

Endothelin B Receptor Blockade Inhibits Dynamics of Cell Interactions and Communications in Melanoma Cell Progression

Anna Bagnato,¹ Laura Rosanò,¹ Francesca Spinella,¹ Valeriana Di Castro,¹ Raffaele Tecce,¹ and Pier Giorgio Natali²

Laboratories of ¹Molecular Pathology and Ultrastructure and ²Immunology, Regina Elena Cancer Institute, Rome, Italy

ABSTRACT

Phenotypic and genotypic analyses of cutaneous melanoma have identified the endothelin B receptor (ET_BR) as tumor progression marker, thus representing a potential therapeutic target. Here, we demonstrate that activation of ET_BR by endothelin-1 (ET-1) and ET-3 leads to loss of expression of the cell adhesion molecule E-cadherin and associated catenin proteins and gain of N-cadherin expression. Exposure of melanoma cells to ET-1 leads to a 60% inhibition in intercellular communication by inducing phosphorylation of gap junctional protein connexin 43. Additionally, activation of the ET_BR pathway increases $\alpha_5\beta_3$ and $\alpha_2\beta_1$ integrin expression and matrix metalloproteinase (MMP)-2 and MMP-9, membrane type-1-MMP activation, and tissue inhibitor MMP-2 secretion. The ET_BR pathway results into the downstream activation of focal adhesion kinase and extracellular signal-regulated kinase 1/2 signaling pathways, which lead to enhanced cell proliferation, adhesion, migration, and MMP-dependent invasion. The small molecule A-192621, an orally bioavailable nonpeptide ET_BR antagonist, significantly inhibits melanoma growth in nude mice. These findings demonstrate that ET-1 and ET-3 through ET_BR activation trigger signaling pathways involved in events associated with disruption of normal host-tumor interactions and progression of cutaneous melanoma. Pharmacological interruption of ET_BR signaling may represent a novel therapeutic strategy in the treatment of this malignancy.

INTRODUCTION

Mouse genetics and molecular profiling of human tissues have identified pathways relevant to melanocyte migration and differentiation as well as to melanoma progression. The study of melanocyte development in knock-out mice has provided insight into the functions of endothelins (ETs) and their receptors in this cell lineage (1–4). The ET family of molecules comprises three isopeptides, ET-1, ET-2, and ET-3, of 21 amino acids that bind to two highly homologous G-protein-coupled receptors, ET_A receptor (ET_AR) and ET_B receptor (ET_BR), which mediate a variety of physiological activity in different cell types (3). In the adult, ET-1 secreted by keratinocytes stimulates proliferation, chemotaxis, and pigment production in melanocytes (5, 6) and plays a central role in UV-B light-induced pigmentation (7, 8). Increasing evidence points to ETs as relevant mediators in tumor progression in a variety of malignancies (9). Melanoma cells express both ET_AR and ET_BR (10). Gene expression analysis (11) and immunophenotyping of human cutaneous melanoma (12) have recently identified ET_BR as critical in the progression of this malignancy. Through the same receptor, ET-1 acts as antiapoptotic factor for melanoma cells and melanocytes (13). Thus, ET_BR blockade by the ET_BR peptide antagonist BQ788 resulted in growth inhibition and death of melanoma cells *in vivo* and *in vitro* (14). Although these

studies define a relevant role of the ET-1/ET_BR pathway in the biology of melanocytic tumors, the molecular events underlying this activity have not been investigated. Early melanoma growth is the result of disrupted intercellular homeostatic regulation (15). Once this balance is lost and malignant transformation has occurred, microenvironmental factors such as cell adherence to extracellular matrix, host-tumor interactions, degradation of matrix components, migration, and invasion became essential for the tumor progression to the metastatic phenotype (15). Changes in cadherin, connexin (Cx), matrix metalloproteinase (MMP), and integrin expression have emerged as key factors in melanoma progression (16–17). Therefore we analyze the role played by ETs and ET_BR in melanoma cell proliferation, cell-cell adhesion and communication, migration, tumor proteinase activation, and invasion. Because activation of focal adhesion kinase (FAK) and of extracellular signal-regulated kinase (Erk) are essential molecular signaling in melanoma progression (18–20), we analyzed the effect of ETs on these ET_BR-mediated signal transduction pathways. In view of the availability of orally active nonpeptide ET_BR antagonists (9, 21), which are more suitable in clinical setting, we use these compounds to assess their antitumor activity *in vivo*.

MATERIALS AND METHODS

Cells. The human cutaneous melanoma cell line 1007 established from a primary lesion was a generous gift of Dr. G. Parmiani (National Cancer Institute, Milan, Italy). The melanoma cell lines SK-Mel28 (American Type Culture Collection, Rockville, MD) M10 and Mel120 were derived from a metastatic melanoma (22). Cells were grown in RPMI 1640 containing 10% FCS. All culture reagents were from Invitrogen (Paisley, Scotland, United Kingdom). Melanoma cells were starved for 24 h in serum-free medium and then incubated for indicated times with ET-1 and ET-3 (Peninsula Laboratories, Belmont, CA). When the effects of the antagonists [BQ123 and BQ788 (Peninsula Laboratories) and A192621 (Abbott Laboratories, Abbott Park, IL)] were studied, they were added 15 min before agonist. Pretreatment of cells with PD98059 (10 μ M; Calbiochem-Novabiochem Corporation, San Diego, CA) or Ionomast (10 μ M; Chemicom International, Temecula, CA) was performed for 20 min before the addition of ET-1 or ET-3.

Reverse Transcription-PCR. Total RNA was prepared using the TRIzol reagent (Invitrogen) as recommended. The reverse transcription-PCR was performed using a SUPERSRIPT One-Step RT-PCR System (Invitrogen). The primer sets used were 5'-TGCTCCTGCTCGTCCCTGATGGATAAAGAG-3' and 5'-GGTCACATAACGCTCTCTGGAGGGCTT-3' for ET-1, 5'-4 CACTGGTTGGATGTGTAATC-3' and 5'-GGAGATCAATGACCACATAG-3' for ET_AR, 5'-TCAACACGGTGGTGTCTGCTGC-3' and 5'-ACTGAATAGCCACCAATCTT-3 for ET_BR, 5'-AACAGGATGGCTGAAGGTGA-3' and 5'-AAAATCCAAGCCCTTTGCTG-3' for E-cadherin, and 5'-TTCCAGCAGCCCTACGACCAG-3' and 5'-GCCTTCCCACTAGTCTCATC-3' for *Snail*. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control and the primer sets used were 5'-TGAAGGTCGGTGTCAACGGA-3' and 5'-GATGGCATGGACTGTGGTCAT-3'. The cDNA was amplified for 35 cycles of a denaturation step at 94°C for 1 min; a primer annealing step at 64°C (E-cadherin), 55°C (*Snail*), and 54°C (ET-1, ET_AR, ET_BR, and GAPDH) for 30 s; and an extension step at 72°C for 1 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide.

Northern Blotting. Total RNA from 1007 cells was separated on 2% denaturing formaldehyde agarose gel (15 μ g RNA/lane) and transferred to a nylon membrane. The membranes were hybridized in the QuickHyb hybrid-

Received 7/30/03; revised 11/4/03; accepted 12/17/03.

Grant support: Associazione Italiana Ricerca sul Cancro, Ministero della Salute, and Consiglio Nazionale delle Ricerche-Ministero dell'Istruzione, dell'Università e della Ricerca.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Notes: F. Spinella is the recipient of a fellowship from FIRC. A preliminary report of this work may be found in the proceedings of the 93rd annual meeting of the American Association for Cancer Research, p. 22, 111, 2002.

Requests for reprints: Anna Bagnato, Molecular Pathology and Ultrastructure Laboratory, Regina Elena Cancer Institute, Via delle Messi d'Oro 156, 00158 Rome, Italy. Phone: 39-0652662565; Fax: 39-0652662505; E-mail: bagnato@ifio.it.

ization solution (Stratagene, La Jolla, CA). The cDNA probe used for analysis of the E-cadherin, *Snail*, and GAPDH mRNA was prepared using reverse transcription-PCR products. Probes were labeled with [α -³²P]dCTP using a random primer oligolabeling kit (Amersham Biosciences, Little Chalfont Buckinghamshire, United Kingdom). Densitometric scanning was performed with a Mustek MFS-6000CX apparatus, and the data were analyzed with Phoretix 1D software and normalized to those of GAPDH.

