

Stanniocalcin 2 enhances mesenchymal stem cell survival by suppressing oxidative stress

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To overcome the disadvantages of stem cell-based cell therapy like low cell survival at the disease site, we used stanniocalcin 2 (STC2), a family of secreted glycoprotein hormones that function to inhibit apoptosis and oxidative damage and to induce proliferation. STC2 gene was transfected into two kinds of stem cells to prolong cell survival and protect the cells from the damage by oxidative stress. The stem cells expressing STC2 exhibited increased cell viability and improved cell survival as well as elevated expression of the pluripotency and self-renewal markers (Oct4 and Nanog) under sub-lethal oxidative conditions. Up-regulation of CDK2 and CDK4 and down-regulation of cell cycle inhibitors p16 and p21 were observed after the delivery of STC2. Furthermore, STC2 transduction activated pAKT and pERK 1/2 signal pathways. Taken together, the STC2 can be used to enhance cell survival and maintain long-term stemness in therapeutic use of stem cells. [BMB Reports 2015; 48(12): 702-707]

INTRODUCTION

With respect to cell-based therapy, there is no doubt that stem cells represent very special and excellent therapeutic tools due to their multipotent capacity to differentiate into various tissues, their self-renewal abilities, and their high degree of plasticity (1). Stem cells are also a smart delivery system to express or secrete therapeutic factors in damaged organs as therapeutic agents and may represent a potential targeted gene delivery system. However, their clinical applications are limited despite their high therapeutic potential.

One of the major obstacles in the use of stem cells for cell

therapy is the low survival rate in tissues and the limited expansion number. Stem cells are faced with dangerous challenges, such as oxidative stress, nutrient deprivation, host immune response, and oxygen deprivation when injected at the disease area, especially ischemic sites (2). Moreover, stem cells need to expand to obtain sufficient cell numbers (routinely 10-400 million mesenchymal stem cells per treatment) to achieve efficient therapeutic effects *in vivo* (3, 4). Among various factors responsible for the successful treatment of stem cell therapy is a reduction in the length of the cell expansion period *in vitro* and increased cell survival before the transplantation or injection. All primary cells, including stem cells or various origins, are limited in the number of cell divisions they can undergo under culture conditions (5, 6).

Recent studies indicate that the overexpression of anti-apoptotic and antioxidant proteins, such as the telomerase catalytic subunit or lipocalin 2, promotes stem cell resistance against ischemic stresses, thus increasing their viability and survival under harsh conditions (2, 7, 8). Thus, increased stem cell therapeutic efficacy is required to maintain their long-term cell survival and to reduce the limitation of the time-dependent cell passage and cell expansion period *in vivo*.

Stanniocalcin 2 (STC2) is a member of the stanniocalcin family and is a peptide hormone regulating calcium and phosphate homeostasis (9, 10). The function of stanniocalcin 1 (STC1) is known to have various biological effects involving the inhibition of apoptosis and oxidative damage in various human cancers (11-13). For example, STC1 was reported to protect retinal ganglion cells by inhibiting apoptosis and oxidative damage (14), and STC1 secreted from MSCs protected bovine intestinal epithelial cells from oxidative damage (15).

However, whether STC family members are pro- or anti-apoptotic remains a controversial issue. Some reports indicate that STC1 is pro-apoptotic in chondrocytes during bone development (16). On the other hand, another report demonstrated anti-apoptotic function in heart and brain under hypoxia conditions (17). Although positive effects of STC1 are well-known in various cancers, the action and mechanism of STC2 in human cells, including stem cells, has yet to be fully understood. Furthermore, the relationship between STC2 and stem cells has not been reported thus far, and there are no

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<http://dx.doi.org/10.5483/BMBRep.2015.48.12.158>

Received 23 July 2015, Revised 13 August 2015,
Accepted 25 September 2015

Keywords: Mesenchymal stem cells, Oxidative stress, Stanniocalcin-2, Stem cell-based therapy

studies concerning how STC affects stem cells.

Therefore, we investigated the biological function and mechanism of STC2 in adipose-derived stromal cells (ADSC), and umbilical cord blood-derived mesenchymal stem cells (UCB-MSC) under oxidative damage to explore potential therapies to overcome the limitation of stem cell-based therapy.

