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Live imaging of transgenic mice expressing FRET biosensors

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Abstract—**In recent years, fluorescence imaging has received particular attention, due to increasing availabilities of fluorescent proteins and dyes, which had driven the development of novel biosensors. Genetically-encoded biosensors based on the principle of Förster resonance energy transfer (FRET) have been widely used in biology to visualize the spatiotemporal dynamics of signaling molecules. Despite the increasing multitude of these biosensors, their application has been mostly limited to cultured cells with transient biosensor expression, due to difficulties in stable expression of FRET biosensors. In this study, we report efficient generation of transgenic mouse lines expressing heritable and functional biosensors for ERK and PKA. These transgenic mice were generated by the cytoplasmic co-injection of Tol2 transposase mRNA and a circular plasmid harboring Tol2 recombination sites. Observation of these transgenic mice by two-photon excitation microscopy yielded real-time activity maps of ERK and PKA in various tissues, with greatly improved signal-to-background ratios. Our transgenic mice may be bred into diverse genetic backgrounds; moreover, the protocol we have developed paves the way for the generation of transgenic mice that express other FRET biosensors, with important applications in the characterization of physiological and pathological signal transduction events in addition to drug development and screening.**

I. Introduction

Fluorescence imaging provides important information about cellular events that cannot be obtained by other methods. Genetically-encoded biosensors based on the principle of Förster resonance energy transfer (FRET) are useful tools to visualize the spatiotemporal dynamics of signaling molecules [1-4]. The majority of the biosensors reported previously are based on the pairing of FRET-optimized variants of cyan- (CFP) and yellow-fluorescent (YFP) proteins as FRET donor and acceptors, respectively (Fig. 1A) [4]. Through the efforts of many researchers, FRET biosensors have been developed to visualize the changing concentrations of ions, sugars, and phospholipids, and to monitor the activities of GTPases, kinases and proteases [5]. However, applications of FRET biosensors have been mostly restricted to cultured cells with transient biosensor expression, due to some practical problems in the generation of transgenic mice expressing FRET biosensors [6]. In our experience, one of the major problems is frequent recombination between the cDNAs of CFP and YFP, which share high sequence similarity. In addition, low biosensor expression and low efficiency of transgenic mouse production have been technical problems. In other studies that reported the generation of transgenic mice expressing FRET biosensors, analysis was mainly performed with isolated cells in vitro and only a few of them succeeded in FRET imaging in living tissues [7-12]. Recently, Yamaguchi et al. succeeded in the generation of transgenic mice expressing a FRET biosensor for Caspase3 by introducing insulator sequences [12]. The authors suggested that the low expression of FRET biosensors is caused by silencing of the transgene. We recently reported an effective method to produce transgenic mice expressing FRET biosensors with Tol2 transposon-mediated gene transfer [13]. Observation of these transgenic mice by two-photon excitation microscopy (TPEM) yielded real-time activity maps of ERK and PKA in various tissues, with greatly improved signal-to-background ratios.

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II. Methods

Microscopy and image processing

For TPEM, we used a BX61WI/FV1000 upright microscope equipped with a x25 water-immersion objective lens (XLPLN25XW-MP; Olympus) or an IX81/FV1000 inverted microscope equipped with a x30 silicon oil-immersion objective lens (UPLSAPO 30xS; Olympus), both of which were connected to a Mai Tai DeepSee HP Ti:sapphire Laser (Spectra Physics). The excitation wavelength for CFP was 840 nm. Acquired images were analyzed with MetaMorph software (Universal Imaging) and Imaris Software (Bitplane).

Observation of auricular skin of transgenic mice

Mice were anaesthetized initially with pentobarbital (intraperitoneal injection, 37 mg/kg) and later with isoflurane (1.5% inhalation, 0.5 L/min). Then, after depilation, mice were placed on a microscope stage maintained at 30 °C. For laser ablation, the laser power was increased up to 30-50%, and the pre-determined regions were scanned 2-5 times. For the induction of skin inflammation, 20 μl of 10% arachidonic acid in acetone was topically applied to the auricle. After 3-4 hours, the mice were anaesthetized and observed as described [13]. An LTB4 receptor inhibitor LY293111 was injected at 4 mg/kg via intraorbital space. A MEK inhibitor PD0325901 in PBS was injected at 5 mg/kg via intraorbital space.

Observation of the small intestine

Mice were anaesthetized as described. An incision was made in the median line of the abdominal wall. The small intestine was pulled out of the abdominal cavity and filled with

phosphate buffered saline to minimize peristalsis. The serosa of the small intestine was positioned on the cover glass of the stage maintained at 37 °C. For the stimulation of PKA, 12 mg/kg theophylline and 0.5 g/kg dbcAMP were sequentially administered via the right jugular vein.

