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Trefoil factor family protein 3 affects cancellous bone formation in the secondary centers of ossification of mouse tibiae

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Abstract

Background and Purpose: *Trefoil factor family (TFF) is a small family of peptides, which comprises three peptides, TFF1, TFF2 and TFF3. The primary sites of TFF expression and synthesis are mucous epithelia, with gastrointestinal mucosa being their main localization. They have a role in mucosal restitution and strengthening of the normal mucosal barrier. Although not present in healthy articular cartilage, TFF3 is expressed in osteoarthritis and septic arthritis affected cartilage and promotes cartilage degradation in such conditions. Also, TFF3 is present in endochondral ossification during embryonic development. Since this implies a role for TFF3 in the ossification process, we evaluated the histomorphometric parameters of the trabecular bone in the epiphyses of mouse tibiae from wild type mice and TFF3 knock-out mice.*

Materials and Methods: *Tibiae of wild-type and knock-out mice were isolated, fixed in 4% paraformaldehyde, paraffin embedded and cut into 6µm sections, which were stained using Masson's trichrome stain. Digital photographs were taken for histomorphometry of the epiphyseal cancellous bone.*

Results: *Trabecular bone volume density, trabecular bone surface density and trabecular number were significantly decreased in TFF3 knock-out mice, when compared to wild-type mice. Trabecular separation was significantly higher in TFF3 knock-out mice, and trabecular thickness did not differ significantly.*

Conclusions: *In addition to its impact on the cartilage degradation, our present study shows that TFF3 might also have a role in the formation of cancellous bone and its properties.*

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INTRODUCTION

Trefoil factor family (TFF) is a small family of peptides, which comprises three peptides, TFF1, TFF2 and TFF3. Their name originates from a three-looped motif in their peptide chain, which is believed to be responsible for their resistance to proteolysis and hydrolysis (1–3). While TFF2 is a „natural dimer", containing two trefoil domains, TFF1 and TFF3 are monomers that contain only one, but TFF1 and TFF3 are able to form homodimers and heterodimers via disulphide bonds *(4–7).*

The primary sites of TFF expression and synthesis are mucous epithelia, with gastrointestinal mucosa being their main localization *(3);*

nevertheless, they are also found in many other organ systems, including nervous system, where a function of a neuropeptide is proposed *(8–10)*. Trefoil factors have a role in strengthening the normal mucosal barrier, and at the same time are important for mucosal restitution after different types of injury *(11–13)*. Mechanisms of TFF peptides' action include an effect on cell motility and apoptosis, modulation of cell-cell and cell-surface contacts, promoting angiogenesis and an effect on immunity *(14–16)*. Such properties are invaluable in mucosal repair, however, they also have a role in the pathogenesis and advancement of different tumors, such as breast, prostate, lung and colorectal cancer *(17–23)*. For this reason, trefoil factors in serum or other body fluids are being discussed as diagnostic markers of different diseases, including tumors, and are also viewed as potential therapeutic agents or targets *(5, 21, 23–26)*. The value of such application is yet to be fully confirmed, however, current research seems promising.

TFF3 protein is found in several avascular tissues. For example, its expression is found to be induced in corneal wounds, and to promote healing of such wounds upon topical administration *(27)*. Another study showed that although not present in healthy articular cartilage, TFF3 is expressed in osteoarthritis and septic arthritis affected cartilage, where it induces several matrix metalloproteinases, promotes apoptosis, and consequently, cartilage degradation *(28)*. Cartilage degradation occurs not only under pathological circumstances, but also in physiological conditions such as endochondral ossification during embryonic development, and it was interesting to see how, amazingly similar to arthritic degradation, TFF3 is expressed in all zones of endochondral ossification, except for the resting zone (analogous to healthy adult cartilage) *(29)*.

Since our previous research implied that TFF3 has a role in the endochondral ossification, we were prompted to take the research further and to investigate if there are differences in bone quality between wild type and TFF3 knock-out mice, particularly in the trabecular bone resulting from endochondral ossification in the secondary ossification centers of epiphyses.

