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Autophagy regulation in pancreatic acinar cells is independent of epidermal growth factor receptor signaling

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ABSTRACT

Autophagy is an intracellular degradation system in eukaryotic cells that occurs at a basal level. It can also be induced in response to environmental signals including nutrients, hormones, microbial pathogens, and growth factors, although the mechanism is not known in detail. We previously demonstrated that excessive autophagy is induced within pancreatic acinar cells deficient in Spink3, which is a trypsin inhibitor. SPINK1, the human homolog of murine Spink3, has structural similarity to epidermal growth factor (EGF), and can bind and stimulate the EGF receptor (EGFR). To analyze the role of the EGFR in pancreatic development, in the regulation of autophagy in pancreatic acinar cells, and in cerulein-induced pancreatitis, we generated and examined acinar cell-specific *Egfr*-deficient ($Egfr^{-/-}$) mice. $Egfr^{-/-}$ mice showed no abnormalities in pancreatic development, induction of autophagy, or cerulein-induced pancreatitis, suggesting that Egfr is dispensable for autophagy regulation in pancreatic acinar cells.

1. Introduction

Serine protease Kazal type 1 (SPINK1) was initially discovered as a trypsin-specific inhibitor in the pancreas [1]. Spink3 is the mouse homolog of human *SPINK1*. SPINK1/Spink3 is secreted by acinar cells into the pancreatic juice, binds rapidly to trypsin and thus inhibits its activity. Interestingly, there are some structural similarities between SPINK1 and the potent growth factor epidermal growth factor (EGF). In fact, our group and others have shown that SPINK1/Spink3 acts as a growth factor in the pancreas [2,3]. We have also demonstrated that SPINK1 can bind to the EGF receptor (EGFR) and that the resulting growth signal is primarily mediated by the MAPK/ERK pathway [2]. Furthermore, we previously reported that excessive autophagy was induced within acinar cells in the exocrine pancreas of *Spink3*-deficient mice [4]. Thus, a third function of Spink3 is to suppress autophagy within pancreatic acinar cells.

Autophagy is a cellular pathway involved in protein and organelle degradation, with a large number of connections to human disease and physiology. Autophagy occurs at a basal level, and can also be induced in response to environmental signals including nutrients, hormones and growth factors [5,6]. The best-characterized regulatory pathway for autophagy includes a class I phosphatidylinositol 3-kinase (PI3K) and target of rapamycin (TOR), both of which inhibit autophagy, although the mechanism is not known [5,6]. A class III PI3K is needed for the activation of autophagy. TOR activity is probably regulated in part through feedback loops to prevent insufficient or excessive autophagy [6]. For example, p70S6 kinase is a substrate of TOR that may act to limit TOR activity, ensuring the basal levels of autophagy that are critical for homeostasis. It is known that some growth factors, such as an insulin [6], can stimulate the TOR–autophagy pathway via activating its receptors. However, the relationship between autophagy and EGFR has not yet been examined. *In vivo* and *in vitro* experiments have shown that pancreatic acinar cells respond with conspicuous autophagic activity to various experimental or pathological conditions such as cerulein-induced pancreatitis [7–9]. We revealed the connection between the suppressive effect of Spink3 on autophagy and the role of autophagy in pancreatitis, thus showing that autophagy is involved in trypsin activation [9]. Spink3 therefore has dual roles in preventing pancreatitis: direct inhibition of trypsin activity by binding, and indirect inhibition of trypsin activation

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through suppression of autophagy [2,10]. However, the precise mechanism of suppression of autophagy by Spink3 is not yet known.

EGFR belongs to a family of receptor tyrosine kinases that includes three other members (erbB2/HER-2, erbB3/HER-3, and erbB4/HER-4) [11]. It is expressed throughout the developing pancreas, as are its ligands, particularly transforming growth factor-alpha [12]. Activation of EGFR leads to initiation of intracellular signaling via several pathways including MAPK/extracellular signal-regulated kinase 1/2 (ERK1/2) (MAPK/ERK), signal transducer and activator of transcription 3 (STAT3), and class I PI3K/v-Akt murine thymoma viral oncogene homolog (AKT). The (PI3K/AKT) pathway is involved in cell growth, apoptosis resistance, invasion, and migration. In addition, the serine/threonine protein kinase AKT, also known as protein kinase B (PKB), a downstream effector of PI3K, was shown to be a critical mediator of mTOR activity [13].

