Expression of Semaphorin-3A and its receptors in endochondral ossification: potential role in skeletal development and innervation.

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ABSTRACT

Bone tissue is densely innervated, and there is increasing evidence for a neural control of bone metabolism. Semaphorin-3A is a very important regulator of neuronal targeting in the peripheral nervous system, as well as angiogenesis, and knockout of the Semaphorin-3A gene induces abnormal bone and cartilage development. We analysed the spatial and temporal expression patterns of Semaphorin-3A signaling molecules during endochondral ossification, in parallel with the establishment of innervation. We show that osteoblasts and chondrocytes differentiated in vitro express most members of the Semaphorin-3A signaling system (Semaphorin-3A, Neuropilin-1, Plexins-A1 and -A2). In vitro, osteoclasts express most receptor chains but not the ligand. In situ, these molecules are all expressed in the periosteum and by resting, pre-hypertrophic and hypertrophic chondrocytes in ossification centers before the onset of neurovascular invasion. They are detected later in osteoblasts and also osteoclasts, with differences in intensity and regional distribution. Semaphorin-3A and Neuropilin-1 are also expressed in the bone marrow. Plexin-A3 is not expressed by bone cell lineages in vitro. It is detected early in the periosteum and hypertrophic chondrocytes. After the onset of ossification, this chain is restricted to a network of cell processes in close vicinity to the cells lining the trabeculae, similar to the pattern observed for neural markers at the same stages. After birth, while the density of innervation decreases, Plexin-A3 is strongly expressed by blood vessels on the ossification front. In conclusion, Semaphorin-3A signaling is present in bone and seems to precede or coincide at the temporal but also spatial level with the invasion of bone by blood vessels and nerve fibres. Expression patterns suggest Plexin-A3/Neuropilin-1 as a candidate receptor in target cells for the regulation of bone innervation by Semaphorin-3A.

INTRODUCTION

Early work as well as recent immunocytochemical studies have highlighted the rich innervation of bone (Calvo and Forteza-Vila, 1969; Serre et al., 1999). The expression of neuromediators in skeletal nerve fibres, and of their receptors in osteoblasts and osteoclasts has been established. The presence in bone of cell processes labeled with neural tip markers like synaptophysin has also been documented. Overall, these data provide evidence for the existence of functional nerve fibres within the bone tissue (Gronblad et al., 1984; Hohmann et al., 1986; Bjurholm et al., 1988; Serre et al., 1999). Some of these nerve fibres are in contact with cells lining the trabeculae (osteoblasts and osteoclasts), suggesting a direct control of bone cell function (Serre et al., 1999). Several studies have indeed shown evidence for a neural control of bone growth. Developmental growth in the rat foot is reduced after surgical denervation (Edoff et al., 1997). The remodelling of bone is also affected by surgical or chemical sensory and sympathetic denervations in rat (Sandhu et al., 1987; Hill et al., 1991; Cherruau et al., 1999). Furthermore, a leptin-dependent regulation of bone mass by the central nervous system has been demonstrated in mice, which appears to be mediated by sympathetic innervation (Ducy et al., 2000; Takeda et al., 2002). The role of sympathetic innervation in bone remodelling is also supported by the changes in bone mass observed in mice deficient in genes coding for dopamine β-hydroxylase, dopamine transporter and Y2 receptors (Bliziotes et al., 2000; Baldock et al., 2002; Takeda et al., 2002).

As early as embryonic day (E)15, nerve fibres appear in the perichondrium of the diaphysis of rat long bones. They become functional at E18-19, when neural tips are formed and neuropeptide expression starts (Sisask et al., 1995; Sisask et al., 1996). There is little understanding of the molecular mechanisms by which neurons reach their targets in bones. In the central nervous system, many studies described several families of proteins which are

involved in wiring and allow axons and dendrites to locate and reach their appropriate targets. The most prominent families of neural signaling molecules are Semaphorins, Netrins, Slits and Ephrins. The Semaphorins, a large family of cell surface and secreted guidance molecules, is one of the best understood. In particular, it has been shown that Semaphorin-3A (Sema-3A) signaling is very important for neuronal targeting in the peripheral nervous system (PNS), as shown for trigeminal and dorsal root ganglia which are involved in bone innervation (Kitsukawa et al., 1997; Taniguchi et al., 1997; Ulupinar et al., 1999). Sema-3A, a soluble molecule, signals through multimeric receptors always combining at least one of the 3 plexins (Plx) and neuropilin-1 (NP-1). NPs do not appear to have a signaling function. Plx contribute to both ligand specificity and signal transduction, activating RhoA through poorly known mediators (Goshima et al., 2002). Recently the expression of Sema-3A and NP-1 was demonstrated in osteoblast and osteoclast cell lines, and NP-1 was shown to be expressed in bone (Togari et al., 2000; Harper et al., 2001). Furthermore, abnormal bone and cartilage development, with vertebral fusions and partial rib duplication, has been observed after a targeted mutation of Sema-3A in mouse, indicating that this signaling pathway may play a role in bone development (Behar et al., 1996).

The aim of our study was to investigate the expression of the molecules in the Sema-3A signaling network during skeletal development, focusing on the spatial and temporal relationship with nerve fibre growth.

RESULTS

Multiple bone cell lineages express components of the Semaphorin axon guidance system

Messenger RNA for Sema-3A, NP-1, Plx-A1 and Plx-A2 but not Plx-A3 were detected in osteoblastic cells. They were also present, with the exception of Sema-3A, in differentiated osteoclasts (fig. 1A). Sema-3A, NP-1 and Plx-A1 and -A2 mRNA are expressed at all stages of chondrocyte differentiation, including the final hypertrophic stage, reached under the highest dose of BMP-2 (fig. 1B). Plx-A3 was not detectable in chondrocytes at any stage. As shown on figure 2, NP-1, Plx-A1 and A2 proteins are expressed by both osteoblasts and osteoclasts. Sema-3A expression was only detected in osteoblasts, confirming RT-PCR data. Only a very faint staining was observed in MC 615 cells for all markers (not shown).

Sema-3A and its receptor chain, NP-1, have the same pattern of expression during endochondral ossification

We observed that Sema-3A and NP-1 displayed nearly identical patterns of expression at all stages of bone development. At E17 Sema-3A (fig. 3C) and NP-1 (not shown) were detected in the outer perichondrium of the diaphysis and metaphysis, and in the pre-hypertrophic and hypertrophic chondrocytes within the future primary ossification center (fig.3 C, enlargement in D). Staining was also present in resting chondrocytes within the epiphysis, the site of the future secondary ossification center (fig. 3C). At E19 NP-1 (fig. 3F) and Sema-3A (not shown) presented a patchy staining in the periosteum of the diaphysis, restricted to the area lining the primary ossification center. At this stage NP-1, but not Sema-3A, was also expressed in the bone collar (fig. 3F) and in blood vessels in the primary ossification center (Fig. 3G). At E21, staining for Sema-3A (fig. 3H) and NP-1 (not shown) was located in

osteoblasts and in the bone marrow and blood vessels, in trabecular bone and only for Sema-3A in the endosteum (fig. 3H). NP-1 (fig. 3J) and Sema-3A (fig. 3K) at P14 were both still located in osteoblasts lining the bone trabeculae but only under the growth plate. Both molecules were also expressed in pre-hypertrophic and hypertrophic chondrocytes of the growth plate (NP-1: Fig. 3L, enlarged in M and M; Sema-3A: data not shown). At P14, Sema-3A (fig. 3O) and NP-1 (fig. 3P) were also detected in large multinucleated (DAPI staining of nuclei in insets) osteoclasts along the trabeculae under the growth plate.