Bone strength correlates to the material and structural properties of bone tissue, as well as the direction of the biomechanical load that acts on it. The structural properties depend on the trabecular bone architecture, bone volume and porosity, while material properties of bone tissue are primarily dependent on the quality of collagen fibers and minerals incorporated in the bone *(30, 31)*. Although the very complex process of endochondral ossification has been investigated extensively, the origin of osteoblasts that produce the cancellous bone trabeculae still isn't fully elucidated. Different sources of these cells have been considered, including perichondrial cells, hypertrophic chondrocytes, pericytes of cartilage-penetrating blood vessels, and circulating progenitor cells *(32)*. Recent research suggests that about sixty percent of the osteoblasts in endochondral bone originates from hypertrophic chondrocytes in endochondral ossification, and that, hence, hypertrophic chondrocytes have the ability to differentiate into osteoblasts *(33)*. Also, it is known that one of the inductors of TFF3 expression in chondrocytes is tumor necrosis factor alpha, which is also important for endochondral ossification *(28, 34, 35)*. However, results of previously published studies do not provide the information about the influence of TFF3 expression on the bone formation, as well as would histomorphometric parameters of the bone differ in TFF3 knock-out mice.

Therefore, we hypothesized that the lack of TFF3 protein in the knock-out mice would alter the morphological properties of endochondral bone, for example, trabecular bone in the secondary centers of ossification, a direct product of endochondral ossification. To test this hypothesis we evaluated the histomorphometric parameters of the trabecular bone in the epiphyses of mouse tibiae from wild type mice and TFF3 knock-out mice.

MATERIALS AND METHODS

Animals and tissue preparation

Experiments were carried out according to the guidelines of the Ethical Committee, Faculty of Medicine at the J. J. Strossmayer University in Osijek, Croatia, and in accordance with Croatian law regarding the handling and treatment of laboratory animals.

Overall 10 male mice were used for the experiment; 5 of them were wild-type inbred mice developed by crossing 129/Sv and c57bl/6j mice, and another 5 TFF3 knock-out mice (for deatails about the TFF3 knock-out mice, see *(36)*). Animals were bred and kept at the Animal Facility of Medical Faculty Osijek, under the conditions of 12 h light/dark cycles, at the average temperature of 23.5 °C and relative humidity 50-60%, with food and water available *ad libidum*. Animals were killed by cervical dislocation at one month of age (28-31 days) and hind limbs were removed and transferred to 1×PBS (pH=7.4). Femur and tibia were carefully separated by cutting the ligaments, after which musculature and fibula were carefully removed, as well as the foot.

The tibiae were fixed in freshly made 4% paraformaldehyde for at least 24 hrs, after which they were decalcified using nitric acid. Bones were embedded in paraffin blocks, after which consecutive sagittal sections (6 µm thick) were cut using a Leica RM550 rotary microtome (Leica, Vienna, Austria). Representative sections of the middle two-thirds of the tibial epiphyses were chosen for the experiment, in order to expose centers of secondary ossification of proximal tibiae. The sections were deparaffinized and stained with Masson's trichrome stain. The system for obtaining digital photographs for histomorphometric measurements of the bone sections consisted

