# miR-24-3p Regulates CDX2 During Intestinalization of Cardiac-Type Epithelium in a Human Model of Barrett´s Esophagus

Short Title: miR-24-3p downregulation and CDX2 expression

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Abbreviations: BE, Barrett’s esophagus; cDNA, complementary DNA; deoxyribonucleic acid; FFPE, formalin-fixed and paraffin-embedded; GERD, gastroesophageal reflux disease; IHC, immunohistochemistry; IM, intestinal metaplasia; IRB: institutional review board; ISH, in situ hybridization; miRNAs, microRNAs; mRNA, messenger RNA; NSCE, non-specialized columnar epithelium; PCR: polymerase chain reaction; RNA: ribonucleic acid; RT: reverse transcription; SD, standard deviation; UV: ultraviolet.

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## ABSTRACT (word count 257)

**Background:** Cardiac-type epithelium has been proposed as the precursor of intestinal metaplasia in the development of Barrett´s esophagus. Dysregulation of microRNAs (miRNAs) and their effects on CDX2 expression may contribute to intestinalization of cardiac-type epithelium. The aim of this study was to examine the possible effect of specific miRNAs on the regulation of CDX2 in a human model of Barrett's esophagus.

**Methods:** Microdissection of cardiac-type glands was performed in biopsy samples from patients who underwent esophagectomy and developed cardiac-type epithelium in the remnant esophagus. OpenArray™ analysis was used to compare the miRNAs profiling of cardiac-type glands with negative or fully positive CDX2 expression. CDX2 was validated as a miR-24 mRNA target by the study of CDX2 expression upon transfection of miRNA mimics and inhibitors in esophageal adenocarcinoma cell lines. The CDX2/miR-24 regulation was finally validated by *in situ* miRNA/CDX2/MUC2 co-expression analysis in cardiac-type mucosa samples of Barrett's esophagus.

**Results:** CDX2 positive glands were characterized by a unique miRNA profile with a significant downregulation of miR-24-3p, miR-30a-5p, miR-133a-3p, miR-520e-3p, miR-548a-1, miR-597-5p, miR-625-3p, miR-638, miR-1255b-1 and miR-1260a, as well as upregulation of miR-590-5p. miRNA-24-3p was identified as potential regulator of *CDX2* gene expression in three databases and confirmed in esophageal adenocarcinoma cell lines. Furthermore, miR-24-3p expression showed a negative correlation with the expression of CDX2 in cardiac-type mucosa samples with different stages of mucosal intestinalization.

**Conclusion:** These results showed that miRNA-24-3p regulates CDX2 expression, and the downregulation of miRNA-24-3p was associated with the acquisition of the intestinal phenotype in esophageal cardiac-type epithelium.


**Keywords:** Barrett's esophagus; CDX2 transcription factor; intestinal metaplasia; cardiac-type epithelium; microRNAs.

## Introduction

The development of metaplasia of non-intestinal columnar type (cardiac-type) represents the earliest morphological appearance of glandular differentiation in the distal esophagus damaged by chronic gastroesophageal reflux disease (GERD). This cardiac-type metaplasia is characterized by monotonous mucous cells, which may evolve with time into an specialized intestinal type of mucosa (villiform surface, mucous cells and goblet cells) in Barrett´s esophagus.[1-4] Its pathogenesis remains to be elucidated with no agreement about the cells of origin.[4] An interesting recent finding is the increased expression of BMP-4 and activation of its pathway in BE and in esophagitis caused by GERD.[5] In fact, the upregulation of BMP-4 excretion in the mesenchyme of the esophagus determines the activation of BMP-4 signalling pathway and modulates the gene expression of the proliferating stem cells located at the basal layer of the esophageal epithelium, leading to a simple type of columnar metaplasia.[5,6] In addition, simultaneous or sequential activation of intestinal-related pathways, such as upregulation of CDX2 may direct the cardiac-type epithelium towards an intestinal phenotype. Lavery et al.[7] suggested that CDX2 expression in a stem cell of Barrett´s glands can lead to progenitors with intestinal phenotype (TFF3/MUC2+). Eventually, niche succession may fill the niche with TFF3+/MUC2+ stem cells giving rise to the fully intestinalized gland seen in BE. Using immunohistochemistry (IHC) methods, CDX2 expression has been demonstrated in 100% of biopsy specimens from intestinal metaplasia, although in cardiac-type epithelium appear in only 30% of the cases.[8] This finding suggests that the expression of CDX2 could be totally or partially inhibited at the squamous- and cardiac-type epithelium stages, respectively. In addition, a lack of methylation at the level of the *CDX2* gene promoter was found in the specialized intestinal metaplasia of BE, while methylation was maintained in normal squamous cell epithelium. However, methylation of the *CDX2* promoter has not been associated with mRNA expression in gastric cancer cell lines.[9,10]

Of note, posttranscriptional regulation of CDX2 expression by microRNAs (miRNAs) may represent another mechanism of inhibition of this gene. miRNAs are an abundant class of endogenous, small (19-25 nucleotides long) noncoding RNAs with a primary role implicated in posttranscriptional gene

silencing.[11] In fact, through their either perfect or imperfect binding to the 3´-untranslated region (3´-UTR) of target transcripts, microRNAs trigger messenger RNAs (mRNA) degradation or translational repression.

