

INVITED REVIEW

Modeling craniofacial and skeletal congenital birth defects to advance therapies

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Abstract

Craniofacial development is an intricate process of patterning, morphogenesis, and growth that involves many tissues within the developing embryo. Genetic misregulation of these processes leads to craniofacial malformations, which comprise over one-third of all congenital birth defects. Significant advances have been made in the clinical management of craniofacial disorders, but currently very few treatments specifically target the underlying molecular causes. Here, we review recent studies in which modeling of craniofacial disorders in primary patient cells, patient-derived induced pluripotent stem cells (iPSCs), and mice have enhanced our understanding of the etiology and pathophysiology of these disorders while also advancing therapeutic avenues for their prevention.

Introduction

The craniofacial complex is one of the most intricate and sophisticated parts of the human body. Its patterning and morphogenesis involve a dynamic interplay between the ectoderm, mesoderm, and endoderm, and a critical role is played by neural crest cells, which give rise to the majority of skeletal and connective tissues in the craniofacial region. These interactions are established and maintained by numerous genes, including those encoding a variety of transcription factors, growth factors, and receptors (1,2). Disruption of gene expression or function results in devastating craniofacial anomalies, which have a

collective incidence rate of 1 in 600 births (3). Much of our current understanding of the etiology and pathophysiology of craniofacial disorders has been uncovered through the use of model systems. Mice are considered by many to be the gold standard for disease modeling, as they are anatomically and physiologically comparative to humans and can be genetically manipulated to mimic human phenotypes (4). Primary patient cells and patient-derived iPSCs have proven to be a valuable complement to the mouse model system by either highlighting species-specific differences or further validating the observations already made in mice (5). The modeling of craniofacial

Received: May 4, 2016. Revised: May 24, 2016. Accepted: May 24, 2016

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disorders has not only informed genetic risk assessment and patient prognosis but also identified potential targets for pharmaceutical intervention. In this review, we highlight recent efforts that have provided new information to advance the treatment of five general classes of human genetic disorders, with particular emphasis on the craniofacial region. In addition, we discuss how this information furthers our understanding of the molecular mechanisms regulating normal craniofacial development.

Craniosynostosis

The cranial sutures are fibrous joints that form between the five principal flat bones of the skull vault during embryogenesis. From the early fetal period through the first years of life, the cranial sutures are primary sites of bone growth and allow the skull vault to expand with the growing brain. Formation and maintenance of the suture, which include the osteogenic fronts of the jointed bones and their interposed mesenchyme, is critical to its function as a growth center. Dysfunction in genes that regulate the organization, proliferation, and/or differentiation within the suture can lead to its premature fusion, a relatively common birth defect known as craniosynostosis (6). Serious clinical problems associated with craniosynostosis include craniofacial deformities, increased intracranial pressure, and impaired brain development leading to learning difficulties or developmental delay. Treatment plans involve surgery to remove and reshapes large areas of the calvaria; however, for many patients suture re-fusion necessitates repeated surgeries (7). Thus, there is a clinical need to develop less invasive, more effective therapies for craniosynostosis. Recent studies that apply our current knowledge of the molecular players in normal and abnormal suture development have advanced the potential for these new therapies.

The pathogenesis of syndromic craniosynostosis is commonly associated with gain-of-function mutations in Fibroblast Growth Factor receptors (FGFRs) 1-3. FGF signaling promotes proliferation and differentiation in osteogenic cells, most notably in the cranial sutures (8,9). Apert syndrome is usually caused by dominant mutations in FGFR2 that increase ligand-dependent activation and subsequently enhance osteoblast differentiation (10). New findings show that expression or nanogel-mediated delivery of a soluble form of FGFR2 harboring the Apert mutation S252W blocks enhanced FGFR2 signaling and inhibits craniosynostosis in a mouse model for Apert syndrome (11,12).

The Bone Morphogenetic Protein (BMP) pathway plays a critical role in the development of the skull vault. Increased BMP signaling is associated with craniosynostosis (13–15), and antagonists of the pathway are being tested as a possible treatment to prevent post-operative re-fusion. In a recent study, delivery of the BMP antagonist GREMLIN1 via hydrogel that rapidly polymerized upon injection prevented bone re-growth in a mouse model for re-synostosis (16).

