Proteomic Profiling of Mesenchymal Stem Cell Responses to Mechanical Strain and TGF- β 1

Running Head: Mesenchymal Stem Cell Responses to Mechanical Strain and TGF- β 1

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ABSTRACT

Mesenchymal stem cells (MSCs) are a potential source of smooth muscle cells (SMCs) for constructing tissue-engineered vascular grafts. However, the details of how specific combinations of vascular microenvironmental factors regulate MSCs are not well understood. Previous studies have suggested that both mechanical stimulation with uniaxial cyclic strain and chemical stimulation with transforming growth factor β 1 (TGF- β 1) can induce smooth muscle markers in MSCs. In this study, we investigated the combined effects of uniaxial cyclic strain and TGF- β 1 stimulation on MSCs. By using a proteomic analysis, we found differential regulation of several proteins and genes, such as the up-regulation of TGF- β 1-induced protein β 3 (BGH3) protein levels by TGF- β 1 and up-regulation of calponin 3 protein level by cyclic strain. At the gene expression level, BGH3 was induced by TGF- β 1, but calponin 3 was not significantly regulated by mechanical strain or TGF- β 1, which was in contrast to the synergistic up-regulation of calponin 1 gene expression by cyclic strain and TGF- β 1. Further experiments with cycloheximide treatment suggested that the up-regulation of calponin 3 by cyclic strain was at post-transcriptional level. The results in this study suggest that both mechanical stimulation and TGF- β 1 signaling play unique and important roles in the regulation of MSCs at both transcriptional and post-transcriptional levels, and that a precise combination of microenvironmental cues may promote MSC differentiation.

KEY TERMS

Proteomics, uniaxial cyclic strain, micropatterning, gene expression, differentiation, smooth muscle cells, cell engineering

INTRODUCTION

The availability of suitable and abundant cell sources is one of the primary limiting factors in vascular tissue engineering¹⁷. Human mesenchymal stem cells (MSCs) are expandable, can resist platelet adhesion⁵, and have the potential to differentiate into smooth muscle cells (SMCs)^{1, 7, 20}, which make MSCs a unique cell source for the construction of tissue-engineered vascular grafts. Therefore, it is important to understand how MSCs respond to vascular mechanical and biochemical factors upon implantation or in a bioreactor. While previous studies have verified the importance of mechanical stimulation as a potential mechanism for driving MSCs toward an SMC phenotype^{3, 4, 11, 19}, particularly with regard to the effects of anisotropic mechanical sensing by MSCs¹¹, mechanical stimulation alone has not been enough to fully drive MSC differentiation into SMCs. For example, in a previous publication we showed that micropatterned grooves can be used to induce contact guidance in MSCs in order to keep the cells elongated and aligned with the axis of cyclic uniaxial mechanical strain, which more accurately simulates the anisotropic mechanical forces existing within a blood vessel wall¹¹. This anisotropic mechanical stimulation alone was sufficient to upregulate expression of calponin 1 (CNN1), an intermediate marker of SMC differentiation, but was not enough to induce expression of late-stage SMC markers such as Myosin Heavy Chain 11 or Myocardin. It is likely that multiple vascular factors (both mechanical and biochemical) are involved in the complete MSC to SMC transition.

Transforming growth factor- β 1 (TGF- β 1) is known to be involved in the regulation of cellular growth, differentiation, migration, and extracellular matrix production^{2, 15, 16, 24, 25}. It has been shown that TGF- β 1 can induce either smooth muscle (SM) markers or chondrogenic

differentiation in MSCs, depending on the specific culture conditions involved^{3, 8, 9, 13, 25}. These findings suggest that TGF- β 1 could play an important role in driving MSCs toward an SMC fate, possibly in combination with other factors. Additionally, TGF- β 1 has been implicated in several studies investigating the response of SMCs to mechanical strain^{10, 12, 18, 22}. Here, we hypothesized that a combination of mechanical strain and TGF- β 1 might induce greater changes in MSCs toward an SMC phenotype.

