HOXA9 mediates and marks premalignant compartment size expansion in colonic adenomas

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Abstract

The transformation of normal colonic epithelium to colorectal cancer (CRC) involves a relatively ordered progression, and understanding the molecular alterations involved may aid rational design of strategies aimed at preventing or counteracting disease. Homeobox A9 (HOXA9) is an oncogene in leukemia and has been implicated in CRC pathology, although its role in disease etiology remains obscure at best. We observe that HOXA9 expression is increased in colonic adenomas compared with location-matched healthy colon epithelium. Its forced expression results in dramatic genetic and signaling changes, with increased expression of growth factors IGF1 and FLT3, super-activity of the AKT survival pathway and a concomitant increase in compartment size. Furthermore, a reduced mRNA expression of the epithelial to mesenchymal transition marker N-cadherin as well as reduced activity of the actin cytoskeletal mediator PAK was seen, which is in apparent agreement with an observed reduced migratory response in HOXA9-overexpressing cells. Thus, HOXA9 appears closely linked with adenoma growth while impairing migration and metastasis and hence is both a marker and driver of premalignant polyp growth. Colonic polyps grow but remain premalignant for up to decades. Here, we show that HOXA9 drives growth in premalignant polyps, but simultaneously prevents further transformation.

Introduction

Colorectal cancer (CRC) is the second most common cancer in men and the third in women (1). Its incidence has slightly risen in The Netherlands over the past decades while prognosis improved (2). In Europe, high CRC prevalence has resulted in the initiation of many national CRC screening programs (3). These programs are continuously evaluated and improved (4, 5). In Europe, incidence rates are declining, probably due to changes in lifestyle and more intensive screening. However, CRC is still the second leading cause of cancer death in Europe (6). Obviously, better understanding of the molecular pathways that mediate progression from normal colonic epithelium to invasive carcinoma would aid efforts aimed at improving prevention and treatment of CRC.

Better definition of molecular markers that relate to the natural history of early CRC would prove exceedingly useful. About 30 years ago, Vogelstein et al. described the importance of premalignant lesions and their role in the adenoma–carcinoma sequence (7). The prevalence of these premalignant lesions is considered to be 25% at the age of 50 years and increases up to 50% at the age of 70 (8–11). It is fair to say that the mechanisms that drive growth of premalignant lesions remain poorly understood. Also stratifying early lesions into those that are truly benign to those that are at risk to undergo micrometastasis is not yet possible. Identifying the molecular determinants involved defines a major question in contemporary preclinical cancer research.
Molecular pathways that underlie carcinogenesis are often aberrations of normal cellular physiology. Carcinogenesis can be seen as an aberrant form of organogenesis (12–14). Homeobox genes, which include the HOX gene clusters, regulate important pathways with relation to both embryogenesis and carcinogenesis (15). The evolutionary well-conserved mammalian HOX genes encode for transcription factors regulating the formation of tissues, structures and organs along the longitudinal body axis during embryology (15–17). Thus far, 39 HOX genes have been identified in humans, which are organized in four clusters (A to D) on separate chromosomes (7, 17, 12, and 2, respectively). During embryogenesis, the different HOX clusters are expressed with temporal and spatial collinearity (18). The nested pattern of HOX genes along the length of the human body’s axis is most clearly observed in segmented structures like the vertebral, branchial arches and limbs (19–21). However, position-specific expression of HOX genes is also present in discreet organs, including the human gut (22, 23). However, the specific functionality of expression of single HOX genes in gastrointestinal pathophysiology remains to be established.

As HOX genes are important regulators of tissue growth and differentiation, it is conceivable that they also play a role in malignant transformation. This has led to an increasing interest in HOX expression patterns in different forms of cancer (24–30). Interestingly, when screening for the expression of HOX family members in gastrointestinal pathophysiology, we observed markedly high expression of HOXA9 in esophageal adenomas when compared with normal esophagus (unpublished data). HOXA9 overexpression as a result of the NUP98–HOXA9 translocation-derived fusion gene is seen in patients with the premalignant Myelodysplastic Syndrome as well as overt myeloid leukemia (31). In MDS, in particular patients with refractory anemia with excess of blasts in transformation (REAB-t) show HOXA9 fusion genes (32). In line with the fact that the NUP98–HOXA9 fusion transcript has been shown to induce hematopoietic hyperproliferation (33), this suggests that it drives the transformation process of MDL to AML. HOXA9 overexpression was also shown to be the strongest factor associated with poor prognosis in AML (34). In addition to hematopoietic malignancies, HOXA9 has a pro-oncogenic effect in epithelial ovarian cancer, osteosarcoma, breast and oral squamous cell cancer (35–37). Moreover, an upregulation of HOXA9 is already present when compared with control cells by qRT–PCR (Supplementary Figure 1, available at Carcinogenesis Online).

