J. Biochem. 2011;150(1):15–22 doi:10.1093/jb/mvr068

JB THE JOURNAL OF BIOCHEMISTRY

JB Review

Ectodomain shedding and remnant peptide signalling of EGFRs and their ligands

Received March 10, 2011; accepted May 9, 2011; published online May 24, 2011

Shigeki Higashiyama[1,2,*], Daisuke Nanba[1,3], Hironao Nakayama[2], Hirofumi Inoue[1,2] and Shinji Fukuda[2]

[1]Department of Cell Growth and Tumor Regulation, Proteo-Medicine Research Center (ProMRes), Ehime University; [2]Department of Biochemistry and Molecular Genetics, Ehime University Graduate School of Medicine; and [3]Senior Research Fellow Center, Ehime University, Shitsukawa, Toon, Ehime 791-0295, Japan

*Shigeki Higashiyama, Department of Biochemistry and Molecular Genetics, Ehime University Graduate School of Medicine, Shitsukawa, Toon, Ehime 791-0295, Japan. Tel: 089-960-5253, Fax: 089-960-5256, email: shigeki@m.ehime-u.ac.jp

Both receptor tyrosine kinases epidermal growth factor receptors (EGFRs) and their ligands are transmembrane proteins. It has been known that ligand binding activates cytoplasmic tyrosine kinase domains of EGFRs, resulting in the transduction of signals for cell proliferation, migration, differentiation or survival. In an EGFRs-ligands system, however, signal transduction occurs not only unidirectionally but also bidirectionally, which is regulated by cell–cell contact and proteolytic cleavage. Recent studies of proteolytic cleavage 'ectodomain shedding' of EGFRs and their ligands mediated by membrane-type metalloproteinases, a disintegrin and metalloproteinases have been unveiling novel functions and molecular mechanism of their remnant peptides. In addition, the study of the remnant peptide signalling would be essential for understanding the physiological and pathological relevance of anti-shedding therapeutic strategies for diseases such as cancer.

Keywords: ADAM/ectodomain shedding/EGFR/EGFR ligand/remnant peptide signalling.

Abbreviations: ADAM, a disintegrin and metalloproteinases; AREG, amphiregulin; BTC, betacellulin; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GPCR, G-protein coupled receptor; HB-EGF, heparin-binding EGF-like growth factor; NRG, neuregulin; TGF-α, transforming growth factor-α.

Various types of transmembrane proteins on cell surface, especially receptors bearing protein kinase domain are crucial players to regulate cell behaviour and fate. Among them, the epidermal growth factor (EGF) receptor (EGFR/ErbB) family are transmembrane tyrosine kinases and have been spearheading the study of cell signal transduction. In order to activate tyrosine kinase domain of EGFRs, the binding of core domain (EGF-like domain) of ligands to EGFRs is essential. The ligand family of EGFRs consist of 13 members, all of which have two characteristic domains in common, a functional EGF-like domain characterized by around 50 amino acid and 3 disulphide bridges and a transmembrane domain with hydrophobic amino acid stretch in addition to N-terminal signal sequence. The extracellular EGF-like domains of all ligands are enough potent in principle to activate receptors and induce cell growth and proliferation. In growth factor research field, the impact of this potency was too impressive, which had left behind the understanding of physiological meaning as membrane proteins. Therefore, it took long period to highlight the ligands as membrane proteins.

Two kinds of landmark events have been leading the study of proteolytic cleavage of EGFRs and their ligand family members, named as 'ectodomain shedding'; one is the identification of a metalloproteinase responsible for release of tumour necrosis factor-α (TNF-α) from its transmembrane precursor, TNF-α converting enzyme (TACE), which belongs to A Disintegrin And Metalloproteinases (ADAMs) (*1*, *2*) and the other the molecular study of EGFR transactivation by G-protein coupled receptor (GPCR) ligands (*3*), 'Triple Membrane-Passing Signal' (Fig. 1) (see the next section). Furthermore, it has been shown that ectodomain shedding of plasma membrane proteins triggers intra-membrane cleavage mediated by γ-secretase (*4*) (Fig. 2), which made the event of ectodomain shedding more attractive for researchers and advanced its study. For example, the ectodomain of ErbB4 is shed by ADAM17 (TACE), which is a prerequisite step for subsequent γ-secretase cleavage of the remnant peptide to release intracellular domain (ICD) fragment. Pro-Neuregulin (proNRG) 1 and pro-betacellulin (proBTC), ligands of EGFRs, also produce ICDs by the sequential processing with ADAMs and γ-secretase. On the contrary, in the case of pro-heparin-binding EGF-like growth factor (proHB-EGF) and pro-amphiregulin (proAREG), also ligands of EGFRs, their ectodomain shedding triggers endocytosis of carboxy-terminal (C-terminal) remnant fragment with transmembrane domain instead of proceeding to the second cleavage by γ-secretase (Fig. 2).

Here, we review ectodomain shedding of EGFRs and their ligands by ADAMs and highlight on the potent role of their remnant peptides in intracellular signalling produced by ectodomain shedding.

Featured Article

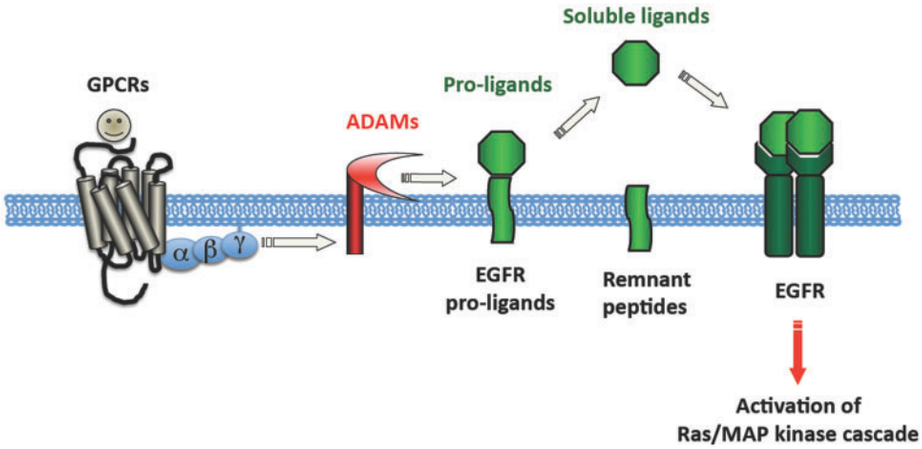

Fig. 1 Triple membrane-passing signal mechanism of EGFR transactivation by GPCR. GPCR-induced EGFR transactivation requires ectodomain shedding of EGFR ligands. ProHB-EGF is a representative ligand shed by GPCR activation.

