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Deletion of IKKβ in activated fibroblasts promotes tumor progression in melanoma

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ABSTRACT

Cancer-associated fibroblasts (CAFs) are a major component of the tumor microenvironment and play critical roles in tumorigenesis. CAFs consists of multiple subpopulations, which have diverse functions. The detailed mechanism, including the role of NF-κB, a critical transcription factor for inflammation and cell survival, in CAFs has not been adequately explored. In this study, we examined the roles of IKKβ, a key kinase for NF-κB activation, in activated CAFs by using mice (KO mice) with deletion of IKKβ in activated fibroblasts (aFbs). We found that melanoma cells implanted in KO mice showed significantly more growth than those implanted in control mice. To exclude the effects of deletion of IKKβ in cells other than aFbs, we implanted a mixture of melanoma cells and IKKβ-deleted aFbs in wild-type mice and observed that the mixture showed greater growth than a mixture of melanoma cells and normal aFbs. In exploring the mechanisms, we found that conditioned medium from IKKβ-deleted aFbs promotes the proliferation of melanoma cells, and the expression of growth arrest-specific 6 (GAS6) and hepatocyte growth factor (HGF), which are major tumor-promoting factors, was upregulated in IKKβ-deleted aFbs. These results indicated the tumor-suppressing function of IKKβ in activated CAFs.

1. Introduction

The tumor microenvironment (TME) is the internal environment of tumors that consists of stromal components, including vascular endothelial cells, immune cells and fibroblasts [1,2]. Fibroblasts in the TME, namely, cancer-associated fibroblasts (CAFs), are the most abundant cell type in the TME and play critical roles in tumorigenesis and development [3,4]. Studies have indicated that CAFs promote the survival and proliferation of tumor cells by secreting a variety of growth factors [5,6] and promoting ECM remodeling [7,8]. In addition, CAFs stimulate angiogenesis within tumors and regulate the immune system [9,10].

CAFs are heterogeneous in origin and function, and they have been divided into multiple subpopulations with different markers [11,12]. For instance, Li et al. identified three types of CAFs in colorectal tumors by single-cell RNA-seq: one type of CAFs consists of normal fibroblasts, the second is activated CAFs consisting of myofibroblasts (aFbs), which are positive for αSMA or SM22α (TAGLN), and the third type is CAFs expressing genes related to extracellular matrix remodeling such as MMP2 [13]. Among them, activated CAFs function more predominantly in regulating tumor growth [14].

NF-κB is a transcription factor involved in various cell responses, including inflammation and cell survival. This molecule is activated mostly through kinase-dependent (IKK-dependent) phosphorylation of I-κB and its subsequent degradation. The IKK complex consists of IKKα, IKKβ and IKKγ [15]. Among them, IKKβ is an essential kinase for NF-κB activation [16].

The activation of NF-κB has been reported to be a cause and indicator of tumor progression [17,18]. However, its role in CAFs has been controversial. For instance, contradictory results were reported regarding the function of NF-κB-IKK signaling in CAFs in intestinal cancer progression [19,20], which indicated that different subpopulations of fibroblasts in the TME can have different effects on tumor regulation. This finding indicates the importance of focusing on specific subtypes of CAFs. In this study, we examined the roles of NF-κB in activated CAFs in melanoma progression by generating mice with deletion of IKKβ in aFbs.

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2. Materials and methods

2.1. Mice

All animal experiments were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University and performed in accordance with the institutional guidelines of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University (Kyoto, Japan). IKKβ flox/flox mice were generously provided by Professor Michael Karin and mated with Sm22αCre mice (#004746 from The Jackson Laboratory, Bar Harbor, ME) to generate myofibroblast-specific IKKβ knockout mice. Mice were born according to Mendelian frequencies. In the mating protocol for all kinds of mice, we could have Cre-negative mice within littermates; therefore, we used littermate WT mice as the control mice in all experiments. We used only female mice in this study.

