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(54) Title: TENOGENIC MEDIUM FOR MESENCHYMAL STEM CELLS DIFFERENTATION INTO TENOGENIC LINEAGE

(57) Abstract: Tenocytes are highly differentiated cells which have a limited potential for replication. Therefore, in vitro propagation of tenocytes may still not be able to supply enough seed cells for tendon tissue engineering. Bone marrow mesenchymal stem cells (MSCs) are able to self-renew and capable to differentiate into multi lineage including tenocytes. Therefore, it is possible that MSCs could serve as seed cells in tendon tissue engineering. In the present invention, method and culture medium for promoting human bone marrow mesenchymal stem cells (MSCs) differentiation into tenogenic lineage is provided.

TENOGENIC MEDIUM FOR MESENCHYMAL STEM CELLS DIFFERENTATION INTO TENOGENIC LINEAGE

FIELD OF THE INVENTION

The present invention relates to a culture medium for promoting human bone marrow mesenchymal stem cells differentiation into tenogenic lineage. The present invention further relates to a method to differentiate human bone marrow mesenchymal stem cells into tenogenic lineage.

BACKGROUND OF THE INVENTION

Tendon damage following trauma contribute to a large proportion of soft tissue injuries reported each year (Butler *et al.*, 2004). The use of surgical technique to repair damaged tendon is presently necessary to ensure that tissue integrity is restored to its pre-injured state. Thereafter, natural tissue healing is expected to take place albeit with limited potential. This is mainly because tendon do not undergo self-repair when damaged due to its hypovascularized nature (Bergljung, 1970) which in turn has a tendency to develop tissue degeneration with aging (Romeo *et al.*, 1999). Recent endeavours in tendon tissue engineering have harnessed the potential application of cell-based therapy for tendon regeneration which overcomes the limitations present in previous tissue regeneration controlled processes (Obaid and Connell, 2010). Mesenchymal stem cells (MSCs) present a great potential for this type of treatment modality due to its multi-potential differentiation ability (Lee and Hui, 2006) while avoiding excessive morbidity to the donor site. However, it has been reported that uncommitted-MSCs may produce complications when used in clinical applications such as ectopic bone formation (Harris *et al.*, 2004). As such, tissue engineering approaches are presently being developed to induce tenogenic differentiation in human MSCs (hMSCs) or to produce controlled differentiation of hMSCs into the desired tenogenic lineage prior to transplantation.

The use of growth factors has been described for MSCs differentiation in stem cell based tissue engineering (Farng *et al.*, 2008). Recent progress in the understanding of growth factors important to the healing of lacerated tendons (Lou *et al.*, 2001) has led

to the use of these biological factors to induce tenogenic differentiation in MSCs (Sharma *et al.*, 2010). Among these growth factors, growth differentiation factor-5 (GDF-5) which is a member of human bone morphogenetic protein (BMP) family, has been identified as a key biological molecule which can accelerate tendon healing (Aspenberg *et al.*, 1999). Although the use of GDF-5 have shown to alter tendon formation rate and function in vivo (Aspenberg *et al.*, 1999), little has been described about the effects of GDF-5 on hMSCs proliferation and differentiation in vitro.

Until today, there is no invention in the field of tendon tissue engineering using GDF-5 as a medium supplement in primary hMSC *in vitro* culture. Therefore, the present invention addresses the use of GDF-5 as an induction factor and other optimal culture condition for hMSC proliferation and tenogenic differentiation in vitro. This may be important when considering that the results may provide a reference data for MSCs culture conditions in laboratory, which can lead to a wider implication for clinical applications.

SUMMARY

An object of the present invention is to provide a culture medium for promoting human bone marrow mesenchymal stem cells differentiation into tenogenic lineage. The culture medium not only initiated tenogenic transformation in MSCs but also maintains the production of tendon matrix substances which is inherently important for tendon repair.

A further object of the present invention is to provide a method to differentiate human bone marrow mesenchymal stem cells into tenogenic lineage.

The present invention will now be described in more details with reference to exemplary embodiments thereof as shown in accompanying figures.

