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β-adrenoceptors are upregulated in human melanoma and their activation releases pro-tumorigenic cytokines and metalloproteases in melanoma cell lines

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Recent studies sight β-adrenergic receptor (AR) antagonists as novel therapeutic agents for melanoma, as they may reduce disease progression. Here within, we evaluated the expression of β-ARs in a series of human cutaneous melanocytic lesions, and studied the effect of their endogenous agonists, norepinephrine (NE) and epinephrine (E), on primary and metastatic human melanoma cell lines. Using immunohistochemistry, we found that both β1- and β2-ARs are expressed in tissues from benign melanocytic naevi, atypical naevi and malignant melanomas and that expression was significantly higher in malignant tumours. Melanoma cell lines (human A375 primary melanoma cell line and human Hs29-4T metastatic melanoma cell lines) also expressed β1- and β2-ARs by measuring transcripts and proteins. NE or E increased metalloprotease-dependent motility, released interleukin-6 and 8 (IL-6, IL-8) and vascular endothelial growth factor (VEGF). These effects of catecholamines were inhibited by the unselective β-AR antagonist propranolol. The role of soluble factors elicited by catecholamines seemed pleiotropic as VEGF synergized with NE increased melanoma invasiveness through 3D barriers, while IL-6 participated in stromal fibroblast activation towards a myofibroblastic phenotype. Our results indicate that NE and E produce *in vitro via* β-ARs activation a number of biological responses that may exert a pro-tumorigenic effect in melanoma cell lines. The observation that β-ARs are upregulated in malignant melanoma tissues support the hypothesis that circulating catecholamines NE and E, by activating their receptors, favour melanoma progression *in vivo*.

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Melanoma represents the most aggressive type of skin cancer, with an increasing incidence found especially in young adults. A significant reduction in mortality has been not observed, despite a noteworthy improvement in early diagnosis achieved in recent years.[1] At present, no medical option can cure metastatic melanoma (MM) and the only effective treatment for the eradication of the disease is early-phase surgery.[2] Hence, increased knowledge of the biological pathways underlying the process of melanoma dissemination and metastasis is crucial in order to identify new therapeutic targets.

Previous studies have shown that various human solid tumours, such as breast, colon, prostatic, ovary, nasopharyngeal and oral cancer, express β2-adrenoceptor (β2-AR), raising the possibility that such receptors may affect invasion and dissemination processes.[3–8] Moreover, some stress neurotransmitters, such as norepinephrine (NE) and epinephrine (E), have been demonstrated to contribute to the regulation of tumour cell invasion, at least in part through β-AR activation.[6,7,9] Interactions between tumour cells and soluble factors originated from the nervous system has recently been proposed to favour metastasis formation.[10] Improved survival rates have been demonstrated in mice with metastatic tumour by combined administration of β-AR antagonists.[11] In addition, recent evidence suggests a dramatic role of β-AR blockers in reducing metastases, tumour

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recurrence and specific mortality in breast cancer patients.[12] More recently, the use of β-blockers for concomitant disease was associated with a reduced risk of progression of thick melanoma[13] and with an increased survival time of melanoma patients,[14] suggesting that the interaction of catecholamines with β-ARs could be a useful target in this disease.

Expression of β-ARs has been found in melanoma cell lines and in human melanoma biopsies and NE was demonstrated to enhance cytokine production from melanoma cells.[15] However, no detailed information regarding β-ARs expression in human cutaneous benign and malignant melanocytic lesions or catecholamine influence on melanoma cell migration has been provided so far. Our aim was to evaluate the expression of β-ARs on a series of human cutaneous melanocytic naevi and malignant melanoma, and assess the potential influence of NE and E on the malignant behaviour of human melanoma cell lines. We could demonstrate a significant upregulation of β-ARs expression in melanoma *in vivo* and the activation of pro-tumorigenic biological responses induced by NE and E *in vitro*.

MATERIALS AND METHODS

Histologic Samples

Forty human cutaneous melanocytic lesions from 40 different patients were evaluated. Tissue samples were retrieved from the archives of the Division of Pathological Anatomy, Department of Critical Care Medicine and Surgery, University of Florence, Florence, and from the Division of Pathology, Pistoia Hospital, Pistoia, Italy.

The study series included five common melanocytic naevi (CN) (two females, three males, age 28–54 years, mean 35.8 years); five atypical (so-called dysplastic) melanocytic naevi (AN) (two females, three males, age 30–47 years, mean 40.4 years); five *in situ* primary melanoma (PM) (two females, three males, age 37–55 years, mean 44.2 years; site: three trunk, one lower extremity); nine superficial spreading (SS) PM (seven females, two males, age 41–82 years, mean 58.4 years; site: four trunk, three leg, two arm; thickness 0.30–1.90 mm, mean 0.82 mm; five level II, three level III); six nodular (N) PM (three females, three males, age 53–76 years, mean 61.5 years; site: three trunk, one leg, two arm; thickness 1.40–17 mm, mean 5.2 mm; two level III, three level IV, one level V), ten MM, five cutaneous and five lymph-nodal (one female, nine males, age 59–87 years, mean 77.1 years).

Materials

Rabbit polyclonal anti-β1- or anti-β2-AR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Chemicon (Temecula, CA, USA), strepatdvidin-biotin peroxidase complex from Ultravision (LabVision, Fremont, CA, USA), DAKO EnVision System HRP from Dako (Carpenteria, CA, USA). For *in vitro* experiments, unless specified all reagents were obtained from Sigma (St Louis, MO) except PVDF membrane (Millipore, Bedford, MA); Matrigel (BD Biosciences, Bedford, MA); Diff-Quik staining kit (Medion Diagnostics, Miami, FL); Transwell (Corning Incorporated, Corning, NY); ilomastat (Chemicon International, Bedford, MA); p38 MAPK (mitogen-activated protein kinase), phospho-p38 MAPK (Thr180/Tyr182), p44/p42 MAPK and phospho-p44/p42 (T202/Y204) monoclonal antibodies (Cell Signalling, Danvers, MA). The Amplite TM Universal Fluorimetric matrix metalloproteinase (MMP) Activity Assay Kit-Red Fluorescence was supplied by AAT Bioquest, Sunnyvale, CA.

Immunohistochemistry

The specimens were obtained by surgical resection in all cases and fixed in 10% formalin before being processed in paraffin. Haematoxylin-eosin stained sections from each histological specimen were reviewed to confirm the histological diagnosis. The protocol was approved by the Institutional Review Board for use of human tissues.

