The Cytoskeletal Mechanisms of Cell-Cell Junction formation in Endothelial Cells

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The Cytoskeletal Mechanisms of Cell-Cell Junction formation in Endothelial Cells

Abstract
Intercellular adhesions are essential for compartmentalization and integrity of tissues in an organism, cell-cell communication, and morphogenesis. The actin cytoskeleton and associated proteins play a vital role in establishing and maintaining cell-cell adhesion. However, the procedure by which cells establish adherens junctions remains largely unclear.

We investigated the dynamics of cell-cell junction formation and the corresponding architecture of the underlying cytoskeleton in cultured human umbilical vein endothelial cells (HUVECs). We show that the initial interaction between cells is mediated by protruding lamellipodia. Upon their retraction, cells maintain contact through thin bridges formed by filopodia-like protrusions connected by VE-cadherin-rich junctions.

Bridges share multiple features with conventional filopodia, such as an internal actin bundle associated with fascin along the length and VASP at the tip. Strikingly, unlike conventional filopodia, transformation of actin organization from the lamellipodial network to filopodial bundle during bridge formation occurs in a proximal-to-distal direction and is accompanied by recruitment of fascin in the same direction. Subsequently, bridge bundles recruit nonmuscle myosin II and mature into stress fibers. Myosin II activity was important for bridge formation and accumulation of VE-cadherin in nascent adherens junctions. Our data reveal a mechanism of cell-cell junction formation in endothelial cells utilizing lamellipodia as the initial protrusive contact, subsequently transforming into filopodia-like bridges connected through adherens junctions. Moreover, a novel lamellipodia-to-filopodia transition is employed in this context.

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THE CYTOSKELETAL MECHANISMS OF CELL-CELL JUNCTION FORMATION IN ENDOTHELIAL CELLS

Matthew Hoelzle

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in
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Presented to the Faculties of the University of Pennsylvania
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Degree of Doctor of Philosophy

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DEDICATION

This dissertation is dedicated to my wife, Barbara, and to my children Benjamin and Claire, whose love has supported me and inspired me.
ACKNOWLEDGEMENT

I thank my family for so much; especially my wife Barbara who, in addition to putting up with me, has supported me in both good and stressful days and without whom, this was not possible. I am truly thankful to my parents who always inspired me; if I can be half as kind, generous, and intelligent as they are, I will consider my life a success. I do not think I could have found a more supportive mentor and advisor in Tatyana Svitkina; for her example of hard work, patiently considering all aspects of experiments, and her support, I am extremely indebted. I thank all of the other members of the Svitkina lab for their advice and friendship, especially Changsong for teaching me all the laboratory techniques, on numerous occasions. I thank Wei Guo for first helping me put on my Penn sweatshirt. I thank Brian Keith for his guidance and example in many aspects of my graduate work including how to be a good teacher, scientist, and person. I thank the Cytoskeleton Journal Club for our weekly gatherings. I also thank my thesis committee members - Roberto Dominguez, Kim Gallagher, Mark Goulian, Wei Guo, and Brian Keith - for their invaluable guidance and advice during this process. I thank Colleen Gasiorowski and all the staff of the Penn Biology Department.
ABSTRACT

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Matthew Hoelzle
Supervisor: Tatyana Svitkina

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Bridges share multiple features with conventional filopodia, such as an internal actin bundle associated with fascin along the length and VASP at the tip. Strikingly, unlike conventional filopodia, transformation of actin organization from the lamellipodial network to filopodial bundle during bridge formation occurs in a proximal-to-distal direction and is accompanied by recruitment of fascin in the same direction. Subsequently, bridge bundles recruit nonmuscle myosin II and mature into stress fibers.
Myosin II activity was important for bridge formation and accumulation of VE-cadherin in nascent adherens junctions. Our data reveal a mechanism of cell-cell junction formation in endothelial cells utilizing lamellipodia as the initial protrusive contact, subsequently transforming into filopodia-like bridges connected through adherens junctions. Moreover, a novel lamellipodia-to-filopodia transition is employed in this context.
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1.1 Endothelial Cell Biology

Lining the inside of the entire vasculature of the blood and lymphatic systems, the endothelium plays a vital role in physiological processes such as haemostasis and angiogenesis by forming a semipermeable barrier between the blood and surrounding tissues. Dysregulation of proper endothelium function is observed in pathological process such as inflammation and tumor development (Dejana et al., 2009). The functional contribution of the endothelium to proper development and function, as well as many diseases and pathological conditions, calls for a greater understanding of how vessels are formed and maintained.

Endothelial cells constitute the lining of the endothelium and in the mature vascular system are surrounded by layers of contracting smooth muscle cells responsible for fluid movement within the vasculature. In addition to providing the contractile force for fluid movement, smooth muscle cells also synthesize much of the connective tissue of the vessel wall, with an extracellular matrix composed of collagen, elastin, and proteoglycans. Depending on surrounding vessel context, endothelial cells within the vascular lining in vivo exhibit a wide heterogeneity of morphology (Hillen, 2006).

Vessel phenotype can be classified into three categories: continuous, fenestrated, or discontinuous. In most tissues, the full lining of the vessel wall is considered as continuous where there is typically little space between cells, approximately 5-10 nm. In contrast, fenestrated capillaries often have small openings called fenestrae about 80-100 nm in diameter covered by a semipermeable diaphragm separating individual endothelial
cells. These capillaries are found in the small intestine, endocrine glands, and the kidney. Discontinuous capillaries, also known as sinusoids, exhibit wide intercellular spaces approximately 100 nm wide with no fenestrae or diaphragm between adjacent cells. Such vessels exist in the spleen, liver, and bone marrow and display the greatest level of permeability. This wide heterogeneity of morphology lends to various levels of capillary permeability, depending on cell context. Furthermore, endothelial cells also exhibit a range of functional heterogeneity with roles in blood coagulation and anticoagulation, vasoconstriction and vasodilatation, as well as antigen presentation and leukocyte homing during infections (Hillen, 2006; Muller, 2011).

1.1.1 Angiogenesis

During development, \textit{de novo} growth of blood vessels occurs through the process of vasculogenesis, whereby angioblasts differentiate into endothelial cells forming a primary capillary plexus. In contrast to vasculogenesis, the formation of blood vessels from preexisting vessels by sprouting, branching, and invading surrounding tissue is referred to as angiogenesis, through which a blood supply is established (Hillen, 2006). During the angiogenic cascade, following degradation of the extra-cellular matrix by matrix metalloproteases, endothelial cells originating from stable vessels proliferate and migrate in a tightly regulated fashion. Angiogenesis is required in many physiological conditions such as embryonic development and wound healing, but is also proposed to be the rate-limiting step in tumor development (Lamalice \textit{et al.}, 2007; Kerbel, 2008). Dysregulation of appropriate angiogenesis is seen in most, if not all, progressive solid
tumors and is considered to be one of the ‘hallmarks of cancer’ (Hanahan and Weinberg, 2000).

In general, to form a multicellular organism, cells must interact with similar and dissimilar cell types for organ formation and compartmentalization, respectively. More specifically in the endothelium during angiogenesis, cells must collectively migrate into a new tissue exhibiting a specific architecture and morphology characterized by numerous cell-cell contacts. However, during this process, the endothelium serves as a selective permeability barrier to functionally separate the blood and surrounding tissue, yet still endothelial cell-cell junctions must remain dynamic to allow for processes including leukocyte transmigration.

In the endothelium, the angiogenic signaling cascade directly leads to the formation of actin-based leading edge protrusions leading to changes in cell motility and morphology. As explained later in this chapter, actin-based protrusions, lamellipodia and filopodia, have been implicated in the initial steps of cell-cell junction formation necessary for the generation of a functional barrier between underlying tissue and fluid encapsulated. However, little detail is known about the transitional actin architecture and respective modifying proteins involved in this process.

1.2 Actin Cytoskeleton

The actin cytoskeleton and associated proteins is the primary force generating machinery for eukaryotic cells. Composed of a network of individual filaments, the actin cytoskeleton provides the necessary pushing, pulling, and resistance forces essential in various cellular processes. The dynamic organizational remodeling of the actin
cytoskeleton allows the cell to adopt various morphologies and behaviors for processes including cell migration and motility, cytokinesis, cargo trafficking, as well as cell-cell junction formation and maintenance. Generating this higher order structure of the actin cytoskeleton is a system of individual actin filaments generated from a cellular pool of monomeric G-actin, nearly always in a state of flux (Figure 1-1) (Insall and Machesky, 2009).

In the eukaryotic cell, actin is the most abundant intracellular protein comprising between 1-5% of total protein by weight in non-muscle cells, and upwards of 10% in muscle cells. A highly evolutionarily conserved 42 kDa protein, G-actin exists in monomeric form, but can also self-assemble into filamentous helical filaments, known as F-actin whose diameter is approximately 7 nm. Due to the intrinsic polarity of each G-actin monomer, the entirety of the subsequent F-actin polymer is also a polar structure. Filamentous actin polarity can be determined by electron microscopy high-resolution structural analysis of actin filaments after myosin subfragment-1 (S1) decoration. When S1 fragments are bound to F-actin, a characteristic arrowhead pattern appears with one end of the filament characterized by a pointed, arrowhead-like appearance, whereas the opposite end of the filament appears barbed (Svitkina and Borisy, 1999a; Campbellone and Welch, 2010).

In vitro experiments of actin assembly reveal three phases of filament assemblage. The initial phase is known as the lag or nucleation phase, where two G-actin monomers interact relatively weakly and may subsequently disassemble. However, if before disassembly occurs a third ATP-bound monomer is added, a trimer known as the nucleus is formed with increased stability and can undergo further monomer addition thereby
generating an actin filament. The nucleation phase can be circumvented by the initial addition of preformed actin seeds, a nucleus of several monomeric actin subunits. Once nucleation occurs, filament elongation via the addition of ATP-bound G-actin preferentially occurs at one end of the filament yielding a polar structure. Actin is an active ATPase, therefore the assembly of ATP-bound monomer into filament results in ATP hydrolysis leading to the majority of actin in a filament as ADP-bound subunits (Lee and Dominguez, 2010).

The intrinsic structural polarity of individual actin filaments within the cytoskeleton provides the network directional force, in that the barbed end (or plus end) is structurally and biochemically different than the pointed end (or minus end), thereby exhibiting more rapid growth. Due to the lower critical concentration of G-actin incorporation at the barbed end than at the pointed end, when the concentration of free G-actin is intermediate, the simultaneous loss of G-actin at the pointed end and incorporation at the barbed end, with a readily available ATP supply, results in a phenomenon known as treadmilling (Insall and Machesky, 2009).

Actin polymerization has been and continues to be readily observed in vitro, independent of additional cellular proteins. However, in the cell, the fate of actin filaments is controlled by a number of actin-associated proteins (also known as actin-binding proteins; ABP) that can bind G-actin, F-actin, or both thereby altering respective actin properties. In addition to other actin regulatory functions, the roles of specific actin-binding proteins may include to; 1) promote the nucleation of actin filaments, 2) inhibit barbed end growth by capping the filament ends, 3) promote the elongation of filamentous actin, 4) promote F-actin disassembly through filament severing, and 5)
crosslink actin filaments. Localized spatial and temporal regulation of ABP function can transform the actin cytoskeleton into a complex network of dynamic filaments with specialized functions at specific locations in the cell (Chhabra and Higgs, 2007).

During migration, cells undergo a characteristic sequence of events including leading edge membrane protrusion, adhesion of the newly protruding structure to the substrate, forward movement of the cell body and retraction of the trailing edge. Early in the migration cascade, actin polymerization and network assembly just under the plasma membrane provides the membrane pushing force necessary to drive cellular protrusive structures, such as lamellipodia and filopodia (Figure 1-2) (Borisy and Svitkina, 2000; Mattila and Lappalainen, 2008; Small et al., 2008).
Figure 1-1: **Cell Morphology and Actin Cytoskeleton** - A polarized, migrating cell on the left displays a broad leading edge, exhibiting a dense actin network (upper right). Within this network are a series of branched actin filaments undergoing protrusion driven by actin polymerization pushing on the cell membrane. Each actin filament (bottom) is composed of a double helix polymer consisting of individual globular actin proteins (extreme right).

Courtesy of Tatyana Svitkina
Figure 1-2: Structure of Distinct Actin Cytoskeletal Leading Edge Features – The actin cytoskeleton in lamellipodia and filopodia are displayed in higher resolution under platinum replica electron microscopy analysis. The lamellipodia is a thin, dendritic network of branched actin filaments. Often originating from lamellipodia, filopodia exhibit a distinctly different actin structure of tightly bundled filaments. Location of lamellipodia and filopodia are shown in the schematic of the cell.