**Materials and methods**

**Sample collection and preparation**

Participants were recruited at the Havenziekenhuis (Rotterdam, The Netherlands) in the context of the nationwide screening for CRC. Asymptomatic patients, aged between 55 and 75 years, with a positive immunologic fecal occult blood test (iFOBT) were referred to this hospital for colonoscopy. If during colonoscopy a premalignant lesion was found, biopsies were taken; one from the center of the premalignant lesion and one from healthy mucosa located in the vicinity of the lesion. Biopsies were immediately stored in RNAlaterTM (Qiagen, Germany) at 4°C and stored at −80°C until RNA was extracted. After the biopsy was taken, the remainder of the colonic polyp was resected and examined by a pathologist. Only biopsies from lesions classified as tubular adenoma with low-grade dysplasia were included for further examination. Thus, only early stages in the adenoma–carcinoma sequence were studied (7). The biopsies were collected as part of the ‘biobank for premalignant colorectal lesions’ and material collection was approved by the medical ethical committee of both the Erasmus Medical Center (Rotterdam, The Netherlands; MEC-2015-199) and the Havenziekenhuis. Written informed consent was obtained from all participants.

**Transduction**

A GeneArt bacterial plasmid (Thermo Fisher Scientific, Waltham, MA) containing the HOXA9 gene and a kanamycin-resistance gene were used for the construction of the lentiviral vector. Firstly, the HOXA9 gene was cloned into the pEN_TmiR3 plasmid, which was a kind gift from Iain Fraser (California Institute of Technology, CA). Subsequently, the HOXA9 insert was transferred into a pSLIK-Hygro plasmid, also received from Iain Fraser (plasmid #2573; Addgene, USA), using a Gateway reaction. The same procedure was followed to create a control plasmid, lacking the HOXA9 insert. All created plasmids were sequenced by LGC Genomics (LGC Genomics GmbH, Germany) and were confirmed to be sequence correct. The pSLIK-Hygro plasmid was transiently transfected in HEK293T cells together with three packaging plasmids (pSV-G, MD and REV). After 2 days, the medium was harvested and viral particles were collected by ultracentrifugation. Caco-2 cells were transduced with the concentrated virus, after 1 day the transduced cells were selected by adding hygromycin B (Thermo Fisher Scientific, The Netherlands) (400 µg/mL) for a period of 1 week. Expression of HOXA9 was confirmed after stimulation doxycycline hyclate by qRT–PCR.

**Cell culture**

The monthly short tandem repeat identity-verified (verification commercially performed by the molecular pathology department of the Erasmus MC) and American Type Culture Collection (ATCCC, Manassas, VA)-obtained mycoplasma-free (monthly commercially checked by GATC Biotech, Konstanz, Germany) human CRC cell line Caco-2 was cultured at 37°C in a 5% CO2 incubator using Dulbecco’s modified Eagle’s medium (DMEM; Lonza, Basel, Switzerland) containing 10% fetal calf serum (FCS; Sigma–Aldrich) and 1% Penicillin/Streptomycin (antibiotics). The same procedure was followed to create a control plasmid, lacking the HOXA9 insert. All created plasmids were sequenced by LGC Genomics (LGC Genomics GmbH, Germany) and were confirmed to be sequence correct. The pSLIK-Hygro plasmid was transiently transfected in HEK293T cells together with three packaging plasmids (pSV-G, MD and REV). After 2 days, the medium was harvested and viral particles were collected by ultracentrifugation. Caco-2 cells were transduced with the concentrated virus, after 1 day the transduced cells were selected by adding hygromycin B (Thermo Fisher Scientific, The Netherlands) (400 µg/mL) for a period of 1 week. Expression of HOXA9 was confirmed after stimulation doxycycline hyclate by qRT–PCR.

