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GDF15 Promotes the Osteogenic Cell Fate of Periodontal Ligament Fibroblasts Affecting Their Mechanobiological Response

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# Equal contribution

Abstract: Periodontal ligament fibroblasts (PdLFs) exert important functions in oral tissue and bone remodeling following mechanical forces, which are specifically applied during orthodontic tooth movement (OTM). Located between the teeth and the alveolar bone, mechanical stress activates the mechanomodulatory functions of PdLFs including the regulation of local inflammation and activation of further bone-remodeling cells. Previous studies suggested the growth differentiation factor 15 (GDF15) as important pro-inflammatory regulator during the PdLFs mechanoresponse. However, the precise mechanism remains to be clarified, as GDF15 may act both intracrine and by receptor binding, potentially also in an autocrine manner. The extent to which PdLFs are susceptible to extracellular GDF15 has not yet been investigated. Thus, our study aims to examine the influence of GDF15 exposure on cellular properties of PdLFs and their mechanoresponse, which seems particularly relevant regarding disease- and aging-associated elevated GDF15 serum levels. Therefore, in addition to investigating potential GDF15 receptors, we analyzed its impact on proliferation, survival, senescence, and differentiation of human PdLFs, demonstrating a pro-osteogenic effect upon long-term stimulation. Furthermore, we detected an altered force-related inflammation and impaired osteoclast differentiation. Overall, our data suggest a major impact of extracellular GDF15 on PdLFs differentiation and their mechanoresponse.

Keywords: GDF15; orthodontic tooth movement; periodontal ligament fibroblasts; osteoblast differentiation; mechanobiological response; inflammation; osteoclast activity

1. Introduction

Orthodontic treatment of tooth malocclusions aims to improve and prevent medically relevant diseases of the teeth and supporting tissues such as the periodontium [1]. Not only highly prevalent in relation to the development of caries and periodontal inflammatory diseases, malocclusions and niches resulting from crowding of teeth may also lead to root recession and tooth loss in the long term [2,3]. As prophylactic therapy, orthodontic tooth movement (OTM) plays an important role in limiting the development and progression of these diseases. However, orthodontic therapy is associated with risks, including tooth root resorption, attachment or even tooth loss [4–6]. The underlying causes of increased occurrence of these risks appear to be multifactorial. In addition to genetic and epigenetic variations, changes in oral and systemic health as well as treatment-specific conditions are particularly reported [7,8]. In particular, extrinsic factors including hormones and growth factors also appear to influence the mechanofunctional remodeling processes of tissue and bone required for tooth movement [9].

The periodontal ligament (PdL) is a fibrous connective tissue located around the tooth root that serves to anchor the tooth in the alveolar bone and modulates the remodeling processes of tissue and

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bone induced by orthodontic forces leading to tooth movement [10]. The heterogeneous tissue includes diverse cell populations such as fibroblasts, osteoblasts, epithelial cell remnants of Malassez, macrophages and undifferentiated mesenchymal stem cells [11]. The predominant cell type are PdL fibroblasts (PdLFs), which, however, are characterized by many osteoblast-like properties as they express osteogenic markers like alkaline phosphatase and osteocalcin and are able to form mineral-like nodules in vitro [12–14].

During OTM, mechanical stimuli in form of tensile and compressive strains are generated, which trigger force-specific mechanobiological functions of PdLFs fostering a favorable microenvironment for tissue and bone remodeling [15–17]. This includes the modulation of aseptic transient inflammatory responses by specific pro- and anti-inflammatory cytokines and the activation of bone-remodeling cells. In this context, the RANKL/RANK/OPG signaling pathway is particularly crucial as it modulates osteoclast differentiation and activity [18–21]. In response to specific stimuli, receptor activator of NF-kB ligand (RANKL) can be secreted by cells of osteogenic origin, stimulating the differentiation of osteoclast precursors by binding to their transmembrane-receptor RANK. Osteoclastogenesis can be blocked by binding of RANKL to the decoy receptor osteoprotegrin (OPG), which can be also secreted by osteoblast-like cells. Unfavorable RANKL/OPG values are not only relevant in terms of bone remodeling defects, but also in root resorption as well as attachment and tooth loss [22].

Tensile forces particularly promote bone formation by increasing secretion of the anti-inflammatory interleukin IL-10 and stimulation of osteoblast differentiation [23,24]. Due to the intrinsic potential of PdL fibroblasts to differentiate into osteoblasts, increased expression of osteogenic markers such as alkaline phosphatase (ALP) and runt-related transcription factor 2 (RUNX2) as well as increased calcium deposits were detected in this context [25,26]. Contrary, compression promotes a bone-resorbing microenvironment by inducing hypoxia and the secretion of pro-inflammatory cytokines including IL-6, IL-8 and prostagladine E2 (PGE2), as well as RANKL by PdLFs [27–30].

As stress-dependently expressed and secreted multifunctional regulator [31,32], we recently identified growth differentiation factor 15 (GDF15) as important pro-inflammatory modulator in the mechanoresponse of PdLFs to compressive forces [33,34]. As member of the transforming growth factor-β (TGF-β)/bone morphogenic protein (BMP) superfamily [31], GDF15 exerts important functions in the regulation of various cellular processes [32,35]. Synthesized as precursor protein, GDF15 undergoes disulfide-linked dimerization prior to secretion [36]. Extracellular GDF15 can bind to cell membrane receptors, with the α-like receptor of the GDNF family (GFRAL) being the best characterized receptor exclusively expressed in the central nervous system [37]. Additionally, it has been reported that members of the activin receptor-like kinase (ALK) family also bind GDF15 and mediate autocrine and paracrine functions [38–40]. Besides receptor-mediated signaling of GDF15, unprocessed GDF15 has also been reported to modulate gene expression after translocation into the nucleus [41,42].

GDF15 serum levels are highly elevated in association with various diseases as well as aging and have been implicated as potential biomarkers [43,44]. Considering its crucial role in the mechanoresponse of PdL fibroblasts [34,45], prolonged exposure to GDF15 could impair their functions and thus increase the risks for these patients in the context of orthodontic therapy. This seems particularly relevant considering that GDF15 has been shown to modulate osteoblast differentiation and function [46,47], thereby potentially also altering cell fate and mechanoresponse of PdLFs. Therefore, this study aims to investigate the effect of an extended GDF15 exposure on the cellular properties and mechanobiological functions of PdL fibroblasts.

2. Results

2.1. hPdLFs express potential receptors for GDF15

Considering the neuron-specific expression of the best-characterized GDF15 receptor GFRAL, members of the ALK protein family are discussed as possible receptors in other cell types [38–40].

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We analyzed their expression in hPdLFs by quantitative PCR indicating gene expression of all family members (*ALK1-7*; Figure 1a). Western Blot analysis validated the expression of all members of the ALK family on protein levels (Figure 1b). Thus, extracellular GDF15 might potentially also stimulates PdL fibroblasts by ALK binding.

