



Trefoil factor family protein 3 (TFF3) is present in cartilage during endochondral ossification in the developing mouse fetus

Nikola Bijelić^{a,*}, Tatjana Belovari^a, Mirela Baus Lončar^{b,c}

^a Department of Histology and Embryology, School of Medicine, University of Osijek, J. Huttlera 4, 31000 Osijek, Croatia

^b Department of Clinical Chemistry and Biochemistry, School of Medicine, University of Osijek, J. Huttlera 4, 31000 Osijek, Croatia

^c Department of Molecular Medicine, Institute Ruđer Bošković, Bijenička 54, 10000 Zagreb, Croatia

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ABSTRACT

Trefoil factor family protein 3 (TFF3) is found in cartilage affected by osteoarthritis and septic arthritis, whereas no TFF3 presence is observed in healthy cartilage. During endochondral ossification, bone tissue replaces degenerating cartilage. There is no data about the role of TFF3 in this process. Our aim was to study the localization of TFF3 in cartilage during endochondral ossification in the mouse fetus. CD1 mouse fetuses, days 14–17, were isolated, fixed, and paraffin embedded. Fetuses were cut into 6 μm sections, and processed for immunohistochemical staining with affinity purified polyclonal rabbit anti-TFF3 antibody. TFF3 was present in cartilage chondrocytes undergoing endochondral ossification, particularly in zone of proliferation, hypertrophy and calcification as well as in zone of cartilage degeneration during the monitored fetal period. Resting cartilage showed no presence of TFF3, while during endochondral ossification TFF3 localization showed an analogous pattern to that reported in cartilage affected by osteoarthritis and septic arthritis. Our data indicate that the role of TFF3 in these pathological conditions is similar to its role in the physiological process of endochondral ossification.

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Introduction

The trefoil factor family of peptides (TFFs) comprises three peptides (TFF1, 2 and 3), and is named after a specific clover-like domain in their peptide chain (Thim, 1989; Wright et al., 1997). TFFs are found in various mammalian tissues. They are predominantly secreted by mucin-secreting goblet cells situated in mucous membranes of the gastrointestinal tract (TFF1 in stomach and colon, TFF2 in stomach, and TFF3 in the intestine) (Hoffman, 2005; Madsen et al., 2007). TFFs participate in a number of different physiological and pathological processes. They have an important role in protection and repair processes of mucous membranes, and are up-regulated in conditions of gastrointestinal injury. These peptides are also involved in carcinogenesis, promote metastasis, and are associated with invasive phenotype of tumors, and their expression is increased in some inflammatory processes, especially those of the gastrointestinal mucosa (Baus-Lončar and Giraud, 2005; Regalo et al., 2005; Kjellev et al., 2006). This is in line with findings that TFFs affect mucus viscosity (Thim et al., 2002; Kjellev et al., 2006), cell migration (Hoffman, 2005), apoptosis (Rösler et al., 2010; Schulze et al., 2010) and immune response (Baus-Lončar et al., 2005). Minute amounts of TFFs are found in the

brain, and therefore, a potential neurotransmitting or neuromodulating role was debated, especially for TFF3, which is co-localized with oxytocin in the hypothalamus (Hoffman et al., 2001). Some investigators have proposed TFFs as clinical biomarkers in different pathological conditions such as nephrolithiasis, kidney tubular injury and cholangiocarcinoma (Rinnert et al., 2010; Yu et al., 2010; Kosriwong et al., 2011). TFF3 is important for gastrointestinal, oral and corneal wound restitution (Hoffman, 2005; Peterson et al., 2009; Schulze et al., 2010), and is also found in the epithelium of airways, where it induces ciliogenesis and promotes the differentiation of ciliated cells (LeSimple et al., 2007).

