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IMPORTANCE OF SELECTED PROTEINS OF COMPACT BONE TISSUE IN POULTRY

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ABSTRACT

Within modern poultry lines, the integrity of the skeleton is subjected to increasing genetic and production stress, which in many cases leads to different health problems of the skeletal system, including problems with osteoporosis and the development of fractures. The role of genetics in bone integrity has been demonstrated by several studies, while the knowledge gained from the targeted study of genes, i.e., proteins that play an important role in bone metabolism, is of great value both for skeletal health and may provide new clues to the biological processes underlying diseases leading to the weakening of the bones. In addition to summarizing basic knowledge about bone metabolism, this review provides insight into the structure and function of proteins that are part of compact bone tissue, focusing on non-collagenous proteins and proteins that are encoded by genes involved in signaling pathways that play an important role in bone metabolism.

Keywords: bone, bone metabolism, protein, gene, signaling pathway

INTRODUCTION

Birds are adapted morphologically and physiologically very well to locomotion, when their typical movement is flight. The whole range of these adaptations makes them a very specific and quite diverse group of vertebrates (**Serrano *et al.*, 2020**). Therefore, the skeleton of birds must be lightweight to minimize the metabolic expenditure of flight and strong enough to withstand the forces with which it comes into contact. The evolution of skeletal structures is governed by these principles, where both the shape of bones and the material properties of bone tissue make bones strong and rigid, but also fragile. There was gradual reduction, loss, and fusion of many skeletal elements and expansion of pneumatized spaces in some bones (**Dumont, 2010**).

BONE STRUCTURE

Bone, as a very dynamic organ, has several functions within the skeleton. In addition to fulfilling the function of structural support of the body, where it is exposed to various loadings of movement, it is furthermore the interface between muscles and protection of internal organs. It is a reservoir of calcium and phosphorus as well, with almost 99 % of the body's total calcium and 80 % of phosphorus found in the skeleton, which is very important in the terms of reproduction (**Conti *et al.*, 2023; Wu *et al.*, 2021**). Approximately one-third of bone is made up of organic compounds, while two-thirds is made up of the inorganic part, i.e., the mineral matrix. Collagen (primary type I) makes up approximately 85 to 90 % of the organic component of bone. It gives bones resistance against tensile forces when resistance against compressive forces is provided by proteoglycans. Another organic components are non-collagenous proteins such as osteocalcin and osteonectin and glycoproteins such as osteopontin. Mineral matrix consists of small crystals of hydroxyapatite, calcium phosphate and mineral calcium salts, which give the bones the right mechanical properties and stiffness. Up to 70 % of inorganic salts are arranged in the hydroxyapatite lattice structure, within cortical bone. The bone structure, thus the necessary components of the bone, must be stored in such a form that it can be easily removed and replaced by cellular control depending on the organism's requirements, while the mineral component must remain insoluble. Bone can respond to load, including stress, by remodeling, so its internal organization can vary considerably, even if the general shape is controlled and given genetically **(Anatomy and radiography, 2022; Henry & Bordoni, 2022; Johnsson *et al.*, 2015; Sobczak *et al.*, 2009**).

TYPES OF BONE TISSUE

Cortical and cancellous bone, i.e., trabecular, or spongy, which are synonyms for cancellous bone, are classifications of two types of bone that can be distinguished at the macroscopic level. Strength, stiffness, and movement are ensured by compact cortical bone tissue. Trabecular bone tissue is soft, flexible, while providing a reduction in total bone mass and cortical bone support (**Koju *et al.*, 2022**). The microscopic structural unit of compact bone is called an osteon or Haversian system. Each osteon is composed of concentric rings of calcified matrix called lamellae. A central canal or Haversian canal runs through the center of each osteon, which contains blood vessels, nerves, and lymphatic vessels. The structure of trabecular bone makes its surface area per unit volume larger, but more labile, while the rate of metabolic activity is also higher, compared to cortical bone, which has a higher true volumetric density. Cancellous bone is found throughout the bird's skeleton as thin, intersecting lamellae within cortical bone. In adults, it is filled with fatty bone marrow, or forms air-filled cavities, while in young, growing individuals, it is filled with red bone marrow (**Anatomy and radiography, 2022; Caon, 2018; Prince & Draper, 2000**). Another type of special endosteal tissue is non-structural medullary bone, which is naturally formed under the influence of estrogenic and androgenic hormones in the hematopoietic medullary cavities of bones only in female birds during egg-laying, i.e., in the reproductive phase, and serves as a calcium reservoir that can be quickly mobilized for the formation of eggshells (**Prondvai, 2017**). This highly vascularized bone tissue closely resembles cancellous, or embryonic bone, while lacking the Haversian system. It is formed as a trabecular meshtwork, when individual spicules grow from the endosteal surface of long bones, which are connected to each other. Also due to its function, it is characterized by containing less collagen compared to cortical and trabecular bone (**Canoville *et al.*, 2020**).