In this study, micropatterning techniques were used to control MSC morphology and induce MSC alignment to coincide with the axis of uniaxial cyclic mechanical strain on elastic poly(dimethyl siloxane) (PDMS) membranes. Our previous studies have shown that MSCs on smooth surfaces have transient up-regulation of SMC markers, while maintaining MSC alignment in the direction of uniaxial mechanical strain induces sustained expression of SMC markers^{11, 19}. Additionally, this mechanical stimulation was combined with TGF- β 1 chemical stimulation to create a more complex representation of the microenvironmental factors acting on SMCs in the vasculature.

The results in this study show that micropatterned guidance maintains MSC alignment with the axis of strain either with or without additional chemical stimulation by TGF- β 1. Subsequent proteomic analysis revealed several interesting protein changes, such as the up-regulation of TGF- β 1-induced protein ig-h3 (BGH3) with TGF- β 1 stimulation and the up-regulation of calponin 3 (CNN3) with cyclic mechanical strain. By further investigating the effects of these factors on MSC gene expression, we found that the change in BGH3 appears to be controlled at the transcriptional level, while the increase in CNN3 may be controlled post-transcriptionally, in contrast to the synergistic increase of the smooth muscle contractile marker,

calponin 1 (CNN1), by both cyclic strain and TGF- β 1. These results suggest both mechanical stimulation and TGF- β 1 signaling play unique and important roles in the regulation of MSCs at both transcriptional and post-transcriptional levels, and that mechanical stimulation can function as a co-factor to promote TGF- β 1-induced MSC differentiation.

RESULTS

MSCs on parallel-oriented microgrooves remained aligned with the axis of strain during both mechanical and chemical stimulation

When MSCs were subjected to concurrent mechanical and chemical stimulation on micropatterned PDMS membranes, MSCs remained aligned with the axis of uniaxial cyclic stretch. Fluorescent staining and confocal microscopy were used to examine the alignment of the F-actin filaments. Figure 1A-B shows that under conditions of uniaxial cyclic strain, stress fibers in MSCs remained well aligned with the direction of the micropatterned grooves, and hence, the axis of strain. Furthermore, addition of TGF- β 1 to the culture media did not interfere with this maintenance of alignment (Figure 1C-D). These results verify that this system is useful for investigating the combined effects of uniaxial cyclic mechanical strain (on micropatterned surface) and TGF- β 1 stimulation on MSCs.

Mechanical stimulation and TGF- β 1 caused differential changes in MSC protein expression

To investigate the overall effects of mechanical strain and TGF- β 1 stimulation on MSCs, we employed a proteomic analysis of our experimental samples. Figure 2 provides representative 2-D gel images from each of the four experimental groups. In general, protein spots from the 2-D gels displayed excellent separation and focusing, and the overall pattern of protein expression appears to be similar for all four groups, suggesting that most protein levels did not change significantly after 24-hour stimulation.

Further quantitative analysis of the 2-D gel images revealed differences in several protein spots when comparing between individual samples based on a volume ratio fold-change cutoff value of at least 1.4. Using this criterion, we found 12 protein spots of interest that were then further analyzed with mass spectrometry to identify the proteins (Table 1).

In particular, proteomic analysis confirmed at least two protein changes (eukaryotic translation elongation factor 2/EF2 and BGH3) previously known to be induced by TGF- β 1, and revealed the changes of several cytoskeletal proteins (CNN3, tropomyosin, actin-related protein 3/ARP3 and γ -actin) induced by mechanical strain, as elaborated upon in the Discussion. For the remainder of the study, we chose to focus on the following protein changes: up-regulation of BGH3 with TGF- β 1 stimulation, and up-regulation of SM protein CNN3 with cyclic mechanical strain. These two spots of interest are circled on the cyclic strain gel in Figure 2 for reference and are labeled with their respective spot numbers from table 1.

