Chapter 8

CCN Family: Matricellular Proteins in Cartilage and Bone Development

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Abstract

The extracellular matrix is the intricate scaffolding which surrounds and supports cells and helps to organize them into tissues and organs. The CCN family of matricellular proteins helps to regulate and modulate production, degradation, and remodeling of the extracellular matrix. In this chapter, we review the extracellular matrix of cartilage and bone, including an overview of chondrogenesis and skeletogenesis, and summarize the importance of the CCN proteins in establishment of the skeletal system. CCN proteins have both positive and negative regulatory roles in skeletal development, and their abnormal expression is related to the pathogenesis of several diseases observed in cartilage and bone that arise when inflammation or tissue injury becomes chronic, including fibrosis, arthritis, and cancer. Understanding the biological functions of the CCN proteins within this context offers opportunities for developing therapeutics by targeting CCN functions.

Keywords: matricellular proteins, CCN family, chondrogenesis; osteogenesis, extracellular matrix

1. Introduction

The extracellular matrix (ECM) is the intricate scaffolding, which surrounds and supports cells, and helps to organize them into tissues and organs. The composition of the extracellular matrix varies based on tissue type and organ function, and this matrix affects many cellular behaviors including proliferation, differentiation, and wound healing. Matricellular proteins, present in the immediate environment of the cell, help to regulate and modulate these functions. We

present a discussion of the important matrix elements of cartilage and bone, and the supporting connective tissues of the body, with regard to their function in health and disease. The major aim of this book chapter is to summarize the role of extracellular matrix proteins and their roles in chondrogenesis and osteogenesis. Additionally, we will discuss the importance of the CCN proteins—a family of secreted extracellular matrix-associated proteins—in functional pathways of chondrogenesis and osteogenesis, and include a discussion of the role of CCNs in pathophysiology of these tissues. The reader will gain an increased awareness of the importance of CCN proteins in a wide range of important functional pathways, providing in-depth focus on their role in bone and cartilage development and repair. These proteins have also been implicated in many human diseases and thus are important targets for drug discovery and development.

2. Overview of chondrogenesis

Cartilage is a specialized type of supporting connective tissue that contains cartilage cells, or chondrocytes, and a specialized extracellular matrix. The type of matrix is specific to the type of cartilage. Depending on the type of cartilage, there may be a perichondrium present, which is a connective tissue layer that contains mesenchymal cells or fibroblasts, as well as chondroblasts. Cartilage develops from mesenchyme cells that commit to become chondrocytes. Cartilage is first seen in the developing embryo in the areas that serve as templates for endochondral ossification, as well as in Meckel's cartilage in the head area [1]. Differentiated chondrocytes are surrounded by matrix and can appear to have space around them and they retain their proliferative capacity within their matrix. Development of cartilage involves several different transcription factors, such as Sox 9, that control this process, as well as growth factors that interact with cellular receptors to facilitate various cellular functions [2]. Interaction with the extracellular matrix has been implicated in development as well. Various epigenetic mechanisms and several different microRNAs also are important in the development of cartilage [3,4]. Chondrocytes may remain as cartilage in specific regions of the body or may be used as a template for the formation of many of the bones in the body during endochondral ossification. Cartilage remains in the adult in several specific places including the bridge of the nose, the trachea and bronchi, the ear, the intervertebral discs of the spinal column, and on the ends of bones as articular cartilage [5]. Chondrocytes are usually considered quiescent in the adult, although this can change during pathological processes such as osteoarthritis. Cartilage has very little capacity to regenerate, even though progenitor cells are present in the adult [6]. This lack of repair ability causes a number of disease issues as humans age, including the most prevalent form of degenerative joint disease, osteoarthritis [7].

2.1. Types of cartilage

Three kinds of cartilage have been identified: hyaline, elastic, and fibrous based on differences in matrix and cellular arrangement and function [5,8]. Hyaline is present in the bronchi, parts

of the trachea, and also as articular cartilage. It also forms the model for bone formation during endochondral ossification. Another elastic cartilage is present in the oropharynx and ear, among other regions of the body. Both of these types of cartilage are typically surrounded by a connective tissue layer and a chondrogenic layer of the perichondrium. Cells from this layer can undergo proliferation and can differentiate into chondrocytes for growth or renewal of cartilage, a type of growth that is appositional in that the newly formed chondrocytes come from outside the cartilage matrix. In addition, cells within the cartilage can proliferate, forming isogenous groups of similar chondrocytes, which is termed interstitial growth. By contrast, articular cartilage, at the surface, is not covered by a perichondrium. This lack of a perichondrium results in the proliferation of articular cartilage occurring only from within the matrix itself. An unfortunate consequence of this lack of a perichondrium is that the articular cartilage, continually exposed to stress from the movement of the joint, is less able to repair itself since no proliferation can occur from the perichondrium. Aging also leads to decreased healing ability and thus damage to cartilage may not repair sufficiently to prevent disease [9]. Damage to the articular cartilage leads to the most common [10] affliction of the aging population, osteoarthritis.

The third type of cartilage, fibrocartilage, is present as a transitional tissue generally connecting two other structures or tissues. Fibrocartilage does not have a perichondrium and thus only grows interstitially. Due to stress on this cartilage, the chondrocytes are typically arranged in a linear fashion [5]. One prominent place where fibrocartilage is present is in the annulus fibrosus of intervertebral discs. It is present in other areas such as part of the knee joint as well. Damage to this tissue may lead to herniation of the intervertebral disc, another major health issue as this may cause back pain [11].

Chondrocytes are surrounded by a thin pericellular matrix containing specific proteins, termed matricellular proteins, which have significantly different properties than the majority of the cartilage matrix [12]. This matrix and the associated chondrocyte are termed a chondron [13]. The matricellular proteins may confer the ability to withstand degradation by matrix metalloproteinases (MMPs), and this may be due to the localization of type VI collagen in this region [14,15]. This collagen is not broken down by many matrix metalloproteinases. Surrounding the pericellular matrix is the matrix termed histologically the territorial matrix and in between groups of chondrocytes is the interterritorial matrix [5].

3. Overview of skeletogenesis

The skeletal system is primarily composed of bone and cartilage, both mesodermal-derived tissues formed through the differentiation and cellular function of osteoblasts and chondrocytes. Skeletogenesis is a complex, multistep process involving the coordinated actions of osteoblast gene expression and cellular activity that are regulated by a multitude of systemic and locally produced growth factors, as well as complex extracellular matrix interaction [16]. Osteoblasts are highly differentiated bone lining cells responsible for the production of bone through secretion and mineralization of ECM constituents. Osteoblasts are protein-producing

cells characterized morphologically by a round nucleus, an intensely basophilic cytoplasm, a prominent Golgi complex, and a well-developed rough endoplasmic reticulum. Osteoblast precursors originate from local pluripotent mesenchymal stem cells from the bone marrow stromal mesenchymal stem cells. Under specific conditions, including ECM interactions, osteoblast precursors will proliferate and differentiate into pre-osteoblasts cells that are committed to differentiate into mature osteoblasts [16–18]. The majority of these mature synthetic osteoblasts will become osteocytes as they become embedded in their secreted ECM. Osteocytes are the most abundant cell type in the bone and have the ability to resorb and synthesize bone matrix but in a more limited capacity compared to osteoclasts and osteoblasts, respectively [19]. These osteocytes form an interconnected network called the lacunocanalicular system that is embedded within the mineralized tissue. The matrix directly surrounding the lacunocanalicular system is not mineralized and has fluid-like mechanical properties and contains ECM proteins secreted by osteocytes [20]. This system is thought to play an active role in bone remodeling and adaptation by sensing and responding to biomechanical and other systemic stimuli. In addition, the ECM proteins produced here also play a role in mineralization [21,22]. Osteoclasts are bone-lining cells that are responsible for bone resorption. Osteoclasts are characterized morphologically as very large, multinucleated cell with abundant Golgi complexes and numerous transport vesicles loaded with lysosomal enzymes. The zone of contact with bone is characterized by the presence of a ruffled border where bone resorption takes place [16,18]. Osteoclasts originate from hematopoietic precursors of the mononuclear/phagocytic lineage that are capable of multilineage differentiation as well as self-renewal. Thus, skeletogenesis results from the total contribution of all of these cell types and occurs as the result of two distinct processes in vivo: endochondral and intramembranous ossification.

