Evidence for a functional role of epigenetically regulated midcluster *HOXB* genes in the development of Barrett esophagus

Massimiliano di Pietro[a], Pierre Lao-Sirieix[a], Shelagh Boyle[b], Andy Cassidy[c], Dani Castillo[d], Amel Saadi[c], Ragnhild Eskeland[b,1], and Rebecca C. Fitzgerald[a,2]

[a]Medical Research Council Cancer Cell Unit, Hutchison Medical Research Council Research Centre, CB2 0XZ Cambridge, United Kingdom; [b]Medical Research Council Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, EH4 2XU Edinburgh, United Kingdom; [c]Cancer Research United Kingdom, Cambridge Research Institute, Li Ka Shing Centre, CB2 0RE Cambridge, United Kingdom; and [d]Section of Gastrointestinal Surgery, Hospital Universitari del Mar, Universitat Autónoma de Barcelona, 08003 Barcelona, Spain

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Barrett esophagus (BE) is a human metaplastic condition that is the only known precursor to esophageal adenocarcinoma. BE is characterized by a posterior intestinal-like phenotype in an anterior organ and therefore it is reminiscent of homeotic transformations, which can occur in transgenic animal models during embryonic development as a consequence of mutations in *HOX* genes. In humans, acquired deregulation of *HOX* genes during adulthood has been linked to carcinogenesis; however, little is known about their role in the pathogenesis of premalignant conditions. We hypothesized that *HOX* genes may be implicated in the development of BE. We demonstrated that three midcluster *HOXB* genes (*HOXB5*, *HOXB6*, and *HOXB7*) are overexpressed in BE, compared with the anatomically adjacent normal esophagus and gastric cardia. The midcluster *HOXB* gene signature in BE is identical to that seen in normal colonic epithelium. Ectopic expression of these three genes in normal squamous esophageal cells in vitro induces markers of intestinal differentiation, such as *KRT20*, *MUC2*, and *VILLIN*. In BE-associated adenocarcinoma, the activation midcluster *HOXB* gene is associated with loss of H3K27me3 and gain of AcH3, compared with normal esophagus. These changes in histone posttranslational modifications correlate with specific chromatin decompaction at the *HOXB* locus. We suggest that epigenetically regulated alterations of *HOX* gene expression can trigger changes in the transcriptional program of adult esophageal cells, with implications for the early stages of carcinogenesis.

metaplasia | Homeobox | cancer | chromatin compaction | epigenetics

Metaplasia is an acquired phenotypic switch from one adult cell type into another, which is thought to result from a change in the expression of a few key developmental genes (1). In humans metaplasia can occur in different anatomical sites and it can be a precancerous condition (1). Hence, understanding the molecular basis of metaplasia is relevant to cancer biology and therapy. Barrett esophagus (BE) is a metaplasia of the distal esophagus where the squamous epithelium is replaced by cells with intestinal-type differentiation (2). BE occurs in the context of gastro-esophageal reflux disease and predisposes to esophageal adenocarcinoma (EAC) (2). Ex vivo exposure of esophageal cells to reflux agents induces genotoxic and epigenetic events, which can alter the expression of developmental and proliferation-related genes (3, 4). However, relatively little is still known about individual genes or pathways involved in BE development.

The Caudal-related Homeobox genes *CDX2* and *CDX1* have been investigated in BE (5). These genes play a crucial role in the differentiation of the endoderm into the primordial gut (6). Transgenic mice expressing either *CDX2* or *CDX1* in the stomach develop gastric intestinal metaplasia (7, 8). Conditional knockout of *CDX2* in the bowel causes gut malformations, failure to activate intestinal markers, and persistence of foregut genes (9). However, to date, unlike in the stomach, there is no evidence that *CDXs* on their own are sufficient to induce an intestinal phenotype in the mouse esophagus.

An intestinal-like epithelium in the esophagus is reminiscent of homeotic transformations, which have been linked to mutations of *HOX* genes (10). The 39 human *HOX* genes are divided into four clusters (HOXA, HOXB, HOXC, and HOXD) and have a collinear expression during development along the anterior-posterior (A-P) axis, whereby 3′ end genes are activated earlier and are more abundant in the anterior parts (11, 12). Collinearity is achieved through epigenetic mechanisms (13–15), whereby transcription correlates with active marks [e.g., histone H3 lysine 4 trimethylation (H3K4me3) and acetylation (AcH3)], whereas gene silencing associates with repressive marks [e.g., histone H3 lysine 27 trimethylation (H3K27me3)]. Histone modifications ultimately affect the degree of chromatin compaction and the accessibility to the transcriptional machinery (14, 16). Developmental functions of *HOX* genes include regulation of cell cycle, apoptosis, cell migration and differentiation and, not surprisingly, alteration of *HOX* gene expression has been shown in cancer (17). However, the expression of *HOX* genes in precancerous conditions, and in particular in BE, has not been investigated to date. Furthermore, despite evidence that alteration of higher-order chromatin structure can occur in human disease, such as cancer, it is not clear whether epigenetically regulated chromatin structural modifications have a causal role in pathogenesis of diseases through changes in gene expression (18).

