TG mucosa while no expression was noted in the WT mucosa (Figure 1c, left panel). In contrast, endogenous mouse TGFα mRNA was similarly expressed in both the WT and TG mucosae by real time PCR (Figure 1c, right panel). TGFα is a 50 amino acid peptide, derived from its 160 amino acid membrane-spanning precursor cleaved by tumor necrosis factor-α-converting enzyme (TACE) [27,28]. With the surplus expression of the human TGFα-transgene in the TG mucosa, an approximately 20 kDa TGFα precursor and its intermediate forms were increased by immunoblotting (Figure 1d).

Since both the precursor and processed forms were equally active for stimulating EGF-R [28], we further examined EGF-R localization in the mucosa. EGF-R was localized heavily in the lower chief cell cluster region and moderately in the mucous neck cell region and in the foveolar pit region in both the WT and TG mucosae (Figure 1e), as was reported previously for the rat gastric mucosa [15]. Since the TGFα transgene was controlled under the mouse metallothionein gene [20] and metallothionein has been reportedly produced in the gastric mucosa [29], we examined the metallothionein-expressing cell-types in the mucosa. Immunohistochemically, we noted metallothionein staining mostly along the lower glandular region and scatteringly along the pit region of the control CD1-strain gastric mucosa (Figure 1f). Thus, the distribution of TGFα-expressing cell-types is similar to that of metallothionein-expressing cells in the TG gastric mucosa (Figures 1a and 1f).

In the WT mouse mucosa, the H^+/K^+-ATPase-positive parietal cells were spread widely over the glandular region except for the top-pit layer, whereas the H^+/K^+-ATPase-positive glandular region was reduced in height in the TG mucosa (Figure 1e). Indeed, the isthmus at the base of the pit region, which was confirmed by PCNA staining, was moved down to the middle of the TG mucosa (Figure 1g). Moreover, PCNA-positive cells were thickly distributed in the middle of the mucosa. Consistently, the PAS-staining-positive foveolar pit was elongated in the TG mucosa in contrast to the short pit in the WT mucosa (Figure 1h). Thus, although the height of the gastric mucosa was similar between the WT and TG mucosae, the proliferative zone was moved down and the foveolar pit region was more elongated with a reduced glandular region in the TG mocosa. Since the growth of pit cells is strongly enhanced by gastrin [25,30], we compared the serum gastrin levels between the WT and TG mice but found no increase in the levels of serum gastrin in the TG mouse (WT, 213

± 41 pg/ml; TG, 263 ± 47 pg/ml, n=5, p=0.438).

Ethanol-induced Gastric Injury in the WT and TG Mouse Mucosae

Orogastric administration of acidified ethanol (100 μl) caused hemorrhagic injury in the TG mucosa (Figure 2a, left). Histologically the injury peeled off the pit and isthmus regions and reached to the mid-glandular region (Figure 2b, left). In contrast, this sort of injury in the TG mouse mucosa was not notable (Figure 2a, right). The injury was histologically limited to the luminal pit region (Figure 2b, right). In the WT mucosa, the injured area increased rapidly with a peak at 6 h after the ethanol administration, whereas in the TG mucosa the area increased slowly and minimally without a marked peak (Figure 2c). Consistently, apoptotic cells shown by the TUNEL staining distributed deeply to the lower glandular region 3 h after the ethanol administration in the WT mucosa, whereas the apoptotic cells were confined to the pit region but did not extend to the glandular region in the TG mucosa (Figure 2d).

Gastric Mucosal Blood Flow in the Ethanol-treated Mucosa

Although a number of cytoprotective regulatory factors have been reported, including NO, CGRP, COX2, and HSP70 [12,13,31,32], mucosal blood flow has been proposed as a primary factor for the protection and restitution of ethanol-induced gastric injury [1,33]. We measured gastric blood flow by placing a probe of the laser Doppler flowmeter on the gastric mucosa accessed via an exposed abdominal wall in the anesthetized mouse. Gastric blood flow decreased marked by 43.5 ± 6.37% after the ethanol administration on the gastric mucosa of the WT mouse (Figure 3). In contrast, it only decreased by 17.2 ± 9.21% after the ethanol administration on the TG mucosa (Figure 3). Thus, the gastric blood flow was well maintained in the TG mucosa even after ethanol treatment. Since mucosal ischemia is one of the most important ulcerogenic factors [33], the maintenance of gastric blood flow does appear to be an essential factor for mucosal protection.

