Live visualization of a functional RET-EGFP chimeric receptor in homozygous knock-in mice

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学 位 論 文 の 内 容 要 旨

Live visualization of a functional RET-EGFP chimeric receptor in homozygous knock-in mice

ホモ接合型ノックインマウスにおける機能的な RET-EGFP キメラ受容体の生体内可視化

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SUMMARY

Introduction

Signaling receptors within the plasma membrane play various roles in maintaining homeostasis in living cells and tissues. Among them, the receptor tyrosine kinases (RTKs) for growth factors exert essential functions in organogenesis during development. Upon ligand binding to RTKs, ligand-RTK complexes are internalized into cells via endocytosis, transported to specific subcellular regions within the cells, and exert their physiological functions to regulate appropriate cell behaviors and physiological functions. For example, Trk receptor tyrosine kinases, which are signaling receptors for neurotrophins, show long-range retrograde transport within the growing axons. This process is required for neuronal survival and subsequent elongation of axons. Thus, the appropriate trafficking of ligand-RTK complexes supports the physiological functions of living cells. However, actual visualization of RTK transport were made possible by in vitro systems which often employs overexpression of RTKs. It remains unknown how RTKs are trafficked in a physiological condition and how RTK trafficking regulates cell behaviors in vivo. To address these questions, it is essential to develop a system that allows the detection of RTK trafficking in vivo.

RET is a RTK that can act as the signaling receptor for another neurotrophic factor family, the GDNF family ligands. RET is essential for development of the enteric nervous system (ENS) and kidneys in mice. Mutations in the human RET gene cause multiple diseases such as Hirschsprung disease (congenital absence of the ENS) and neuroendocrine tumors. Although previous studies revealed that RET regulates multiple processes in organogenesis including cell proliferation, migration and differentiation, little is known about the subcellular localization and trafficking of RET in the living cells and tissues. To address this issue, we generated a new knock-in mouse line that expresses the RET-EGFP chimeric receptor under the *Ret* promoter.

Methods

The RET-EGFP knock-in mouse line was generated by inserting a cassette composed of human RET51 cDNA fused with EGFP cDNA into the mouse *Ret* locus via homologous recombination in embryonic stem cells. To confirm the expression of the RET-EGFP receptor, total RNAs were isolated from embryonic guts and kidneys of $Ret^{RET\text{-}EGFP/RET\text{-}EGFP}$ mice, and RT-PCR was performed. Histological analyses (Acetylcholinesterase, X-gal and immunofluorescence staining) were conducted to examine the phenotypes of $Ret^{RET\text{-}EGFP/RET\text{-}EGFP}$ mice and the distribution pattern of RET-EGFP proteins in cells or tissues. To visualize RET-EGFP protein in living cells, time-lapse imaging was performed in a culture of enteric neural crest-derived cells (ENCCs), motor neurons and sympathetic neurons of $Ret^{RET\text{-}EGFP/RET\text{-}}$

^{EGFP} mouse embryos.

Results

By homologous recombination in mouse ES cells, we inserted the RET-EGFP chimeric receptor cDNA under the *Ret* promoter. $Ret^{RET\text{-}EGFP/RET\text{-}EGFP}$ mice grew to adult with no overt abnormality, which contrasted to the neonatal lethality observed in *Ret*-deficient mice. RT-PCR analysis revealed that $Ret^{RET\text{-}EGFP/RET\text{-}EGFP}$ mice expressed only human but not mouse *Ret* mRNA. These results demonstrate that *Ret* deficiency was rescued by the *RET-EGFP* allele and suggest that RET-EGFP exert the biological function of RET in organogenesis.

