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Single-Cell Imaging of Mechanotransduction in Endothelial Cells

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Abstract

Endothelial cells (ECs) are constantly exposed to chemical and mechanical microenvironment in vivo. In mechanotransduction, cells can sense and translate the extracellular mechanical cues into intracellular biochemical signals, to regulate cellular processes. This regulation is crucial for many physiological functions, such as cell adhesion, migration, proliferation, and survival, as well as the progression of disease such as atherosclerosis. Here, we overview the current molecular understanding of mechanotransduction in ECs associated with atherosclerosis, especially those in response to physiological shear stress. The enabling technology of live-cell imaging has allowed the study of spatiotemporal molecular events and unprecedented understanding of intracellular signaling responses in mechanotransduction. Hence, we also introduce recent studies on mechanotransduction using single-cell imaging technologies.

1. INTRODUCTION

Mechanical forces play crucial roles in regulating pathophysiological processes, e.g., atherosclerosis, the leading cause of death in the USA and most developed countries.[1] Shear stress without a clear direction can lead to endothelial cell (EC) dysfunction and atherogenesis,[2–4] but there is a lack of understanding on how ECs perceive the spatiotemporal cues and trans-duce them into biochemical activities to regulate cellular functions. The underlying pathology of atherosclerosis involves a chronic inflammatory process of vessel wall[5] due to endothelial dysfunction with increased permeability and recruitment of immune cells, including monocytes, resulting from upregulation of adhesion molecules and cytokine secretion by ECs.[5] Atherosclerosis occurs preferentially at vascular curvature and branch sites where the vessel walls and ECs are exposed to disturbed flow, which has been reported to facilitate atherogenesis.[6–8] It is possible that the greater spatiotemporal heterogeneity of shear-stress distribution under atheroprone disturbed flows than that of atheroprotective laminar flows contributes to the pathophysiological modulation of EC responses in the subsequent mechanotransduction.[2,9–15]

Since the plasma membrane provides an interface between the cell and environment, it is expected to be an important subcellular structure in mechanotransduction. Transmembrane receptors such as integrins, G-protein-coupled receptor (GPCR), platelet endothelial cell adhesion molecule (PECAM-1), and transient receptor potential channels (TRPC6) can be activated by shear to regulate downstream signals, i.e., membrane-associated tyrosine kinases, Ca^{2+}, and small GTPases.[16–18] Shear stress has been shown to activate Src, which leads to ERK activation and translocation between nucleus and cytosol, and subsequent

atheroprone genetic changes.[19–21] TRPC6 can also sense shear and trigger Ca^{2+} influx to regulate EC permeability.[22,23] The plasma membrane also consists of different microdomains,[24] including lipid rafts,[25] which play important roles for mechanotransduction.[26] Various mechanosensing elements can localize at or proximal to the plasma membrane to regulate downstream intracellular functions.[27]

The wide applications of fluorescence proteins (FPs) in single live-cell imaging have revolutionized the whole research field of cell biology, including mechanotransduction in ECs. Fluorescence resonance energy transfer (FRET)-based biosensors have been engineered and applied to molecular activities in live cells.[28–37] The ratiometric measurement of the biosensors utilizes the ratio of the donor to acceptor fluorescence intensity to represent the target molecular activity. This measurement is self-normalizing and independent of the heterogeneous biosensor expression level among various cells.[38] The genetically engineered FRET biosensors also allow subcellular localization to cytosol, plasma membrane, or organelles, which can provide versatile measurement of local molecular activities. As a result, the FRET-based biosensors have been widely used in live cells to monitor molecular signals in real time.[39,40]

Utilizing fluorescence imaging technologies including FPs and FRET biosensors, a large amount of video imaging data of live cells have been collected. In order to precisely and efficiently interpret the underlying biological mechanism, automated, intelligent, and objective image analysis tools are in high demands.[39] Automatic methods for the accurate detection of cell and subcellular features are crucial for the high-throughput image analysis and quantification of subcellular molecular interactions. For example, the water algorithm has been widely used to detect FAs in fluorescent images.[41–45] Quantitative image-based analysis can also reveal the hidden spatial pattern and temporal sequence of signaling events.[46,47] Integrated computational tools are needed to quantify the activity and localization of intracellular molecules, which can provide the basis for the quantitative analysis elucidating subcellular molecular interactions and therefore contribution to EC migration and atherosclerosis.[39,47–49]

2. ATHEROSCLEROSIS, EC WOUND HEALING, AND MECHANOTRANSDUCTION

Atherosclerosis is a cardiovascular disease characterized by the patchy deposit of fatty materials in the arterial walls and reduced/blocked blood flows.[50] It occurs preferentially at vascular curvature and branch sites where the vessel walls are exposed to disturbed flows, but not at the straight parts of vessels where laminar flows dominate.[50] Vascular ECs, which form a mono-layer of endothelium lining along and protecting the vessel wall from the circulating blood,[51] are continuously exposed to shear stress resulted from these different flows. It has been shown that ECs subjected to disturbed flows, but not to laminar flows, tend to have a high and sustained permeability, which facilitates the formation of atherosclerosis.[52,53] Recent evidence also indicates that the effect of disturbed flows on ECs is pro-inflammatory, whereas that of laminar flows is anti-inflammatory.[50,54–57] For example, disturbed flows induced the expression of pro-inflammatory BMP-4 and cytokines

and adhesion receptors such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1).[58–60] In contrast, laminar flows can inhibit the inflammatory signaling cascades induced by TNFα.[61–63]

