

## Research Article

# Insulin Enhances the In Vitro Osteogenic Capacity of Flexor Tendon-Derived Progenitor Cells

**Sushmitha S. Durgam** , **Nadine N. Altmann**, **Haley E. Coughlin**, **Audrey Rollins**,  
and **Laura D. Hostnik**

*Department of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University, 601 Vernon L. Tharp Street, Columbus, OH, 43210, USA*

Correspondence should be addressed to Sushmitha S. Durgam; [durgam.3@osu.edu](mailto:durgam.3@osu.edu)

Received 4 August 2019; Revised 17 November 2019; Accepted 30 November 2019; Published 27 December 2019

Academic Editor: Federico Mussano

Copyright © 2019 Sushmitha S. Durgam et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

There is increased incidence of tendon disorders and decreased tendon healing capacity in people with diabetes mellitus (DM). Recent studies have also suggested pathological ossification in repair tendon of people with DM. Therefore, the objective of this study is to investigate the effects of insulin supplementation, an important pathophysiologic stimulus of DM, on tendon progenitor cell (TPC) proliferation and in vitro osteogenic capacity. Passage 3 TPCs were isolated from collagenase-digested, equine superficial digital flexor tendons. TPC proliferation was measured via MTT assay after 3 days of monolayer culture in medium supplemented with 0, 0.007, 0.07, and 0.7 nM insulin. In vitro osteogenic capacity of TPCs (Alizarin Red staining, osteogenic mRNA expression, and alkaline phosphatase bioactivity) was assessed with 0, 0.07, and 0.7 nM insulin-supplemented osteogenic induction medium. Insulin (0.7 nM) significantly increased TPC proliferation after 3 days of monolayer culture. Alizarin Red staining of insulin-treated TPC osteogenic cultures demonstrated robust cell aggregation and mineralized matrix secretion, in a dose-dependent manner. Runx2, alkaline phosphatase, and Osteonectin mRNA and alkaline phosphatase bioactivity of insulin-treated TPC cultures were significantly higher at day 14 of osteogenesis compared to untreated controls. Addition of picropodophyllin (PPP), a selective IGF-I receptor inhibitor, mitigated the increased osteogenic capacity of TPCs, indicating that IGF-I signaling plays an important role. Our findings indicate that hyperinsulinemia may alter TPC phenotype and subsequently impact the quality of repair tendon tissue.

## 1. Introduction

Tendinitis and tendinopathies are common and debilitating injuries in people [1–4]. Recent studies show that people with diabetes mellitus (DM) have increased incidence of tendon disorders and are mediated in part due to structural alterations [5, 6]. In addition, diabetic patients with tendon injuries have decreased tendon healing capacity and are at a greater risk for recurrent injury [5–7]. Increased tendon size [8, 9], disorganized collagen fibers [8, 9], and aberrant ossification in repair tendon [10, 11] are some pathologies reported in people with DM. Increased calcium deposition at Achilles tendon insertions has been observed in people with DM, which subsequently leads to tendon rupture. Pathological ossification within repair tendon reduces tendon

elasticity, increases risk of repeat injury, and is a source of chronic pain and discomfort in people [12, 13]. While recent studies indicate increased tendon pathology in people with DM, the biological and cellular mechanisms responsible are poorly understood.

The cellular fraction of tendons, consisting of mature tenocytes and multipotent tendon progenitor cells (TPCs), is very small (<1%) but is responsible for the synthesis and turnover of extracellular matrix (ECM), which forms the bulk of tendon tissue [14]. Tendon progenitor cells demonstrate universal stem cell characteristics of clonogenicity, multipotency, and self-renewal, and their bioactivity is heavily dependent on their local environment/matrix interactions [14–16]. The in vitro and in vivo phenotypes of tendon progenitor cells in healthy and pathological tendon

tissue have shown to be markedly different [15–17]. In vivo, TPCs in repair tendon are implicated in chondrodegeneration noted during healing [17, 18]. Similarly, the in vitro multipotency of TPCs isolated from injured tendon is restricted and shifts towards a chondroosteogenic phenotype [17, 19]. A recent study by Shi et al. demonstrated that TPCs isolated from DM have a decreased tenogenic phenotype and express higher levels of chondroosteogenic genes [20]. Taken together, aberrant differentiation of TPCs under the influence of pathological stimuli present in DM may be responsible for pathological calcified tissue present in injured/healing tendon.

Exogenous insulin is used in clinical management of DM. In addition, systemic hyperinsulinemia secondary to impaired insulin sensitivity can occur during early DM. While the tenogenic phenotype of TPCs in injured DM tendon tissue and during in vitro culture with high-glucose concentration is significantly decreased, the effects of high-insulin concentrations on TPCs have not been investigated [20, 21]. Both exogenous insulin and recombinant insulin-like growth factor-I (IGF-I) increase the in vitro osteogenic capacity of osteoblasts and periodontal ligament fibroblasts [22, 23]. Therefore, we hypothesize that exogenous insulin affects TPC proliferation and enhances their in vitro osteogenic capacity. In the first set of experiments, the effects of exogenous insulin (Humulin™ 0, 0.07, and 0.7 nM) on proliferation and in vitro osteogenic capacity of equine flexor tendon-derived TPCs were assessed. Secondly, in order to determine if differential effects of insulin on insulin/IGF-I signaling exist, osteogenesis experiments were conducted in the presence of insulin and picropodophyllin (PPP), a selective inhibitor of IGF-I receptor.

## 2. Methods

**2.1. Tendon Progenitor Cell (TPC) Isolation.** TPCs were isolated from superficial digital flexor tendons of three healthy (4–6 years of age) adult horses using protocols previously described [14–16, 24]. These cells were characterized via cell surface marker expression (CD 44<sup>+</sup>, CD29<sup>+</sup>, CD90<sup>+</sup>, and CD45<sup>-</sup>) and in vitro trilineage differentiation as previously described [15, 16, 24]. An intact forelimb superficial flexor tendon was harvested from young adult horses euthanized for reasons unrelated to musculoskeletal disease. A 1–2 cm length of the midsubstance tendon specimen, free of peritendinous tissue, was diced into 0.25 cm<sup>3</sup> pieces and digested in 0.2% collagenase (Worthington) in DMEM supplemented with 2% fetal bovine serum (Gemini Biomedical) at 37°C for 16 hours. The released cells were isolated by filtration and centrifugation, and the cells were seeded at 500 cells/cm<sup>2</sup> in monolayer cultures in high-glucose DMEM supplemented with 10% fetal bovine serum, 37.5 µg/mL of ascorbic acid, 300 µg of L-glutamine/mL, 100 U of sodium penicillin/mL, and 100 µg of streptomycin sulfate/mL (basal medium). These cells were seeded onto cell culture flasks and incubated at 37°C with 5% CO<sub>2</sub> to enable colony formation. Medium was replaced every 3 days. Once discernible colonies were formed (>200 cells/colony), the cells were detached with 0.02% EDTA and 0.05% trypsin. Cell numbers were calcu-

lated by counting an aliquot of the resulting suspension using a haemocytometer and an inverted light microscope. Trypan blue dye exclusion was used to assess cell viability. The primary cell isolates were reseeded at 5 × 10<sup>3</sup> cells/cm<sup>2</sup> and passaged twice at 80–90% confluence to expand cell numbers and enrich for progenitor cells.

