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ORIGINAL ARTICLE

Transient TWEAK overexpression leads to a general salivary epithelial cell proliferation

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OBJECTIVES: Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a multifunctional cytokine that has pro-apoptotic, pro-angiogenic and pro-inflammatory effects. In liver, TWEAK leads to proliferation of progenitor oval cells, but not of mature hepatocytes. This study evaluated the hypothesis that TWEAK overexpression in salivary glands would lead to the proliferation of a salivary progenitor cell.

METHODS: A recombinant, serotype 5 adenoviral vector encoding human TWEAK, AdhTWEAK, was constructed, initially tested *in vitro*, and then administered to male Balb/c mice via cannulation of Wharton's duct. TWEAK expression *in vivo* was monitored as protein secreted into saliva and serum by enzyme-linked immunosorbent assays. Salivary cell proliferation was monitored by proliferating cell nuclear antigen staining and apoptosis was monitored using TUNEL staining.

RESULTS: AdhTWEAK administration led to a dose-dependent, transient TWEAK protein expression, detected primarily in saliva. Salivary epithelial cell proliferation was generalized, peaking on ~days 2 and 3. TWEAK expression had no detectable effect on apoptosis of salivary epithelial cells.

CONCLUSION: Transient overexpression of TWEAK in murine salivary glands leads to a general proliferation of epithelial cells *vs* a selective stimulation of a salivary progenitor cell.

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Keywords: TWEAK; gene transfer; adenoviral vector; cell division; progenitor cell

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Introduction

Salivary gland epithelial cells are considered to be well-differentiated, slowly dividing, generally postmitotic cells (Denny *et al*, 1993; Redman, 1995). Acinar cells are thought to be the sole site of fluid movement in these glands, as well as the cells responsible for most exocrine protein secretion (Baum, 1993; Turner and Sugiya, 2002). Therefore, when a significant loss of acinar cells occurs in an adult salivary gland, for example following radiation for head and neck cancers and with Sjögren's syndrome, patients experience considerable morbidity because of the resulting loss of saliva production (Fox *et al*, 1985; Vivino *et al*, 1999; Nagler, 2002). As a result of this circumstance, there has been a considerable effort to develop novel therapies to regenerate or repair damaged salivary glands (e.g. Baum *et al*, 1999; Kagami *et al*, 2008). One strategy to correct such gland dysfunction involves use of salivary progenitor cells to re-populate the gland (Kagami *et al*, 2008). Although salivary glands, like other parenchymal tissues, are considered to contain progenitor cells (e.g. Zajicek *et al*, 1989; Denny *et al*, 1997; Okumura *et al*, 2003; David *et al*, 2008), the exact source of these cells is not understood.

Chicheportiche *et al* (1997) first reported the discovery of tumor necrosis factor-like weak inducer of apoptosis (TWEAK), which is a member of the tumor necrosis factor-α family. The TWEAK gene encodes a protein of 249 amino acids, which binds specifically to its receptor, fibroblast growth factor-inducible 14 kDa protein (Fn14; Wiley and Winkles, 2003; Vince and Silke, 2006). Since 1997, many studies have examined TWEAK's role in the biology or pathology of numerous tissues, including liver, mammary gland, muscle, the vasculature, synovial joints, and gingiva (e.g. Jakubowski *et al*, 2002, 2005; Michaelson *et al*, 2005; Girgenrath *et al*, 2006; Hosokawa *et al*, 2006; Perper *et al*, 2006). Indeed, TWEAK is now considered a multifunctional cytokine that can have pro-apoptotic, pro-angiogenic and pro-inflammatory effects. TWEAK's multiple, often contradictory, effects appear to be particularly important during inflammation and

oncogenesis (Vince and Silke, 2006; Burkly *et al*, 2007). As a result of the latter roles, TWEAK has become a potential therapeutic target in rheumatoid arthritis and epithelial cancers (e.g. Michaelson *et al*, 2005; Perper *et al*, 2006; Watts *et al*, 2007).