RESULTS

STC2 expression under oxidative stress

After the decision of the sub-lethal concentration of H₂O₂ to stem cells (Supplementary Fig. 1), to access STC2 expression levels under conditions of the oxidative stress and to determine whether STC2 plasmid transfection into stem cells is expressed under the same conditions, RT-PCR was conducted using RNA extracted from cells treated with NC, H₂O₂, pcDNA+H₂O₂, and pcDNA/STC2+H₂O₂. Under the normal condition (NC group), each stem cell expressed low levels of STC2. However, STC2 expression was significantly decreased in both cells in the presence of H₂O₂, as shown in inset graphs of Fig. 1 (P < 0.05 in ADSC). However, the cells transfected with the pcDNA/STC2 plasmid exhibited significantly high levels of STC2 expression compared with those transfected with pcDNA. These results demonstrate that the STC2 plasmid is well expressed in stem cells even under conditions of oxidative damage.

Increased cell proliferation by STC2 overexpression

We next tested the effect of STC2 on cell growth because the STC family is known to play an anti-apoptotic role in response to oxidative stress (9). Accordingly, we sought to determine

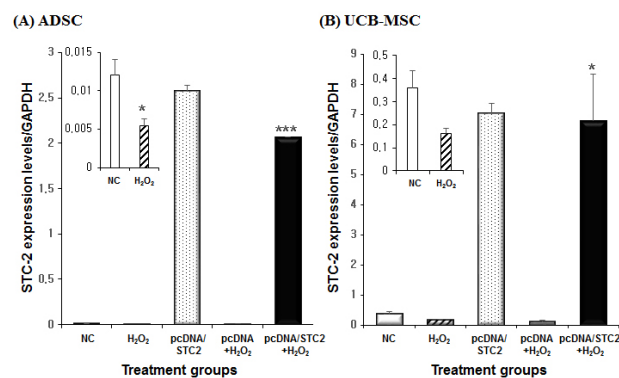


Fig. 1. Evaluation of STC2 expression levels in stem cells. Cells were transfected with pcDNA or pcDNA/STC2 plasmids. After 48 hrs, cells were treated with 100 mM H₂O₂ for 3 hrs, and then RNA was extracted and cDNA was synthesized, RT-PCR was conducted to detect STC2 expression in ADSC (A) and in UCB-MSC (B). Data represent the means and standard errors of triplicate experiments. *P < 0.05 for comparison of H₂O₂ against NC groups and ***P < 0.01 versus pcDNA+H₂O₂ (A), and *P < 0.05 versus H₂O₂ (B).

whether STC2 expression in ADSCs and UCB-MSCs can increase cell proliferation.

Fig. 2A depicts the cell morphologies after H₂O₂ treatment in STC2-expressing ADSCs. Cell death was induced in the cells treated with H₂O₂ compared with untreated cells in normal condition (NC) (37% or 78% cell viability in ADSCs or UCB-MSCs, respectively, Fig. 2B for ADSCs and Supplementary Fig. 2A for UCB-MSCs). Similar rates of cell death were observed in the cells treated with the pcDNA vector in ADSCs. However, STC2-expressing cells exhibited a 1.8-fold increase in cell sur-

