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FGF signaling: its role in bone development and human skeleton diseases

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1. ABSTRACT

Fibroblast growth factors (FGFs)/Fibroblast growth factor receptors (FGFRs) signaling has recently been found to play very important roles in bone development and diseases. Missense mutations in several FGFs and FGFRs were found in human to cause a variety of congenital bone diseases including chondrodysplasia syndromes, craniosynostosis syndromes, and syndromes with dysregulated phosphate metabolism, *etc*. In addition to its role in bone development and genetic diseases, FGF signaling is also involved in the maintenance of adult bone homeostasis and fracture healing, *etc*. Understanding the underlying molecular mechanisms for the role of FGFs/FGFRs in the regulation of bone development and diseases will ultimately lead to better prevention and treatment of FGF signaling-related bone diseases. In this review, we are going to give a summary of the role of FGFs/FGFRs in skeleton development and diseases, and pay more attention to those recent progresses.

2. INTRODUCTION OF SKELETON DEVELOPMENT

There are two major modes of bone formation, intramembranous ossification and endochondral ossification. The former occurs when mesenchymal precursor cells directly differentiate into bone-forming osteoblasts, a process through which all flat bones including calvarias are formed. In endochondral ossification, mesenchymal cells first differentiate into chondrocytes to form a cartilaginous template, which is eventually replaced by the bone. Appendicular skeleton including limbs (long bone), vertebrae, facial bones, and the medial clavicles are formed through endochondral ossification. Skeletogenesis is a highly coordinated process involving multiple complex interactions among a variety of genes. Dysregulated signaling involved in skeleton development can lead to skeleton anomaly (1-8).

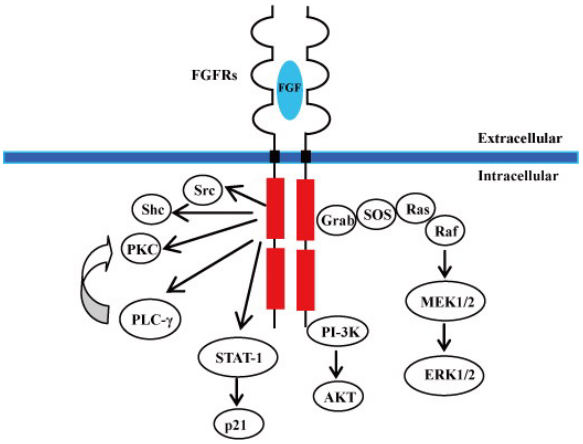

Figure 1. Structure of FGFRs and the major signaling pathways of FGF signal. FGFRs contain an extracellular ligand-binding domain, a transmembrane region and a highly conserved intracellular signaling domain. Activated FGFRs (red rectangles) stimulate multiple pathways such as PLC-γ, Ras-MAP kinase and PI-3 Kinase/AKT pathway. In some cells, FGF signaling also phosphorylates the Shc and Src protein. Additionally, activation of FGFR3 can stimulate STAT1-p21 pathway in chondrocytes.

3. INTRODUCTION OF FGFS/FGFRS

Fibroblast growth factor (FGF) family is currently composed of 23 genes that encode structurally related secreted proteins (9-12). Fibroblast growth factor receptors (FGFRs) are high affinity receptors for FGFs. A typical FGFR contains an extracellular ligand-binding domain, a transmembrane region and a highly conserved intracellular signalling domain.

FGFRs normally exist as an inactivated monomer. Upon activation, heparin/heparan sulfate, FGF, and FGFR form a trimolecular complex (13, 14). The binding of FGFs to the extracellular IgII and IgIII of FGFRs causes dimerization of FGFR monomers (15, 16), leading to the autophosphorylation of intrinsic tyrosine residues.

In general, FGFRs mediate extracellular FGF signals by at least two independent pathways. First, FGFRs directly or indirectly bind to SH2-containing molecules, such as PLC-γ, Crk and Src (17). Secondly, FGFRs are linked to SNT-1/FRS2 (18, 19), which, by binding to adaptor protein Grb2/Sos (19, 20), links FGFRs to Ras-Raf-MAPK (mitogen-activated protein kinases) pathways. In brief, FGFs/FGFRs signaling is mainly mediated by MAPK pathways that include the extracellular signal-related kinase1/2 (ERK1/2), p38 MAPK, stress-activated protein kinase /c-Jun N-terminal kinase (SAPK/JNK), protein kinase C (PKC), and phosphatidylinositol 3 kinase (PI-3K) pathways, *etc*(12) (Figure 1).

Besides those classical signaling molecules involved in the signal transduction of tyrosine kinase receptors, there are other genes such as *Shp2, Spry* (*Sprouty*), *sef* (similar expression to *fgf* genes), *XFLRT3*, *Mkp1*, *2*, *SOCS*, *etc*(21-32) that can positively or negatively regulate FGF signaling at the levels of FGFRs or their downstream signaling molecules. Working together, these molecules act at different levels of the signaling transduction cascades to precisely and tightly regulate FGF signaling activities (28). So far there is very limited information about their interaction with FGF signaling in the skeleton development and diseases.

4. FGFS/FGFRS SIGNALING IN CHONDRODYSPLASIA, CRANIOSYNOSTOSIS AND OTHER HUMAN GENETIC BONE DISEASES

4.1. Introduction of chondrodysplasia and craniosynostosis

One of the most important progresses in bone field in the recent decades is the identification of the roles of FGF signaling in the bone development and diseases. Mutations in FGFR1, 2 or 3 were found responsible for more than a dozen of human skeletal dysplasia that can be basically classified into two types: chondrodysplasia and craniosynostosis syndromes (Table 1) (33-36). Chondrodysplasia affects primarily the appendicular skeletons that are formed through endochondral ossification, resulting in short-limbed dwarfisms, while craniosynostosis affects mainly the intramembranously formed calvarial bones, leading to premature fusion of cranial sutures and the resultant craniofacial malformation.

4.2. FGFR3 and chondrodysplasia

There are three types of chondrodysplasia caused by gain-of-function mutations of FGFR3, achondroplasia (ACH), hypochondroplasia (HCH), and thanatophoric dysplasia (TD) (Table 1). Among them, ACH is the most common form of genetic dwarfism with a frequency of approximately 1 in 20,000 live births. ACH is clinically characterized by rhizomelic dwarfism, lumbar lordosis, macrocephaly, and depressed nasal bridge (37-39).

HCH is phenotypically similar to, but much milder than ACH, while TD is the most severe form of FGFR3 mutation-related chondrodysplasia characterized by severely shortened limbs and perinatal or neonatal death. Based on the gross phenotypes of femora and skulls, TD is subgrouped into TD-I (with straight femur and severe cloverleaf skull) and TD-II (has curved femur without severe cloverleaf skull). Severe Achondroplasia with Developmental Delay & Acanthosis Nigricans (SADDAN) was recently found to be associated with K650M mutation in FGFR3. In addition to severe skeletal dysplasia, patients with SADDAN also exhibit acanthosis nigricans and anomalies in central nervous system (40, 41).

Histologically, these chondrodysplasia patients have distinct abnormalities in their growth plates, which mainly include narrowed zone of proliferating and hypertrophic chondrocytes, disorganized proliferating chondrocyte columns, and premature closure of growth plates (42). Consistent with the severity of clinical phenotypes, the growth plates of TD patients are most severely affected.

Although the underlying molecular mechanisms for the activation of FGFR3 caused by various mutations are

Table 1. FGFs /FGFRs mutations in human and related mouse models

Gene	Human syndromes	Mutations in human genes	Mutations in mouse models	References
FGFR1	PS	P252R	P250R	177
		P252R	P252R BAC transgenic	222
	KS	G97D, Y99C, A167S		
		C277Y, V607M, R622X, W666R, M719R, P772S		
	OD	Y372C		56
FGFR2	AS	S252W	S250W	187
		P253R	P253R	Chen *et al.* unpublished data
	CS/PS	C342Y	C342Y	221
		N/A	Gain-of -function in IIIc	223
		C342R, C342S, C342W,		
	BS	S372C, Y375C		
	CS	Y105C, S252L, S267P, Y281C, Q289P, W290G, W290R, L292E, W301C, Y328C, N311I, G338E, G338R, Y340H, C342F, A344G, S347C, S354C		
	JWS	A344G, C342R,		
	PS	W290C, A315S, D321A, T341P, A344P, S351C, V359F		
	SCS	FGFR2/IgIIIa: VV269-70 del		
FGFR3	ACH	G380R(TM)	G374R	108, 224
			G374R transgenic (Col II promoter)	107
			hG380R transgenic (FGFR3 promoter)	225
		G375C	G369C	44
		G346E		
	CATSHL syndrome	p.R621H(partial loss of FGFR3 function)	FGFR3 knockout mice	60, 132
	CDC	A391E		
	HCH	N328I, N540K		
	NSC,MS	P250R, P350L		
	TD	S371C	S365C	106
		K650E	K644E	109
			K644E cDNA knock in	139
		R248C, S249C, G370C, Y373C		
	SADDAN	K650M	K644M	110
FGF2	Dwarfism	N/A	FGF2 transgenic mice	154
FGF3/FGF4	Craniosynostosis	N/A	Up-regulation of FGF3/4 caused by retroviral insertion	226
FGF23	ADHR	Gain-of-function mutation	Transgenic mice	77, 227
	TC	Impaired action of full length FGF23	Disruption/null	71, 89, 90, 228

