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Phenotypic responses to mechanical stress in fibroblasts from tendon, cornea and skin

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Running Title: Phenotypic comparison of mechanically stressed fibroblasts

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Synopsis

Primary fibroblasts isolated from foetal mouse cornea, skin and tendon were subjected to linear shear stress and analysed for morphological parameters and by microarray as compared with unstimulated controls. Approximately 350 genes were either up or down regulated by a significant amount, with 51 of these being common to all 3 cell types. Approximately 50% of altered genes in tendon and cornea fibroblasts were changed in common with one of the other cell types, with the remaining approximately 50% being specific to tendon or cornea. In skin fibroblasts however, less than 25% of genes whose transcription was altered were specific only to skin. The functional spectrum of genes that were up or down regulated was diverse, with apparent house-keeping genes forming the major category of up-regulated genes, however a significant number of genes associated with cell adhesion, extracellular matrix and matrix remodeling, as well as cytokines and other signaling factors were also affected, somewhat surprisingly in these latter categories the trend was towards a reduction in mRNA levels. Verification of the mRNA quantity of a subset of these genes was performed by RT-PCR and was found to be in agreement with the microarray analysis. These findings provide the first in-depth analysis of phenotypic differences between fibroblast cells from different tissue sources and reveal the responses of these cells to mechanical stress.

Key words: Fibroblast, mechanical stimulation, phenotype, microarray, mechanotransduction

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Introduction

Fibroblasts are stromal cells that constitute the predominant cell type in mesenchymal tissues and are considered to be the primary source of most extracellular matrix (ECM) components[1]. The ECM is composed of a variety of proteins, including collagens, glycosaminoglycans, proteoglycans, and adhesive proteins such as fibronectin and laminin, all of which are secreted by fibroblasts and are eventually assembled into an organized meshwork based upon the functional requirement of the given tissue. Fibroblasts are also capable of producing and secreting various proteolytic enzymes, such as collagenases[2, 3] and serine-proteases[4], as well as some of their specific inhibitors[5], which allows fibroblasts to exert further control on the composition and turnover of the ECM. In addition to the synthesis and regulation of the ECM, fibroblasts play essential roles in wound healing and have been found to be involved in the pathogenesis of fibrotic processes[6].

Despite their broad functional roles and wide-spread use in culture, fibroblasts are very poorly defined. To date, there is no known marker for fibroblasts, mainly due to the fact that all potential markers have also been identified in other members of the connective tissue cell family, such as osteoblasts, smooth muscle cells and adipocytes[7]. To make matters more complicated, the term “fibroblast” sometimes refers to a phenotype or a fibroblast-like cell, rather than a specific cell type. This morphological definition is based upon the fact that fibroblasts are traditionally considered to have a relatively uniform morphology, appearing elongated and spindle-shaped, with clear leading and trailing edges, a morphology sometimes shared in cells that aren’t of fibroblast origin.

The observation that all fibroblasts are created morphologically equal has since been shown to be untrue, as demonstrated by the morphological differences observed between fibroblasts analyzed *in vivo* and those cultured *in vitro*[1, 8, 9]. Phenotypic plasticity in fibroblasts is further supported by findings that fibroblasts isolated from distinct tissues demonstrate unique behavior in culture, such as sensitivity to trypsin and EDTA, replication rate, saturation density, attachment efficiency, and proliferative capacity[10-13], discernible morphology[14-16], differential synthesis of extracellular matrix proteins[11, 13, 17], and distinct cell-surface antigen presentation and surface receptors[18, 19].

In order to test the hypothesis that fibroblasts from different tissues are phenotypically distinct from one another, we have subjected tendon, skin and corneal

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fibroblasts to mechanical stimulation by fluid flow, a technique previously shown to alter morphology, cell adhesion, calcium transients, gene expression, cell alignment and protein secretion in fibroblasts[20-23]. Following stimulation, microarray technology and semi-quantitative RT-PCR were used to analyze the transcriptional responses of the cells. From this study, it is apparent that fibroblasts demonstrate unique gene expression in response to an identical stimulus, supporting the possible differentiative capacity of fibroblasts from diverse tissues.

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Experimental

Fibroblast Isolation and Culture

All cell culture reagents were obtained from Gibco (Paisley, UK). Embryos used for fibroblast isolation were obtained from a time mated CD1 mouse. At 19-days post conception, the pregnant mouse was euthanized by CO_2 asphyxiation and immediately swabbed with 70% ethanol in a sterile hood. Tendon, corneal and skin fibroblasts were isolated according to Spector *et al* [24]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FCS in a 5% CO_2 humidified atmosphere at 37 °C, and were subcultured when they reached approximately 80% confluence. Cells were cultured until five population doublings, at which point they were seeded onto a gelatin-coated (1% w/v) glass plate (7cm x 10cm) and allowed to adhere for approximately seven hours prior to stimulation.

Mechanical stimulation

Fluid flow was applied to cells using a parallel plate flow chamber described previously [25]. Wall shear stress (τ_w) was calculated according to the equation $\tau_w = 6\mu Q/(bh^2)$, where μ is the fluid viscosity (0.001 Pa s), Q is the fluid flow rate (ml/s), and b and h are the width (5.5 cm) and height (0.04 cm) of the flow channel, respectively. The assembled system was maintained at 37 °C in a 5% CO_2 humidified incubator. In this study, tendon, corneal and skin fibroblasts were subjected to a shear stress of 0.1 dynes/cm^2 for 14 hours, with a flow perfusate of DMEM supplemented with 2% FCS and 1% penicillin/streptomycin.

RNA isolation and purification

RNA extractions were carried out with the Absolutely RNA RT-PCR Miniprep kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Isolated total RNA was quantified and qualified by measuring its optical density at 260 nm and 280 nm. RNA samples were stored at -80°C until use.

Microarray analysis

RNA processing and microarray analysis was carried out at the Sir Henry Wellcome Functional Genomics Facility (SHWFGF, University of Glasgow, UK). RNA samples from 3 separate control and stimulated experiments for each of the three tissue

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types were analysed on the Affymetrix GeneChip Mouse Expression Set 430 using standard Affymetrix protocols.

Statistical analysis

Gene expression in the *stimulated* and *control* groups for each of three tissues were compared using FunAlyse, a newly-established automated pipeline in SHWFGF (http://www.gla.ac.uk/functionalgenomics/rp/affy_analysis.html). As a first step of this analysis, all 18 samples were normalized using Robust Multichip Average (RMA) method [26] implemented as module affy 1.2 in the Bioconductor library (http://www.bioconductor.org/). Subsequently, differentially expressed genes were identified using the Rank Products (RP) method [27] that is particularly powerful for experiments involving small number of biologically replicated samples. For every comparison, this method ranks the genes according to differential expression measured by the non-parametric RP-statistic and assesses the statistical significance by producing false discovery rates (FDR). The differentially expressed functional gene classes assigned using GeneOntology annotations (http://www.geneontology.org/) were identified using iterative Group Analysis (iGA) [28]. The A and B arrays of the Affymetrix GeneChip Mouse Expression Set 430 were analysed separately.

