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REVIEW ARTICLE

Signaling pathways in osteogenesis and osteoclastogenesis: Lessons from cranial sutures and applications to regenerative medicine

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Abstract One of the simplest models for examining the interplay between bone formation and resorption is the junction between the cranial bones. Although only roughly a quarter of patients diagnosed with craniosynostosis have been linked to known genetic disturbances, the molecular mechanisms elucidated from these studies have provided basic knowledge of bone homeostasis. This work has translated to methods and advances in bone tissue engineering. In this review, we examine the current knowledge of cranial suture biology derived from human craniosynostosis syndromes and discuss its application to regenerative medicine.

Introduction

Ideal model systems for studying biological processes require three components: simplicity, controllability, and physiologic relevance. In the investigation of bone homeostasis, few models have been more useful than the cranial suture. In terms of simplicity, there is no other model system that exists for bone that can be isolated down to the bare essentials for intramembranous ossification. Due to the limited number of cell types and minimal changes in mechanical forces that occur at cranial sutures, this system allows for direct evaluation of the interactions between osteoprogenitors, osteocytes, osteoclasts, the dura, and the extracellular matrix. Mechanical load on the calvarium is relatively limited considering that the skull is not a weight bearing entity. Muscular pull on the bones is minimal

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in that there are only two muscles directly attached to the cranial bones. In terms of control of the system, *in vitro*, *ex vivo*, *in vivo*, investigation using multiple species, and human syndromes with significant phenotypes can all be used to systematically evaluate single molecular mechanism. Finally, the significance of human phenotype based on single gene mutations cannot be underscored enough. The relevance of processes affecting osteogenesis in cranial sutures is clearly not just an artificial laboratory entity but an actual physiologic process with developmental consequences. These revelations also inspired significant avenues of investigation in bone tissue engineering. In this review, we discuss several major pathways governing bone homeostasis derived from craniosynostosis syndromes and describe its translation to skeletal regeneration.

Cranial suture development and fusion

The mammalian cranial vault contains five bones: paired frontal bones, paired parietal bones, and the occipital or interparietal bone (Fig. 1). Four cranial sutures separate the five bones: the sagittal suture exists between the two paired parietal bones, the coronal suture between the frontal and parietal bones, the metopic between the two frontal bones, and the lambdoid between the occipital and parietal bones. Malleability of the skull imparted by the cranial sutures is essential for the birthing process and subsequent growth of the brain. Growth of the calvarium is typically perpendicular to the direction of the sutures as the brain expands. In the event of a stenosed suture, the compensatory growth occurs parallel to the stenosed suture by expansion at the unaffected sutures. Ossification of the skull occurs via intramembranous ossification from the interplay between the suture mesenchyme and the dura. With the exception of the metopic suture which closes around 18 months of age, all other sutures close after completion of cranial growth well into adulthood. Similar to the human calvarium, the murine posterior frontal suture, analogous to the metopic suture, is the only suture in the mouse to fuse at about 40 days after birth.[1,2]

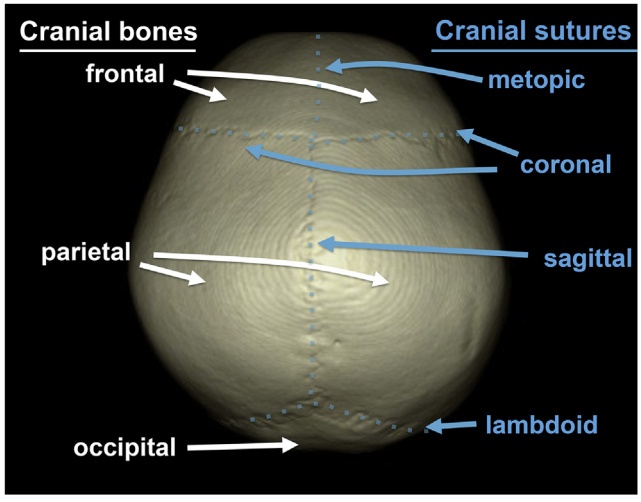

Figure 1 Cranial bones and cranial sutures.

Murine transgenic reporter gene models have now demonstrated that development of the skull is derived from a combination of neural crest and mesodermal lineages. Using two different transgenic mice that labeled cell types with galactosidase under either the Wnt-1 or Mesp-1 promotors, Morriss-Kay and colleagues were able to differentiate the origins of bony development of the skull between the neural crest or mesodermal lineages.[3,4] Their landmark studies definitively demonstrated that the frontal bone is neural crest in origin, the parietal bones are mesodermal, and the occipital bone is a combination of the two. During embryonic development, the coronal suture contains a boundary between the neural crest derived frontal bone on one side and the mesoderm-derived suture mesenchyme and parietal bone on the other side.[3,4] Similarly, at the sagittal suture, there is also a boundary between neural crest and mesodermal lineages. This boundary is likely important for the timing of suture patency versus fusion.

Craniosynostosis, or early fusion of cranial sutures, occurs in approximately 1 in 2000—2500 live births of which the majority are nonsyndromic in nature.[5,6] Single suture nonsyndromic craniosynostosis accounts for over 80% of all craniosynostosis. Sagittal synostosis is the most common form accounting for 40%—50% of all nonsyndromic craniosynostosis with a prevalence of about 1.5 in 10,000 live births and a male to female ratio of 2.5:1. Unicoronal craniosynostosis accounts for 0.7 in 10,000 live births with a male to female ratio of 1:2.3.[7] Metopic synostosis occurs in 0.8 in 10,000 live births with a male to female ratio of 3.3:1. Lastly, lambdoid synostosis occurs in about 0.7 in 10,000 live births with a male to female ratio of 2.2:1.

