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Cathepsin K-upregulation in fibroblasts promotes matrigel invasive ability of squamous cell carcinoma cells via tumor-derived IL-1 $\alpha$

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## **Abstract**

**Background:** Cathepsin K (CTSK), a cysteine protease with strong collagenolytic properties, is involved in extracellular matrix turnover. In the previous studies, CTSK expression was detected in peritumoral fibroblasts (Fbs) around squamous cell carcinoma (SCC), but not in those surrounding benign epidermal tumors. However, the mechanism governing CTSK expression in epidermal tumors remains unclear.

**Objective:** To study the regulatory mechanisms of fibroblastic CTSK expression in the SCC–stromal interaction.

**Methods:** We examined dynamic interactions of Fbs with tumorigenic SCC cells (A431 and A253) or normal human keratinocytes.

**Results:** SCC cells and normal keratinocytes did not synthesize CTSK, while Fbs constitutively expressed CTSK. When cocultured, SCC cells upregulated fibroblastic CTSK expression more potently than did normal keratinocytes, which was mainly attributable to SCC-derived IL-1 $\alpha$ . Coculturing Fbs with SCC cells significantly augmented the matrigel invasive ability of SCC cells, which was downregulated when cocultured with CTSK knockdown Fbs or in the presence of neutralizing anti-IL-1 $\alpha$  antibody.

**Conclusion:** The CTSK-upregulated Fbs generated by SCC-derived IL-1 $\alpha$  may play a crucial role in the progression and invasion of SCC.

## **Introduction**

Cancer cells acquire cell-autonomous capacities to undergo limitless proliferation and survival through the activation of oncogenes and inactivation of tumor suppressor genes. Nevertheless, the formation of a clinically relevant tumor requires support from the surrounding stroma, also referred to as the tumor microenvironment, which includes extracellular matrix, basement membrane and fibroblasts (Fbs) [1-3]. Recent studies have revealed that Fbs have a more profound influence on the development and progression of carcinoma. During invasion and metastasis of epithelial tumors, tumor cells detach from the primary site, migrate, and penetrate several structural barriers, e.g. basement membranes and the interstitial connective tissue, and invade the surrounding connective tissue of the dermis. A series of studies revealed that tumor cells are not individual units within a tumor, but rather depend on the cross-talk with neighboring stromal cells and matrix components [4, 5].

The best-characterized proteinases in the degradation of matrix components are matrix metalloproteinases, which are capable of degrading essentially all extracellular matrix (ECM) [6]. Cathepsin K (CTSK) is a cysteine protease with strong collagenolytic activity, first characterized as an important mediator of bone resorption by osteoclasts [7]. CTSK has a uniquely high potency to degrade a wide range of collagens at multiple sites within the triple helix as well as at extra-helical regions [8]. Previous reports have shown that CTSK expression is implicated in the invasive growth of primary tumors in prostate, breast, thyroid and cutaneous squamous cell carcinoma (SCC), where CTSK overexpression by stromal Fbs is associated with an increased invasive potential [9-14].

However, no reports have explained the specific regulatory mechanisms of

fibroblastic CTSK expression in the tumor–stromal interaction. Cytokines and growth factors have been shown to affect cell adhesion, motility, proliferation, and matrix degradation. Soluble factors such as IL-1 $\alpha$ , IL-6, IL-8 and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) secreted from cancer cells may stimulate stromal Fbs, potentially leading to CTSK expression and tumor progression. In skin, previous studies have found that IL-1 $\alpha$  was a plausible candidate for the regulation of CTSK in skin Fbs [15]. In the present study, we examined in vitro dynamic interactions of Fbs with tumorigenic SCC cells or normal keratinocytes in order to clarify the mechanism governing CTSK expression. We found that SCC-derived IL-1 $\alpha$  augmented the CTSK expression of Fbs, and that the Fbs facilitated the matrigel invasive ability of SCC in the coculture system, which was partly attributable to the CTSK overexpression.