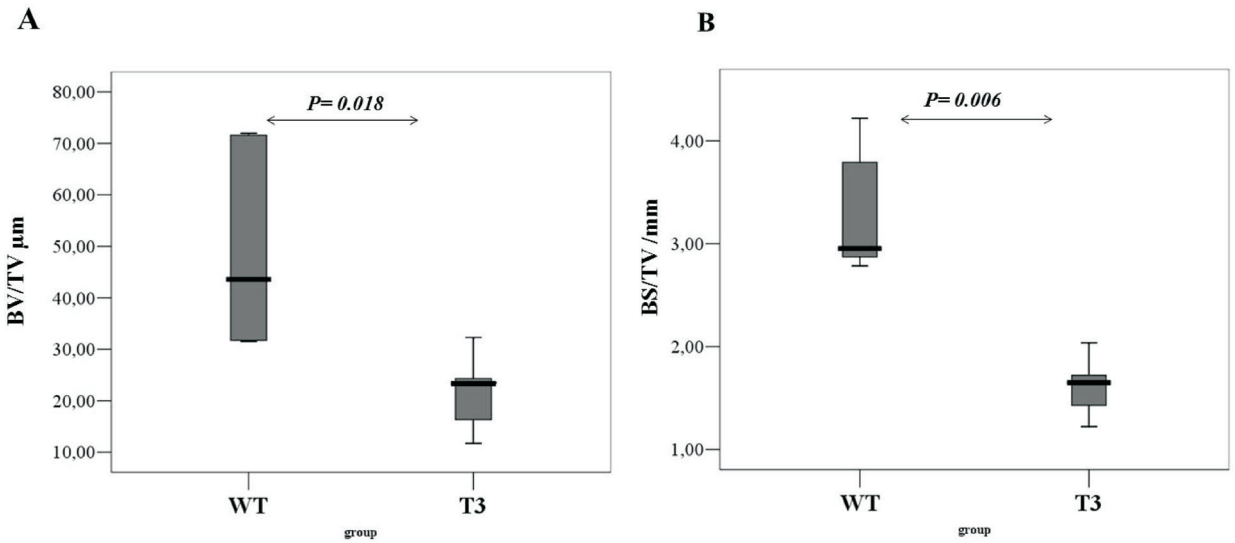

Figure 1. *Trabecular bone volume density and trabecular bone surface density in tibial epiphyses of a 1 month old mice. (A) BV/TV (%) = trabecular bone volume density; (B) BS/TV (/mm) = trabecular bone surface density. WT = wild-type mice, T3 = TFF3 knock-out mice.*

of Olympus BHA microscope (Olympus, Tokyo, Japan) and Pulnix digital camera (Pulnix, Yokohama, Japan) connected to a personal computer. Digital images were captured under 100 × magnification and stored until the measurements were performed.

Bone Histomorphometry

Digital photographic images were analyzed using a semiautomatic image analysis system that employed Issa software (VAMS, Zagreb, Croatia). According to the American Society of Bone and Mineral Research, trabecular bone volume density (BV/TV, in %) corresponds to the amount of trabecular bone within the spongy space. BV/TV was derived from two-dimensional (2D) measurements of bone area (B.Ar) and trabecular tissue area (T.Ar) using Parfitt's formula, as follows *(37)*:

$$BV/TV = 100B.Ar/T.Ar$$

Trabecular bone surface density (BS/TV, in /mm) was then calculated from the values for the perimetry of trabecular bone (B.Pm) and the area of trabecular bone (B.Ar)

$$BS/TV = B.Pm/B.Ar$$

In addition, three more values were calculated to evaluate the architecture of trabecular bone:

(1) Trabecular thickness (Tb.Th, in µm) was derived from measurements of the trabecular perimeter (B.Pm) and bone area (B.Ar) according to Parfitt's formula:

$$Tb.Th = (B.Ar/B.Pm)(\pi/2)$$

(2) Trabecular number (Tb.N, in/mm) was derived from measurements of the trabecular perimeter (B.Pm) and total tissue area (T. Ar) according to the formula:

$$Tb.N = (B.Pm/T.Ar)\times 10$$

(3) Trabecular separation (Tb.Sp, in µm) was derived according to the formula:

$$Tb.Sp = (1000\times T.Ar - B.Ar)/B.Pm$$

Statistical Analyses

All results are expressed as median value. The data were analyzed using Mann-Whitney U test, and difference was considered significant at $p<0.05$

RESULTS

Trabecular Bone Volume Density and Trabecular Bone Surface Density

Median value of trabecular bone volume density (BV/TV, %) was significantly higher in wild-type mice group (44.23 %) than in the TFF3 knock-out group (23.36 %) (Figure 1A).

Trabecular bone surface density (BS/TV, /mm) measurements showed significantly different values in the two groups, being higher in wild-type group (2.78 /mm), than in the TFF3 knock-out group (1.72 /mm) (Figure 1B).