Endochondral ossification is one of two main types of bone formation, the other being intramembranous ossification. Endochondral ossification involves a process in which cartilaginous models of bones are being replaced by bone tissue. It starts during embryonic development, and continues in the postnatal period for a certain period of time, depending on the species. This is a complex process, during which chondrocytes undergo proliferation, hypertrophy, and finally death. Also, significant changes occur in the matrix of cartilage, where hydroxyapatite and non-crystalline calcium-phosphate are accumulating. In some details, endochondral ossification is similar to some pathological conditions of cartilage, such as osteoarthritis, where cartilaginous tissue is destroyed during the process of inflammation and pathological ossification occurs (Kawaguchi, 2008). High expression of TFF3 is found in cartilage of patients with osteoarthritis, and also in

* Corresponding author.

E-mail address: nbijelic@mefos.hr (N. Bijelić).

mouse models of osteoarthritis and septic arthritis, whereas no TFF3 expression is observed in healthy human articular cartilage. The same study shows that TFF3 acts as a proapoptotic effector and that it promotes cartilage degradation *in vitro* (Rösler et al., 2010). Since TFF3 is found in cartilage affected by arthritic degradation, the logical question arises, whether TFF3 would also be present in the physiological process of cartilage degradation. There is no data about the role of TFF3 in this process. Therefore, the aim of this study was to investigate the presence of TFF3 in cartilage undergoing endochondral ossification.

Materials and methods

Care and use of the animals and the experimental protocol were reviewed and approved by the Croatian board for scientific animal experiments and approved by a local ethical committee. A total of 19 CD1 mouse fetuses, day 14, 15, 16 and 17 (Theiler stage 22, 23, 24 and 25) were isolated immediately after pregnant females were killed by cervical dislocation. Fetuses were fixed in 4% paraformaldehyde, and embedded in paraffin. Sagittal sections 6 µm thick were cut. Slides were mounted, dried, deparaffinized and rehydrated. Antigen retrieval was performed by microwave heating using 0.01 M citric acid (pH 6) for approximately 5 min. Endogenous peroxidase blocking with 0.3% hydrogen peroxide for 15 min and non-specific protein blocking with SuperBlock® (Thermo Scientific, Rockford, USA) for 30 min was performed. Affinity purified primary polyclonal rabbit anti-TFF3 antibody (proprietary, self-made) was used for incubating slides overnight at 4 °C (1:5000), with phosphate-buffered saline (PBS) (pH 7.4) as a negative control (primary antibody omitted). Sections of adult mouse intestine were used as a positive control. Slides were rinsed with PBS+0.05% Tween (Sigma–Aldrich, St. Louis, MO, USA) four times, 5 min each. Next, biotinylated goat anti-rabbit secondary antibody (Dako, Glostrup, Denmark) was applied for 30 min at room temperature. After another four 5-min washes in PBS+Tween, slides were incubated with Streptavidin-HRP (Dako) for 30 min at room temperature. Slides were further rinsed 4 times in PBS+Tween incubated with DAB (3,3'-diaminobenzidine) (Sigma–Aldrich), washed with PBS+Tween, dehydrated, counterstained with hematoxylin and mounted. Pictures were taken with an Olympus® C-5050 digital camera on an Olympus® BX50 microscope and QuickPHOTO PRO imaging software (Promicra s.r.o., Prague, Czech Republic).

Results

Sagittal sections through fetuses showed different parts of the skeletal system, with many centers of endochondral ossification revealed at different stages. They were found in bones of the neurocranium, viscerocranium, vertebrae, ribs, sternum, forelimbs, hindlimbs and pelvis. Localization of hyaline cartilage, centers of ossification, and formed bone were as expected in observed stages. All zones of endochondral ossification were exposed and available for examination.

