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CD26-mediated regulation of periostin expression contributes to migration and invasion of malignant pleural mesothelioma cells

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ABSTRACT

Malignant pleural mesothelioma (MPM) is an aggressive malignancy arising from mesothelial lining of pleura. It is generally associated with a history of asbestos exposure and has a very poor prognosis, partly due to the lack of a precise understanding of the molecular mechanisms associated with its malignant behavior. In the present study, we expanded on our previous studies on the enhanced motility and increased CD26 expression in MPM cells, with a particular focus on integrin adhesion molecules. We found that expression of CD26 upregulates periostin secretion by MPM cells, leading to enhanced MPM cell migratory and invasive activity. Moreover, we showed that upregulation of periostin expression results from the nuclear translocation of the basic helix-loop-helix transcription factor Twist1, a process that is mediated by CD26-associated activation of Src phosphorylation. While providing new and profound insights into the molecular mechanisms involved in MPM biology, these findings may also lead to the development of novel therapeutic strategies for MPM.

1. Introduction

Malignant pleural mesothelioma (MPM) is an aggressive malignancy arising from mesothelial lining of pleura [1]. It is generally associated with a history of asbestos exposure and has a very poor prognosis [2]. Once rare, the incident of MPM has increased in industrialized nations as a result of past wide spread exposure to asbestos [1,2]. The incident is predicted to increase further in the next decades, especially in developing countries where asbestos has not yet been prohibited [2]. Due to the lack of efficacy of conventional treatments, novel therapeutic strategies are urgently needed to improve outcomes [3].

CD26 is a 110-kDa type II transmembrane glycoprotein with known dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) activity in its extracellular domain and is capable of cleaving N-terminal dipeptides with either L-proline or L-alanine at the penultimate position [4]. CD26 has an important role in T cell biology and overall immune function [5]. In addition, CD26 is expressed in various cancers and is involved in cancer biology [6]. CD26 itself appears to be a novel therapeutic target, and anti-CD26 monoclonal antibody (mAb) treatment resulted in both *in vitro* and *in vivo* anti-tumor activity against several tumor types, including lymphoma and renal cell carcinoma [7–9]. Of note is that CD26 expression is associated with both pro- or anti-tumor effects in different cancers [6].

Recently, we showed that mesothelioma cells expressing high level of CD26 displayed high proliferative activity and invasiveness, and microarray analysis of CD26 knockdown and CD26-transfected mesothelioma cells showed that CD26 expression was closely linked to expression of genes contributing to cell proliferation and cell cycle regulation [10]. More recently, we demonstrated that CD9 suppressed cell adhesion by inhibiting CD26–α5β1 integrin complex through its negative regulation of

Abbreviations: bHLH, basic helix-loop-helix; csh, control shRNA; csi, control siRNA; FAS1, fasciclin I; CD26/10Chi, stable transfectant of MPM cell line, MSTO-211H with CD26–CD10 chimeric receptor; CD26WT, stable transfectant of MPM cell line, MSTO-211H with a full-length CD26; DPPIV, dipeptidyl peptidase IV; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial–mesenchymal transition; mAb, monoclonal antibody; MPM, malignant pleural mesothelioma; MSTO-P, MPM cell line MSTO-211H parental cells; pAb, polyclonal antibody; qPCR, quantitative real-time RT-PCR; shRNA, short hairpin RNA; siRNA, small interference RNA; TBP, TATA binding protein.

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CD26 [11]. These observations suggest that CD26 regulates the interaction of MPM cells with the extracellular matrix (ECM) via yet-to-be-determined integrin adhesion molecules. Meanwhile, with proximal signaling events associated with the cytoplasmic 6 amino acid residues of CD26 being characterized in normal human T lymphocytes [12], it is conceivable that similar CD26-mediated proximal signaling events, which remain to be clarified, play a pivotal role in MPM cell motility.

In the current study, we extended our previous studies on the enhanced motility and increased CD26 expression level in MPM cells to demonstrate CD26 upregulates expression and secretion of periostin (or osteoblast-specific factor 2) by MPM cells, leading to augmented migratory and invasive activity of MPM cells. Moreover, we showed that upregulation of periostin resulted from nuclear translocation of Twist1, a basic helix-loop-helix (bHLH) transcription factor, via CD26-associated phosphorylation of Src.