For immunohistochemical analyses, a representative section of 3 μm for each lesion was selected. All sections were deparaffined in Bio-Clear (Bio-Optica, Mi, Italy) and hydrated with grade ethanol concentrations until distilled water. Antigen retrieval was performed by calibrated water bath capable of maintaining the Epitope Retrieval Solution EDTA (pH 9.0) at 97 °C for 15 min. The sections were then allowed to cool down to room temperature (RT) for 20 min. To block endogenous peroxidase activity, slides were treated with 3.0% hydrogen peroxidase in distilled water for 10 min and subsequently washed two or three times with PBS. Then polyclonal antibodies anti-β1- or anti-β2-AR were incubated for 1 h at RT at 1:100 dilution or at 1:30 dilution, respectively. Immunohistochemical analysis was performed using the streptavidin-biotin peroxidase complex for β2-AR, or DAKO EnVision System HRP for β1-AR. Finally, aminoethilcarbazole (LabVision) was applied for 5 min as chromogen.

Normal eccrine sweat glands intensely express β2-AR, and this parameter was used as a positive internal control.[16] Negative control was performed by substituting the primary antibody with a non-immune serum at the same concentration. The control sections were treated in parallel with the samples. The sections were lightly counterstained with Mayer's haematoxylin.

Immunostaining was independently assessed by two observers (DM, SM). Discrepancies in the reading were resolved by a second parallel reading of the slides. The percentage of positive cells per lesion was scored according to semi-quantitative criteria. As the percentage of positive naevus melanocytes/melanoma cells was always higher than 50%, semi-quantitative results were expressed as score 1 (50–80% positive naevus melanocytes/melanoma cells), score 2 (81–90% naevus melanocytes/melanoma cell staining) and score 3 (91–100% melanoma cell staining). The cell staining intensity was scored on a scale as weak, moderate, strong, very strong.

For statistical analysis, non-parametric tests were used to determine significant differences between groups. The distribution of the scored values of positive lesions after immunological staining in each group was the unit of analysis. Groups were: naevi, PM, MM. Statistical evaluation was performed also comparing CN *vs* AN, naevi *vs* malignant lesions, and *in situ* PM plus SSPM *vs* NPM and MM. Differences were assessed using the non-parametric Mann–Whitney *U*-test for independent samples and were considered significant at $P \leq 0.05$.

Cell Lines and Culture Conditions

Human dermal fibroblasts (HDFs) isolated from a surgical explantation taken from healthy patients, human A375 PM cell line and human Hs29-4T MM cell line were cultivated in DMEM supplemented with 10% FCS at 37 °C in a 5% CO_2 humidified atmosphere. All experiments were performed with 70–80% confluent cultures, following 18-h incubation in serum-free culture medium. Cells were then stimulated with NE or E, at 1 μM concentration. Where needed, cells were pre-treated with unselective β-AR antagonist propranolol (Sigma-Aldrich) (1 μM). After 1 h, medium was removed and cells were stimulated with NE 1 μM with or without propranolol 1 μM. For fibroblast activation, cells were grown to sub-confluence and treated for 24 h with the indicated cytokines. Fresh serum-free medium was added for an additional 24 h before collection of conditioned medium (CM), in order to obtain CM free from cytokines (but conditioned by their earlier administration). HDFs cells were then incubated with the obtained CM for 24 h and then used for western blot analyses.

Western Blot Analysis

Cells were lysed for 20 min on ice in 500 μl of complete RIPA lysis buffer (50 mmol/l Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1% NP40, 2 mmol/l EGTA, 1 mmol/l sodium orthovanadate, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Lysates were clarified by centrifuging, separated by SDS-PAGE and transferred onto nitrocellulose. The immunoblots were incubated in 3% bovine serum albumin, 10 mmol/l Tris-HCl (pH 7.5), 1 mmol/l EDTA and 0.1% Tween 20 for 1 h at RT and were probed first with specific antibodies and then with secondary antibodies. For chemiluminescence detection, we used Gel Logic 2002 Kodak Imaging System, equipped with a charge-coupled device camera, which guarantees high linearity. Quantity One software (Bio-Rad) was used to obtain quantitative analyses.

Invasion Assay

Transwell system, equipped with 8-μm pore polyvinylpirrolidone-free polycarbonate filters, was used. Cells (5×10^4 in 300 μl) were loaded into the upper compartment in serum-free growth medium with or without 50 μmol/l ilomastat. The upper sides of the porous polycarbonate filters were coated with 50 μg/cm^2 of reconstituted Matrigel basement membrane and placed into six-well culture dishes containing 1 ml of complete growth medium. After 24 h of incubation at 37 °C, noninvading cells and the Matrigel layer were mechanically removed using cotton swabs, and the microporous membrane was fixed in 96% methanol and stained with Diff-Quick solution. Chemotaxis was evaluated by counting the cells that migrated to the lower surface of the polycarbonate filters (six randomly chosen fields, mean ± s.d.).

Real-Time PCR

Total RNA was extracted from Hs29-4T and A375 derived from our experimental conditions using the RNeasy Minikit kit. Total RNA (1 μg) was reverse-transcribed using the Quantitect Reverse Transcription Kit. Reverse transcription was performed in a final volume of 20 μl containing reverse transcriptase, real-time buffer 1 × and real-time primer mix. The amplification was carried out at 42 °C for 2 min, then 42 °C for 15 min and 95 °C for 3 min. Measurement of gene expression was performed by quantitative real-time PCR (7500 Fast Real-Time PCR System, Applied Biosystems), using the Qiagen Quantifast SYBR Green PCR kit. For each sample, 1 μg of cDNA was added to 25 μl of PCR mix. The samples were then subjected to 40 cycles of amplification at 95 °C for 10 s and 60 °C for 30 s. RNeasy Minikit, Quantitect Reverse Transcription Kit, all primer/probe mixes and Qiagen Quantifast SYBR Green PCR Kit were from Qiagen, except the following primers:

ADRB1 FW: 5′-CAGGTGAACTCGAAGCCCAC-3′
ADRB1 REV: 5′-CTCCCATCCCTTCCCAAACT-3′
ADRB2 FW: 5′-ACGCAGTGCGCTCACCTGCCAGACT-3′
ADRB2 REV: 5′-GCTCGAACTTGGCAATGGCTGTGA-3′
VEGF FW: 5′-TACCTCCACCATGCCAAGTG-3′
VEGF REV: 5′-ATGATTCTCCCTCCTCCTTC-3′
IL-8 FW: 5′-CTGGCCGTGGCTCTCTTG-3′
IL-8 REV: 5′-TTAGCACTCCTTGGCAAAACTG-3′
MMP-2 FW: 5′-ACGACCGCGACAAGAAGTTAT-3′
MMP-2 REV: 5′-ATTTGTTGCCCAGGAAAGTG-5′ (NM_00453; Digestive and liver disease 37 (2005) 584–592)
MMP-9 FW: 5′-GACAAGCTCTTCGGCTTCTG-3′
MMP-9 REV: 5′-TCGCTGGTACAGGTCGAGTA-5′
IL-6 FW: 5′-AGTTCCTGCAGTCCAGCC-3′
IL-6 REV: 5′-TCAAACTGCATAGCCACTTTC-3′.

