Downregulation of HOPX Controls Metastatic Behavior in Sarcoma Cells and Identifies Genes Associated with Metastasis

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Abstract

Comparing the gene expression profiles of metastatic and nonmetastatic cells has the power to reveal candidate metastasis-associated genes, whose involvement in metastasis can be experimentally tested. In this study, differentially expressed genes were explored in the v-src-transformed metastatic cell line PR9692 and its nonmetastatic subclone PR9692-E9. First, the contribution of homeodomain only protein X (HOPX) in metastasis formation and development was assessed. HOPX-specific knockdown decreased HOPX expression in the nonmetastatic subclone and displayed reduced cell motility in vitro. Critically, HOPX knockdown decreased the in vivo metastatic capacity in a syngeneic animal model system. Genomic analyses identified a cadre of genes affected by HOPX knockdown that intersected significantly with genes previously found to be differentially expressed in metastatic versus nonmetastatic cells. Furthermore, 232 genes were found in both screens with at least a two-fold change in gene expression, and a number of high-confidence targets were validated for differential expression. Importantly, significant changes were demonstrated in the protein expression level of three metastatic-associated genes (NCAM, FOXG1, and ITGA4), and knockdown of one of the identified HOPX-regulated metastatic genes, ITGA4, showed marked inhibition of cell motility and metastasis formation. These data demonstrate that HOPX is a metastasis-associated gene and that its knockdown decreases the metastatic activity of v-src-transformed cells through altered gene expression patterns.

Implications: This study provides new mechanistic insight into a HOPX-regulated metastatic dissemination signature. Mol Cancer Res; 11(10); 1235–47. ©2013 AACR

Introduction

The metastatic process comprises a complex sequence of events including invasion of the surrounding matrix, intravasation to blood or lymphatic circulation, anchorage independent survival, and extravasation into foreign tissues (1). This multistep cascade is driven by genetic and epigenetic alterations that induce motility, invasiveness, resistance to apoptosis/anoikis, and self-renewal potential in a subpopulation of tumor cells (2, 3). In carcinomas, the metastatic progression requires trans-differentiation of epithelial to mesenchymal phenotype, a process releasing cells from adherent junctions and increasing their ability to migrate through extracellular matrix (2). This, however, is not the rule in sarcomas, which usually do not form metastases despite the mesenchymal character of sarcoma cells (4). Additional changes are necessary for the sarcoma metastasis and the knowledge of this regulatory level could contribute to our incomplete understanding of the metastatic propensity and development of effective therapeutics (5).

Being an addition to the noninvasive cancer, the metastatic gene expression program can be estimated subtractively by comparison of the gene expression profiles between metastatic and nonmetastatic cancers. Therefore, the microarray or next generation sequencing transcriptome technologies have proven as a valuable source of candidate metastasis-associated genes (6–9). We recently used the oligonucleotide microarrays to perform high-throughput screening of global gene expression in a chicken metastatic cell line derived from v-src-induced sarcoma and its nonmetastatic subclone (10). More precisely, the v-src-transformed tumor cell line PR9692 (11) is highly metastatic upon inoculation into syngeneic chickens but rare subclones of this cell line have lost their ability to induce metastases and gave rise to nonmetastatic cell line PR9692-E9 (10). An animal model combining the acquisition and reversion of the invasion-prone phenotype enables identification and study of in vivo effects of candidate metastasis-associated genes. Our experimental model provides a particular advantage, thanks to the availability of inbred chickens (12) syngeneic with the

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PR9692 and PR9692-E9 tumor cell lines, which eliminates the noise of different genetic backgrounds. Chicken as a model resembles other higher vertebrates and is an important complement to mouse model systems; however, it is genetically more distant to human than mice and we could possibly identify genes that are basically new in metastasis. Furthermore, the chicken genome is less redundant, with a lower number of gene family members of similar effect, which again could help in identification of metastasis-associated genes neglected in mice.

Although the molecular biology behind metastatic phenotype in our chicken model has not been fully clarified, we found that, for example transcription factor EGR1, actomyosin contractility factor MYL9, filopodia initiator myosin X (MYO10), extracellular matrix depositors HAS2 and COL6A1-3, cell adhesion protein NCAM1 or extracellular matrix adhesive integrin α6 (ITGA4) are involved in sarcoma progression (10). At the same time, the gene for homeodomain-only protein X (HOPX) was found to be downregulated in cells that lost the ability to metastasize (10). This gene encodes an unusual homeodomain protein that lacks certain conserved residues required for DNA binding. HOPX interacts with serum response factor (SRF) and modulates SRF-dependent cardiac-specific gene expression and cardiac development (13). HOPX was shown to be a human lung cancer and chorionicarcinoma suppressor gene involved in cytrophoblast differentiation (14, 15). Its epigenetically decreased expression level was found in other human malignant tissues, including colorectal cancer or esophageal squamous cell carcinoma (16, 17).

In this study, we studied the role of HOPX in the metastatic process and found that the metastatic cell line PR9692 lost its ability to form metastases after HOPX gene knockdown regardless of the ability to form primary sarcomas. Furthermore, the underlying gene expression changes include both genes that are strongly associated with metastasis and novel candidate metastasis-associated genes. These findings contribute to the definition of metastatic dissemination and provide a manageable list of candidate genes from which to choose targets for further studies of the mechanisms involved in metastasis.