## **2.1 Reagents**

Monoclonal anti-CTSK antibody was purchased from Biovendor, USA. Neutralizing monoclonal anti-IL-1 $\alpha$  antibody was obtained from PeproTech, London, UK. Control mouse IgG was purchased from Santa Cruz Biotechnology. Monoclonal anti-CD10 antibody was purchased from Novocastra, Newcastle, UK. Recombinant IL-1 $\alpha$ , IL-6, IL-8 and TGF- $\beta$ 1 were obtained from PeproTech. ELISA kits for IL-1 $\alpha$  were obtained from Invitrogen. Hiperfect Transfection Reagent was obtained from Qiagen, Valencia, USA.

## **2.2 Cell culture, preparation of culture supernatants and coculture models**

Normal human epidermal keratinocytes (Clonetics-Bio Whittaker, San Diego, CA, USA) were cultured with serum-free keratinocyte growth medium (Lonza, Walkersville,

MD, USA) supplemented with epinephrine, transferrin, hydrocortisone, insulin, human recombinant epidermal growth factor, and bovine pituitary extract. Two tumorigenic SCC cell lines, A431 (ATCC) and A253 (ATCC), and a normal human dermal Fb cell line (ATCC CC-2509) were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (FCS), 2 mM non-essential amino acid solution, 2 mM pyruvate, and 2 mM HEPES buffer in 5% CO<sub>2</sub> at 37°C in a humidified atmosphere. In the indirect non-contact coculture model, 20000 epithelial cells (normal keratinocyte or SCC cell line) were cultured upon transwell inserts (0.4-µm pores, Corning Life Science, NY, USA) with 20000 Fbs seeded in 6-well plates, thus allowing bi-directional diffusion of soluble molecules.

### **2.3 Immunoblot analysis**

Total cellular protein was extracted from cultured cells using complete Lysis-M, EDTA-free (Roche Diagnostics GmbH, Roche Applied Science, Germany) and protein concentration was measured with the BCA protein assay kit (Thermo Scientific, USA). Sample proteins (3 µg each) were subjected to electrophoresis in NuPage 4–12% Bis-Tris gradient gels (Invitrogen, Carlsbad, CA, USA), and gels were blotted onto a polyvinylidene difluoride membrane (Invitrogen). Western blot was conducted using the WesternBreeze Chemiluminescent detection kit (Invitrogen).

### **2.4 Real-time reverse transcription PCR**

Total RNA was isolated using the RNeasy® Mini kit (Qiagen, Valencia, USA), and RNA (10 ng/ul) was reverse transcribed to cDNA with the PrimeScript® RT reagent kit (Perfect Real Time, Takara, Japan) according to the manufacturer's protocol.

Quantitative PCR was carried out with an Mx3000PTM (Stratagene) Sequence Detection System using the PrimeScript®RT reagent kit (Perfect Real Time, Takara, Ohtsu, Japan) according to the manufacturer's protocol, with 30 cycles of 5 seconds at 95°C, 20 seconds at 60°C. Each sample was run in triplicate. All primer sets amplified fragments <200 bp long. The amount of each target gene in a given sample was normalized to the level of GAPDH in that sample. Reaction products were separated on 2% agarose gel in some experiments. To determine the expression levels, the density of the bands was measured with Gel Dox™ XR, Bio-rad.

## **2.5 Immunohistochemistry of skin tumors**

Paraffin-embedded specimens of 20 SCCs were obtained from the archives of the Department of Dermatology, Kyushu University, Japan. The archival paraffin-embedded tissue blocks were cut into 4 µm-thick serial sections and mounted on silanated slides. The sections were deparaffinized with xylene and rehydrated through graded ethanol-water solutions. Antibody-binding epitopes of the tissue sections were retrieved by pressure-cooking in 1 mM ethylene diamine tetra acetic acid buffer (pH 6.0) (Yotoron, Tokyo, Japan) for 10 min, and nonspecific binding was blocked using 10% goat serum. The sections were then incubated with antibodies against CTSK (mouse mAb, clone 3F9; 2 µg/ml; Biovendor, USA) and anti-human IL-1α Antibody (1:50 dilution, R&D Systems) at 4°C overnight. Immunodetection was conducted by an avidin-biotin horseradish peroxidase method with 3,3-diaminobenzidine as chromogen followed by light counterstaining with hematoxylin. Washes with Tris-buffered saline or phosphate-buffered saline were performed between each step according to the manufacturer's protocols. CTSK and IL-1α were assessed as

