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Gas6 induces cancer cell migration and epithelial–mesenchymal transition through upregulation of MAPK and Slug

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ABSTRACT

Binding of Gas6 to Axl (Gas6/Axl axis) alters cellular functions, including migration, invasion, proliferation, and survival. However, the molecular mechanisms underlying Gas6-mediated cell migration remain poorly understood. In this study, we found that Gas6 induced the activation of JNK and ERK1/2 signaling in cancer cells expressing Axl, resulting in the phosphorylation of activator protein-1 (AP-1) transcription factors c-Jun and ATF-2, and induction of Slug. Depletion of c-Jun or ATF-2 by siRNA attenuated the Gas6-induced expression of Slug. Slug expression was required for cell migration and E-cadherin reduction/vimentin induction induced by Gas6. These results suggest that Gas6 induced cell migration via Slug upregulation in JNK- and ERK1/2-dependent mechanisms. These data provide an important insight into the molecular mechanisms mediating Gas6-induced cell migration.

1. Introduction

The invasive nature of tumor cells is the major prerequisite to cancer metastasis. For invasion, cancer cells require three prominent properties: increased cell migration (motility); a loss of cell–cell adhesion along with altered cell-matrix adhesion; and degradation of matrix proteins, which releases cells from the primary tumor mass [1]. Cell migration plays a critical role in cancer cell invasion and metastasis, and is initiated via tumor cell activation of epithelial–mesenchymal transition (EMT) [2–6]. EMT is characterized by the loss of cell polarity and cell–cell interactions, modulation of cell-matrix adhesion, enhanced proteolytic activity such as extracellular matrix degradation, reorganization of the cytoskeleton, and acquisition of cell motility [4]. During EMT, epithelial cells gradually lose their epithelial structures such as E-cadherin-mediated cell–cell adhesion while concomitantly acquiring mesenchymal characteristics such as upregulation of vimentin [4]. Downregulation of E-cadherin is a well-known hallmark of EMT and correlates positively with tumor cell invasion and metastasis [4]. Downregulation of E-cadherin is usually mediated by E-cadherin transcriptional repressors, including the Snail superfamily of zinc-finger factors (Snail and Slug), the ZEB family (ZEB1 and ZEB2), and basic helix–loop–helix factors (Twist and E47) [3,7].

Gas6, the product of the *growth arrest-specific gene 6*, is a member of the vitamin K-dependent family of Gla proteins homologous to the blood coagulation protein S [8]. However, Gas6 has not been found to play any coagulation roles [9]. Instead, Gas6 in general serves as a mitogen (growth factor) and survival factor that protects many non-transformed cell types and tumor cells from serum starvation-induced apoptosis [9,10]. Gas6 is the ligand for the TAM (Axl, Tyro3/Sky and Mer) subfamily of receptor tyrosine kinases [9]. In many systems, binding of Gas6 to Axl alters cellular functions, including migration, proliferation, and survival; a recent study showed that the Gas6/Axl axis induces prostate cancer cell invasion and survival during metastasis to bone marrow [11] whereas other groups have reported that Gas6/Axl induces proliferation in prostate cancer cells [12,13]. In breast cancer, Axl is required for breast cancer cell invasion as well as EMT and breast cancer progression [14], and drives cell migration, neovascularization, and tumor growth [15]. However, molecular mechanisms underlying Gas6-mediated cell migration and invasion are not fully understood.

In this study, we investigated the role of Gas6 in the induction of cell migration and its underlying mechanism. We found that Gas6 induced Slug gene expression in JNK- and ERK1/2-dependent mechanisms via the AP-1 (activator protein-1) transcription factors c-Jun and ATF-2, leading to E-cadherin reduction/vimentin induction and cell migration. These data provide evidence that the Gas6/Axl-Slug axis might be exploited as a target for potential anti-cancer metastasis therapy.

Abbreviations: MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK1/2, extracellular signal-regulated kinase 1/2; AP-1, activator protein-1; EMT, epithelial–mesenchymal transition; siRNA, small interfering RNA; MEK, ERK kinase.

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2. Materials and methods

2.1. Cell culture

Human embryonic kidney 293E (HEK293E) cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were maintained in DMEM with 10% FBS at 37 °C/5% CO_2. DU145 (prostate cancer) and A431 (skin cancer) cells (ATCC) were maintained in RPMI1640 with 10% FBS.

2.2. Plasmid constructs

A cDNA encoding wild-type full-length human Gas6 was provided by the National Genome Information Center (Daejeon, Korea). A cDNA fragment with the sequence encoding Gas6 without leader sequence (amino acid residues from 34 to 678) was inserted into a pCMV-Fc-myc vector [16] to produce pCMV-Gas6-Fc-myc expressing an Fc- and myc-tagged fusion of Gas6.

2.3. Transfection

HEK293E cells were transfected with pCMV-Gas6-Fc-myc or a vector expressing Gas6 (pcDNA3.1-gas6; a kind gift from Dr. Sassan Hafizi, University Hospital Malmö (UMAS), Sweden) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). At 48 h after transfection, the medium was changed to serum-free medium containing vitamin K_1 (10 μg/ml). Conditioned media were collected for 2 days. Secreted Fc- and myc-tagged Gas6 fusion protein (rhGas6-Fc) from the pCMV-Gas6-Fc-myc construct and Gas6 protein from the pcDNA3.1-gas6 construct were analyzed along with purified recombinant human Gas6 protein (R&D systems, Minneapolis, MN) by immunoblotting using an anti-Gas6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For siRNA transfection, cells were transfected with siRNA specific to either c-Jun (5′-GAUGGAAACGACCUUCUAUTT-3′), ATF-2 (5′-AAUGAAGUGGCACAGCUGATT-3′) or Slug (5′-GAGGAAAGACTACAGTCCAAGTT-3′) using Lipofectamine 2000 for 24 h in the absence of serum, and then treated with rhGas6 or subjected to the cell migration assay.