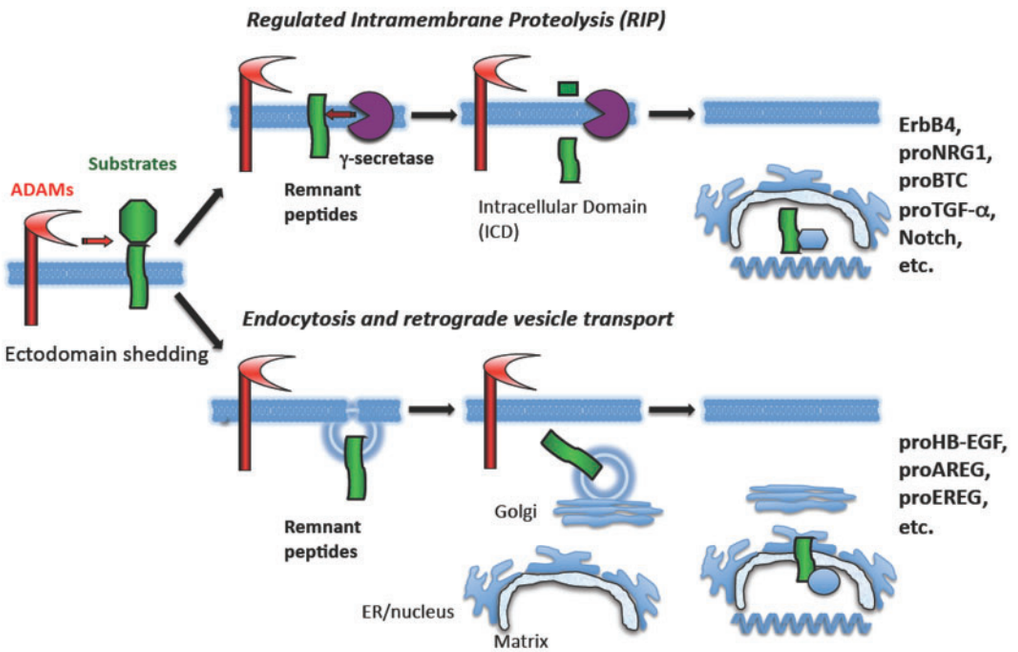

Fig. 2 Remnant peptide signalling pathways. Ectodomain shedding triggers the second cleavage by γ-secretase to produce the ICD peptides of ErbB4, proNRG-1, proBTC, proTGF-α (Higashiyama, S., unpublished observation), Notch, etc. On the contrary, ectodomain shedding of proHB-EGF, proAREG and proepiregulin (proEREG) (Higashiyama, S., unpublished observation) induces endocytosis of the remnant peptides instead of the second cleavage by γ-secretase. The remnant peptides regulate transcriptional factors in different ways.

Significance of ectodomain shedding by ADAMs in an EGFRs-ligands system

The EGFR family, receptor tyrosine kinases, comprise four members EGFR (ErbB1), ErbB2, ErbB3 and ErbB4 and are well known to be driver force of cell proliferation, migration, differentiation and survival. Dysregulation of them directly links to developmental abnormalities and diseases such as cancer. The activation of EGFRs triggered by binding of their specific ligands which consists of 13 members [EGF, transforming growth factor-α (TGF-α), AREG, HB-EGF, betacellulin (BTC), epiregulin, epigen, neuregulin-1 (NRG-1), NRG-2, NRG-3, NRG-4, NRG-5 and NRG-6] (*5*) induces their homo- and heterodimer formation and evoke the following phosphorylation cascade of adaptor molecules to lead to transcriptional activation of essential genes for cell response. Intriguingly, both EGFRs and their ligands are type I transmembrane proteins, which makes us imagine that an EGFRs-ligands system functions in a juxtacrine manner by cell–cell contact as shown in a Notch signalling system (*6*). However, even under physiological conditions, the ligands have been detected in body fluids such as blood, urine and saliva, suggesting that the ligands are available as released soluble forms rather than membrane-anchored forms and the activation of EGFRs seems to be controlled by

post-translational processing of the ligands, ectodomain shedding. Physiological significance of ectodomain shedding of EGFR ligands *in vivo* has been demonstrated clearly in the study of proHB-EGF by generation of soluble *hb-egf* and uncleavable *hb-egf* gene knock-in mice (*7*), the former of which constitutively released HB-EGF and the latter was highly resistant for ectodomain shedding, respectively. Soluble *hb-egf* knock-in mice showed severe hyperplasia of keratinocytes in skin and cradiomyocytes in heart and died in chimeric condition or heterozygote. On the other hand, uncleavable *hb-egf* gene knock-in mice lived in homozygote, but survived no longer over 8 months, because of severe heart failure characterized by similarity to the phenotype of dilated cardiomyopathy. These observations emphasize that ectodomain shedding of proHB-EGF must be a tightly regulated event in development, even more in pathological conditions. These observations would also indicate physiological significance of ectodomain shedding of not only other EGFR ligands, but also a wide range of plasma membrane proteins.

