Biomimetic Regeneration of Elastin Matrices Using Hyaluronan and Copper Ion Cues

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Current efforts to tissue engineer elastin-rich vascular constructs and grafts are limited because of the poor elastogenesis of adult vascular smooth muscle cells (SMCs) and the unavailability of appropriate cues to up-regulate and enhance cross-linking of elastin precursors (tropoelastin) into organized, mature elastin fibers. We earlier showed that hyaluronan (HA) fragments greatly enhance tropo- and matrix-elastin synthesis by SMCs, although the yield of matrix elastin is low. To improve matrix yields, here we investigate the benefits of adding copper (Cu^{2+}) ions (0.01 M and 0.1 M), concurrent with HA (756–2000 kDa), to enhance lysyl oxidase (LOX)-mediated elastin cross-linking machinery. Although absolute elastin amounts in test groups were not different from those in controls, on a per-cell basis, 0.1 M of Cu^{2+} ions slowed cell proliferation (5.6 ± 2.3 -fold increase over 21 days vs 22.9 ± 4.2 -fold for non-additive controls), stimulated synthesis of collagen (4.1 ± 0.4 -fold), tropoelastin (4.1 ± 0.05 -fold) and cross-linked matrix elastin (4.2 ± 0.7 -fold). LOX protein synthesis increased 2.5 times in the presence of 0.1 M of Cu^{2+} ions, and these trends were maintained even in the presence of HA fragments, although LOX functional activity remained unchanged in all cases. The abundance of elastin and LOX in cell layers cultured with 0.1 M of Cu^{2+} ions and HA fragments was qualitatively confirmed using immunofluorescence. Scanning electron microscopy images showed that SMC cultures supplemented with 0.1 M of Cu^{2+} ions and HA oligomers and large fragments exhibited better deposition of mature elastic fibers ($\sim 1 \mu\text{m}$ diameter). However, 0.01 M of Cu^{2+} ions did not have any beneficial effect on elastin regeneration. In conclusion, the results suggest that supplying 0.1 M of Cu^{2+} ions to SMCs to concurrently (a) enhance per-cell yield of elastin matrix while allowing cells to remain viable and synthetic and not density-arrested in long-term culture because of their moderating effects on otherwise rapid cell proliferation and (b) provide additional benefits of enhanced elastin fiber formation and cross-linking within these tissue-engineered constructs.

Introduction

ELASTIN IS A VITAL architectural component of connective tissues that critically endows the characteristics of elasticity and resilience, long-range deformability, and passive recoil without energy input.¹ These properties allow blood vessels to undergo repetitive extension and recoil. Studies have also shown that elastin biochemically regulates behavior of vascular cells to maintain them in a quiescent, non-synthetic, healthy phenotype. Thus, loss of vascular elastin due to excessive availability and activity of elastolytic enzymes,² defects in the elastin gene or elastic fiber assembly process, inflammatory vascular disease, or direct mechanical injury, can lead to vessel wall weakening and rupture and vascular abnormalities.^{3,4} In such cases, failure to restore a healthy vascular elastin matrix can severely compromise blood vessel homeostasis. In this context, implantation of synthetic elastomers or assembly of elastin peptides can replicate

the mechanics of native elastin, but the absence of associated cell-signaling proteins (e.g., fibrillin) prevents the constructs from eliciting native responses from vascular smooth muscle cells (SMCs).^{5–8} An alternative approach is to actively regenerate elastin structures *in vivo* and within tissue-engineered constructs although the current unavailability of scaffolds that can provide cellular cues necessary to up-regulate elastin synthesis and regenerate faithful mimics of native elastin limits this approach.^{9,10}

As mentioned above, elastic fibers are among the most difficult matrix structures to repair or regenerate because they contain other non-elastin protein components (e.g., fibrillin, elaunin) and have a highly regulated recruitment and deposition pattern and a multi-step hierarchical assembly process.¹¹ It has been well established that elastic fiber formation at the molecular level involves recruitment and patterned coacervation of cell-derived soluble tropoelastin precursors onto pre-formed templates of fibrillin-rich

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microfibrils¹² and their stabilization by lysyl oxidase (LOX)-catalyzed desmosine cross-linking.¹³ Hence, cues for elastin regeneration must be able to mimic the spatio-temporal sequence of these events, leading to elastin matrix assembly.

Because earlier studies have suggested a close association between several glycosaminoglycan (GAG) types (e.g., hyaluronic acid (HA), heparin sulfate), proteoglycans (e.g., versican), and elastin within pulmonary and vascular tissues,^{14,15} we investigated the elastin regenerative potential of HA.^{16–19} Most recently, we demonstrated that the elastogenic effects of HA are highly fragment specific, with HA fragments (<1 MDa) and shorter oligomers (<1 kDa) being more cell interactive and more highly elastogenic²⁰ than the relatively bioinert long-chain high molecular weight (HMW) HA (>1 MDa). Our results,^{16,17} however, suggest that the latter might facilitate elastin cross-linking and matrix deposition via purely physical interactions.²¹ Overall, these outcomes indicate the tremendous potential of HA and its fragments in elastin matrix regeneration.

A key impediment to creating highly mature elastin matrices is the absence of cues to improve production and cross-linking efficiency of elastin precursors (tropoelastin) by vascular SMCs. Although our studies show that HA significantly increases tropoelastin and total elastin (matrix elastin + tropoelastin) amounts on a per-cell basis, the net yield of cross-linked matrix elastin relative to the total elastin produced remained low.^{16,17} This stresses the need to provide other exogenous cues that will facilitate and improve the efficiency of tropoelastin recruitment and cross-linking into elastin matrix structures. One possible strategy to achieve this is to enhance cellular production or activity of the LOX enzyme that catalyzes elastin cross-linking.^{22–24} Because extracellular LOX availability and activity are dependent on the presence of Cu²⁺ ions, we hypothesized that the simultaneous delivery of HA and Cu²⁺ cues may enhance tropoelastin synthesis, recruitment, and cross-linking into mature elastin matrix. Thus, the objective of the current study was to evaluate the benefits of Cu²⁺ ion delivery concurrent with elastogenic cues, as represented by HA fragments and oligomers, on elastin cross-linking in a culture model of adult rat aortic SMCs (RASMCs).

