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Intracellular IL-33 Attenuates Extracellular IL-33-induced Cholangiocarcinoma Cell Proliferation and Invasion *via* NF-κB and GSK-3β Pathways

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Abstract. *Background/Aim: The functions of interleukin 33 (IL-33) in cholangiocarcinoma (CCA) are unclear. This study aimed to evaluate the roles of IL-33 in CCA progression. Materials and Methods: The effect of intracellular IL-33 using shIL-33 knocked down KKU-055 (IL-33KD-KKU-055) compared to parental (Pa) KKU-055 and extracellular IL-33 using recombinant human IL-33 (rhIL-33) treatment on the proliferation and invasion of CCA cells grown in 3D cultures was studied. Relevant markers were determined by western blot or ELISA. Results: IL-33KD-KKU-055 cells showed increased proliferation and invasion in 3D cultures compared to Pa-KKU-055 cells, with NF-κB and IL-6 up-regulation. Treatment with 2 ng/ml rhIL-33 promoted Pa-KKU-055 cell proliferation by inducing NF-κB and IL-6 expressions. Upon GSK-3β inactivation and increased nuclear full-length IL-33 (flIL-33), 20 ng/ml rhIL-33 had no effect on proliferation. Both 2 and 20 ng/ml rhIL-33 induced proliferation and invasion of IL-33-negative KKU-213 cells in 3D cultures, as well as NF-κB and IL-6 up-regulation. Conclusion: Intracellular and extracellular IL-33 have distinct roles in the mechanisms of CCA progression.*

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Interleukin 33 (IL-33) is a cytokine belonging to the IL-1 superfamily (1), located inside the nucleus of endothelial cells, fibroblasts, and epithelial cells (2). Intracellularly, the full-length IL-33 isoform (flIL-33) contains an N-terminus nuclear binding domain, and acts as a transcriptional regulator through its chromatin motif by binding to NF-κB transcription factor subunits (p65 and p50) (3-5). Alternatively, released flIL-33 is enzymatically processed, generating a mature IL-33 (mtrIL-33) (6, 7), which binds to the cell surface ST2 receptor (ST2L) expressed on T (8), NK (9), type 2 innate lymphoid cells (10), and macrophages (11). ST2L activation promotes NF-κB, ERK, MAPK, and p38 signaling pathways leading to cell proliferation and cytokine production (12, 13).

In cancer, IL-33 functions as either a good or a bad prognostic marker (14-18), with high serum and tissue IL-33 correlating with lymph node involvement and metastasis in breast cancer (14). Moreover, increased IL-33 relates to the expression of vascular endothelial growth factor, matrix metalloprotease (MMP)-11, and platelet-derived growth factor-C in breast cancer patients (15). IL-33 has also been associated with poor prognosis and high tumor metastasis in hepatocellular carcinoma (16). Furthermore, IL-33 directly affects cancer cell migration and invasion *via* NF-κB, ERK, and AKT signaling pathways in lung, ovarian, and gastric cancers (19, 20). Strangely, IL-33 has been considered a good prognostic marker in patients with lung cancer (17) and hepatocellular carcinoma (18). IL-33 positive staining in normal liver tissues is predominantly located in the nucleus, however, IL-33 in hepatocellular carcinoma tissues is mostly

restricted to the cytoplasm (18). Extracellular IL-33, either flIL-33 (passively released from damaged cells) or mtrIL-33, binds to ST2L enhancing NF-κB, AKT and ERK pathways, resulting in cancer cell progression (12, 13); whereas intracellular flIL-33 suppresses NF-κB-regulated gene transcription by binding to p65 and p50 (3-5). Therefore, it is possible that IL-33, either extracellular or intracellular, may be involved in cancer progression.

Cholangiocarcinoma (CCA) is the second most common hepatic cancer with high mortality and recurrence rates (21). High IL-33 levels correlate with less aggressive clinicopathological parameters and are an independent good prognostic marker in large bile duct CCA (22). In mice, IL-33 promotes biliary repair and carcinogenesis *via* an interleukin 6 sensitive mechanism (23, 24). Accordingly, our group has recently reported that IL-33 was an independent good prognostic marker in CCA patients. Patients with high IL-33 levels in cancer cells and cancer-associated fibroblasts (CAFs) showed longer survival compared to those with low IL-33 (25). These results suggest that extracellular IL-33 could promote CCA cell migration, while intracellular IL-33 seems to suppress CCA cell migration (25); however, the mechanisms related to these effects are unclear.

In this study, the role of intracellular and extracellular IL-33 on CCA cell proliferation and invasion and their related signaling pathways were investigated. Intracellular flIL-33 suppressed CCA cell proliferation and invasion through NF-κB inactivation, resulting in IL-6 reduction. In contrast, extracellular IL-33 promoted proliferation and invasion of IL-33-negative CCA cells, by enhancing NF-κB activation, leading to IL-6 production and GSK-3β activation. However, in IL-33-positive CCA cells, high extracellular IL-33 inactivated GSK-3β, but activated NF-κB, surprisingly leading to enhanced nuclear flIL-33, inhibiting extracellular IL-33-mediated cell proliferation and invasion. Treatment with low extracellular concentrations of IL-33 activated the NF-κB pathway, causing CCA cell proliferation and invasion. These findings demonstrate the cellular mechanisms of function of IL-33 in CCA patients, and that inhibition of IL-33-mediated NF-κB pathway may be an alternative treatment choice in patients having none-to-low levels of IL-33 in cancer cells.

Materials and Methods

CCA cell lines. CCA cell lines, including KKU-055 and KKU-213, were purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Osaka, Japan). Cells were maintained in Dulbecco's Modified Eagle's medium high glucose (DMEM) (Gibco, Thermo Scientific, Waltham, MA, USA) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) at 37˚C in a 5% CO_2 incubator.

shRNA against IL-33 for IL-33 knockdown. IL-33-positive KKU-055 cells and Pa-KKU-055 cells were plated at 2.5×10^5 cells/well in 6-well plate and allowed to adhere overnight to reach 70-80% confluency. IL-33 shRNA plasmid (Santa Cruz Biotechnology, Dallas, TX, USA) was transfected into cells using Lipofectamine 3000 (Invitrogen, Waltham, CA, USA) following the manufacturer's instructions. Briefly, 3.75 μl of Lipofectamine 3000 was diluted in Opti-MEM Medium (Gibco) before being mixed equally with 0.5 μg of IL-33 shRNA plasmid, after incubating at room temperature for 15 min, the DNA-lipid complex was added to KKU-055 cells for 24 h. The stable clone was selected in complete medium containing 1.5 μg/ml puromycin (Santa Cruz Biotechnology). IL-33 knockdown (KD) KKU-055 cells were collected and checked for IL-33 levels using western blot analysis.