III. Results

Transgenic mice are commonly generated by the microinjection of linearized DNA constructs into pronuclei of fertilized mouse oocytes. However, this traditional technique gives physical damage to the oocytes, resulting in less than a 10% survival rate among injected eggs. Recently, we found that the efficiency of transgenic mouse production could be markedly improved by the cytoplasmic co-injection of Tol2 transposase mRNA and a circular plasmid harboring Tol2 recombination sites [14]. Meanwhile, using a PiggyBac transposon system, we succeeded in establishing stable cell lines expressing full-length FRET biosensors [15]. These technical advances encouraged us to employ Tol2-mediated gene transfer to generate transgenic mice expressing FRET biosensors. Here, we reported the efficient establishment of transgenic mouse lines with heritable and functional biosensors for protein kinase A (PKA) and ERK [13]. To monitor ERK and PKA activities, we used the ERK biosensor EKAREV and the PKA biosensor AKAR3EV, respectively [15, 16]. In addition to the cyan and yellow fluorescent proteins used as the FRET donor and acceptor, EKAREV and AKAR3EV include specific phosphorylation substrate, phospho-threonine binding domain linked with a long flexible linker and nuclear export signal (NES) as shown in

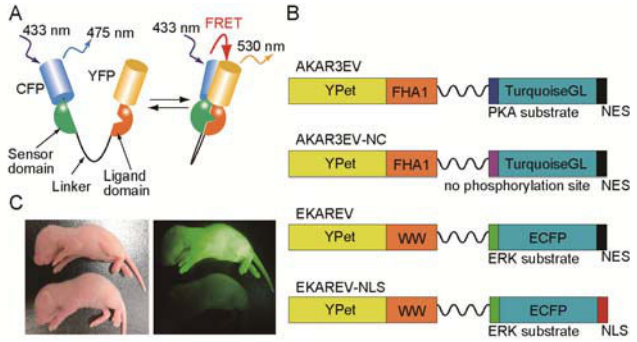

Figure 1 Generation of transgenic mice expressing FRET biosensors.

(A) The mode of action of a FRET biosensor. Phosphorylation of the biosensor by each kinase induces conformational change and increases in FRET. (B) Structure of FRET biosensors. AKAR3EV and EKAREV are FRET biosensors for PKA and ERK, respectively. YPet, a yellow fluorescent protein used as a FRET acceptor; TurquoiseGL and ECFP, cyan fluorescent proteins used as FRET donor; FHA1 and WW, phospho-threonine recognition domains; PKA and ERK substrates, substrates specific for PKA and ERK, respectively. NC, NES, and NLS denote negative control, nuclear exclusion signal, and nuclear localization signal, respectively. (C) A newborn transgenic mouse expressing AKAR3EV and a control mouse were inspected under blue light.

Fig. 1B. We also used two additional FRET biosensors, nucleus-targeted EKAREV (EKAREV-NLS) and a negative control, AKAR3EV-NC, in which alanine was substituted for the threonine in the substrate peptide of AKAR3EV. After co-injection of oocytes with the pT2A-derived expression plasmids and Tol2 mRNA, mice were born efficiently, ranging from 23% to 47% (Table 1). The CAG promoter-driven expression of the FRET biosensors was sufficient to identify newborn transgenic mice by visual inspection of green fluorescence in the skin (Fig. 1C). The integration rate ranged from 5% to 41%, which is a simple estimate based on visual inspection (Table 1). Importantly, the transgenes were transmitted to offspring by Mendelian inheritance. Until the eighth generation of the transgenic mouse lines, the FRET biosensors were functional without any detectable change in the expression level. Therefore, the transposon-mediated gene transfer may not only inhibit recombination between cDNAs of CFP and YFP, but also prevent gene silencing of the FRET biosensor gene.

TABLE I. Efficient Generation of Transgenic Mice Expressing Functional FRET Biosensors

Target	FRET biosensor	Injected	Born	Positive
PKA	AKAR3EV	239	105 (43%)	19 (18%)
None	AKAR3EV-NC	73	17 (23%)	7 (41%)
ERK	EKAREV	93	44 (47%)	2 (5%)
ERK	EKAREV-NLS	63	22 (34%)	7 (30%)

Fertilized eggs derived from Jcl:B6C3F1 (B57BL/6N Jcl X C3H/HeN Jcl) mice were microinjected with a mixture of Tol2 mRNA and respective pT2A-derived FRET biosensor vectors. Thirteen to fifteen eggs were transplanted to each foster mouse

Observation of these transgenic mice by TPEM could yield the real-time maps of PKA and ERK activities in skin, enteric mucosa in normal or inflamed states, with greatly improved signal-to-background ratios. Firstly, we demonstrated the utility of the transgenic mice expressing FRET biosensors by visualizing the activation of PKA and ERK in the epidermis. Upon localized laser ablation of the auricular epithelium, PKA was rapidly and diffusely activated in the squamous epithelium, as monitored by the increase in FRET/CFP ratio (Fig. 2A). This observation suggested that the immediate response in PKA was probably caused by the heat, and that the sustained activation was