2. Materials and methods

2.1. Cell lines, antibodies and reagents

The human MPM cell line MSTO-211H (MSTO-P) was obtained from the American Type Culture Collection. JMN cells were a kind gift from Dr. Brenda Gerwin (Laboratory of Human Carcinogenesis, National Institutes of Health, Bethesda, MD, USA). MSTO-P were stably transfected with a full-length CD26 (CD26WT), CD26–CD10 chimeric receptor (CD26/10Chi) or vector alone (MSTO-Mock), which were developed in our laboratory [13]. Anti-CD26 goat polyclonal antibody (pAb) (AF1180) was from R&D Systems (USA), anti-Src kinase rabbit monoclonal antibody Ab (mAb) (36D10) and anti-phospho-Src family (Tyr416) (p-Src) rabbit mAb (D49G4) were from Cell Signaling Technology (USA), anti-Twist mouse mAb (Twist2C1a) and anti-TATA binding protein (TBP) mouse mAb (mAbcam 51841) from Abcam (UK), and anti-β-actin mouse mAb (AC-74) from Sigma–Aldrich (USA). Src specific inhibitor (PP2) was from Merk Millipore (USA) and stocked in 10 mM with DMSO. Measurement of periostin in supernatants was performed using commercially available enzyme-linked immunosorbent assay (ELISA) kit (Aviscera Bioscience, USA). The supernatants were harvested for measurement 48 h after (1×10^5/ml of each cell type were plated in the culture dish.

2.2. Short hairpin RNA (shRNA) and small interference RNA (siRNA)

To deplete endogenous CD26, two shRNAs were used (reference sequence: NM_001935) [14]. To deplete endogenous periostin, two siRNAs were used (reference sequence: NM_006475). The sequences of CD26-shRNAs and periostin-siRNAs are shown in Supplementary Table S1. shRNA and siRNA were obtained from Sigma–Aldrich. Knockdown experiments in MPM cell lines using shRNA or siRNA were achieved as described previously [11].

2.3. Cell migration and invasion assays

For cell migration assay, cells (500 μl of 1×10^5/ml in 0.1% FBS-RPMI1640) were seeded onto uncoated filters in a 24-well Transwell chamber (8-μm pore size; Costar, USA) with 750 μl of indicated medium in the lower cell, and allowed to migrate at 37 °C in 100% humidifier. For cell invasion assay, cells (500 μl of 1×10^5/ml in 0.1% FBS-RPMI1640) were seeded onto filters of a 24-well Transwell chamber that were coated with Matrigel (BD Biosciences, USA) with 750 μl of indicated medium in the lower cell. Migration or invasion of the cells through the chamber to the underside of the filter was assessed as described previously [11].

2.4. Western blotting and lipid raft fractioning

Total cell lysates, cytosolic and nuclear extracts for Western blot analysis were prepared as described elsewhere [15]. To obtain the lipid raft membrane fraction, indicated cell types (each, 1×10^8) were lysed on ice with 1 ml 1% Triton X-100 and 1 mM PMSF in MNE buffer (25 mM MES [pH 6.5], 150 mM NaCl, 5 mM EDTA), followed by sucrose-gradient ultracentrifugation as described previously [16]. Samples were submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and Western blot analysis using the indicated specific antibodies.

2.5. Quantitative real-time RT-PCR (qPCR) assay

In experiments assessing expression of mRNA of MPM cells, extraction of total RNA, generation of mRNA and quantification of mRNA were performed as described previously [17]. Expression levels of mRNA were calculated on the basis of standard curves generated for each gene and hypoxanthine phosphoribosyltransferase 1 (HPRT1) mRNA was used as an invariant endogenous control. Sequences of primers used in qPCR analysis are shown in Supplementary Table S2.

2.6. Statistical analysis

All experiments were performed in triplicates and repeated at least 3 times. Data were expressed as mean values ± SEM (standard error of the mean) and analyzed by one-way or two-way ANOVA followed by the Tukey–Kramer *post-hoc* test. The level of significance was $P < 0.05$. The calculations were conducted using Prism6.0 software (GraphPad Software, USA).

