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# Preferential tendon stem cell response to growth factor supplementation

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## Abstract

Tendon injuries are increasingly prevalent around the world, accounting for more than 100 000 new clinical cases/year in the USA alone. Cell-based therapies have been proposed as a therapeutic strategy, with recent data advocating the use of tendon stem cells (TSCs) as a potential cell source with clinical relevance for tendon regeneration. However, their *in vitro* expansion is problematic, as they lose their multipotency and change their protein expression profile in culture. Herein, we ventured to assess the influence of insulin-like growth factor 1 (IGF-1), growth and differentiation factor-5 (GDF-5) and transforming growth factor- $\beta$ 1 (TGF $\beta$ 1) supplementation in TSC culture. IGF-1 preserved multipotency for up to 28 days. Upregulation of decorin and scleraxis expression was observed as compared to freshly isolated cells. GDF-5 treated cells exhibited reduced differentiation along adipogenic and chondrogenic pathways after 28 days, and decorin, scleraxis and collagen type I expression was increased. After 28 days, TGF $\beta$ 1 supplementation led to increased scleraxis, osteonectin and collagen type II expression. The varied responses to each growth factor may reflect their role in tendon repair, suggesting that: GDF-5 promotes the transition of tendon stem cells towards tenocytes; TGF $\beta$ 1 induces differentiation along several pathways, including a phenotype indicative of fibrocartilage or calcified tendon, common problems in tendon healing; and IGF-1 promotes proliferation and maintenance of TSC phenotypes, thereby creating a population sufficient to have a beneficial effect. Copyright © 2013 John Wiley & Sons, Ltd.

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**Keywords** tendon stem cell phenotype maintenance; tendon stem cell differentiation; growth factor supplementation; tendon stem cell culture; tenogenesis; tendon healing

## 1. Introduction

In the USA, tendon and ligament injuries account for approximately 50% of all musculoskeletal conditions, with approximately 100 000 new cases/year (Zeugolis *et al.*, 2011a, 2011b). Tendon autografts, the gold standard in clinical practice, which have demonstrated limited success in knee and anterior cruciate ligament surgery (Crossett *et al.*, 2002; Krych *et al.*, 2008), still present the disadvantages of creating a secondary injury

site during harvesting to only partially repair the injured tissue (Ikeda *et al.*, 2011; James *et al.*, 2011a, 2011b), and there is not sufficient supply to treat degenerative conditions (Nakamura and Katsuki, 2002; Chu *et al.*, 2008). Allograft and xenograft alternatives are characterized by delayed remodelling and substantial stability and still face the risk of potential transmission of infectious diseases (Stone *et al.*, 2007). To address the clinical need for functional tendon regeneration, numerous synthetic and natural biomaterials have been developed in recent years (Zeugolis *et al.*, 2011a, 2011b). However, synthetic substitution results in a non-functional and significantly thinner and weaker neotissue and/or in a disordered fibrous capsule (Cao *et al.*, 2006). Natural biopolymers, such as collagen (Kato *et al.*, 1991; Cavallaro *et al.*, 1994; Zeugolis *et al.*, 2009; Kew *et al.*, 2011; Kishore

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*et al.*, 2011), chitosan (Bagnaninchi *et al.*, 2007), fibrin (Hohendorff *et al.*, 2008) and silk (Kardestuncer *et al.*, 2006; Sahoo *et al.*, 2010) have also been assessed for tendon and ligament reconstruction, with promising *in vitro* and *in vivo* results. However, only a few products have been clinically approved to date (Derwin *et al.*, 2006). In fact, there is little evidence to suggest that the invasive new therapies have improved the 4–13% failure rate observed with autografts (Longo *et al.*, 2010; Shearn *et al.*, 2011).

To this end, cell-mediated strategies are under development for tendon and ligament regeneration. It is believed that the limited number of resident tendon cells might explain the limited inherent regeneration capacity of tendon, and therefore delivery of an appropriate cell population may be able to encourage regeneration whilst preventing the development of fibrocartilage regions (Richardson *et al.*, 2007; Sutter 2007; Bullough *et al.*, 2008; Yin *et al.*, 2010). Mesenchymal stem cells (MSCs) (Hoffmann *et al.*, 2006; Krampera *et al.*, 2006; Kryger *et al.*, 2007; Ben-Arav *et al.*, 2009; Schnabel *et al.*, 2009) and adipose-derived stem cells (Park *et al.*, 2010; Uysal and Mizuno, 2010, 2011) have been shown to have greater proliferation rates than tenocytes, whilst animal models and clinical trials have demonstrated significantly improved biomechanical, biological and biochemical characteristics in response to stem cell injection (Caplan *et al.*, 1998; Young *et al.*, 1998; Awad *et al.*, 1999; Wang *et al.*, 2005; Juncosa-Melvin *et al.*, 2006). However, ectopic bone formation has been observed previously after MSCs implantation in tendon models (Harris *et al.*, 2004; Bi *et al.*, 2007; Lui *et al.*, 2011). Moreover, the lack of tendon-specific markers hampers quantification of tenogenic differentiation of these cells (Richardson *et al.*, 2007). The recent discovery of tendon stem cells (TSCs) and their characterization in terms of multipotency, clonogenicity and self-renewal (Bi *et al.*, 2007; Rui *et al.*, 2010) may provide a valuable solution towards tendon regeneration. However, TSCs lose their phenotype *in vitro* with time and passageing (Tan *et al.*, 2012a, 2012b), which significantly limits their clinical potential.

Herein, it was hypothesized that treatment of TSCs with the appropriate concentration of growth factors will facilitate *ex vivo* tendon stem cell phenotype maintenance in terms of multipotency, proliferation, marker expression and extracellular matrix (ECM) production over longer intervals than previously possible. The rationale of using growth factors to achieve this relies on the fact that one of the most potent techniques for maintaining cell phenotype, or indeed inducing cells to differentiate towards a new phenotype, is treatment with growth factors, alone or in combination (Mitchell *et al.*, 1993; Tateno and Yoshizato, 1999; Huang *et al.*, 2009). Induction of tenogenic differentiation in embryonic, adipose-derived or bone marrow-derived stem cells has been attempted using a variety of growth factors, including insulin-like growth factor-1 (IGF-1), growth and differentiation factor-5 (GDF-5) and transforming growth factor- $\beta$ 1 (TGF $\beta$ 1) (Farnig *et al.*, 2008; Kapacee *et al.*, 2010;

Okamoto *et al.*, 2010; Schneider *et al.*, 2011). These three growth factors were selected because they represent different areas of growth factor-mediated effects. Specifically, IGF-1 is known to play a pivotal role in the maintenance of tenocyte phenotype (Costa *et al.*, 2006; Qiu *et al.*, 2012) and tendon repair after injury (Klein *et al.*, 2002a, 2002b; Qiu *et al.*, 2012; Raghavan *et al.*, 2012) and has been used in a clinical trial to enhance collagen synthesis in the patellar tendon of Ehlers–Danlos patients and healthy controls (ClinicalTrials.gov; Trial ID: NCT01446783). GDF-5 supplementation has been shown to maintain tenocyte phenotype (Wolfman *et al.*, 1997; Hogan *et al.*, 2011; Keller *et al.*, 2011), promote tenogenic differentiation (Hayashi *et al.*, 2011; James *et al.*, 2011a, 2011b; Tan *et al.*, 2012a, 2012b) and is known to play a critical role in tendon development (Francis-West *et al.*, 1999; Clark *et al.*, 2001; Chhabra *et al.*, 2003). TGF $\beta$ 1 is highly upregulated during development (Pryce *et al.*, 2009) and after tendon injury (Molloy *et al.*, 2003; Kashiwagi *et al.*, 2004; James *et al.*, 2008) and has been used as a supplement in a variety of tenocyte culture studies (Wolfman *et al.*, 1997; Klein *et al.*, 2002a, 2002b; Schneider *et al.*, 2011; Chen *et al.*, 2012; Heisterbach *et al.*, 2012; Laumonier *et al.*, 2012; Mendias *et al.*, 2012). Furthermore, it is a key component of platelet-rich plasma (PRP), an increasingly popular treatment for tendon strain and tendinopathy (Chen *et al.*, 2012).

## 2. Materials and methods

### 2.1. Materials

All primary cells were obtained in accordance with the ethical guidelines of the National University of Ireland, Galway, from male Lewis rats aged 8–9 weeks (Charles River, UK). Growth and differentiation factor-5 (GDF-5) and insulin-like growth factor-1 (IGF-1) were obtained from ProSpec (Israel). Transforming growth factor- $\beta$ 1 (TGF $\beta$ 1) was obtained from Millipore (Ireland). Unless otherwise specified, all other consumables and cell culture media were obtained from Sigma-Aldrich (Ireland) or Fisher Scientific (Ireland).

### 2.2. Tendon stem cell isolation and characterization

Rat patellar tendon-derived stem cells (TSCs) were harvested as described previously (Bi *et al.*, 2007). Briefly, the tendons were explanted and cleaned aseptically with a scalpel to remove all paratendon, fat and muscle. The tendons were then minced using a scalpel into 1 mm-thick slices and placed in a 0.3% w/w collagenase solution (*Clostridium histolyticum*, > 125 CDU/mg; Sigma-Aldrich, Ireland). After 30 min, the partially digested tendon was placed on a 70  $\mu$ m cell strainer and washed with Hank's balanced salt solution (HBSS). The tendon was then placed in fresh 0.3% collagenase solution and digested

at 37°C for 2 h. The liquid portion after this digest was filtered through a 70 µm cell strainer and the remaining tendon was washed in basal TSC medium (Vornia Biomaterials Ltd, Ireland). The filtered fraction was centrifuged at 300 × g for 5 min; the supernatant was removed, then resuspended in basal TSC medium, placed in a tissue culture flask and allowed to attach for 5 days without disturbance. After 10 days, colonies were identified and the morphology compared to the literature.

