Histol Histopathol (2012) 27: 1579-1587
DOI: 10.14670/HH-27.1579

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Expression and localization of VEGF receptors in human fetal skeletal tissues

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Summary. During development the vertebrate skeleton is the product of deriving cells from distinct embryonic lineages. The craniofacial skeleton is formed by migrating cranial neural crest cells, whereas the axial and limb skeletons are derived from mesodermal cells.

The Vascular Endothelial Growth Factors (VEGFs) / receptors (VEGFRs) system plays an important role in angiogenesis, as well as osteogenesis, during bone development, growth, and remodeling, attracting endothelial cells and osteoclasts and stimulating osteoblast differentiation. Recent evidence has shown that during development VEGFR-3 is also expressed in neural and glial precursors of forebrain and cerebellum, as well as in the eye.

In this study, we found that VEGFR-1, VEGFR-2 and VEGFR-3 are expressed in human bone both in fetal and adult life. The gene expression levels were significantly higher in fetal samples especially in mandibles. In addition, higher levels of VEGFR-3 in orofacial district were confirmed by western blotting analysis. We also observed that in fetal mandibular samples VEGFRs colocalized in several osteoblasts, osteoclasts and osteoprogenitor cells. Furthermore, some cells coexpressed VEGFR-3 and ET-1, a marker of neural crest cells. The results demonstrated different expression of VEGFRs in human mandibular and femoral bones which could be correlated to their different structure, function and development during organogenesis. VEGFR-3 might represent a specific signal for ectomesenchymal lineage differentiation during early human development.

Key words: VEGFRs, VEGFR-3, Skeletogenesis, Development, Neural crest

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Introduction

Bone formation during skeletal development involves complex interactions between multiple cell types, tissues and signalling pathways that govern ossification (Colnot, 2005). In particular, the vertebrate skeleton develops from distinct embryonic cell lineages. The axial and limb skeleton originates from mesodermal cells: the axial skeleton is derived from paraxial mesoderm (somites) and the limb skeleton is the product of lateral plate mesodermal cells. The craniofacial skeleton is mostly of neural crest (NC) origin; in fact NC cells migrate from the dorsal aspect of the neural tube into the branchial arches and the frontonasal mass, and contribute to a variety of tissues, including cartilage and bone (Olsen et al., 2000). Cranial neural crest (CNC) cells migrate to the facial region, in which, in response to ecto-mesenchymal interactions, they differentiate into skeletogenic progenitor cells, which form condensations capable of differentiating along either the chondrogenic or osteogenic lineage (Helms and Schneider, 2003; Dupin et al., 2007).

Advances in skeletal genetics and molecular biology have increased the insights into bone development and led to the identification of novel genes and pathways required to build bone (Olsen et al., 2000). These genetic studies were largely the result of experimental animal studies and investigations on humans with inherited disorders in skeletal morphogenesis, organogenesis and growth. It was reported that the organogenetic phase of skeletal development is controlled by transcription factors, extracellular matrix molecules and growth factor-cytokines that play an autocrine-paracrine role in bone morphogenesis (Olsen et al., 2000; Deng et al., 2008).

In this context, current findings showed that the VEGFs/VEGFRs system plays a key role both in angiogenesis and in bone formation (Zelzer et al., 2002;

Filvaroff, 2003). Moreover, this system was shown to be essential in skeletogenesis during development and postnatal life, participating in bone formation, remodeling and repair (Zelzer and Olsen, 2005).

The complexity of VEGFs biology is paralleled by the emerging complexity of interactions between their receptors (Dai and Rabie, 2007). Three different type III tyrosine kinase receptors mediate the biological effects of VEGFs. They are known as VEGFR-1 or fms-like tyrosine kinase-1 (Flt-1), VEGFR-2 or kinase insert domain-containing receptor (KDR) fetal liver kinase-1 (Flk-1), and VEGFR-3 or fms-like tyrosine kinase-4 (Flt-4) (Deckers et al., 2000; Otrock et al., 2007). Each VEGF subtype selectively binds some of these receptors, often with different affinities and selectivities, demonstrating the diversity of their biological functions. In fact, VEGFR-1 binds VEGF-A and VEGF-B, VEGFR-2 binds VEGF-A, -C, -D and -E, and VEGFR-3 binds VEGF-C and -D (Ferrara et al., 2003; Yamazaki and Morita, 2006; Otrock et al., 2007). VEGFR-1 seems to play an important role in vascular maintenance and in the recruitment of endothelial cell precursors during vasculogenesis. Moreover, it was shown that it is also expressed by both osteoblasts and osteoclasts (Tombran-Tink and Barnstable, 2004). In addition, mice that were deficient in VEGFR-1 signaling had very depleted numbers of osteoclasts and osteoblasts, and, consequently, also a deficiency in bone marrow cavity formation. The findings suggested that VEGFR-1 signaling could be essential for osteoblast activity during bone formation and remodeling both in animals and humans (Nakagawa et al., 2000; Mayr-Wohlfart et al., 2002; Dai and Rabie, 2007; Otomo et al., 2007). VEGFR-2 appears to mediate the differentiation and proliferation of endothelial cells and it is expressed in osteoblasts and osteoclasts (Byun et al., 2007). In particular, VEGFR-2 plays an important role during differentiation of mature osteoclasts (Yang et al., 2008). VEGFR-3 is involved in the development of lymphatic and blood vessels and recently seems to be also implicated in the developing forebrain (Le Bras et al., 2006). In fact, the expression of VEGFR-3 was detectable in developing retina (Choi et al., 2010) and rat cerebellum (Hou et al., 2011). VEGFR-3 has also been demonstrated to be involved in bone development: VEGFR-3 was detected in osteoblasts of the bones of newborn mice and primary human osteoblasts (Orlandini et al., 2006).