TFF3 was present in chondrocytes in the zone of proliferation, zone of hypertrophy, and zone of calcification and cartilage breakdown (Figs. 1–4). Signal was found in the cytoplasm of chondrocytes and was mostly mild or moderate, apart from a few exceptions, where the signal was either weak or missing, or strong. In the zone of cartilage calcification, it was harder to determine the localization of TFF3 in apoptotic chondrocytes (Figs. 1, 2A, 3A and 4A). In addition, TFF3 was detected in the zone of ossification. Positive cells were aligned along the newly formed osseous tissue in a characteristic pattern, thus showing morphological characteristics of osteoblasts (Fig. 1). Sections through the

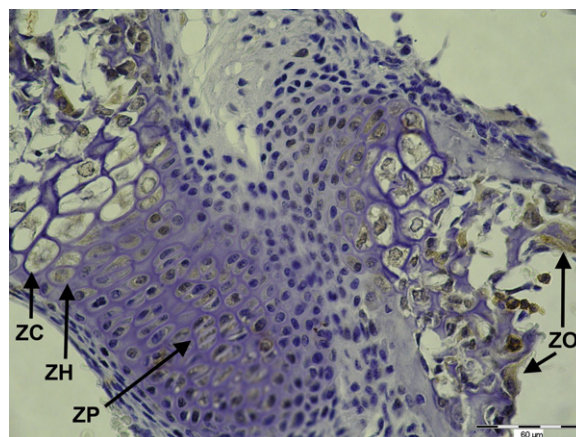


Fig. 1. TFF3 localization during endochondral ossification of vertebral column in a 17-day old mouse fetus. TFF3 is found in chondrocytes occupying the zone of proliferation (ZP), zone of hypertrophy (ZH), zone of calcification and cartilage breakdown (ZC), and in the osteoblasts found in the zone of ossification (ZO).

newly formed bone showed TFF3 presence inside the fetal bone marrow in cells that morphologically correspond with myeloid cells.

TFF3 immunostaining was detected in all stages of fetal development included in this investigation (E14–E17). However, no

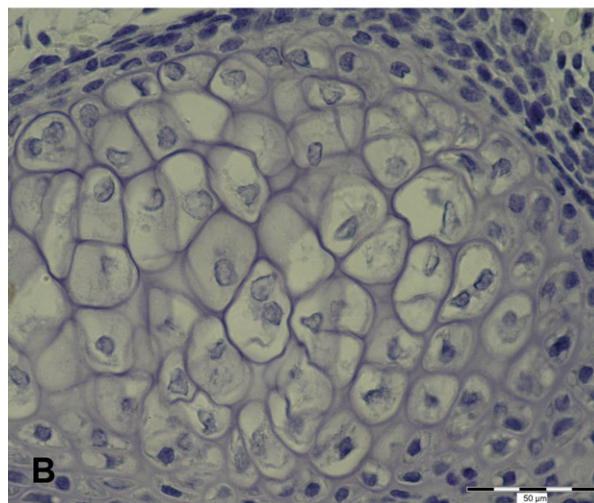
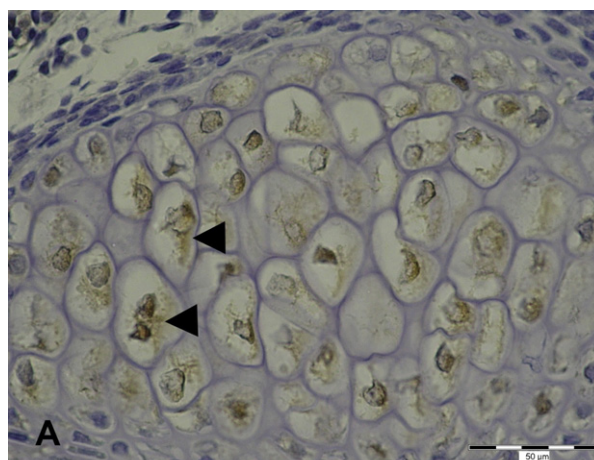


Fig. 2. TFF3 in chondrocytes during endochondral ossification of vertebra in a 15-day old mouse fetus. (A) TFF3 immunostaining is visible in cytoplasm of chondrocytes occupying the zone of hypertrophy and calcification (arrow heads). (B) Negative control.