Characterization for multipotency, clonogenicity, surface marker expression and extracellular matrix (ECM) production (see below) was carried out when the cells reached 80% confluency (approximately 14–21 days). Standard chondrogenic, osteogenic and adipogenic induction protocols were followed, using a Mesenchymal Stem Cell Functional Analysis kit according to the manufacturer's protocol (SC010, R&D Systems, UK). Osteogenic and adipogenic induction experiments were conducted by culturing TSC monolayers in media supplemented with the osteogenic or adipogenic supplements provided with the kit. Adipogenic induction could be observed as soon as 10 days after induction. Osteogenic induction required 21 days. To analyse chondrogenic potential, TSC pellets were treated with chondrogenic induction medium for 28 days and then the pellet was fixed and cryosectioned. Immunohistochemical staining for fatty acid binding protein-4 (FABP4), osteonectin and collagen type II (R&D Systems) was used to quantify the fraction of cells that were successfully induced (adipogenic, osteogenic and chondrogenic induction, respectively) and oil red O, alizarin red and toluidine blue staining was used to confirm the antibody staining qualitatively.

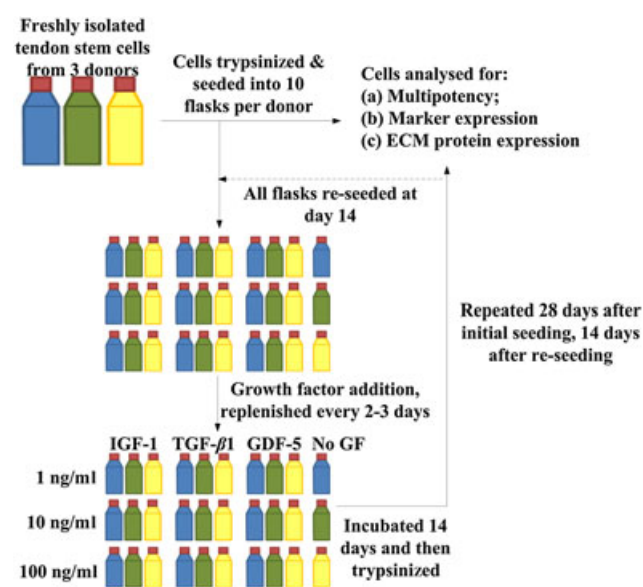
Standard clonogenicity assays were carried out to confirm clonogenicity over time. Briefly, TSCs were plated at density of 10 cells/cm<sup>2</sup> and cultured for 7 days. The cells were then stained with 0.5% crystal violet (Sigma-Aldrich) and counted. Surface marker expression was analysed via flow cytometry (BD FACS Canto, UK) and immunohistochemical staining. For flow cytometry, trypsinized cells (5 × 10<sup>5</sup>) were stained with APC-Cy7 anti-CD90<sup>+</sup>, AlexaFluor 647 anti-CD44<sup>+</sup>, PerCP anti-CD34<sup>+</sup> and FITC anti-CD31<sup>+</sup>. These results were confirmed with immunohistochemical staining with unlabelled analogues (Santa Cruz, USA) and a FITC-labelled donkey anti-mouse secondary antibody (Invitrogen, Ireland). Tendon-specific markers and ECM molecules were also analysed immunohistochemically, using tenomodulin (TNMD) and scleraxis (SCX) or decorin (DCN), collagen type I and tenascin-c (TEN-C), respectively. The expression of collagen type II, osteonectin and FABP4 were also analysed immunohistochemically without any induction, using the same antibodies as in the multipotency analysis.

For the cell surface markers, the positive cell fraction (i.e. fraction of cells expressing the given markers) was quantified using flow cytometry gated to threshold levels using positive and negative controls. These measurements were then confirmed using immunohistochemical staining and image analysis software (Image Proplus v 5, Media Cybernetics), with thresholds set using positive

and negative controls. For markers such as collagen type II and osteonectin, which are secreted proteins, only immunohistochemical analysis was used, as the secreted proteins were not associated with the cell membrane enough to be quantified using flow cytometry.

### 2.3. Growth factor treatment study outline

TSC populations from three donors were used in all studies to form a biological replicate of three. The study outline is illustrated in Figure 1. At time 0 (approximately 18 days after the cells were extracted), 200 000 cells were seeded into 30 flasks (10 flasks/donor). At this point, a subset of these cells was set aside for multipotency analysis and their baseline expression of markers and ECM molecules was quantified. One flask/donor was selected for treatment with 1, 10 and 100 ng/ml of each growth factor and one flask/donor was set aside and treated with basal tendon cell medium (0 ng/ml growth factor). The cells in the treatment groups were treated with the appropriate growth factor-supplemented medium every 2–3 days for 14 days. At this time point, the cells were trypsinized and subsets of the cells from each flask (200 000/flask) were seeded into a new flask and cultured to continue the study. The remaining cells were preserved in RIPA buffer (Thermo Scientific, Germany) for ELISA analysis, seeded for multipotency analysis, or seeded onto optical plates for immunohistochemistry. This procedure



**Figure 1.** Diagram depicting study donors. These cells were trypsinized at day 0 and either seeded into new flasks for the study or analysed for multipotency, marker expression and ECM protein expression. The seeded flasks were treated with growth factors (replenished every 2–3 days) for 14 days. At this point, the flasks were all trypsinized and either seeded into new flasks and cultured for another 14 days (28 total) or used for analysis. After 28 days, all cells were analysed for multipotency, marker expression and ECM protein expression



was repeated after 28 days without reseeding of the flasks. Thus, all of the cells had been trypsinized three times by the end of the 28 days.

## 2.4. Multipotency studies

At each time point, cells were trypsinized and seeded for multipotency analysis. As described in Section 2.2, standard osteogenic and adipogenic induction protocols were followed, using the R&D Systems (UK) Functional Analysis Kit. Immunohistochemical staining for FABP4, osteonectin and collagen type II was used to quantify the fraction of cells that were successfully induced, and oil red O, alizarin red and alcian blue staining were used to confirm the antibody staining qualitatively. The fraction of cells expressing each marker was analysed in order to compare the efficacy of each growth factor in phenotype maintenance.

## 2.5. Immunohistochemistry analysis

Immunohistochemical analysis was used to determine the expression of CD31, CD34, CD44, CD90, DCN, SCX, osteonectin, collagen types I and II, FABP4 and TEN-C. In all cases, the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Anti-CD44 and anti-CD90 were obtained from BioLegend (UK), anti-collagen II and anti-FABP4 from R&D Systems (UK) and the rest from Santa Cruz (USA). The four CD markers were selected in light of the work previously conducted to demonstrate the TSC phenotype in rats (Rui *et al.*, 2010; Zhang *et al.*, 2011). The antibodies to these markers were raised in mice, as was the anti-FABP4; anti-TNMD was raised in rabbit; anti-collagen type II was raised in sheep; and the antibodies to DCN, SCX, osteonectin, collagen type I and TEN-C were raised in goat. The secondary antibodies used were: AlexaFluor 488 donkey anti-mouse (Invitrogen); AlexaFluor 488 mouse anti-sheep (Invitrogen); Texas red donkey anti-rabbit (Invitrogen, Ireland); FITC rabbit anti-goat (Vector Laboratories, UK); and AlexaFluor 594 donkey anti-goat (Invitrogen). When dual staining was used, the antibodies were tested on control selections to ensure negligible interactions.

## 2.6. Direct enzyme-linked immunosorbent assays (ELISAs)

As suitable antibody pairs for sandwich ELISA were not available for most of the target proteins, direct ELISA protocols were optimized instead, using the peptides against the antibody-binding sequences as a standard. These peptides are approximately 20 amino acids in length and are matched to the antibody. It should be noted that the measured concentration refers to the mass of antibody-binding sequences/volume, not the mass of the full protein; in the case of large proteoglycans, such as DCN, this is an important distinction. The

exception to this was collagen type II, as a collagen type II standard was available at a high purity and concentration.

Briefly, antigen-containing samples were coated onto an immunoplate (Thermo Scientific, Germany) overnight at room temperature. The following day, samples were blocked with 5% bovine serum albumin (BSA) and 0.1% Tween in phosphate-buffered saline (PBS), treated with the appropriate primary antibody at a 1:250 dilution, treated with anti-goat HRP at 1:500 dilution, and developed using One-step™ Ultra TMB-ELISA (Thermo Scientific). The protein concentration was normalized to total protein content using a BCA assay (Thermo Scientific).

## 2.7. Histological staining

Standard oil red O, alizarin red and toluidine blue staining protocols were used to confirm the results of the immunohistochemical multipotency analysis and check for unintended differentiation of the stem cells at each time point. Briefly, adipogenic cultures were fixed, rinsed, incubated for 5 min with 60% isopropanol and incubated for 5 min with oil red O stock solution. The oil red O was then removed and the cells washed thoroughly with tap water before counterstaining with haematoxylin. Osteogenic cultures were fixed, washed with PBS, incubated for 20 min in alizarin red working solution, washed with distilled water, and washed again with PBS. Sections of chondrogenic pellets were hydrated in distilled water and stained in the toluidine blue working solution for 3 min. The sections were then washed in distilled water, dehydrated and mounted.

## 2.8. Statistical analysis

All statistical analyses were conducted using IBM SPSS v. 20. Normality was calculated using the Kolmogorov–Smirnov test and/or the Shapiro–Wilk test. Non-parametric data were compared using the independent samples Mann–Whitney U-test or the independent samples Kruskal–Wallis test. Normal data with equal variance were analysed using one-way analysis of variance (ANOVA) to determine statistical significance within groups, and the Bonferroni *post hoc* test was used to determine where the differences lay. Outliers that lay outside of three interquartile ranges were removed from the dataset. Significance was set at  $p < 0.05$  and all graphs are presented as mean  $\pm$  standard error of the mean (SEM).