the number of cells with positive staining in the cytoplasm and scored as follows: 0 (no positive cells), 1+ (positive cells<25%), 2+ (26–50%), and 3+ (>51%) in high power fields.

## **2.6 Matrigel invasion assay**

Invasive ability of SCC was assessed by matrigel invasion assay (16). In brief, the upper surface of a filter (pore size 8.0  $\mu\text{m}$ , BD Biosciences, Heidelberg, Germany) was coated with basement membrane matrigel (BD Biosciences).  $1 \times 10^4$  SCC cells with or without  $1 \times 10^4$  Fbs were added to the upper chamber and incubated for 12 to 36 hrs. After incubation, the remaining cells on the upper surface of the filter were removed by wiping with cotton swabs, and the invading cells on the lower surface were fixed with 70% ethanol for more than 10 minutes, and incubated with 10% goat serum for 10 min. The sections were then incubated with antibodies against CD10 (1:100 dilution; Novocastra) at 4°C overnight. Immunodetection was conducted by an avidin-biotin horseradish peroxidase method with 3,3-diaminobenzidine as chromogen followed by light counterstaining with hematoxylin. CD10 immunostaining was useful in discriminating CD10-positive Fbs from CD10-negative SCC cells. SCC cells that had migrated from the upper to the lower side of the filter were counted under a light microscope. Each experiment was carried out in triplicate wells.

## **2.7 Transfection of siRNA**

The siRNA for CTSK (SASI\_Hs01\_00021520) and scrambled control (Mission-SIC-001-s; Mission-SIC-as) were purchased from Sigma-Aldrich Technologies, USA. Fbs were transfected with siRNA by using HiPerfect Transfection Reagent according to the manufacturer's instructions. 60000 cells in 0.5-ml medium

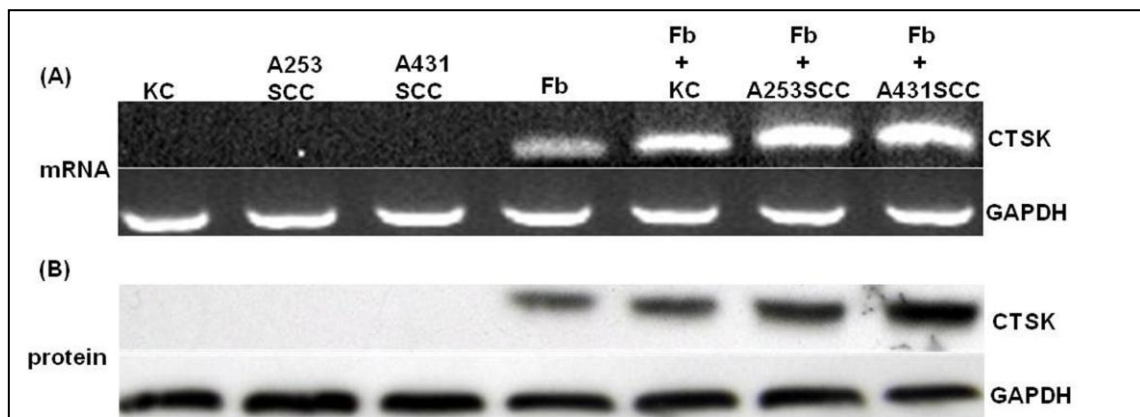


were seeded in 24-well plates. The siRNA was then gently introduced into the cells by mixing with the required amount of Hiperfect Transfection Reagent. In our study, the final concentration of siRNA was 10 nM. Nonsilencing scrambled siRNA served as the control. Experiments were performed using Fbs at 48 hrs post-transfection.