Expression of Cytoprotective Proteins: NO Synthase, COX-2, and HSP70

To explore the mechanism of high blood flow maintenance in the TG mucosa, we examined the expression of NO synthases, which makes NO from arginine, and relaxes vascular smooth

muscles via cGMP-dependent protein kinase [34]. There are three types of NO synthase (NOS): neural NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). nNOS and eNOS are constitutively expressed, whereas iNOS is induced upon gastric injury [35]. nNOS was distributed similarly to the PAS-staining over the foveolar pit region (Figure 1g); it was observed over the elongated pit of the TG mucosa and was limited to the luminal pit of the WT mucosa (Figure 4a). eNOS was weakly scattered over the entire gastric mucosa similarly in both the WT and TG mucosae (data not shown). The iNOS staining was negligible in the WT mucosa, although it was highly induced in the lower glandular region after the mucosa was exposed to ethanol (Figure 4b). In the TG mucosa, iNOS was constitutively expressed in the lower glandular region similarly before and after the injury (Figure 4b), which appears to further contribute to the maintenance of high blood flow.

Prostaglandins were proposed as a major cytoprotective factor over three decades ago [8] and their roles have been extensively studied [36,37]. The role of the prostaglandin-forming enzymes, COX-1 and COX-2, has been controversial in the mucosal protection against gastric irritants [7,11,32]. We examined the expression of COX in the WT and TG mucosae during ethanol injury. The expression of COX-1 did not differ between the WT and TG mucosae, nor before or after the ethanol injury (Figure 5b). COX-2 was not detectable in the WT mucosa, but its expression became evident 6 h after ethanol injury, and its staining was localized to the lower glandular region (Figure 5a and b). In the TG mucosa, the COX-2 immunostaining was intense at the same region even before the injury and remained positive after the injury (Figure 5a). Since COXs have been shown to be expressed in mesenchymal cells such as fibroblasts and mononuclear cells in the gastric mucosa [38], and the streptoavidin/biotin/HPR method sometimes presents false-positive staining, we performed immunofluorescent staining using a frozen section which confirmed COX expression similarly in the lower glandular region of the TG mucosa (data not shown). By immunoblotting, COX-2 increased after the injury in the WT mucosa, whereas it was highly elevated to a similar extent before and after the injury (Figure 5b). Consistently, prostaglandin E_2 levels were significantly higher in the TG mucosa than in the WT mucosa (Figure 5c). Furthermore, NFκB, a transcription factor responsible for COX-2 gene expression, was

localized to the lower glandular region of the TG mucosa even before the ethanol treatment (Figure 5d), suggesting that TGFα transgene causes the constitutive expression of NFκB [21].

Gastric irritants are known to induce heat shock protein 70 (HSP70), which chaperones denatured proteins for renaturation or clearance [39]. HSP70 was not detected in the WT mucosa, but after ethanol injury it was observed extensively over the glandular region, and scatteringly in the pit region of the WT mucosa (Figure 6a). In contrast, it was visible at the lower glandular region of the TG mucosa similarly before and after ethanol treatment (Figure 6a). By immunoblotting, HSP70 was not detected before ethanol injury but appeared 3 h to 12 h after the injury in the WT mucosa (Figure 6b). In contrast, it was already elevated before the injury and increased moderately after the injury (Figure 6b).

Collectively, the three cytoprotection-associated proteins, iNOS, COX-2, and HSP70, were negligible in expression before the injury and highly induced after the injury in the WT mucosa, but they stayed elevated similarly before and after the injury in the TG mucosa.