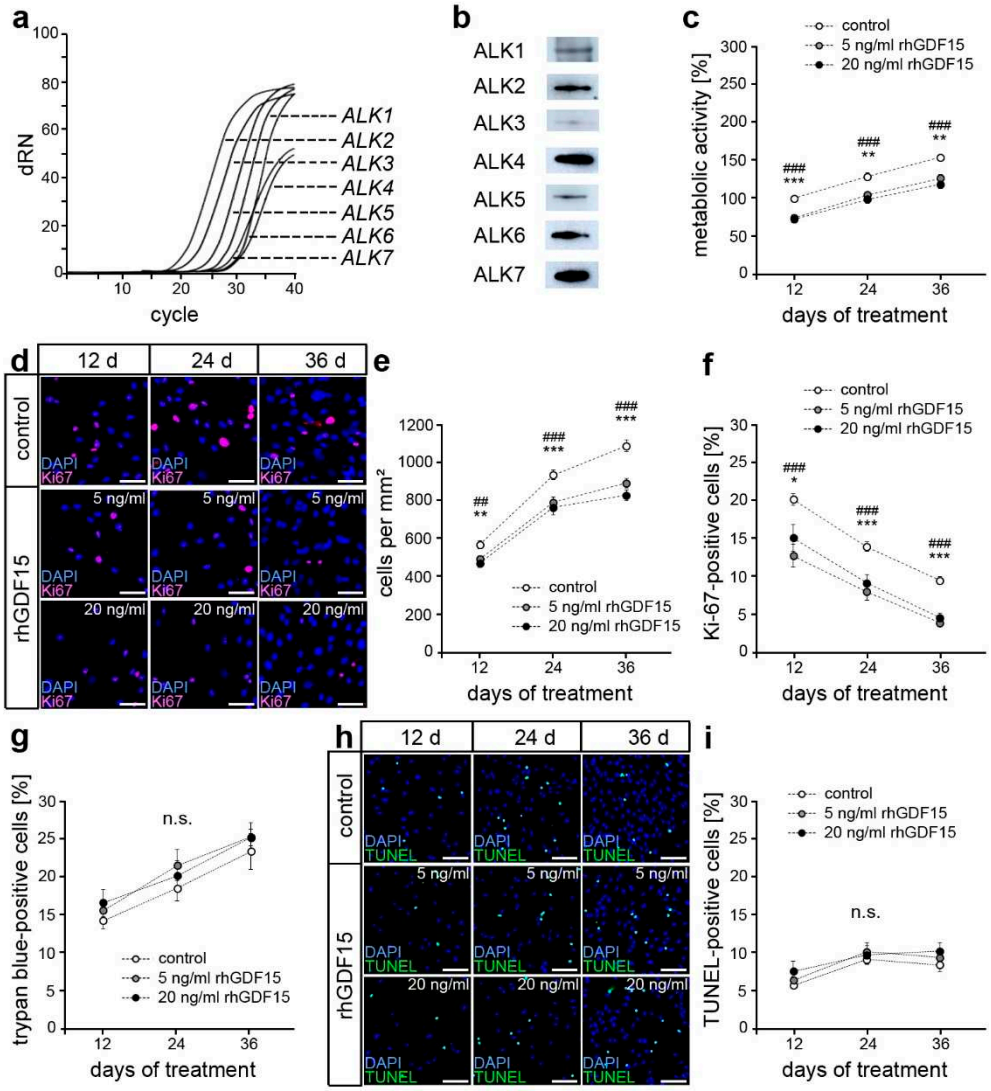

Figure 1. GDF15 limits cell proliferation of hPdLFs without affecting cell survival in the long-term. **(a, b)** RNA (a) and protein (b) expression of activin receptor-like kinases (ALK1-7) in hPdLFs. **(c)** Metabolic activity of hPdLFs after stimulation with 5ng/ml and 20 ng/ml recombinant human GDF15 (rhGDF15) for 12, 24 and 36 days (d) displayed in relation to the 12-days control. **(d-f)** Ki-67-positive hPdLFs (magenta) after stimulation with rhGDF15 with cell nuclei (blue; d). The number of cells per mm² is displayed in (e) and (f) shows the proportion of Ki-67-positive hPdLFs. **(g)** The number auf trypan blue-positive hPdLFs after rhGDF15 stimulation indicating the proportion of dead cells. **(h, i)** TUNEL-positive hPdLFs (green, h) after rhGDF15 stimulation with cell nuclei (blue) and displayed as proportion to the cell number (i). * $p < 0.05$; **/## $p < 0.01$; ***/### $p < 0.001$; */**/*** control in relation to 5 ng/ml rhGDF15; ##/### control in relation to 20 ng/ml rhGDF15; one-way ANOVA and Tukey post hoc test. Scale bar: 20 µm in (d) and 25 µm in (h). dRN, difference between baseline and measured fluorescence; n.s., not significant.

2.2. GDF15 exposure limits cell proliferation without affecting the survival of hPdLFs

To evaluate the impact of GDF15 on hPdLFs, we first analyzed metabolic activity after 12, 24, and 36 days of stimulation with 5 ng/ml and 20 ng/ml recombinant human GDF15 protein (rhGDF15;

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Figure 1c). A 12-day stimulation already resulted in reduced metabolic activity, which was also maintained with longer stimulation duration. Since changes in metabolic activity may be based on differences in cell number and thus proliferation level, we subsequently determined cell density (Figure 1d, e). After 12 days of stimulation, a significant decrease in cell density was observed, which became even more pronounced with increasing duration of exposure, but independent of the applied concentration of rhGDF15. To determine the proportion of proliferative cells, immunofluorescence staining of the proliferation marker Ki-67 was performed after 12, 24, and 36 days of rhGDF15 exposure (Figure 1d, f). Under all conditions, a decreasing proportion of Ki67-positive cells was observed over the culture period, resulting from either growth senescence due to limited space or induction of differentiation programs. However, this decrease was significantly stronger in rhGDF15-stimulated hPdLFs resulting in the lowest numbers of Ki-67-positive cells after 36 days of exposure compared to the control. A significant difference between both concentrations of the applied rhGDF15 could not be detected at any of the analyzed time points. Since GDF15 was reported to effect cell survival, we additionally analyzed the proportion of damaged cells using trypan blue staining (Figure 1g) and apoptosis using TUNEL assay (Figure 1h, i), respectively. However, we neither detected changes in cell survival in relation to culture duration nor caused by exposure to rhGDF15. Together, GDF15 limits the proliferative capacity of hPdLFs without affecting cell survival.

2.3. Long-term exposure to GDF15 promotes osteogenic cell fate of hPdLFs

The reduced proliferation of hPdLFs could be caused by increased cellular senescence or osteogenic differentiation, which are both potentially affected by GDF15, as previously shown in other studies [48,49]. Quantitative analysis of the immunofluorescent staining intensity of p21, an early senescence marker, revealed significantly higher expression levels after 12 and 24 days of rhGDF15 stimulation at both concentrations (Figure 2a, b). However, this increase could not be detected on the latest stimulation point, where p21 levels were not significantly different from the corresponding control. These findings were confirmed by analysis of β-galactosidase activity (Figure 2c, d). Thus, our data suggest enhanced activation of cellular senescence after 12 days of stimulation with rhGDF15, but not with prolonged exposure.

