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*Cancer Res* 2001;61:6532-6539.

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# HOXB7: A Key Factor for Tumor-associated Angiogenic Switch<sup>1</sup>

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## ABSTRACT

We had demonstrated previously a functional bridge between altered homeobox (HOX) gene expression and tumor progression through HOXB7 transactivation of basic fibroblast growth factor.

Here, we have studied whether HOXB7, in addition to basic fibroblast growth factor, may induce other genes directly or indirectly related to neoangiogenesis and tumor invasion. Parental,  $\beta$ -galactosidase-transduced, and HOXB7-transduced SkBr3 cell lines were examined for the expression of several growth factors and growth factor receptors involved in the proliferative and angiogenic processes. Vascular endothelial growth factor, melanoma growth-stimulatory activity/growth-related oncogene  $\alpha$ , interleukin-8, and angiopoietin-2 were up-regulated by HOXB7 transduction. The exception was angiopoietin-1 expression that was abrogated. Additional analyses included the expression levels of enzymes such as matrix metalloprotease (MMP)-2 and MMP-9 and heparanase, capable of proteolytic degradation of extracellular matrix and basement membranes. Results showed an induction of only MMP-9.

The functional implication of such a finding was tested using an *in vitro* coculture assay in a three-dimensional matrix. A delay of differentiation with persistent nests of proliferating cells was found in endothelial cells cocultured with HOXB7-transduced SkBr3 cells. Tumorigenicity of these cells has been evaluated *in vivo*. Xenograft into athymic nude mice showed that SkBr3/HOXB7 cells developed tumors in mice, either irradiated or not, whereas parental SkBr3 cells did not show any tumor take unless mice were sublethally irradiated. Comparison of tumor nodules for vascularization by CD-31 and CD-34 immunostaining revealed an increased number of blood vessels in tumors expressing HOXB7. Together, the results indicate HOXB7 as a key factor up-regulating a variety of proangiogenic stimuli. Thus, HOXB7 gene or protein is a target to aim at to inhibit tumor-associated neoangiogenesis, considering the number and the redundancy of proangiogenic molecules that should be targeted one by one to theoretically achieve the same effect.

## INTRODUCTION

Tumor growth and progression largely depends on angiogenesis. During this process, endothelial cells proliferate, degrade the basement membrane, migrate throughout the stroma, and finally differentiate into the tubular structures of new vessels (1-3). Cytokines such as bFGF,<sup>3</sup> tumor necrosis factor  $\alpha$ , and VEGF are the most potent inducers of angiogenesis; however, their precise role in the coordinate regulation of this process remains veiled (4).

Products of HOX genes are transcription factors responsible for

tissue remodeling. Besides their function in embryonic development, inappropriate HOX gene expression has been associated with different neoplasias (5, 6). HOXB7-enforced expression in hematopoietic progenitor/stem cells, purified from adult human peripheral blood, markedly modulated the proliferative/differentiative program of this population inducing a prolonged proliferation of a discrete population of blast cells and granulocytic-oriented cells (7). These results suggested a potential preleukemic immortalization step attributable to HOXB7 overexpression. This oncogenic potential has been already demonstrated by *in vitro* and *in vivo* transformation assays for several murine Hox genes (8). Moreover, HOXB7 has been shown to be constitutively expressed in both melanoma primary lesions and cell lines (5). Investigating downstream genes targeted by HOXB7, we identified bFGF as its main target. Indeed, HOXB7 binds and transactivates bFGF, whereas HOXB7 inhibition reduces bFGF expression and cell proliferation in melanoma cell lines. The screening of several tumor cell lines indicated SkBr3 from a breast adenocarcinoma (9) as the sole negative sample for the expression of both HOXB7 and bFGF genes. Retroviral-mediated transduction of the sole HOXB7 cDNA into SkBr3 cells resulted in transcription of both HOXB7 and bFGF and in several phenotypic changes including increased cell growth rate, independence from serum withdrawal, and ability to form colonies in semisolid medium (10).

HOX genes likely regulate vasculogenesis and perhaps angiogenesis. Indeed, HOXD3 has been linked to the mechanism converting endothelial cells from a resting to angiogenic/invasive state (11), whereas HOXB3 is required for the subsequent capillary morphogenesis of the new vascular sprouts (12).

Because tumor cells elaborate growth factors that act on the surrounding endothelial cells to induce new blood vessel formation, we studied whether HOXB7, besides bFGF activation, could affect neoangiogenesis through a direct or indirect regulation of growth factors and growth factor receptors.

Our data indicate that HOXB7-enforced expression in SkBr3 cells increased the transcription of the analyzed growth factor genes but not that of the corresponding receptors. The proangiogenic molecules produced by transduced SkBr3 cells are not used or sequestered by the tumor and remain available to the endothelial cells. Accordingly, *in vitro* and *in vivo* evaluation of the angiogenic activity showed an increased and persistent number of newly formed capillaries and an increased tumorigenicity, respectively, of HOXB7/SkBr3 cells. The possible role of HOXB7 as a key activator of tumor-associated neoangiogenesis and its targeting for tumor gene therapy are discussed.

## MATERIALS AND METHODS

**Cell Line Culture and Transduction.** The HOXB7 and  $\beta$ -gal cDNAs encompassing their complete coding sequences were cloned into the retroviral vector LXS.N. The  $\beta$ -gal gene was used as an internal control. The ecotropic GP+ E86 and the amphotropic GP+ env AM12 packaging cell lines were produced. Amphotropic cells were selected in the presence of 0.8 mg/ml of G418 plus 0.2 mg/ml of hygromycin and used to generate helper-free virus-

Received 3/15/01; accepted 6/21/01.

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<sup>1</sup> Supported by the Italian Association for Cancer Research (AIRC) and in part by Ministero della Sanità and The Italy-USA Special Project on Therapy of Tumors. F. F. was supported by a fellowship from Fondazione Italiana per la Ricerca sul Cancro.