Expression of apoptosis-associated proteins in the gastric mucosa

As shown with the TUNEL staining in Figure 2d, the TGFα-expressing mucosa was strongly resistant to ethanol injury. Initially, we examined the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax by immunoblotting. Bcl-2 and Bax were slightly expressed in the WT mucosa before the injury; after the injury, Bax increased intensively whereas Bcl-2 increased to a lesser extent (Figure 7a). In the TG mucosa, Bcl-2 was expressed even before the injury and remained elevated after the injury, whereas Bax decreased to a negligible level after the injury. By immunostaining, Bcl-2 was positive in the lower glandular region of the TG mucosa before the injury, but no staining was visible in the WT mucosa. After the ethanol injury, Bcl-2 appeared strongly along the periphery of the injured lesion in the WT mucosa, in contrast, it stained similarly to the pretreatment level in the TG mucosa (Figure 7b). Thus, the constitutive expression of Bcl-2 may contribute to the anti-apoptotic feature of the TG mucosa against ethanol injury. Evidence of the active form of caspase-3, an executive caspase, was consistently absent in the TG mucosal cell extract before and after the injury, whereas it was

intensively detected in the WT mucosa after the injury, although the proform remained similarly before and after the injury in both the WT and TG mucosae (Figure 7c).

Discussion

EGF family growth factors including TGFα have been proposed as integrative cytoprotective factors against gastric injury [2,4]. The effects of TGFα have been tested by administrating it to animals or culture cells for a short term, and thus the reported cytoprotective mechanisms of TGFα have been somewhat variable [7,12,24,31,40-42]. TGFα is known to be a potent growth-promoting factor leading to hypertrophic growth in the gastric mucosae [19,20]. In contrast, the EGF-R signal is known to activate non-proliferative functions such as the induction of potent cytoprotective trefoil peptides, which are produced from the gastrointestinal mucosa, through an EGF-R/MAP kinase pathway [43]. Therefore, although high levels of TGFα can cause hypertrophic gastropathy, appropriate levels appear to maintain mucosal resistance to gastric injury. However, since the broad presence of PCNA-positive cells shown in Figure 1g indicates the proliferative action of TGFα to gastric precursor cells in the TG mucosa, it does not seem appropriate to categorize the action of TGFα as either proliferative or non-proliferative for gastric protection. More appropriate is to evaluate the diverse effects of TGFα using a long-term TGFα-expressing model. Using such a TGFα-transgene-expressing TG mouse model whose gastric mucosa showed a strong resistance to ethanol injury (Figure 2), we found that the integrative mechanisms of TGFα involve at least three categories of cytoprotective regulators: NO synthases in relation with gastric blood flow, COX-2, and apoptosis-associated regulators such as Bcl-2 and Bax.

Gastric blood flow is known to contribute substantially to mucosal protection against gastric injury [33]. In the present study, a laser Doppler flowmeter analysis revealed a significant maintenance of blood flow when acidified ethanol was applied onto the mucosal surface of the TG mouse (Figure 3). Since vascular dilation occurs even in the high dose capsaicin-denervated gastric mucosa where increased levels of NO contribute greatly [13], we confirmed the expression of NOS in the mucosa. nNOS was expressed at the luminal pit region as previously reported [35]. Since the pit region is elongated to half of the gastric gland in length in the TG mucosa, the nNOS-expressing region was much wider than that in the WT mucosa. Furthermore, iNOS was constitutively expressed along the lower glandular region in the TG mucosa, in contrast to the negligible expression of iNOS unless ethanol was applied to the WT mucosa (Figure 4). nNOS has been reported to be induced by EGF in guinea pig

gastric mucosal primary-cultured cells [44], and in human HaCaT keratinocytes [45]. In contrast, iNOS, a key regulator of oxidant-induced epithelial barrier disruption, has been reportedly downregulated through an EGF-R downstream ζ-isoform of protein kinase C for gastric protection using colorectal carcinoma-derived Caco-2 cells [46]. We postulate that the long-term effect of TGFα on iNOS induction may be different from its short-term effect seen in culture cells.

Previously, prostaglandins have been shown to increase in parallel with the expression of COX-2 protein after ulcer formation in rats [32]. However, intraperitoneal administration of TGFα did not induce prostaglandin E_2 release into the gastric juice nor did it increase gastric tissue levels of prostaglandin E_2 [7]. The protective effect of TGFα to ethanol-induced mucosal injury was not interrupted by indomethacin-induced depletion of prostaglandin E_2 in the rat gastric mucosa [42]. Although the roles of prostaglandins in gastric protection are controvertial, we found that COX-2 and its upstream transcription factor NFκB are markedly expressed in the TGFα-expressing TG mucosa (Figure 5a and d). Our findings suggest that COX-2 is induced by NFκB under the control of TGFα in gastric mucosal cells. Consistently, prostaglandin E_2 levels were muchi more elevated in the TG mucosa than in the WT mucosa (Figure 5c).

