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Elastic Fiber Formation: A Dynamic View of Extracellular Matrix Assembly Using Timer Reporters

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To study the dynamics of elastic fiber assembly, mammalian cells were transfected with a cDNA construct encoding bovine tropoelastin in frame with the Timer reporter. Timer is a derivative of the DsRed fluorescent protein that changes from green to red over time and, hence, can be used to distinguish new from old elastin. Using dynamic imaging microscopy, we found that the first step in elastic fiber formation is the appearance of small cell surface-associated elastin globules that increased in size with time (microassembly). The elastin globules are eventually transferred to pre-existing elastic fibers in the extracellular matrix where they coalesce into larger structures (macroassembly). Mechanical forces associated with cell movement help shape the forming, extracellular elastic fiber network. Time-lapse imaging combined with the use of Timer constructs provides unique tools for studying the temporal and spatial aspects of extracellular matrix formation by live cells. J. Cell. Physiol. 207: 87–96, 2006.

Our understanding of the extracellular matrix (ECM) has expanded greatly over the past two decades. In addition to its well-known structural function, we now know that the ECM plays major biological roles in cellular adhesion, differentiation, and migration. A central function of the ECM, however, remains the creation and maintenance of tissue architecture and mechanical function. An underlying principle of matrix formation is the self-assembly of complex structures wherein monomeric units bind to each other to form oligomers and polymers. The information for proper assembly is encoded in the monomeric units themselves, yet there is increasing evidence that assembly of ECM structures is assisted directly by the cell (Yurchenco et al., 2004).

Cellular control of matrix polymer assembly has been shown for several matrix proteins, including fibronectin (McKeown-Longo and Mosher, 1985; McDonald et al., 1987; Panayotou et al., 1988; Wierzbicka-Patynowski and Schwarzbauer, 2003), collagen (Birk and Trelstad, 1986; Canty et al., 2004), and laminin (Lohikangas et al., 2001; Yurchenco et al., 2004). Collagen assembly has been proposed to occur within specialized plasma membrane channels that provide a confined environment conducive to the ordered assembly of supramolecular structures. Assembly of all three proteins requires an interaction with β1 integrins and perhaps other cell surface receptors (McKeown-Longo and Mosher, 1985; Pankov et al., 2000; Lohikangas et al., 2001; Ohashi et al., 2002).

Like other extracellular matrix proteins, elastin assembly occurs extracellularly in a process directed by the elastin-producing cell. Elastin is secreted from the cell as a soluble monomer (tropoelastin) that must be crosslinked into a functional polymer (Franzblau, 1971). The first step in the cross-linking reaction is the formation of the δ-aldehyde, allysine (Partridge, 1962; Franzblau and Faris, 1981), through oxidation of lysyl ε-amino groups by a member of the lysyl oxidase enzyme family. Approximately 40 lysine residues in 16 crosslinking domains eventually participate in forming the bi-, tri-, and tetra-functional crosslinks that help make the resulting product a polymer with reversible deformation and high resilience. For proper crosslinking to occur, the tropoelastin monomers must be correctly aligned so that the appropriate crosslinking sequences are in proper register. Studies to determine how this alignment takes place, however, have yielded only limited information about alignment requirements and specificity (Brown-Augsburger et al., 1995; Wise et al., 2005).

Our current understanding of elastic fiber assembly has been greatly influenced by ultrastructural analysis of developing elastic tissues and by the physical properties of the tropoelastin precursor protein. Electron micrographic studies have identified two components within elastic fibers: amorphous crosslinked elastin and 10–15 nm microfibrils (Fahrenbach et al., 1966; Greenlee

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et al., 1966; Ross and Bornstein, 1969; Cleary et al., 1983). The microfibril (Sakai et al., 1986; Gibson and Cleary, 1987) has been suggested to play a morphogenetic role in determining the presumptive shape and direction of the forming elastic fiber by serving as an extracellular scaffold that binds tropoelastin monomers in preparation for crosslinking into the functional polymer. This has led to the supposition that an interaction between tropoelastin and microfibrils is the first (and a required) step in formation of an elastic fiber. In support of this argument are studies showing that tropoelastin specifically binds components of the microfibril, including fibrillin and MAGP, and sites of interaction within the various proteins have been identified (Brown-Augsburger et al., 1994, 1996; Trask et al., 2000a,b; Jensen et al., 2001; Rock et al., 2004). The counter argument that the initial step in elastin assembly may not require the assistance of microfibrils is based upon the recognition that molecules of tropoelastin can self-associate through a process called coacervation (Cox et al., 1974; Volpin et al., 1976; Bressan et al., 1983; Vrhovski et al., 1997). Ultrastructural analysis of the tropoelastin coacervates has identified fibers similar to those found in coacervates of α-elastin (Cox et al., 1974; Bressan et al., 1983), suggesting that interactions between tropoelastin monomers may be sufficient for proper domain alignment prior to cross-linkage.

In this report, we use time-lapse microscopy to image the synthesis and assembly into fibers of bovine tropoelastin fused to the fluorescent Timer protein. Timer, a derivative of the DsRed fluorescent protein (DsRed-E5), changes from green to red over time (Terskikh et al., 2000; Wiegand et al., 2003) and, hence, can be used to distinguish new from old elastin. Thus, Timer functions as a fluorescent clock giving both temporal and spatial information about elastin secretion and assembly. Our results suggest that the first step of elastic fiber formation is the organization of small globules of elastin on the cell surface followed by globule aggregation to form the final functional fiber. These findings suggest that elastin self-association on the cell surface is the initial step in elastic fiber assembly (a process that we term microassembly). Microassembly is followed by fusion of elastin globules into larger fibrillar structures directed by long linear microfibrils in the extracellular space (macroassembly). Consistent with what has been shown for assembly of other ECM molecules, the cell plays a direct role in shaping the final fiber through mechanical forces associated with the cellular cytoskeleton, cell movement, and global matrix deformation.

METHODS

Cell culture

FBC-180 cells (Lee et al., 1994a,b) were grown in DMEM (Cellgro, Herndon, VA) supplemented with 10% Cosmic Calf Serum (Hyclone, Logan, UT) and RFL-6 cells (ATCC, Manassas, VA) were grown in Ham's F12 (Sigma, St. Louis, MO) medium containing 20% Cosmic Calf Serum. Each was supplemented with non-essential amino acids (Cellgro), sodium pyruvate (Cellgro), and Penicillin-Streptomycin (Washington University Tissue Culture Supply Center, St. Louis, MO). Cells were maintained in a 37°C humidifying CO_2 incubator.

