expressed SOX2. However, in this second cohort the survival of the patients with the SOX2-positive tumors was not significantly different from the SOX2-negative CRCs (p=0.56).

Next, we compared both study groups, to determine whether there was a difference in study population that might explain the difference in survival (Table 1).

No statistically significant differences were found for the median age of the patients, patient's sex, or left- versus right-sided localization of the tumor. Furthermore, the differentiation grade and tumor stage were also comparable

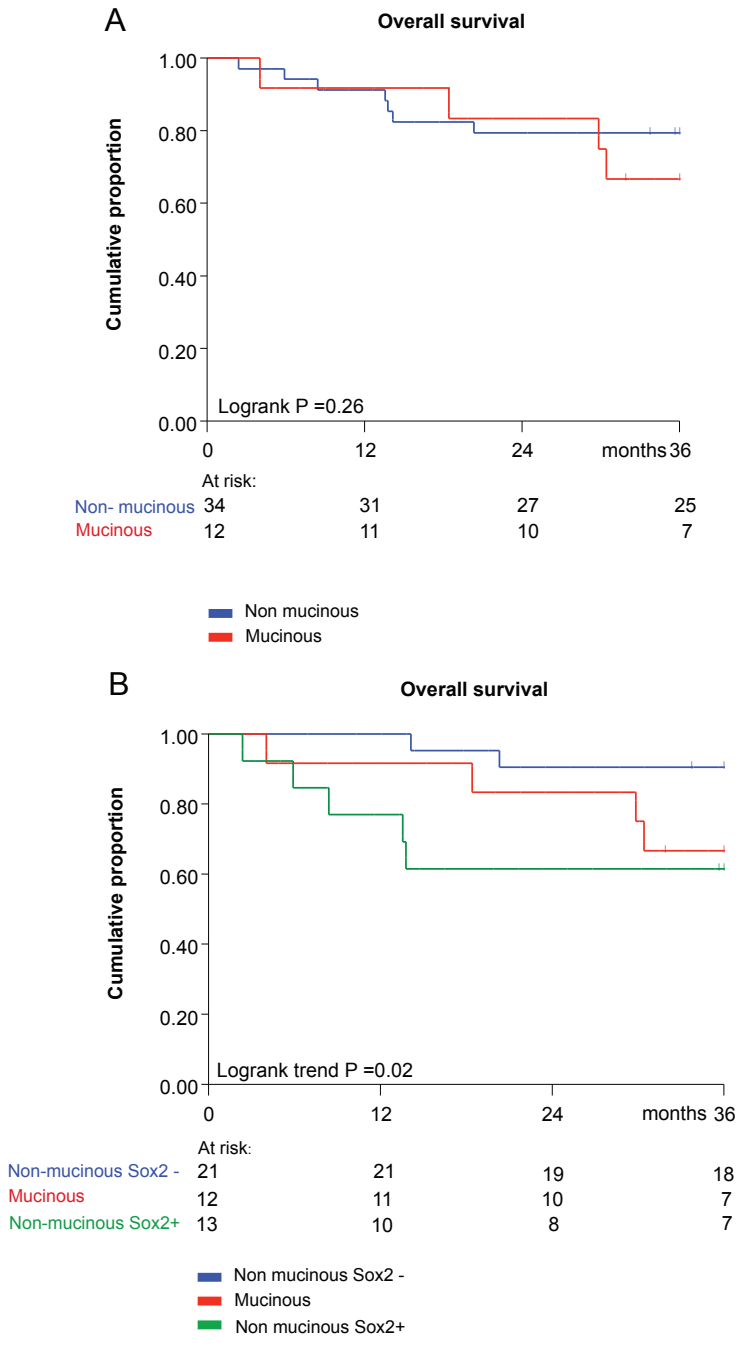

Figure 4: SOX2 expression in non-mucinous CRC correlates with poor survival.

(A) Kaplan Meier survival curves of all mucinous (red) and non-mucinous (blue) CRC of our first test cohort, reveals no significant difference in life expectancy (p=0.26). In (B) Kaplan Meier curves are shown of the same patients in which the non-mucinous CRC are separated in a SOX2-negative group (blue) and SOX2-positive (green) one. The non-mucinous SOX2-positive group showed a significant worse survival compared to both other groups (p=0.02).

between both groups. Lastly, in both study groups SOX2 was expressed in approximately one third of the tumors.

In conclusion, SOX2 is expressed in approximately one third of the non-mucinous CRCs. In the first group we tested, the SOX2-positive group showed a significantly shorter survival, compared to the SOX2-negative group. However, in a second cohort this correlation was not found. The difference between both results is not due to differences in patient or tumor characteristics.

Table 1: Patient and tumor characteristics for each study and test results for difference between studies.

	Mucinous N=12	non-mucinous 1 N=34	Non-mucinous 2 N=56	Significance Non-mucinous1 vs non-mucinous2
		Median(iqr)		
Age in years	60(6)	60(11)	62(10)	0,37*
		N(%)		
Sex M	5(36)	21(62)	31(55)	
V	7(64)	13(38)	25(45)	0,55#
Side L	10(91)	28(82)	43(77)	
R	1(9)	6(18)	13(23)	0,53#
Differentiation				
Good	0	3(9)	2 (4)	
Moderate	4(33)	24(71)	49(88)	
Poor	0	7(21)	4(7)	0,1[0#]#
Unknown	8(67)	0(0)	1(2)	
Distant metastasis				
No	10(83)	27(79)	51(91)	
Yes	2(17)	7(21)	5(9)	0,2&
Stage				
I	2(17)	7(21)	14(25)	
II	4(33)	12(35)	21(38)	
III	1(8)	7(21)	15(27)	
IV	2(17)	7(21)	5(9)	0,58#
unknown	3(25)	1(3)	1(2)	
Sox2 No	11(92)	21(62)	37(66)	
Yes	1(8)	13(38)	19(34)	0,68#

* Mann Whitney U-test
\# Chi-squared test
\## Chi-squared test, good+moderate combined and unknowns excluded
& Fisher exact test
Due to rounding percentages do not always add up to 100%.

Discussion

Colorectal cancer (CRC) can be subdivided in mucinous and non-mucinous CRC. A recent study suggested a putative link between SOX2 expression observed selectively in mucinous CRC, which portend to have a worse clinical prognosis [7]. Recently, aberrant Sox2 expression was associated with worse prognosis in lung and esophagus cancer [10], attributed to the induction of a precursor-like state induced by Sox2. As such, the ectopic SOX2 expression observed in CRCs may likewise affect tumor progression. In this study we aimed to further elucidate the expression behavior of SOX2, CDX2 and MUC5AC in both mucinous and non-mucinous CRC, and to examine whether SOX2 expression can be correlated with specific tumor characteristics and prognosis.

Our results show that SOX2 is expressed in approximately one third of the colorectal cancers. In stark contrast to the results of Park et al.we did however not observe any correlation with mucinous differentiation[7]. In fact, the proportion of non-mucinous cancers showing SOX2-positivity was significantly higher than their mucinous counterparts (13/34 versus 1/12). An explanation for these different observations, may reside in the use of different antibodies or different scoring of SOX2-positivity or mucinous differentiation.

In the normal gastro-intestinal tract SOX2 and CDX2 are expressed in a mutually exclusive manner, i.e. CDX2 marks all the intestinal epithelial cells, whereas SOX2 is expressed in the stomach and esophagus. Here we report that the SOX2-positive patches in colorectal cancers retain normal expression of CDX2, meaning that both proteins are co-expressed in the same cells. We previously showed that co-expression of Sox2 and Cdx2 in the developing embryonic mouse intestine resulted in a loss of Cdx2 binding to its target sequences, leading to loss of intestinal identity and the acquisition of a gastric-like phenotype[13]. As such, the aberrant SOX2 expression could likewise affect the differentiation direction of the colorectal cancer cells. However, histological evaluation of the SOX2-positive cells did not provide any indication of differences in the cellular phenotype of SOX2-positive and negative cells, as both showed the typical dysplastic features of colorectal cancer.

Previously, a second link between SOX2 and the acquisition of gastric features was made by showing within colorectal cancers concordant expression of SOX2 with the gastric foveolar mucin MUC5AC[7]. In support of this, SOX2 was also shown to activate MUC5AC reporter constructs and to increase MUC5AC expression in COS-7 cells[7-8]. MUC5AC expression is however observed in a large proportion of colorectal cancers, far exceeding the fraction of SOX2-positive

tumors[5-6]. Also in our case, we observed between 65-83% of MUC5AC-positive tumors. Moreover, in our sample set we observed numerous examples of cells aberrantly expressing MUC5AC independent of SOX2, indicating that although SOX2 may contribute to MUC5AC expression, it is not absolutely required. Taken together, our data do not support a role for aberrant SOX2 in the induction of gastric features within colorectal cancer.

Aberrant SOX2 expression has however also been linked to the induction of precursor-like features in various cancer types, associated with bad prognosis[10]. Therefore, we tested the survival of patients with SOX2-positive and SOX2-negative tumors in two different cohorts. In the first cohort, the survival of the SOX2-positive subgroup was significantly less compared to the SOX2-negative group. This is in support of several recent publications showing that increased SOX2 expression in colorectal cancers correlated with lymph node metastases and distant recurrence[16-18]. In the second cohort we could not detect differences in survival. These apparent contradicting results were not due to differences in age or sex of the patients in both cohorts, nor were significant differences found in tumor localization, differentiation grade of the tumor, or stage of the disease. Since the majority of the SOX2 positive cases do not show a uniform SOX2 expression, but have patchy staining patterns, it is conceivable that in a significant number of cases the SOX2 positive are missed in case only a small portion of the tumor is available for analysis. Therefore, SOX2 should be validated in a bigger cohort to test whether SOX2 indeed imposes a worse prognosis.

In conclusion, we show that SOX2 is expressed in non-mucinous CRC rather than mucinous CRC. Expression of SOX2 coincides in all cases with CDX2, whereas MUC5AC is not exclusively expressed in SOX2-positive colorectal cancer cells. Additionally, we have indications that SOX2 expression in non-mucinous CRC is associated with a worse survival.

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CHAPTER 7

Induced non-canonical Wnt5a expression perturbs embryonic outgrowth and intestinal elongation, but is well-tolerated in adult mice

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Abstract

Wnt5a is essential during embryonic development, as indicated by mouse *Wnt5a* knockout embryos displaying outgrowth defects of multiple structures including the gut. The dynamics of Wnt5a involvement in these processes is unclear, and perinatal lethality of *Wnt5a* knockout embryos has hampered investigation of Wnt5a during postnatal stages in vivo. Although in vitro studies have suggested a relevant role for Wnt5a postnatally, solid evidence for a significant impact of Wnt5a within the complexity of an adult organism is lacking. We generated a tightly-regulated inducible *Wnt5a* transgenic mouse model and investigated the effects of Wnt5a induction during different time-frames of embryonic development and in adult mice, focusing on the gastrointestinal tract. When induced in embryos from 10.5 dpc onwards, Wnt5a expression led to severe outgrowth defects affecting the gastrointestinal tracts, limbs, facial structures and tails, closely resembling the defects observed in *Wnt5a* knockout mice. However, Wnt5a induction from 13.5 dpc onwards did not cause this phenotype, indicating that the most critical period for Wnt5a in embryonic development is prior to 13.5 dpc. In adult mice, induced Wnt5a expression did not reveal abnormalities, providing the first in vivo evidence that Wnt5a has no major impact on mouse intestinal homeostasis postnatally. Protein expression of Wnt5a receptor Ror2 was strongly reduced in adult intestine compared to embryonic stages. Moreover, we uncovered a regulatory process where induction of Wnt5a causes downregulation of its receptor Ror2. Taken together, our results indicate a role for Wnt5a during a restricted time-frame of embryonic development, but suggest no impact during homeostatic postnatal stages.

Introduction

Whereas the relevance of canonical Wnt/β-catenin signaling in intestinal homeostasis and cancer is well established [1-2], so-called non-canonical Wnt signaling is far less understood. Non-canonical Wnt signaling is represented by multiple alternative signaling routes that act independent of β-catenin transcriptional activity and are typically activated by non-canonical Wnt ligands. Wnt5a is the most extensively studied non-canonical ligand and has gained substantial attention over the last years as it is being implicated in various human diseases including cancer, inflammatory diseases and metabolic disorders [3-5]. Signaling pathways that can be activated by Wnt5a include Wnt/Ca^{2+} and Wnt/JNK pathways, also referred to as the planar cell polarity pathway, are primarily known to mediate oriented cell movements during development [3-4 6-8]. Additionally, Wnt5a can inhibit β-catenin signaling, although promoting effects have also been demonstrated in receptor overexpression experiments [9]. Importantly, which pathway is actually activated by Wnt5a greatly depends on the context, especially dictated by receptor presence. The best characterized Wnt5a receptor is receptor tyrosine kinase-like orphan receptor 2 (Ror2) [9-12]. Members of the family of Frizzled receptors, Ror1 and receptor-like tyrosine kinase Ryk have also been implicated to act as (co)receptors in Wnt5a signaling [4 9 13-14].