Abbreviations: Achondroplasia (ACH), Autosomal dominant hypophosphatemic rickets (ADHR), Apert syndrome (AS), Beare-Stevenson cutis gyrata (BS), Camptodactyly, tall stature, and hearing loss (CATSHL), Crouzon syndrome (CS), Crouzonodermoskeletal syndrome (CDS), Hypochondroplasia(HCH), Jackson-Weiss syndrome (JWS), Kallmann syndrome (KS), Muenke syndrome (MS), Non-syndromic craniosynostosis (NSC), Osteoglophonic Dysplasia (OD) , Pfeiffer syndrome (PS), Saethre-Chotzen-like syndrome(SCS), Severe achondroplasia with developmental delay & acanthosis nigricans (SADDAN), Thanatophoric dysplasia (TD), Tumoral calcinosis(TC), N/A ,not available. Modified from (35, 122).

not the same (43-45), all the mutations causing the above-mentioned chondrodysplasia are gain-of-function mutations. The variable severities of the skeleton phenotypes in these chondrodysplasia patients are actually correlated with the degree of receptor activation. Among all the mutations causing TD, ACH and HCH, mutations responsible for TD lead to the highest activation of FGFR3, whereas mutation leading to HCH results in the least FGFR3 activity (43, 44).

4.3. FGFRs and craniosynostosis

Craniosynostosis is a clinical syndrome characterized by premature fusion of one or several calvarial sutures (46). It occurs with a prevalence of approximately 1 in 2100–3000 live births. In addition to the characteristic malformed skulls, patients with syndromic craniosynostosis may have other extracranial abnormalities including appendicular skeleton malformations, such as syndactyly of the hands and feet (Apert syndrome, AS) or broad thumbs and big toes (Pfeiffer syndrome, PS), and internal organ defects, *etc*.

Based on the sutures affected and the presence of non-cranial features, several distinct clinical entities of craniosynostosis syndromes have been established, which include Apert, Pfeiffer syndromes, Saethre-Chozen-like syndrome(SCS), Crouzon syndrome(CS)and Jackson-Weiss syndromes(JWS), *etc* (47-50).

A variety of mutations in FGFR1, FGFR2 and FGFR3 have been associated with several clinically distinct craniosynostosis syndromes. In addition to the single mutation in FGFR1 (P252R) that causes Pfeiffer syndrome (51), and two mutations in FGFR3, P250R(52, 53) and A391E(54), that cause Muenke syndrome (MS) and Crouzonodermoskeletal syndrome(CDS) (55), respectively, majority of those FGFRs-related craniosynostosis are caused by mutations in FGFR2. Among them, Apert

syndrome is only caused by S252W or P253R mutation of FGFR2, and is mainly characterized by premature closure of coronal sutures and syndactyly of the hands and feet. Like those chondrodysplasia-causing mutations in FGFR3, most CS-related mutations in FGFRs are sporadic, dominant gain-of-function missense mutations.

4.4. Other FGFs/FGFRs-related genetic diseases with abnormal skeleton development

In addition to the chondrodysplasia and craniosynostosis described above, other types of human genetic diseases with skeleton defects have been found to be associated with FGFs/FGFRs mutations or polymorphisms.

Osteoglophonic dysplasia (OD) is an autosomal dominant disorder characterized by craniosynostosis, rhizomelic dwarfism, and nonossifying bone lesions. White and colleagues sequenced FGFR1 of four OD patients, and identified N330I, Y372C and C379R mutation. Three of these patients were found hypophosphatemic, two of them showed low serum 1, 25-dihydroxyvitamin D concentration. One patient had increased serum level of FGF23. *In vitro* reporter gene assay showed that Y372C is a gain-of-function mutation. Since OD patients had long bone defect and hypophosphatemia, this study demonstrated that activation mutation of FGFR1 is responsible for OD, and FGFR1 is a negative regulator of long-bone growth and phosphate metabolism (56).

Kallmann's syndrome (KS) is characterized by idiopathic hypogonadotropic hypogonadism (IHH) and anosmia. Patients may also have cleft palate and other facial dysmorphisms. Heterozygous missense (loss-of-function) mutations in the FGFR1 gene were identified in some KS cohort with a high frequency of cleft palate and facial dysmorphisms(57-59), suggesting that FGFR1 signaling is also involved in palate and facial development.

Mutations in human FGFR3 are mainly gain-of-function mutations. Toydemir found p.R621H substitution in the tyrosine kinase domain of FGFR3 in human lead to a novel disorder characterized by camptodactyly, tall stature, scoliosis, and hearing loss (CATSHL syndrome). The phenotypes of CATSHL syndrome are very similar to those of the *Fgfr3* knockout mouse. Toydemir proposed that the p.R621H substitution of FGFR3 is partial loss-of-function caused by a dominant negative mechanism (60). Consistently, a loss-of-function mutation in sheep FGFR3 was found to cause Ovine hereditary chondrodysplasia, or spider lamb syndrome (SLS), which is characterized by severe skeletal abnormalities (skeletal overgrowth)(61, 62). Lambs homozygous for the SLS allel have deformed bone, whilst hyterozygous lambs (FGFR3 SLS/+) appear normal, but are physically larger than normal lambs.

Nonsyndromic cleft lip and palate (NS CLP) is a common congenital birth defect affecting around 1/700 live births worldwide. Riley sequenced the coding regions and performed association testing on FGFR1, FGFR2, FGFR3, FGF2, FGF3, FGF4, FGF7, FGF8, FGF9, FGF10, and FGF18. They identified seven likely disease-causing mutations, and found associations between NS CLP and SNPs in FGF3, FGF7, FGF10, FGF18, and FGFR1, suggesting that the FGF signaling pathway may contribute to as much as 3-5% of NS CLP(63).

Lacrimo-auriculo-dento-digital (LADD) syndrome is characterized by lacrimal duct aplasia, malformed ears and deafness, small teeth and digital anomalies. Rohmann recently found heterozygous mutations in FGF10, FGFR2 and FGFR3 in LADD families (64).

Considering the important roles of FGF signaling in bone development and diseases, the polymorphisms in FGFs/FGFRs are supposed to affect the development of skeleton. Toydemir suggests that human stature might be influenced by FGFR3 activity in a dose dependent fashion (60). Coussens screened nonpathologic populations for genetic polymorphisms that may be associated with normal craniofacial variation. The htSNP g.8592931G-->C was found to have a significant negative correlation with the cephalic index, further suggesting that FGFR1 is involved in the determination of craniofacial phenotypes (65).

4.5. FGF23 and dysregulated phosphate metabolism

Maintenance of proper serum phosphate levels is very critical for the extracellular matrix mineralization, normal skeletal development and bone integrity. Moreover, phosphate is also an important molecule involved in multiple cellular activities such as energy metabolism, DNA/RNA synthesis, and regulation of intracellular signaling by protein kinase and phosphatase, *etc* (66). Phosphate homeostasis is primarily maintained by its reabsorption mediated by type 2a and 2c sodium-phosphate co-transporters on the brush border membrane of proximal tubules in kidney, and absorption in intestine (67, 68).

In the last several years, FGF23 has been identified as a very essential regulator of phosphate and vitamin D metabolism (66, 68-73).

Although FGF23 was expressed in multiple tissues, it is mainly expressed in mineralized tissue cells including osteoblasts, cementoblasts, and odontoblasts (70, 74). Unlike other FGFs that function locally as growth factors regulating function of nearby cells, FGF23 acts as a hormone that derives mainly from bone and circulates to kidney and other organs to regulate their function.

Recombinant FGF23 was found to inhibit the expression of type 2a and 2c sodium-phosphate co-transporters in proximal tubules, and to reduce serum 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] level by simultaneously suppressing the expression of 25-hydroxyvitamin D-1α-hydroxylase, and enhancing the expression of 25-hydroxyvitamin D-24-hydroxylase(67). FGF23 thus reduces serum phosphate level by suppressing both the phosphate re-absorption in proximal tubules of kidney and phosphate absorption in intestine (68).

Investigations using genetically modified mouse strains further disclosed the role of FGF23 in phosphate and vitamin D metabolism. Homozygous *Fgf23* null mice

(*Fgf23*$^{-/-}$) had significantly increased serum levels of phosphate, calcium and 1,25$(OH)_2$D resulting from up-regulated renal phosphate reabsorption and enhanced expression of renal 25-hydroxyvitamin D-1alpha-hydroxylase respectively (71). The *Fgf23* null mice also exhibit premature aging-like phenotypes including reduced lifespan, infertility, osteoporosis, renal dysfunction, *etc* (71). In contrast, *Fgf23* transgenic mice have opposite features such as low serum vitamin D level, hypophosphatemia and no sign of premature aging-like phenotypes (75). Elimination or reduction of vitamin-D activity from *Fgf23* null mice, either by vitamin-D-deficient diet or introduction of 1a-hydroxylase deficient background could rescue the premature aging-like features and ectopic calcifications. These *in vivo* experimental data strongly supported the very essential role of FGF23 in the regulation of phosphate homeostasis, Vitamin D activity, and in the pathogenesis of premature aging (69).