Courtesy of Tatyana Svitkina
1.2.1 Leading Edge Protrusive Actin Structures: Lamellipodia and Filopodia

During cell migration, it is proposed that lamellipodia and filopodia act as effectors activated downstream of extracellular signals to direct and promote whole cell migration (Mattila and Lappalainen, 2008). The lamellipodia, containing a broad, flat network of actin filaments, undergo protrusive cycles resulting from local induction of actin polymerization. Within the lamellipodium, the actin filaments form a branched network with filament barbed ends facing the leading edge. Key structural and cytoskeleton-remodeling proteins of lamellipodia include the Arp2/3 complex, a 7-member protein complex that nucleates new actin filaments as branches on pre-existing filaments. The Arp2/3 complex binds to the side of existing actin filaments known as the mother filament and initiates the assembly of a daughter filament sprouting at a characteristic 70° angle. Normally existing in an inactive state, Arp2/3 complex is activated by nucleation promoting factors, namely the COOH-terminal domain of the Wiskott-Aldrich syndrome protein (WASP)-family of proteins. Furthermore, the protein cortactin facilitates and stabilizes the Arp2/3 complex at sites of filament branching (Le Clainche and Carlier, 2008; Chesarone and Goode, 2009; Campellone and Welch, 2010).

Initially proposed in 1998 and subsequently further defined, the dendritic nucleation models describes the process by which lamellipodia are formed at the cell’s leading edge (Mullins et al., 1998). External migration cues, including chemotactic agents such as growth factors, bind to receptors and activate signaling pathways often including GTPases and phosphatidylinositol. This is followed by the activation of the WASP family proteins, which in turn activate the Arp2/3 complex. Activated Arp2/3 complex nucleate branched daughter filaments possessing open barbed ends available for
polymerization (Ideses et al., 2008). The force generated by actin polymerization through the activity of elongation factors, including formins and Ena/VASP, pushes on the cell membrane. The pool of available actin monomers is replenished by the depolymerization and/or severing of older filaments near the lamellipodial rear through the activity of ADF/cofilin (Chhabra and Higgs, 2007; Le Clainche and Carlier, 2008; Campellone and Welch, 2010).

Structurally distinct from lamellipodia, filopodia, often originating from and embedded within lamellipodia, are thin, tight bundles of long, parallel actin filaments oriented with their barbed ends toward the tip of the filopodium. Through integrin-extracellular matrix interactions along the shaft or at the tip, filopodia protruding from lamellipodia create points of adhesion that promote migration. Subsequently, components of the focal adhesions, specifically talin, paxillin, and focal adhesion kinase, are recruited to generate mature focal adhesion sites. In addition to serving as one of the critical initiating steps in cell migration, elongated filopodia serve as a means to reach distant targets. Upon reaching their target, filopodia may differentiate into other structures and perform various functions such as serving as a means of distant cell-cell communication and establishing cell-cell junctions (Svitkina et al., 2003; Mattila and Lappalainen, 2008).

Typical markers of filopodia are the actin bundling protein fascin and proteins promoting elongation of barbed ends while protecting them from capping, such as proteins of the formin and Ena/VASP families (Mattila and Lappalainen, 2008). A working model of filopodia initiation proposes a convergence of a subset of processively elongating barbed ends of actin filaments in lamellipodia at the plasma membrane.
mediated, in part, via elongating, anti-capping, and barbed end cross-linking activities of VASP or formins. Stabilizing the newly polymerized structure is the actin bundler fascin that joins together converging actin filaments starting from the filopodial tip into the proximal direction (Figure 1-3) (Svitkina et al., 2003). Alternatively, another reasonable, but not yet proven model suggests that a cluster of membrane-associated formins nucleates a set of autonomous filopodial filaments unconnected to lamellipodia and subsequently maintains their elongation. Indeed, evidence exists of filopodia formation independent of lamellipodia influence. Therefore, it has been proposed that distinct signaling pathways lead to different types of filopodia utilizing disparate core machineries, yet producing the same structure. Still, the co-existence and dependence of filopodia with lamellipodia is often observed in a variety of cell types (Mattila and Lappalainen, 2008; Insall and Machesky, 2009; Xue et al., 2010). Clearly, further investigation is required regarding the transition between two very distinctly different actin cytoskeletal architectures.
**Figure 1-3: Model of Filopodia Formation** - This schematic shows the localization and implied function of various proteins involved in filopodia formation. The working model begins with subset of uncapped actin filaments in the Arp2/3 regulated lamellipodia network converging at the plasma membrane via elongating, anti-capping, and barbed end cross-linking activities of Ena/VASP or formins. Fascin stabilizes and bundles the newly polymerizing structure, while proteins such as IRSp53 promote membrane curvature further promoting filopodia protrusion. Myosin-X traverses the filopodia bundle to deliver filopodia elongation components to the distal tip.

(Figure Adapted from Svitkina et al., 2003)
In both lamellipodia and filopodia leading edge protrusions, the speed of actin polymerization is often greater than the speed of the membrane protrusion. When this occurs, actin subunits within filaments undergo a phenomenon known as retrograde flow where actin filaments slide backward relative to the substrate toward the rear of the lamellipodia. The speed of retrograde flow depends on a variety of factors including actin polymerization speed, membrane tension, strength of substrate adhesion, and actin filament stability. Recently, it was proposed that ADF/cofilin increases the rate of retrograde flow by generating a greater G-actin monomer pool (Ohashi et al., 2011). The increase in free monomeric actin leads to enhanced polymerization. If membrane tension is not alleviated, the majority of force generated in actin polymerization is converted into retrograde flow (Welch et al., 1997; Ohashi et al., 2011).

1.2.2 Ena/VASP and Fascin Modulation of the Actin Cytoskeleton: Necessary in Filopodia Formation

A major regulator of leading edge actin cytoskeletal dynamics in migrating cells is the Ena/VASP family of proteins. In mammals, the family members include Mena the mammalian homologue of the drosophila Ena, VASP, and EVL (Pula and Krause, 2008). Consistent in the Ena/VASP family are several structural protein domains separated by unstructured linkers, namely an N-terminal EVH1 domain, a central proline rich domain, and a C-terminal EVH2 domain. Closest to the N-terminus is the EVH1 domain responsible for binding FP4 motifs involved in Ena/VASP targeting to focal adhesions, filopodia, and lamellipodia. The central proline rich region binds to the SH3 and WW domains of several signaling and scaffolding proteins. The coiled-coil region in the
terminal EVH2 domain supports Ena/VASP tetramerization, while the remaining region within EVH2 is responsible for binding both monomeric actin (GAB: globular actin binding) and filamentous actin (FAB: filamentous actin binding) (Ferron et al., 2007). Given the complex structure, the exact function of Ena/VASP in actin filament dynamics is unresolved. In general, Ena/VASP is involved in actin elongation through the recruitment of profilin-bound actin, where in contrast to free globular actin, profilin-bound actin cannot spontaneously nucleate, but rather can only be added to the barbed ends of actin filaments. Furthermore, Ena/VASP tetramerization aids in accelerating barbed end growth, while remaining processively engaged with the actin filament barbed end. The processive association with the growing barbed end antagonizes actin capping proteins, including CapZ (Bear and Gertler, 2009; Hansen and Mullins, 2010). Reduced actin branching has also been observed, though this effect may be tied to simply sequestering actin monomers from being utilized as a cofactor in Arp2/3 mediated branching. Interestingly, due to its ability to become a tetramer, Ena/VASP is further proposed to act as an actin bundler, though, for example in filopodia, strictly remaining at the distal tip of the actin bundle (Bear and Gertler, 2009).

A well recognized actin bundler, particularly in newly polymerized structures such as filopodia, is the evolutionarily conserved protein fascin, of which the vertebrate genome codes for three forms: 1) fascin-1 in a range of migratory cells; 2) fascin-2 in photoreceptor cells of the retina; and 3) fascin-3 expressed exclusively in the testes (Adams, 2004). A 55-kD monomeric protein, fascin bundles actin in vitro, while in vivo localizes to filopodia protrusions (Vignjevic et al., 2006). Two actin-binding sites allow for actin filament crosslinking and this bundling activity can be inhibited through
phosphorylation by PKC at the actin-binding site nearest the N-terminus. Fascin-actin binding dynamics exhibit a relatively short binding half-life, of approximately 6-9 seconds, suggesting cycles of phosphorylation and dephosphorylation contribute to filopodia dynamics (Mattila and Lappalainen, 2008). Whereas fascin does not efficiently bundle preformed actin filaments, fascin can bundle actin filaments as they polymerize. As filaments within filopodia exhibit parallel polarity, fascin specifically bundles parallel filaments (Svitkina et al., 2003). Mechanistically, fascin provides stiffness and rigidity for actin filament bundles to resist the compression forces of the membrane pushing against the protrusive structure. Very clearly, depletion or inhibition of fascin results in reduced filopodia formation as well as reduced cell migration (Vignjevic et al., 2006).

1.2.3 Contractile Actin Structure: Stress Fibers

In addition to actin providing the protrusive force in cell migration and morphology, the actin cytoskeleton can also provide pulling and resistance forces necessary for motility and cell shape maintenance through cell adhesion to the substrate and contraction of the trailing rear. Classically, in non-muscle cells, cellular contractile forces are driven by the actin cytoskeleton’s association with myosin motors in complex structures known as stress fibers associated with specialized adhesion sites, specifically focal adhesions. Briefly, at the core of focal adhesions are the transmembrane integrin family of proteins, capable of combining in different combinations of heterodimers. The extracellular domain of integrin binds the extracellular matrix, while the cytoplasmic portion associates with actin through cytoskeletal binding and adapter proteins, such as vinculin and talin (Tadokoro et al., 2003; Hotulainen and Lappalainen, 2006).
Responsible for maintaining cell shape particularly during migration, stress fibers are highly ordered structures composed of anti-parallel actin filaments, actin associated proteins, and non-muscle myosin II (Ridley et al., 2003). Stress fibers in eukaryotic cells are typically divided into three categories; 1) transverse arcs, 2) dorsal stress fibers, and 3) ventral stress fibers (Figure 1-4). Transverse arcs are curved structures laterally extending equally from the leading edge, which during cell migration, flow from the leading edge toward the cell center. While not directly connected to the substratum, transverse arcs contribute to the contractile force through association with dorsal stress fibers. This second class, dorsal stress fibers attach to the substrate through focal complexes via integrin-ECM engagement and through actin polymerization elongate one end of the fiber towards the cell center. The third class, ventral stress fibers anchor to focal adhesions on both ends of the actomyosin bundle (Hotulainen and Lappalainen, 2006).
Figure 1-4: Schematic of Stress Fibers in Cells – Three distinct types of stress fibers composed of actin myosin arrays are displayed. Dorsal stress fibers (in red) typically have one end associated with a focal adhesion and often attach to transverse arcs on the other side. Transverse arcs (in yellow) do not associated with focal adhesion. Ventral stress fibers (in green) connect with focal adhesions on both ends.

(Adapted from Hotulainen and Lappalainen, 2006)
Stress fiber formation and function is dependent upon myosin motor activity. A large superfamily of actin binding proteins, the myosin motors are capable of binding and moving along actin filaments through the conversion of ATP chemical energy into mechanical work (Figure 1-5). Typically, the myosin molecule consists of one or two heavy chains and a variable number of light chains. The heavy chains contain a motor domain as well as a cargo-binding tail. Myosin-ATP hydrolysis-cycle force generation in the motor domain subsequently produce specific myosin conformational changes resulting in altered actin affinity (Vicente-Manzanares et al., 2009).

In addition to the canonical transportation system of myosins walking on actin filament tracks for cargo delivery, notably nonmuscle myosin II (NMII) can assemble into bipolar filaments (~0.3 µm in length) with motor domains at both ends. Therefore, due to the presence of multiple actin binding sites at both poles of the bipolar filament, when assembled into bipolar filaments nonmuscle myosin II also has the ability to crosslink and bundle actin filaments. In contrast to the actin filament bundling activity of fascin where tightly compacted bundles are generated, myosins typically crosslink anti-parallel actin filaments. Therefore, rather than walking along actin filaments in a single direction, when in the presence of anti-parallel actin filaments, such as within stress fibers, bipolar NMII filaments pull on actin filaments in opposing directions thus contracting the actomyosin structure (Vicente-Manzanares et al., 2009).
Figure 1-5: Bipolar Myosin Filament – Platinum replica electron microscopy reveals high-resolution architecture of myosin filaments (colored). Following gelsolin treatment, which removes actin filaments, myosin II structures remain, approximately 300 nm in length.
Nonmuscle myosin II is regulated through the phosphorylation status of two conserved residues, namely Ser19 and Thr18 in the regulatory light chain of the myosin hexameric molecule. When in the unphosphorylated state, NMII exhibits a folded conformation effectively hiding the ATP and actin binding regions. Furthermore, when unphosphorylated, NMII cannot assemble into polymerized structures. Phosphorylation at Ser19 can restore all of these characteristic functions and further phosphorylation at Thr18 enhances NMII activity (Matsumura, 2005).

