Functional Rescue of Elastin Insufficiency in Mice by the Human Elastin Gene
Implications for Mouse Models of Human Disease

Eiichi Hirano, Russell H. Knutsen, Hideki Sugitani, Christopher H. Ciliberto, Robert P. Mecham

Abstract—Diseases linked to the elastin gene arise from loss-of-function mutations leading to protein insufficiency (supravalvular aortic stenosis) or from missense mutations that alter the properties of the elastin protein (dominant cutis laxa). Modeling these diseases in mice is problematic because of structural differences between the human and mouse genes. To address this problem, we developed a humanized elastin mouse with elastin production being controlled by the human elastin gene in a bacterial artificial chromosome. The temporal and spatial expression pattern of the human transgene mirrors the endogenous murine gene, and the human gene accurately recapitulates the alternative-splicing pattern found in humans. Human elastin protein interacts with mouse elastin to form functional elastic fibers and when expressed in the elastin haploinsufficient background reverses the hypertension and cardiovascular changes associated with that phenotype. Elastin from the human transgene also rescues the perinatal lethality associated with the null phenotype. The results of this study confirm that reestablishing normal elastin levels is a logical objective for treating diseases of elastin insufficiency such as supravalvular aortic stenosis. This study also illustrates how differences in gene structure and alternative splicing present unique problems for modeling human diseases in mice. (Circ Res. 2007;101:523-531.)

Key Words: elastin ■ supravalvular aortic stenosis ■ vascular disease ■ transgenic mice

Mutations within the elastin gene lead to several elastinopathies in humans that affect large blood vessels, the skin, and the lung. For example, loss-of-function mutations that produce haploinsufficiency have been linked to supravalvular aortic stenosis (SVAS-MIM185500), a congenital narrowing of the ascending aorta and other vessels. SVAS can occur sporadically or as a familial condition with autosomal-dominant inheritance. More than 50 different mutations have been described that lead to isolated SVAS. SVAS is also a component of Williams-Beuren syndrome (WBS-MIM194050), a frequent heterozygous deletion of a ~1.6 Mb segment at chromosome 7q11.23 that includes the elastin gene.

In contrast to the loss-of-function mutations typical of SVAS, evidence suggests that autosomal dominant cutis laxa (ADCL-MIM123700) occurs through a dominant-negative mechanism. ADCL is characterized by lax skin with other internal organ involvement. Most elastin mutations associated with this disease are single nucleotide deletions near the 3' end of the gene resulting in missense sequence that alters the character of a biologically important domain at the end of the tropoelastin molecule. ELN has also been suggested to be a susceptibility gene for hypertension, emphysema, and intracranial aneurysms.

ELN encodes a protein made up of alternating hydrophobic and crosslinking domains. This repeating arrangement reflects the exon structure of the gene, with each type of domain encoded by distinct exons. Alignment of human and mouse elastin cDNA sequences shows 64.5% identity at the nucleotide level and 64.1% identity and 72.6% similarity at the amino acid level. This is below the average identity of 85% at the nucleotide level and 78.5% at the amino acid level for human and mouse cDNAs. There are also major differences in the exon content in human and mouse elastin genes. In most mammalian species, the elastin gene is composed of 36 exons distributed throughout approximately 40 kb of genomic DNA. Rat and mouse Eln have 37 exons attributable to an additional short exon inserted after exon 4. The human ELN gene, however, has only 34 exons attributable to the sequential loss of 2 exons (34 and 35) during primate evolution. In addition, although still contained within the human gene, exon 22 is rarely included in the elastin gene transcript. It is unclear what, if any, selective advantage is conferred on the protein by the loss of 2 exons and the silencing of a third in primate lineages, but these differences in gene structure, together with divergent amino acid sequences between elastin in primates and other species, has important implications for the use of animal models to study diseases associated with mutations in this important human gene.

This report describes a humanized elastin mouse with elastin production being controlled by the human elastin gene encoded by distinct exons.
in a bacterial artificial chromosome (BAC). Elastin from the human transgene is able to reverse the cardiovascular phenotype associated with elastin haploinsufficiency and rescues the lethality of the null phenotype. Importantly, the human BAC elastin transgene retains the human alternative splicing pattern, which makes the human BAC mouse a suitable model to study how ELN mutations lead to human disease. This study also illustrates how differences in gene structure and alternative splicing present unique problems for modeling human diseases in mice and the need for caution in extrapolating information from mouse models to human disease.

