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Mammary tumors derive from mammary epithelial cells that lost the control for both cell growth and differentiation. Activation of a G protein coupled receptor (GPCR) transactivates the EGF receptor (EGFR) growth signal by inducing metalloprotease dependent release of the EGFR ligand HB-EGF. Here, we demonstrated that expression of a membrane bound ADAM Kuzbanian (KUZ) in COS-7 cells stimulates the transactivation of EGFR by bombesin, while a dominant negative form of KUZ (DNK), which lacks the protease domain blocked endogenous protease mediated tranactivation. This transactivation process was very sensitive to the inhibitors of ADAM type metalloprotease, the EGFR autophosphorylation, and HG-EGF. The role of KUZ in this transactivation appeared to be specific, since evolutionarily close related ADAM TACE did not elicit the transactivation. These results show that KUZ is the physiologically relevant metalloprotease that mediates the transactivation of EGFR by GPCR through enhancing the supply of HB-EGF. To investigate the effect of blocking KUZ in mammary neoplasia, transgenic mice conditionally expressing DNK, mutant TIMP3 and activated Notch4 have been constructed.

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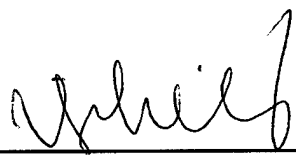

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Introduction

Mammary tumors derive from mammary epithelial cells that lost the control for both cell growth and differentiation. To cease the neoplastic growth in mammary epithelial cells, it is crucial to identify a key molecular switch as a target. In this project, I propose that matrix metalloprotease Kuzbanian (KUZ) is a valid target for molecular intervention, because it is the only metalloprotease known so far that operates in both the cell fate determination and the cell growth control.

KUZ belongs to the ADAM family, which has both a matrix metalloprotease domain and a disintegrin domain. Genetic analysis identifies KUZ as a key regulator of Notch pathway in making cell fate decision. KUZ is responsible for the cleavage of extra-cellular domain of Notch receptors and cell surface ligand Delta, which activates the Notch signaling pathway. Excess activation of Notch4 invariably leads to the development of mouse mammary tumor. In addition, KUZ has been shown to cleave growth factor precursors to generate the mature growth factor.

Binding of mature growth factors, i.e. EGF, to receptor EGFR, triggers the phosphorylation of EGFR and the recruitment of adaptor proteins, such as SHC, Grb2 and Gab1. This complex then turns on the Raf / Ras growth signal cascade, which function as a major cell growth pathways.

Activation of G-protein-coupled receptor (GPCR) also leads to the phosphorylation of EGFR. A process called **transactivation**. Recently, it has been reported that GPCR transactivates EGFR by stimulating the production of HB-EGF, a potent mitotic EGFR ligand. Metalloprotease inhibitors block the transactivation; however, the specific metalloprotease that mediates the transactivation remains elusive.

Results (body)

mKUZ mediates EGFR transactivation by GPCR.

To determine the metalloprotease responsible for transactivation, COS-7 cells were transfected with mouse Kuzbanian (KUZ), and the EGFR activation was examined after treating cells with a GPCR ligand bombesin. In COS-7 cells, agonist binding to bombesin receptor, which coupled to Gq heterotrimeric G protein, elicits the mitogenic effect through the activation of MAP kinase pathway. This effect can be attributed to the transactivation of EGFR by GPCR (1). Indeed, treatment of COS-7 with bombesin lead to a drastic increase of EGFR phosphorylation. This induction of EGFR phosphorylation by GPCR was further enhanced in cells transfected with mKUZ, suggesting that mKUZ positively affected the transactivation process (Figure 1, 2). This is in contrast to the report that MDC9, a very similar ADAM, does not enhance the transactivation of EGFR (1).

On the other hand, transfection of COS-7 with a dominant negative mutant of mKUZ (DNK), which lacks the metalloprotease domain, resulted in clear reduction in the GPCR induced EGFR phosphorylation. In fact, after bombesin treatment, the level of EGFR phosphorylation in the DNK transfected cells were lower than that in cells transfected by a vector plasmid, indicating that DNK may block the endogenous KUZ mediated EGFR transactivation in COS-7.