BONE REMODELING AND CELLS INVOLVED IN THIS PROCESS

Bone, as a living tissue, is not inert and undergoes constant change during life. This process of change, which continues with varying intensity throughout life, is known as remodeling. It includes the bone formation and destruction, i.e., the resorption of old or damaged bone, all depending on the degree of mechanical load, which leads either to the strengthening of the bone architecture or to the weakening of the bone layers, while anabolic and catabolic processes are usually balanced. It therefore protects the structural integrity of the bone system, while also helping to maintain the balance of calcium and phosphorus. In the case of adults, there is a loss of bone mineral density due to the predominance of destructive processes of the skeleton. In remodeling, two primary cells are mainly important, which are

osteoblasts and osteoclasts, but osteocytes are also significantly involved (**Rowe *et al*., 2022**; **Wawrzyniak & Balawender, 2022; Zhou *et al.,* 2008).** These cells originate from the mesenchymal and hematopoietic lineage of stem cells, while this fact emphasizes both the very close relationship between bone and the immune system and the unique regulation of bone homeostasis (**Morgan *et al.,* 2013**). Two types of ossification take place during the bone formation process. Within both types, mesenchymal progenitors condense and initiate evolutionary schemes, including chondrogenesis and osteoblastogenesis. The first type is endochondral ossification, where the cartilage growth plate is gradually replaced by bone. This cartilage growth plate was formed by chondrocytes that differentiated from mesenchymal cells. The second type is intramembranous ossification, which lacks the cartilage intermediate and in which bone organization occurs by direct differentiation of mesenchymal stem cells into osteoblasts. The balance between osteoblasts, which are responsible for bone formation, and osteoclasts, which resorb bone, maintains bone integrity and function (**Shahi *et al*., 2017; Chen *et al*., 2012; Zhang, 2010**).

In the case of physiological conditions, the basic and primary role of osteoblasts is the formation of new bone, both in the developing skeleton and in the remodeling process by synthesizing and then depositing organic bone matrix proteins, which further mineralize. These proteins are, e.g., collagen type I, osteocalcin, bone sialoprotein and osteopontin. Osteoblast precursors are two lineages of embryonic mesenchymal stem cell populations. These initially differentiate into osteoprogenitor cells in a process that requires the action of transcription factors. One such that has been identified as essential for their differentiation is Runt-related transcription factor 2 (Runx2) of the Runx family of transcription factors, osterix or core-binding factor alpha-1 (CBFA1). Subsequently, osteoprogenitor cells differentiate into osteoblasts under the influence of bone morphogenic proteins, insulin-like growth factor-1, -2, various enzymes, hormones, and other growth factors. After the formation of new bone, some osteoblasts die by apoptosis. Even before the establishment of cell differentiation, the apoptosis of osteoblast precursor cells can be suppressed through Wnt proteins, which enables the subsequent differentiation of osteoblasts, when bone formation is supported precisely by the Wnt1 protein. Other osteoblasts become quiescent lining cells on the surface of the bone after changes in shape, with the majority differentiating into osteocytes. Both osteocytes and osteoblasts communicate with each other through extensive cytoplasmic processes. They are also a significant source of receptor activator of nuclear factor-κB ligand (RANKL), osteoprotegerin (OPG) and macrophage colony-stimulating factor (M-CSF), which regulate the differentiation of osteoclasts (**Henry & Bordoni, 2022; Maeda *et al.,* 2022; Ottewell, 2016; Soltanoff *et al.*, 2009**). Up to 95% of bone tissue is made up of a cell type that is derived from the osteoblast cell line, osteocytes, where the mechanisms that cause only some osteoblasts to differentiate into osteocytes are not fully understood. From the bodies of these cells, which are located in an ellipsoidal space called lacuna, distinct dendritic processes emerge and connect with each other, as well as with other cells on the surface of the bone and with nearby blood vessels. Dendritic processes reside in canaliculi, which are small cylindrical canals. According to some authors, osteocytes play a key role in the regulation of the dynamic nature of bones. Osteocytes are also the source of the RANKL, when it was proven through experiments in mice that those lacking the *RANKL* gene had significantly increased bone mass. They also produce a number of other proteins such as dentin matrix protein 1, phosphate-regulating neutral endopeptidase on chromosome X (PHEX), matrix extracellular phosphoglycoprotein, sclerostin, fibroblast growth factor-23 (FGF-23) and OPG. This makes osteocytes cells that are involved in many processes related to mineral regulatory functions and bone remodeling (**Maeda *et al*., 2022; Takano-Yamamoto, 2014; Schaffler & Kennedy, 2012; Nakashima *et al*., 2011).**

Osteoclasts, as multiple giant cells, are the only cells capable of degradation, i.e., resorption of mineralized matrix. Osteoclast precursors are monocyte/macrophage haematopoietic lineage. Differentiation of osteoclasts, which are then found only on the surface of calcified matrix, requires the expression of the RANKL and the M-CSF cytokines, which is strictly controlled by osteoblasts and osteocytes. Through intracellular kinase cascades and nuclear genetic programs coordinated by basic transcription factors, which is initiated by a RANKL-RANKL interactions, there is terminal differentiation to osteoclasts. For the purposes of suppressing bone resorption, through the inhibition of the RANKL-RANK interaction, osteoblasts and osteocytes exclude OPG. The importance of the discovery of the RANK signaling pathway in the bone formation process does not need to be emphasized, and further study of this pathway may provide an understanding of the disease that is related to the loss of bone mass (**Maeda *et al*., 2022; Yahara *et al*., 2022, Ikeda & Takeshita, 2016; Boyle *et al*., 2003**).

The cells that form bone and the bone itself exhibit many signaling mechanisms that are not yet fully understood. There are also many signaling pathways that maintain the balance between osteoblasts and osteoclasts. In addition to the mentioned RANK-RANKL-OPG pathway, others are the WNT/β-Catenin pathway (canonical Wnt), Parathyroid Hormone (PTH) & Parathyroid Hormone Related Peptide (PTHrP) Pathways, Notch and Hedgehog (**Henry & Bordoni; 2022**).