3.1. Intramembranous ossification

Intramembranous ossification occurs in the flat skull bones and the clavicle and involves the condensation and direct differentiation of osteochondral progenitors into bone-forming osteoblasts [23]. The process begins with mesenchymal cell proliferation and condensation into compact nodules at the future sites of bone formation. As these condensations begin to form, mesenchymal cells within the interior of these nodules stop replicating and undergo changes in gene expression (i.e., Runx2/Cfba 1; Osx) [24,25] and associated morphological changes that are characteristic of osteoprogenitor cells. These osteoprogenitor cells begin to secrete a collagen-proteoglycan, pre-bone matrix termed osteoid that eventually becomes mineralized through deposits of minerals such as calcium and phosphates. Typically, osteoblasts remain separated from this mineralized matrix by a layer of the osteoid matrix they secrete; however, some osteoblasts remain trapped in this mineral matrix and become osteocytes. As this process proceeds further, bony spicules are formed and begin to radiate from the region where ossification began. Compacted mesenchymal cells on the exterior of these nascent bony spicules also begin to form a dense layer of vascularized connective tissue that surrounds the mineralized tissue to form the periosteum. The periosteum consists of two primary histological layers: a fibrous layer that has dense collagenous tissue and a cambium layer that is cellular and functions to provide osteoblast cells during bone expansion, regen-

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eration, and fracture repair [26–28]. The cells that remain on the inner surface of the periosteum also differentiate into osteoblasts and deposit osteoid matrix parallel to that of the existing spicules forming bone layers.

3.2. Endochondral ossification

Endochondral ossification occurs for most axial and appendicular bones (e.g., long bones) and involves the differentiation of osteochondral progenitors into chondrocytes that form a cartilage template (anlagen) that will eventually be transformed into bone tissue [23]. Endochondral ossification also begins with condensations of mesenchymal stem cells that migrate to the site of future bone formation and resemble the eventual shape of the bone that will be formed [29]. The distinction between intramembranous and endochondral bone formation lies in the fact that the mesenchymal cells found within the interior of these condensations express cartilage-specific genes [30,31] and differentiate into chondrocytes to form cartilaginous models. N-cadherin appears to be important in the initiation of these condensations, and N-CAM also seems to be critical for their maintenance [32,33]. These chondrocytes also secrete collagen II, aggrecan, and other matrix molecules that constitute the ECM of hyaline cartilage [29]. As development proceeds, further chondrocyte differentiation in the center of these anlagen results in chondrocyte hypertrophy, expression of collagen type X and fibronectin, and subsequent mineralization of the surrounding matrix. Exteriorly located perichondrial cells differentiate into osteoblasts and stimulate the invasion of blood vessels that initiate the conversion of the perichondrial layer into the periosteum where osteoblasts differentiate and secrete collagen type I and other bone matrix-specific proteins. This collar will eventually mineralize by means of intramembranous bone formation forming a bone collar of cortical bone around the periphery of the tissue [29]. The combination of matrix mineralization and vascular invasion promotes the invasion of osteoblast precursor cells, osteoclasts, blood vessel endothelial cells, and hematopoietic cells from the perichondrium into the hypertrophic cartilage. This results in the resorption of the hypertrophic cartilage, osteoblast differentiation, and bone formation, and the hematopoietic and endothelial cells establish the bone marrow stroma in what becomes the primary ossification center [34]. Eventually, this primary ossification center expands and secondary ossification centers form at the distal ends of the developing bone. This results in the development of epiphyseal growth-plate cartilage, responsible for the longitudinal growth of bones [35,36]. The epiphyseal growth plate contains chondrocytes that are organized into structural and functional zones, each with distinct gene expression patterns [37]. In the reserve zone, chondrocytes are spherical with large amounts of matrix consisting of collagen type II and proteoglycans. This zone transitions into a zone of chondrocyte proliferation, where chondrocytes appear discoid and columnar. Elongation of the cartilaginous anlagen mainly occurs from the proliferation of this zone. This zone transitions into the zone of maturation where chondrocytes become prehypertrophic. Here proliferation ceases; however, cell size increases as a result of growth. As this zone progresses, chondrocytes continue to hypertrophy and secrete a collagen X-rich

matrix until eventually they undergo apoptosis leaving behind spaces for subsequent vascular invasion and osteoblast differentiation and bone formation [29].

4. Cartilage and bone extracellular matrix

4.1. Cartilage ECM

The extracellular matrix of cartilage varies based on the type of cartilage. All cartilage types have the aggregated proteoglycan formed by the glycosaminoglycan hyaluronic acid, the chondroitin sulfate/keratan sulfate proteoglycan aggrecan, and link protein, as well as other types of proteoglycans. Cartilage tissues vary in types of collagen present, and may contain elastic fibers. The predominant fibrillar collagen in hyaline and elastic cartilage is type II collagen, while in fibrocartilage the predominant fibrillar collagen is type I. Elastic cartilage has elastic fibers comprising fibrillin and elastin [5]. Differences in the distribution of these matrix molecules are observed in cartilage, with the pericellular matrix, territorial matrix, and interterritorial matrix having distinct matrix compositions [8,38]. In addition, zones in articular cartilage (superficial, intermediate, deep, and calcified) have a distinct organization of matrix. The pericellular matrix of a chondrocyte contains proteins termed "matricellular proteins" to distinguish them from matrix proteins present in other locations. A chondrocyte with its pericellular matrix is termed a chondron [13] and the type of matrix determines the mechanophysical properties of the cartilage [12,39]. These major differences in the most prevalent matrix molecules of the cartilage are not the only distinctions between the various matrices of cartilages. Other fibrillar collagens present in cartilage include types III [40,41], V, and XI [41, 42]. Collagens IX, XII, and XIV (members of the FACIT group of collagens, or Fibril Associated Collagens with Interrupted Triple helices) are present in cartilage matrix [42,43], as is type X collagen during hypertrophy of cartilage in endochondral ossification [42]. Type IV, present in basal laminae, is found in cartilage [44], as are type VI [8,42], type VII, type VIII, and type XVIII collagen. Other proteins present in cartilage include fibronectin [45], thrombospondin or COMP (cartilage oligomeric matrix protein) [46], SPARC or osteonectin, tenascin-C, laminin [44], and the subject of this chapter, CCNs [47]. Proteoglycans in cartilage consist predominantly of the aggregated proteoglycan, with hyaluronic acid and aggrecan associated through link protein. Other proteoglycans are observed including perlecan, decorin, lumican, fibromodulin, syndecan, glypican, biglycan, and epiphycan; all may be present in cartilage in addition to aggrecan [48]. The interaction of chondrocytes with their environment, and particularly the matricellular molecules, can regulate proliferation, differentiation, shape changes, apoptosis, and motility [38,47]. In addition to direct interaction of chondrocyte cellular receptors, such as integrins, with matricellular proteins, the matrix of cartilage can bind and release soluble molecules such as growth factors that regulate many cellular functions [49].

