

Article

Acid Dentin Lysates Increase Amelotin Expression in Oral Epithelial Cells and Gingival Fibroblasts

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Abstract: Amelotin (AMTN) is a secretory calcium-binding phosphoprotein controlling the adhesion of epithelial cells to the tooth surface, forming a protective seal against the oral cavity. It can be proposed that signals released upon dentinolysis increase AMTN expression in periodontal cells, thereby helping to preserve the protective seal. Support for this assumption comes from our RNA sequencing approach showing that gingival fibroblasts exposed to acid dentin lysates (ADL) greatly increased AMTN expression. In the present study, we confirm that acid dentin lysates significantly increase AMTN in gingival fibroblasts and extend this observation towards the epithelial cell lineage by use of the HSC2 oral squamous and TR146 buccal carcinoma cell lines. AMTN immunostaining revealed an intensive signal in the nucleus of HSC2 cells exposed to acid dentin lysates. Acid dentin lysates mediate their effect via the transforming growth factor (TGF)- β type 1 receptor kinase as the antagonist SB431542 abolished the expression of AMTN in the epithelial cells and fibroblasts. Similar to what is known for fibroblasts, acid dentin lysate increased Smad-3 phosphorylation in HSC2 cells. HSC2 cells also respond to the AMTN-stimulating activity of the dentin lysate when adsorbed to gelatin. When simulating regenerative approaches, enamel matrix derivative, TGF- β 1, and bone morphogenetic protein-2 also caused a robust increase in SB431542-dependent AMTN expression in HSC2. Taken together, we show here that acid dentin lysate uses the TGF- β -dependent signaling pathway to support the AMTN expression in epithelial cells, possibly helping in maintaining the protective seal against the oral cavity.

Keywords: amelotin; AMTN; epithelial cells; HSC2; Emdogain; TGF- β ; BMP-2; dentin; periodontal health



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1. Introduction

The periodontium is the tooth-supporting tissue that not only anchors the tooth roots within the alveolar bone, but also seals erupted teeth towards the oral cavity [1,2]. This sealing holds microorganisms and toxins away from the sensitive periodontal ligament, being the main trigger for inflammatory osteolysis, which culminates in tooth loss [3]. Attachment of the junctional epithelial cells to the enamel tooth crown made and the cementum layer covering the root dentin is of imperative importance for maintaining the integrity of the periodontium [3,4]. This seal arises from the later stages of tooth development when parts of the epithelial enamel organ fuse with the oral epithelium forming the junctional epithelium [5]. The specialized basal lamina serves as a glue between the junctional epithelium and the tooth surface [6]. The molecular signals causing the periodontal cells to produce the specialized basal lamina are now beginning to be discovered.

Amelotin (AMTN) is a secretory calcium-binding phosphoprotein that is critical for enamel mineralization during development and for maintaining the specialized basal lamina [6–9]. AMTN is, thus, an integral component of the junctional epithelium [7,10]. AMTN is increasingly expressed under simulated inflammation in vitro [11,12] and protects

the periodontium under inflammatory conditions in vivo [10]. In gingival epithelial cells, TGF- β increased AMTN expression through activation of Smad3 signaling [13–15]. In patients, AMTN is among the candidate genes involved in periodontitis [16] and AMTN was detected at the surfaces of the sulcular epithelium and the junctional epithelium in healthy and inflamed gingiva samples [17]. Even though accumulating evidence has provided insights into how AMTN is involved in the organization of the specialized basal lamina, little is known about the triggering factors that make epithelial cells and mesenchymal cells produce AMTN.

Among the triggering factors for AMTN might be the release from dentin, which can be caused by odontoclasts or as a consequence of dental erosion by acid exposure [18,19]. This scenario can be simulated by preparing acid lysates of dentin [20–22]. Proteomics, RNA sequencing, and bioassays involving SB431542, an inhibitor of TGF- β type 1 receptor kinase, have identified transforming growth factor- β (TGF- β) to be a major component of acid dentin lysates (ADL) [21]. This screening approach further revealed that AMTN is among the genes being expressed in gingival fibroblasts via TGF- β type 1 receptor kinase when exposed to acid bone [23] and dentin [21] lysates. In mice with epithelial cells lacking TGF- β 1, the expressions of AMTN is dramatically decreased [24], whereas in mice with constitutively active TGF- β -activating kinase 1, the expression of AMTN is increased [25]. Consistently, Smad3-deficient mice showed less AMTN in the junctional epithelium compared with wild-type mice [15]. Considering that acid dentin lysates contain TGF- β 1 [21], we propose here that ADL is also capable of driving the expression of AMTN in the epithelium cell lineage. Therefore, the aim of this study is to determine whether ADL is able to upregulate the expression of AMTN in epithelial cells and investigate if this requires TGF- β receptor type 1 kinase signaling.

