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Cripto Enhances Proliferation and Survival of Mesenchymal Stem Cells by Up-Regulating JAK2/STAT3 Pathway in a GRP78-Dependent Manner

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Abstract

Cripto is a small glycosylphosphatidylinositol-anchored signaling protein that can detach from the anchored membrane and stimulate proliferation, migration, differentiation, vascularization, and angiogenesis. In the present study, we demonstrated that Cripto positively affected proliferation and survival of mesenchymal stem cells (MSCs) without affecting multipotency. Cripto also increased expression of phosphorylated janus kinase 2 (p-JAK2), phosphorylated signal transducer and activator of transcription 3 (p-STAT3), 78 kDa glucose-regulated protein (GRP78), c-Myc, and cyclin D1. Notably, treatment with an anti-GRP78 antibody blocked these effects. In addition, pretreatment with STAT3 short interfering RNA (siRNA) inhibited the increase in p-JAK2, c-Myc, cyclin D1, and BCL3 levels caused by Cripto and attenuated the pro-survival action of Cripto on MSCs. We also found that incubation with Cripto protected MSCs from apoptosis caused by hypoxia or H_2O_2 exposure, and the level of caspase-3 decreased by the Cripto-induced expression of B-cell lymphoma 3-encoded protein (BCL3). These effects were sensitive to down-regulation of BCL3 expression by BCL3 siRNA. Finally, we showed that Cripto enhanced expression levels of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF). In summary, our results demonstrated that Cripto activated a novel biochemical cascade that potentiated MSC proliferation and survival. This cascade relied on phosphorylation of JAK2 and STAT3 and was regulated by GRP78. Our findings may facilitate clinical applications of MSCs, as these cells may benefit from positive effects of Cripto on their survival and biological properties.

Key Words: Cripto, Mesenchymal stem cell, Bioactivity, GRP78, JAK2/STAT3

INTRODUCTION

Adult mesenchymal stem cells (MSCs) are multipotent cells able to differentiate into several types of specialized mesenchymal cells, such as osteoblasts, chondrocytes, adipocytes, tenocytes, and others (Caplan, 1991). Human MSCs possess special self-renewal capacity, making them a suitable cell source for potential cell therapy and regeneration strategies (da Silva Meirelles *et al*., 2009). Specifically, in *ex vivo* culturing conditions, differentiation of MSCs into specific cell types can be guided by applying appropriate growth factors or chemicals (Pittenger *et al*., 1999). However, precise understanding of the regulatory mechanisms that mediate various biological events, including proliferation, targeted migration, and differentiation, as well as availability of high-quality input material are needed to utilize human MSCs safely in the clinical setting. Traditionally, MSCs are isolated from tissues such as umbilical cord, endometrial polyps, menses blood, bone marrow, and adipose tissue, because it is easy to harvest MSCs from such preparations and sufficient quantities of cells can be reliably obtained for clinical and experimental applications (Ding *et al*., 2006, 2007). In contrast, *de novo* reproduction of MSCs has been challenging because only a small number of cells could be

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produced in practice. Thus, appropriate knowledge that would allow reliable manipulation of MSC reproduction would be a significant breakthrough in the clinical application of MSCs.

Cripto is a small glycosylphosphatidylinositol-anchored signaling protein that regulates cell survival, proliferation, differentiation, and migration during development upon its release from the membrane to which it is anchored (Kohlmeier *et al*., 1992; Bianco *et al*., 2002; Minchiotti, 2005; Shen, 2007; Andersson *et al*., 2008; Papageorgiou *et al*., 2009; Gray and Vale, 2012). Previous studies have suggested that Cripto also facilitates epithelial-mesenchymal stem cell transition, promotes cell proliferation, and enhances reconstitution capacity (Klauzinska *et al*., 2014; Spike *et al*., 2014). As Cripto plays a critical role in regulating stem cell functions, it has been identified as a potential candidate for manipulations aimed to stimulate proliferation of MSCs. In our study, we aimed to assess the effect of Cripto and its recently characterized molecular pathway on cell proliferation. Expression levels of GRP78, a Cripto binding protein, are known to increase in response to cellular stress. Up-regulation of GRP78 leads to enhanced cytoprotection and chemoresistance of cells in the tumor microenvironment (Li and Lee, 2006), promoting cell proliferation, survival, and resistance to apoptosis (Misra *et al*., 2011; Li *et al*., 2013; Zhang *et al*., 2013). Previous studies also demonstrated that GRP78 was required for the proliferation and survival of embryonic inner cell mass cells that are precursors of pluripotent stem cells (Luo *et al*., 2006). In addition, it has been suggested that in response to GRP78 activation, components of the JAK2/STAT3 pathway become phosphorylated and induce the expression of various factors related to cell proliferation, vascularization, and angiogenesis (Lee *et al*., 2015a). Thus, in our study we sought to examine the precise mechanism by which GRP78 and JAK2/STAT3 affect the Cripto pathway. We aimed to study the effects of Cripto on MSC activity and molecular signaling mechanisms mediating observed positive effect of Cripto on MSC proliferation.

MATERIALS AND METHODS

Cell culture

MSCs derived from human adipose tissue were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). MSCs were confirmed to be pathogen- and mycoplasma-free; they expressed cell surface markers, such as cluster of differentiation (CD) 73 and CD105, but not CD31, and exhibited adipogenic and osteogenic differentiation potential when cultured in specific differentiation media. MSCs were cultured in α-minimum essential medium (Hyclone, Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (Hyclone), 100 U/mL penicillin, and 100 μg/mL streptomycin. MSC cultures were grown in a humidified incubator in the atmosphere of 95% air and 5% CO_2 at 37°C.

MSC differentiation

For MSC differentiation, cells were grown in StemPro adipogenic, osteogenic, or chondrogenic culture medium (Thermo Fisher Scientific, Waltham, MA, USA). Cells were grown in the adipogenic medium for 1 week, osteogenic medium for 1 weeks, and chondrogenic medium for 2 weeks. Adipocytes were stained with oil red O (Sigma-Aldrich, St. Louis, MO, USA) for 10 min, osteoblasts were stained with alkaline phosphatase stain kit (Sigma-Aldrich) for 10 min, and chondrocytes were stained with Safranin O (Sigma-Aldrich) for 5 min. The samples were visualized by inverted microscopy (Nikon, Tokyo, Japan).