Though the signaling pathways for NMII activation have been significantly elucidated, it is still unclear how myosin II is recruited and incorporated into stress fibers, and more generally how stress fibers are formed within a cell. It is known that the assembly of stress fibers occurs concomitantly with maturation of newly formed focal adhesions and the two processes are interdependent, but follows a rather complex proposed mechanism. In the lamella, a relatively less dense actin zone at the base of the lamellipodia, NMII begins to assemble into bipolar filaments. As protrusion of the actin network in the lamellipodia propel the cell forward, newly formed nascent focal adhesions and filamentous actin structures from the lamellipodia undergo retrograde flow entering the lamella. NMII begins to reorganize the actin filament network, preferentially at sites of precursor structures including filopodia bundles and focal complexes (Verkhovsky et al., 1995; Svitkina et al., 1997; Hotulainen and Lappalainen, 2006). Indeed, it was recently suggested that filopodia serve as a seed for sites of future stress fiber formation utilizing NMII (Anderson et al., 2008). Further forming the stress fiber, greater force of NMII-generated contraction promotes a load-dependent NMII recruitment and polymerization to sites of high tension for stabilization, but this is still
under investigation (Fernandez-Gonzalez et al., 2009). During cell migration, the remodeling of the actin network within stress fibers is less dynamic than at the leading edge, yet turnover is necessary for cell-substrate adhesion formation and tail retraction. Conversely, in more quiescent cells, stress fibers contribute to the integrity of cell shape. (Vicente-Manzanares et al., 2009).
1.3 Cell-Cell Junctions

In addition to its fundamental role in cell migration and morphology, the actin cytoskeleton is also necessary in the formation and maintenance of cell-cell junctions to form a cohesive network of cell-cell interactions. Proper intercellular cohesion and communication is essential in the development of multicellular organisms. Tissue and organ formation requires specific adhesion between distinct cell types throughout embryonic development. Furthermore, in the adult, specific cell-cell junction maintenance and remodeling is essential for tissue integrity and function (Yamada and Nelson, 2007). For example, the outer lining of the skin and intestines exhibits continual turnover allowing the incorporation of new cells without the loss of barrier function. Likewise, during wound healing and tissue repair cells must establish new contacts when the wound closes. Additionally, endothelial cells that line the blood vasculature must remain dynamic to allow for leukocyte transmigration to sites of infection (Bazzoni and Dejana, 2004). Cells accomplish these functions through the interactions between various proteins localized to specific adhesion sites.

Large, multiprotein complexes assemble at sites of cell-cell junctions to adhere cells to one another, transmit signals, and generate barriers between tissues. Classically, there are four types of functionally distinct cell-cell interactions and junctions: 1) signal relaying, 2) channel-forming, 3) occluding, and 4) anchoring (Green et al., 2010). For example, signal relaying junctions include neuronal synapses formed between an axon and a dendrite where, in response to an action potential, neurotransmitters are released from the presynaptic terminal to initiate a new action potential in its neighboring cell (Yamada and Nelson, 2007). Gap junctions, the principal form of channel forming
junctions, serve as intercellular connections for the passage of molecules and ions. The primary purpose of occluding junctions, namely tight junctions, is to function as a selective barrier to particular solutes. Anchoring junctions, primarily adherens junctions, mechanically attach cells to their respective cytoskeletons. Tight junctions and adherens junctions are formed from different components, but have overlapping features (Figure 1-6). In both cases, junctional complexes are composed of transmembrane and intracellular molecules contributing to structural integrity of the cell shape, but adherens junctions are of primary importance with respect to the actin cytoskeleton in the context of cellular junctions (Cavey and Lecuit, 2009).
Figure 1-6: Cell-Cell and Substrate Adhesion – Various types of cell-cell and cell-substrate adhesion are described in this schematic. Typically associated with the actin cytoskeleton are adherens junctions, characterized by extracellular cadherin homophilic binding.

(Adapted from Alberts et al., 2002)
1.3.1 Cadherins Family Proteins

At the core of adherens junctions are the transmembrane cadherin family proteins. In mammals, the cadherin protein family consists of at least 80 members each with differential expression depending on cell and tissue type, with the most well documented and studied subfamily known as classical cadherins. Classical cadherins are single pass transmembrane proteins characterized by long extracellular and intracellular domains. The extracellular portion of cadherin mediates calcium dependent homophilic interaction with cadherins from adjacent cells through a series of five characteristic subdomains (EC1-5) (Nelson, 2008).

Immediately following initial contact between two cells, cadherins are proposed to cluster together and stack laterally by cis interactions. The assembly and disassembly of cadherin extracellular adhesion is still controversial, as crystal structure analysis of classical cadherins reveal two different dimeric arrangements known as the strand-swapped model and X dimer model. In the former model, the N-terminal beta-strands of EC-1 domain swap between partner molecules whereas in the latter model, residues near the EC1-EC2 calcium binding site contact one another and the beta-strands do not swap (Nagar et al., 1996; Pertz et al., 1999; Boggon et al., 2002; Haussinger et al., 2004). Recent evidence suggests assembly of cadherin molecules occurs via the strand-swapped model to form strong adhesion, whereas disassembly occurs utilizing the less stable X dimer configuration as the transition state (Hong et al., 2011).

It is proposed that the major factor in determining which extracellular configuration the cadherins will adopt is the intracellular portion of cadherin. Whereas the extracellular domains of cadherins exhibit structural variability between the various
members of the family, much less variability is observed between the intracellular domains. The cytoplasmic cadherin tails links with an assortment of intracellular proteins thereby connecting the adhesion complex to the actin cytoskeleton network (Figure 1-7) (Nelson, 2008; Meng and Takeichi, 2009). Interestingly, physical measurements of the collective adhesive forces among cadherins indicate a very weak interaction. It has been proposed that the actin cytoskeleton supplies the missing force (Mege et al., 2006).
Figure 1-7: Calcium Mediated Cadherin Interaction and Intracellular Linkage to the Cytoskeleton – Calcium mediated cadherin interactions form zipper-like structures. Transmembrane cadherins link to the actin cytoskeleton through multiprotein complexes including α-catenin and β-catenin.

(Adapted from (Rudini and Dejana, 2008))
1.3.2 Cadherin Linkage to the Actin Cytoskeleton

As the actin cytoskeleton provides rigidity, regulating the architecture of the cytoskeleton may be the critical component in generating and maintaining cell-cell contacts. Early in the study of the cadherin-actin cytoskeleton complex, biochemical analysis revealed a direct binding of β-catenin to cadherin, as well as binding to α-catenin (Aberle et al., 1994; Rimm et al., 1995). It was also shown that α-catenin can interact with actin filaments. Furthermore, through microscopy investigation, both β-catenin and α-catenin colocalize to sites of cadherin interaction, giving rise to the model of a multiprotein complex directly connecting cadherins to the actin cytoskeleton. A great deal of investigation into the cellular role of cadherin has revealed that it can modify cytoskeletal architecture through its intracellular protein components of β-catenin, α-catenin, p120, and others, although the mechanism is still unclear (Huber and Weis, 2001; Gates and Peifer, 2005). This model has been recently challenged by evidence that α-catenin cannot simultaneously bind actin filaments when complexed with cadherin and β-catenin (Drees et al., 2005; Yamada et al., 2005).

Reconciling this seemingly conflicting evidence, two additional models have been proposed. First, strong evidence has shown α-catenin to bind other actin binding proteins such as members of the formin family as well as a protein known as EPLIN (epithelial protein lost in neoplasm), whereby these proteins act as the direct linkage between the actin cytoskeleton and the cadherin-catenin(s) complex (Abe and Takeichi, 2008). A second, non-exclusive model suggests a localized increase in α-catenin concentration during cadherin clustering is sufficient to drive α-catenin dimerization, where the
homodimer exhibits a much stronger affinity for binding and bundling actin filaments. This model requires the $\alpha$-catenin association with the cadherin/$\beta$-catenin complex to be labile, which the evidence suggests (Nelson, 2008). Interestingly, further studies have shown $\alpha$-catenin to compete with Arp2/3 complex binding to actin filaments, thereby suppressing the Arp2/3 complex F-actin branching activity (Drees et al., 2005). Indeed, a number of actin binding proteins involved in cell migration are also associated with cell-cell junction initiation and maintenance (Mege et al., 2006; Harris and Tepass, 2010).

1.3.3 Models of Cell-Cell Junction Formation

The procedure by which cells establish adherens junctions remains largely unclear. Observations of cell-cell contact formation in various epithelial cells, which form cohesive cellular sheets, suggest different models. The first model proposes that the initial contact commences with filopodia that extend and interdigitate with similar protrusions emanating from adjacent cells. These actin based protrusions then establish a series of initial point contacts, which subsequently zipper into a continuous cell-cell junction. This work was observed in vitro keratinocytes after stimulation by calcium to promote cadherin-cadherin interaction from adjacent cells (Figure 1-8A) (Vasioukhin et al., 2000).

In the original evidence supporting the filopodia initiating model it was seen that the total number of filopodia always increased upon calcium stimulation, but were much higher at intercellular junctions. Therefore it is unclear how relevant filopodia formation is necessary in cell-cell junction formation. Furthermore, early times points of junction formation were not observed as cells filopodia were observed hours after calcium
stimulation, therefore the early transitional architecture of the actin cytoskeleton is completely unknown. Lastly, it is unclear if this filopodia initiating observation is cell type specific. Indeed, several players of the filopodia machinery are necessary for cell contact formation including Ena/VASP and formins (Vasioukhin et al., 2000; Kobielak et al., 2004; Furman et al., 2007; Benz et al., 2008). It is possible through the activity of Ena/VASP, formins, and other actin remodeling proteins, the protrusive cytoskeletal machinery drives cells closer together, essentially zippering the plasma membrane between the points of contact.

Work from the Nelson laboratory proposed a different model, suggesting that lamellipodia establish the initial junction, while subsequent contact-dependent inhibition of protrusion in both cells contributes to stable contacts. Indeed, very clear studies in MDCK cells have shown dynamic lamellipodia to protrude and essentially probe the adjacent cell initiating cell-cell cadherin interaction. Actin polymerization and reorganization slows as cadherins begin to cluster further. In the latter stages of contact formation, myosin II contractile activity pulls the edges of the contacting membranes outwards, thus expanding the zone of cell-cell contact (Figure 1-8B) (McNeill et al., 1993; Adams et al., 1998; Harris and Tepass, 2010). However, other data suggest that the initial contact between cells stimulates lamellipodia formation through activation of Rac1, a GTPase locally regulating actin nucleators including the Arp2/3 complex (Ehrlich et al., 2002; Baum and Georgiou, 2011). Furthermore, the lamellipodia initiating model has yet to be thoroughly investigated in other cell types.
Figure 1-8: Proposed Models Describing the Initiating Events of Cell-Cell Adhesion

(A) Schematic representing the filopodia initiating model, also known as the zippering model. Environmental cues stimulate filopodia extension initiating cadherin based contact with adjacent cells (Originally proposed by (Vasioukhin et al., 2000; Vasioukhin and Fuchs, 2001)).

(B) Observations in MDCK cells and drosophila embryos suggest cells initiate contact through lamellipodia extensions. Clustering of adhesion proteins lead to the reorganization of the actin cytoskeleton laterally expanding the point of contact (diagram from (Krendel and Bonder, 1999)).
It has been shown that Ena/VASP is recruited by cadherin clustering to sites of early cell-cell junction formation (Vasioukhin et al., 2000; Baum and Perrimon, 2001; Scott et al., 2006). Considering Ena/VASP acts as an actin elongation factor, as well as proposed to have F-actin anti-branching activity by virtue of monomer sequestering, Ena/VASP may be necessary in the transition between expanding contacts associated with Arp2/3 regulated lamellipodia and F-actin cables at mature junctions. However, the effect of Ena/VASP on specific actin cytoskeletal architecture at early cell-cell junctions is unknown.