Hypophosphatasia, a metabolic disorder with craniosynostosis, is caused by loss-of-function mutations in *ALPPL*, the gene encoding Tissue-nonspecific Alkaline Phosphatase (TNAP). TNAP is an osteoblast surface protein that induces hydroxyapatite crystal growth by increasing inorganic phosphate (17,18). A recent report shows that craniosynostosis in *Alppl* knockout mice is rescued by subcutaneous injection of a mineral-targeted form of recombinant TNAP (19).

Craniofacial Dysmorphologies

Dysmorphic craniofacial features can often be quantified as anthropometric measurements outside the normal variance, and these can be isolated or occur in a syndrome. As such, current treatments are directed towards addressing the specific anomalies on a patient-by-patient basis. While craniofacial dysmorphologies can have phenotypic overlap, the underlying mechanism of disease is quite disparate. Due to their genetic heterogeneity, an exciting frontier in the treatment of craniofacial dysmorphologies is the possibility of targeted therapeutics such as genome editing (20–23).

Brachio-ocular-facial (BOF) syndrome is associated with missense mutations in the *TFAP2A* gene encoding AP-2 α , a transcription factor with early roles in neural crest cell specification and survival (24–26). Generation of the first fully penetrant cleft lip and palate mouse model caused by mutations in *Tfap2a* revealed that one cause of clefting can be subtle changes in FGF pathway gene expression in the facial prominences. Manipulation of *Fgf8* gene dosage partially rescued the phenotype, suggesting that FGF signaling and/or downstream effectors may be possible targets of pharmacological intervention in BOF syndrome and nonsyndromic cases of clefting associated with *TFAP2A* mutations (27).

Heterozygous mutations in *BRAF* are found in 50-75% of patients with cardio-facio-cutaneous (CFC) syndrome (21). *BRAF* is a serine threonine kinase that regulates the RAS-MAPK signaling pathway, and therefore CFC syndrome is classified as a RASopathy (28). *Braf*^{Q241R/+} mice exhibit embryonic/neonatal lethality with liver necrosis, edema, and craniofacial abnormalities, effectively mimicking the phenotypes of human patients. Interestingly, co-treatment with MEK inhibitors and histone demethylase inhibitors rescued the pathophysiology (29). This finding has implications not only for prospective therapies of CFC syndrome but for other RASopathies as well. It will be important to examine the epigenetic contributions to heart and skeletal defects in these disorders to inform upon the treatment potential of combined inhibition of HRAS signaling and histone demethylases.

Treacher Collins syndrome (TCS) is an autosomal dominant disorder which presents with hypoplasia of the facial bones, cleft palate, and low set, malformed ears (30). In a mouse model of TCS, haploinsufficiency of the *Tcof1* gene encoding the nucleolar phosphoprotein treacle reduces ribosome biogenesis, causing deficient proliferation and extensive apoptosis of neuroepithelial cells via a nucleolar stress-induced, p53 pathway (31,32). The recent discovery that treacle also functions in DNA damage response/repair to limit oxidative stress-induced neuroepithelial cell death identified a novel underlying contributor to the pathogenesis of TCS. Excitingly, *in utero* treatment with antioxidants prevented DNA damage and minimized cell death in the neuroepithelium to substantially ameliorate the craniofacial anomalies in *Tcof1*^{+/-} embryos (33). While previous work has shown that genetic and pharmacological inhibition of p53 can suppress the neuroepithelial apoptosis in *Tcof*^{+/-} embryos, maternal antioxidant dietary supplementation may be a safer potential therapeutic for patients with TCS, given the risk of tumorigenesis associated with p53 manipulation (32,33).

Dental Anomalies

Developmental dental anomalies are defined as marked deviations from the normal color, contour, size, number, and degree of formation of teeth. These malformations can occur either as

part of a syndrome or as an isolated finding (34). In Costello syndrome (CS), a RASopathy associated with craniofacial, cardiac, musculoskeletal, and neurodevelopmental abnormalities, characteristic dental phenotypes include class III malocclusion, enamel hypomineralization, and soft tissue hyperplasia (35). Nearly all individuals with CS have a heterozygous mutation in *HRAS* that results in constitutive activation of Ras signaling (36,37). A CS mouse model expressing *HRas*^{G12V} phenocopies many aspects of the syndrome and was used to understand the cellular mechanism underlying the hypomineralization of the enamel (38). In this model, enamel-forming ameloblasts lack polarity, and the ameloblast progenitor cells are hyperproliferative. Inhibition of MAPK led to complete rescue of the dental phenotype, whereas modulation of either MAPK or PI3K signaling corrected the defect in progenitor cell proliferation in CS mice (38). This work defined for the first time distinct roles of Ras signaling in tooth development and provided additional evidence for the use of Ras inhibitors in treating CS and other RASopathies.

