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Characterization of the Transglutaminase Gene Family in Zebrafish and in vivo Analysis of Transglutaminase-Dependent Bone Mineralization

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Abstract

We have characterized the protein cross-linking enzyme transglutaminase (TGs) genes in zebrafish, *Danio rerio*, based on the analysis of their genomic organization and phylogenetics. Thirteen zebrafish TG genes (zTGs) have been identified, of which eleven show high homology to only three mammalian enzymes –*TG1*, *TG2* and *FXIIIa*. No zebrafish homologues were identified for mammalian TGs 3–7. Real-time PCR analysis demonstrated distinct temporal expression profiles for zTGs in larvae and adult fish. Analysis by *in situ* hybridization revealed restricted expression of zTG2b and zFXIIIa in skeletal elements, resembling expression of their mammalian homologues in osteo-chondrogenic cells. Mammalian TG2 and FXIIIa have been implicated in promoting osteoblast differentiation and bone mineralization *in vitro*, however mouse models lacking either gene have no skeletal phenotype likely due to a compensation effect. We show in this study that mineralization of the newly formed vertebrae is significantly reduced in fish grown for 5 days in the presence of TG inhibitor KCC-009 added at 3–5 days post fertilization. This treatment reduces average vertebrae mineralization by 30%, with complete inhibition in some fish, and no effect on the overall growth and vertebrae number. This is the first *in vivo* demonstration of the crucial requirement for the TG-catalyzed cross-linking activity in bone mineralization.

Keywords

Transglutaminase; Inhibitors; Zebrafish; Bone Development

Introduction

The comprehensive identification and understanding of both systemic and local bone anabolic factors is essential for the development of new therapeutic targets to treat bone diseases and fractures. Previous *in vitro* studies from ours and other groups have demonstrated that enzymes transglutaminases (TGs) promote osteoblast differentiation and enhance deposition of mineralized matrix (Aeschilamm *et al.*, 1993; Nurminskaya *et al.*, 2003). TGs (R-glutaminylpeptide: amine- γ -glutamyl transferases, EC 2.3.2.13) are multifunctional Ca^{2+} dependent proteins that are crucial for the formation of ϵ -(γ -glutamyl)-lysine-protein cross-links (Lorand and Graham, 2003). In mammals, the family of catalytically active TG is comprised of eight proteins, TGs1–7 and FXIIIa, with most of the mammalian TGs being expressed in a tissue-specific manner.

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Two mammalian TGs, TG2 and FXIIIa, have been reported to be up-regulated in the osteochondrogenic lineage (Aeschlimann *et al.*, 1993; Nurminskaya and Linsenmayer, 1996; Borge *et al.*, 1996; Rosenthal *et al.*, 1997; Nurminskaya and Linsenmayer, 2002; Summey, Jr. *et al.*, 2002; Al-Jallad *et al.*, 2005). Both enzymes are expressed in pre-hypertrophic and hypertrophic chondrocytes of the growth plate and in the "borderline chondrocytes" that are localized to the lateral edges of the growth plate (Nurminskaya and Kaartinen, 2006). These "borderline chondrocytes" are thought to regulate the formation of the bony collar (Bianco *et al.*, 1998), suggesting that extracellular chondrocyte-derived TGs may mediate the coordination of osteoblast and chondrocyte differentiation - a key event in proper bone formation (reviewed in Karsenty, 2001). This hypothesis has been confirmed *in vitro* by the ability of TG2 and FXIIIa to promote differentiation in osteoblasts (Nurminskaya *et al.*, 2003; Becker *et al.*, 2008), and osteoblast-like transformation in vascular smooth muscle cells (Faverman *et al.*, 2008). Nevertheless, despite the *in vitro* evidence, genetic ablation of either enzyme has no effect on skeletal phenotype in mouse models (Nanda *et al.*, 2001; Lauer *et al.*, 2002; Koseki-Kuno *et al.*, 2003). A plausible explanation for the discrepancy between the *in vitro* and *in vivo* studies accounts for functional redundancy between TGs due to high similarity in their substrate specificity (Achyuthan *et al.*, 1996), and as a result, functional compensation for loss of each isoform by other TGs in embryonic development. Thus, compensatory activation of FXIIIa in the TG2^{-/-} cells supports total TG activity and the pattern of protein cross-linking identical in TG2^{-/-} and wild type cartilage (Nurminskaya and Kaartinen, 2006) (Nurminskaya *et al.*, 2006; Tarantino *et al.*, 2008). In addition, TG5, TG1 and TG7 have been postulated to compensate for the loss of TG2 in various tissues (Grenard *et al.*, 2001; Johnson *et al.*, 2008).