**2.2. Cell Proliferation.** Passage 3 TPCs were plated at 3 × 10<sup>3</sup> cells/cm<sup>2</sup> in basal medium in 96-well plates. After 1-day culture, insulin (at 0 nM, 0.007 nM, 0.07 nM, and 0.7 nM concentrations; Humulin™ U-100, Lilly, USA) treatments were added and incubated for 3 days. Three replicate wells for each treatment group were used to measure the cell numbers via a mitochondrial metabolic assay (Cell Titer MTT 96 aqueous one solution cell proliferation assay, Promega) which was used in accordance with the manufacturer's instructions. In brief, 20 µL of the assay reagent containing tetrazolium was added into each well of the 96-well plate containing 100 µL of fresh media and incubated at 37°C for 2.5 hours. Absorbance was measured at 490 nm in a microplate reader (Tecan™ Infinite 200 PRO plate reader) to detect concentrations of the metabolic product, formazan. The mean value was calculated from replicate wells to provide a single data point. These optical density values from plated TPCs from each horse were reported.

**2.3. Osteogenic Differentiation.** Passage 3 TPCs were plated at 5 × 10<sup>3</sup> cells/cm<sup>2</sup> in 6-well cell culture plates and maintained in complete DMEM until they reached 80–90% confluence. Complete DMEM was then substituted with osteogenic medium (complete DMEM supplemented with 10 mM β-glycerolaldehyde-3-phosphate, 50 µg/mL ascorbic acid, 100 nM dexamethasone) or osteogenic medium with 0.07 or 0.7 nM insulin (Humulin® U-100, Lilly, USA). The medium was replaced every 48–72 hours [15, 16]. The cultures were maintained for 14 days. In addition, TPC osteogenic cultures (with 0, 0.07, and 0.7 nM insulin) were also maintained with or without 100 nM picropodophyllin (PPP; Selleckchem, # S766802), a small molecule inhibitor of IGF-I receptor. Control osteogenic cultures were cultured (+/- insulin) with an equal amount of DMSO.

**2.4. Alizarin Red Staining of Osteogenic Cultures.** At days 0, 7, and 14 of osteogenic culture, the medium was aspirated from the cell cultures and the cell monolayers were fixed with 1 mL of 4% formalin at room temperature for 30 minutes. After fixation, the cell layers were washed three times with PBS. One mL of 2% Alizarin Red (Sigma-Aldrich) solution (2 g of Alizarin Red dye dissolved in 100 mL of Milli-Q water; pH adjusted to 4.2) was added to each well and incubated at room temperature for 15 minutes. The unbound stain was then removed, and the cells were washed 3–4 times with water until the rinse solution was clear. Mineral deposits within the cell layers stained bright red. Low-magnification (10x) images were obtained prior to osteogenic differentiation and at days 7 and 14 of osteogenic culture. An inverted light microscope (Leica Microsystems, Leica Application Suite-LAS-version 6.0) was used to assess mineralized matrix deposition.

**2.5. RNA Isolation and Quantitative RT-PCR.** Total RNA was isolated using a previously described protocol [15, 25]. The samples were homogenized in guanidinium thiocyanate-phenol-chloroform solution reagent (TRIzol, Invitrogen) according to the manufacturer's suggested protocol. The resultant pellet was purified using RNeasy silica columns that included on-column DNase digestion. The concentration of RNA was determined by measuring the absorbance at 260 nM (A260) and 320 nM (A320) in NanoDrop One/One® (Thermo Fisher Scientific). One  $\mu\text{g}$  of RNA from each sample was reverse-transcribed (Superscript IV, Invitrogen) using oligo (dT) primers. Equine gene-specific primers were designed from published sequences in Genbank and using ClustalW multiple sequence alignment (available at <http://www.ebi.ac.uk>) (Table 1). Primer specificity was confirmed by cloning and sequencing the amplicons during optimization experiments, as previously described. PCR amplifications were catalyzed by Taq DNA polymerase (ABI QuantStudio 3™, Thermo Fisher Scientific) in the presence of SYBR green. Relative gene expression was quantified using the  $2^{-\Delta\Delta\text{CT}}$  method, normalized to expression of the reference gene, elongation factor-1 $\alpha$  (EF1 $\alpha$ ) [26].

**2.6. Alkaline Phosphatase (ALP) Bioactivity Measurements.** ALP bioactivity was measured as previously described [27]. Briefly, prior to and at day 14 of osteogenic culture, the cells were harvested in 300  $\mu\text{L}$  of lysis buffer (20 mM Tris HCl, 150 mM NaCl, and 1% Triton X-100). Each sample was homogenized, centrifuged at 600 rcf for 15 minutes at 4°C, and kept on ice for 30 minutes. The supernatants were assayed for ALP activity using a commercially available ALP assay kit (Wako Chemicals) that measured the conversion of p-nitrophenylphosphate to p-nitrophenol. The intensity of yellow generated by this reaction after 10 minutes of incubation was measured at 405 nm wavelength (Tecan® Infinite 200 PRO plate reader).

The relative ALP bioactivity of each sample was determined by normalizing the measured p-nitrophenol release/minute to the corresponding DNA content.

**2.7. DNA Measurement.** DNA content was measured using the Quant-iT PicoGreen dsDNA kit (Invitrogen). Samples were diluted 1:5 in 1X TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.5). Serial dilutions of lambda DNA were used to generate a standard curve. Duplicate 100  $\mu\text{L}$  aliquots of each sample and the standards were transferred to a black 96-well microplate. One hundred  $\mu\text{L}$  of PicoGreen reagent (1  $\mu\text{L}$  PicoGreen reagent diluted in 200  $\mu\text{L}$  of 1X TE buffer) was added to each sample and standard. The microplate was placed in the dark to prevent reagent photodegradation. Following 5 minutes of incubation, the fluorescence was measured at 485 nm wavelength (Tecan® Infinite 200 PRO plate reader).

**2.8. Statistical Analysis.** The normal distribution of quantitative data (relative mRNA expression, alkaline phosphatase activity) was confirmed using the Kolmogorov-Smirnov test using SigmaPlot 14 software (Systat Software®). The data were representative of at least three independent experi-

ments, each done in triplicate. All results are expressed as the mean  $\pm$  standard deviation. One-way ANOVA was used to compare 0.07 and 0.7 nM insulin supplementation with untreated osteogenic cultures at day 14 of osteogenesis. A  $p$  value of  $\leq 0.05$  was considered statistically significant.