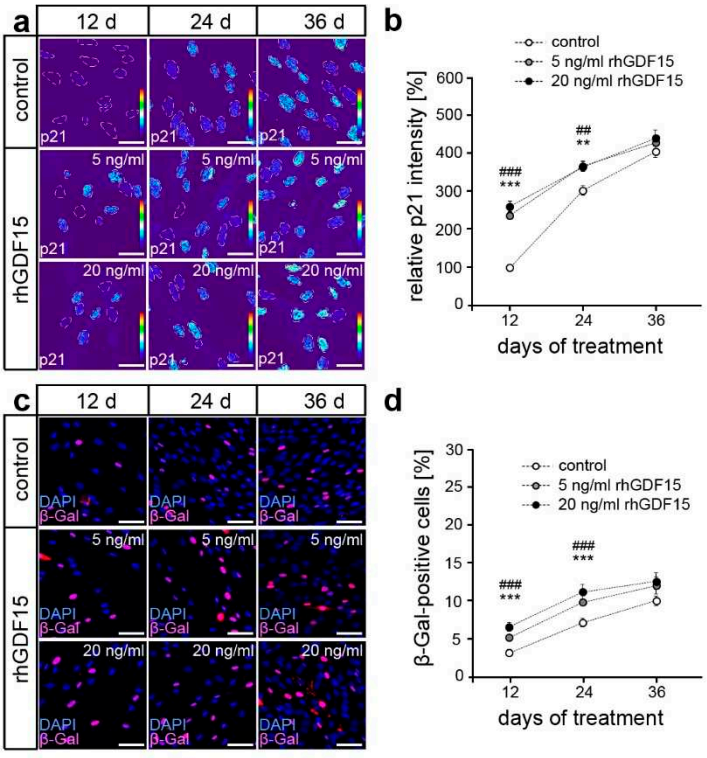

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Figure 2. GDF15 promote cellular senescence of hPdLFs. **(a, b)** p21 intensity in hPdLFs stimulated with 5 ng/ml and 20 ng/ml rhGDF15 for 12, 24 and 36 days (d) shown as thermal LUT (a). Grey scattered lines surround the cell nuclei. Mean p21 intensity in shown in relation to the 12-days control in (b). **(c, d)** β-galactosidase (β-Gal)-positive hPdLFs (magenta) after rhGDF15 stimulation with cell nuclei (blue, c). The proportion of β-Gal-positive hPdLFs is displayed in (d). **/## $p < 0.01$; ***/### $p < 0.001$; */**/*** control in relation to 5 ng/ml rhGDF15; ##/### control in relation to 20 ng/ml rhGDF15; one-way ANOVA and Tukey post hoc test. Scale bar: 10 µm in (a) and 25 µm in (c).

In the following, we aimed to address the osteogenic differentiation and potential alterations by GDF15, which may be a considerable reason for the diminished proliferative capacity of stimulated hPdLFs apart from senescence. To this end, quantitative expression analysis of the osteogenic marker genes *ALPL* and *RUNX2* were performed and showed increased levels after 12 and 24 days of rhGDF15 exposure (Figure 3a, b). However, compared to the corresponding control, no difference in osteogenic gene expression was detected at the last time point analyzed. Further analysis of alkaline phosphatase activity as a characteristic of osteogenic differentiation, clearly showed enhanced levels after 36 days of rhGDF15 exposure (Figure 3c, d), while no significant changes were detected at earlier time points (data not shown; p-values < 0.05). We used hPdLFs stimulated with dexamethasone and β-glycerol phosphate for 36 days as differentiation control [50].

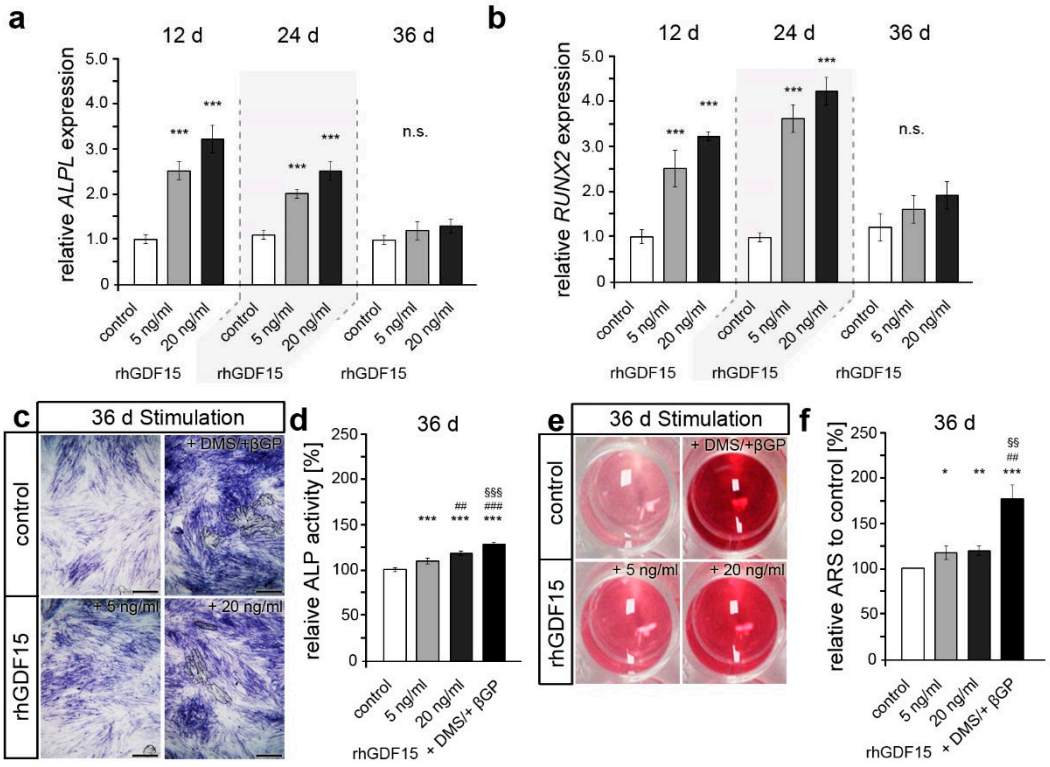

Figure 3. Long-term exposure to GDF15 foster the osteogenic differentiation of hPdLFs. **(a, b)** *ALPL* and *RUNX2* expression encoding osteoblast-related markers in hPdLFs stimulated with 5ng/ml and 20 ng/ml recombinant human GDF15 (rhGDF15) for 12, 24 and 36 days (d) displayed in relation to the control at each time point. **(c, d)** ALP activity (dark blue; c) of 36-days rhGDF15-stimulated hPdLFs displayed in relation to the control in (d). **(e, f)** Alizarin red intensity of hPdLFs in wells stimulated 36 d with rhGDF15 (e) displayed in relation to the control in (f). Stimulation with DMS and βGP was used as positive control. * $p < 0.05$; **/##/§§ $p < 0.01$; ***/###/§§§ $p < 0.001$; */**/*** in relation to control; ##/### in relation to 5 ng/ml rhGDF15; §§/§§§ in relation to 20 ng/ml rhGDF15; one-way ANOVA and Tukey post hoc test. Scale bar: 25 µm in (c).

Considering that ALP activity is critical for mineralization, we further analyzed calcium deposit formation as a marker of active osteoblasts at 36 days of rhGDF15 stimulation (Figure 3e, f). Likewise

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as for ALP activity, a slightly increased formation of mineralized deposits was observed upon long-term stimulation with rhGDF15, which also appeared to be concentration-independent. However, the increase was significantly higher for the osteogenic differentiation control.

Taken together, our data suggest that GDF15 promotes osteogenic differentiation of hPdLFs upon long-term exposure, whereas cellular senescence is more prominent with shorter stimulation periods.