Co-receptors Plexins A1 and A2 are expressed by chondrocytes and osteoblasts

At E17, Plx-A1 was expressed in the perichondrium of the diaphysis and the metaphysis (fig 4A), in hypertrophic chondrocytes at mid-diaphysis and also in the resting chondrocytes in the epiphysis (not shown). Plx-A1 was still present at E19 in hypertrophic chondrocytes (fig 4B), especially those facing the osteogenic perichondrium. It was expressed at lower levels in the periosteum and by bone collar forming osteoblasts (4B, short arrows) and in the primary ossification center (not shown). Plx-A1 was expressed by osteoblasts lining all bone trabeculae at E21 (fig. 4C, arrows) and only those under the growth plate at P14 (fig. 4D, arrows). At the latter stage, it was also expressed by periosteal osteoblasts (fig. 4D, arrowheads), by some osteocytes (fig. 4D, dotted line circles) and in osteoclasts (fig. 4E). At E17, Plx-A2 was expressed in hypertrophic chondrocytes, in resting chondrocytes of the epiphysis and also at lower levels in the perichondrium (fig 4F). At E19, staining extended to the primary ossification center (not shown). At E21, Plx-A2 (Fig. 4G) was present in the periosteum (short arrows) and in some osteoblasts (long arrows) and osteocytes (dotted line circles). At P14, expression of Plx-A2 (fig 4H) was mostly restricted to endosteal (arrows) and periosteal osteoblasts (short arrows) and osteocytes (dotted line circles). At this stage it

was also detected in a few osteoblasts and in osteoclasts under the growth plate (fig. 4I). Both markers were also expressed by other cell types in the marrow and the periosteum (4D, H).

Plx-A3 presents a restricted pattern of expression during endochondral ossification

At E17, Plx-A3 was expressed in the perichondrium of the metaphysis (fig. 5A), where some profiles seemed to outline blood vessels (5A, arrows), and by hypertrophic as well as some resting chondrocytes (not shown). Staining at E19 (fig. 5B) was located in discrete profiles within the periosteum at the mid-diaphysis, some of which suggestive of vascular staining (5C, arrow). Plx-A3 was also detected in the bone collar (5D) and in hypertrophic and at lower levels in resting chondrocytes (not shown). No labeling was observed at this stage within the primary ossification center. At E21, Plx-A3 was expressed in the secondary spongiosa as a loose network along bone trabeculae and in endothelial cells (fig. 5E, enlarged in F). It was also present in the periosteum and hypertrophic and some resting chondrocytes (not shown). At P14, Plx-A3 was strongly expressed in endothelial cells of vascular buds in the ossification front under the growth plate (fig. 5G and H), and in capillaries in the metaphysis (fig. J).

The pattern of expression of neural cell markers in developing rat femur parallels the development of endochondral ossification

To correlate the in vivo expression of Sema-3A and its receptor subunits with the appearance of nerve fibres, we analysed the expression of nerve markers during bone development. Neurofilament heavy chain (NH) is a general nerve marker, while tyrosine hydroxylase (TH), an enzyme involved in the synthesis of catecholamines such as noradrenalin, is a specific marker for sympathetic nerves fibres. The staining patterns of both markers were identical at all development stages, although TH gave a higher background at late stages. At E17, both

markers were expressed only in the perichondrium of the metaphysis (fig. 6A, F). At E19, NH and TH positive fibres were found in the periosteum of the diaphysis along the primary ossification center (fig. 6B, G). At this stage, we also found NH and TH labeling in the bone collar (fig. 6B, G, long arrows). At E21, the nerve fibres were located along the bone trabeculae in the diaphysis (fig. 6C, H, short arrows), in close vicinity to osteoblasts and lining cells. At P14, labeling was weaker in bone and was still present in the periosteum of the diaphysis (NH in fig. 6E, not shown for TH).

DISCUSSION

Three functional receptor complexes have been described for Sema-3A: NP-1/Plx-A1, NP-1/Plx-A2, NP-1/Plx-A3 (Nakamura et al., 2000; Raper, 2000). These complexes also bind other ligands in a competitive fashion: Sema-3B, 3C and 3F, and vascular endothelial growth factor-165 ($VEGF_{165}$). Target genes and signal transduction pathways for this signaling have not been fully elucidated, but in neural cells are known to involve the reorganisation of the cytoskeleton via Rho GTPases, which enables axonal guidance (Castellani and Rougon, 2002). In this study, we show that all components of the Sema-3A signaling system are expressed in mature stages of all 3 bone cell lineages (chondrocytes, osteoblasts and osteoclasts), with the exception of Plx-A3. Although osteoclastic cells from RAW 264.7 cultures do not express Sema-3A, we found labelling in resorbing osteoclasts within the bone tissue. Others reported its expression in osteoclast-like cells from human bone marrow cultures (Togari et al., 2000). This may reflect specificities of immortalised cell lines and/or culture conditions, which could also explain the low levels/absence of Sema-3A signaling proteins in the MC 615 chondrogenic cells, in spite of messenger expression. It was previously reported that MC3T3-E1 osteoblastic cells express NP-1 mRNA and that several human osteoblastic cell lines express Sema-3A mRNA and protein (Togari et al., 2000; Harper et al., 2001). Expression of NP-1 mRNA was also shown in human osteoarthritic cartilage (Enomoto et al., 2003). To the best of our knowledge we are the first to show the expression of Plexins-A1 and –A2 in osteoblasts, osteoclasts and chondrocytes, of NP-1 in osteoclasts, and of Sema-3A in chondrocytes.

During bone development, we first observed Sema-3A and all components of its receptor system in pre-hypertrophic and hypertrophic chondrocytes in ossification centers, and also in the facing periosteum, before and after the onset of endochondral ossification and vascular

invasion. We found in particular that Plx-A1 is more strongly expressed by the outermost layer of chondrocytes of the mid-diaphysis, in direct contact with the osteogenic perichondrium (fig. 4B). Such "borderline" chondrocytes have been suggested to interact with perichondral osteoblasts for the regulation of ossification (Bianco et al., 1998). Thus, Sema-3A signaling precedes or coincides with the invasion of bone by blood vessels and nerve fibres (Table 2). At later stages, Sema-3A and its receptor components (except Plx-A3) are co-expressed by osteoblasts and osteoclasts, with regional differences. Such co-expression is compatible with an autocrine/paracrine pathway, such as has been shown for instance in breast carcinoma cells (Bachelder et al., 2003). However, expression of Sema-3A receptors in bone is not restricted to osteoblast and osteoclasts, but is also found in blood vessels and probably nerve processes. Thus, Sema-3A signaling molecules are in a position to modulate the vascularisation of bone, and the innervation of osteoblasts and osteoclasts during bone development and remodelling.

