Heliyon

Received:
19 May 2017
Revised:
29 September 2017
Accepted:
20 October 2017

Cite as: Kenji Tago, Satoshi Ohta, Masaki Kashiwada, Megumi Funakoshi-Tago, Jitsuhiro Matsugi, Shin-ichi Tominaga, Ken Yanagisawa. ST2 gene products critically contribute to cellular transformation caused by an oncogenic Ras mutant. Heliyon 3 (2017) e00436.
doi: 10.1016/j.heliyon.2017.e00436

ST2 gene products critically contribute to cellular transformation caused by an oncogenic Ras mutant

Kenji Tago [a,*], Satoshi Ohta [a], Masaki Kashiwada [b], Megumi Funakoshi-Tago [c], Jitsuhiro Matsugi [a], Shin-ichi Tominaga [b], Ken Yanagisawa [a]

[a] *Division of Structural Biochemistry, Department of Biochemistry, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan*

[b] *Medical Biochemistry, Department of Biochemistry, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan*

[c] *Department of hygienic chemistry, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan*

* Corresponding author.
E-mail address: ktago@jichi.ac.jp (K. Tago).

Abstract

The ST2 gene was originally identified as a primary responsive gene, and the expressions of its gene products are induced by stimulation with growth factors and by oncogenic stresses. In this study, we observed that oncogenic Ras mutant induced the expression of ST2 and ST2L proteins. Interestingly, the enforced expression of ST2 gene products in NIH-3T3 murine fibroblasts remarkably enhanced Ras (G12V)-induced cellular transformation. Furthermore, when the expression of ST2 gene products was silenced by RNA-interference technique, Ras (G12V)-induced cellular transformation was drastically suppressed. According to these observations, it was indicated that the oncogenic Ras-induced expression of ST2 gene products is required for the acceleration of cellular transformation, and this seems to be independent of the stimulation with IL-33, a ligand for ST2/ST2L. Interestingly, knockdown of ST2 gene products caused a reduction in Rb phosphorylation in transformed murine fibroblasts, suggesting the functional involvement of ST2 gene products in cell cycle progression during cellular

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

transformation. Our current study strongly suggests the importance of ST2 gene products in cellular transformation, and the presence of novel mechanism how ST2 gene products affect the cellular transformation and cell proliferation.

Keywords: Cancer research, Cell biology

1. Introduction

ST2 (IL1RL1, also called as T1) was originally identified as a primary responsive gene, which was highly induced by growth stimulation and oncogenic Ras-induced signaling in quiescent murine fibroblasts [1, 2]. The ST2 gene produces two main different mRNAs by alternative splicing [3]; these mRNAs encode a soluble secreted protein (ST2), and a membrane bound protein (ST2L) [1, 4, 5]. By structural analysis of the ST2 cDNAs, ST2 and ST2L exhibit marked similarity with the IL-1 receptor (IL-1R), suggesting that the ST2 gene products, especially membrane bound ST2L, induce similar signaling cascades to IL-1R. After identification of Interleukin-33 (IL-33) as the specific ligand for ST2/ST2L, ST2L stimulated by IL-33 was demonstrated to activate the transcription factor NF-κB and the MAP kinase family through common signaling molecules to IL-1R [6, 7, 8, 9]. On the other hand, soluble ST2 protein functions as a decoy receptor for IL-33 and regulates the signaling pathway of ST2L [10, 11]. However, the functional relationship between growth stimulation and the expression of ST2 gene products still remains further to investigate.

Among the *RAS* family, the closely related members such as *KRAS*, *NRAS*, and *HRAS* genes are the most frequently mutated in various types of human cancer [12, 13]. Ras family is also understood to harbor the pivotal roles in the regulation of cell growth, apoptosis, and cell differentiation in a wide variety of mammalian cells [14]. It is well understood that Ras proteins are converted from their GDP state to their GTP state in response to stimulation by various growth factors and cytokines, and GTP-bound form of Ras exhibits the activity to stimulate cell growth [14]. The substitution of glycine to valine at amino acid 12 (G12V) or glutamine to leucine at amino acid 61 (Q61L), which are well known as oncogenic mutations found in various tumors, decreases the GTPase activity of Ras protein, and these mutations cause the constitutive activation of Ras protein [15]. To accelerate cell proliferation, Ras interacts with various effector proteins such as c-Raf-1 kinase, phosphatidylinositol-3 kinase, phospholipase C-ε, etc. [12].

The acceleration of the cell proliferation by Ras (G12V) requires the induction of several genes such as cyclin D1, and c-Fos, and these expressions are caused by the activation of a number of transcription factors including Ets, β-catenin and AP-1 [16, 17, 18]. On the other hand, oncogenic Ras also caused the expression of Noxa and Beclin-1, inducers of autophagic cell death, and Ets also activates the promoter of p21, an inhibitor protein for cyclin-dependent kinase [19, 20]. These findings

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

raise a question how oncogenic Ras overcomes the expressions of these anti-proliferative genes, urging us to address the functional analysis of Ras-induced genes.

The precursor protein of IL-33 was originally identified as a nuclear protein, nuclear factor in high endothelial venules (NF-HEV). NF-HEV was reported to associate with heterochromatin, and functions as a transcriptional repressor [21]. Furthermore, it was reported that NF-HEV regulates the activity of transcription factors such as NF-κB [22, 23], suggesting that NF-HEV functions as a fine tuner for the transcription. Recently, we reported that NF-HEV contributes to the cellular transformation provoked by oncogenic Ras through the critical role in the protein synthesis of cyclin D1 [24]. The action of NF-HEV seems to be independent of ST2/ST2L, however it is unclear how ST2/ST2L behave in the cellular transformation induced by oncogenic Ras. We already understand that ST2 but not ST2L contributes to cell proliferation of murine untransformed fibroblasts [25]. However, it is still unclear whether ST2/ST2L harbor the functional importance for the cellular transformation, and if so, what is the significance of ST2/ST2L for the cellular transformation.

In the current study, we investigated the roles of ST2 gene products in the cellular transformation provoked by oncogenic Ras. Although the detailed mechanism still remains to be elucidated, our observations emphasize the functional relationship between oncogenic signal and the expression of ST2 gene products.

2. Materials and methods

2.1. Cell culture

Murine fibroblast NIH-3T3 and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 units/ml penicillin and 100 μg/mL streptomycin.

2.2. Antibodies

Anti-FLAG antibody (M2) was purchased from Merck Millipore (St. Charles, MO, USA). Anti-cyclin A (SC-751), cyclin D1 (SC-753), and β-actin (SC-130301) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-phosphorylated Rb (Ser-807/811, #9308) was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-Rb antibodies were purchased from Cell Signaling Technology (#9309) and BD Biosciences (San Jose, CA, USA, 554136). Anti-ST2 antibody was prepared as described previously [26].

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

2.3. Retrovirus production and infection

Retroviruses were prepared as described previously [27]. HEK293T cells were transfected with helper retrovirus plasmids together with MSCV-ires-GFP or MSCV-ires-Puro encoding the indicated proteins. Viruses were harvested 24–60 h post-transfection, pooled, and stored on ice. Exponentially growing cells (1×10^5 cells per 60-mm-diameter culture dish) were infected twice at 2 h intervals with 2 ml of fresh virus-containing supernatant in complete medium containing 1.0 μg/ml polybrene (Merck Millipore). For the control sample, cells were infected with retrovirus prepared by the transfection with empty vector. Twenty-four hours later, the infected cells were selected by puromycin selection. The puromycin selection was performed for 72 h, and then viable cells were utilized for the experiments.