2.4. Preceding long-term exposure of hPdLFs to rhGDF15 affect their mechanoreactivity

In the following, we aimed to determine the extent to which the shift towards an osteogenic cell fate by rhGDF15 exposure affects the functionality of hPdLFs responding to mechanical stimuli. To this end, we first applied biaxial tensile force for 24 hours on hPdLFs stimulated for 36 days with rhGDF15. Subsequent analysis of genes encoding the anti-inflammatory markers IL-10 (*IL10*; Figure 4a) and IL-1RA (*IL1RN*; Figure 4b), which are increased in hPdLFs following tensile forces [51], revealed no relevant changes due to rhGDF15 exposure. Even though the expression of *IL1RN* appears increased in GDF15-stimulated fibroblasts after tensile force, the baseline levels (red lines) are already enhanced, resulting in comparable fold changes of all conditions to the corresponding tensile-stressed cells (control: 2.42 ± 0.06; 5 ng/ml rhGDF15: 2.21 ± 0.07, p-value to control 0.99847; 20 ng/ml rhGDF15: 2.69 ± 0.09, p-value to control 0.98791). We next analyzed adherent THP1 cells used to visualize the inflammatory response by determining the activation of monocytic immune cells (Figure 4c, d). Whereas tensile force promoted an anti-inflammatory response with reduced THP1 activation, prior rhGDF15 exposure blocked this response.

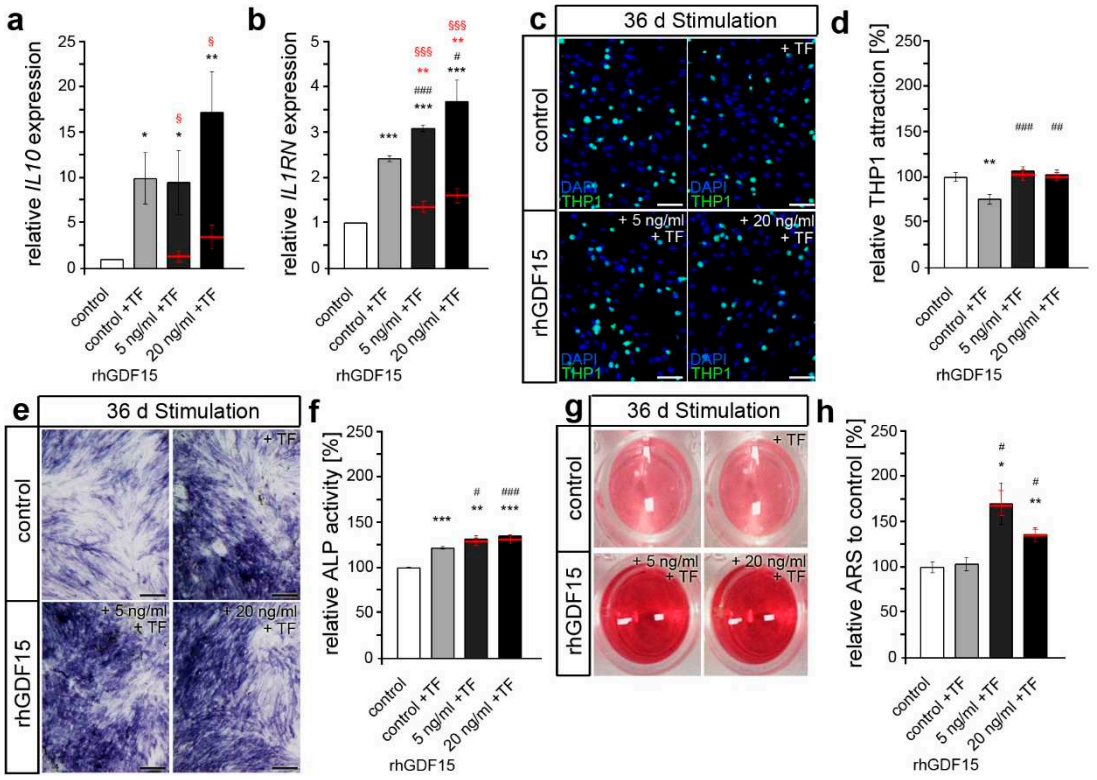

Figure 4. Long-term GDF15-exposed hPdLFs show a reduced anti-inflammatory and pro-osteogenic mechanoresponse to tensile forces. **(a, b)** Quantitative expression levels of *IL10* (a) and *IL1RN* (b) encoding anti-inflammatory markers in hPdLFs stimulated with 5ng/ml and 20 ng/ml recombinant human GDF15 (rhGDF15) for 36 days (d) and stressed with tensile forces for 24 hours (+TF) displayed in relation to the untreated control. (**c, d**) Adherent (activated) THP1 cells (green) on stimulated and stressed hPdLFs (blue, cell nuclei) displayed as number of THP1 cells per hPdLFs and in relation to the untreated control in (d). **(e, f)** ALP activity (dark blue) of 36-days rhGDF15-stimulated hPdLFs stressed by TF (c), displayed in relation to the control in (d). (**g, h**) Alizarin red staining intensity of

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hPdLFs in wells stimulated 36 d with rhGDF15 and stressed by TF (g), displayed in relation to the control in (h). Stimulation with DMS and βGP was used as positive control. Red lines showing baseline levels of the respective condition. */#/§ $p < 0.05$; **/##/§§ $p < 0.01$; ***/###/§§§ $p < 0.001$; */**/*** in relation to control; #/##/### in relation to control +TF; §§/§§§ baseline (red) in relation to 5 ng/ml or 20 ng/ml rhGDF15 +TF; one-way ANOVA and post hoc test (Tukey). Scale bars: 25 μm in (e).

Since bone formation and the activation of osteoblast activity are key features for the tensile site, we further analyzed ALP activity induction in hPdLFs after 24 hours of biaxial tensile stress (Figure 4e, f). Whereas the control treatment displayed increased ALP activity due to the application of tensile force, this was not detected for rhGDF15-stimulated hPdLFs pointing to a limited activation of further osteoblasts by mechanical stress. Of note, ALP activity in rhGDF15-exposed stretched hPdLFs was still significantly higher than in stressed control cells due to increased baseline levels (Figure 4f; (5 ng/ml rhGDF15 to control: p-value $0{,}0001 \times 10^{27}$, ***; 20 ng/ml rhGDF15 to control: p-value 0.0002×10^{5}, ***). Furthermore, we examined the formation of calcium deposits after application of tensile stress (Figure 4g, h). While the previously detected GDF15-dependent increase in calcium deposit formation was also detected in force-stressed cells, neither the control cultures nor rhGDF15-stimulated hPdLFs showed tensile-related differences compared to unstressed hPdLFs, which might be expected due to the short duration of the stress application.

Based on its pivotal role in modulating the pro-inflammatory response to compressive stimuli [34], we next examined the effects of preceding long-term stimulation on the mechanoresponse of compressed hPdLFs. To this end, we performed quantitative expression analysis of important genes encoding the pro-inflammatory cytokines IL-6 (*IL6*) and COX2 (Figure 5a, b). Whereas no GDF15-dependent changes were detected in the expression of *COX2*, *IL6* levels were significantly increased in compressed rhGDF15-stimulated hPdLFs. Of note baseline levels were not changed by rhGDF15 exposure. To investigate whether those changes in *IL6* expression also cause an altered inflammatory response and thus activation of immune cells, we analyzed the adhesion of THP1 monocytic cells accordingly (Figure 5c, d). Thus, compared with controls, increased activation of monocytic cells by compressed hPdLFs was detected during long-term stimulation with rhGDF15.