In this study, the expression of VEGFR-1, VEGFR-2 and VEGFR-3 was investigated in developing and mature human bone from mandibular and femoral bones. The results suggested that the different expression of VEGFRs could be correlated to distinct skeletogenesis of the orofacial and appendicular districts.

Materials and methods

Tissue collection

Human fetal and adult bone biopsies were obtained from 55 donors. The fetal bone fragments, 10 mandibular and 10 femoral bones from 10 to 12 weeks of gestation, were obtained from 20 pregnant women who underwent a therapeutic or voluntary abortion. Informed consent was obtained from each woman. The use of human fetal tissues for research purposes was approved by the Ethical Committee for investigation in humans of the Azienda Ospedaliero-Universitaria Careggi, Florence, Italy (protocol n° 6783-04). The fetal bone fragments were collected and prepared by a stereomicroscope provided with a millimetric scale. In particular, the mandibular body and the diaphysis of femoral bone were isolated.

The adult bone fragments, 20 mandibular and 15 femoral bones, were obtained from 35 subjects (age range: 40-50 years old) during maxillofacial or orthopedic surgery early after trauma without any clinical or radiographic evidence of degenerative pathology. The samples were obtained with written consent from each subject, in accordance with the recommendations of the Ethical Committee on human experimentation (Ronconi et al., 2009).

Some specimens were fixed in formalin solution, routinely processed, embedded in paraffin and utilized for immunohistochemistry and morphological details. For immunofluorescence, RT-PCR and Western blot analyses, other specimens were immediately immersed in liquid nitrogen and then stored at -80°C until use.

Human fetal tissues, such as spinal cord, eye, dermis-hypodermis of head, and dermis-hypodermis of body and human placenta in first quarter of gestation were also analysed by Western blot.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Semiquantitative RT-PCR analysis was carried out as described previously (Tricarico et al., 2002; Gallina et al., 2010). In brief, tissue samples were homogenized in Quiazol Lysis Reagent and total mRNA was extracted using EZ1 RNA universal tissue kit and Biorobot EZ1 System (Qiagen, Milan, Italy), according to the manufacturer's recommendations. Total RNA quantity and quality was measured with Nanodrop-1000 spectrophotometer. Total RNA (200 ng) was reverse transcribed using TaqMan RT-PCR kit (Applied Biosystems, Forster City, CA, USA). Reverse transcription was performed in a final volume of 80 μl containing 500 mM KCl, 0.1 mM EDTA, 100 mM Tris-HCl, pH 8.3, 5.5 mM $MgCl_2$, 500 μM of each dNTP, 2.5 μM of random examers, 0.4 U/μl of RNase inhibitor, 1.25 U/μl of Multiscribe Reverse Transcriptase according to the manufacturer's recommendations. The profile of the one-step reverse transcription reaction was 10' at 25°C, 30' at 48°C and 2' at 95°C. The measurement of VEGFR-1, VEGFR-2 and VEGFR-3 mRNA was performed by using a quantitative realtime PCR method, based on Taqman technology. Probe and primers were provided by Applied Biosystems. For each sample 2.5 μl of cDNA were added to 10 μl of Primers-

Probe Mix. Plates were treated for 2' at 50°C, 10' at 95°C and then submitted to 40 cycles of amplification at 95°C for 15 sec, 60°C for 60 sec in the ABI PRISM 7700 sequence detector (Applied Biosystems). The results for the three genes were expressed as relative expression to GAPDH mRNA with ΔΔCt method. Data are reported as mean ± SD from at least three separate experiments.

