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REVIEW

New Insights into Elastic Fiber Assembly

Jessica E. Wagenseil* and Robert P. Mecham

Elastic fibers provide recoil to tissues that undergo repeated stretch, such as the large arteries and lung. These large extracellular matrix (ECM) structures contain numerous components, and our understanding of elastic fiber assembly is changing as we learn more about the various molecules associated with the assembly process. The main components of elastic fibers are elastin and microfibrils. Elastin makes up the bulk of the mature fiber and is encoded by a single gene. Microfibrils consist mainly of fibrillin, but also contain or associate with proteins such as microfibril associated glycoproteins (MAGPs), fibulins, and EMILIN-1. Microfibrils were thought to facilitate alignment of elastin monomers prior to cross-linking by lysyl oxidase (LOX). We now know that their role, as well as the overall assembly process, is more complex. Elastic fiber formation involves elaborate spatial and temporal regulation of all of the involved proteins and is difficult to recapitulate in adult tissues. This report summarizes the known interactions between elastin and the microfibrillar proteins and their role in elastic fiber assembly based on in vitro studies and evidence from knockout mice. We also propose a model of elastic fiber assembly based on the current data that incorporates interactions between elastin, LOXs, fibulins and the microfibril, as well as the pivotal role played by cells in structuring the final functional fiber. **Birth Defects Research (Part C) 81:229–240, 2007.** © 2008 Wiley-Liss, Inc.

INTRODUCTION

Elastic fibers provide elastic recoil to tissues such as the large arteries, lung, and skin. They store energy during the cardiac and respiratory cycles. The main component of elastic fibers is the protein elastin. During early stages of fiber development, elastin deposits are usually associated with microfibrils, but it is not known exactly how microfibrils and elastin interact during elastic fiber assembly. Elastic fibers are assembled primarily during early development and adult tissues are not capable of properly recapitulating the process. Damaged elastic fibers in adult tissues are often repaired with improperly organized material that does not function normally. This leads to tissues that are too stiff and can result in cardiac, cardiovascular, and pulmonary disease. Understanding the assembly process will help us design interventions for proper elastic fiber repair in adult tissues.

Ultrastructural analysis of developing elastin-rich tissues shows that elastin assembly typically occurs in two phases. First, small microfibril-rich elastin globules appear near the surface of the cell early in the process. Second, these coalesce to form larger structures, which can be sheet-like lamellae in blood vessels or filaments in skin, lung, and other tissues. As the fiber matures, elastin becomes the major component, with a small number of microfibrils detectable on the periphery of the structure.

During the past few years, new insight into elastic fibers and their component proteins has been provided by sometimes surprising phenotypes in knockout mice. These studies identified several unexpected proteins that play a significant role in elastic fiber assembly. Gene array studies of developing mice show the coordinated expression patterns of elastic fiber proteins and emphasize the critical spatial and temporal timing of protein expression. In addition, the ability to visualize extracellular matrix (ECM) assembly through video microscopy highlights the important role that the cell plays in structuring the elastic fiber. This report will summarize the current information on interactions between elastin and the expanding family of microfibrillar proteins, and the role of each protein in elastic fiber assembly. We also present a model of elastic fiber assembly that incorporates information from in vitro and in vivo studies and highlights

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the dynamic role played by the cell in organizing elastin in the ECM.

ELASTIC FIBER COMPONENTS

Elastic fibers are the largest structures in the ECM and consist of two morphologically distinct components. The major component is elastin, which is a cross-linked polymer of the monomeric secreted form of the protein tropoelastin (Sandberg et al., 1971). The second component is visualized as small, 10–15-nm "microfibrils" that localize to the periphery of the fiber in adult tissues. While elastin arises from a single gene, the composition of microfibrils is more complex. The major structural element of microfibrils is contributed by the fibrillins (Sakai et al., 1986; Zhang et al., 1994). Numerous other proteins associate with microfibrils or with elastin itself, including the microfibril associated glycoproteins (MAGPs), fibulins and EMILIN-1 (for a complete list see Kielty et al., 2002). Based on ultrastructural studies, microfibrils are thought to provide a scaffold that facilitates elastin molecular alignment and subsequent cross-linking, which is catalyzed by one or more members of the lysyl oxidase (LOX) gene family (Csiszar, 2001; Kagan and Li, 2003; Lucero and Kagan, 2006).

It is still uncertain how each of the elastic fiber components contribute to the formation of a functional fiber, but clues can be obtained from in vitro studies with isolated proteins and from knockout mouse models. A summary of the biological properties of the major proteins known to be associated with elastic fibers or with elastic fiber assembly is presented below. Particular attention is given to the phenotypes apparent in mice in which the genes have been inactivated.

Tropoelastin/Elastin

Tropoelastin is a 60–70-kDa protein composed of alternating hydrophobic and lysine-containing cross-linking domains (Gray et al., 1973). Of the ~40 lysine residues in the secreted monomer, all but approximately five are modified by LOX to form cross-links. This high degree of cross-linking is responsible for the stability and insolubility of the protein. Tropoelastin is capable of undergoing coacervation under physiological conditions in a process thought to facilitate self-assembly (Cox et al., 1974; Volpin et al., 1976; Clarke et al., 2006). Coacervation is the result of specific interactions between the hydrophobic domains induced by an increase in temperature (Toonkool et al., 2001). Studies suggest that coacervation is an important prerequisite for cross-linking (Narayanan et al., 1978), but it is not known at which point in assembly this occurs. The accepted model has been that interactions between tropoelastin and microfibrils in the extracellular space serve to facilitate alignment of cross-linking domains within tropoelastin prior to cross-linking (Kielty et al., 2002). Alternatively, tropoelastin may self assemble in the absence of microfibrils (e.g., through coacervation) on the cell surface and then be transferred as aggregates to microfibrils. In this case, cross-linking could occur on the cell surface within the microaggregate, once the microaggregate is transferred to the microfibril, or, most likely, at both steps (see model in Fig. 7). The propensity of tropoelastin to self-assemble (i.e., coacervate) also raises the possibility that some of the associated proteins thought to promote fiber assembly may actually serve to limit assembly so that large elastin aggregates do not form (Kozel et al., 2006).