Cell proliferation assay

Cell proliferation was examined by 5-bromo-2′-deoxyuridine (BrdU) incorporation assay. MSCs were cultured in 96-well cultured plates (3,000 cells/well). MSCs were exposed to Cripto (0, 1, 10, 50, 100, 200 ng/mL) for a fixed period of 24 h or to 100 ng/mL Cripto for 12, 24, or 48 h. BrdU incorporation into newly synthesized DNA of proliferating cells was assessed by an enzyme-linked immunosorbent assay (ELISA) colorimetric kit (Roche, Germany). To perform ELISA, 100 μg/mL BrdU was added to MSC cultures and incubated at 37°C for 3 h. An anti-BrdU antibody (100 μL) was added to MSC cultures and incubated at room temperature for 90 min. Then, 100 μL of substrate solution was added and 1 M H_2SO_4 was used to stop the reaction. Light absorbance of the samples was measured by a microplate reader (BMG labtech) at 450 nm.

Single-cell cultivation assay

MSCs were trypsinized to prepare single cell suspensions in growth media. A limiting dilution assay was used to aliquot single MSCs into individual wells of 96-well culture plates. Briefly, cell suspensions containing 1×10^3 cells in 10 mL of growth media were diluted tenfold, and 100 μL of the diluted sample (approximately 1 cell/100 μL) was seeded into 96-well plates. Control MSCs and MSCs transfected with STAT3 short interfering RNA (siRNA) in the presence or absence of 100 ng/mL Cripto were then cultured in a humidified incubator for 10 days.

siRNA transfection

MSCs were grown up to 70% confluence in 60-mm cultured plates and washed twice with phosphate buffered saline (PBS). MSCs were transfected for 48 h with SMART pool siRNAs (100 nM) specific for STAT3 or BCL3 mRNA or scramble siRNA by using Lipofectamine 2000 reagent (Thermo Fisher Scientific) in serum-free αMEM media according to the manufacturer's protocols and then were treated with 100 ng/mL Cripto.

Propidium iodide/annexin V flow cytometry analysis

The proportion of apoptotic cells was determined by flow cytometry analysis. After MSCs were treated with 200 μM H_2O_2 for 6 h or exposed to hypoxic (2% O_2, 5% CO_2, and 93% N_2) conditions at 37°C for 96 h in the presence or absence of Cripto and BCL3 siRNA, the cells were stained with annexin V-FITC and propidium iodide (PI) (FITC Annexin V Apoptosis Detection Kit; BD Pharmingen, Franklin Lakes, NJ, USA), and evaluated using a Cyflow Cube 8 FACS instrument (SysmexPartec, Görlitz, Germany). Data were analyzed using standard FSC Express software (De Novo Software, Los Angeles, CA, USA).

Western blot analysis

Total protein was extracted from MSCs using RIPA lysis buffer (Thermo Fisher Scientific). Cell lysates (20 μg protein) in sample buffer were separated by electrophoresis in 8-12% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose membranes for probing with antibodies. After washing with Tris-buffered saline/Tween-20 buffer (0.05% Tween-20, 150 mM NaCl, 10 mM Tris–HCl; pH 7.6), membranes were blocked with 5% bovine serum albumin for 1 h at room temperature and then incubated with primary antibodies