Western Blotting. Conditioned medium and protein extracts were separated by SDS-PAGE and revealed by Western blot using antibodies (Abs) to MMP-2 and MMP-9 (NeoMarkers, Fremont, CA), tissue inhibitor metalloproteinase (TIMP)-2, membrane type 1 (MT1)-MMP (Chemicon), phospho-ERK1/2 (Cell Signaling Technology, Inc., Beverly, MA), ERK1/2 (Cell Signaling Technology), α_v , α_2 , β_1 , β_3 (Chemicon), E-cadherin, N-cadherin, β -catenin, p120-catenin, Cx43 (BD Transduction Laboratories, Heidelberg, Germany), and β -actin (Oncogene Research Products, Boston, MA). Blots were developed with an enhanced chemiluminescence detection system kit (Amersham Biosciences).

Gelatin Zymography. The 1007 and M10 cell supernatants were electrophoresed for analysis in 9% SDS-PAGE gels containing 1 mg/ml gelatin. The gels were washed for 30 min at 22°C in 2.5% Triton X-100 and then incubated in 50 mM Tris (pH 7.6), 1 mM ZnCl₂, and 5 mM CaCl₂ for 18 h at 37°C. After incubation, the gels were stained with 0.2% Coomassie Blue. Enzyme-digested regions were identified as white bands on a blue background. Molecular sizes were determined from the mobility, using gelatin zymography standards (Chemicon).

Immunoprecipitation and SDS-PAGE. Serum-starved 1007 melanoma cells were treated with ET-1 and ET-3 for different times. Lysates were immunoprecipitated with anti-FAK (Upstate Biotechnology) or anti- β -catenin or anti-p120-catenin. The blots were incubated for 1 h with antiphosphotyrosine mAb (PY20; BD Transduction Laboratories) or with anti-FAK (Upstate Biotechnology) and developed with an enhanced chemiluminescence system.

Flow Cytometry Analysis. Serum-starved 1007 melanoma cells were treated with ET-1 and ET-3 (10 nM) for 24 h and incubated with primary Ab to α_1 , α_2 , α_4 , α_6 , α_v , β_1 , β_3 , β_6 , or $\alpha_v\beta_3$ and $\alpha_2\beta_1$ (Chemicon) for 40 min at 4°C. FITC-conjugated secondary Ab (Chemicon) was applied to the cell for 30 min at 4°C. Labeled cells were scanned on a FACScan cytometer (Becton Dickson).

ELISA. MMP-2 and TIMP-2 levels in conditioned medium were measured using a Biotrak Human MMP-2 Elisa kit (Amersham Biosciences) and a human TIMP-2 Immunoassay Kit (Chemicon), respectively. TIMP-2 expression was measured by an ELISA capable of recognizing TIMP-2 complexes with active MMP-2. The range of detection of the assays was of 1.5–24 ng/ml for MMP-2 and 20–320 ng/ml for TIMP-2. The experiments were performed in duplicate and repeated three times.

Scrape Loading/Dye Coupling. Levels of gap junction intercellular communication (GJIC) in control and treated cultures were determined using the scrape-loading/dye transfer (SL/DY) technique, applying a mixture of fluorescent dyes: 0.5% lucifer yellow (LY; Sigma) and 0.5% rhodamine-dextran (Molecular Probes, Eugene, OR). 1007 and M10 cells, cultured as described previously, were washed thoroughly with PBS in which Ca²⁺ was omitted to prevent uncoupling of the cells due to high Ca²⁺. The mixture was added to the cells, and scrape loading was performed applying two or three cuts on cell monolayer with a razor blade. The dye mixture was rinsed away 1 min after the scrape. Cells were washed three times with PBS and fixed with 4% paraformaldehyde, and cells stained with LY alone and or with rhodamine-dextran were detected by fluorescence emission with an inverted fluorescent microscope equipped with a camera and counted. Junctional permeability was measured after the scrape by taking five successive images per trial. Cells that received the LY from the scrape-loaded cells, excluding the rhodamine-dextran-stained cells, were considered communicating. GJIC capacity was expressed as percentage of the control.

Cell Proliferation Assay. [³H]thymidine incorporation was measured as described previously (23). Responses to all agents were assayed in sextuplicate, and results were expressed as means of three separate experiments.

Adhesion Assay. Serum-starved cells were treated with ET-1 or ET-3 in the absence or in the presence of antagonists for 24 h and labeled by incubation with ⁵¹Cr-labeled sodium chromate (50 μ Ci for 10⁶ cells; DuPont, New England Nuclear Research Products, Wilmington, DE) for 1 h at 37°C. As indicated, cells were incubated with Abs to $\alpha_2\beta_1$ and $\alpha_v\beta_3$ subunits (10 μ g/ml;

Chemicon) for 2 h at 37°C with gentle shaking. Labeled cells (5 \times 10⁴) were plated in 96-well plates precoated with type I collagen (10 μ g/ml; BD Transduction Laboratories) for 120 min at 37°C. The percentage of cell adhesion was calculated as follows: cpm adherent cells/cpm adherent + cpm nonadherent cells \times 100. The assay was performed in sextuplicate, and results were expressed as means of three separate experiments.

Chemotaxis and Chemoinvasion Assay. Chemotaxis and chemoinvasion were assessed with a 48-well modified Boyden chamber (NeuroProbe, Pleasanton, CA) as described previously (24). After 4 (chemotaxis) or 6 h (chemoinvasion) of incubation at 37°C, the filters were removed and stained with Diff-Quick (Merz-Dade, Dudingon, Switzerland), and the migrated cells in 10 high-power fields were counted. Each experimental point was analyzed in triplicate. Pretreatment of cells with Abs to $\alpha_v\beta_3$ and $\alpha_2\beta_1$ (Chemicon) was performed for 60 min at 37°C before the chemotaxis assay.

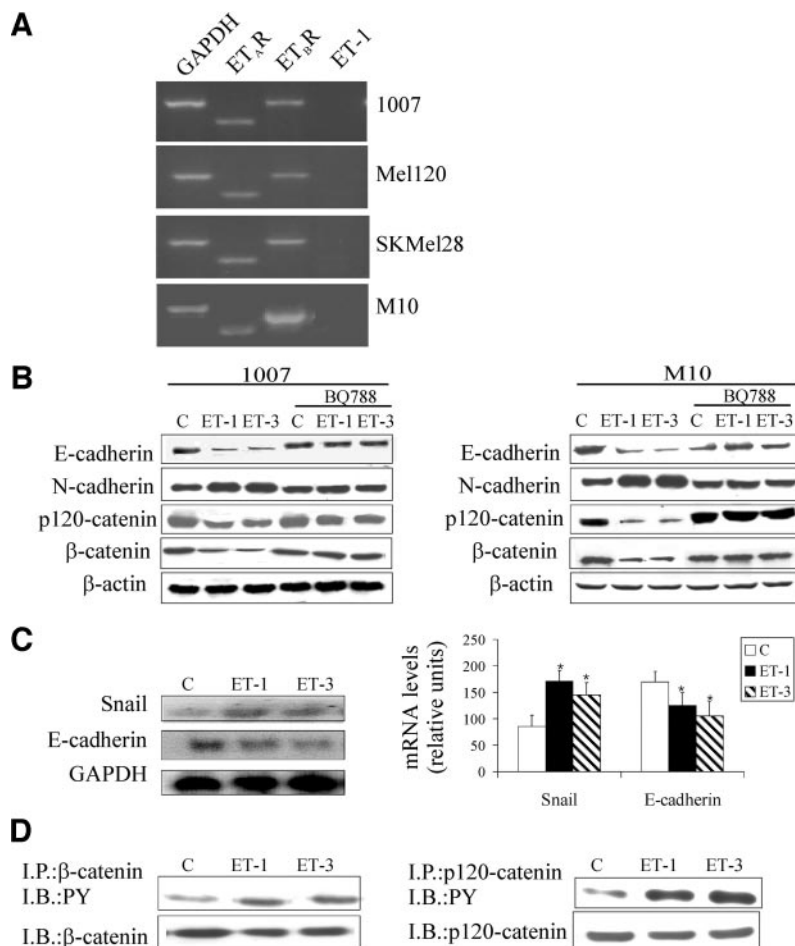
M10 Melanoma Xenografts. Female athymic (nu⁺/nu⁺) mice, 4–6 weeks of age (Charles River Laboratories, Milan, Italy), were handled according to the institutional guidelines under the control of the Italian Ministry of Health. Mice received s.c. injection on one flank with 1.5 \times 10⁶ viable M10 cells expressing ET_BR. The mice were randomized in groups (n = 10) to receive treatment i.p. for 21 days with two different doses of A-192621 (10 mg/kg/day and 20 mg/kg/day), and controls received injection of 200 μ l of drug vehicle (0.25 N NaHCO₃). The treatments were started 7 days after the xenograft, when the tumor was palpable (\sim 0.03 cm³). Each experiment was repeated three times. Tumor size was measured with calipers and was calculated using the formula $\pi/6 \times$ larger diameter \times (smaller diameter)².