2.4. Immunoblot analysis

Whole-cell lysates were prepared using RIPA buffer as described previously [17], and analyzed using the following primary antibodies: anti-GAPDH and anti-β-actin (Santa Cruz Biotechnology); anti-E-cadherin (BD Biosciences, San Jose, CA); anti-vimentin (Sigma); anti-Slug, anti-phospho-Axl (Y702), anti-phospho-c-Jun (S63), anti-c-Jun, anti-phospho-ATF-2 (T71), anti-phospho-JNK (T183/Y185), anti-JNK, anti-phospho-ERK1/2, and anti-ERK1/2 (Cell Signaling, Danvers, MA, USA); anti-Axl and anti-E-cadherin (R&D systems, Minneapolis, MN). Cells were serum-starved for 24 h and then treated with conditioned medium containing recombinant human Gas6 fusion protein (rhGas6–Fc), mock-conditioned medium or purified recombinant human Gas6 protein (rhGas6; R&D systems) for 10 min or 48 h prior to lysis. Where indicated, serum-starved cells were pre-treated with pharmacological inhibitors, including 20 μM PD098059 or 8 μM SP600125 (Sigma) or 0.2% DMSO for 30 min and then treated with 0.2 μg/ml Gas6 in the presence of inhibitors for 10 min or 48 h before lysate preparation.

2.5. Promoter reporter assay

Cells were transfected with Lipofectamine 2000 (Invitrogen). For transfection, 2×10^5 cells were seeded on 6-well plates. After incubation for 24 h, cells were transfected with 2 μg of reporter plasmid DNA for 24 h and then treated with 0.2 μg/ml of purified recombinant human Gas6 protein. At 9 h post-treatment, firefly luciferase activity was measured using a Dual-luciferase reporter assay system (Promega, Southampton, UK). Transfection efficiency was normalized by measuring Renilla luciferase activity, encoded by the co-transfected Renilla luciferase vector (pRL-TK). The Slug promoter (−981/+174) construct in a pGL4-luciferase reporter vector was kindly provided by Dr. Shuang Huang (Medical College of Georgia, USA) [18].

2.6. Cell migration assay

Cells were plated in serum-free medium on Transwell inserts (Corning, NY, USA), and serum-free medium containing 0.2 μg/ml or 0.5 μg/ml of rhGas6 (R&D systems) was put in the lower chamber. After incubation for 48 h at 37 °C/5% CO_2, the inserts were fixed with 3.7% paraformaldehyde/PBS and stained with 2% crystal violet. The number of cells that had migrated was counted in five representative (×100) fields per insert.

2.7. Reverse transcription-PCR

Total RNA was isolated using Trizol (Invitrogen), and cDNA was synthesized using reverse transcriptase (Bioneer, Daejeon, Korea). Semi-quantitative PCR amplification was performed using Snail-specific primers (5′-TGAGGCCAAGGATCTCCAGG-3′ and 5′-GGGCAGGTATGGAGAGGAAG-3′), Slug-specific primers (5′-AGACCCCCATGCCATTGAAG-3′ and 5′-GCTTCGGAGTGAAGAAATGC-3′), ZEB1-specific primers (5′-GTGTTACAGATGCAGCTGACTGTGAAGGTG-3′ and 5′-GATGTCTTGAGTCCTGTTCTTGGTCG-3′), ZEB2-specific primers (5′-TCTCGCCCGAGTGAAGCCTT-3′ and 5′-GGGAGAATTGCTTGATGGAGC-3′) and β-actin-specific primers (5′-CCACTGGCATCGTGATGGAC-3′ and 5′-GCGGATGTCCACGTCACACT-3′). Real-time quantitative PCR was performed using SYBR Green (PKT, Seoul, Korea) with Slug-specific primers (5′-ATACCACAACCAGAGATCCTCA-3′ and 5′-GACTCACTCGCCCCAAAGATG-3′) and GAPDH-specific primers (5′-CATGACCACAGTCCATGCCAT-3′ and 5′-AAGGCCATGCCAGTGAGCTTC-3′) with an annealing temperature of 61 °C.

2.8. Immunocytochemistry

Cells were plated on serum-coated coverslips and incubated with Gas6 for 48 h. The cells were then fixed for 5 min in 3.7% formaldehyde/PBS and permeabilized in 0.3% (vv-1) Triton X-100 for 3 min. Next, the cells were incubated with anti-E-cadherin antibody (R&D systems) followed by a fluorescein-conjugated secondary antibody and with rhodamine-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR). The cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Sigma). Mounted samples were visualized using a fluorescence microscope (IX81; Olympus, Tokyo, Japan).

2.9. Statistical analysis

Statistical analyses were performed using Student's *t*-tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Gas6 induced cell migration and E-cadherin downregulation in DU145 cells

To prepare recombinant Gas6, human Gas6 (residues 34–678) was tagged with Fc and myc and then expressed and secreted from HEK293E cells. Immunoblot analysis of the conditioned medium revealed the presence of a ~100-kDa band (rhGas6–Fc; Fig. 1A).