Timer elastin construct

The bovine tropoelastin-Timer construct was generated from an existing tropoelastin-GFP plasmid by swapping Timer for GFP. To generate GFP tagged tropoelastin, a full-length bovine tropoelastin cDNA in the pCIneo vector (pCIneo-bTE-FL) (Kozel et al., 2003) was cut with *Nhe*I and *Bgl*II. *Nhe*I cuts just 5′ of the start site and BglII cuts midway through exon 28. The pEGFP vector (Clontech, Mountainview, CA) was also cut with these enzymes and the tropoelastin fragment was inserted upstream of GFP (pEGFP-bTE1-28). To complete the tropoelastin transcript, amplification of the 3′ portion of the molecule, from the *Bgl*II site up to, but not including the stop site was required. Primers used were forward 5′-CCTTGGAGGGGTTGGAGAT-CTTGGTGGA-3′ and reverse 5′-GTGGCGACCGGTGTCTTT-CTCTTCCGGCCACA-3′. The resulting PCR product and pEGFP-bTE1-28 were cut with *Bgl*II and *Age*I and ligated together. This process results in the tropoelastin molecule being inserted in the pEGFP vector in frame with the GFP tag. GFP was removed from the bTE-GFP vector by cutting with *Age*I and *Xba*I and the Timer fluorescence marker was inserted after being cut from the pTimer® vector (Living Colors® Fluorescent Timer, BD Biosciences Clontech) using the same enzymes.

Transfection of RFL-6 cells

Transient transfections of RFL-6 cells were performed using Lipofectamine 2000 (Gibco, Carlsbad, CA) according to the manufacturer's instructions. Five times 10^5 cells/well were plated into 35-mm Bioptechs culture dishes (see below) and allowed to reach confluency. Construct DNA (5 μg) and 5 μl of transfection reagent were placed in separate tubes containing serum free medium. After 5 min, the two reagents were mixed for 15 min. The transfection mixture was then added to the cell layers in the presence of 20% Cosmic Calf serum (Hyclone, Logan, UT). Transfection was continued for 6 h before imaging was initiated.

Dynamic imaging of fluorescent proteins

Transiently transfected RFL-6 cells were maintained using standard culture conditions (37°C and 5% CO_2/95% air atmosphere) in a Bioptechs (non-liquid perfused) Delta T culture system, consisting of a heated, indium-tin oxide coated glass dish attached to a calibrated Bioptechs micro-perfusion peristaltic pump. The culture was observed with the 20× objective (NA = 0.4) of an inverted automatized wide-field epifluorescence/ DIC microscope (Leica DMIRE2, Leica Microsystems, Wetzlar, Germany). An objective lens heater was used to improve temperature homogeneity. Images (608 × 512 pixels spatial and 12 bit intensity resolution) were recorded with a cooled Retiga 1300 camera (Qimaging, Burnaby, BC, Canada) in 2 × 2 binned acquisition mode, using 100–300 msec long exposures. Image acquisition and microscope settings were controlled by software described in Czirok et al. (2002). Briefly, 4–10 pre-selected microscopic fields were visited in each scanning cycle. In each of these fields, images were taken in a number of microscopy modes: first DIC, followed by one or two epifluorescent channels. The fluorescent channels differ in the filter sets: 41007 HQ:Cy3 and 41001 HQ:FITC (Chroma Technology Group, Rockingham, VT) were used for green excitation/red emission and for blue excitation/green emission, respectively. For each field and microscopy mode, 3–10 images were acquired in multiple focal planes, separated by 10 μm. The acquisition of the corresponding z-stacks was accomplished within a short period of time (typically a minute) insuring the correct spatial registration of the DIC/epifluorescence images. The practical result of this technology is that no feature moves out of focus during the extended (2–4 days) recording time.

For image processing, the best-focused optical plane was selected from the acquired "z-stacks," as described in Czirok et al. (2002). Thermal camera noise was reduced in the low intensity fluorescence images by applying a median filter (Chen et al., 1995). Most of the images were locally normalized (Czirok et al., 2002, 2004) to compensate for uneven background and photobleaching, and to maximally enhance image details. Some images (shown in Movie B), however, were not locally normalized to preserve the ratio of red:green fluorescence intensities. In these cases, uneven illumination backgrounds were removed using a high-pass filter (Chen et al., 1995), then images were converted from 12 to 8 bit intensity

resolution by the same linear scaling for each frame, which transformed 1% of the total pixels to 255 (white) and 5% of the pixels to 0 (black).

Antibody labeling and imaging

The antibody N6-17 generated against an amino-terminal region of mouse tropoelastin encoded by exons 6–17 was labeled with the dye Cy3 using the FluoroLink antibody labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ). Five times 10^5 RFL-6 cells were plated onto glass coverslips in 35-mm dishes and grown in Hams F12 medium containing 20% Cosmic Calf Serum until confluence. The medium was then removed and medium containing labeled N6-17 antibody was added and incubated with the cells for 30 min. The medium was then replaced with Hams F12 medium without antibody and imaging was initiated. Images were taken every 20 min for 24 h.

Electron microscopy

FBC-180 cells were prepared for electron microscopy as previously described (Robb et al., 1999). RFL-6 cells were grown to confluence in 35-mm tissue culture dishes (Fisher Scientific Co., Pittsburg, PA). Nine days after visual confluence, the medium was removed and the cell layers were washed with phosphate buffered saline (PBS) prior to final fixation in 1% glutaraldehyde in 0.1M sodium cacodylate buffer for 15min on ice. Labeled and fixed cell layers were washed three times with 0.1 M sodium cacodylate then sequentially stained with 1.25% osmium tetroxide and 2% tannic acid, both in 0.1M sodium cacodylate. After washing in 0.1 M sodium cacodylate, the cell layers were rinsed twice with 15% ethanol followed by 30-min incubation with 4% aqueous uranyl acetate before sequential dehydration with ethanol. The samples were then embedded in PolyBed812 (Poly-Sciences)-filled gelatin capsules and baked at 60°C for 18 h. Thin sections (60 nm) were counterstained with uranyl acetate and lead citrate and examined on a Zeiss 902 electron microscope. Preparation and imaging of aortic tissue from an embryonic day 14 mouse was performed as described (Davis, 1993).