Quantitative MMP Activity Assay

MMPs activity was measured with Amplite TM Universal Fluorimetric MMP Activity Assay Kit according to the manufacturer's instructions. Briefly, serum-free medium from confluent monolayer of cells was collected and 5 μl were added to 4-aminophenylmercuric acetate (AMPA; 1 nmol/l) at 37 °C for 1 h to detect MMP-2 activity and at 37 °C for 2 h to detect MMP-9 activity. A 50 μl portion of the mixture was then added to 50 μl of MMP Red substrate solution. After 60 min of incubation the signal was read by fluorescence

microplate reader with excitation (Ex)/emission (Em) = 540 nm/590 nm.

Statistical Analysis

In vitro data are presented as means ± s.d. from at least three experiments. Results were normalized *vs* control expression levels. Statistical analysis of the data were performed by Student's *t*-test. $P \leq 0.05$ was considered statistically significant.

RESULTS

Expression of β-ARs in Tissue Samples

The presence of β-ARs was demonstrated in all melanocytic lesions examined. The staining for β1-AR was confined to the cell cytoplasm in naevus melanocytes and melanoma cells; the reaction for β2-AR was also confined to the cell cytoplasm in all cases, with an additional peripheral membrane pattern in some AN melanocytes and malignant cells. The cell staining intensity was always weak with regard to the reaction for β1-AR, whereas the immunostaining for β2-AR appeared to be weak in CN, moderate (except one very-strong reaction), in AN; moderate or strong in *in situ* PM; from weak to moderate or strong in SSPM, and from strong to very strong in NPM and MM, with no difference between cutaneous and nodal metastasis.

The immunostaining of each lesion taking into account both reaction intensity and percentage of positive cells is shown in Figure 1. In regards to β1-AR expression (Figure 1a), score 1 was evaluated in both CN and AN, score 2 was found in a minority (3 *in situ* and 2 SS) of PM, whereas in the other PM and MM score 3 was detected. β1-AR expression was significantly higher in malignant than in benign lesions ($P \leq 0.0001$) and in PM or MM than in naevi ($P \leq 0.0001$ and $P \leq 0.0001$). No difference was observed between CN and AN, or between *in situ*/SSPM compared with NPM/MM.

With regards to β2-AR expression (Figure 1b), score 1 was observed in CN, score 2 in AN and score 3 was detected in all PM and MM but one (SSPM), which exhibited score 2. β2-AR reactivity was significantly higher in malignant lesions than in naevi ($P \leq 0.0001$), and in PM or MM, respectively, than in naevi ($P \leq 0.0001$ and $P \leq 0.0001$). AN exhibited a significantly higher reactivity compared with CN ($P \leq 0.003$), and no difference was observed between *in situ*/SSPM and NPM/MM.

In addition, no significant difference was detected between PM and MM for both receptors.

Examples of reactions of melanocytic lesions for β1- and β2-AR are shown in Figure 2. Epidermal keratinocytes were lightly coloured for β2-AR, as previously described.[17] Endothelial and stromal cells exhibited heavy reactivity for β2-AR in malignant lesions, and to some extent, in AN.

Taken together, our data showed that β1- and β2-ARs were variably expressed in human melanocytic lesions with a significant upregulation in PM and MM, and, at least for β2-AR, a significant upregulation was also observed in AN *vs* CN.

Effects of Catecholamines on Cancer Cell Motility

In order to confirm the correlation between sensitivity to catecholamines and progression towards a malignant phe-

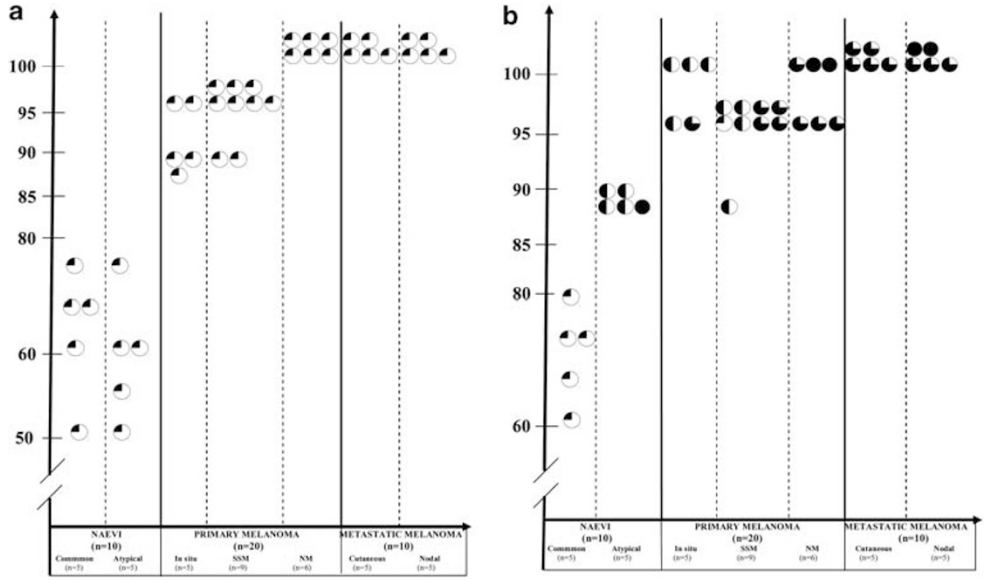

Figure 1 Immunohistochemical expression of β1-AR (**a**) and β2-AR (**b**) in cutaneous human melanocytic lesions: percentage of positivity and staining intensity in each lesion. Each circle represents the percentage of stained cells for one lesion. A quarter-black circle indicates positive weak staining; a half-black circle indicates positive moderate staining; a three-quarter-black circle indicates positive strong staining; a solid-black circle indicates very-strong staining.