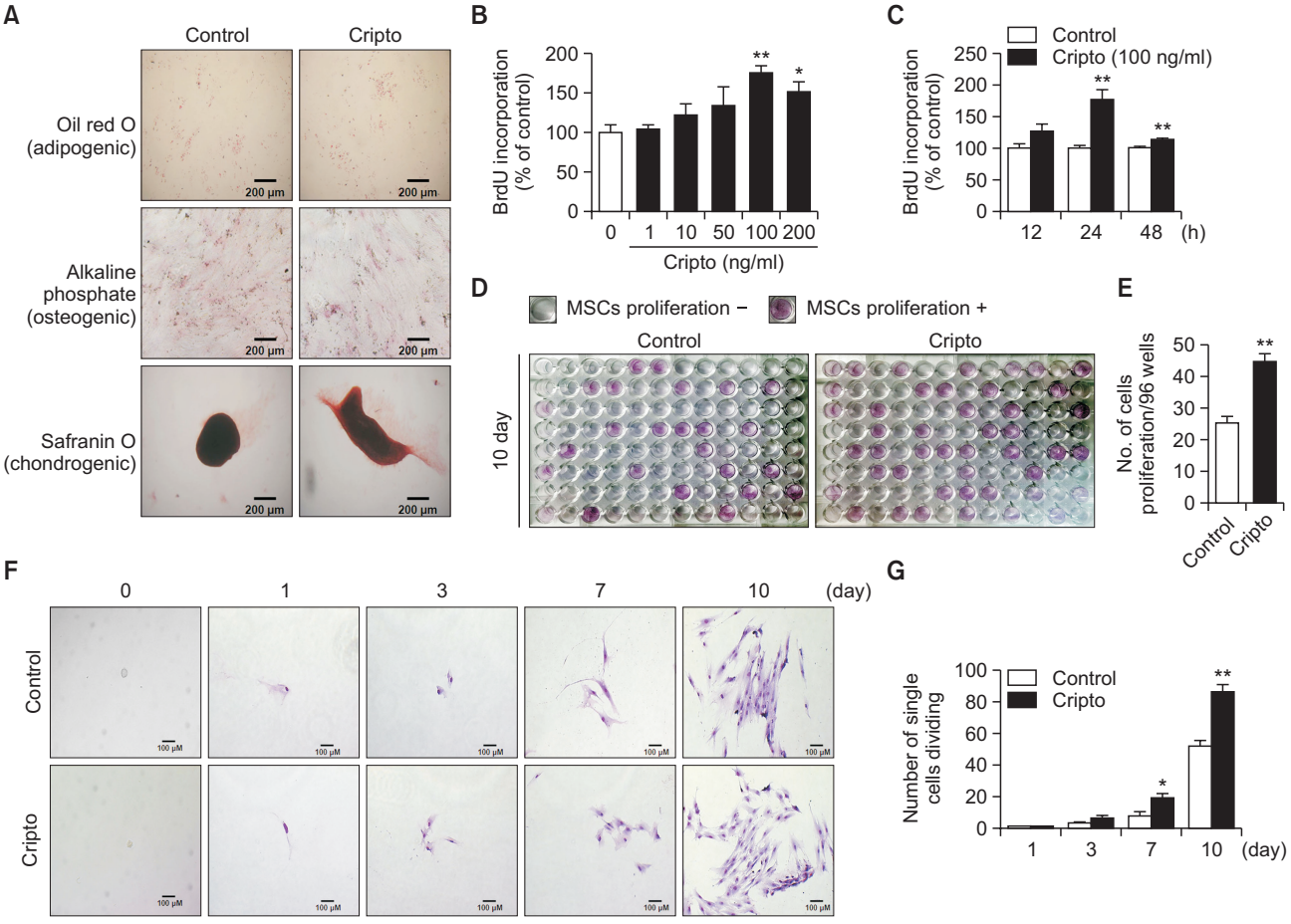

Fig. 1. Effect of Cripto on MSC differentiation and proliferation. (A) MSCs were differentiated into adipocytes, osteocytes, and chondrocytes in the presence of Cripto, and examined by Oil Red O, alkaline phosphatase, and Safranin O staining, respectively. Scale bar=200 μm. Concentration- (B) and time- dependent (C) effects of Cripto on BrdU incorporation are illustrated. MSCs were incubated with Cripto at different concentrations (0-200 ng/mL) for 24 h or with 100 ng/mL Cripto for variable time periods (0–48 h) and then, BrdU incorporation was examined using light absorbance measurements (n=3). (D) Representative image of a 96-well culture plate of a single cell assay with proliferating MSCs stained with Giemsa stain. MSCs were treated with 100 ng/mL Cripto for ten days. (E) Plot of the number of proliferating cells per 96-well plate (n=3). (F) Representative image of a single cell assay for 1 to 10 days in 96-well plate stained with Giemsa stain. Scale bar=100 μm. (G) The number of cells per field of view in each well of a 96-well plate is plotted. The number of dividing cells was significantly higher in MSCs exposed to Cripto than in MSCs treated with vehicle (n=3). Data are expressed as the mean ± SEM of three independent experiments each performed in triplicate dishes. ANOVA followed by post hoc Bonferroni-Dunn test was used for multiple group comparisons. *p<0.05, **p<0.01 vs. control.

against phosphorylated JAK2, STAT3, phosphorylated STAT3, c-Myc, cyclin D1, GRP78, BCL3, cleaved caspase-3, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and β-actin (all from Santa Cruz Biotechnology, Dallas, TX, USA). After incubation of the membranes with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), bands were detected using enhanced chemiluminescence reagents (Amersham Biosciences, Little Chalfont, UK) in a dark room.

Detection of human growth factors

Concentrations of VEGF, FGF, and HGF in MSC lysates were determined using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendations. MSC lysates were quantified using the bicinchoninic acid assay (Thermo Fisher Scientific). Three hundred micrograms of total protein from MSC lysates was used for these experiments. Triplicate measurements were performed for all ELISA assays. Expression levels of growth factors were quantified by measuring absorbance at 450 nm using a microplate reader (BMG Labtech, Ortenberg, Germany).

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). All experiments were analyzed by one-way analysis of variance (ANOVA). If a significant effect of treatment was revealed by one way ANOVA in comparisons involving ≥ 3 groups, Bonferroni-Dunn *post hoc* tests were used to reveal inter-group differences. Differences were considered statistically significant if p<0.05.