### 3. Results

**3.1. Insulin Increases In Vitro TPC Proliferation (Figure 1).** After 3 days of monolayer culture with insulin, TPC proliferation with 0.7 nM insulin was significantly increased (~2-fold,  $p = 0.003$ ) compared to untreated control. There was no significant difference in TPC proliferation with 0.007 and 0.07 nM insulin compared to untreated control.

**3.2. Insulin Increases Osteogenic Differentiation of TPCs In Vitro (Figure 2)**

**3.2.1. Alizarin Red Staining.** Insulin increased aggregate formation and associated Alizarin Red staining in TPC osteogenic cultures (Figure 2(a)). Larger mineralized nodules were noted in osteogenic cultures containing 0.07 and 0.7 nM insulin, in a dose-dependent manner, at both day 7 and day 14 compared to untreated controls. By day 14, intense staining of mineralized nodules, indicative of robust osteogenic differentiation, was evident in osteogenic cultures containing insulin compared to untreated controls.

**3.2.2. Osteogenic Gene Expression.** In day 14 of osteogenic cultures, the Runx2 mRNA level was significantly increased ( $p < 0.001$ ) with increasing insulin concentrations (Figure 2(b)). The Runx2 mRNA level of osteogenic cultures with 0.7 nM insulin (5.5-fold  $\pm$  1.25) was significantly higher than that with 0.07 nM insulin (2.8-fold  $\pm$  0.9;  $p = 0.019$ ) and untreated control (1;  $p = 0.002$ ). There was no significant difference in the Runx2 mRNA level of 0.07 nM insulin and control osteogenic cultures. Similarly, the ALP mRNA level of day 14 of osteogenic cultures with 0.7 nM insulin (6.0-fold  $\pm$  2.2) were significantly higher than that with 0.07 nM insulin (2.56-fold  $\pm$  0.49;  $p = 0.045$ ) and untreated control (1;  $p = 0.010$ ). There was no significant difference in the ALP mRNA level of 0.07 nM insulin and control osteogenic cultures. The Osteonectin mRNA level in day 14 of osteogenic cultures with 0.7 nM insulin (5.8-fold  $\pm$  1.04) was significantly higher than that with 0.07 nM insulin (3.3-fold  $\pm$  0.35;  $p = 0.004$ ) and untreated control (1;  $p < 0.001$ ). The Osteonectin mRNA level in day 14 of osteogenic cultures with 0.07 nM insulin was significantly higher ( $p = 0.003$ ) than untreated control.

**3.2.3. Alkaline Phosphatase (ALP) Activity.** Alkaline phosphatase bioactivities in the osteogenic cultures were consistent with the transcriptional outcomes reported above. ALP bioactivity (mmol/L p-nitrophenol) of day 14 of osteogenic culture with 0.7 nM insulin (23.0 + 4.24) was significantly higher than that with 0.07 nM insulin (6.25  $\pm$  1.49;  $p = 0.009$ ) and untreated control (4.47 + 1.73;  $p = 0.009$ ). There was no significant difference in ALP bioactivity of 0.07 nM insulin and control osteogenic culture.

TABLE 1: Primers used for real-time PCR amplification.

Gene		Sequence	Amplicon (bp)
Runx2	S	5' CAG ACC AGC AGC ACT CCA TA	177
	A	5' CAG CGT CAA CAC CAT CAT TC	
Alkaline phosphatase	S	5' TGG GGT GAA GGC TAA TGA GG	221
	A	5' GGC ATC TCG TTG TCC GAG TA	
Osteonectin (OSN)	S	5' AAC CTT CTG ACC GAG AAG CA	190
	A	5' TGG GAC AGG TAC CCA TCA AT	
Insulin receptor	S	5' CGA GGA CTA TCT GCA CAA TG	182
	A	5' ACC GTC ACA TTC CCG ACA TC	
IGF-I receptor	S	5' TCC TAA CCC TGA CTT CGG CG	212
	A	5' TTC TTG GCA TGT CTG TGT GG	
EF1-alpha	S	5' CCC GGA CAC AGA GAC TTC AT	328
	A	5' AGC ATG TTG TCA CCA TTC CA	

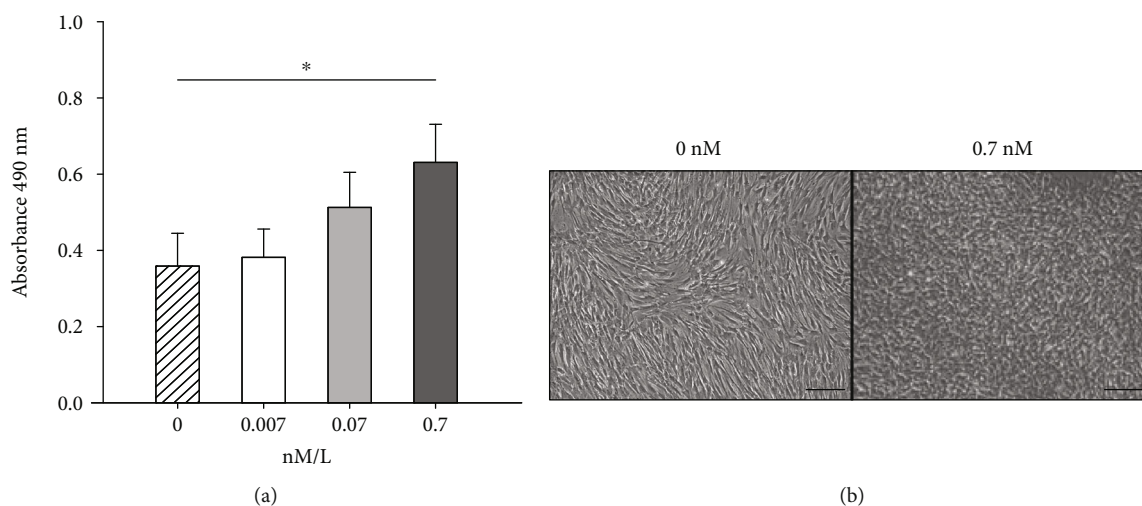


FIGURE 1: (a) TPC proliferation measured via MTT assay after 3 days of monolayer culture in basal medium supplemented with 0, 0.007, 0.07, and 0.7 nM/L insulin. \* represents a significant difference between 0 and 0.7 nM/L insulin ( $p \leq 0.05$ ). (b) Representative phase-contrast photomicrographs of TPC monolayer cultures maintained in basal medium and basal medium with 0.7 nM insulin for 3 days. A significant increase in TPCs is seen in insulin-supplemented medium. Scale bar = 100 microns.