PROTEINS OF ORGANIC BONE MATRIX

Organic bone matrix consists of several types of proteins. The 90 % of them are collagen proteins, with the greatest representation of collagen type I and then small acidic proteins, binding to Ca-protein, called non-collagenous proteins (NPCs), which include proteins such as bone sialoprotein, osteopontin, osteonectin, fibronectin, bone morphometric proteins (BMPs) and other growth factors. In addition to these proteins, small leucin-rich proteoglycans also play an important role, where decorin, biglycan, osteoaderin, lumican and several serum proteins are belonging (**Florencio-Silva *et al.,* 2015; Fujisawa & Kuboki; 1998**).

Collagen proteins

Collagen, as a fibrous protein, serves as mechanical support and is important both in bones and in skin and connective tissue. Out of a total of twenty-eight known types of collagen, collagen type I is predominant. Within collagen, not just collagen type I, are characterized by their triple helical domains, polypeptide helices. These form fibrils that interact with other proteins, whether collagen or non-collagen. Fibril bundles and fibers of higher order fibrils are formed. More precisely, collagen type I is thus made up of three chains, which are encoded by two different genes on different chromosomes. There are two α1 chains and one α2 chain, and these chains are secreted as propeptides. Subsequent cleavage by proteinases occurs. The C- and N- telopeptides remain attached to the triple helix, thereby creating, through post-translational processing, important cross-linking sites that can determine differences between tissue collagens. The elastic stiffness, bending strength and deflection capacity of bone can be reduced due to a decrease in mature cross-links, whereas, due to the denaturation of type I collagen, there is a loss of bone toughness and strength. Another abundant collagen within the bone extracellular matrix is collagen type III and V, which regulate the diameter of collagen type I fibers and its fibrogenesis. Any mutation in its structure, or even its lack, can affect the strength of the bones, thereby increasing the risk of fractures (**Naomi *et al.,* 2021; Lin *et al.,* 2020; Licini *et al.,* 2019; Omokanwaye *et al.,* 2010; Zylberberg, 2004**). The collagen type I consists of *COL1A1* and *COL1A2* genes in chicken, with the *COL1A1* gene on chromosome 27 and gene *COL1A2* on chromosome 2 (***Gallus gallus* Ensembl genome browser, n.d.**).

Non-collagenous proteins

Effect on bone modeling and bone geometry, bone matrix mineralization and significant structural roles in bones. All this is a list of important properties that NPCs possess. By regulating the activity of bone cells, NPCs influence the geometry of bones, but also their microstructure. NPCs are necessary for bone strength, i.e., resistance to fractures, when by removing these proteins from the bone matrix causes changes in cortical and trabecular bone that affect both bone diameter and thickness. Various conceptual models also assume that the arrangement of NPCs in the bone matrix makes these proteins into structural elements that act at the collagen-mineral interface, as a kind of intermediate member to increase toughness, thereby determining the mechanical properties of bone. NPCs also control cell-matrix interactions, the formation of collagen fibrils, and hydroxyapatite crystallites (**Ikegame *et al.,* 2019; Morgan *et al.,* 2015**).

TYPES OF NON-COLLAGENOUS PROTEINS

Small integrin-binding ligands N-linked glycoproteins (SIBLINGs), glycoproteins, proteoglycans, and γ-carboxyglutamate-containing proteins. These are the four groups into which NPCs can be divided (**Lin *et al.,* 2020**). The distribution of NPCs may vary among different authors, for example **Carvalho *et al*.** (**2021**) divides these proteins into two large groups, namely glycoproteins and γ-carboxyglutamate-containing proteins. Therefore, the inclusion of individual proteins within individual types may overlap across authors.

Small integrin-binding ligands N-linked glycoproteins

The family of SIBLINGs includes five important proteins. They are osteopontin (OPN), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1) and matrix extracellular phosphoglycoprotein (MEPE). The designation SIBLING do not refer to their identical functional activity, but to the uniform genetic and biochemical characteristics of the gene family in general, which is a family of five identically oriented tandem genes located on chromosome 4 in poultry. They appear to be poorly conserved, by comparison with each other themselves at the amino acid level. Functionally, they play an important role in mineralization by controlling hydroxyapatite mineralization and crystal growth. These proteins are secreted and soluble, containing integrin-binding ligands, primarily located in bone and dentin. They can also be modulators of cell adhesion (**Dab *et al.,* 2022; Staines *et al.,* 2012; Bellahcène *et al.,* 2008; *Gallus gallus* Ensembl genome browser, n.d.**).

The phosphoprotein OPN was first described in 1979 by Senger and later precisely named and identified in 1985 by Franzén and Heinegård, who isolated it from bovine bone matrix. This hydrophilic protein, which has a high ability to bind calcium, is secreted into all body fluids and is produced by various types of cells

and tissues, accounts for about 2 % of non-collagenous bone in the bone marrow. It has approximately 300 amino acid residues and is encoded by the *SPP1* gene. In the central part of this protein is an integrin-binding glycine-arginine-glycine-aspartic acid (GRGD) sequence that is highly conserved in all vertebrates. It is the calcium binding site and the two heparin binding domains. Two major cell membrane surface receptors that are highly correlated with many physiological and pathological processes are CD44 (hyaluronic acid receptor) and integrins (αvβ1, αvβ3, αvβ5, αvβ6, α4β1, α4β7, α5β1, α9β1). These processes are various immune reactions, biomineralization, inflammation, wound healing, fibrosis, cell migration and adhesion. Abnormal expression of OPN has been shown to be involved in the development of many bone diseases, such as osteoporosis, rheumatoid arthritis, and osteosarcoma (**Kitamura, 2021; Si *et al.*, 2020; Mazzali *et al.*, 2002; O'Regan & Berman, 2000**).