Materials and Methods

BAC DNA Purification and Generation of Transgenic Mice

A human BAC clone (CTB-51J22) containing the complete human elastin gene was obtained from Research Genetics. BAC circular DNA was isolated using a Large-construct kit (Qiagen). After the last ethanol precipitation step, DNA was dissolved in injection buffer (10 mmol/L Tris–HCl, pH 7.4, 10 mmol/L NaCl, 0.25 mmol/L EDTA) and then dialyzed on a floating 0.1 μm Millipore membrane filter against the injection buffer.

Isolated BAC DNA was injected at a concentration of 1 ng/μL into fertilized mouse oocytes from C57BL/6 mice, which were implanted into the uterus of pseudopregnant foster mothers. After birth, potential founders were screened for the presence of the transgene using PCR with human elastin specific primers (see online supplemental data, available at http://circres.ahajournals.org, for primer sequences). Animals positive for the transgene were mated to WT (C57Bl/6) animals to stabilize the line. The generation and characterization of Eln+/- mice has been previously reported.22

RNA quantitation, RNase protection assays, histology, protein assays, and vessel physiology assessment were all done using standard techniques, details of which can be found in the expanded Materials and Methods section in the online data supplement.

Results

In this report, hBAC refers to the homozygous human elastin transgene (ELN+/+) expressed from a BAC and Eln to the endogenous mouse elastin gene. WT refers to nontransgenic mouse wild-type (Eln+/+). Compound phenotypes are indicated by the presence or absence of each allele. For example, hBAC-mWT would be homozygous for both the human transgene and mouse endogenous gene (ELN+/+, Eln+/+), hBAC-mHET would be homozygous for the human transgene and heterozygous for the mouse gene (ELN+/+, Eln+/−), and hBAC-mNULL would be homozygous for the human transgene and null for the mouse gene (ELN+/−, Eln−/−).

Generation of hBAC Mouse

Because the elastin gene in humans is structurally different from the murine ortholog, we developed a humanized elastin mouse with elastin production being controlled by the human elastin gene in a bacterial artificial chromosome. The BAC contains no known genes upstream of ELN and only a fragment of LIMK1 3′ to ELN. Thus, ELN is the only functional gene in the BAC (Figure 1A). Mice from C57Bl/6 oocytes injected with the BAC were screened for incorporation of the transgene by Southern blot analysis and 6 founder lines were identified (Figure 1B), with lines 2 and 4 having the highest copy number. Expression of the human elastin gene was detected in all founder lines except for line 6. All mice were maintained in the C57Bl/6 background.

Transgene expression was evaluated by RNase protection assay of RNA from aorta, lung, heart, and skin taken from animals 4 weeks of age. The human elastin transgene was expressed in all elastin-containing tissues with highest expression levels in the aorta (Figure 1C). The pattern of expression over the developmental time course in all examined tissues was similar to that observed for the endogenous mouse gene (Figure 1D). These findings show that the temporal and tissue-specific expression pattern of the human gene is regulated similarly to the endogenous mouse gene, indicating positional-independent control of expression of the human BAC transgene. mRNA expression levels, however, did not correlate with transgene copy number; founder line 3 had the highest mRNA expression levels with a gene copy number about 4 times lower than line 4 (based on Southern analysis). Line 1 showed the lowest expression values even though its gene copy number was approximately equivalent to line 5 (data not shown).

Elastin From the Human Transgene Associates With Mouse Elastin to Form Functional Elastic Fibers That Reverse Characteristics of Elastin Insufficiency

Immunofluorescence analysis using species-specific elastin antibodies showed that elastin from the hBAC transgene associated with the mouse protein to form elastic fibers in arterial vessel walls (Figure 2A). To determine whether mouse elastic fibers containing human elastin are functionally equivalent to those containing only mouse elastin, hBAC-mWT animals from founder lines 3 and 4 were bred with Eln+/− mice to generate the hBAC-mHET genotype. Levels of insoluble elastin were assessed through quantitation of desmosine, which is a unique crosslinking amino acid whose level is proportional to the amount of insoluble, mature protein. Desmosine values showed a ≈40% increase in insoluble elastin in line 3 (high expresser) transgenic animals, thereby confirming that the human protein undergoes proper crosslinking and contributes to the insoluble elastin pool. As expected from the difference in RNA expression, little change in desmosine levels was detected in line 4 (low expresser) animals (Figure 2B).