The metalloprotease activity of KUZ was directly responsible for the elevated transactivation seen in KUZ transfected cells. Treatment of cells with either the general metalloprotease inhibitor GM6001 or the broad spectrum ADAM-type metalloprotease inhibitor TAPI all inhibited the phosphorylation of EGFR initiated with GPCR (Figur 4). In the catalytic site of metalloprotease family, the key metal-binding residues are invariable. Mutation of a glutamic acid into an alanine in this conserved region destroys the protease activity of the MMP. When such a catalytically inactive KUZ was introduced in COS7 cells, the transactivation of EGFR was reduced suggesting the catalytic activity is responsible for the transactivation.

The kinase activity of EGFR directed the phosphorylation of EGFR after treating cells with the GPCR ligand, because the specific EGFR autophosphorylation inhibitor AG1478 inhibited the transactivation of EGFR, confirming the previous observation (1). And the activation of EGFR was directly related to the HB-EGF in the cells, since the HB-EGF antigonist CRM194 eliminated the transactivation of EGFR (Figure 3). All the evidences suggested that GPCR activate KUZ, which in turn releases the HB-EGF as a potent EGFR stimulator.

The ability of the KUZ to stimulate the EGFR activation by GPCR was also manifested in the activation and recruitment of EGFR adapter proteins, such as SHC and Gab1. Activation of all three forms of SHC was enhanced in the cells transfected with KUZ, but reduced in the cells transfected with DNK (Figure 2). The similar enhancement was seen in the phosphorylation and binding of Gab1 to the EGFR and SHC complex. These results further validated that KUZ is a mediator of GPCR induced EGFR activation.

To confirm the growth signal pathway is activated by KUZ through the GPCR transactivation of EGFR, activation of Ras is detected using a fusion Raf1 protein that pulls down only the activated Ras. When cells were treated with

bombesin, there was a significant increase of activated Ras, and this increase was further elevated by the expression of KUZ, but not DNK. The results demonstrated that the metalloprotease activity of KUZ contributes to the activation of Ras-mediated growth signaling pathway.

The role of KUZ in transactivation appeared to be specific, since another evolutionarily close-related ADAM, the TNF-a-Converting Enzyme (TACE) did not elicit the transactivation (figure 5). More than one membrane bound metalloproteases in ADAM family can process cell surface ligand such as HB-EGF. For example, ADAM9 specifically cleaves the HB-EGF under the regulation of PKC in Vero cells (2), but it does not involve in the transactivation, since neither the wild type nor the dominant negative ADAM9 affects the GPCR induced EGFR phosphorylation (1). Cells transfected with TACE did not elevate the EGFR phosphorylation upon stimulation by bombesin. Unlike DNK, the protease domain deleted TACE had no effect on the EGFR phosphorylation. In addition, TACE did not affect the recruitment of SHC, or Grb2, and the activation of Ras. These results strongly suggested that TACE do not involve in the transactivation process. The results also support the notion that KUZ may be a physiologically relevant mediator of GPCR transaction of EGFR.

Test the role of ADAM in mammary neoplasia in transgenic mice.

To test the role of ADAM type metalloproteases in mammary neoplasia, I have used the strategy of conditional inhibition of KUZ in mammary gland. The strategy seeks to control the KUZ activity in the mammary gland by conditional expressing the dominant negative KUZ (DNK) and the tissue inhibitor of metalloprotease 3 (TIMP3). Therefore, the effect of blocking KUZ in mammary neoplastic growth can be investigated *in vivo* in the presence of regulated amount of these KUZ inhibitors.

The experiment deviated from the original design, which is to introduce TIMP3 mutant directly under the control of mammary specific promoter. Instead, the DNK and TIMP3 mutant are placed under the Tetracycline Responsible Element (TRE), so that their expression is regulated by the presence of tetracycline transactivator (tTA). The level of function tTA, in turn is regulated by amount of tetracycline in the drinking water for mice. The expression of the tTA can be specified to mammary gland by using tissue specific promoter, such as the whey acidic protein (WAP) promoter.

I constructed TRE-DNK, TRE-TIMP3 and TRE-Notch4. TIMP3 has been shown to inhibit the ADAM *in vitro*, and Notch4 is the downstream signaling component of the KUZ. Together, this set of transgenic mice can be used to explore the mechanism of KUZ function in the initial stage of the neoplastic growth. These constructs were linearized, and DNAs purified. The resulting DNAs were introduced into the pronuclei of fertilized egg by microinjection. Five founder lines for TRE-DNK, two lines of TRE-TIMP3 and two lines of TRE-Notch4 of transgenic mice were generated.

