IDENTIFICATION OF SIGNALLING PATHWAYS INVOLVED IN CDX2 REGULATION IN METAPLASTIC LESIONS

IDENTIFICAÇÃO DE VIAS DE SINALIZAÇÃO ENVOLVIDAS NA REGULAÇÃO DE CDX2 EM LESÕES METAPLÁSICAS

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À memória do meu Avô

[Weeping willow with your tears running down,
Why do you always weep and frown?
Is it because he left you one day?
Is it because he could not stay?

On your branches he would swing,
Do you long for the happiness that day would bring?
He found shelter in your shade.
You thought his laughter would never fade.

Weeping willow, stop your tears.
There is something to calm you fears.
You think death has ripped you forever apart.
But I know he'll always be in your heart.

In *My Girl*, 1991]

Abstract

Intestinal metaplasia (IM) of the stomach is a pre-neoplastic lesion that appears following *Helicobacter pylori* infection and that confers increased risk for gastric cancer development. IM is induced by *de novo* expression of the intestine-specific transcription factor CDX2 in the gastric mucosa. However, the regulatory mechanisms and molecular pathways involved in the triggering and maintenance of CDX2 expression in the stomach are yet to be fully unravelled. Taking into account the importance of the BMP pathway in normal intestinal differentiation, we were able to show that key elements of this pathway not only co-localized with CDX2 in IM but also positively regulated CDX2 in an *in vitro* context. Further, we showed that in Juvenile Polyps, an intestinal differentiation phenotype somehow inverse to gastric IM, loss of BMP pathway activity is related to decreased CDX2 expression and loss of intestinal differentiation, thus reinforcing our previous results. Since IM reversibility is still controversial but an issue of fundamental importance, we studied this in a setting of *H. pylori* eradication and in relation to the virulence of the infecting bacteria. In fact, IM is rather stable and infection with high-virulence *H. pylori* strains associate with irreversibility. Following *H. pylori* infection, CDX2 appears ectopically expressed in the stomach, so we tackled, and proved correct, the hypothesis of a direct regulation of CDX2 expression by bacterial interaction with epithelial cells in an *in vitro* co-culture model. Lastly, a regulatory haplo-insufficiency as well as an autoregulatory mechanism for CDX2 had been suggested. These results, taken together with the apparent stability of the metaplastic phenotype, led us to hypothesize that CDX2 is regulating its own expression through an autoregulatory loop. We were able to demonstrate that not only does CDX2 bind to and transactivate its own promoter but also is it positively regulating its own expression in gastrointestinal human carcinoma cell lines. Furthermore, we showed that CDX2 is bound to its own promoter in human IM and in the mouse intestine, thus providing a major contribution to understanding the relevance of this regulatory pathway *in vivo* and its implication in stability of IM.

Altogether, our results put forward some of the regulatory mechanisms involved in CDX2 regulation in the gastric context, thus contributing to unveil molecular pathways implicated in the establishment/maintenance of IM of the stomach.

Resumo

A metaplasia intestinal do estômago é uma lesão pré-neoplásica que aparece na sequência da infecção por *Helicobacter pylori* e confere risco aumentado para o desenvolvimento de cancro gástrico. Esta lesão é induzida pela expressão *de novo* na mucosa gástrica do factor de transcrição intestinal CDX2. No entanto, não são ainda totalmente conhecidos os mecanismos de regulação e vias moleculares envolvidos na iniciação e manutenção da expressão de CDX2 no estômago. Tendo em conta a importância da via dos BMPs na diferenciação intestinal normal, demonstrámos que elementos-chave desta via não só co-localizam com CDX2 na metaplasia intestinal, como também regulam positivamente a sua expressão *in vitro*. Mostrámos também que em pólipos juvenis – um fenótipo intestinal de certo modo inverso da metaplasia intestinal do estômago – a perda de actividade da via dos BMPs está relacionada com uma expressão diminuída de CDX2 e com a perda de diferenciação intestinal, assim reforçando os nossos resultados anteriores. Uma vez que a reversibilidade da metaplasia intestinal é ainda controversa mas um problema fulcral, estudámos esta questão num contexto de erradicação da infecção por *H. pylori*, relacionando-a com a virulência da estirpe bacteriana. De facto, a metaplasia intestinal é relativamente estável e a infecção por estirpes de alta virulência tem uma associação significativa com a irreversibilidade da lesão. A infecção por *H. pylori* leva ao aparecimento de expressão ectópica de CDX2 no estômago e, assim, testámos e confirmámos a hipótese de uma regulação directa da expressão de CDX2 pela interacção da bactéria com as células epiteliais gástricas, num modelo *in vitro* de co-cultura. Por fim, tinha sido já sugerido um mecanismo auto-regulatório para o CDX2 bem como uma haplo-insuficiência regulatória. Estes resultados, juntamente com a aparente estabilidade do fenótipo metaplásico, levaram-nos a colocar a hipótese do CDX2 estar a regular a sua própria expressão através de um ciclo auto-regulatório. Conseguimos, assim, demonstrar que não só o CDX2 liga e transactiva o seu próprio promotor, mas está também a regular positivamente a sua expressão em linhas celulares gastrointestinais. Demonstrámos ainda que o CDX2 liga o seu próprio promotor na metaplasia intestinal humana e no íleo do ratinho, assim contribuindo para sustentar a relevância desta via regulatória *in vivo* e a sua implicação na estabilidade da metaplasia intestinal. No seu conjunto, os nossos resultados propõem alguns dos mecanismos envolvidos na regulação da expressão de CDX2 no contexto gástrico, contribuindo deste modo para

se desvendar as vias moleculares implicadas no estabelecimento e manutenção da metaplasia intestinal do estômago.

1 GENERAL INTRODUCTION
AIM AND OBJECTIVES

● Intestinal Metaplasia of the stomach

Definition and examples of metaplastic lesions

The classical textbook definition for metaplasia is "*a reversible change in which one adult cell type (epithelial or mesenchymal) is replaced by another adult cell type"* [1] and it was recently updated to the more cautious but less informative *"replacement of one type of cell with another type"* [2]. In fact, the former definition had been questioned because of two controversial aspects: on the one hand, that metaplasias are cellular rather than tissue transdifferentiation processes and always having a differentiated cell as a starting point, and, on the other hand, its reversibility. As for the first part, two cellular origins for metaplasia may be envisioned: i) the transdifferentiation of a tissue-specific differentiated cell into another cell type or ii) a switch in the commitment of a tissue-specific stem cell. Thus, a perhaps more accurate definition of metaplasia would be the conversion, in post-natal life, of one cell or tissue type into another, including transdifferentiation of differentiated cells and conversion between tissue-committed stem cells [3-4]. Concerning reversibility, from a strict molecular point of view, and considering that the transdifferentiation consists in switching off a set of genes/transcription factors and switching on another set, the process should be reversible by removal of the newly expressed genes. Nevertheless, not only do *in vitro* transdifferentiated cells seem to be stable once formed, even after removal of the initial trigger [5-6], but also conflicting results have been obtained when actual regression of human metaplastic lesions has been assessed (reviewed in [7]).

Naturally occurring metaplasias nearly always arise in tissues that have been subjected to chronic trauma/inflammation, infection or abnormal hormonal stimulation, and hence undergoing processes of wound healing and continuous regeneration. In epithelial tissues, metaplasias may follow a glandular-to-squamous, a squamous-to-glandular or a glandular-to glandular pathway. An example of the first is the frequent squamous metaplasia of the respiratory epithelium in cigarette smokers [8], whereas the classical example of the second type is Barrett's oesophagus, which appears subsequently to gastro-oesophageal reflux disease [9]. The prototype of a glandular-to-glandular

metaplasia is the intestinal metaplasia of the stomach [10]. Nonetheless, metaplastic lesions are not exclusive of epithelial tissues, and mesenchymal tissues, such as bone, cartilage and adipose tissue can be encountered ectopically in soft tissues or in other organs (e.g. heart) [2]. Many of these metaplastic lesions appear as an adaptive response, conferring a certain protection against the injury agent. However, this phenomenon places a "normal" tissue in an abnormal environment, possibly rendering it more prone to acquiring growth abnormalities and thus creating a susceptible ground for neoplastic transformation, since most metaplasias are pre-neoplastic lesions. Some of these metaplasias therefore bear clinical significance in that they predispose individuals to cancer development – lung cancer usually arises from the ectopic patches of squamous epithelium [8], oesophageal adenocarcinoma generally emerges in areas of Barrett's metaplasia [9] and the great majority of sporadic gastric cancers appear in the context of intestinal metaplasia [11].

Besides being an extremely interesting biological phenomenon, the study of the mechanisms involved in the onset of metaplasias acquires great importance for three main reasons, i) it is likely to extend the knowledge on normal developmental processes, ii) many metaplasias are cancer precursors, and iii) it might provide insight on stem cell reprogramming for transplantation purposes.

Histological and molecular characterization of Intestinal Metaplasia of the stomach

Gastric intestinal metaplasia is a multifocal regenerative lesion that appears following infection- and inflammation-induced gastric gland atrophy. Morphologically, it is characterized by the presence of intestinal cell types, such as goblet, Paneth and absorptive cells, alone or in combination, within the gastric mucosa [10] (Figure 1). The ectopic intestinal glands show a complete reorganization, with displacement of the proliferative zone from the neck region down to the base of the crypt, thus resembling the normal intestine, concomitant with alterations in the stromal sheath surrounding the metaplastic gland which also acquires an intestinal phenotype [12]. Interestingly, experimental data show that implantation of intestinal foetal mesenchyme beneath the gastric epithelial layer induces remodelling of the epithelial architecture with development of immature villi and crypts [13]. Together, these results favour intestinal metaplasia as a tissue, rather than a single cellular, transdifferentiation event.

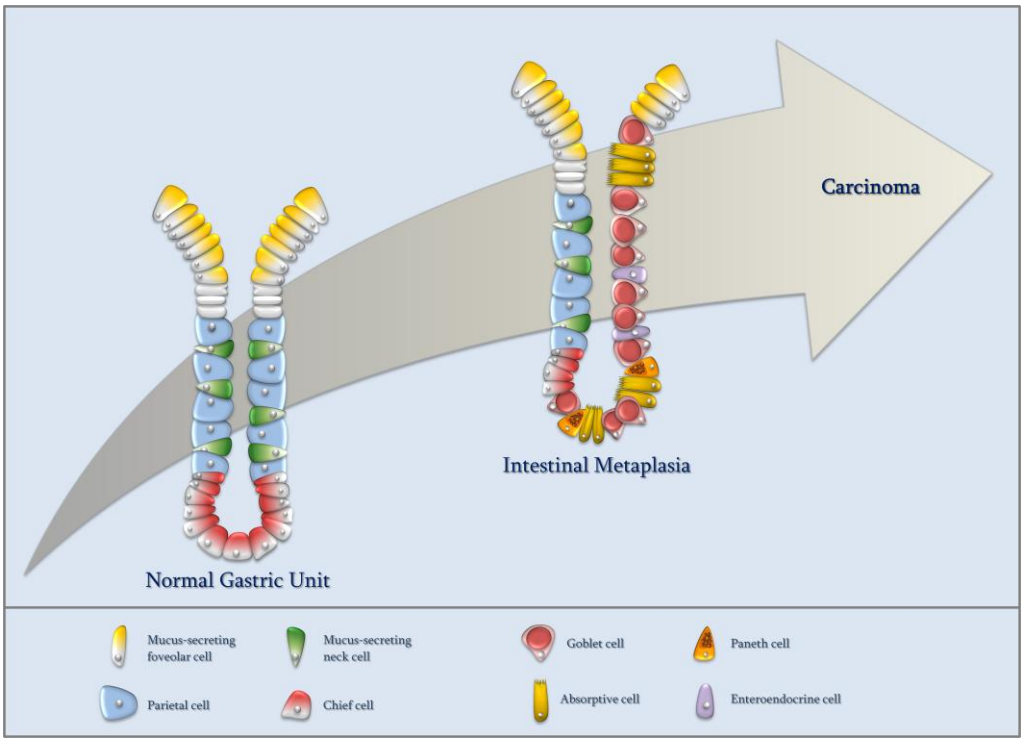


Figure 1 > Schematic representation of normal gastric mucosa and an intestinal metaplastic gland. The normal gastric unit depicted is representative of the corpus, although intestinal metaplasia in *H. pylori* infected stomach usually appears in the antrum or in incisura angularis.

Classification of intestinal metaplasia (IM) has evolved over time. Initially, histochemically-based methods were used allowing the definition of three different types: i) type I, or complete IM, containing absorptive enterocytes, Paneth cells, and sialomucin-secreting goblet cells; ii) type II, incomplete IM, with co-existence of goblet and columnar cells secreting sialomucins and iii) type III, also incomplete IM, with presence of goblet and columnar cells secreting sulphomucins [14]. Later, after the molecular alterations in intestinal metaplasia were described, mainly in terms of loss and gain of differentiation markers, namely mucins, a new classification emerged. Accordingly, two main types of intestinal metaplasia were defined: i) complete or intestinal IM, characterized by complete loss of gastric mucin markers (MUC1, MUC5AC and MUC6) and gain of the intestinal mucin MUC2 and ii) incomplete or gastrointestinal mixed IM, where the intestinal mucin MUC2 is found together with maintenance of the gastric mucins [15]. Interestingly, this mixed phenotype can occur at the single cell level, with co-expression of MUC5AC and MUC2 [16]. From the molecular standpoint, common to both metaplasia types is the *de novo* expression of

the intestinal transcription factor CDX2 [17-21], normally restricted to the intestine. This is also true for the CDX2 homologue CDX1, whose normal expression in adults is also restricted to the intestine but appears ectopically expressed in intestinal metaplasia lesions [20, 22].

Other markers have been described to be differentially expressed in normal gastric epithelium and in intestinal metaplasia. On the one hand, intestinal-specific markers such as Sucrase.-Isomaltase [23], Villin [16], Liver-Intestine-Cadherin (LI-Cadherin) [24], Defensin 5 [25], Alkaline phosphatise [26], intestinal trefoil factor TFF3 [27], amongst others, have been shown to become expressed in intestinal metaplasia lesions. Also the POU homeodomain protein OCT-1 [28] and the transcription factors PDX-1 [29] and MATH1 [30] were shown to be overexpressed in intestinal metaplasia. On the other hand, SOX2 [31], Sonic Hedgehog [32], RUNX3 [33], and TFF1 and TFF2 [34] have been demonstrated to be decreased or lost during the transition from normal gastric to intestinal metaplasia mucosa. Apart from altered gene expression, also several genetic alterations have been described in intestinal metaplasia. Aneuploidy [35], p53 mutations [36-37], microsatellite instability [38-39] and mitochondrial microsatellite instability [40], among others, were all shown to be present in a subset of intestinal metaplasia lesions, mostly in the incomplete type.

Intestinal Metaplasia, *Helicobacter pylori* and Gastric Cancer

Even though gastric cancer incidence has been decreasing in western countries over the last century [41], it remains a serious health burden worldwide, and it is still a major health concern in Portugal [42]. Gastric cancer presents a pronounced incidence variation across geographical areas [43] but it is still the fourth most common cancer and the second leading cause of cancer-related death worldwide [44]. Worsening this scenario, gastric cancer survival is globally low and has not significantly improved during the past decades [45-46]. Pelayo Correa proposed in 1992 a model for the gastric carcinogenesis pathway [47], where infection with *Helicobacter pylori*, together with other environmental aggressions, triggers a stepwise process of progressively more severe lesions in the gastric mucosa, ultimately leading to intestinal-type gastric adenocarcinoma (Figure 2). *H. pylori* was thus classified as a type I carcinogen by the International Agency for Research on Cancer [48], and considered the primary cause

for gastric cancer development. According to the aforementioned model, precancerous steps following infection are chronic gastritis, multifocal atrophic gastritis, intestinal metaplasia and dysplasia [47, 49]. However, some controversy exists concerning the relevance of the latter, since it is rarely seen either prior to cancer or surrounding the carcinoma [10].

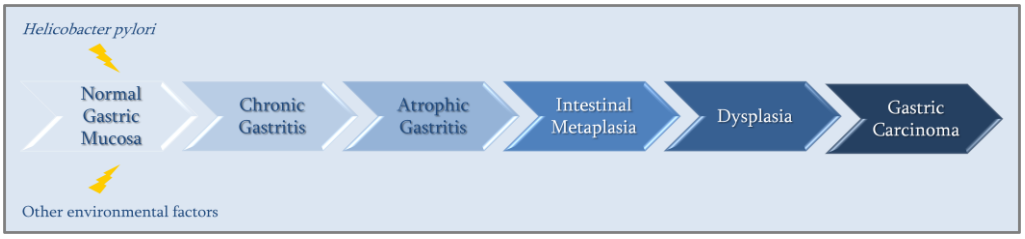


Figure 2 > Gastric carcinogenesis pathway.

Intestinal metaplasia is thus generally accepted as a pre-neoplastic lesion conferring increased risk for gastric cancer development [11], and its cause-effect relation with *H. pylori* infection is indisputable. However, intestinal metaplasia arises in only approximately 30% of infected individuals, from which only about 7% will develop gastric cancer [11]. Although low, these percentages acquire particular importance in countries where the prevalence of infection remains very high, such as Portugal [50], south American [51] and far east countries [52], where still approximately 80% of the population is infected. The variable outcome of *H. pylori* infection suggests that intestinal metaplasia results from distinct adaptive responses in adverse circumstances, with the interplay between individual genetic variation and environmental exposures being determinant for individual susceptibility. Moreover, the existence of different intestinal metaplasia types may aid to understand the variable incidences of gastric cancer in individuals presenting this pre-neoplastic lesion. The relative risks for cancer development ascribed to the presence of different intestinal metaplasia subtypes differ considerably, with type III incomplete type conferring about 4-fold increased risk as compared to type I [14].

The first human study suggesting putative genetic susceptibility for intestinal metaplasia development identified an increased prevalence of this lesion in relatives of gastric cancer patients [53]. Further evidence came from studies associating genetic

polymorphisms to this lesion's outgrowth. Examples of host genetic characteristics conferring increased risk for intestinal metaplasia development are polymorphisms in genes encoding components of the mucus, such as the MUC1 mucin gene [54-55], in genes encoding pro-inflammatory cytokines such as IL-1β and its receptor antagonist [56-58] or even the Insulin-like growth factor binding protein 3 [59].

Genetic characterization of *H. pylori* allowed the association of different bacterial genotypes to diverse gastric histopathological outcomes. High virulence strains, harbouring the *cagA*+, *vacA s1*, *vacA m1* and *babA2* genetic virulence markers, associated with increased risk for strong inflammatory responses and pre-neoplastic lesion development, including intestinal metaplasia [60-63].

H. pylori is probably not the only environmental-associated risk factor for intestinal metaplasia development/progression. The most extensively studied lifestyle exposure is tobacco smoking, and the epidemiological data gathered so far mostly shows that it confers increased risk for both intestinal metaplasia development [64-69] and progression towards carcinoma[70]. Nevertheless, some studies failed to verify this association [71]. The causal role of alcohol [64, 72-73] and salt intake [64, 70-71, 74] for intestinal metaplasia outgrowth is still unsettled, in spite of high salt intake inducing intestinal metaplasia in a Mongolian gerbil model [75]. Nevertheless, these three environmental exposures, tobacco, alcohol and salt, are generally accepted to play deleterious roles. On the other hand, the protective effect of dietary antioxidants and anti-inflammatory nutrients also remains elusive [66, 71, 73, 76-77].

Another aspect of intestinal metaplasia that has been largely debated concerns its putative reversibility. Despite the large number of studies, both observational and experimental, regarding intestinal metaplasia regression after *H. pylori* eradication, the results obtained this far are still conflicting. The first placebo-controlled randomized eradication study supported irreversibility of pre-neoplastic lesions, since cancer risk only decreased upon eradication in subjects with non-atrophic gastric mucosa at baseline [78]. However, other human experimental studies, both of *H. pylori* eradication alone or in combination with alimentary supplements, favoured intestinal metaplasia reversibility [76, 79-80]. During the course of this work, a cohort with 17-years follow-up was described with results supporting irreversibility of this lesion [81].

Animal models of Intestinal Metaplasia

Over the past two decades, several animal models developing intestinal metaplasia have been reported. The one that best recapitulates all gastric histological events following *H. pylori* infection leading to intestinal metaplasia and ultimately gastric cancer, thus corroborating the causal role of infection on pre-neoplastic lesion and cancer appearance, is a Mongolian gerbil model. Several studies show that these animals, after long-term infection, develop intestinal metaplasia lesions that resemble the human ones regarding the cell types that are present [82-84]. Further, these metaplastic lesions evolve to, or at least surround, gastric adenocarcinomas. However, complete molecular characterization, namely with respect to well established markers of the human intestinal metaplasia such as CDX2 and MUC2, among others, is still lacking.

Mouse models vary notably on susceptibility to infection by the different *Helicobacter* species and strains, and the few studies with *H. pylori* infection described so far use mouse-adapted strains [85]. Mice subjected to *H. felis* or *H .pylori* infection fail to develop the classical Cdx2-dependent intestinal metaplasia present in the human disease, with absorptive enterocytes and goblet cells, but rather produce a different type of metaplasia, SPEM – spasmolytic polypeptide-expressing metaplasia [86]. SPEM is characterized by the presence of antral-like glands in the corpus, which express spasmolytic polypeptide (TFF2), a trefoil peptide usually present in the normal intestinal mucosa and gastric antrum. Mice developing SPEM progress to atrophy and evolve to dysplasia and cancer, in what they are consistent with the human carcinogenic events [86-87]. Although SPEM is also found in humans, especially following oxyntic atrophy in the setting of inflammation, and despite the association with *H. pylori* infection and gastric cancer development, it has a much lower significance for human pathology [88-89]. Furthermore, it was also demonstrated that these mice show regression of established mucosal changes, namely atrophy and metaplasia (SPEM), upon early *Helicobacter* eradication [87]. Contrary to what is generally conceived, the same model suggested that gastric pre-neoplastic lesions and intraepithelial cancer originate from bone marrow-derived cells, which are recruited and repopulate the stomach upon infection-induced mucosal injury [90].

The induction of ectopic intestinal phenotype in the stomach was also achieved in animal models by manipulating further downstream events in the carcinogenic cascade.

Two models of transgenic mice with forced *Cdx2* expression in the gastric mucosa were developed [91-92]. Using different promoters to direct Cdx2 expression in the gastric mucosa they assured onset of Cdx2 expression either during embryonic development (when linked to the *Foxa3* promoter) [92] or postnatally (under the control of *H^+/K^+-ATPase β-subunit* gene promoter) [91]. They both displayed extensive intestinal metaplasia, with all intestinal cell types but Paneth cells, as well as a number of intestine-specific gene products typical of the different lineages. Interestingly, these two models put forward two separate paths for metaplasia development. Expression of Cdx2 during foetal development may affect the undifferentiated endodermal cells of the foregut, normally devoid of this protein, and thus interfere with cell fate determination. This possibly results in induction of intestinal rather than gastric differentiation in a subset of these cells. Conversely, the *de novo* expression of Cdx2 associated to differentiated parietal cells suggests a cellular transdifferentiation, loosing expression of gastric markers and gaining intestinal ones. Furthermore, one of the mouse models spontaneously progressed to gastric carcinoma [93], hence indicating that intestinal metaplasia itself plays a significant role in cancer genesis. However, the other failed to confirm intestinal metaplasia progression, even when in conjugation with a high salt diet [94]. A similar model was developed with the *Cdx2* homologue *Cdx1*. Ectopic gastric *Cdx1* expression was directed by the promoter of the *H^+/K^+-ATPase β-subunit* gene and mice developed extensive intestinal metaplasia, but with presence of all four intestinal cell types [95]. The *Cdx1*-induced gastric intestinal metaplasia, however, differed from the *Cdx2*-induced one in differentiation (cell lineages present and absence of pseudopyloric gland metaplasia), structure (the mucosa of *Cdx1* transgenics was significantly thicker than that of the *Cdx2* transgenic or wild-type mice) and proliferation (with *Cdx1* inducing proliferating cells scattered all over intestinal metaplasia and *Cdx2* inducing their concentration at the base of the metaplastic glands) [95].

Other mouse models were shown or suggested to exhibit aberrant intestinal development in the stomach. However, they are poorly studied. The *Gastrin* knock-out mouse shows achlorhydria and develops intestinal metaplasia, with Cdx2 expression, and gastric tumours [96-97]. The homozygous mutation of the SHP2-binding site in the IL-6 family receptor gp130 led to the development of two metaplastic lineages – intestinal-like cells, as judged by the presence of acidic mucins, and clear brush border morphology, but with no evidence of goblet cells, and SPEM [98]. This mouse further

supports predisposition for lesion development in a context of achlorhydria and hypogastrinemia also described in the *Gastrin* knock-out mice. Intestinal transformation of the foetal stomach was found in *Sonic Hedgehog* homozygous null embryos [99], as well as in *Gli3* null ones, which lack this downstream effector of the Hedgehog signalling [100]. Finally, intestinal differentiation with goblet cells and expression of Cdx2 appeared in subcutaneously grafted gastric cells derived from *Runx3*$^{-/-}$ mice [101].

The relevance of genetic susceptibility for intestinal metaplasia development observed in humans was also tackled in two animal models. On the one hand, mice strains with differential Th1 and/or Th2 patterns of immune responses showed different propensity for mucosal damage and metaplasia development upon *Helicobacter sp.* infection [102-103]. On the other hand, it was reported that the rat strain significantly influenced the intestinal metaplasia outcome following X-irradiation [104].

• The *Caudal*-related homeobox gene 2 – *CDX2*

Caudal homologues: structure and function, similarities and role in development

The *caudal* gene was first identified in Drosophila and held responsible for both early posterior segment patterning and later development and maintenance of the hindgut primordium [105-107]. Since then, *caudal* homologues have been identified in a wide range of organisms, from *C. elegans* to mice and humans [108-110], and shown to play critical roles in intestinal epithelium development and maintenance [107, 111]. Furthermore, they have also been associated to carcinogenic processes, not only in the gut but also in non-intestinal tissues, such as stomach, oesophagus, extrahepatic bile duct and hematopoietic lineages [17, 22, 112-116].

In mice and humans three *caudal* homologues are found, *Cdx1* [117], *Cdx2* [118] and *Cdx4* [119]. They locate to the ParaHox gene cluster and share a very high homology between them, with highly conserved domains. The largest and most conserved of them is the homeodomain, encompassing 60-63 aminoacids arranged in a helix-loop-helix structure, which accounts for their DNA-binding activity and coining the name to the homeobox gene family. Further, Cdx1 and Cdx2 also harbour an activation domain at

the N-terminus, conferring them gene transactivation activity, their primary function as transcription factors. All three proteins are expressed early during embryogenesis and bear a role in patterning of the anterior-posterior axis [120]. *Cdx2* heterozygous null mice present an anterior homeotic shift of the vertebrae, along with stunted growth and tail abnormalities [121]. A similar phenotype was described for *Cdx1* null mice [122]. *Cdx4*$^{-/-}$ mice have no morphological defects, and the subtle contribution of this gene to axial patterning was only evident in *Cdx1/Cdx4* and *Cdx2/Cdx4* compound mutants [123]. However, ablation of *Cdx2* was the sole to result in an intestinal phenotype [121]. Later studies demonstrated that both Cdx1 and Cdx2 are important in intestinal development [111] whereas the spatial distribution and function of Cdx4 in post-embryonic life is yet unclear. Lastly, Cdx2 is expressed from day E3.5 in trophectoderm [108] and involved in this lineage's specification, consequently playing a crucial role in implantation [124]. In fact, this precludes the assessment of the *Cdx2* null phenotype due to pre-implantation embryonic lethality [121].

CDX proteins in gut development and intestinal differentiation

Cytodifferentiation into intestinal epithelium occurs early during embryogenesis, with the four epithelial lineages – absorptive enterocytes, Goblet, Paneth and enteroendocrine cells – developing shortly after. In late embryonic and early post-natal life, villi lengthen and crypts are formed from the intervillus regions, and colonic epithelial development occurs even later [125]. Thus, correct development of a mature gut epithelium requires an accurate spatial and temporal expression of a number of gene products. On the other hand, for continuous intestinal epithelium renewal, a tight regulation of stem cell proliferation and daughter cell differentiation is also fundamental. *Cdx2* in conjunction with *Cdx1* may well contribute to this balance. Cdx1 and Cdx2 proteins exhibit a different yet overlapping topographical distribution, both along the anterior-posterior gut axis and the crypt-villus axis. Cdx1 expression increases along the anterior posterior-axis, with maximal expression in distal colon. On the other hand, Cdx2 presents highest expression in the proximal colonic epithelium, and diminishes in both directions [22, 111, 118]. Cdx1 expression markedly varies vertically, being highest in the crypt [22, 111] whereas no gradient in protein level along the crypt-villus axis is observed for Cdx2, but it rather appears differentially phosphorylated along this axis, thus suggesting activity modulation by post-translational mechanisms [126-128]. Temporally, all

evidences are in favour of *Cdx1* being switched on after *Cdx2* in the developing gut [108, 111, 118, 129].

The fundamental importance of CDX2 in regulation of intestinal differentiation is now widely accepted and proven by several lines of evidence. The first evidence was obtained by Suh and colleagues when demonstrating that conditional expression of Cdx2 in IEC-6 undifferentiated intestinal cell line led to the formation of multicellular structures with a well-formed columnar layer. These cells presented characteristics of intestinal epithelial cells, either enterocytic-like polarized cells, with apical microvilli and associated glycocalyx, or cells with a goblet cell-like ultrastructure [130]. Later, similar results were obtained with Cdx1 in the same context [131]. Other studies confirmed both Cdx2- and Cdx1-induced intestinal differentiation *in vitro* [132-134]. The crucial role of Cdx2 in directing intestinal cell fate was further confirmed when *Cdx2* heterozygous null mice were shown to develop non-cancerous polyp-like lesions along the intestine [121]. These polyps arise early during embryonic development and are characterized by complete depletion of Cdx2 expression, with no evidence for any structural alteration (mutation, deletion) in the remaining allele [121, 135]. The mechanism for Cdx2 expression loss is not known, but the variable phenotype among littermates suggests a dosage-dependence or haploinsufficiency for gene expression. Very interestingly, the loss of Cdx2 expression is accompanied by appearance of gastric and oesophageal differentiation, with evidence for intercalary regeneration around the metaplastic lesions [136]. Of note, Cdx1 expression is also lacking in this Cdx2 loss-induced foregut-type heterotopias [137]. In addition, mice with conditional homozygous *Cdx2* knock-out in the intestine presented several intestinal abnormalities, ectopic activation of a foregut differentiation program and had intestinal terminal differentiation severely impaired [138-139]. After demonstration that it is necessary for normal development of the intestinal phenotype, Cdx2 was shown to be sufficient to drive intestinal transdifferentiation of the gastric mucosa. Transgenic mice with forced Cdx2 expression in the stomach, either during embryonic development or postnatally, develop extensive intestinal metaplasia [91-92]. Furthermore, CDX2 appears ectopically expressed in every focus of aberrant intestinal differentiation occurring in the human body [17-20, 140-144]. As in normal intestinal development, also in intestinal metaplasia lesions does CDX2 expression precede that of CDX1 [145], and Cdx1 is induced in mice with Cdx2-driven intestinal metaplasia [146]. In contrast, transgenic mice with Cdx1-induced intestinal metaplasia development do not acquire gastric Cdx2

expression [95]. Altogether, this presents evidence for CDX2 being the master gene for intestinal differentiation, both in normal and aberrant locations. Nonetheless, CDX1 is also implicated in intestinal differentiation, however with a less well defined role [131, 147]. Contrary to the *Cdx2*$^{+/-}$, the *Cdx1* knock-out mice present no intestinal abnormalities [122], nor do the mice with *Cdx1* overexpression in the gut [148-149]. However, not only is CDX1 also expressed in foci of aberrant intestinal differentiation [20, 150-151] but also, surprisingly, transgenic mice with ectopic gastric expression of this gene develop intestinal metaplasia as well [95].