RESULTS

Unlike collagen that assembles into fibers of uniform diameter, elastin assembly can result in fibers of varying size and shape. Many studies support a model wherein small elastin fibrils or aggregates coalesce to form larger fibers, which assemble into even larger fibers in tissues like ligament or skin, or into sheets as occurs in the elastic lamellae in blood vessels [e.g., see the many reports in Sandberg et al. (1977)]. Unfortunately, the term elastic 'fiber' has been used throughout the literature more as a term for assembled, crosslinked elastin than as a reference to a uniform, definable structure. In this report, we use elastic fiber to mean accumulations of elastin outside the cell that adopt a fiber-like appearance, irrespective of size. In this context, the term elastic fiber does not have the same restrictive meaning as does reference to a collagen or fibronectin fiber.

Elastin assembly by RFL-6 cells transfected with tropoelastin-timer

RFL-6 cells are a useful in vitro model to study elastin gene regulation and elastic fiber assembly (Kozel et al., 2004). These cells make abundant amounts of tropoelastin and are able to form fully crosslinked elastin in their extracellular matrix. To study the dynamics of elastin assembly, confluent RFL-6 cells were transfected with a cDNA construct encoding bovine tropoelastin in frame with the Timer reporter at the carboxy-terminus (bTE-Timer). When expressed in mammalian cells, green fluorescence indicates recently translated protein while red fluorescence denotes matured protein. By calibrating the color shift of the Timer product with time, we found that the green to red transition occurs at around 6 h post synthesis. Yellow fluorescence results when protein species with green and red fluorophores are both present. The experiments described in this report are from three different experiments conducted on different days and thus represent independent imaging sessions. The results are typical of what we have seen using still and live imaging in over 20 transfection studies with 6 different cell types, including RFL6 cells, PE cells, fetal bovine chondrocytes (FBC), fetal calf ligament fibroblasts (FCL), bovine aortic smooth muscle cells, and COS7 cells.

Figure 1 is a representative image showing the green and red phases of the bTE-Timer color change and the relationship of the fluorescent structures with the transiently transfected cells. Yellow color occurs when green and red fluorophores are in the same structure. Image sequences of the early stages of elastic fiber assembly, best appreciated from the time-lapse movies included as supplemental material, showed bTE-Timer as small aggregates associated with the cell and fibrous forms in the extracellular matrix. The cell-associated forms were mostly green in color and had the characteristic peri-nuclear appearance of components of the secretory pathway. Patches and small globules of yellow color were also associated with the cell, but these were

Fig. 1. Composite immunofluorescence and DIC images of RFL-6 cells transiently transfected with bovine tropoelastin (bTE)-Timer construct. Timer is a derivative of the DsRed fluorescent protein that changes from green to red over time and, hence, can be used to distinguish new from old elastin. Green fluorescence indicates recently translated protein while red fluorescence denotes matured protein. Cells expressing the bTE-Timer construct show both green and yellow particles whereas fibers in the extracellular matrix are mostly yellow and red. The image was taken ~36 h after transfection. Fluorescence intensities are gamma-corrected (gamma = 0.5). Space bar = 25 μm.

fewer in number, were of irregular size, and were deposited onto fibers of extracellular elastin that were always red or yellow-red, indicating that they had been produced at an earlier time. The majority of the green globules remained associated with the cell through cell division whereas the yellow globules dissipated into the ECM when the cell divided. This difference in globular behavior provides further evidence that the green structures are located intracellularly and contain newly formed elastin whereas the yellow globules represent elastin on the cell surface. That the intracellular globules remain green through the imaging period suggests that the secretory rate of elastin is relatively rapid and efficient such that the newly synthesized fusion protein is secreted before the Timer reporter can change from green to red. We would occasionally see cells with intracellular red vesicles, but these cells were not motile and were most likely dead or dying.

The yellow elastin aggregates on the cell surface were initially quite small but could be seen to coalesce into larger globular structures, much like the formation of a large snowball from smaller ones. This process is shown in Figure 2, where small yellow globules distributed over the cell surface coalesce with the larger structure (arrow), which is eventually deposited into the extracellular space.

Movie A shows a typical time-lapse series for transiently transfected RFL-6 cells. Microscopy was started 6 h after transfection with bTE-Timer and continued at 20 min intervals over 14 h. What is immediately obvious when viewing the movie is the extensive cell motion occurring in a confluent RFL6 cell culture. Most cells move rapidly and cover relatively large distances whereas other cells remain somewhat stationary over the imaging period. Several cells can be seen to detach from the matrix as they round up and divide. Few cells remain motionless. All cells seem to be affected by the composite motion of the cell mass and are pushed and pulled by surrounding cells. As will be discussed below, these cell movements and traction forces within the cell layer are important for shaping the forming extracellular matrix. Movie B is a more detailed view of the field near the center of movie A. To help orient the reader/viewer to the cell and matrix structures that we discuss and show in subsequent movies, cells, elastin globules, and elastic fibers that change color are specifically indicated by boxes and other marks.

Globular elastin on the cell surface is transferred to elastic fibers in the ECM

Movie C and representative parts extracted from the movie compiled in Figure 3 show cells containing newly produced (yellow/green) elastin interacting with yellow-red fibrous elastin. The arrow in Figure 3 points to a cell-associated globule of yellow elastin that is eventually transferred onto an extracellular elastic fiber as the cell moves away. The yellow globule eventually incorporates with other globules as the fiber is stretched and pulled by surrounding cells.

Throughout the culture period, it was common to see elastin-producing cells move into and become associated with networks of pre-existing elastic fibers. During this period, the elastic fiber network would assume the motion of the cell, suggesting that components of the fiber were binding to the cell. An interaction with the cell was also evident from the stretching and pulling motions of the fiber seen as the cell moved away.

In addition to providing a physical link between the growing fiber and the elastin-producing cell, the interaction of extracellular elastic fibers with cells may also serve a signaling role. Movie D and representative parts shown in Figure 4 show an elastin-producing cell with the typical green and yellow globules. When the cell interacts with a pre-existing (red) elastic fiber, the yellow globules on the cell surface (arrow) are redistributed toward the site of elastic fiber contact and eventually become incorporated into the fibrous structure. This redistribution suggests that the cell recognizes the fiber as a target for elastin deposition. It is also

