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## Loss of Mechanical Strain Impairs Abdominal Wall Fibroblast Proliferation, Orientation and Collagen Contraction Function

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### Abstract

**Background**—Laparotomy wound load forces are reduced when dehiscence and incisional hernia formation occur. The purpose of this study is to determine the effects of strain loss on abdominal fascial fibroblast proliferation, orientation and collagen compaction function.

**Methods**—Cultured rat linea alba fibroblasts were subjected to continuous cyclic strain (CS), cyclic strain interrupted at 24 or 48 hours followed by culture at rest (IS-24 and IS-48) or were cultured without mechanical strain (NS). Cell number was measured and images analyzed for cell orientation. Fibroblasts from these groups were seeded onto the surface of (FPCL-S) or mixed into (FPCL-M) a collagen gel matrix and gel area was measured over time.

**Results**—Continuous strain stimulated proliferation when compared to the non-strained cells. The loss of strain (IS) delayed proliferation compared to CS throughout ( $P < 0.05$ ). CS fibroblasts aligned perpendicular to the direction of strain within 12 hours. Within 12 hours of strain loss, IS-48 fibroblasts became significantly less aligned ( $P < .0001$ ), and appeared similar to the randomly organized NS fibroblasts 48 hours after strain removal. The CS and IS-24 groups demonstrated faster and greater overall FPCL-M compaction than both the IS-48 and NS groups ( $P < .0002$ ). The CS group contracted the gel faster than the NS group in FPCL-S ( $P = .029$ ).

**Conclusions**—Mechanical strain rapidly induces a proliferative, morphological and functional response in abdominal wall fibroblasts that is dependent on the continued presence of the strain signal and quickly lost when the load force is removed. The loss of wound edge tension that occurs during laparotomy wound separation and hernia formation may contribute to impaired wound healing through loss of a key stimulatory mechanical signal with important implications for abdominal wall reconstruction.

### Introduction

Laparotomy wound failure progressing to incisional hernia formation is the most frequent complication of abdominal surgery, with an overall estimated incidence of 11 to 16% following abdominal operations.<sup>1–5</sup> The true incidence is probably underestimated, as 8–29% of incisional hernias are asymptomatic and may not be detected without careful physical examination.<sup>4, 6, 7</sup> Despite many advances in surgical technique and patient care, the rate of primary incisional hernia formation has not changed appreciably in 75 years.<sup>8</sup>

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Although the use of mesh has improved hernia repair, recurrence rates remain unacceptably high,<sup>9–11</sup> worsening with each subsequent reoperation<sup>12</sup>.

Incisional abdominal hernias most frequently develop from early laparotomy wound separation and failure within the first postoperative month, often clinically occult<sup>1, 13</sup> The cause of wound failure may be multifactorial – related to closure technique, wound ischemia, infection, excessive straining from coughing or weight lifting, or patient comorbidities such as older age, obesity, malnutrition, diabetes or chronic steroid therapy.<sup>3, 14–21</sup> These same factors may prevent reestablishment of fascial healing once separation has occurred, and contribute to the increased frequency of recurrence following hernia repair, however the exact mechanism of progression from wound separation to hernia formation is unknown.<sup>22</sup>