Further confirming the necessity of the actin cytoskeletal network, myosin II plays a fundamental role in establishing cell-cell junctions. It has been shown that myosin II activity is required for recruitment and stability of cadherins at adherens junctions, as well as establishing mature adherens junctions, but the mechanism is still controversial (Shewan et al., 2005; Ivanov et al., 2007; Fernandez-Gonzalez et al., 2009; Smutny et al., 2010). All told, the presence of several actin remodeling proteins including members of Ena/VASP family and nonmuscle myosin II suggest a dynamic interplay of actin filament formation, stabilization, rearrangement, and turnover during cell-cell junction formation. According to either proposed models of cell-cell junction formation, extensive reorganization the actin cytoskeleton is required during early stages of adherens junction formation, but specific actin cytoskeleton architecture during these stages is unclear.
1.4 Research Objective

While both lamellipodia and filopodia drive cellular protrusions with the potential to initiate cell-cell contact, these structures exhibit very distinct architectures. Yet both lamellipodia and filopodia have been implicated in the initiating phases of cell-cell junction formation. Therefore, the role of cytoskeletal protrusions in cell-cell junction formation requires analysis. Particularly in endothelial cells, further investigation into the reorganization of the actin cytoskeleton during cell-cell junction formation is required. Finally, it is unknown how actin binding proteins involved in both lamellipodia/filopodia formation and adherens junctions dynamics eventually remodel the actin cytoskeleton architecture. Therefore, to better understand the function of the actin cytoskeleton in cell-cell junction formation, we used live cell imaging combined with platinum replica transmission electron microscopy (TEM) to determine actin architecture at different stages of cell-cell junction formation in primary human umbilical vein endothelial cells (HUVECs) at high resolution. Additionally, we investigated the roles of actin remodeling proteins implicated in lamellipodia and filopodia formation, namely Ena/VASP, fascin, and nonmuscle myosin II, among others in both a high resolution and temporal manner. We uncovered a novel mechanism of cell-cell junction formation in which intercellular contact is initiated by lamellipodia followed by formation of interdigitating filopodia-like structures, which we term bridges. Furthermore, we discovered a new mechanism of the lamellipodia-to-filopodia transition within the context of cell-cell adhesion. Our work helps to reconcile previous competing studies of the mechanisms of cell-cell adhesion formation and furthers our understanding of the contribution and interplay of lamellipodia and filopodia to this process.
CHAPTER 2: MATERIALS AND METHODS

**Cells and Reagents:** Human umbilical vein endothelial cells (Lonza) were maintained in Endothelial Cell Basal Medium supplemented with recommended reagents (Lonza) and cultured for 6 passages maximally. For experiments, HUVECs were plated on collagen-coated substrates at approximately 5 ug/cm² collagen concentration. Rat-tail collagen was purchased from BD Biosciences. The following primary antibodies were used: mouse anti-cadherin-5 monoclonal antibody (1:200; BD Bioscience), rabbit polyclonal antibody to nonmuscle myosin II from bovine spleen (1:10; (Verkhovsky et al., 1987), mouse monoclonal anti-fascin (1:100; clone 55K2, Chemicon International), rabbit anti-VASP (1:500; gift of Frank Gertler, Massachusetts Institute of Technology, Cambridge, MA), mouse monoclonal anti-cortactin (1:200; clone 4F11, Upstate Biotechnology), mouse monoclonal anti-tubulin (1:200; clone DM1A, Sigma). Secondary fluorescently labeled antibodies were from Molecular Probes or Jackson Laboratories. Myosin subfragment-1 (S1) was a gift from Yale Goldman (University of Pennsylvania, Philadelphia, PA). Rhodamine labeled actin protein, purified from rabbit skeletal muscle, was purchased from Cytoskeleton. Active (-)- and inactive (+)-blebbistatin (Toronto Research Chemical Inc.) was prepared from 10 mM stock in DMSO. For experiments, cells were plated on collagen-coated substrates a day before the experiment, treated with (-)- or (+)-blebbistatin at 50 uM concentrations in culture medium for 30 min and then processed for live imaging or immunofluorescence.
**Plasmids and constructs:** Human enhanced green fluorescent protein (EGFP)-Ena/VASP was a gift of James Bear (University of North Carolina, Chapel Hill, NC), EGFP-fascin was a gift of Josephine Adams (Case Western Reserve University, Cleveland, Ohio), and EGFP-myosin II-RLC was a gift of T.L. Chew and R. Chisholm (Northwestern University). For transfection, HUVECs were subjected to nucleofection (Nucleofector I, Amaxa protocol for HUVECs, Lonza). Approximately $5 \times 10^5$ cells per nucleofection reaction with 50-70% transfection efficiency were then plated on collagen-coated dishes with fresh media given 1 day after transfection. Cells were analyzed 2-3 days after initial transfection.

**Immunofluorescence:** Cells were quickly washed with PBS before extraction or fixation. For VE-cadherin staining, cells were fixed in 4% paraformaldehyde in PBS for 30 minutes. For nonmuscle myosin II immunostaining, cells were extracted with 1% Triton X-100 in PEM buffer (100 mM PIPES-KOH, pH 6.9, 1 mM MgCl$_2$, and 1 mM EGTA) containing 4% polyethelene glycol (PEG mol. wt. 35,000) for 5 minutes followed by fixation in 4% paraformaldehyde. Fascin staining was performed after methanol fixation (10 min) without extraction followed by 2% glutaraldehyde fixation. For VASP staining, cells were treated with a mixture containing 0.1% glutaraldehyde and 0.5% Triton X-100 in PEM buffer and then fixed in 2% glutaraldehyde. For cortactin staining, cells were pretreated in a solution of 1% Triton-100, 2% PEG, and 2 mM phalloidin for 10 minutes followed by fixation in 2% glutaraldehyde. The actin incorporation assay (barbed end assay) was performed as previously described (Lorenz et al., 2004). Briefly, wells were incubated with 0.4 mM Rh-actin, 0.25 mM ATP, and 0.1% saponin in P-
buffer (10 mM PIPES-KOH, pH 6.9, 138 mM KCl, 4 mM MgCl₂, 3 mM EGTA) for 2 minutes, washed in P-Buffer, and fixed in 4% paraformaldehyde. For detection of F-actin, staining with fluorescently labeled phalloidin was performed after fixation during incubation. For quantification of VE-cadherin localization in bridges, integrated and average fluorescence intensity of VE-cadherin immunostaining in individual puncta was measured after background subtraction and thresholding of identically acquired images using Metamorph imaging software (Molecular Devices).

**Microscopy:** Light microscopy was performed using an inverted microscope (Eclipse TE2000; Nikon, Tokyo, Japan) equipped with Planapo 100 x 1.3 and Cascade 512B charge-coupled device camera (Roper Scientific, Trenton, NJ) driven by Metamorph imaging software (Molecular Devices, Sunnyvale, CA). For live cell imaging, cells were plated on collagen-coated glass-bottomed dishes and kept at 37°C for the duration of the experiment. For phase-contrast time-lapse microscopy in the presence of blebbistatin a red filter was inserted into the transmitted light path to prevent photoinactivation and phototoxicity of blebbistatin.

Samples for platinum replica TEM and for correlative light and TEM were processed essentially as described previously (Svitkina and Borisy, 2006). Briefly, detergent-extracted samples were fixed with 2% glutaraldehyde, tannic acid, and uranyl acetate, critical point dried, coated with platinum and carbon, and transferred onto TEM grids for observation. Detergent extraction was performed for 5 min at room temperature with 1% Triton X-100 in PEM buffer containing 2% PEG, and 2 mM unlabeled phalloidin. For determination of cell surface topography, extraction step was omitted and cells were
directly fixed. For gelsolin treatment, detergent-extracted unfixed cells were incubated with 0.4 µg/ml gelsolin (gift of Dr. A. Weber, University of Pennsylvania) in buffer containing 50 mM MES (pH 6.3), 2 mM MgCl₂, 0.1 mM CaCl₂, and 2 µM paclitaxel for 10 min at room temperature and fixed with 2% glutaraldehyde. For myosin S1 decoration, detergent-extracted unfixed cells were incubated with 0.25 mg/ml S1 in PEM buffer with 5 uM phalloidin for 30 minutes at room temperature and fixed with 2% glutaraldehyde. For immunogold VASP and cortactin staining, cells were treated in the same fashion as for immunofluorescence, substituting secondary fluorescent antibodies with 18-nm gold-conjugated antibodies incubated overnight and fixed with 2% glutaraldehyde. Samples were analyzed using JEOL 1200EX (JEOL, Tokyo, Japan) transmission TEM operated at 100 kV. Images were captured by ORIUS 835.10W CCD camera (Gatan, Warrendale, PA) and presented in inverted contrast. Identification of gold particles was performed at high magnification after contrast enhancement to distinguish them from other bright objects in the samples.
CHAPTER 3: RESULTS

The procedure by which cell-cell junction formation is accomplished is still controversial, as two competing models exist. The first model proposes the initiating event in adherens junction formation is the extension of interdigitating actin based filopodia (Vasioukhin et al., 2000; Vasioukhin and Fuchs, 2001). The second model suggests the initiating event is lamellipodia interaction followed by cadherin based junction stabilization (McNeill et al., 1993; Adams et al., 1998; Krendel and Bonder, 1999). However, it is unclear whether these models are mutually exclusive as often filopodia are generated from within lamellipodia. Furthermore, it is possible that the mechanism of cell-cell junction formation is cell type dependent. Therefore, we decided to investigate the initial events and mechanism of cell-cell junction formation, utilizing human umbilical vein endothelial cells (HUVECs) as our model system.

3.1 The lamellipodia to filopodia-like bridge formation is the predominant event in the initial stages of cell-cell junction formation

To analyze the initial phases of cell-cell junction formation, we utilized phase contrast live cell imaging of passage 3-4 HUVECs plated on collagen-coated dishes and analyzed at subconfluency. The dynamic leading edges of cells exhibiting no contact with an adjacent cell expressed typical lamellipodia and occasional filopodia undergoing characteristic cycles of protrusion and retraction. Cell-cell contacts in these cultures were invariably initiated by protruding lamellipodia generated by one or both cells (n=59). Following retraction of the lamellipodia, thin cytoplasmic bridges connecting two cells
were revealed at the point of the lamellipodial contact in 75% of collision events (Figure 3-1A and 3-1D, Movie S1). In the remaining ~25% cases, lamellipodia-initiated contact did not result in bridge formation, either because lamellipodia did not retract during the 10 min period of observation or, more frequently, because cells separated completely upon retraction (Figure 3-1D, right). In cases where bridges were formed, cells subsequently resumed protruson at the site of the bridge or broke the bridge-mediated cell-cell contact, with similar frequencies of both outcomes (Figure 3-1D, left). The lamellipodia retraction could occur synchronously in both contacting cells resulting in a broad gap between cells crossed by long bridges (Figure 3-1C). Alternatively, only one of two colliding cells retracted and formed a bridge terminating on the flat surface of the other cell (Figure 3-1B). During their formation, bridges often transiently acquired an hourglass shape characterized by a broad proximal base, a narrow stalk and a widened distal tip possessing a persistent mini-lamellipodium (Figure 3-1C). In other cases, mini-lamellipodia were not obvious and the bridges morphologically resembled filopodia in a contact with a neighboring cell throughout the process of their formation (Figure 3-1B).
Figure 3-1: The Lamellipodia-to-Bridge Transition during Initiation of Cell-Cell Junction in HUVECs

(A-C) Phase-contrast time-lapse sequence of junction formation. (A) Low magnification image of two non-contacting cells at time 0 is shown in the left-most panel. Montage of individual frames from the boxed region is shown in right panels. Lamellipodia of two cells form initial contact (3:20) followed by lamellipodia retraction and concomitant bridge formation (7:49 – 16:28). Time in min:sec. Boxed regions in the right-most panel in A are shown at higher spatial and temporal resolution in B and C. (B) Filopodia-like bridges are formed primarily through retraction of the left cell. (C) Bridges are formed through retraction of both cells with intermediate formation of mini-lamellipodium (13:08, lower bridge). Scale bar: 15 µm

(D) Quantification of cell-cell collision events (n=59). Bars show the number of lamellipodia-initiated cell-cell contacts leading to bridge formation (left) or not (right). Grey, transient contacts are broken within 10 min after initiation. Black, stable contacts are defined as greater than 10 min abutting the adjacent cell.
3.2 HUVECs exhibit different modes of contact with adjacent cells

Thin membrane bridges mediating cell-cell communication in various cell types may represent tunneling nanotubules through which cells share the cytoplasm, or filopodial bridges that maintain cells in contact without membrane fusion (Sherer and Mothes, 2008). Light microscopy of bridges formed by HUVECs during junction formation does not allow to distinguish these possibilities and clearly demarcate the boundaries of two interacting cells. Therefore, we applied platinum replica TEM to unextracted HUVECs with preserved plasma membrane to characterize various displays of cell-cell contact at high resolution. We were able to clearly define cell edges in different bridge configurations indicating distinct cytoplasmic pools of contacting cells, as opposed to nanotubules (Figures 3-2A-G).

Examples of contacting lamellipodia of two adjacent cells, which likely corresponded to a very early stage of cell-cell interaction or a case of continuous interplay between contacting lamellipodia, revealed that lamellipodia interacted with one cell completely atop the adjacent cell’s lamellipodia or formed interlocking configurations (Figure 3-2A and 3-2B). High-resolution analysis of long thin bridges revealed that many were formed by filopodia-like protrusions exhibiting extensive lateral contact with similar structures from the adjacent cell (Figure 3-2C and 3-2D). Alternatively, a filopodium extending from one cell made a tip contact with the body of the adjacent cell (Figure 3-2E, top and bottom bridges). Some of these latter bridges exhibited an extensive expansion of the filopodial tip at points of contact resembling mini-lamellipodia, which we observed by time-lapse microscopy (Figure 3-2E, second from the top bridge, F and G). It should be noted that mini-lamellipodia at the tips of
filopodia-like structures were only seen in the context of cell-cell contact, whereas filopodia at free edges possessed a narrow pointed tip. We also observed interesting cases where the membrane of a recipient cell engulfed the tip of a filopodium-like protrusion of an adjacent cell (Figure 3-3). Similar observations were also made in the previous studies of junction formation in skin keratinocytes, which suggested that interdigitating filopodia physically embed into the adjacent cell’s membrane (Vasioukhin et al., 2000).
Figure 3-2: The Lamellipodia-to-Bridge Transition during Initiation of Cell-Cell Junction in HUVECs (cont.)