Of particular interest to us were the studies by Jakubowski *et al* (2005), which clearly showed that in mouse liver, TWEAK leads to the proliferation of unique progenitor cells, termed oval cells, rather than to a general proliferation of mature hepatocytes such as is seen following a partial (70%) hepatectomy (see also Fausto, 2005). Many investigators have examined mammalian salivary glands for the presence of progenitor cells (e.g. Zajicek *et al*, 1989; Schwartz-Arad *et al*, 1991; Denny *et al*, 1993, 1997; Redman, 1995). However, a specific cell type capable of replenishing gland parenchymal cells has not been unequivocally identified, although recently it has been suggested that salivary gland epithelial cells expressing the $\alpha 6\beta 1$ integrin may have stem cell capabilities (Okumura *et al*, 2003; David *et al*, 2008). As salivary glands are known to express TWEAK receptor Fn14 (Wiley and Winkles, 2003), we hypothesized that TWEAK overexpression could lead to the proliferation of a unique cell population in this tissue, i.e. indicating a potential progenitor cell type.

Materials and methods

Construction and purification of AdhTWEAK

A plasmid containing the human (h) TWEAK cDNA (pORF5hTWEAK v.21) was purchased from Invivogen (San Diego, CA, USA). This plasmid was digested with *Nco*I and *Nhe*I to obtain the hTWEAK cDNA, which was then inserted at the *Sal*I and *Bam*HI sites of the shuttle plasmid pAC-CMV-pLpA to obtain pAChTWEAK. A first-generation, $E1^-$, serotype 5 recombinant adenovirus (Ad5) encoding hTWEAK was constructed as previously reported (Delporte *et al*, 1996) by cotransfection with pJM17 into 293 cells to obtain the recombinant Ad5 vector AdhTWEAK. This vector employed the cytomegalovirus promoter/enhancer and a SV40 polyadenylation signal. AdhTWEAK was purified from lysates of transduced 293 cells by two rounds of CsCl gradient centrifugation, as described (Baum *et al*, 2002). Purified vectors were dialyzed against 4 l of dialysis buffer containing 10% glycerol, 0.1 M Tris (pH 7.4), 5 mM $MgCl_2$, for 4 h at 4°C and stored in aliquots at −80°C for later use. The vector titer (3.8×10^{11} particles ml^{-1}) was determined by quantitative PCR using an ABI Prism 7700 (Applied Biosystems, Foster City, CA, USA) with primers from the E2 region of adenovirus, E2q1 (5′-GCAGAACCACCAGCACAGTGT-3′) and E2q2 (5′-TCCACGCATTTCCTTCTAAGCTA-3′).

In vitro *demonstration of hTWEAK expression*

Expression of hTWEAK initially was evaluated in 293 cells, which were grown in improved minimal essential medium (Eagle's) supplemented with 10% bovine serum, 100 U ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin (all from Biosource, Camarillo, CA, USA) at 37°C in a humidified, 5% CO_2 atmosphere, incubator. AdhTWEAK was used to transduce 293 cells at a multiplicity of infection (MOI) of either 10 or 100 particles per cell. After 24 h, the medium was collected, centrifuged at 2568 ***g*** for 3 min and the resulting supernatants used for an hTWEAK enzyme-linked immunosorbent assay (ELISA; Bender MedSystems, Burlingame, CA, USA). To examine the molecular mass of the transgenic protein, A5 cells, a rat submandibular epithelial cell line grown as described previously (Brown *et al*, 1989), were transduced, in serum-free media, with either AdhTWEAK, or a similar vector expressing human leptin, at a MOI of 200. After 48 h, the secreted proteins in the media were collected and precipitated in 80% ethanol essentially as previously described (He *et al*, 1998). Eight micrograms of ethanol-precipitated protein were electrophoresed with 12.5% precast gels (Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose membranes (Bio-Rad). The membranes were treated as described previously (Wang *et al*, 2005), except that blotting grade blocker (Bio-Rad) and donkey anti-goat horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000; Amersham Biosciences, Arlington Heights, IL, USA) were used. Membranes were incubated with anti-human TWEAK/TNFSF12 (1:200; R&D Systems, Minneapolis, MN, USA). We used the Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) to detect bound antibodies, followed by 10-min exposure to X-ray film and development.

