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Running title: **Inhibition of TGF β 1-dependent angiogenesis**

Key words: **TGF β , angiogenesis, uPAR, PAI-1, SMAD**

TGF β 1 antagonistic peptides inhibit TGF β 1-dependent angiogenesis

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ABSTRACT

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5 The role of transforming growth factor-beta (TGF β) in tumor promotion and in
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7 angiogenesis is context-dependent. While TGF β prevents tumor growth and
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9 angiogenesis in early phases of tumor development, evidence is accumulating about its
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11 pro-angiogenic and tumor promotion activities in late stages of tumor progression. Here
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13 we have studied, in an experimental context previously reported to disclose the pro-
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15 angiogenic effects of TGF β , the blocking activity of TGF β antagonist peptides. In
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17 agreement with previous results, we have observed that TGF β exerts a powerful pro-
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19 angiogenic activity on human normal dermal microvascular endothelial cells (MVEC),
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21 by promoting invasion and capillary-morphogenesis in Matrigel. No apoptotic activity of
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23 TGF β was observed. By RT-PCR we have shown that TGF β up-regulates expression not
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25 only of plasminogen activator inhibitor type-1 (PAI-1), but also of the urokinase-type
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27 plasminogen activator receptor (uPAR), whose inhibition by specific antibodies blunted
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29 the TGF β angiogenic response *in vitro*. The SMAD-2/3 and FAK signaling pathways
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31 were activated by TGF β in MVEC, as an early and late response, respectively. The use
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33 of two different TGF β 1 antagonist peptides, derived from TGF β type III receptor
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35 sequence and 15-mer phage display technology, inhibited the signaling and pro-
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37 angiogenic response *in vitro*, as well as uPAR and PAI-1 up-regulation of MVEC
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39 following TGF β challenge. The anti-angiogenic properties of both inhibitors were
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41 evident also in the *in vivo* TGF β Matrigel sponge assay. These results may be relevant to
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43 develop a potentially fruitful strategy for the therapy of late-stage-associated tumor
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45 angiogenesis.
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57 **Key words: TGF β , angiogenesis, capillary morphogenesis, uPAR, PAI-1, SMAD**
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1. Introduction

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5 Transforming growth factor beta-1 (TGF β 1, referred to as TGF β throughout the paper),
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7 is one of the most important growth factors acting in the tumor microenvironment, that
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9 has recently emerged as a putative therapeutic target against cancer and cancer-related
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11 angiogenesis [1]. TGF β is a pleiotropic cytokine with opposing modes of action: its
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13 property to inhibit cell proliferation is cell type-dependent, and its role in adult tissue
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15 homeostasis and during embryonic development has frequently been reported to be the
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17 opposite in different cell types or in subsequent developmental stages [2]. In neoplastic
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19 disease, TGF β suppresses the progression of early lesions, but later this effect is lost and
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21 cancer cells themselves produce TGF β that promotes the metastatic process [3]. TGF β
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23 has been recently shown to modulate a set of pro-metastatic genes that govern the
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25 pattern of osteoclast activation in the sites of bone colonization of lung cancer cells and
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27 its blockade reduces bone tumor burden and osteolytic metastases [4]. Also angiogenesis
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29 regulation reflects the opposite activities of TGF β . TGF β induces expression of selected
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31 members of the Vascular Endothelial Growth Factor (VEGF) family in normal
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33 endothelial [5] and carcinoma cells [6]. Knockouts for TGF β and its receptors show
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35 clear defects in angiogenesis, which often eventuate with death *in utero* [2]. On the other
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37 hand, TGF β inhibits endothelial cell proliferation [7], migration and proteolytic activity
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39 [8]. VEGF, an endothelial cell pro-survival factor, has been shown to act in concert with
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41 TGF β to induce endothelial cell apoptosis [9]. In conclusion, TGF β acts either as
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43 inhibitor or as enhancer of neo-vascularization. The opposing effects of TGF β on
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45 angiogenesis have been tentatively explained by the differential involvement of two
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47 distinct TGF β signaling cascades within endothelial cells, the ubiquitous activin
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49 receptor-like kinase (ALK)5-SMAD2/3 pathway and the endothelial cell-restricted
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ALK1-SMAD1/5 pathway [2, 10]. Additionally, endothelial cells have TGF β type III receptors, whose occupancy by TGF β is essential to favor ALK activity and a successful angiogenic process [11].

Among other activities, phosphorylated SMAD proteins stimulate the production of plasminogen activator inhibitor type 1 (PAI-1), which maintains ECM integrity and favors its accumulation by inhibiting fibrinolysis and preventing plasmin-activation of pro-matrix metallo-proteases (MMPs) [12]. It is now firmly established that a proper activity of the cell-associated plasminogen activation (PA) system, composed by the urokinase-type plasminogen activator (uPA), its receptor (uPAR), and its type-1 inhibitor (PAI-1), is required in the invasion and capillary tube formation steps of angiogenesis [13]. Many data, starting with the pioneer observations of Rifkin and coworkers in early 1990s, indicate a regulative role of TGF β on uPA and PAI-1 expression in endothelial cells and other cell lines [14-19]. However, despite the well established role of uPAR in angiogenesis, information about angiogenic growth factor-dependent regulation of uPAR expression in endothelial cells is limited to Vascular Endothelial Growth Factor (VEGF) [20] and Fibroblast Growth Factor-2 (FGF2) [21]. Here we studied the pro-angiogenic activity of TGF β on human microvascular endothelial cells (MVEC), as related to TGF β -dependent modulation of uPAR and of other members of the PA system, and inhibited TGF β -dependent angiogenesis *in vitro* and *in vivo* by using two peptides, derived from human TGF β type III receptor and phage display technology, with a high affinity for soluble TGF β and potent inhibitory effects on TGF β binding to its receptors [22, 23]. The same peptides have been previously used successfully in controlling bleomycin-induced skin fibrosis in C3H mice [24] and in reducing bone colonization of lung cancer cells in a mouse model [4].

2. Materials and Methods

2.1. Endothelial cell preparation and characterization

For patients selection, ethics approval and patient consent, as well as for endothelial cell isolation from skin biopsies, refer to D'Alessio et al. [25]. Microvascular endothelial cells (MVEC) were prepared from skin biopsies of 15 healthy patients undergoing surgery for traumatic events at the hands. Where present, cell colonies were detached with EDTA, and CD31-positive cells were subjected to immuno-magnetic isolation with Dynabeads CD31 (DynaL ASA, Oslo, Norway) [25]. Isolated cells were further identified as MVEC by labeling with anti-factor VIII-related antigen, anti-CD105 (endoglin), and re-probing with anti-CD31 antibodies. MVEC resulted negative for immune-staining of CD45 (Leukocyte Common Antigen, LCA). Cells were maintained in complete MCDB medium, supplemented with 30% FCS, 20- μ g/ml endothelial cells growth supplement (ECGS), 10 μ g/ml hydrocortisone, 15 UI/ml heparin, and antibiotics (100 UI/ml penicillin, 100 μ g/ml streptomycin, 50 μ g/ml amphotericin), as described [25]. MVEC were used between the 3rd and 7th passage in culture.

2.2. Migration assays

The Boyden chamber (blind wells) was used to evaluate spontaneous and stimulated invasion (chemoinvasion) through Matrigel matrix (BD Biosciences, Two Oak Park, Bedford, MA)-coated porous filters, as described [25]. In some experiments Matrigel matrix Growth Factors Reduced (GFR) (obtained from the same source) has been used. For spontaneous invasion, 200 μ l of cell suspension (6.25×10^3 cells) were placed in the upper compartment of the chamber and migration was allowed to occur for 6 hours. For

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chemoinvasion, test solutions, containing increasing or fixed concentrations of the
chemoattractant (recombinant human TGF β 1, cat. 100-21, PeproTech, Rocky Hill, NJ),
were dissolved in serum-free medium and placed in the lower wells. In the experiments
with TGF β antagonists, each molecule was added to the lower well, together with the
chemotactic substance. In the experiments with anti uPAR and anti PAI-1 antibodies,
each antibody was added to the upper well (1,5 μ g/ml), with the cell suspension. At the
end of the migration time the filter was removed and fixed in methanol. Non-migrating
cells on the upper surface of the filter were removed with a cotton swab, while migrated
cells, adherent on the lower filter surface, were stained with Diff-Quick (Mertz-Dade
AG, Dade International, Milan, Italy) and counted by a light microscope (40 X) on the
whole migration surface per each well. Mobilization was measured by the number of
cells moving across the filter. Experiments were performed in triplicate. Migration was
expressed as mean \pm SD of the number of total cells counted/filter or as the percentage
of basal response.

