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ORIGINAL ARTICLE

Influence of leptin and compression in GAS-6 mediated homeostasis of periodontal ligament cell

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Abstract

Growth arrest-specific protein 6 (GAS-6) regulates immunomodulatory and inflammatory mechanisms in periodontium and may participate in obesity predisposition. This study aimed to determine whether GAS-6 is associated with the homeostasis of periodontal ligament (SV-PDL) cells in the presence of adipokines or compressive forces. The SV-PDL cell line was used. Western blots were employed for TAM receptors detection. Cells were stimulated using different concentrations of GAS-6. The migration, viability, and proliferation were measured by a standard scratch test, MTS assay, and immunofluorescent staining. The mRNA expression was analyzed by RT-PCR. Release of TGF-β1, GAS-6, and Axl were verified by ELISA. Western blot shows that TAM receptors are expressed in SV-PDL cells. GAS-6 has a promoting effect on cell migration and proliferation. RT-PCR analysis showed that GAS-6 induces *Collagen-1*, *Collagen-3*, *Periostin*, and *TGF-β1* mRNA expression whereas it reduces *Caspase-3*, *Caspase-8*, *Caspase-9*, and *IL-6* mRNA expression. Further, secreted GAS-6 in SV-PDL is reduced in response to both compressive forces and leptin and upregulated by IL-6. Additionally, ADAM-10 inhibition reduces GAS-6 and Axl release on SV-PDL cells. TAM receptors especially Axl are identified as the receptors of GAS-6. GAS-6/TAM interactions contribute to periodontal ligament cells homeostasis. Leptin inhibits the GAS-6 release independently of ADAM-10 metalloprotease.

KEYWORDS
ADAM-10, growth arrest-specific 6, leptin, obesity, TAM receptors

1 | INTRODUCTION

Growth arrest-specific 6 (GAS-6) was identified by Schneider et al. (Schneider et al., 1988) in 1988, being described as a vitamin K-dependent protein expressed during growth arrest in serum-starved NIH3T3 cells. GAS-6 is expressed by many cell types including oral mucosa epithelial cells (Nassar et al., 2017) as well as in diverse tissues including brain, bone marrow, and vascular endothelium (Ekman et al., 2010).

GAS-6 acts by binding to the TAM receptors: Tyro3 protein tyrosine kinase (Tyro3), Axl receptor tyrosine kinase (Axl), and Mer tyrosine kinase protooncogene (Mer) (van der Meer et al., 2014). As a ligand of the TAM receptors, GAS-6 regulate several biological mechanisms involved among others in cell survival (Goruppi et al., 1997; Nakano et al., 1996; Sainaghi et al., 2005), cell proliferation (Sainaghi et al., 2005), and cell migration (Fridell et al., 1998). Not long ago it has been described that GAS-6 plays a fundamental role in maintaining oral mucosal homeostasis (Nassar et al., 2017). Chen

et al. (2015) reported that secondary Sjögren syndrome is associated with decreased GAS-6 concentration in plasma and labial salivary glands.

The GAS-6 protein has attracted special research interest in relation with obesity (Robins et al., 2013; Wu et al., 2015). Scroyen et al. (2012) showed in an animal-based study that GAS-6 levels where increased in obese mice compared to normal weighted animals. Clinical studies demonstrate that the concentration of circulating GAS-6 and soluble Axl is significantly increased in obese individuals, indicating a positively correlation between GAS-6 and body fat mass (Hsiao et al., 2013).

In dentistry, there has been a special focus on the effects of GAS-6 on the periodontal structures. Zhang et al. (2020) showed that GAS-6 promotes in vitro the osteogenic induction on human periodontal ligament cells. Considering the fact that elevated GAS-6 levels are present in overweighed individuals (Wu et al., 2015), periodontal homeostasis is impaired in the presence of obesity and could react differently to mechanical factors such as masticatory or parafunctional forces. Thus, the aim of the present study is to analyze in vitro the biological effects that GAS-6 exerts on periodontal ligament cells stimulated with different adipokines or compressive forces.

2 | MATERIAL AND METHODS

2.1 | Cell culture and reagents

The mouse immortalized periodontal ligament cell line (SV-PDL) was kindly provided by Prof. M. Somerman (Laboratory of Oral Connective Tissue Biology, NIH, Bethesda, MD, USA). Cells were seeded onto 6-well plates at a density of 3×10^4 cells/well until confluence in D-MEM (31885-023, Gibco) containing 10% fetal bovine serum (FBS) (10270-106, Gibco), 1% penicillin/streptomycin (15140-122, Gibco), 1% L-glutamine (25030-024, Gibco), 1% pyruvate (11360-039, Gibco), and 1% HEPES (15630-056, Gibco) in a humidified atmosphere of 5% CO_2 at 37°C.

The cells were stimulated using mouse recombinant mouse leptin protein (Cyt-351, Cat. N°: 108MLEP01, Prospec Inc.), mouse adiponectin/Asp30/ADIPOR protein (His Tag, Cat. N°: 50636-M08H, Sino Biological Inc.), recombinant mouse resistin (SRP4560, Sigma-Aldrich), visfatin (SRP4908, Sigma-Aldrich), ghrelin (494127, Sigma-Aldrich), recombinant mouse IL-6 (406-ML-005, Cat. N°: NUQ3220031, R&D systems), recombinant mouse GAS-6 (Cat. N°:8310-GS, R&D systems), and ADAM-10 inhibitor (GI254023X, Sigma-Aldrich). To induce GAS-6 release, the incubation medium was supplement with 5 μg/ml Konakion® (Vitamin K1, CHRPLAPHARM GmbH).

2.2 | Compressive forces application

The SV-PDL cells were seeded into 6-well plates (657160, Greiner Bio-One). After reaching 60%–70% confluence, compressive forces were applied as described by Kanzaki et al. (2002) and Proff et al. (2014). Briefly, 33 mm diameter glass cylinders with pulled surfaces were made by Reichmann Feinoptik Inc. (Brokdorf, Germany). Cylinders of different volumes were fabricated in order to reach pressure forces of 2.4 and 3.6 gf/cm^2, respectively. The cells were covered with the glass cylinders as soon as 60%–70% confluency was reached (Yong et al., 2021).

2.3 | Quantitative mRNA expression assay

RNA was isolated employing RNeasy Mini Kit (Cat. N° 74104, Qiagen) and treated with RNA-Free DNase Set (Cat. N° 79254, Qiagen). RNA concentrations were measured using a spectrophotometer (Nanodrop 2000, Thermo scientific). One-microgram RNA was transcribed into cDNA using iScript™ CDNA Synthesis Kit (Cat. N° 170–8891, BioRad) according to the manufacturer protocol.