3D spheroid proliferation and invasion assays. CCA cells (1×10^3 cells) were mixed with pre-cooled 2.5% Matrigel® Basement Membrane Matrix (Corning, Glendale, AZ, USA) in 1% FBS DMEM in total volume 200 μl then seeded into pre-cooled ultra-low attachment 96-well plate (Corning) before centrifugation at 300 × *g* at 4˚C for 5 min. The spheroids were established for 4 d (4-d spheroid) at 37˚C in a 5% CO_2 atmosphere incubator. For three-dimensional (3D) tumor proliferation, the 4-d spheroid culture plate was placed on ice, then 1% FBS DMEM containing mature rhIL-33, (mtrIL-33), was added every 3-4 d. The spheroid size was measured under the inverted microscope Olympus IX71, using Olympus CellSens Standard software on days 0, 3, and 5 after the first rhIL-33 treatment. The sphere volume was calculated using the longest radius in the formula: sphere volume=$4/3\pi r^3$. Cultures not treated with IL-33 were used as negative controls.

For 3D invasion assay, the 4-d spheroid culture plate was placed on ice, then the Matrigel with or without rhIL-33 [diluted with ice cold 1% FBS DMEM (1:2)] was added. Afterwards, plates were centrifuged at 300 × *g* at 4˚C for 3 min and incubated at 37˚C in 5% CO_2 for 2 h. After Matrigel was solidified, 100 μl of 1% FBS DMEM with or without rhIL-33 was added on top. Invading cell images were taken on days 0, 3, and 5 after treatment, and only the invaded area was measured using the formula: Volume of invading spheroid=Volume of whole spheroid – Volume of spheroid core.

Whole cell lysates, cell fractionation and conditioned-medium collection. Whole cell lysates (WCL) of Pa-KKU-055, IL-33KD-KKU-055, and KKU-213 cells cultured in complete medium were collected to determine endogenous expressions of IL-33, ST2, IL-6, and signaling molecules. Cells were starved in 1% FBS DMEM for 24 h, and then collected at specific times after rhIL-33 treatment. The cells were lysed in RIPA buffer (Santa Cruz Biotechnology) containing lysis buffer, PMSF, sodium orthovanadate, and protease inhibitor cocktail, followed by centrifugation at 12,000 × *g* for 10 min at 4˚C. Moreover, the rhIL-33 treated CCA cells were fractionated into cytoplasmic (Cy) and nuclear (Nu) fractions using Cell Fractionation Kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions. Briefly, 2.5×10^6 cells were lysed in Cytoplasmic Isolation Buffer (CIB) buffer. The lysate was centrifuged at 500 × *g* for 5 min. The supernatant, which contains the cytoplasmic fraction, was collected. The nuclear fraction was then isolated by adding Cytoskeletal/Nuclear Isolation Buffer (CyNIB) buffer to the remaining cell pellet and sonicating it. The conditioned-medium (CM) was collected from cells grown to 80-90% confluence in complete medium for western blot analysis or in 1% FBS DMEM for ELISA. After centrifugation at 2,000 × *g* at 4˚C for 10 min, CM was concentrated using Vivaspin® 6, 5 kDa

MWCO Polyethersulfone filters (GE Healthcare, Chicago, IL, USA) by centrifugation at 40,000 × *g* for 90 min at 4˚C. The protein concentration of both cell lysates and concentrated CM was determined using the Bradford assay and Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA).

IL-33, ST2, IL-6 and signaling molecule detection by western blot. Cell lysates and CM were prepared in sample buffer containing 10% SDS, 1.0 M Tris-HCl pH 6.8, 8% glycerol, and 0.05% (w/v) bromophenol blue. After boiling for 5 min, 30 μg of cell lysate and 250 μg of CM were loaded per lane in 12% SDS-PAGE, and the proteins were separated at 120 V for 90 min and transferred to PVDF membrane (GE Healthcare) at 37 mA for 90 min. The PVDF membrane was blocked with 5% skim milk (Sigma-Aldrich, St. Louis, MO, USA) or 5% BSA in 1 X TBST, and then incubated with 1:500 goat anti-human IL-33 antibody (AF3625, R&D System, Minneapolis, MN, USA), 1:250 goat anti-human ST2 antibody (AF523, R&D System), 1:1,000 rabbit anti-human GSK-3β antibody (9315S, Cell Signaling Technology), 1:1,000 rabbit anti-human pS9GSK-3β antibody (9323S, Cell Signaling Technology), 1:1,000 rabbit anti-human IκBα antibody (9242, Cell Signaling Technology), 1:1,000 mouse anti-human pS32/36IκBα (9246, Cell Signaling Technology), 1:1,000 rabbit anti-human AKT antibody (9272, Cell Signaling Technology), 1:1,000 rabbit anti-human pS473AKT antibody (9271, Cell Signaling Technology), 1:2,000 rabbit anti-human IL-6 antibody (ab6672, Abcam, Cambridge, UK), 1:500 mouse anti-human histone H1 antibody (sc-8030, Santa Cruz Biotechnology) or 1:5,000 mouse anti-human β-actin (sc-47778, Santa Cruz Biotechnology) at 4˚C overnight or RT for 2 h (for ST2 and IL-6). After washing, the membrane was incubated with 1:1,000 rabbit anti-goat IgG-HRP antibody (HAF017, R&D System), 1:2,000 horse anti-mouse IgG-HRP antibody (7076, Cell Signaling Technology) or 1:2,000 goat anti-rabbit IgG-HRP antibody (ab6721, Abcam) at RT for 1 h. The immunoreactive signals were visualized by Clarity Western ECL (Bio-Rad). The β-actin protein levels were used as an internal control determining equal amounts of loading proteins from WCL and cytoplasmic proteins, whereas histone H1 was used as the internal control for the nuclear protein fraction. The densitometric values of all protein bands were normalized with that of β-actin and quantified using Image J (version 1.52a, National Institutes of Health, Bethesda, MD, USA).