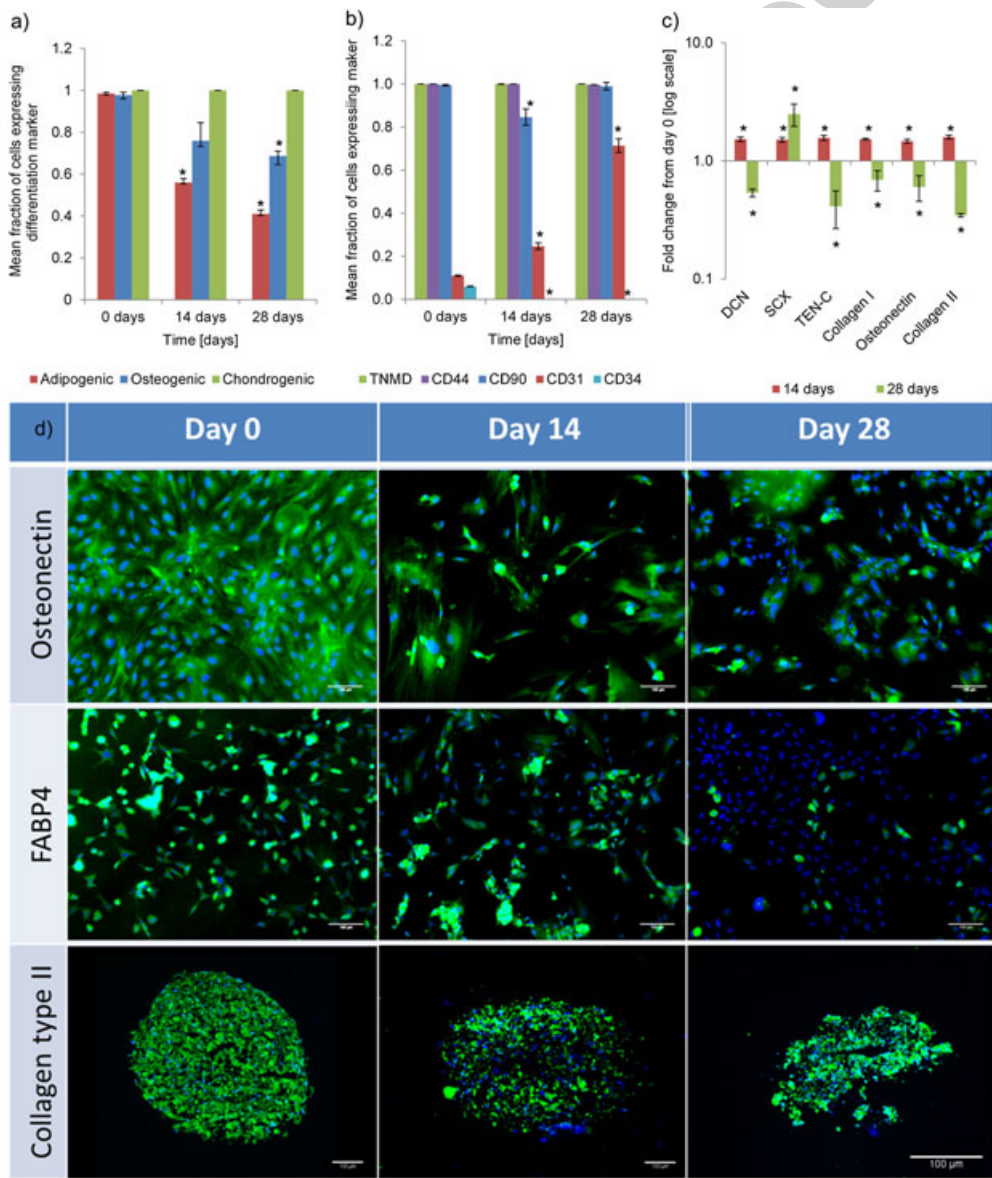
# 3. Results

## 3.1. TSC differentiation potential and marker expression *in vitro*

When extracted from tendon, TSCs rapidly lost their differentiation potential. A significant loss of adipogenic

( $p < 0.0001$ ) and osteogenic ( $p < 0.0001$ ) potential was observed in terms of the fraction of cells expressing F2 FABP4 and osteonectin, respectively (Figure 2a). There was also a decrease in the fraction of cells expressing CD90 after 14 days ( $p < 0.001$ ) and significant increases in the fractions of cells expressing CD31 after 28 days ( $p < 0.0001$ ), implying significant changes in the phenotype of the cells (Figure 2b). CD34 expression significantly decreased from approximately 6% to negligible between day 0 and day 14 ( $p < 0.001$ ) and CD44 expression did not significantly change from 100% at

any time point. Representative images used for these analyses are included in Figure S1 (see supporting information). Protein expression analysis on day 14 revealed an increase in all assayed proteins, including DCN, SCX, TEN-C, collagen type I, osteonectin and collagen type II (Figure 2c). Subsequent protein evaluation on day 28 revealed that, besides SCX, the expression level of the other proteins decreased significantly when compared to freshly isolated cells, as well as to the levels on day 14 ( $p < 0.0001$ ). The expression of SCX was significantly higher on day 28



**Figure 2.** TSC phenotype is altered over time. The fraction of cells expressing differentiation markers (a) decreased over time in the adipogenic and osteogenic groups, although there was no loss in chondrogenic potential over time. In terms of marker expression (b), there was no decrease in the fraction of cells expressing tenomodulin or CD44, while there was a significant decrease in the fraction of cells expressing CD34 (from 6% to 0%) and a temporary decrease in the fraction of cells expressing CD90. There was also a significant increase in the fraction of cells expressing CD31 over time, suggesting a change in phenotype. The protein expression (c) in the untreated cells was variable with time, with significant increases in all of the proteins after 14 days, but only SCX and collagen type II had significant changes after 28 days. Qualitatively (d), the expression of the markers was significantly reduced over time and, although collagen type II-expressing chondrogenic pellets did form, the collagen was less dense and the pellets were not as well formed. Data are expressed as mean  $\pm$  SEM; \*significant change from the values for freshly isolated cells.

compared to day 14 ( $p < 0.0001$ ). Similarly, Figure 2d shows that cells in pellets were capable of expressing osteonectin, FABP4 and collagen type II after 0, 14 and 28 days. However, the densities of cells expressing these markers appeared to decrease with time in the untreated cells (Figure 2d).

### 3.2. TSC multipotency in the presence of IGF-1

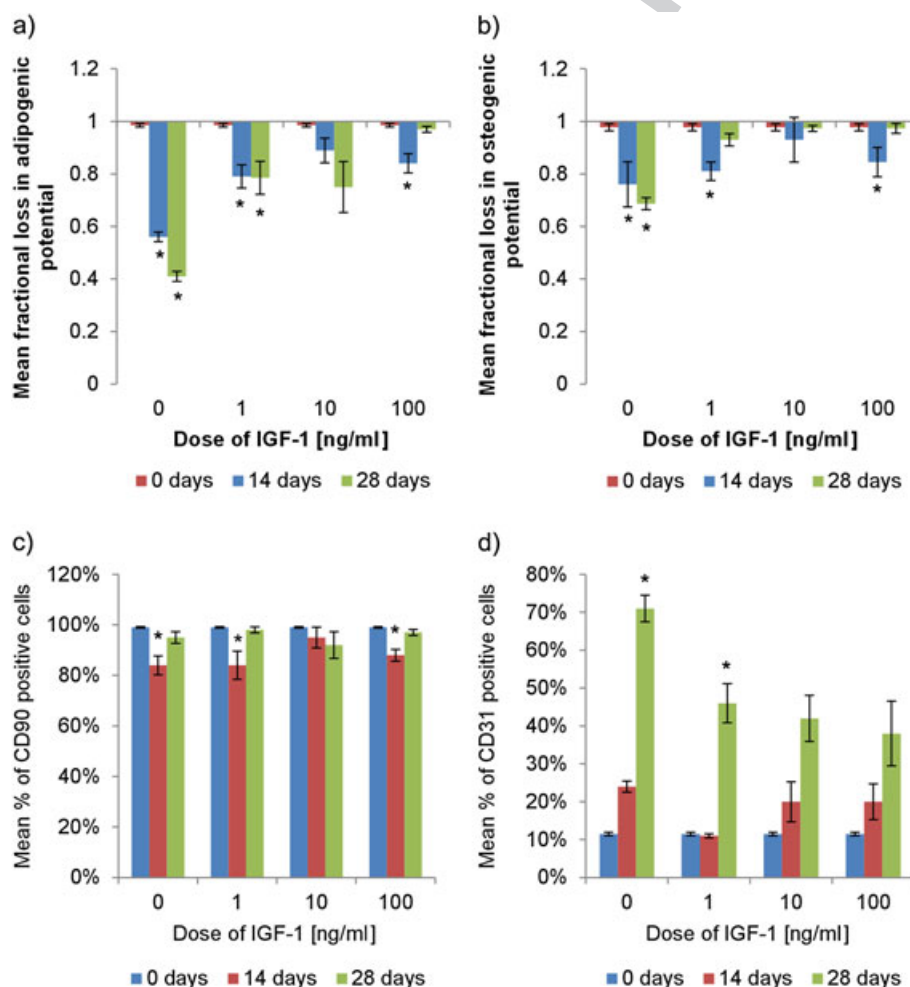
After treatment for 14 and 28 days with 10 ng/ml IGF-1, there was no significant loss in adipogenic ( $p > 0.05$ ;

**F3** Figure 3a) or osteogenic ( $p > 0.05$ ; Figure 3b) potential. Osteogenic potential was maintained with all doses of IGF-1 after 28 days. There was a transient decrease in the fraction of cells expressing CD90 in the untreated cells and in cells treated with 100 ng/ml IGF-1 after 14 days ( $p < 0.001$  and  $p < 0.003$ , respectively; Figure 3c) but this effect was not statistically significant after

28 days. Furthermore, while the fractions of untreated cells expressing CD31 significantly increased after 14 and 28 days in comparison to the day 0 cells ( $p < 0.0001$ ; Figure 3d), there were no significant increases in the groups treated with 10 or 100 ng/ml IGF-1. After 28 days, TSCs from all IGF-1-treated groups were capable of forming collagen type II-expressing pellets (see supporting information, Figure S2a).