Mice lacking the elastin gene (eln−/−) die within a few days of birth of vascular obstruction due to smooth muscle cell (SMC) overproliferation (Li et al., 1998a). The vessel wall is thickened at the time of death in these animals and the SMCs are misarranged. Mice having one elastin allele (eln+/−), in contrast, live a normal lifespan despite substantial hypertension and smaller vessels with thinner walls and altered mechanical properties (Wagenseil et al., 2005). Surprisingly, they also have an increased number of lamellar units in the arterial wall (Li et al., 1998b; Faury et al., 2003) (a lamellar unit is defined as a layer of elastin with its associated SMCs). Lamellar units are established in early development during formation of the vessel wall and lamellar number is linearly related to wall tension in most mammals (Wolinsky and Glagov, 1967). Our recent morphologic studies of developing eln−/− and +/− aorta show that the changes in wall structure occur in the last few days before birth (between embryonic day 18 [E18] and postnatal day 0 [P0]) just as the pressure and blood flow are significantly increasing.

Fibrillins

Fibrillins are large (~350 kDa), cysteine-rich glycoproteins that make up the major structural element of microfibrils (Sakai et al., 1991). A total of three fibrillins (fib-1, -2, and -3) have been identified (Sakai et al., 1986; Lee et al., 1991a; Corson et al., 2004), although the gene for fibrillin-3 has been disrupted in rodents due to chromosome rearrangement (Corson et al., 2004). The primary structure of fibrillin is dominated by calcium binding epidermal growth factor (EGF)-like domains that develop a rod-like structure in the presence of calcium (Downing et al., 1996). Fibrillin-1 isolated from human fibroblasts appears as linear fibrils with a beaded periodicity (Sakai et al., 1991). Fibrillins have Arg-Gly-Asp (RGD) sequences that interact with integrins (Pfaff et al., 1996; Sakamoto et al., 1996; Bax et al., 2003) and heparin-binding domains that interact with cell surface heparan sulfate proteoglycans (Tiedemann et al., 2001; Ritty et al., 2003), suggesting that fibrillins directly signal cells through these receptors. Furthermore, in vivo assembly of fibrillin may require cell-surface receptors in a similar manner to fibronectin (Wu et al., 1995). Fibrillins also

have a major role in binding and sequestering growth factors, such as TGF-β, into the ECM (Neptune et al., 2003).

While microfibrils are most noted for their association with elastic fibers, they can also be found without elastin in the ciliary zonules of the eye and in the circulatory system of invertebrates where they serve a mechanical role in those tissues. The addition of elastin is an evolutionary adaptation in vertebrate animals to handle the high pulsatile pressures of a closed circulatory system (Faury, 2001). Fibrillin-1 and -2 bind tropoelastin in solid phase binding assays (Trask et al., 2000b).

Mutations in the fibrillin-1 gene lead to Marfan syndrome, while fibrillin-2 mutations lead to congenital contractural arachnodactyly (Lee et al., 1991b; Park et al., 1998; Chaudhry et al., 2001). Mice lacking the fibrillin-1 gene (fbn1$-/-$) die within two weeks of birth from vascular and pulmonary complications, including ruptured aortic aneurysms, impaired breathing and diaphragmatic collapse. The elastic fibers in the aorta are abnormally thin and fragmented (Carta et al., 2006). Mice lacking the fibrillin-2 gene (fbn2$-/-$) develop syndactyly, but show no defects in the vascular and pulmonary systems and have a normal lifespan. The elastic fibers in the aorta look normal (Arteaga-Solis et al., 2001; Chaudhry et al., 2001; Carta et al., 2006). Mice deficient in both fibrillins (fbn1$-/-$;fbn2$-/-$) die in utero. At E14.5, there are traces of elastic fibers between SMC layers in fbn1$-/-$:fbn2$-/-$ mice, but nothing close to the organized lamellar units visible in wild-type mice. Half of fbn1$+/-$:fbn2$-/-$ mice die in utero and show a more severe vascular phenotype than fbn1$-/-$ mice. The elastic fibers in the aorta are even thinner and more fragmented than fbn1$-/-$ mice. This indicates that fibrillin-1 may compensate for the loss of fibrillin-2 and that fibrillin-2 may be involved in the initial assembly of aortic microfibrils (Carta et al., 2006).

MAGP-1 and MAGP-2

MAGP-1 was the first microfibrillar protein isolated and completely characterized (Gibson et al., 1986). A second member of the MAGP family, MAGP-2, was subsequently identified (Gibson et al., 1996) (the gene symbols for MAGP-1 and -2 are mfap2 and mfap5, respectively). The MAGPs are small ($\sim$20 kDa) glycoproteins that localize to the beaded region of microfibrils (Henderson et al., 1996). MAGP-1 has been localized to most microfibrils and is thought to be the only protein besides fibrillin that is a constitutive component of the microfibril.

Specific interactions between MAGP-1 and fibrillin-1 have been shown by coimmunoprecipitation studies (Trask et al., 2000a) and recombinant MAGP-1 binds to recombinant fibrillin-1 and to recombinant tropoelastin (Brown-Augsburger et al., 1994; Jensen et al., 2001) in ligand blotting assays. MAGP-1 has also been shown to bind to fibrillin-2 in yeast two-hybrid, ligand blotting, and solid phase binding assays (Werneck et al., 2004). MAGP-2 binds to fibrillin-1 and -2 in yeast two-hybrid studies (Penner et al., 2002) and in solid phase binding assays (Hanssen et al., 2004). The ability of MAGP-1 to bind both tropoelastin and fibrillin suggested that it might play an important role in elastic fiber assembly by serving as a bridging molecule between its two binding partners. In mice lacking the MAGP-1 gene (mfap2$-/-$), however, elastic fiber assembly is normal and there are no structural abnormalities in any of the elastin-rich tissues (our unpublished results). Hence, MAGP-1 is not necessary for normal elastic fiber assembly.

The mfap2$-/-$ mice developed in our laboratory were originally in a mixed background of Black Swiss (BSw) and 129. The mice showed phenotypes of variable penetrance that included increased body weight, skeletal deformations, and bone lesions. When the mice were back-crossed into the C57Bl/6 background, most of these phenotypes disappeared. When back-crossed into the BSw background, many of the original phenotypes reemerged. These phenotypes include a bleeding diathesis in both large and small vessels, an increase in body fat and muscle mass, bone lesions, delayed wound healing, and altered cell adhesion (Weinberg JS, Broekelmann TJ, Pierce RA, Werneck CC, Segade F, Knutsen RH, Mecham RP, unpublished results). All of the phenotypes show variable penetrance with the exception of the bleeding diathesis, which is completely penetrant in all backgrounds, including C57Bl/6. The phenotypes observed in the BSw mfap2$-/-$ mouse are similar to matricellular proteins that bind matrix proteins, but do not contribute to the structural integrity of the ECM, such as thrombospondin (Kyriakides et al., 1998) and secreted protein acidic and rich in cysteine (SPARC) (osteonectin) (Bradshaw et al., 2003).

