Cyclic Strain Disrupts Endothelial Network Formation on Matrigel

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Abstract

Most forms of tissue healing depend critically on revascularisation. In soft tissues and *in vitro*, mechanical stimuli have been shown to promote vessel-forming activity. However, in bone defects, increased interfragmentary motion impairs vascular regeneration. Because these effects seem contradictory, we aimed to determine whether a range of mechanical stimuli exists in which angiogenesis is favoured. A series of cyclic strain magnitudes were applied to a Matrigel-based "tube formation" assay and the total lengths of networks formed by human microvascular endothelial cells measured at 24 hours. Network lengths were reduced at all strain levels, compared to unstretched controls. However, the levels of pro-angiogenic matrix metalloproteases-2 and -9 in the corresponding conditioned media were unchanged by strain, and vascular endothelial growth factor was uniformly elevated in stretched conditions. By repeating the assay with the addition of conditioned media from mesenchymal stem cells cultivated in similar conditions, paracrine stimuli were shown to increase network lengths, but not to alter the negative effect of cyclic stretching. Together, these results demonstrate that directly applied periodic strains can inhibit endothelial organisation *in vitro*, and suggest that this may be due to physical disruption rather than biochemical modulation. Most importantly, the results indicate that the straining of endothelial cells and their assembly into vascular-like structures must be studied simultaneously to adequately characterise the mechanical influence on vessel formation.

Keywords:

mechanical strain; vasculogenesis; tube formation; endothelial cells

Introduction

Revascularisation is a critical process in most forms of tissue regeneration. In ovine bone healing, excessive interfragmentary movement inhibits or delays revascularisation, and results in the impaired restoration of the tissue's mechanical properties (Claes et al., 2002; Lienau et al., 2005; Lienau et al., 2006). Conversely, increased blood flow and tissue stretching have been shown to stimulate angiogenesis (Brown and Hudlicka, 2003; Ichioka et al., 1997; Pietramaggiori et al., 2007), and rigid stabilisation diminishes the vascular response in fracture healing (Sarmiento et al., 1984). Therefore the mechanism and threshold of mechanical impairment during bone healing remain uncertain. Possible explanations include alterations in paracrine signalling on the one hand, and the physical disruption of newly formed vessels by excessive local stresses on the other hand (Carter et al., 1998; Rhinelander, 1974).

In vitro studies have shown that the formation of vessel-like structures by endothelial cells can be stimulated by prior mechanical conditioning (Morrow et al., 2007; Von Offenberg Sweeney et al., 2005). Additionally, angiogenic activity is enhanced by conditioned media from other cells cultured under strain (Kasper et al., 2007; Zheng et al., 2001). However, studies of the direct effects of stress or strain on vessel formation are scarce. No enhancement of endothelial sprouting is reported when cyclic strain is applied directly in gel-based assays; rather, a change in "vessel" morphology and/or orientation is observed (Joung et al., 2006; Matsumoto et al., 2007). In contrast, cyclic stretching of a confluent endothelial cell layer resulted in a strain-dependent contraction of the monolayer into a reticulated network (Shukla et al., 2004). While this suggests that increasing strain may enhance vessel formation, the mechanism of network formation differs fundamentally from those of routinely used angiogenesis / vasculogenesis assays (in which "vessel-like" structures either sprout from

clusters or assemble from separated cells in relatively low-density cultures), making direct comparison difficult. To date, such a quantitative assessment of vessel-like network formation across a range of applied cyclic strain levels has not been made in a standard assay.

To address this issue, we used a commercially available mechanical strain system for cell cultures to apply a periodic stretch to the well-established "tube formation" assay (Kubota et al., 1988). Pro-angiogenic responses were quantified by the length of tube-like networks formed by endothelial cells on Matrigel. As an initial test for a mechano-regulation mechanism for the results, vascular endothelial growth factor (VEGF) and matrix metalloproteases-2 and -9 (MMP-2 and MMP-9 – gelatinases) – all of which may stimulate and/or regulate angiogenic behaviour *in vitro* (Fang et al., 2000; Ferrara and Davis-Smyth, 1997; Jadhav et al., 2004; Schnaper et al., 1993; Zheng et al., 2001) – were quantified in the conditioned media from these cultures. Finally, because mesenchymal stem cells (MSCs) are able to enhance angiogenesis by mechanically regulated paracrine signalling (Kasper et al., 2007), we further investigated whether this could compensate for the negative effects of strain on the endothelial response.

Materials and Methods

Cell Culture

Immortalised human microvascular endothelial cells (HMEC-1) were kindly donated by Prof. G. Schönfelder (Institut für Klinische Pharmakologie und Toxikologie, Charité – Universitätsmedizin Berlin, Germany) and cultured in MCDB 131 (Gibco, Invitrogen, Karlsruhe, Germany) with 5% foetal bovine serum (FBS; Biochrom AG, Berlin, Germany), 2mM L-Glutamine (Biochrom) and 100IE/ml penicillin + 100µg/ml streptomycin

(Biochrom). During routine culture, 1µg/ml hydrocortisone (Sigma-Aldrich Chemie GmbH, Munich, Germany) was added, but was omitted in assays.

MSCs were isolated from bone marrow aspirates from patients (with informed consent) and characterised as reported previously (Kasper et al., 2007). Investigations using these cells were approved by the Ethics Commission of the Charité–Universitätsmedizin Berlin (application number EA1-072-08). The MSCs were cultivated in either RPMI-1640 (Invitrogen, Karlsruhe, Germany) with 5mM HEPES (Sigma-Aldrich, Steinheim Germany), 2mM L-glutamine (Biochrom) and 50µM beta-mercaptoethanol (Sigma-Aldrich, Munich, Germany) or Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Karlsruhe, Germany), both supplemented with 10% FBS and penicillin/streptomycin as above.

"Tube Formation" Assay with Cyclic Stretch

Each well of collagen-I-coated Bioflex 6-well culture plates (Flexcell, Dunn Labortechnik GmbH, Asbach, Germany) was coated with 500µl ice-cold Matrigel (BD Biosciences, Heidelberg, Germany), diluted to 8mg/ml with complete culture medium (to reduce viscosity). The solution was allowed to gel for 1 hour at 37°C. HMEC-1 cells were delivered onto the gels at 2×10^5/well (208 cells/mm^2) and allowed to attach for one hour before commencing the stretching regime.

