Thyroid Hormone Receptor β1 Acts as a Potent Suppressor of Tumor Invasiveness and Metastasis

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Abstract

Loss of thyroid hormone receptors (TR) is a common feature in some tumors, although their role in tumor progression is currently unknown. We show here that expression of TRβ1 in hepatocarcinoma and breast cancer cells reduces tumor growth, causes partial mesenchymal-to-epithelial cell transition, and has a striking inhibitory effect on invasiveness, extravasation, and metastasis formation in mice. In cultured cells, TRβ1 abolishes anchorage-independent growth and migration, blocks responses to epidermal growth factor, insulin-like growth factor-I, and transforming growth factor β, and regulates expression of genes that play a key role in tumorigenicity and metastatic growth. The receptor disrupts the mitogenic action of growth factors by suppressing activation of extracellular signal-regulated kinase and phosphatidylinositol 3-kinase signaling pathways that are crucial for cell proliferation and invasiveness. Furthermore, increased aggressiveness of skin tumors is found in genetically modified mice lacking TRs, further demonstrating the role of these receptors as inhibitors of tumor progression. These results define a novel role for the thyroid hormone receptor as a metastasis suppressor gene, providing a starting point for the development of novel therapeutic strategies for the treatment of human cancer. [Cancer Res 2009;69(2):501–9]

Introduction

The actions of the thyroid hormone triiodothyronine (T3) are mediated by the thyroid hormone receptors TRα and TRβ that give rise to different receptor isoforms (1). TRs belong to the nuclear receptor superfamily that act as ligand-dependent transcription factors by binding to DNA-response elements (TRE) located in regulatory regions of target genes (2). TRs can also alter expression of genes that do not contain a TRE through modulation of the activity of other transcription factors that are target of different signaling pathways. For example, TRs can negatively regulate target gene promoters that carry AP-1 or CRE sites. The receptors do not bind to these DNA motifs themselves but are tethered to the promoter through protein-protein interactions (3, 4). Because the AP-1 complex regulates the expression of genes involved in oncogenic transformation and cellular proliferation (5), down-regulation of AP-1 activity by the receptors could oppose unregulated cell growth. In addition, we have shown that TRs can block transformation by oncogenic *ras*, suggesting that they could play a relevant role as suppressors of *ras*-dependent tumors (6) and reduced expression of TRs and alterations in TR genes are common events in cancer, particularly in hepatocarcinoma and breast tumors (7–11). However, the role of TRs in tumor progression or metastatic growth is currently unknown. For this reason, we have reexpressed TRβ1 in hepatocarcinoma and breast cancer cell lines, which show elevated Ras activation (12, 13), and analyzed their tumorigenic and metastatic ability. The results obtained show that this receptor acts as a strong suppressor of invasiveness and metastasis formation, suggesting that this receptor represents a potential therapeutic target in cancer.

Materials and Methods

Cell lines. SK-hep1 (ATCC: HTB-52) cells were maintained in DMEM with 10% fetal bovine serum (FBS). Cells derived from MDA-MB-468 cells (ATCC: HTB-132), selected by presenting a high efficiency of lung metastasis formation in immunodeficient mice, were a kind gift from Dr. A. Fabra. These cells were grown in DMEM and HAMS F12 (1:1) with 10% FBS.

Retroviral infections. HEK293T cells were cotransfected with 10 μg of pLPCX or pLPCX-TRβ that encodes the human TRβ1 isoform (6), 3.5 μg VSV, and 6.5 μg gag-pol constructs (a gift from Dr. P.M. Comoglio) using calcium phosphate. Viral supernatants were harvested 48 h posttransfection, filtered, and used for infections of SK-hep1 (SK) cells or MDA-MB-468 (MDA) cells in the presence of 4 μg/mL of polybrene. Cells were selected with 2 μg/mL puromycin. Pools of resistant cells (SK, SK-TRβ, MDA, and MDA-TRβ) were cultured in 10% FBS depleted of thyroid hormones by treatment with resin AG-1-X8 (Bio-Rad).

Transfection and reporter assays. A fusion of ELK1 with the DNA binding domain of GAL4 was cotransfected with a reporter plasmid containing four binding sites for GAL4 (pE1b 4 × UAS-luc) to determine the activity of this transcription factor. TK-*Renilla* luciferase plasmid (Promega) was included for correction of transfection efficiency. Cells were transfected with cationic liposomes, and luciferase activity was determined in control cells and in cells treated for 36 h in serum-free medium in the presence and absence of 5 nmol/L T3, 25 ng/mL epidermal growth factor (EGF), or 15 nmol/L insulin-like growth factor-I (IGF-I). Each experiment was performed in triplicate and was repeated at least thrice. Data are mean ± SD and are expressed as fold induction over the values obtained in the untreated cells.

Western blotting. Primary antibodies (listed in supplementary material) were used at 1:2,000 dilution, except for the TRβ antibody that was used at 1:500.

Cell proliferation assays. Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed as recommended by the manufacturer (Roche) in cells inoculated in 24-well plates (8×10^3 per well). After 24 h, cultures were shifted to serum-free medium and incubated for 48 h with the indicated compounds. Absorbance was read at 630 and 570 nm.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-08-2198

Anchorage-independent growth. For colony formation in soft agar, 2×10^4 cells were resuspended in 1.5 mL of 1% agar and 2× DMEM containing 20% FBS. Cells were seeded in 60-mm plates on 2 mL of the same mixture, and serum-free DMEM, with or without T3 (100 nmol/L), was added every 4 d. The number of colonies was scored after 40 d by counting 10 fields in each plate. Treatments were done in triplicate, and experiments were repeated thrice. For growth in suspension, 2.5×10^5 cells were inoculated in 30 mL of serum-free DMEM and kept under agitation for 24 or 48 h. Cells were then plated in 24-well plates and, after 4 h, were used for MTT assays. Results were expressed relative to MTT activity obtained in cells inoculated in parallel without agitation.

Invasion assays. Matrigel (2 μg; BD Biosciences) was placed on top of Transwell plates (24-well plates, Costar). Cells (1×10^5) previously cultured in serum-free medium for 24 h in the presence and absence of 5 nmol/L T3 were inoculated in the upper chamber, and the conditioned medium from NIH3T3 cells was placed in the lower chamber. Migration lasted for 16 h, also with or without T3. Conditioned medium was obtained from fibroblasts incubated for 48 h in serum-free medium. Media were centrifuged and kept frozen until use. Medium containing 2% bovine serum albumin was used as a negative control. After migration, filters were fixed with glutaraldehyde and stained with crystal violet following the manufacturer's specifications.

