Common skeletal features in rare diseases

New links between ciliopathies and FGF-related syndromes

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Congenital skeletal anomalies are rare disorders, with a subset affecting both the cranial and appendicular skeleton. Two categories, craniosynostosis syndromes and chondrodysplasias, frequently result from aberrant regulation of the fibroblast growth factor (FGF) signaling pathway. Our recent work has implicated FGF signaling in a third category: ciliopathic skeletal dysplasias. In this work, we have used mouse mutants in two ciliopathy genes, *Fuzzy (Fuz)* and *orofacial digital syndrome-1 (Ofd-1)*, to demonstrate increase in *Fgf8* gene expression during critical stages of embryogenesis. While the mechanisms underlying FGF dysregulation differ in the different syndromes, our data raise the possibility that convergence on FGF signal transduction may underlie a wide range of skeletal anomalies. Here, we provide additional evidence of the skeletal phenotypes from the *Fuz* mouse model and highlight similarities between human ciliopathies and FGF-related syndromes.

Fibroblast growth factors are well-studied signaling molecules that are critical for embryonic development.[1,2] In humans, 22 structurally related FGF ligands have been identified; most of these are secreted proteins that bind with varying affinities to tyrosine kinase receptors (FGFR1–4). The majority of FGF ligands can bind promiscuously to multiple different FGFRs; further complexity is generated by alternative splicing of FGFR1, 2 and 3. Ligand binding induces receptor dimerization and cross-phosphorylation, which then initiates a broad range of intracellular signaling cascades such as PI3 kinase (PI3K), MAPK, phospholipase C (PLCγ) or JAK/STAT. Activation of the pathway elicits diverse cellular responses, including proliferation and differentiation of multiple cell types. In recent years, developmental studies, as well as identification of human alleles, has made it clear that precise temporal and spatial activation of FGF signaling is necessary for normal development.

The FGF signaling pathway is a key regulator of skeletal development. In humans, mutations in the FGF receptors (FGFRs) are a hallmark of two classes of skeletal anomalies: craniosynostoses and chondrodysplasias (reviewed by Ornitz et al.).[1] Craniosynostosis syndromes are characterized by premature fusion of the cranial sutures and are frequently accompanied by malformations of the axial skeleton. In contrast, chondrodysplasias feature truncation of the appendicular skeleton, due to an increase in FGF signaling during endochondral ossification. Both craniosynostosis and chondrodysplasia syndromes are associated with autosomal dominant mutations of the FGF receptors, which result in increased activation of the signaling pathway.[1] The array of phenotypes correlates with specific mutations in different receptors, but the net effect appears to be that sustained or increased FGF signaling tips the balance between critical steps in osteoblast differentiation.[1,2]

