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Craniofacial and Long Bone Development in the Context of Distraction Osteogenesis

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Abstract

Background—Bone retains regenerative potential into adulthood, and surgeons harness this plasticity during distraction osteogenesis (DO). The underlying biology governing bone development, repair, and regeneration are divergent between the craniofacial and appendicular skeleton. Each type of bone formation is characterized by unique molecular signaling and cellular behavior. Recent discoveries have elucidated the cellular and genetic processes underlying skeletal development and regeneration, providing an opportunity to couple biological and clinical knowledge in order to improve patient care.

Method—A comprehensive literature review of basic and clinical literature regarding craniofacial and long bone development, regeneration, and DO was performed.

Results—The current understanding in craniofacial and long bone development and regeneration are discussed, and clinical considerations for the respective DO procedures are presented.

Conclusions—DO is a powerful tool to regenerate bone and thus address a number of craniofacial and appendicular skeletal deficiencies. The molecular mechanisms underlying bone regeneration, however, remain elusive. Recent work has determined that embryological morphogen gradients constitute important signals during regeneration. Additionally, striking discoveries have illuminated the cellular processes underlying mandibular regeneration during DO, showing that skeletal stem cells reactivate embryological neural crest transcriptomic processes to carry out bone formation during regeneration. Furthermore, innovative adjuvant therapies to complement DO utilize biological processes active in embryogenesis and regeneration. Additional research is needed to further characterize the underlying cellular mechanisms responsible for improved bone formation through adjuvant therapies and the role skeletal stem cells play during regeneration.

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INTRODUCTION

The skeleton possesses unprecedented biomedical research potential, and translational research in skeletal biology may significantly benefit patients. The incidence of skeletal dysplasia is 1:5,000 live births[1,2] while traumatic fracture, osteoporosis, and arthritis are the predominant skeletal disorders during aging[3–5]. The annual healthcare cost of fracture repair resulting from osteoporosis is approximately $17 billion in the United States alone[3,6]. With the large burden of skeletal pathologies permeating all stages of life, uncovering the fundamental biological processes driving skeletal development and regeneration is essential in the development of novel targeted therapies.

The skeleton consists of specialized connective tissues including ossified and non-ossified elements, bone marrow stroma, and supportive tissues[7]. Numerous cell types make up these tissues, including osteocytes, chondrocytes, hematologic, and stromal cells. The common progenitor cell that gives rise to the bone, cartilage, and stromal elements during development, repair, and regeneration is the skeletal stem cell (SSC)[8,9]. Recent discoveries highlight the significant role of the SSC as the enactor of mandibular regeneration during distraction osteogenesis (DO) – the process of lengthening bone through endogenous tissue engineering using a guided mechanical environment[10]. Additionally, morphogens expression is tightly regulated to provide signaling gradients necessary for skeletal growth. We review the current knowledge of the developmental and regenerative biology of the craniofacial and appendicular skeleton.

DEVELOPMENT OF CRANIAL BONES

Most cranial bones arise from ectodermal neural crest cells (NCCs), which originate from the dorsal margins of the closing neural tube (Figure 1). During neurulation, the borders of the neural plate converge at the dorsal midline to form the neural tube. At this point, the NCCs from the roof plate undergo epithelial-to-mesenchymal transition during nueralation[12–14], including delamination and migration events (Figure 1). Delamination begins with the dorsal expression of bone morphogenetic proteins (BMP), which lead to decreased expression of occludins and cadherins resulting in reduced cellular adhesion[13,15]. Occludins are an integral component of tight junctions, while cadherins are important in the formation of adherens junctions[16,17]. Both occludins and cadherins are important in maintaining cell-to-cell adhesion. Concurrently, NCCs secrete matrix metalloproteinases, which break down the overlying basal lamina[13,18,19]. The permeable basal lamina and decreased cellular attachments enable NCCs to migrate throughout the embryo.

NCCs migrate from rostral to caudal due to repulsive guidance between molecular signals, extracellular matrix interactions, and cellular contact inhibition[20,21]. Expression of the Eph receptor tyrosine kinase by NCCs allows them to bind to the ephrin transmembrane ligand, leading to cytoskeletal rearrangement and cellular repulsion[22]. In general, tyrosine kinases catalyze the phosphorylation of tyrosine residues, which cause a functional change in the protein[23] (Figure 2). Both Eph and ephrin ligands are membrane-bound proteins which require direct cell-cell interactions for activation. NCCs, additionally, express integrin $\alpha 5\beta 1$ which guides migration by binding to ligands such as collagen, laminin, and fibronectin on

the extracellular matrix[24,25]. Integrins are transmembrane receptors that activate signal transduction pathways mediating cellular-extracellular matrix interactions and intracellular cytoskeleton rearrangements[26].