The RP-generated lists of differentially expressed genes cut using FDR 10% were further compared manually to identify the highest up- and lowest down-regulated genes in one or more cell lines. Altered genes were manually classified into one of nine, broad functional groups based upon their functional annotation in the SOURCE [29], GenBank [30], and Mouse Genome Informatics [31] databases.

Semi-quantitative RT-PCR

RNA isolated from mechanically stimulated and control fibroblasts was reverse transcribed and amplified using the Titan One Tube RT-PCR System (Roche, Lewes, East Sussex, UK) according to the manufacturer's instructions. 10 ng of total RNA was used as the template in all reactions. The reverse transcription reaction was carried out at 50 °C for 30 minutes, followed by denaturation for 2 min at 94 °C. The thermocycling conditions included 35 cycles of 10 seconds at 94 °C, 30 seconds at 45-65 °C (depending upon the melting temperature of the primer pairs used), 45 seconds at 68 °C, which was increased by 5 seconds for each cycle during cycles 11-25, and a

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final, prolonged elongation cycle of 7 minutes at 68 °C. PCR was performed using primers specific for each target gene (Supplementary Table I http://www.BiochemJ.org/bj/vvv/bjvvvppppadd.htm) and conditions were chosen such that all of the RNAs analysed were in the exponential phase of amplification. Aliquots of the PCR reactions were electrophoresed on 1% agarose gels containing 80 ng/ml ethidium bromide and photographed. The integrated density of the resultant bands was quantified for each sample (NIH Image).

Western Blot Analysis

Western blotting was used to confirm the developmental origin of isolated fibroblasts as well as evaluate levels of lumican, dyxin, CRP1, and neogenin, since the genes encoding these proteins were shown to be differentially regulated with shear stress. Cells were lysed in ice-cold lysis buffer (50 mM Tris pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol) containing protease inhibitors (1 μM pepstatin, 1 mM PMSF, 100 μM TPCK, 260 μM TAME, 10 mM benzamidine) and sonicated on ice. The protein concentration of cell extracts was quantified using the Micro BCA Protein Assay (Perbio Ltd, Cramlington, UK), and 10 μg were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [32] and electrophoretically transferred to a polyvinylidene difluoride membrane. Western blotting was carried out as described previously[33] with one of eight primary antibodies: mouse monoclonal anti desmin (1:200; Sigma), guinea pig polyclonal anti keratin (1:200; Sigma), goat polyclonal anti vimentin (1:400), rabbit polyclonal anti lumican (1:500 dilution; a generous gift from A Oldberg, University of Lund, Sweden), rabbit polyclonal anti dyxin (1:50 dilution; kindly provided by R Crosbie, UCLA), mouse monoclonal anti CRP1 (1:100 dilution; BD Transduction Laboratories), rabbit polyclonal anti neogenin (1:50 dilution; Santa Cruz Biotechnology Inc.), goat polyclonal anti-actin was used as loading control (1:500; Santa Cruz Biotechnology Inc.). Blots were developed with an appropriate secondary antiserum and detected with chromogenic or ECL substrates as previously described [33].

Microscopy

Morphology of control and stimulated fibroblasts was assessed by phase contrast microscopy on a DM IRE2 with Plan 10x/0.3 objective (Leica) at room temperature.

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Images were captured with a DC350F CCD camera (Leica) using Leica QFluoro software (version 1.2.0) and Adobe Photoshop 7.0.

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Results

Isolated cells are of mesenchymal origin and demonstrate fibroblastic morphology

Because there is no known universal marker for fibroblasts, we used the presence and/or absence of intermediate filament proteins to substantiate the mesenchymal origin of the isolated tendon, corneal and skin cell lines. Cell lysates of HeLa, Swiss 3T3 cells, C2C4 myoblasts and the isolated tendon, corneal and skin fibroblasts were resolved on 12% polyacrylamide gels and immunoblotted for desmin, keratin and vimentin (Fig. 1A). The isolated cell lines lack desmin and keratin intermediate filaments, markers for cells of muscle and epithelial origin, respectively, demonstrating a lack of any substantial contamination by these cell types. Tendon, corneal and skin fibroblasts did, however, contain a significant amount of vimentin, an intermediate filament prevalent in cells of mesenchymal origin. Cells appeared to have a typical fibroblast-like morphology in culture with a flattened irregular outline, on contact inhibition cells remained in the same overall pattern with no significant polarization or change in density as seen with many epithelial derived cells data not shown, see also Fig. 1B.

In order to investigate the possible differential responses of tendon, corneal and skin fibroblasts, all three cell lines were subjected to mechanical stimulation by shear stress. Cells were subcultured until five population doublings, seeded onto gelatin-coated glass plates, and were either subjected to 0.1 dynes/cm^2 of laminar fluid flow for 14 hours (stimulated), or maintained under standard tissue culture conditions (control). Both control and stimulated fibroblasts from each cell line were imaged after treatment (Fig. 1B). Upon initial inspection, it is evident that the magnitude of stimulation did not displace cells from their substrate, and tendon, corneal and skin fibroblasts maintain typical elongated, spindle-like morphology even after stimulation. Furthermore, it does not appear that the stimulated fibroblasts re-oriented in the direction of flow. Rather, cells seem to be randomly oriented on the gelatin-coated substrate and display no overt morphological changes as a consequence of the mechanical stimulation (Fig. 1B).

Tendon, corneal and skin fibroblasts demonstrate differential gene regulation when subjected to an identical mechanical stimulus

To gain a comprehensive sense of the genetic responses of tendon, corneal and skin fibroblasts to mechanical stimulation, gene regulation was examined in stimulated and control cells by microarray analysis. Following stimulation, cells were lysed

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directly on the glass plate, and total RNA was isolated and amplified prior to analysis. The probe array used was the GeneChip Mouse Expression Set 430 (Affymetrix, High Wycombe, UK), which enabled the simultaneous quantification of expression of 34,000 genes in parallel.

Microarray analysis revealed that 344 genes were significantly differentially regulated with stimulation (Fig. 2a). Of these altered genes, 15% were common to all three cell lines, 17% were identified in two of the three cell lines, and 69% were differentially regulated in one cell line only. Interestingly, 34% and 27% of the total genes identified were found to be unique to either tendon or corneal fibroblasts, respectively. In contrast, only 7% of the genes identified were unique to skin fibroblasts suggesting a more specific phenotypic expression in tendon and corneal cells than in skin. Upon closer inspection, it is apparent that tendon and corneal fibroblasts have approximately 6% of the identified genes in common, while tendon and skin fibroblasts share 8% of differentially regulated genes. Given that corneal and skin fibroblasts maintain only 3% of the altered genes in common, this hints to the possibility that skin and corneal fibroblasts demonstrate the most differential phenotypic response to stimulation.