Past studies show that mice heterozygous for the elastin gene have unusual cardiovascular properties resulting from elastin haploinsufficiency.13,23 The best-characterized changes, also found with variable penetrance in humans with SVAS and Williams-Beuren syndrome,22,24 include hypertension, mild cardiac hypertrophy, and an increased number of smooth muscle layers (elastic lamellae) in the arterial wall. Figures 2C and 2D show that both hypertension and altered lamellar number are partially reversed in line 3 hBAC-mHET animals. Mean arterial blood pressure decreased from ≈125 mm Hg to ≈100 mm Hg—values close to WT levels. The number of smooth muscle cell layers (ie, lamellar units) also decreased from ≈10 found in the Eln+/− animals to ≈8 (WT levels) in the ascending aorta of line 3 hBAC-mHET mice. In contrast, there was only a small diminution in mean blood pressure in the low expressing hBAC-mHET line 4,
with no change in lamellar number, confirming that elastin dosage from the transgene is responsible for the blood pressure and structural wall changes in these animals.

The effect of human elastin on the mechanical properties of large vessels is documented in Figure 3. We have previously shown that Eln<sup>+/+</sup>/H11001/H11002 vessels have decreased compliance compared with WT animals attributable to a decrease in the elastin to collagen ratio, which makes vessels stiffer.13,23 The vessels in hBAC-mHET mice, however, show mechanical properties intermediate between Eln<sup>+/+</sup>/H11001/H11002 and WT, suggesting that elastin from the human transgene is altering vessel compliance toward normal values by increasing the elastin concentration. These results confirm that human elastin from the hBAC transgene is functional and can augment mouse elastin to reverse elastin insufficiency.

The Human Elastin Transgene Retains the Human Alternative Splicing Pattern

Extensive coding diversity is generated from the single-copy ELN gene by alternate splicing of elastin pre-mRNA.25–27 Alternative splicing of human and mouse elastin mRNA was compared using RT-PCR and RNase protection assay. In mRNA from the hBAC gene, DNA sequences from RT-PCR products as well as RNase protection studies found deletion in all transcripts of exons 22 and 26A, minor amounts of exon 23 deleted (Figure 4A), and deletion of exon 32 in 50% of the transcripts (Figure 4B). The mouse, in contrast, showed no splicing in this region of the gene. These results demonstrate that the hBAC transgene retains the elastin gene-splicing pattern normally observed in humans11,28 and that both the pattern and extent of exon splicing in the human gene is retained in the mouse.

Human Elastin From the BAC-Transgene Rescues the Null Phenotype

To ascertain whether the human elastin gene could completely substitute for the endogenous murine gene, we introduced the BAC transgene into the homozygous Eln knockout background by backcrossing line 3 hBAC-mHET animals (Figure 5A). hBAC-mNULL mice were viable, indicating rescue of the perinatal lethality normally observed with elastin deficiency by the human elastin gene.
No animals of the genotype ELN<sup>−/−</sup>, Eln<sup>−/−</sup> were recovered, however, indicating that the dosage of the hBAC gene on the homozygous knockout background has a direct effect on survival. Immunofluorescence staining of cultured dermal fibroblasts using species-specific elastin antibodies confirmed the absence of mouse elastin in hBAC-mNull animals (supplemental Figure I).

Rescued hBAC-mNull mice were interbred to establish humanized rescued colonies. Although mice were born at the expected Mendelian ratio, ≈15% died prematurely between birth and day 5 and another ≈45% died between 3 and 5 months of age (supplemental Figure II). Autopsy revealed significant cardiomegaly, suggesting heart failure as a probable cause of death. Surviving hBAC-mNull mice, however, were fertile with long-term survivability (>2 year).