(A-G) TEM of unextracted HUVECs showing different types of cell-cell contacts, such as overlapping lamellipodia (A and B), interdigitating filopodia (C,D), or filopodial tips (E) or mini-lamellipodia (E-G) contacting surface of adjacent cell. Scale bars: 1 µm (A,F); 500 nm (C-E, G), and 100 nm (B).
Figure 3-3: Adjacent Cell Membrane Can Engulf Bridge Tips

(A) TEM of unextracted cells reveals embedding of bridge tip into the adjacent cell. Boxed regions are zoomed in lower panels. Scale bar: 1 µm.
3.3 Intercellular actin architecture reveals structural similarity of bridges to free edge filopodia

Actin is the predominant cytoskeletal component of the leading edge protrusions as well as adherens junctions (Mege et al., 2006; Insall and Machesky, 2009). Although actin architecture is relatively well described in lamellipodia and filopodia, it is unclear how the actin cytoskeleton is organized in cell-cell junctions, particularly at the various initial stages of their formation. Platinum replica TEM analysis of detergent-extracted HUVECs revealed that free leading edges in HUVECs displayed typical dendritic network in lamellipodia characterized by branched actin filaments (Figure 3-5A) and thin bundles of long actin filaments in occasional filopodia protruding from lamellipodia (Figure 3-5B). In the context of cell-cell contacts, as expected, the actin network in overlapping lamellipodia of adjacent cells also displayed relatively short branched actin filaments, but few, if any, preexisting filopodial bundles (Figure 3-6A). However, intercellular bridges displayed tight bundles of long actin filaments similar to those in traditional filopodia (Figure 3-4A and 3-4B).

Further extending the similarity with filopodial bundles, bridge bundles splayed apart at the base of the bridge and merged with the proximal actin network (Figure 3-4A and 3-4B). Although cell boundaries were no longer recognizable after membrane extraction, some bridges displayed two or more similarly organized sub-bundles suggesting that they might correspond to bridges formed by laterally interacting filopodia-like protrusions (Figure 3-4A and 3-4B). However, in other cases the interaction between interdigitating bundles was too tight to demarcate a boundary between them (Figure 3-4C). When mini-lamellipodia were observed at the tips of
bridges, they displayed a dendritic actin network typical for lamellipodia, although the shaft of the bridge contained long parallel actin filaments, similar to conventional filopodia or bridges without mini-lamellipodia (Figure 3-4D). We also observed structural equivalents of bridges embedding into the adjacent cell’s membrane in extracted cells that appeared as an actin network engulfing the distal tip of bridges (Figure 3-6B).

Two major known types of actin bundles, filopodial bundles and stress fibers, are different in predominant orientation of their actin filaments. In contrast to uniform orientation in filopodia, actin filaments in stress fibers display mixed polarity with the exception of the focal adhesion area, where all filaments are oriented with barbed ends to the membrane (Cramer et al., 1997; Verkhovsky et al., 1997). To determine whether bridge bundles resemble filopodia or stress fibers with respect of actin filament polarity, we decorated actin filaments in HUVECs with myosin subfragment 1 (S1) and determined filament polarity in contact areas. Myosin S1 decoration revealed that actin filaments in overlapping lamellipodia had barbed ends facing the direction of the leading edge, similar to free leading edge lamellipodia (Figure 3-7A and 3-7B). In bridges formed by a single filopodium-like protrusion landing on the surface of the adjacent cell, barbed ends of each filament with detectable polarity were oriented toward the tip of this filopodium, similarly to free edge filopodia (Figure 3-4E). In bridges that appeared to derive from interdigitating filopodia produced by both contacting cells, actin filaments at the bases of the bridge were uniformly oriented with barbed ends toward the middle of the bridge, whereas the central region might contain filaments with mixed polarity (Figure 3-4F), consistent with an idea that the bridge was formed by two filopodia.
protruding from neighboring cells and forming a junction in the middle of the bridge. Therefore filaments within bridges are similar in both structure and actin polarity to free filopodia.
Figure 3-4: Structural Organization of Actin Filaments in Intercellular Bridges

(A - C) Bridges contact tight bundles of long actin filaments, sometimes splaying apart at the base of the bridge (A,B), similar to free edge filopodia. Bridges may contain slightly separated sub-bundles (B) or an apparently single bundle. Boxed regions in main panels are enlarged at right (A,C) or bottom (B). Scale bars: 1 µm (A and B, main panels), 500 nm (A, zoom; C, main panel) and 200 nm (B and C, zooms).

(D) Mini-lamellipodia at the tips of bridges (brown) contain a branched actin network, as are in free-edge lamellipodia. Boxed region in main panel is enlarged at right. Scale bars: 2 µm (main panel), 500 nm (zoom).

(E and F) Myosin S1 decoration of actin filaments within bridges demonstrates barbed end orientation away from the bridge base in individual (E) and interdigitating (F) bridges. Boxed regions in main panels are enlarged at bottom. Arrowheads show direction of pointed ends to left (green) or right (red). Right inset in F shows an individual decorated actin filament with polarity indicated by an arrowhead. Scale bars: 1 µm (E), 500 nm (F), 200 nm (E and F, zooms), and 50 nm (F, inset).
Figure 3-5: Actin Organization in Leading Edge Protrusions in HUVECs

(A and B) TEM of free-edge protrusions demonstrate typical dendritic actin network of short filaments in lamellipodia (A) and bundled long filaments in filopodia (B). Scale bars: 500 nm.
Figure 3-6: Actin Organization in Cell-Cell Contacts

(A) TEM of interacting lamellipodia at cell-cell contacts demonstrates similar structural organization to free-edge lamellipodia. Scale bar: 500 nm.
(B) TEM of a bridge embedding into adjacent cell cytoskeleton. Scale bar: 500 nm.
Figure 3-7: Actin Filament Polarity Revealed in Free Edge and Overlapping Lamellipodia

(A and B) In both overlapping lamellipodia at cell-cell contact (A) and free-edge lamellipodia (B) actin filaments are oriented with barbed ends facing the leading edge. Scale bars: 500 nm (A) and 200 nm (B).
3.4 Bridges contain VE-cadherin and molecular markers of filopodia

The structural organization of the actin cytoskeleton implies participation of certain molecular components of the cytoskeleton. To further characterize the bridges and confirm our interpretation of the structural data, we determined their molecular components by immunofluorescence microscopy. Since bridges appear as the first manifestation of cell-cell junction formation in HUVECs, we investigated the localization of VE-cadherin, a key component of adherens junctions in endothelial cells. Confirming the presence of adherens junctions at sites of bridge formation, VE-cadherin was highly enriched in bridges. Its distributions ranged from continuous staining along the shaft of the bridge to single or multiple punctate accumulations (Figure 3-8A). These findings are similar to previously reported VE-cadherin localization to filopodia-like structures connecting two adjacent endothelial cells (Almagro et al., 2010). In contrast, VE-cadherin antibody stained uniformly and with lower intensity in free or overlapping lamellipodia (Figure 3-9A), suggesting a relocalization of VE-cadherin to bridges upon cell-cell contact initiation. Furthermore, bridges were primarily actin-rich structures with no microtubules present (Figure 3-9B).

Since bridges were similar in structure and filament polarity to traditional filopodia, we tested whether bridges share other key features with filopodia. First, we determined the localization of a bundling protein fascin, which is considered to be a key marker of filopodia. Consistent with the filopodia-like morphology of bridges, immunostaining of fascin revealed its localization to bridges (Figure 3-8B). In contrast, the lamellipodial marker, cortactin (Ren et al., 2009a), was present in overlapping
lamellipodia, but not in mature bridges (Figure 3-9C), as also confirmed by immunogold labeling (Figure 3-10A).

Next, we performed an actin incorporation assay to examine whether bridges possessed open barbed ends at their tips similar to conventional filopodia. Rhodamine-actin readily incorporated at the edges of free lamellipodia and tips of filopodia, as expected (Figure 3-9D). Importantly, open barbed ends were also found in bridges (Figure 3-8C) suggesting actin bundles within these structures to be actively polymerizing. The sites of Rhodamine-actin incorporation were often distributed along a significant distance over the bridge in addition to dot-shaped sites of incorporation, suggesting that open barbed ends may not be always focused at the filopodial tip, but were distributed along the junction.

The barbed end-associated elongating and anti-capping protein VASP is another conventional filopodial marker, which is enriched at filopodial tips, but also localizes to the leading edge of lamellipodia, cell-matrix adhesions at the tips of stress fibers, and adherens junctions (Krause et al., 2003). Immunostaining of HUVECs with VASP antibody revealed the expected VASP localization to lamellipodial edges and filopodial tips at free cell edges (Figure 3-10B and 3-10C, respectively), but also at the edge of mini-lamellipodia and in 68% (N=25) of bridges (Figure 3-8D). Immunogold labeling to establish high-resolution localization of VASP by TEM confirmed that VASP localized to mini-lamellipodia at the distal tips of bridges (Figure 3-8E-G). A slightly lower fraction of VASP-positive bridges (44%, N=25) detected by TEM might be due to higher spatial precision of TEM or limited access to dense cytoskeletal regions for gold-labeled antibodies. By both techniques, VASP was usually absent at longer established bridges.
without mini-lamellipodia (Figure 3-10D) suggesting a transient requirement of VASP for actin polymerization in bridges or transient elongation of bridges. Together, these data suggest an active branched actin network in mini-lamellipodia at the tip of bridges, while proximal actin bundles at the bridge base bearing molecular features of filopodia.
Figure 3-8: Bridges Contain Adherens Junctions and Filopodial Markers

(A) Immunofluorescence staining of VE-cadherin (red) and F-actin by phalloidin (green).
(B) Immunofluorescence staining of fascin shown individually (upper left) and as overlay of phase contrast image (upper right). Boxed region is zoomed at bottom.
(C) Actin incorporation assay. Bridges contain uncapped barbed ends incorporating rhodamine-labeled actin (red) into pre-existing F-actin structures labeled by phalloidin (green).
(D) Immunofluorescence attaining of VASP (red) and F-actin staining by phalloidin (green) are shown at low magnification in left panel. Boxed region is zoomed in right panels as individual channels and as a merged image. VASP localizes to bridge tips (white arrow) and along edge of mini-lamellipodia (black arrow).
(E-G) Immunogold staining of VASP. Low magnification image (E) shows several bridges between interacting cells. Enlarged boxed region from E (F,G) shows structures belonging to two adjacent cells in different colors (F) and position of gold particles (G, yellow). Scale bars: 10 μm (A-D), 1 μm (E), and 200 nm (F and G).
Figure 3-9: Immunofluorescence staining of Cytoskeletal Proteins in HUVECs.

(A) Immunostaining of VE-cadherin and F-actin shows intense staining along shaft of bridges and weaker staining in overlapping lamellipodia (yellow arrow). Scale bar: 10 mm.

(B) Immunostaining of tubulin and F-actin shows that bridges are primarily actin-based structures, while microtubules may occasionally penetrate into a bridge base. Scale bar: 10 um.

(C) Immunostaining of cortactin and F-actin demonstrates bright staining in lamellipodia (white arrow), but no staining in bridge shaft (black arrow). Scale bar: 5 um.

(D) Rhodamine-labeled actin (red) incorporates along the free edge of lamellipodia and tips of free filopodia. Scale bar: 5 μm.
Figure 3-10: Immunogold TEM of Cortactin and VASP

(A) Immunogold labeling of cortactin reveals its presence in lamellipodia, but not along the bridge shaft. Scale bar: 500 nm.
(B-D) Immunogold labeling of VASP demonstrates its presence along leading edge of free lamellipodia (B) and tips of free filopodia (C). Established bridges no longer contain VASP at the tip or along the shaft (D). Boxed region is magnified at right. Gold particles are highlighted in yellow. Scale bars: 50 nm (B,C) and 500 nm (D).
3.5 Dynamics of molecular markers during bridge formation

During formation of conventional filopodia at the cell leading edge by a convergent elongation mechanism, fascin is initially recruited to the tip of the filopodial precursor and then propagates along the forming bundle in proximal direction, while its steady state distribution is characterized by highest concentration at the tip and a gradual decrease toward the base of the filopodium (Svitkina et al., 2003). To elucidate the dynamics of fascin during bridge formation, GFP-fascin transfected HUVECs were analyzed by time-lapse fluorescence microscopy. During cell-cell junction formation, GFP-fascin was not enriched in lamellipodia, but following lamellipodia collapse, was concentrated in the forming bridges (Figure 3-11A, Movie S2). In contrast to leading edge filopodia, the peak of GFP-fascin enrichment was typically found along the shaft of bridges, while the distal and proximal portions of the bridge had dim fluorescence. In hourglass-like protrusions, likely corresponding to mini-lamellipodia-containing bridges, fascin was enriched at the neck of the protrusion (Figure 3-11A, 1:39 time point). Due to uneven distribution of GFP-fascin along the length of the bridge, it was possible to observe persistent movement of individual fluorescent features in the proximal direction towards the bridge base (Figure 3-11B, Movie S3), suggesting retrograde flow in bridges. Following fascin rearward movement, new regions of GFP-fascin enrichment appeared along the shaft distally, but not necessarily at the tip of protrusions, suggesting propagation of the bundle in distal direction. In contrast to continuous retrograde flow at a fairly constant rate of 2.0 ± 0.4 μm/min, fascin arrival to bridges usually occurred in bursts, when the region of fascin enrichment suddenly expanded in the distal direction by 0.6 ± 0.4 mm (N=10 bursts, 6 bridges) within one frame of the movie (10 sec) with an
average interval of 4.3 ± 1.0 min between bursts. In 4 out of 10 bridges GFP-fascin displayed only retrograde flow without bursts of distal expansion.