To date, limited data are available on miRNA expression during the progression from esophagitis to cardiac-type epithelium and intestinal metaplasia. In a previous study, Fassan et al.[12] have compared the miRNA expression profiling of cardiac-type epithelium vs. intestinal metaplasia, and demonstrated a similar miRNA expression signature except for the significant downregulation of miR-18a/miR-203/miR-205 and the upregulation of miR-192/miR-215. On the other hand, using a human model of BE, a previous study of our group found variable CDX2 protein expression levels in 63% of cardiac-type biopsies from the remnant esophagus in patients undergoing esophagectomy.[3] A spectrum ranging from CDX2 negative glands, glands with scattered positive nuclei or fully stained CDX2 glands was observed at follow-up endoscopic biopsies. However, microdissection of glands showing different patterns of CDX2 expression may better characterize the role of specific miRNAs in the modulation of CDX2 expression before the development of intestinal metaplasia.

This study aimed to identify differentially expressed miRNAs in the cardiac-type epithelium at two different stages of CDX2 immunoexpression before the appearance of intestinal metaplasia and to confirm the regulation of CDX2 by the specific miRNAs identified.

## METHODS

*Study Population and Biopsy Specimens*

A series of 21 patients who underwent esophagectomy for cancer between 2007 and 2013 were enrolled for this study. All these patients agreed to participate in a prospective endoscopic surveillance study with endoscopies and multiple biopsies performed every 6 months during the first two years after surgery and every year afterwards. Only patients who developed areas of histologically proven non-specialised columnar metaplasia (cardiac-type epithelium) in the remnant esophagus (12 out of 21) were further considered in the study. Biological samples were stored and later on obtained from Parc de Salut Mar Biobanc (MARBiobanc).

A second series of 12 patients with an endoscopic and histopathologic diagnosis of long-segment BE were selected from the archives of the surgical pathology unit of the University of Padua. Two matched biopsy samples were selected from each of these 12 patients according to the presence of intestinal metaplasia. In particular, a biopsy was representative of cardiac-type metaplasia (i.e. no intestinal metaplastic glands detected) and the other was characterized by complete intestinalization of the metaplastic epithelium.

The study was approved (13/5286/I) by the Institutional Review Board (IRB) of the Hospital del Mar Medical Research Institute (Barcelona, Spain) and the validation study by the IRB of Padua University Hospital (Padua, Italy).

*Tissue Processing and Immunohistochemistry*

All biopsies were formalin-fixed and paraffin-embedded (FFPE) for pathological diagnosis. One 3μm thick section of each biopsy was stained with haematoxylin/eosin and evaluated by an expert gastrointestinal pathologist for the presence of non-specialized columnar epithelium (NSCE) and intestinal metaplasia. The diagnosis of NSCE was made when the glands were composed of mucous cells only, with no parietal or chief cells. The diagnosis of intestinal metaplasia (IM) required the presence of definitive goblet cells within glands. Standard immunohistochemistry (IHC) for CDX2 was performed on 3 μm thick sections of FFPE tissue as previously described.[13]

*Immunoguided Laser Assisted Microdissection*

Microdissection of glands was performed using a Leica LMD 6000 Laser Microdissection Microscope (Leica Microsystems, Wetzlar, Germany). Membrane slides PEN 2.0 µm (Leica) were irradiated with ultraviolet (UV) light at 254 nm for 80 min. After UV treatment, each slide was coated with poly-L-lysine 0.1% (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. These two steps improve tissue adhesion to the membrane.[14] For drying, the slides were placed in a side holder for 60 min at room temperature. Three 7 µm consecutive tissue sections were cut in a microtome and mounted on the pre-treated membrane slides and preserved at -80ºC until the immunostaining and microdissection.

CDX2 immunostaining was performed with minor modifications. Antigen retrieval was performed using DAKO PT link reagent (Agilent Technologies, Santa-Clara, CA, USA) during 20 min at 97ºC, because loss of tissue with the standard method was detected. The remaining procedure until developing with 3'3'-diaminobenzidine was carried out using standard IHC methods. Microdissection was performed in three consecutive sections containing glands fully expressing CDX2 and adjacent areas without expression. Microdissected tissue was immediately placed into a collection vessel containing a minimum of 160 µL of deparaffinization solution (Qiagen, Hilden, Germany) and finally stored at -80ºC.

*RNA Extraction and Quantification in Microdissected Samples*

Total RNA including miRNAs from microdissected cardiac-type glands showing different patterns of CDX2 expression (negative and positive) was isolated using miRNeasy FFPE Kit (Qiagen, Hilden, Germany). The quantity and quality of the extracted RNA was measured running the RNA 6000 Pico Lab-on-a-Chip on the Bioanalyzer 2100 equipment (Agilent Technologies, Santa-Clara, CA, USA). A filter of < 200 nt was applied in order to obtain a more accurate quantification of small RNAs.