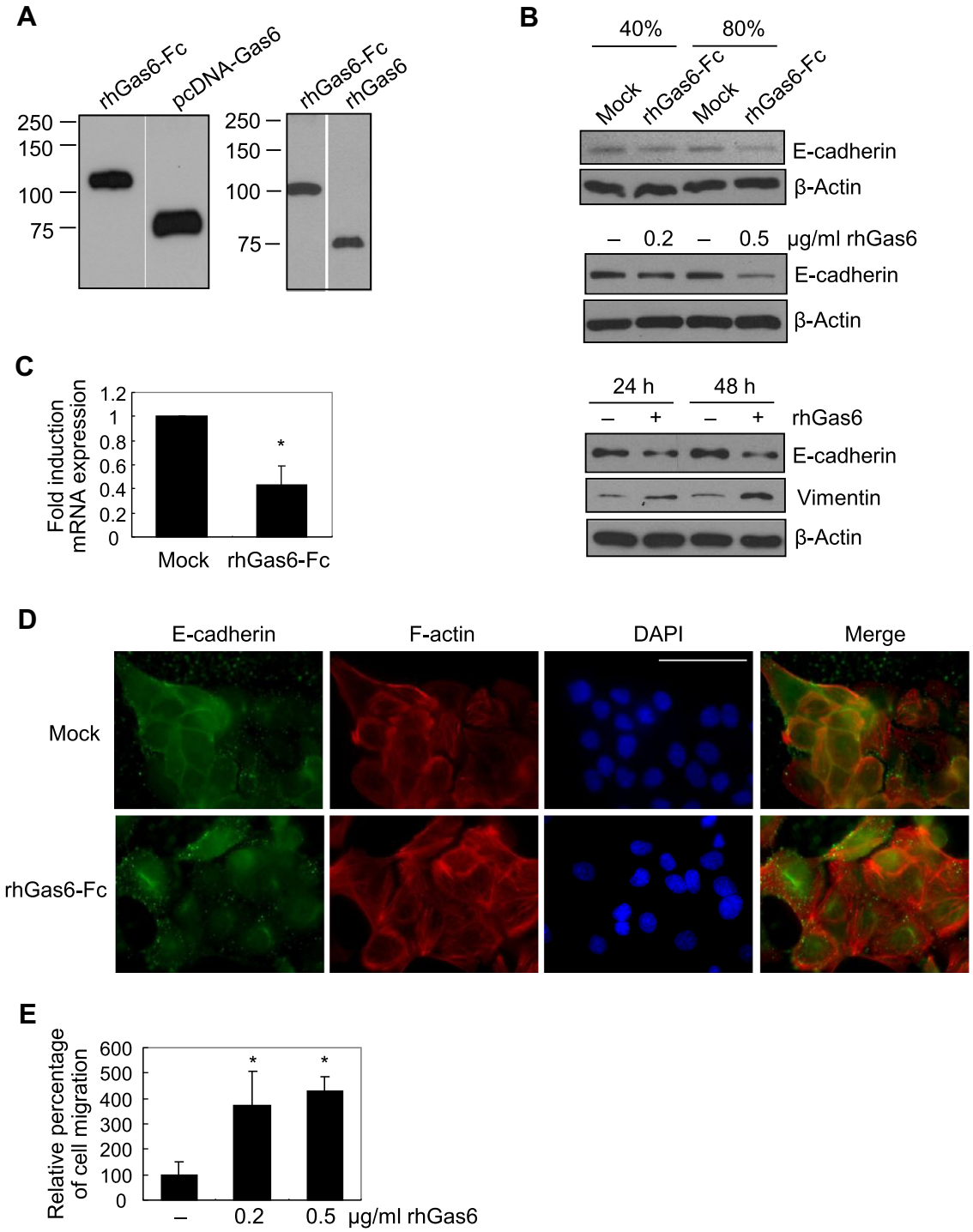

Fig. 1. Gas6 induced E-cadherin downregulation and cell migration. (A) Left, HEK293E cells were transfected with the pCMV-Gas6-Fc-myc (rhGas6–Fc) or pcDNA3.1-gas6 (pcDNA-Gas6) construct for 48 h. Conditioned medium in the absence of serum was collected for an additional 48 h. Each 40 μl of conditioned medium was analyzed by immunoblotting using anti-Gas6 antibody. Right, 20 μl of conditioned medium containing rhGas6–Fc (from (A) left) was analyzed along with 20 ng of purified recombinant human Gas6 protein (rhGas6; R&D systems) by immunoblotting with anti-Gas6 antibody. (B) Lysates from DU145 cells treated with Gas6 were analyzed by immunoblotting. Upper, Cells were serum-starved for 24 h and then treated with 40% and 80% of the conditioned medium containing rhGas6–Fc fusion protein or mock-conditioned medium for 48 h. Middle, Serum-starved cells were treated with 0.2 μg/ml and 0.5 μg/ml of the purified recombinant human Gas6 protein for 48 h. Lower, Serum-starved cells were treated with 0.2 μg/ml of the purified recombinant human Gas6 for 24 h and 48 h. (C and D) Serum-starved DU145 cells were treated with 40% of the conditioned medium containing rhGas6–Fc fusion protein or mock-conditioned medium for 48 h. (C) E-cadherin mRNA levels were determined by real-time quantitative PCR analysis. (D) Immunofluorescence analysis of E-cadherin and F-actin. Cells were stained with anti-E-cadherin followed by FITC-conjugated secondary antibody and rhodamine-labeled phalloidin for F-actin and the nucleus was counterstained with DAPI. Scale bar, 50 μm. (E) DU145 cells were subjected to the Transwell migration assay. Cells (4×10^4) were allowed to migrate toward 0.2 μg/ml and 0.5 μg/ml of purified rhGas6 protein for 48 h. Values represent mean ± SD. $^*P < 0.05$.