Cyclic stretching was applied using the Flexercell Tension Plus FX-4000T system (Flexcell International Corp., Hillsborough, NC, USA) with 25mm BioFlex Loading Stations (Flexcell). Nominal strains ranging from 2.5–20% were employed at a frequency of 1Hz. A further set of experiments at 2.5% nominal strain used UniFlex plates (collagen-coated; Flexcell) to apply a uniaxial strain to the cultures; due to the uneven surfaces of the UniFlex membranes, 600µl of Matrigel solution was used for coatings. Experiments contained

duplicate strained and unstrained samples, with additional cell-free controls. Each strain level was tested in three separate experiments.

After 24 hours of stretching, endothelial networks were photographed in five fields per well with a 5× objective magnification. The lengths (in pixels) of cords of interconnecting cells were measured using ImageJ (version 1.39u, 2007, W.S. Rasband, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/) and totalled for each condition (+/– strain).

Analysis of Conditioned Media

At the conclusion of each experiment, a sample of conditioned medium (CM) was collected from each well and stored at –80°C and subsequently assayed for MMPs involved in angiogenesis. CM samples from each strain level, and corresponding controls, were assayed for MMP-2 and MMP-9 using gelatin zymography. 10% Novex gels (Invitrogen) were used according to manufacturer's instructions, with 15µl CM per lane. The resulting band densities were measured using ImageJ. A subset of CM samples were also assayed for cell viability using an LDH assay (LDH-Cytotoxicity Assay Kit II, BioCat, Heidelberg, Germany), according to the manufacturer's instructions.

An enzyme-linked immunosorbent assay (ELISA) was carried out to probe conditioned media from each experiment for VEGF. The assay was conducted on 200µl samples according to the manufacturer's instructions (Quantikine® Human VEGF Immunoassay, R&D Systems, Minneapolis, USA).

Supplementation with Conditioned Media from Mesenchymal Stem Cells

MSCs (passage 5, 81-year-old male patient) were cultured for three days under identical conditions to the "tube formation" assays, i.e. in Matrigel-coated BioFlex wells, with and without a nominal strain of 2.5%. Two millilitres of the conditioned medium (DMEM-based) from each sample was added to endothelial cells, seeded in 1ml of the routine MCDB 131-based medium. Controls with both types of non-conditioned medium were also tested. The "tube formation" assays then proceeded as described above.

Further, to allow comparison with previously published results (Kasper et al., 2007), conditioned media from MSCs in loaded and unloaded fibrin constructs were also tested in the same way. The MSCs were obtained from a 52-year-old female and used at passage 3. One million cells were suspended in each 700µl fibrin construct (fibrinogen and thrombin-S solutions (TISSUCOL-Kit 2,0 Immuno, Baxter, Unterschleißheim, Germany) both diluted 1/4 with culture medium) and the resulting gels sandwiched between bone spongiosa chips of 15mm diameter. These were placed in a purpose-built bioreactor (Matziolis et al., 2006) with 25ml culture medium (RPMI-1640-based) and subjected to a 1Hz cyclic load, corresponding to a 20% compression of the gel, for 3 days. Unloaded controls were cultured in otherwise identical conditions. The conditioned media were aliquoted and stored at –80°C before use in the "tube formation" assay as described above.

Validation of Strain Levels on Coated Plates

Digital image correlation was used to quantify local strains across the BioFlex membranes (Bieler et al., 2009; Boerboom et al., 2008). After Matrigel coating, a fine paint speckle pattern was applied to both gels and uncoated membranes using an airbrush. Each whole well was then digitally photographed over a range of nominal strains from 0–20%. The paint spots

were digitally correlated between relaxed and stretched images using Vic-2D software (version 4.4.1, 2006, Correlated Solutions, Inc., Columbia, SC, USA), to calculate strain levels across the flat region of the loading post. The tensions were characterised as first principal strains, averaged over the surface.

Statistics

Statistical evaluations used SPSS (version 12.0, SPSS Inc., USA). In each experiment, differences between network lengths over ten fields were tested using the Mann-Whitney U-test, with significance judged as $p \leq 0.05$. To compare results between different strain levels, total network lengths for strained samples were normalised to unstretched controls and analysed by Mann-Whitney U-tests and the Kruskal-Wallis test. Measured strain magnitudes (first principal strains) with and without gel coatings were tested for linear correlation against the corresponding nominal strain levels. Zymography results were tested for significant differences in each of the four bands (MMP-2 and -9, active and pro- forms) using paired Student's *t*-tests. Wilcoxon tests were used to compare data pooled across all strain levels, (conditioned media compared to corresponding cell-free control media, and media from stretched versus unstretched samples).

Results

Strain Level Validation

Digital image correlation confirmed that all strains were transmitted through the gels, with excellent correlation between strains on Matrigel-coated and uncoated membranes ($R^2 = 1.00$, comparing average first principal strains). While the measured strains consistently exceeded the nominal magnitudes, these also showed a highly linear correlation ($R^2 = 1.00$, on both

uncoated and coated membranes). When tested after 24 hours' stretching at 20% nominal strain, the correlation was somewhat lower for the coated membranes ($R^2 = 0.88$ *vs*. set point; $R^2 = 0.95$ *vs*. uncoated), predominantly due to the poorer transmission of strain levels above 10% (both $R^2 = 1.00$ when higher strains excluded).

Endothelial Network Formation Under Cyclic Strain

In all experiments, the application of cyclic strains at 1Hz resulted in a significantly lower HMEC-1 network length ($p < 0.03$; Figure 1), except for one instance at 10% stretch ($p = 0.08$). The inhibition tended towards a lesser severity at 2.5%, with all other strain levels comparable (variability with strain level: $p = 0.09$). The suppression of network formation was equivalent for uniaxial and biaxial strain conditions ($p = 0.10$). No preferential alignment of the cells or networks could be observed under either uniaxial or biaxial strain.

Conditioned Medium Analyses

Gelatin zymography (Figure 2a) revealed no significant differences in MMP-9 levels (for either pro- or active forms) between conditioned media (CM) from any of the HMEC-1 cultures (stretched or unstretched) and their corresponding cell-free controls ($p > 0.09$); this indicated that the predominant source of this protease was the serum in the culture medium and/or the Matrigel. Levels of both MMP-2 forms were significantly higher in CM from cell-containing cultures (mean ratio of CM : cell-free controls ± SD: 1.1±0.2 (pro), 1.2±0.3 (active); $p \leq 0.03$); however, no significant differences were detected between the various strain levels and unstretched cultures ($p \geq 0.17$).

