

## Dual Targeting of Tumor and Endothelial Cells by Gonadotropin-Releasing Hormone Agonists to Reduce Melanoma Angiogenesis

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We showed previously that GnRH receptors are expressed in melanoma cells; their activation reduces cell growth and metastatic behavior. Here, we investigated whether GnRH agonists might affect the expression of genes involved in melanoma progression. By genome-wide transcriptomic and real-time PCR analysis, we first observed that GnRH agonists decrease the expression of the pro-angiogenic factor vascular endothelial growth factor (VEGF) (all isoforms) in BLM melanoma cells. Then, we demonstrated that GnRH agonists specifically decrease the expression of the VEGF<sub>165</sub> isoform as well as its secretion from BLM cells. These data suggested that activation of GnRH receptors might reduce the pro-angiogenic behavior of melanoma cells. To verify this hypothesis, we treated BLM cells with a GnRH agonist; the conditioned medium from these cells was tested to assess its capability to stimulate human umbilical vein endothelial cell (HUVEC) motility. The migration of HUVECs towards the conditioned medium of GnRH agonist-treated BLM cells was significantly lower than the migration of HUVECs toward the conditioned medium of untreated cells. Thus, GnRH agonists reduce the pro-angiogenic behavior of melanoma cells through a decreased production of bioactive VEGF. We then found that GnRH receptors are also expressed on HUVECs and that GnRH agonists reduce their ability to proliferate and to form capillary-like tubes when stimulated by VEGF. These findings suggest that GnRH agonists exert an anti-angiogenic activity indirectly by decreasing VEGF secretion from tumor cells and directly by counteracting the pro-angiogenic activity of the growth factor. These data might lead to the development of novel targeted approaches for melanoma. (*Endocrinology* 151: 4643–4653, 2010)

The incidence of cutaneous melanoma keeps increasing (1); thus, despite the improving in early diagnosis allowing curative surgical strategies, this pathology remains the leading cause of skin cancer deaths in developed countries (2, 3). In its late stages, cutaneous melanoma is characterized by a high proliferation rate and by a strong ability to give rise to metastases (4, 5). In this context, the process of angiogenesis

plays a crucial role by inducing formation of a new vasculature (6). New blood vessels provide the adequate supply of oxygen and nutrients to melanoma cells and allow their dissemination to distant organs (6). Accordingly, melanoma cells have been shown extensively to secrete high amounts of vascular endothelial growth factor (VEGF), the most selective stimulator of rapid angiogenesis (7, 8).

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Abbreviations: FBS, Fetal bovine serum; GnRH-A, GnRH agonist Goserelin cetate; GnRH-Ant, GnRH antagonist Antide; GnRH-R, GnRH receptor; HUVEC, human umbilical vein endothelial cell; RMA, robust multichip analysis; VEGF, vascular endothelial growth factor

The prognosis of highly aggressive, metastatic melanoma is still very poor because of the resistance of the disseminated tumor to standard chemotherapy (9, 10). Cytotoxic compounds, such as dacarbazine, have been reported to be associated with a modest antitumor activity and significant side effects (11). Immunotherapy has demonstrated response rates of approximately 15–20% with only a slightly more durable response than that found with chemotherapy (10, 12, 13). Based on these disappointing observations, major efforts are now given to unraveling the molecular mechanisms underlying melanoma progression toward its most proliferative, metastatic, and pro-angiogenic stage. Specifically, a better understanding of the mechanisms controlling the expression/activity of the VEGF system might stimulate the development of innovative, and hopefully more successful, targeted therapeutic strategies. To this purpose, it must be underlined that clinical trials based on VEGF-targeted therapies are at present ongoing in melanoma patients (14, 15).

GnRH is the hypothalamic decapeptide that is well known for its central role in the control of the reproductive axis (16). GnRH exerts its activity by binding to  $G\alpha_q$ /phospholipase C-coupled receptors (GnRH-R) on pituitary gonadotropes (16). Chronic administration of GnRH agonists induces desensitization of GnRH-R, thus suppressing the function of the pituitary-gonadal axis. Based on this mechanism of action, GnRH analogs are widely and successfully used for the treatment of hormone-dependent pathologies, such as tumors of the reproductive tract (prostate, breast, endometrium) (17–19). GnRH is also well known as an autocrine/paracrine factor that is expressed, together with its receptors, in tumors of the reproductive system, in which it acts as a local regulator of tumor growth (20–25). In particular, our laboratory has extensively demonstrated that GnRH agonists significantly reduce the growth and the metastatic behavior of prostate cancer cells, both androgen dependent and androgen independent (23–25), and that tumor GnRH-R are specifically coupled to the  $G\alpha_i$ -cAMP intracellular signaling pathway (26). Similar observations have been widely reported also for tumors of the female reproductive tract, such as ovarian and endometrial cancers (27, 28). Thus, it is suggested that these compounds might exert an additional and direct antitumor activity on tumors expressing GnRH-R.

In previous studies, we demonstrated that  $G\alpha_i$ -coupled GnRH-R are also expressed in melanoma cells; activation of these receptors significantly reduces the proliferative as well as the migratory/invasive behavior of these cells (29–31). The possible effect of GnRH agonists on the process of angiogenesis in melanoma has never been investigated.

Here, we demonstrate that GnRH agonists significantly decrease the expression of VEGF in melanoma cells,

followed by reduction of its secretion and activity. Surprisingly, we found that GnRH-R are also expressed in human umbilical vein endothelial cells (HUVECs) and that their activation interferes with VEGF-induced cell proliferation and ability of tube formation. Thus, we demonstrate a dual targeting of both cancer and endothelial cells by GnRH agonists and hypothesize its role in reducing the angiogenic behavior of melanoma cells.

## Materials and Methods

### Chemicals

The GnRH agonist Goserelin acetate [D-Ser(tBu)<sup>6</sup>Aza-Gly<sup>10</sup>-GnRH; Zoladex] (GnRH-A) was kindly provided from AstraZeneca Pharmaceuticals (Macclesfield, Cheshire, UK). The GnRH antagonist Antide [N-Ac-D-Nal<sup>1</sup>-D-Cpa<sup>2</sup>-D-Pal<sup>3</sup>-Ser<sup>4</sup>-Lys(Nic)<sup>5</sup>-D-Lys(Nic)<sup>6</sup>-Leu<sup>7</sup>-Ilys<sup>8</sup>-Pro<sup>9</sup>-D-Ala<sup>10</sup>-NH<sub>2</sub> (GnRH-Ant)] was purchased from Sigma (St. Louis, MO). The pro-angiogenic factor VEGF<sub>165</sub> was from R & D System (Minneapolis, MN).