37 **2.3. Proliferation studies**

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Cell growth was quantified in subconfluent cell monolayers, as described [25]. MVEC
were seeded onto 24 multi-well plates (Sarstedt, Verona, Italy) (15×10^3 cells/well) in
complete MCDB medium and were left to adhere overnight. Cells were then extensively
washed in phosphate buffered saline and maintained for 24 h in 1% FCS-MCDB
medium. Medium was removed and cells were incubated with 1% FCS-MCDB medium
containing increasing concentrations of TGF β for 48 hours. The proliferative effect of
30% FCS was defined as an optimal growth. Cells were fixed by adding 1 ml of ice-cold
methanol, stained with Diff-Quick and counted. Experiments were performed in
triplicate. Values were expressed as percent increase/decrease over basal response.

2.4. *In vitro* capillary morphogenesis assay

Matrigel (0.5 ml; 10-12 mg/ml) was pipetted into 13-mm (diameter) tissue culture wells and polymerised for 30 min to 1 h at 37°C, as described [25]. MVEC were plated (60×10^3 /ml), in complete MCDB medium, supplemented with 30% FCS, and 20 µg/ml ECGS. Capillary morphogenesis was also performed in the presence of 1 ng/ml TGFβ and/or 100-150 µg/ml antagonist peptide, and/or 1,5 µg/ml of anti-uPAR (cat. 3936, American Diagnostica Inc., Stamford, CT), and anti-PAI-1 (cat. sc-59633, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Positive controls were obtained upon stimulation with 10 ng/ml recombinant human VEGF (cat. 100-20 A, PeproTech). Some experiments of capillary morphogenesis were performed also with Matrigel matrix GFR. The effects on the growth and morphogenesis of endothelial cells were recorded after 6 and 24 hours with an inverted microscope (Leitz DM-IRB) equipped with CCD optics and a digital analysis system. Results were quantified at 6h by measuring the percent field occupancy of capillary projections. Six to nine photographic fields from three plates were scanned for each point.

2.5. RT-PCR analysis of uPAR, uPA and PAI-1

mRNA levels of uPAR, uPA and PAI-1 were determined by an internal-based semi-quantitative RT-PCR, using procedures previously described [26]. The primers' sequences, the size of products and cycling profile are reported in Table 1. The reaction products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under ultraviolet illumination using Polaroid positive/negative instant films, and quantified as reported [26].

2.6. Western Blotting

For signal transduction experiments N-MVEC were grown to 70% confluence and were serum-starved overnight in MCDB supplemented with 2% FCS. Specific treatments (TGF β alone, antagonist alone, and TGF β with each antagonist) were performed either for 60 min or 24 hours upon MVEC starvation. Confluent monolayers of MVEC, before and after specific treatments, were lysed in a 10 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 15% glycerol, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g of aprotinin per 100 ml. 40-100 μ g of the cell extract protein were subjected to electrophoresis in SDS-(10% or 12%) polyacrylamide gel under reducing conditions and then blotted to a polyvinylidene difluoride membrane (Hybond-C Extra; Amersham Biosciences, Piscataway, NJ) for 3 h at 35 V. The membrane was incubated with 5 % skim milk in 20 mM Tris buffer, pH 7.4, for 1 h at room temperature to block non-specific binding and then probed with primary antibody to phospho-ERK (p42/p44) (200 μ g/ml, 1:500) (cat. 9101S, Cell Signaling, Beverly, MA), ERK-2 (200 μ g/ml, 1:500) (cat. sc-154),

1 JNK (200µg/ml, 1:500) (cat. sc-571) and phospho-JNK (200µg/ml, 1:500) (cat. sc-6254)
2 (Santa Cruz Biotechnology), p38^{MAPK} (250µg/ml, 1:200) (cat. AB3188, Chemicon
3 International, Temecula, CA), phospho-p38^{MAPK} (250µg/ml, 1:500) (cat. 44-684G,
4 Biosource International, Camarillo, CA), SMAD5 (100µg/ml, 1:500) (cat. 9517),
5 SMAD2/3 (100µg/ml, 1:500) (cat. 3102), phospho-SMAD1/5 (Ser 463/465) (100µg/ml,
6 1:500) (cat. 9516), and phospho-SMAD2 (Ser 465/467) (100µg/ml, 1:500) (cat. 3108)
7 (Cell Signaling Technology, Inc., Danvers, MA), phospho-SMAD3 (1:500) (cat.
8 AB3226, R&D System, Minneapolis, MN), phospho-FAK (tyr576/577) (100µg/ml,
9 1:500) (cat. 3281, Cell Signaling), FAK (200µg/ml, 1:500) (cat. Sc-56901), overnight at
10 4 °C. After incubation with horseradish peroxidase-conjugated donkey anti-mouse or
11 anti-rabbit IgG (1:5,000) for 1 h (Amersham Bioscience), immune complexes were
12 detected with the enhanced chemiluminescence ECLTM detection system (Amersham
13 Bioscience). The membranes were exposed to autoradiographic films (Hyperfilm MP;
14 Amersham Bioscience) for 1-30 min.

15 Also TGFβ-dependent apoptosis of MVEC was evaluated by Western blot, using the
16 following monoclonal antibodies: anti-caspase-3 (100 µg/ml, 1:500) (cat. Sc-7272, Santa
17 Cruz Biotechnology) and anti-PARP-1 (100 µg/ml, 1:500) (cat. ALX-804-210, Alexis
18 Biochemicals, Farmingdale, NY). MVEC were grown to 70% confluence and were
19 serum-starved overnight in MCDB medium supplemented with 2% FCS. Specific
20 treatments (increasing concentration of TGFβ: 10 pg/ml, 100 pg/ml, 1 ng/ml, 10 ng/ml)
21 were performed for additional 6, 24 and 72 hours after MVEC starvation. Treatment
22 with H₂O₂ (200 mM final concentration, apoptosis positive control) was performed for 1
23 hour after starvation.

2.7. Immunocytochemistry

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2 TGF β -dependent apoptosis of MVEC was also evaluated by immunocytochemistry. N-
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4 MVEC were grown on glass slides to 70% confluence and were serum-starved overnight
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6 in MCDB medium supplemented with 2% FCS. Cells were incubated in the presence or
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8 absence of increasing concentrations of TGF β (10 pg/ml, 100 pg/ml, 1 ng/ml, 10
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10 ng/ml). α -bisabolol (Fluka and Riedel-De Haen, Sigma Chemical Company, Milan,
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12 Italy) (initial concentration 250 μ M), was diluted 1:80 in order to induce apoptosis in
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14 MVEC over a 6 to 24 hours treatment (positive control). Treatment with H₂O₂ for 1
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16 hour was used as a further positive control for apoptosis. MVEC were then stained with
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18 the fluorescent DNA-binding dye Hoechst 33342 (10 μ g/ml) (Invitrogen, San Giuliano
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20 Milanese, Italy) for 15 minutes at room temperature. Condensed and fragmented nuclei,
21
22 characteristic of apoptotic cells, were counted with a fluorescence microscope (Leyca
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24 DC-200, Leyca Microsystem Imaging Solutions Ltd, Cambridge, UK).
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34 **2.8. Antagonist TGF β peptides**

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39 We have used two TGF β antagonist peptides developed by Digna Biotech (Pamplona,
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41 Spain), one derived from its type III receptor, as previously reported [22]: peptide p144
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43 (TSLDASIIWAMMQN, 1580.86 kDa); the other one derived from phage display library
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45 technology, as previously reported [23]: peptide p17 (KRIWFIPRSSWYERA, 1995.6
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47 kDa). While p17 is water soluble, p144 is hydrophobic. The p144 stock solution was
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49 usually sonicated to reduce the particle size and to increase solubility before *in vitro*
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51 assays. After sonication, around 20% of p144 is dissolved in aqueous solutions (J.
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53 Dotor, personal communication). The final concentration of 100 μ g/ml antagonist
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1 peptides was chosen on the basis of previous results [22, 23] and of preliminary data
2 indicating that at 250 µg/ml each peptide became toxic to MVEC.
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7 **2.9. Matrigel Sponge Assay (*in vivo* angiogenesis)**