Quantitative real-time PCR. Total RNA was extracted using Tri Reagent (Sigma), and mRNA levels were analyzed by quantitative RT-Q-PCR. RT was performed with 2 μg of RNA following specifications of SuperScript First-Strand Synthesis System (Invitrogen Life Technologies). PCR reactions were performed using a MX3005P instrument (Stratagene) and detected with Sybr Green. Data analysis was done using the comparative computed tomography method, and data were corrected with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Primers used are indicated in the supplementary material.

Animal studies. All animal work was done in compliance with the European Community Law (86/609/EEC) and with the approval of the Ethics Committee of the Consejo Superior de Investigaciones Cientificas. Groups of eight athymic nude mice 4 to 6 wk old were used for xenografting studies. Hepatocarcinoma cells SK or SK-TRβ (1×10^6 in 100 μL PBS) were injected s.c. into each flank. For studies of breast cancer cell tumorigenesis, a small incision was made to reveal the second abdominal right mammary gland. MDA and MDA-TRβ cells (1×10^6 cells in 100 μL PBS) were inoculated directly into the mammary fat pad, and the incision was closed with wound clips. Primary tumor outgrowth was monitored twice a week by taking measurements of the tumor length (L) and width (W) with a digital caliper. Tumor volume was calculated as $\pi LW^2/6$. Only tumors with a diameter of >0.3 cm were considered. Mammary gland tumors were surgically resected after 30 d, and mice were monitored for the appearance of spontaneous metastasis 30 d after resection. For formation of experimental metastasis in lung, 1×10^6 cells were injected into the lateral tail vein. Animals were sacrificed 30 d after injection of hepatocarcinoma cells or 40 d after injection of breast cancer cells. $TR\alpha^{-/-}/TR\beta^{-/-}$ double knockout (KO) mice in a hybrid genetic background of 129/OLa+129/Sv+BALB/c+C57BL/6 and wild-type ($TR\alpha^{+/+}/TR\beta^{+/+}$) animals with the same background (14) were genotyped and used for studies of skin carcinogenesis. For the two-stage carcinogenesis protocol, dorsal skin of mice was shaved at an age of 8 to 10 wk and skin was treated 3 d later with a single dose of 7,12-dimethylbenz(a)anthracene (DMBA; 32 μg/200 μL in acetone). Eight days after initiation, skin was subsequently treated twice a week with 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 12.5 μg/200 μL in acetone) for 30 wk. The number of tumors was counted, and their maximum diameter

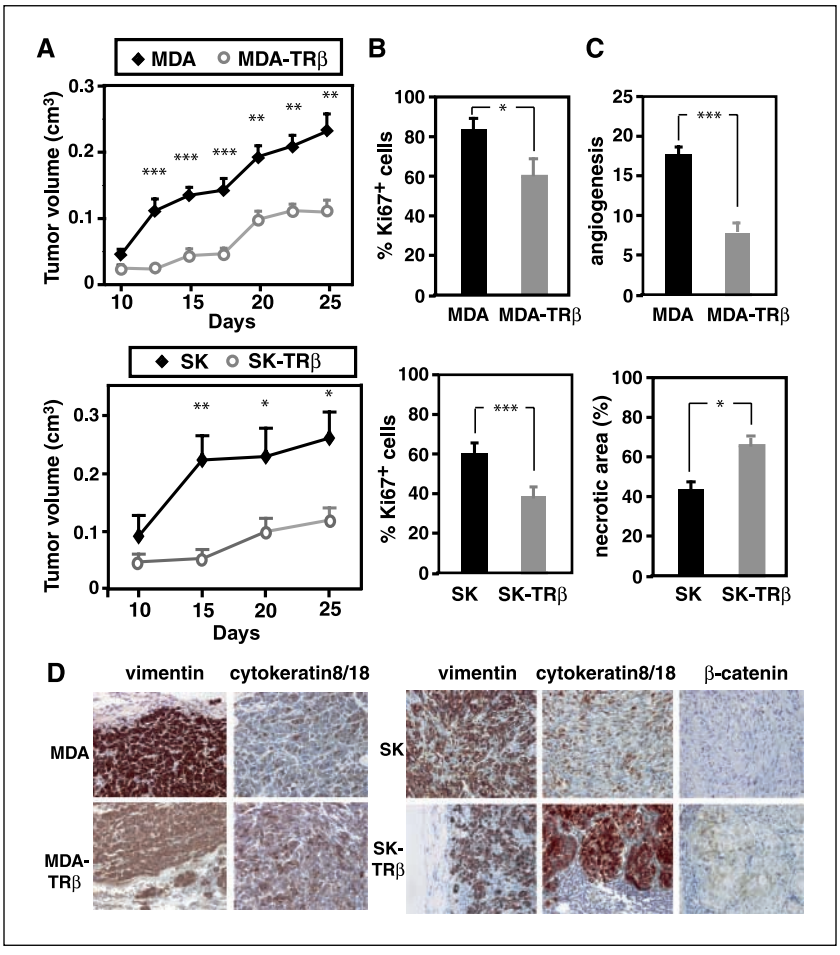

Figure 1. TRβ1 inhibits tumor growth and causes a partial mesenchymal-to-epithelial transition. *A,* nude mice were injected with parental and TRβ1-expressing hepatocarcinoma (SK) and breast cancer (MDA) cells, and tumor volume was measured at different time points after inoculation. *B,* tumors were excised at 30 d, and the percentage of cells expressing Ki67 was determined. *C,* TRβ1 reduced tumor angiogenesis, determined by counting the number of neoformed blood vessels/field (*top*), and increased the necrotic area determined from trichromic staining (*bottom*). *Columns,* mean; *bars,* SE. *D,* immunohistochemical staining for vimentin, cytokeratin 8/18, and β-catenin.

was measured twice a week. Only tumors bigger than 2 mm were taken into account for analysis.

