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Collagen and heparan sulfate coatings differentially alter cell proliferation and attachment *in vitro* and *in vivo*

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Abstract

Tissue engineering is an innovative field of research applied to treat intestinal diseases. Engineered smooth muscle requires dense smooth muscle tissue and robust vascularization to support contraction. The purpose of this study was to use heparan sulfate (HS) and collagen coatings to increase the attachment of smooth muscle cells (SMCs) to scaffolds and improve their survival after implantation. SMCs grown on biologically coated scaffolds were evaluated for maturity and cell numbers after 2, 4 and 6 weeks *in vitro* and both 2 and 6 weeks *in vivo*. Implants were also assessed for vascularization. Collagen-coated scaffolds increased attachment, growth and maturity of SMCs in culture. HS-coated implants increased angiogenesis after 2 weeks, contributing to an increase in SMC survival and growth compared to HS-coated scaffolds grown *in vitro*. The angiogenic effects of HS may be useful for engineering intestinal smooth muscle.

Keywords

Smooth Muscle; Tissue Engineering; Biomaterials; Intestine; Polycaprolactone; Heparan Sulfate; Collagen

INNOVATION

Biomaterials are often critical for delivering cells into new environments within living systems. As such, understanding the differential responses of biomaterials during *in vitro* and *in vivo* tissue engineering applications is necessary for favorable medical outcomes. Collagen is a common extracellular matrix protein in mammals and is among the most studied biomaterial coatings used in tissue engineering applications. Collagen generally increases beneficial characteristics such as attachment and proliferation for cultured cells. However, collagen may not have optimal properties for the complex *in vivo* environment. An alternative biomaterial coating, heparan sulfate (HS), improves vasculogenesis and survival of SMCs *in vivo* despite reduced cell attachment and proliferation characteristics *in vitro*. Using a novel technique for tissue engineering smooth muscle that utilizes a porous scaffold made from degradable polymer fibers, this report demonstrates important biomaterial characteristics to consider during the translation of *in vitro* culture to *in vivo* experiments.

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INTRODUCTION

Short bowel syndrome (SBS) is a disease characterized by loss of functional intestine, resulting in malnutrition associated with high morbidity, mortality and healthcare costs[1–3]. Current treatments for SBS include total parenteral nutrition and intestinal transplantation, but both are associated with unacceptable rates of complications[4–7]. Newer treatments have attempted to increase intestinal transit time or intestinal length, thereby improving nutrient absorption[8–10], but have limited regenerative ability. Tissue engineering is an emerging field of biomedical research that aims to regrow or create tissues or organs and may be a viable alternative to current treatments for SBS[11]. Engineered intestine is often limited by expansion of SMCs and the contractile forces they generate[12,13]; therefore the survival of thick, contractile smooth muscle tissues are essential to the ability to regenerate intestine. Current tissue engineering methods have been unable to generate forces similar to native intestine, i.e. approximately 30,000 millinewtons/gram during induced contraction, with a baseline contraction frequency of 24–33 contractions per minute[14].

Some intestinal engineering approaches have grown SMCs on scaffolds derived from natural polymers such as collagen[15], chitosan[16] or decellularized submucosa[17,18] as a substrate. Unique challenges arise with extensive use of these naturally derived materials *in vivo* due to increased inflammation, scaffold contraction and inconsistency with the source material compared to synthetic materials. Polycaprolactone (PCL) and similar combinations of poly-lactic/poly-glycolic acid (PLGA) scaffolds provide a more consistent product and have been used extensively to engineer a variety of intestinal tissues[19–21], but require chemical or biological modification to overcome hydrophobic interactions with media and cells which greatly reduce cell attachment and survival[22].

In order to improve cellular attachment on hydrophobic materials, scaffolds can be made more hydrophilic with plasma etching, allowing for homogenous surface modification of the intricate polymer scaffolds without changes to bulk material properties such as stiffness, fiber size or fiber alignment[22]. Specifically, plasma treatment converts hydrophobic polymer chains on the surface of the PCL fibers to more hydrophilic carboxylate groups[23]. Plasma etching is known to affect surface charges and can lead to changes in release of various surface-adsorbed proteins[24].

Biological coatings have also been employed to promote cellular attachment. Collagen has been used in many tissue engineering applications, often in conjunction with other treatments[25,26], and specifically for intestine[27,28] and intestinal smooth muscle[29–31]. Tissue-engineered smooth muscle constructs have used collagen extensively, both as a substrate[15] and as a coating to enhance cell attachment to polymers[29]. HS is an easy to use and well-studied biological coating that is inexpensive compared to most growth factors, and has been shown to maintain healthy intestine in a variety of studies[16,32,33]. Further, HS is known to restrict the immature, proliferative phenotype of cultured aortic smooth muscle and promote a contractile phenotype[34,35]. HS has many traits beneficial to tissue engineering, including the sequestration of circulating growth factors[36], activation of ligand/receptor complexes[37], and stimulation of angiogenesis[38] which increases survival of transplanted intestinal smooth muscle[30].

We sought to compare the beneficial cell attachment and proliferation effects of collagen with the pro-angiogenic effects of HS. Scaffolds were coated with collagen or HS to determine cell numbers and maturity on cultured and implanted polymer implants.

MATERIALS AND METHODS

Fabrication and coating of electrospun poly-ε-caprolactone scaffolds

The scaffold formation protocol was described in detail previously[30]; briefly, polymer solution was made from 11% w/w ester capped PCL (Lactel Absorbable Polymers, Birmingham, AL) in hexafluoro-2-propanol (Sigma, St. Louis, MO). Electrospinning was performed at approximately 3,000 rpm with 18 kV and 15 cm between the 18-gauge blunt-tipped needle and collector, and approximately 165 mg PCL was deposited per scaffold. After electrospinning, scaffolds were coated with either collagen or HS to better facilitate cell adhesion. In short, approximately 150 μm of thick electrospun PCL scaffolds were cut in to a rectangle of size 25 mm × 9.5 mm, with 250-μm diameter pores laser-cut in an arrayed pattern.

Heparan sulfate coating

PCL scaffolds were sterilized for 30 minutes in 70% ethanol, rehydrated with a decreasing sequence of ethanol rinses prior to three washes in sterile distilled water. Scaffolds were coated with HS sodium salt derived from bovine kidney (Sigma, St. Louis, MO) as described previously[39] using 2-(N-Morpholino)ethanesulfonic acid buffer (0.05M, pH 5.5, Sigma), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 0.5M, Sigma) and N-hydroxysuccinimide (NHS, 0.5M, Sigma). Briefly, 1 mg/mL heparan was crosslinked on to scaffolds with EDC and NHS chemistry for 2 hours before three consecutive rinses with deionized water. The coated scaffolds were subsequently dried and kept at 4°C until use. For visualization experiments, a 1:100 ratio of FITC-heparan to unlabeled heparan was used confirm heparan crosslinking fluorescently.

Collagen coating

Prior to collagen coating, dry PCL scaffolds were plasma etched (Harrick Plasma, Ithaca, NY) in air for 90 seconds at high power and 500 mTorr pressure. Scaffolds were then sterilized in 70% ethanol for 30 minutes, rehydrated with a decreasing sequence of ethanol rinses and rinsed three times in deionized water with a final rinse of PBS. Each scaffold was coated with 500 μL of 0.25 mg/mL collagen type I solution (Purecol, Advanced Biomatrix, San Diego, CA) neutralized to physiological pH with 0.1N sodium hydroxide and diluted in PBS. Scaffolds were then stored overnight in a 37°C humidified incubator and rinsed three times in PBS before cell seeding.

Scaffold characterization

Scanning electron microscopy—The surface of the HS- and collagen-coated scaffolds was assessed using low-vacuum scanning electron microscopy at 5 keV (Nova NanoSEM 230 FEI, Hillsboro, OR) after mounting on aluminum stubs.

Fourier transform infrared spectroscopy—Functional groups in uncoated PCL, HS-coated PCL and collagen-coated PCL were compared via attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) using an Avatar 360 Thermo Nicolet Spectrometer (Thermo Scientific, Waltham, MA). Scaffolds were placed in contact with the diamond ATR crystal to obtain the absorbance spectra between 1,500 cm^{-1} and 4,000 cm^{-1} wavenumber. Results were converted to percent transmittance, and the functional group region between 1,500 cm^{-1} and 4,000 cm^{-1} was analyzed.

Isolation and culture of neonate intestinal smooth muscle cells

Primary green fluorescent protein (GFP)-expressing cells were harvested as described previously[30] from neonatal transgenic GFP-expressing Lewis rats generously gifted by Reichardt at the University of Würzburg, Germany. The GFP-expressing rats were maintained by UCLA's Department of Laboratory Animal Medicine and were sacrificed with approval of the UCLA Institutional Review Board. In short, 5- to 7-day-old animals were sacrificed and muscle strips were gently teased away from the small intestine. Muscle tissue was digested at 37°C for 30 minutes in 0.01% collagenase IV (Sigma), rinsed in Hank's buffered saline solution, filtered through a 70-μm cell strainer, counted with a hemacytometer and seeded with a density of 250,000 cells/cm^2 as determined in earlier experiments[30], either on circular scaffolds within a well plate for *in vitro* experiments, or on rectangular scaffolds for *in vivo* experiments within a custom polydimethylsiloxane (PDMS, Dow Corning, Elizabethtown, KY) cell seeding tray shaped to fit our scaffolds. Control cells were seeded directly on to a 24-well plate at 25,000 cells/cm^2 density. For *in vivo* experiments, the cells were allowed to grow on the scaffolds in these conditions for 2 weeks prior to implantation (day 0), or maintained *in vitro* until collection of implants at 2 weeks or 6 weeks after implantation. For *in vitro* experiments, the scaffolds were collected at 2, 4 or 6 weeks following seeding. Cells were maintained in a humidified incubator kept at 37°C in DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1× antibiotic/antimycotic.

