Steroid sulfatase (STS) is an enzyme responsible for the hydrolysis of aryl and alkyl sulfates. STS plays a pivotal role in the regulation of estrogens and androgens that promote the growth of hormone-dependent tumors, such as those of breast or prostate cancer. However, the molecular function of STS in tumor growth is still not clear. To elucidate the role of STS in cancer cell proliferation, we investigated whether STS is able to regulate the integrin signaling pathway. We found that overexpression of STS in HeLa cells increases the protein and mRNA levels of integrin β1 and fibronectin, a ligand of integrin α5β1. Dehydroepiandrosterone (DHEA), one of the main metabolites of STS, also increases mRNA and protein expression of integrin β1 and fibronectin. Further, STS expression and DHEA treatment enhanced phosphorylation of focal adhesion kinase (FAK) at the Tyr 925 residue. Moreover, increased phosphorylation of ERK at Thr 202 and Tyr 204 residues by STS indicates that STS activates the MAPK/ERK pathway. In conclusion, these results suggest that STS expression and DHEA treatment may enhance MAPK/ERK signaling through up-regulation of integrin β1 and activation of FAK.

Key Words: Steroid sulfatase, Dehydroepiandrosterone, Integrin β1, FAK, MAPK/ERK pathway
ular signaling pathways such as NF-κB (Lamb et al., 2011), Akt (Oloumi et al., 2004), and MAPK/ERK pathways (Lee et al., 2012), and induces cell growth, polarity, motility, and differentiation (Guo and Giancotti, 2004).

Irregular expression and activity of integrins are characteristic of cancer cells and contribute towards their growth, migration, and differentiation. For instance, integrin αvβ3 expression in metastatic breast cancer cells is higher than that in normal breast cells (Liapis et al., 1996), and promotes motility in breast cancer cells by interacting with MMP-9 (Rolli et al., 2003). Integrin α5β1 also confers invasiveness to breast tumors by regulating the activity of MMP-2 (Morozевич et al., 2009).

Furthermore, androgen receptor activation induces integrin α6β1 expression to promote prostate cell survival through activation of the NF-κB pathway and expression of Bcl-xL (Lamb et al., 2011).

Since integrin lacks intrinsic tyrosine kinase activity, it requires non-receptor tyrosine kinases, such as Src and focal adhesion kinase (FAK), to transduce extracellular cues to intracellular signaling pathways (Guo and Giancotti, 2004). A fibronectin-integrin complex recruits FAK to the focal contact area, which induces autophosphorylation of FAK at Tyr 739 residue. Next, the SH2 domain of Src binds to phosphorylated FAK, causing further phosphorylation of FAK at its Tyr 925 residue. Subsequently, the SH2 domain of Grb2 binds to phosphorylated Tyr 925 residue of FAK and forms a complex with SOS to activate Ras GTPase and induce the MAPK/ERK signaling pathway (Schlaepfer et al., 1994; Schlaepfer and Hunter, 1996). Activation of this signaling pathway contributes to cell proliferation, migration, and invasion of cancer cells (Wang et al., 2005; Cheung et al., 2008; Meng et al., 2009).

Expression of integrin α5β1 and its ligand fibronectin was found to be induced by estrogen and progesterone in normal mouse mammary glands (Woodward et al., 2001). In addition, expression of integrin α5 and β1 is promoted by 17β-estradiol through activation of ER-α (Cid et al., 1999; Sisci et al., 2010). Various tissues of female reproductive organs, such as ovarian tissue, have relatively high STS activity (Haning et al., 1993). The growth and proliferation of these organs are significantly increased by activation of ER (Park et al., 2008; Ahn et al., 2014; Kim et al., 2015). STS may support tumor growth of female reproductive tracts as well as breast and prostate tissues (Yamamoto et al., 1993). For example, malignant endometrial tumor tissues have 12-fold higher STS activity compared to normal endometrial tissues (Yamamoto et al., 1993). Moreover, urine samples from patients with cervical cancer contain higher amounts of DHEA compared to those from healthy females (Lee et al., 2003). Fournier and Poirier (2009) also reported a strong activity of STS in HeLa cells, a human cervical cancer line. Since STS is the major enzyme used to increase 17β-estradiol levels in female reproductive organs, we hypothesized that STS activates integrin-mediated signaling. Therefore, STS may play an important role in growth of human cervical cancer cells by enhancing the expression of integrin α5β1 and fibronectin by production of DHEA, a precursor for 17β-estradiol.