Mice lacking *Egfr* die between mid-gestation and postnatal day 20 depending on their genetic background, with defects in placenta, brain, bone, skin, and lung [14–18]. The early lethality of *Egfr*-deficient ($Egfr^{-/-}$) mice has hampered a careful analysis of *Egfr* function in the adult pancreas. We produced conditional $Egfr^{-/-}$ mice by mating mice carrying a floxed *Egfr* allele [19] with $Spink3^{Cre/+}$ mice in which Cre was expressed under the control of the *Spink3* promoter [20]. This has enabled us to study the role of EGFR signaling in pancreatic development, the regulation of autophagy in pancreatic acinar cells, and cerulein-induced pancreatitis.

2. Materials and methods

2.1. Animal protocol and experimental design

Mice were kept under specific-pathogen-free conditions with free access to food and water in a 12 h light/dark cycle. C57BL/6N mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). All animal experiments were performed with approval of the Kumamoto University Institutional Animal Care and Use Committee.

2.2. Generation of Egfr-deficient mice in the pancreatic acinar cells

Mice with a floxed EGFR allele ($Egfr^{f/f}$) [19], in which the promoter and the first exon of the *Egfr* gene are flanked by *lox*P sites, and $Spink3^{Cre/+}$ mice were generated previously [20]. In $Spink3^{Cre/+}$ mice, the cre recombinase gene is expressed only in acinar cells, but not in islet or ductal cells, in the pancreas [20]. We first generated $Egfr^{f/-}$ mice by crossing $Egfr^{f/f}$ and pCAGGS-Cre mice. We then crossed $Egfr^{f/-}$ and $Spink3^{Cre/+}$, and generated $Egfr^{f/+}$;$Spink3^{Cre/+}$ mice as controls, and $Egfr^{f/-}$;$Spink3^{Cre/+}$ mice.

2.3. Cerulein-induced acute pancreatitis

After an overnight fast, 8-week-old mice were given hourly intraperitoneal injections of either saline (control) or cerulein, which is a cholecystokinin (CCK) analog (50 μg/kg) (Sigma–Aldrich Corp, Tokyo, Japan) for 6 or 9 h. One hour after the last injection, serum and pancreas samples were collected and used for the following studies. Serum was used to measure pancreatic amylase activity using the substrate 2-chloro-4-nitrophenyl-4-galactopyranosylmaltoside (Gal-G2-CNP) (CicaLiquid-N p-AMY, Kanto Chemical Co., Inc., Tokyo, Japan).

2.4. Histological and immunohistochemical analysis and pathologic scoring

For histological analysis, pancreatic tissue was fixed overnight in 15% formalin (Wako, Osaka, Japan), embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). Pathological score was estimated using a method described previously with minor modifications [21]. Statistical significance was determined using the Mann–Whitney *U*-test. The differences were considered to be statistically significant at $P < 0.05$.

2.5. Trypsin activity in the pancreas

Pancreas tissue was disrupted using the Multi-Beads Shocker system (Yasui Kikai Corp., Osaka, Japan), and cold buffer containing 5 mM 2-morpholinoethanesulfonic acid (pH 6.5), 1 mM $MgSO_4$, and 250 mM sucrose was added. Trypsin activity in homogenates was measured fluorimetrically using Boc-Gln-Ala-Arg-MCA (Code; 3135-v, Peptide Institute Inc., Osaka, Japan) as the substrate according to the method of Kawabata et al. [22] using the Infinite 200 PRO multimode microplate reader (Tecan, Männedorf, Switzerland). Trypsin activity in each sample was determined using a standard curve for purified trypsin (Product Number T1426, Sigma–Aldrich Corp). One unit was defined as producing a ΔA_{253} of 0.001 per min at pH 7.6 at 25 °C using *N*-benzoyl arginine ethyl ester as a substrate.

