Society For Biomaterials

Materials composed of the *Drosophila melanogaster* protein ultrabithorax are cytocompatible

Jan L. Patterson,[1*] Colette A. Abbey,[1*] Kayla J. Bayless,[1] Sarah E. Bondos[1,2]

[1]Department of Molecular and Cellular Medicine, College of Medicine, Texas A&M Health Science Center, College Station, Texas 77843-1114

[2]Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77005

Received 7 January 2013; revised 13 February 2013; accepted 15 February 2013
Published online 18 April 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.34675

Abstract: The *Drosophila melanogaster* Hox protein ultrabithorax (Ubx) has the interesting ability to hierarchically self-assemble *in vitro* into materials that have mechanical properties comparable to natural elastin. Ubx materials can be easily functionalized by gene fusion, generating potentially useful scaffolds for cell and tissue engineering. Here, we tested the cytocompatibility of fibers composed of Ubx or an mCherry-Ubx fusion protein. Fibers were cultured with three primary human cell lines derived from vasculature at low passage: umbilical vein endothelial cells, brain vascular pericytes, or aortic smooth muscle cells. No direct or indirect toxicity was observed for any cell line, in response to fibers composed of either plain Ubx or mCherry-Ubx. Cells readily adhered to Ubx fibers, and cells attached to fibers could be transferred between tissue cultures without loss of viability for at least 96 h. When attached to fibers, the morphology of the three cell lines differed somewhat, but all cells in contact with Ubx fibers exhibited a microtubular network aligned with the long axis of Ubx fibers. Thus, Ubx fibers are cytocompatible with cultured primary human vascular cells.
 J Biomed Mater Res Part A: 102A: 97–104, 2014.

Key Words: protein-based materials, biocompatible, cytocompatible, ultrabithorax, Hox

How to cite this article: Patterson JL, Abbey CA, Bayless KJ, Bondos SE. 2014. Materials composed of the *Drosophila melanogaster* protein ultrabithorax are cytocompatible. J Biomed Mater Res Part A 2014:102A:97–104.

INTRODUCTION

Ultrabithorax (Ubx) is a *Drosophila melanogaste*r Hox protein which functions *in vivo* by binding DNA[1,2] and subsequently activating or repressing transcription.[3–5] Although Ubx can bind DNA as a multimer,[1,6] large oligomers have not been observed *in vivo*. Surprisingly, Ubx hierarchically self-assembles into biomaterials.[7] Ubx first forms subnanoscale protofibrils, which align to form nanoscale fibrils.[8] Nanoscale fibrils assemble at the air–water interface to form films, which can be drawn into fibers with microscale diameters.[7]

The mechanical properties of Ubx fibers can be tuned by altering their diameter, which is in turn controlled by manipulating protein concentration and material assembly time.[9] This approach was used to generate fibers with a breaking stress and breaking strain comparable to natural elastin. Because Ubx fibers can mimic the mechanical properties of the extracellular matrix protein elastin and cells respond to the mechanical properties of their surrounding matrix,[10] Ubx materials may be useful as scaffolds for tissue engineering or cell culture studies using primary human cells.

Ubx materials have an additional useful trait: they can be readily functionalized with full-length proteins by fusing DNA encoding the functional protein to the *ubx* gene.[11] The resulting fusion gene produces a single polypeptide chain comprising both the appended protein and Ubx. Many factors that control cell behavior *in vivo* either are proteins, or bind to proteins, and thus could be easily incorporated and patterned within the Ubx scaffold to generate a rich array of functionalized materials. Proteins that direct cell behavior often require their full-length sequence and a folded structure to retain their full activity,[12] a requirement which can be easily achieved with Ubx materials.[11] Because Ubx self-assembles rapidly in mild aqueous buffers, proteins fused to Ubx are incorporated into the resulting materials in a native, fully functional state.[11] In addition, multiple Ubx fusion proteins, and consequently their corresponding

Additional Supporting Information may be found in the online version of this article.
*These authors are cofirst authors.
Correspondence to: S. E. Bondos; e-mail: sebondos@tamhsc.edu
Contract grant sponsors: NSF CAREER 1151394 and TAMHSC RDEAP to SEB
Contract grant sponsor: National Heart Lung and Blood Institute to KJB; contract grant number: HL-095786

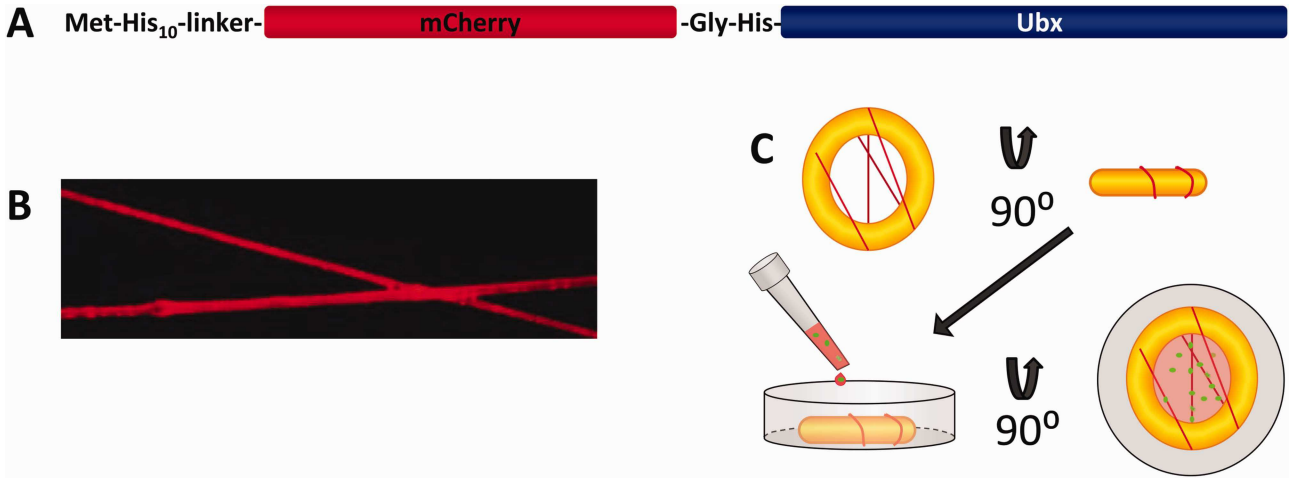


FIGURE 1. mCherry-Ubx fibers and experimental design. A, Bar graph of the sequence of the Ubx and mCherry-Ubx fusion protein. Segments listed from N- to C-terminus: methionine, a 10× His-tag, a hydrophilic linker (SerSerGlyGlyHisAspAspAspLysHis), mCherry, a two amino acid GlyHis linker, and the Ia splice variant of Ubx. B, Micrograph demonstrating that mCherry fibers are fluorescent. C, A schematic depicting how fibers were supported in culture with cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

functionalities, can be patterned within the material by manipulating the method of assembly.[11]

