

Lactoferrin and parathyroid hormone are not harmful to primary tenocytes *in vitro*, but PDGF may be

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Summary

Introduction: Recently, bone-active factors such as parathyroid hormone and lactoferrin, have been used in pre-clinical models to promote tendon healing. However, there is limited understanding of how these boneactive factors may affect the cells of the tendon themselves. Here, we present an *in vitro* study assessing the effects of parathyroid hormone and lactoferrin on primary tendon cells (tenocytes), and compare their responses to the tenogenic factors, PDGF, IGF-1 and TGF- β .

Materials and Methods: Tenocyte proliferation and collagen production were assessed by alamarBlue® and Sirius red assays, respectively. To assess tenocyte trans-differentiation, changes in the expression of genes important in tenocyte, chondrocyte and osteoblast biology were determined using real-time PCR.

Results: Parathyroid hormone and lactoferrin had no effect on tenocyte growth or collagen production, with minimal changes in gene expression and no detrimental effects observed to suggest trans-differentiation away from tendon cell behaviour. Tenogenic factors PDGF, IGF-1 and TGF all increased tenocyte collagen production, howev-

er, the gene expression data suggests that PDGF promotes severe de-differentiation of the tenocytes.

Discussion: Our findings suggest that using parathyroid hormone or lactoferrin as a singular factor to promote tendon healing may not be of benefit, but for use in tendon-bone healing there would be no detrimental effect on the tendon itself.

KEY WORDS: tendon, growth factors, healing, lactoferrin, parathyroid hormone.

Introduction

The potential of growth factor treatments to improve the poor healing capacity of tendon is a much studied area of research, with little clinical uptake to date^{1, 2}. Factors such as platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF) and members of the transforming growth factor beta (TGF- β) superfamily are all upregulated during the healing process, and have been extensively studied using tendon cells and explants *in vitro*, and using tendon defect models *in vivo*^{3, 4}. In addition, application of a high concentration growth factor cocktail in the form of platelet rich plasma (PRP) showed early promise in pre-clinical studies, but clinical results have been mixed^{5, 6}.

Recently there has been a number of small animal studies looking at the use of bone-active factors to improve tendon healing, either in models of flexor tendon injury, or in the more complex tendon-bone defects. Parathyroid hormone (PTH) treatment was shown to increase the expression of key matrix genes in the flexor tendon, but had poor effect on joint mobility⁷, while in both ACL reconstruction and rotator cuff healing, PTH increased the amount of bone present at the tendon-bone interface, and therefore increased total pull-out strength of the repaired defect^{8, 9}. Peptides derived from lactoferrin, a potent bone anabolic factor, have also been shown to improve the healing outcomes of flexor tendons, by reducing focal adhesions^{10, 11}.

However, there is still poor understanding of how PTH may affect the cells of the tendon themselves. This is especially important as hypothyroidism has been linked to musculoskeletal problems in humans¹², and clinically increased serum PTH is associated with poor tendon health¹³⁻¹⁵. Lactoferrin,

meanwhile, has been shown to increase human tenocyte survival and growth *in vitro*¹⁶. Yet, given the tendency of tenocytes to trans-differentiate down non-tenocytic lineages, a major contributing factor in tendinopathy¹⁷, it is important to understand how these cells respond to potential therapeutic factors at a genetic level, as this will allow us to infer the long-term clinical consequences of potential treatments. Here we have assessed the effects of PTH and lactoferrin on primary tenocytes and compared their responses to that of better studied tenogenic factors, PDGF, IGF-1 and TGF- β . These were chosen as PDGF is among the most widely studied tendon factors, and IGF-1 and TGF- β are both present within the tendon matrix. Furthermore, all are upregulated during tendon healing, and are known to enhance tendon cell growth, collagen production and matrix remodelling, *in vitro*¹⁸⁻²⁶. Furthermore, we have focussed on not only classical tendon-related outputs, such as cell proliferation and collagen production, we have also assessed the tenocyte gene expression profile, assessing genes important in tenocyte, chondrocyte and osteoblast biology. We hypothesise that while these factors may be anabolic to bone tissues, and elicit positive effects in tendon cells, they may result in the trans-differentiation of these cells into a more osteoblastic-type cell.

