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Convergence of Calcium Channel Regulation and Mechanotransduction in Skeletal Regenerative Biomaterial Design

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Abstract

Cells are known to perceive their microenvironment through extracellular and intracellular mechanical signals. Upon sensing mechanical stimuli, cells can initiate various downstream signaling pathways that are vital to regulating proliferation, growth, and homeostasis. One such physiologic activity modulated by mechanical stimuli is osteogenic differentiation. The process of osteogenic mechanotransduction is regulated by numerous calcium ion channels—including channels coupled to cilia, mechanosensitive and voltage-sensitive channels, and channels associated with the endoplasmic reticulum. Evidence suggests these channels are implicated in osteogenic pathways such as the YAP/TAZ and canonical Wnt pathways. This review aims to describe the involvement of calcium channels in regulating osteogenic differentiation in response to mechanical loading and characterize the fashion in which those channels directly or indirectly mediate this process. The mechanotransduction pathway is a promising target for the development of regenerative materials for clinical applications due to its independence from exogenous growth factor supplementation. As such, also described are examples of osteogenic biomaterial strategies that involve the discussed calcium ion channels, calcium-dependent cellular structures, or calcium ion-regulating cellular features. Understanding the distinct ways calcium channels and signaling regulate these processes may uncover potential targets for advancing biomaterials with regenerative osteogenic capabilities.

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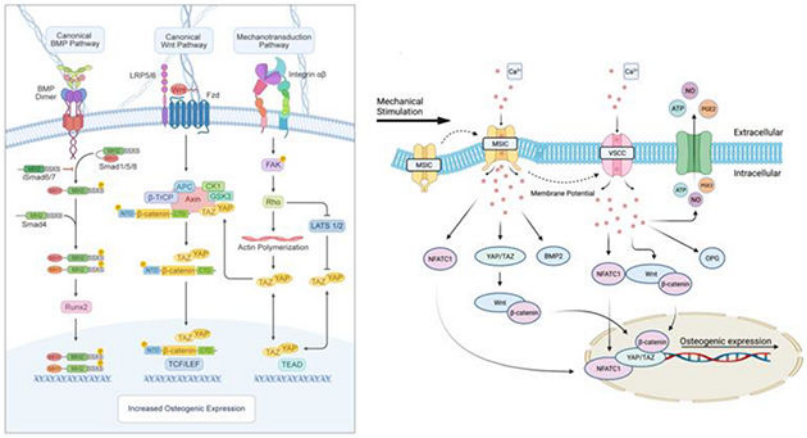
Graphical Abstract



This work summarizes notable calcium channels that regulate mechanotransduction signaling pathways and their relevance in skeletal biomaterial design. The various calcium channels, structures, and associated pathways that are relevant to osteogenic differentiation and regeneration are thus discussed in this review. Ultimately, the purpose of this work is to inform the next generation of biomaterial development through calcium centered strategies.

Keywords

Calcium Channels; Cellular Mechanotransduction; Biocompatible Materials; Osteogenesis; Bone Regeneration

1. Introduction

In recent years, utilization of the mechanotransduction pathway has garnered increasing attention in biomaterial design, especially as it pertains to stimulating skeletal regeneration for clinical applications.[1, 2] Our previous work has summarized examples of biomaterial strategies employed in order to engineer microenvironments capable of activating the individual mechanical signaling pathways that underly osteogenesis.[3] Some of the materials utilized include substrates composed of collagen, polydimethylsiloxane (PDMS), hydrogels, inorganic ions, glass coverslips, biodegradable patches, and porous scaffolding. Materials are often modified with special coatings such as fibronectin,[4] or adhesive peptides such as arginine-glycine-aspartate (RGD)[5] in order to promote regenerative potential. The variety of material modifications and combinations allows for unique, tunable variations in construct stiffness, geometric composition, ligand functionalization, and elasticity. Each of these material properties influences osteogenic growth and repair in a distinct fashion. By coordinating osteogenic gene expression, cellular proliferation, and differentiation, calcium ion (Ca^{2+}) signaling is critical to driving these changes. Further, Ca^{2+} ions are known to play a significant role in bone matrix mineralization by interacting with PO_4^{3-} to form hydroxyapatite crystals within osteoblast matrix vesicles. Subsequent propagation of hydroxyapatite along collagen fibrils promotes matrix mineralization, thus transforming immature osteoid bone into novel bone formation (Figure 1).[6] Due to the

diverse roles Ca^{2+} ions play in regulating cellular activity and bone mineralization, an understanding of the Ca^{2+} channels that govern mechanotransduction are relevant to the development of bone regenerative constructs.

The intricate process by which cells transduce mechanical stimuli into osteogenic responses has been elucidated through prior work by Dupont et al. and others.[7–9] Examples of mechanical stimuli utilized by osteocytes to sense their microenvironment include forces derived from extracellular matrix (ECM) stiffness, cell-to-cell adhesion, cytoskeletal tension, and fluid shear stress from canalicular networks. In response to these forces, key pathways are triggered that mediate osteogenesis; namely, the Yes-associated protein/transcriptional coactivator with PDZ-binding domain (YAP/TAZ), Canonical BMP, and Wnt/β-catenin pathways (Figure 2). [7, 10]

We review the Ca^{2+} channels intricately connected to these pathways in the context of osteogenic differentiation and regeneration. Some Ca^{2+} channels are coupled to fundamental organelles, such as cilia, or facilitate cell-to-cell interactions, like gap and adherens junctions.[11] Other types of Ca^{2+} channels include mechanosensitive ion channels (MSIC) and voltage-sensitive calcium channels (VSCC), which localize to the plasma membrane.[12] Ca^{2+} channels also coordinate intracellular processes, such as endoplasmic reticulum (ER) activity.[13] This review aims to describe the relevant Ca^{2+} channels and Ca^{2+} channel-linked cellular structures associated with mechanotransduction and transcription of osteogenic genes. Also discussed are biomaterial-informed strategies that target or involve the specified Ca^{2+} channels and Ca^{2+}-coupled features in the regulation of those processes.

2. Calcium Channels and Mechanotransduction

2.1 Cilia

Cilia can be classified as either motile or primary.[14] Motile cilia are confined to specific populations of epithelial cells, such as those in the airway, digestive tract, middle ear, and ovaries. They present in large numbers at the cell membrane and beat in a coordinated fashion that often serves to circulate extracellular material. In the lungs, motile cilia help clear mucus and debris, while in the gut, they serve to regulate bile flow. In contrast, primary cilia are nonmotile and singular structures that typically possess sensory functions and coordinate the activation of different cellular mechanisms. Primary cilia can be associated with Ca^{2+} channels such as Transient Receptor Potential subfamily V member 4 (TRPV4),[15–17] and have been reported to regulate elements such as the canonical hedgehog pathway, G protein-coupled receptors, Wnt, tyrosine kinase receptors, and transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP) signaling. [18] Several studies have demonstrated the role of primary cilia in mechanically-induced mineralization, maturation, and osteoblastic differentiation (Figure 3). Li et al. demonstrated that subjecting MC3T3-E1 osteoblasts to chloral hydrate treatment—a well-characterized process of chemically removing primary cilia—results in inhibition of osteoblast survival and osteogenesis, as demonstrated by decreased expression of bone matrix markers type 1 collagen (COL1A1), osteopontin (OPN), and osteocalcin (OCN).[19] Decreases in alkaline phosphatase (ALP) activity, osteoblastic differentiation, and mineralization were also observed, thus demonstrating the regulatory nature of primary cilia in bone development.

Additionally, subjecting MLO-A5 murine osteoblasts to fluid shear stress has been shown to induce prostaglandin E2 (PGE_2) release, which promotes bone formation, as well as mineral deposition.[20] On the contrary, damage to or removal of primary cilia via chloral hydrate can inhibit PGE_2 release and osseous mineralization.[21]

Pertaining to material approaches, McMurray et al. demonstrated the use of surface topography to modulate primary cilia activity and structure, and in turn influence Wnt signaling.[22] Compared to mesenchymal stem cells (MSC) cultured on flat surfaces, those cultured on microgrooved surfaces underwent primary cilia elongation that resulted in negative regulation of Wnt signaling; this was demonstrated by a weaker response to Wnt3a, accompanied by reduced β-catenin and Axin-2 gene expression, giving way to reduced proliferation. Similarly utilizing surface topography to modulate primary cilia length, Zhang et al. showed that MSCs cultured *in vitro* on tricalcium PO_4^{3-} ceramic disks with submicron-scale topography—in contrast to micron-scale topography—displayed increased primary cilia length and TGFβ recruitment, resulting in enhanced osteogenic differentiation indicated by upregulation of ALP and osteopontin (OPN).[23] Among scaffolds harvested following 12 weeks of implantation within the para-spinal muscles of a canine model, *in vivo* bone formation was observed within scaffolds featuring submicron, but not micron topography.

Specific structural components of cilia also suggest targets for modulating mechanotransduction. For example, TRPV4 —a mechanosensitive calcium channel—plays a critical role in MSC mechanotransduction and is a member of the transient receptor potential (TRP) channel family. Corrigan et al. concluded that TRPV4 localizes to cellular areas that experience high strain under fluid shear, especially at the primary cilium.[16] The authors showed that TRPV4 protein expression is significantly increased with oscillatory fluid shear and mediates calcium deposition in response to flow. Correspondingly, this study also demonstrated that the TRPV-specific antagonist GSK205 downregulates mechanoinduced intracellular calcium signaling and impairs osteogenic gene expression of cyclooxygenase 2 (Cox2) and OPN following oscillatory fluid flow (OFF). Moreover, activation of TRPV4 with the small molecule agonist GSK101 leads to increased expression of Cox2 and OPN, suggesting that GSK101 can mimic the anabolic effect of mechanical loading while highlighting the role of TRPV4 in mechanotransduction.[16]

In addition to modulating TRPV4 with chemical agents, local material architectures may also be employed. For example, Hou et al. demonstrated that culturing MSCs on titanium surfaces with nano- to micro-sized surface topographies—in comparison with polished titanium surfaces—promoted enhanced TRPV4 expression and activation, driving an increase in intracellular Ca^{2+}.[24] This upregulation was suggested to ultimately result in increased osteoblastic gene expression and MSC differentiation by driving Wnt/β-catenin signaling and nuclear translocation of nuclear factor of activated T cells 1 (NFATc1). The authors further postulated that the observed increase in Ca^{2+} influx afforded by upregulated TRPV4 activation is secondary to surface topography-induced changes to primary cilia and the cytoskeleton. Collectively, these findings suggest that cilia and TRPV4 play a central role in mediating mechanotransduction in MSCs by regulating calcium deposition and osteogenic gene expression.