To overcome complications associated with this compensation mechanism in the genetic loss-of-function mammalian models and to obtain insight into the role of TG-mediated cross-linking in bone formation, we employed the *in vivo* analysis of bone development in zebrafish (*Danio rerio*). Several physiologic features, such as early transparency, short maturation period, and high reproductive capacity, make this model ideal for studying developmental processes (Brittijn *et al.*, 2009). Additionally, numerous zebrafish developmental mechanisms, including bone development, share common factors with mammalian systems. Furthermore, the presence of orthologues for genes commonly seen in human diseases makes zebrafish especially useful for preliminary *in vivo* drug studies (Brittijn *et al.*, 2009). However, transglutaminase enzymes in zebrafish have not been studied on either genetic or functional levels. In the present study, we analyzed the zebrafish genome for TG (zTGs) genes, and have identified thirteen isoforms eleven of which are highly similar to one of the three human TGs (FXIIIa, TG2 and TG1). Taking into consideration that two of these mammalian homologues have been implicated in the regulation of mammal tissue calcification, we analyzed regulation of bone formation in zebrafish in which total TG activity was inhibited during vertebrae mineralization. Our study demonstrates a crucial role for TG-mediated cross-linking in bone calcification.

Material and Methods

BLAST Search, Sequence Alignments and Phylogenetic Analysis

NCBI database of *Danio rerio* protein sequences was searched with the blastp algorithm using the NCBI Blast server. We aligned the sequences with CLUSTAL-W (<http://www.ebi.ac.uk/Tools/clustalw2>) and constructed a phylogenetic tree using maximum parsimony algorithm with protpars tool in the PHYLIP 3.5 package (<http://www.es.emblnet.org>). We also aligned sequences and constructed a phylogenetic tree using the COBALT tool at NCBI (<http://www.ncbi.nlm.nih.gov/tools/cobalt>). Further, we used the phylogeny.fr package (<http://www.phylogeny.fr/version2.cgi/index.cgi>) for alignment and phylogenetic analyses.

Embryo Generation and Maintenance

Wild type zebrafish were maintained at the zebrafish facility of the Aquaculture Research Center, Center of Marine Biotechnology University of Maryland, as previously described (Du *et al.*, 2001). Embryos were obtained from natural mating, staged according to morphology or by days post-fertilization (dpf), and kept at 28.5°C on a 14 hour light 10 hour dark cycle. During inhibitor treatment the zebrafish larvae were kept in 6-well plates with 5 fish in 5ml of water per well. Inhibitors were dissolved to a stock concentration of 10mM in 100% Dimethyl Sulfoxide (DMSO) (Sigma, MO) and added directly to the fish water to a final concentration of 30µM. Fish water, supplemented with paramisol and inhibitor, was changed once per day.

Ex vivo identification of TG Cross-linking Activity in Zebrafish

Transglutaminase cross-linking activity in zebrafish was visualized *ex vivo* by incubation with 5mM rhodamine-conjugated synthetic substrate Pro-Val-Lys-Gly (SY2011) (Kim *et al.*, 1997) in PBS for four hours at 28°C. Zebrafish were then washed with PBS and left overnight, to allow all unincorporated substrate to diffuse out, at room temperature in PBS. Zebrafish then were fixed in 4% PFA at room temperature for 2 hours. Images were acquired by a Leica DMIL FLUO microscope equipped with a SPOT RT slider real-time CCD camera (Diagnostic Instruments, Inc.).

Whole Mount In situ Hybridization

Whole mount *in situ* hybridization was adapted from the previously described protocol (Thisse *et al.*, 2007). Embryos were fixed in 4% PFA and bleached with 3% Hydrogen Peroxide in 0.5% Potassium Hydroxide in PBS-T for 15 minutes. This was followed by Proteinase K (Novagen) digestion (10µg/ml) for 30 minutes, second fixation in 4% PFA and acetone treatment for 8 minutes at -20°C. The DIG-labeled antisense RNA probes were used at 2ng/µl concentration in the hybridization buffer. Hybridized DIG-RNA probes were detected with an anti-DIG fab fragments antibody fused to alkaline phosphatase (Roche, CA) at a 1:4000 dilution followed by colorimetric assay with NBT/BCIP solution (Roche, CA) in the dark for 24–72 hours until color developed. Staining progression was monitored with Nikon AZ100 microscope and analyzed with Nikon Elements software.