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12 TGFβ (1 ng/ml), alone or in addition to heparin (50 U/ml), in the presence or absence of
13 the TGFβ inhibitor peptides P144 and P17, was added to unpolymerized Matrigel at 4°C
14 at a final volume of 0.6 ml. The Matrigel suspension was carefully injected
15 subcutaneously into the flanks of C57/BL6 male mice (Charles River, Calco [Lecco],
16 Italy) using a cold syringe. As the matrigel warms to body temperature, it polymerizes to
17 a solid gel, which then becomes vascularized within 4 days in response to the angiogenic
18 substance. The extent of vascularization is quantified by measuring the hemoglobin
19 content of the recovered sponges. Groups of 4 to 8 pellets were injected for each
20 treatment. The reagents for all treatments were added to the Matrigel solution prior to
21 injection. Four days after injection, the pellets were removed, minced and diluted in
22 water to measure the hemoglobin content with a Drabkin reagent kit (Sigma). Samples
23 were also fixed in formalin and embedded in paraffin, for histological analysis and
24 stained with hematoxylin-eosin.
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46 **2.10. Statistical analysis**

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51 Results are expressed as means ± SD for (n) experiments. Multiple comparisons were
52 performed by the Student-Newman-Keuls test, after demonstration of significant
53 differences among medians by nonparametric variance analysis according to Kruskal-
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3. Results

3.1 *TGF β promotes Matrigel invasion and capillary morphogenesis of human MVEC*

The property of MVEC to move within ECM is the basic requirement to form ordered cords of endothelial cells appointed to give origin to blood vessels. The left panel of Fig. 1A shows the results of the Matrigel chemoinvasion assay under the effect of increasing TGF β concentrations in the lower well of the migration chamber. Since the maximal effect was obtained at 1 ng/ml TGF β , such a concentration was used throughout the whole study. The insets of the left panel of Fig. 1A shows the appearance of the lower side of the Matrigel-coated porous filter after scraping of the upper face and staining of migrated MVEC. Invasion assays performed with Matrigel matrix GFR gave similar results. On the contrary, TGF β did not stimulate MVEC proliferation (right panel of Fig. 1A). The final angiogenic event requires MVEC to organize in interconnecting tubular structures to form the new capillaries. Other studies have previously shown that TGF β stimulates a capillary tube patterning by endothelial cells embedded in collagen type I [27] and fibrin matrices [28]. To assay whether also in our experimental conditions TGF β stimulated capillary morphogenesis in MVEC, we have studied the capillary tube patterning in Matrigel, a method currently used in our laboratory [25]. In this assay MVEC produce elongated processes which eventuate in the formation of anastomosing cords of cells resembling a capillary plexus [25]. Fig. 1B shows that, after 6 hours from plating on Matrigel, MVEC produced abundant networks of branching cords of cells (upper left panel). By 24 hours (lower left panel), MVEC formed an interconnected network of anastomosing cells that, by low power light microscopy, had a “honeycomb”

1 appearance. The ability to form a complete network can be strengthened by the addition
2 of angiogenic growth factors to culture medium. In the presence of 1 ng/ml TGF β the
3 capillary morphogenesis increased by about 40% and 59% at both 6 and 24 hours,
4 respectively (central panels), an effect that was comparable to that elicited by
5 exogenous addition of 10 ng/ml VEGF, used as a positive control (right panels). The use
6 of Matrigel matrix GFR gave similar results.
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13 **3.2 TGF β does not induce apoptosis of MVEC.**

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21 TGF β has been shown to promote endothelial cell apoptosis *in vitro* [9], which has been
22 suggested to be required for pruning the formation of vascular networks. We have
23 checked for TGF β -dependent apoptosis in MVEC. Fig. 2A shows the negative results
24 obtained with Hoechst 33342 dye staining, using α -bisabolol and H₂O₂ treatment of
25 MVEC as positive controls. It is noteworthy that under our experimental conditions
26 neither TGF β nor the peptide inhibitors alone, as well as the association of TGF β and
27 peptide inhibitors, stimulated apoptosis of MVEC. Results obtained with caspase-3
28 activation, shown in the Western blot reported in Fig. 2B, were in agreement with those
29 obtained with immunocytochemistry. The use of anti-PARP-1 antibodies in Western
30 blotting gave results similar to those obtained with anti-caspase-3 (not shown).
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49 **3.3. TGF β -induced MVEC invasion and capillary morphogenesis depend on up-** 50 **regulation of uPAR**

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56 Left panel of Fig. 3A shows uPAR and PAI-1 mRNA increase 24 h after TGF β
57 stimulation of MVEC, as revealed by RT-PCR. No modification was observed for uPA
58 mRNA. The use of TGF β peptide antagonists prevented TGF β -dependent uPAR and
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1 PAI-1 up-regulation (right panel of Fig. 3A). TGF β -stimulated MVEC increase of
2 Matrigel invasion (Fig. 3B) and capillary morphogenesis (Fig. 3C) were inhibited by
3 incubating MVEC in the upper well of the migration chamber and upon seeding on the
4 Matrigel bed, respectively, with the anti-uPAR antibody (1,5 μ g/ml, American
5 Diagnostica, cat. 3936), which inhibits uPA/uPAR interaction. Also the treatment with
6 anti-PAI-1 antibody produced results similar to those obtained with anti-uPAR antibody,
7 either in MVEC invasion (Fig. 3B) or in MVEC capillary morphogenesis assay (Fig.
8 3C). Collectively, these data indicate that TGF β stimulates the invasive and
9 differentiation steps of *in vitro* angiogenesis of MVEC mainly by uPAR and PAI-1 up-
10 regulation. The results obtained with TGF β stimulation and with peptide inhibitors of
11 TGF β -TGF β receptor interaction suggest that PAI-1 is mainly a product of MVEC
12 themselves, rather than being picked-up from Matrigel, which has been reported to
13 contain small amounts of entrapped PAI-1 and uPA [29].
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34 ***3.4. TGF β antagonist peptides inhibit TGF β -dependent Matrigel invasion and*** 35 ***capillary morphogenesis of MVEC.*** 36 37 38 39 40

41 Fig. 4A shows the results of Matrigel chemoinvasion experiments performed by
42 stimulating MVEC invasion with TGF β added to the lower well of the chamber, in the
43 presence or in the absence of various TGF β antagonist peptides added to the cell
44 suspension in the upper well of the migration chamber throughout the whole experiment.
45 It is evident that both TGF β antagonist peptides efficiently inhibited TGF β -stimulated
46 MVEC invasion. It is interesting that each peptide showed a basal MVEC invasion
47 inhibiting activity even in the absence of exogenous TGF β addition. It is likely that such
48 an effect has to be related to antagonization of the TGF β constitutively present within
49 the Matrigel [29], which forms a very thin layer over the migration filter. Also capillary
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1 morphogenesis was inhibited by TGF β antagonist peptides, as shown in Fig. 4B. In this
2 case, in the absence of TGF β stimulation antagonist peptides did not show any inhibiting
3 activity, which was fully expressed following exogenous TGF β addition. It is likely that
4 the thickness of the gel prevents antagonist peptides to reach Matrigel-entrapped TGF β ,
5 which is instead easily inhibited once added together with inhibiting peptides.
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11 **3.5. TGF β -dependent signalling in MVEC is inhibited by antagonist TGF β peptides.**

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19 The signalling pathways to be studied under TGF β stimulation were selected on the
20 basis of data recently reviewed by Leask [30]. Data shown in Fig. 5A indicate that after
21 1 hour of TGF β stimulation only the classic SMAD2/3 pathway was triggered in MVEC.
22 However, upon a 24 hour TGF β stimulation only the FAK pathway resulted activated,
23 while all the others, including SMAD2/3, did not show any change. Therefore, inhibiting
24 experiments with TGF β antagonist peptides were focused only on phospho-SMAD2/3
25 inhibition after short-term treatment and on phospho-FAK inhibition after long-term
26 treatment. All the peptides inhibited SMAD2/3 and FAK phosphorylation at the relevant
27 times (Fig. 5B). These data, coupled with the property of TGF β antagonist of inhibiting
28 TGF β -induced uPAR and PAI-1 up-regulation (Fig. 3A), suggest that TGF β promotes
29 MVEC invasion and capillary morphogenesis by uPAR and PAI-1 up-regulation which
30 follows phospho-SMAD2/3 and FAK phosphorylation, and that TGF β antagonists
31 inhibit TGF β pro-angiogenic effects by preventing SMAD2/3 and FAK phosphorylation
32 and the subsequent uPAR and PAI-1 up-regulation.
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56 **3.6. TGF β inhibitor peptides inhibit angiogenesis in Matrigel Sponge Assay in vivo.**

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In order to measure TGF β *in vivo* angiogenesis, we performed a Matrigel Sponge Assay. Vascularization was seen in samples recovered from injected TGF β -containing matrigel sponges. In contrast, vascularization was inhibited by the addition of P144 and P17 as shown by decreases in hemoglobin content. Angiogenesis was induced by the addition of TGF β alone (Fig. 6A), however, the efficiency was improved by the co-addition of heparin (Fig. 6B). The angiogenic effect of TGF β was blocked by addition of the inhibitory peptides P144 and P17 (Fig. 6A-B). Both peptides alone have not effect when used in absence of pro-angiogenic stimuli (Fig. 6C).