The implantation procedure has been described previously[30] and was performed in accordance with UCLA's Animal Research Committee protocol #2004-197-21. Briefly, scaffolds were wrapped twice around a #6 silicone catheter, sutured with prolene, and wrapped and sutured in place in the omentum of Lewis rats.

Immunohistochemistry

Immunostaining of GFP-expressing SMCs—Immunohistochemical determination of the implants has been described previously[30]. Constructs were retrieved and sectioned after 2 or 6 weeks implantation, then stained with antibodies to SMA or DES (Dako, Carpinteria, CA; 1:50 dilution), myosin heavy chain (MHC; Santa Cruz Biotechnology, Dallas, TX; 1:50 dilution), GFP (Clontech, Mountain View, CA; 1:200 dilution) or von Willebrand Factor (vWF; Dako; 1:200 dilution). Antibodies were then labeled with fluorescent secondary antibodies (Life Technologies; 1:500 dilution) except for vWF, which was visualized with DAB (3,3-diaminobenzidine; Vector Laboratories) and counter-stained with hematoxylin. Scaffolds without primary antibodies did not show any significant amount of staining (Supplementary Fig. 1).

RNA and DNA quantification

RNA and DNA extraction and quantification was described in a previous publication[30]. In short, RNA was extracted from scaffolds and implants using an RNeasy kit (Qiagen, Germantown, MD) and quantified with real-time PCR on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Life Technologies). Primer/probe mixtures for SMA, DES, MHC and GAPDH were purchased from Applied Biosystems (GAPDH – Rn01775763_g1; SMA – Rn01759928_g1; MHC – Rn01530321_m1; DES – Rn00574732_m1). GFP primer/probes were custom sequences purchased from Operon (Huntsville, AL; Forward: 5′-ACTACAACAGCCACAACGTCTATATCA-3′; Reverse: 5′-GGCGGATCTTGAAGTTCACC-3′; Probe: 5′-(6-FAM) CCGACAAGCAGAAGAACGGCATCA(Tamra-Q)-3′). All samples were normalized to GAPDH and to RNA extracted from muscle strips from rat pups 3–5 days after birth.

Statistics

Differences between groups were evaluated using a Student's *t*-test. A p-value <0.05 was considered statistically significant.

RESULTS

Scaffold characterization

Fibers of the electrospun PCL scaffolds showed a uniform thickness and directionality (Fig. 1a). Electrospun PCL formed a dense network of smooth fibers that was highly porous (Fig. 1b). HS-coated scaffolds contained sub-micron roughness on PCL fibers (Fig. 1c), which was characteristic of scaffolds with HS coating[38]. After coating with collagen, scaffolds showed a fine network of collagen fibers interspersed among the larger PCL fibers (Fig. 1d).

Scaffolds coated were labeled with FITC-HS and were immunofluorescently stained for collagen (Fig. 2). FITC-labeled HS was visible on HS-coated scaffolds only (Fig. 2a,b). Scaffolds coated with collagen only fluoresced red from the secondary antibody labeling to anti-collagen type 1 (Fig. 2c,d).

FTIR spectra of the scaffolds contain absorption peaks at various spectra associated with functional groups (Fig. 3). All scaffolds contain peaks at 2,970 cm^{-1} and 2,890 cm^{-1}, indicative of $–CH_2–$ stretching, as well as a distinct carbonyl peak at 1,720 cm^{-1} consistent with bare PCL (Fig. 3a). The HS-coated scaffolds created a peak at 3,350 cm^{-1}, indicating a secondary amine N-H stretch (Fig. 3b), and a peak at approximately 1,660 cm^{-1} from a new amide C=O bond. Heparan contains a sulfate group, which is normally observed between 1,350 cm^{-1} and 1,450 cm^{-1}; however, artifacts of measurement in the PCL groups overshadowed these peaks[40]. The collagen-coated scaffolds (Fig. 3c) contained a broad peak around 3,400 cm^{-1} from a stretch in an –OH group.

Immunohistochemical analysis

Scaffolds grown in culture stained positively for smooth muscle actin (SMA) after culturing for 2, 4 and 6 weeks on all coatings (Fig. 4). SMCs grown in culture stain consistently high for SMA, which is a general marker for intestinal SMCs. Cells grown on collagen-coated

scaffolds showed the greatest amount of SMA at all points, while HS-coated scaffolds increased SMA staining consistently from 2–6 weeks. Collagen-coated scaffolds showed an increase in cell attachment at all time points, and SMCs densely covered the surface of the scaffold after only 2 weeks. The high levels of SMC attachment observed on the collagen-coated scaffolds were consistent with qPCR results (Fig. 7). HS-coated scaffolds showed reduced staining for SMA, indicating fewer SMCs attaching after initial seeding and reduced proliferation during culture. SMC attachment on HS-coated scaffolds were similar to collagen-coated scaffolds after plasma treating HS-coated scaffolds, although this technique also reduced the angiogenic effects of HS-coated scaffolds *in vivo* (data not shown).

Desmin (DES) is an intermediate marker for smooth muscle maturity. DES staining (Fig. 5) on HS-coated scaffolds increased from weeks 2–6, but was reduced compared to either collagen-coated scaffold. Both collagen- and HS-coated scaffolds showed comparable results, with slight increases in DES staining, and collagen-coated scaffolds overall had greater DES expression at all points.

MHC is a marker of advanced maturity in SMCs. MHC expression (Fig. 6) was consistently low on all scaffolds at all time points, but had increased slightly by week 6 on collagen-coated scaffolds, perhaps due to greater overall cell numbers on these scaffolds at this time point. HS-coated scaffolds did not show the same increase.

Analysis of RNA expression

All scaffolds and cells grown in well plates showed a decrease in expression for SMA, DES and MHC at all time points, for all genes, compared to expression in primary muscle strips (Fig. 7). Collagen coated scaffolds showed an increase in gene expression for SMA, DES and MHC compared to HS-coated scaffolds after 2 weeks. Conversely, cells seeded on to a well plate showed an increase in gene expression for the same markers compared to any of the scaffolds at 2 weeks.

By week 4, collagen-coated implants still showed an increase in expression of SMA and DES, but there was no increase in MHC compared to HS-coated scaffolds. Cells grown in well plates also showed an increase in SMA and DES compared to any scaffold, but only had increased expression of MHC compared to HS-coated scaffolds.

By week 6, there was no statistical difference between any set of scaffolds, including cells grown on a well plate only, for any marker.

Immunohistochemistry of implants

Histological staining with hematoxylin and eosin was performed after recovering explants following 2 or 6 weeks in the rat omentum (Fig. 8). HS-coated explants (**a**, 2 weeks *in vivo*; **c**, 6 weeks *in vivo*) and collagen-coated implants (**b**, 2 weeks *in vivo*; **d**, 6 weeks *in vivo*) both showed cell infiltration throughout the scaffolds.

Implants were sectioned circumferentially and imaged with the outer, omental-facing layers in the bottom of the image and inner layers, luminal layers at the top of the image as described previously[30]. Immunohistological staining for SMA and DES (Fig. 9) and MHC

(Fig. 10) showed an increase in both markers from 2–6 weeks. SMA stained strongly, but sporadically at 2 weeks in all implants; however, actin staining was uniform and dense throughout the scaffolds by 6 weeks (Fig. 9a–d). DES staining increased slightly between 2 and 6 weeks (Fig. 9e–h). MHC showed a slight increase in staining from 2 weeks (HS, Fig. 10a; collagen, Fig. 10b) to 6 weeks in all scaffold groups (HS, Fig. 10c; collagen, Fig. 10d). GFP staining of SMCs (Fig. 11) remained constant from 2 weeks (HS, Fig. 11a; collagen, Fig. 11b) to 6 weeks (HS, Fig. 11c; collagen, Fig. 11d).

GFP-expressing DNA quantification

During *in vitro* culture, scaffolds coated in collagen showed a statistically higher number of GFP-expressing SMCs as determined by qPCR compared to HS-coated scaffolds at all time points (Fig. 11e). HS-coated scaffolds had about the same number of cells from the initial seeding by week 6, while collagen-scaffolds had reached this number by the day of implantation. Implants were recovered after 2 and 6 weeks and GFP+ cells were quantified with qPCR to GFP DNA. After 2 weeks *in vivo*, HS-coated implants had more GFP+ cells than HS-coated scaffolds *in vitro*, but there were no significant differences between HS- and collagen-coated implants at either week 2 or week 6.

Quantification of angiogenesis

Angiogenesis was quantified with staining for vWF, which stains blood vessels a brown/black circle when positive (Fig. 12). There was a significant increase in blood vessels in HS-coated implants compared to collagen-coated implants after 2 weeks (HS, Fig. 12a; collagen, Fig. 12b), however after 6 weeks (HS, Fig. 12c; collagen, Fig. 12d) the differences were no longer significant (Fig. 12e).

DISCUSSION

The goal of this study was to improve cellular attachment, proliferation and maturity by coating scaffolds with either HS or collagen. While collagen led to rapid SMC proliferation and maturation *in vitro*, HS increased the speed of angiogenesis and improved survival of implanted SMCs *in vivo*.