Western blot analysis

Tissue samples were homogenized in ice-cold lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton, 0.25% SDS) supplemented with a

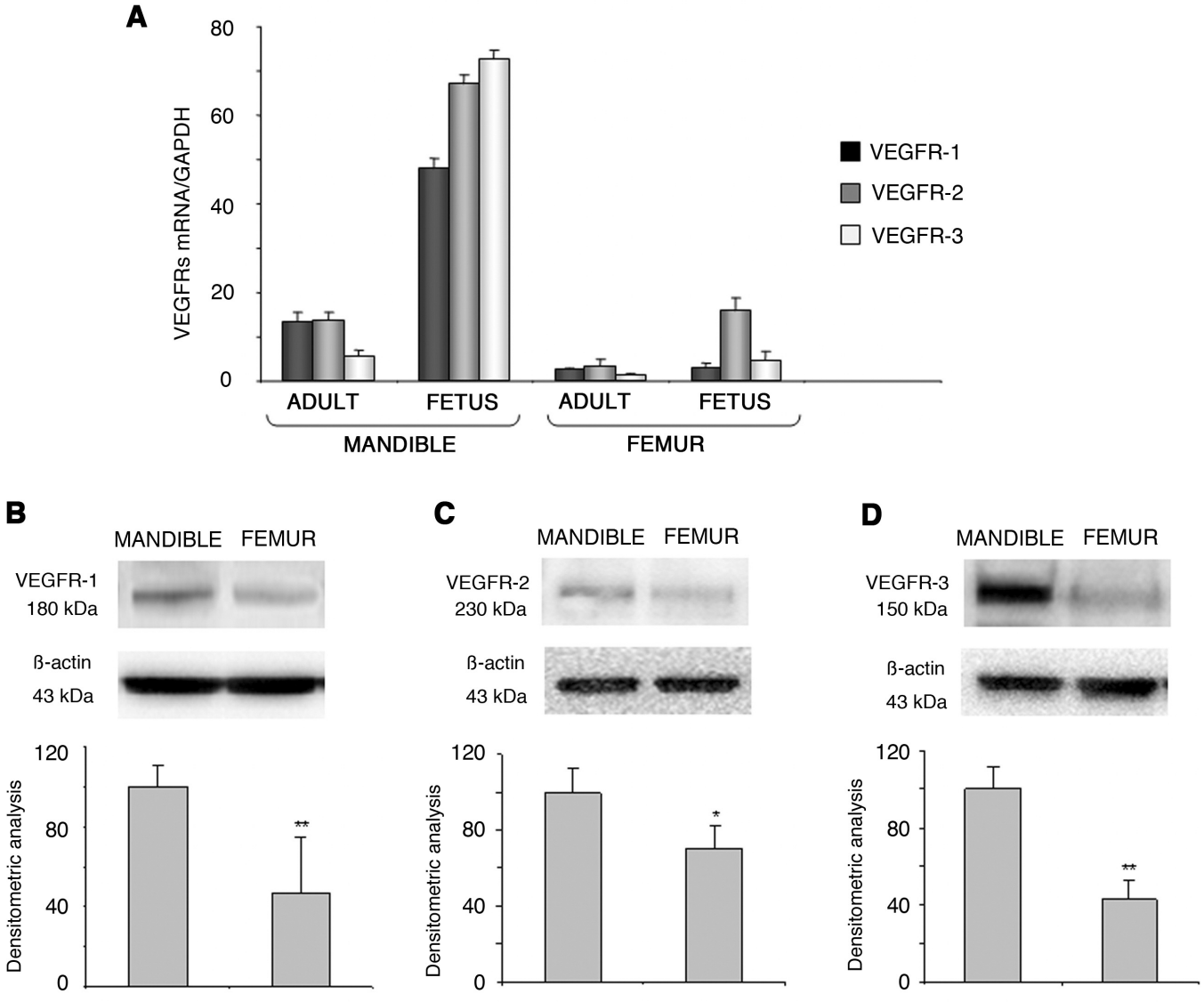

Fig. 1. A. VEGFRs gene expression. Histogram illustrating the mRNA levels of VEGFRs in the human bone samples determined by quantitative RT-PCR, as detailed in materials and methods. Results were calculated according to the comparative cycle threshold method using GAPDH as reference gene for normalization. Data are reported as mean ± SD from at least three separate experiments. Note the significantly higher levels in fetal mandibular tissues compared with adult tissues ($p<0.01$) and fetal femur ($p<0.01$). VEGFR-3 was mostly expressed in fetal mandible ($p<0.01$). **B-D**. VEGFR protein expression in human fetal bone samples. Western blot analysis of fetal bone samples showed a significantly higher expression of VEGFRs in mandible samples compared with femur. The densitometric quantification of bands corresponding to the proteins examined by Western blot was expressed as a percentage over mandibular tissues (taken as 100%). ß-actin signal was used for the normalization of each protein expression. Western blot images are representative of three samples and data are reported as mean ± SD (*$p<0.05$ mandible vs femur; ** $p<0.01$ mandible vs femur).

protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Electrophoresis and protein transfer to membranes were performed as previously described (Crescioli et al., 2008). Membranes were incubated at 4°C overnight with primary antibodies (anti-VEGFR-1 1:800; anti-VEGFR-2 1:500; anti-VEGFR-3 1:1000; anti-ß actin 1:10000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in TTBS, followed by peroxidase conjugated secondary IgG (Santa Cruz Biotechnology). The reacted proteins were revealed by the enhanced chemi-luminescence system (ECL plus; Amersham Bioscience, Little Chalfont, UK). Densitometric analysis was carried out using the Quantity One software (Bio-Rad Labs, Hercules, CA, USA). Data were obtained from three samples of three separate experiments and were expressed as mean ± SD, taking mandible as 100%.