Here we show that three genes in the core of the *HOXB* cluster are activated in BE, with a gene signature identical to that of the colon. We provide evidence that overexpression of midcluster *HOXB* genes in squamous esophageal cells activates markers of intestinal differentiation. Finally, we show that the activation of midcluster *HOXB* genes in BE-related neoplasia correlates with changes in the histone posttranslational modifications and the degree of chromatin compaction.

Results

Midcluster *HOXB* Genes Are Up-Regulated in BE. To test whether *HOX* genes are differentially expressed in BE compared with

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[1]Present address: Department of Molecular Biosciences, University of Oslo, N-0316 Oslo, Norway.

[2]To whom correspondence should be addressed. E-mail: rcf@hutchison-mrc.cam.ac.uk.

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normal adjacent epithelia, we analyzed by microarray the expression of the human *HOX* genes in BE, normal esophagus, and gastric cardia. Only minor changes were seen in *HOXA* and *HOXD* clusters, whereas several *HOXB* and *HOXC* genes were significantly overexpressed in BE compared with the adjacent tissues (Fig. 1). In particular *HOXB5* and *HOXB6* showed the largest fold-induction (>3.5-fold) and *HOXB7* was also significantly increased, although to a lesser extent. Small changes were also seen for three neighboring genes in the *HOXC* cluster (*HOXC9*, *HOXC10*, and *HOXC11*). We analyzed the expression of these two groups of genes in a previously published and independent microarray dataset profiling normal esophagus, nondysplastic BE, and BE with either low-grade dysplasia (LGD) or high-grade dysplasia (HGD) (19). This dataset confirmed the up-regulation of *HOXB5*, *HOXB6*, and *HOXB7*, which was statistically significant for *HOXB5* and *HOXB7* (Fig. S1). In contrast, none of the three *HOXC* genes showed significant change and, therefore, we focused on the midcluster *HOXB* genes only. We tested by quantitative PCR (qPCR) their expression in an independent sample set comprising normal esophagus, nondysplastic BE, BE with LGD and HGD, as well as BE-associated EAC (Fig. 2). *HOXB5*, *HOXB6*, and *HOXB7* were overexpressed in all of the stages of BE disease compared with normal esophagus, whereas the flanking genes, *HOXB4* and *HOXB8*, did not show any change in the esophageal tissues. In situ hybridization confirmed higher mRNA levels of *HOXB5*, *HOXB6*, and *HOXB7* in BE compared with gastric cardia and normal esophagus (Fig. S2*A*). Comparison with adjacent sections stained with columnar and squamous markers (Fig. S2*B*) showed that *HOXB* RNA was localized in the epithelial compartment, although the stroma also showed some background expression. A higher expression of HOXB6 and HOXB7 in BE compared with normal esophagus was also seen at the protein level (Fig. S2*C*), but HOXB5 could not be validated because of lack of specificity of commercially available antibodies. With the exception of markers of intestinal differentiation and CDXs (5, 20), little is known about similarities in gene-expression patterns between BE and other normal gut epithelia. In addition only one study has looked at *HOX* expression levels in the human adult gut (21). We tested the expression of midcluster *HOXB* genes also in other normal gastrointestinal (GI) epithelia, such as stomach, duodenum, and colon (Fig. 2). As previously reported in the murine GI tract (22), we found loss of collinearity in some *HOXB* genes. Interestingly we found that the expression of *HOXB4–8* in BE was identical to that of the colon. We therefore concluded that *HOXB5*, *HOXB6*, and *HOXB7* are activated in BE, and that the midcluster *HOXB* gene signature in BE most resembled the colon rather than other GI epithelia.