4.2. Bone ECM

Bone ECM is a composite material that provides the bulk, shape, and strength of bone tissue through a combination of mineral, collagen, and non-collagenous protein components. However, bone ECM provides much more than just mechanical and structural support, due to its role in the developmental patterning of bone and by providing a spatial context necessary for regulating cellular behavior important for bone development and maintenance. ECM proteins can signal through cell surface receptors on bone cells to regulate cell functions, and factors contained within the ECM including growth factors, cytokines, chemokines, and extracellular enzymes can also modulate the activity of bone cells and/or affect the ECM itself [50]. Bone ECM contains numerous components (over 100 proteins) and various bone tissue compartments (e.g., periosteum, marrow stroma, epiphyseal growth plate, etc.) have unique ECM environments that play diverse roles in directing bone development through regulating the differentiation process of mesenchymal stem cells and remodeling of bone through the coupled activity of osteoclasts and osteoblasts [20]. Bone ECM can be generally broken down into collagenous components that represent the majority of the ECM proteins and non-collagenous components including proteoglycans, glycosylated proteins, small-integrin-binding, N-glycosylated proteins (SIBLINGs), Gla-containing proteins, numerous MMPs, matricellular proteins, and cell-associated proteins such as integrins and cadherins. Non-collagenous components have multifactorial roles in organizing the ECM, coordinating cell-cell and mineral-matrix interactions, and regulating the mineralization process [51]. Here, we summarize some of the key components of bone and cartilage ECM, describe their role in various tissue compartments and their contributions for bone and cartilage development, maintenance, and disease.

4.2.1. Collagenous proteins

Collagen type I—Collagen type I is by far the most abundant ECM protein found in the organic component of the bone matrix accounting for roughly 90% and is the basic building block of the bone matrix fiber network. It serves as a scaffolding substrate for mineralization and also binds and orients other matrix proteins that nucleate the mineral depositions [51]. Collagen type I is secreted by committed pre-osteoblast cells and primarily determines the material strength attributes of the skeleton, but is also involved in osteoblast lineage progression. Collage type I can bind with integrins on pre-osteoblasts to initiate signaling cascades that activate Runx2 (a master transcriptional activator of osteoblast differentiation), which controls the differentiation of osteoblasts and expression of other important bone-specific ECM proteins [e.g., osteopontin (OPN), bone sialoprotein (BSP), etc.] [52–54]. Human mutations in collagen type I result in phenotypic features of osteogenesis imperfecta (OI) [55]. OI is characterized by bone brittleness leading to a higher rate of fracture in patients with the disorder potentially from thinner or osteoporotic-like bone mass; however, the exact cause of these symptoms remains an active area of investigation [20,56]. Animal models of the disease display brittle/mechanically weak bones that possibly result from impaired or improper collagen mineralization [57] and defects in the microarchitecture of the bone structure [58,59]. More recently, recessive forms of OI have confirmed that while the quantity and structure of type I collagen

is critical to maintaining proper bone strength, posttranslational modification and assembly of type I collagen into its normal lattice structure are key regulators of bone strength as well [60].

Collagen type II—Collagen type II is the major structural protein in cartilage ECM (~85%) and the major collagen found in the growth plate during endochondral ossification [61]. It is primarily found in the matrix secreted by the reserve zone chondrocytes. More than a hundred human mutations of collagen type II have been identified; however, only heterozygous mutations causing autosomal-dominant phenotypes have been described to date due to phenotypic variations and age-dependent phenotypic transitions [62]. Type II collagen mutations give rise to a spectrum of phenotypes predominantly affecting cartilage and bone that range from severe disorders that are perinatally lethal to the milder conditions that are recognized in the postnatal period and childhood. These can include skeletal abnormalities (e.g., Stickler syndrome a.k.a. hereditary progressive arthro-opthalmopathy) or chondrodysplasias that are characterized by disproportionate short stature, eye abnormalities, cleft palate, and hearing loss [62]. Mouse models where collagen II is deleted phenotypically resemble human achondrogenesis type II and die immediately before or at birth and are smaller than their littermates [63]. Long bones from these animals lack endochondral bone and the epiphyseal growth plate and intervertebral discs are not developed [63,64]. Other mouse models with mutations of collagen type II also display growth-plate anomalies and chondrodysplasia [65]; some of these mice have similar phenotypes found in the human forms of the disease including short bones, cleft palate, and respiratory failure [62,65].

Collagen type III and collagen type V—Collagen type III is a fibrillar collagen that is found in extensible connective tissues such as vascular system, skin, gut, and lung, frequently in association with type I collagen. Collagen type V is a minor fibrillar collagen found in skin, tendons, and ligaments [66]. Both collagen type III and type V are found in bone and in trace amounts and may play a role in regulating collagen diameter. Collagen type III is expressed particularly during bone healing [51]. Human mutations in collagen type III and V are associated with Ehlers-Danlos syndrome, a disease characterized by defects in the structure, production, or processing of collagen that leads to wide-ranging symptoms in the digestive, excretory, and particularly the cardiovascular systems [66]. Collagen type III mutations are also associated with aortic and intracranial arterial aneurysms. Collagen type III null mice are embryonic lethal, but analysis of null cells in culture or heterozygous mice suggests that type III collagen may promote bone differentiation [67].

Collagen type VI is a major matricellular protein present in cartilage and most connective tissues [8, 42, 68–70]. In cartilage, collagen type VI is present in the pericellular matrix and its localization determines the boundary of this region [14]. Both integrins and the integral membrane proteoglycan NG2 can bind to this collagen in cartilage [71–74]. Differentiated chondrocytes express collagen type VI, while this expression is lost when chondrocytes undergo dedifferentiation [75]. Human mutations of collagen type VI lead to a range of disorders from a milder Bethlem myopathy to a more severe Ullrich muscular dystrophy associated with muscle weakness [76]. Knockout mice lacking type VI collagen show altera-

tions in the skeleton, including less density of bone, delayed secondary ossification, and faster development of osteoarthritis [70].

Collagen type X—type X collagen is found in cartilage, but due to its expression in hypertrophic chondrocytes found in bone at the epiphyseal growth plate, it is important for endochondral ossification [77–80]. Human mutations in COL10A1 lead to autosomal-dominant Schmid metaphyseal dysplasia [81]. Schmid metaphyseal dysplasia is phenotypically characterized by long bones that are short and curved, with widened growth plates and metaphyses [82]. Interestingly, initial studies of collagen type X null mice showed no obvious abnormalities in the development and growth of long bones [38]; however, phenotypic changes that in part mimicked Schmid metaphyseal dysplasia were observed in a second, separate collagen X null mouse line [83]. In these mice, the height of the resting zone of growth-plate chondrocytes was reduced and trabecular bone architecture was altered in the chondro-osseous junction as well as differences in the distribution and organization of growth-plate ECM components [83]. In transgenic mice models expressing dominant negative collagen type X, endochondral ossification was also effected displaying variable phenotypes [84,85]. In all of these models, the hypertrophic zone of the growth plate was compressed and the degree of compression correlated with phenotype severity [84,85]. Additionally, two other collagen X models the first where mice express collagen X containing a deletion similar to one found in human Schmid metaphyseal dysplasia patients and a second knock-in mouse with a collagen X Asn617Lys mutation displayed shortened limbs, consistent with a role in the epiphyseal growth plate [86–88].

4.2.2. Non-collagenous proteins

4.2.2.1. Proteoglycans

Bone ECM contains several proteoglycans that, other than collagens, represent the major constituents of the bone ECM. Proteoglycans are macromolecules that contain a central core protein and one or more acidic polysaccharide side chains (glycosaminoglycans) [51,89]. Proteoglycans exhibit diverse biological functions including cell proliferation, adhesion, migration, and differentiation and act as structural components in tissue organization. They can also interact with growth factors and cytokines, as well as with growth factor receptors, and are implicated in cell signaling [90]. Bone ECM contains several classes of proteoglycans that include small leucine-rich proteoglycans (SLRP), aggrecan, heparin sulfate proteoglycans (HSPGs), and hyaluronic acid [90].