Other *in vivo* and *in vitro* studies also implicate TRPV4 as a major regulator of mechanotransduction and bone formation. In mice models, knockout of Kif3a—a subunit of the kinesin II intraflagellar transport protein required for primary cilia formation—resulted in decreased formation of new bone in response to mechanical loading.[25] Likewise, *in vitro* siRNA knockdown of TRPV4 or treatment with chloral hydrate in osteocyte-like MLO-Y4 cells exposed to OFF resulted in blocked Cox2 mRNA expression and reduced ratios of osteoprotegerin to receptor activator of nuclear factor NF-κB ligand (OPG/RANKL), additionally suggesting that TRPV4 may be important in both anabolic and catabolic processes in bone homeostasis.[26, 27] The authors also performed siRNA knockdown of other Ca^{2+}-permeable channels that localize to the primary cilium, including polycystin-2 (PC2 or PKD2) and Piezo1, which are discussed further in subsection 2.7. siRNA-mediated knockdown of TRPV4—but not PC2 or Piezo1—negatively altered flow-induced ciliary Ca^{2+} increases and load-induced Cox2 mRNA increases, suggesting that TRPV4 is a major Ca^{2+} channel involved in cilia-mediated mechanotransduction. It should be noted that TRPV4 knockdown demonstrated a reduction in flow-induced Ca^{2+} movement but did not impair cytosolic Ca^{2+} peaks, implying that different mechanisms may serve to maintain normal cytosolic Ca^{2+} levels. Indeed, knockdown of polaris—an intraflagellar transport component required for primary cilium biogenesis—in MC3T3-E1 osteoblasts subjected to flow retained Ca^{2+} flux, which persisted even after inhibition of stretch-sensitive ion channels or chelation of Ca^{2+}. These observations suggest the existence of Ca^{2+}-independent osteogenic responses.[27] Tummala et al. additionally showed that polaris knockdown reduces mRNA expression of the transcription factors Runx2 and Sox9, which regulate bone formation and chondrogenic differentiation, respectively. mRNA expression of proliferator-activated receptor γ (PPARγ)—a nuclear hormone receptor induced in adipocytes—was also reduced.[28] This emphasizes that primary cilia are integral components in mediating multiple tissue differentiation pathways beyond osteogenic fates.

While cilia and TRPV4 have been implicated in the regulation of bone mineralization, calcium signaling, and osteogenic differentiation, the specific downstream targets of this cilia-TRPV4 axis remain unclear. Two potential downstream targets that have been proposed include the ERK and β-catenin pathways.[29] This perspective is based on the notion that cilia-mediated Ca^{2+} influx inhibits the activity of adenylyl cyclase 6, a cAMP producer localized to the ciliary microdomain,[30, 31] and therefore reduces intracellular cAMP levels.[30, 32] This in turn reduces activation of protein kinase A (PKA), a family of enzymes with cAMP dependency which has been shown to localize to the primary cilium.[33] PKA has been demonstrated to inhibit GSK-3β-mediated degradation of β-catenin, a transcription factor associated with osteoblastic differentiation.[34] Therefore, Ca^{2+} influx via TRPV4 may prevent the inhibition of β-catenin degradation and promote osteogenic expression. However, the link between primary cilia and the β-catenin pathway remains the subject of scientific discourse, as data exists both in support of,[35] and against this relationship.[36]

2.2 Polycystins

Polycystins are a class of proteins with mechanosensory properties,[15, 37, 38] and make up their own subfamily (TRPP) within the larger TRP channel family (Figure 3). The TRPP family is further subdivided into two groups: polycystic kidney disease 1 (PKD1 or TRPP1),

and polycystic kidney disease 2 (PKD2 or TRPP2).[39] These proteins localize to cilia,[40] the plasma membrane as part of ion channel complexes,[41] cell junctions,[42] the ECM,[43] and the ER.[44] Furthermore, both PKD1 and PKD2 are involved in intracellular calcium signaling and entry.[37, 44, 45] Prior work has shown that PKD1 and PKD2 complex with each other,[46, 47] and that this interaction is required for PKD2 to function as an ion channel within the plasma membrane.[46] Of note, it is not yet clear whether PKD1 also functions as an ion channel or whether it acts solely as a chaperone for PKD2.

Xiao et al. showed that polycystins interact with TAZ and can mediate osteoblastogenesis and Runx2 expression via a calcium-dependent mechanism.[48, 49] Specifically, the authors found that increasing ECM stiffness and applying mechanical stretch to multipotent mesenchymal stem cells stimulated the nuclear translocation of the PKD1/TAZ complex, leading to increased osteogenic Runx2 expression. Correspondingly, polycystin deficiencies have been shown to result in impaired bone mineral density and cortical thickness, ultimately causing osteopenia.[50] Notably, polycystin-regulated osteoblastogenesis is itself regulated through an interaction between polycystins and the transcriptional coactivator, TAZ. [48, 51] TAZ is a major protein of the Hippo pathway—involved in the regulation of cell growth, proliferation, and apoptosis—[52] and is also regulated by mechanical stimuli to induce osteoblastogenesis as part of the YAP/TAZ mechanotransduction pathway.[7, 42, 53] Thus, via interactions with TAZ and Runx2 expression, polycystins play important roles in mechanically-mediated bone formation and MSC differentiation.

Direct activation or inhibition of polycystin activity may be of interest to the biomaterials field, specifically in regard to the design of bone regenerative materials. Sibilla et al. showed that the addition of powdered Biostite—a biomaterial composed of chondroitin sulfate, synthetic hydroxyapatite, and type I collagen—to 72 hour *in vitro* cultures of osteoblast-like cell lines MG-63 and SaOS-2 led to a three-fold increase in intracellular levels of PKD2, and a significant reduction in cell proliferation, compared to cells cultured without Biostite. [54] RT-PCR analysis revealed elevated expression of ALP and osteonectin among Biostite-treated cells, and the authors suggested that the observed osteoblastic differentiation could be attributed in part to PKD2-mediated mechanosensory mechanisms.

2.3 Gap Junctions and Hemichannels

Connnexin43 (Cx43) is a protein that composes osteocytic gap junctions[55] and hemichannels (Figure 3).[56] Cx43 is additionally known to regulate bone matrix production *in vitro*[57] and bone formation *in vivo,*[58] as well as coordinate release of the osteogenic-promoting factor, PGE_2, in response to mechanical strain.[23][59] Furthermore, Cx43 mediates mechanotransduction via intercellular Ca^{2+} signaling. Jørgensen et al. demonstrated that osteoblastic rat cell lines require gap junctional communication for transmission of slow intercellular calcium waves incited by a micromanipulator.[60] With administration of α-glycyrrhetinic acid—a gap junction inhibitor—the spread of intercellular calcium signals was blocked as measured by a fluorescent calcium indicator, fura-2. In the absence of this inhibitor, stimulation of a single osteoblast via micromanipulator caused a rise in intracellular Ca^{2+} that was transmitted to neighboring cells. Further, nifedipine, an L-type voltage-gated calcium channel inhibitor, blocked Ca^{2+}

propagation across the osteoblastic cells suggesting that the gap junction-dependent signal spreading relies on influx of extracellular Ca^{2+} through plasma membrane channels.[60] D'Andrea and colleagues demonstrated similar findings in articular chondrocytes subjected to mechanical stimulation, supporting the relationship between mechanical stimulation and gap junction-regulated Ca^{2+} signaling.[61] Collectively, these findings suggest that mechanically-stimulated expression of Cx43 allows for increased intracellular Ca^{2+} levels through cell-to-cell and extracellular-to-cell spread. However, regulation of intracellular Ca^{2+} gap junction signaling is potentially subject to other regulatory pathways. Indeed, Saunders et al. revealed that intracellular Ca^{2+} concentrations in both gap-junction deficient and control MC3T3-E1 osteoblastic cells increased following stimulation by OFF, and that the degree of change was not significantly different between treatment groups.[62] This work suggested that gap junction mediated Ca^{2+} signaling may operate independently from other signal transduction elements.

Gap junctions such as Cx43 may be targets for material-directed modulation of MSC activity. By co-culturing hMSCs and human umbilical endothelial cells on substrate surfaces modified via lithography to feature bone tissue-inspired nanopatterns, Kim et al. showed increased osteogenesis—as indicated by the observation of mineralization and immunostaining of OCN—relative to co-cultures on flat substrates.[63] A maximal increase in osteogenesis from baseline measurements represented by the flat substrate was observed for grooved surfaces with a 1:3 ratio of ridge to groove width. Expression levels of Cx43 were measured in order to investigate cell-cell interactions and were found to be significantly higher among cells cultured on nanopatterned substrates relative to flat substrates. The authors suggest that this observed increase in Cx43—in addition to observed increases in BMP-2 secretion, nuclei elongation, and average focal adhesion size—enhanced hMSC osteogenesis.[63] Zhao et al. similarly demonstrated the influence of substrate patterning on Cx43 activity by engineering hydroxyapatite-based ceramics featuring hybrid surface structures composed of nanorods and micropatterns. [64] RT-qPCR and immunostaining detected significantly increased Cx43 levels among human bone marrow stromal cells (hBMSCs) cultured on surfaces featuring micro/nano-hybrid structures compared to flat surfaces. This enhanced cell-cell communication via Cx43 was essential to the overall increased osteogenic differentiation, proliferation, and adhesion measured among hBMSCs cultured on the patterned surfaces compared to non-patterned surfaces.