Materials and Methods

Experimental animals

Tumor induction experiments were carried out with the inbred chicken line CC.R1 (12) maintained at the Institute of Molecular Genetics, Prague. Hens and cockerels were kept separately in individual cages with free access to food and water. The photoperiod of 12-hour light and 12-hour dark was applied. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the Academy of Sciences of the Czech Republic.

Analysis of tumor growth and metastasis

Chickens were inoculated by injection into the outer area of pectoral muscle at 6 weeks of age with 10⁴ tumor cells or 10⁵ tumor cells with HOPX or ITGA4 knockdown taken directly from the tissue culture and resuspended in 0.2 mL of culture medium. The progression of tumors was monitored as described previously (11) and birds were sacrificed before reaching the terminal stage. 20 to 49 days after inoculation. The spontaneous metastatic activity of each tumor cell line was determined by examining autopsied chickens. Metastases were observed by gross inspection and using a dissection microscope.

Cell culture and transfection

v-src-transformed metastatic cell line PR9692 and non-metastatic cell line PR9692-E9 were cultured as described previously (10, 11). PR9692-shHOPX, PR9692-shITGA4, and PR9692-shMOCK cells were derived from PR9692 cells by stable transfection of parental cells in culture with plasmids pDNA3shHOPX, pDNA3-shITGA4, and pDNA3shMOCK using FuGENE HD Transfection reagent (Promega) according to the manufacturer’s instructions. Cells were selected for 2 weeks using 500 μg/mL G418.

PR9692-E9-HOPX cells were derived from PR9692-E9 cells by infection with retroviral particles produced by AviPack packaging cells (18) transfected by the pSFCV-HOPX retrovirus vector. The transient transfection was conducted using X-tremeGENE HP DNA Transfection Reagent (Roche) according to the manufacturer’s instructions.

Quantitative RT-PCR

Total RNA was isolated from approximately 10 million cells using RNAzol RT (Molecular Research Center). One microgram of total RNA was reverse-transcribed using random hexamers and M-MLV reverse transcriptase (Promega). Gene expression was then evaluated by quantitative RT-PCR (qRT-PCR) using the MESA GREEN qPCR Master Mix Plus for SYBR assay (Eurogentec) in the Chromo 4 cycler (Bio-Rad). See Supplementary Material for the primer sequences (Supplementary Table S1). Signals were normalized to the corresponding GAPDH controls. Data analysis was conducted with the aid of the Opticon Monitor software (Bio-Rad). The relative abundances of mRNAs in the samples were approximated with $E^{ΔΔCT}$ values and presented as mean normalized expression (MNE). Significance of differences was estimated with the Welch t test for pairs PR9692 versus PR9692-E9 and PR9692-shMOCK versus PR9692-shHOPX, respectively.

Bisulfite analysis of CpG methylation

Sodium bisulfite treatment of DNA was conducted using EZ DNA Methylation Kit (Zymo Research) according to the manufacturer’s instructions. Isolation of DNA samples, seminested PCR, and sequencing of PCR clones were conducted essentially as described previously (19). See Supplementary Data for the sequence of primers for PCR of the HOPX promoter region from bisulfite-treated DNAs (Supplementary Table S1). Only PCR clones with at least 95% conversion of cytosine outside of CpG dinucleotides were taken for analysis.
DNA constructs

For short hairpin RNA (shRNA) knockdown experiments, pDNA3shRNA was constructed by removing the Spel CMV promoter fragment from pCDNA3 plasmid (Invitrogen) and by introducing the full-length microRNA (miRNA) expression cassette (20) using HindIII and BamHI restriction sites. The cassette contains RNA pol III promoter and chicken microRNA flanking sequences; the common loop comes from the human miRNA 30. pSFCV-HOPX expression retrovirus vector was constructed by introducing the full length coding sequence of the chicken HOPX cDNA into HindIII and EcoRI restriction sites of the empty pSFCV-LE vector (21).

To select anti-HOPX (AAGCTAGATGCTGGAGTGTA) and anti-ITGA4 (AGTTGCAAGTAAGAGCGAGCA) shRNAs, the HOPX and ITGA4 sequences were analyzed using a web-based siRNA-design tool (http://www.genscript.com).

For construction of the anti-HOPX and anti-ITGA4 shRNA expression plasmids (pDNA3-shHOPX and pDNA3-shITGA4) and the control shRNA expression vector (pDNA3-shMOCK), we used the oligonucleotides for shRNA synthesis, Firsthairpin primer 5 and Firsthairpin primer 3 (20). Briefly, the miRNA30-like hairpin+chicken microRNA-flanking sequence was generated by PCR using the shRNA-specific primer pair (shHOPX, shITGA4, and shMOCK) plus the general primer pair (Firsthairpin primer 5 and Firsthairpin primer 3). All constructs were verified by sequencing. After digestion with NheI and MluI, the PCR product was ligated into the pDNA3shRNA vector digested with the same enzymes. Sequences of all used oligonucleotides are shown in Supplementary Table S1.

Western blotting analysis

Proteins from 4 × 10^5 cells were resolved on 8% to 12% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membrane, which was consequently blocked in 5% skim milk. The membrane was then probed with 1:300 HOPX Antibody (C-term, Abgent) and the HRP-conjugated secondary anti-rabbit antibody (Cell Signaling Technology). The luminescent reaction was conducted using LumiGLO (Cell Signaling Technology). ITGA4, FOXG1, pVAV3, and NCAM1 proteins were detected using the same procedure and 1:1,000 antibodies anti-ITGA4 (Millipore), anti-FOXG1B (Ab2; Sigma), anti-VAV3 (phospho Y173; Abcam), and anti-NCAM1 (clone 5e-a; Developmental Studies Hybridoma Bank, http://dshb.biology.uiowa.edu/Order-forms), respectively. Equal protein loading and transfer was verified by GAPDH immunodetection (mouse monoclonal antibody 6C5, 1:4,000 dilution; Invitrogen) on the same membrane.

