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Review

Regulation of human cranial osteoblast phenotype by FGF-2, FGFR-2 and BMP-2 signaling

P.J. Marie, F. Debiais and E. Haÿ

INSERM U349, Hopital Lariboisière, Paris, France

Summary. The formation of cranial bone requires the differentiation of osteoblasts from undifferentiated mesenchymal cells. The balance between osteoblast recruitment, proliferation, differentiation and apoptosis in sutures between cranial bones is essential for calvarial bone formation. The mechanisms that control human osteoblasts during normal calvarial bone formation and premature suture ossification (craniosynostosis) begin to be understood. Our studies of the human calvaria osteoblast phenotype and calvarial bone formation showed that premature fusion of the sutures in non-syndromic and syndromic (Apert syndrome) craniosynostoses results from precocious osteoblast differentiation. We showed that Fibroblast Growth Factor-2 (FGF-2), FGF receptor-2 (FGFR-2) and Bone Morphogenetic Protein-2 (BMP-2), three essential factors involved in skeletal development, regulate the proliferation, differentiation and apoptosis in human calvaria osteoblasts. Mechanisms that induce the differentiated osteoblast phenotype have also been identified in human calvaria osteoblasts. We demonstrated the implication of molecules (N-cadherin, Il-1) and signaling pathways (src, PKC) by which these local factors modulate human calvaria osteoblast differentiation and apoptosis. The identification of these essential signaling molecules provides new insights into the pathways controlling the differentiated osteoblast phenotype, and leads to a more comprehensive view in the mechanisms that control normal and premature cranial ossification in humans.

Key words: Osteoblasts, BMP-2, FGF-2, Human, Calvaria, Signaling, Osteogenesis, Suture

Offprint requests to: Pierre. J. Marie, Laboratory of Osteoblast Biology and Pathology, INSERM Unité 349 affiliated CNRS, Hopital Lariboisière, 2 rue Ambroise Paré, 75475 Paris Cedex 10, France. Fax: 33 1 49 95 84 52. e-mail: pierre.marie@inserm.lrb.ap-hop-paris.fr

Introduction

Most calvarial bones are formed by intramembranous ossification which occurs without previous cartilage formation. During development, the condensation of mesenchymal cells is followed by their progressive differentiation into osteoblasts that form a mineralized matrix in ossification centers. These calvarial bones expand during development but do not fuse at the junction with other cranial bones, allowing skull expansion during growth (Hall and Miyake, 2000). The junction between calvarial bones is a functional structure formed of two bone plates separated by cells with different functions. This structure, called suture, is responsible for the maintenance of separation between the two membrane bones, and thereby is essential for the growth of the skull. Any perturbation between these processes induces premature or delayed fusion of the sutures and abnormal formation of cranial bones. The events occuring at the suture level and controlling the cell behaviour are therefore highly important in the control of membranous ossification (Cohen, 1997).

Several cell types are involved in the control of suture formation. Most cells surrounding the suture are mesenchymal cells. In the vicinity of the suture, a minority of these cells differentiate into pre-osteoblasts. These cells then differentiate into mature osteoblasts which are found along the bone trabeculae that they are forming (Fig. 1). At the end of the formation period, osteoblasts die by apoptosis or are embedded in the matrix, becoming osteocytes, which then undergo apoptosis at the end of their life (Marie, 1999a,b). The early commitment of mesenchymal stem cells into osteoblasts requires expression of Cbfa1/Runx2, a transcription factor that regulates several genes in osteoblasts, such as the (α1)I collagen chain (COLIA1), bone sialoprotein (BSP), osteopontin, transforming growth factor (TGF-ß) and osteocalcin (OC) (Ducy et al., 1997; Karsenty, 2000). In addition, other families of transcription factors, such as AP-1 (Fos/Jun), Msx2 and Dlx5, play important roles in osteoblast differentiation and bone formation (reviewed in Marie, 2001).

