

# Localization of trefoil factor family peptide 3 (TFF3) in epithelial tissues originating from the three germ layers of developing mouse embryo

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## ABSTRACT

Trefoil factor family (TFF) peptides are involved in the maintenance of epithelial integrity and epithelial restitution. Mature epithelial tissues originate from different embryonic germ layers. The objective of this research was to explore the presence and localization of TFF3 peptide in mouse embryonic epithelia and to examine if the occurrence of TFF3 peptide is germ layer-dependent. Mouse embryos (14-18 days old) were fixed in 4% paraformaldehyde and embedded in paraffin. Immunohistochemistry was performed with affinity purified rabbit anti-TFF3 antibody, goat anti-rabbit biotinylated secondary antibody and streptavidin-horseradish peroxidase, followed by 3,3'-diaminobenzidine. TFF3 peptide was present in the gastric and intestinal mucosa, respiratory mucosa in the upper and lower airways, pancreas, kidney tubules, epidermis, and oral cavity. The presence and localization of TFF3 peptide was associated with the embryonic stage and tissue differentiation. TFF3 peptide distribution specific to the germ layers was not observed. The role of TFF3 peptide in cell migration and differentiation, immune response, and apoptosis might be associated with specific embryonic epithelial cells. TFF3 peptide may also be considered as a marker for mucosal maturation.

KEY WORDS: TFF3; digestive system; embryonic development; epidermis; epithelium; germ layers; immunohistochemistry; mice; respiratory system; trefoil factor; urogenital system

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## INTRODUCTION

Trefoil factor family (TFF) peptides are involved in the maintenance of epithelial integrity and epithelial restitution. TFF3 peptide is found in the epithelia of various human organ systems. The main site of TFF3 peptide expression is gastrointestinal mucosa, where it is excreted from the granules together with mucins onto the epithelial surface, which contributes to the formation of the protective mucosal layer [1,2]. Following mucosal injury, TFF peptides act as motogens by reducing cell-cell and cell-surface contacts [3]. Antiapoptotic and pro-angiogenic effects of TFF3 peptide have been described [4,5], as well as its effect on the immune system [6,7].

TFF3 peptide is involved in regeneration of epithelial injuries in the digestive system, and it stimulates the differentiation of ciliated cells, which is important in maintaining the normal

function of the respiratory system [8]. In the urinary system, TFF3 peptide is found in the kidney tissue and is assumed to play an important role in repairing the damage of kidney tubular epithelium [9]. TFF3 peptide is also found in the epithelia of several other organs (e.g. eye, inner ear, uterus, mucous glands, pancreas, liver, breast, etc.) [10-12], as well as in urine, serum, amniotic fluid, and breast milk [13]. In addition, TFF3 peptide was detected in the nervous system and some avascular tissues [14,15].

The localization and function of TFF3 peptide have been monitored in different pathological conditions. A number of studies identified the oncogenic potential of TFF3 peptide. Higher TFF3 peptide levels in the serum were associated with a more malignant form of tumor, existence of distant metastases, and a worse prognosis for patients with colon, stomach, and breast tumors [16-18]. TFF3 peptide is assumed to stimulate the proliferation of tumor cells, extend the survival, and facilitate their migration, which results in the formation of distant metastases [17]. TFF3 peptide was also investigated in the context of inflammatory bowel disease and shown to have a role in protection and repair of the epithelium [19,20]. In

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addition, local and systematic application of TFF dimers mitigates the course of ulcerous colitis [21]. Recent research shows that TFF3 peptide levels are increased during inflammatory processes of the respiratory system, such as chronic obstructive pulmonary disease and asthma [22].