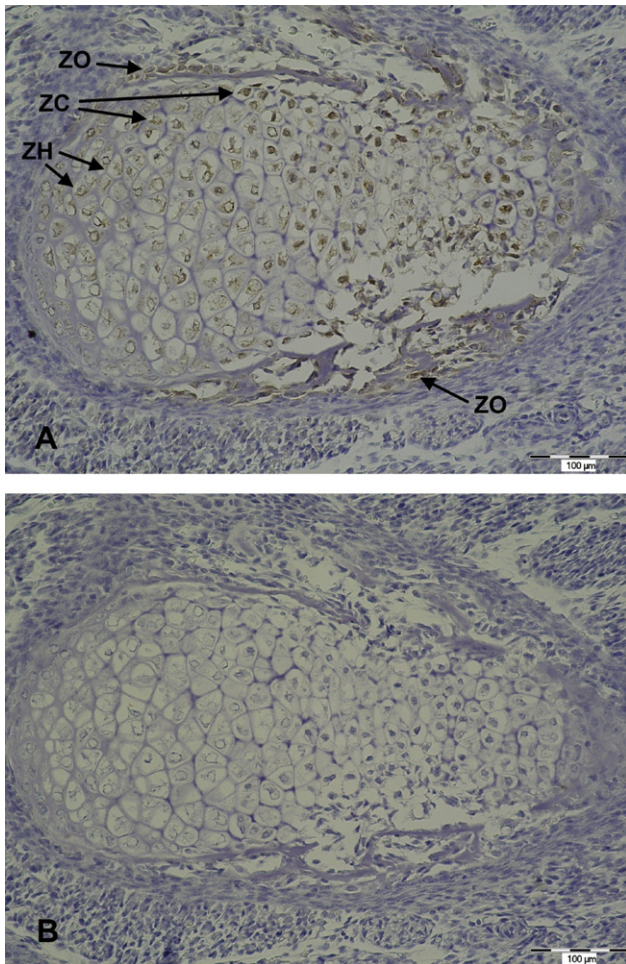


Fig. 3. Localization of TFF3 during endochondral ossification in humerus of a 14-day old mouse fetus. (A) Cytoplasmic signal is visible in the cells occupying zone of hypertrophy (ZH) and zone of ossification (ZO). Apoptotic chondrocytes in the zone of calcification (ZC) do not share clear cytoplasmic pattern of signal distribution, but rather the whole cell appears positive. (B) Negative control from serial section showing no signal.

signal was observed in the zone of resting cartilage, as well as in other cartilaginous formations that normally do not undergo endochondral ossification, such as ventral parts of ribs and bronchial cartilage (Figs. 4 and 5). Also, negative controls showed no staining (Figs. 2B, 3B, and 4B).

Discussion

Trefoil factors have been a subject of many analyses in different contexts. Primary focuses of TFF investigation were their effects on mucous membranes and expression in certain tumors. In recent years, research on TFF3 in avascular tissues, such as cornea and cartilage, were conducted, and showed different effects of TFF3 on such tissues. For example, TFF3 is shown to promote restitution of corneal cells *in vitro*, and also *in vivo*, where exogenously applied TFF3 accelerates corneal wound healing, while in TFF3 knock-out mice corneal wound healing is prolonged (Göke et al., 2001; Paulsen et al., 2008; Schulze et al., 2010). Rösler et al. (2010) showed that TFF3 induces matrix metalloproteinases (MMPs), especially MMP-3, which break down the collagen network in the extracellular matrix of cartilage. They also observed pro-apoptotic effects of TFF3 in chondrocytes. On the other hand, TFF3 shows anti-apoptotic effect in normal colon cell lines, and also in colon cancer cell lines, whilst in TFF3 knock-out mice, intestinal apoptosis is