In order to examine the transcriptional response of these cell lines to stimulation more carefully, altered genes were manually classified into one of nine, broad functional groups based upon their functional annotation in the SOURCE[29], GenBank[30], and Mouse Genome Informatics[31] databases. An automated Iterative Group Analysis classification system[28] was also used with qualitatively similar results, data not shown. Upon initial inspection, it is clear that the functional spectrum of altered genes was diverse; transcripts were found to be implicated in apoptosis, cell division, ECM and cytoskeletal remodeling, housekeeping, cell signaling, stress response, transcription or cellular transport (Fig. 2b). Tendon fibroblasts demonstrated approximately equal numbers of up- and down-regulated genes with stimulation, whereas the majority of genes were down-regulated in corneal fibroblasts. When compared with the other two cell lines, skin fibroblasts showed a significantly lower number of altered genes with stimulation, the majority of which were up-regulated. It is also clear that housekeeping genes comprise the major category of up-regulated genes, while a significant number of genes associated with cell division, extracellular matrix and cytoskeletal remodeling, as well as signaling factors, showed reductions in mRNA levels.

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The proportional contribution of the functional spectrum of differentially regulated genes was also calculated (Fig. 2C). Upon consideration of the total number of altered genes, the proportion of altered apoptosis- and transcription-related genes was within 3% in all three cell lines. Tendon and corneal fibroblasts show a significantly higher proportion of alterations in genes involved in cell division and ECM and cytoskeleton remodeling as compared with skin fibroblasts. Furthermore, corneal fibroblasts demonstrate a lower contribution of genes involved in signaling when compared with the other two cell lines. Of the genes differentially regulated with stimulation in tendon and corneal fibroblasts, approximately 4% were involved in transport. This is in contrast to an approximate 8% contribution demonstrated by skin fibroblasts. Signaling- and housekeeping-related genes were altered in similar proportions in tendon and skin fibroblasts, whereas the proportion of signaling-related genes changed with stimulation in corneal fibroblasts was markedly less. Of the transcripts implicated in stress responses, skin fibroblasts demonstrated the largest proportion, followed by tendon and then corneal cells.

If one considers differentially up- and down-regulated genes separately, it becomes apparent that the majority of housekeeping, stress response and transport related transcripts are up-regulated with stimulation, whereas apoptosis-, cell division- and signaling-related genes are largely down-regulated. Tendon fibroblasts have the largest proportion of up-regulated genes involved in ECM/cytoskeletal remodeling, whereas skin fibroblasts have a markedly larger number of up-regulated genes involved in signaling and transport. As far as down-regulated genes are concerned, all three cell lines show a similar proportion of genes involved in apoptosis and transport-related genes, yet maintain differences in the proportions of other functional classifications. Tendon fibroblasts, for example, have a significantly higher proportion of ECM/cytoskeletal remodeling-, housekeeping- and signaling-related genes down-regulated with stimulation, whereas skin fibroblasts have a higher proportion of up-regulated stress response- and transcription-related genes.

RT-PCR and quantification

In order to validate the changes in gene expression identified by microarray analysis, a subset of 14 genes was examined by semi-quantitative RT-PCR, representing eight down-regulated and six up-regulated genes. These particular genes were chosen based upon their differential regulation in one or more cell lines and/or possible role in

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ECM biogenesis or cytoskeletal organization. RNA previously isolated from three replicate mechanical stimulation experiments for each cell line was used as a template for RT-PCR. The resultant products were electrophoresed and the volume integration of each product, visualized by ethidium bromide staining, was quantified with NIH Image (Fig 3). In all cases, the generated RT-PCR products migrated at their expected size, and semi-quantitative RT-PCR substantiated the up- or down-regulation of transcription initially revealed by the microarray data; the direction of regulation (i.e. up- or down-regulation) is conserved in all cases between RT-PCR and the microarray data. On occasion, the relative magnitude of regulation determined by RT-PCR appears to be different between different genes, but not within genes with respect to the microarray data. This is partly due to the limitations of determining a useful dynamic range for the number of PCR cycles in the RT-PCR that could be applied across all samples. For example, the RNA levels determined by RT-PCR for Neogenin and Serpin1b were relatively low. Consequently, upward changes with stimulation are disproportionately large compared to genes with a more robust RT-PCR signal, such as Gsta4 and Hmox1. Accordingly, quantification of the RT-PCR reactions are listed in a semi-quantitative manner (Fig. 3).

Protein levels do not directly correlate with mRNA levels, but do change, in stimulated fibroblasts

In an effort to gain a more global understanding of the response of fibroblasts to mechanical stimulation, levels of expression of four proteins were also investigated (Fig. 4, Table I). To achieve this, control and mechanically stimulated fibroblasts were lysed immediately after treatment, sonicated and the protein concentration of the resulting lysate determined. Equal concentrations of lysates were electrophoresed and subsequently visualized by western blotting, using antibodies directed to lumican, dyxin, CRP1, and neogenin, and quantified using a β-actin loading control.

The lumican antibody identified four distinct bands, indicating proteins with apparent molecular weights of 73, 62, 30 and 26 kDa, corresponding to the intact glycoprotein, an alternatively glycosylated form, and two isoforms of the core protein typically found in fibroblasts, respectively[34, 35]. After quantification, it appears that stimulation has no effect on the levels of the 73, 30 or 26 kDa species in all three cell lines. However, the 62 kDa species, corresponding to the alternatively deglycosylated form of lumican, shows an increase of approximately 6-, 3.5-, and 7-fold with

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stimulation in tendon, corneal and skin fibroblasts, respectively. The apparent increase in the 62 kDa lumican species is at odds with the decreased levels of lumican mRNA with stimulation, as determined by both microarray and RT-PCR. However, the appearance of an alternative glycosylated form upon stimulation is in itself interesting. This points to a post-translational pathway that has effects in a different direction to the transcriptional control, which may reveal an important functional insight into the role of lumican. This aspect of the study warrants further investigation.

The dyxin antibody revealed a band of approximately 40 kDa, which is in agreement with previous reports[36]. After quantification and normalization against β-actin, all three cell lines appear to demonstrate an increase in protein expression with stimulation. Skin and tendon fibroblasts show the greatest increase in protein levels, with an approximate increase of 2-fold when compared to controls. This increase in dyxin protein levels is in contrast to reducing amounts of mRNA transcripts following stimulation.

Probing cell lysates with the CRP1 antisera revealed a band of approximately 20 kDa. Levels of transcripts encoding CRP1 were found to decrease in stimulated tendon fibroblasts, and this trend correlated with a concomitant 3.8-fold decrease in CRP1 protein levels. Furthermore, despite the fact that differential expression of CRP1 mRNA was not shown to be significantly altered in stimulated corneal or skin fibroblasts, levels of CRP1 protein in these cell lines was also shown to decrease by approximately 3-fold.