Methods

Reagents and ethical approval

This study was conducted in accord with international standards and meets the ethical standards of the Muscle, Ligaments, and Tendons Journal²⁷. Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM: F-12) and Penicillin/Streptomycin mixture (10,000U/ml) were supplied by Gibco (ThermoFisher Scientific Inc., Waltham, MA). Fetal bovine serum (FBS) was obtained from HyClone Laboratories (GE Healthcare Life Sciences, Logan, UT). Bovine serum albumin (BSA) was obtained from Immuno-Chemical Products Ltd. (Auckland, New Zealand). Rat platelet-derived growth factor (PDGF-BB) was from R&D Systems (Minneapolis, MN). Lactoferrin was kindly donated by Dr. Kate Palmano (Lactopharma, Auckland, NZ). Transforming growth factor- β (TGF- β) was obtained from Sigma-Aldrich (St. Louis, MO). Parathyroid hormone (PTH 1-34) was obtained from Bachem (Bubendorf, Switzerland). Insulin-like growth factor (IGF)-1 was kindly donated by Dr. Jian Guan (Liggins Institute, University of Auckland, Auckland, NZ). Primary rat tenocytes were isolated in accordance with the Animal Ethics Committee of The University of Auckland, New Zealand.

Primary tenocyte cell culture

Primary rat tenocytes were isolated from tendon fascicles teased from tails of mature female Wistar rats, as previously described. Briefly, tendon was cut into <1cm pieces and digested in 0.5mg/ml dispase and

0.5mg/ml collagenase (both from Sigma-Aldrich) in DMEM: F-12 with 10% FBS at 37°C for up to 18 hours until most of the extracellular matrix had been digested. The cell suspension was then passed through a cell strainer, washed and resuspended in enzyme-free media. Cells were cultured in DMEM: F-12 with 10% FBS in 75cm² flasks (Corning Inc., Corning, NY) and incubated at 37°C with 5% CO₂ until confluent.

Cell growth assays

Primary rat tenocytes were seeded in 24-well plates (Greiner BioOne, Kremsmünster, Upper Austria), at a density of 7.5×10⁴ cells/well and cultured in DMEM: F-12 with 5% FBS. After 48 hours media was changed to DMEM: F-12 with 0.5% FBS. Cells were then incubated with the treatment compounds [Lactoferrin, PTH (1-34), PDGF, TGF- β , and IGF-1] for a further 72 hours. Cell growth was measured by adding alamarBlue® (Life Technologies, ThermoFisher Scientific Inc.) at 5% of final concentration in well for 4 hours at 37°C. Following this incubation, 200 μ l of the alamarBlue® conditioned medium was transferred to a 96-well plate (Greiner Bio-One) and fluorescence (excitation 540nm; emission 630nm) was read using a Synergy 2 multi-detection microplate reader (BioTek Instruments Inc., Winooski, VT). The change in alamarBlue® is expressed here as a ratio of the untreated control fluorescence readings.

Collagen deposition

Cells were cultured and treated as described above. At the end of the treatment period (72 hours after the addition of growth factors), cells were fixed with Bouin's solution (71% saturated picric acid, 24% formalin, 5% 0.5M acetic acid) for 30 minutes and then stained with 0.1% Sirius red dissolved in saturated picric acid for 1 hour. At the end of this incubation, cells were washed with 0.01M hydrochloric acid five times and left to air dry. The dye was released using 0.1M sodium hydroxide and 200 μ l of the released dye was transferred to a 96-well plate. Absorbance was measured at 570nm using a Synergy 2 multi-detection microplate reader. There were 6 wells in each treatment group and measurements were calibrated to a standard curve made using rat tail collagen type I (Corning).

Gene expression

For analysis of gene expression, cells were cultured and treated as described above. Total cellular RNA was extracted from cultured cells and purified using the RNeasy minikit (Qiagen, Venlo, The Netherlands). Genomic DNA was removed using RNase-free DNase set (Qiagen). The quantity and purity of the RNA were measured using a NanoDrop Lite spectrophotometer (Thermo Scientific, Victoria, Australia). Reverse transcription (500ng RNA used for each sample) was carried out using SuperScript III (Life Technologies) and cDNA was used for real-time PCR. Primer-probe sets were purchased as TaqMan® Gene Expression Assays from Applied Biosystems (Thermo Fisher Scientific). Multiplex PCR was

performed with FAM-labelled probes specific for the genes of interest (collagen Ia1: Rn00801649_g1; collagen IIIa1: Rn01437681_m1; decorin: Rn01503161_m1; scleraxis: Rn01504576_m1; tenomodulin: Rn00574164_m1; RUNX2: Rn01512298_m1; alkaline phosphatase: Rn0001516028_m1; aggrecan: Rn00573424_m1; sox9: Rn01751069_mH), and VIC-labelled 18S rRNA endogenous control probes according to the company's instructions, using an ABI PRISM 7900HT sequence detection system (Applied Biosystems). Samples were assayed in triplicates. The relative level of mRNA expression was determined using the $\Delta\Delta C_t$ calculation method, normalized to values of the non-treated cells (control).

Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA) with *post hoc* Dunnet's test using GraphPad Prism Software (GraphPad Software, San Diego, CA).

Results

PDGF treatment increased tenocyte cell growth, bone-active factors had no effect

Following 72 hours treatment, neither lactoferrin nor PTH(1-34) had an effect on primary rat tenocyte growth in this assay (Fig. 1). PDGF, however, significantly increased tenocyte cell growth in a dose-dependent manner, with the highest concentration (20ng/mL) increasing cell growth by 60% ($p < 0.0005$). TGF- β and IGF-1 had no effect.

PDGF, IGF-1 and TGF- β increased tenocyte collagen deposition, bone-active factors had no effect

In primary rat tenocyte cultures, all concentrations of PDGF and TGF- β significantly increased collagen deposition, as quantified by Sirius red dye release. PDGF increased collagen deposition by 40-60% ($p < 0.05$), while TGF- β increased collagen by 25-40% ($p < 0.05$). The lower IGF-1 concentration significantly increased collagen deposition (24%, $p < 0.05$), whereas higher concentrations of IGF-1 treatment did not significantly increase collagen deposition (16-19%). Neither lactoferrin, nor PTH (1-34) had any effect on collagen deposition in these cultures (Fig. 2).

Lactoferrin had little effect on tenocyte gene expression

There were minimal changes observed in the gene expression levels of primary rat tenocytes treated with lactoferrin. The higher concentration of lactoferrin (100 μ g/mL) induced a marginal, but statistically significant, increases in tenomodulin expression, and decreased RUNX2 expression levels ($P < 0.05$) (Fig. 3).

PTH (1-34) increased gene expression associated with matrix assembly and decreased chondrocyte marker expression

Gene expression levels of decorin were significantly increased in primary rat tenocytes following treatment with 10^{-9} M and 10^{-8} M PTH (1-34), increasing 6-fold compared to control with the higher concentration of PTH (1-34) ($P < 0.001$). High concentrations of PTH (1-34) treatment also decreased the expression of the chondrocyte genes aggrecan and SOX9 by 2-3 fold,