2.4. RNA interference for ST2 gene products

Annealed oligonucleotides encoding a short hairpin RNA (sh-RNA) for murine ST2 gene products were inserted into pSUPER-retro-puro retroviral plasmid (Oligoengine, Seattle, WA, USA). The sequences of oligonucleotides used for constructing the sh-RNA retroviral vector against mouse ST2 gene products were as follows: 5′-GATCCCCgggaagagctgcaaggtggTTCAAGAGACCACCTTGCAGCTCTTCCCTTTTTA-3′ and 5′-AGCTTAAAAAgggaagagctgcaaggtggTCTCTTGAACCACCTTGCAGCTCTTCCCGGG-3′ (underlined sequences correspond to the sequence [1] [19-mer] of murine ST2, from 876 to 894 in ORF). The sequences of oligonucleotides used for constructing the sh-RNA retroviral vector against human ST2 gene products were as follows: 5′-GATCCCCGgaaagaaatcgtgtgtttTTCAAGAGAAAACACACGATTTCTTTCCTTTTTA-3′ and 5′-AGCTTAAAAAGgaaagaaatcgtgtgtttTCTCTTGAAAAACACACGATTTCTTTCCGGG-3′ for sh-human ST2 #1, and 5′-GATCCCCCgtaagactaagtaggaaaTTCAAGAGATTTCCTACTTAGTCTTACGTTTTTA-3′ and 5′-AGCTTAAAAACgtaagactaagtaggaaaTCTCTTGAATTTCCTACTTAGTCTTACGGGG-3′ for sh-human ST2 #2 (underlined sequences correspond to the sequences of human ST2). The infection with retroviruses including these sh-RNA into NIH-3T3 cells was performed at the same time with the retrovirus harboring oncogenic Ras.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted by using TRI reagent (Merck Millipore). Single stranded cDNA was synthesized by reverse transcription from 2 μg of total RNA using ReverTra Ace (TOYOBO, Osaka, Japan). Quantitative PCR by using KAPA SYBR Fast qPCR kit (KAPA Biosystems, Wilmington, MA, USA) was performed in a LightCycler 96 (Roche Diagnostics, Indianapolis, IN, USA) with PCR cycles set at 94 °C for 10 sec, 50 °C for 15 sec, and 72 °C for 1 min. The nucleotide

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

sequences of primers used for the quantitative PCR were as follows: ST2 (forward 5′-CAAGAAGAGGAAGGTCGAAATG-3′, reverse 5′-ATGTGTGAGGGA-CACTCCTTAC-3′), ST2L (forward 5′-CAAGAAGAGGAAGGTCGAAATG-3′, reverse 5′-AGCAACCTCAATCCAGAACAC-3′).

2.6. Focus formation, colony formation, and anoikis assays

A total of 3×10^3 infected NIH-3T3 cells were mixed with 1×10^5 uninfected cells and seeded onto a 60 mm-diameter dish. Two-to-three weeks later, transformed foci were stained with Giemsa and photographed. The focuses exhibiting a diameter of 1 mm or more were counted using NIH ImageJ software, and results were shown in the graph. For the colony formation assay, infected NIH-3T3 cells were seeded onto soft agar at 1×10^4 cells per 35 mm-diameter dish, and grown for 2–3 weeks. The visible colonies showing a diameter of 1.0 mm or more were counted using NIH ImageJ software, and results were shown in the graph. In several experiments, colonies were visualized by the treatment with MTT (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). To evaluate the resistance to anoikis, cells were suspended in DMEM including 1% FBS, and then seeded onto the 24-well plates treated with poly-HEMA (2-hydroxyethyl methacrylate; Sigma-Aldrich). Twenty-hour hours later, cell viability was measured using a WST-1 assay (Roche Diagnostics, Basel, Switzerland).

2.7. Immunoblotting analysis

After selection with puromycin, the infected cells were cultured in DMEM including no FBS for 24 h, since serum stimulation could induce the expression of ST2, ST2L, cyclin D1, and the phosphorylation of Rb. Presence of serum could enhance the background of these signals. Cells were suspended in RIPA buffer (10 mM sodium phosphate [pH 7.2], 150 mM NaCl, 3 mM $MgCl_2$, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.2 U/ml aprotinin, and phosphatase inhibitors) and briefly sonicated on ice. Then, lysates were cleared by sedimentation in a microcentrifuge at 16,400 × *g* for 10 min. Twenty-five μg of whole cell lysates were loaded in each lane of SDS-polyacrylamide gel, and the electrophoresis was performed. And then, the separated proteins were transferred to polyvinylidene difluoride membrane (Merck Millipore). Proteins were detected by immunoblotting analysis with the indicated antibodies. To detect the endogenous ST2 and ST2L proteins, the proteins in conditioned media and whole cell lysates were concentrated by Heparin Sepharose 6 Fast Flow (GE healthcare life sciences, Little Chalfont, UK) and TCA precipitation, respectively. Especially, we adjusted the amount of conditioned medium according to the protein concentration extracted from the cells from which the conditioned medium was collected, and utilized them for column chromatography and immunoblots. Then, concentrated fractions were treated with N-endoglycosidase F (Takara Bio, Shiga, Japan) to remove the sugar

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

chains conjugated with ST2 and ST2L proteins. De-glycosylated ST2 and ST2L were detected by immunoblot with antiserum against mouse ST2 prepared from rabbit [26]. The probed proteins were visualized by the treatment with ECL (GE healthcare life sciences).

2.8. Enzyme-Linked Immune Sorbent Assay (ELISA)

Conditioned medium was collected from each culture dish of infected cells. The concentrations of murine ST2 and IL-33 were measured by using Quantikine ELISA for mouse ST2 (MST200) and mouse/rat IL-33 (M3300) (R&D system, Minneapolis, MN, USA), respectively.

2.9. Cell cycle analysis

Infected NIH-3T3 cells were introduced into G_0 phase by serum starvation for 24 h. Then, the cells were released into the cell cycle by splitting the cells onto new dishes. After the indicated periods, cells were harvested for the immunoblotting analysis and cell cycle analysis. Immunoblotting analysis was performed with primary antibodies against cyclin D1, cyclin A, c-Myc. $p27^{Kip1}$, and β-actin. For the DNA content analysis, cells were fixed and suspended with the lysis buffer including 25 μg/ml propidium iodide. The cell suspension was analyzed using an LSRFortessa (Becton Dickinson, Franklin Lakes, NJ, USA).

2.10. Statistical analysis of data

In the case of colony formation assay and cell proliferation assay, we performed the experiment individually three times in triplicates, and gained similar results. Among all experiments performed, we showed the results from one experiment in triplicate. In other experiments, we performed the experiment individually three times, and showed the data. In the graph, error bar means standard deviation (S.D., $n = 3$), and the results of calculation of independent t-test were shown.

2.11. Original immunoblot analysis data

In the supplementary file labeled as “Original blots for Ras & ST2”, we show all of the data of full-length immunoblots, which were used for the prepared figures.

3. Results

3.1. Ras (G12V) causes the protein expression of ST2 and ST2L in murine fibroblasts

It was reported previously that an oncogenic Ras mutant causes the expression of the soluble form of ST2 in NIH-3T3 murine fibroblasts [2]. And recently, we also reported that oncogenic Ras induced the mRNA expressions of not only ST2 but

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

also ST2L [24]. To analyze the effect of an oncogenic Ras mutant on the expression of ST2 gene products, we infected NIH-3T3 cells with a retrovirus harboring oncogenic H-Ras mutant named Ras (G12V) and then, total RNA was prepared. Comparably to previous two reports, we observed a significant induction of the mRNA expression of both ST2 and ST2L (Fig. 1A). We additionally attempted to detect the induction of endogenous proteins of ST2/ST2L by Ras (G12V). ST2 is soluble protein, and secreted into conditioned media. To enhance the sensitivity of the immunoblot analysis, ST2 in conditioned media was concentrated by Heparin Sepharose affinity chromatography, and then treated with