We next focused on a possible impact on the activation of bone-resorbing osteoclasts by analyzing *RANKL* and *OPG* expression of force-stressed hPdLFs, which encode key players in the regulation of osteoclastogenesis (Figure 5e, f). Whereas compression promoted a pronounced increase in *RANKL* levels and a significant decrease in *OPG* levels in controls, these changes were not observed in rhGDF15-stimulated hPdLFs. To functionally address changes in osteoclast activation, macrophages resulting from pre-stimulated THP1 monocytic cells were cultured for six days with the medium supernatant of force-stressed hPdLFs to stimulate their differentiation into osteoclasts [52] (Figure 5f, g). Using TRAP assay, enhanced differentiation into mononuclear pre-osteoclasts and matured multinuclear osteoclasts was detected after stimulation with the medium supernatant of compressed control fibroblasts compared to the unstressed control. Consistent with the RANKL and OPG expression data, a decreased number of osteoclast activation was observed. However, it was still enhanced compared to the non-stressed control and the rhGDF15-stimulated fibroblasts.

Together, our data indicate a relevant impact of long-term stimulation with GDF15 on the cellular properties and the pro-inflammatory mechanobiological response of human PdL fibroblasts resulting in increased inflammation due to compressive forces but reduced osteoclast activation potentially via modulation of RANKL/OPG.

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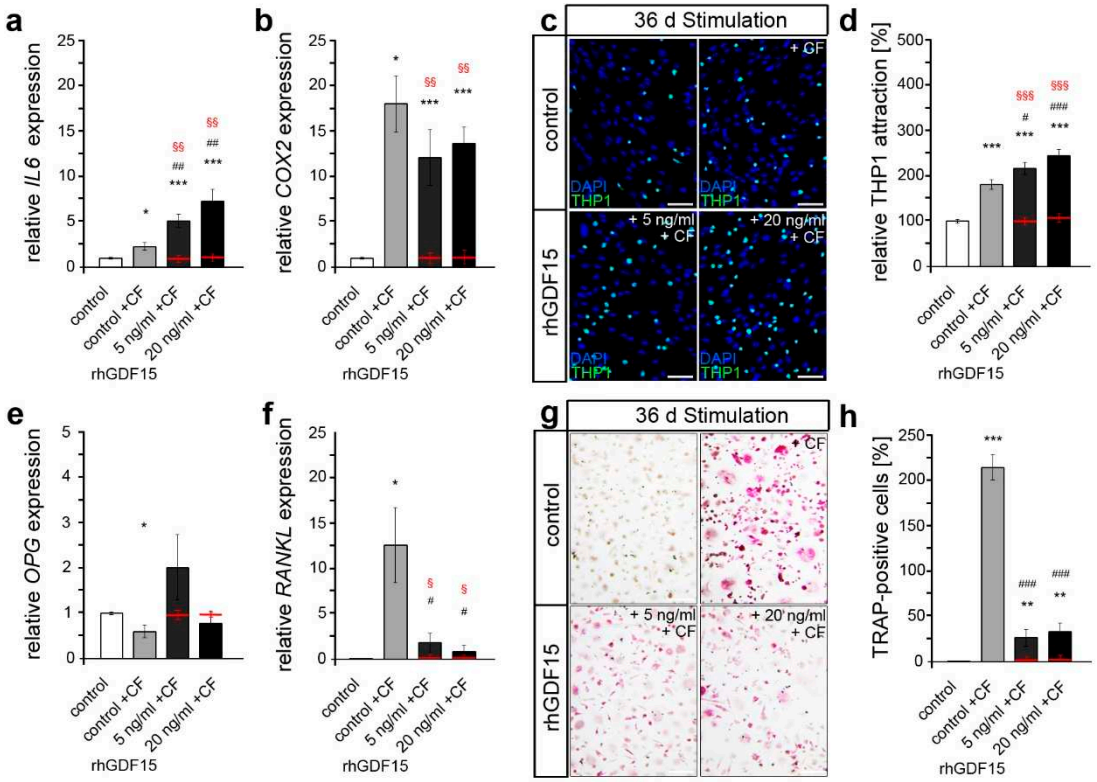

Figure 5. Long-term GDF15-exposed hPdLFs show an increased pro-inflammatory response to compressive stimuli with limited activation of osteoclasts. (a, b) Quantitative expression levels of *IL6* (a) and *COX2* (b) encoding pro-inflammatory markers in hPdLFs stimulated with 5ng/ml and 20 ng/ml recombinant human GDF15 (rhGDF15) for 36 days (d) and stressed with compressive forces for 24 hours (+CF), displayed in relation to the untreated control. (c, d) Adherent (activated) THP1 cells (green) on stimulated and stressed hPdLFs (blue, cell nuclei) displayed as number of THP1 cells per hPdLFs and in relation to the untreated control in (d). (e, f) Quantitative expression levels of *OPG* (e) and *RANKL* (f) in stimulated and stressed hPdLFs displayed in relation to the untreated control. (g, h) TRAP-positive THP1 cells (magenta; g) indicating the differentiation into osteoclasts, when stimulated with the medium supernatant of 36-days rhGDF15-stimulated hPdLFs additionally stressed by CF. The proportion of TRAP-positive osteoclasts is displayed in (h). Red lines showing baseline levels of the respective conditions. */#/§ $p < 0.05$; **/##/§§ $p < 0.01$; ***/###/§§§ $p < 0.001$; */**/*** in relation to control; #/##/### in relation to control +TF; §§/§§§ baseline (red) in relation to 5 ng/ml or 20 ng/ml rhGDF15 +TF; one-way ANOVA and Tukey post hoc test. Scale bars: 25 µm in (c) and (g).

3. Discussion

The interdependent and interrelated responses of periodontal ligament cells to mechanical forces generated during orthodontic treatment are modulated by a variety of key factors [27,53]. A change in their balance or functionality due to extrinsic and intrinsic factors can affect tooth movement. Recently, we demonstrated a relevant pro-inflammatory role of GDF15 in the mechanosignaling of PdLFs [34]. However, elevated GDF15 serum levels have been detected in various diseases and within the orthodontic context more relevant in obesity and aging [44,54], which might affect tooth movement in those patient groups.

Thus to uncover potential effects of an increased GDF15 exposure on the mechanofunctionality of periodontal cells, we stimulated PdL fibroblasts up to 36 days with recombinant GDF15 protein prior subsequent analysis of the response to mechanical forces. Thereby, GDF15 stimulation resulted in significantly decreased proliferation correlating with an increased rate of cellular senescence at shorter exposures, while longer stimulation resulted in a more prominent level of osteogenic differentiation. Surprisingly, compressed hyperinflammatory PdLFs did not display correlated

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excessive osteoclast activation, in fact, it was significantly attenuated due to long-term GDF15 exposure.

GDF15 is known for its multiple functions in tissue proliferation and differentiation and has also been detected in fibroblasts. Kim et al. demonstrated an inhibiting effect of GDF15 on renal fibroblast growth in primary fibroblasts isolated from mouse kidney subjected to ureteral obstruction-induced fibrosis [55]. In addition, decreased growth and activation of lung fibroblasts due to GDF15-associated alterations in the TGF-Smad pathway have been described [56]. Contrarily, Guo et al. recently revealed that GDF15 can promote proliferation in newborn rat cardiac fibroblasts after irradiation, which was confirmed by the increased cell proliferation rate and increased expression of fibrosis markers (Col1α and αSma) after transfection with GDF15 in vitro [57]. However, our data support an anti-proliferative effect of GDF15, specifically on hPdLFs, in terms of decreased Ki67, whereas we could not detect an impact on apoptosis rate of hPdLFs.