Mechanotransduction, the perception of cellular signaling occurring in response to mechanical force, may also play a role in laparotomy wound healing. Many different cells throughout the body experience mechanical forces on a frequent basis such as vascular endothelial and smooth muscle cells,<sup>23–25</sup> cardiac myocytes and fibroblasts,<sup>26, 27</sup> pulmonary alveolar cells,<sup>28</sup> bone osteoblasts,<sup>29</sup> tendon and ligament fibroblasts,<sup>30, 31</sup> and fibroblasts and myofibroblasts during the contraction phase of cutaneous wound healing.<sup>32, 33</sup> Mechanical strain is known to signal repair or regenerative responses of cells in many of these same tissues. The abdominal wall is subjected to intermittent and constant strain caused by breathing, coughing, weight bearing, etc.<sup>34</sup> Models of tendon injury suggest that load-bearing tissues like the abdominal wall are dependent on mechanical strain to signal repair.<sup>35, 36</sup> This is believed to occur through mechanotransduction pathways that activate wound repair fibroblasts.<sup>30, 37, 38</sup> *In vitro*, strain induces uniform alignment in many cell types,<sup>23, 25, 39–41</sup> and the parallel arrangement of fibroblasts and collagen fibers in normal abdominal fascia contributes to its strength and is required for optimal fascial wound healing.<sup>42</sup> Fibroblasts play an essential role in wound repair, migrating into the wound bed, proliferating, synthesizing growth factors, producing and remodeling new extracellular matrix, and contracting the wound.<sup>33, 43</sup> Our previous work found that a fibroblast wound healing defect is induced when laparotomy wounds are mechanically disrupted and form incisional hernias.<sup>44, 45</sup> It may be that the ideal abdominal wall reconstruction reestablishes physiological load much like a tendon following repair.

The aims of this study are to confirm the stimulatory proliferative and cell alignment effects of strain on abdominal wall fibroblasts, and then measure the fibroblast response when the mechanical signal is removed in terms of proliferation, change in cell orientation and fibroblast populated collagen lattice (FPCL) contraction function.

## Materials and Methods

### Materials

Low glucose Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin antibiotics were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories, Inc. (Logan, UT). Rat tail collagen was purchased from BD Biosciences (San Jose, CA).

### Abdominal wall musculotendinous fibroblast (abMTF) cell culture

Biopsies of normal SD rat linea albae were treated in 0.0125% trypsin at 37 °C for 30 min. The peritoneal side of the biopsies was scraped to remove mesothelial cells. Biopsies were minced in culture medium, spread on the bottom of T-75 cm<sup>2</sup> flasks and allowed to stand undisturbed for two hours at 37 °C in 5% CO<sub>2</sub> until the tissue pieces adhered to the flask bottom. The medium was then gently drained. DMEM culture medium containing 15% FBS,

1,000  $\mu$ /mL penicillin G and 1,000  $\mu$ g/mL streptomycin sulfate solution was then slowly added to the culture flask and the tissue samples were incubated at 37 °C in 5% CO<sub>2</sub>. The medium was changed every two days and the abMTF primary cell cultures were grown to confluence for passage. Experimental abMTFs were used at passage three for Flexcell experiments and passage four for FPCL assays.

### Application of mechanical strain

$2.5 \times 10^5$  fibroblasts were plated in the third passage on BioFlex six-well plates coated with type I collagen (Flexcell International Corp., Hillsborough, NC). The cells were cultured in DMEM culture medium containing 15% FBS, 1,000  $\mu$ /mL penicillin G and 1,000  $\mu$ g/mL streptomycin sulfate for 48 h followed by 24 h FBS starving in serum free medium. The culture medium was then replaced with low glucose DMEM containing 2.5% FBS, 1,000  $\mu$ /mL penicillin G and 1,000  $\mu$ g/mL streptomycin sulfate. In order to minimize the effects of factors contained in serum, we used the lowest FBS concentration, 2.5%, that we previously identified as allowing significant strain response compared to unstrained controls. Strained cells were exposed to continuous cycles of strain/relaxation generated by a cyclic vacuum produced by a computer driven system (Flexcell 4000; Flexcell International Corp., Hillsborough, NC). The strain is transmitted to adherent cells cultured on the upper surface of the membrane. The membranes were deformed by a sine-wave vacuum force from below to a peak of 10% extension at 12 cycles/minute (cpm) for up to 192 hours (8 days). These extension and rate settings were determined to produce optimal fibroblast growth responses in our preliminary investigations. For cell proliferation experiments, cells in the Cyclic Strain (CS) group were subjected to this strain regimen for the entire 192 hours, cells in the Interrupted Strain (IS-48) group were strained for 48 hours followed by culture at rest for 144 hours until the 192 h time point and cells in the No Strain (NS) group were cultured at rest for the entire 192 hour period. The 48 hour time point for interrupting strain allowed for a significant and established response to strain while remaining in log phase growth prior to strain removal. For FPCL experiments, the same CS, IS-48 and NS groups were used and collected at 96 h, with an additional group, IS-24, subjected to strain for 48 hours followed by culture at rest for 24 hours and collected at 72 h. All six-well plates were incubated at 37°C with 5% CO<sub>2</sub>. The medium was changed after the first 48 hours and then every 24 hours.