We show here that ADL pushes HSC2 oral squamous carcinoma cells to increase AMTN via TGF- β type 1 receptor kinase, together with Smad-3 phosphorylation. We also show that enamel matrix derivative (EMD), as a clinically applied material [26] with strong TGF- β activity [27], increases AMTN in HSC2 cells. Likewise, recombinant bone morphogenetic protein 2 (BMP-2), which is used for oral bone augmentation [28], causes a robust SB431542-dependent increase of AMTN expression in HSC2. Considering that autogenous teeth are increasingly used for the reconstruction of alveolar ridge deficiencies [29], our observations are of possible clinical relevance, as the treatment not only supports bone formation but may also support the regain of soft tissue seals.

2. Methods

2.1. Acid Dentin Lysate Preparation

As describe previously [20–22] teeth were extracted from adult pigs less than 6 hours following sacrifice (Fleischerei Leopold Hödl, Vienna, Austria). Pigs were not sacrificed for the purpose of our experiments. Periodontal ligaments and soft tissue attachment were removed from the teeth with a surgical blade (Swann-Morton, Sheffield, United Kingdom). Furthermore, enamel was removed using a manual grinding and polishing device (Metaserv 2000, Cleveland, OH, USA). Subsequently, the pulp chamber was cleaned with a dental probe (Instrapac, Worksop, UK) and the teeth were crushed using a hammer. One gram of wet dentin was incubated while being stirred overnight at room temperature with 10 mL of 0.1 N HCl (10% weight/volume). The resulting acid dentin lysate (ADL) was centrifuged and the pH was neutralized using NaOH. Following sterile filtration (0.2 μ m pore size; Starlab, Blakelands, UK), ADL aliquots were kept frozen at -20°C . The stocks were thawed immediately before each experiment. To prepare crown- and root-derived ADL, crowns were separated from the root using a cutting and grinding device (Exakt Apparatebau, Norderstedt, Germany).

2.2. Cell Culture

All experiments were performed in accordance with the relevant guidelines and regulations for human and animal research. Human gingiva samples were taken from

extracted wisdom teeth of patients who had signed an informed consent form. The harvesting procedure was approved by the Ethics Committee of the Medical University of Vienna (EK NR 631/2007). Experiments were performed using three different strains of fibroblasts derived from the explants, passaged less than 10 times. The oral squamous cell carcinoma cell line HSC2, originally obtained from Health Science Research Resources Bank (Sennan, Japan), was kindly provided by Prof. Rausch-Fan, Department of Periodontology, Medical University of Vienna, Austria. The oral squamous cell carcinoma cell line TR146 from the European Collection of Authenticated Cell Cultures (ECACC) was provided in cooperation with Wienfried Neuhaus from the Competence Unit Molecular Diagnostics, Center of Health and Bioresources, Austrian Institute of Technology GmbH. HSC2, TR146, and gingival fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Invitrogen) and antibiotics (Invitrogen) at 37 °C, 5% CO₂, and 95% humidity. ADL was used at 5% for all experiments. The inhibitors for the TGF- β receptor I kinase 10 μ M SB431542 (Billerica, MA, USA), 300 ng/mL BMP-2 and 30 ng/mL TGF- β 1 (both ProSpec-Tany TechnoGene Ltd., Rehovot, Israel), 100 μ g/mL Emdogain (Enamel matrix derivative, EMD; Straumann, Basel, Switzerland), and 100 ng/mL PDGF-BB (R&D Systems, Inc., Minneapolis, MN, USA) were used at the indicated concentrations.

2.3. RT-PCR

Total RNA was extracted (ExtractMe, Blirt S.A., Gdańsk, Poland) and exposed to reverse transcription (LabQ cDNA synthesis kit; LabConsulting, Vösendorf, Austria). RT-PCR was done according to the manufacturer's instructions (LabQ SYBR green master mix) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Primer sequences were h18s-F: ccg att gga tgg ttt agt gag, h18s-R: agt tgc acc gtc ttc tca gc; hAMTN-F: gat caa ctc ggt cat tac cac a, hAMTN-R: tgg ttt ggt agt gtt ccc tga, h AMG-F: gaa atg ggg acc tgg att tt, hAMG-R: ccc agg atg agg tgg tag ag, hAMBN-F: gag ttt tgc agt gcc gtt c, h AMBN-R: gcagacttcccaactgtctca, hENAM-F: aca acc acc atg gca aat tc, hENAM-R: att ggt ggg cgt cca taa c. Calculation of the relative gene expression was based on the delta delta CT method using the appropriate software (CFX Maestro™, Bio-Rad Laboratories).

2.4. Immunostaining

Immunofluorescent analysis of AMTN was performed on HSC2 plated onto glass slides (Merck, Darmstadt, Germany) treated with EMD, TGF- β , BMP-2, PDGF, and ADL for 24 h. Cells were fixed in paraformaldehyde and blocked in 1% BSA and 0.3% Triton X100 in PBS at room temperature for one hour. Cells were subsequently incubated with rabbit antiamelotin antibody (ab122312, Abcam, Cambridge, UK) for another hour. Alexa Fluor 488 secondary antibody (Cell Signaling Technology, Danvers, MA, USA) was applied for one hour. Cells were washed and fluorescent images were captured (Euromex, Oxion fluorescence, Arnhem, The Netherlands).