**3.3. Insulin Differentially Upregulates IGF-I Receptor mRNA during Osteogenic Differentiation of TPCs (Figure 3).** Osteogenic differentiation of TPCs (without insulin supplementation) did not induce upregulation of IGF-I receptor or insulin receptor mRNA. In day 14 of osteogenic cultures, both 0.07 and 0.7 nM insulin groups had significantly increased (8.1-fold  $\pm$  1.1;  $p = 0.02$ ; 13.3-fold  $\pm$  1.3;  $p = 0.001$ ) IGF-I receptor mRNA levels. In contrast, insulin supplementation to TPC osteogenic cultures did not upregulate insulin receptor mRNA level.

#### 3.4. Effect of Picropodophyllin (PPP) on Insulin-Mediated Osteogenic Differentiation of TPCs (Figure 4)

**3.4.1. Alizarin Red Staining (Figure 4(a)).** Similar osteogenic cultures of TPCs with 0.07 and 0.7 nM insulin were also maintained in the presence of 100 nM PPP, a specific inhibitor of IGF-I receptor. Addition of PPP to control osteogenic cultures did not prevent cell aggregation and secretion of

mineralized matrix. However, addition of PPP to osteogenic cultures containing insulin prevented aggregation and deposition of mineralized matrix. There was minimal Alizarin Red stain uptake in day 14 of osteogenic cultures treated with PPP and 0.07 and 0.7 nM insulin.

**3.4.2. Osteogenic Gene Expression.** Given that, 0 and 0.07 nM insulin groups were not consistently different from each other, and the effect of PPP was assessed in the 0.7 nM insulin group. In day 14 of osteogenic cultures treated with PPP and 0.7 nM insulin, mRNA levels of Runx2, ALP, and Osteocalcin were significantly reduced compared to the 0.7 nM insulin group. The mRNA levels of all 3 osteogenic markers in day 14 of osteogenic cultures treated with PPP and 0.7 nM insulin and osteogenic control group were not significantly different from each other.

**3.4.3. ALP Bioactivity.** Consistent with transcriptional results, ALP bioactivity in day 14 of osteogenic cultures

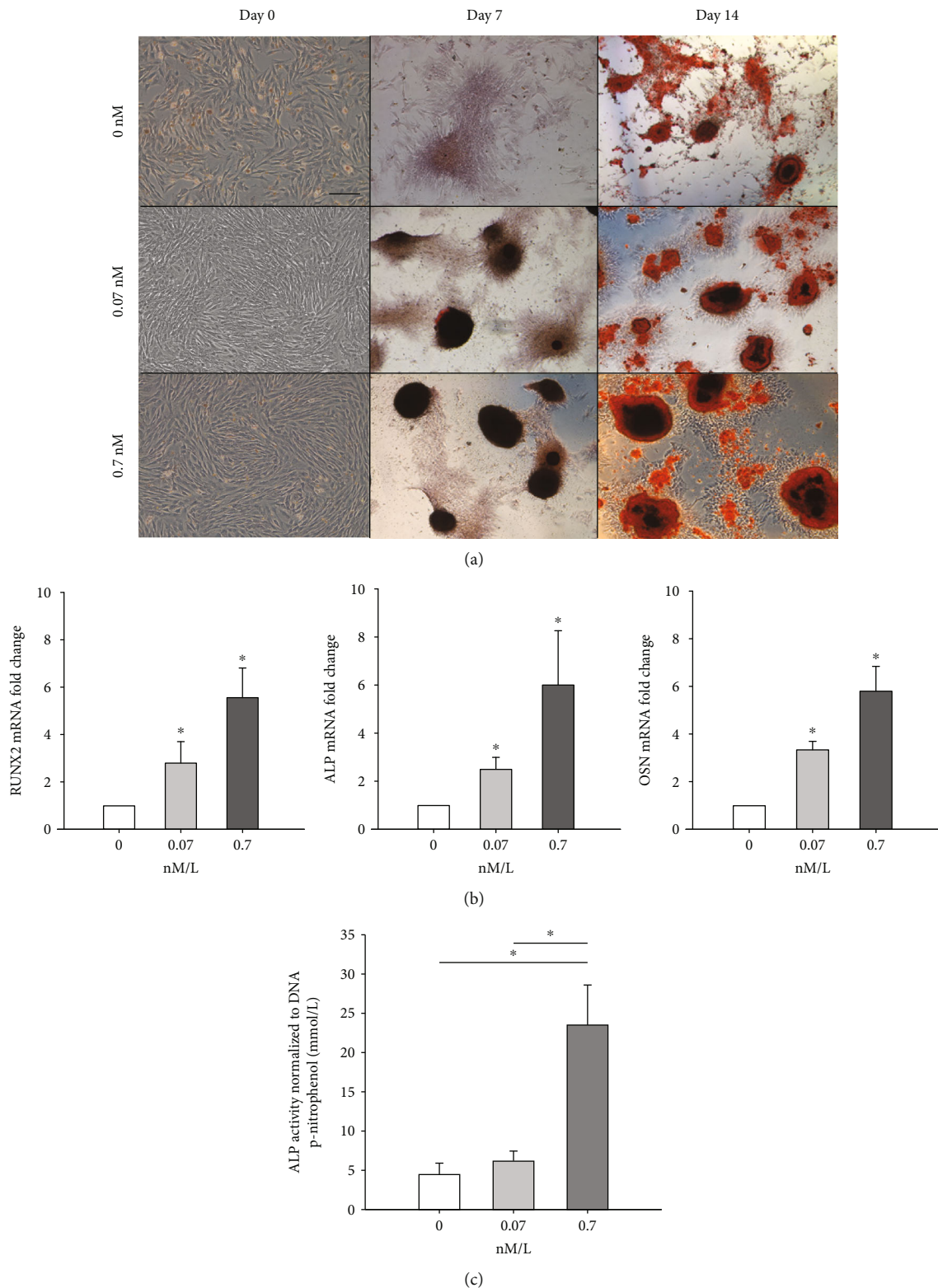


FIGURE 2: (a) Alizarin Red-stained TPC osteogenic cultures supplemented with 0, 0.07, and 0.7 nM/L insulin. Insulin promoted cell aggregation and mineralized matrix secretion as evidenced by intensity of Alizarin Red stain uptake. Scale bar = 500 microns. (b) mRNA levels (normalized to EF1a) of RUNX2, ALP, and Osteonectin (OSN) in TPC osteogenic cultures supplemented with 0, 0.07, and 0.7 nM/L insulin. Insulin treatment significantly increased osteogenic gene expression. \* represents a significant difference ( $p \leq 0.05$ ) between treatment groups. (c) Alkaline phosphatase bioactivity (normalized to total DNA content) of TPC osteogenic cultures supplemented with 0, 0.07, and 0.7 nM/L insulin. \* represents a significant increase in alkaline phosphatase activity ( $p \leq 0.05$ ).