The indispensable nature of Wnt5a during embryogenesis is indicated by the perinatal lethality of homozygous *Wnt5a* knockout mice [8]. During development, Wnt5a expression is predominantly detected in structures undergoing extensive outgrowth, including the limbs, tail and facial structures [8 15]. Expression in these structures is in a graded fashion with highest levels towards the outer tips and most pronounced between 10 and 14 days post coitum (dpc) [8 15]. Loss of *Wnt5a* results in a drastic shortening of these structures in mouse embryos [8]. Importantly, the embryonic phenotype of *Ror2* homozygous knockout mice closely resembles the *Wnt5a* knockout phenotype, although appearing milder [16-17]. Thus, during embryonic development both Wnt5a and its main receptor Ror2, are essential for the proper outgrowth of multiple structures. This also applies to the gastrointestinal tract. Normal gut development involves temporal and spatial specific expression of Wnt5a and Ror2. Both Wnt5a and Ror2 expression peak between 10.5 and 13.5 dpc, a critical period when midgut elongation occurs. Wnt5a expression is restricted to the mesenchyme of the caudal midgut and rostral hindgut (i.e. future distal small intestine and proximal large intestine), suggested to correlate with regions of the small intestine that undergo elongation [18-20]. Ror2 expression shows a complex region-specific pattern in both the mesenchyme and epithelium

[20]. Homozygous knockout of either *Wnt5a* or *Ror2* results in shortened embryonic intestines, in addition to an imperforate anus in *Wnt5a* knockout embryos [18]. The dynamics of Wnt5a functioning are not completely understood to date. Given that endogenous Wnt5a expression in the gut is most prominent between 10.5-13.5 dpc and diminishes after that might be an indication that Wnt5a is most crucially involved in developmental elongation processes in this specific period. However, it remains to be clarified whether the functioning of Wnt5a extends beyond this stage. Relevant postnatal roles of Wnt5a have been suggested based upon associative Wnt5a expression studies and in vitro studies. Hence, enhanced Wnt5a expression has been observed in a variety of human diseases, including inflammatory conditions [21-22], metabolic disorders [4-5 23] and a multitude of cancer types, among which colorectal cancer [3 24-25]. Also, a wide variety of in vitro studies have implicated Wnt5a in multiple cellular processes, including cell growth, differentiation, migration and invasion [3-4 7 26]. Despite these supportive data, solid in vivo evidence for a significant postnatal impact of Wnt5a is lacking. To date, it has not been possible to modulate and investigate the activities of Wnt5a in an adult organism. We have generated an inducible *Wnt5a* transgenic mouse model, enabling in vivo determination of the relevance and activities of Wnt5a during desired time-frames. Transgenic Wnt5a expression was induced during specific time-frames of embryonic development and during adulthood, followed by a phenotypic analysis focusing on the intestinal tract.

Materials and methods

Generation of inducible Wnt5a mouse model and in vivo doxycyline administration

To generate the TetO-*Wnt5a* construct, a 1.1 kb XbaI fragment was isolated from a TetO-FLAG-*Wnt5a* vector (kindly provided by Dr. R. Nusse, Stanford University, CA), and inserted into the pTRE-Tight vector containing an additional 0.68 kb b-globin intron. The 2.4 kb expression cassette was XhoI digested from the plasmid backbone, gel-purified and injected into C57Bl/6J oocytes by standard procedures. TetO-*Wnt5a* positive founders were identified by PCR using transgene specific primers SCT2F (5'-CTACACCCTGGTCATCATCC) and mWnt5AFLAG-R (5'-TCGTACCTAGAGACCACCAAG).

TetO-*Wnt5a* mice were interbred with hnRNP-*rtTA2S-M2* mice [27], obtaining double transgenic mice. Wildtype and single transgenic mice were generated simultaneously and served as control animals. Doxycycline (dox) was administered via the drinking water (2 mg/ml dox, 5% sucrose). Induction during embryogenesis was performed by administering dox to timed pregnant females.

All mice were generated, bred and maintained under specific pathogen-free conditions at the animal facility of the Erasmus MC University Medical Center. All animal experiments were approved by the Institute's Animals Ethics Committee and were carried out in accordance with Dutch legislation.

Cell culture and treatments

Tail fibroblasts were isolated from adult mice by incubating tail tips in Dulbecco's modified essential medium (DMEM, Lonza) complemented with 0.14 U/ml Blendzyme (Roche), 1% antibiotics/antimycotics (Gibco) and 0.5% fetal bovine serum (FBS, Sigma Aldrich) for 3 hours at 37°C. Mixture was put on a cell strainer and cells were cultured in DMEM with 1% antibiotics/antimycotics and 10% FBS at 37°C, 5% CO_2. Mouse embryonic fibroblasts (MEFs) were isolated from embryos of 13.5-15.5 dpc and cultured and transfected as previously described.[28] When indicated, cells were induced with 1 ug/ml dox. HEK293T cells were cultured in DMEM with 10% FBS, 1% p/s at 37°C, 5% CO_2.

β-catenin reporter assay

β-catenin reporter assay in MEFs was performed as previously described [28]. A Ror2 expression vector (kindly provided by Dr. A. Kikuchi, Osaka University, Japan) was co-transfected to assure sufficient receptor availability for the transgenic Wnt5a. Transfected cells were provided with 1 ug/ml dox for 24 hours of which the final 16 hours cells were simultaneously stimulated with either 25% of L-control or L-Wnt3a conditioned medium. Luciferase activities were measured using the dual-luciferase reporter assay system (Promega) and a LUMIstar luminometer. Assays were performed in duplicate and performed twice.

Immunoblotting

Cultured cells and snap-frozen intestinal tissue specimens were lysed in Laemmli sample buffer containing 0.1M DTT and incubated at 95 °C for 10 min. Western blotting was performed according to standard chemoluminescent procedures or using fluorescent Odyssey immunoblotting (LI-COR Biosciences, Lincoln, NE, USA) [28]. Antibodies are indicated in Supplementary Table I. Quantification was performed using Odyssey Li-Cor software.

Skeletal preparations and staining

Embryos were skinned, eviscerated, fixed overnight in 96% ethanol containing 1% glacial acetic acid. Cartilage was stained overnight in 0.5 mg/ml Alcian Blue. Soft tissues were dissolved in 1.5% potassium hydroxide for 3 hours followed by staining of bone overnight in Alizarin Red and destaining in 0.5% potassium hydroxide/20% glycerol.

Tissue processing and histology

Adult intestines, whole embryos or embryonic intestines were isolated, washed in PBS and fixed overnight in 4% PBS-buffered paraformaldehyde (4% PFA) at 4°C. Fixed embryonic intestines were pre-embedded in 5% bacto-agar (BD) in PBS and cut into 6-8 pieces enabling proper identification of the different regions. All mouse materials were embedded in paraffin according to routine protocols.

Haemotoxylin eosin (HE), Periodic Acid Schiff (PAS) and Alkaline Phosphatase (AP) stainings were performed according to routine protocols. Immunohistochemistry was performed using antigen retrievals and antibodies

as indicated in Supplementary Table II. Visualization was performed using the Envision+ System or StreptABCcomplex/HRP (Dako).

RNA isolation, cDNA synthesis and quantitative PCR

RNA was isolated from snap-frozen embryonic intestines and adult duodenum tissues using Nucleospin RNA II Kit (Machery-Nagel, Düren, Germany), followed by cDNA generation using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed using Sensimix SYBR Green (Bioline) or TaqMan (in case of *Axin2/Actb*) Gene Expression Assays (Applied Biosystems) and run in the IQ5 Real time PCR detection system (Bio-Rad). Primers are shown in Supplementary Table III. Expression levels were corrected for expression of *Actb*, averaged and presented as fold changes.

Results

Tight regulation of functionally active Wnt5a protein in TetO-Wnt5a transgenic mouse model

We generated TetO-*Wnt5a* mouse founder lines carrying the mouse *Wnt5a* gene under the control of a Tet-inducible promoter. This promoter is inducible by the rtTA transcription activator in a doxycyline (dox) dependent manner. TetO-*Wnt5a* founders were crossed with the hnRNP-*rtTA* transgenic mouse line, which drives ubiquitous expression of the *rtTA2S-M2* gene [27]. Dox administration allows transgenic Wnt5a induction only in double transgenic mice carrying both TetO-*Wnt5a* and hnRNP-*rtTA* constructs. Wnt5a expressing founders were identified by culturing tail fibroblasts, providing a founder with

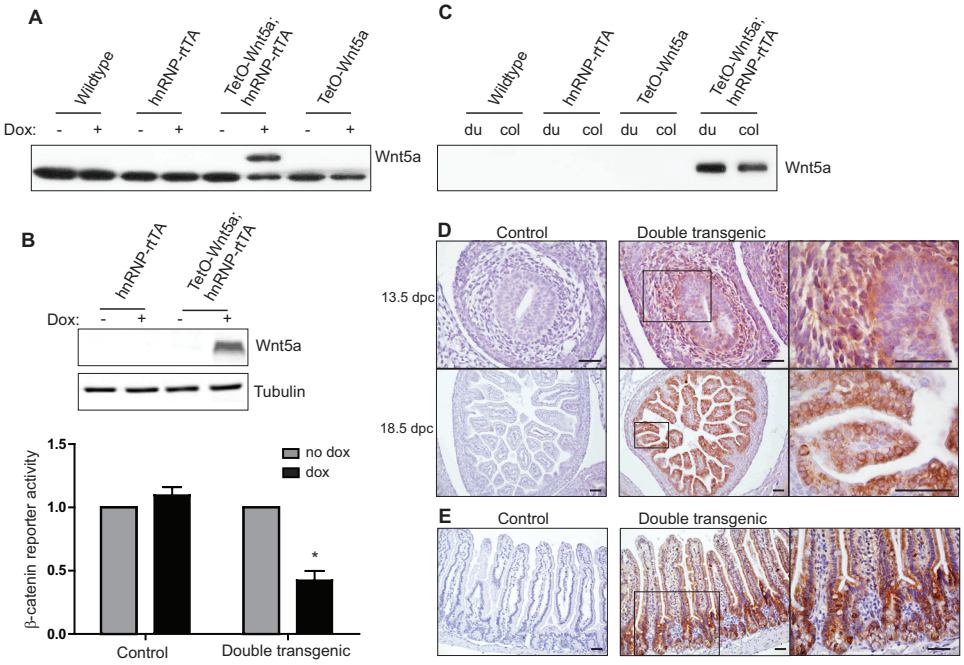

Figure 1. Tight regulation of functionally active Wnt5a protein in TetO-*Wnt5a* transgenic mouse model.

(A) Wnt5a immunoblot of tail fibroblasts and (B) MEFs. Wnt5a antibody only detects overexpressed Wnt5a because of low sensitivity. β-catenin reporter assay showing that Wnt3a-induced β-catenin signaling is reduced following transgenic Wnt5a induction, * p=0.015. Two individual MEF lines were used for each genotype. (C) Intestine of adult mice induced for 1 week analyzed by Wnt5a immunoblot (du=duodenum, col=colon) and by (D) immunohistochemical Wnt5a staining. Abundant Wnt5a expression is revealed in double transgenic embryos (dox from 10.5 dpc onwards). and (E) adult intestine.

robust Wnt5a protein expression upon dox administration without displaying Wnt5a expression in controls (figure 1A).

Similar results were observed in mouse embryonic fibroblasts (MEFs) (figure 1B and S1A). To validate the functionality of the transgenic Wnt5a protein, we determined its capability to inhibit canonical Wnt/β-catenin signaling. MEF lines of control and double transgenic genotypes were stimulated with Wnt3a to enhance β-catenin signaling and doxycyline was added to induce transgenic Wnt5a (figure 1B, upper panel). β-catenin mediated transcription was significantly decreased upon induction of transgenic Wnt5a (figure 1B, lower panel). This indicates that upon dox induction in double transgenic MEFs, the cells produce functionally active Wnt5a protein.

In vivo dox administration successfully induced transgenic Wnt5a expression in double transgenic mice exclusively, as shown by immunoblotting and immunohistochemistry (figure 1C-E). Induction was a rapid process since transgenic Wnt5a was detected already within 24 hours following dox administration (figure S1B). In adult and embryonic mice, expression of transgenic Wnt5a protein was verified along the entire intestinal tract by immunohistochemistry (data not shown). When induction was performed during embryonic development from 10.5 dpc onwards, transgenic Wnt5a expression was observed in gut mesenchyme and epithelium at 13.5 dpc, shifting towards a predominant epithelial expression pattern at 18.5 dpc (figure 1D). In the adult intestine, induced Wnt5a was most prominent in the epithelial cells and stromal expression was clearly present although less abundant, displaying an overall diffuse staining in addition to several intensely stained individual cells (figure 1E). Wnt5a expression was not detected in muscle cells. Although all following experiments were performed with above described TetO-*Wnt5a* founder line unless mentioned differently, we validated a second TetO-*Wnt5a* founder which displayed a mosaic Wnt5a expression pattern (figure 6C and S1C).

Induced Wnt5a expression deregulates embryonic development

Wnt5a is indispensable during mouse development, since its absence results in defective embryonic outgrowth and perinatal lethality [8]. We induced Wnt5a expression during several time-frames of embryogenesis to investigate the developmental consequences of overexpressing Wnt5a. Dox-induced double transgenic mice will be further referred to as Wnt5a^{ind} and compared to dox-induced control mice. Wnt5a induction from 6.5 dpc to 13.5 dpc resulted in severe developmental defects (figure 2A).

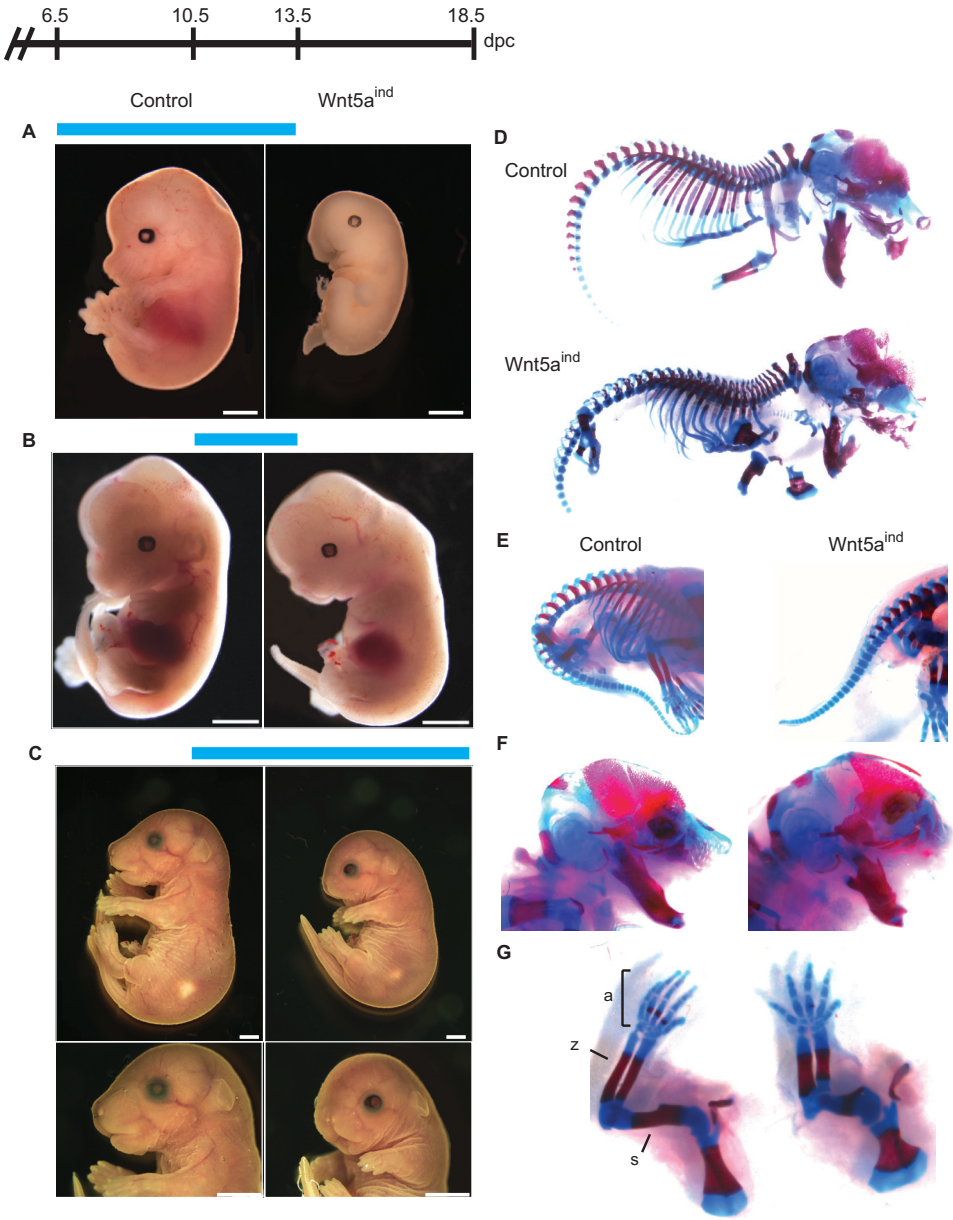

Figure 2. Induced Wnt5a expression deregulates embryonic development.