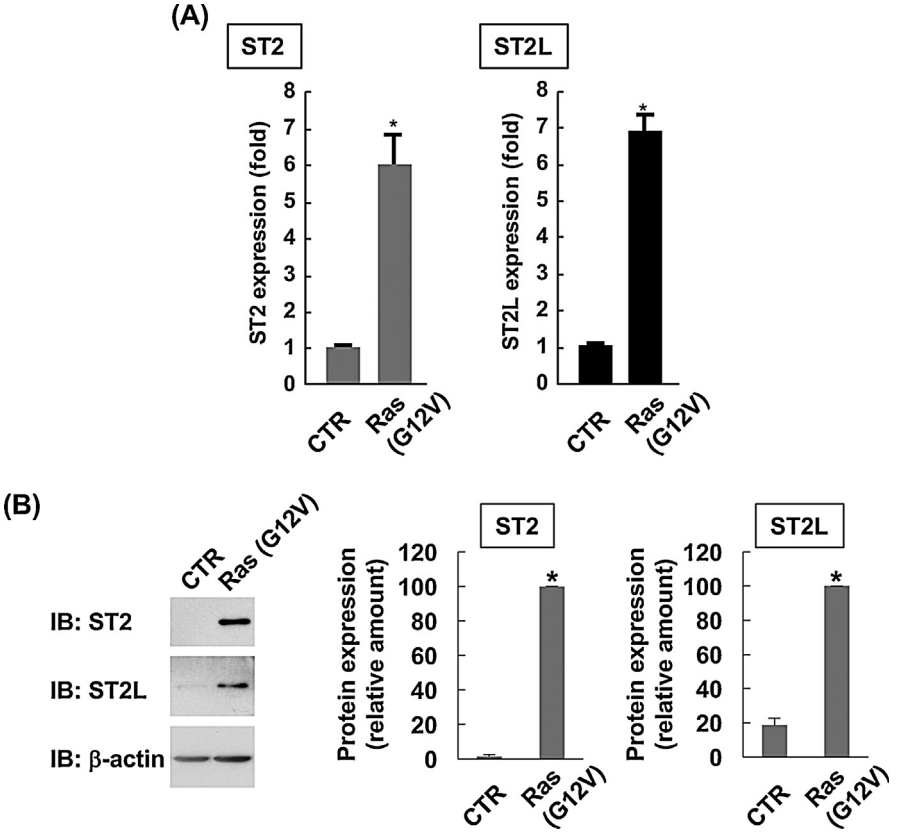

Fig. 1. Ras (G12V) causes the expression of ST2 gene products in NIH-3T3 murine fibroblasts. (A) NIH-3T3 cells were infected with the empty retrovirus (CTR) or retrovirus harboring Ras (G12V), and infected cells were selected by the treatment with puromycin (7.5 μg/ml) for 72 h. Then, total RNA was extracted from infected cells, and the expression levels of ST2 gene products were analyzed by quantitative PCR. In the graph, error bars = S.D. (n = 3, *P < 0.005). (B) Conditioned medium and whole cell lysates were concentrated, and treated with N-glycosidase F. Then, ST2 and ST2L proteins induced by Ras (G12V) were detected by immunoblot analysis with anti-mouse ST2 antibody, and β-actin was also stained as a loading control for the immunoblot analysis. The intensities of bands were quantified, and shown in the graphs. In the graph, error bars = S.D. (n = 3, *P<0.005). Original blots are shown in supplementary file named as "Original blots for Ras & ST2".

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

N-glycosidase F to remove the sugar chains from ST2 protein. In the case of ST2L, whole cell lysates were concentrated by TCA-precipitation, and treated with N-glycosidase F. As shown in Fig. 1B, oncogenic Ras significantly induced the expression of ST2 and ST2L proteins.

3.2. Co-expression of ST2 gene product augments Ras (G12V)-induced cyclin D1 expression

To clarify the physiological roles of ST2 gene products in cellular transformation, we attempted to establish the cells expressing the Ras (G12V) alone and the combination including Ras (G12V) and ST2 or ST2L. We utilized retroviruses to forcibly express Ras (G12V) by adjusting the amount of retroviruses, such as 5-, 15-, and 50-fold dilution to monitor the expression level of Ras (G12V) protein. Then, to express ST2 or ST2L together, cells were co-infected with control retrovirus and retroviruses harboring ST2 and ST2L, respectively. We first analyzed the protein expression of endogenous and exogenous ST2 and ST2L in NIH-3T3 cells by immunoblot analysis using anti-ST2 and M2 antibody, respectively. In the whole cell lysates, ST2 and ST2L exhibited multiple bands, which are considered to be due to the heterogeneous glycosylation. Interestingly, Ras (G12V) augmented the expression level of exogenous ST2 and ST2L proteins depending on the protein levels of Ras (G12V) (Fig. 2A). However, endogenous ST2 and ST2L failed to be detected by immunoblot using anti-ST2 antibody. Comparing these observations with the results shown in Fig. 1, it is suggested that the glycosylation of ST2 could be an obstacle for antibody to recognize the core protein of ST2 and ST2L. Therefore, the glycosidase treatment is required for the detection of endogenous ST2 and ST2L. On the other hand, expression of Ras protein was not altered by co-expression with neither ST2 nor ST2L (Fig. 2A and Fig. 3A). When ST2 and ST2L were forcibly expressed with Ras (G12V), the expression of cyclin D1 protein was apparently elevated; however, Retinoblastoma protein (Rb) phosphorylation at Ser-807/811 was barely altered (Fig. 2A and 2B). To verify the validity of our experiments, we evaluated the expressional ratio between cyclin D1 and exogenous oncogenic Ras, since the expression level of Cyclin D1 may represent the correlation with the expression amount of Ras protein. However, in the presence of ST2, the ratio between cyclin D1 and exogenous oncogenic Ras was clearly higher than other samples, indicating that ST2 clearly accelerates the expression of cyclin D1 induced by oncogenic Ras (Fig. 3B). Next, we tested the effect of ST2/ST2L on the mRNA expression of cyclin D1 induced by oncogenic Ras. As shown in Fig. 3C, although Ras (G12V) significantly induced the expression of cyclin D1 mRNA, the enforced expression of ST2 or ST2L could not enhance the mRNA expression of cyclin D1 induced by oncogenic Ras.

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

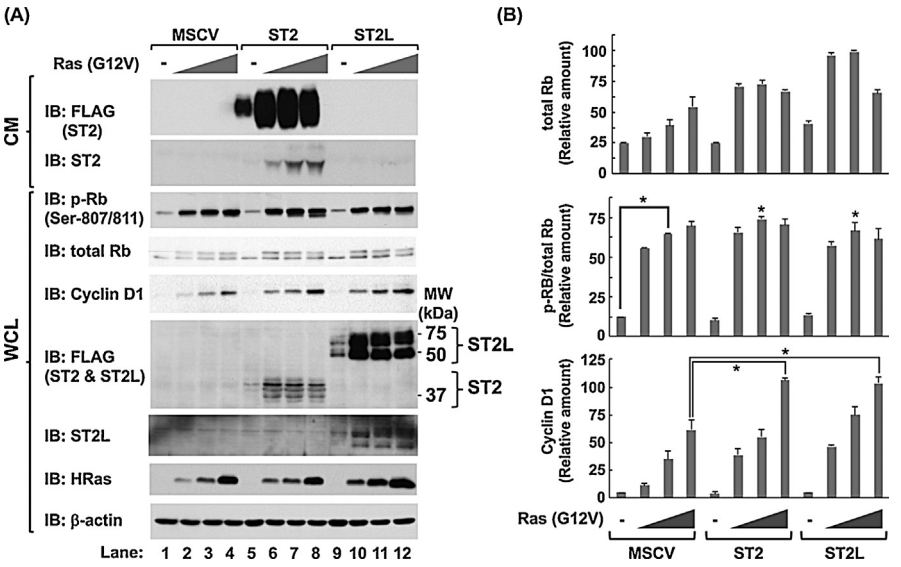

Fig. 2. Enforced expression of ST2 gene products in NIH-3T3 cells transformed by oncogenic Ras. (A) We diluted retroviruses to forcibly express the various amount Ras (G12V) by adjusting the amounts of retroviruses harboring gene of Ras (G12V), such as 50- (lane 2, 6, and 10), 15- (lane 3, 7, and 11), and 5-fold (lane 4, 8, and 12) dilution, and infected cells were selected by the treatment with puromycin for 72 h. To co-express ST2-FLAG and ST2L-FLAG, we then infected the retroviruses harboring ST2-FLAG and ST2L-FLAG, respectively. After selection with puromycin, cells were treated with serum starvation for 24 h. Then, whole cell lysates (WCL) and conditioned media (CM) were prepared, and analyzed by immunoblotting with the primary antibodies such as anti-FLAG (M2), H-Ras, cyclin D1, Rb, phosphorylated Rb (Ser-807/811), and β-actin antibodies, respectively. To detect secreted ST2, the culture supernatant (conditioned media: CM) was also analyzed. The positions of bands for ST2L and ST2 are indicated. Original blots are shown in supplementary file named as "Original blots for Ras & ST2". (B) The intensity of the expression of total Rb protein, the ratio between Rb phosphorylation and total Rb, and the expression of cyclin D1 protein were quantitated, calculated, and then results were shown in graphs. In the graph, error bars = S.D. (n = 3, *P<0.005).

3.3. Enforced expression of ST2 gene products accelerates cellular transformation induced by Ras (G12V)

We next tested the effects of ST2 and ST2L on cellular transformation. Before performing the experiment, we tested whether Ras could induce cellular transformation in Ras expression level-dependent manner. It was reported that Ras onco-protein could provoke the cellular transformation in its dose-dependent manner [28], however, our results failed to reproduce their results. As shown in Fig. 3D, we infected retroviruses into NIH-3T3 cells to forcibly express Ras (G12V) by adjusting the amount of retroviruses, such as 5-, 15-, and 50-fold dilution, and tested the colony formation abilities. Significantly, 5-fold diluted retrovirus seemed to express the excess amount of Ras (G12V), and exhibited the lower transforming activity than the cases of 50- and 15-fold dilution. This could be explained by a previous report [29]. Ras protein is modified with farnesyl group, and this modification contributes to the membrane translocation of Ras protein.