Real-Time PCR

Total RNA was isolated from unfertilized eggs, 4dpf, 13dpf and torso of adult zebrafish with Trizol reagent (Invitrogen) and used for first-strand cDNA synthesis. Primers for zTGs were designed using NCBI primer design software (Table 1). The real-time PCR was run on a Roche LightCycler 480 II following the manufactures instructions for heat activation, amplification and melting curves for 45 cycles. Expression levels were normalized to β-actin mRNA.

TG Activity Assay

Total TG cross-linking activity in whole fish lysates was assayed by incorporation of the biotinylated pentylamine Ez-link Pentylamine-Biotin (Pierce, IL) into *N,N'*-Dimethylcasein (Sigma-Aldrich, MO) in the ELISA-like assay as previously described (Trigwell *et al.*, 2004). The 96-well microtiter plates (Maxisorp NUNC, UK) were incubated overnight with 250µl of 1mg/ml *N,N'*-Dimethylcasein (Sigma-Aldrich, MO) in 5mM Sodium Carbonate (pH 9.8), and blocked with 200µL of 0.1% bovine serum albumin (BSA) (HyClone, Ut) in 5mM Sodium Carbonate (pH 9.8) for one hour at 37°C. Whole 14dpf zebrafish mated larvae were lysed in 5mM Tris-HCl pH 7.5, 0.25M Sucrose, 0.2mM MgSO₄, 2mM DTT, 0.4mM PMSF, 5µg/ml Leupeptine and 0.4% Triton X-100 (lysis buffer), centrifuged and TG-containing supernatant was used for further assays. Purified guinea pig liver

transglutaminase 2 (gplTG2) (Sigma-Aldrich, MO) was used as a standard for activity tests. For inhibitory studies, zebrafish lysates (20ng total protein) or purified gplTG2 (75ng purified protein) were pre-incubated with 100 μ M inhibitors for one hour at 28°C. Reaction was carried out in 100mM Tris-HCl pH 8.5, 6.7mM CaCl₂, 13.3mM DTT and 2.5mM Ez-link Pentylamine-Biotin (Pierce, IL) for one hour at 37°C. Incorporated Ez-link Pentylamine-Biotin was detected with 1:5000 ExtrAvidin-Peroxidase (Sigma, MO) and Super AquaBlue ELISA Substrate (eBioscience, CA) followed by reading the absorbance at 405nm on a Polarstar Optima plate reader.

Calcein Staining of mineralized vertebrae

Protocol was adapted from Du *et al.*, 2001. Zebrafish were transferred from fish water to 0.2% Calcein Stain (Sigma, MO) in water, pH 7, and incubated in the dark for 10 minutes at room temperature. Fish were washed in ocean salt water (0.3g/L aquarium salt) and then transferred into ocean salt water and incubated for 10 minutes in the dark at room temperature. After incubation zebrafish were euthanized with 0.4% Tricaine (Sigma, MO), 20mM Tris pH 9.0 and mounted in 3% methyl-cellulose. Images of the fluorescently stained vertebrae were taken 5 minutes after staining to avoid bleaching. Photos were taken with a Leica DMIL FLUO microscope equipped with a SPOT RT slider real-time CCD camera (Diagnostic Instruments, Inc.). Intensity of the calcein staining was analyzed for each vertebra with Photoshop.

Data and Statistical Analysis

Data was collected and analyzed by both Microsoft Excel and Photoshop. Statistical significance was calculated by the student's T-test and the error bars demonstrate the standard error mean.

Results

Identification of Zebrafish TG Genes

To identify zebrafish TG genes (zTGs) we analyzed the current version of the NCBI zebrafish proteome database with blastp searches. By using human TG1, TG2, TG4, and FXIIIa protein sequences as queries, in each case we found the same set of fourteen homologous zebrafish proteins. Among these, one protein and corresponding gene LOC793095 initially described as a partial TG-homologous sequence has been removed from the genome assembly as not supported by sufficient evidence. Consistent with this, we did not detect expression of this gene through zebrafish development by real-time PCR. Therefore, our analysis identified thirteen zTGs (Table 2). To further clarify phylogenetic relationship between the identified zTGs and human TGs, we performed multiple alignments of protein sequences and constructed phylogenetic trees using the following approaches: (i) TGs sequences were aligned with CLUSTAL-W and a phylogenetic tree was constructed using maximum parsimony algorithm with protpars tool in the PHYLIP 3.5 package, additionally (ii) sequence alignment and phylogenetic tree construction were done using the COBALT tool at NCBI, and (iii) phylogeny.fr package was used to align the sequences using the MUSCLE tool and to employ the maximum likelihood or neighbor-joining algorithm to construct the trees and analyze them with bootstrapping (1,000 iterations), or alternatively to use the Bayesian tree building algorithm with 1,000 iterations and burnin parameter of 50. All of these approaches resulted in the same tree configuration (a representative example of Bayesian tree is shown on Fig. 1a). We found that five zTGs (LOC's 793448, 100535918, 555962, 100334173, and 566581) are strikingly close to each other and to the human TG1 and probably originated by gene amplification after divergence of their common ancestor from the human TG1 (Fig. 1a). Also, three zTGs (LOC's 558353, 767742, and 561287) are similar to each other and to the human FXIIIa, and this group of