The ELISA showed a trend of increased levels of VEGF in the media from strained HMEC-1 cultures ($p > 0.09$). The concentration did not vary significantly between strain levels ($p = 0.58$); when compared between pooled strained vs. unstrained conditions, a significant

elevation was detected (mean ratio of stretched : static = 1.2 ± 0.1, $p < 0.01$; Figure 2b). No VEGF was detected in media from Matrigel-coated wells without cells.

Furthermore, an LDH-based cytotoxicity assay on conditioned media indicated no differences in cell death between strained and unstrained cultures at any strain level (data not shown).

Network Formation with Conditioned Medium from MSCs

Under all stretched and static conditions, CM from MSCs cultured on Matrigel enhanced network formation by the endothelial cells ($p < 0.01$), as shown in Figure 3(a). This stimulation occurred despite the inhibitory effect of the (fresh) DMEM-based medium ($p = 0.04$; data not shown). Additionally, loading enhanced the pro-angiogenic stimulus from MSCs cultured in fibrin constructs ($p = 0.01$, Figure 3(b)). Despite these marked paracrine stimuli, endothelial cultures supplemented with CM from either strained or unstrained MSCs still showed significantly diminished network formation under cyclic stretching ($p < 0.01$). Stretching of MSC cultures on Matrigel had no significant paracrine effect on HMEC-1 network length ($p \geq 0.39$).

Discussion

Previous research has shown that angiogenesis can be stimulated by a certain measure of mechanical stimulation (Brown and Hudlicka, 2003; Ichioka et al., 1997; Sarmiento et al., 1984), but also that increased strain inhibits or delays this process during fracture healing (Claes et al., 2002; Lienau et al., 2005; Lienau et al., 2006). To address this apparent discrepancy, we examined endothelial structuring under direct mechanical strain.

By applying a cyclic stretch directly to Matrigel-based "tube formation" assays, we have demonstrated that a broad range of mechanical strains are able to reduce endothelial network

formation *in vitro*. This result contrasts with that of Shukla *et al.* (2004), who report an increasing degree of network formation with cyclic strain levels. However, it must be noted that, in this previous study, the networks formed by contraction from a confluent cell layer, whereas in Matrigel-based "tube formation" assays, they form by co-alignment and assembly from a relatively sparse cell population. In a confluent culture, small, stretch-induced ruptures in the cell layer or underlying extracellular matrix would progressively expand under inter-cellular (or applied) tension, promoting the contraction of cells into a reticular network (Vernon and Sage, 1995). On the other hand, disruption of the more limited adhesions within cord-like assemblies of initially separated cells could result in collapse of the structures under cell-generated tension. Likewise, increased contractile force generation within endothelial cells promotes network formation from a confluent network (Hoang et al., 2004), but inhibits it when the cells are seeded at sub-confluent density (Mavria et al., 2006). Alternatively, continuous cyclic strain may inhibit the formation of the required attachments.

The inhibition of network formation in the current study was not consistent with the slightly elevated VEGF levels in CM from strained HMEC-1 cultures, by which findings from earlier studies (Salani et al., 2000; Yahata et al., 2003; Zheng et al., 2001) would lead us to expect enhanced "tube formation" on Matrigel.

MMP-2 and MMP-9 levels were also unchanged by stretching, despite the inhibition of network formation and previous work showing their contributions to "tube formation" on Matrigel (Jadhav et al., 2004; Schnaper et al., 1993) and their mechanical regulation in endothelial cells (Haseneen et al., 2003; Magid et al., 2003; Milkiewicz et al., 2007; Shukla et al., 2004; von Offenberg Sweeney et al., 2004; Wang et al., 2003). Furthermore, MMP-2 up-regulation has been shown to mediate the pro-angiogenic effect of pre-straining endothelial cells (Von Offenberg Sweeney et al., 2005). However, in none of the previous studies

showing mechanical regulation of MMPs were the endothelial cells cultivated on Matrigel, on which Schnaper *et al.* (1993) found that HUVECs down-regulated their MMP-2 expression. The cited studies also measured responses in confluent cultures, which would perhaps better correspond to the conditions in intact vessels than the tissues into which new capillaries sprout. The levels in the serum and Matrigel may also have masked the differences in the cell-derived proteases.

Because the strain over the BioFlex / UniFlex membranes is not uniform (Bieler et al., 2009; Shukla et al., 2004; Vande Geest et al., 2004), not all cells in the culture will be subjected to identical conditions. The soluble factors measured therefore represent the average of a range of responses, which may mask subtle changes. However, any paracrine regulation would be a response to these concentrations, to which all cells are exposed. The strain level validation data also suggest that the gel may have partially separated from the membrane after being subject to maximal cyclic stretching, resulting in a reduced ability to transmit the higher levels of strain in full; of the studies conducted, though, this would have only potentially reduced strains over time in experiments conducted at the maximum strain level of 20%.

In agreement with previous reports (Ghajar et al., 2006; Gruber et al., 2005; Kasper et al., 2007), a paracrine enhancement of network assembly by CM from MSCs was confirmed. Interestingly, stretching of MSCs cultured on Matrigel did not alter this enhancement, whereas MSCs within a fibrin gel construct required mechanical stimulation (cyclic compression) to achieve this endothelial response, as previously reported (Kasper et al., 2007). This discrepancy may relate to the considerable difference in mechanical conditions between these two cases, and also, the different extracellular matrices present. It may, however, also indicate a change in the cells' ability to transduce the strain on Matrigel, e.g. due to differences in cell adhesion and its downstream signalling (Hirayama and Sumpio,

2007). This may also account for the lack of preferential alignment observed under uniaxial strain. Despite the stimulatory effects, none of the conditioned media prevented a significant reduction in network formation by stretching of the HMEC-1 cultures.

Taken together, these data demonstrate that, even in otherwise permissive or favourable conditions, the applied strain interfered with the formation and/or maintenance of the endothelial networks, suggesting a physical disruption of the process, rather than a biochemical regulation. Indeed, earlier reports (Morrow et al., 2007; Shukla et al., 2004; Von Offenberg Sweeney et al., 2005) demonstrate that the biochemical response to strain can enhance "tube formation". This concept of a physical disruption is consistent with the primarily mechanical nature of endothelial organisation on Matrigel, as evidenced by the dependence on cell-generated lines of traction in the soft matrix guiding migration and alignment (Vernon et al., 1992; Vernon and Sage, 1995), rather than transcriptional or translational changes (Zimrin et al., 1995). It is plausible that a biaxial strain might therefore over-saturate the inter-cellular tension in the matrix, although this would lead us to expect an enhancement in network formation parallel to an applied uniaxial strain, which was not apparent. In either configuration, though, the requirement for decoupling between the cytoskeleton and matrix for the cellular reorganisation (Deroanne et al., 2001) and the observation of tubular structures tethered only at their ends (Maciag et al., 1982) also suggests that the cells may be particularly susceptible to the disruption of adhesions during network formation. It is, however, possible that the "tube formation" assay thus presents an artificial sensitivity to such disruption. Assays using more natural, three-dimensional matrices will enable any such Matrigel-specific effects to be clarified, and provide further insights into both the mechanisms of mechano-regulation and the range of conditions in which angiogenesis is favoured.