In response to environmental or physiological stress, such as exposure to heat, ethanol, heavy metals, and water-immersion stress, heat shock proteins such as HSP70 are induced in the gastric mucosa [39,47]. HSPs are known to play a role in refolding of denatured proteins and facilitate the recovery of damaged cellular functions. HSP70 is also thought to interrupt stress-induced apoptosis pathways at several steps, including a cytochrome C releasing step from mitochondria, and a pro-caspase activation step by directly interacting with Apaf-1 [48]. In the present study, HSP70 was already elevated in the TG mucosa before ethanol injury, in contrast to the almost completely lack of HSP70 expression without injury in the WT mucosa (Figure 6). Together with HSP70, anti-apoptotic Bcl-2 remained similarly elevated before and after the ethanol injury, but pro-apoptotic Bax was down-regulated in the TG mucosa after the injury (Figure 7). Bcl-2 is an anti-apoptotic protein which resides on the mitochondrial outer membrane and inhibits channel formation of Bax, a pro-apoptotic protein of the same Bcl-2 family, through which cytochrome C leaks to the cytoplasm [49,50]. Thus, constitutive

expression of Bcl-2 by TGFα may overcome unfavorable ethanol-exposure stress at least by suppressing Bax activation. Once the Bcl-2 function is suppressed, cytochrome C and other apoptogenic factors are released through the Bax channel, and cytochrome C associates with Apaf-1 to promote the activation of the initiator caspase, pro-caspase 9, whose active form subsequently activates the effecter caspase, pro-caspase 3. Caspase 3 cleaves the inhibitor of caspase-activated DNase (ICAD) to release CAD, which then causes DNA fragmentation [50,51]. In the present study, active 17 kDa caspase 3 was not formed in the TG mucosa, whereas it was markedly formed in the WT mucosa after the injury. Thus, we propose that the inhibitory effect of Bcl-2 on the release of cytochrome C, and the HSP70 block of cytochrome C-initiated apoptosome formation to produce activated caspase 9, resulted in the suppression of caspase 3 formation from its precursor [50,52].

The strong resistance of TGFα-expressing gastric mucosa to ethanol injury appears to involve at least three cytoprotective mechanisms: a well-maintained gastric blood flow partly due to NO synthase expression by TGFα; constitutive expression of COX-2, which produces prostaglandins that may recruit GSM precursor cells to the injured pit region [53]; and upregulation of anti-apoptotic proteins such as HSP70 and Bcl-2, downregulation of pro-apoptotic Bax, and inhibition of pro-caspase 3 activation. These distinct cytoprotection mechanisms appear to connect in an integrated manner.

Conclusions

We propose that non-pathological levels of long-term TGFα expression may enhance the protective capacity of the gastric mucosa against injury and may help maintain the integration of mucosal functions by activating other essential cytoprotective factors such as mucosal blood flow, COX-2, and anti-apoptotic regulators such as HSP70 and Bcl-2 through these interconnecting pathways.

List of abbreviations

TGFα, transforming growth factor α; TG, transgenic; WT, wild-type; EGF-R, epidermal growth factor receptor; COX, cyclooxygenase: NOS, nitric oxide synthase; HSP, heat shock protein; GSM, gastric surface mucosa; HRP, horseradish peroxydase

Competing interests

The authors declare that they have no competing interests.

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Authors contributions

TK performed immunohistochemistry, Western blotting, Northern blotting, maintained transgenic mice, and performed genotype analysis with some help from SK, NS, NH and KS. MY performed gastric blood flow measurements. HT, TT and MM supervised the first author TK. All authors read and approved the final manuscript.