2.6. Western blot analysis

Pancreas samples were homogenized in lysate buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, protease inhibitor cocktail [1:100 dilution; Nacalai Tesque Inc., Kyoto, Japan] and phosphatase inhibitor cocktail [1:100 dilution; Nacalai Tesque]). Extracts (15 μg of protein per lane) underwent polyacrylamide gel electrophoresis and were transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Darmstadt, Germany). After 1 h of incubation at room temperature in blocking buffer (TBS, 5% non-fat dry milk, 0.1% Tween-20), the membranes were incubated overnight at 4 °C with the appropriate primary antibodies diluted in wash buffer (TBS, 0.1% Tween-20) containing 5% non-fat dry milk. All primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Membranes were then washed three times (5 min each) and incubated with secondary antibody (anti-rabbit immunoglobulin G antibody, 1:5000 dilution) diluted in wash buffer (TBS, 0.1% Tween-20) containing 5% non-fat dry milk for 1 h at room temperature followed by three washes (10 min each). Blots were developed using ECL Plus reagents (GE Healthcare UK Ltd., Buckinghamshire, England). Densitometric quantification was carried out using Image J software (http://rsb.info.nih.gov/ij/).

2.7. Statistical analysis

Each experiment was performed at least three times. The statistical significance of the data was determined by applying the two-tailed Student *t* test or the log rank test. The differences were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Effect of Egfr deficiency on pancreatic development

We used $Spink3^{Cre/+}$ mice to delete the *Egfr* gene. $Egfr^{f/+}$;$Spink3^{Cre/+}$ mice carrying one normal allele of *Egfr* were used as a control, and $Egfr^{f/-}$;$Spink3^{Cre/+}$ carrying both targeted alleles of *Egfr* as the deficient mouse. Western blot analysis of pancreas revealed that EGFR protein was present in $Egfr^{f/+}$;$Spink3^{Cre/+}$ mice, but was almost undetectable in $Egfr^{f/-}$;$Spink3^{Cre/+}$ mice at 8 weeks of age (Fig. 1A). $Egfr^{f/-}$;$Spink3^{Cre/+}$ mice were born at the expected Mendelian ratio and did not show abnormal growth compared to $Egfr^{f/+}$

;*Spink3*$^{Cre/+}$ mice (Fig. 1B). A very small amount of EGFR was detected in the pancreas of *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice, probably owing to the expression of *Egfr* in islet and duct cells of the pancreas, because Cre is not expressed in these cells. There was no histological abnormality in either *Egfr*$^{f/+}$;*Spink3*$^{Cre/+}$ or *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice (Fig. 1C). These data suggest that *Egfr* is dispensable for development of the pancreas.

3.2. Effect of Egfr deficiency on induction of autophagy

As shown previously, strong induction of autophagy was observed in the absence of Spink3. If Spink3 down-regulates autophagy through EGFR, autophagy should be induced in the absence of EGFR. However, there was no histological abnormality in the pancreas at 8 weeks of age (Fig. 1C). Autophagic activity was examined by Western blot using anti-LC3 and anti-p62 antibodies. The ratios of LC3-II/LC3-I were similar in control and *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice, although LC3-II increased after starvation in the pancreas of both strains as expected. Autophagy is important for clearance of protein aggregates that form in cells. The ubiquitin- and LC3-binding protein "p62" regulates the formation of protein aggregates [6]. Because we have previously shown that p62 increased in cerulein-induced pancreatitis (unpublished data), we examined whether *Egfr* deficiency affects p62 expression. However, the level of p62 expression was constant in control and *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice under both fed and starved conditions (Fig. 2A).

There are three major pathways controlled by EGFR: the MAPK/ERK pathway, the PI3K/AKT pathway, and the STAT pathway [11]. The MAPK/ERK pathway is a critically important route that regulates cell proliferation and survival and the PI3K/AKT-mammalian target of rapamycin; the mTOR pathway is important for regulation of acinar cell protein synthesis [23]. In pancreatic acinar cells, *Egfr* deficiency did not affect the amount or phosphorylation of either ERK1/2 or AKT (Fig. 2B), suggesting that *Egfr* deficiency does not influence these intracellular signaling pathway in acinar cells. We also analyzed the expression of other ERBB family members, HER2 and HER3; however, there was no difference in the expression of these (Fig. 2C). Taken together, these data suggested that *Egfr* is not involved in autophagy regulation by Spink3.

