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SOX2 and CDX2 in gastric intestinal metaplasia and dysplasia: impact on differentiation

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INTRODUCTION

Gastric Cancer – Overview

Gastric cancer was the most common and most lethal cancer in the world during most part of the last century. Despite a gradual decrease in its incidence, it is still the fifthmost common malignancy (6.8% of all cancers), after lung, breast, colo-rectum and prostate and the third leading cause of cancer death (8.8% of total) after lung and liver cancers. More than 70% of the cases occur in developing countries.[1]

In Portugal, the scenario is similar. Despite a slow decrease in stomach cancer mortality, it still represents one of the most important oncological problems.[2] According to IARC Cancer Base, gastric cancer is the fifth most incident cancer in Portugal (6.1% of all cancers) and the third most deadly (9.5% of total). These values surpass the ones of WHO Europe Region (4.3% and 6.5% respectively).[1, 3]

More than 90% of gastric tumours are adenocarcinomas.[4] According to Lauren's classification, these can be further divided into two main histopathological entities: intestinal and diffuse types.[5] They differ in epidemiologic, clinic, pathologic and molecular patterns.[6]

Intestinal type gastric carcinoma is the most common subtype and it is preceded by a prolonged precancerous cascade, most frequently initiated by *Helicobacter pylori*, that is still not fully understood.[6, 7] Morphologically, it forms glands that range from well differentiated to moderately differentiated tumours and it typically arises on a background of chronic atrophic gastritis with intestinal metaplasia.[6]

Diffuse type gastric carcinoma is not preceded by well-defined precancerous lesions.[7] It also shows an association with *H. pylori* but, unlike intestinal type, it develops without passing through the intermediate step of intestinal metaplasia.[8, 9] It may occur in an hereditary context, but most frequently it is sporadic. In both cases, loss of expression

of the cellular adhesion protein E-cadherin results, morphologically, in poorly cohesive cells diffusely infiltrating the gastric wall with little or no gland formation.[6]

Gastric Cancer – Carcinogenesis Pathways

Nowadays, the most accepted model of gastric carcinogenesis of the intestinal type still has its foundations on Correa's model. According to Correa, a decades-long, multistep and multifactorial cascade starting with chronic gastric inflammation, progressing to gland loss and their replacement by intestinal-type epithelium, followed by loss of cellular differentiation, leads ultimately to the development of invasive gastric adenocarcinoma (figure 1).[7, 10-12]

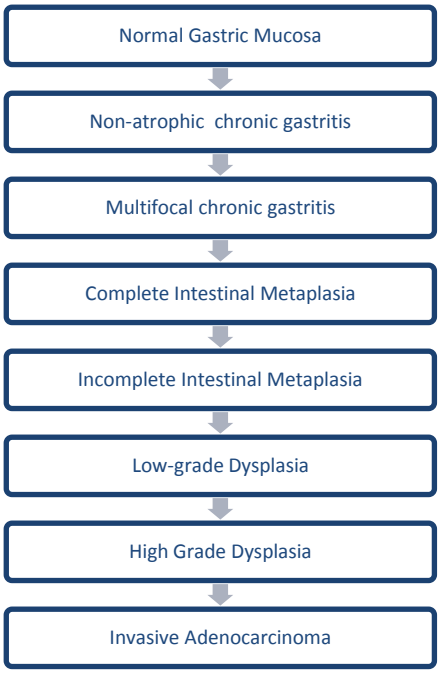

Figure 1 - Correa's Carcinogenesis Model

Tsukashita et al. have suggested that the tumourigenesis of low-grade dysplasia particularly with an intestinal phenotype may be different from high-grade dysplasia and intramucosal carcinomas with gastric phenotype.[13] Nishimura et al. tested this hypothesis and concluded that non-invasive low-grade neoplasms of the intestinal lineage progress to non-invasive high-grade neoplasms but rarely to intramucosal adenocarcinomas, whereas intramucosal adenocarcinomas of the gastric lineage largely arise *de novo* from the proper gastric mucosa and are partially derived from non-invasive high-grade neoplasms.[14]

These two models are not necessarily mutually exclusive so, although gastric carcinogenesis is not fully understood, we can admit that adenoma-carcinoma sequence is responsible for a subset of gastric cancers and that the rest of them may arise *de novo*.

Risk Factors and Determinants of Gastric Carcinogenesis

In its gastric carcinogenesis model, Correa also proposed that both deleterious and protective factors could modulate the progression/regression of this cascade.[10]

Helicobacter pylori is the most frequent cause of gastritis, being classified by IARC as a type I (definite) human carcinogen in 1994.[15] Two proposed mechanisms to step in the carcinogenic cascade are the immune response elicited by *H. pylori* and the damage resulting from oxidative stress.[16] The severity of inflammation varies both with the virulence of *H. pylori* strains, given by *vacA* and *cagA* genotypes, and with proinflammatory host genetic polymorphisms in the *IL1β*, *IL1RN* and *TNFα* genes.[17-19] Eradication with antibiotic treatment can reverse this initial inflammatory reaction but intestinal metaplasia reversibility is still a controversial issue.[20] Furthermore, intestinal metaplasia arises in only 30% of infected individuals, from which only around 7% will develop gastric cancer,[9, 21] so the presence of other genetic/environmental factors modulating progression towards cancer has to be considered.

Other risk factors for the development of sporadic gastric cancer include salt and nitrous compounds of the diet and tobacco smoking. On the other side, fresh fruits and vegetables may play a protective role.[22]

Pre-malignant conditions and lesions - metaplasia and dysplasia

Multifocal atrophic gastritis and intestinal metaplasia confer a high risk for the development of gastric cancer, thus they are considered to be precancerous conditions. [23]

Intestinal metaplasia represents a phenotypic change from the normal epithelial cell of gastric mucosa to an intestinal phenotype. One of its classifications divides IM into complete or incomplete type, both characterized by the presence of intestinal markers such as the transcription factor CDX2 and the mucin MUC2. Complete intestinal

metaplasia displays goblet and absorptive cells and no expression of gastric mucins. Incomplete intestinal metaplasia displays goblet and columnar non-absorptive cells, in which gastric mucins (MUC5AC, and MUC6) are co-expressed with the intestinal markers.[24] This classification has clinical relevance since incomplete intestinal metaplasia confers a higher risk of progression to gastric cancer.[25]

Gastric dysplasia (also known as intraepithelial neoplasia or non-invasive neoplasia) represents the penultimate stage of the gastric carcinogenesis sequence. Histologically, it is defined by unequivocal neoplastic epithelium without evidence of tissue invasion, and is thus a direct neoplastic precancerous lesion.[23] The term adenoma refers to a raised circumscribed dysplastic lesion protruding above the mucosal surface.[26]

