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力传导中亚细胞水平的分子 FRET 成像

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摘要:体内细胞受到含有化学和力学因素的生理和病理生理的刺激,故研究这些因素在细胞和器官水平如何调节功能就尤为重要。有关细胞和器官对化学因素的反应已开展诸多研究,而力学因素的影响却鲜有报道。近年来,荧光蛋白和显微镜技术的发展已成为阐明力传导过程的有用工具,先进的信号活细胞成像技术促进了力学生物学中分子机制的时空因素研究。本文综述荧光蛋白的基本知识以及其在生物学研究中的应用,特别讨论了以荧光共振能量迁移(fluorescence proteins and microscopy,FRET)技术为基础的生物传感器的发展和特征。基因编码的 FRET 生物传感器能够实现分子时空活动的成像和定量,使得活细胞中生物化学信号在力学刺激下的反应和传导可视化。同时,本文重点阐述分子水平力学刺激下的活细胞信号传导。

关键词:荧光共振能量迁移;荧光蛋白;力学刺激;信号传导;生物传感器;力学生物学
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FRET imaging of molecular hierarchy at subcellular levels in mechanotransduction

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Abstract: Cells in the body are exposed to physiological and pathophysiological stimuli that encompass both chemical and mechanical factors. It is important to understand how these factors modulate functions at cellular and organ levels. Compared to the large amount of information on cellular or organ responses to chemical factors, there is a paucity of knowledge on the effects of mechanical factors. Recent advances of fluorescence proteins and microscopy make it a very useful tool for elucidating the mechanotransduction processes; the state-of-the-art technologies for live-cell imaging of signaling is particularly valuable for investigating the spatial and temporal aspects of molecular mechanisms in mechanobiology. This review will cover the basic knowledge of fluorescence proteins and their application for biological research. In particular, the development and characterization of biosensors based on fluorescent resonance energy transfer (FRET) will be discussed. Genetically encoded FRET biosensors, which allows the imaging and quantification of tempo-spatial activation of molecules, will be introduced to demonstrate how the initiation and transmission of biochemical signals in response to local mechanical stimulation can be visualized in live cells. Specific emphasis will be on the elucidation of molecule hierarchy of signaling transduction in live cells upon the mechanical stimulation.

Key words: Fluorescent resonance energy transfer (FRET); Fluorescent protein; Mechanical stimulation; Signaling transduction; Biosensor; Mechanobiology

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Atherosclerosis is the leading cause of death in the United States and most other developed countries[1]. In fact, sudden death caused by atherosclerosis occurs to about half million people each year in the United States[2] with a related economic cost estimated to be more than 150 billion in 2007[3]. Atherosclerosis occurs preferentially at vascular curvature and branch sites where the vessel walls are exposed to disturbed flow, but not at the straight parts of vessels where laminar flow dominates[4]. Vascular endothelial cells (ECs) which form the interface between the circulating blood and the arterial wall are constantly exposed to these hemodynamic forces and the ensuing shear stress, the tangential component of hemodynamic forces acting on ECs. Ample evidence has indicated that the different flows and the ensuing shear stresses regulate the cell-cell junctions of vascular endothelial cells (ECs) and the subsequent endothelium permeability, the major cause of atherosclerosis. Despite the tremendous advancements of our understanding on the role of mechanical force in pathophysiological processes during past decades, it remains unclear about the detailed molecular mechanism by which cells sense the spatiotemporal characteristics of these mechanical stimuli and coordinate the molecular hierarchy at subcellular levels to determine patho-physiological consequences. Fluorescence resonance energy transfer (FRET) technology and genetically encoded biosensors have provided powerful tools for visualizing active molecular events with high spatiotemporal resolutions in live cells. In this paper, we will introduce the basic concept of mechanotransduction, signaling transductions at subcellular regions, FRET technology and biosensor designs based on FRET, as well as their application for the visualization of mechanotransduction. We will specifically discuss the application of biosensors for the visualization of Src signaling pathways and their roles in regulating the mechanotransduction. Src is activated by mechanical force and plays central roles in a variety of cellular processes at subcellular regions[5-6]. Hence, the visualization of Src during mechanotransduction should advance our understanding on how cells sense mechanical cues and convert into biochemical signals. We will also discuss multiple molecular signals simultaneously visualized in the same cells utilizing two independent FRET biosensors with distinct colors to elucidate the signaling hierarchy in regulating cellular functions. This simultaneous visualization of multiple signaling cascades at subcellular levels is particularly important as it becomes clear that molecular interactions and their biological functions in live cells are largely dependent on their subcellular location/environment, possibly due to the different sets of mediator molecules at different subcellular locations[7-8]. As such, the integration of multi-color FRET biosensors for the direct visualization of the molecular interaction and regulation at subcellular levels will bring revolutionary advancement to the fundamental understanding of intracellular signaling networks. The results will shed new lights on the molecular mechanism by which different flows regulate cardiovascular diseases, such as atherosclerosis.