*miRNA Expression in Microdissected Glands using OpenArray™Platform*

Reverse transcription (RT) and preamplification were performed on all samples using Megaplex™ Primer Pools A v2.1 and Megaplex™ RT Primers, Human Pool B v3.0 (Thermo Fisher Scientific, Waltham, MA, USA) with the recommended protocol (optimized protocol with low sample input for profiling human microRNA using the OpenArray™ platform. Version 5 February 2013) for TaqMan™ OpenArray™ Human microRNA panel (Thermo Fisher Scientific, Waltham, MA, USA).

The cDNA was prepared using total RNA obtained as previously described using Taqman™MicroRNA Reverse Transcription kit and Megaplex™ RT Primer Pools (Thermo Fisher Scientific, Waltham, MA, USA) in a 7.5 μL final volume. The cycling conditions used for RT were those recommended by the manufacturer. Preamplification for all samples was performed using the entire 7.5 μL of RT product in a final volume of 40 μL, with 16 cycles of preamplification performed in samples.

We prepared the OpenArray™ load plate with the preamplified product diluted 1:2 with Taqman™ OpenArray™ Real-Time PCR Master Mix (PN 4462164) in a final volume of 6μl. OpenArray™ Plates were loaded with AccuFill using standard AccuFill method (OpenArray™ AccuFill™ System User Guide, Thermo Fisher Scientific, Waltham, MA, USA). Quality control was performed on OpenArray's raw data using the ExpressionSuite Software v1.0.3 (Thermo Fisher Scientific, Waltham, MA, USA). All samples were included in posterior analyses and data exported were analyzed in R environment v 3.2.3. Global normalization was performed on all samples to obtain ΔCt measures[15]. Mean of expression was obtained for technical replicates.

*Cell Lines, Transfection Experiments and Reagents*

The human esophageal adenocarcinoma cells JH-EsoAd1, FLO-1, KYAE-1, OE19 and OE33 were kindly provided by Dr. Winand Dinjens (Erasmus University Medical Center Rotterdam, The Netherlands). JH-EsoAd1 and FLO-1 were cultured in DMEM medium supplemented with 10% FBS, and KYAE-1, OE19 and OE33 in RPMI-10% FBS medium. Western blot analysis was performed as previously described[13] using polyclonal anti-CDX2 (EPR27644 Abcam) and monoclonal anti-β-ACTIN (clone AC15, Sigma-Aldrich, St. Louis, MO, USA) antibodies.

FLO-1 and KYAE-1 cell lines were selected for transfection experiments based on their CDX2 and miRNA expression levels. Cells were transfected with 30nM of hsa-mir-24-3p *mir*Vana™ miRNA mimic, hsa-miR-24-3p *mir*Vana™ miRNA inhibitor or *mir*Vana™ miRNA Inhibitor negative control using Lipofectamine RNAiMax (all ThermoFisher Scientific, Waltham, MA, USA).

*RNA Isolation and Quantitative Real-Time PCR*

RNA from cell lines was extracted using RNeasy Plus Micro kit (Qiagen, Hilden, Germany) following reverse transcription using the Transcriptor First Strand cDNA Synthesis Kit (Roche). RNA from FFPE samples was extracted using the RecoverAll kit (Ambion, Austin, TX, USA). qRT-PCR was performed using the QuantStudio System (ThermoFisher Scientific, Waltham, MA, USA). The specific *CDX2* primers were: Fw: GGAGCTGGAGAAGGAGTTTCAC and Rv: GCTCTGCGGTTCTGAAACCA. HPRT was analysed as an internal control using the primers: Fw: GGCCAGACTTTGTTGGATTTG and Rv: TGCGCTCATCTTAGGCTTTGT. Primers for hsa-miR-24-3p qRT-PCR were purchased from TaqMan™ Advanced Assay miRNAs (Thermo Fisher Scientific, Waltham, MA, USA). For normalization we used miRNAs ath-miR-159a, cel-Lin-4 and cel-miR-2 as spike-in controls (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR for FFPE derived RNA was performed by using the NCode miRNA method (Invitrogen, Carlsbad, CA) on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Normalization was done with the small nuclear RNA U6B. PCR reactions were run in triplicate including no-template controls. The data were analyzed using the comparative Ct method.

*miR-24-3p/CDX2 Co-expression and MUC2 Analysis*

Protein/miRNA co-expression analysis was carried out as previously described with minor modifications.[16] In situ hybridization (ISH) for miR-24-3p and the positive control U6 were performed using the 5' digoxigenin-tagged LNA probe (Exiqon). Negative controls included the omission of the probe and the use of a scrambled LNA probe (Exiqon). Only cytoplasmic miR-24-3p intensity was retained for scoring. After ISH, we performed the immunohistochemistry for CDX2 (clone EP25; Leica Biosystems, Milan, Italy), using the Benchmark LT

automated system from Leica Microsystems BOND-MAX according to the manufacturer's instructions. Protein/miRNA expression was quantified analysing chromogen-specific intensity with ImageJ (NIH). IHC analysis for CDX2 and MUC2 (clone CCP58; Leica Biosystems) was performed on a Benchmark LT automated system from Leica Microsystems BOND-MAX according to the manufacturer's instructions.