2.4 Integrins

Integrins are trans-membrane proteins that facilitate interactions between the ECM and cytoskeleton (Figure 3), regulating activities including proliferation, migration, and death. [65] Additionally, integrins play an important role in many signal transduction pathways, [66] including mechanotransduction.[67] They are comprised of an α and β dimer, which undergo conformational changes in response to mechanical stimuli.[5] Upon changing conformation, integrins are thought to activate the transcriptional co-activators YAP/TAZ,[68] in conjunction with the focal adhesion kinase (FAK), Src, phosphoinositide 3-kinase, and c-Jun N-terminal kinase (JNK) pathways.[69, 70] In support of this, recent work demonstrated that integrin-Src signaling is vital for nuclear localization of YAP/TAZ,[68, 70] and is therefore critical for downstream bone development.[71] Accordingly, numerous studies

have implicated integrins as an integral component of mechanically-mediated osteogenic differentiation.[72]

In addition to playing a role in YAP/TAZ activation, integrins are also suggested to be critical in intracellular Ca^{2+} regulation. For instance, integrin binding to fibronectin in fibroblasts induces phosphatidylinositol biphosphate synthesis and subsequent hydrolysis to inositol triphosphate (IP_3), which is vital for increasing intracellular Ca^{2+} via ER release of Ca^{2+}.[73] Moreover, engagement of integrin $\alpha_5\beta_1$ with fibronectin has been shown to enhance plasma membrane Ca^{2+} channel current,[74] indicating that integrins can modulate Ca^{2+} signaling through both intracellular and extracellular sources. Not only do integrins influence Ca^{2+}, but they also respond to Ca^{2+}. Specifically, Xiang et al. found that an extracellular Ca^{2+} of 0.5 mM promotes osteoclast migration and adhesion; a finding that was suggested to be partially explained cytoskeleton organization via integrin $\alpha_v\beta_3$ activity. [75] Additionally, osteoclast migration and adhesion was found to be less efficient in an extracellular Ca^{2+} concentration of 1.2 mM, although YAP/TAZ activity was increased at this elevated concentration.

In the design of bone regenerative biomaterial constructs, stiffness is one target parameter capable of controlling integrin activity. Prior reviews have discussed the static, dynamic, and biophysical properties of ECM-based biomaterials and their influences on various biochemical processes including cell adhesion, spreading, migration, growth, and differentiation. In brief, material stiffness (defined as the resistance of an object to deformation or material rigidity), activates integrin protein clusters with subsequent biochemical signaling that enhances phosphorylation of FAK. Downstream of this event, nuclear transcription factors YAP/TAZ concentrate in the nucleus and mediate the cellular mechanoresponse.[2, 76] Du et al. demonstrated that matrix stiffness can be tuned in order to modulate integrin-regulated Wnt1 expression.[77] By increasing the Young's modulus of a collagen-coated polyacrylamide matrix upon which bone marrow mesenchymal stem cells (BMMSCs) were cultured, integrin activation was correspondingly increased, leading to enhanced expression of members of the Wnt/β-catenin pathway. The resulting accumulation of β-catenin was demonstrated to increase Wnt1 expression, and therefore establish a positive feedback loop. Compared to a soft matrix (0.5–1 kPa), the stiff matrix (100 kPa) led to increased integrin-activated β-catenin/Wnt pathway activity, and ultimately drove osteogenic lineage specification. [77] In addition to matrix stiffness, nanotopographical features are another design consideration for integrin control. For example, Bello et al. demonstrated increased focal adhesion length and maturity, and adhesion per cell area among MC3T3-E1 cells cultured over 3 days on nanoporous titanium surfaces compared to smooth titanium surfaces. [78, 79] Further, cells on the nanoporous substrate demonstrated significantly greater expression of focal adhesion markers, including various integrins and paxillin, and were determined by scanning electron microscope (SEM) to feature a greater abundance of filopodia. By achieving a nanoporous surface featuring pore diameters of 20 ± 5 nm, the authors increased focal adhesions and in turn improved osteogenic cell proliferation and expression, suggesting a novel modality for guiding tissue regeneration and integration of implants.[78]

2.5 Cadherins

Cadherins are adhesive proteins that exist at intercellular junctions and are essential for tissue homeostasis and cell-to-cell adhesion (Figure 3). Despite not having a directly associated Ca^{2+} channel, the cadherins still play a role in mediating mechanotransduction and have indirect influences on downstream Ca^{2+} signaling. Cadherins can be categorized into two subtypes: E- and N-cadherin. During embryonic development of vertebrates, cells of the ectoderm express E-cadherin, allowing an epithelial cell layer to form as cells adhere together.[80] Cells can switch from E-cadherin to N-cadherin expression, which has different adhesive properties. N-cadherin-expressing cells then form a new cell layer which forms the embryonic neural tube. These transmembrane molecules are calcium-dependent and play an important role in solid tissue formation. [81] Indeed, *in vitro* experiments show that cadherin expression can induce cell aggregation in the presence of Ca^{2+}.[82]

Furthermore, cadherins have been found to interact with cytoplasmic proteins called catenins.[83] The catenins, α-catenin, β-catenin, and γ-catenin, are the three major cytoplasmic partners of E-cadherin. Cadherin-catenin complexes are essential for coordinating cytoskeleton reorganization and cell-to-cell contact,[84] and therefore contribute to the process of tissue remodeling. β-catenin has been shown to be a transcriptional coactivator of the Wnt signaling pathway[85] and a regulator of osteoblastic cell differentiation and proliferation in response to mechanotransduction.[86] When subjected to mechanical strain, β-catenin and E-cadherin interactions are disrupted, causing a dissociation of the complex and subsequent translocation of β-catenin into the nucleus. Other studies have demonstrated that calcium is critical to increasing β-catenin stability, enhancing β-catenin transcription, and augmenting Wnt/β-catenin gene expression.[87, 88]

Numerous material strategies have been explored in order to direct cadherin-mediated mechanotransduction. For example, Zhu et al. decorated a porous, hyaluronic acid (HA)-based 3D hydrogel with N-cadherin mimetic peptide (Cad) in order to drive osteogenesis of hMSCs embedded within the matrix, and therefore increase bone matrix deposition.[89] By incorporating Cad, the authors sought to recapitulate cell-cell interactions essential to osteogenesis in the bone marrow niche. Following 12 weeks of *in vivo* implantation to treat rat calvarial defects, MSC-seeded, porous Cad hydrogels—compared to control hydrogels—revealed significantly greater bone formation as measured via micro computed tomography. In a later study, Zhu et al. again made use of Cad to instead decorate a titanium substrate surface upon which hMSCs were seeded.[90] The Cad-functionalized biomaterial surface was demonstrated to promote activation of the Wnt/β-catenin pathway across seeded hMSCs, and correspondingly drive osteogenic differentiation, in-situ bone formation, and integration at the bone-implant interface both *in vitro* and *in vivo*. Other biomaterial approaches to direct osteogenic differentiation and bone regeneration by modulating or mimicking cadherin/catenin have included micro/nanotopographies,[91] matrix dimensionality,[92] Sr^{2+}-modified surfaces,[93] and polyelectrolyte-based matrices.[94]

2.6 Cytoskeleton

The cytoskeleton of a cell functions to organize intracellular content, to connect the cell to an external environment, and to generate forces that allow for cell movement (Figure

3). Actin filaments, microtubules, and intermediate filaments compose the cytoskeleton structure. Physical forces can act upon this network of proteins to influence cellular function.[95] For instance, mechanical strain can induce cytoskeletal stiffening,[96] impair microtubule growth rate,[97] and influence actin growth velocity.[98] Essentially, these forces modulate the tension of the intracellular cytoskeleton, which has downstream implications on cellular development, differentiation, and disease.[99]

The cytoskeleton additionally has implications in mediating osteocytic mechanotransduction and Ca^{2+} signaling. In rat osteoclasts, Ca^{2+} has been shown to regulate actin formation. Specifically, Kajiya et al. demonstrated that treatment of rat osteoclasts with protein tyrosine kinase inhibitors (PTKI) results in impaired actin formation, as well as inhibition of bone resorption. While the mechanism connecting PTKI to osteoclast resorption activity was unclear, treatment with the inhibitors led to increased cytosolic Ca^{2+} concentrations, suggesting that PTKI-induced Ca^{2+} entry may act as an inhibitory signal for osteoclastic resorption. Further, other studies support the role of osteoclast actin rings in mediating osteoclast functionality.[100] Taken together, the results of these works indicate that actin rings are required for osteoclast resorption and that this process is regulated by Ca^{2+} signaling. One study identified spectrin as an important player in the regulation of MLO-Y4 osteocytes.[101] Chemical disruption of the spectrin network impaired cell cortex stiffness, nitric oxide (NO) secretion (indicated to promote bone development), and cell-to-cell connections. This break in the network also caused significant Ca^{2+} influx, suggesting that the cytoskeleton not only senses mechanical stimuli, but also mediates Ca^{2+} signaling, similar to integrins. Supporting this, extracellular Ca^{2+} has been found to regulate actin crosslinking,[102] polymerization,[103] and stabilization.[104] Collectively, these findings provide evidence that the cytoskeleton is a key intermediary of mechanotransduction, Ca^{2+} signaling, and bone mineralization.