2.5. Gelatin Coating

Gelatin powder from porcine skin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in double distilled water at a final concentration of 0.1%. Next, 24-well culture plates were coated with gelatin 0.1% and placed into the incubator. Following this, the liquid was removed and wells were vigorously washed using PBS. Undiluted ADL and serum-free media were applied onto the gelatin coating plates, which were returned to the incubator overnight. After vigorous washing using PBS, cells were plated and after 24 h underwent RNA extraction.

2.6. Western Blot

HSC2 samples seeded at 30,000 cells/cm² were exposed overnight to 5% ADL and 30 ng/mL TGF- β . Protein extracts in SDS buffer containing protease and phosphatase

inhibitors (cOmplete ULTRA Tablets and PhosSTOP; Roche, Mannheim, Germany) were separated by SDS-PAGE and transferred onto Polyvinylidene fluoride (PVDF) membranes (Roche Diagnostics, Mannheim, Germany). The binding of rabbit p-Smad3 antibody (1:1000, phospho S423 + S425, (EP823Y), Abcam, Cambridge, UK) and mouse Smad3 antibody (1:1000, sc-101154, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was detected with peroxidase-labeled antibodies (1:10,000, CS-7074, antirabbit IgG, Cell Signaling Technology) and (1:10,000, 7076, antimouse IgG, Cell Signaling Technology), respectively. Peroxidase was visualized with Clarity Western ECL Substrate (Bio-Rad Laboratories) and signals were detected with the ChemiDoc imaging system (Bio-Rad Laboratories).

2.7. Statistical Analysis

The experiments were repeated at least three times. Every single data point is representative of an independent experiment, with data points being individually obtained from the teeth of different pigs. Statistical analysis was based on Mann–Whitney U test and one-way ANOVA Kruskal–Wallis test without Dunn’s multiple comparisons correction. Analyses were performed using Prism v8 (GraphPad Software, La Jolla, CA, USA). Significance was set at $p < 0.05$.

3. Results

3.1. Acid Dentin Lysates Induce Expression of the AMTN but Not the Enamel Matrix Proteins

To evaluate the effects of ADL on the expression of AMTN and three main matrix enamel proteins, namely amelogenin (AMG), ameloblastin (AMBN), and enamelin (ENAM), RT-PCR analysis of the respective genes was conducted in human oral squamous cell line (HSC2), buccal carcinoma cell line (TR146), and gingival fibroblast (GF). ADL significantly induced the expression of AMTN but no changes were noticed for the expression of the matrix enamel proteins (Figure 1). This observation is in agreement with our RNA sequencing data showing that the expression levels of AMG, AMBN, and ENAM did not reach the level of significance in gingival fibroblasts exposed to ADL [21]. Interestingly, the expression of odontogenic ameloblast-associated protein (ODAM) [6] as an interacting protein with AMTN was also not regulated by ADL. These findings have identified AMTN to be a target gene of the various oral cell types when exposed to ADL.

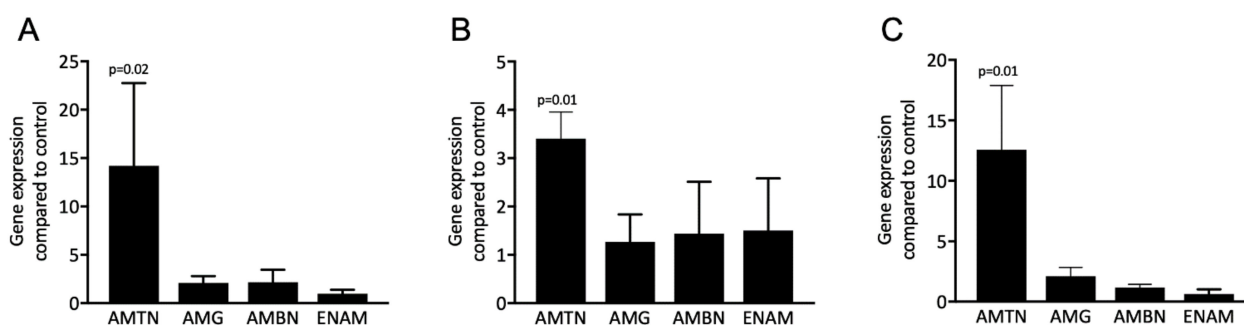


Figure 1. ADL modulates expression of AMTN but not AMG, AMBN, or ENAM. RT-PCR of AMTN, AMG, AMBN, and ENAM in (A) human oral squamous (HSC2), (B) buccal carcinoma (TR146), and (C) gingival fibroblast (GF) cell lines revealed significant changes in the expression of AMTN, but not EMPs using ADL. N = 3. Statistics based on one-way ANOVA Kruskal–Wallis test. Values are the means $\pm$ SD of the treated samples versus untreated control.