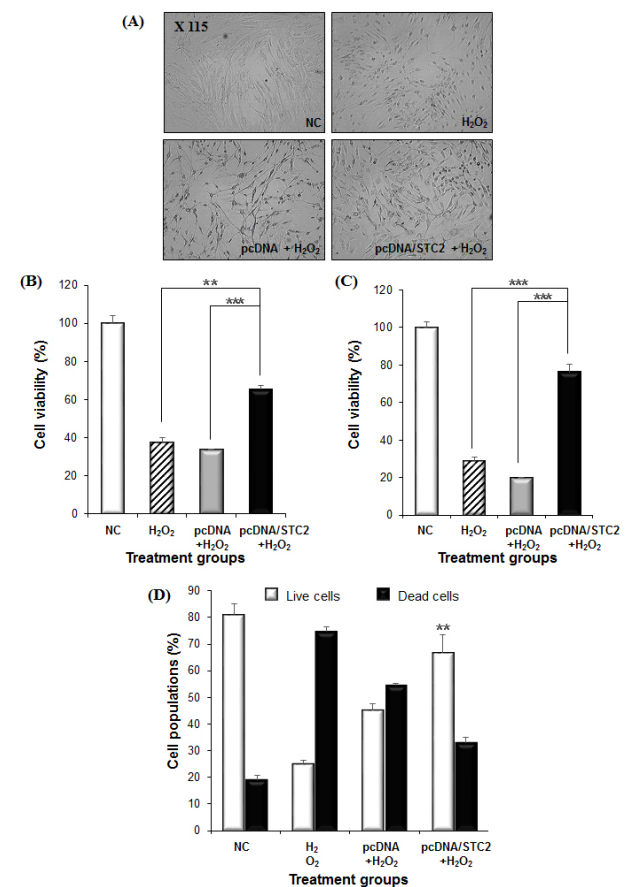


Fig. 2. Increased cell proliferative activity and enhancement of live/dead cell populations by STC2 expressed. At 48 hrs post-STC2 delivery, H₂O₂ was treated to cells for 3 hrs and then cell morphology was observed (A) and MTT assay were performed (B). Another groups were assessed at 4 days post-media change of H₂O₂ treatment (C). Increased cell proliferation and viability were observed in the cells transfected with STC2 plasmid. Data represent the means and standard errors of triplicate experiments. **P < 0.02 and ***P < 0.01 for comparison of pcDNA/STC2+H₂O₂ groups against H₂O₂ or pcDNA+H₂O₂ groups, respectively. Also, cell viability of ADSCs was observed by Arthur image-based cytometer in cells stained with PI solution after the treatment of STC2 and H₂O₂ (D). Dead cells were stained by PI as red color. **P < 0.02 versus H₂O₂ groups.

vival in response to H₂O₂ stress. Four days after the treatment, increased cell death upon H₂O₂ stress was observed in the pcDNA-treated group compared with the results in Fig. 2B (Fig. 2C for ADSCs and Supplementary Fig. 2B for USC -MSCs). The pattern of results at this time was similar to that Fig. 2A, demonstrating that STC2 improves cell viability and proliferation upon H₂O₂ challenges. Taken together, these results indicate that STC2 can overcome oxidative stress-induced cellular damage.

Live and dead cell populations

Because STC can act as a factor to regulate cell survival, we evaluated cell viability after cellular damage. Cell viability was evaluated by counting the number of cells stained by PI, which is capable of discriminating live and dead cells. After staining, cells were analyzed by Arthur imaged-based cytometry. The quantification of live and dead cells treated with varying concentrations of NC, H₂O₂, pcDNA+H₂O₂, and pcDNA/STC2+H₂O₂ is reported as the percentage of the cell population.

Fig. 2D shows the percent of cell stained by PI with respect to the total population as well as the percent of the total population that is both viable and expresses PI. The live and dead populations represented 81 and 19% of the parental cells, respectively. Larger dead cell populations were observed in the cells treated with H₂O₂ (25% for live and 75% for dead cells). However, increased live cell and decreased dead cell populations were observed in the cells treated with pcDNA/STC2+H₂O₂ (67 and 33%) compared with those of the cells treated with pcDNA+H₂O₂ (45 and 55%). These data demonstrate that STC2 expression in ADSCs elicits enhanced cell survival compared with empty vector transfected cells in response to oxidative stress.

Biological potential of ADSCs expressing STC2

The increased cell proliferation induced by STC2 may also be caused by the up-regulation of cell cycle-related proteins. To compare the expression levels of cell cycle regulators, we measured the levels of cyclin-dependent kinases (CDKs) and their inhibitor proteins p16 and p21 by RT-PCR in ADSCs treated with NC, H₂O₂, and pcDNA/STC2+H₂O₂.

As shown in Fig. 3A, CDK2 or CDK4 expression was increased in the cells treated with pcDNA/STC2+H₂O₂ under oxidative conditions. CDK2 and CDK4 expression were about 5.8- and 3.2-fold higher, respectively, in the cells transfected with pcDNA/STC2 than in the cells treated with H₂O₂ alone. These results were also confirmed in UCB-MSCs (Supplementary Fig. 3). In the case of the CDK inhibitor proteins, CDK inhibitor 2A and 1A (also known as p16 and p21, respectively), the cells transfected with pcDNA/STC2+H₂O₂ exhibited approximately 2.6- and 1.1-fold lower p16 and p21 expression than those of parental cells when treated with H₂O₂ (Fig. 3B). These results demonstrate that elevated expression of CDK2 and CDK4 and decreased expression p16 is at least responsible for the increased cell viability induced by STC2.