Histology and immunohistochemistry. Tumors and tissues were processed for histopathologic procedures by fixing in 4% buffered formalin and were embedded in paraffin wax. Sections (4–5 μm) were stained with H&E or Masson trichromic or processed for immunohistochemistry. Immunohistochemistry was performed using standard protocols on deparaffinized sections. Sections were digested with 0.1% pepsin in 0.5 mol/L acetic acid for 20 min at 37°C to enhance antigenic exposure and incubated with primary antibodies (dilution 1:100) at 4°C overnight, followed by incubation with avidin-biotin peroxidase complex, and were revealed with diaminodenzidine (Sigma) and counterstained with Harris' hematoxylin. Antibodies used are indicated in the supplementary material. CD31 was used to determine tumor angiogenesis (number of neoformed vessels/area field). Ki67 was used to determine the proliferation index (Ki67-positive cells/total cells). To analyze cell proliferation *in vivo*, mice were injected i.p. with bromodeoxyuridine (BrdUrd; 0.1 mg/g weight in 0.9% NaCl), 1 h prior sacrifice.

Statistical analysis. The Kaplan-Meier method was used to estimate the percentage of tumor-free animals, and the Breslow test was used to test for differences between curves using SPSS 12.0. ANOVA was used to evaluate statistical significance in tumor volume curves, proliferation, angiogenesis grade, and necrotic area. Results are expressed as the mean ± SE of the indicated number of experiments. The 95% confidence intervals were calculated based on SE. *In vitro* results are expressed as the mean ± SD. Statistical significance was estimated with Student's *t* test for unpaired observations (in all cases $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Results

TRβ1 reduces tumor growth and causes partial mesenchymal-epithelial transition. Hepatocarcinoma SK-hep1 (SK) cells and mammary adenocarcinoma MDA-MB-468 (MDA) cells have lost TRβ expression. To analyze a possible effect of TRβ1 on tumorigenesis, we generated cells that stably reexpress this receptor (SK-TRβ and MDA-TRβ, respectively). The receptor levels obtained were in the same order of those found in normal mouse liver (Supplementary Fig. S1*A*). Expression of TRβ1 resulted in gain of ligand-dependent transcription, as analyzed in transient transfection assays with a TRE-containing reporter construct (Supplementary Fig. S1*B*). When these cells, as well as cells infected with the empty vector, were inoculated into nude mice, it was observed that tumor volume was significantly reduced in mice injected with cells that express TRβ1 (Fig. 1*A*). Immunohistochemistry for Ki67 showed that TRβ1 reduced the number of cells expressing this proliferation marker (Fig. 1*B*). Reduced proliferation accompanied by increased expression of the cyclin kinase inhibitor p27 was also found in explants of SK-TRβ tumors (Supplementary Fig. S2*A* and *B*). Tumors formed by hepatocarcinoma cells were poorly vascularized and had a large necrotic central area that was significantly higher in tumors that originated from cells expressing TRβ1 (Fig. 1*C*). Angiogenesis was, however, clearly detectable in the breast cancer cell xenografts, and expression of TRβ1 significantly reduced the number of neoformed blood vessels (Fig. 1*C*). In addition, receptor expression did not induce apoptosis in breast cancer or hepatocarcinoma tumors, as analyzed by either TUNEL assay or expression of cleaved caspase-3 (Supplementary Fig. S2*C*).

TRβ1 conferred a more differentiated tumor phenotype (Supplementary Fig. S2*D*) and caused an important decrease in the levels of the mesenchymal marker vimentin and a strong increase of the epithelial marker cytokeratin 8/18 (Fig. 1*D*). Another epithelial marker, β-catenin, was absent both before and after TRβ1 expression in MDA cells, but it was present with a characteristic epithelial pattern at the plasma membrane of TRβ1-expressing hepatocarcinoma cells (Fig. 1*D*). These results show that TRβ1 can trigger a partial mesenchymal to epithelial transition.

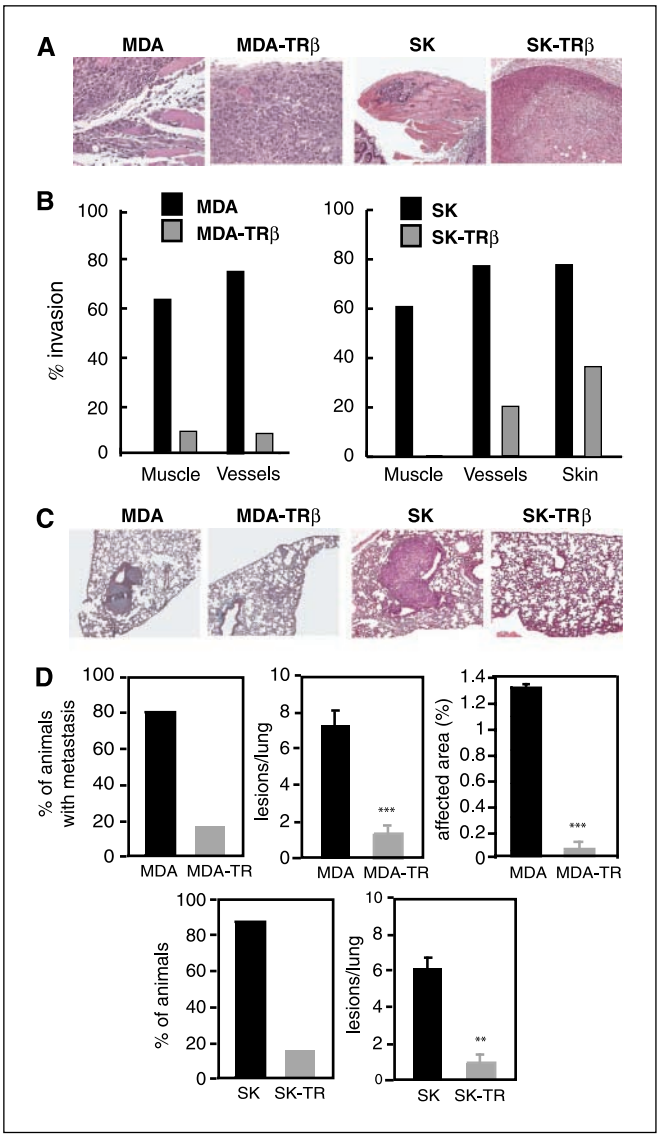

Figure 2. TRβ1 reduces tumor invasiveness and metastatic growth. *A,* representative H&E staining of tumors formed by parental and TRβ-expressing cells, showing that tumors originated from cells that express the receptor were less invasive. *B,* quantification of the percentage of tumors that infiltrated surrounding tissues, such as muscle, blood and lymph vessels, and skin. *C,* representative H&E of lungs from mice injected with parental and TRβ1-expressing cells 30 (for MDA cells) or 40 (for SK cells) d before into the tail vein. *D,* percentage of animals bearing metastatic lesions, number of lesions/lung, and area of lung parenchyma affected (mean ± SE).