## 2.8 Statistical analysis

Student's t-test was used to analyze data for significant differences. Values of  $P < 0.05$  were regarded as statistically significant.

(Fig.1)



## 3. Results

### 3.1 Preferential induction of CTSK in Fbs by SCC compared to normal keratinocytes

We first examined whether CTSK expression in Fbs was modified using the transwell coculture system with normal keratinocytes, and with A253 and A431 SCC cells (Fig. 1A). Both normal keratinocytes and SCC cells did not express CTSK mRNA and protein, whereas Fbs constitutively expressed CTSK (Fig. 1B and 1C). When cocultured, keratinocytes and SCC cells enhanced the fibroblastic CTSK expression. Compared to normal keratinocytes, tumorigenic SCC cells more potently augmented the

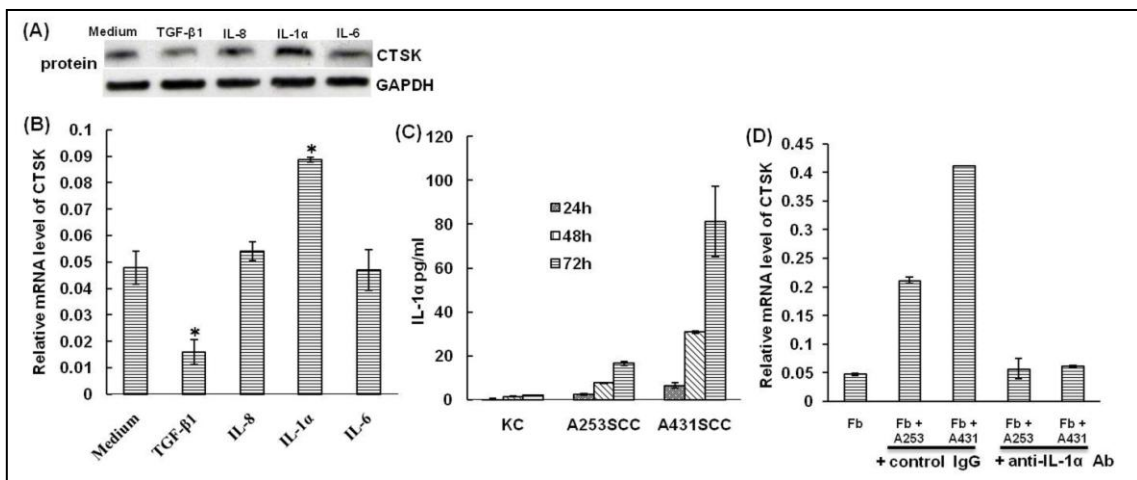
CTSK expression in Fbs (Fig. 1B and 1C). Moreover, coculture with A431 induced a higher level of fibroblastic CTSK expression than did A253. These results suggested that SCC cells secreted some soluble factor(s) that upregulated CTSK expression in Fbs.