HS-coated scaffolds had improved *in vivo* characteristics despite the reduced cellular attachment exhibited *in vitro*. Vascularization of tissue-engineered implants remains a significant limitation to development of larger tissues and organs. In our experiments, we used laser-cut pores to improve cellular infiltration and vascular perfusion *in vivo*, both characteristics that should also be improved by HS coating[38]. HS-coated implants showed a significant increase in angiogenesis after 2 weeks post-implantation, which corresponded to an increase in GFP+ SMCs on implants (678,000 ± 273,000 after 2 weeks; 871,000 ± 278,000 after 6 weeks) compared to scaffolds *in vitro* (168,000 ± 42,000 after 2 weeks; 511,000 ± 178,000 after 6 weeks). This result suggests that the rapid vascularization into HS-coated implants might aid SMC survival or proliferation after implantation compared to collagen-coated implants. Although not statistically significant, more GFP+ SMCs were measured on HS-coated implants after 6 weeks compared to either 2-week implants or 6-week HS-coated scaffolds *in vitro*, but vascularization did not change noticeably between 2

and 6 weeks implantation. A possible explanation for this observation is that early infiltration of blood vessels may be critical to sustaining recently implanted cells, and continues to support proliferating SMCs after angiogenesis tapers off.

Conversely, GFP+ cells on collagen-coated implants did not change noticeably (498,000 ± 195,000 after 2 weeks; 668,000 ± 135,000 after 6 weeks) after implantation and may have even decreased compared to scaffolds grown *in vitro* (604,000 ± 193,000 after 2 weeks; 1,439,000 ± 570,000 after 6 weeks). Collagen-coated implants had significantly fewer infiltrating blood vessels compared to HS-coated implants (287 ± 58 blood vessels with HS-coated implants after 2 weeks; 195 ± 44 blood vessels on collagen-coated implants after 2 weeks), leading to a decrease in surviving SMCs immediately after implantation.

Characteristics of scaffolds with each coating *in vitro* were very different than the implants with each coating *in vivo*. Although the collagen-coated scaffolds were nearly confluent after 2 weeks *in vitro*, desmin expression did not increase appreciably until 4 weeks, suggesting a lag in the restoration of maturity following confluency and the proliferative state of the SMCs. This finding would seem to agree with the widely held belief that SMCs can transition between a proliferative, synthetic state and a slow-growing, mature state following confluency[20]. There was only a small increase in MHC staining within the 6-week timeframe of this study on collagen-coated scaffolds compared to the HS-coated scaffolds. Seemingly, the small increases in *SMA* gene expression observed with qPCR on collagen-coated scaffolds accumulate over the course of weeks. This led to improved staining of DES and MHC compared to HS-coated scaffolds at 6 weeks. There was very little positive staining for either desmin or MHC on either coating after implantation, suggesting that the cultured SMCs may have lost their natural phenotype.

The effects of both biological coatings follow the expected effects seen in other literature. Collagen-coated PLGA had increased cell attachment and proliferation of esophageal SMCs[29], and intestinal SMCs proliferated in collagen sponges *in vivo*[15]; however, excessive amounts of collagen I has been associated with colitis[41] and has maintained SMCs in the synthetic phenotype after implantation[20], Conversely, heparan proteoglycans are known to immobilize and activate growth factors in the extracellular matrix adjacent to cells[36] such as bFGF[35,42,43], a known regulator of SMC proliferation and maturity[20]. In radiation-induced models of fibrosis, increases in collagen were associated with the proliferative state of SMCs, which was ameliorated by a heparan mimetic[33]. Heparan's angiogenic properties are well studied[38] and further enhance the survival of SMCs after implantation. Scaffolds that were plasma etched after coating with HS did not exhibit angiogenic properties (data not shown). HS is a highly charged biopolymer, and plasma etching alters surface charges and surface groups[44]. Any interference with the charge density, distribution or overall charge would likely reduce the ability to bind circulating growth factors through electrostatic interactions, thereby reducing the intrinsic angiogenic properties[45]. In a previous publication, we have shown through high magnification tunneling electron microscopy that HS crosslinks around PCL fibers[38]. An alternative method to combine the angiogenic properties of HS with increased cell attachment would introduce HS, or a short heparan ligand, linked to the collagen-coated PCL. Other surfaces have been used to engineer smooth

muscle tissue, including fibrin[46], fibronectin[47], vitronectin[48,49], chitosan[16,50], laminin[51], matrigel[52] and RGD adhesive peptides[53].

Both collagen and HS coatings could contribute to the development of a thick layer of engineered contractile smooth muscle. One potential area of future research could introduce attachment ligands to HS-coated implants to improve growth of SMCs. An important consideration is maintaining the negative charges on heparan that generate the growth factor binding characteristics of heparan[45]. Attempts to coat collagen on to HS-coated scaffolds rendered characteristics indistinguishable from collagen-coated scaffolds alone, *in vivo* and *in vitro* (data not shown).

CONCLUSION

Our results show that collagen performs better than HS in maintenance of SMC attachment and proliferation *in vitro*. Confluency is rapidly reached on collagen-coated scaffolds compared to HS coating. We also showed that maturity increased immediately after cells reached confluency on the collagen-coated scaffolds, suggesting a transition between synthetic and mature phenotypes *in vitro*. However, implanted HS-coated implants showed no difference in GFP+ cell numbers compared to collagen-coated implants, and had a significant increase in blood vessels after 2 weeks of implantation. These results show that HS-coating has beneficial effects when used *in vivo* that are inadequate to support SMCs *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Donohoe CL, Reynolds JV. Short bowel syndrome. Surg J R Coll Surg Edinburgh Irel. 2010; 8(5): 270–279.
2. Bines JE. Intestinal failure: A new era in clinical management. J Gastroenterol Hepatol. 2009; 24(Suppl. 3):S86–S92. [PubMed: 19799705]
3. Spencer AU, Kovacevich D, Mckinney-Barnett M, Hair D, Canham J, Maksym C. Pediatric short-bowel syndrome: The cost of comprehensive care. Am J Clin Nutr. 2008; 88(6):1552–1559. [PubMed: 19064515]
4. Fishbein TM. Intestinal transplantation. N Engl J Med. 2009; 361(10):998–1008. [PubMed: 19726774]
5. Scolapio JS, Fleming RC, Kelly DG, Wick DM, Zinsmeister AR. Survival of home parenteral nutrition-treated patients: 20 years of experience at the Mayo Clinic. Mayo Clin Proc. 1999; 74(3): 217–222. [PubMed: 10089988]
6. Fryer JP. Intestinal transplantation: Current status. Gastroenterol Clin North Am. 2007; 36(1):145–159. vii. [PubMed: 17472880]
7. Pironi L, Joly F, Forbes A, Colomb V, Lyszkowska M, Baxter J, et al. Long-term follow-up of patients on home parenteral nutrition in Europe: Implications for intestinal transplantation. Gut. 2011; 60(1):17–25. [PubMed: 21068130]

8. Sullins VF, Wagner JP, Suwarnasarn AT, Lee SL, Wu BM, Dunn JCY. A novel biodegradable device for intestinal lengthening. J Pediatr Surg. 2014; 49(1):109–113. discussion 113. [PubMed: 24439592]
9. Chang PCY, Mendoza J, Park J, Lam MM, Wu B, Atkinson JB, et al. Sustainability of mechanically lengthened bowel in rats. J Pediatr Surg. 2006; 41(12):2019–2022. [PubMed: 17161196]
10. Safford SD, Freemerman AJ, Safford KM, Bentley R, Skinner MA. Longitudinal mechanical tension induces growth in the small bowel of juvenile rats. Gut. 2005; 54(8):1085–1090. [PubMed: 15840689]
11. Dunn JCY. Tissue engineering and regenerative science in pediatrics. Pediatr Res. 2008; 63(5): 459–460. [PubMed: 18360308]
12. Shi XZ, Sarna SK. Cell culture retains contractile phenotype but epigenetically modulates cell-signaling proteins of excitation-contraction coupling in colon smooth muscle cells. Am J Physiol Gastrointest Liver Physiol. 2013; 304(4):G337–G345. [PubMed: 23238936]
13. Nair DG, Han TY, Lourenssen S, Blennerhassett MG. Proliferation modulates intestinal smooth muscle phenotype *in vitro* and in colitis *in vivo*. Am J Physiol Gastrointest Liver Physiol. 2011; 300(5):G903–G913. [PubMed: 21311027]
14. Mendoza J, Chang C, Blalock CL, Atkinson JB, Wu BM, Dunn JCY. Contractile function of the mechanically lengthened intestine. J Surg Res. 2006; 136(1):8–12. [PubMed: 16979663]
15. Nakase Y, Hagiwara A, Nakamura T, Kin S, Nakashima S, Yoshikawa T, et al. Tissue engineering of small intestinal tissue using collagen sponge scaffolds seeded with smooth muscle cells. Tissue Eng. 2006; 12(2):403–412. [PubMed: 16548698]
16. Zakhem E, Raghavan S, Gilmont RR, Bitar KN. Chitosan-based scaffolds for the support of smooth muscle constructs in intestinal tissue engineering. Biomaterials. 2012; 33(19):4810–4817. [PubMed: 22483012]
17. Qin HH, Dunn JCY. Small intestinal submucosa seeded with intestinal smooth muscle cells in a rodent jejunal interposition model. J Surg Res. 2011; 171(1):e21–e26. [PubMed: 21937060]
18. Lee M, Chang PCY, Dunn JCY. Evaluation of small intestinal submucosa as scaffolds for intestinal tissue engineering. J Surg Res. 2008; 147(2):168–171. [PubMed: 18406427]
19. Chen DC, Avansino JR, Agopian VG, Hoagland VD, Woolman JD, Pan S, et al. Comparison of polyester scaffolds for bioengineered intestinal mucosa. Cells Tissues Organs. 2006; 184(3–4): 154–165. [PubMed: 17409741]
20. Lee M, Wu BM, Stelzner M, Reichardt HM, Dunn JCY. Intestinal smooth muscle cell maintenance by basic fibroblast growth factor. Tissue Eng Part A. 2008; 14(8):1395–1402. [PubMed: 18680389]
21. Grikscheit TC, Siddique A, Ochoa ER, Srinivasan A, Alsberg E, Hodin RA, et al. Tissue-engineered small intestine improves recovery after massive small bowel resection. Ann Surg. 2004; 240(5):748–754. [PubMed: 15492554]
22. Siri S, Wadbua P, Amornkitbamrung V, Kampa N, Maensiri S. Surface modification of electrospun PCL scaffolds by plasma treatment and addition of adhesive protein to promote fibroblast cell adhesion. Mater Sci Technol. 2010; 26(11):1292–1297.
23. Martins A, Pinho ED, Faria S, Pashkuleva I, Marques AP, Reis RL, et al. Surface modification of electrospun polycaprolactone nanofiber meshes by plasma treatment to enhance biological performance. Small. 2009; 5(10):1195–1206. [PubMed: 19242938]
24. Lee M, Chen TT, Iruela-Arispe ML, Wu BM, Dunn JCY. Modulation of protein delivery from modular polymer scaffolds. Biomaterials. 2007; 28(10):1862–1870. [PubMed: 17184836]
25. Truong YB, Glattauer V, Briggs KL, Zappe S, Ramshaw JAM. Collagen-based layer-by-layer coating on electrospun polymer scaffolds. Biomaterials. 2012; 33(36):9198–9204. [PubMed: 23036963]
26. Shen H, Hu X, Yang F, Bei J, Wang S. Combining oxygen plasma treatment with anchorage of cationized gelatin for enhancing cell affinity of poly(lactide-co-glycolide). Biomaterials. 2007; 28(29):4219–4230. [PubMed: 17618682]
27. Jabaji Z, Sears CM, Brinkley GJ, Lei NY, Joshi VS, Wang J, et al. Use of collagen gel as an alternative extracellular matrix for the *in vitro* and *in vivo* growth of murine small intestinal epithelium. Tissue Eng Part C Methods. 2013; 19(12):961–969. [PubMed: 23566043]