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<sup>3</sup> The abbreviations used are: bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; MGSA/GRO $\alpha$ , melanoma growth-stimulatory activity/growth-related oncogene  $\alpha$ ; HOX, homeobox;  $\beta$ -gal,  $\beta$ -galactosidase; RT-PCR, reverse transcription-PCR; IL, interleukin; MMP, matrix metalloprotease; HUVEC, human umbilical vein endothelial cell; Ang, angiopoietin; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

containing supernatant. The viral titer of the selected clones was  $1.3 \times 10^6$  pfu/ml for HOXB7 and  $2 \times 10^6$  pfu/ml for  $\beta$ -gal. For target infection, SkBr3 cells were treated twice for 4 h with undiluted packaging cell supernatants in the presence of 8  $\mu$ g/ml of Polybrene. Cells were grown for 48 h and then selected with G418 (1.6mg/ml; Ref. 10).

**RNA Analysis.** RT-PCR analysis and RNase protection were performed according to standard procedures (5, 10, 13). The sequences of primers, the PCR products, and the annealing conditions are reported in Table 1. All of the amplified fragments were hybridized to a 30-base end-labeled oligonucleotide according to Southern blot standard procedures.

For the RNase protection assays, DNA fragments to be transcribed consisted of a specific sequence for VEGF exon 3 of 130 bp. IL-8 was a 227-bp *EcoRI-SacI*, and GRO $\alpha$  was a 250-bp *BamHI*. Fragments were cloned in pCRII vector (Invitrogen BV, Groningen, the Netherlands). The  $\beta$ -actin insert was a 93-bp *RsaI* fragment, always labeled in the presence of cold GTP to obtain a low specific activity. The expression levels were analyzed by the Image Quant software (Molecular Dynamics, Sunnyvale, CA).

**Antisense Experiments.** Antisense inhibition analysis was carried out as described previously (5). Briefly, cells ( $2 \times 10^5$ ) of each sample were incubated in triplicate in 96-well plates in 0.2 ml of culture medium supplemented with 10% fetal bovine serum, heat-inactivated for 30 min at 65°C to destroy nucleases, in the presence or absence of antisense and scrambled phosphorothioate oligomers. The antisense oligomers were complementary to the translation start site. Oligomers at concentrations of 30  $\mu$ M were added 24 h after cell plating, and proliferation was tested on day 7. Antisense inhibition was calculated relative to that of scrambled-treated cells, the inhibition of which never exceeded 20% of the proliferation rate shown by their untreated counterparts. The specificity of the inhibitory effect was ensured by a series of controls: (a) the sense, scrambled, and antisense sequences were tested against sequences from the European Molecular Biology Laboratory database to demonstrate the absence of toxicity and the specificity of inhibition; and (b) the abrogation of the specific messages only in the antisense-treated cells was confirmed by RT-PCR, whereas unrelated control mRNAs were not modified. For RT-PCR analysis, on the last day of culture, cells from the third well of each sample were lysed for total RNA extraction by a modification of the guanidine-thiocyanate CsCl gradient made in a microultracentrifuge (TL-100; Beckman Inst., Palo Alto, CA) in the presence of rRNA as carrier.

**ELISA.** Intracellular and secreted protein (bFGF, VEGF, IL-8, and GRO $\alpha$ ) were quantified by ELISA kits from R&D Systems (Minneapolis, MN). Different concentrations of cells ranging from  $10^5$ /ml to  $10^6$ /ml were cultured for 24 h and 48 h, and their cell lysates and culture media were collected and stored at  $-80^\circ\text{C}$  until use. To prepare cell lysates, cells were washed in PBS and resuspended in 10 mM Tris-HCl (pH 7.4), containing 1 M NaCl, in the presence of protease inhibitors. The cells were disrupted by three cycles of freeze-thawing, and the homogenate was centrifuged at  $30,000 \times g$  for 20 min.

**Western Blot.** The expression of MMP-9 was evaluated in cell lysates by Western blot according to standard procedures. Because the latent forms of metalloproteases are activated by treatment *in vitro* with proteinases like trypsin, to avoid artifactual results tumor cells were detached from plastic by scraping. Anti-MMP-9 monoclonal antibody (Oncogene Science Research, Boston, MA), which recognizes both the latent and active forms of the protein, was used at 1:400 dilution.

**Coculture of Tumor and Endothelial Cells: *in Vitro* Capillary-like Structure and Cell Growth Assays.** HUVEC, isolated from umbilical cord vein according to standard procedures (PromoCell, Heidelberg, Germany), were used at passage 2–6. HUVEC were kept in culture on gelatin-coated plastic in M199 medium as reported (14). For coculture experiments,  $10^5$  endothelial cells were plated in 6-well plates coated with Matrigel Growth Factor Reduced (Collaborative Research) and allowed to adhere. Inserts (0.4- $\mu$ m pore transwell inserts; Falcon; Becton Dickinson, Franklin Lakes, NJ) containing  $6 \times 10^5$  parental or HOXB7-transduced SkBr3 cells were then applied on the plates containing the HUVEC. *In vitro* angiogenesis was monitored after 24 h and 48 h, and plates were photographed with a Zeiss microscope. For cell growth assay,  $3 \times 10^4$  HUVEC were plated in 24-well plates coated with gelatin in M199 medium containing 10% FCS and 10% newborn serum without factors. After 24 h, the medium was removed and replaced with M199 medium containing 2.5% FCS without factors. After an additional 24 h, transwell inserts containing  $5 \times 10^4$  tumor cells in M199 with 2.5% FCS were applied on the plates containing endothelial cells. For quantification of cell growth, after 40 h of coculture, endothelial cells were photographed with a Zeiss microscope and then trypsinized and counted. Coculture experiments in both proliferative and differentiative conditions of endothelial cells were performed also in the presence of increasing amounts (0.5–2.5  $\mu$ g/ml) of a blocking anti-VEGF antibody (R&D Systems).

***In Vivo* Assay.** SkBr3 or SkBr3/HOXB7 cells in exponential growth phase were injected s.c. at dose of  $3 \times 10^6$  and  $10^7$  into adult athymic nude mice purchased from Charles River (Calco, Italy) and maintained at the Istituto Nazionale Tumori (Milan, Italy) under standard conditions according to institutional guidelines. Tumor growths were monitored twice a week. At different time points, tumors were excised and fixed for histopathological examination.