BRIEF DESCRIPTION OF THE DRAWING/FIGURES

Figure 1: Alamar Blue (AB) cell proliferation assay for hMSCs cultured in different concentration of GDF-5 (ng/ml). Cell proliferation in hMSCs showed no significant differences at varying concentration of GDF-5.

Figure 2: Total collagen content analysis for dose response of hMSCs treated with different concentration of GDF-5.

(a) At 96 hours in culture medium supplemented with GDF-5 at different concentrations, the total collagen expression in hMSCs was significantly increased at 100 ng/ml of GDF-5 (*$p<0.05$).

(b) At day 4, day 7 and day 10 in culture medium supplemented with GDF-5 at 0, 50 and 100ng/ml, the total collagen expression was significantly elevated in hMSCs on day 4 and day 7, when treated with 100 and 50 ng/ml of GDF-5 respectively (*$p<0.05$). No significant difference was observed on day 10 between hMSCs treated with 50 and 100 ng/ml of GDF-5 (*$p<0.05$).

Figure 3: Gene expression analysis of candidate tenogenic markers: type-I collagen (*Col-I*) scleraxis (*Scx*), type-III collagen (*Col-III*), decorin (*Dec*, and nucleostemin (*Nst*) in hMSCs treated with GDF-5 (0, 50 and 100 ng/ml). *Col-I*, *Scx* and *Col-III* showed significant up-regulation (*$p<0.05$ and **$p<0.01$), at 100 ng/ml of GDF-5.

DETAIL DESCRIPTION OF THE PRESENT INVENTION

hMSC Isolation and characterization

Human bone marrow was harvested from patients (n=3) undergoing intramedullary nailing in University Malaya Medical Centre (UMMC). Informed consent was obtained from all donors. hMSC were isolated from bone marrow procured and expanded *in vitro*. Two millilitre (2ml) of bone marrow was diluted with 2 ml of phosphate buffered saline (PBS, pH 7.2) and layered in 3 ml of Ficoll-Paque Premium

(GE Healthcare, Sweden) before undergoing gradient centrifugation at 2200 rpm for 30 minutes (Eppendorf 5810R).

The mononuclear layer (second top layer) was then collected and washed twice with Dulbecco's Modified Eagle's Medium (DMEM) low glucose (DMEM-LG) (Invitrogen-Gibco, USA) supplemented with antibiotic/antimycotic 1% (v/v) (Invitrogen-Gibco, USA). The isolated mononuclear cells were cultured in growth medium (DMEM-LG supplemented with 10% FBS, antibiotic/antimycotic 1% (v/v) and 2 mM L-glutamin; all from Invitrogen-Gibco (USA) and transferred into T75 tissue culture flasks (Nunc™, USA). The medium was changed at day five to remove non-adherent cells with subsequent medium change conducted at three-day intervals.

To determine whether the cells obtained consisted of pure MSCs, various tests including immunohistochemical staining for specific cell surface markers, cell morphological analyses and the ability of the isolated cells to undergo tri-lineage differentiation i.e. chondrogenic, tenogenic and osteogenic differentiation were conducted.

Result of hMSCs Isolation and characterization

Three to five days following the plating of bone marrow mononuclear cells onto plastic surface cell culture flasks, fibroblastic like cell colonies formed in these cultures demonstrated a low degree of heterogeneity. However, the numbers of fibroblastic appearance like cells became increasingly noticeable over time while in contrast, the heterogeneous cells (such as polygonal cells) became lesser. In the presence of serum (or FBS), adherent fibroblastic hMSCs isolated from human bone marrow grew in size and numbers while the non-adherent cells were quickly removed when media changes were performed which happened every three-days. The final

homogeneous cell population obtained at end of passage 2 (P2) cell culture was used for downstream differentiation assay.

Isolated cells appear to conform to the characteristics expected of MSCs. Cells appears to have (1) spindle shaped plastic adherent features, (2) demonstrated positive markers for CD45, CD105 and CD166 while being absent for CD34 and CD45 and (3) able to undergo tri-lineage differentiation.