Fibulins

Fibulins are 50–200 kDa in size (Timpl et al., 2003) and have a series of calcium binding EGF-like domains followed by a carboxy-terminal fibulin-like domain (Argraves et al., 2003). There are five members of the fibulin family. Fibulin-1 associates with some basement membranes and with the amorphous elastin core of elastic fibers, but not with individual microfibrils (Roark et al., 1995). Fibulin-2 also associates with basement membranes (Pan et al., 1993) and strongly binds tropoelastin (Sasaki et al., 1999). Unlike fibulin-1, fibulin-2 binds directly to fibrillin-1 and has been shown to localize to the interface between the amorphous elastin core and microfibrils in skin (Reinhardt et al., 1996). Fibulin-3 does not bind to fibrillin-1 (El-Hallous et al., 2007) and has been shown to only weakly interact with tropoelastin (Kobayashi et al., 2007). Fibulin-3 is expressed in capillaries, but not large blood vessels (Giltay et al., 1999) and most likely does not have a role in elas-

tic fiber assembly. Fibulin-4 binds fibrillin-1 (El-Hallous et al., 2007) and shows moderate affinity for tropoelastin. Fibulin-4 has been localized to microfibrils (Kobayashi et al., 2007). Fibulin-5 colocalizes with elastic fibers in vitro (Nakamura et al., 2002) and can be seen at the elastin/microfibril interface in the aorta (Yanagisawa et al., 2002). Fibulin-5 binds tropoelastin and shows weak affinity for the C- and N-terminus of fibrillin-1 (Yanagisawa et al., 2002). Fibulin-5 also binds cell surface integrins (Nakamura et al., 2002).

The splice D variant of fibulin-1 has been implicated in human syndactyly (Debeer et al., 2002). Mice deficient in fibulin-1 (fbln1−/−) die within 24–48 hr of birth and exhibit spontaneous bleeding as early as E12.5. Fbln1−/− mice show defects in the kidney, lung and capillaries, but the heart and large elastic arteries look normal. These findings suggest that fibulin-1 is not involved in elastic fiber assembly, but plays a role in organizing basement membrane and microfibrils in angiogenesis and capillary formation. Other fibulins, such as fibulin-2, may also compensate for the loss of fibulin-1 in these mice (Kostka et al., 2001). No known human diseases have been associated with fibulin-2 and a fibulin-2 knockout mouse has no obvious elastin phenotype (Chiu, M-L, personal communication). A mutation in fibulin-3 has been implicated in a macular dystrophy (Stone et al., 1999). A fibulin-3 knockout mouse has not yet been described.

A mutation in the fibulin-4 gene has been recently linked to an autosomal recessive form of cutis laxa. The patient had loose skin, emphysema, aortic tortuosity, and an ascending aortic aneurysm (Hucthagowder et al., 2006). Mice lacking the fibulin-4 gene (fbln4−/−) show the most severe phenotype of the existing fibulin knockout mice. Fbln4−/− mice die perinatally with severe lung and vascular defects. The aorta is narrowed by E12.5 and tortuous by E15.5. The elastic fibers contain irregular elastin aggregates with evenly distributed rod-like filaments visible within the usual amorphous globules. Elastin cross-links are greatly reduced, but the expression of tropoelastin and LOX is normal (McLaughlin et al., 2006). The rod-like filaments in the fbn4−/− elastic lamellae resemble those observed in the aorta of chicks fed β-aminopropionitrile (BAPN), an irreversible LOX inhibitor. BAPN prevents the proper cross-linking of elastin by LOX. The rod-like filaments in the BAPN-fed chicks have been identified as proteoglycans (Contri et al., 1985). The similar appearance of fbn4−/− mouse lamellae and poorly cross-linked chick lamellae suggests that fibulin-4 facilitates cross-linking of elastin at some stage in the assembly process. Like fibulin-4, mutations in the fibulin-5 gene have also been linked to cutis laxa (Loeys et al., 2002; Markova et al., 2003; Hu et al., 2006). Fibulin-5 knockout (fbln5−/−) mice show similar phenotypes to the fibulin-4−/− mice, but with less severity as they live a normal lifespan. Fbln5−/− mice have loose skin, tortuous aortas, and disrupted elastic fibers with abnormal elastin aggregates (Nakamura et al., 2002; Yanagisawa et al., 2002).

Elastin Microfibril Interface Located Protein (EMILIN)-1

EMILIN-1 is one of four members of the EMILIN protein family that have similar structural features including an EMI domain, an α-helical domain and a gC1q domain (Colombatti et al., 2000). EMILIN stands for ''elastin microfibril interface located protein.'' As the name suggests, EMILIN-1 is found in elastin-rich tissues and is localized to the interface between amorphous elastin and microfibrils. Anti-EMILIN-1 antibodies promote the formation of elastin aggregates rather than fibers in SMC cultures (Bressan et al., 1993). EMILIN-1 binds elastin in solid-phase binding assays and both elastin and fibulin-5 in immunoprecipitation assays (Zanetti et al., 2004).

No known human disease has been linked to mutations in EMILIN-1. EMILIN-1 knockout (emilin1−/−) mice live a normal lifespan, but show alterations in the elastic fibers of the skin and aorta. The amorphous elastin core appears irregularly shaped and split and the cells appear to have lost their connections to the elastic fibers. EMILIN-1−/− embryonic fibroblasts form elastin fibers in vitro that are thinner and straighter than control fibroblasts and lack the complete colocalization of elastin and fibulin-5 observed in controls. Fibulin-2 and fibrillin-1 fibers appear normal (Zanetti et al., 2004). Similar to eln+/− mice, emilin1−/− mice have significant high renin-induced hypertension and vessels with smaller diameters and thinner walls (Faury et al., 2003; Zacchigna et al., 2006). The absence of EMILIN-1 increases TGF-β signaling and, interestingly, the vascular phenotype can be reversed by inactivating one TGF-β allele (emilin-1−/−; tgf-β+/− mice) (Zacchigna et al., 2006).

