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PROSPECTS

FGF and FGFR Signaling in Chondrodysplasias and Craniosynostosis

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Abstract The first experimental mouse model for FGF2 in bone dysplasia was made serendipitously by overexpression of FGF from a constitutive promoter. The results were not widely accepted, rightfully drew skepticism, and were difficult to publish; because of over 2,000 studies published on FGF-2 at the time (1993), only a few reported a role of FGF-2 in bone growth and differentiation. However, mapping of human dwarfisms to mutations of the FGFRs shortly, thereafter, made the case that bone growth and remodeling was a major physiological function for FGF. Subsequent production of numerous transgenic and targeted null mice for several genes in the bone growth and remodeling pathways have marvelously elucidated the role of FGFs and their interactions with other genes. Indeed, studies of the FGF pathway present one of the best success stories for use of experimental genetics in functionally parsing morphogenetic regulatory pathways. What remains largely unresolved is the pleiotropic nature of FGF-2. How does it accelerate growth in one cell then stimulate apoptosis or retard growth for another cell in the same type of tissue? Some of the answers may come through distinguishing the FGF-2 protein isoforms, made from alternative translation start sites, these appear to have substantially different functions. Although we have made substantial progress, there is still much to be learned regarding FGF-2 as a most complex, enigmatic protein. Studies of genetic models in mice and human FGFR mutations have provided strong evidence that FGFRs are important modulators of osteoblast function during membranous bone formation. However, there is some controversy regarding the effects of FGFR signaling in human and murine genetic models. Although significant progress has been made in our understanding of FGFR signaling, several questions remain concerning the signaling pathways involved in osteoblast regulation by activated FGFR. Additionally, little is known about the specific role of FGFR target genes involved in cranial bone formation. These issues need to be addressed in future in in vitro and in vivo approaches to better understand the molecular mechanisms of action of FGFR signaling in osteoblasts that result in anabolic effects in bone formation. J. Cell. Biochem. 96: 888–896, 2005.

Key words: FGF2; FGF receptors; chondrodysplasias

FGF SIGNALING IN CHONDRODYSPLASIAS

Endochondral bone growth at the epiphysial plate requires constant growth, replacement, differentiation, and death of chondrocytes. Several gene families have been implicated in the intricate regulation of chondrocyte differentiation as they progress from the resting zone to the hypertrophy zone. These include transcription factors, growth factors, and hormones that interact with a variety of extracellular matrix proteins that form cartilage and, ultimately, mature bone [Hurley et al., 2002; Karsenty and Wagner, 2002]. Principle among the growth factors regulating endochondral bone growth are the FGFs [Liu et al., 2002]. Human dwarfisms have been mapped to the FGFRs [DeMoerlooze and Dickson, 1997]. These mutations are autosomal dominant, sporadic-point mutations clustered around the third Ig domain, the transmembrane domain, and the tyrosine kinase domain of the FGFRs. Each of

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the four FGFR presents a different class of human dwarfism when mutated. Mutation of FGFR1 causes Pfeiffer's syndrome [Muenke et al., 1994], different mutations of FGFR2 cause Jackson–Wiess, Crouzon, and Apert syndrome [Jabs et al., 1994; Reardon et al., 1994] and various mutations of FGFR3 cause achondroplasia, hypochondroplasia, and thanadophoric dysplasia [Shiang et al., 1994; Tavormina et al., 1995]. All of these mutations are believed to result in aberrant or amplified signal transduction from the tyrosine kinase domain of the FGFRs [Ornitz, 2005] Some of the syndromes resulting from mutations in FGFR1 and FGFR2 primarily affect the axial skeleton, particularly the facial and head bones, while mutation of FGFR3 appears to predominantly affect the distal skeleton and/or the long bones [Liu et al., 2002] but does include a distinct macrocephaly.