Statistical Analysis. Statistical evaluations of data were made by the two-sided Student's test and by two-way ANOVA as appropriate.

RESULTS

ET-1 and ET-3 Alter Cell Adhesion Molecule Expression through ET_BR. ET-mediated activities were assessed on the primary melanoma 1007 cells and in the metastatic cell lines M10, SK Mel 28, and Mel 120, which express ET_AR and ET_BR mRNA but no ET-1 mRNA (Fig. 1A). The tumor suppressor E-cadherin, which is often down-regulated in most melanoma (25), has recently been shown to be modulated by ET-1 (26). In 1007 and M10 cells, ETs induced E-cadherin down-regulation upon 24 h of treatment (Fig. 1B). Because the catenins are cytoplasmic proteins that bind E-cadherin and are critical for cellular adhesion, we investigated the effects of ETs on β -catenin and p120-catenin. In both melanoma cell lines, ET-1 and ET-3 stimulation for 24 h decreased the protein levels of β - and p120-catenin paralleling those of E-cadherin down-regulation (Fig. 1B). The shift in cadherin profile from E- to N-isofom during melanoma progression has been described both *in vitro* and *in vivo*, as a mechanism that not only frees melanoma cells from control by keratinocytes but also provides new adhesion properties enhancing the malignant phenotype (16). In 1007 and M10 melanoma cells, we observed that ET-1- and ET-3-induced progressive loss of E-cadherin was concomitant with gain of N-cadherin expression (Fig. 1B), which may enable melanoma cells to interact with N-cadherin-positive neighboring melanoma cells, fibroblasts, and endothelial cells. BQ788, a specific ET_BR antagonist, reversed these effects, indicating that ET_BR activation is required for these responses (Fig. 1B). The transcription factor *Snail*, a master regulator of epithelial to mesenchymal transition, has recently been found to repress E-cadherin expression. Screening of a panel of melanoma cells from different progression stages revealed that the expression of *Snail* negatively correlates with expression of E-cadherin (15, 27). By Northern blot analysis, we demonstrated that ET-treatment of 1007 cells induced a significant increase in the expression of *Snail* mRNA that closely correlated with down-regulation of E-cadherin at mRNA and protein levels, suggesting that down-regulation of E-cadherin protein involved transcriptional mechanisms (Fig. 1C). Tyrosine phosphorylation of catenin proteins may result in a reduced interaction with both

Fig. 1. A, ET_AR and ET_BR mRNAs detected by reverse transcription-PCR in melanoma cell lines. PCR products of 367 bp for ET_AR, 529 bp for ET_BR, and 533 bp for GAPDH are shown as visualized by ethidium bromide. All of the melanoma cell lines tested, 1007, M10, SKMel 28, and Mel120, expressed mRNA for ET_AR and ET_BR but no ET-1 mRNA. B, down-regulation of E-cadherin and catenin proteins and up-regulation of N-cadherin by ET-1 and ET-3 in melanoma cells. Serum-starved 1007 and M10 cells were stimulated with 10 nM ET-1 or ET-3 for 24 h in the absence or presence of 100 nM BQ788. Lysates were immunoblotted with Abs to E-cadherin, N-cadherin, β -catenin, or p120-catenin and for internal control Ab to β -actin. C, *Snail* mRNA levels expression inversely correlate with E-cadherin after ET-treatment. *Snail* and E-cadherin mRNA were analyzed by Northern blotting in 1007 cells treated with ET-1 or ET-3 (10 nM) for 6 h. GAPDH mRNA expression levels were used as a loading control. The relative density of mRNA content from C was statistically analyzed and represents the average value of three independent Northern blots \pm SD; *, $P < 0.001$ compared with control. D, ET-1 and ET-3 induce the tyrosine phosphorylation of catenin proteins. Serum-starved 1007 cells were incubated for 5 min with 10 nM ET-1 or ET-3. Cell lysates were immunoprecipitated with anti- β -catenin and anti-p120-catenin and then immunoblotted with antiphosphotyrosine. The membranes were reprobbed with the specific anti- β - and p120-catenin to ensure equal amounts of proteins.



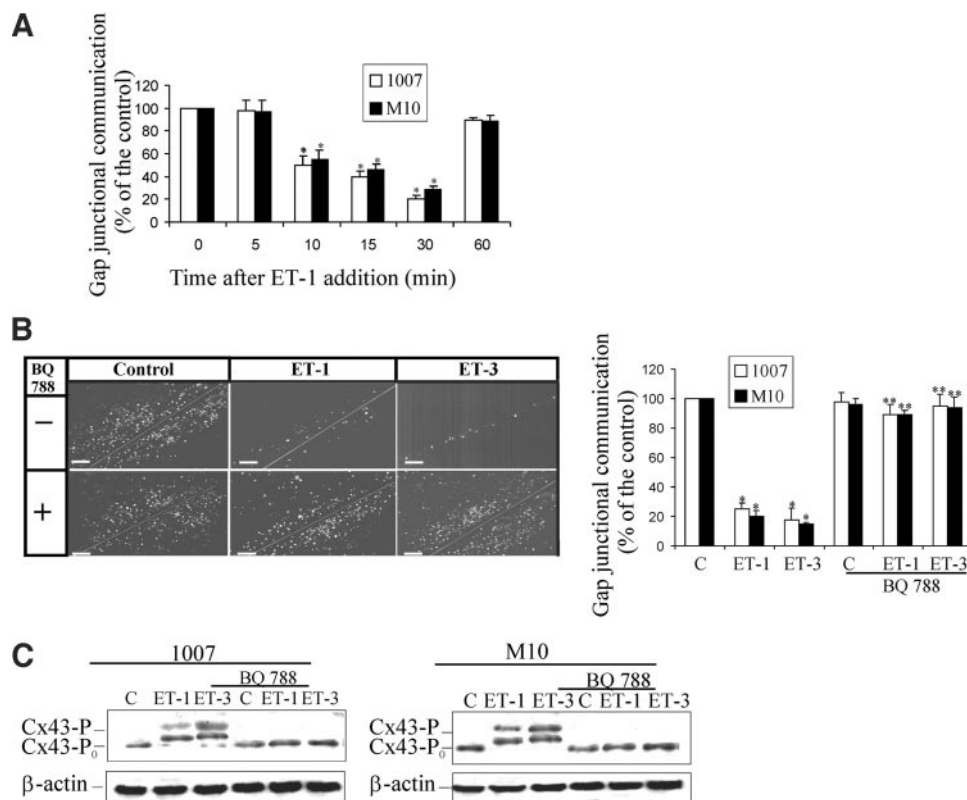
E-cadherin and actin-cytoskeleton (28). In this context, we observed that in serum-starved 1007 cells, both isopeptide ET-1 and ET-3 (10 nM) induced an increase in the tyrosine phosphorylation of β -catenin and p120-catenin that contributes to loss of functional cell adhesions (Fig. 1D).

Intercellular Communication Is Impaired by ET-1 and ET-3 through ET_BR Binding. Loss of GJIC has been shown to occur during melanoma progression. We studied the effects of ETs on GJIC using SL/DT methods in 1007 and M10 cells. For the evaluation of GJIC function, only cells stained with LY, excluding the artificially damaged rhodamine-dextran-stained cells, were counted. Addition of 10 nM ET-1 resulted in a transient and time-dependent reduction in GJIC. Intercellular transfer of LY was still detectable 5 min after ET-1 addition, whereas after 30 min, the dye remained confined to the wounded cells and GJIC were inhibited by 60–70% with respect to the control. GJIC of melanoma cells returned to basal level within 1 h (Fig. 2A). To determine whether ET_BR was responsible for ET-induced disruption of GJIC, the ET_BR antagonist was used in SL/DT experiments. ET-1- and ET-3-induced dye transfer inhibition was completely prevented by BQ788 (Fig. 2B). Gap junction formation requires Cx molecules aligning in hemi-channels. Both melanocytes and keratinocytes express Cx43 in coculture (25). The phosphorylation of Cx43 is believed to be casually linked with disruption of GJIC (29). Recent evidence shows that ET-1 decreases GJIC in ovarian carcinoma cells by inducing Cx43 phosphorylation (30). Therefore to evaluate the effect of ETs on Cx43 expression in melanoma cells, 1007 and M10 cells were exposed to ET-1 and ET-3 (10 nM). Non-phosphorylated Cx43 was detected in untreated 1007 and M10 cells. ETs significantly induced increase in electrophoretic mobility shift of

Cx43 (Fig. 2C). Exposure of melanoma cells to 10 nM ET-1 and ET-3 resulted in a rapid induction of two phosphorylated species that we refer to collectively as Cx43-P (Fig. 2C). BQ788 blocked this effect, indicating that ET_BR activation results into phosphorylation of Cx43 and disruption of GJIC.