### Cell cultures

The human melanoma BLM cell line was kindly donated by Dr. G. N. van Muijen (Department of Pathology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands). BLM cells were cultured in DMEM (Seromed Biochrom KG, Berlin, Germany), supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Paisley, Scotland, UK), glutamine (1 mM), antibiotics (100 IU/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate) and sodium pyruvate (100 nM). Cells were grown at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air.

A panel of human melanoma cell lines (kindly provided by Meenhard Herlyn, Wistar Institute, Philadelphia, PA) derived from tumors at different stages of progression were available at the Department of Oncology, Mario Negri Institute (Milan, Italy) (32). WM793, WM902, WM1341, and WM1552 cells were isolated from nonmetastatic primary lesions; WM115, WM278A, and WM 983A cells derive from cutaneous primary lesions that originated metastatic disease from which WM239A, WM1617, and WM983B cells were isolated, respectively. WM373 and WM9 cells were also from metastases. All the cell lines were grown in RPMI 1640 medium (Invitrogen) supplemented with 5% heat-inactivated FBS and 1% L-glutamine.

HUVECs were obtained as described previously (33) and grown in M199 medium supplemented with 20% FBS, glutamine (1 mM), antibiotics (100 IU/ml penicillin G sodium and 100 µg/ml streptomycin sulfate), 20 mM HEPES, 6 IU/ml heparin (Bio Spa, Milan, Italy), and endothelial cell growth supplement (BD Biosciences, San Jose, CA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. All cultures were verified to be *Mycoplasma* free.

### RT-PCR analysis of GnRH-R mRNA

Total RNA was prepared with the use of the RNeasy mini kit (Qiagen, Chatsworth, CA), according to the instructions of the manufacturer. RT was performed on 1 µg of total RNA. cDNA synthesis was performed using the Gene AMP Gold RNA PCR Reagent kit (Applied Biosystems, Foster City, CA), with an oligo(dT)<sub>16</sub> as a primer for the RT. Samples containing cDNAs

were then amplified in a 50  $\mu$ l solution containing PCR buffer (150 mM Tris-HCl, 100 mM KCl, 1.75 mM MgCl<sub>2</sub>, 0.8 mM dNTP, and 2.5 U of AmpliTaq Gold DNA). PCR was performed for 35 cycles (1-min denaturation at 94 C, 1-min primer annealing at 58 C, and 2-min primer extension at 72 C) in the presence of the following primers: 5'-GACCTTGCTCTGGAAAGATCC-3' (sense, -25 to -5; 30 pmol) and 5'-CAGGCTGATCACCAC-CATCA-3' (antisense, 844–860; 30 pmol) (34, 35). After PCR, the amplified cDNA products (319 bp) were separated on 1.5% agarose gel and stained with ethidium bromide.  $\beta$ -Actin was amplified, as a loading control.

### Microarray analysis in BLM cells

BLM melanoma cells were seeded ( $5 \times 10^5$  cells/10-cm tissue culture dishes); after 48 h, cells were treated with GnRH-A ( $10^{-6}$  M) for 24 h, and total RNA was prepared as described above. After quality control using a bioanalyzer (Agilent 2100), RNAs were labeled according to the Affymetrix protocol using One Cycle Labeling kit (Affymetrix, Santa Clara, CA). Fifteen micrograms of resulting cRNAs were hybridized onto whole-genome microarray U133 Plus 2.0. After hybridization on Affymetrix HG-U133 Plus 2.0 chips, gene expression values were estimated for each probe set using packages in the Bioconductor suite (36). Genes were normalized and analyzed with both the robust multichip analysis (RMA) method within the affy package (37, 38) and corrected for GC content with the GCRMA package (39). The differences in log expression levels for both RMA and GCRMA normalized data were evaluated by the two-tailed *t* test as implemented in the limma package (40). Genes with *P* values < 0.05 and the absolute expression fold change greater than 2 (log difference of 1) were considered as significantly differentially expressed between treated and untreated cells. A gene list was generated by taking the overlap of significantly differentially expressed genes generated by limma from both the RMA and GCRMA normalization methods. The probes used to analyze VEGF expression were selected to detect all VEGFA isoforms.

### Real-time PCR analysis for VEGF expression in BLM cells

To confirm the effects of GnRH-A on VEGF expression in BLM cells, real-time PCR analysis was performed initially on the same RNA samples submitted to microarray analysis and then on new experimental setups. In these subsequent experiments, BLM melanoma cells were seeded ( $5 \times 10^5$  cells/10-cm tissue culture dishes) in 10% FBS-supplemented medium. After 48 h, cells were treated with GnRH-A ( $10^{-6}$  M) for different time intervals (6, 12, 24, and 36 h). At the end of the treatment, RNA was extracted and reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems). The resulting cDNAs were amplified by real-time PCR using TaqMan polymerase and primers Hs 00903129m1 (Applied Biosystems). The primers were designed by the manufacturer using Primer Expression version 1 (Applied Biosystems) based on the sequence of the human VEGFA from the GenBank database (accession no. NM\_003376-4). The probes were selected to detect all VEGFA isoforms. The 18S ribosome RNA was used as a normalizing unit for each reaction, using a set of primers purchased as a kit (TaqMan Ribosomal RNA Control Reagents kit; Applied Biosystems).

Real-time PCR analysis was performed by the Real Time PCR Service, Cogentech (Fondazione Italiana per la Ricerca sul Can-

cro, Institute of Molecular Oncology Foundation, European Institute of Oncology Campus, Milan, Italy).

### RT-PCR analysis of VEGF<sub>165</sub> and VEGF<sub>121</sub> isoform expression in BLM cells

Experiments were performed to verify whether the different VEGF isoforms (VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>165</sub>, and VEGF<sub>189</sub>), derived from alternative splicing of the same pre-mRNA might be expressed in BLM melanoma cells. RNA (1  $\mu$ g) extracted from BLM cells was reverse transcribed, as described above. cDNA amplification was then performed in the presence of a set of primers (sense, 5'-TCGGGCCTCCGAAACCATGA-3'; antisense, 5'-CCTGGTGAGAGATCTGGTTC-3') recognizing all the VEGF isoforms (41).