The mechanisms by which the suture is formed and maintained begin to be understood. The formation as well as maintenance of the suture is highly dependent on the recruitment, proliferation, differentiation and apoptosis of osteoblasts and osteocytes (Most et al., 1998; Rice et al., 1999; Opperman, 2000; Morris-Kay et al., 2001). Experimental studies indicate that these events are under the control of several regulatory factors, including TGF-ß, Fibroblast Growth Factors (FGFs) and Bone Morphogenetic Proteins (BMPs). These molecules are secreted locally and regulate cell differentiation and apoptosis. Most information on the control of membranous ossification by local factors have been drawn from experimental studies in rodent models, and little is known about the regulation of calvarial osteoblasts in humans. Our laboratory has previously developed human endosteal osteoblast cultures to study the regulation of trabecular bone formation (reviewed in Marie et al., 1994; Marie, 1995). These models led us to determine the cellular and molecular alterations of osteoblasts in human disorders of bone formation (reviewed in Marie, 1999a). More recently, we developed a new model of human calvaria cell cultures with the aim of establishing the mechanisms of regulation of human cranial ossification and its regulation by local factors. In particular, we determined the roles of FGF-2, FGFR-2 and BMP-2 on human calvaria osteoblast proliferation, differentiation and apoptosis and identified some signaling pathways involved in these effects. In this review, we have summarized our present knowledge on the mechanisms of action and signaling of FGF-2, FGFR-2 and BMP-2 on osteoblast proliferation, differentiation and apoptosis in human calvaria osteoblasts.

Human calvaria osteoblasts

The morphology and histology of cranial sutures have been extensively studied in rats and mice. In these models, the effects of local factors such as TGF-ßs and FGFs have been documented (Kim et al., 1998; Iseki et al., 1999; Rice et al., 1999; Opperman, 2000). In contrast to these animal models, little is known about the formation and regulation of human cranial bones and about the regulation of the osteoblast phenotype in normal and pathological conditions. We developed and characterized human calvarial cell cultures with the aim of establishing the mechanisms of cranial bone formation in humans (de Pollak et al., 1997). Bone cells isolated from human calvaria in neonates display characteristics of the osteoblast phenotype such as alkaline phosphatase (ALP) activity, osteocalcin production, expression of bone matrix proteins (type I collagen, osteonectin, osteopontin), and responsiveness to calciotropic hormones. In addition to expressing these osteoblast phenotypic characteristics, human calvaria cells express parathyroid hormone-related peptide (PTHrP) as well as functional PTH/PTHrP receptors, and respond to PTHrP, suggesting a possible paracrine mechanism of action of PTHrP (Lomri et al., 1997). Human calvaria cells also express FGF and FGF receptors (FGFR)-1, -2 and -3 (Debiais et al., 1998), as well as BMP-2 and BMP receptors (Haÿ and Marie, unpublished data), indicating that they are target cells for these factors. One of the typical characteristics of mature osteoblasts is the ability to form a mineralized bone-like structure in vitro. We found that human calvaria cells cultured in the presence of ascorbic acid and phosphate first proliferate, then synthesize type I collagen and form a mineralized matrix (Debiais et al., 1998; Haÿ et al., 1999). During these progressive stages of development, ALP activity decreases, and type I collagen synthesis increases whereas calcium is incorporated into the deposited matrix (Fig. 2). In these two-dimensional culture conditions, matrix mineralization appears as a diffuse phenomenon. When human calvaria cells were cultured in aggregates, however, a calcified nodular bone-like structures is formed, providing a model to study human osteogenesis in vitro (De Pollak et al., 1997; Haÿ et al., 2000). Thus, human calvaria osteoblasts express the full osteoblast phenotype, which provided us with a unique model for analysis of normal and abnormal calvarial bone formation in humans.

Thanks to this model, we determined the age-related changes in the growth characteristics and osteoblast phenotype in relation to bone formation during human postnatal calvaria osteogenesis (De Pollak et al., 1997). Histomorphometric analysis of normal calvaria samples obtained from children aged 3 to 18 months showed an age-related decrease in the extent of bone surface covered with osteoblasts and newly synthesized collagen, demonstrating a progressive decline in bone formation during postnatal calvaria osteogenesis. To determine the cellular mechanisms involved, we derived calvaria cells from normal sutures and determined their

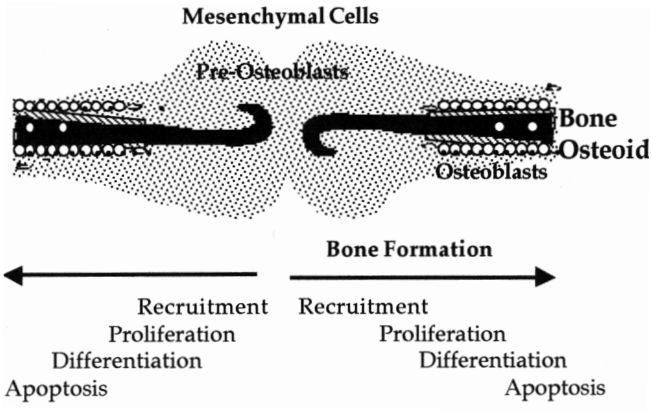

Fig. 1. Schematic representation of a developing human fetal coronal suture. Indifferentiated mesenchymal cells present in the suture separating the two bone edges differentiate into pre-osteoblasts and mature osteoblasts that form the bone matrix. At the end of the formation period, osteoblasts die by apoptosis or are embedded in the matrix, becoming osteocytes. Bone formation and maintenance of the suture are highly dependent on the recruitment, proliferation, differentiation and apoptosis of osteoblasts.

