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Transglutaminases in mineralized tissues

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1. ABSTRACT

Bone development and formation during embryogenesis as well as postnatally during bone remodeling is a complex process controlled systemically and locally by hormones, growth factors and matrix molecules. Transglutaminases (TGases) are the protein cross-linking enzymes, which have long been implicated in bone development and formation. Two members of TGase family, TG2 (also called tissue transglutaminase) and FXIIIA (the enzymatic A subunit of coagulation factor XIII), are expressed in chondrocytes and osteoblasts. The results of analyses *in vivo* and *in vitro* accumulated to date indicate an important role of these enzymes in promoting chondrocyte and osteoblast differentiation and matrix mineralization. These effects could be mediated by protein cross-linking activity of TGases, by GTPase activity of TG2 or via non-catalytic signaling effects. The aim of this review is to summarize the available data regarding the expression, localization and activity of TG2 and FXIIIA in mineralizing tissues and to discuss a number of mechanisms by which TGases could exert their promineralizing effects.

2. INTRODUCTION

The human skeleton is composed of over 200 bones of different sizes and shapes with each of them having a distinct purpose in maintaining locomotive and protective functions as well as regulating calcium homeostasis. For decades, researchers have been trying to understand the mechanisms that regulate the formation, growth and remodeling of bones. Each bone is constructed, mineralized and located at pre-determined sites in the body to function in a highly coordinated manner. The skeleton is also remodeled throughout life in a process that maintains bone mass and quality, and any defects in this cycle lead to degenerative bone diseases such as osteoporosis and arthritis. Although tremendous progress has been made, the regulatory networks controlling bone formation, growth and remodeling have not been fully elucidated. A characteristic feature of the skeleton is that it is an organ made of two distinct tissues, cartilage and bone. There are specific cell types in each of these tissues: chondrocytes in cartilage, and osteoblasts, osteocytes, and osteoclasts in bone, with osteoblasts being the major cell type responsible for bone formation. Chondrocytes and osteoblasts derive

from the common mesenchymal progenitor cells. Many genes and genetic pathways influencing skeletal development have now been established (reviewed in (1); (2)). Nevertheless, many links are still missing, many pathways are incomplete and the roles of a number of proteins are unclear as new players in bone formation are continuously being identified. Recent studies have implicated the transglutaminase enzymes (TGases) in the regulation of differentiation of both chondrocytes and osteoblasts. This review will summarize available data on the expression patterns of TGases in the skeleton, present evidence for TGase-induced hard tissue cell differentiation and discuss possible molecular pathways mediating this regulation. Understanding the roles of TGase enzymes in bone biology will allow development of potential novel therapeutic drugs for intervention in bone pathologies, such as osteoarthritis, osteoporosis and tumor metastasis to bone in cancer.

3. BONE DEVELOPMENT

During embryonic and fetal development, skeletal elements can be formed via one of two independent yet coupled pathways. Intramembranous ossification involves the direct commitment of mesenchymal progenitor cells to differentiate into osteoblasts given the proper environment and molecular signals. Osteoblasts deposit an extracellular matrix rich in type I collagen, glycoproteins and proteoglycans that is competent for mineralization. The bones of the skull, part of the clavicle and the bony collars of long bones are formed by this mechanism. Alternatively, bones may be formed by endochondral ossification where deposition of bone matrix occurs on a pre-existing cartilage matrix. Bones of the axial and appendicular skeleton (long bones) and most of the facial bones are formed this way. Endochondral ossification starts with the initial formation of cartilaginous elements. The chondrocytes within each element become organized into linear arrays within growth plates and progress through the resting, proliferative, pre-hypertrophic and hypertrophic phases of differentiation. The hypertrophic cartilage subsequently mineralizes and is invaded by chondroclasts (multinucleated giant cells of the macrophage lineage which cause cartilage resorption), osteoblasts, marrow cells and vascular progenitor cells from the adjacent perichondrium ((1)-(3)).

Just prior to the cellular invasion of the hypertrophic cartilage, bone deposition begins by the creation of a bony collar by conversion of the perichondrium (the thin layer of mesenchyme-derived flattened cells surrounding the mid-portion of the cartilaginous anlagen) into periosteum in areas immediately adjacent to hypertrophic chondrocytes. The distal and proximal borders of the periosteum are aligned with the zones of pre-hypertrophic chondrocytes due to coordinated differentiation of hypertrophic chondrocytes and periosteal osteoblasts (reviewed in (4); (5); (6)). The membranous bony collar around the cartilaginous anlagen is critical for determining the shape and the size of the developing bone. ((7); (5)). Similar mechanism of intramembranous bone formation is utilized in bone remodeling, which occurs throughout life to maintain proper bone mass and quality.

During endochondral ossification, a large number of chondrocyte-derived signaling factors may regulate osteoblast differentiation. These include the secreted protein Indian Hedgehog (acting through the parathyroid hormone-related protein) ((8); (9); (10)), proteins of the Wnt family ((11); (12); (13)), proteins of the TGF□/BMP family, □FGF, inorganic pyrophosphate, and homeobox-containing transcription factors such as Dlx5 and Fos-related proteins ((5); (8); (9); reviewed in (14); and (15); (16); (17); (18); (19); (20); (21)).

Recent reports have implicated TGases in the regulation of chondrocyte and osteoblast differentiation during skeletal development, as well as in bone remodeling. This review focuses on these recent data and discusses the distribution of specific TGases in mineralized tissues and their substrates, as well as provides an overview of the potential mechanisms by which TGases could mediate their function in the skeleton ((22); (23); (24); (25); (26); (27)).

4. TRANSGLUTAMINASE EXPRESSION AND ACTIVITY DURING BONE FORMATION – TG2 and FXIIIA

4.1. Expression of TGases in the cartilaginous growth plate

To date, nine members of the TGase family have been identified (28). Only two of them – TG2 and FXIIIA – have been detected in cartilaginous and osseous tissues. Up-regulation of TG2 was first demonstrated in the hypertrophic zone of the growth plate in juvenile rats by immunohistochemistry (29). Subsequently, a significant increase in FXIIIA expression was identified in the hypertrophic zone of the avian embryonic growth plate by subtractive hybridization (30). To better understand the potential roles of these TGases in skeletal biology, we analyzed the expression patterns of TG2 and FXIIIA in the early embryonic limb development using a chicken model. The formation of the tibia in the chicken embryo begins around day 4 in the regions of mesenchymal condensation. A stacked cell layer, surrounding the cartilaginous anlagen, forms the perichondrium ((31); (32)). By 6.5 days, chondrocytes in the middle of the cartilaginous anlagen begin to hypertrophy and the perichondrial cells differentiate into osteoblasts and form a bony collar ((31); (10); (33)). At this time (day 6.5) the enzymes TG2 and FXIIIA are both present throughout the areas of chondrocyte condensation (Figure 1). An identical pattern of expression was identified for TG2 (using the antibody against purified guinea pig liver TG2 (Upstate Biotechnology), and for FXIIIA (using the antibodies against human FXIIIA (Calbiochem) and against chicken FXIIIA (34)). Higher levels of both proteins are detected in the hypertrophic zone and epiphyseal regions of the long bone, while a slight decrease in expression is restricted to the zone of chondrocyte proliferation (boxed area in Figure 1A). Later in development (at day 9), the zone of TGase expression is even more restricted (Figure 1B), with a down-regulation in expression both in proliferative and resting chondrocytes. At the epiphyses, TGases are expressed only in the superficial layers of cells which will