Ciliopathies are a heterogeneous group of disorders that arise from abnormal formation or function of the cilium.[3] Cilia are finger-like organelles at the cell surface
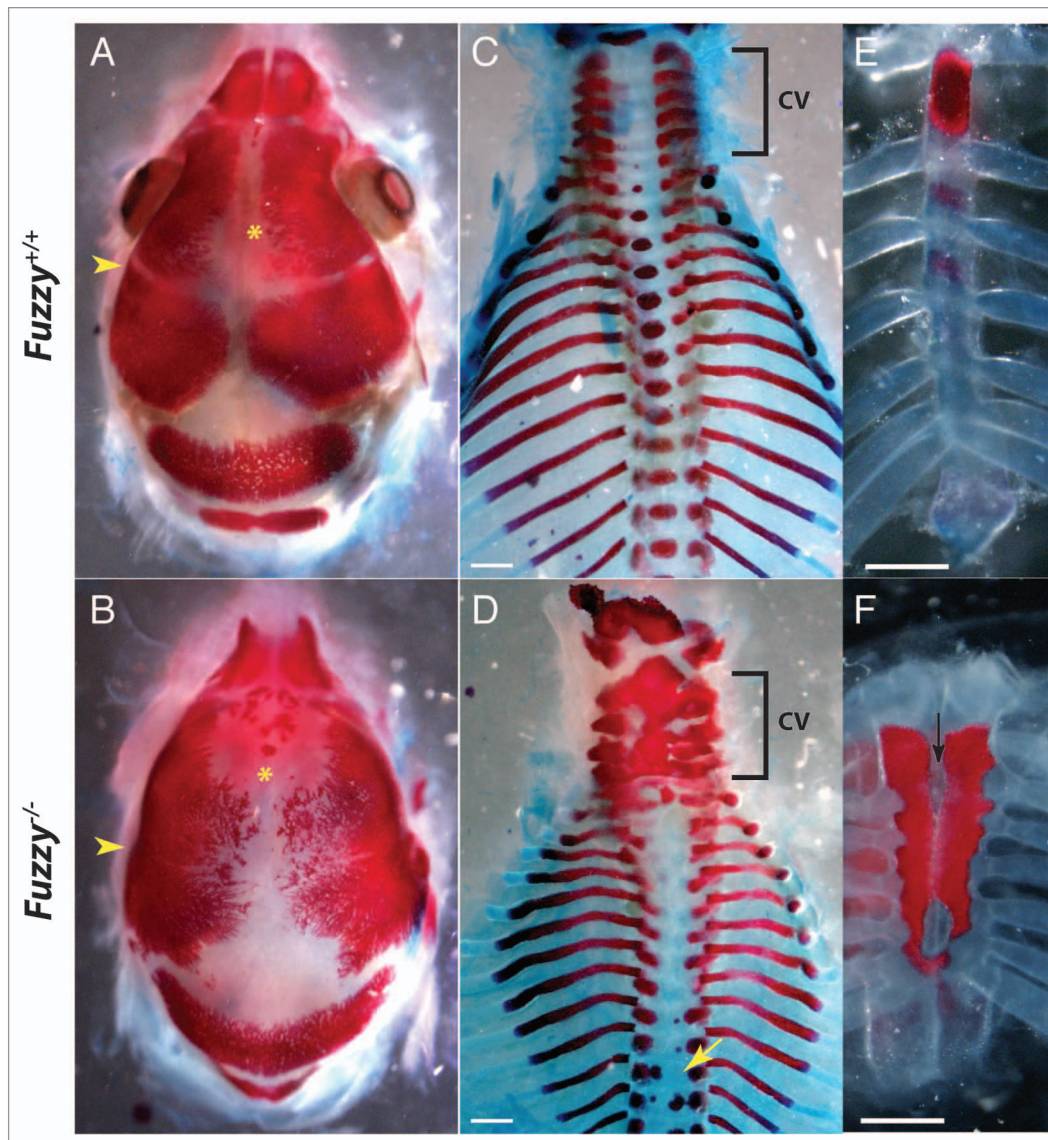
Figure 1. Skeletal preparations of wild-type and *Fuz*⁻/⁻ embryos at E18.5. Alizarin red staining marks the bone. Alcian blue staining marks the cartilage. (**A and B**) Dorsal views of the skull. (**A**) Control. (**B**) Mutant mice display synostosis of the coronal suture (yellow arrowhead) as well as an open anterior fontanelle (yellow asterix). (**C and D**) Dorsal view of the axial skeleton. (**C**) Control. (**D**) In mutant animals, the cervical vertebra (cv, bracket) are fused. Ossification of the centrum in thoracic vertebra is lost or aberrant (yellow arrow). (**E and F**) Frontal views of the sternum. (**E**) Control. (**F**) In mutants, the sternum is shorter, hyperossified and cleft/bifid (black arrow).

comprised of a microtubule axoneme attached to a basal body. Depending on the microtubule arrangement, a cilium may be motile or immotile. Disorders of the motile cilia frequently involve fluid flow; for example, patients with primary ciliary dyskinesia have difficulty clearing mucus from their lungs due to defects in the multi-ciliated epithelium.[4] Immotile or primary cilia stand alone and are required for function of many signaling pathways.[5] To date, ciliopathic skeletal phenotypes have mainly been attributed to changes in primary cilia-dependent transduction of Hedgehog signals.[6] This has been best studied in the long bones; however, the phenotypic range of ciliopathies is extremely broad. As a consequence, it can be difficult to diagnose or treat ciliopathies, and the underlying etiology is often unclear.

Ciliopathies affecting the skeleton are rare syndromic anomalies. Some patients exhibit limb phenotypes such as syndactyly or polydactyly [in the case of Bardet-Biedl syndrome (MIM #209900)].[7,8] Dysplasia of the ribs, and occasional shortened limbs, are also seen, as in Jeune asphyxiating thoracic dystrophy (MIM #208500).[9] Frequently, ciliary defects also lead to changes in the craniofacial skeleton in humans and mice,[10-12] with craniosynostosis observed in Sensenbrenner syndrome or cranioectodermal dysplasia (MIM #614378).[13] The broad range of systems affected suggests varied molecular causes. Thus, grouping similar skeletal phenotypes across multiple disorders, and

Table 1. Skeletal phenotypes observed in ciliopathies and FGF syndromes. Included are selected human disorders and animal models. Unfortunately, due to space constraints, we regret that we are unable to cite all relevant papers.