**Immunohistochemistry.** Tumor fragments were embedded in OCT compound (Miles Lab., Elkert, IN), snap-frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$ . Immunohistochemical analysis using the peroxidase-antiperoxidase method was performed as described (15). Briefly, 5- $\mu$ m cryostat sections were fixed in acetone and immunostained with rat antimouse monoclonal antibodies CD31/PECAM-1 (Mec 13.3 hybridoma) and CD34 (RAM34; PharMingen, San Diego, CA). Sections were preincubated with rabbit serum and sequentially incubated with optimal dilutions of primary antibodies, rabbit antirat IgG (Zymed Laboratories, Inc., San Francisco, CA), and rat peroxidase antiperoxidase (Abbot Laboratories, North Chicago, IL). Serial sections from the tumors

Table 1 Primers, PCR products, and annealing conditions

Gene	Upper primer (5' to 3')	Lower primer (5' to 3')	Product size (bp)	AT (C°) <sup>a</sup>
VEGF	gaagtgggtgaagttcattgatgac	cgatcgttctgtatcagctcttcc	540	58
bFGF	atccaaaacagggacctgggc	aactccatcaccagcagag	664	51
GRO $\alpha$	ggatccaaagcaaatggccaatgag	tgttctaagccagaaa cact	250	60
Ang-1	ggtcacactgggacagca	cgtaaggagtaactgggc	244	51
Ang-2	tgctaagctgctggtttatt	ctctgcaaccgagctcatgta	248	54
IL-8	atgacttccaagctggccctggct	tctcagccctctcaaaaactctc	289	60
Flt-1	gaagcaacagctcaatggg	gatggtccactcctaca	332	50
Flt-4	ccagatgaagacattgag	gaaaatgctgacgtatgctg	192	53
KDR	agactttgagcatggaag	ccattccaccaaaagatg	312	50
FGF-R1	aaccaccagcccaaccaca	cgacaccagcccagctt	687	57
FGF-R2	tcctatgacattaacctgtt	cacataaacggcagtgtaaa	702	57
FGF-R3	gcctggactactcctcg	gtttgccaccagcctgct	661	57
FGF-R4	ggctgatgctgagtgctg	actaagcgccttgctcca	474	55
Tie-1	agctgctgctttcgccagt	tcataagctcggcagaggt	331	52
Tie-2/TEK	agcagctctctcacttgcct	acaagtcaccccgagtagg	320	55
IL-8R1	cagttacagctctaccctgcc	gctgtctttggccagggagtc	964	60
IL-8R2	cgccagcttaccatcaaaaca	ggttaacacgatgactgccaag	488	60
MMP-2	tcgcccacatcaaatgctc	gtgatctgttcttctcc	301	53
MMP-9	aaccaatctcaccgacag	caaaggctctgcaatca	317	55
HPSE	atctcaagtaaacaggaga	accaacatcaggaccata	453	50
S26	gcctccaagatgacaag	ccagagaatagcctgct	397	56
GAPDH	acatcaagaaggtggaagcagg	ctctcctctgtgctcttctgctg	282	56

<sup>a</sup> AT, annealing temperature.

were analyzed, and the number of vessels was evaluated in the entire growing area at  $\times 400$  magnification. The statistical analysis was performed by Student's *t* test.

## RESULTS

**HOXB7-mediated Up-Regulation of Proangiogenic Factors, except Ang-1.** As described previously (10), the breast adenocarcinoma cell line SkBr3, characterized by lack of both HOXB7 and bFGF gene expression, became capable of expressing them upon transduction of HOXB7 cDNA alone. The transduced SkBr3 cells increased the growth rate and partially loose growth factor dependence.

Parental,  $\beta$ -gal-transduced, and HOXB7-transduced SkBr3 cells were analyzed by RNase protection and/or RT-PCR to test whether enforced expression of HOXB7 may affect genes, in addition to bFGF, having a direct or indirect role in neoangiogenesis. The expression level of VEGF, GRO $\alpha$ , IL-8, Ang-1, Ang-2, and the corresponding receptor genes was evaluated (Figs. 1-3). All of the examined growth factors, except Ang-1 that was sharply down-regulated, were increased in the HOXB7-transduced cells. A 3–10-fold increase of VEGF, Ang-2, GRO $\alpha$ , and IL-8 was found in SkBr3/HOXB7 cells.

**HOXB7 Regulation of VEGF-specific Isoforms.** RT-PCR amplification of VEGF with primers spanning exon 3 and exon 8 allowed us to analyze the proportion of the different transcripts resulting from alternative splicing (16). Fig. 1A shows a clear increase of VEGF in the HOXB7-transduced cells ranging from 1.5-fold for VEGF<sub>121</sub> to 7- and 12-fold for VEGF<sub>165</sub> and VEGF<sub>189</sub>, respectively. To see whether such pattern of transcripts might be regulated by HOXB7, we analyzed melanoma cell lines that constitutively express HOXB7 (5). In the A375 and 665/1 melanoma cells, antisense phosphorothioate oligomers to HOXB7 specifically reduced VEGF<sub>165</sub> and VEGF<sub>189</sub> of 5-

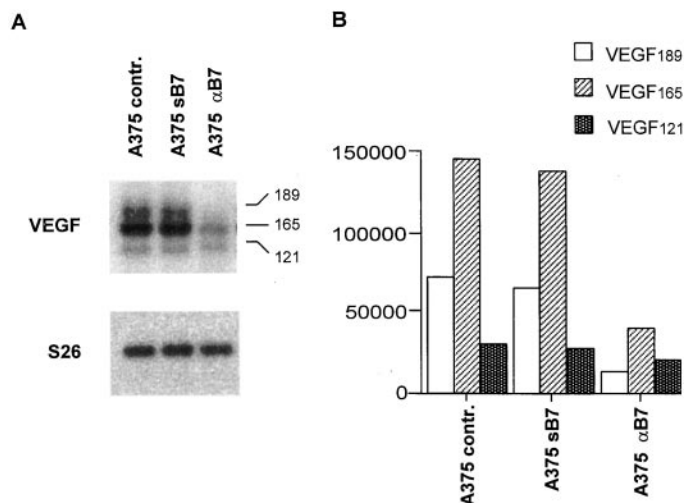


Fig. 2. Expression analysis of differentially spliced VEGF transcripts in the A375 melanoma cell line. A, RT-PCR analysis of untreated (Lane 1), 30  $\mu$ M scrambled (s-B7; Lane 2), and 30  $\mu$ M anti-HOXB7 ( $\alpha$ -B7; Lane 3)-treated cells. S26 housekeeping gene was the internal control. B, densitometric analysis (PhosphorImager; Molecular Dynamics, Sunnyvale, CA) of VEGF transcripts, normalized to the S26 internal control.

and 10-fold, respectively, whereas VEGF<sub>121</sub> remained the same (see Fig. 2).