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

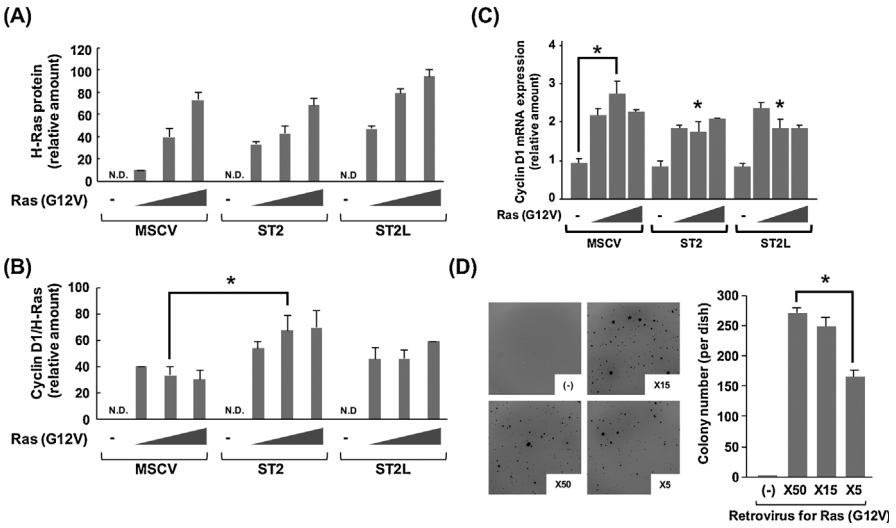

Fig. 3. Effect of enforced expression of ST2 gene products on the expression of Ras protein and cyclin D1 mRNA. (A) In the cells analyzed in Fig. 2, the intensity of the expression of H-Ras protein, which were normalized with the expression of β-actin, and then results were shown in graph. In the graph, error bars = S.D. (n = 3). (B) The expressional ratio between cyclin D1 and H-Ras was calculated, and then results were shown in graph. In the graph, error bars = S.D. (n = 3). (C) Infected cells were utilized for the total RNA extraction, and analyzed by qPCR for evaluating the effect of Ras (G12V) and ST2 gene products on the expression of cyclin D1 mRNA. The results were shown in graph. In the graph, error bars = S.D. (n = 3, *P < 0.005). (D) In the cells analyzed in Fig. 2, infected cells were seeded onto soft agar at 1×10^4 cells per 35 mm-diameter dishes, and grown for 2–3 weeks. The colonies were visualized by the treatment with MTT, and the results were shown in graph (magnification: 4 ×). In the graph, error bars = S.D. (n = 3, *P<0.005).

Excess amount of Ras expression caused the presence of unmodified form of Ras protein, and this unmodified Ras protein that is probably localized in cytosol functions as dominant negative. Then, we chose the condition evaluated in Fig. 2, such as 15-fold diluted retrovirus harboring Ras (G12V) for similar expression levels, and utilized them for following experiments. We first analyzed focus formation. As shown in Fig. 4A and B, ST2 and ST2L exhibited no ability to form a focus by themselves. However, enforced expression of ST2, but not ST2L accelerated Ras (G12V)-induced focus formation. Furthermore, we performed the analysis of colony formation in soft agar media, and we observed that the enforced expression of soluble ST2 significantly enhanced the colony formation by NIH-3T3 cells transformed by Ras (G12V) (Fig. 4C and D). The enforced expression of ST2L also caused the moderate enhancement of colony formation induced by Ras (G12V), therefore we concluded that the enforced expression of both ST2 and ST2L accelerates the cellular transformation provoked by Ras (G12V). It was unexpected that both ST2 and ST2L enhanced the cellular transformation activity of Ras (G12V). Although we do not have suitable explanation for this observation, we guess that ST2 and ST2L may affect the cellular transformation through the different machineries. As a supportive information, we observed that the enforced expression of ST2L enhanced the Ras

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

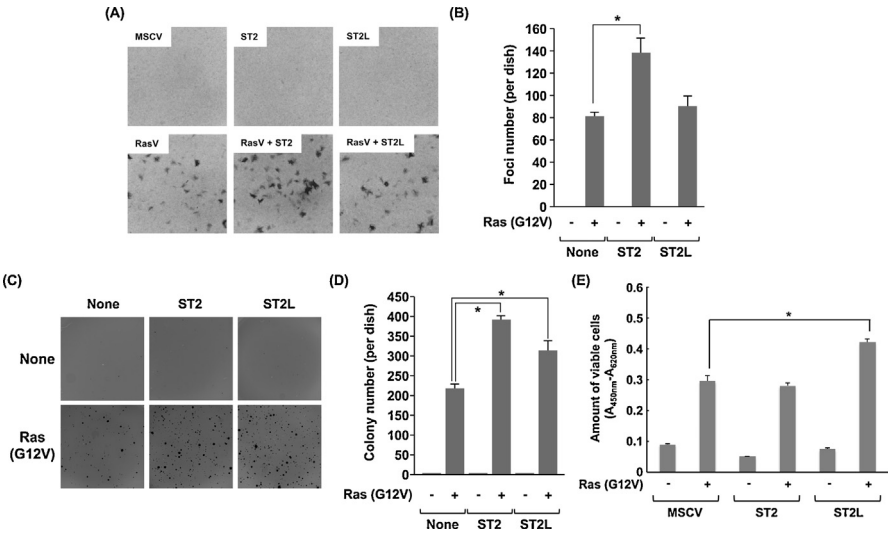

Fig. 4. Enforced expression of ST2 gene products accelerates cellular transformation induced by Ras (G12V). (A) NIH-3T3 cells were infected with the indicated combination of retroviruses harboring Ras (G12V), ST2-FLAG, and ST2L-FLAG, and infected cells were selected by the treatment with puromycin for 72 h. Then, a total of 3×10^3 infected cells were mixed with 1×10^5 uninfected cells and seeded onto 60 mm-diameter dishes. Two weeks later, transformed foci were stained with Giemsa, and then photographed at 4 × magnification. (B) The numbers of foci shown in (A) were counted, and the results were shown in the graph. In the graph, error bars = S.D. (n = 3, *P < 0.01). (C) Infected NIH-3T3 cells were seeded onto soft agar at 1×10^4 cells per 35-mm dish, and grown for 2–3 weeks. Grown colonies were photographed at 4 × magnification, and the numbers of colonies were counted. (D) The number of colonies of transformed NIH-3T3 cells counted in (C) is shown in the graph. In the graph, error bars = S.D. (n = 3, *P < 0.005). (E) To evaluate the resistance to anoikis, cells were suspended in DMEM including 1% FBS, and then seeded onto the 24-well plates treated with poly-HEMA. Twenty-hour hours later, cell viability was measured using a WST-1 assay. In the graph, error bars = S.D. (n = 3, *P<0.01).

(G12V)-provoked resistance against anoikis (Fig. 4E). On the other hand, ST2 failed to affect Ras (G12V)-induced survival ability.

In the previous report [24], we mainly performed the focus formation assay to evaluate the transforming activity, and concluded that the recombinant ST2 protein did not affect the focus formation caused by Ras-transformed NIH-3T3 cells. On the other hand, in the current study, we otherwise performed colony formation assay in soft agar-media, and calculated the statistics for these samples. As results, we concluded that the Ras-provoked cellular transformation was accelerated by the enforced expression of soluble ST2 protein.

3.4. Soluble ST2 accelerated the cellular transformation in paracrine- and autocrine-dependent manner

In Fig. 4, the enforced expression of ST2L exhibited the ability to enhance the colony formation in soft agar media, however failed to accelerate the formation of

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

foci. To negate the possibility that poor expression efficiency of ectopic ST2L in NIH-3T3 cells may cause the result such as lack of ability to accelerate the formation of foci, we evaluated the expression of ST2L by immunostaining analysis. As shown in Fig. 5A, almost 80 to 90% of cells expressed FLAG-tagged ST2L, and its expression was strongly enhanced by co-expression with Ras (G12V). On the other hand, it was difficult to detect ST2-FLAG protein, since majority of the protein seemed to be secreted from the cells. By performing ELISA, we measured the concentration of soluble ST2 secreted into conditioned