Materials and Methods

Cell culture

HA with molecular weights of 2000 kDa, 20 kDa (Lifecore Technologies, Chaska, MN), and 0.76 kDa, designated as HMW, very low molecular weight (VLMW), and oligomers, respectively, were dissolved in sterile culture medium before being added to cell cultures. HA oligomer mixtures containing predominantly 4-mers (75 ± 15 % w/w, with 6-mers and 8-mers forming the balance) were prepared in the laboratory using protocols previously reported.¹⁷ Briefly, 20 mg of HMW HA (Genzyme Biosurgery, Cambridge, MA) was enzymatically digested with bovine *hyaluronidase testicular* (3.6 mg, 451 U/mg; Sigma-Aldrich, St. Louis, MO) in 4 mL of digest buffer (150 mM sodium chloride (NaCl), 100 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA) disodium salt, pH 5.0) for 18 h at 37°C. The enzyme activity was terminated by boiling the mixture in a water bath for 5 min after digestion, and the mixture was dialyzed against water for 12 h and frozen until use at –20°C.

Low-passage (3–5) adult RASMCs (Cell Applications, San Diego, CA) were selected for the current study because their lower levels of tropoelastin production than that of neonatal cells makes them more relevant to regeneration of elastin in adult blood vessels. RASMCs were seeded onto 6-well tissue culture plates (Becton Dickinson Labware, San Jose, CA) at a density of 3 × 10⁴ cells per well and treated with Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) containing 10% v/v fetal bovine serum and 1% v/v penicillin-streptomycin (VWR International, Westchester, PA). HA fragments prepared in serum-rich medium were added to cell cultures at an ultimate dose of 0.2 µg/mL. Copper sulfate (CuSO₄; Sigma-Aldrich) was dissolved in distilled water and added exogenously to the culture wells at final doses of 0.1 M or 0.01 M, except in control cultures, which received no supplements. The culture medium was replaced twice weekly, and the spent medium from each well was pooled over the 21-day culture period and frozen for further biochemical analysis. To isolate the observed effects on RASMCs from Cu²⁺ ions and not SO₄²⁻ ions, sodium sulfate (Na₂SO₄; Sigma-Aldrich; 0.01 and 0.1 M) was added instead of CuSO₄ to control cultures.

DNA assay for cell proliferation

The DNA content of cell layers was estimated using a fluorometric method described by Labarca and Paigen.²⁵ Briefly, cell layers at 1 and 21 days of culture were detached with 0.25% v/v trypsin-EDTA, pelleted using centrifugation, and resuspended in 1 mL of NaCl/Pi-buffer (4 M NaCl, 50 mM disodium phosphate, 2 mM EDTA, 0.02% sodium-azide, pH 7.4), and a 100-µL aliquot was assayed. The DNA in the sonicated aliquot was measured using the fluorometric assay, and the cell count was used to normalize the measured amounts of synthesized matrix for reliable comparison between experimental cases.¹⁶ Actual cell counts were calculated on the basis of an estimated 6 pg of DNA/cell.

Hydroxyl-proline assay for collagen

Collagen incorporated within cell layers cultured over 21 days and collagen released by the cells into the pooled medium over the same period were quantified using a hydroxyl-proline assay, as described previously.¹⁶ Briefly, the cell layers were homogenized in distilled water, pelleted using centrifugation (10,000 g, 10 min), and digested with 1 mL of 0.1 N sodium hydroxide (NaOH; 1 h, 98°C). The digestate was centrifuged to isolate a mass of insoluble, cross-linked elastin. The supernatant containing solubilized collagen and immature matrix elastin was neutralized with an equal volume of 12 N of hydrochloric acid, and divided into two equal volumetric halves. One half-volume was hydrolyzed at 110°C for 16 h and dried in a constant stream of N₂ gas overnight, and 20-µL aliquots of the reconstituted residue were assayed for hydroxy-proline content. Standards (0–300 µg/mL) were prepared using trans-4-hydroxyproline (Sigma-Aldrich). Standards and test samples (20 µL) were mixed with 250 µL of chloramine-T reagent (1.41 g chloramine-T in 10 mL of deionized water, 10 mL of n-propanol, and 80 mL of OH-Pro buffer, pH 6.0), 250 µL of a stock solution containing 15 g p-dimethyl amino benzaldehyde (Sigma-Aldrich) mixed in 60 mL of n-propanol and 26 mL of 70% v/v perchloric acid, heated to 60°C for 15 min, and the

absorbance measured at 558 nm. The OH-Pro buffer contained 50 g of citric acid, 120 g of sodium acetate, 24 g of NaOH, dissolved in 1.2 L of distilled water containing 12 mL of glacial acetic acid and 300 mL of n-propanol. The amounts of matrix-derived collagen (in the cell layers) and soluble collagen (in pooled medium fraction) were calculated based on the 13.2% content of hydroxyl-proline in collagen.

Fastin assay for elastin

The amount of elastin present in deposited matrix as alkali-soluble and cross-linked alkali-insoluble fractions and in pooled medium aliquots as elastin precursor (tropoelastin) was quantified using a Fastin assay (Accurate Scientific Corp, Westbury, NY) as described previously.¹⁶ Because the Fastin assay quantifies only soluble α -elastin, the insoluble elastin was first reduced to a soluble form. To do this, the elastin pellet, obtained by digesting the harvested cell layer with 0.1 N of NaOH over 1 h, was dried to a constant weight and solubilized three times with 0.25 N of oxalic acid (1 h/cycle, 95°C), and the pooled digests were then filtered in microcentrifuge tubes fitted with low molecular weight cut-off membranes (10,000 Da). The insufficiently cross-linked, soluble elastin fraction retained in the oxalic acid-free fraction and in the water-reconstituted hydrolysate (from the collagen assay above) were also quantified using the Fastin assay. Spent fractions of media pooled twice a week over the 3-week culture period were lyophilized and processed for tropoelastin using the Fastin assay. Standards (0–100 μ g) for the Fastin assay were prepared using bovine α -elastin (1 mg/mL) provided with the assay kit. Quantification of the α -elastin was based on release of a bound elastin-specific dye (tetraphenyl porphine sulfonate (TPPS) in citrate phosphate buffer, pH 7) upon treatment with a destaining reagent (methanol-ammonia) and measurement of its absorbance at 513 nm. The volume-corrected amounts of synthesized matrix were normalized to the respective DNA amounts to provide a reliable basis of comparison between samples and to broadly assess whether the observed changes in the amount of matrix synthesized could be due to increases in elastin production on a per-cell basis.

Western blot analysis of LOX

Western blot analysis was performed using methods described previously¹⁷ to semi-quantitatively assess benefits, if any, to LOX protein synthesis. Briefly, aliquots of spent medium from cultures were pooled twice a week over the 21-day culture period, lyophilized, and assayed for protein content using a DC protein assay kit (Biorad Corporation, Hercules, CA) to further optimize sample volumes for sodium dodecyl sulfate polyacrylamide gel electrophoresis Western blot. Protein bands were detected with primary rabbit and rat polyclonal antibodies to the 31-kDa active LOX protein (Santa Cruz Biotechnology, Santa Cruz, CA), visualized, and quantified using a Chemi-Imager IS 4400 system (Alpha Innotech, San Leandro, CA).