In spite of continuous research, the exact molecular mechanism of TFF3 peptide in the restitution and regeneration of gastric mucosa is still unclear [23]. TFF3 peptide acts through various signaling cascades in cells [24]. The peptide stimulates the activation of extracellular signal-regulated kinase/mitogen-activated protein kinase and activates serine phosphorylation of Akt, a kinase associated with apoptotic pathways [25]. Intestinal restitution induced by TFF3 peptide is also connected with IL-6/Gp130/STAT signaling [26]. TFF3 peptide activates STAT3 [27] that exerts anti-apoptotic and mitogenic effects [28]. It modulates the E-cadherin/catenin cell adhesion complex in various ways. For example, exogenous TFF3 peptide in HT-29 cells reduces the level of E-cadherin,  $\beta$ -catenin,  $\alpha$ -catenin and the adenomatous polyposis coli (APC) protein, leading to significant alterations in cell aggregation, detachment from the substratum, and translocation of APC from the cytoplasm to nucleus [29].

Mature epithelial tissues originate from all three embryonic germ layers. Ectodermal derivatives are epidermis and cutaneous appendages, as well as olfactory and mouth epithelium. The epithelium of the pelvic/colorectal system and ureters and epithelium of the kidney collecting duct system and renal tubules (nephrons) differentiate from the intermediate mesoderm. Endothelium and mesothelium originate from the lateral plate mesoderm. Endoderm-derived epithelium lines the digestive tube, trachea, bronchi, lungs, tympanic cavity, auditory tube, urinary bladder, and urethra. In addition, epithelial cells in the liver, gallbladder, pancreas, thymus, thyroid gland and parathyroids also develop from the endoderm [30].

All or individual TFF peptides have been reported in the embryonic nervous and gastrointestinal system, as well as in the process of endochondral ossification in the mouse embryos [31-35]. However, no systematic overview of TFF peptide expression in embryonic tissue was published to date. The objective of this study was to explore the presence and localization of TFF3 peptide in mouse embryonic epithelia and to examine if the occurrence of TFF3 peptide is germ layer-dependent.

## MATERIALS AND METHODS

CD1 mouse embryos, 14, 15, 16, 17 and 18 days old, were taken from the 4% paraformaldehyde fixed and paraffin-embedded embryonic sample collection of the Department of Histology and Embryology, Faculty of Medicine Osijek. The research has been approved by the local Ethical Committee

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The embryos in the collection were obtained from dams on a specific gestational day, following cervical dislocation. Overall, 23 mouse embryos were used (3-6 per stage, due to a varying number of embryos per pregnancy and the fact that a few embryos were excluded for inadequate fixation). The paraffin blocks were cut on a microtome (Reichert-Jung 2400, Vienna, Austria) into 6  $\mu$ m sagittal sections, placed on adhesive Menzel-Gläser Polysine slides (Thermo Scientific, Rockford, USA) or equivalent.

Slides were deparaffinized and rehydrated using 100% xylene, 100%, 96%, and 70% ethanol and tap water. Peroxidase block was performed using 0.3% hydrogen peroxide for 15 minutes, after which antigen retrieval was done by microwave heating in citrate buffer (pH 6) for 3-5 minutes. Non-specific binding was blocked with SuperBlock<sup>®</sup> solution (Thermo Scientific, Rockford, SAD) for at least 30 minutes. Affinity-purified polyclonal rabbit anti-TFF3 antibody was used (dilution 1:5000) for overnight incubation at 4°C. Specificity of the primary antibody was tested on TFF3 knockout mouse intestine where no staining was found. Detection system specificity was tested by omitting primary antibody. Wild-type mouse intestine was used as the positive control. The antibody has been raised against a specific epitope comprising the last 15 amino acid (AA) positions (45-59) [VPWCFKPLQEA ECTF].

After primary antibody incubation, the slides were repeatedly rinsed in phosphate buffered saline (PBS) with 0.05% Tween (Sigma-Aldrich, St. Louis; MO, SAD). Following the rinsing, biotinylated goat anti-rabbit antibody (Dako, Glostrup, Denmark) was used as a secondary layer, dilution 1:300 for 30 minutes. Rinsing in PBS was repeated, and then streptavidin-horseradish peroxidase (Dako or Sigma-Aldrich) was applied in 1:300 dilution for another 30 minutes. Following another rinsing series, immunocomplexes were visualized with 3,3'-diaminobenzidine solution (Sigma-Aldrich or Vector Laboratories, Burlingame, CA, USA). After another series of rinsing in PBS, the slides were counterstained with hemalaun, rinsed in tap water, dehydrated and permanent slides were made using Canada balsam. Olympus<sup>®</sup> C-5050 digital camera mounted on an Olympus<sup>®</sup> BX-50 microscope (Olympus, Tokyo, Japan) was used for taking digital photographs with the QuickPHOTO Pro software (Promicra s.r.o., Prague, Czech Republic).