IL-6 and sST2 detection by ELISA. The CM of CCA cells with or without rhIL-33 were collected after 24 h of treatment and concentrated as described above. A total of 250 μg of CM (50-100 μl) was used to determine the levels of secreted IL-6 and sST2 using Human IL-6 Quantikine ELISA Kit (R&D System) and Human ST2/IL-33R Quantikine ELISA Kit (R&D System) following the manufacturer's instructions. Recombinant human tumor necrosis factor alpha (rhTNF-α) (ImmunoTools, Friesoythe, Germany) was used as a positive control for IL-6 stimulation (26).

Statistical analysis. Mean spheroid volume and mean densitometric values of signaling molecules in western blot were compared between several time periods and treatment groups using one-way analysis of variance (ANOVA) by Tukey test. All statistical analyses were performed using GraphPad Prism program (version 5.01) (San Diego, CA, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

Knockdown of intracellular IL-33 enhances cell proliferation, invasion, activation of NF-κB and IL-6 production. The CCA cell line KKU-055 endogenously expresses flIL-33, although mtrIL-33 was not found in the CM (Figure 1A), whereas KKU-213 cells could not produce IL-33. Membrane and decoy IL-33 receptor, ST2L and sST2, were detected in both KKU-055 and KKU-213 cells. Herein, KKU-055 is designated as an IL-33-positive and KKU-213 as an IL-33-negative cell line.

To determine whether intracellular IL-33 plays a role in CCA cell proliferation and invasion, IL-33 knockdown KKU-055 cells (IL-33KD-KKU-055; IL-33KD) were generated from parental KKU-055 cells (Pa-KKU-055; Pa) (Figure 1B). Morphology (Figure 1C) and viability of IL-33KD-KKU-055 were similar to Pa-KKU-055 cells. IL-33 levels in IL-33KD-KKU-055 were estimated to be 38.7% of those in the parental cells (Figure 1D), with higher NF-κB activation (increased pIκBα/IκBα) compared to Pa-KKU-055 ($p<0.01$) (Figure 1B and E). Knockdown of intracellular IL-33 profoundly promoted IL-6 expression ($p<0.0001$) (Figure 1B and F), confirmed by higher IL-6 ($p<0.0001$) (Figure 1G) and sST2 levels in the media ($p<0.05$) (Figure 1H) of IL-33KD-KKU-055 cells compared to those of Pa-KKU-055, as detected by ELISA.

When comparing the effect of intracellular IL-33 on the proliferation and invasion of IL-33KD-KKU-055 cells grown in 3D cultures to those of parental cells, increased proliferation was observed in both (time-dependent) (Figure 1I and K), with enhanced spheroid proliferation on day 3 ($p<0.05$) and 5 ($p<0.01$) in IL-33KD-KKU-055 cells versus parental cells (Figure 1I and K). IL-33KD-KKU-055 cells exhibited increased invasion capability in a time-dependent manner compared to Pa-KKU-055 cells ($p<0.05$) (Figure 1J and L).

Low extracellular IL-33 drives IL-33-positive cell NF-κB-dependent proliferation, attenuated by high IL-33 in a GSK-3β-dependent mechanism. Results showed that 2 ng/ml rhIL-33 induced 3D proliferation of Pa-KKU-055 cells on day 3 and 5 ($p<0.01$), although this effect was diminished by exposure to 20 ng/ml rhIL-33 ($p<0.01$) (Figure 2A and B). However, low dose extracellular IL-33 had no effect on IL-33KD-KKU-055 cells at any time point (Figure 2A and C). Notably, untreated IL-33KD-KKU-055 cells had higher intrinsic proliferation rate than Pa-KKU-055 cells (Figure 2B and C), supporting previous findings mentioned above (Figure 1I and K).

Both high and low rhIL-33 concentrations enhanced NF-κB activation, represented by the pIκBα/IκBα ratio in both Pa-KKU-055 and IL-33KD-KKU-055 cells, compared to untreated controls ($p<0.01$ and $p<0.0001$) (Figure 2D and E). In Pa-KKU-055, 2 ng/ml rhIL-33 activated NF-κB after 30 min ($p<0.05$), whereas 20 ng/ml rhIL-33 had no effect