28. Jabaji Z, Brinkley GJ, Khalil HA, Sears CM, Lei NY, Lewis M, et al. Type I collagen as an extracellular matrix for the *in vitro* growth of human small intestinal epithelium. PLoS One. 2014; 9(9):1–9.
29. Zhu Y, Chan-Park MB, Sin Chian K. The growth improvement of porcine esophageal smooth muscle cells on collagen-grafted poly(DL-lactide-co-glycolide) membrane. J Biomed Mater Res B Appl Biomater. 2005; 75(1):193–199. [PubMed: 16025463]
30. Walthers CM, Nazemi AK, Patel SL, Wu BM, Dunn JCY. The effect of scaffold macroporosity on angiogenesis and cell survival in tissue-engineered smooth muscle. Biomaterials. 2014; 35:5129–5137. [PubMed: 24695092]
31. Kobayashi M, Lei NY, Wang Q, Wu BM, Dunn JCY. Orthogonally oriented scaffolds with aligned fibers for engineering intestinal smooth muscle. Biomaterials. 2015; 61:75–84. [PubMed: 26001072]
32. Vachon PH, Durand J, Beaulieu JF. Basement membrane formation and redistribution of the beta 1 integrins in a human intestinal co-culture system. Anat Rec. 1993; 235(4):567–576. [PubMed: 8465988]
33. Alexakis C, Guettoufi A, Mestries P, Strup C, Mathé D, Barbaud C, et al. Heparan mimetic regulates collagen expression and TGF-beta1 distribution in gamma-irradiated human intestinal smooth muscle cells. FASEB J. 2001; 15(9):1546–1554. [PubMed: 11427486]
34. Newcomb PM, Herman IM. Pericyte growth and contractile phenotype: Modulation by endothelial-synthesized matrix and comparison with aortic smooth muscle. J Cell Physiol. 1993; 155(2):385–393. [PubMed: 8482730]
35. Castellot JJ, Favreaug LV, Karnovskys MJ, Rosenbergfjgll RD. Inhibition of vascular smooth muscle cell growth by endothelial. J Biol Chem. 1982; 257(19):11256–11260. [PubMed: 7118883]
36. Bernfield M, Götte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J. Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem. 1999; 168:729–777.
37. Kramer KL, Yost HJ. Heparan sulfate core proteins in cell-cell signaling. Annu Rev Genet. 2003; 37:461–484. [PubMed: 14616070]
38. Singh S, Wu BM, Dunn JCY. The enhancement of VEGF-mediated angiogenesis by polycaprolactone scaffolds with surface cross-linked heparin. Biomaterials. 2011; 32(8):2059–2069. [PubMed: 21147501]
39. Singh S, Wu BM, Dunn JCY. Accelerating vascularization in polycaprolactone scaffolds. Tissue Eng Part A. 2011; 17(13–14):1819–1830. [PubMed: 21395445]
40. Morterra C, Cerrato G. Bronsted acidity of a superacid sulfate-doped ZrO2 system. J Phys Chem. 1994; 98:12373–12381.
41. Mahavadi S, Flynn RS, Grider JR, Qiao LY, Murthy KS, Hazelgrove KB, et al. Amelioration of excess collagen IαI, fibrosis, and smooth muscle growth in TNBS-induced colitis in IGF-I(+/−) mice. Inflamm Bowel Dis. 2011; 17(3):711–719. [PubMed: 20722057]
42. Yayon A, Klagsbrun M, Esko JD, Leder P, Ornitz DM. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell. 1991; 64(4): 841–848. [PubMed: 1847668]
43. Gallagher JT, Turnbull JE. Heparan sulphate in the binding and activation of basic fibroblast growth factor. Glycobiology. 1992; 2(6):523–528. [PubMed: 1472758]
44. Pieper JS, van Wachem PB, van Luyn MJA, Brouwer LA, Hafmans T, Veerkamp JH, et al. Attachment of glycosaminoglycans to collagenous matrices modulates the tissue response in rats. Biomaterials. 2000; 21(16):1689–1699. [PubMed: 10905410]
45. Mammadov R, Mammadov B, Guler MO, Tekinay AB. Growth factor binding on heparin mimetic peptide nanofibers. Biomacromolecules. 2012; 13:3311–3319. [PubMed: 22963465]
46. Somara S, Gilmont RR, Dennis RG, Bitar KN. Bioengineered internal anal sphincter derived from isolated human internal anal sphincter smooth muscle cells. Gastroenterology. 2009; 137(1):53–61. [PubMed: 19328796]
47. Costa-silva B, Coelho M, Rosene F, Mendes C, Alvarez-silva M. Fibronectin promotes differentiation of neural crest progenitors endowed with smooth muscle cell potential. Exp Cell Res. 2009; 315(6):955–967. [PubMed: 19331824]

48. Kuemmerle JF. Occupation of avb3 -integrin by endogenous ligands modulates IGF-I receptor activation and proliferation of human intestinal smooth muscle. Am J Physiol Gastrointest Liver Physiol. 2006; 341:1194–1202.

49. Hazelgrove KB, Flynn RS, Qiao LY, Grider JR, Kuemmerle JF. Endogenous IGF-I and alpha v beta3 integrin ligands regulate increased smooth muscle growth in TNBS-induced colitis. Am J Physiol Gastrointest Liver Physiol. 2009; 296:G1230–G1237. [PubMed: 19359426]

50. Zakhem E, Raghavan S, Bitar KN. Neo-innervation of a bioengineered intestinal smooth muscle construct around chitosan scaffold. Biomaterials. 2014; 35(6):1882–1889. [PubMed: 24315576]

51. Hayashi K, Saga H, Chimori Y, Kimura K, Yamanaka Y, Sobue K. Differentiated phenotype of smooth muscle cells depends on signaling pathways through insulin-like growth factors and phosphatidylinositol 3-kinase. J Biol Chem. 1998; 273(44):28860–28867. [PubMed: 9786887]

52. Rodriguez A, Karen J, Gardner H, Gerdin B, Rubin K, Sundberg C. Integrin α1β1 is involved in the differentiation into myofibroblasts in adult reactive tissues *in vivo*. J Cell Mol Med. 2009; 13(9):3449–3462. [PubMed: 19397781]

53. Hahn MS, McHale MK, Wang E, Schmedlen RH, West JL. Physiologic pulsatile flow bioreactor conditioning of poly(ethylene glycol)-based tissue engineered vascular grafts. Ann Biomed Eng. 2007; 35(2):190–200. [PubMed: 17180465]