### Table 1. The sequences of the PCR primers used in this study

| Target gene | Sequence                  |
|-------------|---------------------------|
| STS         | 5’-CCCTCTAGTCTCTCTTCTGTGGG-3’ |
| Anti-sense  | 5’-GGTCAATGGGGAAGCCTGTA-3’ |
| Integrin α5 | 5’-GCTGTTGAGTACAAGTCTCTT-3’ |
| Anti-sense  | 5’-AATTCCGGTGAAGTTATCTGTG-3’ |
| Integrin β1 | 5’-CGATGTGGTGTTACGATGAC-3’  |
| Anti-sense  | 5’-CAAGATCTAGGGTTTCATGCTG-3’ |
| Fibronectin | 5’-GTGGGGCAACTCTGTC-3’      |
| Anti-sense  | 5’-TCGAGGCAAGTTCCAGTG-3’    |
| GAPDH       | 5’-TGACGGGAAGTTCAGTG-3’     |
| Anti-sense  | 5’-TCCACCACCCCTGTTGCTGA-3’  |

### MATERIALS AND METHODS

#### Reagents

DHEA and DHEAS were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-STS antibody was purchased from Abcam (Cambridge, MA, USA). Antibodies against cyclin D1, ERK-1, GAPDH, integrin β1, MEK-1, phospho-Akt 1/2/3 (Ser 473), and phospho-MEK 1/2 (Ser 218/Ser 222) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against FAK, phospho-FAK (Tyr 925), and phospho-p44/42 MAPK (Thr202/Tyr204) were purchased from Cell Signaling Technology (Beverly, MA, USA). HRP-conjugated goat anti-rabbit IgG was obtained from Bethyl (Montgomery, TX, USA). Enhanced chemiluminescence (ECL) detection reagent was purchased from Thermo Scientific (Rockford, IL, USA). Ex Taq polymerase was purchased from TaKaRa Bio (Shiga, Japan). SYBR® Green PCR Master Mix was purchased from QIAGEN (Hilden, Germany).

#### Cell culture

HeLa cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were grown in MEM/EBSS medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. For treatment of HeLa cells with DHEA, 1×10⁶ cells were seeded in MEM/EBSS medium supplemented with 10% FBS as a monolayer on to 100-mm dish plates and cultured under standard incubation (37°C in a humidified atmosphere with 5% CO2). Twenty-four hours after seeding, the growth media was changed to MEM/EBSS medium supplemented with 10% charcoal-stripped FBS for 24 h and the samples underwent serum starvation in serum-free MEM/EBSS medium for 24 h. Subsequently, cells were treated with the designated concentrations of DHEA for 24 h.

#### Transient transfection of plasmid DNA

STS overexpression vector pcDNA 3.1/Zeo including STS-encoding sequence was used in transfection. HeLa cells (1×10⁶) were transfected with 2 μg of plasmid DNA, using the Neon™ transfection system (Invitrogen, Carlsbad, CA, USA), and cultured in 100-mm dishes in antibiotic-free MEM/EBSS media with 10% FBS for 48 h.

#### RT-PCR and qRT-PCR

Total RNA was extracted using Ribospin™ (GeneALL, Seoul, Korea). Total RNA (1000 ng) was reverse transcribed...
at 37°C for 1 h in 25 μl total volume containing 5X RT buffer, 10 mM dNTPs, 40 U RNase inhibitor, 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase, and 100 pmole of oligo-dT primer. Reaction mixtures (0.8 μl) from each sample were amplified with 10 pmole of each oligonucleotide primers, 0.2 mM dNTPs, 1.5 mM MgCl₂, and 1.25 U of Ex Taq polymerase. Amplification was conducted as follows: one cycle at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 10 sec, annealing at 58°C for 15 sec, and extension at 72°C for 15 sec. Primer sequences are listed in Table 1. PCR products were run on a 2% (w/v) agarose gel by gel electrophoresis, and visualized with ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). Quantitative RT-PCR (qRT-PCR) was conducted using the Rotor-Gene SYBR® PCR Kit (QIAGEN), following the manufacturer’s instructions, and analyzed using QIAGEN Rotor-Gene Q Series software. Each reaction included 10 μl of 2X SYBR® Green PCR Master Mix, 2 μM oligonucleotide primers for specific target gene, and 2 μl of cDNA in a final volume of 20 μl. Amplification was performed as follows: one cycle at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 5 sec, and annealing and extension at 58°C for 10 sec.