### 3.2 Upregulation of CTSK by IL-1 $\alpha$ in Fbs

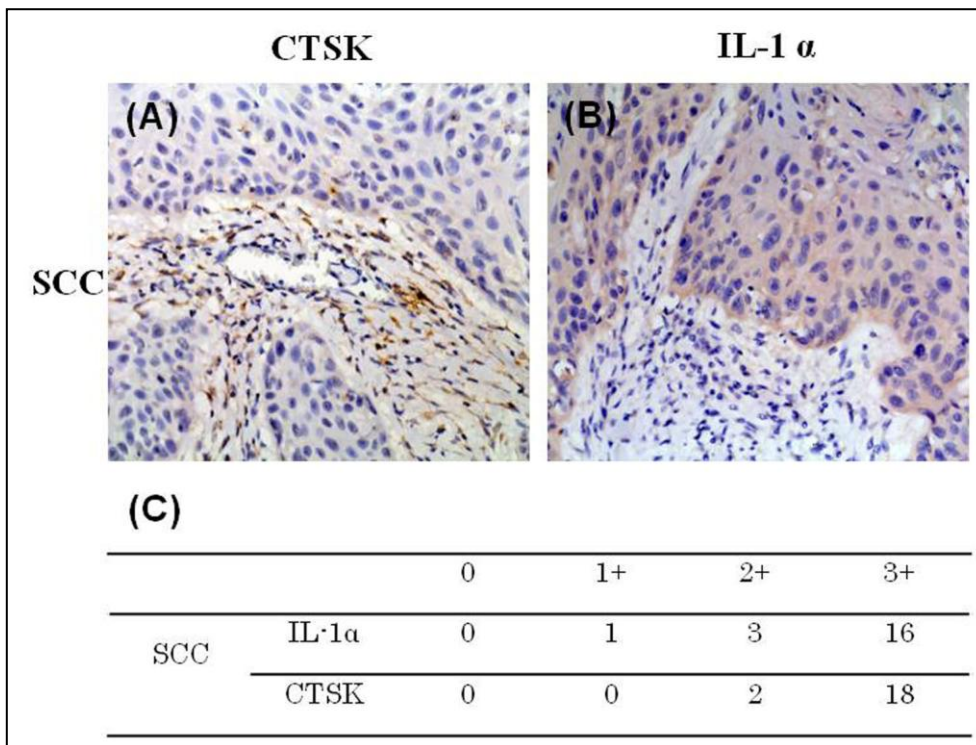
Squamous cells are known to secrete various cytokines such as IL-1 $\alpha$ , IL-6, IL-8 and TGF- $\beta$ 1. In order to examine whether these cytokines affect the CTSK expression of Fbs, we cultured Fbs in the presence or absence of these cytokines. Both protein (Fig. 2A) and mRNA (Fig. 2B) levels of CTSK were upregulated by exogenous IL-1 $\alpha$ , but not by IL-6, IL-8 and TGF- $\beta$ 1; rather, TGF- $\beta$ 1 downregulated the CTSK expression (Fig. 2A and 2B).

We next examined whether normal keratinocytes or SCC cells secrete measurable amounts of IL-1 $\alpha$ . We could detect a small amount of IL-1 $\alpha$  in the culture supernatant of normal keratinocytes, whereas A253 and A431 SCC cells produced larger amounts of IL-1 $\alpha$  than normal keratinocytes in a time-dependent manner. A253 SCC secreted less IL-1 $\alpha$  than did A431 SCC (Fig. 2C), which might explain the lower CTSK-inducing capacity of A253 than A431. We next evaluated the effect of SCC-derived IL-1 $\alpha$  on the fibroblastic CTSK expression by adding neutralizing anti-IL- $\alpha$  antibody to the transwell coculture model. As shown in Fig. 2D, the anti-IL- $\alpha$  antibody significantly inhibited the upregulation of fibroblastic CTSK mRNA expression induced by cocultured A431 SCC and A253 SCC. These results indicated that SCC-derived IL-1 $\alpha$  was the major factor responsible for fibroblastic CTSK overexpression during SCC and Fb interaction in our experimental model.