behaviour in vitro. We found that the age-related decrease in bone formation was associated with a decline in osteoblastic cell proliferation in vitro during postnatal human calvaria osteogenesis. In contrast, ALP activity and osteocalcin production increased with age in basal conditions and in response to $1,25(OH)_2$ vitamin D3, indicating increased osteoblast differentiation. These in vivo/in vitro studies therefore revealed a reciprocal relationship between human osteoblast cell growth and differentiation during human postnatal osteogenesis (De Pollak et al., 1997).

Increased osteoblast differentiation in non syndromic craniosynostosis

Craniosynostosis occurs by premature fusion of cranial sutures. However, the pathogenesis of this premature cranial suture ossification is not known. Our working hypothesis is that premature fusion of the sutures results from an imbalance between calvaria cell proliferation and differentiation, leading to precocious osteoblast differentiation. We tested this hypothesis by evaluating the histological indices of bone formation and the characteristics of osteoblastic cells derived from normal and affected cranial sutures in infants and children with nonsyndromic craniosynostosis (De Pollak et al., 1996a,b). The histomorphometric analysis showed that bone formation was higher in fused sutures compared with normal sutures in the same patients, showing that bone formation activity at the suture site is locally increased in craniosynostosis. To determine the mechanisms involved, calvaria cells were derived from normal and fused sutures and their in vitro behaviour was determined. We found that osteoblast differentiation markers (ALP activity and osteocalcin production in basal conditions and after stimulation with 1,25-dihydroxyvitamin D) were higher in osteoblasts from fused sutures than in normal sutures. In contrast, cell proliferation did not differ in the two groups. This showed for the first time that premature suture ossification in human nonsyndromic craniosynostosis results from an increased maturation of osteoblastic cells (De Pollak et al., 1996a,b), a finding that was recently confirmed by other investigators (Shevde et al., 2001). Although the underlying cellular and molecular mechanisms involved are not known, one can postulate that increased osteoblast differentiation may result from alterations in the production or responsiveness to local growth factors such as FGFs, and BMPs (Iseki et al., 1999; Rice et al., 1999; Oppermann, 2000). It was therefore of crucial interest to determine the regulation of human calvaria cell behaviour by these factors.

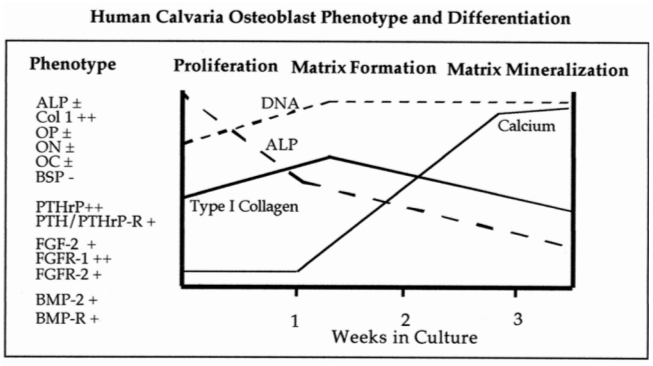

Fig. 2. Phenotypic characteristics of human calvarial cells in vitro. Bone cells isolated from human calvaria express characteristics of the osteoblast phenotype such as alkaline phosphatase (ALP), osteocalcin (OC), type I collagen, osteonectin (ON), osteopontin (OP), but not bone sialoprotein (BSP), parathyroid hormone-related peptide (PTHrP) and its receptor (PTH/PTHrP-R), FGF-2 and FGF receptors (FGFRs) as well as BMP-2 and BMP receptors (BMP-R). During osteogenesis in vitro, human calvaria cells first proliferate, then ALP activity decreases, type I collagen synthesis increases whereas calcium is incorporated into the deposited matrix.