Members of the SLRP family are composed of core proteins of leucine-rich repeats that are approximately 25 amino acids in length and represent the most abundant type of proteoglycans in bone. Some of the most studied in bone include biglycan decorin, fibromodulin, and lumican and these studies have demonstrated that SLRPs are involved in the structural organization of the bone ECM and regulation of growth factor activity [90]. Biglycan is distributed evenly throughout bone ECM [91] and can bind collagen type I and several important bone growth factors such as transforming growth factor-beta 1 (TGF-β1) [89]. Biglycan null mice have reduced biomechanical bone strength [92] and fail to achieve peak

bone mass due to a decrease in bone formation due to low osteoblast numbers and activity [92]. These mice also have an age-related reduction in capacity to produce bone marrow stromal cells (MSCs), reduced responses to TGF-β1, reduced collagen synthesis, and relatively more cellular apoptosis [93]. Additionally, quantitative variations in the range, mean, and distribution profiles of the collagen fibril diameters were detected [93]. Decorin null mice have no skeletal defect, with no major phenotypic changes in bone at macroscopic or histological levels; however, changes in collagen fibril size and shape in bone have been observed. However, decorin/biglycan double null mice have a more severe osteopenia than in biglycan-deficient mice, with earlier onset and severely reduced cortical and trabecular bone mass [93]. TGF-β1-binding experiments demonstrated that it bounds to decorin with high affinity and that this interaction may increase TGF-β1-receptor interaction to enhance its bioactivity [94]. Fibromodulin is a small keratan sulfate proteoglycan that is found in bone in a pericellular fashion near late-hypertrophic chondrocytes of the secondary ossification centers and in the growth plate suggesting a role during endochondral ossification. Fibromodulin null mice have no apparent skeletal phenotype but abnormal and fewer collagen fibril bundles in the tail than in wild-type animals [95,96]. Fibromodulin possesses the capacity for TGF-β1 binding [97] and levels have been correlated with decreased TGF-β1 expression in multiple fetal and adult rodent models. Recent studies suggest that fibromodulin coordinates temporospatial distribution of TGF-β ligands and receptors to modulate TGF-β bioactivity [98]. Lumican is secreted specifically by differentiating and mature osteoblasts and is a significant proteoglycan component of the bone matrix, playing an essential role in the regulation of collagen fibril formation [99]. Lumican null mice display altered collagen fibril structure [100]; however, no alteration in bone structure was reported in these mice. Interestingly, double null lumican/fibromodulin mice are smaller than their wild-type littermates and display age-dependent osteoarthritis [100].

Aggrecan is a large chondroitin sulfate proteoglycan and is the major proteoglycan component of cartilage [101,102], but it is also expressed in developing bone tissue [103]. Human mutations in aggrecan cause two types of spondyloepiphyseal dysplasia, an autosomal-dominant Kimberley type and autosomal-recessive Aggrecan type, resulting in dwarfism, skeletal abnormalities, and problems with vision and hearing, and an autosomal-dominant familial osteochondritis dissecans, which displays abnormal cartilage formation and joint issues. In mice, a natural truncating mutation of aggrecan leads to an autosomal-recessive cartilage matrix-deficiency syndrome with abnormal craniofacial structures, shortened limbs and tail, and perinatal lethality [88]. In these mice, chondrocytes are disorganized and the amount of hypertrophic chondrocytes is significantly reduced and expression of other ECM genes is altered in the growth plates of these mutant mice [104].

HSPGs act as regulators of skeletal patterning, differentiation, growth, and homeostasis and are a critical component of the hematopoietic stem cell niche within the growth plate and bone marrow [105]. Studies suggest that osteoblast precursors and osteoblasts synthesize HSPGs that both membrane- and matrix-associated HSPG are found in bone tissue and may play an important role in cell-cell interactions between fibroblast-like cells and osteoclast-lineage cells by interacting with heparin-binding growth factors, growth factor receptors, and/or other heparin-binding adhesion molecules, such as fibronectin [90,106]. HSPGs act as co-receptors

for numerous signaling molecules, such as fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), TGF-β1, and TGF-β2 in addition to various other cytokines [107,108]. The binding of these signaling molecules to HSPGs can serve a variety of functional purposes including immobilization of these factors or cytokines and/or protection against proteolytic degradation, thereby affecting their biological availability and/or modulation of their biological activity [90].

One example of the role that HSPGs can have on skeletal development can be seen with perlecan. Perlecan is a large heparan sulfate/chondroitin sulfate proteoglycan and is a ubiquitous component of basement membranes and articular cartilage, and in bone it is present in the extracellular matrix of the growth plate where it plays an important role in bone structure [109–112]. Mutations in the human perlecan gene cause autosomal-recessive skeletal disorders including the severe and lethal Silverman-Handmaker type characterized by a flat face, disorganized growth plate, cleft palate, and death at birth to the milder Rolland-Desbuquois type dyssegmental dwarfism skeletal dysplasia [81]. Schwartz-Jampel syndrome (myotonic chondrodystrophy) is a milder, progressive disease as a result of reduced perlecan levels characterized by abnormalities of the skeletal muscles (myotonic myopathy), bone dysplasia, joint contractures, and/or growth delays resulting in dwarfism [81,113]. While most perlecan null mice are embryonic lethal, surviving embryos have defects in skeletal development starting at E14.5 with disorganized growth plates, reduced proliferation of chondrocytes, reduced endochondral ossification, and reduced numbers of type II collagen fibrils [114,115]. The mice also die shortly after birth [114,115]. Additionally, it has been shown that perlecan mediates binding and delivery of FGF-2 to FGF receptors [116] and that it regulates VEGF signaling and is essential for vascularization during endochondral bone formation [117].

Hyaluronic acid (hyaluronan) is a non-sulfated linear polysaccharide present in the extracellular matrix of every vertebrate's tissue important for bone regeneration [118]. Large amounts of hyaluronan are synthesized during bone formation and it plays a role in enhancing chondrogenic and osteogenic differentiation potentials of mesenchymal stem cells [118] by regulating expression of chondrogenic markers including sulfated glycosaminoglycans, SOX-9, aggrecan, and collagen type II and osteogenic markers alkaline phosphatase (ALP), osterix, runx2, and collagen type I [119–121]. Additionally, hyaluronan synthase-2 (Has2) knockout mouse model demonstrates that it is important for spine development [122].

4.2.2.2. Glycosylated proteins (glycoproteins)

The majority of bone ECM proteins are modified posttranslationally with either N- or O-linked oligosaccharides. In addition, many glycoproteins found in bone ECM contain an arginine-glycine-aspartic acid (RGD) sequence that facilitates integrin binding important for mechanotransduction [89]. Thus, glycoprotein proteins represent an ever-growing group of bone ECM proteins with diverse functions and thus we will only focus on some key proteins in this chapter.

Osteonectin (SPARC, BM-40) is a secreted, multifunctional calcium-binding glycoprotein that participates in tissue remodeling, morphogenesis, and bone mineralization and is secreted by many different types of cells including osteoblasts [123,124]. Osteonectin can initiate miner-

alization by binding to type I collagen and synthetic hydroxyl apatite and mediating mineralization of the type I collagen [123]. It has also been demonstrated that osteonectin can bind growth factors and regulate their activity of growth factors including platelet-derived growth factor (PDGF), FGF, or (VEGF) [125,126]. It also regulates ECM and matrix metalloprotease production [127,128]. Osteonectin also regulates cell proliferation, promotes osteoblastogenesis [129], and it can stimulate angiogenesis [89]. Osteonectin-deficient mice display decreased bone remodeling with a marked negative bone balance that leads to osteopenia in older animals [20]. Bone turnover is decreased as a result of both reduced osteoclast and osteoblast surface with consequential development of low bone mass [20]. Expression of osteonectin in the intervertebral disc decreases with age [130]; mice lacking osteonectin have disc herniations [131] and may exhibit increased pain [132]. Osteonectin immunostaining is increased in osteoarthritis in cartilage as compared to age-matched controls [133], indicating a role for this protein in the pathogenesis of osteoarthritis. Pseudoachondroplasia, which is related to mutations in osteonectin, is caused by retention of this protein within the endoplasmic reticulum. This disease is an autosomal-dominant disorder that causes dwarfism [134].