After cranial NCCs colonize the facial prominences, the cells aggregate, condense, and differentiate in response to signals from the surrounding niche[27]. NCCs that colonize the first arch form the maxilla and mandible[21]. The transcriptional profile of NCC populations uniquely corelate with their origination on the neural tube anterior-posterior axis. For example, *Hox* gene expression in the pharyngeal arch cell populations increases in the posterior direction[28,29]. *Hox* genes are homeobox genes that specify body plan regions along the head-tail axis[30]. The Hox proteins ensure the correct structures form in the correct places of the body. Overexpression of *Hoxa2* in the first pharyngeal arch limits mandible formation[31–33] (Figure 3A). Furthermore, *Hoxa2* has been shown to suppress the expression of *Runx2*, which is important in skeletogenesis. *Runx2* promotes bone differentiation, and inhibition of *Runx2* through *Hoxa2* expression limits bone formation[34–36] (Figure 3B). As craniofacial bone development proceeds through intramembranous ossification, absence of *Hoxa2* expression is critical for bone formation in the first pharyngeal arch (Figure 3C)[35].

Intramembranous ossification is a process distinct to bone development of the mandible, clavicle, and most bones of the skull. Intramembranous ossification initiates during fetal development *in utero,* and the skull and clavicles are not fully ossified at birth[37]. These bones fully ossify at different post-natal time points and follow a similar ossification paradigm: (1) formation of ossification center, (2) matrix formation, (3) periosteum weaving, and (4) compact bone formation[37]. A concentration of mesenchymal cells differentiate into bone-depositing osteoblasts that cluster to form an ossification center[38]. Next, the osteoblasts secrete collagenous matrix proteins, or osteoids, which calcify and confine the osteoblasts. Once the osteoblasts are embedded onto the osteoid, the osteoblasts develop into osteocytes. Synchronously, osteogenic cells from adjacent connective tissue differentiate into osteoblasts on the periphery of the growing bone. Ongoing bone deposition allows collections of osteoids to congregate near capillaries, forming the trabecular matrix of spongy bone. Osteoblasts on the periphery of the spongy bone develop into the periosteum. This newly formed periosteum produces compact bone around the spongy bone, while the spongy bone surrounding nearby blood vessels condenses into bone marrow[37]. Intramembranous ossification thus results in formation of the bone without an intermediate cartilaginous anlage.

DEVELOPMENT OF LONG BONES

Long bone development begins with outgrowth of the limb buds from the trunk (Figure 1) in the presumptive forelimb and hindlimb locations. Cells from the lateral plate mesoderm migrate to create a mass of proliferative bone progenitor cells known as the limb field. Three areas of significance form to pattern the growing limb bud: apical ectodermal ridge (AER), progress zone, and zone of polarizing activity (ZPA) (Figure 1). The AER is a structurally distinct ridge of epithelium located at the distalmost extent of the limb bud. The AER bisects the dorsal and ventral aspects of the growing limb bud and is necessary for limb outgrowth[39]. Second, the progress zone is a mass of cells found underneath the AER[40,41].

The progress zone is necessary for limb type specification, with cells in this zone harboring intrinsic properties to determine limb type. The third structure is the ZPA, which is restricted to the posterior aspect of the bud and provides signals directing limb bud growth along the anterior-posterior axis[42].

Morphogenetic signaling gradients are central to direct limb length and patterning during development. For example, proximal-distal specification relies upon the antagonistic relationship between retinoic acid (RA) and fibroblast growth factor (FGF) 8[43,44]. Cell fates are influenced by a proximal source of RA originating from the embryonic trunk and a distal source of FGF-8 originating from the AER[45]. Anterior-posterior specification occurs through Sonic hedgehog, BMP, and Gremlin signaling, which originates in the ZPA domain[42,46–48], while dorsal-ventral specification relies upon a gradient of WNT and BMP[49]. Long bone development further continues through endochondral ossification.

Endochondral ossification is responsible for bone formation of all skeletal elements other than the craniofacial bones and clavicle. This begins when progenitor cells differentiate into chondrocytes and synthesize extracellular matrix abundant in Type II collagen[37,50–54]. This cartilaginous model prefigures the shape of ossified bone and enlarges through chondrocyte proliferation. The chondrocytes are divided into three zones during this process. First is the zone of proliferation, located in the center and contains rapidly dividing chondrocytes. These cells stop proliferating in the zone of maturation. The outermost, hypertrophic zone is composed of chondrocytes that secrete a distinct matrix containing Type X collagen[37,50,54–59]. Concomitantly, the hypertrophic chondrocytes direct the cells in the perichondrium to differentiate into osteoblasts[60–62]. Moreover, angiogenesis of the hypertrophic zone and perichondrium allow ossification of the cartilage matrix by the invading osteoblasts[11,63–67].

Our understanding of the cellular basis in limb development has greatly advanced with the recent discovery of the SSC. These cells were first identified in the femoral growth plates of mice, and possess the ability to self-renew and differentiate into bone, cartilage, and stromal subtypes[8]. Further evidence supporting the intrinsic ability of the mouse SSC (mSSC) and its downstream progenitor cells to generate these tissue types included production of ossicle, cartilage, and marrow after transplantation of purified cells into a kidney capsule niche[8] (Figure 4A). The corresponding human SCC was subsequently isolated from the femoral growth plate, exhibiting similar properties of self-renewal and differentiation into each skeletal tissue type[9] (Figure 4B).