ET-1 and ET-3 Induce Secretion and Activation of MMPs through ET_BR. A critical step during tumor invasion is the degradation of the extracellular matrix (ECM) by MMPs. To identify the role of ETs on the activation status of MMP-2 and MMP-9, conditioned media of 1007 and M10 cells were analyzed by gelatin zymography. When 10 nM ET-1 and ET-3 was added, zymography showed that both melanoma cell lines secreted high levels of gelatinolytic proteases corresponding to the active forms of MMP-2 and MMP-9 (Fig. 3A). These results were confirmed by Western blotting, demonstrating that treatment with ET-1 and ET-3 induced an increased secretion of both latent and active forms of MMP-2 and MMP-9 in the conditioned medium of 1007 cells as compared with untreated cells (Fig. 3B). MT1-MMP is a transmembrane MMP known to bind and activate MMP-2 at the cell surface (31). ET-1 and ET-3 enhanced expression of both the latent MT1-MMP and to an even greater extent, the activated form of MT1-MMP. Recent studies have demonstrated that activation of pro-MMP-2 by MT1-MMP depends upon the presence of critical amounts of TIMP-2, which is required for the formation of the ternary complex that leads to the activation of MMP-2 (32). Both ET-1 and ET-3 induced a significant increase of TIMP-2 expression as demonstrated by Western blotting (Fig. 3B). By ELISA, we confirmed that both isopeptides were capable of eliciting MMP-2 and TIMP-2 secretion in the conditioned medium of 1007 cells. Addition of BQ788 completely blocked the ET-induced conversion of

Fig. 2. ETs induce GJIC inhibition in melanoma cells. **A**, serum-starved 1007 and M10 cells were treated with 10 nM ET-1 for up to 60 min, and GJIC capacity was assayed by SL/DT method. Gap junction function was evaluated by analyzing net transfer of LY, excluding dextran-stained cells, as described in "Materials and Methods." The data, reported as the relative percentage of the control, represent the average value of three different assays each performed in triplicate samples \pm SD; *, $P < 0.005$ compared with control. **B**, serum-starved 1007 and M10 cells were incubated for 30 min with ET-1 or ET-3 (10 nM) in the absence or in presence of BQ 788 (100 nM). Photographs show a representative experiment of SL/DT assay performed on 1007 cells; bar = 100 μ m. The data, reported as the relative percentage of the control, represent the average value of three different assays each performed in triplicate \pm SD; *, $P < 0.005$ compared with control; **, $P < 0.0001$ compared with ET-1 or ET-3. **C**, ET-1 and ET-3 induce the phosphorylation of Cx43. Serum-starved 1007 and M10 cells were incubated for 5 min with 10 nM ET-1 or ET-3 in the absence or in the presence of BQ788 (100 nM). Cell lysates were immunoblotted with anti-Cx43 and with anti- β -actin for internal control.

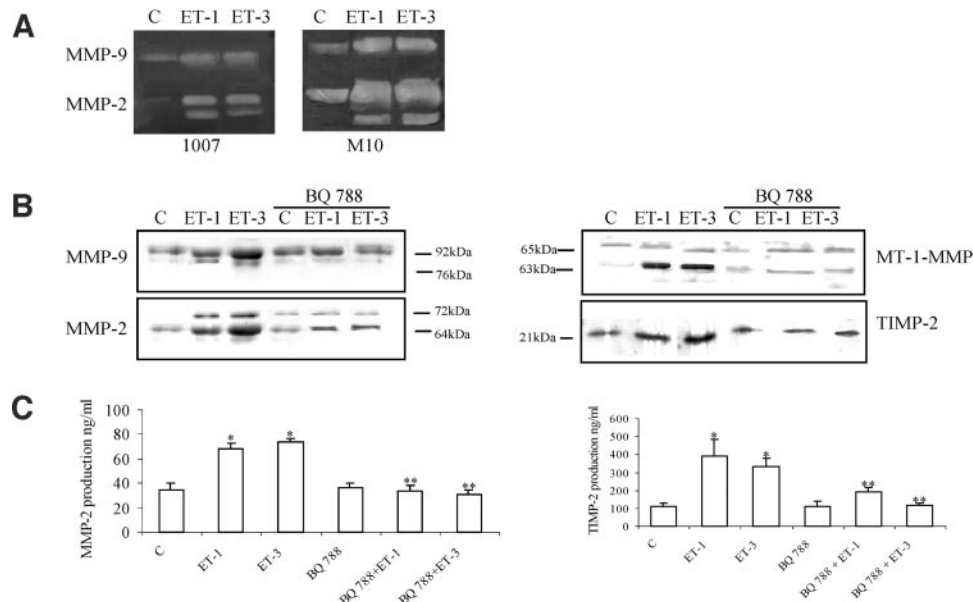


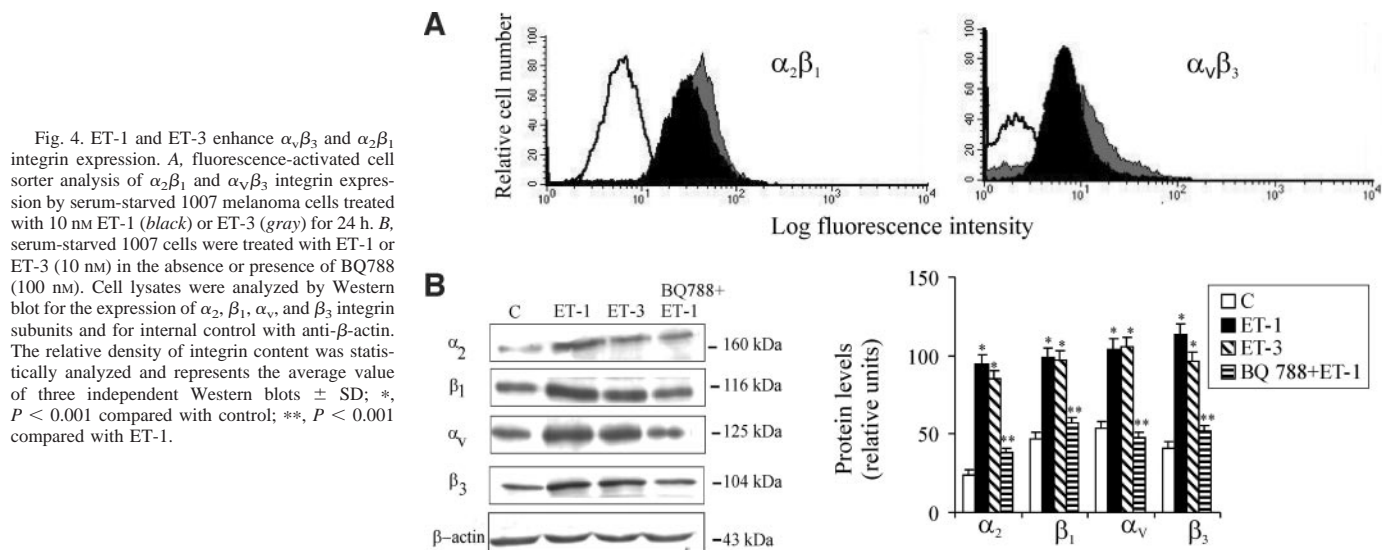
latent MMP-2, MMP-9, and MT1-MMP to their active form and TIMP-2 expression (Fig. 3C), as analyzed by Western blot and ELISA (Fig. 3, B and C). These data indicate that the activation of MMP, involved in a complex proteolytic cascade, is mediated by ET_BR activation.

ET-1 and ET-3 Increase $\alpha_2\beta_1$ and $\alpha_v\beta_3$ Integrin Expression. Because tumor cell invasiveness and metastasis formation depend on cell adhesive properties to the ECM (33–35), we investigated whether ETs were capable of modulating integrin expression on 1007 melanoma cells. Among the integrin subunits evaluated by flow cytometry, the levels of α_1 , α_4 , α_6 , β_5 , and β_6 integrin subunit expression in cells

stimulated with ET-1 and ET-3 (10 nM) for 24 h remained unaltered, whereas the levels of α_v , β_3 , α_2 , and β_1 expression were up-regulated (data not shown). Thus, in 1007 melanoma cells, ET-1 and ET-3 were able to significantly increase the heterodimeric $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrin expression (Fig. 4A), which play a major role in melanoma progression (33–35). These results were confirmed by Western blotting (Fig. 4B), demonstrating that both peptides significantly increased α_v , β_3 , α_2 , and β_1 expression that was blocked by BQ788. These results suggested that ETs through ET_BR activated $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins that could promote rapid adherence and increased motility of melanoma cells.