Observation of different degrees of cellular and architectural atypia allows classification into low and high grade dysplasia.[27] Most patients harboring lesions classified as high grade dysplasia are at high risk for either synchronous invasive carcinoma or its rapid development.[28, 29]

Dysplasia can also be classified, according to its phenotypical characteristics, into adenomatous/intestinal or foveolar/gastric.[30] These morphological types can be confirmed by immunohistochemistry techniques. Intestinal type has positivity to MUC2, CD10 and CDX2 and gastric type has positivity to MUC5AC and MUC6, lack of CD10 and low CDX2 expression. Those which show both gastric and intestinal phenotypes are classified as hybrid/mixed phenotype while those showing neither gastric nor intestinal markers are grouped as null type.[6]

Although the literature on the clinical relevance of this morphological classification is limited, some clues have aroused. Park et al have showed that adenomatous type dysplasia is associated with IM showing a complete intestinal phenotype, while foveolar and hybrid dysplasias are closer to incomplete IM.[31] Also, Tsukashita et al

demonstrated that the majority of low-grade dysplasia (81.8 %) expresses intestinal markers and generally expresses no gastric markers, whereas more than half of high-grade dysplasia expresses gastric markers (72.2%).[13, 31]

Molecular Pathology

The research on the determinants of gastric cancer precursors has been less extensive than for the cancer endpoints and there are currently no immunohistochemical or molecular assays that can help stratify the risk of progression of gastric dysplastic lesions.

The genetic and molecular abnormalities occurring during gastric carcinogenesis include chromosomal instability, CpG-island methylation and microsatellite instability (MSI).[30]

Chromosomal instability refers to copy number variations and is the predominant type of genomic instability, being present in more than 60% of gastric cancers.[32, 33] Loss of heterozygosity at the *TP53* locus was demonstrated in 14% of a series of metaplastic lesions, in 22% of gastric dysplastic lesions and 30% to 50% of gastric carcinomas, consistent with their role in cancer progression.[30]

Aberrant DNA hypermethylation occurs preferentially in CpG islands.[33] Among gastric adenomas, it was described in genes involved in cell cycle regulation (*p14, p16, COX2*), signal transduction (*APC*), DNA repair (*hMLH1* and *MGMT*) and invasion and metastasis (*E-cadherin* and *TIMP3*).[30] *RUNX3* is a recently recognized tumor suppressor gene for gastric cancer. An increase in the proportion of its promoter methylation along gastric carcinogenesis was described, with 16% in chronic atrophic gastritis, 37% in IM, 42% in gastric adenoma, 55% in dysplasia, and 75% in GC tissues, suggesting its importance at the later stages of gastric carcinogenesis.[30, 33]

MSI phenotype results from accumulating genomic mutations due to inactivation of the DNA mismatch repair pathway, through promoter hypermethylation and loss of expression of *MHL1*. MSI has been documented both in intestinal metaplasia and in dysplastic lesions (~20%) being therefore considered among the early molecular events in gastric carcinogenesis.[32]

Based on gene expression profiling, three gastric tumor subtypes were identified. They were classified as proliferative, mesenchymal, and metabolic and they show differential sensitivities to chemotherapy agents.[34]

Although molecular events underlying gastric carcinogenesis are not fully uncovered, it is becoming evident that the balance between gastric and intestinal differentiation has a significant impact, at least in the early steps of the gastric carcinogenesis cascade, with the most frequent pre-malignant condition resulting from a switch of gastric to an intestinal differentiation profile.

CDX2 – An intestinal differentiation transcription factor

CDX2 is a transcription factor member of the caudal-related homeobox gene family with an important role for intestinal epithelial development.[35] In normal conditions, CDX2 is expressed in small intestinal and colonic epithelia, but not in gastric epithelium.[36] However, in certain unfavourable conditions to the normal gastric mucosa environment – usually elicited by *H. pylori* infection – gastric epithelial cells may acquire ectopic expression of CDX2 and transdifferentiate into an intestinal phenotype – intestinal metaplasia.[37-40]

The strength of these observations largely come from transgenic mouse models studies, in which *CDX2* was under the control of promoters from different gastric-specific genes (*Foxa3* or *H+/K+-ATPase b-subunit*). Mutoh et al. and Silberg et al. reported that the ectopic expression of CDX2 in the gastric mucosa of transgenic mice alone was sufficient to induce a complete transformation of the gastric mucosal glands

to intestinal-like mucosa, confirming the crucial role of CDX2 in intestinal differentiation.[37, 38] A posterior paper of Mutoh et al. also showed that long-term intestinal metaplasia of the *CDX2* transgenic mice induced invasive gastric carcinoma.[41]

Some degree of this CDX2 abnormal expression is maintained in the progression to gastric dysplasia and gastric cancers.[39, 42] Although Kim et al demonstrated a positive correlation between CDX2 expression and the increasing grade of dysplasia and carcinoma,[43] more recent papers described the opposite association, with CDX2 expression progressively reduced in gastric dysplasia and cancer.[42, 44, 45] Other authors reported a significantly decreased CDX2 expression in incomplete intestinal metaplasia compared with complete intestinal metaplasia,[39, 44] and an inverse association between CDX2 expression in gastric cancer and the expression of gastric mucins MUC5AC and MUC6.[42, 44] Also, CDX2-positive tumours have a better outcome than CDX2-negative tumours, with less invasiveness, fewer lymph node metastases and a higher 5-year survival.[42, 45] Based on these findings, a tumour suppressor role was suggested for *CDX2* in human gastric carcinogenesis,[42, 45] similarly to colorectal cancer.[46, 47]

SOX2 – A gastric differentiation transcription factor

SOX proteins are important transcription factors with pleiotropic functions – stem and progenitor cell fate determination, cell differentiation, proliferation, reprogramming, tissue homeostasis and repair.[48] They constitute a large family of genes – with 20 different *SOX* genes identified until now[49] – and they have in common the HMG domain similarity with *Sry* (sex-determining region Y).[48]

In normal tissues, SOX2 is expressed in the foregut-derived organs such as pharynx, oesophagus and stomach, but not in hindgut-derived organs such as intestine, where the CDX1 and CDX2 proteins assume the responsibility of cell differentiation.[50, 51]

Several other tissues also have been shown to express SOX2, among them are trachea, lung, tongue, cervix, brain, skin and bone.[48]