*Statistical Analysis*

Data are expressed as mean and standard deviation (SD) or median and interquartile range (IQR) according to their distribution. Differences between groups were tested by applying a paired *t*-test on the ΔCts values and the Wilcoxon matched-pairs signed-rank test for the validation study. *P* values were adjusted for multiple comparisons using false discovery rate.[17] Adjusted *P* values < .05 were considered statistically significant. Data were analyzed using R environment v3.2.3. statistical program.

## Results

Multiple tissue samples (from 4 to 13) were obtained during follow-up endoscopies in 12 patients with known columnar metaplasia developed in the remnant esophagus after esophagectomy procedures. The mean length of the columnar metaplasia segment was 15.5 mm (range 5-30 mm). The median follow-up to the development of cardiac-type mucosa and to the first detection of CDX2 expression was 19.5 (IQR 12-27) and 24.6 (IQR 12-24) months, respectively. IHC analysis was performed to detect CDX2 positive samples and to confirm scattered nuclear expression in biopsies with cardiac-type epithelium at different time periods (Table 1). In 15 out of 55 biopsies (27%) from 5 patients, isolated glands fully expressing CDX2 surrounded by glands with interspersed CDX2 positive nuclei or negative CDX2 expression were found. Only these biopsies were considered and used for microdissection (Figure 1). The total number of tissue samples microdissected is shown in Table 2.

*OpenArray™ Expression Data*

Considering that the amount of RNA obtained from microdissected glands was limited and lower than that recommended for the OpenArray™ analysis, we aimed to identify a threshold of concentration able to detect more than 75% of the endogenous miRNAs. Therefore, we used three Barrett´s intestinal metaplasia samples (not included in the study) to establish the most adequate threshold of detection. A concentration threshold of 0.052 ng/μL/sample resulted as the most adequate for our purposes.

The OpenArray™ analysis was performed in 4 samples with CDX2 positive glands (A) and 4 samples with CDX2 negative glands (B) (Table 3). The results of the OpenArray™ analysis are shown in Figure 2. Overall, a total of 11 differentially expressed miRNAs comparing groups A and B was observed. CDX2 positive glands showed significant downregulation of 10 miRNAs (miR-24-3p, miR-30a-5p, miR-133a-3p, miR-520e-3p, miR-548a-1, miR-597-5p, miR-625-3p, miR-638, miR-1255b-1 and miR-1260a) and upregulation of miR-590-5p.

Examining four databases (MicroCosm Targets Version 5, miRanda Human miRNA Targets, TargetScan Human 7.1 and microRNA.org) several putative miRNAs binding sites within the 3´-UTR of *CDX2* mRNA were found. Also, three out of the four databases predicted miR-9, miR-22 and miR-24-3p as potential regulators of *CDX2* gene expression. Since miR-24-3p was also identified by OpenArray™ analysis, this miRNA for further validation in tissue samples and functional studies in cell lines was selected.

*Expression of CDX2 and miRNA-24-3p in Esophageal Adenocarcinoma Cell Lines*

In gastroesophageal adenocarcinoma cell lines, *CDX2* gene and protein expression was evaluated by quantitative real-time PCR (qRT-PCR) and Western blot, respectively. Figure 3A shows that *CDX2* was expressed in all five gastroesophageal adenocarcinoma cell lines at different protein levels, with KYAE-1 showing the highest expression and JH-EsoAd1 the lowest expression. The expression of *CDX2* was also evaluated by qRT-PCR; overall, comparable levels of mRNA have been found in all cell lines, except for the KYAE-1 cell line that showed significantly higher *CDX2* expression (Figure 3B). Interestingly, an inverse relationship between the abundance of *CDX2* mRNA and the levels of

miRNA24-3p in the KYAE-1 cell line respect to the FLO-1 cell line was found. The remaining cell lines showed no differences in miRNA24-3p expression compared to the KYAE-1 cell line (Figure 3C).

*CDX2 Regulation by miR-24-3p*

To elucidate the effect of miRNA-24-3p expression on *CDX2* expression, KYAE-1 and FLO-1 cell lines were transfected with a synthetic double-stranded RNA that mimics the cellular precursor of miRNA-24-3p (miRNA-24 mimic) and a single-stranded RNA oligonucleotide that has been designed to bind and inhibit the activity of the endogenous miR-24-3p (miRNA-24 inhibitor). After transfection of KYAE-1 cell line with the miRNA-24 mimic, the expression levels of miR-24-3p markedly increased compared to the mock transfection or the negative control. Instead, transfection of KYAE-1 cell line with the miRNA-24 inhibitor resulted in reduced levels of miR-24-3p, confirming its inhibitory action (Figure 4A). Concerning CDX2 protein expression, mock transfected cells and cells transfected with either miRNA-24 inhibitor or the negative control showed comparable protein levels, while CDX2 levels strongly decreased in cells transfected with miRNA-24 mimic (Figure 4B). Similar results were obtained using the FLO-1 cells (see Supplementary Figure 1). Notably, for both KYAE-1 and FLO-1 cell lines, no significant differences in *CDX2* mRNA expression have been found comparing cells transfected with the miRNA-24 mimic or inhibitor and the negative control (Figure 4C, Supplementary Figure 1).