A slight shift in the molecular mass was detected after PNGase F treatment, suggesting the presence of glycosylation (data not shown). This recombinant human Gas6 fusion protein (rhGas6–Fc) in the conditioned medium was comparable with intact Gas6 (pcDNA-Gas6) in conditioned medium from HEK293E cells transfected with pcDNA3.1-gas6 (Fig. 1A, left), and was also comparable

with purified recombinant human Gas6 protein (rhGas6; R&D systems) (Fig. 1A, right). To examine effects of Gas6 on cancer cell migration and EMT, we treated cells expressing endogenous Axl with conditioned medium containing recombinant human Gas6 fusion protein (rhGas6–Fc) or purified recombinant human Gas6 protein (rhGas6; R&D systems).

Because downregulation of E-cadherin is a key hallmark of EMT, regulation of E-cadherin by Gas6 was examined. Immunoblot analysis showed that E-cadherin expression was substantially reduced in DU145 cells in a dose-dependent manner following treatment with conditioned medium containing recombinant human Gas6 fusion protein (rhGas6–Fc) when compared with the mock-conditioned medium, or purified recombinant Gas6 protein (rhGas6) compared with no treatment (Fig. 1B, upper and middle). In addition, vimentin was also substantially upregulated in a time-dependent manner by Gas6 treatment (Fig. 1B, lower). Using real-time quantitative PCR analysis, we also observed that E-cadherin mRNA was significantly reduced by Gas6 treatment (Fig. 1C). Immunofluorescence analysis showed that cells displayed substantially reduced E-cadherin levels at the cell boundary when treated with conditioned medium containing recombinant Gas6 fusion protein, whereas cells maintained E-cadherin expression in organized cell junctions when treated with mock-conditioned medium (Fig. 1D). We also observed that E-cadherin was moderately reduced by Gas6 treatment in A431 cells, which express endogenous Axl (Supplementary Fig. S1A). In contrast, E-cadherin levels were not altered by Gas6 treatment in WiDr and HCT15 cells (data not shown), which do not normally express Axl (Supplementary Fig. S1B). On the other hand, E-cadherin was reduced and vimentin was moderately induced in HCT15 cells overexpressing Axl following Gas6 treatment (Supplementary Fig. S2), suggesting that Gas6-mediated effects are Axl-dependent. In addition, Gas6 induced DU145 cell migration in a dose-dependent manner (Fig. 1E).

3.2. Gas6 activated JNK and ERK1/2 signaling pathways and upregulated Slug gene expression

To explore the molecular basis underlying Gas6-mediated E-cadherin reduction and cell migration, we first investigated the activation of several signaling pathways and E-cadherin transcriptional repressors. As expected, Axl phosphorylation was induced by Gas6 treatment in DU145 and A431 cells, both of which express endogenous Axl (Fig. 2A). Among the molecules we screened, phosphorylation of ERK1/2 and JNK was increased by Gas6 treatment (Fig. 2A). In addition, we observed that Slug was increased by Gas6 treatment (Fig. 2A). Slug mRNA level was also increased by Gas6 in DU145 cells, whereas mRNA expression levels of other E-cadherin transcriptional repressors, including Snail, ZEB1, ZEB2