The identification of the SSC has illuminated important skeletal biology. For example, downstream progenitor subsets that are derived from the mSSC have been shown to execute long bone fracture repair[68]. Following femoral fracture, these cells exhibited increased cell frequency, viability, and enhanced osteogenic function. Intriguingly, the injury-responsive mSSC transcriptional profile showed upregulation of the same genes and signaling morphogens important in long bone embryogenesis, such as *BMP* and Hedgehog[68]. These data highlight the molecular overlap between long bone embryogenesis and regeneration.

With regards to the role of the SSC in regenerative contexts, a recent study explored the behavior of mSSCs during mandibular distraction osteogenesis (MDO). This work revealed the bone regenerate was clonally derived from mSSCs[10]. The pathway by which mSSCs respond to the mechanical force of MDO involves upregulation of focal adhesion kinase (FAK) signaling[10] (Figure 5). The underlying genetic programs responding to FAK signal transduction in mSSCs included activation of transcriptional elements characteristic of primitive NCCs[10,69]. This genetic reversion of mandibular mSSCs back to primitive NCCs characteristics underscores the importance of understanding bone embryogenesis as it applies to bone regeneration biology. Furthermore, the fact that MDO can proceed in patients with Type II collagenopathy harken back to intramembranous ossification, which occurs without a cartilage intermediate[70]. In fact, mSSCs are capable of forming new mandibular bone through intramembranous ossification during MDO, thereby recapitulating developmental processes during regeneration[10].

ADJUVANT THERAPIES FOR MANDIBULAR DISTRACTION OSTEOGENESIS

MDO is an advantageous strategy to treat mandibular hypoplasia, which is a feature of multiple clinical problems related to mandibular deficiencies. While generally performed in children, MDO may be applied to adult populations, as well[71,72]. MDO is a dynamic process rather than a single intervention. Common corticotomies used include the oblique body/angle cut, vertical ramus cut, and inverted-L ramus cut, which vary in use based upon age and mandibular anatomy[71,73]. Postoperatively, the distraction protocol begins with a variable period of 0–5 days. After latency, distraction occurs with a total rate of 1 to 2 mm of distraction per day divided over a frequency of 1 to 4 times per day. The final phase of MDO involves consolidation of the regenerate over a time period of 6 to 12 weeks[71,74]. The overall complication rate ranges from 20–40%, and a technical learning curve may exist with increased complication rates noted earlier in a surgeon's experience[75]. Major postoperative compilations of MDO include malunion/nonunion, premature consolidation, and relapse[75].

The ability of adjuvant therapies to enhance the MDO surgeries has been studied using various animal models. Deferoxamine (DFO) accelerates bone consolidation in rats undergoing MDO[76] by chelating iron, which result in the stimulation of the hypoxia inducible factor 1-α (HIF-1α) pathway. HIF-1α is a subunit of the heterodimeric transcription factor HIF-1, which is considered the master transcriptional regulator for cellular and developmental response towards hypoxia[77–79]. With regards to regeneration, the upregulation of HIF-1α led to improved wound healing of damaged tissue in mice, while down-regulation of HIF-1α resulted in diminished wound closure[80]. Specifically, in terms of bone regeneration, mSSC chromatin architecture sequencing revealed that the HIF-1α transcriptional network plays a substantial role during MDO[10]. Additionally, a recent case report showed improved pterygomaxillary area and density in a patient receiving DFO during MDO after irradiation, highlighting the potential clinical relevance of DFO as an adjuvant therapy during MDO[81].

Another prominent molecule that supports MDO bone regeneration is BMP, with high levels of expression confirmed during distraction and subsequent decline during consolidation[82]. BMP interacts with cell surface receptors known as BMP receptors. The interaction leads to signal transduction resulting in the mobilization of members from the SMAD family of proteins, which are essential for fracture repair and bone growth[83]. Recombinant human BMP-2 (rhBMP-2) has been approved for patient administration to improve fracture repair in the tibia and for specific spinal indications. Contraindications for use include any type of anterior cervical spine fusion and soft tissue swelling near the esophagus and trachea, given the propensity of rhBMP to cause swelling in this region. Other reports have also highlighted problems with ectopic bone growth and variability in dosage delivered with current carrier systems[84]. Aside from BMP, vascular endothelial growth factor and FGF-2 expression have also been found to increase during consildation[85,86]. Exogenous growth factor administration along similar timelines holds potential to promote improved bone formation during MDO[87].

Alternatively, mesenchymal stem cells (MSC) have been studied as a cellular therapeutic to enhance bone consolidation in the setting of MDO. Sheep hemi-mandibles treated with MSCs on the first day of consolidation had greater total and compact bone ratio in the regenerate zone[88]. Another study examined endogenous recruitment of MSCs to the site of bone formation using a rat model of MDO; the stromal cell-derived factor-1/chemokine receptor-4 pathway activation was found to promote migration of MSCs to the distraction site[89]. However, these studies were not able to determine contribution efficiency of the recruited MSCs to the distraction regenerate.