*miRNA-24-3p/CDX2 Inverse Correlation*

The negative correlation between miR-24-3p and CDX2 expression was further investigated by miR-24-3p ISH and CDX2 immunohistochemical analyses on same biopsies used for the OpenArray™ analysis (Figure 5). The intestinal metaplastic glands were characterized by positive staining of CDX2 and a faint or negative staining of miR-24-3p (Figure 5A, C). In intestinalized mucosa that has coexistence of both intestinal and cardiac-type columnar epithelia, higher expression of miR-24-3p was consistently observed in the CDX2 negative cells compared to the CDX2 positive surrounding cells (Figure 5B). On the other hand, non-intestinalized CDX2 negative glands showed a moderate miR-24-3p expression (Figure 5B, C). These data were confirmed by image software

analysis (ImageJ) showing a significant miR-24-3p deregulation in intestinalized glands ($P < .001$; Figure 5D).

Moreover, miR-24-3p expression was assessed by both ISH and qRT-PCR in a series of matched biopsies obtained from 12 patients with long segment Barrett's esophagus. miR-24-3p levels were significantly upregulated in cardiac-type mucosa as compared to those observed in intestinalized CDX2/MUC2 positive epithelia (Figure 6A). MUC2, a *bona fide* target gene of CDX2, was used as a marker of the transcriptional activity of CDX2.[18,19] Image software analysis showed a significantly downregulation of miR-24-3p in all but three matched samples of intestinal metaplasia ($P = .004$; Figure 6B). qRT-PCR analysis performed on the same samples confirmed a significant downregulation of miR-24-3p in all but one of the CDX2/MUC2 positive intestinal metaplasia samples ($P = .001$; Figure 6C).

## Discussion

Clinical observations strongly support that cardiac-type epithelium composed of non-goblet columnar glands represents an intermediate step during the process leading to intestinal metaplasia in BE.[3,20] Recent evidences indicate that the cardiac gland contains originally gastric cell lineages and subsequently evolves into a mixture of both gastric and intestinal lineages that are clonal.[7,21] *De novo* expression of CDX2 in a stem cell will give rise to intestinal lineages that converts a gastric gland into a partially (mixed glands) and, eventually, fully intestinalized gland.

It has been shown that more than 50% of patients undergoing esophagectomy and reconstruction with gastric conduit develop cardiac-type epithelium in the remnant esophagus.[22] Using this human model of BE, we previously reported the early appearance of CDX2 expression in cardiac-type glands, ranging from scattered positive nuclei within a gland to glands fully expressing CDX2 and presence of goblet cells.[3] These findings fit well with the model about the clonal acquisition of the intestinal phenotype in BE where CDX2 plays a critical role.

The molecular mechanisms driving CDX2 activation in non-goblet columnar epithelium are poorly characterized. Some studies focused on miRNA dysregulation in each step of Barrett´s carcinogenesis have shown specific

miRNA expression signatures associated with cancer progression.[23-25] By contrast, the molecular profiling of BE-related metaplastic changes remains unclear. The single study of Fassan et al.[12] has addressed the characterization of the molecular profile of non-goblet columnar metaplasia (cardiac-type epithelium) versus intestinal metaplasia in BE. They described different miRNA expression pattern in cardiac-type epithelium versus intestinal metaplasia, in particular the downregulation of miR-203 and miR-205.

In this study, we have investigated the differential miRNA expression in cardiac-type epithelium of glands fully expressing CDX2 and glands negative for CDX2. Ten miRNAs (miR-24-3p, miR-30a-5p, miR-133a-3p, miR-520e-3p, miR-548a-1, miR-597-5p, miR-625-3p, miR-638, miR-1255b-1 and miR-1260a) were found downregulated in CDX2 positive glands, while a single miRNA (miR-590-5p) was selectively upregulated.

Among the downregulated miRNAs, miRNA-24-3p appears in three databases as potential regulator of the *CDX2* gene so we further validated its putative regulatory role. Transfection of miRNA-24-3p mature RNAi in the esophageal adenocarcinoma-derived cell lines FLO-1 and KYAE-1 resulted in a reduction of CDX2 protein expression in both cell lines. It may be speculated that this reduction is due to the interference of miRNA-24-3p in the *CDX2* translation, since we showed that the *CDX2* mRNA levels are not affected by the transfection with the miRNA-24-3p mimic (Figure 4, Supplementary Figure 1). Translational repression by miRNA-24-3p has been described for other target mRNAs such as protein coding *cingulin*,[26] *p130*$^{Cas}$,[27] or *ARNT*.[28] However, miRNA-24-3p modulates gene expression through different mechanisms; in fact for other genes, such as *BIM*[29] or *FSCN1*,[30] it acts by regulating their mRNA levels, while for others, like *CSN5/Jab1*, both types of mechanisms can coexist.[31] Of note, miR-24-3p was identified as a regulator of both Notch1 and its Delta-like ligand 1 (Dll1),[32] which were recently described as master regulators of differentiation of gastric cardia progenitor cells in a mouse model of BE.[33] Thus, further studies should investigate whether the effect of miR-24-3p down-regulation is multi-targeting affecting a series of important gene expressions in the acquisition of the intestinal phenotype.