Neogenin immunoblots revealed a prominent band at approximately 193 kDa; this most likely differs from the protein's theoretical molecular weight of 150 kDa because of glycosylation[37]. Shear stress resulted in the differential regulation of neogenin by skin and corneal fibroblasts. Neogenin was found to increase by approximately 2-fold upon the application of mechanical stimulation in skin fibroblasts, which correlated well with a 1.67-fold increase in neogenin transcript levels, as determined by microarray analysis. Corneal fibroblasts, on the other hand, displayed an almost 3-fold decrease in neogenin following stimulation. In this case, the decrease in protein levels occurs without any apparent change in gene expression. Tendon fibroblasts did not show any change in neogenin protein levels with stimulation.

Discussion

Whilst the word fibroblast is a relatively generic term applied to connective tissue cells of diverse origins, these cells non-the-less possess features specific to their

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tissue of origin. In this study we have attempted to define the genes in common and genes that define that specificity by examining the response of fibroblasts from different tissues to mechanical stress.

Of the 344 genes shown to be differentially regulated in the three cell lines, a surprisingly low 15% of these were common to all three and less than a third were shared between any two cell lines. A summary of some of these genes are shown in Table II. Furthermore, the spectrum of genes that are altered in response to mechanical stress are perhaps intuitively not what one might have expected.

A number of genes were found that have a role in the protection of cells against oxidative stress, a phenomenon generated upon the production of reactive oxygen that can lead to subsequent changes to cellular macromolecules including nucleic acids, proteins and lipids[38]. The overwhelming majority of these genes, glutathione S-transferase (Gsta2), heme oxygenase 1 (Hmox1), glutamate-cysteine ligase, modifier subunit (Gclm), xanthine dehydrogenase (Xdh) aldo-keto reductase family 1, member B3 (Akr1b3), secretory leukocyte protease inhibitor (Slpi), were up-regulated in various cell lines after stimulation. Given the method by which these cells were stimulated, it is not surprising that all three cell lines responded by up-regulating oxidative response genes. An increase in these transcripts most likely serves to protect the cells from reactive oxygen species produced as a consequence of mechanical stimulation. It is not so much the fact that the cells are all mounting a similar stress response that is of particular interest, but rather the differential regulation of unique oxidative-stress genes.

All three cell lines demonstrated the differential regulation of genes implicated in adipocyte differentiation, which is of particular interest given the capability of fibroblast precursors to differentiate along various connective tissue cell-type lineages, such as adipocytes[7]. In the case of carbonic anhydrase 3 (Car3) or lipocalin 2 (Lcn2), which have both been found to be expressed at high levels in mature adipocytes [39, 40], it appears that stimulation is effectively reinforcing the fibroblast phenotype, since levels of these genes were down-regulated with fluid flow. In contrast, however, angiopoietin-like 4 (Angptl4) and ectonucleotide pyrophosphatase/phosphodiesterase 2 (Enpp2) have been shown to be up-regulated during adipocyte differentiation[41-43]. Because Angptl4 and Enpp2 were both up-regulated in this study in response to stimulation, this hints to the possibility that shear stress is inducing an adipogenic-shift in tendon, corneal and skin fibroblasts. While this picture is currently conflicting and

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difficult to interpret, the implication of these genes in mesenchymal differentiation is nonetheless interesting and should be investigated further.

Because the method of stimulation used in this study was mechanical, genes that were implicated in mechanotransduction, or the conversion of mechanical signals into biochemical response via direct or indirect connections between the internal actin cytoskeleton and the ECM, were investigated. Not surprisingly, numerous studies have shown that cells subjected to mechanical stimuli alter both the gene and protein expression of ECM and cytoskeletal components[44-47]. Given the importance of the cytoskeletal - ECM linkage in maintaining cellular integrity and physically resisting mechanical stress the number of genes altered in this category were surprisingly few, accounting for less than 5% of genes overall. ECM structure might be expected to be altered in response to shear stress. Two genes in this category were shown to be markedly altered with stimulation, however lumican (Lum) was downregulated, whereas chondroitin sulphate proteoglycan 4 (Cspg4) was upregualted.

Of the relatively few genes implicated in ECM/cytoskeleton remodeling, three were found to encode LIM domain-containing cytoskeletal adaptor proteins: cysteine and glycine-rich protein (Crp1), LIM and cysteine-rich domains 1 (Dyxin) and LIM domain containing preferred translocation partner in lipoma (Lpp). The LIM domain is implicated in protein-protein interactions, may target proteins to distinct subcellular locations, and is thought to mediate the assembly of multimeric protein complexes[48]. Levels of Crp1, which itself has been shown to interact with alpha-actinin[49], as well as associate with and possibly stabilize adhesion plaques through its binding to zyxin[50, 51], were down-regulated in tendon fibroblasts with stimulation. Both Dyxin and Lpp were found to be down-regulated in corneal fibroblasts after stimulation.

Other genes encoding proteins that either directly or indirectly interacted with the actin cytoskeleton were also found such as myosin light chain kinase (Mlck), Mlck transcripts were reduced in tendon fibroblasts upon stimulation. We have identified Mlck previously as a shear stress-response gene in endothelial cells[52], however in this case, Mlck was up-regulated. Furthermore, in Mlck-inhibited cells, myosin light chain phosphorylation was blocked at the cell periphery but not at the centre of the cell, and zyxin-containing adhesions were not assembled at the cell periphery but focal adhesions were maintained in the centre of the cell. These cells continued to generate membrane protrusions around the cell, but turned over more frequently and migrated less effectively[53]. It is possible, then, that tendon fibroblasts demonstrated a reduction in

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Mlck levels in order to reduce migration under such mechanical stresses. Corneal fibroblasts also demonstrated a decrease in the level of transcripts for the FERM domain containing 3 (Frmd3) gene. Interestingly, only one gene implicated in ECM/cytoskeleton remodeling, Adamts5, was common to two or more cell lines after mechanical stimulation, with the rest being unique to one of the three lines tested. This trend is seen only in this functional classification and suggests that ECM/cytoskeleton-related genes may contribute more specifically to the differential response of tendon, corneal and skin cell lines to stimulation.

The fact that protein levels showed such variable correlation to microarray data is not surprising and has been documented in previous reports[54]. Such poor correlations between protein and mRNA levels most likely results from (1) the possibility of regulating protein levels at either transcriptional or translational levels, and/or (2) the rate of protein turnover *in vivo*[55]. This study provides the first in-depth analysis of the tissue-specific transcriptional response of a cell type to a mechanical stimulation and its comparison to the same cell type in other tissues. These findings will provide an invaluable resource for further study of the factors that control cell and tissue specific mechanical stress responses and may provide avenues for the manipulation and improvement of tissue engineered prostheses and implants for reconstructive surgery.