### Phenotypic Differences Between Rescued and Normal Mice

Arterial smooth muscle cells in the aorta of hBAC-mNull animals formed concentric layers within the arterial wall (Figure 6A). Thin elastic lamellae were evident between the smooth muscle layers, although they were discontinuous and fragmented when compared with lamellae in WT animals. All hBAC-mNull mice had significantly thickened ascending aortic walls (Figure 6A), with less integrated elastic lamellae. The wall of the abdominal aorta in hBAC-mNull animals, in contrast, was similar to WT mice except for thinner lamellae. The decreased vessel wall elastin content suggested by the histology was confirmed through quantification of desmosine. Desmosine levels in the abdominal aorta were one-half WT values and those in the ascending aorta approximately one-third WT levels (Figure 6B). Collagen content as...
assessed as nmole hydroxyproline per mg protein was elevated by \( \approx 20\% \) (40.8 \pm 4.8 nmol compared with WT values of 33.9 \pm 2.8 nmol) in hBAC-mNULL ascending aorta relative to WT control. In contrast, collagen levels in the abdominal aorta of hBAC-mNULL animals were lower compared with WT (72.0 \pm 3.7 nmoles hydroxyproline in hBAC-mNull compared with 98.4 \pm 5.9 nmoles in WT).

Comparison of 3-month-old hBAC-mNULL and Eln\(^{-/-}\) mice revealed a similar average body weight (\( \approx 25 \) g), with no statistically significant difference \( (P>0.02) \) (supplemental Table I). No statistically significant difference in heart rate could be detected between the 2 genotypes (both were in the range of 760 beats per min measured under mild anesthesia). Blood pressure measurements showed a marked elevation in systolic, diastolic, and mean blood pressures in the rescued mice (Figure 6C) relative to WT levels. Total heart weight to body weight, LV+septum to body weight, and RV to body weight ratios were elevated (15% and 13%, respectively) in hBAC-NULL animals (supplemental Table I). Measurement of plasma renin concentrations showed elevation of active renin in rescued mice (60 \pm 12 ng/mL/hr for hBAC-mNULL compared with 12 \pm 2 ng/mL/hr for hBAC-mWT), suggesting that renovascular mechanisms are active in maintaining the elevated pressures, as has been demonstrated in Eln\(^{+/−}\) mice.\(^{13}\)

As was the case in the aorta, lungs in hBAC-mNULL animals showed a \( \approx 65\% \) reduction in elastin levels. As a result, the animals manifest a form of congenital emphysema characterized by grossly enlarged thoraces, airspace enlargement, and altered mechanics. The lung phenotype has been described in detail in Shifren et al.\(^{29}\)

**Human BAC Elastin Gene Expression Is \( \approx 60\% \) Lower Than the Endogenous Mouse Gene**

To determine whether reduced human elastin levels in the transgenic animals result from decreased expression from the
human gene or increased turnover of the human mRNA relative to the mouse message, semiquantitative PCR analysis was used to assess elastin mRNA levels in the aorta or smooth muscle cells of transgenic animals. Figure 7A shows that hBAC mRNA levels were decreased relative to expression of the endogenous mouse gene. Densitometric scanning of the signal after 26 cycles of PCR suggest a decrease of approximately 60%. This is in agreement with elastin protein values as assessed by desmosine and explains why elastin replenishment in the Eln\textsuperscript{+/+} background does not reach WT levels (eg, see Figure 2B). Figure 7B shows results from RNA turnover studies in vascular smooth muscle cells demonstrating that human elastin mRNA is more stable than the mouse transcript. Thus, differences in elastin mRNA turnover do not contribute to the low mRNA levels measured for human elastin.