1 Mechanotransduction, atherosclerosis, and shear stress

Atherosclerosis is a cardiovascular disease characterized by the patchy deposit of fatty materials in the arterial walls and the subsequently reduced/blocked blood flow[4]. It occurs preferentially at vascular curvature and branch sites where the vessel walls are exposed to disturbed flow, but not at the straight parts of vessels where laminar flow dominates[4]. Vascular endothelial cells (ECs), which form a monolayer of endothelium lining along and protecting the vessel wall from the circulating blood[9], 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[10-11]. Recent evidence also indicates that the effect of disturbed flows on ECs is pro-inflammatory whereas that of laminar flows is anti-inflammatory[9,12-14]. For example, laminar flows can inhibit the inflammatory signaling cascades induced by TNFα[15-17]. In contrast, 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)[18-20]. It remains unclear, however, how cells sense the distinct spatiotemporal characteristics of these mechanical stimuli and determine the corresponding pathophysiological consequences.

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 migration at the wounding edge followed by EC proliferation, serves as the critical step to restore the endothelium integrity[21]. Cell

junctions in EC monolayers can be disrupted by shear stress and recover if continuously exposed to laminar flows for long-term as the cells adapt to the new environment[10-11]. However, it is expected that this transient disruption of cell junctions under laminar flows will last longer during the wound healing process than in normal conditions because it takes time for cells to repair the wound and restore a stable monolayer and adapt to the new environment. The junction disruption would keep the ECs disengaged from each other and free to move. This laminar-flow-directed protrusion and migration of ECs at the upstream side of wounded area can be maintained before the wound closure, thus facilitating the EC migration towards the wounded area. At the downstream side, the laminar-flow-induced protrusion along the flow direction negates the shear-induced motility enhancement. Although a wound per se can 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[22]. This asymmetric effect of laminar flows results in an increased net speed of wound healing comparing to a wound healing process without flow. In fact, both *in vitro* and *in vivo* experiments indicate that laminar flows enhance EC migration and consequently wound healing[23-25]. Laminar flows have been shown to enhance the wound healing by modulating cell-cell and cell-ECM adhesions, in particular, VE-cadherin-mediated AJ[26-27] and β1 integrins[28]. Membrane fluidity, cytoskeleton, and tyrosine kinases also appear to be important for the laminar-flow-induced EC wound healing[22,29-30]. Interestingly, disturbed flows induce sustained disruption of AJ[10-11]. However, the EC migration speed toward the wounded area under disturbed flows is comparable to that without flow[22]. It was revealed that ECs under disturbed flows have strong staining of focal adhesion proteins due to the unstable mechanical environment[22], which may impair the cell detachment from the substrate necessary for EC migration and hence wound closure[31]. At the current stage, there is a lack of study on how ECs coordinate the multiple signaling events in space and time under different flows to regulate 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, including atherosclerosis[32-34]. For example, a wild range of signaling and structural molecules, including plasma membrane, membrane proteins/receptors (e. g. integrins[5,35], GPCR[36], Cadherin[11,37], PECAM-1[37-39], VCAM-1[40-41], ICAM-1[41], and ion channels[42], actin filaments[34], microtubules[43-44], and intermediate filaments[45-47] were identified to play important roles in transmitting shear stress into biochemical signaling cascades, i. e. mechanotransduction. Evidence also indicates that both the temporal and spatial gradient of shear stress can affect the cellular functions[48-55]. In fact, it was realized that the subcellular characteristics of shear stress are heterogeneous in a single cell and impact significantly on cellular responses[5,49,56-57]. Calcium flux in response to shear stress showed significant subcellular directionality[58-59]. The lateral diffusion coefficient of lipids in the plasma membrane also increased at regions upstream of nucleus while decreased at the downstream regions upon shear stress application[60].

2 The plasma membrane and src kinase in mechanotransduction

The plasma membrane is not uniform in structure[61] and has different microdomains, e. g. lipid rafts, which are rich in cholesterol, sphingomyelin, and saturated fatty acids[62]. These microdomain structures and their interaction with cytoskeleton are involved in the regulation of intracellular signaling[63]. Indeed, Src family kinase members, including Fyn and Lyn, are anchored to lipid rafts to become activated following N-terminal palmitoylation and myristoylation[64], whereas H-Ras depends on the non-rafts anchoring at the plasma membrane to be functional[64-65]. Evidence also indicates that the plasma membrane and its microdomains are involved in mechanotransduction. For example, different subtypes of G-proteins are partitioned into membrane microdomains to regulate the mechanical-activated signaling molecules, e. g. nitric oxide production[66] and MAPK activity[67]. The modulation of the plasma membrane fluidity also altered the shear stress-induced MAPK signaling pathway[60], further underscoring the importance of the plasma membrane in mechanotransduction.