Together, these properties suggest Ubx materials could be a useful scaffold for cell biology or tissue engineering experiments. However, any scaffold must be both nontoxic and able to promote cell interactions in culture for an extended period of time. Unlike scaffolds created from extracellular matrix proteins such as elastin or fibronectin, Ubx materials do not exist in nature, and therefore cells have not evolved to bind these materials. Consequently, the studies presented here focused on whether Ubx materials are compatible with cells and promote the attachment of cells in culture. In addition to generating fibers with unfunctionalized Ubx, in some experiments we also used an mCherry-Ubx fusion protein[11] [Fig. 1(A,B)] to increase monomer production in *Escherichia coli* and help visualize the fibers.

Because Ubx fibers mimic the mechanical properties of elastin, which is enriched in the extracellular matrix of skin and large arterial vessels,[13,14] we initially focused this study on cells that participate in vasculogenesis. Vascularization of three-dimensional (3D) scaffolds represents a major hurdle in tissue engineering, since cell growth is limited by the availability of oxygen and nutrients.[15,16] We tested here whether Ubx-based materials are toxic to three primary human cell lines: umbilical vein endothelial cells (ECs), aortic smooth muscle cells (SMCs), or brain vascular pericytes (PCs). In general, cells can be combined with materials by direct application,[17] cell layering,[18] entrapment,[19] or cell electrospinning.[20,21] For the purpose of cytotoxicity studies, we applied cells and media directly to mCherry-Ubx or Ubx fibers. No evidence of either direct or indirect cytotoxicity was observed for any cell line cultured with Ubx fibers. Rather, all of these cell types interacted readily with mCherry-Ubx and Ubx materials. Adherent morphologies similar to those seen on tissue culture plastic were observed for cells bound to Ubx fibers, and the fibers appeared to promote cell elongation along the fiber axis. Cells attached to Ubx fibers remained attached after transfer to separate culture dishes, and remained viable and associated with those fibers for up to 96 h. Tubulin staining revealed a normal cytoskeletal arrangement, roughly aligned in the direction of the fiber axis. These observations demonstrate that Ubx fibers are not toxic to human vascular cells; rather, Ubx fibers promote sustained cellular interactions in culture.

MATERIALS AND METHODS

Production of mCherry-Ubx fibers

Fibers composed of mCherry-Ubx were used for these experiments due to the higher expression and yield of this fusion relative to plain Ubx. A His-tagged mCherry-Ubx fusion [sequence schematic in Fig. 1(A)] was expressed in Rosetta *E. coli* (Novagen) as previously described.[7,11] After induction with 1 m*M* isopropyl β-D-1-thiogalactopyranoside, cells were fermented 18 h at 25°C, harvested by centrifugation at 3500*g* for 30 min at 4°C, and stored at −20°C. Frozen cell pellets were lysed in 15 mL of lysis buffer (50 m*M* NaH_2PO_4, 5% glucose (w/v), 500 m*M* NaCl, 20 m*M* imidazole pH 8.0, 1 ethylenediaminetetraacetic acid-free protease inhibitor tablet (Roche), and 0.8 mg/L DNase I). Cell lysates were centrifuged at 34,000*g*, for 30 min at 4°C. The supernatant was loaded onto a Ni-nitrilotriacetic acid agarose resin (Qiagen) column that was previously equilibrated with 100 mL of equilibration buffer (50 m*M* NaH_2PO_4, 5% glucose (w/v), 500 m*M* NaCl, 20 m*M* imidazole, and pH 8.0). The column was washed four times with equilibration buffer containing 0, 20, and 40 m*M* imidazole (10 column volumes each) and finally washed with 5–10 column volumes of equilibration buffer containing 80 m*M* imidazole. Purified protein was eluted in 10 column volumes of elution buffer (200 m*M* imidazole dissolved in equilibration buffer),

stored at 4°C, and used within 24 h. Fibers were produced using the buffer reservoir method described by Huang et al.[11] Fibers were wrapped multiple times around a 10 μL sterile plastic inoculation loop and the handle was cut away. The resulting loop was stored in a sterile tissue culture dish until used in cell culture experiments the following morning. Although no steps were taken to sterilize the fibers, we did not observe bacterial or fungal contamination of the subsequent cell cultures.

Cell culture

Primary human umbilical vein ECs, passages 3–6 (Lonza, C2517A), were cultured on gelatin-coated (1 mg/mL) tissue culture flasks and passaged once per week. Growth media consisted of M199 medium (Gibco) containing 100 μg/mL heparin (Sigma), 0.4 mg/mL lyophilized bovine hypothalamic extract (Pel-Freeze Biologicals, Rogers, AK), 15% heat inactivated fetal bovine serum (FBS; Invitrogen), 1× antibiotic-antimycotic mix (Gibco), and 1 μg/mL gentamycin (Gibco). The same medium was used for experiments with ECs, but cells were cultured on tissue culture plastic without gelatin coating.

Primary human brain vascular PCs (ScienCell, #1200) were cultured on gelatin-coated (1 mg/mL) tissue culture flasks and passaged once per week in PC medium (ScienCell), 1× PGS (ScienCell), 10% FBS (ScienCell), 1× antibiotic-antimycotic mix, and 1 μg/mL gentamycin. The same medium was used for experiments with PCs, but cells were cultured on tissue culture plastic without gelatin coating.

Primary human aortic SMCs (Lonza, CC-2571) were cultured on tissue culture flasks and passaged once per week in SMC medium (Lonza) supplemented with 1× antibiotic/antimycotic mix (Gibco), 1 μg/mL gentamycin, 10% FBS (Invitrogen), 1× sodium pyruvate (Invitrogen), and 1× nonessential amino acid (Invitrogen).

Live/dead assays

Inoculation loops, each wrapped multiple times with a single fiber [Fig. 1(C)] were placed in separate wells in NUNC 5-well plates. Cells were cultured to confluence, trypsinized, and resuspended in growth media at approximately 80,000 cells/250 μL for ECs and SMCs, or 100,000 cells/250 μL for PCs and subsequently added to the interior of the inoculation loops. After incubation for either 24 or 48 h, the media was removed and the wells were rinsed three times with phosphate-buffered saline (PBS) containing 0.05 m*M* Mn^{2+} and 0.5 m*M* Mg^{2+} followed by the addition of 2 μ*M* calcein acetoxymethylester and 4 μ*M* ethidium homodimer-1 (Live/dead viability/cytotoxicity kit; Molecular Probes). The live/dead reagents were incubated with cells for 30 min at room temperature in the dark, prior to imaging with an Olympus CKX41 microscope equipped with a Q color 3 Olympus camera.