CDX2 regulation of intestine-specific gene expression

CDX2 is primarily a transcription factor, and its role in intestinal differentiation encompasses its positive regulatory activity on several genes involved in proliferation, differentiation and intestinal cell fate specification. Typically, CDX2 target genes enclose one or several copies of a consensus Cdx-binding motif, frequently defined as TTTAT(A/G) [152], but with some degree of nucleotide variance, being mainly characterized as an AT-rich sequence, both in proximal promoters [153-154] or distal elements [155-157]. Oftentimes, these Cdx-binding sites locate in close proximity to [158-160] or even overlapping with [161-163] the TATA-box. The first gene identified as a Cdx2 transcriptional target was the enterocyte-specific enzyme Sucrase-Isomaltase (SI) [132, 153, 164]. Following SI, several CDX2 downstream targets have been identified, most of which are characteristic of intestinal cells' structure and activity. Still concerning genes involved in enterocytic activity, Lactase-phlorizin hydrolase (LPH) [154, 158-159], Guanylyl cyclase C (GC-C) [165], intestinal PhospholipaseA/lysophospholipase (PA/LP) [160], Alkaline phosphatise(ALP) [157], Vitamin D receptor [166], Calbindin-D9K [155-156, 161] and Villin [167] were shown to be regulated by CDX2. This protein was also demonstrated to play a role in goblet cell differentiation by regulating MUC2 [162, 168] and KLF4 [169-170]. Further, CDX2 also regulates the expression of gene products that mediate cellular adhesion, including LI-Cadherin [171], Claudin 2 [172], Claudin 3 and 4 [173], and other intestinally-expressed markers such as A33 antigen [174], Carbonic anhydrase I [175], Clusterin [163], Trefoil factor 3 (TFF3) [176], NADPH oxidase 1 (NOX1) [177] and Insulin receptor substrate 2 (IRS2) [178]. In addition, CDX2 was shown to regulate genes associated to growth inhibition, regional migration and differentiation. Examples of these genes include KLF4[170], MAPK/MAK/MRK overlapping

kinase (MOK) [179] and p21 [180], which are involved in growth arrest and differentiation, Engulfment and cell motility 3 (ELMO3) [181], which is implicated in cell migration, and MATH1 [30], which is involved in cell fate determination of the intestinal secretory lineage [182-183]. A still unclarified target of CDX2 regulation is its homologue CDX1. *Cdx1* expression is switched on after *Cdx2* both in the developing gut [108, 111, 118, 129] and in intestinal metaplasia [145]. Additionally, Cdx1 is also lost, together with Cdx2, in the intestinal polyps of *Cdx2*$^{+/-}$ mice [137] and in conditional *Cdx2* knock-outs' ileum [138], and transgenic mice with gastric Cdx2 expression are shown to onset endogenous *Cdx1* expression [146]. This suggests that Cdx2 expression not only appears prior but is also required for Cdx1 expression. In contrast to this, CDX1 expression has also been suggested to be downregulated by CDX2 in cell line models [132, 184].

For some of these CDX2 targets, the regulation was demonstrated to be cell-type specific. In addition, and at the intestinal level, CDX2 target genes are expressed in a well-arranged pattern both along the gut and in the different intestinal cell-types. This fine-tuning of the CDX2-induced gene transcription, and activity in general, is likely to rely on CDX2 interaction with other transcription factors [158, 164, 177, 185-186] – possibly cell- or tissue-specific –, as well as on post-translational modifications such as phosphorylation [126-128, 187].

Regulation of *CDX2* expression

CDX2 regulation during embryonic development and in post-natal life relies upon binding of numerous transcription factors to *cis*-regulatory elements, and probably subsequent integration of their positive and negative transcriptional effects. The promoter region containing the first intron and extending up to -5kb upstream of the transcriptional start site was shown to be sufficient for Cdx2 activation at gastrulation and later inactivation at midgestation, although additional 4kb upstream were required for maintenance of Cdx2 expression in intestinal endoderm/epithelium [188]. This region provided binding sites for a series of transcription factors, of which Hepatocyte Nuclear Factor 4 alpha (HNF4α), GATA6, β-Catenin and Tcf4 exerted a synergistic stimulatory effect on the *Cdx2* promoter in a colonic cell line. HNF4α, which is a transcription factor important for intestinal development, was later confirmed to bind to

and transactivate the *CDX2* promoter [189]. In this same context, SOX2 and SOX9 act as repressors, blunting the abovementioned positive regulation [188]. In line with this, SOX9 had already been suggested as a CDX2 transcriptional repressor [190] and further data confirmed the repressive effect of SOX2, as decreasing its expression led to an upregulation of CDX2 in gastric cells [31]. In the intestinal context, other regulatory pathways for CDX2 have been put forward. PTEN and TNF-α proved to differentially regulate CDX2 expression through a NF-κB-dependent pathway [191]. NF-κB p65/p50 dimers reduce *CDX2* promoter activity and this effect is prevented by binding of p50/p50 dimers. TNF-α and PI3K favour p65/p50 binding whereas PTEN, by inhibition of the PI3K pathway, shifts the balance towards p50/p50. Involvement of the intestinal epithelium microenvironment was suggested in the direct CDX2 expression regulation. Sodium butyrate, a byproduct of colonic bacteria fermentation, stimulated CDX2 expression [192]. A stimulation of CDX2 by myofibroblast-produced Wnt5a through its receptor Ror2 was also reported [193]. Also Laminin-α1, a basement membrane component, positively modulates CDX2 expression [132]. Conversely, Collagen type I, through β1-integrin signalling, transcriptionally represses CDX2 [194], correlating with the observed CDX2 decrease in budding tumour cells. Also disruption of members of the Retinoblastoma pathway led to increased CDX2 expression [195]. Recently, colorectal carcinoma cells revealed Protein kinase C (PKC)-dependent CDX2 loss upon activation of the Raf-MEK-ERK1/2 [196].

Other regulatory mechanisms and transcription factors have been suggested though with conflicting results and high degree of tissue/context-specificity. Long-term acid exposure was shown to induce Cdx2 expression in cultured oesophageal keratinocytes [197] but an opposite effect was reported in the duodenal context [198]. Jin and Li presented data on positive regulation of CDX2 by OCT1 in pancreatic and intestinal cell lines [199] which was not reproduced later in the gastric model, where promoter binding but not transactivation was demonstrated [28]. Mechanisms of CDX2 regulation by cyclic AMP [200] as well as autoregulation of the CDX2 promoter [201-202] exhibit a strict cell type-specificity in pancreatic and colonic cell lines. It was also shown that acid and/or bile acid treatments were able to activate CDX2 through promoter demethylation in human oesophageal cells [203], and methylation had been linked to reduced expression in colorectal cell lines [204]. However, although CDX2 methylation status was suggested to vary from gastric cancers to surrounding normal epithelium [205], it failed to relate do CDX2 expression in normal gastric mucosa, intestinal

metaplasia foci and gastric carcinoma cell lines [206]. Lastly, and during the course of this work, in an *in vitro* co-culture model, *H. pylori* increased CDX2 expression [207], by means yet to be unraveled.

● The Bone Morphogenetic Protein (BMP) pathway

Bone Morphogenetic Proteins (BMPs) comprise multifunctional secreted growth factors that compose the largest sub-family of the Transforming Growth Factor β (TGFβ) superfamily [208]. They were first identified by their ability to induce *in vivo* ectopic bone and cartilage formation [209-211], and named after this finding [212]. However, the name proved to be misleading since BMPs have been implicated, thereafter, in processes as diverse as proliferation, differentiation, apoptosis and cell-fate determination in multiple tissues, and shown to exert pleiotropic biological effects, from regulation of early embryonic growth and development to organogenesis (reviewed in [213-215]).

The canonical BMP signalling pathway follows a well established cascade of events from binding of the extracellular ligands to interference with gene transcription. BMP homodimers initiate signalling by binding to preformed heterotetrameric receptor complexes. These are composed of a dimer of type I receptors, BMP receptor 1A (BMPR1A) [216] or BMP receptor 1B (BMPR1B) [217], and a dimer of type II receptors, BMP receptor II (BMPRII) [218], both of which are serine-threonine kinase receptors. Upon ligand binding, the type I receptor is phosphorylated by the type II receptor kinase, becoming active and able to further phosphorylate intracellular transducers [219]. The BMP intracellular signalling molecules, named SMADs, are grouped into three sub-classes: signal transducer receptor-regulated SMADs (R-SMADs), which include SMAD1, SMAD5 and SMAD8; common mediator SMAD (co-SMAD), SMAD4; and inhibitory SMADs, comprising SMAD6 and SMAD7. Activated type I receptors phosphorylate the R-SMADs, which then form heterodimeric complexes with the co-SMAD and translocate to the nucleus, where they recruit co-activators and/or co-repressors to regulate specific target genes (reviewed in [220-221]). Binding to the DNA occurs at specific sequences, known as BMP/SMAD-responsive elements, falling into CAGA [222] or GCCG [223] sequences. The inhibitory SMAD6 and SMAD7 play their role by interfering both with R-

SMAD phosphorylation and with R-SMAD/co-SMAD heterodimerization (reviewed in [220-221]). Other mechanisms of pathway inhibition have been suggested, including an autoregulatory negative feedback loop [224], the non-signalling pseudoreceptor BAMBI [203], the ubiquitination-inducers SMURFs [225-226] and a series of extracellular antagonists, namely Noggin [227]. The activity of BMPs is thus tightly regulated by gene expression, growth factor protein processing from the precursor polypeptide and binding to secreted soluble BMP antagonists, and, at the intracellular level, by phosphorylation inhibition or induced degradation of intracellular transducers (Figure 3). An alternative SMAD-independent signalling has also been proposed, occurring upon ligand-induced heteromeric receptor complex formation and activation of the MAPK pathway [228].

We will focus on the involvement of the BMP pathway in intestinal differentiation. The precise function of this pathway in the intestine remains to be fully unravelled. However, it has been implicated in establishment and maintenance of normal intestinal epithelial architecture and differentiation in *Xenopus* [229] and mammalian [230] models, being a key factor in the mediation of epithelial-mesenchymal interactions. Long-range signalling from the intervillus region induces BMP2 and BMP4 expression in the intestinal mesenchyme. These molecules feed back to the epithelium at the villi, which express their receptor, BMPR1A, as well as the hallmark of an active pathway, the phosphorylated forms of SMADs 1, 5 and 8. Here BMP signalling induces differentiation and inhibits crypt-like behaviour. On the other hand, mesenchymal expression of the BMP inhibitor Noggin in the area surrounding the crypt prevents BMP action thus warranting persistence of proliferation [231-232]. Furthermore, the BMP pathway has been suggested to cross-talk with other active signalling pathways, as the Wnt [233] and Hedgehog [229, 234] pathways. During the course of this work, *in vitro* studies have further shown that BMP4 was able to promote intestinal differentiation in an oesophageal model as assessed by expression of columnar cytoketarins [235].

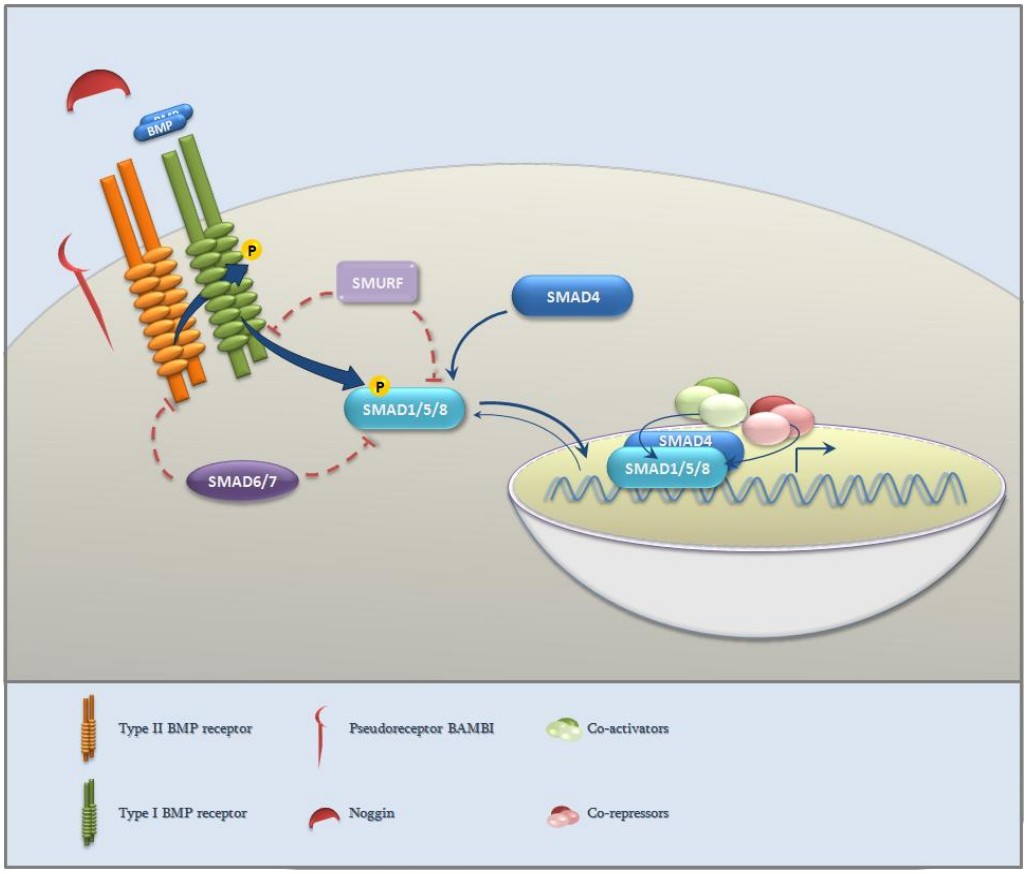


Figure 3 > Schematic representation of the canonical Bone Morphogenetic Protein pathway.

Mice models with impairment of the BMP pathway confirmed its pivotal role in normal intestinal morphogenesis. Homozygous null mutation of *Smad4* leads to embryonic lethality, but heterozygous mutants are viable and appeared normal in the first year of life. However, mice older than one year develop multiple gastric and intestinal polyps highly similar to those in Juvenile Polyposis [236]. Interestingly, a later study suggested that the impairment of Smad signalling in T-cells rather than in the epithelium, would account for the tumourigenesis in *Smad4*$^{+/-}$ mice as well as in Juvenile Polyposis patients [237]. Furthermore, conditional *Bmpr1a* ablation in the mouse intestine resulted in an hyperproliferation of the crypt compartment and a defective terminal differentiation of cells from the secretory lineage [233, 238]. A similar phenotype was observed in a mouse model with Noggin overexpression in the intestine, where BMP pathway inhibition led to the appearance of numerous ectopic crypts and atrophy of the differentiated compartment together with polyp-like growth [230, 232]. Altogether, this ascertains the role of the BMP pathway in the balanced production of the different

epithelial cell lineages together with the maintenance of intestinal proliferative/differentiated compartments' homeostasis.

Interestingly, deficiencies in this pathway are also found in a human gastrointestinal disease, the cancer-predisposing Juvenile Polyposis Syndrome, where mutations in *SMAD4* and *BMPR1A* account for about 40% of the familial cases [239]. This dominant autosomal and genetically heterogeneous disorder is characterized by the appearance of numerous hamartomatous polyps in the intestine and throughout the gastrointestinal tract. The Juvenile polyps present a distorted architecture, with a normal epithelium overlaying a markedly expanded lamina propria enclosing dilated cystic glands, inflammatory cells and a prominent stroma [240], a phenotype closely resembling that of the mouse models with intestinal loss of the BMP pathway.

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Aim and Objectives

Intestinal metaplasia of the stomach is a lesion consisting in the aberrant differentiation of the gastric mucosa into an intestinal phenotype, where glands resemble the intestinal epithelium both morphologically and functionally. It is a pre-neoplastic lesion that appears in the gastric carcinogenic pathway following *Helicobacter pylori* infection, and it confers increased risk for gastric carcinoma development, which remains the second leading cause of cancer-related death worldwide. Furthermore, intestinal metaplasia was shown to be dependent on ectopic expression of the homeobox gene CDX2.

CDX2 is a transcription factor essential for intestinal development and phenotype maintenance in adults, and its post-embryonic expression is restricted to the gut. Animal models with loss of CDX2 expression in the intestine and forced expression in the stomach demonstrated that this gene product plays a crucial role in intestinal differentiation in both normal and aberrant locations. Accordingly, CDX2 is expressed in every focus of aberrant intestinal differentiation in the human body, namely gastric intestinal metaplasia. However, the molecular mechanisms governing the onset and maintenance of its ectopic expression in this setting are yet largely unknown.

The general aim of this work is to unravel novel molecular mechanisms involved in the regulation of CDX2 expression in the gastric context and relate them to intestinal metaplasia establishment and maintenance.

Specific objectives

1) Characterize the Bone Morphogenetic Protein (BMP) pathway in intestinal metaplasia and in study its role in CDX2 regulation

The BMP pathway is active in the intestine and has been extensively linked to maintenance of the differentiated compartment in this organ. Mouse models with loss of BMP signalling show hyperproliferation of the intestinal crypts and a deficient differentiated compartment, and fail to terminally differentiate the intestinal secretory

lineage. On the other hand, CDX2 is also responsible for intestinal cell specification and differentiation, namely by regulating a series of intestine-specific genes. Interestingly, BMP-expressing inflammatory cells are recruited to the stomach following *H. pylori* infection. We thus hypothesized that the BMP pathway might regulate CDX2 expression in the gastric context and consequently in intestinal metaplasia. In Chapter 2 we characterize the expression of CDX2 and key elements of the BMP pathway in human intestinal metaplasia and use an *in vitro* model of gastric carcinoma cell lines to study the regulation of CDX2 by this pathway.

2) Extend the link between the BMP pathway and CDX2 to another human disease model – the Juvenile polyps

The Juvenile Polyposis Syndrome is a human condition characterized by multiple polyps along the gastrointestinal tract and predisposing to cancer progression. Genetically, a subset of cases has been linked to mutation in genes of the BMP pathway, namely *SMAD4* and *BMPR1A*. We hypothesized that in Juvenile polyps loss of BMP signalling might down-regulate CDX2 expression, leading to loss of intestinal differentiation, in a reverse model of intestinal metaplasia. In accordance, in Chapter 3 we use an immunohistochemical and an *in situ* hybridization approach to characterize the expression of elements of the BMP pathway and gastric and intestinal differentiation markers in familial and isolated cases of Juvenile Polyposis.

3) Evaluate the (ir)reversibility of intestinal metaplasia and predictive value of ectopic CDX2 expression for lesion development

H. pylori infection leads to a series of sequential gastric lesions, namely intestinal metaplasia. Bacterial virulence factors play a role, together with other environmental exposures and host genetic make-up, in determining the histological outcome of the infection. To what extent intestinal metaplasia regresses upon clearance of the infection is not yet established. Also, CDX2 is expressed in non-metaplastic gastric cells, but the biological significance of this observation remains unknown. In Chapter 4 we tackle these issues in a patient-based study of *H. pylori* eradication and follow-up, where we

relate histological outcome of the gastric lesions, namely intestinal metaplasia, to *H. pylori* virulence and CDX2 expression.

4) Assess the direct regulation of CDX2 by *Helicobacter pylori*

In the context of *H. pylori* infection there is a concomitant gastric inflammation, and the subsequent cellular events are probably the result of the combined participation of inflammation and bacterial interaction with host cells. In fact, *H. pylori* attachment to gastric cells is known to alter the expression of several host genes. Upon bacteria-epithelial cell interaction, the bacterial protein cagA is injected in the host cell and modulates a series of cellular responses. In Chapter 5 we used an *in vitro* co-culture model to study the effect of *H. pylori* on CDX2 expression, namely with regard to participation of the bacterial cagA protein.

5) Unveil the putative CDX2 autoregulation

The phenotype presented by the *Cdx2*$^{+/-}$ mice, with focal loss of Cdx2 expression without loss of heterozygosity, suggests a regulatory haplo-insufficiency which is reconcilable with an autoregulatory mechanism. In agreement with this result, CDX2 was shown to transactivate its own promoter in a cell type-specific manner. Since the regulatory pathways for CDX2 uncovered this far were not sufficient to fully explain CDX2 expression maintenance in the gastric context, especially after *H. pylori* eradication and clearance of the inflammation, we hypothesized that CDX2 autoregulation might be relevant in the gastric context. To test this hypothesis we performed a series of *in vitro* cellular assays, together with *in vivo* validation in human intestinal metaplasia and in the mouse intestine, to assess the effect of CDX2 on its own expression. This study is presented in Chapter 6.

In Chapter 7 we present a review of the current knowledge on CDX2 regulation within the pathophysiology of intestinal metaplasia of the stomach.

KEY ELEMENTS OF THE BMP/SMAD PATHWAY CO-LOCALIZE WITH CDX2 IN INTESTINAL METAPLASIA AND REGULATE CDX2 EXPRESSION IN HUMAN GASTRIC CELL LINES

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Original Paper

Key elements of the BMP/SMAD pathway co-localize with CDX2 in intestinal metaplasia and regulate CDX2 expression in human gastric cell lines

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Abstract

Helicobacter pylori **infection induces intestinal metaplasia of the stomach, a preneoplastic lesion associated with an increased risk for gastric cancer development. Intestinal metaplasia is induced by the intestine-specific transcription factor CDX2 but the mechanisms responsible for this ectopic expression have never been described. We hypothesized that the BMP/SMAD pathway has a role in CDX2 regulation, in this context, for the following reasons: (1) the BMP pathway is crucial for normal intestinal differentiation and (2) there is an influx of BMP2 and BMP4-producing cells to the stomach upon *Helicobacter pylori* infection. We evaluated the expression of key elements of the BMP pathway in human stomach specimens with IM. Growth factor treatments, with BMP2 and BMP4, were performed in cultured cells and a knock-down experiment of SMAD4 was done using RNAi. We showed overexpression in IM of BMP2/4, BMPR1A, and SMAD4 in 56% of IM foci, and pSMAD1/5/8 in 100% of IM foci as compared to adjacent mucosa. *In vitro*, treatment of AGS cells with BMP2 and BMP4 increased endogenous CDX2 expression as well as the intestinal differentiation markers MUC2 and LI-cadherin. On the other hand, SMAD4 knock-down led to decreased endogenous CDX2, MUC2, and LI-cadherin in AGS. Treatment of the SMAD4 knock-down cells had no influence on CDX2 expression as opposed to wild-type cells. A 9.3 kb CDX2 promoter could be transactivated by SMAD4 and SMAD1 in a cell-dependent manner. In conclusion, we identified for the first time that the BMP pathway is active in intestinal metaplasia and that BMP2 and BMP4 regulate CDX2 expression and promote intestinal differentiation through the canonical signal transducers.**
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Keywords: **CDX2; BMP; SMAD4; intestinal metaplasia; gastric carcinoma**

Introduction

Intestinal metaplasia (IM) of the stomach is a preneoplastic lesion consisting in the transdifferentiation of gastric mucosa to an intestinal phenotype. IM occurs in the gastric carcinogenesis pathway following *Helicobacter pylori* infection, which induces chronic inflammation, IM, and, in a subset of cases, gastric carcinoma [1,2]. The intestinal differentiation process depends on the expression of the homeobox gene CDX2. CDX2 is a transcription factor crucial for intestinal differentiation both in normal and in aberrant locations. In normal mucosa, CDX2 is present in the adult intestine, regulating the expression of intestinal proteins [3], including the mucin MUC2 [4] and LI-cadherin [5]. $Cdx2^{+/-}$ heterozygous mice develop polyp-like lesions in the intestine, with the appearance of gastric mucosa, which result from the local loss of function of Cdx2 [5,6]. Furthermore, several studies have shown that CDX2 is *de novo* expressed in human intestinal metaplasia of the stomach, oesophagus, liver, and gall bladder [7,8]. The primary role played by CDX2 in the phenotypic transformation towards intestinal-type differentiation in these metaplasias has been demonstrated using transgenic mice which ectopically express Cdx2 in the gastric epithelium and subsequently develop IM [9,10]. It is of most importance, in this setting, to identify the mechanisms that regulate CDX2 expression in IM establishment and/or maintenance.

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Bone morphogenetic proteins (BMPs) were originally identified as peptides that induce ectopic bone and cartilage production [11]. BMPs constitute the largest subfamily of the TGF-beta superfamily of growth factors and exert pleiotropic biological effects, ranging from regulation of early developmental processes to organogenesis [12,13]. Like other members of the TGF-beta family of growth factors, BMP signals are initiated by the binding of the ligand to the specific transmembrane serine/threonine kinase receptors. Upon ligand binding, type II receptor kinase phosphorylates type I receptor and activates its kinase domain. Then activated type I receptor kinase phosphorylates the SMAD proteins, which are downstream signal transducers. Upon BMP stimulation, SMAD1, −5, and −8 are phosphorylated by the receptor, form a complex with SMAD4, and translocate to the nucleus, where they act as transcriptional regulators [14–16]. In the intestine, the BMP signalling pathway, through BMP2 and BMP4, is a key mediator of epithelial–mesenchymal interactions, which are fundamental for the maintenance of the differentiation and architecture of the intestinal epithelium. BMP2 and BMP4 are expressed in the mesenchyme, whereas the receptor BMPR1A is expressed in the epithelium [17]. This pathway is active in the villi, as shown by the expression of SMAD4 and phosphorylated SMAD1, −5, and −8. Mutations in SMAD4 and BMPR1A receptor are responsible for the Juvenile Polyposis Syndrome, characterized by the appearance of several hamartomatous polyps throughout the gastrointestinal tract [18,19]. The same phenotype is observed in SMAD4 knock-out mice [20], whereas in BMPR1A knock-out mice there is hyperproliferation of the crypt compartment [21]. The same result was observed in another mouse model, with inhibition of the BMP pathway in the intestine [22]. Furthermore, it has been shown that there is an influx of BMP2 and BMP4-producing cells to the stomach upon *H. pylori* infection [23] and it has been recently demonstrated that BMP2 and BMP4 induce intestinal differentiation in oesophageal and intestinal models [24,25]. In this work, we explored the relationship of this pathway with IM of the stomach and with intestinal differentiation through CDX2 in gastric cell lines.

Materials and methods

Immunohistochemistry

Thirty-seven surgical specimens from patients with gastric carcinoma, operated at Hospital S João, Porto, Portugal, were included in this study. The use of retrospective samples where informed consent cannot be obtained is authorized for research studies by the Portuguese law. Paraffin-embedded specimens from antrum mucosa with IM foci ($n = 29$) and from carcinomas ($n = 19$) were evaluated. Carcinomas were histologically classified according to Laurén's classification [26]. Immunohistochemistry was carried out as previously described [27]. The antibodies used were as follows: mouse monoclonal anti-CDX2 (Biogenex, 1 : 40); rabbit polyclonal anti-BMPR1A (sc-20 736, 1 : 35), mouse monoclonal anti-SMAD4 (sc-7966, 1 : 50), from Santa Cruz Biotechnology, Inc; rabbit polyclonal anti-pSMAD1/5/8 (Millipore/Chemicon, AB3848, 1 : 75) and mouse monoclonal anti-BMP2 (R&D, 1 : 200). Staining results were expressed in IM versus normal mucosa regarding both localization in the gastric mucosa compartments and the intensity of staining, as increased (overexpression) or decreased, using a subjective evaluation by the consensus of three observers (RB, LD, and RA). In gastric carcinomas, staining results were semi-quantitatively expressed as negative, positive in less than 50% of the cells, and positive in more than 50% of the cells.

Cell culture and treatments

Human gastric carcinoma cell lines, AGS (ATCC), MKN45 (JCRB0254, The RIKEN Cell Bank, Japan), GP202 [28], GP220 [28], and KatoIII (ATCC) were cultured under standard conditions. Cells were treated for 24 h with BMP2 and BMP4 (R&D), at 100 ng/ml and 50 and 100 ng/ml, respectively.

Protein extraction and western blot

Cells were lysed and the protein content was measured using standard methods. Protein extracts (50 μg) were analysed by standard SDS-PAGE, transferred to a nitrocellulose membrane (Amersham Biosciences), and blotted with primary antibodies (anti-CDX2, 1 : 500, Biogenex; anti-SMAD4, 1 : 750, SCBT; anti-pSMAD1/5/8, 1 : 500, Chemicon; anti-actin, 1 : 8000, SCBT) in 5% non-fat milk in 1× TBS 0.02% Tween-20 (Sigma). Antibodies for BMPR1A (1 : 400, SCBT), BMPR1B (1 : 125, R&D) and BMPR2 (1 : 250, R&D) were diluted in 5% BSA. Peroxidase-conjugated secondary antibodies (for CDX2, SMAD4 and BMPR1B, goat anti-mouse-HRP, 1 : 2000; for pSMAD1/5/8 and actin, goat anti-rabbit-HRP, 1 : 2000; for BMPR1A and BMPR2, donkey anti-goat-HRP, 1 : 2000; all from SCBT) were used and developed with the ECL detection kit (BioRad). Quantification of the western blots was performed using Bio-Rad Software Quantity One.

RNA extraction and real-time PCR

Total RNA was extracted using TRI Reagent (Sigma). Reverse transcription was performed with 3 μg of total RNA. CDX2, MUC2, villin1, LI-cadherin, Id1, Id2, and 18S were amplified with SYBR Green (Applied Biosystems) in a fluorescence reader ABI Prism 7000. The 18S level was used for normalization and relative mRNA levels were calculated [29]. The primers used are listed in Supplementary Table 1 (available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2369.html). Each experiment was carried out in triplicate at least twice; the results are expressed as means ± SD of representative triplicates.

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Immunofluorescence

Cells were grown for 24 h on slides, fixed, and blocked with non-immune serum diluted 1 : 5 in 10% BSA. Slides were incubated overnight with anti-CDX2 (Biogenex) diluted 1 : 50 in 5% BSA. After three washes with 1× PBS, slides were incubated with FITC-labelled secondary antibody (DAKO) diluted 1 : 100 in 5% BSA and mounted in Vectashield with DAPI (Vector).

RNA interference

SMAD4 knock-down cells were established using a retroviral short hairpin RNA expression system. Target sequences and scrambled control were chosen according to standard RNAi rules [30]. Oligos for *SMAD4* exon 4 and scrambled were inserted in pSUPER-retro.puro vectors (Oligoengine), transfected into the PhoenixGP packaging cell line, and virus-containing media were collected and used to infect target AGS cells. Six and 12 h post-infection, selective puromycin-containing media were added to the cells. The oligos used are listed in Supplementary Table 1 (available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2369.html).

Transient transfection and luciferase assay

The reporter plasmid pCdx2-9Luc containing 9.3 kb of the murine Cdx2 promoter [31] was used for cell transfection with Lipofectamine2000 (Invitrogen) and luciferase assays. Cells were co-transfected with expression vectors for SMAD4 and SMAD1 (kind gifts from Professor Joan Massagué, Sloan-Kettering Institute) and SMAD6, SMAD7, and pCMV-β-gal (1 µg of DNA : 1.5 µl of Lipofectamine). Total extracts were prepared using 1× Reporter Lysis Buffer (Promega), according to the manufacturer's instructions, and luciferase activity was measured in a 1450 Microbeta luminescence counter (Wallac). β-Galactosidase activity was measured to correct for transfection efficiency. Each experiment was carried out in triplicate at least twice; the results are expressed as means ± SD of representative triplicates.

Chromatin immunoprecipitation assay (ChIP)

ChIP was carried out with the EZ ChIP Kit (Upstate Biotechnology, Millipore), according to the manufacturer's guidelines, using 90–95% confluency AGS cells cultured in 75 cm^2 flasks and previously transfected with a FLAG-SMAD4 expression vector. Semi-quantitative PCR was performed on the extracted DNA using CDX2 promoter primers (see Supplementary Table 1, available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2369.html).

Results

Expression of CDX2 and key elements of the BMP/SMAD pathway in intestinal metaplasia and gastric carcinomas

In order to see if the BMP pathway could be involved in the process of intestinal metaplasia (IM) of the stomach, we characterized the expression pattern of CDX2 and key elements of this pathway in IM and adjacent gastric mucosa. In all cases, CDX2 was expressed exclusively in IM cells (Figure 1). Proteins of the BMP/SMAD pathway included BMP2/4 (the antibody is directed to BMP2 but cross-reacts with BMP4), BMPR1A, the activated (phosphorylated) form of receptor-regulated SMADs, pSMAD1/5/8, which are specific of BMP activation, and the downstream signalling transduction factor SMAD4. All proteins were strongly expressed in all 29 IM cases. BMP2/4 was expressed in the cytoplasm of epithelial cells as well as in stromal inflammatory cells and was overexpressed in IM compared with adjacent normal mucosa. BMPR1A localized to the membrane and cytoplasm; pSMAD1/5/8 localized to the nucleus; and SMAD4 localized to the cytoplasm and nucleus (Figure 1). The expression of these proteins in the non-metaplastic gastric mucosa was evaluated in 18 cases and the pattern of expression was more variable: BMPR1A and SMAD4 localized exclusively in the antrum gastric glands in ten cases (56%), whereas in the remaining eight cases (44%), expression occurred throughout the whole mucosa. pSMAD1/5/8 was expressed in all cases in the neck region of the gastric mucosa (Figure 1). BMPR1A and SMAD4 were overexpressed in IM compared with adjacent gastric mucosa in 56% of the cases, whereas pSMAD1/5/8 was overexpressed in IM compared with adjacent gastric mucosa in 100% of the cases. Nineteen gastric carcinomas were evaluated for CDX2 and pSMAD1/5/8 expression as a hallmark of an active BMP/SMAD pathway (see Supplementary Figure 1, available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2369.html). CDX2 was observed in 15/19 cases (79%) and pSMAD1/5/8 was observed in 16/19 cases (84%). There was a significant association ($p < 0.05$) between expression of CDX2 and pSMAD1/5/8, with 14 cases expressing both proteins, two cases expressing only pSMAD1/5/8, and one case expressing only CDX2 (see Supplementary Figure 1, available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2369.html). We did not observe a significant association between pSMAD1/5/8 and CDX2 expression and the histological type of gastric carcinoma.