**Western blot**

Whole cells were harvested and lysed in 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 1% nonidet P-40, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin for 30 min at 4°C followed by centrifugation at 22000×g for 15 min at 4°C. Protein concentrations were measured using BCA Protein Assay Reagents (Thermo). Extracted cellular proteins (20 μg) were separated on 10% SDS-PAGE at 100 V and electrophoretically transferred onto 0.45 μm PVDF membrane. Nonspecific binding was blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% tween-20 (TBS-T) for 2 h at 4°C, and

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*Fig. 1.* STS overexpression increases the expression of integrin β1 in HeLa cells. (A) Cells were transfected with pcDNA3.1-STS for 24 h. Total RNA was isolated, and STS, integrin α5, and integrin β1 genes were individually amplified with specific primers. Expression of GAPDH mRNA was determined as an RNA control. Real-time PCR was performed in triplicate. The data represents the mean ± SD. *p<0.05. (B) Cells were transfected with pcDNA3.1-STS for 24 h. Total cellular lysates were prepared and used for western blot analyses with antibodies against STS, integrin α5, or integrin β1. GAPDH was used as a loading control. Cellular lysates were loaded in duplicate on each blot. (C) Confocal microscopic analysis of HeLa cells transfected with pcDNA3.1-STS for 24 h. Scale bar: 20 μm.
then incubated overnight with specific primary antibody at a 1:1000 dilution in TBS-T. Horseradish peroxidase (HRP)-conjugated secondary antibody was incubated at 4°C for 2 h. Proteins were visualized with ECL (Thermo) and the band intensity was measured using ChemiDoc XRS densitometer and quantified by Quantity One software (Bio-Rad).

**Immunofluorescence**

Cells were grown on coverslips and rapidly washed with PBS after incubation with chemicals for the designated times (24 h) and fixed with 3.7% (w/v) paraformaldehyde for 30 min at room temperature. After washing with PBS, the cells were blocked for 30 min in PBS containing 5% goat serum and 0.2% Triton X-100, and then incubated with specific primary antibodies overnight. Next, the cells were washed extensively and stained with Texas Red-conjugated goat anti-rabbit IgG (1:500) for 2 h. After additional washes, the coverslips were mounted onto glass slides, using 3 μl of UltraCruz™ Mounting Medium containing DAPI. Fluorescence signals were analyzed using an LSM700 Confocal Laser Scanning Microscope (Carl Zeiss, Jena, Germany).

**Cell viability assay**

Cells (7×10^3 cells /well) were added onto a 96-well microplate, and were incubated for the specified times at 37°C. After treatment with chemicals for the designated time, the cells were treated with 10 μl of EZ-CyTox (Daeil Lab Service, Seoul, Korea) for 30 min. The absorbance was measured at 450 nm using a GENios Pro microplate reader (TECAN, Männedorf, Switzerland).

**Statistical analysis**

One-way analysis of variance and statistical analysis were performed followed by Dunnett’s Multiple Comparison t-test using Graph-Pad Prism Software (GraphPad Software Inc., San Diego, CA, USA) when appropriate. The difference was considered statistically significant at p<0.05.

**RESULTS**

**STS increases the expression of integrin β1 in HeLa cells**

STS may be involved in tumor growth by producing precursors for estrogen synthesis (Reed et al., 2005). Previously, 17β-estradiol was found to induce expression of integrin α5 and β1 (Woodward et al., 2001). To investigate the ways in which STS expression plays a critical role in tumor growth, we examined whether STS promotes integrin α5 and β1 expression. When HeLa cells were transfected with pcDNA 3.1-STS plasmid, mRNA levels and protein levels of integrin β1