The human dwarfisms have been reproduced in mice through experimental genetics. Indeed, Coffin et al. were the first to produce a dwarf mouse by overexpression of FGF2 through a constitutive phosphoglycerate kinase promoter [Coffin et al., 1995]. The chondrodysplasia resulting from FGF2 overexpression was unexpected and difficult to interpret. There was a relatively small literature base describing the effects of FGF2 on chondrocyte differentiation in vitro [Trippel et al., 1993] and some expression data for FGF2 and FGF receptors at the epiphysial growth plate [Patstone et al., 1993; Coffin et al., 1995]. Targeted deletion of FGFR1 and FGFR2 in mice predated the human mapping and null mutations of both FGFR1 and FGFR2 resulted in embryonic lethality [Deng et al., 1994; Yamaguchi et al., 1994]. However, subsequent mapping of the human dwarfisms as sporadic, autosomal dominant phenotypes put the TgFGF2-related dwarfism in mice in perfect biological context. Since the human mutations are gain of function, it follows that over-expression of the ligand produces a similar result where FGF2 is a negative regulator of bone growth. Then, targeted deletion of FGFR3 in mice subsequent to the human mapping data showed that loss of FGFR function resulted in excessive longitudinal bone growth [Colvin et al., 1996; Deng et al., 1996]. More genetic data for this model were generated when transgenic overexpression of wild-type FGFR3 caused no phenotype, while overexpression of FGFR3 containing a G380R mutation common to human dwarfisms resulted in achondroplasia and dwarfism [Naski et al., 1998]. Finally, knock-ins of various mutations (including G380R) into the endogenous FGFRs gene have again reproduced the mouse dwarf/chondroplasias [Chen et al., 1999; Li et al., 1999; Iwata et al., 2000; Chen et al., 2001; Iwata et al., 2001]. Collectively, human genetics and experimental murine genetics have provided solid evidence demonstrating that FGF2 is a negative regulator of endochondral bone growth with a specific effect on chondrocyte differentiation at the epiphysial growth plate.

Given this data, a reasonable hypothesis stated that the FGF2 knockout would produce longer bones, much like the FGFR3 null mouse. However, deletion of FGF2 produced no effect in bone length [Zhou et al., 1998]. Montero et al. have observed some expansion of the hypertrophy zone in these mice, but there is no apparent net effect on longitudinal bone growth. Obviously there is some compensatory mechanism that checks excessive longitudinal growth in the absence of FGF2. Either one of the other 22 FGF family members is compensating for the loss of FGF2, or there is a physiological mechanism related to ossification that counters FGF2 loss at the growth plate. The latter is a definite possibility since Montero et al. have found osteopenia in the FGF2KO mice, related to osteoblast differentiation, that was previously undetected [Montero et al., 2000]. Additional studies are required to determine the compensatory growth factors or mechanisms that mitigates this loss of FGF2 in bone. The best candidate appears to be FGF18 because among FGF family members, only FGF2 and FGF18 cause an expansion of the proliferation zone chondrocytes as a feature of the phenotype [Ohbayashi et al, 2002; Ornitz, 2005].

THE FGF REGULATORY PATHWAY FOR ENDOCHONDRAL BONE GROWTH

A putative regulatory pathway for FGF2 mediation of endochondral bone growth is outlined in Figure 1 [Wagner and Karsenty, 2001]. The FGF regulatory pathways for bone growth, beyond the ligands and receptors, are now under intense scrutiny. Genetic results, in vitro data, and gene expression data all provide clues about putative regulatory molecules. As expected, FGF2 and FGFR3 are both expressed at the epiphysial growth plate with concentrations in the proliferation and hypertrophic zones [Coffin