3.2. Acid Dentin Lysate-Induced Expression of AMTN Is Based on Activation of the TGF- β Receptor Type 1 Kinase

To confirm the data from our RNAseq approach showing AMTN to be among the most upregulated gene by ADL and dependent on activation of TGF- β receptor type 1 kinase [21], expression of AMTN was evaluated in HSC2, TR146, and GF. We show here that ADL significantly increased the expression of AMTN in HSC2, TR146, and gingival fibroblasts. This increase was fully dependent on TGF- β receptor type 1 kinase, since

SB431542 blocked ADL-induced expression of AMTN even below the levels of untreated cells (Figure 2). These findings led us to conclude that it requires activation of the TGF- β receptor signaling, allowing ADL to drive the expression of AMTN in the various oral cell types.

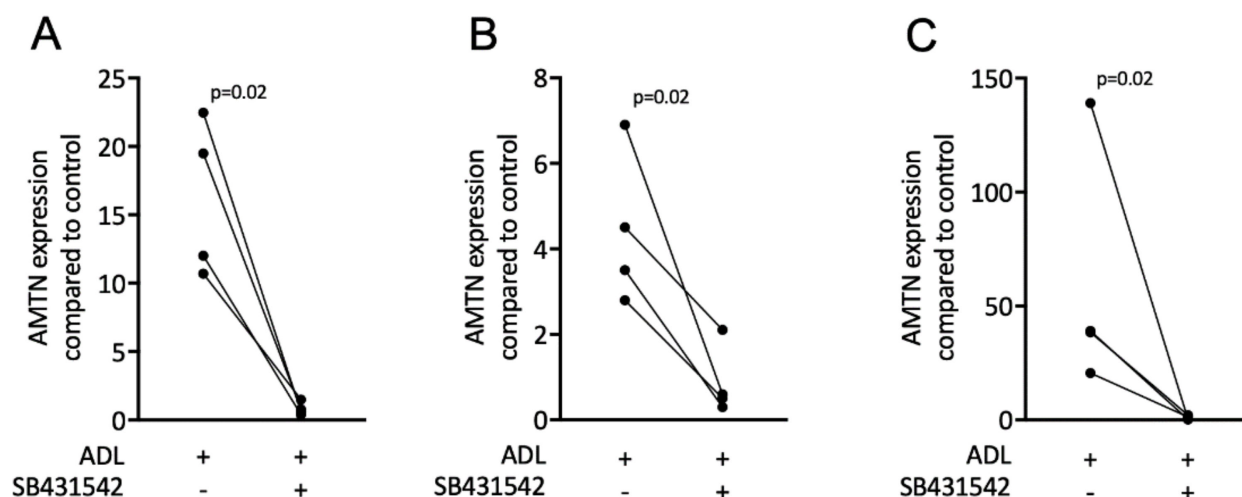


Figure 2. Acid dentin lysates induced-expression of AMTN is TGF- β receptor type 1 kinase-dependent. RT-PCR analysis of (A) HSC2, (B) TR146, and (C) GF exposed to ADL with and without SB431542 showed an increase in T β RI-dependent expression of AMTN. Statistics based on two-tailed Mann–Whitney test.

3.3. TGF- β and BMP-2 Can Induce Expression of AMTN

We then evaluated the effects of recombinant growth factors TGF- β 1, BMP-2, and PDGF-BB, which might affect the expression of AMTN in this setting. The results from RT-PCR analysis of HSC2 cells showed a significant upregulation of AMTN by TGF- β and BMP-2 but not by PDGF-BB. In TR146 cells and gingival fibroblasts, only BMP2 and TGF- β 1, respectively, increased AMTN expression (Figure 3). These data confirm the relevance of TGF- β signaling as well as the related BMP-2 signaling in driving the expression of AMTN, particularly in the HSC2 cells.

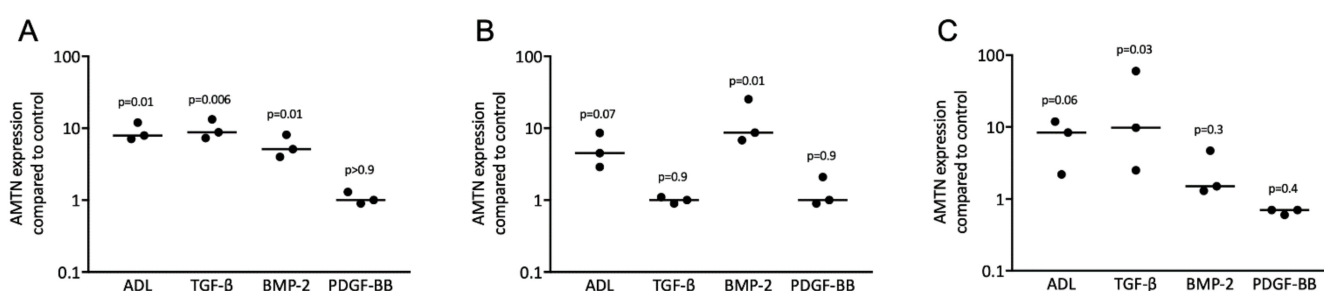


Figure 3. TGF- β and BMP-2 upregulate AMTN. RT-PCR analysis of the AMTN gene in the presence of ADL, TGF- β , BMP-2, and PDGF-BB showed upregulation induced by TGF- β and BMP-2 in (A) HSC2, while for (B) TR146 only BMP-2 and for (C) GF only TGF- β increased AMTN expression, in comparison to the untreated controls. Statistics based on one-way ANOVA Kruskal–Wallis test.