FGF-2 regulates human calvaria osteoblast proliferation and differentiation

FGFs are a family of polypeptides that are important factors controlling bone development, growth, remodeling and repair (reviewed in: Hurley et al., 2001; Marie et al., 2001). The cellular actions of FGFs are known to be mediated by interactions with FGFRs, a family of tyrosine kinase receptors (Wang et al., 1994). FGF binding to FGFR induces intrinsic tyrosine phosphorylation and activation of signal transduction pathways such as Raf, mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinases (Erks), src and p38 MAP kinases, phospholipase Cγ (PLCγ), and protein kinase C (PKC), among other signaling pathways (Givol and Yahon, 1992; Jaye et al., 1992). The intracellular signaling mediated by FGF/FGFR leads to activation of genes involved in cell proliferation, migration, differentiation and survival (Burke et al., 1998; Naski and Ornitz, 1998).

Given the possible role of FGFs in the regulation of human calvaria bone formation, we performed studies to identify the effect and signaling pathways involved in the FGF-2 actions in human calvaria osteoblasts. We found that FGF-2 effects on human calvaria cells are dependent on the stage of osteoblast maturation. Indeed, short-term treatment with FGF-2 increased cell growth and decreased ALP activity, type I collagen, osteocalcin synthesis and matrix mineralization in less mature human calvaria osteoblasts. In contrast, FGF-2 increased OC production and matrix mineralization in more mature cells, indicating that FGF-2 modulates human calvaria osteoblats by acting at distinct stages of cell maturation (Debiais et al., 1998). These studies were conducted with primary culture of human osteoblasts which have some limitations (Marie et al, 1994). One of these limitations is that extensive assessment of cellular or molecular mechanisms do require a large number of osteoblastic cells that cannot be provided by postnatal tissue samples in humans. We therefore developed human fetal or neonatal calvaria cell cultures immortalized with the SV-40 large T antigen.

Theoretically, it is possible that immortalization per se may alter the properties of the cells. However, we always found that the osteoblast phenotype (differentiation or apoptosis) in immortalized human calvaria cells is consistent with the phenotype obtained with primary human calvaria cells in several experimental settings as well as in vivo, indicating that immortalized human calvaria osteoblasts reflected the phenotype in primary cultures (Lomri et al., 1998; Haÿ et al., 2001; Lemonnier et al., 2001a; 2001b). Using immortalized human calvaria osteoblasts, we found that FGF-2 modulates cell-cell adhesion, suggesting an effect of FGF-2 on cell-cell adhesion molecules. Cadherins are transmembrane glycoproteins that mediate homophilic, calcium-dependent cell-cell adhesion (Takeichi, 1990). A variety of cadherins are expressed in osteoblastic cells, albeit that this varies with the cell type (reviewed in Civitelli, 1995; Marie, 2002). We found that human calvaria osteoblasts express N-cadherin, E-cadherin, and neural cell-adhesion molecule (N-CAM) in vitro (Haÿ et al., 2000; Debiais et al., 2001) and in vivo (Lemonnier et al., 2000). Interestingly, FGF-2 specifically increases N-cadherin expression and function in human calvaria osteoblasts, and this effect involves PKC- and src-signaling pathways (Debiais et al., 2001). This identified signaling mechanisms by which FGF-2 can act on human calvaria osteoblasts (Fig. 3). Interestingly, recent data point to a role of cadherins in osteoblast differentiation (Cheng et al., 1998; Lemonnier et al., 1998; Ferrari et al., 2000; Haÿ et al., 2000; reviewed in Marie, 2002). Thus, the control of N-cadherin by FGF-2 signaling may be important in human calvaria osteogenesis, as detailed below.