2.2. Antibodies and reagents

The antibodies used included anti-IKKβ (Millipore, Billerica, MA, USA), anti-I-κB (Ser32/36), anti-phospho-p65 (Ser536) (Cell Signaling Technology, Boston, MA, USA), anti-p65 (Santa Cruz), anti-mouse IgG HRP-linked and anti-rabbit IgG HRP-linked antibodies (Cell Signaling Technology, Boston, MA, USA), and anti-β-actin (Cell Signaling Technology, Boston, MA, USA).

2.3. Cell culture

B16–F0 melanoma cells were purchased from ATCC (CRL-6322). Skin fibroblasts were isolated from the WT and IKKβ-KO mice and cultured in DMEM with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with an atmosphere containing 5% CO_2 at 37 °C. For all experiments, cells were used at passages 4–5.

2.4. Western blot

Protein was extracted from cultured fibroblasts using a cell lysis buffer (9803S; Cell Signaling) with a protease inhibitor cocktail. Blots were visualized by ECL Western Blotting Detection kit (GE Healthcare). The expression level of β-actin was used as the iloading control. Densitometric analysis was performed by ImageJ software. All experiments were performed at least in triplicate.

2.5. Tumor transplantation experiments

Six-to eight-week-old WT and KO mice were anesthetized, and 1×10^5 B16 cells were implanted subcutaneously. Tumor sizes were measured at different timepoints with calipers, and tumor volumes were calculated by the formula $X^2 \times Y \times 0.5$ (X: smaller diameter, Y: larger diameter). For implantation of B16 cells and fibroblasts, we used B16 cells (2.5×10^4) alone or intermixed with skin fibroblasts (2.5×10^5) from WT and KO mice.

2.6. Histology

The tumor tissues were divided into two halves and fixed in 4% paraformaldehyde for histological analyses. We cut tumor tissues into 7-μm sections, deparaffinized them in xylene and serially dehydrated them in decreasing concentrations of ethanol. Sections were stained with hematoxylin and eosin.

2.7. TUNEL assay

To evaluate apoptosis in melanoma tissue, we performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining by in situ apoptosis detection kit (TaKaRa Bio, Japan) according to the manufacturer's protocol. Five microscopic fields randomly selected in each group were used to calculate the ratio of TUNEL-positive cells.

2.8. Coculture experiments

Coculture experiments were performed in 24-well plates using a transwell system (Falcon 353495, 0.4 μm pore size). Melanoma cells ($2X10^4$) and fibroblasts ($2X10^4$) were seeded in the lower and upper wells, respectively, in medium containing 1% bovine serum and incubated at 37 °C in standard incubator conditions.

2.9. Conditioned medium assay

WT or KO fibroblasts ($5X10^5$) were seeded in a 6-well culture dish in 3 ml of medium for 24 h. The medium was collected under sterile conditions, followed by centrifugation at 1500×*g* for 10 min, and then, the supernatant was collected as the conditioned medium (CM) and stored at −80 °C as WT-CM and KO-CM. Plain medium (PM), which consisted of medium without cell incubation, served as the control under the same experimental conditions. After that, B16–F0 melanoma cells were seeded in 6-well dishes and incubated for 24 h in CM and PM, and the cell number was counted. These experiments were repeated four times, and the mean cell number was analyzed by a *t*-test (two-tailed).

2.10. Real-time PCR

Total RNA was extracted from fibroblasts using TRIzol (Thermo Fisher Scientific). cDNA was generated from the total RNA by using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO) according to the manufacturer's instructions. Quantitative polymerase chain reaction was performed by using Thunderbird SYBR qPCR Mix (TOYOBO) and a real-time PCR system (StepOnePlus; Thermo, Japan). The relative mRNA levels of IKKβ, GAS6 and HGF were normalized to the Rn18s rRNA level in the same sample. The following primer sequences were used: IKKβ: forward: 5′-CAGCCCAAAGAACAGAGACC-3′, reverse: 5′- ACCACATTGGGATGGTTCAG-3′, GAS6: forward: 5′- GGGCCTAAAACTATCCCCAGA-3′, reverse: 5′-GGTACAAGGACTTCACGCTCT-3′, HGF: forward: 5′- AAAGGGACGGTATCCATCACT-3′, reverse: 5′- GCGATAGCTCGAAGGCAAAAAG-3'.