**Discussion**

In this report we describe a humanized mouse expressing the human elastin transgene in the form of a BAC. Expression of the human and mouse genes in all founder lines except for line 6 showed a similar pattern in multiple tissues, suggesting appropriate temporal and spatial expression of the human transgene. Interestingly, large differences in the human:mouse mRNA ratio were observed, with the human transgene expressed at significantly lower levels. That the human gene retains appropriate spatiotemporal regulation in the mouse when overall gene expression is greatly reduced suggests that the regulatory elements controlling these processes are distinct. It is possible that the elements controlling tissue- and time-specific expression are conserved in the 2 genes, whereas control of expression intensity is regulated by a nonconserved element(s).\textsuperscript{30} A comparison of transcription factor binding sites (identified using TESS and TFSEARCH software) in the promoter region up to 2 kb upstream of the ATG shows similarities and differences between the mouse and human gene, consistent with this possibility. In addition, expression profiling of developing mouse aorta\textsuperscript{31,32} found that several transcription factors with binding sites in the human,
boundaries of the BAC. Such long-range regulation has base deletions that lead to missense sequence. All are mutations associated with ADCL, for example, are single base deletions that lead to missense sequence. The single base deletions that have been identified occur within, or in close proximity to, exons that undergo alternative splicing in humans (particularly exons 30 or 32). Whether the mutation produces a missense sequence with a 3’terminally extended open reading frame depends on the splice pattern. For example, a reported single base deletion in exon 30 results in a frameshift that leads to missense sequence and premature termination in exon 32.11 If exon 32 is spliced out, which occurs ~50% of the time in humans and in the transgenic BAC, missense sequence continues into the 3’UTR until a new stop codon is encountered downstream of the normal translation termination site. Splicing out of the exon containing the mutation results in normal protein. Because exons 30 and 32 are not spliced in the mouse, mutations in these exons in human, if generated in mice, may have a different biological effect and may not be relevant for functional analysis.

The structural differences in the coding region of the human and mouse genes, combined with differences in alternative splicing, raised the question of whether the human protein could complement mouse elastin or whether the differences in sequence and structure would interfere with normal elastic fiber assembly when expressed in the mouse background. The issue was addressed directly by showing that expression of the human elastin BAC in the Eln<sup>−/−</sup> background raised total vascular insoluble elastin levels by ~40% and partially reversed the high blood pressure, vessel wall remodeling, and altered vessel compliance that is characteristic of elastin insufficiency. Furthermore, no change in any of these physiological parameters was detected when the human gene was expressed in the WT background (hBAC-mWT), confirming that the human protein was not acting to disrupt mouse fiber assembly. These results provide evidence that human elastin can combine with the mouse protein to form functional fibers despite compositional differences in the 2 proteins. Reversal of the complex traits associated with elastin insufficiency in the hBAC-mHET mouse also confirms that reestablishing normal elastin levels is a logical objective for treating SVAS.

Rescue of the null phenotype by elastin from the human gene supports the functional studies described above in suggesting that assembly of the human protein into a functional elastic fiber is unaffected by the mouse background. However, although levels of elastin in the hBAC-mNULL animals are sufficient to rescue the lethality associated with absence of elastin, they are significantly below normal levels. Quantitation of insoluble elastin in lung and large vessels of these animals shows protein levels to be around one-third the normal values, which is lower than the 50% reduction seen in the mELN<sup>−/−</sup> phenotype. The fact that no animals of the genotype ELN<sup>−/−</sup>, Eln<sup>−/−</sup> were recovered indicates that functional elastin levels cannot drop below the 30% level and still support viability.

As predicted from the extremely low elastin levels, rescued hBAC-mNULL mice show a more severe phenotype than what is observed in Eln<sup>−/−</sup> animals. The walls of elastic vessels in hBAC-mNULL animals are thicker, contain less elastin, and are less compliant that those in Eln<sup>−/−</sup> mice. Although systemic blood pressure is high in both genotypes,
hBAC-mNULL animals have higher diastolic pressure, which is consistent with their stiffer vessels. As expected from the elevated blood pressure, hBAC-mNULL mice show cardiac hypertrophy characterized by a ≈15% increase in total heart weight and a ≈20% increase in left ventricular weight. Interestingly, there was a ≈40% increase in right ventricular mass, suggesting significant pulmonary hypertension. These changes in ventricular hypertrophy are higher than what was observed in Eln+/− animals (≈13% increase in left ventricular weight, and ≈25% increase in right ventricular weight),13 consistent with greater cardiac stress in the hBAC-mNULL animals. In previous studies we showed that the hypertension associated with elastin insufficiency was correlated with high active renin levels and not attributable to dysfunction of the resistance vasculature. 13 It is interesting that active plasma renin levels were found to be elevated in hBAC-mNULL animals to values similar to those found in mEln+/− mice,13 suggesting a role for the kidney and the renin-angiotensin system in maintaining high blood pressure in both genotypes. The characteristics of the mice described in this study indicate how the phenotypic traits associated with elastin insufficiency directly correlate with elastin levels, which may help explain the phenotypic variability associated with SVAS in humans. Our results also show that restoring elastin to fetal and early postnatal periods when elastin production is highest. 31,32 It will be interesting to determine whether in-creasing vessel wall elastin in the adult periods has beneficial effects on vessel function.