In order to modulate mechanotransduction through cytoskeleton-influencing material cues, Pan et al. engineered a hierarchical material architecture composed of nanowires and macropores that was demonstrated to promote enhanced cytoskeleton development, elongation, spreading, and tension when coated with MSCs.[9] This increased cytoskeletal tension was suggested to cause a measured increase in YAP activity among the seeded MSCs, and was necessary for the YAP-mediated mechanotransduction that drove osteogenic differentiation. Elsewhere, Dupont et al. too made use of cytoskeleton-influencing material cues in order to modulate YAP/TAZ-mediated mechanotransduction; namely, subcellular YAP/TAZ localization was shown to depend on cytoskeletal tension.[7] By increasing the rigidity of fibronectin-coated acrylamide hydrogels from soft (0.7–1 kPa) to stiff (15–40 kPa), the authors were able to increase YAP/TAZ activity and nuclear localization among hMSCs cultured on the material surfaces. Further, adhesive patterns were also shown to influence cytoskeletal organization and, therefore, YAP/TAZ activity; by increasing the size of micropatterned fibronectin islands on the substrate surface from 300 μm^2 to 10,000 μm^2, YAP/TAZ activity was indicated to almost entirely re-localize from the cytoplasm to the nucleus.[7]

Staehlke et al. sought to elucidate the involvement of Ca^{2+} mobilization in topography-induced cytoskeleton activity that regulates osteogenic differentiation.[105] In addition to

featuring shortened actin cytoskeletons, intracellular Ca^{2+} concentration was reduced in MG-63 cells cultured on micropillar structures compared to those cultured on flat surfaces. Further, the Ca^{2+} mobilization potential in response to ATP stimulation was significantly reduced in the cells cultured on the pillars. Collectively, these results indicate that Ca^{2+} signaling plays a role in mediating topography-induced cellular mechanotransduction.

2.7 Mechanosensitive Ion Channels (MSIC)

MSICs are channels that change conformation in response to mechanical stimuli in order to accommodate ion influx (Figure 4). Of the various MSICs, Piezo1 and Piezo2 have been identified as key mechanical sensors required for bone development and osteoblast differentiation.[106, 107] Between the two channels, Piezo1 has greater mRNA expression in bone,[108] and is required for mechanically-induced gene expression in osteocytes.[109, 110] While Piezo1 plays a larger role in bone growth, Piezo2 still shares some redundant functionality in guiding skeletal development. Indeed, both Piezo1 and Piezo2 have been shown to regulate Ca^{2+} influx in response to fluid shear stress (FSS) to stimulate calcineurin, which in turn promotes activation of YAP, β-catenin, and NFATC1 transcription factors.[107, 111] Concerted activation of the NFAT-Yap1-β-catenin transcription factor network is vital in controlling osteoblast cell fate. As discussed earlier, YAP/TAZ are essential effectors of cell proliferation in response to mechanical stimuli[7] and bone development.[71] Similarly, the Wnt/β-catenin pathway is essential for bone formation and maintenance in response to mechanical loading.[112] The NFAT family of transcription factors are integral substrates of protein phosphatase 3 catalytic subunit alpha (Ppp3ca) and regulate gene expression. Further, NFATs are involved in promoting differentiation of both osteoclasts[113] and osteoblasts,[114] as well as mediate mechanically-induced Cox2 expression.[115]

Piezo1 stimulation via the chemical agonist, Yoda1, and hydrostatic pressure both lead to increased expression of bone morphogenetic protein 2 (BMP2) and subsequent osteoblast differentiation.[116] These findings demonstrate that Ca^{2+} influx via Piezo1/2 has downstream effects on multiple pathways that jointly promote osteogenesis. Biomaterial strategies may be devised in order to modulate these channels within tissue-engineered constructs. By engineering a cell-laden 3D hydrogel consisting of unidirectional microfilament networks (FLight), Liu et al. demonstrated an upregulation of Piezo1 activity compared to the cells cultured within a 3D bulk hydrogel or atop a 2D FLight hydrogel surface.[117] Further, Xing et al. showed that treatment of human dental follicle cells with the Piezo1 agonist, Yoda1, resulted in cellular proliferation, Ca^{2+} nodule formation, and increased expression of ALP, Runx2, OCN, and β-catenin.[118] As such, the construction of biomaterials that seek to not only activate Piezo1 and other MSICs through mechanical stimuli, but also through chemical activation may be considered.

Transient receptor potential melastatin 7 (TRPM7) is another MSIC which is involved in skeletal development,[119] cell migration,[120] and determination of MSC fate.[121] Liu et al. demonstrated that 1) FSS increases TRPM7 expression and membrane localization, 2) TRPM7 knockdown or inhibition impairs Osterix, Dlx5, ALP, and Col1a1 osteogenic expression, and that 3) TRPM7 is required for osteogenic differentiation of MSCs.[121] TRPM7 also regulates actomyosin function via a Ca^{2+}-dependent mechanism, thus

influencing cell spreading and adhesion, and ultimately affecting the mechanical forces experienced by cells.[122] TRPM7 is additionally involved in increasing Ca^{2+} influx from the ER, and promoting NFAT nuclear localization and osteogenesis.[123] The influence of TRPM7 on these pathways may be utilized in the development of bone regenerative materials. Using a 3D-printed scaffold composed of Sr^{2+}-Ca^{2+} silicate and Mg^{2+}-Ca^{2+} silicate, Lin et al. demonstrated that the release of both $Sr^{2=}$ and Mg^{2+} led to an upregulation of the Akt and Wnt pathways, and increased protein levels of TRPM7 among MSCs cultured within the scaffold.[124] The increased TRPM7 signaling was suggested to play a key role in further promoting osteogenic differentiation of the embedded MSCs.

2.8 Voltage-gated or voltage-sensitive Calcium Channels (VGCC/VSCC)

VSCCs transduce alterations in membrane potential into intracellular Ca^{2+} currents (Figure 4). The L-type VSCCs are involved in the regulation of osteogenic mechanotransduction and are more highly expressed in osteoblasts than osteocytes.[125] Many of the subunits associated with these channels play a role in this regulation. The L-type VSCC α1 or pore-forming subunit is one such fundamental subunit, which is coded for by four different genes. These genes are $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, and $Ca_v1.4$. Of these isoforms, $Ca_v1.1$, $Ca_v1.2$, and $Ca_v1.3$ have been found to be expressed in osteoblasts.[126] However, the role of $Ca_v1.1$ is better understood in skeletal muscle excitation-contraction coupling[127] with less exploration of its role in osteogenic mechanotransduction.[128]

$Ca_v1.2$ or Cacna1C regulates osteogenesis of bone marrow-derived MSCs through the canonical Wnt/β-catenin pathway[129] and additionally modulates OPG expression.[110, 130] $Ca_v1.2$ has also been found to be downregulated in aged mouse models, leading to compromised osteogenic capacity as measured by decreased expression of ALP, Runx2, and OCN.[129] In contrast, $Ca_v1.2$ upregulation in transgenic ovariectomized female mice has been shown to mitigate symptoms of osteoporosis by promoting osteogenesis and inhibiting osteoclast activity through OPG activity.[131] Furthermore, $Ca_v1.2$ is expressed in the first and second pharyngeal arches of mice and regulates embryonic jaw development through Ca^{2+} influx and has also been shown to regulate the NFAT signaling pathway and influences mandibular chondrocyte hypertrophy in zebrafish.[132] Upstream, $Ca_v1.2$ is regulated by the biologically active form of vitamin D, 1,25-dihydroxyvitamin D3, otherwise known as calcitriol.[133]

$Ca_v1.3$ is another subunit that plays a role in the skeletal response to mechanical loading and has demonstrated expression in osteoblasts. Li et al. showed that, similar to $Ca_v1.2$, $Ca_v1.3$ downregulation results in decreased bone mineralization in *in vivo* models.[128] However, the authors found that $Ca_v1.3$ (−/−) mice can still maintain a sufficient osteogenic response in response to mechanical loading, which is potentially explained by the finding that both $Ca_v1.1$ and $Ca_v1.2$ expression significantly increases in the absence of $Ca_v1.3$, with $Ca_v1.2$ increases being greater than $Ca_v1.1$. Thus, while $Ca_v1.3$ may play a role in bony mineralization, this channel may not strongly mediate osteoblast activity in response to mechanical stimuli. Altogether, these findings suggest that of the three isoforms, $Ca_v1.2$ plays a central role in mediating osteogenic mechanotransduction.

In addition to the various α1 pore-forming subunits, Ca^{2+} channel protein complexes are also comprised of auxiliary subunits. However, the role of these auxiliary subunits in mechanotransduction and osteogenesis is not yet clear. For instance, the β4-subunit has been linked to down-regulation of the Wnt/β-catenin pathway and impaired cell division; however, this has only been shown in hepatoma cell lines.[88] Potential influences on mechanically mediated osteogenesis from the α2, β, γ, or δ-subunits are largely unknown. Although Ca^{2+} channels can be formed without the presence of any auxiliary subunits,[134] future studies may seek to investigate the role of these components, given that these subunits can fine-tune Ca^{2+} channel conductance and gating characteristics.[135]

Ca^{2+} influx via L-type channels influences different cellular signals in response to mechanical stimulation. Particularly, production of NO,[136, 137] Cox2,[138] PGE_2,[139] and adenosine triphosphate (ATP)[20, 140] can all be mediated by Ca^{2+} influx and mechanical stress. Cellular assembly of these products affects many downstream targets including the canonical Wnt pathway,[141] RANKL/OPG expression,[142] Cx43 expression,[143] and regulation of osteocyte apoptosis,[144] ultimately influencing bone formation and regulation.