LOX

There are five members of the LOX family: LOX and four LOX-like proteins (LOXL1–4). Each protein contains an N-terminal signal peptide, a variable region in the center of the molecule, and a C-terminal region that shows significant sequence similarity within the family. LOX and LOXL1 are most similar to each other, while LOXL2–4 appear to belong to a separate LOX subfamily (Lucero and Kagan, 2006). Enzyme activation is dependent on processing from the pro forms by bone specific proteases, such as bone morphogenic protein-1 (BMP-1) (Borel et al., 2001). LOX and LOXL1 cross-link both elastin and collagen. In elastin, lysine residues are modified to form bi-, tri-, and tetra-functional cross-links, with desmosine and isodesmosine being unique to elastin in vertebrates (Partridge et al., 1963, 1964). LOX can cross-link recombinant tropoelastin in vitro without the presence of any other elastic fiber proteins (Bedell-Hogan et al., 1993). LOX localizes to the elastin/microfibril interface in aorta (Kagan et al., 1986) and LOX and

LOXL1 colocalize in most elastic tissues (Hayashi et al., 2004). LOXL1 specifically localizes to sites of elastogenesis and interacts with fibulin-5 (Liu et al., 2004). The pro region plays a role in directing LOX and LOXL1 to elastic fibers in vitro, and sequence differences in the pro regions may account for some of the functional differences between the enzymes (Thomassin et al., 2005).

As might be expected for an enzyme so important for collagen and elastin maturation, numerous disease states have been linked to alterations in LOX activity. These include copper metabolism or transport disorders (Sacks, 2003), as well as Alzheimer disease (Gilad et al., 2005), amyotrophic lateral sclerosis (ALS) (Li et al., 2004), and even cancer progression (Erler and Giaccia, 2006). Recently, single nucleotide polymorphisms in LOXL have been identified as risk factors for glaucoma (Thorleifsson et al., 2007).

Mice lacking the LOX gene (lox−/−) die at the end of gestation or just after birth with large aortic aneurysms and diaphragmatic collapse. The elastic lamellae in the aorta are fragmented and discontinuous and the vessel wall is thicker with a decreased lumen size. Desmosine cross-links are reduced approximately 60% in the aorta and lungs (Maki et al., 2002; Hornstra et al., 2003). Lox−/− mice also show impaired airway development in the lungs and abnormal collagen and elastin fibers in the skin (Maki et al., 2005). Mice lacking the LOXL gene (loxl1−/−) live a normal lifespan and do not show the obvious vascular and pulmonary defects apparent in lox−/− mice. However, postpartum loxl1−/− mice develop pelvic organ prolapse, loose skin, enlarged airspaces and vascular deformities. Therefore, LOXL1 may play a unique role in elastic fiber homeostasis (Liu et al., 2004).

ELASTIC FIBER ASSEMBLY

The proteins necessary for elastic fiber assembly must be regulated both spatially and temporally. This complex regulatory process can be examined with gene array and morphologic studies of developing elastic tissues. Elastic fiber assembly can also be recapitulated in vitro and watched in real time using fluorescently-labeled proteins or antibodies. Live imaging studies have highlighted the active role of the cell in elastic fiber assembly as it coordinates and directs proteins to the appropriate locations.

Coordinated Gene Expression

Most of the elastic fiber proteins are expressed from the second half of development (around E14 in the mouse) and show a steady rise through the early postnatal period (around P7–14 in mice). This is followed by a decrease in expression to low levels that persist through adulthood (Kelleher et al., 2004). In adult tissue, elastic fibers are difficult to repair and elastin often does not polymerize or form functional three-dimensional (3D) fibers (Chrzanowski et al., 1980; Shifren and Mecham, 2006). This is probably because the complicated temporal and spatial regulation is difficult to recapitulate in adult tissues.

Our laboratory has recently completed a gene expression study of developing mouse aorta from E12 to P180. The data was generated following similar protocols to our previous array studies (Kelleher et al., 2004; McLean et al., 2005), but was completed using the Affymetrix Mouse 430v2 chip (39,000 genes) (Santa Clara, CA) that includes more matrix probes. All of the elastic fiber genes mentioned above, except for fibrillin-2 (fbn2) and MAGP-1 (mfap2), show an increase in expression from E14 through ~P14 followed by a decrease to low levels where expression remains steady from P60 to P180. Fbn2 and mfap2, on the other hand, start out with high expression values and are probably turned on prior to E14. Expression of these proteins remains high until P0 when they quickly drop to lower levels and oscillate around this value through adulthood. The fbn2 expression pattern supports the hypothesis that fibrillin-2 is involved in the initial assembly of microfibrils, but plays a lesser role in elastic fiber assembly (Carta et al., 2006). The relative amounts of each protein on the gene array may explain some phenotypes in the knockout mice. For example, lox and loxl1 show almost identical expression patterns, but lox amounts are substantially higher than loxl1 and the lox−/− mouse has a much more severe phenotype than loxl1−/−.

The coordinated expression pattern of all the proteins, except fibrillin-2 and MAGP-1, implies that each protein is necessary for normal elastic fiber assembly. It also suggests that homologous proteins in the same family may compensate for each other in knockout mouse models. For example, if fibulin-1 is absent, fibulin-2 may be able to compensate during development and produce a relatively normal aorta because the temporal expression pattern is correct. The severity of the phenotype may depend on gene dosage of total fibulins instead of the presence or absence of specific fibulin proteins. Our laboratory has recently shown that mice with decreasing amounts of elastin (from 100 to 30% normal levels) have increasingly severe vascular (Hirano et al., 2007) and pulmonary (Shifren et al., 2007) phenotypes. If elastin amounts fall below 30%, the mice cannot survive postnatally (Hirano et al., 2007).