### 3.3. TSC marker expression in the presence of IGF-1

IGF-1 supplementation did not affect DCN, TEN-C and osteonectin levels (Figure 4a, c, f, respectively;  $p > 0.05$ ). **F4** SCX expression (Figure 4b) was slightly increased at all doses after 28 days ( $p < 0.002$ ), whilst collagen type I expression (Figure 4d) was slightly decreased at all doses after 14 days and at 10 and 100 ng/ml



**Figure 3.** IGF-1 supplementation reduced the loss in stem cell phenotype observed in the TSCs. There was no significant decrease in adipogenic differentiation (a) in the group treated with 10 ng/ml at either time point and no change after 28 days in the 100 ng/ml group. The loss in osteogenic potential (b) was also reduced to negligible in the 10 ng/ml group and was not significant after 28 days at any dose. Chondrogenic potential was also evaluated and all groups were capable of forming collagen type II-expressing pellets at all time points. The temporary decrease in CD90 expression (c) observed after 14 days in the untreated cells was not observed in the group treated with 10 ng/ml IGF-1, neither was there any significant increase in CD31 expression (d) in either the 10 or 100 ng/ml groups at any time point. The 1 ng/ml dose did not appear sufficient to completely prevent changes in phenotype, but it was partially effective as compared to unsupplemented medium. Data are expressed as mean  $\pm$  SEM; \*significant change from the values for freshly isolated cells



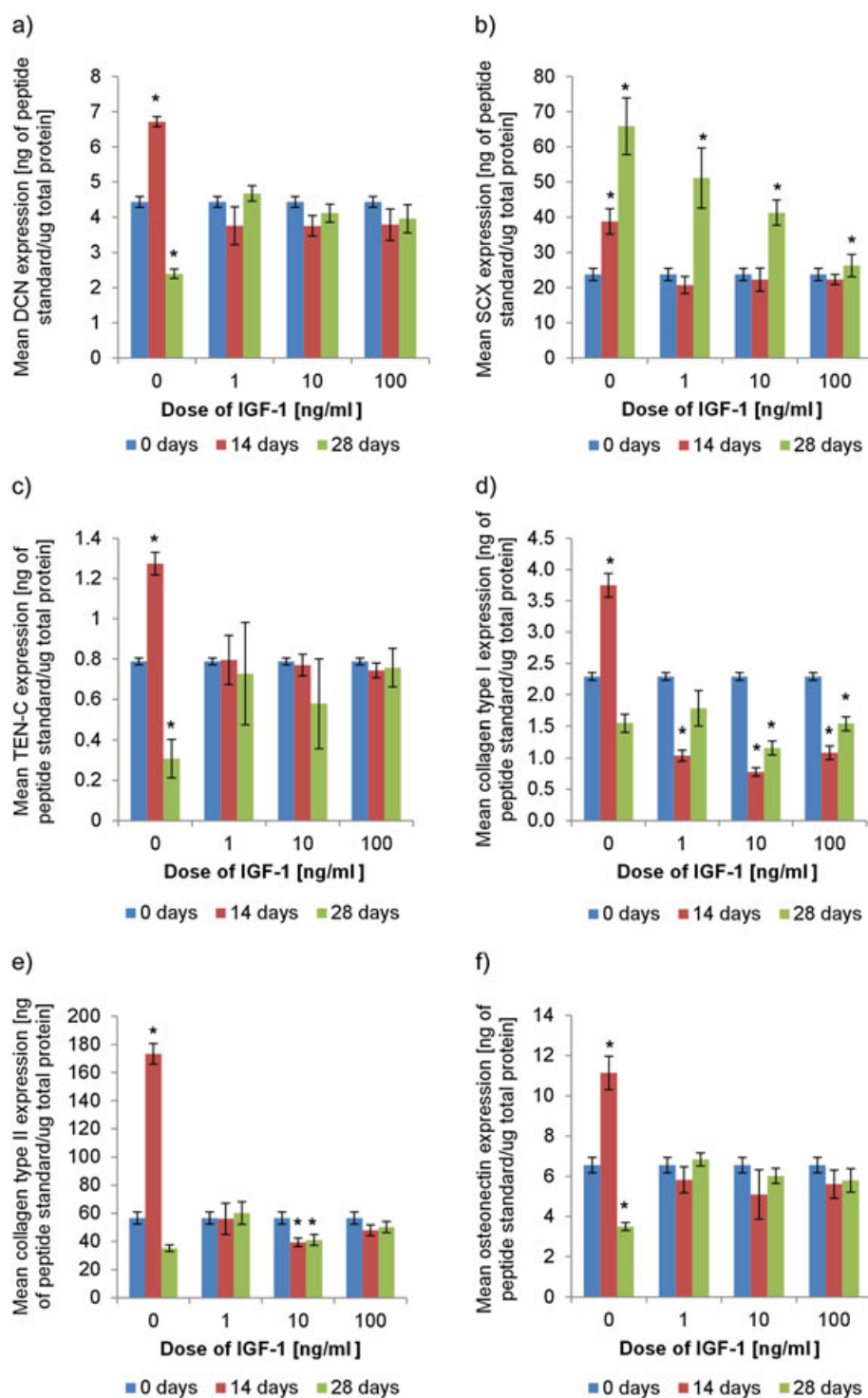


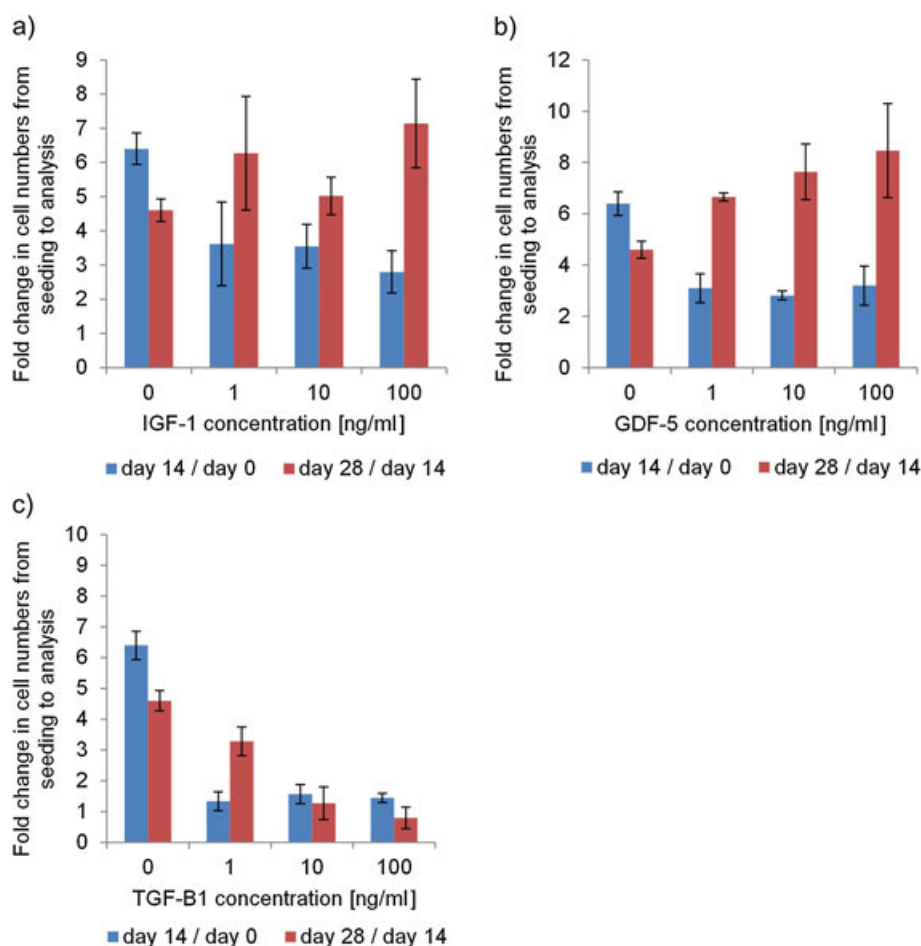
Figure 4. IGF-1 supplementation maintained the levels of some, but not all, tendon-, bone- and cartilage-related proteins. SCX expression slightly increased over time in all groups (b), but DCN (a), TEN-C (c) and osteonectin (f) expression did not significantly change in the groups treated with IGF-1 at any dose. Collagen type I expression decreased at all doses, although this effect was only statistically significant after 28 days in the 10 and 100 ng/ml groups. There was also decreased collagen type II expression in all groups at both 14 and 28 days. Data are expressed as mean  $\pm$  SEM; \*significant change from the values for freshly isolated cells

after 28 days ( $p < 0.003$  and  $p < 0.001$ , respectively). Markers of adipogenic differentiation were not observed (see supporting information, Figure S3a) and a statistically significant downregulation of collagen type II expression was observed at all doses ( $p < 0.006$ ; Figure 4e). No significant decrease in proliferation was observed after 28 days ( $p > 0.05$ ; Figure 5a).