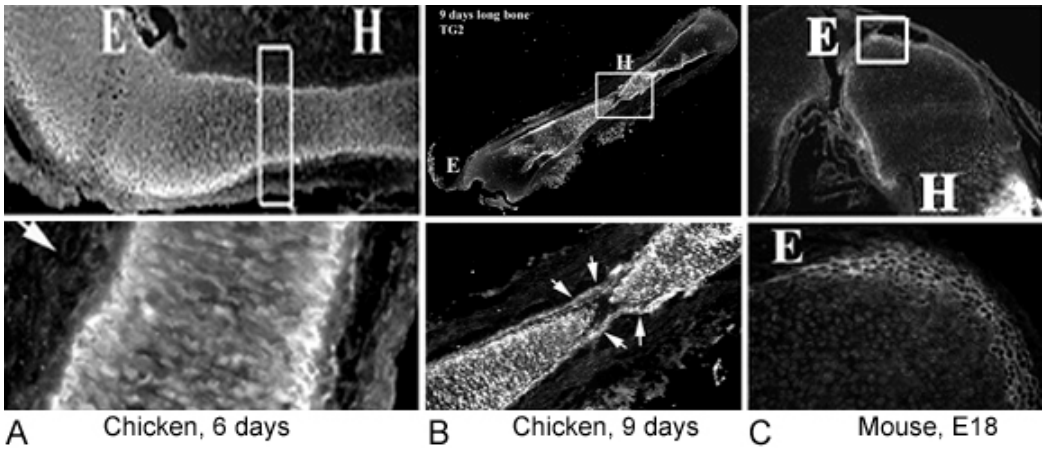

Figure 1. Expression of FXIIIA analyzed by immunohistochemistry using anti-chicken FXIIIA antibody A3 (34). A – chicken wing, B – chicken tibia, C – mouse tibia. Upper panels – 10x magnification. Boxed areas are shown at higher magnification in the power panels. Arrows point at perichondrium/periosteum. H – zone of hypertrophy, E – epiphysis. The same pattern of TG2 expression is detected with the anti-TG2 antibody (Upstate Biotechnology).

form the articular cartilage. This restriction is more pronounced by the end of embryonic development (a similar pattern of TGases expression has been identified in chicken and mouse embryos ((35); (36)) (Figure 1C). The protein distribution corresponds to the pattern of mRNA expression as analyzed by *in situ* hybridization (37). In conclusion, the expression of two TGases – TG2 and FXIIIA – is activated early in the condensing limb mesenchyme. In skeletal elements of mid-stage embryos, where chondrocytes differentiate in a spatially ordered manner, the expression of these proteins is restricted to mitotically active chondroblasts close to the epiphyses, and to the post-mitotic pre-hypertrophic and differentiated hypertrophic chondrocytes.

Development of endochondral bone together with the surrounding periosteal bony collar is a highly synchronized process. Bony collar growth takes place at its non-mineralized advancing edges, which surround the zones of pre-hypertrophy or early hypertrophy of the growth plate where two TGases (TG2 and FXIIIA) are up-regulated. The levels of FXIIIA are especially high in the "borderline chondrocytes", located at the very lateral edges of the growth plate (Figure 2, the cell layer marked with asterisks). These chondrocytes are characteristically positioned at the physical border of the cartilaginous and non-cartilaginous tissue (perichondrium, marked with arrows in Figure 2). These cells are thought to regulate bony collar and themselves demonstrate signs of dual differentiation: cartilage cells in a cartilage matrix, with osteoblast-like phenotypic properties (38). On the contrary, in the hypertrophic cartilage underlying the fully mineralized periosteal bone no borderline chondrocytes are present, and FXIIIA localizes throughout the cartilaginous matrix uniformly.

4.2. Expression of TGases in the perichondrium/ periosteum

In addition to chondrocytes of the growth plate, osteoblasts of the perichondrium/periosteum also express both TG2 and FXIIIA, as detected by immunohistochemistry (identical pattern of expression was detected for both proteins, although the antibodies employed exhibited high specificity by Western blot analysis (data not shown)). At 6 days of development, the perichondrium is TGase-negative (Figure 1A, arrow) while at 9 days the area of perichondrium surrounding the primary center ossification, now called periosteum, is TGase-positive (Figure 1B, arrows). Cells in this region of the periosteum start to differentiate into osteoblasts, expressing the transcription factor Cbfa1, and to form the mineralized bony collar around the cartilaginous core (39). These immunohistochemical data support our previous results obtained by PCR on expression of TG2 and FXIIIA in periosteal osteoblasts (22). Thus, initiation of TGase synthesis by osteoblasts correlates with deposition of mineralized bone matrix.

Similarly, the same two TGases are expressed in the mammalian mineralized bone tissue (from rats and mice), as revealed by enzymatic assay (Figure 3; thrombin (or trypsin)-induced increase of TGase activity is indicative of the presence of zymogene form of FXIIIA in the analyzed samples}. The biochemical data is supported by the PCR analysis and immunohistochemistry ((40); (25)). In adult cortical bone, TG2 is found by immunohistochemistry in the osteoid layer, but not in the mineralized matrix compartment implying its inactivation and/or masking of epitopes by mineral (25). TG2 is also localized in the pericellular matrix surrounding osteocytes, thus suggesting an involvement of this enzyme in the osteocyte formation. Expression of TGases in osteoblasts has also been extensively studied on the mRNA level by PCR analysis. Primary osteoblasts (41) and several osteoblastic cell lines such as human osteosarcoma cells (HOS and MG-63) (41), rat osteosarcoma (ROS) (40) and mouse pre-osteoblastic cell line MC3T3-E1 subclone 14 (26); (27)) have been analyzed. All these cell lines express TG2, consistent with the broad expression of this enzyme in a wide variety of cells and tissues (reviewed in 28; 131). In addition, similar to chondrogenic cells, ROS and MC3T3-E1/C14 pre-osteoblast cells express FXIIIA ((40);

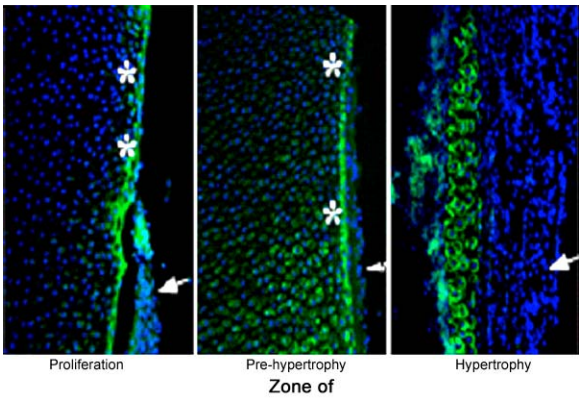

Figure 2. Expression of FXIIIA in 14-day chicken growth plate. FXIIIA detected with the A3 antibody (34), and secondary antibody labeled with FITC. DNA counterstained with Hoechst dye (blue staining). Arrows mark perichondrium/periosteum; asterisks mark borderline chondrocytes.