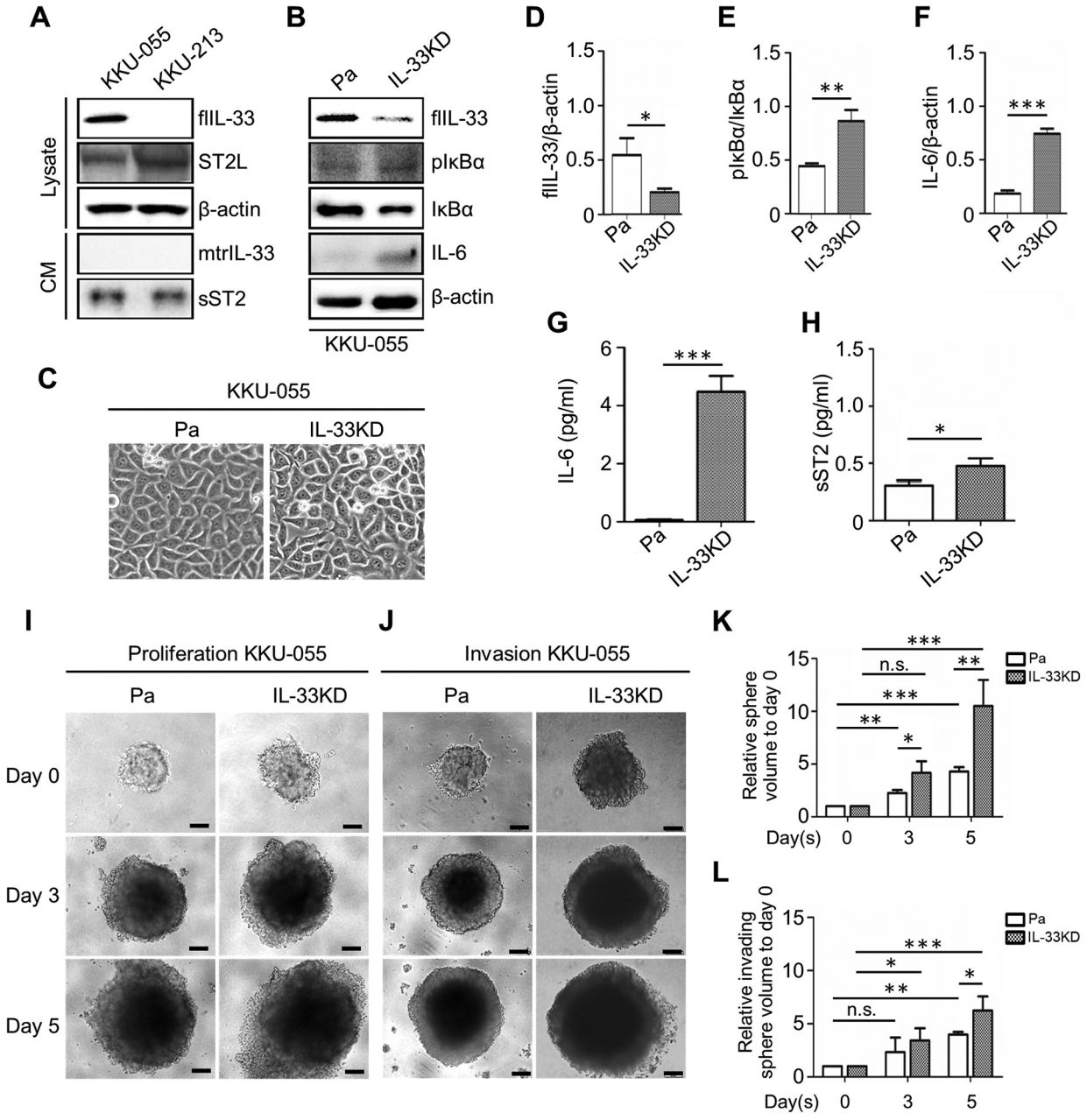

Figure 1. *IL-33 and ST2 expression in CCA cells and the effect of intracellular IL-33 on cell proliferation and invasion. Western blot analysis of (A) endogenous IL-33, ST2L and sST2 in CCA cell lysates and conditioned-medium (CM) and (B) signaling molecules. (C) The morphology of Pa-KKU-055 and IL-33KD-KKU-055 cells. Original magnification of 200×. Densitometric values of (D) flIL-33/β-actin (E) pIκBα/IκBα (F) IL-6/β-actin in Pa-KKU-055 cells compared with IL-33KD-KKU-055 cells. ELISA results of (G) IL-6 and (H) sST2 in Pa-KKU-055 cell CM compared with IL-33KD-KKU-055 cell CM. (I and K) 3D proliferation and (J and L) 3D invasion of Pa-KKU-055 and IL-33KD-KKU-055 cells on days 0, 3 and 5. Original magnification of 100×. Three independent experiments were performed. Bars represent mean±SD. *p<0.05, **p<0.01, ***p<0.0001.*

(Figure 2D and E). Remarkably, high rhIL-33 (20 ng/ml) induced NF-κB activation in IL-33KD-KKU-055 cells.

Regarding AKT pathway regulation, delayed (90 min) reduction of AKT phosphorylation occurred with high rhIL-33 concentration in both Pa-KKU-055 and IL-33KD-KKU-055 cells ($p<0.05$) (Figure 2D and F). In relation to the effect of rhIL-33 on the pS9GSK-3β/GSK-3β ratio (representing GSK-3β inactive status), low concentration of