As expected, *Fgf23*$^{-/-}$ mice also showed distinct skeleton phenotypes such as severe growth retardation and abnormal bone development. The 7-week-old *Fgf23* null mice had very short limb and marked reduction in mineral content, and severely disorganized growth plates with absence of hypertrophic chondrocytes.

Furthermore, the transgenic mice carrying a chicken β-actin promoter-driven *Fgf23* had rachitic bone including shorter femur, markedly decreased bone mineral density. These changes indicate that there is impaired mineralization of bones formed by both endochondral ossification and membranous bone formation in *Fgf 23* transgenic mice (75).

The critical roles of FGF23 in the regulation of bone development and phosphate metabolism have been further demonstrated in human studies. FGF23 has recently been found to be involved in human hyperphosphatemic tumoral calcinosis and several hypophosphatemic diseases including familial tumoral calcinosis(76), autosomal dominant hypophosphatemic rickets (ADHR) (77), autosomal recessive hypophosphatemic rickets/ osteomalacia (ARHR)(78), tumor-induced osteomalacia (TIO)(79), renal phosphate wasting in McCune–Albright syndrome/fibrous dysplasia (FD)(74), and X-linked hypophosphatemic rickets (XLH) (68, 70, 80).

ADHR and TIO are characterized by hypophosphatemia and low serum 1, 25-dihydroxyvitamin D [1, 25$(OH)_2$ D] levels. ADHR and TIO were proposed to be caused by enhanced FGF23 activity. FGF23 was found abundantly expressed in tumors causing TIO (79, 81). Mutations of FGF23 found in ADHR patients were postulated to result in increased full-length biologically functional FGF23 protein.

In addition to ADHR and TIO, FGF23 was also involved in other hypophosphatemic diseases including XLH, MAS and ARHR. Inactivating mutations in phosphate-regulating gene with homologies to endopeptidases on the X-chromosome (PHEX) are responsible for XLH (82). FGF23 level is increased in most patients with XLH (80, 83) and in Hyp mice, murine homologue of XLH caused by inactivation mutation of *Phex* gene (84). Hyp mice had increased FGF23 expression and hypophosphatemia, which was reversed by introducing FGF23 null mutation into Hyp mice (85, 86), indicating that enhanced FGF23 activity is responsible for the hypophosphatemia in patients with XLH and Hyp mice.

ARHR was recently found to result from missense mutations in dentin matrix protein-1 (DMP-1) gene. *Dmp1*-null mice exhibit hypophosphatemic rickets and osteomalacia similar to ARHR patients (78, 87). Both *Dmp1*-null mice and individuals with ARHR show elevated serum FGF23 levels. Considering the role of FGF23 in ADHR and other hypophosphatemic diseases, ARHR is proposed to be associated with excessive actions of FGF23.

In addition to its role in hypophosphatemic diseases, FGF23 was also found to be involved in hyperphosphatemic diseases. Hyperphosphatemic familial tumoral calcinosis was a relatively rare genetic disease characterized by enhanced renal tubular phosphate reabsorption and elevated serum phosphorus and 1, 25-dihydroxyvitamin D levels, as well as paraarticular calcific tumors (88). Multiple mutations in FGF23 gene that led to decreased FGF23 activity were identified in patients with hyperphosphatemic familial tumoral calcinosis (76, 89, 90).

Mutations in GALNT3, a gene encoding UDP-N-acetyl-alpha-Dgalactosamine: polypeptide N-acetylgalactosaminyltransferase 3 (ppGaNTase-T3) involved in mucin-type O-glycan synthesis, was also demonstrated responsible for familial tumoral calcinosis. Mucin-type O-glycation of serine or threonine residue is the most prevalent posttranslational modification of proteins. These patients had significantly decreased serum level of full length FGF23, suggesting that insufficient full-length FGF23 as a result of enhanced processing of FGF23 due to mutations in GALNT3 may mediate the hyperphosphatemia (91-93).

5. MECHANISM OF FGFS/FGFRS IN BONE DEVELOPMENT AND GENETIC DISEASES

5.1. Role of FGFs/FGFRs in endochondral ossification

5.1.1. FGFs/FGFRs expression

Appendicular bones are formed through endochondral ossification, in which FGFs/FGFRs play important roles.

Expression of several FGF ligands including FGF2, FGF5, FGF6, FGF7 and FGF9 has been observed in mesenchyme surrounding the condensation (94). In developing long bone, FGF2 was expressed in growth plate chondrocytes and osteoblasts of long bone. Using a more sensitive quantitative PCR, it's found that rat tibia perichondrium expressed FGF1, 2, 6, 7, 9, and 18 and, at lower levels, FGF 21 and 22. While growth plate expressed FGF 2, 7, 18, and 22 (95).

In limb bud, FGFR1 and FGFR2 were expressed in condensing mesenchyme, while no FGFR3 was detected (96, 97). Later, FGFR2 appeared as the first marker of prechondrogenic condensations, and FGFR3 was first expressed in chondrocytes differentiated initially from the center of the mesenchyme condensation. In developing

bone, FGFR2 was predominantly localized to perichondrial and periosteal tissue and weakly to endosteal tissue and trabecular bone (98). Besides its expression in the perichondrium and trabecular region of developing bone (primary spongiosa) (99), FGFR1 was also expressed in prehypertrophic and hypertrophic chondrocytes. Whereas FGFR3 was primarily expressed in the reserve and proliferating zone of growth plates (97, 99-103).

Using quantitative real-time RT-PCR, Lazarus found that rat tibia growth plates express all FGFRs, i.e., FGFR1, 2, 3 and 4. Among them, FGFR1 and 3 were expressed at greater levels, with FGFR1 up-regulated in hypertrophic chondrocytes and FGFR3 in both proliferating and hypertrophic chondrocytes. In contrast, FGFR2 and 4 were expressed at earlier stages of differentiation, with FGFR2 up-regulated in the resting zone and FGFR4 in the resting and proliferative zones of growth plates (95).

5.1.2. Role of FGFs/FGFRs in endochondral ossification

Genetically modified mouse models have been used extensively to study the role of FGFs and FGFRs in bone development and diseases. It's found that although many FGFs express in bone tissue, only few of them showed obvious bone phenotypes when they were genetically modified in mouse (Table 2).

Fgf2 null mice showed no obvious cartilage abnormalities (104), suggesting that endogenous FGF2 is not critical for the normal chondrogenesis. Contrastingly, *Fgf2* transgenic mice driven by phosphoglycerate kinase promoter exhibited dwarf phenotypes including premature closure of the growth plate and shortening of bone length(105), similar to that caused by gain-of-function mutation in FGFR3(44, 106-110). These data suggested that FGF2, when overdosed, is an inhibitory factor for chondrogenesis. Consistently, in cultured bone, FGF2 treatment resulted in a decrease in bone elongation, hypertrophic differentiation and chondrocyte proliferation (106).

Although *Fgf2* deficient mice showed no gross bone phenotypes, its bone marrow stromal cells do have decreased mineralization. Micro-CT examination and histomorphometry further revealed markedly reduced plate like trabecular structures, trabecular bone volume, mineral apposition, and bone formation rates in $Fgf2^{-/-}$ mice, indicating that endogenous FGF2 is essential for the maintenance of bone mass as well as structure integrity (104). Consistently, FGF2 was found by Yao to improve trabecular bone connectivity and bone strength in the lumbar vertebral body of ovariectomy-induced osteopenic rats (111). PTH significantly increased the bone formation and BMD in femur from $Fgf2^{+/+}$ mice when compared with $Fgf2^{-/-}$ mice, indicating that endogenous FGF2 is important for the maximum bone anabolic effect of PTH in mice (112).

Very interestingly, adult transgenic mice carrying FGF2 cDNA driven by phosphoglycerate kinase promoter showed decrease in trabecular bone, bone formation rates, and osteoclasts. These results suggest that FGF2 functioning as a negative regulator of postnatal bone growth and remodeling (105). The inconsistent results between *Fgf2* null and transgenic mice described above suggest that either absence of FGF2 or non-physiologically enhanced activity of FGF2 will lead to dysregulated postnatal bone homeostasis. Osteogenesis is coupled with chondrogenesis, since FGF2 overexpression mice driven by phosphoglycerate kinase promoter also showed inhibited chondrogenesis. It's also possible that the decreased bone formation is secondary to the retarded chondrogenesis.

Although FGF5, FGF6 and FGF7 express in mesenchyme surrounding the condensation, knockout mice lacking FGF5, 6, 7, however, showed no apparent skeletal phenotypes, suggesting the redundancies between these FGFs (113-118).

FGF8 is well known for its role in the outgrowth and patterning of the limbs (119). In addition, FGF8 is widely expressed in the developing skeleton. FGF8 treatment at early stage of osteoblastic differentiation stimulated the initial proliferation and subsequent osteoblast-specific ALP production, bone nodule formation, and calcium accumulation. FGF8 also stimulated the proliferation of MG63 osteosarcoma cells. These results demonstrate that FGF8 effectively predetermines bone marrow cells to differentiate into osteoblasts and increases bone formation *in vitro* (120).