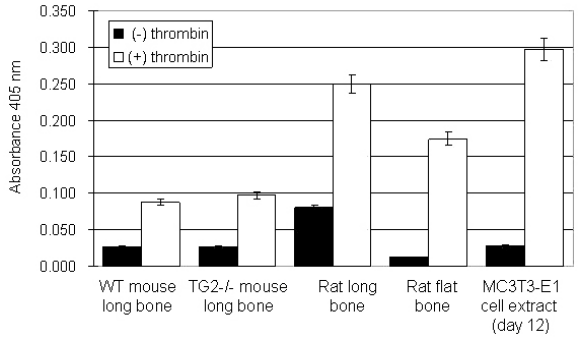

Figure 3. TGase activity assays for TG2 and FXIIIa on rat and mouse bone extracts and differentiated mouse MC3T3-E1/C14 osteoblasts. Bone extracts were prepared as described in (25). Enzymatic activity was measured using biotin-(pentyl)amine incorporation into fibronectin in a microplate assays in the presence or absence of thrombin which reveals the activity of FXIIIa.

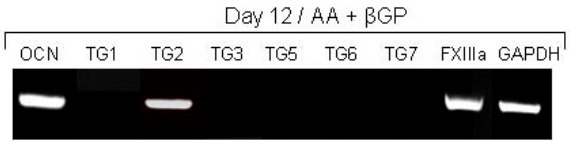

Figure 4. RT-PCR analysis of expression of different TGases in differentiated MC3T3-E1/C14 mouse osteoblast cell line. Osteocalcin (OCN) represents a late marker of osteoblast differentiation. GAPDH was used as a positive control for the PCR reaction.

(27)), while other TGases are either absent or expressed only at extremely low levels not detectable by PCR analysis (Figure 4), (26); (27)).

The evolutionarily conserved pattern of FXIIIA and TG2 expression in osteoblasts suggests an involvement of these proteins in bone formation and functioning. Overall, the expression and localization patterns of both enzymes in the osseous tissues are consistent with a role for these enzymes in the regulation of cell differentiation and extracellular matrix mineralization (reviewed earlier by Aeschlimann and Thomazy (42)).

4.3. Distribution of TGase enzymatic activity in bone tissues and resident cells

The enzymatic activities of the two TGases present in bone tissues are regulated by different mechanisms. Intracellular and circulating FXIIIA is a zymogen, which requires proteolytic activation for catalyzing the calcium-dependent transamidation reaction (the active enzyme is usually referred to as FXIIIa). Indeed, we have previously shown that hypertrophic chondrocytes possess a cell type-specific mechanism for proteolytic activation of FXIIIA (43). In contrast, activity of TG2 depends on the levels of Ca2+ and nucleotides in its microenvironment. The intracellular TG2 is present primarily in a catalytically latent form in the cytosol (44). The cytosolic latency of TG2 is maintained through its binding to guanine nucleotides, especially to GDP (45). The intracellular influx of Ca2+ may cause conformational changes to support the transamidating activity of TG2 (45). Alternatively, the externalization of TG2 has been proposed to promote its activity due to translocation from high intracellular (468+/-224 mM) to lower extracellular (0.4-6 mM) GTP microenvironment; ((24); (45); (46)).

Enzymatic analysis of TGases in the extracts/lysates of chondrocytes and in the bone extracts prepared by EDTA extraction (contains only mineral-bound extracellular matrix proteins) shows abundant TGase activity associated with the extracellular matrix (Figure 3) (43). This activity in cartilage pieces, chondrocytes and osteoblast cell cultures is dramatically increased upon trypsin (or thrombin) pre-treatment, indicating that the zymogen of FXIIIA provides a pool of latent extracellular TGase activity ((47); (43); (45)), (Figure 3). Immunohistochemical analysis demonstrated that the FXIIIA zymogen, detected with the Ac16 antibody against the activation peptide of the FXIIIA protein (34) is localized mostly intracellularly with very low levels detectable in the extracellular matrix (Figure 5, left panel). On the contrary, matrix-associated FXIIIA is present mostly in the active form (FXIIIa), as detected with the A3 antibody, which recognizes the C-terminal portion of the molecule in both inactive zymogen and the active enzyme (34). In addition, extracellular localization of enzymatically active TGases was shown by the incorporation of a fluorescence-labeled peptide TGase substrate into the matrix of hypertrophic and articular cartilage (36).

Taken together, these data confirm the presence of extracellular TGase activity in cartilaginous and bony matrices, although both TGases expressed by chondrocytes and osteoblasts (TG2 and FXIIIA) lack a secretory signaling peptide. Mechanisms of TGases' externalization are not clearly established yet. Our previous studies suggested that intracellular proteolytic activation of FXIIIA into FXIIIa by the hypertrophic chondrocytes is followed by cell death and externalization of both the active and zymogenic forms of FXIIIA into the matrix (43).

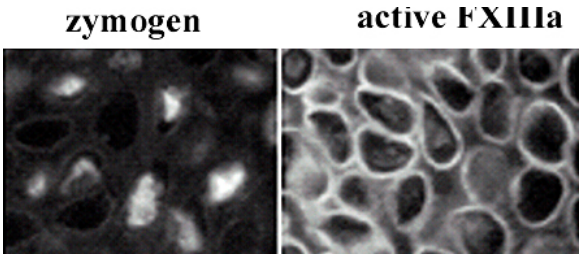

Figure 5. Distribution of FXIIIA in the hypertrophic region of the growth plate in a 20-day-old chicken embryo. Zymogen was detected with the Ac16 antibody, while total levels of FXIIIA (including both the active form, FXIIIa, and the zymogenic FXIIIA) were detected with the A3 antibody. Both antibodies were generated against chicken FXIIIA (34).

Nevertheless, other mechanisms of externalization may be also involved, especially in the pre-hypertrophic zone where the rate of cell death is still insignificant. Recent mutagenesis-based studies provided some insights into the mechanisms of TG2 externalization. These studies showed the importance of Tyr-274 in mediating the release of TG2 into the extracellular matrix and demonstrated the independence of this process from the transmidating enzymatic activity ((48); (24)).

