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ORIGINAL ARTICLE

FXIIIA and TGF-β over-expression produces normal musculo-skeletal phenotype in TG2-/- mice

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Abstract Transglutaminase (TGs) enzymes and proteins crosslinking have for long time been implicated in the formation of hard tissue development, matrix maturation and mineralization. Among the TGs family members, in the context of connective tissue formation, TG2 and Factor XIII are expressed in cartilage by hypertrophic chondrocytes. Here, we analyse the morphological consequences of TG2 deficiency, during the development of skeletal elements. When TG2 is absent, there are not gross abnormalities in the development of the skeletal system, probably from compensatory mechanisms resulting in increased expression of FXIIIA and TGF-ß 1. In vivo other TGs may be involved in promoting chondrocytes and osteoblast differentiation and matrix mineralisation

Keywords Endochondral ossification · Transglutaminase

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Introduction

The process of endochondral bone development in vertebrates is characterised by replacement of a cartilage anlagen by bone: chondrogenesis and osteogenesis are tightly coordinated in time and space (Erlebacher et al. 1995; Marks and Hermey 1996). Three tissues appear to be the main players in the initiation of endochondral ossification: the cartilage, the adjacent perichondrium, and the invading vasculature. Interactions among these tissues are synchronised, and a large number of secreted and intracellular factors acting in this process have been recently identified (Colnot 2005). Hypertrophic growth plate chondrocytes provide a scaffold for subsequent formation of trabecular bone by mineralising their surrounding matrix and inducing a bone collar, the precursor of cortical bone, in the adjacent perichondrium (Chung and Lanske 1998). In mice, endochondral ossification starts at 14.5-day embryo. By 19-day embryo, ossification is complete (Wirtschafter 1966).

Transglutaminase (TGs) enzymes and proteins crosslinking have been implicated in the formation of hard tissue, matrix maturation and mineralisation (Lorand and Graham 2003; Aeschlimann et al. 1993, 1996; Aeschlimann and Thomazy 2000; Candi et al. 2005; Melino et al. 2000). TGs are a nine member family of calcium-dependent enzymes which catalyse the formation of covalent γ-(glutamyl)-ε-lysyl bonds (isopeptide bonds or crosslinks) among protein-bound lysines and glutamines, thus forming large polymers or proteins. TGs can also use as substrate primary amines (putrescine, spermine and spermidine) to specific glutamine residues (Lorand and Graham 2003). TGs crosslinking activity can take place in the cytosol of the cells, at the cells surface, and/or the bone extracellular matrix compartments (Lorand and Graham 2003).

Many TG substrates directly or indirectly related to matrix maturation and mineralisation have been identified in vitro so far, including collagen I-V-X-XI, fibronectin, osteopontin, osteonectin and bone sialoprotein (Mosher and Schad 1979; Jeong et al. 1995; Kaartinen et al. 1999; Lorand et al. 1998; Turner and Lorand 1989; Prince et al. 1991; Sorensen et al. 1994; Kaartinen et al. 2002; Esposito and Caputo 2005; Johnson 2007). However, the function of the crosslinking is not yet understood. Among the TGs family members, in the context of connective tissue formation, TG2 and Factor XIII are expressed in cartilage by hypertrophic chondrocytes (Aeschlimann et al. 1996; Aeschlimann and Thomazy 2000; Nurminskaya and Linsenmayer 2002; De Laurenzi and Melino 2001; Nurminskaya et al. 1998). In addition, several reports correlate in vitro TG2 expression, chondrocyte differentiation and matrix mineralization (Aeschlimann et al. 1996; Aeschlimann and Thomazy 2000; Nurminskaya et al. 1998; Nurminskaya and Linsenmayer 2002; Johnson and Terkeltaub 2005). Also, TGF-ß family is involved during endochondral ossification and bone remodelling (Janssens et al. 2005). However, the mechanisms behind these phenomena mediated by TGs, and in particular those mediated by TG2, are not known.

We characterised the morphological consequences of TG2 deficiency produced by a gene-targeting approach in mice (De Laurenzi and Melino 2001) during the development of skeletal elements.

Materials and methods

The local Animal Experiment Committee approved all procedures performed in this study.

Tissue preparation

Five newborns mice of TG2 knock-out and wild type were stained with 0.5% alizarin red and 0.015% alcian blue were performed as described previously (Hogan et al. 1994).