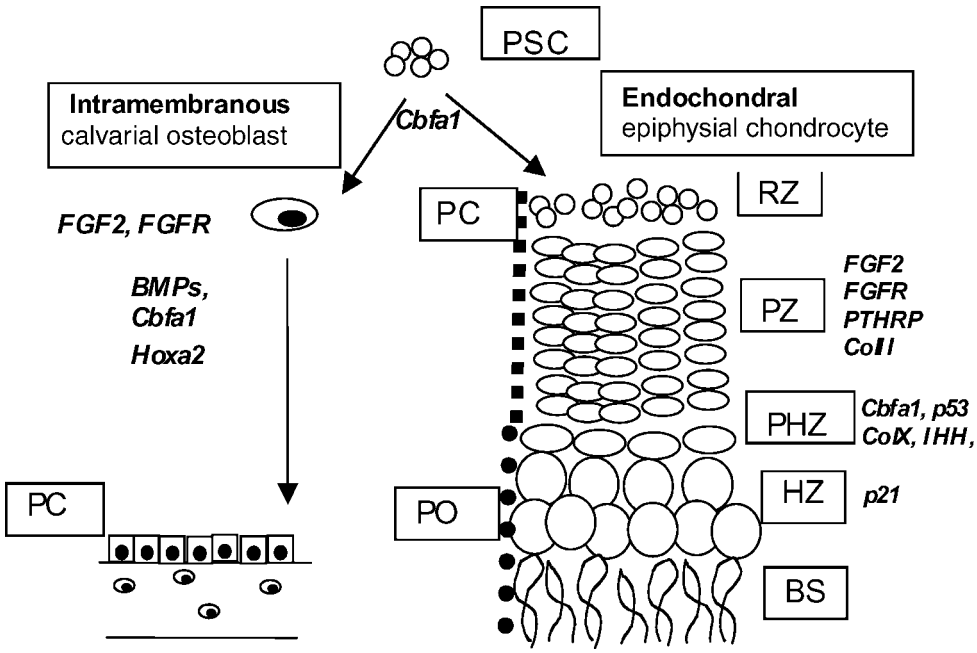

Fig. 1. Schematic diagram for molecular regulation of intramembranous (left) and endochondral-bone growth (right). PSC, primative stem cell; PC, perichondrium; PO, periosteum; RZ, resting zone; PZ, proliferation zone; PHZ, prehypertrophy zone; HZ, hypertrophy zone; BS, bone spicules (modified from Wagner and Karsenty).

et al., 1995; Deng et al., 1996]. Along with FGF2, BMP4, growth hormone, Indian Hedgehog (IHH), Insulin Growth Factor-1 (IGF-1), and Parathyroid Hormone related Protein (PTHrP) are ligands that have been shown to affect the growth plate through gain or loss of function [Karsenty and Wagner, 2002]. PTHrP and PTHrPR null mice also show chondrodysplasia/dwarf-related phenotypes, suggesting that they, similar to FGF2 are negative regulators of bone growth. IHH reciprocally regulates PTHrP expression [Vortkamp et al., 1996]. Similar to FGF2, the PTHrPR is expressed in the growth plate [Vortkamp et al., 1996]. FGFR signaling in bone is thought to function through the Stats, which upregulate cell-cycle inhibitors [Sahni et al., 1999]. Indeed, studies have shown that the TgFGF2 long bone-dwarf phenotype is extensively rescued when the TgFGF2 mouse is crossed with the Stat1−/− mouse [Sahni et al., 2001].

THE FGF REGULATORY PATHWAY FOR INTRAMEMBRANOUS BONE GROWTH

FGF2 is an important regulator of bone cell function in both endochondral growth (described above) and intramembranous growth as studied extensively [Hurley et al., 2002]. Published data show that while continuous FGF2 treatment stimulates osteoblast replication, decreases the differentiation markers alkaline phosphatase and type 1 collagen, and stimulates osteoclast formation and bone resorption [Hurley et al., 2002], Intermittent FGF2 treatment stimulates bone formation both in vitro [Zhang et al., 2002] and in vivo [Hurley et al., 2002]. FGF2 mRNA and protein levels in osteoblasts are regulated by hormones and local factors including parathyroid hormone (PTH) [Hurley et al., 1999], transforming growth factor β one (TGFβ), prostaglandins (PGs), and interleukin-1β (IL-1β) [Hurley et al., 2002]. Figure 1 juxtaposes our most current regulatory models for intramembranous and endochondral-bone growth. The most obvious difference lies in the functional cell types regulated by the FGF regulatory pathway, the chondrocyte for endochondral bone growth, and the osteoblast for intramembranous growth. Both are mesodermal derivatives, but it was shown that the osteoblast is much more sensitive to hormonal and eicosanoid regulation [Kawaguchi et al., 1995; Hurley et al., 1999; Zhang et al., 2002]. An interesting result, in this context, comes from crossing the TgFGF2 mice