The consequences of early cranial suture fusion are both visible and functional. With the exception of mild cases, the majority of patients with craniosynostosis have characteristic head shapes depending on the type of synostoses that is present. This congenital anomaly is not only distressing to parents, but it may also harbor functional consequences. In multi-suture or syndromic cases, suture fusion has clearly been related to increased intracranial pressure with potential consequences in brain development.[8,9] In nonsyndromic cases, several landmark studies have now demonstrated that functional consequences also occur. Persing and colleagues have recently published their prospective, multi-center studies using a battery of neuropsychiatric testing to show that total cranial vault remodeling before 6 months of age improves outcomes. In addition, their work also showed that minimally invasive endoscopic strip craniectomies are definitively inferior to total cranial vault remodeling even when completed at an early age.[10—12] Although these studies do not consider intermediate surgical techniques such as the *pi* procedure in cranial vault reconstruction, their work is of great significance in surgical decision making and states that a minimally invasive correction for nonsyndromic sagittal synostosis adversely affects the future intelligence and neuropsychological function of a child with craniosynostosis.

The etiology of craniosynostosis is varied. A number of monogenetic alterations have been described, however, factors such as advanced maternal age, advanced paternal age, race, birth plurality, and gender have all been

associated with an increased risk of craniosynostosis.[5–7,13] In terms of syndromic synostosis, more than 100 different mutations have been described in relationship to craniosynostosis. In the Oxford cohort, 27% of 300 cases of craniosynostosis have the following mutations: FGFR2 8.3%, FGFR3 6.4%, Twist1 4.9%, EFNB1 1.8%, FAM20C $<$ 1%, LMX1B $<$ 1%, structural chromosome anomalies 3.7%, TCF12 $\sim$ 1%, ERF 2.9%.[14–16] In general, the genes contributing to craniosynostosis can be categorized to genes encoding molecules that effect osteogenic upregulation, osteoclastogenic downregulation, cell patterning, extracellular matrix, apoptosis, cell proliferation, or vascular function.[13,16–18]

Anatomic and cellular components of bone homeostasis

Normal bone biology, like all biology, is a process that has both positive and negative regulators. At the anatomic level, bone formation and resorption occurs as part of two major mechanisms of bone homeostasis: modeling and remodeling. Bone modeling generates a net positive quantity of bone at specific surfaces separate from the resorptive surfaces. One physiologic example is provided by Sarnat and colleagues who described that mandibular ramal growth occurred at the posterior and inferior borders, while resorption was prominent at the anterior border.[19] In contrast, bone remodeling couples osteoblast and osteoclast activity and is a process common in adult bone. This coupling of bone formation and resorption is spatially enclosed within specialized anatomic structures called basic multicellular units (BMUs).[20] The BMU is comprised of osteoblasts, osteoclasts, and mature osteocytes within mineralized bone matrix. Membrane bound and secreted factors affecting both bone formation and resorption are in a balance within the three major cell types. As we now know from the craniosynostosis and pathologic bone diseases, this balance can easily be dysregulated with preference towards either process.

At the cellular level, two major cell lineages are responsible for bone homeostasis: osteoblasts and osteoclasts.[21,22] Mesenchymal stem cells (MSCs) are pluripotent cells that have the ability to differentiate into chondrocytes, adipocytes, osteoblasts, and myoblasts. The osteoblastic lineage begins with the differentiation of MSCs into a fibroblast colony forming unit (CFU-F), which is further differentiated into pre-osteoblasts through the action of a number of signaling pathways that are discussed further below. Central to the signaling pathways is the activation of Runx2, a master transcription factor in osteoblast differentiation. Osteoblasts are responsive to mechanical stimuli and growth factor receptor-mediated signals.[23] Following secretion of bone matrix, a population of osteoblasts undergoes further differentiation to osteocytes and remains embedded within the matrix. Osteocytes, the most abundant cell type in bone, are interconnected via dendritic processes and gap junctions to each other, osteoblasts, and endosteal lining cells with close proximity to osteoclasts. Although the exact purpose of the osteocyte network is controversial, it likely has a role as mechanical sensors for stress and injury.[24,25] In addition, the discovery that osteocytes are the major source of sclerostin, a Wnt antagonist, as well as RANKL suggest that osteocytes may be the cell type holds the balance between bone formation versus resorption.

Unlike the osteoblast lineage of cells, osteoclasts are multinucleated cells differentiated from hematopoietic stem cells (HSCs) that function to resorb and remodel bone.[21] Following differentiation of the HSC to monocyte colony forming units (CFU-M), osteoclastogenesis is stimulated by the action of macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (NF-κB) (RANK). Osteoclasts exert their function by direct contact with bone, resulting in the formation of resorption pits or tunnels.