**RNA isolation, cDNA synthesis and PCR array/qRT–PCR**

Total RNA was extracted with the NucleoSpin® RNA kit (Machery-Nagel, Germany). For cDNA preparation, the reverse transcription system from Takara (TAKARA BIO INC) was used according to the manufacturers manual. A cDNA concentration of 10 ng/µl for patient material and 30 ng/µl for Caco-2 cells was used for quantitative polymerase chain reaction (qPCR).

| Abbreviations | Description |
|---------------|-------------|
| CRC | colorectal cancer |
| DMEM | Dulbecco’s modified Eagle’s medium |
| qPCR | quantitative polymerase chain reaction |
controls. In addition, potential interesting candidates as derived from the array as well as HOXA9 targets identified in literature but not present in the array were tested separately (for primers and conditions, see Supplementary Table 1, available at Carcinogenesis Online) in three independent experiments. Quantitative PCR was performed with SYBR Green (Applied Biosystems, USA) in an iQ5 PCR machine (Bio-Rad, Hercules, CA). To establish a loading control, TPT1, UBC, and GAPDH were used as reference genes (42). The ΔCT method was used to calculate expression values.

(Phospho)protein profiling
Caco-2 control and HOXA9-transduced cell lines were seeded in a Petri dish (60 cm) at 500 000 cells/dish and treated with 0.5 mg/ml doxycycline hyclate. After 72 h, proteins were extracted in 500 μL Laemmli Buffer [SDS 4%, glycerol 20%, Tris-Cl (pH 6.8) 120 mM, bromophenol blue 0.02% (wt/vol) and DTT 0.1 M] and the protein concentrations were determined using a commercial kit (RC DC Protein Assay—Bio Rad). Western blotting was performed as described (43, 44). In short, 40 μg protein was resolved by SDS-PAGE and blotted onto Immobilon FL PVDF membranes (Millipore, Bedford, MA). Membranes were blocked in Odyssey Blocking Buffer (PBS) and incubated overnight at 4°C with appropriate primary antibody (Supplementary Table 2, available at Carcinogenesis Online for details), followed by the appropriate Alexa-linked secondary antibodies, at 1:5000 dilution, in Odyssey Blocking Buffer for 1 h. The fluorescent bands were detected using fluorescent Odyssey Imaging System and densitometric analysis was performed with ImageJ (45). All blots were re-probed for actin to control for equal loading and normalized results are represented as ratios of protein of interest over actin levels per lane. Three independent experiments were performed, run together on one blot, and heat maps of the phospho-protein profile (46) in the six samples were constructed with CIMminer (Genomics and Bioinformatics Group, Laboratory of Molecular Pharmacology, Center for Cancer Research, National Cancer Institute) (47).

Cell count and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
For these experiments, four·10⁵ cells of the HOXA9-transduced and control-transduced cell lines were seeded and cultured for 6 days in separate T75 Cellstar culture flasks. After 6 days of culturing, the number of cells in both flasks was measured using a CellometerTM Auto T4 cell counter (Nexcelom Bioscience LLC, USA). After 6 days of stimulation, the total number of HOXA9-overexpressing cells in the T75 flask was calculated relative to the number of cells in the control cell line. This experiment was repeated four times. MTT assays were performed as described previously (48). Transduced Caco-2 cells were seeded in a 96 well plate, each well containing 1 000 cells. After 24, 48, 72 and 96 h, cell metabolic activity and viable cells were detected by firstly adding 10 μl 5mg/ml MTT to 100 μl DMEM, followed by 3 h incubation at 37°C and replacing the DMEM by dimethyl sulfoxide (DMSO; Sigma–Aldrich). Intensity of color was measured in a Model 680 XR microplate reader (Bio-Rad, USA). This experiment was repeated eight times.

3D Spheroid-based cell expansion assay
Cytodex-3 microcarrier beads (Sigma–Aldrich) were mixed with 5 × 10⁵ Caco-2 HOXA9 overexpression and control cell suspensions, at a density of 40 cells per bead and incubated at 37°C for 6 h with gentle mixing. The suspension was transferred to 25 cm² flasks and incubated for 48 h. Coated beads were embedded in 1.6 mg/ml collagen gel (collagen: modified Eagle’s medium: 7.5% wt/vol NaHCO₃ in the ratio 11:8.3), put in plates, incubated at 37°C for 2 h to polymerize and covered with 500 μl DMEM with 10% FBS, 1% p/s and 5 ng/ml doxycycline. Spheroid growth was measured by quantifying the cell layer extending from the surface of the bead. Ten coated beads were photographed every 24 h with a ×10 objective. All measurements were performed using AxioVision 4.5 software and assays were performed three times independently. Data were statistically analyzed by two-way ANOVA.