The results suggest that the cause of impaired revascularisation in early stages of fracture healing seems to be a physical disruption of endothelial structure assembly, e.g. due to breakage of cell–matrix and/or cell–cell adhesions, rather than a phenotypic modulation. When compared to previously reported positive effects of pre-treatment with periodic stretching (Morrow et al., 2007; Von Offenberg Sweeney et al., 2005), the present data suggest that the characterisation of mechanical effects on angiogenic activity must address the stimulation and "vessel" formation simultaneously, rather than as isolated events, to avoid misinterpretation.

The positive effect of mechanically loaded MSCs seems to be opposed by a negative effect of strain on endothelial cell assembly. The former is a paracrine effect (Kasper et al., 2007), while the latter is rather a direct mechanical disruption. Thus, in a clinical setting, the window of mechanical stability that allows a positive paracrine stimulus while not leading to disruption of the newly organising tissue must be controlled.

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Figure Legends

Figure 1 Effect of cyclic stretching on endothelial cell network formation on Matrigel. (*a*–*c*) Representative micrographs of "tube formation" assays after 24 hours (original magnification 5×) under (*a*) static conditions and cyclic stretching: (*b*) 5% equibiaxial and (*c*) 2.5% uniaxial. The line in (*c*) indicates the direction of applied strain; no preferential alignment of cell networks was observed. Scale bar: 250μm. (*d*) Total network length measured in 10 fields over duplicate wells, normalised to static controls. The plot summarises results from three independent experiments per strain condition. ***** $p < 0.05$ (stretched *vs.* static). No significant differences were detected between the different strain levels ($p = 0.09$) or between biaxial and uniaxial conditions ($p = 0.10$).

Figure 2 Conditioned medium analyses. (*a*) Representative gelatin zymogram. Zymography showed no significant differences in MMP-9 levels between any of the samples tested ($p > 0.09$); slight, but significant, increases in both pro- and active forms of MMP-2 were detected in CM from cell-containing cultures, compared to cell-free controls ($p \leq 0.03$), but no differences were detected between CM from any stretched and unstretched cultures ($p \geq 0.17$). (*b*) VEGF levels determined by ELISA, with concentrations in CM from stretched samples expressed relative to corresponding unstretched controls. No significant differences were detected between stretched and unstretched samples ($p > 0.09$), or between the different strain levels ($p = 0.58$). However, the apparent trend of elevated VEGF levels in CM from stretched cultures was significant when data were pooled (i.e. comparing unstretched to stretched, irrespective of strain magnitude: $p < 0.01$).

Figure 3 Endothelial network formation under cyclic strain, supplemented with CM from mesenchymal stem cell (MSC) cultures. MSCs were cultured for 3 days under static and mechanically stimulated (1Hz) conditions: (*a*) on Matrigel ± 2.5% stretching in the FlexerCell

system, and (*b*) in 3D fibrin constructs ± approx. 20% compressive strain in a purpose-built bioreactor. HMEC-1 "tube formation" assays were then supplemented with either conditioned media from each of these samples, or the regular culture medium, MCDB131, and subjected to 24h of stretched (2.5%) or static conditions. Results are reported as total length of HMEC-1 networks, measured in 10 fields over duplicate wells for each combination. * $p < 0.02$, comparing HMEC-1 +/– strain; $^{\#}$ $p < 0.01$ comparing added media for identical HMEC-1 strain conditions (○ and ◊ represent outliers).

Figure 1(a)

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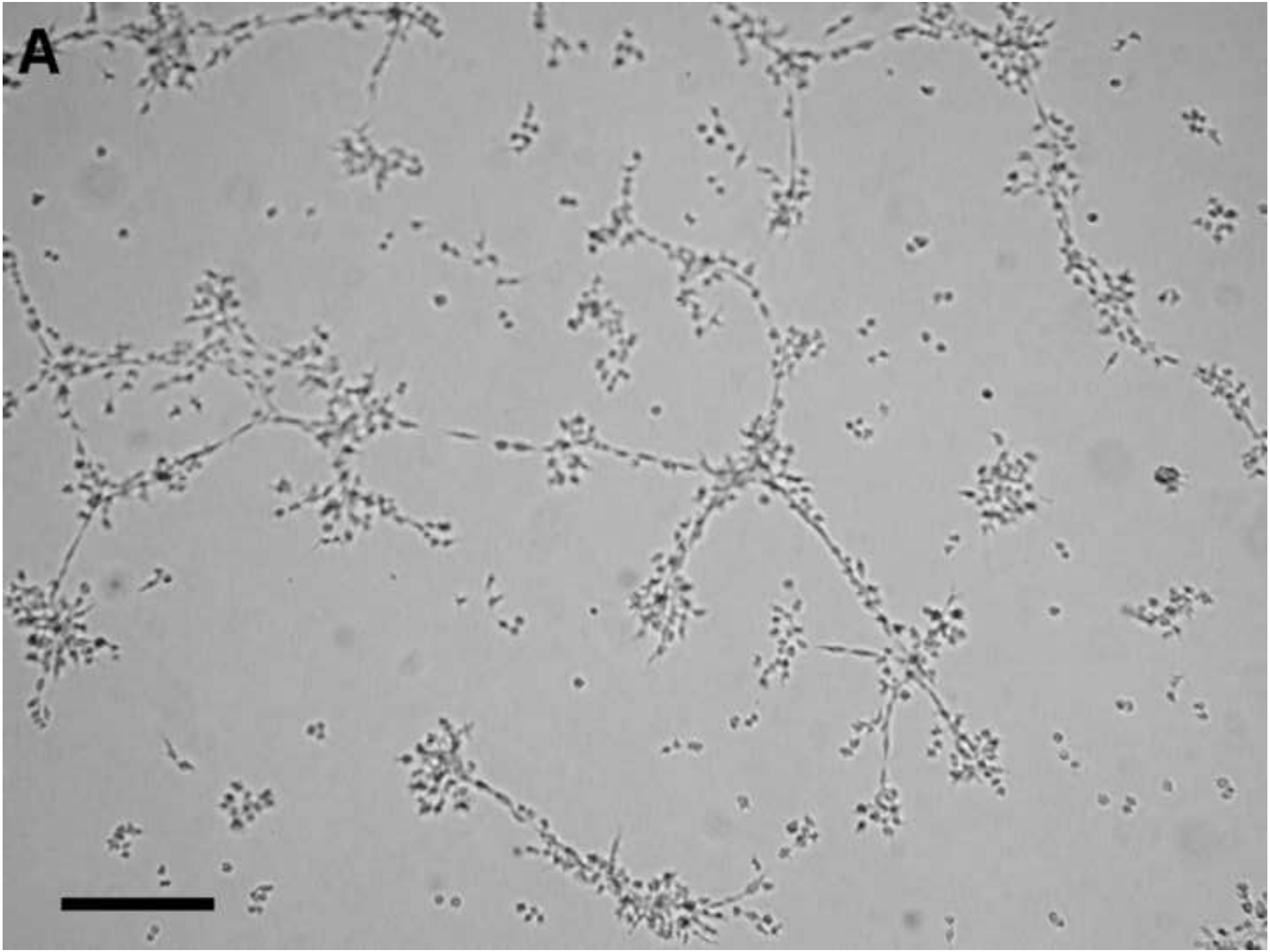