To analyze vascularization and cellular content, we used a hemotoxylin-eosin staining of sections from the recovered samples seen in Fig. 6B. In matrigel sponges containing TGF β , we observed invasive inflammatory cells and vascularization (Fig. 6D, upper panel), however, both vascularization and inflammation were reduced by the presence of P144 as well as P17 (Fig. 6D, medium and lower panels).

4. Discussion

We have shown that two peptides, derived from human TGF β type III receptor and phage display technology respectively, with a high affinity for soluble TGF β and potent inhibitory effects on TGF β binding to its receptors [22, 23] inhibited TGF β -dependent MVEC invasion and capillary morphogenesis, by inhibiting TGF β -dependent SMAD2/3 and FAK phosphorylation and up-regulation of uPAR and PAI-1. The same peptides exhibited a noticeable anti-angiogenic activity in the Matrigel Sponge Angiogenesis model *in vivo*. The *in vivo* anti-angiogenic properties of p17 and p144 were evident both in TGF β -dependent angiogenesis and in heparin-enhanced TGF β -dependent angiogenesis. Additionally, we observed that TGF β promotes angiogenesis *in*

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vitro without exerting any pro-apoptotic effect. Antibodies against of uPAR and PAI-1 resulted in inhibition of TGF β -dependent *in vitro* angiogenesis. Collectively, these evidences suggest that TGF β antagonist peptides exert a strong inhibition on TGF β -induced angiogenesis, which depends on inhibition of TGF β binding to its receptor, reduction of SMAD2/3 and FAK phosphorylation and of the subsequent up-regulation of uPAR and PAI-1 expression in MVEC.

To study the angiogenic activity of TGF β we have used a Matrigel-based model, both *in vitro* (invasion, capillary patterning) and *in vivo* (Matrigel sponge assay). Our data are in agreement with previous results obtained with TGF β in collagen type I capillary morphogenesis of rat epididimal fat pad endothelial cells [27], and with those reported in fibrin gels for bovine aortic endothelial cells [28]. The similarity of results we have obtained with Matrigel matrix and Matrigel matrix GFR simplifies the interpretation of the data.

The best-described classical TGF β signaling pathway involves TGF β binding to its receptor, a type I/II heteromeric serine/threonine kinase, and induction of Smad 2/3 phosphorylation, which we have observed upon TGF β stimulation in our human MVEC. Smads have intrinsic transcription-inducing activity [31]. In the basal state, Smad2/3 are found in the cytoplasm but after activation, they form a complex with Smad4 and are shuttled to the nucleus where they bind DNA at a number of transcription sites such as AP-1 or Sp-1 [32]. TGF β has also been reported to regulate gene expression via activation of mitogen-activated protein kinases (MAPK), particularly p38 [33]. Although the stress-activated protein kinase p38 has been shown to be activated by TGF β in endothelial cells from other species and tissues [34-38], human MVEC did not show any p38 activation under our experimental conditions. Similarly, we did not observe JNK activation, which has been previously described in TGF β stimulation of bovine aortic endothelial cells [28]. FAK activation occurring 24 h after TGF β challenging in MVEC

1 is consistent with previous data [39]. Levels of FAK protein expression and/or activation
2 have been correlated to phenotypic changes that affect cell differentiation and function,
3 notably adhesion and migration, in a number of tissues [40-43]. Targeted FAK deletion
4 in vascular endothelial cells leads to apoptosis and aberrant cell movement whereas FAK
5 overexpression results in increased angiogenesis [44, 45].
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11 It is now generally accepted that TGF β promotes late stage tumor progression [46]. The
12 ability of TGF β to promote tumorigenesis was initially attributed to its activity on
13 tumor-associated fibroblasts [47], able to stimulate the growth of epithelial cells and
14 angiogenesis by direct cell-to-cell contact and by release of soluble factors, such as
15 TGF β . The loss of TGF β responsiveness in mouse fibroblasts results into the loss of
16 invasion of invasive prostate and squamous cell carcinoma of the forestomach [48].
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Angiogenesis stimulation is considered one of the major tumor-promoting activity of
TGF β . The pro-angiogenic activity of TGF β presumably combines direct and indirect
effects. TGF β induces fibroblasts to over-express VEGF, which acts on endothelial cells
to stimulate proliferation and migration [49]. In endothelial cells ALK5 mediates a
TGF β -dependent recruitment of ALK1 into a TGF β receptor complex and is required for
optimal ALK1 activation and subsequent angiogenesis stimulation [10]. The type III
TGF β accessory receptor is essential for vascularity and is required for efficient
TGF β /ALK1-Smad 1/5 signaling, which indirectly inhibits TGF β /ALK5-Smad 2/3
signaling [11]. Here we have shown that there are differences in the level of inhibition of
p-Smad 2 and p-Smad 3 by the two antagonist peptides used. As shown in figure 5B,
coincubation with TGF β and p144 or p17 inhibited p-Smad 3, but not as much p-Smad
2. This is in line with a previous report showing pro-angiogenesis activity of the former
[50]. The intense search for identification of the switch in TGF β signaling pathway from
one of tumor suppression to one of tumor promotion has led to development of TGF β
antagonists for inhibition of tumor progression and of TGF β -dependent angiogenesis

1 [for a review, 46]. However, systemic inhibition of TGF β raises important safety
2 concerns, since this factor displays pleiotropic effects including a well established anti-
3 tumor activity, whose inhibition could result into tumor progression [51]. Therefore,
4 results on TGF β inhibition in the control of cancer-associated angiogenesis must be
5 considered in their contextual dependence. From the TGF β standpoint the most
6 important issue is how to restore lost tumor suppressor function while eliminating or
7 preventing acquired pro-oncogenic/angiogenic effects. Therefore, late-stage invasive,
8 metastatic disease, which is typically characterized by locally or systemically elevated
9 TGF β levels, coupled with diminished responsiveness of tumor cells to its suppressor
10 functions [52, 53], has the chance to be the appropriate target for a therapeutic use of
11 TGF β antagonist peptides. Peptide p17 may have a better clinical implication due to its
12 aqueous solubility. Experiments are in progress to evaluate the efficacy of each peptide
13 and of their combination in the control of tumor development in mice. Immediately after
14 homogeneous preparations of TGF β were available, its role as a multifunctional
15 regulatory molecule became appreciable and the contextual dependence of the role of
16 TGF β during carcinogenesis and angiogenesis represents a paradigm [3, 54].
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Table 1 PCR primers and cycling conditions			
Primers	Sequence	Size (bp)	Cycling profile
uPA	5'-AAAATGCTGTGTGCTGCTGACC-3' (sense) 5'-CCCTGCCCTGAAGTCGTTAGTG-3' (antisense)	704	94°C, 1 min 56°C, 1 min 72°C, 1 min 35 cycles total
uPAR	5'-GGTCACCCGCCGCTG-3' (sense) 5'-CCACTGCGGTACTGGACA-3' (antisense)	910	94°C, 1 min 56°C, 1 min 72°C, 1 min 35 cycles total
PAI-I	5'- GAACAAGGATGAGATCAGCACC-3' (sense) 5'- ACTATGACAGCTGTGGATGAGG-3' (antisense)	780	94°C, 1 min 56°C, 1 min 72°C, 1 min 35 cycles total
GAPDH	5'-CCACCCATGGCAAATCCATGGCA-3' 5'-TCTAGACGGCAGGTCAGGTCCACC-3'	598	94°C, 1 min 56°C, 1 min 72°C, 1 min 35 cycles total

LEGEND TO FIGURES

Fig. 1 - *In vitro* angiogenic activity of TGF β on MVEC. (A) the left part of the panel shows the concentration-dependence of TGF β -chemoinvasion of MVEC through Matrigel-coated porous filters in blind-well Boyden chamber assay. Data are expressed as percent increase of cell migration by TGF β , with respect to the migration of control unstimulated cells considered as 100%. Insets show the typical aspect of the lower face of the filters in control unstimulated MVEC and under stimulation with 1 ng/ml TGF β . The right part of the panel shows MVEC growth under the effect of TGF β at the shown concentrations. Stimulation with 30% FCS and with 10 ng/ml VEGF were used as positive controls. Data are expressed as percent increase of cell number with respect to the number of control unstimulated cells considered as 100%. Data are the mean (\pm SD) of three different experiments performed in triplicate. * $p < 0.05$, significantly different from control. (B) Capillary morphogenesis assay of MVEC seeded on Matrigel. 60×10^3

1 cells were plated on Matrigel-coated (0.5 ml; 10-12 mg/ml) 13 mm tissue culture wells
2 and photographed after 6 and 24 hours from plating. Positive control was obtained upon
3 stimulation with 50 ng/ml VEGF (panels C and F). Six to 9 photographic fields from 3
4 plates were scanned for each point, considering as 100% the area occupied by untreated
5 MVEC. The data are expressed as percent modification (\pm SD) with respect to control. *
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p < 0.05, significantly different from control. The results shown are representative of 3 different experiments. Two experimental replicas for invasion capillary morphogenesis assays performed with Matrigel matrix GFR gave similar results.