Hereditary conditions involving nonsyndromic enamel conditions are referred to as amelogenesis imperfectas (AIs). The X-linked form of hypoplastic AI is associated with missense mutations in Amelogenin, an extracellular matrix protein secreted by ameloblasts (39,40). The murine Y62H Amelogenin mutation similarly results in the eruption of malformed tooth enamel with severely compromised mechanical properties (41). Recent work has demonstrated that this specific mutation disrupts proper intracellular trafficking of amelogenin and induces ER stress-related apoptosis in ameloblasts, classifying AI as a protein conformational disease for the first time (42). Treatment with 4-phenylbutyrate, which can act to relieve conformational abnormalities of the protein, rescued the enamel phenotype in affected female mice by promoting cell survival over apoptosis, offering a potential therapeutic option for patients with this form of AI (42,43).

Skeletal Dysplasias

Skeletal dysplasias represent one of the largest classes of birth defects, with over 450 recognizable conditions (44). The craniofacial defects in these disorders result from the combinatorial interactions of transcription factors, growth factors, and receptors responsible for the intricate genetic patterning and morphogenesis of craniofacial structures (45). With the advent of next-generation DNA sequencing, clinical phenotypes can be linked to key cellular processes of skeletal development, including proliferation, differentiation, and apoptosis. Dominant missense mutations in *FGFR3* that reduce chondrocyte proliferation are associated with achondroplasia (ACH) and thanatophoric dysplasia (TD), the most common genetic forms of dwarfism (46–48). Craniofacial findings include macrocephaly, frontal bossing, and midface hypoplasia in ACH, and macrocrania, cloverleaf skull, and frontal bossing in TD. The severity of these chondrodysplasias is linked with the degree of constitutively activated *FGFR3* signaling through MAPK or STAT1, and as such, therapeutic strategies have focused on decreasing excessive downstream signaling (49,50). Recent work in patient-specific iPSCs has identified statins as a potential drug to treat *FGFR3*-mediated chondrodysplasias. Treatment with statins rescued cartilage formation in chondrogenically differentiated TD1 and ACH iPSCs and led to significant recovery of bone growth in an ACH mouse model (51). While the precise mechanism of action remains to be determined, the success of statin treatment

highlights a previously unappreciated role for anabolic activity during chondrogenesis (52–54).

Maintaining the proper balance between proliferation and differentiation is also critical for bone formation. Examination of the pathophysiology of Bent Bone Dysplasia Syndrome (BBDS) revealed an unexpected nuclear route for FGF signaling to regulate osteoprogenitor cell proliferation and differentiation via ribosome biogenesis (55). BBDS is a dominant disorder characterized by bent long bones in the lower extremities and craniofacial abnormalities including poorly mineralized calvaria, craniosynostosis, midface hypoplasia, micrognathia, low-set ears, and prenatal teeth. BBDS results from mutations in the transmembrane domain of *FGFR2* that redistribute the receptor from the plasma membrane to the nucleolus, where it activates ribosomal DNA transcription by halting *RUNX2*-mediated repression (55,56). Inhibition of ribosomal RNA synthesis by small molecules has been shown to be effective in preclinical cancer models in mice and may be a potential therapeutic strategy to specifically target the pro-proliferative nucleolar role *FGFR2* in BBDS and other *FGFR2* gain-of-function disorders (57–60).