Signaling bone formation

Multiple pathways have been identified to contribute to osteogenesis. In cranial suture fusion, the most infamous signaling pathway is the fibroblast growth factor receptor (FGFR) family due to its frequent association to craniosynostosis syndromes such as Crouzon, Apert, and Pfeiffer syndromes (Table 1). For example, Apert syndrome is caused by FGFR2 mutations and accounts for 40% of all craniosynostosis syndromes.[6] FGFR signaling is crucial to both intramembranous and endochondral ossification and is involved in proliferation, differentiation, and tissue healing.[40] FGF receptors all contain an extracellular domain with either 2 or 3 immunoglobulin like domains, a transmembrane domain, and a cytoplasmic tyrosine kinase domain (Fig. 2). Most of the significant craniosynostosis mutations occur in the extracellular domain resulting in a dominant active effect. FGF receptors are expressed by osteoprogenitor cells and FGF ligands are expressed by both mesenchymal cells and osteoblasts. The FGFR family has drawn the most attention as it has been shown to be involved in the majority of syndromic cases of craniosynostosis. A mutation in these genes ultimately results in a gain of function which manifests in either enhanced ligand affinity or less discrete binding.[41,42]

Within the FGFR signaling pathway, downstream mediators have also been linked to craniosynostosis. Msx2 is part of the highly conserved Msx homeobox gene family that causes Boston type craniosynostosis.[36,43] Msx2 expression is

Table 1 Genes affected in major craniosynostosis syndromes.

Craniosynostosis	Gene	Reference
Crouzon	FGFR2, FGFR3, IL-11R	26–30
Apert	FGFR2	31
Saethre-Chotzen	Twist1, TCF12	14,32,33
Muenke	FGFR3	17
Pfeiffer	FGFR1, FGFR2	34,35
Boston-type	Msx2	36
Beare-Stevenson	FGFR2	37
Craniofrontonasal dysplasia	EFNB1	38
Jackson-Weiss	FGFR2	39

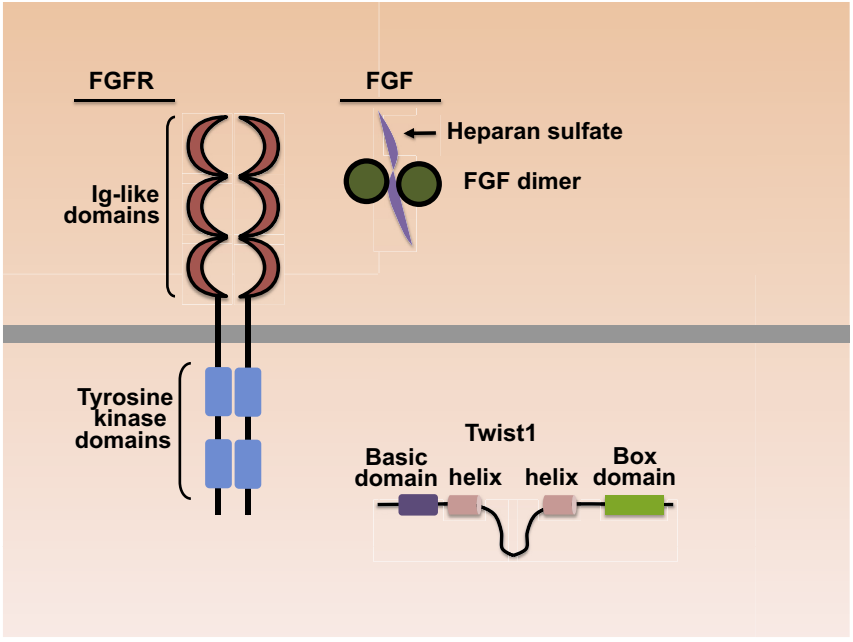

Figure 2 Members of the FGFR signaling pathway. FGFR receptors are characterized by two to three extracellular Ig-like domains and two tyrosine kinase domains that mediate downstream signaling. The FGF ligand is typically bound to heparan sulfate in the extracellular matrix in dimeric form. This allows potentially for binding of multiple FGFR. One of the negative regulators of FGFR signaling is Twist, a basic helix-loop-helix transcription factor.

increased in response to FGF-2.[44] Overexpression of Msx genes in calvarial osteoprogenitor cells results in proliferation instead of differentiation of the cells.[43,45] In the mouse, Msx2 is expressed in the neural crest population that gives rise to the calvarial bones and the dura. Runx2, a major osteogenic effector, is also induced downstream of the FGFR signaling pathway. Runx2 is essential for both endochondral and intramembranous ossification.[46] In craniosynostosis, additional copies of Runx2 have been detected in a small number of individuals causing pansynostosis and significant midface hypoplasia.[47]