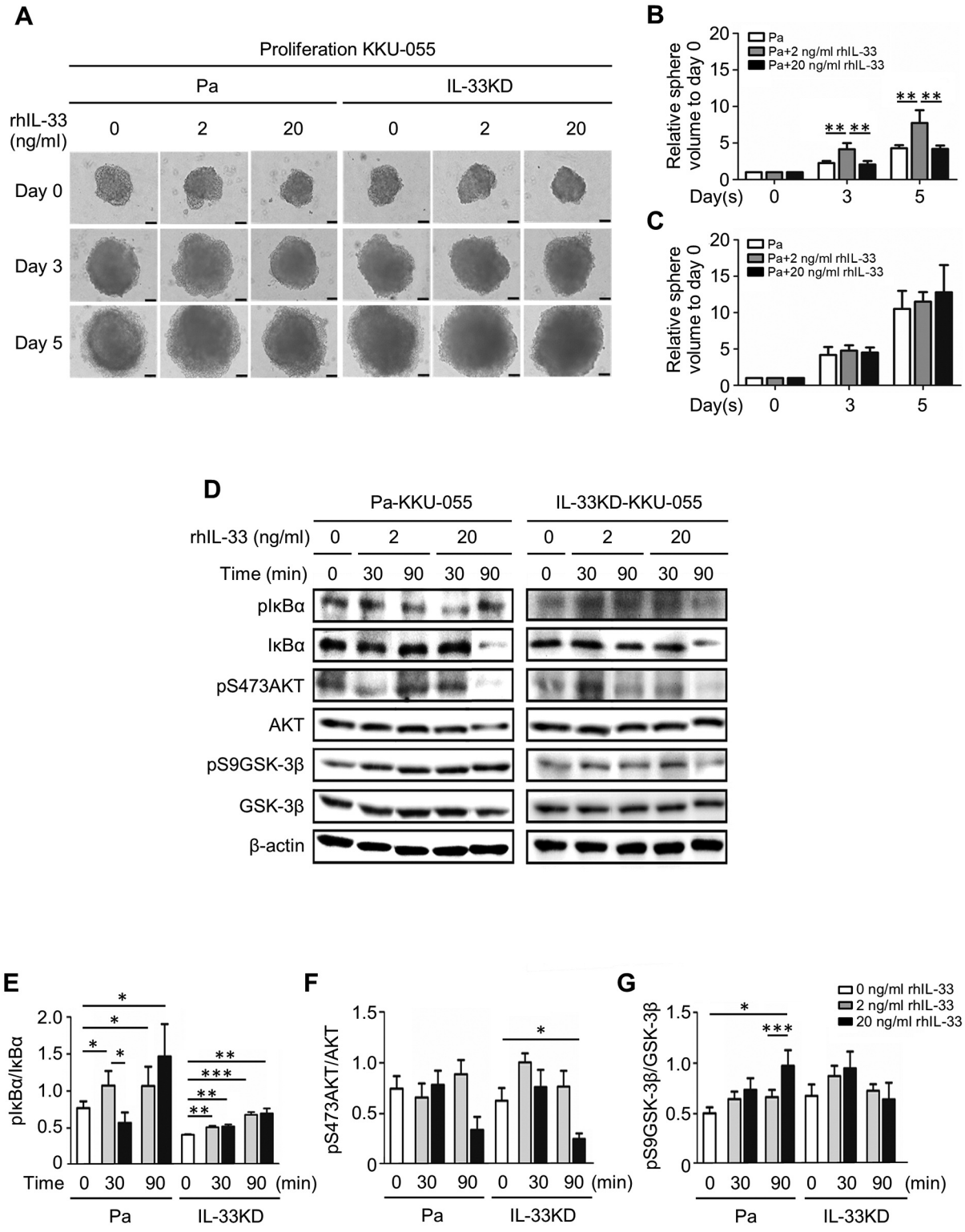

Figure 2. *The effect of rhIL-33 on cell proliferation and signaling molecules involved in its response. (A) proliferation of rhIL-33 treated Pa-KKU-055 and IL-33KD-KKU-055 grown in 3D cultures. Original magnification of 100×. Relative 3D proliferation of rhIL-33 treated (B) Pa-KKU-055 and (C) IL-33KD-KKU-055 cells. (D) Western blot analysis of signaling molecules. Densitometric values of (E) pIκBα/IκBα, (F) pS473AKT/AKT (G) pS9GSK-3β/GSK-3β in rhIL-33 treated Pa-KKU-055 and IL-33KD-KKU-055 cells. Three independent experiments were performed. Bars represent mean±SD. *p<0.05, **p<0.01, ***p<0.0001.*

rhIL-33 (2 ng/ml) had no effect whereas high concentration of rhIL-33 (20 ng/ml) increased it at 90 min in Pa-KKU-055 cells compared to untreated ($p<0.05$) and low concentration treated cells ($p<0.0001$) (Figure 2D and G). Contrastingly, in IL-33KD-KKU-055 cells, both rhIL-33 concentrations had no effect on GSK-3β function (Figure 2D and G).

Dual role of IL-33 on IL-6, ST2 variants and nuclear flIL-33 production in IL-33 positive CCA cells. The results revealed that in whole cell lysates (WCLs), both the low and high concentrations of rhIL-33 had no effect on intracellular flIL-33 levels in Pa-KKU-055 cells (Figure 3A and C), but in IL-33KD-KKU-055 cells, where basal IL-33 expression is dramatically reduced, extracellular rhIL-33 dose-dependently induced intracellular IL-33 expression ($p<0.01$) (Figure 3B and C). Additionally, low and high rhIL-33 concentrations had no effect on intracellular flIL-33 levels in cytoplasmic (CY) fractions of Pa-KKU-055 cells (Figure 3E and F), whereas treatment with a high concentration of rhIL-33 increased the levels of nuclear (NU) flIL-33 ($p<0.0001$) (Figure 3E and G) in Pa-KKU-055 cells. Only extracellular high concentrations of rhIL-33 induced ST2L in Pa-KKU-055 cells ($p<0.01$), although this was not observed in IL-33KD-KKU-055 cells (Figure 3A and D). Interestingly, sST2 levels were reduced in the CM of Pa-KKU-055 cells ($p<0.01$) treated with low rhIL-33, however, they remained unaltered after high rhIL-33 treatment (Figure 3I). As expected, both low and high concentrations of rhIL-33 had no effect on sST2 extracellular levels in IL-33KD-KKU-055 cell cultures compared to control (Figure 3I).

Treatment with both low and high rhIL-33 concentrations increased extracellular IL-6 levels in Pa-KKU-055 cultures ($p<0.0001$ and $p<0.01$), similar to the TNF-α treatment used as a positive control ($p<0.01$) (Figure 3H). In contrast, this induction effect was not observed in IL-33KD-KKU-055 cells (Figure 3H).

Extracellular IL-33 promotes proliferation and invasion via NF-κB activation, GSK-3β inactivation and IL-6 production in IL-33-negative CCA cells. The effect of extracellular IL-33 on IL-33-negative KKU-213 cell proliferation and invasion was determined along with the signaling pathways involved. Both rhIL-33 concentrations promoted cell proliferation ($p<0.05$) (Figure 4A and B) and invasion ($p<0.05$) (Figure 4A and C) on day 5 post-treatment.

Extracellular rhIL-33 promoted pIκBα/IκBα at 30-, 60-, and 90-min post-treatment, representing NF-κB-dependent pathway activation (Figure 4D and E). Only the low concentration of rhIL-33 at 90 min post-treatment activated the AKT pathway, by increasing the pS473AKT/AKT ratio ($p<0.01$) (Figure 4D and F). Decreased pS9GSK-3β/GSK-3β ratio, representing GSK-3β pathway activation, was observed in KKU-213 cells treated with both rhIL-33 concentrations at 60 and 90 min ($p<0.05$) (Figure 4D and G). Both low and high concentrations of rhIL-33 had no effect on intracellular IL-33 and ST2L levels in KKU-213 cells (Figure 4H and I), whereas treatment with low concentration of rhIL-33 induced IL-6 (Figure 4J), without affecting the levels of sST2 in KKU-213 cell CM (Figure 4K).

Discussion

High IL-33 levels in cancer and stromal CAFs correlate with a favorable prognosis of patients with CCA (25), however, the mechanism of the suppressive effect of IL-33 on CCA aggressive properties remains unclear. As intracellular and extracellular IL-33 have different functions (27), using IL-33-positive and negative cells, we showed their distinct effects on CCA cell proliferation and invasion. Intracellular IL-33 prevented cell proliferation and invasion by inhibiting NF-κB/IL-6 pathway, while extracellular IL-33 activated NF-κB/IL-6 and/or GSK-3β signaling pathways, depending on its levels. Low IL-33 activated proliferation and invasion in both IL-33-positive and IL-33-negative CCA cells, whereas high levels facilitated cell proliferation and invasion of IL-33-negative cells, but not of IL-33-positive cells. The possible mechanisms of the effects of extracellular IL-33 on CCA cells with or without endogenous IL-33 through NF-κB and GSK-3β signaling pathways were examined.