Cellular Activity

Cells are actively involved in assembling many ECM molecules, including fibronectin (Mao and Schwarzbauer, 2005) and collagen (Birk and Trelstad, 1986; Kadler, 2004), but their role in elastic fiber assembly has been overlooked. Recent live imaging studies in our laboratory show that cells are actively involved in the organization and alignment of elastin aggregates into linear structures and the deposition of elastin aggregates onto preexisting fibers (Kozel et al., 2006; Czirok et al.,

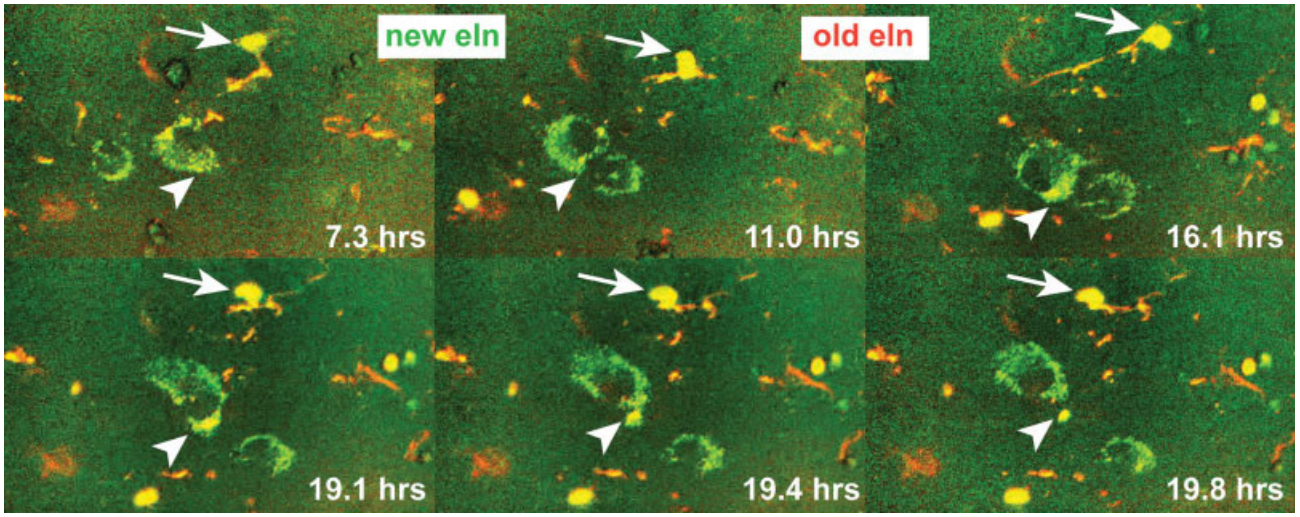

Figure 1. Assembly of elastin on the cell surface and transfer to extracellular microfibrils. Frames from a live imaging study with rat fetal lung fibroblast (RFL-6) cells transfected with pTimer-tropoelastin. A movie with all of the time frames is in the online Supplemental Material (Movie A). Imaging was started 1 day after transiently transfecting confluent cells. Cells were imaged for 24.5 hr with 22 min between frames. pTimer-tropoelastin starts out with green fluorescence (new eln) and changes to red after about 8 hr (old eln). Yellow staining indicates colocalization of new and old eln. The arrow shows a cell with yellow elastin staining on its surface that moves toward an existing elastin fiber, tracks along it to the right, stretches it, then follows it to the left before releasing the fiber. The arrowhead shows small green cell-associated elastin aggregates that coalesce into a large yellow aggregate that is released by the cell into the ECM. Additional cell and elastin aggregate and fiber movement can be seen in Movie A.

2006). In these studies, cells were transfected with an expression construct encoding tropoelastin fused with pTimer, which is a fluorescent protein that changes from green to red fluorescence over about eight hours, allowing us to discriminate between new (green), old (red) and combined new and old (yellow) elastin. In transfected cells, small green aggregates of newly formed elastin are visible on the cell surface. These aggregates coalesce into larger structures and remain in contact with the cell long enough for some molecules to make the green to red transition. The large, yellow aggregates move on the cell surface toward preexisting red fibers where they are deposited and the cell moves away. The initial formation of small aggregates is termed ''microassembly'', while the transfer of the older, sometimes larger aggregates to existing fibers is termed ''macroassembly'' (Kozel et al., 2006). Pulse-chase immunolabeling of the fibroblast-like rat fetal lung fibroblast (RFL-6) cells demonstrates that tropoelastin globules aggregate in a hierarchical manner, creating progressively larger fibrillar structures. By analyzing the correlation between cell and ECM movements, both the aggregation process and shaping the aggregates into fibrillar form was found to be coupled to cell motion. The motion of nonadjacent cells becomes more coordinated as the physical size of elastin-containing aggregates increases, indicating that the formation of elastic fibers involves the concerted action and motility of multiple cells (Czirok et al., 2006).

Frames from one of the pTimer-tropoelastin live imaging studies are shown in Figure 1. A cell with yellow elastin on its surface can be seen tracking, stretching, and releasing an extracellular elastin fiber. Another cell that starts out with green elastin aggregates, gathers the aggregates into a larger structure that turns yellow and is deposited in the ECM, presumably on an unstained preexisting fiber. A movie with all the time frames for Figure 1 is in the online Supplemental Material (Movie A{MOVA}; available online at http://www.interscience.wiley.com/jpages/1542-975X/suppmat). Unlike cellular assembly of collagen and fibronectin, the nature of the cellular interaction with elastin is relatively unknown. Possible binding partners include elastin-binding protein (Hinek et al., 1988; Wrenn et al., 1988), integrins (Rodgers and Weiss, 2004), and cell-surface glycosaminoglycans (Broekelmann et al., 2005).

Additional studies in our laboratory have focused on determining the interactions between elastin and other elastic fiber proteins during in vitro assembly. To determine if LOX is present at the early stage of elastin assembly, cells were incubated with BAPN to inhibit LOX activity and elastin assembly was followed using video imaging. The presence of BAPN decreased in vitro elastin matrix assembly in a dose dependent manner (Fig. 2), suggesting that cross-linking is important for aggregate formation on the cell surface and that LOX or some other member of the LOX family is present at elastin assembly sites. Live imaging studies have also been instructive as to the participation of other elastic fiber proteins in fiber formation. For example, imaging studies with fluorescently labeled