In keeping with a number of recent studies showing evidence that bone modelling and remodelling are controlled by the nervous system (Sandhu et al., 1987; Hill et al., 1991; Edoff et al., 1997; Cherruau et al., 1999; Takeda and Karsenty, 2001; Takeda et al., 2002), we previously showed a dense innervation of bone in close contact with osteoblasts and osteoclasts, although no typical synapses were observed (Serre et al., 1999). We observed here the same labeling pattern and density for NH, a general nerve marker and TH, a specific marker for sympathetic fibres. This suggests that the sympathetic system, which is known to innervate blood vessels and suspected to play a part in the regulation of bone remodelling, is the major one in bone, at least at the stages that we investigated (Ducy et al., 2000; Takeda et al., 2002; Levasseur et al., 2003). In ossified areas, we found staining for NP-1 not only in osteoblasts and osteoclasts, but also in endothelial cells and within the bone marrow. Such generalised expression did not allow identification of a "network" pattern similar to nerve

markers; nonetheless, expression of NP-1 in nerve processes is amply documented in the literature (Yu and Bargmann, 2001). In contrast, the expression pattern of Plx-A3 in developing bone strikingly parallels the one observed for nerve markers from E17 to E21. It is, however, also localised in the endothelial cells of capillaries, particularly at the ossification front in later stages. Peripheral nerves and blood vessels have similar patterns of development in the forelimb, and Sema-3A may function as a common signal molecule for the establishment of the two systems (Bates et al., 2003). Our unablility to detect Plx-A3 expression within the primary ossification center at E19 could be explained by a down-regulation allowing vascular penetration (see below). Overall, our observations suggest that Plx-A3 is the candidate partner of NP-1 for the regulation of neurovascular patterning by Sema-3A.

Sema-3A has been shown in numerous studies to be a repulsive neural guidance cue, crucial for the normal patterning of nerves fibres in vivo (Schwarting et al., 2000; Kawasaki et al., 2002). However, this action can be counteracted by competitive inhibitors. For instance, the competition for NP-1 occurring between Sema-3A and $VEGF_{165}$, which is expressed by human hypertrophic, but not proliferative chondrocytes of the growth plate (Petersen et al., 2002), seems to play a key role in mechanisms of innervation and angiogenesis. The effect of $VEGF_{165}$ on both peripheral nerves and blood vessels opposes the effect of sema-3A (Nakamura et al., 2000). $VEGF_{165}$ enhances endothelial cell survival and mobility and increases axonal outgrowth and neural survival in vitro (Castellani and Rougon, 2002; Petersen et al., 2002; Enomoto et al., 2003). In contrast, Sema-3A inhibits endothelial cells mobility and survival, as well as axonal outgrowth (Deckers et al., 2000; Castellani and Rougon, 2002; Bachelder et al., 2003; Serini et al., 2003). An hypothesis suggested by our observations on early bone development is that the Sema-3A/Plx-A3/NP-1 pathway would inhibit neurovascularisation of the cartilage anlage early in skeletal development. This block

could be removed later and locally by the down regulation of key receptor chains (e.g. Plx-A3), the elimination of Sema-3A producing cells (e.g. hypertrophic chondrocytes, cf. POC at E19, Table 2) or by the expression of competitors of Sema-3A (such as $VEGF_{165}$), for instance by perichondral cells and hypertrophic chondrocytes. Inhibition of Sema3A signaling would then allow blood vessel and nerve fibre invasion of the diaphysis, and thus the onset of endochondral ossification together with the start of local neural and/or vascular regulation of bone formation. Later, during the ossification process, Sema-3A may provide a repulsive guidance allowing the growing nerve fibres to be directed to their targets in the bone tissue. However, other guidance sytems are certainly involved in bone neurovascular development and are likely to interfere with the Sema-3A pathway.

In conclusion, we show that the Sema-3A signaling network appears to be well positioned to regulate the development of bone innervation, and perhaps other aspects of bone development and remodelling.

MATERIALS AND METHODS

In vitro models

We analysed the expression of the Sema-3A signaling molecules in in vitro models of osteoblasts, osteoclasts and chondrocytes. In mature (day 15) rat calvaria (RC) cell cultures, osteoprogenitors form bone colonies expressing high levels of osteoblast markers, osteocalcin and bone sialoprotein (Malaval et al., 1999)(Fig. 1A, 2B). As an osteoclast model, we used the RAW 264.7 cell line, which, when treated with the cytokine RANKL, fully differentiate in multinucleated osteoclast-like cells expressing calcitonin receptor and tartrate resistant acid phosphatase (Hsu et al., 1999)(Fig 1A, 2D). As a chondrocyte model we used the immortalised mouse cell line MC 615, which can differentiate into hypertrophic chondrocytes when treated with BMP-2, as documented by the concomitant expression of Collagen Xα1 and osteocalcin (Fig 1B)(Valcourt et al., 2002).

In situ model

To analyse the spatial expression of sema-3A and NP-1 during bone development, we performed immunocytochemical studies on developing rat femurs at days E17 and E19 (beginning of endochondral ossification), at E21 (birth) and 14 days after birth (P14, end of the endochondral ossification). At E17 the bone is still a cartilage model in the center of which (mid-diaphysis) some chondrocytes become hypertrophic. It is limited by a layer of undifferentiated osteogenic cells, the perichondrium (termed periosteum at later stages). At E19 the bone collar has formed, and vascular invasion and endochondral ossification has begun in the mid-diaphysis (primary ossification center). The growth plates are formed soon after E19, separating the epiphysis from the metaphysis. In fully developed bone, trabecular bone tissue is restricted to the metaphysis and the epiphysis; the midshaft (diaphysis)

contains only bone marrow. At E21 vascular invasion of the epiphysis and development of the secondary ossification center has begun, and is well established at P14. Histologically, osteoblasts are observed lining the outer cortical bone, as the basal cell layer of the periosteum (fig. 3A, short arrows). They also line the inner cortical bone (endosteum) and are present as cuboidal cells along bone trabeculae in the metaphysis (fig. 3B, short arrows). Osteoclasts are larger, multinucleated cells, usually in direct contact with the bone matrix (Fig 3A, B, long arrows).