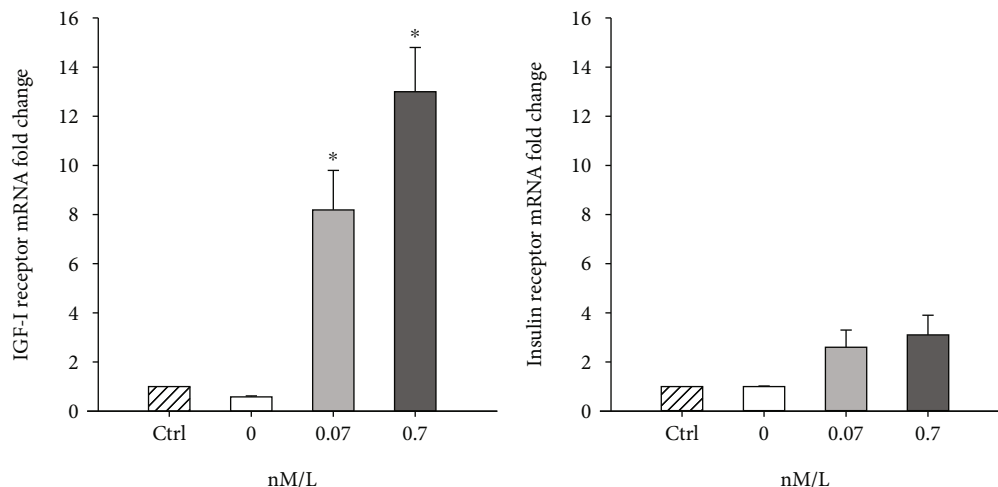


FIGURE 3: mRNA levels (normalized to EF1a) of IGF-I receptor and insulin receptor of TPC control monolayer cultures maintained in basal medium (Ctrl) and TPC osteogenic cultures supplemented with 0, 0.07, and 0.7 nM/L insulin. \* represents a significant increase ( $p \leq 0.05$ ).

treated with PPP and 0.7 nM insulin was significantly reduced compared to insulin alone and was similar to osteogenic control.

#### 4. Discussion

Given insulin/IGF-I's role in mesenchymal stem cell (MSC) osteogenesis [22, 23, 28–30], we hypothesized that insulin promotes proliferation and in vitro osteogenic differentiation of TPCs. Our results indicate that insulin significantly increased TPC proliferation after 3 days of monolayer culture. Secondly, insulin significantly increased the in vitro osteogenic capacity of TPCs compared to control osteogenic cultures, in a dose-dependent manner. These outcomes support our hypothesis. Interestingly, adding picropodophyllin, a selective IGF-I receptor inhibitor, to insulin-treated cultures mitigated the enhanced osteogenic differentiation capacity of TPCs and returned to baseline levels as control osteogenic cultures.

Our experiments were focused on evaluating the impact of sustained hyperinsulinemia or impaired insulin sensitivity on TPCs. The insulin concentrations used in this study were extrapolated from plasma insulin concentrations of people with hyperinsulinemia/insulin resistance [31, 32]. Insulin has been shown to have a pro-proliferative effect on several cell types, including mesenchymal stem/progenitor cells [33–38]. Insulin and IGF-I ligands and their respective receptors share marked similarities in their signaling properties in eukaryotic cells [22]. Insulin increased cell proliferation by binding to both insulin and/or IGF-I receptors and subsequent activation of Akt and/or ERK pathways [33, 34, 37]. Similarly, IGF-I inhibited cell cycle arrest in TPCs via the Akt pathway and consequently increased cell proliferation [39–42]. Although we have not investigated the mechanism involved in pro-proliferative effects of insulin on TPCs, similar mechanisms are likely involved. On the other hand, Lin et al. demonstrated that high glucose decreases in vitro TPC proliferation and induces apoptosis [21]. A subsequent study by the same group showed that TPCs isolated from rats with

experimental diabetes had a decreased rate of in vitro proliferation compared to control TPCs. In both studies, hyperglycemia alone as a pathophysiologic stimulus in DM on characteristics of TPCs was evaluated. In light of these findings, assessing the combined effects of high insulin and high glucose on TPC viability/proliferation is warranted.

Insulin plays a critical role in skeletal bone formation and maintenance [43]. Insulin stimulates endogenous IGF-I signaling by binding to IGF-I receptor, also a key factor for osteogenic differentiation and bone development [44, 45]. In this study, insulin enhanced the TPC osteogenic capacity by upregulating mRNA expression of Runx2, a master transcriptional regulator of osteogenic differentiation, in a dose-dependent manner. Runx2 upregulation is necessary for subsequent expression of critical matrix proteins such as collagen type I, Osteocalcin, and Osteopontin. The osteogenic differentiation and mineralization mediated by IGF-I ligand demonstrated in other MSC types occurs via activation of Akt and/or ERK pathways [22, 23, 34, 46]. Given that insulin and IGF-I activate similar intracellular signaling pathways, insulin potentially enhances TPCs' osteogenic capacity via activation of Akt and/or ERK pathways.

Our data indicates that insulin supplementation (at the concentrations used in this study) during TPC osteogenesis differentially upregulates IGF-I receptor mRNA expression without marked changes in insulin receptor mRNA expression. To this end, PPP added to insulin-treated TPC osteogenic cultures prevented the increased osteogenic differentiation (osteogenic mRNA levels, mineralized matrix secretion assessed with Alizarin Red staining, and alkaline phosphatase bioactivity) of TPCs. Since PPP did not interfere with the basal osteogenic capacity of TPCs, a complex interplay between conditions favoring ossification at the expense of tenogenesis is likely. A recent study by Jiang et al. demonstrated that IGF-I potentiated the osteogenic differentiation induced by BMP9 in bone marrow-derived MSCs [47]. Accepting the role of bone morphogenetic protein (BMP) signaling dysregulation (upregulated BMP receptor-1a, BMP receptor 2, Smad1, and Smad5 mRNAs) in tendon