Fibronectin is a high-molecular weight glycoprotein dimer that is synthesized by numerous connective tissues throughout the body and contains three alternative spliced domains. Fibronectin is produced from a single gene but as a result of alternative splicing exists in vivo in two forms: a soluble, circulating form known as plasma fibronectin that is synthesized in hepatocytes and a cellular form produced by a number of cell types including osteoblasts and gets incorporated into the bone matrix [135,136]. Fibronectin has been shown to bind to extracellular matrix components including collagen, fibrin, and HSPGs [137]. Fibronectin can also bind to 11 different integrins, six of which are expressed by osteoblasts; however, the primary adhesion integrin that fibronectin binds to on osteoblasts remains an active area of investigation [138]. It appears that fibronectin's key function is in the assembly of collagen as fibronectin is critical for collagen polymerization and matrix integrity [139,140]. Osteoblasts express fibronectin in multiple stages of their differentiation including during proliferation and differentiation concurrently with collagen type I expression. FN null mice die in utero at embryonic day 8.5, prior to skeletal development [141]. Studies using conditional null animals found that while conditional deletion of fibronectin in differentiating osteoblasts failed to show a decrease in fibronectin in the bone ECM, conditional deletion of fibronectin in the liver showed a marked decrease in fibronectin content in bone ECM associated with a decreased mineral-to-matrix ratio and changed biomechanical properties [138]. These studies suggest that while osteoblast-derived fibronectin affected osteoblast differentiation and function, fibronectin found in bone ECM originates from the liver [138].

Thrombospondins are a family of ECM glycoproteins that consist of thrombospondins 1–5 and can be divided into two subgroups: A, which contains thrombospondins 1 and 2, and B, which contains thrombospondins 3–5 (a.k.a. cartilage oligomeric protein or COMP) [142]. We will focus on thrombospondins 1, 2, and 5 (COMP) in this section. Thrombospondin 1 functions in a wide variety of physiological functions including platelet aggregation, inflammatory responses, and the regulation of angiogenesis during wound repair and tumor growth. Thrombospondin 1 binds a variety of cell receptors including CD36, CD47 (integrin-associated

protein), numerous integrins, proteoglycans, and calcium [142–145]. Thrombospondin 2 has similar physiological as thrombospondin 1, but it also plays a role in the assembly of connective tissue ECM [142], while COMP is primarily expressed in cartilage and certain other connective tissues and has roles in chondrocyte attachment, differentiation, and cartilage ECM assembly [146]. Thrombospondins 1 and 2 can each bind latent TGF-β, and unlike thrombospondin 2, thrombospondin 1 can control TGF-β bioactivity by releasing it from its latency complex [20]. Thrombospondin 1 null mice are reported to have minor trabecular bone abnormalities [147], mild spine deformation [148], and mild growth-plate cartilage disorganization [149] in addition to enhanced angiogenesis [150]. Additionally, these models suggest that thrombospondin 1 plays a role in bone homeostasis, both in mediating bone matrix integrity and by regulating OC formation as a matrix-derived paracrine signaling molecules [151]. Conversely, thrombospondin 2 null mice have an increase in MSC number, suggesting that it serves as an inhibitor of MSC proliferation [152,153]. Human mutations in thrombospondin cause a pseudoachondroplasia and a type of epiphyseal dysplasia [81]. COMP mutations cause a type of pseudochondrodysplasia and an epiphyseal dysplasia [81,154,155]. Mice lacking this protein tend to appear normal with regard to their skeleton [156], but do have issues with wound healing [157,158]. COMP may affect cellular functions by affecting the interaction of matrix and growth factors with chondrocytes [46]. Thrombospondins function primarily as anti-angiogenic proteins [159]. Thrombospondin is present in fibrocartilage of the intervertebral disc, and lack of this protein causes disorganization of this region [160]. No disc herniation was noted in these mice. Thrombospondins help to organize the growth plate [149]. In osteoarthritis, inappropriate angiogenesis within the cartilage may cause changes in the articular cartilage that lead to degradation of the matrix. Gene transfer of thrombospondin 1 using an adenoviral vector into knee joints suppressed osteoarthritis disease progression, with concomitant reductions in new vessel growth and inflammatory cell infiltration [161].

Periostin is a disulfide-linked, heparin-binding *N* terminus-glycosylated secreted protein that appears to be essential for proper ECM synthesis, particularly with respect to collagen I fibrillogenesis [162,163]. Expression of periostin occurs in many tissues including bone and cartilage and under many pathologic states. It is expressed in osteoblasts and is present in the intervertebral disc and its expression increases in degenerated human discs [130,164]. Overall, the function of periostin in cartilage is not well understood [165]. Periostin expression is also known to be prominent in fibrotic conditions, including subepithelial fibrosis in bronchial asthma [166] and in bone marrow fibrosis [167]. Periostin null mice display severe growth retardation, suggesting that periostin is essential for postnatal development. Histological analysis of the periostin knockout mice demonstrated severe incisor enamel defects, periodontal disease, a lack of trabecular bone, cartilage, and cardiac valve defects; however, some of these phenotypes might be secondary due to eating difficulties as a result of the lesions in the periodontium [168].

Alkaline phosphatase is a glycoprotein enzyme that is found both bound to cell surfaces and also within the mineralized matrix [51]. Human ALP is classified into four isoenzyme types, tissue-nonspecific (TNAP), intestinal, placenta, and germ cell of which the TNAP type is ubiquitously expressed in many tissues, including bone and is the form implicated in its

biomineralization [169]. Mineralization of cartilage, bone, and teeth occurs by a series of physicochemical and biochemical processes that facilitate hydroxyapatite deposition (mineralization) into collagen fibrils of the matrix [170] and/or within the lumen of chondrocyte and osteoblast-derived matrix vesicles [171,172] through hydroxylation of pyrophosphate providing inorganic phosphates to promote mineralization [169]. Deactivating mutations in the TNAP gene causes the inborn error of metabolism known as hypophosphatasia [173], characterized by poorly mineralized cartilage (rickets) and bones (osteomalacia), spontaneous bone fractures, and elevated extra-cellular inorganic pyrophosphate (PPi) concentrations [174,175]. TNAP null mice also display skeletal disease at approximately 10 days of age and featured worsening rachitic changes, osteopenia, and fracture [176]. Histologically, these mice displayed developmental arrest of chondrocyte differentiation in epiphyses and in growth plates with diminished or absence of hypertrophic zones with progressive osteoidosis from defective skeletal matrix mineralization [176].

4.2.2.3. Small-integrin-binding, N-glycosylated proteins (SIBLINGs)

SIBLINGs are classified by the presence of the RGD sequence and large amounts of sialic acid known as small-integrin-binding, N-glycosylated proteins (SIBLINGs) [29]. SIBLINGs, along with other matrix proteins, are thought to play a role in cell attachment facilitated by transient or stable focal adhesions to ECM molecules mediated by cell surface receptors. They also play a role in intracellular signaling. Although these SIBLINGs were initially only found in mineralized tissue, many of them can now be found in other tissues [177,178]. Bone cells synthesize at least five SIBLING members including OPN, BSP, dentin matrix protein-1, dentin sialoprotein, and matrix extracellular phosphoprotein. Here, we discuss on OPN and BSP.