3.3. Effect of Egfr deficiency on severity of cerulein-induced pancreatitis

We and other groups have reported that autophagy is strongly induced in cerulein-induced pancreatitis [9,24]. In order to investigate the effect of *Egfr* deficiency on the severity of acute pancreatitis, we administered saline (control) or cerulein, which is a cholecystokinin (CCK) analog, intraperitoneally to *Egfr*$^{f/+}$;*Spink3*$^{Cre/+}$ and *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice. In the *Egfr*$^{f/+}$;*Spink3*$^{Cre/+}$ and *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice, pancreatic enlargement due to edema was observed macroscopically after cerulein administration. In addition, HE staining and pathologic scores showed features of acute pancreatitis such as edema, necrotic acinar cells, and inflammatory cell infiltration in the pancreas of both genotypes (Fig. 3A and B). Serum amylase activity and trypsin activity (markers of acute pancreatitis) were increased in these mice (Fig. 2C and D). However, there was no significant difference between *Egfr*$^{f/+}$;*Spink3*$^{Cre/+}$ and *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice. These results

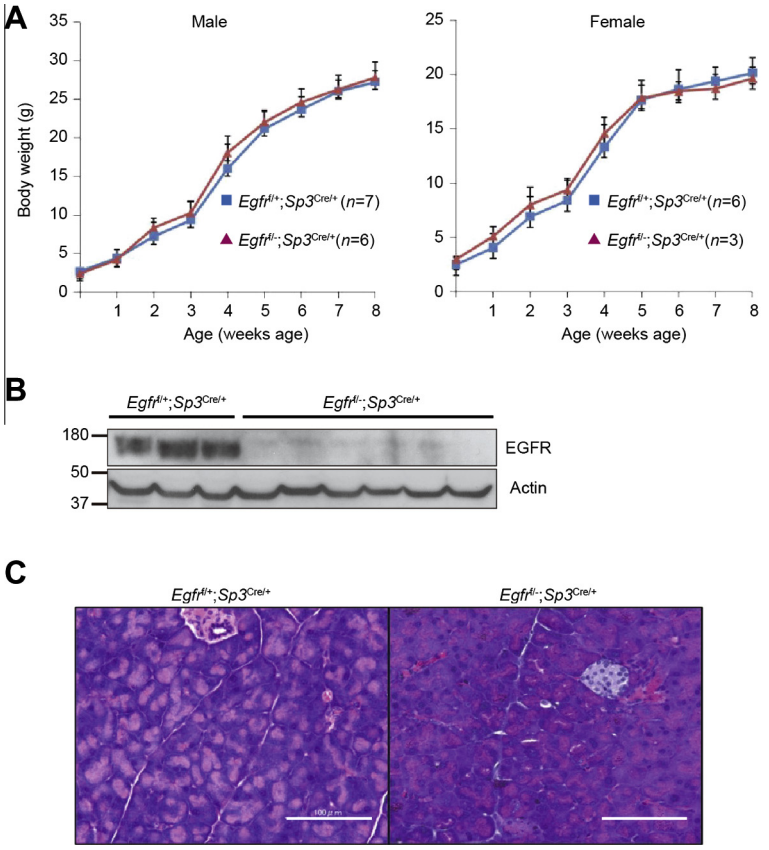

Fig. 1. Generation of *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice. (A) Body weight of *Egfr*$^{f/+}$;*Spink3*$^{Cre/+}$ (*Egfr*$^{f/+}$;*Sp3*$^{Cre/+}$) and *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ (*Egfr*$^{f/-}$;*Sp3*$^{Cre/+}$) mice at various times after birth (left panel, male; right, female; n = 3–7 mice). Results represent the mean ± standard deviation. (B) Western blot analysis of EGFR in the pancreas of 8 week old *Egfr*$^{f/+}$;*Spink3*$^{Cre/+}$ and *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice (n = 3–6 mice). Actin was used as a loading control. (C) HE staining of mouse pancreas at 8 weeks of age. Scale bars indicate 100 μm. Results are representative of two independent experiments.