In addition to L-type VSCCs, T-type VSCCs are also sensitive to mechanical stimuli. However, contrary to the L-type channels, T-type VSCC expression is more predominant in osteocytes than in osteoblasts.[125] Lu et al. showed that treatment with NNC55–0396—a T-type VSCC inhibitor—decreased fluid shear-induced Ca^{2+} influx in osteocytes, suggesting that T-type VSCCs play a role in propagating mechanical stimuli and indicating their importance in Ca^{2+}-mediated gene expression, metabolism, and proliferation.[145] Despite T-type channels being more prevalent than L-type channels in osteocytes, L-type channels still serve a role in mediating osteocyte mechanotransduction. Elsewhere, Li et al. demonstrated that, indeed, *in vivo* inhibition of L-type VSCCs with antagonists such as verapamil or nifedipine substantially reduces mechanically induced bone formation but does not completely abolish bone formation.[146]

VSCCs are another potential target or mediator for bone regenerative biomaterial strategies. Wood et al. demonstrated that L-type VSCCs can be directly activated through a porous dihydropyridine-releasing poly(L-lactide) scaffold.[147] In their study, Wood showed that the dihydropyridine agonist, Bay K8644, acts to increase the opening time of L-type VSCCs resulting in increased osteoid production and bone mineralization in human bone cell-seeded constructs subjected to load. Seeking to characterize the influence of implant surface charge on osteogenesis, Mao et al. cultured MSCs atop BaTiO3-based piezoelectric ceramics featuring surface potentials generated through high-voltage polarization.[148] Compared to positive substrate surfaces, those with negative surfaces were shown to enhance MSC attachment, migration, and osteogenic differentiation. Greater VSCC activation was observed among cells cultured upon the negative surfaces, reflected by a 2.5-fold increase in intracellular Ca^{2+} concentration relative to the cells cultured on non-polarized surfaces. The authors postulated that the increase in Ca^{2+} concentration, in addition to enhanced integrin activity, were key to the observed osteogenic differentiation. In addition to static biomaterial cues and features, stimuli-dependent biomaterials may also be relevant to bone regeneration. Espinosa et al. designed Janus scaffolds composed of polycaprolactone (PCL) and polylactide (PLA) that—under the external application of ultrasound—generate

nanovibrations that are transmitted to seeded cells.[149] In comparison to non-stimulated PCL/PLA scaffolds, stimulated scaffolds were shown to drive enhanced matrix development, and proliferation and osteogenic differentiation of MSCs primarily through increasing the formation and activation of VSCCs. It was found that expression of genes encoding subunits of L-type VSSCs was increased 3-fold among cells cultured on Janus scaffolds, and dihydropyridine (DHPR) staining revealed L-type VSSCs both formed and coupled to ryanodine receptors (RyR) only on Janus scaffolds. Indeed, when MSCs atop these scaffolds were treated with nifedipine, the application of ultrasound had no effect on proliferation or differentiation.

2.9 Mechanosensitive Store-operated Calcium Entry (SOCE): Orai1, STIM1, and TRPC1

Store-operated calcium entry (SOCE) is a Ca^{2+} pathway that is activated in response to depleted ER Ca^{2+} stores (Figure 5) and was first described in 1986.[150] Since its discovery, the regulation of intracellular Ca^{2+} via SOCE has also been shown to play critical roles in various physiological functions, including cardiac contraction and vascular tone,[151] skeletal muscle contraction,[152] and cancer biology.[153] The major components of the SOCE pathway include transient receptor potential canonical 1 (TRPC1), stromal interaction molecule 1 (Stim1), and the calcium release-activated calcium (CRAC) channel, Orai1. TRPC1 is a plasma membrane Ca^{2+} channel that facilitates extracellular Ca^{2+} entry in order to replete ER Ca^{2+} levels.[154] Additional activity by sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and IP_3 allows for control of Ca^{2+} entry and secretion from the ER, thus balancing intracellular Ca^{2+} concentrations.

Stim1 is an ER protein that regulates TRPC1 functionality. Following ER Ca^{2+} store depletion Stim1 binds to TRPC1 and subsequently displaces Caveolin-1 (CAV1) (TRPC1 inactivator), resulting in TRPC1 activation.[155–157] Knockdown of Stim1 has been shown to substantially reduce TRPC1-mediated Ca^{2+} current, further supporting the role of Stim1 as a regulator of TRPC1.[155, 158]

TRPC1 is additionally regulated by the plasma membrane CRAC channel, Orai1, a finding reported by Ong and colleagues.[157, 158] Similar to Stim1, knockdown of Orai1 induces a strong elimination of TRPC1-mediated SOCE. Chen et al. showed that Stim1 and TRPC1 overexpression in HEK293 cells induces a 2.4-fold increase in SOCE relative to control cells; however, siRNA knockdown of Orai1in these cells led to an >50% attenuation of SOCE, suggesting that Orai1 is a functional requirement of the SOCE pathway. Several studies have demonstrated that TRPC1 and Orai1 together form a heteromeric channel, and that Ca^{2+} entry via Orai1 activates TRPC1.[157, 159]

Activity of the SOCE pathway and the TRPC1/Stim1/Orai1 complex have important ramifications for osteogenic differentiation. Lee et al. found that Orai1 mediates osteogenic differentiation via BMP signaling in bone marrow MSCs. [136] The authors observed that bone marrow MSCs from Orai1(−/−) mice displayed reduced phosphorylation of Smad1/5/8, the immediate effectors of BMP signaling. Moreover, the project demonstrated that the bone marrow MScs possess impaired alkaline phosphatase activity, alizarin red S staining, and Runx2 expression. Furthermore, work by Robinson et al. has shown that Orai1(−/−) mice lack multinucleated osteoclasts resulting in decreased bone resorption, as well as

impaired cortical ossification with thinned trabeculae suggesting a role in both cortical and trabecular bone.[160] Additionally, the TRPC1/Stim1/Orai1 complex appears to play a regulatory role in organizing osteoblast polarity in response to gel substrate stiffness.[161] Some evidence suggests that the SOCE pathway also interacts with the osteogenic transcriptional coactivators YAP/TAZ.[162] Taken together, the TRPC1/Stim1/Orai1 complex regulates bone physiology through control of various mechanisms.

Although there are many regulators of SOCE,[163] lipid rafts are a unique director of the pathway as these microdomains additionally play a part in mechanosensation.[164] Lipid rafts are plasma membrane microdomains enriched in cholesterol and sphingolipids, and are intricately connected to various signaling complexes including those related to host defense, vascular inflammation, neurological disease, kidney disease, obesity, and cancer.[165] Within SOCE, lipid rafts have been demonstrated to govern Ca^{2+} entry along with the TRPC1/Stim1/Orai1 complex. When lipid rafts are disrupted by methyl-β-cyclodextrin, cellular intake of Ca^{2+} via SOCE is impaired in a manner dependent on extracellular Ca^{2+} concentration.[166] Disruption of lipid rafts is also associated with downregulation of osteoblastic cell polarity.[161]

Biomaterial strategies may consider the link between material cues and the SOCE pathway. Li et al. sought to compare Ca^{2+} signaling among MC3T3-E1 cells cultured on smooth titanium surfaces to those on titanium surfaces featuring topographical micro/nano-texture cues endowed via anodization and etching.[167] For cells cultured on the topographical architecture, RNA sequencing revealed significant Ca^{2+} signal pathway enrichment, and observation of intracellular Ca^{2+} oscillations through real-time fluorescence microscopy demonstrated higher Ca^{2+} peaks and more rapid influx and outflux. Significant upregulation of Orai1 expression and osteogenic differentiation was induced by nano-textured topography. Two causes were indicated to account for the increased cytosolic Ca^{2+}: Ca^{2+} release from the ER lumen due to topography-induced ER stress, and subsequent activation of Orai1-mediated SOCE to recover ER Ca^{2+}. These data collectively suggested that SOCE facilitated by Orai1 plays a role in mediating mechanotransduction and osteogenic differentiation in response to topographical cues.[167]

3. Implications and Future Directions for Materials-based Bone Regeneration

The design of biomaterials to induce osteogenesis via mechanotransduction is well studied,[1, 2, 168] and offers a promising skeletal regenerative strategy. We have previously demonstrated that nanoparticulate mineralized collagen glycosaminoglycan scaffolds efficiently induce osteoprogenitor differentiation and skull healing by way of activating the mechanotransduction pathway (Figure 6).[169] Important mediators of this regenerative process include β-catenin, BMP, and YAP/TAZ. As described here, Ca^{2+} channels and their linked cellular components are also involved in mechanotransduction and osteogenic differentiation.

For the fields of materials science and regenerative medicine, the influences of these channels can be considered during biomaterial development and interpretation of scientific

studies. Included across this text are examples of approaches that have considered the aforementioned Ca^{2+} channels, signaling, and associated pathways in the design or analyses of bone regenerative biomaterials. Yet, this area of research is nascent, and further efforts are required to elucidate the implications of the relationship between these proteins, Ca^{2+} signaling, and material cues on bone formation. Indeed, literature examining the relationships for potential regulatory interactions between the various Ca^{2+} channels we describe is sparse. In addition, Ca^{2+} ions are known to be vital in regulating other mechanisms beyond osteogenesis, such as angiogenesis. In particular, Ca^{2+} is known to promote fibrinogen polymerization by interacting with nodule sites along the glycoprotein, thereby promoting thrombosis formation and impairing blood flow in a manner that may affect bone regeneration.[170] Within the field of biomaterials, some literature has reviewed the methodology employed in studying and quantifying both osteogenesis and angiogenesis in bioactive glass experiments.[171] However, the relationship between Ca^{2+} signaling and materials-induced angiogenesis remains to be fully explored. Future research may seek to further develop an understanding of the underlying molecular signaling that governs osteogenic differentiation and the interplay of Ca^{2+} channels may leverage the full potential of the osteogenic capacity of biomaterials.