Cherubism is a condition caused by excessive osteoclast activity in the mandible and maxilla, which drives progressive proliferation of fibrous tissues and leads to severe facial deformities. Spontaneous regression of bone lesions is usually observed at puberty, and surgical intervention is only considered when functional or aesthetic concerns arise (61,62). Recently, two independent studies presented promising pharmacological therapeutic approaches to inhibit or delay the progression of cherubic lesions. Most patients with cherubism have gain-of-function mutations in the gene encoding *SH3BP2*, an adapter protein involved in the immune response. *Sh3bp2* knock-in mice develop massive infiltration of macrophages into skeletal elements, including the jaw, which can be rescued by genetic inhibition of *TNF- α* expression (63). Consistent with the role of *TNF- α* , treatment with the anti-*TNF- α* inhibitor etanercept significantly reduced facial swelling and bone loss in neonatal mice. Furthermore, this phenotypic rescue was not recapitulated in adult mice, emphasizing the importance of early diagnosis and treatment of cherubism (64). An effective therapy for patients with actively growing and established inflammatory lesions may be bone marrow (BM) transplants. Transplantation of wild type BM cells to *Sh3bp2* knockin mice rescued the systemic inflammation and bone loss in adult cherubism that could not be ameliorated by etanercept treatment (65). Treatment with tacrolimus, an immunosuppressor that has been shown to inhibit activation of the calcineurin/NFATc pathway and osteoclastogenesis (66–68), led to significant clinical improvement in a 4-year old boy with an aggressive form of cherubism; specifically noted was stabilization of jaw size and intraosseous osteogenesis (69). Future studies are needed to determine the precise mechanism of action of tacrolimus and whether combined treatment with anti-inflammatories may further ameliorate the pathophysiology of cherubism.

Bone Mineral Density

Bone mineral density (BMD) is determined by the relative rates of bone deposition and resorption, which are carried out by osteoblasts and osteoclasts, respectively. Mutations in the genes controlling osteoblast and osteoclast function cause congenital disorders with abnormal BMD. While these conditions present with generalized skeletal abnormalities, the craniofacial findings have important clinical complications. In osteopenic and osteoporotic disorders, where bone resorption exceeds

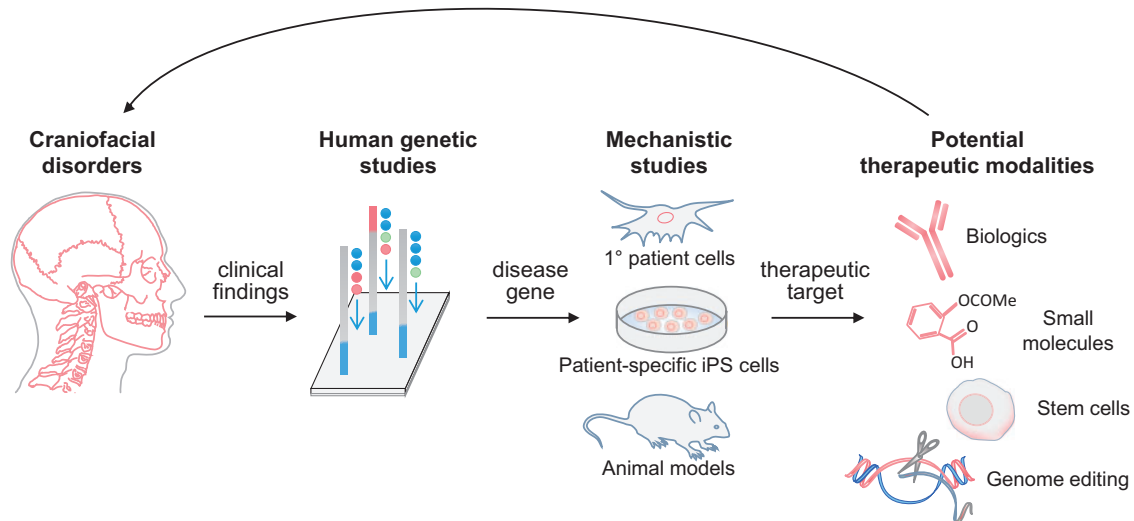


Figure 1. Pipeline for transforming insights from disease models into potential therapeutics for craniofacial disorders. Human genetic studies identify critical genes linked to craniofacial disease. Mechanistic studies, using primary patient cells, patient-specific iPS cells, and/or animal models, probe the disease gene's role in craniofacial biology. Once the biological function of the gene is discovered, therapeutic targets can be identified. Having an in-depth view of the target's biology aids in selecting therapeutic modalities, such as biologics, small molecules, stems cells, and possibly gene editing.

deposition, calvaria are undermineralized, malformed, and fractured. In osteopetrotic disorders, where bone deposition outpaces resorption, there is focal or widespread thickening of the calvaria, skull base, and facial bones. Recent studies have supported the use of biologics to restore the balance between bone anabolism and catabolism in congenital BMD disorders.