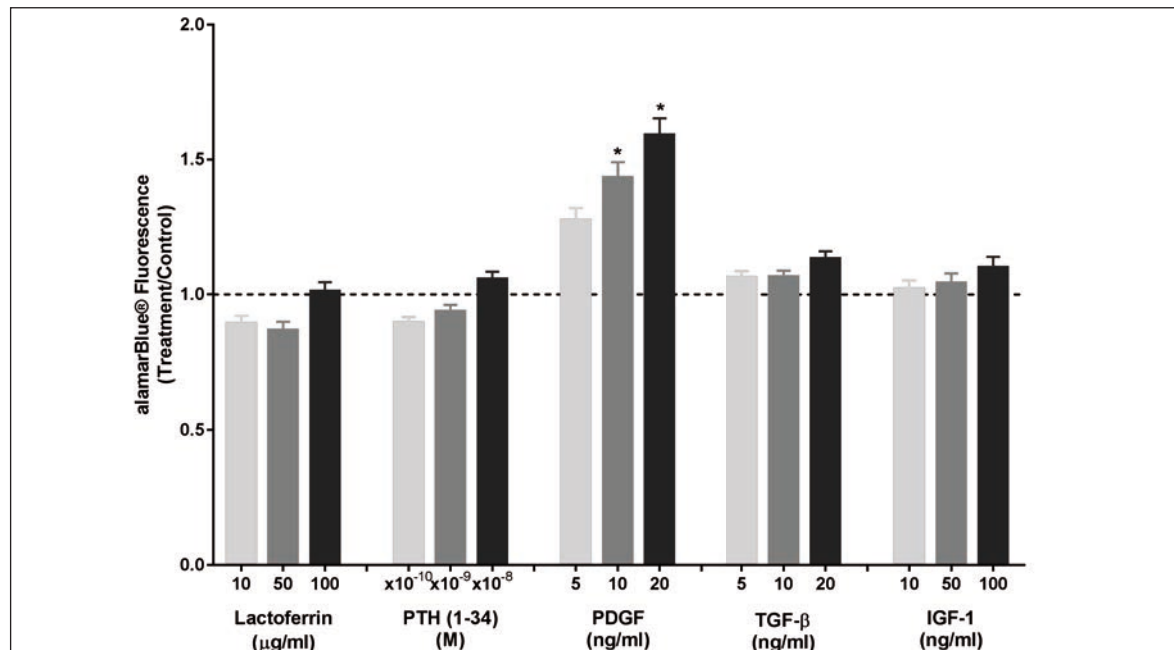


Figure 1. PDGF increases tenocyte cell growth. Effects of the bone active factors lactoferrin and PTH (1-34), and the tenogenic factors PDGF, TGF- β and IGF-1 on primary rat tenocyte cell growth, determined by alamarBlue® assay. Data are presented as mean ratio of control \pm SEM. $n \geq 4$. * = significantly different from control ($P < 0.05$).

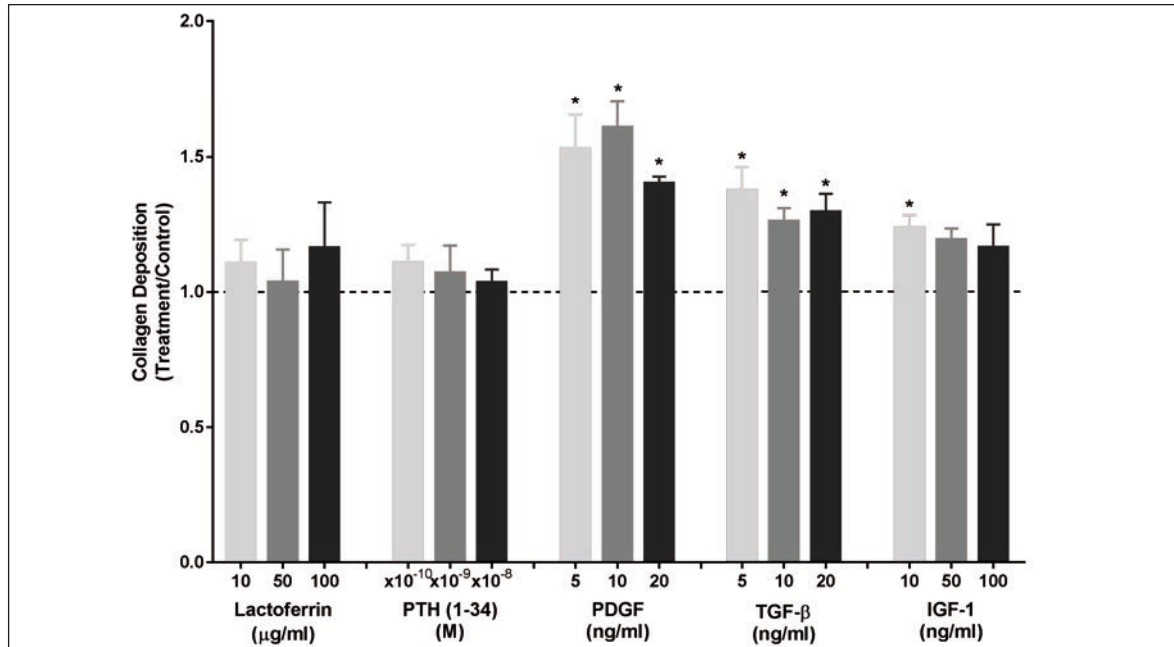


Figure 2. Tenocyte growth factors increase primary rat tenocyte collagen deposition. Effects of the bone active factors lactoferrin and PTH (1-34), and the tenogenic factors PDGF, TGF-β and IGF-1 on primary rat tenocyte collagen production, determined by sirius red assay. Data are presented as mean ratio of control ± SEM. n =3. *=significantly different from control (P < 0.05).