Five skeletons of 13.5- to 18.5-day embryo of TG2 knock-out and wild type mice, plus five newborn mice of TG2 knock-out and wild type mice were euthanised with an overdose of chloroform (total numbers of animals 70). After some skin incisions, embryos were processed. All samples were fixed in 10% buffered formalin for 12 hours, and decalcified for 48 hours in Decalcifier II (Surgipath Medical Industries, Richmond, IL, USA). After usual processing and paraffin embedding, 5 µm section were placed on SuperFrost Plus glass slides (Menzel-Gläser, Braunschweig, Germany) and used for haematoxylin–eosin or immunohistochemical stainings. Femurs were selected for this study.

Immunohistochemical study

Slides were heated in a dry oven overnight at 60°C, de-paraffinised in xylene, and rehydrated in graded concentrations of ethanol. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide and methanol. Slides were re-hydrated in phosphate-buffered saline. Non-specific antibody binding was blocked by incubation with normal goat serum (Dako Cytomation, Glostrup, Denmark). Optimal anti-TG2 antibody (Covalab, Vinci-Biochem, Firenze, Italy) dilution was found to be 1:75 for 30 min. Slides were then incubated with a biotin-labeled goat anti-rabbit secondary antibody (Dako Cytomation, Glostrup, Denmark), followed by a streptavidin-horseradish peroxidase conjugate. Bound antibody was revealed with the use of the substrate 3,3′-diaminobenzidine. Sections were counterstained with haematoxylin, washed, dehydrated with graded concentrations of ethanol, cleared in xylene, mounted, and examined at light microscopy. Human breast carcinoma was included with each batch of sections as a positive control. The location of the TG2 was determined by comparing each immunohistochemical section with the adjacent slice stained with haematoxylin–eosin.

Western blotting

Western Blots were performed using the whole sample derived from a mouse femur, lysed in Laemli Buffer. The blots (polyvinylidene difluoride membrane) were kept in blocking solution (10% dried milk) for 2 h at room temperature. Blots were incubated shaking for 2 h at room temperature with the antibodies to TG2 (dilution 1:500, Covalab); anti-FXIII (dilution 1:1,000, Santa Cruz); anti-TGFβ1 (dilution 1:500, Santa Cruz); anti-β tubulin (dilution 1: 1,000, Santa Cruz). After three washes in PBS-tween 20 (0.05%), secondary antibody peroxidase conjugated (dilution 1:10,000) was added in blocking solution, shaking for 1 h at room temperature. Proteins were detected using the enhanced chemiluminescence method (ECL, Amersham).

Results

The size of the skeletal elements and the relative ratio of cartilage to bone in TG2-deficient mice were indistinguishable from those of new born wild-type animals (Fig. 1a–c). Haematoxylin–eosin stained sections showed that the wild-type fetal growth plate consists of three major layers of chondrocytes: proliferating, prehypertrophic, and hypertrophic layers. The proliferating layer contained two distinct types of chondrocytes. Proliferating chondrocytes