Genetic studies of congenital BMD disorders have demonstrated that the Wnt/LRP5 pathway increases bone density by promoting osteoblast production and function. Loss-of-function mutations in the Wnt co-receptor LRP5 cause the low bone mass disorder osteoporosis-pseudoglioma syndrome (OPPG), while LRP5 gain-of-function mutations cause higher bone mass disorders Van Buchem disease, osteosclerosis, and osteopetrosis (70–74). LRP5 mutations in higher bone mass disorders increase the co-receptor activity by disrupting the binding of the inhibitor sclerostin, which is inactivated in higher bone density disorder sclerosteosis (75–78). These studies laid the groundwork for development of an inhibitory antibody against sclerostin that is now in phase 3 clinical trials for the treatment of postmenopausal osteoporosis (79). New evidence supports repurposing anti-sclerostin to treat the very syndromes that advanced its discovery: depletion of sclerostin, either genetically or through the use of anti-sclerostin, increases the BMD of mouse models for OPGG (80,81). These findings also provide the rationale for use of a recombinant Wnt/LRP5 inhibitor or inhibitory antibody against LRP5 to block bone overgrowth in the osteopetrotic disorders.

There is strong evidence to suggest that anti-sclerostin will increase BMD in other skeletal fragility syndromes as well, such as osteogenesis imperfecta (OI) and hereditary hypophosphatemic rickets, despite differences in the molecular pathologies. While OI is largely caused by deficiencies in type I collagen production, modification, or secretion, mouse models for OI gain a significant increase in bone mass and strength when Wnt/LRP5 signaling is increased, through either expression of LRP5 gain-of-function mutation or treatment with anti-sclerostin (82–86). Additionally, anti-sclerostin significantly improved osteomalacia in *DMP1* knockout mice, a model for hereditary hypophosphatemic rickets (82).

Mouse models with reduced BMD have enabled identification of promising new targets for protein-based therapies. Defective type I collagen biosynthesis in OI increases the bioavailability of TGF β , leading to excessive TGF β signaling (87). Promotion of osteoclast bone resorption by TGF β signaling provides a rationale for the use of inhibitory antibodies against TGF β . Indeed, anti-TGF β treatment improved bone mass in mouse models for OI (87). Knockout of *Nell1*, which codes for a secreted bone-inducing factor, leads to age-related osteoporosis (88). Correspondingly, delivery of recombinant NELL1 was shown to increase bone formation via the Wnt pathway in both small and large animal models of osteoporosis (89).

Future Directions

Studies that model congenital disorders in primary patient cells, iPSCs, and mice have advanced therapeutic opportunities for craniofacial disorders (Fig. 1). New technologies such as CRISPR/Cas9 that increase the speed, efficiency, and simplicity in genome editing will allow for rapid generation of cell lines and animal models that carry human disease-causing mutations (90–93). Specifically, genome-editing techniques offer a way to model Mendelian disorders in large animals, whose disease states may more closely resemble humans than the mouse models. Indeed, strategies for CRISPR/Cas9-modification in monkey, pig, and goat embryos have recently been reported (94–96). Genome editing will also aid in the study of congenital disorders associated with allelic or locus heterogeneity, which can complicate the diagnosis and treatment of these conditions (44,97). Introducing patient-specific mutations will help to identify genotype-phenotype correlations and subtle differences in the mechanistic effects of specific mutation. One of the most exciting clinical applications of genome editing is the possibility of correcting disease-causing genes. The therapeutic potential of CRISPR/Cas9 is currently being investigated in patient-derived iPSCs, organoid cultures, and mouse models (98–103). These studies raise high hopes for improving the clinical diagnosis, treatment, and outcome of patients with craniofacial and skeletal malformations.

Acknowledgements

A.E.M. and O.D.K. co-supervised the writing of this review.

Conflict of Interest Statement. None declared.

Funding

This work was supported by the National Institutes of Health [R01DE025222 to A.E.M, U01-DE024440 and R01-DE024988 to O.D.K]; and March of Dimes [#6-FY15-233 to A.E.M.].

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