Figure 3 provides close-up images of the protein spots of interest from Figure 2 for easier visual comparison. Spots #3 and #6 were analyzed separately with mass spectrometry and both were identified as BGH3 (Figure 3). Gel images clearly revealed that both spots had greater intensity in the samples with TGF- β 1 stimulation compared to those without. No obvious differences in these two spots were observed between samples with and without mechanical strain. Two adjacent protein spots were both identified as CNN3 by mass spectrometry, and were labeled as spot #5 (Figure 3). Gel images revealed that the two circled spots had greater intensity with uniaxial cyclic strain than without. No obvious differences in these two spots were observed when comparing conditions of TGF- β 1 stimulation.

Mechanical stimulation and TGF- β 1 caused differential changes in MSC gene expression

To further investigate whether the proteomic changes were regulated at a transcriptional level in MSCs, we performed quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis to examine gene expression of two representative proteins: BGH3 induced by TGF- β 1 and CNN3 induced by mechanical strain. As shown in Figure 4, BGH3 gene expression was increased by TGF- β 1 stimulation but not by mechanical strain, consistent with the change in protein level. However, the gene expression of CNN3 did not show statistically significant changes in response to either mechanical or chemical stimulation, demonstrating a difference in transcriptional vs. post-transcriptional regulation of this protein.

Additionally, we examined the gene expression of another CNN isoform, CNN1, due to its previously demonstrated gene- and protein-level induction by mechanical strain¹¹. A change in CNN1 protein level was not found in this proteomic analysis, possibly due to its low relative abundance in the gel, but our results show that both mechanical strain and TGF- β 1 alone increased CNN1 gene expression (Figure 4). Furthermore, the combination of the two stimuli had synergistic effects at the gene level; the increase in CNN1 gene expression in response to simultaneous exposure to TGF- β 1 and mechanical strain was significantly greater than the CNN1 increase in response to either stimulus alone.

Mechanical regulation of CNN expression was dependent on protein synthesis

To further investigate how mechanical strain regulated CNN3 expression at post-transcription level, we treated cells with cycloheximide, an inhibitor of protein synthesis. As shown in Figure 5, CNN3 protein expression was induced by mechanical strain, which was

inhibited by cycloheximide, suggesting that mechanical strain regulates the protein synthesis of CNN3.

DISCUSSION

In this study, we simultaneously applied mechanical and chemical stimulation to MSCs *in vitro*, while maintaining cell alignment with the axis of uniaxial cyclic strain via micropatterned contact guidance. Proteomic analysis of the effects of combined uniaxial cyclic strain and TGF- β 1 stimulation revealed that fewer changes were induced on the proteomic level after only 24 hours of simultaneous stimulation, compared to the >2-fold changes of over 60 proteins in response to 4-day of TGF- β 1 stimulation in our previous study²⁵. It is expected that experiments with longer time period would show more significant changes. However, the changes in proteins and genes at 24-hour time point have manifested several interesting points, and the changes in protein expression, although less than 2-fold, may contribute to greater overall changes in the assembly of actin filaments and cell differentiation in the long term. For example, mechanical strain upregulated CNN3 and γ -actin while downregulating ARP3, which could enhance the assembly of contractile elements but decrease actin branching in the cells. Our previous study with a longer time course (4 days) showed that TGF- β 1 upregulated α -actin while downregulating gelsolin to promote actin assembly²⁵. Further studies need to be performed to monitor temporal changes of the MSC protein profile, which will enable a systems biology analysis of interactions and coordination between different signaling pathways (e.g., the increase of CNN3 and α -actin and the decrease of ARP3 and gelsolin) and provide insight into the underlying mechanisms.