Like FGF2, FGF9 has stimulatory effects on osteogenesis; it showed differentiation stage-dependent mitogenic effect on cultured calvarial bone cells. It can also induce expression of BMP-2 and inhibit noggin expression (121). Consistently, the skeletons of newborn $Fgf9^{-/-}$ mice are slightly smaller than their wild type littermates, and their proximal skeletal elements are disproportionately short (122). In contrast to its promoting effects on osteogenesis, FGF9, when over expressed in chondrocytes of mice, leads to dwarf phenotypes resulting from inhibited chondrocyte proliferation (123). It's also observed that, in transgenic mice, overexpression of FGF9 in cranial mesenchymal cells leads to a switch from intramembranous to endochondral ossification in calvarial bones, indicating that FGF9 may regulate bone development by affecting the differentiation direction of mesenchyme(124).These data suggest that FGF9 may have distinct roles in the development of mesenchyme, osteoblasts and chondrocytes.

Fgf18-deficient mice showed phenotypes in both endochondral and intramembranous bones (125, 126). *Fgf18* null mice exhibit a growth plate phenotype similar to that observed in mice lacking *Fgfr3*, which includes expanded zones of proliferating and hypertrophic chondrocytes, and increased chondrocyte proliferation. Developing long bone of *Fgf18* null mice also displayed delayed ossification and decreased expression of osteogenic markers such as osteopontin and osteocalcin. Considering the expression pattern and role of FGFR2, FGFR3 in chondrogenesis and osteogenesis, it's proposed that FGF18 may regulate cell proliferation and differentiation positively

Table 2. Mouse models with mutations of FGFs/FGFRs showing skeleton phenotypes

Gene	Mutation	Survival	Phenotype	Reference
FGFR1	Disruption/null	Lethal, E7.5–9.5	Growth retardation, defect of mesodermal patterning	229, 230
	FGFR1-deficient ES chimeras	Lethal during gastrulation	Defective cell migration through primitive streak, malformation of chimeric limb buds	231, 232
	Disruption/α-isoforms	Lethal, E9.5–12.5	Distal truncation of limb bud, lethal at E9.5–12.5 due to posterior embryonic defects	233
	Disruption of IIIb	Viable	No obvious phenotype	234
	Disruption of IIIc	Lethal	Gastrulation defects	234
	Chondrocyte-specific deletion using Col2a□Cre	Viable	Increased proliferation, but delayed osteoblast differentiation and increased proliferation, increased bone mass at adult stage	99
	Osteoblast-specific deletion using Col I- Cre	Viable	Accelerated differentiation and enhanced mineralization, increased bone mass at adult stage	99
	P250R mutation	Viable	Pfeiffer syndrome, just like P250R mutation in human FGFR1	177
	P252RBAC transgenic	Viable	Pfeiffer syndrome, P250R mutation in human FGFR1	222
FGFR2	Deletion/null	Lethal E4.5–5.5	The growth of the inner cell mass stopped, no visceral endoderm formed, trophoblast defects	235
	Deletion of IIIb and IIIc	Lethal E10.5	Failure of limb bud initiation and placenta formation, tetraploid rescued embryos die at birth without limbs	236, 237
	Deletion of IIIb	Lethal P0	Agenesis or severe dysgenesis of multiple organs	238
	Deletion of IIIc	Viable	Delayed ossification in the sphenoid region of the skull base, dwarfism in the long bones and axial skeleton	239
	Conditional/dermol-Cre	Viable	Skeletal dwarfism and decreased bone density	98
	RNAi/EIIA-Cre	Lethal	Displayed limb defects	240
	S250W mutation	Viable	Apert syndrome, just like S252W mutation in human FGFR2	187
	P253R mutation	Viable	Apert syndrome, just like P253R mutation in human FGFR2	Chen *et al.* unpub. data
	C342Y mutation	Viable	Crouzon syndrome/Pfeiffer syndrome, just like C342Y mutation in human FGFR2	221
	Gain-of-function in IIIc	Viable	Crouzon syndrome/Pfeiffer syndrome	223
FGFR3	Disruption/null	Viable	Bone overgrowth, inner ear defect	102, 103
	Deletion of IIIb	Viable	No obvious phenotype	133
	Deletion of IIIc	Viable	Skeletal overgrowth, decreased bone mineral density	133
	G374R knock in	Viable	ACH	108, 224
	G374R Transgenic (Col II promoter)	Viable	Mice are dwarfed, with axial, appendicular and craniofacial skeletal hypoplasia	107
	hG380R Transgenic (FGFR3 promoter)	Viable	Disproportionate dwarfism similar to those of human achondroplasia	225
	G369C knock in	Viable	ACH	44
	S365C knock in	Viable	TD	106
	K644E knock in	Neonatal Lethal	TD	109
	K644EcDNA knock in	Viable	TD	139
	K644M knock in	Viable	SADDAN	110
FGFR4	Disruption/null	Viable	Morphologically normal, No obvious defects in Skeleton	241
FGFR3/FGFR4	Cross of FGFR3 and FGFR4 mutants	Viable	Neonatal growth retardation, lung abnormalities.	241
FGF1	Disruption/null	Viable	No obvious phenotype	242
FGF2	Disruption/null	Viable	Impaired cerebral cortex development, blood pressure regulation and wound healing, decreased bone mass	104, 243
	Haplo-insufficiency	Viable	Decreased bone mass	244
	Phosphoglycerate kinase promoter transgenic (TgFGF2)	Viable	Dwarf mouse with premature closure of the growth plate and shortening of bone length□defective bone mineralization and osteopenia	105
FGF3	Disruption/null	Viable	Inner ear, tail outgrowth	245
FGF3/FGF4	Up-regulation of FGF3/4 caused by retroviral insertion	Viable	Craniosynostosis	226
FGF8	Disruption/null	Lethal E8.5	Many phenotypes including gastrulation, brain, heart and craniofacial development	246
FGF9	Disruption/null	Lethal P0	Lung hypoplasia, xy sex reversal, inner ear morphogenesis defect, slightly smaller than their wild type littermates	122, 247-249
	Conditional null allele	Viable	No obvious phenotype	250
	Transgenic mice (ColII-promoter)	Viable	Dwarfism, reduced proliferation and terminal differentiation of chondrocytes, bone growth defect	123, 124
	Transgenic mice, (αA-crystallin promoter)	Viable	The parietal bones in these mice show a switch from intramembranous to endochondral ossification	123, 124
FGF10	Disruption/null	Lethal P0	Many phenotypes including limbs, lungs, kidneys and others	251, 252
FGF18	Disruption/null	Lethal P1	Delayed ossification and increased chondrocyte proliferation, decreased alveolar spaces in the lung	125, 126, 129, 253
FGF23	Disruption/null	Viable	Hyperphosphatemia, hypoglycemia, chronic kidney disease, reduced bone density and infertility	71, 228
	Transgenic mice (a chicken β-actin promoter)	Viable	Rachitic bone including shorter femur, decreased bone mineral density	75
	Transgenic mice (Col I-promoter)	Viable	Growth retardation, osteomalacia, and disturbed phosphate homeostasis	227

Modified from (12, 35, 215)

in osteogenesis through FGFR2c and FGFR3c, and negatively in chondrogenesis through FGFR3c(126).

In vitro studies have also been carried out to explore the role of FGF18 in chondrogenesis and osteogenesis. FGF18 treatment resulted in increased Brdu incorporation into perichondrial cells of cultured metatarsals and enhanced proliferation of cultured mouse primary osteoblasts, osteoblastic MC3T3-E1 cells(127), supporting the promoting effect of FGF18 on osteogenesis.

In vitro bone culture experiment further demonstrated the role of FGF18 in chondrogenesis found in *Fgf18* null mice. Treatment of cultured mouse metatarsals with FGF18 resulted in decrease in bone length, hypertrophic differentiation of chondrocytes, and Brdu incorporation into chondrocytes (128).

In contrast to the inhibitory effect of FGF18 on chondrocyte proliferation found *Fgf18* null mice or FGF18-treated cultured bone, FGF18 treatment, however, stimulated the proliferation of cultured primary chondrocytes, and prechondrocytic ATDC5 cells, which can be blocked by specific ERK or p38 MAPK inhibitor (127). FGF18 treatment also showed inhibitory actions on the differention of chondrocytes (127). These seemingly contradictory data derived from mouse models and cultured cells suggest that the *in vivo* role of FGF18 in chondrogenesis need to be further studied.

In addition, FGF18 has also been found to regulate bone development by inducing skeletal vascularization and subsequent recruitment of osteoblasts/osteoclasts through regulation of early stages of chondrogenesis and VEGF expression, and by stimulating osteoclast formation and function (127, 129).