Vector administration to male Balb/c mice

The National Institute of Dental and Craniofacial Research Animal Care and Use Committee and the National Institutes of Health Biosafety Committee approved all animal experiments. Balb/c mice were anesthetized with a mixture of 100 mg ml^{-1} ketamine (Fort Dodge Animal Health, Fort Dodge, IA, USA) and 20 mg ml^{-1} xylazine (Fort Dodge Animal Health) given intramuscularly (1 μl g^{-1} of body weight). For each animal, both Wharton's ducts of the submandibular gland were cannulated with modified polyethylene tubing (Intramedic PE-10; BD Diagnostic Systems, Sparks, MD, USA), and atropine (intramuscular injection, 0.5 mg kg^{-1} body weight; Sigma, St. Louis, MO, USA) was administered to decrease salivary flow. After 10 min, different doses of AdhTWEAK (10^8–5×10^9 vector particles per gland) were administered by retrograde ductal delivery into the cannulated glands in a 50-μl volume. Typically, saline was administered to control mice, although for some experiments an irrelevant control Ad5 vector encoding human erythropoietin, AdhEpo, was administered (see Results and discussion). At various times thereafter (see Results and discussion), mice were again anesthetized and given a subcutaneous injection of pilocarpine (0.5 mg ml^{-1}, 1 μl g^{-1} body weight; Sigma) to stimulate salivary flow. Whole saliva was collected from the oral cavity with a microhematocrit capillary tube (Fisher Scientific, Hampton, NH, USA). Blood samples were obtained from mice by retro-orbital plexus bleeding.

Thereafter, saliva and serum were stored at −80°C until assayed for hTWEAK by the above ELISA. For dose–response experiments, there were four mice per group, while for time-course studies there were 8–12 mice per group.

Detection of salivary cell proliferation and apoptosis

We used proliferating cell nuclear antigen (PCNA) staining to monitor salivary cell proliferation and terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) to monitor apoptosis. Mouse glands (four mice per experimental group, i.e. eight glands) were fixed in 10% neutral buffered formalin and embedded in paraffin. Five micrometer sections were cut for both histological evaluations. After deparaffinizing and rehydration, the slides were blocked for endogenous peroxidase activity with 3% H_2O_2 in methanol for 10 min. PCNA staining was performed with the ZYMED PCNA staining kit (Invitrogen Corporation, Carlsbad, CA, USA) on sections from control glands (saline- and AdhEpo-treated), as well as AdTWEAK-treated glands. Before antibody labeling, the slides were treated three times with a citrate buffer solution (9 ml of 0.1 M citric acid and 41 ml of 0.1 M sodium citrate, plus 450 ml of distilled water) in a 600 W microwave for 5 min. Thereafter, slides were processed with routine indirect immunoperoxidase techniques. Three examiners independently counted the absolute number of PCNA positive ductal, acinar and granular convoluted duct (GCD) cells in a blinded manner in three randomly chosen fields/section. All other gland cell types present (i.e. myoepithelial cells, excretory duct, interstitial) were considered together and exhibited no treatment-related changes (data not shown). For each treatment group, the mean of all PCNA positive cells was then calculated. The TUNEL staining was performed with the ApopTag Plus peroxidase *in situ* apoptosis detection kit (Chemicon International Inc., Temecula, CA, USA), on sections from control (saline treated) and AdTWEAK-treated glands, according to the manufacturer's instructions, as previously described (Lodde *et al*, 2006). An oral pathologist, blinded as to the treatment group, counted the number of TUNEL-positive apoptotic cells per section.