An interesting finding in this study is that cyclic uniaxial strain (but not TGF- β 1) upregulated the level of CNN3 protein in MSCs, but this expression was not regulated at the gene expression level, suggesting that other mechanisms at a post-transcriptional level may

account for the changes CNN3 protein. In contrast, both mechanical strain and TGF- β 1 induced an increase in CNN1 gene expression, and the combination of the two factors further induced a synergistic increase in CNN1 gene expression. These results suggest that mechanical strain and TGF- β 1 may regulate the expression of CNN1 and CNN3 through different mechanisms, and that the combination of mechanical strain and TGF- β 1 can promote MSC differentiation into SMCs more effectively than either stimulus alone. It is noted that CNN1 (basic isoform) is only expressed in the SMC lineage while CNN3 (acidic isoform) is expressed in both smooth muscle and other non-muscle tissues¹⁴. This indicates that the signaling pathways involved in the transcriptional activation of these two calponin isoforms are quite different. Although the transcriptional factors involved in CNN1 and CNN3 have not been clearly identified, our data suggests that the promoter of CNN1, but not CNN3, has the binding sites regulated by TGF- β 1-mediated signaling, e.g., Smads and/or extracellular-regulated kinases, which awaits more in-depth investigations.

It was previously shown that BGH3 expression is inducible by TGF- β 1^{6,21}, and may play an inhibitory role in osteoblast differentiation²³. Proteomic analysis from this study confirmed the up-regulation of BGH3 in MSCs via TGF- β 1 stimulation, and BGH3 induction is also regulated at the transcriptional level. However, cyclic uniaxial strain appeared to have no additional effect on BGH3 expression at either the gene or protein level. Interestingly, BGH3 appeared as two spots of varying molecular weight on the 2-D gels, suggesting the presence of a potential degradation product of BGH3 at the lower molecular weight. Since an increase in BGH3 could potentially lead to a decrease in bone-related gene expression²³, this suggests that

our culture conditions may help to guide MSC differentiation away from an osteogenic phenotype.

TGF- β 1 appears to be an important chemical factor for driving MSCs toward an SMC phenotype as previously hypothesized²⁵. Long-term treatment of MSCs with TGF- β 1 induces the expression of myocardin, a master transcriptional factor in SMCs (unpublished observation). However, the addition of mechanical strain to the culture conditions has additional effects on intermediate contractile markers, suggesting that mechanical strain can function as a co-factor in driving MSCs toward a contractile SMC phenotype.

Lastly, since we have previously shown that cell orientation affects cell responses to uniaxial strain¹¹, we cannot exclude the possibility that other modes/directions of mechanical strain could also work together with TGF- β 1 to regulate MSC functions. In general, the results of this study demonstrated that MSC response to growth factor exposure and mechanical strain can be a complex function of multiple stimuli, and the results warrant further investigation with regard to various combinations of chemical and mechanical regulators of vascular differentiation.

MATERIALS AND METHODS

Microfabrication and Soft Lithography

To create patterned membranes with parallel microgrooves (10 μm wide, 3 μm deep, 10 μm distance between each groove), we used microfabrication techniques as described in a previous publication¹¹.

Cell Culture and Cyclic Strain

Human MSCs were acquired from Cambrex Corp and were maintained for growth without differentiation as previously described¹¹. To stimulate MSCs with cyclic strain, MSCs were first seeded on micropatterned membranes within a custom-built uniaxial stretch machine as previously described¹¹. “+cyclic strain” samples were subjected to 5% uniaxial cyclic strain at a frequency of 60 cycles per minute (cpm). To account for fluid shear stress effects, “-cyclic strain” samples also moved back and forth at 60 cpm, but with no strain on the membrane. Additionally, TGF- β 1 was added to the appropriate samples as described in the next section.