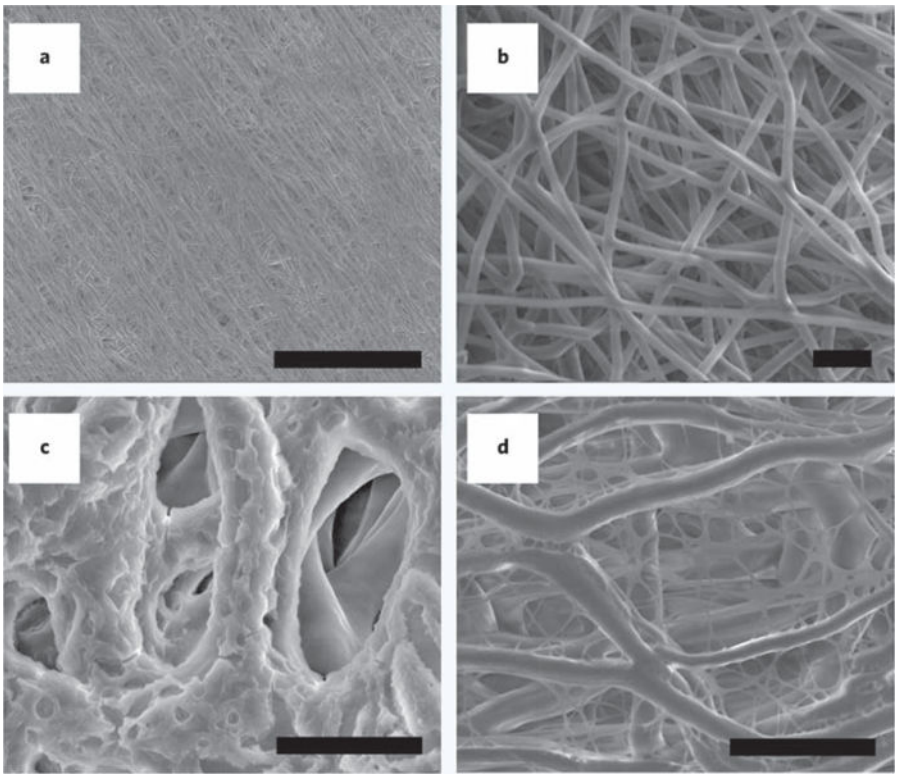

Figure 1.

Scanning electron microscope (SEM) analyses of uncoated PCL scaffolds at 1,000× magnification (**a**) and 10,000× magnification (**b**), HS-coated scaffolds (**c**) and collagen-coated scaffolds (**d**) Scale bar = 100 μm (**a**) and 10 μm (**b–d**).

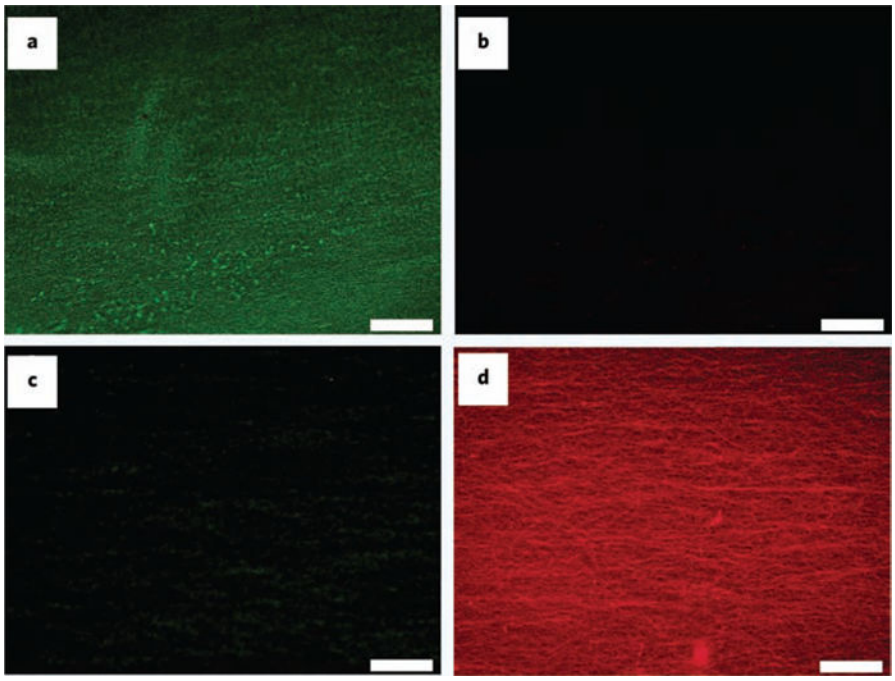

Figure 2.

FITC-heparan immobilization on polycaprolactone (PCL) electrospun scaffolds. Scaffolds coated with FITC-HS (**a**) showed only green fluorescence and no red (**b**), and collagen-coated scaffolds (**c**) were negative for FITC (**c**) but had uniform red fluorescence from RITC-labeled secondary antibodies to anti-collagen (**d**). Scale bar = 200 μm.

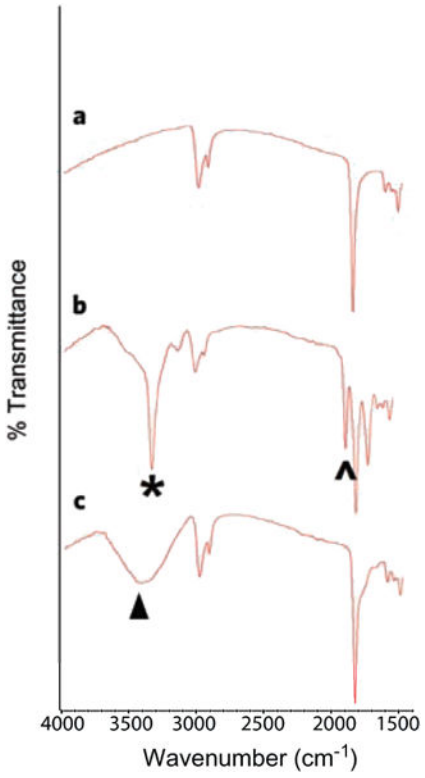

Figure 3.

Fourier transform infrared spectroscopy (FTIR) transmittance spectra of uncoated PCL (**a**), HS-coated PCL (**b**) and collagen-coated PCL (**c**). The asterisk (*) indicates a peak at 3,350 cm^{-1} from stretching amines in HS, a carrot (^) indicates a peak at 1,660 cm^{-1} from an amide bond in HS and the arrow head indicates hydroxyl stretching at 3,400 cm^{-1} due to collagen.

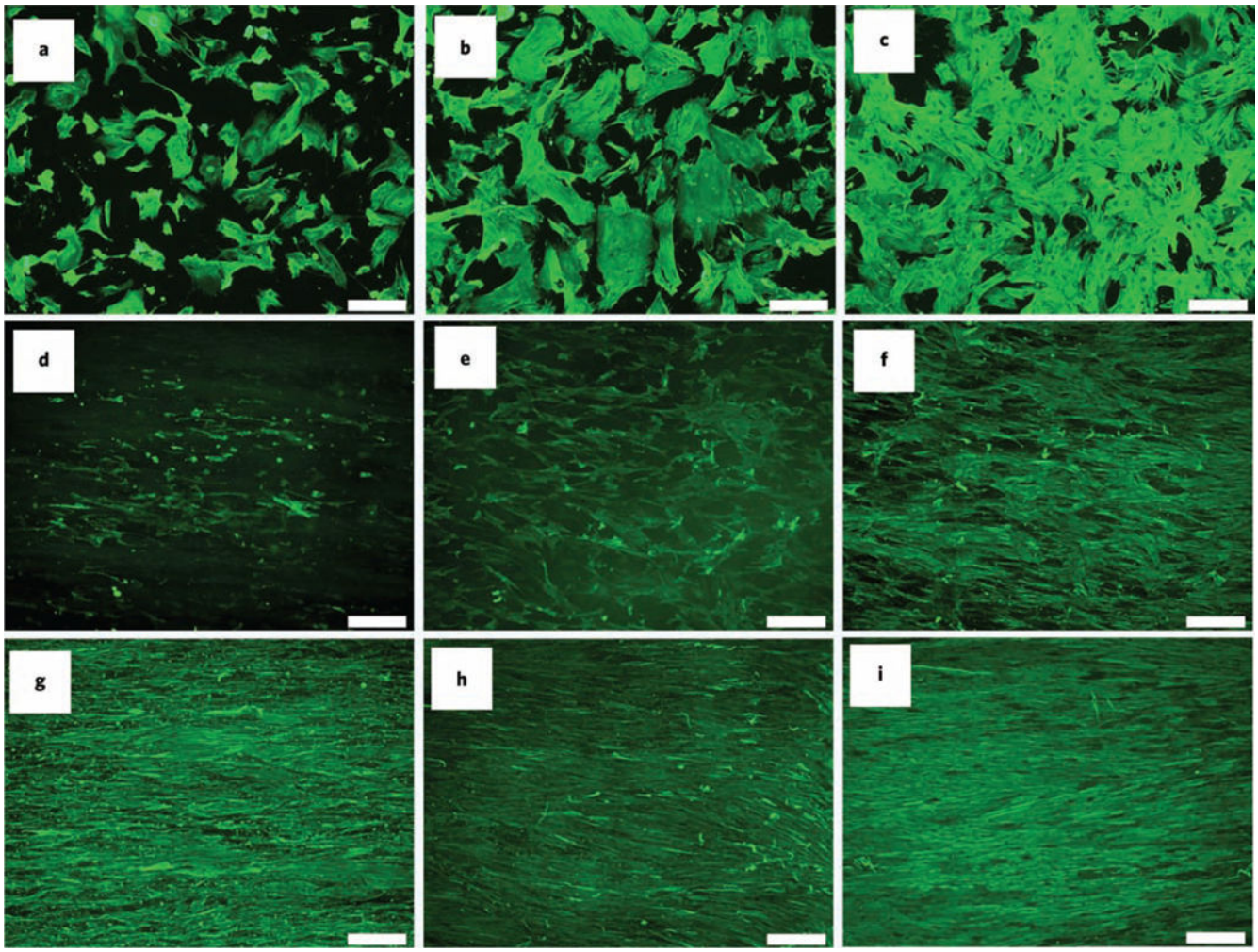

Figure 4.