Results and discussion

Initially, we tested AdhTWEAK expression in 293 cells *in vitro*. At 24 h after transduction, cells exposed to the vector, at either a MOI of 10 or 100 particles per cell, secreted high levels of hTWEAK in the media (22 785 ± 2167 and 63 195 ± 8470 pg ml^{-1}; mean ± s.e.m.), respectively. When cell culture media from transduced A5 cells were electrophoresed and subjected to Western blot analyses, a single immunoreactive band of ~23 kDa was observed (Figure 1). Conversely, no immunoreactive protein bands were found in lysates prepared from A5 cells transduced with a control adenoviral vector, AdhLeptin, encoding human leptin, or from non-transduced cells.

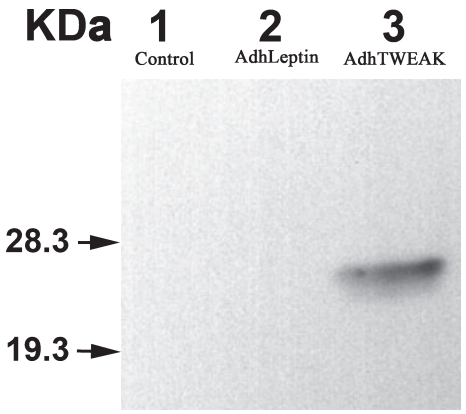

Figure 1 Western blot showing secreted hTWEAK from A5 cells *in vitro*. A5 cells were non-transduced (lane 1), or transduced by an irrelevant vector (AdhLeptin; lane 2) or AdhTWEAK (lane 3) at a multiplicity of infection of 200. Immunoreactive hTWEAK protein is shown in lane 3. The arrows to the left indicate the molecular mass (in kDa) of protein standards. See Materials and methods for additional details

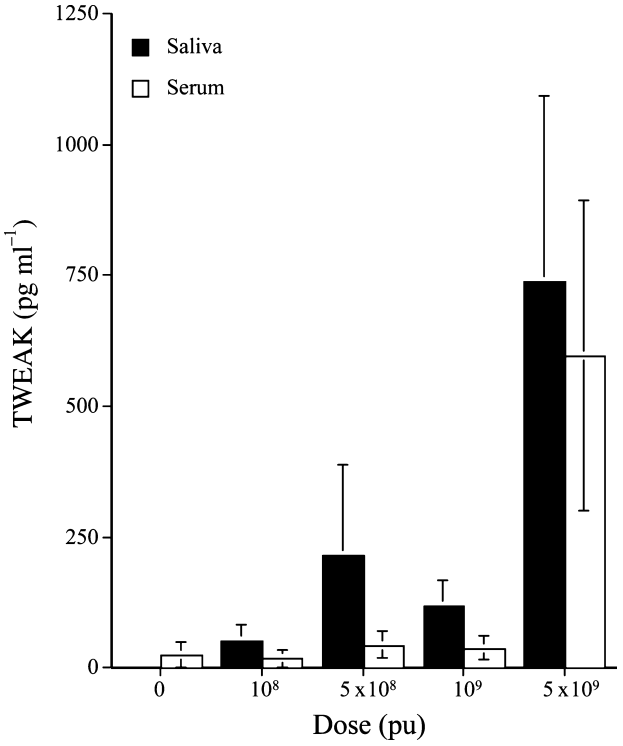

Figure 2 Effect of the AdhTWEAK dose administered on the detection of hTWEAK in murine saliva and serum. Mice were administered AdhTWEAK to their submandibular glands at the indicated doses and hTWEAK was measured by enzyme-linked immunosorbent assays after 72 h in both saliva and serum. Data shown are the mean ± s.e.m. of results from four mice. See Materials and methods for additional details

We next examined hTWEAK expression *in vivo* after AdhTWEAK delivery to the submandibular glands of Balb/c mice. AdhTWEAK was administered to glands at different doses (from 10^8 to 5×10^9 particles per gland), and hTWEAK assayed in saliva and serum