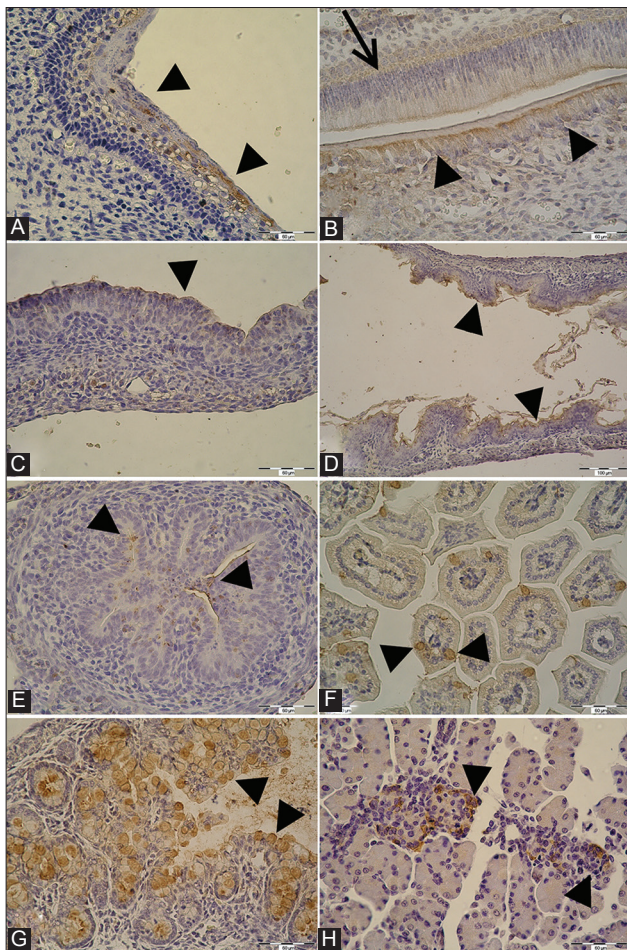
## RESULTS

### Gastrointestinal mucosa

TFF3 peptide was differently distributed along the gastrointestinal tract, depending on the gestational day.

In the oral cavity, mild to moderate signal intensity for TFF3 peptide was observed in stratified squamous epithelium, in the five monitored embryonic stages (Figure 1A). The sections of the developing teeth showed the presence of TFF3 peptide in the cells of both epithelial and mesenchymal origin; for example, TFF3 signal was observed in the stellate reticulum, odontoblasts, and enamel epithelium (Figure 1B). Although still visible, TFF3 signal in the oral cavity was the weakest on embryonic day 14 (E14).

In the gastric mucosa, weak or no immunostaining was detected in E14 and E15 stages (Figure 1C). In E16 stage, TFF3



**FIGURE 1.** Localization of trefoil factor family 3 (TFF3) peptide in the mouse embryonic gastrointestinal system. (A) TFF3 peptide signal in the stratified squamous epithelium of the oral cavity; 16-day-old mouse embryo. (B) Odontoblasts (arrow heads) and enamel epithelium (arrow) positive for TFF3 peptide in a developing tooth of an 18-day-old mouse embryo. (C) Discrete TFF3 peptide signal in the undifferentiated endodermal epithelium of the gastric mucosa; 15-day-old mouse embryo. (D) TFF3 peptide staining in the differentiated nonglandular stomach of an 18-day-old embryo. (E) Scattered TFF3 peptide signal in the endodermal epithelium undergoing differentiation in the small intestine; 15-day-old mouse embryo. (F) Localization of TFF3 peptide in the differentiated goblet cells of the small intestine of a 17-day-old mouse embryo. (G) Strong TFF3 staining in the differentiated goblet cells of the colonic mucosa; 17-day-old mouse embryo. (H) TFF3 peptide present in the tissue between the exocrine acini, probably developing islets of Langerhans. Scale bar: 60  $\mu\text{m}$ , except for (D) 100  $\mu\text{m}$ .