Fig. 2 - TGF β -dependent apoptosis in MVEC. (A) Immunocytochemistry of MVEC stained with the fluorescent DNA-binding dye Hoechst 33342 in the conditions reported in the figure and described under Materials and Methods. Condensed and fragmented nuclei, characteristic of apoptotic cells, were evaluated with a fluorescence microscope. (B) Western blotting for caspase-3 activation. Lane 1: control unstimulated MVEC; lane 2: pro-apoptotic effect of H₂O₂ (1 hour); lane 3: pro-apoptotic effect of α -bisabolol (6 hours); lane 4, stimulation with TGF β (1 ng/ml for 6 hours); lane 5: p144 alone (150 μ g/ml for 6 hours); lane 6: p144 (150 μ g/ml) and TGF β (1 ng/ml) for 6 hours; lane 7: p17 alone (100 μ g/ml for 6 hours); lane 8: p17 (100 μ g/ml) and TGF β (1 ng/ml) for 6 hours. The results obtained by incubating MVEC for longer times (6, 24 and 72 h) with the relevant substances gave similar results, as well as Western blotting with anti-PARP-1 antibodies (not shown). Molecular weights are shown on the left. α -tubulin was used as a reference loading control. The results shown are representative of three different experiments performed in triplicate.

Fig. 3 - TGF β -dependent up-regulation of uPAR and PAI-1 and their involvement in TGF β -stimulated *in vitro* angiogenesis. (A) Left part: RT-PCR of uPA, uPAR and

1 PAI-1 mRNA in MVEC, following 24 h of stimulation with TGF β (1 ng/ml). 3 μ g of
2 total RNA were reversely transcribed, and then amplified using the primers reported in
3 table 1. GAPDH served as a control. Numbers on the right represent the size of each RT-
4 PCR reaction product in terms of base pairs. Right part: RT-PCR showing modulation of
5 TGF β -dependent up-regulation of uPAR and PAI-1 by TGF β antagonist peptides. Each
6 experimental condition is reported in the figure and in the text. Refer to the figure of the
7 left side for symbols. The results shown in the panel are representative of 3 different
8 experiments. (B) uPAR and PAI-1 function in both spontaneous and TGF β -dependent
9 stimulation of MVEC Matrigel invasion. The left side of the panel shows the aspect of
10 the lower side of the migration filters of a typical experiment performed in the reported
11 conditions. Migration is expressed as % modification (\pm SD) of the number of invasive
12 cells with respect to control unstimulated MVEC considered as 100%. The right side of
13 the panel shows the quantification of 3 different experiments performed in triplicate. * p
14 < 0.05, significantly different from control. (C) uPAR and PAI-1 function in TGF β -
15 dependent stimulation of MVEC capillary morphogenesis. The percent decrease/increase
16 of capillary morphogenesis is referred to control unstimulated cells taken as 100%. * p <
17 0.05, significantly different from control. The results shown are representative of 3
18 different experiments.

19 **Fig. 4 - Effect of TGF β antagonist peptides on TGF β -dependent angiogenesis *in***
20 ***vitro*.** (A) Activity of TGF β antagonist peptides on MVEC invasion. Data shown
21 represent the mean % modification (\pm SD) of the number of invasive cells with respect
22 to control unstimulated MVEC, obtained in three different experiments performed in
23 triplicate. * p < 0.05, significantly different from control. (B) Activity of TGF β
24 antagonist peptides on MVEC capillary morphogenesis. The panels of the figure show
25 capillary-like organization of MVEC after 24 hours under each experimental condition.

1 The data show a typical experiment out of three experiments performed in triplicate. Six
2 to 9 photographic fields from 3 plates were scanned for each point, considering as 100%
3 the area occupied by untreated MVEC. The data are expressed as percent modification
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5 (\pm SD) with respect to control. * $p < 0.05$, significantly different from control.
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11 **Fig. 5 - TGF β signaling pathways in MVEC and activity of TGF β antagonist**
12 **peptides.** (A) TGF β -stimulated signaling pathways in MVEC. Involvement of signaling
13 pathways was studied following short-term (60 min) and long-term (24 h) stimulation
14 MVEC with TGF β . Molecules of each pathway are shown as total and phosphorylated
15 form (p-). (B) Activity of TGF β antagonist peptides on SMAD2/3 and FAK signaling
16 pathways. In both panels molecular weights are reported in kDa. Results are indicative
17 of 5 different experiments.
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31 **Fig. 6 - Matrigel Sponge Assay (*in vivo* angiogenesis).** (A) Angiogenesis in a Matrigel
32 Sponge Assay by the addition of matrigel containing TGF β (1ng/ml) alone or in the
33 presence of the inhibitory peptides P144 and P17 (100 μ g/ml and 150 μ g/ml
34 respectively). (B) Effect on TGF β -induced angiogenesis of the inhibitory peptides P144
35 and P17 in the presence of heparin. Both peptides significantly blocked the
36 proangiogenic effects of TGF β (graphs are shown as mean \pm SE; *** $P < 0.001$, Student
37 t-test). On the right of the panel: representative photographs of individual Matrigel
38 sponges recovered at autopsy in the experimental conditions shown on the left side of
39 the panel. (C) The effect on TGF β -induced angiogenesis of the inhibitory peptide P17 in
40 the presence of heparin. Both peptides have not effects in absence of proangiogenic
41 factors. The right side of the panel shows representative photographs of individual
42 Matrigel sponges recovered at autopsy shown in the left part of the panel. (D)
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1 experiments shown in panel B. TGF β -induced invasion of inflammatory cells and
2 angiogenesis in the matrigel sponge. In the presence of P144 or P17 the proangiogenic
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4 effects of TGF β are reduced.
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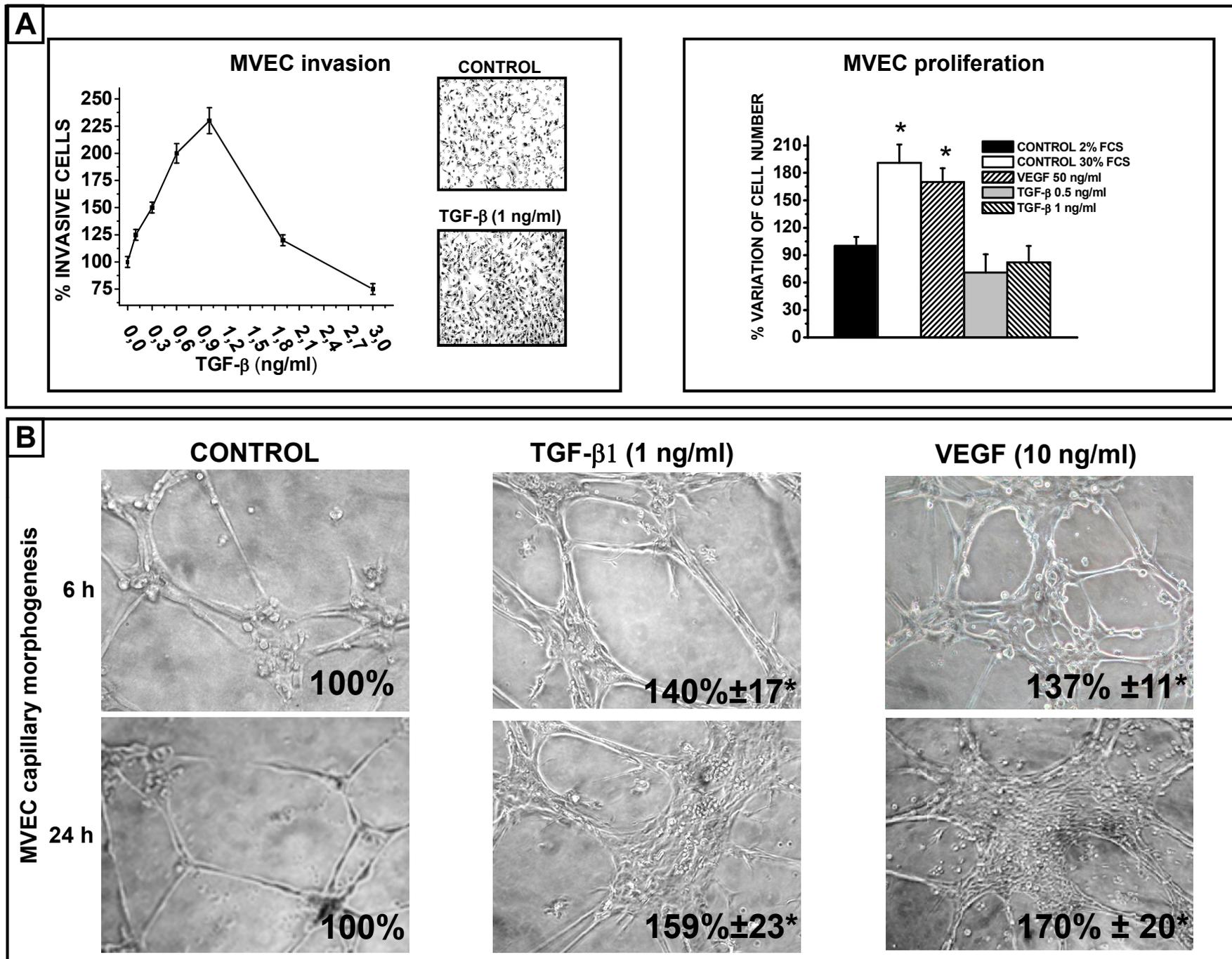