Real-time polymerase assays were performed utilizing SsoAdvanced™ Universal SYBR® Green Supermix (1723271, Bio-Rad) and used the specific primers: *TGF-β1* (qMmuCED0044726); *Collagen-1* (qMmuCED0044222); *Collagen-3* (qMmuCID0006332); *Periostin* (qMmuCID0046524); *IL-6* (qMmuCED0045760); *Caspase-3* (qMmuCED0047599); *Caspase-8* (qMmuCED0005542); *Caspase-9* (qMmuCED0046922); and *ADAM-10* (qMmuCED0050303). Target gene expressions were normalized to the expression of *GAPDH* (qMmuCED0027497, Bio-Rad) as housekeeping gene. Thresholds were amplified and detected using CFX96™ Real-Time System Cycler (Bio-Rad). Results were analyzed using the Bio-Rad CFX Manager 3.1 software. Each experiment was repeated successfully at least three times.

2.4 | Cell migration assay

SV-PDL cells were plated at a density of 8×10^3 cells/well in a 6-well plate, incubated in D-MEM containing 10% FBS, and cultured until confluence. Cells were preincubated for 12 h with starvation medium (0.5% FBS) and wounded by scratching using a 100 μl tip. Through this procedure, a single standardized scratched area was created in the center of the cell layer. Afterward, all non-adherent cells were washed with 1× phosphate-buffered saline (PBS) (10010023, ThermoFisher). The wounded cell monolayers were incubated in the presence and absence of different concentrations of GAS-6 (50, 100, and 250 ng/ml) (Cat. N°:8310-GS, R&D systems) for 12 h. Wounded-area images were taken immediately after wounding and 12 h after scratching. Cell conditioned medium (0.5% FBS or 10% FBS) were added to the control groups, respectively, which served as the negative control and positive control. The wounded cell layers were photographed at 10× magnification (Leica Inc.), and the percentages of wound closure area between cell layer borders were analyzed and calculated over time using the Image J software (National Institutes of Health and University of Wisconsin, USA).

2.5 | Cell viability assay

The possible effect that GAS-6 (Cat. N°:8310-GS, R&D systems) may exert on the viability of SV-PDL cells was examined calorimetrically using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96@ Aqueous One Solution Cell Proliferation Assay, G3582, Promega) according to manufacturer's instructions. Briefly, mouse SV-PDL cells at a density of 5×10^3 cells/well at a passage 3–5 were seeded in a 96-well plate containing 5% FBS overnight to allow adherence. Then, cells were washed twice with 1× PBS and treated with different concentration of GAS-6 (R&D systems, Cat. N°:8310-GS) in D-MEM containing 0.5% FBS over a period of 24 h. Thereafter, 20 µl of the MTS reagent was added into each well, and the cells were incubated during 2 h at 37°C in a 5% CO_2 atmosphere. Plates were read by 490 nm using a 96-well microplate reader (BioTek) to measure the amount of formazan by cellular reduction of MTS.

2.6 | Cell proliferation assay

Cell proliferation was assessed using Ki-67 and PCNA immunofluorescent staining. Briefly, SV-PDL cells (5×10^3 cells/well) were seeded onto sterile Falco™ Chambered Cell Culture Slides (354108, Fisher Scientific) in 1 ml of growing medium overnight and then treated with GAS-6 (R&D systems, Cat. N°:8310-GS) at varying concentrations (0, 50, 100, and 250 ng/ml) for 24 h in starvation medium. The treated cells were then fixed with fixation buffer containing 4% paraformaldehyde (554655, BD Cytofix™) for 15 min at room temperature (RT) and washed twice with 1× PBS with 0.02% Tween-20 (PBST) (P1379, Sigma-Aldrich). For permeabilization, Triton™ X-100 (T-9284, Sigma-Aldrich) was added at 0.2% (v/v, in PBS) for 10 min at RT. After washing twice with 1× PBST, cells were blocked with immunofluorescence blocking buffer (#12411, Cell Signaling Technology) for 20 min at RT. Afterward, rabbit anti-Ki-67 antibody (ab70362, Cell Signaling Technology) (dilution 1:50) or rabbit anti-PCNA antibody (ab70362, Cell Signaling Technology) (dilution 1:400) was added to cells and incubated overnight at 4°C. SV-PDL cells were washed three times with 1× PBST and further stained with DyLight@ 488 polyclonal goat anti-rabbit IgG H&L (ab96899, Abcam) (dilution 1:500) for 1 h in the dark at RT. After washing twice with 1× PBST, SV-PDL were exposed for 5 min to 0.1% DAPI (ab104139, Abcam) for blue nuclear staining and mounted with a coverslip. Slides were imaged with confocal high-resolution fluorescence microscopy (Leica Microsystems), and positively stained cells were counted manually. A minimum of 4 wells was analyzed for each concentration of GAS-6.

2.7 | Protein extraction and Western blot analysis

Cells were collected in RIPA buffer Pierce™ (89901, Thermo Scientific) supplemented with 3% phosphatase and protease inhibitors (78442,