**Growth Factor Receptor Analysis.** The analysis of the corresponding receptors included bFGF receptors from 1 to 4, VEGF receptors Flt-1, KDR and Flt-4, IL-8 receptors and Tie-1 and Tie-2/Tek. The expression of all of them remained generally unchanged or slightly down-regulated (Fig. 3 and Ref. 10). Parental SkBr3/ $\beta$ -gal and SkBr3/HOXB7 cells constitutively expressed bFGF R-2, -3, and -4 (10) and IL-8 high affinity receptors (Fig. 3), whereas they were negative for bFGF receptor-1 (10) and for Tie-2/Tek (Fig. 3). In parental cells, the three VEGF receptors, Flt-1, KDR, and Flt-4, were expressed from low to high level, respectively, whereas they were shut off in the SkBr3/HOXB7 cells. IL-8R1 was down-regulated, whereas IL-8R2 was expressed at constant level (Fig. 3). Only Tie-1, which has no known ligand, showed a 5–10-fold increase in transduced cells. Lack or down-regulation of these receptors in the presence of their respective ligands, being against any possible autocrine loop, may rather suggest a strategy by which tumor cells avoid to sequester proangiogenic factors making them available to the neovessels. Accordingly, VEGF did not show any proliferative effect on SkBr3/HOXB7, and the addition of a blocking antibody up to 72 h did not change the cell growth rate (Fig. 4). The specificity of the antibody was confirmed by measuring a reduction in the level of detectable VEGF from 1580 to 54  $\mu$ g/ml in culture supernatants as assayed by an ELISA.

**HOXB7 Stimulates MMP-9, But Not MMP-2 and Heparanase, Expression.** RT-PCR analysis of proteases involved in tumor progression (17, 18), including MMP-2, MMP-9, and heparanase, was also performed. No relevant modulation was found for MMP-2 and heparanase (barely or not detectable in both parental and transduced cells), whereas an increase of 35–40-fold of MMP-9 was detected (Fig. 5A). This up-regulation was confirmed at protein level by Western blot analysis as shown in Fig. 5B and quantified by densitometric analysis. MMP-9 protein was barely detectable in the SkBr3 parental cells, whereas in the HOXB7-transduced cells we observed a 38-fold increase of the mature  $M_r$  84,000 enzyme and a 16-fold increase of the  $M_r$  68,000 form, attributable to autocatalytic cleavage of the COOH terminus.

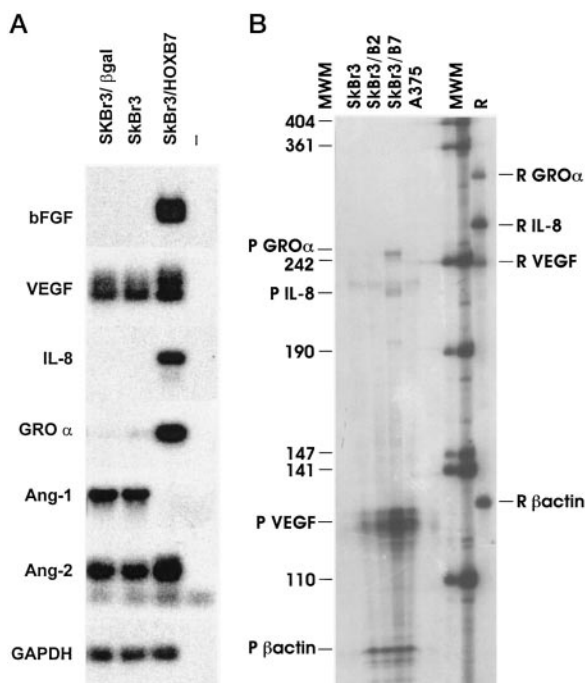


Fig. 1. Expression of growth factor genes in SkBr3 mammary carcinoma,  $\beta$ -gal-transduced, and HOXB7-transduced SkBr3. A, RT-PCR analysis. Growth factor genes were amplified for 35 cycles, whereas control GAPDH remained in the exponential phase at 18 cycles. (–) is the negative control, including all of the reaction reagents but RT-RNA. B, RNase protection analysis. Riboprobes (R) are shown on the right, protected fragments (P) and molecular size (bp) markers (MWM; pGEM4Z *Hpa*II) are shown on the left.  $\beta$ -actin expression is shown as internal control.



**Effective Induction of Growth Factors in the HOXB7/SkBr3 Cells.** Among the examined growth factors, the exception is bFGF, which seems to promote an autocrine loop in HOXB7-transduced SkBr3 cells without being released (Fig. 6 and Ref. 10). The secretion of the other proangiogenic factors may likely activate the cell surface receptors present on endothelial cells. ELISA assays detected production of VEGF, GRO $\alpha$ , and IL-8 (Fig. 6) in supernatants of SkBr3/HOXB7. A 10-fold increase of VEGF was found in the secreted fraction of the transduced *versus* the parental cells (Fig. 6), according to up-regulation of VEGF mRNA (Fig. 1). IL-8 was undetectable in parental cells, whereas SkBr3/HOXB7 produced up to  $\sim 2$  ng/ml of IL-8 that was totally found in the extracellular secreted fraction. Finally, GRO $\alpha$  was found in the secreted fraction of the transduced cells, up-regulated of 40-fold as compared with the original cell line (Fig. 6).