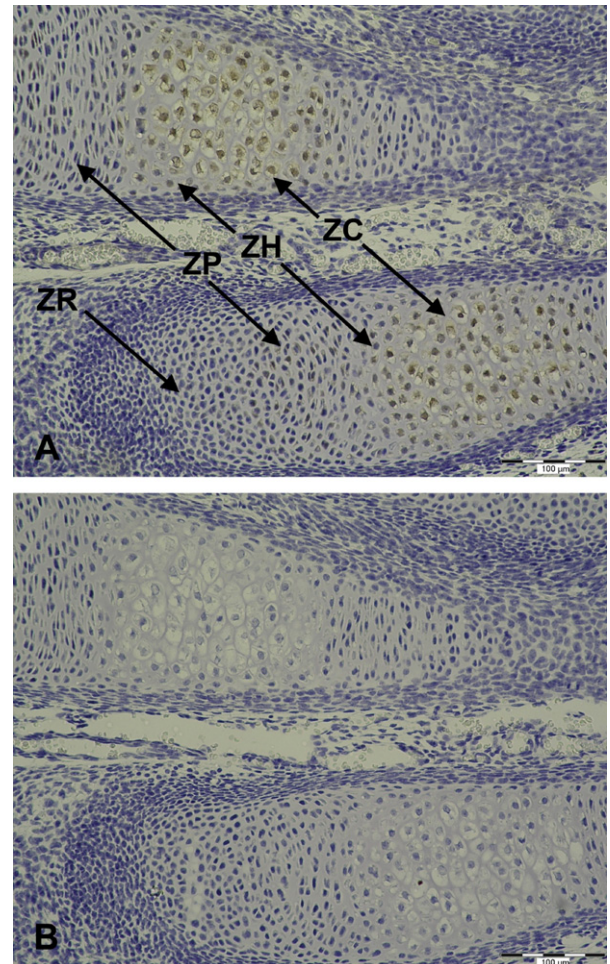


Fig. 4. TFF3 presence during endochondral ossification in metatarsal bones of a 16-day old mouse fetus. (A) Positive staining in the zone of proliferation (ZP), hypertrophy (ZH) and calcification (ZC) No signal in the zone of resting cartilage (ZR). (B) No visible staining in negative control.

increased (Taupin et al., 2000; Regalo et al., 2005; Lubka et al., 2009). Rösler et al. (2010) have also debated a potential anabolic function of TFF3, and effects on cell migration in cartilage, which is in line with the fact that TFF3 and other members of TFF family reduce cell-to-cell contacts and cell-matrix interaction (Hoffmann and Jagla, 2002; Hoffman, 2005; Regalo et al., 2005). Therefore, whether it is involved in proliferation or degradation of the tissue, TFF3 plays an important role. In simple terms, TFF3 seems to be a tool which can be used as an aid in building as well as destroying. A recent study by Ahmed et al. (2012) supports this conclusion. These researchers described the presence of TFF3 in normal breast and breast cancer. They demonstrate that in normal breast epithelial cells, TFF3 is associated with normal functionality, but in case of poorly differentiated carcinoma it is associated with tumor progression and metastases. Also, localization of TFF3 by immunohistochemical analysis showed different pattern in well-differentiated tumor cells (luminal edge of the cell), and in poorly differentiated ones (towards the stroma). This means that in different tissues and in different circumstances, the effects of TFF3 on angiogenesis, apoptosis, cell-to-cell contacts can be exploited to protect mucosal integrity in the case of normal cells, and to promote aggressive tumor behavior in the case of poorly differentiated carcinoma. Although at first glance, these effects seem to contradict one another, they are most probably the result of the same, above described mechanisms.