Recently, inter-receptor cross-talk has received significant attention as an essential element in understanding the increasingly complex signalling networks identified within cells. Among them, transactivation of EGFR has been shown to play a crucial role in signalling by GPCRs, cytokine receptors, receptor tyrosine kinases and integrins to a variety of cellular responses. It has been reported that transactivation of EGFR is evoked by two kinds of signalling ways, ligand-dependent and independent pathways. The ligand-dependent pathway requires ectodomain shedding of ligands mediated by activation of ADAMs to release soluble ligands into the extracellular space. The released ligands activate EGFR in an autocrine manner. This system is named as 'Triple Membrane-Passing Signal' (Fig. 1) (*3*, *8*). The ligand-independent transactivation of EGFR is mediated intracellular signal molecules such as Ca^{2+}, Ca^{2+}-calmodulin-dependent protein kinase, protein kinase C and Src family kinases (*8*, *9*). It has been shown that cellular Src (c-Src) phosphorylates the cytoplasmic tyrosine residues, Tyr-845 and Tyr-1101 of EGFR, and that the Tyr-845 phosphorylation is critical for the mitogenic response to both the EGFR and a GPCR for lysophosphatidic acid (*10*). Therefore, the ligand-dependent and independent pathways of EGFR transactivation would be distinguishable by metalloproteinase inhibitors (*11*, *12*). However, recent reports showed that β2-adrenargic receptor and muscarinic M2-induced EGFR transactivation was ligand-independent and resulted in an incomplete downstream signalling involving extracellular signal-regulated kinase (ERK), but not phospholipase C-gamma 1 (PLCγ1) and Akt (*11*, *12*). These findings indicate that the ligand shedding-dependent and independent pathways of EGFR transactivation are appropriately activated according to the properties of ligands.

Thirteen members of human ADAMs have been characterized as active metalloproteinases (*13*) and ADAM9, 10, 12, 15, 17 and 19 have been characterized in ectodomain shedding of EGFRs and the ligands. Among these ADAMs, ADAM10 and 17 seem to be key molecules in physiological shedding of the seven EGFR-ligands. Based on studies with cells lacking various ADAMs, ADAM17 is considered the principal sheddase for pro-forms of TGF-α, HB-EGF, AREG, epiregulin and epigen, whereas ADAM10 was identified as the major sheddase for pro-forms of EGF and BTC (*14*). The relevance of ADAM17 in shedding of proTGF-α, proHB-EGF and proAREG *in vivo* was corroborated by studies in mice lacking *adam17*, which resemble mice lacking *tgf-α* in that they have open eyes at birth and wavy whiskers, or mice lacking *areg* with respect to defects in mammary ductal development, or mice lacking *hb-egf* with respect to defects in heart valve development. On the other hand, substrate specificity of ADAMs for EGFR ligands is still obscure, even though many different kinds of interactive factors of ADAM cytoplasmic tails have been reported (*13*). An attractive hypothesis has been that ADAMs selectively target a subset of substrates, perhaps via an interaction between the extracellular domains of the enzyme and substrate, and that this accounts, at least in part, for the substrate selectivity. For further understanding of the substrate recognition mechanism of ADAMs, crystallography and computational analysis of ADAMs or ADAM/substrate complexes might be essential. Recent review by Takeda (*15*) might give a good insight to understand how ADAMs recognize substrates. ADAMs have distinct characteristic domains, pro-peptide (P), metalloproteinase (M), disintegrin-like (D), cysteine-rich (C), epidermal growth factor-like (EGF), transmembrane (TM) and cytoplasmic (CT) domains. The mammalian ADAMs are most closely related to the P-III class of snake venom metalloproteinases (SVMPs). Vascular apoptosis-inducing protein-1 (VAP1) was the first P-III SVMP structure to be resolved by X-ray crystallography (*16*), and contains M, D and C (MDC) domains, which are shared by ADAMs. Takeda found an interesting region designated hyper-variable region (HVR) in C domain which opposed to the catalytic site and putatively assigned protein-binding site. He indicated the intriguing possibility that the HVR creates an exocite for binding substrates and proposed substrate recognition models of ADAMs mediated by the HVR (*15*). Recently, regulatory molecules to interact with both ADAMs and EGFR ligands at extracellular space have been reported. Nardilysin (*N*-arginine dibasic convertase, also known as Nrd1 and NRDc) mediates interaction between ADAM17 and HB-EGF or NRG1 and regulates its shedding event (*17*, *18*). AREG-regulated protein 35 (ARP 35) also bound both ADAM17 and AREG and regulated its shedding event (Nakayama, H., and Higashiyama, S., unpublished data). These observations would support the attractive substrate recognition model through the HVR of ADAMs. Further study will be required to understand the basis for the substrate selectivity and regulation of ADAMs under various physiological and pathological conditions.

Remnant peptide signalling of ErbB4

Among the EGFRs, ectodomain shedding is unique for jm-a ErbB4 isoform which has a longer stalk region (16 amino acid residues) and seems simply to permit accessibility of ADAM17 (*19*). ErbB4 shedding can be activated by phorbol ester TPA in all types of cells tested and ligand (neuregulin) binding in some type of cells (*20*, *21*). While shed ErbB4 ectodomain with 120 kDa may simply neutralize neuregulin activity, which is not demonstrated yet, the remnant 80 kDa transmembrane peptide was subsequently subjected to intramembranous cleavage by γ-secretase and is converted to a soluble 80-kDa ICD peptide. ErbB4-ICD was found in the cytoplasm, nucleus and mitochondria (*22*, *23*). ErbB4-ICD interacts with TAB2 and thereby with N-CoR, a corepressor, and chaperons to become a complex to translocate into the nucleus. This system is required for correct control of astrogenesis in mouse development (*24*). ErbB4-ICD also binds STAT5 and seems to function in mammary differentiation (*25*). It is also reported that ErbB4-ICD is functionally involved in the development of mammary gland and breast cancer (*26*). While ErbB4 nuclear localization has been observed in normal and tumour mammary tissues and exogenous ErbB4-ICD expression provokes differentiation events, it has not yet been demonstrated that the ErbB4 cleavage and the production of endogenous ErbB4-ICD is physiologically relevant in this tissue. ErbB4-ICD also binds to other transcriptional regulators, ETO2 (*27*), YAP (*28*) and oestrogen receptor (*29*), and regulates their nuclear localization. In these models, ErbB4-ICD is proposed to function as a chaperon of transcriptional regulators. ErbB4-ICD is still active as a tyrosine kinase (*30*) and phosphorylates Mdm2, a regulator of p53 (*31*). Interestingly, intact ErbB4 does not phosphorylate Mdm2. These results, many of which have been produced in experimental cell systems only, suggest that proteolytic processing of ErbB4 by ADAM and γ-secretase allows new signalling functions to occur.