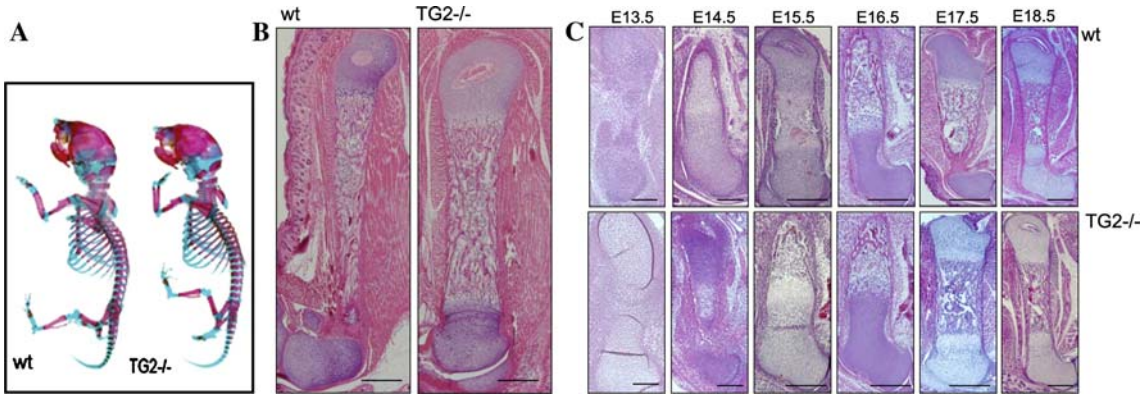

Fig. 1 Cartilage and bone develop normally in newborns lacking TG2. The skeletons from newborn littermates of wild-type and TG2-/- were stained with alcian blue and alizarin red, detecting cartilage and bony structures, respectively (**a**). Haemotoxylin–eosin stained sections of femur of new born wild type and TG2-/- mice (**b**). Analysis of histological embryos femurs development from wild type and TG2-/- mice from 13.5 to 18.5 days stained with haematoxylin–eosin (**c**). The primary ossification centers and periosteal cuffs are fully formed. The secondary ossification center of the proximal epiphysis is already visible at the 14.5 days in the knock-out embryo, and fully formed in both the panels at day 18.5. *Bars* 200 µm for wild type and TG2-/- at E13.5 and E14.5. *Bars* 250 µm for wild type and TG2-/- at E15.5, E16.5 and E17.5, *bars* 500 µm for wild type and TG2-/- at E18.5

close to the articular surface were round and randomly scattered, with the typical features of peri-articular chondrocytes. Proliferating chondrocytes immediately below the peri-articular proliferating chondrocytes were flat, and formed longitudinal columns (Fig. 2a, b): they were columnar chondrocytes.

TG2-/- animals were indistinguishable in size and behavior from wild-type mice in all the embryonic stages analysed, and up to an age of 1 year (data not shown). Adult mice lacking TG2 displayed no obvious skeletal abnormalities, and no skeletal elements were missing or deformed. Immunohistochemistry confirmed the expression of the TG2 in the hypertrophic chondrocytes of wild type mice, and its absence in knock-out mice (Fig. 2c, d). In knock-out mice, deficiency of TG2 causes no obvious consequences during embryonic skeletal development and in adulthood. The observation that TG2-/- mice have no overt skeletal phenotype, suggest that at least a second TG enzyme must influence matrix assembly and mineralisation. Previously, it has been described that, in addition to TG2, cultured osteoblasts also express the plasma transglutaminase Factor XIIIA (FXIIIA; Nurminskaya and Linsenmayer 2002; De Laurenzi and Melino 2001; Nurminskaya and Kaartinen 2006). To verify whether Factor XIIIA is involved, we analysed by western-blot its expression on protein extracts obtained from wild type and TG2-/- femurs (Fig. 3). The distal femoral epiphysis at the 18.5 day post conception of wild type and TG2-/- mice were isolated, and proteins were extracted as indicated in "Materials and methods". Western blot analysis indicate that FXIIIA protein is increased of about fivefold (Fig. 3), as evaluated by densitometry analysis normalised over the loading control (ß-tubulin). We also determined the levels of transforming growth factor beta-1 (TGF-ß1). Western blot analysis showed a marked increase of the TGF-ß 1 protein level in TG2-/- bones as compared with wild type (Fig. 3).

Discussion

TG2 and Factor XIIIA are the only identified transglutaminases in human, mouse, and chick growth plates. These locations led to speculation that transglutaminases regulate endochondral ossification in particular both terminal cell differentiation and matrix calcification (Johnson 2007; Nurminskaya and Linsenmayer 2002; Nurminskaya et al. 2002; Johnson et al. 2001) also FXIIIA-null mice show a dramatic decrease in COL I deposition in osteoblast cultures (Al-Jallad et al. 2006). Indeed, many investigators confirmed this hypothesis in vitro, but to our knowledge no studies on the endochondral ossification of TG2-/- mice during are available.

Recently, a 37 kDa form of Factor XIIIA was discovered. It is located exclusively in cartilage and bone, and not in plasma. This shortened form of Factor XIIIA may be controlling mineralisation or have unique functions (Nakano et al. 2007), similar to those of the shortened isoform of TG2 in brain (Antonyak et al. 2006).

Latent TGF binding protein-1 is particularly interesting because it release the active TGF-ß1 only after its TG2-catalysed linkage to the matrix (Nunes et al. 1997). This cytokine affects many aspects of bone formation (Janssens et al. 2005), and TGs may regulate the extracellular levels