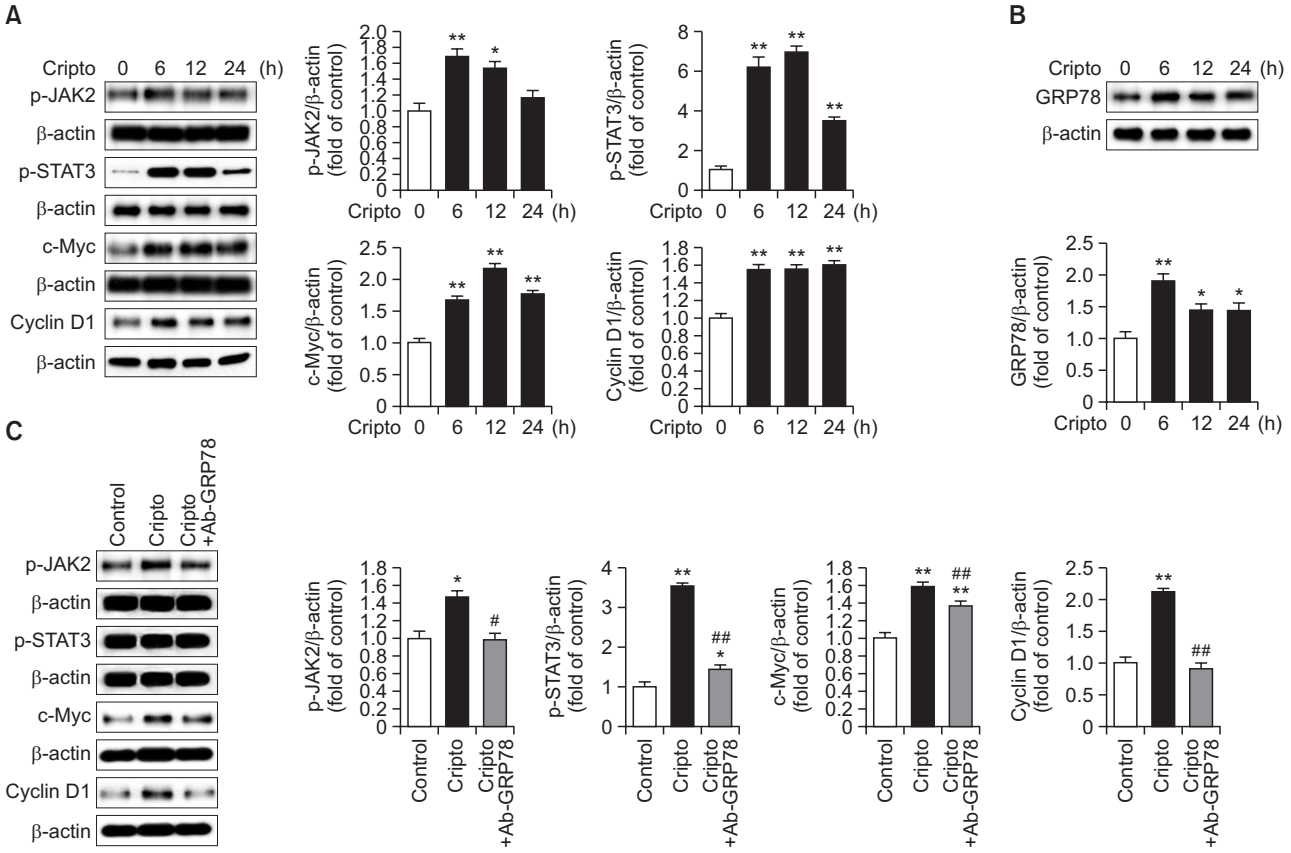

Fig. 2. Effects of Cripto on the expression levels of cell cycle regulatory proteins and phosphorylation of JAK2 and STAT3 are dependent on GRP78. MSCs were treated with 100 ng/mL Cripto for 0–24 h. (A) Total protein was extracted and immunoblotted with antibodies against phosphorylated JAK2, phosphorylated STAT3, c-Myc, and cyclin D1. Amounts of β-actin were used as internal loading controls. Panel on the right illustrates mean normalized levels (± SEM) of phosphorylated JAK2, phosphorylated STAT3, c-Myc, and cyclin D1 (n=3). Statistical significance of differences is indicated as follows: *p<0.05, **p<0.01 vs. control. (B) Total protein was extracted and immunoblotted with antibodies against GRP78. Amounts of β-actin were used as internal loading controls. Panel on the right illustrates mean normalized levels (± SEM) of GRP78 (n=3). Statistical significance of differences is indicated as follows: *p<0.05, **p<0.01 vs. control. (C) MSCs were pretreated with a neutralizing antibody against GRP78 (100 ng/mL) for 24 h before the treatment with 100 ng/mL Cripto for another 24 h. Total protein was extracted and immunoblotted with antibodies against phosphorylated JAK2, phosphorylated STAT3, c-Myc, and cyclin D1. Amounts of β-actin were used as internal loading controls. Panel on the right illustrates mean normalized levels (± SEM) of phosphorylated JAK2, phosphorylated STAT3, c-Myc, and cyclin D1 (n=3). Statistical significance of differences is indicated as follows: *p<0.05, **p<0.01 vs. control, $^{\#}p$<0.05, $^{\#\#}p$<0.01 vs. Cripto alone.

RESULTS

Effect of Cripto on MSC differentiation and proliferation

There was no significant difference in MSC differentiation when cells were cultured with Cripto, as determined by Oil Red O (adipogenesis), alkaline phosphatase (osteogenesis), and Safranin O (chondrogenesis) staining (Fig. 1A). MSCs were incubated with Cripto at different concentrations (0-200 ng/mL) for 24 h or with 100 ng/mL Cripto for varying time periods (0-48 h) to reveal concentration- and time dependent effects of this protein on cell proliferation. As shown in Fig. 1B and 1C, the maximum increase in the level of BrdU incorporation was observed after 24-h incubation with 100 ng/mL Cripto. To assess the effects of Cripto on the division of individual cells, we first seeded individual MSCs onto a culture plate, and then confirmed cell division in a time-dependent manner for 0-10 days. To assess the effects of Cripto on MSC viability and proliferation, the Giemsa stain was used. As shown in Fig. 1D and 1E, treatment with 100 ng/mL Cripto increased cell proliferation, with the number of single cell divisions increasing after seven days of incubation with Cripto (Fig. 1F, 1G). These results suggested Cripto has a strong potentiating effect on MSC proliferation.

Involvement of GRP78-dependent JAK/STAT3 pathway activation and cell cycle regulatory protein expression in MSC proliferation

To assess the role of GRP78 and related pathways in the potentiation of MSC proliferation by Cripto, we examined whether Cripto induced the expression of cell cycle regulatory proteins, and GRP78 in MSCs. As seen in Fig. 2A, 100 ng/mL Cripto significantly increased the levels of phosphorylated JAK, phosphorylated STAT3, c-Myc, and cyclin D1. In addition, Cripto significantly increased the expression of GRP78 (Fig. 2B). However, the levels of phosphorylated JAK, phosphorylated STAT3, cyclin D1, and c-Myc decreased significantly following pretreatment with a GRP78 neutralizing antibody (Fig. 2C). These results showed that Cripto-mediated