Midcluster *HOXB* Genes Activate Intestinal Markers. To investigate whether midcluster *HOXB* genes are linked to an intestinal phenotype, we used the normal esophagus-derived NES cell line as an in vitro model of squamous esophageal cells (23). These cells are characterized by very low or no expression of the intestinal markers cytokeratin 20 (KRT20), cytokeratin 8/18 (KRT8/18), and VILLIN, and express cytokeratin 13 (KRT13) (Fig. S3). Transfection of NES cells with vectors coding for *HOXB5*, *HOXB6*, and *HOXB7* (Fig. S4 *A–C*) induced several intestinal markers (e.g., *KRT20*, *MUC2*, and *VILLIN*), whereas CDX2, KRT8 and KRT18 remained unchanged (Fig. 3*A*). The regulation of KRT20, villin, and MUC2 was also tested at protein level. KRT20 was induced only in *HOXB7*-transfected cells and in a dose-dependent fashion (Fig. 3*B* and Fig. S4*D*). Villin showed only a subtle change, which also appeared to be dose-dependent in cells transfected with HOXB6 (Fig. S4*D*), whereas MUC2 was detected by immunofluorescence in cells transfected with each of the three HOXB vectors (Fig. 3*B*). To confirm the specificity of the regulation of *KRT20* and *MUC2* by HOXB7, transfected NES cells were rescued with an siRNA against HOXB7 (Fig. 3*D*). siRNA treatment reduced the overexpression of *HOXB7* by 60%, almost completely abolished the transcriptional effect of HOXB7 on *KRT20* and reduced by ~50% the up-regulation of *MUC2*, whereas no effect was seen on *VILLIN*. We also transfected NES cells with an expression vector coding for CDX2, which has been previously shown to activate intestinal markers in different cellular models (24, 25). As expected, we observed an induction of some intestine-specific genes, such as *MUC2* and *KRT20*, and to a lesser extent *VILLIN* (Fig. S5 *A–C*), but no effect on midcluster *HOXB* genes. Finally, to investigate whether midcluster *HOXB* genes can be modulated in vitro by conditions mimicking gastro-esopahgeal reflux, as previously showed for *CDX2* in a different cellular model (24), NES cells where treated with a mixture of conjugated bile salts and/or acidified medium (pH5). No induction of *HOXB* genes and CDX2 was observed under these experimental conditions (Fig. S4*E*). Taken together, these data show that midcluster *HOXB* genes transcriptionally regulate markers of intestinal differentiation, with an overall effect comparable to *CDX2*.

Midcluster *HOXB* Genes Are Epigenetically Regulated in BE-Associated Neoplasia. *HOX* genes are regulated by epigenetic mechanisms involving the histone code during ES cell differentiation and mouse development (13–15). Recently *HOX* gene expression in primary and malignant prostate cells was shown to correlate with

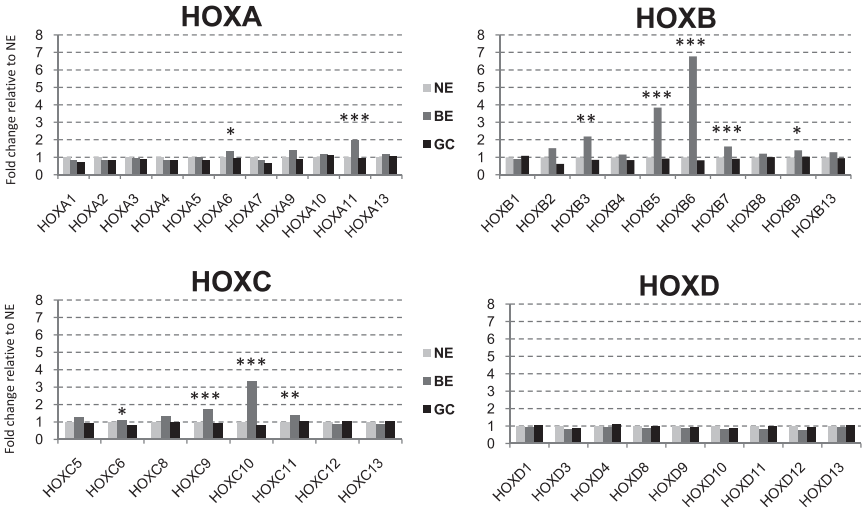

Fig. 1. Screening of *HOX* gene expression in BE. Expression of the human *HOX* genes was assessed by microarray in endoscopic biopsies of normal esophagus (NE, $n = 8$), BE ($n = 10$), and gastric cardia (GC, $n = 10$). *HOXC4* was not represented in this microarray platform; therefore, this gene was not included in the analysis. Data are plotted as normalized values relative to NE. For statistical analysis, ANOVA was performed with the false-discovery rate (FDR) Step Up method for the multiple test correction. ***$P < 10^{-4}$; **$P < 10^{-3}$; *$P < 10^{-2}$ in both BE vs. NE and BE vs. GC.