2.11. Statistical analysis

Data are expressed as the mean ± standard deviation. ANOVA was performed, followed by Student's *t*-test (which was used to compare two datasets). The level of significance was established at $P \leq 0.05$. The statistical analysis was carried out using GraphPad Prism 9.

3. Results

3.1. Generation of mice with deletion of IKKβ in activated fibroblasts

We generated mice with deletion of IKKβ in aFbs (KO mice) by using SM22α-Cre since SM22α is a known marker for aFbs [21–25]. SM22α-Cre mice and IKKβ-floxed mice were mated to generate KO mice in which IKKβ was deleted in SM22α-expressing cells. First, we examined the activity of NF-κB in IKKβ-deleted aFbs. Since fibroblasts were found to be activated when cultured on hard dishes [26], we harvested fibroblasts from mouse skin and cultured them on hard dishes. As shown in Fig. 1A and B, IKKβ was successfully knocked out in cultured fibroblasts harvested from the skin of KO

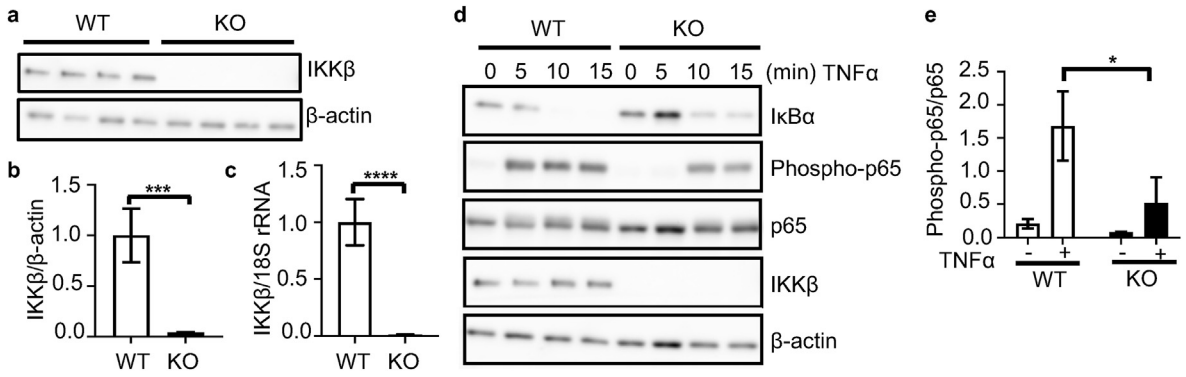

Fig. 1. IKKβ was successfully knocked out in activated fibroblasts from KO mice.
a) and b) Representative western blots and densitometry analysis of IKKβ and β-actin expression in activated fibroblasts (aFbs) isolated from WT and KO littermate mice (n = 4). c) Results of quantitative real-time PCR (RT–qPCR) for the expression of the IKKβ gene in WT and KO aFbs normalized to the Rn18S rRNA level. Bars represent the mean ± SD (*t*-test, n = 4). ****P < 0.0001. d and e) Representative western blots and densitometric analysis of phosphorylated p65/total p65 in aFbs stimulated with TNF-α (10 ng/mL) for 0, 5, 10, and 15 min. Data represent the mean ± SD (*t*-test, n = 3). *$P < 0.05$.

mice (KO fibroblasts), as expected. The mRNA expression level of IKKβ was also eliminated in the KO aFbs (Fig. 1C). Next, we examined the NF-κB activity in KO aFbs. IKKβ activates NF-κB through the phosphorylation and degradation of IκB and phosphorylates p65, the major subunit of NF-κB. We found that both the degradation of IκBα and the phosphorylation of p65 were suppressed in the KO aFbs (Fig. 1D and E). These results confirmed that the activity of NF-κB is suppressed in KO aFbs.