On the other hand, other EGFRs including jm-b ErbB4 isoform, EGFR, ErbB2 and ErbB3 have relatively short stalk regions (6–9 amino acid residues) and are not likely to be subjected to a significant level of ADAM-mediated ectodomain shedding.

Remnant peptide signalling of pro-neuregulin 1

NRG is the subfamily of EGFR ligands and directly binds to ErbB3 and/or ErbB4. The NRG family consists of six members (NRG1–6) (*5*). The prototype and best-studied member of the family is NRG1. From the single NRG1 gene, various isoforms are produced by alternative splicing and categorized to four major forms (Types I–IV) (*32*). Type II isoform is a secreted form and Types I, III and IV are transmembrane forms. In transmembrane NRGs (proNRGs) derived from the NRG1 gene, the intracellular domain differs in both the length and the primary sequence (*33*). Another divergent region of proNRGs is located between the fifth cysteine residue of the EGF-like unit and the transmembrane domain and distinguishes between α and β proNRG isoforms (*34*). This juxtamembrane domain extends from the sixth cysteine residue to the beginning of the transmembrane domain. The juxtamembrane region is also highly variable in both the length and the sequence between NRG isoforms of the same subfamily. Thus β2 isoforms contain the shorter juxtamembrane region, β4 has the larger and β1 has an intermediate length. The β3 isoforms are characterized by the presence of a stop codon in the juxtamembrane region that prevents their association to the plasma membrane and produces a soluble form (*34*). Thus, variation of juxtamembrane region affects the susceptibility for ectodomain shedding by ADAMs. Therefore, the mechanisms and proteolytic components responsible for shedding of proNRGs are still veiled largely, although ADAM17 and ADAM19 have been characterized as sheddases of proNRG1 in cultured cells (*35*). Recently, an interesting report has come out presenting that nardilysin enhanced ectodomain shedding of proNRG1, and was essential for axonal maturation and myelination in the central and peripheral nervous system in mouse, in part, through the modulation of NRG1 shedding (*18*).

Ectodomain shedding of proNRG1 is evoked by the interaction with soluble ErbB4 (*36*). However, within the context of cell–cell contact, it has not yet been demonstrated that intact ErbB4–NRG1 interaction triggers ectodomain shedding of ErbB4 and/or proNRG1. ProNRG1 shedding by ADAMs is followed by the cleavage at the transmembrane domain by γ-secretase and the released intracellular domain (NRG-1-ICD) enters the nucleus to repress the expression of several regulators of apoptosis (*36*). The same group has shown that in the mouse cochlea, synaptic activity increases the level of nuclear NRG-ICD and up-regulates post-synaptic density protein-95 (PSD-95), a scaffolding protein that is enriched in post-synaptic structures. NRG-ICD enhances the transcriptional activity of the PSD-95 promoter by binding to a zinc-finger transcription factor Eos. The NRG-ICD–Eos complex induces endogenous PSD-95 expression *in vivo* through a signalling pathway that is mostly independent of γ-secretase regulation. This upregulation of PSD-95 expression by the NRG-ICD–Eos complex provides a molecular basis for activity-dependent synaptic plasticity (*37*).

Remnant peptide signalling of pro-HB-EGF

Ectodomain shedding of proHB-EGF is frequently observed in many types of cells in culture, involves different ADAMs in different types of cells and has been characterized highly sensitive and lower selective to extracellular stimuli. Phorbol ester TPA is the strongest inducer of proHB-EGF shedding (*38*) and known as an activator of ADAM17 (*14*). TPA-induced proHB-EGF shedding was abrogated in *adam17*-deficient cells (*14*). Mice lacking *hb-egf* showed the defects in heart valve development, which was overlapped with the phenotype of *adam17*-deficient mice (*7*, *39*). These observations implicate that ADAM17 is a prominent candidate among proHB-EGF

sheddases. On the other hand, various GPCR agonists such as bombesin, phenylephrine, angiotensin II, lysophosphatidic acid, thrombin, serotonin, endothelin I and some chemokines are more physiological stimuli of proHB-EGF shedding. GPCR-activated shedding of proHB-EGF are mediated by different ADAM members including ADAM10, 12, 15 as well as 17 (*40–42*), suggesting that proHB-EGF would be a favourable target of ADAMs and an intermediary factor of GPCR signals. Shed HB-EGF can activate EGFR in paracrine and autocrine fashions. Thus, GPCRs pass its signal in part to EGFR via release of HB-EGF by ADAM-mediated ectodomain shedding in an autocrine fashion (Fig. 1). This system would function as a signal amplifier.

Intriguingly, ectodomain shedding of proHB-EGF does not trigger γ-secretase cleavage, which is different from other cases as shown in ErbB4, proNRG1 and proBTC described here. Remnant transmembrane peptide, proHB-EGF-carboxy terminal fragment (HB-EGF-CTF) is internalized as vesicles. HB-EGF-CTF translocates from the plasma membrane into the reticular network of the endoplasmic reticulum (ER) and the nuclear envelope by retrograde membrane trafficking (*43*, *44*). Immunoelectron microscopy revealed the localized HB-EGF-CTF in the inner nuclear membrane (*44*). Since this process is impaired by the inhibition of metalloproteinase activity, translocation of HB-EGF-CTF into the nuclear envelope is a shedding dependent event. Furthermore, promyelocytic leukaemia zinc finger (PLZF) and B-cell leukaemia 6 (Bcl6) proteins have been identified as binding partners of the cytoplasmic region of proHB-EGF (*43*, *45*). PLZF and Bcl6 are transcriptional repressors with structure domain homology of other BTB/POZ and C2H2-type zinc-finger proteins and suppress cyclin A, c-Myc and HoxD and macrophage inflammatory protein 1-α, CD69 and Cyclin D2 expression, respectively (*5*). These repressors negatively regulate the cell cycle. PLZF and Bcl6 produce transcriptional repression through recruitment of a repressor complex that contains N-CoR, SMRT, Sin3a and histone deacetylases (*46*). Internalized HB-EGF-CTF associates with nuclear PLZF and Bcl6, which might occur at the nuclear periphery. Various recent studies have shown transcriptionally silent genes are located at or translocated to the nuclear periphery upon silencing. Indeed, interaction of HB-EGF-CTF with PLZF or Bcl6 results in the reversal of decreased expression of their target genes (*43*, *45*). Whether HB-EGF-CTF containing a transmembrane domain would directly regulate genes silenced by PLZF and Bcl6 at the nuclear periphery is still unknown.