Fig. 3. ET-1 and ET-3 increase the secretion and activation of MMP-9, MMP-2, and MT1-MMP and the secretion of TIMP-2. **A**, serum-starved 1007 and M10 melanoma cell lines were stimulated for 24 h with 10 nM ET-1 or ET-3. Enzymatic activity of MMP-2 and MMP-9 was studied in conditioned media of melanoma cells by SDS-PAGE gelatin zymography, and gelatin lysis bands show migration positions of pro-MMPs and active MMPs. **B**, serum-starved 1007 cells were stimulated with 10 nM ET-1 or ET-3 for 24 h in the absence or presence of 100 nM BQ788. Conditioned media (MMP-2, MMP-9, and TIMP-2) or cell lysates (MT1-MMP) from melanoma cells were tested for the expression of MMP-9 (the M_r 92,000 proform and the M_r 76,000 active form), MMP-2 (the M_r 72,000 proform and M_r 64,000 active form), and MT1-MMP (the M_r 65,000 proform and the M_r 63,000 active form) and for TIMP-2 (M_r 21,000) by Western blotting. **C**, MMP-2 and TIMP-2 were also measured in conditioned medium from 1007 cells treated with 10 nM ET-1 or ET-3 in the absence or presence of BQ788 (100 nM) for 24 h using ELISA kit. Data are represented as means of results from three experiments each performed in duplicate. Bars indicate \pm SD. *, $P \leq 0.001$ compared with control; **, $P < 0.0001$ compared with ET-1 and ET-3.





ET-1 and ET-3 Induce FAK and MAPK Activation through ET_BR. Several lines of evidence have implicated that Erk and FAK pathways play a critical role in the oncogenic behavior of malignant melanoma (18–20). Because Erk and FAK activation represents a key event in ET-1-induced mitogenic signaling pathway (36), we investigated the effect of ETs on these ET_BR-mediated pathways in melanoma cells. ET-1 and ET-3 (10 nM) treatment resulted in a time-dependent induction of Erk1/2 and FAK phosphorylation, which reached a peak after 5 min and returned to baseline levels by 60 min (Fig. 5A). In presence of the constitutive activation of FAK and Erk1/2 in metastatic melanoma cell lines (18, 19), ET-1 and ET-3 were capable of increasing FAK and Erk1/2 activation in all four cell lines deriving from either primary (1007) or metastatic (M10, SKMel

28, and Mel120) tumors (Fig. 5B). The phosphorylation of Erk1/2 induced by ETs was inhibited by PD98059 (10 μ M), a specific MAPK kinase inhibitor (Fig. 5B). To investigate which receptor subtype mediates FAK and Erk1/2 phosphorylation, BQ123 (100 nM), a specific ET_AR antagonist, and BQ788 (100 nM) were used in the presence or in absence of ET-1 and ET-3 (10 nM). BQ788, but not BQ123, was able to completely block ET-1- and ET-3-induced FAK and Erk1/2 activation (Fig. 5C), indicating that these signaling pathways are mediated through ET_BR. Furthermore, by treating 1007 adherent cells with cytochalasin-D to disrupt actin filaments, a reduced level of FAK phosphorylation after ET-1 and ET-3 (10 nM) stimulation was observed (Fig. 5D), indicating that the integrity of the cytoskeleton is required for ET-1- and ET-3-induced FAK activation in melanoma cells.

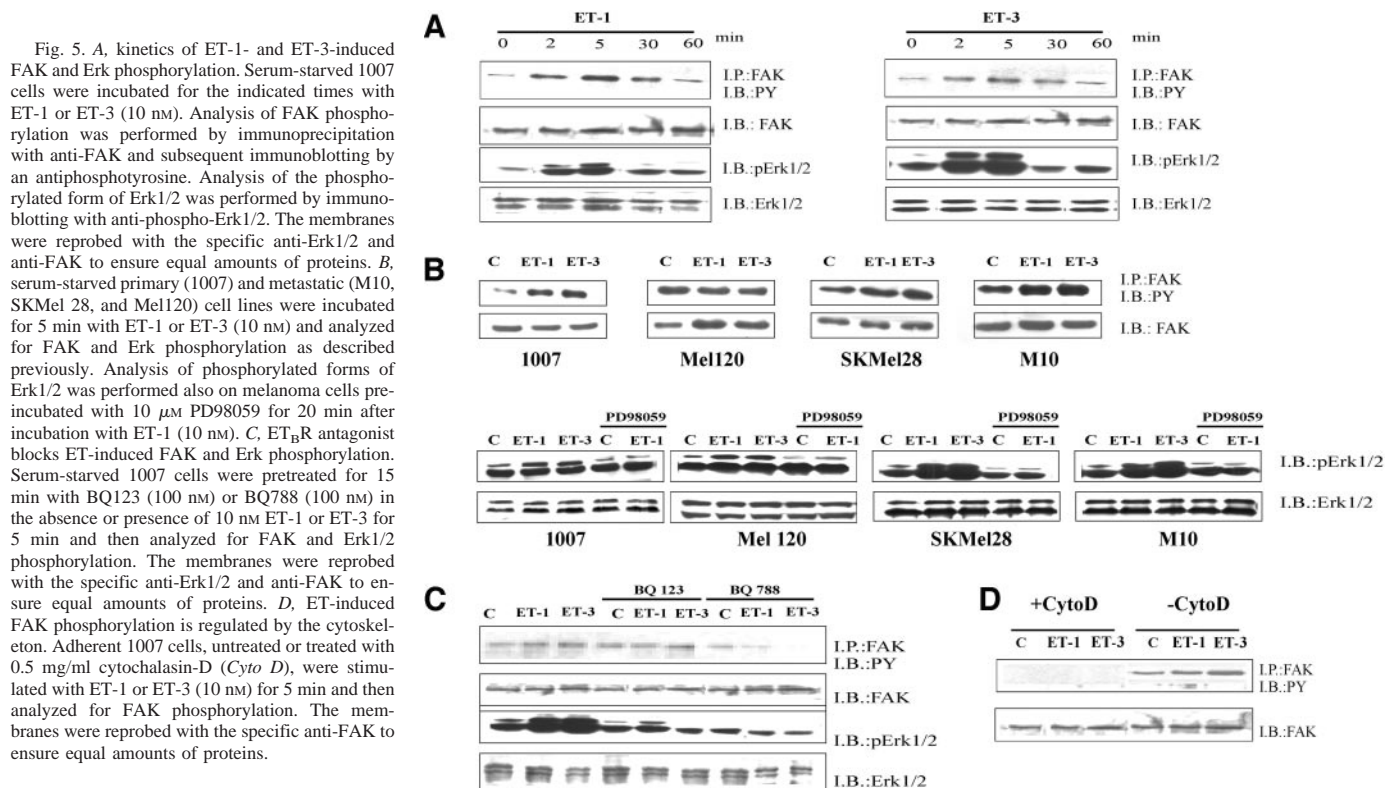


Fig. 5. A, kinetics of ET-1- and ET-3-induced FAK and Erk phosphorylation. Serum-starved 1007 cells were incubated for the indicated times with ET-1 or ET-3 (10 nM). Analysis of FAK phosphorylation was performed by immunoprecipitation with anti-FAK and subsequent immunoblotting by an antiphosphotyrosine. Analysis of the phosphorylated form of Erk1/2 was performed by immunoblotting with anti-phospho-Erk1/2. The membranes were re-probed with the specific anti-Erk1/2 and anti-FAK to ensure equal amounts of proteins. **B**, serum-starved primary (1007) and metastatic (M10, SKMel 28, and Mel120) cell lines were incubated for 5 min with ET-1 or ET-3 (10 nM) and analyzed for FAK and Erk phosphorylation as described previously. Analysis of phosphorylated forms of Erk1/2 was performed also on melanoma cells pre-incubated with 10 μ M PD98059 for 20 min after incubation with ET-1 (10 nM). **C**, ET_BR antagonist blocks ET-induced FAK and Erk phosphorylation. Serum-starved 1007 cells were pretreated for 15 min with BQ123 (100 nM) or BQ788 (100 nM) in the absence or presence of 10 nM ET-1 or ET-3 for 5 min and then analyzed for FAK and Erk1/2 phosphorylation. The membranes were re-probed with the specific anti-Erk1/2 and anti-FAK to ensure equal amounts of proteins. **D**, ET-induced FAK phosphorylation is regulated by the cytoskeleton. Adherent 1007 cells, untreated or treated with 0.5 mg/ml cytochalasin-D (Cyto D), were stimulated with ET-1 or ET-3 (10 nM) for 5 min and then analyzed for FAK phosphorylation. The membranes were re-probed with the specific anti-FAK to ensure equal amounts of proteins.