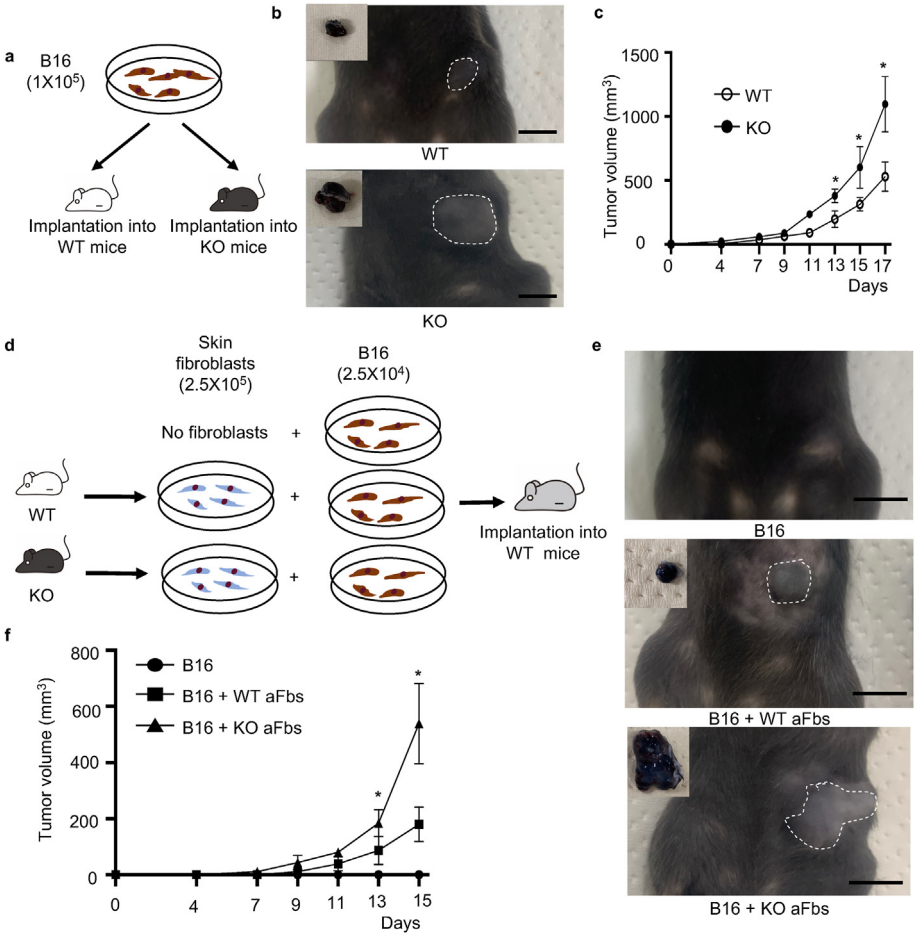

Fig. 2. Tumor progression of melanoma cells implanted into WT/KO mice and the mixture of melanoma cells and WT/KO activated fibroblasts.
a) Schematic representation. WT and KO mice were injected with the same number of melanoma cells. b) Representative images of melanoma in WT and KO mice. Scale bar: 10 mm. c) The time-course change in melanoma tumor size in the WT and KO mice. The points are the mean ± SD of 5 tumors for the WT group and 5 tumors for the KO group. d) Schematic representation. Melanoma cells intermixed with WT or KO aFbs were implanted into normal mice. e) Representative images of each tumor. Scale bar: 10 mm. f) The time-course change in the size of each tumor. (n = 5 for each group), *$P < 0.05$. Scale bar: 10 mm.