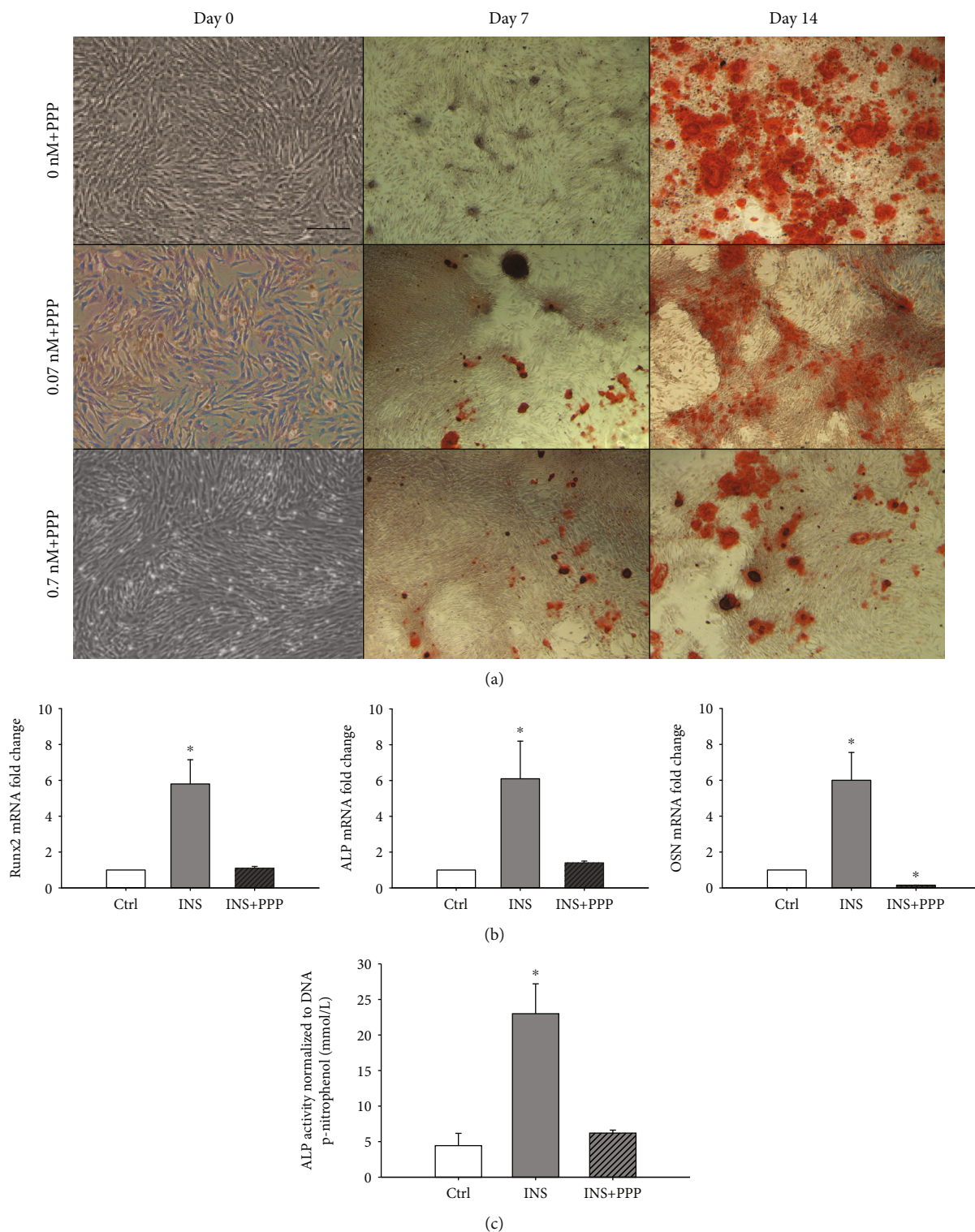


FIGURE 4: (a) Alizarin Red-stained TPC osteogenic cultures with 0, 0.07, and 0.7 nM/L insulin and 100 nM of picropodophyllin (PPP). PPP mitigated aggregation and mineralized matrix secretion of TPCs seen with insulin treatment. Scale bar = 500 microns. (b) mRNA levels (normalized to EF1a) of RUNX2, ALP, and Osteonectin (OSN) in TPC osteogenic cultures without insulin (Ctrl), supplemented with 0.7 nM/L insulin (INS) and with 0.7 nM/L insulin and 100 mM picropodophyllin (INS+PPP). PPP returned the increased osteogenic gene expression seen in insulin-treated TPCs back to baseline. \* represents a significant increase between treatment groups ( $p \leq 0.05$ ). (c) Alkaline phosphatase bioactivity (normalized to DNA) of TPC osteogenic cultures without insulin (Ctrl), supplemented with 0.7 nM/L insulin (INS) and with 0.7 nM/L insulin and 100 mM picropodophyllin (INS+PPP). \* represents a significant increase between treatment groups ( $p \leq 0.05$ ).

pathological ossification and TPCs undergoing in vitro and in vivo osteogenesis [12, 48, 49], assessing BMP signaling in insulin treated TPCs is warranted.

The clinical importance of these in vitro outcomes requires further investigation, given that impaired insulin sensitivity/hyperinsulinemia is frequently present in combination with hyperglycemia in diabetic patients. With regard to TPC proliferation, our study, among others, demonstrates that hyperinsulinemia is pro-proliferative, whereas hyperglycemia decreases proliferation. In contrast, the overall osteogenic capacity of TPCs is increased under both pathophysiologic stimuli of DM (high insulin and high glucose) and may support TPC-mediated pathological ossification. Accepting the critical roles of tendon ECM and mechanical loading on tendon homeostasis and TPC bioactivity, these outcomes will need to be confirmed in vivo. Impaired insulin sensitivity alone or in combination with hyperglycemia represents an important trigger that underlies degenerative processes in cardiovascular tissues, specifically, disturbed ECM assembly leading to calcification diabetic patients. Although the mechanisms resulting in aberrant TPC phenotypes under inflammatory/healing conditions differ widely, these experiments represent the first step in determining the effect of hyperinsulinemia on TPC activity and phenotype.

## 5. Conclusion

In summary, insulin increased the proliferation and osteogenic capacity of flexor tendon-derived progenitor cells in vitro. Addition of picropodophyllin, a selective IGF-I receptor, mitigated the increased osteogenic capacity of TPCs, indicating that IGF-I signaling plays an important role in insulin-mediated osteogenic differentiation of TPCs. These findings suggest that hyperinsulinemia may alter TPC phenotype and subsequently impact the quality of repair tendon tissue. Further studies are necessary to identify the cellular and biological processes involved in diabetic tendinopathy.

## Data Availability

All data used to support the findings of this study are included within the article.

## Disclosure

This paper was presented in part as a poster at the Orthopaedic Research Society 2019 Annual Meeting, Austin, TX (February 2 to 5).

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Acknowledgments

The authors would like to acknowledge the body of work described in Durgam SS. 2016 "In Vitro Characterization and In Vivo Assessment of Equine Tendon-Derived Progenitor Cells" Dissertation submitted to the University of Illinois in partial requirement for Doctor of Philosophy.