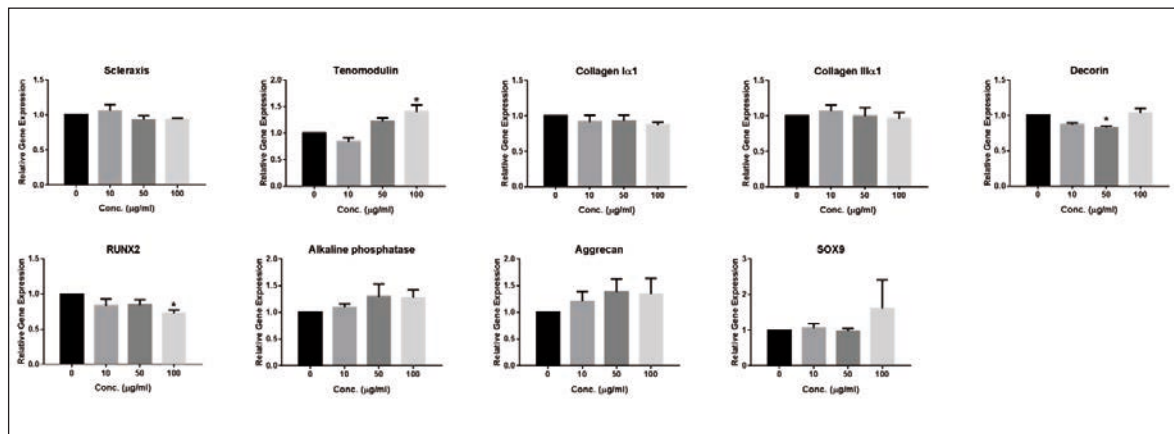


Figure 3. Lactoferrin had little effect on tenocyte gene expression. Effects of increasing concentrations of lactoferrin on the expression of tendon and non-tendon related genes in primary rat tenocytes. Data are presented as means ± SEM. n=3. *=significantly different from control (P < 0.05).

compared to the control (P<0.05). There were no changes in the expression of other tenocyte or osteoblastic gene markers (Fig. 4).

PDGF caused a de-differentiation of tenocytes

PDGF treatment led to significant decreases in the expression of key tenocyte markers scleraxis and tenomodulin, as well as the osteoblastic marker alkaline phosphatase, and the chondrocytic markers aggrecan and SOX9 in primary rat tenocytes (Fig. 5).

TGF-β enhanced tenomodulin expression and decreases osteoblastic marker gene expression

Rat tenocytes treated with all concentrations of TGF-β had significantly increased gene expression levels of tenomodulin compared to control, but significantly decreased levels of decorin (P<0.05). Similarly, all concentrations of TGF-β decreased the expression of alkaline phosphatase (Fig. 6).

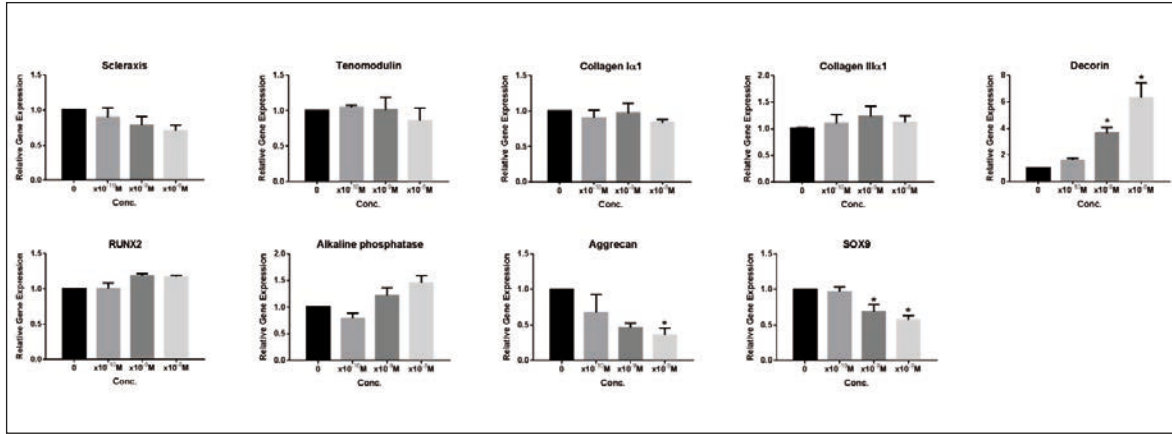


Figure 4. PTH (1-34) increased gene expression associated with matrix assembly and decreased chondrocyte marker expression. Effects of increasing concentrations of PTH (1-34) on the expression of tendon and non-tendon related genes in primary rat tenocytes. Data are presented as means \pm SEM. n=3. *=significantly different from control ($P < 0.05$).