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Disclosures
None.

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Supplemental Methods

**Genotyping BAC Transgenic Mice**

The generation and characterization of *Eln*+/- mice has been previously reported. The genotype at the mouse *Eln* locus was assessed by PCR analysis using specific primer sets: Primer A (DL1), 5'-GGTTGTCAGACTACAATCTGACC-3'; primer B (DL2), 5'-CAACTTTGCCCCAATGACTCTCC-3'; primer C (DL5), 5'-GAGAGGTATAGGGGAAGACTTGTGCT-3. Used together, primers B and C are specific to the wild-type mouse *Eln* locus and yields a 477-bp fragment, whereas primer A is specific to the targeted mouse *Eln* locus and yields a 250-bp fragment when used with primer B.

To identify the human BAC transgene by Southern analysis, 10mg of genomic DNA was digested with Ssp I and subjected to electrophoresis in agarose and transferred to nitrocellulose membranes according to standard procedures. Radioactive probes were produced using a random priming kit obtained from Amersham, and the blots were hybridized with 32P-labeled 340-bp human elastin cDNA probe produced by PCR (forward 5'-GAAAGCCTTGCCGGAAGAA-3' and reverse 5'-CGTGTAGCAGAAGGCCCTGTA-3'). After washing at 65°C in 0.1 M NaCl, 1.5 mM sodium citrate pH 7.0, the membranes were dried and exposed to x-ray film at -70°C for a suitable time.

**Total RNA isolation and RNase Protection Assay**

Total RNA was prepared using a TRIzol reagent kit (Invitrogen). A 420 bp fragment of the human elastin cDNA was subcloned into pGEM-3Z (-) (Promega). The plasmid was linearized with Sal I and a [32P]UTP -labeled antisense riboprobe was generated using T7 polymerase and a Maxi Script *in vitro* transcription kit according to the manufacturer's instructions (Ambion, Austin, TX). The human elastin riboprobe was gel purified on a 5% polyacrylamide/8 M urea gel and eluted for 2 hrs at 37 °C.

Ribonuclease protection assays were then performed using a RPA III kit according to the manufacturer's directions (Ambion). Briefly, 2 x 10^5 cpm of gel-purified human elastin
riboprobe was co-precipitated with 10 µg of RNA and hybridized overnight at 42 °C. Samples were digested with RNase A/T1 at 1:100 dilution for 30 min at 37 °C and then inactivated and precipitated. Samples were solubilized in 8 µl of gel loading buffer. One-half the volume was loaded onto a 5% polyacrylamide/8 M urea gel run at 250 V for 1 hr. Riboprobes of known sizes were run alongside RNA samples to verify the size of the probe and protected fragments. Protected fragments for human elastin and mouse β-actin (Ambion, Austin, TX) were 510 and 245 bp, respectively.

**Semi-quantitative RT-PCR**

The expression levels of various mRNAs were determined by semi quantitative RT-PCR. Three independent PCR reactions were performed using different cDNA reactions by mouse ELN-specific primer (TCCTCCATAGGGCTTCGGGGT), human ELN-specific primer (AGGCAGGCCCCACCTGGGAAAAT), or β-actin [20-mer oligo (Promega, Madison WI)]. Total RNA was reverse-transcribed using a 20-mer oligo (dT) primer and Superscript III reverse transcriptase (Invitrogen) following the manufacturer's instructions. Briefly, 1 µg of RNA from tissues was incubated at 65°C for 10 min in the presence of 1 µl of oligo (dT) primer (0.5 µg). After a brief centrifugation, 4 µl of first strand buffer, 2 µl of dithiothreitol (0.1 M), 1 µl of dNTP mixture (10 mM) and 1 µl of reverse Superscript transcriptase (200 U) were added and incubated at 55°C for 60 min and followed by a second incubation at 50°C for 60 min. The reaction was terminated by incubation at 70°C for 15 min. PCR was performed on the cDNA products using the following primer sets:

**hELN**
- **forward:** 5’-TGCCGCCAAGGCTGCAAGTA-3’
- **reverse:** 5’-ATTTTGCTGCAGCTGCAACTCCACCA-3’

**mEln**
- **forward:** 5’-TGCCGCCGCGCCAAGCTGCTAAGTAT-3’
- **reverse:** 5’-TGTCAGCTGCTTGGCAGCAAGAT-3’

Amplification using Ready-To-Go beads (Pharmacia, Piscataway, NJ) was performed for 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. Semi-quantitative RT-PCR using primers specific for mouse elastin, human elastin and β-actin. 0.5 µl of cDNA product was subjected to increasing cycles of PCR to determine the linear range of the PCR reaction. For hybridization, oligonucleotide probes for mouse
elastin (5'-CCAGGTGCAGTACCAGGTGCACTGCCAGGTGCAGT-3'), human elastin (5'-CCAAGGCTGCAAAGTACGGAGTGGGGAACCCCAGCA-3'), and β-actin (5'-TCATGAAGTGTGACGTTGACATCCGT-3') were labeled with [γ-32P]ATP using T4 polynucleotide kinase. After transfer to nylon membranes, blots were hybridized with radioactive probes.

**Relative quantification of BAC transgene and endogenous mEln expression.**

RT-PCR was performed using oligo-dT primers and Superscript III (Invitrogen). PCR amplification was performed using primers cELN Fw (5’-CGACCTCATCAACGTTGGTGCTAC-3’) and cELN Rv (5’-ACCCTTCCCTCCTCCCTCCC-3’), both of which are fully complementary to mouse and human cDNA. The human transcript was discerned from the murine transcript on the basis of an AvrII restriction site, which is not present in mouse-derived cDNA amplicons. Varying cycle-number amplifications were performed for 18–30 cycles to determine the region of exponential increase of each amplicon. Digestion products were separated on an agarose gel, and then Southern blot was performed. Hybridization was performed using a [γ-32P]ATP-labeled cELN Fw oligonucleotide.

**mRNA stability**

Postconfluent aortic smooth muscle cells from hELN-mHET mice were treated for 24 hours with 50 µM of the RNA polymerase inhibitor 5,6-dichloro-1-D-ribofuranosylbenzimidazole dissolved in DMSO or 0.1% DMSO alone. Total RNA was then isolated using the TRIzol reagent kit (Invitrogen). Mouse and human tropoelastin expression was assessed by semi-quantitative RT-PCR with primers that interact with both human and mouse transcripts (exon 33 to 3’UTR): 5'-GCAGCAAGACCTGGCTT-3’ (exon 33 forward primer) and 5'-GCTGGAGTAGTTGCAACCACGATG-3’ (3’UTR reverse primer). PCR products for these primers are 215 bp for mouse and 134 bp for human. The amplification products were separated on a 1.5% agarose gel and quantified by scanning densitometry. Values are expressed as percentage of mRNA levels in untreated human or mouse cells.
Immunohistochemistry, histology, and protein analysis

Frozen sections (10 µm) of ascending mouse aorta were stained with antibodies specific for mouse or human elastins followed by detection with Alexa Fluor 488 goat anti-rabbit IgG diluted 1:200. After washing with PBS, the sections were treated with 0.5% Sky blue in PBS for 5 min to suppress autofluorescence from existing elastic fibers. Fluorescence was stabilized using anti-fade Vectashield (Vector) mounting medium containing 4’,6 diamidino-2-phenylindole (DAPI, Vector). A Zeiss Axioscope microscope was used for fluorescence microscopy, and the images were captured with an Axiocam digital camera using Axiovision software. All images are shown at magnification ×63 except where noted. For routine histology, mouse tissues were fixed in 4% paraformaldehyde overnight at 4°C. The fixed tissues were embedded in paraffin and 5-µm sections were stained with Verhoeff von Gieson stain using standard protocols.