Although a number of pathways essential to bone homeostasis have not been directly associated with craniosynostosis, disruptions in FGFR signaling affect the regulation of pathways such as the bone morphogenetic protein (BMP) signals. BMPs, members of the transforming growth factor-β (TGF-β) superfamily of growth factors, are heterogeneous growth factors that have been shown to promote osteogenesis, chondrogenesis, and organogenesis via signaling through heteromeric BMP receptor (BMPR) complexes.[48] The BMPs are particularly significant in clinical practice because two of its members (BMP-2 and -7) are currently approved for use in therapeutic applications.[49] BMPs are first synthesized as precursor proteins that dimerize intracellularly. Upon dimerization, they are cleaved at the consensus Arg-x-x-Arg site, yielding carboxy-terminal mature dimers that are secreted (Fig. 3). Following secretion, BMP dimers activate intracellular processes by binding to BMPR complexes.[50] Both type I and type II BMPR are transmembrane serine threonine kinases.[51] Type I BMPR are generally considered to be the high affinity receptors that determine the specificity of BMP signaling and type II receptors are the constitutively active receptors that activate downstream processes after binding to type I receptors. However, this general rule has been challenged by the binding patterns of certain BMPs such as BMP-9.[52] The mode of BMPR oligomerization at the cell surface is a determinant in downstream signaling pathways.[53,54] In the BMP-mediated signaling complex (also called the BMP-induced signaling complex or BISC), BMP dimers bind to type I BMPR dimers and recruit type II BMPR dimers to the complex. This complex is internalized in caveolae and results in the activation of ERK, p38 MAPK, and PI3K pathways without Smad activation. In contrast, type I and type II BMPR can exist in a tetrameric preformed complex. When BMP dimers bind to the tetrameric preformed complex, the receptor Smads (Smad 1/5/8 or Smad 2/3) are recruited and phosphorylated by the receptor. Internalization occurs through a clathrin dependent endosomal route. Phosphorylated receptor Smads associate with co-Smad (Smad 4) and translocate to the nucleus to activate Smad-dependent genes such as Id1-3. Crosstalk between the two pathways occurs. Both ERK and p38 MAPK are activated by the Smad independent pathway and both have the capabilities to target receptor Smads for proteasomal degradation.[55] Both canonical and non-canonical pathways can induce osteogenic genes.

Inhibitors of BMP signaling such as Noggin are now known to be important for suture patency.[56] This is believed to occur via a paracrine fashion from neural crest derived cells in the dura. High FGF-2 activity in murine sutures has been found to downregulate noggin expression at certain time points which then permits endogenous BMP signaling and subsequent fusion of the suture.[57,58] Genetic disruptions of the BMP pathway members have not been described in human craniosynostosis, however, dysregulation of the pathway is a byproduct of known craniosynostosis mutations.

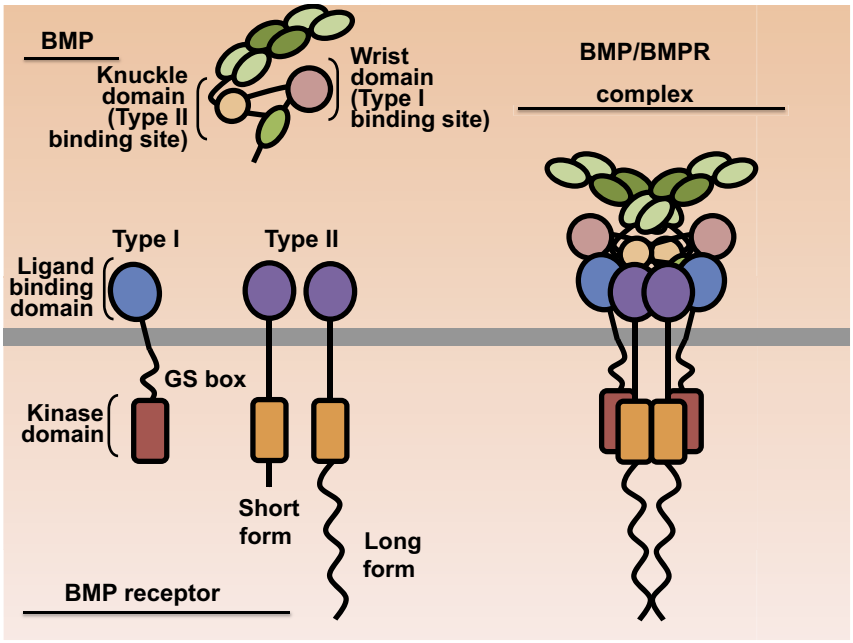

Figure 3 Members of the BMPR signaling pathway. BMPR are heterotetrameric receptors. The type I receptor is a high affinity receptor that directs specificity of BMP binding. The BMP ligand is secreted and binds its cognate receptor in dimeric form.

Loss of function mutations in negative regulators of the FGFR pathway are also found in craniosynostosis syndromes. Twist1, a basic helix-loop-helix transcription factor, is related to FGFR signaling in that it acts to direct mesenchymal cell fate during skeletal development towards both the chondrogenic and osteogenic lineages. Twist1 has been found to have over 100 different mutations and the majority of them causing a loss of function. The mechanism of Twist1 directed lineage determination is based on negative regulation of FGFR2 expression thereby prolonging suture patency.[59,60] The importance of Twist1 in maintaining suture patency is further highlighted by a recent description that, TCF12, the heterodimeric binding partner for Twist1, is a cause for coronal synostosis.[14] Heterozygous loss of function mutations resulting in haploinsufficiency are associated with Saethre—Chotzen syndrome for both Twist and TCF12. Twist is upstream of the ephrins, which are membrane bound ligands for Eph family receptor kinases known to regulate cell adhesion and migration during development. Specifically, the Twist-Ephrin axis has been demonstrated in murine models to be essential to the neural crest-mesoderm boundary in the developing cranial suture.[61] Missense mutations of EFNB-1 are the most commonly found abnormalities contributing to the development of craniofrontonasal dysplasia (also called craniofrontonasal syndrome) and other craniosynostoses.[38,62]

While osteoblast activity has been at the forefront of much of these studies, there still exists a fine balance between the cellular interaction of osteoblasts and osteoclasts which is at the core of bone biology and development. All of these studies have led to a better understanding of cranial suture genetics and biology, but little light has been shed upon the role of the osteoclast and its activity in suture patency.