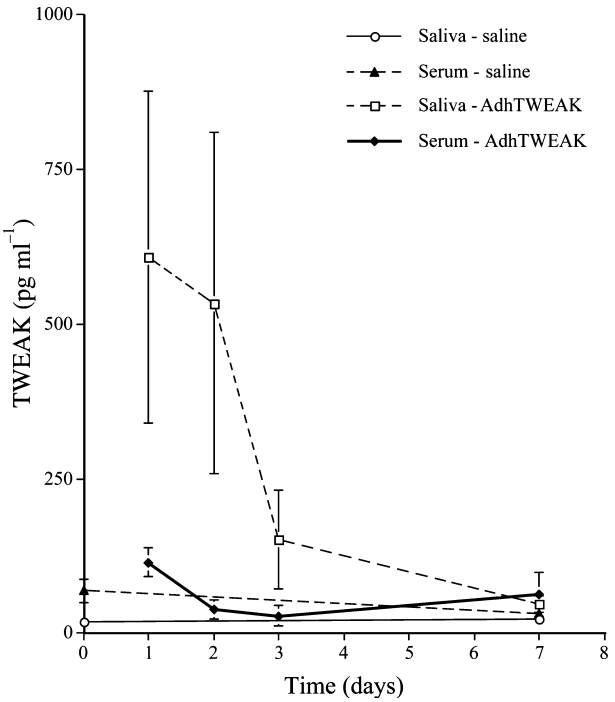

Figure 3 Time course of hTWEAK detection in saliva and serum after AdhTWEAK administration to murine submandibular glands. AdhTWEAK (10^9 particles per gland), or saline, was delivered to both submandibular glands and hTWEAK was measured by enzyme-linked immunosorbent assays in both saliva and serum at the indicated times. Data shown are the mean ± s.e.m. of results from 12 mice (saliva) and eight mice (serum). See Materials and methods for additional details

samples after 72 h. As shown in Figure 2, the detection of hTWEAK was vector dose-dependent, and the expressed hTWEAK was primarily found in saliva except at the highest dose administered. Based on these results, we decided to evaluate the time course of hTWEAK expression at a dose of 10^9 particles per gland. This dose was chosen because the distribution of secreted hTWEAK in serum and saliva appeared altered at the 5×10^9 particles per gland dose (Figure 2), which could be a result of (i) transgene overexpression and saturation of the exocrine secretory pathway by the newly synthesized hTWEAK (Marmorstein *et al*, 2000) and/or (ii) the likelihood of increased glandular inflammation at the higher dose (Adesanya *et al*, 1996).

As shown in Figure 3, high levels of hTWEAK were seen in saliva 1–2 days following AdhTWEAK administration. Thereafter, transgene expression decreased markedly; i.e. to ~25% peak levels by day 3 and near background levels by day 7 (Figure 3; saline-treated mice; ~20 pg ml^{-1} saliva). This pattern of transgene expression is commonly observed following Ad5 vector administration to mouse salivary glands (Voutetakis *et al*, 2005). In general, little hTWEAK was found in serum from these animals, with levels greater than background only seen on day 1.

Next, we examined the salivary glands of the AdhTWEAK-treated mice for evidence of biological activity, assaying both cellular proliferation by PCNA staining and apoptosis by TUNEL staining. As shown in Figure 4a, in saline-treated mice on day 0, only low levels of PCNA staining were evident, consistent with established evidence that salivary gland epithelial cells normally divide very slowly (e.g. Denny *et al*, 1997). However, as early as 1-day post-AdhTWEAK delivery higher levels of PCNA staining were seen in all transduced glands (Figure 4b). PCNA staining increased further on days 2 and 3 (~14- and 30-fold; Figures 4c,d and 5), with especially higher changes seen in acinar cells (Figure 5). By day 7, the total number of proliferating cells was markedly decreased, and not significantly different from the saline control (Figure 5). Administration of an irrelevant Ad5 vector, AdhEpo