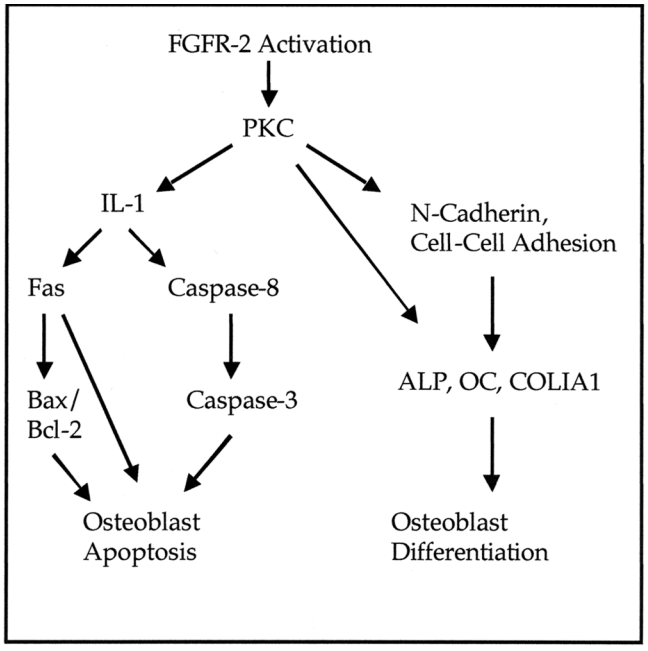

Fig. 3. Mechanisms and signaling pathways involved in FGF/FGFR-2 interactions in human calvaria osteoblasts. PKC activated by FGF-2 or FGFR-2 promotes expression of N-cadherin and enhances cell-cell aggregation. PKC activation also promotes osteoblast phenotypic genes, and induces the cascade of events leading to apoptosis in human calvaria osteoblasts.

FGFR-2 signaling regulates human calvaria osteoblast differentiation

The important role of FGF/FGFR signaling in human calvaria osteogenesis was revealed by the finding that mutations in FGFRs induce premature cranial ossification (craniosynostosis) (Muenke and Schelle, 1995; Wilkie et al., 1995; Hehr and Muenke, 1999). In Apert syndrome, an autosomal dominant disorder characterized by coronal craniosynostosis, mutations in FGFR-2 produce missense substitutions in the linker region between the second and third extracellular Ig domains (Robertson et al., 1998). Experimental analyses indicate that some FGFR-2 mutations induce constitutive activation of the receptor or alterations of ligand binding (Anderson et al., 1998; Plotnikov et al., 2000; Yu et al., 2000; Ibrahimi et al., 2001; Yu and Ornitz, 2001). The phenotypic consequences of these abnormalities in osteoblasts were unknown until we identified the phenotype of human calvaria osteoblasts affected by FGFR-2 mutations (Lomri et al., 1998; Lemonnier et al., 2000). We showed that the premature ossification induced by FGFR-2 constitutive activation in fetus and neonate calvaria with Apert syndrome results from an increased extent of subperiosteal bone formation. This effect results from increased type 1 collagen, osteocalcin and osteopontin expression, and increased production of mineralized matrix by mutant osteoblasts. In contrast, cell growth in basal conditions or in response to exogenous FGF-2 is not affected in mutant cells in vitro or in vivo. Thus, activation of FGFR-2 signaling in Apert syndrome induces premature calvaria cell differentiation, leading to increased subperiosteal bone matrix formation and accelerated calvaria ossification (Lomri et al., 1998; Lemonnier et al., 2000; Marie et al., 2000).

To gain further insight into the cellular mechanisms involved in this effect, we examined the effects of Apert FGFR-2 mutations on the expression of FGFRs in human calvaria osteoblasts. We found that FGFR-2 mutations were associated with downregulation of FGFR-2 in mutant osteoblasts whereas FGFR-1 and FGFR-3 levels were not decreased. FGFR-2 downregulation resulted from receptor internalization rather than by changes in receptor mRNA. We then characterized the signal transduction pathways induced by the S252W FGFR-2 mutation, the prominent mutation in Apert syndrome. Mutant osteoblasts showed increased phospholipase C gamma (PLC gamma), protein PKC alpha phosphorylation and PKC activity (Lemonnier et al., 2001a). Further studies showed that PKC alpha (Fragale et al., 1999) and mRNA expression (Lomri et al., 2001) were increased in Apert mutant osteoblasts. Using specific inhibitors of FGF-signaling

molecules, we showed that the premature osteoblast differentiation induced by the FGFR-2 mutation is associated with a PKC-independent downregulation of FGFR-2 in human calvaria cells (Lemonnier et al., 2000). This shows that a single mutation in a growth factor receptor in human calvaria osteoblasts induces alterations of FGF signaling and subsequent changes in the expression of the receptor and of the osteoblast phenotype (Lomri et al., 1998; Lemonnier et al., 2000; Marie et al., 2000).