Balancing bone formation to bone resorption

A controlled amount of resorption in bone regeneration and remodeling is part of normal bone physiology. However, the balance of formation versus resorption is precarious and can be tipped in favor of the former or the latter depending on the local microenvironment. In pathological circumstances such as chronic inflammation or bony metastases in cancer, the ratio of osteoclastogenic factors to osteoclast inhibitors increases and a net resorption of bone occurs.[63,64] Osteoclast differentiation is supported by mesenchymal cells through cell-to-cell contact and paracrine effects which are regulated by both macrophage colony-stimulating factor (M-CSF) and the receptor activator of NF-κB (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) axis (Fig. 4).[65—67] Unlike osteoprogenitors, osteoclasts derive from hematopoietic progenitors. However, a number of different cell types are necessary to direct monocyte to osteoclast differentiation by supplying RANKL. These cell types include osteoblasts, osteocytes, stromal cells, B and T cells, synovial fibroblasts, hypertrophic chondrocytes, and other osteoclasts. Analogous to lymphocytes, the mechanism for differentiation and activation is a two-signal process requiring both M-CSF and RANK receptors. Although both cytokines are essential for osteoclastogenesis, M-CSF is primarily involved in stimulating the survival and proliferation of osteoclast progenitors via upregulation of Bcl-xL while RANKL is responsible for stimulating their differentiation.[68]

Described by its ability to inhibit osteoclast development, the first molecule within the axis to be discovered was OPG. OPG is a member of the tumor necrosis factor receptor (TNFR) superfamily that is secreted due to a lack of the transmembrane domain.[69] As a soluble protein, OPG binds and sequesters RANKL from binding RANK, thereby inhibiting osteoclast activation. Transgenic mice that

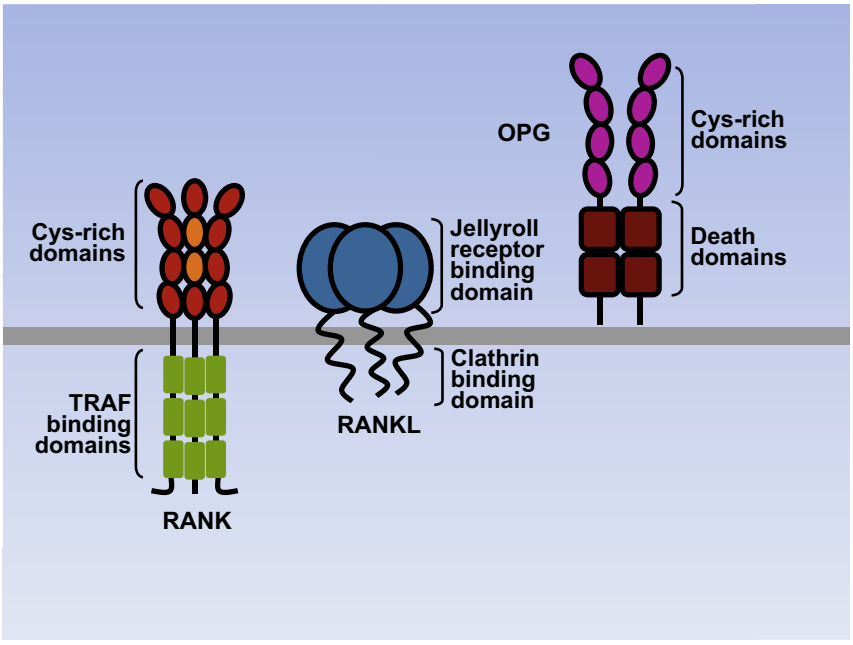

Figure 4 Members of the RANK signaling pathway. RANK is a member of the TNFR superfamily that is essential for osteoclastogenesis and function. After binding, TRAF adaptor proteins are recruited to the receptor complex to activate downstream intracellular mediators. Like the other member of the TNFR family, RANK binds RANKL as a trimer to activate intracellular signaling pathways. OPG, a decoy receptor, negatively regulates this pathway by binding and inactivating RANKL.

overexpress OPG demonstrated both an increase in bone density as well as osteopetrosis. Conversely, OPG knockout mice exhibit profound osteoporosis.[70–72] Not only did these studies show a crucial role for OPG in the maintenance of bone mass but they also suggested that OPG may neutralize a TNF-related factor that could stimulate osteoclast development.[73] This factor was identified as RANKL or originally called TRANCE (TNF-related activation-induced cytokine).