Several recent studies reported the genetic dissection of the role of FGFR 1, 2, 3 in mouse bone development. Due to the embryonic lethality of *Fgfr1* null mice, the *in vivo* role of FGFR1 in skeleton development was poorly understood. Recently Jacob, using Cre/loxp system in mice, found that although adult mice lacking *Fgfr1*, either in progenitor cells or in differentiated osteoblasts, exhibited increased bone mass, the role of FGFR1 in these two cell lineages is different. Inactivation of FGFR1 in osteo-chondro-progenitor cells lead to delayed osteoblast differentiation, whereas inactivation of FGFR1 in differentiated osteoblasts resulted in accelerated differentiation and enhanced mineralization. In immature osteoblasts, *Fgfr1* deficiency increased proliferation and delayed differentiation and matrix mineralization (99). Jacob proposed a model in which the FGFR1 promotes the differentiation of mesenchymal progenitor cells into preosteoblasts, and inhibits the mesenchymal progenitor cell proliferation, preosteoblast maturation and mineralization.

Using a similar conditional gene deletion approach, Yu deleted *Fgfr2* in Dermo1-expressing cells that will give rise to both chondrocyte and osteoblast lineages including perichondrial and periosteal cells surrounding cartilage. The resultant mutant mice showed skeletal dwarfism accompanied by significant decrease in bone density, trabecular bone and diaphyseal cortical thickness. The mutant mice also exhibited decreased proliferation of osteoprogenitors. Although the mutant appear to have no obvious changes in osteoblast differentiation, their osteoblasts were less active, and some of them looks like atrophic-appearing osteoblasts.

These data indicated that FGFR2 is essential for the proliferation of osteoprogenitor cells and the anabolic function of mature osteoblasts, and that endogenous FGFR2 signaling is required for the maintenance of bone homeostasis (98).

Bone development is accomplished through coordinated chondrogenesis and osteogenesis. The role of FGFR3 in chondrogenesis is well studied. The expression of FGFR3 in osteoblasts/osteocytes indicates that it may also be involved in the osteogenesis during bone development and remodeling (130, 131). Furthermore, mice mimicking human achondroplasia showed increased expression of osteoblast differentiation markers and advanced bone collars (44), indicating the potential effects of FGFR3 on osteogenesis.

Valverde-Franco found that young adult *Fgfr3* null mice are osteopenic due to reduced cortical bone thickness, lack of trabecular connectivity and defective trabecular bone mineralization. The *Fgfr3* null mice also exhibited increased expression of osteocalcin and MMP-13, markers of differentiated osteoblasts, and increased numbers of TRAP-positive osteoclasts. Primary cultures of adherent bone marrow-derived cells from *Fgfr3* null mice developed fewer mineralized nodules than FGFR3 wild type cultures of the same age. These data demonstrate that FGFR3 is required for post-natal bone growth and metabolism, and that modulation of FGFR3 may be a potential therapeutic approach for osteopenic disorders and those associated with defective bone mineralization (132).

Eswarakumar has made the epithelial *Fgfr3b* isoform deficient (*Fgfr3b* null mice) and the mesenchymal *Fgfr3c* isoform deficient mouse model(*Fgfr3c* null mice), and found no obvious skeleton changes in *Fgfr3b* null mice, but observed dramatic skeletal overgrowth and decreased bone mineral density in the cortical and trabecular bone, which resembling the phenotype of *Fgfr3* null mice. These data demonstrated that the mesenchymal FGFR3c isoform is responsible for regulating normal skeletal development (133).

In addition to cartilage at growth plates, long bone also has articular cartilage, which is normally existed in an undifferentiated status to protect the long bones and joints (134). Although the role of FGFR3 in growth plate cartilage is extensively studied, the role of FGFR3 in articular cartilage is elusive. Valverde-Franco showed that *Fgfr3* null mice exhibited premature articular cartilage degeneration and early arthritis, indicating that endogenous

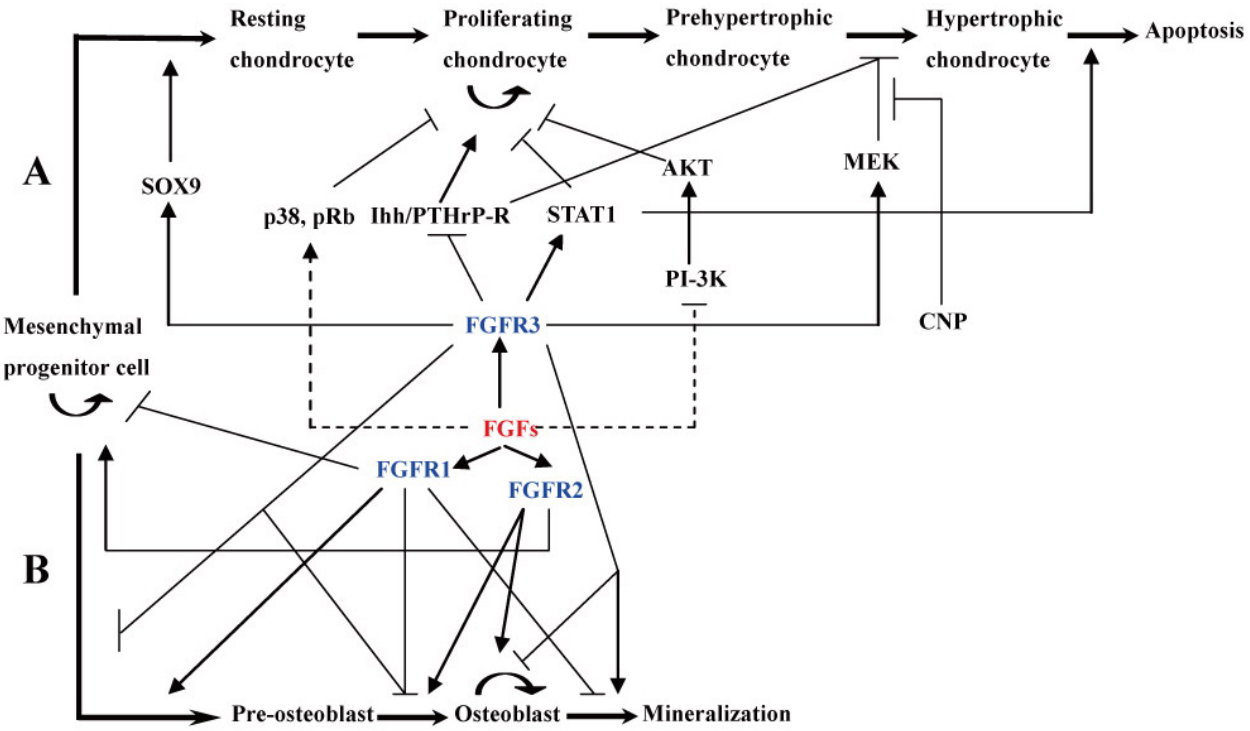

Figure 2. Model for the regulation of chondrogenesis (A) and osteogenesis (B) by FGF signaling based on several studies(14,98,99,106,132,143,150,151,153,155) (A) In chondrogenesis, FGFR3 plays very important role in the regulation of proliferation, differentiation and apoptosis of chondrocyte through several signaling pathways. (B) In osteogenesis, FGFR1 inhibits proliferation and promotes differentiation at early stages of osteoprogenitor development, as well as prevents further differentiation and mineralization. However, FGFR2 is a positive regulator of osteogenesis. FGFR3 promotes mineralization, but inhibits the proliferation, differentiation of osteoblast. The effects of FGFR1 and FGFR2 on chondrogenesis are not mentioned here, please see the details in references (98, 99, 220).

FGFR3 signaling is very essential for the maintenance of normal articular cartilage metabolism (135).

From these data, it appears that FGFR1, 2 and 3 are involved in the regulation of both chondrogenesis and osteogenesis, although each FGFR has distinct effects on them (Figure 2).

5.2. FGFR3 in chondrodysplasia

Our understanding of the molecular mechanism of chondrodysplasia resulting from FGFR3 mutations has been deepened greatly in the last decade. Besides human-related studies, generation of genetically modified mouse models mimicking these human diseases has played essential roles in our efforts to explore the mechanisms (Table1, 2). A number of mouse models carrying a variety of FGFR3 mutations, corresponding to their human equivalent, have been generated (136). In general, these mice phenocopied the skeleton features of corresponding human patients (136). Compared with their wild type littermates, the mutant mice exhibited smaller body size, shortened long bone, and dome-shaped skull, *etc*. Histologically, growth plates of these mutant mice have disorganized chondrocyte columns accompanied by shortened proliferating and hypertrophic zones. Consistent to what have been found in patients, the severity of the mutant mice is determined by the activity of the mutated FGFR3. Mice carrying K644E (equivalent to K650E that causes TD-II in humans) exhibited the most severe phenotypes, and died within few hours after birth, whereas mice harboring G369C (equivalent to G375C mutation in human) exhibited much milder phenotypes than those carrying FGFR3 S365C mutation (equivalent to human S731C mutation that causes TD-I).

Chondrodysplasia affects mainly appendicular bones that are formed through highly coordinated chondrogenensis at growth plates and osteogenesis at bone collar and trabecular bones. FGFR3 is mainly expressed at cartilage of growth plates, suggesting that the major target of FGFR3 mutations is chondrogensis. In fact, chondrodysplasia resulting from FGFR3 mutations affects primarily the chondrogenesis of endochondral bones such as appendicular bones and cranial base synchondroses. Consistently, detailed analysis of samples from human patients or chondrodysplasia mouse models indicates that FGFR3 mutations affect all the major stages of chondrogenesis at growth plates. The mutant growth plates showed irregular and shorter proliferating chondrocyte columns, and decreased Brdu or ^{3}H-thymidine incorporation, indicating inhibited chondrocyte proliferation. The faster and prolonged endochondral bone

growth, as well as the increased length of appendicular bones and enhanced proliferation of chondrocytes of the growth plate of *Fgfr3*-null mice (102, 103) further support the inhibitory effects of FGFR3 on chondrocyte proliferation. Furthermore, treatment of FGF2 and FGF18, ligands that can activate FGFR3, resulted in retarded bone elongation and decreased chondrocyte proliferation in the cultured metatarsal rudiments from embryonic wild type mice (44, 106, 128). These data demonstrated that FGFR3 is a negative regulator of bone growth.