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Figure legends

Figure 1

Characterization of the TGFα-expressing TG mucosa. Scale in each figure indicates 100 μm. (a) Distribution of TGFα-expressing cells. Brown-colored TGFα-positive cells were visible along the luminal pit region in the WT mucosa, the elongated foveolar pit region, and the lower one-third of the glandular region in the TG mucosa. (b) Overlap of red-colored TGFα-positive cells and green-colored H^+/K^+-ATPase-positive parietal cells. Lower TGFα-positive cells in cluster are distinct from the green-colored parietal cells in the TG mucosa. (c) Northern blot of human TGFα mRNA and PCR-amplified mouse TGFα mRNA. Human TGFα mRNA is expressed in the TG mucosa alone (left panel), but PCR-amplified mouse TGFα mRNA is expressed similarly in both WT and TG mucosae (right panel). (d) Western blot of TGFα intermediate forms. TGFα precursor (20 kDa) and its intermediate forms are visualized more intensively in the TG mucosa than in the WT mucosa. (e) Localization of EGF receptor in the gastric mucosa. EGF receptor (EGF-R) was stained with Cy3 (red) and H^+/K^+-ATPase was stained with FITC (green). EGF-R was immunostained moderately in the upper pit region and strongly in the lower glandular region similarly in both the WT and TG mucosae. (f) Distribution of metallothionein in the gastric mucosa. Metallothionein is immunostained strongly in the lower glandular region, and moderately along the foveolar region. (g) Elongation of the gastric pit of the TG mucosa. PCNA-positive cells are distributed at the upper-third region in the WT gastric glands, whereas they are distributed more numerously and broadly in the middle glandular region. (h) PAS staining shows elongated pit in the TG mucosa.

Figure 2

Ethanol-induced gastric injury in the gastric mucosae. (a) Macroscopic view of the gastric mucosa. Scale: 5 mm. Both mucosae are 6 h after ethanol administration. Note that the WT mucosa displays extensive hemorrhagic lesions, in contrast, the TG mucosa looks undamaged after ethanol administration. (b) Hematoxylin and eosin staining of ethanol-injured WT mucosa (left) and TG mucosa (right). Note the deep ulcer formation in the WT mucosa. Scale: 100 μm. (c) Time course of ethanol-induced gastric injury. Injured area is

plotted against time up to 12 h after the ethanol administration. Each point represents an average of five mice. $p < 0.05$ (d) TUNEL staining of WT mucosa (left) and TG mucosa (right). Both sections are consecutive to those in 2*B*, respectively. Apoptotic cells are visible with dark-brown nuclei. Numerous apoptotic cells are noted in the WT mucosa. All photos were 6 h after the ethanol administration. Scale: 100 μm.

Figure 3

Assessment of gastric mucosal blood flow in the gastric mucosae. Gastric mucosal blood flow was assessed by comparing the blood flow ratio before and after the ethanol treatment. The ratio was obtained as a percent against the blood flow before the treatment, which was much larger in the WT mucosa than in the TG mucosa. $*p < 0.03$

Figure 4

Immunostaining of NO synthases in the gastric mucosa. Scale: 100 μm. (a) nNOS immunostaining. nNOS was visible along the pit region, resulting in its wider distribution along the elongated pit in the TG mucosa. (b) iNOS immunostaining. iNOS was induced in the lower glandular region after ethanol injury in the WT mucosa, whereas it was constitutively expressed in the same region of the TG mucosa irrespective of ethanol injury.

Figure 5

COX-2 and NFκB expression in the gastric mucosa. (a) Immunostaining of COX-2. COX-2 was intensively induced after the injury at the lower glandular region of the WT mucosa, whereas it was constitutively expressed in the same region of the TG mucosa even before the injury. Ethanol-injured mucosa was stained 6 h after the ethanol administration. Scale: 100 μm. (b) Immunoblotting of COX-2 and COX-1. The blots were measured 0, 3, and 6 h after the injury. Actin filaments were used as a control. Note that COX-2 was intensively expressed before the injury in the TG mucosa. (c) Measurement of prostaglandin E_2. Prostaglandin E_2 levels were measured before the ethanol treatment in the WT and TG mucosae. $n = 5$, $p = 0.013$. (d) Immunostaining of NFκB. NFκB was intensively induced after the injury at the same lower glandular region of the WT mucosa as was COX-2. In contrast,

NFκB was constitutively expressed in the same region of the TG mucosa even before the injury. Scale: 100 μm.