**Induction of Growth and Angiogenesis in a Three-dimensional *in Vitro* Model.** To examine the actual function of the growth factors induced by HOXB7 in the SkBr3 cells on endothelial cell differentiation into capillary-like structures, an *in vitro* assay reproducing the interactions between tumor and endothelial cells has been used. Endothelial cells were plated on a three-dimensional matrix of Matrigel growth factor reduced and cocultivated with parental *versus* HOXB7/SkBr3 cells or medium alone. The analysis at both 20 and 40 h showed clear morphological differences. At 20 h, endothelial cells formed thin interconnecting cords in all of the conditions, but the HUVEC grown on HOXB7-expressing cells showed a delay in the differentiative process with nests of undifferentiated proliferating cells after 2–3 days of culture (Fig. 7). The experiment was made also by adding an anti-VEGF blocking antibody. Fig. 7D shows how the inhibition of the SkBr3/HOXB7-secreted VEGF could modify endothelial cell morphology reducing the differentiative delay we have observed. No clear differences

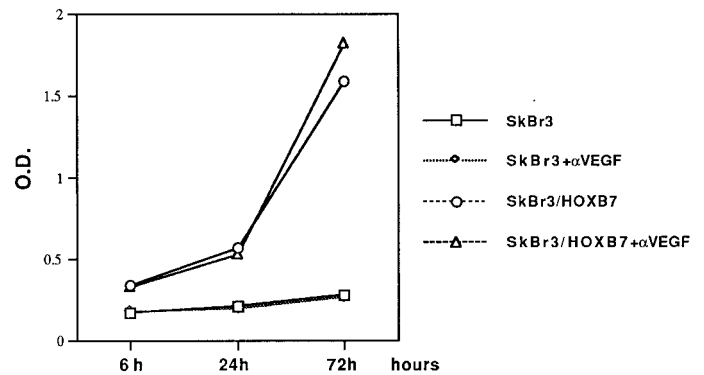


Fig. 4. Effects of anti-VEGF blocking antibody (1  $\mu$ g/ml) on parental and HOXB7-transduced SkBr3 cells. Growth curves, evaluated by methylene blue inclusion, were compared.

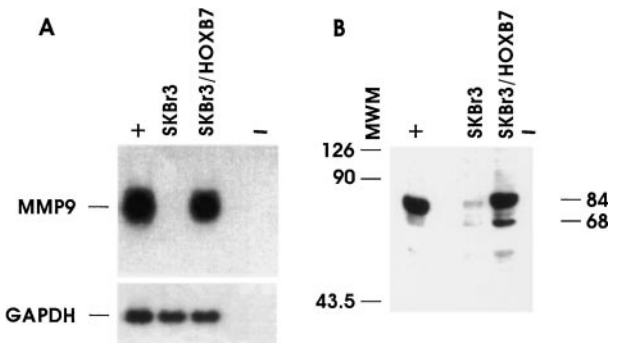


Fig. 5. MMP-9 expression studies. A, RT-PCR analysis in parental and HOXB7-transduced cells. B, Western blot analysis on the same samples. Sizes are Da (shown in thousands). PMA-induced HL-60 were used as a positive control (37).

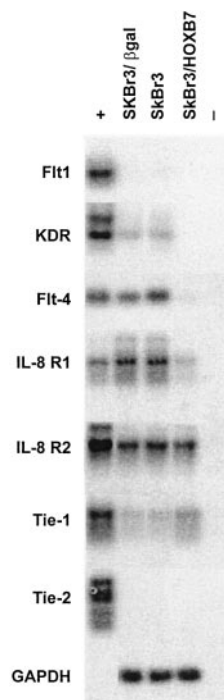


Fig. 3. RT-PCR analysis of growth factor receptors in parental,  $\beta$ -gal-transduced, and HOXB7-transduced SkBr3 cell lines. (–) is the negative control; from top to bottom, positive controls are U937 (33), A375 (34), HEL (35), HL60 for both IL-8 receptors (36), A375 and IMR90 cell lines, respectively. SkBr3 and SkBr3/B7 samples are the same as in Fig. 1A. GAPDH housekeeping gene expression is shown as internal control. Receptor genes were amplified for 35 cycles. cDNA used for Tie-1 amplification was one tenth of the amount used for the other genes.

were found by adding the anti-VEGF antibody in HUVEC cocultured with the parental cell line (data not shown). The growth stimulatory activity of parental *versus* HOXB7/SKBr3 on endothelial cells was compared in a cocultivation assay (Fig. 8). An increased number (3–5-fold) of HUVEC grown in the presence of HOXB7/SKBr3-expressing cells was observed in four independent experiments. Assays were repeated in the presence or absence of an anti-VEGF blocking antibody. The proliferative rate of the endothelial cells cocultured with SkBr3/HOXB7 cells and the anti-VEGF antibody was 48% of that obtained in its absence. The same assay performed with HUVEC cocultured with parental SkBr3 showed a proliferation rate of 32% of that obtained with SkBr3/HOXB7 that was further reduced to 20% in the presence of the blocking antibody (Fig. 8C).

**HOXB7 Increases Angiogenesis *in Vivo*.** We tested whether SkBr3/HOXB7 cells were more tumorigenic and whether tumor vascularization was increased by injecting s.c.  $3 \times 10^6$  and  $10^7$  cells into athymic nude mice (Table 2). Although parental cells originated barely or not detectable tumors, all of the mice that received SkBr3/HOXB7 injections showed tumors growing at the injection site. To obtain tumors from the parental cell line to be compared for vessels content with the HOXB7-transduced nodules, recipient mice were irradiated with 400 rad the day before the injection. The analysis of incipient SkBr3 tumors from irradiated mice showed poor vascularization with most of the tissue necrotic. Vessels were outside the tumor, and the presence of muscular walls was suggestive of residual s.c. vessels (Fig. 9A). Immunohistology of SkBr3/HOXB7 tumor nodules showed increased vascularization, as evaluated by anti-CD31 and anti-CD34 immunostaining.