signal was observed in several sections, while in E17 and E18 stages it was clearly visible in all sections, especially in the nonglandular stomach (Figure 1D). On the other hand, in the glandular stomach, the signal was weak or missing (data not shown).

In the small intestine, TFF3 signal was absent in E14 stage, mild signal intensity was detected in E15 stage (Figure 1E), and a strong TFF3 signal, typical for the goblet cells, was observed in E16 to E18 stages (Figure 1F), comparable to the results for the adult intestinal mucosa. Similar pattern of TFF3 signal intensity was seen in the colon, except in the E16 stage; in some cases, the goblet cells were not completely differentiated, thus the signal was not consistent in all sections. A strong TFF3 signal was observed in all stages where the goblet cells were clearly differentiated, especially in E17 and E18 stages (Figure 1G). In the rectum, TFF3 signal was strong in the later stages (i.e., E17 and E18).

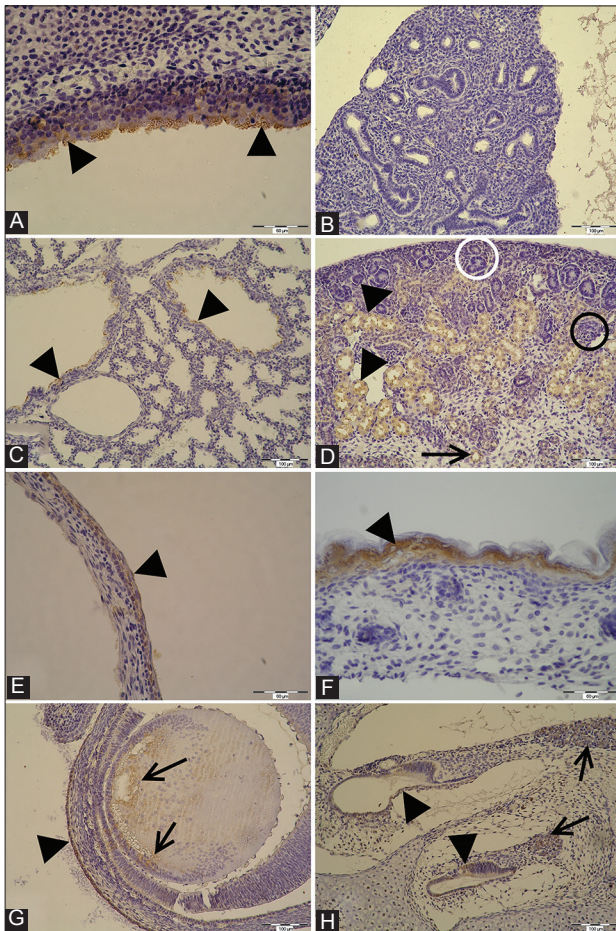
The pancreatic acinar cells were negative for TFF3 peptide, while the positive signal was detected in the tissue between acinar cell areas, which might correspond to pancreatic islets of Langerhans (Figure 1H). TFF3 signal was present to some extent in the liver, but was not distributed homogeneously. The salivary glands were mostly negative for TFF3 peptide, except for the ducts, where moderate TFF3 signal intensity was occasionally observed.

### Respiratory system

The nasal respiratory epithelium was TFF3-positive, especially in the embryos in E16 to E18 stages (Figure 2A). The signal was mostly present in the goblet cells and on the surface of ciliated cells (cilia). In embryos 14 and 15 days old, mild TFF3 signal intensity was sporadically detected (i.e., not in all sections). Similar results were obtained for the lungs (Figure 2B and C). For example, in the lungs, TFF3 peptide was mostly localized in the respiratory epithelium of the bronchi and bronchioles, in E17 and E18 stages. TFF3 signal was not observed in small bronchiolar branches. Similarly, TFF3 signal was not detected in the earlier embryonic stages (i.e. E14-E16), except for a weak signal in several sections.