The AGS gastric carcinoma cell line is responsive to BMP2 and BMP4 activation

To evaluate the responsiveness of gastric cell lines to the BMP/SMAD pathway, a panel of five gastric

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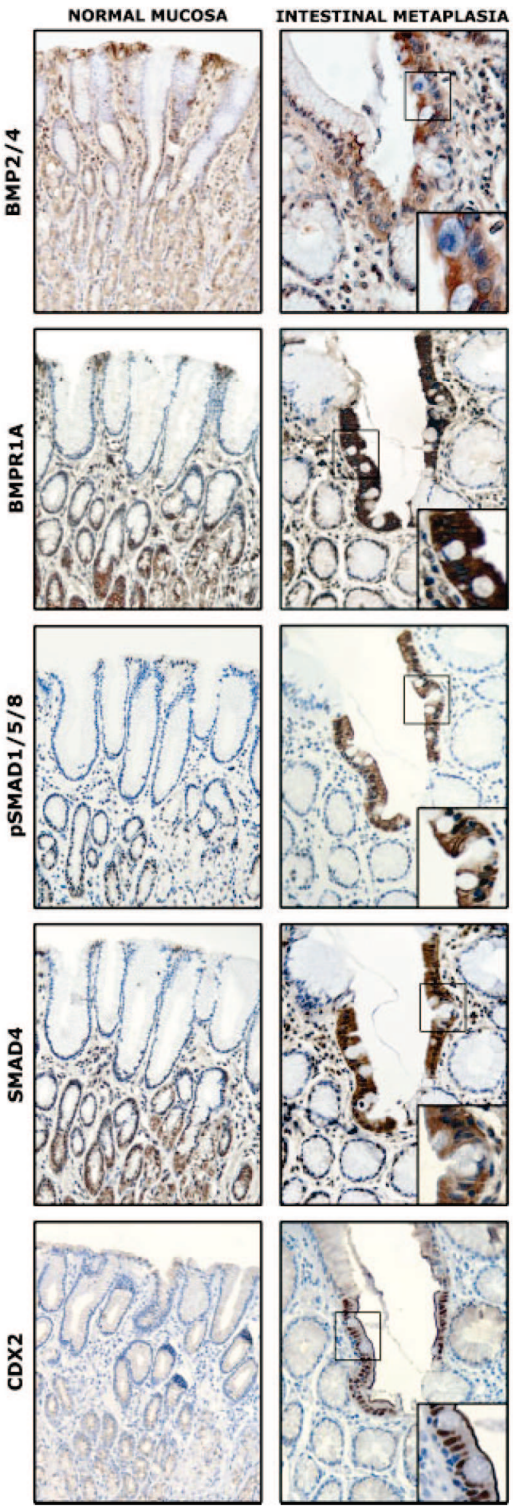


Figure 1. Immunohistochemical analysis of key elements of the BMP/SMAD signalling pathway and CDX2 in human gastric epithelium. BMP2/4, BMPR1A, pSMAD1/5/8, SMAD4, and CDX2 expression in normal gastric mucosa (left panel), and intestinal metaplasia (right panel). Inserts show subcellular localization of the proteins

carcinoma cell lines, AGS, MKN45, GP202, GP220, and KatoIII, was characterized regarding expression of BMP receptors, signal transduction molecules, and CDX2. Furthermore, cell lines were treated with BMP2 and BMP4, and the expression of two canonical targets of this pathway, Id1 and Id2 [32], was assessed to control the activity of the pathway. The results showed that three cell lines, AGS, GP202, and GP220, expressed the key elements of the BMP/SMAD pathway (Figure 2A). In contrast, SMAD4 was missing and BMPR1B and pSMAD1/5/8 were very low in MNK45 cells. In KatoIII, pSMAD1/5/8 was absent, suggesting that this pathway is inactive, perhaps related to the low level of BMPR1B. Figure 2A also illustrates that two of the three cell lines expressing every component of the BMP pathway, AGS and GP220, also expressed CDX2, whereas the third cell line, GP202, failed to produce CDX2, but this cell line also showed very low levels of BMPR1B. Upon treatment with BMP2 and BMP4, all the cell lines responded to some extent, with increases in Id1 and Id2 expression, but only AGS reacted strongly (Figure 2B); therefore this was the cell line selected for further experiments.

Exogenous addition of BMP2 and BMP4 increases CDX2 expression as well as intestinal differentiation markers in AGS

To evaluate whether the BMP/SMAD pathway regulates CDX2 expression, AGS cells were treated with exogenous BMP2 and BMP4. In AGS, although both untreated and treated cells express CDX2, an increase in CDX2 mRNA was observed upon treatment with BMP2 and BMP4 of 2.7- and 2.3-fold, respectively (Figure 3A). Increased CDX2 protein expression in the treated cells was also observed by western blot and immunofluorescence (Figures 3B and 3C). In addition, the expression of three intestinal differentiation markers, MUC2, villin1, and LI-cadherin, was assessed. The MUC2 and LI-cadherin genes are known downstream targets of CDX2 [4,33], whereas no evidence exists that it is the same for villin1. The results showed significantly increased expression of MUC2 and LI-cadherin with both BMPs, whereas villin1 showed only a slight increase (Figure 3D), confirming the functionality of CDX2 stimulation by BMPs.

SMAD4 knock-down decreases CDX2 expression as well as intestinal differentiation markers in AGS

To confirm the regulation of CDX2 expression by BMPs, the pathway was abrogated by knocking down the fundamental signal transducer SMAD4, using RNA interference in AGS. Short hairpin RNA was directed to exon 4 of *SMAD4* and a corresponding scrambled control was designed. After infection and selection, two independent polyclonal cellular mixtures for SMAD4 knock-down and one for the scrambled control were used in the subsequent studies. The results showed that SMAD4 was inhibited (Figure 4A). For the same cells, the level of CDX2 protein (Figure 4A) was reduced and mRNA was decreased to around 60%, compared with the scrambled-infected cells (Figure 4B). In addition, expression of the two downstream targets of CDX2,

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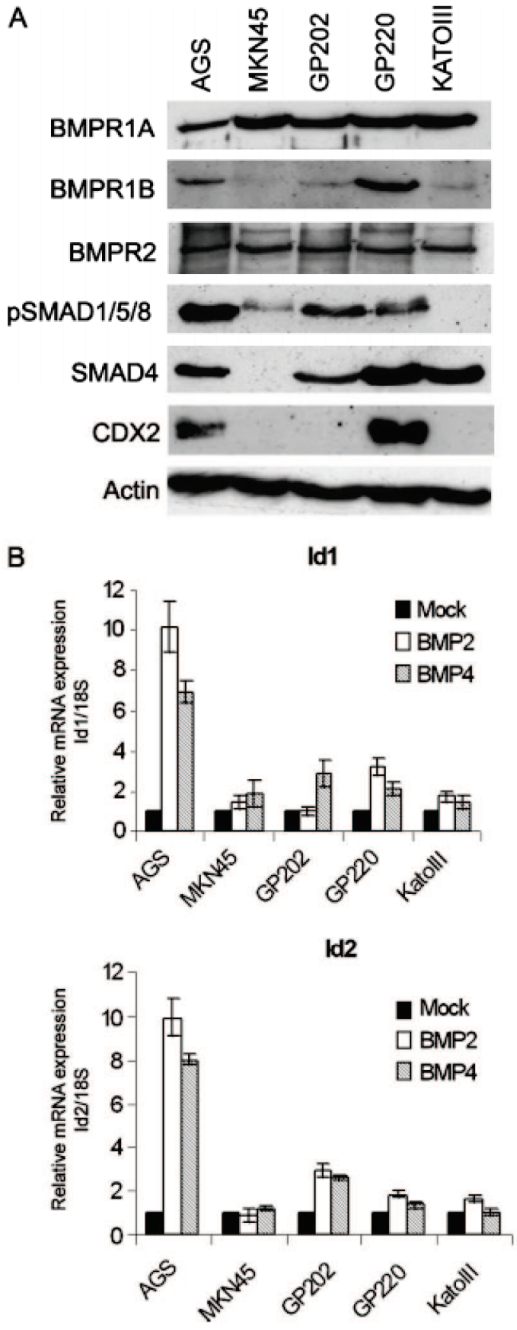


Figure 2. Expression of key elements of the BMP/SMAD pathway and CDX2 in gastric cell lines and their responsiveness to BMP treatment. (A) Western blot showing endogenous levels of BMPR1A, BMPR1B, BMPR2, pSMAD1/5/8, SMAD4, and CDX2 in a panel of five gastric carcinoma cell lines. β-actin was used as the loading control. (B) Fold increase of the inhibitors of differentiation/DNA binding proteins 1 and 2 (Id1 and Id2), in the same panel of cell lines, treated with exogenous BMP2 and BMP4 in the concentration of 100 ng/ml and 50 ng/ml, respectively. The values obtained with mock-treated cells were referred to as 1. mRNA levels were normalized with the corresponding 18S mRNA level

MUC2 and LI-cadherin, was significantly decreased compared with the scrambled control, whereas villin1 showed only a slight decrease (Figure 4C), which corroborated the functional effect of the SMAD4 knock-down on CDX2. The results obtained in the two independent polyclonal cellular mixtures were of similar range, ruling out any possible selection bias for these experiments.

BMP2 and BMP4-induced increase in CDX2 expression is abrogated in SMAD4 knock-down cells

To investigate if the BMP-mediated induction of CDX2 expression followed the canonical BMP pathway through SMAD4, SMAD4 knock-down cells were treated with exogenous BMP2 and BMP4. In scrambled-infected cells, an increase in mRNA expression was observed upon treatment with BMP2 and BMP4 of 1.9- and 1.5-fold, respectively (Figure 5A), whereas in the SMAD4 knock-down cells, treatment did not have any effect on CDX2 expression (Figure 5A). The expression level of the BMP receptors was evaluated by western blot and immunofluorescence (data not shown), to discard unspecific knock-down of the receptors (Figure 5B). Thus, SMAD4 knock-down alone impaired the effect of BMPs on CDX2 expression, without affecting the upstream elements of the pathway.

SMAD4 and SMAD1 regulate the Cdx2 promoter in gastric carcinoma cell lines

To evaluate if the regulation of CDX2 expression by SMAD4 is through transcriptional activation of the CDX2 gene, the reporter plasmid pCdx2-9Luc containing the 9.3 kb promoter of the murine Cdx2 gene was used for transfection and luciferase assays in the five gastric cell lines, in combination with the SMAD4 expression vector or the control empty vector. The results showed that the Cdx2 promoter reacted differently to SMAD4 in the five cell lines. In KatoIII, the activation of the Cdx2 promoter was 2.5-fold, whereas in AGS it was only 1.6-fold and non-significant in the other cell lines (Figure 6A). Of note, the basal activity of the Cdx2 promoter was about 30-fold higher in AGS cells than in KatoIII cells (data not shown), which led us to hypothesize that the limited stimulatory effect exerted by SMAD4 in AGS cells is linked to the fact that the activity of this promoter is already maximal in this cell line and thus poorly responsive to further stimulation by SMAD4. To confirm this experimentally, AGS cells were transfected with the inhibitory SMADs, SMAD6 or SMAD7, to reduce SMAD signalling in these cells. The results showed that the activity of the Cdx2 promoter decreased by 25% and 40% in the presence of SMAD6 and SMAD7, respectively (Figure 6B), which is in line with a transcriptional effect of SMAD signalling on the Cdx2 promoter. To support this conclusion further, we reasoned that SMAD4 is translocated to the nucleus when bound to the receptor-regulated SMADs and that KatoIII does not express pSMAD1/5/8. We therefore addressed the effect of SMAD4 + SMAD1 in KatoIII cells. Figure 6C shows that combining SMAD4 + SMAD1 had a stronger effect on the Cdx2 promoter than SMAD4 alone.

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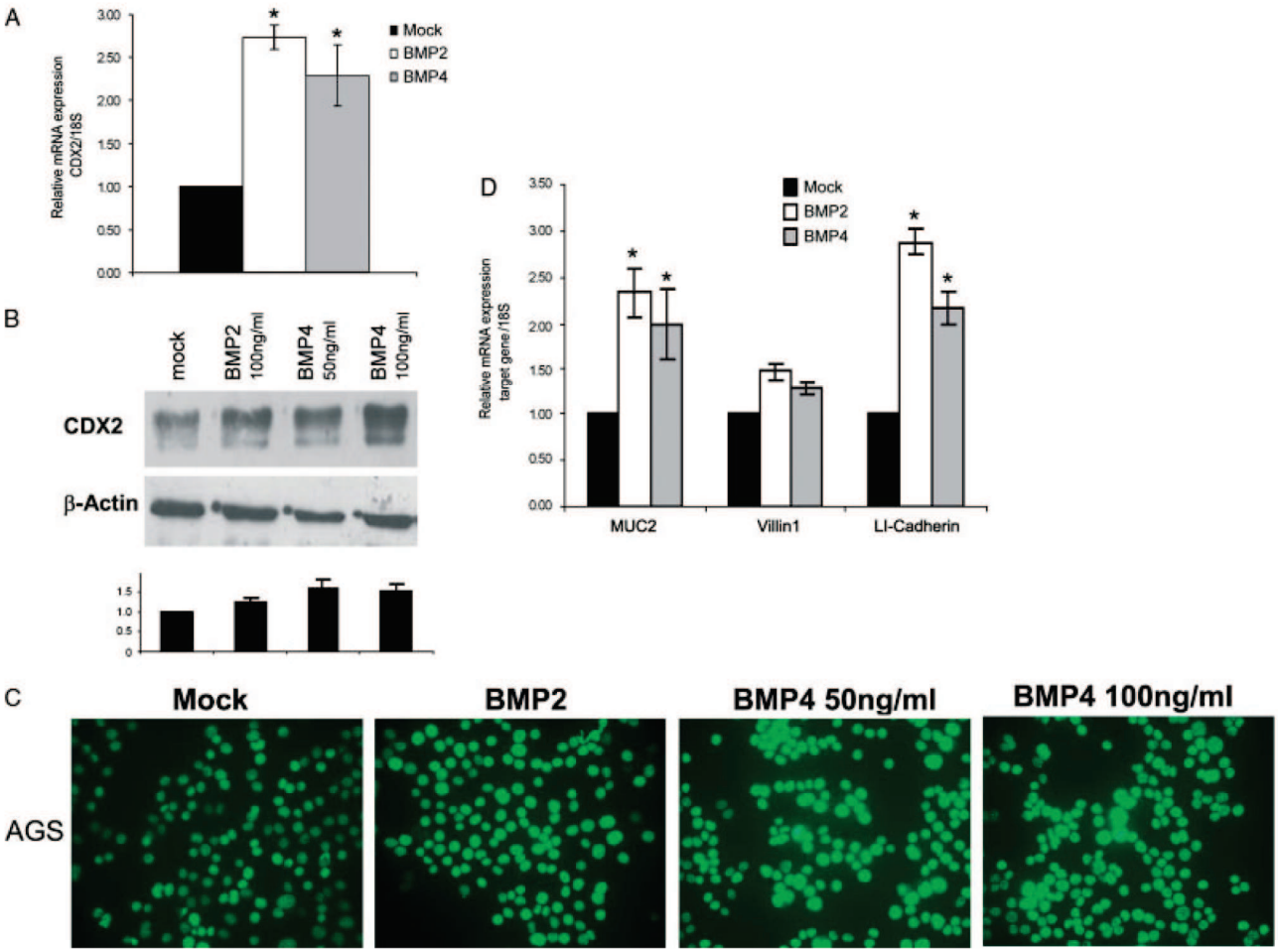


Figure 3. Effect of BMP2 and BMP4 treatment on endogenous CDX2 and intestinal differentiation markers. (A) Fold increase of CDX2 mRNA in AGS cells treated with exogenous BMP2 and BMP4 in the concentration of 100 ng/ml and 50 ng/ml, respectively. The values obtained with mock-treated cells were referred to as 1. CDX2 mRNA levels were normalized with the corresponding 18S mRNA level. $^{*}p < 0.005$. (B) Western blot showing endogenous levels of CDX2 with the same treatments performed in A. Mock-treated cells were used as the experiment control and β-actin was used as the loading control. The lower panel in B shows quantification of the western blot. (C) Fluorescent immunocytochemistry of CDX2 in cells treated with BMP2 (100 ng/ml) and two concentrations of BMP4 (50 and 100 ng/ml). Mock-treated cells were used as a control. (D) Fold increase of MUC2, villin1, and LI-cadherin mRNA in AGS cells treated as in A. The values obtained with mock-treated cells were referred to as 1. mRNA levels were normalized with the corresponding 18S mRNA level. $^{*}p < 0.005$

Identification of a SMAD4 *cis*-element within the CDX2 promoter

In order to demonstrate that the SMAD4 transcription factor binds to the CDX2 promoter, chromatin immunoprecipitation (ChIP) was performed in AGS cells. A highly conserved putative binding sequence for SMAD4 was found within 1200 nucleotides of the CDX2 proximal promoter (nucleotides −348/−344) and therefore evaluated in the precipitated DNA. The results showed that SMAD4 protein is present in the most conserved binding sequence of the proximal CDX2 promoter (Figure 6D). To examine the functional role of this SMAD4 consensus binding, site-directed mutagenesis was performed. A wild-type and a mutated CDX2 promoter construct, −871/−1 and −871mS4, were co-transfected with SMAD4 in KatoIII cells, in which the CDX2 promoter was more efficiently transactivated by SMAD4 (see Supplementary Figure 2, available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2369.html). The results obtained showed that mutation of the putative SMAD4 binding site identified by ChIP abrogates transactivation.

Discussion

Intestinal metaplasia (IM) of the stomach is a preneoplasic lesion for gastric cancer, with a high incidence in several countries including Japan and Portugal. IM is linked to ectopic expression of the intestine-specific transcription factor CDX2, known to drive intestinal-type differentiation [5,10], but the mechanism(s) involved in this ectopic expression remain elusive. In this study, we demonstrated first, increased expression of several elements of the BMP/SMAD pathway in a collection of human intestinal metaplasias of the stomach, co-localizing with CDX2; second, that ectopic CDX2 expression in gastric cancer cell lines,

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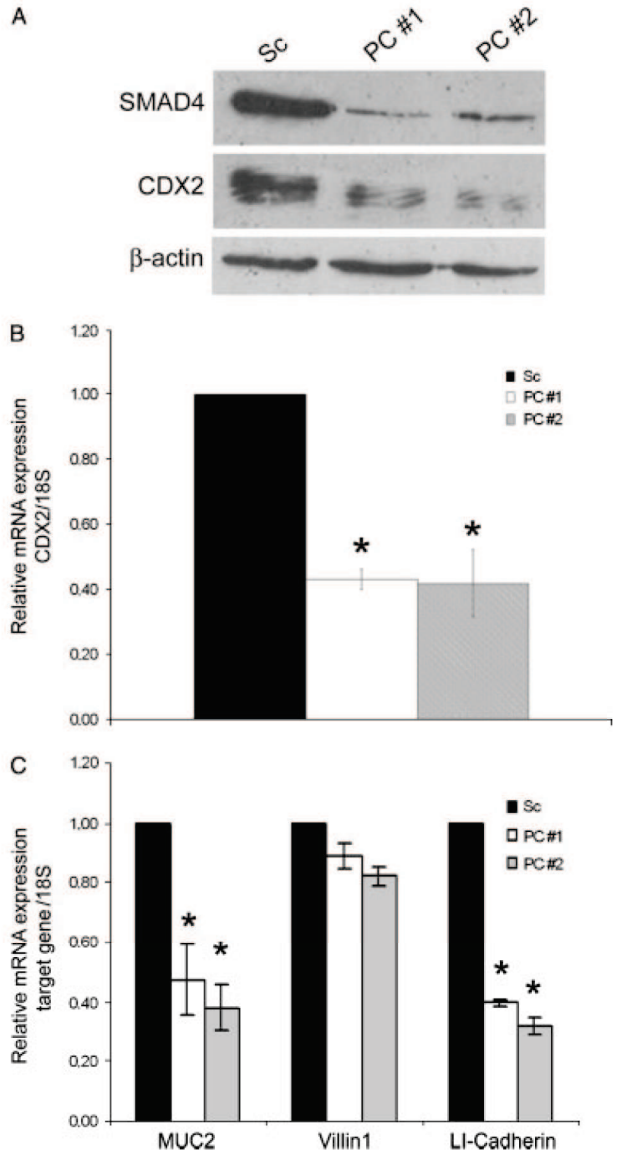


Figure 4. Effect of SMAD4 knock-down on endogenous CDX2 and intestinal differentiation markers. (A) Western blot showing endogenous levels of SMAD4 and CDX2 in two independent polyclonal AGS cellular mixtures infected with SMAD4-directed RNAi retroviruses, compared with scrambled-infected cells. β-actin was used as the loading control. (B) Fold reduction of CDX2 mRNA in the same cells compared with the scrambled-infected cells. The values obtained with scrambled-infected cells were referred to as 1. CDX2 mRNA levels were normalized with the corresponding 18S mRNA level. $^{*}p < 0.001$. (C) Fold reduction of MUC2, villin1, and LI-cadherin mRNA in the same cells compared with the scrambled-infected cells. The values obtained with scrambled-infected cells were referred to as 1. mRNA levels were normalized with the corresponding 18S mRNA level. $^{*}p < 0.001$

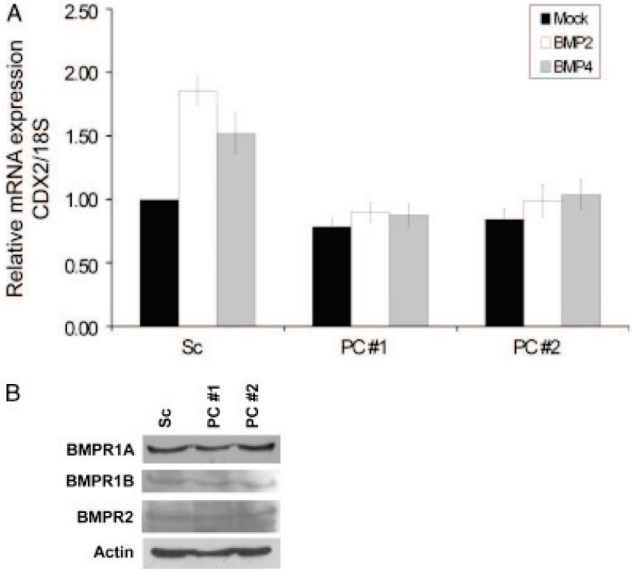


Figure 5. Effect of BMP2 and BMP4 treatment on endogenous CDX2 mRNA in SMAD4 knock-down cells. (A) Fold increase of CDX2 mRNA in two independent polyclonal AGS cellular mixtures infected with SMAD4-directed RNAi retroviruses and in scrambled-infected cells treated with exogenous BMP2 and BMP4 in the concentration of 100 ng/ml and 50 ng/ml, respectively. The values obtained with mock-treated cells were referred to as 1. CDX2 mRNA levels were normalized with the corresponding 18S mRNA level. (B) Western blot for BMPR1A, BMPR1B, and BMPR2. β-actin was used as the loading control

and also in gastric carcinomas, correlates with overexpression of the BMP/SMAD pathway; and third, that CDX2 expression is up-regulated by BMP2 and BMP4 through the canonical intracellular transducer SMAD4 in a gastric cell line characterized by active BMP/SMAD signalling.

CDX2 regulatory mechanisms have been previously addressed but not fully clarified. A general mechanism involving promoter demethylation, eventually mediated by bile acid reflux, was shown to be a prerequisite for CDX2 expression in human oesophageal epithelial cells, contributing to the development of Barrett's oesophagus [34]. In colon cancer cells, it was shown that PTEN/PI3K and TNFα/NFκB can target the CDX2 promoter [35] and, in Barrett's oesophagus, two NFκB binding sites were also shown to be necessary for bile acid activation of the CDX2 promoter [36]. Furthermore, the CDX2 promoter is activated in endocrine, proglucagon-producing cells, but not in colon cancer cells, by the second messenger cAMP via the Epac pathway [37]. On the other hand, CDX2 can be transcriptionally repressed by Sox9 in colonic cells [38]. However, regulatory mechanisms activating CDX2 transcription in gastric cells are lacking, although Manzo *et al* have shown that *H. pylori* induces CDX2 expression by an unidentified mechanism [39]. We have previously shown that OCT1 co-localizes with CDX2 in gastric IM and binds to the CDX2 promoter in gastric cells. However, we could not see transcriptional activity of OCT1 on the CDX2 promoter [27], as previously demonstrated in colon cancer cells [40]. Our present results are therefore a major contribution to the identification of the BMP/SMAD pathway as a candidate mechanism to regulate CDX2 transcription and consequently intestinal differentiation in gastric cells. It is also interesting that the pathway that we identified involves signalling from non-epithelial cells, as the main source of BMPs. However, we have also shown that epithelial cells from IM overexpress BMP2/4 together with their receptors,

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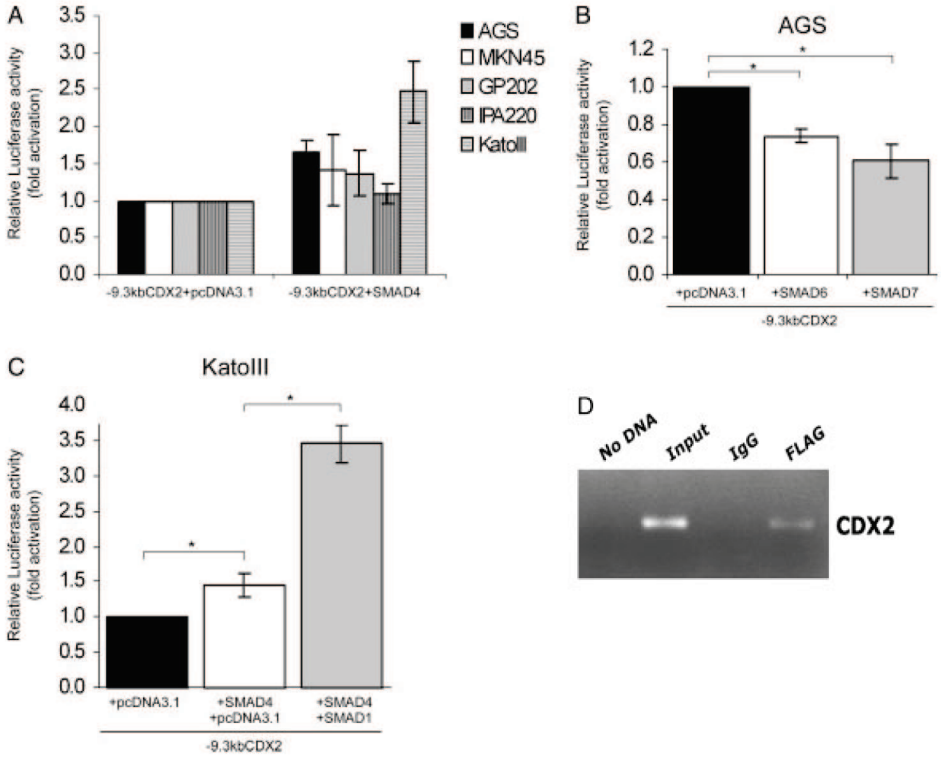


Figure 6. Regulation of a 9.3 kb CDX2 promoter by SMAD4 and SMAD1. (A) Co-transfection experiments were performed in the presence of a 9.3 kb CDX2 promoter construct and a SMAD4 expression vector in a panel of five gastric carcinoma cell lines. (B) Co-transfection experiments were performed in the presence of a 9.3 kb CDX2 promoter construct and the inhibitory SMADs, SMAD6 and SMAD7, in AGS. (C) Co-transfection experiments were performed in the presence of a 9.3 kb CDX2 promoter construct and a SMAD4 and a SMAD1 expression vector in KatoIII. The values obtained with transfection of the empty vector were referred to as 1. $^{*}p < 0.05$. (D) SMAD4 associates with CDX2 promoter in AGS cells. ChIP assay was carried out with an isotype control immunoglobulin G (IgG) and with an anti-FLAG antibody (FLAG) after transfection with a FLAG-SMAD4-expressing vector. PCR amplification of a proximal CDX2 promoter region was performed to detect SMAD4-bound DNA. A fraction of the chromatin preparation (1%) was used as input in the reaction

which, as previously reported in the colon [41], suggests an autocrine regulatory mechanism.

Epithelial–mesenchymal interactions are necessary for intestinal development as well as for cell renewal in the mature organ [42,43]. BMP2 and BMP4 are involved in the maintenance of the proliferation and differentiation compartments of the intestine [25,44]. Furthermore, it has been shown that the BMP pathway plays a role in the transformation of normal oesophageal cells to an intestinal phenotype in a model of Barrett's oesophagus [24]. In accordance with this, and in line with our results, BMP2 and BMP4 become abundantly present in the gastric mucosa upon *H. pylori* infection, whereas in the normal gastric mucosa they are expressed at very low levels [23]. Our work suggests that the role played by the BMP pathway in intestinal differentiation could be, at least partially, through regulation of CDX2. Several observations support this hypothesis: key elements of the BMP pathway are overexpressed in IM and the ability to promote intestinal transformation was shown *in vitro*, by increasing CDX2 expression as well as MUC2 and LI-cadherin upon BMP treatment. Beyond CDX2 itself, these two proteins, known to be ectopically expressed in IM, follow the changes in CDX2 levels induced by BMP treatment or SMAD4 knockdown. Despite the fact that CDX2 overexpression, induced by BMP treatment, was capable of increasing the expression of MUC2 and LI-cadherin, and not villin or sucrase-isomaltase (data not shown), we cannot extrapolate that the BMP/SMAD4 pathway specifically induces goblet cell differentiation, since other regulatory elements may be involved in the enterocytic differentiation regulated by CDX2 [45].

In this study, we investigated how BMP molecules induce CDX2 expression, showing that the canonical pathway via SMAD4 is used. In AGS cells, which express both SMAD4 and CDX2, SMAD4 knockdown, using an exon 4 and also an exon 1 target (not shown), not only reduces CDX2 expression, but also prevents CDX2 induction by BMPs. In fact, AGS has high endogenous levels of the proteins involved in the whole pathway and behaves in accordance with our hypothesis that BMPs regulate CDX2 through SMAD4. Furthermore, our results suggest that this regulation is through the CDX2 promoter because SMAD4 is present in the CDX2 promoter in AGS cells and is able to transactivate a CDX2 promoter construct in KatoIII cells. We hypothesized that the low transactivation observed in AGS might be due to the fact that this cell line has high endogenous levels

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of the proteins involved in the whole pathway. The fact that we were able to reduce the basal activity of a CDX2 promoter construct in AGS with inhibitory SMADs supports this hypothesis. In addition, we confirmed that the SMAD4 binding site identified by ChIP is active because when it is mutated, transactivation of the CDX2 promoter is abrogated. We cannot exclude the possibility that other sites might be present and active in more distal regions.

In conclusion, we identified that the BMP pathway is active in IM and that BMP2 and BMP4 regulate CDX2 expression and promote intestinal differentiation through the canonical signal transducers.

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Supplementary material

Supplementary material may be found at the web address http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2369.html.

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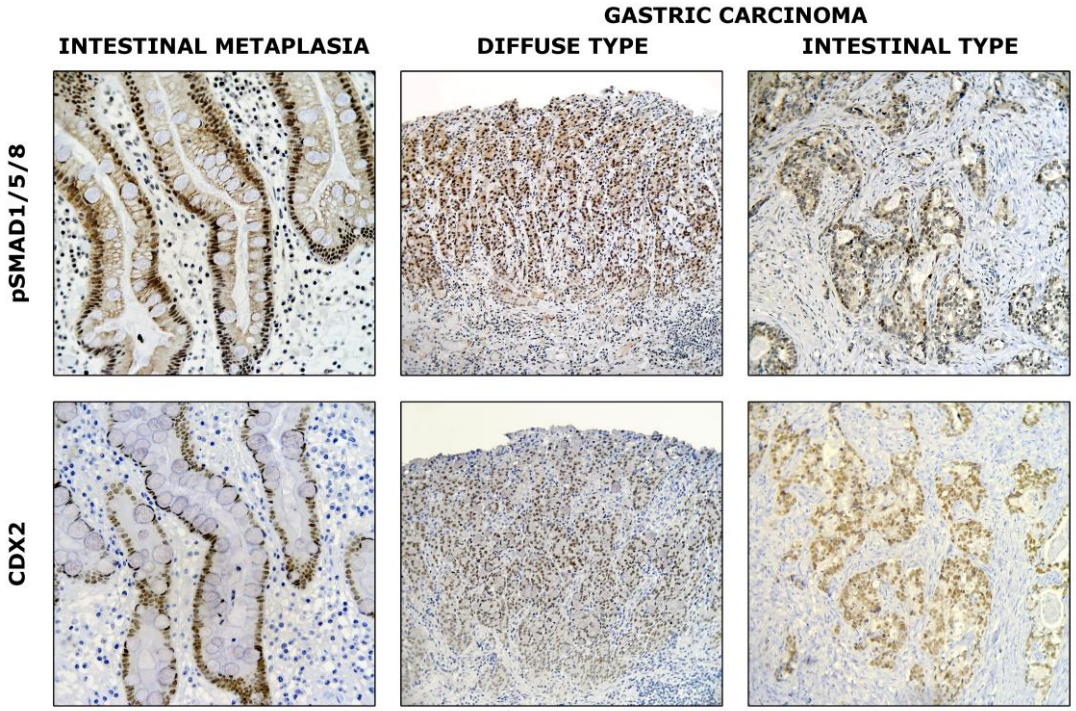


Fig. 1 (Suppl.) - Immunohistochemical analysis of pSMAD1/5/8 and CDX2 in gastric carcinomas. A diffuse and an intestinal type gastric carcinoma expressing both pSMAD1/5/8 and CDX2 are shown. An area of intestinal metaplasia adjacent to one of the gastric carcinoma also exhibits staining for both elements.