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Figure Legends

Figure 1. **Confirmation of mesenchymal origin of isolated fibroblasts and morphological examination after mechanical stimulation**. *A*, Lysates of cultured mouse tendon, corneal and skin fibroblasts were resolved by SDS-PAGE and submitted to immunoblot analysis with the indicated intermediate filament anti-sera. Lysates of HeLa cells, NIH 3T3 cells, and C2C4 myoblasts serve as positive controls for keratin, vimentin and desmin, respectively. Fibroblast cells from tendon, cornes and skin expressed only vimentin demonstraing a lck of contamiantion by epithelial or muscle tissues. *B*, Tendon, corneal and skin fibroblasts were seeded onto gelatin-coated glass plates and placed either in a single well plate (control) or subjected to 0.1 dynes/cm^2 fluid flow for 14 h (stimulated). Cells were subsequently imaged with phase contrast microscopy in order to examine cell directionality and morphology. Scale bar = 100 μm. Arrow = direction of fluid flow.

Figure 2. **Total number and functional classification of genes up- and down-regulated in tendon, corneal and skin fibroblasts following mechanical stimulation.** mRNA isolated form mechanically stimulated tendon, corneal and skin fibroblasts was subjected to microarray analysis. *A*, Venn Diagram representing 344 up- and down-regulated genes in tendon, corneal and skin fibroblasts. The number of genes identified in a given cell line are designated, and overlap regions denote genes common to the indicated cell lines. *B*, Number and functional classification of genes up- or down-regulated in three fibroblast cell lines in response to stimulation. Genes were categorized into one of nine broad categories based upon their functional annotation (GenBank). *C*, Proportional contribution of the functional classifications of genes to the total numbers of genes up- or down-regulated in response to fluid flow in each individual cell type.

Figure 3. **Validation of microarray data by semi-quantitative RT-PCR**. Mouse skin, tendon, and corneal fibroblasts were subjected to 14 hours of laminar flow at a rate of 0.1 dynes/cm^2. Total RNA was isolated from three replicate experiments of control (C) and stimulated (S) tendon, corneal and skin fibroblasts and subjected to reverse transcription for 14 genes of interest. Samples were taken during the linear phase of amplification, resolved by electrophoresis on 1% agarose gels and visualised by

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ethidium bromide staining. The intensity of the resultant bands was quantified (NIH Image). Genes are represented as being up- or down-regulated in skin (S), tendon (T), or corneal (C) fibroblasts by less than 2-fold (-/+), greater than or equal to 2-fold, but less than 10-fold (- -/ + +), or greater than or equal to 10-fold (- - -/ + + +). As means of comparison, the fold-change in gene expression as determined by microarray analysis (where statistically significant) is listed alongside the semi-quantitative integrated density results from RT-PCR.

Figure 4. **Expression of extracellular matrix- and cytoskeleton-related proteins in mechanically stimulated fibroblasts**. Western blot analysis of control (C) and stimulated (S) tendon, corneal, and skin fibroblasts. Cell lines were subjected to 14 hours of laminar flow at a rate of 0.1 dynes/cm^2. Western blots were probed with the antibodies against the indicated proteins; immunoblotting with an antibody directed against β-actin serves as a loading control. Detection was by ECL.

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Protein	Species	Tendon	Cornea	Skin
Lumican	62 kDa	6.07 ± 1.16	3.50 ± 0.49	7.21 ± 4.80
Dyxin	40 kDa	1.98 ± 0.44	1.24 ± 0.19	2.08 ± 0.69
Crp1	20 kDa	-3.79 ± 1.62	-3.08 ± 0.93	-2.78 ± 1.22
Neogenin	193 kDa	–	-2.98 ± 0.97	2.16 ± 1.15

Table I. Quantification of immuno-blotting of ECM/cytoskeleton-related proteins differentially regulated with stimulation. Values are represented as the mean fold change between stimulated and control cells ± SEM.

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Gene description	Function	Cell Line T	Cell Line C	Cell Line S	Assession #
A. Highest up-regulated genes					
heme oxygenase (decycling) 1 (Hmox1)	Housekeeping	20.2	25.3	18.2	NM_010442
glutathione S-transferase, alpha 2 (Gsta2)	Stress Response	18.7	27.9	13.3	NM_008182
neoplastic progression 3 (Npn3)	Transcription	11.9	9.8		NM_029688
angiopoietin-like 4 (Angptl4)	Housekeeping	11.4		8.6	NM_020581
glutamate-cysteine ligase , modifier subunit (Gclm)	Housekeeping	11.1	9.3	8.5	NM_008129
heat shock protein 1A (Hsp72)	Stress Response		9.0		NM_010479
proliferin (Plf)	Signalling			14.0	NM_031191
B. Lowest down-regulated genes					
CCAAT/enhancer binding protein delta (Cebpd)	Transcription	-4.3			NM_007679
carbonic anhydrase 3 (Car3)	Housekeeping	-4.3			NM_007606
chemokine (C-C motif) ligand 20 (Ccl20)	Signalling	-4.0		-2.5	NM_016960
pentaxin related gene (Ptx3)	Stress Response	-4.0		-2.6	NM_008987
early growth response 3 (Egr3)	Transcription	-3.5			NM_018781
forkhead box P2 (Foxp2)	Transcription		-3.6		NM_053242
follistatin (Fst)	Signalling		-3.4		NM_008046
cyclin E2 (Ccne2)	Cell Division		-3.3		NM_009830
GULP, engulfment adaptor PTB domain containing 1 (Gulp1)	Apoptosis		-3.3		BB138485
solute carrier family 4 (anion exchanger), member 4 (Slc4a4)	Transport		-3.1		NM_018760
chemokine (C-X-C motif) ligand 5 (Cxcl5)	Signalling			-4.9	NM_009141
thymic stromal lymphopoietin (Tslp)	Signalling			-2.8	NM_021367
lipocalin 2 (Lcn2)	Housekeeping			-2.6	NM_008491
C. Highest up-regulated genes found in only one cell line					
dual specificity phosphatase 4 (Dusp4)	Signalling	4.8			NM_176933
xanthine dehydrogenase (Xdh)	Housekeeping	4.5			NM_011723
mitogen activated protein kinase kinase kinase 4 (Map3k4)	Signalling	3.9			NM_011948
dystonin (Dst)	ECM/Cytoskeleton	3.7			NM_133833
growth arrest specific 5 (Gas5)	Cell Division	3.5			NM_013525
gamma-aminobutyric acid receptor-associated protein-like 1 (Gabarapl1)	ECM/Cytoskeleton		4.4		NM_020590
aldo-keto reductase family 1, member B3 (Akr1b3)	Housekeeping		3.7		BB469763
sphingosine kinase 1 (Sphk1)	ECM/Cytoskeleton		3.3		NM_011451
cytochrome b-5 (Cyb5)	Housekeeping		2.9		NM_025797
ectonucleotide pyrophosphatase/phosphodiesterase 2 (Enpp2)	Housekeeping		2.8		NM_015744
BTB (POZ) domain containing 11 (Btbd11)	Transcription			3.5	BC072592
neogenin (Neo)	ECM/Cytoskeleton			2.7	NM_008684
secretory leukocyte protease inhibitor (Slpi)	Stress Response			2.5	NM_011414
endothelin receptor type B (Ednrb)	Signalling			2.5	NM_007904
centaurin, gamma 2 (Centg2)	Transport			2.5	NM_178119
D. Lowest down-regulated genes found in only one cell line					
carbonic anhydrase 3 (Car3)	Housekeeping	-4.3			NM_007606
ELAV-like 2 (Hu antigen B) (Elavl2)	Cell Division	-3.0			NM_207685
chemokine (C-X-C motif) ligand 14 (Cxcl14)	Signalling	-3.0			NM_019568
early growth response 2 (Egr2)	Transcription	-3.0			NM_010118
receptor activator of NF-κB Ligand (RANKL)	Signalling	-2.9			NM_011613
forkhead box P2 (Foxp2)	Transcription		-3.6		NM_053242
solute carrier family 4, member 4 (Slc4a4)	Transport		-3.1		NM_018760