FGFR-2 signaling regulates human calvaria osteoblast adhesion

During the course of these studies, we found that, in addition to increase osteoblast differentiation, the activating FGFR-2 mutation increased cell-cell aggregation in vitro. This effect was suppressed by specific neutralizing anti-N- and anti-E-cadherin antibodies, suggesting a role of cadherins in the phenotype induced by mutated FGFR-2. We found that mutant osteoblasts express increased N- and E-cadherin, but not N-CAM mRNA and protein levels in vitro and in vivo (Lemonnier et al., 2001a). Moreover, neutralizing anti-N-cadherin antibody or N-cadherin antisense oligonucleotides reduced ALP activity as well as ALP, COLIA1, and OC mRNA overexpression in mutant osteoblasts, suggesting that N-cadherin is involved in the abnormal phenotype induced by the mutation. In addition, inhibition of PKC activity, which is increased in mutant osteoblasts, suppressed N-cadherin as well as overexpression of osteoblast marker genes in mutant cells, indicating that PKC signaling is involved in the increased N-cadherin and osteoblast gene expression induced by the activating FGFR-2 mutation in human calvaria osteoblasts (Lemonnier et al., 2001a) (Fig. 3). This effect is consistent with the activation of PKC-mediated activation of N-cadherin synthesis by FGF-2 (Debiais et al., 2001). We therefore tested the possibility that N-cadherin expression and function may be directly regulated by activation of PKC in normal human calvaria osteoblasts. We found that phorbol 12,13-dibutyrate, which transiently increased PKC activity, also increased N-cadherin mRNA and protein levels whereas E-cadherin expression was not affected. Transient treatment with phorbol ester also increased cell-cell aggregation in normal human calvaria osteoblasts, which was suppressed by neutralizing N-cadherin antibodies. Moreover, we found that phorbol ester dose-dependently increased ALP activity similarly to the promoting effect of BMP-2. These data further support a role for PKC in N-cadherin-mediated control of human calvaria osteoblast differentiation (Delannoy et al., 2001) (Fig. 3).

FGFR-2 signaling regulates human calvaria osteoblast apoptosis

At the end of the formation period, osteoblasts die by apoptosis recognized by chromatin condensation, nuclear fragmentation, DNA degradation, and formation of membrane blebbing. Apoptosis is essential for the elimination of osteoblasts during skeletal development and remodeling and this phenomenon controls osteoblast lifespan and thereby bone formation (Manolagas, 2000). Because apoptosis normally occurs in suture during developement, any perturbation in the number of apoptotic cells may lead to premature or delayed suture closure (Bourez et al., 1997; Rice et al., 1999). Some regulatory molecules were recently found to control osteoblast apoptosis (Manolagas, 2000). Among them, FGF-2 was reported to activate apoptosis in mouse cranial sutures (Mansukhani et al., 2000), suggesting a possible role for FGF signaling in the control of osteoblast apoptosis. However, whether FGF-2 regulates human calvaria osteoblast apoptosis is unknown. We tested the hypothesis that activation of the FGFR-2 receptor in Apert syndrome may affect apoptosis in human calvaria osteoblats. We found that the mutation increased apoptosis in mature osteoblasts and osteocytes in the Apert suture compared to normal coronal suture (Lemonnier et al., 2001b). Mutant osteoblasts showed increased DNA fragmentation due to a constitutive increase in caspase-8 and effector caspases (-3, -6, -7) activity. This was related to PKC activation because inhibition of PKC inhibited caspase-8, effector caspases, and apoptosis in mutant osteoblasts. Further analysis of the underlying mechanisms revealed increased expression of interleukin (IL)-1 alpha, IL-1 beta, Fas, and Bax, and decreased Bcl-2 levels in mutant cells. Interleukin I plays a major role in FGFR-2-induced apoptosis because neutralizing anti-IL-1 antibody reduces caspase-3, -6, -7 and apoptosis in mutant cells. Thus, activation of FGFR-2 in Apert syndrome promotes apoptosis in human osteoblasts through activation of protein kinase C, overexpression of IL-1 and Fas, activation of caspase-8, and an increase in Bax/Bcl-2 levels, leading to increased effector caspases and DNA fragmentation. This identifies a novel FGFR-2 signaling pathway involved in the premature apoptosis induced by FGFR-2 activation in human calvaria osteoblasts (Lemonnier et al., 2001b) (Fig. 3).

BMP-2 signaling regulates human calvaria osteoblast differentiation

In addition to FGF/FGFR interactions, BMPs are involved in the control of cranial bone formation. BMPs are known to play important roles in cartilage and bone formation (Wozney, 1992; Urist, 1997). BMP-2, a prototype of BMPs, exerts multiple effects on cells of the osteoblastic lineage (Marie, 1997; Yamaguchi et al., 2000; Baylink et al., 2001). BMP-2 induces the differentiation of mesenchymal cells into osteoblast precursors, and promotes the maturation of osteoblasts by increasing the expression of Cbfa1/Runx2 and osteoblast marker genes. BMPs signal through type I and II serine/threonine kinase receptors which phosphorylate

downstream target protein called Smads (Heldin et al., 1997; Nakao et al., 1997). Activation of type I BMP-receptor phosphorylates Smad1 and Smad5 which can then associate with Smad4 in a heteromeric complex. This complex is then translocated to the nucleus where it can activate gene transcription. Although the Smad proteins are important molecules in the BMP pathway, other signaling pathways, such as extracellular signal-regulated kinase (ERK1/2), PKC and cAMP-dependent protein kinase A (PKA) may also play a role in BMP-2-induced osteoblast differentiation (Yamaguchi et al., 2000).