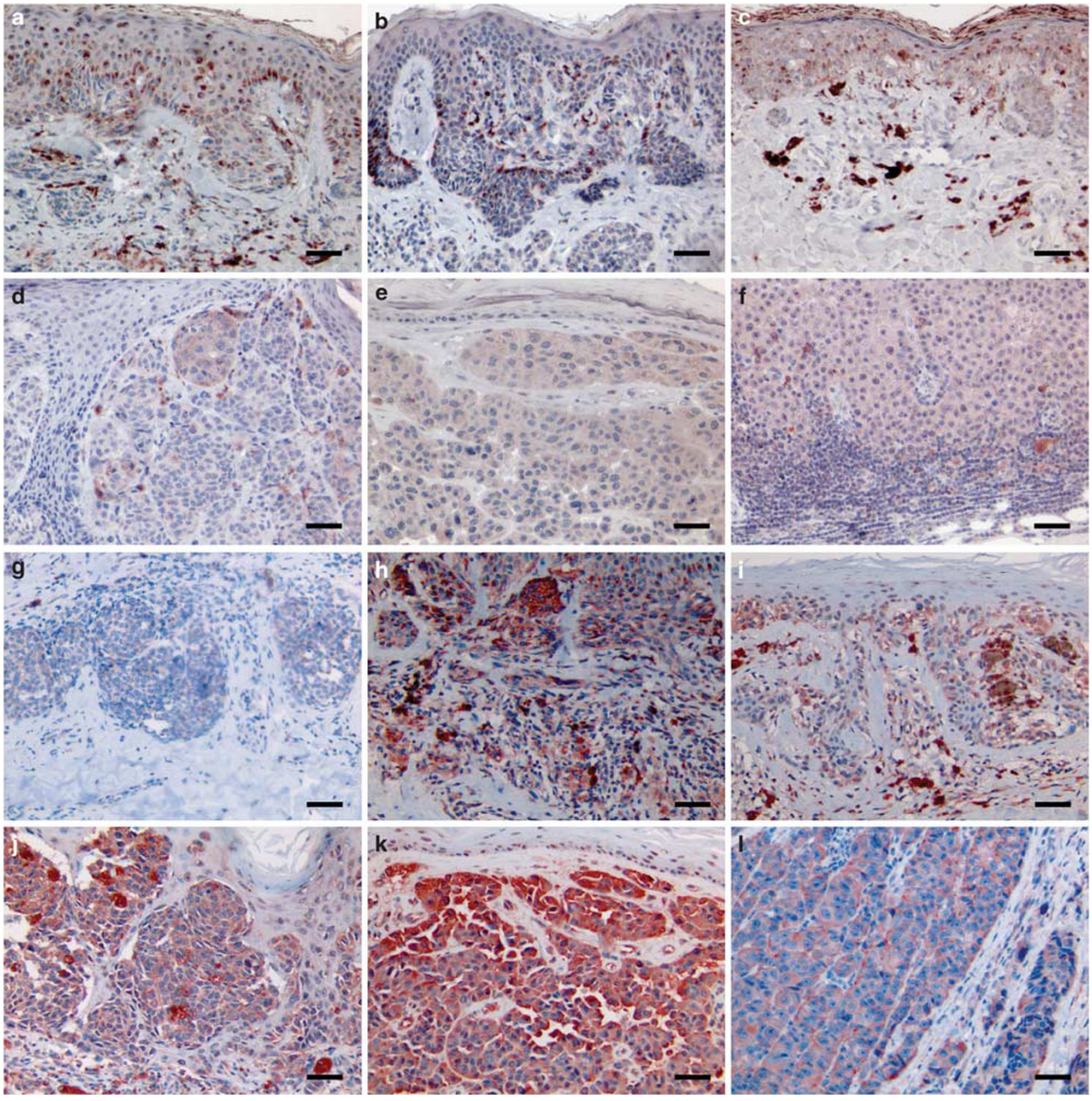

Figure 2 Expression of β1-AR and β2-AR in human cutaneous melanocytic lesions. β1-AR immunostaining in CN (**a**), AN (**b**), *in situ* PM (**c**), SSPM (**d**), NPM (**e**), nodal MM (**f**): all lesions show a low reaction intensity, confined to the cell cytoplasm. β2-AR immunoreactivity in CN (**g**), AN (**h**), *in situ* PM (**i**), SSPM (**j**), NPM (**k**), nodal MM (**l**): except the weak staining intensity observed in CN, a moderate to strong or very-strong reactivity intensity is detected in all the other lesions. While CN cells show only cytoplasmic staining for β2-AR, AN and melanoma cells show cytoplasmic and membranous positivity; stromal cells show some reactivity in malignant lesions and AN (Scale bar, 50 μm).

notype of melanoma cells, we treated with NE or E two human melanoma cell lines, namely Hs29-4T cells, selected from a metastatic lesion, and A375 cells, derived from PM. We observed that both cell lines express low and comparable levels of β1-AR, as shown in Figure 3, while they both express higher amounts of β2-AR (Figures 4a and b), with the primary A375 melanoma cell line exhibiting a significantly higher expression of β2-AR compared with the metastatic Hs29-4T cell line. Both cell lines are able to respond to catecholamine stimulation with protein kinase A (PKA) phosphorylation, a known trait of β-AR stimulation (Figures 4c and d). In addition, we evaluated the effects of both catecholamines on the MAPK pathways, as few studies until now have investigated the effects of β-adrenergic signalling on these molecular pathways in melanoma models. As shown in Figure 5, both NE and E are able to induce activation,

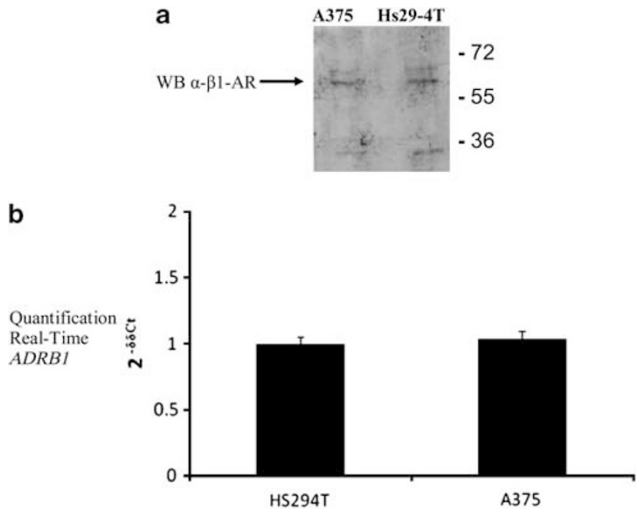

Figure 3 Expression of β1-AR in melanoma cell lines. (**a**) Analysis of β1-AR expression by immunoblot in primary (A375) and metastatic (Hs29-4T) melanoma cell lines. (**b**) Amount of *ADRB1* mRNA by real-time PCR. The amount of target, normalized to the endogenous reference (18S RNA), was given by the $2^{-\Delta\Delta ct}$ calculation and was reported as $2^{-\Delta\Delta ct}$. Both immunoblots and real-time PCR are the mean of three independent assays.

although with different kinetics, of p42-p44 and p38 MAPK in both cell lines (Figures 5a and b).

We then analysed the 3D motility of these cells upon catecholamine stimulation. The invasion assay, carried out by Boyden chambers covered with Matrigel to mimic a 3D barrier, revealed that both NE and E are able to elicit invasive behaviour in both metastatic or PM cells. Both NE and E appear to be more effective in PM cells with respect to MM cells. In addition, E is the most efficient catecholamine in eliciting invasiveness of the metastatic Hs29-4T cell line (Figures 6a and b). In both cells lines the increase in 3D invasiveness is sensible to treatment with ilomastat, a broad range inhibitor of MMPs (Figures 6a and b). The pro-invasive effect of both NE and E is strongly sensitive to propranolol, thereby confirming the involvement of β subtypes of AR. The last finding suggests the involvement of a proteolytic degradation of the Matrigel barrier during invasion. We, therefore, analysed by Real-Time PCR the expression of MMP-2 and MMP-9, the main proteolytc enzymes expressed by Hs29-4T and A375 cell lines, during stimulation with catecholamines. Figures 6c and d reveal that, while NE and E do not influence MMP-9 production, the expression of MMP-2 is increased by NE in A375 PM cells and by E in metastatic Hs29-4T. More importantly, both NE and E are able to maintain a high activation state of secreted MMP-2 and MMP-9 following catecholamines stimulation for 24 h on both cell lines (Figures 6e and f).