Table 1 (Suppl.) – Immunohistochemical results of CDX2 and SMAD1/5/8 in gastric carcinomas.

	SMAD1/5/8					
CDX2	Negative n (%)		Positive in <50% n (%)		Positive in >50% n (%)	
Negative	2	(66.7)	2	(22.2)	0	(0)
Positive in <50%	1	(33.3)	2	(22.2)	0	(0)
Positive in >50%	0	(0)	5	(55.6)	7	(100)
Total	3	(100)	9	(100)	7	(100)

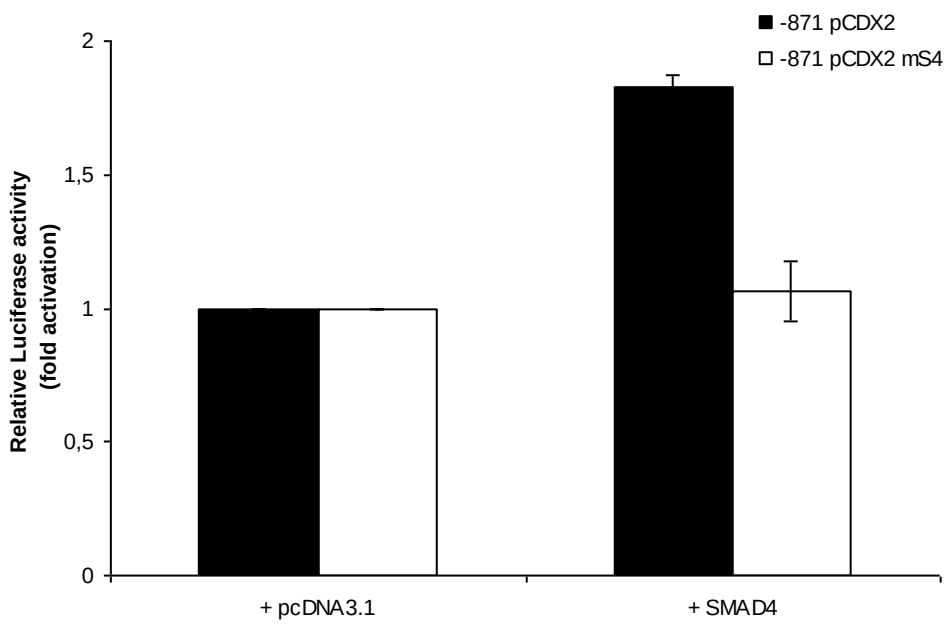


Fig. 2 (Suppl.) - Co-transfection experiments with SMAD4 were performed in KatoIII cells in the presence of -871/-1 CDX2 promoter construct and the same construct mutated for the SMAD4 binding element located within nucleotides -348/-344. The values obtained with transfection of the empty vector were referred to as 1. The results are means ± S.D. of triplicates in a representative experiment. The experiment was done separately at least twice.

Material and Methods:

Site-directed mutagenesis - To introduce a point mutation in the -871 CDX2 gene promoter region the QuickChange Site-directed mutagenesis protocol (Stratagene) was used. The oligos used for this purpose were the following: S: GACGTCTCCAACCATTGGTG***AGC***GTGTCATTACTAA and AS: TTAGTAATGACAC***GCT***CACCAATGGTTGGAGACGTC. The mutated nucleotides are bold, italicized and underlined. The PCR cycles were set as follows: 95ºC for 30sec; 12 cycles of 95ºC for 30sec, 55ºC for 1min, 68ºC for 5min. After the PCR reaction, the products were incubated with *Dpn I* for 1h at 37ºC, to degrade the methylated template, and transformed to *E. coli* competent cells. All the plasmids were checked by sequencing.

Table 2 (Suppl.) – Sequences of the oligonucleotides used for real-time PCR, RNAi and ChIP.

Primers used for real-timePCR	Sequence (5'→3')
CDX2	A:TTC ACT ACA GTC GCT ACA TCA CCA T
	AS: TTG TTG ATT TTC CTC TCC TTT GCT
MUC2	A: GCC TGC AGA GCT ATT CAG AAT TC
	AS: ATC TTC TGC ATG TTC CCA AAC TC
Villin1	S: TGC CCA GCA GAA GAT GGT AGA T
	AS: GGA ATC CAC AGG TAC CAG CTC TAG
LI-Cadherin	S: CGA AGG CTC AGT AAG GCA GAA
	AS: CAT CCA GGT CTG TGG CAT TG
SMAD4	A: CCA CCA AAA CGG CCA TCT T
	AS: TGG AAA TGG GAG GCT GGA A
Id1	S: CAG GGA CCT TCA GTT GG
	AS: TCA GCG ACA CAA GAT GC
Id2	S: CTA TTG TCA GCC TGC ATC AC
	AS: CAG TGC TTT GCT GTC ATT TG
18S	S: CGC CGC TAG AGG TGA AAT TC
	AS: CAT TCT TGG CAA ATG CTT TCG
Oligonucleotides used for RNAi	**Sequence (5'→3')**
Ex1 Scrambled	S: GAT CCC CTA TTT CTC AGG CGG AAA CAT TCA AGA GAT GTT TCC GCC TGA GAA ATA TTT TTA
	AS: AGC TTA AAA ATA TTT CTC AGG CGG AAA CAT CTC TTG AAT GTT TCC GCC TGA GAA ATA GGG
Ex1	S: GAT CCC CAT GGA GCT CAT CCT AGT AAT TCA AGA GAT TAC TAG GAT GAG CTC CAT TTT TTA
	AS: AGC TTA AAA AAT GGA GCT CAT CCT AGT AAT CTC TTG AAT TAC TAG GAT GAG CTC CAT GGG
Ex4 Scrambled	S: GAT CCC CCA TCG CTC GTT ATT GAT AGT TCA AGA GAC TAT CAA TAA CGA GCG ATG TTT TTA
	AS: AGC TTA AAA ACA TCG CTC GTT ATT GAT AGT CTC TTG AAC TAT CAA TAA CGA GCG ATG GGG
Ex4	S: GAT CCC CAG GAT TTC CTC ATG TGA TCT TCA AGA GAG ATC ACA TGA GGA AAT CCT TTT TTA
	AS: AGC TTA AAA AAG GAT TTC CTC ATG TGA TCT CTC TTG AAG ATC ACA TGA GGA AAT CCT GGG
Primers used for ChIP	**Sequence (5'→3')**
CDX2	S: CGA GGG GTT GTG CGT AGA GT
	AS: ACA GGC TGG CGT GCG GA

JUVENILE POLYPS HAVE GASTRIC DIFFERENTIATION WITH MUC5AC EXPRESSION AND DOWNREGULATION OF CDX2 AND SMAD4

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ORIGINAL PAPER

Juvenile polyps have gastric differentiation with MUC5AC expression and downregulation of CDX2 and SMAD4

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Abstract CDX2 is a homeobox transcription factor that works as a master gene in intestinal differentiation, both in the colon and in aberrant locations such as intestinal metaplasia (IM) of the stomach. Transgenic mice with Cdx2 expression in the stomach develop IM and $Cdx2^{+/-}$ mice develop hamartomatous polyps in the colon presenting gastric differentiation. We previously observed regulation of CDX2 by the BMP/SMAD pathway in the gastric context. Here, we hypothesized that juvenile polyps, which are hamartomatous polyps caused by mutations in members of the BMP/SMAD pathway, might recapitulate the gastric differentiation observed in $Cdx2^{+/-}$ mice due to SMAD4 and CDX2 downregulation. We characterized SMAD4 and CDX2 expression in a series of 18 solitary juvenile polyps and 2 polyps from juvenile polyposis (JP) patients, one with a germline *SMAD4* mutation and one with a germline *BMPRIA* mutation, as well as the expression of an intestinal differentiation marker, MUC2 (by immunohistochemistry and in situ hybridization), and gastric differentiation markers, MUC5AC and MUC6 (by immunohistochemistry). We observed that juvenile polyps have a heterogeneous expression of CDX2, MUC2 and SMAD4, with negative areas, and 15 of the 18 solitary polyps and the JP case with *SMAD4* mutation exhibit de novo expression of MUC5AC but not MUC6. In conclusion, juvenile polyps have gastric transdifferentiation associated with downregulation of CDX2 and SMAD4, lending support to the role of the BMP/SMAD pathway in CDX2 regulation.

Keywords CDX2 · Juvenile polyps · SMAD4 · MUC5AC · Metaplasia

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Introduction

CDX2 is a homeobox transcription factor that plays a master role in intestinal differentiation and homeostasis, both in the colon and in aberrant locations (Chawengsaksophak et al. 1997; Mutoh et al. 2002; Silberg et al. 2000, 2002). CDX2 is expressed in intestinal metaplasia (IM) of the stomach (Almeida et al. 2003), being a master gene for its establishment and maintenance (Mesquita et al. 2006). It regulates MUC2 expression both in colon and in IM (Mesquita et al. 2003; Yamamoto et al. 2003) and transgenic mice with ectopic expression of Cdx2 in the gastric mucosa develop IM (Mutoh et al. 2002; Silberg et al. 2002). Conversely, $Cdx2^{+/-}$ mice develop, upon loss of expression of the second allele, hamartomatous polyps in the colon with gastric differentiation (Chawengsaksophak et al. 1997; Beck et al. 1999; Tamai et al. 1999).

We recently showed that key elements of the BMP/SMAD pathway co-localized with CDX2 in IM (Barros et al. 2008). Further, we demonstrated that treating a gastric cell line with exogenous BMP2 and BMP4 resulted in an increase of CDX2 and intestinal mucin MUC2 expression (Barros et al. 2008). Inhibition of SMAD4 in the same cells not only led to a reduction in CDX2 expression but also prevented the stimulatory effect of the exogenous BMPs, thus showing that this pathway regulates CDX2 expression in vitro (Barros et al. 2008).

A human model with loss of BMP pathway activity in the gastrointestinal tract is the juvenile polyposis (JP) syndrome. Juvenile polyps are hamartomatous polyps of the colon characterized by the presence of an expanded lamina propria containing dilated mucus-filled glands, inflammatory cells and prominent stroma, with a normal overlying epithelium (Merg and Howe 2004). About 40% of sporadic and familial cases are caused by mutations in members of the TGF-β/BMP/SMAD pathway, specifically by mutations in SMAD4 (20%) and in BMPR1A (20%) genes (Howe et al. 2001). The remaining 60% of the cases have yet unclarified causes.

We hypothesized that in juvenile polyps the loss of the BMP/SMAD pathway could be down-regulating CDX2, putatively leading to the appearance of gastric differentiation within the polyp, hence representing a reverse model of IM. The putative involvement of CDX2 in the onset of JP has been previously addressed, revealing that CDX2 is not mutated in JP cases (Woodford-Richens et al. 2001). However, expression of this protein has never been characterized.

In this study, we characterized SMAD4 and CDX2 expression in a series of solitary juvenile polyps and in two polyps from JP patients, as well as the expression of an intestinal differentiation marker, MUC2 mucin, and gastric differentiation markers, MUC5AC and MUC6 mucins (Reis et al. 1999).

Materials and methods

Material

A total of 18 solitary colorectal juvenile polyps, endoscopically or surgically resected from 1990 to 2004 in Hospital S. João, Porto, Portugal were investigated. The use of retrospective samples where informed consent cannot be obtained is authorized for research studies by the Portuguese law. The ages of the patients ranged from 3 to 64 years. Two polyps from patients with JP, one with a confirmed *SMAD4* germline mutation, 1244_7delACAG (Howe et al. 1998), and the other with a confirmed germline *BMPRIA* promoter and first non-coding exon deletion (unpublished) were also included. All samples were fixed in 10% buffered formalin and embedded in paraffin. Sections were cut at 4 μm for hematoxylin and eosin staining and for immunohistochemical detection of the homeodomain protein CDX2, gastric mucins MUC5AC and MUC6, intestinal mucin MUC2 and SMAD4.

Immunohistochemistry

Prior to immunostaining with all antibodies, sections were deparaffinized with xylene. For CDX2 and SMAD4 staining, sections were boiled with an antigen retrieval Citrate pH 6 solution for 20 min; for MUC2 staining, sections were incubated with Neuraminidase for 2 h at 37°C and for MUC5AC and MUC6 staining there was no pretreatment. After this step, sections were incubated with 3% hydrogen peroxide in methanol for 10 min, followed by an incubation for 15 min with Ultra V block solution (Labvision) and incubated overnight at 4°C with primary antibodies: anti-CDX2 (BioGenex) diluted 1:20 in 5% BSA, and anti-SMAD4 (B8, Santa-Cruz Biotechnology), anti-MUC5AC (Reis et al. 1997) anti-MUC2 (Reis et al. 1998) and anti-MUC6 (Reis et al. 1999). Subsequently, the UltraVision Detection System, anti-polyvalent-HRP (Labvision) was used following the manufacturer's protocol. Slides were developed with DAB, counterstained with Mayer hematoxylin, dehydrated and mounted with Entellan.

Double staining for CDX2 and MUC5AC was used to better visualize expression patterns. Sections were first stained for CDX2 following the protocol previously described until the final step with DAB. Slides were then washed in water for 10 min and incubated in Tris-buffered saline (TBS, pH 7.6) for 30 min at 60°C. For the second incubation, sections were treated with nonimmune serum for 20 min, followed by overnight incubation with MUC5AC monoclonal antibody at 4°C. MUC5AC was visualized with a biotinylated secondary antibody, an avidin/biotin detection system (Vectastain ABC kit, Vector Laboratories, Burlingame CA) and Fast Red as substrate. Slides were mounted in Glycergel (DAKO).

Double immunofluorescence staining was also used to visualize CDX2 and MUC2. Sections were first stained for CDX2 following the protocol previously described using a rabbit-anti mouse FITC secondary antibody (DAKO). MUC2 was developed with Texas Red. Slides were mounted in Vectashield (Vector).

In situ hybridization

Sense and antisense synthetic oligonucleotides (48mers) corresponding to the tandem repeat sequence of MUC2 gene were used (López-Ferrer et al. 2000). Oligonucleotides were labeled with digoxigenin (DIG) following the

Table 1 Clinicopathological features of the cases with solitary juvenile polyps

Patients	16
Age	
Range (years)	3–64
Average ± SD (years)	19.8 ± 19.7
Lesions	18
Location	
Colon	10
Rectum	8
Size	
Range (cm)	0.8–2.5
Average ± SD (cm)	1.5 ± 0.6

manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Briefly, sections were deparaffinized, rehydrated and digested with proteinase K (1 µg/ml in 0.1 M Tris HCl, pH 7, 0.5 M EDTA) for 15 min at 37°C. Samples were fixed with 4% PFA for 15 min and treated with 0.1 M triethanolamine, pH 8, and 0.25% acetic anhydride for 10 min. After prehybridization in 4 × SSPE with 1% Denhardt's at 42°C for 1 h; sections were dehydrated and hybridized overnight at 40°C with DIG labeled oligonucleotides (200–400 µg/ml) diluted in 4 × SSPE, 1% Denhardt's, 50% formamide, 20 mM DTT, 0.1 M sodium phosphate, pH 7.2, 1% sarkosyl, and tRNA (250 µg/ml). Post-hybridization washes were done stepwise from 4 × SSPE at 20°C to a final wash with 0.1 × SSPE at 42°C. Sections were rehydrated, dried, and incubated with 5% normal horse serum. AP conjugated sheep anti-DIG antibodies (Boehringer Mannheim) were incubated overnight at 4°C. AP was developed overnight at 4°C using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (Promega, Wisconsin, USA) in 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris HCl, pH 9.5. Slides were rinsed and mounted in Aquatex.

Results

Clinicopathological characteristics of the sporadic cases are described in Table 1.

All 18 solitary polyps showed typical features of juvenile polyps, with distorted architecture, branching glands with hyperplasia of goblet cells, inflammation, occasionally with dilated cysts (Figs. 1a, 2a, 3a). Most of the polyps showed focal erosion of the superficial epithelium. In addition to these commonly described characteristics, all polyps showed *foci* with a distinct morphology, characterized by scarce goblet cells and predominance of columnar cells (Figs. 1b, 2b), either mucus-secreting or with eosinophilic cytoplasm, without criteria of dysplasia, usually described as regenerative. These *foci* were more frequently localized underneath the outer surface of the polyps, in areas of intense inflammation. The JP case with a *SMAD4* mutation is morphologically identical to the sporadic cases, including the presence of hyperplastic and regenerative features (Fig. 4a). The polyp with a *BMPRIA* mutation showed only hyperplastic features (Fig. 5a).

Analysis of CDX2 and MUC2 expression, two intestinal differentiation markers, revealed that both proteins are expressed in all solitary polyps, displaying a "patchy", heterogeneous pattern (Figs. 2c, 3b, c,). CDX2 was found in the nucleus whereas MUC2 was found in goblet cells, both in the cytoplasm and in the goblet vacuole. Heterogeneous expression of MUC2 was confirmed by in situ hybridization (Figs. 2d, 3d). SMAD4 followed a similar pattern of expression with heterogeneous nuclear staining (Fig. 3e).

Regarding the expression of the gastric differentiation markers, it was observed that 15 of the 18 solitary juvenile polyps (83%) had *foci* with MUC5AC expression (Figs. 2e, 3f) in the cytoplasm. MUC5AC was predominantly identified in glands with the distinct morphologic features, as described above, and was localized mainly in the peripheral areas of the polyps. MUC5AC expression coincided with

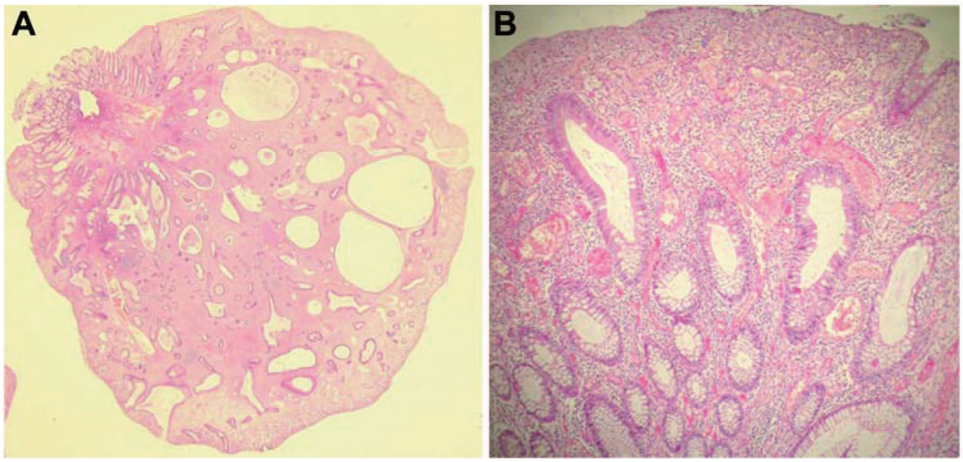


Fig. 1 H&E staining of a juvenile polyp. **a** Low power view with cystic glands; **b** Peripheral area of the polyp with glands characterized by predominance of columnar cells

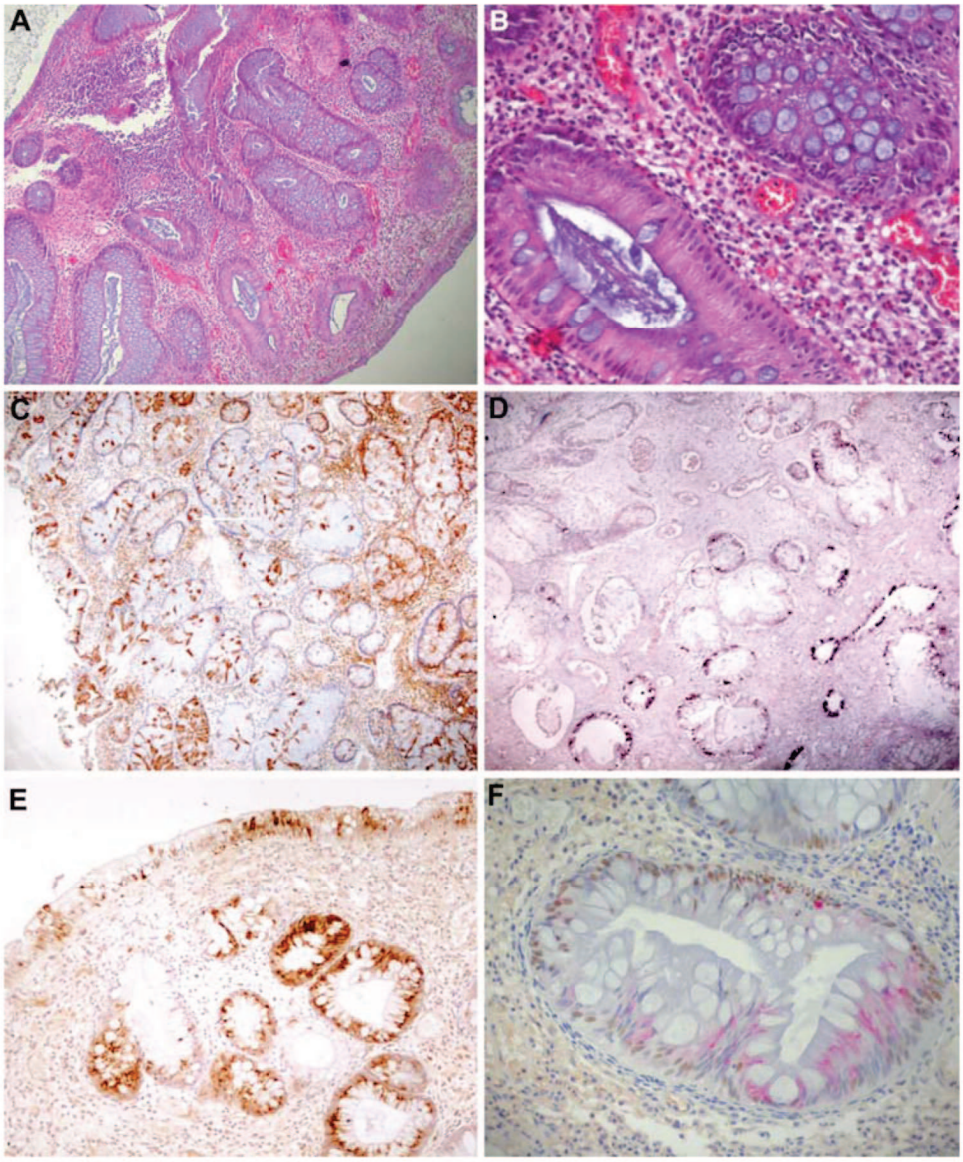


Fig. 2 H&E and immunohistochemistry in one solitary juvenile polyp. **a** H&E low magnification showing hyperplastic, goblet cell-rich glands; **b** H&E showing one peripheral gland with predominance of columnar cells; **c** MUC2 immunohistochemistry showing a heterogeneous staining pattern; **d** MUC2 in situ hybridization with heterogeneous expression; **e** Strong expression of MUC5AC in a peripheral area of the polyp. **f** Double staining for CDX2 (*brown* staining in the nuclei) and MUC5AC (*red* staining in the cytoplasm). Cells positive for MUC5AC have decreased CDX2 nuclear staining

decreased SMAD4 and CDX2 expression (Fig. 2f). MUC6 expression was not detected in these polyps.

The JP case with a *SMAD4* mutation has an expression profile identical to the solitary polyps (Fig. 4). The JP case with a *BMPRIA* mutation is distinct, showing vestigial MUC5AC expression, heterogeneous expression of SMAD4, with no evident loss, heterogeneous expression of MUC2, and no loss of CDX2 (Fig. 5).

Both solitary and *SMAD4* mutated JP case showed extensive co-localization of the alterations described above: decreased expression of SMAD4, CDX2 and partially also MUC2 and de novo expression of MUC5AC (Figs. 2, 4). This modified expression profile was mainly observed in the so-called regenerative areas, where the normal large bowel epithelium was substituted by columnar eosinophilic cells with interspersed goblet cells. The exception was the JP case with *BMPRIA* mutation where the alteration in the expression profile was restricted to vestigial MUC5AC expression, in areas without obvious regenerative characteristics.

Discussion

In this study we describe the expression of MUC5AC, concomitantly with decreased expression of CDX2, SMAD4 and MUC2, in *foci* with predominance of columnar cells, localized in superficial areas of juvenile polyps that are considered as regenerative.

Our hypothesis was that, since the BMP/SMAD pathway regulates CDX2 in vitro, we could see loss or decreased CDX2 expression in a human condition with impairment of

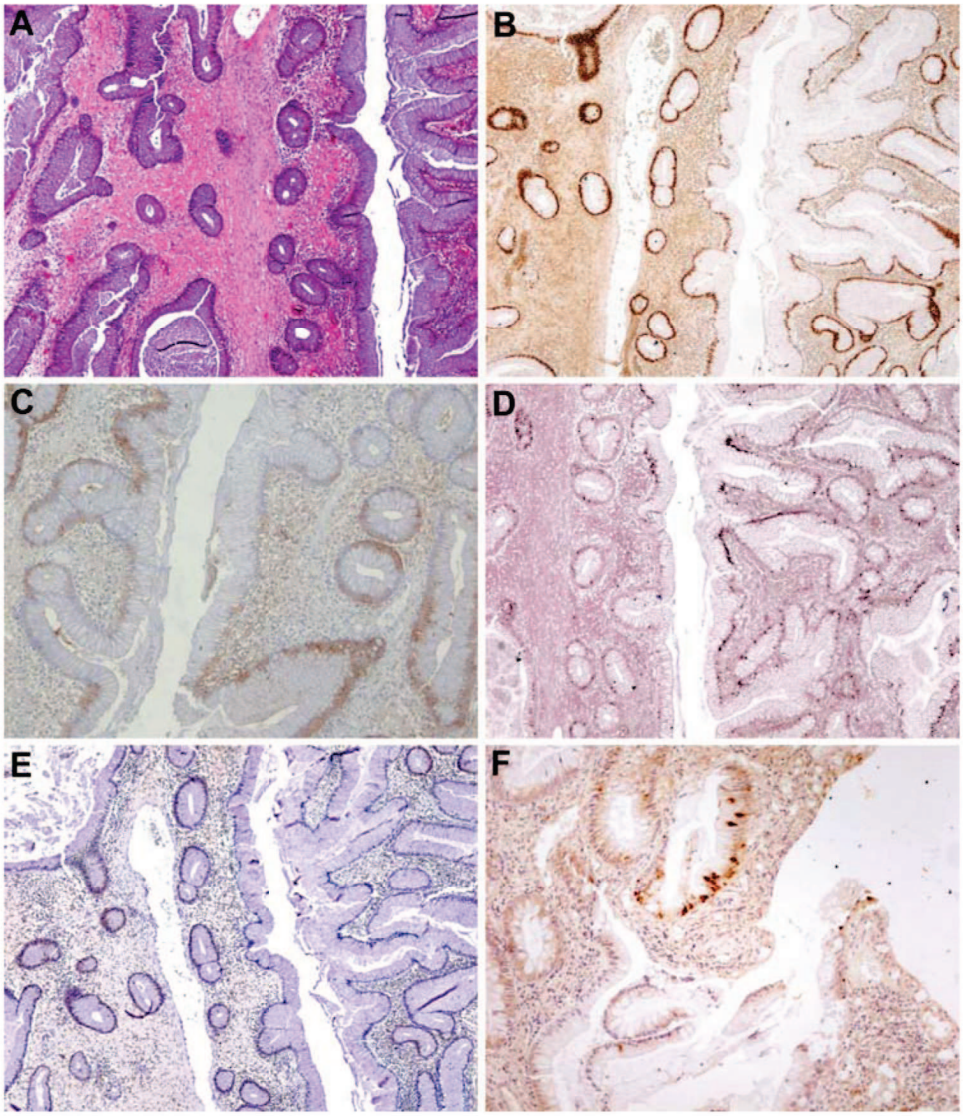


Fig. 3 H&E and immunohistochemistry in one solitary juvenile polyp. **a** H&E low magnification showing hyperplastic, goblet cell-rich glands; **b** CDX2 immunohistochemistry showing heterogeneous nuclear staining; **c** MUC2 immunohistochemistry showing an heterogeneous staining pattern with predominantly perinuclear cytoplasmic staining; **d** MUC2 in situ hybridization with heterogeneous expression; **e** Heterogeneous nuclear staining for SMAD4; **f** Expression of MUC5AC in a peripheral area of the polyp. Images **a**–**e** correspond to serial sections of the same area of the polyp

this pathway, such as juvenile polyps. Furthermore, the hallmark of CDX2 loss of expression is the loss of intestinal differentiation, here characterized by MUC2 expression, and appearance of a gastric phenotype, here characterized by the expression of MUC5AC. The fact that loss of CDX2 expression leads to the appearance of a gastric phenotype was well demonstrated in the $Cdx2^{+/-}$ mouse model (Beck et al. 1999).

A similar pattern of altered differentiation has been previously described in hyperplastic polyps (Bartman et al. 1999; Koike et al. 2003), which for that reason, have been designated by some authors as metaplastic, and in serrated adenomas (Biemer-Huttmann et al. 1999; Jass 1999; Mochizuka et al. 2007; Yao et al. 1999). Also in juvenile polyps, Koike et al. (2003) showed gastric differentiation. Our results extend these observations and demonstrate that gastric metaplasia also occurs in juvenile polyps, both in solitary and in cases with germline mutations. We further demonstrate that this metaplastic phenotype is associated with focal loss of CDX2 related with an altered BMP pathway, assessed by loss/decreased SMAD4 expression. The JP case with a *SMAD4* germline mutation supports this hypothesis by showing a phenotype switch that is identical (morphologically and by immunohistochemistry) to the solitary cases, namely with focal reduction/absence of SMAD4 expression in the areas with gastric differentiation. The JP case with *BMPRIA* germline mutation is morphologically distinct, with absence of metaplastic glands, and shows vestigial MUC5AC expression without obvious involvement of the BMP/SMAD4 pathway (no evident reduction of SMAD4) and of CDX2. This might be interpreted by a less effective mechanism of impairment of the pathway, because, in this case, the mutation occurs in the regulatory region of the gene and therefore may not be completely deleterious for the protein function. Furthermore, we cannot exclude the possibility that other BMP

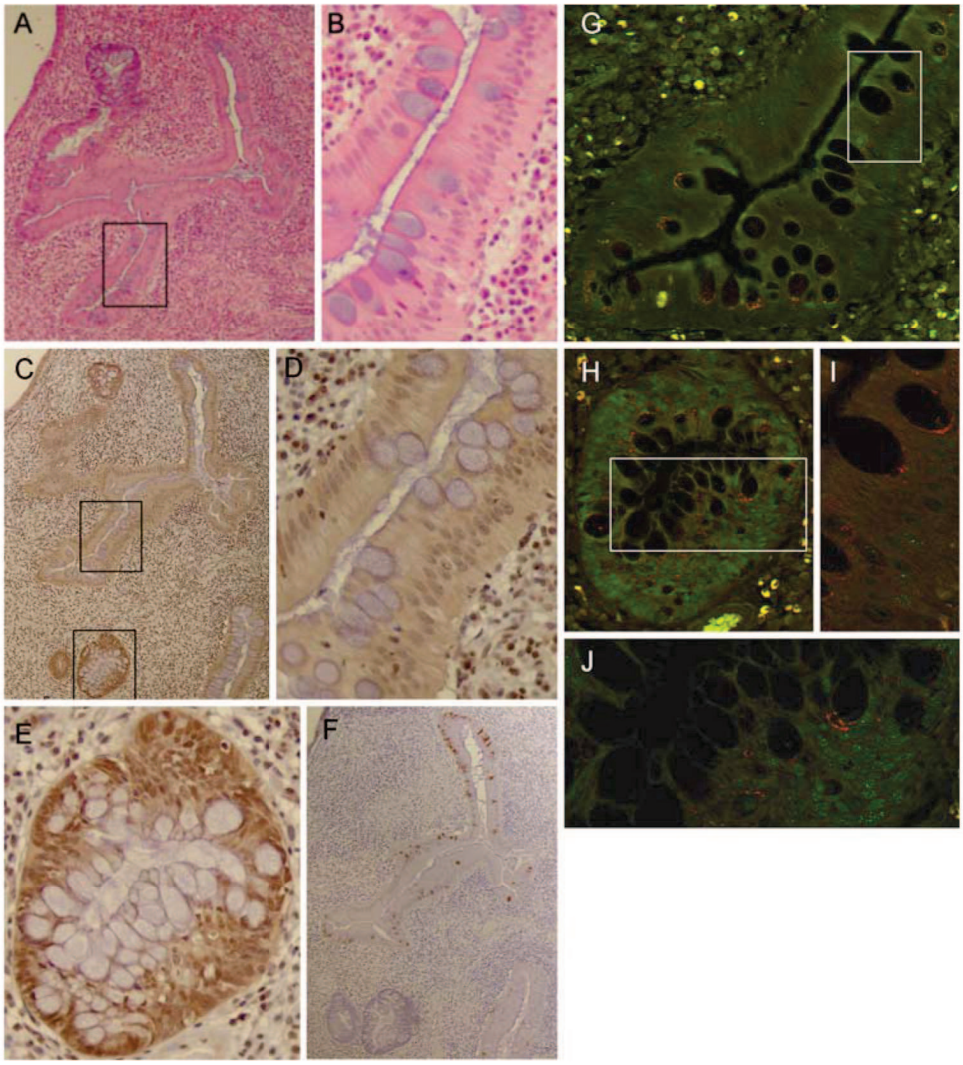


Fig. 4 H&E and immunohistochemistry in the juvenile polyposis case with a *SMAD4* mutation. **a** H&E showing a gland with columnar cells and scarce goblet cells; **b** Detail of **a**; **c** Same gland showing low expression of SMAD4 and adjacent gland with higher SMAD4 expression; **d**, **e** Detail of **c**; **f** Same gland as in **a** showing MUC5AC expression; **g** Same gland as in **a** showing low CDX2 expression (*green*) and vestigial MUC2 expression localized in the perinuclear region (*red*); **h** Adjacent gland, as in **c**, showing stronger CDX2 expression (*green*) and vestigial MUC2 expression localized in the perinuclear region (*red*); **i**, **j** Detail of **g** and **h**, respectively

receptors are active and replacing BMPRIA function (Attisano and Wrana 2002).