Cell culture

Culture reagents were from Sigma (Saint-Quentin Fallavier, France) unless otherwise indicated. 21 days pregnant female Wistar rats were killed by cervical dislocation, in accordance with the local Ethical Committee recommendations, and the fetuses were collected through C-section. Fetal rat calvaria (RC) cells were enzymatically isolated by sequential digestion with collagenase as described previously (Bellows et al., 1986; (Malaval et al., 1999); populations obtained from digests II through V were kept and plated separately. After 24 h, cells were trypsinised (0.01% trypsin in citrate saline), counted, populations II-V were pooled, and cells were plated at 3 x 10^3 cells/cm^2 in 35 mm dishes for RNA isolation and on 1cm^2 glass coverslips for immunostaining. RC cells were grown in α-MEM medium containing antibiotics, 10% FBS, 50µg/ml ascorbic acid, and 10 mM sodium β-glycerophosphate. The medium was changed every 2-3 days. All dishes were incubated for 15 days at 37°C in a humidified atmosphere of 95%air/5% CO_2. The RAW 264.7 monocytic cell line was cultured in 25cm^2 flasks or on glass coverslips, in DMEM medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1% glutamate and 0.5% fongizone. The cultures were kept for 6 days to differentiate into osteoclasts in whole medium described above supplemented with 30ng/ml Rank Ligand (kindly provided by

Amgen, Thousands Oaks, CA, USA). The mouse chondrogenic MC 615 cell line has been characterised previously (Mallein-Gerin and Olsen, 1993; Valcourt et al., 2002). MC 615 cells were maintained in 1:1 high glucose DMEM/Ham's F-12 containing 10% FBS and supplemented with 2mM L-glutamine, 100 units/ml penicillin, 100µg/ml streptomycin (all product from in Vitrogen). For differentiation into hypertrophic chondrocytes, MC 615 cells were grown for 3 days in flasks or on glass coverslips as above, except that FBS was reduced to 1% and BMP-2 (0-100ng/ml) was added to the medium. Recombinant human BMP-2 was produced and purified by Genetics Institute, Inc. (Cambridge, MA, USA). The culture medium supplemented with BMP-2 was replaced each day. At the end of the culture time, all cell types were either processed for RNA isolation or fixed and permeabilised for immunostaining.

RNA isolation and RT-PCR

Cells were scraped in lysis buffer and total RNA was isolated using the Rneasy^{R} minikit (Qiagen SA, Courtaboeuf, France). 2µg of total RNA were reverse-transcribed using Omniscript reverse transcriptase from Qiagen SA (Courtaboeuf, France) and oligo $(dT)_{12\text{-}18}$ primers (Amersham Pharmacia Biotech, Orsay, France). Polymerase Chain Reaction (PCR) was performed using 2µl (equivalent to 0.2µg of RNA) of each RT reaction as template with the HotStarTaq^{TM} DNA polymerase kit (Qiagen SA). PCR primers were designed from published DNA sequences (Table I). Efficiency and specificity of amplification with the designed primer pairs were controlled using mRNA isolated from mouse or rat brain.

Tissue processing

The fetuses were collected as described in the Cell Culture section. The femurs of fetuses and born rats were rapidly excised and cleaned of skin and muscles, then cut longitudinally at the

diaphysis level (for E18 and older fetuses) and immediately fixed in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.3 at 4°C for 24h (McKee et al., 1991). The samples were decalcified in 4% di-sodium ethylenediamine-tetraacetic acid (EDTA) for 1 up to 5 weeks before dehydratation through a graded ethanol series and embedded in paraffin. Sections (5µm) were cut on a Leica RM2145 microtome (Leica instruments, Heidelberg, Germany) and processed for immunocytochemistry.

Immunocytochemistry

Cultures on glass coverslips were fixed in 3.7% formaldehyde, permeabilised in –20°C methanol and stored in PBS. Cytoenzymology for TRAP activity in RAW cells was performed with a commercial kit (#386-A, Sigma). Sections from decalcified samples were laid on silanised slides and dried overnight at 37°C. After two baths of methylcyclohexane and rehydration, the sections were treated, along with coverslips, for 15min with 100mM glycine and 50mM ammonium chloride in Tris buffer, pH 7.6, in order to saturate free aldehydic groups. Endogenous peroxidase activity was blocked by incubation for 15min with 1% sodium azide and 1.5% H_2O_2 in 50% methanol. Sections and cultures were then incubated overnight at 4°C with primary antibodies diluted in Tris Buffer Saline (TBS: Tris-HCl 50mM pH: 7.6, 0.9% NaCl, 0.01% BSA) containing 1% normal rat serum to saturate non-specific sites. All goat polyclonal antibodies against Sema-3A and its receptor components were purchased from Santa-Cruz Biotechnology (Santa-Cruz, CA, USA). On sections, the anti rat-Sema-3A (sc1148) and anti human-NP-1 (sc7240) were used at 2µg/ml, the anti human-Plx-A1 (sc10139) at 0.4µg/ml, the anti human-Plx-A2 (sc10144) at 4µg/ml and the anti human-Plx-A3 (sc10134) at 0.8µg/ml. The rabbit anti rat-Tyrosine Hydroxylase (TH) (AB151), used at 1/5000, and the rabbit anti rat-Neurofilament Heavy Chain (NH) (AB1991), used at 1/2000, were purchased from Chemicon (Temecula, CA, USA). The

production and use of the rabbit antiserum directed against bone sialoprotein (BSP) have been described previously ((Malaval et al., 1999) and references therein). Controls were incubated with non-immune goat or rabbit serum. For goat primary antibodies on sections, we used a second step incubation with a rabbit anti-goat IgG antibody (Dako, Copenhagen, Denmark) for 30 min. Both goat and rabbit antibody-antigen complexes were detected with a rabbit Dako EnVision + K4002 kit (Dako). For coverslips, the incubation time of primary antibodies was reduced to 30 minutes at room temperature, and the signal was enhanced by the use of a biotin labeled anti-goat secondary antibody with an additional incubation step with streptavidin-peroxidase. Labeling was revealed with 3-3' diaminobenzidine tetrahydrochloride (DAB) (Sigma) in Tris buffer containing 0.01% H_2O_2. All washings were done with TBS containing 0.02% Triton X100. Some labeled sections were counter-stained either with toluidine blue or with the DNA labeling compound, 4', 6 diamidino-2-phenylindole (DAPI, Sigma); some unlabeled sections at P14 were stained with Goldner's trichrome for histological visualisation. Sections were dehydrated, mounted in Xam (Gurr-BDH Laboratory, Poole, UK) and then observed on a Leica DMRB microscope (Leica, Paris, France). Coverslips were mounted in Fluoprep (Biomérieux, Marcy l'Etoile, France).

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Table 1: Primers sequences and PCR conditions.

Target	Origin	Primer sequences (5' to 3')	Accession Number	Product length (bp)	Number of cycles	Annealing temperature (ºC)
Sema-3A	Rat	GTGTTCCTTGG CCATATGCTC CAGCAGTTGAGCCAATGT CAG	NM_017310	472	35	48
NP-1	Mouse	CTGGTGAGCCCTGTGGTCTATTCC ATATCATCCACAGCAATCCCACCG	AF 018957	272	35	55
Plx-A1	Mouse	TCACTCACCTGGTGGTTCA CACAAAGCCAAACATGTCAGC	D 86948	505	35	50
Plx-A2	Mouse	CATGTCTGAGAGACAGGTCACCA CCAGGTGAGATGCAGATGACTTG	D 86949	360	35	55
Plx-A3	Mouse	GCTGTTGATGGCAAGTCTGA GAAGGAGCACTGACAAAGC	NM_008883	198	40	52
ColXa1	Mouse	GCAACTAAGGGCCTCAATGG GAGCCACTAGGAATCCTGAG	NM_009925	596	35	57
OCN	Mouse	CAAGTCCCACACAGCAGCTT AAAGCCGAGCTGCCAGAGTT	X 04142	371	30	58
OCN	Rat	AGGACCCTCTCTCTGCTCAC AACGGTGGTGCCATAGATGC	X04141	294	25	60
BSP	Rat	ATGGAGATGGCGATAGTTCG TGAAACCCGTTCAGAAGGAC	NM_012587	504	30	60
CTR	Mouse	GTCTTGCAACTACTTCTGGATGC AAGAAGAAGTTGACCACCAGAGC	U18542	255	40	55
TRAP	Mouse	CTCTCTGACCACCTGTGCTTCCTC GAACCTCTTGTCGCTGGCATCGTG	NM_007388	292	26	55
GAPDH	Mouse	ATCACTGCCACCCAGAAGAC ATGAGGTCCACCACCCTGTT	AK015422	443	25	57

Table 2 – Expression of Sema-3A signaling molecules during endochondral ossification.