### Urinary system

A moderate to strong signal was detected in the developing kidney tubules. Although TFF3 signal was detected to some extent in E14 stage, a weak TFF3 signal was present mostly from E15 stage onward (Figure 2D). TFF3 immunostaining was predominantly visible in the cortical tubules, which morphologically correspond to proximal and distal convoluted tubules. It was also present in some of the medullary tubules, morphologically corresponding to the thick limb of the Henle's loop and collecting ducts. Tubules originating from renal vesicles showed stronger staining for TFF3 peptide than the collecting



**FIGURE 2.** Trefoil factor family 3 (TFF3) peptide in different embryonic epithelial tissues. (A) Presence of TFF3 peptide in the nasal respiratory epithelium; 16-day-old mouse. (B) Developing lungs of a 14-day-old mouse embryo devoid of any TFF3 immunostaining. (C) Respiratory epithelium in the bronchioles of a 17-day-old mouse embryo positive for TFF3 peptide. (D) Localization of TFF3 peptide in the kidney of a 17-day-old mouse embryo. TFF3 peptide is present in the cortical tubules (arrow heads) and in the medullary tubules as well (arrow). Glomeruli (black circle) and the developing tubular structures of the nephrogone zone (white circle) are negative for TFF3 peptide. (E) Mild TFF3 staining in the developing epidermis of a 15-day-old mouse embryo. (F) Epidermal signal confined to *stratum granulosum* and *stratum spinosum* of a 17-day-old mouse embryo. (G) Developing eye in a 15-day-old mouse embryo. The signal is visible in the cornea (arrow head) and in the lens (arrow). (H) Inner ear in a 17-day-old mouse embryo. TFF3 is present in the cochlear epithelium (arrow heads). Spiral ganglion is also positive (arrows). Scale bar: (A), (E), (F) – 60 µm, (B), (C), (D), (G), (H) – 100 µm.

ducts. The glomeruli were negative for TFF3 peptide in all development stages, as well as the sections of the developing structures of the nephrogone zone. Similarly, the urinary bladder sections were negative for TFF3 peptide, with only mild signal intensity observed on several sections, which was probably the experimental artifact (weak background staining).

### The skin and sensory organs

In E14 and E15 stages, the typical epidermal layer structure was still not visible, while in E16, and especially in E17 and E18 stages, the epidermal layers were completely formed, which

was in line with the normal embryonic development. A mild to moderate, diffuse TFF3 signal was observed in the developing epidermis, in E14 and E15 stages (Figure 2E). As the epidermal layers formed, the signal became confined to the epidermal layers that are in the middle (i.e., *stratum spinosum* and *stratum granulosum*), while occurring only occasionally in *stratum corneum*, in E18 stage (Figure 2F). *Stratum basale* was negative for TFF3 peptide in almost all sections. The cells in the hair follicles were also positive for TFF3 peptide. The distribution of TFF3-positive cells in the hair follicles was the same as in the epidermis. No TFF3 immunostaining was detected in the dermis.