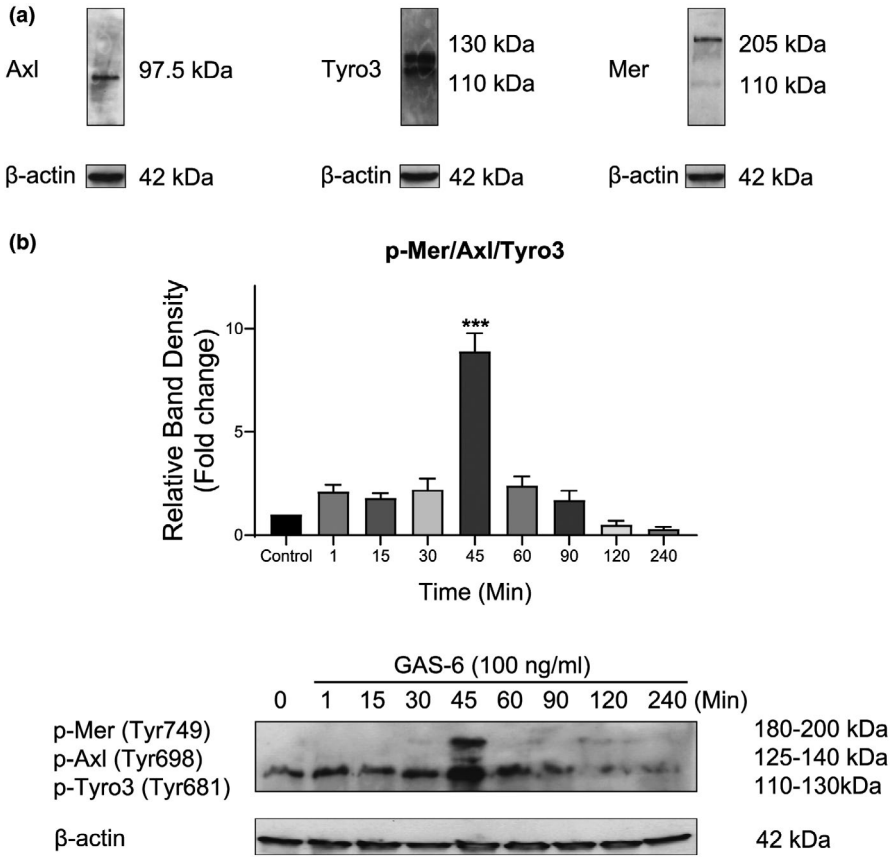


FIGURE 1 TAM receptors are expressed in periodontal ligament cell line. (a): Western blot analysis was performed using Axl, Mer, and Tyro3 antibodies to show the expression of TAM receptors in mouse periodontal ligament cell line. β-actin was used as a loading control. (b): Analysis of GAS-6 stimulation of TAM receptors on SV-PDL cells. Kinetic Western blot analysis of Axl, Tyro3, and Mer phosphorylated proteins. Graphics represent the relative expression values of p-Axl/Tyro3/Mer normalized to control cells at time 0. $n = 3$ independent biological replicates. Graphs = mean ± SD. ns $p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Thermo Scientific). Protein concentration was measured using Pierce™ BCA Protein Assay Kit (23225, Thermo Scientific) on a Nanodrop 2000 Spectrophotometer (Thermo Scientific). Protein aliquots were separated by electrophoresis on SDS polyacrylamide gels and blotted to a nitrocellulose membrane (1704271, Bio-Rad) using Trans-Blot Turbo Transfer System (Bio-Rad). Ponceau S solution (P7170, Sigma-Aldrich) staining was employed to visualize the transferred protein bands.

Membranes were blocked with 5% non-fat milk (T145.1, ROTH) and incubated for 1 h at room temperature employing the following antibodies: mouse Mer (#108921, R&D systems), dilution 1:1000; mouse Tyro3 (AF759, R&D systems), dilution 1:1000; Axl (AF7793, Affinity Biosciences) dilution 1:1000; Phospho-Axl (Tyr698)/Mer (Tyr749)/Tyro3(Tyr681) (#44463, Cell Signaling Technology), dilution 1:500. As loading control, β-actin (ab8227, Abcam) dilution 1:2000 was employed. The secondary antibodies: Polyclonal Goat Anti-Rabbit (P0448, Dako); Rabbit Anti-Goat (P0160, Dako); and Polyclonal Goat Anti-Mouse (P0447, Dako) Immunoglobulins/HRP at a dilution 1:2000 were used. The membranes were developed utilizing Amersham ECL Western Blotting Detection Reagents (9838243, GE Healthcare) and detected with Amersham Hyperfilm ECL (28906836, GE Healthcare) on OPTIMAX X-Ray Film Processor (11701-9806-3716, PROTEC GmbH).

2.8 | Enzyme-Linked Immunosorbent assay (ELISA)

GAS-6 and TGF-β1 release were measured on cell supernatants using a highly sensitive GAS-6 ELISA kit (Mouse) (OKBB00533, BIOZOL) and TGF-β1 ELISA Kit (OKBB00255, BIOZOL) following the manufacturer's instructions. The secreted Axl levels on cell supernatants were detected using the Axl ELISA kit (ABIN625112, online-antibodies Inc.). Plates were read at 405 nm, and results were analyzed with xMark™ Microplate Absorbance Spectrophotometer software (1681150, Bio-Rad).

For the analysis of the assay results, the relative OD_{405} was calculated for each sample by plotting the mean replicate OD_{405} of each standard serial dilution vs. the respective standard concentration. The concentration of GAS-6, TGF-β1, and Axl was interpolated by using linear regression of the relative OD_{405} against the standard curve. The measurements were performed with 3 biological replicas.

2.9 | Data analysis

Statistical analyses were performed using GraphPad Prism 8.0 software (GraphPad software). All values are expressed as means ± standard deviation (SD) and analyzed using paired Student's *t* test or ANOVA test. A p value <0.05 was considered as statistically significant. Data analyzed were averaged from a minimum of 3 independent experiments on separate primary cultures.

3 | RESULTS

3.1 | Periodontal ligament cells express TAM receptors

First, we aimed to elucidate whether SV-PDL cells express functional GAS-6/TAM receptors. Our WB analysis revealed that Axl, Tyro3, and Mer receptors are present on this cell line (Figure 1a).

The exogenous stimulation with 100 ng/ml GAS-6 (Cat. N°:8310-GS, R&D systems) induces the phosphorylation of the three TAM receptors after a time period of 45 min (Figure 1b).