We presumed, that increased cellular senescence and osteogenic differentiation could be reasons for the anti-proliferative effect of GDF15 on hPdLFs. GDF15 is highly upregulated with age and associated with many age-related diseases [58]. It is thus suggested as a biomarker for aging [44]. Besides, GDF15 levels correlate positively with cellular senescence induced by ionizing radiation in human aortic endothelial cells [48]. However, the pleiotropic regulator GDF15 is also associated with anti-senescence effects. Thus, Li et al. reported that GDF15 downregulates p21, a senescence marker, via activation of both PI3K/AKT and MAPK/ERK signaling pathways, resulting in cervical cancer cell proliferation [59]. We detected higher p21 levels and increased ß-galactosidase activity due to GDF15 stimulation in hPdLFs, rather supporting the function of GDF15 as a promoter of cellular senescence.

Apart from cellular senescence, we considered osteogenic differentiation as a potential reason for GDF15-related reduction of hPdLF proliferation. In this study, we provide supporting evidence regarding the osteogenic potential of GDF15 in PdL fibroblasts. Quantitative expression analysis of osteogenic marker genes *ALPL* and *RUNX2* showed increased levels after 12 and 24 days of rhGDF15 exposure. ALP activity increased accordingly, but showed a temporal shift compared to expression pattern with its highest activity at 36 days of exposure. We further detected an increase in mineralized deposits of rhGDF15 exposed cells compared to control condition, which undermines the osteogenic effect of GDF15.

Members of the TGF-ß-family are known to regulate osteoblast and osteoclast differentiation [60,61]. In addition to our own previous results in primary osteoblasts [33], a pro-osteogenic effect of GDF15 on various cell types has been observed in a number of studies. Thus, Uchiyama et al. demonstrated, that the number of bone marrow-derived mesenchymal stem cells (BM-MSCs) increased significantly after 7 days of stimulation with rhGDF15 [49]. Further analysis showed, that protein levels of representative markers of osteoblastic differentiation RUNX2 and OSX were increased in rhGDF15-treated human BM-MSCs compared to control cells. Moreover, transfection with *GDF15* cDNA was shown to promote osteoblast differentiation in prostate cancer cells in vitro [62]. According to Siddiqui et al. [47], prostate cancer (PCa) secreted GDF15 promotes bone metastases and bone turnover. Further, rhGDF15 promotes osteogenic differentiation of mouse calvarial osteoblasts and GDF15 deletion inhibits PCa-mediated osteoblast differentiation and mineralization, suggesting an essential role of GDF15 in the stimulation of osteoblast differentiation by PCa. It should be noted that Westhrin et al. also reported an anti-osteogenic effect of GDF15 on human bone marrow-derived mesenchymal stem cells with reduced alkaline phosphatase activity, matrix mineralization and mRNA levels of *RUNX2*, Type I collagen and osteocalcin due to GDF15 stimulation for 17 days [46]. However, altered cell fate might influence cellular responses to mechanical forces.

We recently reported that *GDF15*-deficient hPdLF showed a significantly reduced secretion of the pro-inflammatory cytokines IL6, PGE2, and TNFα upon mechanical compression [34]. Combined with lower numbers of activated monocytic THP1 cells, our previous data suggest a proinflammatory role for GDF15 in hPdLFs. However, we left one relevant question unexplored. What is the role of intracellular GDF15 activity versus its effects by extracellular stimulation through receptor binding? Via different protein states, GDF15 can trigger intracrine signaling by translocation to the nucleus

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and auto- or paracrine signaling through membrane-bound receptors [39,41]. Artz et al. identified ALK-5 as a receptor expressed in mouse leucocytes that inhibits integrin activation triggered by GDF15 [40]. Moreover, GDF15 has been found to trigger analgesia in rat primary sensory neurons via ALK-2 receptor [39]. Here, we detected all members of the ALK family in human PdLFs.

Extending our previous study, our new data now suggest that the pro-inflammatory effect of GDF15 in compressed hPdLFs is mediated by auto- or paracrine action via protein-receptor binding. Thus, increased IL6 levels and activated monocytes were detected due to GDF15 stimulation. Interestingly, GDF15-stimulated mechano-controls showed comparable transcription levels of *IL6*, which is in contradiction to Li et al. reporting increased *IL6* expression levels in unforced hPdLFs due to GDF15 exposure [45]. A plausible explanation might relate to the concentration of GDF15 used for cell exposure. While we stimulated with 5 to a maximum of 20 ng/ml GDF15 protein, which already corresponds to high concentrations secreted by various cells upon different stresses [63–67], Li et al. used an even higher concentration of 100 ng/ml. Nevertheless, both studies confirm a pro-inflammatory influence of GDF15 in hPdLFs.

Compressed fibroblasts also usually show an increased RANKL and a decreased OPG secretion leading to enhanced osteoclast activation [68–70]. Our data indicate, that long-term exposure to 5-20 ng/ml rhGDF15 significantly reduced those mechano-related effects as reduced *RANKL/OPG expression* ratios and osteoclast activation were detected. In contrast to this, short-term stimulation with significantly higher dose of 100 ng/ml GDF15 protein resulted in an increased *RANKL/OPG* expression ratio in human PdL cells mainly due to reduced OPG levels [45]. Since we stimulated cells with GDF15 for 36 days driving an osteogenic cell fate, the mechanoreactivity might differ due to cell differentiation. Comparable to our results, GDF15 stimulation of mouse calvarial osteoblasts (MCOs) also failed to induce changes in *Opg* expression levels [47]. In contrast to hPdLFs, GDF15-exposed MCOs showed increased *Rankl* levels when stimulated with GDF15 concentrations higher than 10 ng/ml. However, osteoclast activation was not functionally validated in both previous studies within this context [45,47], which limits comparison with our study in terms of possible differences between RNA and protein expression as well as secretion. Furthermore, considering the limited sequence homology of GDF15 between humans and rodents [71], possible effects of nonspecies-matched stimulation may account for differences to findings of Siddiqui, who used human recombinant GDF15 to stimulated mouse MCOs. Nevertheless, GDF15 stimulation of PdL fibroblasts and (PdL-derived) osteoblasts appears to affect the activation of osteoclasts by these cells, although this seems to depend on the specific conditions.

In summary, our results strongly suggest a relevant impact of GDF15 on the cell fate of PdL fibroblasts promoting osteogenic differentiation in the long-term. Moreover, GDF15 seems to impact the force-related inflammatory mechanoresponse of those cells and subsequently affected their activation of osteoclast. Since we used a simplified in vitro model to simulate orthodontic tooth movement with compressive and tensile strain, future studies should focus on these findings in vivo. Altogether, our study provides evidence that long-term elevated GDF15 levels, such as those associated with specific diseases as well as with aging [43,44], indeed impact the mechanoreactivity of PdL cells and thus may be relevant in orthodontic treatment of those patients.

4. Materials and Methods

4.1. Cell Culture

Human periodontal ligament fibroblasts (hPdLF, Lonza, Basel, Switzerland) were cultured in culture medium containing Dulbecco's modified Eagle's medium (DMEM, Capricorn Scientific, Ebsdorfergrund, Germany), 4.5 g/L Glucose, 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Carlsbad, CA, USA), 100 U/mL penicillin, 100 mg/L streptomycin and 50 mg/L L-ascorbic acid at 37°C, 5% CO2 and 95% humidity. Cells were passaged at 75% confluence with 0.05% Trypsin/EDTA (Thermo Fisher Scientific, Carlsbad, CA, USA). Cells of passages six to nine were used for the experiments.