Non-invasive therapies that aid in bone formation during MDO include low-level laser (LLL) therapy and low-intensity pulsed ultrasound (LIPUS). LLL therapy consists of an 800 nm wavelength range daily laser treatments during distraction and/or consolidation showing improved bone regeneration in rabbits[90]. How LLL promotes bone formation from a mechanistic standpoint, however, remains poorly understood. Compared to LLL therapy, there is more insight into the cellular mechanism of LIPUS in promoting of bone formation. The technology utilizes low intensity and pulsed mechanical waves in order to induce regenerative and anti-inflammatory effect on bone, cartilage, and tendon[91]. The mechanism by which LIPUS induces regenerative effects is unknown; however, one theory is the non-thermal phenomena, where the mechanical waves cause changes in cellular physiology[92–94]. As such, FAK, which was shown to be upregulated in mSSCs during mouse MDO, may have an important role as mechanical signals are transduced into cellular signals[10].

ADJUVANT THERAPIES FOR LIMB DISTRACTION OSTEOGENESIS

Just as MDO may be used to address a wide range of underlying pathology, limb distraction osteogenesis (LDO) is applied to various bone and soft tissue deficits of the appendicular skeleton. These include limb length discrepancies, oncologic resection, traumatic deformity correction, and treatment of ankle osteoarthritis[95–98]. Clinical procedures for LDO are similar to MDO, and preoperative imaging and planning are the first steps. Careful evaluation of bone vascular health and surrounding soft tissue is important for LDO[99]. Postoperative protocols also mirror MDO distraction strategies, with 5–7 days of latency and

subsequent distraction carried out at 0.75–1.0 mm per day, paired with ongoing physical therapy into the consolidation phase[100,101]. The complication rate of appendicular distraction appears to be higher than mandibular distraction. The most frequent complications are frame-related followed by nonunion[99]. The data are heterogenous, with some retrospective studies noting total number of complications exceeding the number of patients enrolled in the study[102,103].

Similar to MDO, bone marrow MSCs have been studied to determine their therapeutic role in reducing the treatment time of LDO. One rabbit study utilized autogenous bone marrow MSCs from the tibia, with transplantation of one million cells into the distraction gap after four to six *ex vivo* passages[104]. Another study in dogs used allogenic bone marrow MSCs from the tibia and transplanted one million cells into the distraction gap after three passages[105]. While MSC administration led to faster bone formation, heterogenous cell culture and administration methodology precludes clinical application at this time.

LIPUS has also been investigated in the setting of tibial DO. In the study, twenty minutes of therapy at a frequency of 1.5 MHz and impulse length of 200 μsec daily throughout the distraction period demonstrated an increased radiologic callus density by 33%[106]. Another study applied the same parameters of LIPUS therapy during both distraction and consolidation, which led to faster healing[107]. While the use of LIPUS as a potential adjuvant therapy in LDO is promising, quantification of bone formation is inconsistent impairing direct comparisons.

Overall, data surrounding the use of adjuvant therapies to augment LDO are not robust, and many strategies are extrapolated from their use in MDO. For example, LLL therapy has not been studied in the context of LDO. However, given its promising results in MDO, LLL therapy holds potential to enhance bone formation during LDO. Overall, understanding the biological basis of DO will continue to illuminate potential translational therapies to improve bone regeneration and thus clinical outcomes. Furthermore, interpreting these strategies from the perspective of resident SSCs in long bone, from which they were first described, will be key for appreciating how these approaches may be clinically translated.

CONCLUSION

Although progress has been achieved in skeletal biology research, the fundamental understanding of regulatory mechanisms governing bone development and regeneration remain elusive. Studies have uncovered the morphogens and transcription factors necessary for skeletal growth; however, the role of these factors in bone regeneration has yet to be been determined. Discovery of the SSC has improved our understanding of the cellular underpinnings of bone regeneration, highlighting that recapitulation of developmental processes in various postnatal processes. Harnessing these cells as therapeutic targets may prove a powerful tool in addressing the clinical challenges of both MDO and LDO, as well as other skeletal disorders. Further research should seek to better understand the molecular biology of SSCs, along with their precise behavior during tissue production, maintenance, and repair. Developmental mapping of skeletal tissue, including characterization of cellular, molecular, and genetic patterns giving rise to craniofacial and long bones are crucial in

understanding bone regenerative processes and the role of adjuvant therapies during treatment. Current data examining adjuvant therapies to enhance bone formation during DO are preliminary; continued work to determine the biological basis of DO will inform the development of innovative surgical techniques and adjunctive treatments. Furthermore, the biological understanding of MDO is more advanced than LDO in regard to development, regeneration, and adjuvant therapy outcomes, which presents a key opportunity to progress the scientific knowledge surrounding LDO. Advances in our grasp of skeletal regenerative and developmental biology hold potential for translation of clinical interventions to provide patients with improved solutions for skeletal defects and injury.