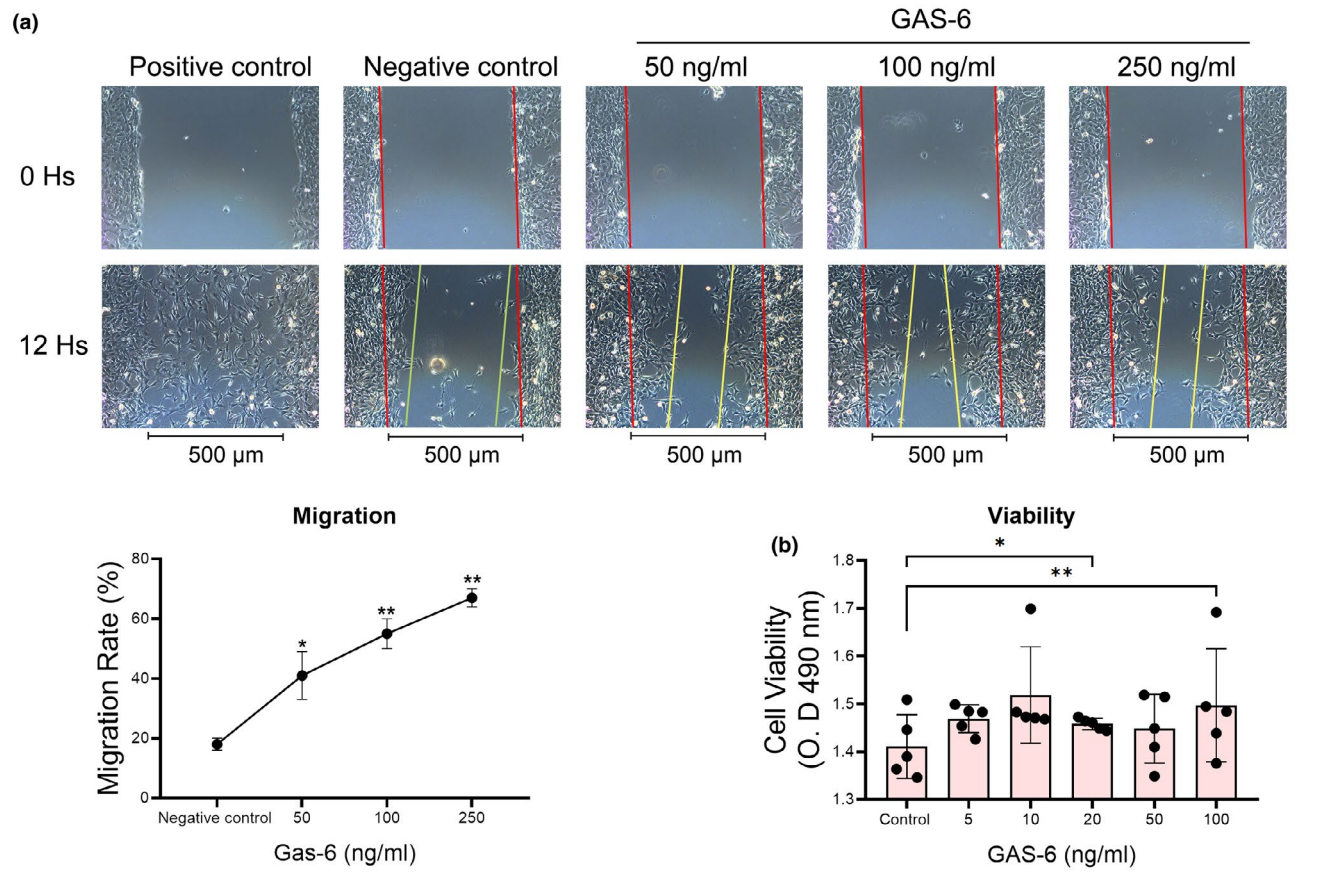
3.2 | GAS-6 promotes cell migration and proliferation

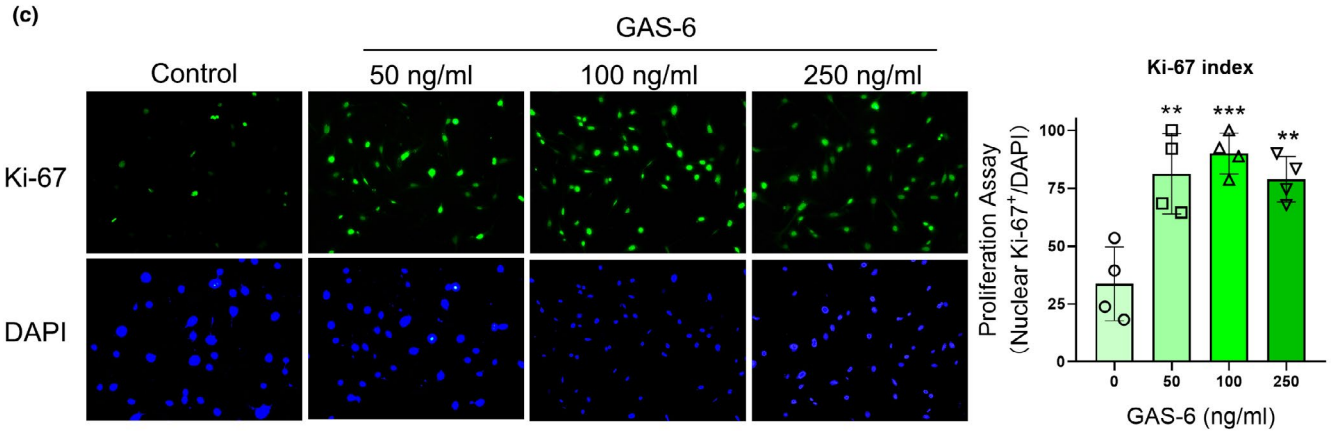
Second, we aimed to analyze the effect that exogenous stimulation with GAS-6 exerts on SV-PDL cells during a period of 12 h. The migration tests indicate that the addition of 50, 100, and 250 ng/ml of this protein into cell culture significantly increases the ratio of cell migration up to 23.6 ± 8.0% ($*p < 0.05$), 37.7 ± 3.0% ($**p < 0.01$), and 48.9 ± 12.36% ($**p < 0.01$) in comparison with unstimulated cells (Figure 2a).

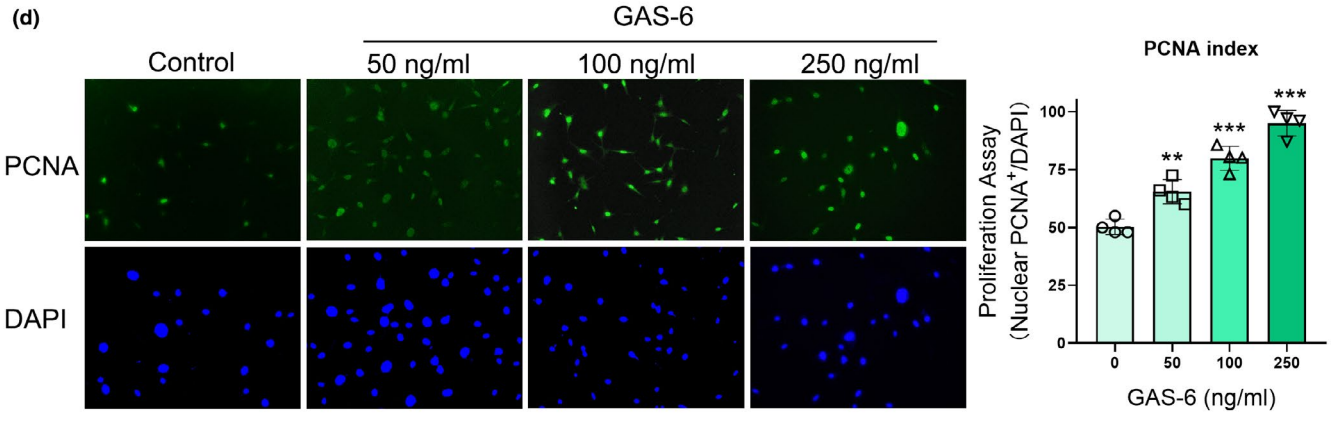
The colorimetric analysis revealed that GAS-6 stimulation of periodontal ligament cells significantly increases cell viability using MTS assay even at low protein concentrations over a period of 24 h (Figure 2b).