Immunohistochemistry

Immunohistochemical studies were performed on tissue sections, as previously described (Marini et al., 2007; Morelli et al., 2008). Briefly, the slides were stained for indirect immunoperoxidase technique using rabbit polyclonal anti-VEGFR-1 (1:80 dilution; Santa Cruz Biotechnology), mouse monoclonal anti-VEGFR-2 (1:80 dilution; Santa Cruz Biotechnology) or rabbit polyclonal anti-VEGFR-3 (1:100 dilution; Santa Cruz Biotechnology) as primary antibodies. Moreover, mouse monoclonal anti-RANKL (1:200 dilution; Santa Cruz Biotechnology) and anti-osteonectin (1:200 dilution; Santa Cruz Biotechnology), specific markers of bone cells (Undale et al., 2010), were used.

Sections were rinsed in PBS, incubated with the prediluted biotinylated secondary antibody and then with the streptavidin–biotin peroxidase complex following the manufacturer's instructions (Ultravion large volume detection system anti-polyvalent, Lab-Vision, Fremont, CA, USA). The development reaction of the product was performed using 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) as chromogen.

The specificity of the anti-VEGFR antibodies was

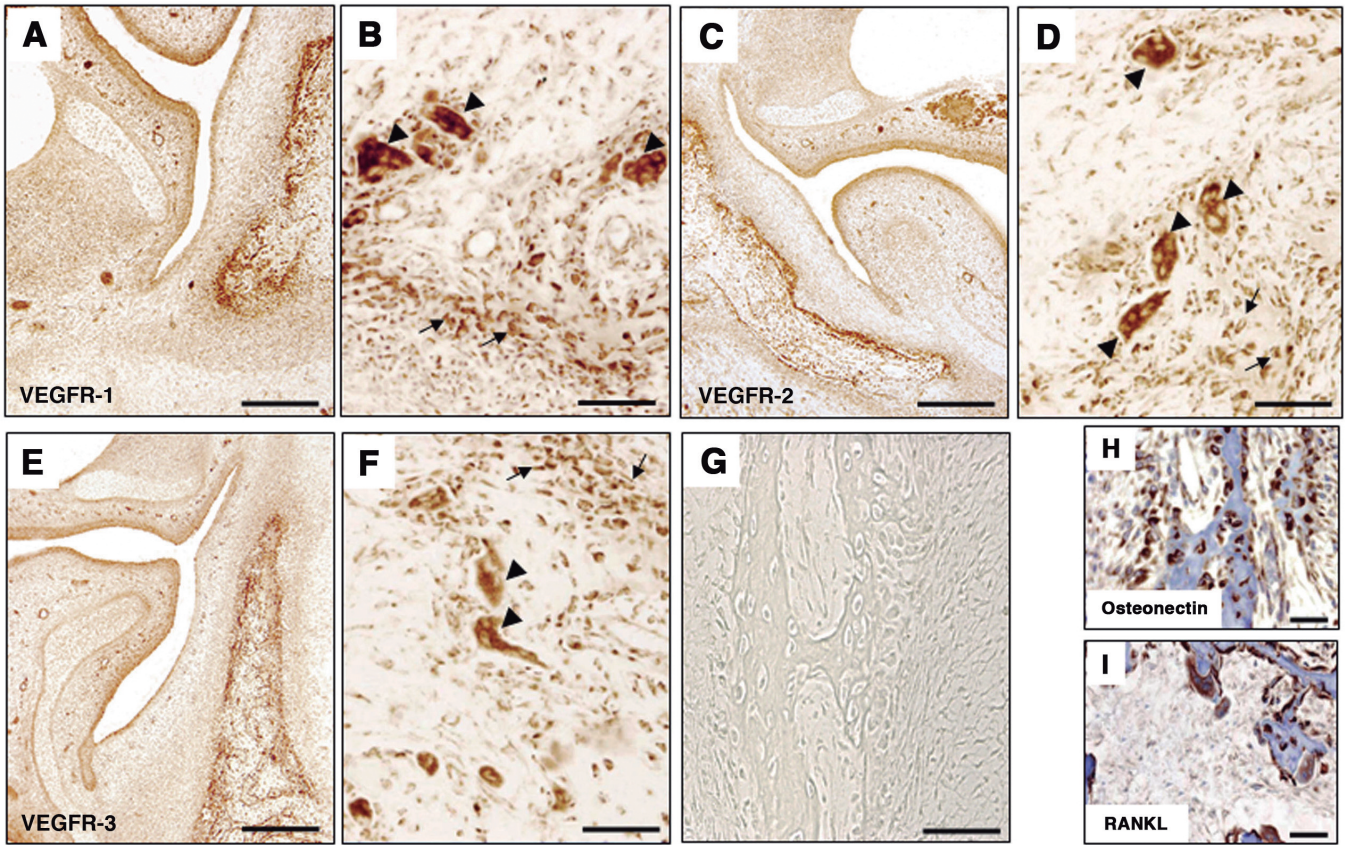

Fig. 2. VEGFR immunolocalization in fetal mandibular samples. VEGFR-1 (**A, B**), -2 (**C, D**) and -3 (**E, F**) immunopositivity is detectable in the osteoblasts (arrows), osteoclasts (arrowhead) and their precursors. Representative micrograph of a 10.5-week-old human fetus. Not counterstained. **G.** Representative micrograph of no immunolabeling when anti-VEGFR-3 antibody was preabsorbed with corresponding peptide (10.5-week-old human fetus). Not counterstained. **H, I.** Immunoreactivity for markers of bone cells: immunopositivity for osteonectin is detected in pre- and osteoblasts **(H)**; in close proximity to bone surfaces, pre-osteoclasts and osteoclasts are positive to RANKL **(I)**. Haematoxylin counterstained. Scale bar: A, C, E, 200 μm; B, D, F-I, 50 μm.