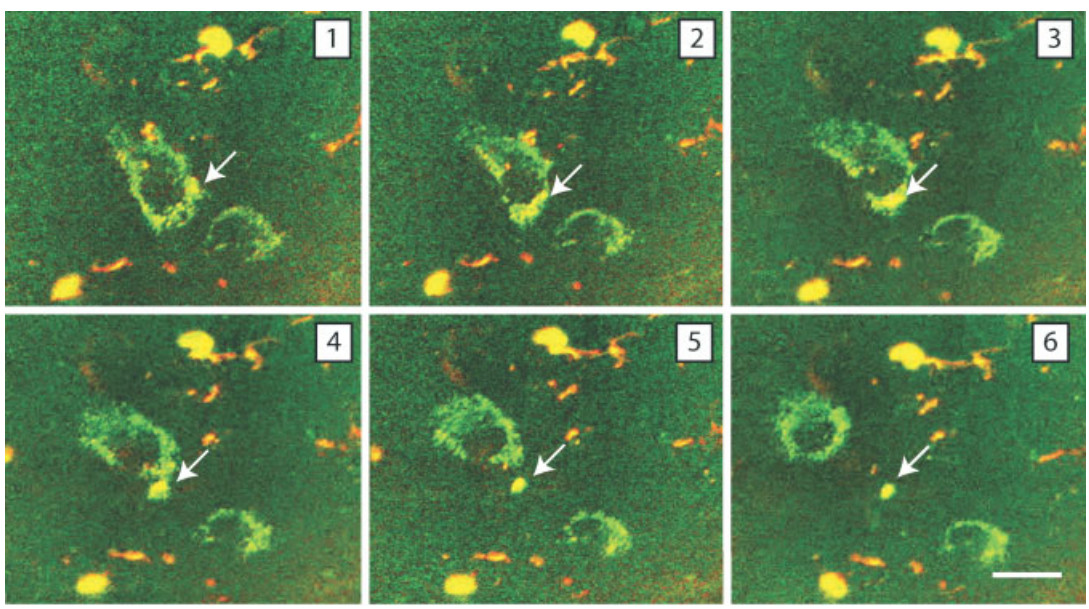

Fig. 2. Elastin aggregates are assembled into globules on the cell surface. Parts 1–6 represent six time points from a 20-h time-lapse sequence showing the formation of a large yellow globule from smaller yellow particles on the surface of a RFL-6 cell expressing bTE-Timer. The particle is eventually deposited into the extracellular matrix as the cell moves away. Each image is an overlay of the red and green fluorescence channels. Time points when images were taken: (1) 24 h 20 min, (2) 24 h 42 min, (3) 25 h 04 min, (4) 25 h 26 min, (5) 25 h 48 min, (6) 26 h 32 min. Fluorescence intensities are gamma-corrected (gamma = 0.5). Space bar = 20 μm.

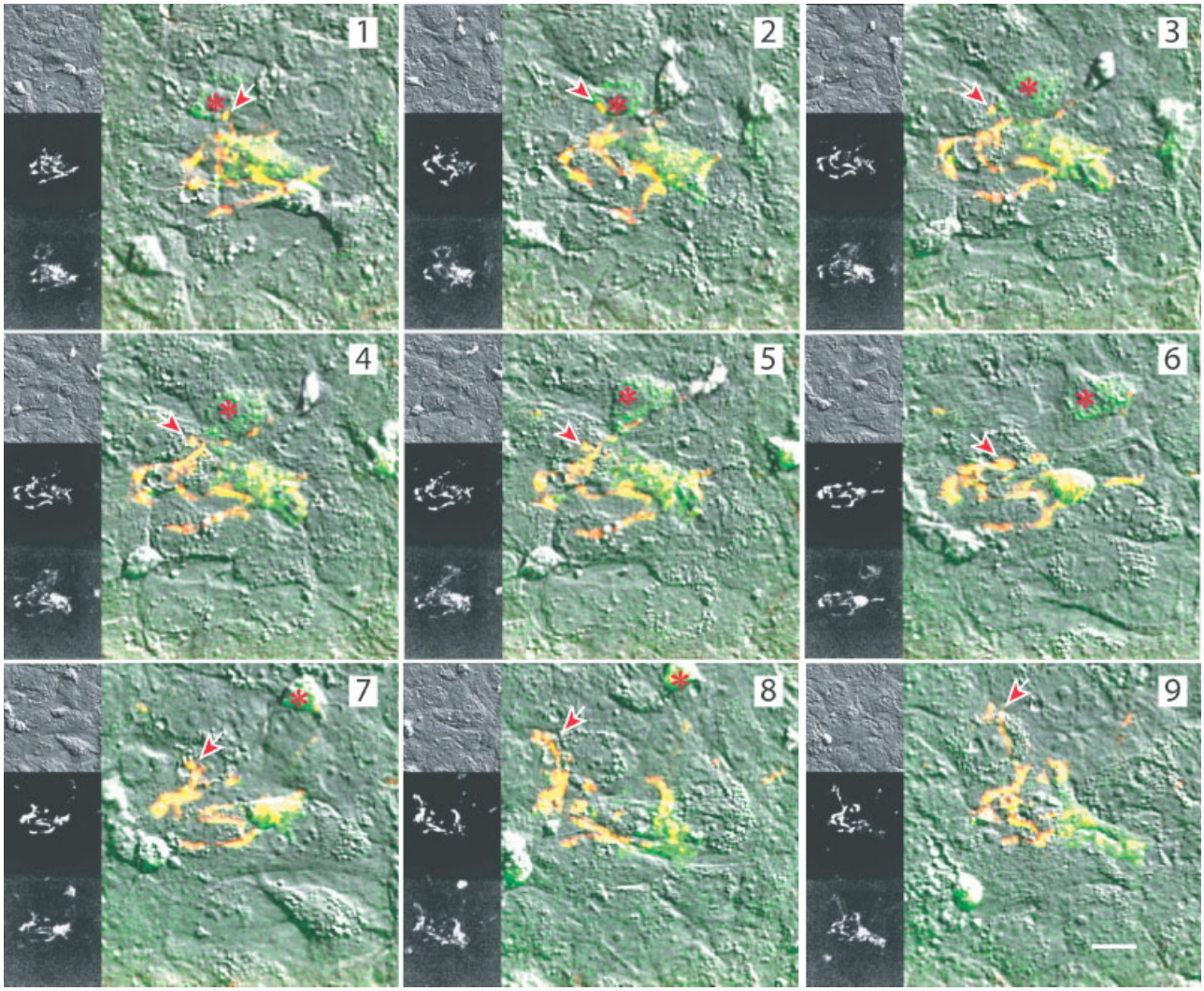

Fig. 3. Globular elastin on the cell surface is transferred to elastic fibers in the ECM. A globule of yellow bTE-Timer (arrow) on the surface of the cell marked with * is transferred from the cell surface to a growing extracellular elastic fiber. After transferring the globule of elastin, the cell moves away and the motion of surrounding cells stretches the fiber. On the left of each part are the DIC image (**top**) and images from the red (**middle**) and green (**bottom**) fluorescence channels. The parts were extracted from movie B. Time points when images were taken: (1) 23 h 36 min, (2) 25 h 48 min, (3) 27 h 16 min, (4) 27 h 38 min, (5) 28 h 00 min, (6) 30 h 34 min, (7) 31 h 28 min, (8) 33 h 40 min, (9) 35 h 52 min. Fluorescence intensities are gamma-corrected (gamma = 0.5). Space bar = 20 μm.

interesting to note how the nucleus in the cell to the left of the elastin-producing cell rotates (Movie D) as the pre-existing elastic fiber passes over it, suggesting a physical interaction between the cell and the fiber.