We next analyzed the expression of Ki-67 and PCNA, markers of cellular proliferation to determine whether GAS-6 at different doses (50, 100, and 250 ng/ml) for 24 h regulates SV-PDL proliferation (Figure 2c–d). The IF staining showed that GAS-6 significantly increased the expression of Ki-67 and PCNA ($**p < 0.01$) (Figure 2c–d).

FIGURE 2 GAS-6 promotes SV-PDL cell migration, viability and proliferation. (a): The migration effect of GAS-6 was measured by standard scratch test. GAS-6 (50, 100, and 250 ng/ml) promotes SV-PDL cell migration. Data are presented as percentage of wound recovery and expressed as mean ± SD. (b): Cell viability assay performed by MTS assay. Stimulation with different concentrations of GAS-6 lead to significantly increased cell viability of SV-PDL cells compared to negative control group (0.5% FBS). Data are expressed as mean ± SD. (c–d): immunofluorescence microscopy images shown representative proliferation markers Ki-67 and PCNA. SV-PDL were exposed to different doses of GAS-6 during 24 h and then underwent immunofluorescence staining for Ki-67 or PCNA to visualize cells in the proliferation stage. The proportion of proliferating cells for each group was quantified. Data are expressed as mean ± SD. $n = 3$ independent biological replicates. Graphs = mean ± SD. ns $p > 0.05$ $*p < 0.05$, $**p < 0.01$, $***p < 0.001$







3.3 | Increased *Collagen Type I*, *III* and *Periostin* mRNA levels after GAS-6 stimulation

Next, we performed a kinetic mRNA analysis of relevant genes for periodontal ligament cell biology. The exogenous stimulation with 100 ng/ml GAS-6 significantly upregulates the levels of *Collagen-1* and *Collagen-3* as well as *Periostin* over a period from 15 to 45 min, whereas the levels of *TGF-β1* reach statistical significance after 45 min (Figure 3).

On the contrary, we observed that the mRNA levels of *IL-6* and *Caspase-3* reach a non-significant peak after 15-min GAS-6 addition, suggesting an initial pro-inflammatory effect of GAS-6 on periodontal ligament cells. However, these levels show a significant reduction after a period of 45 min. Alike, the mRNA expressions of *Caspase-9* and *Caspase-8* significantly decrease after a period of 45 min in the presence of exogenous GAS-6 (Figure 3a).

Further, we observed a significant increase in the released amount of TGF-β1 protein over a period of 24 and 48 h in the presence of exogenous GAS-6 (*$p < 0.05$) (Figure 3b).

3.4 | Periodontal ligament cells release GAS-6, and its expression is reduced by compression as well as by exogenous leptin

Because different studies have described the involvement of GAS-6 in the homeostasis of obesity and systemic inflammation (Wu et al., 2015), we further aimed to analyze whether SV-PDL can release GAS-6 and whether its expression can be altered by co-stimulation with different adipokines or compressive forces. For this purpose, the cells were maintained in cell culture medium added with vitamin K1 (5 μg/ml) in the presence of different adipokines (leptin, adiponectin, resistin, visfatin, and ghrelin) as well as IL-6 at a concentration of 100 ng/ml or stimulated with compressive forces of 2.4 gf/cm^2 and 3.6 gr/cm^2 during 24 h. As result, we observed that SV-PDL cells have the property to release GAS-6 (30.43 ± 4.43 pg/ml measured on supernatants) under normal conditions, and this release is significantly reduced by leptin (*$p < 0.05$) as well by the application of compressive forces of 2.4 and 3.6 gr/cm^2 (**$p < 0.01$) (Figure 4a).

3.5 | ADAM-10 pharmacological blockade can effectively reduce GAS-6 as well as Axl secretion on SV-PDL cells

It was described that ADAM-10 exerts a regulatory role in the expression of GAS-6 (Orme et al., 2016). To analyze the possible relation of ADAM-10 metalloprotease in the regulation of GAS-6 on periodontal ligament cells, we first explore the *ADAM-10* mRNA expression in response to adipokines and compressive forces stimulation. The results show that *ADAM-10* expression is significantly upregulated by all exogenous stimuli applied (**$p < 0.05$). The groups treated with leptin, visfatin, and resistin or maintained under compression showed a high increase (***$p < 0.001$) of *ADAM-10* mRNA levels whereas groups treated with adiponectin or ghrelin showed a lower but significantly increase (Figure 4b).

The application of an ADAM-10 antagonist on periodontal ligament cells effectively reduces the release of GAS-6: The cell pretreatment with 10 μM GI254023X (Sigma-Aldrich) during 1 h and further stimulation with leptin, adiponectin, resistin, visfatin, ghrelin, and Il-6 or by the application of compressive forces notably influence the ratio of GAS-6 release into cell culture medium. The groups treated with adiponectin, resistin, visfatin, ghrelin, and Il-6 as well as the unstimulated cells showed a significantly reduction in the GAS-6 levels due to ADAM-10 blockade; however, this effect was not observed in the presence of leptin. The GAS-6 levels did not significantly change in the cells cultivated under compressive forces despite ADAM-10 blockade (Figure 4c).

ADAM-10 antagonism also exerts a strong inhibitory effect on Axl release: The cell pretreatment with 10 μM GI254023X (Sigma-Aldrich) during 1 h significantly reduces the Axl release on SV-PDL cells cultivated under normal conditions over a period of 24 h. Alike, the cells treated with leptin, adiponectin, resistin, ghrelin, and Il-6 or maintained under compressive forces of 2.4 gf/cm² showed a significant reduction in the Axl levels measured on cell supernatants. A moderate Axl release downregulation (p = ns) was observed in the group treated with visfatin (Figure 4d).

4 | DISCUSSION

In the current study, we show that SV-PDL cells are a target of GAS-6. In vitro, GAS-6 promotes SV-PDL cell migration, viability, and proliferation, inducing the upregulation of *TGF-β1*, *Collagen-1*, *Collagen-3*, and *Periostin* expression. We also observed a downregulation of *Caspase-3*, *Caspase-8*, *and Caspase-9* as well as a reduction in the expression of *IL-6* in response to GAS-6, suggesting a possible protective effect on these cells. Additionally, the inhibition of ADAM-10 attenuates GAS-6 release, an effect that was partially reversed by leptin. Finally, we demonstrated that ADAM-10 blockade has a significant inhibitory effect on Axl release.

TAM receptors are widely expressed on different cells as osteoclast (Mahasandana et al., 1990; Ruiz-Heiland et al., 2014) or endothelial vascular smooth muscle cells (O'Donnell et al., 1999). TAM receptors are involved in many important mechanisms principally in the regulation of cell hemostasis and inflammation (Camenisch et al., 1999).