LOX-functional activity

Spent culture medium pooled at day 21 of RASMC culture was assayed for LOX enzyme activity using a fluorometric assay based on generation of hydrogen peroxide (H_2O_2)

when LOX acts on a synthetic substrate. H_2O_2 was detected using an Amplex red kit (Molecular Probes, Eugene, OR) as described previously.²⁶ The fluorescence intensities were recorded with excitation and emission wavelengths at 560 and 590 nm, respectively.

Immunofluorescence detection of elastin, fibrillin, and LOX

Immunofluorescence techniques were used to confirm elastin, fibrillin, and LOX expression by cells that were cultured under conditions deemed necessary to upregulate elastin synthesis (from biochemical analysis). RASMCs were seeded in 4-well sterile chamber slides at 5×10^3 cells per well and cultured with HA and $CuSO_4$ cues, as described earlier. At 21 days, the cell layers were fixed with 4% w/v paraformaldehyde for 10 min and labeled with Alexa 488 Phalloidin (Molecular Probes; 1:20 dilution; 20 min, 25°C), a marker for SMC actin. The target proteins were detected with polyclonal antibodies to elastin, fibrillin-1 (Elastin Products Inc., Owensville, MO), and LOX (Santa Cruz Biotechnology) and visualized using a rhodamine-conjugated donkey anti-rabbit immunoglobulin G secondary antibody (Chemicon, Temecula, CA) on a fluorescence microscope. Cell nuclei were visualized with the nuclear stain 4', 6-diamino-2-phenylindole dihydrochloride contained in the mounting medium (Vectashield; Vector Labs, Burlingame, CA).

Matrix ultrastructure

Scanning electron microscopy was used to discern the structural organization of matrix elastin within cell layers cultured with or without exogenous HA cues and various provided doses of copper ions. For sample preparation, medium-aspirated cell layers were incubated in 1 N of NaOH for 2 h at 60°C to digest the cells and the non-elastogenous matrix, fixed with 2% w/v glutaraldehyde (4°C, 1 h), and

FIG. 1. Proliferation ratios of rat aortic smooth muscle cells supplemented with sodium sulfate alone, copper sulfate alone, or together with HA fragments (0.2 μ g/mL). Data shown represent means $\pm$ standard deviations of cell count after 21 days of culture, normalized to initial seeding density and further normalized to control cultures that received no additives ($n=3$ /case). * $P < 0.05$ represents significant differences from controls.

FIG. 2. Effects of exogenous copper sulfate with or without hyaluronan fragments (0.2 μ g/mL) on collagen (A), tropoelastin (B), alkali-soluble matrix elastin (C), and cross-linked alkali-insoluble matrix elastin (D), synthesized by adult rat aortic smooth muscle cells. Values (means $\pm$ standard deviations) are shown normalized to the DNA content of the respective cell layers at 21 days of culture ($n=3$ /case) relative to control cultures. * $P < 0.05$ represents significant differences relative to control cultures.

treated with increasing ethanol gradient series (60–100% v/v, each for 15 min). The dried matrix layers were sputter gold-coated at 30 mA for 1 min and visualized in a Hitachi S4800 field emission scanning electron microscope (Hitachi High Technologies America, Pleasanton, CA).

Statistical analysis

All experiments were performed in triplicate and quantitative results reported as means $\pm$ standard deviations (SDs). Statistical significance between and within groups was determined using two-way analysis of variance. Results were deemed to be significantly different from controls at $p < 0.05$.

Results

Cell proliferation

Proliferation ratios of RASMCs cultured in the presence of CuSO₄ alone or together with HA fragments and oligomers are shown in Figure 1. The effects of Na₂SO₄ addition are also shown for comparison. Addition of Na₂SO₄ alone had no effect on RASMC morphology or proliferation ($p=0.4$ vs controls), irrespective of the added dose. CuSO₄ (0.1 M) in-

duced significant rounding of RASMCs in the first 3 days after its addition, although the cells recovered sufficiently to exhibit normal morphology throughout the rest of the culture period. When 0.01 M of CuSO₄ was added, the temporal change in cell morphology induced by the 0.1 M CuSO₄ was not observed. At 3 weeks of culture, the cell proliferation ratio (ratio of cell number at day 21 to day 1) for 0.1 M CuSO₄ cultures was 5.6 ± 2.3 -fold, whereas those in cultures that received 0.01 M of CuSO₄ and in non-additive controls were 44.5 ± 6.7 -fold, and 22.9 ± 4.2 -fold, respectively. Thus, in effect, cell numbers in 0.1 M and 0.01 M CuSO₄-supplemented cultures at 21 days were 0.24 ± 0.1 and 1.9 ± 0.3 times greater than that in non-additive controls ($p=0.005$ and 0.001 vs control). Cell proliferation ratios in the presence of both HA fragments (all sizes) and CuSO₄ (0.1 M or 0.01 M) were not different from those in cultures that received the respective doses of CuSO₄ alone.

Matrix synthesis

The data shown in Figure 2 represent means $\pm$ SDs of protein synthesized ($n=3$ /case) in test groups normalized initially to their respective cellular DNA contents at 21 days

TABLE 1. ABSOLUTE AMOUNTS OF TOTAL COLLAGEN, TROPOELASTIN, SOLUBLE MATRIX ELASTIN, AND CROSS-LINKED MATRIX ELASTIN PRODUCED BY RAT AORTIC SMOOTH MUSCLE CELLS SUPPLEMENTED WITH SODIUM SULFATE ALONE OR COPPER SULFATE WITH AND WITHOUT HYALURONAN FRAGMENTS (OLIGOMERS, VERY LOW MOLECULAR WEIGHT AND HIGH MOLECULAR WEIGHT) OVER THE 21-DAY CULTURE PERIOD