Migration assay
Migration assays were performed as described (49). Briefly, a barrier is inserted in a culture chamber and this prevents cells from entering a defined area occupied by the barrier. Cells were seeded around this barrier to form a monolayer, the barrier is removed, and migration into the defined cell-free area is measured.

cBioportal query
We performed a query on 15 April 2018 on http://www.cbioportal.org (50, 51). We selected all eight studies from the category ‘bowel’ including a total of 3 477 cases. This includes four published studies (52–55), and ‘colorectal adenocarcinoma [TCGA, Provisional]’, ‘Colon adenocarcinoma [TGOA, PanCancer Atlas]’, ‘Rectum adenocarcinoma [TGOA, PanCancer Atlas]’ and ‘Targeted sequencing of 1134 samples from metastatic colorectal cancer samples (MSK, Cancer Cell 2018)’.

Statistics
Relative expression of potential target genes was calculated comparing the transduced cell line overexpressing HOXA9 to the control cell line. The one-sample t-test was used to test for statistical significance. The Komarov-Smirnov test, the D’Agostino and Pearson omnibus normality test and the Shapiro-Wilk normality test were used for each target gene to see if the results came from a Gaussian distribution. If this assumption was violated, the relative expression of the target gene was displayed using the median and the interquartile range. In those cases, the Wilcoxon Signed-Rank Test was used to test for the difference in expression. The Student’s t-test was used to test for statistical significance in phospho-protein profile. To test for significance of the observed difference in cell number after 6 days of stimulation, the paired t-test was used (Graphpad Prism 5; GraphPad Software, Inc., USA). A two-way analysis of variance (ANOVA) was used to test for significant difference at each time point in the MTT assay. P values < 0.05 were considered to be statistically significant. Given that the biological significance of a given fold-change probably depends on the gene and on the experimental context, no fixed fold change cut-off was employed in this study.

Results
HOXA9 is overexpressed in colonic adenomas
HOX genes have been linked to cancer development and especially HOXA9 is interesting in this respect as it functions as an oncogene in various hematological malignancies (56). It has been reported that HOXA9 contributes to self-renewal and over-accumulation of cancer stem cells in CRC (57). Thus, we decided to investigate whether HOXA9 mRNA expression is deregulated in colorectal premalignant tissue. To this end, we collected 27 biopsies from colon adenomas and location-matched healthy tissue and determined HOXA9 expression by qPCR. A direct comparison between the paired adenoma and healthy tissues demonstrates significantly increased HOXA9 mRNA expression levels in the adenoma samples (fold change (FC) 1.95; P < 1.0·10⁻⁷; Figure 1). Of note, five patients did not adhere to this trend of upregulated HOXA9 in their adenomatous tissue; however, no differences in clinical parameters (age, gender, ethnicity or type of polypy) could be detected and no follow-up data were available to assess long-term consequences of differences in HOXA9 between patient groups. We concluded that pre-malignant colonic polyps are characterized by an abundance of HOXA9 expression when compared with healthy colonic tissue.

HOXA9 overexpression substantially alters the oncogenic mRNA profile
Having shown that HOXA9 upregulation is an early event during colonic carcinogenesis, we next investigated the molecular consequences of this upregulation by overexpressing HOXA9 in the CRC model cell lines. Transduction of such cells with an inducible HOXA9 lentiviral vector results in a ~30–70 fold increase in HOXA9 mRNA expression upon doxycycline induction, when compared with cells transduced with a control plasmid (Supplementary Figure 1, available at Carcinogenesis Online). Next,
the effect of HOXA9 overexpression on potential target genes was examined. To identify potentially interesting HOXA9 targets, we employed the Cancer Pathway Finder RT™ Profiler PCR Array (Figure 2A). Intriguingly, analysis of the differentially expressed genes showed that the most drastically downregulated gene was CCL2, which encodes for the chemokine MCP-1, and is a well-known mediator of tumor metastasis. Indeed, high levels of CCL2 are associated with poor outcome in CRC patients due to high incidence of metastasis (58, 59). Thus, this result implies that HOXA9 expression might be specific to pre-metastatic lesions.