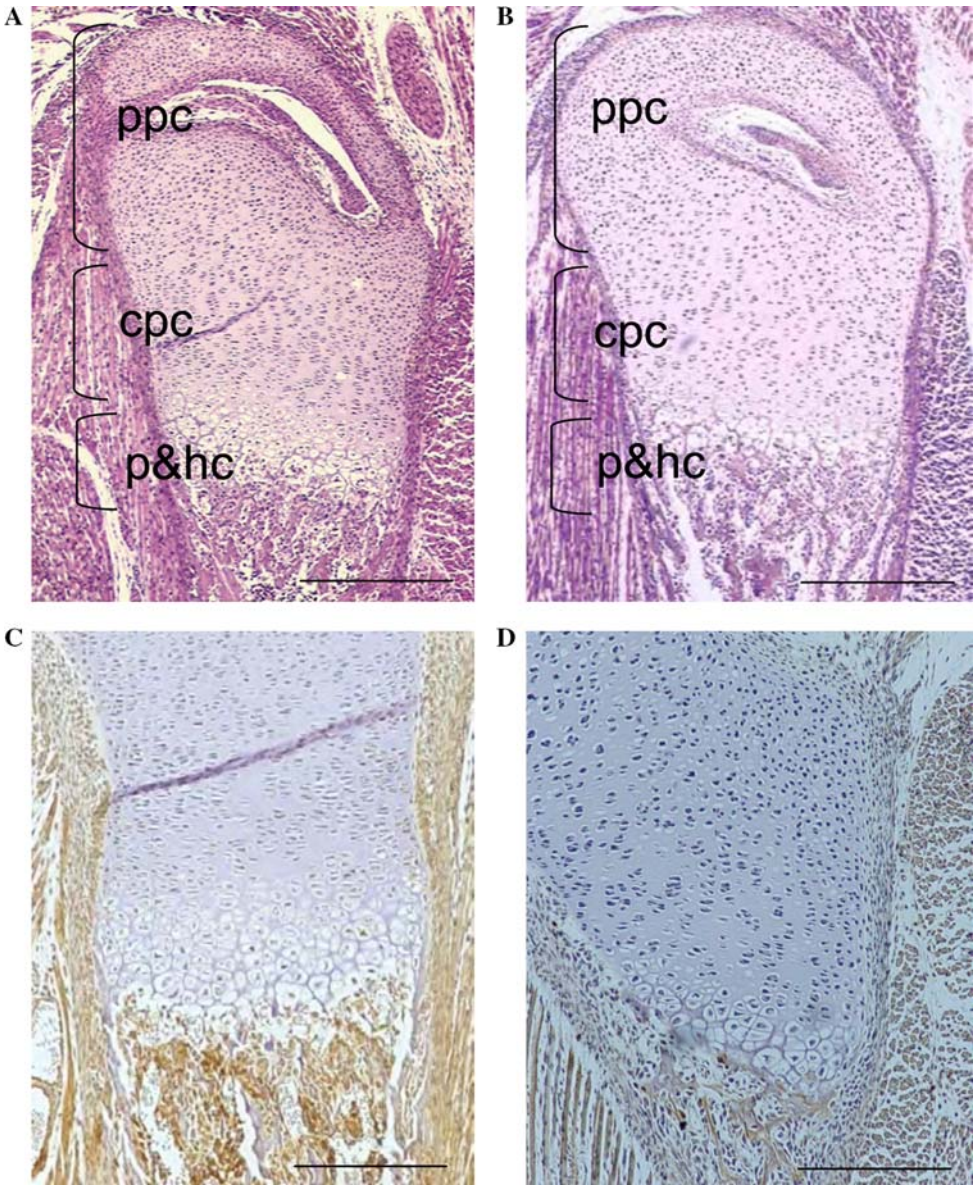

Fig. 2 Haematoxylin–eosin staining of sections of wild type (**a**) and TG2-/- (**b**) proximal femoral growth plates from the 18.5-day embryo. Brackets indicate the approximate lengths of the growth plates. Periarticular proliferating chondrocytes (ppc), columnar proliferating chondrocytes (cpc), prehypertrophic and hypertophic chondrocytes (p&hc) in the growth plate are regularly arranged both in wild type anf TG2-/- samples. *Bars* 500 µm. Expression of TG2 in proximal femur of wild type (**c**) and TG2-/- (**d**) mice. Immunohistochemistry of proximal femoral growth plate sections from 18.5-day embryo. Note in the wild type the increased expression of TG intracellularly and in the interterritorial matrix of columnar proliferating chondrocytes. *Bars* 500 µm

of TGF-ß. In addition, TGF-ß1 null mice show severe skeletal defects (Geiser et al. 1998).

TGF-ß is expressed in chondrocytes, osteoblast and osteocytes (Janssens et al. 2005). Other studies have shown that low levels (5–200 pg/ml) of TGF-ß2 promoted mineralisation in the co-cultures, but they demonstrated a slightly lower level of activated latent TGF-ß after treatment with recombinant TG2. This suggests that the mechanism of TG2 induced mineralisation did not involve TGF-ß (Nurminskaya et al. 2003).

To study in vivo the involvement of TG2 in skeletal development, and to exclude possible developmental delay or deformities, we focused on the early development of skeletal structures in embryos from the stage embryo 13.5 to newborn.