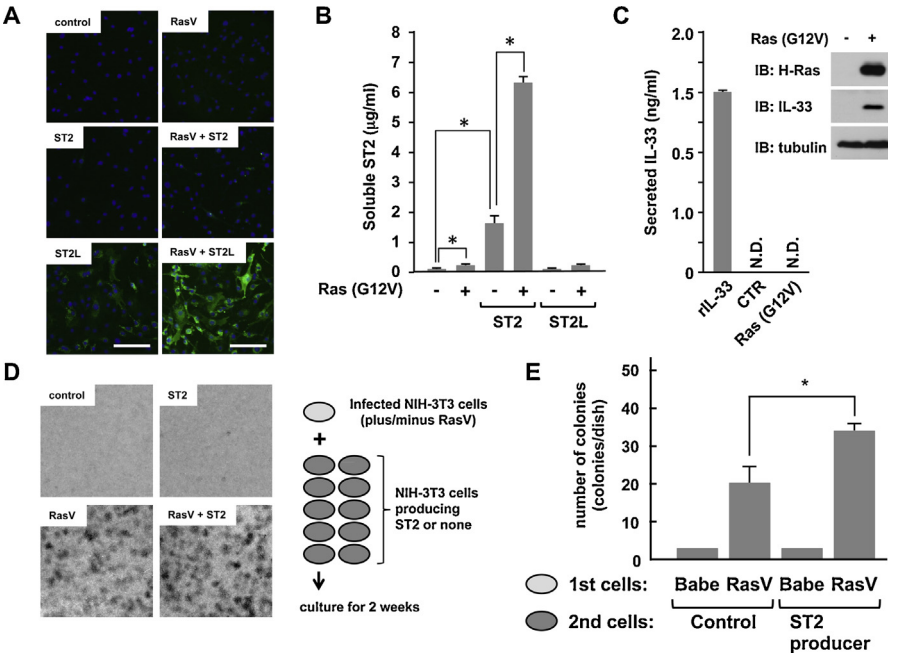

Fig. 5. Soluble ST2 accelerated the cellular transformation in paracrine- and autocrine-dependent manner. (A) Then infected NIH-3T3 cells analyzed in Fig. 4 were seeded on slide glasses, and fixed with paraformaldehyde. Then, the expression of FLAG-tagged ST2 and ST2L were detected by immuno-staining analysis using anti-FLAG (green). Nucleus was also stained with DAPI (blue). In bottom photographs, scale bars were added (length: 153 μm) (B) Conditioned medium was also collected from the infected NIH-3T3 cells analyzed in (A). Then, concentration of ST2 was analyzed by ELISA. In the graph, error bars = S.D. (n = 3, *P < 0.001). (C) Using conditioned medium from control NIH-3T3 and Ras (G12V)-transformed NIH-3T3 cells, ELISA for IL-33 was also performed. Recombinant IL-33 (rIL-33) was utilized as a positive control. In the graph, error bars = S.D. (n = 3, *P < 0.005). N.D. means "Not detected". The expressions of Ras (G12V) and intracellular IL-33 were detected by immunoblot analysis. The blot for β-tubulin is a loading control. Original blots are shown in supplementary file named as "Original blots for Ras & ST2". (D) To test the paracrine- and autocrine-dependent effect of ST2 on cellular transformation, 3×10^3 untransformed NIH-3T3 cells or transformed cells provoked by Ras (G12V) were mixed with 1×10^5 normal NIH-3T3 (top photograph), NIH-3T3 producing soluble ST2 (middle photograph), or NIH-3T3 cells expressing ST2L (bottom photograph), and then cultured in 60-mm diameter dishes during 2 weeks. Foci were visualized by staining with Giemsa reagent, and then photographed at 4 × magnification. (E) The numbers of foci shown in (D) were counted, and the results were shown in the graph. In the graph, error bars = S.D. (n = 3, *P<0.005).

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

media. As shown in Fig. 5B, enforced expression of Ras (G12V) resulted in approximately 4-fold enhancement of concentration of soluble ST2 in conditioned media. On the other hand, enforced expression of ST2 resulted in the secretion of large amount of soluble ST2 protein, and its concentration was almost 30-fold higher than the endogenous soluble ST2 produced by Ras (G12V). Furthermore, Ras (G12V) enhanced the concentration of ectopic soluble ST2, and these observations were well fit with the results shown in Fig. 2. On the other hand, the enforced expression of ST2L did not affect the concentration of soluble ST2 in conditioned medium. In addition, we also tested the concentration of IL-33 in conditioned medium. Although Ras (G12V) induced the expression of IL-33 precursor, NF-HEV, which was detectable by immuno-blot analysis, the secretion of IL-33/NF-HEV in conditioned medium was lower than the detectable level by ELISA (Fig. 5C), supporting our conclusion that ST2/ST2L accelerate the cellular transformation in IL-33-independent manner. Since soluble ST2 is secreted from producer cells, and then could affect the neighbor cells, we next investigated whether secreted soluble ST2 could accelerate the cellular transformation provoked by oncogenic Ras. Expectedly, when Ras (G12V)-transformed NIH-3T3 cells were co-cultured with untransformed NIH-3T3 cells producing exogenous ST2 protein, number of foci was effectively increased (Fig. 5D and E), suggesting that Soluble ST2 accelerates the cellular transformation in paracrine- and autocrine-dependent manner.

3.5. Silencing the expression of ST2 gene products suppresses Ras (G12V)-provoked cellular transformation

Next, to clarify the functional involvement of endogenous ST2 gene products in Ras-provoked cellular transformation, we tested the effect of knockdown of ST2 gene products on the cellular transformation using retroviruses that express short hairpin RNA (sh-RNA). As shown in Fig. 6A, sh-RNA against ST2/ST2L suppressed Ras (G12V)-induced expression of both ST2 and ST2L. Furthermore, we confirmed that the expression of proteins of ST2 and ST2L by performing immunoblot analysis. The expression of soluble ST2 protein was completely silenced by sh-RNA against ST2/ST2L, however ST2L protein was partially remained (Fig. 6B and C). We do not have suitable explanation for these different results of the effects of shRNA against ST2/ST2L. While ST2 is a secreted protein, ST2L is a membrane-spanning protein. Life span of protein is affected by a number of conditions such as localization. Membrane-spanning ST2L protein maybe more stable than soluble ST2, this maybe the reason why the expression of ST2L protein was not completely silenced by shRNA against ST2/ST2L.

Strikingly, when the expression levels of ST2 gene products were reduced, Ras (G12V)-provoked transformation was drastically diminished, suggesting that ST2 gene products harbor an essential role in Ras (G12V)-provoked cellular transformation (Fig. 7A and B). Interestingly, when ST2 gene products were knocked down, the

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

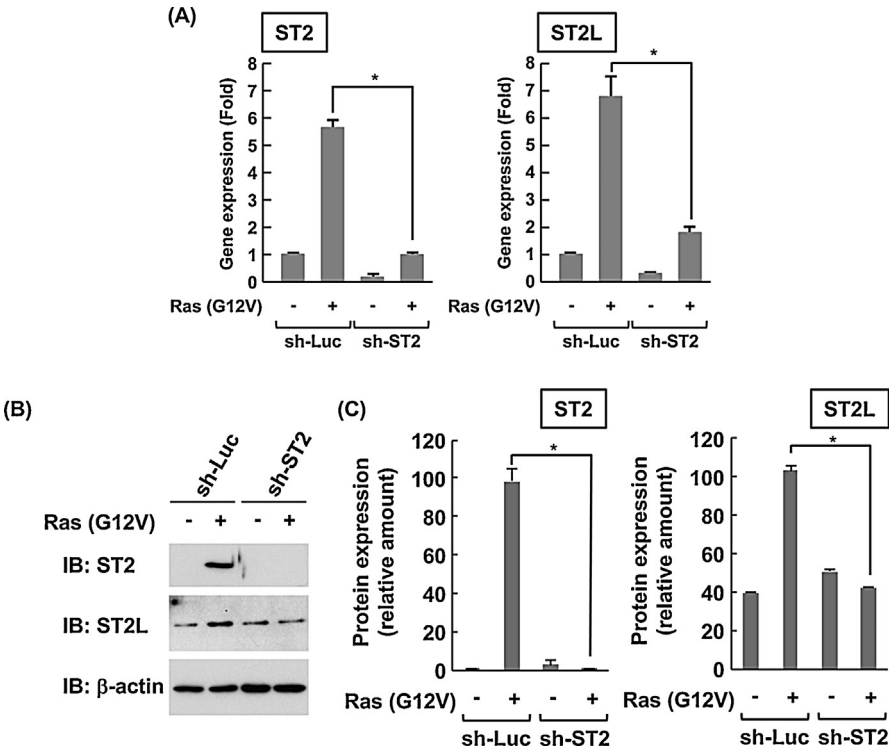

Fig. 6. Knockdown of the expression of ST2 gene products in normal and transformed NIH-3T3 cells. (A) NIH-3T3 cells were infected with the indicated retroviruses including Ras (G12V), sh-luciferase (sh-Luc), and sh-ST2/ST2L (Referred to as sh-ST2), and infected cells were selected by the treatment with puromycin for 72 h. And then, total RNA was extracted from infected cells, and the expression levels of ST2 gene products were analyzed by quantitative PCR. In the graph, error bars = S.D. (n = 3, *P < 0.005). (B) The reduction of the protein expression of ST2 and ST2L by sh-RNA was detected by immunoblot analysis. Original blots are shown in supplementary file named as "Original blots for Ras & ST2". (C) The results of (B) were shown in the graphs, respectively. In the graph, error bars = S.D. (n = 3, *P<0.005).

phosphorylation of Rb protein at Ser-807/811 was significantly suppressed, while the expression of cyclin D1 protein was not altered significantly (Fig. 7C and D). These observations strongly suggested that endogenous ST2 gene products have critical roles in Ras (G12V)-provoked cellular transformation through affecting the cell cycle progression, especially the phosphorylation of Rb protein.