Another protein from the SIBLINGs family that is highly expressed by osteoblasts, osteoclasts and hypertrophic chondrocytes in the growth plate is BSP. In poultry, the gene encoding this protein (*IBSP*) is located between the *DMP1* and *MEPE* genes and compared to OPN, its gene expression is more limited. Beyond the skeleton, it can also be expressed in tooth odontoblasts, cementoblasts, placental trophoblasts and strongly upregulated in many malignant tumors. Its exact role is not entirely clear, however, in in vitro experiments, it stimulates the formation of hydroxyapatite and enables interactions between cells through an integrin binding site. BSP may be a potential marker of bone turnover due to the fact, that small amounts of it are found in the circulation. This could help in the early detection of various bone disorders, as well as bone metastases (**Bouet *et al.*, 2015; McKee & Cole, 2012; Cremers *et al.*, 2008**).

DSSP as a large precursor protein, is encoded by a large mRNA transcript, where dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are expressed as that one a single mRNA transcript. These proteins are not unique to teeth, but DSPP has recently been found to be present in osteoblasts and bone, and when comparing teeth versus bone, there are very different regulatory mechanisms controlling DSPP expression (**Qin *et al*., 2003**).

A specific highly phosphorylated protein, originally identified from bone dentin, is DMP1, which is essential for both the proper biomineralization of cementum, dentin, and enamel, as well as bone and cartilage. As part of various chemical analyzes of proteins, it was found that DMP1, as a precursor, was cleaved into two forms in its entire length, namely C-terminal and N-terminal fragments. It is also a molecule that, by transcription in the nucleus, initiates the differentiation of osteoblasts and, in the later stages of their maturation, extracellularly organizes the formation of a mineralized matrix. Also, the research of various mutations within human medicine led to the discovery of a new disease: autosomal recessive hypophosphatemic rickets. Through the discovery of a new hormone that is released from bones and targeted in the kidneys, the hormone FGF23, DMP1 can regulate phosphate homeostasis, which sets a new direction of research in the connection of biomineralization with phosphate regulation (**Qin *et al.*, 2007; Kim *et al.*, 2006; Narayanan *et al.*, 2003**).

MEPE, as one of the phosphoglycoproteins involved in bone mineralization, in the bone and dentine mineral matrix, is primarily expressed in osteocytes of adult bone, and in osteoblasts *in vitro* experiments during mineralization. In rodent experiments where this gene has been knocked out, MEPE can be considered a regulator of bone metabolism, primarily inhibiting bone formation. Originally, this gene was identified for high expression in tumors that cause oncogenic hypophosphatemic osteomalacia (OHO) (**Lu *et al.*, 2004; Nampei *et al.*, 2004**).

Glycoproteins

Another group of NPCs includes glycoproteins, which on the protein chain contain covalently attached carbohydrate molecules in various combinations and positions. This includes, for example, alkaline phosphatase (ALP), osteonectin, thrombospondins (TSPs) and fibronectin (FN), which are formed at various stages of osteoblast maturation. They are immediately involved in several processes, which are cell-matrix interaction, cell proliferation, hydroxyapatite deposition (**Lin *et al.*, 2020; Robey, 2002**).

To a group of glycoproteins that bind to the plasma membrane, specifically the surface of the cell membrane of osteoblasts by glycosylphosphatidylinositol includes the ectoenzyme ALP. In part, bone ALP is released into the circulation, in several isoforms that show the same enzymatic activity, only the content of carbohydrates and sialic acid is different. The isoform found in normal bone is B1x. Other isoforms, such as B1 and B2, have different enzymatic activity within different types of bone tissue, with cancellous bone having higher B1 activity, while the total activity (B1 and B2) is lower, and the opposite within trabecular bone, with higher B2 activity bone ALP. Through the hydrolysis of inorganic pyrophosphate, which plays the role of a natural inhibitor of bone mineralization, bone ALP can be considered an important regulator of this process. As already indicated, there are several types of alkaline phosphatase, and this large family is encoded by the *ALPL* gene. A non-tissue-specific form (liver/bone/kidney), which is referred to as *TNAP*, was tested in experiments on rodents, where its inactivation resulted in abnormally short growth plates and the appearance of hypomineralized areas within the bone (**Sharma *et al.*, 2014; Szulc & Bauer, 2013**).

Glycoprotein that is the most abundant non-collagenous protein within mineralized tissues and is also expressed in osteoblasts, endothelial cells and fibroblasts is secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin. This protein plays a role in osteoblast differentiation and regulates the formation and assembly of collagen fibers, where its affinity for collagen and hydroxyapatite, thereby participating in bone formation, is known. SPARC consists of several domains. The first is the N-terminal low-affinity, high-capacity domain, where the mineral binding region is located, this is the calcium binding domain. The second is a cysteine-rich domain, followed by a hydrophilic region, while the last, fourth domain, is a domain with an E-F hand motif at the C-terminus (extracellular Ca^{2+} (EC) domain), including a collagen-binding domain. The E-F hand motif consists of two α helices and one short loop region (a helix-loop-helix structure, characteristic of certain calcium-binding proteins). Within poultry, the *SPARC* gene is located on chromosome 13. The hydroxyapatite and collagen-binding domains interact, yet are separated, and this separation is hypothesized to facilitate better mineralization of collagen during bone formation. Glycosylation of SPARC is different within different tissues, for example the glycosylated form of SPARC expressed in platelets binds collagen with less affinity than the form present in bone. Experiments on SPARC-null rodents showed a reduced number of bone cells, while bone formation was reduced by almost 50%. The development of deep osteopenia, where bone loss occurred, was noted (**Roumeliotis *et al.*, 2020; Rosset & Bradshaw, 2016; Dole *et al*., 2015; Delany & Hankenson; 2009; *Gallus gallus* Ensembl genome browser, n.d.)**.