In normal stomach, SOX2 has a nuclear expression, mainly in the neck region of the gastric glands, both at the body and antrum regions. Starting with *H. pylori* stimuli,[40] a progressive reduction of the expression of SOX2 from normal gastric mucosa to incomplete intestinal metaplasia, then to complete intestinal metaplasia[51] and to adenocarcinoma,[50, 52] has been demonstrated, along with an inverse increase of CDX2 expression.[51] This *CDX2-SOX2* interplay was comprehensively demonstrated both with in vitro[40, 53, 54] and with transgenic mouse models studies. Conditional knock-out of the *CDX2* gene in mice showed increased SOX2 expression in the regions where CDX2 expression was deficient, with a consequent shift from an intestinal to a gastric/esophageal differentiation.[55, 56] The same was observed when ectopic expression of SOX2 was induced in the intestine.[57] Also, it has been shown that *SOX2* up-regulates stomach-specific expression of *pepsinogen A*[58] and *MUC5AC* genes.[59] Another finding is the higher SOX2 expression in adenocarcinomas expressing gastric mucins compared to adenocarcinomas expressing intestinal mucins.[50, 60] For all these reasons, *SOX2* is a highly suspected candidate for gastric differentiation.

SOX2 expression has been positively associated with tumour grade and with worse prognosis in a variety of tumours.[61] Concerning gastric adenocarcinoma, conflicting results were published. Matsuoka et al found a statistically significant correlation between SOX2-positive expression and depth of tumour invasion, lymph node metastasis and lymphatic invasion.[62] On the other hand, Otsubo et al reported that SOX2 expression is frequently down-regulated in human gastric cancer, through aberrant DNA methylation, associating with a significantly shorter survival time. Also, they showed that SOX2 inhibits proliferation, induces cell-cycle arrest and apoptosis in gastric cell lines, suggesting a tumour suppressor role for *SOX2* in gastric carcinogenesis.[52]

Aim

This work aims to better characterize the balance of gastric vs. intestinal differentiation in the precancerous conditions and lesions of the gastric carcinogenesis cascade through a systematic description of SOX2 expression in the normal gastric mucosa, complete IM, incomplete IM and dysplasia.

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Differentiation reprogramming in gastric intestinal metaplasia and dysplasia: role of SOX2 and CDX2

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ABSTRACT

Gastric cancer remains a major health concern worldwide. Intestinal metaplasia (IM), resulting from *de novo* expression of CDX2, and dysplasia are precursor lesions of gastric cancer associated with increased risk for cancer development. Multiple evidences suggest that SOX2 is a transcription factor with a role in gastric differentiation and its interplay with CDX2 may determine gastric carcinogenesis. Our aim was to assess SOX2 involvement in gastric premalignant conditions and lesions in an attempt to establish its relationship with CDX2 and with the differentiation reprogramming that characterizes gastric carcinogenesis. Characterization of gastric (SOX2, MUC5AC, MUC6) and intestinal (CDX2 and MUC2) markers in normal gastric mucosa, in 55 foci of IM and in 26 foci of dysplasia was performed by immunohistochemistry. *In vitro* models were used to study the putative cross-regulation between SOX2 and CDX2. SOX2 was expressed in the normal gastric mucosa, in the presumptive stem cell compartment, and was maintained in 7% of the complete and 85% of the incomplete IM subtypes, the latter defined by MUC5AC expression. 12% of the dysplastic lesions expressed SOX2 and the association with MUC5AC was lost. Conversely, CDX2 was present in all IM and dysplastic lesions. Finally, SOX2 negatively regulated CDX2 in a cell-type specific manner. In conclusion, SOX2 associates with gastric differentiation and is lost in the progression to dysplasia, whereas CDX2 is *de novo* acquired in IM and maintained in dysplasia. This suggests that the balance between gastric and intestinal differentiation programs impacts on progression of the gastric carcinogenic cascade.

Keywords: SOX2, CDX2, intestinal metaplasia, dysplasia, gastric cancer

INTRODUCTION

Gastric cancer is the fourth most common malignancy and the second-leading cause of cancer-related deaths worldwide.[1] The majority of gastric cancers is diagnosed late, and as such has a dismal prognosis, which is reflected by a 5-year survival rate of no more than 25%.[2] Thus, the most promising strategies to control the disease are based on prevention and early diagnosis. Gastric cancer is usually initiated by an inflammatory process associated with infection by *Helicobacter pylori*, which may lead to multifocal atrophic gastritis, intestinal metaplasia (IM), dysplasia and finally cancer.[3] IM is a switch from a gastric to an intestinal differentiation profile and is the most frequent premalignant condition of the stomach, appearing adjacent to more than 80% of gastric cancers.[4] It is an heterogeneous lesion with two subtypes, complete and incomplete, both characterized by the presence of intestinal markers such as the transcription factor CDX2 and the mucin MUC2. The complete IM subtype is defined by the absence of gastric differentiation markers, such as mucins MUC5AC and MUC6, whereas in the incomplete IM subtype there is concomitant expression of gastric and intestinal markers.[5] This classification has clinical implications because the incomplete subtype is more frequently associated with gastric cancer and shows an increased risk for cancer progression.[4,6]

CDX2 is a homeobox transcription factor critical for intestinal differentiation [7-8]and is a specific biomarker of the early steps of the gastric carcinogenic cascade, driving the onset of IM.[9-10] The key role of CDX2 in the metaplastic transformation of the gastric mucosa was categorically demonstrated by two transgenic mouse models with ectopic expression of CDX2 in the gastric epithelium and subsequent development of IM with absorptive, goblet, and enteroendocrine cell types.[11-12]

On the other hand, an increasing number of evidences support the involvement of the transcription factor SOX2 in gastric differentiation. SOX2 is a sex-determining region Y-box 2 gene, a member of the high mobility group (HMG) domain proteins that

is essential to maintain pluripotency in embryonic stem (ES) cells and also to reprogram fibroblasts into induced pluripotent (iPS) cells.[13-14] More recently, it has also been identified as an adult stem cell marker in mice.[15] Furthermore, modulating SOX2 expression in mice demonstrated the importance of this protein in the differentiation of the esophageal epithelium and proper morphogenesis of the esophagus, trachea and lung.[16-17] In the digestive tract, it was observed in chick and in mouse gut that SOX2 and CDX2 expression are mutually exclusive.[18-19] *In vitro* studies showed that SOX2 negatively regulated the CDX2 promoter by hampering the action of other transcription factors in an intestinal context and that SOX2 downregulation led to CDX2 overexpression in a gastric context.[20-21] In accordance with these observations, we have demonstrated that SOX2 and CDX2 can be inversely regulated by the BMP pathway and *Helicobacter pylori.*[22] Moreover, two mouse models with conditional CDX2 loss of function, showed increased SOX2 expression in the regions where CDX2 was abrogated, with a consequent shift from an intestinal to a gastric/esophageal differentiation.[23-24] The same was observed when forced expression of SOX2 was induced in the intestine,[25-26]suggesting that the interplay of these two transcription factors is critical for the balance between gastric and intestinal differentiation.