The mechanisms of control of human cranial ossification by BMPs are not known. We therefore studied the mechanisms of action of BMP-2 on human calvaria osteoblasts. We first showed that BMP-2 promotes human calvaria osteoblast marker gene expression, and this effect was sufficient to induce optimal matrix mineralization independently of changes in cell growth and type 1 collagen expression (Haÿ et al., 1999). Similar effects were found in immortalized human calvaria osteoblasts (Haÿ et al., 2000), indicating that BMP-2 promotes human calvaria osteoblast differentiation and bone formation. Subsequent studies were therefore developed to determine the mechanisms involved in the control of BMP-2 in calvaria cell differentiation and osteogenesis. Because cadherins were previously found to be target genes for BMP-2 in cartilage (Haas and Tuan, 1999), we investigated the effects of BMP-2 on cadherin expression in human osteoblasts. We found that BMP-2 transiently increases N- and E-cadherin mRNA and protein levels in human calvaria osteoblasts during in vitro osteogenesis, and that transcription is necessary for this effect. N- and E-cadherin antibodies not only blocked cell-cell adhesion induced by BMP-2, but also reduced the effect of BMP-2 on ALP expression, Cbfa1/Runx2 and osteocalcin expression, showing that the BMP-2-induced early promotion of cell-cell adhesion is required for the expression of osteoblast marker genes in human calvaria cells (Haÿ et al., 2000) (Fig. 4). These studies revealed that N- and E-cadherins are targets of BMP-2 signaling in human calvaria osteoblasts, and further emphasized the role of N-cadherin in human calvaria osteoblast differentiation and osteogenesis (reviewed in Marie, 2002).

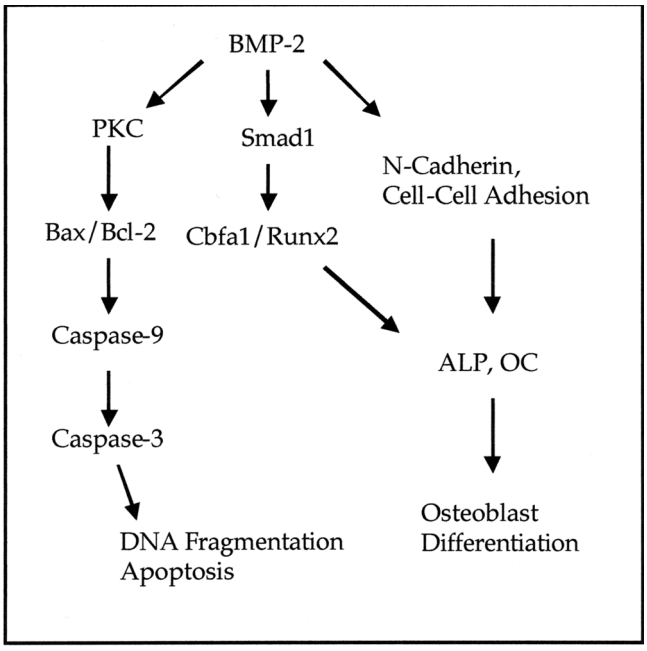

Fig. 4. Mechanisms and signaling pathways involved in BMP-2 actions in human calvaria osteoblasts. BMP-2 activates signaling pathways such as Smad1 which leads to expression of Cbfa1/Runx2, and PKC that leads to the enhancement of N-cadherin expression, cell-cell aggregation and osteoblast gene expression, and to the increase in osteoblast apoptosis mediated by Bax, caspases-9 and -3.