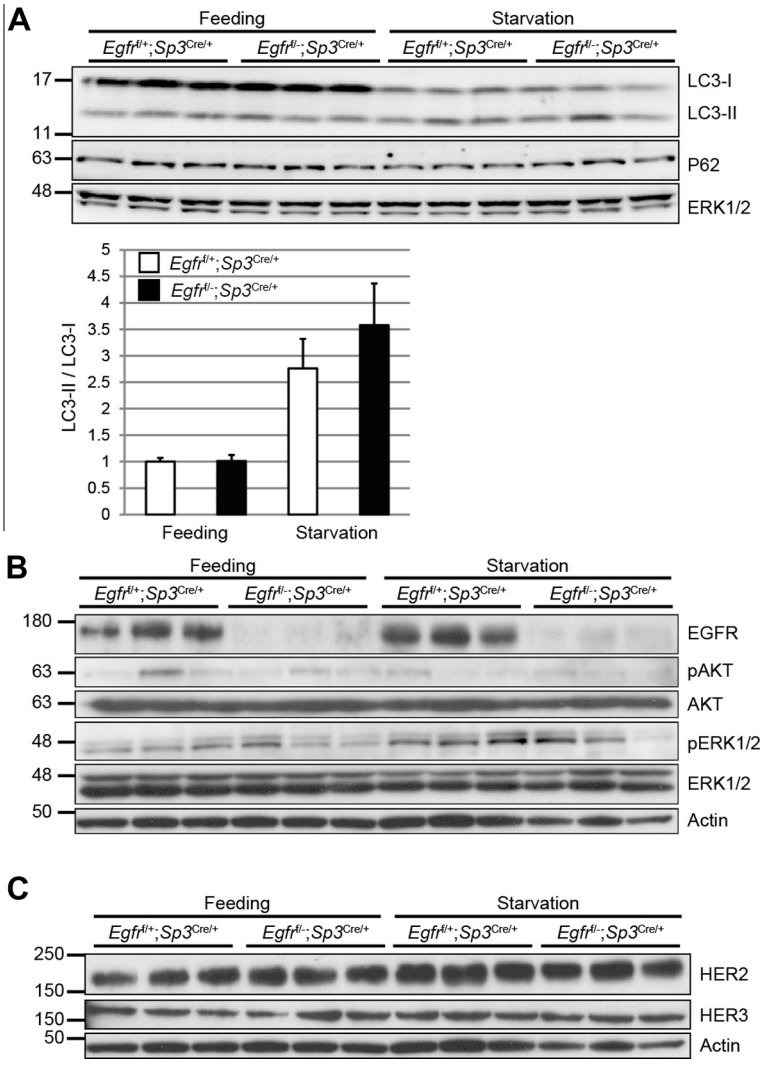

Fig. 2. Western blot analysis after fasting. (A) Pancreas extracts were analyzed by immunoblotting with anti-LC3, anti-p62, or anti-ERK antibodies in the indicated genotypes at 8 weeks of age during normal feeding, and after fasting for 24 h (n = 3 mice). ERK1/2 was used as a loading control. (B) Western blot analysis of EGFR, phosphorylated AKT (p-AKT), and p-ERK in pancreas tissue from 8 week old *Egfr*$^{f/+}$;*Spink3*$^{Cre/+}$ and *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice during normal feeding, and following fasting for 24 h (n = 3 mice). Actin was used as a loading control. The results represent mean ± standard error for three mice. (C) Western blot analysis of Her2 and Her3 in pancreas tissue from 8 week old *Egfr*$^{f/+}$;*Spink3*$^{Cre/+}$ and *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice during normal feeding and following fasting for 24 h (n = 3 mice). Actin was used as a loading control. Results are representative of two independent experiments.

suggest that the severity of pancreatitis induced by cerulein is independent of *Egfr*.

3.4. Effect of Egfr deficiency on autophagy induction by cerulein

It is known that autophagy is enhanced in cerulein-induced pancreatitis. In fact, autophagy markers such as LC3-II and p62 were strongly elevated by cerulein administration in the pancreas of not only *Egfr*$^{f/+}$;*Spink3*$^{Cre/+}$ mice, but also of *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice (Fig. 4A). We also analyzed the expression of EGFR and its downstream signaling molecules by Western blot analysis. EGFR was strongly induced by the administration of cerulein in *Egfr*$^{f/+}$;*Spink3*$^{Cre/+}$ mice, but not in *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice, as was expected (Fig. 4B). AKT was not elevated after cerulein treatment. In contrast, ERK1/2 was activated by administration of cerulein, but there was no obvious difference in activity of the downstream signaling molecules between the two genotypes (Fig. 4B). EGF–EGFR and CCK (including cerulein) can activate the MAPK cascade in pancreatic acinar cells, leading to activation of ERK1/2, by distinct mechanisms [25]. Activation of ERK1/2 may be induced by the CCK (cerulein)–CCK receptor pathway. The expression level of HER2 protein, but not HER3, was decreased in both *Egfr*$^{f/+}$;*Spink3*$^{Cre/+}$ and *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice by administration of cerulein (Fig. 4C).