	Skull/Face	Palate	Limb/Hand	Vertebra	Rib/Thorax	Sternum	Refs.
				Affected structure			
Ciliopathies							
Human							
Meckel	encephalocele	C	PD	?	-	-	20
Bardet-Biedl	Shape change	HA	PD, SD	scoliosis	-	-	37
Joubert	-	C?	-	Cervical fusion	-	-	28
OFD1	Shape change	HA	PD	-	-	-	20
Jeune	-	-	PD	Cervical stenosis	irregular	bulge	9, 38
Sensenbrenner	SS	HA	SL, PD	-	Short ribs	-	13, 39–41
Ellis-Van Creveld	-	-	SL, PD	-	Short ribs	-	42
Mouse Models							
Meckel: *MKS1^{hlb614}/MKS1^{krc}*	-	C	-	-	-	bifid, fused	35, 43
Ellis-van Creveld: *EVC*	-	-	SL	Fusions	Short ribs	-	44, 45
Orofacial Digital: *OFD1*	-	C	SL, PD	-	-	bifid, fused	46, 47
Sensenbrenner: *WDR35*	-	-	-	-	Short ribs	-	48
Ciliopathy: *Fuz*	CS	HA	SL, PD	Cervical fusion	Short ribs	Fused, bifid	14, 16–18
Craniosynostosis							
Human							
Apert	CS	HA	SD	Cervical fusion	-	-	19, 49
Crouzon	CS	HA	SL, SD	Cervical fusion	-	-	21, 50
Pfeiffer	CS	HA	SD	Cervical fusion	-	-	22, 23
Mouse Models							
Crouzon/Pfeiffer: *Fgfr2c^{C342Y}*	CS	C	-	-	-	Fused	51, 52
Pfeiffer: Tg(*Fgfr1^{P252R}*)	CS	-	PD	Homeotic transformation	-	Fusions	27
Apert: *Fgfr2$^{IIIc/\Delta}$*	CS	-	-	-	-	Fused	29, 53
Apert: *Fgfr2^{S252W}*	CS	C	-	-	-	Fusions	30
Chondrodysplasia							
Human							
Chondrodysplasia punctata 2	variable	-	SL	Scoliosis fusions	calcified	calcified	20
Hypochondroplasia	variable	-	SL	stenosis	-	-	53
Thanatophoric dysplasia	-	-	SL	flattened	Short ribs	-	54
Achondroplasia	Small base	-	SL	stenosis	Small chest	-	55
Mouse Models							
Achondroplasia: *Fgfr3^{G374R}*	-	-	SL	Cervical fusion	-	-	56
Thanatophoric Dysplasia: FGFR3369	-	-	SL	-	Short ribs	-	57

Abbreviations: SS, sagittal synostosis; CS, coronal synostosis; C, cleft palate; HA, high arched palate; PD, polydactyly; SD, syndactyly; SL, short limbs

comparison with animal models, may provide useful insight into the underlying molecular events.

The *Fuzzy* gene is associated with neural tube defects and has previously been shown to be a ciliopathy gene.[14-17] In our current work, we examine the requirements for *Fuz* in development of the craniofacial structures.[18] Craniofacial defects include craniosynostosis and facial anomalies (**Fig. 1A and B**).[18] As documented in Tabler et al., *Fuz* mutant mice have a complete synostosis of the coronal suture, as well as an open anterior fontanelle, reminiscent of Apert syndrome synostoses (MIM #101200) (**Fig. 1A-B**).[19] Our analysis of the *Fuzzy* mutant also showed broader defects of the skeleton. Consistent

with ciliopathic Hedgehog phenotypes, *Fuz* mutants have polydactyly and shortened long bones.[17] Most interesting, we also found anomalous elements in the axial skeleton. The cervical vertebra (cv) were frequently fused (**Fig. 1C and D**, bracket), while ossification of the centrum in the thoracic vertebra is generally absent. Occasionally, small islands of ectopic ossification are seen (**Fig. 1C and D**, yellow arrow). As in ciliopathies such as Jeune Syndrome (MIM #208500), the ribs are shorter. Surprisingly, the sternum is hyperossified, shorter, and bifid (**Fig. 1E and F**, black arrow marks cleft). In earlier stages, cartilaginous joints are formed (data not shown); by embryonic day (E)18.5, the sternal joints have been obliterated (**Fig. 1E and F**).