Techniques that have been used to modulate Ca^{2+} channels and movement in other cell lines may be adopted or appropriated in the design of future bone regenerative efforts. For example, Li et al. designed a study where laser-induced cavitation microbubbles were used to stimulate HEK293T embryonic kidney cells.[172] The study investigated the influence of Piezo1 on mediating Ca^{2+} influx in response to microbubble stimulation via knockdown or transfection of Piezo1. Further, the study demonstrated that integrin attachment to RGD-coated polystyrene microbeads enhanced the Ca^{2+} response via local drag effects created by the beads. Ion-doped brushite cements have also been reviewed as a biomaterial strategy for bone grafting.[173] Specifically, these Ca^{2+}-PO_4^{3-} based cements can induce osteogenesis and angiogenesis via cell-material interactions that are dependent upon the effects of its ionic constituents. Further, the effects of Ca^{2+}-PO_4^{3-} cements can be further augmented by the presence of other bioactive ions within the material, such as Mg^{2+}, Sr^{2+}, Zn^{2+}, Cu^{2+}, Li^{+}, and Co^{2+}.

Additionally, of the current regenerative biomaterial approaches that involve Ca^{2+} channel-mediated mechanotransduction, the majority have relied primarily upon the presentation of topographical patterns and cues. Other material features can be further studied for their potential uses in dictating this process. In addition to expanding the palette of current biomaterial approaches, this would give way to synergistic strategies that combine different material cues to more intricately recreate and repair an osteogenic niche. One example that can be further characterized is matrix stiffness. When human MSCs are seeded upon polyacrylamide hydrogels, osteogenic differentiation has been shown to increase in a process that is mediated by integrins.[174] Matrix stiffness further regulates osteogenic differentiation by regulating SOCE[161] and plays a role through concerted activation of NFAT-Yap1-β-catenin.[107]

Another direction for the design of bone regenerative materials is to give greater attention to the non-canonical Wnt signaling pathway, which has been shown to require the participation

of voltage-gated Ca^{2+} channels.[175] Among previously reported bone regeneration and healing approaches, the canonical Wnt pathway has been more extensively studied.[176] However, biomaterial strategies have been employed that indicate that characterization and utilization of non-canonical Wnt signaling may provide valuable therapeutic applications for osteogenesis. Utilizing a methacrylated hyaluronic acid (MeHA)-based scaffold functionalized with Foxy5—a Wnt5a mimetic hexapeptide—Li et al. designed a biomaterial strategy that activated non-canonical Wnt signaling in order to facilitate MSC mechanotransduction and osteogenesis, and enhance bone regeneration *in vivo* when used to treat rat calvarial defects.[177] The authors demonstrated that increased intracellular Ca^{2+} levels contributed to the promoted osteogenesis observed for Foxy5-presenting hydrogel samples.

4. Conclusions

The regulation of mechanotransduction via Ca^{2+} channels and signaling plays a major role in mediating osteogenic differentiation. There are a multitude of channels that participate in this process; the channels range from those that are closely coupled with plasma membrane components such as cilia, gap junctions, integrins, and cadherins to MSICs and VSCCs. Intracellular channels—such as those associated with SOCE—are also a fundamental part of mediating mechanical stimuli. Multiple osteogenic pathways are linked to these channels and involve the Runx2 axis, canonical and non-canonical Wnt signaling, BMP signaling, YAP/TAZ mechanotransduction, and the ERK pathway. Other regulatory products are intertwined with Ca^{2+} channel activity like PGE_2, NO, ATP, and OPG. In short, Ca^{2+} channels are intricately linked to numerous osteogenic pathways in a manner dependent upon the intensity of mechanical load and available Ca^{2+} concentration. Thus, Ca^{2+} channels and Ca^{2+} movement carry considerable implications for the design of skeletal regenerative materials. The amount of bony mineralization, osteogenic gene expression, and MSC differentiation that occurs in response to mechanical stimuli is facilitated by the presence and functionality of these channels and their ionic messengers. Numerous studies have shown that biomaterial cues can be designed to tune the cellular movement and flow of Ca^{2+}, and therefore modulate those processes.

Still, the role of Ca^{2+} channels in coordinating osteogenesis is only one part of the whole. The role of other ions circulating throughout the bone microenvironment such as Zn^{2+}, Sr^{2+}, Mg^{2+},Ti^{4+}, and PO_4^{3-} should also receive further study. For instance, TRPM7 plays a central role in not only Ca^{2+} signaling but also Mg^{2+} and Zn^{2+} homeostasis.[178] Additionally, while determination of Ca^{2+} waves via gap junction communication is fairly well studied in multiple cell types, gap junctions may not be specifically selective to Ca^{2+} ions and could also transmit other cations/anions and small molecules (e.g. cAMP, NO, and prostaglandins). There is a close relationship between Mg^{2+} ion and Ca^{2+} ion in physiologic function. Mg^{2+} is the second most abundant intracellular cation and is also important in bone homeostasis.[179] Determinations of Mg^{2+} flux between cells may yield insights on possible influences of Mg^{2+} signaling on Ca^{2+} signaling. Moreover, biocompatible Ca^{2+}-PO_4^{3-} coatings of materials demonstrate improved cellular adhesion, proliferation, and differentiation in bone regeneration in *in vitro*, *in vivo*, and clinical trials further illustrating the role of PO_4^{3-} in promoting tissue regeneration.[180]

Additional investigations of other aspects of Ca^{2+} control, like nuclear pore regulation and nuclear trafficking,[181] may yield more insights into the mechanics of mechanotransduction. Ca^{2+} driven nuclear trafficking is known to have implications on breast cancer gene 1 (BRCA1) and ovarian adenocarcinoma, but similar mechanisms are not well-studied within mechanotransduction. Furthermore, Ca^{2+} signaling is not only regulated by changes in cytosolic concentrations, but also on the frequency of Ca^{2+} oscillations which can be seen with some agonists and with mechanical perturbations. It has been demonstrated that mechanically induced Ca^{2+} oscillations in osteocytes lead to the release of extracellular vesicles and enhanced bone formation.[182]

The considerable influence of Ca^{2+} entry and signaling on osteogenesis contends that these underlying molecular mechanisms are relevant to the therapeutic potential of bone regenerative biomaterial constructs. As such, further characterizing the various channels and pathways involved in mechanotransduction can advance biomaterial approaches seeking to recapitulate endogenous bone formation, and regenerate structures more closely representing native bone tissue.

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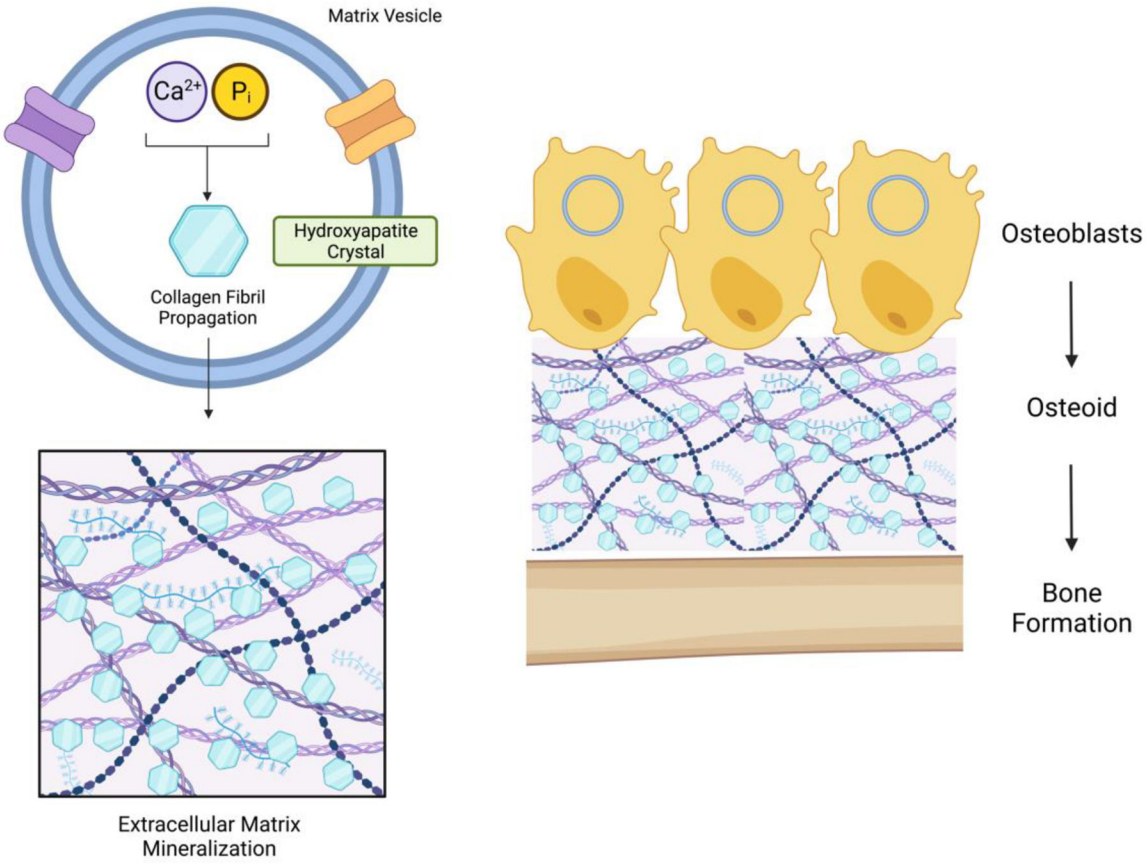

Figure 1: Ca^{2+} ion contribution to bone matrix mineralization.

Ca^{2+} and PO_4^{3-} are transported into osteoblast matrix vesicles from the cytoplasm and combine to form hydroxyapatite crystals. Upon cellular release, hydroxyapatite subsequently prorogates along collagen fibrils within the ECM. In this fashion the ECM is mineralized with hydroxyapatite, thus converting unmineralized osteoid into newly mineralized bone.