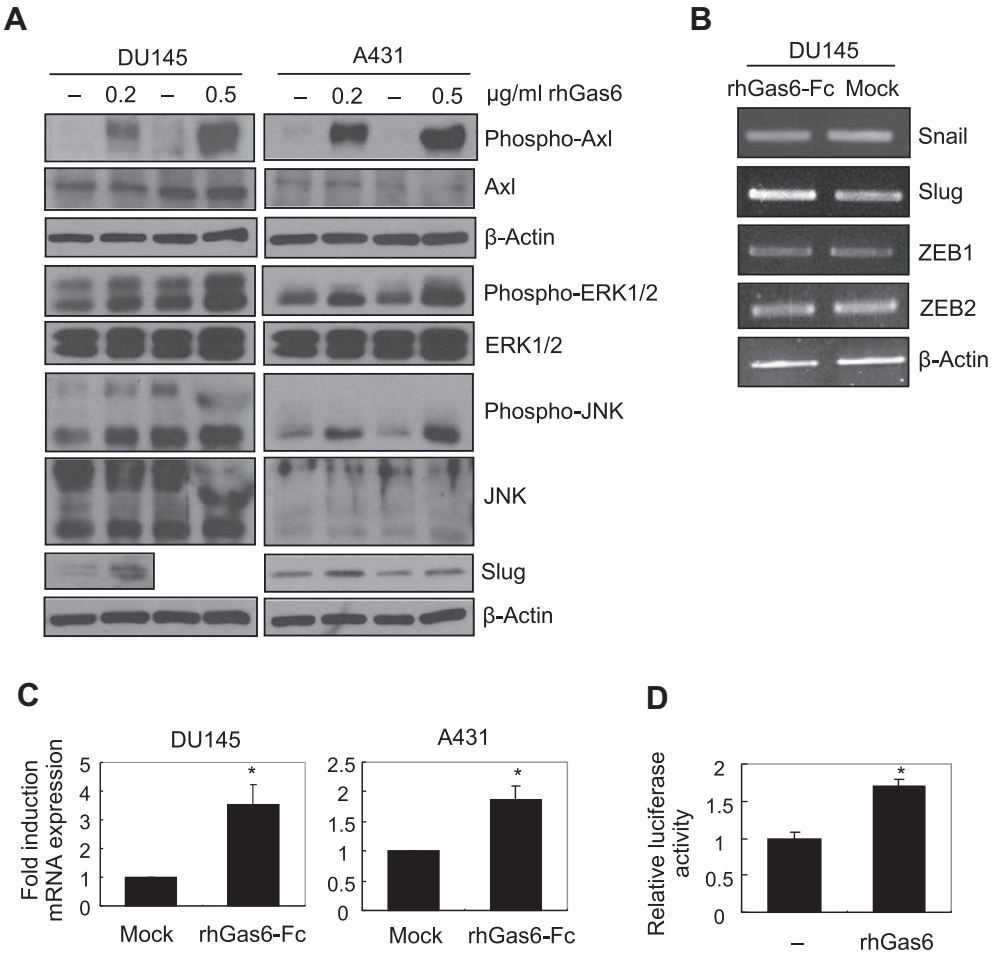

Fig. 2. Gas6 activated JNK and ERK1/2 signaling pathways and upregulated Slug gene expression. (A) Cells were serum-starved for 24 h and then treated with rhGas6 protein for 10 min (phospho-Axl, phospho-ERK1/2, phospho-JNK) or 48 h (Slug) prior to lysis. Whole-cell lysates were analyzed by immunoblotting. (B,C) Cells were serum-starved for 24 h and then treated with 40% of the conditioned medium containing rhGas6–Fc fusion protein or mock-conditioned medium for 48 h before total RNA isolation. (B) Semiquantitative reverse transcription-PCR analysis. (C) Real-time quantitative PCR analysis of Slug mRNA levels. (D) Cells were transfected with the Slug promoter (−981/+174) construct in the pGL4 vector for 24 h and then treated with 0.2 μg/ml of rhGas6 for 9 h. Firefly luciferase activity representing Slug promoter activity was measured and normalized to Renilla luciferase activity to measure transfection efficiency. Values represent mean ± SD. *$P < 0.05$.

(Fig. 2B), and Twist1 (data not shown), did not change substantially following Gas6 treatment. Real-time quantitative PCR analysis confirmed that Slug mRNA was significantly enhanced by Gas6 treatment in DU145 and A431 cells (Fig. 2C).

To determine whether Gas6 upregulates Slug gene transcription, we transiently transfected DU145 cells with a reporter plasmid driven by the Slug promoter (−981/+174) containing AP-1 site (+26/+34) and treated cells with Gas6. Consistent with the data shown in Fig. 2A–C, Gas6 treatment induced a 1.7-fold increase in Slug promoter activity in DU145 cells (Fig. 2D).

3.3. Slug was upregulated by Gas6 through c-Jun and ATF-2 in ERK1/2- and JNK-dependent manners

To determine the role of JNK and ERK1/2 signaling in Gas6-mediated Slug upregulation, cells were treated with the following pharmacological inhibitors: 0.2% dimethyl sulfoxide (vehicle), 20 μM PD098059 (a specific MEK/ERK1/2 inhibitor), and 8 μM SP600125 (a specific JNK inhibitor). Immunoblot analysis showed that Gas6 treatment increased phosphorylation of c-Jun and ATF-2 in DU145 and A431 cells, respectively (Fig. 3A). Inhibition of MEK/ERK1/2 or JNK reduced phosphorylation and expression of c-Jun induced by Gas6 in DU145 cells, while inhibition of MEK/ERK1/2 or JNK reduced ATF-2 phosphorylation induced by Gas6 in A431 cells (Fig. 3A). Furthermore, Gas6-induced Slug expression was efficiently attenuated by MEK/ERK1/2 and JNK inhibitors in both DU145 and A431 cells (Fig. 3A). Of note, MEK/ERK1/2 inhibitor also reduced phosphorylation of JNK, implying that MEK/ERK1/2 is possibly involved in JNK activation in DU145 and A431 cells. We then examined whether c-Jun and ATF-2 are required for Slug expression induced by Gas6. Immunoblot analysis showed that knockdown of c-Jun and ATF-2 by using siRNA in DU145 and A431 cells, respectively, substantially reduced the induction of Slug by Gas6 (Fig. 3B). These results indicate that JNK and ERK1/2 signaling activities are required for Gas6-induced Slug expression through the AP-1 transcription factors c-Jun and ATF-2.

3.4. Gas6 induced cell migration and EMT through a Slug-dependent mechanism

We then determined the role of Slug in Gas6-induced cell migration. Transwell migration assay results showed that siRNA-