We further examined the biological function of the RET-EGFP receptor in organogenesis by crossing the *RET-EGFP* mice to the *Ret-tauLacZ* mice (*Ret*-null) to visualize overall *Ret*-expressing tissues by X-gal staining. $Ret^{tauLacZ/+}$ embryo, which show no deficit, was used as control. As reported previously, $Ret^{tauLacZ/tauLacZ}$ embryo showed complete absence of the ENS and kidneys, and impaired development of sympathetic chain ganglia. In contrast, $Ret^{tauLacZ/RET\text{-}EGFP}$ embryo displayed normal ENS, kidneys and sympathetic chain ganglia. To investigate the ENS phenotype in more details, we performed acetylcholinesterase staining and PGP9.5 staining of the gut in adult $Ret^{RET\text{-}EGFP/RET\text{-}EGFP}$ mice. No difference in enteric neuron numbers was detected in all of the gut regions examined (duodenum, ileum and colon) between wild type (wt) and $Ret^{RET\text{-}EGFP/RET\text{-}EGFP}$ mice. These results collectively indicate that RET-EGFP exerts the physiological function of RET.

To explore the subcellular distribution of the RET-EGFP molecules in cells, we performed immunohistochemical analyses of *Ret*-expressing cells in various tissues derived from $Ret^{RET\text{-}EGFP/RET\text{-}EGFP}$ mice. In developing ENS, we found that the native fluorescent signals of RET-EGFP were detected in all of differentiating enteric neurons. Furthermore, fluorescent signals of RET-EGFP were detected not only at the plasma membrane but also in the cytoplasm, which displayed a punctate pattern. We further investigated the distribution of RET-EGFP in spinal nerves and sympathetic nerves in newborn $Ret^{RET\text{-}EGFP/RET\text{-}EGFP}$ mice by anti-neurofilament and anti-tyrosine hydroxylase antibodies, respectively. A punctate pattern of RET-EGFP proteins was observed within these axons. Together, these results suggest that RET-EGFP proteins are transported within the neuronal cell bodies and axons.

To investigate the dynamics of the RET-EGFP molecules in living cells, we performed time-lapse imaging of axons of sensory, motor and sympathetic neurons in culture. These analyses revealed an active movement of RET-EGFP puncta in axons of all neuronal types. RET-EGFP puncta appeared to be transported primarily in the retrograde direction in sensory and motor axons. We observed that the velocity of RET-EGFP transport was significantly higher in motor axons than that in sympathetic axons. To examine whether retrograde transport of RET-EGFP particles occur in vivo, we performed time-lapse imaging of the thoracodorsal nerve of $Ret^{RET\text{-}EGFP/RET\text{-}EGFP}$ embryos. The analysis revealed a robust retrograde movement of RET-EGFP particles. These results confirmed that RET-EGFP is actively transported within these axons in vivo.

Because the migration of ENS precursors (ENCCs: enteric neural crest-derived cells) requires RET signaling, we also examined the trafficking of RET-EGFP in ENCCs by time-lapse imaging. RET-EGFP signals were detected both in the cell membrane and cytoplasm, as were observed in immunohistochemical analyses. Although RET-EGFP was widely distributed in the cytoplasm, an accumulation of GFP signals in the frontal perinuclear region was observed. We speculated these RET-EGFP to be in the endoplasmic reticulum and Golgi apparatus. Moreover, RET-EGFP displayed a punctate pattern that actively transported in both endo- and exo-cytic directions. To confirm whether RET-EGFP is actually trafficked by endocytic pathway, we performed double immunostaining of ENCCs with anti-GFP, and with anti-Rab5 and –Rab11 antibodies, which detected early and recycling endosomes, respectively. Signals of GFP overlapped partially with those of Rab5 or Rab11. These results demonstrate that the trafficking of RET-EGFP is indeed mediated by the endocytic pathways.

Conclusion

In this study, to examine the spatiotemporal pattern of subcellular localization of RET in living cells, we generated a new knock-in mouse line that expresses the RET-EGFP chimeric receptor under the *Ret* promoter. Because $Ret^{RET\text{-}EGFP/RET\text{-}EGFP}$ mice display normal organogenesis, RET-EGFP serves as the functional RET receptor. We succeeded in visualization of RET-EGFP protein in living cells and found that RET is actively transported within neuronal axons and cell bodies. This transport is at least partly mediated by endocytic pathway. RET-EGFP mice provide a unique platform that enables for examining the dynamics and physiology of RET trafficking. Future studies using this system will contribute to elucidation of the biological significance of RET transport in RET-dependent organogenesis and pathogenesis.