5. TRANSGLUTAMINASES REGULATE CELL DIFFERENTIATION IN BONE TISSUES.

5.1. Regulation of cell differentiation and matrix mineralization during endochondral ossification.

The expression and localization patterns of TG2 and FXIIIA in the developing bones are consistent with a role for these enzymes in the regulation of cell differentiation and extracellular matrix mineralization (as discussed above). We hypothesize that TGases expressed in the pre-hypertrophic and hypertrophic zones of the growth plate directly regulate the growth of the periosteal bony collar and deposition of the osseous mineralized matrix.

To determine whether chondrocytes regulate mineralization of the leading edge of the bony collar, and, if so, whether chondrocyte-derived TGase is one of the factors regulating this process, we devised a co-culture system for rapid analysis of extracellular matrix mineralization. In this system, cells from the non-mineralized, leading edge of the bony collar are cultured along with hypertrophic chondrocytes, resulting in rapid and extensive mineralization (22). The adherent cells from the non-mineralizing edge are defined as pre-osteoblasts as they express certain molecules characteristic of early osteoblast development (such as alkaline phosphatase, osteopontin and osteonectin), but not the markers indicative of late osteoblast differentiation (for example, bone sialoprotein [BSP] and osteocalcin) ((49); (50)). When cultured in a standard mineralization medium containing β-glycerophosphate and ascorbic acid, these cells are able to produce some mineralized matrix. However, the extent of mineralization is limited, and it appears only after an extended period in culture. When pre-osteoblast cells are co-cultured along with hypertrophic chondrocytes, mineralization is extensive (averaging approximately 150 mineralized nodules in a 35-mm dish) and rapid (5-6 days). Co-cultures of mouse hypertrophic chondrocytes with chicken preosteoblast cells allowed for analysis of species-specific expression of BSP and osteocalcin, two markers of advanced osteoblast differentiation, associated with mineralization of the matrix. At the time of mineralization, only chicken BSP and osteocalcin were detected by RT-PCR, with none detectable from mouse. These results confirm osteoblastic differentiation of chicken pre-osteoblast cells, and suggest a regulatory/stimulatory role for chondrocytes in pre-osteoblast maturation (22).

To test whether TGases can function in regulating matrix mineralization, TGase activity in the pre-osteoblast/hypertrophic chondrocyte co-cultures was altered both by decreasing the endogenous enzymatic activity through the addition of the competitive inhibitor putrescine (1,4-diaminobutane), and by increasing the total activity through the addition of exogenous TGase. Mineralization in the presence of purified guinea pig liver TG2 increased by 3- to 5-fold over the untreated cultures. Consistent with these results, inhibition of the endogenous TGase activity by putrescine produced an opposite effect, significantly reducing mineralization by 30-50% (22).

Thus, the ability of exogenous enzyme to promote differentiation of the early periosteal osteoblasts *in vitro* combined with the localization of endogenous TGases in the embryonic long bones suggests a role chondrocytes-derived TGases in the synchronized growth of the cortical and endochondral bones.

5.2. Regulation of cell differentiation and matrix mineralization during intramembranous ossification

Osteoblast differentiation is a sequential process consisting of early mesenchymal condensation, followed by cell proliferation and then a gradual maturation whereby cells begin deposition of a collagenous matrix, which undergoes mineralization. Matrix mineralization is one 'marker' related to fully mature osteoblasts. Whereas some osteoblasts die by apoptosis or become bone-lining cells, others become dendritic osteocytes, which function as mechanosensors deep within the mineralized bone and communicate with each other and osteoblasts by yet unidentified mechanisms. The two earliest and most specific molecular markers of osteoblast differentiation are the transcription factors Cbfa1 (Runx2) ((49); (2)) and Osterix (Osx or Sp7), the latter acting downstream of Cbfa1 (51). In addition to mineralization, expression of alkaline phosphatase (ALP), bone sialoprotein (BSP) and osteocalcin (OCN) are characteristic features of osteoblast maturation ((50); (52)).

To test directly whether TGases induce an increase in mineralization of osteoblast cultures, we examined the expression of BSP in cultures of preosteoblast cells treated with exogenous TG2. Control cultures of preosteoblast cells showed slight mineralization and had little, if any, mRNA for BSP. However, when these cells were grown in the presence of exogenously added

enzyme (0.01 U/ml), mRNAs for BSP and OCN were clearly detectable. TGase-induced changes in the gene expression pattern were accompanied by matrix mineralization in the preosteoblast cultures (22). Similar results were observed in cell cultures of the mouse osteoblast cell line MC3T3-E1 subclone 14. Differentiation and mineralization of these cells was induced by ascorbic acid and β-glycerophosphate. Perturbation of TGase activity in the cell cultures by cystamine resulted in reduced collagen production and alkaline phosphatase levels and complete abrogation of mineralization indicative of arrested osteoblast differentiation (27). Thus, early osteoblasts of evolutionarily divergent species (such as chicken and mouse) respond in a similar manner to the extracellular TGase activity by enhanced deposition of mineralized matrix, while the inhibition of the endogenous TGase enzymatic activity results in reduced levels of mineralization. The mechanisms by which TGases could effect mineralization are discussed below.

6. MECHANISMS FOR TGase-MEDIATED CELL DIFFERENTIATION AND MINERALIZATION

6.1. Regulation of mineralization

Matrix mineralization results from the precipitation of apatitic mineral from available calcium and phosphate ions in the permissive extracellular matrix deposited by differentiated cells (such as osteoblasts in bone, cementoblasts in teeth and hypertrophic chondrocytes in the growth plate). Physiological mineralization of the connective tissue generally involves the presence of a collagen-containing matrix with nanometer-sized hydroxyapatite crystals being deposited within and between collagen fibrils. Mechanisms that regulate mineral deposition at the molecular level have been under investigation for decades and a number of theories have emerged. Experimental and genetic studies support the possibility of two different mechanisms regulating matrix mineralization. Mouse genetic studies suggest control of mineralization by the presence, or absence, of inhibitors (53). Several candidate protein inhibitors such as matrix Gla protein and alpha$_2$-HS-glycoprotein (also known as fetuin) have been identified from the phenotypes of knockout mice showing extensive mineralization of soft tissues ((54); (55)). Recent work by Karsenty and colleagues also suggests that mineralization is an active process regulated by the co-expression of type I collagen and alkaline phosphatase, and by extracellular levels of pyrophosphate (56). *In vitro* and *in vivo* studies have demonstrated that some matrix noncollagenous proteins and/or proteoglycans may act as mineral nucleators to assist in mineral deposition by binding to the gap regions of striated collagen fibrils where crystal growth begins ((57); (58)). Despite the progress already achieved, it is likely that other determinants of mineralization remain to be discovered. The mineralization process clearly requires a rather complex interplay between tissue-resident molecules (both nucleators and inhibitors) and local adjustment of ion concentration. While the role of TGases in matrix mineralization is becoming clearer, the mechanisms behind these actions remain unknown. Below we discuss the potential pathways by which TGases exert their pro-mineralizing activity.