controlled by omitting the primary antibody, and by preabsorption of the primary antibodies with the respective peptides (100 μg/ml) at room temperature for 1h. Third trimester human placenta was used as positive control (Marini et al., 2007). For each antibody, all sections were treated with the same batch to eliminate inter-batch variations. The slides were evaluated and photographed using a Nikon Microphot-FXA microscope (Nikon, Tokyo, Japan).

Confocal laser scanner microscopy

Colocalization studies of VEGFRs expression in human bone cryosections were performed with confocal microscopy, as already described (Chavalmane et al., 2010). Triple immunofluorescence labelling was performed using anti-VEGFR-1, anti-VEGFR-2 and anti-VEGFR-3 antibodies, already described, followed by A-11001 Alexa Fluor 488 goat anti-rabbit IgG (1:200, Molecular Probes, Eugene, OR, USA), R6393 rhodamine red goat antimouse IgG (1:200, Molecular Probes) and Cy5 goat anti-rabbit IgG (1:100, Chemicon, Temecula, CA, USA), respectively. The percentage of positive cells was calculated by counting the number of stained cells over the total cells in at least 15 separate fields per slide.

Double immunostaining was performed using anti-VEGFR-3 and mouse monoclonal anti-ET-1 (1:100, Thermo Fisher Scientific, Rockford, IL, USA) antibodies, and revealed using the following conjugated antibodies: Cy5 and R6393 rhodamine red, respectively. The slides were observed under a Bio-Rad MRC 1024 ES Confocal Laser Scanning Microscope (Bio-Rad), equipped with a 15 mW Krypton/Argon laser source for fluorescence measurements.

Statistical analysis

The results were expressed as mean ± SD. Statistical analysis was performed by the Mann-Whitney test or by ANOVA with post-hoc test (Bonferroni correction), as appropriate. $p<0.05$ was considered significant.

Results

The real time quantitative RT-PCR results of VEGFR gene expression is reported in figure 1A. The results showed that VEGFR mRNAs were present in all the fetal and adult bone samples, but levels were significantly higher in fetal mandibular tissues compared