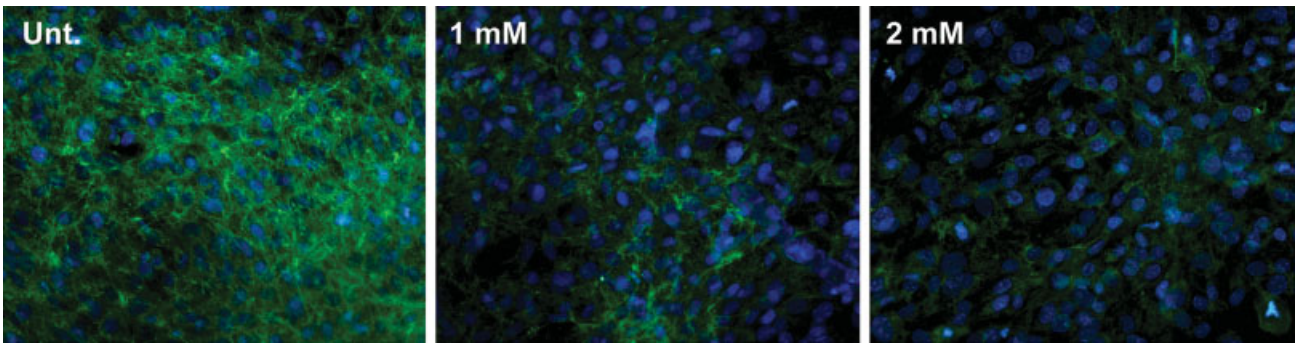

Figure 2. Inhibition of LOX activity blocks elastin assembly. RFL6 cells untreated or treated with 1 or 2 mM BAPN, an inhibitor of LOX, for five days after plating at 50% confluence. Cells were fixed with methanol and stained with a polyclonal antibody to mouse tropoelastin (green) and Hoechst nuclear stain (blue).

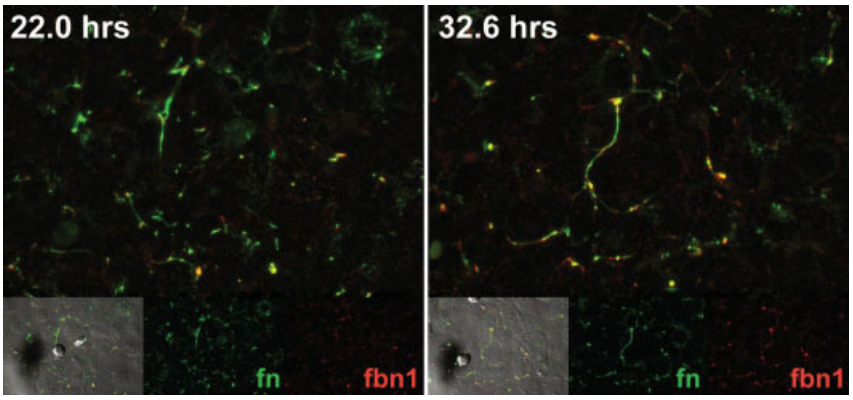

Figure 3. Colocalization of fibronectin and fibrillin during fiber assembly. Frames from a live imaging study of bovine pigmented epithelial (PE) cells stained with a polyclonal antibody to fibronectin (fn, green) and a monoclonal antibody to fibrillin-1 (fbn-1, red). A movie with all of the time frames is in the online Supplemental Material (Movie B). Imaging began one day after the cells were plated at 50% confluency. Cells were imaged for 68 hr with 40 min between frames. Primary and secondary antibodies were added sequentially to the cells for 1 hr each, then rinsed before imaging. The secondary antibodies alone showed no signal and the secondary antibodies did not cross-react. Antibodies were added and rinsed again 22 and 32 hr after imaging began. At the start of the imaging period (not shown), several small fn fibers and a few fbn-1 aggregates are visible. By 22 hr, more fn fibers and fbn-1 aggregates can be seen with fbn-1 aggregates colocalizing with fn fibers. By 32.6 hr, several small fn fibers have combined to make larger fibers and some of the fbn-1 aggregates have formed fibers that colocalize with previously formed fn fibers. In Movie B, the cells can be seen moving, combining and breaking fibers.

antibodies show that elastin fibers are formed at the same location as previously existing fibronectin, fibrillin-1, MAGP-1 and fibulin-5 fibers. The proteins are temporally arranged into fibers in that order: fibronectin, fibrillin-1, MAGP-1, fibulin-5, and elastin in a variety of elastin producing cells. Figure 3 shows frames from a live imaging study where the cells have been incubated with antibodies to fibronectin and fibrillin-1. At the beginning of the imaging period, only fibronectin fibers are apparent. After 32 hr of imaging and two subsequent antibody incubations, fibrillin-1 fibers are visible and are forming in the same location as previously existing fibronectin fibers. A movie with all of the frames from the live imaging study is included in the online Supplemental Material (Movie B{MOVB}). Fibronectin is not generally considered an elastic fiber protein, but an initial fibronectin scaffold is necessary for collagen type I and type III matrix assembly in vitro (Velling et al., 2002) and may be associated with elastic fiber assembly, at least in vitro. LOX binds to fibronectin with a similar affinity to tropoelastin, but does not act on fibronectin enzymatically. In fibronectin null (fn−/−) fibroblasts, LOX processing from the pro form to the active form is reduced; therefore, fibronectin may serve as a scaffold for enzymatically active LOX (Fogelgren et al., 2005). Fn−/− mice die before E14.5 with extensive systemic defects (George et al., 1993).

Figure 4 shows frames from a live imaging study in which the cells have been incubated with antibodies to fibulin-5 and tropoelastin. At the start of the imaging period several thin fibulin-5 fibers and numerous aggregates are visible. There is one elastin fiber that colocalizes with a fibulin-5 fiber and there are a few elastin aggregates. After 27 hr, both fibulin-5 and elastin show numerous colocalized aggregates that are linearly arranged in fibers. Not all fibulin-5 and elastin aggregates are colocalized, but approximately half are, indicating the fibulin-5 may play a role in the microassembly step where elastin aggregates are formed on the cell surface. During the imaging period, the fibers shift location in the imaging plane due to active rearrangement by the cells. This can be better appreciated in the complete movie in the online Supplemental Material (Movie C{MOVC}).