Studies have been carried out to further uncover the mechanism for the inhibiting effects of FGF signaling on chondrocyte proliferation (Figure 2).

FGF treatment or ectopic expression of constitutively activated mutant FGFR3 (K644E in mouse) was found to induce nuclear translocation of STAT1 and expression of cell-cycle inhibitor p21 (WAF1/CIP1)(137, 138). Up-regulated expression of cell cycle inhibitors (p16, p18, p19 and p21) and STAT proteins (STAT1, STAT 5a, and STAT 5b) were also found in chondrocytes of mutant growth plates(106, 139). STAT1 activation and increased p21 (WAF1/CIP1) expression were also found in the cartilage cells from the TDII fetus (137). These data suggested that a number of cell cycle inhibitors and STATs such as p21, STAT1 may mediate the inhibitory effects of FGFR3 mutation on chondrocyte proliferation.

The involvement of STAT1 in the regulation of bone development by FGFR3 was further studied using *Stat1* null mice. Growth plates of *Stat1* null mice showed a transient expansion of the proliferative zone and acceleration of longitudinal bone growth (140). FGF2 treatment failed to induce chondrocyte growth inhibition in primary chondrocytes derived from *Stat1* null mice (138), while it normally does in chondrocytes from wild type mice. Furthermore, crossing *Stat1* null background into transgenic mouse over expressing human FGF2 (TgFGF) leads to a significant correction of the reduced proliferation in chondrocytes of TgFGF mice (140). These data demonstrated that activated FGFs/FGFRs function through STAT1 mediated pathway to inhibit chondrocyte proliferation.

Recently, suppressors of cytokine signaling (SOCS) proteins that normally inhibit the activity of STATs were found to interact with FGFR. Ben-Zvi showed that activation of FGFRs in chondrocytes induced the expression of SOCS1 and SOCS3 mRNA, and that these proteins are constitutively associated with FGFR3. In chondrocytic cell line over expressing SOCS1, STAT1 phosphorylation is repressed, MAPK phosphorylation is elevated and prolonged (29). These data suggested that FGFR signaling can be regulated at different levels, but the effect of the interaction between SOCSs and FGFR3 on chondrocytes, and bone development *in vivo* need to be further studied.

In addition to the activation of STAT1, pRb family protein p107 and p130 have also been found involved in chondrocyte growth arrest induced by FGF signaling (141, 142).

PI-3K is one of the major downstream signaling pathways of FGF signaling. Priore found that Akt phosphorylation is down-regulated in chondrocytes treated with FGF. Forced expression of a constitutively activated Akt (myr-Akt) in the RCS chondrosarcoma cell line partially counteracts the inhibitory effect of FGF signaling, suggesting that Akt mediates the inhibitory effect of FGF signaling on chondrocyte proliferation (143).

PTHrP-R and IHH are expressed at growth plate chondrocytes and can stimulate chondrocyte proliferation (144-147). FGF2 treatment directly down-regulates the mRNA level of IHH and PTHrP-R (106). Mice mimicking human ACH and TD have markedly decreased expression of IHH and PTHrP-R (107). It was thus suggested that the inhibitory effects of FGFR3 on chondrocyte proliferation is partially mediated by its down regulation of IHH and PTHrP-R (4, 106).

In contrast to the inhibitory effect of FGFR3 on chondrocyte proliferation found in most mouse models carrying FGFR3 mutations, mutant embryos harboring FGFR3-K644E mutation displayed enhanced or normal proliferation of growth plate chondrocytes during early (E14-15) or late stages of endochondral ossification, suggesting that the inhibitory effects of FGFR3 signaling on chondrocyte proliferation might be developmental stage dependent (109).

FGF signaling also affects chondrocyte differentiation. Chondrocytes are originally differentiated from mesenchyme. FGFR3 has been used as a marker of mesenchyme (148), and forced expression of FGFR3 in C3H10T1/2, a pluripotent murine mesenchymal stem cell line, is sufficient for chondrogenic differentiation, indicating that FGFR3 could induce the differentiation of mesenchyme into chondrocyte lineage (149). Enhanced FGF signaling including activation of FGFR3 was also found to cause up-regulation of the chondrogenic *Sox*9 gene in C3H10T1/2 and primary chondrocytes (150). There are controversies about the effect of FGFR3 on chondrocyte differentiation into hypertrophic chondrocytes. Based on the narrowed zone of hypertrophic chondrocytes in either cultured wild type bone treated with FGF2/FGF9 or in long bone of mutant mice carrying gain-of-function mutation of FGFR3, FGFR3 is generally considered as an inhibitor of chondrocyte differentiation (33, 35).

Constitutive activation of MEK1 in chondrocytes of mice leads to dwarf phenotype similar to achondroplasia (151). The mutant mice exhibited incomplete chondrocyte hypertrophy in the growth plates and delayed endochondral ossification, whereas chondrocyte proliferation was unaffected. Introduction of this constitutively activated MEK1 into chondrocytes of *Fgfr3*-deficient mice rescued the skeletal overgrowth characteristic of *Fgfr3* null mice. In addition, loss of *Stat1* restored only the reduced chondrocyte proliferation, but not the reduced hypertrophic zone and the achondroplasia-like phenotype in mice mimicking achondroplasia. A model was proposed in which MAPK mediates the inhibitory effect of FGFR3 on chondrocyte differentiation, while STAT1 mainly mediates

the inhibition of chondrocyte proliferation by FGFR3 (14, 151).

However, in cultured chondrocyte cell line (RCS) treated with FGF1, it's was suggested that FGF signaling accelerates the differentiation of chondrocytes by regulating the expression of some chondrocyte differentiation related genes, for example, inhibiting the expression of Ids. Mice harboring an activating FGFR3 mutation also showed decreased expression of Id 1, 3 in growth plates(142). Moreover, Minina and colleagues (152), using embryonic bone culture model, proposed a model in which the FGF signaling promotes both the onset of chondrocyte differentiation, through the down-regulation of the IHH/PTHrP pathway, and the acceleration of chondrocyte differentiation process in an IHH/PTHrP-independent manner.

Hypertrophic chondrocytes will finally undergo apoptosis. Increased apoptosis was found in chondrocytes isolated from patients with TD (153), and in chondrocytes from mice over expressing FGF2 under the control of a constitutive phosphoglycerate kinase promoter (TgFGF mice) that exhibits skeletal dwarf phenotype (140, 154). Loss of *Stat1* (*Stat1*$^{-/-}$) in this *Fgf2*-overexpression transgenic mice can rescue the excessive apoptosis of chondrocytes to near-normal levels (140), suggesting that STAT1 may mediate the increased chondrocyte apoptosis induced by over activated FGF signaling. The presence of STAT1 in the nuclei of hypertrophic chondrocytes in thanatophoric dysplasia further supports the role of STAT1 in mediating the apoptosis induced by FGFR3 activation (153).

A number of genes were found to interact with FGFR3 to regulate the bone development. TGF-β treatment resulted in increased tyrosine phosphorylation of FGFR3 and FGF18 mRNA level in cultured bone. The inhibitory effects of TGF-β on hypertrophic differentiation and elongation of cultured metatarsal bone were alleviated by pretreatment with FGF signaling inhibitors. These results suggest that TGF-β can regulate FGF signaling during embryonic bone formation (128).

In a limb culture system, Minina demonstrated that BMP treatment can alleviate the achondroplasia phenotype by rescuing the reduced domains of proliferating and hypertrophic chondrocytes in mouse model for achondroplasia (FGFR3 G380R), indicating that manipulation of the BMP signaling pathway would be a novel approach to alleviate the achondroplasia phenotype (152).

C-type natriuretic peptide (CNP) stimulates endochondral ossification and rescues the dwarf phenotype of mouse model carrying constitutive active FGFR3 by correcting the decreased extracellular matrix synthesis in the growth plates through inhibition of MAPK pathway (155). In ATDC5 cells, FGF2 and FGF18 markedly reduced CNP-dependent intracellular cGMP production through a partially MAPK-dependent pathway. Conversely, CNP and 8-bromo-cGMP dose-dependently inhibited the induction of ERK phosphorylation by FGF2 and FGF18 without changing the level of FGFR3. In the cultured fetal mouse tibias, CNP and FGF18 counteracted on the longitudinal bone growth. These results suggest that the CNP/GC-B pathway constitutes the negative cross talk between FGFs/FGFRs and the activity of MAPK (156).