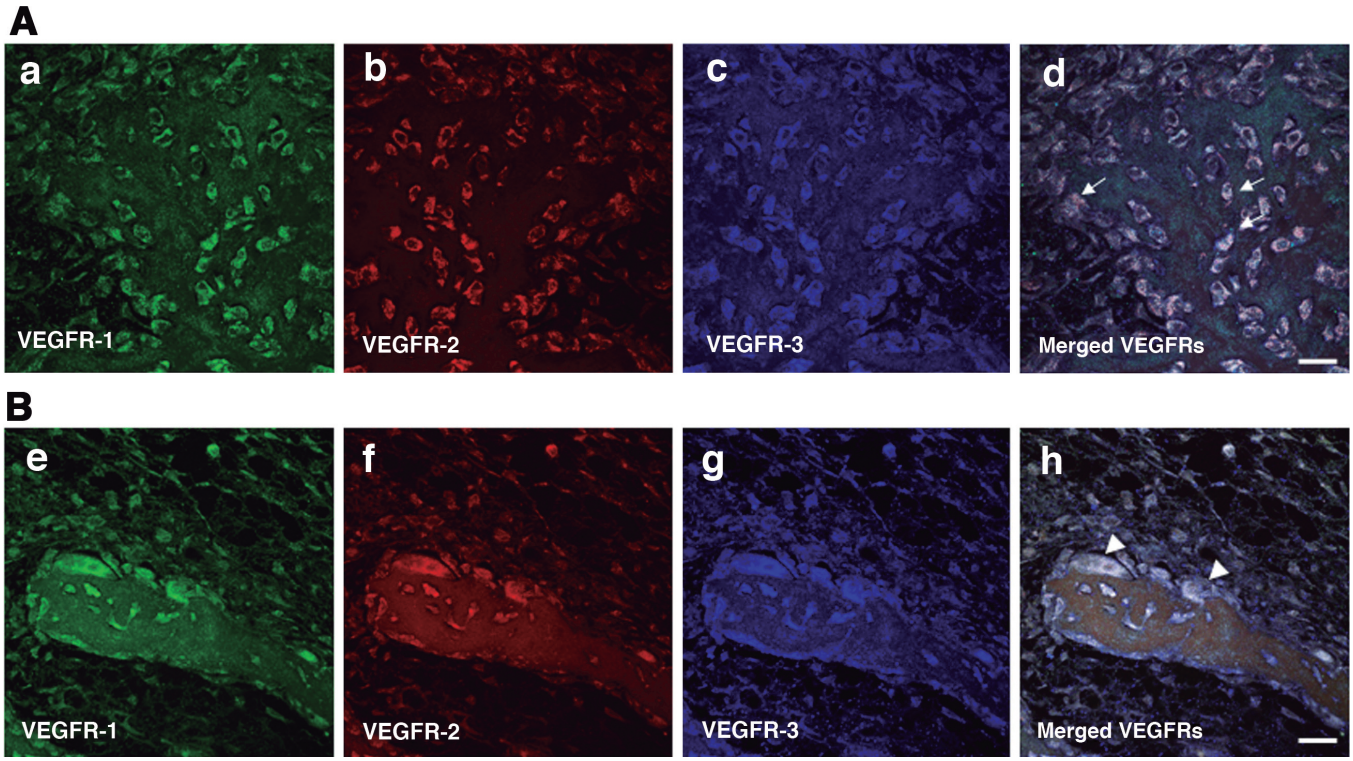

Fig. 3. A, B. VEGFR colocalization in fetal mandibular samples. Coexpression of VEGFR-1 **(a, e)**, -2 **(b, f)** and -3 **(c, g)** in coronal sections of fetal mandible assessed by confocal microscopy. Immunolocalization was performed by triple labelling with specific antibodies (green, A-11001 Alexa Fluor 488 for anti-VEGFR-1; red, R6393 rhodamine for anti-VEGFR-2 and blue, Cy5 for anti-VEGFR-3). In the merged images **(d, h)**, white color indicates colocalization of three receptors in osteoblasts (arrows) **(d)** and osteoclasts (arrowhead) **(h)**. Representative micrograph of mandible sections from a 11-week-old human fetus. Scale bar: 43 μm.

with adult tissues ($p<0.01$) and fetal femur ($p<0.01$). In particular VEGFR-3 was mostly expressed in fetal mandible ($p<0.01$). Levels of VEGFRs were higher in the fetal femur when compared with adult femur ($p<0.01$). mRNA levels of VEGFR-1, -2 and -3 in the adult mandibular samples were significantly higher with respect to adult femur ($p<0.01$).

Differentially expressed VEGFRs in fetal bone tissues, between the mandible and femur, were further confirmed by western blotting analysis. As shown in Fig. 1B,C,D, the densitometric analysis revealed that VEGFR-1, -2 and -3 expression was significantly higher in fetal mandible compared with fetal femur (*$p<0.05$ mandible vs femur; ** $p<0.01$ mandible vs femur).

Immunohistochemical analysis, performed to localize VEGFR expression, demonstrated positivity for VEGFR-1, -2 and -3 in bone cells in fetal mandibular samples (Fig. 2A,C,E); in particular VEGFR expression was observed in osteoblasts, osteoclasts and their precursors (Fig. 2B,D,F). The specificity of antibodies was assessed by incubating sections in antiserum preabsorbed with the corresponding peptide (100 μg/ml) (Fig. 2G). Human placenta was used as positive control (not shown). We used monoclonal antibodies against osteonectin and RANKL to confirm that VEGFR immunopositivity was specific for osteoblasts, osteoclasts and their precursors (Fig. 2H,I). Fig. 2 B,D,F and Fig. 2 H,I showed two populations that were virtually identical. Triple labelling confocal microscopy analysis identified that VEGFR expression was