Figure 1

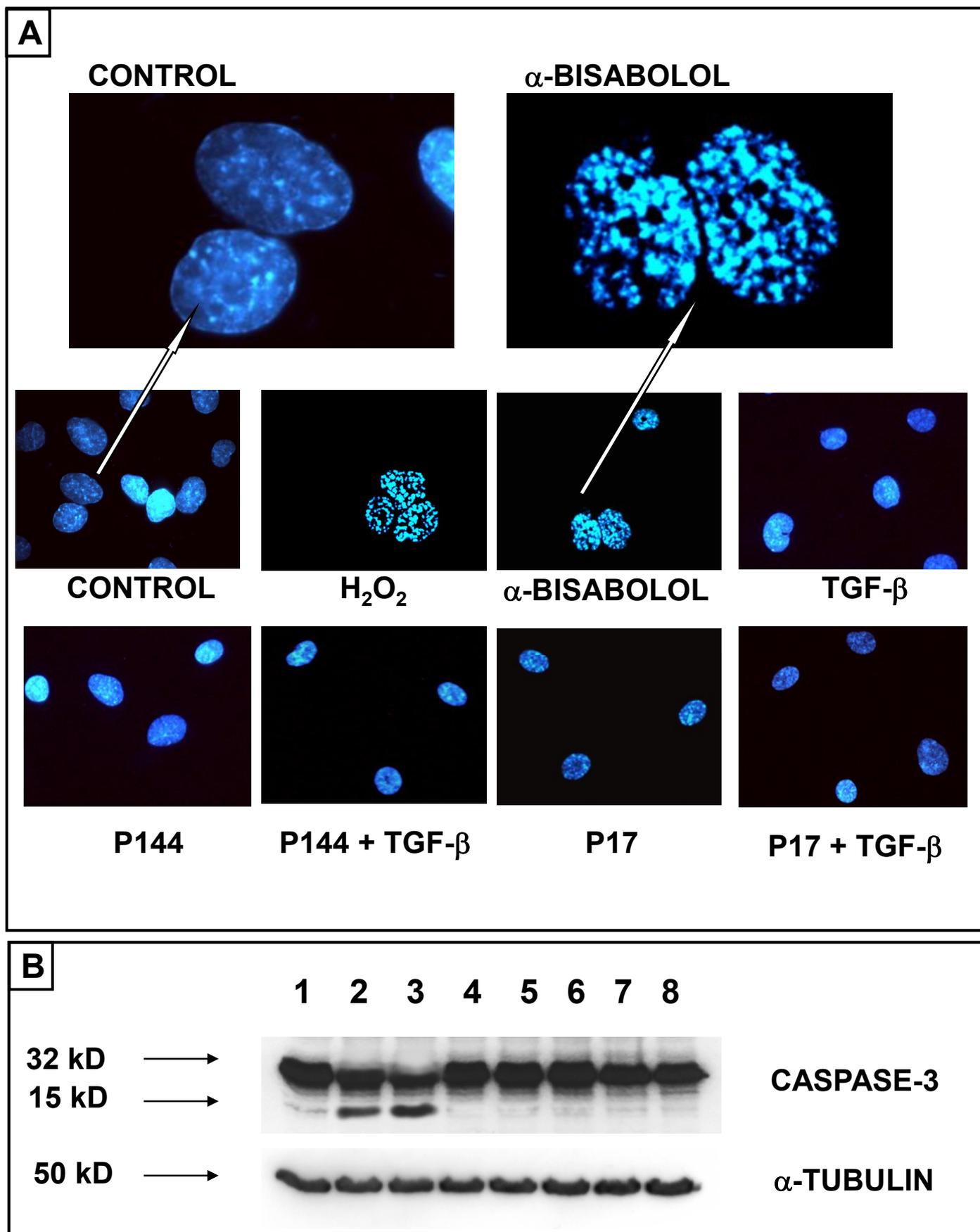


Figure 2

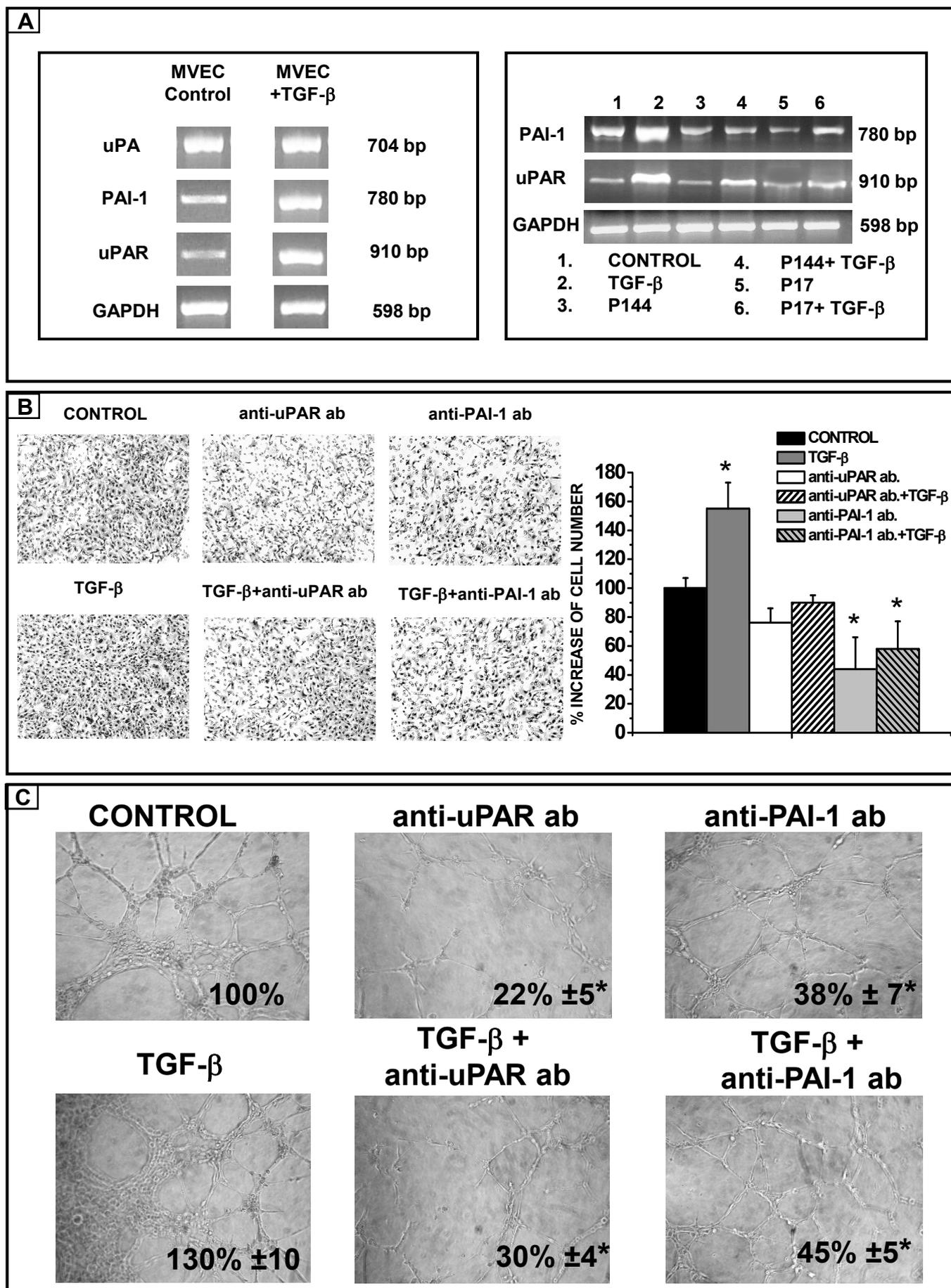