For quantification of elastin and collagen levels, the ascending or abdominal aorta collected from 3-month-old mice was hydrolyzed 48 hours at 110°C in 6N HCl. HCl was removed under vacuum and the dried samples resuspended in 200 µl of amino acid sample buffer and filtered using Ultrafree-MC (0.45µm) centrifugal filters (Millipore). For hydroxyproline analysis, 100 µl of the filtered sample was diluted to 0.5 ml with sample buffer and 50 µl analyzed using a Beckman 6300 amino acid analyzer with the first buffer (Beckman Buffer Na-E) adjusted to pH 2.2 and a column temperature of 60°C. Under these conditions, hydroxyproline elutes as a distinct peak in front of aspartic acid. For desmosine analysis, 50 µl from the initial 200µl dilution was used. The program for resolving desmosine crosslinks consists of 0.2 M sodium citrate, pH 3.28 (Beckman Buffer Na-E) for 14 min, 0.2 M sodium citrate, pH 4.25 (Beckman buffer Na-F) for 12 min, Beckman micro-column citrate buffer (pH 5.26) for 10 min, and 1.0 M sodium citrate, pH 6.40 (Beckman buffer Na-D) for 22 min. Column temperature was maintained at 49°C for the first 11 min and then elevated to 75°C for the remainder of the run. Column flow rate was 20 ml/h. Under these conditions isodesmosine and desmosine elute as distinct peaks at 35.7 and 36.7 minutes, respectively. Ninhydrin was turned on only during the crosslink elution period.
Blood pressure, plasma renin, heart weights, and vessel extensibility measurements

hBAC transgenic mice from a range of ages were used for blood pressure and heart rate measurements. The animals were anesthetized using a ketamine/xylazine (87 mg/kg and 13 mg/kg, respectively) cocktail and were then restrained on a heated holder to maintain body temperature. A Millar pressure transducer was inserted into the right carotid artery and moved to the ascending aorta where heart rate, systolic, and diastolic blood pressure were monitored. Active renin levels in mouse plasma were determined by the clinical laboratories at Barnes Jewish Hospital, Washington University Medical Center (St. Louis, Missouri, USA). For heart weight measurements, hearts from 11 hBAC-mNULL and 6 WT 3-month old animals were dissected, washed, and weighed (wet weight). The left ventricle plus septum and right ventricle weights were obtained using dissected tissue.

For vessel extensibility measurements, isolated vessels were cannulated and mounted on a pressure arteriograph (Danish Myotechnology, Copenhagen, Denmark). Vessels were transilluminated under an inverted microscope connected to a charged-coupled device camera and computerized system, allowing a continuous recording of changes in vessel diameter. Experimental details are described in 3, 4.
Supplemental Table I

Measurement of heart weight in WT and hBAC-mNULL mice at 3 months of age.

<table>
<thead>
<tr>
<th></th>
<th>WT (n=6)</th>
<th>hBAC-mNULL (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal Weight (g)</td>
<td>25.5 ±1.2</td>
<td>25.1±1.7</td>
</tr>
<tr>
<td>Total heart weight (mg)</td>
<td>120±1</td>
<td>135±1*</td>
</tr>
<tr>
<td>Left ventricle + septum (mg)</td>
<td>90.6±1</td>
<td>108±1*</td>
</tr>
<tr>
<td>Right ventricle (mg)</td>
<td>19.1±1</td>
<td>27.1±2*</td>
</tr>
</tbody>
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All values are mean ± SD. * indicates significant difference (p<0.05) as determined by unpaired t-test.
References


Legends for Supplemental Figures

Supplemental Figure 1
Detection of elastin protein in cells established from the skin of newborn hBAC-mWT and hBAC-mNULL animals. Elastin in confluent cultures of mouse skin fibroblasts was visualized using human or mouse species-specific elastin antibody followed by a fluorescent secondary antibody. An extensive extracellular elastic fiber network was visualized with the antibody to human elastin in cells from both genotypes and with the antibody to mouse elastin in hBAC-mWT cells. No elastin fibers were detected with the mouse elastin antibody in hBAC-mNULL fibroblasts, confirming the functional knockout of both mouse elastin alleles in this genotype.

Supplemental Figure 2:
Kaplan-Meier curve showing survival of hBAC-mNULL mice compared to WT controls. Approximately 15% of the mice die between birth and 5 days of age. Survival is then stable out to 90 days when all but about 45% of the remaining mice die. Those that survive past this point show long-term survival.
Supplemental Figure 1
Supplemental Figure 2