	Total collagen produced, mg		Tropoelastin produced, mg		Soluble matrix elastin, mg		Crosslinked matrix elastin, mg	
	0.1 M	0.01 M	0.1 M	0.01 M	0.1 M	0.01 M	0.1 M	0.01 M
	Mean $\pm$ Standard Deviation							
Controls	5.4 $\pm$ 0.5		82.6 $\pm$ 1.4		22.2 $\pm$ 1.5		0.51 $\pm$ 0.08	
Sodium sulfate	5.7 $\pm$ 0.9	5.8 $\pm$ 0.8	81.7 $\pm$ 1.2	81.3 $\pm$ 0.7	22.2 $\pm$ 0.8	23 $\pm$ 0.5	0.77 $\pm$ 0.1	0.6 $\pm$ 0.05
CuSO ₄	5.5 $\pm$ 0.5	5.4 $\pm$ 0.5	83.7 $\pm$ 1.4	82.5 $\pm$ 2.3	22.1 $\pm$ 0.7	21.6 $\pm$ 2.5	0.52 $\pm$ 0.1	0.58 $\pm$ 0.1
CuSO ₄ -Oligos	5.1 $\pm$ 0.4	5.0 $\pm$ 0.3	89.3 $\pm$ 2.6	84.8 $\pm$ 0.3	19.7 $\pm$ 3.2	23.6 $\pm$ 0.8	0.58 $\pm$ 0.1	0.87 $\pm$ 0.2
Very-low molecular-weight CuSO ₄	5.3 $\pm$ 0.9	4.9 $\pm$ 0.3	81.7 $\pm$ 0.9	83.4 $\pm$ 0.7	24.3 $\pm$ 1.2	5.2 $\pm$ 0.2	0.79 $\pm$ 0.2	0.68 $\pm$ 0.1
High-molecular-weight CuSO ₄	5.5 $\pm$ 0.7	5.0 $\pm$ 0.7	82.4 $\pm$ 0.7	82.6 $\pm$ 0.9	16.6 $\pm$ 3	21.2 $\pm$ 0.3	0.73 $\pm$ 0.1	0.98 $\pm$ 0.4

The data shown here are not normalized to DNA content (i.e., cell number).
CuSO₄, copper sulfate.

and further normalized to the corresponding protein content in non-additive control cultures. The absolute (not normalized to DNA) amounts of total collagen, tropoelastin, and matrix elastin produced in each case are shown in Table 1. As evident from Figure 2A, on a per-cell basis, 0.1 and 0.01 M doses of Na₂SO₄ did not significantly affect total (matrix + soluble) collagen output by RASMCs (0.94 $\pm$ 0.15 and 1.1 $\pm$ 0.13 times greater than controls; p = 0.2 and 0.1, respectively). When 0.1 M CuSO₄ alone was provided, synthesis of collagen (on a per-ng of DNA basis) was 4.1 $\pm$ 0.4 times greater than with non-additive controls (1332 $\pm$ 140 ng/ng of DNA), whereas addition of 0.01 M CuSO₄ suppressed collagen production to a 0.5 $\pm$ 0.04 fraction of controls (p < 0.001 vs controls). In the presence of oligomers, VLMW and HMW HA, 0.1 M CuSO₄ enhanced control levels of collagen synthesis by 4.6 $\pm$ 0.4, 4.5 $\pm$ 0.8 and 4.5 $\pm$ 0.6 times, respectively, whereas 0.01 M CuSO₄ consistently suppressed collagen synthesis in all cases (p < 0.001 vs controls). However, absolute (not normalized to DNA content) collagen production levels in all cases were not different from those in non-additive control cultures (Table 1).

The trends in tropoelastin production by RASMCs (Fig. 2B) closely mirrored those observed for collagen synthesis under identical conditions. Na₂SO₄ had no effect on control levels of tropoelastin production by RASMCs (20,074 $\pm$ 1,240 ng/ng of DNA), irrespective of added dose (p = 0.5 vs controls). In the absence of HA, 0.1 M CuSO₄ enhanced control levels of tropoelastin production on a per-ng of DNA basis by 4.1 $\pm$ 0.05 times (p < 0.001 vs control), whereas 0.01 M CuSO₄ inhibited the same to a 0.5 $\pm$ 0.04 fraction of control values (p < 0.001 vs controls). When provided together with HA fragments, 0.1 M CuSO₄ likewise enhanced tropoelastin production, whereas 0.01 M CuSO₄ marginally decreased it (p < 0.03 in all cases vs controls). No HA fragment size-dependent effects were noted in either case, although in the presence of 0.1 M CuSO₄, HA oligomers stimulated significantly more tropoelastin production (1.3 $\pm$ 0.01 times) than in cultures that received CuSO₄ alone (p < 0.001). As apparent in Table 1, the absolute amounts of tropoelastin produced (not normalized to DNA amounts) in all cases, irrespective of CuSO₄ dose and HA fragment size, were almost identical to those of controls.

Elastin incorporated into the matrix was measured as a sum of two individual fractions (i.e., a highly cross-linked,

alkali-insoluble elastin pellet and an alkali-soluble fraction). As shown in Figure 2C, irrespective of provided dose, Na₂SO₄ had no effect on control production levels of alkali-soluble and insoluble matrix elastin on a per-ng of DNA basis (p > 0.4 vs controls). Addition of 0.1 M CuSO₄ alone increased soluble elastin synthesis dramatically by 4.1 $\pm$ 0.1 times, whereas 0.01 M CuSO₄ inhibited the same to a 0.5 $\pm$ 0.4 fraction of non-additive control values (5388 $\pm$ 363 ng/ng of DNA; p = 0.001 and 0.27 vs controls, respectively). In the presence of HA fragments, 0.01 M CuSO₄ consistently suppressed production of alkali-soluble matrix elastin (p < 0.01 vs controls; per ng of DNA basis), whereas addition of 0.1 M CuSO₄ dramatically increased the same by 4.47 $\pm$ 0.7, 5.02 $\pm$ 0.25 and 3.38 $\pm$ 0.6 times upon concurrent addition of HA oligomers, VLMW, and HMW HA, respectively (p < 0.05 vs controls). Differences in outcomes between these cases were deemed to be statistically insignificant.

On a per-ng of DNA basis, addition of Na₂SO₄ did not influence control production levels of alkali-insoluble, cross-linked matrix elastin (i.e., structural elastin) (Fig. 2D). When 0.1 M of CuSO₄ was delivered alone, cross-linked matrix elastin synthesis (122 $\pm$ 19 ng/ng of DNA) was 4.2 $\pm$ 0.7 times greater than control levels, whereas concurrent addition of oligomers and VLMW and HMW HA increased the same by 5.8 $\pm$ 0.7, 7.2 $\pm$ 1.4, and 6.5 $\pm$ 0.9 times, respectively (p < 0.001 vs controls in all cases). However, when 0.01 M CuSO₄ was supplemented alone or together with HA fragments, there was no benefit to cross-linked matrix elastin synthesis (on a per-ng of DNA basis), over controls (0.6 $\pm$ 0.1, 1.2 $\pm$ 0.2, 0.8 $\pm$ 0.1 and 1.02 $\pm$ 0.4 times in the presence of 0.01 M CuSO₄ alone or together with oligomers and VLMW and HMW HA, respectively; p > 0.05 vs controls in all cases). However, irrespective of added HA fragment size and CuSO₄ dose, the absolute production levels (not normalized to DNA amounts) of alkali-soluble and -insoluble matrix elastin were not different from control cultures (see Table 1).