zebrafish TGs also originated by a relatively recent gene amplification after their common ancestor diverged from the human FXIIIa. Further, three zTGs are similar to human TG2. Among these, two genes (LOC's 323856 and 447909) originated after their common ancestor split from human TG2, and another one (LOC559012) is more ancient. Finally, two zebrafish TGs have been annotated as TG2-like (LOC559691) and TG5 (LOC565984). However, these zebrafish proteins are similarly distant from all human TGs based on pairwise comparisons (Fig. 1b) and their association with human homologs suggested by annotations, or with other human TGs, is not well supported by phylogenetic analysis. Therefore, based on the sequence analysis these two zTGs should be considered as novel TG isoforms, and we refer to these henceforth as zTG-91 and zTG-84 (Table 2).

Chromosomal arrangement of the zTG genes (Fig. 2a; schematic diagram of the chromosomal arrangements of TG genes in human and zebrafish genomes is shown in Fig 2b) is dramatically different from the TGs gene clustering pattern in humans (Grenard *et al.*, 2001) where TG2, TG3, and TG6 form a cluster on chromosome 20, TG5 and TG7 are clustered on chromosome 15, and TG1, TG4, and FXIIIa are present on different chromosomes. In zebrafish, the TG1/TGK-like transglutaminase genes are located on two chromosomes. The most ancient gene zTG1-81 (LOC566581) is located on chromosome 2, and the other four genes are located on chromosome 23. Among these, the two most recently diverged genes zTG1-18 (LOC100535918) and zTG1-48 (LOC793448) are separated by 1 Mb, and the others are located more distantly (Fig. 2a, b). A similar arrangement exists for the FXIIIa-like transglutaminases, where the most ancient gene LOC561287 is located on chromosome 7 and the two recently diverged genes LOC558353 and LOC767742 are both located on chromosome 24 separated by a mere 7 kb. In contrast, all three zTG2 genes are located on different chromosomes with the most ancient zTG2 genes (LOC559012) being located on chromosome 18, and LOC323856 on chromosome 6 versus LOC447909 on chromosome 23 despite their recent divergence. The remaining zebrafish TG genes that are not closely related to other TGs (LOC565984 and LOC559691) are located on the opposite ends of chromosome 6. All zTG genes have introns and hence are likely not the products of retrotranspositions. Thus, the expansion of the TG2-like subfamily and initial expansions of the TG1-like and FXIIIa-like subfamilies in zebrafish may have been associated with gene duplications and inter-chromosomal rearrangements, while further amplification of the FXIIIa-like and TG1-like genes probably occurred by local gene duplications which in the case of TG1-like genes were followed with intra-chromosomal rearrangements. These findings, and the absence of overt *TG3*, *TG4*, *TG5*, *TG6* and *TG7* homologs in zebrafish, raise a possibility that amplification of the human TG genes occurred after the evolutionary split between these species. Accordingly, it appears that the expansion of the TG1-like, TG2-like, and FXIIIa-like gene families in zebrafish occurred independently during the same time period.

Expression and activity of TGs in zebrafish

The expression profiles for all identified zTG genes were analyzed by real-time PCR in both embryonic development stages and adult fish. Since our study aimed to determine the role of zTGs in bone calcification which begins around 5dpf and is completed by 16dpf when calcification of all vertebrae can be visualized with vital calcein staining (Du *et al.*, 2001), we analyzed expression of zTGs in the 4 dpf larvae just before the initiation of vertebral calcification, and in the 13 dpf larvae when most vertebrae have already been calcified. In addition, expression levels for zTG genes were analyzed in adult torso presenting homeostatic bone. Expression of each zTG was normalized to the housekeeping β -actin gene and compared to unfertilized eggs. This analysis identified five zebrafish TGs (zFXIII-87, zTG2c, zTG2-12, zTG1-81, and zTG1-73) induced in larvae as compared to the non-fertilized eggs, but almost completely silenced in adulthood (Fig 3a), implicating their

involvement in the developmental processes. In contrast, zFXIIIa-53 and zFXIIIa-42 are both up-regulated in adult tissue (Fig. 3b), suggesting a putative role for these enzymes in body homeostasis rather than in growth and development. A minor role in fish growth and development is also suggested for two zTG1 genes - zTG1-62 and zTG1-18 - based on their dramatic down-regulation after fertilization.