### 3.4. TSC multipotency in the presence of GDF-5

After 14 and 28 days of TSC culture in media supplemented with 1, 10 or 100 ng/ml GDF-5, significant losses in TSC multipotency were observed (Figure 6). Significant decreases in adipogenic potential were observed after only 14 days at all doses ( $p < 0.009$ ) and after 28 days at





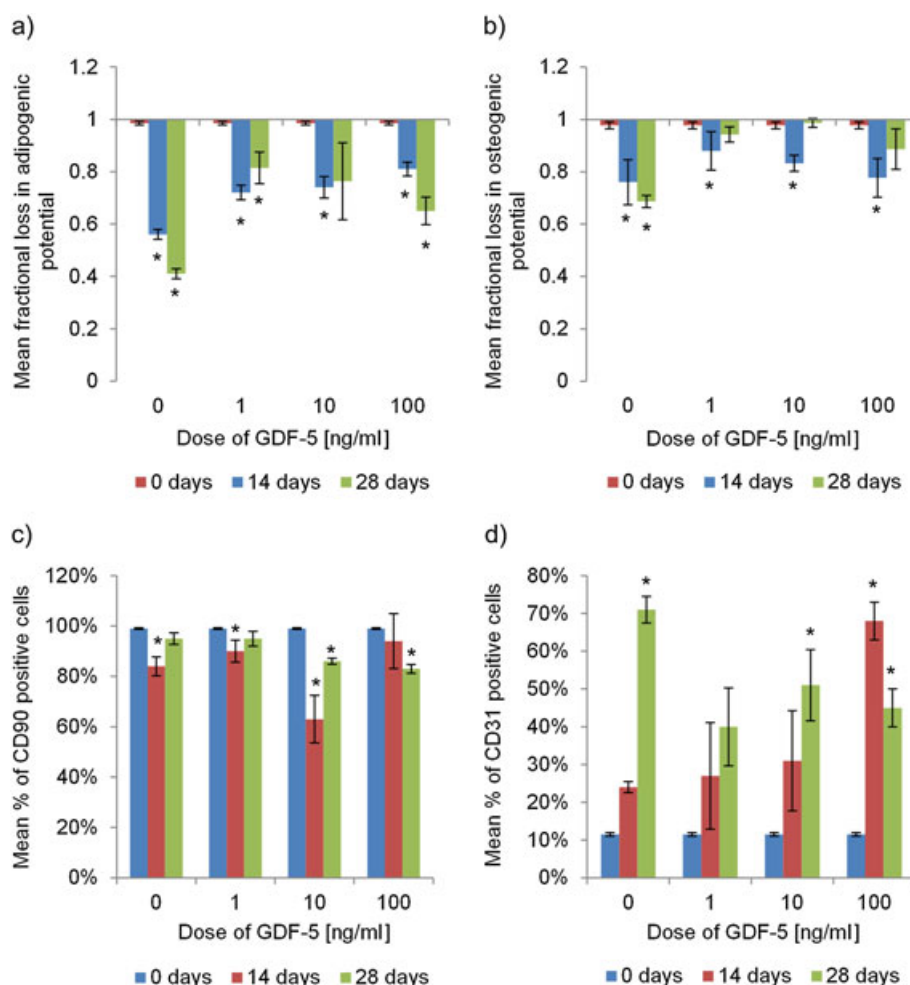
**Figure 5.** Proliferation as a function of time and growth factor dose. The fold change in cell numbers was used as a measure of proliferation. The cell proliferation rate between day 28 and day 14 did not significantly decrease as compared to the fold change between day 14 and day 0 in the cells treated with IGF-1 at any dose (a), although the fold change in cell numbers from day 0 to 14 was significantly less in the 100 ng/ml group than in the untreated cells. The proliferation rate temporarily decreased in the cells treated with 1–100 ng/ml GDF-5 (b; as compared to the untreated cells) but recovered after 28 days to be greater than that of the untreated cells. The proliferation rate of TSCs treated with TGF-β1 (c) significantly decreased as a function of dose after 28 days and was significantly increased in all groups after 14 days. Data are expressed as mean  $\pm$  SEM; \*significant change from the values for freshly isolated cells

1 and 100 ng/ml doses of GDF-5 ( $p < 0.008$ ; Figure 6a). Osteogenic potential was significantly decreased after 14 days in all groups ( $p < 0.026$ ; Figure 6b), but this effect was no longer significant after 28 days. CD90 expression was also reduced after 14 days in cells treated with 0–10 ng/ml GDF-5 doses ( $p < 0.001$ ) and after 28 days in groups treated with 10 and 100 ng/ml doses of GDF-5 ( $p = 0.022$ ) and CD31 expression was increased ( $p < 0.035$ ). Chondrogenic pellets formed from TSCs treated for 28 days with 10 and 100 ng/ml GDF-5 dissolved before culture was complete, suggesting a significant decrease in chondrogenic differentiation potential at the higher GDF-5 doses (see supporting information, Figure S2b).

### 3.5. TSC marker expression in the presence of GDF-5

GDF-5 treatment of TSC cultures induced statistically significant changes in expression levels of ECM and tendon-

related proteins. DCN expression was slightly increased after 28 days in the 1 ng/ml group ( $p < 0.025$ ; Figure 7a), while SCX expression was elevated compared to the untreated controls after 28 days in all groups ( $p < 0.003$ ; Figure 7b). TEN-C was downregulated in the 10 ng/ml groups after 28 days ( $p < 0.042$ ; Figure 6c) and collagen type I expression was significantly decreased in all groups after 14 days ( $p < 0.004$ ; Figure 6d), but unchanged after 28 days. Significant decreases were observed in collagen type II expression at the 1 and 100 ng/ml doses (Figure 6e). After 14 days, osteonectin expression was significantly increased in the 1 ng/ml group and decreased in the 100 ng/ml group ( $p < 0.002$  and  $p < 0.039$ , respectively; Figure 6f). After 28 days, however, no significant changes in osteonectin expression were observed in TSCs treated with GDF-5 ( $p > 0.05$ ). FABP4 expression was negligible in all samples (see supporting information, Figure S3b). No significant change in proliferation was observed after 28 days ( $p > 0.05$ ; Figure 5c).



**Figure 6.** GDF-5 supplementation did not maintain the stemness of the TSCs in terms of either adipogenic (a) or osteogenic (b) differentiation. Furthermore, TSCs treated with 10 or 100 ng/ml GDF-5 formed chondrogenic pellets but they dissolved before culture was complete, making it impossible to assess their collagen type II production. Significant decreases in CD90 expression were observed at both 10 and 100 ng/ml doses after 28 days (c) and CD31 expression increased significantly over time at the 10 and 100 ng/ml doses as well. Data are expressed as mean  $\pm$  SEM; \*significant change from the values for freshly isolated cells

### 3.6. TSC multipotency in the presence of TGF $\beta$ 1

Significant losses in adipogenic potential were observed in response to treatment with all doses of TGF $\beta$ 1 after 14 days (Figure 8a). Osteogenic potential was also decreased in all groups after 14 days ( $p < 0.048$ ), but this effect was not statistically significant after 28 days (Figure 8b). CD90 expression was significantly decreased after 14 days in all treatment groups ( $p < 0.019$ ; Figure 8c) but not after 28 days, while CD31 expression significantly increased after 28 days in all groups ( $p < 0.004$ ) and after 14 days in the group treated with 100 ng/ml TGF $\beta$ 1 ( $p < 0.03$ ; Figure 8d). All groups at all time points formed collagen type II-expressing chondrogenic pellets (see supporting information, Figure S2c).

### 3.7. TSC marker expression in the presence of TGF $\beta$ 1

After 28 days, there were significant changes in the expression of several markers. DCN was significantly upregulated

after 28 days in the group treated with 1 ng/ml TGF $\beta$ 1, as compared to initial DCN expression levels ( $p < 0.003$ ; Figure 9a). SCX expression after 28 days was significantly upregulated in comparison to time 0 at all doses of TGF $\beta$ 1 ( $p < 0.014$ ; Figure 9b). A significant increase in TEN-C expression was observed in the 100 ng/ml group after 28 days ( $p < 0.04$ ; Figure 9c). Collagen type I expression was also increased after 28 days in the 1 and 100 ng/ml groups ( $p < 0.047$ ; Figure 9d). Collagen type II expression was significantly increased (levels more than doubled) at all doses after 28 days in comparison to TSCs at day 0 ( $p < 0.005$ ; Figure 9e). Osteonectin expression was significantly increased when treated with 1 ng/ml TGF $\beta$ 1 and decreased after 14 days in response to both 10 and 100 ng/ml TGF $\beta$ 1 ( $p < 0.048$ ; Figure 9f). These changes are suggestive of a chondrocyte-like phenotype in these cells or in a subpopulation of the cells, which was further supported by the rounded shape of many of the cells and the staining for sulphated glycosaminoglycans in these cells (toluidine blue) (Figure 9g). A significant decrease in cell proliferation as compared to untreated TSCs was observed at both

