Intravital imaging of mice expressing Förster resonance energy transfer (FRET) biosensors

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Background

In the past half century, there has been tremendous progress in the understanding of oncogene (or growth factor) signal transduction cascades. Growth factors activate receptor tyrosine kinase, which turns on Ras and triggers activation of the serine threonine kinase cascades consisting of Raf, MEK, and ERK (1). This signal transduction cascade has been extensively characterized by the use of biochemical techniques in cancer cell lines, generally under the assumption that cancer cells are a homogenous population. We now know cancer cells are heterogeneous and change their properties via their interaction with stromal cells. Therefore, we need techniques to examine the activity of signaling molecules at the single cell resolution in vivo.

One such technique is using genetically encoded biosensors based on the principle of Förster resonance energy transfer (FRET) to monitor the activity changes of signaling molecules in living cells (2, 3). Despite the increasing ubiquity of these biosensors, their application has mostly been limited to cultured cells that transiently express the FRET biosensor constructs, due to particular difficulties in the development of cell lines and transgenic mice with FRET biosensors. In previous studies on the generation of transgenic mice expressing FRET biosensors, the analysis has usually been conducted in vitro with cells isolated from the tissues of transgenic mice (4–8), and only a few studies have successfully pursued FRET imaging in living tissues (7, 8), probably due to the low levels of expression.

Discussion

The typical structure of genetically encoded FRET biosensors comprises the sensor and ligand domains, which are sandwiched by cyan-fluorescent protein (CFP) and yellow-fluorescent protein (YFP) (Fig. 1, upper panel). The sensor domain serves to monitor the activity changes of signaling molecules. Upon conformational change caused by the molecule of interest, the sensor domain binds to the ligand domain. In the case of the biosensor for ERK, for example, the sensor domain is the substrate peptide of ERK (9, 10). The phosphorylated-sensor domain binds to the WW phosphothreonine-binding domain, which brings CFP in close proximity to YFP and causes FRET.

The inefficiency in the development of transgenic mice expressing the FRET biosensor was recently overcome by three groups using different methods. First, by using insulators, Yamaguchi, et al. expressed FRET biosensors for caspase activity (11). Johnsson, et al. expressed
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Fig. 1. The structure of a typical FRET biosensor is shown in the upper panel. YFP denotes the yellow fluorescent protein used as a FRET donor, and CFP denotes the cyan fluorescent proteins used as FRET acceptors. Upon conformational change of the sensor domain induced by, for example, phosphorylation or GTP binding, the ligand domain binds to the sensor domain, bringing CFP and YFP in close proximity and, thereby, increasing the Förster resonance energy transfer. FRET biosensors can be expressed in transgenic mice, yielding a model known as the FRET mouse. The FRET mouse is a versatile tool for understanding the cancer cell heterogeneity, drug sensitivity, and stromal cell response. Two-photon microscopy can be used for the live imaging of signaling molecule activity in this model. The cancer cells can be sorted according to the activity of the signaling molecules by FACS, which will open the way for biochemical or genetic analyses.

Lastly, we found that transposon-mediated gene transfer ensured high expression of FRET biosensors and a biosensor for Rac1 by inserting the expression unit into the ROSA26 locus (12).
developed more than ten transgenic mouse lines, which are collectively called FRET mice (13). It is worth noting that this transposon-mediated gene transfer was originally established to generate cell lines expressing FRET biosensors (10).

What questions are currently being answered by intravital imaging with FRET biosensors? Intravital imaging has been extensively used to examine cancer invasion and metastasis (14, 15). In both invasion and metastasis, Rho-family GTPases are known to play critical roles (16); however, the activity changes of Rho-family GTPases in vivo were not known until recently. By observing glioblastoma cells that stably express FRET biosensors for Rho-family GTPases, Hirata, et al. showed that glioblastoma cells invading at the front of a tumor mass exhibit higher Rac1 activity than trailing cells (17). The Rac1 activity in glioblastoma cells fluctuated over a timescale that was substantially longer than that of the cell cycle, suggesting that the intrinsic heterogeneity in Rac1 activity may facilitate the generation of glioblastoma cells with a high invasion capacity (18).