Here, we sought to characterize SOX2 expression and the relationship with CDX2 in gastric cancer precursor lesions in an attempt to establish its involvement in the differentiation reprogramming that characterizes gastric carcinogenesis.

MATERIAL AND METHODS

Material

Normal gastric mucosa was studied in 10 biopsies representative of the body (n = 5) and the antrum (n = 5) regions obtained from individuals with no gastric pathologies. IM (55 foci) and the adjacent gastric mucosa were studied in 11 surgical specimens obtained from patients with gastric carcinoma undergoing surgery at Centro Hospitalar S. João, Porto, Portugal. IM foci were classified into complete (n = 29) or incomplete (n = 26) type according to the pattern of mucin expression.[5] Twenty-six foci of dysplasia were studied in 22 samples obtained by Endoscopic Submucosal Dissection (ESD) at the same hospital. Foci were considered distinct when they were separated by a stretch of gastric glands. The use of retrospective samples from which informed consent cannot be obtained is authorized for research studies by the Portuguese law.

Immunohistochemistry

Paraffin–embedded specimens were subjected to immunohistochemistry for SOX2, CDX2, MUC5AC, MUC6 and MUC2 using the antibodies described in Table 1. Detection of CDX2, MUC5AC and MUC2 was performed by incubation with a biotin-labeled rabbit anti-mouse secondary antibody (DAKO, 1:100) followed by incubation with an avidin/biotin detection system (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) and development with 3,3′-diaminobenzidine (DAB). Detection of SOX2 was done using the Dako REAL™ Envision™ Detection System Peroxidase/DAB+ (DAKO, Glostrup, Denmark) according to the manufacturer's instructions.

For double staining (SOX2/MUC5AC or SOX2/MUC6), immunohistochemistry was first performed for SOX2 using the Dako REAL™ Envision™ Detection System

Peroxidase/DAB+ (DAKO, Glostrup, Denmark) and immediately followed by immunohistochemistry for MUC5AC or MUC6 using the Envision™ G2 System/ AP (Permanent Red) (DAKO), according to the manufacturer's instructions. Tissue counterstaining was performed using Mayer hematoxylin. Cases were considered positive when more than 5% of the cells were stained with each antibody.

Cell Culture

The human gastric carcinoma cell lines AGS (ATCC) and MKN45 (JCRB0254, The RIKEN Cell Bank) were maintained in RPMI1640-GlutaMAx (Gibco, Invitrogen), whereas the human colon carcinoma cell line Caco-2 (ATCC) was maintained in DMEM (Gibco, Invitrogen). Media were supplemented with 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, USA) and 1% antibiotics (10U/mL penicillin and 10μg/mL streptomycin) (Gibco, Invitrogen, Carlsbad, USA). Cells were maintained at 37ºC in a humidified 5% CO2 incubator.

Transient transfection assays

For AGS and MKN45, one day prior to transfection, cells (7.5x105) were seeded in 6-well plates. Transfection of the expression vector CMV/SOX2, (1ug/well) or the empty vector was carried out using Lipofectamine 2000 (Invitrogen) (1μg DNA:1.5μl lipofectamine ratio) in serum and antibiotic free OPTI-MEM (Gibco, Invitrogen). Cells were recovered 48h after transfection. Caco-2 cells were subjected to two consecutive rounds of transfection. Cells were seeded in 6-well plates and transfected either with the expression vector CMV/SOX2 or with the empty vector (1ug/well), as described above. The medium was changed and a second similar transfection was performed 72 hours after platting. Cells were recovered 48h after the second transfection.

Protein Extraction and Western Blot

Whole cell extracts were obtained by ressuspension of cell pellets in RIPA buffer (50mM TrisHCl pH 7.4, 150mM NaCl, 2mM EDTA, 1% NP-40, 0.1% SDS) in the presence of Complete protease inhibitors cocktail, (Roche, Indianopolis, USA) and quantification of total protein was determined by BCA protein assay (Pierce, Illinois, USA). Protein extracts were then subjected to standard SDS-PAGE, transferred to a nitrocellulose membrane (Amersham, GE Healthcare, UK) and blotted with primary antibodies (anti-CDX2,1:500, Biogenex; anti-SOX2, 1:4500, SIGMA; anti-β-actin, 1:8000, SCBT) in 5% BSA (Sigma, St. Louis, USA) in TBS 0.01% Tween-20 (Sigma, St. Louis, USA). Peroxidase-conjugated secondary antibodies were used and developed with the ECL detection kit (Amersham, GE Healthcare, UK). Quantification of the western blots was performed using the Software Quantity One (BioRad, CA, USA).

RNA extraction and Real-Time PCR

Total RNA was extracted using TRI Reagent (Sigma, St. Louis, USA) and converted to cDNA using the SuperScript® II Reverse Transcriptase (Invitrogen, Carslbad, USA). CDX2 or 18S were amplified with SYBR Green (Applied Biosystems, Foster City, USA) in a fluorescence reader ABI Prism 7500. The levels of 18S were used for normalization and relative mRNA levels were calculated. Each experiment was carried out in triplicates at least twice; the results are expressed as means ± SD of representative triplicates.

Statistical analysis

Statistical analysis was performed using the StatView program (version 5.0). Distributions were compared by χ^2 and Student's *t*-test and significance was assumed whenever *p* values were <0.05.

RESULTS

SOX2 expression in the normal gastric mucosa

We first characterized SOX2 expression in the normal gastric mucosa. For that purpose, we performed immunohistochemistry in 10 normal gastric mucosas and in 11 gastric mucosas adjacent to IM. The pattern of expression was similar in all samples. SOX2 was mainly expressed in the nuclei of the cells in the neck region of the gastric glands, both at the body and antrum (Fig 1) and expression was progressively lost towards the surface. SOX2 expression is also less marked in the deep glands particularly in the body region (Fig 1).