This array of phenotypes suggested a similarity to several classes of FGFR-dependent skeletal anomalies, including craniosynostosis syndromes and chondrodysplasias.[20] All of these syndromes arise from dysregulation of FGF receptors;[1] however, the status of FGF signaling in ciliopathy mutants has not been well explored. **Table 1** catalogs the skeletal malformations ciliopathies and FGF related syndromes. We found significant overlap across the range of disorders.

Many craniosynostosis patients have a progressive fusion of the cervical spine, with two thirds of Apert patients exhibiting complex fusions in the C5-C6 segment.[21] C2-C3 fusions are also quite common in these syndromes, with additional reports from Saethre-Chotzen (MIM #101400) and Pfeiffer syndromes (MIM#101600).[22-24] To our knowledge, FGF-induced chondrodysplasias are not associated with spinal stenosis; however, cases have been reported in rhizomelic chondrodysplasia punctata patients.[25] Interestingly, congenital scoliosis due to vertebral defects has also been linked to aberrant *FGF* signaling during development.[26] In addition, in a mouse model of Pfeiffer syndrome, vertebral homeotic transformations have been noted.[27] Thus, phenotypes seen in

the different mouse models, combined with some reported anomalies of the cervical vertebra in Joubert Syndrome (MIM #213300), suggest that ciliopathy patients could be assessed for spinal aberrations.[28]

Sternal abnormalities, a common feature of ciliopathies and FGF syndromes, are another striking phenotype seen in *Fuz* mutants. Premature or ectopic fusion of the sternum is seen in a number of mouse models, including Apert/Pfeiffer Syndrome and achondroplasia mice.[27,29-31] The current data suggest that hyperactivation of FGF receptors leads to an impairment in sternal joint formation and subsequent hyperossification. In humans, premature ossification of the sternum is a hallmark of Noonan syndrome.[32] The causative mutation in Noonan syndrome is *PTPN11*, which encodes SHP-2, a key regulator of the FGF-Ras-MAPK pathway.[33] Finally, sternal anomalies are also observed in ciliopathic animal models.[34,35]

Taken together, our data suggest that the skeletal anomalies described may all converge on deregulation of the FGF signaling pathway. Indeed, we found that a subset of phenotypes in our ciliopathic mouse mutants, *Fuz* and *OFD-1*, are attributable to increased *Fgf8* gene expression and genetic reduction of *FGF8* rescued these phenotypes.[18]

Our approach of cataloguing human phenotypes, and comparison to animal models, led us to a surprising role for FGFs in ciliopathies. However, clearly, FGFs alone, or in combination with Hedgehog signaling, cannot be the sole molecular players in skeletal dysplasias. FGF signaling plays roles at multiple steps in both endochondral and intramembranous ossification.[1] For example, during long bone formation, signaling via FGFR2 and FGFR3 promotes chondrocyte condensation and differentiation respectively. Later in this process, FGFR3 is needed to limit the amounts of proliferative pre-hypertrophic chondrocytes. In intramembranous ossification, as seen in

the calvaria, FGFs are involved in every step of osteoblast differentiation and subsequent ossification. *FGFR1* and *2* are expressed at the osteogenic front, and are necessary for osteoblast differentiation. *FGF2* is expressed in the sutures, and signals to the adjacent osteogenic cells. Thus, specific phenotypes observed in the human syndromes described can be attributed to inappropriate temporal or spatial activation of the pathway. For example, hyperactivation of FGFR3 severely reduces regions of pre-hypertrophic chondrocyte proliferation resulting in short long bones. Conversely, FGFR1 and 2 dysregulation leads to premature osteoblast differentiation and craniosynostosis.

Dysregulation of Gli processing is also known to cause a variety of skeletal defects, notably in the long bones, vertebra and sternum. *Gli2* mouse mutants have shortened long bones and absence or malformation of vertebral bodies, while *Gli3* mutants have slightly shortened long bones accompanied by polydactyly, as well as fusions of the cervical vertebra and bifid, hyperossified sterna.[36] Thus, there is substantial phenotypic overlap between *Gli* mutants and other animal models of skeletal syndromes. As described above, it is likely that correct timing and location of a suite of signals is critical for shaping the skeleton. Because pathological mutations can lead to changes at multiple levels during development, we propose that further comparison of human phenotypes and animal models can provide important insights into the genetic networks governing overlapping disease phenotypes.

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