To investigate the temporal localization of VASP during bridge formation, GFP-VASP transfected primary HUVECs were analyzed with live cell imaging. In overlapping lamellipodia and in early bridges associated with mini-lamellipodia, we observed the enrichment of GFP-VASP at the leading edge of lamellipodia and mini-lamellipodia (Figure 3-11C upper bridge, 0:00 time point, Movie S4). During lamellipodia retraction and bridge formation, GFP-VASP began to condense to distinct puncta or streaks in the forming bridge (Figure 3-11C, upper bridge, 4:16 – 7:31). Weak dot-shaped puncta could be found at the tips of filopodial-like protrusions in bridges, suggesting that they were homologous to VASP at tips of conventional filopodia. In contrast, streaks were more commonly located along the length of the bridge slightly away from the tip, suggesting that they might correspond to the junctional pool of VASP. In mature bridges, dot-shaped tip puncta of GFP-VASP were usually not detectable, whereas streaks persisted over longer time, suggesting that early filopodia-like bridges might acquire characteristics of junction-associated stress fibers during their maturation.
Figure 3-11: Dynamics of Filopodial Markers in Bridges

(A,B) Time-lapse fluorescence microscopy of EGFP-fascin dynamics in nascent (A) and established (B) bridges shown as EGFP fluorescence (upper right) and fluorescence-phase overlay (lower right). Colored arrows in A mark individual protrusions. Arrow in B points to a retrogradely moving feature within the bridge. Overviews of the fields are shown in left panels.

(C) Time lapse fluorescence microscopy of EGFP-VASP dynamics in nascent bridges shown as EGFP fluorescence (upper right) and fluorescence-phase overlay (lower right). Overview of the field is shown in left panel. Time in min:sec. Scale bars: 10 µm.
3.6 Bridge formation occurs via a novel lamellipodia-to-filopodia transition

Characterization of cell-cell contacts utilizing TEM reveals the actin cytoskeleton architecture in detail, but does not yield information regarding the temporal sequence in which the initial lamellipodia-like configuration of the cytoskeleton in the protruding edge transforms into filopodia-like bundles in bridges. Therefore, we performed correlative TEM to link cell behavior to high-resolution actin cytoskeletal architecture in HUVECs. The goal of this technique is to correlate the dynamics of cell motile behavior through low-resolution light microscopy with high-resolution structural analysis utilizing electron microscopy of the same cell. Specifically for our experiment, following phase contrast live-cell imaging of cell-cell contact establishment, the same cells were permeabilized with detergent, fixed, and processed for TEM analysis.

In the example shown in Figure 12, live cell imaging (Figure 3-12A, Movie S5) showed that lamellipodia of two initially separated cells came into a contact starting from upper right corner of the frame (1:15 time point) and expanding toward lower left (1:15 – 4:15). After some interplay between two lamellipodia (4:15 – 5:30), retraction began (6:30) and also progressed from upper right to lower left corner of the frame generating three bridges, among which the top bridge is the oldest, followed by the middle bridge and the lowest bridge is the youngest. Two lower bridges maintained a mini-lamellipodia at the tip by the time of extraction (7:15), whereas the top bridge did not. Following cell extraction and fixation, TEM investigation of the same region (Figure 3-12B) showed that the mini-lamellipodia at the tips of two younger bridges consisted of a branched dendritic network similar to conventional lamellipodia, whereas the bridge shafts contained tight actin filament bundles with seamless transition between two cytoskeletal
arrangements (Figure 3-12C-E). The oldest bridge in this region contained a typical filopodial bundle without a mini-lamellipodium at the tip (Figure 3-12F). Together, our data from correlative light and TEM showed that bridge formation occurred via collapsing of the lamellipodial actin network into a tight actin bundle starting proximally in the bridge shaft and propagating toward the tip along the entire bridge shaft to form mature bridges containing typical filopodial bundles.
Figure 3-12: Reorganization of the Actin Cytoskeleton during Bridge formation revealed by Correlative light and TEM of Bridge Formation.

(A) Phase contrast time-lapse sequence showing following events during formation of nascent bridges: protruding lamellipodia (0:00), initiation of a contact (1:15), expansion of the contact and interplay of lamellipodia (1:15-4:15), beginning of cell edge retraction (5:30), and formation of nascent bridges at the time of extraction and fixation (6:30-7:15). Time in min:sec.

(B-F) Platinum replica TEM of the same region. (B) Low magnification image colorized to demarcate cell-cell boundary. Boxes indicate regions magnified in C, D, and F. Actin network in mini-lamellipodia at the tips of bridges (C,D) gradually transforms into proximally located bundles. Boxed region in D is further magnified in E to show the structure of the transition zone. Mature bridge (F) contains a tight actin bundle without mini-lamellipodia. Scale bars: 2 \( \mu \text{m} \) (A,B), 1 \( \mu \text{m} \) (C), 500 nm (D, F) and 200 nm (E).
3.7 Nonmuscle myosin II gradually invades mature bridges

The formation of bridges concomitantly with the cell edge retraction suggests that cellular contractile forces may contribute to early stages of the process, whereas the distributions of open barbed ends and VASP in mature bridges suggests that bridges may eventually transform into stress fibers. Supporting the latter idea, it has recently been shown that in endothelial cells, actin stress fibers in adjacent cells are linked via adherens junctions (Millan et al., 2010).

Our structural TEM data also showed that bridges were sometimes continuous with stress fibers (Figure 3-14). Because of these considerations, we investigated the localization and dynamics of nonmuscle myosin II, an indicator of contractile bundles and networks in nonmuscle cells. Immunostaining of non-contacting, free edge lamellipodia revealed an absence of myosin II localization (Figure 3-15A). Furthermore, high-resolution TEM analysis of the HUVEC cytoskeleton following gelsolin treatment, which dissolves actin filaments while still preserving myosin II, showed myosin II bipolar filaments oriented parallel to the leading edge (Figure 3-15B). However in bridges, by immunostaining, though not observed in majority of bridges, there was an accumulation of Myo-II in a fraction of bridges, particularly at the bridge base (Figure 3-13A). Gelsolin treatment and EM investigation showed that when Myo-II filaments were present in bridges they were aligned with the bridge axis (Figure 3-13B-D). To follow the time course of Myo-II appearance in bridges, we transfected HUVECs with GFP tagged myosin light chain (MLC), a subunit of the hexameric Myo-II molecule. GFP-MLC localized to stress fibers in a punctate pattern and formed individual puncta in cell lamellae, but was absent from lamellipodia.
We have previously shown that Myo-II puncta in lamella correspond to clusters of
Myo-II bipolar filaments (Svitkina et al., 1997; Svitkina and Borisy, 1999b). Here we
found that in both non-contacting cells and in contacting cells during lamellipodial
interplay or beginning of retraction, GFP-MLC puncta in lamellae were found at a
significant distance from the leading edge. At later time points, Myo-II puncta began to
accumulate at the bridge bases and gradually invaded the shaft of the bridge, but not
extended all the way to the tip. In this process, new GFP-MLC puncta appeared distally
relative to pre-existing puncta, suggesting Myo-II accumulation occurred through de
novo assembly of Myo-II filaments in bridges similar to the analogous process in cell
lamellae (Svitkina et al., 1997; Svitkina and Borisy, 1999b). Subsequently, the entire
array underwent retrograde flow freeing space for new Myo-II puncta (Figure 3-13E and
3-13F, Movie S6). The rate of myosin II retrograde flow (1.7 ± 0.4 µm/min; N=12) was
not significantly different from the rate of retrograde flow measured in GFP-fascin-
expressing cells. Ensuing bridge maturation was characterized by Myo-II augmentation
at the base and along the shaft, supposedly as the bridge transitions from filopodia-like
structure to extension of a stress fiber.
**Figure 3-13: Myosin II Incorporates into the Shaft of Mature Bridges**

(A) Immunofluorescence staining of nonmuscle myosin II (red) and phalloidin staining of F-actin (green) demonstrates presence of myosin II in some bridge bases, but not in the bridge shaft.

(B-D) TEM of bridges following actin filament removal by gelsolin to expose myosin II filaments. Boxed region in B is magnified in C; boxed region in C is further magnified in D to show a stack of myosin II bipolar filaments oriented along the bridge axis at the base of a major bridge. Myosin II is increasingly absent toward the bridge tip.

(E and F) Time-lapse sequence of EGFP-MLC dynamics in intercellular bridges. Boxed region from E is shown at greater spatial and temporal resolution in F. Bright puncta of EGFP-MLC are present in proximal regions of bridges (E); they undergo retrograde flow (arrows in F), while new EGFP-MLC puncta appear distally. Time in min:sec. Scale bars: 10 μm (A,E), 2 μm (B), 500 nm (C), 100 nm (D).
Figure 3-14: Bridges May Be Continuous with Stress Fibers Running the Length of the Cell

*(A and B)* TEM of contacting cells reveal bridges as a continuation of stress fibers. Scale bar: 2 μm (A) and 1 μm (B).
Figure 3-15: Myosin II Localization In Non-Contacting Cells

(A and B) Myosin II incorporates into stress fibers running the length of the cell, but not at free edge lamellipodia under light microscopy (A) and platinum replica electron microscopy (B) investigation.
3.8 Myosin II activity is essential for bridge formation and VE-cadherin accumulation

Contractile cellular processes are typically driven by myosin II, suggesting a myosin II dependent mechanism of bridge formation in the course of cell edge retraction. To test this possibility, we examined the effect of blebbistatin, a nonmuscle myosin II inhibitor, on bridge formation. In the control treatment by an inactive (+)-blebbistatin, the lamellipodia to bridge transition was the predominant event of initial cell-cell encounter (Figure 3-16A, Movie S7), similar to untreated cells (Figure 3-1B). However, when myosin II was inhibited by active (-)-blebbistatin treatment, the frequency of bridge formation was decreased, whereas contacting cells exhibiting sustained lamellipodia interplay with no cellular retraction was the predominant result of initial cell-cell contact (Figure 3-16A). When bridges did form following collision events in (-)-blebbistatin-treated cells, cells either resumed protrusion or broke the bridge-mediated cell-cell contact entirely with frequencies similar to those in control samples (Figure 3-16A and Figure 3-1B).

Previously studies have shown the recruitment of VE-cadherin to established adherens junctions to be stimulated by myosin II dependent tension force (Liu et al, 2010). To characterize the role of myosin II in VE-cadherin localization during early stages of cell-cell junction formation, immunostaining of VE-cadherin was performed following blebbistatin treatment. VE-cadherin recruitment to cell-cell contacts was dramatically decreased as a result of myosin II inhibition, but not entirely abolished (Figure 3-16B). Quantification of the intensity of VE-cadherin immunostaining demonstrated a drastic decrease of the total amount of VE-cadherin per bridge after blebbistatin treatment (Figure 3-16C), whereas the average intensity of VE-cadherin per
junction area was only slightly diminished (Figure 3-16D). These results indicate that myosin II stimulates recruitment of VE-cadherin to nascent junctions, primarily by increasing the size of adherens junctions with a smaller effect on VE-cadherin density within the junction.
Figure 3-16: Myosin II Inhibition Results in Reduced Bridge Formation and VE-Cadherin Accretion

(A) Quantification of cell-cell collision events following treatment with active (-)-blebbistatin ("Blebbistatin", n=40) or inactive (+)-blebbistatin ("Control", n=28). Bars show the number of lamellipodia-initiated cell-cell contacts leading to bridge formation or not. Grey, transient contacts are broken within 10 min after initiation. Black, stable contacts are defined as greater than 10 min abutting the adjacent cell.

(B) Immunofluorescence of VE-cadherin (red) and phalloidin staining of F-actin (green) following 30 minute treatment with control inactive (+)-blebbistatin (left) or active (-)-blebbistatin (right) reveal reduced size of VE-cadherin puncta following myosin II inhibition. Insets are magnified with equal ratios relative to boxed regions in original image. Scale bar: 10 μm

(C) Quantification of VE-cadherin staining integrated intensity in bridges. Data are represented as mean ± SEM, n=42 for active (-)-blebbistatin; n=31 for inactive (+)-blebbistatin. **p<0.001, as determined by a two sample t-test.

(D) Quantification of VE-cadherin staining average intensity in individual puncta. Data are represented as mean ± SEM, n=42 for active (-)-blebbistatin; n=31 for inactive (+)-blebbistatin. *p<0.05 as determined by a two sample t-test.
CHAPTER 4: DISCUSSION

In the present study, we show that HUVECs establish initial cell-cell junctions via a previously unknown mechanism utilizing distinct actin-based structures at different stages of the process. We characterized the molecular composition and high-resolution cytoskeletal architecture of these junctional structures and how they transition from one to the other.