Figure 6

HSP70 expression in the gastric mucosa. (a) Immunostaining of HSP70. HSP70 was induced along the necrotic region and the lower glandular region of the WT mucosa after ethanol injury, whereas it was constitutively expressed in the lower glandular region of the TG mucosa before and after the injury. Ethanol-injured mucosa was stained 6 h after the ethanol administration. Scale: 100 μm. (b) Immunoblotting of HSP70. HSP70 blot was assessed 0, 3, 6, 12 h after the ethanol injury. HSP was induced after ethanol injury in the WT mucosa, while it was constitutively expressed in the TG mucosa before and after the injury.

Figure 7

Expression of apoptosis-associated proteins. (a) Immunoblot of Bax and Bcl-2. Bax was strongly induced at the 3 h and 6 h time points in the WT mucosa, whereas it was reduced to a negligible level in the TG mucosa after ethanol injury. Bcl-2 was stably expressed similarly before and after the injury in the TG mucosa, whereas it was increased to some extent at 6 h after the injury in the WT mucosa. (b) Immunostaining of Bcl-2. Bcl-2 was not visible in the TG mucosa before the injury, but appeared along the border region of necrotic area after the injury. In contrast, Bcl-2 was constitutively induced over the TG mucosa even before the injury, and the staining was confined to the lower glandular region after the injury. Scale: 100 μm. (c) Immunoblot of caspase 3. After the injury, a 17 kDa active form of caspase 3 appeared in the WT mucosa, whereas it did not in the TG mucosa. A 32 kDa proform was unchanged both in the WT and TG mucosae.

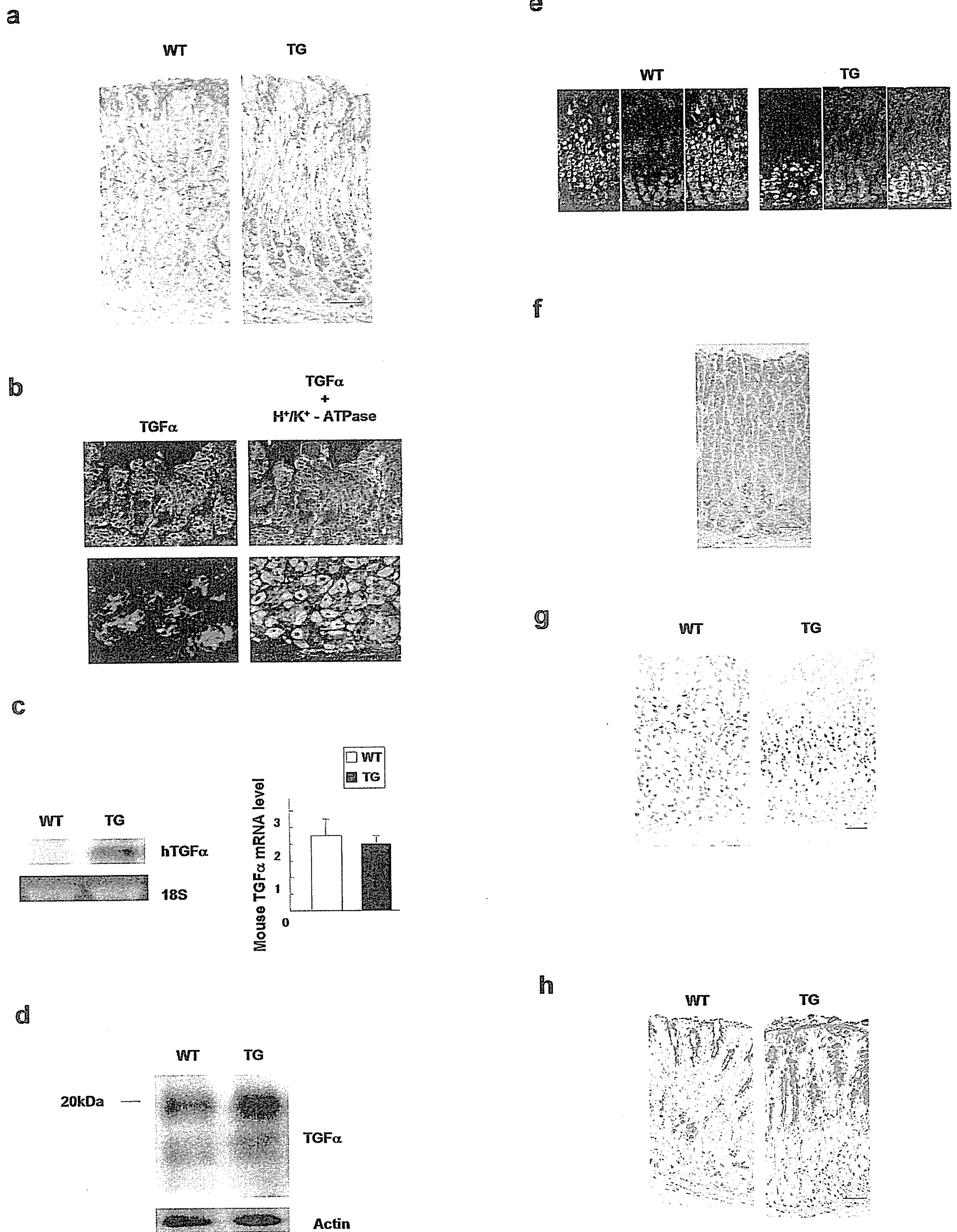
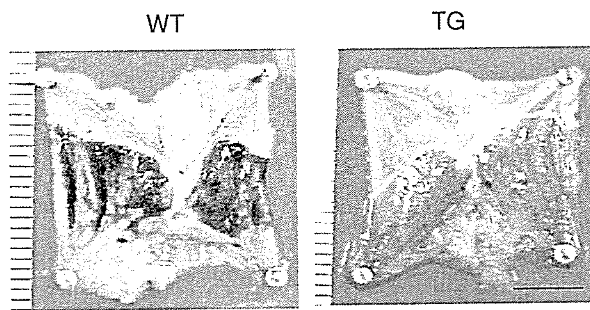


