Materials and Methods

Cells and Cell Culture

The melanoma cell lines A375 and MUM-2B were obtained from China Infrastructure of Cell Line Resources (Beijing, China). A375 cells were cultured in DMEM (Hyclone), and MUM-2B cells were cultured in RPMI 1640 (Hyclone) at 37°C in a 5% CO_2 atmosphere. All cell media were supplemented with 10% fetal bovine serum (Gibco, New York, USA) and penicillin/streptomycin (100 U/mL/100 g/mL).

Reagents and Antibodies

Antibodies against ST2 (ab25877), MMP-9 (ab76003), recombinant human IL-33 protein (ab155721) and MKK inhibitor U0126 (ab120241) were purchased from Abcam (Cambridge, UK). Antibodies against p-ERK1/2 (Thr202/Tyr204) were purchased from CST. Antibodies against MMP-2 (0373-2-AP) for Western blotting were purchased from Proteintech™. Antibodies against GAPDH (sc-47724), ERK1/2 (sc-514302), HRP-conjugated goat anti-rabbit IgG and anti-mouse IgG secondary antibodies were obtained from Santa Cruz (Dallas, TX, USA). MTT cell proliferation Detection Kit (KGA311-KGA312) and DMSO (KGT5131) were purchased from KeyGen BioTech.

Immunohistochemical Staining and Assessment

Case data and paraffin-embedded melanoma tissue from 66 cases were collected from Tianjin Cancer Hospital of China between 1984 and 2000. All experiments using human tissue were approved by the Institutional Research Committee. The specific experimental operation steps are as described in a previous report [29]. The antibodies and concentrations used are as follows: ST2 (1:200), MMP-2 (1:200).

shRNA and Plasmid Transfection

MUM-2B and A375 cells were infected with a lentivirus carrying the ST2 silencing plasmid (shRNA catalog No. HSH022242-LVRU6GP; GeneCopoeia™) and its negative control plasmid (catalog No. CSHCTR001-LVRU6GP) using a Lenti-Pac HIV Expression Packaging Kit (catalog No. HPK-LvTR-40). Western blotting analysis was used to verify transfection efficiency.

Three-Dimensional (3D) Cultures

24-well plates coated with 50 µl of Matrigel matrix (BD Biosciences, San Jose, CA, USA) were incubated for 48 h at 37°C. 2–3 × 10^5 cells of the experimental group and the control group were cultured separately and incubated with media containing IL-33 (50 ng/mL) at 37°C for 24 h. Subsequently, the formation of VM was observed under a phase-contrast microscope (×100).

Cell Proliferation Assay and Colony Formation Assay

A375 and MUM-2B cells were plated in three 6-well dishes (10^3 cells per dish) with or without IL-33, cultured for 8 days, and stained with crystal violet solution. The number of colonies with >50 cells was recorded.

Plant A375 and MUM-2B cells (1 × 10^4 cells) were plated into 96-well dishes separately with or without IL-33, in triplicate. The cells were incubated for 1, 2, 3 and 4 days. Then, 50 µL of MTT reagent were added to each well, including controls, and plates were returned to the cell incubator for 4 h. After that, 150 µL of DMSO were added to each well, including controls. All samples were swirled gently. The optical density (OD) was determined at 540 nm using a BioTekELx800.

Cell Migration and Invasion Assay

Cell migration and invasion ability was measured using a transwell (Corning) chamber in vitro. For migration assays, after treatment with or without IL-33 (10 ng/mL, 50 ng/mL), about 1×10^5 cells in serum-free medium were added to the upper chamber, and 500 µl of complete medium was added to the bottom chamber. For the invasion assay, the upper chambers were coated with Matrigel. After 16 h (migration) and 48 h (invasion), cells that migrated through the chamber membrane were fixed and stained with 1% crystal violet solution, then observed under an inverted light microscope (Nikon ×100) and counted. Each experiment was performed in triplicate.

Wound Healing Assay

A375 and MUM-2B cells were seeded into 24-well plates (2×10^5/well), in triplicate. Using a 10-µL pipette tip, the cell monolayer was scratched, and subsequent cells were maintained in fresh medium containing IL-33 (50 ng/mL). Cell migration was recorded at 0, 24 and 48 h. Migration speed was calculated using software.

Western Blotting Analysis

Samples were lysed on ice, separated by electrophoresis and electroblotted onto a PVDF membrane (Millipore, Darmstadt, Germany). For experiments using the phospho-ERK1/2 antibody, membranes were incubated in antibody solution containing BSA, TBST and Tween-20 at 4°C overnight. For the other antibodies, the membranes were incubated with primary antibodies diluted with nonfat milk overnight at 4°C. The antibodies and dilution factors were as follows: ST2 (1:200), ERK (1:200), MMP-2 (1:500) and MMP-9 (1:500). Secondary antibodies (1:2,000) were incubated for 2 h. The bands were exposed using a C-DiGit Blot Scanner (LI-COR) and analyzed using Image-Pro Plus software.

Real-Time PCR

Total RNA was isolated with TRNzol-A+ reagent (Tiangen, Beijing, China) and subjected to reverse-transcriptase PCR using the PrimeScript™ RT reagent kit with gDNA eraser (TaKaRa, Dalian, China). The following real-time PCR primers obtained from Sangon Biotech were used: MMP-2 (forward: 5'-GCGACCACAGCCAACTACGATG-3', reverse: 5'-GTGCCAAGGTCAATGTCAGGAGAG-3'), MMP-9 (forward: 5'-GAATGGCATCCGGCACCTCTATG-3', reverse: 5'-CCACTTGTCGGCGATAAGGAAGG-3'). Real-time PCR Master Mix (SYBR Green) was used for all experiments. Signals were detected with an ABI 7500 Real-Time PCR System (Applied Biosystems, CA, USA).

Statistical Analysis

All data were analyzed with SPSS version 17.0 (SPSS, Chicago, IL, USA). The measured data are expressed as the mean with standard deviation (SD) or standard error of the mean (SEM) A p value of <0.05 was defined as significant. Differences between two groups were assessed using Student's t test, whereas multiple groups were compared by ANOVA.