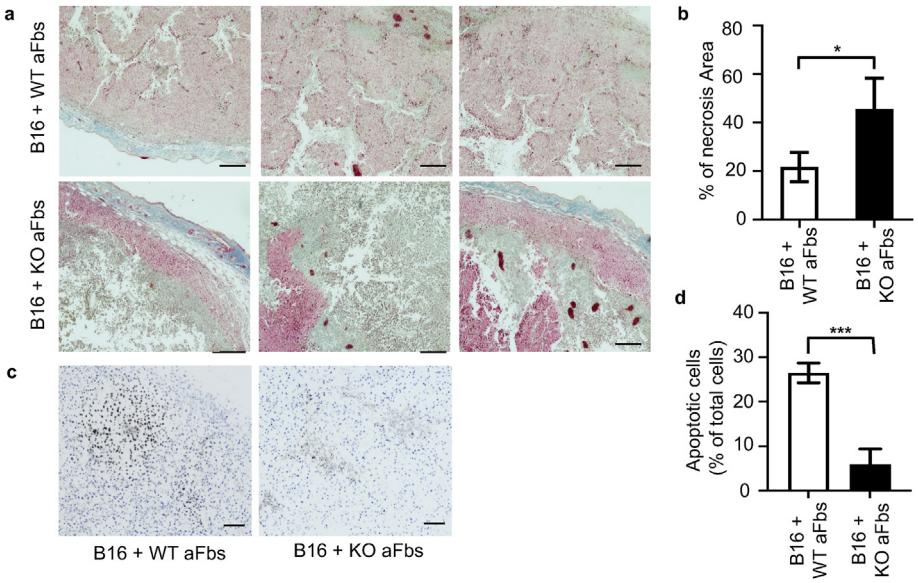

Fig. 3. Deletion of IKKβ in activated fibroblasts augmented necrosis and suppressed apoptosis in tumors.
a) Histological images of tumors composed of WT/KO aFbs and melanoma cells (hematoxylin and eosin stain). Scale bar: 200 μm. Images from three independent tumors are shown. b) Necrosis-to-tumor area ratio in tumors composed of WT/KO aFbs and melanoma cells. Numerical data are presented as the mean value ± SD (*t*-test, n = 5). c) Apoptotic cells in the tumor tissues were detected by a TUNEL assay. TUNEL-positive nuclei are stained brown, and TUNEL-negative nuclei are stained blue. Scale bar: 100 μm. d) Quantitative analysis of apoptosis in tumors composed of WT/KO aFbs and melanoma cells. The percentage of TUNEL-positive cells was determined by ImageJ software. Five sections were counted per tumor. n = 5; Error bars, mean ± SD; ***P < 0.001. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