Trabecular Structure

Trabecular thickness values measured in the wild-type group (246.65 µm) were lower than those obtained from the TFF3 knock-out group (253.55 µm), however, this difference was not statistically significant (Figure 2A). Trabecular number and trabecular separation were found to be significantly different when comparing wild-type and TFF3 knock-out groups. (Figure 2, B and C). The trabecular number in the wild-type group was higher (1.48/mm) than in the TFF3 knock-out group (1.14 /

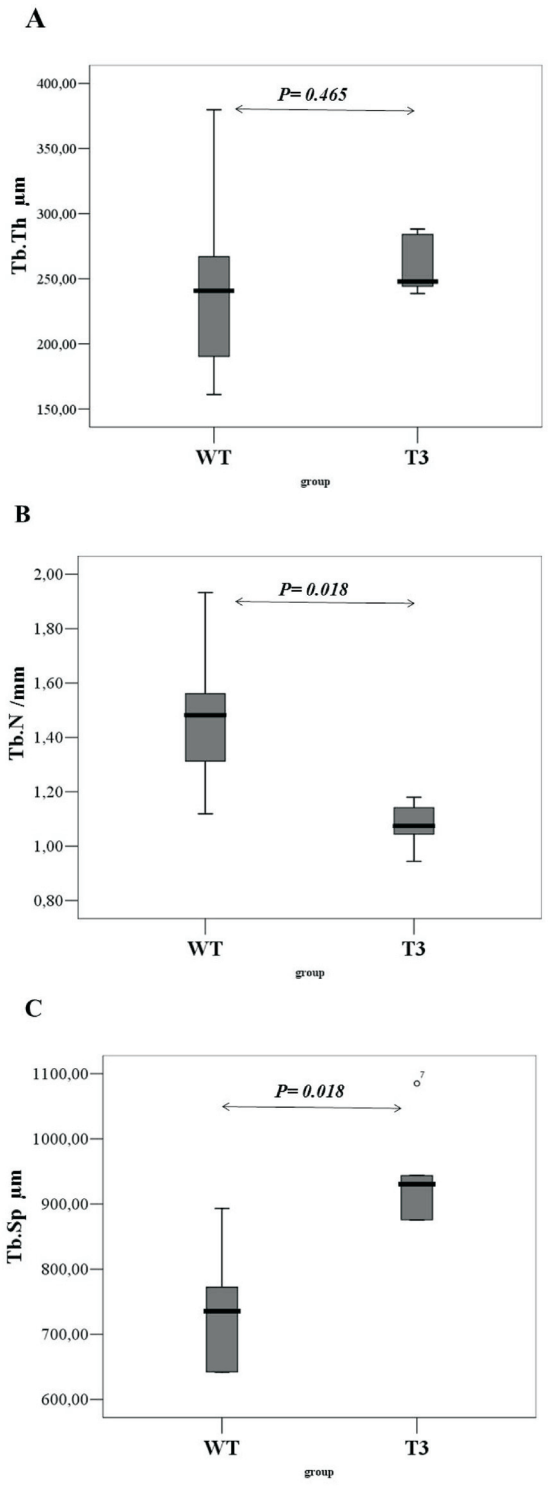

Figure 2. *Trabecular thickness, number and separation in the tibial epiphyses of a 1 month old mice. (A) Tb.Th (µm) = trabecular thickness; (B) Tb.N (/mm) = trabecular number; (C) Tb.Sp (µm) = trabecular separation. WT = wild-type mice, T3 = TFF3 knock-out mice.*

mm), and the trabecular separation was significantly higher in the TFF3 knock-out (952.14 µm), than in the wild-type group (736.88 µm).