ET-1 and ET-3 Induce Melanoma Cell Proliferation, Adhesion, Migration, and Invasion through ET_BR. To examine the effects of ETs on cell proliferation, primary (1007) and metastatic (M10, SK-Mel28, and Mel120) cell lines were treated with different concentrations of ET-1 and ET-3. As shown in Fig. 6A, a significant and dose-dependent increase in [³H]thymidine incorporation was observed in all melanoma cell lines tested.

To assess the effect of ET-1 and ET-3 on cell adhesion, 1007 cells were cultured with ET-1 and ET-3 for 24 h, and adhesion to type I collagen-coated surfaces was measured. A significant increase in cell adhesion was observed in ET-1- and ET-3-treated cells relative to control (Fig. 6B). We therefore examined the effect of ET-1 and ET-3 on the functional consequences of enhanced integrin expression and MMP activity on cellular events associated with metastatic spreading. Addition of 1 up to 100 nM ET-1 and ET-3 to melanoma cells induced a marked and dose-dependent increase in cell motility (Fig. 6C) and invasiveness (Fig. 6D). We investigated the ability of anti- $\alpha_2\beta_1$ and anti- $\alpha_v\beta_3$ Abs to interfere with adhesion and migration of 1007 melanoma cells. Abs to the $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins strongly reduced adhesion to type I collagen, as well as cell migration induced by ETs. Moreover, ET-1-stimulated-invasion was reduced to the control level in the presence of a potent chemical broad-spectrum MMP inhibitor, such as Ilomastat (10 μ M), and in the presence of MEK inhibitor, such as PD98059 (10 μ M), demonstrating that ETs are able to induce MMP-dependent invasion through MAPK signaling pathways. The stimulatory effect of 10 nM ET-1 and ET-3 on 1007 cell proliferation, adhesion, migration, and invasion was completely blocked by BQ788, whereas BQ123 only partially inhibited ET-induced effects, indicating ET_BR as a relevant receptor in these events associated with melanoma progression.

A-192621, a Selective ET_BR Antagonist, Inhibits Cell Proliferation and Melanoma Growth in Nude Mice. On the basis of the previous findings, we investigated the effect of the potent A-192621, an orally active nonpeptide ET_BR antagonist (37), on the *in vitro* and *in vivo* growth of M10 cells, which rapidly grow in nude mice. M10 cell line was incubated with ETs and/or with A-192621. The ET-induced proliferation was significantly inhibited in the presence A-192621 (100 nM; $P \leq 0.01$; Fig. 7A). We translated these results into a model of nude mice xenografted with M10 melanoma cells. Treatment with A-192621 produced a 60% inhibition of M10 tumor growth with either low (10 mg/kg/day) or high (20 mg/kg/day) doses (Fig. 7B). A-192621 treatment was generally well tolerated with no signs of acute or delayed toxicity, even at the highest A-192621 dose. Comparison of time course of tumor growth curves by two-way ANOVA with group and time as variables showed that the group-by-time interaction for tumor growth was statistically significant ($P < 0.0001$). Furthermore, the tumor growth inhibition obtained with A-192621 persisted for up to 3 weeks after the termination of the treatment.

DISCUSSION

Progression of cutaneous melanoma requires the accumulation of a variety of phenotypical alterations that free the transformed melanocytes from the control of the surrounding normal microenvironment and enable them to acquire a stable invasive phenotype (15). Due to the resistance of melanoma to current therapies, the identification of molecular mechanisms underlying local and metastatic growth is mandatory for the development of novel treatments. Here, we have investigated whether the G-protein-coupled receptor activated by ETs may represent a relevant therapeutic target in this malignancy.

Loss of interactions between tumor cells and the surrounding normal microenvironment characterizes the progression of a number of

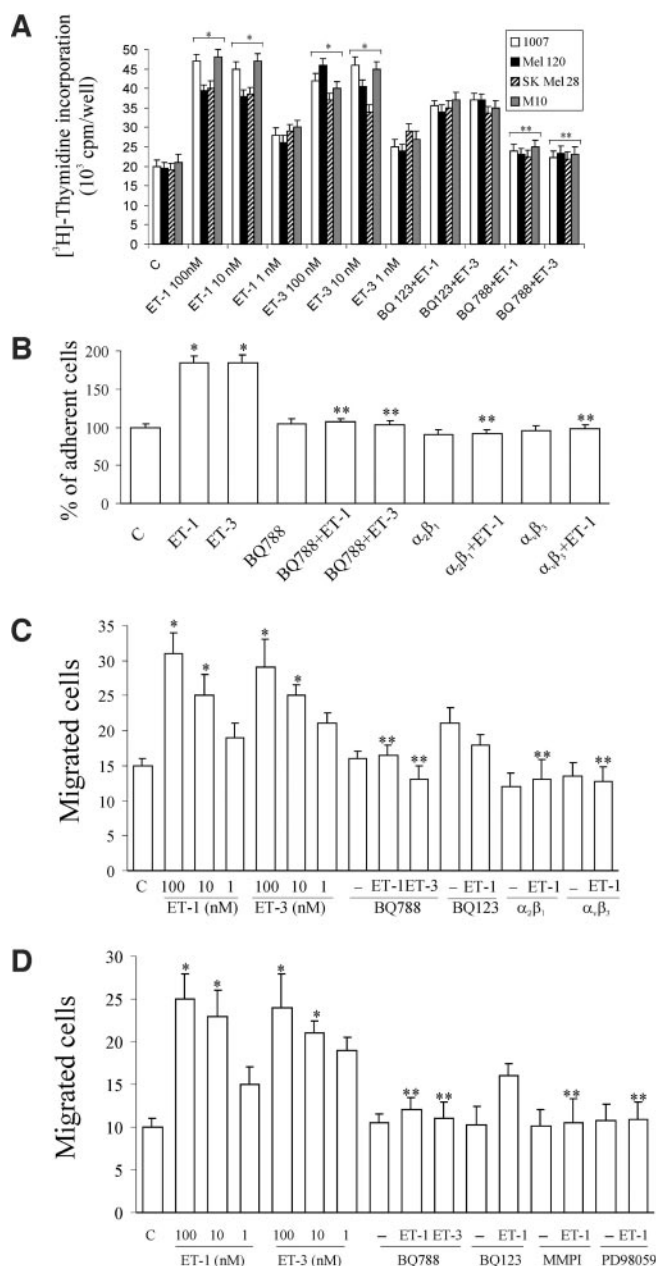


Fig. 6. Modulation of melanoma cell proliferation, adhesion, migration, and invasion by ET-1 and ET-3. A, ETs stimulate cell proliferation. Serum-starved 1007, M10, Mel120, and SKMel28 cells were treated with different concentrations of agonists for 24 h. Cells were treated with ET-1 or ET-3 (10 nM) in the absence or in the presence of BQ788 (100 nM) or BQ123 (100 nM) as indicated. Bars = mean \pm SD of data from three independent experiments each performed in sextuplicate; *, $P \leq 0.0001$ compared with control; **, $P \leq 0.005$ compared with ET-1 or ET-3. B, ET-1 and ET-3 increase cellular adhesion to type I collagen. Serum-starved 1007 cells were cultured for 24 h with ET-1 or ET-3 (10 nM) in the absence or in the presence of BQ788 (100 nM). Inhibition of ET-1-stimulated 1007 cell adhesion to substrate by Abs $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrin (10 μ g/ml) was analyzed. Data represent the means and SD from triplicate experiments each performed in sextuplicate. *, $P \leq 0.0001$ compared with control; **, $P \leq 0.005$ compared with ET-1 or ET-3. C, ET-1 and ET-3 induce melanoma cell migration (C) and invasion (D). Serum-starved 1007 cells (5×10^5 cells/ml) were treated with different doses of ET-1 or ET-3. Cells were treated with ET-1 or ET-3 (10 nM) in the absence or in the presence of BQ788 (100 nM) or BQ123 (100 nM), anti- $\alpha_2\beta_1$ and anti- $\alpha_v\beta_3$ integrins (10 μ g/ml), MMP inhibitor (Ilomastat; 10 μ M), or PD98059 (10 μ M), as indicated. Data represent the means of results from three experiments each performed in triplicate. *, $P \leq 0.001$ compared with control. **, $P \leq 0.001$ compared with ET-1 or ET-3.

malignancies (17). Changes in cell surface adhesion molecules that modulate these interactions are likely to be a prerequisite for invasive growth. The shift in cadherin molecules from the E- to N-isotype has been correlated with tumor cell motility, invasiveness, and capability