Cell Proliferation Analysis of hMSC Under GDF-5 Induction

In order to assess cell proliferation, hMSCs (P2) were seeded in standard 96-well culture plates at cell density of 10^4 cells/ml immersed in 250 µl of culture medium. GDF-5 supplemented medium (either 0, 5, 25, 50, 100 or 500 ng/ml) were added to the cultures three days after seeding. Cells were incubated for an additional two days before 25 µl of alamar blue reagent (Invitrogen-Gibco, USA) was added to the medium. Culture plates were protected from light using aluminium foil. Absorbance readings at 570 nm and 600 nm were read using a spectrophotometer (Epoch, Biotek, USA) at 0, 2, 4, 6, 12 and 24, 36, 48 and 60 hours. Untreated hMSCs cultured in MSCs growth medium were used as controls.

Dose and Temporal Effects in hMSC Proliferation Using GDF-5 Induction

The dose and temporal effects of GDF-5 on the proliferation rate of hMSCs were determined using Alamar blue assay. The results of the cell proliferation assays demonstrated no significant differences between the proliferation of hMSCs cultured with or without GDF- 5 (Figure 1). This finding demonstrates that GDF-5 did not alter the proliferation rate of tenogenic-hMSCs.

Tenocyte culture for comparison in total collagen expression

Native tenocytes were isolated and cultured from similar donors. The method used to isolate and characterize the primary tenocyte were described elsewhere (Tan *et al.*, 2008). These cells were used for comparison in subsequent experiment.

In vitro Tenogenic Differentiation and Total Collagen Quantification

hMSCs (P2) were seeded in standard 6-well culture plates at a density of 2 x 10^4 cells per well using serum-free DMEM supplemented with either 0, 5, 25, 50, 100 or 500 ng/ml of recombinant hGDF-5 (R&D Systems, Inc., Minneapolis, MN). Tenocytes isolated as previously described were seeded in similar density to that of hMSCs and were used for comparison. These cells were not supplemented with GDF-5. For dose response analysis, total collagen expressions were measured at 96 hours. Based on the result obtained from this experiment, only three concentrations i.e. 0, 50 and 100 ng/ml of GDF-5 were selected for further analysis which determines the collagen and gene expression levels over time (i.e. day 4, 7 and 10).

For time response experiments, total collagen assays were conducted at day 4, 7, and 10 in hMSCs culture supplemented with 0, 50 and 100 ng/ml of GDF-5. Total collagen expression in cultured tenogenic-hMSC was quantified using Sircol™ soluble collagen assay kit (Biocolor, Ireland). Cell culture medium mixed with 1ml of Sircol dye reagent was agitated vigorously in a 1.5 ml microcentrifuge tube for 30 minutes. The mixtures were then centrifuged for 10 minutes at 10,000xg to collect the collagen-dye complex at the bottom of the centrifuge tubes. The unbound dye solutions were later removed by draining the tubes. Subsequently, 1ml of the alkaline reagent was added to each microcentrifuge tube and mixed. When the unbound dye was later dissolved and the absorbance of the samples was measured at 540 nm. The collagen content in the medium was calculated based on the standard curve plot using type-I collagen supplied with the kit. All samples were processed in triplicates and mean values were used for analysis. Statistical analysis was analyzed using SPSS (ver. 17) software. To determine the means differences, one-way analysis of variance (ANOVA) was employed. Statistical significance was accepted with p value of less than 0.05 ($P<0.05$).

Dose Response of GDF-5 Induced Total Collagen Expression in Tenogenic Differentiation of hMSC

The dose response of hMSCs to GDF-5 in their total collagen expression was determined using different amounts of GDF-5 in cell culture medium. In this

quantitative analysis, the results of total collagen assay revealed that at 100 ng/ml, GDF-5 induce significant elevated response (9.983±1.695 μg/ml: $p<0.05$, Table 1) as compared to 0, 5 and 25 ng/ml of GDF-5 (Figure 2a). This expression level was comparable to that observed in tenocyte culture (10.387±2.316 μg/ml). No significant differences were observed in the collagen concentration between cultures supplemented with 50, 100 or 500 ng/ml of GDF-5 (Table 1).