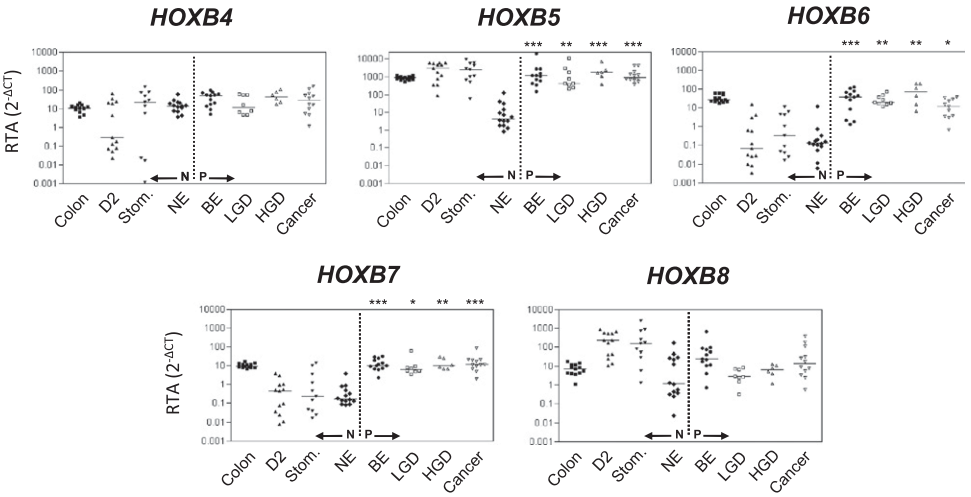

Fig. 2. External RNA validation of midcluster *HOXB* genes in BE and normal gut epithelia. Expression of the midcluster *HOXB* genes was assessed by qPCR in endoscopic biopsies of colon (n = 14), duodenum (D2, n = 13), gastric body (Stom, n = 11), NE (n = 14), BE (n = 13), BE with LGD (n = 8), BE with HGD (n = 6), as well as in surgical specimens of EAC (Cancer, n = 11). N = normal tissues arranged from distal to proximal. P = pathological tissues arranged from benign to malignant. Data were normalized to *GaPDH*. Statistical analysis was performed comparing pathological tissues to NE with one-way ANOVA test, with Dunn's multiple correction test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

H3K4me3 and H3K27me3 (26). To test whether these epigenetic mechanisms occur at the *HOXB* gene promoters during BE carcinogenesis, we performed ChIP in surgical specimens of EAC and normal esophagus. Cancer tissues were chosen for this experiment for two reasons. First, the expression of midcluster *HOXB* genes in EAC and BE was identical (Fig. 2). Second, the chromatin yield of endoscopic biopsies of BE was too low for successful ChIP. In keeping with the gene expression data, we found loss of H3K27me3 and gain of AcH3 in EAC compared with normal esophagus at the promoter of *HOXB5*, *HOXB6*, and *HOXB7*, but not of *HOXB4* and *HOXB8* (Fig. 4 *A* and *B*). Interestingly, we observed little and non significant changes in the levels of H3K4me3 (Fig. 4*C*). Hence, we concluded that activation of midcluster *HOXB* genes in BE-associated EAC correlates with levels of H3K27me3 and AcH3 at gene regulatory regions.

***HOXB* Cluster Undergoes Progressive Decompaction in BE-Related Carcinogenesis.** Changes in histone posttranslational modifications can alter the degree of chromatin compaction with effect on gene expression (16, 27). Moreover, during ES differentiation *HOX* activation is accompanied by loss of H3K27me3 and local chromatin decompaction (28, 29). We therefore asked whether differential expression of midcluster *HOXB* genes in BE and BE-associated EAC correlated with different degrees of chromatin compaction. To this end, we measured chromatin compaction by 3D FISH in tissue specimens of normal esophagus, BE, and BE-associated