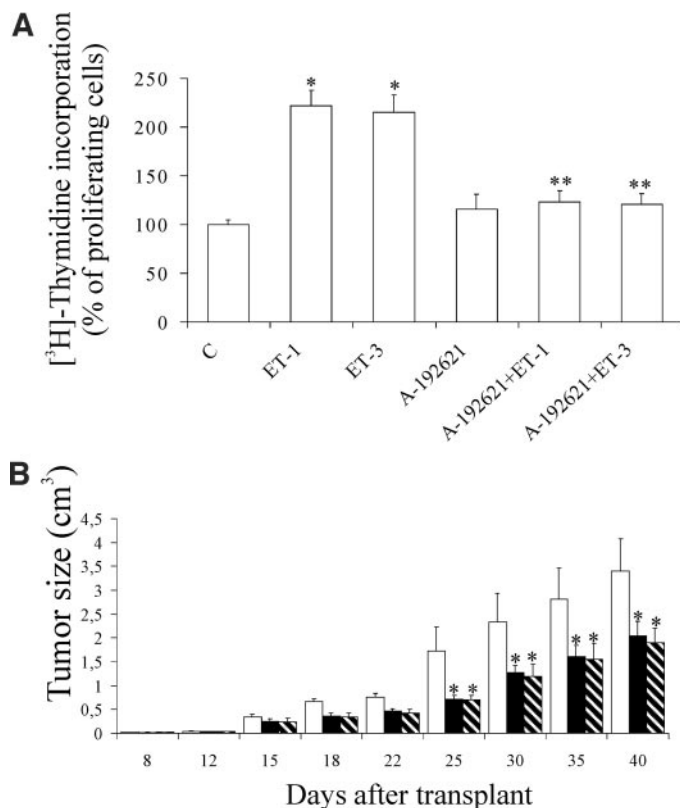


Fig. 7. A-192621, a selective ET_BR antagonist, inhibits cell proliferation and tumor growth *in vivo*. **A**, A-192621 blocks cell proliferation. Ten nM ET-1 or ET-3 was added to serum-starved M10 cells. A-192621 (100 nM) was incubated 15 min before the addition of ETs. Data are means of results from three experiments each performed in sextuplicate. *, $P \leq 0.001$ compared with control. **, $P \leq 0.01$ compared with ET-1 or ET-3. **B**, antitumor activity of ET_BR antagonist treatment on established M10 human melanoma xenografts. Mice received injection s.c. with 1.5×10^6 cells. Seven days after the tumor injection, mice were treated i.p. for 21 days with vehicle or with A-192621 (10 mg/kg/day, ■, or 20 mg/kg/day, ▩). Controls are indicated as □. Three different experiments were performed. Data represent the averages. Bars indicate \pm SD. *, $P < 0.001$.

to interact with wider stromal counterparts in breast carcinoma (38). ET-1-induced down-regulation of E-cadherin, which is responsible for contact-mediated regulatory control by keratinocytes (16), has been described previously. Here, we demonstrate that ET-1 and ET-3 through ET_BR induce down-regulation of E-cadherin and associated catenin proteins, such as β -catenin and p120-catenin, with a parallel up-regulation of N-cadherin. This latter change allows homotypic adhesive contact as well as heterotypic (*i.e.*, fibroblasts, endothelial cells) melanoma cell-cell interactions (15).

Our results now document one pathway regulating transcription of E-cadherin. Consistent with the role of the transcriptional repressor *Snail* in melanoma (27), we demonstrate that ETs induce a significant up-regulation of *Snail* mRNA that concurs with the E-cadherin mRNA down-regulation. In addition, ETs can suppress the adherent junctional function by the tyrosine phosphorylation of β - and p120-catenins, resulting in increased levels of catenin-free pools that cause a decreased cell-cell adhesion.

Of interest, ETs appear to impair the gap junction communication system by inducing a transient and time-dependent reduction of GJIC and Cx43 phosphorylation, enabling melanoma cells to move into new cellular and stromal microenvironments (28).

Remodeling of the immediate ECM, which is a necessary step in tumor local invasion, requires the presence of active MMPs as well as their subcellular redistribution. With regard to melanoma, ET-1 can induce MMP secretion and activation as result of increased expression and activation of MT1-MMP and the concomitant up-regulation of

TIMP-2 (32). Melanoma cell invasiveness relies on a promigratory subset of cells at the tumor leading edge, which is characterized by a molecular program capable of silencing neighboring cells via cell-to-cell signaling and concomitant polarization of $\beta 1$ -integrin expression (39). Melanoma cells at the leading margin engage and cluster $\beta 1$ -integrins in anterior protrusions and show an increased expression and activity of MT1-MMP and MMP-2, leading to polarized ECM degradation and a collective movement of migration and invasion (15). The present study demonstrates that ETs are also capable of up-regulating $\beta 1$ integrin, MT1-MMP, and MMP-2 expression, which is accompanied by increased rates of adhesion to ECM molecules and by increased migratory and invasive capacity. Moreover, in all of the melanoma cell lines tested ET-1 and ET-3 activated, through the ET_BR, FAK, and ERK, which are the signaling pathways claimed to contribute to the high metastatic potential of this tumor (18–20). Consistent with the role of ERK in regulating invasive activity, treatment of melanoma cells with the MEK inhibitor PD98059 reduced the MMP-dependent matrigel invasion of 1007 cells.

The present findings and a recent gene array profiling of melanoma has identified that ET_BR is a new marker associated with multiple aggressive phenotypes including the plasticity of melanoma cells to engage in vasculogenic mimicry (11). Screening a large series of benign and malignant pigment cell lesions from different progression stages using immunohistochemistry and quantitative reverse transcription-PCR analysis reveals ET_BR as a tumor progression marker in malignant melanoma and points to ET_BR as clinically relevant target especially in consideration of the development of small molecules capable of antagonizing ETs. Blockade of ET_BR by the peptide antagonist, BQ788, in fact has been demonstrated to inhibit melanoma cell growth *in vivo* and *in vitro* (14). In view of the potential use in clinical settings, we showed that the specific nonpeptidic orally active ET_BR antagonist A-192621 displays antitumor activity against established melanoma expressing ET_BR. In conclusion, we have identified multiple molecular pathways elicited by ET-1 and ET-3 that regulate melanoma local and metastatic growth. Because all of the molecular effectors involved in melanoma progression including cell-cell adhesion and cell-cell communication molecules, tumor proteases, and integrins are triggered by the ET_BR activity, blockade of this receptor by small molecules results in inhibition of melanoma growth *in vitro* and *in vivo*, thus offering the possibility of exploring targeted therapy in this malignancy. In view of known resistance of melanoma to current therapies, this knowledge may be of clinical relevance to assess the extent to which ET_BR blockade can be exploited in integrated treatments.

ACKNOWLEDGMENTS

We warmly acknowledge our lost friend Raffaele Tecce for having pioneered this study. We are grateful to Dr. Perry Nisen of Abbott Global Oncology Development for kindly providing A-192621 and to Giacomo Elia, Rocco Fraioli, and Maria V. Sarcone for excellent technical and secretarial assistance.

REFERENCES

- Shin, M. K., Levorso, J. M., Ingram, R. S., and Tilghman, S. M. The temporal requirement for endothelin receptor-B signalling during neural crest development. *Nature (Lond.)*, 402: 496–501, 1999.
- Herlyn, M., Berking, C., Li, G., and Satyamoorthy, K. Lessons from melanocyte development for understanding the biological events in naevus and melanoma formation. *Melanoma Res.*, 10: 303–312, 2000.
- Levin, E. R. Endothelins. *N. Engl. J. Med.*, 333: 356–363, 1995.
- Reid, K., Turnley, A. M., Maxwell, G. D., Kurihara, Y., Kurihara, H., Bartlett, P. F., and Murphy, M. Multiple roles for endothelin in melanocyte development: regulation of progenitor number and stimulation of differentiation. *Development*, 122: 3911–3919, 1996.