Table 1: Statistical analysis of total collagen expression in hMSCs culture medium at different concentration of GDF-5 at 96 hours of cell culture. Summary of least significant differences (LSD) analysis with Bonferroni adjustment for multiple pairwise comparisons of mean total collagen differences in the culture medium of hMSCs supplemented with different amount of GDF-5. The p-value was presented at 95% confidence interval and significant value was denoted with an asterisk (** Highly significant difference (1%), *Significant difference (5%).

		p-Value				
		Different concentration of GDF-5 (ng/ml)				
		5	25	50	100	500
Different concentration of GDF-5 (ng/ml)	0	0.515	0.978	0.157	**0.008****	0.187
	5		0.520	0.426	**0.035***	0.485
	25			0.153	**0.007****	0.183
	50				0.178	0.921
	100					0.149

The ensuing time response experiments showed a significant increase in the total collagen expression from hMSCs supplemented with 100 ng/ml of GDF-5 at day-4, -7 and -10 as compared to untreated cultures (Figure 2b). A significant increase in total collagen expression was only observed at day-7 onwards in hMSCs treated with 50 ng/ml of GDF-5. This finding demonstrates that 100 ng/ml of GDF-5 is sufficient to induce tenogenic response from hMSCs as early as day-4, but with the use of 50 ng/ml of GDF-5, a longer time period is required.

Relative Gene Expression Analysis by Quantitative Polymerase Chain Reaction (qPCR)

To induce expression of tenogenic-specific phenotypes, hMSCs were cultured in DMEM supplemented with GDF-5 at 0, 50 and 100ng/ml. After 4 days, the degree of cell differentiation was determined by using real-time PCR (qPCR). This was achieved by measuring scleraxis (*Scx*), type-I collagen (*Col-I*), type-III collagen (*Col-III*), decorin (*Dec)* and nucleostemin (*Nst*) gene expressions. Total RNA was extracted from the hMSCs cultured with and without GDF-5. One microgramme of total RNA was reverse-transcribed into cDNA using the transcriptor high fidelity cDNA Synthesis kit (Roche Diagnostics GmbH; Mannheim, Germany). Quantitative polymerase chain reaction (qPCR) was performed using a Bio-Rad CFX96™ Real-time detection system (Bio-Rad Laboratories, Inc., Hercules, CA) in a final volume of 20 µl with10 uL iQ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA), 0.6 µL RT samples and 0.2 µM of each primer (for type-I collagen (Col-*I*), type-III collagen (*Col-III*), scleraxis (*Scx*), decorin (*Dec*), nucleostemin (*Nst*); Supplementary Material #1). The amplification protocol was as follows: an initial denaturation and activation step at 95°C for 30s followed by 40 cycles of 95°C for 15s and 61°C for 45s. A melting curve program was carried out routinely to confirm the presence of a single product (55-95°C with a heating rate of 0.5°C per second and a continuous fluorescence measurement). The annealing temperature at 61°C was derived empirically using temperature gradients. To estimate amplification efficiency, a standard curve was generated for each target molecule via 5-fold serial dilution of a cDNA pool containing the target gene sequences. Data was analyzed using the CFX manager software. A relative quantification method (with corrected PCR efficiency (Pfaffl, 2001)) was performed. All the data was normalized to GAPDH which was used as the reference gene after correcting for differences in amplification efficiency (as recommended in the CFX manager package). Data was presented as log10-fold change (±standard deviation) of relative quantification of target mRNA relative to control samples (untreated MSCs). To compare the fold changes between the untreated and GDF-5 treated samples, Student's t-tests were employed. For all

comparisons, the statistical significance was accepted at 95% confidence interval ($p<0.05$).

Result of Relative Gene Expression Analysis of hMSC Tenogenic Differentiation

Relative gene expression levels for types I and type III collagen (*Col-I* and *Col-III*), decorin (*Dec*), scleraxis (*Scx*) and nucleostemin (*Nst*) for hMSCs grown in 0, 50 and 100 ng/ml of GDF-5 at 96 hr showed significant differences (Figure 3). At 100 ng/ml of GDF-5, candidate tenogenic-markers, *Col-I* and *Scx* were significantly upregulated (2.31± 0.27 and 2.30±1.81 fold increase respectively) (Figure 3).