No expression was detected by the real-time PCR analysis for zTG1-48 at any stage (data not shown), implicating that this locus maybe a silent pseudogene, however more detailed studies are required to test this further. Conversely, novel genes zTG-84 and zTG-91, in addition to zTG2b, are almost ubiquitously expressed in zebrafish tissues at all analyzed stages (Fig. 3b, zTG-84 is not included in the figure because all data in this figure is normalized to non-fertilized eggs in which zTG-84 is not expressed), resembling the ubiquitous expression of mammalian TG2 enzyme. The most noticeable distinction between these three ubiquitous zTGs is the complete absence of zTG-84 transcript in the non-fertilized eggs, suggesting its reduced role in early embryogenesis.

Localization of zTG expression by in situ hybridization

The expression data from the real-time PCR analysis was expanded by *in situ* hybridization studies on the 2–3 dpf zebrafish. Although many zTGs were not detectable at these stages by *in situ* hybridization indicating relatively low levels of expression, expression of several genes was observed in a tissue-specific pattern (Fig. 4a top panel). Thus, zTG2c expression is restricted to muscles while zTG1-81 is expressed in the muscle and, likely, in the notochord. Expression of zTG2b is restricted to notochord and zFXIIIa-87 is detected in the pectoral fin. Sense RNA probes were used as negative controls (Fig. 4a, low panel). The identified muscle-specific expression of zTG1-81 and zTG2c in 2–3 dpf fish suggests a role for these enzymes in muscle development. While zTG2c has been detected in the muscle even earlier, at 1 dpf fish (ZFIN

(<http://zfin.org/cgi-bin/webdriver?Mlval=aa-xpatslect.apg>), further analysis is needed to investigate in more detail the stage-specific and muscle-type specific expression of zTG1-81. A novel finding of our study is the notochord-specific expression of zTG2b in the 2–3 dpf fish. Earlier in development, at 1 dpf, this gene is ubiquitously expressed throughout the embryo (ZFIN (<http://zfin.org/cgi-bin/webdriver?Mlval=aa-xpatslect.apg>), suggesting that it may be expressed in the progenitor cells which give rise to the osteo-chondrogenic lineage. This pattern of expression corresponds to that seen for the TG2 gene in avian mesenchymal limb bud cells (Nurminsky et al., 2010) and suggests a role for this enzyme in skeletal formation. In addition, expression of zFXIIIa-87 was detected in the developing fins, which are enriched with skeletal ray elements, implicating this enzyme in bone formation. To determine whether zTGs expressed in the skeletal and muscle tissue are enzymatically active, we employed a rhodamine-labeled peptide Pro-Val-Lys-Gly, also called SY2011, which is a substrate for TGs (Kim et al., 1997). Decapitated fish were incubated with 5 mM Rho-SY2011 for *ex vivo* incorporation through TG-mediated cross-linking. Incorporated peptide was visualized by rhodamine fluorescence (Fig. 4b) to detect tissue with active zTGs. In agreement with the results of *in situ* hybridization, SY2011 was cross-linked into striated muscles of the torso, into periosteal bone of the vertebrae and in the in large blood vessels.

Together, these results demonstrate presence of the enzymatically active TGs in the developing skeleton of zebrafish justifying this organism as a good model to analyze the role of TGs in bone formation.

KCC009 is a potent inhibitor of TGase Cross-Linking Activity in zebrafish tissues

To analyze the role for the TG-mediated cross-linking in bone formation, we employed a pharmacologic approach to inhibit total catalytic activity of TGs with a small molecule inhibitor KCC-009 which inhibits the deamidation step of the cross-linking reaction (Poster et al., 1981; Choi *et al.*, 2005). Using pentylamine-based activity assay, we tested the potency of KCC-009 in inhibiting total TG cross-linking activity in total tissue lysates from 19dpf zebrafish. Purified guinea pig liver TG2 was used as a control in these studies, which demonstrated the complete inhibition of the TG-mediated cross-linking in zebrafish tissues (Fig. 5a). We also found that KCC-009 was able to inhibit protein cross-linking in adult tissue extracts (data not shown), indicating that it can be used to study the role of TGs both in fish development and in adult tissue homeostasis.