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RAD51 homolog (*S. cerevisiae*) (Rad51)	Stress Response		-2.7		NM_011234
Nik-related kinase (Nrk)	Signalling		-2.7		NM_013724
anillin, actin binding protein (Anln)	ECM/Cytoskeleton		-2.6		NM_028390
lipocalin 2 (Lcn2)	Housekeeping			-2.6	NM_008491
odd-skipped related 1 (*Drosophila*) (Osr1)	Transcription			-2.3	NM_011859
endothelin 1 (Edn1)	Signalling			-2.3	NM_010104
E. Genes of interest identified in one cell line					
lumican (Lum)	ECM/Cytoskeleton	-2.5			NM_008524
LIM and cysteine-rich domains 1 (Dyxin)	ECM/Cytoskeleton		-2.5		NM_144799
cysteine and glycine-rich protein 1 (Crp1)	ECM/Cytoskeleton	-2.4			NM_007791
myosin, light polypeptide kinase (Mlck)	ECM/Cytoskeleton	-2.0			NM_139300
LIM domain containing preferred translocation partner in lipoma (Lpp)	ECM/Cytoskeleton		-2.1		NM_178665
FERM domain containing 3 (Frmd3)	ECM/Cytoskeleton		-2.0		NM_172869
A disintegrin and metalloprotease domain 33 (ADAM33)	ECM/Cytoskeleton	2.1			NM_033615
chondroitin sulfate proteoglycan 4 (Cspg4)	ECM/Cytoskeleton	3.4			NM_139001
F. Genes of interest identified in all cell lines					
a disintegrin-like & metalloprotease w/thrombospondin type 1 motif, 5 (Adamts5)	ECM/Cytoskeleton	-1.8	-2.2	-1.9	NM_011782
serine (or cysteine) proteinase inhibitor, clade B, member 1b (Serpinb1b)	Housekeeping	5.6	4.5	6.7	NM_173052
glutathione S-transferase, alpha 4 (Gsta4)	Stress Response	6.6	4.3	5.9	NM_010357

Table II. Genes differentially regulated in tendon, corneal and skin fibroblasts with mechanical stimulation. The gene expression profiles of tendon (T), corneal (C) and skin (S) fibroblasts were assessed as described in "Experimental Procedures." The tabulated genes were manually clustered into the indicated functional groups based upon their annotation. The numerical intensity values shown are the fold-changes calculated by the FunaAlyse software as antilog of mean between-chip log-ratios, positive values correspond to up-regulation whilst negative values correspond to down-regulation. Triplicate microarray experiments were performed with each fibroblast cell line.