Firstly, we have determined which protrusive organelles initiate cell-cell junction in endothelial cells. Previously, it has been observed that contacts between adjacent epithelial cells are initiated either by lamellipodia, as in MDCK and IAR-2 cells (Krendel and Bonder, 1999; Ehrlich et al., 2002), or by filopodia, as in keratinocytes (Vasioukhin et al., 2000), or asymmetrically by lamellipodia of one cell and stress fibers within another cell, as in CHO cell line (Brevier et al., 2008). In contrast, our data demonstrate that endothelial cells utilize both types of protrusive structures, but sequentially. Specifically, cell-cell interaction is initiated by lamellipodia of adjacent cells that meet during the protrusive part of their protrusion-retraction cycles, whereas subsequent retraction of lamellipodia leads to the formation of filopodial-like bridges at the points of contact. By applying blebbistatin treatment, we found that the retraction phase is a myosin II-dependent event and that it is required for bridge formation. Incomplete inhibition of cell edge retraction and bridge formation may reflect partial inhibition of myosin II in these conditions. The contractile network of actin and myosin II filaments in the cell lamella is likely responsible for the cell edge retraction and subsequent bridge formation.
We confirmed the identity of bridges as filopodia-like structures based on their cytoskeletal architecture and the presence of conventional filopodial markers, fascin and VASP. The structural and molecular features of bridges and their formation in the course of cell retraction makes bridges similar to substrate-attached retraction fibers, which are also filopodia-related structures (Cramer and Mitchison, 1995; Svitkina et al., 2003). Although usually perceived as a byproduct of cell retraction, retraction fibers may in fact represent functional cellular organelles probing the substrate stiffness and adhesion strength, as required for proper cell and tissue morphogenesis (Janmey and Miller, 2011). Filopodia-like bridges may function in this capacity during adherens junction formation. In cells exiting mitosis, retraction fibers have been shown to facilitate cell spreading by guiding the extending lamellipodia toward pre-existing adhesions (Cramer and Mitchison, 1993; Thery and Bornens, 2006). The re-spreading of mitotic cells is very similar to the resumed lamellipodial protrusion along inter-cellular bridges toward nascent bridge junctions, suggesting functional similarity between bridges and retraction fibers.

The distinct roles of lamellipodia and filopodia in junction initiation revealed here do not necessarily contradict earlier observations (Krendel and Bonder, 1999; Vasioukhin et al., 2000; Ehrlich et al., 2002; Harris and Nelson, 2010), but may rather reflect differences in the mode of junction initiation in endothelial versus epithelial cells. These differences, in fact, seem to extend beyond the junction initiation phase. Indeed, the initial junctional complexes in epithelial cells appear to persistently expand or zipper into a continuous linear junction (Harris and Nelson, 2010), whereas endothelial cells undergo multiple rounds of bridge formation followed by resumed lamellipodial protrusion and
bridge formation again (Millan et al., 2010). Although endothelial cells may eventually establish cohesive cellular sheets, HUVECs did not reach this stage within the time frame of our experiments. The differences in dynamic behavior of contacting endothelial and epithelial cells may have functional consequences for relevant tissues. While both types of cells line the tissue surfaces and therefore should form cohesive sheets to resist mechanical challenges and maintain tissue integrity, endothelial sheets also must remain dynamic to permit solute exchange and especially paracellular transmigration of leukocytes.

In response to a leukocyte engagement, the adhesion molecules VCAM-1 and ICAM-1 are enriched at the endothelial cell border. Following leukocyte induced VCAM-1/ICAM-1 molecule clustering, adherens junctions diminish in strength (Muller, 2009b). Among a range of VCAM-1/ICAM-1 signaling effects, the weakening at least partially occurs via VCAM-1 signaling resulting in Rac1 stimulation, a known activator of lamellipodia formation, as well as adherens junction disassembly via ICAM-1 induced phosphorylation of VE-cadherin (van Wetering et al., 2003). While still mechanistically controversial as to whether paracellular or transcellular migration is utilized, leukocytes may exploit the gaps transiently provided as a result of promoting intercellular lamellipodia protrusion/retraction. Thus, following lamellipodia retraction, cells still maintain contact via bridge formation for resolution of a stable cell-cell junction, thereby functionally opening and closing the gate for passage. Besides spontaneous activity, the junctions may open in vivo in a regulated manner in response to a leukocyte engagement (van Wetering et al., 2002; Turowski et al., 2008; Muller, 2009a).
Secondly, we have discovered a novel mechanism of lamellipodia-to-filopodia transformation during bridge formation. Two previous models of filopodia formation were formulated for filopodia emerging from the free leading edge of migrating cells (Yang and Svitkina, 2011). According to the convergent elongation model, free edge filopodia are initiated from lamellipodia through coalescence of processively elongating barbed ends of a subset of lamellipodial filaments leading to convergence of these filaments that are subsequently bundled by fascin (Svitkina et al., 2003). Nascent filopodia formed by this mechanism are nested within the lamellipodial network at their roots, while the actin bundle appears to form in a tip-to-base direction. Another reasonable, but not yet proven model suggests that a cluster of membrane-associated formins nucleates a set of autonomous filopodial filaments unconnected to lamellipodia and subsequently maintains their elongation (Steffen et al., 2006; Faix et al., 2009).

In contrast to both models, filopodia-like junctional bridges form via the collapse of the lamellipodial actin network into filopodial bundles starting at the base of the lamellipodium and followed by the elongation of bundling towards the distal tip of the protrusion. Accordingly, accumulation of fascin in bridge bundles progresses not in the tip-to-base direction, as in free edge filopodia (Svitkina et al., 2003), but in a shaft-to-tip fashion. Since fascin is believed to be recruited to preformed parallel bundles (Brill-Karniely et al., 2009; Courson and Rock, 2010), the mode of fascin accumulation in bridges supports an idea of bundle assembly in a proximal-to-distal direction. Interestingly, fascin arrival to bridges occurs in discontinuous bursts, suggesting a similar mode of bundle formation. Mini-lamellipodia persist at the bridge tips as intermediates,
but disappear as the bridge matures into a tight actin bundle with structural and molecular features of leading edge filopodia.

The exact origin of long filaments forming the bridge bundle upon lamellipodial collapse remains unclear. However, tight correlation between their appearance and cell retraction suggests force-dependent mechanism(s) of actin cytoskeleton reorganization. In fact, similar mechanisms may also function during formation of retraction fibers, filopodia-related structures attached to extracellular matrix and developing at the free cell edge concomitantly with edge retraction (Cramer and Mitchison, 1997; Svitkina et al., 2003).

One possibility is that the stretch applied to the lamellipodium during retraction promotes debranching and subsequent annealing of short lamellipodial filaments (Skau et al., 2009; Okreglak and Drubin, 2010), which would simultaneously cause loss of lamellipodia and formation of long filaments, as we observed. Classical experiments in actin filament dynamics showed the ability of short actin filaments to anneal end-to-end in vitro, leading to an increase in filament length (Murphy et al., 1988). Furthermore, modeling simulations predict a necessary annealing mechanism to account for rapid lamellipodia protrusion (Sept et al., 1999; Fass et al., 2004). It has been suggested that Arp2/3 detachment of short daughter filaments in lamellipodia uncaps pointed ends, which can lead to annealing of the free pointed end with a growing barbed end (Fass et al., 2008).

Further considering the origin of long filaments after lamellipodia collapse, rather than annealing, another non-exclusive possibility is that a subset of long actin filaments pre-existing in the lamellipodial network undergoes fast force-assisted elongation, as
proposed for formin-associated actin filaments (Kozlov and Bershadsky, 2004), but may also apply to VASP. For this mechanism to work, the elongation factor, formin or VASP, should be strongly attached, for example at the adherens junction, which would also target barbed end elongation to adhesion sites. Consistent with this idea, open barbed ends in bridges are arranged in the same pattern as VE-cadherin clusters and as VASP streaks formed concomitantly with VASP departure from the lamellipodial leading edge. Filament elongation may also be triggered by more complex signaling events from force-induced VE-cadherin clustering to actin remodeling proteins involved in lamellipodia-to-filopodia reorganization, such as fascin, VASP, formins. Regarding cell-cell junctions, this regulation may also affect myosin-X, a motor protein enriched at filopodial tips (Berg and Cheney, 2002) and responsible for delivery of VE-cadherin to points of endothelial cell-cell contact (Almagro et al., 2010).

Finally, we have found that the filopodia-like organization of bridges is a transient state, after which bridges mature into stress-fiber-like structures by incorporating myosin II filaments, similar to the previous suggestion that free filopodia act as seeds by which stress fibers are initiated. Though bridges utilize fascin as an actin bundler of parallel filaments, the incorporation of myosin, which typically bundles anti-parallel actin filaments, into bridges suggests active displacement of fascin during the transition of bridge into stress fiber. Indeed, several biochemical in vitro studies have shown NMII to actively unbundle preformed fascin-actin-bundles in a myosin concentration dependent manner (Ishikawa et al., 2003; Haviv et al., 2008; Norstrom et al., 2010). The mechanism by which NMII dependent fascin-actin unbundling occurs is unknown, though may be answered by virtue of steric hindrance. Fascin can bundle actin filaments
very tightly, between 4 – 8 nm in distance between actin filaments, whereas
minifilaments of myosin II are simply too large to coexist with fascin. Furthermore, the
forces produced by the myosin motors may break the bonds between fascin and the actin
filaments functionally separating the filaments in the actin bundle.

Whereas newly formed bridges are devoid of myosin II accumulations, discrete
myosin II spots begin to appear in sustained bridges. The mode of myosin II arrival to
bridges also closely mimics the pattern of myosin II assembly in lamellae (Verkhovsky et
al., 1995; Svitkina et al., 1997). In both cases, new myosin II spots are first formed at a
distance from the leading edge but in front of pre-existing myosin II structures, then
undergo retrograde flow, and eventually coalesce with pre-existing myosin II
accumulations to form actin-myosin II bundles. Subsequently, the mature myosin II-
positive bridges likely give rise to fully formed stress fibers associating with adherens
junctions in confluent endothelial cells (Millan et al., 2010). Interestingly, a similar
filopodia-to-stress fiber progression with concurrent accumulation of myosin II has been
reported for free filopodia in migrating cells (Anderson et al., 2008; Nemethova et al.,
2008).

Whereas myosin II has roles in both the construction and deconstruction of F-
actin structures (Medeiros et al., 2006; Haviv et al., 2008; Wilson et al., 2010), with
respect to cell-matrix adhesions (Chrzanowska-Wodnicka and Burridge, 1996;
Bershadsky et al., 2006) or cell-cell junctions (Liu et al., 2010), it is generally thought to
mediate force-dependent expansion and stabilization of adhesions. Myosin II also plays a
role during initiation of cell-substrate adhesions, although its motor activity is not
absolutely required at this stage and the cross-linking activity is sufficient (Choi et al.,
2008). In analogy with these data, we have found that myosin II also functions during initiation of cell-cell junctions in the course of bridge formation, although the relative contributions of motor and cross-linking activities of myosin II to this process remain to be determined. We suppose that initial cell retraction driven by the contractile actin-myosin II network in the lamella may provide force to induce nascent adherens junctions. When myosin II filaments are subsequently assembled within bridges, they generate greater local force applied to these nascent junctions leading to junction strengthening and growth.

One possible explanation of stimulated assembly of myosin II filaments in bridges is the result of increased tension there. Indeed, it has been shown that myosin II preferentially accumulates at sites of artificially applied mechanical strain (Ren et al., 2009b) Consistent with this hypothesis, recent work showed cadherin, specifically E-cadherin, acts as a mechanosensor that can transmit force between the extracellular environment and the actin and myosin cytoskeletal network (le Duc et al., 2010). Additionally, in living drosophila embryos, increased mechanical tension was exclusively sufficient to promote myosin II localization to sites of cell-cell adhesion (Fernandez-Gonzalez et al., 2009). Therefore, myosin II not only produces tension in the actin network through its own intrinsic motor activity, but additionally can be recruited to sites of increased mechanical tension. Thus, our data suggests a positive feedback mechanism where the initial VE-cadherin clustering in response to weak retraction-mediated force generates local tension, which promotes further myosin accumulation subsequently inducing increased VE-cadherin clustering. Cadherins have been shown to aggregate
when membrane tension increases, although proposed to occur independent of myosin II activity (Delanoe-Ayari et al., 2004), however our data suggests otherwise.

Conventional nonmuscle myosin II consists of two isoforms, myosin IIA and myosin IIB (Vicente-Manzanares et al., 2009; Smutny et al., 2010). Our technique utilizing a fluorescent light chain incorporated equally well into both isoforms cannot distinguish between activity of IIA and IIB. However, recent observations have shown distinct functions of IIA and IIB at adherens junctions. It was shown that myosin IIA is required for cadherin clustering and concentration, whereas myosin IIB regulated filamentous actin stability and turnover at adherens junctions (Smutny et al., 2010). While IIA and IIB share many biochemical properties, one major difference is the measure of proportion of time spent bound to actin, known as their duty ratio. The duty ratio of IIB is more than three times that of IIA and is therefore more likely to stay linked with filamentous actin. Therefore, while myosin IIA is necessary for junction formation via its regulation of cadherin function, myosin IIB is more likely the specific motor necessary for tension produced at adherens junction.