Migration assay

Cells in the amount of 8 × 10^5 were plated on an uncoated 6-well plate in the PR9692-shHOPX knockdown experiment or on a plate coated with fibronectin (Millipore) in the PR9692-shITGA4 experiment and cultured overnight to confluence. Using a sterile 200-µL pipette tip, 3 separate wounds on each well were scratched. Cells were washed with 40°C culture medium. The plate was then placed in a BL109 incubator (PeCon GmbH) assembled on an Leica DMi6000B microscope, preheated to 40°C. The photographs were taken at 1-minute intervals in the course of 24 hours using a Leica DFC360 FX camera. Images from time points 0, 10, 12, 20, and 24 hours were further analyzed quantitatively. The distance of each scratch closure was measured using the ImageJ software (http://rsb.info.nih.gov/ij/). Multiple positions were followed at the same time.

Three-dimensional invasion assay

Cell invasion was evaluated using the three-dimensional (3D) collagen invasion assay (22) with slight modifications. In brief, 10 µL of collagen solution (Serva) in cultivation medium was added into each well of µ-Slide Angiogenesis plate and polymerized for 30 minutes at 37°C. A total of 5 × 10^5 cells in 50 µL were added on the surface of collagen gel and allowed to invade for 48 hours at 40°C. After 1 day, the growth medium was replaced with serum-free medium. Images of 10 µm optical sections were taken using ProgRes MF Cool camera (Jenoptik) and Nikon-Eclipse TE2000-S (20×/0.40 HMC objective). For each cell line, 8 fields of view were analyzed using VirtualDub software and average invasion depth was normalized to that of PR9692 cells. The significance of differences was analyzed with ANOVA followed by Tukey honest significant difference test. The analysis was conducted in R version 2.15.3 (R Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/).

Cell-cycle and cell-proliferation analyses

Cells in the amount of 5 × 10^5 were plated on 60-mm dishes and incubated for the analyses of DNA content and doubling time. After 48 hours, cells were trypsinized and resuspended in PBS. Cell suspension was supplemented with absolute ethanol up to the final concentration of 70%. The cells were incubated on ice for 15 minutes. Samples were then centrifuged at 200 × g for 10 minutes and the ethanol decanted. Cells were resuspended in PBS and stained with 5 µg/mL propidium iodide at room temperature for 30 minutes. The staining intensity was measured by flow cytometry (BD LSRII, excitation and emission wavelengths of 536 and 617 nm, respectively) and data analyzed using FlowJo. The cell doubling time was calculated from the cells trypsinized and counted after 72 hours of cultivation using the equation 1/log Nh – log Ni)/hours, where Nh is the number of cells harvested and Ni is the number of cells inoculated.

Microarray analysis and data processing

Total RNA from 2 independent cultures isolated as described earlier was labeled according to the Expression Analysis Technical Manual (Affymetrix, 2001), hybridized to the Affymetrix GeneChip Chicken Genome Array and scanned using the Affymetrix GeneChip Scanner 3000. The exact procedures of reverse transcription, T7-driven in vitro

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transcription, hybridization, and washing were described in detail previously (10). Data were processed in Partek Genomics Suite (Partek Inc.) using GC-RMA normalization. Selection criteria of differential gene expression were set as at least 2-fold change and treatment effect P-value below 0.05. As a large proportion of the standard annotation of the Chicken GeneChip is just numbers of expressed sequence tags (EST) we tried to assign these ESTs to genes by bioinformatics methods. In many cases, we were able to unequivocally assign the ESTs to 3′-untranslated regions of known genes. We manually modified the probe set annotation accordingly (Supplementary Table S2). Microarray data have been deposited at GEO (GEO ID: GSE42516). All data are MIAME compliant.

Analysis of actin cytoskeleton organization
To examine the changes in actin cytoskeleton organization, 5 × 10^5 cells were plated on type I rat tail collagen (Millipore)-coated coverslips in the 12-well plate. Cells were allowed to adhere at 40°C overnight, washed twice with PBS, fixed with 4% paraformaldehyde for 20 minutes, and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. Subsequently, the cells were stained with 200 units/mL solution of Alexa Fluor 488 phalloidin (Invitrogen) for 50 minutes, then washed twice with PBS, and observed at the Leica DMI6000/TCS SP5 AOBS TANDEM confocal microscope. Images were taken with the Leica DFC420C digital camera.

Adhesion assay
To analyze the adhesive behavior of cells, 1 × 10^5 cells were plated on fibronectin-coated 6-well plate (Millipore) blocked with 0.5% bovine serum albumin (BSA) for 30 minutes to prevent nonspecific binding. Cells were left to adhere for 2 hours at 40°C and the plate was then shaken at 2,000 rpm for 15 seconds. Released cells were removed by 3 washes with 0.1% BSA in cultured medium. Adhered cells were fixed with 4% paraformaldehyde and stained with Crystal Violet. The plate was left to dry up completely. After that, 2% SDS was added followed by incubation for 30 minutes. The plate was then shaken and read at 550 nm.