It has previously been shown that GAS-6 interacts with different cell lines (Espindola et al., 2018; Nakano et al., 1997; Zhang et al., 2020) and regulates their cell biological response in vitro. For example, Nakano et al. (1997) described that GAS-6 promotes vascular smooth muscle cells proliferation induced by $Ca2^+$ mobilizing growth factors. Espindola et al. (2018) showed that GAS-6/TAM receptor activity contributes to the activation of pulmonary fibroblast. Recently, Zhang et al. (2020) described that GAS-6 promotes increased migration as well as mineralized nodule formation

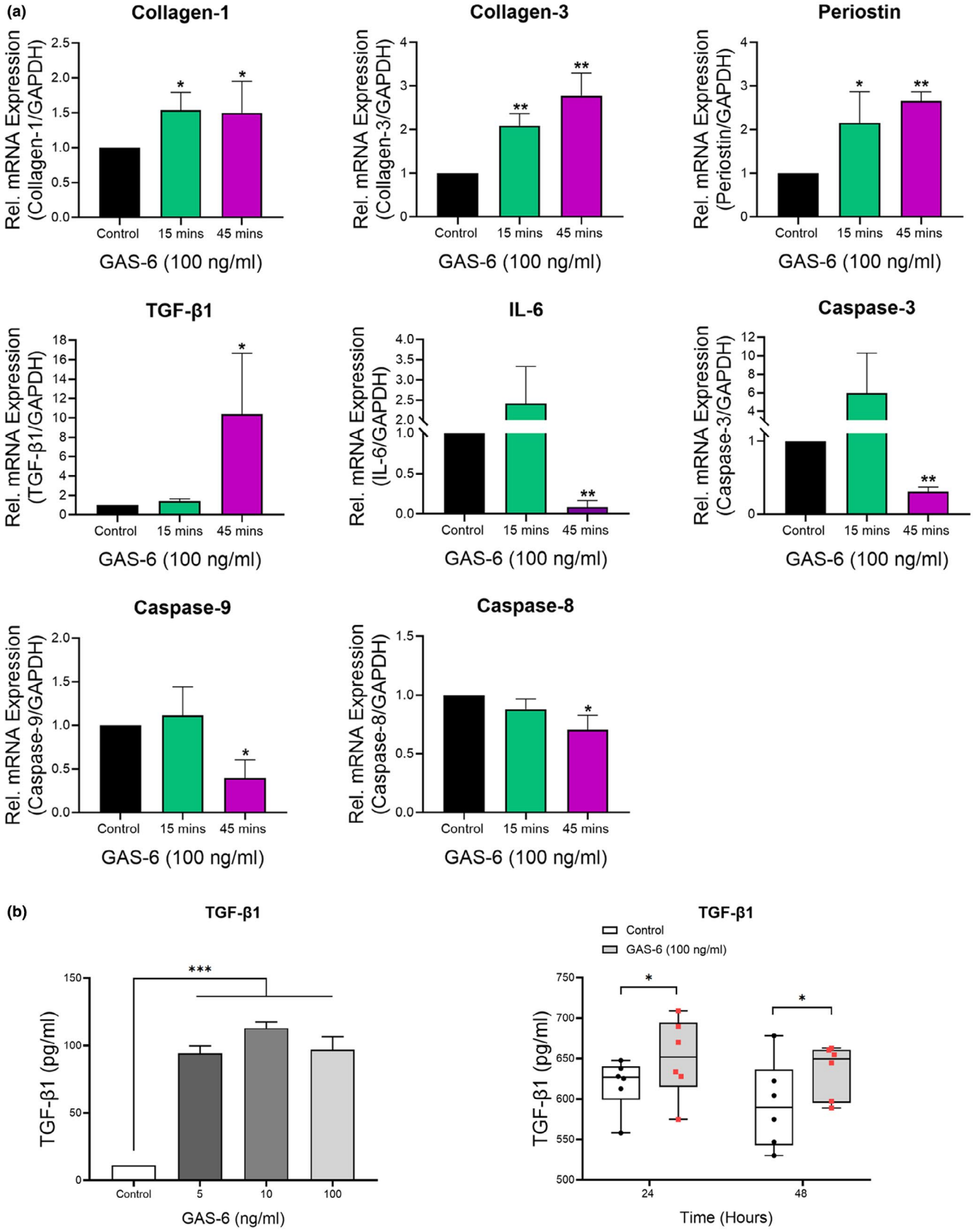


FIGURE 3 Effect of GAS-6 on the mRNA expression of *Collagen-1*, *Collagen-3*, *Periostin*, *TGF-β1*, *IL-6*, *Caspase-3*, *Caspase-8*, *Caspase-9*, and TGF-β1 release in mouse periodontal ligament cells. (a): RT-PCR was performed on extracts from GAS-6-treated SV-PDL cells cultured after 15 and 45 min. *GAPDH* was used as housekeeping gene. Results were analyzed based on $2^{-\Delta\Delta Cq}$ method. (b): TGF-β1 release after GAS-6 stimulation measured by ELISA assay. GAS-6 promotes TGF-β1 release measured on supernatants after 24 and 48 h. $n = 3$ independent biological replicates. Graphs = mean ± SD. ns $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