Effects of Catecholamines on Cytokine Production and Tumour Microenvironment

Cancer cells secrete many cytokines, chemokines and growth factors that can affect their own aggressiveness, in terms of proliferation, invasion or survival, as well the reactivity of the surrounding stroma. To address the role of catecholamine stimulation in these features we first analysed the expression of a panel of cytokines/growth factors by real-time PCR. We found that catecholamine stimulation leads to an increase in the expression of VEGF, IL-6 and IL-8 (Figures 7a–c). Interestingly the two catecholamines used show differential effects for VEGF, IL-6 and IL-8 in Hs29-4T and A375 cells. In A375 PM cells both NE or E are able to elicit expression of VEGF, IL-6 and IL-8. Conversely, the Hs29-4T metastatic cell line senses NE to increase expression of IL-6 and IL-8, and E to express VEGF.

The role of VEGF, IL-6 and IL-8 in tumour progression varies from increase in invasiveness/scattering and growth of angiogenic sprouting for VEGF and IL-8, to activation of stromal and/or inflammatory cells for IL-6.[18–20] We observed that VEGF stimulation increases the invasive spur induced by NE in A375 PM cells, while IL-6 does not have a role (Figure 8a). On the other side, we analysed the ability of IL-6, in association with NE, to activate dermal fibroblasts. We observed that IL-6 is able to activate dermal fibroblasts, as demonstrated by their ability to express α-smooth muscle actin (α-SMA), an acknowledged marker of myofibroblasts.[21,22] In addition, in dermal fibroblasts, the CM of NE-treated A375 cells elicits an activation state very similar with respect to treatment with IL-6 alone. Conversely, VEGF treatment is almost ineffective in eliciting a reactivity of fibroblasts (Figure 8b).

Taken together, these data suggest that *in vitro* the treatment of human melanoma cells with catecholamines dramatically affects their aggressiveness, inducing expression of MMP-2, VEGF, IL-6 and IL-8. These factors orchestrate a feed-forward loop leading to increase of proteolytic invasiveness of tumour cells, as well as activating surrounding fibroblasts.

DISCUSSION

The present study shows that the immunohistochemical expression of β-1 and β-2 ARs is significantly upregulated in melanoma tissues. Interestingly, all tested melanocytic lesions exhibited some immunoreactivity, suggesting that both benign and malignant lesions can theoretically be affected by catecholamines *in vivo*. However, since β-ARs staining significantly increases in PM and MM compared with melanocytic naevi, it appears that malignant lesions can be more deeply influenced by catecholamines than benign counterparts. The staining intensity for β2-AR progressively increased from CN, towards AN, to PM and MM, whereas the reaction intensity for β1-AR was weaker, in all groups of lesions. Such a difference can rely on a different reactivity of the used antibodies, but it is likely that β1-AR is expressed on naevus melanocytes and melanoma cells of sections at a lower level than β2-AR. This hypothesis was supported by PCR and western blot analysis of primary and MM cell lines,

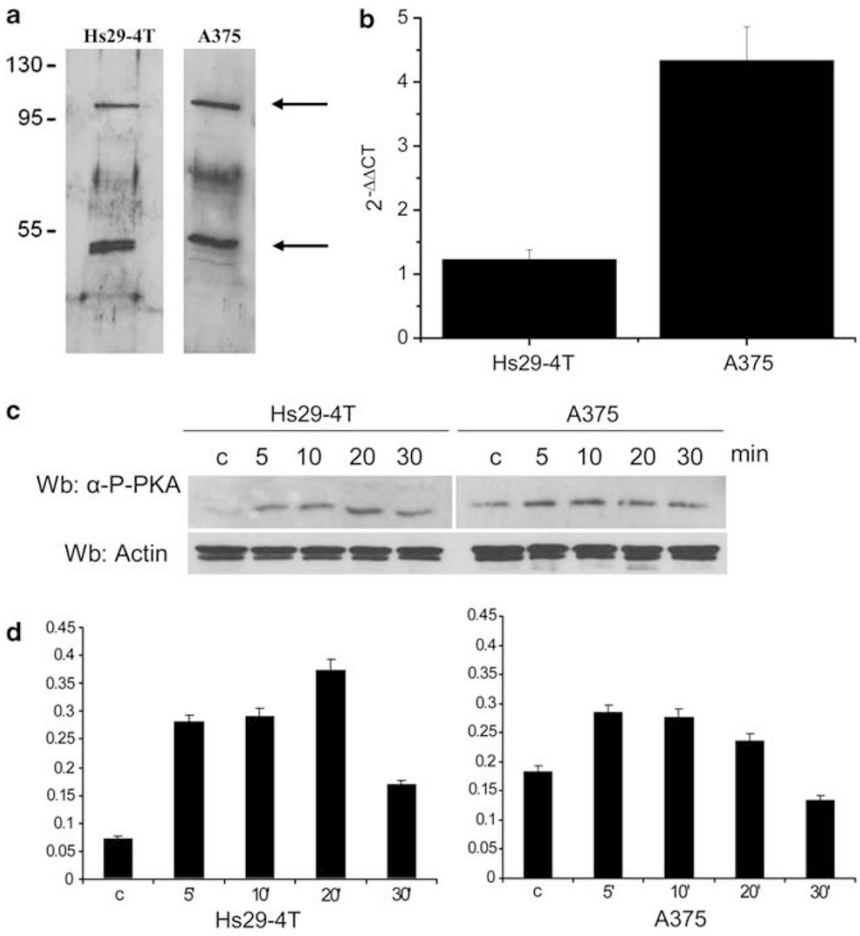

Figure 4 Expression of β2-AR in melanoma cell lines and analysis of the signalling pathway activated by NE stimulation. (**a**) Analysis of β2-AR expression by immunoblot in primary (A375) and metastatic (Hs29-4T) melanoma cell lines. (**b**) Amount of *ADRB2* mRNA by real-time PCR. The amount of target was given by the $2^{-\Delta\Delta ct}$ calculation and was reported as $2^{-\Delta\Delta ct}$. Immunoblot and real-time PCR are the mean of three different experiments. (**c**, **d**) Analysis of activation of PKA. Melanoma cell lines were serum-deprived overnight and then stimulated with NE (1 μM) for the indicated period and an immunoblot analysis for the detection of the phosphorylation level of p-PKA was shown. Actin immunoblot was used for normalization. The bar graph below represents the phosphorylation level of PKA in four different experiments. $^*P < 0.005$.

both of which exhibited a lower expression of β1-AR *vs* β2-AR.