SOX2 expression in gastric premalignant lesions

We next evaluated the expression pattern of SOX2 in gastric premalignant conditions and lesions: intestinal metaplasia and dysplasia. We performed immunohistochemistry in 26 foci of incomplete IM and 29 foci of complete IM, from a total of 21 cases. Complete and incomplete IM were defined, as previously described,[5] by the expression pattern of gastric mucins, with the complete type characterized by the absence of gastric mucins MUC5AC and MUC6 whereas the incomplete type expresses at least one of these mucins. SOX2 exhibited a distinct expression pattern according to the IM subtype, being expressed in 85% (22/26) of the incomplete IM foci and in 7% (2/29) of the complete IM foci (Table 2). Double immunostainings showed co-expression of SOX2 and MUC5AC in incomplete IM (Fig 2) and absence of both proteins in complete IM (Fig 2). MUC2 and CDX2 were expressed in all IM foci, as previously described (data not shown).[9]

We next evaluated the expression pattern of SOX2 in 26 foci of dysplasia: low grade dysplasia (LGD) – 20 foci; high grade dysplasia – 6 foci. SOX2 was expressed in 12% of the foci (3/26), all of low grade dysplasia (Table 3). Fig 3 shows dysplastic glands with SOX2 expression adjacent to negative ones. To further assess the differentiation characteristics of these dysplastic lesions we studied CDX2, MUC2, MUC5AC and MUC6 expression. The results are displayed in Table 3 and showed that all dysplastic foci expressed CDX2, 46% (12/26) expressed MUC2, 58% (15/26) expressed MUC5AC and 68%(17/25) expressed MUC6. Moreover, CDX2 expression was uniform throughout the lesions, similarly to IM, whereas expression of all mucins was scattered and heterogeneous (Fig 3).

SOX2 and CDX2 cross-regulation

Different studies with mice models suggest that CDX2 negatively regulates SOX2 and also the reverse.[20-21,25-26] In order to determine if there is a direct cross-regulatory mechanism between SOX2 and CDX2, which could be relevant in the onset of IM, we transiently transfected two gastric carcinoma cell lines (AGS and MKN45) and one colonic carcinoma cell line (Caco-2) with a CMV/SOX2 vector and the gastric carcinoma cell lines with a CMV/CDX2 expression vector (this experiment was only performed in the gastric cell lines since Caco-2 expresses CDX2 at high levels). The results obtained showed that in Caco-2 cells, increased SOX2 levels led to a decreased CDX2 expression both at the mRNA and the protein level (Fig 4) whereas in none of the cell lines CDX2 regulated SOX2 expression (data not shown).

DISCUSSION

We have shown that SOX2 is consistently expressed in the normal gastric mucosa, mostly in the neck region, and in incomplete intestinal metaplasia (IM) conceivably explaining the maintenance of a gastric differentiation program in these cells.

Gastric carcinogenesis is complex and so far largely elusive at the molecular level. However, understanding this process is key in order to design more effective screening and surveillance approaches or even revert preneoplastic lesions. One of the major pathways unfolds in a background of chronic atrophic gastritis and is associated with reprogramming of differentiation leading to IM. It is consensual that IM is an heterogeneous lesion, though different classification criteria have been proposed throughout the years, either based on morphological features or on mucin expression profiles. The mucin expression profile can be determined by classical histochemical methods using the periodic acid-Schiff, Alcian blue, and high iron diamine staining,[28] or using immunohistochemistry,[5] which was the approach applied in this study. Notwithstanding, all the methods lead to the classification of IM into complete and incomplete subtypes, based on loss or persistence of gastric differentiation together with acquisition of the intestinal one as currently accepted by most authors. [29] Moreover, incomplete IM, regardless of the classification method used, is consistently more frequently associated with progression to cancer.[4,6] The involvement of the transcription factor CDX2 in the transformation process leading to both types of IM is undisputable and now an explanation for the gastric phenotype observed in incomplete IM is suggested by the differential expression of SOX2.

Our results clarified the profile of SOX2 expression, both in normal stomach and in gastric lesions, reinforcing its relationship with gastric differentiation.[30-31] We show, for the first time, that SOX2 localizes predominantly to the neck region of the normal gastric epithelium, which is the proliferative compartment of the mucosa and the

expected location of gastric stem cells.[32] This observation complies with a role of this transcription factor in the maintenance of the adult gastric epithelium, previously observed in mice[15] and is in accordance with observations that SOX2 regulates MUC5AC and pepsinogen A, both of which are gastric differentiation markers.[33-34]

Here, we also show that SOX2 is able to repress CDX2 expression, which might be one of the molecular mechanisms involved in maintaining normal gastric differentiation and, when deregulated, in IM onset. In fact, it was shown in other models that SOX2 downregulates CDX2 expression via SOX21.[25] This adds to other molecular mechanisms that might also be involved in IM, namely the BMP pathway, that is overexpressed in gastric IM, upregulates CDX2 and downregulates SOX2 in a gastric carcinoma cell line.[22,35] The potential interactions and or synergies of these different molecular mechanisms need further clarification.

Finally, we disclosed the expression pattern of SOX2 and CDX2 in gastric dysplasia. We showed that the majority of dysplastic lesions do not express SOX2 whereas all of them express CDX2, which is in accordance with Rugge *et al.*[36] These two events suggest that the progression from normal gastric mucosa to dysplasia occurs concomitantly with gain of intestinal and loss of gastric differentiation, favoring the theory that dysplasia arises from metaplasia. This complies with the conclusions of Gutierrez-Gonzalez *et al*. showing that IM and dysplasia share genetic mutations in tumor suppressor genes, either APC or p53, in three cases.[37] Likewise, Barrett's esophagus which is a very similar lesion to gastric IM, is considered a bona fide preneoplastic lesion where dysplasia ensues. Mutations in p53 or p16 were identified in Barrett's esophagus and dysplasia, further supporting the morphological evidences.[38] Our results thus reinforce the link between IM and dysplasia in the gastric setting, based on the expression of SOX2 and CDX2. This observation, strengthened by previous findings that IM is a lesion difficult to revert or even a "point of no return"[39] has clinically relevant implications namely regarding surveillance of patients with IM, which

is in line with recent epidemiological data.[40] Curiously, SOX2 and CDX2 no longer associate with mucin expression in dysplastic foci. A possible interpretation could be that these genes become subjected to regulatory mechanisms associated with the transformation process.

In this study we have demonstrated that SOX2 is expressed in the normal stomach and is maintained in incomplete IM explaining the gastric differentiation still observed in these lesions. Given that SOX2 expression is lost both in dysplasia and in the complete IM subtype, we hypothesize that incomplete IM is a transient state that precedes the onset of both premalignant lesions (Fig 5). In conclusion, the balance between gastric and intestinal differentiation has a significant impact at least in the early steps of gastric carcinogenesis.

Conflict of interest statement

The authors disclose no conflict of interests.

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FIGURE LEGENDS

Figure 1- SOX2 expression in the normal gastric mucosa. A and C) H&E staining of gastric mucosa from incisura and antrum, respectively. (B and D) Immunodetection of SOX2 (brown) in the same regions of the gastric mucosa.