Following the atherosclerosis, the injury of endothelium and loss of ECs after bypass surgery or balloon angioplasty is the main cause of restenosis. The EC wound healing process, which involves EC migration at the wounding edge followed by EC proliferation, serves as the critical step to restore the endothelium integrity and prevent restenosis.[64] Shear stress has been shown to affect the EC wound healing process by modulating EC junctions. EC junctions in a monolayer can be disrupted by shear stress and recovered if continuously exposed to laminar flows for long term as the cells adapt to the new environment.[52,53] The transient disruption of cell junctions under laminar flows will last long during the wound healing process because it takes time for cells to repair the wound, restore a stable monolayer, and adapt to the new environment. This junction disruption would hence keep the ECs disengaged from each other and free to move. As such, the laminar flow-directed protrusion and migration of ECs at the upstream side of wounded area can be promoted and maintained before the wound closure, thus facilitating the EC migration toward the wounded area. At the downstream side, the pushing effect of laminar flows on cells along the flow direction negates the junction disruption-induced motility enhancement. Although a wound per se can also induce a migration of cells at both wounding edges to close the wound, results have revealed that laminar flows promote cells at the upstream side to migrate into the wounded area faster, whereas the rate of cell migration downstream of flow is comparable to that without flow.[65] This asymmetric effect of laminar flows results in an increased net speed of wound healing comparing to that without flow. In fact, both in vitro and in vivo experiments indicate that laminar flows enhance EC migration and consequently wound healing.[66–68] Laminar flows have been shown to enhance the wound healing by modulating cell–cell and cell–extracellular matrix (ECM) adhesions, in particular, vein endothelial (VE)-cadherin-mediated adherens junction (AJ)[69,70] and β1 integrins.[71] Membrane fluidity, cytoskeleton, and tyrosine kinases also appear to be important for the laminar flow-induced EC wound healing.[65,72,73] Interestingly, disturbed flows induce sustained disruption of AJ.[52,53] However, the EC migration speed toward the wounded area under disturbed flows is comparable to that without flow.[65] It was revealed that ECs under disturbed flows have strong staining of focal adhesion proteins due to the unstable mechanical environment,[65] which may impair the cell detachment from the substrate necessary for EC migration and hence negate the motility enhancement due to the junction disruption.[74] It remains an intriguing question how ECs coordinate the multiple signaling events in space and time under different flows to regulate AJ, protrusion, motility, migration, and wound healing.

It has been well documented that shear stress can activate a variety of signaling cascades and gene expressions to regulate EC functions and pathophysiological processes.[7,8,75] For example, a wide range of signaling molecules and structures, including the plasma membrane,[76,77] membrane proteins/receptors (e.g., integrins,[16,19] GPCR,[17] cadherin,[18,53] PECAM-1,[18,78,79] VCAM-1,[80,81] ICAM-1,[80] and ion channels[75]), actin filaments,[82] microtubules,[83,84] and intermediate filaments[85–87] were identified to play important roles in transmitting shear stress into biochemical signaling cascades, i.e., mechanotransduction. Atheroprone disturbed fluid shear stress has been reported to induce the expression of

fibronectin (FN) gene in human ECs and enhance its assembly into fibril matrix.[88] In particular, the PECAM-1/NF-κB pathway was found to be essential for FN accumulation in atheroprone regions of the aortic arch, which positively feedbacks and promotes the activation of NF-κB and atherogenesis.[88] Evidence also indicates that both the temporal and spatial gradient of shear stress can affect the cellular functions.[2,10–12,14,15,76,89] In fact, it was realized that the subcellular characteristics of shear stress are heterogeneous in a single cell and impact significantly on cellular responses.[12,19,90,91] Calcium flux in response to shear stress showed significant subcellular directionality.[92,93] The lateral diffusion coefficient of lipids in the plasma membrane also increased at regions upstream of the nucleus while decreased at the downstream regions upon shear-stress application.[94]