### Cell proliferation

Cell counts were obtained for the CS, IS-48 and NS groups at 48, 96, 144 and 192 h. The cell monolayer was washed with PBS. The cells were detached with 950  $\mu$ l 0.05% Trypsin at 37 °C for 5 min. 50  $\mu$ l FBS was added to each well to stop the trypsin reaction. Fibroblasts were washed from the well bottom, transferred to 1.5 ml Eppendorf tubes and saved on ice. Cells numbers were counted using a hemocytometer.

### Cell orientation

To assess cell orientation in relation to strain, a Nikon TMS inverted phase contrast microscope with a Nikon Coolpix 5000 high resolution digital camera (Nikon Corp., Japan) was used to obtain digital microphotographs at 160 $\times$  magnification. Images of individual wells were taken at 24, 48, 60, 72, 84 and 96 h. Images were centered at 1.0 cm from the center of the well, the approximate location of maximal radial strain.<sup>46</sup> Images were analyzed using ImageJ software (NIH, USA). Each image was divided into four quadrants and 8 cells in each quadrant were identified. The angle of the fibroblast cell body axis in relation to the direction of radial strain was measured from 0 to 90 degrees. A total of 6 wells across two separate experiments totaling 192 cells for each group at each time point were analyzed.

## Fibroblast-populated collagen lattice (FPCL)

Collagen lattices were prepared from type I rat tail collagen per the manufacturer's directions (BD Biosciences, San Jose, CA). Trypsinized abMTFs obtained from the CS, IS-24, IS-48 and NS groups at the previously described time points were suspended in cold DMEM. 60-well tissue culture plates were precoated with 4% BSA in PBS for 24 h. For FPCL with fibroblasts cultured on the surface of the collagen gels (FPCL-S), 1.3 mL DMEM plus 0.2 mL FBS plus 0.5 mL type I collagen stock solution were mixed to yield a final concentration of 1.25 mg/mL of collagen. This collagen working solution (2 ml) was added to each well. After polymerization at 37°C, 10<sup>5</sup> abMTFs were poured on each collagen gel in 2 ml DMEM containing 10% FBS. The plates were incubated in a cell culture incubator for 2 hours until cells adhered to the gel surface. The gels were then detached from the well sidewalls by gently scoring around the circumference with a fine pipette tip. For mixed fibroblast collagen gels (FPCL-M), 10<sup>5</sup> fibroblasts in 1.3 mL DMEM suspension plus 0.2 mL FBS plus 0.5 mL type I collagen stock solution were mixed to yield a final concentration of 1.25 mg/mL of collagen. This collagen/abMTF mixture (2 ml) was then added to each well. After polymerization at 37°C, 2 mL DMEM containing 10% FBS was added to each well. The plates were incubated for 2 hours and then the gels were detached as above. After detachment, gel diameter was measured with a ruler and the gel area was calculated at designated time points.

## Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA), R version 2.10.1 (R Foundation, www.r-project.org) and SPSS (IBM, Chicago, IL, USA). One-way ANOVA with Tukey post hoc test was used to analyze cell proliferation and FPCL data. The Kolmogorov-Smirnov test for two populations was used to compare abMTF orientation distributions in relation to the strain direction. A one population Kolmogorov-Smirnov test was used to compare the NS group to a uniform distribution at each time point. Significance level was set at  $P < 0.05$  for all statistical analyses.