with Stat1−/− (null) mice. Both the chondrodysplasia and macrocephaly of the TgFGF2 mice is associated with increased apoptosis of chondrocytes and osteoblasts, respectively. Virtually, all of the phenotypic reversal from the dwarf TgFGF2 mouse toward normal stature lies in the endochondral tissues. Little of the macrocephaly, a hallmark of intramembranous TgFGF2 phenotype, is reversed in the Stat1−/−xTgFGF2 mouse [Sahni et al., 1999]. These data suggest that Stat1 primarily functions in the endochondral tissues and not the intramembranous tissues for signal transduction in the FGF2 regulatory pathway. Moreover, the data reveal a fundamental difference in the FGF2 regulatory pathways between the two tissues. Studies are needed to determine how Stat1 functions in the FGF2 regulatory pathway to differentially affect both forms of bone growth and remodeling physiologically and to further define the function of Stat1 and other related regulatory molecules in the FGF2 regulatory pathway.

FGF2 LOSS OF FUNCTION IN OSTEOPENIA

The production of the FGF2 null or "knockout" (FGF2KO) mouse [Zhou et al., 1998] allowed for further studies of the role of FGF2 in bone. Surprisingly, screening and examination of bone tissues in these mice revealed no apparent phenotype. However, a more careful study revealed decreased bone formation and bone mineralization, that is osteopenia, in the FGF2KO mouse that is dependent on aging [Montero et al., 2000]. These effects appear to result from differences in osteoblast function during growth and remodeling. Most interesting, however, is the revelation that osteopenia (decreased bone-mineral density and bone formation) also occurs in the TgFGF2 mouse [Sobue et al., 2005]. Hypothetically, bone-mineral density and bone formation would be increased in the FGF2 transgenic (overexpression) mouse, consistent with a traditional gain and loss of function scenario. However, the osteopenia in the TgFGF2 mice is not dependent on aging as it is in the FGF2KO mouse. Thus, the data suggest that the osteopenia in the two lines of mice occur through different mechanisms; but the osteopenia in both cases is mediated by FGF2, making this a compelling line of investigation because comparing these two forms will reveal important clues on how osteopenia occurs and the underlying molecular mechanisms for degenerative bone diseases.

Another intriguing aspect is the role of the isoforms of FGF2 in osteopenia. Significantly, there are multiple isoforms of FGF-2 protein. In humans, there are three high molecular wt protein (HMW-22, 23, 24 kDa) isoforms that have nuclear targeting sequences and a low molecular weight (18 KDA–18 kDa) isoform that is exported from cells and stored in bone matrix. As discussed above, non-targeted over expression of all isoforms of FGF-2 in transgenic TgFGF2 mice resulted in a dwarf phenotype as well as osteopenia. Similarly knockout of all isoforms of FGF2 in mice also results in decreased bone mass. However, there are no data on whether a specific isoform (s) is responsible for decreased bone mass in these mice or the role that FGF-2 isoforms play in bone remodeling.

FGF AND FGFR SIGNALING IN CRANIOSYNOSTOSIS: DIVERGENCES AND CONCENSUS

During membranous bone formation, the proliferation, differentiation, and apoptosis of cells of the osteoblastic lineage are dependent on the temporal expression and activity of high affinity FGF receptors (FGFRs) [Ornitz and Marie, 2002]. Studies of genetic models in mice and humans with FGFR mutations have provided evidence that FGFR activation results in premature cranial-suture fusion, or craniosynostosis. Several gain-of-function FGFR mutations induce premature ossification of the cranial sutures and most mutations in the FGFR gene family are gain-of-function [Ornitz and Marie, 2002]. However, activating FGFR mutations induce variable functional effects in human and murine models. Studies in primary calvarial cells derived from patients with Apert syndrome showed that osteoblastic cell proliferation is not increased in vitro or in vivo by the natural S252W and P253R Apert activating FGFR2 mutations, which are responsible for nearly all Apert cases [Lomri et al., 1998; Fragale et al., 1999]. In addition, Apert activating FGFR2 mutations in fetal or postnatal human calvarial cells in vitro and in vivo increase the expression of osteoblast differentiation gene markers and bone formation [Lemonnier et al., 2001; Tanimoto et al., 2004; Baroni et al., 2005], which is also found in human non-syndromic craniosynostosis [DePollack