Complete loss of CDX2/SMAD4 and MUC2 is focal so these polyps largely retain intestinal differentiation. The gastric differentiation is of the foveolar type, characterized by the expression of the gastric mucin MUC5AC, normally present in gastric foveolar epithelium, and absence of MUC6, normally present in gastric glands, differently from serrated polyps where MUC6 is also present. Moreover, if these *foci* are regenerative, they undergo a metaplastic regeneration, which shows that a normal intestinal differentiation is more difficult to achieve in this context. Interestingly, a similar gastric and intestinal-mixed phenotype occurs in IM of the stomach (Reis et al. 1999; Silva et al. 2002). Some IM foci acquire a "complete" intestinal phenotype regarding mucin expression because they express MUC2 and completely lose MUC5AC and MUC6; at variance, some IM foci have a gastric and intestinal-mixed phenotype expressing MUC2, and retaining gastric differentiation (MUC5AC and MUC6) (Reis et al. 1999). In both cases the onset of an intestinal phenotype in the stomach is due to an aberrant expression of the homeobox gene CDX2 (Almeida et al. 2003). In gastric IM, aberrant CDX2 expression is regulated by the BMP/SMAD pathway, leading to the development of an intestinal phenotype; we hypothesize that here the inverse phenomenon could be the trigger for gastric metaplasia.

A striking observation in the current study is the heterogeneous/focal reduction in expression of SMAD4/CDX2 (accompanied by de novo MUC5AC expression) within the polyps. This might reflect a complex regulation of CDX2, but also the absence of complete inactivation of SMAD4, even in the JP case with a *SMAD4* germline mutation, suggesting a dose-dependent modulation of the BMP/SMAD4 pathway. This is more evident in the JP case with BMPRIA mutation. Complete loss of the pathway appears to not be essential for polyp formation but further reduction/loss is required for gastric metaplasia within the polyp.

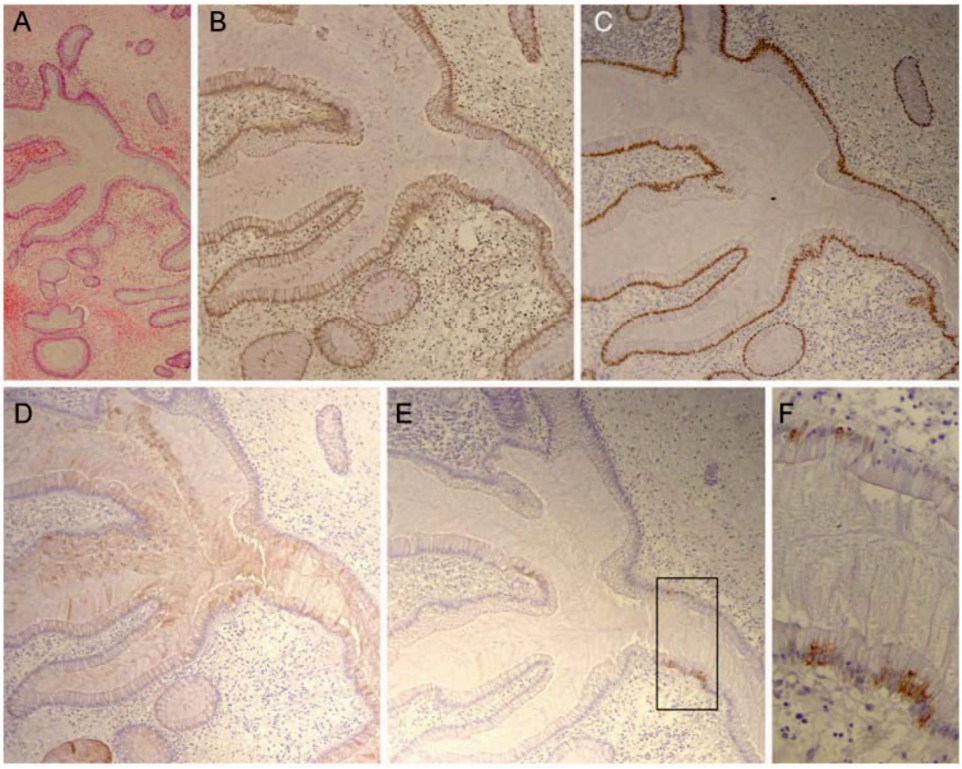


Fig. 5 H&E and immunohistochemistry in the juvenile polyposis case with a *BMPRIA* mutation. **a** H&E showing hyperplastic, goblet cell-rich glands; **b** Same gland with strong and homogeneous SMAD4 expression; **c** Same gland with strong and homogeneous CDX2 expression; **d** Same gland with heterogeneous MUC2 expression; **e** Same gland with vestigial MUC5AC; **f** Detail of **e**

In conclusion, we demonstrate that juvenile polyps have, in addition to altered proliferation, an altered differentiation, namely gastric/intestinal-mixed differentiation linked to deficient CDX2/SMAD4 expression. Our results provide further evidence on the association between CDX2 expression and the BMP/SMAD pathway as recently demonstrated in gastric cells and IM (Barros et al. 2008).

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4

RELEVANCE OF HIGH VIRULENCE *HELICOBACTER PYLORI* STRAINS AND FUTILITY OF CDX2 EXPRESSION FOR PREDICTING INTESTINAL METAPLASIA AFTER ERADICATION OF INFECTION

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ORIGINAL ARTICLE

Relevance of high virulence *Helicobacter pylori* strains and futility of CDX2 expression for predicting intestinal metaplasia after eradication of infection

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Abstract

Objective. Different *Helicobacter pylori* genotypes are associated with distinct inflammatory responses and consequent development of pre-neoplastic lesions, namely intestinal metaplasia (IM), which is dependent on the expression of CDX2. We aimed to evaluate IM progression/regression in the context of *H. pylori* eradication, bringing into play the effect of the virulence of infecting *H. pylori* strains and the hypothesis that CDX2 expression might be a marker for later development of IM. ***Material and methods.*** Sixty-five male volunteers were evaluated by endoscopy before *H. pylori* eradication and after a median six-year follow-up. Histological diagnosis was performed at baseline and follow-up, and baseline *H. pylori* genotypes and CDX2 expression in non-metaplastic foci were also assessed. ***Results.*** Fifty-one individuals remained free from infection at follow-up. Six out of 27 who had no metaplastic lesions at baseline developed IM. CDX2 nuclear expression was observed in 15 of the 21 cases (71.4%) showing no progression to IM, and in three out of six cases (50%) with progression to IM ($p = 0.367$). Six of the 24 cases with IM at baseline showed regression to less severe outcomes, which was less frequent in those infected with high-virulence strains (7.7% vs. 50%, $p = 0.047$). In the latter there is a significant persistence of lymphoid follicles. ***Conclusions.*** Our results support that under infection with high virulence *H. pylori* strains, IM is a point of difficult return in the gastric carcinogenic pathway. The appearance of CDX-expressing cells in non-metaplastic foci was not associated with the development of IM during the six-year follow-up.

Key Words: *CDX2*, *chronic gastritis*, *gastric carcinoma*, Helicobacter pylori, *intestinal metaplasia*

Introduction

There is a large body of observational evidence on the association between *Helicobacter pylori* infection and gastric cancer and its pre-neoplastic lesions [1]. *H. pylori* is a well-established carcinogen [2] and nearly two-thirds of all gastric cancers are attributed to infection with these bacteria [3]. Nevertheless, the pathways from infection to gastric cancer remain elusive.

In the presence of other environmental exposures and host intrinsic factors, *H. pylori* infection may lead to sequentially more severe lesions in the gastric mucosa from chronic gastritis, through intestinal metaplasia (IM), and ultimately to gastric carcinoma [4]. Different *H. pylori* genotypes result in distinct gastric histopathological outcomes in a way that strains with high virulence (*cagA*+, *vacA* s1 and *vacA* m1) confer an increased risk for strong inflammatory responses and pre-neoplastic lesion development, namely IM [5–7].

A recent cohort study with 17-year follow-up supports IM irreversibility [8] and a previous placebo-controlled randomized eradication study suggested

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the irreversibility of the pre-neoplastic lesions, since only subjects with non-atrophic gastric mucosa at baseline had their risk of gastric cancer reduced by eradication [9]. Nonetheless, other human experimental studies favored reversibility of IM [10–12]. Regression of established IM lesions upon eradication was shown in a mouse model of *Helicobacter felis* infection [13], but these mice develop spasmolytic polypeptide expressing metaplasia, which substantially differs from the classical human IM, both in morphology and cell types present.

Gastric IM is a pre-neoplastic lesion consisting of the transdifferentiation of the gastric mucosa to an intestinal phenotype [4,14]. The intestinal differentiation process, both in normal and aberrant locations, was proven to depend on the expression of the homeobox gene *CDX2* [15]. In normal mucosa, CDX2 is only present in the adult intestine, but it is aberrantly expressed in IM and induces intestinal differentiation in animal models [16,17]. Since both CDX2-positive foci and isolated cells appear in the normal stomach of individuals infected with *H. pylori* [18,19] and *in vitro H. pylori* positively regulates CDX2 expression [20], it is possible that expression of CDX2 in morphologically non-metaplastic cells might be related to the subsequent development of IM.

This study aims to evaluate how IM evolved in the context of *H. pylori* eradication and in particular the influence of the virulence of the infecting bacteria and the presence of CDX2 expression in non-metaplastic foci as a putative marker for later development of IM.

Materials and methods

This study was conducted on a Caucasian population from northern Portugal, where IM and gastric carcinoma are highly frequent [6,21]. Workers from the Viana do Castelo shipyard were invited to participate in a gastric pathology survey in 1998. Four hundred and sixty, nearly 40% of all workers, volunteered for the study. Participants completed a physician-administered questionnaire on digestive symptoms and had a blood sample drawn. An upper digestive endoscopy was performed in 354 participants presenting dyspeptic symptoms and/or serum IgG antibodies to *H. pylori*, as previously described [6].

From the 354 *H. pylori*-positive subjects, either by serology or histopathology criteria, 178 were referred to standard eradication treatment (Figure 1), according to the Maastricht Consensus Report [22]. They presented a clinical diagnosis of dyspepsia or peptic ulcer, and/or incomplete or mixed IM and/or

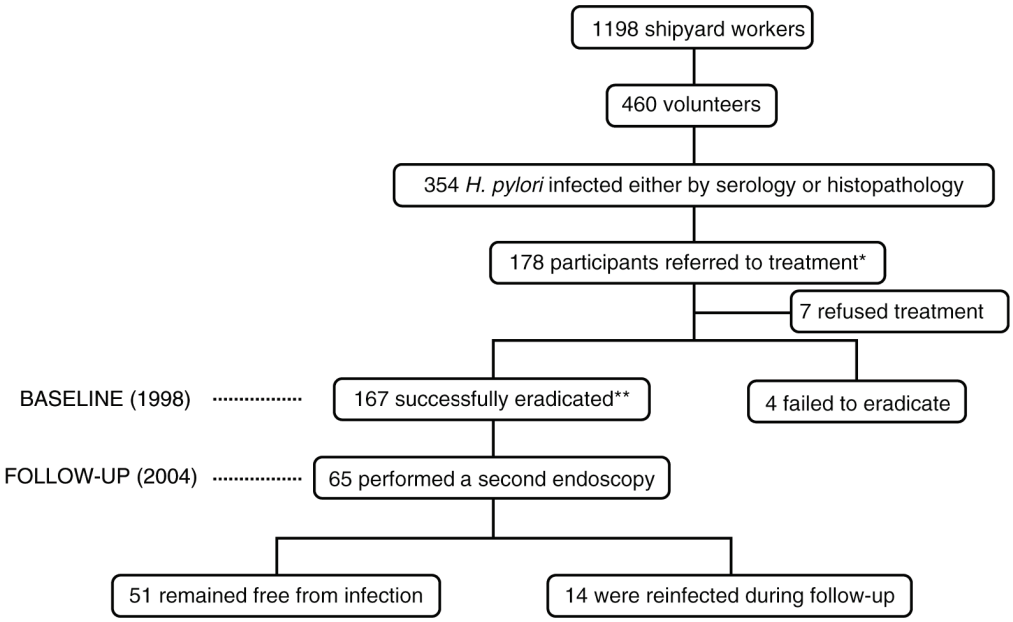


Figure 1. Study sample and participants' evaluation.
*Participants presenting at least one of the following conditions: dyspepsia or peptic ulcer in the clinical diagnosis; incomplete or mixed intestinal metaplasia or erosions/ulcerations in their histopathological diagnosis at any location; or endoscopic ulceration at any location.
**First line therapy: omeprazole 20 mg, clarithromycin 500 mg, and amoxicillin 1000 mg or tinidazole 500 mg, twice daily for a one-week period; Second line therapy: omeprazole 20 mg, tetracyline 500 mg, bismuth subcitrate 120 mg, and metronidazole 250 mg, twice daily for a two-week period.

erosions/ulcerations in any of the biopsy specimens, and/or an endoscopic diagnosis of ulcer.

Eradication was successfully accomplished in 167 subjects (97.7%), with first (n = 148) or second line therapies (n = 19), as confirmed by a ^{13}C-urea breath test. Sixty-five of those successfully eradicated remained at the working place and performed a second endoscopy in 2004 (median follow-up: 6.4 years; range: 5.3–6.9 years), and were selected as participants for the present investigation. At follow-up, 51 were free from infection and 14 were diagnosed *H. pylori*-positive, either by histology or polymerase chain reaction (PCR) and thus considered as re-infected.

No significant differences were found between those who were followed and those for whom a follow-up evaluation was not accomplished, regarding gender (92.3% vs. 94.1% males, p = 0.646), median age (44 years vs. 45 years, p = 0.874), and education (32.8% vs. 31% with less than four school years, p = 0.298). The flow of the participants is represented in Figure 1.

Histopathological study

Four biopsy specimens (one from corpus, one from antrum, and two from *incisura angularis*) were taken from each subject, both at baseline and follow-up. *H. pylori* density, chronic inflammation, polymorphonuclear activity, glandular atrophy, and IM were scored according to the updated Sydney system [23]. The biopsies were formalin-fixed and paraffin-embedded, and sections were then stained with hematoxylin and eosin and with modified Giemsa staining for histopathological and *H. pylori* evaluations, respectively.

Regarding inflammatory indicators, each subject was assigned the most severe score observed in any of the three gastric locations evaluated, and the histological diagnosis was based upon the most severe lesion, following this descending order: IM, chronic atrophic gastritis (CAG), and chronic non-atrophic gastritis (CNAG). No cases of cancer or dysplasia were observed. Subjects were further classified as having complete, incomplete, or mixed IM (if features of both complete and incomplete types were observed in the same biopsy or in different regions of the stomach) [24].

Assessment of H. pylori infection by PCR

Total DNA was extracted from frozen antral biopsy specimens using the method described by Boom et al. [25] and *H. pylori* detection was performed by multiplex amplification of *vacA* and *cagA* genes followed by reverse hybridization on a line probe assay as described earlier [26].

The virulence of the *H. pylori* infecting strains at baseline was assessed in 48 of the 51 individuals remaining free of infection at follow-up and in 12 of the 14 subjects re-infected during follow-up. For data analysis, each subject was classified as infected with high or low virulence strains, according to criteria described in Table I.

Immunohistochemistry

Sections from gastric biopsy specimens from the incisura angularis (n = 23) or the antrum (n = 4) were used. Immunohistochemistry was carried out using a monoclonal antibody against CDX2 (Biogenex), diluted 1:50, as previously described [16].

Participants were classified as negative or positive for CDX2 expression, irrespective of the extent of the staining.

Data analysis

The 65 study subjects were grouped according to the baseline absence (normal gastric mucosa or CNAG or CAG) or presence of IM and according to the

Table I. Criteria used for classification of the virulence of the *Helicobacter pylori* infecting strains, according to virulence factors *cagA* and *vacA*.

		vacA	
	cagA	s Strains	m Strains
High virulent	+	s1	m1
	+	s1 and s2*	m1 and m2*
	+	s1	m2
	+	s1 and s2*	m2
	+	s2	m1
	+	s2	m1 and m2*
Low virulent	-	s1	m1
	-	s1 and s2*	m1 and m2*
	-	s1 and s2*	m1
	-	s1	m1 and m2*
	-	s1	m2
		s1 and s2*	m2
	-	s2	m1
	-	s2	m1 and m2*
	-	s2	m2
	+	s2	m2

*Infection with multiple strains.

infection status at follow-up (infected vs. not infected). The analyses were conducted separately for those remaining free of infection at follow-up (n = 51) or re-infected (n = 14).

Subjects with normal gastric mucosa, CNAG, or CAG at baseline were further classified according to the progression to IM (progression vs. unchanged), and those with IM at baseline were classified taking into account the regression of IM to less severe lesions (regression vs. unchanged). There were no statistically significant median age differences between groups (no progression: 45 years, progression: 44 years, regression: 48 years, no regression: 43 years; $p = 0.821$).

Proportions were compared across groups using the χ^2 or the Fisher's exact test, as appropriate, and within each group the changes between baseline and follow-up evaluations were compared using the McNemar test on paired proportions.

Results

Histopathological analysis of the gastric mucosa in subjects undergoing baseline successful eradication therapy

Among the 51 individuals who remained infection-free during follow-up, 24 (47.1%) presented IM with absent/mild atrophy (n = 17, 70.8%) or moderate/abundant atrophy (n = 7, 29.2%). From these, 24 cases with IM, six (25%) showed regression to a normal gastric mucosa, CNAG, or CAG. The 27 participants with no IM at baseline had absent/mild glandular atrophy and during follow-up, six (22.2%) developed IM (Table II).

Fourteen individuals were considered re-infected with *H. pylori* during follow-up. Seven presented IM at baseline, from which one (14.3%) showed regression to CAG. Three (42.9%) developed IM during follow-up (Table II).

Amongst participants with no IM at baseline (n = 34), the percentage of subjects with progression to IM among those remaining free of infection at follow-up was approximately half the observed in those being re-infected, though the difference was not statistically significant (22.2% vs. 42.9%, $p = 0.534$). No significant differences were also observed among participants with IM at baseline (n = 31) regarding the percentage of those showing regression of IM, according to infection status at follow-up (25% vs. 14.3%, p = 0.934) (Table II), though regression was more frequent in those who did not re-infect.

Baseline CDX2 expression in subjects with no intestinal metaplasia at baseline, among those undergoing successful eradication and remaining free of infection at follow-up

The presence of CDX2 positive cells was assessed in the 27 participants with no IM at baseline and without re-infection during follow-up. CDX2 nuclear expression was observed in 15 of the 21 cases (71.4%) showing no progression to IM during follow-up and in three of the six cases (50%) with IM diagnosed at follow-up (p = 0.367) (Figure 2).

Table II. Histopathological diagnosis at baseline and during follow-up in the individuals undergoing baseline successful *Helicobacter pylori* eradication, according to virulence of the baseline infecting strains and the infection status at follow-up.

		At follow-up					
		H. pylori-negative			*H. pylori*-positive		
At baseline		Regression	Unchanged	Progression	Regression	Unchanged	Progression
IM	*H. pylori* strains	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Absent	All	-	21 (77.8)	6 (22.2)	-	4 (57.1)	3 (42.9)
	Low virulent†	-	15 (79)	4 (21)	-	1 (33.3)	2 (66.7)
	High virulent†	-	6 (75)	2 (25)	-	3 (75)	1 (25)
Present	All	6 (25)	18 (75)	-	1 (14.3)	6 (85.7)	-
	Low virulent†⋆	4 (50)	4 (50)	-	0 (0)	3 (100)	-
	High virulent†⋆	1 (7.7)	12 (92.3)	-	1 (50)	1 (50)	-

⋆The virulence of the *H. pylori* infecting strains at baseline could not be assessed for five subjects.
†Criteria described in Table I: high virulent (simultaneously *cagA*+ and *vacA* s1/m1, *vacA* s1/m2 or *vacA* s2/m1); low virulent (otherwise); subjects simultaneously infected with *vacA* s1 and s2 strains or *vacA* m1 and m2 strains were considered infected with high-virulent strains. IM = intestinal metaplasia; No progression = IM absent at baseline + IM absent at follow-up; No regression = IM present at baseline + IM present at follow-up; Progression = IM absent at baseline + IM present at follow-up; Regression = IM present at baseline + IM absent at follow–up.

Evolution of the histopathological diagnosis, according to virulence of H. pylori infecting strains, among those undergoing successful eradication and remaining free of infection at follow-up

A similar percentage of cases progressed to IM in the presence of high or low virulence *H. pylori* strains (25% vs. 21.0%, $p = 0.822$). However, regression of IM was significantly more frequent in those infected with low virulence strains (50% vs. 7.7%, $p = 0.047$) (Table II).

From the four subjects showing progression to IM infected with low virulence strains, one developed complete IM, two developed incomplete IM, and one developed mixed-type IM. One individual infected with a high virulence strain developed complete IM and another incomplete IM. One subject infected with high virulence strain had regression of incomplete IM, and from the four individuals infected with low virulence strains, two regressed from complete IM and two from mixed-type IM.

Inflammatory indicators at baseline and at follow-up, according to the presence of IM at baseline and at follow-up, among those undergoing successful eradication and remaining free of infection at follow-up

While almost all subjects presented moderate/abundant disperse chronic lymphoid infiltrate at baseline (96%), this was absent at follow-up in all of the eradicated subjects (Table III). There was also a very low frequency of neutrophilic activity at follow-up, varying from 0% in the group progressing to IM to 22% in the group without regression of the metaplastic lesions, despite that neutrophilic activity was high at baseline in all the groups (Table III).

Lymphoid follicles were observed in nearly all participants at baseline (98%) and were observed in approximately two-thirds of all subjects at follow-up, regardless of the presence of IM at baseline and progression or regression. In those progressing to IM, all presented lymphoid follicles both at baseline and follow-up evaluations (Table III).

Discussion

Among subjects undergoing successful eradication of *H. pylori* and remaining free of infection after a six-year follow-up, regression of the metaplastic lesions was observed in less than 10% of cases previously infected with high-virulence strains, while half of the infected with less virulent strains showed regression to less severe lesions or normal gastric mucosa. Progression to IM did not significantly differ according to the infection status at follow-up or CDX2 expression status at baseline.

The relatively small sample evaluated in this study limits the interpretation of the results on the relation between re-infection and gastric lesions' evolution, especially according to IM sub-types. Although false negative results in the assessment of infection status after eradication treatment are unlikely, due to the high reliability of the urea breath test [27], the potential for misclassification of the histopathologic characteristics due to sampling error in biopsy collection further limits the interpretation of our findings. Nevertheless, this study enabled us to show for the first time an association between virulence of *H. pylori* strains and regression of IM. The histological consequences of eradication with respect to inflammatory markers are well established [9] and may be used as an internal control of eradication effectiveness. As expected, eradication resulted in an almost complete abrogation of disperse chronic lymphoid infiltrate and neutrophylic activity. Interestingly, lymphoid follicles, which are largely retained even after infection is

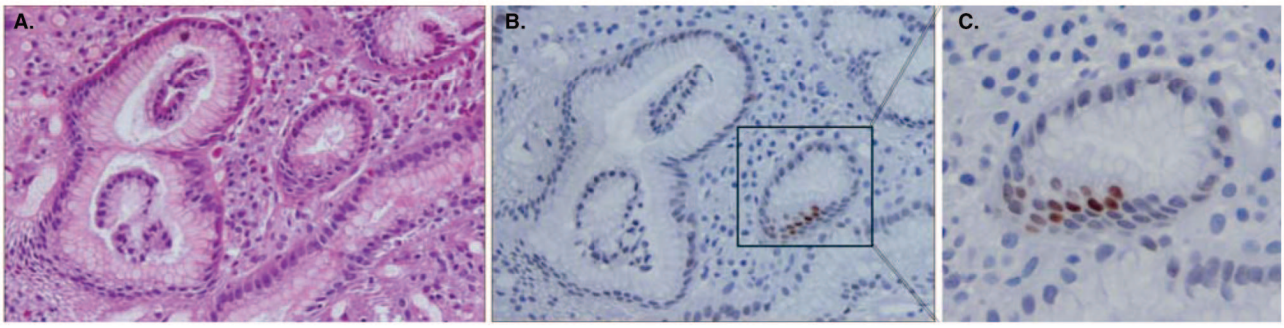


Figure 2. Expression of CDX2 in histologically non-mestaplastic cells. (A) H&E staining (× 200); (B) CDX2 immunostaining (× 200); (C) higher magnification (× 400) of a gland with CDX2 nuclear staining.
H&E = hematoxylin and eosin.

Table III. Inflammatory indicators at baseline and at follow-up, according to the presence of IM at baseline and at follow-up, in subjects undergoing successful eradication of *Helicobacter pylori* infection and remaining free of infection at follow-up.

	Disperse chronic lymphoid infiltrate, *n* (%)				Lymphoid follicles, *n* (%)				Neutrophilic activity, *n* (%)			
		Follow-up				Follow-up				Follow-up		
	Baseline	A/M	M/A	*P**	Baseline	–	+	*P**	Baseline	–	+	*P**
No progression	A/M	0 (0)	0 (0)	<0.001	–	0 (0)	0 (0)	0.016	–	0 (0)	0 (0)	<0.001
	M/A	21 (100)	0 (0)		+	8 (38.1)	13 (61.9)		+	19 (90.5)	2 (9.5)	
Progression	A/M	0 (0)	0 (0)	0.031	–	0 (0)	0 (0)	>0.999	–	0 (0)	0 (0)	0.031
	M/A	6 (100)	0 (0)		+	0 (0)	6 (100)		+	6 (100)	0 (0)	
Regression	A/M	0 (0)	0 (0)	0.031	–	0 (0)	0 (0)	0.500	–	0 (0)	0 (0)	0.062
	M/A	6 (100)	0 (0)		+	2 (33.3)	4 (66.7)		+	5 (83.3)	1 (16.7)	
No regression	A/M	2 (11.1)	0 (0)	<0.001	–	0 (0)	1 (5.6)	0.125	–	1 (5.6)	0 (0)	<0.001
	M/A	16 (88.9)	0 (0)		+	6 (33.3)	11 (61.1)		+	13 (72.2)	4 (22.2)	

**P* value from McNemar test (null values taken as 0.5 for *p* estimation).
A/M = absent/mild; IM = intestinal metaplasia; M/A = moderate/abundant; No progression = IM absent at baseline + IM absent at follow-up; No regression = IM present at baseline + IM present at follow-up; Progression = IM absent at baseline + IM present at follow-up; Regression = IM present at baseline + IM absent at follow-up; – = absent, + = present.

cleared, show some degree of persistence among eradicated subjects but do not correlate with the evaluated outcomes.

We did not find differences in the grades of the inflammatory markers according to virulence of the infecting strains at baseline, which may be partly explained by the fact that the lower virulence group does not include only the strains with the lowest possible virulence (considering the virulence factors *cagA* and *vacA*). Furthermore, infection has probably persisted for many years prior to analysis, and the characteristics observed in adults may not reflect those of an early phase of infection, since DNA alterations such as mutations or altered methylation profiles [28,29] may have already occurred, especially among subjects infected with high virulence strains.

IM is triggered by the expression of the intestinal master gene CDX2, which is normally expressed only in the adult intestine and is *de novo* expressed in metaplastic lesions of the stomach, esophagus, liver, and gall bladder [16,30]. Furthermore, ectopic CDX2 expression in the stomach was shown to be sufficient to induce extensive IM development in a mouse model [17]. Since both CDX2-positive foci and isolated cells appear in the normal stomach of individuals infected with *H. pylori* [18,19] and, *in vitro*, *H. pylori* positively regulates CDX2 expression [20], we addressed the possibility that expression of CDX2 in morphologically non-metaplastic cells was related to the subsequent development of IM. However, our results showed no significant association between the presence of CDX2 protein in the normal gastric glands and the subsequent progression to IM. The follow-up may have not been long enough to uncover differences in the progression to IM according to the presence of CDX2 expressing cells but we may also speculate that CDX2 stable expression in the gastric stem cells might be a requisite for the appearance of metaplastic glands. Stable expression may happen less frequently and at a later stage in the cascade of events, rather than its sporadic activation in gastric differentiated cells, which might be a frequent phenomenon without biological significance. To fully address this issue, it would be necessary to evaluate if the progression to IM was associated with double staining of CDX2 with a gastric stem cell marker, which, at the present time, is not well established.

In summary, our results show an association between infection with high virulence *H. pylori* strains and the non-reversibility of IM, together with a persistence of lymphoid follicles, supporting the hypothesis that IM is a rather stable phenotype that rarely reverts upon eradication of the infection in the subjects at higher risk for cancer development. No significant association was observed between the appearance of foci of CDX2-expressing cells and the development of IM during the follow-up period of this study.

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CDX2 EXPRESSION IS INDUCED BY *HELICOBACTER PYLORI* IN AGS CELLS

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LETTER TO THE EDITOR

CDX2 expression is induced by *Helicobacter pylori* in AGS cells

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To The Editor: In the paper by Vauhkonen et al. [1], recently published in the Scandinavian Journal of Gastroenterology, the investigators observe that CDX2 is expressed focally and at low intensity in normal stomach, particularly in the antrum, and that this expression is enhanced in Helicobacter pylori gastritis and reversible after eradication of the bacteria. In accordance with the results by Vauhkonen et al., we observed, in vitro, an increase in CDX2 expression upon infection of a gastric carcinoma cell line (AGS) with a cag PAI-positive H. pylori strain, 26695. In our experiment, AGS cells were infected at two different ratios of bacteria to epithelial cells, 100:1 and 200:1, during 3 h and 8 h. We observed an increase in CDX2 expression, determined by reverse transcription-polymerase chain reaction (RT-PCR), after 8 h of infection (Figure 1A). Next, we evaluated the putative involvement of the CagA gene in the regulation of the CDX2 promoter, by co-transfecting three gastric carcinoma cell lines with the proximal CDX2 promoter (-871/-1) [2] and a CagA expression vector [3]. However, we did not observe any increased transcriptional activity of the CDX2 promoter (Figure 1B) by CagA.

CDX2 is a marker and, more importantly, a master gene for intestinal differentiation both in the intestine [4] and in aberrant locations, such as intestinal metaplasia (IM) of the stomach [5–7], which is a preneoplastic lesion that is strongly associated with *H. pylori* infection, in epidemiological [8] and *in vivo* studies [9]. Vauhkonen et al. address two very interesting points on the regulation of CDX2 in the stomach, the association with *H. pylori* infection and the reversibility of CDX2 expression. Our results complement the ones obtained by Vauhkonen et al., since we show that the presence of *H. pylori* is sufficient to increase CDX2 expression in a gastric carcinoma cell line, and that this regulation is not directly through CagA in an *in vitro* system. The importance of this demonstration stems from the fact that, *in vivo* (in tissues), it is not possible to separate the role played by the bacteria and by the concomitant inflammatory process. In fact, the circumstance that CDX2 is also aberrantly expressed in other locations, such as Barrett's oesophagus [10], and even in autoimmune gastritis, strongly suggests that the inflammatory process may play an important role.