Figure 1(b)

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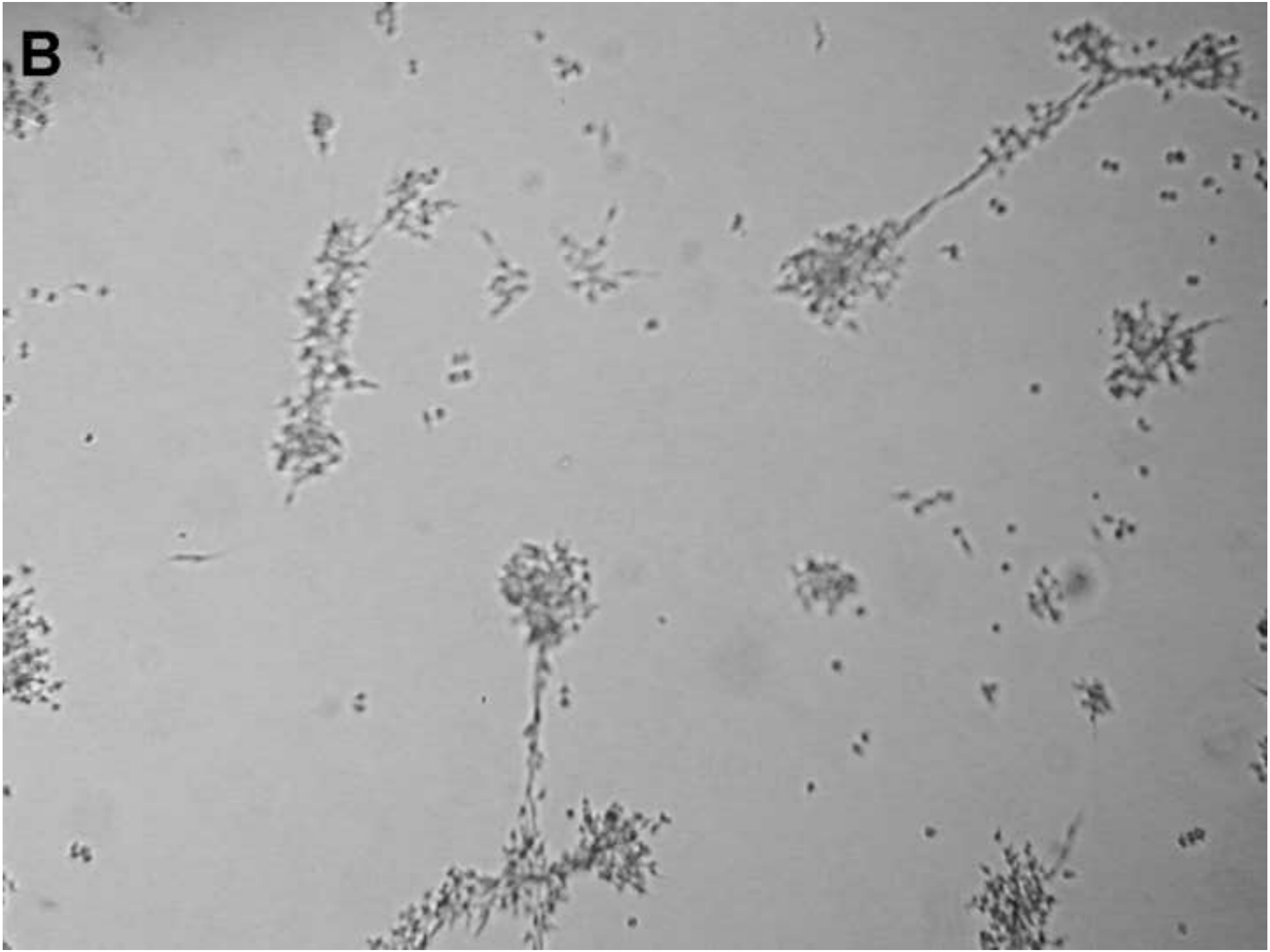

Figure 1(c)