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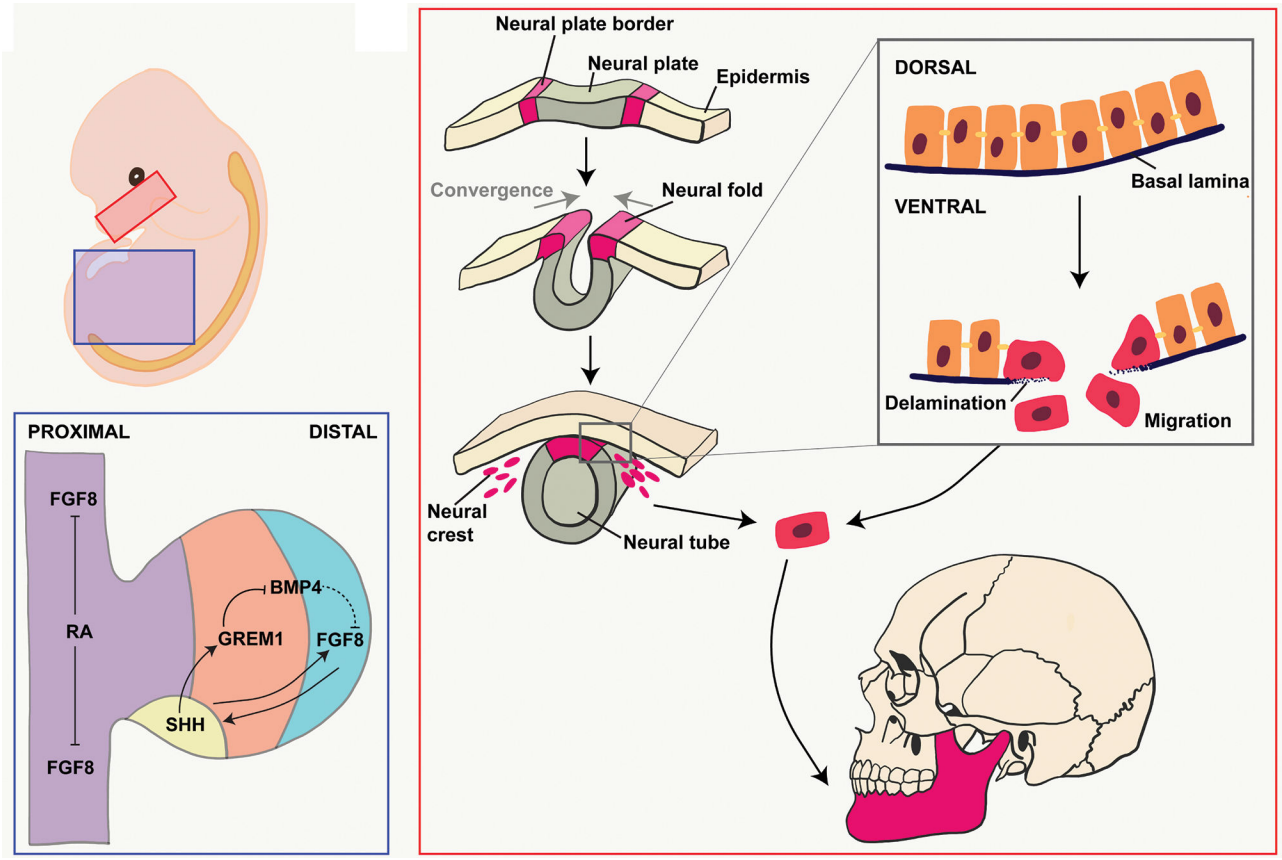

Figure 1. Embryonic View of Bone Development.

(A) Developing mandible (red box) and lower limb (blue box). **(B)** Endothelial-to-mesenchymal transition of cranial neural crest cells. **(C)** Signaling throughout the limb bud trunk (purple), zone of polarizing activity (yellow), progress zone (orange), and apical endodermal ridge (blue).