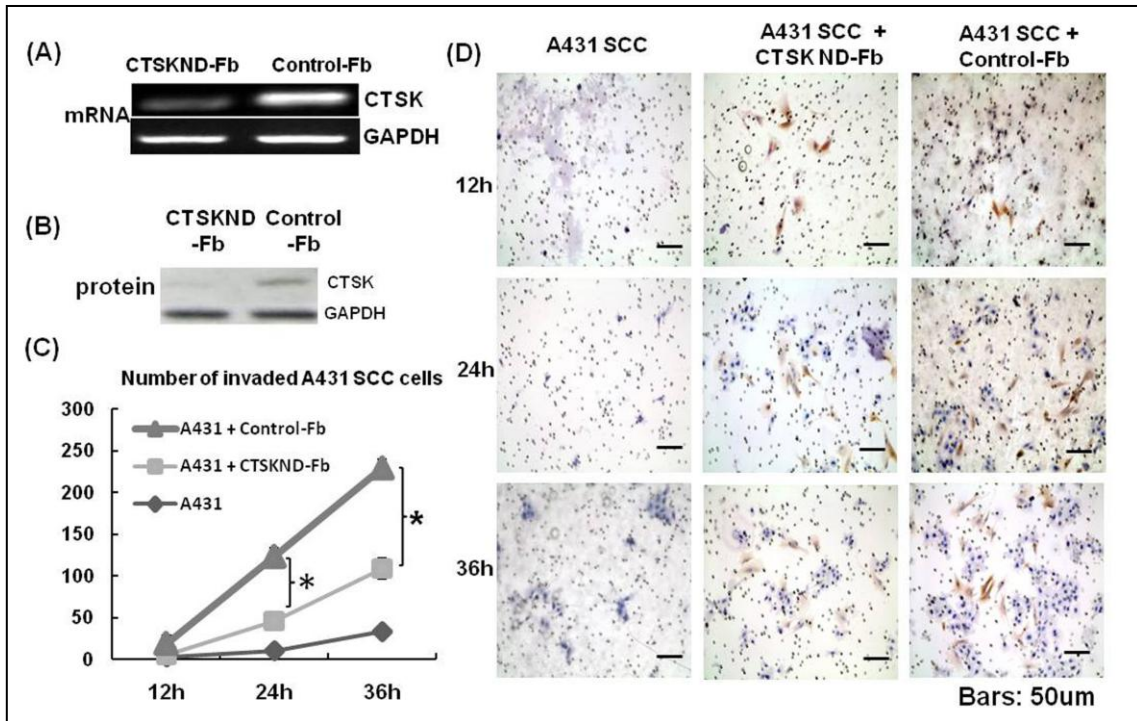
(Fig.2)



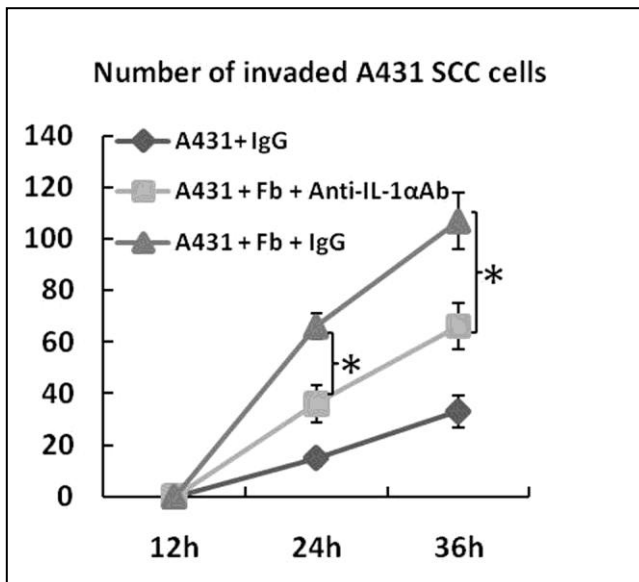
(fig.3)



(Fig.4)



(Fig.5)



### 3.3 IL-1 $\alpha$ and CTSK expression in SCC in vivo

To further confirm the relationship between tumor cell-derived IL-1 $\alpha$  production and fibroblastic CTSK expression in vivo, we next immunohistochemically examined the CTSK and IL-1 $\alpha$  expression in 20 SCCs. As previously demonstrated (14), the stromal Fbs strongly expressed CTSK in SCCs (Fig. 3A and 3C). Likewise, IL-1 $\alpha$  was also observed in the cytoplasm of SCC cells (Fig. 3B and 3C). These results indicated that the present in vitro coculture model mimics, at least in part, in vivo tumor and Fb (IL-1 $\alpha$ /CTSK) interaction.