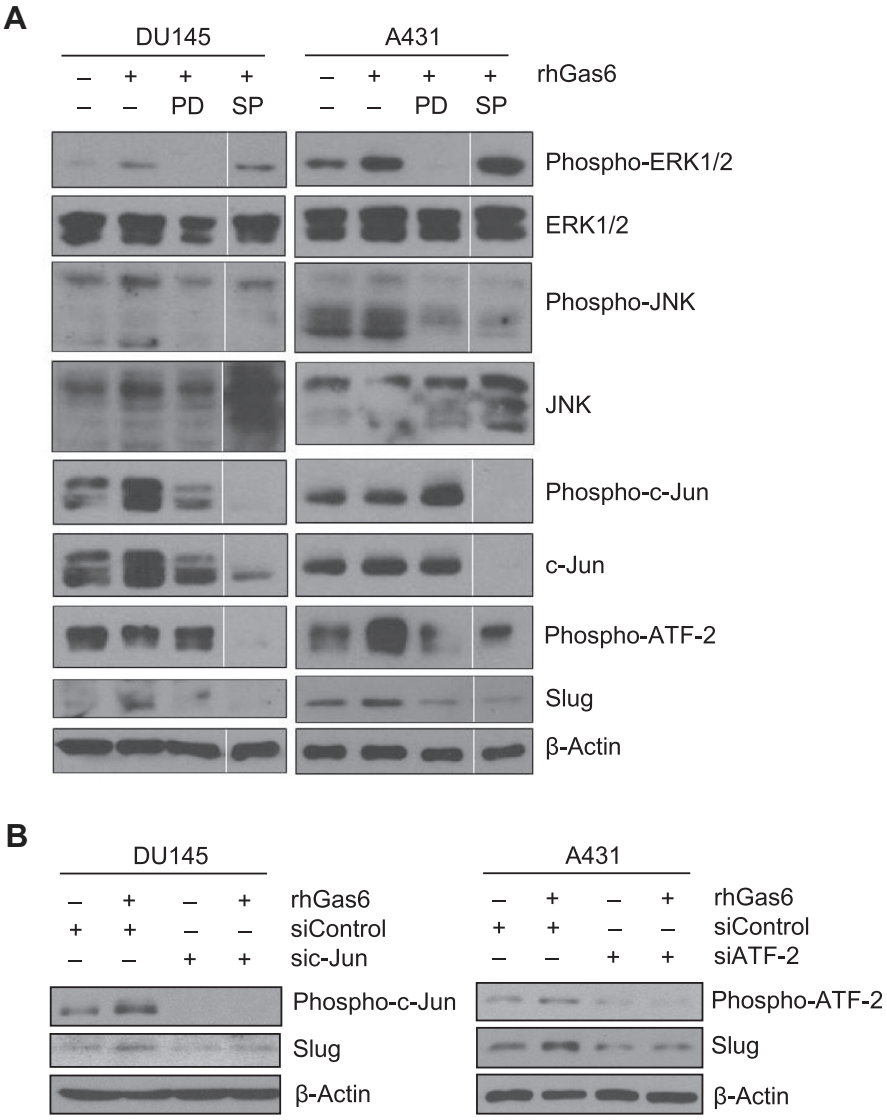

Fig. 3. JNK and ERK1/2 activation was required for Gas6-induced Slug expression. (A) Serum-starved cells were pretreated with 0.2% DMSO, 20 μM PD098059, or 8 μM SP600125 for 30 min and then treated with 0.2 μg/ml of Gas6 in the presence of DMSO or inhibitors for 10 min (phospho-ERK1/2, phospho-JNK) or 48 h (phospho-c-Jun, phospho-ATF-2, Slug). Whole-cell lysates were analyzed by immunoblotting. (B) Cells were transfected with siRNA for 24 h and then treated with 0.2 μg/ml of Gas6 for 48 h. Whole-cell lysates were analyzed by immunoblotting.

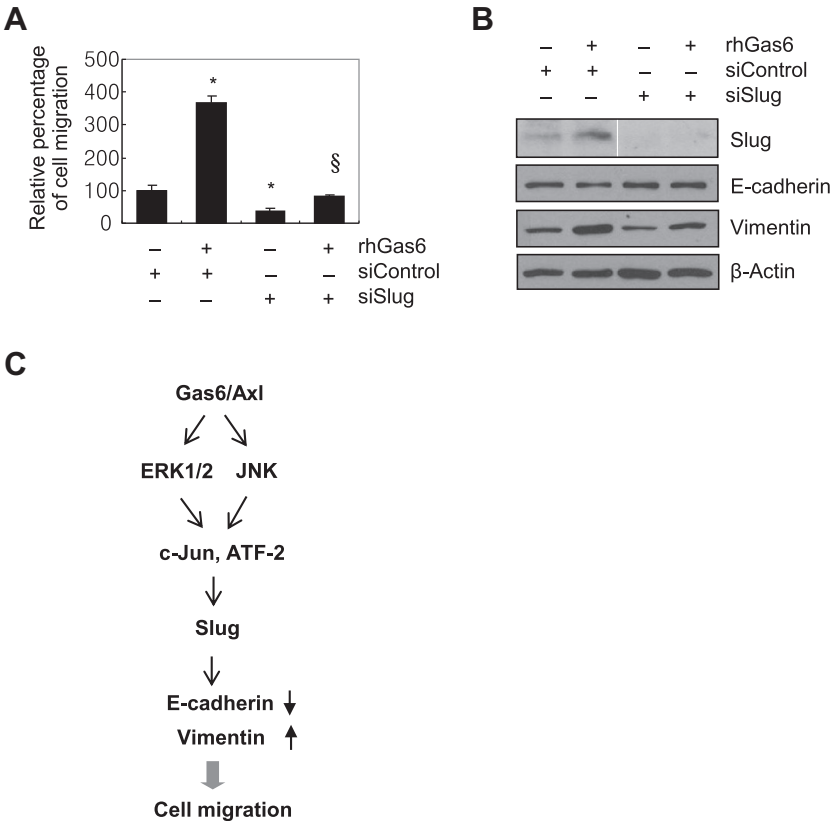

Fig. 4. Gas6 induced cell migration and EMT in a Slug-dependent mechanism. (A) DU145 cells were transfected with siRNA for 24 h and then subjected to the Transwell migration assay. Transfected cells (4×10^4) were allowed to migrate toward 0.5 μg/ml of rhGas6 for 48 h. Values represent mean ± SD. $^*P < 0.05$, compared with control siRNA (siControl); $^{\S}P < 0.05$, compared with control siRNA + rhGas6. (B) DU145 cells were transfected with siRNA for 24 h and then treated with 0.2 μg/ml of rhGas6 for 48 h. Whole-cell lysates were analyzed by immunoblotting. (C) A schematic representation illustrating the pathways for Gas6-mediated EMT events (E-cadherin reduction/vimentin induction) and increased cell migration in human cancer cells.

mediated depletion of Slug in DU145 cells resulted in a significant suppression of basal motility compared with control siRNA (Fig. 4A; lane 1 vs. lane 3). Furthermore, suppression of Slug expression substantially reduced Gas6-mediated cell migration (Fig. 4A; lane 2 vs. lane 4). Immunoblot analysis demonstrated that Gas6 reduced E-cadherin expression and induced vimentin expression; these effects were substantially reversed following the suppression of Slug expression (Fig. 4B). These results suggest that Gas6 induces cell migration and EMT marker regulation through a Slug-dependent mechanism.