## Results

### Cell proliferative response to strain

Cell counts are shown in Figure 1A–E for each group measured after 48, 96, 144 and 192 hours. Continuous strain and strain interrupted after 48 hours significantly stimulated fibroblast proliferation ( $P < .05$ , one-way ANOVA) over time. Further between groups comparison confirmed the proliferative response to strain, with cell number in the CS group higher than in the NS group at all time points ( $P < .0001$  at 48 h,  $P = 0.022$  at 96 h,  $P = .0002$  at 144 h and  $P < .0001$  at 192 h, post hoc Tukey test; Fig. 1A–E). This difference increased with time, with CS cell count  $2.05 \times 10^5$  compared to  $1.45 \times 10^5$  in the NS group at 48 h and  $3.07 \times 10^5$  compared to  $1.90 \times 10^5$  at 192 h. Removing strain resulted in decreased cell proliferation. IS-48 cell number was significantly lower than the CS group at 96 h, 48 hours after strain removal ( $1.95 \times 10^5$  vs.  $2.50 \times 10^5$ ,  $P = .041$ ; Fig. 1C) and this difference persisted through 192 h ( $P = .030$  at 144 h and  $P = .019$  at 192 h; Fig. 1D–E). There was no significant difference in cell number between the IS-48 and NS groups at 96 h ( $P = .64$ ; Fig. 1C), however the IS-48 group was higher than the NS group at 144 h ( $P = .009$ ; Fig. 1D) and 192 h ( $P = .010$ ; Fig. 1E). Interestingly, at 96 h the IS-48 group cell number appeared to have decreased compared to at 48 h ( $1.95 \times 10^5$  vs.  $2.05 \times 10^5$ ; Fig. 1A), however this change was not significant ( $P = .37$ , t-test).

### Cell orientation response to strain

Cell orientation relative to the direction of strain from 0–90 degrees was measured at 24, 48, 60, 72, 84 and 96 hours. A summary of cell orientation distributions are shown in Figure 2. As expected, the NS group cells maintained a uniform distribution at all time points ( $P > 0.05$  for null hypothesis; Fig. 2A). Within 24 hours of strain application, the CS group cells had shifted significantly toward 90 degree alignment in relation to the strain force ( $P < 0.0001$ , Fig. 2B). Maximal alignment was achieved by 48 h. Following strain removal, IS-48 cells became increasingly less aligned, significant within 12 hours ( $P < 0.0001$ ; Fig. 2C). At 96 h, 48 hours after strain removal, the IS-48 cell group orientation appeared similar to the NS group, however the difference in distributions remained significant ( $P = 0.010$ ). Representative images of the fibroblasts at the 96 h time point are shown in Figure 3. The IS-48 cell orientation, 48 hours after strain removal, appears similar to the randomly oriented NS cells, while the CS cells are oriented perpendicular to the direction of strain.

### FPCL

FPCL is an accepted model of granulation tissue and wound contraction.<sup>47</sup> To determine if loss of cyclic strain impairs fibroblast function, the intrinsic kinetic activity of abMTFs in terms of ability to bind to and remodel a collagen matrix was measured and compared among all four groups. FPCL contraction was measured in both mixed (FPCL-M; Fig. 4) and surface (FPCL-S; Fig. 5) models. The majority of contraction in both models occurred within the first 24 hours, reaching maximal contraction by approximately 72 hours (Fig. 4A and 5A). In FPCL-M, the CS and IS-24 groups demonstrated faster (gel area at 12 hours,  $P < .0001$ ; Fig. 4B) and greater overall (gel area at 96 hours,  $P < .0001$ ; Fig. 4C) contraction than both the IS-48 and NS groups. FPCL-S contraction was faster in the CS compared to the NS group ( $P = .029$ ; Fig. 5B), but no difference was seen in overall contraction ( $P = .44$ , one-way ANOVA; Fig. 5C). There was no difference between the CS and IS-24 groups ( $P > 0.05$ ) or the IS-48 and NS groups ( $P > 0.05$ ) for these measures in either FPCL-M or FPCL-S.