EGFR signalling promotes G1-phase progression in the cell cycle by regulating the expression of cyclin D and c-Myc via the Ras-MAPK signalling cascade. Therefore, proteolytic cleavage of proHB-EGF by ADAMs generates two types of mitogenic signalling molecules in an autocrine fashion and the coordination of the dual intracellular signals mediated by HB-EGF and HB-EGF-CTF may be important for cell cycle progression. In fact, EGFR and FGFR-mediated c-Myc induction and cell cycle progression in primary cultured mouse embryonic fibroblasts are abrogated by knockout of *hb-egf* gene, or by a metalloproteinase inhibitor, although downstream molecules of the receptors are activated (*47*). Induction of c-Myc expression by EGF or bFGF is recovered in *hb-egf*$^{-/-}$ mouse embryonic fibroblasts by overexpression of wild-type proHB-EGF, but no recovery was observed with an uncleavable mutant of proHB-EGF. The uncleavable mutant also inhibited EGF-induced acetylation of histone H3 at the mouse c-Myc first intron region, which could negatively affect transcriptional activation. Thus, the signal transduction initiated by generation of HB-EGF-CTF in the shedding event plays an essential intermediary role in growth factor-induced cell cycle progression. *PLZF* and *Bcl6* as well as *HB-EGF* are expressed in a large number of tissues including the heart. This suggests that deregulation of PLZF and Bcl6 repression activities mediated by proHB-EGF shedding is involved in regulation of cell proliferation and differentiation in various signalling cascades during the development and maintenance of adult tissues.

Heart failure observed in HB-EGF-null and uncleavable proHB-EGF knock-in mice (*7*, *48*, *49*) might, in part, be due to the loss of HB-EGF-CTF-PLZF/Bcl6 signalling in the heart, where PLZF and Bcl6 are highly expressed and are suggested to be involved in maintaining cardiac function (*50*). Furthermore, it has been reported that cardiomyocytes expressing an uncleavable proHB-EGF mutant in culture underwent apoptosis under normoxic conditions, which distinctly increased under hypoxic conditions due to the increased caspase-3 activity, reactive oxygen species accumulation and an increased c-Jun N-terminal kinase activity (*51*). The induced apoptosis was not rescued by the addition of recombinant soluble HB-EGF. Therefore, the molecular mechanism underlying this phenomenon might be explained by the loss of HB-EGF-CTF function. BAG-1 has been reported as the binding protein of the cytoplasmic domain of proHB-EGF and its interaction with proHB-EGF leads to decreased cell adhesion, increased resistance to apoptosis and rapid secretion of soluble HB-EGF (*52*). BAG-1 is a multifunctional protein that interacts with a diverse array of molecular targets including the Bcl-2 apoptosis regulator, the 70-kDa heat shock proteins, Hsc70 and Hsp70, nuclear hormone receptors, the RAF kinase, components of the ubiquitinylation/proteasome machinery and DNA (*53*). These findings suggest that BAG-1 might be a target and functional partner of HB-EGF-CTF in heart.

Remnant peptide signalling of pro-amphiregulin

Ectodomain shedding of proAREG is mediated by ADAM17 (*14*) and yields a transmembrane-cytoplasmic fragment (AREG-CTF), as well as a soluble AREG, which closely resembles proHB-EGF shedding and HB-EGF-CTF. The proAREG-shedding stimuli trigger endocytosis of not only AREG-CTF, but also unshed proAREG without activation of

γ-secretase and subsequent intramembrane proteolysis (*54*). AREG-CTF and unshed proAREG translocates from the plasma membrane to the lysosome and/or to the inner nuclear membrane via retrograde membrane trafficking. What determines the different destination of AREG-CTF? C-terminal truncation of AREG-CTF to expose RKKL241 sequence at C-terminal end, which subsequently activated the ER-retrieval signal and allowed the nuclear envelope localization of AREG-CTF. The truncated form of proAREG interacts with A-type lamin and is retained at the inner nuclear membrane. Heterochromatin formation is then induced and global transcription is transiently suppressed. This study gives new insight into epigenetic chromatin organization in mammalian cells: a plasma membrane-anchored growth factor is targeted to the inner nuclear membrane where it participates in dynamic chromatin organization and control of transcription.

Remnant peptide signalling of pro-betacellulin

Ectodomain shedding of proBTC is mediated by ADAM10 (*55*) to produce transmembrane-cytoplasmic fragment (BTC-CTF). BTC-CTF is immediately subjected to intramembranous cleavage by γ-secretase to generate an intracellulear-domain fragment (BTC-ICD), which differs from the cases of proHB-EGF and proAREG described above. Cytoplasmic domain of proBTC was S-palmitoylated, which was required for the stabilization, γ-secretase-dependent production and the nuclear-membrane translocation of BTC-ICD (*55*). The function of BTC-ICD localized at the nuclear-membrane was estimated by overexpression of an extracellular domain-deletion mutant of proBTC, which showed the cell growth inhibition. The detail of this mechanism, however, still remains unclear.