3. SIGNALING MOLECULES INVOLVED IN MECHANOSENSING AND MECHANOTRANSDUCTION

TRPCs at the plasma membrane can regulate Ca^{2+} entry and play crucial roles in regulating EC actomyosin contractility and permeability[22,23]; increased TRPC6 expression has been revealed as a hallmark of atherosclerosis[95] and atheroprone flow shear stress has been shown to induce TRPC6 expression in ECs.[96] TRPC6 can also interact with caveolin,[97] which is localized at VE-cadherin-mediated AJs.[98] In fact, TRPC4, a close family member of TRPC6, has been shown to colocalize with VE-cadherin at AJs.[99] Therefore, the Ca^{2+} influx mediated by TRPC6 may affect the local [Ca^{2+}] in the intercellular junction, at least transiently, and modulate VE-cadherin functions to regulate EC permeability. While TRPC6 can mediate the effect of shear stress on triggering the Ca^{2+} influx under different stimuli,[100] it remains controversial whether TRPC6 can be directly activated by mechanical tension[101] or indirectly via GPCRs and their effector phospholipase C (PLC).[102,103] Since shear stress has been reported to stimulate GPCRs and their coupled G-proteins,[17,104,105] it is possible that shear stress utilizes GPCR/PLC pathway to activate TRPC6 besides imposing direct mechanical impact.

Src kinase is recruited to the plasma membrane and activated differentially at the different membrane microdomains under various conditions (Fig. 2.1).[34,106] Shear stress can activate Src[19,107,108] through plasma membrane mechanosomes containing PKG/Shp2.[109] This shear-induced Src can then lead to the phosphorylation and activation of ERK[21,110,111] via Shc/Grb2/SOS/Ras/MEK pathway.[109] The phosphorylated ERK can translocate into the nucleus[112] and undergo an oscillatory translocation between nucleus and cytosol[20] to regulate downstream gene expression of MCP-1 for monocyte recruitment.[113] It has been hypothesized that the spatiotemporal patterns of these oscillatory activities of transcription factors can regulate the strength and time course of the target gene expression.[20] Recent evidence indicates that molecular functions are dependent on its subcellular localization. For example, Src inhibits Rho GTPase at the focal adhesion sites,[114] whereas it activates Rho GTPase at podosomes.[115] Furthermore, junction remodeling takes place at a molecularly and phenotypically distinct subset of VE-cadherin adhesions, focal AJs, and vinculin associates with focal AJs and stabilizes the junctions from opening during their force-dependent remodeling.[116] Therefore, the different spatiotemporal characteristics of disturbed and laminar flows may cause differential TRPC6 activation and/or Src localization/function at

membrane microdomains to regulate AJs and MCP-1, which modulate endothelial permeability and monocyte recruitment, respectively.[5]

4. THE EFFECT OF SUBCELLULAR STRUCTURE ON MECHANOTRANSDUCTION

The plasma membrane is not uniform in structure[24] and has different compartments, e.g., lipid rafts, which are rich in cholesterol, sphingomyelin, and saturated fatty acids.[25] These compartment structures and their interaction with cytoskeleton are involved in the regulation of intracellular signaling.[26] Indeed, Src family kinase (SFK) members, including Fyn and Lyn, are anchored at lipid rafts to become activated following N-terminal palmitoylation and myristoylation,[117] whereas H-Ras depends on the non-rafts anchoring at the plasma membrane to be functional.[117,118] Evidence also indicates that the plasma membrane and its compartments are involved in mechanotransduction. For example, different subtypes of G-proteins are partitioned into membrane compartments to regulate the mechanical-activated signaling molecules, e.g., nitric oxide production[119] and MAPK activity.[120] The modulation of the plasma membrane fluidity also altered the shear stress-induced MAPK signaling pathway,[94] further underscoring the importance of the plasma membrane in mechanotransduction.

Src kinase is a nonreceptor tyrosine kinase critical for a variety of cellular processes.[114] At resting state, Src localizes at the microtubule-associated perinuclear regions[34,121–126] and/or at the nonrafts regions on the plasma membrane.[127–129] Recent evidence indicates that Rho small GTPases and the associated actin network can facilitate the transportation of Src from perinuclear area to actin-associated cell periphery,[130–132] possibly through the Src SH3 domain, but not the catalytic domain.[133,134] The inhibition of RhoA or actin stress fibers but not microtubules resulted in the blockage of Src translocation/activation in response to various stimuli.[130–132] The compartmental structures at the plasma membrane are also involved in the localization and regulation of Src kinase.[26,135] Indeed, SFKs can be trans ported to distinct compartments of plasma membrane through different types of endosomes.[136] SFK members such as Lyn and Fyn can reside in lipid rafts of the plasma membrane,[137] via their N-terminal myristoylation and palmitoylation sites.[118] Src kinase itself has only myristoylation motif and it is not clear whether Src kinase localizes within the lipid rafts at the plasma membrane.[127–129,138–140] In mouse fibroblasts, Src was shown to be excluded from the detergent-resistant membrane (DRM) fractions in one study, while another publication suggested that Src resides in DRM fraction.[127,140] Different groups also reported different Src localizations in PC12 cells.[129,139] This inconsistency is likely attributed to the controversial effects of nonionic detergents and the detergent extraction method used in these reports for isolating DRMs.[141,142] Advanced methods are hence needed to visualize the Src translocation and activation at different compartments on the plasma membrane in live cells.