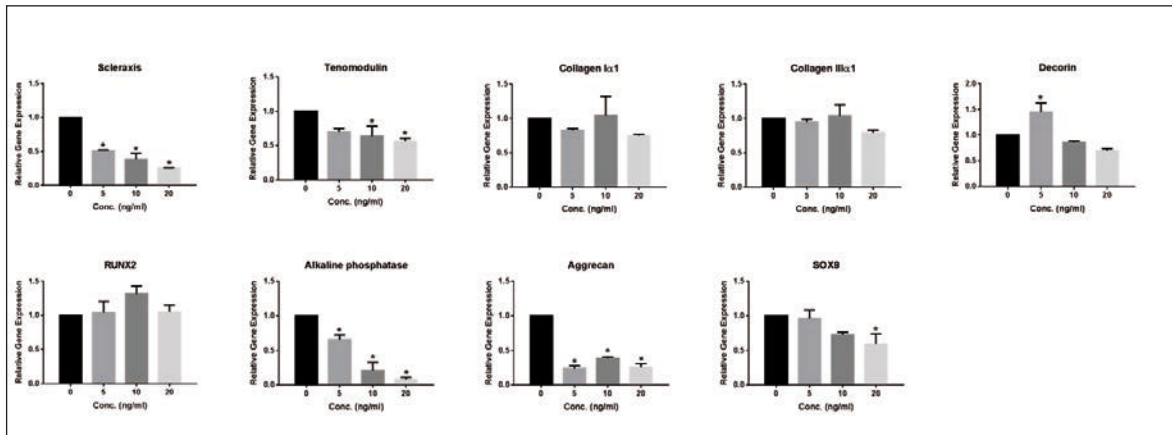


Figure 5. PDGF caused a de-differentiation of tenocytes. Effects of increasing concentrations of PDGF on the expression of tendon and non-tendon related genes in primary rat tenocytes. Data are presented as means \pm SEM. n=3. *=significantly different from control ($P < 0.05$).

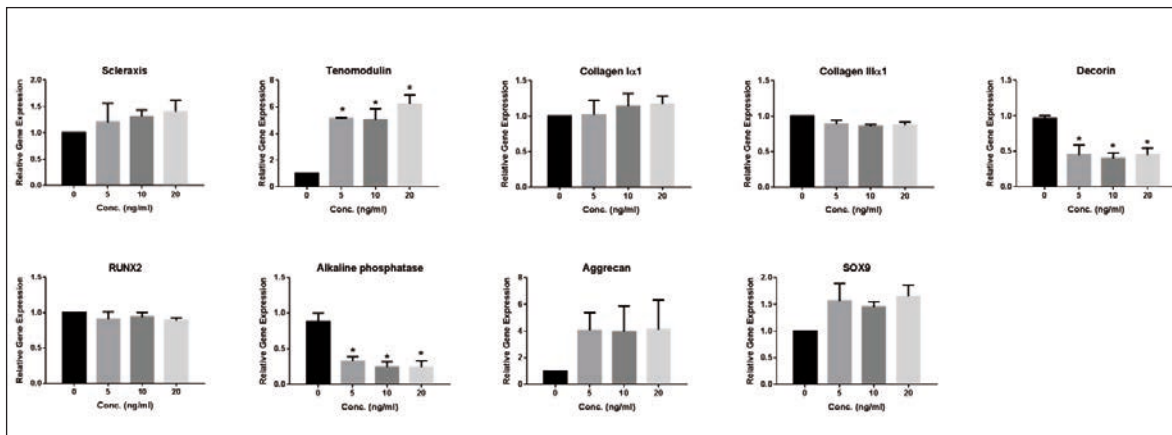


Figure 6. TGF- β enhanced tenomodulin expression and decreases osteoblastic marker gene expression. Effects of increasing concentrations of TGF- β on the expression of tendon and non-tendon related genes in primary rat tenocytes. Data are presented as means \pm SEM. n=3. *=significantly different from control ($P < 0.05$).