4. Discussion

In this study, we have shown that (1) *Egfr* is not necessary for development and maintenance of pancreatic acinar cells during development or at maturity, (2) *Egfr* is not involved in regulation of autophagy by Spink3, and (3) *Egfr* is not related to the severity of pancreatitis or induction of autophagy by cerulein.

EGFR has been implicated in the development of organs requiring epithelial–mesenchymal interactions in the lung [26]. Without functional EGFR, lungs and mammary gland exhibit impaired branching [27,28]. The pancreas also develops through branching morphogenesis and the EGF family has been implicated in this process. In this study, using *Spink3*$^{Cre/+}$ mice, murine *Spink3* mRNA was detected at 11.5 days post coitus (dpc), before formation of the typical shape of the exocrine structure of the pancreas, and was clearly expressed in acinar cells by 13.5 dpc [29]. In adult mice, *Spink3* is expressed only in pancreatic acinar cells, but not in islet or ductal

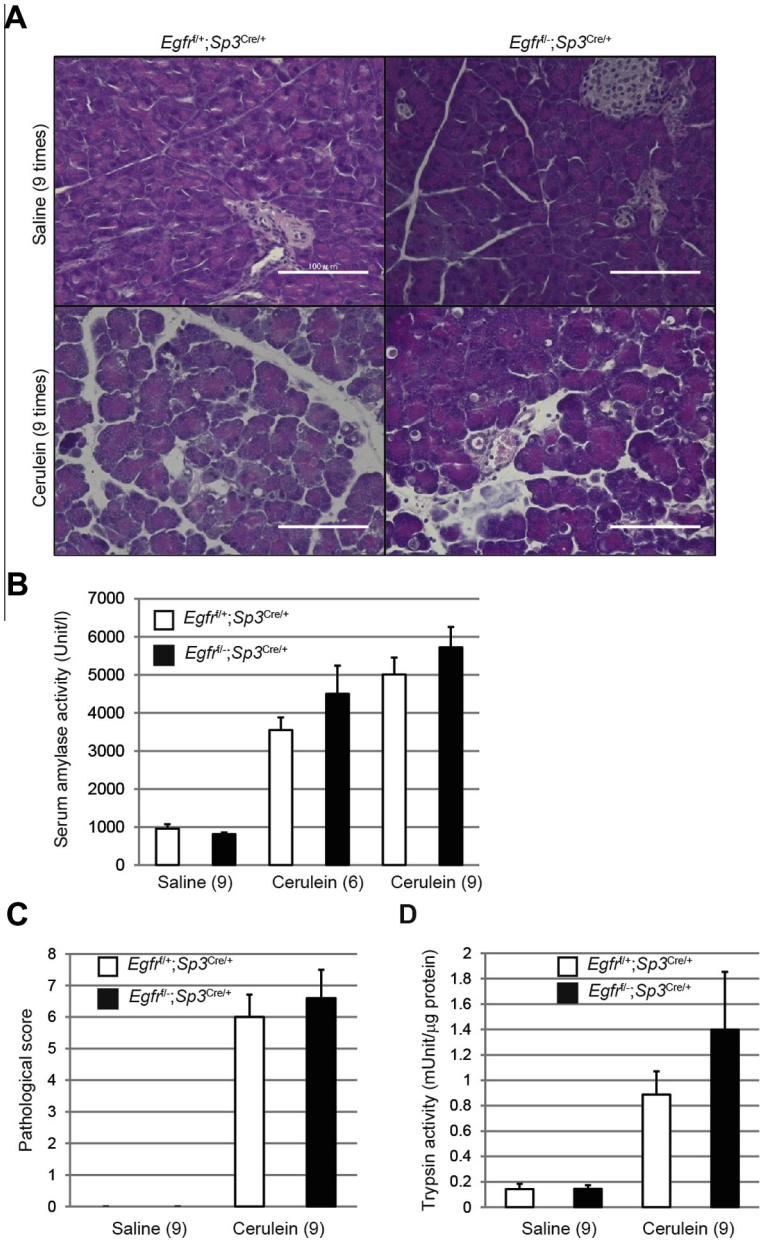

Fig. 3. Analysis of cerulean-induced pancreatitis in $Egfr^{f/-}$;$Spink3^{Cre/+}$ mice. (A) HE staining of the pancreas; (B) serum amylase activity; (C) pathological scoring in the pancreas; (D) trypsin activity in the pancreas of the indicated genotypes in cerulein-induced pancreatitis ($n = 6$ mice). Saline (control) and cerulein were given hourly intraperitoneally 6 and 9 times, respectively. Scale bars indicate 100 μm. The results represent mean ± standard error of the mean of six mice.

cells [20]. However, our data show that *Egfr* is not essential for acinar cell development.