Figure 2- SOX2 expression in intestinal metaplasia. H&E staining showing gastric mucosa with intestinal metaplasia (A). Double immunodetection of SOX2 (brown) and MUC5AC (red) showing the presence of complete (MUC5AC negative) and incomplete (MUC5AC positive) IM glands (B and inset).

Figure 3- Expression of SOX2, CDX2, MUC5AC and MUC2 in gastric dysplasia. Dysplastic foci were immunostained for the gastric differentiation markers SOX2 (A and inset) and MUC5AC (D) and intestinal differentiation markers CDX2 (B) and MUC2 (D). In (A) arrowhead indicates dysplastic glands with SOX2 expression whereas the arrow indicates dysplastic glands without SOX2 expression. In (D) arrow indicates IM with homogeneous MUC2 expression as opposed to dysplasia where expression is scattered (arrowhead).

Figure 4- SOX2 overexpression in Caco-2 cells. Caco-2 colonic cell line was transiently transfected either with an empty vector or a SOX2 expression vector. The effect of SOX2 expression on CDX2 protein and RNA levels was assessed by western blotting (right) and qRT-PCR (left), respectively.

Figure 5- Schematic representation of gastric carcinogenesis.

Table 1 – Primary antibodies and immunohistochemistry conditions used in this study

Antibody	Clone	Antigen Retrieval Buffer	Antigen Retrieval Conditions	Dilution	Incubation time (min)	Localization	Source
CDX2	CDX2-88	Citrate Buffer 10mM pH6.0	40 minutes at 98ºC	1:50	Overnight (4ºC)	Nuclear	Biogenex, San Ramon, CA
MUC2	PMH1	0,1 U/mL Neuraminidase[a]	2h at 37ºC	undiluted	Overnight (4ºC)	Cytoplasmatic	Supernatant [27]
MUC5AC	CLH2	none	none	1:10	Overnight (4ºC)	Cytoplasmatic	Supernatant [27]
MUC6	CLH5	none	none	1:10	Overnight (4ºC)	Cytoplasmatic	Supernatant [27]
SOX2	SP-76	EDTA 10mM pH8.0	40 minutes at 98ºC	1:50	1h (Room Temperature)	Nuclear	Cell Marque, Rockling, CA

[a]Neuraminidase from *Clostridium perfringes* type VI (Sigma) was diluted in sodium acetate buffer (pH 5.5)

Table 2 - Expression of SOX2 in gastric IM.

	SOX2		
Parameter	**Negative n (%)**	**Positive n (%)**	***p* value**
Complete IM foci (n=29)	27 (93%)	2 (7%)	<0.0001
Incomplete IM foci (n=26)	4 (15%)	22 (85%)	

Table 3 - Expression of SOX2, CDX2, MUC5AC, MUC2 and MUC6 in dysplasia

	Dysplasia (n=26)	
Parameter	**Positive n (%)**	**Negative n (%)**
SOX2	3 (12%)	23 (88%)
CDX2	20 (100%)	0 (0%)
MUC5AC	15 (58%)	11 (42%)
MUC2	12 (46%)	14 (54%)
MUC6[a]	17 (68%)	8 (32%)