Fibers are shaped by cellular forces

When observed over time, elastic fibers in the ECM of cultured RFL-6 cells showed movements that are correlated with attachment to moving cells. In Movie B, there are several elastin-producing (green) cells toward the right side of the image that contact and interact with a preexisting elastic fiber and seem to envelop themselves with the fiber for a period of time. Below the two cells is a yellow-red fiber (white asterisk) that moves toward the bottom of the image and is stretched by passing cells. Throughout the culture, fibers can be seen to stretch (Fig. 5, Movie E, Fig. 3, parts 7–9) and show elastic recoil. In most instances, cellular movements were important in shaping the fiber network where small fibers could be seen to merge to form larger units.

Globular elastin confirmed by direct antibody staining

To insure that the globular forms of elastin observed in the transfection studies were not artifacts arising from aggregation induced by the Timer tag, non-transfected RFL-6 cells were incubated with an elastin-specific antibody conjugated with Cy3. Time-lapse microscopy was conducted as described above. In experiments where the antibody was added for 30 min followed by the washing out of unbound antibody (equivalent to a pulse-chase experiment), elastin globules similar to those observed with the Timer construct were evident throughout the culture (Fig. 6). Time-lapse imaging showed that the globules were initially associated with the cell but eventually formed fibrous aggregates in the ECM away from the cell. In Movie F, the elastin globules are seen to outline and move with the cell that they are attached to. With time, the outline of the cell disappears as the antibody-labeled globules leave the cell and localize to discrete elastin-containing fibers. The trajectories of individual particles shown in Movie G reveal

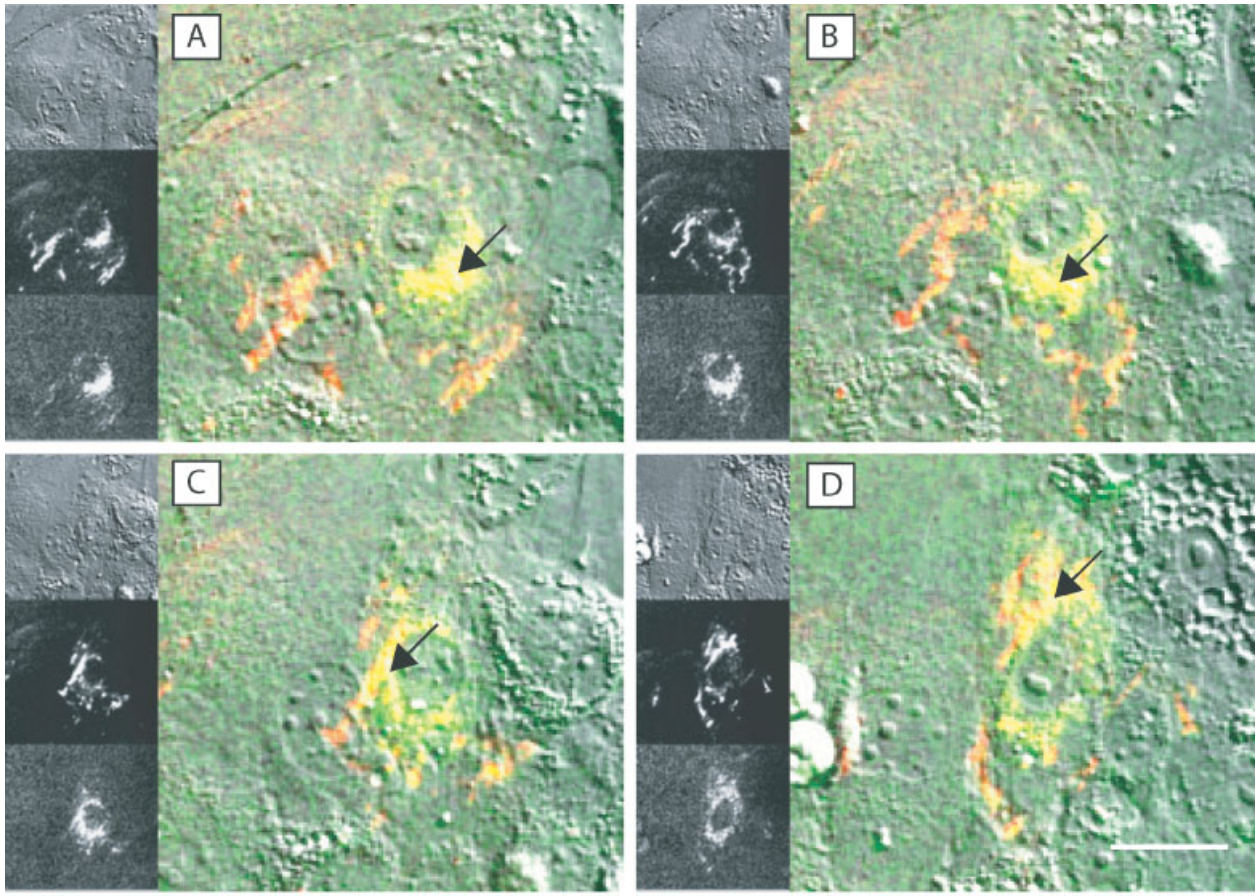

Fig. 4. Cells interact directly and transiently with elastic fibers during elastic fiber formation. Parts from time-lapse movie C showing an elastin-producing cell (bTE-Timer) interacting with a pre-existing elastic fiber (orange in color) that moves towards the cell. Elastin globules on the cell surface (yellow fluorescence—arrow) are redistributed toward the elastic fiber and eventually end up as part of the fiber. On the left of each part are the DIC image (**top**) and images from the red (**middle**) and green (**bottom**) fluorescence channels. Time points when images were taken: **A**: 32 h 46 min, (**B**) 34 h 14 min, (**C**) 26 h 04 min, (**D**) 41 h 12 min. Fluorescence intensities are gamma-corrected (gamma = 0.5). Space bar = 30 μm.

that particles in early culture show uncoordinated, erratic movements with frequent changes in direction. Later, the motion becomes less erratic and more coordinated as the particles coalesce into fibers. The antibody studies confirm that elastin globules are the basic building unit of extracellular elastic fibers and that the globular structures are initially associated with the plasma membrane of cells. A detailed discussion of the globule to fiber transition can be found in (Czirok et al., 2005). This manuscript also contains a computational analysis of the motion of tropoelastin-containing filaments compared to cell motility, demonstrating that motile cells help build elastic fibers by moving and connecting progressively larger segments.