In the previous study, we already reported that ST2L is not required for the cellular transformation provoked by oncogenic Ras [24]. Therefore, we concluded that ST2 but not ST2L seems to contribute to the cellular transformation provoked by Ras (G12V).

3.6. ST2 gene products may modulate the regulation mechanism of the cell cycle

We also found that the knockdown of ST2 gene products slowed down cell proliferation [25]. To determine whether ST2 gene products might positively

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

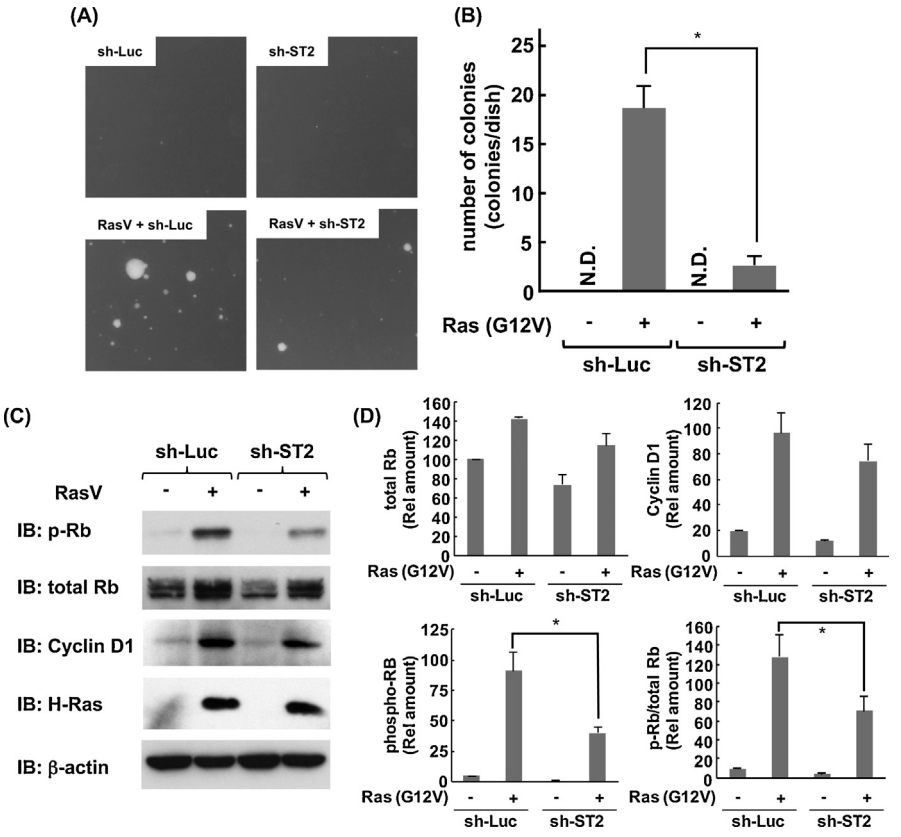

Fig. 7. Silencing the expression of ST2 gene products suppresses the cellular transformation induced by Ras (G12V). (A) Infected NIH-3T3 cells were seeded onto soft agar at 1×10^4 cells per 35-mm dish, and grown for 2–3 weeks. Grown colonies were photographed at 4 × magnification, and the numbers of colonies were counted. In the labels of photographs, RasV means Ras (G12V) mutant. (B) The number of colonies of transformed NIH-3T3 cells counted in (A) is shown in the graph. In the graph, error bars = S.D. (n = 3, *P < 0.005). N.D. means "Not detected". (C) Whole cell lysates were prepared from the infected cells and analyzed by immunoblotting with the primary antibodies indicated on the left of the image. Original blots are shown in supplementary file named as "Original blots for Ras & ST2". (D) The intensity of Rb phosphorylation, total Rb, cyclin D1, and the ratio between phosphorylated Rb and total Rb were quantitated, calculated, and then results were shown in graphs, respectively. In the graph, error bars = S.D. (n = 3, *P<0.005).

regulate cell cycle progression, we silenced the expression of ST2 gene products in NIH-3T3 cells using sh-RNA and examined its effects on cell cycle progression from G_0 to G_2/M phase. Infected NIH-3T3 cells with sh-RNA against control luciferase or ST2 gene products were serum starved to induce growth arrest and then re-stimulated with serum to synchronously enter the cell cycle. Then, cells were harvested for cell cycle analysis and immunoblotting analysis for marker proteins of cell cycle progression such as $p27^{Kip1}$, cyclin D1, cyclin A, and c-Myc. As shown in Fig. 8A, the knockdown of ST2 gene products effectively caused the retardation of cell cycle progression. Especially, the entry into S phase was apparently delayed, when ST2 gene products were knocked down (Fig. 8B).

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

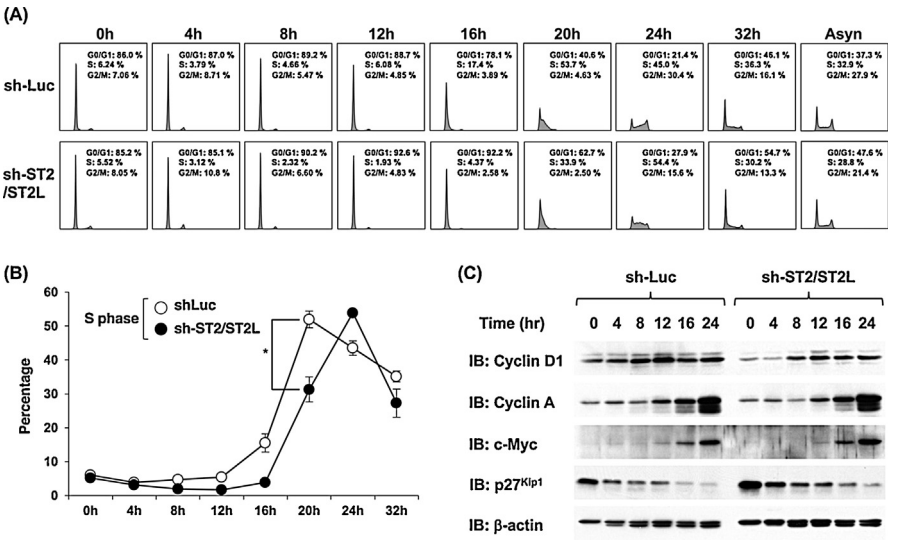

Fig. 8. ST2 gene products may modulate the regulation of the cell cycle**.** (A) NIH-3T3 cells were infected with the indicated retroviruses. Infected cells were held in G_0 phase by serum starvation for 24 h. Then, the cells were released into the cell cycle by dividing the cells onto new dishes. At the indicated time points, cells were harvested and stained with propidium iodide. Cell suspension was analyzed by flow cytometry. (B) The percentage of cells entering into S-phase was shown in graph. Significant difference of the cell population entering to S-phase at 20 hr after cell cycle release between ST2-knockdowned cells and control cells was statistically determined by the Student's *t*-test. In the graph, error bars = S.D. (n = 3, *P < 0.005). (C) At indicated time points, cells were harvested and analyzed by immunoblot with primary antibodies, as indicated at the left of the image. Original blots are shown in supplementary file named as "Original blots for Ras & ST2".

Although the expression of cyclin D1 protein was slightly decreased, the expression levels of other marker proteins, cyclin A and c-Myc were not altered by the knockdown of ST2 gene products. Interestingly, the expression level of $p27^{Kip1}$ protein was obviously increased at the growth arrest phase in cells in which ST2 gene products were knocked down (Fig. 8C). In the previous report [25], we showed that the addition of recombinant ST2 exhibited no effect on cell cycle progression. In the current study, we utilized the different experimental condition, in which endogenous ST2 gene products were knocked down by sh-RNA, and finally could observe the functional involvement of endogenous ST2/ST2L, probably ST2 in the cell cycle progression. Although we do not have direct evidence, knockdown of ST2 gene products may cause the effective delay in the cell cycle through the increased expression of $p27^{Kip1}$ and maybe slight reduction of cyclin D1 protein. The effect of ST2 gene products on cellular transformation might be due to cumulative alteration of the cell cycle progression.