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**Fig. 2.** DHEA treatment induces the expression of integrin β1 in HeLa cells. (A) Cells were treated with DHEA (100 nM) for 24 h. Total RNA was isolated, and integrin β1 genes were amplified with specific primers. Expression of GAPDH mRNA was determined as an RNA control. Real-time PCR was performed in triplicate. The data represents the mean ± SD. *p<0.05. (B) Western blot analyses. Cells were treated with DHEA (100 nM) for 24 h. Total cellular lysates were prepared and used for western blot analyses with antibodies against integrin β1. GAPDH was used as a loading control. Cellular lysates were loaded in duplicate on each blot. (C) Confocal microscopic analysis of HeLa cells treated with DHEA (100 nM) for 24 h. Scale bar: 20 μm.
increased, however, neither mRNA nor protein levels of integrin α5 increased (Fig. 1A, 1B). Confocal microscopic analysis also showed that overexpression of STS strongly induces integrin β1 expression (Fig. 1C).

**DHEA induces the expression of integrin β1 in HeLa cells**

To elucidate the method by which STS induces integrin β1 expression, cells were treated with DHEA, a major metabolic product of STS, and we examined the mRNA and protein levels of integrin β1. Cells were cultured in serum free medium for 24 h prior to DHEA treatment for serum starvation to maximize the effect of DHEA. After serum starvation was completed, HeLa cells were treated with 100 nM of DHEA for 24 h. We found that DHEA effectively increased both mRNA (Fig. 2A) and protein levels (Fig. 2B) of integrin β1 in HeLa cells. Focal microscopic analysis also showed that treatment with DHEA induced integrin β1 expression (Fig. 2C). These data indicate that the activity of STS may play an important role in STS-induced integrin β1 expression.

**Increase of fibronectin expression by STS**

The ECM protein fibronectin is a specific ligand for the integrin α5β1 and induces tumor growth (Pankov and Yamada, 2002). Since fibronectin participates in integrin signaling and plays a crucial role in tumor growth, we examined the effects of STS and DHEA on fibronectin expression levels. When STS was overexpressed in HeLa cells, the mRNA level of fibronectin increased (Fig. 3A). Similarly, mRNA levels of fibronectin significantly increased in cells treated with DHEA (100 nM) (Fig. 3B). These results indicate that STS and DHEA promote...
Activation of integrin signaling by up-regulation of integrin β1 and fibronectin.

**STS or DHEA induces the MAPK/ERK pathway, but not the Akt pathway in HeLa cells**

To investigate which intracellular signaling pathway is up-regulated by STS and DHEA, we examined the activity of the MAPK/ERK and Akt pathways. Notably, when cells were over-expressed with STS or treated with DHEA, MEK phosphorylation at Ser 218 and Ser 222 residues and ERK phosphorylation at Thr 202 and Tyr 204 increased significantly (Fig. 4A, 4B). In addition, STS overexpression and DHEA treatment caused an increase in expression of c-fos, a major transcriptional target of the MAPK/ERK pathway (Muller et al., 1997) (Fig. 4A, 4B). However, neither STS overexpression nor DHEA treatment induced phosphorylation of Akt at Ser 473, indicating that the Akt pathway was not activated (Fig. 4C, 4D). Consistent with this finding, the expression levels of cyclin D1, a major target of Akt signaling (Gera et al., 2004; Yang et al., 2007), were not affected by STS or DHEA (Fig. 4C, 4D).

**Activation of FAK plays an important role in STS-mediated MAPK/ERK signaling**

Since integrin has no intrinsic catalytic activity, it may relay extracellular cues to intracellular signaling pathways through the phosphorylation of Src or FAK. When Src and FAK are recruited to the intracellular domain of integrin, they are phosphorylated at Tyr 416 or Tyr 925 residues, respectively, and phosphorylated FAK causes the activation of the MAPK/ERK pathway (Lee et al., 2012). To elucidate the association between STS-induced integrin β1/fibronectin up-regulation and activation of the MAPK/ERK pathway, we examined phosphorylation of FAK at Tyr 925. When HeLa cells were over-expressed with STS or treated with DHEA, phosphorylation of FAK at Tyr 925 increased (Fig. 5). These results indicate that STS activates the MAPK/ERK pathway through FAK phosphorylation, which is promoted by integrin β1/fibronectin up-regulation.

**STS or DHEA increases the cell viability of HeLa cells**

Since STS or DHEA is able to activate the MAPK/ERK pathway, we examined cell viability of HeLa cells following STS overexpression or DHEA treatment. We measured an approximately 2-fold increase in cell viability following STS overexpression (Fig. 6A). Similarly, cell viability of DHEA-treated cells also increased. In contrast, DHEAS had no detectable effect on cell proliferation (Fig. 6B). These results indicate that STS mediates cell survival through activation of integrin signaling.