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Figure 1A

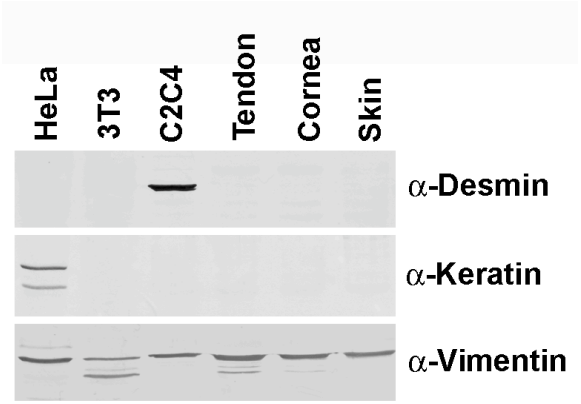

Figure 1B

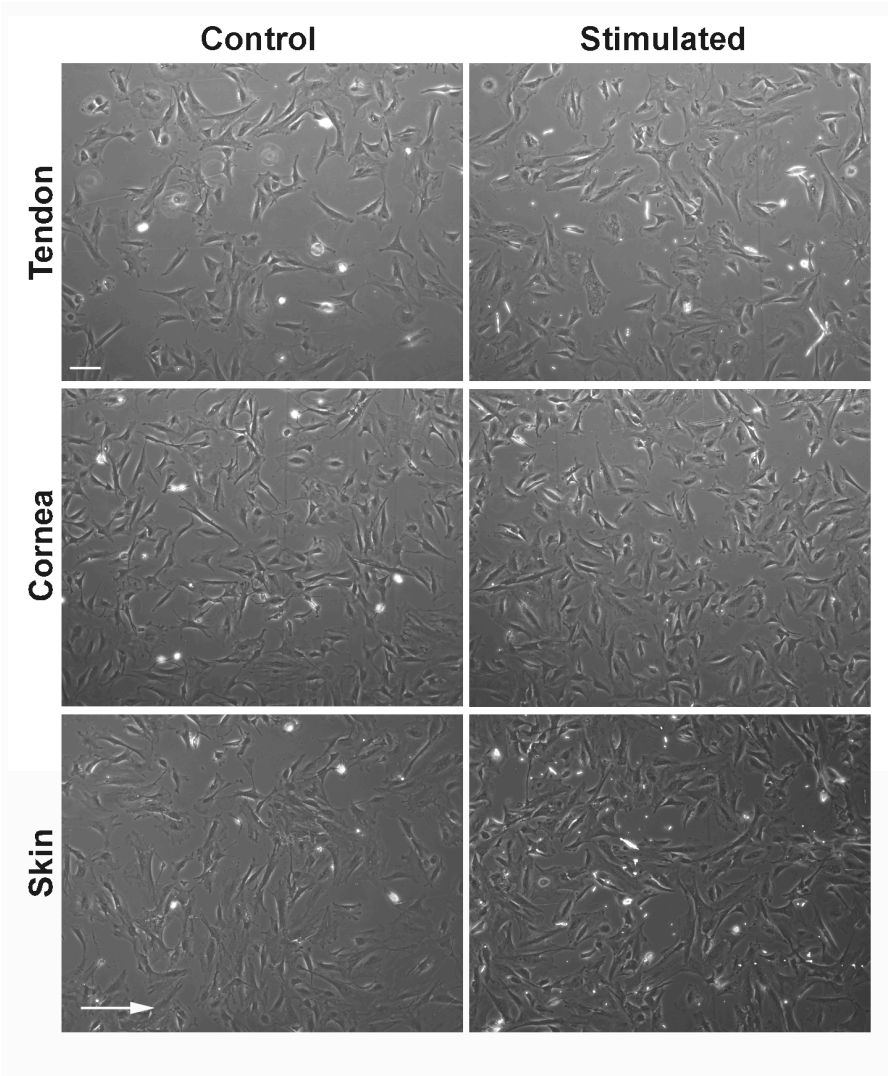

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Figure 2A

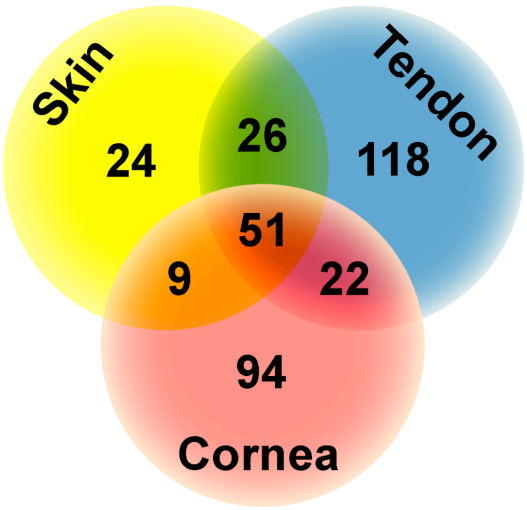

Figure 2B

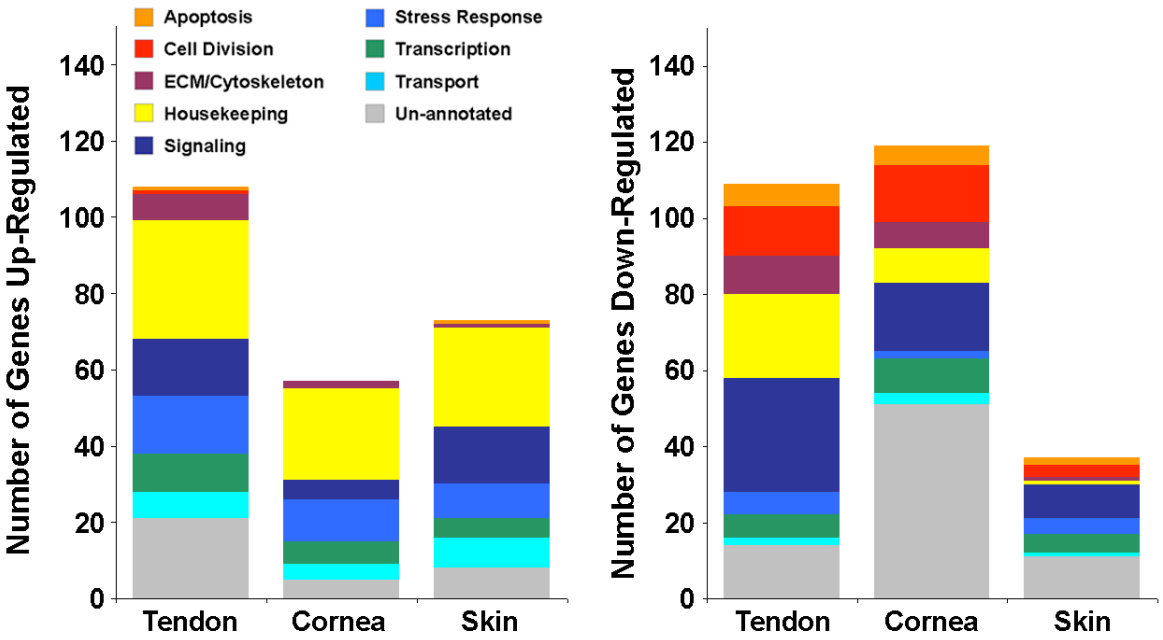

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Figure 2C

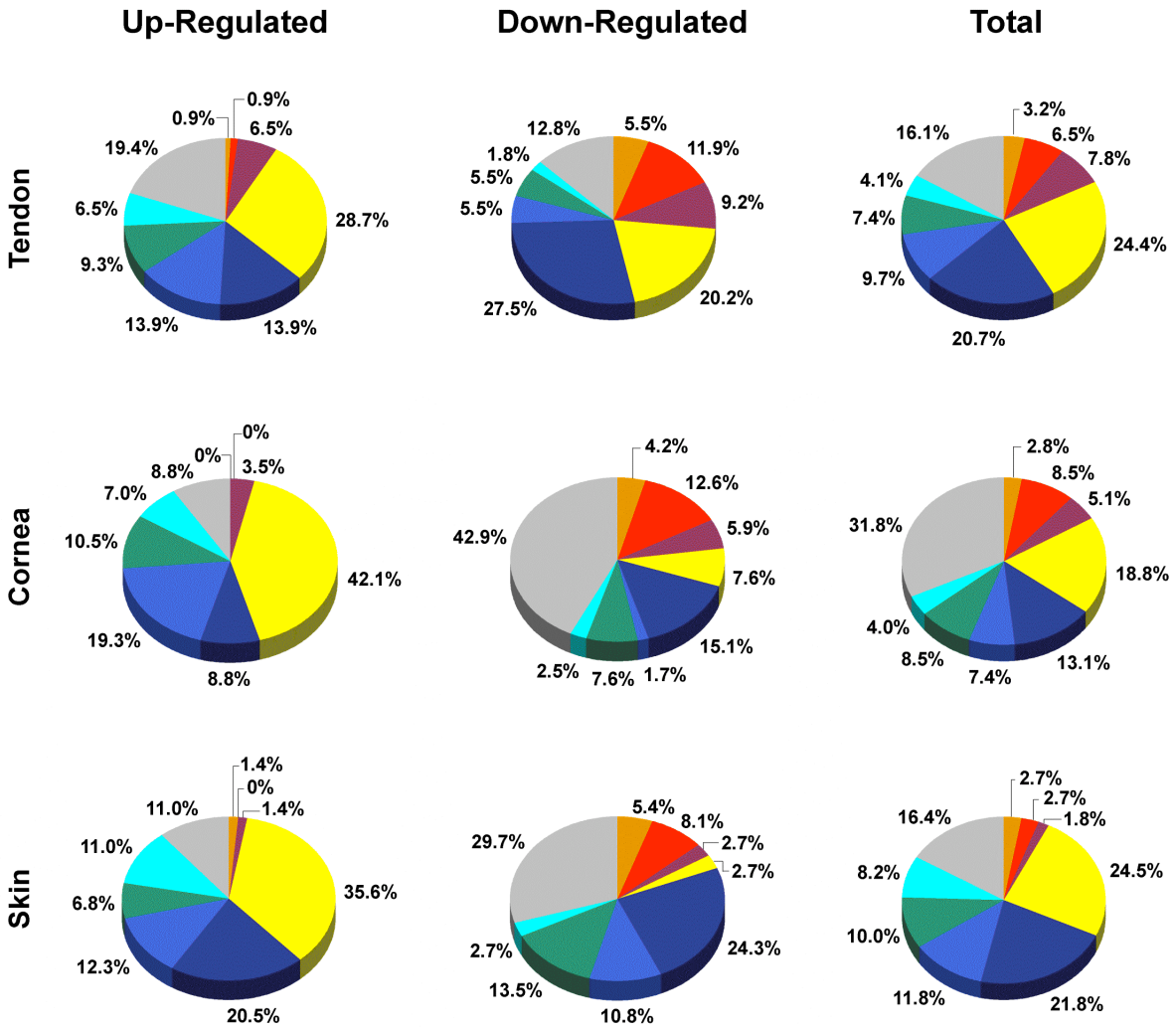

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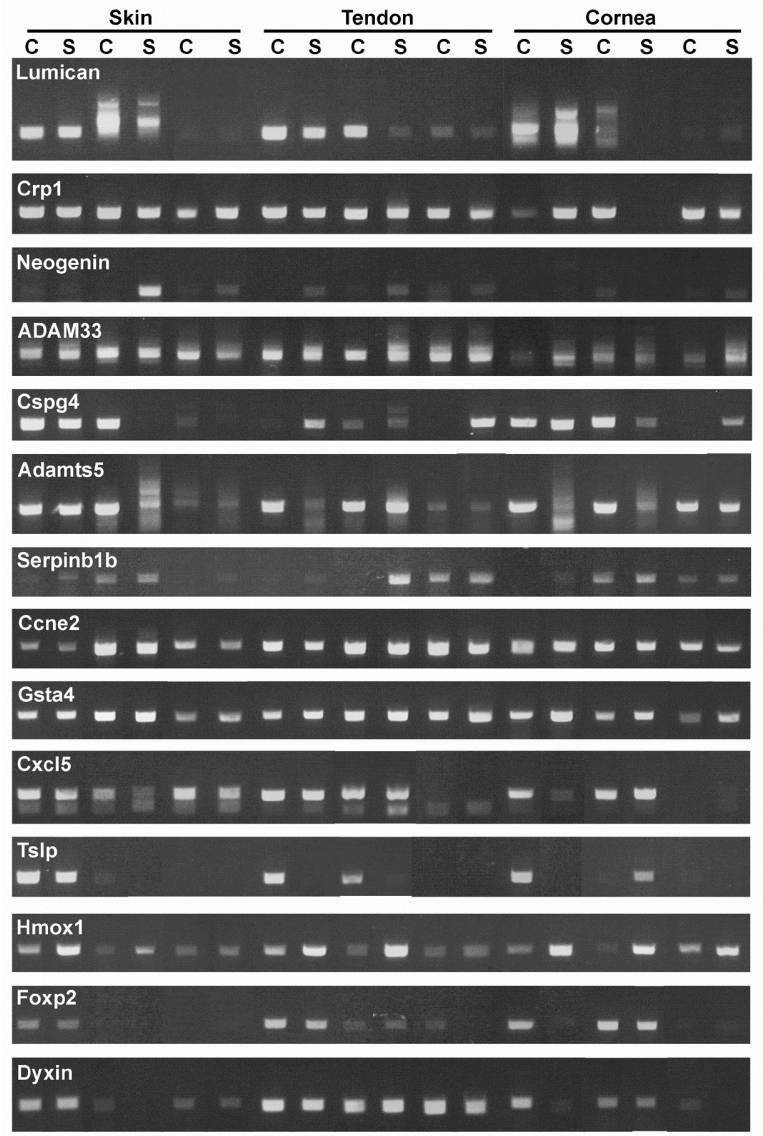
Figure 3