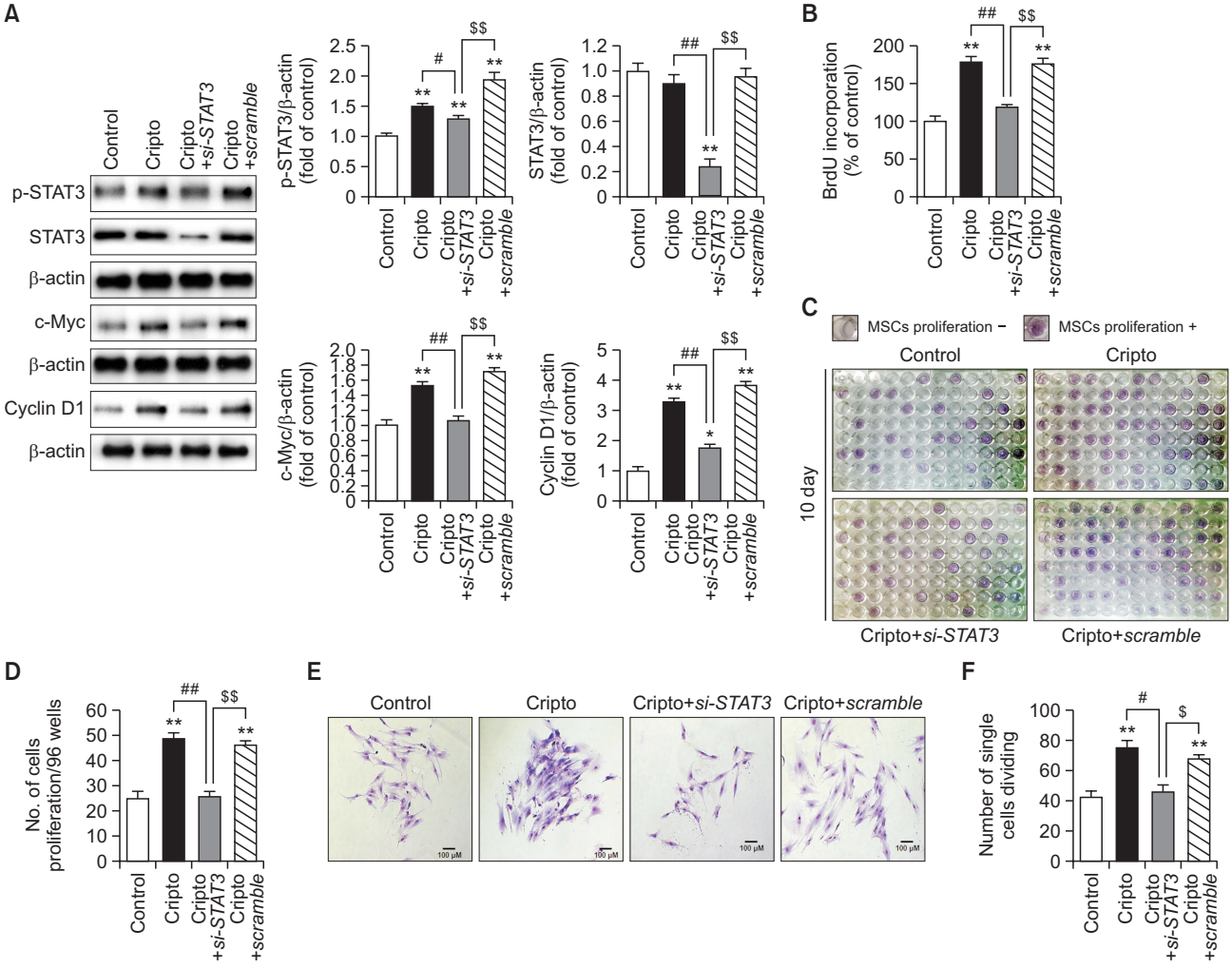

Fig. 3. Modulation of MSC proliferation by Cripto is dependent on STAT3. MSCs were pre-transfected with STAT3 siRNA or non-targeting scrambled siRNA for 24 h before the treatment with 100 ng/mL Cripto for 24 h. (A) Total protein was extracted and immunoblotted with antibodies against phosphorylated STAT3, total STAT3, c-Myc, and cyclin D1. Amounts of β-actin were used as internal loading controls. Panel on the right illustrates mean normalized levels (± SEM) of phosphorylated STAT3, c-Myc, and cyclin D1 (n=3). (B) BrdU incorporation was measured using fluorescence detection (n=3). (C) Representative image of a 96-well culture plate of a single cell assay with proliferating MSCs stained with Giemsa stain. (D) Plot of the number of proliferating cells per 96-well plate (n=3). Data are expressed as the mean ± SEM of three independent experiments each performed in triplicate dishes. ANOVA followed by post hoc Bonferroni-Dunn test was used for multiple group comparisons. (E) Representative image of a single cell assay after 10 days in 96-well plate, stained with Giemsa stain. Scale bar=100 μm. (F) The number of cells per field of view in each well of a 96-well plate is plotted (n=3). (A, B, D, F) Statistical significance of differences is indicated as follows: **p*<0.05, ***p*<0.01 vs. control, $^{\#}p$<0.05, $^{\#\#}p$<0.01 vs. Cripto alone, $^{\$}p$<0.05, $^{\$\$}p$<0.01 vs. Cripto with STAT3 siRNA.

GRP78 expression was important for the expression of cell cycle-related proteins.

Effect of Cripto on MSC proliferation through STAT3

To elucidate the role of the STAT3 pathway in MSC proliferation induced by Cripto, MSCs were pretreated with STAT3 siRNA prior to the incubation with Cripto and then, cell proliferation and western blot assays were carried out. As seen in Fig. 3A, pretreatment with STAT3 siRNA reduced the observed increase in the levels of phosphorylated STAT3, c-Myc, and cyclin D1 caused by Cripto. In addition, pretreatment with STAT3 siRNA inhibited the increased rates of BrdU incorporation (Fig. 3B), cell proliferation (Fig. 3C, 3D), and single cell division (Fig. 3E, 3F) induced by Cripto. This result suggested that STAT3 plays an important role in the effect of Cripto on MSCs.

Cripto potentiates MSC survival via up-regulation of BCL3 expression and inhibition of caspase-3 levels

STAT3 is usually translocated to the cell nucleus, where it acts as a transcription activator (Akira *et al*., 1994). To investigate the effect of STAT3 activation by Cripto on cell survival, we examined the level of the survival factor BCL3 in MSCs. Cripto increased the level of BCL3, which was inhibited by pretreatment with STAT3 siRNA (Fig. 4A, 4B). As Cripto increased BCL3 level apparently through the up-regulation of STAT3, we hypothesized that incubation with Cripto could have a protective effect against H_2O_2- or hypoxia-induced cell death that can be prominent because of oxidative stress in various diseases. We therefore explored whether the increase in BCL3 expression induced by Cripto affected caspase-3 levels augmented by the oxidative stress induced by H_2O_2 or