4. Discussion

In the current study, we observed that oncogenic Ras induced the expression of both ST2 and ST2L proteins. In the previous report, we found that oncogenic Ras

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

induced the expression of mRNA of ST2 and ST2L (Ref 24, also shown in Fig. 1A). However, the current observation in Fig. 2 suggested that oncogenic Ras enhanced the protein amount of exogenous ST2 and ST2L which were expressed by constitutively active retroviral system. These observations suggested that oncogenic Ras not only stimulates the promoter of ST2/ST2L gene, but also stabilized the ST2 and ST2L protein. These two products were generated by alternative splicing of mRNA, however the detailed mechanism is still unknown. In addition, it was unclear whether cell growth stimulation-induced ST2 gene products are involved in cell proliferation and carcinogenesis. In the previous study, when purified soluble ST2 protein was added to a culture of human glioblastoma T98 G cells, their colony formation in soft agar was effectively diminished [30]. Recently, Akimoto and colleagues also reported that ST2 inhibits colorectal cancer malignant growth [31]. On the other hand, it was also reported that the IL-33/ST2 signaling pathway promotes epithelial cell transformation and breast tumorigenesis via accelerated activation of AP-1 and STAT3 [32]. Furthermore, Yu and colleagues reported that the IL-33-ST2L signaling axis contributes to cancer cell invasion and migration of gastric cancer cells through the activation of Extracellular Signal-regulated kinase (ERK) [33]. These reports supported the possibility that ST2 gene products contribute to cellular transformation and tumor progression, although these reports include several problems to be solved about the cellular context, especially the status of tumor suppressor genes, because those such as Arf/Ink4a, p53, and Rb have to be inactivated or lost for tumorigenesis [34, 35].

We showed that the knockdown of ST2 gene products reduced the tumor formation by NIH-3T3 cells transformed by Ras (G12V) (Fig. 7). The knockdown of ST2 gene products caused the reduction of phosphorylation of Rb protein, suggesting that ST2 gene products seem to contribute to cell cycle progression. On the other hand, we already reported that ST2L was dispensable for the cellular transformation [24]. Taking these observations together, it was suggested that ST2 more critically contributed to cellular transformation than ST2L, and this is well fit with our previous report [25].

In the current study, we observed that the knockdown of ST2 gene products caused the suppression of Rb phosphorylation at Ser-807/811 (Fig. 7C and D). The phosphorylation of Rb at Ser-807/811 is catalyzed by cyclin-dependent protein kinase 4 and 6 (CDK4/6) complexes, which interact with an essential activator for CDK4/6, cyclin D [36]. On the other hand, the enforced expression of ST2 and ST2L caused the enhancement of cyclin D1 expression (Fig. 2). The expression level of cyclin D1 induced by Ras (G12V) seems to be enough for inducing the phosphorylation of Rb protein, therefore the enforced expression of ST2/ST2L failed to enhance the phosphorylation of Rb caused by Ras (G12V). In the case of breast cancer, the connection between cyclin D1 and estrogen receptor (ER) was

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

reported [37, 38]. Cyclin D1 protein forms a direct physical complex with ER and exhibits a strong synergy with estrogen to activate ER [39]. It should be noted that cyclin D1 not only interacts with ER but also with several members of the steroid receptor coactivators (SRCs) [40, 41], suggesting that similar events could happen in other types of cellular transformation. Cyclin D proteins were also reported to regulate the abilities of DMP1, a Myb-like protein, and these effects were also connected to the acceleration of cell proliferation [42, 43]. Although speculative, excess amount of cyclin D1 expression might contribute to the acceleration of cell growth mediated by these machineries.

As shown in Fig. 3D, the enforced expression of ST2/ST2L failed to enhance the Ras (G12V)-induced expression of cyclin D1 mRNA. Now, we could not explain how ST2 protein enhances the expression of cyclin D1 protein. According to a number of reports, the expression of cyclin D1 protein is complexly regulated by Ras signaling pathway. Ras signal stimulates the cyclin D1 promoter, and induces the expression of cyclin D1 mRNA through the activation of transcription factors such as Ets-2 and AP-1 [44, 45]. Ras signaling pathway also utilizes protein kinase Akt to stabilize cyclin D1 protein via the inhibition of GSK-3β, which functions as triggering kinase for the ubiquitination of cyclin D1 protein [46, 47]. Furthermore, our current study clearly showed that oncogenic Ras accelerates the protein synthesis of cyclin D1 through the functional involvement of intracellular IL-33/NF-HEV [24].

In the current study, we showed that ST2 gene products, especially soluble ST2 accelerates the cell proliferation, and contribute to Ras (G12V)-induced cellular transformation (Fig. 9). IL-33 has been understood to function as an alarmin,

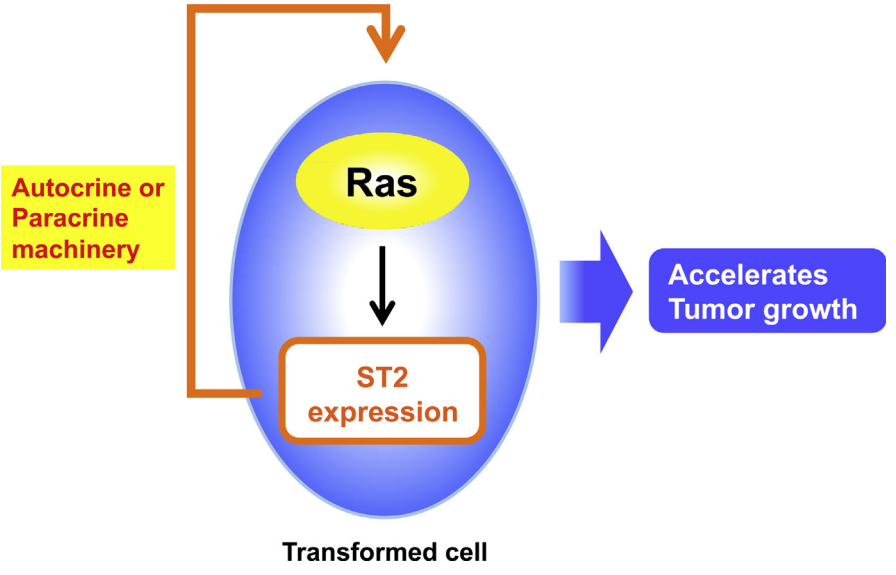

Fig. 9. Working hypothesis for the mechanism how ST2 gene products contribute to the tumor growth and progression.

http://dx.doi.org/10.1016/j.heliyon.2017.e00436

which is secreted from necrotic cells [48]. However, we could not detect the presence of secreted IL-33 in culture supernatant (unpublished data), suggesting that the enhancing effect of ST2 on the oncogenic signals seem to be independent on IL-33. Several research group including us suggested the presence of binding protein for soluble ST2 on the surface of target cells [49, 50, 51]. To clarify the mechanism how ST2 accelerates the cellular transformation, the identification of ST2 receptor would be an important clue.

Declarations

Author contribution statement

Kenji Tago: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Satoshi Ohta, Masaki Kashiwada: Performed the experiments.

Megumi Funakoshi-Tago: Contributed reagents, materials, analysis tools or data.

Jitsuhiro Matsugi: Analyzed and interpreted the data.

Shin-ichi Tominaga: Conceived and designed the experiments; Analyzed and interpreted the data.

Ken Yanagisawa: Analyzed and interpreted the data; Wrote the paper.

Funding statement

This work was supported by Grants-in-Aid for Scientific Research, KAKENHI from the JSPS (Japan Society for the Promotion of Science) (Grant numbers are 26440062 and 26460376), MEXT-Supported Program for the Strategic Research Foundation at Private Universities (S1311029), and JKA (21-1-042-8) through its promotion funds from KEIRIN RACE.

Competing interest statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at http://dx.doi.org/10.1016/j.heliyon.2017.e00436.

Acknowledgements

We thank Dr. Scott Lowe for providing the pBabePuro-Ras (G12V). We also thank Ms. Chihiro Aoki-Ohmura and Ms. Natsue Nakayama for their helpful assistance.

References

[1] S. Tominaga, A putative protein of a growth specific cDNA from BALB/c-3T3 cells is highly similar to the extracellular portion of mouse interleukin 1 receptor, FEBS Lett. 258 (1989) 301–304.

[2] A.K. Werenskiold, S. Hoffmann, R. Klemenz, Induction of a mitogen-responsive gene after expression of the Ha-Ras oncogene in NIH 3T3 fibroblasts, Mol. Cell. Biol. 9 (1989) 5207–5214.

[3] G. Superti-Furga, G. Bergers, D. Picard, M. Busslinger, Hormone-dependent transcriptional regulation and cellular transformation by Fos-steroid receptor fusion proteins, Proc. Natl. Acad. Sci. USA 88 (1991) 5114–5118.

[4] K. Yanagisawa, et al., Presence of a novel primary response gene ST2L, encoding a product highly similar to the interleukin 1 receptor type 1, FEBS Lett. 318 (1993) 83–87.