Because cell viability against oxidative stress-induced cellular damage was increased by STC2, we next assessed whether the multipotency of stem cells is maintained by STC2 expression in ADSCs. Our results revealed that pcDNA/STC2+H₂O₂ exhibited higher expression of stemness factors (Nanog and Oct4) than the H₂O₂ groups alone ($P < 0.02$ for Nanog and $P < 0.05$ for Oct4) (Fig. 3C). Intriguingly, Nanog and Oct4 expression in cells treated with STC2 were more highly induced under oxidative conditions than under normoxic conditions ($P < 0.02$ for Oct4). These data imply that STC2 transfection leads to maintained biological potency in stem cells

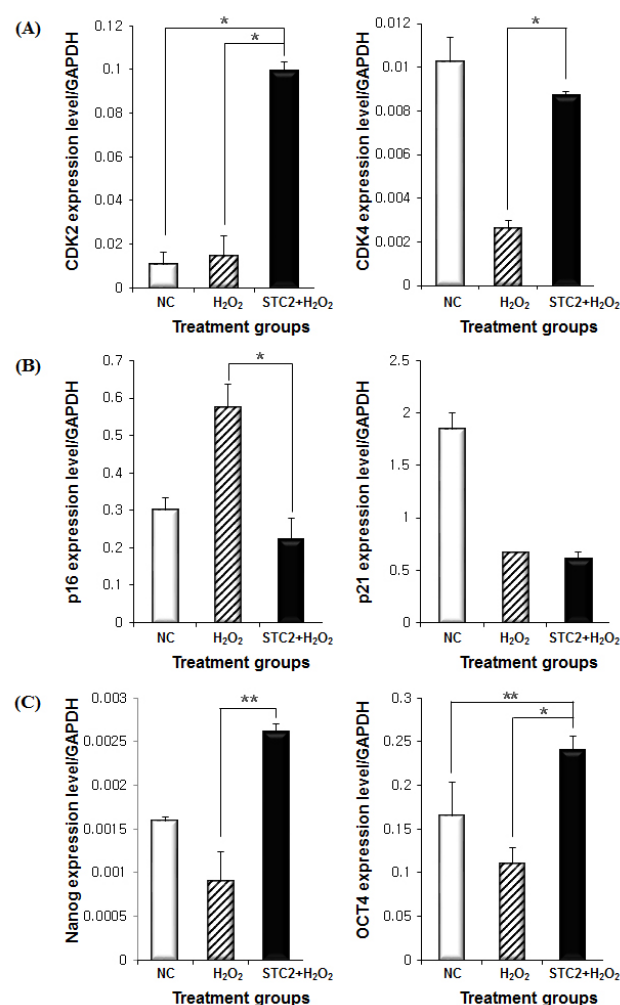


Fig. 3. Biological activity of STC2 expressed in ADSC. The expression levels of cell cycle regulator proteins, CDK2 and 4 (A), inhibitor proteins, p16 and p21 (B), and stemness factors, Nanog and Oct4 (C). ADSCs were treated with NC, H₂O₂, and pcDNA/STC2+H₂O₂, and then got oxidative damage for 3 hrs. After RNA extraction and cDNA synthesis, the expression of each factors were measured by RT-PCR. Data represent means \pm SE. * $P < 0.05$ and ** $P < 0.02$ for remarked groups.

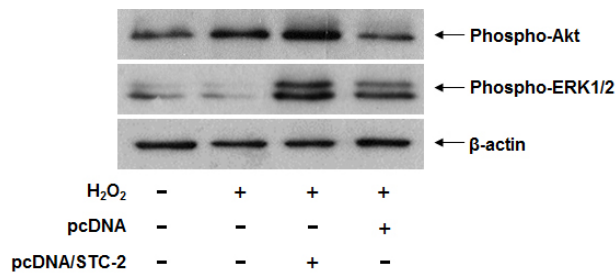


Fig. 4. Investigation of the mechanism about improved positive effects in stem cells. Cellular signal pathway was examined by western blot. Phosphorylated Akt and ERK1/2 levels up-regulated in STC2 expressed ADSC under oxidative stress. pcDNA+H₂O₂ groups were used as a negative control and β -actin was used as internal control.

subjected to cellular damage caused by oxidative stress.

STC2 signaling pathway confers improved stem cell survival under oxidative stress

To explore the mechanism by which STC2 induces improved stem cell viability against oxidative stress, the expression levels of phospho-protein kinase B (pAkt) and phospho-extracellular signal-regulated kinase 1/2 (pERK 1/2), which play major roles in cell survival and proliferation, were examined by western blot analysis.