Generation of the remnant peptide signalling of EGFR ligands is characterized by γ-secretase-dependent and -independent pathways. It is still unclear, however, how second cleavage by γ-secretase would be regulated. The study of proBTC might give an insight on this matter. The cytoplasmic domain of proBTC is S-palmitoylated as well as proTGF-α (*56*), both of which are susceptible to γ-secretase. On the other hand, neither S-palmitoylation nor the second cleavege by γ-secretase have been reported on the other EGFR ligands. It is also known that two subunits of γ-secretase complex, APH-1 and nicastrin are S-palmitoylated and that palmitoylation plays a role in raft localization (*57*). These circumstantial evidences might indicate the crucial role of modification of the cytoplasmic tails such as S-palmitoylation to select the pathways.

Conclusion

Ectodomain shedding of transmembrane proteins on cell surface is a tightly regulated event, and its dysregulation leads to developmental abnormalities and diseases. As ectodomain shedding of transmembrane proteins provides signal molecules bidirectionally, remnant peptides produced by this event has given rise to more attractive factors to understand signals in intracellular space. We believe that the knowledge obtained from the study of the EGFRs and their ligands provides new insights into the understanding the role of ectodomain shedding and remnant peptide signalling of other transmembrane proteins as well.

Acknowledgements

We gratefully acknowledge all members of Higashiyama's lab for helpful discussion.

Funding
Grant-in-Aid for Scientific Research from Ministry of Education, Culture, Sports, Science and Technology, Japan #20390082 and #1704168 (to S.H.).

Conflict of interest
None declared.

References

1. Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J.L., Wolfson, M.F., Castner, B.J., Stocking, K.L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K.A., Gerhart, M., Davis, R., Fitzner, J.N., Johnson, R.S., Paxton, R.J., March, C.J., and Cerretti, D.P. (1997) A metalloproteinase disintegrin that releases tumor-necrosis factor- from cells. *Nature* **385**, 729–733
2. Moss, M.L., Jin, S.L.C., Milla, M.E., Burkhart, W., Carter, H.L., Chen, W.J., Clay, W.C., Didsbury, J.R., Hassler, D., Hoffman, C.R., Kost, T.A., Lambert, M.H., Leesnitzer, M.A., McCauley, P., McGeehan, G., Mitchell, J., Moyer, M., Pahel, G., Rocque, W., Overton, L.K., Schoenen, F., Seaton, T., Su, J.L., Warnaer, J., Willard, D., and Becherer, J.D. (1997) Cloning of a disintegrin metalloprotease that processes precursor tumor-necrosis factor-α. *Nature* **385**, 733–7336
3. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402**, 884–888
4. Hartmann, D., Tournoy, J., Safting, P., Annaert, W., and De Strooper, B. (2001) Implication of APP secretases in notch signaling. *J. Mol. Neurosci.* **17**, 171–181
5. Higashiyama, S., Iwabuki, H., Morimoto, C., Hieda, M., Inoue, H., and Matsushita, N. (2008) Membrane-anchored growth factors, the epidermal growth factor family: beyond receptor ligands. *Cancer Sci.* **99**, 214–220
6. Bray, S.J. (2006) Notch signaling: a simple pathway becomes complex. *Nat. Rev. Mol. Cell Biol.* **7**, 678–689
7. Yamazaki, S., Iwamoto, R., Saeki, K., Asakura, M., Takashima, S., Yamazaki, A., Kimura, R., Mizushima, H., Moribe, H., Higashiyama, S., Endoh, M., Kaneda, Y., Takagi, S., Itami, S., Takeda, N., Yamada, G., and Mekada, E. (2003) Mice with defects in HB-EGF ectodomain shedding show severe developmental abnormalities. *J. Cell Biol.* **163**, 469–475
8. Prenzel, N., Zwick, E., Leserer, M., and Ullrich, A. (2000) Tyrosine kinase signalling in breast cancer. Epidermal growth factor receptor: convergence point