Experiments were then performed to verify whether GnRH-A might affect the expression of the VEGF isoforms. Specifically, we focused our attention on VEGF<sub>165</sub>, the key protein in angiogenesis induction, and VEGF<sub>121</sub>, the most abundantly expressed isoform in BLM cells, according to our results. To this purpose, BLM melanoma cells were seeded ( $7 \times 10^5$  cell/10-cm tissue culture dishes) in 10% FBS-supplemented medium. After 48 h, cells were treated with GnRH-A ( $10^{-6}$  M) for different time intervals (6, 12, 24, and 36 h). At the end of the treatment, RNA was extracted and reverse transcribed. Preliminary experiments were performed to choose the most appropriate PCR conditions (temperatures and number of cycles) (data not shown). For VEGF<sub>165</sub> cDNA amplification, 2  $\mu$ g RNA was reverse transcribed, and PCR conditions were as follows: 25-s denaturation at 95 C, 25-s primer annealing at 58 C, and 25-s primer extension at 72 C, for 27 cycles, using the common sense VEGF primer (5'-CCCTGATGAGATCGAGTACATCTT-3') and the VEGF<sub>165</sub>-specific antisense primer (5'-GCCTCGGCTTGTCACATTTT-3') (41). For VEGF<sub>121</sub> cDNA amplification, 1  $\mu$ g RNA was reverse transcribed, and PCR conditions were as follows: 25-s denaturation at 95 C, 25-s primer annealing at 58 C, and 25-s primer extension at 72 C, for 30 cycles, using the common sense VEGF primer (5'-CCCTGATGAGATCGAGTACATCTT-3') and the VEGF<sub>121</sub>-specific antisense primer (5'-AGCAAGGCCACAGGGATTT-3') (41).  $\beta$ -Actin expression was amplified, as a control. After PCR, the amplified cDNA products were separated on 1.5% agarose gel and stained with ethidium bromide. The experiments were repeated three times. The bands were quantified by densitometry, and the ratios between either VEGF<sub>165</sub> or VEGF<sub>121</sub> and  $\beta$ -actin levels were taken into account for statistical analysis.

### ELISA assay for VEGF<sub>165</sub> secretion from BLM cells

BLM cells were seeded ( $2 \times 10^4$  cells per well) in 24-well plates and cultured in 10% FBS-supplemented medium for 48 h. The medium was then changed to 1% FBS-supplemented medium, and cells were treated with GnRH-A ( $10^{-6}$  M) for different time intervals (6, 24, 48, and 72 h). At the end of each incubation period, the medium was collected and centrifuged at  $1000 \times g$  for 10 min. VEGF protein levels were then evaluated, in collected supernatants, using an ELISA (Quantikine Human VEGF Immunoassay; R & D Systems), specifically recognizing the VEGF<sub>165</sub> isoform, according to the instructions of the manufacturer. An enzyme immunoassay multi-well reader set to read at an emission of 450 nm was used to quantify the results. The sensitivity of the assay was 5 pg/ml. Three separate experiments were performed.

## HUVEC chemotactic assay

To evaluate the motility of HUVECs toward different chemoattractants, chemotactic assays were conducted using the 48-well Boyden's chamber (Neuroprobe, Cabin John, MD), in which each pair of wells were separated by polyvinylpyrrolidone-free polycarbonate porous membrane (8  $\mu\text{m}$  pore size) (29). HUVECs were seeded ( $2.5 \times 10^4$  cells/50  $\mu\text{l}$ ) in the open-bottom wells of the chemotaxis chambers. The lower compartments of the chamber were filled with the following chemoattractants: 1% FBS medium, GnRH-A ( $10^{-6}$  M) in 1% FBS medium; VEGF<sub>165</sub> (10 ng/ml) in 1% FBS medium. The chambers were then incubated for 4 h at 37 C in the cell culture incubator. Cells that had migrated through the pores and found adherent to the underside of the membrane were fixed and stained (Diff-Quick kit; Dade, Dudingon, Switzerland). Six random objective fields of stained cells were counted for each well. The ability of HUVECs to migrate toward the culture medium of BLM cells, treated or not with GnRH-A, was investigated. To this purpose, BLM cells ( $5 \times 10^5$  cells per dish) were seeded in 6-cm tissue culture dishes, in 10% FBS-supplemented medium. After 48 h, the medium was changed to 1% FBS-supplemented medium, in either the absence or presence of GnRH-A ( $10^{-6}$  M) for 24, 48, or 72 h. At the end of the treatment, the media (conditioned medium) were collected and centrifuged at  $1000 \times g$  for 10 min and assayed as chemoattractant. A chemomigration assay of HUVECs was then performed, as described above, by filling the lower compartment of the Boyden's chamber with conditioned media from BLM cells, either untreated or treated with GnRH-A. Each experiment was performed three times.

## HUVEC proliferation studies

HUVECs were plated ( $2.5 \times 10^4$  cells per dish) in 6-cm dishes in culture medium. Cells were allowed to attach and start growing for 24 h; the seeding media were then changed to serum-starved media and treated with VEGF<sub>165</sub> (10 ng/ml, dose selected on the basis of preliminary experiments), in either the absence or presence of GnRH-A ( $10^{-6}$  M), for 4 d. The specificity of the activity of GnRH-A was assessed by treating HUVECs with both VEGF and GnRH-A, in the presence of the GnRH-A antagonist GnRH-Ant ( $10^{-6}$  M). At the end of the treatment, cells were collected and counted by hemocytometer.

## Tube formation assay

The ability of HUVECs to form capillary-like structures was measured on a layer of reconstituted basement membrane matrix (Matrigel; BD Biosciences) added to the 96-well tissue culture plate (13.47 mg/ml, 100  $\mu\text{l}$ /well) at 4 C. HUVECs were trypsinized, counted, resuspended in culture medium, and seeded on the top of the reconstituted basement membrane ( $1 \times 10^4$  cells per well). After 30 min at 37 C, the medium was changed, and cells were treated with VEGF (10 ng/ml), either alone or in the presence of GnRH-A ( $10^{-6}$  M). The specificity of the activity of GnRH-A was assessed by treating HUVECs with both VEGF and GnRH-A, in the presence of GnRH-Ant ( $10^{-6}$  M). After 18 h, capillary tube formation was assessed, as described previously (42), under an inverted microscope at 40 $\times$  magnification. Quantification of tube formation was performed by counting nine random microscopic fields (40 $\times$ ) per sample, with each condition being assessed in triplicate.

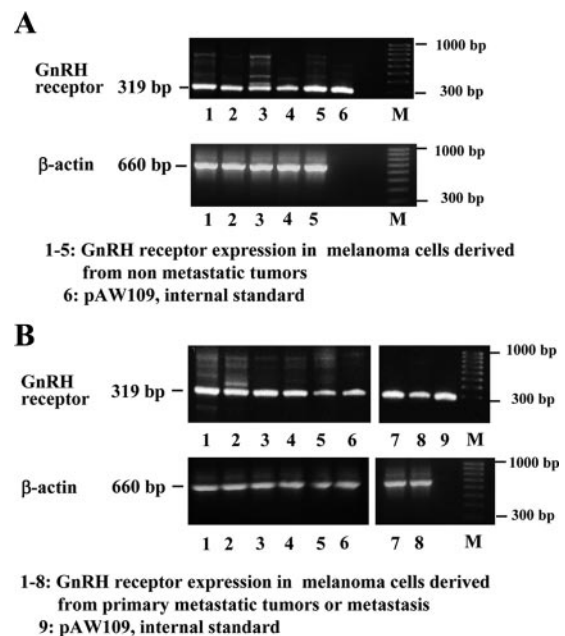
## Statistical analysis

When appropriate, data were analyzed by Bonferroni's test after one-way ANOVA.