Stage	Site	Sema-3A/NP-1	Plx-A1/Plx-A2	Plx-A3	Nerve markers
E17	Periosteum	+	++/+	+	Along the metaphysis
	HC	++	++	+	-
E19	Periosteum	++	+	+	Ubiquitous
	POC	NP-1	+	-	-
E21	Periosteum	++	+	+	+
	Cortical bone	+	+	+	+
	Metaphysis	++	+	+	++
P14	Periosteum	+	+	+	+
	Cortical bone	-	Osteoblasts Osteocytes	+	+
	Metaphysis	Osteoblasts and osteoclasts under the growth plate	Osteoblasts (few for A2) and osteoclasts under the growth plate	Endothelial cells under the growth plate	Loose network in the marrow

+: Expressed, -: Not detected. HC: Hypertrophic chondrocytes; POC: Primary ossification center.

LEGENDS OF FIGURES

Figure 1: **Expression of RNA for Sema-3A signaling molecules by osteoblasts, osteoclasts and chondrocytes in vitro.** A: RT-PCR analysis of differentiation markers and Sema-3A signaling molecules in mature osteoblastic (15 days of culture) RC cell cultures and osteoclasts differentiated from RAW 264.7 cells under RANKL treatment for 6 days. B: RT-PCR analysis of differentiation markers and Sema-3A signaling molecules in the mouse chondrogenic cell line MC 615 grown with BMP-2. OCN: osteocalcin, BSP: bone sialoprotein, CTR: calcitonin receptor, TRAP: tartrate-resistant acid phosphatase, CollXα1: collagen Xα1.

Figure 2: **Immunodetection of Sema-3A signaling molecules in osteoblasts and osteoclasts in vitro.** Osteoblasts differentiated from RC cells (A, B, E, G, I, K) and osteoclast-like cells differentiated from RAW 264.7 cells (C, D, F, H, J, L) were immunolabeled for the antigens indicated. Controls (A, C) were incubated with non-immune antibodies. D: Cytoenzymology for the osteoclast marker TRAP.

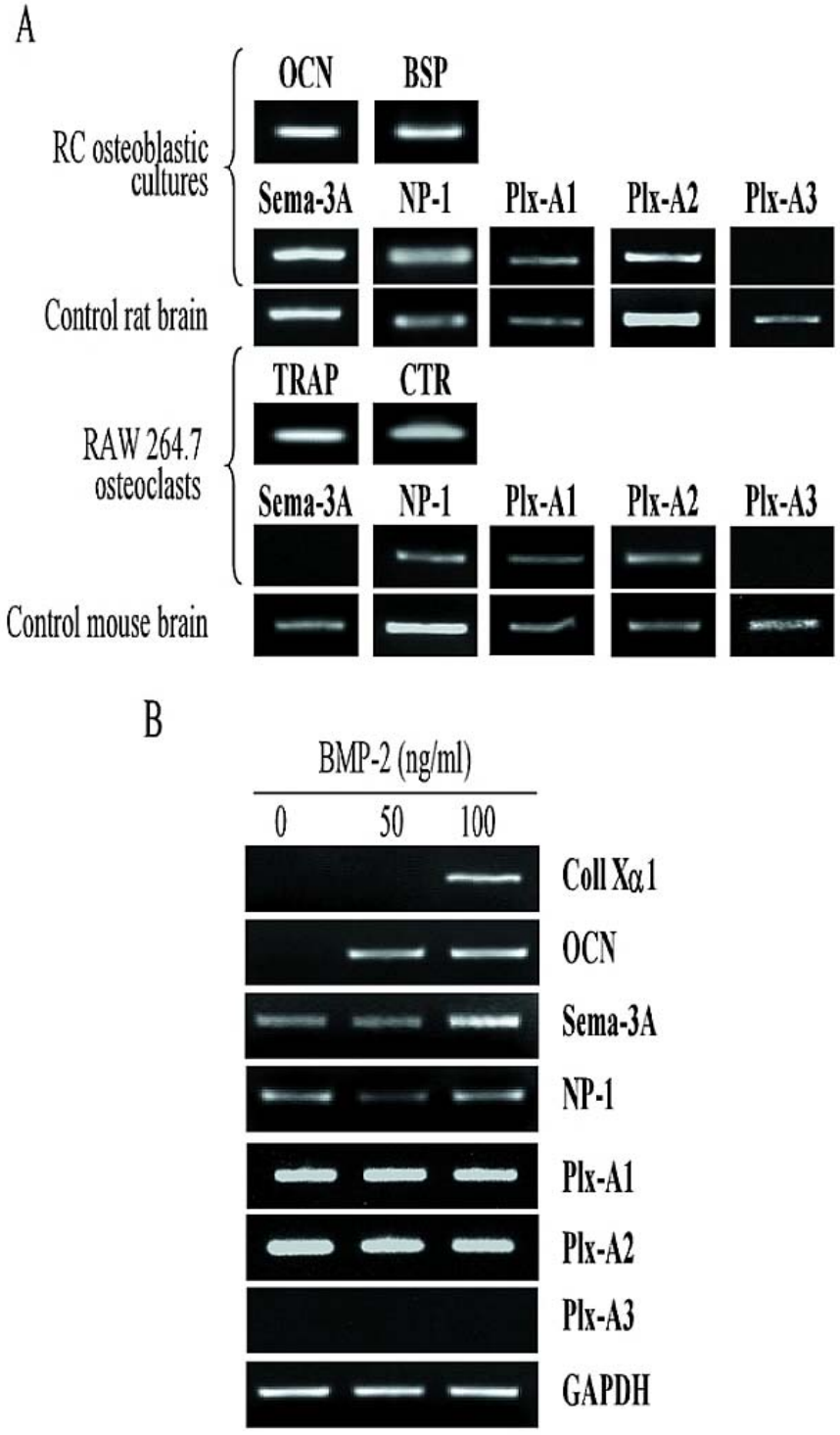
Figure 3: **Immunodetection of sema-3A and NP-1 during endochondral development of rat femur.** A and B: trichrome histological staining at P14, showing cortical (A) and trabecular areas (B); short arrows: osteoblasts, long arrows: osteoclasts (X2000). C: Sema-3A at E17; arrows: periosteum, dotted line: pre-hypertrophic and hypertrophic chondrocytes in mid-diaphysis, dotted circle: resting chondrocytes in the epiphysis (X100); mid-diaphysis enlarged in D. E: Non-immune control at E17. F: NP-1 at E19, diaphysis; short arrows: staining in the periosteum, long arrows: staining in the bone collar (X800). G: NP-1 at E19, primary ossification center; stars: blood vessels (X800). H: Sema-3A at E21, diaphysis;