Western blots for LOX synthesis

Spent medium fractions pooled over 21 days from test and control cultures were analyzed using Western blot, and the DNA-normalized intensities of the LOX-protein bands within test cultures were further normalized to those in

FIG. 3. Lysyl oxidase (LOX) protein amounts in pooled medium aliquots collected over 21 days of culture. Shown are means $\pm$ standard deviations of DNA-normalized intensities, measured from representative sodium dodecyl sulfate polyacrylamide gel electrophoresis Western blots containing bands corresponding to LOX produced in the respective cases.

controls (Fig. 3). Exogenous CuSO₄ (0.01 M) alone and together with HA fragments did not enhance LOX protein synthesis more than in controls; there were no differences in LOX synthesis between cultures that received different-sized HA fragments either, but LOX protein synthesis was 2.7 ± 0.3 greater in the presence of 0.1 M CuSO₄ alone and up to 3.5 times greater when HA fragments were also provided ($p < 0.01$ vs controls, in all cases).

LOX functional activity

Figure 4 shows the effect of addition of CuSO₄ alone or together with HA fragments on LOX enzyme activity. LOX activity was measured in the spent culture medium after 21 days of culture. Addition of 0.1 M CuSO₄ alone and concurrent with HA fragments had no significant effect on basal LOX functional activity. LOX activities measured in cultures that received a 0.01-M dose of CuSO₄ were significantly higher than that in controls in most cases (1.4 ± 0.2 , 1.12 ± 0.1 , 1.4 ± 0.02 , and 1.3 ± 0.17 for 0.01 M CuSO₄ alone and with oligomers and VLMW and HMW HA, respectively; $p < 0.05$ vs control).

Immunodetection of elastin, fibrillin, and LOX in cell layers

Immunofluorescence micrographs of 21-day-old cell layers (Fig. 5) confirmed the presence of elastin, fibrillin, and LOX (red fluorescence) in cultures that received 0.1 M CuSO₄ alone or together with HA fragments. Fluorescence intensity due to elastin was visibly greater in cultures supplemented with CuSO₄, particularly those that also received HA fragments, than in control cultures. Fluorescence intensity due to fibrillin was also greater in CuSO₄-supplemented cultures than in controls, although it was most pronounced in cultures that also received VLMW and HMW HA. However, fluorescence due to LOX was relatively weak in all cultures.

FIG. 4. Lysyl oxidase (LOX) enzyme activities in cultures treated with copper sulfate and hyaluronan fragments. Values (means $\pm$ standard deviation) are shown normalized to the LOX activity measured in control cell layers at 21 days of culture ($n = 3$ /case). * $P < 0.05$ represents significance in differences relative to controls.

Structural analysis of matrix elastin

Figure 6 shows representative scanning electron micrographs of elastin matrices isolated from 21-day cultures. Addition of CuSO₄ with or without other HA fragments appeared to enhance matrix elastin amounts much more than the non-additive control cultures where elastin was sparingly deposited as amorphous clumps (Fig. 6A). However, addition of 0.01 M CuSO₄ alone or together with HMW HA resulted in featureless clump-like deposits, probably of amorphous elastin (Fig. 6B, C). CuSO₄ (0.1 M) and HA oligomers together promoted deposition of elongated, aggregating elastin fibrils (Fig. 6E), different from the discrete clumps of amorphous elastin that were uniformly distributed within cell layers when 0.1 M CuSO₄ was provided alone (Fig. 6D). When 0.1 M of CuSO₄ and HMW HA were provided together, elastin fiber formation was likewise favored, with the matrix containing more apparently fully formed fibers ($\sim 1 \mu\text{m}$ diameter; Fig. 6F) than in cultures provided with HA oligomers.

Discussion

Our long-term research goal is to develop exogenous or biomaterial-based cues to stimulate regeneration of structural and functional mimics of vascular elastin matrices on demand. Despite a variety of prior strategies to tissue engineer elastin-rich constructs,^{27–30} poor tropoelastin mRNA expression by adult cells have severely limited positive outcomes.^{31,32} As mentioned in the Introduction, our ongoing study of HA cues for elastin regeneration is driven by prior work that suggested that several GAG types (HA, heparin sulfate) have roles in elastin synthesis, maturation, and organization *in vivo*.³³ For example, it has been suggested that HA has strongly binds to versican,³³ probably to facilitate its further interaction with elastin-associated microfibrils to form higher-order structures important for elastin fiber assembly.^{34,35} Also, it has been suggested that

FIG. 5. Immunodetection of elastin, fibrillin and lysyl oxidase (LOX; red) within rat aortic smooth muscle cell layers after 21 days of culture in the presence of copper sulfate alone (0.1 M) or together with hyaluronan-fragments (0.2 $\mu\text{g}/\text{mL}$); control cultures received no additives. Immunolabeling controls received no primary antibodies and exhibited no background fluorescence when treated with the fluorophore-labeled secondary probe. Color images available online at www.liebertonline.com/ten.

anionic HA chains coacervate soluble tropoelastin to facilitate its cross-linking locally into a stable matrix.^{36,37} Because the physico-chemical and biological properties of HA are often dependent on its size,³⁸ we have sought to investigate these differences in the context of stimulating elastin matrix synthesis.^{16,17}

In other studies, copper ions (Cu^{2+}) have been shown to minimize vascular defects,³⁹ promote angiogenesis,⁴⁰ and enhance LOX enzyme activity.⁴¹ Deficiency of nutritional copper has been linked to vascular lesions and development of aneurysms.^{42–44} One study showed that, in the presence of HA, low doses (0.01 M) of CuSO_4 enhance tissue vascularization.⁴⁵ Dahl *et al.* investigated the singular effects of increasing medium Cu^{2+} ion concentration on matrix cross-linking efficiency in an engineered vascular-like tissue and showed that increasing levels of cross-links formed.⁴⁶ However, the synergistic benefits of HA and Cu^{2+} on elastin matrix regeneration by improving tropoelastin and matrix

elastin synthesis and cross-linking has not been elucidated. Thus, in addition to investigating this in an *in vitro* culture model, we have sought to identify the Cu^{2+} doses that provide greater benefits to elastin matrix synthesis.