Well plates and scaffolds stained with anti-smooth muscle actin (SMA) at 2, 4 and 6 weeks post-seeding. Well plates (top row) increased in SMA-positive cells from 2 weeks (left column) through 6 weeks (right column), and SMA expression remained consistently high. HS-coated scaffolds (middle row) increased expression from 2, 4 and 6 weeks. Collagen-coated scaffolds (bottom row) showed consistently high SMA staining. Scale bar = 200 μm.

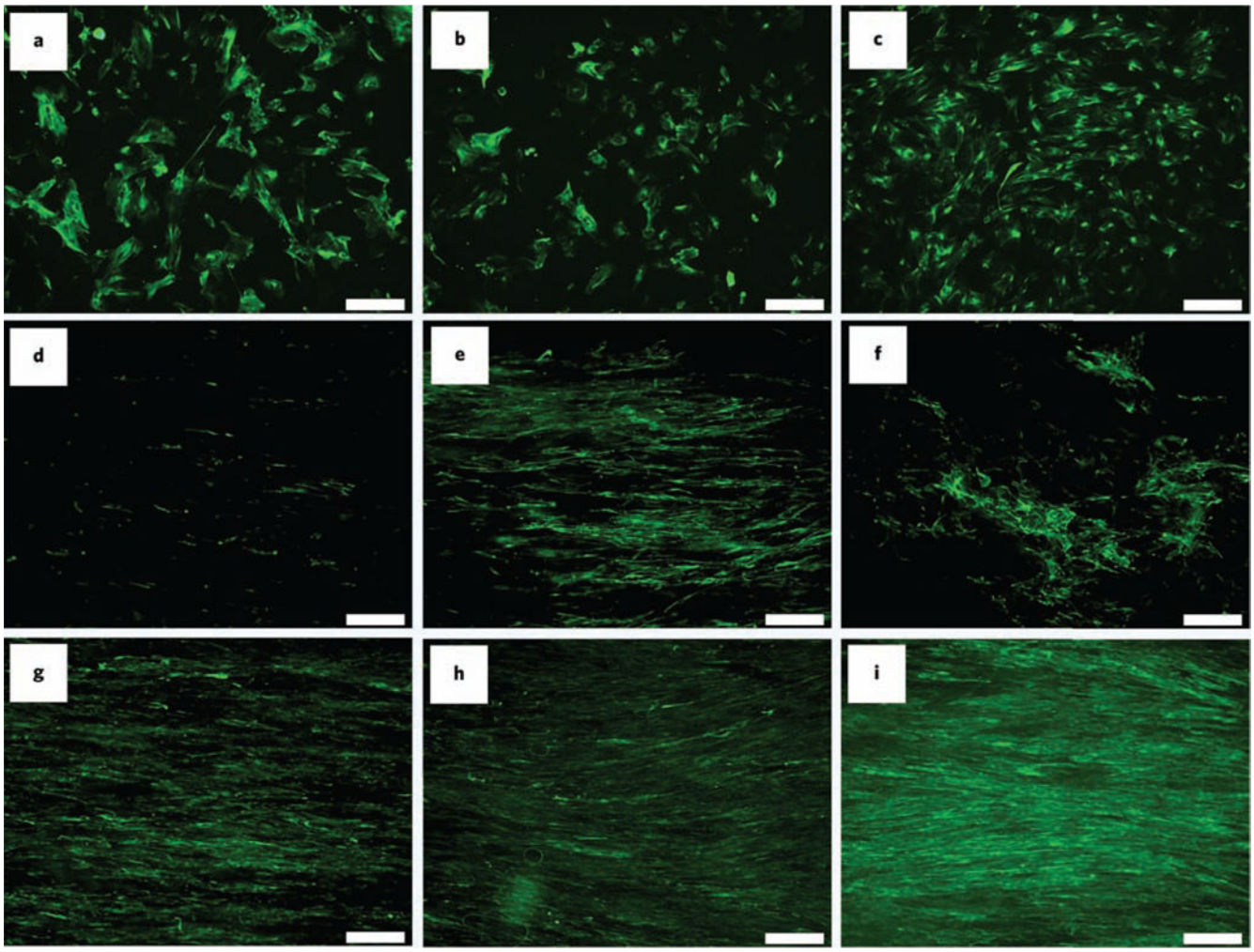

Figure 5.

Well plates and scaffolds stained with anti-desmin (DES) at 2, 4 and 6 weeks post-seeding. Well plates (top row) increased in DES-positive cells from 2 weeks (left column) through 6 weeks (right column). HS-coated scaffolds (middle row) increased expression from 2, 4 and 6 weeks. Collagen-coated scaffolds (bottom row) showed consistently high DES staining, and increased slightly over the 6-week duration. Scale bar = 200 μm.

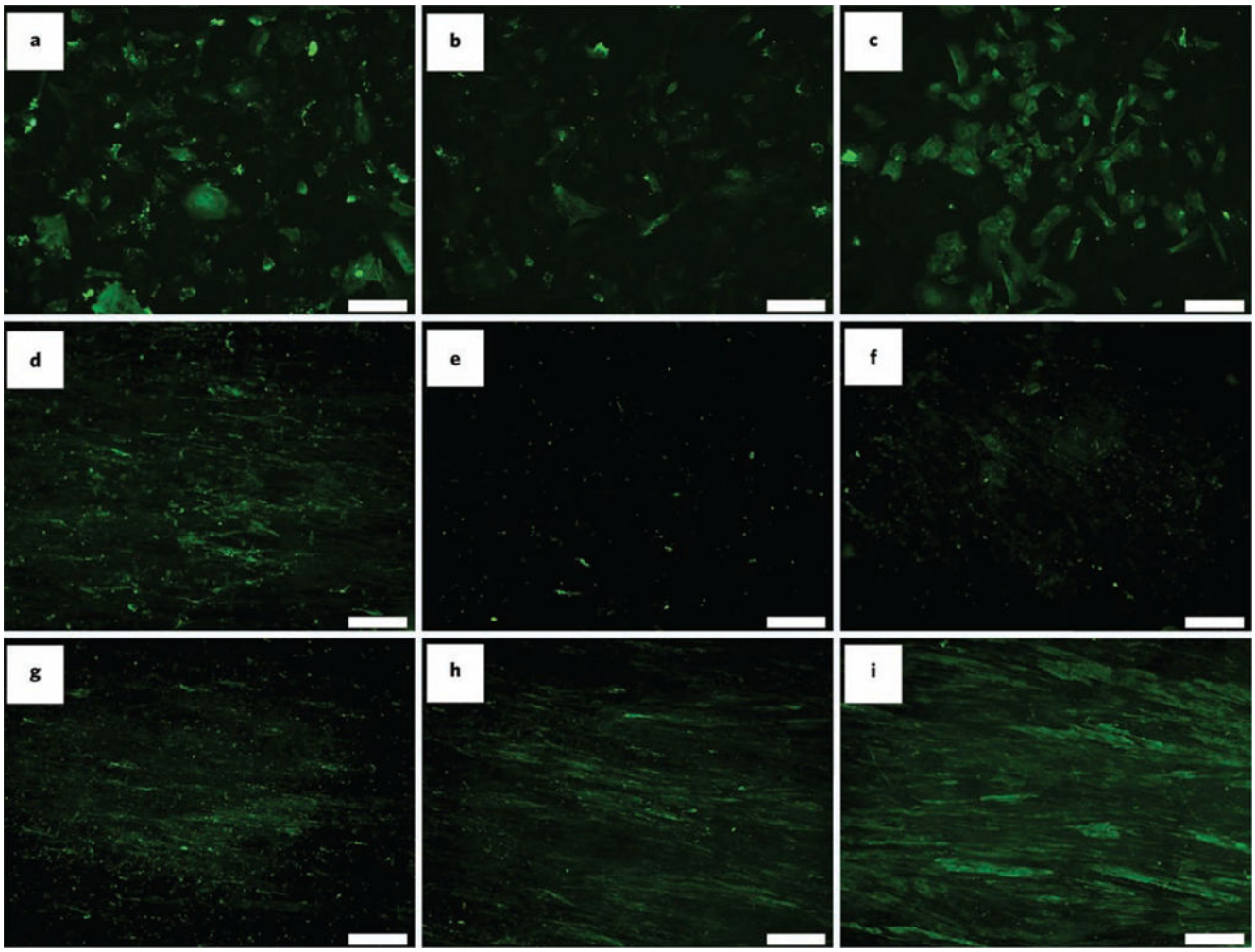

Figure 6.

Well plates and scaffolds stained with anti-myosin heavy chain (MHC) at 2, 4 and 6 weeks post-seeding. Well plates (top row) had consistently low numbers of MHC-positive cells from 2 weeks (left column) through 6 weeks (right column). HS-coated scaffolds (middle row) had low levels of MHC expression from 2, 4 and 6 weeks. Collagen-coated scaffolds (bottom row) had some MHC expression that increased in week 6. Scale bar = 200 μm.