β1-integrin null mice showed chondrodysplasia phenotype accompanied by disorganized proliferating chondrocyte columns and decreased chondrocyte proliferation in growth plates. The mutant chondrocytes also showed upregulated expression of FGFR3, p16, p21 and nuclear translocation of STAT1 and STAT5a, suggesting that β1-integrin regulates bone development by negatively regulating the FGFR3-related pathways (157).

5.3. The role of FGFs/FGFRs in intramembranous ossification

5.3.1. FGFs/FGFRs expression

Multiple FGFs and FGFRs are expressed during the development of craniofacial sutures (33, 34, 36). All FGFs, except FGF3 and FGF4, are identified in coronal suture of E17.5 embryos by RT-PCR (158). Among them, FGF2 is expressed in osteogenic mesenchymal cells and osteoblasts (159-161). FGF9 is also expressed in mesenchyme of suture in early craniofacial development stages (160). During intramembranous bone formation, FGF18 is expressed strongly in osteogenic mesenchymal cells and differentiating osteoblasts at sutures and rims of the calvarial bones as well as cartilaginous base of the cranium. Expression of FGF20 extends to the rims of all calvarial bones, including the coronal sutures (125, 126, 158).

FGFR1 is expressed mainly in the calvarial mesenchyme and later in osteoblasts. In contrast, FGFR2 is expressed in proliferating osteoprogenitor cells (162), and in differentiating osteoblasts (96, 126, 163-165). FGFR3 is not found in calvarial mesenchymal cells or periosteal cells, it expresses primarily at the cranial cartilage, and at low levels in the osteogenic front of suture at later development stages (96, 166, 167). FGFR1, FGFR2 and FGFR3 are all intensely expressed in the cartilages of the cranial base (167, 168). Actually, different sutures have distinct spatial and temporal expression patterns of FGFs/FGFRs. For example, FGF2 expression is significantly stronger in posterofrontal cells than that in sagittal suture mesenchymal cells. While FGFR1 mRNA level is significantly higher in the posterior frontal suture than the sagittal suture (169).

5.3.2. FGFs/FGFRs signaling in intramembranous ossification

FGFs/FGFRs signaling plays an important role in intramembranous ossification (Figure 2), which is strongly supported by the fact that mutations in FGFR1, FGFR2 and FGFR3 cause craniosynostosis (33, 34, 170). Majority of these mutations are gain-of-function mutations, suggesting that, although there are controversies about the role of FGF signaling in the proliferation, differentiation and apoptosis of osteogenic and osteoblast cells (34), FGF signaling in

general promotes intramembranous ossification. In fact, many other evidences support this viewpoint. Application of FGF2 or FGF4 soaked beads to developing mouse coronal suture leads to synostotic coronal sutures accompanied by induction of apoptosis, collagen type I expression and mineralization (171). Local blocking of endogenous FGF2 activity in chicken prevents cranial osteogenesis in a dose dependent manner (172). In contrast, forced expression of dominant-negative FGFR1 gene inhibits suture fusion in rat calvaria(173).

Fgf18-deficient mice showed defect ossification in calvaria. The mutants have delayed suture closure accompanied by decreased proliferation of calvarial osteogenic mesenchymal cells and delayed terminal differentiation of mesenchyme to calvarial osteoblasts. Mutant also had decreased calvarial bone mineralization (126).

Sutures are formed through multiple coordinated processes including mesenchymal cell condensation, preosteoblast proliferation, osteoblast differentiation, mineralization and apoptosis. FGF signaling regulates all these processes.

Increased proliferation is supposed to cause premature closure of sutures. FGF signaling may lead to increased proliferation of suture cells. FGF4 treatment was found to cause premature suture fusion with increased cell proliferation in cultured calvaria and in mice, respectively (160). Conversely, disruption of FGF2 gene in mice results in decreased calvarial bone formation and bone mass (104). Consistently, blocking of endogenous FGF2 activity leads to decreased cell proliferation in cultured chick cranial vaults (172).

FGF signaling also regulates the differentiation of calvarial cells, although its actual role is still not very clear. Local FGF2 treatment induced suture closure accompanied by locally decreased cell proliferation and increased osteoblast differentiation, suggesting that enhanced FGF signaling leads to a shift of suture cells from proliferation to differentiation (162). Many other studies also indicate that FGF signaling leads to enhanced osteoblast differentiation (174-177).

However, forced expression of mutant FGFR2 containing either S252W or C342Y caused blocked differentiation/mineralization (178). Mansukhani proposed that FGF signaling has dual effect on osteoblast proliferation and differentiation. Immature osteoblasts respond to FGF signaling with increased proliferation, whereas differentiating osteoblasts undergo apoptosis. The intensity of FGF signaling may also affect the functional status of calvarial cells. In embryonic chick cranial vaults, minor reduction of endogenous FGF2 by local application of beads containing FGF2 neutralizing antibody led to a switch of skeletogenic cells from a differentiative to a proliferative mode. Whereas further blocking of FGF2 function resulted in abolishing of both proliferation and differentiation (172).

Differentiation of osteoblasts is driven mainly by Cbfa1/Runx2, a master transcription factor that controls osteoblastogenesis. Human cleidocranial dysplasia is caused by haploinsufficiency mutation of Cbfa1/Runx2 (179). *Cbfa1/Runx2* null mice have completely no osteoblasts (180, 181). FGF signaling affects the expression and activity of Cbfa1/Runx2. Expression of Cbfa1/Runx2 can be induced by FGF2/FGF8 treatment or transfection of FGFR1 cDNA containing Pro250Arg mutation, a mutation causing human Pfeiffer syndrome, in C3H10T1/2(177), indicating that Cbfa1/Runx2 can be induced even in non-osteoblasts by FGF activity. Choi found that FGF2 treatment of developing bone fronts stimulated expression of BMP2 and Cbfa1 gene, and Cbfa1 is a major mediator of FGF2-induced BMP2 expression in cranial bone development (182).

FGF2 treatment also enhances the binding of Cbfa1/Runx2 to Cbfa1-binding consensus sequence in the promoter of osteocalcin, a specific marker of mature osteoblasts, which further supports that FGF signaling induce osteoblast differentiation through Cbfa1/Runx2 (183). ERK and PKC are involved in the FGF stimulated phosphorylation and transcriptional activity of Cbfa1/Runx2 (183-185).

Actually, different FGFs/FGFRs may have distinct effects on the suture development. The spatiotemporaral expression patterns of FGFRs at sutures also indicate that different FGFRs may have distinct effects on the proliferation and differentiation of calvarial cells. Iseki *et al.* found that the differentiation process of osteogenic cells into osteoblasts is preceded by the downregulation of FGFR2, and upregulation of FGFR1. Once differentiation is completed, FGFR1, osteonectin and alkaline phosphatase (AKP) are down-regulated. Iseki proposed that FGFR2 signaling regulates stem cell proliferation, whereas FGFR1 is involved in the osteogenic differentiation process but not in maintaining the differentiated state (162).

There are also controversies about the role of FGF signaling in the apoptosis of calvarial cells. Debiais found that FGF2 protected cultured human calvarial osteoblasts from serum starvation-induced apoptosis through activation of PI-3K and inhibition of caspases (186). FGF4 or FGF2 administration induces apoptosis, collagen type I expression, and mineralization in the developing mouse coronal suture (171). *Fgf2* over expression transgenic mice showed increased apoptosis. The apoptotic cells are concentrated at the osteogenic front representing differentiating calvarial osteoblasts, while immature cells in the middle of the suture are less apoptotic (178). Moreover, enhanced FGFR2 activity resulting from S252W in mice and cultured cells caused significantly increased apoptosis (178, 187).

Actually, the final effects of FGF signaling on the apoptosis of calvarial osteoblasts are also depended on the different stage of osteoblasts and the duration and intensity of FGF signaling. It's possible that acute FGF signaling may reduce apoptosis of immature osteoblasts, whereas

continuous FGF signaling may promote apoptosis in more mature osteoblasts.

Many molecules are involved in the regulation of osteoblast apoptosis by FGF signaling. Enhanced expression or activity of IL-1,Fas, PKC, caspase-8 and Bax were found involved in the promotion of osteoblast apoptosis by FGFs/FGFRs signaling(187-190).

The mechanism for the regulation of suture development by FGF signaling is very complicated. During normal suture development, there is a balance among the proliferation, differentiation, and apoptosis of the osteogenic precursor cells (171, 191). It appears that FGF signaling regulates suture closure by affecting this balance. But the actual effects of FGF signaling on cranial osteogenesis are determined by a variety of factors such as the intensity and duration of FGF activity, and the differentiation stage of osteoblasts/ osteogenic cells (162).

In general, FGF signaling appears to increase the proliferation of immature cells, while promotes the progression of undifferentiated cells toward mature osteoblasts and induces apoptosis of mature osteoblasts in developing calvaria (178). Lower FGF signaling activity is associated with proliferation, while enhanced FGF signaling will lead to differentiation and apoptosis (162, 172).

Apoptosis decreases the number of osteoblasts and their precursors (192). In craniosynostosis such as Apert syndrome caused by gain-of-function mutations in FGFR2, osteogenic precursor cells may be forced by enhanced FGF signaling to undergo premature apoptosis at the expense of proliferating and differentiated cells, which lead to more narrowed gap between the overlapping frontal and parietal bones, and eventually premature suture obliteration (35, 187). Decreased apoptosis, however, has also been proposed to be responsible for the occurrence of craniosynostosis (191, 193, 194). These seemingly contradictory data suggest that the effect of FGF signaling on the differentiation and apoptosis of osteogenic cells need to be further studied.