### 3.4 Augmentation of matrigel invasive ability of SCC cells by coculturing CTSK-expressed Fbs

In order to assess the biological role of CTSK-bearing Fbs, we next generated CTSK knockdown Fbs (CTSKND-Fbs) by transfecting siRNA for CTSK. The mRNA and protein levels of CTSK were markedly inhibited in CTSKND-Fbs compared to control-Fbs transfected by scrambled RNA (control-Fbs) (Fig. 4A and 4B). We then examined the matrigel invasive ability of A431 SCC cells in the presence or absence of CTSKND-Fbs or control-Fbs (Fig. 4C). A431 SCC cells invaded matrigel in a time-dependent manner (Fig. 4D). When cocultured with control-Fbs, the matrigel invasive ability of A431 SCC cells markedly increased in a time-dependent manner, however, the invasive ability of A431 SCC was downregulated when incubated with CTSKND-Fbs (Fig. 4C and 4D). Similar results were obtained in another SCC cell line, A253 (data not shown). We also added neutralizing anti-IL-1 $\alpha$  antibody to the matrigel invasive assay. The anti-IL-1 $\alpha$  antibody significantly inhibited the invasive ability of A431 SCC cells (Fig. 5).

#### 4. Discussion

Tumor invasion and metastasis are indispensable for the progression of malignant tumors. These processes are facilitated by upregulation of various types of proteinases, which induce the escape of cancer cells from the primary site, breaking down connective barriers of the extracellular matrix and basement membranes [17]. Cathepsins are a group of papain-cysteine proteases with collagenolytic activity. The human cysteine cathepsin family includes 11 members which share a conserved activated site, and they primarily function as intracellular proteases mediating terminal nonspecific bulk proteolysis in the acidic environment of lysosomes via phagocytosis [18]. CTSK degrades components of the ECM and basement membranes, and is overexpressed in stromal Fbs at the invasive front of various malignant tumors [9-14, 19, 20]. However, the regulatory mechanism and implication of fibroblastic CTSK expression remain largely unknown.

In this study, SCC and normal keratinocytes did not express CTSK, while its constitutive and consistent expression was detected in normal cultured Fbs. SCC cells induced Fbs to enhance CTSK production more significantly than did keratinocytes in the transwell coculture system, suggesting that soluble factors play a critical role in this phenomenon. Among the squamous cell-derived cytokines, IL-1 $\alpha$  induced CTSK production in Fbs. We also confirmed that SCC cells, but much lower amounts in keratinocytes, indeed secreted a substantial amount of IL-1 $\alpha$ , and that the addition of anti-IL-1 $\alpha$  potentially neutralized the CTSK upregulation in Fbs induced by SCC coculture. These results emphasize the critical role of SCC-derived IL-1 $\alpha$  in the fibroblastic CTSK upregulation in the tumor–stroma microenvironment. We also

confirmed the *in vivo* expression of IL-1 $\alpha$  with consistent stromal fibroblastic CTSK expression in SCC, indicating that our *in vitro* SCC-Fb coculture model mimics and represents, at least to some extent, the *in vivo* tumor-stroma microenvironment.

Fbs are known to internalize extracellular collagen via phagocytosis. Previous studies have found that CTSK in normal Fbs is localized in lysosomes, to which Fbs internalize extracellular collagen I and IV, and thus CTSK degrades collagens with much higher potency than other matrix metalloproteinases [15, 21]. Since collagen IV is one of the major basement membrane components [22], we examined the effects of CTSK-bearing Fbs on the matrigel invasive ability of SCC cells using matrigel invasion coculture assay. The matrigel invasive ability of SCC cells was markedly enhanced in the presence of control-Fbs, however, this enhancing effect was significantly downregulated when SCC cells were cocultured with CTSKND-Fbs, suggesting that the CTSK-bearing Fbs promoted the invasive ability of SCC cells.