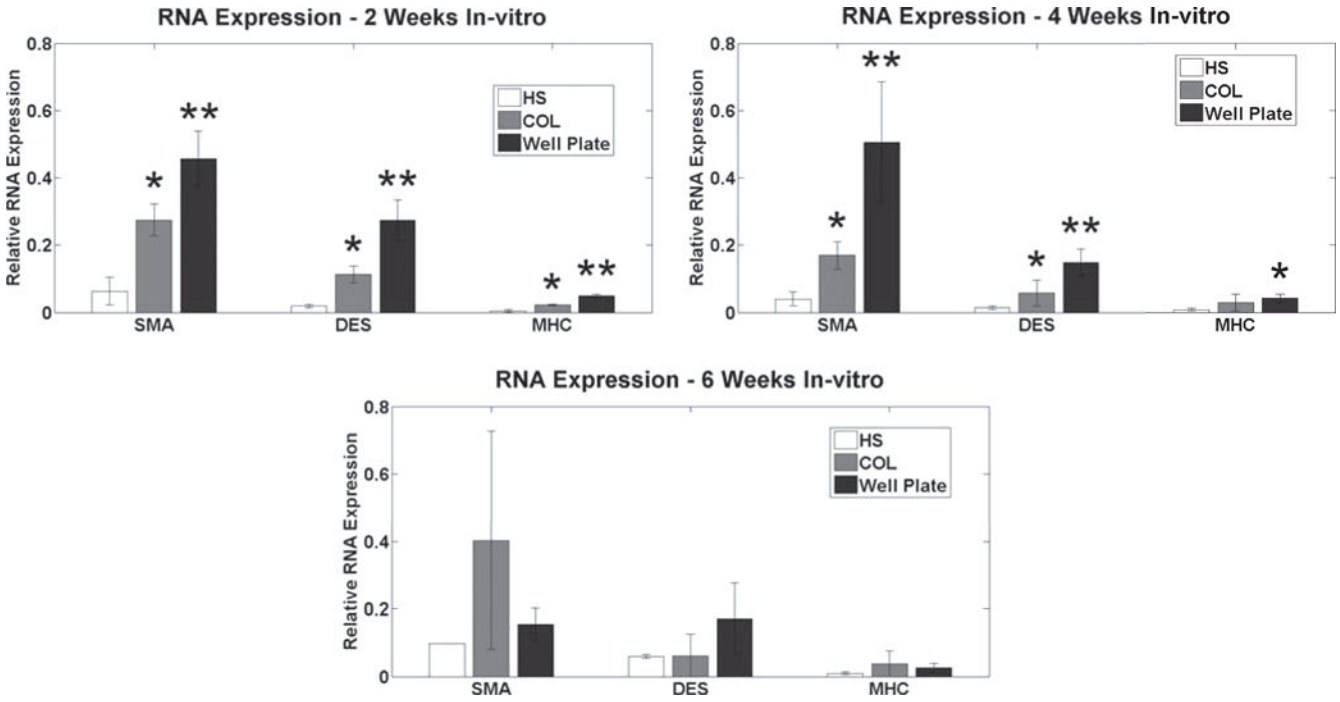

Figure 7.

RNA expression 2, 4 and 6 weeks after seeding as quantified with qPCR and normalized to strips of smooth muscle. HS, HS-coated; COL, collagen-coated. After 2 and 4 weeks in culture, well plates had significantly greater expression of SMA, DES and MHC compared to both HS-coated and collagen-coated scaffolds. Collagen-coated scaffolds had significantly more expression of SMA, and DES at both 2 and 4 weeks, and MHC at 2 weeks compared to HS-coated scaffolds. By 6 weeks, there were no statistically significant differences between any of the culture conditions. (*denotes $p < 0.05$ compared to HS-coated scaffolds, **denotes $p < 0.05$ compared to collagen-coated scaffolds).

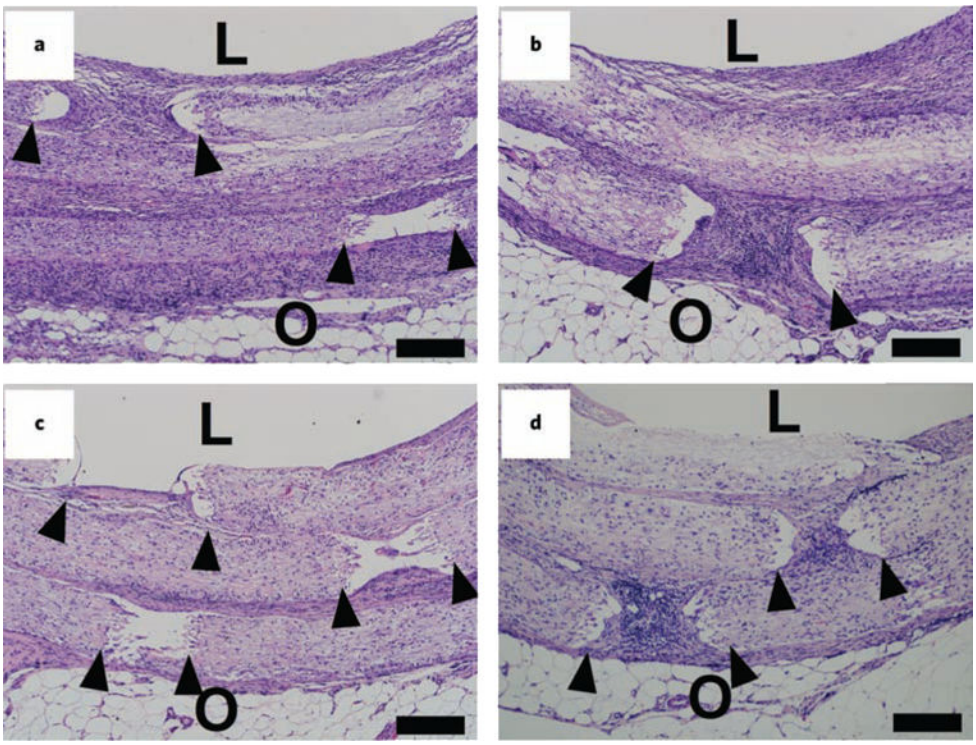

Figure 8.

H&E stained implants collected 2 and 6 weeks post-implantation. HS-coated implants (**a,c**) and collagen-coated implants (**b,d**) were harvested at either 2 weeks (HS, a; collagen, b) or 6 weeks (HS, c; collagen, d) *in vivo*. Scaffolds are labeled with omentum (O) and luminal (L) labels on the outer- and innermost layers, respectively, and arrows mark the edges of laser-cut holes through the PCL layers. Scale bar = 200 μm.

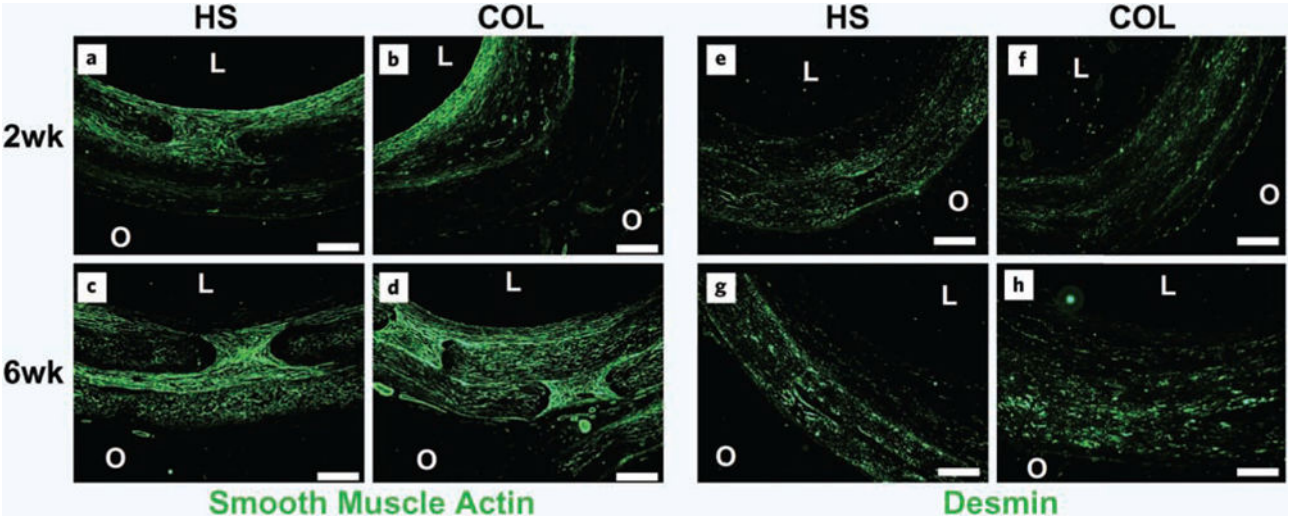

Figure 9.

Scaffolds harvested after implantation for 2 or 6 weeks and labeled for SMA (left) or DES (right). HS-coated implants (**a,c,e,g**) and collagen-coated implants (**b**,**d**,**f**,**h**) were harvested at either 2 weeks (**a,b,e,f**) or 6 weeks (**c,d,g,h**) *in vivo*. There was consistently high expression of SMA in both scaffolds and at both time points. Between 2 and 6 weeks, there was a slight increase in DES immunofluorescence in both HS- and collagen-coated implants. Scale bar = 200 μm. Omentum (O) and luminal (L) are labeled adjacent to the outer- and innermost layers, respectively.