Based on our data, we propose a model of cell-cell junction formation in endothelial cells (Figure 4-1), according to which cell-cell interaction is initiated via contact of protruding lamellipodia of adjacent cells. Following cell edge retraction, lamellipodia begin to collapse starting from the rear, which produces an hourglass-shaped bridge with a mini-lamellipodium at the tip. Whereas the mini-lamellipodium still contains a branched actin network typical for lamellipodia, the shaft of the bridge contains a filopodium-like actin bundle. At the next stage of the process, the continuing collapse of the lamellipodia network from the rear leads to elongation of the shaft bundle.
towards the bridge’s distal tip. Bundle formation is accompanied by progressive accumulation of fascin, redistribution of VASP from the lamellipodial leading edge toward the bundle tip, and incorporation of myosin II into the bridge base at the later stages of the process. We propose that there is a positive functional relationship between initial point contacts made by lamellipodia and the initiation of bridges at these sites, while induced bridges may strengthen the nascent junction and maintain the cells close to each other, which would increase the chances of junction expansion.

We speculate the activity of α-catenin to be vital in this process. Known to localize to nascent adherens junctions, when α-catenin is not complexed with β-catenin and at a high enough concentration, α-catenin can dimerize and function to bind to and bundle actin filaments. Therefore α-catenin may be responsible for the bundling of the branched actin network within mini-lamellipodia into tight actin bundles within bridges (Rimm et al., 1995). Furthermore, it has been reported that under tension at adherens junction, α-catenin can undergo a conformational change, thereby revealing a potential binding site for the protein vinculin, an F-actin binding protein that can recruit other actin modifying proteins, including Ena/VASP (Yonemura et al., 2010). I hypothesize α-catenin to be necessary for the bundling of the lamellipodial network into bridges.

In conclusion, our study gives new insight into the dynamics and architecture of the actin cytoskeleton during cell-cell junction formation in endothelial cells, which will help to more clearly understand how endothelial cells control permeability essential for nutrient flow during embryonic development as well as biological phenomena such as leukocyte transmigration.
Figure 4-1: Model of Cell-Cell Adhesion Formation in Endothelial Cells

Stage 1. Initial cell-cell collision is mediated by protruding lamellipodia containing a branched actin network and utilizing typical lamellipodial proteins including VASP at the leading edge.

Stage 2. Cell retraction promotes VE-cadherin clustering and adherens junction formation. Lamellipodia begin to collapse from the rear forming a nascent bridge. Actin filaments are bundled by fascin in the bridge shaft, while an active lamellipodial network remains at the distal tip in mini-lamellipodium.

Stage 3. Mini-lamellipodial actin network completely collapses. Fascin is progressively recruited to the distal region of the bridge and undergoes retrograde flow with the actin bundle. VASP redistributes from the edge of the mini-lamellipodium to the tip of the bridge bundle. Myosin II enters the bridge base.

Stage 4. Myosin II filaments continuously assemble within the bridge and undergo retrograde flow with the bridge bundles. Accumulating myosin II displaces fascin from the bridge and gradually transforms the bridge into a stress fiber.
CHAPTER 5: FUTURE DIRECTIONS

5.1 Future Direction Introduction

This work describes a novel mechanism of the initiating events in cell-cell junction formation utilizing two very distinct actin cytoskeletal structures. We have observed, in vitro, lamellipodia initiate cell-cell contact, followed by the subsequent generation of filopodia-like bridges utilizing a novel transitional architecture. Bridges employ the elongation factor VASP, the actin bundler fascin, and can incorporate myosin II. Inhibition of myosin II results in reduced bridge formation as well as reduced adherens junction size.

5.2 Adherens Junction Dependent Bridge Formation

Bridges generated following lamellipodia contact exhibit rich VE-cadherin staining. However, it is unclear whether the phenomenon of bridge formation is primarily the result of VE-cadherin-cadherin interaction. Considering the actin cytoskeleton is principally associated with adherens junctions with respect to cell-cell adhesion, it is reasonable to speculate that the lamellipodia to filopodia-like bridge transition is exclusively adherens junction dependent. To investigate this question, a number of techniques can be utilized. We propose to utilize the following two techniques to inhibit cadherin function: 1) monoclonal VE-cadherin function blocking antibody; and 2) knockdown of VE-cadherin utilizing siRNA.

In both in vitro and in vivo studies, VE-cadherin function was lost after application of a VE-cadherin-specific monoclonal antibody (Corada et al., 1999; Heupel et al., 2009). While two types of cadherin are expressed in HUVECs, neural (N-) and
vascular endothelial (VE-), with respect to endothelial cell-cell interaction VE-cadherin is primarily utilized (Dejana et al., 1995). Therefore functional blocking antibody application specifically disrupts VE-cadherin-VE-cadherin interaction, thereby inhibiting endothelial cell-cell adherens junction based formation. However, there is a potential that the endocytosis of VE-cadherin bound to monoclonal blocking antibody may result in too transient an effect. Therefore, alternatively, siRNA knockdown of VE-cadherin may be more beneficial for our purposes creating a longer window of VE-cadherin function inhibition. If indeed the lamellipodia to bridge transition is adherens junction, then the addition of VE-cadherin Ab in a semi-confluent culture or the knockdown of VE-cadherin should inhibit this transition.

To mimic cadherin interaction on single cells, we propose to utilize one of two current techniques to coat glass coverslips with specific areas of collagen or cadherin. The first technique, micropatterning, offers reproducibly rigid control over the spatial organization of various substrates on a range of surfaces. However, this powerful technique also requires specialized equipment and significant time for specific protocol optimization (Javaherian et al., 2011). For our purposes, we propose utilizing the more commonly used stripe assay where intermittent stripes of VE-cadherin are layered upon a collagen coated surface (Knoll et al., 2007). Therefore, when crawling on collagen, HUVECs should exhibit conventional cell migratory behavior, yet when VE-cadherin coated portions of the coverslips are encountered, cell engagement, signaling, and subsequent architectural rearrangements are cadherin dependent. We anticipate these cytoskeletal rearrangements to mimic our cell culture observations exhibiting lamellipodia extension and subsequent bridge formation.
5.3 Cotransfection of MyoII and Fascin

Our results suggest filopodia-like structures bundled by fascin to be a transient state, followed by stress fiber-like structure maturation incorporating myosin II. Although fascin and myosin II both participate in bridge formation, it is unclear at this point whether these actin bundlers can coexist within the same structure in vivo. Indeed, a similar phenomenon has been observed in stress fibers showing that α-actinin, an additional actin bundler within stress fibers, and myosin II localize in an alternating punctate fashion and therefore cannot occupy the same space along the fibers (Hotulainen and Lappalainen, 2006).

Considering previous in vitro studies have shown myosin II to actively disassemble preformed fascin-actin-bundles (Ishikawa et al., 2003; Haviv et al., 2008; Norstrom et al., 2010), we propose that in the context of bridge formation and maturation, myosin II localization leads to the displacement of fascin within bridges. Observation of live cells cotransfected with both fluorescent myosin II and fascin will directly address our hypothesis. We predict fascin and myosin to localize to the same long actin structure, but to exhibit little to no overlap and colocalization. Furthermore, we can utilize correlative EM to visualize protein dynamics and localization under light microscopy followed by processing for EM to examine the corresponding high-resolution actin cytoskeleton architecture. This, in combination with either S1 decoration or gelsolin treatment will yield information at sites of fascin and myosin localization regarding actin polarity and myosin bipolar filament architecture, as well as the cell behavior leading to the generation of these structures. Information relating the actin polarity and myosin architecture correlating to areas of fascin and myosin localization
will grant further insight into both fascin and myosin protein activity. Moreover, this information will also give new consideration into how leading edge characteristics, specifically filopodia and bridges, associate with structures often considered independent of leading edge dynamics, specifically stress fibers.

5.4 Examine the effect of shear stress of myosin II accumulation in cell-cell junctions

Recent studies have suggested myosin II recruitment to be tension dependent (Fernandez-Gonzalez et al., 2009; Ren et al., 2009b). Given that following cell retraction and subsequent bridge formation, we observed myosin II accumulation, we propose to examine high-resolution myosin II architecture in the context of cell-cell junction formation under various degrees of artificially induced stress. In vivo, endothelial cells are constantly exposed to a wide range of biological forces including fluid shear stress, which can influence endothelial cell morphology and function. Under artificially induced shear stress, endothelial cells have been observed to elongate and change their shape by the reorganization of the actin cytoskeleton in response to the direction, and furthermore degree, of shear stress (Tarbell, 2010). A number of in vitro systems have been utilized for this purpose of artificially applied shear stress, but most often, parallel flow chambers are used. We propose that increased shear stress will result in increased myosin II localization to sites of bridge formation under TEM analysis following gelsolin treatment. Furthermore, in the presence of blebbistatin, we propose myosin II localization to bridges will be dramatically reduced as inhibition of myosin II generated tension will impede further myosin II accumulation. Likely, VE-cadherin analysis will further reveal reduced junction formation under blebbistatin treatment.
Under the conditions of 48 hour shear stress application, Noria et al. observed adherens junction remodeling in endothelial cells and found that in the initial phase of shear stress application, VE-cadherin, α-, and β-catenin, localization was disrupted (8.5 hour)(Noria et al., 1999). However, at the 48-hour phase, they observed the recovery of all proteins to adherens junction suggesting that their disassembly and reassembly likely mimics adherens junction formation. Under such conditions, we anticipate the de novo assembly of adherens junctions will likely replicate our observation of early cell-cell junction formation utilizing the lamellipodia-to-bridge transition. Making use of our specialized technique of platinum replica TEM, we can analyze at high-resolution the architecture of the actin cytoskeleton in relation to increased stress at various times during the remodeling and formation of adherens junctions. Furthermore, following gelsolin treatment, myosin II structure can also be analyzed utilizing this technique. We propose a positive relationship between increased stress and myosin II accumulation to sites of bridge formation and mature stress fibers.

5.5 Examine effects of Ena/VASP on actin architecture at later stages of cell-cell junction formation

In addition to our proposal that Ena/VASP is utilized in establishing nascent cell-cell contacts, evidence suggests that Ena/VASP stabilizes established endothelial cell-cell adhesion resulting in a decrease in permeability (Furman et al., 2007). It is speculated that loss of Ena/VASP results in chronic relaxation of force exerted by the actin cytoskeleton on stable cell-cell contacts. However, the specific architecture of the actin cytoskeleton at stable cell-cell contacts is yet to be elucidated. Indeed, our initial results
indicate a very different actin architecture at stable cell-cell contacts. Rather than the observed lamellipodia-to-bridge transition utilized in early cell-cell junction formation we uncovered, at later stages, adjacent HUVECs exhibit thick actin bundles running parallel (Figure 5-1).
Figure 5-1: F-actin Bundles in Quiescent Contacting HUVECs
(A) High resolution investigation reveals actin architecture. Boxed region is magnified. Scale bar: 200 µm
To determine the role of Ena/VASP in maintaining established endothelial cell-cell contact, we propose to investigate utilizing the following techniques: 1) transfect HUVECs with a GFP-fusion Ena/VASP protein to examine cellular location in established cell-cell contacts; 2) perform EM to determine the cytoskeletal structure at stable cell-cell interfaces; 3) perform immunoEM staining of Ena/VASP to determine high-resolution location in stable contacts; and 4) generate and express a GFP-FP4-Mito construct to regulate Ena/VASP function followed by cytoskeletal structural analysis.

Previous studies investigating the role of Ena/VASP have taken advantage of a GFP-fusion to FP4, a ligand specific for binding to the EVH1 domain of Ena/VASP. This fusion protein is tagged with a mitochondrial targeting sequence, thereby sequestering Ena/VASP proteins to the mitochondrial surface (Bear et al., 2000; Bear et al., 2002). When generated, the effect of Ena/VASP ablation specifically in the context of cell-cell contact maintenance can be ascertained. To control for Ena/VASP independent effects upon expression of GFP-FP4-Mito, the phenylalanine of FP4 necessary for EVH1 binding will be mutated to alanine (AP4-mito) to inhibit Ena/VASP interaction. To establish the effects of targeting Ena/VASP to the membrane, a GFP-FP4-CAAX construct may also be utilized, with a AP4-CAAX construct as control.

As previously stated, Ena/VASP is suggested to function as an actin anti-capping and anti-branching factor. We propose that in the presence of GFP-FP4-Mito, the actin cytoskeleton at the cell-cell interface will no longer form parallel F-actin bundles as seen through TEM analysis. The functional consequences of Ena/VASP modulation of the actin cytoskeleton at stable cell-cell contacts can be examined through a number of permeability assays. In HUVECs, cell permeability is most often observed measuring the
passage of a membrane impermeant molecule between two compartments, separated by an endothelial cell monolayer. Therefore, in our case, cell permeability can be examined under the aforementioned conditions of Ena/VASP modulation utilizing a fluorescently tagged dextran molecule.
Bibliography