3.4. EMD Induces Expression of AMTN

Emdogain-containing EMD exerts promising clinical outcomes regarding periodontal regeneration [30], while EMD has TGF- β activity [27]. Based on previous findings, we tested whether EMD modulates the expression of AMTN in vitro. Gene expression analysis showed an SB431542-dependent expression of AMTN by EMD in HSC2 cells. In TR146 and gingival fibroblasts, EMD was not able to consistently increase the expression of

AMTN (Figure 4). These observations indicate that the TGF- β activity of EMD is capable of stimulating AMTN expression in HSC2 cells.

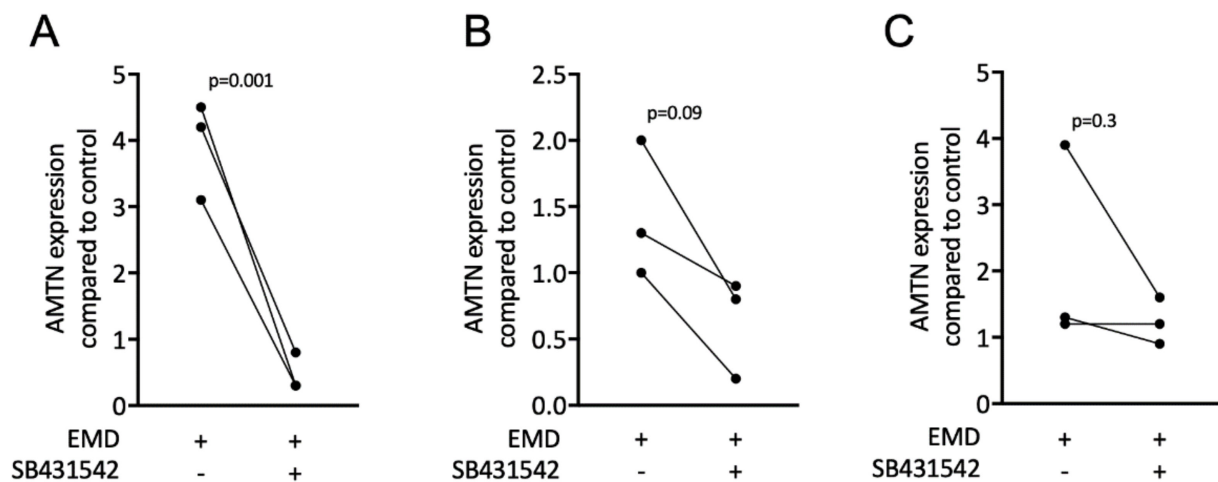


Figure 4. EMD increases the expression of AMTN, dependent on SB431542. RT-PCR of AMTN showed increased expression by EMD, which was blocked by SB431542 in (A) HSC2. An increase of AMTN was not observed in (B) TR146 or (C) GF. Statistics based on two-tailed Mann–Whitney test.

3.5. ADL, TGF- β 1, and BMP-2 Increase Positive Staining of AMTN in Nucleus

To confirm the regulation of AMTN by ADL, TGF- β 1, and BMP-2 in HSC2 cells, an immunofluorescent analysis was performed. Immunostaining revealed an increase of nuclear staining of AMTN by TGF- β , ADL, and BMP-2 in comparison to the untreated control (Figure 5). These observations showing AMTN at the protein level support the data from gene expression analysis with HSC2 cells.