In vivo, KCC-009 showed low toxicity in mice and cancer xenografts (Choi et al., 2005; Yuan *et al.*, 2007; Satpathy *et al.*, 2009). Similarly, KCC-009 had no toxic effects on zebrafish as evident by unaffected viability (Fig. 5b), standard length (SL) as a reflection of development (Parichy et al., 2009) (Fig. 5c) and total vertebrae number (Fig. 5d) in zebrafish treated with 30 μ M KCC-009 for 5–6 days when treatment was initiated before 6dpf (Fig. 5). Moreover, when KCC-009 treatment was started at 4dpf we observed a slight but significant increase in vertebrae number from an average of 18 vertebrae in control fish up to 21 vertebrae in the KCC-009 treated animals (Fig. 5d) ($p < 0.05$, $n = 9$).

In vivo inhibition of TG activity with KCC-009 affects bone formation

In order to analyze the role of TGs in bone formation, we grew zebrafish in the continuous presence of 30 μ M KCC-009 over 5 days starting at 3–5dpf. Control fish were grown in the presence of 0.3% DMSO which served as the vehicle for KCC-009. Bone mineralization was analyzed by *in vivo* calcein staining (Du *et al.*, 2001). A representative pair of calcein stained vertebrae for both the control and KCC-009 treated zebrafish is shown in Fig. 6a, demonstrating the dramatic decrease in vertebrae mineralization for the KCC-009 treated group. Quantitative analysis of calcein staining intensity of the mineralized vertebrae revealed a significant average decrease of 30% in mineralization for the KCC009-treated fish (Fig 6b; $p < 0.05$, $n = 45$), although there was a high variation in the degree of KCC-009 induced reduction in vertebrae calcification. Due to KCC-009's potent ability to inhibit the cross-linking activity of all zTGs (Fig 5a), these results suggest that TG-mediated cross-linking plays a critical role in vertebrae ossification.

Discussion

In this study, we have characterized the new family of TG genes in zebrafish, *Danio rerio*. Thirteen zebrafish TG genes homologous to mammalian TGs have been identified, of which five are homologous to human TG1 (hTG1), three are homologues of hTG2 and three show homology to hFXIIIa, as revealed by phylogenetic analysis. Two zebrafish TGs show little similarity to any of the human TGs and have therefore been classified as novel TGs with the names zTG-84 and zTG-91, in spite of previous annotations for these genes as homologues of hTG5 and hTG2.

Expression pattern of the zTGs analyzed at 4dpf, 13dpf and in adult fish by real-time PCR shows ubiquitous expression of zTG-84, zTG-91 and zTG2b genes, resembling the ubiquitous expression of mammalian TG2 enzyme, which is implicated in many biological processes including bone formation. In addition, zFXIIIa-87, zTG2c, zTG1-81, zTG1-73 and zTG2-12 are up-regulated during stages in development that are significant to bone mineralization, implicating their potential role in bone development. Our data from *in situ* hybridization further supports this hypothesis by demonstrating the notochord-specific

expression of zTGb, restricted expression of zFXIIIa-87 to the pectoral fins and presence of zTG1–81 RNA in the notochord. In contrast, mRNA for zTGc was identified only in skeletal muscle in agreement with earlier studies (Thisse et al., 2004) and supporting specificity of this analysis. Although RNAs for several zTGs were not detected by *in situ* hybridization in this study, likely due to low levels of their expression, the data obtained clearly demonstrates expression of TG2 and FXIIIa genes in the skeletal tissues. Mammalian homologues of these genes have been implicated as potent regulators of osteoblast differentiation *in vitro* (Nurminskaya et al., 2003; Becker et al., 2008), supporting a notion that TGs may also regulate bone development *in vivo*.

The original finding of this study is the demonstrated reduction in vertebrae calcification in zebrafish grown in the presence of TG-specific irreversible acivicin-derived inhibitor KCC-009. No signs of general toxicity of KCC009 were observed in zebrafish grown from 3–4 dpf until 9–10 dpf in its presence, suggesting that tissue-specific inhibition of TG-mediated cross-linking in the skeleton underlies this effect. Our initial *in situ* analysis on 2–3 dpf fish has identified expression of three zTG genes in the skeletal tissues, including zTG2b, zFXIIIa-87 and zTG1081. However, other zTGs may be also expressed in the mineralizing bone later in development, and this should be studied in more details for comprehensive understanding of the molecular control of tissue calcification. Although the demonstrated importance of TG activity in vertebrae mineralization is an important contribution towards this goal, the weak selectivity of KCC-009 toward individual zTGs is a limitation of this study. The KCC-009 compound efficiently inhibits total TG activity in the zebrafish lysates in which expression of at least nine isogenes have been identified at each analyzed stage of growth. This precludes identification of the roles of individual zTGs in the regulation of bone calcification. In future, we plan to generate transgenic fish to establish the roles of individual enzymes in bone mineralization, taking into consideration previous *in vitro* studies which have implicated a major role for FXIIIa (Nurminskaya et al., 1998; Nakano et al., 2007).