TRβ1 reduces invasiveness and metastasis formation. Tumors formed by MDA cells present a diffuse highly infiltrative growth pattern. In contrast, MDA-TRβ cells give rise to tumors with a more compact structure and surrounded by a pseudocapsule of collagen and inflammatory cells (Fig. 2*A*) and lack tumor cell infiltration of adjacent muscle or lymph and blood vessels (Fig. 2*B*). In agreement with the inhibitory effect of TRβ1 on tumor invasiveness, 40% of the implanted MDA cells caused the appearance of distant nodular metastasis in the forelimbs 30 days after extirpation of the mammary tumors, whereas no metastasis were found when MDA-TRβ cells were used. In hepatocarcinoma

cell tumors, expression of TRβ1 also changed the infiltrative and diffuse growth pattern to a nodular growth (Fig. 2*A*), inhibiting totally muscle infiltration and strongly reducing invasion of vessels and skin (Fig. 2*B*).

To analyze the effect of the receptor in formation of experimental metastasis, parental and TRβ1-expressing cells were injected into the tail vein of nude mice. Examination of the lungs at necropsy showed that TRβ1 had a potent inhibitory effect on metastasis formation (Fig. 2*D*). Whereas most mice inoculated with MDA or SK cells developed metastasis, only 20% of mice injected with TRβ1-expressing cells had metastatic lesions. Furthermore, large nodular metastasis were found in lungs of mice injected with parental cells, whereas cells that express the receptor at most gave rise to micrometastasis with a pattern of interstitial cells (Fig. 2*C*). The number of lesions was also strongly reduced by TRβ1, and quantification of parenchymal involvement showed that TRβ1 drastically decreased the area of the lungs affected by metastatic growth (Fig. 2*D*).

To determine if TRβ1 affects extravasation, both hepatocarcinoma and breast cancer cells were incubated with labeled [^{125}I]deoxyuridine before injection into the tail of nude mice. Expression of TRβ1 reduced the amount of radioactivity in the lungs as soon as 4 hours postinjection in the case of MDA cells and 8 hours in the case of SK cells (Supplementary Fig. S3). This indicates that the receptor has antimetastatic activity by blocking not only the ability of cancer cells to proliferate and colonize the lung parenchyma but also by limiting cancer cell extravasation.

TRβ inhibits anchorage-independent growth and migration. The ability of cancer cells to survive and proliferate in the absence of a solid substrate is an important component of the acquisition of an invasive and metastatic phenotype. TRβ1 blocked colony formation by MDA and SK cells in soft agar (Fig. 3*A*) and prevented their ability to grow in suspension under rocking conditions (Fig. 3*B*). In addition, migration assays through Matrigel showed that receptor expression markedly impaired *in vitro* invasiveness (Fig. 3*C*). In breast cancer cells these effects were already maximal in the absence of exogenously added T3, whereas in hepatocarcinoma cells total inhibitions were only obtained in T3-treated cells.

TRβ abolishes growth factor–induced proliferation. Autocrine factors, as well as paracrine signals produced by stromal cells, induce proliferation, survival, and tumor cell invasiveness (15, 16). We, therefore, tested if TRβ1 affects the response of cancer cells to growth factors. Whereas EGF or IGF-I triggered proliferation of parental hepatocarcinoma and breast cancer cells (Fig. 4*A*), expression of TRβ1 abolished this response. The main signaling pathways responsible for growth factor stimulation of cell proliferation are the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways. As shown in Fig. 4*B*, extracellular signal-regulated kinase (ERK) phosphorylation in response to EGF and IGF-I was reduced in TRβ1-expressing cells and AKT phosphorylation was also blunted in MDA cells. In addition, EGF and IGF-I increased significantly ERK-dependent gene expression in parental cells, and this response was blocked in cells expressing the receptor (Fig. 4*C*). Furthermore, transcriptional activation of ATF-2, a downstream target of the MAPK pathway, by these factors was also blocked by TRβ1 (Supplementary Fig. S4*A*). Repression of transcription by growth factors of a plasmid-containing FOX0 binding sites was also abolished by TRβ1 in MDA cells (Supplementary Fig. S4*B*), confirming that the receptor antagonizes activation of the PI3K pathway.

The proliferative response of hepatocarcinoma and breast cancer cells to transforming growth factor β (TGFβ) was also abolished by TRβ1 (Supplementary Fig. S5*A* and *B*). Many actions of TGFβ are mediated by SMAD activation but these factors can also induce MAPK and PI3K activation (17, 18), and TRβ also blocked stimulation of these pathways by TGFβ (Supplementary Fig. S5*C* and 5*D*).

In agreement with the reduced MAPK activity and the lack of growth factor–induced proliferation, the levels of AP-1 complexes analyzed in gel retardation assays were strongly reduced in MDA-TRβ cells and did not increase in response to EGF, IGF-I, or TGFβ (Supplementary Fig. S6). Taken together, these results show that TRβ antagonizes stimulation of signaling pathways by growth and transforming growth factors that play a key role in tumor progression and invasiveness.