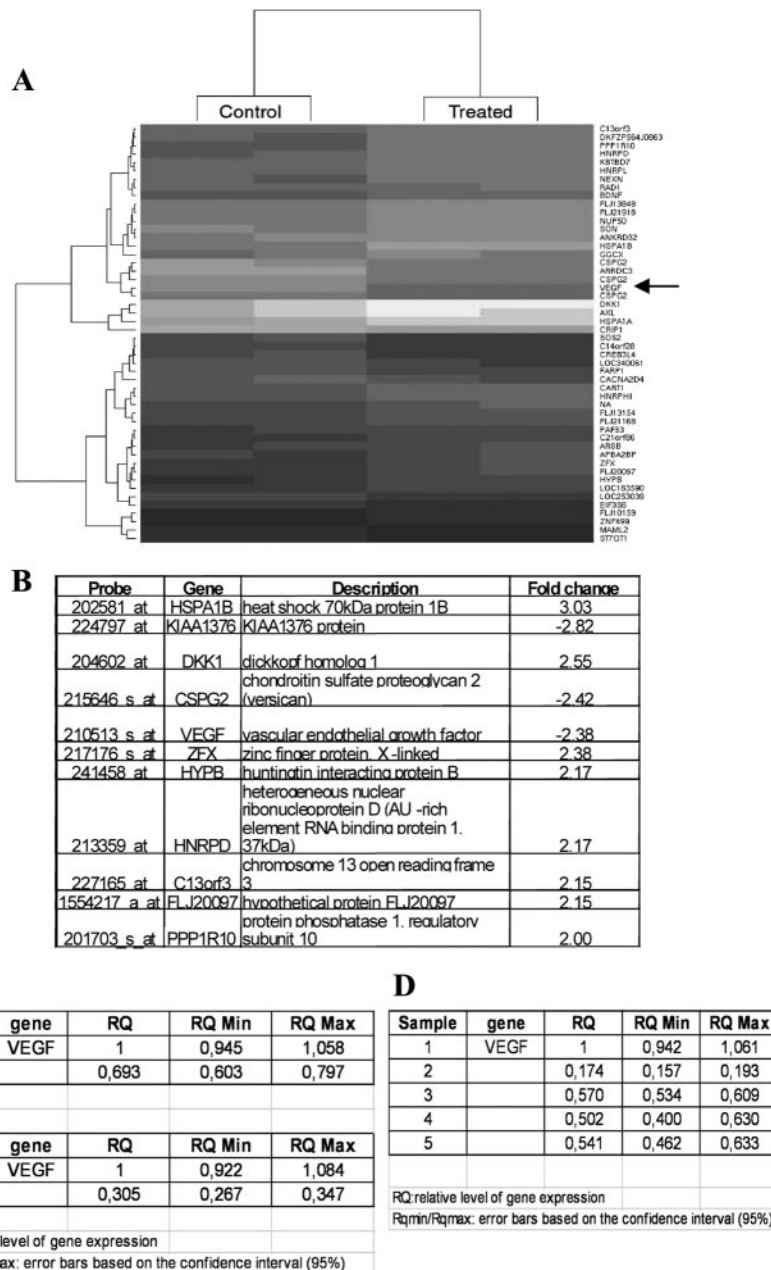
## Results

### Expression of GnRH-R in melanoma cell lines from tumors at different stages of progression

We showed previously that GnRH-R are expressed, at both mRNA and protein level, in BLM melanoma cells (29). Here, we investigated the expression of the receptor in a panel of cell lines derived from human melanomas at different stages of the disease. By RT-PCR, we could show that the mRNA coding for the GnRH-R is expressed in cells derived from nonmetastatic tumors (Fig. 1A, lanes 1–5) and in cells derived from malignant melanomas (Fig. 1B, lanes 1–8). Figure 1B also shows that the level of expression of the receptor seems to be similar in cells from primary lesions and from metastases, indicating that no loss of GnRH-R takes place during the metastatic process, confirming previous observations (43).



**FIG. 1.** Expression of GnRH-R in a panel of melanoma cell lines derived from tumors at different stages of progression. A, Expression of GnRH-R in melanoma cells derived from nonmetastatic tumors. Lanes 1–5, BLM, WM793, WM902, WM1341, WM1552; lane 6, pAW109, internal standard; M, molecular weight marker (1000–100 bp). B, Expression of GnRH-R in melanoma cells derived from primary metastatic melanomas or metastasis. Lanes 1, 2, WM115 and WM239A (primary and metachronous metastasis); lane 3, WM373 (metastasis); lanes 4, 5, WM278A and WM1617 (primary and metachronous metastasis); lanes 6, 7, WM983A and WM983B (primary and synchronous metastasis); lane 8, WM9 (metastasis); lane 9, pAW109, internal standard; M, molecular weight marker (1000–100 bp).



**FIG. 2.** GnRH agonists decrease VEGF expression in BLM cells. A, B, BLM cells were treated with GnRH-A ( $10^{-6}$  M) for 24 h. RNA was extracted and changes in gene expression profile were evaluated by genome-wide transcriptomic analysis (Affymetrix Human Genome U133 Plus 2.0 Array), as described in *Materials and Methods*. A, Representative heat map showing changes in gene expression. Data were compiled for gene expression of two separate controls and two separate treated samples. The arrow points to VEGF as a downregulated gene after treatment. B, Analysis with Bioconductor shows gene expression values estimated for each probe set using the RMA and corrected for GC content with the GCRMA method applied to all samples simultaneously. Absolute fold change greater than 2 is expressed as log difference of 1 above background control. Genes with  $P$  values  $< 0.05$  and the absolute expression fold change greater than 2 (log difference of 1) were considered as significantly differentially expressed between treated and untreated cells. C, Real-time PCR analysis of VEGF expression in BLM cells was performed on the same RNA samples submitted to microarray analysis. C, Untreated controls; GnRH-A, BLM cells treated with GnRH-A for 24 h. D, Real-time analysis of VEGF expression in BLM cells treated with GnRH-A ( $10^{-6}$  M) for different time intervals. Lane 1, Untreated controls; lane 2, 6 h; lane 3, 12 h; lane 4, 24 h; lane 5, 36 h. The 18S ribosome RNA was used as a normalizing unit for each reaction. RQ, Relative level of gene expression; RQ min/RQ max, error bars based on the confidence interval (95%). Results of one from three experiments performed are reported.