Src kinase is a non-receptor tyrosine kinase critical for a variety of cellular processes[7]. Before stimulation, Src

localizes at microtubules-associated perinuclear regions in various types of cells[68-74]. 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[75-77], possibly through the Src SH3 domain, but not the catalytic domain[78-79]. The inhibition of RhoA or actin stress fibers but not microtubules resulted in the blockage of Src translocation/activation in response to various stimuli[75-77]. The compartmental structures at the plasma membrane are also involved in the localization and regulation of intracellular signaling molecules, including Src kinase[63,80]. Src family kinases (SFKs) can be transported to distinct compartments of plasma membrane through different types of endosomes[81]. SFK members such as Lyn and Fyn can reside in lipid rafts of the plasma membrane[82], via their N-terminal myristoylation and palmitoylation sites[65]. Src kinase itself has only myristoylation motif and it is not clear whether Src kinase localizes within the lipid rafts at the plasma membrane[83-88]. 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[83-84]. Different groups also reported different Src localizations in PC12 cells[85,88]. This inconsistency is likely attributed to the controversial effects of non-ionic detergents and the detergent extraction method used in these reports for isolating DRMs, which, however, may not exactly correspond to the lipid rafts in living cells and may include membranes that do not contain rafts before detergent extraction[89-90]. The development of advanced methods is needed to visualize the Src translocation and activation at the plasma membrane in live cells.

In the inactive state, the SH3 and SH2 domains of Src kinase are coupled together by intramolecular interactions, and the catalytic kinase domain of Src is masked by its interaction with the C-terminal tail, thus preventing its action on substrate molecules. Shear stress can induce the engagement of integrins[35], which may subsequently activate Src[7,91]. In fact, shear stress has been shown to activate Src in different cell types[5-6]. Several putative mechanisms have been proposed for the Src activation induced by shear stress and integrin-engagement: ① The shear-modulated cytoplasmic tail of integrin β3 may directly recruit Src kinase through its SH3 domain. This action disrupts the intramolecular interactions between different domains of Src and activate Src[92]; ② Shear-activated integrins cause the myristoylation-mediated translocation of Src to focal adhesion sites[7]. FAK Y397, in a high-affinity $_{p}$YAEI context, competes with the Src C-terminal $_{p}$Y527 for its intramolecular interaction and thus activate Src[7,93]; ③ Recent evidence has shown that integrin-engagement leads to the association of integrin αvβ3 with RPTPα, a well-characterized activator of Src family kinases[94]. It is possible that shear-regulated integrins recruit RPTPα to dephosphorylate the Y527 on the C-terminal tail of Src and release it from the kinase domain, thus activating Src[94]. It becomes clear that the Src activation and its biological functions are tightly controlled by the subcellular locations. For example, Src induces the p190GAP activation and inhibits Rho GTPase at the focal adhesion sites[7], whereas it activates Rho GTPase at podosomes[8]. Hence, the visualization of Src activation with high spatiotemporal resolution in live cells at subcellular levels should advance our understanding of the Src functions in response to mechanical stimulations.

3 Src and adherens junction (AJ)

The permeability of endothelium and consequently atherosclerosis involves EC junctions[26,95]. Among the three major types of intercellular connections, viz., adherens junction (AJ), gap junction, and tight junction[96], AJ is the most ubiquitous[97]. In ECs, AJ is mainly comprised of a membrane receptor VE-cadherin, with its intracellular domain separated into the juxtamembrane 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[96,98-99].

Active Src perturbs the cadherin-mediated cell-cell adhesion. For example, AJ was severely deteriorated in v-Src transformed fibroblasts[100-101]. Constitutively active Src protein also caused the tyrosine phosphorylation of E-cadherin and a concurrent loss of cell-cell contact[102]. Further, ERK is constitutively activated in Src-transformed cells[103-104]. The SH2/SH3 domains of Src can recruit[105] and activate ERK, resulting in the phosphorylation of a ERK substrate molecule myosin light chain kinase (MLCK)[64]. The phosphorylation of MLCK ultimately leads to the phosphorylation of myosin light chain (MLC) and acti-

vation of actomyosin machinery to cause the *in situ* contractility and the breakage of AJ[105]. Shear stress has been shown to activate both Src and ERK in BAECs[5-6,106]. It is, however, not clear how mechanical force activates Src, ERK and MLCK in space and time to regulate AJ.