In summary, although the mechanism has not been clarified, both the report by Vauhkonen et al. and our own data show that CDX2 expression in the stomach is regulated directly by *H. pylori.*

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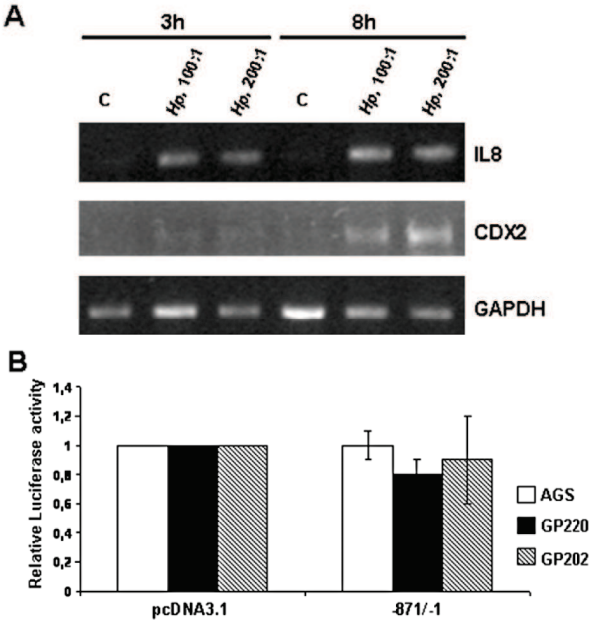


Figure 1. Role of *Helicobacter pylori* in regulating CDX2 expression and promoter activity in AGS cells. A. AGS cells were infected with *H. pylori* at two different ratios of bacteria to epithelial cells, 100:1 and 200:1, during 3 and 8 h. RNA was extracted with Tri reagent (Sigma) and 5 μg was used for cDNA synthesis. 3 μl cDNA was subsequently used to amplify each gene. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis shows expression of IL8 (known to be strongly induced by *H. pylori* and therefore used as a control) after 3 h and 8 h of infection, and CDX2 expression mainly after 8 h of infection. GAPDH was used as a control. B. AGS cells were co-transfected with pGL3(-871/-1)CDX2 promoter construct and a CagA expression vector, using Lipofectamine 2000 reagent (Invitrogen). Total cell extracts were prepared after 48 h and Luciferase activity determined. -Galactosidase activity was measured to normalize for transfection efficiency. The results, expressed as fold increase in Luciferase activity relative to the empty vector, show that CagA does not transactivate CDX2 (-871/-1) promoter in any of the three gastric carcinoma cell lines.

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CDX2 AUTOREGULATION IN HUMAN INTESTINAL METAPLASIA OF THE STOMACH: IMPACT ON THE STABILITY OF THE PHENOTYPE

6

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Gut (accepted manuscript)

CDX2 autoregulation in human intestinal metaplasia of the stomach: impact on the stability of the phenotype

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ABSTRACT

Background & Aims: Intestinal metaplasia (IM) is a gastric pre-neoplastic lesion that appears following *Helicobacter pylori* infection and confers increased risk for cancer development. IM is induced by gastric expression of the intestine-specific transcription factor CDX2. The regulatory mechanisms involved in triggering and maintaining gastric CDX2 expression are not fully unravelled. The CDX2+/- mouse develops intestinal polyps with gastric differentiation and total loss of CDX2 expression, in absence of structural loss of the second allele, suggesting a regulatory defect. This putative haplo-insufficiency together with the apparent stability of IM, led us to hypothesize that CDX2 is regulating its own expression through an autoregulatory loop in both contexts.

Methods: Gastrointestinal cell lines were co-transfected with wild-type or mutated Cdx2 promoter constructs and CDX2 expression vector for luciferase assays. Transfection experiments were also used to assess endogenous CDX2 autoregulation, evaluated by RT-PCR, qPCR and western blotting. ChIP was performed in a cell line, mouse ileum and human IM.

Results: We demonstrate that CDX2 binds to and transactivates its own promoter and also that it positively regulates its expression in gastrointestinal human carcinoma cell lines. Furthermore, we show that CDX2 is bound to its promoter in the mouse ileum and in human gastric IM, thus providing a major contribution to understand the relevance of this autoregulatory pathway *in vivo*.

Conclusion: We demonstrate another layer of complexity in CDX2 regulation, by an effective autoregulatory loop, which may have a major impact on the stability of human IM possibly resulting in inescapable continuance of the gastric carcinogenesis pathway.

INTRODUCTION

Intestinal Metaplasia (IM) of the stomach is a pre-neoplastic lesion conferring increased risk for the development of gastric carcinoma, which remains the second leading cause of cancer death worldwide (1). IM occurs most frequently in the gastric carcinogenic cascade following *Helicobacter pylori* infection, which leads to the appearance of a chronic gastritis, atrophy, progression to IM and, ultimately, gastric cancer (2). Eighty percent of the gastric carcinomas appear in the context of IM (3) and the presence of this pre-neoplastic lesion confers an increased risk, ranging from 2-fold to about 6-fold, for subsequent cancer development (3-5). Furthermore, animal models, both of *H. pylori* infection and subsequent lesions or induced gastric IM, also recapitulate the progression from IM to gastric cancer (6-9). Therefore understanding the mechanisms behind IM establishment, maintenance and progression is of utmost importance.

IM consists on the transdifferentiation of the gastric mucosa to an intestinal phenotype and depends on the expression of the homeobox transcription factor CDX2, the master gene for intestinal differentiation (10-11). Under normal conditions, CDX2 expression in adults is restricted to the intestine, but it becomes ectopically expressed in human intestinal metaplasia lesions, not only of the stomach (12-13), but also of oeasophagus (14-15) and gallbladder (16).

Cdx2 homozygotic null mutant mice are not viable because embryos fail to implant, whereas *Cdx2*$^{+/-}$ mice develop non-cancerous polyp-like lesions with focal loss of Cdx2 expression and appearance of gastric differentiation (17-18). Conversely, forced expression of Cdx2 in the stomach of transgenic mice led to extensive IM, with subsequent progression to gastric cancer (9, 19-20). Further, CDX2 has been directly implicated in transcriptional regulation of intestinal terminal differentiation markers, such as MUC2 (21), LI-Cadherin (22) and Sucrase-Isomaltase (23), among others. However, the molecular mechanisms regulating CDX2 expression, namely in IM establishment and maintenance, are not completely disclosed. Evidences so far suggest a complex regulation with involvement of multiple regulatory pathways. We recently demonstrated that key elements of the BMP pathway co-localized with CDX2 in IM and positively regulated CDX2 in gastric cell lines (24), and we showed a direct regulation of CDX2 expression by *H. pylori* interaction with epithelial cells in an *in vitro* co-culture model (25). Both mechanisms fail to give insight on the maintenance of CDX2 expression and on the generally observed low reversibility of IM even after *H. pylori* eradication and consequent clearance of the inflammatory response (26-28).

Xu *et al* showed that CDX2 is able to transactivate its own promoter *in vitro* in a cell type-specific manner (29), suggesting a positive autoregulatory loop, but the importance of this process for CDX2 regulation *in vivo*, namely in IM, is yet to be established. Furthermore, the phenotype observed in the *Cdx2*$^{+/-}$ mice clearly suggests a dosage-dependence on Cdx2 transcriptional activity compatible with a self-regulatory mechanism, since the germline loss of one allele with no structural second hit leads to total loss of expression of this

gene focally (17). These results, together with the apparent stability of the CDX2-dependent IM phenotype, led us to hypothesize that CDX2 regulates its own expression through a positive autoregulatory loop in the gastric and intestinal contexts.

METHODS

Cell culture, transient transfection and luciferase assays

Human gastric carcinoma cell lines, AGS (ATCC), MKN45 (JCRB0254, The RIKEN Cell Bank, Japan), GP202 (30) IPA220 (30), KatoIII (ATCC), and MKN28 (JCRB0253, The RIKEN Cell Bank, Japan), and intestinal carcinoma cell lines, HCT116 (ATCC), HT-29 (ATCC), HCT15 (ATCC) and CO115 (31), were cultured under standard conditions. The reporter plasmids pCdx2-1Luc and pCdx2-9Luc, containing 1kb and 9.3 kb of the murine Cdx2 promoter respectively (32), were used for cell transfection with Lipofectamine2000 (Invitrogen) and luciferase assays. Cells were co-transfected with an expression vector for CDX2 or the corresponding empty one, and a CMV-β-gal vector (1 µg of DNA : 1.5 µl of Lipofectamine). AGS cells were also co-transfected with the CDX2 expression vector, or the corresponding empty one, and the pCdx2-1luc wild-type, single and double mutated. Total extracts were prepared using 1×Reporter Lysis Buffer (Promega), according to the manufacturer's instructions, and luciferase activity was measured in a 1450 Microbeta luminescence counter (Wallac). β-Galactosidase activity was measured to correct for transfection efficiency. Each experiment was carried out in triplicate at least twice; the results are expressed as means ± SD of representative triplicates.

AGS, MKN45, HCT116 and HeLa (ATCC) were transfected with a CDX2 expression vector, containing two FLAG tags at the N-terminus, or the corresponding empty one, for RNA and protein analysis. MKN45 and HCT116 were also transfected with the CDX2 expression vector alone or together with the dominant negative truncated CDX2 form, miniCDX2 (33) (Vanier et al, manuscript in preparation) for RNA expression analysis. The amounts of DNA in each of these experimental conditions were equalized by adding the corresponding empty vector.

Site-directed mutagenesis

To introduce point mutations in the pCdx2-1luc gene promoter region the QuickChange Site-directed mutagenesis protocol (Stratagene) was used. The oligos used for this purpose are listed in Table 1. The double mutant was obtained by introducing the mutation MUT2 in the pCdx2-1luc MUT1. The PCR cycles were set as follows: 95ºC for 30sec; 16 cycles of 95ºC for 30sec, 55ºC for 1min, 68ºC for 5min. Following PCR reaction, the products were incubated with *DpnI* for 1h at 37ºC and transformed to *E. coli* competent cells. All the plasmids were checked by sequencing.

RNA extraction and RT-PCR

Total RNA was extracted using TRI Reagent (Sigma) and reverse transcription was performed with 5µg of total RNA. PCR amplification was performed using the primer sets outlined in Table 1 and the products were separated in 1.5% agarose gels. GAPDH levels were used as internal control. qPCR was performed using SYBR Green (endogenous CDX2) or Taqman (Applied Biosystems) reagents. TBP levels were used for normalization and relative mRNA levels were calculated using the Ct method. The primers and probes used are listed in Table 1. Each experiment was carried out in duplicate at least twice; the results are expressed as means ± SD.

Table 1 – Sequences of the oligonucleotides used for site-directed mutagenesis, RT-PCR, q PCR and ChIP.

Site-directed mutagenesis	5' → 3'
Mutation 1*	S: CGATTGTTTAATGTAATAGTTT***GGC***AAAATGAATTGCTTTCC AS: GGAAAGCAATTCATTTT***GCC***AAACTATTACATTAAACAATCG
Mutation 2*	S: TCTTGTAAACACTCGTT***CC***TCACGGAAGGCCGCCG AS: CGGCGGCCTTCCGTGA***GG***AACGAGTGTTTACAAGA
RT-PCR	5' → 3'
Endogenous CDX2	F: CCAGGACGAAAGACAAATATCGA R: GACTCTGCTAGACTCCTCAG
Exogenous CDX2	F: CCAGGACGAAAGACAAATATCGA R: CGACAAGCTTGACATTGAAGC
GAPDH	F: ACCATCTTCCAGGAGCGAG R: GGATGACCTTGCCCACAG
qPCR	5' → 3'
Endogenous CDX2	F: GTGCTAAACCCCACCGTCAC R: GACTCTGCTAGACTCCTCAG
LI-Caherin	TaqMan Gene Expression Assay CDH17 Hs00184865_m1
MUC2	TaqManGene Expression Assay MUC2, Hs03005094_m1
TBP	TaqMan Gene Expression Assay TBP, Hs99999910_m1
human ChIP	5' → 3'
pCDX2 Region 1	F: GCATTAGCAGAAATTCTCTTTTC R:GCATGTGGTAGAAGTTAGGCT
pCDX2 Region 2	F: CTAACTTCTACCACATGCCCA R: GCATCTCTGACTTCATCTTACA
pCDX2 Region 3	F: GAGTTTCTTGACCGCCCTCTT R: CCTCCAATCACAGGTTCAAAGA
pCDX2 Region 4	F: CTTTGAACCTGTGATTGGAGGT R: CTCTACGCACAACCCCTCGAA
pCDX2 Region 5	F: CGAGGGGTTGTGCGTAGAGT R: ACAGGCTGGCGTGCGGA
pSucrase- Isomaltase	F: GGCTGGTAAGGGTGCAATAA R: GCCTGTTCTCTTTGCTATGTTG
mouse ChIP	5' → 3'
pCdx2 Region 1	F: CAACGGTGGATTCATTCCG R: GGAAGTATTTGTGCTGACACC
pCdx2 Region 2	F: GGTGTCAGCACAAATACTTCC R: GTAATTAGTGGATGGCTGGG
pCdx2 Region 3	F: CCCAGCCATCCACTAATTAC R: GAAAAGACGATTCTACCTCCAG
pCdx2 Region 4	F: CGTTTCCAAACCCAGCTTCC R: CCCCCAGAAACACGATTTGC
pCdx2 Region 5	F: GCAAATCGTGTTTCTGGGGG R: GCGGCCTTACGTGATTAACG
pSucrase-Isomaltase	F: GATAGGCTTGTGAAAGTGCAAT R: CCTGTAGTATCTGCTGTGTTG

* Mutated nucleotides are in bold, italicized and underlined.

Protein extraction and Western Blot
Cells were lysated and the protein content was measured using standard methods. Protein extracts (50 µg for AGS and HCT116 and 70 µg for MKN45) were analysed by standard SDS-PAGE, transferred to a nitrocellulose membrane (Amersham Biosciences), and blotted with primary antibodies (anti-CDX2, 1:500, Biogenex; anti-actin, 1:8000, SCBT) in 5% non-fat milk in 1× TBS 0.02% Tween-20 (Sigma). Peroxidase-conjugated secondary antibodies (for CDX2 goat anti-mouse-HRP, 1:2000, and for actin goat anti-rabbit-HRP, 1:2000, both from SCBT) were used and developed with the ECL detection kit (BioRad). Quantification of the western blots was performed using Bio-Rad Software Quantity One.

Chromatin Immunoprecipitation in a gastric carcinoma cell line
ChIP was carried out using 90–95% confluency IPA220 cells. Cells were washed with 1xPBS and DNA/protein crosslinking induced with 1% formaldehyde for 10 min at room temperature and terminated with glycine at 125mM final concentration. After being washed in 1xPBS containing protease inhibitors (Complete protease inhibitor cocktail; Sigma), the samples were sonicated in lysis buffer (50mM Tris pH 8.0, 10mM EDTA, 1% SDS). The solubilized chromatin was incubated with anti-CDX2 antibody (BioGenex) or control IgG overnight at 4ºC. Concomitantly, Protein G-Agarose beads (Roche) were blocked with 1%BSA. Immunoprecipitated chromatin was then incubated with the beads for 2h at 4ºC, washed with low salt buffer (20mM Tris pH 8.0, 150mM NaCl, 2mM EDTA, 0.1% SDS, 1% Triton X-100), high salt buffer (20mM Tris pH 8.0, 0.5M NaCl, 2mM EDTA, 0.1% SDS, 1% Triton X-100), LiCl buffer (10mM Tris pH 8.0, 0.25M LiCl, 1mM EDTA, 1% NP-40, 1% NaDOC) and TE buffer, and eluted with elution buffer (0.1M N_a2CO_3, 1%SDS). Reversion of the crosslink was performed overnight at 65ºC and DNA was recovered using GFX DNA purification columns (GE Healthcare). The DNA was analyzed by PCR using the primer pairs listed in Table 1.

Immunohistochemistry in frozen tissue sections
Frozen sections were fixed in cold methanol and hydrated, followed by immunostaining as previously described (12). The primary antibody against CDX2 (BioGenex) was diluted 1:50 in 5% BSA.

Chromatin Immunoprecipitation in mouse ileum and in human intestinal metaplasia (IM)
For mouse tissue ChIP, a wild-type mouse was sacrificed and the intestine removed and washed in cold NaCl 9‰. The distal ileum mucosa was scrapped using a glass slide and fixed in 1% formaldehyde. After this, the Magna ChIP Kit (Upstate Biotechnology, Millipore) was used according to the manufacturer's guidelines. Immunoprecipitation was performed with 2µg of mouse monoclonal anti-CDX2 antibody (Biogenex) and normal mouse IgGs (Invitrogen) were used as negative control. Extracted DNA was then analyzed by qPCR with primers covering the mouse Cdx2 proximal promoter (Table 1) and the values normalized against the IgG negative control.

The IM tissue samples were collected from the stomach of three gastrectomized patients diagnosed with gastric adenocarcinoma, operated in Hospital S. João, Porto, Portugal, and authorized by informed consent to the Tumour Bank of Hospital S. João, Porto, Portugal. The whole surgical specimens were briefly washed with water for removal of blood. For case #1, four tissue samples were collected in the proximal and distal areas adjacent to the tumour and for cases #2 and #3, two samples were collected from each. The mucosa was separated from the muscle layer, washed in cold 1x PBS with Complete protease inhibitor cocktail (Roche). The tissue segment was then cut in smaller pieces to facilitate fixation and fixed in 1% formaldehyde and fast frozen in liquid nitrogen. Without thawing, the tissue was pulverized using a Biopulverizer (Biospec Products) to facilitate posterior lysis. Following this step, the EZ Magna ChIP Kit (Upstate Biotechnology, Millipore) was used according to the manufacturer's guidelines. Immunoprecipitation was performed using the same antibody and control as above. Extracted DNA was then analyzed by qPCR with primers covering the human CDX2 proximal promoter (Table 1) and the values normalized against the IgG negative control. A second sample was collected adjacent to each of the previous ones, frozen in OCT, stained with hematoxylin and eosin to check for presence of IM.

RESULTS

CDX2 regulates its own promoter in gastric and intestinal carcinoma cell lines

To evaluate if CDX2 is able to transcriptionally activate its own promoter, the reporter plasmids pCdx2-1Luc and pCdx2-9Luc containing a 1kb and a 9.3kb promoter fragment of the murine Cdx2 gene were used for transfection and luciferase assays in a panel of gastric and intestinal carcinoma cell lines, in combination with a CDX2 expression vector or the corresponding control empty vector. The cell lines used were AGS, MKN45, GP202, KatoIII and MKN28 (gastric) and HCT116, HT-29, HCT15 and CO115 (intestinal). Cdx2 transactivates its own promoter at different levels, dependent on the cellular context. Transcriptional inductions of the 9.3kb promoter ranged from 1.4-fold in CO115 up to about 9-fold in MKN45, KatoIII and HCT116 and for the 1kb promoter they ranged from 1.4-fold in MKN28 up to about 7-fold in KatoIII and HCT116 (Figure 1). Using a series of deletion mutants, comprising 2kb up to 8kb of the Cdx2 promoter, we were able to observe CDX2 transactivation of its own promoter in all the constructs. There was no obvious pattern of increasing transactivation along increasing promoter lengths that could contribute to mapping potential CDX2 binding sites. The levels of transactivation varied among cell lines independently of basal CDX2 expression, as already found for the 1kb and 9.3kb promoters (data not shown).

Identification of active CDX2 cis-elements within the CDX2 promoter

In order to demonstrate that the CDX2 transcription factor binds directly to its own promoter, Chromatin Immunoprecipitation (ChIP) was performed in IPA220 cells, which is the cell line with the highest CDX2 expression (24). Since CDX2 was able to transactivate its own promoter already in the 1kb construct, we studied the most proximal region of the CDX2 promoter and found several putative binding sequences for CDX2, more or less conserved relatively to the consensus CDX2 binding sequence (34). Five regions within the first 1200 nucleotides were evaluated in the precipitated DNA. The results show that CDX2 protein is present in 3 of the regions – the most proximal and the two most distal ones (Figure 2A). To examine the functional role of these CDX2 binding sites, we chose two sequences, conserved between mouse and human to mutate by site-directed mutagenesis. These two sites (-954/-952 and -293/-292) were mutated independently and together in the -1kb mouse Cdx2 promoter construct. The wild-type and the three mutated Cdx2 promoter constructs were co-transfected with CDX2 in AGS cells and promoter activity evaluated by a luciferase assay. The results obtained show that each independent mutation of the putative CDX2 binding sites located within the positive binding regions identified by ChIP leads to a decreased promoter activity of about 40%, whereas the double mutant leads to complete abrogation of the promoter activation (Figure 2B).

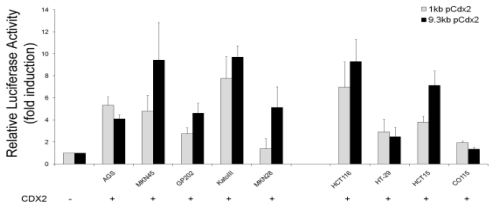


Figure 1. Regulation of a 1kb and a 9.3kb Cdx2 promoter by CDX2. Co-transfection experiments were performed in the presence of a 1kb or a 9.3kb Cdx2 promoter construct and a CDX2 expression vector in a panel of five gastric and four intestinal carcinoma cell lines. The values obtained were corrected for transfection efficiency with β-galactosidase activity and the luciferase activities obtained with transfection of the empty expression vector were referred to as 1.

Transfection of CDX2 increases the endogenous level of CDX2 in a panel of cell lines

In order to study the ability of CDX2 to regulate its own expression in the endogenous context of cell lines, the gastric AGS and MKN45 and the intestinal HCT116 cell lines were transfected with CDX2 expression vector, or the corresponding empty one, and endogenous CDX2 levels were evaluated both at the RNA and at the protein level. The endogenous and vector mRNA gene products are distinguished using specific primer pairs and corresponding proteins are discriminated by molecular weight, which differs due to the two FLAG tags encoded by the expression vector. The results show that there is a clear increase in endogenous CDX2 mRNA expression upon CDX2 transfection in MKN45 and HCT116 yet a slighter effect is detectable in AGS (Figure 3A). The same results are observed at the protein level, where endogenous CDX2 increases 2.2, 2.7 and 3.7 times in AGS; HCT116 and MKN45 cells, respectively (Figure 3B). To evaluate if this effect was cell context specific, the same transfection experiment was performed in the non-gastrointestinal HeLa cell line, which does not express CDX2 endogenously. RT-PCR results show that CDX2 transfection activates the endogenous expression of CDX2 in these cells (Figure 3A).

A dominant-negative form of CDX2 abrogates its own regulation

Aiming at confirming the positive effect of CDX2 in regulating its own endogenous expression, we set up an experimental context using a dominant negative form of CDX2 (33). This is a truncated form of the CDX2 protein, miniCDX2, which competes for the DNA binding with its wild-type counterpart but lacks the transactivation activity. When transfecting the miniCDX2 together with the wild-type form in two cell lines, MKN45 and HCT116, we were able to observe an almost complete abrogation of the inducing activity of CDX2 both on its targets MUC2 (Figure 4A) and LI-Cadherin (Figure 4B), thus confirming its dominant negative activity, and on endogenous CDX2 (Figure 4C), in accordance with the previous results.

Cdx2 is bound to its own promoter in the mouse ileum and in human intestinal metaplasia (IM)

In order to confirm the relevance of the autoregulatory mechanism *in vivo*, we performed a Chromatin Immunoprecipitation (ChIP) against Cdx2 in the mouse distal ileum, which is one of the intestinal segments with highest Cdx2 expression(32). A series of primer pairs covering about 1200 of the Cdx2 proximal promoter region was designed, together with one encompassing the Cdx2 binding site on the Sucrase-Isomaltase promoter (32) to use as positive control. The results show that Cdx2 is bound to the promoter region detected with the primer pair 5 (Figure 5A), which covers a region corresponding to the more distal positive region detected by ChIP in the human cell line. As expected, Cdx2 was also detected bound to Sucrase-Isomaltase promoter.

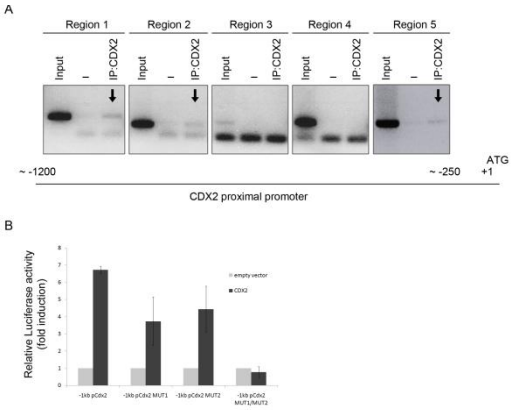


Figure 2. Active CDX2 cis-elements on the CDX2 promoter. A, ChIP assay was carried out with an isotype control immunoglobulin G (IgG) and with an anti-CDX2 antibody. PCR amplification of five regions within the proximal CDX2 promoter was performed to detect CDX2-bound DNA. A fraction of the chromatin preparation (1%) was used as input in the reaction. B, Site-directed mutagenesis was used to introduce the mutations 1 and 2, alone or together, in a construct of 1kb of the mCdx2 proximal promoter. Co-transfection experiments were then performed in the presence of the 1kb promoter construct, wild-type, single or double mutated, and a CDX2 expression vector in AGS cells. The values obtained were corrected for transfection efficiency with β-galactosidase activity and the luciferase activities obtained with transfection of the empty expression vector were referred to as 1.

We further aimed to confirm the relevance of this regulatory pathway in human IM, so we carried out a ChIP experiment in samples of IM. Since CDX2 is present in the stomach exclusively in foci of IM we performed ChIP from the whole mucosa fragment, containing both normal and metaplastic glands. As depicted in Figure 5B for case #1, we collected four mucosa samples (numbered 1 through 4) in different areas surrounding the tumor. After observation of the samples immediately adjacent to each of the previous ones, we excluded samples 1 and 2, which did not have clear foci of IM and performed ChIP in samples 3 and 4, both containing several foci of IM (Figure 5C and D). We confirmed, as previously described (12), that CDX2 protein is only present in areas of IM (Figure 5C3 and D3). The results obtained from the immunoprecipitated DNA in sample 3 show that CDX2 is bound to its promoter in two regions, one more proximal and one further upstream, separated by a region with no binding (Figure 5E). The regions that were positive in IM correspond to those found in the human cell line IPA220. The most distal regions found in human cells correspond to the region found positive in the mouse ileum. Furthermore, CDX2 is bound to the human Sucrase-Isomaltase promoter, which may be used as positive control also in IM (Figure 5E). As for sample 4, the results show roughly the same, although promoter region 3, which was negative for sample 3, shows positive binding even though at a much lower level compared to the other regions (Figure 5F).

The results for cases #2 and #3 are in line with the ones for case #1 (Supplementary material), showing an enrichment in immunoprecipitated DNA in IM samples and, as expected, negative results for the normal gastric mucosa, which is devoid of CDX2.

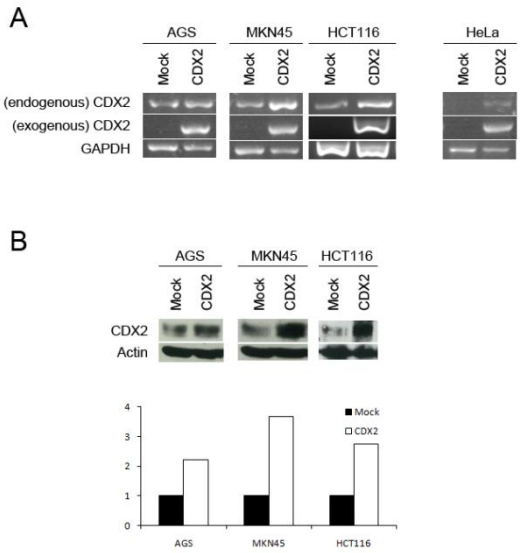


Figure 3. Effect of CDX2 transfection on endogenous CDX2 levels in gastrointestinal and non-gastrointestinal cell lines. A, Endogenous and exogenous CDX2 mRNA level in AGS, MKN45, HCT116 and HeLa cell lines upon transfection with either an empty or a CDX2-expressing vector. GAPDH was used as mRNA level control. B, Western blots showing endogenous levels of CDX2 in AGS, MKN45 and HCT116 cell lines upon transfection with either an empty or a CDX2-expressing vector. β-actin was used as loading control. The lower panel in B shows quantification of the western blots.

DISCUSSION

In the present study, we show that CDX2 is autoregulated *in vivo* and behaves as its own transcriptional target in the gastric and intestinal context. Our results confirm and extend the previously shown CDX2 transactivation of its own promoter in a pancreatic and in an intestinal cell line (29, 35). In this work, CDX2 is able to transactivate a 9.3kb Cdx2 promoter, which better mimics the endogenous promoter by covering a much more extensive regulatory region, in a panel of gastrointestinal cell lines. Nevertheless, we were able to see transactivation in the same panel of cell lines from the much shorter 1kb Cdx2 promoter, indicating that activity of CDX2 on its own promoter happens from the most proximal sequences. The levels of transactivation varied among cell lines so we cannot exclude the participation of context-specific co-factors, either activators or repressors, intervening in the autoregulatory process. CDX1, which is also autoregulated (36), was shown to depend on direct interaction with LEF1 and subsequent complex binding to LEF/TCF response elements on the CDX1 promoter for autoregulation to occur (37).

CDX2 usually binds AT-rich sequences, more or less conserved with the consensus TTTAT, which are often in close proximity to the TATA box in many CDX2 target genes. We used this criterion to define the putative CDX2 binding sites within the regions presenting CDX2 binding as assessed by ChIP, which were selected for posterior mutational analysis. Our results show that the two elected sites have some degree of redundancy in CDX2 autoregulation, since mutation of each of them alone led to only about 40% reduction in CDX2 transactivation of its own promoter. However, when mutating both sites together we were able to abrogate CDX2 positive activity on its own promoter, thus showing that CDX2 not only binds to but also directly transactivates its promoter *in vitro*.

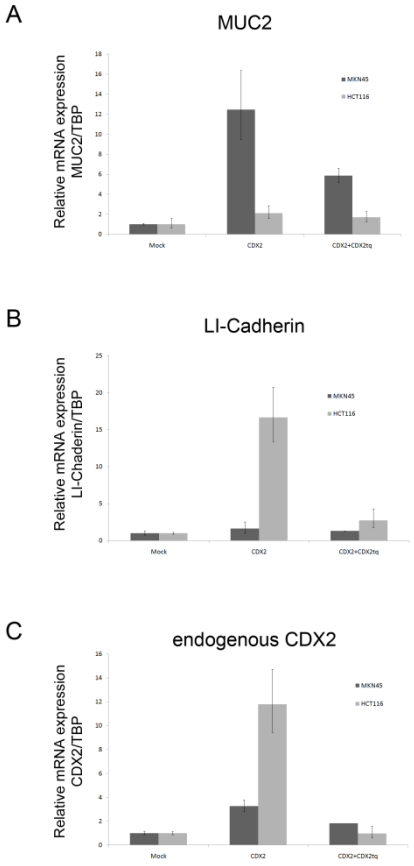


Figure 4. Effect of a dominant negative form of CDX2 on CDX2 targets and its endogenous expression. Fold increase of A, MUC2, B, LI-Cadherin and C, endogeneous CDX2 mRNA in MKN45 and HCT116 cells transfected with CDX2 alone or together with a dominant negative form, evaluated by qPCR. The values obtained with mock-transfected cells were referred to as 1. mRNA levels were normalized to the corresponding TBP mRNA level.

We further studied this autoregulation by assessing CDX2 activity on its endogenous levels. CDX2 not only increases its own endogenous expression in gastric and intestinal cell lines but it was also capable of initiating its own mRNA expression in the non-CDX2-expressing cervical HeLa cell line. Analyzing the results obtained for the gastrointestinal cell lines, it is worth noting that the lower the starting endogenous CDX2 level, the striker the effect. This is in agreement with previous reports (38) stating that cell lines cannot cope with a too high expression of CDX2, possibly due to induction of proliferation arrest and apoptosis (11). On the other hand, the observation made in the HeLa cell line showed that CDX2 is able to initiate its own expression in a non-gastrointestinal setting and without further stimulus, which suggests that the autoregulatory mechanism is sufficient to maintain its expression. The results obtained with the miniCDX2 reinforce this observation showing that CDX2 behaves like its other targets.