Other downregulated genes included the metabolism genes ACLY and ACSL4 and the apoptosis genes CASP2 and CASP7. Among the most prominently upregulated genes were the cell cycle genes CCND2, CCND3, SKP2 and MKI67 and the growth factor FGF2, suggesting that HOXA9 overexpression provokes a proliferative phenotype. Another highly upregulated gene is the insulin growth factor binding protein 7 (IGFBP7). This gene is part of the category of IGFI signaling modulating genes, and while often considered tumor suppressive, has also been shown to be upregulated in some cancers, and may have growth-stimulatory effects in CRC (60–62). HOXA9 overexpression led to overexpression of the HMG-box gene SOX10. This gene is best known for its role in neural crest differentiation during embryogenesis, but its ectopic expression in tumors has also been shown to confer tumor aggressiveness in some tumor types (63–66), although a tumor-suppressive role has also been reported (67). In conjunction, these results are best interpreted as indicating that HOXA9 expression may stimulate adenoma growth and is responsible for compartment expansion but concomitantly would be associated with non-metaplastic behavior.

Taken together, this exploratory analysis suggests a decreased migratory phenotype, with reduced apoptosis, and increased proliferation markers. Next, we expanded on these findings by designing qPCR primers for a range of target genes not included in the array, but with specific connection to HOXA9 as identified in literature search including all tissues and model systems (Supplementary Table 1, available at Carcinogenesis Online). In addition, we also verified the main interesting finding of the Cancer Pathway Finder array, CCL2 expression using alternative primers. The statistically significantly regulated genes with the highest upregulation were FLT3 (mean FC = 3.8), IGF1 (mean FC = 2.8), PTGS2 (mean FC = 2.4) and WNT5a (mean FC = 1.8).FLT3, IGF1 and WNT5a are all associated with compartment expansion (Figure 2B). FLT3 is a tyrosine kinase receptor, IGF is a growth factor which stimulates phosphorylation-dependent kinase cascades via the IGF-receptor and WNT5a activates intracellular signaling through ligation to the Ror2/FRizzled receptors. Overexpression of PTGS2 (better known as COX2) has been associated with adenomatous changes and its inhibition is well-established to counteract colorectal polyp formation (73, 74). The genes with the highest relative downregulation were CYBB (mean FC = 0.2), encoding for the NADPH oxidase complex protein NOX2 and CCL2 (mean FC = 0.2). Interestingly, a switch in expression from NOX1 to NOX2 induced a migratory invasive phenotype in CRC cells (75), which, together with the decreased CCL2 expression, suggests that HOXA9 overexpression decreases CRC migratory behavior. Genes associated with epithelial to mesenchymal transition (EMT) (BMP1, BMP2, KRT19, VIM and ZEB) measured in this study showed little to no difference in expression as a result of HOXA9 overexpression. Overall, a picture emerges that HOXA9 is associated with polyp formation but also counteracts malignant progression.

Changes in cellular phosphoprofile support a role for HOXA9 in polyp growth but not malignant expansion

As HOXA9 overexpression modulates genetic transcription patterns towards an early proliferative phenotypes, we next sought to determine to what extent HOXA9-induced changes are translated to altered signal transduction patterns. We performed extensive (phospho) protein profiling to quantify the expression and activation status of several important signal transduction molecules (Supplementary Table 2, available at Carcinogenesis Online). Analysis of constitutive expression of signaling molecules did not reveal significantly discriminative patterns between HOXA9 overexpressing and mock-transduced cell pools (Supplementary Figure 2A and B, available at Carcinogenesis Online). However, a distinct phosphoprofile upon HOXA9 overexpression was seen, as evidenced by the clustering of control and overexpression samples (Figure 3).