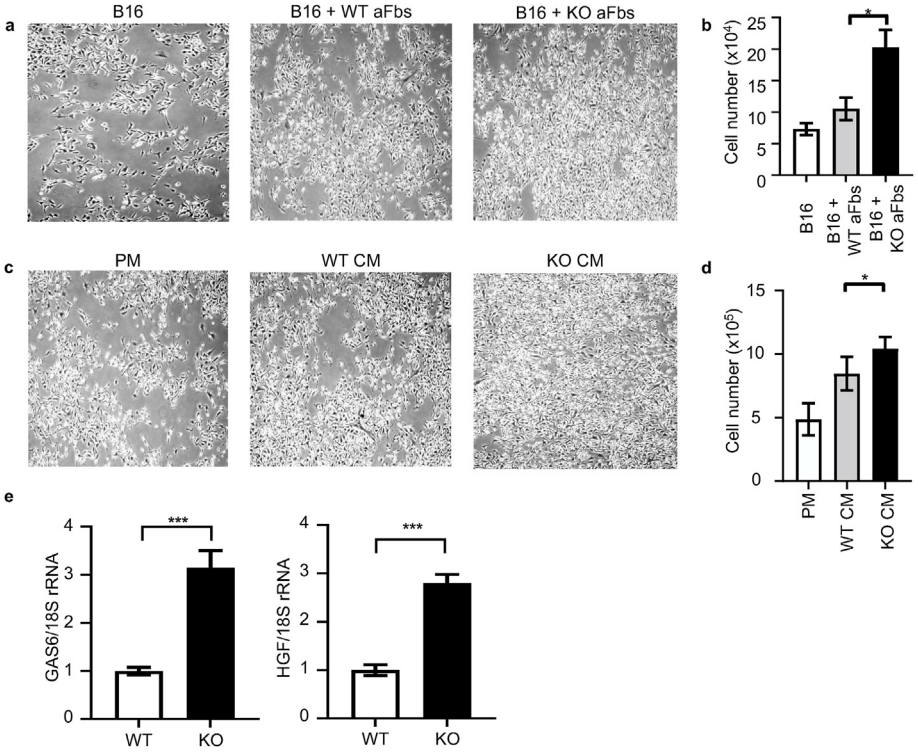

Fig. 4. Conditioned medium from IKKβ-deleted active fibroblasts promoted the proliferation of melanoma cells
a) Representative images of melanoma cells cocultured with WT/KO aFbs. b) The number of melanoma cells cocultured with WT/KO aFbs. Melanoma cells without cocultured aFbs were used as controls. The results are represented as the mean ± SD (*t*-test, n = 4). *p < 0.05. c) and d) Representative images of melanoma cells cultured in plain medium (PM) and conditioned medium (CM) from the WT/KO aFbs. After 24 h of incubation, the cell number was measured. Error bars, mean ± SD; *, $P < 0.05$ (*t*-test, n = 6). e) Results of qRT−PCR for the mRNA expression of GAS6 and HGF in WT and KO aFbs. Error bars, mean ± SD, ***$P < 0.001$ (*t*-test, n = 4).

3.2. Deletion of IKKβ in activated fibroblasts promotes tumor growth

To explore the roles of NF-κB in activated CAFs during melanoma tumorigenesis, we implanted melanoma B16–F0 cells into WT and KO mice (Fig. 2A) and found that tumors implanted in the skin of the KO mice showed significantly greater growth than that of tumors implanted in the skin of the WT mice (Fig. 2B and C).

SM22α is a known marker for aFbs but is also expressed in vascular smooth muscle cells (VSMCs) [27]. Therefore, IKKβ is also deleted in VSMCs in KO mice, as shown in our previous report [28], which could impact blood circulation to the tumor and subsequent tumor growth. To exclude this possibility, we implanted a mixture of melanoma cells and WT/KO aFbs into normal mice (Fig. 2D). As another control, only melanoma cells were injected into normal mice. Consistent with previous studies reporting the tumor-promoting functions of fibroblasts [29,30], tumors derived from melanoma and WT aFbs showed greater growth than those derived from melanoma cells alone (Fig. 2E and F), and interestingly, tumors derived from melanoma and KO aFbs showed greater growth than those derived from melanoma and WT aFbs.

3.3. Deletion of IKK β in activated fibroblasts augmented necrosis and suppressed apoptosis in tumors

Necrosis and apoptosis are histological indicators of tumor characteristics. Necrosis in tumors is recognized as a sign of malignancy since it is observed when tumors grow rapidly [31,32]. Moreover, apoptosis in tumors is used as an indicator of the effects of anticancer therapy [33,34]. Previous reports indicated that tumor-promoting CAFs inhibit the apoptosis of tumor cells, and their deletion resulted in decreased necrosis [5,6,10]. Based on these results, we evaluated tumor necrosis and apoptosis. Histological analysis indicated that necrosis was much more severe in the tumors consisting of melanoma and KO aFbs than in those consisting of melanoma and WT aFbs (Fig. 3A). Quantification of the necrotic area indicated that it occupied more than half of the area in the tumor with melanoma and KO aFbs, which was almost twice as much as that of the tumor with melanoma and WT aFbs (Fig. 3B). We also evaluated apoptosis by TUNEL staining and found that it was lower in melanoma and KO aFbs (Fig. 3C and D). These results indicated that IKKβ in activated CAFs suppresses necrosis and promotes apoptosis.