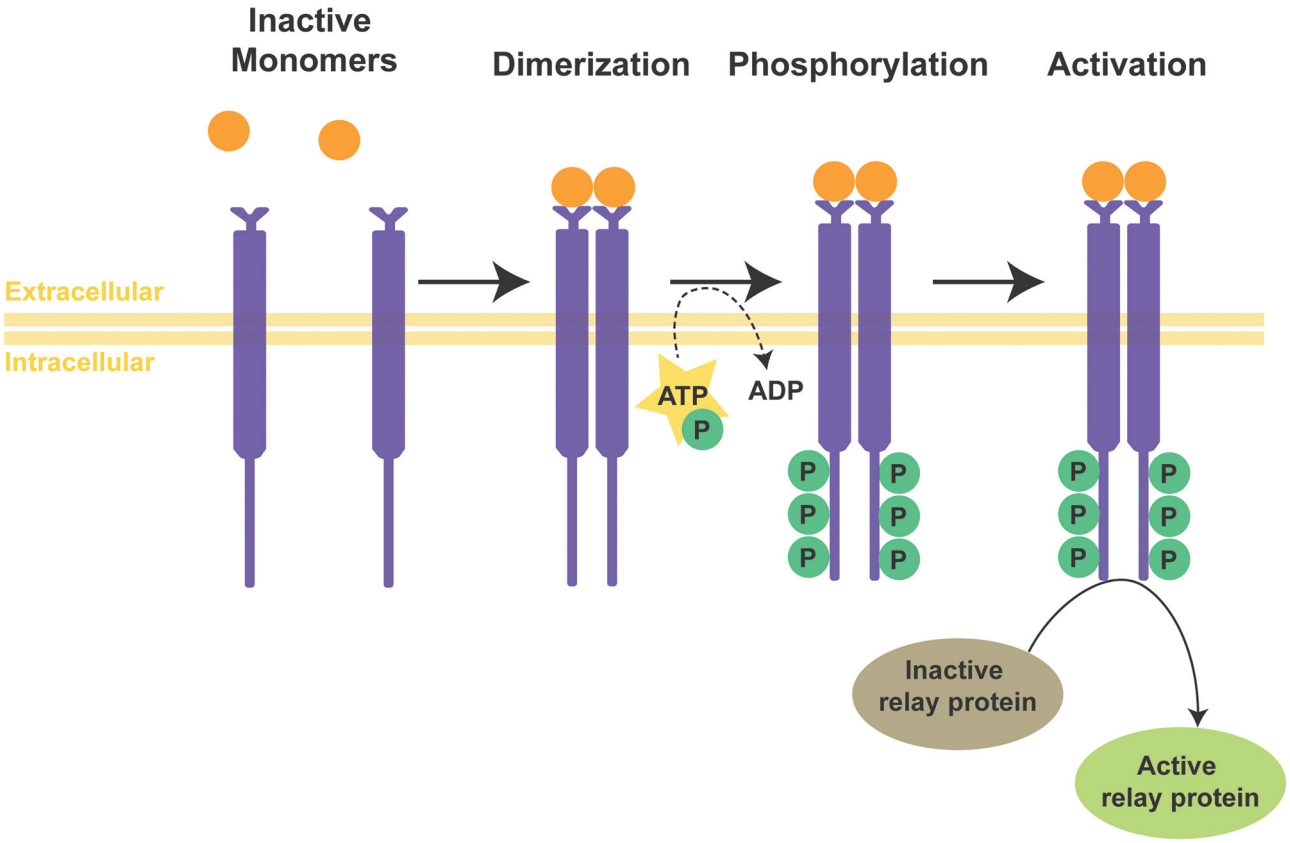

Figure 2. Tyrosine Kinase Pathway.

The single pass, type I receptor tyrosine kinase resides in the plasma membrane. The receptor tyrosine kinase is activated through the binding of a ligand leading to a ligand-induced dimerization with the cytoplasmic tyrosine kinase domain. The dimerization results in autophosphorylation of the tyrosine residues inducing conformational changes which stabilize the active site of the kinase. The phosphotyrosine residues act as recruitment sites for downstream signaling proteins.