RANK, a tumor necrosis factor superfamily receptor originally identified in T lymphocytes and osteoblasts, is now known to be essential for osteoclast differentiation and activation upon binding to its cognate ligand.[74,75] Knockout mice generated in these studies show that both RANK and RANKL deficiencies resulted in osteopetrosis due to a complete absence of osteoclasts.[70,76] The binding of RANKL with RANK results in the trimerization of the receptor and recruitment of the adaptor protein tumor necrosis associated factor 6 (TRAF6) to the receptor complex. Although the RANK complex can interact with other TRAFs, only TRAF6 has been found to be essential for osteoclast function.[77,78] This interaction then activates a cascade of intracellular signaling pathways including nuclear factor of activated T cells 1 (NFATc1), mitogen activated protein kinases (MAPKs) such as Jun N terminal kinase (JNK) and p38, and NF-κB.[66,77,79,80]

The discovery of the RANK-RANKL-OPG axis has been a critical breakthrough in the understanding of osteoclast differentiation.[81–84] While macrophage colony forming units have been known to be a precursor to macrophages, RANK activation induces differentiation into osteoclasts. Most importantly, the characterization of RANKL allowed the differentiation of osteoclasts *in vitro* and the ability to perform mechanistic studies on osteoclasts. Due to the direct relationship between the RANK-RANKL-OPG axis and osteoclast activation, targeted therapies against the axis are under investigation for fracture healing and other conditions requiring a net osteogenic state.[85]

Osteoclast activation in craniosynostosis

Beyond the physiological aspect that the RANK-RANKL-OPG axis imparts on osteoclastogenesis, overactivity of the axis is significant in the pathogenesis of certain diseases. In terms of genetic descriptions, duplications in the signal peptide of RANK have been linked to Paget's disease, which is characterized by focal areas of increased bone remodeling. Another disease called familial expansile osteolysis is a rare autosomal dominant disorder that causes erosion of long bones by progressive osteoclastic resorption. Furthermore, juvenile Paget's disease is thought to be caused by mutations affecting the ligand binding domain of OPG.[86] These patients suffer from excessive osteoclastic bone resorption and irregular bone formation that leads to bone pain and deformities. Rheumatoid arthritis has also been linked to osteoclast formation. More specifically, RANKL expression in fibroblasts within the synovia has been shown to lead to the formation of osteoclasts causing the bone loss seen in the disease. In animal models, inhibition of RANKL through OPG did not influence the severity of inflammation but the treatment did stop the loss of bone in inflamed joints of arthritic rats in a dose-dependent manner and untreated animals saw a large increase in osteoclast numbers.[87]

Unlike work on long bones and joints, alterations in osteoclasts are found less frequently in craniosynostosis. It is likely that the reason this occurs is that a lack of osteoclast

function rather than excessive osteoclast activity results in a severe systemic alteration which may not be compatible with life. Nevertheless, heritable osteopetroses have been reported in human diseases. Autosomal recessive osteopetrosis (ARO) is a rare, heterogeneous disease that presents soon after birth and frequently leads to postnatal death.[88,89] The incidence of ARO is 1:250,000, albeit the incidence is higher in some geographical areas due to consanguinity. The clinical manifestations of ARO are secondary to defective bone resorption, which has been reported as a constellation of presentations including craniosynostosis, macrocephaly, foraminal narrowing of the skull base, structural brain malformations, frontal bossing, exophthalmos, hypertelorbitism, and micrognathia. One of the major problems facing patients with ARO is the decreased immune response leading to a high risk for osteomyelitis and other infections. The perturbations in bony development of the calvarium have been documented both radiographically and clinically. The characteristic facies of ARO has been described to include frontal bossing, exophthalmos, hypertelorbitism, midface hypoplasia, and craniosynostosis. In fact, radiographic studies on the disease have demonstrated the characteristic harlequin sign bilaterally signifying bicoronal craniosynostosis.[90,91] ARO can be divided into the subset of osteopetroses that are osteoclast rich versus the subset that has a paucity of osteoclasts. In the majority of the ARO forms where the defect is intrinsic to osteoclast function, the definitive treatment is a bone marrow transplant for rescue. The exception to the rule is when the defect affects RANKL, in which a proof of principle investigation has suggested that RANKL infusion may rescue the phenotype.[92]

The evidence that osteoclasts are important to suture patency has been confirmed in an experimental *ex vivo* murine model. Recent studies have demonstrated a novel role for RANK-RANKL-OPG axis in cranial suture biology as well as the development nonsyndromic craniosynostosis through the fine balance of osteoblast and osteoclast function. Using a well established model for cranial suture biology, CD1 mice were examined for RANK expressions in various cranial sutures. Immunohistochemical analysis showed strong RANK staining at the junction of coronal and sagittal sutures up to 12 weeks of age. Both of these sutures are known to stay patent past 5 weeks of age. In contrast, posterior frontal sutures which close *in vivo* around 5 weeks of age showed decreased RANK staining. Similar results were found when pathologically fused and patent sutures were examined in human subjects. RANK expression in patients with known nonsyndromic craniosynostosis were examined in the same manner as the murine model and comparable results were found.[93] Patent human coronal sutures displayed high levels of RANK staining at the suture junction in contrast to the low levels found within the pathologically fused human sutures. Unlike the RANK receptor, the expression pattern for RANKL does not correlate with suture patency. Both fused and patent sutures express RANKL equally. Furthermore, levels of isolated RANK mRNA were also examined at each time point in the respective sutures as well as calvarial bone without suture. Interestingly, levels of RANK mRNA decreased in a temporal fashion regardless of the suture, suggesting that RANK expression is not regulated at the transcription level but potentially controlled at the posttranslational level. Lastly, RANK knockdown in calvarial strip suture cultures demonstrated an increase in bone density within patent sutures after transduction with a small interfering RNA specific for RANK.[93]

In summary, a model for bone homeostasis from the pathways affected in craniosynostosis can be synthesized (Fig. 5). Within the basic multicellular unit, osteogenic signals are received by the osteoblast via FGF/FGFR and BMP/BMPR pathways. Activation of both FGFR and BMPR signaling pathways results in transactivation of osteogenic genes. This transactivation is negative regulated by Twist1. At the same time, activation of osteogenic genes simultaneously activates RANKL and OPG expression. RANKL binds to RANK on the osteoclast to stimulate osteoclastogenesis while OPG negatively regulates this process. The relative quantity of RANKL to OPG is likely to be an important factor in osteoclast activity. Within mineralized bone, osteocytes also secrete RANKL and OPG via its canalicular network. Although not discussed in this review, osteocytes are also the main sources for sclerostin, which is an important regulator of the Wnt pathway.