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論文審査の結果の要旨

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（要旨は１，０００字～２，０００字程度）

Receptor tyrosine kinases (RTKs), signaling receptors for growth factors regulate various aspects of cellular physiology in organogenesis. Upon ligand binding to RTKs, ligand-RTK complexes are internalized into cells, transported to specific subcellular regions, and exert their physiological functions for regulating appropriate cell behaviors. The RET receptor tyrosine kinase acts as the signaling receptor for GDNF family ligands and play an important role in development of the enteric nervous system (ENS) and kidneys. RET regulates multiple cellular processes, such as cell proliferation, migration and differentiation in organogenesis, suggesting that appropriate localization and trafficking of RET are crucial for its physiological functions. However, little is known about the subcellular localization and RET transport in living cells and tissues.

To address this issue, we generated a *RET-EGFP* knock-in mouse line that expresses the RET-EGFP chimeric receptor under the *Ret* promoter. $Ret^{RET\text{-}EGFP/RET\text{-}EGFP}$ mice were viable and grew to adults, which contrasted to *Ret*-deficient mice that die after birth due to absence of the enteric nervous system (ENS) and kidneys. RT-PCR analysis revealed expression of RET-EGFP but not the endogenous mouse *Ret* mRNA in $Ret^{RET\text{-}EGFP/RET\text{-}EGFP}$ embryos. These results suggest that the RET-EGFP allele exerts function equivalent to that of wild-type *Ret* allele. To further examine the biological function of RET-EGFP allele in organogenesis, we crossed RET-EGFP mice to the *Ret-tauLacZ* mice (*Ret*-null) to visualize gross morphologies of *Ret*-expressing tissues by X-gal staining. Although $Ret^{tauLacZ/tauLacZ}$ embryos displayed the absence of the ENS and kidneys, $Ret^{tauLacZ/RET\text{-}EGFP}$ embryos exhibited no obvious deficit in these organs, and the patterns of X-gal staining were comparable between $Ret^{tauLacZ/RET\text{-}EGFP}$ and $Ret^{tauLacZ/+}$ embryos. We also examined the phenotypes of the ENS in adult $Ret^{RET\text{-}EGFP/RET\text{-}EGFP}$ mice, and found that there was no difference in enteric neuron numbers between wild-type and homozygous mutant mice. These results collectively indicate that the RET-EGFP exerts the physiological function of wild-type RET.

To explore the subcellular distribution of the RET-EGFP molecules, we performed immunohistochemical analyses of *Ret*-expressing cells in various tissues derived from $Ret^{RET\text{-}EGFP/RET\text{-}EGFP}$ mice. In developing ENS, fluorescent signals of RET-EGFP were detected both on the cell membrane and in the cytoplasm of enteric neural crest-derived cells (ENCCs). In spinal nerves and sympathetic nerves, a punctate pattern was observed within these axons. Time-lapse imaging of cultured neurons and migrating ENCCs revealed active movement of RET-EGFP puncta in neuronal axons and cell bodies, which suggests that RET-EGFP proteins are transported within the neuronal cell bodies and axons. Immunostaining of ENCCs with anti-Rab5 or anti-Rab11 antibodies (detecting early or recycling endosomes, respectively) revealed that EGFP signals partially colocalized with Rab5 or Rab11, suggesting that the trafficking of RET-EGFP is partly mediated by the endocytic pathway.

In summary, the RET-EGFP mice provide a unique platform that enables visualization of the spatiotemporal dynamics of functional RET protein in living cells. Future studies using this system will contribute to elucidation of the biological significance of RET trafficking in Ret-dependent organogenesis and pathogenesis.

The candidate, having completed studies on RET functions in organogenesis, with a specialty in live imaging of subcellular localization of RET protein, and having advanced the field of knowledge in the area of developmental cell biology, is hereby recognized as having qualified for the degree of Ph.D. (Medicine).