Currently, experiments are on going to establish the expression profile of transgene in these mice by mating them with Tet-o-tTA mice, which express tTA in all the tissue when there is no tetracycline present. After the profiles of the transgene expression in these mice have been established, these mice will be used to mate with mice expressing the tetracycline transactivator (tTA) in mammary gland, such as mice that express tTA under the control of the whey acidic protein

promotor (WAP-tTA). The resulting double transgenic mice will have the transgene—dominant KUZ or inhibitor TIMP3--express in the mammary gland when the tetracycline is withdraw from their drinking water, which turns on the tTA expression. Thus the role of KUZ in normal mammary gland development and in different stages of mammary gland neoplastic growth can be examined in detail *in vivo*.

Key Research accomplishments:

Demonstrated that ADAM-type metalloproteases KUZ mediates the cross-talk between the G-protein coupled receptor and EGF receptor signaling pathways, two major pathways that control cell behavior.

Developed three types of transgenic mice that carry the dominant negative KUZ, its downstream effector INT3 and its inhibitor TIMP3, respectively.

Reportable Outcomes:

Three types of transgenic mice that carry the dominant negative KUZ, its downstream effector INT3 and its inhibitor TIMP3, respectively r.

Conclusions:

Researches for the reporting period established the role of ADAM-type metalloproteases KUZ as a mediator for the two major signaling pathways, which are crucial for both growth control and differentiation. The discovery essentially proved that KUZ is one of the best targets to control the neoplastic growth of mammary epithelial cells.

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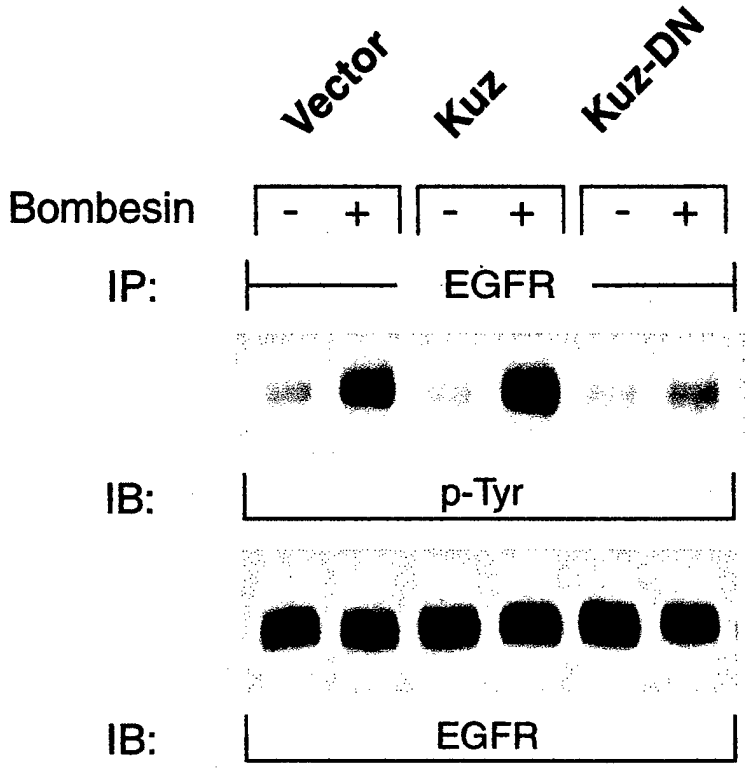
Figure 1