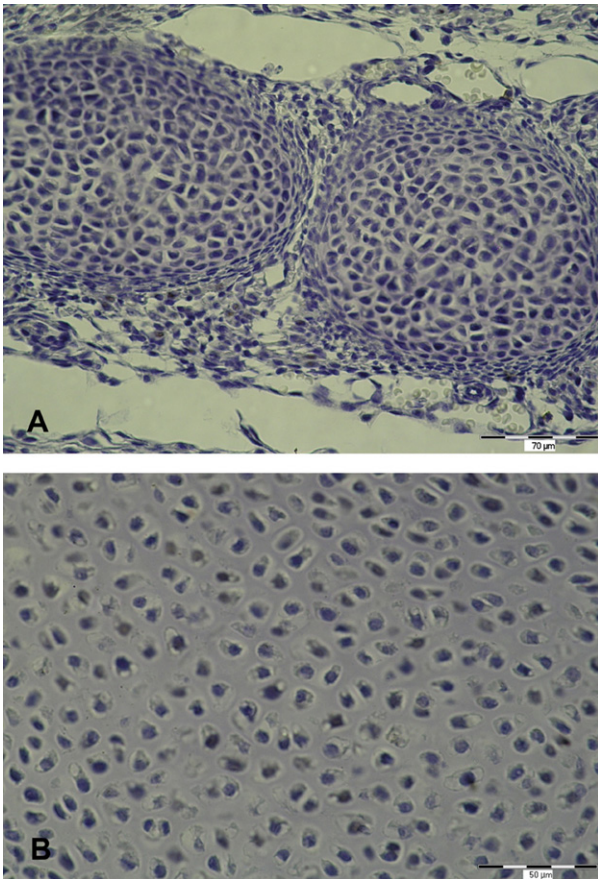


Fig. 5. Resting cartilage showing no presence of TFF3. (A) Non-ossified rib cartilage in 16-day old mouse fetus. (B) Unossified cartilage in the distal humeral epiphysis of a 16-day old mouse fetus.

Our results demonstrated that the localization pattern of TFF3 during endochondral ossification in mouse fetuses shows an interesting analogy to that found in cartilage affected by osteoarthritis and septic arthritis. TFF3 expression in chondrocytes is induced by tumor necrosis factor alpha (TNF α) and interleukin-1 beta (IL-1 β) administration *in vitro* (Rösler et al., 2010). Furthermore, TNF α was shown to play an active role in endochondral ossification (Lehmann et al., 2005; Lukić et al., 2005). These findings lead to the conclusion that similar mechanisms are involved both in endochondral ossification as a physiological process, and in osteoarthritis or septic arthritis as pathological processes (Kawaguchi, 2008). Moreover, since TFF3 expression is induced in all these conditions, but not in healthy articular cartilage, nor in the zone of resting cartilage during endochondral ossification, there is reason to believe that TFF3 plays an important role in cartilage degradation processes, physiological or pathological, and that it is tightly connected with the effects of TFF3 on apoptosis and matrix metalloproteinases. These mechanisms require further investigation. The fact that TFF3 was also detected in sites of new bone formation, particularly in fetal osteoblasts, points to TFF3 as not only having a role in cartilage degradation, but also in the formation of the new bone.

It is unclear, though, to what extent TFF3, IL-1 β and TNF α are connected in the process of endochondral ossification. Although researchers have shown an important role of TNF α in endochondral ossification and an up-regulating effect on TFF3 expression by TNF α and IL-1 β in chondrocytes, there is also strong evidence that the very same cytokines suppress TFF3 expression in gastrointestinal cells, and these signaling mechanisms have also been described

(Dossinger et al., 2002; Baus-Lončar et al., 2003, 2004). Considering this, we can conclude that this effect could be cartilage-specific for yet unknown reasons, and that signaling mechanisms underlying these phenomena are more complex than are currently understood.

When considered in the context of embryonic and fetal development, osteoarthritis could in fact be a pathologically re-awakened process of endochondral ossification, since it seems to employ similar mechanisms in cartilage destruction. A better understanding of these mechanisms is a potential key to earlier diagnosis and more effective treatment of osteoarthritis as a degenerative disease. Also, potential roles of TFF1 and TFF2 in cartilage physiology and pathology are yet unknown and would be interesting to investigate. Such research is of particular interest because some scientists have also described tumors as having various features of embryonic tissue (Monk and Holding, 2001). In this manner, TFFs are one of the links between embryonic and fetal development and various important diseases of modern civilization, such as tumors and, in this case, osteoarthritis. Our results show that an understanding of embryological processes could be the key to understanding debilitating diseases.

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