Osteopontin (OPN; BSP-1) is a highly negatively charged bone ECM protein that can undergo extensive posttranslational modification mainly phosphorylation [179]. OPN is one of the most extensively studied SIBLING proteins and has broad physiological and pathological functions including development [180], bone remodeling [181], immune function [182], fibrosis [183], and cancer [184]. It is expressed by a wide range of cells and promotes attachment, proliferation, migration, chemotaxis, and apoptosis of macrophages, lymphocytes, osteoblasts, and a range of tumor cells [185–187]. OPN binds calcium and is a key regulator of hydroxyapatite nucleation and is produced by osteoblasts during their terminal differentiation prior to matrix mineralization. OPN regulates bone mass and overall bone quality by minimizing strain-induced fatigue damage and microcrack propagation in bone [188]. In addition, it can also mediate the attachment of osteoclasts and can affect the shape and size of hydroxyapatite crystals in the bone ECM [189–191]. OPN can mediate signaling through integrins, for example, it can bind to $\alpha 4\beta 1$ integrin and trigger a cell type-specific integrin-mediated signaling cascade [192]. OPN null mice showed no bone phenotype [193], but in stress situations, such as oophorectomy, the mice do not develop osteoporosis [91]. Recent data confirm and extend this observation that the skeleton of OPN null mice does not respond properly under stress underlining the importance of OPN in bone metabolism [194,195]. However, other studies have demonstrated that OPN null mice do have a bone phenotype also under physiological

conditions [196–198]. More recent OPN null models demonstrate that while bone volume was normal in young null animals, the volume and number of osteoclasts were increased, but that osteoclasts from these mice have a lower resorptive capacity providing evidence for a role of osteopontin in osteoclast activity [199].

BSP-2 is a SIBLING glycoprotein and is expressed in chondrocytes, hypertrophic cartilage, and in osteoblasts at the onset of mineralization and in osteoclasts [200]. BSP is highly expressed at sites of primary bone formation [201], and it coincides with the initial formation of membranous and endochondral bone, the maximal level being reached during the formation of embryonic bone [202]. BSP-2 binds calcium; however, it does not nucleate the hydroxyl apatite found in the bone ECM and BSP-2 mediates cell attachment through interaction with vitronectin receptor [29]. BSP-2 null mice are shorter than their wild type counterparts and display a low level of bone remodeling, with both bone formation and mineralization severely impairing in vivo and in vitro models [203]. These mice also have lower osteoclast numbers and surfaces in vivo; osteoclast recruitment and activity in vitro were impaired and impairment of chondrocyte proliferation was suspected [203,204].

4.2.2.4. Gla-containing proteins

Gla-containing proteins refer to a group of endogenously made bone ECM proteins that undergo vitamin K-dependent gamma-carboxylase modification. The dicarboxylic glutamyl (gla) residues enhance calcium binding. The formation of gamma-carboxy-glutamic acid (Gla) occurs via a unique posttranslational modification of specific peptide-bound glutamate residues, which is required for the biological activities of these proteins.

Osteocalcin (OCN) is one of the most abundant non-collagenous proteins of the bone ECM. It is produced by differentiated osteoblasts [205,206] and once transcribed undergoes posttranslational modifications within osteoblasts that include the carboxylation of three glutamic residues [207]. Vitamin D stimulates osteocalcin transcription and vitamin K regulates the carboxylation processes. Various growth factors, hormones, or cytokines can also modulate osteocalcin production [206]. Osteocalcin is secreted by osteoblasts during active bone formation and can bind with the mineralized bone ECM [207]; however, its exact role in bone physiology remains an active area of investigation. Osteocalcin promotes the recruitment and differentiation of circulating monocytes and osteoclast precursors, suggesting its role on osteoblast-osteoclast interaction and bone resorption [206–208] and other studies have shown that osteoclast resorption is impaired in OCN null bone [208]. However, OCN null mice have a higher bone mineral density without any change in bone resorption and mineralization [209]. Recently, tissue-specific transgenic mice with osteoblast-specific overexpression or reduced OCN production suggested that OCN might have an important endocrine function for glucose metabolism and lipid homeostasis [210,211].

5. The CCN family

The CCN family makes up a group of six highly conserved, secreted, extracellular matrix-associated proteins that regulate diverse cellular functions, including skeletal development, wound healing, fibrosis, and cancer. Originally named after the three identified members—cysteine-rich 61 (Cyr61, CCN1), connective tissue growth factor (CTGF, CCN2), and nephroblastoma overexpressed (Nov, CCN3)—this family also includes the Wnt-induced secreted proteins 1–3 (i.e., WISP1/CCN4, WISP2/CCN5, and WISP3/CCN6). Members of the CCN family share a unique and conserved modular structure and interact with and orchestrate cellular responses to extracellular factors via direct binding to cell surface receptors, including integrins, Notch1, neurotrophic tyrosine kinase receptor type 1 (TrkA), low-density lipoprotein receptor-related proteins (LRPs), and HSPGs. CCN proteins can also mediate biological functions by interacting with growth factors such as tumor growth factor beta (TGF-β), vascular endothelial growth factor, and bone morphogenetic proteins (BMPs) and by associating with other ECM proteins including fibronectin and fibulin 1C. Through these interactions, CCN proteins serve both distinct and overlapping biological roles. Consequently, deregulation of their expression or activities contributes to the pathobiology of several diseases, many of which may arise when inflammation or tissue injury becomes chronic, including vascular diseases, fibrosis, arthritis, and cancer.

5.1. Structure and function of CCN family members

CCN proteins are cysteine-rich and share a modular structure (Modules I–IV), with an N-terminal secretory peptide followed by four conserved domains with sequence homologies to insulin-like growth factor-binding proteins (IGFBPs), von Willebrand factor type C repeat (VWC), thrombospondin type I repeat (TSP1), and a carboxyl-terminal domain (CT) that contains a cysteine-knot motif [212]. The order of these modules has been strictly conserved during evolution, suggesting that it is critically important for these proteins. Each module is involved in protein binding and contains conserved hydrophobic, polar, and cysteine residues. Module I shares 32% sequence homology with the N-terminal cysteine-rich regions of the IGF-binding proteins and contains a GCGC-CXXC motif that is involved in IGF binding. Module II includes a stretch of 70 amino acids with sequence identity to von Willebrand factor as well as various thrombospondins, collagens, and mucins [212]. This domain has been shown to mediate protein oligomerization [213]. Module III is a TSP1 repeat that contains the WSXCSXXCG motif, which is thought to be implicated in the binding of sulfated glycoconjugates and to be important for cell attachment [212,214]. The last module, Module IV, occurs at the carboxy-terminus of various extracellular proteins and is the least conserved of the four domains at the nucleotide sequence level. It consists of several cysteine residues that adopt a cysteine-knot motif. This motif comprises a complex structure of two-stranded β-sheets that lie face to face and are linked by three interlocking disulfide bridges [215] and occurs in TGF-β, PDGF, and nerve growth factor (NGF). It is critical for several of the biological functions of CCN proteins and is thought to mediate dimerization and binding to cell surface receptors. CCN5 is the only family member that lacks the CT domain [216]. A variable, central hinge

region that is susceptible to proteolytic processing by MMPs and other proteases links the amino-terminus and carboxy-terminus of these proteins, yielding two halves that bind distinct cell surface receptors [217]. It is not clear whether the individual properties of each of the four modules govern the biological properties of the CCN protein or if it is the combination of the modules and other sequences within the protein that do so. However, all of the modules are highly interactive with a number of other molecules, which include cell surface receptors, ECM components, growth factors, and structural proteins [218].

5.2. Cell surface receptors mediating CCN functions in cartilage and bone

Despite the structural similarities that CCN proteins have to other protein domains as described above, their interactions are unique because of their ability to bind extracellular factors via their modular domains. CCN proteins have been shown to interact specifically with cell surface receptors such as HSPGs, integrins, and LRPs, accounting for their ability to regulate numerous cellular functions. CCN2, the most studied of the CCN family members, shares common functionality with CCN1 with respect to interaction with integrins and HSPGs, causing comparable biological effects. LRP1 is another common receptor shared by CCN2 and CCN1; however, the target cell and biological consequence differ between the two [219,220].