TGF- β 1 Stimulation

Prior to stimulation with cyclic mechanical strain, MSCs on the micropatterned elastic PDMS membranes were cultured for 24 hours in a medium with Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% fungizone (all from Invitrogen Corp.) in the absence or presence of TGF- β 1 (5 ng/mL) (PeproTech, Rocky Hill, NJ). Then the samples were kept as un-stretched controls or subjected to cyclic strain for additional 24 hours. The experimental setup resulted in a total of four

different experimental groups: (1) -strain, -TGF- β 1, (2) +strain, -TGF- β 1, (3) -strain, +TGF- β 1, or (4) +strain, +TGF- β 1.

Immunofluorescent Staining and Confocal Microscopy

Immunostaining and confocal microscopy were used to visualize the structure of the cytoskeleton in MSCs. MSCs were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained for actin filaments by using rhodamine-conjugated phalloidin.

A Leica TCL SL confocal microscopy system, including He/Ne laser sources and a Leica DM IRB microscope, was used to capture multiple Z-section images (~0.4 μ m thick sections over a range of ~6 μ m) for a given specimen. These sections were subsequently projected to a single plane to create an overall image of the specimen. All images in a given group were collected with the same hardware and software settings.

Two-Dimensional Differential In-Gel Electrophoresis (2D-DIGE) and Immunoblotting Analysis

Cells were lysed with 100 μ L lysis buffer (containing 25 mM Tris, pH 7.4, 0.5 M NaCl, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, 10 μ g/mL leupeptin, and 1 mM Na_3VO_4) per membrane. Protein lysates were centrifuged to pellet cellular debris, and the supernatant was removed and quantified using DC Protein Assay (Bio-Rad Inc.).

Protein samples were processed in urea buffer and subjected to 2D-DIGE analysis (Applied Biomix, Inc, Hayward, CA). Briefly, samples of equal loading were labeled with Cy2, Cy3, or Cy5 dyes (GE Healthcare/Amersham, Piscataway, NJ) on ice for 30 minutes. Labeled

samples were mixed with a rehydration buffer and then subjected to isoelectric focusing (IEF) on a 13-cm precast IPG (immobilized pH gradient) strip (pH 3-10, GE Healthcare/Amersham) using an Amersham Pharmacia IPGPHOR unit with a power supply (EPS 3501XL) in gradient mode. The samples were then separated in the second dimension by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Subsequent 2-D gels were scanned using Typhoon Trio scanner (GE Healthcare/Amersham), and images were analyzed using ImageQuant and DeCyder software (GE Healthcare/Amersham). Individual fluorescent dye signals were converted to black and white images corresponding to the individual samples, and gel images are presented in Figure 2.

To obtain a comprehensive analysis of the various protein spot comparisons between each of the four groups, samples were run in one of the following sets of gels, with three samples in each set: (1) -strain/-TGF, +strain/-TGF, and +strain/+TGF (2) -strain/-TGF, -strain/+TGF, +strain/+TGF. Quantitative comparisons were then made between two individual samples for each of the three possible combinations in the two separate gels (total of 6 crosswise comparisons between samples). In each gel, the individual volume ratios (samples 1 vs. 2, samples 1 vs. 3, and samples 2 vs. 3) were calculated for each protein spot and used to determine relative protein expression. To simplify the presented data, only comparisons to the negative control sample (i.e., “-strain/-TGF”) are presented.

Immunoblotting analysis was performed as described previously¹¹. The antibodies against CNN3 and actin were obtained from Santa Cruz Biotechnologies, Inc.

Protein Spot Identification with Mass Spectrometry

To determine the identities of the differentially-regulated proteins within the 2-D gel spots, the gels were subjected to in-gel tryptic digestion and peptide extraction, followed by mass spectrometry analysis as described in a previous publication²⁵. Briefly, the protein spots of interest were first manually removed from the gels using a razor blade and then digested with trypsin²⁵. The digested protein samples were then subjected to Q-TOF mass spectrometry using a hybrid quadrupole/orthogonal time-of-flight mass spectrometer, Q-TOF API US (Waters), interfaced with a capillary liquid chromatography system (Waters), which constitutes a complete liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis²⁵. MS/MS spectra were obtained in a data-dependent acquisition mode, and mass spectra were processed by using MassLynx 4.0 software²⁵. Protein identities were determined using the Protein Global Server 1.0/2.0 software, and were further confirmed by Mascot (www.matrixscience.com) by using the MS/MS peak lists exported from MassLynx²⁵.