Knockdown of IL-33 in the IL-33-positive cell line KKU-055, which does not secrete IL-33, resulted in enhanced proliferation and invasion. This effect was supported by similar experiments in ovarian cancer cells, where siIL-33-treated cells showed enhanced cell proliferation and migration (28). Moreover, NF-κB and IL-6 were up-regulated in IL-33KD-KKU-055 cells in comparison to parental cells. Consequently, intracellular IL-33, both nuclear and cytoplasmic, inhibited the NF-κB-regulated expression of genes, such as tumor necrosis factor (TNF)-α (4) and IL-6 (29). In contrast, IL-6 increased in siIL-33-treated pulmonary arterial endothelial cells (29), therefore, the regulation of this inflammatory gene by IL-33 is cell- or tissue-dependent. As NF-κB and IL-6 are aberrantly expressed in CCA and correlate with cell growth and anti-apoptosis (30-32), intracellular IL-33 possibly suppresses proliferation and invasion *via* the NF-κB/IL-6 pathway. In contrast, over-expression of intracellular IL-33 increased osteosarcoma cell survival *via* the PI3K/AKT pathway (33) and promoted skin cancer development *via* Smad6 inhibition (34), but had no effect on glioma cell proliferation (35). Therefore, intracellular IL-33 possibly activates different pathways in cancers, leading to either activation or suppression of cellular proliferation, migration, and invasion.

Additionally, extracellular IL-33 stimulated NF-κB, ERK, and AKT signaling pathways, as well as matrix metalloproteases (MMPs) and IL-6 production, promoting

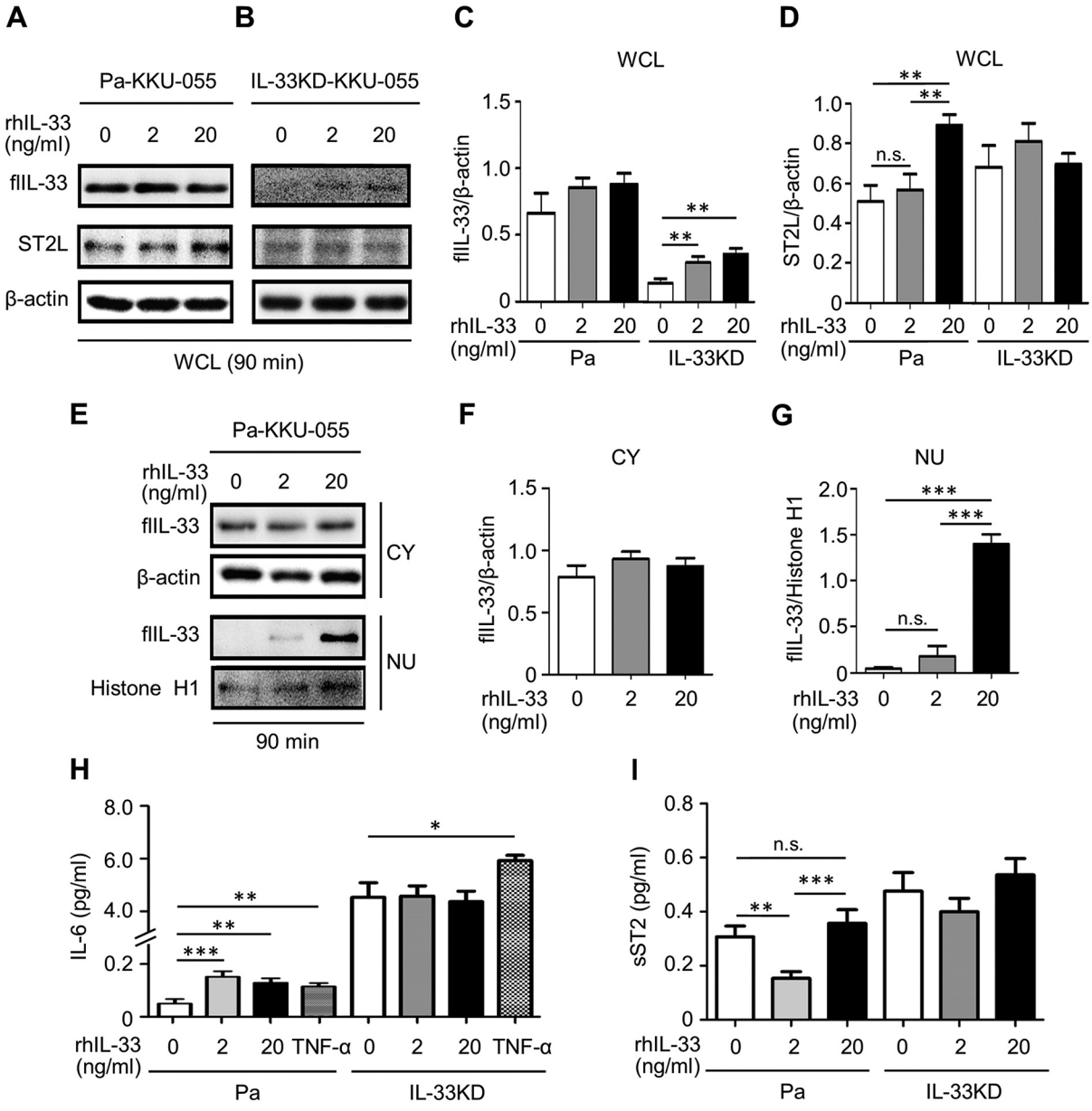

Figure 3. *Intracellular IL-33, ST2L, sST2 and IL-6 in rhIL-33 treated Pa-KKU-055 and IL-33KD-KKU-055 cells. Western blot analysis in WCLs of rhIL-33 treated (A) Pa-KKU-055 and (B) IL-33KD-KKU-055 cells. Densitometric values of (C) flIL-33/β-actin and (D) ST2L/β-actin in WCLs of Pa-KKU-055 and IL-33KD-KKU-055 cells. (E) Western blot analysis of fractionated Pa-KKU-055 cells treated with rhIL-33. Densitometric values of flIL-33/β-actin in (F) cytoplasmic and (G) nuclear fractions of rhIL-33 treated Pa-KKU-055 cells. ELISA results of (G) IL-6 and (H) sST2 in rhIL-33 treated Pa-KKU-055 cell CM compared with that of IL-33KD-KKU-055 cells. Three independent experiments were performed. Bars represent mean±SD. *p<0.05, **p<0.01, ***p<0.0001.*

cancer cell migration and invasion in lung cancer (19, 20), as we demonstrated in IL-33-negative CCA cell and has been reported in colorectal cancer (36), *via* NF-κB-mediated COX2/PGE2 (37). Additionally, as we demonstrated in IL-33-negative CCA cells, extracellular IL-33 has been shown to stimulate GSK-3β activity in mouse lung epithelial cells (38). GSK-3β levels have been shown to be associated with metastasis and poor prognosis in CCA patients (39); with increased cell proliferation, migration, invasion, and survival in various cancers (40-42). Thus, extracellular IL-33 promotes CCA cell proliferation and invasion through activating NF-κB/IL-6 and GSK-3β. Interestingly, a low