Proliferating chondrocytes undergo a limited number of divisions and then lose the capacity by beginning to hyperthrophy. This switch is regulated by parathyroid hormone-related peptide (Chung and Lanske 1998). Prehyperthrophic and hypertophic chondrocytes increase dramatically their height, volume and matrix mass, and become more rounded until they have an egg like configuration (Fig. 2a, b).

In this study, we demonstrated no gross skeletal abnormalities or developmental delay in bone formation in the absence of TG2 in the femora of mice. Therefore, in vivo other TGs may be involved in promoting chondrocytes and osteoblast differentiation and matrix mineralisation. Among the TGs, FXIIIA has been described to contribute to TGs activity observed in both hypertrophic chondrocytes and

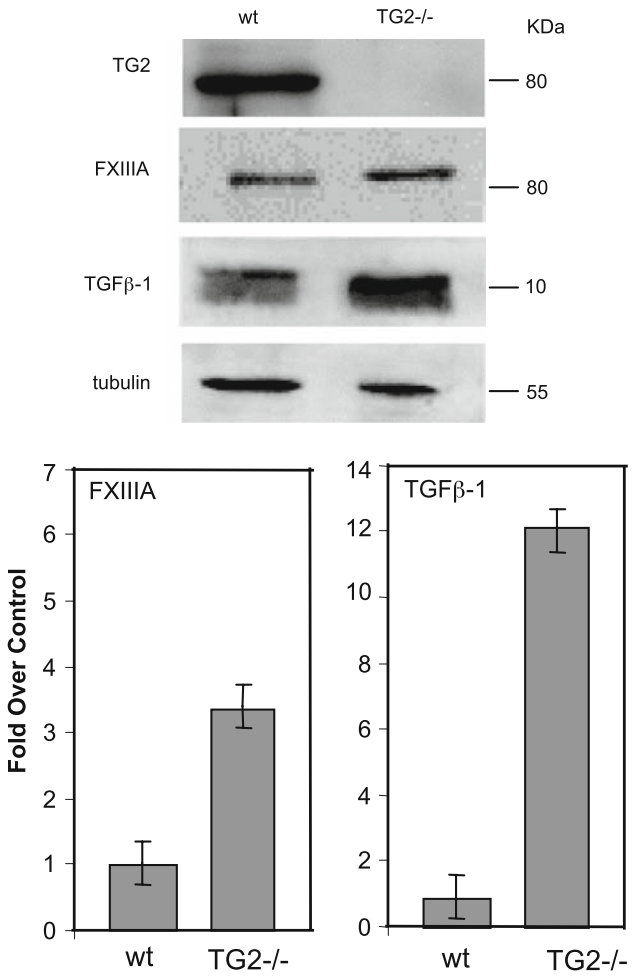

Fig. 3 Expression of FXIII and TGF-β1 in femora of wild type and TG2-/- mice. Western blot analysis was performed on isolated distal femoral epiphysis at 18.5 days (**a**), with β-tubulin as loading control. The densitometric analysis (**b**) shows the relative expression of FXIIIA and TGF-β1. A representative experiment of three is shown. The analysis was normalized over the loading control

osteoblasts. Interestingly, protein level of FXIIIA increases fivefold in protein extracted by TG2-/- femurs compared with wild type. Therefore, FXIIIA is expressed in vivo in bone, and we suggest that TG2 and FXIIIA work sinergically in these processes. In addition, we observed an increased level of TGF-ß1 protein in TG2-/- bones in comparison with wt. TGF-ß1 is a potent multifunctional regulators of osteoblast differentiation, and TGF-ß1 activation and upregulation could be another parallel compensatory mechanism responsible for the absence of abnormal skeletal phenotype in TG2-/- mice. Therefore, results obtained in vitro can contradict in vivo observations.

In the absence of TG2, we were not able to detect phenotypical abnormalities in the femora of embryonic, young, and adult TG2-/- mice. This may result from compensatory mechanisms involving increased expression of FXIIIA and TGF-ß 1.

Several questions remain open and are subject to future research. For example, are there other TG isoforms supplying the TG2 activity during endochondral ossification? Are the recent short forms of FXIIIA and TG2 in vivo the truly protagonist of endochondral ossification? Which mechanisms activate in vivo the short forms of FXIIIA and TG2? Are other parallel mechanisms involving TGs and time depending cytokines and/or growth factors important in mineralisation and stabilisation of extracellular matrix during endochondral ossification? The development of further knock-out mice for TG2, Factor XIIIA and TGF-ß 1 may help answer at least some of these questions.

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