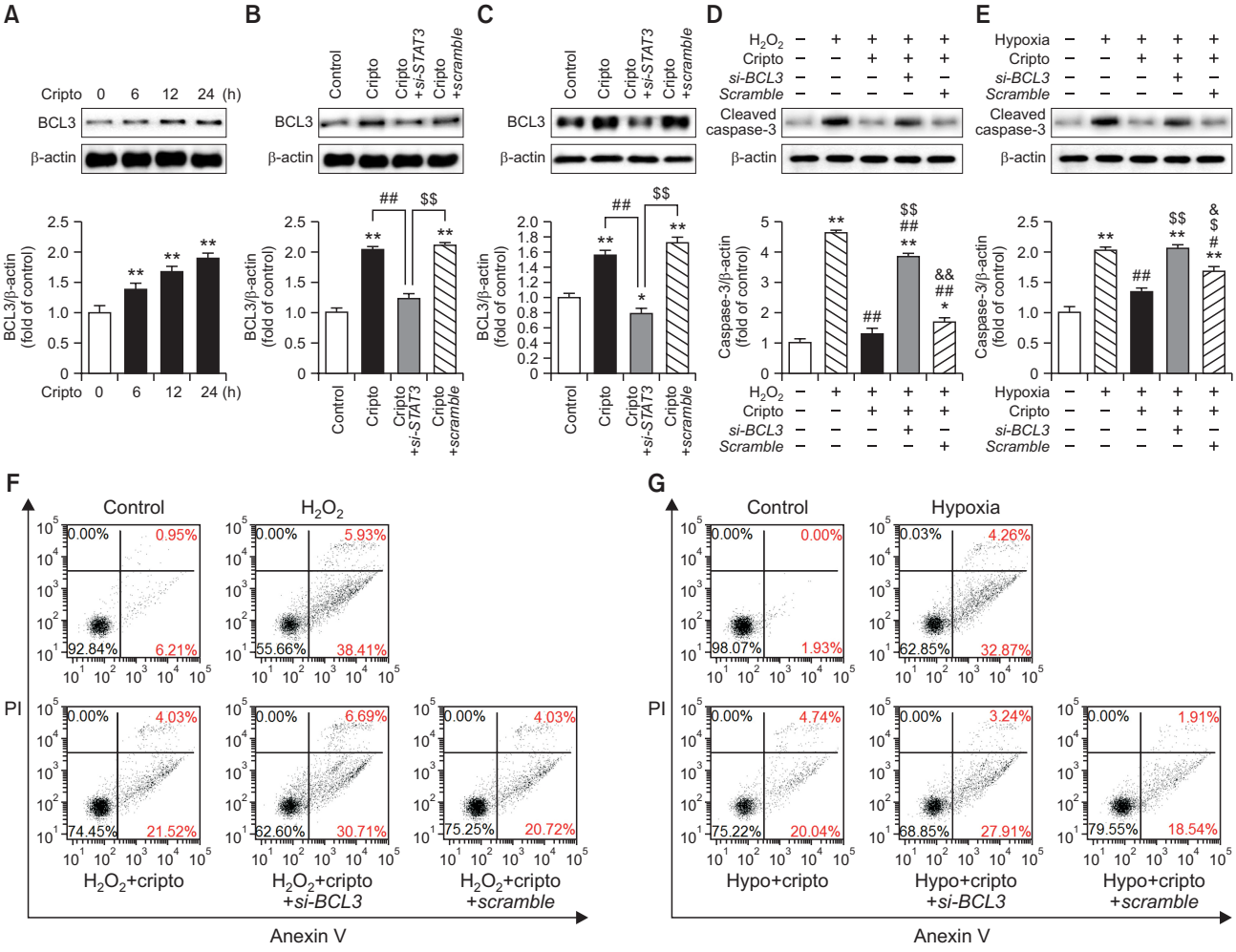

Fig. 4. Up-regulation of BCL3 expression and inhibition of hydrogen peroxide-induced MSC apoptosis by Cripto. MSCs were treated with 100 ng/mL Cripto for 0-24 h. (A) Total protein was extracted and immunoblotted with an antibody against BCL3. Amounts of β-actin were used as internal loading controls. Lower panel indicates mean normalized levels (± SEM) of BCL3 (n=3). Statistical significance is indicated as follows: $^{**}p<0.01$ vs. control. (B) MSCs were pre-transfected with STAT3 siRNA or non-targeting scrambled siRNA for 24 h before the treatment with 100 ng/mL Cripto for 24 h. Total protein was extracted and immunoblotted with an antibody against BCL3. Amounts of β-actin were used as internal loading controls. Lower panel indicates mean normalized levels (± SEM) of BCL3 (n=3). Statistical significance is indicated as follows: $^{**}p<0.01$ vs. control, $^{\#\#}p<0.01$ vs. Cripto alone, $^{\$\$}p<0.01$ vs. Cripto with STAT3 siRNA. (C) MSCs were pre-transfected with BCL3 siRNA or non-targeting scrambled siRNA for 24 h before treatment with 100 ng/mL Cripto for 24 h. Total protein was extracted and immunoblotted with an antibody against BCL3. Amounts of β-actin were used as internal loading controls. Lower panel indicates mean normalized levels (± SEM) of BCL3 (n=3). Statistical significance is indicated as follows: $^{*}p<0.05$, $^{**}p<0.01$ vs. control, $^{\#\#}p<0.01$ vs. Cripto alone, $^{\$\$}p<0.01$ vs. Cripto with STAT3 siRNA. (D, E) MSCs were transfected with BCL3 siRNA for 24 h followed by the treatment with 100 ng/mL Cripto for 24 h, and then exposed to 200 μM H_2O_2 for 6 h or hypoxic condition for 96 h. Total protein was extracted and immunoblotted with an antibody against caspase-3. Amounts of β-actin were used as internal loading controls. Lower panels in (D) and (E) indicate mean normalized levels (± SEM) of caspase-3 (n=3). Statistical significance is indicated as follows: $^{*}p<0.05$, $^{**}p<0.01$ vs. control, $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. H_2O_2 or hypoxia, $^{\$}p<0.05$, $^{\$\$}p<0.01$ vs. Cripto under H_2O_2 or hypoxia, $^{\&}p<0.05$, $^{\&\&}p<0.01$ vs. Cripto with BCL3 siRNA under H_2O_2 or hypoxia. (F, G) Apoptosis of MSCs was measured using propidium iodide (PI)/annexin V staining and flow cytometry. PI/annexin V double-negative cells were considered live cells, PI-negative/annexin V-positive cells were considered early apoptotic cells, and PI/annexin V double-positive cells were considered late apoptotic cells (n=3).

hypoxia. We also determined the efficiency of BCL3 siRNA transfection using a BCL3 western blot (Fig. 4C). As shown in Fig. 4D and 4E, Cripto reduced the increase in caspase-3 level induced by exposure to H_2O_2 and hypoxia. Notably, in both cases, the effects of Cripto were inhibited by pretreatment with BCL3 siRNA. The extent of cell death in response to H_2O_2 or hypoxia was monitored by using the annexin V/PI apoptosis assay and flow cytometry measurements. As shown in Fig. 4F and 4G, both incubation with H_2O_2 and hypoxic conditions increased the proportion of apoptotic MSCs. However, a pretreatment with Cripto rescued MSC viability from detrimental effects of H_2O_2 and hypoxia. As in the case with caspase-3 dynamics, this effect of Cripto was inhibited by the pretreatment with BCL3 siRNA. These results suggested that up-regulation of BCL3 expression caused by Cripto has a key role in the protection against MSC apoptosis induced by oxidative stress.