From suture biology to skeletal tissue engineering

The strides in molecular genetics and signaling achieved through the understanding of cranial suture biology and congenital craniosynostoses have been extensive in the past 25 years. Despite all of these advances, craniosynostosis remains a surgical disease. Especially in syndromic cases, the abilities to decompress elevated intracranial pressure or protect the exophthalmic globe from exposure are not yet in the realm of pharmacologic or genetic therapies. However, these basic studies have been instrumental in effecting advances in skeletal regenerative technologies which have the potential of clinical relevance beyond rare congenital conditions.

The conceptual approach to bone tissue engineering is to direct regeneration by applying osteogenic factors to a specific three-dimensional space. Current methods used to accomplish this concept usually include three elements: osteogenic cells, scaffolding material, and growth factors.[94,95] Strategic placement of the proper combination of cells and growth factors can support both recruitment of osteogenic cells from the host environment and osteogenesis on the scaffold. Scaffolding material, traditionally thought to be an inert structure, is now known to have differential abilities to support osteogenesis depending on the material, porosity, and ability to mimic the normal extracellular matrix.[94] Contemporary bone tissue engineering research aims to optimize the delivery of the three components to generate a stable quantity of bone that fully integrates into the human body and sustains the test of time.

Advances in bone tissue engineering have historically generated great excitement initially; however, most attempts at transfer have largely failed. In 2001, Vacanti and colleagues excited the field of tissue engineering with the implantation of an engineered distal phalanx containing periosteum-derived cells on a phalanx shaped