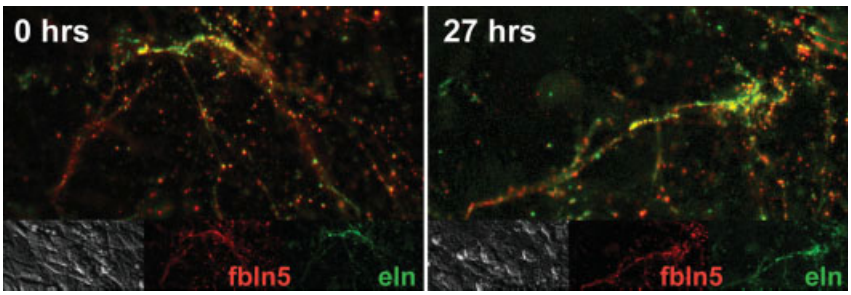

Figure 4. Distribution of fibulin-5 and tropoelastin in cultured cells. Frames from a live imaging study of fetal calf ligament (FCL) cells stained with a monoclonal antibody for fibulin-5 (fbln-5, red) and a polyclonal antibody for tropoelastin (eln, green). A movie with all of the time frames is in the online Supplemental Material (Movie C). Imaging began five days after the cells were plated at 50% confluence. Cells were imaged for 40 hr with 30 min between frames. The secondary antibodies alone showed no signal and the secondary antibodies did not cross-react. Primary and secondary antibodies were added sequentially to the cells for 1 hr each, then rinsed before imaging. At the start of the imaging period, several thin fibers and numerous fbln-5 aggregates are visible. One eln fiber and several eln aggregates colocalize with the fbln-5 structures. By 27 hr, most of the fbln-5 and eln aggregates are aligned in colocalized fibers. This coordinated reorganization can be followed in Movie C.

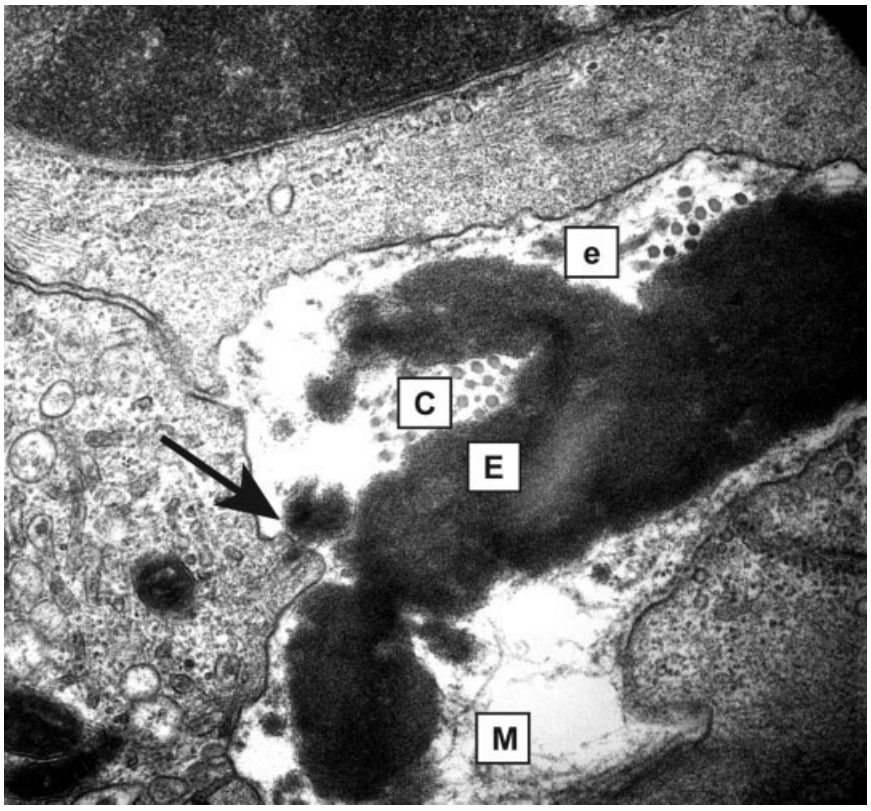

Figure 5. Electron micrograph of elastic fiber assembly in the developing mouse aorta. P0 mouse aorta showing secretion and organization of elastin by cells. Collagen fibers (C) can be seen in cross-section near the large amorphous elastin (E) lamella. Microfibrils (M) are located between the cell and the elastin. Smaller elastin aggregates (e) decorate some of the microfibrils. An elastin aggregate appears to be forming within a plasma membrane protrusion (arrow) to join a larger aggregate at the cell surface.

Live imaging studies with light microscopy are useful for studying the dynamics of fiber assembly, but do not have sufficient resolution to examine interactions between individual cells and elastic fibers. Electron microscopy (EM) has the resolution to study cellular interactions and has brought new understanding to the role of cells in the assembly of other matrix molecules, like collagen. EM studies have shown that collagen fibrillogenesis is initiated within plasma membrane recesses, presumably under close cellular regulation (Birk and Trelstad, 1986). Similar structures, given the name fibripositors, have been described in ligament cells (Canty et al., 2004). Our laboratory has recently completed an EM study of developing mouse aorta from E14 to P0. The images show the dramatic increase in elastin deposition during the last few days of development and are consistent with the gene array studies (Kelleher et al., 2004). Figure 5 is an EM image of a P0 aorta taken near the center of the vessel wall. Cross-sections of individual collagen fibers and a network of microfibrils can be seen next to the amorphous elastin lamella. Small globules of elastin are also present at the cell surface and interspersed through the microfibrillar network. There appears to be an elastin aggregate being secreted directly from a cell membrane protrusion to join a larger aggregate at the cell surface. This behavior is consistent with our live imaging studies (Kozel et al., 2006) and is reminiscent of collagen fibripositors (Canty et al., 2004).

Model

Figure 6A summarizes the known binding interactions between elastin and the microfibrillar proteins discussed in this review. Figure 6B shows the localization of each protein as described in microscopy studies of elastic tissues. Fibulin-1 is the only protein, besides elastin, that has been localized to the amorphous core of elastic fibers using antibodies in EM studies (Roark et al., 1995). Ironically, the fbn1−/− mouse seems to have normal arteries, but severe capillary defects (Kostka et al., 2001). Obviously, assembly of elastic fibers is a complex process that

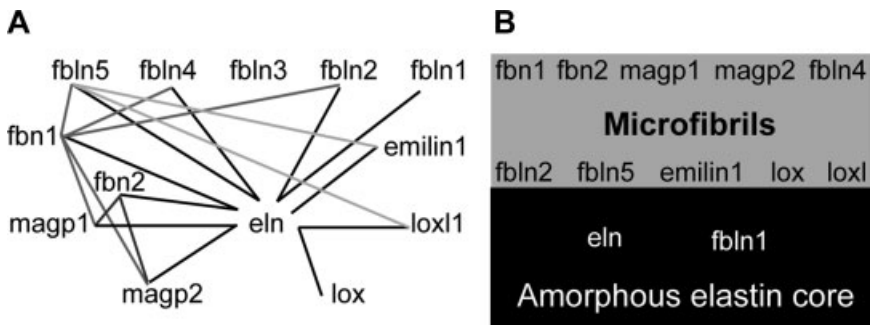

Figure 6. Binding interactions and spatial localization of elastic fiber proteins. **A**: Known binding interactions between elastin and the microfibrillar proteins. **B**: Observed localization of elastin and the microfibrillar proteins with relationship to the amorphous elastin core and the associated microfibrils.