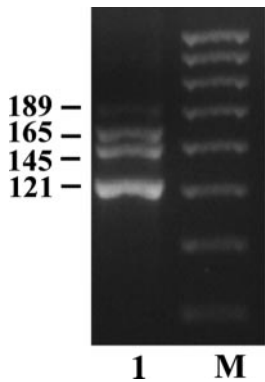
### GnRH agonists decrease VEGF expression in melanoma cells

We reported previously that GnRH agonists significantly reduce the growth and the motility of BLM cells (29, 31). Experiments were performed to investigate whether GnRH agonists might affect the expression of genes involved in the malignant progression of melanoma. BLM cells were treated with GnRH-A ( $10^{-6}$  M) for 24 h, and changes in gene expression profile were evaluated by genome-wide transcriptomic analysis (Affymetrix Human Genome U133 Plus 2.0 Array).

A representative heat map showing changes in gene expression is reported in Fig. 2A. Data were compiled for gene expression of two separate controls and two treated samples. Genes with  $P$  values  $< 0.05$  and the absolute expression fold change greater than 2 (log difference of 1) are considered as significantly differentially expressed between treated and untreated cells (Fig. 2B). The arrow points to VEGF as a downregulated gene after the treatment (Fig. 2A). For a color version of these data, see Supplemental Fig. 1 (published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). To confirm this observation, real-time PCR analysis was first performed on the same RNA samples analyzed previously by microarray. Figure 2C confirms that, in BLM cells, the expression of VEGF mRNA is significantly reduced 24 h after GnRH-A treatment. In subsequent experiments, the expression of VEGF at different time intervals after the treatment with GnRH-A (6–36 h) was analyzed. Figure 2D shows that the expression of VEGF is substantially decreased at all the time intervals considered (lanes 2–5 vs. lane 1).

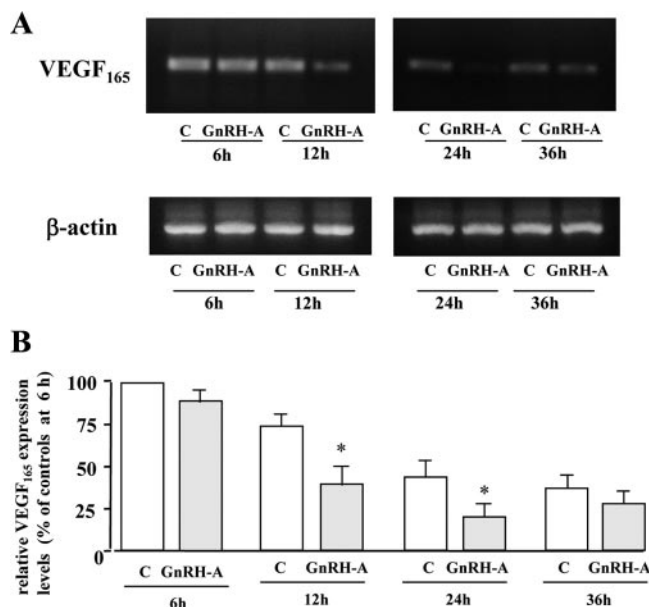
### GnRH agonists decrease VEGF<sub>165</sub>, but not VEGF<sub>121</sub>, isoform expression in melanoma cells

It is known that different VEGF isoforms are generated by alternative splicing of the same primary transcript

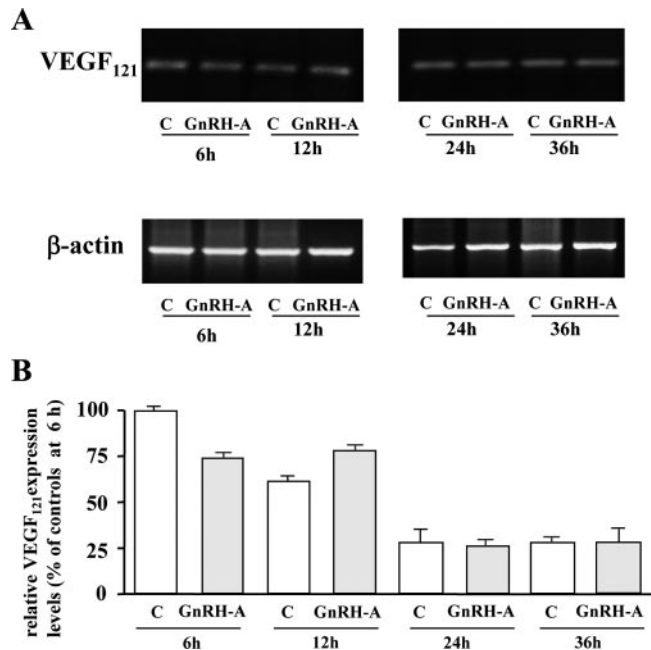


**FIG. 3.** RT-PCR analysis showing the expression of the different VEGF isoforms in BLM cells (lane 1). M, Molecular weight marker (1000–100 bp).

(44). By RT-PCR, we first demonstrated that the four VEGF isoforms, VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>165</sub>, and VEGF<sub>189</sub> (corresponding to 530, 590, 680, and 700 bp) are expressed in BLM cells (Fig. 3). Then, by using specific primers (41), we investigated the effects of GnRH-A specifically on the expression of the VEGF<sub>165</sub> isoform, the key protein in angiogenesis induction, and of the VEGF<sub>121</sub>, the most abundantly expressed isoform in BLM cells (Fig. 3). Cells were treated with GnRH-A (10<sup>-6</sup> M) for different time intervals (6, 12, 24, and 36 h). Figure 4, A and B, shows that GnRH-A significantly decreases VEGF<sub>165</sub> expression at 12 and 24 h of treatment. On the contrary, GnRH-A does not significantly affect the expression of VEGF<sub>121</sub>, at any



**FIG. 4.** GnRH agonists decrease VEGF<sub>165</sub> isoform expression in BLM cells. A, RT-PCR analysis of the expression of the VEGF<sub>165</sub> isoform in BLM cells treated with GnRH-A (10<sup>-6</sup> M). β-Actin was amplified as a control. One representative of three different experiments is shown here. B, Densitometric analysis of the data reported in A. The bands were quantified by densitometry, and the ratios between VEGF<sub>165</sub> and β-actin levels were taken into account for statistical analysis. The results are expressed as a percentage of the relative VEGF<sub>165</sub>/β-actin ratios in untreated controls (C) at 6 h. \*, P < 0.05 vs. controls (C).