We and other groups have reported that SPINK1 can bind to and stimulate EGFR, thus inducing cell proliferation and migration [2,30]. In contrast, Niinobu et al. predicted the presence of a SPINK1-specific receptor other than the EGFR [3]. They studied specific binding sites for SPINK1 on 3T3 Swiss albino cells using radio-iodinated recombinant SPINK1 (^{125}I-SPINK1). Based on their observations, they predicted the presence of a SPINK1-specific receptor, and suggested that the biological effect of SPINK1 is mediated by high-affinity plasma membrane receptors. In this study, we showed that EGFR is not involved in Spink3-mediated regulation of autophagy in pancreatic acinar cells. These observations suggest that the proposed SPINK1/Spink3-specific receptor may regulate autophagy via the PI3K–AKT–TOR pathway. It has been reported that when EGFR activity is inhibited, a compensatory signal occurs via other ERBB family molecules [31–33]. These data indicate that such compensatory mechanisms mask the phenotype of $Egfr^{f/-}$;$Spink3^{Cre/+}$ mice. The expression level of Her2 and Her3 proteins was approximately the same in the $Egfr^{f/-}$;$Spink3^{Cre/+}$ and $Egfr^{f/}$;$Spink3^{Cre/+}$mice. Thus, it is not plausible that a compensatory mechanism acts in the absence of EGFR in the regulation of autophagy. Further studies will be required to clarify the molecules involved in the regeneration of autophagy in the pancreas.

High doses of CCK or its analog cerulein are widely used to study of these responses in rat and mouse acute pancreatitis models [34]. CCK is a major physiologic regulator of digestive enzyme secretion by pancreatic acinar cell; however, supra-physiological doses of CCK are harmful to the pancreatic acinar cells, resulting