6.2 TGase-mediated protein cross-linking

6.2.1. Substrates of TGases in mineralized tissues

Externalization of TG2 and the active form of FXIIIA (FXIIIa) results in enrichment of the extracellular matrix of bone and hypertorphic cartilage with enzymes capable of cross-linking various proteins. The isopeptide bond (a gamma-glutamyl-epsilon-lysine or polyamine bond) generated by TGases is resistant to regular proteolysis, and accordingly TGases are thought to increase biochemical stability of tissues by cross-linking matrix molecules. In addition to stabilization of the matrix, TGase-induced polymerization of pericellular skeletal matrix calcium-binding proteins has been proposed to promote nucleation and/or growth of calcium-containing crystals (reviewed in (42)).

Protein substrates for the TGases belong to two categories: 1) glutamine acyl donor substrates, which allow the substrate protein to polymerize into large, homotypic macromolecular structures or into heterotypic assemblies with other cross-linking partners, and 2) lysine acyl acceptor substrates, which allows a protein to function as a 'passive' cross-linking partner to a glutamine acyl donor substrate. TGases generally are highly selective for glutamine acyl donor substrates, although it is not clear how TGases recognize and react with the particular glutamine residues amongst many glutamines present in most proteins, since there is no obvious sequence homology between the protein substrates. However, some trends have been recently observed such as the vicinity of proline, glycine and asparagine residues (reviewed in (59)). On the contrary, the required lysines are thought to be less specific and can be provided by a number of proteins (or primary amines). While dozens of TGase substrates have been identified across a multitude of tissues, relatively few are expressed in mineralized tissues. These include collagens I and II, fibronectin and a variety of calcium-binding proteins, but the understanding of their function in bone, cartilage and teeth is rudimentary.

6.2.1.1. Collagens

Perhaps the best-known and most important TGase substrates of the mineralized tissue are collagens. Collagen is the main fibrous component of most connective tissues, and defects in collagen fibrillogenesis and cross-linking (including pyridinoline, pyrrole and aldol cross-links) result in defective matrix assembly and mineralization as seen in osteogenesis imperfecta and dentinogenesis imperfecta. Both collagen type I (characteristic collagen of bone) and type II (characteristic for cartilage) serve as substrates for TGase activity *in vitro.* In addition to intramolecular collagen cross-linking, cross-linking between collagen and non-collagenous extracellular matrix proteins might also occur *in vivo* ((60); (61); (62); (63); (27)).

A fibrillar collagen network is necessary for matrix mineralization. Reintroduction of the fibrillar type II collagen in ROS 17/2.8 cells, which do not express type I collagen, resulted in the deposition of a mineralized matrix in the presence of beta-glycerophosphate (56). TGases have an ability to promote collagen synthesis ((64); (65); (27)).

Collagen synthesis and assembly further stimulates collagen production and thus promotes cell differentiation ((66); (67); (68); (69); (70); (71)). In addition, TGase-catalyzed cross-links confer increased stability and resistance to degradation to the modified extracellular proteins ((72); (73); (74)). Given the importance of a collagenous network for mineral deposition, the mechanisms of TGase-induced matrix mineralization could be linked to the ability of TGases to increase collagen production by modulating collagen synthesis and by stabilizing the collagen network.

Although necessary, a collagenous network alone is not sufficient to induce bone mineralization (56). Moreover, concentration of TGase-catalyzed epsilon-(gamma-glutamyl)lysine cross-links in the cartilaginous matrix is higher in the non-collagenous proteins, and is associated for example, with microfibrillar fraction of tissues (75). Several non-collagenous bone and cartilage matrix proteins have been identified as TGase substrates and will be discussed below.

6.2.1.2. Fibronectin

Fibronectin (FN) is an important cell adhesion protein on the cell surface and in the extracellular matrix. The molecule of FN contains both TGase-reactive glutamines and lysines, and both TG2 and FXIIIa promote the formation of a fibrillar FN network ((76); (77)). The cross-linked insoluble FN fibrils regulate osteoblast differentiation and bone formation, i.e., collagen deposition on the FN scaffold matrix and mineralization ((69); (78); (66); (67); (68)). In addition to TGases' ability to support FN network formation by cross-linking, TGases may also affect the RGD- and beta-1 integrin-dependent promineralizing effects of fibrillar FN (79) by enhancing FN binding to the cell surface. TG2 binds to the cell surface and to FN via the non-catalytic N-terminal domain, acting as an integrin-binding co-receptor for FN ((80); (81); (82); (83)).

The close relationship between TGases and FN in cell adhesion and matrix assembly implies that TGases could partake in all FN maturation steps, starting from focal contacts, then leading to focal adhesions, to fibrillar adhesions and finally to matrix fibrils. The latter induce further matrix formation and mineralization (84).

6.2.1.3. SIBLING proteins

A number of the non-collagenous proteins from hard tissues belong to the SIBLING family (Small Integrin-Binding Ligand N-linked Glycoprotein) (85). SIBLING proteins all exhibit prominent mineral-and cell-binding capacities and are highly expressed in hard tissues. For example, osteoblasts, hypertrophic chondrocytes and osteocytes express osteopontin (OPN), BSP and dentin matrix protein-1 (DMP-1); the tooth forming odontoblasts express dentin phosphoprotein (DPP), dentin sialoprotein (DSP) and BSP; cementocytes express OPN, BSP and DMP-1 (86). Common among SIBLING proteins is their high level and diversity of posttranslational modifications (phosphorylation, glycosylation and sulphation). In addition to these modifications, OPN, BSP, DPP and DMP were found in polymeric forms in teeth and bone extracts implying that they are also covalently cross-linked. *In vitro*, all of these SIBLING proteins served as TG2 substrates ((87); (88); (89); (90); (91); (92)).

SIBLING proteins may support collagen mineralization *in vivo* by binding to collagen fibrils, as has been shown for DPP, OPN and BSP ((91); (93); (94); (95)). TGases have a potential to stabilize these interactions *in vivo*. Additionally, TGase-induced polymerization of acidic bone proteins, such as OPN and osteonectin (ON), may nucleate mineralization, as has been proposed earlier ((61); (42); (28)). Indeed, polymeric assemblies of DMP-1, most likely stabilized by TGases (92) can promote hydroxyapatite formation *in vitro* (96) and in the teeth, where they are abundant (92). However, based on *in vitro* crystal growth studies, on the analyses of the OPN -/- mice and of the MGP-OPN double knockout mice, as well as on current data on OPN in pathological calcification, it is unlikely that OPN polymers contribute to mineral nucleation in bone, cartilage or dentin ((97); (98); (99); (100)). More likely, another SIBLING protein, BSP, could function as a nucleator of mineralization when covalently linked to collagen fibrils as it is capable of promoting mineral formation in nucleation assays *in vitro* (101). Until the skeletal elements of BSP -/- mice are analyzed in detail, its role as a mineral nucleator *in vivo* is yet unconfirmed.