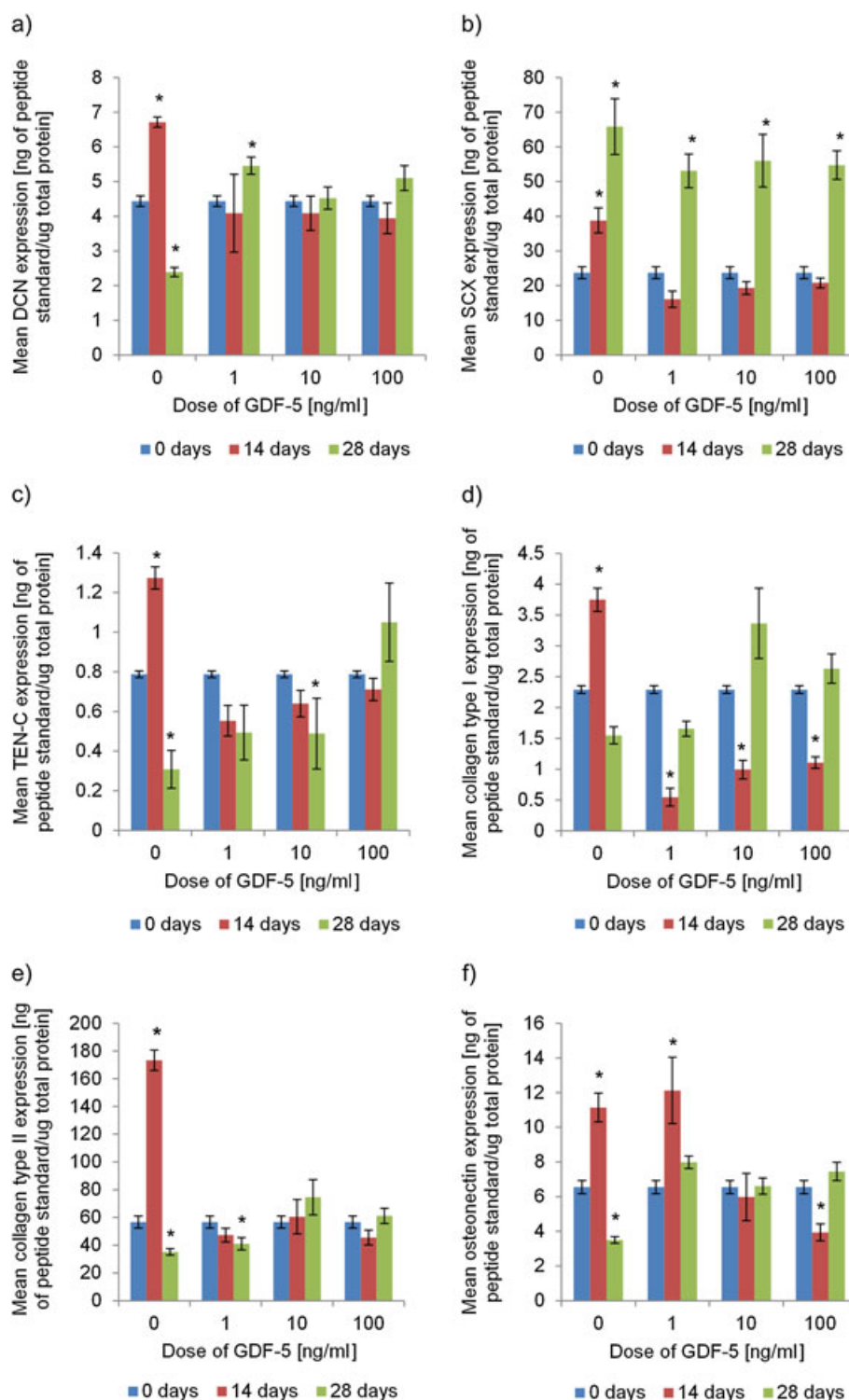


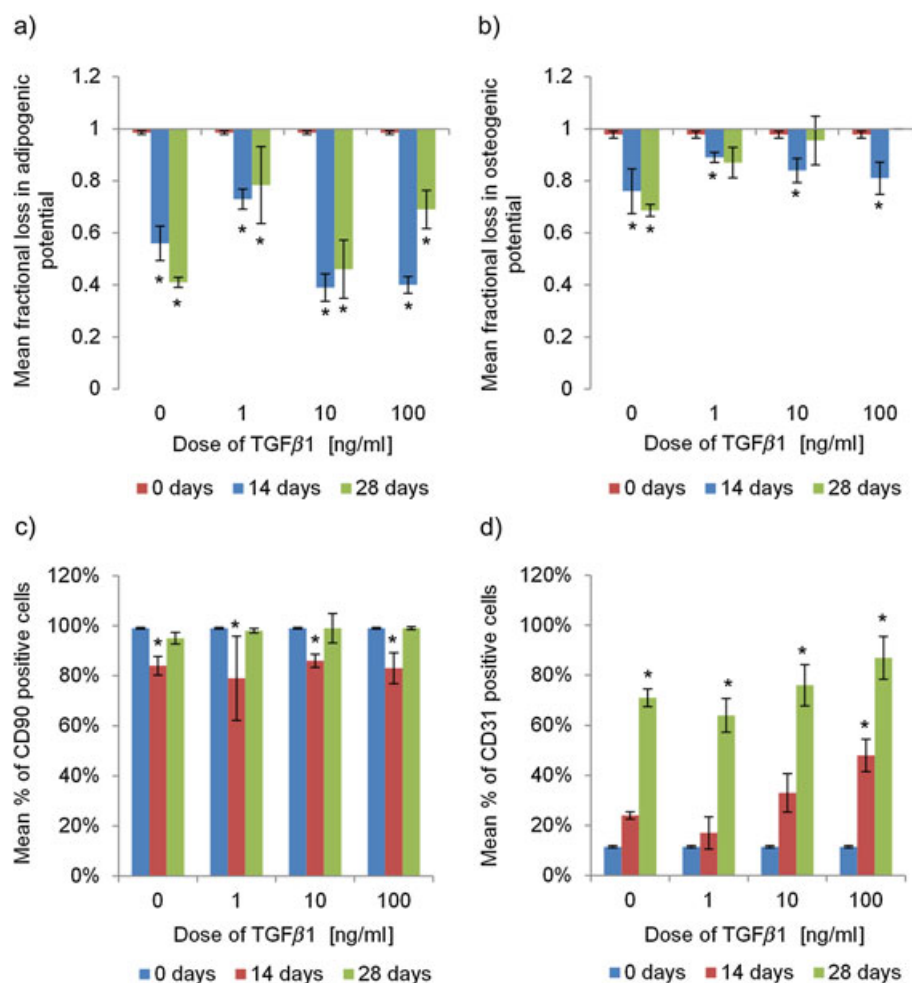
Figure 7. GDF-5 supplementation increased the expression of tendon-related markers. After 28 days, DCN expression (a) increased significantly at the 1 ng/ml dose, while SCX expression increased significantly at all doses (b). TEN-C expression (c) decreased marginally in response to the 10 ng/ml dose after 28 days, while collagen type I expression was downregulated temporarily at day 14 in all groups, but recovered after 28 days (d). Collagen type II expression decreased in both the 1 and 100 ng/ml groups (e), while there were temporary changes after 14 days in osteonectin expression that were not statistically significant after 28 days (f). No FABP4 expression or fat droplets were observed in any group at any time point. Data are expressed as mean  $\pm$  SEM; \*significant change from the values for freshly isolated cells

time points in the 10 and 100 ng/ml groups, suggesting the acquisition of a less proliferative phenotype ( $p < 0.0001$ ; Figure 9h). FABP4 expression was negligible in all groups (see supporting information, Figure S3c).

## 4. Discussion

Although cell-based therapies are becoming increasingly important in the field of tissue engineering and regenerative medicine, widespread acceptance and clinical