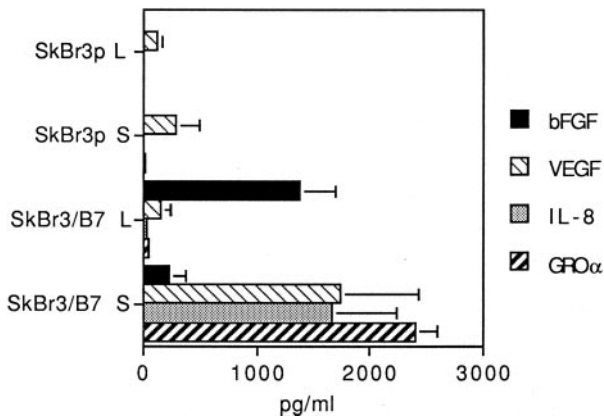


Fig. 6. Growth factor production in parental and HOXB7-transduced SkBr3 cells maintained in 10% serum. The ELISA analysis was performed on both cell lysates and supernatants obtained from  $5 \times 10^5$  cells in 24 h. Values represent mean  $\pm$  SE of three separate experiments.

Vascular lacunas, unicellular and small vessels without an organized wall, as well as muscular vessels, were present within the tumor site and in the peritumoral area (Fig. 9B). In tumors, beside the increased number of new vessels, HOXB7 expression seems to directly or indirectly up-regulate CD31 expression to a bright immunodetection (Fig. 9). The number of vessels were counted in the entire growing areas, and the differences, evaluated by Student's *t* test, were statistically significant (Table 3).

## DISCUSSION

Proliferation index and extent of tumor-associated neoangiogenesis are important prognostic variables of carcinomas (3, 19). Their func-

tional role in tumor progression has been largely studied but not completely understood. Growth factor and growth factor receptor interplay is more relevant in the context of tumor and its stroma (20). Because neoangiogenesis is a "common denominator" in the pathogenesis of several diseases, antiangiogenic therapies seem to be extremely appealing in that they should have minimal side effects, should not develop pharmacological resistance, and should result in a wide effect because each capillary supplies a lot of cells (4, 21). The remaining problem is the complex cross-talk among redundant angiogenic factors underlying this process and possibly interfering with any therapeutic applications using single or few antagonists of such angiogenic factors. A solution would be the identification of an upstream gene acting as a regulator of most, if not all, of the main angiogenic molecules, and, based on our data, HOXB7 might be proposed as an attractive target for a broad inhibition.

We have reported (5) the direct relationship between a homeogene, *HOXB7*, and bFGF in melanomas and in the SKBr3 breast carcinoma cell line. SKBr3 cells upon transduction of the sole *HOXB7* gene, became bFGF-positive, increased the proliferative rate, and acquired independence from serum and the ability to form foci in semisolid agar (10).

In this study, we show that several genes involved in the proliferative and angiogenic processes are also affected by HOXB7 expression. Growth factors and growth factor receptors, known to have partially overlapping but specific roles in controlling the growth of new vessels, were analyzed. In particular in the HOXB7-transduced SkBr3 cells, besides the induction of bFGF, we found an increase of 3–10-fold of VEGF, GRO $\alpha$ , IL-8, and Ang-2 at both mRNA (see Figs. 1–2) and, when possible, protein levels (Fig. 6). On the contrary, Ang-1 was totally abrogated as outlined by the use of primers that can amplify all of the Ang-1 isoforms identified

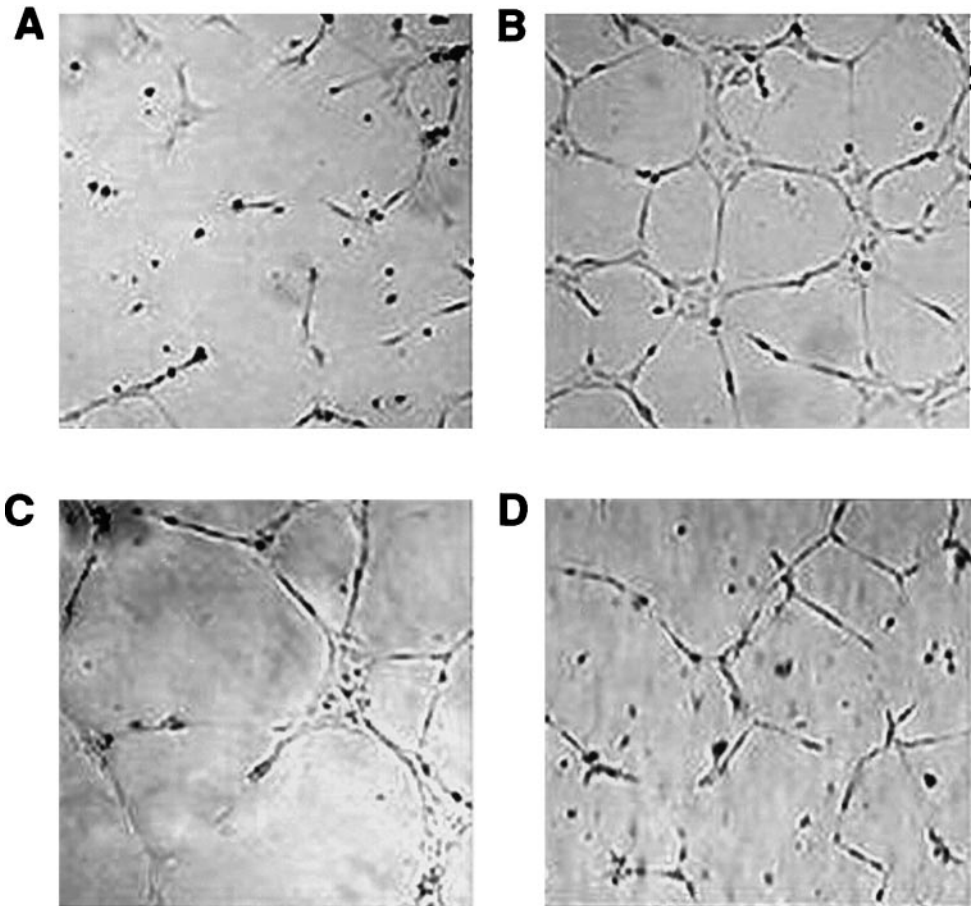


Fig. 7. Endothelial cell morphology in a three-dimensional growth factor-reduced Matrigel system. HUVECs were cocultured with (A) SkBr3 or (B–D) HOXB7/transduced SkBr3 cells. A blocking anti-VEGF antibody (1  $\mu$ g/ml) was added to the SkBr3/HOXB7 culture (D). Photographs were taken after 40 h. Magnifications A, B, and D,  $\times 25$ ; C,  $\times 100$ . Results are representative of five independent experiments.