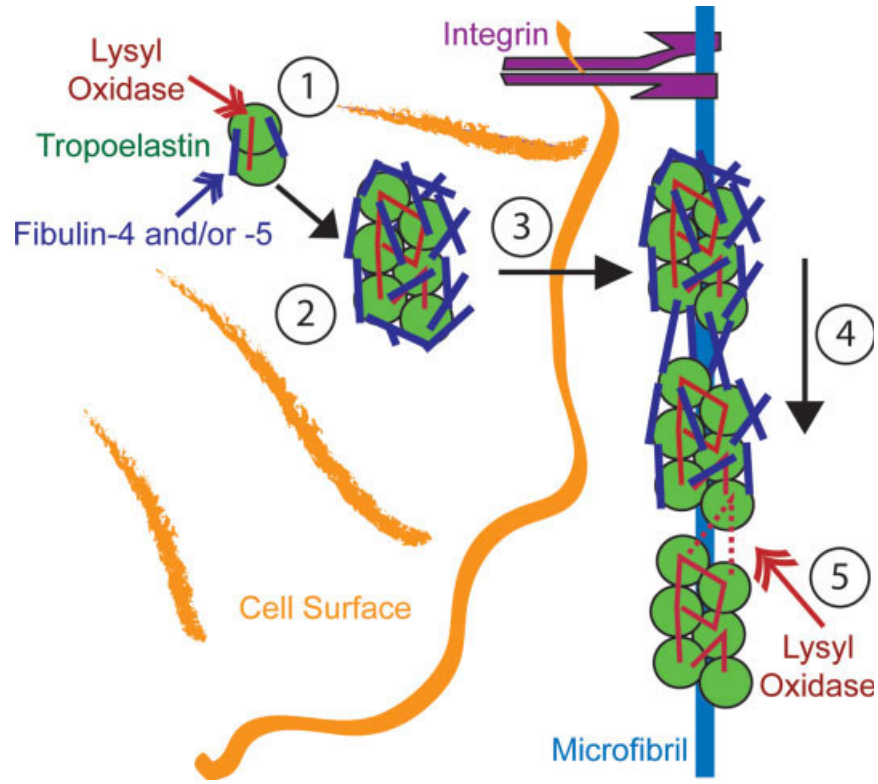

Figure 7. Model of elastic fiber assembly. (1) Tropoelastin is transported to assembly sites on the plasma membrane where it is organized into small aggregates that are cross-linked by a LOX. Cell surface receptors/binding proteins such as heparan sulfate proteoglycans at these sites may serve to assist with the initial assembly steps. Interaction with Fibulin-4 and/or -5 may facilitate cross-linking or possibly help limit the size of the aggregates. (2) The aggregates remain on the cell surface while newly secreted elastin is added to increase the size. (3) The aggregates are then transferred to extracellular microfibrils, which interact with the cell through integrins. Fibulin-4 and/or -5, or another microfibril-associated protein, may assist the transfer of elastin aggregates to the microfibril. (4) Elastin aggregates on the microfibril coalesce into larger structures. Fibulin-4 and/or -5 may facilitate this process. (5) The elastin aggregates are further cross-linked by LOX to form the complete elastic fiber.

involves interactions between numerous proteins and binding, and localization studies, combined with mouse knockout data on these proteins, are not always consistent. Based on the published data and our unpublished studies, however, we have developed a hypothetical model for elastic fiber assembly that includes pivotal roles for individual cells, elastin, LOX and/or LOXL, fibulin-4 and/or -5, and microfibrils (Fig. 7).

In our model, tropoelastin is secreted by the cells and organized into cross-linked aggregates on the cell surface (Kozel et al., 2006), possibly through interactions with cell surface glycosaminoglycans (Broekelmann et al., 2005) and one or more LOX family members. Fibulin-4 and/or -5 interact with these aggregates to facilitate cross-linking (McLaughlin et al., 2006) or to limit the aggregate size. The elastin aggregates remain on the cell surface long enough for new elastin to be secreted from the cell and added to the aggregates (Kozel et al., 2006). The cell actively collects small aggregates on its surface into larger aggregates that are then transferred to preexisting microfibrils. The microfibrils are composed primarily of fibrillin-1 and/or -2, but probably contain other microfibril-associated proteins, such as MAGPs. The microfibrils bind to the cell surface through integrins. Fibulin-4 and -5 both bind fibrillin-1 (El-Hallous et al., 2007; Yanagisawa et al., 2002) and may help transfer the elastin aggregates to the microfibril. The elastin aggregates on the microfibril coalesce into larger structures and are further cross-linked by LOX and/or LOXL to form the complete and functional elastic fiber. Fibulin-4 and/or -5 may facilitate the cross-linking step. It is important to note that few of the proteins in the model can be detected by antibodies within the complete, mature, amorphous elastic fiber. They may be coated with elastin so that the epitope is unavailable, they may be physically displaced and moved to the edge of the elastic fiber or they may be degraded by proteases during the final cross-linking step.

CONCLUSIONS

We have summarized the current data on elastin and elastic fiber proteins and their role in elastic

fiber assembly. We have also presented a working model that assimilates much of the current information obtained through both in vivo and in vitro methods. The next step in understanding elastic fiber formation will be identifying how key elastic fiber molecules interact at the cell surface, how these molecules are sorted within the cell and transported to sites of assembly, how the cell regulates the overall assembly process, and how multiple cells cooperate to structure a functional fiber. The answers to these questions will require new experimental approaches that combine the power of dynamic imaging with specific antibodies, novel protein constructs, and knockout cell lines.

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