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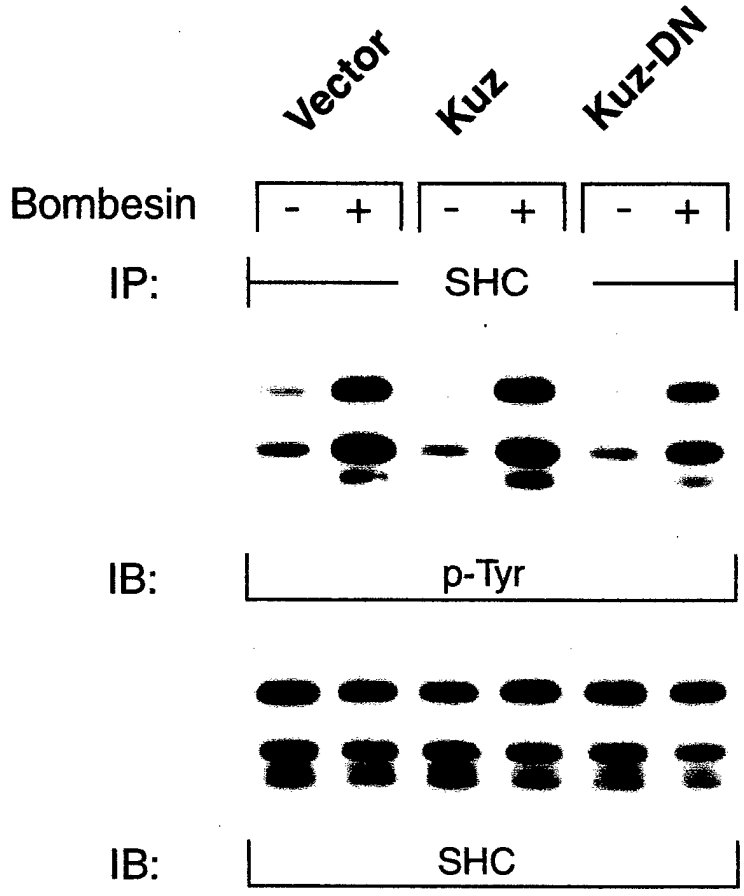

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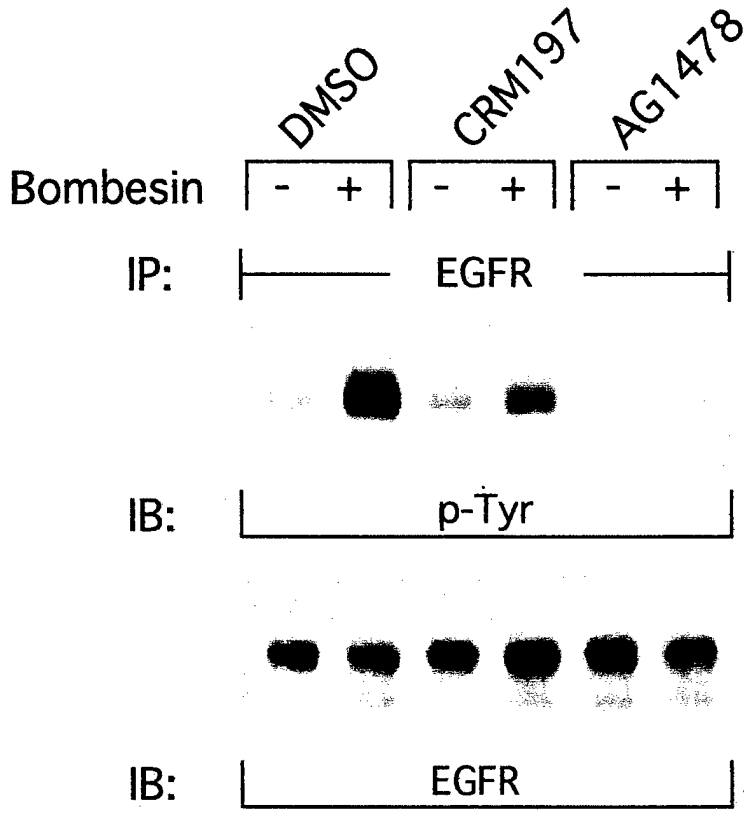