Macroscopic appearance of dox-induced control versus double transgenic embryos. Time-frames of dox administration are indicated by blue bars against timeline. (A) Wnt5a^{ind} embryos at 13.5 dpc, induced from 6.5 dpc onwards, were already necrotic. (B,C) Embryos at respectively 13.5 and 18.5 dpc, induced from 10.5 dpc onwards, showing deficient outgrowth of face, limbs and tail. (D-G) Skeletal stainings of 17.5-18.5 dpc embryos, comparing similarly aged embryonic structures. (D) Overview of control (upper) and Wnt5a^{ind} (lower) phenotype. Limbs and tails are not complete in this figure panel. Clear shortening is observed affecting (E) tail (F) head (G) forelimb (a=autopod, z=zeugopod, s=stylopod).

Externally, primarily the head, limbs and tail showed outgrowth defects. As the Wnt5a^{ind} embryos had already died in utero at 13.5 dpc, necrosis precluded a more detailed examination of these embryos. Starting induction of transgenic Wnt5a later, i.e. at 10.5 dpc, did not result in embryonic lethality but led to facial deformities and shortened limbs and tail (figure 2B-C). Similar defects were observed using our mosaic Wnt5a founder (figure S2A). Later dox inductions, from 13.5 dpc to 18.5 dpc, did not lead to apparent developmental defects in Wnt5a^{ind} embryos (figure S2B). Examination of skeletal stainings provided insight regarding the basis of the externally visible defects in late-stage embryos that had been induced from 10.5 dpc onwards (figure 2D). The tails of Wnt5a^{ind} embryos were clearly shortened, caused by a decreased number of vertebrae (figure 2E). In the head, the most severely shortened bones are those in the upper jaw, while lower jaw elements were mildly shortened (figure 2F). Limb shortening was chiefly caused by deficient outgrowth of individual skeletal elements, including stylopodal and zeugopodal bones which appear thicker than normal as well (figure 2G). Autopods appeared underdeveloped with shorter and thicker digits and a delay of ossification events, but no obvious loss of skeletal elements as has been reported for *Ror2* and *Wnt5a* knockout embryos was observed. Together, these experiments revealed that Wnt5a overexpression causes embryonic defects resembling those observed in *Wnt5a* KO and *Ror2* KO embryos, affecting the structures whose progenitors normally express Wnt5a in a graded fashion. Time dependency, i.e. before 13.5 dpc, of the Wnt5a overexpression phenotype matches the period in which endogenous Wnt5a is normally expressed most abundantly [8 15 18-20].

Induced Wnt5a expression disturbs embryonic intestinal elongation

In the developing gastrointestinal tract, Wnt5a expression is detected in the mesenchyme of the caudal midgut, rostral hindgut and stomach fundus [18-20]. Loss of *Wnt5a* leads to severely shortened gastrointestinal tracts [18]. Interestingly, we found that time-specific Wnt5a overexpression from 10.5 dpc until 18.5 dpc also resulted in drastic shortening of the small and large intestine, cecum and stomach (figure 3A-C).

Furthermore, Wnt5a^{ind} embryos displayed an imperforate anus (figure 3D), as has also been observed in *Wnt5a* KO embryos [18]. A similar phenotype was revealed using the mosaic TetO-*Wnt5a* founder line, albeit less drastic (figure S2C). The phenotype was not observed when Wnt5a expression was induced from 13.5 dpc onwards (figure S2D). This indicates that the activities

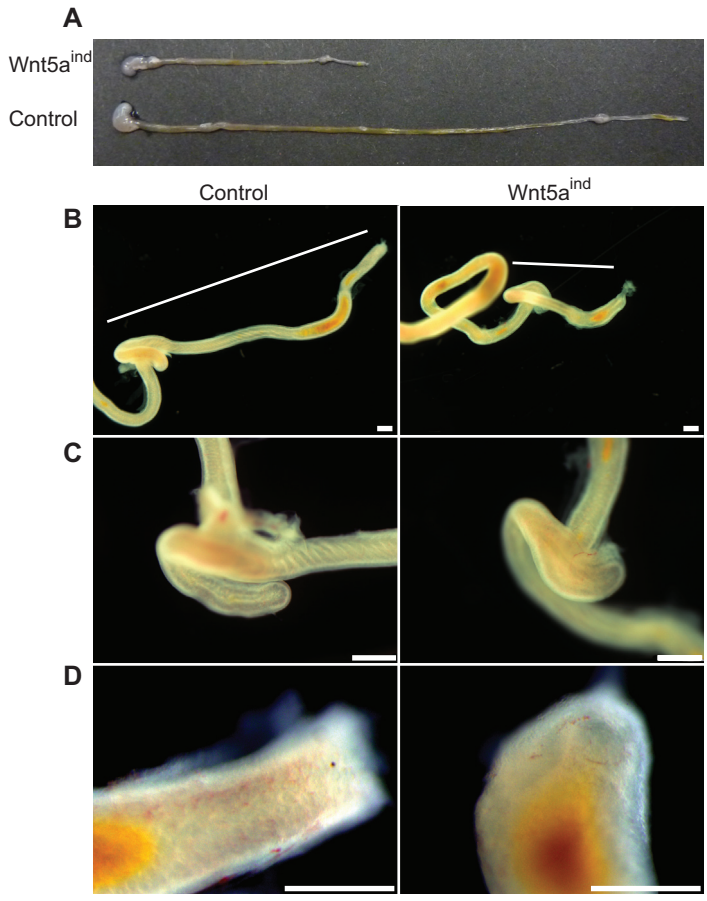

Figure 3. Induced Wnt5a expression disturbs embryonic intestinal elongation.

Gastrointestinal structures of 18.5 dpc embryos induced from 10.5 dpc onwards. (A) Overview of gastrointestinal tracts (B) Higher magnification of the large intestine and (C) cecum. (D) The Wnt5a^{ind} embryonic anus is imperforated.

by which induced Wnt5a interferes with intestinal elongation mainly take place between 10.5 and 13.5 dpc, which is the phase of intestinal elongation when a rapid increase in length occurs and when Wnt5a is normally expressed most abundantly. Together, our data demonstrate that ubiquitous Wnt5a overexpression causes gastrointestinal elongation defects resembling those observed in *Wnt5a* KO and *Ror2* KO embryos, when induced from 10.5 dpc but not from 13.5 dpc onwards, reflecting the time-frame when Wnt5a is normally expressed at highest levels [8 15 18-20].

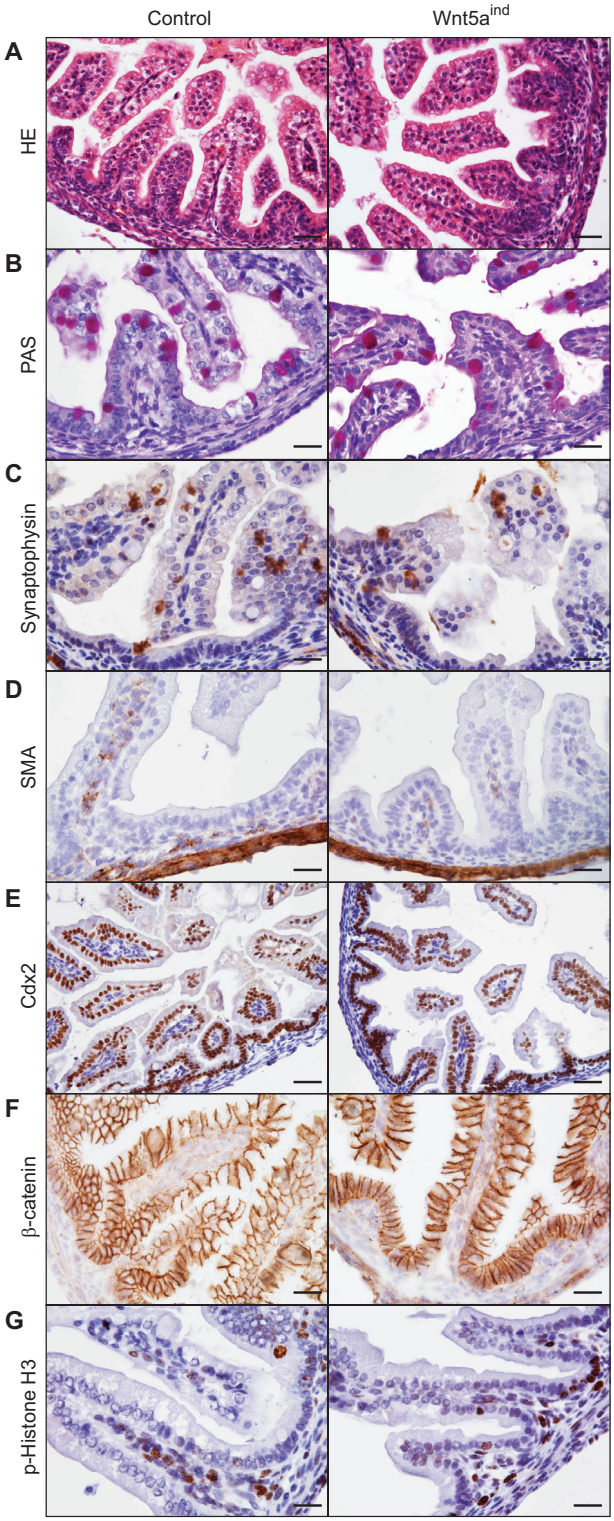

Figure 4. Wnt5a does not alter cell fate during intestinal development.

Stainings of intestinal cross-sections of 18.5 dpc embryos of control versus double transgenic genotypes, all induced from 10.5 dpc onwards. (A) HE and (B) PAS staining show unaffected histology and goblet cell presence. (C) Staining for synaptophysin and (D) SMA reveal no differences in enteroendocrine cells and smooth muscle cells respectively. (E-G) Cdx2, β-catenin and phospho-Histone H3 appear unaffected.

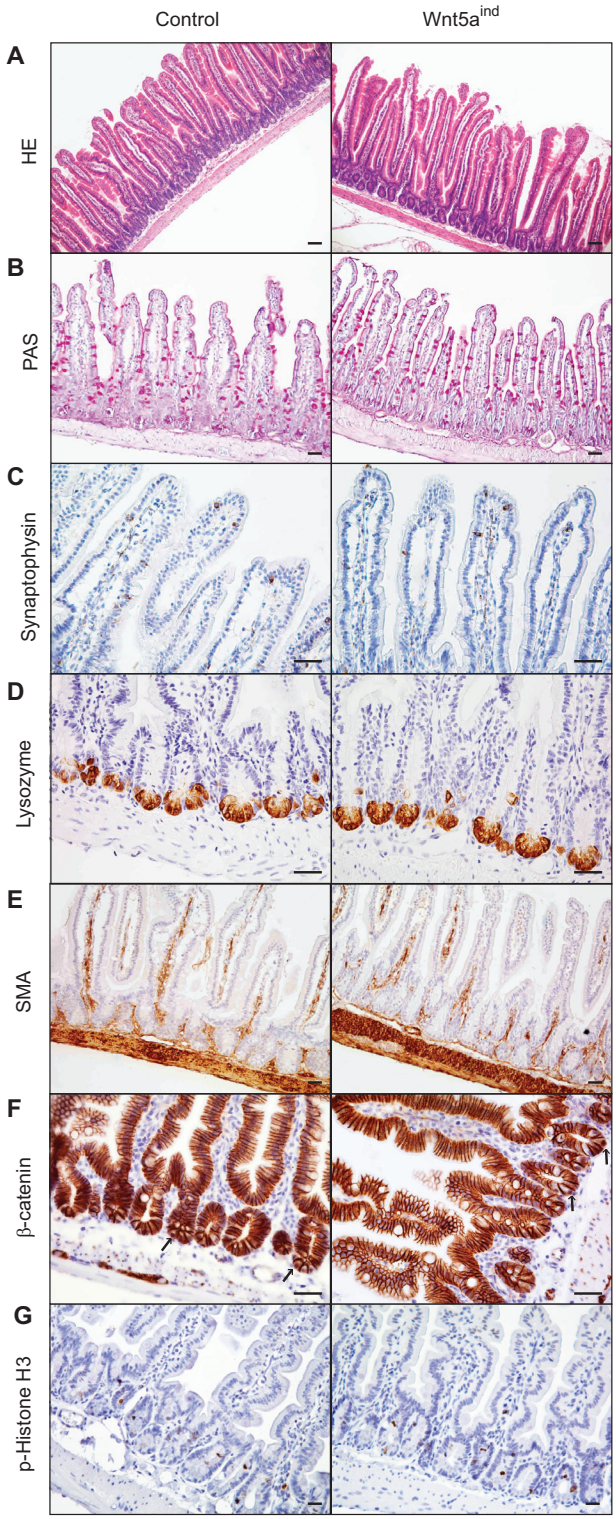

Figure 5. Induced Wnt5a expression is well-tolerated in the adult mouse intestine.