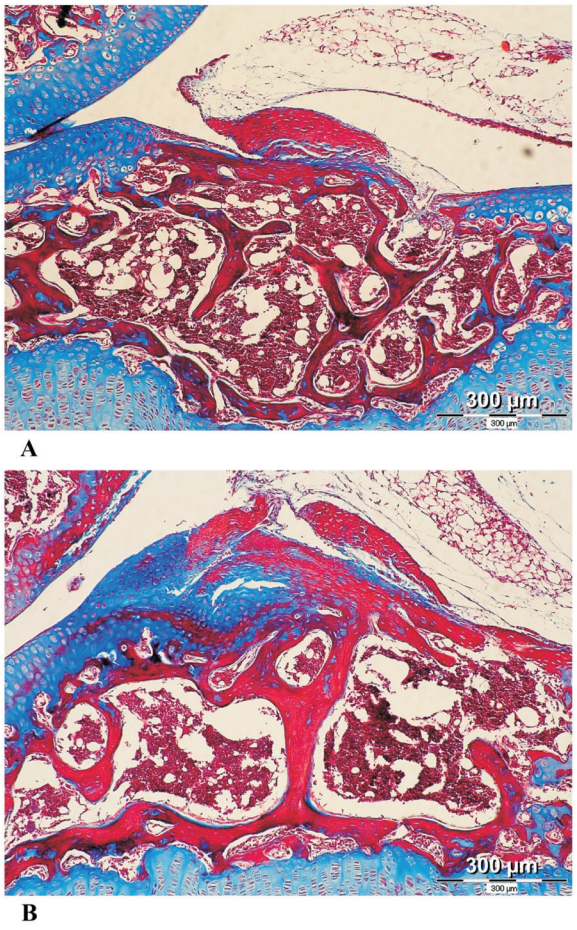

Figure 3. *Histological sections of mouse tibial epiphyses, Masson's trichrome stain, magnification 100×. (A) Wild-type mouse. (B) TFF3 knock-out mouse.*

Histological examination

Examination of the slides under the microscope, after the staining, reveals an obvious difference between wild-type and TFF3 knock-out mice. TFF3 knock-out mice had less trabeculae in the tibial epiphysis, larger parts of bone marrow space was devoid of trabeculae, although, the thickness of the trabeculae present in the secondary ossification centre of the knock-out mice was similar to that of wild-type mice, in some animals even slightly thicker than that of wild-type mice. Overall, the trabeculae of wild-type mice appeared more rugged (Figure 3).

DISCUSSION

In this study we demonstrated that almost all of the examined morphometry parameters of cancellous bone inside the proximal tibial epiphysis were changed significantly in TFF3 knock-out mice when compared to wild-type mice. Overall, trabecular bone surface density and volume density were significantly decreased in the knock-out animals; the trabeculae were approximately of the

same thickness, however, trabecular separation was more pronounced, and the overall number of trabeculae was smaller. Therefore, our results point to the fact that the lack of TFF3 protein impairs bone formation in the secondary ossification centers, at least to some extent. Influence of TFF3 on chondrocyte apoptosis and the activity of several matrix metalloproteinases *(28)* is missing in TFF3 knock-out animals, and this might interfere with the natural process of cartilage degradation which would then contribute to reduced amount of „foundation material" for cancellous bone formation in the epiphyseal secondary ossification centers. Since TFF proteins are shown to influence or to be influenced by transdifferentiation of different cells in the respiratory system *(38–40),* it may be hypothesized that they also play a role in transdifferentiation of the hypertrophic chondrocytes into osteoblasts during ossification.

While TFF3 is shown to have an impact on the cartilage catabolism and is present in endochondral ossification *(28, 29)*, our present study shows that this impact of TFF3 on endochondral ossification seems to exceed that of affecting cartilage degradation, and possibly includes a role in the formation of cancellous bone. Some of the recent research showed that changes in cartilage and subchondral bone are closely tied during osteoarthritis *(41, 42)*. Furthermore, since TFF3 is involved in the process of cartilage degradation and bone formation, this would imply possible therapeutic usage of this phenomenon.

Our knowledge about TFF peptides is constantly growing, and research on this small family of peptides has somewhat moved to areas different from that of mucosal surfaces, with new sites of TFF3 expression having developmental and clinical significance. Further experiments are needed in order to more fully understand the impact of TFF3 on bone formation and histomorphometric parameters of the bones in TFF3 knock-out mice. More detailed studies on the embryonic and postnatal endochondral bone formation would definitely give more insight into the connection of TFF3 with this process. Also, similar experiments on older mice (5 or 6 months) would give additional insight of the final result of endochondral ossification. However, this is the first time that TFF3 protein was reported to be connected with morphological parameters of cancellous bone, and that its impact on bone formation is implied. Therefore, we can say that our preliminary data opened some interesting and possibly fruitful perspectives in the research about the TFF proteins.

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