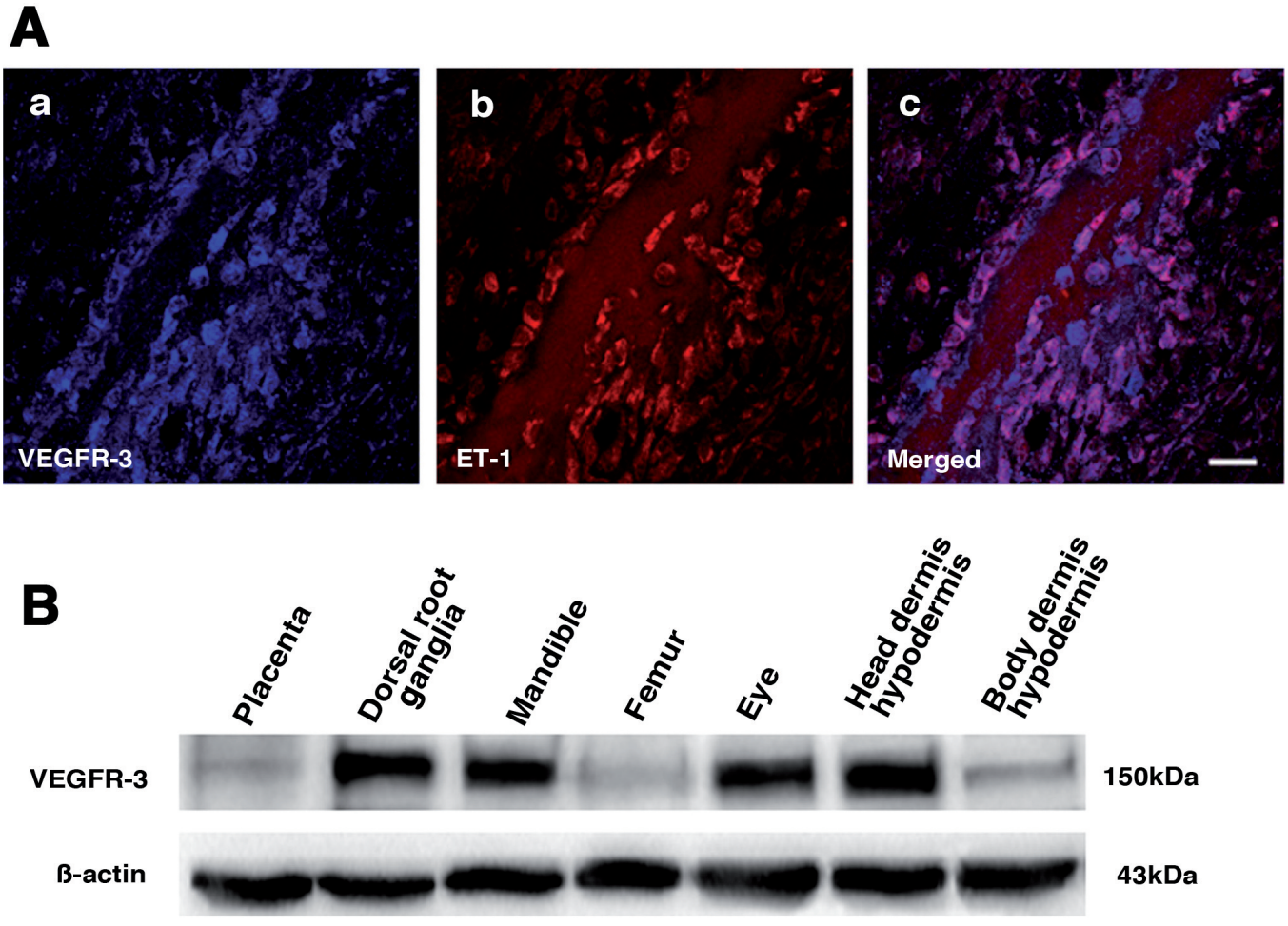

Fig. 4. A. Immunofluorescence study of VEGFR-3 and ET-1 in fetal mandible. Coexpression of VEGFR-3 **(a)** and ET-1 **(b)** in sections of fetal mandible as assessed by confocal microscopy. Immunolocalization was performed by double labelling with specific antibodies (blue, Cy5 for anti-VEGFR-3; red, R6393 rhodamine for anti-ET-1). Colocalization of VEGFR-3 and ET-1 is evident in the merged image (magenta) **(c).** Representative micrograph of a 10-week-old human fetus. Scale bar: 43 μm. **B.** VEGFR-3 western blot analysis of human fetal tissues. VEGFR-3 protein is widely expressed in human fetal tissues of known neuroectodermal origin such as dorsal root ganglia, eye, mandible, dermis-hypodermis of the head and neck; while weak expression is observed in tissues of different embryological origin such as femur and body dermis-hypodermis. Human placenta in first quarter of gestation was used as positive control. Data were obtained from tissues of three different fetuses. Representative experiment of a 10.3-week-old human fetus.

colocalized in bone cells (30% ± 2,3%) (Fig. 3A,B).