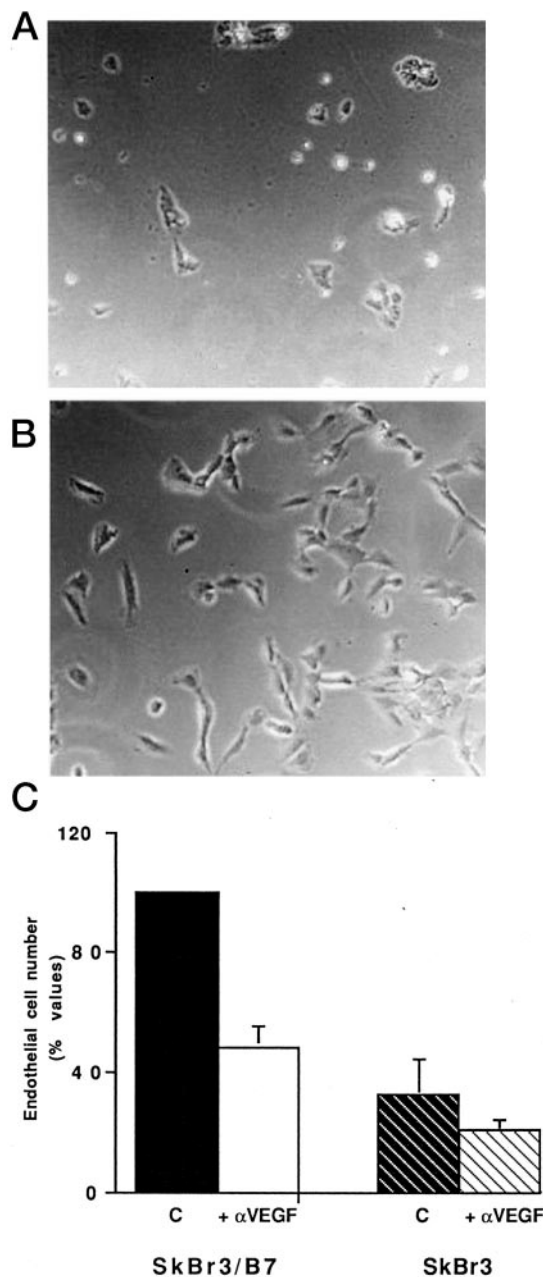


Fig. 8. Proliferation assay of endothelial cells in a transwell cocultured system. Cells were analyzed 4 days after plating. Results are representative of four independent experiments. Morphological coculture results of HUVEC grown with (A) SkBr3 and (B) HOXB7/SkBr3 are shown. Phase contrast; magnification 25 $\times$ . C, quantitative data showing endothelial cell number after coculture with SkBr3/HOXB7 versus SkBr3, with or without 1  $\mu$ g/ml of the anti-VEGF blocking antibody. Values, given as percentage of the control (*i.e.*, the number of HUVEC cocultured with SkBr3/HOXB7 cells) are mean  $\pm$  SD from four separate experiments.

thus far (22). This finding could appear in contrast to transgenic studies (23) where overexpression of Ang-1 was associated to increased skin vascularization. In agreement with our results, recent data showed that 90% of breast primary tumors were negative for Ang-1 transcription. Moreover, enforced expression of Ang-1 in a breast carcinoma cell line reduced tumor growth, suggesting an inhibitory action in this cellular model (24). In the highly disordered tumor vasculature, the reported stabilizing effect of Ang-1 might interfere with the intense sprouting of neovessels (24). An interplay between VEGF and Angs in regulating tumor angiogenesis has been hypothesized. High-grade malignant tumors, showing a lot of immature tumor vessels, do not express

Ang-1, but Ang-2 (25), a natural antagonist of Ang-1, and VEGF. Ang-1/Tie-2 interaction seems to stabilize microvessels, and a down-regulation of Ang-1 may prime tumor vasculature for active propagation, likely through cooperation between VEGF and Ang-2 (26). Also, the HOXB7-transduced HeLa cell line showed VEGF and Ang-2 up-regulation paralleled by Ang-1 down-regulation (data not shown), thus confirming a possible specific role of HOXB7 in regulation of vasculogenesis during embryonic formation and in neoangiogenesis during tumor progression when its expression is deregulated.

Our results underscore the selective induction of VEGF<sub>165</sub> and VEGF<sub>189</sub> isoforms by HOXB7. In fact, although expression of VEGF<sub>121</sub> remained substantially unchanged, VEGF<sub>165</sub> and <sub>189</sub> were expressed at a ratio of 1:7.5 and 1:12 in SkBr3 and SkBr3/HOXB7, respectively (see Fig. 1). The same pattern was observed in the A375 and 665/1 melanoma cell lines after treatment with antisense oligomers targeting HOXB7. This treatment down-regulated selectively the expression of VEGF<sub>189</sub> and VEGF<sub>165</sub> (7-fold and 4-fold decrease, respectively), but not of VEGF<sub>121</sub> (Fig. 2).

Table 2 HOXB7 induces tumorigenicity to SkBr3 cell upon injection into athymic nu/nu mice

Cell type	No. of cells injected into nu/nu mice		
	$3 \times 10^6$	$10^7$	$3 \times 10^6$ in X-rad <sup>a</sup>
SkBr3	1/6	1/6	4/8
SkBr3/HOXB7	6/6	4/4	8/8

<sup>a</sup> Recipient mice were irradiated with 400 rad the day before tumor cell injection.