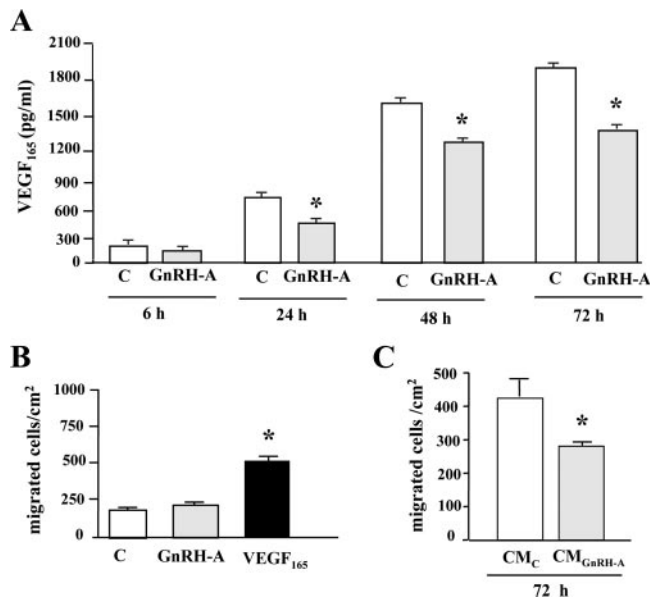
**FIG. 5.** GnRH agonists do not affect VEGF<sub>121</sub> isoform expression in BLM cells. A, RT-PCR analysis of the expression of the VEGF<sub>121</sub> isoform in BLM cells treated with GnRH-A (10<sup>-6</sup> M). β-Actin was amplified as a control. One representative of three different experiments is shown here. B, Densitometric analysis of the data reported in A. The bands were quantified by densitometry, and the ratios between VEGF<sub>121</sub> and β-actin levels were taken into account for statistical analysis. The results are expressed as a percentage of the relative VEGF<sub>121</sub>/β-actin ratios in untreated controls (C) at 6 h.

time intervals considered (Fig. 5, A and B). Interestingly, the levels of VEGF<sub>165</sub> (and also those of VEGF<sub>121</sub>) expression decrease over time in control cells (Figs. 4, A and B, and 5, A and B). The reason for this decrease is unclear; however, we might speculate that changing the culture media, at the beginning of the treatment, with fresh media might stimulate the growth factor expression. This stimulation then decreases over time.

**GnRH agonists decrease VEGF<sub>165</sub> secretion from melanoma cells**

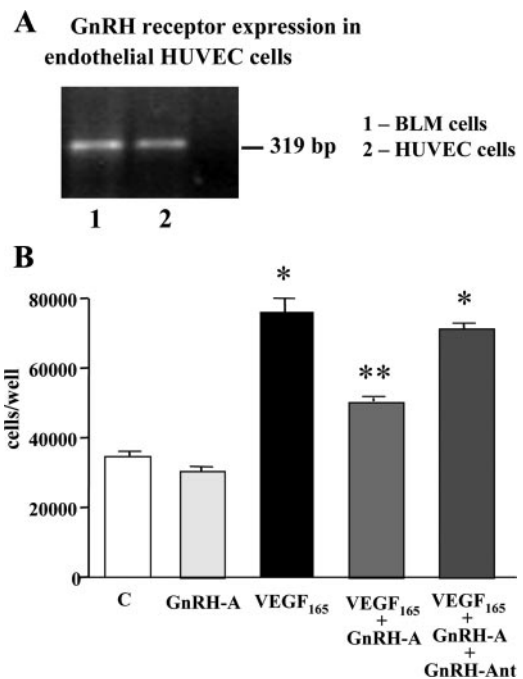
To confirm the impact of the changes in VEGF mRNA levels on its translation and release, we analyzed VEGF<sub>165</sub> levels in the conditioned media of BLM cells treated with GnRH-A (10<sup>-6</sup> M) for different time intervals (6, 24, 48, and 72 h). Figure 6A shows that the secretion of the VEGF<sub>165</sub> isoform from BLM cells is significantly reduced after 24, 48, and 72 h of treatment. Thus, GnRH agonists exert an inhibitory effect on VEGF synthesis and on its subsequent secretion from melanoma cells.

Given the decreased secretion of VEGF from GnRH-A-treated BLM cells, we next investigated the chemoattractant effects of the conditioned media of GnRH-A-treated cells on the migratory behavior of HUVECs, a biological function mediated by VEGF (45). We first con-



**FIG. 6.** GnRH agonists decrease VEGF<sub>165</sub> secretion from BLM cells. A, BLM cells were treated with GnRH-A (10<sup>-6</sup> M) for 6, 24, 48, or 72 h. VEGF levels were analyzed, in collected supernatants, using an ELISA, specifically recognizing the VEGF<sub>165</sub> isoform. Data are expressed as picograms per milliliter of VEGF<sub>165</sub> in the culture medium and represent the results from three separate experiments. \*, *P* < 0.05 vs. untreated controls (C). B, Chemotactic assay performed to evaluate the ability of HUVECs to move toward VEGF<sub>165</sub>. HUVECs were seeded in the open-bottom well of a chemotaxis chamber. The lower compartments were filled with the following: 1% FBS-RPMI medium (C), GnRH-A (10<sup>-6</sup> M) in 1% FBS-RPMI medium; VEGF<sub>165</sub> (10 ng/ml) in 1% FBS-RPMI medium. After 4 h at 37 C, the cells that had migrated through the pores and found adherent to the underside of the membrane were fixed and stained. Six random objective fields of stained cells were counted for each well. Data are expressed as number of migrated cells per square centimeters and represent the results from three separate experiments. \*, *P* < 0.05 vs. untreated controls (C). C, Chemotactic assay performed to evaluate the ability of HUVECs to move toward medium conditioned by BML cells. Cells were treated for 72 h with GnRH-A (10<sup>-6</sup> M). The conditioned media from both untreated (controls, CM<sub>C</sub>) and treated (CM<sub>GnRH-A</sub>) cells were collected and used as the chemoattractant in the lower compartments of the chemotaxis chamber. Chemotactic assay was performed as described in B. Data are expressed as number of migrated cells per square centimeters and represent the results from three separate experiments. Values are represented as the mean ± SE. \*, *P* < 0.05 vs. untreated controls (C).

firmed that VEGF<sub>165</sub> represents a chemotactic stimulus for HUVECs, whereas in the same assay, GnRH-A was devoid of any effect (Fig. 6B). Then, BLM cells were treated with GnRH-A (10<sup>-6</sup> M) for 24, 48, or 72 h; the conditioned media were placed in the lower well of the Boyden's chamber, as the chemoattractant. Figure 6C shows that the number of HUVECs migrating toward the medium of BLM cells pretreated (72 h) with GnRH-A is significantly lower than the number of cells migrating toward the conditioned medium of untreated cells. At shorter treatments (24 and 48 h), the conditioned medium of BLM cells does not affect the migration of HUVECs (data not shown). To explain this observation, we hypothesize that other factors in-