Co-expression analyses using in situ hybridization (IH) and immunohistochemistry (IHC) showed that the levels of miR-24-3p were higher in

CDX2 negative cardiac-type glands compared to adjacent glands fully expressing CDX2, which is consistent with a repressive effect of miR-24-3p on CDX2. These observations were validated in an independent set of samples from patients with long segments of BE, where an inverse pattern of expression for miR-24-3p and CDX2 in cardiac-type glands with and without intestinal phenotype was confirmed. Moreover, the levels of MUC2, a downstream target for CDX2, were high in CDX2-positive fully intestinalized glands, while MUC2 was negative in CDX2 negative cardiac-type epithelium expressing miR-24-3p.

miR-24-3p was previously found to be overexpressed in several types of cancer.[26,34,35] Also, Viswanathan et al.[36] noted that miR-24-3p was strongly induced upon blastocyst-derived trophoblastic stem cells differentiation in parallel with a decline in the levels of CDX2, suggesting that miR-24-3p may contribute to CDX2 repression, although no functional studies were performed.

To our knowledge, this is the first study investigating the regulation of CDX2 by miRNAs in BE. In gastric cell lines, miR-9 can repress CDX2 expression promoting cell proliferation[37] and in bile acids-induced gastric intestinal metaplasia, miR-92a-1-5p increases CDX2 expression by targeting FOXD1.[38] In addition, Matsuzaki et al.[39] observed increased levels of miR-221 and miR-222 in human esophageal adenocarcinoma tissues compared with areas of BE. They found that the exposure of esophageal cells to bile acids activates *FXR* and increases the levels of miR-221 and miR-222, which in turn reduces the levels of their target mRNA $p27^{Kip1}$, promoting the degradation of CDX2 by the proteasome.

The role of the other miRNAs in the development of BE remains uncharacterized. A tumour suppressive as well as an oncogenic role has been described for miR-30a-5p.[40] In the context of esophageal squamous cell carcinoma, low expression of miR-30a-3p/5p has been associated with advanced tumour stages and poor prognosis.[41] Kan et al.[42] found increasing levels of miR-30a-5p, miR-590-5p and miR-625-3p in the sequence from normal esophagus epithelium-Barrett's esophagus-adenocarcinoma. Although, the role of these three miRNAs have not been investigated in the present study, results are consistent with upregulation of miR-590-5p in fully intestinalized glands, which are abundant in BE and more prone to neoplastic transformation.

In relation to miR-625-3p, Wang et al.[43] have described its downregulation in esophageal tumors compared to normal epithelium, which also correlated with advanced tumor stages and the presence of metastasis. Although in this study[41] the histological tumor type is not reported, it is tempting to establish a correlation between these results and the downregulation of miR-625-3p we have observed in the fully intestinalized glands more prone to malignant transformation.

The present findings should be interpreted taking into account some limitation, such as the fact that three out of the four pairs of samples analyzed for miRNA differential expression were obtained from the same patient. However, it is important to consider that the first foci of intestinal metaplasia needs 3-5 years to develop in the human model of BE, so the time constraints and the limitations of the microdissected samples are important challenges to this type of studies. However, validation of the finding of the regulatory role of miRNA-24-3p on CDX2 expression in two series of patients with BE overcomes this limitation. Future studies should investigate miR-24-3p during BE patients' follow-up and its deregulation within the phenotypic carcinogenetic cascade occurring in the Barrett's mucosa. On the other hand, altering CDX or miRNA expression in an organoid model of the cardia would be interesting to address the question if CDX expression determines differentiation of columnar cells (origin likely in the cardia) into goblet cell containing intestinal metaplasia or another type of BE tissue.

In summary, an early involvement of miRNAs in the acquisition of the intestinal phenotype in metaplastic epithelia was observed. Downregulation of miRNA-24-3p plays a relevant role in the regulation of CDX2 expression and the consequent acquisition of the intestinal phenotype in BE. Further studies are needed to confirm these results and to explore the prognostic value of miRNAs signatures in tailoring secondary prevention strategies in BE patients.

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**Author´s Contributions:**

All authors included in this paper fulfil the criteria of authorship and have approved the submission of this manuscript. Specifically, design: MP, CdB, MC LG, LN, MF. Experiments: CdB, MG, LN, RA, CB, MF. Tissue: MC, MP, MG, MI, VG, CdB, CB, MF. Analysis: LN, RA, CB, MP, MF, GG, LG. Writing manuscript: MP, MF, CdB, LN, GG, LG. Critical review of manuscript: all authors.