In melanoma tissue sections a strong reactivity for β2-AR was also observed in most endothelial and stromal cells, including macrophages, suggesting the possible influence of catecholamines on cells of the tumour microenvironment and the chance of further potential biologic loops capable of affecting metastatic behaviour of neoplastic cells.

We also demonstrated that A375 primary and Hs29-4T MM cell lines respond to catecholamine stimulation enhancing motility and invasion, and producing molecules closely related to neoplastic progression. In keeping, we show that NE and E are able to elicit activation of p42/p44 and p38 MAPKs, acknowledged to have mandatory roles for cell growth, survival and invasive ability, in both primary and metastatic cell lines. This observation is in agreement with the findings of Pak *et al.*,[23] also indicating the Ras-MAPKs pathway as a target of β-adrenoceptors. These data could be of striking interest in order to find new strategies for melanoma treatment. In fact, Meier *et al.*[24] showed that combined targeting of the p42/p44 and Akt signalling pathways significantly inhibited growth and enhanced apoptosis in melanoma cell cultures.

Moreover, NE and E exhibited a diverse stimulation capacity on the two cell lines. Concerning the invasion assay, the primary cell line responded to NE (and, at a lesser degree, to E), while the metastatic cell line showed a clear reaction only to E. The inhibition induced by ilomastat strongly suggests a proteolytic degradation of Matrigel and the probable intervention of MMPs. In fact, we demonstrate that MMP-2, rather than MMP-9, is produced at a significantly higher level compared with baseline, by A375 cells under NE stimulus and by Hs29-4T cells under E stimulation. Furthermore, our data show that both NE and E are able to elicit

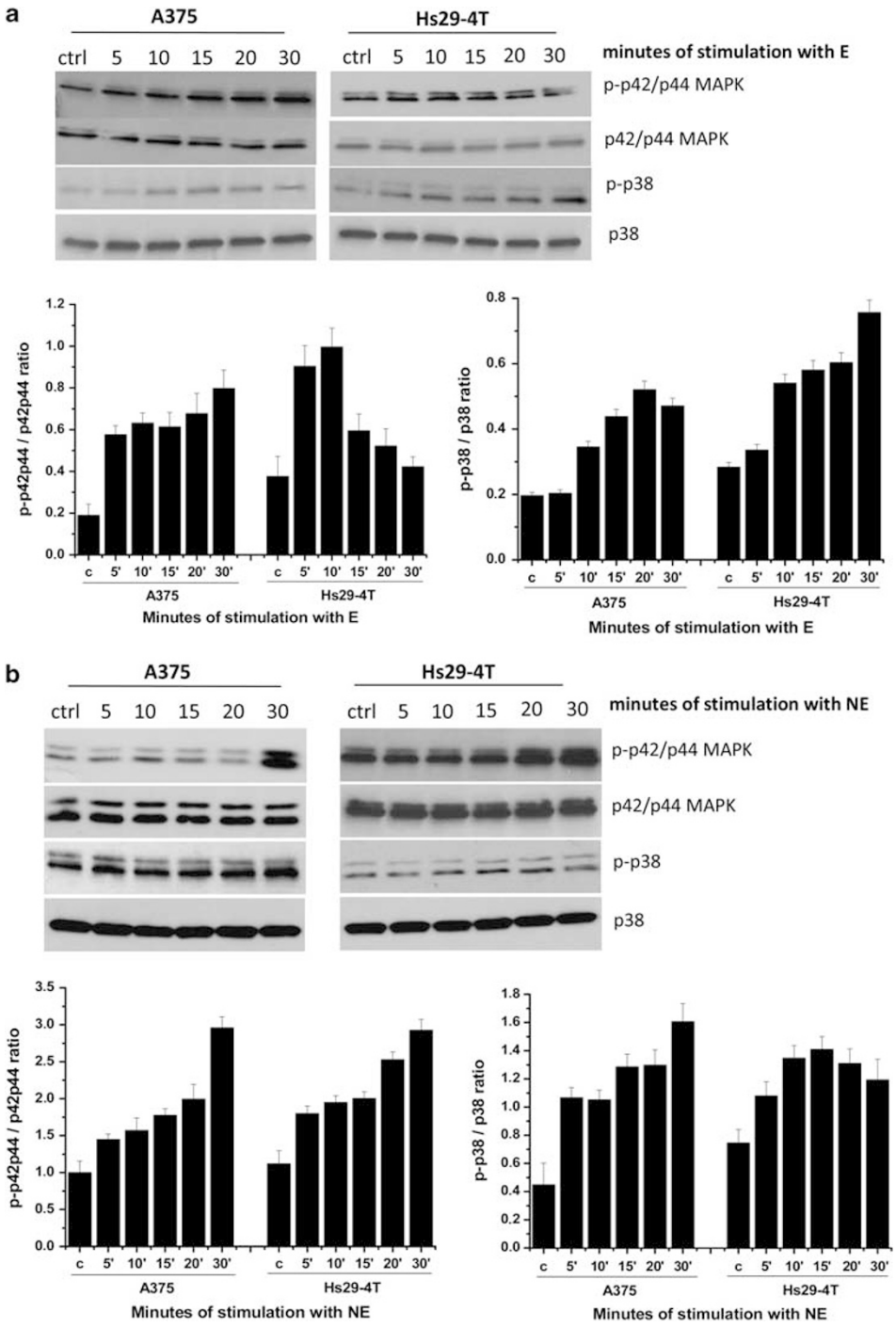

Figure 5 Analysis of activation of p42/p44 and p38 MAPK. Melanoma cell lines were serum-deprived overnight and then stimulated with E (**a**) or NE (1 μM) (**b**) for the indicated period and an immunoblot analysis for the detection of the phosphorylation level of MAPKs were shown. Total p42/p44 and p38 MAPK immunoblot were used for normalization. The bar graphs below represent the phosphorylation level of MAPKs in four different experiments. *$P<0.005$.

sustained activation of MMP-2 and MMP-9 both in primary and metastatic cell lines. Thus, increased motility of melanoma cells seems to be due to a proteolytic invasive capacity, typical of a mesenchymal phenotype,[25,26] and catecholamines seem able to influence MMPs activity both at a transcriptional and at a post-translational level. The migration of neoplastic cells appears to be increased through activation of β-ARs, because it is completely abolished by propranolol.