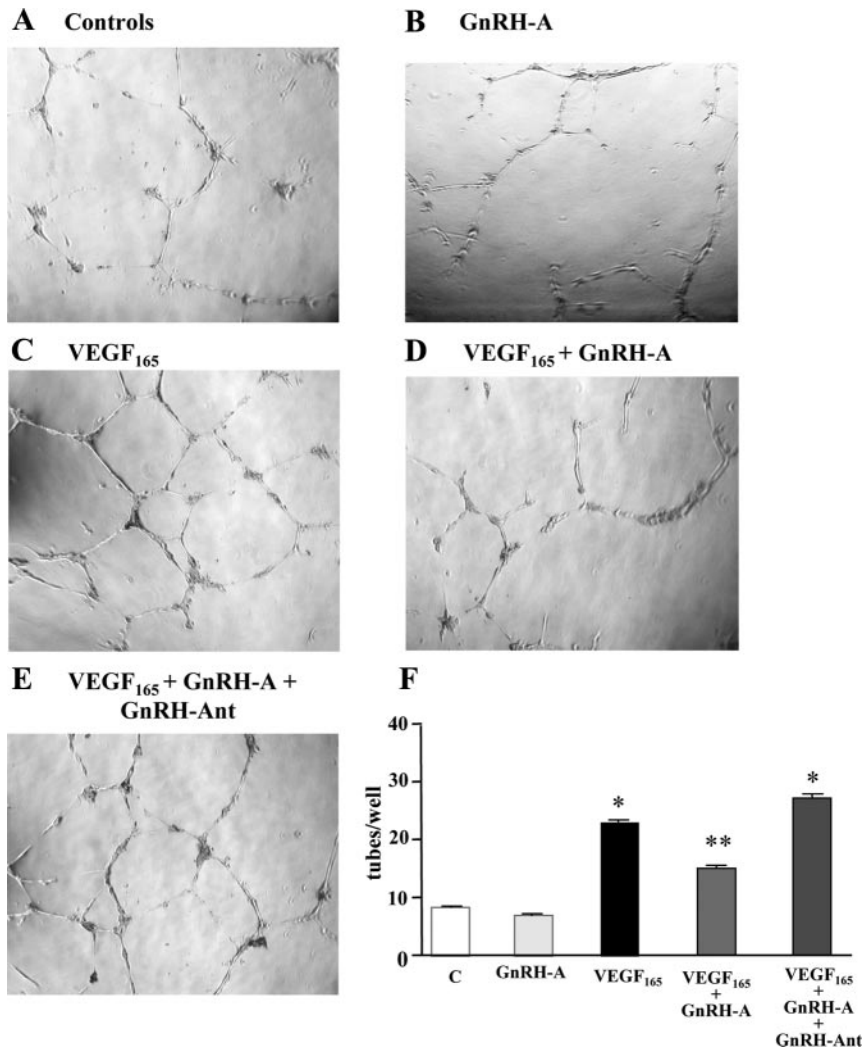
**FIG. 7.** GnRH agonists counteract the proliferative activity of VEGF<sub>165</sub> on HUVECs. A, RT-PCR analysis showing the expression of GnRH receptors in HUVECs. Lane 1, BLM cells used as a control; lane 2, HUVECs. B, HUVECs were treated daily, for 4 d, with VEGF<sub>165</sub> (10 ng/ml) and GnRH-A (10<sup>-6</sup> M), either alone or in combination. The specificity of GnRH-A activity was assessed by cotreating the cells with a GnRH antagonist (GnRH-Ant). Results are expressed as the number of cells per well and represent the results from three separate experiments. Values are represented as the mean ± SE. \*, *P* < 0.05 vs. untreated controls (C). \*\*, *P* < 0.05 vs. VEGF<sub>165</sub>-treated cells.

terfering with the activity of secreted VEGF might be present in the conditioned medium of BLM cells.

### GnRH agonists counteract VEGF-stimulated HUVEC proliferation

To study whether GnRH agonists could directly affect the angiogenic properties of endothelial cells, the expression of the GnRH-R was investigated in HUVECs. By RT-PCR, we found that the mRNA coding for this receptor is expressed in HUVECs (Fig. 7A, lane 2), as well as in BLM cells (Fig. 7A, lane 1), used as a control. This suggests that GnRH agonists might exert an additional, direct effect on tumor angiogenesis.

To investigate whether GnRH agonists might affect endothelial cell proliferation, HUVECs were treated, for 4 d, with VEGF<sub>165</sub> (10 ng/ml), either alone or in the presence of GnRH-A (10<sup>-6</sup> M). As expected, VEGF<sub>165</sub> significantly stimulated HUVEC proliferation, whereas GnRH-A, given alone, did not affect endothelial cell growth. In contrast, the GnRH agonist significantly counteracted the proliferative activity of VEGF<sub>165</sub> (Fig. 7B). The activity of GnRH-A is specific because it is completely counteracted by the cotreatment of the cells with the GnRH antagonist GnRH-Ant (Fig. 7B).



**FIG. 8.** GnRH agonists counteract the ability of VEGF<sub>165</sub> to induce HUVECs tube formation. A, HUVECs were seeded on the top of a reconstituted basement membrane and treated with VEGF<sub>165</sub> (10 ng/ml) and GnRH-A (10<sup>-6</sup> M), either alone or in combination, for 18 h (A–D). The specificity of GnRH-A activity was assessed by cotreating the cells with a GnRH antagonist (GnRH-Ant) (E). F, Number of tubes was measured, and data represent the results from three separate experiments. Data were analyzed by ANOVA followed by Bonferroni's test. Values are represented as the mean ± SE. \*, *P* < 0.05 vs. untreated controls (C). \*\*, *P* < 0.05 vs. VEGF<sub>165</sub>-treated cells.

### GnRH agonists counteract VEGF-stimulated endothelial cells *in vitro* tube formation

Having established that GnRH-A counteracts VEGF-induced HUVEC proliferation, we then examined its implication in the angiogenic process *in vitro*. For these studies, we used a conventional angiogenesis test, based on the ability of endothelial cells to spontaneously form capillary-like structures, when incubated on an extracellular basement membrane matrix preparation (Matrigel). Figure 8A shows that, in the absence of serum, HUVECs lack the ability to undergo alignment into capillary-like structures. VEGF strongly induces the process of tube formation (Fig. 8C), whereas GnRH-A, given alone, is not able to affect the formation of capillaries (Fig. 8B). GnRH-A substantially counteracts the effects of VEGF (Fig. 8D);

the activity of GnRH-A is specific because it is completely counteracted by the cotreatment of the cells with the GnRH antagonist GnRH-Ant (Fig. 8E). Tube formation was quantified by counting the number of endothelial cell structures, as summarized in Fig. 8F. These results demonstrate that the activation of locally expressed GnRH-R interferes with the VEGF-induced capability of endothelial cells to form capillary-like structures.