TSPs include a family of five multidomain glycoproteins, which, thanks to their structure, can interact with several ligands, but also with other proteins, cytokines, or proteases. They are involved in a number of processes, such as wound healing, angiogenesis, connective tissue organization, cell proliferation and migration, bone remodeling, osteolysis, and osteogenesis. Subgroup of A family, which exhibits comparable properties, among which we include the ability to organize extracellular matrix (ECM), modulation of collagen fibrillogenesis, antiangiogenic activity and others, includes thrombospondin 1(TSP-1) and thrombospondin 2 (TSP-2). Although the two proteins are very similar in that they are trimeric in structure, a major difference between them is that only TSP-1 contains the sequence required to activate the otherwise latent transforming growth factor-beta (TGF-β), where TSP-2 is the antagonist of activation. Another subgroup B of the thrombospondin family are the pentameric thrombospondins 3-5 (TSP3-5/COMP), where TSP-5 is known as oligomeric cartilage matrix protein (COMP). TSPs oligomerization is stabilized using intersubunit disulfide bonds. In the case of trimeric TSPs, these are formed between cysteine residues adjacent to the animo-terminal end of seven-membered repeats and within pentameric TSPs with a carboxy-terminal end **(Alford *et al.*, 2021; Carminati & Taraboletti, 2020; Adams & Lawler, 2011, 2004)**.

Another key regulator in the bone repair process is the evolutionarily conserved protein FN, a dimer that is structurally composed of one or more alternatively spliced domains. These are type I, II, III (FNI-III) units and C-terminal dimerization site. Alternative splicing of one pre-mRNA molecule can produce up to twenty different isoforms of this protein. FN is encoded by the *FN1* gene, which is located on chromosome 7 in poultry. There are two forms of FN, the soluble form or plasma FN (pFN) and the cellular form (cFN). These forms differ from each other structurally, by the presence of selected domains and their splicing. FN can bind to up to eleven different integrins, of which up to six are expressed by osteoblasts (a4p1, a5p1, a8p1, avp1, avp3 and avp5), but which integrin(s) is the primary adhesion molecule for osteoblast binding to fibronectin is not known. In addition to being an important protein during bone repair, it is also important in dynamic tissue remodeling during embryonic development, as confirmed by experiments with FN knockout rodents that died in uterus by the tenth embryonic day. It is also a ubiquitous component of the ECM in all tissues, which is associated with effects on cell adhesion, growth, and differentiation (**Dinesh *et al.*, 2022; Klavert & van der Eerden, 2021; Bentmann *et al.*, 2010; *Gallus gallus* Ensembl genome browser, n.d.**).

Proteoglycans

Proteoglycans are another large group of NPCs characterized by the presence of glycosaminoglycan (GAG) residues, which are covalently bound to the core of the protein by means of identical linkages via O-link to serine residues. These are GAG residues such as dermatan sulfate, chondroitin sulfate, heparan sulfate, hyaluronic acid, keratan sulfate, and heparin, where hyaluronic acid is the only one of these residues that is linear, with an unbranched backbone that repeats disaccharide units without sulfates groups. The biological function of proteoglycans is determined by the composition of the GAG chains. An important family of proteoglycans that have been described in association with bone and in all processes of bone formation, whether it was cell proliferation, cell matrix deposition, remodeling, and hydroxyapatite formation, but also collagen fibrilogenesis, are small leucine-rich proteoglycans (SLRPs). In order for these secreted extracellular proteins to regulate normal as well as pathological cell behavior, these secreted extracellular proteins interact with cell surface receptors and cytokines. Important bone proteoglycans include, for example, biglycan (BGN), decorin (DCN), keratocan (KTN), osteoadherin (OSAD), osteoglycin/mimecan (OGN), fibromodulin (FMOD), proline/arginine-rich end leucine-rich repeat protein (PRELP), and lumican (LUM). If there was a loss of SLRP due to unregulated proteolysis, or even a change in their expression profile, a whole range of bone diseases could

occur due to their involvement in bone morphogenesis and homeostasis (**Sorvina *et al.*, 2023; Carvalho *et al.*, 2021; Chen *et al.*, 2021; Coulson-Thomas *et al.*, 2015**).

DCN and BGN are very similar SLRPs, which are similarly located extracellularly and fall into class I. Their GAG chain is made of chondroitin sulfate, or dermatan sulfate, where DCN has one and BGN has two side chains. Early bone matrix deposition initiates DCN expression, while during cell proliferation and mineralization BGN expression occurs and is paused during bone matrix deposition. Both proteoglycans share a common binding site for collagen type I and collagen type IV, where BGN binds with lower affinity for collagen type I. However, in case DCN is absent, BGN functionally compensates for decorin by its overexpression. Furthermore, DCN binds collagen type II and III. It is not entirely clear whether, in the case of changes in the mechanical properties and organization of collagen, it is an overexpression of BGN, the absence of DCN, or certain combinations, given their overlapping functions during development. DCN is encoded by the *DCN* gene located on chromosome 1 in poultry, while the *BGN* gene for BGN is located on chromosome 13 (**Appunni *et al.*, 2019; Robinson *et al.*, 2017; Zanotti *et al.*, 2005; *Gallus gallus* Ensembl genome browser, n.d.**).