Cell transfer experiments

ECs and SMCs were resuspended in growth media at 80,000 cells/250 μL and PCs were resuspended at 75,000 cells/250 μL. These cells were added to 5-well plates (NUNC) containing inoculating loops wrapped with Ubx fibers and incubated at 37°C with 5% CO_2. After 24 h, inoculating loops and fibers were transferred to a new dish containing fresh growth media without cells. After an additional 24–96 h in culture, the cells that transferred attached to the fibers were subjected to live/dead assays as described above.

Immunostaining experiments

Cells were incubated with Ubx fibers wrapped around inoculation loops as described above and cultured for 24 or 48 h. A freshly made 6% paraformaldehyde stock in PBS (500 μL) was added to the existing culture media at a final concentration of 4%, and samples were fixed overnight. Samples were washed twice for 15 min with 25 m*M* Tris, 200 m*M* glycine solution. Each sample was permeabilized with 500 μL of 0.5% Triton X-100 solution in PBS for 20 min. Wells were aspirated and blocked overnight in 500 μL 0.1% Triton X-100, 1% BSA, 0.2% sodium azide, and 5% goat serum at 4°C. Primary antibodies raised against α-tubulin (Sigma T6199) were diluted 1:50 in blocking solution and incubated in the wells for 3 h. Loops were washed three times for 10 min each in 0.1% Triton X-100 in PBS (500 μL) and incubated with (1:200 dilution) goat anti-mouse Alexa 488 conjugated secondary antibodies (Molecular Probes) in blocking solution for 1 h. Loops were washed three times in 0.1% Triton X-100 in PBS (500 μL), counterstained with 10 μ*M* 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes), placed on a 22 mm × 55 mm coverslip and imaged immediately using confocal microscopy on a Nikon Eclipse Ti equipped with NIS Elements AR 4.10.01 software.

RESULTS AND DISCUSSION

Ubx fibers are compatible with primary human ECs in culture

Because Ubx materials have properties that could be useful in tissue engineering studies,[9,11] we tested whether these materials are directly and indirectly compatible with cells in culture. For these experiments, we tested Ubx fibers, which allow simultaneous comparison between cells growing on Ubx materials and cells growing on tissue culture plastic, thus providing an internal negative control in each experimental replicate. The goal is to determine whether Ubx fibers are either indirectly or directly toxic to cells in culture.

For live/dead assays, we used fibers composed of an mCherry-Ubx fusion protein which are fluorescent and have a similar absorption–emission as free mCherry in solution [Fig. 1(A,B)].[11] By including mCherry, we could visualize the fibers in the assay, and thus more easily determine whether cells were in contact with Ubx fibers. mCherry-Ubx is also produced at higher yields than Ubx in *E. coli* (data not shown), making these fibers more efficient to generate. Because mCherry is a small structured protein and Ubx is a much larger protein with long unstructured regions,[1] it is unlikely that mCherry can completely mask Ubx from contact with the cells.

Initial studies were conducted with human umbilical vein ECs. mCherry-Ubx fibers were wrapped around inoculation loops and placed in culture before adding a nearly confluent layer of ECs to allow abundant cell–fiber contacts

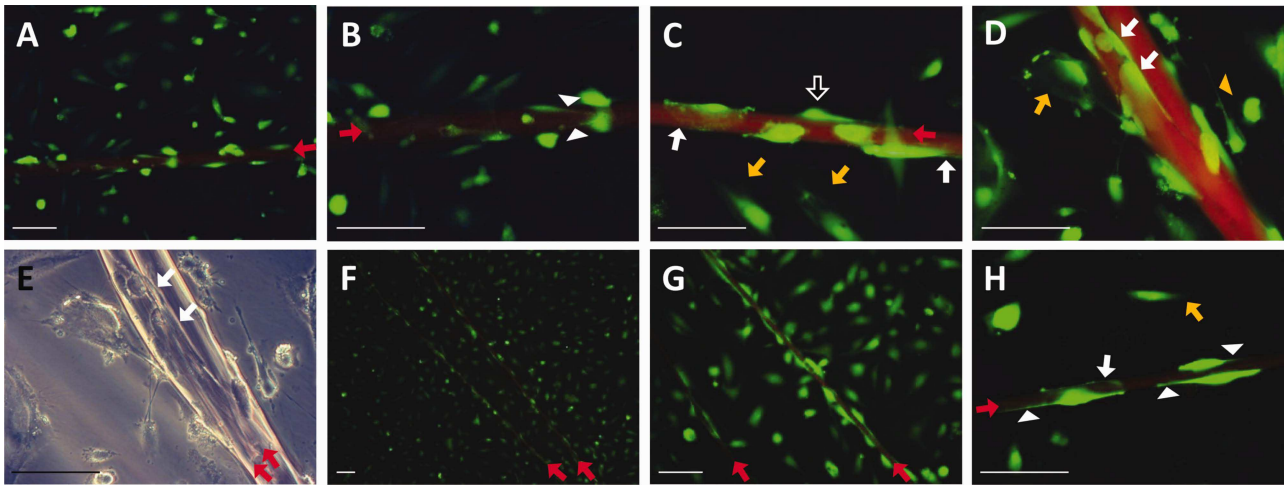


FIGURE 2. Ubx fibers are not toxic to endothelial cells and are capable of supporting normal cell morphologies. Live/dead assays of cells incubated on or in the presence of fibers for either 24 h (A–E) or 48 h (F–H). mCherry-Ubx fibers are marked by red arrows. A, Live/dead assays reveal no evidence of dead (red) cells, and thus no cytotoxicity either for cells cultured in the presence of mCherry-Ubx fibers or for cells in contact with fibers. B, Long processes are generated by cells growing on fibers (white arrowheads). C, Spread morphologies are also observed for cells grown both on (white arrows) and off (yellow arrows) fibers. An EC attached to the side of a fiber is marked by a black arrow. D, Fluorescence microscopy and E) phase microscopy of cells (white arrows) growing in the crevice between two partially fused fibers. Processes are also occasionally observed for cells growing on tissue culture plastic (orange arrowhead in panel D). ECs are also able to use lamellipodia to wrap around mCherry-Ubx fibers (bottom white arrow in D and E). F, After 48 h in culture with Ubx fibers, a greater density of cells was observed in contact with the fiber. G and H, After 48 h, fewer processes and more spread out cells are observed both on fibers and on tissue culture plastic. Scale bar equals 100 μm in all panels. Viability of ECs on fibers was 99%, and for ECs on tissue culture plastic was 98%. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