Electron microscopy shows elastin globules in the ECM of cultured cells

Immunofluorescence microscopy studies of elastin-producing cells have often described elastin deposited into the extracellular matrix as assuming a beaded appearance in early stages of fiber assembly (Robb et al., 1999; Kozel et al., 2004), consistent with the observations described above. The elastin beads were shown to co-localize with fibrillin-positive elements of the matrix, suggesting that microfibrils were somehow involved in elastin deposition. To determine whether globules were, indeed, the form of elastin elaborated by the cell, cultures of RFL-6 and FBC cells were analyzed by electron microscopy.

Figure 7 shows images of the extensive elastin globular network formed in FBC (part A) and RFL-6 (part B) cells in culture. Immunogold labeling with an antibody to elastin confirmed that the particles were elastin (data not shown). Globules could be seen associated with the cell surface and in the extracellular space where they were invariably connected to long, straight filamentous structures. Analysis of the images suggested that the larger elastin structures arise through aggregation and condensation of surrounding smaller globules (Fig. 7C), which is consistent with our dynamic imaging and numerous other studies. Toselli et al. (1981), for example, showed that immature elastic fibers in cultured smooth muscle cells consist of "small conglomerates of amorphous material" that are distributed among bundles of microfibrils. Over time, the fibers take on a more filamentous quality with an increase in size of the fiber and loss of the small conglomerates.

Elastin globules in intact tissue

To investigate whether elastic fiber assembly occurs through globular intermediates in intact tissue, standard transmission microscopy was used to visualize early stages of elastin assembly in the developing mouse aorta. Similar to what was found in our in vitro experiments and what has been described in numerous ultrastructural studies (Haust et al., 1965; Fahrenbach et al., 1966; Albert, 1972), early forms of elastin produced by

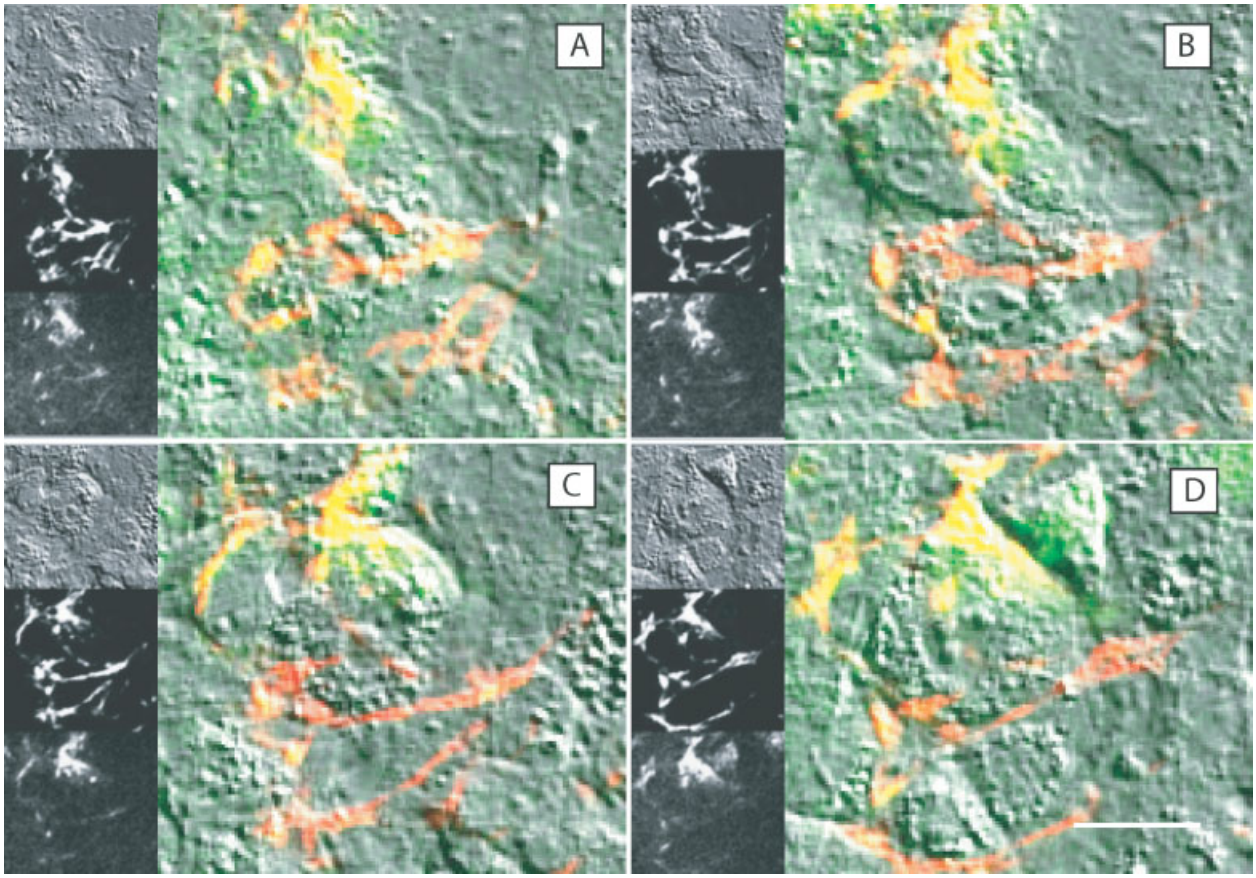

Fig. 5. Mechanical forces associated with cell movement help shape the forming elastic fiber network. **Parts A–D** show the stretching of an elastic fiber produced by RFL-6 cells transfected with bTE-Timer. During this period, the fiber matures as indicated by the change in color from yellow-red (Part A) to completely red (**Parts C, D**). The parts were extracted from movie E. Time points when images were taken: (A) 30 h 34 min, (B) 32 h 46 min, (C) 33 h 52 min, (D) 37 h 54 min. Fluorescence intensities are gamma-corrected (gamma = 0.5). Space bar = 20 μm.

aortic wall cells consist of small globules of darkly staining elastin closely associated with the plasma membrane. The elastin globules were embedded in an orderly array of filaments and were seen to fuse with adjacent globules to form larger structures (Fig. 7D).