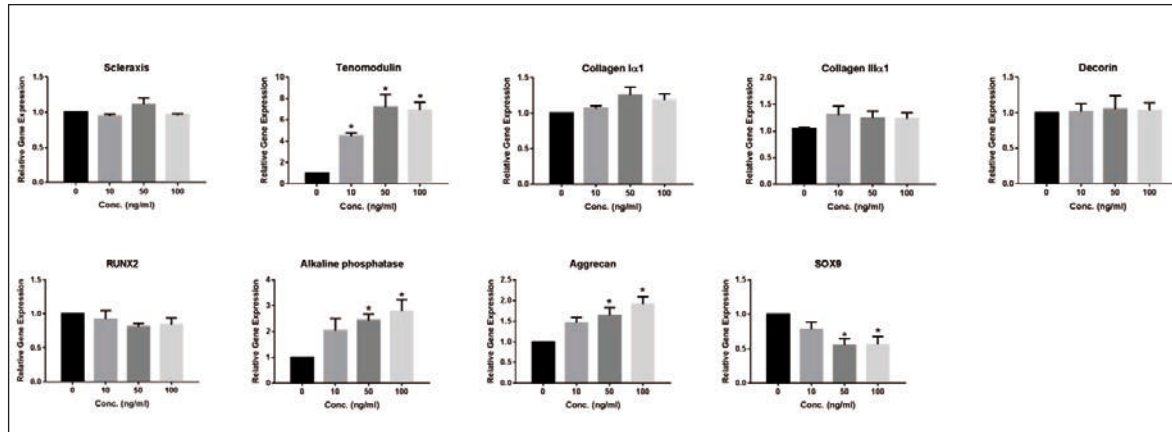


Figure 7. IGF enhanced tenomodulin expression in tenocytes, as well as non-tenocytic gene markers. Effects of increasing concentrations of IGF-1 on the expression of tendon and non-tendon related genes in primary rat tenocytes. Data are presented as means \pm SEM. n=3. *=significantly different from control (P < 0.05).

IGF-1 enhanced tenomodulin expression in tenocytes, as well as non-tenocytic gene markers

Tenocytes treated with all concentrations of IGF-1 had significantly higher gene expression levels of tenomodulin. There were no other changes in tenocyte-related gene expression (Fig. 7). The expression of the osteoblastic marker alkaline phosphatase was significantly increased with the higher concentrations of IGF-1 (50 and 100ng/mL). These concentrations of IGF-1 also increased the expression of aggrecan, but decreased the expression of SOX9.

Discussion

Here, we have demonstrated that the bone-active factors PTH and lactoferrin have little effect on tendon cells directly, with increases seen in the expression of genes related to collagen fibrillogenesis, but no detrimental effects which may lead to tenocyte trans-differentiation. Our proliferation and collagen production results with PDGF, IGF-1 and TGF β treatment were in keeping with previous findings, however, the gene expression data from PDGF treatment suggests severe de-differentiation of the tenocytes. Lactoferrin is a multipotent factor with strong bone anabolic effects, which is also known to increase chondrocyte proliferation²⁸. Previous studies have demonstrated that lactoferrin is capable of increasing tenocyte growth and collagen synthesis¹⁶. We hypothesised that being a bone-active factor, treatment of tenocytes with lactoferrin would cause trans-differentiation down non-tenocytic lineages. However, we did not see this, nor did we see any increases in tenocyte growth or collagen production. Interestingly however, high concentrations of lactoferrin did increase the expression of tenomodulin, a key factor involved in tendon development. This is the first time the effect of lactoferrin has been demonstrated on tenocyte gene expression. Given the limited effects demonstrated here on tenocytes, lactoferrin may not

be the most potent factor for promoting tendon healing. However, when taken into context with previously published data, it may have a use in treating tendon-bone defects, where tendon, fibrocartilage and bone occur within millimetres.

Clinically, hypothyroidism has been linked to musculoskeletal problems in humans¹², and increased serum PTH is associated with tendon laxity and spontaneous tendon rupture¹³⁻¹⁵, although pre-clinical *in vivo* studies using PTH suggest it may have some benefit in tendon healing⁷⁻⁹. In our study PTH had no effect on tendon proliferation or collagen production, but did increase the expression of decorin, which plays a role in matrix assembly and could have direct implications on collagen fibrillogenesis. Interestingly, PTH treatment reduced the expression of the chondrocytic genes aggrecan and sox9, suggesting that it may also help in the maintenance of a tenocytic lineage. The clinical correlation between high serum PTH and poor tendon health is likely due to continuous exposure to PTH and increased bone resorbing osteoclast activity at the tendon-bone interface^{14, 15}, rather than the short or intermittent administration discussed in this paper and the pre-clinical studies published to date⁷⁻⁹. These results suggest that PTH may have a slight beneficial effect on tendon healing, particularly in the tendon-bone area as pre-clinical studies have suggested.