Figure 3

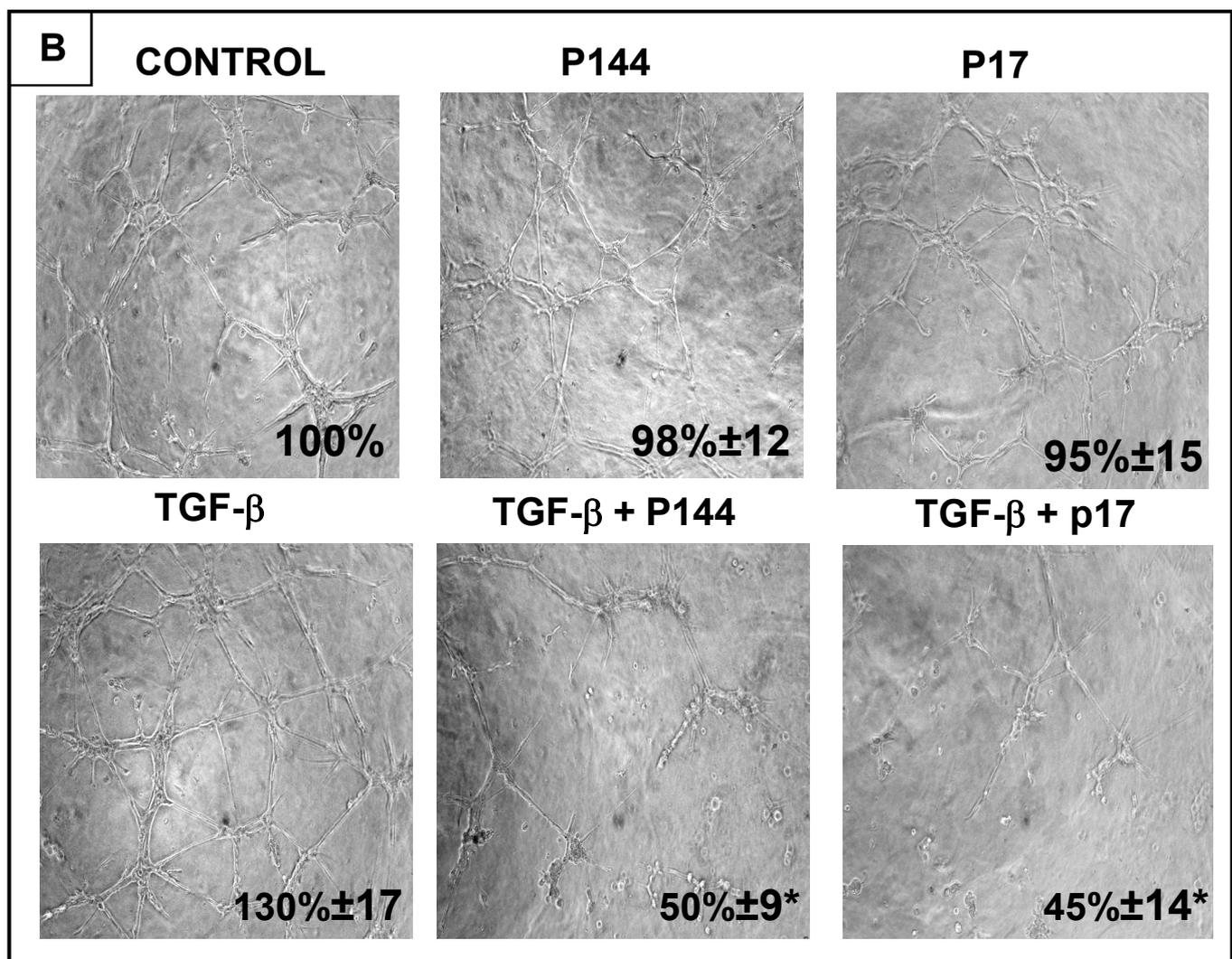
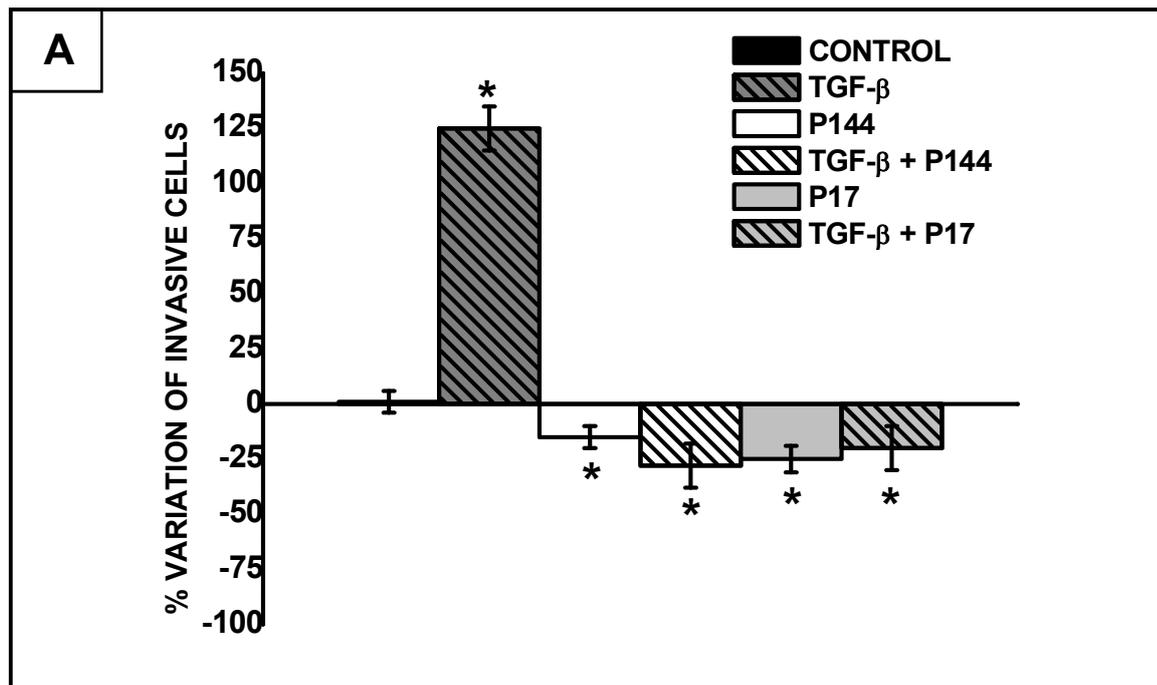