Results
Decreased HOPX expression in the nonmetastatic PR9692-E9 cells
In our previous study (10), the gene expression analysis using Affymetrix Chicken Gene Chips revealed tens of genes differentially expressed in the metastatic PR9692 and nonmetastatic PR9692-E9 cells, with HOPX gene downregulated in PR9692-E9 cells. In this report, we focused on the HOPX gene because to date, HOPX has not been involved in metastasis formation. We verified the microarray data using qRT-PCR (Fig. 1A) and found a significant decrease in the HOPX mRNA level in PR9692-E9 cells. Transcriptional suppression of HOPX expression has repeatedly been correlated with promoter hypermethylation (15, 16, 23, 24). To test the epigenetic character of HOPX suppression, we examined the HOPX methylation status in PR9692 and PR9692-E9 cells by bisulfite sequencing. We found densely methylated HOPX promoter in PR9692-E9 and slightly hypomethylated HOPX promoter in the metastatic PR9692 cells (Fig. 1B). In conclusion, we showed epigenetic HOPX gene downregulation in nonmetastatic cell line PR9692-E9.

Knockdown of HOPX decreases cell migration, invasion, and proliferation of PR9692
To analyze the possible involvement of HOPX in metastasis, we used shRNA to knockdown endogenous HOPX gene expression in the PR9692 cell line and observed in vitro correlates of the processes leading to metastases. HOPX expression in PR9692 cells significantly dropped at the mRNA and protein levels after stable transfection with pDNA3shHOPX plasmid (Fig. 2A and B), whereas the control shRNA (shMOCK) did not influence HOPX expression. The HOPX knockdown was stable for at least 6 weeks of continuous in vitro culture. Vice versa, we...
overexpressed the chicken HOPX in PR9692-E9-HOPX cell line created by infection of the nonmetastatic cell line PR9692-E9 with pSFCV-HOPX expression retrovirus vector (Fig. 2A and B).

Changes in cell motility after the knockdown of HOPX were examined using the scratch wound healing assay. Control PR9692 and PR9692-shMOCK cells spread over a significantly larger area of the wound gap than PR9692-shHOPX cells (Fig. 2C). At 10 hours of migration, the reduction observed in PR9692-shHOPX was 1.38-fold, \( P = 0.01 \). The wound gaps in control plates were nearly filled within 20 hours, whereas the gaps remained widely open in plates with the PR9692-shHOPX cells (Fig. 2D).

To rule out the effect of different cell-proliferation rates in wound healing migration assay, we analyzed the ability of cells to invade 3D collagen in vitro. In this assay, invasion of individual cell is assessed and correlated with total number of counted cells. Cells were placed on the

**Figure 2.** Decrease of HOPX expression and its in vitro effects on cell migration, invasiveness, and proliferation. A, HOPX knockdown at the mRNA level measured by qRT-PCR in parental PR9692 cells (black), PR9692-shMOCK (dark gray), PR9692-shHOPX cells (white gray), and PR9692-E9-HOPX (vertical stripes). MNE \( \pm \) SE calculated from 3 experiments each conducted in triplicate (*, \( P < 0.05 \), **, \( P < 0.01 \)). B, knockdown of HOPX at the protein level analyzed by Western blotting. GAPDH blot is shown as the loading control. C, cell migration analyzed by scratch assay and shown as cell migration area in time. The widths of the gaps from 5 measurements were analyzed. PR9692, black diamonds; PR9692-shMOCK, dark gray squares; PR9692-shHOPX, light gray triangles. D, phase-contrast images of wound areas 0 and 20 hours after wounding of PR9692-shMOCK (top) and PR9692-shHOPX (bottom) cell monolayer. E, 3D invasion capacity of cells assayed in 3D collagen matrix. The results are shown as mean percentage ± SE of 3 independent experiments (**, \( P < 0.01 \)). F, cell proliferation rate shown as doubling time in hours. Data represent means ± SE of 3 parallel cell cultures (**, \( P < 0.01 \), value above the bar represents the increase in doubling time after HOPX knockdown in percent). G, cell-cycle analysis of PR9692 (left column), PR9692-shMOCK (middle column), and PR9692-shHOPX (right column); the proportions of cells in G1 (black), S (light gray), and G2 (white) phases obtained by FACS were calculated as means of 3 independent experiments.
surface of collagen matrix and allowed to invade for 48 hours. The PR9692 and PR9692-shMOCK cell lines displayed high invasive activity, whereas the ability of PR9692-shHOPX cells to invade 3D collagen matrix was reduced by 62% ($P < 0.05$) in comparison with PR9692 cells. To further confirm the effect of HOPX on invasion, we analyzed the invasive activity of PR9692-E9-HOPX cells. Increased HOPX expression resulted in enhanced invasion almost up to the level of metastatic line PR9692 (Fig. 2E). Notably, HOPX knockdown inhibited invasion of PR9692-shHOPX cells and HOPX upregulation rescued invasiveness of PR9692-E9-HOPX cells.

The effect of HOPX knockdown on cell proliferation was estimated as a doubling time of the cell culture. The results showed a significantly ($P = 0.007$) longer doubling time of PR9692-shHOPX cells compared to PR9692 and PR9692-shMOCK cells (Fig. 2F). To further confirm this result, we analyzed the cell-cycle distribution by propidium iodide staining of intact nuclei. HOPX knockdown in the PR9692-shHOPX cells correlated with an increase in G1-phase population, and decrease of the S- and G2-phase populations in comparison with the PR9692 and PR9692-shMOCK cells (Fig. 2G). This result is consistent with G1-S phase cell-cycle arrest.