The use of growth factors for treating tendon disorders has failed to have a clinical impact. PDGF is one of the most widely studied factors for improving tendon healing, and *in vitro* results have been especially positive, with potent increases in tendon cell and explant proliferation and collagen production observed^{19, 20, 26}. When studied in small animal models of tendon healing, however, results were varied²⁹⁻³¹. In this study, treatment of tenocytes with PDGF reduced the expression of the major tenocyte genes, scleraxis and tenomodulin, and previous studies also demonstrated that PDGF may have a detrimental effect on tendon gene expression¹⁹. Clinically, PDGF receptor

expression is increased in diseased tendons, and is associated with hypercellularity³², and taken into context with our findings on the poor effects of PDGF on tendon-related genes, this suggests that PDGF may not be the most suitable factor for improving tendon healing outcomes.

IGF-1 and TGF- β are both present within the tendon matrix¹⁸, and *in vitro* have been shown to be involved in tendon cell growth, collagen production and matrix remodelling¹⁹⁻²⁵. Developmental studies suggest a key role for TGF β 1 in tendon development³³, and inhibiting TGF β 1 has generally resulted in poor healing outcomes in *in vivo* tendon defect models³⁴. Gene expression studies, however, suggest TGF β 1 treatment may favour a chondrocytic response over a tenogenic one^{22, 25, 35}, with our results suggesting similar movement away from a tenogenic lineage. IGF-1, on the other hand, appears to promote the expression of tendon genes, particularly scleraxis, tenomodulin and collagen Ia1^{21, 22}, and has been used clinically to enhance collagen synthesis in the patellar tendon of healthy patients, and in patients with Ehlers-Danlos syndrome, who have abnormal collagen fibril formation^{36, 37}. In our study, low concentration of IGF-1 increased collagen production and tenocyte gene expression, but did not increase expression of non-tenocyte markers. Although, higher concentrations of IGF-1 did increase the expression of both osteoblastic and chondrocytic gene markers, suggesting concentration dependent effects.

There were differences between some of our research findings and those previously published, particularly with respect to the lack of proliferative response observed following IGF-1 and lactoferrin treatment. However, not all studies have reported a proliferative response in tendon cells following IGF-1 treatment²², and IGF-1 is known to be less responsive than PDGF^{19, 20}. It should also be noted that culture conditions will vary between studies, and this will inherently affect cell responses.

Indeed, the main limitations of our study relate to our choice of culture conditions. Firstly, we used rat tenocytes as a readily available source of primary cells to screen tendon cell responses, while other studies have used a range of human cells, avian cells and tissue explants. The use of human cells would be ideal for translational observations, and this is an obvious future direction towards understanding tendon cell response to lactoferrin and PTH. Secondly, our study has focussed on the specific individual effects of lactoferrin and PTH on tendon cells, in comparison to more tenogenic factors. A more relevant biological system to study the healing potential of these factors would be to study them in combination with other growth factors, as there is an abundance of evidence suggesting that combined growth factor treatments will have more clinical relevance than singular growth factor administration^{20, 26, 35, 38}, and this also makes sense from a biological perspective as developmental and healing processes rely on a cascade of factors, rather than any one specific factor. Finally, the study is limited by virtue of being purely *in vitro*, as it is well

understood that tendon cells behave differently in culture, compared to *in vivo*^{39, 40}. However, our aim was to understand the specific effects of lactoferrin and PTH on tendon cells, and mechanistically this can only be done *in vitro*.

This is the first study to carry out such a comprehensive analysis of tendon cell response to lactoferrin and PTH and certainly paves the way for future research to study these bone-active factors, likely in combination with other tenogenic factors, in human cells *in vitro* and in animal models of tendon-bone healing, *in vivo*.

Overall, we have demonstrated that neither of the bone-active factors lactoferrin or PTH appear to have detrimental effects on tendon cell behaviour, and while singularly they may not present much benefit for tendon repair, combined with more tenogenic factors, such as IGF-1, they may have benefit in improving tendon-bone defects. Furthermore, while PDGF enhances the basic cell functions of cell growth and collagen production, our data suggests the long-term effects of PDGF may be less than desirable with severe de-differentiation of the host cells occurring.

Conflicts of Interest

The Authors declare that there is no conflict of interest.

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