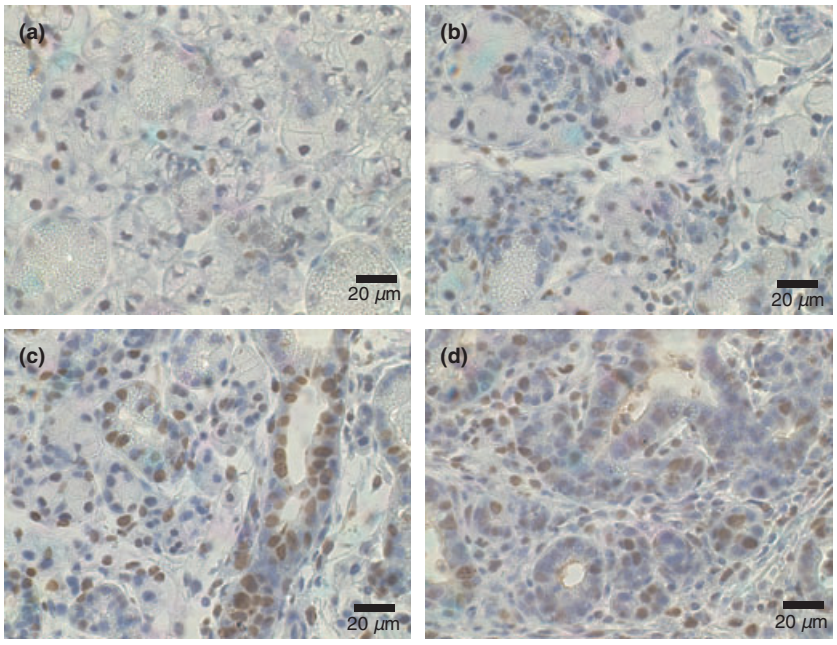

Figure 4 Detection of proliferating cell nuclear antigen (PCNA) staining in submandibular glands of mice following AdhTWEAK administration. AdhTWEAK (10^9 particles per gland), or saline, was delivered to both submandibular glands ($n = 4$ mice per group) and PCNA staining performed on gland sections as described in Materials and methods to evaluate cell proliferation. Brown staining represents PCNA-positive nuclei. Sections are counterstained with hematoxylin. **(a)** Day 0 after saline administration; **(b)** Day 1 after AdhTWEAK administration; **(c)** Day 2 after-AdhTWEAK administration; **(d)** Day 3 after AdhTWEAK administration. Magnification is ×200

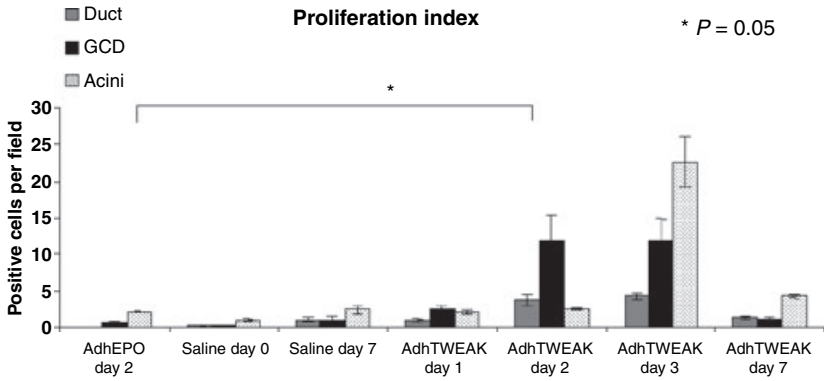

Figure 5 Quantification of AdTWEAK-induced salivary epithelial cell proliferation. Three examiners independently counted proliferating cell nuclear antigen (PCNA)-positive nuclei, such as shown in Figure 4, and determined the number of positive ductal, acinar and granular convoluted duct cells, as described in Materials and methods. AdhTWEAK (10^9 particles per gland), saline or AdhEpo (an irrelevant Ad5 vector encoding human erythropoietin; Voutetakis *et al*, 2005; 10^9 particles per gland) was delivered to both submandibular glands. For each animal ($n = 4$ per group), three different fields per section were evaluated. Data shown are the mean ± s.e.m. of the indicated PCNA positive cell types per field for each treatment group. The level of PCNA staining in AdTWEAK-treated glands on day 2 was significantly different from that seen in AdhEpo-treated glands