Src contributes to cell protrusion and migration in many ways. Src, mediated by focal adhesion kinase (FAK), can phosphorylate p130cas, which recruits Crk and DOCK180 through the interaction of SH3 domain on Crk and PXXP motif on DOCK180. DOCK180

subsequently binds to ELMO and activates Rac, which leads to the activation of Wave1/Scar1.[143] Recent results indicate that Src can also directly phosphorylate Scar1.[144] Activated Scar1 can bind to and activate Arp2/3, which causes the branching growth of actin filaments and the formation of actin arcs adjacent to the plasma membrane.[143] The polymerized actin filaments bend beneath membrane, and the subsequently accumulated thermodynamic energy in situ may eventually promote the protrusion of the lipid layer at the leading edge along migration direction.[145] It has been reported that Src can be activated in live human umbilical VE cells by applying laser-tweezer traction on FN-coated beads adhering to the cells.[34] A rapid distal Src activation and a slow directional wave propagation of Src activation along the plasma membrane have been observed. The wave propagated away from the stimulation site with a relatively constant speed of about 18.1 nm/s. This force-induced directional and long-range activation of Src can be abolished by the disruption of actin filaments or microtubules. Therefore, the transmission of mechanically induced Src activation is considered a dynamic process that directs signals via the cytoskeleton to spatial destinations (Fig. 2.2).[34]

The permeability of endothelium and consequently atherosclerosis involves EC junctions.[69,147] Among the three major types of intercellular connections, viz., AJ, gap junction, and tight junction,[148] AJ is the most ubiquitous.[149] In ECs, AJ is mainly comprised of a membrane receptor VE-cadherin, with its intracellular domain separated into the juxta-membrane domain (JMD) and the catenin-binding domain (CBD). JMD provides putative docking sites for $p120^{ctn}$, which is a substrate molecule for Src. CBD binds directly to β-catenin and γ-catenin, which possibly bridge the VE-cadherin complex to actin-based cytoskeleton.[148,150,151]

Active Src perturbs the cadherin-mediated cell–cell adhesion. For example, AJ was severely deteriorated in v-Src transformed fibroblasts.[152,153] Constitutively active Src protein also caused the tyrosine phosphorylation of E-cadherin and a concurrent loss of cell–cell contact.[154] Further, ERK is constitutively activated in Src-transformed cells.[110,111] The SH2/SH3 domains of Src can recruit[155] and activate ERK, resulting in the phosphorylation of an ERK substrate molecule myosin light-chain kinase (MLCK).[117] The phosphorylation of MLCK ultimately leads to the phosphorylation of myosin light chain and activation of actomyosin machinery to cause the in situ contractility and the breakage of AJ.[155] Shear stress has been shown to activate both Src and ERK in bovine aortic endothelial cells (BAECs).[19,107,156] It is, however, not clear how different flows activate Src, ERK, and MLCK in space and time to regulate AJ.

5. FOCAL ADHESION AND FAK

Focal adhesions are the contact sites of cells to outside ECM through transmembrane proteins integrins. Integrins are heterodimeric receptors containing α and β subunits, and so far 24 different subtypes of integrins have been identified in vertebrates with the combination of 18α and 8β subunits. These integrin subtypes allow the diverse and specific recognition of various ECM proteins, e.g., FN, fibrinogen, collagen, vitronectin, and laminin.[157]

After the ligation of integrins with ECM proteins, many structural and signaling proteins are recruited to focal adhesions. Signaling proteins at focal adhesions include kinases, e.g., Src, FAK, integrin-linked kinase, and phosphatase, e.g., receptor-like tyrosine phosphatase α. Since integrin itself lacks the enzymatic activity, these signaling proteins at focal adhesions are crucial to transfer extracellular mechanical information inside the cells. For example, integrin-mediated activation of Src and FAK can regulate Rho GTPases, which then regulate the organization of actin cytoskeleton.[158,159] Src and FAK coordinate and regulate downstream signals in focal adhesions (Fig. 2.2).[146,160] It has been reported that growth factor-induced FAK activation is mediated and maintained by Src activity, while FAK activation on cell adhesion is independent of and in fact essential for the Src activation.[146] FAK also interacts with integrin receptors and ECM proteins to sense the mechano-environment (Fig. 2.2).[160] Seong et al. found that matrix protein FN-mediated FAK activation is dependent on mechanical tension, which may expose the otherwise hidden FN synergy site to integrin α5. In contrast, the binding motif of type I collagen to its receptor integrin α2 is constitutively exposed. Hence, FAK can be sufficiently activated on type I collagen independent of tension. Therefore, different ECM proteins can differentially transmit or shield mechanical forces from the environment to the functional molecules in the cell and regulate cellular functions (Fig. 2.2).[160]