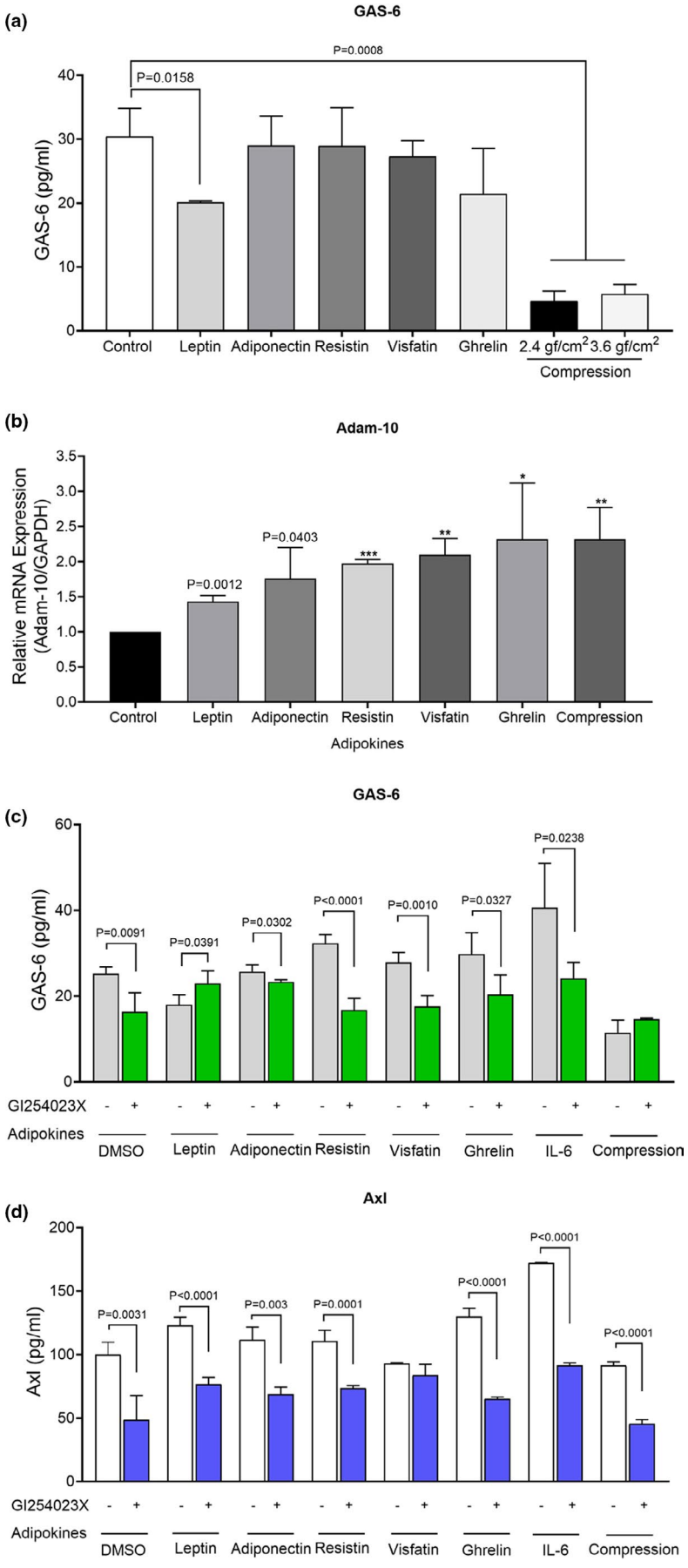


FIGURE 4 Downregulation effect of leptin as well as compressive forces on GAS-6 release is independently regulated by ADAM-10 metalloprotease. (a): SV-PDL cells can release in vitro soluble GAS-6 in the presence of vitamin K1 (5 μg/ml, Konakion®, CHRPLAPHARM GmbH). This effect is decreased by leptin and by compressive forces. Relative to the normal homogenates, soluble GAS-6 is significantly decreased after leptin as well as compressive force addition. (b): Adipokines or compression up-regulate *ADAM-10* mRNA expression on SV-PDL cells. (c): Soluble GAS-6 levels are reduced by ADAM-10 blockade. This effect is not observed when cells are co-cultivated with exogenous leptin. (d): Soluble Axl release is counteracted by ADAM-10 inhibition despite adipokines, IL-6 or compressive co-stimuli. $n = 3$ independent biological replicates. Graphs = mean ± SD. ns $p > 0.05$ $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

in human periodontal ligament cells. Additionally, in accordance with the results published by Zhang et al. (2020), we also observed that GAS-6 strongly induced SV-PDL cell migration, viability, and proliferation especially at a concentration of 100 ng/ml.

Our present results also indicate that GAS-6 strongly induces TGF-β1 release from SV-PDL cells. Transforming growth factor-beta-1 (TGF-β1) is a potent stimulator of tissue regeneration (Shi & Massague, 2003). It has been shown that TGF-β1 improves bone-guided tissue regeneration in animal models (Wikesjo et al., 1998). TGF-β1 promotes connective tissue growth, being involved in extracellular matrix production and collagen-1 stimulation, which is essential for the development and regeneration of periodontal tissue including the periodontal ligament (Asano et al., 2005). In periodontal ligament cells, it has also been described that TGF-β1 positively induce *Collagen-1* and *Periostin* mRNA expression (Watanabe et al., 2014). Periostin is essential for periodontal ligament remodeling during orthodontic treatment (Xu et al., 2017). Considering the fact that TGF-β1 release is strongly promoted by GAS-6, we hypothesized here that GAS-6 protein levels may be crucial for periodontal ligament homeostasis during orthodontics, because its capacity to regulate *TGF-β1*, *Collagen-1*, *Collagen-3*, and *Periostin* expression.

Orthodontic tooth movement (OTM) in obese patients differs from the process in normal weight patients due to the imbalance in the secretion of adipocyte-derived adipokines (Yong et al., 2020,2021). As compressive force to the PDL progresses, the roles of leptin on the PDL cells are miss regulated owing to the concentration changes (Yong, von Bremen, Ruiz-Heiland, et al., 2021).

Force changes in the compression distribution in the periodontium after the application of mechanical forces trigger the remodeling process, which requires inflammation-like reactions, apoptosis, and biologically active substances, such as enzymes and cytokines, responsible for connective tissue remodeling (Dilsiz et al., 2010). The mechanical forces compress the PDL cells in the pressured area. From our result, GAS-6 is regulated by adipokines and compressive forces. Moreover, GAS-6 modulates the gene expression of *Collagen-1*, *Collagen-3*, *Periostin*, *TGF-β1*, *IL-6*, *Caspase-8*, and *Caspase-9* on SV-PDL cells.

TAM receptor activation displays both anti-inflammatory and pro-inflammatory effects, depending on the target cell type (Goruppi et al., 1996). In vitro studies have shown that GAS-6 is able to protect serum-starved NIH3T3 cells from cell death by apoptosis as induced by complete growth factor depletion (Goruppi et al., 1996). Other studies have also revealed the anti-apoptotic effect of GAS-6. For example, Hasanbasic et al. (2004) showed that GAS-6 protect primary endothelial cell from apoptosis as well as Melaragno et al. (2004) demonstrated its protective effect on vascular smooth muscle cells. Grommes et al. (2008) described that GAS-6 inhibits the pro-inflammatory cytokine production in cardiomyocytes. Another cell-based study using monocytes showed that GAS-6 has the property to inhibit IL-6 secretion regulating by PI3K/Akt/GSK3β and NF-κB (Alciato et al., 2010). In light of the current literature, it is evident that the GAS-6/TAM system has anti-inflammatory properties, mainly deriving from the modulation of genes activity such *IL-6* expression (Alciato et al., 2010).

Here, we show that exogenous GAS-6 drastically reduces the mRNA levels of *IL-6* after a short initial upregulation, as well as it negatively regulates caspases mRNA expression. However, further research should be performed to elucidate the possible anti-inflammatory and anti-apoptotic effect of GAS-6 on periodontal cells.