[5] H. Li, et al., The cloning and nucleotide sequence of human ST2L cDNA, Genomics 67 (2000) 284–290.

[6] J. Schmitz, et al., IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines, Immunity 23 (2005) 479–490.

[7] S. Sanada, et al., IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system, J. Clin. Invest. 117 (2007) 1538–1549.

[8] M. Funakoshi-Tago, et al., TRAF6 is a critical signal transducer in IL-33 signaling pathway, Cell Signal. 20 (2008) 1679–1686.

[9] M. Funakoshi-Tago, et al., JAK2 is an important signal transducer in IL-33-induced NF-(B activation, Cell Signal. 23 (2011) 363–370.

[10] H. Hayakawa, M. Hayakawa, A. Kume, S. Tominaga, Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation, J. Biol. Chem. 282 (2007) 26369–26380.

[11] H. Ohto-Ozaki, et al., Characterization of ST2 transgenic mice with resistance to IL-33, Eur. J. Immunol. 40 (2010) 2632–2642.

[12] A.E. Karnoub, R.A. Weinberg, Ras oncogenes: split personalities, Nat. Rev. Mol. Cell Biol. 9 (2008) 517–531.

[13] Y. Kaziro, et al., Structure and function of signal-transducing GTP-binding proteins, Annu. Rev. Biochem. 60 (1991) 349–400.

[14] A.G. Stephen, D. Esposito, R.K. Bagni, F. McCormick, Dragging ras back in the ring, Cancer Cell 25 (2014) 272–281.

 http://dx.doi.org/10.1016/j.heliyon.2017.e00436

[15] M. Krypuy, et al., High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: KRAS codon 12 and 13 mutations in non-small cell lung cancer, BMC Cancer 6 (2006) 295.

[16] C. Albanese, et al., Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions, J. Biol. Chem. 270 (1995) 23589–23597.

[17] O. Tetsu, F. McCormick, β-catenin regulates expression of cyclin D1 in colon carcinoma cells, Nature 398 (1999) 422–426.

[18] W.H. Herman, M.S. Simonson, Nuclear signaling by endothelin-1: A Ras pathway for activation of the c-fos serum response element, J. Biol. Chem. 270 (1995) 11654–11661.

[19] M. Elgendy, C. Sheridan, G. Brumatti, S.J. Martin, Oncogenic Ras-induced expression of Noxa and Beclin-1 promotes autophagic cell death and limits clonogenic survival, Mol. Cell 42 (2011) 23–35.

[20] J.S. Park, et al., A role for both Ets and C/EBP transcription factors and mRNA stabilization in the MAPK-dependent increase in p21 (Cip-1/WAF1/mda6) protein levels in primary hepatocytes, Mol. Biol. Cell 11 (2000) 2915–2932.

[21] E.S. Baekkevold, et al., Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules, Am. J. Pathol. 163 (2003) 69–79.

[22] V. Carriere, et al., IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo, Proc. Natl. Acad. Sci. USA 104 (2007) 282–287.

[23] S. Ali, et al., The dual function cytokine IL-33 interacts with the transcription factor NF-(B to dampen NF-(B-stimulated gene transcription, J. Immunol. 187 (2011) 1609–1616.

[24] S. Ohta, et al., Intracellular NF-HEV/IL-33 harbors essential roles in Ras-induced cellular transformation by contributing to cyclin D1 protein synthesis, Cell Signal. 28 (2016) 1025–1036.

[25] S. Tominaga, S. Ohta, K. Tago, Soluble form of the ST2 gene product exhibits growth promoting activity in NIH-3T3 cells, Biochem. Biophys. Rep. 5 (2016) 8–15.

[26] T. Takagi, et al., Identification of the product of the murine ST2 gene, Biochim. Biophys. Acta 1178 (1993) 194–200.

 http://dx.doi.org/10.1016/j.heliyon.2017.e00436

[27] K. Tago, et al., Arf tumor suppressor disrupts the oncogenic positive feedback loop including c-Myc and DDX5, Oncogene 34 (2015) 314–322.

[28] F. Ciardiello, et al., Transformation of mouse mammary epithelial cells with the Ha-ras but not with the neu oncogene results in a gene dosage-dependent increase in transforming growth factor-alpha production, FEBS Lett. 250 (1989) 474–478.

[29] M. Miyake, S. Mizutani, H. Koide, Y. Kaziro, Unfarnesylated transforming Ras mutant inhibits the Ras-signaling pathway by forming a stable Ras•Raf complex in the cytosol, FEBS Lett. 378 (1996) 15–18.

[30] Y. Haga, et al., The effect of ST2 gene product on anchorage-independent growth of a glioblastoma cell line, T98G, Eur. J. Biochem. 270 (2003) 163–170.

[31] M. Akimoto, R. Maruyama, H. Takamaru, T. Ochiya, K. Takenaga, Soluble IL-33 receptor sST2 inhibits colorectal cancer malignant growth by modifying the tumour microenvironment, Nat. Commun. 7 (2016) 13589.

[32] J.Y. Kim, et al., Interleukin-33/ST2 axis promotes epithelial cell transformation and breast tumorigenesis via upregulation of COT activity, Oncogene 34 (2015) 4928–4938.

[33] X.X. Yu, et al., IL-33 Promotes Gastric Cancer Cell Invasion and Migration Via ST2-ERK1/2 Pathway, Dig. Dis. Sci. 60 (2015) 1265–1272.

[34] C.J. Sherr, Principles of tumor suppression, Cell 116 (2004) 235–246.

[35] C.J. Sherr, et al., p53-Dependent and -independent functions of the Arf tumor suppressor, Cold Spring Harb. Symp. Quant. Biol. 70 (2005) 129–137.

[36] C.J. Sherr, D. Beach, G.I. Shapiro, Targeting CDK4 and CDK6: From Discovery to Therapy, Cancer Discov. 6 (2016) 353–367.

[37] P.J. van Diest, et al., Cyclin D1 expression in invasive breast cancer: Correlations and prognostic value, Am. J. Pathol. 150 (1997) 705–711.

[38] M.F. Buckley, et al., Expression and amplification of cyclin genes in human breast cancer, Oncogene 8 (1993) 2127–2133.

[39] E. Schuuring, et al., Amplification of genes within the chromosome 11q13 region is indicative of poor prognosis in patients with operable breast cancer, Cancer Res. 52 (1992) 5229–5234.

[40] R.M. Zwijsen, et al., CDK-independent activation of estrogen receptor by cyclin D1, Cell 88 (1997) 405–415.

 http://dx.doi.org/10.1016/j.heliyon.2017.e00436

[41] R.M. Zwijsen, et al., Ligand-independent recruitment of steroid receptor coactivators to estrogen receptor by cyclin D1, Genes Dev. 12 (1998) 3488–3498.

[42] B. Ganter, J.S. Fu S. l. Lipsick, D-type cyclins repress transcriptional activation by the v-Myb but not the c-Myb DNA-binding domain, EMBO J. 17 (1998) 255–268.

[43] K. Inoue, C.J. Sherr, Gene expression and cell cycle arrest mediated by transcription factor DMP1 is antagonized by D-type cyclins through a cyclin-dependent-kinase-independent mechanism, Mol. Cell Biol. 18 (1998) 1590-–1600.

[44] C. Albanese, et al., Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions, J. Biol. Chem. 270 (1995) 23589–23597.

[45] I. Treinies, et al., Activated MEK stimulates expression of AP-1 components independently of phosphatidylinositol 3-kinase (PI3-kinase) but requires a PI3-kinase signal to stimulate DNA synthesis, Mol. Cell Biol. 19 (1999) 321–329.

[46] J.A. Diehl, M. Cheng, M.F. Roussel, C.J. Sherr, Glycogen synthase kinase-3β regulates cyclin D1 proteolysis and subcellular localization, Genes Dev. 12 (1998) 3499–3511.

[47] R.C. Muise-Helmericks, et al., Cyclin D.;1 expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway, J. Biol. Chem. 273 (1998) 29864–29872.

[48] C. Moussion, N. Ortega, J.P. Girard, The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? PLoS One 3 (2008) e3331.

[49] K. Yanagisawa, et al., The expression of ST2 gene in helper T cells and the binding of ST2 protein to myeloma-derived RPMI8226 cells, J. Biochem. 121 (1997) 95–103.

[50] M.J. Sweet, et al., A novel pathway regulating lipopolysaccharide-induced shock by ST2/T1 via inhibition of Toll-like receptor 4 expression, J. Immunol. 166 (2001) 6633–6639.

[51] A. Nagata, et al., Soluble ST2 protein inhibits LPS stimulation on monocyte-derived dendritic cells, Cell Mol. Immunol. 9 (2012) 399–409.

http://dx.doi.org/10.1016/j.heliyon.2017.e00436