Stainings of intestinal sections of adult mice induced for 1 week. (A) HE and (B) PAS staining show unaffected histology and goblet cell presence. (C) Staining for synaptophysin and (D) lysozyme reveal no differences in enteroendocrine cells and paneth cell presence respectively. (E) SMA staining reveals no obvious differences. (F) β-catenin staining shows equal membranous and nuclear (arrowheads) expression. (G) phospho-Histone H3 staining indicates unaffected proliferation.

Wnt5a does not alter cell fate during intestinal development

To investigate whether the differentiation state of the embryonic Wnt5a^{ind} shortened intestines was altered, intestines of embryos induced from 10.5 dpc until 18.5 dpc were isolated and analyzed extensively. Hematoxylin eosin (HE) staining revealed no histological aberrations in Wnt5a^{ind} embryonic intestines (figure 4A).

Presence of goblet and enteroendocrine cells was not influenced by Wnt5a induction as determined by Periodic Acid Schiff (PAS), Muc2 and synaptophysin staining (figure 4B-C and S3A). Smooth muscle actin (SMA) staining showed normal smooth muscle layer development and expression of homeobox protein Cdx2 was unaffected (figure 4D-E). Furthermore, no abnormal localization of either β-catenin or E-cadherin expression was observed in the intestines of Wnt5a^{ind} embryonic intestines (figure 4F and S3B). Proliferation of intestinal cells determined by phospho-histone H3 staining revealed no gross differences in proliferation rate among the different genotypes (figure 4G). Altogether this indicated that Wnt5a has no impact on embryonic intestinal cell fate, in line with similar findings in *Wnt5a* knockout embryos [18].

Induced Wnt5a expression is well-tolerated in the adult mouse intestine

To determine the consequences of increased Wnt5a expression postnatally, we performed doxycycline inductions in adult mice for multiple durations, including 1 day, 1 week and 3-5 months. During the induction period, the overall appearance of the mice remained unaffected. Following the inductions, mice were examined for abnormalities with a specific focus on the intestinal tract. No gross abnormalities in length and macroscopic appearance were observed as a consequence of transgenic Wnt5a induction. HE staining revealed no histological abnormalities in the intestines of Wnt5a^{ind} mice (figure 5A).

Staining for specific intestinal cell types including PAS for goblet cells, synaptophysin for enteroendocrine cells, lysozyme for Paneth cells, SMA for muscle and stromal cells and alkaline phosphatase (AP) for enterocytes all showed no changes upon transgenic Wnt5a expression in adult mice (figure 5B-E and S3C). Immunohistochemical staining of β-catenin revealed no aberrant expression, displaying membranous and nuclear β-catenin in Wnt5a^{ind} intestine comparable to corresponding control mice (figure 5F). E-cadherin staining was unaffected (figure S3D) and also proliferation of intestinal cells was unaltered (figure 5G). Altogether, induced Wnt5a expression is tolerated well by adult mice and does not affect postnatal intestinal homeostasis and cell fate.

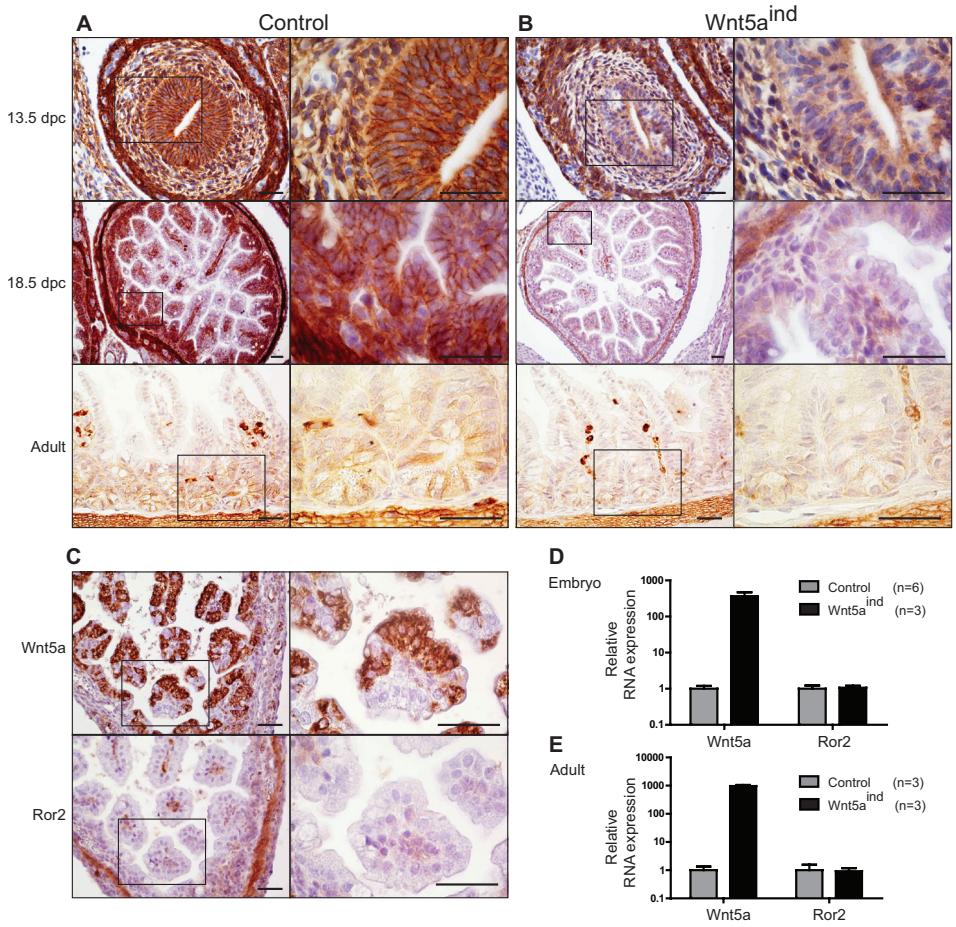

Figure 6. Wnt5a downregulates Ror2 protein in embryonic and adult intestine.

All embryos were subjected to dox induction from 10.5 dpc onwards and adult mice were induced for 1 week. In adults, intensely stained stromal cells assumingly represent IgG producing cells, unintentionally detected by our secondary anti-mouse antibodies. (A) Immunohistochemical Ror2 staining of cross-sections of 13.5 and 18.5 dpc embryonic intestines of control genotypes. Relatively low Ror2 expression is observed in adult intestine of control genotype (lower panel). (B) Drastically reduced Ror2 expression in Wnt5a^{ind} embryonic and adult intestines. (C) Mosaic Wnt5a expression in 18.5 dpc Wnt5a^{ind} embryonic intestine using the alternative TetO-*Wnt5a* founder (upper panel). Ror2 expression is reduced in a general fashion. (D,E) Relative *Wnt5a* and *Ror2* RNA expression in 17.5 dpc embryonic and adult intestine respectively.

Wnt5a downregulates Ror2 protein in embryonic and adult intestine

As Ror2 is an important receptor mediating Wnt5a signaling, we determined Ror2 protein expression by immunohistochemistry. All embryos were subjected to dox induction from 10.5 dpc onwards and adult mice were induced for 1 week. Examination of Ror2 protein expression in 13.5 and 18.5 dpc

embryonic intestines of control genotypes revealed abundant expression in the mesenchyme and muscle layers, and membranous Ror2 in the epithelium (figure 6A).

In the adult intestine of control genotypes, Ror2 protein levels were strongly reduced compared to embryonic stages, presenting mainly in the muscle layers while along the crypt-villus structures only low level expression was observed in crypt compartments (figure 6A, lower panel). Reduced *Ror2* expression in adult compared to embryonic intestine was confirmed on RNA level (data not shown). Interestingly, we found that intestinal Ror2 protein expression was strongly reduced in corresponding Wnt5a^{ind} mice (figure 6B). This Ror2 downregulation was particularly clear at embryonic stages, when a major reduction in all gut compartments, including muscle, epithelium and mesenchyme was observed. In the adult intestine, Wnt5a induction reduced the expression of Ror2 in muscle cells and to barely detectable levels in crypt cells. Notably, using the alternative founder with a mosaic Wnt5a expression pattern we also observed an overall downregulation of Ror2 protein in 18.5 dpc Wnt5a^{ind} intestines, even in regions that do not overexpress Wnt5a. This indicates that secreted transgenic Wnt5a ligands acted in a paracrine fashion (figure 6C). *Ror2* RNA levels were not affected in the Wnt5a^{ind} intestines of both the embryonic and adult stages, despite the clear increase in *Wnt5a* RNA production (figure 6D and E), demonstrating that Wnt5a does not regulate Ror2 expression at the level of RNA production. In contrast to the reduced Ror2 protein levels that we observed in Wnt5a^{ind} embryonic intestines, we did not observe altered Ror2 protein expression in the intestines of homozygous *Wnt5a* knockout mice compared to wildtype or heterozygous littermates (figure S4A-B). We did however observe downregulation of endogenous Ror2 protein on cultured HEK293T cells and MEFs upon Wnt5a stimulation (figure S5). Taken together, our data show that Wnt5a reduces its receptor Ror2 on the protein level in a paracrine fashion.

Induced Wnt5a expression enhances downstream signaling in embryonic intestine

The finding that induced Wnt5a expression reduces Ror2 protein levels raised the question what the consequences are for downstream Wnt5a signaling. We determined the levels of Wnt5a downstream targets in embryonic intestines (induced 10.5-17.5 dpc) by immunoblotting. Ror2 protein levels were strongly reduced in Wnt5a^{ind} mice, confirming the immunohistochemical analyses (figure 7A).

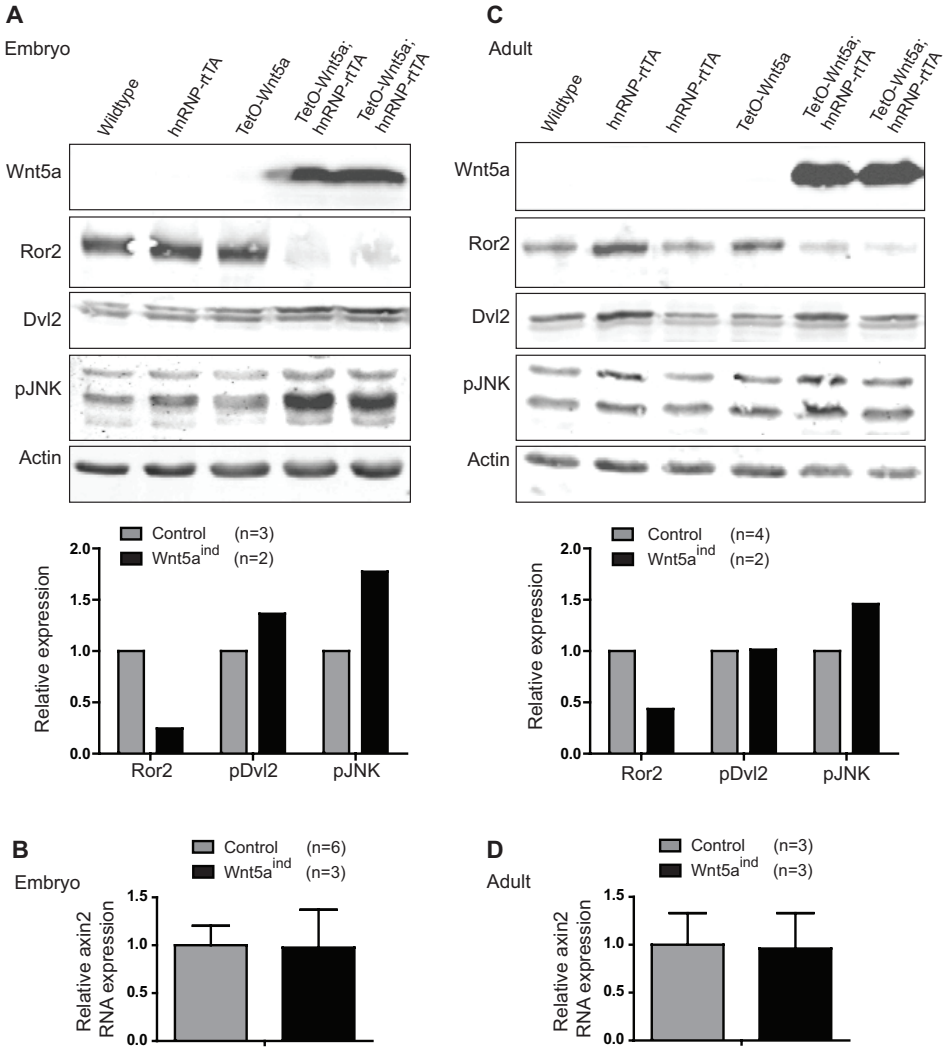

Figure 7. Induced Wnt5a expression enhances downstream signaling in embryonic intestine despite Ror2 downregulation.

Immunoblotting and quantification. Expression levels were corrected for loading controls or in the case of pDvl2, presented as the proportion of pDvl2 of total Dvl2, and averaged. Representative blots are shown. (A) Immunoblotting of 17.5 dpc embryonic intestines induced from 10.5 dpc onwards, showing Ror2 downregulation in Wnt5a^{ind} intestines as well as slightly enhanced levels of pDvl2, represented by the upper band, and increased pJNK levels. (B) Relative *Axin2* RNA expression levels in 17.5 dpc embryonic intestine induced from 10.5 dpc onwards. (C) Immunoblotting of duodenum tissues of adult mice induced for 1 week, showing Ror2 downregulation in Wnt5a^{ind} intestines whereas pDvl2 and pJNK appear less consistently affected. (D) Relative *Axin2* RNA expression levels in duodenum tissues of adult mice.

Several studies have shown that Wnt5a can induce Dvl2 phosphorylation, presumably in a Ror2 dependent manner [13 29-30]. In line with this, we observed a reproducible 1.4-fold increase in phosphorylated Dvl2 levels (upper bands). Moreover, levels of the Wnt5a downstream target pJNK were clearly increased in Wnt5a^{ind} embryonic intestines, showing a 1.8 fold increase. Thus, although Ror2 levels are strongly reduced in Wn5a^{ind} embryos, Wnt5a signaling is enhanced. Despite possible modulation of Wnt/β-catenin signaling by Wnt5a, we observed no changes in RNA expression levels of the β-catenin target gene *Axin2* in Wnt5a^{ind} embryonic intestines (figure 7B).