	Microarray			RT-PCR		
	S	T	C	S	T	C
Lumican		-2.5			- -	
Crp1		-2.4			-	
Neogenin	2.7			+ + +		
ADAM33		2.1			+	
Cspg4		3.4			+ + +	
Adamts5	-1.9	-1.8	-2.2	-	- -	-
Serpinb1b	6.7	5.6	4.5	+ + +	+ + +	+ + +
Ccne2	-2.4	-1.4	-3.3	-	-	-
Gsta4	5.9	6.6	4.3	+	+	+
Cxcl5	-4.9	-3.5		-	-	
Tslp	-2.8	-2.5		- -	- - -	
Hmox1	18.2	20.2	25.3	+ +	+ +	+ +
Foxp2			-3.6			- - -
Dyxin			-2.5			- - -

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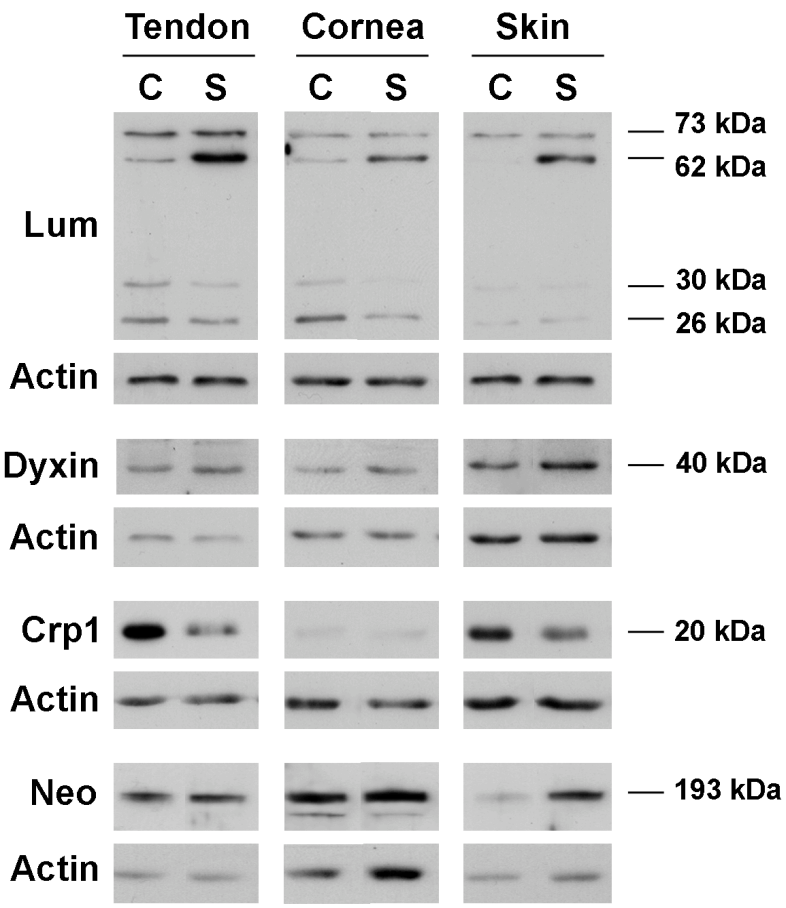
Figure 4



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