It has been suggested that in addition to controlling hydroxyapatite deposition in mineralized tissues, SIBLING proteins could act as "matrix growth factors", promoting hard tissue cell differentiation (102). The role of TGases in this process is relevant as these enzymes co-localize with SIBLING proteins at matrix-cell interfaces. Thus, TG2 co-localizes with OPN and BSP in the osteoid and in the pericellular matrix of osteocytes; it also co-localizes with BSP and DMP-1 in the pericellular matrix of cementocytes, with DMP-1, DPP in the odontoblast and osteocyte processes (92). In addition, TG2 and FXIIIA are co-expressed with DMP-1 in the hypertrophic chondrocytes of the growth plate (103). Further work is required to understand the mechanisms of SIBLING proteins' polymerization in cartilage and bone and their involvement in the regulation of signaling cascades. Recently described signaling capabilities of DPP to regulate fibroblast and osteoblast gene expression and differentiation via integrins and the MAP kinase signaling pathway (101) provide the first experimental support for the proposed roles of SIBLING proteins in hard mineralized tissues.

In summary, multiple SIBLING proteins are TGase substrates and are polymerized *in vivo*, likely by TGase-induced cross-linking. Protein polymerization of BSP, DMP-1 and DPP may affect matrix calcification directly. In addition, polymerized SIBLING proteins could indirectly effect mineralization by promoting cell differentiation. A plausible scenario suggests augmentation of the cell-adhesive and signaling properties of SIBLING proteins upon polymerization by grouping RGD-containing proteins together. Such grouping can promote integrin clustering on the cell surface, thereby affecting further

downstream signaling in the cells, resulting in changes in gene expression and cell differentiation. In this way, TGase-mediated polymerization could represent an activation process for specific roles of these proteins.

6.2.1.4. Other extracellular matrix proteins

Previous studies identified two more bone proteins as TGase substrates - ON, also known as SPARC or BM-40, and fibrillin-1. ON is a calcium-binding, cysteine-rich glycoprotein expressed in a large number of tissues including bone and cartilage (104). ON was identified as a major TGase substrate in cartilage by Aeschlimann and colleagues who speculated that its polymerization promotes matrix stabilization and mineral nucleation (61). However, adult ON-/- mice have no hard tissue developmental defects, but demonstrate an osteopenic phenotype, show decreased bone formation and bone remodeling defects due to the decreased numbers of both osteoblasts and osteoclasts on the bone surface ((105); (106)). In this regard, TGase-mediated ON incorporation into the cartilage matrix may be important for promoting cartilage degradation by chondroclasts/osteoclasts and for adhesion of osteoblasts and bone formation during endochondral ossification.

Fibrillin-1 is a TGase substrate (107) whose function is closely related to long bone development. Mutations in the fibrillin-1 gene cause Marfan syndrome characterized by unusually long limbs, joint laxity and scoliosis. Though fibrillin-1 is most commonly associated with connective tissues rich in elastic fibers, it is also expressed in the perichondrium/periosteum of long bones (108) and in the bone cells localized to osteocytes and to the endosteal surface of the bone. Fibrillin-1 is the major cross-linked protein in the cartilaginous matrix (75). Nevertheless, the specific roles of fibrillin-1 in bone cell differentiation and mineralization are not known.

6.3. TGase-mediated regulation of TGF-beta

Another potential pathway by which TGases may exert their pro-mineralizing activity, involves the recently described ability of these enzymes to regulate the extracellular levels of active transforming growth factors (TGF-beta). TGF-beta belongs to a family of regulators of cell growth and differentiation, which includes three isotypes of TGF-beta (TGF-beta 1, TGF-beta 2 and TGF-beta 3), activins, inhibins and bone morphogenetic proteins (BMPs).

TGF-beta 1-3 are ubiquitous cytokines, also expressed by chondrocytes, osteoblasts and osteocytes. These growth factors affect many aspects of endochondral and intramembranous bone formation ((109); (110); (111)). TGF-beta 1-null mice show a severe skeletal phenotype having short bones with abnormal growth plates and decreased mineral content, decreased number of osteoblasts, and altered chondrocyte proliferation and differentiation ((112); (113)). TGF-beta 2-null mice similarly show a number of cartilage defects, smaller bones and reduced cranial ossification (114). Genetic evidence suggests that endogenous TGF-beta maintains cartilage homeostasis by preventing inappropriate chondrocyte differentiation (115). In addition, TGF-beta 1 and BMP-2 are potent multifunctional regulators of osteoblast differentiation. Their signaling pathways may target the transcriptional activity of Cbfa1, an essential regulator of osteogenesis (reviewed in (21)).

Cells secrete TGF-beta in the form of a latent precursor, which is non-covalently bound to latency-associated peptide (LAP). This complex is further bound to a latent TGF-beta binding protein (LTBP). Activation of the latent complex can occur through a variety of mechanisms in various tissues and cell culture systems, including TGase-mediated cross-linking of LTBP to other matrix proteins with subsequent release of active TGF-beta (reviewed in (116)). Previous studies demonstrated that LTBP1 is a substrate for TG2 *in vitro* (117) and in various cells ((118); (119)), including articular chondrocytes ((120); (121)).

Despite the described ability of TGases to activate latent TGF-beta, our data suggest that TG2-induced mineralization and accelerated maturation of embryonic pre-osteoblasts does not involve activation of TGF-beta (22). Similarly, matrix mineralization in chondrogenic TC28 cells is increased by elevated levels of TG2 or FXIIIA, but not by TGF-beta (122). Experimental data measuring the effects of TGF-beta on matrix mineralization depend on the precise culture conditions and often result in contradictory conclusions. Understanding the relationship between the extracellular TGases and activation of TGF-beta is even more complicated by additional factors, such as age-related differences ((120); (121)); or the nature of the bone tissues, such as the observed differences in the mineral content and crystallinity in the cortical and trabecular bones of TGF-beta 1-null mice (113). Overall, analysis of the TGase/ TGF-beta relationship *in vivo* may be more informative, since these systems preserve the interactions between cells, extracellular matrix and growth factors.

6.4. TGase actions independent from transamidating activity

Although biological functions of TGases are generally attributed to their enzymatic protein-modifying activity, in some instances these proteins may exhibit a specialized non-catalytic activity, as has been shown for TG2 (reviewed in (28)). For example, TG2 acts as an integrin-binding cell adhesion co-receptor independently from its cross-linking activity (80). Similarly, the GTPase activity of TG2 is independent from its transamidating activity (see chapter below).

TG2 is the only dual function family member with transglutaminase (TGase) and receptor signaling activities. Because of its ability to bind and hydrolyze GTP, it is also called G_h/TG2 (for a"novel G protein") (123). TG3 can also bind GTP, but lacks the hydrolyzing activity. G_h/TG2 G2 has been implicated in a wide variety of processes, depending on its localization: intracellular membrane-associated or cytosolic, and extracellular at the cell surface. The two enzymatic activities of TG2 are reciprocally regulated: binding of GTP to TG2 inhibits its

TGase activity, while calcium binding inhibits the GTPase activity, suggesting that microenvironment plays an important role of regulating these disparate activities of TG2 ((124); (125)). In addition to inducing a conformational change in TG2 protein that prevents its transamidating activity, GTP binding also selectively inhibits the proteolytic degradation of TG2, thus providing additional level of regulation of the activity of this TGase (124).