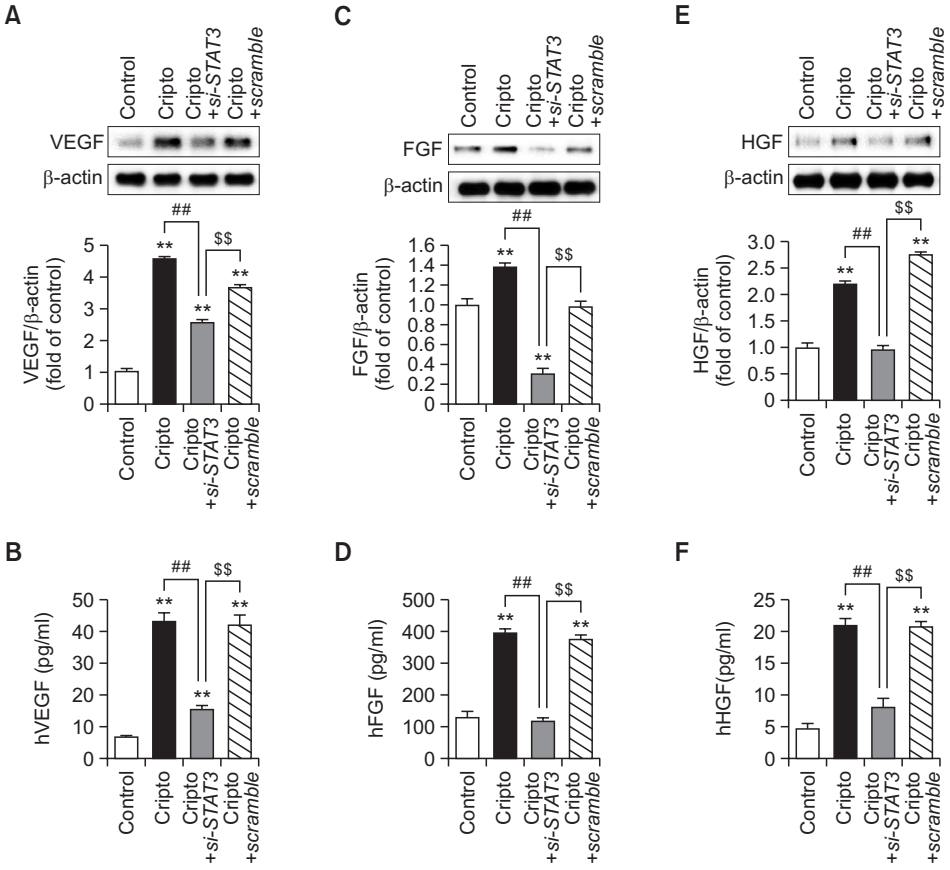

Fig. 5. Effect of Cripto on the expression of vascularization factors. MSCs were pre-transfected with STAT3 siRNA or non-targeting scrambled siRNA for 24 h before the treatment with 100 ng/mL Cripto for 24 h. (A) Total protein was extracted and immunoblotted with an anti-VEGF antibody. Amounts of β-actin were used as internal loading controls. Lower panel indicates mean normalized levels (± SEM) of VEGF (n=3). (B) Measurements of human VEGF concentration in MSCs using ELISA (n=3). (C) Total protein was extracted and immunoblotted with an anti-FGF antibody (n=3). Amounts of β-actin were used as internal loading controls. Lower panel indicates mean normalized levels (± SEM) of FGF. (D) Measurements of human FGF concentration in MSCs using ELISA (n=3). (E) Total protein was extracted and immunoblotted with an anti-HGF antibody. Amounts of β-actin were used as internal loading controls. Lower panel indicates mean normalized levels (± SEM) of HGF (n=3). (F) Measurements of human HGF concentration in MSCs using ELISA (n=3). Statistical significance is indicated as follows: $^{**}p<0.01$ vs. control, $^{\#\#}p<0.01$ vs. Cripto alone, $^{\$\$}p<0.01$ vs. Cripto with STAT3 siRNA.

Cripto up-regulates expression of vascularization factors in a STAT3-dependent manner

It has been known that translocation of STAT3 into the nucleus can affect factors related to angiogenesis, such as VEGF (Tan *et al*., 2016) , FGF (Xue *et al*., 2017), and HGF (Lee *et al*., 2016b). We found that exposure to Cripto increased expression levels of VEGF, FGF, and HGF (Fig. 5A, 5C, 5E). Furthermore, a pretreatment with STAT3 siRNA significantly suppressed the increase in the levels of VEGF, FGF, and HGF induced by Cripto (Fig. 5A, 5C, 5E). To confirm vascularization factors expression after Cripto treatment, we assessed the expression of human VEGF, human FGF, and human HGF. Cripto increased expression levels of VEGF, FGF, and HGF (Fig. 5B, 5D, 5F). Furthermore, a pretreatment with STAT3 siRNA significantly suppressed the increase in the levels of VEGF, FGF, and HGF induced by Cripto (Fig. 5B, 5D, 5F).

DISCUSSION

In the present study, we demonstrated that Cripto strongly potentiated proliferation and survival of MSCs by activating JAK2/STAT3 signaling pathway in a GRP78-sensitive manner. In particular, exposure to Cripto augmented expression levels of cell cycle regulatory proteins, an anti-apoptotic protein, and several vascularization-related proteins.

Although human MSCs have considerable potential to contribute significantly to therapeutic transplantation of malfunctioning tissues or organs, approved protocols that exploit such adaptability have not been worked out. The main limitation for mass production and use of MSCs as therapies is their low availability in human tissues. Thus, several previous studies sought to discover molecular pathways that could potentially stimulate reproductive induction of MSCs *ex vivo* for clinical purposes (Ball *et al*., 2007; Le Blanc *et al*., 2008; Bernardo *et al*., 2011). Accumulating evidence in the scientific literature suggests that Cripto and its numerous downstream molecules could enhance survival of diverse types of cells (Zhang *et al*., 2010). However, the key role of the Cripto-STAT3-BCL3 pathway, revealed by us in the present study, has not been reported in studies of Cripto downstream signaling (Bianco *et al*., 2002; Gray and Vale, 2012; Yao *et al*., 2015). Thus, the novel

protocol for the induction of MSC proliferation and survival by activating Cripto-mediated signaling, uncovers another potential way in which MSC preparations could be optimized for better therapeutic interventions. It has been shown previously that Cripto/GRP78 modulation of the TGF-β pathway enhanced stem cell proliferation, indicating that it could be an attractive therapeutic strategy for disease treatment (Gray and Vale, 2012). However, to the best of our knowledge, our study has demonstrated for the first time that Cripto can stimulate proliferation of MSCs through a novel signaling pathway. Our data showed that Cripto increased proliferation of MSCs in a concentration- and time-dependent manner. These results confirmed our hypothesis about the role of the Cripto pathway in the induction of MSC proliferation and indicated that Cripto may be a convenient target for modulation in mass production of MSCs *ex vivo*, allowing stem cell therapy to be a more effective clinical intervention.

It has been suggested that the effects of Cripto on cellular properties depend on the interaction of Cripto with GRP78 on the cell surface (Shani *et al*., 2008; Kelber *et al*., 2009). Although GRP78 is primarily targeted to endoplasmic reticulum, it can also be localized in the plasma membrane, where it performs a receptor-like function associated with enhanced cellular proliferation and survival (Gonzalez-Gronow *et al*., 2009; Sato *et al*., 2010; Ni *et al*., 2011). In our study, we found that exposure to Cripto significantly increased GRP78 levels, consistent with a previous study (Gray and Vale, 2012). In addition, we were able to discover that GRP78 is one of the membrane receptors for Cripto. It is known that GRP78 overexpression not only induces cell proliferation and survival, but also influences other signal molecules related to cell-proliferation and cell survival (Sato *et al*., 2010; Spike *et al*., 2014). This suggests that Cripto regulates MSC proliferation and survival through enhancing the expression of GRP78. Treatment with a neutralizing anti-GRP78 antibody inhibited Cripto-induced increase in the expression levels of c-Myc, cyclin D1, phosphorylated JAK, and phosphorylated STAT3, suggesting that GRP78 is critical for the action of Cripto on cell proliferation pathways. It has been shown that overexpression of GRP78 increased its membrane expression and enhanced the amount of phosphorylated STAT3 as an immediate downstream effector (Yao *et al*., 2015). That finding suggested that STAT3 phosphorylation is an important event that potentially transduces the effects of Cripto-GRP78 interaction on the expression levels of phosphorylated STAT3, c-Myc, and cyclin D1. In our study, consistent with previous reports, we showed that STAT3 plays a critical role in the effect of Cripto on MSC proliferation: Cripto induced up-regulation of c-Myc and cyclin D1, downstream cell cycle regulatory proteins in the Cripto-GRP78-Jak/STAT3 activation pathway, that was reduced by the pretreatment of MSCs with STAT3 siRNA. In addition, cell division, cell proliferation, as well as BrdU incorporation were all inhibited by STAT3 siRNA, further confirming the key role of STAT3 in the effect of Cripto on MSCs. Other components of the novel Cripto-STAT3 pathway discovered by us need to be revealed in future studies. In addition, it will be of interest to determine whether this pathway is operational in other types of cells.