Altogether, HOPX downregulation led to slower migration, invasion in a 3D environment, and proliferation of PR9692 cells. The decreased migration and invasion does not result from a slower proliferation rate, as we observed individual cells mostly within the period of their first doubling.

### HOPX knockdown inhibits formation of lung metastases

To correlate the in vitro decreased cell migration and invasion of PR9692 cells with metastasis, we examined the in vivo effect of HOPX knockdown on the growth and invasion of sarcomas in our original chicken model (12). Chickens of the inbred line CC.R1, the original host of the PR9692 tumor (11), reproducibly develop sarcomas and subsequently lung metastases after inoculation of PR9692 cells (10). Birds injected with PR9692 cells and PR9692-shMOCK cells were monitored for tumor growth, autopsied 20 to 25 days postinoculation, and inspected for the presence of metastases. As shown in Table 1, all PR9692- and PR9692-shMOCK-inoculated birds developed progressively growing sarcomas and macroscopic analysis of lungs revealed extensive regions of tumor tissue. As expected from the lower proliferation rate of PR9692-shHOPX cells, sarcomas induced by these cells grew more slowly and were autopsied later, on days 36 to 49 postinoculation, than control sarcomas. However, the final size and incidence of PR9692-shHOPX-induced sarcomas was comparable with the controls. In contrast, only few birds developed metastases after inoculation of PR9692-shHOPX cells even in case of the largest primary tumors autopsied after a long period of growth. To further confirm the influence of HOPX level on metastasis, we tested the metastatic potential of PR9692-E9-HOPX cells, in which the HOPX expression was recovered from a retrovirus vector. The increased HOPX expression rescued metastasis by 28% (Table 1). These results indicate that the knockdown of HOPX affects the capacity of PR9692 cells to form lung metastases after intramuscular inoculation.

### Table 1. Number and size of metastases assessed in animals 20–49 days postinoculation (d.p.i.) of cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of inoculated cells</th>
<th>d.p.i.</th>
<th>No. of chicks with primary tumor/No. of chicks inoculated</th>
<th>Mean final tumor weight (g)</th>
<th>No. of chicks with metastases</th>
<th>% of chicks with metastases from formed primary tumor</th>
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<tr>
<td>PR9692</td>
<td>10^4</td>
<td>20</td>
<td>19/19</td>
<td>39.6</td>
<td>2</td>
<td>3</td>
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<tr>
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<td>6/6</td>
<td>67.5</td>
<td>1</td>
<td>1</td>
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<tr>
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<td>4/4</td>
<td>58.7</td>
<td>1</td>
<td>0</td>
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<td>11/11</td>
<td>31.5</td>
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<td>4</td>
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<tr>
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<td>49</td>
<td>9/10</td>
<td>25.7</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>PR9692-shITGA4</td>
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<td>49</td>
<td>6/6</td>
<td>39.7</td>
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<td>70.1</td>
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<tr>
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<td>49</td>
<td>18/18</td>
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The extent of lung metastasis: +, multiple (>10) small and 1–3 medium-sized (3–4 mm) metastases; ++, multiple small and more than 3 medium-sized metastases and/or 1–3 large (4–5 mm) isolated metastases; +++, lungs overgrown with medium to large metastases growing together, frequently more than 50% of lungs formed by tumor tissue, in some chicks metastases seized the majority of the lungs.
implantation in the syngeneic chickens. Moreover, this decrease in metastasis is not the result of longer doubling time of PR9692-shHOPX cells.

**Differential gene expression analysis of PR9692shMOCK and PR9692shHOPX cells**

To describe changes in gene expression accompanying the decreased metastasis after the HOPX knockdown, a comparative gene expression analysis between PR9692-shMOCK and PR9692-shHOPX cells was conducted using the oligonucleotide Affymetrix Chicken GeneChip. The analysis revealed more than 700 differentially expressed genes with at least 2-fold change of gene expression, _P_-value <0.05. To specify genes involved in metastasis, we compared the present results with our previous study (10), showing differences in gene expression between the metastatic PR9692 and spontaneously nonmetastatic PR9692-E9 cells. The overlap of genes differently expressed in both pairs of metastatic and nonmetastatic cell lines (Fig. 3A) points to candidate metastasis-associated genes with promoting or suppressing roles in tumor invasion. We identified 232 genes that show at least 2-fold corresponding change in gene expression in both pairs of cells. Among them, 186 genes were downregulated 2- to 192-fold and 46 were upregulated 2- to 36-fold in nonmetastatic cells (Fig. 3A). Selected genes with the most significant differences in expression between the pairs of cell lines are summarized in Table 2. A complete list of all genes showing at least a 2-fold difference in expression is included in Supplementary Table S2. These genes encode proteins of several functional classes including tumorigenesis (EPB41L3, KIAA1210, SALL3), cell adhesion and migration, cell survival, angiogenesis, and HOPX itself, these findings were confirmed at the protein level (Fig. 3C).

Using the microarray technology, we identified several genes that, to our knowledge, have not been seen in the context with metastasis so far [e.g., 5-hydroxytryptamine receptor 1D (HTR1D), NUDT14, RGS20].

We subsequently validated the microarray results by qRT-PCR of several genes, ADCY7, ARGHD1B, BAMBI, FOXG1, FSCN1, FUCAI, GPFT2, ITPR3, ITGA4, LSP1, NCAM1, NUDT14, RGS20, RHOF, and RIAM, that have already been implicated in cell migration, adhesion, or angiogenesis (28, 33–46). The results from qRT-PCR analysis (Fig. 3B) were in good agreement with the microarray data, corroborating the reliability of the microarray results. Moreover, in the case of ITGA4, NCAM1, FOXG1, and HOPX itself, these findings were confirmed at the protein level (Fig. 3C).