Animal studies have shown that increased GAS-6 can be associated with a predisposition to suffer from obesity. Maquoi et al. (2005) demonstrated in their study using GAS-6-deficient mice that these animals have a significantly less fat mass as their wild-type littermates when they were fed with high-fat diet. This evidence suggests that the GAS-6/TAM system can represent an important pathogenic mechanism for obesity and obesity-associated inflammation.

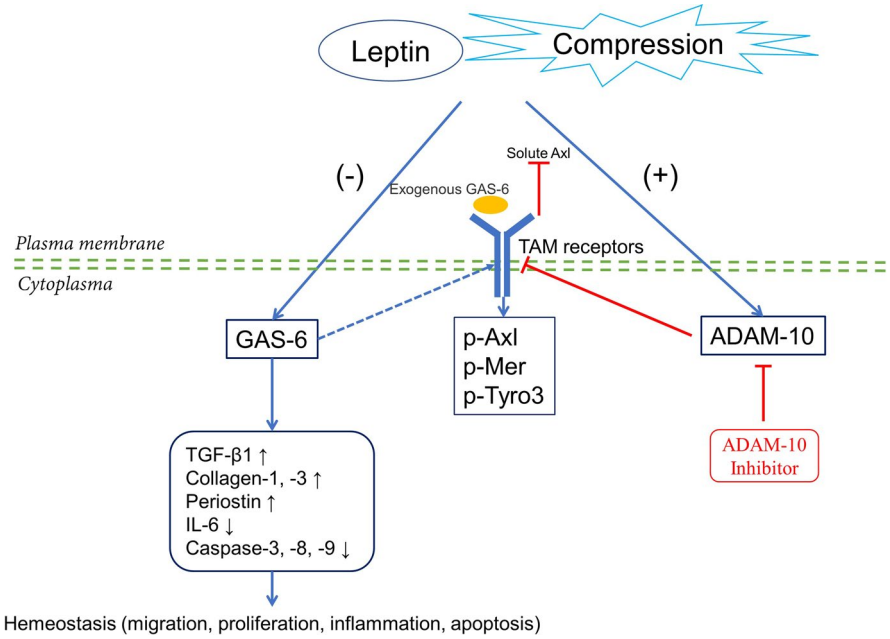
Clinical studies indicate that the plasma concentration of GAS-6 varies between 13 and 23 ng/ml. Hsiao et al. (2013) reported that plasma GAS-6 levels in obese adolescents (13.9 ± 3.9 ng/ml) were significantly higher than those in lean adolescents (12.3 ± 4.4 ng/ml). In the present study, we showed that the release of GAS-6 from PDL-SV cells was not significantly altered by cell cultivation in combination with different adipokines except leptin that significantly reduced GAS-6 expression. The cell cultivation under other conditions as for example application of compressive forces also does significantly reduced GAS-6 release. On the contrary, the addition of IL-6 reduces GAS-6 release on SV-PDL cells.

In the regulation of GAS-6 secretion, A-Disintegrin and Metalloproteinases (ADAMs) such as ADAM-10 play a central role in the cell surface shedding of ectodomains for TAM receptors. ADAM-10 cleaves Axl, the resulting cleaved form of receptors can induce internalization of the receptor and transport of the membrane for recycling (O'Bryan et al., 1995).

The proteolytic cleavage of the TAM receptors Tyro3, Axl, and Mer are catalyzed by metalloproteinases specially ADAM-10 (Flem-Karlsen et al., 2020) and ADAM-17 (Thorp et al., 2011). It has been described that GAS-6 positively correlated with soluble Axl and Mer (Weinger et al., 2009); thus, we further investigated the effect of ADAM-10 blockade in the release of GAS-6 and Axl. Here, we could determinate that SV-PDL cells have the ability to release GAS-6 and Axl in the presence of vitamin K1, effect that can be almost counteracted by ADAM-10 blockade, except in the group treated with leptin, indicating that ADAM-10 promote Axl secretion, and that GAS-6/Axl regulate periodontal ligament cell homeostasis.

Soluble Axl blocked the protective effect of membrane-bound Axl by inhibiting GAS-6 induced tyrosine phosphorylation of Axl (Rothlin et al., 2007). The binding of GAS-6 to TAM receptors acts as an inhibitor of inflammation by inhibiting Toll-like receptor and cytokine receptor cascades (Rothlin et al., 2007). Thus, loss of GAS-6 signaling, along with the dysregulation of balance between soluble Axl and Mer, might detrimentally affect the homeostasis of SV-PDL, especially during OTM.

GAS-6 is decreased by stimulation with leptin or compression. ADAM-10 is significantly elevated in response to adipokines or



SCHEME 1 The proposed model describing that growth arrest-specific 6 protein maintains periodontal ligament cells homeostasis and negative regulated by leptin as well as compression independently of ADAM-10 metalloprotease

compressive force stimulation. Furthermore, ADAM-10 blockade increases the GAS-6 secretion by leptin/compression addition. These results indicate that the regulation effect of ADAM-10 on GAS-6 secretion is independently of leptin/compression (Scheme 1).

Moreover, ADAM-10 blockade decreases soluble Axl in the presence of adipokines, IL-6, or compression. This is in agreement with a previous study (Miller et al., 2016) which revealed that in vitro ADAM-10 most efficiently cleaves Axl and therefore is most likely to be the principal factor responsible for cleaving Axl to its soluble form in vivo. Thus, adipokines/compression regulate the soluble Axl by ADAM-10.

Recently, it has shown that leptin exert a strong inductor of PGE_2 release on radicular cells, acting a high concentrations as a pro-apoptotic inductor on cementoblasts (Ruiz-Heiland et al., 2020). We thus hypothesize that higher leptin concentration in the periodontium due obesity negative correlates with GAS-6 and soluble Axl on periodontal ligament cells. Continued clinical and experimental investigation is required to elucidate the GAS-6 effect during OTM.

To conclude, the periodontal ligament cells express TAM receptors and release GAS-6 protein in vitro. The GAS-6 feedback mechanism on SV-PDL cells is influenced by leptin as well as by compressive forces. ADAM-10 metalloprotease blockade can effectively reduce GAS-6 and Axl release. These findings have pointed out the role of GAS-6/TAM regulation in periodontal ligament cells.

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CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTIONS

Jiawen Yong: Data curation; Formal analysis; Methodology; Resources; Software; Supervision; Validation; Visualization; Writing – original draft. **Sabine Elisabeth. Groeger:** Formal analysis; Project administration; Supervision; Validation; Writing – review & editing. **Sabine Ruf:** Conceptualization; Funding acquisition; Project administration; Supervision; Validation; Writing – review & editing. **Gisela Ruiz-Heiland:** Methodology; Supervision; Validation; Writing – review & editing.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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