Similar analyses were performed on duodenal tissues of adult animals induced for 1 week. Immunoblotting for Ror2 revealed a 2-fold downregulation in Wnt5a^{ind} adult intestines, and confirmed the relatively low expression compared to the embryonic situation (figure 7*C*). No significant differences in pDvl2 were observed upon Wnt5a induction, whereas pJNK levels were at most slightly increased. In accordance with the embryonic intestines, *Axin2* RNA expression levels were not changed in adult Wnt5a^{ind} duodenum (figure 7D). Taken together, despite the drastic downregulation of Ror2 protein in Wnt5a^{ind} intestines, embryonic levels of pDvl2 and pJNK were enhanced, indicating a net gain of Wnt5a signaling. In the adult Wnt5a^{ind} intestine, enhanced Wnt5a signaling was not clearly observed.

Discussion

Wnt5a undoubtedly plays a significant role during embryonic development, as indicated by multiple outgrowth defects affecting limbs, tail, face and the gastrointestinal tract in *Wnt5a* knockout mice [8 18]. However, not much is known on the dynamics of Wnt5a functioning to date. Although endogenous Wnt5a expression in the gut is most prominent before 13.5 dpc and diminishes after that period, it is not known whether Wnt5a is involved beyond this time-frame of development. In addition, relevant postnatal roles of Wnt5a have been suggested based upon associative Wnt5a expression studies and in vitro studies. Unfortunately, it has not been possible to date to modulate and investigate the activities of Wnt5a in an adult organism as a consequence of the perinatal lethality of *Wnt5a* knockout mice. We have generated an inducible *Wnt5a* transgenic mouse model, and induced transgenic Wnt5a expression during specific time-frames of embryonic development and during adulthood, followed by a phenotypic analysis focusing on the intestinal tract.

Regarding the consequences of Wnt5a induction in embryos from 10.5 dpc onwards, the observed defects including shortened snout, limbs and tail resemble those seen in *Wnt5a* and *Ror2* knockouts and correspond to structures in whose progenitors Wnt5a is normally expressed [8 15-17 20]. Our results demonstrate that embryonic Wnt5a gain of function results in a phenotype resembling a Wnt5a loss of function situation, a phenomenon reported more often [31-32]. Importantly, these phenotypic defects were not observed when Wnt5a was induced from 13.5 dpc onwards. Together with the knowledge that Wnt5a is most prominently expressed before 13.5 dpc, this suggests that developmental outgrowth processes involving Wnt5a mainly occur before 13.5 dpc. Similar phenotypical resemblance and time-dependency of the phenotype apply to the gastrointestinal tract. When we induced transgenic Wnt5a from 10.5 dpc onwards, intestinal length was severely reduced, as is observed in *Wnt5a* knockout embryos [18]. Intestinal elongation was not affected when Wnt5a was induced from 13.5 dpc onwards. During normal embryogenesis, intestinal Wnt5a expression is restricted to the mesenchyme of the caudal midgut and rostral hindgut, thereby providing positional information directing intestinal elongation [18-20]. However, both in the Wnt5a overexpression and *Wnt5a* knockout situation, such a regionalized endogenous Wnt5a gradient is lost, resulting in lack of positional cues required for directional elongation, likely explaining the comparable elongation defects. Supportively, time dependency of our Wnt5a-mediated gut phenotype coincided with the period during which endogenous Wnt5a is normally expressed most prominently, i.e. between 10.5

and 13.5 dpc. Together, our data indicate that Wnt5a is involved in intestinal elongation processes that occur before 13.5 dpc and that proper regionalized Wnt5a expression between 10.5-13.5 dpc is required for complete elongation [18-20]. An alternative explanation for the observed intestinal shortening might be presented by the strongly downregulated Ror2 protein levels in our Wnt5a^{ind} embryos, thereby phenocopying a *Ror2* knockout situation. However, we do not consider this a solid explanation, given the residual Ror2 protein expression and increased levels of pDvl2 and pJNK in Wnt5a^{ind} embryonic intestines, indicating a net gain of Wnt5a signaling. Although we cannot exclude that the enhanced signaling is mediated via alternative Wnt5a receptors, such as the Frizzleds, it suggests that Ror2 reduction has not hampered Wnt5a signaling. Ror2 has been shown to undergo clathrin-mediated internalization upon Wnt5a stimulation and to present in early endosomes [33-34]. Subsequent routes taken by Ror2 remain elusive. Other Wnt5a-related receptors including Fz2, Fz4 and Fz5 may undergo lysosomal degradation following internalization [35-37]. Our results support the possibility that Ror2 follows this route as well, involving a process more often observed after receptor-ligand endocytosis where receptors are either recycled to the membrane or directed to lysosomes for degradation [38]. As shown for the EGF receptor, high ligand exposure directs more receptors towards lysosomal degradation as a mechanism to restrict signaling. This seems a likely scenario for Ror2 to follow the high Wnt5a levels induced in our mice.

Beyond the involvement of Wnt5a in embryonic development, relevant postnatal roles of Wnt5a have been suggested based upon associative Wnt5a expression studies and in vitro studies. However, our data show that the adult mouse intestine is not affected by induced Wnt5a expression, neither showing histological aberrations nor changes in β-catenin signaling. This might appear surprising since Wnt5a can potentially interact with multiple signaling pathways and has been attributed modulating capacities with regard to cell proliferation, migration and differentiation [3-4 7 26]. On the other hand, our results imply that the main function of Wnt5a exists during embryonic outgrowth events, which obviously do not occur during the maintenance of adult intestinal homeostasis. Supportively, levels of endogenous Wnt5a expression in adult intestine are relatively low compared to levels in developing gut [18-20 24], also applying to Ror2 expression levels (figure 6A-C). Considering this, it might be speculated that sufficient Ror2 expression is needed to acquire an effect following Wnt5a induction. However, embryonic Wnt5a induction from 13.5 dpc onwards did not result in a phenotype despite abundant Ror2 presence, showing that the phenotypic consequences do not entirely depend on the presence of Ror2.

Extra-intestinal aberrations resulting from postnatal hnRNP-*rtTA*-driven Wnt5a expression were not observed, although other adult organs were not investigated in sufficient detail to allow firm conclusions.

Taken together, our results indicate a role for Wnt5a during a restricted time-frame of embryonic development, but suggest no impact during homeostatic postnatal stages. Conditional *Wnt5a* knockout studies in adult animals could ideally further support our findings. Furthermore, as increased Wnt5a expression is typically observed in diseased situations, additional triggers may be needed to reveal postnatal activities of Wnt5a. In the future, this can be investigated by exposing our newly generated inducible Wnt5a mouse model to specific stress conditions or combining it with disease models.

Acknowledgements

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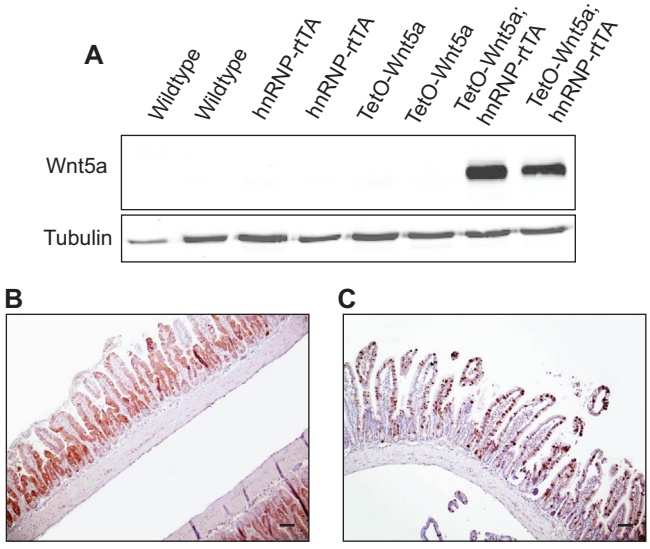
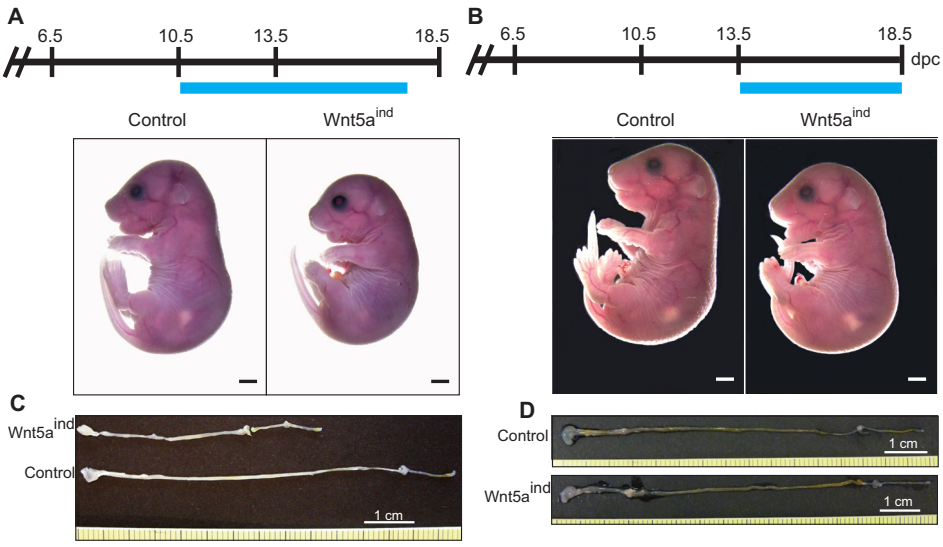
Supplementary figure legends



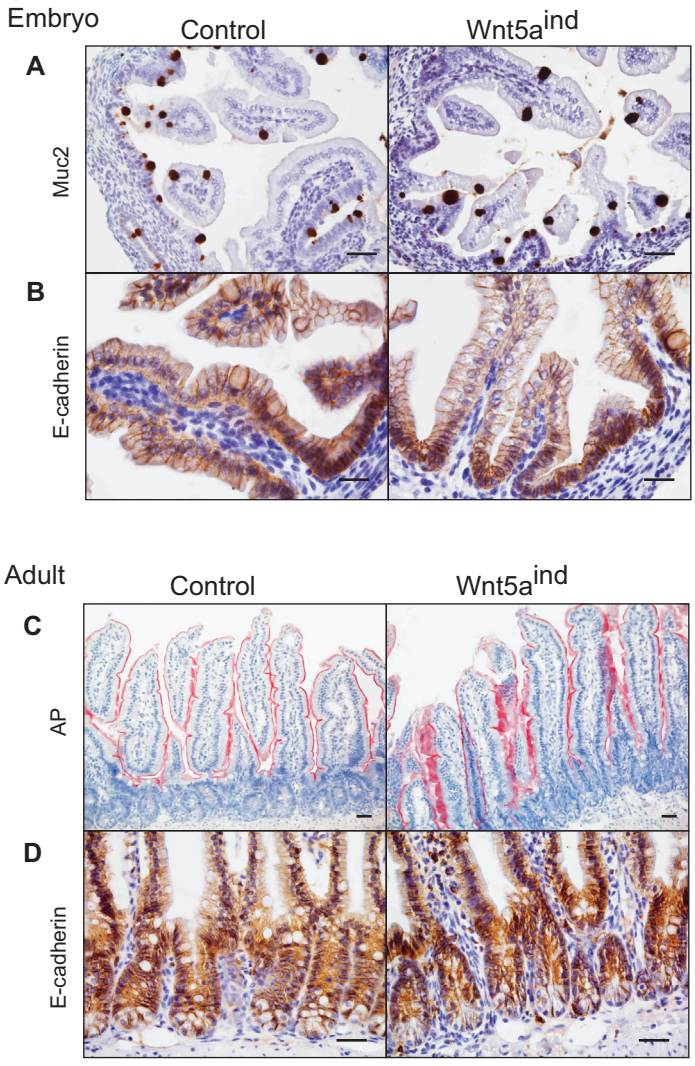
Supplementary figure 1. Further validation of the TetO-*Wnt5a* mouse model.

(A) Wnt5a immunoblot showing multiple MEF lines derived from all different genotypes, all induced in vitro with dox. Wnt5a is detected in double transgenic MEF lines exclusively. (B) Immunohistochemical staining reveals induced Wnt5a expression in double transgenic adult mouse intestine already within 24 hours after dox administration. (C) Double transgenic adult intestine of the alternative TetO-Wnt5a founder induced for 1 week, displaying a mosaic expression pattern of induced Wnt5a protein.



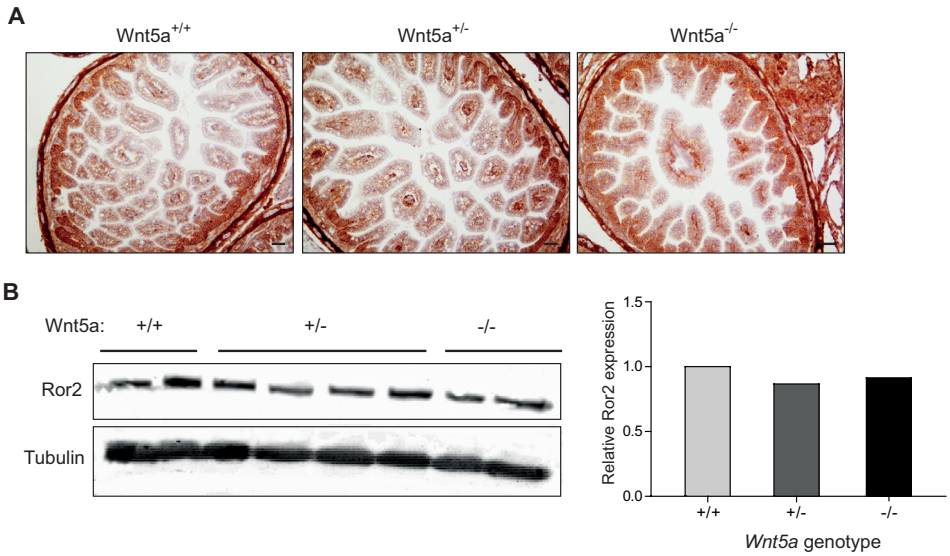
Supplementary figure 2. Induced Wnt5a deregulates embryonic development and intestinal elongation.