KTN plays a role in the process of mineral matrix formation, with knockout rodents showing reduced intensity of mineral deposition and rate of bone formation. It is secreted by osteoblasts, as well as LUM, FMOD and OSAD, which has been described in bovine mineralized bone matrix. LUM and OGN have been described within the medullary bone of birds. Osteoclastogenesis and overall bone resorption were impaired by the proteoglycan PRELP, which was described in conjunctival tissue (**Coulson-Thomas *et al.*, 2015; Igwe *et al.*, 2011**).

Gamma-carboxyglutamate-containing proteins

An important group of NCPs present in serum, dentin, bone matrix and many calcified tissues are γ-carboxyglutamic acid (Gla)-containing proteins. It is an acid produced by a specific post-translational modification that is dependent on vitamin K, while the proteins that contain Gla in bone are osteocalcin (OCN), matrix Gla protein (MGP) and periostin (POSTN) (**Lin *et al.*, 2020**).

The bone γ-carboxyglutamic acid protein (BGLAP), OCN, is a factor that is expressed and secreted by osteoblasts. As soon as OCN, as a mature peptide, undergoes several splicing events and subsequent γ-carboxylation on three residues, the result is a peptide that has a high affinity for bone and the extracellular matrix, but then decarboxylation occurs again due to the low pH inside the osteoclast resorption compartments. In this way, uncarboxylated OCN enters the circulation, while its affinity for bone is reduced. Previous studies looking at various deletions of OCN have shown that its effect on bone metabolism, in terms of mineralization, total bone density and as an inhibitor of bone mass, is less than expected, or no effect at all. It has also been tested as a factor that improves glucose metabolism, maintains muscle mass, and indicates testosterone synthesis in the testes, while more recent studies have not confirmed these roles and its effect on the crystallographic orientation of the c-axis of biological apatite (BAp) has been demonstrated. This axis is normally parallel to the collagen fibrils, but in the case of the OCN knockout rodents, this axis was severely disrupted, leading to compromised bone strength (**Komori, 2020; Moriishi *et al.*, 2020; Moser & van der Eerden, 2019**). From the point of view of protein domains, or even the organization of genes, OCN is structurally very similar to MGP, from which it most likely arose by duplication of a tandem gene. Despite this similarity, both proteins followed different evolutionary strategies to gain different functions. MGP synthesis occurs in both bone and many mesenchymal cells. It is also highly represented by chondrocytes and vascular smooth muscle cells. This calcification inhibitor is known in several species that differ in the intensity of carboxylation and phosphorylation. OCN is encoded by the *BGLAP* gene, also known as *OC*, *BGP*, or *OCN*, and is located on chromosome 25 in poultry. MGP is encoded by the *MGP* gene and is located on chromosome 1 (**Bjørklund *et al.*, 2020; Cancela *et al.*, 2014; *Gallus gallus* Ensembl genome browser, n.d.**).

POSTN is another osteoblast-specific protein that is expressed in bone, but also in other collagen-rich tissues, such as heart valves, tendons, and some tumors, and plays a role in the regulation of bone formation (**Naylor & Eastell; 2015**).

WNT AND RANK/RANKL/OPG SIGNALING PATHWAYS

The importance of the Wnt and RANK/RANKL/OPG signaling pathways in bone metabolism has already been demonstrated several times. Dysregulation of these pathways leads to many bone disorders due to their important role in bone cell differentiation and the processes involved (**Zhu *et al.*, 2021; Wang *et al.*, 2020**).

Wnt signaling pathway and selected proteins involved in Wnt signaling

In the 1980s, one of the evolutionarily highly conserved pathways was discovered, which plays an important role in many biological processes, regulates many aspects of cell fate, and is critical in adult tissue homeostasis and many functions during embryonic development, including bone and cartilage formation. It is a Wnt signaling pathway, which includes a family of proteins, secreted glycoproteins, while the main signaling branches downstream of the Frizzled receptor (FZD) have so far been discovered, which includes canonical and non-canonical signaling. The canonical pathway, or the Wnt/β-catenin dependent pathway, is mediated by β-catenin, which in the absence of Wnt stimulation is phosphorylated, then ubiquitinated and rapidly degraded through the proteasomal system to prevent cytoplasmic accumulation. Conversely, when Wnt is stimulated, cytoplasmic accumulation of β-catenin follows. Expression of target genes occurs after the translocation of accumulated β-catenin into the nucleus. The non-canonical signaling pathway, which is independent of β-catenin, is further divided into a pathway regulating planar cell polarity (Wnt/PCP) and a Wnt/Ca^{2+} pathway influencing the level of Ca^{2+} in the cytoplasm. Activation of the canonical or non-canonical signaling pathway occurs through the binding of ligands, receptors, and co-receptors. In the case of activation of the canonical pathway, it is a combination of Wnt1 and Wnt3a ligands, the FZD and the co-receptors low-density lipoprotein-related receptors 5 and 6 (LRP5/6). Inhibition of the pathways is ensured by binding to a specific region of the coreceptor, which is mediated by proteins of the Dickkopf family (DKK) and sclerostin (SOST) (**Liu *et al.*, 2022; Maeda *et al.*, 2019; Houschyar *et al.*, 2019; Kim *et al.*, 2013; Kobayashi *et al.*, 2008; Komiya & Habas, 2008**). The Wnt family can be divided into two large groups. The first group, Wnt1, includes Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt7a, Wnt8, Wnt8b, Wnt10a. These participate in the canonical signaling pathway. The non-canonical signaling pathway is activated by the Wnt5 category, which includes Wnt4, Wnt5a and Wnt11 (**He *et al.*, 2015**).