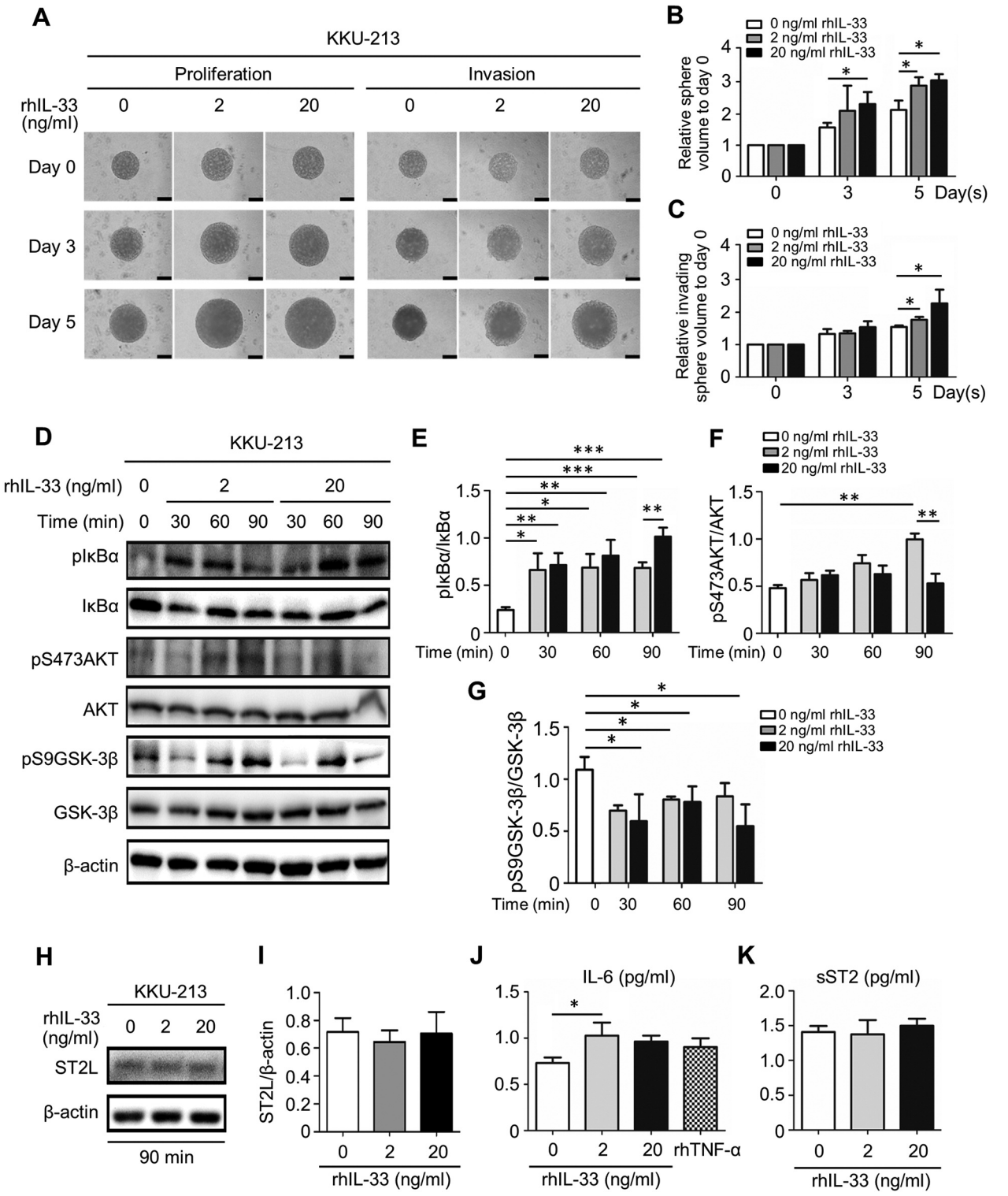

Figure 4. *Proliferation, invasion and signaling molecules in rhIL-33 treated KKU-213 cells grown in 3D cultures. (A) 3D proliferation and 3D invasion of rhIL-33 treated KKU-213. Original magnification of 100×. (B) relative 3D proliferation (C) relative 3D invasion of rhIL-33 treated KKU-213 cells (D) Western blot analysis of signaling molecules in rhIL-33 treated KKU-213 cells. The densitometric values of (E) pIκBα/IκBα, (F) pS473AKT/AKT (G) pS9GSK-3β/GSK-3β in rhIL-33 treated KKU-213 cells. (H) Western blot analysis of flIL-33 and ST2L in WCLs of rhIL-33 treated KKU-213 cells at 90 min. (I) Densitometric value of ST2L/β-actin in rhIL-33 treated KKU-213 cells. ELISA results of (J) IL-6 and (K) sST2 in rhIL-33 treated KKU-213 cells. Bars represent mean±SD. *p<0.05, **p<0.01, ***p<0.0001.*