[a]In one case, MUC6 expression could not be evaluated

Figure 1

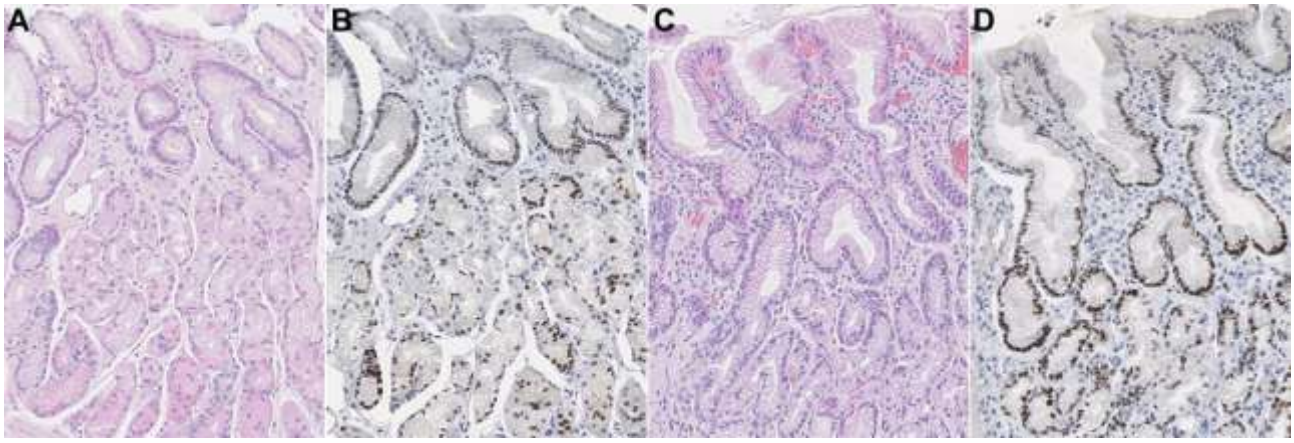


Figure 2

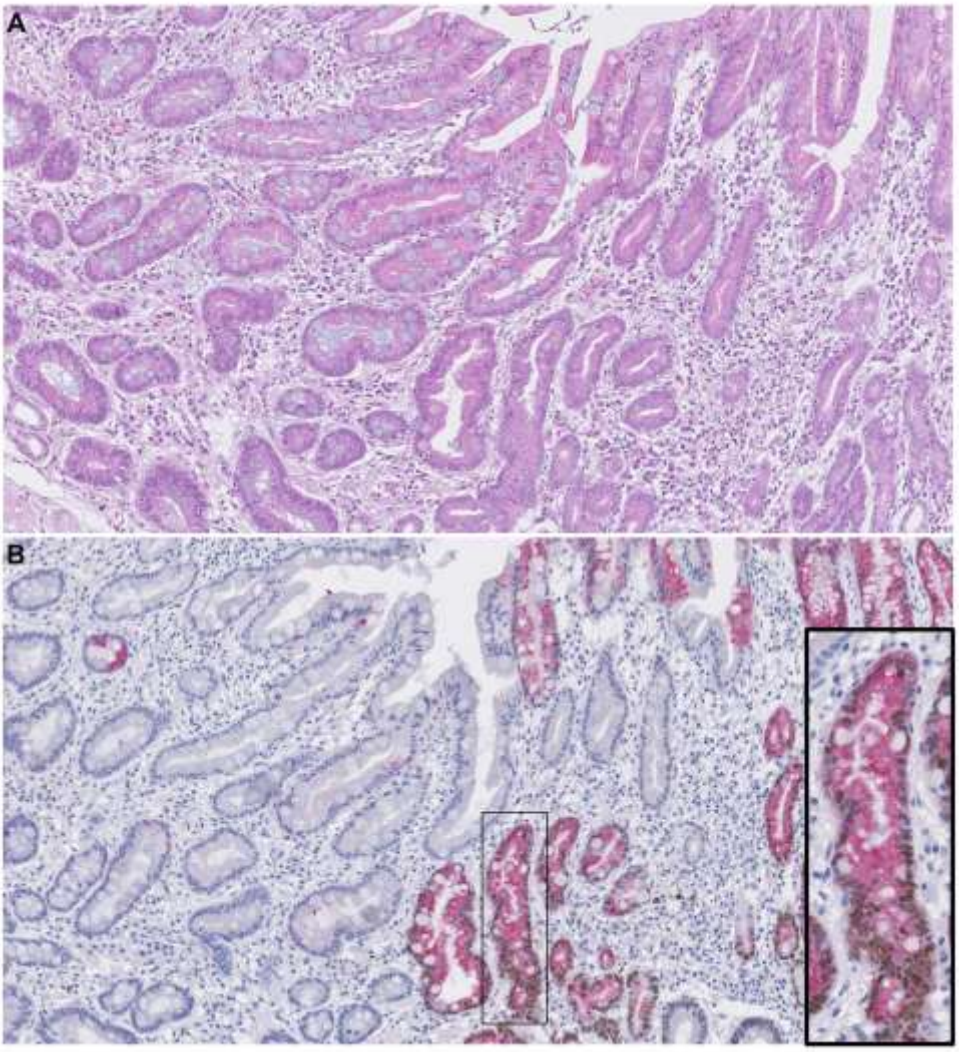


Figure 3

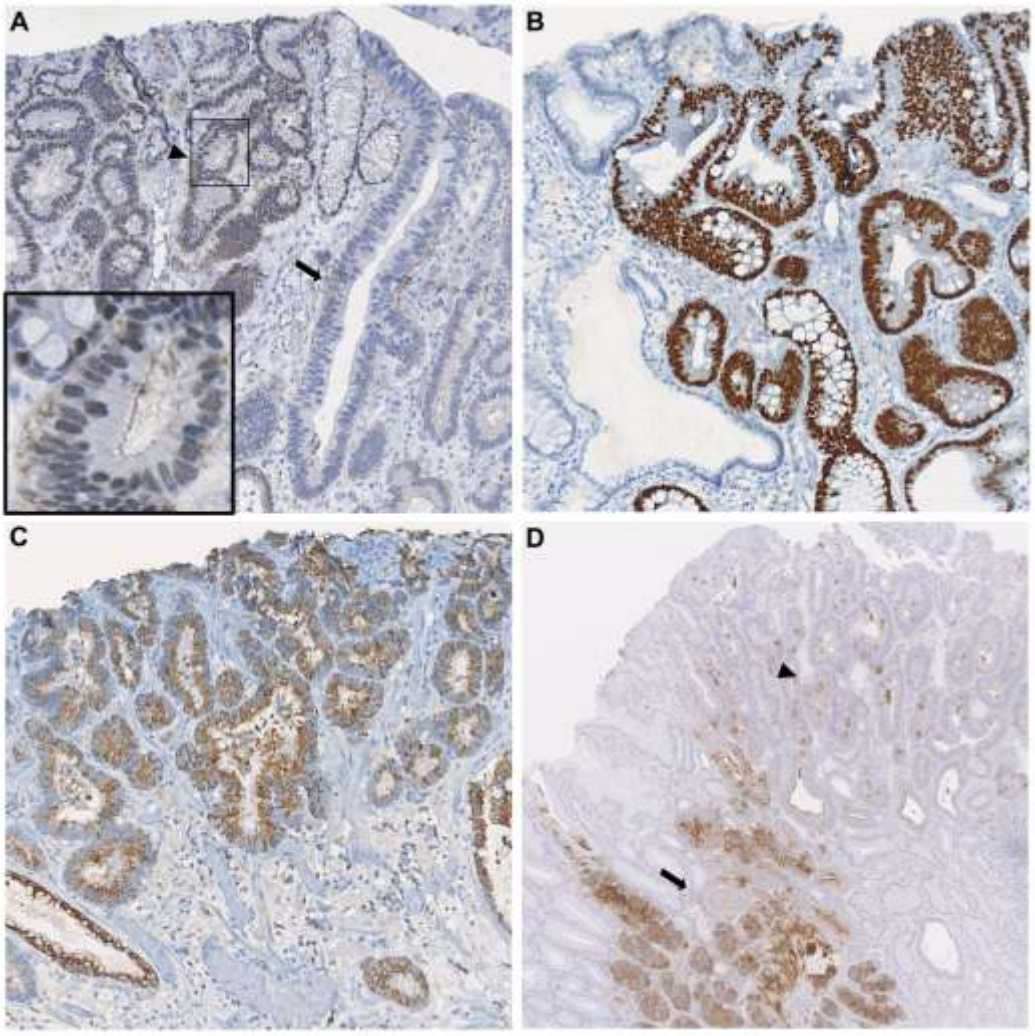
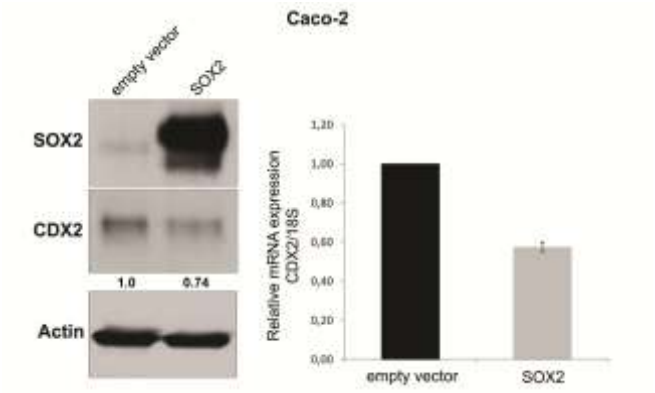