In investigating the effects of exogenous Cu^{2+} ions on cell behavior and matrix synthesis, all experiments were conducted in serum-rich medium. Although culture studies in a serum-free medium would be preferable to isolate the effects of Cu^{2+} ions on cell behavior, the need to stimulate matrix synthesis by cells necessitates that serum-rich conditions be provided. Fetal bovine serum has been shown to contain trace amounts of Cu^{2+} ions ($\sim 10\text{--}20\text{ nM}$),⁴⁶ almost all of which is bound to ceruloplasmin and albumin. Estimations of free Cu^{2+} ions indicate that it is unlikely to exceed picomolar concentrations.⁴⁷ Thus, the background levels of Cu^{2+} ions are far lower than the lowest exogenous dose (0.01 M) used in this study and may be almost identical to Cu^{2+} -free control medium.

FIG. 6. Representative scanning electron microscopy images of 21-day-old rat aortic smooth muscle cell layers for non-additive controls (A); cultured with copper sulfate (CuSO_4) alone (0.01 M (B), 0.1 M (D)), CuSO_4 (0.1 M) with oligomers (E), or together with high-molecular-weight (HMW) hyaluronan (HA) (F). (C) The nature of the elastin matrix when CuSO_4 (0.01 M) was provided together with HMW HA. Additional presence of oligomers or HMW HA enhanced formation of matrix elastin fibers more than 0.1 M CuSO_4 -supplemented cultures, with diameters ranging between 0.5 and 1.0 μm , indicated by arrows.

Our experiments indicated that the addition of Na_2SO_4 had no effect on cell proliferation ratios or matrix synthesis, signifying the absence of any counter-ion (SO_4^{2-})-induced effects on cellular behavior, at least at the doses tested in this study. Previously, we showed that large HA fragments (20-200 kDa) modestly increased cell proliferation in a dose-independent manner, whereas HA oligomers (2 $\mu\text{g}/\text{mL}$) were ineffective.^{16,17} However, in this study, we found that, in the presence of 0.01 M of CuSO_4 , neither HA fragment size nor dose affected cell proliferation, although Cu^{2+} itself enhanced cell proliferation more than non-additive controls. On the other hand, 0.1 M CuSO_4 significantly inhibited cell proliferation, in the presence and absence of HA fragments. These results suggest that SMCs respond in a dose-dependent manner to Cu^{2+} ions in the context of cell proliferation and that interaction between Cu^{2+} ions and SMCs appears to interrupt HA-induced intracellular signaling pathways influencing cell proliferation. Prior studies also showed that much lower doses (0.5 mM) of Cu^{2+} ions do not influence SMC proliferation, although endothelial cells proliferated rapidly.⁴⁸ Here, we show that CuSO_4 in the range of 0.01 M induces SMC proliferation, results that suggest that the effect of Cu^{2+} ions on cells is specific to cell type and dose. At the higher doses of CuSO_4 (0.1 M), although no cell death was observed, cells appeared rounded in the first 3 days after CuSO_4 addition but gradually assumed a more spread morphology. Based on prior reports,^{49,50} we hypothesize that, at this higher tested dose, some degree of free-hydroxyl radical release occurs into the medium, which may suppress cell proliferation and influence cell spreading due to hitherto unknown intracellular signaling pathways. However, the lack of any consistent long-term hyper-trophic

or hypo-trophic phenomena suggests that cellular metabolic pathways are not adversely affected at this tested Cu^{2+} dose, which can be safely applied in alternate tissue-engineering approaches to improve matrix yields.

Although there were no significant differences between the absolute amounts of total elastin and matrix elastin produced in test groups and control cultures (as shown in Table 1), 0.1 M CuSO_4 (with or without HA) improved elastin yield on a per-cell basis approximately 4 times more than control cultures. Qualitatively, cultures that received 0.1 M CuSO_4 (but not 0.01 M) and HA fragments also contained far more elastin fibers than in controls (amorphous clumps only); LOX protein production was also 2.5 times as great and even more so (3.5 times) in the presence of HA fragments. The literature suggests strong interplay between transforming growth factor beta ($\text{TGF-}\beta$) availability and LOX,^{51,52} which in turn may imply that Cu^{2+} ion-induced increases in endogenous $\text{TGF-}\beta$ release may have mediated the observed increases in LOX production. The increases in tropoelastin production in 0.1 M CuSO_4 -supplemented cultures could also be due to the $\text{TGF-}\beta$ -mediated effect of Cu^{2+} ions, although this hypothesis is subject to future validation. Regardless, these results strongly indicate that 0.1 M CuSO_4 , provided with or without HA fragments, makes SMCs far more efficient in generating tropoelastin and matrix elastin and enhances elastin fiber formation. However, cell proliferation, although robust, is somewhat slower in such cultures, because absolute amounts of matrix generated over a defined culture period are similar to that generated by identically seeded control cultures.

Typically, tissue-engineering constructs for clinical and pre-clinical testing and use must be of sufficient size. This

necessitates seeding of scaffolds at higher cell densities and culturing these scaffolds over longer periods to facilitate substantial elastin matrix accumulation,⁵³ especially because the elastin yield by adult vascular cells is limited. During these extended periods of culture, cells frequently proliferate rapidly (as in the control cultures here) to first super-saturate the scaffold pores and surface and may eventually die competing for essential gases and nutrients. In addition, studies have shown that post-confluence, density-arrested SMCs generate much less collagen matrix on a per-cell basis,⁵⁴ which negates the purpose of initial high cell seeding density and long-term culture; this logic likely also holds true for cellular elastin matrix production. Thus, robust production of elastin matrix by cells that remain viable and synthetic in long-term culture demands gradual proliferation and high yields of elastin synthesis on a per-cell basis. In this context, supplying Cu^{2+} ions equivalent to the 0.1 M dose might enable these criteria to be met while providing additional benefits of enhanced elastin fiber formation and cross-linking within these tissue-engineered constructs.

Fluorescence imaging and ultrastructural analyses of synthesized elastin matrices qualitatively supported the biochemical observations. RASMCs cultured with 0.01 M of CuSO_4 alone or together with HA fragments were found to deposit amorphous elastin clumps, as in additive-free control cultures. Additional presence of HA fragments resulted in dramatically greater elastic fiber formation than in 0.1 M CuSO_4 -supplemented cultures, with numerous elastic fibers measuring approximately 0.5 to 1.0 μm in diameter seen in abundance. The literature suggests that tropoelastin precursors are not very capable of spontaneous self-assembly and thus require helper proteins (e.g., microfibrils) to guide their alignment for further cross-linking and fiber assembly.⁵⁵ Once this initial alignment has been achieved, the structure is stabilized against proteolytic degradation using Cu^{2+} ion-dependent LOX, which oxidizes the lysine residues of the aligned elastin molecules and enables cross-linking. Thus, these stabilized and aligned elastin structures act as nucleation sites for further coacervation and cross-linking of more tropoelastin, resulting in organized elastic fiber growth. Moreover, highly anionic GAGs (HA in this case) promote elastic fiber formation by electrostatically binding to the unoxidized lysine residues of newly synthesized tropoelastin during their association with microfibrils, thus preventing their random self-aggregation far away from the site of fiber formation.³⁶ The retention of the tropoelastin molecules by GAGs would thus indirectly facilitate their LOX-mediated cross-linking and encourage elastin fiber growth, as stated above. In direct support of this hypothesis, the simultaneous availability of HA fragments and increases in LOX synthesis (2.7 ± 0.3 times) with the addition of 0.1 M of CuSO_4 , along with significant presence of fibrillin in the cell layers (as shown in Figure 5), might have contributed to the observed increases in the structural quality of elastin fiber formation.