RNA Isolation and qRT-PCR

Cells were lysed with 1.0 mL of RNA STAT-60 reagent (Tel-Test Inc.) per membrane. RNA was extracted using chloroform and phenol extractions, precipitated using isopropanol, and the resulting RNA pellet was washed with 75% ethanol. RNA pellets were resuspended in 20 µL DEPC-treated H₂O and were quantified as described. cDNA was synthesized using two-step reverse-transcription with the ThermoScript RT-PCR system (Invitrogen), followed by qRT-PCR with SYBR-green reagent and the ABI Prism® 7000 Sequence Detection System (Applied Biosystems). Primers for the genes of interest were all designed using the ABI Prism Primer

Express software v.2.0 (Applied Biosystems). Refer to Table 2 for a full list of primer sequences. The gene levels from each sample were normalized to 18S levels from the same sample. Data was analyzed using ABI Prism 7000 SDS software (Applied Biosystems).

Statistical analysis

PCR data was analyzed for statistical significance between all groups using ANOVA followed by a Holm's t-test for comparisons between each individual group. * $P < 0.05$ using one-tail t-test ($n=5$). Data is presented as mean $\pm$ standard deviation. All replicates were obtained from independent experiments.

ACKNOWLEDGEMENTS

We thank Drs. Jerry Shen and Jian Liao at Applied Biomics Inc. for their help in 2D-DIGE analysis. This work was supported in part by grants HL079419, HL078534 and HL083900 from NIH. Work also funded by the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

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TABLES

Table 1. Proteins identified by proteomic analysis

Protein symbol	Protein Name	MW (kD)	PI	Spot Number	[-strain/+TGF]/ [-strain/-TGF] Ratio	[+strain/-TGF]/ [-strain/-TGF] Ratio	[+strain/+TGF]/ [-strain/-TGF] Ratio
EF2	Eukaryotic Translation Elongation Factor 2	96.2	6.4	1	1.26	1.97	1.87
FINC	C-terminus truncated, Fibronectin 1	243.0	5.6	2	1.47	1.11	1.54
BGH3	Transforming growth factor-beta-induced protein ig-h3 [Precursor]	75.2	7.6	3	1.08	1.72	1.77
SERC	Phosphoserine aminotransferase isoform 1	40.8	7.6	4	-1.40	1.19	1.12
CNN3	Calponin 3	36.6	5.7	5	1.62	1.00	1.65
BGH3	N-terminus truncated, Transforming growth factor-beta-induced protein ig-h3 [Precursor]	75.2	7.6	6	1.05	1.61	1.58
CALR	Calreticulin	48.3	4.3	7	-1.56	-1.10	-1.45
AK1C1	Aldo-keto reductase family 1	37.2	7.1	8	-1.01	-1.23	-1.55
TPM1	Tropomyosin 1 alpha chain	32.8	4.7	9	-1.43	-1.12	-1.41
ARP3	ARP3 actin-related protein 3 homolog	47.8	5.6	10	-1.72	-1.24	-1.58
NQO1	NAD(P)H menadione oxidoreductase 1	30.9	8.9	11	1.02	-1.07	-1.45
ACTG	C-terminus half of full length gamma actin	18.7	5.2	12	1.64	-1.28	1.37