Figure 4


Caco-2
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SOX2
SOX2
CDX2
1.0
0.74
Actin
Relative mRNA expression
CDX2/18S
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1,00
0,80
0,60
0,40
0,20
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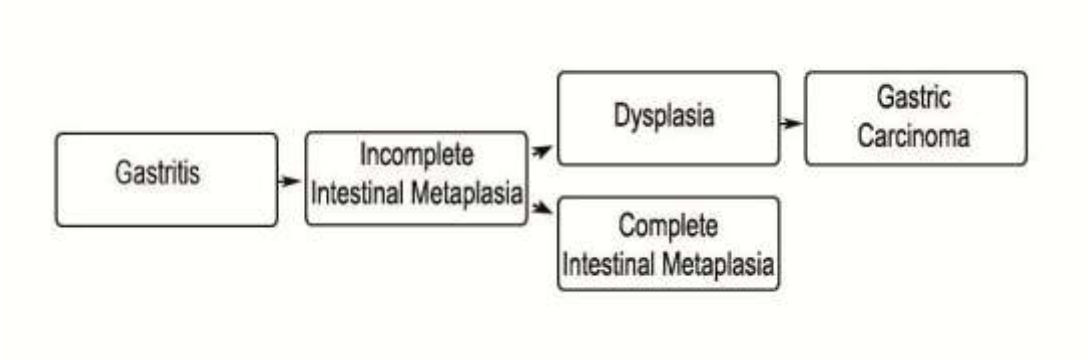
SOX2

Figure 5


Gastritis
Incomplete Intestinal Metaplasia
Dysplasia
Gastric Carcinoma
Complete Intestinal Metaplasia

DISCUSSION

In spite of our well-established understanding of the phenotypic natural history occurring in the shift from native epithelia to invasive intestinal-type carcinoma in the gastric mucosa, the molecular typing of the precancerous changes in gastric mucosa remains elusive.[1] It is, however, becoming evident that the balance between gastric and intestinal differentiation may play a significant role, at least in the early steps of the carcinogenesis cascade. It has been shown that the transcription factors SOX2 and CDX2 are stomach and intestine-specific, respectively,[2, 3] thus they may provide a useful tool to analyse this cascade.

In this study, we performed a systematic characterization of SOX2 expression along the precancerous cascade using immunohistochemistry in a series of 10 cases of normal gastric mucosa obtained from patients with no gastric pathology, 55 foci of IM adjacent to gastric carcinomas or to dysplasia, and 26 foci of dysplasia. We also used in vitro models to determine if there is a cross-regulation between SOX2 and CDX2.

Contrary to other authors, we have used an anti-SOX2 monoclonal antibody, raising the confidence level on the immunohistochemistry method and results. Also, the double immunostaining technique provides valuable information about the co-localization of the different markers. The small number of cases included might be pointed out as a limitation of the study.

We have shown that SOX2 expression is localized especially in the neck region of normal gastric glands, which is similar to other authors' findings.[4] This is the proliferative compartment of the mucosa and the expected location of gastric stem cells,[5, 6] which is consistent with previous papers describing SOX2 role in stem cell maintenance.[7] However, the number of positive cells is greater than expected if SOX2 was only expressed in stem and progenitor cells, which suggests that this transcription

factor is not only required to switch on a gastric differentiation program, but also to maintain it in terminally differentiated cells.

In intestinal metaplasia (IM), SOX2 is expressed in cases that maintain a gastric phenotype, characterized by the presence of the MUC5AC mucin and defining the incomplete IM subtype. By contrast, in complete IM, SOX2 expression is lost concomitantly with loss of MUC5AC. These two aspects are especially evident with the double SOX2/MUC5AC immunostaining. Previous studies have also described SOX2 expression in human IM and, despite a different IM classification, the co-expression of SOX2 with MUC5AC or the absence of both SOX2 and MUC5AC was also observed.[4, 8] These observations are according with the expected role of SOX2 in the maintenance of gastric differentiation in incomplete type IM.

The expression pattern of SOX2 in gastric dysplasia was so far unknown. We showed for the first time that the majority of dysplastic lesions do not express SOX2 (3 out of 26) whereas all of them express CDX2. The positive cases were all low-grade dysplasias. One would be tempted to relate this finding with the previous reports of SOX2 possible tumour suppressor role,[9] but the series of cases is short and it would be a risky shot. Despite our expectation of finding a relation between SOX2 and MUC5AC expression in dysplasia, we could not find such association nor with CDX2 expression in this lesion.

Although gastric carcinogenesis still is a mind-breaking issue, it is recognized that one of its major pathways develops in an *H. pylori*-induced background of chronic atrophic gastritis and is associated with reprogramming of differentiation leading to IM. CDX2 is documented as being a reliable responsible for this intestinal shift, leading to both types of IM. The differential expression of SOX2 in IM subtypes brought out the rationale for the maintenance of gastric differentiation in incomplete type IM.

While CDX2 expression is maintained in the progression to gastric dysplasia, SOX2 expression is substantially lost. These two events suggest that the progression from normal gastric mucosa to dysplasia occurs concomitantly with gain of intestinal and loss of gastric differentiation.

The regulatory mechanisms underlying CDX2 activation and SOX2 downregulation in the gastric setting are closer to be understood. A previous report of this group has shown that the BMP pathway, which is overexpressed in gastric IM, upregulates CDX2 and downregulates SOX2 in a gastric carcinoma cell line.[10, 11] Furthermore, conditional knock-out of the CDX2 gene in mice intestines' showed increased SOX2 expression, with a consequent shift from an intestinal to a gastric/esophageal differentiation.[12, 13] The same was observed when ectopic expression of SOX2 was induced in the intestine.[14] These observations point to a mutual negative regulation of SOX2 and CDX2. In this study, we could not confirm the regulation of SOX2 by CDX2 by simply transfecting cell lines with CDX2, which suggests that co-factors or mediators are required for this process. We could though confirm SOX2 ability to negatively regulate CDX2 expression in one cell line.

One possible interpretation of our results would be that upon adverse events, the normal gastric mucosa evolves to a transient state of incomplete IM induced by CDX2. From this point, both SOX2 expression and gastric differentiation are lost, leading to complete IM with low evidence for further carcinogenic evolution. Otherwise, for unknown reasons, the transient incomplete IM expressing both transcription factors creates an unstable setting, prone to acquire genetic alterations in oncogenes or tumour suppressor genes that induces evolution to dysplasia, while maintaining the progressive loss of SOX2. This is also observed in gastric adenocarcinoma.[9, 15]

In summary, SOX2 is expressed in the normal stomach and is maintained in incomplete IM explaining the maintenance of a gastric differentiation program in these

lesions. SOX2 expression is lost in the dysplastic lesions and in the complete IM subtype, so we hypothesize that incomplete IM may precede the onset of them both. The balance between gastric and intestinal differentiation has a significant impact in gastric carcinogenesis, at least in the early steps.

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ANEXO

NORMAS DE PUBLICAÇÃO

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Introduction

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