for signal integration and diversification. *Breast Cancer Res*. **2**, 184–190
9. Liebmann, C. (2011) EGF receptor activation by GPCRs: an universal pathway reveals different versions. *Mol. Cell. Endocrinol*. **331**, 222–231
10. Tice, D.A., Biscardi, J.S., Nickles, A.L., and Parsons, S.J. (1999) Mechanism of biological synergy between cellular Src and epidermal growth factor receptor. *Proc. Natl Acad. Sci. USA*. **96**, 1415–1420
11. Stirnweiss, J., Valkova, C., Ziesché, E., Drube, S., and Liebmann, C. (2006) Muscarinic M2 receptors mediate transactivation of EGF receptor through Fyn kinase and without matrix metalloproteases. *Cell Signal* **18**, 1338–1349
12. Drube, S., Stirnweiss, J., Valkova, C., and Liebmann, C. (2006) Ligand-independent and EGF receptor-supported transactivation: lessons from β2-adrenergic receptor signalling. *Cell Signal* **18**, 1633–1646
13. Edwards, D.R., Handsley, M.M., and Pennington, C.J. (2008) The ADAM metalloproteases. *Mol. Asp. Med*. **29**, 258–289
14. Sahin, U., Weskamp, G., Kelly, K., Zhou, H.M., Higashiyama, S., Peschon, J., Hartmann, D., Saftig, P., and Blobel, C.P. (2004) Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J. Cell Biol*. **164**, 769–779
15. Takeda, S. (2009) Three-dimensional domain architecture of the ADAM family proteinases. *Semin. Cell Dev. Biol*. **20**, 146–152
16. Takeda, S., Igarashi, T., Mori, H., and Araki, S. (2006) Crystal structures of VAP1 reveal ADAMs' MDC domain architecture and its unique C-shaped scaffold. *EMBO J*. **25**, 2388–2396
17. Nishi, E., Hiraoka, Y., Yoshida, K., Okawa, K., and Kita, T. (2006) Nardilysin enhances ectodomain shedding of heparin-binding epidermal growth factor-like growth factor through activation of tumor necrosis factor-alpha-converting enzyme. *J. Biol. Chem*. **281**, 31164–31172
18. Ohno, M., Hiraoka, Y., Matsuoka, T., Tomimoto, H., Takao, K., Miyakawa, T., Oshima, N., Kiyonari, H., Kimura, T., Kita, T., and Nishi, E. (2009) Nardilysin regulates axonal maturation and myelination in the central and peripheral nervous system. *Nat. Neurosci*. **12**, 1506–1513
19. Rio, C., Buxbaum, J.D., Peschon, J.J., and Corfas, G. (2000) Tumor necrosis factor-|α-converting enzyme is required for cleavage of erbB4/HER4. *J. Biol. Chem*. **275**, 10379–10387
20. Vecchi, M., Baulida, J., and Carpenter, G. (1996) Selective cleavage of the heregulin receptor ErbB-4 by protein kinase C activation. *J. Biol. Chem*. **271**, 18989–18995
21. Zhou, W. and Carpenter, G. (2000) Heregulin-dependent trafficking and cleavage of ErbB-4. *J. Biol. Chem*. **275**, 34737–34743
22. Ni, C.Y., Murphy, M.P., Golde, T.E., and Carpenter, G. (2001) gamma–Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science* **294**, 2179–2181
23. Lee, H.J., Jung, K.M., Huang, Y.Z., Bennett, L.B., Lee, J.S., Mei, L., and Kim, T.W. (2002) Presenilin-dependent gamma-secretase-like intramembrane cleavage of ErbB4. *J. Biol. Chem*. **277**, 6318–6323
24. Sardi, S.P., Murtie, J., Koirala, S., Patten, B.A., and Corfas, G. (2006) Presenilin-dependent ErbB4 nuclear signaling regulates the timing of astrogenesis in the developing brain. *Cell* **127**, 185–197
25. Williams, C.C., Allison, J.G., Vidal, G.A., Burow, M.E., Beckman, B.S., Marrero, L., and Jones, F.E. (2004) The ERBB4/HER4 receptor tyrosine kinase regulates gene expression by functioning as a STAT5A nuclear chaperone. *J. Cell Biol*. **167**, 469–478
26. Jones, F.E. (2008) HER4 intracellular domain (4ICD) activity in the developing mammary gland and breast cancer. *J. Mammary Gland Biol. Neoplasia* **13**, 247–258
27. Linggi, B. and Carpenter, G. (2006) ErbB-4 s80 intracellular domain abrogates ETO2-dependent transcriptional repression. *J. Biol. Chem*. **281**, 25373–25380
28. Komuro, A., Nagai, M., Navin, N.E., and Sudol, M. (2003) WW domain-containing protein YAP associates with ErbB-4 and acts as a co-transcriptional activator for the carboxyl-terminal fragment of ErbB-4 that translocates to the nucleus. *J. Biol. Chem*. **278**, 33334–33341
29. Zhu, Y., Sullivan, L.L., Nair, S.S., Williams, C.C., Pandey, A.K., Marrero, L., Vadlamudi, R.K., and Jones, F.E. (2006) Coregulation of estrogen receptor by ERBB4/HER4 establishes a growth-promoting autocrine signal in breast tumor cells. *Cancer Res*. **66**, 7991–7998
30. Linggi, B., Cheng, Q.C., Rao, A.R., and Carpenter, G. (2006) The ErbB-4 s80 intracellular domain is a constitutively active tyrosine kinase. *Oncogene* **25**, 160–163
31. Arasada, R.R. and Carpenter, G. (2005) Secretase-dependent tyrosine phosphorylation of Mdm2 by the ErbB-4 intracellular domain fragment. *J. Biol. Chem*. **280**, 30783–30787
32. Birchmeier, C. (2009) ErbB receptors and the development of the nervous system. *Exp. Cell Res*. **315**, 611–618
33. Wen, D., Suggs, S.V., Karunagaran, D., Liu, N., Cupples, R.L., Luo, Y., Janssen, A.M., Ben-Baruch, N., Trollinger, D.B., Jacobsen, V.L., Meng, S.U., Lu, H.S., Hu, S., Chang, D., Yang, W., Yanigahara, D., Koski, R., and Yarden, Y. (1994) Structural and functional aspects of the multiplicity of Neu differentiation factors. *Mol. Cell Biol*. **4**, 1909–1919
34. Holmes, W.E., Sliwkowski, M.X., Akita, R.W., Henzel, W.J., Lee, J., Park, J.W., Yansura, D., Abadi, N., Raab, H., Lewis, G.D., Shepard, H.M., Kuang, W.J., Wood, W.I., Goeddel, D.V., and Vandlen, R.L. (1992) Identification of heregulin, a specific activator of p185erbB2. *Science* **256**, 1205–1210
35. Horiuchi, K., Zhou, H.M., Kelly, K., Manova, K., and Blobel, C.P. (2005) Evaluation of the contributions of ADAMs 9, 12, 15, 17, and 19 to heart development and ectodomain shedding of neuregulins β1 and β2. *Dev. Biol*. **283**, 459–471
36. Bao, J., Wolpowitz, D., Role, L.W., and Talmage, D.A. (2003) Back signaling by the Nrg-1 intercellular domain. *J. Cell Biol*. **161**, 1133–1141
37. Bao, J., Lin, H., Ouyang, Y., Lei, D., Osma, A., Kim, T.W., Mei, L., Dai, P., Ohlemiller, K.K., and Ambron, R.T. (2004) Activity-dependent transcription regulation of PSD-95 by neuregulin-1 and Eos. *Nat. Neurosci*. **7**, 1250–1258
38. Goishi, K., Higashiyama, S., Klagsbrun, M., Ishikawa, M., Mekada, E., and Taniguchi, N. (1995) Phorbol ester induces the rapid processing of cell surface heparin-binding EGF-like growth factor: conversion from juxtacrine to paracrine growth factor activity. *Mol. Biol. Cell* **6**, 967–980
39. Jackson, L.F., Qiu, T.H., Sunnarborg, S.W., Chang, A., Zhang, C., Patterson, C., and Lee, D.C. (2003) Defective valvulogenesis in HB-EGF and TACE-null mice is associated with aberrant BMP signaling. *EMBO J*. **22**, 2704–2716
40. Tanida, S., Joh, T., Itoh, K., Kataoka, H., Sasaki, M., Ohara, H., Nakazawa, T., Nomura, T., Kinugasa, Y.,