(A) 17.5 dpc embryos of control versus double transgenic genotypes using the alternative mosaic TetO-*Wnt5a* founder, dox-induced from 10.5 dpc onwards. Developmental defects are similar to the defects observed using the regular TetO-*Wnt5a* founder (B) 18.5 dpc embryos of control and double transgenic genotypes (regular founder) induced from 13.5 dpc do not reveal gross differences in development. (C) Gastrointestinal tracts isolated from 17.5 dpc control and double transgenic embryos using the mosaic TetO-Wnt5a founder, induced from 10.5 dpc onwards. (D) Gastrointestinal tracts isolated from 18.5 dpc control and double transgenic embryos (regular founder), induced from 13.5 dpc onwards.



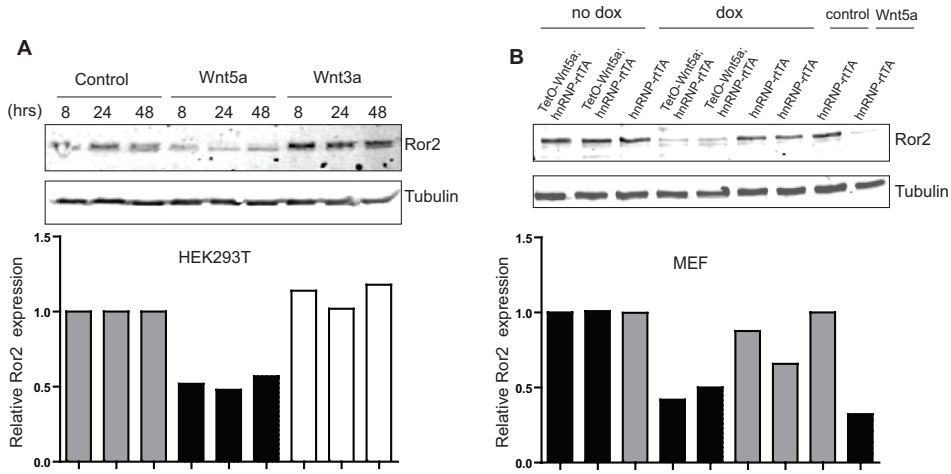
Supplementary figure 3. Induced Wnt5a does not alter cell fate during intestinal development and adult homeostasis.

Immunohistochemical stainings of intestinal sections of 18.5 dpc embryos induced from 10.5 dpc onwards and adult mice induced for 1 week. Representative images are shown from similar regions of the gastrointestinal tract when comparing control and Wnt5a^{ind} intestine within a staining. (A) Muc2 staining indicating unchanged goblet cell presence in Wnt5a^{ind} embryonic intestine. (B) E-cadherin staining showing no effect upon Wnt5a induction in embryonic intestine. (C) Alkaline phosphatase (AP) staining of adult intestine indicating similar appearance of enterocytes in control and Wnt5a^{ind} intestines (D) E-cadherin staining of adult intestines reveals no differences between control and Wnt5a^{ind} intestines.



Supplementary figure 4. Ror2 protein expression is not altered in the intestines of *Wnt5a* knockout embryos.

(A) Immunohistochemical Ror2 staining on the intestines of Wnt5a$^{+/+}$, Wnt5a$^{+/-}$ and Wnt5a$^{-/-}$ 17.5 dpc embryos. (B) Immunoblot and quantification of Ror2 protein in intestines of Wnt5a$^{+/+}$, Wnt5a$^{+/-}$ and Wnt5a$^{-/-}$ 17.5 dpc embryos, corrected for corresponding tubulin levels and averaged.



Supplementary figure 5. Wnt5a downregulates Ror2 protein in HEK293T and MEF cells in vitro.

(A) Ror2 immunoblot of HEK293T cells stimulated with control, Wnt5a or Wnt3a conditioned medium for indicated durations. Endogenous Ror2 protein levels are clearly reduced following Wnt5a stimulation but not in response to control or Wnt3a conditioned medium. Graph shows quantification of corresponding individual lanes. (B) Ror2 immunoblot of MEF lines of hnRNP-*rtTA* and TetO-*Wnt5a*;hnRNP-*rtTA* genotypes with/without dox induction, next to a hnRNP-*rtTA* MEF line stimulated exogenously with control or Wnt5a conditioned medium. Dox induction in TetO-*Wnt5a*;hnRNP-*rtTA* lines and exogenous Wnt5a stimulation on hnRNP-*rtTA* cells reduce endogenous Ror2 protein levels. Graph shows quantification of corresponding individual lanes.

Supplementary materials and methods

Table I Antibodies immunoblotting

Primary antibodies

Antibody	*Dilution*	*Company*
Goat-anti-Wnt5a	1:500	R&D
Mouse-anti-Ror2	1:200	gift R. Nusse (DSHB)
Rabbit-anti-Tubulin	1:10000	Abcam
Rabbit-anti-Dvl2	1:200	Santa Cruz
Rabbit-anti-phospho-SAPK/JNK	1:500	Cell Signaling
Mouse-anti-Actin	1:2500	Santa Cruz

Secondary antibodies

Rabbit-anti-goat-HRP	1:10000	DAKO
Goat-anti-mouse IgG IRDye 680	1:5000	Westburg
Goat-anti-rabbit IgG IRDye 800CW	1:5000	Westburg
Donkey-anti-goat IgG IRDye 680	1:5000	Westburg

Table II Antibodies and antigen retrievals immunohistochemistry

Primary antibodies

Antibody	*Dilution*	*Company*	*Antigen retrieval*
Goat-anti-Wnt5a	1:200	R&D	Citrate pH6
Mouse-anti-Ror2	1:200-1:1000	DSHB (gift R. Nusse)	TE pH9
Mouse-anti-E-cadherin	1:1000	BD Transduction Lab.	Citrate pH6
Rabbit-anti-β-catenin 1247-1	1:2000	Epitomics	TE pH9
Rabbit-anti-lysozyme	1:12500	Dako	5' 0.1% Pronase
Mouse-anti-SMA	1:250	Dako	Citrate pH6
Mouse-anti-Cdx2	1:20	Biogenex	TE pH9
Rabbit-anti-Mucin2	1:400	Santa Cruz Biotechn.	TE pH9
Rabbit-anti-phosphoHistone H3	1:800	Upstate	TE pH9
Rabbit-anti-Synaptophysin	1:250-1:750	Dako	Citrate pH6

Secondary antibodies

HRP-conjugated goat-anti-mouse IgG (EnVision™)	n/a	Dako
HRP-conjugated goat-anti-rabbit IgG (EnVision™)	n/a	Dako
HRP-conjugated goat-anti-rat	1:250	Jackson Immuno Research
HRP-conjugated rabbit-anti-goat	1:250	Dako

Table III Primers quantitative PCR

Wnt5a F	5'cctatgagagcgcacgcatc 3'
Wnt5a R	5'ggagccagacactccatgac 3'
Ror2 F	5'gaaggcccgtggtgctttac 3'
Ror2 R	5'cccatcttgctgccatctcg 3'
Actb F	5'agacctctatgccaacacag 3'
Actb R	5'cacagagtacttgcgctcag 3'

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CHAPTER 8

General discussion

Discussion

The overall aims of the research described in this thesis were to investigate in more detail the role of Sox2 in establishing and maintaining organ identity in the digestive tract, together with the examination of ectopic Sox2 expression in human diseases of the intestinal tract, such as colorectal cancer and various congenital anomalies.

The results of these studies indicate that ectopic expression of Sox2 in the developing intestinal epithelium of the mouse causes an anteriorization event of the intestinal epithelium, leading to a gastric-like phenotype. This effect is however not observed in the mature intestinal tract, suggesting that mature intestinal cells have a lower plasticity. In the mature intestinal epithelium, ectopic expression of Sox2 appears to drive the intestinal cells into a more progenitor-like phenotype. Our findings are of interest in light of human diseases in which ectopic gastric tissue is seen in the intestine, such as Meckel's diverticulum, intestinal duplications and duodenitis. It gives valuable information about the possible aetiology of these diseases and it may provide a new platform for therapeutic strategies.

The developing and mature intestinal tract show different degrees of plasticity towards gastric conversion

One of the earliest steps in patterning the primitive gut is the regionalization into an anterior and posterior domain, which correlates with the expression of Sox2 and Cdx2, respectively. Later in development Cdx2 becomes restricted to the intestinal epithelium with a sharp boundary, marking the transition from stomach to duodenum, while Sox2 is expressed in the stomach and esophagus[1-4]. This apparent reciprocal expression pattern of Sox2 and Cdx2 suggest that these factors influence each other. However, it remains unclear whether Sox2 or Cdx2 has a dominant role in determining the fate of the developing gut endoderm.

We used the mouse line described in **Chapter 2** to specifically induce Sox2 expression in the presumptive intestinal tract during development. The results of this study are described in **Chapter 3** of this thesis. The findings provide strong evidence that expression of Sox2 in the developing gut drives the activation of the foregut transcriptional program and leads to conversion from an intestinal into a premature gastric-like epithelium, despite simultaneous expression of Cdx2. In the mature intestinal tract this effect is not observed (**Chapter 4**), but instead we show that ectopic expression of Sox2 in the mature intestinal tract drives cells into a more progenitor-like state, as aberrantly localized proliferating cells

were observed in the villus compartment. Similar observations were made when Cdx2 was ablated in the developing and mature intestinal tract. When ablated in the developing intestinal tract, this led to a rapid conversion into gastric-like epithelium, whereas Cdx2 deletion in the mature tract fails to reproduce this phenotype[5-7].

We conclude that the developing and mature intestinal tracts differ in their plasticity towards gastric conversion, following modulation of Sox2 or Cdx2 expression during each time frame. This difference in plasticity might be caused by the fact that the mature intestine has more specialized and dedicated niches for the stem cells when compared with the developing gut. In this respect, there may be two important mechanisms restricting the plasticity of the mature intestinal tract. First, one of the main players involved in enforcing stem cell features in the adult gut is the Wnt/β-catenin signalling pathway[8]. Ablation of β-catenin or its down-stream effector Tcf4 specifically in the adult intestinal tract, results in the rapid loss of stem cells and crypt structures[9-10]. Surprisingly, the role of β-catenin signalling in the developing gut appears to be less important, as active β-catenin signalling is only detectable in prospective crypt cells postnatally[11]. As such, the increased activity of this pathway in adults to enforce intestinal identity may reduce the possibilities for gastric conversion by factors like Sox2. Secondly, it has been reported that the intestinal stem cell niche is supported by the Paneth cells, which reside at the base of the crypts. These Paneth cells secrete various growth factors that strongly support stem cell maintenance[12]. As these cells are functional from birth onwards, their presence may further reduce plasticity of the mature intestinal tract.

The outcome of ectopic Sox2 expression will also be determined by the expression levels of both ectopic Sox2 and endogenous Cdx2. It has been shown that Sox2 has dose-dependent effects in development. For example, normal expression levels of Sox2 cause the future esophagus and anterior stomach to form into a stratified squamous epithelium, whereas in mice with lower levels of expression the formation of a more pseudo-stratified, columnar epithelium occurred[13]. Cdx2 on the other hand has varying expression levels along the anterior- posterior axis of the mature gut, with its peak expression in the colon[2]. At sites where Cdx2 expression is relatively low during development, the effect of ectopic Sox2 may be more pronounced. Alternatively, it may be that the reduced plasticity of the mature intestinal tract is related to other factors such as the presence of Paneth cells and the dominant influence of β-catenin signalling.

In contrast to the intestine, where the proliferative zone is restricted to the crypt compartment, the dividing stem cell progeny of the stomach is located more

apically, at the isthmus of the gastric pits[14]. The expanded proliferative zone and the aberrant localization of proliferating cells at the tip of the villi might suggest a partial conversion of the mature intestinal epithelium to a stomach like nature. We may investigate this hypothesis of delayed conversion by administrating suboptimal dosages of doxycycline, which would allow these animals to survive for a longer period, thereby possibly leading to the formation of gastric tissue in the intestine.

In support of the latter, a similar approach was taken by the group of Hryniuk et al., who deleted Cdx2 throughout the complete intestinal epithelium[6]. They described an acute effect following full ablation of Cdx2, and a long-term effect of Cdx2 in only a part of the intestinal epithelium. Acute deletion of Cdx2 caused diarrhea and rapid wasting of the animals within a week, symptoms we also observed by ectopic expression of Sox2 in the intestine. In addition, the morphology of the villi was dramatically affected in the Cdx2 knock-out mice. Furthermore, deletion of Cdx2 caused loss of enterocytes and to a lesser extent loss of enteroendocrine and Paneth cells. Within this one week time frame, no evidence for gastric conversion was observed. On the other hand, long-term deletion of Cdx2 in only a subset of the crypt-villus structures resulted in the formation of patches of metaplasia, which were devoid of the four main intestinal cells, but instead expressed the gastric parietal cell marker H+/K+ ATPase, as well as other stomach-related genes. Using a similar approach, Stringer et al. also reported a partial conversion towards gastric epithelium after 19 weeks follow-up[7]. These results indicate that longer time of Sox2 induction in the intestinal epithelium likewise could cause conversion into stomach cells.

The reason for the different effects of acute and chronic Cdx2 loss might also lie in the crypt compartment, where the stem cells reside. In contrast to the acute loss of Cdx2, chronic loss of Cdx2 caused the formation of aberrant crypt-villus structures. These structures apparently consist of a stem cell population that is independent of the niche containing Paneth cells. The small intestine contains both cycling and quiescent stem cells, which are responsible for tissue renewal and the regenerative response after injury, respectively. In the small intestine four to six stem cells reside in the crypt[15]. The crypt base columnar (CBC) cells, interspersed between Paneth cells, represent the cycling population of stem cells, which are distributed throughout the intestine, and are marked by the G protein-coupled protein Lgr5[16-17]. The quiescent stem cells on the other hand are marked by the Polycomb group protein Bmi1, and are located at the +4 cell position immediately after the uppermost Paneth cells in the crypts of the proximal small intestine[18]. It might be that chronic Cdx2 ablation induced a situation of injury,

inducing the quiescent stem cells to become activated. In a similar way long-term ectopic expression of Sox2 might induce a situation of injury, which would lead to the activation of the quiescent stem cells, leading to different effects of acute and chronic expression of Sox2. We observed the formation of crypt-like structures in the mature intestinal tract and it is plausible that long-term expression of Sox2 in the intestinal tract, results in the formation of metaplasia and conversion into a gastric phenotype.