TABLE I. Compilation of the Divergent Effects of Activating FGFR Mutations on Osteoblast Replication, Differentiation, or Apoptosis in Murine and Human Osteoblasts

Activating FGFR mutation	Osteoblast replication	Osteoblast differentiation	Osteoblast apoptosis
Murine models			
P250R (FGFR1)	+	+	+
S252W (FGFR2)	0; +	0; −	+
C342Y (FGFR2)	+	−	+
Human models			
S252W (FGFR2)	0; −	+	+
P253R (FGFR2)	0; −	+	
C342R (FGFR2)	−	+	

+, positive effect; −, negative effect; 0, no effect.
For details of references, see text.

et al., 1996; Fragale et al., 1999]. In mice, the osteoblast phenotype induced by activating FGFR mutations is less clear (Table I). The sutures of mice carrying a P250R mutation in FGFR1, which is orthologous to the Pfeiffer syndrome mutation in humans, show increased osteoblast proliferation and differentiation [Zhou et al., 2000], whereas the activating S250W FGFR2 Apert mutation does not cause obvious alteration in cell proliferation or differentiation [Chen et al., 2003]. In contrast, expression of Apert and Crouzon activating FGFR2 mutations increase cell proliferation and decrease osteoblast differentiation in murine-osteoblastic cells [Mansukhani et al., 2000, 2005]. Conditional inactivation of FGFR2 affects the proliferation of osteoprogenitors and the function, but not the differentiation, of mature osteoblasts in mice [Yu et al., 2003], also indicating that the phenotype induced by activating FGFR mutations in murine osteoblasts is variable and differs from that found in human craniosynostosis (Table I).

Several hypotheses, not exclusive, can be proposed to explain the discrepancies in the phenotype observed in human and mouse FGFR-mutant osteoblasts. The decreased differentiation induced by FGFR activation in murine cells may be secondary to the increased proliferation observed in these cells [Mansukhani et al., 2000]. Additonally, the cellular responsiveness to FGFR activation is dependent on the stage of osteoblast maturation [Debiais et al., 2004; Mansukhani et al., 2005]. It must be noted that the complexity of FGF signaling controls a large number of genes, such as transcription factors, soluble factors, membranous, and matrix proteins [Marie, 2003], which in turn control cell proliferation, adhesion, differentiation, or apoptosis. Presumably, the gene-expression profile and phenotype induced by punctual FGFR mutations in murine osteoblasts may differ from the natural FGFR mutations in human craniosynostosis because of distinct environmental factors or genetic background. The analysis of the differential expression of genes in FGFR2-mutant human osteoblasts may provide clues in the molecular events controlled by FGFR signaling and resulting in premature cranial-bone formation in humans.

One area of consensus in murine and human genetic models is the common implication of FGFR signaling in the control of osteoblast apoptosis. Although acute FGF signaling reduces apoptosis of immature osteoblasts, continuous signaling promotes apoptosis in more mature osteoblasts [Debiais et al., 2004]. Consistently, constitutive activation of FGFR2 signaling by the C342Y Crouzon and the S252W Apert FGFR-2 mutations promote apoptosis in mouse osteoblasts [Mansukhani et al., 2000; Chen et al., 2003]. Apert FGFR-2 mutations also induce premature apoptosis in human osteoblasts and osteocytes in vivo and in vitro [Lemonnier et al., 2001; Kaabeche et al., 2005]. The upregulation of apoptosis by constitutive FGF signaling may be a significant mechanism controlling osteoblast number and osteogenesis. Apoptosis may be required for eliminating excessive cell number resulting from osteoprogenitor cell replication induced by FGFR in murine osteoblasts [Mansukhani et al., 2000]. Alternatively, apoptosis induced by constitutive FGFR2 activation in mature osteoblasts may be a necessary event compensating for the accelerated osteoblast differentiation induced by FGFR2 signaling [Lemonnier et al., 2001]. Further studies are required to determine the