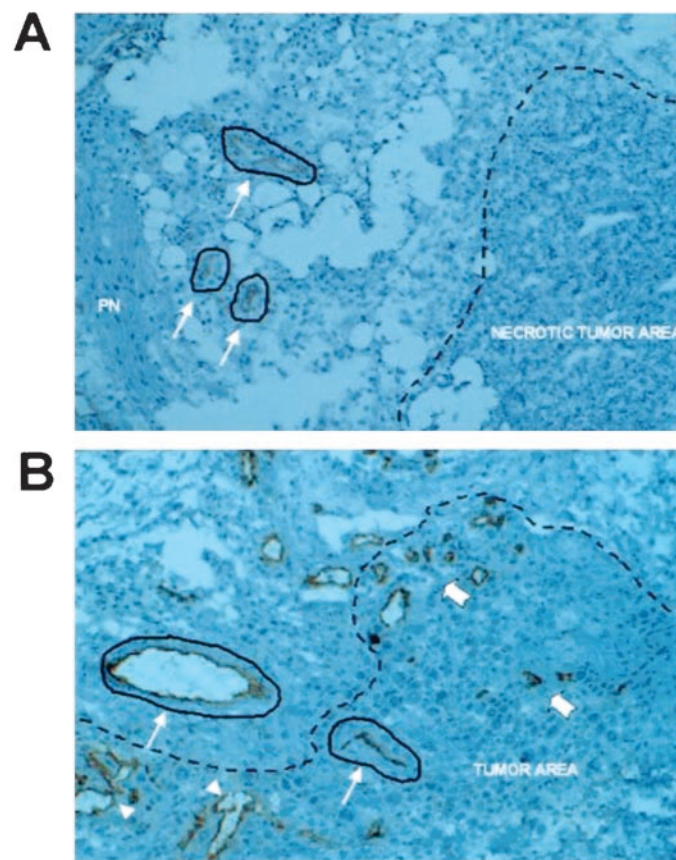


Fig. 9. Immunohistochemical staining of tumors excised from athymic nude mice. (A) SkBr3 (magnification  $\times 300$ ) and (B) SkBr3/B7 (magnification  $\times 400$ ) tissue sections immunostained with an anti-CD31 antibody. Dashed lines delimit the tumor areas. Thin arrows, muscular vessels; thick arrows, small vessels and single cell vessels; arrowheads, vascular lacunas; PN, peripheral nerve.

Table 3 Vascularization analysis of tumors obtained by injecting SkBr3 or SkBr3/HOXB7 cells into athymic nude mice

Tumors from three independent experiments were analyzed at day 7. The entire tumor-growing area was evaluated with the help of a mm<sup>2</sup> grid. Vascularization is expressed as number of capillaries/field at 400× enlargement and reported as mean ± SD.

Anti-CD34		Anti-CD31	
SkBr3	SkBr3/HOXB7 <sup>a</sup>	SkBr3	SkBr3/HOXB7 <sup>a</sup>
3.0 ± 0.6	13.5 ± 3.5	2.7 ± 1.6	13.0 ± 2.0
5.0 ± 2.0	14.6 ± 2.7	4.0 ± 1.0	14.0 ± 2.0
7.0 ± 3.0	19.0 ± 6.3	6.0 ± 3.0	16.0 ± 4.0
4.0 ± 2.0	15.0 ± 2.5	4.0 ± 2.0	16.0 ± 2.0

<sup>a</sup> P < 0.0002.

Because the alternative splicing originated polypeptides with a different secretion pattern and possibly different function, VEGF<sub>189</sub> and <sub>165</sub> may influence tumor progression (27). Although VEGF<sub>121</sub> is a diffusible protein, the longer VEGF<sub>189</sub> isoform is tightly associated with the ECM and can be released as a soluble and bioactive factor by heparin and plasmin. VEGF<sub>165</sub> shows an intermediate behavior (28). The preferential induction of the longer, membrane-bound isoforms by HOXB7 could be related to the role of the HOX genes in ECM remodeling during development (29). Extracellular proteolysis and degradation of the ECM occurring during morphogenesis are thought to be involved in invasiveness and metastases of tumor cells (30). In agreement with these data, we found a strong increase of MMP-9 in the HOXB7-transduced cells (see Fig. 5).

Growth factor receptors have been studied to recognize whether the induction of proliferation and angiogenesis is based on autocrine or paracrine loops. The lack of a marked up-modulation of receptors for angiogenic factors on neoplastic cells suggested that their ligands, except bFGF, did not play autocrine activity. Such ligands, not sequestered by tumor cells, remain free to activate cell surface receptors on endothelial cells. This function was confirmed by the experiments performed by adding an anti-VEGF blocking antibody in the SkBr3/HOXB7 culture medium. Although the presence of this antibody did not interfere with tumor cell proliferation (see Fig. 4), persisting capillary-like structures formed by endothelial cells grown in the presence of HOXB7-producing cells, although still showing a morphology different from SkBr3 parental coculture, did not last as long as in the presence of the antibody (Fig. 7D). Endothelial cells are known to express several members of the receptor tyrosine kinase family, including receptors for VEGF and FGF and Tie-1 and Tie-2/Tek (31, 32). This may explain the induction of angiogenesis and tumorigenesis in xenografted SkBr3/HOXB7 cells. The presence of these endothelial receptors can well account for the induction of the angiogenic phenotype that we observed. Among the receptors only Tie1 was found up-regulated in SkBr3/HOXB7 cells but, because its ligand is unknown, no functional inference could be formulated at this moment.

To assess the actual function of the HOXB7-based microenvironment, *in vitro* and *in vivo* angiogenic assays were performed. In both cases, HOXB7 overexpression correlated with an enhanced angiogenic response, and our data indicate that this effect could be likely attributable to the induction of many different proangiogenic factors.

In this view, HOXB7 might represent a master gene to be targeted to inhibit tumor-associated angiogenesis. Its ability of activating many angiogenic molecules makes it an attractive upstream gene, the inhibition of which could be a highly effective antiangiogenic strategy.

## ACKNOWLEDGMENTS

We thank Giuseppe Loreto for preparation of some of the figures.

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