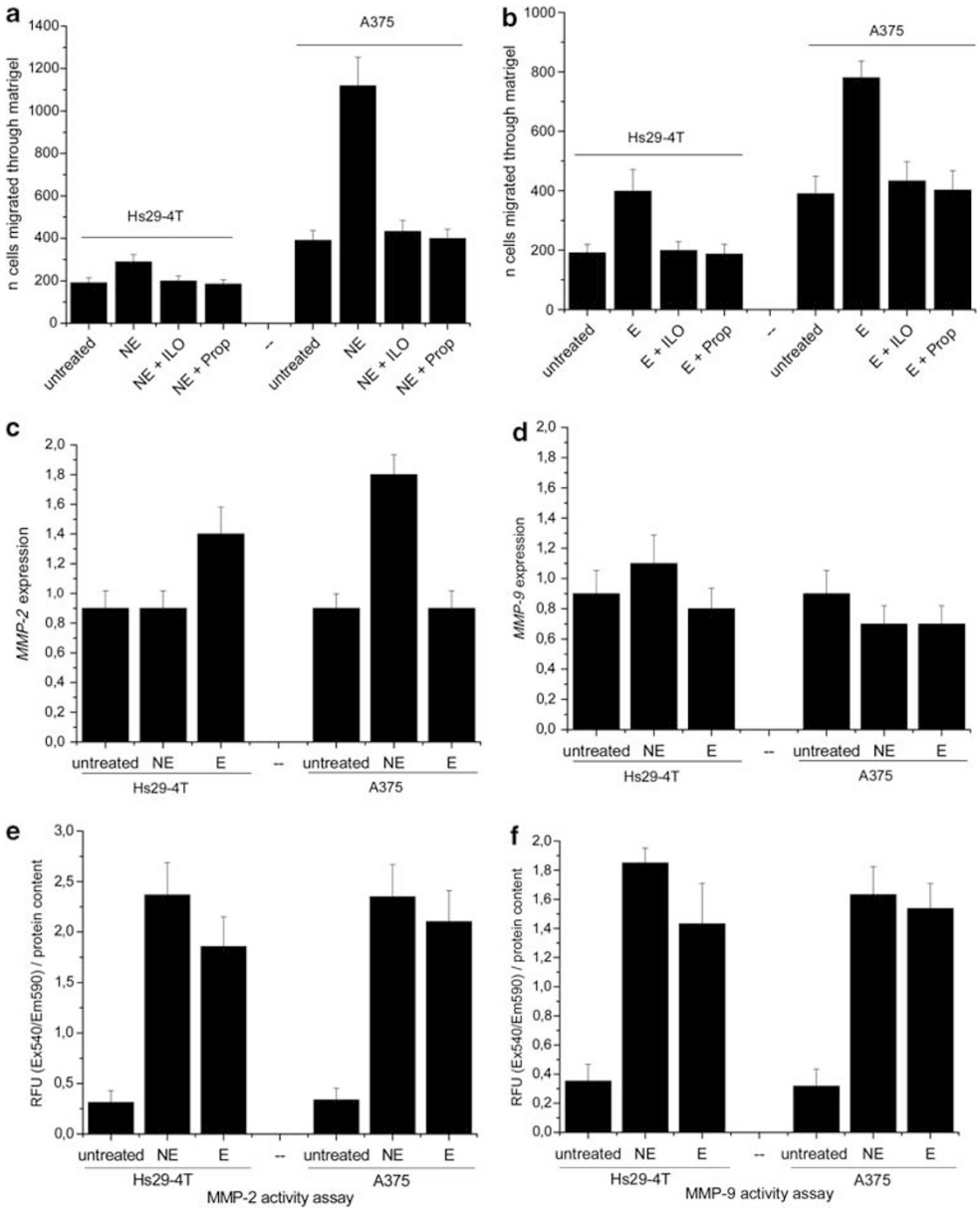

Figure 6 Effects of E and NE stimulation on melanoma cell lines invasion. Melanoma cell lines were serum-deprived overnight and then seeded in the upper Boyden chamber for assay their invasion. (**a**, **b**) NE (**a**) or E (**b**) (1 μM), in the presence or absence of ilomastat (50 μmol/l) or propranolol (1 μM), were added in the upper Boyden chamber. Bar graphs represent the mean of migrated cells counted in six different fields for each experiment. $^*P<0.005$ *vs* untreated. (**c**, **d**). Expression of *MMP-2* (**c**) and *MMP-9* (**d**) mRNA by real-time PCR. Melanoma cell lines were serum-deprived overnight and then stimulated with E or NE (1 μM) for 24 h. The amount of target, normalized to the *GAPDH* mRNA amounts, was given by the $2^{-\Delta\Delta}$CT calculation and was reported as arbitrary units (a.u.). The graphs report data as the mean of three independent assays. (**e**, **f**) MMP-2 and MMP-9 activity assay. Melanoma cell lines were serum-deprived overnight and then stimulated with NE or E (1 μM) for 24 h. The media obtained were then tested for MMPs activity with a fluorimetric kit, following the manufacturer's instructions (see Materials and Methods). Data are presented as RFU *vs* concentration of test compounds. The graphs report data as the mean of four independent experiments. $^*P<0.005$.

With regard to the production of cytokine transcripts, A375 cells significantly increase levels of IL-6 and VEGF under NE and E challenge, whereas Hs29-4T cells increase IL-6 expression under NE stimulus, and produce significant amounts of VEGF, especially under E activation. Concerning the expression of IL-8, the primary cell line responded to both NE and E at a significantly higher degree compared with the metastatic one, and both cell lines reacted more intensely to NE. This result is in agreement with the angiogenic role of IL-8, as *de novo* angiogenesis is particularly useful for primary tumours to escape the hostile microenvironment and disseminate metastasis.[27] In addition, the catecholamine-induced