5. Scott, G., Cassidy, L., and Abdel-Malek, Z. α -Melanocyte-stimulating hormone and endothelin-1 have opposing effects on melanocyte adhesion, migration, and pp125FAK phosphorylation. *Exp. Cell Res.*, *237*: 19–28, 1997.
6. Imokawa, G., Yada, Y., and Miyagishi, M. Endothelins secreted from human keratinocytes are intrinsic mitogens for human melanocytes. *J. Biol. Chem.*, *267*: 24675–24680, 1992.
7. Tada, A., Suzuki, I., Im, S., Davis, M. B., Cornelius, J., Babcock, G., Nordlund, J. J., and Abdel-Malek, Z. A. Endothelin-1 is a paracrine growth factor that modulates melanogenesis of human melanocytes and participates in their responses to ultraviolet radiation. *Cell Growth & Differ.*, *9*: 575–584, 1998.
8. Imokawa, G., Miyagishi, M., and Yada, Y. Endothelin-1 as a new melanogen: coordinated expression of its gene and the tyrosinase gene in UVB-exposed human epidermis. *J. Invest. Dermatol.*, *105*: 32–37, 1995.
9. Nelson, J., Bagnato, A., Battistini, B., and Nisen, P. The endothelin axis: emerging role in cancer. *Nat. Rev. Cancer*, *3*: 110–116, 2003.
10. Yohn, J. J., Smith, C., Stevens, T., Hoffman, T. A., Morelli, J. G., Hurt, D. L., Yanagisawa, M., Kane, M. A., and Zamora, M. R. Human melanoma cells express functional endothelin-1 receptors. *Biochem. Biophys. Res. Commun.*, *201*: 449–457, 1994.
11. Bittner, M., Meltzer, P., Chen, Y., Jiang, Y., Sefror, E., Hendrix, M., Radmacher, M., Simon, R., Yakhini, Z., Ben-Dor, A., *et al.* Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature (Lond.)*, *406*: 536–540, 2000.
12. Demunter, A., De Wolf-Peeters, C., Degreef, H., Stas, M., and van den Oord, J. J. Expression of the endothelin-B receptor in pigment cell lesions of the skin: evidence for its role as tumor progression marker in malignant melanoma. *Virchows Arch.*, *438*: 485–491, 2001.
13. Eberle, J., Fecker, L. F., Orfanos, C. E., and Geilen, C. C. Endothelin-1 decreases basic apoptotic rates in human melanoma cell lines. *J. Invest. Dermatol.*, *119*: 549–555, 2002.
14. Lahav, R., Heffner, G., and Patterson, P. H. An endothelin receptor B antagonist inhibits growth and induces cell death in human melanoma cells *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. USA*, *96*: 11496–11500, 1999.
15. Li, G., Satyamoorthy, K., Meier, F., Berking, C., Bogenrieder, T., and Herlyn, M. Function and regulation of melanoma-stromal fibroblast interactions: when seeds meet soil. *Oncogene*, *22*: 3162–3171, 2003.
16. Hsu, M., Andl, T., Li, G., Meinkoth, J. L., and Herlyn, M. Cadherin repertoire determines partner-specific gap junctional communication during melanoma progression. *J. Cell Sci.*, *113*: 1535–1542, 2000.
17. Rüter, D., Bogenrieder, T., Elder, D., and Herlyn, M. Melanoma-stroma interactions: structural and functional aspects. *Lancet Oncol.*, *3*: 35–43, 2002.
18. Kahana, O., Micksche, M., Witz, I. P., and Yron, I. The focal adhesion kinase (P125FAK) is constitutively active in human malignant melanoma. *Oncogene*, *21*: 3969–3977, 2002.
19. Satyamoorthy, K., Li, G., Gerrero, M. R., Brose, M. S., Volpe, P., Weber, L. B., van Belle, P., Elder, D. E., and Herlyn, M. Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. *Cancer Res.*, *63*: 756–759, 2003.
20. Smalley, K. S. M. A pivotal role for ERK in the oncogenic behaviour of malignant melanoma. *Int. J. Cancer*, *104*: 527–532, 2003.
21. Remuzzi, G., Perico, N., and Benigni, A. New therapeutics that antagonize endothelin: promises and frustrations. *Nat. Rev. Drug Discovery*, *1*: 986–1001, 2002.
22. Golub, S. H., Hanson, D. C., Morton, D. L., Pellegrino, M. A., Sulit, H. L., and Ferrone, S. Comparison of histocompatibility antigens on cultured human tumor cells and fibroblasts by quantitative antibody absorption and sensitivity to cell-mediated cytotoxicity. *J. Natl. Cancer Inst. (Bethesda)*, *58*: 167–171, 1976.
23. Bagnato, A., Salani, D., Di Castro, V., Wu-Wong, J. R., Tecce, R., Nicotra, M. R., Venuti, A., and Natali, P. G. Expression of endothelin-1 and endothelin A receptor in ovarian carcinoma: evidence for an autocrine role in tumor growth. *Cancer Res.*, *59*: 720–727, 1999.
24. Rosanò, L., Varmi, M., Salani, D., Di Castro, V., Spinella, F., Natali, P. G., and Bagnato, A. Endothelin-1 induces tumor proteinase activation and invasiveness of ovarian carcinoma cells. *Cancer Res.*, *61*: 8340–8346, 2001.
25. Hsu, M. Y., Meier, F. E., Nesbit, M., Hsu, J. Y., Van Belle, P., Elder, D. E., and Herlyn, M. E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors. *Am. J. Pathol.*, *156*: 1515–1525, 2000.
26. Jamal, S., and Schneider, R. J. UV-induction of keratinocyte endothelin-1 downregulates E-cadherin in melanocytes and melanoma cells. *J. Clin. Investig.*, *110*: 443–452, 2002.
27. Poser, I., Dominguez, D., Garcia de Herreros, G., Varnai, A., Buettner, R., and Bosserhoff, A. K. Loss of E-cadherin expression in melanoma cells involves upregulation of the transcriptional repressor Snail. *J. Biol. Chem.*, *276*: 24661–24666, 2001.
28. Müller, T., Choida, A., Reichmann, E., and Ullrich, A. Phosphorylation and free pool of β -catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration. *J. Biol. Chem.*, *274*: 10173–10183, 1999.
29. Rivedal, E., and Opsahl, H. Role of PKC and MAP kinase in EGF- and TPA-induced connexin43 phosphorylation and inhibition of gap junction intercellular communication in rat liver epithelial cells. *Carcinogenesis*, *22*: 1543–1550, 2001.
30. Spinella, F., Rosanò, L., Di Castro, V., Nicotra, M. R., Natali, P. G., and Bagnato, A. Endothelin-1 decreases gap junctional intercellular communication by inducing phosphorylation of connexin 43 in human ovarian carcinoma cells. *J. Biol. Chem.*, *278*: 41294–41301, 2003.
31. Brinckerhoff, C. E., and Matrisian, L. M. Matrix metalloproteinases: a tail of a frog became a prince. *Nat. Rev. Mol. Cell Biol.*, *3*: 207–214, 2002.
32. Bernardo, M. M., and Fridman, R. TIMP-2 (tissue inhibitor of metalloproteinase-2) regulates MMP-2 (matrix metalloproteinase-2) activity in the extracellular environment after pro-MMP-2 activation by MT1 (membrane type1)-MMP. *Biochem. J.*, *374*: 739–745, 2003.
33. Natali, P. G., Nicotra, M. R., Bartolazzi, A., Cavaliere, R., and Bigotti, A. Integrin expression in cutaneous malignant melanoma: association of the $\alpha 3 \beta 1$ heterodimer with tumor progression. *Int. J. Cancer*, *54*: 68–72, 1993.
34. Natali, P. G., Hamby, C. V., Felding-Habermann, B., Liang, B., Nicotra, M. R., Di Filippo, F., Giannarelli, D., Temponi, M., and Ferrone, S. Clinical significance of $\alpha(v)\beta 3$ integrin and intercellular adhesion molecule-1 expression in cutaneous malignant melanoma lesions. *Cancer Res.*, *57*: 1554–1560, 1997.
35. Eguchi, H., and Horikoshi, T. The expression of integrin $\alpha 2 \beta 1$ and attachment to type I collagen of melanoma cells are preferentially induced by tumour promoter, TPA (12-*O*-tetradecanoyl phorbol-13-acetate). *Br. J. Dermatol.*, *134*: 33–39, 1996.
36. Bagnato, A., Tecce, R., Di Castro, V., and Catt, K. J. Activation of mitogenic signaling by endothelin-1 in ovarian carcinoma cells. *Cancer Res.*, *57*: 1306–1311, 1997.
37. Griswold, D. E., Douglas, S. A., Martin, L. D., Davis, T. G., Davis, L., Ao, Z., Luttmann, M. A., Pullen, M., Nambi, P., Hay, D. W., *et al.* Endothelin B receptor modulates inflammatory pain and cutaneous inflammation. *Mol. Pharmacol.*, *56*: 807–812, 1999.
38. Hazan, R. B., Phillips, G. R., Qiao, R. F., Norton, L., and Aaronson, S. A. Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. *J. Cell. Biol.*, *148*: 779–790, 2000.
39. Hegerfeldt, Y., Tusch, M., Brocker, E. B., and Friedl, P. Collective cell movement in primary melanoma explants: plasticity of cell-cell interaction, $\beta 1$ -integrin function, and migration strategies. *Cancer Res.*, *62*: 2125–2130, 2002.