Ectopic Sox2 expression in the intestinal tract and progenitor identity

We demonstrated that ectopic expression of Sox2 in the presumptive intestinal epithelium of the developing mouse embryo activated the foregut transcriptional program, causing conversion from an intestinal into a premature gastric epithelium, despite simultaneous expression of Cdx2. In addition to the gastric differentiation, we also observed a high proportion of cells that were actively dividing, suggesting that the ectopic Sox2 induced a progenitor-like phenotype in a substantial proportion of the cells. This also appears to be the case in the mature intestinal tract, based on the aberrantly positioned proliferating cells and the appearance of apparently crypt-like structures in the villi.

Our data that Sox2 might impose progenitor identity within the gut is in concordance with current literature [19-22]. Sox2 is one of the transcription factors shown to be necessary to preserve pluripotency of embryonic stem cells and self-renewal of tissue-specific adult stem cells, together with Oct 3/4, N-Myc and Nanog[23-24]. Null mutations of each of these genes result in embryonic lethality due to the inability to maintain pluripotent cells. Additionally, it was demonstrated that reduction of Sox2 expression in mouse embryonic stem cells induced differentiation into trophoectoderm, strongly indicating that Sox2 function is critical for the maintenance of pluripotency[25-27]. In cooperation with Oct4, N-Myc and Klf4, Sox2 is capable to induce pluripotent stem cell-like properties in terminally differentiated somatic cells, the so-called iPS cells[23-24]. Furthermore, sustained expression of SOX2 in the neural progenitors of the vertebrate nervous system, inhibits neural differentiation and leads to the maintenance of progenitor characteristics, while inhibition of SOX2 results in the early onset of neuronal differentiation[21]. As such, our work seems to provide another example which shows that Sox2 is associated with progenitor identity.

Ectopic expression of Sox2 in the intestinal epithelium causes conversion into gastric-like cells rather than an esophageal phenotype.

Although Sox2 is expressed both in the esophagus and stomach, it is interesting that ectopic expression of Sox2 in the intestinal epithelium, results in conversion specifically into cells with gastric characteristics, rather than towards an esophageal phenotype. A possible explanation may be the specific epithelium-type and the function of these organs, relative to that of the intestine. In contrast to the simple columnar epithelial layer found in the stomach and intestine, the esophagus consists of stratified squamous epithelium, in concordance with its primarily transport function, compared to the secretory and absorptive function of the stomach and intestine. In these functions the stomach and intestine are closer related, and therefore conversion from intestinal towards stomach-like epithelium is more likely to occur, and may require less alterations of their transcriptional program.

Furthermore, Sox2 alone may not be capable of fully anteriorizing the intestine in the absence or presence of other regulatory signals. For example, it has been shown that bile inhibits the squamous differentiation program of esophageal epithelial cells[28]. Bile acids are made in the liver, stored in the gall bladder and then secreted into the intestine. The influence of bile therefore might inhibit the formation of an esophageal phenotype, upon ectopic expression of Sox2 in the intestinal tract.

Ectopic expression of SOX2 in human congenital anomalies of the intestine.

Since little was known about the aetiology of intestinal congenital anomalies in human, we examined the expression pattern of the transcription factors SOX2 and CDX2 in cases of intestinal duplication, persistent omphalomesenteric duct and Meckel's diverticulum, described in **Chapter 5.** In all three congenital anomalies of the intestine the presence of gastric or gastric-like cells in the affected intestinal tissue had been described previously. In general, we found a reciprocal expression pattern of CDX2 and SOX2 in the intestine and gastric tissue, respectively. Interestingly, we observed however three cases that co-expressed CDX2 in the ectopic gastric type heteroplasia, suggesting that the primary reason for the appearance of gastric tissue is not just the lack of CDX2, as was proposed by Martin et al[29], but rather the ectopic expression of SOX2, as we showed in our study in mice.

Sherwood et al described that during mouse development, Sox2 and Cdx2 are expressed from the anterior and posterior endoderm, respectively. They however pointed out a phase at E8.75-E9.25 when cells at the cellular border of stomach and intestine co-express both transcription factors. At E9.5 the cellular border remains, but the cells exclusively express either Sox2 or Cdx2[4]. This phase of embryonic development coincides with the timing of involution of the omphalomesenteric duct, i.e. the temporary connection between the midgut endoderm and the yolk sac. These observations could be of value to understand the expression pattern we observed in the congenital anomalies in human. Our data show that in pathological situations apparently cells are present that express both SOX2 and CDX2. These cells might represent descendants from the population of cells described above, which co-express both factors during a short phase of intestinal development, at the same time when involution of the omphalomesenteric duct takes place. These cells are able to form either intestinal or gastric epithelium. Timing, spacing, cues from the surrounding mesenchyme and the expression level of both transcription factors relative to each other might induce the choice towards either gastric or intestinal tissue. Extrapolating our mouse data to our findings in the human congenital anomalies, we could say that in cases where cells express both SOX2 and CDX2 in an intestinal background, SOX2 is dominant over CDX2 and will lead to a conversion of this cell towards a gastric like cell.

The fact that in humans the ectopic gastric tissue is present only in patches, while in mice we induce a gastric-like phenotype throughout the whole small intestine, might be caused by the fact that in the human situation a limited amount of cells are present, which express both factors. This is in contrast to the situation in the mouse model in which we ectopically expressed Sox2 throughout the whole intestinal epithelium. It is worth mentioning that the dominance of either Sox2 or Cdx2 is dependent on the tissue it is expressed in. We showed that ectopic expression of Sox2 in the intestinal epithelium caused differentiation towards a gastric phenotype. On the other hand, when Cdx2 is ectopically expressed in the mouse stomach, it causes a transformation towards intestinal mucosa, while retaining Sox2 expression[30-31]. A possible explanation is that the relative amount of expression of each transcription factor is critical for determining in which direction a cell will differentiate. For example, when Sox2 is relative lowly expressed and ectopic Cdx2 expression is abundant, then Cdx2 is more likely to overrule the effects of Sox2 and drive cell fate towards intestinal differentiation. In support of this, intestinal metaplasia is predominantly found in the antrum and the proximal lesser curvature of the stomach, where Sox2 is expressed at lower

levels than in other parts of the stomach, and ectopic Cdx2 is more likely to induce the formation of intestinal metaplasia[31-32].

Sox2 expression in human colorectal cancer

In **chapter 6** we showed that SOX2 was ectopically expressed in non-mucinous CRC, in contrast to a previous study, which suggested that SOX2 expression was associated with mucinous differentiation[33]. In addition, we show in concordance with our mouse and human congenital anomaly data, that also in human CRC, cells exist that co-express SOX2 and CDX2. Whereas in the normal intestinal epithelium ectopic SOX2 may contribute to gastric conversion, we do not believe this to be the case in the colorectal cancer cells, despite the expression of the gastric mucin MUC5AC that is observed in a large proportion of CRCs. First of all, aberrant MUC5AC expression is independent of SOX2 (**chapter 6**), and is more likely the result of hyperactivation of the mucin gene cluster in which both MUC5AC and the intestinal mucin MUC2 are located. Secondly, to the best of our knowledge the emergence of gastric cell types have not been reported in CRC.

We showed different effects of SOX2 on patient survival in the two groups that were tested. In our first test group, we showed that ectopic Sox2 was associated with a significantly shorter survival, which is in accordance with some recent publications[34-36]. However, our second test group did not show this association, most likely because the predictive value of the second group is currently too low. Based on studies done in other cancer types, it would however be plausible that SOX2 is involved in inducing a more aggressive kind of tumors[37-38]. For example, it has been shown that overexpression of SOX2 is associated with a worse survival in lung and esophageal squamous cell carcinomas by supporting proliferation and anchorage independent growth[ref38]. For prostate cancer, SOX2 expression levels was shown to correlate with advanced histological grade and to reduce sensitivity to apoptotic stimuli[ref 40], whereas in melanoma SOX2 appears to enhance invasion[ref 39]. Although we did not observe SOX2 specifically at invasive fronts in the CRCs we tested, it remains however possible that ectopic expression of SOX2 induces characteristics of stemness and allows cells to migrate and differentiate beyond their natural niche. A possible way to test the migration capacity of CRC cells upon SOX2 expression is to ectopically express SOX2 in a human CRC cell line and perform a migration assay.

Clinical relevance and future perspectives

In regenerative medicine, knowledge about factors that are able to determine cell fate is of great relevance. During the past years great effort has been made to characterize the intestinal stem cell [16 18] and to culture these stem cells in vitro[39], with eventually the goal to be able to regenerate tissue that might be used for transplantation in patients with intestinal diseases. It would be convenient to be able to generate from stem cells from the intestine, also other tissues for instance stomach, by adding or removing factors. It would be interesting to see whether cultured organoids from intestinal stem cells are able to convert into stomach cells upon Sox2 induction. These cells could then be cultured into functional stomach cells and used for transplantations. The big advantage would be that these intestinal stem cells, which are present in great amounts in the human intestinal tract, might be easily accessible and used as basic cells to create different tissues. Furthermore, problems concerning rejection of donor material, might be overcome when tissue is made from cells from the patients own body.

In this thesis we have investigated in more detail the role of Sox2 in establishing organ identity. In addition, we have investigated the possible role of Sox2 in colorectal tumor formation and progression. The outcome of our research adds more insight in the transcriptional regulation underlying the development of the gastro-intestinal tract. Furthermore, our data improve our understanding of the mechanisms behind metaplasia and provides possibilities for future therapeutic applications within the regenerative medicine field.

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CHAPTER 9

Summary
Samenvatting

Summary

Various molecular factors have been identified which play an essential role in the formation of organs of the digestive tract, including the transcription factors Sox2 and Cdx2. In the digestive tract, Sox2 expression is restricted to the stomach and esophagus, while Cdx2 is exclusively expressed in the intestine. Normally, the expression of these transcription factors is mutually exclusive. Once formed, organ identity is strongly maintained and rarely converted into another tissue type. However, in various diseases ectopic gut tissue arises outside its organ boundaries, including Barrett's esophagus, intestinal metaplasia in the stomach and gastric heteroplasia in the intestinal tract. The mechanisms underlying the formation of this ectopic tissue are not fully understood.

In **chapter 1** we give a general introduction about the organization and function of the gastro-intestinal tract, together with a description of the relevant transcriptional regulators, i.e. SOX2 and CDX2. In mice, conditional ablation of Cdx2 from the developing gut endoderm results in severe malformation of the intestinal tract and anteriorization of the posterior gut. Interestingly, this anteriorization coincided with ectopic expression of Sox2, indicating that Cdx2 and Sox2 influence each other. Therefore, we investigated the role of the transcription factor Sox2 in establishing and maintaining organ identity, together with the examination of ectopic Sox2 expression in various human diseases of the intestinal tract, such as colorectal cancer (CRC) and various congenital anomalies.

Chapter 2 describes the newly generated Villin-rtTA mouse model that we used in our studies for the ectopic induction of Sox2 in the intestinal tract. In **chapter 3** we have used this villin-rtTA transgenic mouse line to demonstrate that ectopic expression of Sox2 in the presumptive intestinal epithelium of the developing mouse embryo activated the foregut transcriptional program, causing conversion from an intestinal to a premature gastric epithelium, despite simultaneous expression of Cdx2. These results indicate that ectopic expression of Sox2 in an intestinal background has a dominant effect over Cdx2.

In order to investigate whether the mature intestinal tract still possesses a degree of plasticity, which would allow conversion of committed intestinal cells to transform into cells which have gastric characteristics, we also induced Sox2 expression in the mature intestinal epithelium. Our results described in **Chapter 4**, suggest that Sox2 expression by itself is not sufficient to aberrantly induce gastric epithelium in the adult intestinal tract, and most likely requires the simultaneous loss of Cdx2 functionality. Our results confirm the limited degree of plasticity of the

adult intestinal epithelium compared to its embryonic counterpart. Furthermore, we show that ectopic expression of Sox2 induces proliferation throughout the entire crypt-villus axis, which likely underlies the formation of aberrant crypts and multilayered epithelium that we observe. This phenotype is in accordance with the induction of pluripotency and progenitor identity that has frequently been attributed to Sox2.

As shown in mice, the embryonic intestinal epithelium has a certain degree of plasticity and ectopic expression of Sox2 results in anteriorization of the intestine, in spite of sustained Cdx2 expression. Contrasting the mouse data, in human little is known about the expression patterns and effects of SOX2 and CDX2 in the gastro-intestinal tract. Therefore, we examined the expression pattern of both transcription factors in several congenital diseases of the intestine, in which ectopic gastric tissue can be present. The results described in **chapter 5** show that ectopic expression of SOX2 in the developing human intestinal tract might underlie the formation of gastric tissue. We demonstrate that in some gastric heteroplasia cases, CDX2 is expressed simultaneously with SOX2, suggesting that in human development the intestinal epithelium is also able to change its identity, in accordance with our mouse studies.

In recent years, SOX2 expression has been linked to tumor progression for various cancer types, and has also been associated with mucinous differentiation in colorectal cancer. In contrast to the latter, we show in **chapter 6** that ectopic expression of SOX2 is present in non-mucinous CRC, rather than in mucinous CRC, and that the gastric mucin MUC5AC is expressed independently of SOX2, in both the tumor as well as morphological normal tissue adjacent to the tumor. Furthermore, we have indications that ectopic expression of Sox2 is associated with a worse survival of these patients.

The canonical Wnt/β-catenin signaling pathway is well-known for its prominent role in regulating development and homeostasis of the intestinal tract. The role of the non-canonical Wnt signaling pathway on these processes is however far less understood. In **chapter 7** of this thesis we investigated the role of Wnt5a induction during different time frames of embryonic gut development and the mature intestinal tract. Our results indicate a role for Wnt5a in intestinal elongation during a restricted time-frame of embryonic development, but suggest no impact during homeostatic postnatal stages. In contrast to Sox2, ectopic expression of Wnt5a during the embryonic phase does not affect intestinal identity.

Lastly, in the general discussion (**chapter 8**) we discuss our findings and indicate their clinical relevance. Moreover, suggestions for additional experiments

that might contribute to our understanding of the role of Sox2 in determining and maintaining organ identity in the gastrointestinal tract are provided.

Overall, we show that although the gut is committed to the intestinal lineage, the developing intestinal tract possesses a certain degree of plasticity, making it possible to change the intestinal fate, both in mice and humans. As a result of ectopic expression of Sox2 conversion of intestinal cells into a cell type found more anteriorly might underlie ectopic gastric tissue formation in several human diseases. Our current knowledge gives new insights in the possible aetiology of these diseases and provides a new base for therapeutic interventions.