Although the precise mechanism of TG-dependent bone calcification remains to be determined, a credible model would account for the formation of cross-linked protein scaffolds, such as a collagen type 1-fibronectin cross-linked network, which has previously been shown to promote matrix mineralization in the MC3T3-E1 osteoblastic cell line (Al-Jallad et al., 2005). A peak in collagen type 1 expression around 2–3 dpf in growing zebrafish (Dubois et al., 2002) could allow for formation of such a scaffold in the vertebrae. This scaffold would then be susceptible to calcification which culminates in visually detectable levels of calcium deposited into vertebrae beginning on 5 dpf (Du et al., 2001). Inhibition of the TG-dependent cross-linking starting at 3 dpf interferes with this process thereby inhibiting vertebrae calcification.

Comprehensive understanding of the molecular mechanisms that govern osteoblast maturation and calcification of bone matrix is crucial to develop novel therapeutics for bone disease. Bone tissue homeostasis requires a delicate balance between bone formation by osteoblasts and bone resorption by osteoclasts, which is disrupted in osteoporosis. The current approach to treatment for osteoporosis relies on anti-resorptive agents to inhibit osteoclast function but do not restore bone mass (Rawadi, 2008). This study provides the first *in vivo* evidence for the critical role of TG-mediated cross-linking in bone mineralization which could lead to a new therapeutic target in osteoporosis and other bone diseases. Furthermore, this study demonstrates the value of zebrafish model for *in vivo* analysis of the multiple biological roles of transglutaminases proposed by the *in vitro* or correlative studies, including various developmental processes and disorders such as neurodegenerative, skin and ocular pathologies as well as cancer (Mione et al., 2010).

Abbreviation List

dpf	Days Post-Fertilization
DMSO	Dimethyl Sulfoxide
FXIIIa	Factor Thirteen A
hTG	Human Transglutaminase
gplTG2	Purified Guinea Pig Liver Transglutaminase 2
SL	Standard Length
TGs	Transglutaminases
zTGs	Zebrafish Transglutaminases

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a **b**

Percent Similarity Between Human and Zebrafish TGs								
	hTG1	hTG2	hTG3	hTG4	hTG5	hTG6	hTG7	hFXIII
LOC559691 TG2-like	53	57	56	54	55	58	54	52
LOC565984 TG5	52	54	54	51	57	54	52	52

Fig. 1.

Phylogenetic analysis of TG genes in Zebrafish. (a) Phylogenetic tree depicting the evolutionary relationships among TG genes of *Danio rerio* and human origin as established by the Bayesian tree building algorithm. Only three out of nine mammalian TGs have an overt zebrafish homologue, including *TG2*, *FXIIIa* and *TG1*. (b) Table showing the similarly low similarity between the two novel zTGs, LOC559691 TG2-like and LOC565984 TG5, and diverse human TGs.

Fig. 2.

Analysis of genomic organization of TG genes in Zebrafish. (a) chromosomal arrangement of zebrafish TG genes. (b) Color-coded schematic of chromosomal clustering of TGs in both human and zebrafish.

a **b** **Fig. 3.**

Expression patterns of TGs during Zebrafish development. Real-time PCR analysis was used to determine TGs expression pattern. Whole bodies were used for analysis at 4dpf, 13dpf and the torso from adult zebrafish. Data was normalized to β -actin and compared to unfertilized eggs. (a) Real-time PCR showing TG genes that are upregulated during development (in 4 and 13dpf zebrafish). (b) Real-time PCR analysis showing the TG genes that have either little change in expression during development or significant upregulation in adult fish.

a **b** **Fig. 4.**

Tissue specific expression of TGs in 2–3 dpf Zebrafish. (a) Representative images from whole mount *in situ* hybridization for zTG2b, zFXIII, zTG2c and zTG1–81 showing the tissue-specific expression patterns for TGs (top) and the negative control hybridization with the sense probes (bottom). (b) *Ex vivo* incorporation of the rhodamine-labeled synthetic substrate Pro-Val-Lys-Gly in zebrafish showing areas with high TG cross-linking activity in the ossifying vertebrae, blood vessels and muscle.