TRβ1 represses coordinately expression of genes involved in tumorigenesis and metastasis formation. Altered expression of growth factor receptors could underlie the reduced response of TRβ1-expressing cells to growth factors. TRβ1 caused a drastic reduction of epidermal growth factor receptor 1 (EGFR1) and ErbB3 mRNAs, without altering transcripts for their heterodimeric partner ErbB2, in both hepatocarcinoma and breast cancer cells

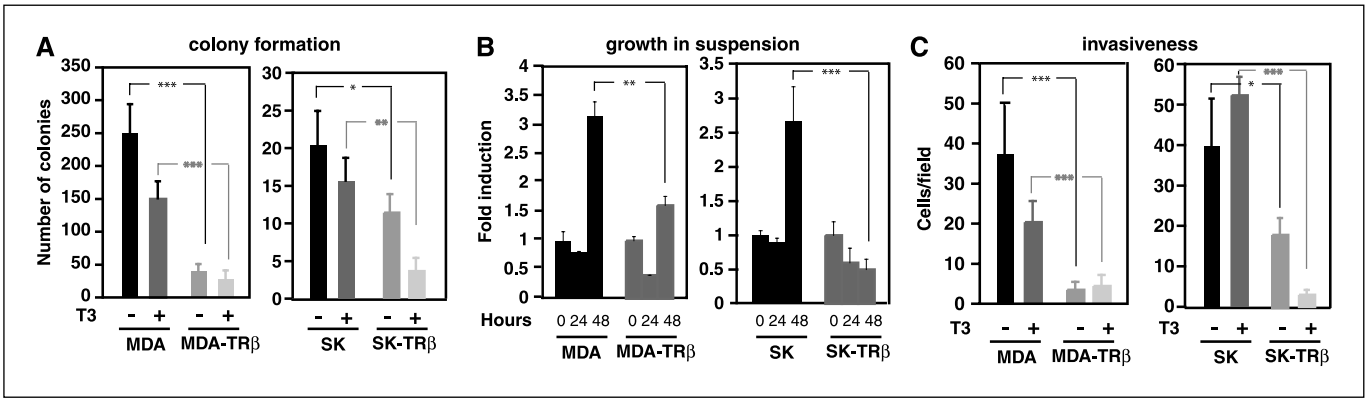

Figure 3. TRβ1 blocks anchorage-independent growth and invasion. *A,* colony formation in soft agar was determined in parental MDA and SK cells and in cells expressing TRβ1. Cells were grown in the presence and absence of 100 nmol/L T3, and the number of colonies (mean ± SD) was scored after 40 d. *B,* cells were grown in suspension under rocking conditions for 0, 24, and 48 h. Data (mean ± SD) are expressed as fold induction with respect to the values obtained at time 0. *C,* cells incubated for 24 h in the presence and absence of 5 nmol/L T3 were inoculated into the upper chamber of Transwell plates containing Matrigel. Migration lasted for 16 h and also occurred in the presence and absence of hormone. Cells that passed through the filter were stained and scored. *Columns,* mean; *bars,* SD.

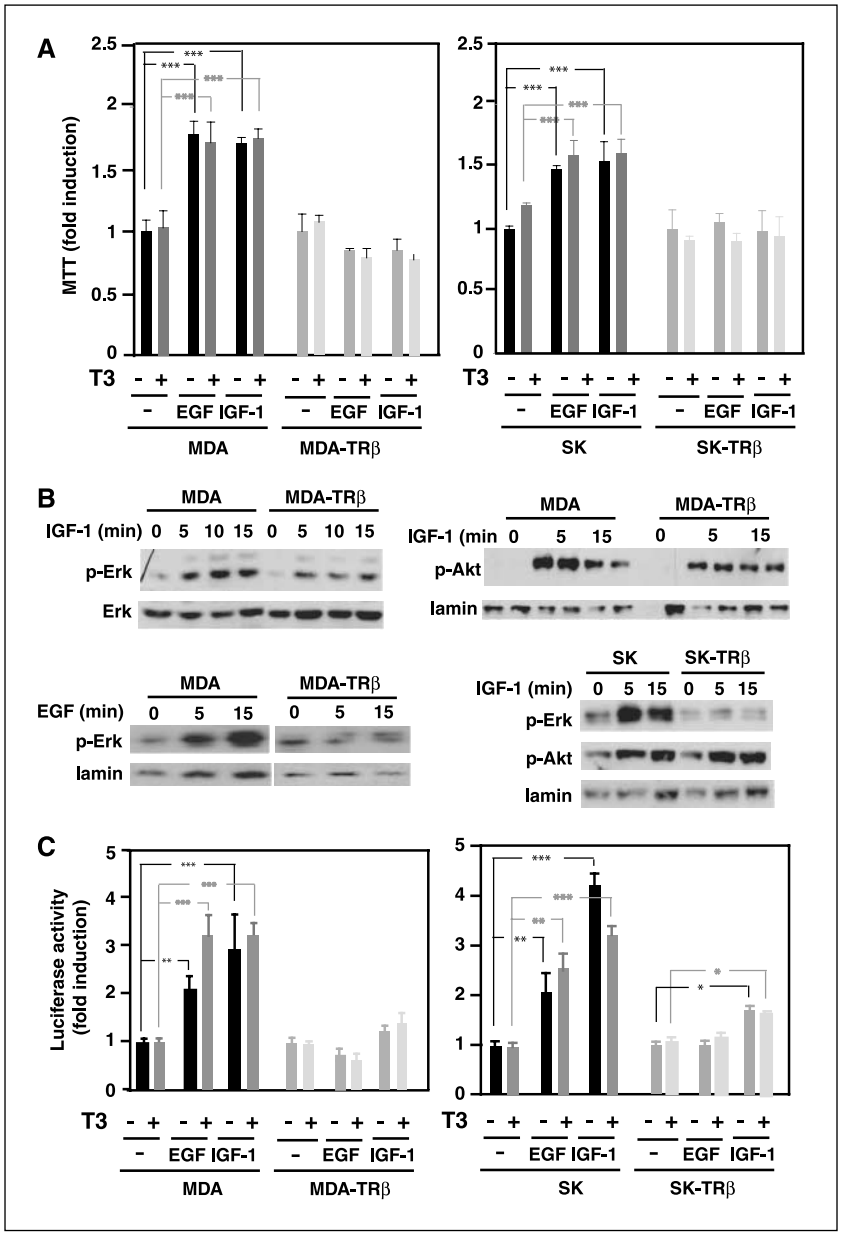

Figure 4. TRβ1 blocks the response of cancer cells to EGF and IGF-I. *A,* parental and TRβ-expressing MDA and SK cells were incubated in serum-free medium alone or in the presence of 5 nmol/L T3, 25 ng/mL EGF, or 15 nmol/L IGF-I for 48 h, and MTT values were determined. Data (mean ± SD) are expressed as fold induction over the values obtained in the corresponding control untreated cells. *B,* TRβ1 reduces activation of ERK and AKT by EGF and IGF-I. Western blotting was performed to detect phosphorylation of ERK and AKT in the different groups of cells incubated for the times indicated with these growth factors. Lamin levels were used as a loading control. *C,* TRβ1 blocks stimulation of ERK transcriptional activity by growth factors. Cells were transfected with a GAL-Elk fusion construct (a direct target of ERK) and the UAS luciferase reporter plasmid. Luciferase activity was determined in control cells and in cells treated for 36 h with T3, EGF, or IGF-I, as indicated. Data (mean ± SD) are expressed as fold induction over the values obtained in the corresponding untreated cells.