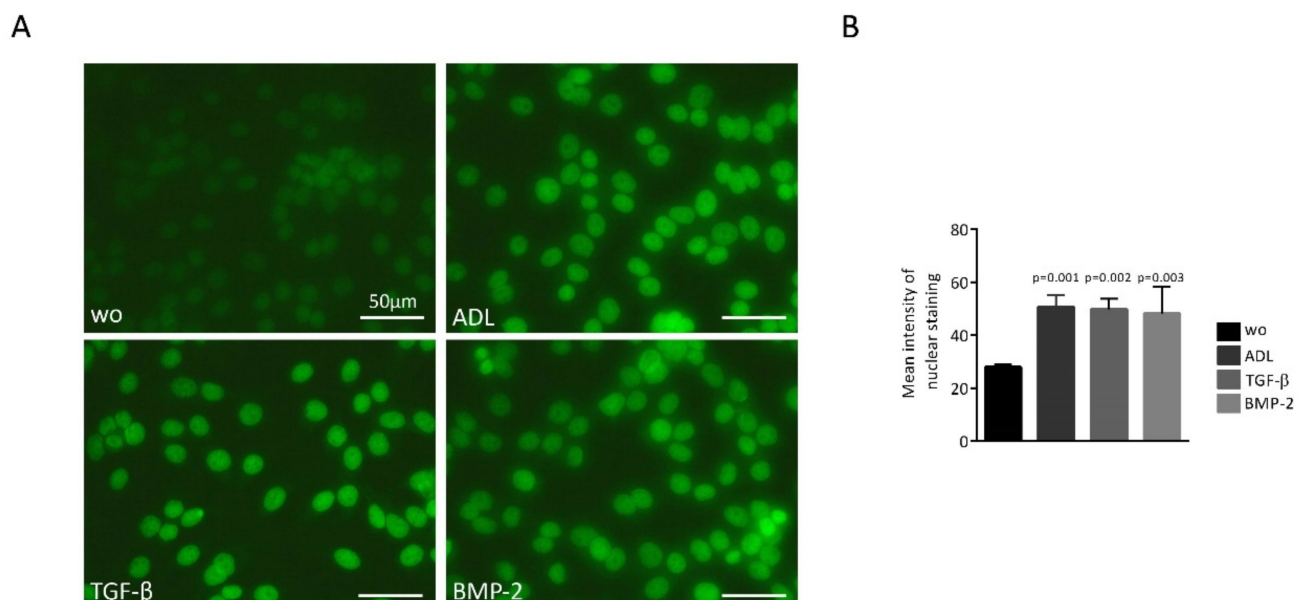


Figure 5. ADL, TGF- β , and BMP-2 increased positive staining of the nucleus for AMTN in HSC2. (A) Immunofluorescent staining of the nucleus for AMTN showed higher intensity in ADL, TGF- β , and BMP-2. (B) Comparison of mean fluorescent intensity of the nucleus by Image J software showed higher mean intensity levels in ADL, TGF- β , and BMP-2, compared to untreated control. Note: “wo” stands for without indicating unstimulated control.

3.6. Smad3 Phosphorylation Is Induced by ADL and TGF- β 1 in HSC2

To investigate whether phosphorylation of Smad3 as a part of TGF- β signaling is activated in HSC2, cells were exposed to ADL and TGF- β for 1 h. Following this, cells were analyzed for the phosphorylation of Smad3 using Western blot analysis. Western blot showed increased phosphorylation of Smad3 by ADL and even more with TGF- β 1 (Figure 6). This analysis supports the previous observations by showing that ADL is capable of activating the canonical TGF- β signaling pathway in HSC2 cells.

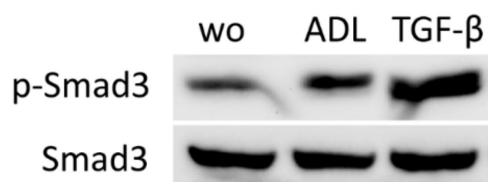


Figure 6. ADL and TGF- β induce phosphorylation of Smad3. Activation of TGF- β signaling in HSC2 was evaluated by Western blot against p-Smad3. The results showed increased phosphorylation of Smad3 by ADL and TGF- β . Note: “wo” stands for without indicating unstimulated control.

3.7. Inhibitors of TGF- β Receptor 1 Kinase Downregulate Expression of AMTN

To evaluate whether blocking of the TGF- β receptor 1 kinase also downregulates the intrinsic TGF- β activity of the cells, expression of AMTN was evaluated in cells exposed to inhibitors of TGF- β receptor 1 kinase SB431542 and LY2157299 for 24 h. Gene expression analysis showed a significant downregulation of AMTN by SB431542 and LY2157299 compared to the untreated control in HSC2 cells (Figure 7). These findings led us to conclude that autocrine TGF- β signaling in HSC2 cells is sufficient to regulate the expression of AMTN.

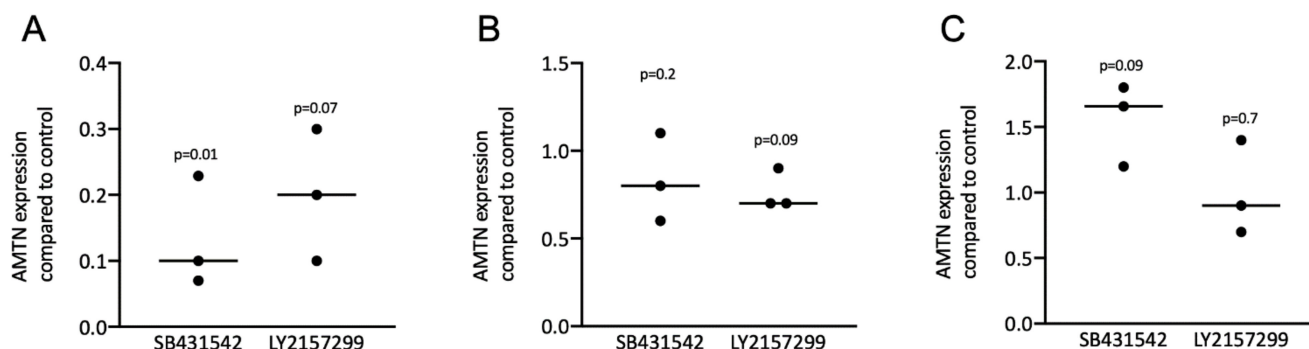


Figure 7. Inhibitors of T β RI downregulate expression of AMTN. RT-PCR analysis of the AMTN gene in the presence of SB431542 and LY2157299 showed decreased expression in comparison to untreated control in (A) HSC2, (B) TR146, and (C) GF. Statistics based on one-way ANOVA Kruskal–Wallis test.