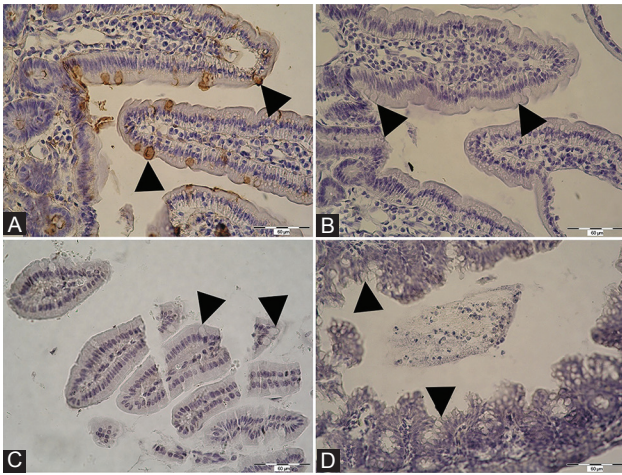
TFF3 was detected in the corneal and conjunctival epithelium and lens (Figure 2G), as well as in the retina and optical nerve (data not shown). The signal was moderate in E14 to E16 stages, mild to moderate in E17 and E18 stages, and was not always consistent in all sections. TFF3 was also detected in the inner ear (Figure 2H), particularly in the neurons of spiral ganglion and in the epithelium covering various portions of membranous labyrinth, mostly cochlea and semicircular ducts. The *crista ampullaris* was observed on several sections and showed a weak TFF3 signal. Sensory nerve fibers coming from the ganglia of the inner ear showed a mild and diffuse signal. The remaining part of the labyrinth, including the connective tissue and cartilage were negative for TFF3 peptide.

Positive and negative controls are presented in Figure 3.

## DISCUSSION

Our results showed that TFF3 peptide was present in different epithelial tissues of developing mouse embryo, from E14 to E18 stages. The intensity of the immunostaining in some tissues depended on the developmental stage. TFF3 peptide-expressing tissues originated from all three germ layers, namely, ectoderm (epidermis, sensory epithelium), mesoderm (kidney tubules), and endoderm (respiratory and gastrointestinal mucosa), therefore, TFF3 peptide expression in embryonic epithelial tissues is probably not embryonic layer-dependent.

Otto and Patel [35] showed by means of *in situ* hybridization that TFF3 mRNA is present in the stomach from day 13 of embryonic development. On days 15 and 16, TFF3 mRNA was observed from the stomach area to colon, and from day 17 it was confined to the small intestine and colon [35]. However, our study showed a weak signal and uneven distribution of TFF3 peptide in the gastric and intestinal epithelium in E14 and E15 stages, which could be due to the fact that the differentiation of endoderm into intestinal epithelium occurred on the day 15 or 16 of embryonic development [36]. This is why the presence of TFF3 peptide was the most pronounced in E17 and E18 stages. Nevertheless, the fact that TFF3 peptide can be found to some extent in the gastrointestinal mucosa



**FIGURE 3.** Examples of control sections. (A) Positive control. Adult mouse small intestine mucosa clearly shows trefoil factor family 3 (TFF3) peptide staining in the goblet cells. (B) Negative control 1. Adult mouse small intestine mucosa with primary antibody omitted. All tissues are negative. (C) Negative control 2. The small intestine of a TFF3 knockout mouse is negative for TFF3. (D) Colonic mucosa of a 17-day-old mouse embryo with primary antibody omitted. Arrow heads show goblet cells on all four images. Scale bar: 60 µm on all images.

before the maturation of the epithelium raises questions regarding its possible role in the differentiation of the main cell lines in this mucosa, since the similar role of TFF1 peptide was already described in gastric epithelium [37,38]. Having in mind the timeframe of TFF3 mRNA expression and peptide presence in the epithelium of the gastrointestinal tract, it may be hypothesized that TFF3 peptide contributes to the differentiation of endoderm to mature epithelium. Nevertheless, since no characteristic cell differentiation disorder is reported for TFF3 knockout mice, we can assume that TFF3 peptide is not crucial for this process. TFF3 peptide was observed in the goblet cells as soon as the epithelium was differentiated, and this finding supports the notion that it is an early marker of gastrointestinal maturation [33]. TFF3 peptide was also present on the mucosal surface and in the scarce intestinal content. Considering its effects on cytokines and defensin expression [13], TFF3 peptide might contribute to the mechanisms of innate immunity and mucosal protection. TFF3 signal found in the embryonic pancreas correlates with what is currently known about its localization in adult pancreas tissue, where it is found in the pancreatic islets and ducts [39]. It is still unclear if TFF3 peptide is involved in the development of pancreas. Similarly, the role of TFF3 peptide in the tooth development is also not understood well, and this should be investigated in the future.