Structural proteins, for example, talin, paxillin, vinculin, and zyxin, link other focal adhesion proteins and actin cytoskeleton. For example, ECM-bound integrins can recruit an adaptor molecule talin to focal adhesions. Talin has the binding sites for another molecule vinculin, which then bind to actin cytoskeleton.[161,162] As such, outside ECM can be physically connected to intracellular cytoskeleton through integrin and focal adhesion proteins. Therefore, due to their physical location at the interface between extracellular microenvironments and intracellular space, focal adhesions play crucial roles in sensing mechanical signals from both outside and inside.[163,164]

6. TOOLS TO MONITOR SIGNAL TRANSDUCTION IN LIVE CELLS

6.1. FPs, FRET, and fluorescence lifetime imaging microscopy

Molecular-tagged FPs and specific FRET biosensors are capable of monitoring cellular events in live cells.[28–37] YPet, a variant of yellow FP, was paired with enhanced cyan fluorescence protein (ECFP) and severalfolds increase in sensitivity was observed for various biosensors.[165,166] These ECFP/YPet-based biosensors, however, only allow the visualization of one active molecular event in a single live cell. Several studies have used one FP as the common donor or acceptor for two FRET biosensors to visualize different molecular signals in the same cell.[167–169] Other pairs, including Ametrine paired with tdTomato,[170] mOrange with mCherry,[171–174] TagFP with mPlum,[175] and T-Sapphire with DsRed,[176] have been explored as a second FRET pair.[171–174] These approaches either require sophisticated means to quantify the signals or are difficult for multi-FRET imaging in a single cell. The potential of mOrange2/mCherry as a new FRET pair, together with ECFP and YPet, was also demonstrated for dual-FRET imaging.[177]

Although mOrange2 and mCherry can act as a new FRET pair to monitor active signaling events,[166] there is some overlap between their excitation spectra to cause nonspecific cross

talk,[171,172] and this may have contributed to the lower sensitivity of a MT1-MMP FRET biosensor in vitro using the mOrange2/mCherry pair.[166] Because fluorescence lifetime imaging microscopy (FLIM) only monitors the donor lifetime to measure FRET signals without the need to measure the acceptor lifetime, it can avoid the nonspecific contamination of acceptor excitation/emission. FLIM is also independent of the local concentrations of fluorescent molecules and can separate the population of "FRETing" donors from those of noninteracting ones based on the lifetime distribution, thus enhancing the accuracy of FRET detection.[178–181] Hence, FLIM is ideal for the visualization of multiple FRET biosensors simultaneously in the same live cell.

Recently, FRET techniques have been applied to visualize signal transduction in response to shear stress. For example, GFP-fused Rac and Alexa568-p21-binding domain of PAK1 (PBD) were used to monitor the Rac activation in live cell by measuring FRET between GFP to Alexa568.[182] With this FRET-based biosensor, shear stress was shown to induce a directional activation of Rac concentrated at the leading edge of the cell along flow direction.[183] Shear stress has also been shown to induce a polarized Cdc42 activation along flow direction visualized by the FRET between a GFP-Cdc42 and an Alexa568-PBD.[184] In another study, a separated pair of ECFP-fused relA and EYFP-fused IκBα was used to monitor the interaction of relA and IκBα. The FRET efficiency between ECFP-relA and EYFP-IκBα decreased upon shear-stress application, indicating a mechanical force-induced dissociation of relA and IκBα.[185] CFP and YFP have also been fused to human B_2 bradykinin receptor, a GPCR, to detect the activation of GPCR. Shear stress was shown to activate B_2 bradykinin GPCR within 2 min, which can be inhibited by B_2-selective antagonist.[17] These results suggest that B_2 bradykinin GPCR may serve as a mechanosensing molecule in response to shear stress. In the mechanosensing and mechanotransducing elements included focal adhesion and the cytoskeleton network, FRET biosensors have been developed for many molecules, including Src, FAK, α-actinin, vinculin, and talin.[34,146,186]

6.2. Quantitative image-based analysis for live cells

The development and application of FPs and related biosensors have greatly advanced our knowledge of signaling transduction in live cells. The vast amount of imaging data produced by these FPs and biosensors demands the development of automated, intelligent, and objective image analysis tools to allow precise and efficient interpretation of complex biological information.[39,44] Automated algorithms integrated with image-based analysis have been developed and utilized to analyze the kinetics of FPs, discretize the intracellular space, track the cell movement, and detect the localization of the FPs. Here, we briefly review the widely used fluorescence recovery after photobleach (FRAP) analysis methods for quantifying the diffusion kinetics of molecules and provide an example of quantitative polarity analysis based on images.