(Fig. 5*A*). An important decrease of IGFIR expression together with an increase in IGFBP3, a binding protein that modulates growth factor availability (19), was also observed in both cell types. In contrast, transcripts for the TGFβRII receptor were not reduced by TRβ1 (Fig. 5*A*). This indicates that additional mechanisms downstream of receptor expression are responsible for the insensitivity to transforming growth factors in TRβ-expressing cells.

Genes that are relevant for metastatic progression have been identified (20–25). Transcripts for the prostaglandin-synthesizing enzyme cyclooxygenase 2 and the transcriptional inhibitor ID1, recently identified among the genes that mediate breast cancer metastasis to the lungs, were strongly reduced by TRβ in MDA cells (Fig. 5*B*). The same occurred in MDA and SK cells with the transcripts for the chemokine receptor CXCR4 or for c-Met, also markers of metastastic growth (Fig. 5*B*). This reduction could explain the reduced invasiveness and engraftment of TRβ1-expressing cells. In addition, in SK cells, TRβ1 caused a significant decrease in the transcripts for the chemokine receptors CCR6 and CCR1, related to tumor dissemination in hepatocarcinoma (26–29). Matrix-remodeling metalloproteinases (MMP) are also important for invasiveness, and MMP-1 and MMP-9 transcripts were reduced after TRβ reexpression (Fig. 5*B*). MMP-9 activity in conditioned medium, both in the absence and presence of T3, was also decreased (Supplementary Fig. S7). In contrast, no changes in MMP-2 transcripts or activity was found in MDA cells, but TGFβ treatment caused MMP-2 activation in parental hepatocarcinoma cells but not in SK-TRβ cells, showing the inability of these cells to respond to this factor (Supplementary Fig. S7). Loss of caspase-1 is associated with metastasic growth, and caspase-1 mRNA was increased in TRβ1-expressing cells (Fig. 5*B*). Repression of gene expression by TRβ is consistent with the antagonism of MAPK and PI3K signaling pathways because incubation of parental MDA cells with the inhibitors UO or LY reduced significantly the levels of these mRNAs (Supplementary Fig. S8).

Role of TRs on chemical skin carcinogenesis in mice. In experimental two-stage skin carcinogenesis, benign and malignant neoplasms can be induced on the backs of mice after exposure to a carcinogen, such as DMBA (initiation), and subsequent chronic regenerative epidermal hyperplasia caused by a promoting agent, such as TPA (promotion; ref. 30). This protocol provides an excellent model to analyze the role of TRs on tumor progression. As shown in Fig. 6*A*, strong TRβ expression was detected in normal skin, as well as in hyperplasic epidermis, obtained after topical application of TPA for 4 days. However, receptor expression was strongly reduced in the papillomas and was totally lost in squamous cell carcinomas (SCC), supporting the notion that TRβ acts as a suppressor of malignant transformation.

We next tested if endogenous TRs can suppress epithelial tumor formation and malignization by comparing the development of skin tumors in wild-type mice and in mice lacking both TRα and TRβ (Fig. 6*B*). Tumor number increased with time, reaching a plateau at ~20 weeks after DMBA treatment in both groups. Unexpectedly, $TR\alpha^{-/-}/TR\beta^{-/-}$ double KO mice developed a significantly lower number of tumors than controls. However, after