FGFs/FGFRs exert their effects on calvarial bone formation by interacting with a variety of other molecules such as transcription factors, growth factors, *etc*. Beside its cross-talking with MSX2, Twist(131, 167, 191, 195), FGF signaling also interacts with Forkhead/winged helix transcription factor, Foxc1.Foxc1 regulates the proliferation of calvarial osteoprogenitor cells by controlling BMP-driven expression of Msx2 and Alx4 during calvarial development(196). *Foxc1* null mice develop only rudimentary calvarial bones (197). Heterozygous *Foxc1* mutants have delayed ossification of calvarial bones. FGF2 can regulate the expression of Foxc1 in calvarial mesenchyme, indicating that Foxc1 may be involved in the regulation of calvarial development by interacting with FGF signaling pathways (196).

Wnt and Sox signaling are very important in the regulation of osteoblastogenesis (198-201). Mansukhani found a striking down-regulated expression of multiple Wnt target genes and a concomitant induction of the transcription factor SOX2 in osteoblasts expressing FGFR2 activating mutations (C342Y or S252W). Wnt signals promote osteoblast function and regulate bone mass. Sox2 is expressed in calvarial osteoblasts *in vivo* and was found to inhibit osteoblast differentiation and cause down-regulation of the expression of numerous Wnt target genes, these results indicate that FGF signaling can regulate osteoblast differentiation through induction of Sox2 and regulation of the Wnt-β-catenin pathway (202).

TGF-β family plays an important role in the suture development (203-205). TGF-β2 and TGF-β3 can regulate the proliferation and apoptosis of osteogenic suture cells (191, 206). TGF-β2 stimulates cranial suture closure through activation of the ERK-MAPK pathway (205). FGF acts downstream of TGF-β signaling in regulating cranial neural crest cell proliferation, and exogenous FGF2 rescues the cell proliferation defect in the frontal primordium of *Tgfbr*2 mutant (207). Prolonged treatment of FGF2 leads to increased TGF-β2 production in human osteoblastic MG-63 cells (208). Conversely, TGF-β regulates FGF2 and FGFRs expression in osteoblastic cells (209). These observations suggest that FGF signaling might also affect suture morphogenesis through interacting with TGF-β signaling (191).

BMP signaling is very essential for the osetogenesis. Unlike the opposite effects between FGF and BMP signaling in chondrogenesis (152), BMP signaling may mediate the effects of FGF signaling on the suture development. FGF2 and FGF9 increased expression of BMP2 in calvarial bone cells isolated from chick embryonic frontal bone. Meanwhile, blocking of the endogenous FGF signaling using a virally transduced dominant-negative FGF receptor resulted in significantly down regulated expression of BMP-2, indicating that endogenous FGF signaling is a positive upstream regulator of the BMP-2 in calvarial osteoblasts(121). Fakhry and others also found that the expression of a BMP antagonist noggin was inhibited by FGF2 and FGF9 (121). Consistently, Warren has also found that the noggin expression is suppressed by FGF2 and FGFR2 with gain-of-function mutations (210). These data suggest that FGF signaling can either directly or indirectly control cranial suture fusion through BMP signaling.

In addition to the molecules mentioned above, FGF signaling also interacts with IGF, HGF and VEGF to affect calvarial osteogenesis *in vivo*. For example, FGF2 up-regulates insulin-like growth factor-I (IGF-I) expression and inhibits the expression of its regulatory binding protein-IGF binding protein-5 in bone cells (211). Moreover, FGFs are also found to increase VEGF production of osteoblasts (212).

5.4. FGFRs in craniosynostosis

A number of mutations in FGFR1, 2 and 3 were found responsible for several types of craniosynostosis. Majority of those mutations in FGFRs lead to constitutive activation of FGFRs by a variety of mechanisms such as ligand-independent dimerization and activation, and

changed ligand-binding specificity of FGFRs (11, 213-219), *etc*. Although our understandings about the molecular mechanisms underlying these craniosynostosis have been deepened using a variety of *in vitro* approaches such as cultured cells, to further explore the mechanisms, *in vivo* approaches including animal models have been used. Several mouse models have been made to mimic human craniosynostosis resulting from mutations in FGFR1, 2(Table 1). Zhou has made a model carrying a P250R mutation in FGFR1, which corresponds to the P252R mutation in human FGFR1 that causes human Pfeiffer syndrome (177). The mutant mice exhibited grossly as well as histologically confirmed premature fusion of the interfrontal, coronal and sagittal suture. The mutant mice had more AKP-positive cells and enhanced expression of Cbfa1, BSP (Bone Sialoprotein), osteopontin and osteocalcin, indicating that premature differentiation of osteoblasts might be responsible for the human Pfeiffer syndrome caused by P252R mutation in FGFR1.

We have generated a knock-in mouse model mimicking human Apert syndrome caused by a S252W mutation in FGFR2 (187). The mutant mice showed premature fusion of the coronal suture. Surprisingly, the coronal suture of mutant mice has no significant changes in cell proliferation, and expression of BSP, AKP, osteocalcin and Cbfa1/Runx2. Conversely, the mutant mice have increased apoptosis and Bax expression in the coronal suture (187). These data suggest that increased cell apoptosis, instead of altered proliferation or differentiation, is responsible for Apert syndrome caused by FGFR2 S252W mutation.

Wang also generated a knock-in mouse model with S252W mutation in FGFR2. This mutant mice exhibit skeleton malformation similar to that in human patients with Apert syndrome, which include a midline sutural defect and craniosynostosis caused by dysregulated osteoblastic proliferation and differentiation. Interestingly, ectopic cartilage at the midline sagittal suture, and cartilage abnormalities in the basicranium, nasal turbinates and trachea were also found. The mutant also showed long bone abnormalities as evidenced by disorganized growth plates and more prominent cartilage mineralization at P1. These data suggest that altered cartilage and bone development play a significant role in the pathogenesis of the Apert syndrome phenotype (220).

In another mouse model with a Cys342Tyr mutation in FGFR2 (equivalent mutation in human causes Crouzon and Pfeiffer syndromes), premature fusion of cranial sutures was accompanied by enhanced expression of Cbfa-1 and osteopontin. There was also increased number of proliferating (osteoprogenitor) cells in the coronal sutures at E14.5 (221), indicating increased osteogenesis in craniosynostosis caused by this mutation.

6. PERSPECTIVES

Animal and clinical studies have shown that FGF signaling plays critical roles during skeleton development and diseases. Nevertheless, many unresolved issues remain to be further studied.

The searching for novel mutations, either gain-of-function or loss-of-function, in FGFs and FGFRs responsible for those etiologically known and unknown skeleton diseases is very critical. The important roles of FGF signaling in bone development and diseases indicate that the genetic polymorphisms in FGFs/FGFRs may affect the development and diseases of human skeleton and be associated with normal variation, which needs to be studied in people of various races.

Although the dysregulated chondrogenesis and osteogenesis are the main abnormalities found in the FGFRs-related chondrodysplasia and craniosynostosis respectively, it appears that both abnormal osteogenesis and chondrogenesis are involved in the pathogenesis of FGF signaling-related chondrodysplasia and craniosynostosis, which need further studies.

There is increasing number of signaling molecules found to regulate endochondral and intramembranous ossification. Interaction between these molecules and FGF signaling pathways during skeletal development and diseases is one of the major unsolved issues in this field. Crossing between mouse strains harboring various FGFs/FGFRs mutations and other mouse models carrying genetic modifications of genes regulating FGF signaling or bone development is extremely important for the genetic dissection of the interactions between FGFs/FGFRs and these molecules. Conditional knock out of FGFR1 at mature osteoblasts leads to increased FGFR3 expression, whereas cultured stromal cells from *Fgfr3* null mice have increased expression of FGFR1 and FGFR2 mRNA. These data suggested that there is compensation or cross-talk between different FGFRs during skeleton development and diseases, which need to be elucidated.

As the development and maintenance of normal bone structure depend on the balance among cartilage-forming chondrocytes, bone-forming osteoblasts and bone-resorbing osteoclasts, the role of osteoclasts in bone development and diseases cannot be underestimated. In this aspect, the regulation of osteoclastogenesis and functions by FGF signaling needs to be further clarified.

Exploration of the pathogenesis and therapeutic approaches of those skeleton diseases resulting from mutations in FGFs/FGFRs is urgently needed. Many state-of-art techniques such as proteomics, genomics, RNAi, genetically modified mouse models, *in vivo* imaging, *etc* will be greatly helpful for these endeavors. For example, introduction of RANi allel specifically against FGFR2 S252W mutation was found to significantly alleviate the skull phenotype of mouse model for Apert syndrome resulting from FGFR2(+/S252W) mutation(254).

Fracture healing, to some extent, recapitulates the skeletal developmental process. They share some similar cellular processes and signaling networks. Our accumulating knowledge about the role of FGF signaling in skeletal development will certainly facilitate our understanding and searching for the therapeutic measures for these diseases.

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