DISCUSSION

Dynamic imaging of elastic fiber formation by cells expressing tropoelastin tagged with the fluorescent Timer construct suggests that the initial step in elastin assembly is the formation of small elastin aggregates on the cell surface. Early after transfection, vesicles containing green material were evident throughout the cell and remained with the cell during cell division, suggesting that these are intracellular vesicles containing newly synthesized elastin. The presumptive intracellular vesicles remained green throughout the imaging period consistent with tropoelastin-Timer being cycled out of the vesicle before the color change can occur. These results are consistent with the known

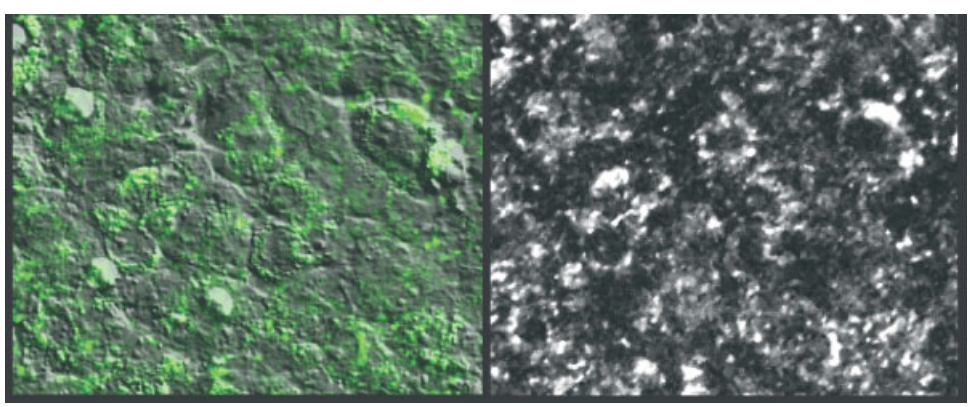

Fig. 6. Globular elastin confirmed by direct antibody staining. To insure that the globular forms of elastin observed in the bTE-Timer transfection studies were not artifacts arising from aggregation of the Timer tag, non-transfected RFL-6 cells were incubated with an elastin-specific antibody conjugated with Cy3. Fluorescence microscopy showed globules throughout the culture similar in appearance to those seen in bTE-Timer-transfected cells. The image on the left is a superimposition of DIC and fluorescent images. The image on the right is the fluorescence image without DIC. The image was extracted from movie F.

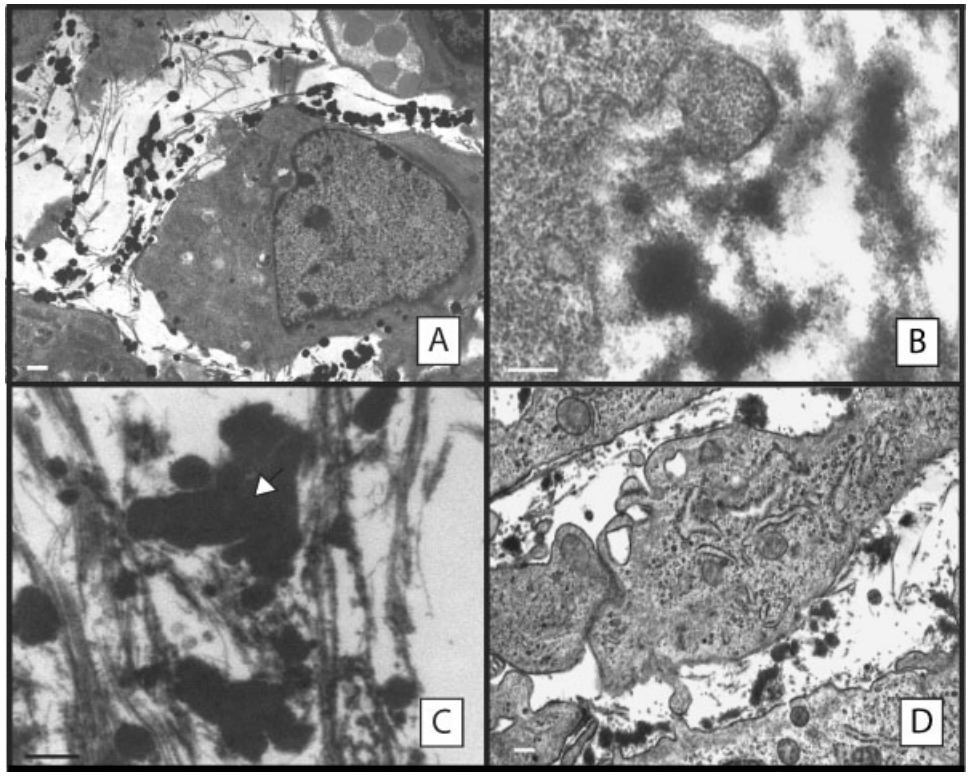

Fig. 7. Elastin globules detected by electron microscopy. Electron micrographs of cultured FBC (**part A**) and RFL-6 cells (**parts B, C**) showing elastin globules in the extracellular matrix. The arrow in part C shows a cluster of elastin globules that are aggregating to form larger units. Elastin secreted by smooth muscle cells in an embryonic day 14 mouse aorta (**part D**) first appears as small aggregates similar to what was found with elastin-producing cells in culture. Bars: A, D, 1 μm; B, C, 0.15 μm.

transit time of ~30 min for tropoelastin synthesis and secretion (Davis and Mecham, 1998) and suggest that tropoelastin does not accumulate for appreciable time within the cell. The yellow globules found on the cell surface could frequently be seen to coalesce into larger globules until they were released into the ECM, usually onto a growing elastic fiber aggregate in the immediate vicinity. The presence of yellow but no completely red globules on the cell surface suggests that newly formed elastin (green) is continuously deposited onto older elastin (red) as long as the particle is associated with the cell. The presence of red Timer in the globules indicates that the particles can remain on the cell surface for greater than 6 h, which is the time required for the green-to-red color change.