### Discussion

We have demonstrated that cyclic strain stimulates abdominal wall fascial fibroblast proliferation and induces uniform alignment perpendicular to the direction of strain. Differences in proliferation were seen within 48 hours of strain application and fibroblast alignment began within 24 hours, reaching a maximum by 48 hours. This is consistent with previous studies finding that increased fibroblast and osteoblast proliferation occurs within 48 hours and fibroblasts, osteoblasts and endothelial cells align perpendicular to the strain force within 12 to 24 hours.<sup>23, 39, 40, 48</sup> Other studies have demonstrated that initial proliferative and mechanical cellular responses begin even earlier, with anti-apoptotic signal and MAP kinase activation occurring within minutes,<sup>49</sup> upregulation of  $\beta$  actin mRNA within one hour,<sup>31</sup> integrin and collagen upregulation within 2 hours following 3 hours of cyclic strain,<sup>31</sup> cell alignment starting within one to three hours<sup>31, 50</sup> and DNA synthesis and PCNA expression increasing within 24 hours of strain application.<sup>49, 51</sup>

There are, however, fewer studies of the effects of strain removal on cellular responses. We believe that there may be a fundamental biological mechanism activated or deactivated during laparotomy wound failure, with implications toward the techniques of abdominal wall reconstruction. We observe here that strain-induced fibroblast proliferation is lost within 48 hours of strain removal and loss of cell alignment begins within 12 hours. Neidlinger-Wilke *et al.*<sup>50</sup> found a significant shift toward perpendicular alignment of dermal fibroblasts after three hours of cyclic strain, which was lost within four hours following strain removal. Most other studies are limited to examining the effects of releasing

intrinsicly-developed static cellular tension within collagen gels. Fibroblasts seeded on the surface of or mixed into attached collagen gels will quickly reorganize and contract the collagen, developing an isometric tension. DNA synthesis, collagen production and cell proliferation is stimulated and cells develop a bipolar morphology and align uniformly.<sup>52</sup> Beginning within one to several hours after gel release, however, DNA and protein synthesis is inhibited, collagen production declines, apoptosis is induced and cell population recedes, response to growth factors diminishes and cells develop a stellate morphology with loss of cell alignment.<sup>53–56</sup> Similar results are seen in epithelial cells in released collagen gels.<sup>57</sup> Although static tensions are certainly present in the abdominal wall, it is a dynamic structure subject to significant cyclic and variable strains, and the forces to which fibroblasts in a healing laparotomy wound are subjected are likely better modeled through application of cyclic strain to cell substrate rather than intrinsic, static strain. In terms of cell proliferation and morphology, however, our results do appear consistent with those of attached static collagen gels.

We have also shown that strain-stimulated fibroblasts are able to contract a collagen matrix faster and to a greater extent than unstrained controls, and that a significant impairment of this ability occurs between 24 and 48 hours following strain removal. These findings were most prominent in the mixed FPCL (FPCL-M) model, in which no difference in the rate of or overall gel contraction was found between cells 24 hours removed from strain (IS-24) and continuously strained cells (CS), whereas 48 hours after strain removal (IS-48) this increased contraction ability was lost and there was no difference from unstrained cells (NS). In surface FPCL (FPCL-S), faster gel contraction was only observed in the cyclic strain (CS) group and no differences in overall contraction were seen. FPCL-M more closely models *in vivo* laparotomy wound healing conditions, allowing three-dimensional fibroblast-matrix interactions, which provides a more tissue-like environment.<sup>58</sup>

To our knowledge, this is the first study to examine the impact of cyclic strain removal on fibroblast contraction function in FPCL. We have previously identified deficits in the kinetic properties of fibroblasts cultured from laparotomy wound and hernia biopsies obtained from a rat model of incisional hernias. It was observed that fibroblasts cultured from normally healing laparotomy wounds caused 80% FPCL contraction over five days, while incisional hernia fibroblasts caused only 50% lattice contraction (unpublished data). Together with our observations of proliferation and orientation loss following strain removal, this suggests that a certain degree of wound tension may be necessary for optimal abMTF function.