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THP1 monocytic cells (DMSZ, Braunschweig, Germany) were cultured at 37 °C, 5% CO2, and 95% humidity in RPMI 1640 medium (Thermo Fisher Scientific, Carlsbad, CA, USA) containing 10% FBS, 100 U/mL penicillin, and 100 g/mL streptomycin. Cells were passaged weekly and seeded at a density of 1 x 10^6 cells in a T175 culture flask (Thermo Fisher Scientific, Carlsbad, CA, USA).

4.2. Stimulation with recombinant human GDF15 protein

For evaluation of GDF15-induced effects, hPdLFs were stimulated for 12, 24, and 36 days with 5ng/ml or 20ng/ml recombinant human GDF15 protein (R&D Systems, Minneapolis, MN, USA) in culture flasks. As osteoblast differentiation control, hPdLFs were stimulated with 100nM dexamethasone and 10mM β-glycerol phosphate. Twelve days prior to the final experiments, 100.000 cells were seeded into 6-well plates. For immunofluorescent analysis and determination of THP1 activation, 5000 cells were seeded onto glass coverslips in 48-well plates and further cultured for 12 days in respective media.

4.3. Application of mechanical forces

At the final day of stimulation, a static compression of 2 g/cm² was achieved by applying sterilized glass plates for 24 hours according to the protocol of Kirschneck et al. [72] and as describe before [33,34,73]. For subsequent analysis of monocyte activation, force application was performed in 24-well plates by centrifugation via two rounds of twelve-hour centrifugations at 30 °C with a force of 7.13 g/cm2. Control cells were cultured at 30 °C for the duration of force application. A three-hour recovery break at 37 °C, under 5% CO2 and 95% humidity was applied between the centrifugation rounds.

For application of static isotrophic tensile force, hPdLFs were seeded on flexible bottomed 6-well plates (BioFlex® Culture Plates, FLEXCELL®, Asbach, Germany) coated with pronectin. Tensile forces of 15,9% were applied by spherical cap silicone stamps of a 25 mm radius and a high of 7,1 mm according to Nazet et al. [74]. The self-made stamps (S4 suhy dental a-silicone; Bisico, Bielefeld, Germany) were clamped into the bottom of the flexible membrane plates for 24 hours.

4.4. RNA expression analysis

For analyzing gene expression, cells were isolated with TRIzol reagent (Thermo Fisher Scientific, Carlsbad, CA, USA). RNA isolation, cDNA synthesis, and quantitative PCR were performed as previously described [33,34,75]. Briefly, 1-bromo-3-chloro-propane (Sigma-Aldrich, St. Louis, MO, USA) and centrifugation was used for separation of RNA. Cleaning of RNA was performed with RNA Clean & Concentrator-5 kit (Zymo research, Freiburg, Germany). The quality and quantity of the RNA were measured with Nanodrop OneC (Thermo Fisher Scientific, Carlsbad, CA, USA). SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, Carlsbad, CA, USA) and Oligo(dt)18 primers (Thermo Fisher Scientific, Carlsbad, CA, USA) were used for cDNA synthesis. Quantitative PCR was performed with Luminaris Color HiGreen qPCR Master Mix (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's protocol and analyzed with qTOWER3 (Analytik Jena, Jena, Germany). Primer design was performed as previously described [33,34][Schuldt, 2022 #803. Quality and specificity of the primers were analyzed using melting curves and agarose gel electrophoresis. Dilution series of cDNA were used to calculate primer efficiency. Table 1 contains all information for the used primers. RPL22 and TBP were used as reference genes for data analysis according to the ΔΔCT method.

Table 1. qPCR primer sequences of human genes indicated in 5`-3` direction. bp, base pairs. Length, amplicon length.

Gene	Gene Symbol	NCBI Gene ID	Primer Sequence	Length
Activin A receptor like type 1	*ACVRL1 (alias ALK1)*	94	fw: GTGGAGTGTGTGGGAAAAGG rev: CATGTCTGAGGCGATGAAGC	180 bp

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Activin A receptor type 1	*ACVR1 (alias ALK2)*	90	fw: GCATTCCCAGAGCACCAATC rev: GGCCACTTCCCACAAAACAA	166 bp
Activin A receptor type 1B	*ACVR1B (alias ALK4)*	91	fw: TTAGTGCCCTCTGACCCTTC rev: ATCATCTTCCCCATCACCCG	122 bp
Activin A receptor type 1C	*ACVR1C (alias ALK7)*	130399	fw: TGTTGGTCTGGTTTACTGGGA rev: TGCTTCACAACTTTGCCACT	181 bp
Alkaline Phosphatase	*ALPL*	249	fw: ACTGCAGACATTCTCAAA rev: GAGTGAGTGAGTGAGCA	190 bp
Bone morphogenetic protein receptor type 1A	*BMPR1A*	657	fw: ACCACTTCCAGCCCTACATC rev: TTGACACACACAACCTCACG	172 bp
Bone morphogenetic protein receptor type 1B	*BMPR1B (alias ALK6)*	658	fw: GCATCAAGAAGTTACGCCCC rev: TGGGACTCTGACATTTTGGC	160 bp
Interleukin 6	*IL6*	3569	fw: CATCCTCGACGGCATCTCAG rev: TCACCAGGCAAGTCTCCTCA	164 bp
Interleukin 10	*IL10*	3586	fw: AGCCATGAGTGAGTTTGACA rev: AGAGCCCCAGATCCGATTTT	141 bp
Interleukin 1 receptor antagonist	*IL1RN*	3557	fw: GATGTGCCTGTCCTGTGTCA rev: ACTCAAAACTGGTGGTGGGG	146 bp
Mitochondrially encoded cytochrome c oxidase II	*MT-COX2 (alias COX2)*	4513	fw: GATGATTGCCCGACTCCCTT rev: GGCCCTCGCTTATGATCTGT	185 bp
Ribosomal protein L22	*RPL22*	6146	fw: TGATTGCACCCACCCTGTAG rev: GGTTCCCAGCTTTTCCGT TC	98 bp
RUNX family transcription factor 2	*RUNX2*	6146	fw: CCCACGAATGCACTATCC rev: GGACATACCGAGGGACA	120 bp
TATA-box binding protein	*TBP*	6908	fw: CGGCTGTTTAACTTCGCTTCC rev: TGGGTTATCTTCACACGCCAAG	86 bp
TNF receptor superfamily member 11b	*TNFRSF11B (alias OPG)*	4982	fw: GAAGGGCGCTACCTTGA rev: GCAAACTGTATTTCGCTC	142 bp
TNF Superfamily Member 11	*TNFSF11 (alias RANKL)*	8600	fw: ATCACAGCACATCAGAGCAGA rev: TCACTTTATGGGAACCAGATGGG	160 bp
Transforming growth factor beta receptor 1	*TGFBR1 (alias ALK5)*	7046	fw: AAAACTTGCTCTGTCCACGG rev: TGCCAGTCCTAAGTCTGCAA	157 bp

4.5. Protein preparation and expression analysis

For analyzing protein expression, cultured hPdLFS were isolated with ice-cold phosphate-buffered saline (PBS) and centrifugation. Further protein isolation and expression analysis by semi-dry western blot was performed as previously described [76]. Following primary antibodies from *Santa Cruz Biotechnology* (Dallas, Texas, USA) were used: rat-anti-ALK1 (sc-101556, 1:100), mouse-anti-ACTR-I (ALK2; sc-374523, 1:100), mouse-anti-BMPR-IA (ALK3; sc-134285, 1:100), mouse-anti-ACTR-IB (ALK4; sc-73677, 1:100), rat-anti-TGFβ-RI (ALK5; sc-101574, 1:100), mouse-anti-BMPR-IB (ALK6; sc-515886, 1:100) mouse-anti-ACTR-IC (ALK7; sc-374538; 1:100). Following secondary antibodies coupled to horseradish peroxidase (HRP) from *Thermo Fisher Scientific* (Carlsbad, CA, USA) were used: goat-anti-mouse IgG HRP (#31430, 1:5000) and goat-anti-rat IgG HRP (#31470, 1:5000).