The genes related to collagen fiber formation, *Col-III*, and matrix assembly (Zhang *et al.*, 2006), *Dec*, were also significantly expressed at 96hr as compared to 0 hour ($p<0.05$). There was 1.84±0.28 and 2.56±0.41 fold increase in *Col-III* expression from time 0 when 50 and 100 ng/ml were used respectively. For *Dec* expression, there was 4.47±0.41 and 4.42±0.57 fold increase when 50 and 100 ng/ml were used respectively. No significant difference in *Nst* gene expression levels was observed in all groups which demonstrates that despite undergoing tenogenic differentiation, cells maintained their original MSC gene expression.

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We claim:

1. A culture medium for promoting human bone marrow mesenchymal stem cells differentiation into tenogenic lineage which comprises: (a) 50 ng/ml to 500 ng/ml of growth differentiation factor-5 (GDF-5) and (b) a serum-free Dulbecco's Modified Eagle's Medium (DMEM) Platelet-rich plasma.

2. The culture medium in claim 1, wherein the preferable concentration of the growth differentiation factor-5 (GDF-5) is 100 ng/ml.

3. The culture medium in claim 1, which do not alter the proliferation rates of the human mesenchymal stem cells but provide optimal tenogenic differentiation response.

4. A method of promoting human bone marrow mesenchymal stem cells differentiation into tenogenic lineage, comprise:

i) harvesting the bone marrow from human bone;

ii) isolating the mononuclear cells from human bone marrow;

iii) culturing the isolated mononuclear cells in a medium comprise: DMEM-LG supplemented with 10% FBS, antibiotic/antimycotic 1% (v/v) and 2 mM L-glutamin;

iv) changing the medium at day five to remove non-adherent cells with subsequent medium change conducted at three-day intervals;

v) characterizing the mononuclear cells obtained to ensure the cells were mesenchymal stem cells in nature;

vi) seeding the Passage 2 mesenchymal stem cells in standard 6-wells culture plates at a density of 2 x 104 cells per well; and

vii) adding a culture medium for promoting human bone marrow mesenchymal stem cells differentiation into tenogenic lineage which comprises, 50 ng/ml to 500 ng/ml of growth differentiation factor-5 (GDF-5) and a serum-free DMEM.

5. The method in claim 4, wherein the preferable concentration of the growth differentiation factor-5 (GDF-5) is 100 ng/ml.

6. The method in claim 4, which do not alter the proliferation rates of the human mesenchymal stem cells but provide optimal tenogenic differentiation response.