Within the components of the Wnt signaling pathway, it was found, that the protein WISP-1 (WNT1-inducible-signaling pathway 1), encoded by the *WISP1* gene, also known as *CCN4*, located on chromosome 2 in poultry, is a new target for modulating osteogenesis and improving bone strength (**Ferrand *et al.*, 2017; *Gallus gallus* Ensembl genome browser, n.d.**). The structure of CCN proteins consists of an amino-terminal secretory signal peptide that is followed by four structural domains. Absences of different domains, in certain variants of CCN proteins, play distinct biological roles, while also being involved in various pathologies. The protein is expressed in many places in the body, be it the lungs, heart, brain, kidneys, ovaries, epithelium, or in osteoblasts or osteoblastic stem cells of the perichondral mesenchyme during embryonic development. Cell death of osteoblasts or their precursors, impaired bone repair, blockage of cell proliferation, progressive spinal cord injury and many others can be caused by loss of Wnt1 signaling (**Gurbuz & Chiquet-Ehrismann, 2015; Maiese, 2014; Ono *et al.*, 2011; French *et al.*, 2004**). Various genetic experiments in mice show that after knocking out this gene, the test subjects have lower total bone volume and cortical bone thickness than wild-type mice. On the other hand, increased mineral density, total bone volume and cortical bone thickness were observed in test subjects with overexpressed WISP1 (**Wang *et al.*, 2018**).

The highly homologous proteins, LRP5/6, play an essential role in canonical Wnt signaling as coreceptors. They are involved in skeletal remodeling, so mutations in the genes encoding these proteins are associated with a number of diseases, such as osteoporosis, but also cancer and metabolic disorders. In poultry, the genes encoding these proteins are located on different chromosomes, in the case of LRP5 on chromosome 5 and LRP6 on chromosome 1. Structurally, they contain large extracellular domains including four β-propeller motifs. These are followed by three low-density lipoprotein (LDL) type 1 ligand binding domains (**Kang & Robling, 2015; Joiner *et al.*, 2013; MacDonald *et al.*, 2011; *Gallus gallus* Ensembl genome browser, n.d.)**.

One of the modulators of the endogenous secreted pathway, which is part of the Wnt pathway, are DKKs and sclerostin SOST. They inhibit the canonical pathway by binding to the LRP5 and LRP6, which also regulates bone mass (**Ueland *et al.*, 2019**). DKKs are a family of soluble LRP5/6 antagonists, where four *DKK* genes (1-4) have been described in human studies, with *DKK1* being the most studied. The family of these proteins shows little sequence similarity, with only two domains highly conserved among individual members. The first is an N-terminal cysteine-rich (Cys1) domain that modulates the interactions of the second domain. That is the C-terminal cysteine-rich domain (Cys2), and this domain inhibits Wnt through its bindings (**Giralt *et al.*, 2021**). When DKK1 is forced to be overexpressed in osteoblasts, the result is osteopenia. Also, activation of DKK1 in osteoblasts can be the cause of osteoporosis, while DKK1 inhibits fracture repair and participates in erosive arthritis, so strengthening Wnt/β-catenin signaling or neutralizing DKK1 could help in the treatment of bone pathologies (**Pinzone *et al.*, 2009**). The *SOST* gene, located in poultry on chromosome 27, expresses a small protein that is exclusively found in osteocytes within bone cells. It is a SOST that is a potent inhibitor of bone formation and was originally included in the DAN family of BMP antagonists but was later shown to bind LRP5/6 with high affinity. After binding to osteoblast receptors, the intracellular signaling cascade is activated, with the final result being the inhibition of osteoblastic bone formation. In human studies, mutations in the *SOST* gene locus are associated with rare skeletal disorders characterized by bone overgrowth, sclerosteosis, and Van Buchem's disease. Structurally, SOST forms a core cysteine knot with three loops that are flanked by highly flexible N- and C-terminal domains (**Kim *et al.*, 2022; Sebastian & Loots, 2017; Lewiecki, 2014; Robling *et al.*, 2008**).

The evolutionarily conserved protein GPR177 (G protein-coupled receptor 177/Wntless) is necessary for the secretion of Wnt ligands, which is encoded by the *GPR177* (*WLS*) gene, located on chromosome 8 in poultry. It is the largest family of membrane receptors that can activate heterotrimeric G proteins, which consist of α, β, and γ subunits, control cell behavior. Also, most of these proteins

are N-glycosylated, while it is not completely known whether this glycosylation is necessary for the interaction within Wnt signaling. GPR177 is involved in a variety of processes such as mammary gland morphogenesis. Also, deletion of GPR177 within mature osteoblasts completely disrupted postnatal bone homeostasis (**Du *et al.*, 2019; Zhong *et al.*, 2015; Das *et al.*, 2012; Jin *et al.*, 2010; *Gallus gallus* Ensembl genome browser, n.d.**).