Fig. 5. KCC-009 is a potent inhibitor of total cross-linking TG activity *in vitro* and has no toxic effects in zebrafish. (a) TG cross-linking activity assayed by pentylamine-biotin incorporation into *N,N'*-dimethylcasein. Total protein lysates from 14dpf zebrafish were used. To inhibit TG activity samples were pre-incubated for 1 hour at 28°C with KCC-009. Purified guinea pig liver TG2 (Sigma) was used as positive control. (* $P \leq 0.05$) (b,c,d) Toxicity of KCC-009 treatment was analyzed by comparing the (b) average survival percent, (c) standard length in millimeters and (d) average total vertebrae number (Day 0 black bar, Control at 9/10dpf light grey bar and KCC-009 treated at 9/10dpf dark grey bar in c and d) for the untreated control zebrafish and fish grown for 5–6 days in the presence of 30 μ M KCC-009. N=9–16 per time point (* $P \leq 0.05$)

Fig. 6.

Inhibition of TGs by KCC-009 dramatically reduces vertebrae mineralization. Zebrafish were grown for 5–6 days starting at 3–5dpf in the presence of KCC-009. (a) Representative calcein stained images of zebrafish, Control (top) and KCC-009 (bottom) treated fish, KCC-009 treatment started at 3dpf and images taken at 9dpf, shown in grayscale. (c) Average change in calcein staining intensity per vertebra in the presence of KCC-009. Intensity of calcein staining for each vertebra was determined with Photoshop. N=16 fish for 5dpf treatment, 9 for 4dpf treatment and 20 for 3dpf experiment. (* $P \leq 0.05$)

Table 1

Primers used for real-time PCR analysis and accession numbers for zTGs.

Target Transglutaminase	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Accession Number
zFXIIIa-87	GTTCGGCCCAAACAGCGGGT	CCTGCGGATGCCGTACGGTG	NM_001077154
zTG2c	ACGCATCTGAACGGTGTGGACA	AAGGCAGATGTCCAGTATTCCGTGC	NM_001004647
zTG2b	GGCAAGTGATCCAACGCCGC	GGCGTCTCTGGTGCTGTAGTCGA	NM_212656
zTG-91	TGCTGATGACGGACGGGTCC	GATCCTCTGTCCCGGGCCGA	XP_688146.5
zTG-84	TGAACGCAGACGTGCGGACC	TGGCGCCCGTCATCTGAGGA	XM_689249
zTG1-81	ATCGCGGTGGAACGGCCTG	CGCAGAGTGCAGGGCAGAGG	XM_689858
zTG1-48	CTCCGGAGCAAGAAGTGCAGAA	CTGTAGCGTCCGATGCACGC	XM_001331878
zTG1-96	TCATGCCTTTTCTCATGCAGCCCA	CAGTGCGTCACAACGCTGAGC	XM_001332039
zTG1-73	ATATCCATCGAACTGAAGCTA	AGCCTTCAACTCTTTACCAAC	XP_002665624
zFXIIIa-53	ATCAATCTCCAACCTCCCGAAC	TCCATCAAGGCGAAGCTCA	XP_686649
zFXIIIa-42	GAATTTAAAGCGACAGTCACC	TGAGCATTAAAGCCATACACA	NP_001070179
zTG1-18	AGCACGTCAAAACCATCCAC	AGCACCTCCAAAATCAGATCG	XP_003201279
zTG2-12	AAGCCTCTGTCATTGTTCG	GCTGTCATTGAGTATATCGC	XP_687398.2

Table 2

Zebrafish TG gene ID numbers and corresponding abbreviations used throughout the text

Gene Name	Gene Abbreviation
LOC555962 TG1	zTG1-62
LOC793448 TKG-like	zTG1-48
LOC100535918 TKG-like	zTG1-18
LOC566581 TKG isoform 1	zTG1-81
LOC100334173 TKG-like	zTG1-73
LOC323856 TG2b	zTG2b
LOC559012TG2-novel	zTG2-12
LOC447909 TG2	zTG2c
LOC767742 hypothetical protein	zFXIIIa-42
LOC558353 FXIIIa chain-like	zFXIIIa-53
LOC561287 Factor XIIIa chain	zFXIIIa-87
LOC559691 TG2-like	zTG-91
LOC565984 PREDICTED TG5	zTG-84