precise role of apoptosis induced by FGFR signaling during cranial suture formation. Microarray analyses revealed that several apoptotic genes are altered by FGFR2 activation in human [Lomri et al., 2001] and murine osteoblasts [Mansukhani et al., 2005]. Whether these genes are commonly involved in the human and mouse premature suture fusion induced by FGFR activation remains to be determined.

SIGNALING PATHWAYS INDUCED BY FGFR ACTIVATION IN OSTEOBLASTS

Another key issue relates to the specific role of FGFR signaling pathways that are involved in craniosynostosis. In human-calvarial osteoblasts, Apert FGFR2 mutations constitutively activate PKC expression, phosphorylation, and activity [Fragale et al., 1999; Lemonnier et al., 2001]. Additionally, the S252W Apert FGFR2 mutation downregulates the expression and activity of Src family members Fyn and Lyn in human osteoblasts, which contributes to the premature differentiation phenotype [Kaabeche et al., 2004] (Fig. 2). This does not rule out that FGFR may induce other important signaling pathways controlling osteogenesis. For example, recent analysis of cell signaling in murine osteoblasts expressing Apert or Crouzon FGFR2 mutations revealed that activation of FGFR downregulates Wnt target genes [Mansukhani et al., 2005] suggesting that Wnt signaling may be involved in the phenotype induced by FGFR2 activation in these murine cells. Further analysis in human mutant osteoblasts may help to determine the signaling pathways that are involved in human craniosynostosis.

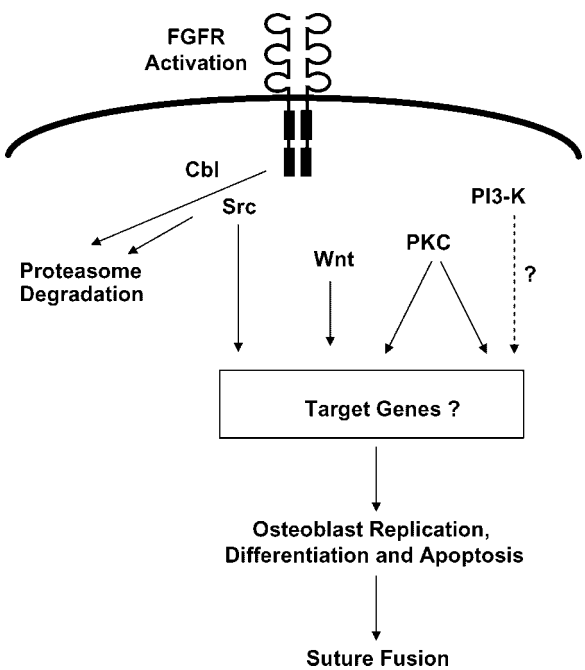

Fig. 2. Model of the mechanisms of action of activating FGFR signaling on murine-and human-osteoblast replication, differentiation and apoptosis. Dotted lines represent putative mechanisms. Question marks identify key issues that need to be investigated in future experimental approaches.