Conclusions

The current study demonstrates the combined utility of 0.1 M of CuSO_4 and HA, particularly HA oligomers (~756 Da) and HMW HA (~2000 kDa), to significantly increase tropoelastin release and improve elastin matrix synthesis, cross-linking, and fiber formation by adult rat vascular SMCs.

Enhanced LOX production and fibrillin scaffold formation with 0.1 M CuSO_4 delivery may orchestrate these benefits, although the cell-signaling pathways involved in these observed increases need to be elucidated. The results obtained might be of tremendous utility in restoring elastin matrix homeostasis in de-elasticized vessels and tissue-engineering constructs *in vivo* and *in vitro* and possibly even serve as an accelerated *in vitro* model to investigate elastogenesis during wound healing in adult tissues.

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References

- Davidson, J.M., and Giro, M.G. Regulation of matrix accumulation. In Mecham, R. P., ed. *Biology and Regulation of Extracellular Matrix*. New York: Academic Press, 1986, pp 178-216.
- Kielty, C.M., Woolley, D.E., Whittaker, S.P., and Shuttleworth, C.A. Catabolism of intact fibrillin microfibrils by neutrophil elastase, chymotrypsin and trypsin. *FEBS Lett* **351**, 85, 1994.
- Sandberg, L.B., Soskel, N.T., and Leslie, J.G. Elastin structure, biosynthesis, and relation to disease states. *N Engl J Med* **304**, 566, 1981.
- Brooke, B.S., Bayes-Genis, A., and Li, D.Y. New insights into elastin and vascular disease. *Trends Cardiovasc Med* **13**, 176, 2003.
- Bellingham, C.M., Lillie, M.A., Gosline, J.M., Wright, G.M., Starcher, B.C., Bailey, A.J., Woodhouse, K.A., and Keeley, F.W. Recombinant human elastin polypeptides self-assemble into biomaterials with elastin-like properties. *Biopolymers* **70**, 445, 2003.
- Mithieux, S.M., Rasko, J.E., and Weiss, A.S. Synthetic elastin hydrogels derived from massive elastic assemblies of self-organized human protein monomers. *Biomaterials* **25**, 4921, 2004.
- Urry, D.W., Pattanaik, A., Xu, J., Woods, T.C., McPherson, D.T., and Parker, T.M. Elastic protein-based polymers in soft tissue augmentation and generation. *J Biomater Sci Polym Ed* **9**, 1015, 1998.
- Wright, E.R., and Conticello, V.P. Self-assembly of block copolymers derived from elastin-mimetic polypeptide sequences. *Adv Drug Deliv Rev* **54**, 1057, 2002.
- Mitchell, S.L., and Niklason, L.E. Requirements for growing tissue-engineered vascular grafts. *Cardiovasc Pathol* **12**, 59, 2003.
- Walls, T., Herden, T., Haverich, A., and Mertsching, H. Influence of scaffold thickness and scaffold composition on bioartificial graft survival. *Biomaterials* **24**, 1233, 2003.
- Kielty, C.M., Sherratt, M.J., and Shuttleworth, C.A. Elastic fibres. *J Cell Sci* **115**, 2817, 2002.
- Mecham, R.P., and Davis, E.C. Elastic fiber structure and assembly. In Yurchenco, P.D., Birk, D.E., and Mecham, R.P., eds. *Extracellular Matrix Assembly and Structure*. New York: Academic Press, 1994, pp 281-314.
- Csiszar, K. Lysyl oxidases: a novel multifunctional amine oxidase family. *Prog Nucleic Acid Res Mol Biol* **70**, 1, 2001.
- Radhakrishnamurthy, B., Ruiz, H., and Berenson, G.S. Interactions of glycosaminoglycans with collagen and elastin in bovine aorta. *Adv Exp Med Biol* **82**, 160, 1977.