Because ET-1, widely expressed in craniofacial tissues, is a marker of NC origin, we evaluated its possible coexpression with VEGFR-3. Double labelling confocal microscopy analysis showed colocalization of VEGFR-3 and ET-1 in some bone cells and in their precursors (Fig. 4A). VEGFR-3 was also widely expressed in human fetal tissues of known neuroectodermal origin such as dorsal root ganglia, eye, mandible and dermis-hypodermis of the head and neck (as shown in Fig. 4B).

Discussion

Previous studies have shown that angiogenesis, as well as osteogenesis, are closely associated processes during bone development, growth, and remodeling, sharing some essential mediators, including VEGFs/VEGFRs (Pufe et al., 2003; Dai and Rabie, 2007; Reumann et al., 2010; Thi et al., 2010).

We demonstrated for the first time that VEGFR-1, -2 and -3 are differentially expressed in the human fetal and adult bone. The gene expression levels were significantly higher in fetal samples, especially in mandibular tissues, and VEGFR-3 was mostly expressed. The higher expression of VEGFRs in fetal bone, compared with adult, could be explained by a major and continuous remodeling process during skeletogenesis. Skeletal development is a complex event, synchronized by a large number of secreted and intracellular factors (Colnot, 2005; Deng et al., 2008). Some of these factors primarily control cartilage differentiation, while others regulate bone formation and/or angiogenesis (Colnot, 2005). Previous studies have shown that the VEGFs/VEGFRs system is an integral part of bone formation and turnover in the growing skeleton (Horner et al., 2001). Moreover, bone formation is a continuous process that begins during fetal development, but is known to persist through life as a remodeling process. This has suggested that the regenerative capacity of adult bone may depend upon re-induction of molecular pathways that mediate chondrogenesis and osteogenesis during fetal development (Ferguson et al., 1999; Axelrad et al., 2007). Therefore, the presence of VEGFR expression in adult bone, although lower with respect to fetus, could play a role in the remodeling process and represent a hypothetical reserve of regulators in healing processes.

Another interesting feature is the increase of VEGFR levels in fetal mandible, with respect to femur. These data were confirmed by western blotting analysis. This might be correlated to their different development during organogenesis. Embryologic development knowledge has revealed that the molecular mechanisms controlling skeletogenesis in the orofacial bones are unique and different from those in the axial and appendicular bones (Yamaza et al., 2011). The orofacial skeletal components have been considered to develop from migrating CNC cells, whereas the axial and limb skeleton are derived from lateral plate mesodermal cells (Chai and Maxson, 2006; Yamaza et al., 2011). The NC cells are a pluripotent population that migrates extensively and gives rise to a vast array of cell types, tissues and organs. In the head and the neck, the NC cells form the craniofacial cartilages, bones, dermis, adipose tissue, and vascular smooth muscle cells, but also differentiate into neurons and glial cells of the peripheral nervous system, melanocytes and endocrine cells in the adrenal and thyroid glands (Trainor et al., 2003; Helms et al., 2005; Dupin et al., 2007). Researchers are questioning if the mechanisms that control skeletogenesis in the craniofacial region are the same elsewhere in the body. The answers lie in the molecular machinery that generates NC cells, controls their migration, and guides their differentiation to cartilage and bone (Helms and Schneider, 2003). Several factors play a role in this process: FGF, TGF-ß, VEGF (Colnot, 2005; Helms et al., 2005). To our knowledge, this is the first report on VEGFR-3 expression in human mandibular tissue. Immunohistochemical analysis demonstrated positivity for VEGFR-3, but also -1 and -2, in bone cells of fetal mandible. In particular, VEGFR expression was observed in osteoblasts, osteoclasts and their precursors. In addition, triple labelling confocal microscopy analysis identified that VEGFR expression was colocalized in several bone cells.

Some researchers have demonstrated a newly discovered role for VEGFR-3 during development: in rat and murine, it was expressed in neural and glial precursors of forebrain and cerebellum, but also in the eye (Maurer et al., 2003; Choi et al., 2010; Hou et al., 2011). Since in the orofacial tissues some cells coexpressed VEGFR-3 and ET-1, a marker of NC (Kurihara et al., 1994; Barni et al., 1998; Ozeki et al., 2004), VEGFR-3 expression might be used as a marker to distinguish those cells of the NC lineage committed to becoming osteogenetic cells. VEGFR-3 expression in fetal mandible was similar to that of human tissues, of known neuroectodermal origin, whereas it was down-regulated in those structures of different embryological origin. We hypothesized that VEGFR-3 might represent a specific signal for ectomesenchymal lineage differentiation during early human development.

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Accepted June 22, 2012