Furthermore, we found that Cdx2 binds its own promoter in the mouse intestine and maybe more interesting for our work, we found that it binds its own promoter in human intestinal metaplasia (IM), thus suggesting that autoregulation of CDX2

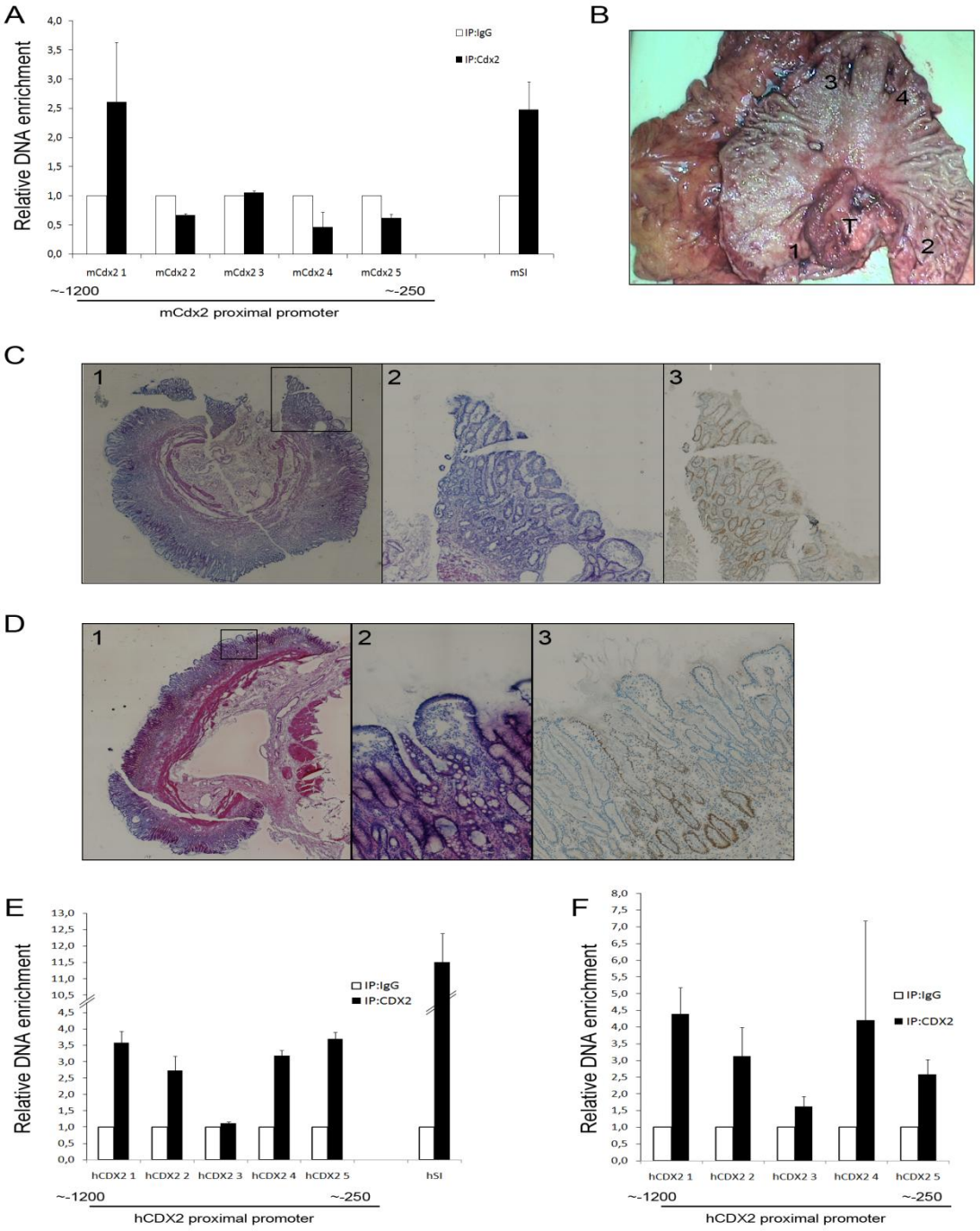


Figure 5. CDX2 binding to its own promoter in mouse ileum and human intestinal metaplasia. ChIP assay was carried out with an isotype control immunoglobulin G (IgG) and with an anti-CDX2 antibody in DNA from mouse ileum (A) and two samples of human intestinal metaplasia (E and F). Purified DNA was analyzed by qPCR using specific primers covering five regions of the proximal mouse and human CDX2 promoters. Fold enrichments are expressed as ratios of the IP:CDX2 signal to that of IP:IgG one, and calculated by extrapolation from a standard curve of Input DNA dilutions. B, Whole gastrectomized stomach. One through 4 mark the samples taken for analysis and T marks the adenocarcinoma. C and D, H&E staining and immunohystochemical detection of CDX2 in fragments 3 and 4, respectively. C1/D1, H&E staining of the whole fragments (magnification 4x); C2/D2, Foci of intestinal metaplasia (magnification 20x) and C3/D3, CDX2 immunostaining in the same areas.

happens *in vivo* and might play a role in the maintenance of this lesion.

The first suggestion that CDX2 autoregulation could be relevant *in vivo* comes from the heterozygous *Cdx2* null mice (18). While lost in the intestinal polyps, Cdx2 expression from one single allele is retained in the surrounding normal intestinal epithelium. These observations suggest that the combination of a single functional allele with lack of some external signal/co-factor might result in failure to reach a certain threshold of expression leading to complete loss of Cdx2 and polyp development, which is in line with a self-regulatory mechanism. Moreover, the extent, time and location of the defects varied among animals, putting forward the possibility of the existence of a critical threshold that has to be reached for autoregulation to take place. In this study, we show that Cdx2 is autoregulated in the intestine, which is in line with these previous observations.

It has been well demonstrated over the past years that CDX2 plays a crucial role in the intestinal phenotype determination during embryogenesis and its maintenance post-natally. Moreover, focal gains and losses of CDX2 expression induce a shift in phenotype from gastric/esophagus to intestinal and vice-versa. One well documented example is IM of the stomach which is triggered by CDX2. Gastric IM is a pre-neoplastic lesion associated with a higher risk of gastric cancer development (3-5). Despite ongoing controversy, once installed gastric IM seems to rarely regress (26-28, 39). This remains true even after the conditions favouring its appearance, such as *H. pylori* infection and the concomitant inflammation, have been treated, and is well evidenced in the studies by Asfeldt *et al*., by Wong *et al*. and our own (26-27). It is in fact impossible to follow the fate of a particular IM focus after *H. pylori* eradication and inflammation clearance, but the aforementioned studies show persistence of IM in most individuals after undergoing eradication therapy. The present study provides a putative explanation for this, based on the maintenance of CDX2 expression through an autoregulatory loop, independently of the initial trigger, thus maintaining the intestinal phenotype. It is tempting to speculate that, in addition to clearing the infection and inflammation, it is probably necessary to interfere with the CDX2 autoregulatory loop in order to revert IM.

In the setting, apparently rare, of loss of CDX2 by the metaplastic glands and their disappearance from the gastric mucosa, further studies will be needed to show if the cells transdifferentiate back to the gastric phenotype or rather are overgrown by the surrounding normal gastric glands (40). From the mechanistic and developmental point of view it would be very interesting to fully discriminate between these two processes. However, from the clinical point of view, the major interest is to be able to interfere with metaplastic maintenance and therefore interrupt the gastric carcinogenic pathway.

Altogether, the results presented in this study clearly demonstrate that CDX2 is regulating its own expression in the gastrointestinal context, and show that it is bound to its own promoter in the mouse intestine and in human IM. This regulatory mechanism may have an impact on the stability of human IM and, possibly, on inescapable progression of the gastric carcinogenesis pathway.

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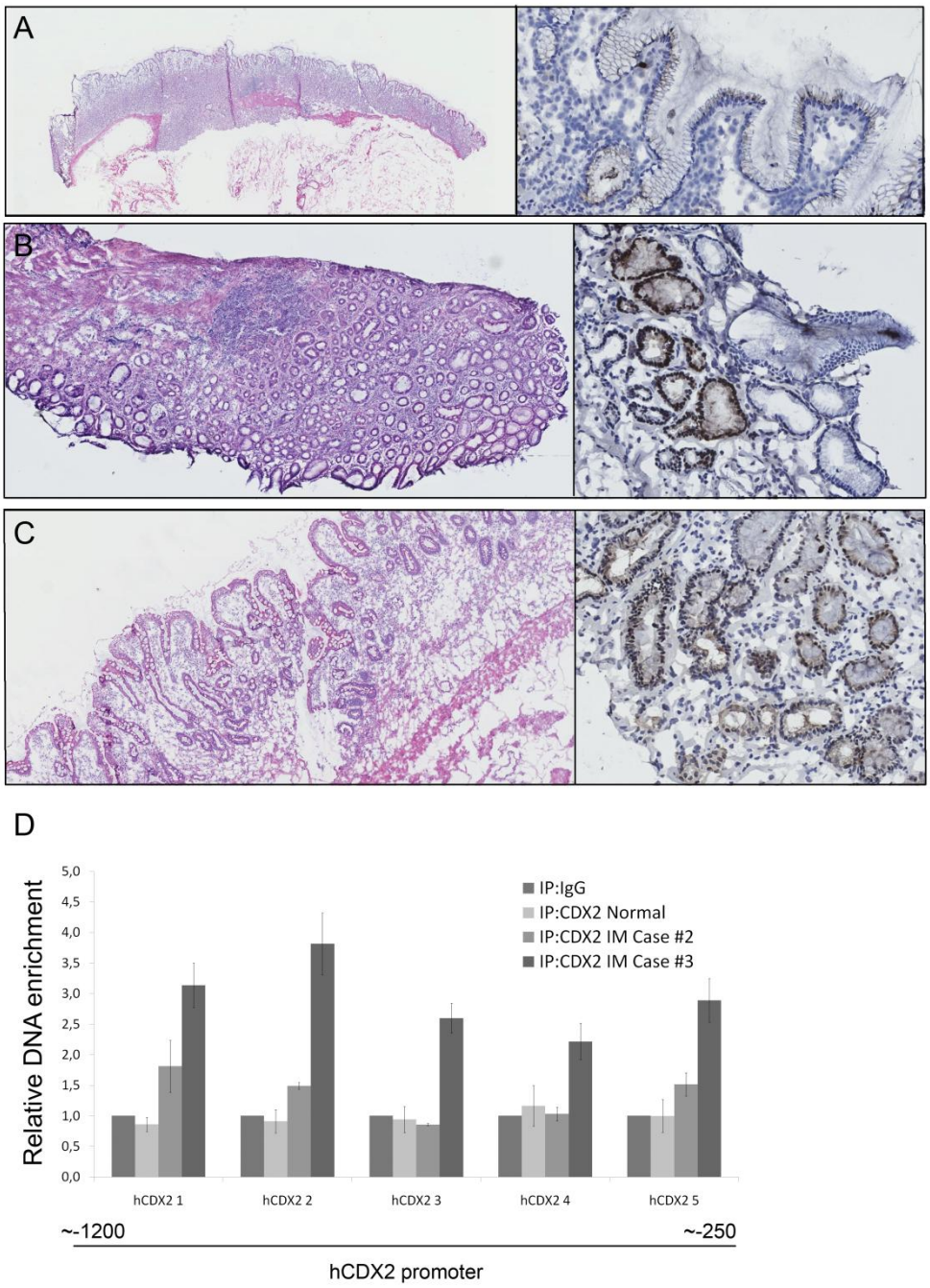
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Supplementary Figure



CDX2 binding to its own promoter in human gastric samples. H&E staining and immunohystochemical detection of CDX2 in normal gastric mucosa (A) and two samples of intestinal metaplasia from different individuals (B and C). ChIP assay was carried out as described in the Methods section and the results are presented as ratios of the IP:CDX2 signal to that of IP:IgG one, and calculated by extrapolation from a standard curve of Input DNA dilutions (D). As expected CDX2 is only observed in metaplastic glands and the normal gastric mucosa gives a negative ChIP in the whole region analysed. The two additional intestinal metaplasia cases reproduce the results presented in Figure 5.

PATHOPHYSIOLOGY OF INTESTINAL METAPLASIA OF THE STOMACH: EMPHASIS ON CDX2 REGULATION

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Pathophysiology of intestinal metaplasia of the stomach: emphasis on *CDX2* regulation

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Abstract

IM (intestinal metaplasia) of the stomach is a pre-neoplastic lesion that usually follows *Helicobacter pylori* infection and that confers increased risk for gastric cancer development. After setting the role played by CDX2 (*Caudal*-type homeobox 2) in the establishment of gastric IM, it became of foremost importance to unravel the regulatory mechanisms behind its *de novo* expression in the stomach. In the present paper, we review the basic pathology of gastric IM as well as the current knowledge on molecular pathways involved in *CDX2* regulation in the gastric context.

Metaplasia: definition and examples

Metaplasia is classically defined as a reversible change in which one epithelial or mesenchymal adult cell type is replaced by another adult cell type [1]. At least two possibilities for the cellular origin of metaplasia can be envisioned: (i) transdifferentiation of a tissue-specific differentiated cell into another cell type; or (ii) the change in commitment of a tissue-specific stem cell. This suggests a perhaps more accurate definition of metaplasia as the conversion, in postnatal life, of one cell type, differentiated or stem, into another [2].

In epithelial tissues, metaplasia follows a glandular–squamous, a squamous–glandular or a glandular–glandular pathway. A frequent example of the first type can be found in the respiratory epithelium of cigarette smokers, whereas a squamous–glandular metaplasia is most frequently observed in Barrett's oesophagus, subsequent to gastro-oesophageal reflux disease. IM (intestinal metaplasia) of the stomach is the prototype of a glandular–glandular metaplasia. Nevertheless, the metaplastic lesions are not exclusive to epithelial tissues, and mesenchymal tissues, such as bone, cartilage and adipose tissue, can be encountered ectopically in soft tissues or other organs (e.g. heart).

The present review examines several aspects of gastric IM, with an emphasis on the molecular pathways that lead to the CDX (*Caudal*-type homeobox) 2-dependent intestinalization of the gastric mucosa.

Intestinal metaplasia of human stomach

Gastric IM is a multifocal lesion characterized, morphologically, by the presence in the gastric mucosa, alone or in combination, of intestinal cell types such as goblet, Paneth and absorptive cells. The metaplastic glands show reorganization of the whole metaplastic crypt, with displacement of the proliferative zone from the neck region to the base of the crypt, and modification of the stroma that surrounds the metaplastic glands [3,4].

Characterization of gastric IM was initially based on histochemical methods that allowed the identification of three distinct types: Type I, containing absorptive cells, Paneth cells and goblet cells secreting sialomucins; Type II with goblet and columnar cells secreting sialomucins; and Type III, with goblet and columnar cells secreting sulfomucins [5].

We and others have contributed to describe the molecular alterations of IM beyond the classical morphological and histochemical characterization. We know more now about the differentiation markers that are lost and gained in the differentiation switch. On the basis of newly identified markers of gastric and intestinal differentiation, namely mucin markers, a new classification of IM emerged. The new approach defines two main IM types: complete, or intestinal IM, and incomplete, or gastrointestinal mixed IM [6–8]. Complete IM is characterized by loss of gastric mucin markers (MUC1, MUC5AC, MUC6) and gain of the intestinal mucin MUC2, whereas incomplete IM is characterized by the maintenance of gastric mucin markers, together with the intestinal mucin MUC2 [6,7]. Interestingly, mixture of the gastric and the intestinal phenotype can also occur at the cellular level. Niwa et al. [9] showed that single cells can express both gastric and intestinal mucins, such as MUC5AC and MUC2.

Common to both types of IM is the *de novo* expression of *CDX2*, a transcription factor that, in normal conditions, is expressed exclusively in the intestine, but is present in every focus of aberrant intestinal differentiation occurring in the human body. It is thus considered the master gene for intestinal differentiation and it is reviewed in detail below.

Key words: bone morphogenetic protein (BMP), *CDX2*, *Helicobacter pylori*, intestinal metaplasia, mucin, stomach.

Abbreviations used: BMP, bone morphogenetic protein; CDX, *Caudal*-type homeobox; IL, interleukin; IM, intestinal metaplasia; SOX2, SRY (sex-determining region Y) box 2.

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Pathophysiology of gastric IM

The pathophysiology of metaplasia in general, and gastric IM in particular, involves diverse aggression mechanisms, but it is generally perceived that the common ground to all metaplasia types is chronic inflammation. Several epidemiological studies, pioneered by Pelayo Correa [10], show that IM occurs in gastric carcinogenesis pathway following *Helicobacter pylori* infection, which induces a chronic gastritis with atrophy and IM. Epidemiological data are supported by Mongolian gerbil models, where IM is also induced by *H. pylori* infection [11–13] and follows the same cascade of events. Mouse models vary considerably in the susceptibility to infection by different *Helicobacter* strains. Those that are susceptible do not develop the classical Cdx2-dependent IM seen in humans, but rather a different type of metaplasia characterized by the presence of antral morphology in the corpus, with expression of spasmolytic polypeptide, a trefoil peptide expressed in normal intestinal mucosa. From this observation was coined the name SPEM (spasmolytic polypeptide-expressing metaplasia) which, nevertheless, progresses from atrophy and evolves to dysplasia and cancer, closely recapitulating the human disease [14,15].

IM of the stomach is generally considered to be a precancerous lesion, and the association with *H. pylori* infection is indisputable. However, IM develops in a small proportion of infected human subjects. Only 30% of infected individuals will develop IM and, among these, only approx. 7% will develop gastric cancer [16]. These values are particularly important in countries where infection with *H. pylori* is still very high, such as Portugal [17] and South American countries [18], with approx. 80% of the population infected. Classification of IM in subtypes is particularly relevant because different risk impacts are attributed to each subtype, with incomplete IM being more associated with development of gastric carcinoma [19].

IM is the outcome of an adaptive response to an adverse environment that affects some, but not all, individuals in similar contexts. Evidence suggests that interactions between individual genetic variations, for instance IL (interleukin)-1β polymorphisms [20], *H. pylori* virulence factors, such as *cagA*$^+$ strains [21,22], and exposure to other environmental carcinogens, are determinant for single individual susceptibility to IM.

H. pylori is probably not the only risk factor for IM development, but the results obtained this far on other environmental risk factors are still conflicting. However, the overall epidemiological information gathered shows that smoking plays a major deleterious role [17], and salty foods are probably harmful [23,24]. On the other hand, studies regarding the protective effect of dietary antioxidants and anti-inflammatory nutrients remain inconclusive [25].

Role of *CDX* genes in the development of IM

Intestinal differentiation is strongly dependent on the expression of the homeobox gene of the ParaHox cluster *CDX2*. This is evident from *in vitro* studies with undifferentiated intestinal cell lines where *de novo* expression of *CDX2* induces intestinal differentiation [26–28]. Furthermore, *Cdx2*$^{+/-}$ heterozygous mice develop non-cancerous polyp-like heteroplasias in the intestine, characterized by a total loss of *Cdx2* expression, that arise early during embryonic development [29] and correspond to an anterior homoeotic shift with appearance of gastric and oesophageal mucosa in the intestine, as well as an anterior homoeotic shift in the vertebrae [30,31]. Conversely, mouse models demonstrated that forced expression of *Cdx2* is also involved in intestinal differentiation of the gastric mucosa. Indeed, transgenic mice with ectopic expression of *Cdx2* in the stomach, either in embryonic development or postnatally, develop gastric IM [32,33], which means that CDX2 is responsible not only for the normal intestinal differentiation, but also for intestinal differentiation in aberrant locations. Several studies have shown that *CDX2* is expressed in human intestinal metaplastic lesions of the stomach [34–38], oesophagus [39–41], liver [42] and gall bladder [43]. Nonetheless, another gene has also been implicated in intestinal differentiation, which is the *CDX2* homologue *CDX1*. The role of CDX1 in intestinal differentiation is not as well defined as that of CDX2 [44,45]. *Cdx1*-knockout mice, in contrast with the *Cdx2*-knockout mouse, and the *Cdx1*-overexpressing mice in the gut are viable and do not exhibit any intestinal malformation [46,47]. *Cdx1* expression is turned on after *Cdx2* in the developing gut endoderm and is absent from the foregut-type heteroplasia resulting from loss of *Cdx2* [48], indicating that *Cdx2* expression is before and is required for *Cdx1* expression. However, *CDX1* is also expressed in intestinal metaplastic lesions of several locations such as stomach [38], oesophagus [49] and liver [50], and a transgenic mouse with *Cdx1* expression in the stomach also develops IM [51].

IM directed both by CDX1 and CDX2 exhibits not only a morphological transformation, but also a functional transformation. They act as transcription factors for a large number of intestinal genes that play major roles in intestinal function, such as those encoding sucrase-isomaltase [52], lactase–phlorizin hydrolase [53] and MUC2 [54]. Moreover, ectopic *Cdx2* expression in the gastric mucosa not only drives the intestine-type differentiation of the epithelium, but also has an impact on the underlying mesenchymal sheet [3].

Regulation of *CDX2* expression in gastric IM

The evidence showing that *CDX2* is a master gene involved in intestinal metaplasia of the gastric mucosa is very convincing. The question that is now being addressed is how it becomes ectopically expressed in gastric mucosa. Considering the context where this occurs, several mechanisms can be envisioned: *CDX2* can be positively regulated by transcription factors whose expression is triggered by molecular pathways that become activated in the complex milieu where IM arises, namely the inflammatory milieu; *CDX2* can be activated directly by *H. pylori* proteins; and/or repression

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of *CDX2* by gastric transcription factors can be released in the molecular context that precedes IM. There is evidence supporting all of these mechanisms, suggesting that *CDX2* regulation is rather complex and most probably involves interaction of several signalling pathways.

BMPs (bone morphogenetic proteins)

We and others [55,56] observed that BMPs, specifically BMP2 and BMP4, become abundantly expressed in the gastric mucosa infected with *H. pylori*, either in inflammatory cells [56] or in epithelial cells [55]. The pathway is active in epithelial cells as demonstrated by the expression of key elements of the BMP pathway: BMP receptors, SMAD4 and the phosphorylated form of SMAD1/5/8. In addition, we demonstrated, in a gastric carcinoma cell line, that BMP2/4, through its canonical signalling transducer SMAD4, up-regulates *CDX2* expression [55]. In accordance, in an *in vitro* model of Barrett's oesophagus [57], oesophageal cells exposed to BMP4 begin to express columnar cytokeratins, not normally found in squamous epithelium. However, in the Barrett's oesophagus model, *CDX2* expression was not assessed.

The precise function of the BMP pathway in the adult intestine is not fully understood, but growing evidence shows that the BMP pathway plays an important role in the maintenance of the intestinal proliferative and differentiated compartments in *Xenopus* [58], as well as in mammalian models [59]. Furthermore, there is a distinct pattern of expression of BMP pathway elements and inhibitors along the crypt–villus axis [60]. Mutations in members of this pathway have been associated with diseases such as juvenile polyposis syndrome [61,62], which is an hamartomatous syndrome characterized by the appearance of distorted crypts with excessive proliferation [63], but also focal loss of intestinal differentiation, and appearance of gastric markers [64]. Ultimately, complete loss of expression of the BMP pathway in the intestine could lead to gastric metaplasia [64]. Recently, two mouse models of BMP pathway inhibition in the intestine were developed: mice with ablated *Bmpr1a* in the intestine showed a mild proliferative defect, and an obvious differentiation defect of the secretory cell lineage with loss of goblet, Paneth and enteroendocrine cell maturation [65]; mice with overexpression of the BMP inhibitor Noggin in the intestine presented numerous ectopic crypts and altered proliferation together with polyp-like growth [59,66]. Altogether, this demonstrates that an intact BMP signalling in the intestinal epithelium is important for the balanced production of epithelial cell lineages, as well as maintenance of the proliferative/differentiation homoeostasis. Furthermore, the BMP pathway has been shown to cross-talk with other active signalling pathways in the intestine, such as the Wnt [67] and the Hedgehog [68] pathways.

H. pylori and SOX2 [SRY (sex-determining region Y) box 2]

Evidence exists that *H. pylori* is involved in the aberrant expression of *CDX2* in the stomach, but very little is known about the molecular mechanisms involved in this process. Several studies have demonstrated that *H. pylori* infection alone [69] or in combination with GORD (gastro-oesophageal reflux disease) [70] leads to expression of *CDX2* in the gastric mucosa, mainly in areas of IM, but also in foci of non-metaplastic cells. We have shown *in vitro* that gastric cell lines co-cultured with *H. pylori* have an increase in *CDX2* expression, suggesting that the direct interaction of the bacteria with epithelial cells is able to induce *CDX2* expression [71]. In the same model, there was an increase in *BMP2* expression [72]. Recently, Asonuma et al. [73] showed that *H. pylori* inhibits IL-4-mediated *SOX2* induction, in a co-culture model similar to the previous one [73]. They also show, as other groups previously had [74], that *SOX2* and *CDX2* are inversely expressed in gastric mucosa and intestinal metaplasia. Finally, *SOX2* is also suggested as a *CDX2* repressor, since down-regulating its levels led to up-regulation of *CDX2* expression [73]. Accordingly, a study characterizing the *Cdx2* promoter, revealed interactions of multiple transcription factors, and *Sox2* appeared to be a repressor, impairing the positive regulation exerted by other transcription factors, whereas HNF4α (hepatic nuclear factor 4α), β-catenin, Tcf4 (T-cell factor 4) and, to a lesser extent, GATA6, all stimulated promoter activity of a Cdx2–luciferase construct in co-transfection experiments in a colonic cell line [75]. SOX2 is crucial for foregut formation during embryogenesis and it has also been suggested as a transcription factor involved in gastric differentiation [76]. However, the role of SOX2 in gastric differentiation remains to be better clarified.

Other regulatory mechanisms

Other CDX2 regulatory mechanisms have been studied in different models, such as intestine and Barrett's oesophagus, but not demonstrated to be relevant in gastric cells. In pancreatic and intestinal cell lines, *OCT-1* was implicated in *CDX2* promoter activation [77], and an autoregulation of *CDX2* was proposed [78] in an intestinal cell line. The former hypothesis was tested in the gastric model, showing that, in fact, *OCT-1* is overexpressed, together with *CDX2*, in intestinal metaplastic lesions, but it is unable to regulate its expression at the promoter level [79]. The putative *CDX2* autoregulation remains to be clarified in the gastric context. However, this regulatory pathway deserves further investigation, since if *CDX2* is in fact able to regulate its own expression in IM, this might contribute to the stability of the phenotype and impair reversion of this gastric cancer precursor lesion.

It has been described that acid and bile acids could activate CDX2 in oesophageal epithelial cells through promoter demethylation, leading to Barrett's oesophagus [80]; however, in gastric cancer cell lines, in normal gastric mucosa and in IM foci, we could not detect a functional relation between the methylation status of the proximal *CDX2* promoter and *CDX2* expression [81], suggesting a tissue-specific role for *CDX2* promoter methylation.

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Concluding remarks

Gastric IM is a tissue phenotype switch initiated by *H. pylori* infection that, in susceptible individuals subjected to particularly aggressive *H. pylori* strains and lifestyle exposures, will trigger *CDX2* expression. This process involves inhibition of gastric transcription factors, with SOX2 being a good candidate, and activation of CDX2 transcription factors, with the BMP pathway playing a central stimulatory role. It is known that, upon *CDX2* expression, a whole set of intestinal genes is up-regulated with the appearance of an intestinal phenotype. The progress in identification of molecular pathways involved in CDX2 regulation has been enormous, but is still limited by the lack of an *in vitro* model of gastric IM and by the lack of a mouse model that recapitulates the CDX2-dependent metaplasia upon *H. pylori* infection. Animal models such as the Mongolian gerbil model, which reproduces the human disease, deserves further study to validate and extend observations with gastric cell lines.

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GENERAL DISCUSSION
SUMMARY AND CONCLUSIONS

8

General Discussion

Gastric cancer is paradigmatic in being one of the few human cancers where the single lesion preceding neoplastic transformation is an aberrant differentiation – intestinal metaplasia – rather than an adenomatous growth, as is the case, for instance, in colorectal cancer. Metaplasia appears in the setting of chronic trauma and consistent regeneration and acquires clinical significance because it predisposes for cancer development. Hence metaplasias are in these cases crucial steps in the multistep progression towards cancer.

Though declining over the past decades, gastric cancer remains a serious health burden worldwide. Despite incidence variation across geographical areas [1], with foci of high incidence in the Latin America, Eastern Asia and Portugal [2], gastric cancer remains the fourth most common cancer and ranks second in cancer-related death worldwide [3]. Gastric cancer survival is globally low and has registered only moderate improvements during the last decades [4-5]. Altogether this shows that much may still be done in this field, and more effective preventive and/or therapeutic strategies will likely rely on a deeper understanding of the molecular events of the carcinogenic cascade, aiming to find putative targets for intervention. Histopathologically, the gastric carcinogenic pathway has now been established and well accepted for almost two decades. In 1992, Pelayo Correa proposed a model consisting of a series of sequential lesions of the gastric mucosa induced by the interplay of environmental aggressions, among which *Helicobacter pylori* infection plays a pivotal role, and individual genetic make-up. These lesions include chronic gastritis, atrophy and intestinal metaplasia, all associated at increasing levels with gastric carcinoma development. The molecular trigger for intestinal differentiation in the gastric context is expression of CDX2, which is common to all intestinal metaplasias [6-10], regardless of the subtype. However, downstream events leading to a complete conversion into an intestinal phenotype (complete or intestinal type) or to retaining of gastric markers together with the new intestinal ones (incomplete of gastrointestinal mixed type) remain to be investigated. Furthermore, the cancer risks conferred by the different subtypes differ [11], raising another interesting question at the molecular standpoint. This issue, though very appealing, falls out of the scope of this work, where we sought for molecular events upstream of CDX2 expression.

After several observational epidemiological studies having ascertained the increased risk for cancer development conferred by intestinal metaplasia [12-13], it became of foremost importance to further study this lesion under two perspectives: first, to understand how intestinal metaplasia arises in the context of the *H. pylori*-infected and inflamed stomach, which may aid in the prevention of the pre-neoplastic lesion appearance in individuals infected with the bacteria; second, to disclose the putative (ir)reversibility of intestinal metaplasia, which would provide insight for prevention of cancer development in subjects already bearing this lesion. In this work, we tackled both issues by studying signalling pathways that could be involved in the establishment and/or maintenance of the aberrant intestinal phenotype in the stomach following *H. pylori* infection. We focused on the pathways involved in regulation of aberrant CDX2 expression and approached the potential of reversibility of the intestinal metaplasia lesion, namely exploring the putative autoregulation of the *CDX2* gene. The two parts of this work will be discussed separately and all the results will then be joined together in a model for gastric ectopic CDX2 expression and subsequent stabilization of intestinal differentiation.

● Part I > BMP pathway and CDX2 – together towards intestinal differentiation

Intestinal metaplasia is a well acknowledged pre-neoplastic lesion of the stomach, conferring increased risk for cancer development and dependent on ectopic expression of the master gene for intestinal differentiation *CDX2*. However, the molecular mechanisms driving *de novo* CDX2 expression in the stomach, and subsequent intestinal differentiation, remain largely elusive. Two major types of pathways may be envisioned to play a part in this process – signalling pathways absent from the normal stomach, but involved in intestinal development and differentiation, that become active in the stomach under specific circumstances and, conversely, pathways involved in the establishment and maintenance of the gastric phenotype which become inactivated. It might thus be expected that CDX2 is subjected to both negative and positive

regulation in the gastric context, and recent data from our work and others' favour this hypothesis. Hence, it is hypothesized that *H. pylori* infection together with the consequent cascade of events in the normal mucosa would release the negative regulation forces and imbalance the equilibrium towards a positive CDX2 regulation. Moreover, since inflammation is one of the most striking infection-induced mucosal injuries, it is probable that abnormal signalling from these newly arrived infiltrating cells play an important, if not determinant, role in instigating the subsequent cellular events. Concordant with this is the presence of intestinal metaplasia in patients with autoimmune gastritis, regardless of their infectious status [14].

From the developmental point of view, the appearance of an aberrant intestinal phenotype in more anterior organs, such as the stomach or the oesophagus, corresponds to a posterior homeotic shift. It is associated to an aberrant expression of the intestinal master gene *CDX2*, which has been shown to be important both for the embryonic specification of the intestine and later maintenance of this phenotype in adults [15-16]. It is legitimate to suppose that, at least to some extent, the molecular mechanisms responsible for intestinal differentiation will be common to intestine itself and metaplastic foci. Accordingly, we started looking for pathways which are active in the intestinal differentiated compartment and important for its maintenance. One that filled these requisites was the Bone Morphogenetic Protein (BMP) pathway. The BMP pathway was shown to signal from the mesenchyme to the villi and to be maintained inactive in the proliferative compartment by the presence, in the mesenchyme surrounding the crypts, of a soluble inhibitor of the extracellular growth factors BMP2 and BMP4, Noggin [17]. In addition, mice with impaired BMP pathway in the intestine, either by homozygous deletion of the BMP receptor *Bmpr1a* [18-19] or by overexpression of the BMP inhibitor Noggin [20-21], show an overproliferation of the crypt compartment and loss of the differentiated lineages. Lastly, and as a first possible link with CDX2, both *Smad4*$^{+/-}$ mice [22], lacking this BMP intracellular transducer, and patients with Juvenile Polyposis Syndrome, a human condition characterized by germline mutations in elements of the BMP pathway [23], bear intestinal polyp-like lesions, resembling to some extent those of the *Cdx2* heterozygous null mice [24-25]. We thus hypothesized that the BMP pathway might be responsible for CDX2 regulation. Further, we extended this by hypothesizing that the role of the BMP pathway in intestinal differentiation, both in normal and aberrant locations, might rely, at least in part, on this positive effect on the expression of the intestinal master gene

CDX2. We used two human models of disease to test this hypothesis – the gastric intestinal metaplasia, which is a model of ectopic intestinal differentiation and our primary object of study, and the Juvenile Polyposis Syndrome, a gastrointestinal disease associated to loss of BMP pathway activity.