BMP-2 signaling regulates human calvaria osteoblast apoptosis

In non-skeletal cell types, BMPs have both pro- and anti-apoptotic effects (Marazzi et al., 1997). We tested the hypothesis that BMP-2 may modulate apoptosis in human calvaria osteoblasts and determined the signaling pathways involved in this effect. We found that BMP-2 promotes apoptosis in primary human calvaria osteoblasts and in immortalized human neonatal calvaria osteoblasts, whereas TGF-beta 2 inhibits apoptosis (Haÿ et al., 2001). Further analyses of the mechanisms of action showed that BMP-2 increases Bax/Bcl-2 ratio, as well as mitochondrial cytochrome c release into the cytosol, leading to increased caspase-9 and caspase-3, -6, and -7 activities. Several signaling pathways, including the Smad proteins, could be involved in this effect. Activation of Smad1 led to increased expression of Cbfa1/Runx2 in human calvaria osteoblasts. However, overexpression of a dominant-negative Smad1 did not inhibit the activation of caspases or apoptosis induced by BMP-2, whereas it blocked the BMP-2-induced expression of Cbfa1/Runx2 in human calvaria osteoblasts. This indicated that the Smad1 signaling pathway is not involved in the BMP-2-induced apoptosis. Likewise, inhibition of PKA, p38 MAPK or MEK had no effect on apoptosis induced by BMP-2. In contrast, BMP-2 increased PKC activity, and a PKC inhibitor blocked the BMP-2-induced apoptosis in human calvaria osteoblasts. This revealed that BMP-2 uses a Smad-independent, PKC-dependent pathway to activate apoptosis in human calvaria osteoblasts via a Bax/Bcl-2 and cytochrome c-caspase-9-caspase-3, -6, -7 cascade (Haÿ et al., 2001) (Fig. 4).

Overall, these studies not only showed that FGF-2, FGFR-2 and BMP-2 play an important role in human calvaria osteoblast proliferation, differentiation and apoptosis, but also revealed cellular mechanisms (cadherins, Il-1) and signaling pathways (src, PKC) by which these factors regulate the human calvaria

osteoblast phenotype and cranial osteogenesis. Although several signaling cascades may be involved in these effects, activation of PKC appears to play a major role in the control of both osteoblast differentiation and apoptosis. These studies therefore provide new insights into the regulation of osteoblasts and bone formation in human calvaria by FGF/FGFR interactions and BMP-2 (Fig. 5).

Conclusions and perspectives

The development of new models of human calvaria osteoblasts led us to determine the cellular mechanisms by which FGF/FGFR interactions and BMP-2 control cell proliferation, differentiation or apoptosis. Signaling mechanisms that play a major role in human calvaria osteoblast differentiation and apoptosis have been identified. The identification of these essential signaling molecules involved in FGF-2 and BMP-2 actions led to a more comprehensive view in the mechanisms that induce the differentiated osteoblast phenotype and regulation of human calvaria bone formation in humans. Moreover, our studies provide a cellular and molecular basis for the pathogenesis of craniosynostosis induced by FGFR-2 mutations.

Despite these advances in understanding FGF-2 and BMP-2 signaling in human osteoblasts, much is to be learned about how these signals act on transcriptional regulators to control gene expression. Osteoblast commitment and differentiation are dependent on the spatial and temporal expression and/or cooperation of several transcription factors. Some of these factors are required for normal cranial ossification. For example, deletion or mutations in Msx-2 (Jabs et al., 1993; Wilkie et al., 2000), Cbfa1 (Mundlos et al., 1997) or Twist (El Ghouzzi et al., 1997; Yousfi et al., 2001) cause defects in skull ossification in humans. It will be therefore of interest to determine how FGF/FGFR and BMP-2 signaling controls the expression and activity of these transcription factors in human calvaria osteoblasts. Finally, we will need to learn more about the signaling pathways emanating from FGF and BMP receptors in human calvaria osteoblasts. The analysis of these pathways, now under way in our laboratory, will lead to a better understanding of the mechanisms that induce the differentiated osteoblast phenotype and regulate cranial osteogenesis in humans.

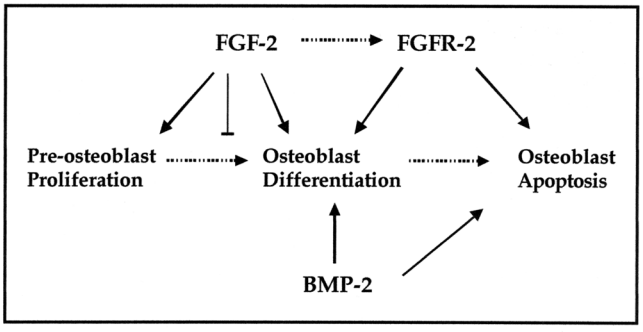

Fig. 5. Present knowledge on the regulation of human calvaria osteoblast phenotype by FGF-2, FGFR-2 and BMP-2. These local factors block (broken line) or activate (black arrow) proliferation, differentiation and apoptosis of human calvaria cells at different stages, leading to control the differentiated osteoblastic phenotype and human calvaria osteogenesis.

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