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## Legends

**Figure 1. Microdissection of CDX2-positive glands.** (A, A') Immunodetection of CDX2 in the cardiac-type epithelium from two biopsies from the remnant esophagus of patients after esophagectomy. Glands fully positive for CDX2 are shown. (B, B') Examples of one of the three consecutive sections of the same gland prepared for microdissection. (C, C') Appearance of the tissue section after laser-mediated microdissection of the glands. The bar in A, B and C is 50 μM and in A', B' and C' is 100 μM.

**Figure 2. MicroRNA Expression in microdissected glands.** The differential expression pattern of miRNAs between CDX2-negative (solid bars) and positive glands (empty bars) was determined using the TaqMan™ OpenArray™ human microRNA platform. Due to the different magnitude of the expression levels, the differential expression of the most abundant miRNAs identified has been plotted using a logarithmic scale (right plot), while a linear scale has been used for those with lower expression levels (left plot). (**P* < .05)

**Figure 3. Expression of CDX2 and miR-24-3p in esophageal carcinoma-derived cell lines.** (A) CDX2 protein levels were measured in lysates of cell lines derived from distal esophageal adenocarcinoma (FLO-1), Barrett-derived esophageal adenocarcinomas (JH-EsoAd1 and OE33), adenocarcinoma of gastric cardia/esophageal junction (OE19) and pleural effusion of a distal esophageal adenocarcinoma (KYAE-1) by Western blot. The levels of β-ACTIN (ACTB) were used as loading control. (B) *CDX2* mRNA levels were measured in the same cell lines by qRT-PCR. The levels of *HPRT* were measured as endogenous control and used for normalization. The mean value of the mRNA levels relative to the KYAE-1 cell line and the standard deviation of at least three independent experiments are plotted. (C) The abundance of miR24-3p was measured by qRT-PCR in the same cell lines. Bars represent the mean value of the miR24-3p levels relative to the KYAE-1 cell line of at least three experiments and the standard deviation. (*ns*, non-significant; **P* < .05; *****P* < .001).

**Figure 4**. **miRNA-24-3p regulates CDX2 protein levels but not mRNA expression levels in KYAE-1 cells.** Measurement of the expression levels of hsa-miR-24-3p by qRT-PCR in the KYAE-1 cell line 48 h after mock transfection (mock) or transfection with miRNA-24 inhibitor negative control (neg. control, 30nM), hsa-mir-24-3p *mir*Vana™ miRNA Mimic (mimic-miR24, 30 nM) or Inhibitor (inhibit-miR24, 30 nM). The levels of HPRT were measured as endogenous control and used for normalization. The mean value and the standard deviation of at least three independent experiments are plotted. (B) The levels of CDX2 expression in KYAE-1 cells mock transfected (mock) and transfected with the negative control (neg. control), mimic-miR24 and inhibit-miR24 were measured by western blotting. The levels of β-ACTIN (ACTB) were used as loading control. (C) The levels of *CDX2* mRNA were measured by qRT-PCR in samples from KYAE-1 cells 48 hours after mock transfection (mock) of after transfection of *mir*Vana™ miRNA inhibitor negative control (30nM, neg. control); hsa-mir-24-3p *mir*Vana™ miRNA Mimic (30 nM, mimic-miR24) or Inhibitor (30 nM, inhibit-miR24). The levels of *HPRT* were measured as endogenous control and used for normalization. The mean value and the standard deviation of at least three independent experiments are plotted. No statistically significant differences were observed between the samples. (*ns*, not significant; ****$P < .001$)

**Figure 5. miR-24-3p in situ hybridization in biopsy sections containing cardiac-type glands with few glands fully expressing CDX2.** miR-24-3p is in blue and CDX2 in red. Scale bars = 100 μm. (A) Coexistence of intestinal and cardiac type columnar mucosa. The intestinal metaplasia is characterized by a positive staining of CDX2 (some cytoplasmic staining is due to the co-staining protocol) and lower miR-24-3p staining in comparison to non-intestinalized glands. (B) In an early-phase intestinalized gland note a CDX2 negative, but miR-24-3p positive cell, which is marked (arrow). (C) Two cases of non-intestinalized (left panels) and intestinalized (right panels) glands with magnifications of selected areas to better evaluate miR-24-3p staining in the two different glands; note the significant lower miR-24 expression in intestinalized glands. (D) Relative miR-24-3p expression as analyzed by image

software analysis in 10 different CDX2-negative (CDX2 neg.) and CDX2-positive (CDX2 pos.) glands. (****$P < .001$)

**Figure 6. miR-24-3p expression in Barrett's esophagus.** (A) miR-24-3p expression was detected by ISH (red signal, left panels) while MUC2 (brown signal, middle panels) and CDX2 (brown signal, right panels) were detected by immunohistochemistry in matched biopsies of long segment Barrett's esophagus displaying gastric metaplasia (upper panels) or intestinal metaplasia (lower panels). Scale bar is 100 μm. (B) Relative miR-24-3p expression in 12 matched CDX2/MUC2-negative and CDX2/MUC2-positive glands. Images were analysed with ImageJ and showed a significant downregulation of miR-24-3p in samples with intestinal metaplasia (Wilcoxon matched-pairs signed-rank test, $P = .004$). (C) Measurements of the levels of miR-24-3p RNA by qRT-PCR in CDX2/MUC2-negative (empty bars) and CDX2/MUC2-positive (solid bars) glands showing a downregulation of the miR-24-3p RNA levels in samples with intestinal metaplasia (Wilcoxon signed-rank test, $P = .001$).