**Knockdown of ITGA4 suppresses cell adhesion, migration, and metastasis formation**

Among the candidate metastasis-associated genes, we chose the _ITGA4_ gene to show its metastatic capacity and validate our screen. We stably decreased the ITGA4 expression level in PR9692 cells by the same shRNA knockdown approach as for HOPX. Very effective knockdown of ITGA4 mRNA level (Fig. 4A) corresponds with the absence of any signal at the protein level (Fig. 4B). To investigate possible changes of cell migration as an effect of ITGA4 knockdown, we used the scratch-wound healing migration assays on fibronectin and compared PR9692 cells, PR9692-shMOCK cells, and PR9692sh-ITGA4 cells. Fibronectin-coated plates were used in this experiment, as fibronectin is a ligand for transmembrane heterodimers α4/β1 and α4/β7 integrins. The wound gap filling was monitored during 24 hours and, as shown in Fig. 4C, the cell-occupied area at 20 hours was larger in PR9692 and PR9692-shMOCK cells than in PR9692shITGA4 cells (2.92-fold reduction, _P_ = 0.027; Fig. 4C). The wound gap had not been filled by PR9692shITGA4 (Fig. 4D).

Integrins are cell adhesion proteins and weakening of the cell-substrate attachment hallmarks the metastasis in some types of cancer (2). Thus, we next investigated whether knockdown of ITGA4 affected cell—substrate adhesion in PR9692 cells using an adhesion assay. We found that the adhesion of PR9692-shITGA4 to fibronectin was reduced by approximately 33% at _P_ < 0.05 when compared with control cells PR9692 and PR9692-shMOCK (Fig. 4E).

In a parallel to HOPX, we examined the effect of ITGA4 knockdown on invasive behavior. The 3D collagen invasion assay showed that in PR9692 cells, the knockdown of ITGA4 led to cell invasion reduced by 72%. These data show that ITGA4 promotes cell migration and invasion (Fig. 2E). In accordance to the changes in motility, substrate adhesion and 3D invasion, ITGA4 level also influenced the organization of actin cytoskeleton. Phalloidin staining of actin showed that ITGA4 knockdown in PR9692 cells resulted in a loss of actin stress fibers and defective cell polarity (Fig. 4F). These changes in morphology on collagen-coated cell substrate plastic in PR9692shITGA4 resembled the phenotype of PR9692-E9 cells.

The effect of ITGA4 knockdown on metastasis formation was tested _in vivo_ in animals injected with PR9692-shITGA4 cells. All birds developed primary sarcomas, and after 37 days they were autopsied and their lungs were inspected for the presence of metastases. Only low-grade spreading of metastases was detected in animals injected with PR9692-shITGA4 cells, whereas multiple metastases were detected in all birds injected with PR9692 or PR9692-shMOCK (Table 1).

Altogether, the results showed that ITGA4 knockdown correlates with decreased cell motility, cell adhesion, and metastasis in PR9692-shITGA4 cells. These findings indicate that ITGA4 plays an important role in the processes that are obligatory for the cells to be metastatic.

**Discussion**

Microarrays provide a powerful tool for expression profiling of tumors and metastases and for uncovering the expression signature of metastasis-associated genes. Doing that, we have to cope with tumor heterogeneity accompanied by difficulties in matching the disseminated cells with the respective tumor samples. Our experimental comparison of the v-src-transformed and highly metastatic chicken sarcoma cell line with its nonmetastatic but fully tumorigenic clonal
Figure 3. Microarray data reveal the candidate metastasis-associated genes with differential gene expression in cells with decreased HOPX expression. A, schematic representation of the overlap between 2 sets of differentially expressed genes in 2 pairs of metastatic/nonmetastatic cells. B, decreased expression of ADCY7, ARGHDIB, BAMBI, FOXG1, FSCN1, FUC1, GFPT2, HOPX, ITPR3, ITGA4, LSP1, NCAM1, NUDT14, RGS20, RHOF, and RIAM in PR9692 (black), PR9692-shMOCK (dark gray), PR9692-shHOPX (light gray), and PR9692-E9 (white) cells determined by qRT-PCR (\( P < 0.05 \)), MNE \( \pm \) SD calculated from 3 independent experiments. C, knockdown of HOPX and decreased expression of NCAM1, ITGA4, and FOXG1 genes in PR9692, PR9692-shMOCK, PR9692-shHOPX, and PR9692-E9 cells at protein level analyzed by Western blotting. GAPDH blot is shown as the loading control.
### Table 2. Selected genes differentially expressed in PR9692-shHOPX vs. PR9692-shMOCK and PR9692-E9 vs. PR9692 cells