**DISCUSSION**

STS hydrolyzes alkyl or aryl steroid sulfates to their free form. For instance, STS hydrolyzes DHEAS to its unconjugated form, DHEA. Since the enzymatic activity of STS can catalyze the synthesis of precursors for steroids such as 17β-estradiol or progesterone, STS contributes to growth of hormone-dependent tumors like that of breast cancer (Reed et al., 2005). A previous study found that hormone-dependent breast tumors have higher STS mRNA expression and enzymatic activity than normal breast tissue (Utsumi et al., 2000). Moreover, other studies found abnormal expression and activity of STS in ER-positive breast tumors, indicating that increased STS expression is closely associated with poor prognosis (Miyoshi et al., 2003), and length of time before relapse (Evans et al., 1993, 1994). Furthermore, Sato et al. (2009) showed that high expression of STS in colon cancer induces strong intratumoral estrogen synthesis and suggested STS expression as a useful prognostic factor for colon cancer. Thus, we can infer that STS largely contributes to tumor growth by regulating endogenous steroid synthesis. Although the function of STS has been well studied, the detailed molecular mechanism by which STS promotes proliferation of cancer cells is still not clear.

In this study, we found that STS and DHEA induced integrin β1 expression in HeLa cells. Integrin α5β1 heterodimer plays a crucial role in tumor growth (Lahlou and Muller, 2011). We found that STS and DHEA induced fibronectin expression, an ECM ligand for the integrin α5β1 (Pankov and Yamada, 2002). In many studies, interaction between integrin α5β1 and fibronectin has significant effects on tumor growth. For instance,
this interaction can activate MMP-1, conferring invasiveness to the breast cancer tissue (Jia et al., 2004). Fibronectin and integrin α5β1 binding can activate various cellular signaling pathways important for cell growth, including the MAPK/ERK pathway (Wilson et al., 2003) and Akt pathway (Nam et al., 2010).

Towards understanding the effect of STS- and DHEA-induced up-regulation of integrin β1 and fibronectin, we found that both STS expression and DHEA treatment were able to induce phosphorylation of ERK and expression of c-fos protein, a well-known transcriptional target of MAPK/ERK pathway (Muller et al., 1997). These results indicate that STS activates the MAPK/ERK pathway through its enzymatic function. We also found that STS expression and DHEA treatment increased the phosphorylation of FAK, which serves as a mediator between integrin and the MAPK/ERK pathway. When FAK is recruited to the cytoplasmic domain of an activate integrin/fibronectin complex, FAK is activated through auto-phosphorylation at its Tyr 397 residue. The SH2 domain of Src binds to activated FAK, causing phosphorylation of the Tyr 925 residue on FAK, which consequently facilitates binding of the SH2 domain of adaptor protein Grb2 to form the FAK-Grb2-SOS complex. Ultimately, this activates the MAPK/ERK pathway through Ras GTPase activation (Schlaepfer and Hunter, 1996; Lahlou and Muller, 2011). Thus, our results indicate that STS expression and DHEA treatment promotes formation of the FAK-Grb2-SOS complex by increasing phosphorylation of FAK at Tyr 925.

In summary, our results indicate that STS enhances HeLa cell proliferation by inducing the expression of integrin β1 and its specific ligand fibronectin, which in turn enhances phosphorylation of FAK and ultimately activates the MAPK/ERK pathway. Moreover, we conclude that the enzymatic function of STS is crucial for integrin signaling because DHEA treatment was sufficient to enhance expression of integrin β1 and activate the MAPK/ERK pathway.

Further studies need to be conducted to elucidate the specific downstream signaling of STS that induces the expression of integrin β1 and fibronectin. In previous studies, urokinase-type plasminogen activator receptor (uPAR), which mediates plasminogen activation through binding with the urokinase-type plasminogen activator, was reported to be closely related with integrin α5β1 (Noh et al., 2013). Since uPAR enhances fibronectin binding to integrin α5β1 (Monaghan et al., 2004), we expect that future studies will show that STS-induced integrin signaling can be mediated through uPAR-related signaling.

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