6.4.1. Regulation of mineralization by non-catalytic actions of TGase

In corroboration, recent studies by others and us suggest that the cross-linking activity of TG2 is not required for acceleration of matrix mineralization and differentiation of bone cells ((22); (24)). In the preosteoblast/hypertrophic chondrocyte co-cultures this is suggested by the following observations: (1) Adding exogenous TG2 enhances mineralization but is not accompanied by any changes in the pattern of protein cross-linking; (2) pre-activation of exogenously added TG2 with DTT and calcium does not increase the levels of TGase-induced mineralization, and (3) exogenous TG2 binds directly to cell-surface proteins both in preosteoblasts and hypertrophic chondrocyte cells without the formation of cross-links in these proteins (as determined by using the biotinylated specific substrate for TGases (data not shown). While *in vitro* results suggest that TG2-mediated acceleration of osteoblast differentiation and enhancement of matrix mineralization is independent from its cross-linking activity, a possibility exists that *in vivo* binding of endogenous TGases to the cell surfaces of preosteoblasts and chondrocytes involves the transamidating activity.

In agreement with the hypothesis that extracellular TG2 may promote maturation and matrix mineralization in preosteoblasts in a transamidation-independent manner, a recent study by Johnson and Terkeltaub (24) demonstrates that externalized GTP-bound TG2 acts as a molecular switch for hypertrophic differentiation and calcification of the chondrocytes in a manner that does not require its TGase enzymatic and GTPase activities. This study also emphasizes the importance of TG2 externalization for induction of matrix mineralization in the chondrocytic cells. FXIIIA derived transamidation activity was not studied.

6.4.2. Regulation of mineralization by GTP binding/GTPase activity of TG2

G_h/TG2 participates in the intracellular signaling through stimulation of phospholipase C (PLC)-mediated inositol phosphate (IP) production in response to agonist activation of $alpha_{1B}$- and $alpha_{1D}$-adrenergic receptors (123) and of thromboxane A_2 receptors (126). It is believed that G_h/TG2 activates the delta-1 isoform of phospholipase C (PLC) (127). Activation of PLC may be an important regulator of differentiation programs both in chondrocytes and osteoblasts. Thus, previous studies have showed that regulation of osteoblastic cell differentiation by basic fibroblast growth factor (b-FGF) is mediated by the main signaling mechanism involving tyrosine phosphorylation of PLC and activation of protein kinase C (PKC) (128). Similarly, PLC and G-proteins mediate the 17-beta-estradiol-induced activation of PKC, which is associated with chondrocyte proliferation, differentiation, and matrix synthesis (129). An important role of Gh/TG2 signaling activity in chondrocyte differentiation is demonstrated by the ability of the wild type TG2 to promote hypertrophic differentiation in chondrocytic CH-8 cells, while GTP-binding mutant TG2 (K173L) lacks such ability (24). These results emphasize the importance of TG2 and its GTPase activity for cartilage maturation. Interestingly, the TG2-induced mineralization in chondrocytic CH-8 cells is independent from both the transamidating and GTPase activities, but relies on the GTP-bound state of the extracellular TG2. Most likely, the GTP-bound conformation supports the fibronectin-independent binding of TG2 to integrins, thus activating the downstream classical signaling through p38 kinase to promote chondrocyte differentiation. This study provides a possible mechanism for the non-catalytic effects of TG2 in the matrix where a TG2/GTP complex could function as a signaling-type molecule interacting with cell surface constituents (24). In addition, TGases may indirectly affect matrix calcification in chondrocytes, by modulating various processes such as signal transduction, cell adhesion, activation of latent transforming growth factor TGF-beta (130); and also the apoptotic process (131), which is pro-mineralizing (37).

6.4.3. Integrin-mediated signaling

The array of cell surface TGase-binding proteins may determine the effects of extracellular TGases (in particular, TG2) on different cells. TG2 can interact with integrins of the beta-1 and beta-3 subfamilies (80). The repertoire of integrin expression may be an important parameter, which defines the effect of external TGases on the cell fate. We have identified distinct patterns of the TG2-binding cell surface proteins (Figure 6) in two cell types, which respond differently to exogenous TG2: the preosteoblasts undergo differentiation to osteoblasts, while hypertrophic chondrocytes retain their phenotype. The roles of these TG2-binding proteins in mediating the effects of the extracellular enzyme on cell differentiation are currently under investigation in our laboratories.

Previous studies showed that extracellular TG2 directly interacts with multiple integrins of the beta-1 and beta-3 subfamilies (80), affecting the integrin-mediated cell adhesion (132). In mechanically strained osteoblastic cells integrins co-localize with TG2 at the plaques of cell-matrix interactions (133), and beta-1 integrin mediates activation of PKA signaling (134). Previous studies have shown regulation of mineralization in osteoblasts by PKA signaling, which involves phosphorylation of specific transcription factors and subsequent effect on the transcription levels of genes, such as BSP, a marker of osteoblastic differentiation (135). PKA has also been shown to mediate the paracrine and/or autocrine effects of parathyroid hormone on cell proliferation and differentiation. In two types of cells capable of biomineralization, e.g., osteoblasts and cementoblasts, activation of the PKA pathway has been reported to

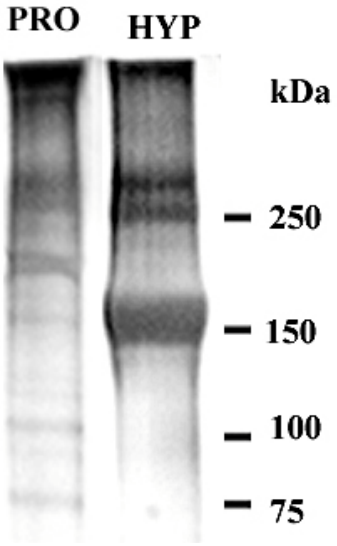

Figure 6. Cell-surface proteins interacting with exogenous TG2. Cells were incubated with purified guinea pig liver TG2 and treated with DTSSP reagent to cross-link TG2 to its receptors. Cell lysates were subjected to co-immunoprecipitation with anti-TG2 antibody, proteins were separated on 4-15% gradient SDS gel and analyzed by silver staining procedure. Molecular mass markers in kDa are shown to the right of the gel. Cell lysates of the pre-osteoblastic cells from the non-mineralized leading edge of the bone collar (PRO) and of the hypertrophic chondrocytes (HYP) were analyzed.

decrease both matrix mineralization and the expression of BSP ((136); (137)). Our studies suggest that TG2-enhanced maturation of osteoblasts involves inhibition of PKA-mediated signaling (22). Further analysis of the TG2-binding proteins and the affected intracellular pathways will significantly enhance general understanding of the programs of chondrocyte and osteoblast differentiation.