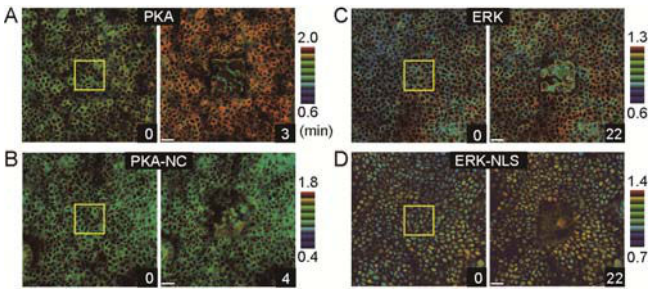

Figure 2 Rapid activation of PKA and ERK in laser-ablated skin.

The auricular epidermis of transgenic mice expressing AKAR3EV (A), AKAR3EV-NC (B), EKAREV (C), or EKAREV-NLS (D) were observed by TPEM. AKAR3EV-NC lacks the PKA phosphorylation site and serves as a negative control. FRET biosensors are localized at the cytoplasm (A, B, C) or the nucleus (D). Laser ablation was performed in the region demarcated by the yellow box. The colored side bars show the FRET/CFP ratio ranges. Scale bar = 20 μm.

caused by chemical mediators, damaged-associated molecular pattern molecules (DAMPs) [17, 18]. In addition, epinephrine is known to activate PKA via the β2-adrenergic receptor in keratinocytes [19]. In transgenic mice expressing the negative control FRET biosensor, no increase in FRET/CFP ratio was observed in the squamous epithelium (Fig. 2B). In basal layer cells adjacent to laser-ablated lesions,

ERK was rapidly activated, followed by a gradual increase in activity. Unlike PKA activation, the initiation of activation was delayed in cells adjacent to the lesion in a distance-dependent manner (Fig. 2C and 2D).

The transgenic mice described here allow direct video-monitoring of agonist/antagonist pharmacodynamics affecting various signaling molecules in different cell types of live mice. The marked activation of ERK in activated granulocytes prompted us to examine the effect of ERK inhibition on the migration of granulocytes induced by arachidonic acid (Fig. 3). ERK activity in granulocytes dropped within a few minutes after intravenous injection of a MEK inhibitor, PD 0325901. Interestingly, granulocytes halted migration approximately ten minutes after the inhibition of ERK activity. Close examination of cell

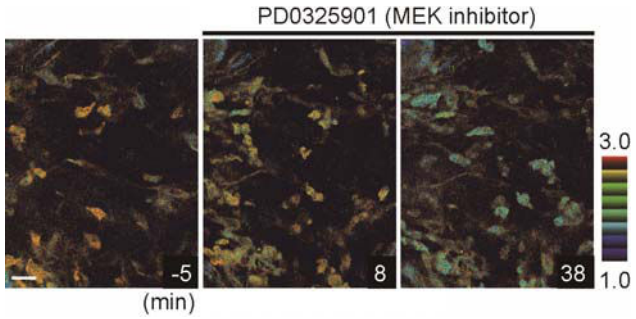

Figure 3 Real-time monitoring of the pharmacodynamics of a MEK inhibitor.

Time-lapse 2P FRET images of the auricular dermis in a transgenic mouse expressing EKAREV, four hours after treatment with 10% arachidonic acid. A MEK inhibitor, PD0325901, was injected intravenously (5 mg/kg) at time zero. The colored side bars show the FRET/CFP ratio ranges. Scale bar = 20 μm

movement in the video images revealed that several minutes after ERK inhibition, each granulocyte stochastically stopped migration, but still continued to extend pseudopodia in many directions. Because granulocyte migration within the connective tissue requires attachment to and detachment from the extracellular matrices, our observation suggests that ERK activity may be required for the detachment process.

The transgenic mouse expressing the FRET biosensors also provides a tool to untangle the upstream signaling pathways that lead to chemotaxis in living mice. The arachidonic acid-induced mobilization and ERK activation of granulocytes in the dermis was suppressed by a competitive antagonist of a chemotactic cytokine LTB4, LY293111, injected at 0 min (Fig. 4). Following laser ablation at 83 min induced rapid ERK activation and chemotaxis of the neighboring granulocytes toward the injured region, indicating that tissue damage-induced chemotaxis does not require LTB4 signaling. Injection of PD0325901 at 95 min suppressed ERK activity and inhibited migration of granulocytes. A second laser ablation of the nearby tissue at 129 min no more activated ERK nor evoked chemotaxis of granulocytes. These data showed the essential role of ERK activity in chemotaxis of granulocytes in live mice.