Particle tracking studies show that the globules remain associated with the cell during early phases of assembly, thereby implying a direct association between elastin and the plasma membrane. This also suggests an active role for the cell in the assembly process. Elastin binds to cells in a specific and saturable manner with kinetics typical of a receptor-ligand interaction (Wrenn et al., 1988). The nature of the elastin binding proteins that participate in assembly, however, is unclear with αvβ3 integrin (Rodgers and Weiss, 2004), several non-integrin proteins (Hornebeck et al., 1986; Mecham et al., 1989; Robert et al., 1989), an elastin binding protein arising from an alternatively spliced form of β-galactosidase (Hinek et al., 1993; Hinek, 1996), and cell surface glycosaminoglycans (Broekelmann et al., 2005) representing possible binding partners. In an earlier study, we showed that elastin-coated gold beads could bind to the surface of live cells and would frequently move to regions of the membrane over cytoskeletal bundles where they were then translocated along these filaments (Mecham et al., 1991). This suggests that cytoskeletal elements may play a role in the early stages of elastin assembly or in the transport of elastin globules over the cell surface.

The dynamic imaging studies described in this report are consistent with a model wherein tropoelastin molecules secreted from the cell assemble on the plasma membrane into aggregates that become globules. The initial organization of tropoelastin monomers into aggregates could occur through self-association when the proteins are brought to assembly sites on the cell surface. Aggregates then associate with fibrillin-containing microfibrils where they coalesce to form larger aggregates and eventually a functional fiber. We noted a similar globule to fiber transition in earlier studies when purified tropoelastin was added to cultures of live or killed cells. In these experiments, the added tropoelastin was observed to form globular structures in association with existing microfibrils in the cellular ECM. With time, the globules fused to form fibers as the matrix matured, even in the absence of live cells (Kozel et al., 2004). Wise et al. (2005) identified elastin 'droplets' similar to our globules when purified tropoelastin was crosslinked by a yeast form of lysyl oxidase. Like the tropoelastin added to cultured cells, the droplets went on to spontaneously fuse to form larger stable structures. These results provide a strong argument that elastin globules have the ability, if not propensity, to fuse to form higher ordered aggregates, and that this elastin self-assembly can drive many, if not most stages of elastin polymerization.

What is not clear from our imaging studies is whether microfibrils play a role in early elastin assembly. As

shown in Figure 7, the elastin globules away from the cell surface are frequently associated with microfibrils, but it is not clear whether these fibers (or fibers of differing composition) are present at the earliest stages of assembly on the cell surface. The unique ability of tropoelastin to self-associate has long been suggested as the mechanism for elastin self-assembly (Cox et al., 1974; Bressan et al., 1983; Vrhovski et al., 1997; Jensen et al., 2000; Wise et al., 2005) and the role of microfibrils has always been viewed as somehow facilitating this process. It is possible, however, that the function of the microfibrils is not to initiate assembly, but to restrict either the extent or rate of tropoelastin aggregation so that elastin fiber formation can be directed and controlled. Microfibrils could also have other important regulatory roles. On the cell surface, microfibrils could provide the cell a target for elastin secretion through their ability to interact with integrins. In the extracellular space, interactions between fibrillar proteins could direct and mediate the coalescence of elastin aggregates to form fibers. It is well known that the ratio of microfibrils to elastin decreases as the elastic fiber matures (Albert, 1972), suggesting that microfibrils are removed during the assembly process. Their removal may be necessary to expose hydrophobic surfaces on the elastin globules to enable the coalescence required to form higher order structures.

Disruption of genes for microfibrillar proteins in mice is beginning to provide new insight into the role of microfibrils in elastin assembly. Inactivation of genes encoding fibrillin-1 and fibrillin-2, the major structural protein of the 10–15 nm microfibrils, has little effect on elastic fiber formation (Pereira et al., 1997; Arteaga-Solis et al., 2001; Chaudhry et al., 2001). Inactivation of the fibulin-5 gene, in contrast, results in large unordered aggregates of elastin instead of the linear dense fibers and lamellar structures normally found in skin and blood vessels (Yanagisawa et al., 2002). This is to be expected if the role of fibulin-5 is to limit the aggregation of globules until directed fusion can occur. It is important to note that in all of the microfibril-related knockout animals (fibrillins, fibulins, MAGPs, and lysyl oxidase), elastin aggregation still occurs. This provides further evidence that the initial formation of elastin aggregates occurs through self-assembly and may not require the assistance of microfibrils.

Elastin forms the most varied and largest structures in the extracellular matrix, ranging from sheet-like lamella in blood vessels to large, irregular contiguous fibers in other elastic tissues, such as lung and skin. The diversity of the architectures suggests different and more varied packing requirements for elastin than what is seen with other ECM components. Similar to what is known for the assembly of fibronectin, laminin, and collagen, the cell plays an active and important role in forming the elastic fiber by secreting elastin to sites where assembly can occur and where the concentration of the elastin monomer is sufficient to facilitate alignment and crosslinking by lysyl oxidase. Whether the initial tropoelastin interactions occur in a unique intracellular compartment or at specialized assembly sites on the plasma membrane, as has been described for collagen (Birk and Trelstad, 1986; Canty et al., 2004) and fibronectin (Peters and Mosher, 1987), is not known, but should be anticipated. Elastin also maintains an intimate association with the plasma membrane throughout the early assembly period, but unlike fibronectin assembly that is dependent upon the unfolding of FN dimers by cytoskeleton-generated tension mediated by integrins (Hynes, 1999), tropoelastin does not interact with β1 integrins (Broekelmann et al., 2005) and there was little evidence that cryptic sites need to be exposed prior to assembly. Our time-lapse data as well as previous studies (Mecham et al., 1991) show, however, that elastin aggregates do move on the cell surface, suggesting an interaction with some binding protein that is linked to the cytoskeleton. It is possible that the microfibril provides the linkage to the cell since both fibrillin and fibulin-5 have been shown to interact with integrins (Pfaff et al., 1996; Sakamoto et al., 1996; Nakamura et al., 2002).

The combination of time-lapse microscopy and Timer-labeled proteins provides a powerful, novel tool for studying extracellular matrix assembly. The relatively slow maturation of Timer, during which the initial green emission changes to red over approximately 6–12 h, is uniquely suited to the time course of ECM assembly. By capturing this color change using dynamic imaging techniques, it is possible to develop a new level of understanding of ECM dynamics in the context of surrounding cells.

Online supplemental material

Movies showing the organization by cultured RFL-6 cells of tropoelastin into elastic fibers are in the online supplemental material. Movies A–E show cells expressing the tropoelastin-Timer construct. Movies F and G show the dynamics of elastic fiber formation using Cy3-labeled elastin antibodies.

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