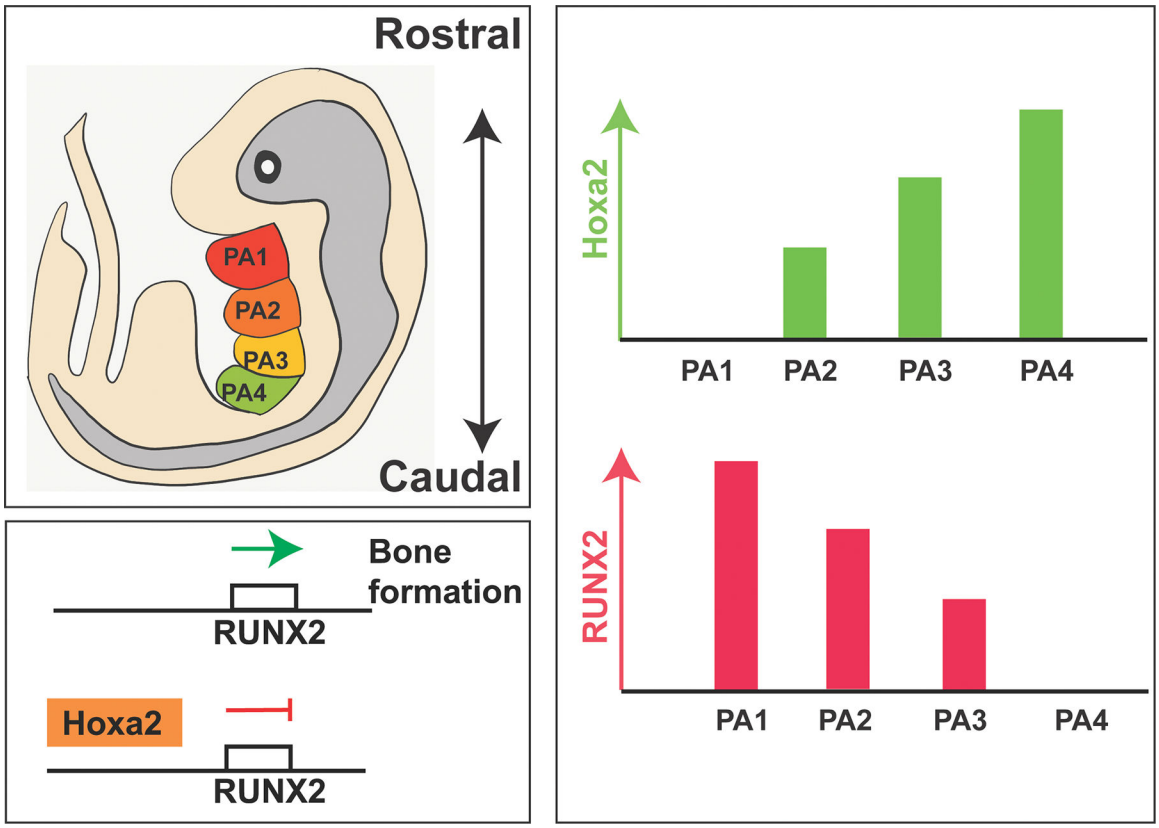

Figure 3. Hoxa2/Runx2 Pathway.

(A) Mouse embryo with developing pharyngeal arches (PA). PA1 (red) gives rise to the muscles of mastication and mandible. PA2 (orange) gives rise to the muscles of facial expression and hyoid bone. PA3 (yellow) gives rise to the greater horn and lower body of the hyoid bone. PA4 (green) gives rise to the thyroid and cricoid cartilage. **(B)** In the absence of Hoxa2, Runx2 activation will occur leading to bone formation. In the presence of Hoxa2, Runx2 will be suppressed limiting bone formation. **(C)** The expression of Hoxa2 increases in the caudal direction of the pharyngeal arches with PA1 not having expression of Hoxa2, while PA4 possesses a high level of Hoxa2 expression. Analogously, the expression of Runx2 decreases in the caudal direction of the pharyngeal arches with PA1 having the greatest expression of Runx2, while PA4 possesses a low level of Runx2 expression.

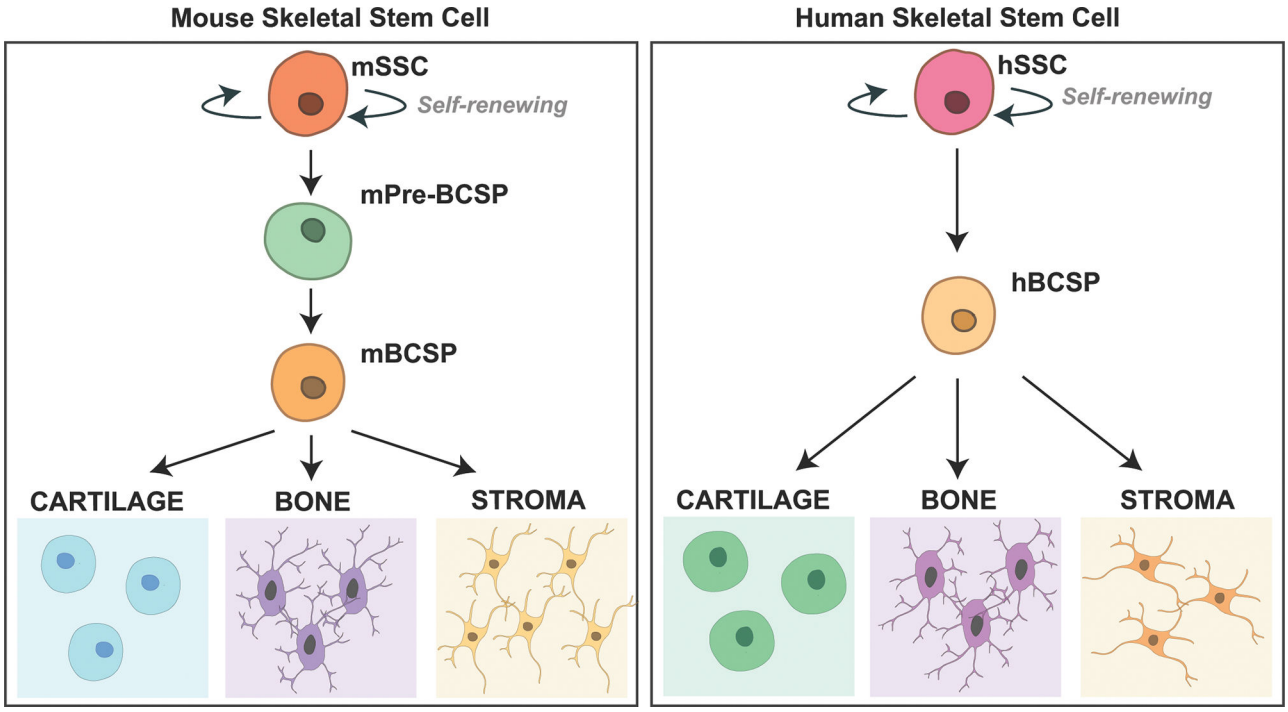

Figure 4. Skeletal Stem Cell Hierarchy.

(A) The skeletal stem cell hierarchy in mice beginning with a self-renewing mouse Skeletal Stem Cell (mSSC) differentiating to lineage-restricted bone, cartilage, and stromal cells through a Bone, Cartilage, Stromal Progenitor (BCSP) cell[8]. **(B)** The skeletal stem cell hierarchy in humans beginning with a self-renewing human Skeletal Stem Cell (hSSC) differentiating to lineage-restricted bone, cartilage, and stromal cells through a Bone, Cartilage, Stromal Progenitor (BCSP) cell[9].