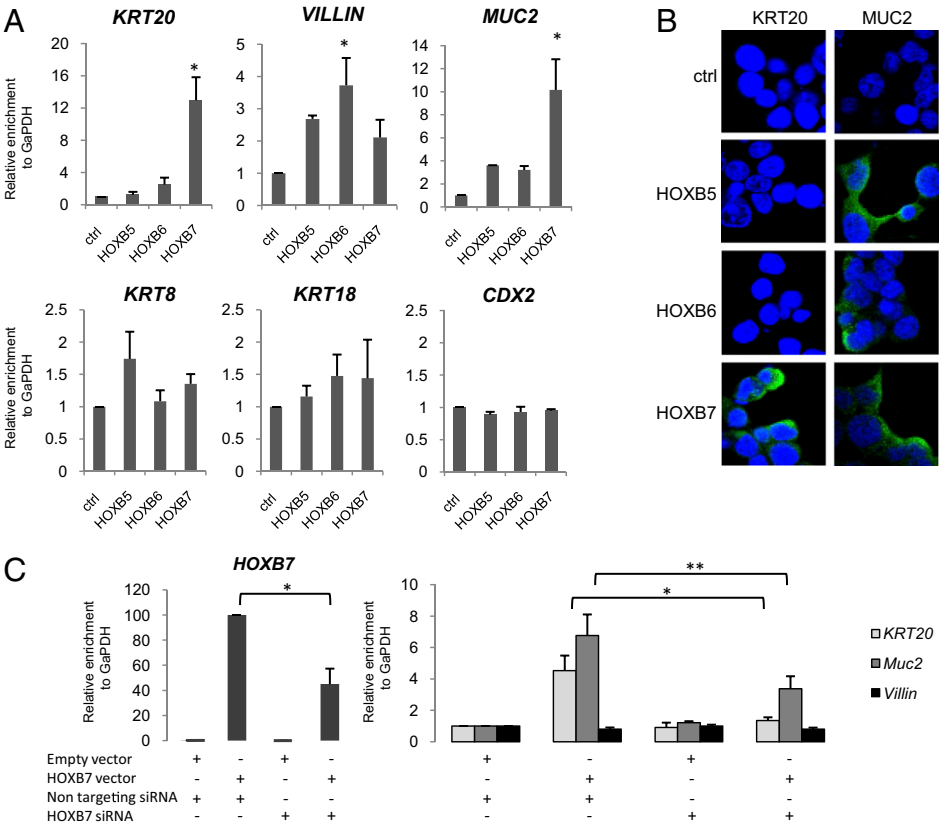

Fig. 3. Midcluster *HOXB* genes activate intestinal markers in normal esophageal cells. (*A*) Expression of intestinal markers (*KRT20*, *VILLIN*, *MUC2*, *KRT8*, and *KRT18*) and *CDX2* was assessed by qPCR in NES cells transfected with empty vector (ctrl) or expression vector coding for *HOXB5*, *HOXB6*, and *HOXB7*. Data are plotted as relative to empty vector. Error bars represent SE of three biological replicates. Statistical analysis was performed with one-way Friedman ANOVA test with Dunn's multiple correction test. *$P < 0.05$. (*B*) Immunofluorescence analysis with antibodies against KRT20 and MUC2 of NES cells transfected as in *A*. (Magnification: 40×.) (*C*) Expression of *HOXB7* (*Left*) and intestinal markers (*Right*) assessed by qPCR in NES cells transfected with either empty vector or *HOXB7* expression vector and cotransfected with either nontargeting siRNA or an siRNA against the coding region of *HOXB7*. Error bars represent SE of three biological replicates. Statistical analysis performed with paired t test. *$P < 0.05$; **$P < 0.01$.

EAC (Fig. 5). The squared interprobe distance (d^2) has been shown to relate to genomic separation in kilobases (30). Three-dimensional FISH revealed a step-wise increase of the median d^2 from normal to BE and from BE to cancer, with an overall significant change in the analysis for trend ($P < 0.001$). When comparing individual histological conditions however, we found statistical significance only in the comparisons normal vs. cancer ($P = 0.0017$) and BE vs. cancer ($P = 0.0431$). This finding suggests that there is progressive chromatin decompaction at the *HOXB* locus during BE carcinogenesis.

Discussion

This study provides evidence that three *HOXB* genes are activated in BE and have a potential role in the development of the disease. In addition, we demonstrate that an epigenetically controlled modification in chromatin structure is linked to this change in gene expression.

BE is reminiscent of homeotic transformations and represents an ideal model to study the involvement of *HOX* genes in the pathogenesis of human disease. *HOX* genes have been studied for their developmental role along the A-P and other embryonic axes, such as female reproductive tract, limbs, and the GI tract (11, 12). In keeping with abnormal gut phenotypes described in *HOX* transgenic mice (31, 32), collinearity of the *HOX* genes occurs in the murine gut during embryogenesis (22). *HOX* gene expression is not restricted to developing tissues, but is also maintained during adulthood, where it may not simply represent maintenance of an early transcriptional activation. In fact, deregulation of *HOX* genes has been shown in human disease (33), in particular cancer (17). However, the timing of *HOX* gene deregulation during carcinogenesis is still poorly understood. Furthermore, very little is known about *HOX* gene-expression changes in premalignant conditions and how *HOX* genes can regulate the development of metaplasia.