Such heterogeneity was also observed in mammary tumors developed in mouse mammary tumor virus (MMTV)-Neu transgenic mice, which are widely used as a mouse model of HER2/Neu-positive luminal-type breast cancer (19, 20). Kumagai, et al. crossed transgenic mice expressing a FRET biosensor for ERK with the MMTV-Neu mice and sorted the resulting mammary tumor cells depending on their ERK activity (21) (Fig. 1). Interestingly, the efficiency of mammosphere formation was higher in the ERKlo population than the ERKhi cell population, suggesting that ERK activity suppresses cancer stem cell (CSC) properties. Therefore, drugs targeting HER2, such as trastuzumab and lapatinib, which culminate in the inhibition of ERK activity, may increase the fraction of cancer stem cells.

In experiments using Eisuke mice, it was recently revealed how growth factor signals are propagated in mouse skin. Hiratsuka, et al. found that ERK activation in the epidermal basal layer cells could be propagated radially to the neighboring cells, a phenomenon called spatial propagation of radial ERK activity distribution, or SPREAD (22). The classical protocol to induce papilloma in the skin, i.e., topical application of 12-O-tetradecanoylphorbol-13-acetate (TPA), significantly increased the frequency of SPREAD. The SPREAD frequency was correlated with the appearance of mitosis, suggesting that ERK activation may regulate G2/M exit to the G1 phase. The initiation of SPREAD depends on epidermal growth factor receptor and ADAM-family matrix metalloproteinases. This observation showed for the first time how growth signals are propagated within the epidermal cells, while also demonstrating the power of intravital imaging of FRET mice. This observation also showed how the so-called autocrine signaling of growth factors works. It would be interesting to examine how the growth signals are propagated in squamous cell carcinoma in situ.

Future Directions

As mentioned above, the intravital imaging of FRET mice will help us to understand the signal transduction pathways underlying cancer cell heterogeneity.

- CSCs: Imaging of cells expressing the CSC markers in vivo will provide clues as to where the CSCs replicate and differentiate. To further explore the regulation of CSCs in terms of not only replication and differentiation, but also migration, invasion, and metastasis, the activities of key signaling molecules must be observed in vivo. ERK and Rho-family...
GTPases are the critical regulators of cell growth and cell migration, respectively; therefore, \textit{in vivo} FRET imaging of these activities in combination with the stem cell markers will be of great interest.

- \textbf{Invasion and metastasis:} \textit{In vivo} imaging of cancer cells revealed several types of invasion (14). The mechanism underlying the switches between different types of migration will be clarified by using cancer cells expressing FRET biosensors.

There are some differences in response to the anticancer drugs between the tissue culture cells and cancer cells \textit{in vivo}. The effect of anticancer drugs can be examined easily using FRET biosensors both \textit{in vitro} and \textit{in vivo}.

- \textbf{Drug sensitivity of cancer cells:} By using a FRET biosensor as a target molecule, real-time monitoring of the effect of the anticancer drug is possible (23). The same approach will also answer which cancer cell population is particularly resistant to the anticancer drug \textit{in vivo}. Even in the case that the proper FRET biosensor is not available for the drug target molecule, FRET biosensors for apoptosis or stress could be used as surrogate markers.

- \textbf{Sensitivity to radiation therapy:} By using a FRET biosensor against DNA damage, for example, a FRET biosensor for ATM (24), it would be possible to monitor the effects of radiation at a single cell resolution. Again, this approach could help explain why a small fraction of cells is resistant to radiation therapy.

Fibroblasts, macrophages, and other stromal cells could promote or inhibit cancer cell growth. However, other than their close proximity, there is no evidence that cancer cells actually interact with the surrounding stromal cells. FRET biosensors are the tools to study such intercellular communications.

- \textbf{Communication between cancer cells and stromal cells:} Time-lapse FRET imaging of the cancer cells and stromal cells will reveal how signals are transmitted between these two cell types.

- \textbf{Optogenetic or chemical biological tools:} A critical problem of intravital imaging is the difficulty of inducing perturbation into the cells. Recently, optogenetic or drug-inducible tools to activate cells of interest have been made available. By combining these techniques, we can observe whether the cell of interest can transduce signals to the neighboring cells. We have shown that ERK activation in an epithelial cell can trigger ERK activation in the neighboring cells \textit{in vitro} (25). Introducing this system to mice will help us to delineate the mechanism of signal propagation \textit{in vivo}.

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