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Description</th>
<th>Fold-change (PR9692-shHOPX vs. PR9692-shMOCK)</th>
<th>P-value (PR9692-shHOPX vs. PR9692-shMOCK)</th>
<th>Fold-change (PR9692-E9 vs. PR9692)</th>
<th>P-value (PR9692-E9 vs. PR9692)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADCY7</td>
<td>Adenylate cyclase 7</td>
<td>-15.013</td>
<td>&lt;0.001</td>
<td>-29.929</td>
<td>&lt;0.001</td>
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<tr>
<td>ANXA7</td>
<td>Annexin A7</td>
<td>-16.042</td>
<td>&lt;0.001</td>
<td>-126.263</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ARHGDIIB</td>
<td>Rho GDP dissociation inhibitor (GDI) beta</td>
<td>-2.597</td>
<td>0.003</td>
<td>-5.320</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BAMB1</td>
<td>BMP and activin membrane-bound inhibitor homolog</td>
<td>-6.627</td>
<td>&lt;0.001</td>
<td>-9.066</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DNM1</td>
<td>Dynamin 1</td>
<td>-8.172</td>
<td>0.001</td>
<td>-5.833</td>
<td>0.001</td>
</tr>
<tr>
<td>FOXG1</td>
<td>Forkhead box G1</td>
<td>-2.687</td>
<td>0.022</td>
<td>-2.155</td>
<td>0.027</td>
</tr>
<tr>
<td>FSCN1</td>
<td>Fascin homolog 1, actin-bundling protein</td>
<td>-13.139</td>
<td>0.001</td>
<td>-6.842</td>
<td>0.002</td>
</tr>
<tr>
<td>FUCA1</td>
<td>Fucosidase, α-L-1, tissue</td>
<td>-24.395</td>
<td>&lt;0.001</td>
<td>-15.404</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GFPT2</td>
<td>Glutamine-fructose-6-phosphate transaminase 2</td>
<td>-55.721</td>
<td>&lt;0.001</td>
<td>-251.417</td>
<td>&lt;0.001</td>
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<tr>
<td>GSR</td>
<td>Glutathione reductase</td>
<td>-42.586</td>
<td>&lt;0.001</td>
<td>-29.441</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HTR1D</td>
<td>5-Hydroxytryptamine receptor 1D, G protein-coupled</td>
<td>-192.015</td>
<td>-9.542</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>ITPR3</td>
<td>Inositol 1,4,5-trisphosphate receptor, type 3</td>
<td>-19.539</td>
<td>&lt;0.001</td>
<td>-21.216</td>
<td>&lt;0.001</td>
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<tr>
<td>KIAA1210</td>
<td>KIAA1210</td>
<td>4.111</td>
<td>&lt;0.001</td>
<td>12.099</td>
<td>&lt;0.001</td>
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<tr>
<td>LAMB1</td>
<td>Laminin, β1</td>
<td>-17.410</td>
<td>0.001</td>
<td>-2.681</td>
<td>0.046</td>
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<tr>
<td>LHFPL5</td>
<td>Lipoma HMGC fusion partner-like 5</td>
<td>-19.196</td>
<td>0.001</td>
<td>-70.501</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LSP1</td>
<td>Lymphocyte-specific protein 1</td>
<td>-17.073</td>
<td>&lt;0.001</td>
<td>-37.097</td>
<td>&lt;0.001</td>
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<tr>
<td>MAPK11</td>
<td>Mitogen-activated protein kinase 11</td>
<td>-14.056</td>
<td>&lt;0.001</td>
<td>-5.475</td>
<td>0.001</td>
</tr>
<tr>
<td>NCA1M1</td>
<td>Neural cell adhesion molecule 1</td>
<td>-12.569</td>
<td>0.001</td>
<td>-31.376</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NUDT14</td>
<td>Nudix-type motif 14</td>
<td>-63.491</td>
<td>&lt;0.001</td>
<td>-18.548</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PAG5</td>
<td>Protein kinase C and casein kinase substrate in neurons</td>
<td>-16.870</td>
<td>0.001</td>
<td>-7.897</td>
<td>0.001</td>
</tr>
<tr>
<td>RAB15</td>
<td>Ras-related protein Rab-15</td>
<td>-70.320</td>
<td>&lt;0.001</td>
<td>-54.899</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RGS20</td>
<td>Regulator of G-protein signaling 20</td>
<td>-18.632</td>
<td>&lt;0.001</td>
<td>-4.004</td>
<td>0.003</td>
</tr>
<tr>
<td>RHOF</td>
<td>Ras homolog gene family, member F</td>
<td>-17.316</td>
<td>&lt;0.001</td>
<td>-7.873</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RIAM</td>
<td>Raps1-GTP-interacting adapter molecule</td>
<td>-43.213</td>
<td>&lt;0.001</td>
<td>-13.357</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SALL3</td>
<td>Sal-like 3 (Drosophila)</td>
<td>3.656</td>
<td>0.043</td>
<td>6.707</td>
<td>0.004</td>
</tr>
<tr>
<td>SLC16A10</td>
<td>Solute carrier family 16, member 10</td>
<td>9.468</td>
<td>0.019</td>
<td>5.911</td>
<td>0.022</td>
</tr>
<tr>
<td>SLC22A3</td>
<td>Solute carrier family 22</td>
<td>-92.347</td>
<td>&lt;0.001</td>
<td>-30.656</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TTC32</td>
<td>Tetrahydrocysteine repeat domain 32</td>
<td>-33.421</td>
<td>&lt;0.001</td>
<td>-10.161</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
derivative (10) overcomes this drawback. Previously, model systems of high- and low-metastatic clonal derivatives of mouse cell lines were used (47) but not pro
duced by microarrays. Our unique chicken model offers syngeneic chickens for in vivo experiments, lower mutability, and better matching of primary tumor with metastases.