As shown in Fig. 4, reduction of pAkt and pERK 1/2 was observed in the cells treated with H₂O₂. In contrast, STC2 transfection increased the protein levels relative to the pcDNA groups despite treatment with H₂O₂. These data indicate that the activation of Akt and ERK 1/2 by STC2 might up-regulate cell cycle regulators and stemness factors and thus increase cell survival upon oxidative stress.

DISCUSSION

Stem cell-based cell therapies derived from various sources have been applied in over 250 ongoing clinical trials (18). Although the benefits of stem cell therapy have been reported for a variety of diseases, some limitations exist that complicate successful clinical trials using stem cells. The major hurdles include how to overcome the heterogeneity of stem cell populations and cell senescence as well as cryopreservation and *in vitro* expansion for clinical application.

Death during the cell expansion period and death induced by environmental conditions, such as hypoxia or oxidative damage, represent additional challenges that may be overcome by taking advantage of the overexpression of anti-apoptotic and antioxidant proteins to improve cell survival. Another approach is to pursue large-scale expansion in bioreactors (19), induce enhanced stem cell trophic functions (20, 21) or encapsulate stem cells with biomaterials such as microcapsules (22-24). Increasing stem cell survival through various

strategies will promote long-term therapeutic efficacy.

Therefore, we focused on a protein up-regulated in cancer, stanniocalcin 2 (STC2). STC2 is highly expressed in hepatocellular carcinoma (25) and breast cancer (26) as well as in human tissues such as skeletal muscle, heart, and pancreas (27). STC2 also promotes anti-apoptotic and pro-proliferative action in cancer. We applied the protein to provide stem cells with the survival properties of cancer.

In the present study, we administered H₂O₂ to two types of stem cells, ADSCs and UCB-MSCs to mimic oxidative stress-induced cellular damage in ischemic sites *in vivo*. In the case of cancer, the expression of stanniocalcin family members is induced under oxidative condition. However, the levels of STC2 in stem cells were down-regulated under the same conditions (Fig. 1). Consequently, the cell viability and proliferation rate were reduced, leading to cell death. Cell damage was accelerated by oxidative stress. However, STC2 can promote recovery against cell damage under oxidative conditions. When STC2 is expressed in stem cells, cell viability and proliferation were increased after exposure to H₂O₂ at early and late time points, as shown in Fig. 2. These patterns were observed in another stem cell, UCB-MSC. The results indicate that STC2 exhibits pleiotropic effects by increasing the resistance of stem cells to microenvironmental damage. These facts were confirmed by measuring the live and dead cell populations in Fig. 2D. The major causes of the low therapeutic effects of stem cells *in vivo* are associated with immune rejection, anoikis, and oxidative damage-mediated apoptosis (3, 28-30). In our study, the live cell population was significantly reduced following treatment with H₂O₂ but was largely unaffected after treatment with STC2, suggesting that STC2 can promote cell survival under stressful circumstances.

The improved cell viability and proliferation may also be caused by increased cell cycle proteins. Cyclin-dependent kinases (CDKs) regulate the cell cycle in complex with their catalytic subunits (31). The activities of CDK2 and 4 are known to be restricted to the G1-S phase of the cell cycle and are essential for the G1/S transition (32, 33). Additionally, CDK2 or 4 are controlled by the CDK inhibitors p21Cip1 (CDKN1A), p27Kip1 (CDKN1B), and p16^{INK4a} (34, 35).

Based on the facts described above, we have evaluated the expression levels of cell cycle-related proteins because CDK2 and 4 as well as p16 and 21 are responsible for G1/S progression with cyclins. The levels of cell cycle proteins were up-regulated by STC2 despite H₂O₂ treatment, but the expression of the CDK inhibitor proteins, p16 and p21, were down-regulated (Fig. 3A and 3B). Hydrogen oxidase-treated cells were arrested in G1 or at G1/S phase compared with the non-treated cells. In addition to their function as CDK inhibitor proteins, p16 and p21 also exhibit roles in senescence and are known to be senescence markers. Cell senescence progressively increases with passage number, leading to cell death. Fig. 3B demonstrates low expression levels of p16 and p21 in STC2-expressing cells after H₂O₂ treatment, which may

indicate greater survival advantages in hazardous circumstance. Taken together, these results indicate that the expression of STC2 induces the up-regulation of stem cell cycle proteins and protects against oxidative stress, leading to increased proliferation.