### Discussion

We showed previously that high-affinity GnRH-R are expressed in two human melanoma cell lines, BLM and Me15392. To confirm this observation, and before further unraveling the role played by these receptors in melanoma progression, we analyzed the expression of GnRH-R in a panel of human melanoma cell lines derived from either nonmetastatic or intermediate/highly metastatic primary tumors and from metastasis. We observed that the receptors are expressed in all the tumor samples tested, independent of their malignant behavior, in agreement with previously published data (43).

We then sought to identify genes whose expression might be specifically regulated by GnRH-R activation. To this purpose, by using the Affymetrix Human Genome U133 Plus 2.0 Array, we investigated the gene expression

profile in melanoma cells treated with a GnRH agonist (GnRH-A). Among the other genes whose expression was increased included the following: HSPA1B, which has been shown to activate T cells recognizing melanoma differentiation antigens (46); and DHK1, the antagonist of the canonical Wnt signaling pathway, endowed with tumor suppressor activity (47). Among the genes whose expression was reduced was CSPG2 (chondroitin sulfate proteoglycan2, versican), a proteoglycan of the extracellular matrix that is overexpressed in melanoma and contributes to melanoma progression, favoring the detachment of the cells and the metastatic dissemination (48). These data look particularly intriguing and certainly deserve additional investigation.



In our genome-wide transcriptomic analysis, we found that GnRH-A significantly reduced the expression of VEGF, and, given the pivotal role of this growth factor in the process of angiogenesis, we focused our attention on this gene. By real-time PCR and comparative PCR, we confirmed that GnRH-A significantly reduces the expression of VEGF, and specifically the VEGF<sub>165</sub> isoform, in BLM cells. Consistently, the amount of VEGF<sub>165</sub> secreted from melanoma cells was also found to be significantly reduced. Thus, we hypothesized that treatment of melanoma cells with GnRH agonists might reduce the capacity of these cells to attract endothelial cells, thus interfering with the process of neo-angiogenesis. Indeed, we found that the ability of HUVECs to move toward the conditioned media of BLM cells was significantly reduced when the cells were pretreated with a GnRH agonist. Together, these observations strongly indicate that GnRH agonists might decrease the pro-angiogenic activity of melanoma cells by reducing the expression/secretion/activity of VEGF. Accordingly, the expression of VEGF has been shown to be significantly reduced in human endometrial cell cultures (49), as well as in human uterine myomas (50) after treatment with GnRH agonists, although in these studies the effect on the pro-angiogenic activity was not reported. Interestingly, VEGF immunostaining has been found to be decreased in prostate cancer tissues of patients undergoing complete androgen blockade therapy (bicalutamide + goserelin acetate); however, a specific direct effect of the GnRH agonist has not been addressed in this clinical study (51). In female rats, GnRH analogs have been reported to regulate follicular development through a down-regulation of VEGF expression in ovarian follicles (52).

In previous papers, we have demonstrated that GnRH analogs significantly inhibit the proliferation as well as the migratory/invasive behavior of BLM melanoma cells (by reducing the expression/activity of  $\alpha 3$  integrin and MMP-2) (29, 31). The data here reported further confirm a direct antitumor activity of GnRH agonists on melanoma cells, while indicating a novel (i.e. anti-angiogenic) mechanism of action, through VEGF. In partial agreement with our observations, Keller *et al.* (43) reported the presence of GnRH-R in melanoma cells; treatment of nude mice bearing human melanoma xenografts with a cytotoxic analog of GnRH (GnRH agonist + chemotherapeutic agent) results in a significant decrease of tumor volume. However, it must be underlined that these authors did not directly address the antitumor activity of GnRH agonists on melanoma growth. In their study, they used the GnRH agonist as the carrier molecule that, by binding to GnRH-R on melanoma cells, can specifically target a cytotoxic compound at the level of tumor cells.

As mentioned, we showed previously that GnRH agonists significantly reduce melanoma cell proliferation (29). Moreover, it is known that tumor cells may express VEGF receptors and that VEGF, through these receptors, may stimulate tumor cell proliferation. Thus, it is possible to speculate that the antiproliferative effect of GnRH agonists might not be direct but mediated by a reduced VEGF secretion. We ruled out this possibility by demonstrating that BLM cells do not express the VEGF receptor and, accordingly, VEGF does not modify BLM cell proliferation (data not shown).

In the past, the direct antitumor activity of GnRH agonists has been extensively demonstrated in endocrine-related tumors by us (23–25) as well as by other investigators (20–22). These observations have later been supported by clinical data, indicating that expression of GnRH-R is a favorable prognostic factor in both prostate (53) and ovarian cancers (54).

Surprisingly, in this paper, we found that GnRH-R are expressed also in HUVECs. These receptors are functional, because their activation reduces VEGF-induced cell proliferation and ability to form capillary-like structures. To our knowledge, this is the first report of a direct effect of GnRH agonists on the angiogenic process. This observation is particularly relevant because it indicates that GnRH agonists might exert an antitumor effect on melanomas (and, in general, on tumors expressing GnRH-R) not only by reducing tumor cell proliferation and metastatic behavior but also by counteracting the process of angiogenesis, both indirectly by reducing VEGF secretion from tumor cells and directly by interfering with the VEGF-induced process of angiogenesis.

In previous papers, we demonstrated that, in prostate cancer cells, GnRH agonists reduce the proliferation and the migratory/invasive behavior of the cells by counteracting the activity of epidermal growth factor (55) and IGF-I (56, 57). Here, we show that these compounds reduce VEGF secretion/activity from tumor cells and interfere with the pro-angiogenic activity of the growth factor on endothelial cells. Thus, GnRH agonists exert a significant inhibitory effect on tumor progression and neoangiogenesis, by interfering with the activity of the growth factors that specifically favor these processes.

In conclusion, in the present paper, we demonstrated that GnRH agonists reduce VEGF secretion from melanoma cells (less growth factor is made available in the tumor microenvironment to stimulate angiogenesis) and in addition counteract the pro-angiogenic effects exerted by VEGF on endothelial cells (proliferation and cord formation). GnRH-R might represent a molecular target for the development of novel therapeutic interventions in melanoma.

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