A potential anti-apoptotic and pro-survival role for BCL3, which interacts with Cripto and STAT3, has been previously described (Lee *et al*., 2015a), although a variety of STAT3-dependent signaling molecules act on cell survival and anti-

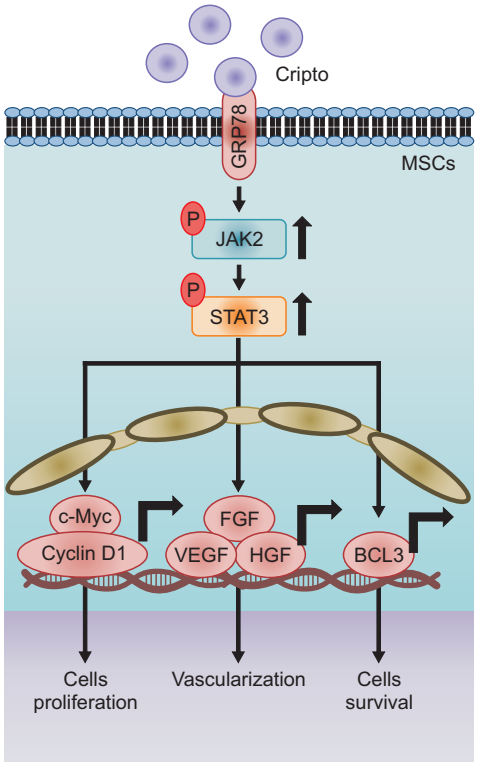

Fig. 6. Schematic representation of the putative mechanisms by which Cripto increases proliferation, survival, and enhances secretion of growth factors in MSCs. Exposure to Cripto increases the proliferative potential of MSCs via the activation of the JAK2/STAT3 pathway and production of growth factors, such as FGF, VEGF, and HGF. In addition, Cripto promotes MSC survival by augmenting BCL3 expression in a STAT3-dependent manner.

apoptosis (Zhou *et al*., 2015; Xu and Tang, 2017). Our previous study suggests that BCL3 is the key factor that protects MSCs from death due to oxidative stress (Lee *et al*., 2015a). Thus, our study focused on Cripto's beneficial effects on cell survival via STAT3-dependent BCL3 and its ability to block downstream apoptotic signaling molecules. Our results were consistent with the notion made by previous studies that phosphorylated STAT3 could act as a transcription factor that regulates the level of BCL3, thereby preventing hypoxia-induced apoptosis mediated by the activation of pro-apoptotic caspase-3. Caspase-3 has been known as an "effector caspase" that when activated, cleaves other protein substrates to trigger apoptosis (Cohen, 1997). In contrast, BCL3 was found to exhibit anti-apoptotic properties (Ahmed and Milner, 2009). We created hypoxic environment by incubating cells with H_2O_2 to enhance cellular caspase-3 level, and determined whether Cripto-BCL3 induction protects MSCs from apoptosis. Western blot results demonstrated that upon exposure to Cripto, BCL3 levels in MSCs increased in a time-dependent manner, whereas caspase-3 levels were negatively correlated with the levels of BCL3. This finding indicated that BCL3 affected the level of downstream caspase-3 in the cell, which resulted in the prevention of apoptosis and prolonged survival of MSCs. In addition, when STAT3 level was reduced by the transfection of STAT3 siRNA, BCL3 protein levels, as well as anti-apoptotic effects of Cripto, were decreased in MSCs, suggesting that

STAT3-mediated modulation of the BCL3 pathway is central to Cripto functions. The observed relationships between Cripto, p-STAT3, BCL3, and caspase-3 confirmed the notion about the involvement of Cripto in cell apoptotic pathways. It has been shown that the translocation of STAT3 into the nucleus triggers the production of FGF, VEGF, and HGF, which are major inducers of vascularization in MSCs (Niu *et al*., 2002; Dudka *et al*., 2010; Gorin *et al*., 2016; Xue *et al*., 2017). Such data prompted us to examine whether the levels of these vascularization factors would be sensitive to the modulation of the Cripto-STAT3 pathway activity. Because the treatment with STAT3 siRNA attenuated the increase in VEGF, FGF, and HGF expression levels caused by Cripto, we concluded that Cripto affected the expression of vascularization factors through the STAT3 pathway. In our previous studies, we elucidated that an increase in VEGF, FGF, and HGF by external stimuli induces vascularization of stem cells in an ischemic and hypoxic model (Lee *et al*., 2015a, 2015b, 2016a; Han *et al*., 2016). Although our study does not explicitly show the effects of Cripto on vascularization, it sufficiently shows that VEGF, FGF, and HGF induced by Cripto could increase the differentiation and regeneration of the stem cells. Delivery of tissue constructs with adult stem cells into injured and ischemic tissues has emerged as a potential therapeutic option for tissue repair and regeneration. Thus, enhanced vascularization may considerably improve clinical efficiency of MSC-based therapies.

In summary, our study revealed a novel method to improve the quality of MSC clinical preparations by using the ability of Cripto to enhance cell proliferation, survival, and vascularization via a novel pathway that involves Cripto, GRP78, STAT3, and BCL3. According to our results, Cripto through its effects on stem cell proliferation and protection could yield better outcomes in degenerative and congenital disease treatment via stem cell therapy, drug delivery, and tissue engineering (Fig. 6). Moreover, given that practical and efficient ex novo generation of MSCs is essential for their clinical applications, modulation of the activity of this novel MSC-stimulating pathway may bolster the efficacy of MSC replantation therapy.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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