Through the Wnt/β-catenin signaling pathway, osteogenic differentiation is activated, with the help of the important regulatory factor Runx2. This member of the Runt domain family of transcription factors controls osteoblast development and maturation into osteocytes by regulating the transcription of many genes. Although it plays an important role in bone cell development and gene transcription, its role in gene expression and new bone formation is generally insufficient, whereas changes in expression levels are associated with skeletal disease. Many bone cancers are caused by its overexpression, while cleidocranial dysplasia is caused by Runx2 haploinsufficiency. In poultry, the Runx2 gene is located on chromosome 3, and some studies in broiler testing suggest the involvement of this gene in the occurrence of necrosis of the femoral head (**Kim *et al.*, 2020; Shen *et al.*, 2020; Haxaire *et al.*, 2016; Paludo *et al.*, 2014; Gaur *et al.*, 2005; Schroeder *et al.*, 2005; *Gallus gallus* Ensembl genome browser, n.d.**).

RANK/RANKL/OPG signaling pathway and selected proteins involved in RANK/RANKL/OPG signaling

A major advance in bone biology occurred with the discovery of the RANK/RANKL/OPG signaling system in the mid-1990s, when a specific factor necessary and sufficient for osteoclast development, produced by preosteoblastic/stromal cells, was discovered. It was the soluble, decoy receptor osteoprotegerin, that elucidated and discovered this system. That the formation of osteoclasts is influenced by osteoblastic stromal cells was already clear a few years before, but that this is done through the expression of members of the tumor necrosis factor (TNF) superfamily was not expected at first. The main molecules of this system are the receptor activator of nuclear factor (NF)-kB-ligand (RANKL), the receptor activator of NF-kB (RANK) and the soluble decoy receptor OPG. RANKL is expressed on the surface of preosteoblastic/stromal cells and binds to the signaling receptor RANK on the surface of osteoclastic precursor cells. This leads to differentiation to mature osteoclasts, as part of the previous fusion to multinucleated cells, whereby osteoclasts further adhere to the bone surface, acid, and lytic enzymes such as cathepsin K and tartrate-resistant acid phosphatase are secreted, thereby supporting bone resorption. This entire system is inactivated by OPG and its prevention of RANKL binding to the RANK receptor. This inhibits osteoclastogenesis and ensures bone protection against excessive resorption through osteoclasts. Several other cytokines and hormones are involved in the entire system, which increases or decreases the production of individual molecules. They are, for example, estrogen (increased production of OPG), parathormone (increased production of RANKL/decreased production of OPG), transforming growth factor β (increased production of OPG), 1,25-dihydroxyvitamin D3 (increased production of RANKL) and glucocorticoids (increased production of RANKL/decreased production OPG). However, the regulation of osteoclasts can also take place directly on the osteoclasts themselves and not through osteoblasts, using estrogen and calcitonin. This signaling pathway is not only involved in bone homeostasis, but also in other physiological processes, such as atherosclerosis, sarcopenia, and various cardiovascular diseases. It also plays an important role in the physiology of the mammary gland, where it is the main downstream mediator of progesterone-controlled mammary epithelial cell proliferation (**Marcadet *et al.*, 2022; Tobeiha *et al.*, 2020; Infante *et al.*, 2019; Boyce & Xing, 2008; Khosla, 2001;**).

RANKL, as a homotrimeric protein, is encoded by a single gene, *TNFSF11* (TNF superfamily member 11), located on chromosome 1 in poultry, with a number of 400 amino acids. Three isoforms are expressed through alternative splicing. In humans, there are two isoforms that are type II transmembrane proteins. The third isoform lacks both the transmembrane and cytoplasmic domains, presenting itself as a soluble ligand (sRANKL). The exact function of individual isoforms is not known, but it is assumed that membrane RANKL ensures cell-cell interaction with osteoclasts and their precursors, and diffusion to activate target cells is ensured by sRANKL. In addition to bone cells, RANKL is expressed by T lymphocytes, B lymphocytes and megakaryocytes. Very similar is the RANK protein, which is encoded by the *TNFRSF11A* (TNF receptor superfamily member 11a) gene, which is on chromosome 2 in poultry. Like RANKL, this protein is widely expressed, including cells of the macrophage/monocyte lineage, including dendritic cells and fibroblasts, in mammary gland, breast and prostate cancer cells. Another member of the TNF receptor family is osteoprotegerin, encoded by the TNFRSF11B gene (TNF receptor superfamily member 11b), located in poultry on chromosome 2. It is expressed in the kidney, liver, spleen, heart, and bone marrow in addition to osteoblasts. The determinant of bone mass is the RANKL/OPG ratio, while OPG expression is also regulated by Wnt/β-catenin signaling within osteoblasts. It follows that bone mass is regulated by two main signaling pathways, RANKL/RANK and Wnt/β-catenin. (**Ono *et al.*, 2020; Liu & Zhang, 2015; Wright *et al.*, 2009; Boyce & Xing, 2008, 2007; *Gallus gallus* Ensembl genome browser, n.d.**).

CONCLUSION

Poultry farming and the resulting production of meat and eggs is an integral part and branch of animal production. The consumption of poultry meat is growing year by year. The intensive selection of poultry improved the genetic gain of important economic properties. However, this also led to many health problems, mainly related to the skeletal system, including problems with osteoporosis and the occurrence of fractures, which represent a big problem in the area of animal welfare, production and subsequently in the economic aspects of breeding. The genetic basis and interrelationship of this issue is not fully explored and understood, which can also be said about the genetic architecture of important bone features and the roles of some proteins within bone metabolism, while their targeted study may reveal new knowledge that can help to understand the processes that lead to skeletal disease. The result of this review is a summary of the knowledge of selected important proteins that are part of compact bone or are involved in signaling pathways that relate to bone metabolism.

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