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4.6. Immunofluorescent Staining

To analyze cells characteristics, hPdLFs cultured on coverslips were fixed after specific treatment with 4% PFA for 10 minutes and washed three times with PBS containing 0.1% Triton™ X-100 (Merck Millipore, Burlington, Massachusetts, USA). Primary antibody incubation was performed for 3 hours in blocking solution consisting of PBS, 0.1% Triton™ X-100 and 4% bovine serum albumin, followed by three washing steps and subsequent secondary antibody incubation for 45 minutes in blocking solution. DAPI (Thermo Fisher Scientific, Carlsbad, CA, USA; 1:10,000 in PBS) was applied for 10 min to stain cell nuclei. The following antibodies were used: rabbit-anti-human p21 (18769S; Cell Signaling Technologies, Danvers, Massachusetts, USA; 1:100), rabbit-anti-human Ki67 (ab15580; Abcam, Cambridge, UK; 1:500), goat-anti-rabbit-Cy5.

4.7. MTT Assay

The cell vitality was assessed via MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay (Sigma Aldrich, Taufkirchen, Germany) according to the manufacturer's instructions and measured the INFINITE M NANO microplate reader (Tecan Austria GmbH, Gröding, Austria).

4.8. Cell death assays

Trypan blue staining was performed on unfixed cells immediately after stimulation with rhGDF15. Cells were briefly washed with pre-warmed PBS, and 0.4% trypan blue solution was added to the PBS at a ratio of 1:2 for 5 minutes. After rinsing twice with PBS, microscopic images were captured immediately.

ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Sigma Aldrich, St. Louis, Missouri, USA) was used according to the manufacturer's protocol to detected apoptotic cells grown to 75% confluency on coverslips.

4.9. Senescence assay

To detect cellular senescence, 75% confluent hPdLFs cultured on coverslips were analyzed with the CellEvent™ Senescence Green Detection Kit according to the manufacturer's protocol.

4.10. Alkaline phosphatase activity assay

To analysis the activity of alkaline phosphatase, cultured hPdLFs were fixed with 4% PFA for 10 min, washed with PBS and incubated with 1-Step™ NBT/BCIP Substrate Solution (Thermo Fisher Scinetific, Carlsbad, CA, USA) for 90 min. After washing with PBS, cells were directly imaged.

4.11. Alizarin red staining

Staining of calcium deposits to detect osteogenic differentiation was performed as previously described [77]. Briefly, cultured hPdLFs were fixed with 10 % PFA for 10 min prior staining with 40 mM Alizarin Red S (Merck Millipore, Burlington, Massachusetts, United States) for 20 min. Following a rinse with water, 10% acetic acid was used to dissolve the stained cells. Subsequent to heating at 85 °C for 10 min, cells were centrifuged at 20000 g for 15 min, and pH of the supernatant was neutralized with 10% ammonium hydroxide. OD405 was measured as duplicates using the *Infinite M nano plate reader* (Tecan Life Science, Männedorf, Switzerland).

4.12. THP1 activation assay

For visualization of the inflammatory response of stressed hPdLF, THP1 activation assay was performed as previously described [73]. To this end, 50×10^3 non-adherent THP1 monocytic cells were stained with Celltracker CMFDA (Thermo Fisher Scientific, Carlsbad, CA, USA) and added to each well of a 24-well plate with cultured hPdLF. Non-adherent cells were removed by washing with prewarmed PBS and coverslips were fixed with 4% paraformaldehyde (PFA) for 10 min. After

washing with PBS, cell nuclei were stained with DAPI (1:10.000 in PBS, Thermo Fisher Scientific, Carlsbad, CA, USA) and coverslips were mounted for microscopy on objects slides.

4.13. Osteoclast activation assay and TRAP staining

For the analysis of osteoclast differentiation induced by stressed hPdLFs, two-days phorbol 12-myristate 13-acetat (PMA, 100 ng/ml)-prestimulated macrophage-like THP1 cells were cultured in 96-well plates with the collected media supernatant of stressed hPdLFs. Subsequently, these cells were prefixed in 4% PFA for 10 min, then fixed for 1 min in 50:50 acetone/ethanol, air-dried and stained for tartrate-resistant acid phosphatase (TRAP). Staining solution consisting of 0.1 mg/ml Naphtol AS-MX phosphate, 0.5 mg /ml Fast Red Violet LB salt, 1 % N,N-dimethyl formamid in 50 mM sodium acetat trihydrate, 50 mM tartrate dehydrate and 0.1 % acetic acid (all Merck Millipore, Burlington, Massachusetts, United States) was applied for 60 min at 37 °C. Finally, cells were washed in PBS and directly imaged. Stimulation was performed with three independent collections of medium supernatant and analyzed on two coverslips per condition in each independent experiment.

4.14. Microscopy, Image Analysis and Statistics

Immunofluorescent stainings, TUNEL and senescence assays as well as THP1 activation assay were imaged with the inverted confocal laser scanning microscope TCS SP5 (Leica, Wetzlar, Germany). Fiji software (https://imagej.net/Fiji, accessed on 01.04.2017) was used for image analysis. Fluorescence intensity of p21 was assesed as previously reported [78]. Briefly, in 270 cells per condition, mean grey values (MGVs) of p21 stainings were measured and the background was subtracted for each measurement. MGV were normalized to control conditions and presented as percent changes. For visual presentation, MGV was displayed as intensity as thermal LUT. Each experimental condition was analyzed at least in biological triplicates with two technical replicates per condition in each independent experiment.

For statistical analysis and figure illustration, Graph Pad Prism (https://www.graphpad.com, accessed on 01.02.2021) and Adobe Photoshop CS5 (https://adobe.com, accessed on 01.02.2013) was used. One-way ANOVA and post hoc test (Tukey) were used as statistical tests. Significance levels: p value < 0.05 */#/§; p value < 0.01 **/##/§§; p value < 0.001 ***/###/§§§.

5. Conclusions

Orthodontic tooth movement is promoted by applied mechanical stimuli and relies on the crucial functions of PdL fibroblasts regarding the modulation of tissue and bone remodeling. Here we could demonstrate, that GDF15 alters the cell fate of PdLFs in an anti-proliferative and pro-osteogenic manner and further affects the mechanoresponse of PdLFs. These findings provide a link for future research to evaluate how orthodontic treatments are affected by elevated GDF15 blood levels, as seen in patients with advanced age and pathologic conditions such as inflammation, myocardial ischemia, and cancer [43].

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