Laparotomy wound healing is a complex process involving interplay between many different types of cells and failure with progression to hernia formation is multifactorial. Caution must be exercised in relating *in vitro* studies of wound healing to the dynamic processes of the abdominal wall in which load forces are variable and technical and patient factors play a role. However, the fibroblast is the dominant cell type during the proliferative and remodeling phases, in which new extracellular matrix including collagen is synthesized and ultimate wound strength is developed. Our findings suggest that early fascial separation and diminished wound tension may lead to loss of a key stimulatory mechanical signal for fibroblast proliferation, alignment and contraction function, resulting in the inability to heal the initial wound failure with subsequent progression to hernia formation. Thus, preventing the initial fascial separation by optimizing patient factors for wound healing and rigorous adherence to technical principles of wound closure are critical to minimizing the risk of hernia formation. Additionally, tension, to a certain degree, may be necessary to stimulate appropriate wound healing, and a truly “tension free” hernia repair may be suboptimal. Where the balance lies is a key question that has yet to be answered.

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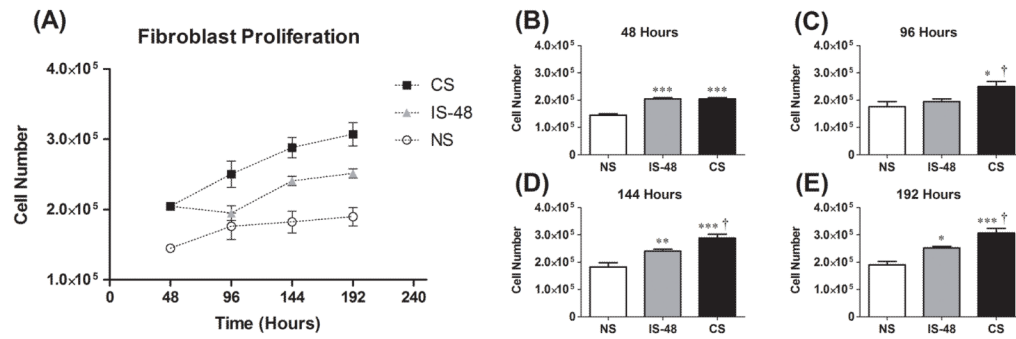
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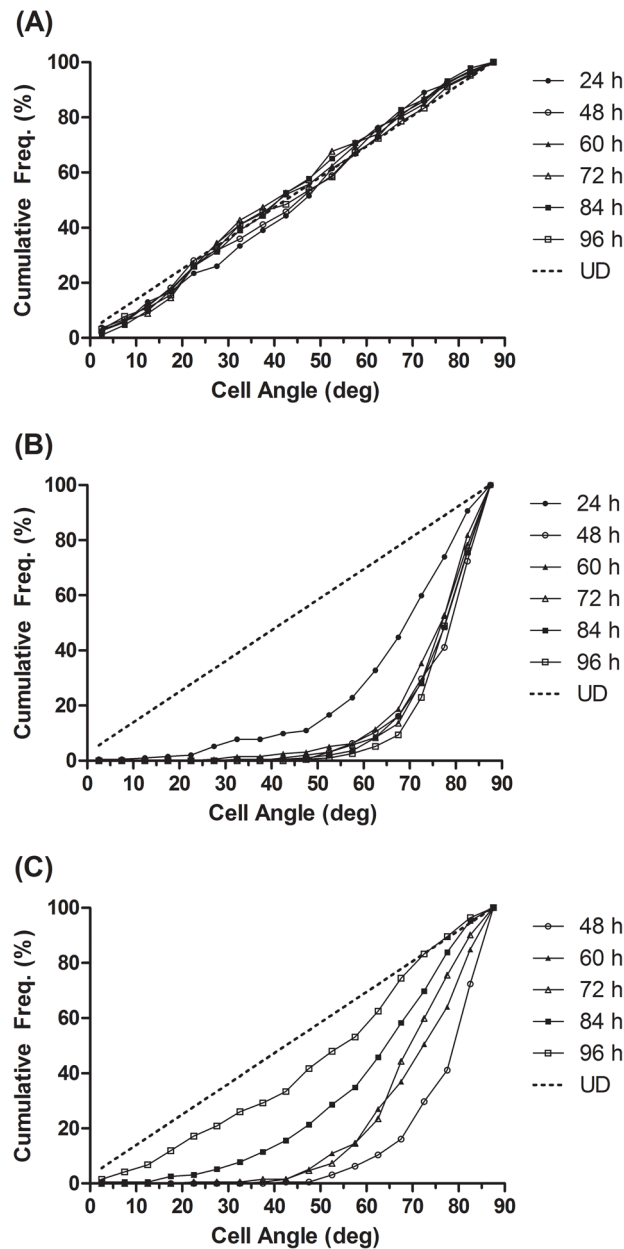