Cancer cells metastasize to distant organs via multiple mechanisms, including basement membrane degradation, migration, adhesion to blood and lymphatic vessels and angiogenesis. IL-1 $\alpha$ , a key inflammatory cytokine, has been reported to be produced by various cancer cells derived from pancreas, lung, ovary and stomach, and to increase the invasive and metastatic capabilities of tumor cells [23, 24]. Our data indicate that IL-1 $\alpha$  secreted by SCC cells may enhance the CTSK expression of the surrounding Fbs in a paracrine fashion, which turns out to augment the invasive ability of SCC cells with support from CTSK-bearing Fbs. The interplay of IL-1 $\alpha$  and CTSK in the SCC/Fbs interface may play a crucial role in tumor progression and may be a potential therapeutic target for disarming malignant tumors.

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## **Legends for Figures**

Figure 1. (A) CTSK mRNA expression level in KC, A253 SCC, A431 SCC, Fb, and Fb indirectly cocultured with KC, A253 SCC, and A431 SCC cells for 48 hrs. (B) Western blot analysis of CTSK protein expression level in KC, A253 SCC, A431 SCC, Fb, and Fb indirectly cocultured with KC, A253 SCC, A431 SCC cells for 48 hrs. KC: keratinocytes

Figure 2. (A) Fbs were cultured with medium only, TGF- $\beta$ 1 (5 ng/ml), IL-8 (5 ng/ml), IL-1 $\alpha$  (5 ng/ml), and IL-6 (5 ng/ml) for 24 hrs, and protein levels of CTSK were measured by western blot. (B) Fbs were cultured with medium only, TGF- $\beta$ 1 (5 ng/ml), IL-8 (5 ng/ml), IL-1 $\alpha$  (5 ng/ml), and IL-6 (5 ng/ml) for 24 hrs, and mRNA levels of CTSK were measured by real-time PCR. (C) Concentrations of IL-1 $\alpha$  were assayed by ELISA in the supernatants of KC and SCC cells cultured for 24, 48 and 72 hrs. (D) The neutralizing anti-IL-1 $\alpha$  antibody significantly reduced the upregulation of CTSK in Fbs induced by SCC in the coculture model.

Figure 3. Immunohistochemical staining for CTSK (A) and IL-1 $\alpha$  (B) in SCC. CTSK was strongly expressed in the majority of stromal Fbs in SCC. In parallel with the stromal CTSK expression, IL-1 $\alpha$  expression was observed in the tumor cells of SCC (C).

Figure 4.(A) mRNA levels of Fbs transfected with siRNA for CTSK (CTSKND-Fbs) and Fbs transfected with control scrambled RNA Fbs (control-Fbs). CTSK mRNA level was markedly downregulated in CTSKND-Fbs. (B) CTSK protein levels in CTSKND-Fbs and control-Fbs. CTSK protein level was reduced in CTSKND-Fbs. (C) Matrigel invasion assay was performed using A431 SCC cells in the presence or absence of CTSKND-Fbs or control-Fbs by 12, 24 and 36 hrs incubation. (D) A431 SCC cells invaded matrigel in a time-dependent manner (◆). Coculturing of CTSK-bearing control-Fbs significantly upregulated the matrigel invasive ability of A431 SCC (▲). However, the upregulation of invasive ability was markedly reduced when cocultured with CTSKND-Fbs (■). A431 SCC cells were counted excluding CD10+Fbs (brown). \*: P<0.05

Figure 5. A431 SCC cells invaded matrigel in a time-dependent manner (◆). Coculturing of Fb significantly upregulated the matrigel invasive ability of A431 SCC (▲). However, the upregulation of invasive ability was significantly reduced when treated with neutralizing anti-IL-1 $\alpha$  antibody (■). A431 SCC cells were counted excluding CD10+Fbs (brown). \*: P<0.05