3.8. AMTN-Induced Activity of ADL Binds to Gelatin

As collagens are among the most applied biomaterials in the field of regenerative dentistry, we raised the question of whether ADL-inductive activity of AMTN binds to gelatin. Therefore, cells were seeded on gelatin-coated plates that were soaked with serum-free media, given as control or undiluted ADL. Gene expression analysis determined a weak but significant increased expression of AMTN by ADL compared to unstimulated control in HSC2. The TGF- β activity was too weak to cause a reliable increase in TR146 or gingival fibroblasts (Figure 8). These findings support the concept that ADL is a source of TGF- β that adsorbes to collagen and can drive AMTN expression in HSC2 cells.

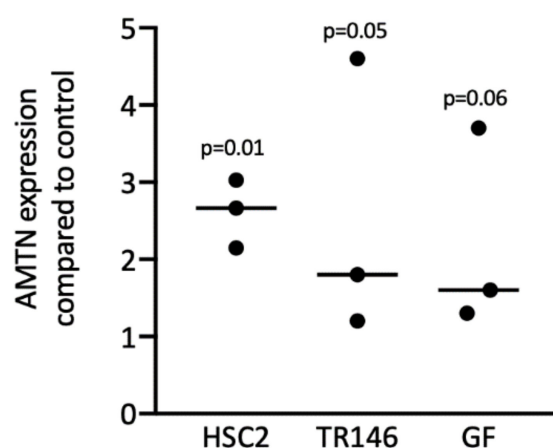


Figure 8. ADL bound to gelatin induces expression of AMTN. RT-PCR analysis showed induced expression of AMTN in comparison to untreated control in HSC2, TR146, and GF when ADL was bound to gelatin. Statistics based on one-way ANOVA Kruskal–Wallis test, comparing treated samples to the untreated control.

3.9. Root- and Crown-Derived ADL Induces Expression of AMTN

To further elucidate the origin of AMTN increase by ADL, the root and crown of the tooth were separated and exposed to acid treatment for ADL preparation. Gene expression analysis identified that in HSC2 cells both R-ADL and C-ADL increased the expression significantly compared to the untreated control (Figure 9). These findings provide the first insights into the capacity of ADL produced from teeth with and without the enamel to drive AMTN expression in HSC2 cells.

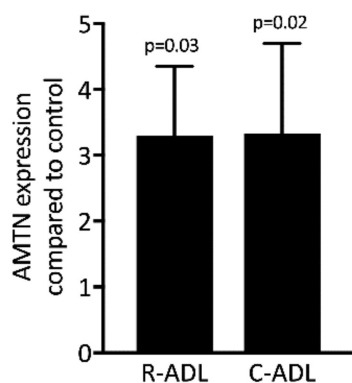


Figure 9. Root-derived ADL (R-ADL) and crown-derived ADL (C-ADL) induce the expression of AMTN. RT-PCR of AMTN shows regulation by R-ADL vs. C-ADL, $n = 3$. Statistics based on one-way ANOVA Kruskal–Wallis test, comparing treated samples to the untreated control.

4. Discussion

The sealing of the periodontal space towards the oral cavity is accomplished by the firm attachment of epithelial cells to the rather smooth tooth surface. It is the specialized basal lamina produced by the junctional epithelium that serves as a molecular adhesive to the hard tissue of the tooth [4,6]. AMTN is among the small spectrum of proteins comprising the specialized basal lamina [2,6–10,17]. It is, thus, not surprising that great research efforts have been made to understand the formation of the basal lamina and its preservation during adulthood. Surprisingly, little is known on how the basal lamina recovers upon catabolic events that are usually caused by chronic inflammation. The present research proposes the injured dentin to release TGF- β , which in turn stimulates the epithelial cells to produce AMTN, thereby supporting regain of the original junctional epithelium seal.

Our research effort was further based on our RNAseq approach, whereby gingival fibroblasts exposed to acid dentin lysates increasingly expressed AMTN [21]. Inspired by this finding, we have now focused on oral epithelial cell lines and gingival fibroblasts to express AMTN, ODAM, and three main matrix enamel proteins; however, only AMTN showed a robust increase in expression, particular in the HSC2 cells. Considering that ADL is a rich source of TGF- β [21] and that recombinant TGF- β 1 was reported to stimulate the expression of AMTN in gingival epithelial cells [14], we now show that SB431542 blocks the AMTN expression in the epithelial cell lines. Our findings that ADL enhances the phosphorylation levels of Smad3 in HSC2 cells further supports the activation of canonical TGF- β signaling. Taken together, it is the TGF- β activity released from the acidified dentin that causes HSC2 epithelial cells to express AMTN.