Figure 4

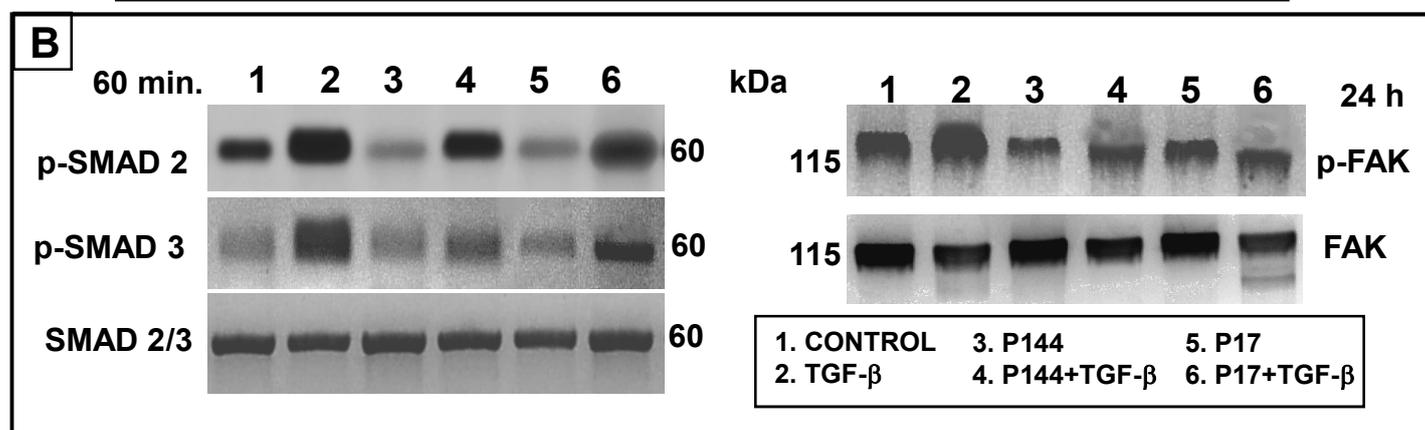
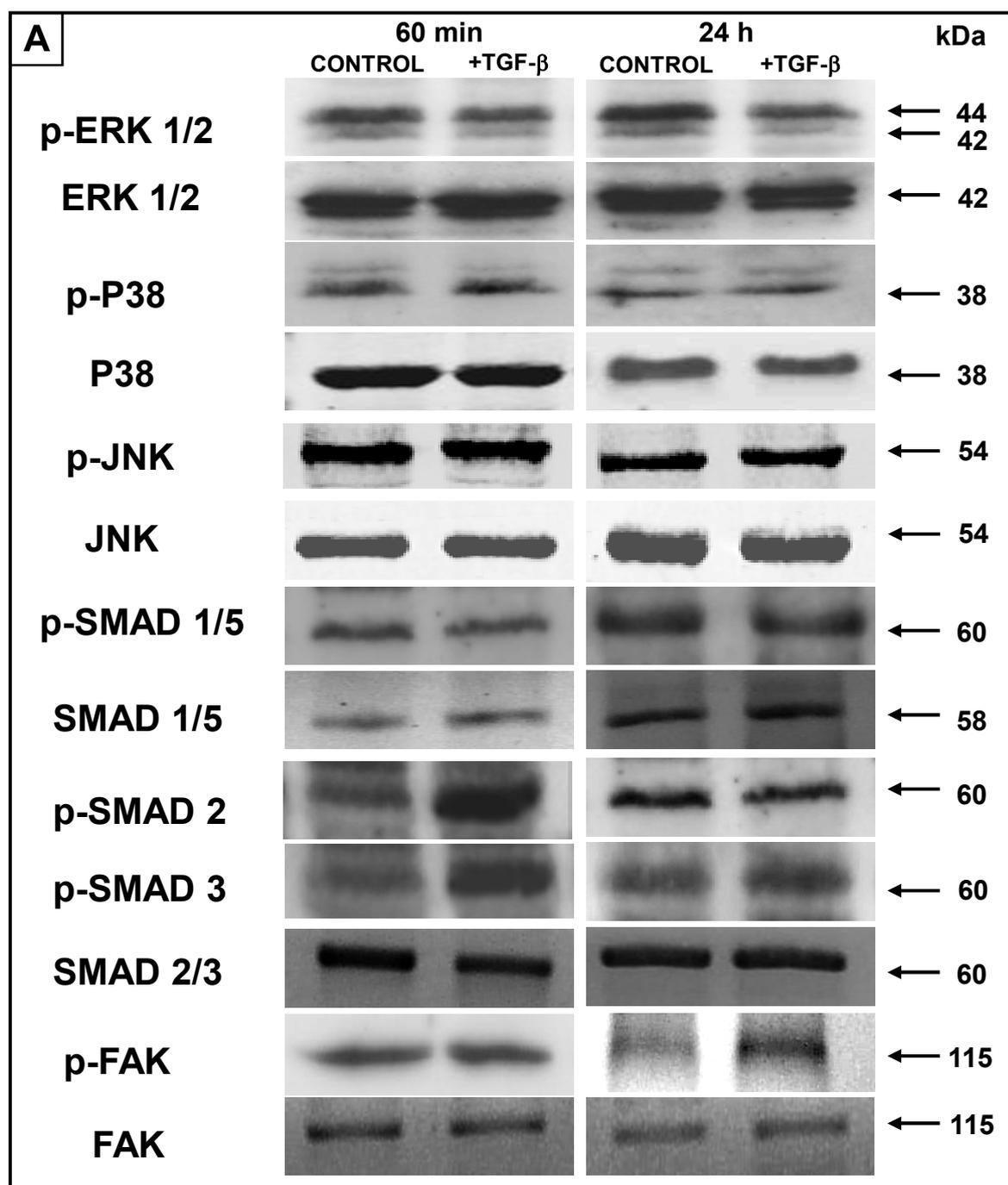


Figure 5

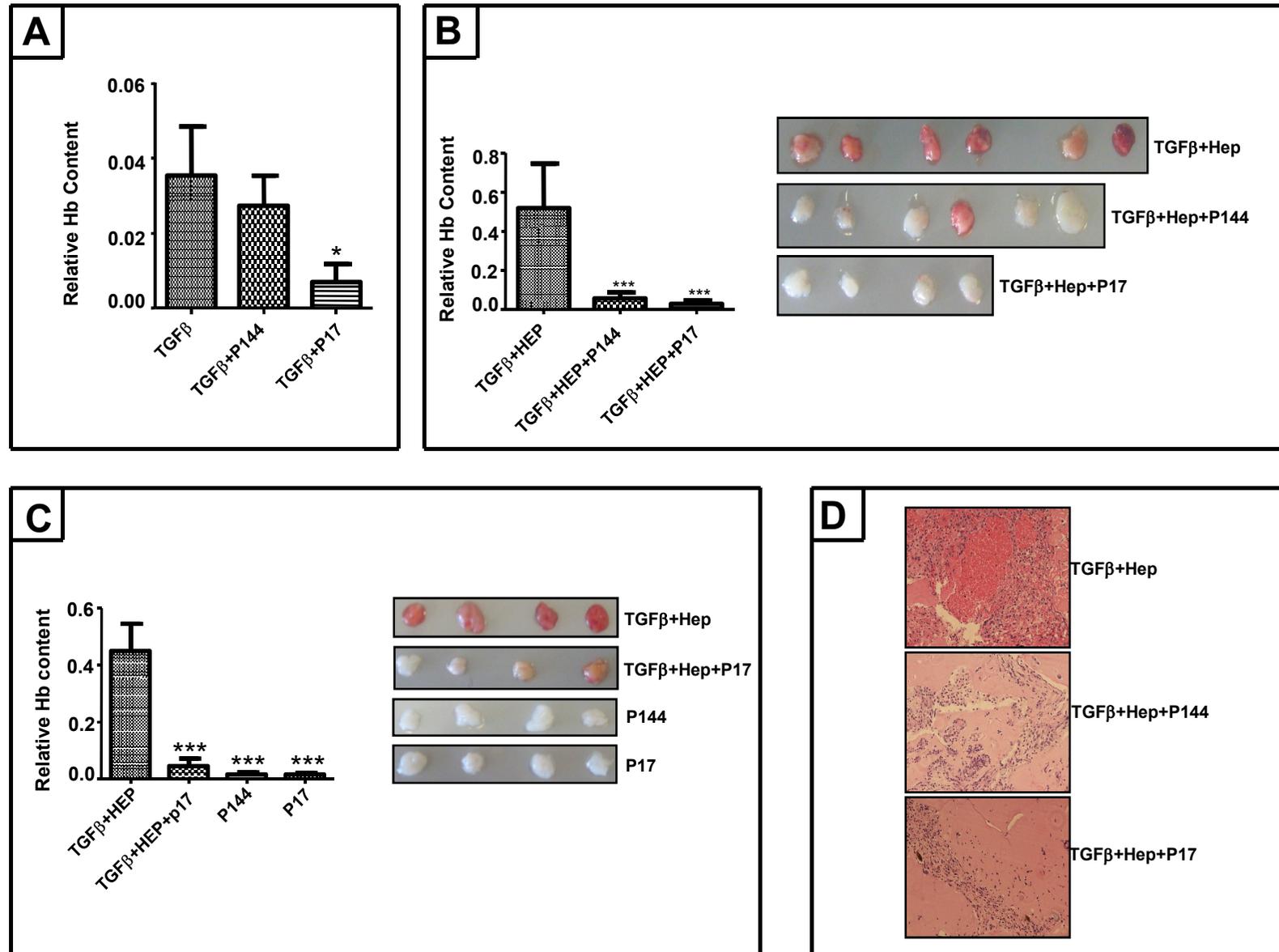


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