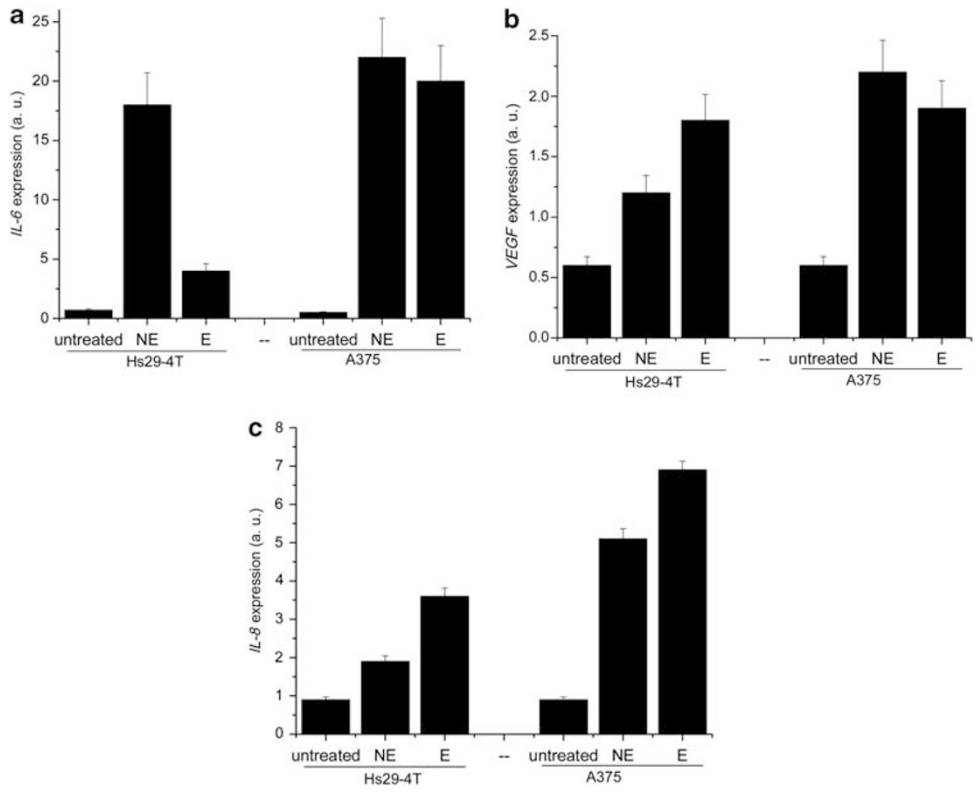

Figure 7 Expression of VEGF, IL-6 and IL-8 in E- and NE-treated melanoma cell lines. Melanoma cell lines were serum-deprived overnight and then stimulated with E or NE (1 μM). Total RNA was used for the amplification of mRNA of *IL-6* (**a**), *VEGF* (**b**) and *IL-8* (**c**), using as housekeeping gene *GAPDH* mRNA. The amount of target, normalized to the *GAPDH* mRNA amounts, was given by the $2^{-\Delta\Delta CT}$ calculation and was reported as arbitrary units (a.u.). The graphs report data as mean of three independent assays. $^{*}P<0.005$.

IL-8 enhancement is in agreement with the described IL-8 stimulation produced by NE in prostate cancer.[10]

We do not know exactly why such a discrepancy exists between primary and metastatic cell line response, but it is possible that at least in part this difference is due to a higher expression of β2-ARs, assessed as protein and transcript, on the PM cell line. Another possibility is that IL-6 and IL-8, whose expression was associated with early malignancy of melanoma *in vivo*[28] are actually secreted more efficiently by a cell line derived from a PM.

In vitro experiments clearly show that catecholamines can augment the malignant behaviour of melanoma cells affecting both invasion capacity and cytokine production.

Our *in vitro* experiments suggest that some pro-metastatic loops could work in melanoma *in vivo* too. We demonstrated that IL-6 and NE in melanoma cells can activate dermal fibroblasts towards a myofibroblastic phenotype, identified by α-SMA expression.[21] It is well known that stromal fibroblasts within tumours undergo a process, commonly called mesenchymal–mesenchymal transition to myofibroblasts, leading them to achieve a more contractile phenotype and allowing a cross talk with tumour cells dramatically increasing their aggressiveness.[29,30] In turn, activated fibroblasts can secrete other pro-metastatic cytokines, such as VEGF,[31] capable of inducing further tumour angiogenesis. In addition, in our experiments, VEGF can increase, particularly when associated with NE, melanoma cell migration and hence invasion capacity.

Previous data support the hypothesis that various types of stress, such as surgical procedure and the immediate post operative period, or neuroendocrine stress due to psychosocial factors, can stimulate tumour progression both in animals and humans.[10] This seems to be true also for melanoma at least in *in vivo* models.[32,33]

Our work provides evidence that stress hormones like NE and E can significantly stimulate the malignancy of melanoma cells at various levels and that β-ARs, likely involved in this response, are largely expressed in melanoma cell lines and cutaneous melanomas. It is the first time, to our knowledge, that β-ARs are demonstrated in a large series of human cutaneous melanocytic lesions, and even in melanocytic naevi, suggesting a potential influence of catecholamines also in benign counterparts. Moreover, the detection of β2-AR also in the stromal cells of melanoma microenvironment implies further possible effects of catecholamines on melanoma progression. Consequently, it is possible that the interaction catecholamines-β-ARs could have a dramatic role during the clinical course of melanoma patients. The efforts

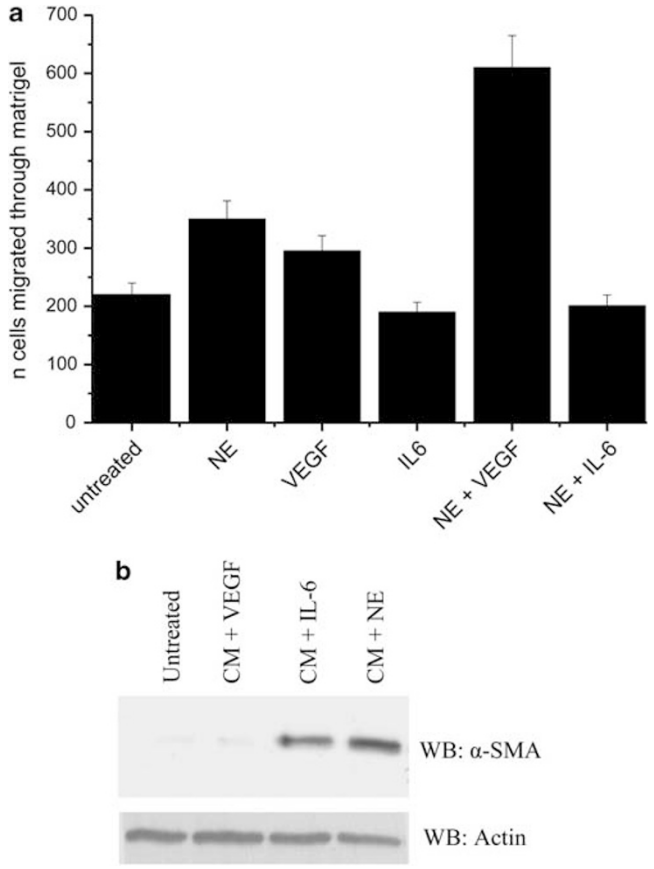

Figure 8 Synergy among NE, cytokines and tumour microenvironment. (**a**) A375 primary melanoma cells were serum-deprived overnight and then seeded in the upper Boyden chamber for assaying their invasion. NE (1 μM), IL-6 (50 ng/μl), VEGF (20 ng/μl), in combination with NE or alone, were added in the upper Boyden chamber. Bar graph represents the mean of migrated cells counted in six different fields for each experiment. *$P < 0.005$ *vs* untreated. (**b**) Analysis of human dermal fibroblasts (HDFs) activation state, through evaluation of α-SMA expression, after treatment with conditioned medium (CM) from NE/IL-6/VEGF-treated A375 cells. A375 cells were grown to sub-confluence and treated for 24 h with 1 μM NE, IL-6 (50 ng/μl) or VEGF (20 ng/μl). Fresh serum-free medium was added for an additional 24 h before collection of CM, in order to obtain CM free from NE, IL-6 or VEGF (but conditioned by their earlier administration). HDFs were then incubated with the obtained CM for 24 h and then used for western blot analysis of α-SMA expression. Actin immunoblot was used for normalization. The blot is representative of three different experiments.

to understand molecular events underlying such an interaction can be very useful for indicating new targets in therapy.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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