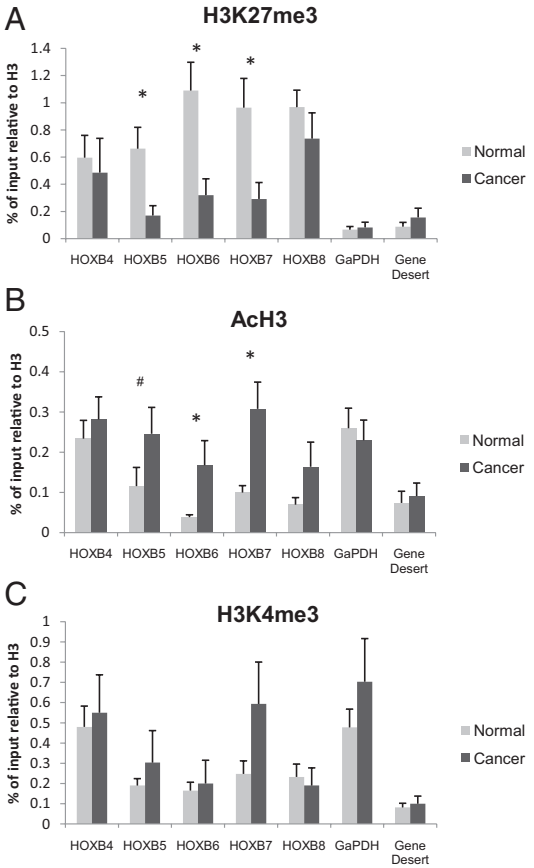

Fig. 4. Epigenetic regulation of midcluster *HOXB* genes in BE-associated EAC. ChIP was performed on surgical specimens of NE ($n = 5$) and EAC (Cancer; $n = 5$) with antibodies against H3K27me3 (*A*), AcH3 (*B*), or H3K4me3 (*C*). Data are expressed as percentage of input relative to histone H3. qPCR was performed with primers generating amplicons within ± 200 bp from the transcription start site or within a locus devoid of genes (Gene Desert) as a negative control. Error bars represent SE. Statistical analysis was performed with Mann–Whitney *U* test. $^*P < 0.05$; $^{\#}P = 0.09$.

In this work we show that *HOXB5*, *HOXB6*, and *HOXB7* are activated in BE, where the midcluster HOXB signature is remarkably similar to that of the colon. There is debate in the field concerning the genetic and phenotypic nature of BE in comparison with physiological intestinal tissues. This similarity to the colon correlates with the known activation in BE of caudal Homeobox genes (5) and the similar pattern of sulfomucins in the globet cells (34). Of note, we found high expression levels of midcluster *HOXB* genes also in BE cases with no histological evidence of globet cells, in keeping with the notion that a pathological diagnosis of BE can be supported by both gastric and intestinal metaplasia (35). With regards to *HOXB* gene levels in normal gut epithelia, our data are in agreement with previous evidence that midcluster *HOXB* genes are less expressed in the esophagus than in posterior segments of the GI tract (21). However, the midcluster *HOXB* profile we found in human adult GI tissues does differ from that found in embryonic murine gut (22). Namely, we found high levels of *HOXB4–8* in the colon, which are silenced in embryonic murine colon. Furthermore the expression gap extending from *Hoxb5* to *Hoxb9*, found in developing murine duodenum, was only restricted in our human data to *HOXB6* and *HOXB7*. Overall, these findings suggest that the expression of *HOX* genes in murine embryogenesis is only partially representative of that of corresponding human adult tissues.

Our data also suggest that midcluster *HOXB* genes have a functional role in the activation of an intestinal transcriptional phenotype. *HOXB5*, *HOXB6*, and *HOXB7* can activate some, but not all the intestinal makers commonly expressed in BE (36–38). Although this finding could depend on the transient nature of the transfection, it indicates that in the cell line model tested these genes do not induce a full intestinal phenotype and that other transcriptional factors may be required. Caudal related Homeobox genes (*CDX2* and *CDX1*) are candidate genes for this. In agreement with previous data (5), we found that *CDX2* expression was activated in our BE and EAC samples to a similar extent as the midcluster *HOXB* genes (Fig. S5*D*). Our data showed that *CDX2* can activate some intestine-specific genes, to an extent that is very similar to that of *HOXB* genes. However, we found lack of cross-activation between *HOXBs* and *CDX2*. This finding is in keeping with previous data in mice harboring a conditional deletion of *CDX2* in the endoderm, where little deregulation of the HOX code was observed, suggesting that *HOX* gene expression in endoderm-derived tissue is largely independent from CDX2 (9). We suggest that the pathological conditions created by reflux disease, in the presence of a favorable genetic background, can induce aberrant activation of multiple transcriptional factors (e.g., *HOXs* and *CDXs*), which act synergistically to determine an intestinal phenotype. Whether the cellular target of this reprogramming is a stem cell or a more committed cellular population remains to be determined. A recent report has shown that *p63*-null mice develop a columnar epithelium in the proximal stomach, which appears to arise from the expansion of progenitor cells located at the squamo-columnar junction (39). Although the molecular profiling of the murine columnar phenotype showed some similarities to that of BE, no activation of *HOX* genes was reported. Specific analysis of *HOX* gene expression in this system will be needed to elucidate how our data fit with the recently proposed murine model.