15. Bartholomew, J.S., and Anderson, J.C. Investigation of relationships between collagens, elastin and proteoglycans in bovine thoracic aorta by immunofluorescence techniques. *Histochem J* **15**, 1177, 1983.
16. Joddar, B., and Ramamurthi, A. Fragment size- and dose-specific effects of hyaluronan on matrix synthesis by vascular smooth muscle cells. *Biomaterials* **27**, 2994, 2006.
17. Joddar, B., and Ramamurthi, A. Elastogenic effects of exogenous hyaluronan oligosaccharides on vascular smooth muscle cells. *Biomaterials* **27**, 5698, 2006.
18. Ramamurthi, A., Vesely, I. Evaluation of the matrix-synthesis potential of crosslinked hyaluronan gels for tissue engineering of aortic heart valves. *Biomaterials* **26**, 999, 2005.
19. Evanko, S.P., Angello, J.C., and Wight, T.N. Formation of hyaluronan- and versican-rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **19**, 1004, 1999.
20. Toole, B.P. Hyaluronan: from extracellular glue to pericellular cue. *Nat Rev Cancer* **4**, 528, 2004.
21. Noble, P.W. Hyaluronan and its catabolic products in tissue injury and repair. *Matrix Biol* **21**, 25, 2002.
22. Eyre, D. Collagen crosslinking amino-acids. *Methods Enzymol* **144**, 115, 1987.
23. Kagan, H.M., and Li, W. Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. *J Cell Biochem* **88**, 660, 2003.
24. Gacheru, S., Trackman, P.C., Shah, M.A., O'Gara, C.Y., Spacciapoli, P., Greenaway, F.T., and Kagan, H.M. Structural and catalytic properties of copper in lysyl oxidase. *J Biol Chem* **265**, 19022, 1990.
25. Labarca, C., and Paigen, K. A simple, rapid, and sensitive DNA assay. *Anal Biochem* **102**, 344, 1980.
26. Palamakumbura, A.H., and Trackman, P.C. A Fluorometric assay for detection of lysyl oxidase enzyme activity in biological samples. *Anal Biochem* **300**, 245, 2002.
27. Alsberg, E., Anderson, K.W., Albeiruti, A., Rowley, J.A., and Mooney, D.J. Engineering growing tissues. *Proc Natl Acad Sci U S A* **99**, 12025, 2002.
28. Mann, B.K., and West, J.L. Tissue engineering in the cardiovascular system: progress towards a tissue engineered heart. *Anat Rec* **263**, 367, 2001.
29. Weinberg, C.B., and Bell, E. A blood vessel constructed from collagen and cultured vascular cells. *Science* **231**, 397, 1986.
30. Niklason, L., and Langer, R.S. Advances in tissue engineering of blood vessels and other tissues. *Transpl Immunol* **5**, 303, 1997.
31. Johnson, D.J., Robson, P., Hew, Y., and Keeley, F.W. Decreased elastin synthesis in normal development and in long-term aortic organ and cell cultures is related to rapid and selective destabilization of mRNA for elastin. *Circ Res* **77**, 1107, 1995.
32. McMahon, M.P., Faris, B., Wolfe, B.L., Brown, K.E., Pratt, C.A., Toselli, P., and Franzblau, C. Aging effects on the elastin composition in the extracellular matrix of cultured rat aortic smooth muscle cells. *In Vitro Cell Dev Biol* **21**, 674, 1985.
33. Wight, T.N. Versican: a versatile extracellular matrix proteoglycan in cell biology. *Curr Opin Cell Biol* **14**, 617, 2002.
34. Isogai, Z., Asperger, A., Keene, D.R., Ono, R.N., Reinhardt, D.P., and Sakai, L.Y. Versican interacts with fibrillin-1 and links extracellular microfibrils to other connective tissue networks. *J Biol Chem* **277**, 4565, 2002.
35. Zimmermann, D.R., Dours-Zimmermann, M.T., Schubert, M., and Bruckner-Tuderman, L. Versican is expressed in the proliferating zone in the epidermis and in association with the elastic network of the dermis. *J Cell Biol* **124**, 817, 1994.
36. Fornieri, C., Baccarani-Contri, M., Quaglino, D. Jr., and Pasquali-Ronchetti, I. Lysyl oxidase activity and elastin/glycosaminoglycan interactions in growing chick and rat aortas. *J Cell Biol* **105**, 1463, 1987.
37. Bressan, G.M., Pasquali-Ronchetti, I., Fornieri, C., Mattioli, F., Castellani, I., and Volpin, D. Relevance of aggregation properties of tropoelastin to the assembly and structure of elastic fibers. *J Ultrastruct Mol Struct Res* **94**, 209, 1986.
38. Lapcik, L. Jr., Lapcik, L., De Smedt, S., Demeester, J., and Chabreck, P. Hyaluronan: preparation, structure, properties, and applications. *Chem Rev* **98**, 2663, 1998.
39. Reiser, K., McCormick, R., and Rucker, R. Enzymatic and nonenzymatic cross-linking of collagen and elastin. *FASEB J* **6**, 2439, 1992.
40. Sen, C.K., Khanna, S., Venojarvi, M., Trikha, P., Ellison, E.C., Hunt, T.K., and Roy, S. Copper-induced vascular endothelial growth factor expression and wound healing. *Am J Physiol Heart Circ Physiol* **282**, H2821, 2002.
41. Rucker, R., Kosonen, T., Clegg, M.S., Mitchell, A.E., Rucker, R.B., Uriu-Hare, J.Y., and Keen, C.L. Copper, lysyl oxidase, and extracellular matrix protein crosslinking. *Am J Clin Nutr* **67**, 996S, 1998.
42. Hunsaker, H.A., Morita, M., and Allen, K.G. Marginal copper deficiency in rats. Aortal morphology of elastin and cholesterol values in first-generation adult males. *Atherosclerosis* **1**, 1, 1984.
43. Tinker, D., Romero-Chapman, N., Reiser, K., Hyde, D., and Rucker, R.B. Elastin metabolism during recovery from impaired crosslink formation. *Arch Biochem Biophys* **278**, 326, 1990.
44. Allen, K.G., Klevay, L.M. Cholesterolemia and cardiovascular abnormalities in rats caused by copper deficiency. *Atherosclerosis* **29**, 81, 1978.
45. Giavaresi, G., Torricellia, P., Fornasari, P.M., Giardina, R., Barbuccib, R., and Leoneb, G. Blood vessel formation after soft-tissue implantation of hyaluronan based hydrogel supplemented with copper ions. *Biomaterials* **26**, 3001, 2005.
46. Dahl, S.L.M., Rucker, R.B., and Niklason, L.E. Effects of copper and cross-linking on the extracellular matrix of tissue-engineered arteries. *Cell Transplant* **14**, 367, 2005.
47. Sorenson, J.R.J. Use of essential metalloelement complexes or chelates in biological studies. *Free Radic Biol Med* **13**, 593, 1992.
48. Hu, G.F. Copper stimulates proliferation of human endothelial cells under culture. *J Cell Biochem* **69**, 326, 1998.
49. Halliwell, B., and Gutteridge, J.M. The importance of free radicals and catalytic metal ions in human diseases. *Mol Aspects Med* **8**, 89, 1985.
50. Rowley, D., and Halliwell, B. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals in the presence of copper salts: a physiologically significant reaction? *Arch Biochem Biophys* **225**, 279, 1983.
51. Shanley, C.J., Gharaee-Kermani, M., Sarkar, R., Welling, T.H., Krieger, A., Ford, J.W., Stanley, J.C., and Phan, S.H. Transforming growth factor-beta 1 increases lysyl oxidase enzyme activity and mRNA in rat aortic smooth muscle cells. *J Vasc Surg* **25**, 446, 1997.
52. Gacheru, S.N., Thomas, K.M., Murray, S.A., Csiszar, K., Smith-Mungo, L.I., and Kagan, H.M. Transcriptional and post-transcriptional control of lysyl oxidase expression in

- vascular smooth muscle cells: effects of TGF-beta1 and serum deprivation. *J Cell Biochem* **65**, 395, 1997.
53. Solchaga, L.A., Tognana, E., Penick, K., Baskaran, H., Goldberg, V.M., Caplan, A.I., and Welter, J.F. A rapid seeding technique for the assembly of large cell/scaffold composite constructs. *Tissue Eng* **12**, 1851, 2006.
54. Majors, A.K., and Ehrhart, L.A. Cell density and proliferation modulate collagen synthesis and procollagen mRNA levels in arterial smooth muscle cells. *Exp Cell Res* **200**, 168, 1992.
55. Augsburger, P.B., Tisdale, C., Broekelmann, T., Sloan, C., and Mecham, R.P. Identification of an elastin cross-linking domain that joins three peptide chains. *J Biol Chem* **270**, 17778, 1995.

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