[Fig. 1(C)]. Cell viability experiments were conducted at 24 h [Fig. 2(A–E)] and 48 h [Fig. 2(F–H)]. There was no evidence of contact inhibition for cells growing adjacent to fibers, and cells that were exposed to but not touching fibers (indirect contact) as well as cells in direct contact with fibers were viable, as evidenced by fluorescent (green) signal [Fig. 2(A)]. No evidence of cytotoxicity was observed after either 24 or 48 h of culture. PCs were 99% viable on mCherry-Ubx fibers, similar to the 97% PC viability on tissue culture plastic. This lack of apparent toxicity was not due to an inability of EthD-1, the "dead" stain, to become activated, since toxicity was observed after a 10 min exposure of cells to ethanol (data not shown) and after a 45 min exposure to room air (Supporting Information Fig. 1). These data demonstrate that Ubx fibers do not leach toxic substances, since cells growing on the tissue culture plastic survive, remain adherent, spread, and show no signs of toxicity (Fig. 2).

ECs readily attach to mCherry-Ubx fibers. Because cells are observed binding to the sides of the fiber, they cannot simply be resting on top of fibers with little interaction [Fig. 2(C) black arrow]. In addition, cells appeared to elongate within crevices between two partially fused fibers [Fig. 2(D,E) white arrows], a situation that may maximize cell–fiber interactions. After 48 h in culture, a high density of cells was observed in contact with mCherry-Ubx fibers [Fig. 2(F,G)]. Cells attached to fibers and appeared to spread (white arrows) and extend (white arrowheads) along the length of the fiber [Fig. 2(B,C,H)]. Cells cultured in monolayers also had spread [Fig. 2(C,H) orange arrows] and extended [Fig. 2(D) orange arrowheads] morphologies. The similarity in cell morphologies both on and off fibers demonstrates Ubx fibers do not negatively affect cell behavior.

Ubx fibers are compatible with primary human SMCs in culture

As for ECs, mCherry-Ubx fibers did not compromise human aortic SMC viability when tested with live/dead viability dyes (Fig. 3). No cell death was observed for any SMCs cultured with Ubx fibers, regardless of whether the cells were in direct or indirect contact with fibers for 24 h [Fig. 3(A–G)] or 48 h [Fig. 3(H–J)]. SMC viability was >99% on mCherry-Ubx fibers and 97% on tissue culture plastic. The morphology of cells enveloping the fiber is adherent [Fig. 3(B,J) white arrows]. SMCs also extend processes that vary considerably in length and appearance [Fig. 3(B,C,G,I) white arrowheads]. Long processes that occur on fibers were consistently oriented along the axis of mCherry-Ubx fibers [Fig. 3(C,D,I) white arrowheads] and sometimes connected multiple SMCs on a single fiber [Fig. 3(E,G) white arrowheads]. Both long (unoriented) and short extensions were also observed for SMCs grown on tissue culture plastic [Fig. 3(B,F) orange arrowheads]. Importantly, toxicity was also not observed in these elongated cells nor in SMCs attached to tissue culture plastic near fibers.

In addition, SMC morphology was not altered in cell monolayers bridging mCherry-Ubx fibers and the tissue culture plastic [Fig. 3(B,G)]. In several cases, SMCs attached to fibers above the monolayer [Fig. 3(E,I)]. This arrangement is likely due to the ring detaching from the bottom of the dish during the live/dead staining process. Interestingly, the SMCs that were attached to fibers and suspended above the

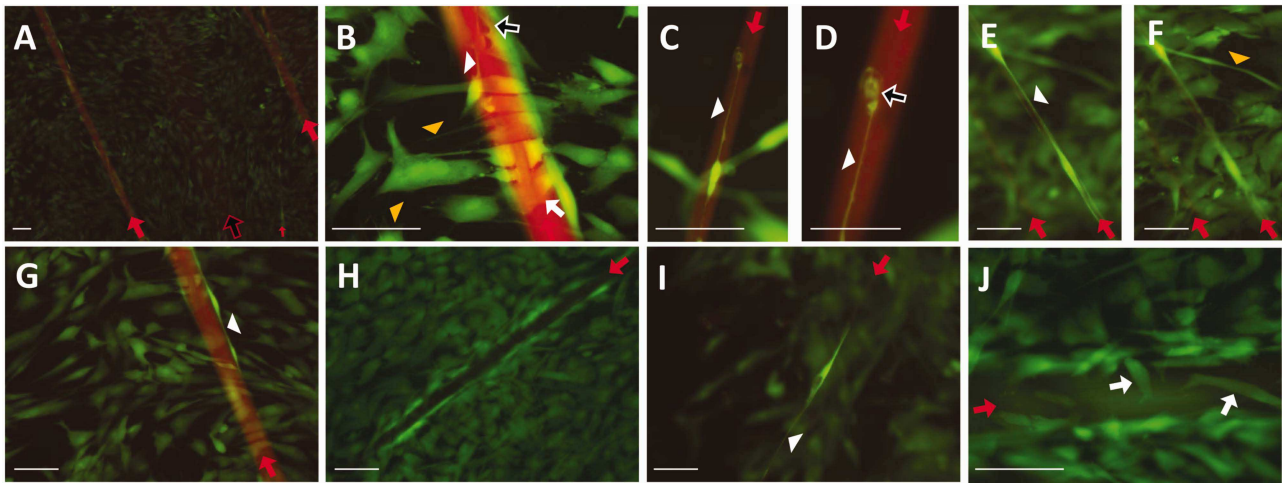


FIGURE 3. mCherry-Ubx fibers are not toxic to smooth muscle cells. Fibers were incubated with cells for either 24 h (A–G) or 48 h (H–J) before live/dead staining. mCherry-Ubx fibers are marked by red arrows. A, After 24 h in culture with SMCs, no cytotoxicity from mCherry-Ubx fibers was observed. An out-of-focus fiber is marked by a black arrow with red outline. B, SMCs have similar morphologies on or off the fibers: spread morphologies (white arrow), scalloped edges (black arrow), and thin processes anchoring the cells (white arrowhead). C and D, When cells form projections along the axis (white arrowheads), these projections can become extremely long and may terminate on the fiber (panel D black arrow) or in contact with neighboring cells (E–G). F, A different focal plane of the image in panel (E), showing that cells with long projections (yellow arrowheads) were also observed on tissue culture plastic. H, Fibers are still not toxic to cells after 48 h of culture. However, SMCs do not cluster around fibers to the extent observed for ECs and pericytes. I, Greatly elongated cells bound to the fiber are still observed after 48 h incubation. J, Cells interact normally when crossing fibers, rather than aligning with them. Scale bars equal 100 μm in all panels except panel 3D, in which the scale bar equals 50 μm. Viability of SMCs on fibers was >99%, and for SMCs on tissue culture plastic was 97%. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cell monolayer were also viable [Fig. 3(C,E,I)]. Such cells, supported only by fibers, were observed both at 24 and 48 h of culture. Thus, in all conditions tested, mCherry-Ubx fibers are not toxic to SMCs.