arrows: labeled endosteum (X400). I: non-immune control at E21. J: NP-1 at P14, growth plate and ossification front (200X). K: Sema-3A labelled osteoblasts under the growth plate at P14 (X2000). L: NP-1 at P14, pre-hypertrophic and hypertrophic chondrocytes (1000X); selected areas enlarged in M and N. O: Sema-3A at P14, osteoclast near the ossification front (2000X). P: NP-1 at P14, osteoclast near the ossification front (2000X). Insets in O and P: counter-staining with DAPI for fluorescent nucleus detection. Sections K, L, M, and N were counter-stained with toluidin blue. BC: bone collar, GP: growth plate, P: periosteum, PHC: pre-hypertrophic chondrocytes, T: trabecula.

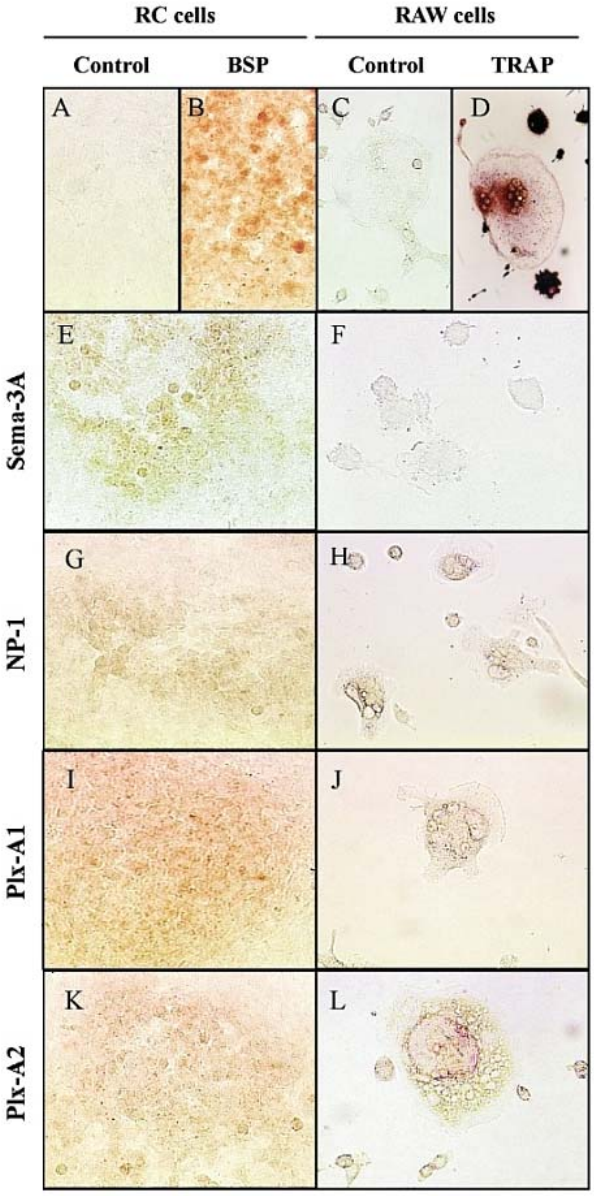
Figure 4: **Immunodetection of Plx-A1 and Plx-A2 during endochondral development of rat femur.** A: Plx-A1 at E17, diaphysis (X400). B: Plx-A1 at E19, diaphysis along the primary ossification center; stars: hypertrophic chondrocytes, long arrows: "borderline" chondrocytes, short arrows: periosteal osteoblasts (X800). C: Plx-A1 at E21; arrows: osteoblasts (X1000). D: Plx-A1 at P14, metaphysis; long arrows: trabecular osteoblasts, short arrows: periosteal osteoblasts (X400). Selected area enlarged in inset; dotted line circles: osteocytes. E: PlxA1 at P14, osteoclast near the ossification front (2000X). F: Plx-A2 at E17; dotted line circle: hypertrophic chondrocytes (X100). G: Plx-A2 at E21, diaphysis; short arrows: staining in periosteum, long arrows: osteoblasts, dotted line circle: osteocytes (X600). H: Plx-A2 at P14, cortical bone of the diaphysis; long arrows: endosteal osteoblasts, short arrows: periosteal osteoblasts, dotted line circles: osteocytes (X600). I: PlxA2 at P14, osteoclast near the ossification front (2000X). J: Non-immune control at P14 (X600). Insets in E and I: Counter-staining with DAPI for fluorescent nucleus detection. Section G was counter-stained with toluidin blue. P: periosteum, CB: cortical bone, T: Trabecula.

Figure 5: **Immunodetection of Plx-A3 during endochondral development of rat femur.** A: E17, arrows: staining in the periosteum (X600); B: E19, diaphysis along the primary ossification centre (X200), selected areas enlarged in C (periosteum) and D (cortical bone); C and D: arrows: labeled profiles (X1000); E: E21, diaphysis (X100), selected area enlarged in F (X600); G: P14, ossification front, arrows: vascular buds (X800), enlarged in H (X1200); I: Non-immune control at E21; J: Trabecular bone in the metaphysis at P14; stars: lumen of labeled capillaries (X1000). All sections were counter-stained with toluidin blue.

Figure 6: **Immunodetection of NH and TH during endochondral development of rat femur.** A: NH at E17; arrows: labeling in the periosteum (X200). B: NH at E19, diaphysis along the primary ossification center; long arrows: staining in the periosteum, short arrows: staining in the bone collar (X800). C: NH at E21, diaphysis (X800). D: Non-immune control at E21 (X600). E: NH at P14, periosteum of the diaphysis; arrows: staining (X800). F: TH at E17, metaphysis; arrows: labeling in the periosteum (X1200). G: TH at E19, diaphysis along the primary ossification center; long arrows: staining in the periosteum, short arrows: staining in the bone collar (X400). H: TH at E21, diaphysis (X1200). I: non-immune control at E21 (X1200). All sections were counter-stained with toluidin blue.