6.3. The FRAP analysis and finite-element-based diffusion analysis

The diffusion kinetics of a FRET biosensor, or a fluorescent molecule, can be examined by FRAP analysis, a widely used technique in estimating the apparent diffusion coefficient of molecules in live cells.[187–190] In classical FRAP analysis, the fluorescence recovery curve is

obtained by monitoring the recovery process of the average fluorescence intensity in the small region, which underwent photobleaching and the subsequent recovery. The apparent diffusion coefficient of fluorescent molecules can be estimated by performing parameter fitting on the recovery curve.[187] Results from the FRAP analysis has been revealed the characteristics of transport kinetics for many important molecules.[190–192] However, there are limitations with this approach on the geometry of the cell, the geometry of the photobleached spot, and the actual photobleaching process that can be analyzed.[187–189,193] Most recently, FRAP analysis using computational approaches, such as the model-based finite-element (FE) and finite-difference methods and the simulation-based computational particle and Monte Carlo methods, has been developed to address these limitations.[192,194–200]

In particular, the FE method is a computational approach for defining a set of linear equations, which approximate the partial differential equations in the value of solutions. It is well known for the flexibility in resolving complex geometry of tissue and cellular structures for approximating the elastic and diffusion equations.[201,202] This method has been used to estimate the apparent diffusion constant in inhomogeneous tissues[200] and model protein transport in single cells.[203] Newly developed FE-based image analysis and simulation tool for FRAP enforce no specific requirement on the geometry of cell, the bleaching light beam, or the photo-bleaching process. This tool provided a general approach for evaluating the accuracy of the diffusion model. With this FE-based tool integrated with diffusion model, the diffusion of FRET-based Src biosensors has been evaluated, simulated, and subsequently subtracted from the biosensor FRET signals in live cells.[46]

6.4. Automatic tracking of moving cells and subcellular features

Image registration is the process of transforming different sets of images into one coordinate system. It is widely used in engineering and science for automatically finding the pixel-wise transformation from the data to reference images by detecting and matching their feature points and the interpolation of values among these feature points.[204,205] For example, it was used in live-cell FRET imaging to physically align the fluorescent images obtained at different wavelengths simultaneously.[31,182,206] However, the registration of the video images of moving cells is a considerably more challenging problem, due to the relatively nonlinear movement of cell body during migration or adhesion. To circumvent the difficulty involved in the image registration of moving cells, some quantification methods have been developed to divide the whole cell into small wedges in the polar coordinated system and quantify the cell motility, protein distribution, or molecular activity in these wedges with different fixed angles.[206–208] These methods are very useful to align and quantify the molecular localization in the angular direction among different cells or in a cell which changes the shape in time. However, a robust and accurate whole-cell image registration method capable of tracking all intracellular pixels is needed for the precise image analysis and model simulation of molecular activities in migrating cells.

In addition to whole-cell analysis, it is very important to detect and track molecular locations and activities within subcellular structures. For example, the image segmentation-based water algorithm has been developed to detect individual FAs based on their intensity

profiles. It was initially used to characterize the molecular heterogeneity between and within FAs and to study the effect of kinase inhibitors on FA disassembly.[41] It was also applied successfully for analyzing the correlation between the local force and the physical property of FAs in subcellular locations, such as orientation, size, and shape.[42] This algorithm has further helped to discover that the FA dynamics is regulated by the pattern of integrin clusters,[209] and to characterize the effect of different drugs on the structure and organization of FAs in a high-throughput fashion.[43]

Polarized molecular activities play important roles in guiding the cell toward persistent and directional migration as those observed in wound healing and the growth of blood vessels. Molecular polarity has been quantified using automated analysis method, and the related time difference among different molecules can be used to compare the time sequence in the signaling pathways (Fig. 2.3).[46,47] Lu et al. showed the polarized distributions of the activities of phosphatidylinositol 3-kinase (PI3K) and the Rac1 small GTPase monitored using chimeric FPs in cells. The cells were constrained on micropatterned strips, with one end connecting to a neigh-boring cell (junction end) and the other end free of cell–cell contact (free end, see Fig. 2.3). The subcellular distribution of FPs and the edge position and velocity at the free end of the cells were quantified to analyze their correlation and interpret the signaling sequence. The initiation of the edge extension occurred before the activation of PI3K, which led to a stable extension of the free end followed by the Rac1 activation.[47] The results showed the power of quantitative image-based analysis in deciphering coordinated sequential signaling events regulating the lamellipodia extension and migration in live cells.[47]

7. CONCLUSION

ECs can sense the mechanical force from the environment and convert it into molecular signals regulating cellular function. Studies on the regulation and function, especially mechanotransduction, of ECs provide important insights into the prevention and treatment of cardiovascular diseases such as atherosclerosis. Many molecules function at the plasma membrane or focal adhesions at the interface between the cell and the environment, acting as mechanosensors. Transmembrane receptors and focal adhesion-associated molecules, such as integrin, CGPR, PECAM-1, TRPC6, Src, FAK, p130Cas, and vinculin, are examples of mechanotransducing molecules sensitive to shear stress, stretch, or the stiffness of the microenvironment. Novel technologies such as fluorescence imaging and FRET biosensors provide powerful tools to study the cellular and molecular functions with high spatiotemporal resolution at the single-cell level. Quantitative image-based analysis tools allow the systematic quantification and inference of single-cell imaging results with rigor and accuracy. Therefore, the future in single-cell imaging of mechanotransduction will be illuminated by genetic engineered fluorescence biosensors, which enables the systematic and quantitative exploration of molecular networks in live cells.