Figure 1

a



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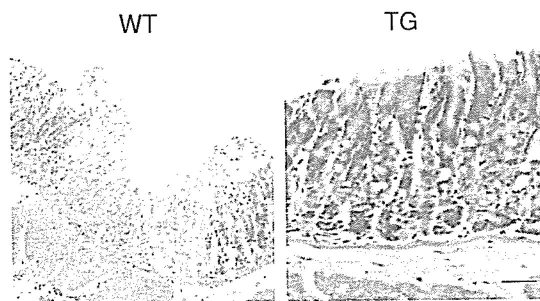

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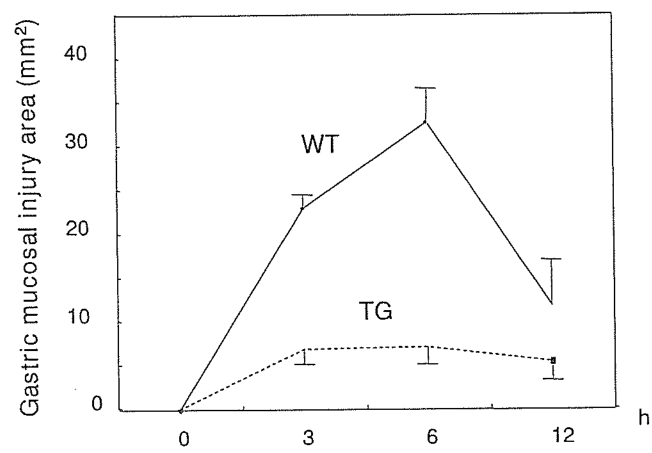

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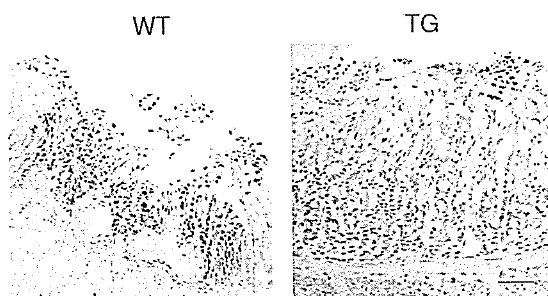

Figure 2