Of note, the midcluster *HOXB* genes had similar levels of expression across different pathological stages of BE including EAC (Fig. 1*A*), suggesting that these genes may not be involved in the progression to cancer. It has been reported that in squamous esophageal cancer several *HOX* genes, including *HOXB7*,

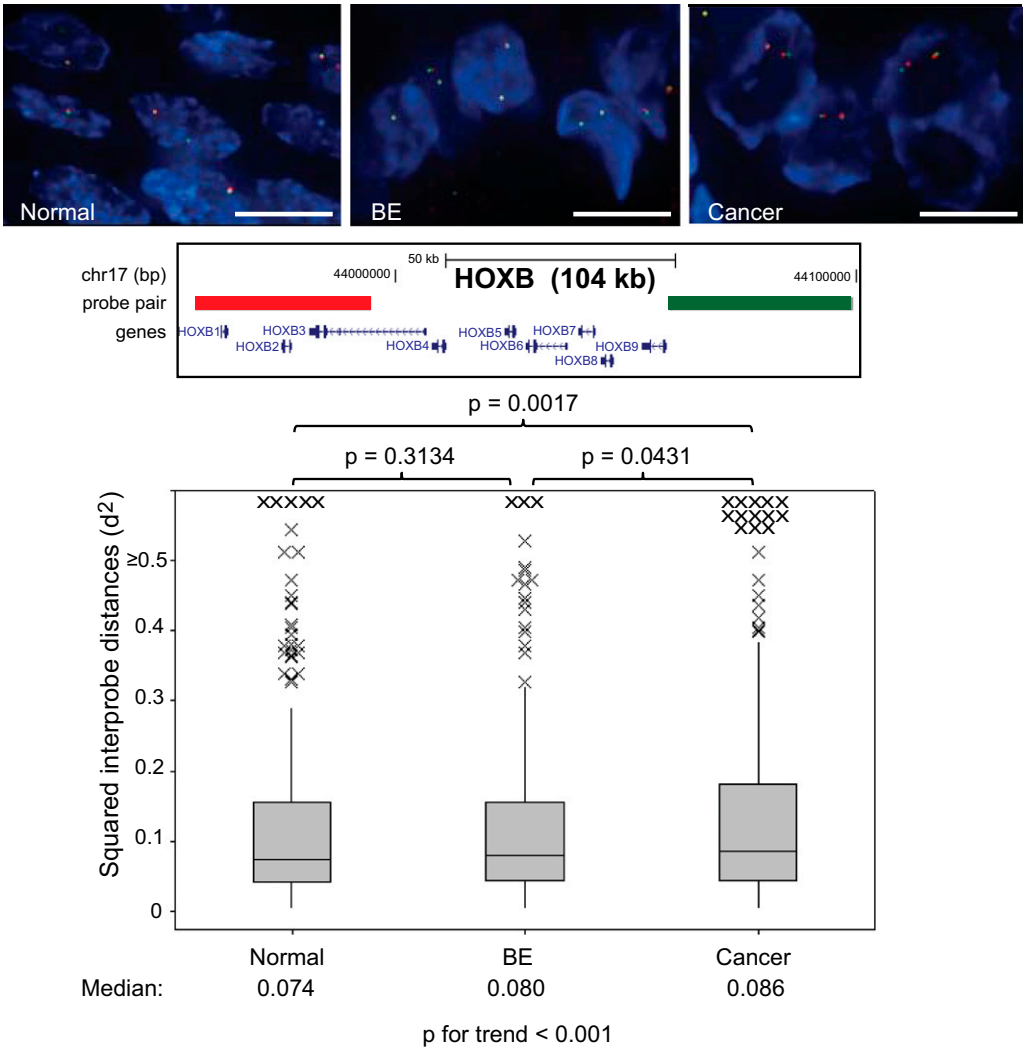

Fig. 5. Progressive *HOXB* locus decompaction occurs in BE carcinogenesis. Three-dimensional FISH with probe pairs spanning HOXB1 and HOXB9 on HSA17 in nuclei from esophageal sections of normal (*Top Left*), BE (*Top Center*), and EAC (*Top Right*) cells counterstained with DAPI (blue). (Scale bars, 5 μm.) (*Middle*) Diagrams show the positions of probes in the University of California Santa Cruz browser [March 2006 Assembly (NCBI36/hg18)]. Genome position is shown in base pairs. (*Bottom*) Box plots show the distribution of squared interprobe distances (d^2) in epithelial cells of NE (n = 5), BE (n = 3), and BE-associated EAC (n = 3). The shaded boxes show the mean and interquartile range of the data; asterisks indicate outliers (n = 55–134 loci). The statistical significance of differences between individual conditions was examined by Mann–Whitney *U* tests. Trend was analyzed by linear regression on logarithmic scale.

are deregulated (40). We cannot exclude that the activation of midcluster *HOXB* gene in BE acts as a priming event, interfering with the regulation of key cellular functions and increasing the susceptibility to EAC. Testing this hypothesis is beyond the scope of this work, but remains an interesting biological question.