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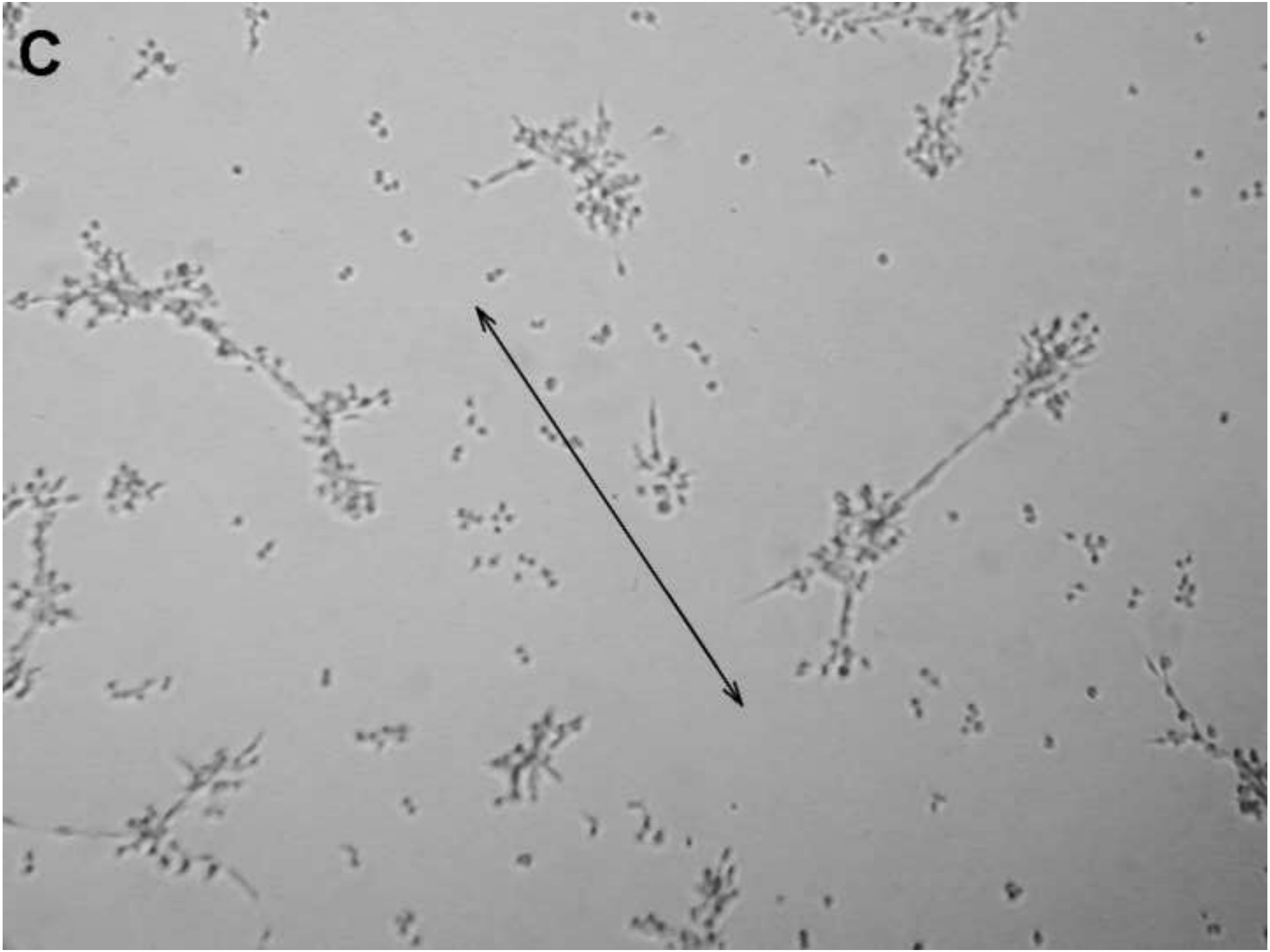
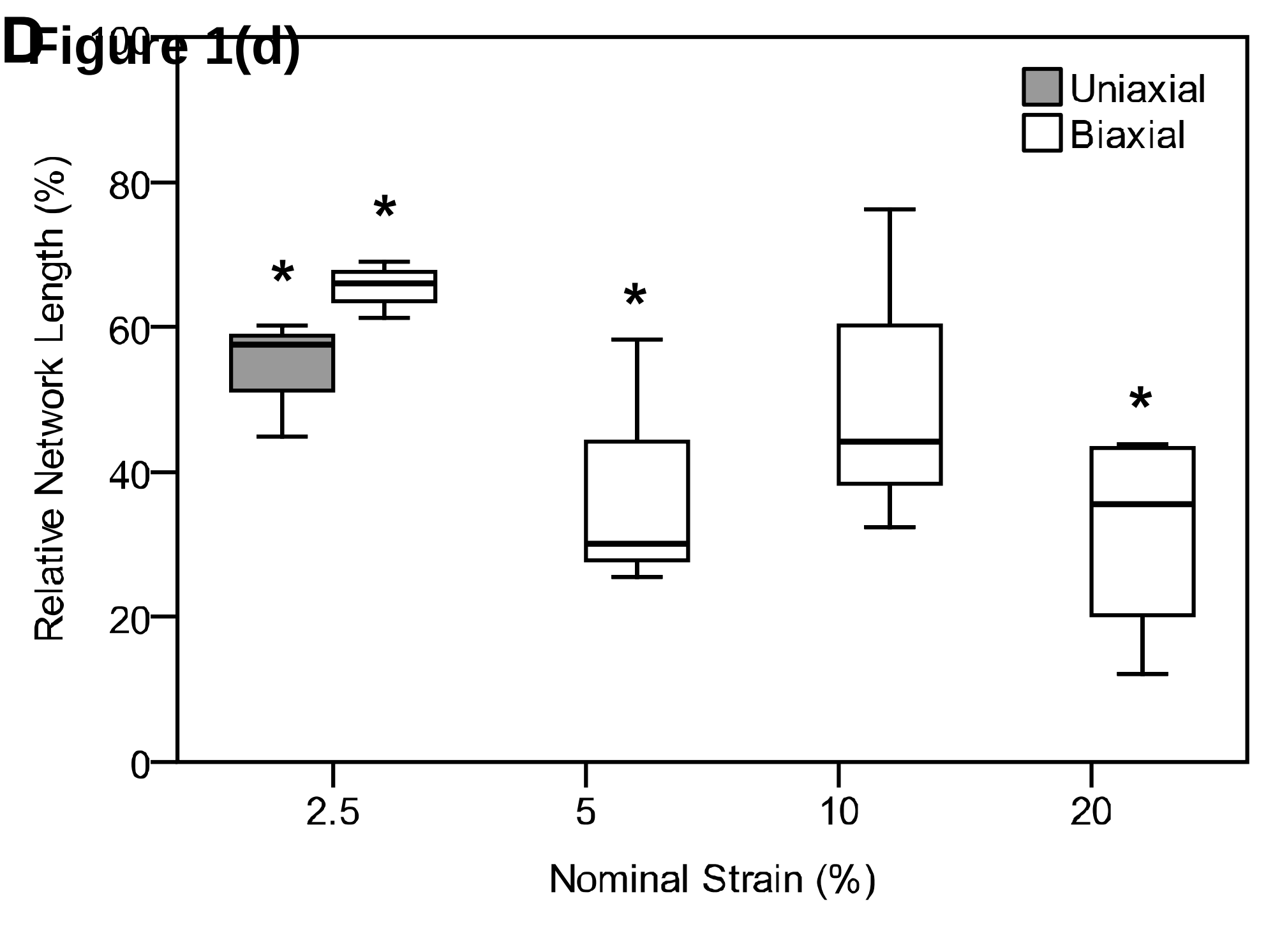