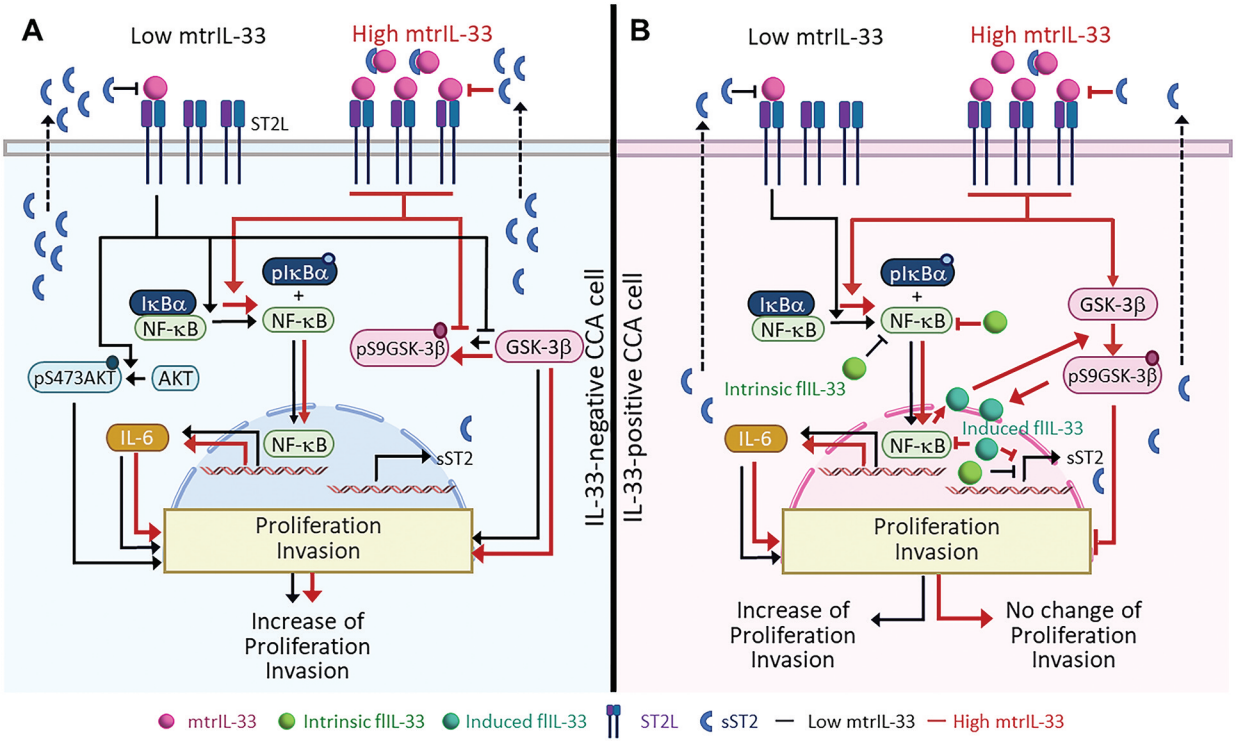

Figure 5. *Schematic diagram of intracellular and extracellular IL-33 roles in CCA progression. Effects of low and high concentrations of extracellular IL-33 on IL-33-negative and IL-33-positive CCA cells. (A) Extracellular IL-33 promotes CCA proliferation and invasion by activating NF-κB and GSK-3β in IL-33-nagative cells. (B) Intracellular IL-33 functions as a tumor suppressor in CCA by inhibiting NF-κB function and IL-6 production. High levels of intracellular IL-33 in CCA cells counteract the effects of high levels of extracellular IL-33 by increasing nuclear IL-33 and inactivating GSK-3β.*

extracellular IL-33 concentration promoted IL-33-negative CCA cell proliferation and invasion through AKT and the NF-κB/IL-6 and GSK-3β pathways, as seen in lung cancer cells (19). Therefore, IL-33 may mediate CCA proliferation and invasion *via* multiple pathways, although further gene expression arrays and/or phosphoproteomic analyses are required to elucidate the extracellular IL-33-mediated signaling pathway in CCA.

The role of intracellular IL-33 in the effects of extracellular IL-33 stimulation was investigated in KKU-055 cells treated with extracellular IL-33. Only low concentrations of extracellular IL-33 promoted NF-κB/IL-6-induced KKU-055 cell proliferation and invasion, and stimulated NF-κB-dependent IL-33 expression in murine macrophages (43). In intestinal epithelial cells, GSK-3β inactivation has been shown to promote intracellular IL-33 expression (44). Furthermore, in CCA cells increased nuclear IL-33 levels in response to high extracellular IL-33 is possibly a consequence of NF-κB activation and GSK-3β inactivation. Thus, increasing nuclear IL-33 *via* extracellular IL-33 stimulation attenuated the effect of high concentration of extracellular IL-33 on the proliferation and invasion of CCA cells. In response to high extracellular concentration of IL-33, GSK-3β inactivation only occurred in IL-33-positive, but not in IL-33-negative cells. No published reports exist regarding intracellular IL-33 modulating GSK-3β inactivation, although, pSTAT3 levels increase in IL-33-positive wild type (WT) mice lung epithelial cells, however, IL-33 deficient cells with lower pSTAT3 levels, failed to induce IL-33 expression (45). Therefore, intracellular IL-33 could suppress the effects of extracellular IL-33 *via* modulating the activation of signaling molecules, such as GSK-3β, in CCA.

However, extracellular IL-33 had no effect on the proliferation and invasion of IL-33KD-KKU-055 cells, although sST2 levels increased compared to parental cells. This is supported by the fact that intracellular IL-33 suppresses the expression of sST2 by binding to its promoter (29), and the fact that sST2 binds to IL-33, inhibiting ovarian cancer cell proliferation, migration, and invasion (28). Thus, high sST2 levels produced by IL-33KD-KKU-055 cells can bind to rhIL-33, neutralizing cell proliferation and invasion.

We reported that clinical CCA samples showed high IL-33 levels in cancer cells and CAFs, denoting good prognosis

(25). Extracellular IL-33 is supposed to be released from CAFs in CCA, as seen in lung cancer (46) and breast cancer (47). Correlating findings in human tissues, high IL-33 levels in CCA cells inhibit cancer cell proliferation and invasion and attenuate high CAFs-derived IL-33-driven CCA cell proliferation and invasion. This may explain why CCA patients with high levels of IL-33 in both cancer cells and CAFs have good prognosis with relatively long survival (Figure 5A and B).

These findings confirm that patients with IL-33 derived from both cancer cells and stromal fibroblasts have good prognosis. Treatment with agents that modulate IL-33-mediated signaling pathways, including anti-IL-33 neutralizing antibody, sST2 agonist, and anti-ST2L antibody (48) may be a treatment option for IL-33-negative CCA patients with stromal CAFs releasing high levels of IL-33.

Conflicts of Interest

The Authors have no conflicts of interest related to this publication.

Authors' Contributions

CT and MH and SY conceived and designed the experiments. SY and ST performed the experiments. SY analyzed the data. PT, KV and SO substantially contributed for analysis and interpretation of the data and supervised the study. SY wrote the manuscript. CT, MH, SO and ST reviewed and edited the manuscript. SY and CT confirmed the authenticity of all the raw data. All Authors read and approved the final manuscript.

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