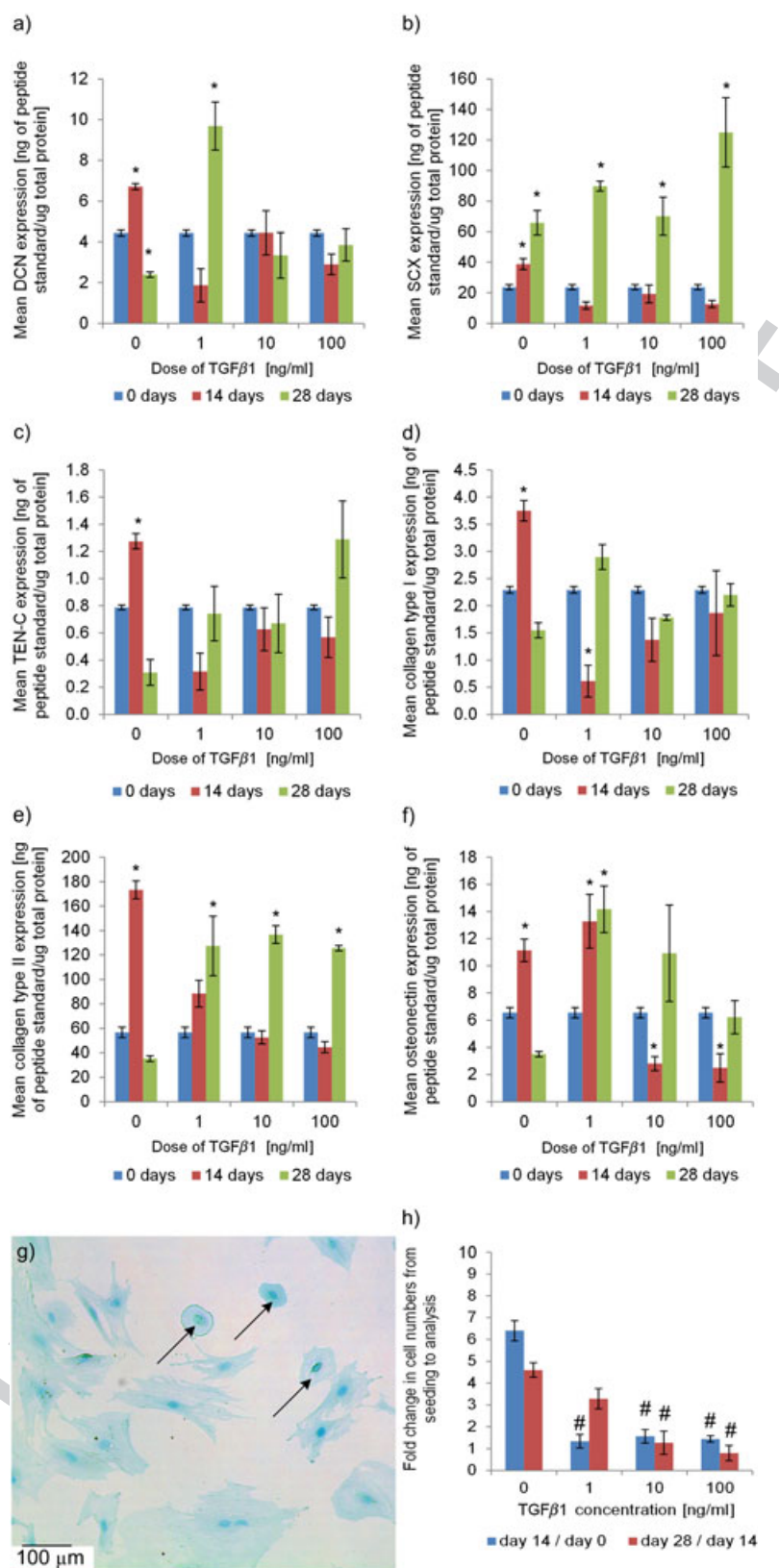


**Figure 8.** TGFβ1 induced differentiation of TSCs away from their native phenotype. At all doses and time points, significant decreases in adipogenic differentiation were observed (a). All groups were able to form collagen type II-expressing pellets. After 14 days, significant decreases in osteogenic potential were also observed (b), but this effect was not significant after 28 days. The temporary decrease in the fraction of cells expressing CD90 (c) observed in the untreated cells was similar to that in TGFβ1-treated cells at all time points, and the increase in the fraction of cells expressing CD31 (d) was more pronounced after treatment with TGFβ1 in all groups after 28 days. Data are expressed as mean ± SEM; \*significant change from the values for freshly isolated cells

translation is still limited, owing to cell phenotype drift in culture and subsequent loss of therapeutic potential. Growth factor supplementation has been advocated to remedy tenocyte phenotypic loss in culture (Klein *et al.*, 2002a, 2002b; Takahashi *et al.*, 2002; Tang *et al.*, 2003; Thomopoulos *et al.*, 2005, 2010; Keller *et al.*, 2011; Qiu *et al.*, 2012; Caliarì and Harley, 2013) and has been extensively studied as means of modulating stem cell differentiation (Musarò *et al.*, 2004; Farnig *et al.*, 2008; Hayashi *et al.*, 2011; Rose *et al.*, 2012; Trosan *et al.*, 2012). In the present study we evaluated the influence of growth factor supplementation on the maintenance of tendon stem cell phenotype, a cell population known to lose its phenotype in culture with time and passaging (Tan *et al.*, 2012a, 2012b).

A significant loss in adipogenic and osteogenic capacity was observed, although the rate of this loss was faster than described elsewhere (within three passages, not five). This could be attributable to the deliberately low seeding numbers and, thus, the longer culture time between passages (i.e. phenotypic loss was likely similar when comparing overall culture time instead of passages).

Further changes in CD90 or CD44 expression may not have been visible until after longer culture times. Interestingly, an increase in the expression of CD31, a molecule that plays key role in angiogenesis, was observed over the 28 day assay period in all groups, particularly with TGFβ1 treatment. CD34 expression, as expected, was observed to be low to negligible at all time points. Importantly, we observed increased expression of SCX at 14 and 28 days, as compared to freshly isolated cells. However, this finding is in contrast to a previous report showing decreased SCX expression with time (Tan *et al.*, 2012a, 2012b). This is also in variance to the apparent biphasic expression of the other proteins evaluated, showing significantly higher expression levels on day 14 compared to day 28. The increased expression of SCX observed in the present study could indicate a shift of TSC from the primitive (stemness) phenotype with capacity to differentiate into bone- and cartilage-like tissues to a more mature tenocyte phenotype with diminished multipotency. This is consistent with the progressive loss in multipotency, as revealed by downregulation of markers for osteogenesis and adipogenesis.



**Figure 9.** TGFβ1 induced significant changes in expression of tenogenic markers and significantly upregulated production of osteogenic and chondrogenic genes. DCN expression (a) was significantly upregulated after 28 days by treatment with 1 ng/ml TGFβ1, but unaffected at all other doses. SCX expression (b) was increased at all doses after 28 days. TEN-C expression (c) was increased only at the 100 ng/ml dose after 28 days, while collagen type I synthesis (d) was upregulated at both the 1 and 100 ng/ml doses after 28 days. Collagen type II expression (e) was significantly upregulated in response to all doses after 28 days, as was osteonectin expression. Microscopic examination showed the presence of small, rounded cells with strong staining for glycosaminoglycans and proteoglycans (g), and estimation of the change in proliferation rate indicated a significant decrease in the proliferation characteristics of terminally differentiated, relatively quiescent cells such as chondrocytes. Data are expressed as mean ± SEM; \*significant change from the values for freshly isolated cells

Considering the influence of the different growth factors in the present study on TSC phenotype and differentiation, IGF-1 treatment at 10 or 100 ng/ml maintained TSC multipotency and phenotype, evident by maintenance in the fraction of cells capable of differentiating down adipogenic, osteogenic and chondrogenic pathways and the expression of CD90, CD31, DCN, TEN-C and osteonectin. This observation is in accordance with previous studies, where blocking the IGF-1 receptor resulted in significantly decreased SSEA3 expression and increased apoptosis in cultures of embryonic stem cells (Bendall *et al.*, 2007), and in decreased Oct-4 and Nanog expression in spermatogonial stem cells (Huang *et al.*, 2009), demonstrating the crucial role of IGF-1 signalling in maintaining stem cells in culture. In a broader context, IGF-1 overexpression attenuated the effects of ageing on cardiac stem cells in mice (Capogrossi, 2004) and may play a role in delaying senescence in humans (Rincon *et al.*, 2004), suggesting a potent role in maintaining multiple cellular functions. In the context of tendons, IGF-1 injections have been investigated as a potential therapeutic tool, both in animal studies (Kurtz *et al.*, 1999) and in a human clinical trial (Trial ID: NCT01446783). The results have been encouraging, suggesting a role of IGF-1 in strengthening the tendinous ECM (Kurtz *et al.*, 1999). In tenocyte cultures, IGF-1 supplementation has been employed to promote tenocyte phenotype maintenance, in terms of scleraxis, decorin and tenomodulin expression (Klein *et al.*, 2002a, 2002b; Qiu *et al.*, 2012) and proliferation (Thomopoulos *et al.*, 2010; Caliarì and Harley, 2013). This may explain the increased scleraxis expression observed in all groups after 28 days.

Treatment with GDF-5, on the other hand, resulted in the preservation of tendon-related phenotype. However, this treatment was unable to preserve adipogenic and chondrogenic potentials. The loss in multipotency was coupled with a significant increase in SCX expression at all time points without significant increases in collagen type II or osteonectin expression, which would be associated with chondrogenic or osteogenic differentiation, respectively. These findings are consistent with other published reports which have used GDF-5 to induce a tendon-like phenotype in mesenchymal stem cells (Farng *et al.*, 2008; Hayashi *et al.*, 2011) and adipose-derived stem cells (Park *et al.*, 2010; James *et al.*, 2011a, 2011b), or to maintain tenocyte phenotype during *in vitro* culture (Hogan *et al.*, 2011; Keller *et al.*, 2011). In some of these studies, GDF-5 supplementation was used in combination with another stimulus, such as topography (James *et al.*, 2011a, 2011b) or mechanical stimulation (Farng *et al.*, 2008). This suggests that GDF-5 may also be an ideal supplement for the culture of tendon-like constructs grown on textured materials or cultured in bioreactors, where they can be mechanically stimulated. Our findings are also consistent with the known importance of GDF-5 in tenogenesis during development (Wolfman *et al.*, 1997; Francis-West *et al.*, 1999; Stone, 2000) and in tendon repair after injury (Stone, 2000; Chhabra *et al.*,

2003). Although the mechanism of GDF-5 action in tendon development and repair is not yet fully identified, these results further support the use of GDF-5 for maintaining tenocyte cultures and potentially inducing tenogenic differentiation.

Finally, although treatment with TGF $\beta$ 1 did not significantly enhance the expression of tendon-related markers in the present study, it appeared to induce a chondrocyte-like phenotype, characterized by increased collagen type II expression, reduced proliferation, increased GAG staining and increased numbers of round-shaped cells, as has been observed previously (Kawamura *et al.*, 2005). Treatment with TGF $\beta$ 1 also increased the fraction of cells expressing CD31 and the levels of osteonectin (at the lowest dose only), suggesting that some osteogenic differentiation may have occurred. Considering the role of TGF $\beta$ 1 as a potent regulator of proliferation, differentiation and cytokine secretion (Molloy *et al.*, 2003; Kashiwagi *et al.*, 2004), it was expected to induce more than one type of differentiation. In this case, the increased expression of markers associated with cartilage, bone and endothelium suggests that TGF $\beta$ 1 upregulation observed after injury may actually contribute to the development of vascularized fibrocartilaginous regions or calcified areas seen in tendinopathy (Xu and Murrell, 2008). As these regions have poor mechanical properties and may contribute to degeneration of the surrounding tendon, this natural upregulation of TGF $\beta$ 1 may be more pathogenic than reparative. In fact, if TGF $\beta$ 1 upregulation causes differentiation of TSCs, it may increase the speed of basic repair by inducing the cells to differentiate and produce more ECM (i.e. DCN, TEN-C and collagens) but limit the overall tendon repair capacity by reducing the numbers of TSCs or redirecting their differentiation towards osteogenesis and chondrogenesis, as has previously been hypothesized (Rui *et al.*, 2011). Thus, it would appear that TGF $\beta$ 1 supplementation of TSC cultures generates an *in vitro* cell population more representative of tendinopathy than healthy tendon, and would be more valuable in generating disease models than in preparation of cells for therapeutic injections.