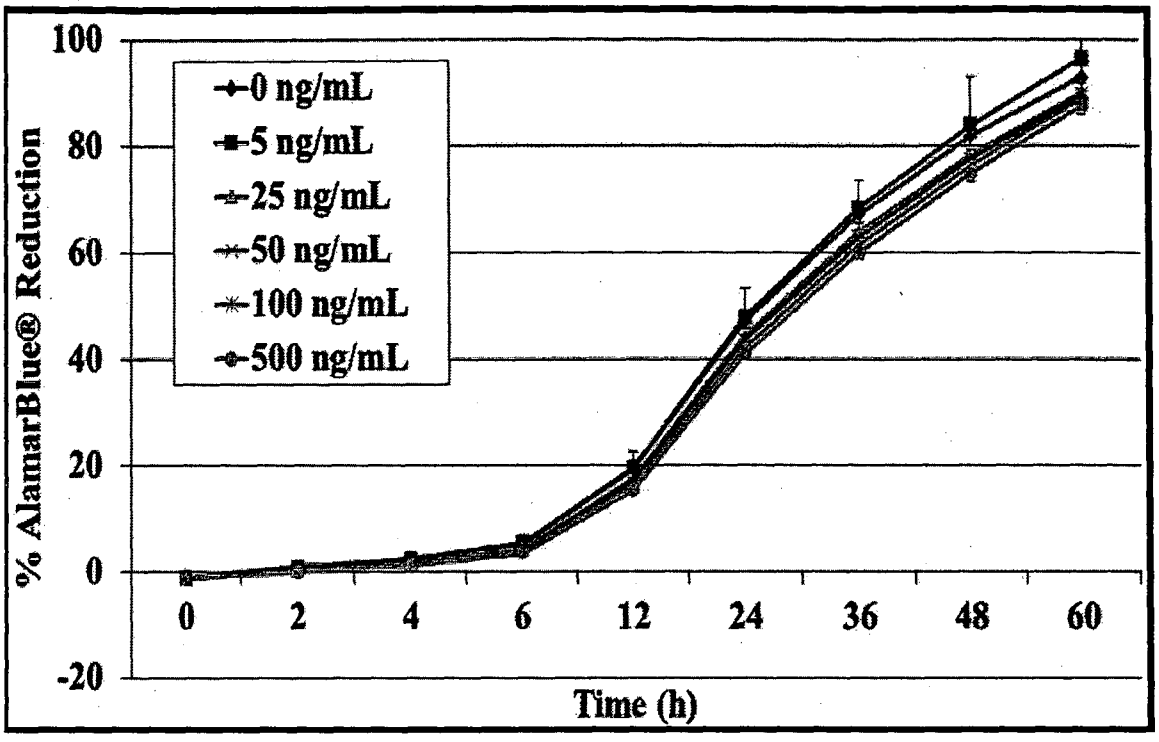
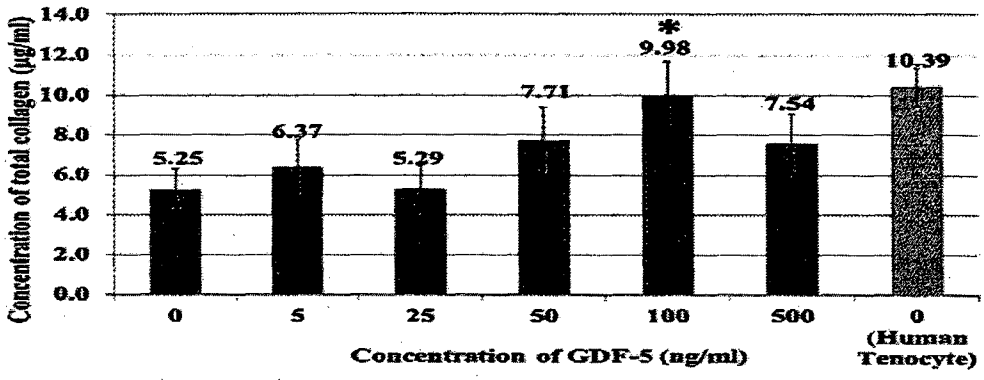

100
80
60
40
20
0
-20
% AlamarBlue® Reduction
0 ng/mL
5 ng/mL
25 ng/mL
50 ng/mL
100 ng/mL
500 ng/mL
0
2
4
6
12
24
36
48
60
Time (h)

Figure 1

a


Concentration of total collagen (µg/ml)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
5.25
6.37
5.29
7.71
9.98
7.54
10.39
*
0
5
25
50
100
500
0 (Human Tenocyte)
Concentration of GDF-5 (ng/ml)