The most discriminate findings were a clustering of downregulated p-S6 (FC = 0.50, \( P < 1.0 \times 10^{-4} \)) and p-PDK1 (FC = 0.45, \( P < 0.0001 \)).
P = 5.9·10⁻³) upon HOXA9 overexpression (see Figure 4 for individual analyses). Canonical signalling dictates that activity of PDK1 results in phosphorylation of the Thr308 residue of AKT, a survival protein, whereas phosphorylation of this protein on its Ser473 residue is dependent on the mammalian target of rapamycin (mTOR) when in association with Rictor in the so-called mTORC2 complex (Figure 5A). Fully activated AKT in turn is known to activate the mTOR/Raptor complex (mTORC1), which results in phosphorylation both the ribosomal S6 kinase and the translation factor 4E-BP regulating cell size and protein synthesis, respectively (76). However, our results indicate an uncoupling with canonical mTOR signaling in cells overexpressing HOXA9: (i) the decreased PDK activity was not accompanied by reduced AKT-thr308 phosphorylation, but corresponded closely to decreased S6 phosphorylation. (ii) Although there was a trend towards lower mTOR phosphorylation, this was not significant, suggesting that the phosphorylation of S6 upon HOXA9 is not a direct effect of reduced mTOR signaling. (iii) 4E-BP phosphorylation was significantly increased (FC = 1.21, P = 2.0·10⁻³), rather than decreased HOXA9-overexpressing cells. Uncoupling between mTOR and its downstream targets is not unprecedented, as a direct activation of S6 via PDK1 has also been described (77, 78), and 4E-BP1 signaling can be independent of mTOR activity in CRC (79). However, this begs the question as to what activates 4E-BP, if not mTOR. Based on our data set, it is tempting to speculate that AKT activity (FC = 1.6, P = 2.12·10⁻²) may bypass mTOR in the phosphorylation of 4E-BP (Figure 5B). Adding a further layer of complexity, it was previously shown that knockdown of Rictor promotes AKT phosphorylation, and it is conceivable that HOXA9 modulates part of the mTORC2 complex rather than mTOR per se (80).

Another interesting feature revealed by the phosphoprofiling was a significant downregulation of PAK activity (FC = 0.75, P = 2.1·10⁻³), a protein involved in cytoskeletal rearrangement and migration. The direct upstream activator of PAK is the GTPase RAC1 (81). Although we did not observe any significant changes in activity of RAC1 as measured by its phosphorylation, there was a trend towards reduced RAC1 phosphorylation levels in HOXA9-overexpressing cells. It should be noted that the role of phosphorylation for RAC1 activity has been questioned, and
its activity is best measured by demonstrating its GTP-loading. However, we have also previously shown that PAK activity can be mediated through AMPK-tubero sclerosis complex (TSC), independent of mTOR and RAC1 (82). In toto support these observations the notion that HOXA9 expression supports benign but not malignant compartment expansion.

**HOXA9 overexpression inhibits cellular migration suggesting that its expression is specific for the adenoma stage in CRC progression**

The reduced expression of chemokines (CCL2) and cytoskeletal modulators (pPAK) suggests that motility of cells might be affected upon overexpression of HOXA9 (84, 85). Therefore, we investigated migratory ability of these cells using 2D ring-barrier assays which, unlike conventional scratch assays, are not influenced by proliferative capacity of cell cultures but use time-lapse microscopy to track individual cell movement. Results show that HOXA9 overexpression leads to a significant reduction in individual cell migration, compromising the total and effective migration after 3 days, as well as in the efficiency and speed of cellular migration (Figure 7).

Tubular adenoma is the precursor to full blown CRC but is considered to have no metastatic potential. The present study shows that HOXA9 is highly expressed in colonic tubular adenomas and this expression can drive compartment expansion in a preclinical model. However, forced expression of HOXA9 also counteracts CCL2 and metastasis. In conjunction, these findings suggest that HOXA9 is a defining molecular marker of specifically the tubular adenoma stage in CRC development. This would fit a recent report that showed that in full blown CRC, tumors are characterized by increased HOXA9 expression but that such expression does not correlate with control cells (P = 0.0272; Figure 6, C1 for quantified results and C2 for illustrations of cells on the beads). Hence, increased HOXA9 expression may be directly related to the compartment size expansion that characterizes the adenomatous epithelium.
Figure 4. HOXA9 induces differential protein expression. (A) Western blots of the five proteins for which the phosphorylation status was significantly altered by HOXA9, their names are depicted on the right, the size of the band is indicated on the left. The HOXA9 status of the samples is depicted on top. (B) The results of densitometric analysis of the fluorescence bands are depicted and expressed in normalized densitometry values (AU, arbitrary units). Blots were reprobed for actin for loading control. Additionally, their corresponding $P$ values are depicted above the panels.