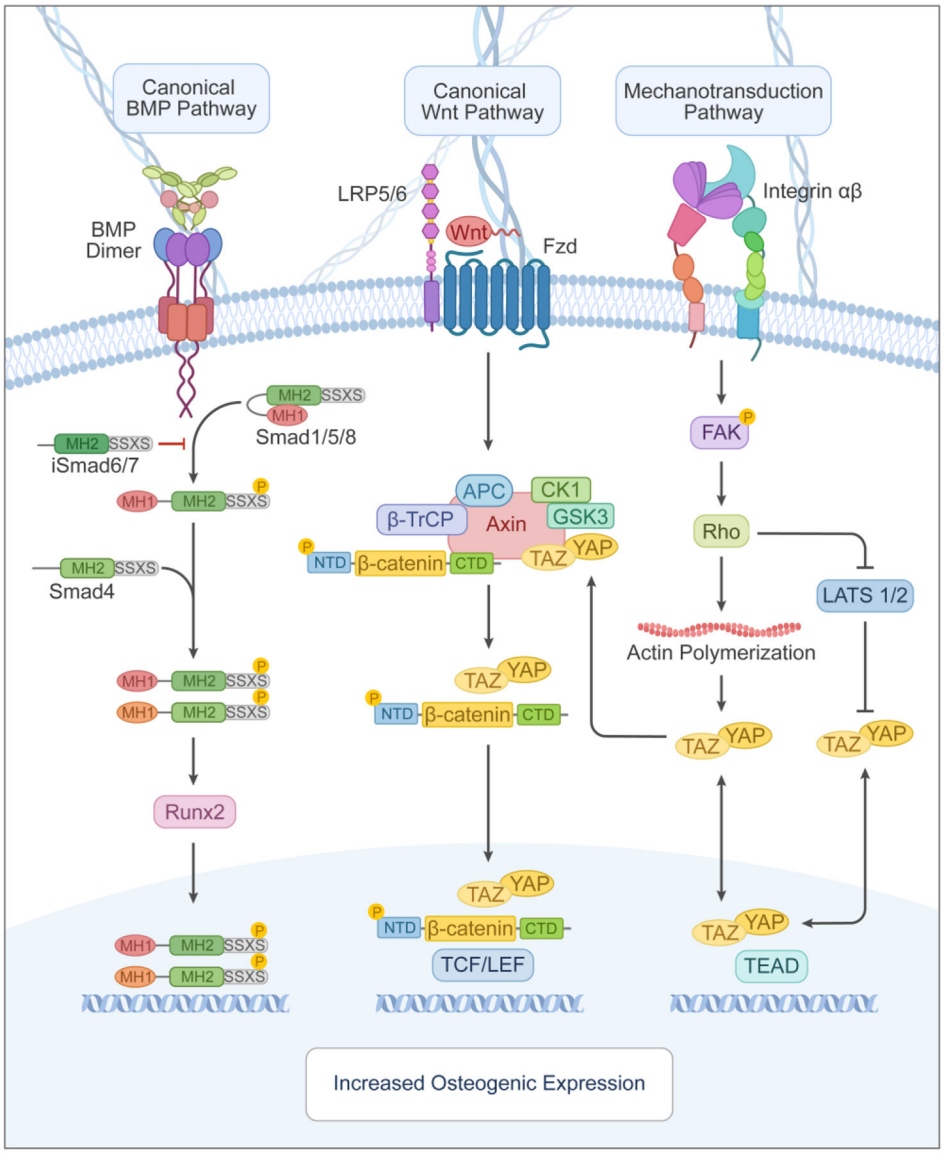

Figure 2: Osteogenic Molecular pathways.

In the Canonical BMP Pathway, BMP ligand dimers interface with BMP receptors, resulting in Smad1/5/8 mediated signal transduction. The transcription factor, Runx2, subsequently translocates to the nucleus and increases osteogenic expression. In the Canonical Wnt Pathway, the Wnt protein binds to the transmembrane receptors, comprised of frizzled proteins and LRP5/6. As a result, the β-catenin degradation complex is inhibited, allowing for stabilized β-catenin to enter the nucleus and target gene expression. In the Mechanotransduction Pathway, integrin αβ receptors sense mechanical stimuli and transmit signals to a focal adhesion kinase complex and the actin cytoskeleton. This initiates YAP/TAZ nuclear migration and ultimately regulates osteogenic expression. YAP/TAZ proteins additionally mediate crosstalk with the Canonical Wnt Pathway.

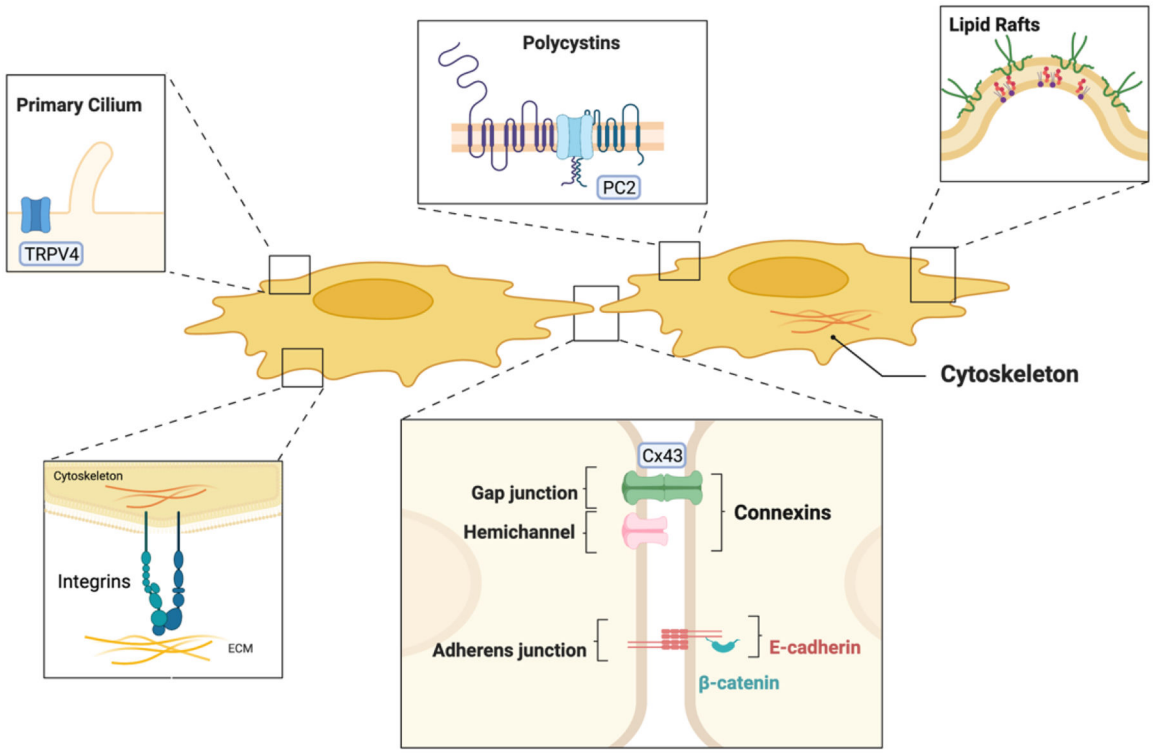

Figure 3: Various Modes of Mechanosensation.

The cell possesses multiple structures that enable the sensation of mechanical stimuli. These include the primary cilium, polycystins, lipid rafts, connexins, adherens junctions, and integrins.

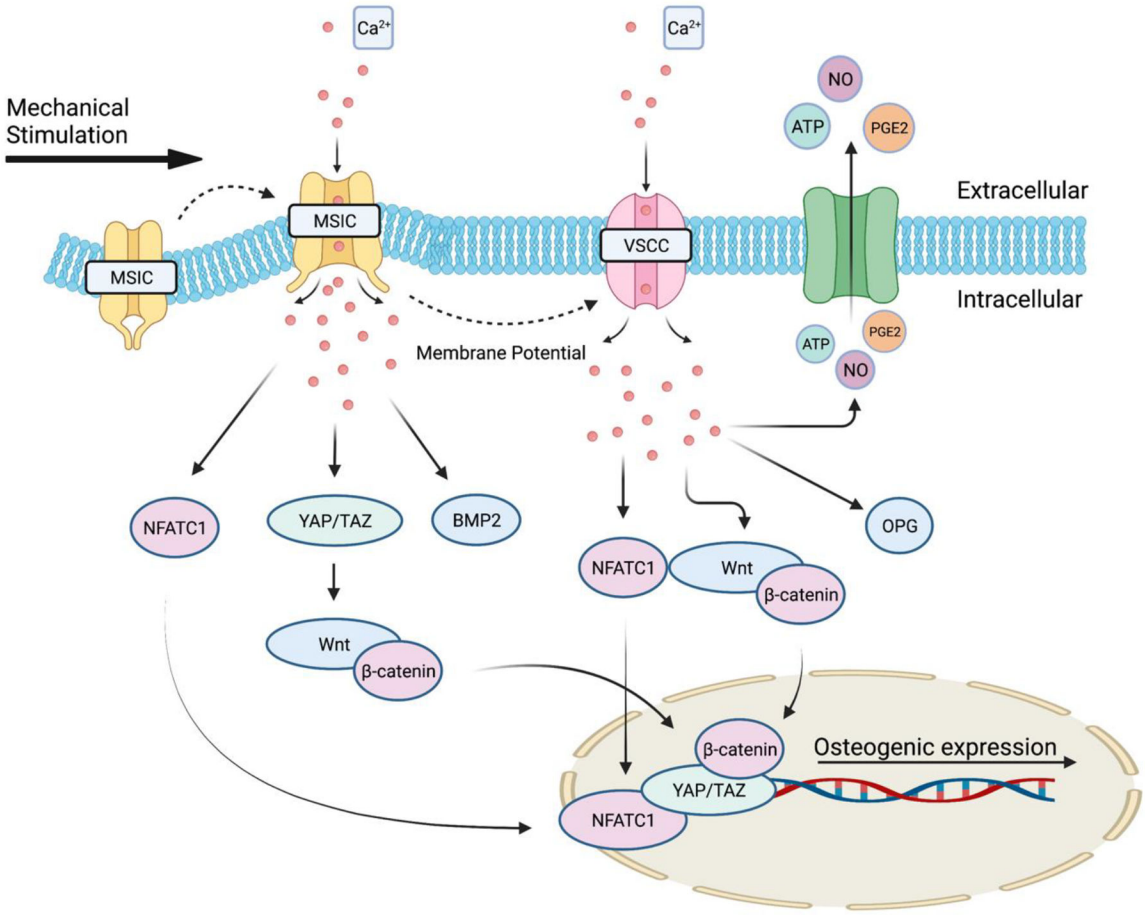

Figure 4: Calcium Channels and Downstream Elements.