Gomez et al., 2005 - Figure 1



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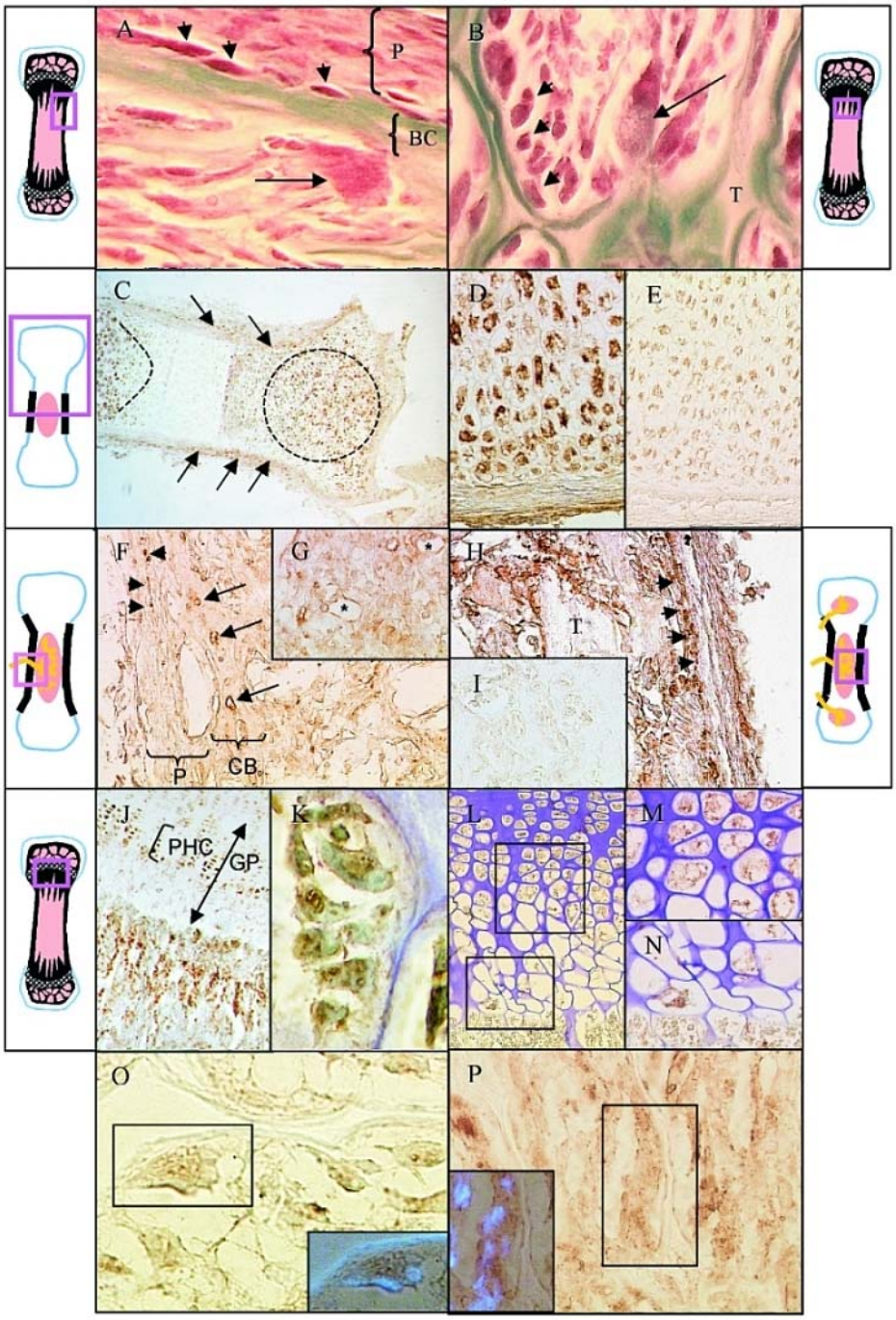
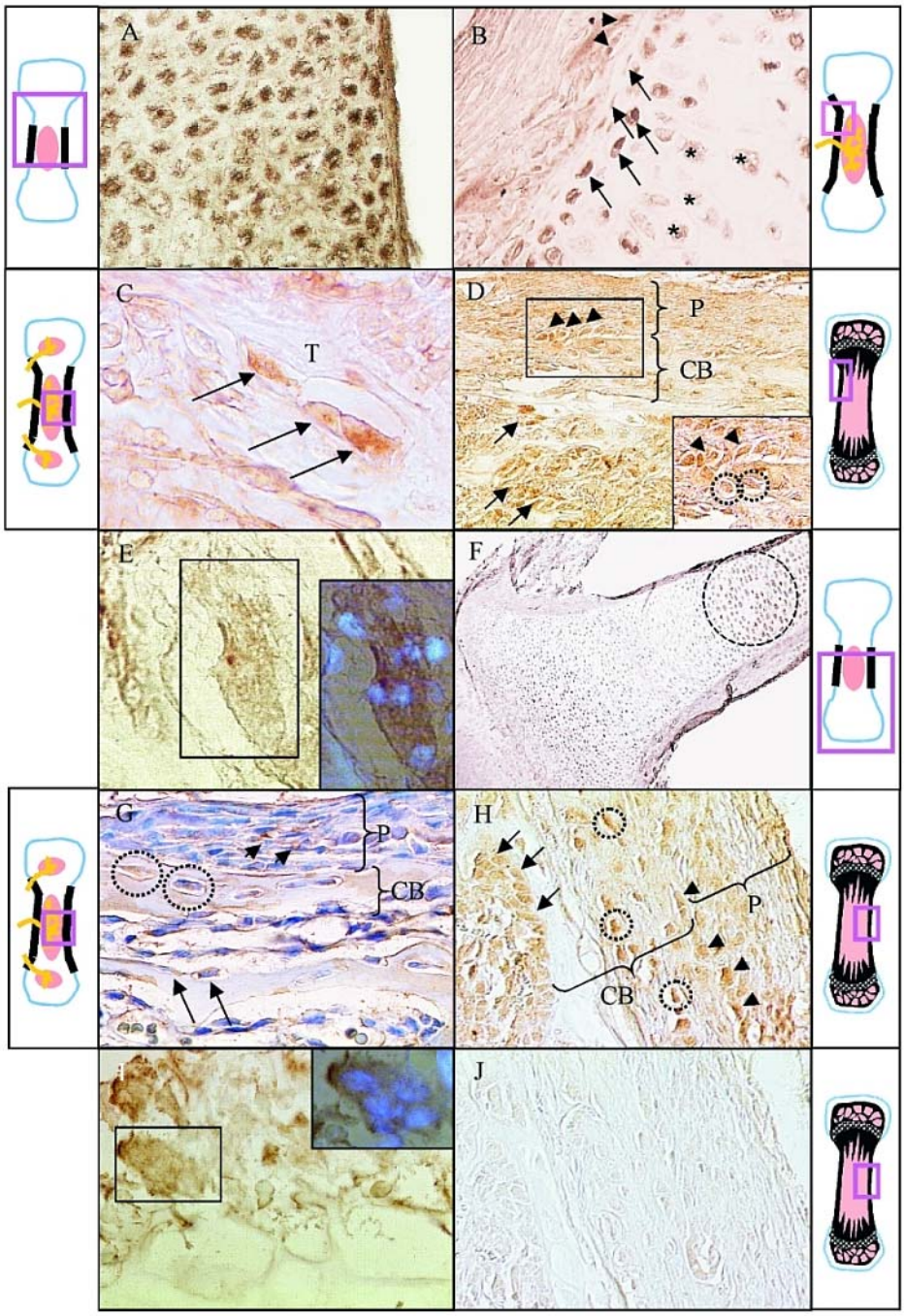