## References

- [1] J. C. Patterson-Kane and T. Rich, "Achilles tendon injuries in elite athletes: lessons in pathophysiology from their equine counterparts," *ILAR Journal*, vol. 55, no. 1, pp. 86–99, 2014.
- [2] M. Gajhede-Knudsen, J. Ekstrand, H. Magnusson, and N. Maffulli, "Recurrence of Achilles tendon injuries in elite male football players is more common after early return to play: an 11-year follow-up of the UEFA Champions League injury study," *British Journal of Sports Medicine*, vol. 47, no. 12, pp. 763–768, 2013.
- [3] T. Nyyssonen, P. Luthje, and H. Kroger, "The increasing incidence and difference in sex distribution of Achilles tendon rupture in Finland in 1987-1999," *Scandinavian Journal of Surgery*, vol. 97, no. 3, pp. 272–275, 2008.
- [4] R. K. W. Smith, H. L. Birch, S. Goodman, D. Heinegård, and A. E. Goodship, "The influence of ageing and exercise on tendon growth and degeneration—hypotheses for the initiation and prevention of strain-induced tendinopathies," *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, vol. 133, no. 4, pp. 1039–1050, 2002.
- [5] A. Aydeniz, S. GURSOY, and E. GUNEY, "Which musculoskeletal complications are most frequently seen in type 2 diabetes mellitus?," *Journal of International Medical Research*, vol. 36, no. 3, pp. 505–511, 2008.
- [6] R. R. de Oliveira, A. Lemos, P. V. de Castro Silveira, R. J. da Silva, and S. R. de Moraes, "Alterations of tendons in patients with diabetes mellitus: a systematic review," *Diabetic Medicine*, vol. 28, no. 8, pp. 886–895, 2011.
- [7] A. E. Goodship, H. L. Birch, and A. M. Wilson, "The pathobiology and repair of tendon and ligament injury," *Veterinary Clinics of North America-Equine Practice*, vol. 10, no. 2, pp. 323–349, 1994.
- [8] M. Akturk, A. Ozdemir, I. Maral, I. Yetkin, and M. Arslan, "Evaluation of Achilles tendon thickening in type 2 diabetes mellitus," *Experimental and Clinical Endocrinology & Diabetes*, vol. 115, no. 2, pp. 92–96, 2007.
- [9] N. Papanas, N. Courcoutsakis, K. Papatheodorou, G. Daskalogiannakis, E. Maltezos, and P. Prassopoulos, "Achilles tendon volume in type 2 diabetic patients with or without peripheral neuropathy: MRI study," *Experimental and Clinical Endocrinology & Diabetes*, vol. 117, no. 10, pp. 645–648, 2009.
- [10] F. Batista, C. Nery, M. Pinzur et al., "Achilles tendinopathy in diabetes mellitus," *Foot & Ankle International*, vol. 29, no. 5, pp. 498–501, 2008.
- [11] N. Maffulli, U. G. Longo, G. D. Maffulli, A. Khanna, and V. Denaro, "Achilles tendon ruptures in diabetic patients," *Archives of Orthopaedic and Trauma Surgery*, vol. 131, no. 1, pp. 33–38, 2011.
- [12] L. Lin, Q. Shen, T. Xue, and C. Yu, "Heterotopic ossification induced by Achilles tenotomy via endochondral bone formation: expression of bone and cartilage related genes," *Bone*, vol. 46, no. 2, pp. 425–431, 2010.
- [13] P. P. Y. Lui et al., "Chondrocyte phenotype and ectopic ossification in collagenase-induced tendon degeneration," *Journal of Histochemistry & Cytochemistry*, vol. 57, no. 2, pp. 91–100, 2009.
- [14] Y. Bi, D. Ehrlichou, T. M. Kilts et al., "Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche," *Nature Medicine*, vol. 13, no. 10, pp. 1219–1227, 2007.