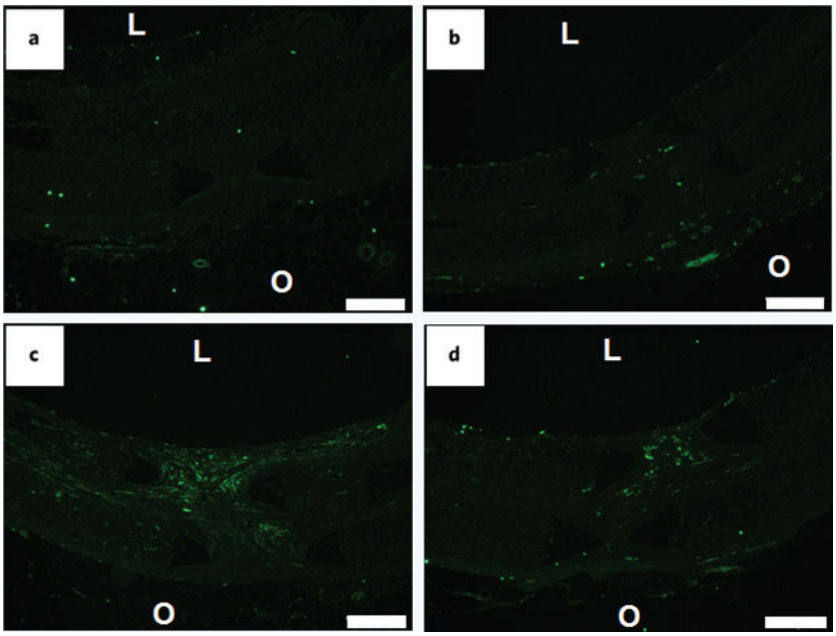

Figure 10.

Scaffolds harvested after implantation for 2 or 6 weeks and labeled for MHC. HS-coated implants (**a,c**) and collagen-coated implants (**b,d**) were harvested at either 2 weeks (HS, a; collagen, b) or 6 weeks (HS, c; collagen, d) *in vivo*. There was very little MHC expression in both HS- and collagen-coated scaffolds at both time points. Scale bar = 200 μm. Omentum (O) and luminal (L) are labeled adjacent to the outer- and innermost layers, respectively.

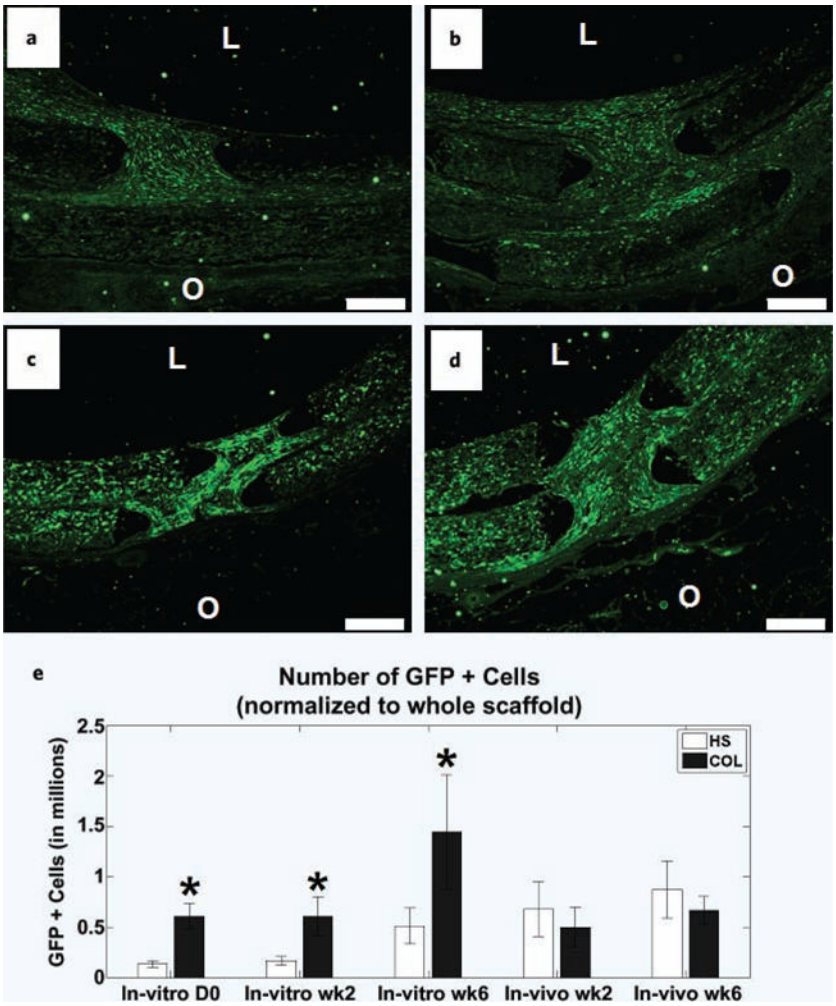

Figure 11.

Scaffolds harvested after implantation for 2 or 6 weeks and labeled for GFP. HS-coated implants (**a,c**) and collagen-coated implants (**b,d**) were harvested at either 2 weeks (HS, a; collagen, b) or 6 weeks (HS, c; collagen, d) *in vivo*. Green fluorescent protein (GFP)-expressing cells were present in both scaffold coatings at 2 weeks and increased at 6 weeks. Scale bar = 200 μm. GFP-positive DNA-quantified by qPCR on scaffolds grown *in vitro* and *in vivo* (**e**). Scaffolds were seeded with 500,000 cells 2 weeks prior to "D0" — simultaneous with day 0 of implantation of rolled scaffolds. "Wk2" corresponds to 2 weeks after "implantation", or 4 weeks after cell seeding. "Wk6" corresponds to 6 weeks after "implantation", or 8 weeks days after cell seeding. Collagen-coated scaffolds grown *in vitro* had significantly more GFP+ cells compared to HS-coated scaffolds at every time point. Scaffolds that were implanted were cell-seeded with 500,000 cells 2 weeks prior to implantation and retrieved after 2 or 6 weeks — simultaneous with 2 or 6 weeks of *in vitro* scaffolds. There was no statistical difference between HS- and collagen-coated scaffolds at either week 2 or week 6. (*denotes $p < 0.05$ compared to HS-coated implants). Omentum (O) and luminal (L) are labeled adjacent to the outer- and innermost layers, respectively.

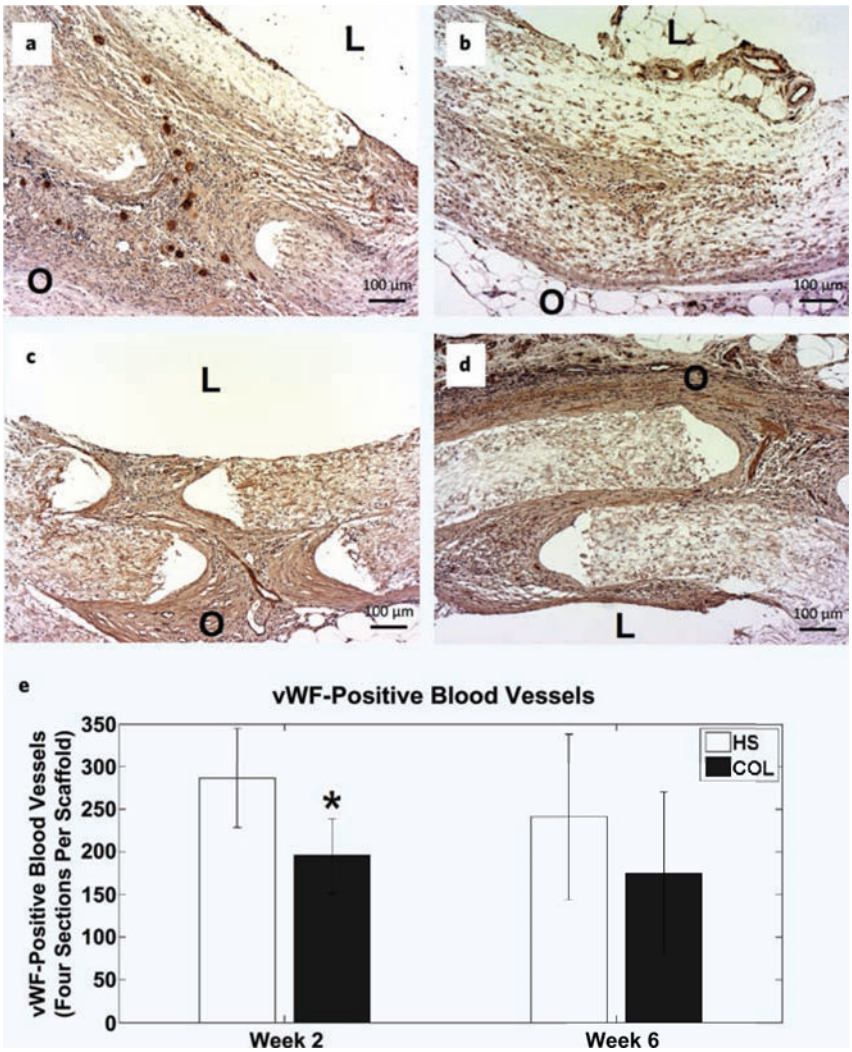

Figure 12.

Immunohistochemical evaluation of implants for von Willebrand factor (vWF) positive blood vessels. Angiogenesis was quantified with anti-vWF antibodies, diaminobenzidine (DAB) stain, and counterstained with hematoxylin A. There was an increase in blood vessels in HS-coated implants (**a**) compared to collagen-coated implants (**c**) after 2 weeks, but there was no difference in blood vessel infiltration in either HS-coated (**b**) or collagen-coated implants (**d**) after 6 weeks. Scale bar = 100 µm. Blood vessels were stained for vWF and positive vessels were quantified (**e**). There was a statistical increases in blood vessel infiltration between the HS- and collagen-coated scaffolds at week 2, but not week 6 ($p < 0.05$, *). Omentum (O) and luminal (L) are labeled adjacent to the outer- and innermost layers, respectively.