Our results showed that TFF3 signal coincided with the appearance of the goblet cells in the epithelium of the conducting zone, including the nasal cavity. Mild signal intensity for TFF3 peptide was observed in several sections before the respiratory epithelium was fully differentiated (E14 and E15

stages). Apart from the goblet cells, TFF3 peptide was located on the surface of respiratory epithelium, particularly in the area covered by the cilia. Previous research demonstrated that TFF3 peptide is associated with the differentiation of respiratory epithelium cells [8], and our results possibly confirm this role. A role of TFF3 peptide as a marker of mucosal maturation is also plausible.

The TFF3 signal was strong in the tubular system of the developing kidney from the embryonic day 15, when mesonephric tubules were developed to some degree. Furthermore, no signal was detected in the nephrogenic zone where mesenchymal-epithelial transition (MET) takes place. This implies that TFF3 peptide has no effect on the MET, nor on the cell differentiation in kidney tubules. The occurrence of TFF3 peptide in the later stages of kidney development might point to a role of TFF3 peptide in the functional maturation of tubular cells, or it could be simply used as a marker of epithelial maturation.

The epidermal stratification in mouse embryos starts around the 15<sup>th</sup> day of embryonic development [40]. Our results revealed the presence of TFF3 peptide from day 14 to 18 of embryonic development. As soon as the epidermis was stratified, TFF3 peptide was observed in the spinous layer, the place of proliferation and functional changes of keratinocytes, and in the granular layer, in which the nuclei and organelles are starting to disappear and keratohyalin is being accumulated. Although TFF3 peptide is not found in the adult skin [11], it might have a role in the differentiation of epidermal cells, but not a crucial one, since no skin abnormalities have been reported in TFF3 knockout mice. Because TFF1 and TFF3 peptides are present in at least one type of skin cancer [41], the association between TFF3 peptide, embryonic development, and cancer pathogenesis might exist.

The presence of TFF3 peptide in the corneal epithelium of mouse embryo is in line with its role in the maintenance and repair of the ocular surface epithelium [12]. Although it was not a primary aim of this research, we also observed TFF3 signal in the structures of neural origin, namely, the retina and optic nerve. Since only TFF2 expression has been reported in the retina so far [42], it would be interesting to further investigate the expression of TFF3 peptide in this context, to determine if this was only a transient expression. TFF3 peptide has been detected in the inner ear, and it is important for normal hearing [10,43]. It is possible that TFF3 peptide contributes to the normal development of the epithelial structures in the eye and ear. TFF3 peptide presence in the cochlear ganglion and nerves might partly explain presbycusis in TFF3 knockout mice. On the other hand, TFF3 peptide could also be important for the maintenance and normal development of the cochlear epithelium. Further research is needed to elucidate the role of TFF3 peptide in the development and function of sensory organs.

Somewhat contradictory opinions have been reported with regard to the beneficial and harmful effects of TFF peptides in various healthy and diseased tissues. Although important in mucosal repair and protection and differentiation of certain cell lines, these peptides also seem to be important in the pathogenesis and advancement of different diseases. The very same mechanisms by which TFF peptides act favorably on the homeostasis and repair of tissues (e.g., the effect on migration, differentiation, apoptosis, angiogenesis, etc.) also seem to contribute to the malignant potential of several types of cancer, their invasiveness and metastases. The presence and localization of TFF3 peptide in the embryonic epithelial tissues described in this work support a balanced perspective on the biological roles of TFF3 and possibly other two peptides. Thus, TFF3 peptide might be used as a molecular “tool” in both normal physiological and pathological conditions. Furthermore, since tumors are known to exhibit properties similar to that of embryonic tissues, better understanding of TFF3 peptide role in embryonic development could improve the understanding of its role in tumor pathogenesis and advancement [32,44]. This might further explain why in certain tissues, such as cartilage and cornea, TFF3 peptide is expressed during embryonic development, disease or injury, but not in healthy adult tissue. Since it is widely expressed in embryonic tissues, the role of TFF3 peptide in embryonic development should be further investigated.

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## DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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