Figure 5. HOXA9 influences mitogenesis, protein translation and actin modulation. (A) Canonical signaling pathways of phosphoproteins. (B) Altered signaling upon HOXA9 overexpression. Red arrows indicate statistically significant directional regulation and pink arrows depict a trend in regulation.
Figure 6. HOXA9 overexpression results in increased growth of the cell pool. (A) Cell count after 6 days stimulation with doxycycline. Amount of control cells after 6 days is the reference value. (B) MTT assay measuring viable cells in HOXA9 and control transduced Caco-2 cells. Results are represented as mean ± SEM of three independent experiments. *P < 0.05, **P < 0.001 compared with control by Student’s T-test. (C1) 3D-migration assay, quantified results of one representative experiment (out of three) with data of ten coated beads. (C2) Photographs of control and HOXA9-overexpressing cells on beads in gelatin at days 1, 2, 3, 4, and 7. 'd' = day. Data were statistically analyzed by two-way ANOVA.

Figure 7. HOXA9 overexpression inhibits cellular migration. Individual cells were tracked in time laps microscopy of cellular migration for control cells (A) and HOXA6-overexpressing cells (B). A minimum of 10 cells were traced for each condition per experiment, and total migration (C), effective migration (D), efficiency (E) and velocity (F) of migration were calculated. Results are represented as mean ± standard error of the mean (SEM) of three independent experiments.
not correlate with clinical outcome (41). Here, we show that this increase is particularly manifest at the adenoma stage, but our mechanistic studies also reveal that for successful metastasis, HOXA9 expression is inhibitory. Thus, increased HOXA9 expression has a specific pro-oncogenic functionality only at early stage of the CRC process and can be considered a marker of these early stages.

Discussion

CRC remains a major health problem. Better definition of molecular markers that relate to the natural history of early CRC would prove exceedingly useful. In this study, we compared HOXA9 expression between adenoma tissue and location-matched healthy colon epithelium and we subsequently decided to investigate the role of HOXA9 in oncological transformation of colonic epithelial cells. We observe that HOXA9 is overexpressed in colonic adenomas and drives compartment expansion but concomitantly counteracts metastasis. Thus, HOXA9 expression emerges as a molecular determinant for pre-micrometastatic colonic adenomas and may support benign polyp growth in the colon.

Potential mechanisms mediating increased HOXA9 expression in adenoma

Based on the data from the publically available cBioPortal (Supplementary Figure 3, available at Carcinogenesis Online), gene amplification appears not to be involved in the increase of HOXA9 levels in CRC (50) (not shown). Given that gene amplifications are rare in colonic adenomas, this is in line with our findings. Also chromosomal translocation, which can drive altered HOXA9 expression in hematological malignancies, does not appear an important factor in this respect (not shown). Furthermore, HOXA9 was not found to be differentially methylated in CRC (86). Bhatlekar et al. found that HOXA4 and HOXA9 are up-regulated in CRC stem cells (57). Their data indicate that HOXA9 aids self-renewal and overpopulation of stem cells in CRC. Multiple reports have described HOXA9 as a pro-oncogenic factor in other solid tumors (87). Hence, increased HOXA9 expression may well be driven by selection of clones that have a competitive advantage because of relatively high HOXA9 expression and the resulting outcompeting of cells not having such high expression. In line with this train of thought is that β-catenin signaling, activated upon WNT5A ligation to its receptor, is required for HoxA9-mediated transformation in the hematopoietic system (88). Additionally, colonic adenomas are almost universally characterized by high levels of β-catenin signaling (89). However, further experimentation is obviously necessary to substantiate this notion.

Conclusion

In conclusion, HOXA9 is overexpressed in colonic adenomas. It inhibits cellular migration which appears to be mediated by effects on PAK activity. Strikingly, the pro-oncogenic phenotype of HOXA9 alteration in hematologic malignancies was also found in this study as HOXA9 stimulates cell growth. This phenotype appears to be mediated through increased IGF1, FLT3, PTGS2 and p-AKT and p-4E-BP1. This is the first mechanistic study into the effect of HOXA9 in a premalignant lesion.

Supplementary Material

Supplementary data are available at Carcinogenesis online.

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