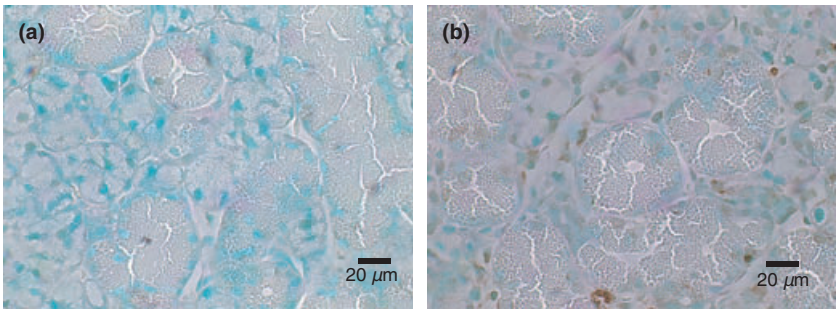

Figure 6 Detection of TUNEL-positive staining in submandibular glands of mice following AdhTWEAK administration. AdhTWEAK (10^9 particles per gland), or saline, was delivered to both submandibular glands ($n = 4$ mice per group) and TUNEL staining performed on gland sections as described in Materials and methods to evaluate apoptosis. Brown staining represents TUNEL-positive (apoptotic) nuclei. Sections are counterstained with methyl green. **(a)** Day 0 after saline administration; **(b)** Day 2 after AdhTWEAK administration. Magnification is ×200

(encoding human erythropoietin; Voutetakis *et al*, 2005) had no effect on PCNA staining in salivary glands (Figure 5).

There was little TUNEL-positive staining seen in salivary epithelial cells on day 0 in the saline-treated glands (Figure 6a), indicating few apoptotic parenchymal cells are normally present. Similarly, at 1 (not shown) and 2 days (Figure 6b) following AdhTWEAK administration, there also were very few TUNEL-positive salivary epithelial cells seen. There was no statistically significant difference in the number of apoptotic ductal, acinar or GCD cells observed between control glands and AdTWEAK-treated samples for any of the time points observed (days 1, 2, 3 and 7). However, in the gland interstitium several TUNEL-positive, non-parenchymal, mononuclear cells were seen after AdTWEAK administration (Figure 6b). This was not surprising given the immune response typically elicited by Ad5 vectors (Adesanya *et al*, 1996).

In conclusion, in the present study we hypothesized that hTWEAK overexpression could lead to the proliferation of a unique progenitor cell type in murine submandibular glands. We constructed a recombinant Ad5 vector to test this hypothesis in mice, the same species in which hTWEAK overexpression has been shown to induce oval cell proliferation in the liver (Jakubowski *et al*, 2005). AdhTWEAK led to considerable hTWEAK expression in murine submandibular glands, with the expressed hTWEAK being primarily secreted into saliva, i.e. via an exocrine route. Impressively, the overexpression of hTWEAK in these glands led to a rapid, but transitory, increase in the proliferation of most salivary epithelial cell types. There was no evidence for a specific increase in proliferation by a unique, potential salivary progenitor cell population, as was found in the liver (Jakubowski *et al*, 2005).

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Author contributions

Dr Takayuki Sugito conceived the idea, designed and performed experiments, helped to analyze data and participated in writing the paper. Dr Ana P. Cotrim performed experiments, helped to

analyze data and participated in writing the paper. Dr Fumi Mineshiba and Dr Changyu Zheng along with Ms Corinne M. Goldsmith performed experiments and helped to write the paper. Dr Bruce J. Baum designed experiments, helped to analyze data and participated in writing the paper.

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