Table 2. Primer sequences used for qRT-PCR

Gene name	Forward Primer (5' to 3')	Reverse primer (5' to 3')
BGH3	AATCTGTGGCAAATCAACAGTCA	TCATATCCAGGACAGCACTCGTA
CNN1	GCATGTCCTCTGCTCACTTCAA	GGGCCAGCTTGTTCTTAACCT
CNN3	GGCTCTAGCAGGTCTGGCTAAA	AATGTCAATGGTTGTATGGAATCCT

FIGURE LEGENDS

Figure 1. Immunofluorescent staining of F-actin cytoskeletal filaments in MSCs. Cells were cultured in the absence or presence of TGF- β 1 (5 ng/mL) for 24 hours under static conditions, followed by additional 24-hours static culture or mechanical stimulation (strain is in left-right direction). Scale bar is 100 μ m.

Figure 2. Representative 2-D gel images of protein samples taken from MSCs after chemical (5 ng/mL TGF- β 1) and/or mechanical stimulation. Original fluorescent signals were converted to black and white images shown here. Spots of particular interest are circled and numbered according to Table 1. Spots 3 and 6 are BGH3. Spot 5 is CNN3.

Figure 3. Comparison of BGH3 and CNN3 spot intensities taken from 2-D gel analysis of MSCs after chemical (5 ng/mL TGF- β 1) and/or mechanical stimulation. All circled spots correspond to spot numbers from Table 1.

Figure 4. Changes in MSC gene expression induced by chemical (5 ng/mL TGF- β 1) and/or mechanical stimulation. Data represents relative mRNA expression levels normalized to 18S. *P<0.05 using one-tail t-test (n=5).

Figure 5. Post-transcriptional regulation of CNN3. Cells were cultured for 1 day, and either subjected to mechanical stimulation or kept as static control in the absence or presence of cycloheximide (10 μ g/mL) for 24 hours. Protein lysates from each sample were subjected to immunoblotting analysis by using an antibody against CNN3 or actin (to show equal loading of proteins).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Table 1. Proteins identified by proteomic analysis

Protein symbol	Protein Name	MW (kD)	PI	Spot No.	[-strain/+TGF]/ [-strain/-TGF] Ratio	[+strain/-TGF]/ [-strain/-TGF] Ratio	[+strain/+TGF]/ [-strain/-TGF] Ratio
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FINC	C-terminus truncated, Fibronectin 1	243	5.6	2	1.11	1.47	1.54
BGH3	Transforming growth factor-beta-induced protein ig-h3 [Precursor]	75.2	7.6	3	1.72	1.08	1.77
SERC	Phosphoserine aminotransferase isoform 1	40.8	7.6	4	1.19	-1.40	1.12
CNN3	Calponin 3	36.6	5.7	5	1.00	1.62	1.65
BGH3	N-terminus truncated, Transforming growth factor-beta-induced protein ig-h3 [Precursor]	75.2	7.6	6	1.61	1.05	1.58
CALR	Calreticulin	48.3	4.3	7	-1.10	-1.56	-1.45
AK1C1	Aldo-keto reductase family 1	37.2	7.1	8	-1.23	-1.01	-1.55
TPM1	Tropomyosin 1 alpha chain	32.8	4.7	9	-1.12	-1.43	-1.41
ARP3	ARP3 actin-related protein 3 homolog	47.8	5.6	10	-1.24	-1.72	-1.58
NQO1	NAD(P)H menadione oxidoreductase 1	30.9	8.9	11	-1.07	1.02	-1.45
ACTG	C-terminus half of full length gamma actin	18.7	5.2	12	-1.28	1.64	1.37

Table 2. Primers for RT-PCR

Gene name	Forward Primer (5' to 3')	Reverse primer (5' to 3')
BGH3	AATCTGTGGCAAATCAACAGTCA	TCATATCCAGGACAGCACTCGTA
CNN1	GCATGTCCTCTGCTCACTTCAA	GGGCCAGCTTGTTCTTAACCT
CNN3	GGCTCTAGCAGGTCTGGCTAAA	AATGTCAATGGTTGTATGGAATCCT