A major reason for the clinical application of stem cell is their multi-pluripotency and self-renewal capacity (36). The long-term expression of pluripotency markers is essential for the improvement of the therapeutic efficacy (37, 38).

Thus, to verify these facts, we assessed whether Nanog and Oct4, representative pluripotency markers, were maintained by STC2 expression in stem cells under the conditions of cellular damage. In Fig. 3C, multipotency markers were significantly induced compared with those of the H₂O₂-treated group despite the harmful environment, indicating that prolonged marker expression may allow STC2-expressing stem cells to improve therapeutic efficacy and regenerative capacity in hazardous environments.

We accordingly evaluated the potential molecular mechanism by which these positive effects were observed upon STC2 expression. Fig. 4 demonstrates that the pERK1/2 and pAkt signal increased after STC2 expression despite H₂O₂ treatment compared with the control groups. Consequently, the enhanced cell survival and proliferation in response to STC2 expression may be caused by elevated activity of the ERK and Akt signaling pathways. Additionally, ERK expression levels are responsible for promoting the G1/S phase of the cell cycle. The expressed STC2 in ADSCs promoted cell cycle progression downstream of increased regulatory proteins (e.g., CDKs) under oxidative stress through ERK activation. Taken together, STC2 promoted enhanced up-regulation of the ERK and Akt pathway accompanied by increased cell survival and proliferative activity, supporting the potential application of STC2 in stem cell therapies.

To improve stem cell-based therapy, stem cells must overcome poor cell survival and the loss of multi-pluripotency during cell expansion before clinical trials as well as ischemic conditions at disease sites to increase therapeutic potency. In the present study, we used STC2 to improve the therapeutic function of stem cells by maintaining stemness factors and increasing cell survival and proliferative activity under oxidative stress-induced cellular damage. This research is the first report focused on the effects of STC2 on stem cells. Increased cell proliferation and survival were observed in ADSCs and UCB-MSCs expressing STC2 after exposure to H₂O₂. When STC2 was delivered into the cells, up-regulation of cell cycle regulator proteins and down-regulation of cell cycle inhibitors was induced relative to the treatment of the cells with H₂O₂ or control vector. Moreover, high expression of pluripotency markers was observed and maintained in the cells treated with STC2 despite oxidative conditions. High activation of ERK and Akt accompanied the improved positive effects on stem cells. Together, our results indicate that the expression of a paradoxical gene in stem cells may be a promising strategy to im-

prove multipotent capacity and to overcome the *in vivo* limitations for a variety of clinical applications, indicating that STC2 may induce the long-term therapeutic efficacy of stem cells.

MATERIALS AND METHODS

Cell lines and STC2 plasmid construct

Human adipose-derived mesenchymal stem cells (ADSC) and human mesenchymal stem cells isolated from umbilical cord blood (UCB-MSC) were kindly provided from EHL Biotechnology Institute and Dr. Kyung Sun Kang at Seoul National University in Seoul, Republic of Korea, respectively. The human UCB-MSC isolation procedure was approved by the Borame Hospital Institutional Review Board and Seoul National University (IRB No. 0603/001-002-07C1). ADSC, UCB-MSC cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Grand Island, NY) with 20% fetal bovine serum (FBS; Gibco-BRL), 10% for H460 and penicillin/streptomycin (Gibco-BRL). They were maintained at 37°C and 5% CO₂.

To create stanniocalcin2 (STC2) expressing pDNA, STC2 cDNA was amplified by PCR from human lung cancer H460 (purchased from ATCC). The forward and reverse primers for STC2 were 5'-GGATCCTACACACGGCTCGCCGAC-3' and 5'-CTCGAGTCACCTCCGGATATCAGA-3', respectively. The *Bam*HI and *Xho*I sites were introduced into the forward and reverse primers, respectively (the enzyme sites are underlined). The amplified STC2 was inserted into pGEM[®]-T Vector (Promega, Madison, WI), and then was digested with *Bam*HI and *Xho*I and then purified by electrophoretic elution from a 1% agarose gel. pcDNA/STC2 was constructed by insertion of the STC2 fragment into the site of pcDNA3.1 (Life technologies, Grand Island). Each cloning steps were confirmed by restriction enzyme digestion (data not shown). Most of contents of Materials and Methods were described in supplementary data.

ACKNOWLEDGEMENTS

This research was supported by grants from the National Research Foundation (NRF) funded by the Ministry of Science, ICT & Future Planning (2012M3A9C6049716) and Education (2013R1A1A2062949).

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