6.5. Synergistic functions of TG2 and FXIIIA – lessons from the knockouts

The observations summarized in this review document the importance of TGase activity in the promotion of *in vitro* bone formation and in both the endochondral and intramembranous ossification process. It is evident that two TGases, TG2 and FXIIIA, contribute to the TGase activity observed in both hypertrophic chondrocytes and osteoblasts, and it is additionally possible that these enzymes work synergistically in these processes. Observations in TG2-deficient mice are consistent with this notion of two or more TGases being active in the skeleton. The phenotypes of TG2 knockout mice were described in 2001 independently by two research groups – with neither mouse reported to have an overt skeletal phenotype ((138); (139)). Nevertheless, detailed analysis *in vitro* identified in the TG2-/- chondrocytes slower responses to retinoic acid- and CXCL1-induced differentiation towards hypertrophy ((24); (140); (141)). The phenotype of FXIIIA knockout mice reported the same year was also without skeletal or dental abnormalities, but with coagulation and bleeding defects ((142); (143)). The absence of a mineralized tissue phenotype in either of these mice, and the normal ultrastructure of cell and extracellular matrix in the TG2-/- bones and teeth detected by electron microscopy (unpublished data), suggest that the transamidating activity of each individual TGase is not critical for hard tissue formation. Nevertheless, the reported effect of non-discriminating TGase inhibitors to cause a blockade in osteoblast differentiation and mineralization indicates that TG2 and FXIIIA can compensate for each other in the null background and have similar functions in hard tissues, with the exception of exclusive GTPase activity of TG2. For example, the observation that TG2-/- chondrocytes are capable of hypertrophy implies that TG2 enzyme alone is not critical for chondrocyte hypertrophy, but does not exclude the possibility that TGase activity would not be. Our work demonstrates that TGase activity assays performed on EDTA-demineralized extracts from TG2-/- bones (to release proteins bound to mineral, after removal of the soft tissue components by guanidine hydrochloride), revealed no difference in TGase activity in the TG2-/- mice bones as compared to similar bone extracts from their wild type counterparts (Figure 7). Similarly, OPN polymerization, indicative of TGase activity, was unaltered as detected by Western blot analysis (Figure 7). Further work is required on the co-localization of two TGases (TG2 and FXIIIA) in the bone tissues to specify their individual functions. Development of TG2/FXIIIA double knockout mice, or conditional knockouts restricted to the skeleton, would significantly advance our understanding of the roles of TGases in bone formation/development.

7. CONCLUSIONS / PERSPECTIVES

This review summarizes the accumulating evidence that extracellular TGases may regulate bone development and normal physiology. Additionally, TGases contribute to bone pathologies associated with/or accompanied by excessive matrix mineralization. For example, levels of TGases are increased in the articular cartilage affected by osteoarthritis, where the enzymes may promote chondrocyte differentiation to hypertrophy, support matrix calcification and thus, stimulate intra-articular inflammation and further damage of the cartilage in osteoarthritis (144). Similarly, age-associated increase in TG2 levels in articular cartilage (145) may enhance inflammation and damage to the joint surfaces.

Characteristic pattern of expression of two TGases (TG2 and FXIIIA) in the mineralizing tissues, combined with the demonstrated ability of TG2 to promote osteoblast maturation and to induce chondrocytes hypertrophy, provokes further analysis of the molecular mechanisms that mediate the TGase-induced cell differentiation. The functions of TG2 and FXIIIA appear to be coupled. Both enzymes contribute to the levels of transamidating activity in these tissues and can compensate for the loss of each other. Given the emerging data on TGases, it is credible to hypothesize that in the mineralized tissue matrices, the major role of TG2 is most likely to regulate cell adhesion, spreading and signaling at the cell surface both in chondrocytes and osteoblasts. Further work

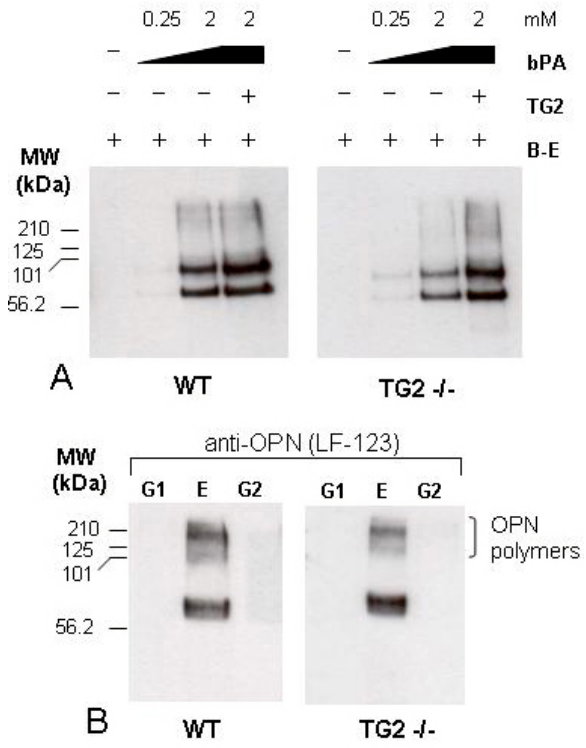

Figure 7. TGase activity and OPN polymerization in bone extracts of wild type and TG2-/- mice. (A) Wild type and TG2-/- mouse bones (marrow and growth plates removed) were pulverized under liquid nitrogen and powder was subjected to sequential extraction using guanidine hydrochloride (G1), EDTA (E) and guanidine hydrochloride (G2). TGase activity as assessed in EDTA-extract (after buffer exchange to 5 mM NH_4HCO_3, pH 8) by primary amine incorporation assay where extracts were incubated with biotin-(pentyl)amine (bPA) in reaction buffer containing 3 mM $CaCl_2$, 1 mM DTT, and 150 mM Tris-HCl, pH 8. TG-mediated biotin-(pentyl)amine labeling of natural TG substrates in the extracts was assessed by Western blotting using ExtrAvidin®-horseradish peroxidase conjugate. Labeling was visualized by enhanced chemiluminescence (ECL kit, Amersham) (B) Wild type and TG2-/- mice bone extracts, prepared as above, were subjected to Western blotting and detected with anti-OPN to visualize OPN polymerization as indicative of TG activity in bone.

on the specific functions of these enzymes in various genetic models would provide key *in vivo* data on the role of TGase activity in bone formation.

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Abbreviations: TGase – transglutaminase; TG2 – tissue transglutaminase; FXIIIA – the A subunit of coagulation factor XIII including both zymogen and active forms; FXIIIa – enzymatically active (proteolytically activated) form of FXIIIA; FN – fibronectin; OPN – osteopontin; ON-osteonectin; OCN – osteocalcin; BSP - bone sialoprotein; DMP-1 - dentin matrix protein-1; DPP - dentin phosphoprotein; DSP - dentin sialoprotein

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