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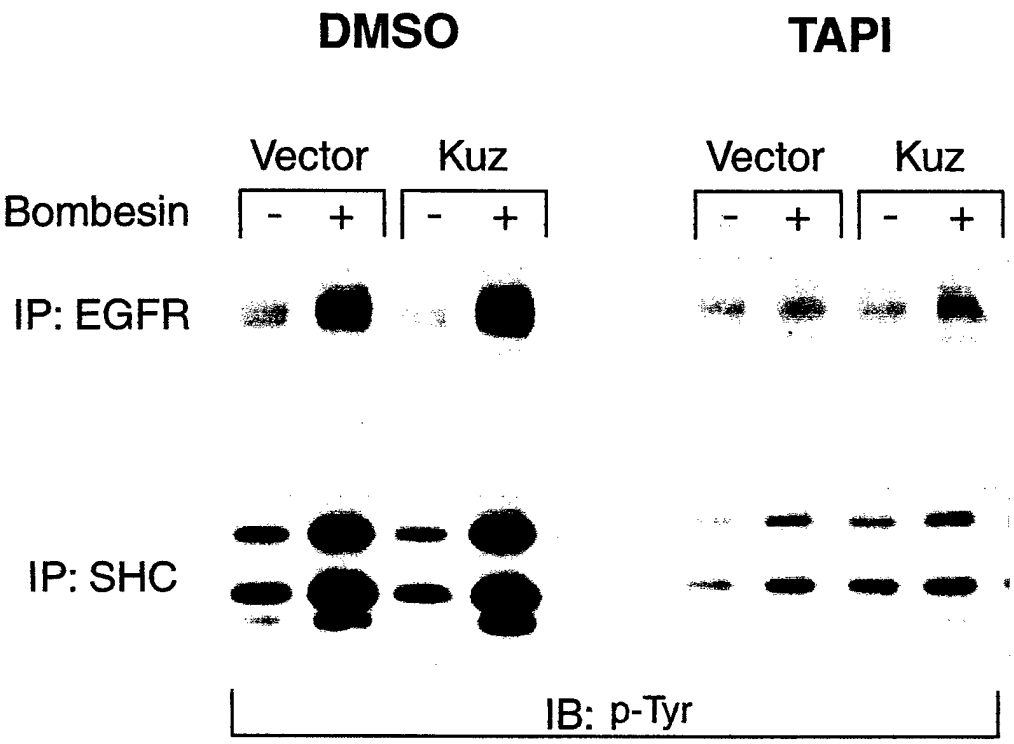
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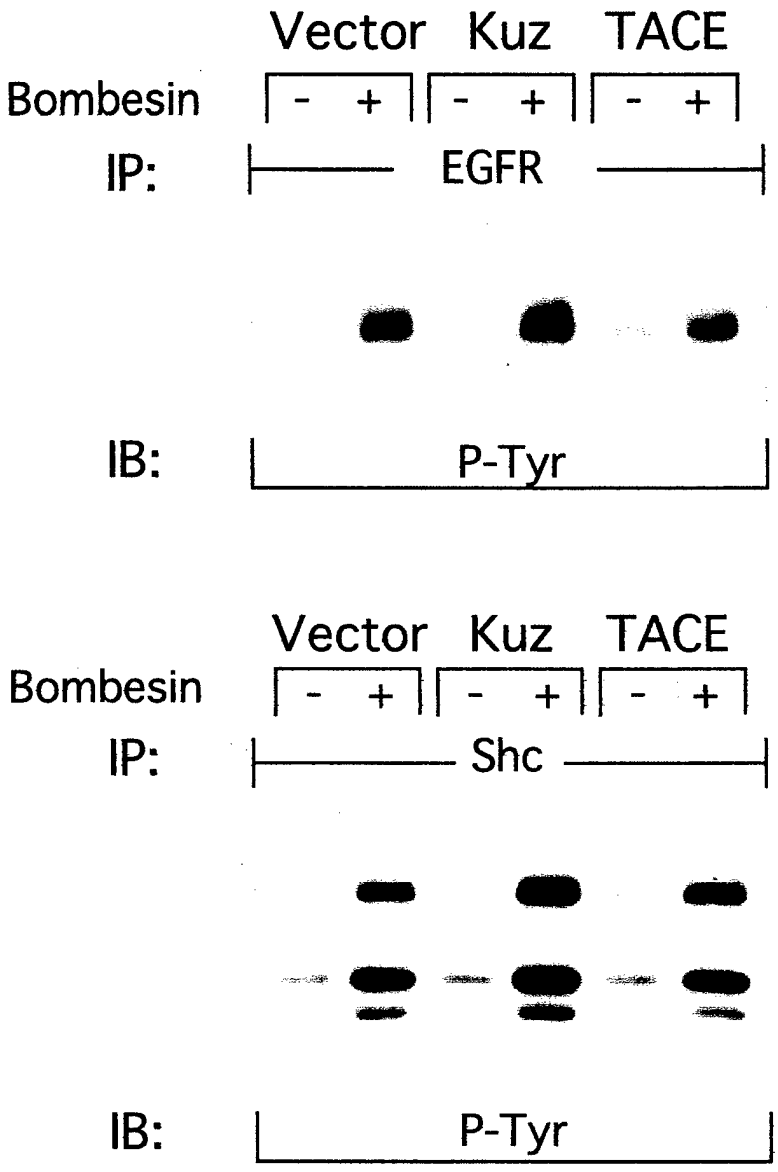
Figure 5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