Gomez et al., 2005 - Figure 3



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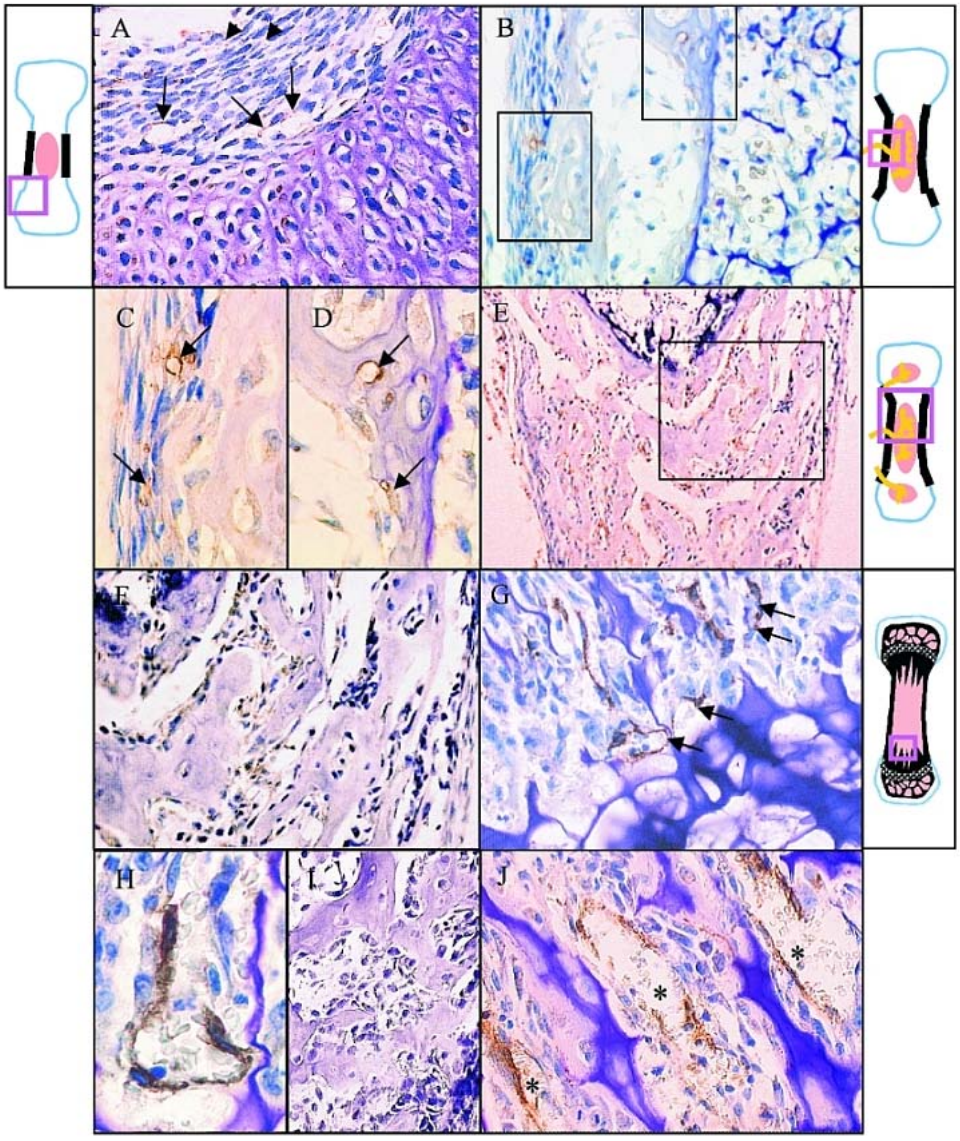
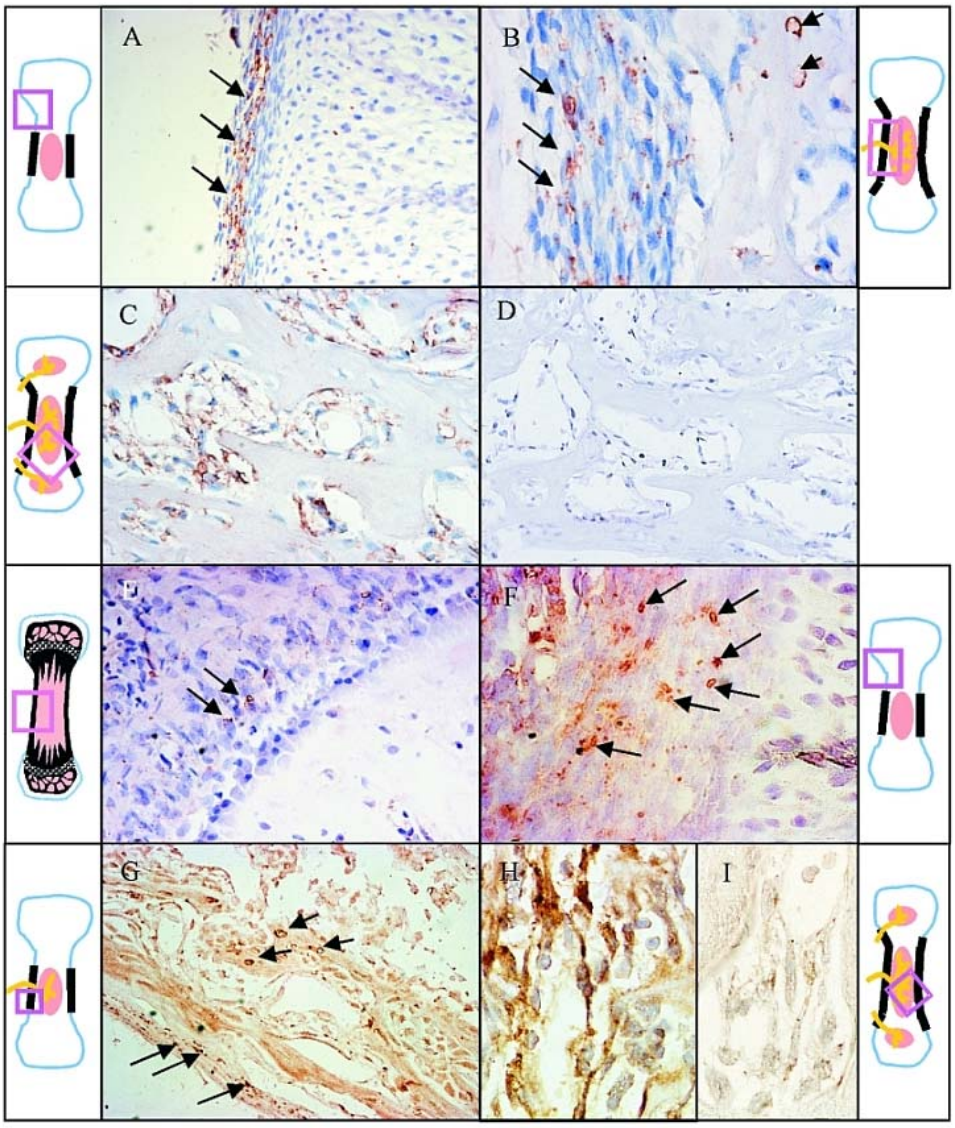


Gomez et al., 2005 - Figure 5



Gomez et al., 2005 - Figure 6