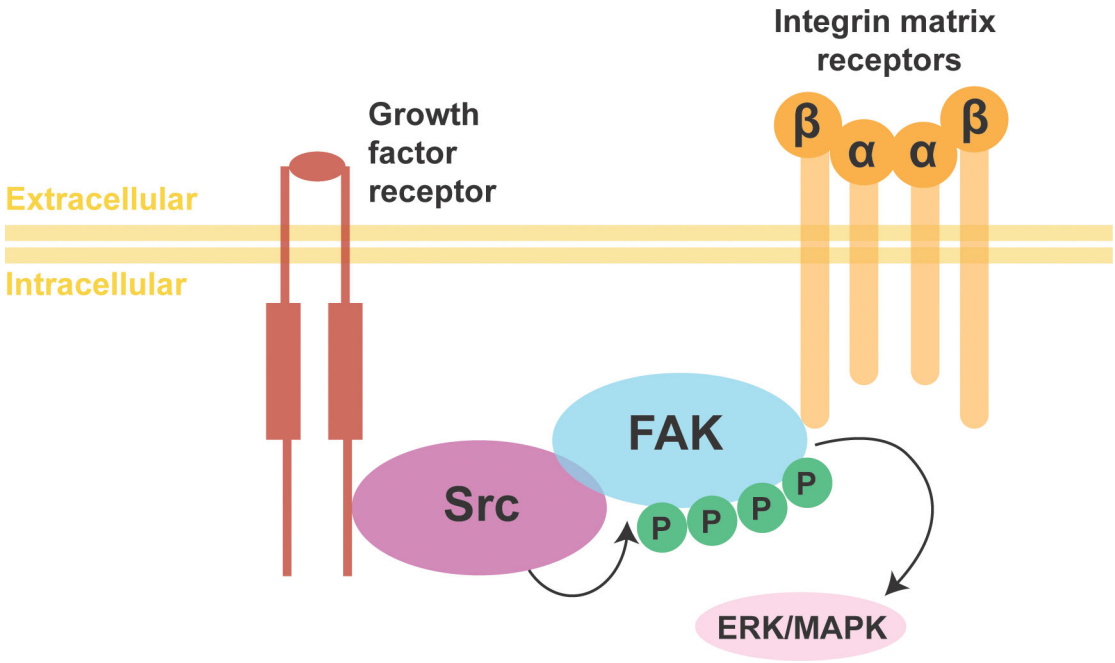

Figure 5. Focal Adhesion Kinase Signaling Pathway.

Cytoplasmic tyrosine kinase FAK becomes activated after interacting with transmembrane integrin proteins allowing FAK to form a complex with Src family kinase. The complex initiates downstream signaling pathways through the phosphorylation of other proteins such as ERK/MAPK.

Table 1.

Table of abbreviations used throughout the review with adjoining definitions.

Abbreviation	Full Name	Definition
DO	distraction osteogenesis	the process of lengthening bone through endogenous tissue engineering using a guided mechanical environment
SSC	skeletal stem cell	reside in the postnatal bone marrow and give rise to cartilage, bone, hematopoiesis-supportive stroma
NCC	neural crest cell	temporary group of cells arising from the embryonic ectoderm cell layer, and giving rise to a diverse cell lineage
BMP	bone morphogenic protein	growth factors consisting of pivotal morphogenetic signals determining tissue architecture throughout the body
AER	apical ectodermal ridge	structure that forms at the distal end of the limb bud acting as a major signaling center to ensure proper development of a limb
ZPA	zone of polarizing activity	area of the developing limb that contains signals which instruct the developing limb bud to form along the anterior/posterior axis
RA	retinoic acid	a metabolite for vitamin A_1 that is required for growth and development
FGF	fibroblast growth factor	Cell signaling proteins that are crucial in normal development
mSSC	mouse skeletal stem cell	skeletal stem cells isolated from mouse bones
MDO	mandibular distraction osteogenesis	a surgical procedure that lengthens the lower jaw
FAK	focal adhesion kinase	protein tyrosine kinase concentrated in focal adhesions forming amongst cells attaching to extracellular matrix
DFO	deferoxamine	a medication that binds iron and aluminum
HIF-1α	hypoxia inducible factor 1-α	transcription factor that respond to hypoxia
rhBMP-2	recombinant human BMP-2	BMPs generated using recombinant DNA technology for clinical use
MSC	mesenchymal stem cell	multipotent stromal cells that can differentiate into a variety of cell types
LLL	low-level laser	application of red and near infra-red light over injuries or lesions to improve wound and soft tissue healing and reduce inflammation
LIPUS	low-intensity pulsed ultrasound	low intensity and pulsed mechanical waves used to induce regenerative and anti-inflammatory effects on bone, cartilage, and tendon
LDO	limb distraction osteogenesis	A surgical procedure that lengthens long bones of the appendicular skeleton including the tibia and femur