We identified HOPX as a candidate metastasis-associated gene in chicken sarcomas. Previous work documented the decreased HOPX expression in several types of human cancer. Explicitly, HOPX was suggested as tumor suppressor in esophageal and gastric cancer (17, 23). These studies, however, did not study the role of HOPX in tumor dissemination and metastasis. We describe here the downregulation of HOPX in nonmetastatic cell line PR9692-E9, which suggests that HOPX takes part in the process of invasion and metastasis of sarcoma. In vitro cell invasion and migration assays as well as in vivo metastasis induction are in good agreement with this assumption.

The subcloning process leading to the nonmetastatic cell line PR9692-E9 could have sampled random polymorphisms of gene expression already present in the parental cell line PR9692. To confirm that HOPX downregulation is associated with the nonmetastatic phenotype and to validate our screen of metastasis-associated genes, we compared the expression profiles of PR9692-E9 and HOPX knockdown PR9692-shHOPX cell lines. Both sets of differentially expressed genes displayed a substantial overlap. Furthermore, the credibility of our system is increased by the finding...
of differentially expressed genes already implicated in cancer dissemination including neuronal adhesion molecule NCAM1, forkhead transcription factor FOXG1, or ITGA4. Downregulation of these 3 genes was also confirmed at the protein level.

NCAM1 stimulates migration and invasion of epithelial ovarian carcinoma cells and melanoma cells and promotes metastatic dissemination in mice. This promalignant function of NCAM1 is mediated by its interaction with fibroblast growth factor receptor (38, 48). FOXG1 acts as an oncoprotein inhibiting p16/CDKN2A/CIP1 transcription (37, 49). Integrins are the most important matrix receptors and their downregulation can promote dissemination of primary tumor but also suppress the attachment of already migrating tumor cells at distant sites. In accordance, downregulation is crucial for the establishment of invasive phenotype in breast cancer (50). Downregulation of ITGA4 in PR9692-E9 cells leading to inhibition of metastatic dissemination can be an example of the second mechanism. In addition, decreased ITGA4 expression can inhibit tumor lymphangiogenesis and prevent metastatic disease (51).

The challenging output of this study is the set of candidate metastasis-associated genes, which were not yet clearly implicated in tumor metastasis and do not belong into the gene families intuitively involved either in carcinogenesis or tumor dissemination. These genes include, for example, nucleoside diphosphate-linked moiety X motif 14 (NUDT14), regulator of G-protein signaling 20 (RGS20), or adenylate cyclase 7 (ADCY7) to mention the genes with most striking differences of expression. We observed NUDT14 downregulated more than 87-fold on average (P < 0.001) in PR9692-E9 cells. The NUDT14 gene encodes a protein that hydrolyzes UDP-glucose to glucose-1-phosphate and UMP- and ADP-ribose to ribose 5-phosphate and AMP. This gene maps to 14q32.33, a chromosomal region of frequently observed cytogenetic alterations in B-cell malignancies (52). It was also reported that NUDT14 is overexpressed in human rectal carcinoma (40). RGS20 belongs to a family of proteins with conserved GTPase-activating domain of approximately 120 amino acid residues. Previous, polymorphisms of RGS2 and RGS6 genes were described in bladder and lung cancers (53, 54). RGS20 itself was reported in stabilizing the microtubule filaments (55). In our study, we observed RGS20 downregulated more than 5-fold on average (P = 0.003) in PR9692-E9. ADCY7 is a calcium-regulated membrane-bound adenylate cyclase, whose upregulation was described in a highly metastatic clone of murine sarcoma cell line (36). In accordance, we found ADCY7 expression downregulated more than 21-fold (P = 0.000006) on average in the nonmetastatic PR9692-E9 cell line. Finally, HTRID is an instance of candidate metastasis-associated gene, which has not yet been reported in tumorigenesis or metastasis at all. The microarray-based overexpression of NUTD14, RGS20, and ADCY7 was confirmed by qRT-PCR (Table 2).

Detailed studies focused on individual candidate genes resulting from our chicken screen have to be carried out in the future to confirm their role in metastasis and explain the mechanisms of their action. We cannot exclude the mere bystander effect without gene-specific silencing analysis as it was conducted with HOPX and ITGA4. Furthermore, there is a concern of opposite effects on cancer progression exerted by a single gene. ITGA4 is a good example. Even classical proto-oncogene c-src, whose activity or expression is elevated in a number of human tumors, suppresses metastasis of bladder cancer (56, 57). Future work should therefore include metastatic models of carcinomas to evaluate whether HOPX is universally associated with metastasis. Quite recently, HOPX and GATA6 were shown to inhibit metastasis in a lineage-specific subtype of lung adenocarcinoma (58). Because HOPX lacks a DNA binding domain, interaction with other sequence-specific transcription factor is necessary to exert any effect at the level of transcriptome. GATA6 specifies the endoderm lineage and, depending on the particular context, either promotes or inhibits cancer. In our model of mesenchymal-derived tumor, HOPX must interact with other factor(s) and acts inversely as promoter of metastasis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: D. Kovárová, J. Plachy, J. Kola, K. Trejbalová, J. Hejnár
Development of methodology: D. Kovárová, J. Hejnár
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Kovárová, J. Plachy, J. Kola
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Kovárová, J. Kola, K. Trejbalová, V. Čermáková, J. Hejnár
Writing, review, and/or revision of the manuscript: D. Kovárová, J. Plachy, J. Kola, V. Čermáková, J. Hejnár
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V. Čermáková, J. Hejnár
Study supervision: J. Hejnár

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References


Downregulation of HOPX Controls Metastatic Behavior in Sarcoma Cells and Identifies Genes Associated with Metastasis

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