Ubx fibers are compatible with primary human PCs in culture

We next tested whether human brain vascular PCs were compatible with mCherry-Ubx fibers. All PCs that were observed after 24 and 48 h were alive and exhibited no evidence of cytotoxicity, either in direct or indirect contact with fibers. PCs appear to readily interact with fibers and exhibit little toxicity, indicated by the predominant green fluorescence following live/dead staining of PCs that nearly or entirely coated mCherry-Ubx fibers [Fig. 4(all panels)]. PCs that grew on mCherry-Ubx fibers and tissue culture plastic were 97% viable. Cells not only attached readily to mCherry-Ubx fibers, but they also appeared to increase in density adjacent to mCherry-Ubx fibers [Fig. 4(A)]. A confluent PC monolayer can also form beneath mCherry-Ubx fibers [Fig. 4(B)]. In areas where abundant PCs are present, processes extended around the upper [Fig. 4(C)] and lower [Fig. 4(D)] surfaces of the mCherry-Ubx fiber in a manner similar to envelopment of capillaries *in vivo*.[22] After 48 h of culture, low power images showed that PCs remained associated with fibers at significantly higher densities than on tissue culture plastic [Fig. 4(E,F)]. PCs were consistently observed on or in a network between fibers at 48 h [Fig. 4(G–I)]. These networks were observed on tissue culture plastic [Fig. 4(B)], on mCherry-Ubx fibers [Fig. 4(D,F–I)], and between cells attached to different mCherry-Ubx fibers [Fig. 4(F–I)]. By 48 h, cells were often able to fill in the Y-shaped gaps between fused fibers [Fig. 4(G)] or partially fill in the space between parallel fibers [Fig. 4(H,I)]. As with SMCs, we observed PCs on fibers suspended above the monolayer [Fig. 4(F)]. Surprisingly, suspended PCs can extend processes connecting cells attached to nearby fibers, even without tissue culture plastic to support these processes [Fig. 4(F, black arrow)]. These results demonstrate that culturing PCs on Ubx materials does not alter either the shape of the cells or their ability to form processes between cells.

Cytoskeletal alignment with fibers reflects cell–fiber interactions

To test whether cells have a normal cytoskeletal architecture when associated with Ubx fibers, we stained for tubulin using indirect immunofluorescence. Because mCherry fluorescence from the fibers is particularly robust, we used plain Ubx fibers for these assays to avoid masking the immunofluorescence signal. Samples were also stained with DAPI to visualize cell nuclei. Filamentous networks of tubulin were detected in all cells associated with Ubx fibers. ECs [Fig. 5(A,B)], SMCs [Fig. 5(C,D)], and PCs [Fig. 5(E,F)] remained associated with fibers throughout the staining process and microtubule networks generally aligned with the Ubx fiber axis [Fig. 5(B) black arrow]. Since cells normally respond to the geometry of the materials upon which they grow,[22–26] it is reasonable to expect the shape of Ubx fibers to influence the configuration of the cytoskeleton. This observable cytoskeletal organization provides additional evidence that all cell types tested can bind and

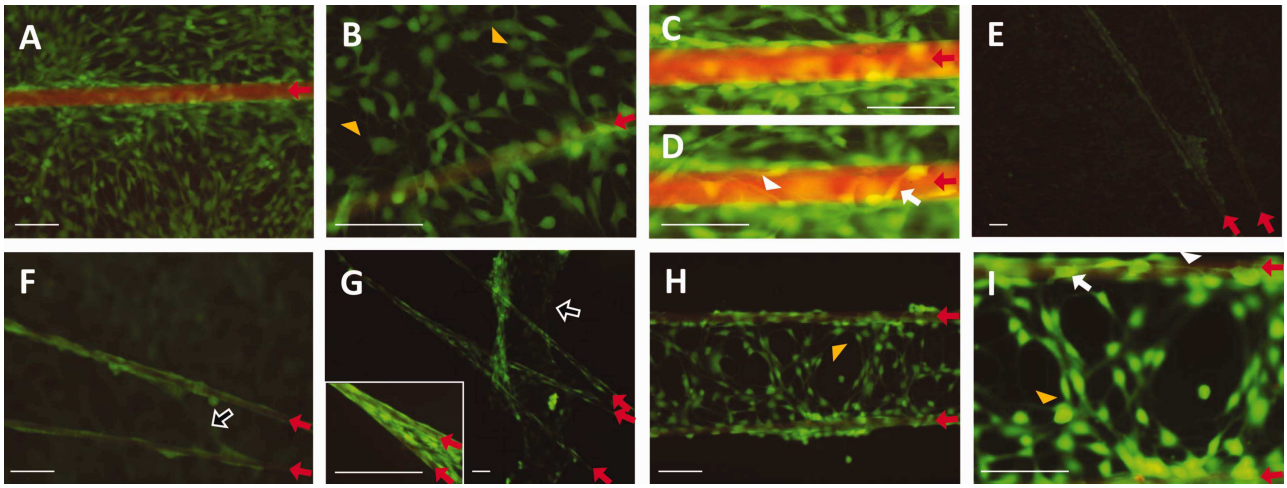


FIGURE 4. Human brain vascular pericytes strongly interact with mCherry-Ubx fibers and show no signs of toxicity. Fibers are incubated with cells for either 24 h (A–D) or 48 h (E–I). mCherry-Ubx fibers are marked by red arrows. A, mCherry-Ubx fibers are not toxic to pericytes, either in direct or indirect contact. After 24 h, cells are already beginning to concentrate on the fiber surface. B, Pericytes create networks of long processes (yellow arrowheads), including cells both on and off the fiber. C and D, Two focal planes, showing cells interacting with the sides (C) and top (D) of mCherry-Ubx fibers. Both thin processes (white arrowhead) and wider cell extensions (white arrow) are observed. E, After 48 h, cells are also more densely distributed at the surface of the fiber, an indication of good cell–fiber interactions. F, Cells on nearby fibers have also created a long process to bridge the two fibers (black arrow), even when the fibers are suspended in media well above the floor of the culture dish. G–I, Cells can use these bridges to fill in the area between the fibers. Even though cells are plated at even densities as in panel (A), cells vacate nearby areas to cluster around fibers. The fiber–cell and cell–cell connections are strong enough to lift a layer of pericytes off the tissue-culture plastic as a sheet (G, black arrow). Pericytes completely coat fibers and will begin to fill in the area between nearby fibers. A portion of panel (G) is magnified in the inset. Scale bar equals 100 μm in all panels. Viability of PCs on both fibers and tissue culture plastic was 97%. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