Ohmoto, H., Ishiguro, H., Yoshino, K., Higashiyama, S., and Itoh, M. (2004) The mechanism of cleavage of EGFR ligands induced by inflammatory cytokines in gastric cancer cells. *Gastroenterology* **127**, 559–569
41. Hart, S., Fischer, O.M., Prenzel, N., Zwick-Wallasch, E., Schneider, M., Hennighausen, L., and Ullich, A. (2005) GPCR-induced migration of breast carcinoma cells depends on both EGFR signal transactivation and EGFR-independent pathways. *Biol. Chem.* **386**, 845–855
42. Asakura, M., Kitakaze, M., Takashima, S., Liao, Y., Ishikura, F., Yoshinaka, T., Ohmoto, H., Node, K., Yoshino, K., Ishiguro, H., Asanuma, H., Sanada, S., Matsumura, Y., Takeda, H., Beppu, S., Tada, M., Hori, M., and Higashiyama, S. (2002) Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. *Nat. Med.* **8**, 35–40
43. Nanba, D., Mammoto, A., Hashimoto, K., and Higashiyama, S. (2003) Proteolytic release of the carboxy-terminal fragment of heparin-binding EGF-like growth factor causes nuclear export of PLZF. *J. Cell Biol.* **163**, 489–502
44. Hieda, M., Isokane, M., Koizumi, M., Higashi, C., Tachibana, T., Shudo, M., Heida, Y., Taguchi, T., and Higashiyama, S. (2008) Membrane-anchored growth factor HB-EGF on cell surface targets to the inner nuclear membrane. *J. Cell Biol.* **180**, 763–769
45. Kinugasa, Y., Hieda, M., Hori, M., and Higashiyama, S. (2007) The carboxy-terminal fragment of proHB-EGF reverses Bcl6-mediated gene expression. *J. Biol. Chem.* **282**, 14797–14806
46. Perissi, V., Jepsen, K., Glass, C.K., and Rosenfeld, M.G. (2010) Deconstructing repression: evolving models of co-repressor action. *Nat. Rev. Genet.* **11**, 109–123
47. Nanba, D., Inoue, H., Shigemi, Y., Shirakata, Y., Hashimoto, K., and Higashiyama, S. (2008) An intermediary role of proHB-EGF shedding in growth factor-induced c-myc expression. *J. Cell. Physiol.* **214**, 465–473
48. Iwamoto, R., Yamazaki, S, Asakura, M., Takashima, S., Hasuwa, H., Miyado, K., Adachi, S., Kitakaze, M., Hashimoto, K., Rabb, G., Klagsbrun, M., Nanba, D., Higashiyama, S., Hori, M., and Mekada, E. (2003) HB-EGF and ErbB signaling is essential for heart function. *Proc. Natl Acad. Sci. USA.* **100**, 3221–3226
49. Nanba, D., Kinugasa, Y., Morimoto, C., Koizumi, M., Yamamura, H., Takahashi, K., Takakura, N., Mekada, E., Hashimoto, K., and Higashiyama, S. (2006) Loss of HB-EGF in smooth muscle or endothelial cell lineages causes heart valve malformation and cardiac hypertrophy. *Biochem. Biophys. Res. Commun.* **350**, 315–321
50. Yoshida, T., Fukuda, T., Hatano, M., Koseki, H., Okabe, S., Ishibashi, K., Kojima, S., Arima, M., Komuro, I., Ishii, G., Miki, T., Hirosawa, S., Miyasaka, N., Taniguchi, M., Ochiai, T., Isono, K., and Tokuhisa, T. (1999) The role of Bcl6 in mature cardiac myocytes. *Cardiovasc. Res.* **42**, 670–679
51. Uetani, T., Nakayama, H., Okayama, H., Okura, T., Higaki, J., Inoue, H., and Higashiyama, S. (2009) Insufficient shedding of proHB-EGF enhances hypoxic cell death in H9c2 cardiomyoblasts via activation of Caspase-3 and c-Jun N-terminal kinase. *J. Biol. Chem.* **284**, 12399–12409
52. Lin, J., Hutchinson, L., Gaston, S.M., Raab, G., and Freeman, M.R. (2001) BAG-1 is a novel cytoplasmic binding partner of the membrane form of heparin-binding EGF-like growth factor: a unique role for proHB-EGF in cell survival regulation. *J. Biol. Chem.* **267**, 30127–30132
53. Townsend, P.A., Cutress, R.I., Sharp, A., Brimmell, M., and Packham, G. (2003) BAG-1: a multifunctional regulator of cell growth and survival. *Biochim. Biophys. Acta.* **1603**, 83–98
54. Isokane, M., Hieda, M., Hamakawa, H., and Higashiyama, S. (2008) Membrane-anchored growth factor amphiregulin on cell surface targets to the inner nuclear membrane. *J. Cell Sci.* **121**, 3608–3618
55. Stoeck, A., Shang, L., and Dempsey, P.J. (2010) Sequential and gamma-secretase-dependent processing of the betacellulin precursor generates a palmitoylated intracellular-domain fragment that inhibits cell growth. *J. Cell Sci.* **123**, 2319–2331
56. Shum, L., Turck, C.W., and Derynck, R. (1996) Cysteines 153 and 154 of transmembrane transforming growth factor-alpha are palmitoylated and mediate cytoplasmic protein association. *J. Biol. Chem.* **271**, 28502–28508
57. Cheng, H., Vetrivel, K.S., Drisdel, R.C., Meckler, X., Gong, P., Leem, J.Y, Li, T., Carter, M., Chen, Y., Nguyen, P., Iwatsubo, T., Tomita, T., Wong, P.C., Green, W.N., Kounnas, M.Z., and Thinakaran, G. (2009) S-palmitoylation of gamma-secretase subunits nicastrin and APH-1. *J. Biol. Chem.* **284**, 1373–1384