Notable mechanosensitive channels include Piezo1, Piezo2, and TRPM7. Noteworthy voltage-sensitive calcium channels include $Ca_v1.2$ and $Ca_v1.3$. Mechanical stimulation alters the conformation of mechanosensitive ion channels, allowing for Ca^{2+} entry. As Ca^{2+} enters the cell, the change in membrane potential induces activation of voltage-sensitive calcium channels. The influx of Ca^{2+} is thus utilized in several different targets including NFATC1, YAP/TAZ, canonical Wnt, BMP2, and OPG. This influx can also induce osteogenic transcription. Ca^{2+} signaling also has important effects on ATP, NO, and PGE_2 release, which in turn promotes osteogenic changes; NFAT, nuclear factor of activated-T cells.

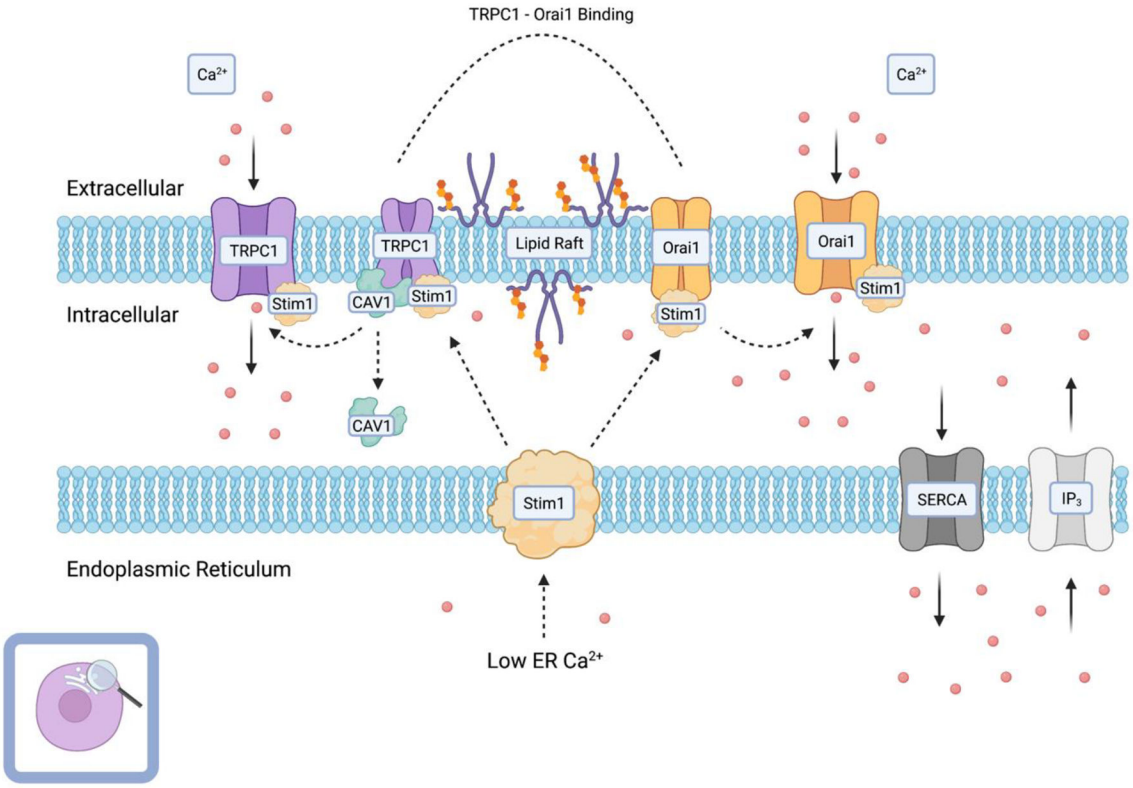

Figure 5: Storage-Operated Calcium Entry (SOCE).

SOCE is primarily regulated by three calcium channels: Orai1, Stim1, and TRPC1. TRPC1 is a plasma membrane channel that facilitates Ca^{2+} entry. TRPC1 functionality requires interaction with both Stim1 and Orai1. Binding of the ER channel Stim1 to TRPC1 displaces the TRPC1 inactivator, Caveolin-1 (CAV1). Binding of Stim1 to Orai1 forms a pore within the calcium release-activated calcium channel, allowing Ca^{2+} to enter. Once Ca^{2+} enters the cell, SERCA can facilitate entry into the endoplasmic reticulum, while IP_3 balances intracellular Ca^{2+} concentrations; SERCA, sarcoendoplasmic reticulum calcium ATPase; IP_3, inositol 1,4,5-triphosphate.

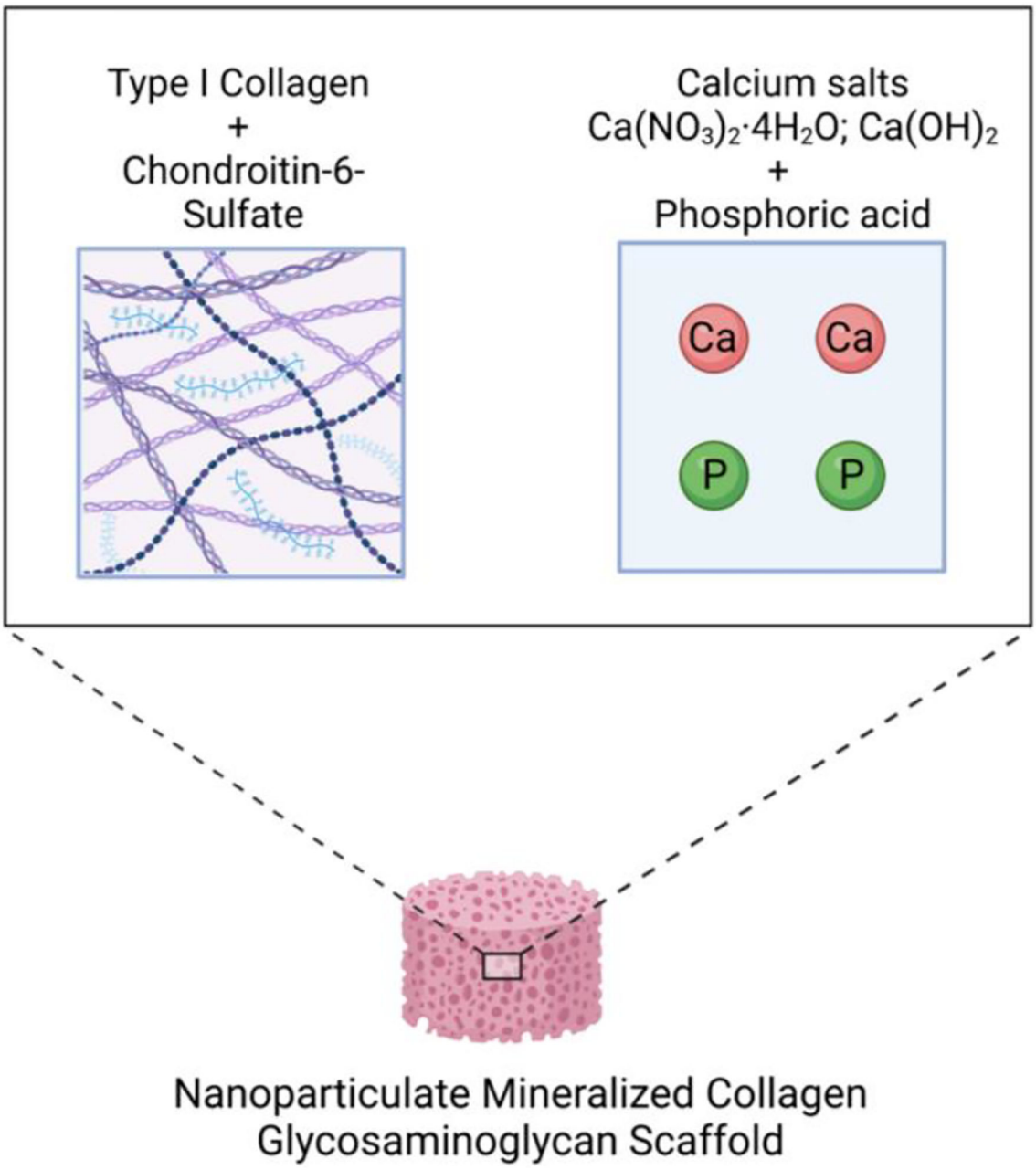

Figure 6: Nanoparticulate mineralized collagen glycosaminoglycan scaffold composition.
The ECM-based scaffold recapitulates both the organic and inorganic components of bone. Type I collagen and the glycosaminoglycan, chondroitin-6-sulfate, form the organic component of the scaffold. Calcium salts and phosphoric acid form the inorganic component of the scaffold.