4. Discussion

The Gas6/Axl axis modulates cellular functions, including migration, proliferation, and survival, and is involved in cancer metastasis and progression. Gas6/Axl induces prostate cancer cell invasion and survival during metastasis to bone marrow [11]. Recently, it has been reported that Axl induces prostate cancer cell proliferation and tumor growth mainly through activation of the Akt/NF-κB signaling pathway [13]. However, molecular mechanisms underlying Gas6/Axl-mediated cell migration and invasion remain poorly understood. In this study, we report that Gas6 treatment and subsequent Axl activation induce cell migration and EMT characteristics (E-cadherin reduction and vimentin induction) via upregulation of Slug. Slug expression mediated by Gas6 is mainly through the AP-1 transcription factors c-Jun and ATF-2 in an ERK1/2- and JNK-dependent manner. These data provide an important insight into the molecular basis mediating Gas6-mediated cell migration. The Gas6/Axl-Slug axis might be exploited as a target for potential anti-cancer metastasis therapy.

On the other hand, we observed that epidermal growth factor (EGF) treatment and subsequent EGF receptor activation increased phosphorylation of c-Jun and ATF-2 in A431 cells. However, Slug was not substantially increased although vimentin was induced following EGF treatment (Supplementary Fig. S3). Therefore, it is possible that Gas6/Axl-AP-1-Slug axis may not be directly involved in general EMT induced by conventional EMT inducers but play a specific role in inducing EMT under certain contexts.

In this study, we expected that Gas6-mediated effects would be largely Axl-dependent because Axl is a major receptor for Gas6 in DU145 cells, which express low levels of Tyro3/Sky and Mer (data not shown) [11], although we could not completely exclude the possibility that Tyro3/Sky or Mer receptor may be at least partially involved in Gas6-mediated effects. Recently, it has been reported that Axl is upregulated by E-cadherin transcriptional repressors, in particular, Snail and Slug in MCF10A immortalized breast epithelial cells that establish an autocrine signaling loop with Gas6, and Axl plays a critical role in breast cancer cell invasion [14]. It has been reported consistently that Axl is upregulated by Slug via vimentin induction and contributes to migration and lung metastasis of breast cancer cells [19]. Therefore, our observation that the Gas6/Axl axis upregulates Slug expression possibly suggests a positive feedback loop between Gas6/Axl and Slug that may contribute to cancer metastasis and progression. It is also possible that cancer cells can dramatically accelerate the aggressiveness of a malignancy by themselves via upregulation of Slug expression by autocrine and/or paracrine Gas6 protein production.

On the other hand, it has been reported that knockdown of Axl in PC3 and DU145 cells resulted in decreased expression of Slug and Snail at the protein level [20]. Therefore, it is possible that

Axl can regulate expression of EMT transcription factors through different/specific pathways in a context-dependent manner (e.g., depending on endogenous vs exogenous Gas6) although this remains to be determined.

Slug is a member of the Snail family and is upregulated in metastatic breast cancer, lung cancer, mesothelioma, and melanoma [7]. Recently, it has been reported that Slug is involved in metastatic prostate cancer cell invasion and migration [21,22]. However, the mechanism by which Slug is induced remains unknown. Slug is induced along with Snail by transforming growth factor-beta (TGFβ) directly through Smad3 in mouse epithelial cultures [23]. Slug is also rapidly induced by fibroblast growth factor (FGF) and hepatocyte growth factor (HGF), and is responsible for EMT [24]. Slug is upregulated in pancreatic cancer cells by TGFβ-induced JNK activation [25]. ERK-dependent expression of c-Jun/Fra-1 upregulates Slug gene expression to modulate breast cancer cell migration [18], indicating that the AP-1 transcription factor is involved in Slug expression. Here, we show that Gas6 induces c-Jun and ATF-2 phosphorylation in DU145 and A431 cells, respectively, through the ERK1/2 and JNK signaling pathways, resulting in Gas6-induced Slug expression. These observations suggest a novel signaling pathway for Slug upregulation.

Taken together, we provide an important insight into the molecular mechanisms mediating the Gas6-induced downregulation of E-cadherin and increased cell migration (Fig. 4C). We propose that Gas6 treatment results in the activation of Axl and activates ERK1/2 and JNK signaling pathways, which subsequently activate c-Jun and ATF-2. The activated AP-1 transcription factors increase the expression of Slug to downregulate E-cadherin and upregulate vimentin, which contributes to Gas6-induced cell migration. This finding suggests that anti-cancer metastasis therapies could be developed by targeting the Gas6/Axl-Slug axis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.082.