The data also led us to raise new research questions. We identified a strong variance in the expression of AMTN in response to ADL and even recombinant TGF- β 1 in gingival fibroblasts from different donors, suggesting that possibly the variation of the donor or the passaging might have affected the outcome. Additionally, the TR146 cells were less responsive than the HSC2 cells with respect to the AMTN regulation induced by ADL. We have no explanation for this but it could be the overall responsiveness to TGF- β —independent of AMTN expression—that distinguishes the various *in vitro* models from each other. Nevertheless, all of our *in vitro* models should be interpreted with care, as they do not necessarily represent the unique situation of the junctional epithelial cells that express AMTN *in vivo*. Future studies should, thus, use freshly isolated junctional epithelial cells or the respective explant cultures to be exposed to ADL, a setting more closely simulating the clinical situation. We have to interpret the findings that ADL supports AMTN expression with respect to the *in vitro* models used.

The clinical relevance also remains hypothetical. It is not clear to which extent TGF- β released from dentin by the resorption of odontoclasts is capable of reaching the junctional epithelium *in vivo*. Root resorption is usually not so pronounced that it releases enough TGF- β and other growth factors to regain tissue homeostasis. Moreover, the macrophages, particular those of the TGF- β -producing lineage, are also supposed to help tissue regeneration [31]. Our research is, thus, an inspiration for future research to decipher the role of macrophages and their release of TGF- β in the process of regaining the specialized basal lamina. Apart from TGF- β , recombinant BMP2, an osteoinductive protein that is approved for augmentation surgery in the sinus [28], increased AMTN in the HSC2 cells. These findings suggest that it is not exclusively the TGF- β signaling that drives AMTN expression.

Clinical relevance might also be linked to the local application of EMD [26], with its strong TGF- β activity [27]. It was, thus, not surprising to see EMD stimulate AMTN expression in HSC2 cells in a SB431542-dependent mode of action. Our *in vitro* observation supports preclinical and to some extent also clinical observations that EMD supports the regain of the junctional epithelium and its reattachment to the tooth surface *in vivo*. Indirect evidence for this can be drawn from preclinical models showing that EMD reduces the downgrowth of the junctional epithelium in rats [32] and canines [33]. Future studies should evaluate whether EMD stimulates AMTN expression in the junctional epithelial cells, thereby supporting the formation of the basal lamina, which reduces the epithelialization of the periodontal pocket.

The clinical aspects discussed above lead us to discuss study limitations. First, even though HSC2 and TR146 are epithelial cells in origin, they have the characteristics of an oral squamous and buccal mucosa cell carcinoma, respectively, and do not necessarily represent the junctional epithelial cells. The findings presented here should, therefore, be considered as the results of a proof-of-principle approach used to identify epithelial cells in response to ADL. Second, we have not tested the functionality of AMTN produced by the HSC2 cells in gluing the epithelial cells towards the passive surface of the tooth. However, we have generated indirect evidence that the TGF- β activity of ADL partially adsorbs to a collagen matrix, which in turn causes HSC2 cells to produce AMTN. It is also possible that

in vivo, collagen becomes a source of TGF- β . Finally, and considering that extracted teeth used as grafts are partially resorbed by osteoclasts [30], the released TGF- β might support epithelial cells to seal off the augmented space towards the oral cavity.

Overall, our in vitro findings show increased expression of AMTN by ADL involving TGF- β receptor I kinase. The present research should be understood as an extension of our recent observations that ADL stimulated AMTN expression in gingival fibroblasts [21], and this information should be translated into the epithelial cell system. These findings basically confirm the role of TGF- β in increasing AMTN under these conditions and extend this principle towards the clinically relevant observation that EMD is also capable of supporting HSC2 epithelial cells in their expression of AMTN.

Author Contributions: J.N. contributed to conceptualization and design, methodology, acquisition, analysis, software, validation, and interpretation; critically revised the manuscript; gave final approval; and agreed to be accountable for all aspects of work. Z.K. contributed to acquisition, analysis, and interpretation; critically revised the manuscript; gave final approval; and agreed to be accountable for all aspects of the work. L.P. contributed to acquisition, analysis, and interpretation; critically revised the manuscript; gave final approval; and agreed to be accountable for all aspects of the work. R.G. contributed to conceptualization and design, acquisition, analysis, and interpretation; drafted and critically revised the manuscript; gave final approval; and agreed to be accountable for all aspects of the work. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The harvesting of gingiva was approved by the Ethics Committee of the Medical University of Vienna (EK NR 631/2007).

Informed Consent Statement: Human gingiva samples were taken from extracted wisdom teeth of patients who had signed an informed consent form.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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