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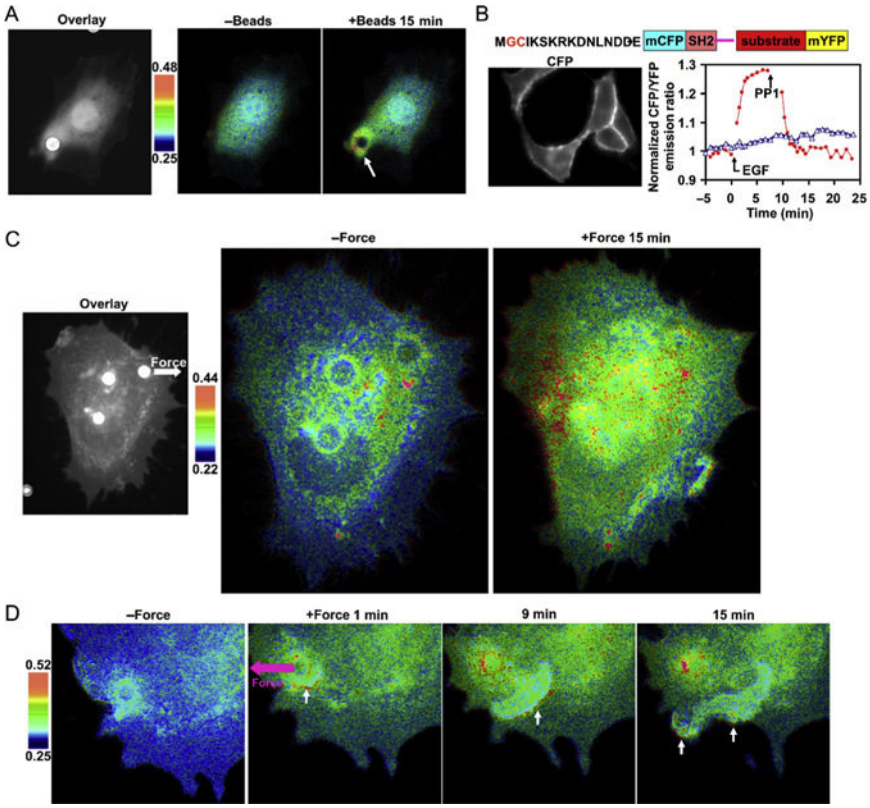

Figure 2.1.

Directional and long-range propagation of Src induced by mechanical force. (A) A fibronectin-coated bead (white spot from phase contrast image overlaid on CFP cell image) induced FRET responses around the bead. White arrow points to the spot with activated Src. Color bar represents CFP/YFP emission ratio values. (B) The schematic diagram in the upper panel shows the design strategy of membrane targeting. The CFP-only image on the left shows the effective tethering of the reporter on the plasma membrane. The EGF-induced FRET responses of the reporter is reversed by PP1 (red line) and prevented by pretreatment with PP1 (blue line). (C) Laser-tweezer traction on the bead at the upper right corner of the cell (shown on the left) caused FRET responses. White arrow represents force direction. (D) FRET responses of a cell with clear directional wave propagation away from the site of mechanical stimulation. This research was originally published in the Nature Journal.[34]