- [15] S. Durgam, B. Schuster, A. Cymerman, A. Stewart, and M. Stewart, "Differential adhesion selection for enrichment of tendon-derived progenitor cells during in vitro culture," *Tissue Engineering Part C-Methods*, vol. 22, no. 8, pp. 801–808, 2016.
- [16] K. A. Williamson, K. J. Lee, W. J. Humphreys, E. J. Comerford, P. D. Clegg, and E. G. Canty-Laird, "Restricted differentiation potential of progenitor cell populations obtained from the equine superficial digital flexor tendon (SDFT)," *Journal of Orthopaedic Research*, vol. 33, no. 6, pp. 849–858, 2015.
- [17] S. Asai, S. Otsuru, M. E. Candela et al., "Tendon progenitor cells in injured tendons have strong chondrogenic potential: the CD105-negative subpopulation induces chondrogenic degeneration," *Stem Cells*, vol. 32, no. 12, pp. 3266–3277, 2014.
- [18] N. Maffulli, J. Reaper, S. W. Ewen, S. W. Waterston, and V. Barras, "Chondral metaplasia in calcific insertional tendinopathy of the Achilles tendon," *Clinical Journal of Sport Medicine*, vol. 16, no. 4, pp. 329–334, 2006.
- [19] K. Zhang, S. Asai, B. Yu, and M. Enomoto-Iwamoto, "IL-1 $\beta$  irreversibly inhibits tenogenic differentiation and alters metabolism in injured tendon-derived progenitor cells in vitro," *Biochemical and Biophysical Research Communications*, vol. 463, no. 4, pp. 667–672, 2015.
- [20] L. Shi, L. Ying-juan, G.-c. Dai et al., "Impaired function of tendon-derived stem cells in experimental diabetes mellitus rat tendons: implications for cellular mechanism of diabetic tendon disorder," *Stem Cell Research & Therapy*, vol. 10, no. 1, p. 27, 2019.
- [21] Y. C. Lin, Y. J. Li, Y. F. Rui et al., "The effects of high glucose on tendon-derived stem cells: implications of the pathogenesis of diabetic tendon disorders," *Oncotarget*, vol. 8, no. 11, pp. 17518–17528, 2017.
- [22] W. Zhang, X. Shen, C. Wan et al., "Effects of insulin and insulin-like growth factor 1 on osteoblast proliferation and differentiation: differential signalling via Akt and ERK," *Cell Biochemistry and Function*, vol. 30, no. 4, pp. 297–302, 2012.
- [23] Y. Yu, J. Mu, Z. Fan et al., "Insulin-like growth factor 1 enhances the proliferation and osteogenic differentiation of human periodontal ligament stem cells via ERK and JNK MAPK pathways," *Histochemistry and Cell Biology*, vol. 137, no. 4, pp. 513–525, 2012.
- [24] S. S. Durgam, A. A. Stewart, M. Sivaguru, A. J. Wagoner Johnson, and M. C. Stewart, "Tendon-derived progenitor cells improve healing of collagenase-induced flexor tendinitis," *Journal of Orthopaedic Research*, vol. 34, no. 12, pp. 2162–2171, 2016.
- [25] S. S. Durgam, A. A. Stewart, H. C. Pondenis, A. C. Yates, R. B. Evans, and M. C. Stewart, "Responses of equine tendon- and bone marrow-derived cells to monolayer expansion with fibroblast growth factor-2 and sequential culture with pulverized tendon and insulin-like growth factor-I," *American Journal of Veterinary Research*, vol. 73, no. 1, pp. 162–170, 2012.
- [26] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.
- [27] Y. W. Chen, E. Caporali, and M. Stewart, "Bone morphogenetic protein 2 stimulates chondrogenesis of equine synovial membrane-derived progenitor cells," *Veterinary and Comparative Orthopaedics and Traumatology*, vol. 29, no. 05, pp. 378–385, 2016.
- [28] B. Reible, G. Schmidmaier, A. Moghaddam, and F. Westhauser, "Insulin-like growth factor-1 as a possible alternative to bone morphogenetic protein-7 to induce osteogenic differentiation of human mesenchymal stem cells in vitro," *International Journal of Molecular Sciences*, vol. 19, no. 6, p. 1674, 2018.
- [29] J. W. Zhou, F. Y. Wei, and Y. Q. Ma, "Inhibiting PPAR $\gamma$  by erythropoietin while upregulating TAZ by IGF1 synergistically promote osteogenic differentiation of mesenchymal stem cells," *Biochemical and Biophysical Research Communications*, vol. 478, no. 1, pp. 349–355, 2016.
- [30] C. Y. Chen, K. Y. Tseng, Y. L. Lai, Y. S. Chen, F. H. Lin, and S. Lin, "Overexpression of insulin-like growth factor 1 enhanced the osteogenic capability of aging bone marrow mesenchymal stem cells," *Theranostics*, vol. 7, no. 6, pp. 1598–1611, 2017.
- [31] M. Bodenlenz, L. A. Schaupp, T. Druml et al., "Measurement of interstitial insulin in human adipose and muscle tissue under moderate hyperinsulinemia by means of direct interstitial access," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 289, no. 2, pp. E296–E300, 2005.
- [32] N. M. Templeman, S. Skovsø, M. M. Page, G. E. Lim, and J. D. Johnson, "A causal role for hyperinsulinemia in obesity," *Journal of Endocrinology*, vol. 232, no. 3, pp. R173–R183, 2017.
- [33] Y. Gong, Y. Ma, M. Sinyuk et al., "Insulin-mediated signaling promotes proliferation and survival of glioblastoma through Akt activation," *Neuro-Oncology*, vol. 18, no. 1, pp. 48–57, 2016.
- [34] P. Li, J. Wei, X. Gao et al., "Insulin promotes the proliferation of human umbilical cord matrix-derived mesenchymal stem cells by activating the Akt-cyclin D1 axis," *Stem Cells International*, vol. 2017, 10 pages, 2017.
- [35] M. Palaniappan, B. Menon, and K. M. J. Menon, "Stimulatory effect of insulin on theca-interstitial cell proliferation and cell cycle regulatory proteins through MTORC1 dependent pathway," *Molecular and Cellular Endocrinology*, vol. 366, no. 1, pp. 81–89, 2013.
- [36] J. I. Selig, D. M. Ouwens, S. Raschke et al., "Impact of hyperinsulinemia and hyperglycemia on valvular interstitial cells - a link between aortic heart valve degeneration and type 2 diabetes," *Biochimica et Biophysica Acta - Molecular Basis of Disease*, vol. 1865, no. 9, pp. 2526–2537, 2019.
- [37] K. Siddle, "Molecular basis of signaling specificity of insulin and IGF receptors: neglected corners and recent advances," *Frontiers in Endocrinology*, vol. 3, 2012.
- [38] K. Straßburger, M. Tiebe, F. Pinna, K. Breuhahn, and A. A. Teleman, "Insulin/IGF signaling drives cell proliferation in part via Yorkie/YAP," *Developmental Biology*, vol. 367, no. 2, pp. 187–196, 2012.
- [39] S. O. Abrahamsson and S. Lohmander, "Differential effects of insulin-like growth factor-I on matrix and DNA synthesis in various regions and types of rabbit tendons," *Journal of Orthopaedic Research*, vol. 14, no. 3, pp. 370–376, 1996.
- [40] M. A. Costa, C. Wu, B. V. Pham, A. K. S. Chong, H. M. Pham, and J. Chang, "Tissue engineering of flexor tendons: optimization of tenocyte proliferation using growth factor supplementation," *Tissue Engineering*, vol. 12, no. 7, pp. 1937–1943, 2006.
- [41] E. A. DesRosiers, L. Yahia, and C. H. Rivard, "Proliferative and matrix synthesis response of canine anterior cruciate ligament fibroblasts submitted to combined growth factors," *Journal of Orthopaedic Research*, vol. 14, no. 2, pp. 200–208, 1996.

- [42] S. S. Durgam, A. A. Stewart, H. C. Pondenis, S. M. Gutierrez-Nibeyro, R. B. Evans, and M. C. Stewart, "Comparison of equine tendon- and bone marrow-derived cells cultured on tendon matrix with or without insulin-like growth factor-I supplementation," *American Journal of Veterinary Research*, vol. 73, no. 1, pp. 153–161, 2012.
- [43] J. L. Fowlkes, R. C. Bunn, L. Liu et al., "Runx-related transcription factor 2 (RUNX2) and RUNX2-related osteogenic genes are down-regulated throughout osteogenesis in type 1 diabetes mellitus," *Endocrinology*, vol. 149, no. 4, pp. 1697–1704, 2008.
- [44] J. He, C. J. Rosen, D. J. Adams, and B. E. Kream, "Postnatal growth and bone mass in mice with IGF-I haploinsufficiency," *Bone*, vol. 38, no. 6, pp. 826–835, 2006.
- [45] D. M. Tiago, M. L. Cancela, and V. Laize, "Proliferative and mineralogenic effects of insulin, IGF-1, and vanadate in fish osteoblast-like cells," *Journal of Bone and Mineral Metabolism*, vol. 29, no. 3, pp. 377–382, 2011.
- [46] X. X. Cong, X. S. Rao, J. X. Lin et al., "Activation of AKT-mTOR signaling directs tenogenesis of mesenchymal stem cells," *Stem Cells*, vol. 36, no. 4, pp. 527–539, 2018.
- [47] H. T. Jiang, C. C. Ran, Y. P. Liao et al., "IGF-1 reverses the osteogenic inhibitory effect of dexamethasone on BMP9-induced osteogenic differentiation in mouse embryonic fibroblasts via PI3K/AKT/COX-2 pathway," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 191, p. 105363, 2019.
- [48] F. Oliva, D. Barisani, A. Grasso, and N. Maffulli, "Gene expression analysis in calcific tendinopathy of the rotator cuff," *European Cells & Materials*, vol. 21, pp. 548–557, 2011.
- [49] Y. F. Rui, P. P. Y. Lui, M. Ni, L. S. Chan, Y. W. Lee, and K. M. Chan, "Mechanical loading increased BMP-2 expression which promoted osteogenic differentiation of tendon-derived stem cells," *Journal of Orthopaedic Research*, vol. 29, no. 3, pp. 390–396, 2011.



**Hindawi**

Submit your manuscripts at  
[www.hindawi.com](http://www.hindawi.com)