3.4. Conditioned medium from IKKβ-deleted activated fibroblasts promoted tumor proliferation

To analyze the mechanism by which IKKβ in aFbs regulates tumor growth, we performed coculture experiments with a transwell system. The results indicated that the melanoma cells cocultured with WT aFbs proliferated more than the melanoma cells without cocultures, which was consistent with previous reports [35,36]. Interestingly, the melanoma cells cocultured with KO aFbs proliferated significantly more than those cocultured with WT aFbs (Fig. 4A and B). To confirm the results, we also examined the effect of conditioned medium on the proliferation of tumor cells. The results indicated that the melanoma cells treated with conditioned medium from the WT aFbs proliferated more than those treated with standard medium, and the melanoma cells treated with conditioned medium from the KO aFbs proliferated even more than those treated with conditioned medium from the WT aFbs (Fig. 4C and D). These consistent results indicated that certain cytokines secreted from KO aFbs promoted tumor proliferation.

CAFs secrete many kinds of cytokines and factors that contribute to tumorigenesis [37]. Among them, hepatocyte growth factor (HGF) and growth arrest-specific 6 (GAS6) were investigated in this study based on previous reports. One study showed that the transcription of Smad7, a negative regulator of TGFβ signaling, is impaired and upregulates hepatocyte growth factor (HGF) expression in IKKβ-deficient fibroblasts, which promoted cancer progression in mice with deletion of IKKβ in ColVI-expressing fibroblasts [19]. GAS6 was reported to promote tumor cell proliferation and is downregulated by NF-κB [38,39]. We examined these molecules in WT/KO aFbs by RT–PCR, and the results indicated that the expression of HGF and GAS6 was significantly upregulated in the KO aFbs (Fig. 4E), which would explain, at least in part, how the deletion of IKKβ in activated CAFs promoted tumor progression.

4. Discussion

CAFs are critical regulators of the maintenance and proliferation of tumors, but their subpopulations have different functions. Additionally, the role of NF-κB in CAFs has not been adequately explored. In this study, we revealed that deletion of IKKβ, a critical kinase for NF-κB activation, in aFbs promotes the proliferation of melanoma cells, and the secretion of GAS6 and HGF, which are major tumor-promoting factors, from IKKβ-deleted aFbs was increased.

Since NF-κB signaling plays diverse roles in tumorigenesis, its function in CAFs has been a major topic of interest for researchers. However, the classification of CAFs has been inherently vague. In the News and Views section in *Nature*, two articles published in the *Journal of Experimental Medicine* were highlighted [40]. Pallangyo et al. inhibited NF-κB-IKK signaling in ColVI-expressing CAFs by deleting IKKβ and found that it promoted the proliferation of cancerous intestinal epithelial cells [19]. On the other hand, Koliaraki et al. reported that the deletion of IKKβ in Col1-expressing CAFs suppressed intestinal tumors [20]. These opposite results indicated that different subpopulations of fibroblasts in the TME have different effects on tumor regulation. Based on these reports and discussions, we conducted this study with a focus on activated CAFs by using SM22α as a marker. Furthermore, we implanted a mixture of tumor cells with WT or KO aFbs into normal mice to exclude the effect of deletion of IKKβ in cells other than activated CAFs, which was not examined in these previous papers.

The results of the coculture and conditioned medium experiments suggested that some cytokines/factors secreted from KO aFbs augmented the proliferation of tumor cells. Several cytokines, including transforming growth factor-β (TGFβ), fibroblast growth factor (FGF), GAS6 and HGF, are reported to have such functions [41–44]. Among them, we focused on GAS6 and HGF since they are regulated by the IKK-NF-κB pathway [38,45]. However, it is possible that there are other cancer-promoting cytokines that were upregulated by deletion of IKKβ in aFbs. RNA-seq analysis of WT/KO fibroblasts and/or proteomics analysis of conditioned medium would be useful for further exploration. We will examine these issues in the near future.

Data availability

All data generated or analyzed during this study are included in this published article.

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Declaration of competing interest

The authors declare no conflicts of interest.

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