Figure 1(d)


Uniaxial
Biaxial
Relative Network Length (%)
100
80
60
40
20
0
2.5
5
10
20
Nominal Strain (%)
*
*
*
*

A

Figure 2(a)

←pro-MMP-9
←active MMP-9
←pro-MMP-2
←active MMP-2
Unstretched
20%
Unstretched
10%
Unstretched
5%
Unstr. Cell-Free
5% Cell-Free

Figure 2(b)

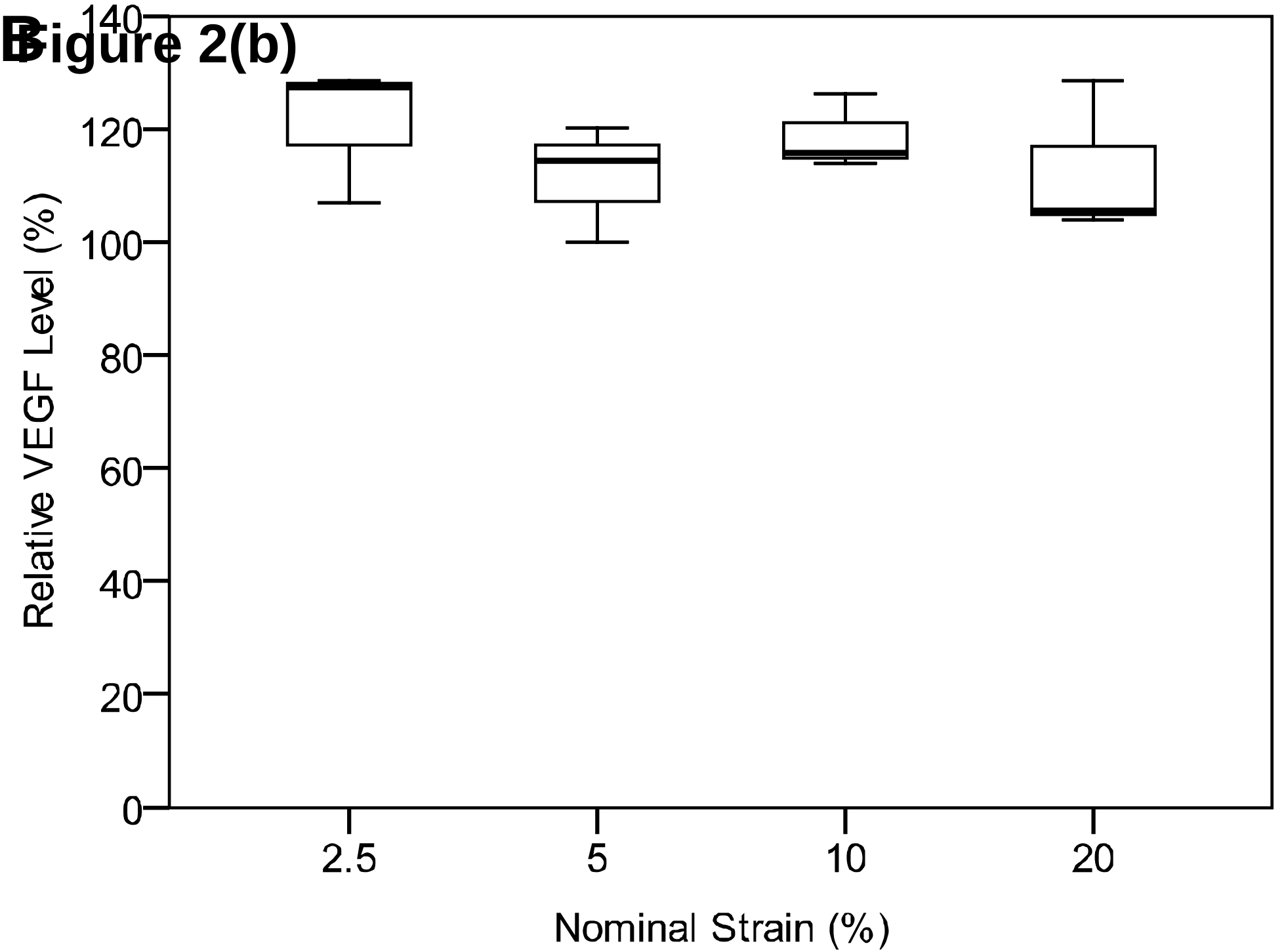
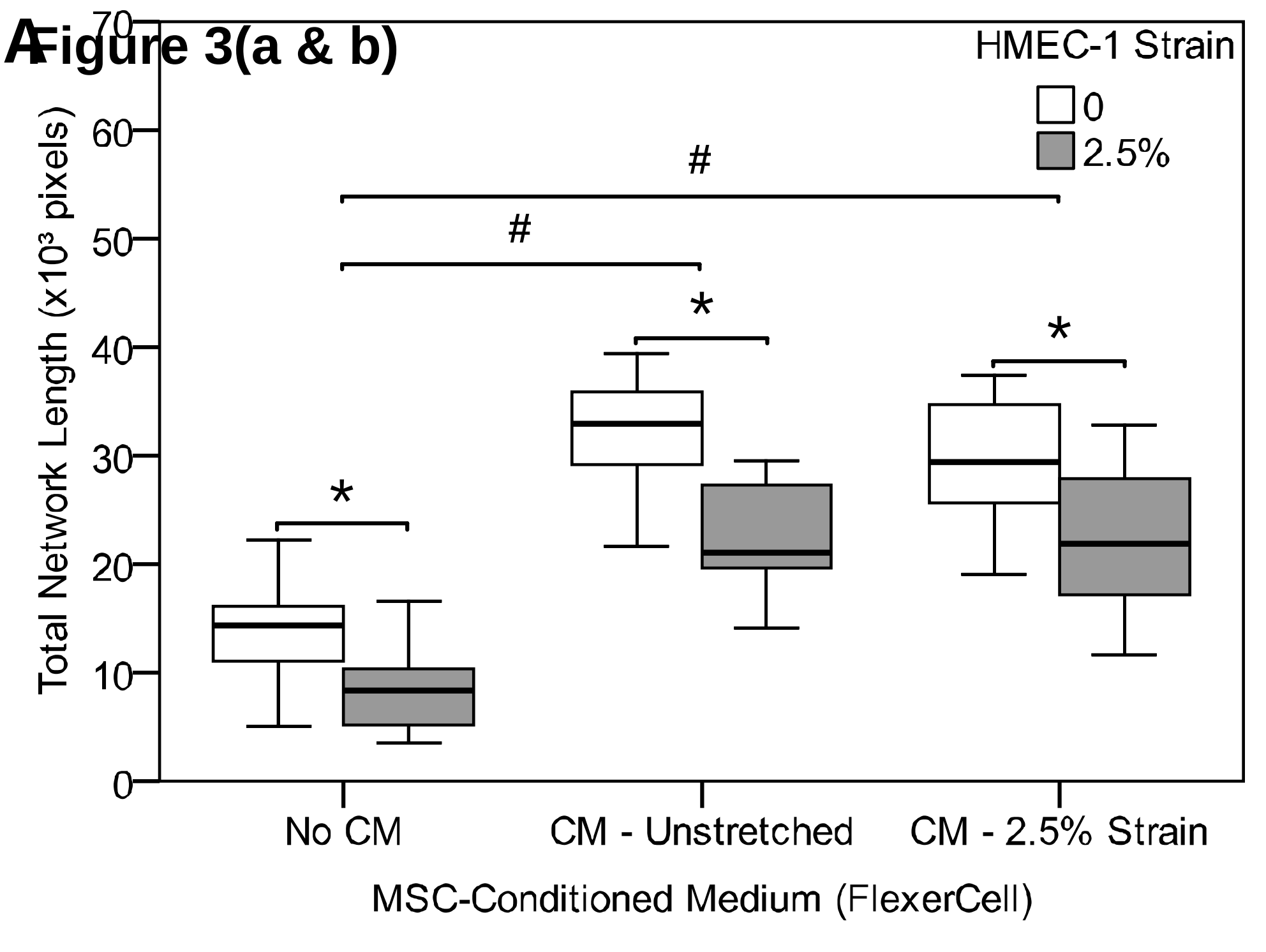
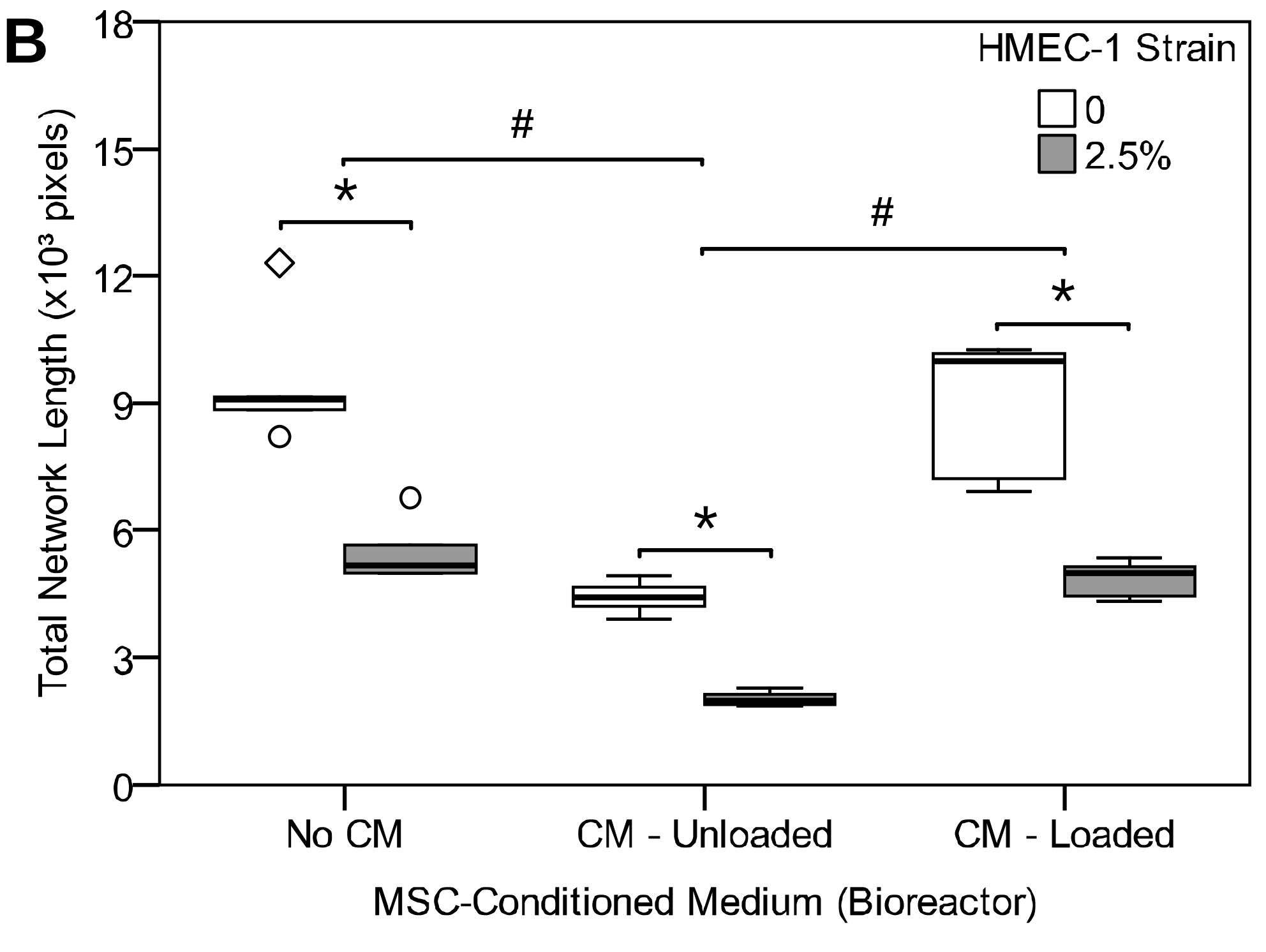

140
120
100
80
60
40
20
0
Relative VEGF Level (%)
2.5
5
10
20
Nominal Strain (%)

Figure 3(a & b)


HMEC-1 Strain
0
2.5%
Total Network Length (x10³ pixels)
70
60
50
40
30
20
10
0
#
#
*
*
*
No CM
CM - Unstretched
CM - 2.5% Strain
MSC-Conditioned Medium (FlexerCell)


B
HMEC-1 Strain
0
2.5%
Total Network Length (x10³ pixels)
18
15
12
9
6
3
0
#
*
#
*
*
No CM
CM - Unloaded
CM - Loaded
MSC-Conditioned Medium (Bioreactor)