Considering the enormous number of drugs that activate/inactivate PKA either directly or indirectly via Gs or Gi-coupled receptors, transgenic mice expressing the PKA FRET biosensor will provide an ideal animal model to study the pharmacodynamics of these pro- or anti-PKA drugs. To investigate this possibility, the cAMP phosphodiesterase inhibitor theophylline and a cAMP analogue, dibutyryl cyclic AMP (dbcAMP), were sequentially administered to these

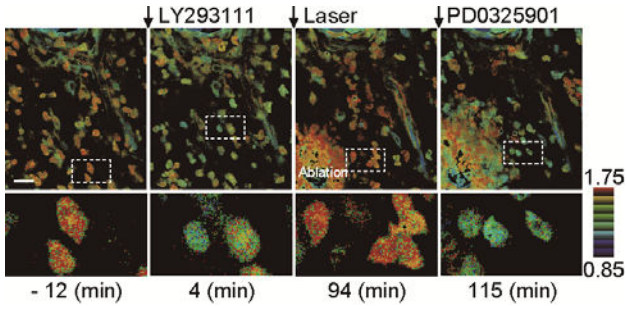

Figure 4 Effect of LTB4 receptor antagonist on granulocyte migration and ERK activity.

Time-lapse FRET images of the auricular dermis in a transgenic mouse expressing EKAREV four hours after treatment with 10% arachidonic acid. Antagonist of LTB_4 receptor, LY293111, was injected intravenously (4 mg/kg) at time zero. PD0325901 was injected intravenously (5 mg/kg) at 96 minutes. Lower images show dotted areas. The colored side bars show the FRET/CFP ratio ranges. Scale bar = 20 μm

transgenic mice (Fig. 5). PKA activity was monitored in the muscular layer of the small intestine, where intermuscular plexus neurons, smooth muscles, and endothelial cells were observed for their sensitivity to the reagents. Intestinal peristalsis was simultaneously quantified by the drift of the plexus neurons. Upon theophylline administration, PKA activity increased modestly in the smooth muscle cells, endothelial cells, and intermuscular plexus neurons. However, this modest increase in PKA was able to suppress the

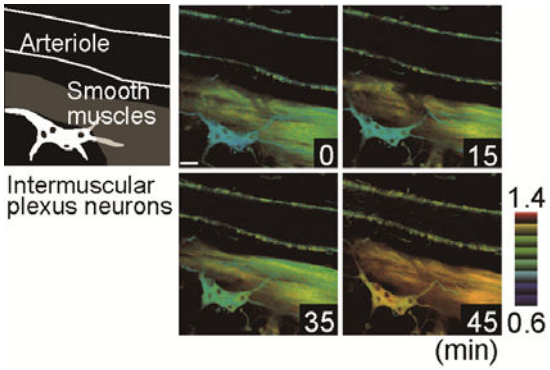

Figure 5 Monitoring the effect of PKA stimulators on PKA activity and peristalsis of the small intestine.

Intravital FRET imaging of the muscular layer of the small intestine of a transgenic mice expressing AKAR3EV. cAMP-specific phosphodiesterase inhibitor, theophylline, and a cAMP analogue, dbcAMP, were administered intravenously at 5 and 35 minutes after acquisition of the images. The colored side bars show the FRET/CFP ratio ranges. Scale bar = 20 μm

intestinal peristalsis only transiently. Additional injection of dbcAMP fully activated PKA in the smooth muscle cells and intermuscular plexus neurons, which resulted in the cessation of peristalsis. Remarkably, dbcAMP increased the PKA activity in the endothelial cells only marginally. Thus, the difference in the sensitivity to PKA agonists was also readily visible in the transgenic mice expressing the PKA FRET biosensor.

IV. Conclusion

Experiments with cultured cells have yielded a great amount of information about signaling pathways over the past half century. However, there remain enormous gaps in our understanding between in vitro and in vivo. The successful generation of transgenic mice expressing FRET biosensors has the potential to elucidate cellular events by direct observation. In this study, we could observe the activities of ERK and PKA in various tissues of the transgenic mice expressing FRET biosensors. However, the high expression of FRET biosensors suffers from two flaws. First, the ubiquitous expression could perturb endogenous signalling pathways and affect mice development of the transgenic mice. In fact, we found that a high and ubiquitous expression of a FRET biosensor for Ras causes infertility of transgenic mice. Second, in solid tissues such as brain, identification of cell types is often difficult by the morphology alone. To circumvent these problems, we have developed a second line of transgenic mice expressing the FRET biosensors in a Cre recombinase dependent manner. Reporter mice expressing additional FRET biosensors will likely become indispensable in the development and screening of drugs targeting specific signaling molecules, and the assessment of both pharmacodynamics and drug toxicity.

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