4 Src and polarized protrusion

Src contributes to cell protrusion and migration in many ways. Src, mediated by 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[107]. Recent results indicate that Src can also directly phosphorylate Scar1[108]. 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 plasma membrane[107]. The polymerized actin filaments bend beneath membrane and the subsequently accumulated thermodynamic energy *in situ* may eventually promote the protrusion of lipid layer at the leading edge along migration direction[108].

5 Fluorescence resonance energy transfer (FRET) and fluorescent proteins (FPs)

FRET occurs when two fluorophores are in proximity, with the emission spectrum of the donor overlapping the excitation spectrum of the acceptor[109-110]. Any change of the distance and/or relative orientation between these two fluorophores can affect the efficiency of FRET and therefore, the ratio of acceptor-to-donor emissions[111]. Previous studies have shown that fusion proteins with interacting peptide partners sandwiched between two different FPs are capable of monitoring various cellular events in live cells with high spatial and temporal resolution[65,74,112-119]. The most popular FPs for FRET pair at the current stage are the cyan FP (CFP) serving as the donor and the yellow FP (YFP) as the acceptor. However, the dynamic range of most genetically-encoded biosensors is limited (typically 20-40% change upon stimulation) and hence has hindered their broad applications.

6 An improved FRET pair

A new FRET pair, CyPet and YPet, was recently developed through the screening of diverse libraries. This new pair allows the ratiometric FRET signals 20 fold higher than its parental ECFP/EYFP pair at room temperature[120]. However, CyPet is deficient at 37 ℃ and not suitable for live cell imaging[121]. We hence paired YPet with ECFP. Several folds increase in sensitivity has been observed in biosensors for a variety of molecules, including Src, MT1-MMP, Ca^{2+}, and the small GTPase Rac1[122]. As such, these ECFP/YPet-based biosensors can provide much improved sensitivities to visualize the spatiotemporal activation of signaling molecules in live cells under different stimulations.

7 Multiple FRET imaging

The ECFP/YPet-based biosensors, however, only allows the visualization of one active molecular event in a single live cell. To visualize multiple molecular events in a simultaneous fashion, biosensors with new FRET pair need to be developed. FPs with different excitation and emission wavelengths have been recently developed through directed evolutionary strategies[123-124]. Among these FPs, mOrange and mCherry appear to have the potential to serve as a new FRET pair besides CFP and YFP. However, mOrange has poor photostability[124]. Four mutantions (Q64H, F99Y, E160K, and G196D) have hence been introduced into mOrange to generate mOrange2, which displays drastically enhanced photostability and almost identical spectral characteristics relative to the original mOrange[124]. Hence, mOrange2 and mCherry may serve as a new FRET pair spectrally distinguishable from ECFP and YPet. We have replaced the ECFP/YPet FRET pair for the FRET biosensors with mOrange2 and mCherry[122]. As such, the ECFP/YPet-based biosensors and mOrange2/mCherry-based biosensors can be simultaneously visualized in a single live cell.

8 Shear stress and FRET imaging in live cells

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[125]. With this FRET-based biosensor, shear stress was shown to induce a directional activation of Rac concen-

trated at the leading edge of the cell along flow direction[126]. 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[127]. 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[128]. CFP and YFP have also been fused to human B_2 bradykinin receptor, a G protein-coupled receptor (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[36]. These results suggest that B_2 bradykinin GPCR may serve as a mechano-sensing molecule in response to shear stress.

9 Conclusion and future directions

Genetically encoded biosensors based on FRET have allowed the successful visualization of various signaling events in live cells with high temporal and spatial resolution[74,112-114]. However, given the limited number of FPs suitable for FRET, most studies conducted to date allow for the visualization of only a single type of active molecular event in live cell systems. Integrating the mOrange2/mCherry and ECFP/YPet FRET pairs, two active signaling events can be visualized simultaneously in a single live cell at subcellular levels. This is particularly important given that molecular interactions and their biological functions in live cells are largely dependent on their subcellular location/environment, possibly due to the different sets of mediator molecules at different subcellular locations. This notion is underscored by recent findings that ① Src induces the p190RhoGAP activation and subsequently inhibits RhoA at the focal adhesion sites[7], whereas Src activates RhoA at podosomes[8]; ② RhoA couples with its downstream molecule ROCK at the cell rear and a contractile region behind lamellipodium, but co-localizes with another substrate molecule mDia at the leading edge of a migrating cell[118,129]. Hence, simultaneous visualization of molecular events in a single cell at subcellular regions will provide invaluable information for our systematic understanding of molecular mechanism and cell signaling in regulating pathophysiological consequences under different flows. Since ECs are critical for cardiovascular diseases, including atherosclerosis and restenosis, the FRET imaging results of ECs should also provide rational basis for developing new ways to treat these diseases. It is expected more and more novel FRET biosensors will be developed and these biosensors will also provide powerful tools for detecting cardiovascular diseases as well as the efficacy of therapeutic drugs.

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