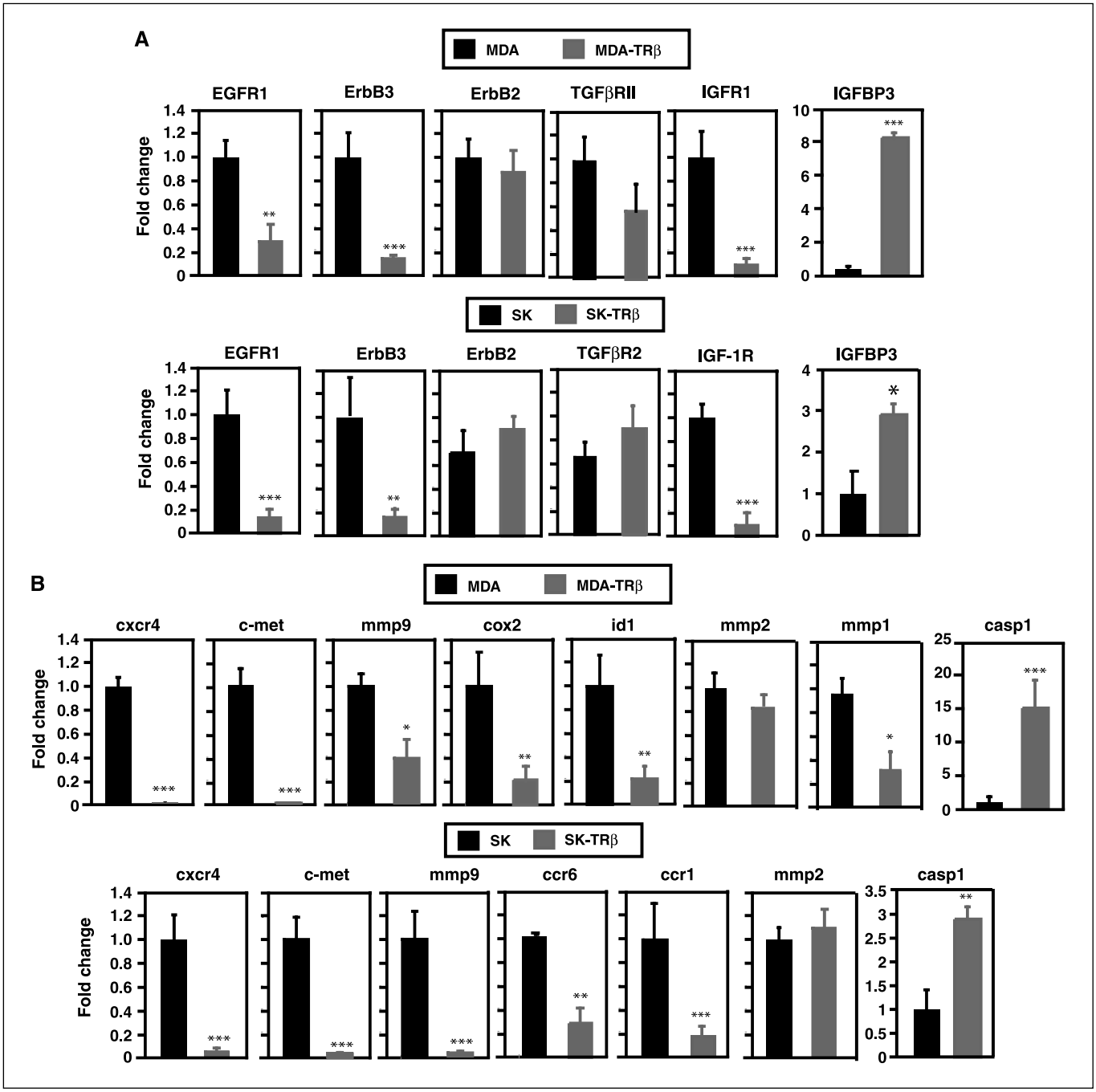

Figure 5. TRβ1 regulates expression of genes involved in invasiveness and metastasis formation. Relative expression levels of transcripts for growth factor receptors (*A*) and prometastatic genes (*B*) were estimated by real-time PCR in MDA, MDA-TRβ, SK, and SK-TRβ cells. Data (mean ± SD) were corrected by the amount of GAPDH mRNA and are expressed as fold change over the values obtained in the corresponding parental cells.

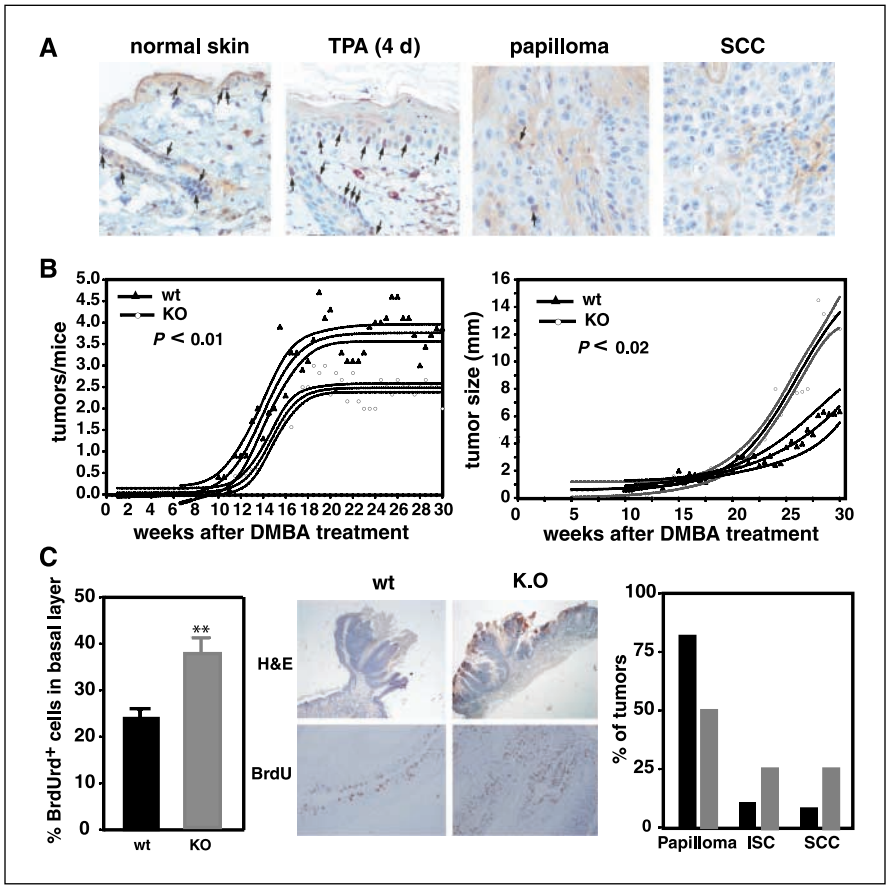

Figure 6. Role of TRs on chemical skin carcinogenesis in mice. *A,* TRβ expression is lost during progression of skin carcinogenesis. TRβ was detected by immunohistochemistry in normal skin, in skin of mice after 4 d of topical treatment with TPA (two applications of 12.5 μg), and in benign papillomas and SCC from mice subjected to the DMBA/TPA chemical skin carcinogenesis protocol. *B,* number and size of tumors during DMBA/TPA two-stage skin carcinogenesis comparing $TR\alpha^{-/-}/TR\beta^{-/-}$ double KO animals with their wild-type (*wt*) controls. *Triangles,* wild-type; *open circles,* $TR\alpha^{-/-}/TR\beta^{-/-}$. The curves represent Bolzmann Sigmoidal nonlinear regression analysis of the data points. The respective 95% confidence intervals are expressed as flanking lines. *C,* the index of tumor proliferation (mean ± SE) represented by the average percentage of $BrdUrd^+$ cells in the basal epithelial cell layer within representative end point skin tumors of KO and wild-type animals (*left*). Representative H&E staining and BrdUrd incorporation of chemically induced skin tumors in wild-type and $TR\alpha^{-/-}/TR\beta^{-/-}$ mice (*middle*). Histologic classification of skin tumors at the end of the experiment. *Columns,* percentage of benign papillomas, *in situ* carcinomas (*ISC*), and SCC of $TR\alpha^{-/-}/TR\beta^{-/-}$ and wild-type animals. Double KO tumors show a more malignant phenotype than wild-type counterparts (*right*).

20 weeks, tumor growth was significantly faster in animals devoid of the receptors. The higher proliferation rate of tumors from mice lacking TRs was reflected by an increase in the percentage of BrdUrd-positive cells in the basal layer of epithelium at necropsy (30 weeks; Fig. 6*C*). Interestingly, skin tumors from $TR\alpha^{-/-}/TR\beta^{-/-}$ mice had a more malignant phenotype. Histologic classification showed that >80% of tumors found in wild-type mice were typically well-differentiated papillomas, whereas in KO mice, 50% of tumors were displayed signs of *in situ* carcinoma and SCC (that reached 25% of the tumors developed), indicating that endogenous TRs suppress malignant progression in this model of epithelial carcinogenesis.