Direct binding of CCN proteins to integrins present on cellular surface drives many of their effects on cartilage and bone. Integrins comprise a large family of cell-cell and cell-matrix receptors that signal both from the ECM to the cytoplasm and from the cell to the matrix (inside-out and outside-in) [221]. Integrins are $\alpha\beta$ heterodimers and can be present in a number of configurations. The different combinations of α and β receptors define what ECM molecules a cell interacts with. These receptors regulate many cellular functions such as proliferation, differentiation, motility, and developmental processes among others [221]. A decrease in the interaction of β integrins with matrix molecules is observed during the pathogenesis of osteoarthritis, and thus the disruption of integrin-matrix interaction causes cellular dysfunction in cartilage [222]. Integrins that have been identified in cartilage include $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_6\beta_1$, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_3\beta_1$. As is the case with matrix molecules, integrins are expressed differentially in specific regions of cartilage and during development and pathogenesis.

Other matrix receptors are present in cartilage. One example is NG2, a transmembrane proteoglycan in cartilage with the matricellular protein type VI collagen as its ligand [72–74]. NG2 interaction with type VI collagen may be an important interaction in determining the progression of sarcomas [223]. Annexin V, or anchorin II, binds to type II collagen and is mainly expressed in the superficial zone of articular cartilage [224]. CD44 binds to hyaluronic acid; blocking of this receptor causes a loss of the matrix of cartilage [225].

As a matricellular protein, CCN2 also binds the fibronectin receptor ($\alpha_5\beta_1$) and aggrecan, which are major components of the ECM [226]. The interaction of $\alpha_5\beta_1$ with fibronectin in cartilage causes loss of the differentiated state [227]. Blocking of this integrin causes a loss of the differentiation of pre-hypertrophic chondrocytes [228]. A knockout mouse that has loss of β_1 specifically in chondrocytes exhibits a disease similar to chondrodysplasia [222]. β? is the most expressed integrin β subunit in osteoarthritic chondrocytes [229].

CCN2 is induced by signaling from retinoids in cartilage in the growth plate [230]. CCN2 causes differentiation of the chondrocytes and is important for matrix deposition and signaling from cellular receptors [226,231]. Retinoids are important signaling molecules involved in the cartilage becoming hypertrophic during endochondral ossification.

5.3. CCNs in chondrogenesis and osteogenesis

The CCN proteins appear to play a critical role in the establishment of the skeletal system and have been shown to have positive and negative regulatory roles in skeletal development, as demonstrated through cell culture experiments and animal models. Collectively, these studies demonstrate that CCN proteins promote differentiation and proliferation of chondrocytes, osteoblasts, and vascular endothelial cells, which are important in both endochondral and intramembranous ossification.

Cartilage is first seen in the developing embryo in the areas that serve as templates for endochondral ossification, as well as in Meckel's cartilage in the head area [1]. Both CCN1 and CCN2 promote chondrogenic and osteoblastic differentiation [232,233]. CCNs can interact with members of the TGF-β and BMP family via the chordin-like homology found in the VWC domain, and modulate their binding affinity for their respective receptors [234]. CCN2 is important during embryogenesis and bone formation, including during proliferation of mesenchymal cells, their differentiation into chondrocytes, and condensation of these cells into the cartilage model that will form bone [1,235]. CCN2 expression is highest in the vascular tissue and the maturing chondrocytes of the embryo. TGF-β, which induces CCN2, directs the condensation of these cells and regulates secretion of other matricellular proteins such as fibronectin [235]. Cells from CCN2 knockout mice do not undergo this condensation process in vitro and have less synthesis of cartilage proteoglycans [236]. Neutralizing antibodies, siRNA, and antisense oligonucleotides have all been used to demonstrate that reduced or absence of CCN2 prevents condensation from occurring [1,235]. Overexpression of CCN2 causes an increase in the length and density of bones [237]. In vertebral bodies, downregulation of CCN2 is necessary for chondrocyte differentiation [235]. These studies demonstrate that the spatial and temporal regulation of CCNs is an important component of development.

Development of cartilage involves several different transcription factors, such as Sox 9, that control this process, as well as growth factors that interact with cellular receptors to facilitate various cellular functions [2]. Interaction with the extracellular matrix has been implicated in development as well. Various epigenetic mechanisms and several different microRNAs also are important in the development of cartilage [3,4].

BMPs are important during skeletogenesis [238] and are known to be regulated by CCN2 [234]. Expression of CCN2 is observed in both the perichondrium and the chondrocytes during development [239]. BMPs play a role in the repair of cartilage as well as during development [240]. BMP2 causes proliferation of chondrocytes during development, while BMP4 does not [241]. CCN2 and CCN3 can bind BMP2 and abrogate its ability to promote chondrogenic and

osteogenic differentiation [242,243], respectively, whereas CCN4 binding to BMP2 enhances its function in osteogenesis [244]. CCN2 also binds BMP4 and blocks its signaling capabilities [234]. This binding thus modulates the activity of BMP-9. CCN2 also modulates the Wnt pathway in *Xenopus* embryos by binding to LRP6 co-receptor [245]. CCN2 expression in chondrocytes is controlled by both Rac 1 and actin pathways that are mediated by TGF-β/Smad signaling. CCN4 is expressed in developing mesenchymal, pre-osteoblastic, and cartilage cells and is believed to regulate skeletal growth and repair [246]. A truncated form of CCN4, WISP1v, has been shown to regulate the differentiation of chondrocytes toward endochondral ossification [247].

Chondrocytes are usually considered quiescent in the adult, although this can change during pathological processes such as osteoarthritis. Cartilage has very little capacity to regenerate, even though progenitor cells are present in the adult [6]. This lack of repair ability causes a number of disease issues as humans age, including the most prevalent form of degenerative joint disease, osteoarthritis [7]. In adult skeletal systems, CCN2 is highly expressed in the osteoblasts lining metaphyseal trabeculae and in osteogenic surfaces lining fracture calluses, suggesting that its upregulation in these areas may contribute to bone growth and fracture repair [248].

5.4. CCNs in cartilage and bone pathology

Several types of pathologies are observed in cartilage and bone. In cartilage, the most common is osteoarthritis, which significantly increases as the population ages. Disorders of the intervertebral disc are observed as well, and also increase with aging. Wounding and subsequent repair of cartilage occurs and can mimic the progression of osteoarthritis. Various types of chondritis, or inflammation of cartilage, are observed in various regions of the body. Congenital defects in cartilage include conditions such as chondrodysplasia, an example of which would be achondroplasia, causing dwarfism. Finally, chondromas or chondrosarcomas, tumors that can either become or are cancer, can occur within this tissue.

5.4.1. Intervertebral disc

CCN2 is highly expressed in the cartilage present in the intervertebral disc, just as it is in other types of cartilage. The intervertebral disc contains the nucleus pulposus, and this is surrounded by the annulus fibrosus composed of fibrocartilage. The annulus fibrosus contains mainly the fibrillar collagen type I, although type II collagen is present along the inner surface as in reference [249]. Proper function of the annulus fibrosus is important in disc function, and lack of intact matrix can lead to disc instability [249]. Herniation of the nucleus pulposus is sometimes implicated as a cause of back pain [11] and increased expression of CCN2 can be correlated with discs that are painful [250].

5.4.2. Osteoarthritis and cartilage wound healing

Osteoarthritis is the most common form of degenerative joint disease [7]. During the disease process, chondrocytes undergo cell death due to lack of ability to renew, particularly with

aging [251]. The initial degenerative changes that occur are termed chondropenia, although this is not often identified as osteoarthritis [252]. Upregulation of the catabolic pathways occurs, causing cartilage degradation, and alteration of the phenotype of the chondrocytes is ultimately responsible for loss of articular cartilage [253]. Much of this change in cartilage structure and function occurs via interactions with matrix and signaling from both matrix and growth factors [254,255]. Initial disease presents with an upregulation of collagen type II synthesis, and a change in collagen type from type II to types I and III [40,256]. A decrease in aggrecan is observed during the pathogenesis of osteoarthritis, while other proteoglycans such as decorin and fibromodulin are increased [257]. Aging causes decreased matrix deposition subsequent to increases in degradation by MMPs as well, causing additional issues with tissue integrity [255]. MMPs and aggrecanase are upregulated during osteoarthritis disease progression [258] causing additional cartilage loss. These processes are very similar to what occurs following an injury to cartilage and its subsequent repair [259].