TSCs have been shown to spontaneously lose their multipotency and self-renewal when cultured *in vitro* (Tan *et al.*, 2012a, 2012b; Zhang and Wang, 2013). Development of *in vitro* cell culture conditions that enhance the maintenance of their stemness and multipotency is critical in the quest to realize their therapeutic potential. In the present study, treatment of TSCs with IGF-1 and, to a lesser extent, with GDF-5 enabled the expression of tendon-related markers during the 28 day culture period. TGF $\beta$ 1, on the other hand, promoted a more chondrogenic phenotype.

## 5. Conclusions

TSC phenotype is lost over time in culture, evident by a reduction in the expression of related stem cell surface



markers, concurrent with a significant upregulation of collagen type II and osteonectin expressions, depicting lineage commitment. Continuous supplementation with 10 ng/ml IGF-1 significantly improved the maintenance of the TSC phenotype for up to 28 days. Whereas supplementation with GDF-5 maintained and promoted markers associated with TSC phenotype (SCX, TEN-C and DCN), it was ineffective in maintaining their multipotency. TGF $\beta$ 1, on the other hand, induced differentiation of a subset of cells towards a chondrogenic phenotype. Therefore, we demonstrate here that continuous supplementation of basic cell culture media with 10 or 100 ng/ml IGF-1 maintains TSC phenotype. This may prove particularly useful in allowing long-term expansion of these cells, thereby greatly improving their therapeutic potential.

## Conflict of interest

The authors have declared that there is no conflict of interest. Q14

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## Supporting information on the internet

Additional supporting information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Images of extracted tendon stem cells, showing CD90- and TEN-C-expressing cells (a), CD44-expressing cells without collagen expression (b), DCN-expressing cells with negligible CD31 expression (c) and scleraxis-expressing cells with negligible CD34 expression (d)

**Figure S2.** Chondrogenic pellets prepared from (a) untreated TSCs at each time point; (b) TSCs treated with 1, 10 or 100 ng/ml IGF-1; (c) TSCs treated with 1, 10 or 100 ng/ml GDF-5; (d) TSCs treated with 1, 10 or 100 ng/ml TGF- $\beta$ 1, stained for collagen type II (green) and counter-stained with DAPI (blue)

**Figure S3.** FABP4 expression as an indicator of adipogenesis in TSCs at each time point



# Author Query Form

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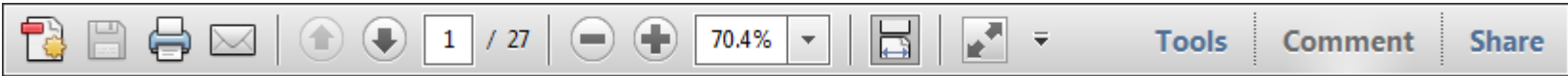
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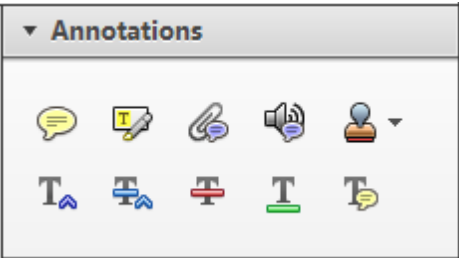
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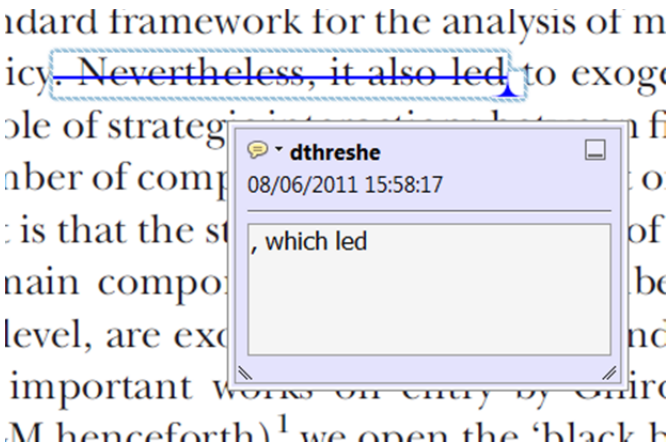
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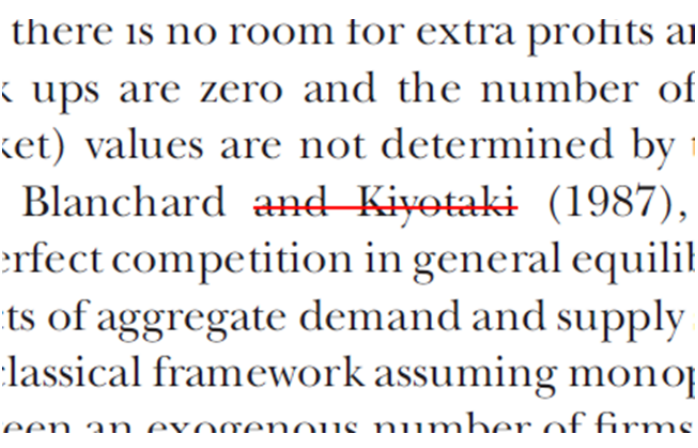
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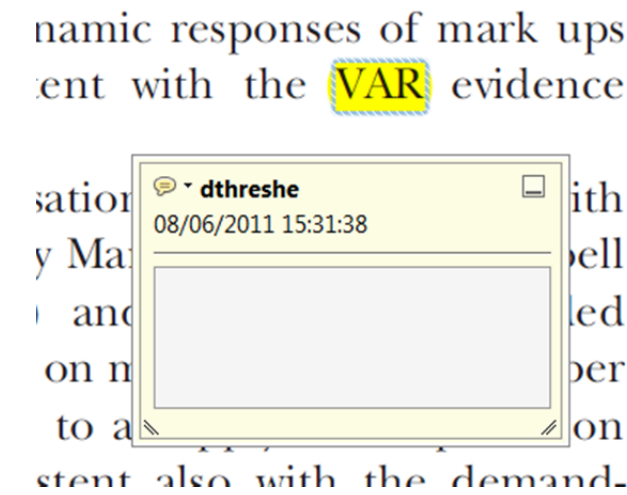
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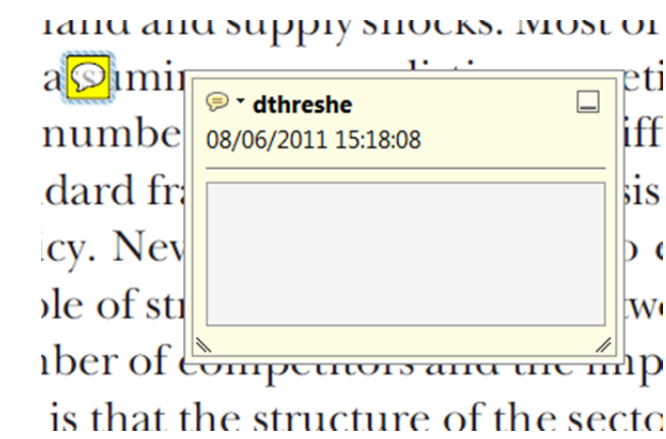
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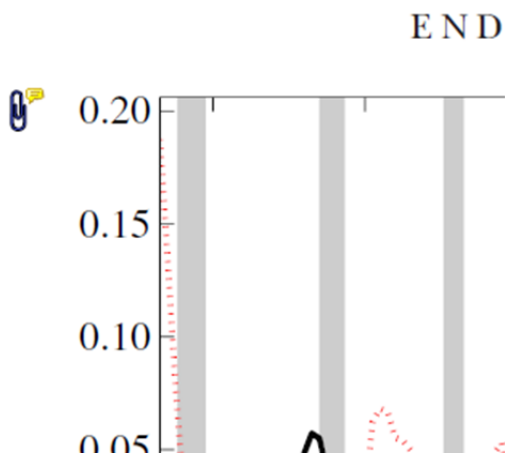
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of the business cycle, starting with the  
on perfect competition, constant returns  
production. In this environment goods  
extra profits and the structure of market  
he number of firms in the individual firm  
etermined by the model. The New-Key  
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general equilibrium models with nominal  
ad and supply shocks. Most of this literat

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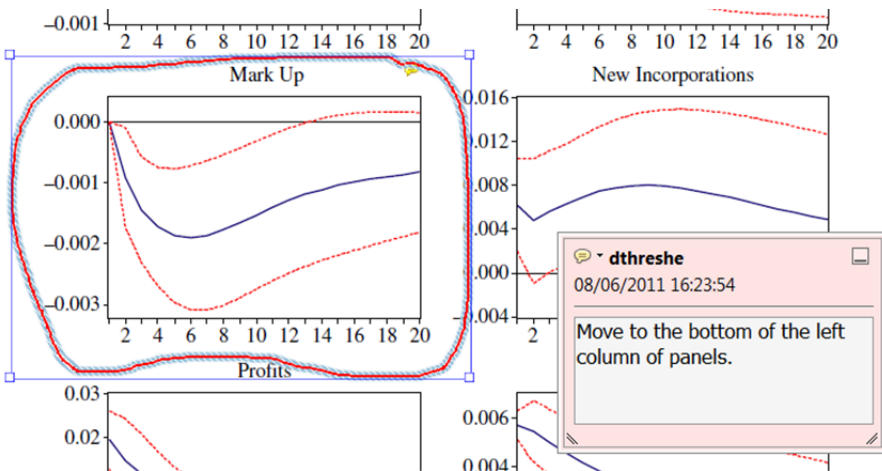


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Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..



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