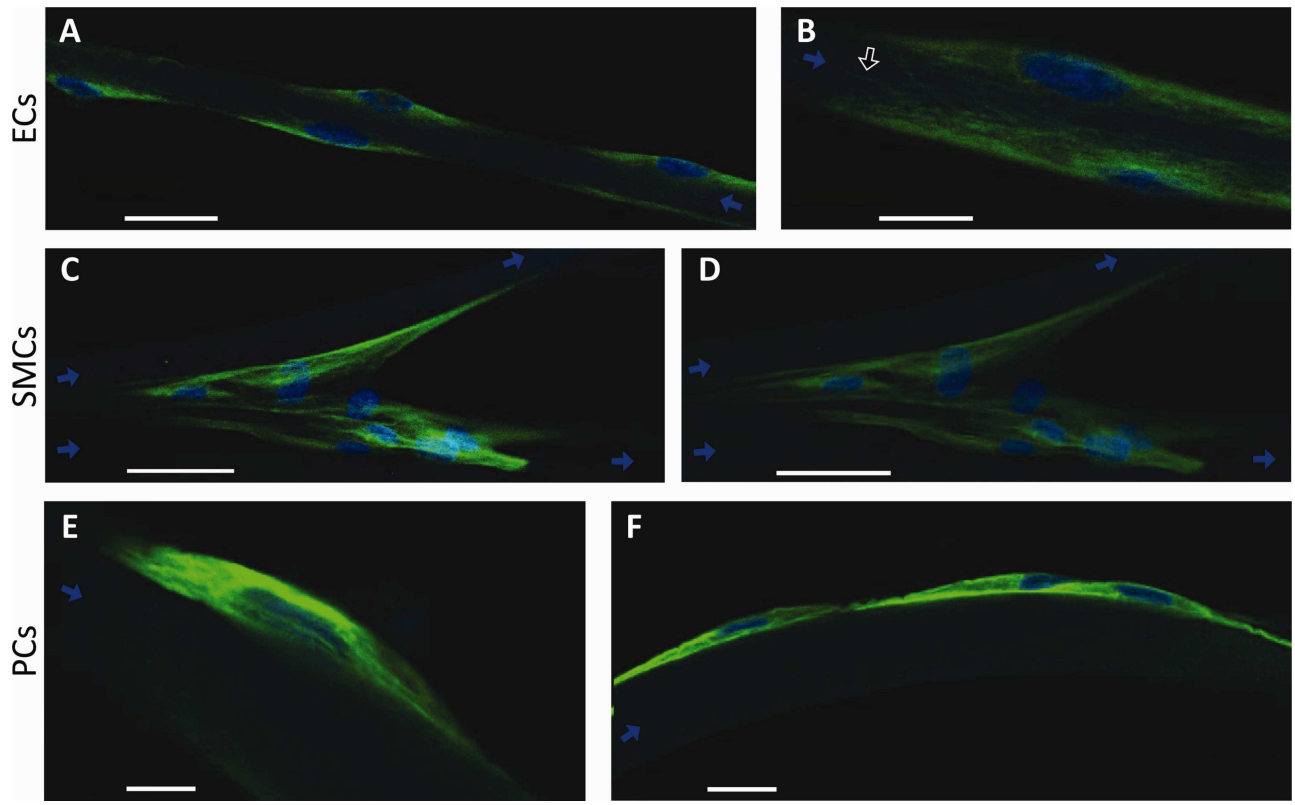


FIGURE 5. Ubx fiber interaction induces cytoskeletal alignment. Ubx fibers, lightly stained by DAPI, are marked with blue arrows. ECs (A and B) SMCs (C and D) and PCs (E and F) growing on plain Ubx fibers were immunostained for tubulin (green) and stained with DAPI (blue). In all cases, the cytoskeletons are normal and roughly aligned with Ubx fibers. Scale bars: (A) 20 μm; (B, E, and F) 10 μm; (C and D) 50 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