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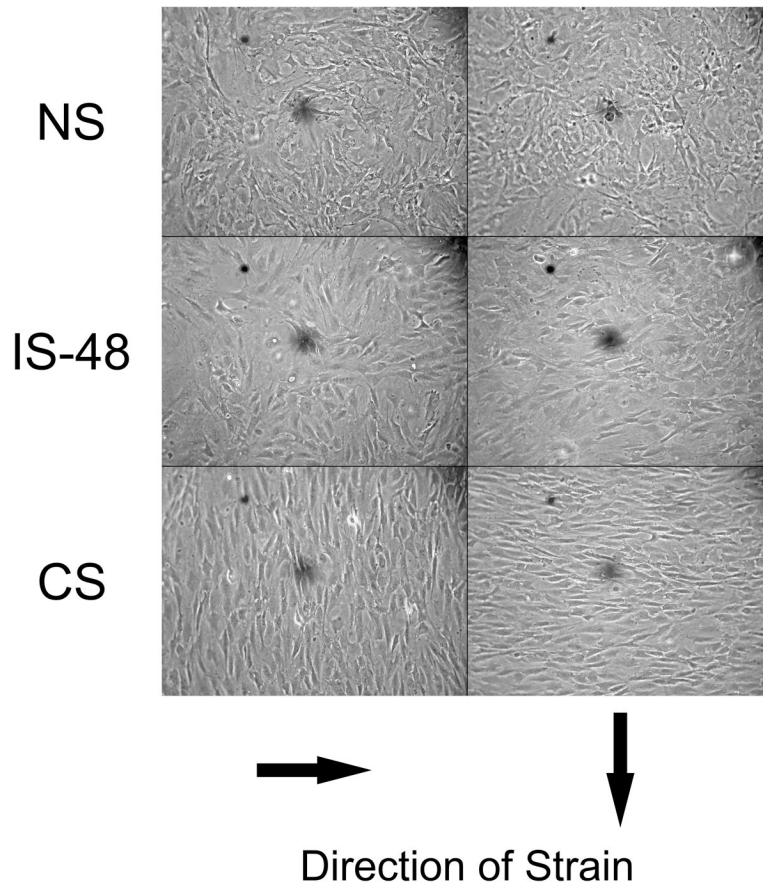


**Figure 1.**

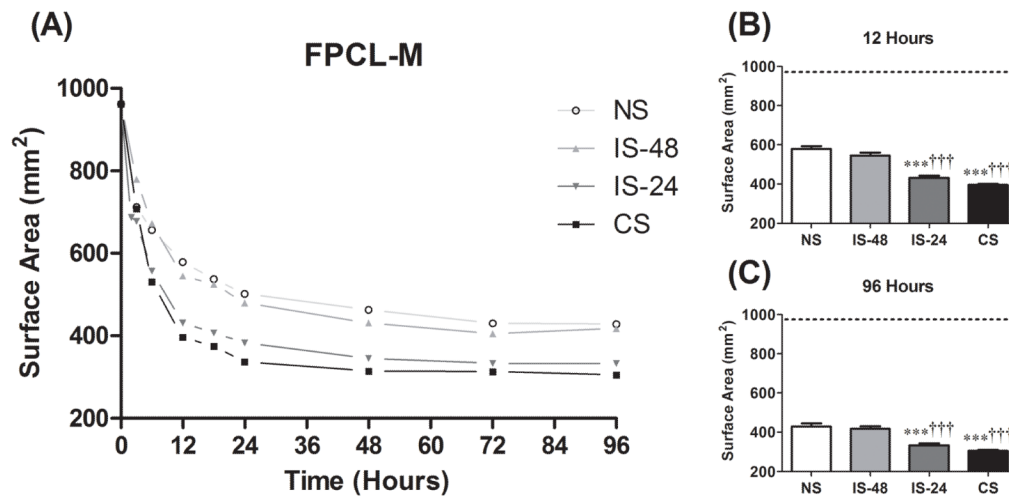
(A) Fibroblast cell number for No Strain (NS), 48 hour Interrupted Strain (IS-48) and continuous Cyclic Strain (CS) groups from 48 – 192 hours. (B)-(E) Cell number at 48 hours (B), 96 hours (C), 144 hours (D) and 192 hours (E). \*:  $P < .05$ , \*\*:  $P < .01$ , and \*\*\*:  $P < .001$  compared to NS group. †:  $P < .05$  compared to IS-48 group.