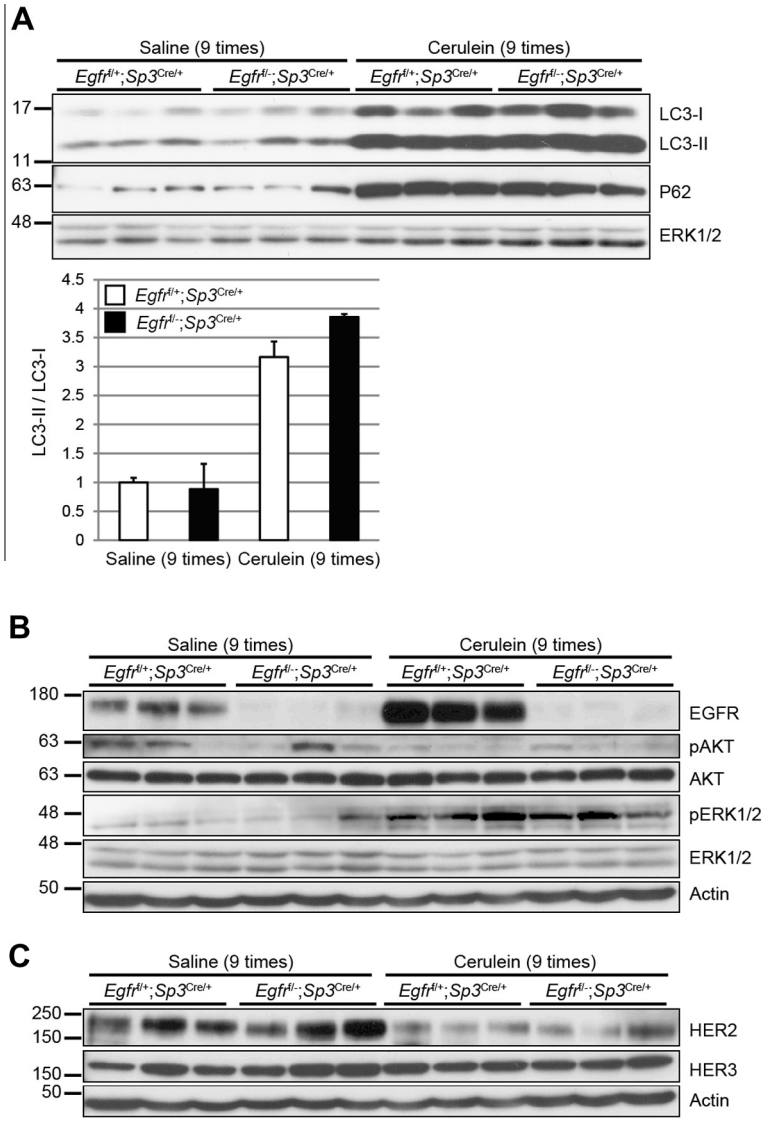

Fig. 4. Western blot analysis after cerulein-induced pancreatitis. (A) Pancreas extracts were analyzed by immunoblotting with anti-LC3, anti-P62, or anti-ERK antibodies in the indicated genotypes at 8 weeks of age after induction of pancreatitis using cerulein ($n = 3$ mice). ERK1/2 was used as a loading control. The results represent the mean ± standard error of the mean of three mice. (B) Western blot analysis of EGFR, p-AKT, and p-ERK in pancreas tissue from 8 week old *Egfr*$^{f/+}$;*Spink3*$^{Cre/+}$ and *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice after induction of pancreatitis using cerulein (9 times hourly injections; $n = 3$ mice). Actin was used as a loading control. (C) Western blot analysis of HER2 and HER3 in *Egfr*$^{f/+}$;*Spink3*$^{Cre/+}$ and *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mouse pancreas at 8 weeks of age after induction of pancreatitis using cerulein ($n = 3$ mice). Actin was used as a loading control. Results are representative of two independent experiments.

in acute pancreatitis. Cerulein-induced pancreatitis is similar to human edematous pancreatitis, manifesting with dysregulation of digestive enzyme production and cytoplasmic vacuolization, the death of acinar cells, edema formation, and infiltration of inflammatory cells into the pancreas. Severity of pancreatitis and induction of autophagy by cerulein were similar in the *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ and *Egfr*$^{f/-}$;*Spink3*$^{Cre/+}$ mice, suggesting that cerulein did not bind to the CCK receptor. The homology between Egfr and the CCK receptor A is 44% and 23% at the mRNA and protein levels, respectively. CCK and cerulein bind to CCK receptors, which are a group of G-protein coupled receptors [35]. There are two different subtypes, CCK receptor A (Cckar) and receptor B (Cckbr), which are ≈50% homologous [36]. Cckar is specific for CCK and this receptor is a major physiological mediator of pancreatic enzyme secretion [23], being the predominant form on rodent acinar cells. CCK acts through intracellular messengers, particularly Ca^{2+}, diacylglycerol (DAG), and cAMP to induce secretion (for a review see [37]). CCK also activates MAPK cascades. Although the function of these pathways is not well understood, the MAPKs may be involved in control of cell growth, differentiation, survival, and apoptosis. The PI3K–mTOR pathway is also activated by CCK and this pathway is important for regulation of acinar cell protein synthesis [38]. Although some of these downstream signaling pathways are shared in both Egfr and Cckar, the deficiency of *Egfr* did not affect the downstream signaling pathway mediated by cerulein.

Overall, our study suggests that the Egfr is not involved in the regulation of autophagy by Spink3, for which we suggest there are three possibilities. The first is that autophagy is regulated by compensatory mechanisms via another EGFR family member. The second is that a novel receptor, which is stimulated by SPINK1/Spink3, can mediate the signal to suppress autophagy. The third and final possibility is that SPINK1/Spink3 can directly regulate autophagy within the cytoplasm of pancreatic acinar cells, leading to suppression of autophagy. Further studies will be required to clarify the mechanisms by which Spink3 can suppress autophagy.

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