Samenvatting

Om nutriënten op te nemen uit voedsel en vloeistoffen, heeft het lichaam een gespecialiseerd orgaanstelsel ontwikkeld, het spijsverteringskanaal, welke ondermeer de mond, slokdarm, maag en darmen omvat. Histologisch gezien is het maag-darmstelsel in principe een holle buis die uit vier verschillende lagen bestaat, waarvan de mucosa het meest gespecialiseerd is. De aanleg van het spijsverteringskanaal en geassocieerde organen wordt tijdens de ontwikkeling bepaald door een verscheidenheid aan factoren. Twee factoren die een centrale rol hebben in de vroege aanleg zijn de transcriptiefactoren Sox2 en Cdx2. Beide factoren zijn betrokken bij de zogenaamde specificatie van het spijsverteringskanaal, waarbij de expressie van Sox2 beperkt is tot de slokdarm en maag, terwijl Cdx2 exclusief tot expressie komt in de darm. Normaliter overlappen Cdx2 en Sox2 expressie elkaar niet in gezond weefsel. Wanneer het spijsverteringskanaal eenmaal is gevormd, blijft de identiteit van de verschillende onderdelen (slokdarm, maag, dunne darm, etc) behouden. Er zijn echter verschillende aandoeningen bekend, waarbij weefsel dat specifiek is voor een bepaald onderdeel van het spijsverteringskanaal op een verkeerde plaats wordt gevonden, zoals Barretts's slokdarm (darmweefsel in de slokdarm), darmmetaplasie in de maag, en maagheteroplasie in de darmen. Het onderliggende mechanisme van de formatie van dit zogenaamde ectopisch weefsel is onduidelijk.

In **hoofdstuk 1** geven wij een algemene introductie betreffende de organisatie en functie van het maag-darmstelsel, tezamen met een beschrijving van de relevante transcriptiefactoren SOX2 en CDX2. In muizen leidt de inactivatie van Cdx2 in het ontwikkelende darmepitheel tot ernstige malformatie van het darmstelsel en tot anteriorisatie van het posteriore deel van de darm, dat wil zeggen het darmepitheel gaat sterk gelijken op het maag- en slokdarmepitheel. Interessant genoeg, valt deze anteriorisatie samen met ectopische expressie van Sox2, suggererend dat Cdx2 en Sox2 elkaar beïnvloeden. Wij hebben onderzoek gedaan naar de rol van de transcriptiefactor Sox2 in het ontstaan en behouden van orgaanidentiteit, evenals naar ectopische Sox2 expressie in verscheidene humane aandoeningen van het darmstelsel, zoals dikke darmkanker en verschillende aangeboren afwijkingen van het maag-darmstelsel.

Hoofdstuk 2 beschrijft het nieuw ontwikkelde Villin-rtTA muismodel die wij hebben gebruikt in onze studies om Sox2 ectopisch tot expressie te brengen specifiek in het darmstelsel. In dit muismodel komt het rtTA eiwit alleen tot expressie in het darmepitheel dankzij de darmspecifieke Villin promoter. De

activiteit van het rtTA eiwit als transcriptiefactor kunnen we vervolgens reguleren door middel van het toedienen van doxycycline aan het drinkwater. Hierdoor kan het rtTA eiwit binden aan het tetracycline responsive regulatory element (TRE) in de promoter regio van het te induceren gen, waardoor dit gen gereguleerd tot expressie kan komen. In **hoofdstuk 3** beschrijven wij dat de geïnduceerde expressie van Sox2 in het epitheel van het ontwikkelende spijsverteringskanaal wat normaal de darm moet vormen, leidt tot de activatie van genen specifiek voor het maagweefsel. Het uiteindelijke resultaat is de conversie van darmepitheel naar een prematuur maagepitheel, ondanks dat Cdx2 tegelijk tot expressie komt. Deze resultaten wijzen erop dat ectopische expressie van Sox2 in een darmachtergrond een dominant effect heeft over Cdx2.

Om te onderzoeken of het volledig ontwikkelde darmstelsel van volwassen dieren dezelfde mate van plasticiteit vertoont, hebben wij ook in het volwassen darmepitheel Sox2 geïnduceerd. Deze resultaten, beschreven in **hoofdstuk 4**, suggereren dat alleen ectopische expressie van Sox2 niet voldoende is om maagepitheel te induceren in het volwassen darmstelsel. Onze resultaten bevestigen een gelimiteerde mate van plasticiteit in het volwassen darmepitheel, in vergelijking tot de ontwikkelende darm in de muis embryo. Tevens tonen wij aan dat ectopische expressie van Sox2 in de volwassen darm leidt tot proliferatie in de gehele crypt-villus unit, de formatie van extra crypten en de formatie van meerlagig darmepitheel. Dit fenotype komt overeen met de inductie van een pluripotente en voorloperidentiteit, welke frequent wordt toebedeeld aan de expressie van Sox2.

Verschillende studies met muismodellen, waaronder ons eigen werk beschreven in hoofdstuk 3, hebben aangetoond dat het embryonale darmepitheel een zekere mate van plasticiteit heeft. Bovendien hebben wij aangetoond dat de ectopische expressie van Sox2 leidt tot een anteriorisatie van de primitieve darm, ondanks dat Cdx2 expressie wordt behouden. In tegenstelling tot beschikbare muisdata, is er weinig bekend over de expressie patronen van SOX2 en CDX2 in het humane maag-darmstelsel. Daarom hebben wij de expressiepatronen van beide transcriptiefactoren onderzocht in verscheidene aangeboren afwijkingen van de darm, waarin ectopisch maagweefsel in de darmen aanwezig kan zijn. De resultaten, welke zijn beschreven in **hoofdstuk 5**, laten zien dat in sommige gevallen van maagheteroplasie, CDX2 tegelijk met SOX2 tot expressie komt. Dit suggereert dat ook tijdens de humane ontwikkeling, het darmepitheel van identiteit kan veranderen, in overeenstemming met onze muisdata.

In de afgelopen jaren is de expressie van SOX2 in verband gebracht met tumorprogressie in verschillende soorten kanker. Bovendien is SOX2 in verband

gebracht met de mucineuze differentiatie van dikke darmkanker. In sterke tegenstelling tot de laatst genoemde observatie, beschrijven wij in **hoofdstuk 6** dat ectopische expressie van SOX2 juist in niet-mucineuze dikke darmkanker aanwezig is. Bovendien tonen wij aan dat de maagspecifieke mucine MUC5ac onafhankelijk van SOX2 tot expressie komt, in zowel de tumor als ook in het omliggende morfologisch normale weesfel. Daarnaast hebben wij indicaties dat ectopische expressie van SOX2 is geassocieerd met een slechtere overleving van deze patiënten.

De canonical Wnt/β-catenine signaaltransductie route is bekend vanwege haar prominente rol in de regulatie van ontwikkeling en homeostase van het darmstelsel. De centrale speler van dit mechanisme is β-catenine. In de afwezigheid van een Wnt signaal, zal β-catenine worden afgebroken. Echter wanneer Wnt liganden hun receptoren binden, zal het degradatiecomplex worden geïnactiveerd en zal β-catenine niet worden afgebroken. Het zal worden getransporteerd naar de celkern, waar het verscheidene transcriptiefactoren kan binden, wat vervolgens leidt tot de activatie van verschillende doelgenen, welke belangrijk zijn voor de homeostase van het darmstelsel. In tegenstelling tot canonical Wnt/β-catenine, is er nog maar weinig bekend over de rol van de non-canonical Wnt signaalroute in darmontwikkeling en homeostase. De non-canonical Wnt pathway functioneert onafhankelijk van β-catenine. De meest bestudeerde non-canonical wnt ligand is Wnt5a. In **hoofdstuk 7** van dit proefschrift hebben wij het effect van Wnt5a inductie onderzocht tijdens verschillende tijdspunten van de embryonale darmontwikkeling en het volwassen darmstelsel. De resultaten wijzen op een rol voor Wnt5a in de verlenging van de darm tijdens een specifiek tijdspunt in de embryonale ontwikkeling, maar suggereert geen effect op het handhaven van de darmstructuur in het volwassen stadium. In tegenstelling tot Sox2, tast ectopische expressie van Wnt5a tijdens de embryonale fase de darmidentiteit niet aan.

Tot slot, bediscussiëren wij in de algemene discussie (**hoofdstuk 8**) onze bevindingen en geven wij de klinische relevatie aan. Bovendien geven wij suggesties voor aanvullende experimenten die kunnen bijdragen aan ons begrip over de rol van Sox2 in het bepalen en behouden van orgaanidentiteit in het maag-darmstelsel.

Samenvattend tonen wij aan dat zowel in muis als mens het ontwikkelende darmstelsel een zekere mate van plasticiteit heeft, wat het mogelijk maakt om de identiteit van de darm te veranderen. Als gevolg van ectopische expressie van Sox2 vindt er conversie plaats van darmcellen naar cellen die meer anterior

worden gevonden in het maag-darmstelsel, wat mogelijk een belangrijke bijdrage kan leveren aan de vorming van ectopisch maagweefsel zoals die gezien wordt in verscheidene humane ziekten. Onze huidige kennis geeft nieuwe inzichten in de mogelijke etiologie van deze ziekten en geeft een nieuwe basis voor therapeutische interventies.

Appendices

Curriculum vitae

Lalini Jasoda Raghoebir werd geboren op 8 maart 1984 te Amersfoort. In 2003 behaalde zij haar eindexamen aan het Johan van Oldenbarnevelt Gymnasium, te Amersfoort. Zij studeerde een jaar lang Biomedische wetenschappen aan de Universiteit van Utrecht, alvorens te starten met de studie Geneeskunde aan de Erasmus Universiteit Rotterdam, waarvoor zij in 2008 haar doctoraal diploma behaalde. Parallel aan de studie geneeskunde deed zij de research master Molecular Medicine, tevens aan de Erasmus Universiteit Rotterdam. In 2009 behaalde zij haar Master of Science graad. Vervolgens startte zij met promotieonderzoek onder leiding van prof. dr. D.Tibboel en onder supervisie van dr. R.J. Rottier en dr. M.J.M.Smits. De resultaten hiervan zijn beschreven in dit proefschrift en gepubliceerd in, of ter publicatie aangeboden aan, vooraanstaande internationale tijdschriften. In het tweede jaar van haar promotietraject is zij begonnen met keuzevakken aan de faculteit rechtsgeleerdheid en heeft het eerste jaar rechten reeds met succes afgerond. Thans heeft zij haar studie geneeskunde hervat en doet zij haar co-schappen.

List of publications

Raghoebir L, Bakker ER, Mills JC, Swagemakers S, Buscop-van Kempen M, Boerema-de Munck A, et al. SOX2 redirects the developmental fate of the intestinal epithelium toward a premature gastric phenotype. *J Mol Cell Biol* 2012.

Bakker ER, **Raghoebir L**, Franken PF, Helvensteijn W, van Gurp L, Meijlink F, et al. Induced Wnt5a expression perturbs embryonic outgrowth and intestinal elongation, but is well-tolerated in adult mice. *Dev Biol* 2012;369(1):91-100.

Roth S, Franken P, van Veelen W, Blonden L, **Raghoebir L**, Beverloo B, et al. Generation of a tightly regulated doxycycline-inducible model for studying mouse intestinal biology. *Genesis* 2009;47(1):7-13.

Lalini Raghoebir, Katharina Biermann, Marjon Buscop-van Kempen, Renee M. Wijnen, Dick Tibboel, Ron Smits, Robbert J. Rottier. Expression pattern of SOX2 and CDX2 in human vitelline duct anomalies and intestinal duplications.*Submitted.*

Lalini Raghoebir, Katharina Biermann, Maxime P. Look, Marjon Buscop-van Kempen, Dick Tibboel, Robbert J. Rottier, Ron Smits. Aberrant SOX2 expression in colorectal cancers shows no direct relation with the expression of the gastric mucin MUC5AC, but correlates with non-mucinous differentiation. Submitted

Raghoebir L, Anne Boerema-de Munck, Marjon Buscop-van Kempen, Dick Tibboel, Ron Smits, Robbert J Rottier. Ectopic expression of Sox2 in the mature intestinal tract induces aberrant proliferation, rather than changes in the intestinal differentiation program. *Manuscript in preparation.*

PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student	Lalini Jasoda Raghoebir
Erasmus Medical Centre Department	Pediatric Surgery/ Cell Biology
Research School	Postgraduate School Molecular Medicine
PhD period	July 2009-July 2012
Promotor	Prof.dr.D. Tibboel
Supervisor(s)	Dr. R.J.Rottier
	Dr. M.M.J. Smits

PhD Training	**Year**
General academic skills	
"Safely working in the laboratory"	2009
English biomedical writing, Rotterdam	2011
Adobe Photoshop and illustrator CS4, Rotterdam	2010
In-depth courses	
Biomedical research techniques, Rotterdam	2008
In vivo imaging "From molecule to organism", Rotterdam	2008
Basic and translational oncology, Rotterdam	2008
From development to disease, Rotterdam	2009
MCB	2009
Epigenetic regulation, Leiden	2010
International conferences	
Winter School of the International Graduiertenkolleg Kleinwalsertal, Austria:	2010
* "transcriptional control in developmental processes" (oral presentation),	2011
* "Chromatin changes in differentiation and malignancies" (oral presentation).	2011
Digestive disease week. Chicago (oral presentation), USA	

PhD Training	Year
Seminars and workshops	
PhD student workshop, Cologne	2010
PhD student workshop, Maastricht (presentation)	2011
Presentations	
Monday Morning Meetings, Cell Biology	2009-2012
Weekly MDL seminar, Rotterdam	2011
Supervising practicals and excursions, Tutoring	
Master students	2010-2012
Social Activities	
Cluster 15 Pantomime commitee	2010-2011

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Eindelijk ben ik bij het dankwoord aangekomen en ik kan het niet helpen constant te glimlachen. Dit komt deels omdat mijn proefschrift nu echt af is, maar ook vanwege de mooie herinneringen die weer boven komen. Onderzoek doe je nooit alleen en ik voel me gezegend dat ik altijd omringd ben geweest door fijne personen, die altijd bereid waren om te mee te denken, te helpen waar nodig of gewoon om een praatje te maken. Hiervoor wil ik jullie allemaal ontzettend bedanken!

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Lalini Raghoebir