**Figure 2.** Cumulative distributions of cell orientation in relation to the direction of strain (0 to 90 degrees) at each experimental time point from 24 to 96 hours. Each point represents a 5 degree interval. UD (Uniform Distribution): Hypothetical uniform distribution of cell angles. Results from all three groups are demonstrated: (A) NS; (B) CS; (C) IS-48. Strain was removed at the 48 hour mark in the IS-48 group (C).

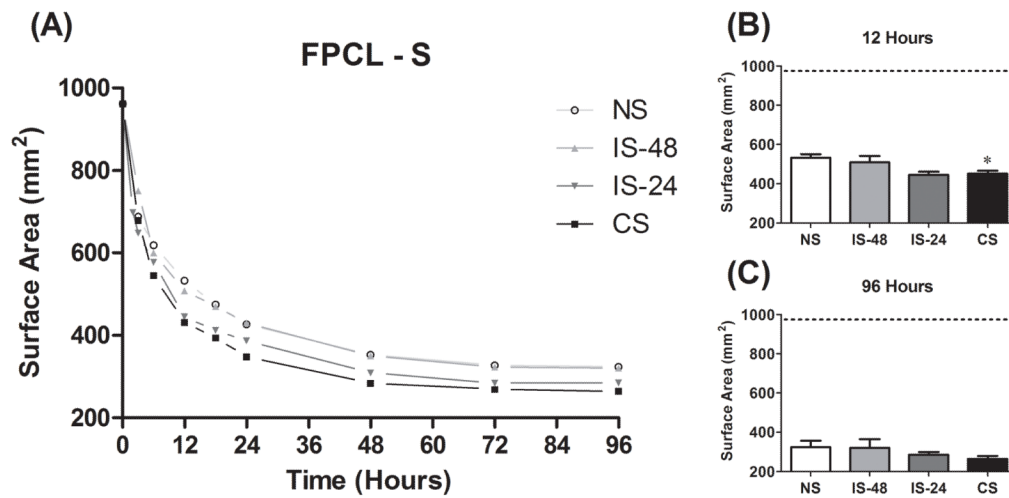


**Figure 3.** Representative images demonstrating cell orientation in relation to the direction of strain (arrows) at 96 hours in the NS, IS-48 and CS groups. This time point is 48 hours after strain removal in the IS-48 group. Images are taken at 160 $\times$  magnification.



**Figure 4.**

(A) Mixed FPCL (FPCL-M) results for change in gel surface area over time from 0 to 96 hours. The 12 hour time point (B) is used as a measure of rate of gel contraction. The 96 hour time point (C) is used as a measure of overall gel compaction. \*\*\*:  $P < .001$  compared to NS group. †††:  $P < .001$  compared to IS-48 group.



**Figure 5.**

(A) Surface FPCL (FPCL-S) results for change in gel surface area over time from 0 to 96 hours. The 12 hour time point (B) is used as a measure of rate of gel contraction. The 96 hour time point (C) is used as a measure of overall gel compaction. \*:  $P < .05$  compared to NS group.