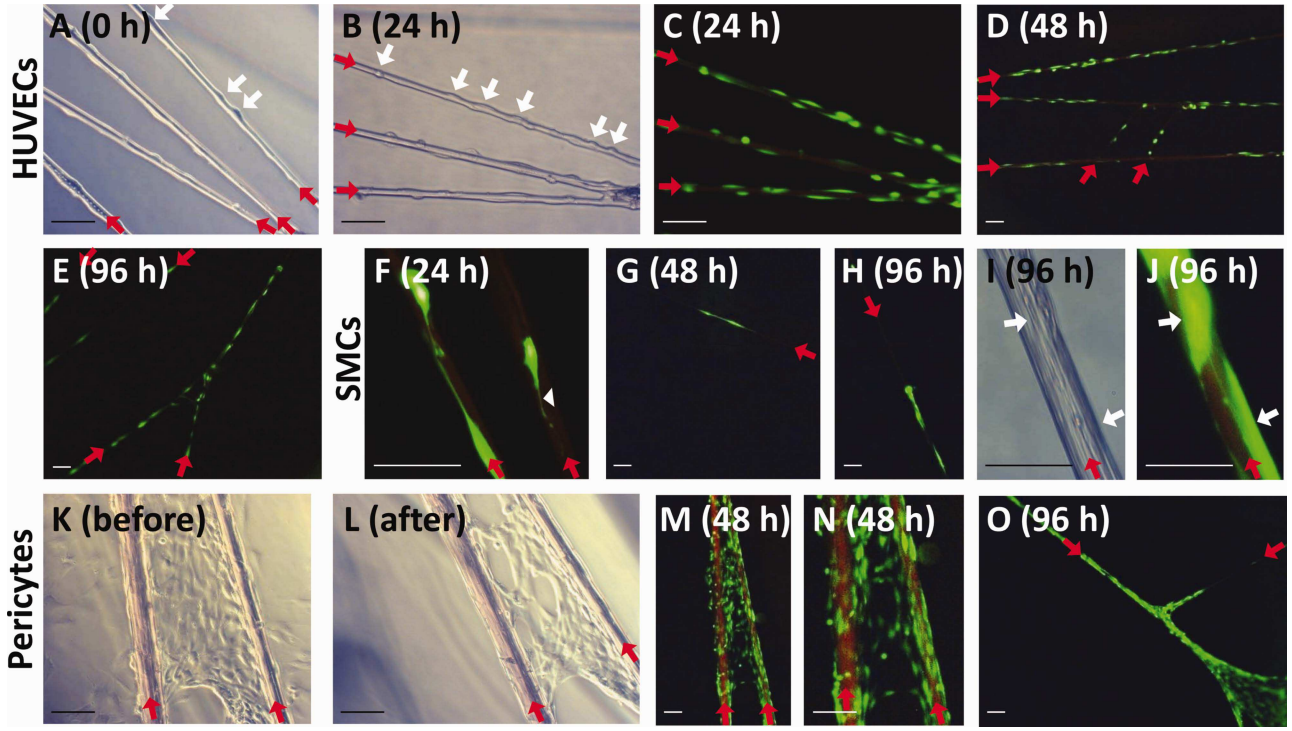


FIGURE 6. Cells bound to fibers can be transferred between cultures. Cells were grown on fibers for 24 h and moved to a new dish with fresh growth media. Cells were monitored by phase microscopy, and cell survival after transfer by live/dead stains at various timepoints marked on each panel. mCherry-Ubx fibers are marked by red arrows. The presence of ECs on fibers immediately after transfer (A) and in the same sample 24 h after transfer demonstrate that transfer does not kill the cells (B and C). Live/dead assays demonstrate cells have a long viability 48 h (D) and 96 h (E) after transfer. F–J, Smooth muscle cells at various times after transfer are also viable and retain the elongated (panels F–H) and spread (panels I and H white arrows) morphologies observed previously. K-N) An apparent sheet of pericytes bounded by two mCherry-Ubx fibers before transfer (K) and 48 h after transfer (L–N) to a new dish. L and M, After 48 h culture in the new dish, a live/dead stain confirms that cells not only survived the transfer, but filled in holes and created an additional connection between the two fibers. O, Live/dead stain of a different sample with pericytes coating and suspended between fibers 96 h after transfer. Scale bars are 100 μm in all panels. Viability for all cell transfer experiments was 100%. Note that fluorescent micrographs originate from different dishes and thus differences in cell density do not necessarily reflect cell growth. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

interact with the fiber. Importantly, the use of plain Ubx fibers, which lack the mCherry fusion, confirms that exposure to Ubx is not cytotoxic to ECs, SMCs, or PCs.

Vascular cells remain viable and associated with Ubx fibers in long-term cultures

To determine whether ECs, SMCs, or PCs maintained long-term interactions with Ubx fibers in culture without support from a cell monolayer, we incubated mCherry-Ubx fibers with ECs for 24 h. After 24 h, fibers and any associated cells were transferred to a new dish with fresh growth media and incubated for an additional 24–96 h (Fig. 6). Phase contrast images taken immediately after transferring fibers show ECs remained associated with the fiber through transfer [Fig. 6(A) white arrows] and 24 h after transfer [Fig. 6(B) white arrows]. This viability persisted for as long as we monitored the ECs [24–96 h; Fig. 6(C–E)]. After the fiber transfer, the total density of viable ECs on mCherry-Ubx fibers did not appear dramatically different at 24, 48, or 96 h [Fig. 6(C–E)]. These data show ECs were consistently alive immediately after transfer and for several days afterward.

SMCs also maintained association with Ubx fibers after transfer and remained viable at 24, 48, and 96 h after transfer [Fig. 6(F–J)]. Most observed SMCs had an elongated morphology with extensions protruding down the length of the axis [Fig. 6(F–H)]. However, spread SMC morphologies were also observed [Fig. 6(I,J) white arrows].

Interestingly, what appeared to be sheets of PCs, supported by multiple mCherry-Ubx fibers, successfully transferred with the fibers [Fig. 6(K,L)]. These PC sheets remained intact [Fig. 6(L)] and viable after transfer [Fig. 6(M–O)]. Many tissue engineering applications require the growth of sheets of cells.[27,28] Thus, PC addition to mCherry-Ubx fibers could provide a new approach to creating and manipulating cell sheets.

Together, these results demonstrate that primary human ECs, SMCs, and PCs remained associated with mCherry-Ubx fibers and were viable for at least 96 h after transfer to a new culture dish. These data reinforce the concept that these cell lines attach to fibers, and that fibers exhibit no toxicity toward any of the primary cell lines tested. Importantly, the fibers are also a sufficient substrate to maintain cell viability in culture. These traits are consistent across multiple cell lines.

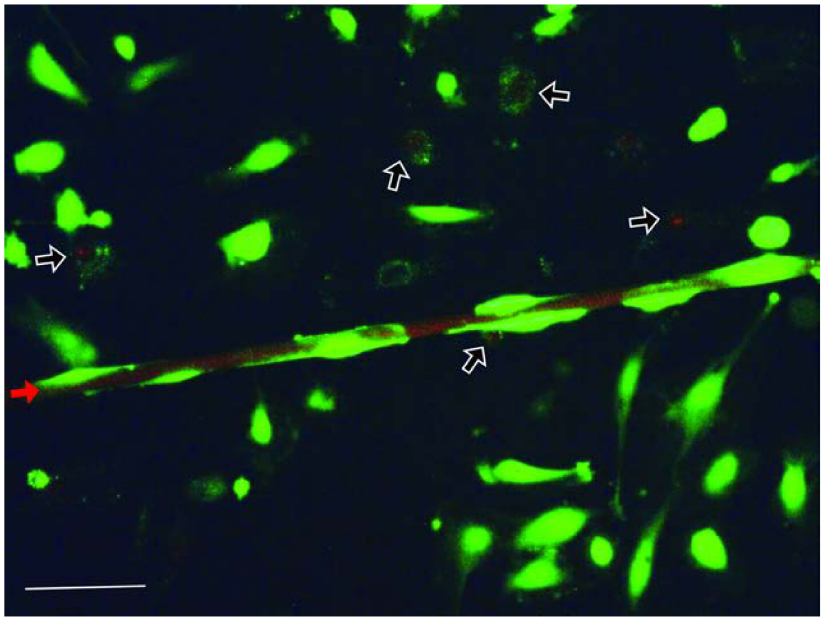
CONCLUSIONS

In this work, we have demonstrated that Ubx fibers are not toxic to a variety of cells. Since dead cells were rarely observed in the live/dead assays for any of the three cell lines, we conclude that cell viability exceeds 97% when cultured in the presence of or in direct contact with Ubx fibers. The cytoskeletons of all three cell lines were robust and intact when in contact with Ubx fibers. The observation of cytoskeletal alignment for all three cell types suggest that cells are not merely resting adjacent to Ubx fibers, but instead are interacting with and responding to the fibers. The ability of all tested cell types to remain attached to fibers and viable when transferred between cultures, combined with their ability to remain bound through staining and washing steps, demonstrates that cell–Ubx interactions are reasonably robust. In addition, cells cultured on Ubx fibers maintain normal morphologies. Together, these data suggest Ubx fibers are cytocompatible and cell adherent, and thus are a potentially useful tool for designing cell or tissue engineering experiments.

