Endothelial cell nanotube insertions into cardiac and skeletal myocytes during coordinated tissue development

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1. Introduction

Tissue development requires coordinated activity across several cell types to properly synchronize cellular structures and function. As nearly all tissues are perfused by blood vessels, communication between capillary endothelial cells (ECs) and neighbouring cells is critical for orchestrating proper timing and arrangement of developing cells within the tissue. Though several paracrine signalling mechanisms have been described, it is still not well-understood how proximity to ECs supports development of adjacent cells.¹ Here, we describe a novel structural association which would permit intercellular communication between capillary ECs and myocytes in developing heart and skeletal muscle *in vivo*.

2. Methods

All procedures were approved by the NHLBI, NIH Animal Care and Use Committee in accordance with guidelines described in the Animal Care and Welfare Act (7 USC 2142 § 13). C57BL/6N mice were anaesthetized with 2% isoflurane continuously via nose cone while lying on a heated bed. After tissue fixation as described previously,² mice were euthanized via exsanguination. Focus ion beam scanning electron microscopy of mouse postnatal heart and skeletal muscle was performed with 10 nm resolution as described previously.² Each contiguous myocyte-associated capillary was counted as a single data point for quantification purposes.

3. Results and discussion

Tubular structures of ~150 nm diameter and 3–5 µm in length were observed within both cardiac and skeletal myocytes on postnatal Day 1 (P1) (Figure 1A and C). Tracking nanotubes in 3D revealed their origins as projections with a continuous cell membrane from adjacent ECs (Figure 1B and D). Upon insertion into myocytes, the distance between respective cell membranes ranged between 0 and 220 nm with 34% of the

nanotube membrane within range for direct, functional contact with the myocyte (within 30 nm).³ Nanotube insertion sites did not overlap with t-tubule openings in the sarcolemma. EC nanotube insertions were found in 50% of myocyte-associated capillaries in P1 heart, 25% at P7 and not observed at all in mice ranging from P14 up to 4 months of age (Figure 1E). EC nanotubes were less frequent in skeletal muscle and found in 20% of myocyte-associated capillaries at P1 and not at all from P7 to adult suggesting this structural association is specific to early striated muscle development.

No organelles, microtubules, or myosin were found within EC nanotubes indicating they are likely actin-based projections. Cellular projections previously described as microtubule nanotubes and primary cilia are known to contain microtubules^{4,5} and, thus, are different from EC nanotubes. Tunnelling nanotubes are another type of cellular projection which can be either microtubule or actin-based, though tunnelling nanotubes are reportedly much longer (50–1000 µm) than EC nanotubes and are also known to transfer organelles.^{4,5} It is more likely that EC nanotube insertions are related to cytonemes, the actin-based projections of up to 700 µm in length which have been shown to facilitate signalling between neighbouring stem cells.⁵ However, cytoneme-mediated signalling has primarily been described in *Drosophila* or cultured cells.⁵ Nanotube connections between endothelial progenitor cells and cardiomyocytes in culture have been reported but were 5–120 µm long and facilitated transfer of mitochondria⁶ indicating they were likely tunnelling nanotubes.⁵ EC nanotube insertions into myocytes are similar in appearance to myoblast protrusions into myocytes as observed during skeletal muscle development (Figure 1F), though one major difference is that EC nanotube insertions do not lead to fusion between adjacent cells. Taken together, the insertion of EC nanotubes into the beating heart and contracting muscle provides a specialized cellular interface unlike previously described cellular projection interactions.

A direct, two-way EC-myocyte communication system would ensure that signal exchange is limited to between coupled cells and offer an additional level of precision over the currently more well-described EC-myocyte paracrine signalling mechanisms.¹ Whereas release of secreted factors into the extracellular space allows for general coordination of developmental signalling across many cells, specific communication

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Figure 1 (A) FIB-SEM image of P1 mouse endothelial cell (EC) nanotube (yellow) within a myocyte (red). (B) 3D rendering of the ECs (yellow, magenta), pericyte (green), and myocyte cell membrane (red) from A. (C) FIB-SEM image of P1 mouse EC nanotube (yellow) entering a cardiomyocyte (red). (D) 3D rendering of ECs (yellow, magenta), pericyte (green), and cardiomyocyte cell membrane (red) from C. (E) Percentage of capillaries with EC nanotubes inserted into myocytes in striated muscle. Heart: $n = 6, 4, 5, 21$ capillaries for P1, P7, P14-21, P42+, respectively. Skeletal muscle: $n = 5, 7, 16, 35$ capillaries for P1, P7, P14-21, P42+, respectively. (F) FIB-SEM image of P1 mouse myoblast (blue) nanotubes within a myocyte (red). Scale bars: 1 μm .

between two adjacent cells through EC nanotube insertions would provide a mechanism for fine tuning local development within the overall tissue.

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