HOX genes are known to be a target of Polycomb and Trithorax group proteins, which regulate developmental genes through modification of histone residues (41). Although there is evidence for this type of epigenetic regulation in cancer (26, 42), the majority of the studies so far have used cell line models. In the present work we have analyzed histone epigenetic modifications in tissue samples and demonstrated that the activation of midcluster *HOXB* genes in BE-associated EAC correlates with levels of H3K27me3 and AcH3. Interestingly, we detected in normal esophagus both H3K4me3 and H3K27me3 at the promoter regions of *HOXB* genes. Coexistence of H3K27me3 and H3K4me3 (bivalency) has been found at regulatory regions of key developmental genes in ES cells (43), where it can poise them to subsequent activation or repression during differentiation. The histone code found at midcluster *HOXB* genes in normal esophagus is reminiscent of that of bivalent genes. Although bivalency was initially thought to be present in pluripotent cells only, in fact about 10% of poised genes remain bivalent in differentiated cells (44). The activation of *HOXB* genes in BE-associated EAC is likely a result of loss of the repressive mark associated with gain of an alternative active mark (histone acetylation) rather than with a significant change in H3K4me3.

In mammalian cells, increased histone acetylation has been shown to cause chromatin decompaction (45) and increased chromatin accessibility (27). Conversely, Polycomb repressive complexes have been implicated in vitro and in vivo in chromatin condensation and repression (29, 46). These phenomena are crucial for collinear activation of *HOX* genes during development (14). Alteration in the higher-order chromatin structure has been described in many human diseases, including cancer (18); however, to date evidence is lacking that chromatin conformational events regulated by epigenetic mechanisms can change the expression of genes involved in the pathogenesis of a human disease (18). We have demonstrated that the chromatin of *HOXB* locus undergoes progressive decompaction from normal esophagus to nondysplastic BE and BE-associated EAC. This finding would suggest that these dynamic chromatin changes start early on during the pathogenesis of benign BE, but become more pronounced as the disease progresses to malignant stages.

Additional questions remain to be addressed. Even though our microarray datasets pointed to the mid HOXB cluster as the most significantly activated locus in BE, we cannot exclude that other *HOX* genes have a role in BE-associated carcinogenesis. In addition, it is still not clear why only the core of the HOXB cluster is activated upon *HOXB* locus decompaction and why the expression of these genes through different stages of disease remains constant despite progressive local chromatin decompaction. A second layer of regulatory mechanisms may be involved in con-

trolling the spreading of active and repressive marks across the locus. Recently the chromatin binding protein CTCF has been shown to associate with the boundaries of H3K27me3 domains in a cell-specific manner (47). It will be interesting to study the binding of CTCF within the *HOXB* locus in normal esophagus and BE to test whether it correlates with the activation *HOXB* genes.

In conclusion, this study provides evidence that *HOX* genes are involved in the development of BE. In particular, we found that midcluster *HOXB* genes are activated in BE with a gene signature identical to that of the colon. In addition, we have demonstrated that midcluster *HOXB* genes transcriptionally regulate markers of an intestinal phenotype and that the activation of these genes is associated with changes in the histone code and the degree of chromatin compaction.

Materials and Methods

Human *HOX* gene expression was profiled by microarray (Affymetrix HuGene-1_0-st-v1) in endoscopic biopsies. Validation of microarray findings was carried out with qPCR, in situ hybridization, and Western blotting. All BE biopsies were taken in patients with a visible segment of BE at least 2 cm in length. NES cells were transfected with expression vectors coding for HOXB5, HOXB6, HOXB7, and CDX2 or with siRNA targeting HOXB7 (Dharmacon, ThermoFisher). Histone modification at *HOXB* gene promoters were analyzed by ChIP and *HOXB* locus chromatin compaction was assessed by 3D FISH in tissue samples of normal esophagus, BE, and BE-associated EAC. Sequences of primers used in qPCR and ChIP are provided in Table S1. Genomic locations of FISH probes are in Table S2.

Details are described in the *SI Materials and Methods*.

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