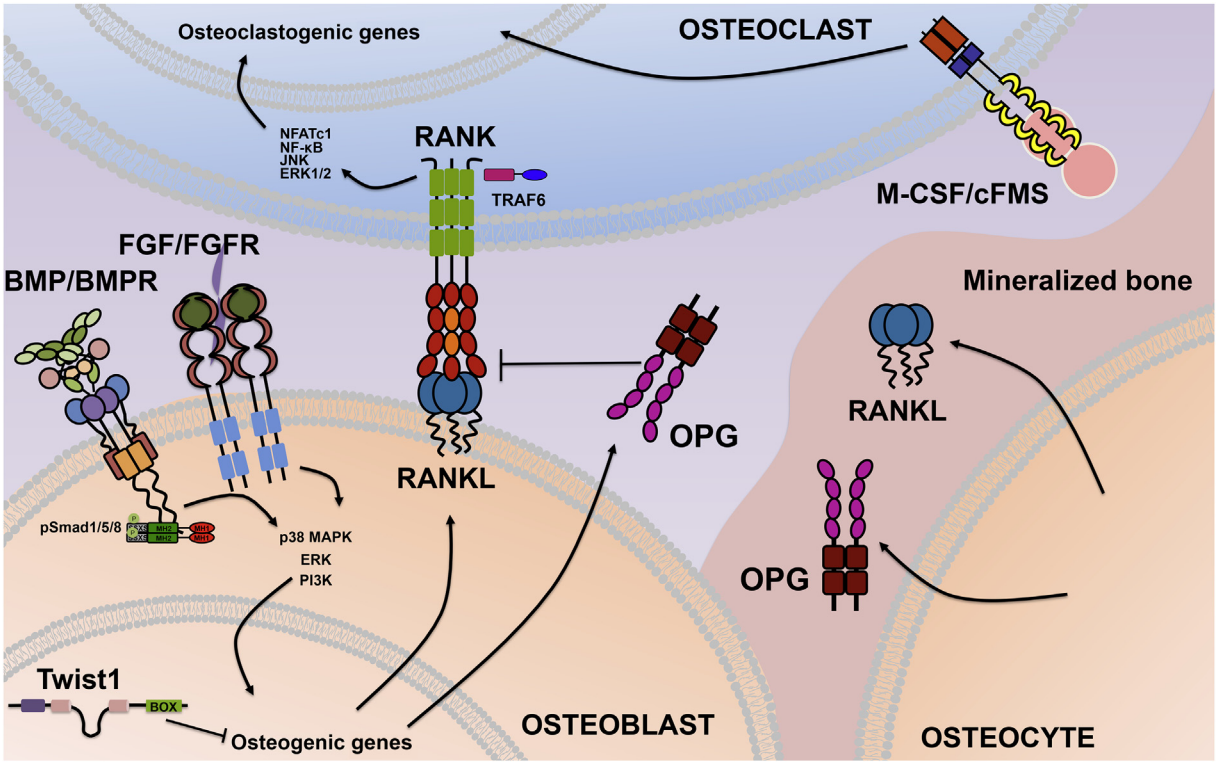

Figure 5 Lessons on bone homeostasis from cranial suture biology. From the many genetic and mechanistic studies on the craniosynostosis and cranial suture biology, contributions on interplay between the basic multicellular unit for bone homeostasis have been elucidated. From the bone formation perspective, the FGFR gain of function mutations and Twist1 loss of function mutations result in dysregulated bone formation. Twist 1 downregulates not only the FGFR signaling but also BMPR signaling. From the osteoclastogenic perspective, RANK axis mutations have severe phenotypes in human disease and are frequently incompatible with life. In the rare surviving patients, RANK or RANKL loss of function inhibits osteoclast function causing craniosynostosis and osteopetrosis. However, these opposing processes are significantly intertwined. Osteogenic gene activation also increases secretion of RANKL and OPG from osteoprogenitors. Similarly, the osteocyte is also a source of RANKL and OPG.

hydroxyapatite scaffold.[96] Similarly, Warnke and colleagues reported implantation of an engineered, autologous human mandible using hydroxyapatite blocks and a titanium mesh scaffold.[97,98] However, the course for this patient was marked with fracture of the mesh/bone junction, infection, and necrotic bone. In this work, engineered bone bores minimal advantages, at best, and some disadvantages in comparison to the bone graft substitutes that currently exist. Furthermore, both groups and other investigators[99,100] have shown data using a hydroxyapatite scaffold, known to be osteoconductive, even in the absence of osteogenic cells. Thus, without a formal direct comparison with hydroxyapatite scaffolds alone, the advantages of these processes are difficult to measure. Smaller bone defects have fared better.[101–103] Hibi and colleagues[103] have reported a process using autologous serum and bone marrow to generate osteogenic material that was able to induce and/or conduct osteogenesis in alveolar clefts. Schimming and Schmelzeisen demonstrated success in approximately 66% of their patients using autologous periosteal osteoprogenitors for maxillary sinus augmentation.[101,102] Both groups demonstrated that in small bone defects, bone engineering has the potential to be accomplished but carries its own morbidity as well as some disadvantages in comparison to surgical bone grafting or bone graft substitutes. In small defects such as alveolar clefts, it is also important to acknowledge that recombinant BMP-2 alone is also capable of healing via osteoinduction.[104,105] Unfortunately, long term follow up of BMP-2 treatment in alveolar clefts showed untoward effects on maxillary growth.[106] This highlights the need for therapies that do not rely primarily on exogenous, supraphysiological quantities of growth factors. Like the study of craniosynostosis, the primary focus of bone tissue engineering has been on making bone with minimal to no consideration for bone breakdown.

From the knowledge gained from studying craniosynostosis, a number of molecules have been utilized to increase *in vitro* osteogenesis and *in vivo* models of bone healing. Within the FGF pathway, a multitude of scaffolds have been combined with FGF-2 for bone regeneration.[107] FGF-2 in combination with BMP-2 have shown the ability to increase osteogenesis of bone marrow derived mesenchymal stem cells in a temporal fashion.[108] In calvarial defects, chemical tunable FGF-2 can induce *in vivo* bone healing in adipose-derived stem cells.[109] Twist1 knockdown in human adipose-derived stem cells have demonstrated an increase in BMP and FGF signaling resulting in osteogenic differentiation.[110] Certainly, the BMP pathway has received the most attention in both the laboratory and the clinical realm for making

bone. Two obstacles that have plagued bone engineering are the presence of inflammation after implantation of bone and long term resorption. In studies with human mesenchymal stem cells, this is frequently overlooked in laboratory investigations in that the human cells and scaffolds are implanted into immunocompromised recipients. However, inflammation is more apparent when non-human, immunocompetent models are utilized. Although the reason for inflammation in syngeneic, immunocompetent models may be multifactorial, it is likely that the scaffolding material may be part of the reason. A multitude of scaffolds have been reported in the literature comprised of biodegradable polymers, extracellular matrix components, or combinations thereof. Polymers made of poly-L-lactic acid and poly-L-glycolic acid have been long tested in our laboratory and others as a scaffold material for bone engineering. Both substances and their derivatives have been in use as resorbable suture material and resorbable plates and screws for skeletal defects. Both substances degrade into acidic metabolites, thereby inducing inflammation via superoxide release from phagocytes.[111,112] In small defects, local inflammation may be relatively inconsequential for bone resorption. However, when the defect is large enough to warrant replacement, prolonged inflammation may resorb the quantity of bone necessary for engraftment. This realization has led many investigators to find ways on decreasing inflammation in an effort to decrease resorption. Interestingly, the initiation of osteogenic differentiation is also related to resorption in that RANKL and OPG expression increase. The amount of increase is temporally dependent and the ratio of RANKL to OPG also varies in a temporal manner (JCL, unpublished observations). Because osteogenesis induces the expression of RANK and OPG in mesenchymal stem cells, additional attention to the RANK axis and osteoclastogenic activation may be an important consideration to the future of tissue engineering.

Conclusions

The lessons learned from cranial suture biology have impacted both an understanding of the pathogenesis of congenital craniosynostosis as well as opened new avenues of investigation in bone tissue engineering. Like all biology, the pathways are complex, the players are varied in cell types, and perturbations of homeostasis can result in different outcomes. The future of reconstructive surgery is regenerative surgery and these lessons are invaluable advances towards clinical solutions.

Conflicts of interest

All authors have none to declare.

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