Intestinal Metaplasia and Juvenile polyps – inverse differentiation models?

We analysed the expression of several elements of the BMP pathway together with that of CDX2 in a series of human gastric intestinal metaplasias (Paper I). The results obtained show an overexpression of elements of this pathway in the intestinal metaplasia foci, thus co-localizing with CDX2, which is exclusively positive in the lesion. Furthermore, we were able to show that both in intestinal metaplasia and in gastric carcinoma cases the expression of CDX2 strictly correlated to that of pSMAD1/5/8, the hallmark of an active BMP pathway. The Juvenile polyps analysed (Paper II), besides presenting all classically described characteristics [23] showed foci of distinct morphology, with scarce goblet cells and predominance of columnar cells and regenerative features. SMAD4, as well as the intestinal markers CDX2 and MUC2, displayed a heterogeneous "patchy" pattern of expression across the polyp. In addition, *de novo* expression of the gastric-type mucin MUC5AC was detected and appeared predominantly in the areas of "distinct morphologic features" described above. Remarkably, MUC5AC expression was coincident with decreased SMAD4, CDX2 and MUC2 expression. This held for both sporadic cases and a familial case with a known *SMAD4* germline mutation. The other familial case, harbouring a mutation in the *BMPR1A*, had only hyperplastic features with no evidence of metaplastic foci, and will be further discussed below. Together, these results provided the first suggestion that the BMP pathway is important for CDX2 regulation. In two opposite human lesions from the differentiation standpoint (Figure 1), CDX2 appeared expressed in areas of an active BMP pathway and downregulated upon loss of expression of a critical element for pathway function. In both cases, like in mice models [24, 26-27], CDX2 expression was the hallmark of intestinal differentiation. Its expression related to an intestinal phenotype both morphologically and as assessed by expression of the intestinal mucin marker MUC2, and CDX2 absence/loss associated to loss of intestinal phenotype and appearance of a gastric phenotype, here characterized by the expression of gastric-specific MUC5AC.

Interestingly, the pathway we here identified involves signalling from non-epithelial cells, as main sources of BMPs, therefore supporting the idea that the initial trigger for aberrant CDX2 expression in the stomach comes from the inflammatory infiltrate. Also in line with this hypothesis, Bleuming and co-workers showed an influx of BMP2- and BMP4-producing inflammatory cells upon *H. pylori* infection of the gastric mucosa [28]. However, and although this is a generally accepted model of signalling for this pathway, we also showed an overexpression of BMP2/4 together with their receptors in the intestinal metaplasia cells. This suggests the existence of an autocrine regulatory mechanism, which had also previously been reported in the colonic context [29], where the cells signal back to themselves upholding a persistently active pathway.

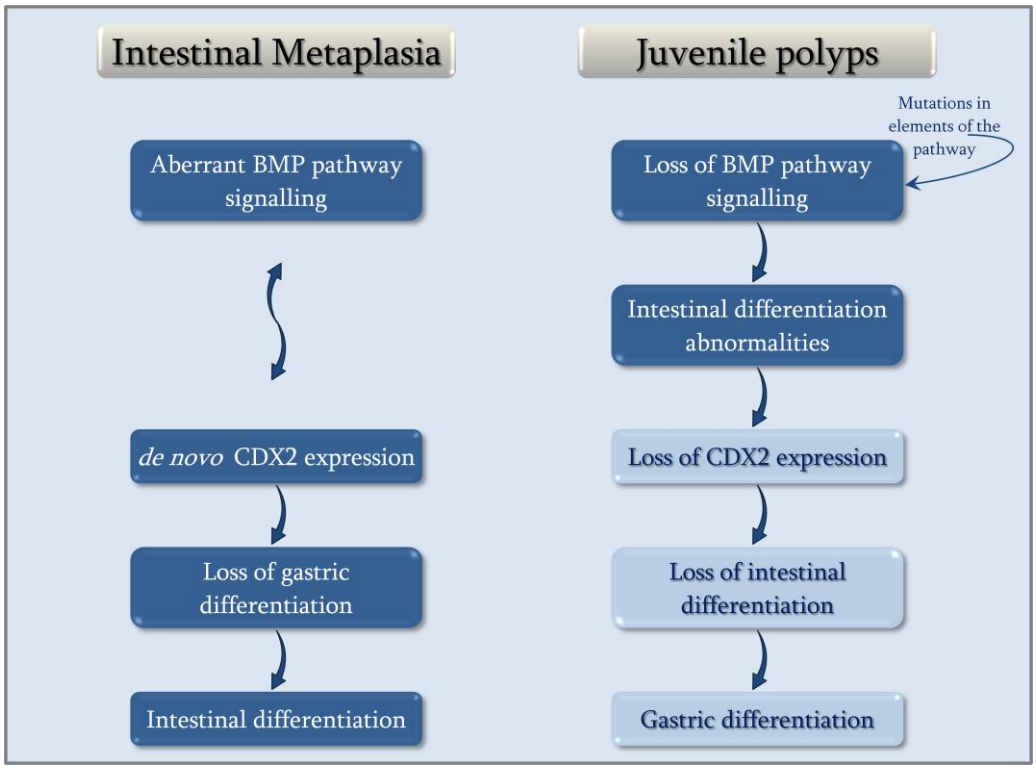


Figure 1 > Intestinal metaplasia and Juvenile polyps as inverse models of differentiation.

CDX2 regulation by the BMP pathway

Aiming to prove that the BMP pathway was in fact regulating CDX2 expression we used an *in vitro* approach with a gastric cell line, AGS, which has an active BMP pathway. We assessed the intact pathway in this cell line by two means. First, we confirmed the expression of all the key elements necessary to transduce the signal from the extracellular binding of BMP ligands to their receptors until nuclear intracellular mediators. Second, upon BMP treatment, this cell line responded with up-regulation of the canonical BMP targets Inhibitor of differentiation (Id) 1 and 2 [30]. Using this cell line we were able to show that treatment with both BMP2 and BMP4 up-regulated CDX2 expression with intervention of the intracellular transducer SMAD4, whose knock-down impaired the effect on CDX2. Furthermore, we demonstrated that the effect of the BMP pathway extends to intestinal differentiation markers like LI-Cadherin and MUC2, with BMP treatment up-regulating them and SMAD4 knock-down decreasing their expression. Of note, both these proteins also appear ectopically expressed in intestinal metaplasia [31-32].

Several regulatory mechanisms have been proposed for CDX2, but in the gastric context they are yet largely unknown. *H. pylori* was suggested to have a positive regulatory effect on CDX2 expression [33], by yet unidentified molecular mechanisms. Conversely, a negative regulation of CDX2 by SOX2 was also proposed [34]. Moreover, this CDX2 inhibitor appears to be downregulated in the *H. pylori*-infected stomach and further in the progression to intestinal metaplasia [35]. Epigenetic modifications of the CDX2 promoter have also been suggested to play a role in CDX2 regulation during progression to Barrett's oesophagus [36]. However, although shown to differ between gastric tumours and normal mucosa [37], CDX2 promoter methylation failed to associate to CDX2 expression in the gastric context [38]. The BMP pathway, like CDX2, has been consistently associated with normal and ectopic intestinal differentiation. BMP2 and BMP4 have been implicated in the differential maintenance of the proliferative and differentiated compartments in the intestine [17-18], and BMP4 in intestinal transformation of normal oesophageal cells [39]. The evidence for recruitment of BMP-producing cells induced by *H. pylori* links together both lines of evidence. Altogether, we provided a major contribution for the identification of the BMP/SMAD pathway as a mechanism regulating CDX2, adding a step further in the understanding of the signalling networks present in the infected and injured stomach. Furthermore,

our results show that, at least to some extent, the positive effect of the BMP pathway in the establishment/maintenance of an intestinal phenotype, in normal or aberrant locations, might be due to positive regulation of CDX2 expression. Our hypothesis is that upon *H. pylori* infection there is an aberrant activation of the BMP pathway in the stomach, which contributes to CDX2 expression. Concomitantly, the infection induces downregulation of the CDX2 repressor SOX2, thus releasing, at least to some extent, the negative regulatory mechanisms to which CDX2 might be subjected in the gastric context (unpublished results from our group). The fact that the CDX2 promoter is not structurally altered by methylation in the stomach favours the possibility that its expression is being regulated solely by the (in)balance of these, and possibly other, molecular pathways.

Further into BMP pathway function and significance in the gastrointestinal context

It is worth mentioning that in the series of intestinal metaplasia cases that we studied, BMP pathway elements were overexpressed in the lesion, probably contributing to its establishment and/or maintenance, but were present in the normal mucosa as well (Paper I). Accordingly, a number of studies show that, to some extent, a basal activity of this pathway in the stomach is important for gastric development and homeostasis. Firstly, BMPs proved to be needed for stomach gland formation in chicken embryos [40]. Further, *in vitro* studies have implicated BMP2/4 in the modulation of gastric cells proliferation [41] and in chief [41] and parietal [42] cell differentiation. Gastric activity of the BMP pathway was also suggested to be required to suppress hyperplasia and tumour formation in mouse models [43-44] and BMP2 expression was suppressed by promoter methylation in gastric cancers [45]. Together with our results, this is in favour of an aberrant and imbalanced rather than a *de novo* and ectopic activation of the BMP pathway in the stomach, contrary to what happens, for instance, with CDX2. In addition, and drawing attention to the importance of multiple signalling in this context, gastric inhibition of the BMP pathway through misexpression of its antagonist Noggin did not result in any gastric phenotype, but together with induction of the Prostaglandin E2 pathway it led to the development of gastric hamartomas [46].

Looking further into the results obtained in our work, other findings might shed light on the better understanding of the BMP pathway function and importance in the

gastric and intestinal contexts. CDX2 has been implicated in expression regulation of a series of intestinal-specific genes, many of them aberrantly expressed in intestinal metaplasia as well (see introduction for details). We thus decided to study some of them in the setting of BMP-treated gastric cells (Paper I). Interestingly, treatment-induced overexpression of CDX2 resulted in increased levels of MUC2 and LI-Cadherin, but had only a marginal or no effect on Villin and Sucrase-Isomaltase expression, respectively. Although tempting, we cannot extrapolate from this to an involvement of the BMP pathway, through CDX2, on goblet but not absorptive cell differentiation, since it is not possible to rule out the importance of other pathways and regulatory elements on CDX2-regulated enterocytic differentiation [47-48]. However, *Bmpr1a* conditional ablation studies report that in fact the BMP pathway affects terminal differentiation of the intestinal secretory lineage but not of absorptive enterocytes [18].

The study of the Juvenile polyps put forward a second line of BMP pathway regulation relying on redundancy of some elements and a dose-dependent modulation. Contrary to what was found for both solitary Juvenile polyps and the Juvenile Polyposis case with a *SMAD4* germline mutation, the one harbouring a *BMPR1A* mutation had only hyperplasic but no metaplastic features (Paper II). The expression of the gastric MUC5AC was only vestigial and with no obvious dependence on loss of BMP pathway (as assessed by SMAD4 expression) or CDX2. This suggests a less effective impairment of the pathway in this case, which might be attributed to at least two mechanisms. On the one hand, the mutation in this gene maps to its regulatory region, likely resulting in a reduction but not a total depletion of the corresponding protein. On the other hand, the hypothesis of other BMP receptors being active and taking over its function cannot be excluded [49]. Interestingly, even in the case with the monoallelic *SMAD4* mutation, the expression of its gene product, as that of CDX2, is very heterogeneous within the polyp only with areas of focal loss. This suggests a complex regulation of CDX2 expression, with involvement of cross-talk between several active pathways in the intestine, and also some dose-dependent modulation of the BMP pathway activity. Taken together, the data presented here favours that in the case of Juvenile polyps, complete BMP pathway loss in epithelial cells is not required for intestinal hyperplasia and polyp growth, but seems to be a requisite for reduction of CDX2 expression and subsequent/concomitant differentiation abnormalities. This suggests that the BMP pathway is exerting two lines of homeostasis control in this context – a CDX2-

independent effect on cell proliferation and a CDX2-dependent one on cellular differentiation.

● Part II > Intestinal metaplasia reversibility and CDX2 autoregulation

For adoption of therapeutic strategies and prognostic evaluation of patients with pre-neoplastic lesions towards cancer prevention, several main issues are to be considered when trying to base decision making on biological evidences. One concerns understanding of the extracellular factors and molecular events that take part in the progression towards neoplastic transformation – mutation-inducing agents, microenvironmental factors, genetic alterations, gene silencing, among others. It is also still not possible from this point of view to fully understand epidemiological data showing that individuals with incomplete intestinal metaplasia are at increased risk as compared to those with only metaplasia of the complete type. However, although not proven, it is conceivable that incomplete metaplasia, in not recapitulating any normal differentiation program of the human body, might be more unstable and more prone to acquire genetic alterations. In fact, it was shown that many reported structural genetic alterations in intestinal metaplasia, such as gene mutation and deletions, aneuploidy and microsatellite instability, have a high predominance [50], when not exclusivity [51], in the incomplete type. The other question remaining unclarified until now concerns the development of complete or incomplete intestinal metaplasia from the common ground of *H. pylori* infection and ectopic CDX2 expression in the stomach. It is not known if, or to what extent, genetic susceptibility and/or differential (micro)environmental exposures contribute to the development of one type or the other. Also yet to be unveiled is if the two types of intestinal metaplasia are "fixed", result of divergent differentiation processes, or "interchangeable". However, these issues are beyond the scope of this work. The other major issue in this setting is the ability of intestinal metaplasia to revert, with a high impact on *H. pylori* eradication strategies.

Helicobacter pylori eradication and intestinal metaplasia reversibility

In the gastric carcinogenic pathway, *H. pylori* infection is accepted as the initial trigger for subsequent mucosal damages and transformations. In this line of thought, the important issue that should be taken into account concerns the putative reversibility of the pre-neoplastic lesions, namely intestinal metaplasia, either spontaneously or by clearance of the initial trigger for their development. This acquires special relevance in countries with high incidence of both *H. pylori* infection and consequent lesions, where it would be a major achievement to be able to block the carcinogenic pathway at this late step, impairing it from reaching the adenocarcinoma stage. Epidemiological studies have documented a decrease in cancer risk upon *H. pylori* eradication in individuals with intestinal metaplasia. However, conflicting results have been reported concerning actual regression of the lesions in this setting. Also in the Mongolian gerbil model there is discrepancy on the beneficial effect of *H. pylori* eradication, with one study describing marked reduction in pre-neoplastic lesions [52] and another stating that early but not late eradication induces mucosal improvement [53]. In a mouse model of *H. felis* infection, eradication reduced SPEM, a metaplastic lesion considerably different from the classical human intestinal metaplasia [54]. On the other hand, it is well accepted that the genotype of the infecting bacteria plays a role in the gastric histological outcome, with high virulence strains having a more deleterious effect, both in terms of inflammatory response and of pre-neoplastic lesion development [55-57]. We thus designed a study dealing with both types of evidence where we assessed reversibility of intestinal metaplasia upon eradication in relation to virulence of baseline infecting *H. pylori* strain (Paper III). The results obtained show an overall low regression of intestinal metaplasia, which was significantly less frequent in individuals infected with high virulence strains as compared to those with low virulence ones. Despite the small sample size which limits confident extrapolations, eventual effects of sampling errors during biopsy collection, and lack of information on the number of years of infection persistence prior to analysis, which are all common potential pitfalls of this type of studies, it provided the first observational association between *H. pylori* virulence and intestinal metaplasia regression.

Significance and consequences of *Helicobacter pylori*-related sporadic gastric CDX2 expression

Controversy also still exists regarding the actual cellular origin of intestinal metaplasia glands. Regardless of the mechanisms suggested to play a part in the differentiation switch process, most studies are based on the hypothesis that tissue stem cells are the ones reprogrammed and thus the initiator cells. However, some authors claim that metaplasia arises from a true transdifferentiation process, involving redifferentiation of dedifferentiated gastric epithelial cells [58] and one study suggested the participation of bone marrow cells in the repopulation of damaged stomach and as initiators in the gastric carcinogenic cascade, including SPEM [59]. The results we obtained favour the hypothesis involving tissue-specific stem cells. Ectopic CDX2 expression in the stomach was shown to be necessary and sufficient to drive intestinal metaplastic transformation of the gastric mucosa. Not only is this transcription factor present in virtually all human cases of ectopic intestinal differentiation [6-7, 60-63] but also the sole gastric expression of Cdx2 in transgenic mouse models led to extensive intestinal metaplasia [26-27]. However, sporadic activation of CDX2 in gastric cells seems to be a rather frequent biological phenomenon, and we found that more than half of the studied population, infected with *H. pylori* but with no intestinal metaplasia, presented scattered positive staining of non-metaplastic cells (Paper III). In agreement with our observations, other studies also report presence of focal CDX2-positive cells in areas without intestinal metaplasia within the *H. pylori*-infected stomach [9, 64-65], reporting, among *H. pylori*-infected subjects, percentages of affected individuals ranging from 14% [9] up to 100% [65]. Interestingly, in an *in vitro* co-culture model, which contrary to the gastric context in the previous studies is devoid of the mesenchymal inflammatory signals, we (Paper IV) and others [33] showed that *H. pylori* infection directly interfered with CDX2 expression in a gastric carcinoma cell line. This effect of the bacteria, separate from the concomitant inflammation, may play a part in the aforementioned observed phenotype. Since intestinal metaplasia is triggered by expression of CDX2, we hypothesized that this sporadic activation of its expression in the infected stomach could be a first step in metaplastic transformation of the mucosa, and perhaps a target stage for intervention. However, we found no association between the presence of CDX2 and the development of intestinal metaplasia, after a mean period of 6 years, since it developed in similar percentages of the subjects with or without CDX2 expression at baseline (Paper III). Two possible explanations for this may come forward.

First, we cannot rule out that the follow-up time might not have been enough to uncover differences between the groups, together with possible sample size issues. However, we may also speculate that CDX2 activation in differentiated cells might be of no consequence, and its stable expression in gastric stem cells might be a requisite for cell reprogramming and actual phenotypic change. This latter possibly happens less frequently and/or at a later stage in the cascade of events, which could also explain the reversion of gastric CDX2 expression upon infection eradication in individuals without intestinal metaplasia [65]. An interesting approach to further understand this issue would be to look for co-localization of CDX2 with gastric stem cell markers and then try to associate it with progression to intestinal metaplasia. However, at the present time, although suggested [66], no such markers are yet well established. Nonetheless, and although in mouse models sole ectopic Cdx2 expression was causal for metaplastic transformation [26-27], one last hypothesis is that CDX2 might be unable to initiate intestinal metaplasia alone in adult gastric cells. Our results therefore support that, at least until we are able to link it to other cellular markers, this sporadic activation in differentiated cells appears to be a frequent event with no biological significance and, thus, no prognostic value.

We were also able to describe that CDX2 expression increased in a gastric cell line upon infection with *H. pylori*, in a time and multiplicity of infection-dependent manner (Paper IV). However, from our study, the molecular mechanisms involved in *H. pylori* upregulation of CDX2 expression remain elusive. Both in our work and in a previous one showing a similar effect [33], the bacterial strains used, 26695 and 60190, respectively, are *cag*PAI-positive, and thus encode for the bacterial protein cagA. This protein has been described to be translocated into the gastric cells through a type IV secretion system and modulate cellular responses [67]. We hypothesized that cagA could be involved in the CDX2 promoter transactivation, but failed to prove so in a co-transfection experiment with a luciferase reporter (Paper IV). However, the cagA protein undergoes tyrosine phosphorylation in the cells to become activated [67], and we were not able to check for protein phosphorylation status after transfection. Therefore, although our results suggest that promoter activity is cagA-independent, we cannot completely exclude that cagA may influence CDX2 expression. Until now, no other studies have addressed this issue, but it would be interesting to further look into it either *in vitro* or trying to epidemiologically associate the *cag*PAI status of infecting

bacteria with the outcome in terms of CDX2 positivity in non-metaplastic areas, as it has previously been positively associated with intestinal metaplasia development [56].

CDX2 autoregulation – putative implication in phenotype stability

As discussed above, reversibility of intestinal metaplasia lesions is still an unclarified topic, but also one of fundamental importance. Not only is it a very interesting and still poorly understood biological event but also would its comprehension be very important to prevent progression along the gastric carcinogenic pathway and for the definition of putative intervention targets. It is well established that CDX2, which is ectopically expressed in human intestinal metaplasia, is pivotal for directing and maintaining the intestinal phenotype, both in normal and aberrant locations, so it is plausible to assume that reversion of the lesion would imply loss of expression of the *CDX2* gene. The CDX2 regulatory mechanisms in the gastric context discussed this far directly involve *H. pylori* bacteria-epithelial cell interaction and signalling from inflammatory cells and are insufficient to explain maintenance of CDX2 expression. This is especially true when we want to justify the retaining of CDX2 expression after infection eradication and inflammation clearance, concomitant with the observed low reversibility of intestinal metaplasia, suggesting the participation of yet another layer of complexity in CDX2 regulation *in vivo*. The phenotype described in *Cdx2*$^{+/-}$ mice, with areas of loss of expression without any structural loss of the second allele, suggested a regulatory haplo-insufficiency of Cdx2. Interestingly, not only might this feature be attributable to a self-regulatory mechanism but also has CDX2 been suggested to regulate its own promoter [68]. Taken together, these evidences led us to hypothesize that an auto-regulatory mechanism might be responsible for maintenance of CDX2 expression in the gastric context. Although it had previously been shown that CDX2 was able to transactivate its own promoter in a pancreatic and in an intestinal cell line [68], the *in vivo* relevance of this regulatory pathway was still unknown. The work presented here confirms and greatly extends these results and we support that autoregulation might have a major impact on stability of the intestinal metaplasia and difficult reversibility of the phenotype.

Using an *in vitro* co-transfection approach, we showed that CDX2 transactivated a 9.3kb fragment of the mouse *Cdx2* promoter in a panel of nine gastrointestinal cell

lines (Paper V). Even though all cell lines showed promoter transactivation, the inductive effect was greatly variable among cell lines, ranging from 1.4- up to 9-fold, which suggests that context-specific co-factors, activators or repressors, may be interfering with this mechanism. Of note, and in line with this hypothesis, autoregulation of the CDX2 homologue CDX1 [69] was proven to be dependent on CDX1 interaction with a co-factor, LEF1, and to occur via LEF/TCF responsive elements on the CDX1 promoter [70]. However, and in agreement with a previous report [68], we demonstrated that CDX2 binds its own promoter in living cells through Cdx2-binding elements, and that these sites are active in the transactivation process, as assessed by site-directed mutagenesis followed by luciferase reporter assays. Importantly, our results further indicate that *CDX2* is a target of its own transactivational activity, regulating its own endogenous expression in the gastrointestinal context and being able to initiate its endogenous expression in the CDX2-negative non-gastrointestinal HeLa cell line. As we assessed the relevance of this mechanism *in vivo*, we presented data confirming that Cdx2 is bound to its promoter in the mouse intestine and, most strikingly, that CDX2 binds its own promoter in human intestinal metaplasia (Paper V).

Altogether, our results sustain that CDX2 is regulating its own expression in the gastro-intestinal context and that it is bound to its promoter in intestinal metaplasia. Linking this back to the apparent stability of this lesion, it is plausible to hypothesize that *H. pylori* infection and consequent gastric inflammation provide the initial trigger for CDX2 expression in the gastric cells. With chronic infection and gastritis, all altered signalling network will induce a certain level of expression, over the self-maintenance threshold, which will allow the gene product to continue its own expression. As a matter of fact, this fits into a model of cell commitment during embryonic development involving regulatory loops of pivotal transcription factors [71]. An extracellular inducing factor causes the expression of the transcription factor which then, directly or indirectly, maintains its own expression conferring stability to the specified state. Interestingly, according to this model, the persistence of the inducing factor becomes superfluous. This could provide an explanation for the observation that the metaplastic lesion does not readily revert upon *H. pylori* eradication and inflammation clearance, the first signals responsible for the onset of CDX2 expression. Our results thus favour that, after being activated, CDX2 is capable of maintaining its own expression via an auto-regulatory mechanism, hence conferring stability to the intestinal phenotype and hampering to some extent its regression. However, reversibility of gastric CDX2

expression, in patients with no evidence for metaplastic transformation, was shown in a human model of *H. pylori* eradication [65]. In fact, what this study showed was disappearance of the sporadic activation upon eradication of the infection, which may easily be reconciled with our results in that it is not the intestinal phenotype, but rather the sporadic CDX2 expression, that disappears. We might speculate, taking all our results into account, that in this setting the threshold for autoregulation had not yet been achieved and/or CDX2 was not stably expressed in order to reprogram gastric stem cells. Therefore, the removal of the infection agent discontinued the inductive signalling and natural clearance of positive cells occurred during the normal cell renewal process.

It is worth noting that this type of auto-maintained systems have a certain lability and may theoretically be reversed if a large fluctuation of the protein level takes it below the self-regulatory threshold. Accordingly, it is reasonable to predict that, in addition to eradicating *H. pylori* infection, it will be necessary to interfere with the CDX2 auto-regulatory loop in order to revert intestinal metaplasia. In case loss of CDX2 in the metaplastic glands actually leads to disappearance of the lesion, further studies would be needed to clarify the biological cascade of events taking part in the process and discriminate between two major hypotheses. Although conceptually very relevant, from the clinical point of view it does not matter if intestinal metaplasia is cleared from the stomach by cellular transdifferentiation back to a gastric phenotype or by loss of growth advantage and repopulation of the mucosa with normal gastric glands. In either case, should we be able to show that interference with CDX2 expression, bringing it below a certain threshold, resulted in a reversion of the phenotype or sufficient growth disadvantage, this would be a major contribution to a putative “cure” of intestinal metaplasia and interruption of the gastric carcinogenic pathway.

● Part III > Establishment and maintenance of intestinal metaplasia through CDX2 – linking pathways together

Metaplasias are a rather rare event in nature, but one with considerable pathological impact since many of these differentiation abnormalities predispose individuals to neoplasia development. Fully understanding how these lesions happen and the cascade of events leading to their appearance might enable the design of methods to deliberately induce alterations in cellular differentiation programs in order to revert harmful metaplasias back to the normal tissue. In the work presented here, we sought for a better understanding of the regulatory mechanisms of a pivotal transcription factor involved in one of these metaplasias – CDX2 (reviewed in paper VI).

Taking together all the results obtained in this work, we built a model for CDX2 expression onset and persistence in the gastric context, contributing to the establishment and maintenance of a rather stable intestinal phenotype in the stomach – the pre-neoplastic lesion intestinal metaplasia (Figure 2).

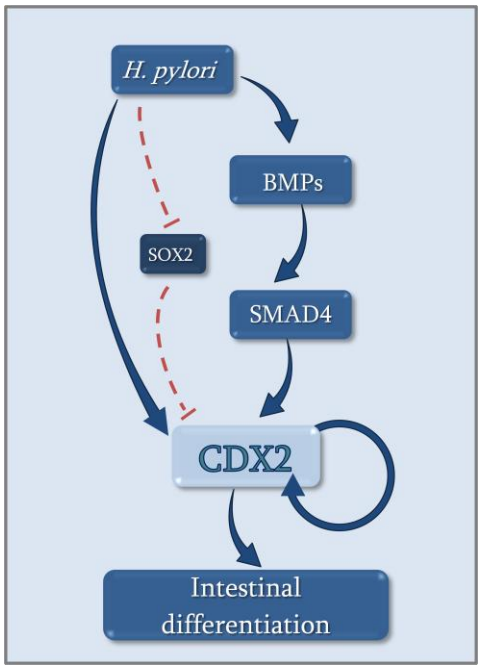


Figure 2 > Model of CDX2 regulation in the gastric context.

H. pylori infection has long been accepted to be directly or indirectly responsible for triggering the mucosal damage and alterations that precede gastric cancer. In light of the current knowledge, we reason that *H. pylori* infection possibly induces two lines of deleterious effects on the gastric mucosa. On the one hand, it provokes long-range effects by inducing strong inflammation, with all the altered cytokine signalling that it accounts for, namely the recruitment of BMP-expressing cells. On the other hand, the bacteria may directly interfere with gene expression in cells to which it attaches. In fact, *H. pylori* produces a dual effect towards increased CDX2 expression – not only does it induce CDX2 (Paper IV) but it also decreases SOX2, a repressor of CDX2 expression (unpublished results from our group). The alterations induced in these cells may perchance then propagate and reprogram neighbouring cells by secondary cell-cell interactions or production and release of specific signalling molecules. We also showed that BMPs, through SMAD4, positively regulated CDX2 expression and that elements of this pathway co-localize with CDX2 (Paper I and II). This inductive signalling may come from BMP-expressing inflammatory cells and/or from BMPs produced by the epithelial cells (Paper I). Lastly, we demonstrated an auto-regulatory loop for CDX2 (Paper V) which might contribute to the maintenance of its expression even upon variations in the remaining inductive signals. Altogether, this regulatory network is probably contributing not only to the development of intestinal metaplasia but also to its apparent stability and difficult reversibility (Paper III).

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Summary and Conclusions

Intestinal metaplasia of the stomach is a pre-neoplastic lesion consisting on the aberrant differentiation of the gastric mucosa into an intestinal phenotype and conferring increased risk for gastric carcinoma development. It appears in the gastric carcinogenesis pathway following long-term *Helicobacter pylori* infection, and it was shown to be dependent on ectopic expression of the intestinal homeobox gene CDX2. The study of this lesion acquires special importance since gastric cancer remains the second most frequent cause of cancer-related death worldwide.

CDX2 is a transcription factor essential for intestinal development and phenotype maintenance in adults, and its post-embryonic expression is restricted to the gut. However, in human pathology it appears ectopically expressed in every focus of aberrant intestinal differentiation, namely gastric intestinal metaplasia. Furthermore, loss of CDX2 expression in the intestine and its forced gastric expression in mice proved the central role played by CDX2 in promoting/maintaining intestinal differentiation in both normal and aberrant locations. Nonetheless, the molecular mechanisms governing the onset of its ectopic expression and maintenance in the gastric setting remain to be fully disclosed.

The general aim of this work was to unravel novel molecular mechanisms involved in the regulation of CDX2 expression in the gastric context and relate them to intestinal metaplasia establishment and maintenance. To achieve this goal we focused on specific objectives from which we were able to draw the following main conclusions:

1) We have found that several elements of the Bone Morphogenetic Protein (BMP) pathway, namely the hallmark of an active pathway pSMAD1/5/8, were overexpressed in intestinal metaplasia lesions as compared to surrounding normal gastric mucosa and co-localized with CDX2, only expressed in this lesion. Furthermore, we have shown that BMP2 and BMP4 induce an upregulation of CDX2 expression through the intracellular signal transducer SMAD4. This effect extended to the intestinal differentiation markers MUC2 and LI-Cadherin. Lastly, we have proved that not only is SMAD4 able to bind the *CDX2* promoter but also do elements of the pathway transactivate (SMAD4 and SMAD1) or repress (inhibitory SMAD6 and SMAD7) its promoter activity. We thus conclude that the BMP pathway regulates CDX2 expression *in vitro* and might be important for its regulation in intestinal metaplasia establishment and/or maintenance.

2) We have described that Juvenile polyps, both sporadic and a familial case with a *SMAD4* germline mutation, in addition to altered proliferation present foci of altered differentiation, with loss of intestinal and gain of gastric phenotype. These foci had decreased or loss of expression of the intestinal markers CDX2 and MUC2 and ectopically expressed the gastric mucin MUC5AC, linked to deficient SMAD4 expression.

A familial case bearing a *BMPR1A* mutation which only downregulated its expression had hyperplastic features with no evidence for differentiation abnormalities. These findings reinforced the ones presented in the previous point, strengthening the close relationship between the BMP pathway and CDX2 expression.

3) We have presented evidence on the association between infection with high virulence *H. pylori* strains and non-reversibility of intestinal metaplasia after a 6-year follow-up. Furthermore, we have shown that *H. pylori* infection regulates CDX2 expression in an *in vitro* co-culture model.

4) We have also shown that CDX2 is sporadically expressed in non-metaplastic cells across the gastric mucosa in *H. pylori* infected individuals. However, and even though CDX2 is the accepted trigger for metaplastic transformation, CDX2 sporadic expression failed to associate with intestinal metaplasia development after a 6-year follow-up.

5) We have found that CDX2 is autoregulated *in vitro* in the gastrointestinal context, actively binding and transactivating its own promoter and regulating its endogenous expression. Additionally, we have shown that it binds its own promoter *in vivo* in the human intestinal metaplasia lesion and in the mouse gut. Together with the aforementioned data we concluded that CDX2 autoregulation might be a major contributor to the apparent stability and low reversibility of intestinal metaplasia.

Altogether, we presented evidence on the participation of novel molecular pathways in the regulation of CDX2 expression. These are compatible with previously described signalling networks in the *H. pylori*-infected stomach and therefore extend the current knowledge on how CDX2 is regulated and on how the CDX2-dependent gastric intestinal differentiation, the intestinal metaplasia lesion, is acquired and maintained.