Discussion

Our results show that TRβ1 is a potent suppressor of tumorigenesis, invasiveness, and metastasis formation. Reexpression of TRβ1 in hepatocarcinoma and breast cancer reduces tumor growth, enhances expression of epithelial markers, such as keratin 8/18 or β-catenin, and diminishes expression of the mesenchymal marker vimentin. Enhanced keratin 8/18 and β-catenin expression is associated with reduced invasiveness (31), whereas high vimentin levels are found in invasive tumors (32). Therefore, TRβ1 seems to facilitate mesenchymal-to-epithelial transition, retarding tumor progression.

To form a tumor, cancer cells must create a self-sustaining environment by secreting proteases and angiogenic factors, elude death, and alter their response to growth stimulatory and inhibitory signals (33). Our results show that TRβ1 alters many of these interrelated processes because it reduced tumor angiogenesis, increased necrosis, blocked the response to growth factors, and reduced expression and/or activity of selected metalloproteases and other genes important for tumor progression.

Growth factors, such as EGF or IGF-I, are potent mitogens (34), and their receptors can be overexpressed in hepatocarcinoma and breast cancer cells (35, 36). Our results show that TRβ1 disrupted the mitogenic action of these factors by suppressing activation of ERK and PI3K signaling pathways that are crucial for cell proliferation and invasiveness (37–39). Down-regulation of growth factor receptors could participate in the insensitivity of TRβ1-expressing cells to EGF and IGF-I. Indeed, transcripts for IGFIR, EGFR, or ErbB3, although not ErbB2, were strongly reduced by TRβ1, and the weaker ERK and PI3K response to these factors could be related to reduced receptor expression. However, other downstream components of the growth factor signaling pathways must be repressed by the receptor, because TRβ1 totally abolished activation of transcription factors, such as ELK1 or ATF-2. This is in accordance with our observations that TRs can repress Ras-mediated responses downstream of ERK (6) and with the demonstration that TRs antagonize activation of genes that do not contain TREs through mechanisms that can involve direct interaction of these receptors with other signaling components and transcription factors (2). Furthermore, our data show that TRβ1 blocks TGFβ-dependent proliferation in hepatocarcinoma and breast cancer cells without altering TGFβRII expression. Interestingly, inhibition of responses to EGF, IGF-I, or TGFβ by TRβ1 can occur in the absence of exogenous T3, suggesting that unoccupied receptors can block the response to growth and transforming

growth factors. The high levels of receptor expressed in MDA-TRβ and SK-TRβ could have contributed to the gain of ligand-independent effects, although ligand dependency was observed when transactivation of a TR-responsive plasmid was analyzed. Interestingly, a mutant receptor unable to bind T3 can cause thyroid tumors in mice (7), and different TR mutants defective in ligand binding were found in hepatocarcinomas (7, 40, 41).

Our results show that TRβ1 had a major inhibitory effect on invasiveness both *in vivo* and *in vitro*. In animals, the receptor strongly diminished local invasion of the host stroma and potently repressed formation of experimental metastasis in lung. The inhibitory effect of TRβ1 on tumor growth could contribute to the reduction of formation of long-distance metastasis. However, the fact that metastatic growth is blocked when cells are directly injected into the tail vein indicates that TRβ, indeed, acts as a metastasis suppressor gene. Not only the presence of a receptor in the cancer cell but also the thyroidal status of the host can modulate tumor growth and metastasis formation. We had previously shown that thyroidal status has some effect on tumorigenesis because tumor formation by *ras*-transformed fibroblasts is slightly retarded in hypothyroid animals (6). Preliminary results indicate that this also occurs with hepatocarcinoma and breast cancer cells. In contrast, metastasis formation by these cells increases in nude mice treated with antithyroidal drugs.[6] This suggests that TR ligand levels could also influence tumorigenic and metastatic growth.

The inhibitory effect of TRβ1 on tumor invasiveness is compatible with the reduced migration observed in cultured tumor cells expressing the receptor. This could restrain their entry into the circulation, as well as their exit from the bloodstream into the lung parenchyma, and accordingly, we have shown that extravasation was reduced by TRβ1. Furthermore, the inability to grow in the absence of a solid substrate could decrease survival of TRβ1-expressing cells in the circulation.

Recent searches for genetic determinants of metastasis have led to the identification of gene sets, or "signatures," for which the expression in primary tumors is associated with high risk of metastasis and poor survival (42–47), and genes that are relevant for metastatic progression of breast cancer have been recently identified (20, 21). Our results show that expression of many of these genes was strongly reduced by TRβ1 in breast cancer cells. In addition, TRβ1 blocked expression of the receptors CXCR4, CCR1, CCR6, and c-met, also involved in metastatic growth and bad prognosis in patients (26–29). The finding that TRβ1 down-regulates coordinately the expression of these genes suggests a common molecular mechanism for this receptor action. Repression of gene expression by TR in many cases involves cross-talk with other factors and signaling pathways, and the finding that inhibitors of MAPK or PI3K inhibit their expression in parental cells indicates that antagonism of these pathways by TRβ1 could be responsible for their transcriptional repression.

That endogenous TRs play a role in the progression from benign tumors to invasive carcinomas was shown by the distinct response of normal and TR-deficient mice to the skin carcinogenesis protocol. TR deficiency seems to inhibit benign tumor formation at early stages of skin carcinogenesis while increasing malignant progression at later stages. Histopathologic evaluation of skin tumors revealed that, in contrast to the well-differentiated papillomas found in control mice, half of the tumors in mice lacking TRs were diagnosed as either *in situ* carcinomas or SCC.

In summary, our results show that TRs have an important role in tumor progression in experimental animals, suggesting that these receptors could constitute a novel therapeutic target in cancer. Elucidation of the role of these receptors in metastatic growth should lead to a better understanding of the biology of metastasis and its susceptibility to treatment in humans.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 6/10/2008; revised 9/26/2008; accepted 10/28/2008.

Grant support: MEC grants BFU2004 03165 and BFU2007-62402, FIS grants RD06/0020/0036, Acción Transversal de Cáncer and PIO40682, and EU Project CRESCENDO grant FP6-018652.

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We thank A. Fabra for valuable help and reagents and C. Sánchez and M. Sánchez-Prieto for technical help.

[6] O. Martínez-Iglesias and A. Aranda, unpublished results.

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