CCN2 is increased in chondrocytes isolated from human osteoarthritis cartilage [260–262]. Increases in this matricellular protein can lead to fibrosis, and it is possible that mechanisms used to deliver CCN2 to degenerating discs may cause some repair to occur [249] similar to what is seen in an experimental model of osteoarthritis [263]. CCN3 has been shown to be upregulated in an osteoarthritis mouse model [264] and in human osteoarthritis as well [265]. Mutation of CCN3 in mice causes a loss of normal joint function and disease that appears similar to osteoarthritis in humans [266].

5.4.3. Chondromas/chondrosarcomas

A chondroma is a benign tumor of cartilage that may be present within bone and can cause fractures due to its growth pattern. Normally, little is done with these tumors unless there is a danger of fracture. Chondrosarcomas are cancerous tumors that can occur at any age, unlike other sarcomas. These are refractory to treatment and can be highly metastatic. The expression of CCNs can be correlated with chondrosarcoma grades and thus may be useful in clinical identification of these tumors [267]. The same may be true for chondromas as well.

5.4.4. Osteosarcomas

Osteosarcoma is the most common type of cancer that develops in bone, occurring most frequently in children and young adults. Most primary tumors develop in the areas of bone that are growing rapidly such as near the ends of long bones surrounding the knee—the distal femur or the proximal tibia. The proximal humerous is the next most common site, although osteosarcoma can develop in any bone. Evidence suggests that osteosarcoma might originate from mesenchymal cells with osteoblastic features [268,269]. CCN1 expression correlates with poor prognosis of osteosarcoma and overexpression of CCN1 increases cell proliferation and metastatic potential of tumor cell lines [270] and CCN1 knockdown reverses this phenomenon [271]. In humans, CCN3 is associated with increased lung metastasis in osteosarcoma patients [272] and CCN4 expression was shown to be higher in bone from osteosarcoma patients compared to normal tissue [273].

5.4.5. Ewing sarcoma

Ewing sarcoma is the second most common type of bone cancer that predominantly affects children. CCN3 is expressed in approximately 30% of Ewing Sarcoma cases and its expression correlates with lower survival [272]. Elevated CCN3 expression also correlated with recurrences and metastases compared to primary tumors in a study that examined 170 human Ewing sarcoma specimens by immunohistochemistry. In this same study, a low level of CCN3 expression was associated with improved patient prognosis [274].

5.4.6. Chondrodysplasias

Mutations in the major collagen present in hyaline and elastic cartilage, type II, cause several chondrodysplasias [81]. Mutation of type XI collagen also causes skeletal issues due to issues with the structure of the growth plate, in mice with this defect [275]. Mice lacking collagen IX appear normal but have alterations in the cartilage forming the growth plate and ultimately these mice develop osteoarthritis when older [276]. Mutations cause skeletal issues in humans as well, as do mutations in type X collagen. Mice lacking collagen X have several phenotypes, including no obvious changes, metaphyseal dysplasia, and death soon after birth [81,83,277].

5.4.7. CCNs in cartilage and bone pathophysiology

The role of CCNs in cartilage function involves several family members of this class of matricellular proteins. CCN1-6 have all been identified in cartilage matrix, and all CCN genes have an increase in their expression in osteoarthritis or rheumatoid arthritis [265]. CCN3 is integral to the proliferation of chondrocytes, while CCN1, CCN2, and CCN6 are involved in later states of maturation, proliferation, and the calcification of cartilage matrix during endochondral ossification. CCN4 and CCN5 also participate in the differentiation and calcification of cartilage [231]. CCN2 causes proper bone strength, shape, and length, while the counteraction by CCN3 regulates these structural processes.

CCN1 knockout mice die during development, making analysis of their skeleton difficult; however, this protein causes proliferation of chondrocytes and secretion of matrix in vitro [278]. CCN1 is present in chondrocytes in the proliferative and pre-hypertrophic zones during endochondral bone formation [278]. CCN1 signals through the WNT signaling pathway to cause maturation of chondrocytes isolated from sternal cartilage, with overexpression of CCN1 causing damage to chondrocytes [279]. Induction of CCN1 by β-catenin causes maturation of chondrocytes, while overexpression using a cartilage-specific promoter causes chondrodysplasia [279]. CCN1 is decreased in expression during the differentiation of mesenchymal stem cells into chondrocytes and osteoblasts, indicating that it may be important in the maintenance of stem cells [280].

CCN2 promotes differentiation and proliferation of chondrocytes as well as osteoblasts (see reference [47]). The presence of CCN2 is an important modulator of the deposition of cartilage matrix. CCN2 also is a major factor in the induction of fibrosis [281]. This growth factor causes secretion of collagen type II and can induce cells to proliferate and subsequently differentiate [282]. Lack of CCN2 causes issues with bone growth due to lack of hypertrophic zone cartilage

growth and loss of angiogenesis [283,284] and causes death immediately after birth due to respiratory failure [239]. CCN2 binds several growth factors, including bone morphogenetic proteins and TGF-β, and can affect cartilage function in this manner [226].

Addition of CCN2 to damaged cartilage in rats can cause enhanced healing [263]. Although exogenous administration of matrix metalloproteases can cause damage to cartilage similar to osteoarthritis [285,286], the use of these inhibitors does not aid in the treatment of the disease [287]. Overexpression of CCN2 using a col2a1 promoter caused reversal of some aging-related changes present in the articular cartilage of aged mice by enhancing matrix deposition and proliferation of chondrocytes. In addition, changes characteristic of cartilage degeneration were reversed, such as the expression of collagens I or X and the presence of MMPs 9 and 13 [288]. These mice also showed greater levels of matrix and faster ossification during endochondral bone growth [237].

CCN2 binds to receptor activator of NF-kappa B (RANK) as seen by plasmon resonance analysis, and enhances RANK signaling. This indicates its importance in the formation of osteoclasts [289].

Fibroblast growth factor causes an increase in BMPs, and this binding causes repair of articular cartilage defects. Different BMPs have differing effects on this repair, but overall BMP3 is an important component during repair of articular cartilage [279].

CCN3 decreases proliferation in several different cell types [290]. CCN3 appears to be downregulated by PTHrP [231], which is involved in the growth of bone. CCN3 may regulate apoptosis under conditions of serum deprivation. The presence of this CCN decreased levels of both proteoglycan and collagen, which may mimic the cartilage environment in which no vascularization occurs and thus conditions are somewhat hypoxic [291]. Bone regeneration in mice that lack CCN3 is enhanced [292]. Loss of CCN2 causes an increase in the expression of CCN3 with a concomitant decrease in proliferation due to the presence of this matricellular protein. This deletion also caused reduced differentiation of chondrocytes due to the upregulation of CCN3 [231]. Loss of CCN2 causes reductions in aggrecan and types II and X collagen during development, a process which mimics the matrix loss that occurs with aging during osteoarthritis development [226]. Overexpression of CCN2 has effects that counter these reductions [288]. Overexpression of CCN4 caused an effect on cartilage differentiation by changing the function of another growth factor, TGF-β3. Mice that completely lacked CCN4 did repair surgical defects well, while mice that expressed CCN4 demonstrated some recovery from this injury [293]. Lack of another member of the CCN family, CCN6, causes a disease in humans that is a form of childhood arthritis, progressive pseudorheumatoid dysplasia [294]. The function of CCN6 in normal cartilage is not well understood, although its expression is high in osteoarthritis [295].

CCN1 and CCN2 expression is elevated during fracture repair in the long bones throughout the reparative phase of the callus, notably in proliferating chondrocytes and osteoblasts [296,297]. Abrogation of CCN1 by antibodies inhibits bone fracture healing in mice [298]. Further, recombinant CCN2 protein promotes the repair of articular cartilage in a rat osteoar-

thritis model [263]. These studies suggest that CCN proteins may play important roles in the homeostasis of bone and cartilage tissues.

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