REFERENCES

1. Liu Y, Matthews KS, Bondos SE. Multiple intrinsically disordered sequences alter DNA binding by the homeodomain of the *Drosophila* Hox protein ultrabithorax. J Biol Chem 2008;283: 20874–20887.
2. Liu Y, Matthews KS, Bondos SE. Internal regulatory interactions determine DNA binding specificity by a Hox transcription factor. J Mol Biol 2009;390:760–774.
3. Tan XX, Bondos S, Li L, Matthews KS. Transcription activation by ultrabithorax Ib protein requires a predicted α-helical region. Biochemistry 2002;41:2774–2785.
4. Galant R, Carroll SB. Evolution of a transcriptional repression domain in an insect Hox protein. Nature 2002;415:910–913.
5. Ronshaugen M, McGinnis N, McGinnis W. Hox protein mutation and macroevolution of the insect body plan. Nature 2002;415: 914–917.
6. Beachy PA, Varkey J, Young KE, von Kessler DP, Sun BI, Ekker SC. Cooperative binding of an ultrabithorax homeodomain protein to nearby and distant DNA sites. Mol Cell Biol 1993;13: 6941–6956.
7. Greer AM, Huang Z, Oriakhi A, Lu Y, Lou J, Matthews KS, Bondos SE. The *Drosophila* transcription factor ultrabithorax self-assembles into protein-based biomaterials with multiple morphologies. Biomacromolecules 2009;10:829–837.
8. Majithia R, Patterson J, Bondos SE, Meissner KE. On the design of composite protein-quantum dot biomaterials via self-assembly. Biomacromolecules 2011;12:3629–3637.
9. Huang Z, Lu Y, Majithia R, Shah J, Meissner K, Matthews KS, Bondos SE, Lou J. Size dictates mechanical properties for protein fibers self-assembled by the *Drosophila* Hox transcription factor ultrabithorax. Biomacromolecules 2010;11:3644–3651.
10. Engler AJ, Sen S, Sweeney HL, Discher SE. Matrix elasticity direct stem cell lineage specification. Cell 2006;126:677–689.
11. Huang Z, Salim T, Brawley A, Patterson J, Matthews KS, Bondos SE. Functionalization and patterning of protein-based materials using active ultrabithorax chimeras. Adv Funct Mater 2011;21: 2633–2640.
12. Woolfson DN, Mahmoud ZN. More than just bare scaffolds: Towards multi-component and decorated fibrous biomaterials. Chem Soc Rev 2010;39:3464–3479.
13. Rosenbloom J, Abrams WR, Mecham R. Extracellular matrix 4: The elastic fiber. FASEB J 1993;7:1208–1218.
14. Wagenseil JE, Mecham RP. Vascular extracellular matrix and arterial mechanics. Physiol Rev 2009;89:957–989.
15. Novosel EC, Kleinhans C, Kluger PJ. Vascularization is the key challenge in tissue engineering. Adv Drug Deliv Rev 2011;63: 300–311.
16. Owen SC, Shoichet MS. Design of three-dimensional biomimetic scaffolds. J Biomed Mater Res A 2010;94:1321–1331.
17. Zhang X, Baughman CB, Kaplan KL. In vitro evaluation of electrospun silk fibroin scaffolds for vascular cell growth. Biomaterials 2008;29:2217–2227.
18. Canolat MF, Tang C, Bernacki SH, Pourdeyhimi B, Khan S. Mammalian cell viability in electrospun composite nanofiber structures. Macromol Biosci 2011;11:1346–1356.
19. Tremblay PL, Hudon V, Berthod F, Germain L, Auger FA. Inoculation of tissue-engineered capillaries with the host's vasculature in a reconstructed skin transplanted on mice. Am J Transplant 2005; 5:1002–1010.
20. Townsend-Nicholson A, Jayasinghe SN. Cell electrospinning: A unique biotechnique for encapsulating living organisms for generating active biological microthreads/scaffolds. Biomacromolecules 2006;7:3364–3369.
21. Jayasinghe SN, Irvine S, McEwan JR. Cell electrospinning highly concentrated cellular suspensions containing primary living organisms into cell-bearing threads and scaffolds. Nanomedicine 2007;2:555–567.
22. Sims DE. The pericyte—A review. Tissue Cell 1986;18:153–174.
23. Liu Y, Franco A, Huang L, Gersappe D, Clark RA, Rafailovich MH. Control of cell migration in two and three dimensions using substrate morphology. Exp Cell Res 2009;315:2544–2557.
24. Malmstrom J, Christensen B, Jakobsen HP, Lovmand J, Foldbjerg R, Sørensen ES, Sutherland DS. Large area protein patterning reveals nanoscale control of focal adhesion development. Nano Lett 2010;10:686–694.
25. Soliman S, Sant S, Nichol JW, Khabiry M, Traversa E, Khademhosseini A. Controlling the porosity of fibrous scaffolds by modulating the fiber diameter and packing density. J Biomed Mater Res A 2011;96:566–574.
26. Thakar RG, Cheng Q, Patel S, Chu J, Nasir M, Liepmann D, Komvopoulos K, Li S. Cell-shape regulation of smooth muscle cell proliferation. Biophys J 2009;96:3423–3432.
27. Nelson CM, VanDuijn MM, Inman JL, Fletcher DA, Bissell MJ. Tissue geometry determines sites of mammary branching morphogenesis in organotypic cultures. Science 2006;314:298–300.
28. Melchels FPW, Domingos MAN, Klein TJ, Malda J, Bartolo PJ, Hutmacher DW. Additive manufacturing of tissues and organs. Prog Polym Sci 2012;37:1079–1104.
29. Mie M, Mizushima Y, Kobatake E. Novel extracellular matrix for cell sheet recovery using genetically engineered Elastin-like protein. J Biomed Mater Res B Appl Biomater 2008;86:283–290.

Supporting Figure 1



Supporting Figure 2