Another important critical issue in FGFR signaling is the role of FGFR2 downregulation in the control of osteoblast phenotype. After ligand binding, FGFR is downregulated by internalisation and degradation, which occurs in part through FGFR ubiquitination. Constitutive activation of FGFR2 by the S252W FGFR2 mutation accelerates FGFR down regulation in mutant osteoblasts in vitro and in vivo [Lemonnier et al., 2000]. The ubiquitin ligase Cbl was found to control FGFR1 degradation after ligand activation [Wong et al., 2002]. Similarly, FGFR2 activation induced by the overactive FGFR2 S252W mutation induces c-Cbl-mediated FGFR2 proteasome degradation, as well as Lyn and Fyn downregulation, which results in increased expression of early markers of osteoblast differentiation [Kaabeche et al., 2004]. Although this reveals a key role for Cbl in the control of FGFR degradation and osteoblast phenotype in Apert osteoblasts, a number of other proteins may interact with FGFR to attenuate FGFR signaling. Future studies are, therefore, needed to identify the role of proteins that may control FGFR internalisation in response to constitutive FGFR receptor activation (Fig. 2).

PUTATIVE TARGET GENES OF FGFR SIGNALING IN OSTEOBLASTS

A remaining important issue concerns the identification of target genes of FGFR signaling in osteoblasts. Several data suggest that Runx2, a master transcription factor that regulates the expression of several genes during osteogenesis, is a target gene for FGFR signaling. Craniosynostosis induced by the activating P250R mutation in FGFR1 in mice is associated with increased expression of Runx2 [Zhou et al., 2000]. Consistently, a gain-of-function C342Y FGFR2 mutation enhances Runx2 expression and causes premature fusion of cranial sutures in mice [Eswarakumar et al., 2002]. The P253R

and S252W FGFR2 mutations were also found to increase Runx2 expression in human calvarial osteoblasts from Apert patients [Tanimoto et al., 2004; Baroni et al., 2005]. Conversely, disruption of FGFR2IIIC, the mesenchymal splice variant of FGFR2, decreases the transcription of Runx2 and retards ossification [Eswarakumar et al., 2002]. However, Runx2 expression was found to be unchanged in mouse osteoblastic cells expressing the C342Y or S252W FGFR2 mutations [Mansukhani et al., 2005]. It is, therefore, unclear whether Runx2 directly or indirectly contributes to the osteoblast phenotype induced by FGFR activation. Other transcription factors are likely to be regulated by FGFR activation. For example, Sox2 was recently shown to be induced by FGFR2 activating mutations in murine osteoblasts [Mansukhani et al., 2005]. Additionally, Twist, a bHLH transcription factor, which was suggested to interfer with FGF signaling during cranial suture formation [Rice et al., 2000], was recently shown to interact with FGFR2 and to contribute to the osteoblast phenotype in Saethre–Chotzen craniosynostosis [Guenou et al., 2005]. Further studies are needed to determine the precise role of these and other transcription factors in the human osteoblast phenotype induced by FGFR activation (Fig. 2).

CONCLUSION

The role of FGF ligands in chondrodysplasias and craniosynostosis has not been clearly defined. However, data from the TgFGF2 and Fgf2 knockout mice supports a role for FGF2 in pathologic disorders associated with both endochondral and intramembranous bone formation and remodeling. Although Stat1 appears to be an important downstream signaling molecule in FGF2 endochondral bone development, it does not appear to be important in the effects of FGF2 on intramembranous bone formation. Studies are needed to identify other signaling pathways that mediate the effects of FGF2 in intramembranous bone formation. In addition, although both overexpression and knockout of FGF2 is associated with osteopenia, the differential time-dependent manifestation of this phenotype suggests that the osteopenia in the two lines of mice occurs through different mechanisms; but the osteopenia in both cases is mediated by FGF2, making this a compelling line of investigation because comparing these two forms will reveal important clues on how osteopenia occurs and the underlying molecular mechanisms for degenerative bone diseases.

Despite major advances in our understanding of the implication of FGFR signaling in membranous bone formation, the role of FGFR in osteoblast biology remains unclear. New areas of research are needed to clarify the controversial effects of FGFR signaling in mouse and human osteoblast models of craniosynostosis. Also, future experimental approaches should aim at determining the implication and cross-talks of specific signaling pathways induced by FGFR in osteoblasts in mice and humans. Finally, future investigations will have to identify specific genes that mediate osteoblast differentiation and apoptosis controlled by FGFR signaling. Such approaches may help to better understand the molecular mechanisms by which FGFR signaling controls osteoblasts and bone formation.

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