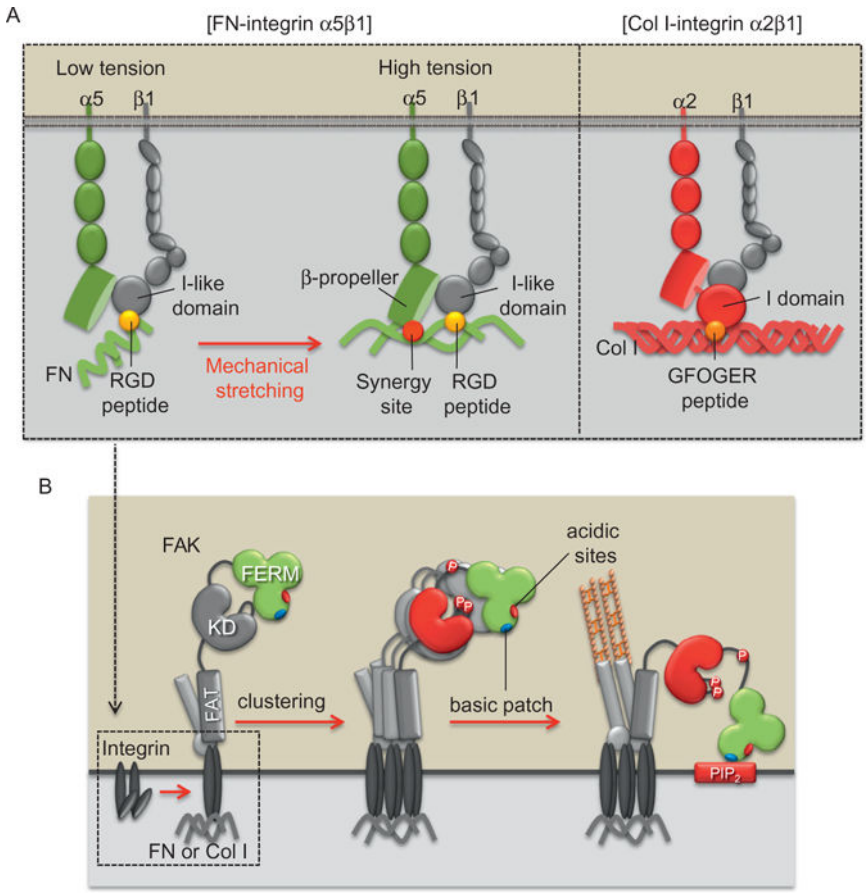

Figure 2.2.

Proposed model of FAK mechanoactivation mechanisms via different ECM and integrin subtypes during cell adhesion process. (A) Integrin α5β1 can be fully activated in the tensioned state where both RGD peptide (yellow circle [white in print version]) and synergy site (red circle [dark gray in print version]) bind to α5 and β1 subunits, respectively. Because FN synergy site is exposed only in the high-tensional state, the FAK activation via integrin α5β1 is dependent on the mechanical environment. In contrast, integrin α2β1 can directly bind to the constitutively exposed GFOGER motif (orange circle [light gray in print version]) in Col I, thus causing the activation of integrin α2β1 and FAK independent of mechanical tension. (B) Integrin activation can recruit and induce the transphosphorylation of FAK. This leads to the FAK activation, which is maintained by the interaction between the FERM basic patch (blue oval [light gray in print version]) and PIP2 to prevent the inhibitory interaction of myosin II with FERM acidic sites (red oval [dark gray in print version]). This research was originally published in Nature Communication.[146]

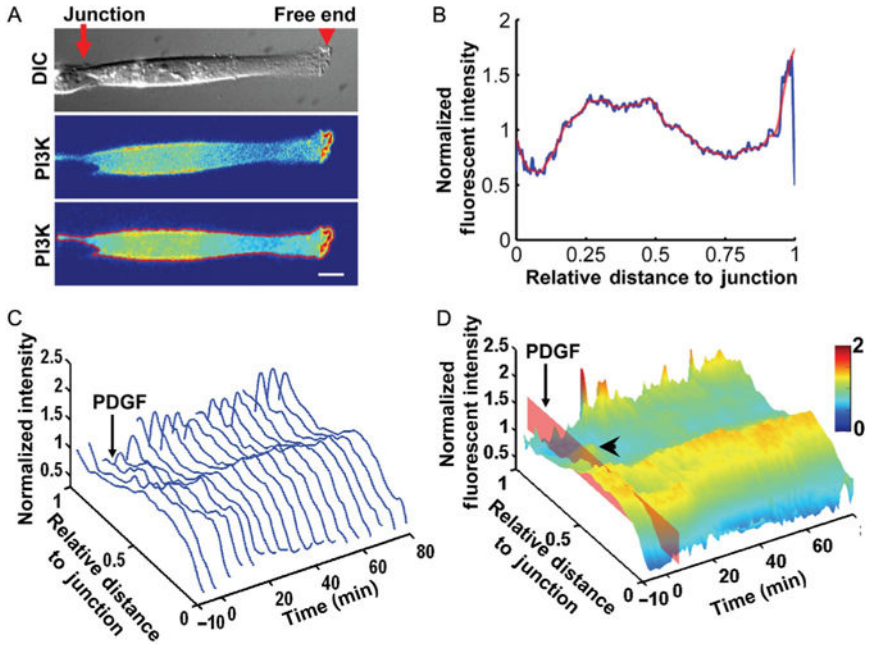

Figure 2.3.

The quantification methods and protein localization results. (A) The alignment and outline of a cell before quantification. Top panel: The DIC image of a cell with a junction connecting to a neighboring cell on a patterned FN strip and a free end capable of lamellipodial protrusion. Middle panel: The fluorescence image shows the intensity distribution of PH-Akt-GFP expressed in the cell of interest. Bottom panel: The cell was rotated and aligned along a horizontal direction, and the boundary of the cell calculated and overlaid in red with the fluorescence intensity image. Scale bar: 10 μm. (B) The normalized fluorescence intensity of PH-Akt-GFP plotted against the relative distance to the junction endwith the blue and redlines representing the rawand smoothed data, respectively. (C) The sequence of the normalized fluorescence intensity curves arranged along the time axis with a 3D view. The platelet-derived growth factor (PDGF) simulation was applied at 0 min. (D) The 3D intensity surface of PI3K activity landscape was color-coded by the fluorescence intensity values and visualized as a function of time and distance to the junction end, based on the data in (C). The time of PDGF stimulation is indicated bythe redplane. A transient secondary peak can be observed between the cell body and the free end (arrowhead). This research was originally published in PLoS One.[210]