b

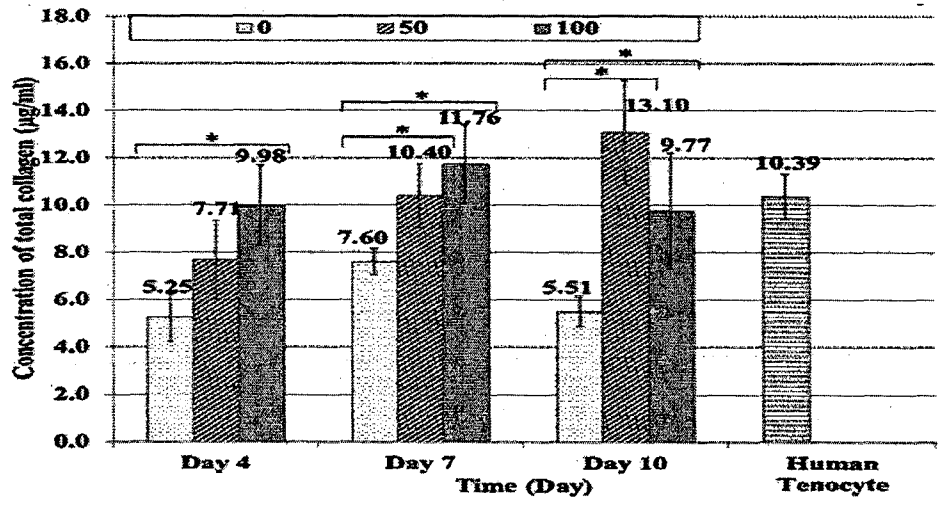

Concentration of total collagen (µg/ml)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
50
100
5.25
7.71
9.98
7.60
10.40
11.76
5.51
13.10
9.77
10.39
*
Day 4
Day 7
Day 10
Human Tenocyte
Time (Day)

Figure 2

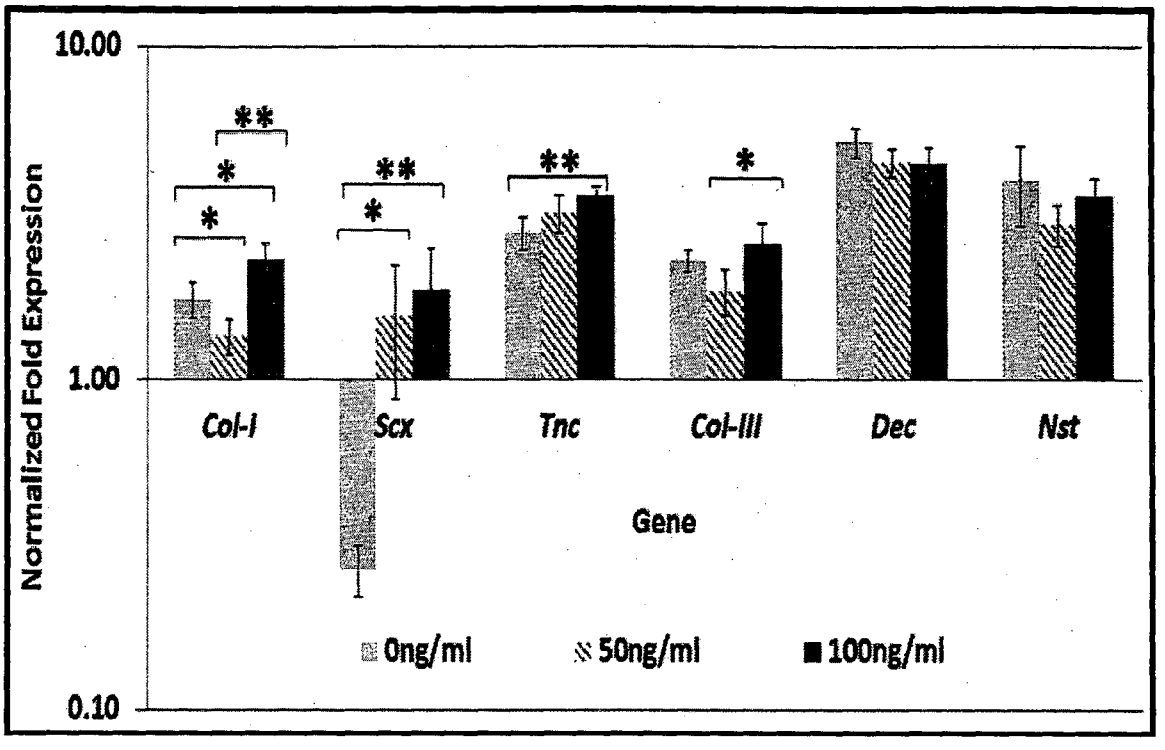

10.00
1.00
0.10
Normalized Fold Expression
**
*
*
**
*
**
*
Col-I
Scx
Tnc
Col-III
Dec
Nst
Gene
0ng/ml
50ng/ml
100ng/ml

Figure 3