References

[1] W.G. Stetler-Stevenson, A.E. Yu, Proteases in invasion: matrix metalloproteinases, Semin. Cancer Biol. 11 (2001) 143–152.
[2] R. Kalluri, R.A. Weinberg, The basics of epithelial–mesenchymal transition, J. Clin. Invest. 119 (2009) 1420–1428.
[3] J.P. Thiery, H. Acloque, R.Y. Huang, M.A. Nieto, Epithelial–mesenchymal transitions in development and disease, Cell 139 (2009) 871–890.
[4] J.P. Thiery, J.P. Sleeman, Complex networks orchestrate epithelial–mesenchymal transitions, Nat. Rev. Mol. Cell Biol. 7 (2006) 131–142.
[5] G. van der Pluijm, Epithelial plasticity, cancer stem cells and bone metastasis formation, Bone 48 (2011) 37–43.
[6] J. Yang, R.A. Weinberg, Epithelial–mesenchymal transition: at the crossroads of development and tumor metastasis, Dev. Cell 14 (2008) 818–829.
[7] H. Peinado, D. Olmeda, A. Cano, Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype?, Nat Rev. Cancer 7 (2007) 415–428.
[8] G. Manfioletti, C. Brancolini, G. Avanzi, C. Schneider, The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade, Mol. Cell Biol. 13 (1993) 4976–4985.
[9] S. Hafizi, B. Dahlback, Gas6 and protein S. Vitamin K-dependent ligands for the Axl receptor tyrosine kinase subfamily, FEBS J. 273 (2006) 5231–5244.
[10] S. Goruppi, E. Ruaro, C. Schneider, Gas6, the ligand of Axl tyrosine kinase receptor, has mitogenic and survival activities for serum starved NIH3T3 fibroblasts, Oncogene 12 (1996) 471–480.
[11] Y. Shiozawa, E.A. Pedersen, L.R. Patel, A.M. Ziegler, A.M. Havens, Y. Jung, J. Wang, S. Zalucha, R.D. Loberg, K.J. Pienta, R.S. Taichman, GAS6/AXL axis regulates prostate cancer invasion, proliferation, and survival in the bone marrow niche, Neoplasia 12 (2010) 116–127.
[12] P.P. Sainaghi, L. Castello, L. Bergamasco, M. Galletti, P. Bellosta, G.C. Avanzi, Gas6 induces proliferation in prostate carcinoma cell lines expressing the Axl receptor, J. Cell Physiol. 204 (2005) 36–44.
[13] J.D. Paccez, G.J. Vasques, R.G. Correa, J.F. Vasconcellos, K. Duncan, X. Gu, M. Bhasin, T.A. Libermann, L.F. Zerbini, The receptor tyrosine kinase Axl is an essential regulator of prostate cancer proliferation and tumor growth and represents a new therapeutic target, Oncogene 32 (2013) 689–698.
[14] C. Gjerdrum, C. Tiron, T. Hoiby, I. Stefansson, H. Haugen, T. Sandal, K. Collett, S. Li, E. McCormack, B.T. Gjertsen, D.R. Micklem, L.A. Akslen, C. Glackin, J.B. Lorens, Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival, Proc. Natl. Acad. Sci. USA 107 (2010) 1124–1129.
[15] S.J. Holland, M.J. Powell, C. Franci, E.W. Chan, A.M. Friera, R.E. Atchison, J. McLaughlin, S.E. Swift, E.S. Pali, G. Yam, S. Wong, J. Lasaga, M.R. Shen, S. Yu, W. Xu, Y. Hitoshi, J. Bogenberger, J.E. Nor, D.G. Payan, J.B. Lorens, Multiple roles for the receptor tyrosine kinase axl in tumor formation, Cancer Res. 65 (2005) 9294–9303.
[16] H. Jung, K.P. Lee, S.J. Park, J.H. Park, Y.S. Jang, S.Y. Choi, J.G. Jung, K. Jo, D.Y. Park, J.H. Yoon, D.S. Lim, G.R. Hong, C. Choi, Y.K. Park, J.W. Lee, H.J. Hong, S. Kim, Y.W. Park, TMPRSS4 promotes invasion, migration and metastasis of human tumor cells by facilitating an epithelial–mesenchymal transition, Oncogene 27 (2008) 2635–2647.
[17] S. Kim, H.Y. Kang, E.H. Nam, M.S. Choi, X.F. Zhao, C.S. Hong, J.W. Lee, J.H. Lee, Y.K. Park, TMPRSS4 induces invasion and epithelial–mesenchymal transition through upregulation of integrin alpha5 and its signaling pathways, Carcinogenesis 31 (2010) 597–606.
[18] H. Chen, G. Zhu, Y. Li, R.N. Padia, Z. Dong, Z.K. Pan, K. Liu, S. Huang, Extracellular signal-regulated kinase signaling pathway regulates breast cancer cell migration by maintaining slug expression, Cancer Res. 69 (2009) 9228–9235.
[19] K. Vuoriluoto, H. Haugen, S. Kiviluoto, J.P. Mpindi, J. Nevo, C. Gjerdrum, C. Tiron, J.B. Lorens, J. Ivaska, Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer, Oncogene 30 (2011) 1436–1448.
[20] A. Mishra, J. Wang, Y. Shiozawa, S. McGee, J. Kim, Y. Jung, J. Joseph, J.E. Berry, A. Havens, K.J. Pienta, R.S. Taichman, Hypoxia stabilizes GAS6/Axl signaling in metastatic prostate cancer, Mol. Cancer Res. 10 (2012) 703–712.
[21] M. Emadi Baygi, Z.S. Soheili, F. Essmann, A. Deezagi, R. Engers, W. Goering, W.A. Schulz, Slug/SNAI2 regulates cell proliferation and invasiveness of metastatic prostate cancer cell lines, Tumour Biol. 31 (2010) 297–307.
[22] B. Uygur, W.S. Wu, SLUG promotes prostate cancer cell migration and invasion via CXCR4/CXCL12 axis, Mol. Cancer 10 (2011) 139.
[23] J. Zavadil, L. Cermak, N. Soto-Nieves, E.P. Bottinger, Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition, EMBO J. 23 (2004) 1155–1165.
[24] P. Savagner, K.M. Yamada, J.P. Thiery, The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial–mesenchymal transition, J. Cell Biol. 137 (1997) 1403–1419.
[25] C. Geismann, M. Morscheck, D. Koch, F. Bergmann, H. Ungefroren, A. Arlt, M.S. Tsao, M.G. Bachem, P. Altevogt, B. Sipos, U.R. Folsch, H. Schafer, S.S. Muerkoster, Up-regulation of L1CAM in pancreatic duct cells is transforming growth factor beta1- and slug-dependent: role in malignant transformation of pancreatic cancer, Cancer Res. 69 (2009) 4517–4526.