**Supplementary Figure 1**. **miRNA-24-3p regulates CDX2 protein levels but not mRNA expression levels in FLO-1 cells.** (A) Measurement of the expression levels of miR-24-3p by qRT-PCR in the FLO-1 cell line 48 h after mock transfection (mock) or transfection of *mir*Vana™ miRNA inhibitor negative control (neg. control, 30 nM); hsa-mir-24-3p *mir*Vana™ miRNA mimic (mimic-miR24, 30 nM) or inhibitor (inhibit-miR24, 30nM). The levels of HPRT were measured as endogenous control and used for normalization. The mean value and the standard deviation of at least three independent experiments are plotted. (B) The protein levels of CDX2 in FLO-1 cells mock transfected (mock) and transfected with the negative control (neg. control), mimic-miR24 and inhibit-miR24 were measured by Western blot. The levels of β-ACTIN (ACTB) were used as loading control. (C) The levels of *CDX2* mRNA were measured by qRT-PCR in samples from FLO-1 cells 48 h after mock transfection (mock) of after transfection of *mir*Vana™ miRNA inhibitor negative control (30 nM, neg. control); hsa-mir-24-3p *mir*Vana™ miRNA mimic (30 nM, mimic-miR24) or inhibitor (30 nM, inhibit-miR24). The levels of *HPRT* were measured as endogenous control and used for normalization. The mean value and the

standard deviation of at least three independent experiments are plotted. (*ns*, not significant; *$P < .05$; ****$P < .001$).

**Table 1** Cardiac-type metaplasia and CDX2 expression detection during endoscopic follow-up after esophagectomy

| Case | Maximum endoscopic follow-up (months) | Cardiac-type epithelium first detected (months) | CDX2 expression first detected (months) |
|---|---|---|---|
| 1 | 24 | 12 | No expression |
| 2 | 108 | 48 | 60 |
| 3 | 36 | 18 | 24 |
| 4 | 48 | 12 | 24 |
| 5 | 24 | 6 | 12 |
| 6 | 48 | 6 | 6 |
| 7 | 84 | 48 | 60 |
| 8 | 84 | 12 | 12 |
| 9 | 48 | 12 | 12 |
| 10 | 48 | 36 | No expression |
| 11 | 36 | 12 | 24 |
| 12 | 18 | 12 | 12 |

**Table 2** Number of endoscopic biopsies with cardiac-type epithelium where glands fully expressing CDX2 were detected

| Case | Follow-up time (months) | Number of cardiac-type biopsies with glands fully expressing CDX2 |
|---|---|---|
| 2 | 84<br>96<br>108 | 1/7<br>5/13<br>5/6 |
| 7 | 84 | 1/4 |
| 8 | 84 | 1/9 |
| 9 | 48 | 1/6 |
| 11 | 36 | 1/10 |
| Total | | 15/55 |

**Table 3** Sample selection process for the OpenArray analysis

| Case | Follow-up (months) | Biopsy # | Microdissected glandular area based on CDX2 pattern | RNA concentration* ng/μl | Definitive samples used for OpenArray** |
|---|---|---|---|---|---|
| 2 | 84 | 1 | Positive | 0.082 | |
| | | | Negative | 0.048 | |
| | 96 | 1 | Positive | 0.009 | A1 |
| | | | Negative | 0.234 | |
| | | 2 | Positive | 0.525 | B1 |
| | | | Negative | 0.009 | |
| | | 3 | Positive | 0.056 | |
| | | | Negative | 0.015 | |
| | | 4 | Positive | 0.016 | |
| | | | Negative | 0.034 | |
| | | 5 | Positive | 0.043 | |
| | | | Negative | 0.002 | |
| | 108 | 1 | Positive | 0.046 | A2 |
| | | | Negative | 0.339 | |
| | | 2 | Positive | 0.043 | B2 |
| | | | Negative | 0.129 | |
| | | 3 | Positive | 0.055 | A3 |
| | | | Negative | 0.282 | |
| | | 4 | Positive | 0.032 | B3 |
| | | | Negative | 0.251 | |
| | | 5 | Positive | 0.025 | |
| | | | Negative | 0.075 | |
| 7 | 84 | 1 | Positive | 0.066 | |
| | | | Negative | 0.015 | |
| 8 | 84 | 1 | Positive | 0.049 | |
| | | | Negative | 0.053 | |
| 9 | 48 | 1 | Positive | 0.033 | |
| | | | Negative | 0.211 | |
| 11 | 36 | 1 | Positive | 0.084 | A4 |
| | | | Negative | 0.101 | B4 |

* A minimum RNA concentration (>0.052) threshold was previously established.
**Samples A1, A2, A3, B1, B2, and B3 were the combination of two RNA isolated from glands with the same pattern of CDX2 expression and from the same patient and same follow-up time.