3. Results

3.1. Upregulation of periostin in CD26-expressing MPM cells

To understand the nature of CD26 association with certain molecules and MPM biology, we previously performed microarray analysis of CD26-depleted and CD26 over-expressed mesothelioma cells, with data having been deposited in NCBI's Gene Expression Omnibus and being accessible through GEO Series accession number GSE52216 [11]. Among these, we focused in the present study on the upregulation of the secretory protein periostin in CD26-expressing MPM cells as being correlated with migratory and invasive activity as integrin adhesion molecules. To determine that CD26 expression is associated with periostin upregulation, we first conducted knockdown experiments using shRNA against CD26 in JMN cells, which express CD26 endogenously. Expression of CD26 was determined by flow cytometry of JMN in the presence of control shRNA (csh) or two different sequences of CD26-shRNAs (sh-1 or sh-2) (Supplementary Fig. S1A). As shown in Fig. 1A, a significant decrease in the level of periostin mRNA was observed in JMN cells in the presence of CD26-shRNAs. In addition, periostin concentration in culture supernatant was significantly decreased in CD26-knockdown JMN cells (Fig. 1B). These data indicate that CD26 and periostin are correlatively expressed in MPM cells.

We next define the crucial role of the CD26 cytoplasmic region in regulating periostin expression. For this purpose, we analyzed periostin expression in MSTO-CD26WT, MSTO-CD26/10Chi and MSTO-Mock cells. The CD26–CD10 chimeric receptor (CD26/10Chi) was composed of the N-terminal cytoplasmic region of human CD10 (1–23 amino acid position) ligated to the transmembrane and extracellular regions of human CD26 (7–766 amino acid position), and this mutant chimeric receptor was shown to

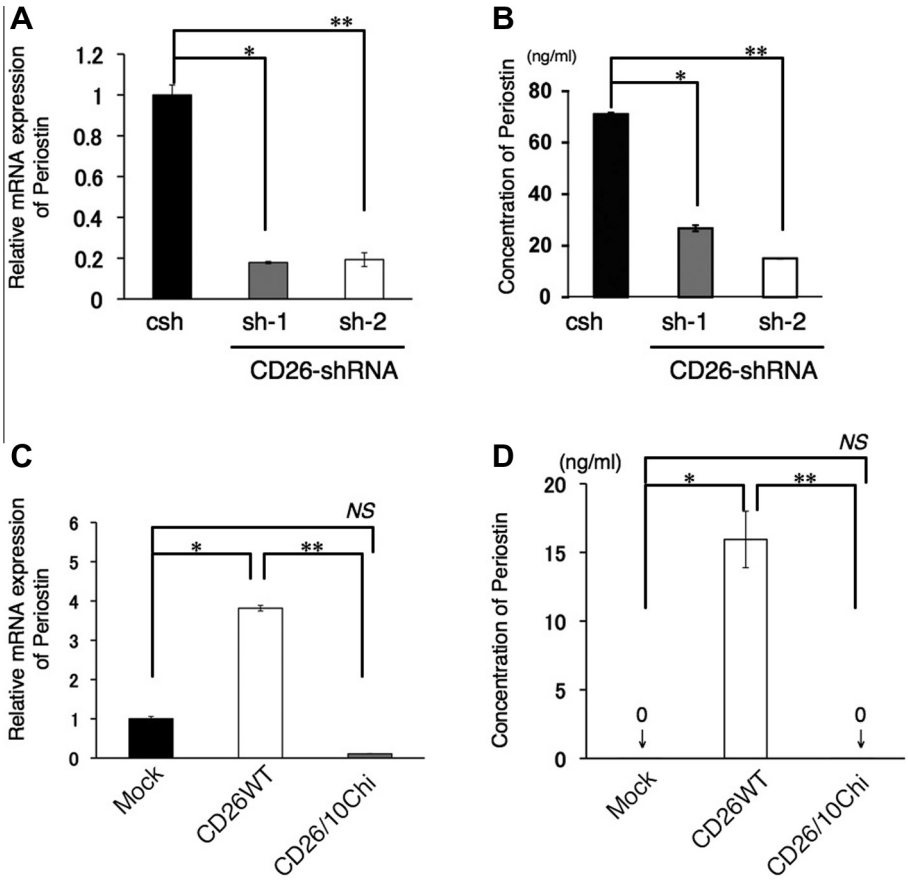

Fig. 1. Upregulation of periostin in CD26-expressing MPM cells. (A) JMN cells were transfected with two different CD26-shRNAs (sh-1 or sh-2) or control shRNA (csh), and mRNA expression of periostin was quantified by real-time RT-PCR. Each expression was normalized to hypoxanthine phosphoribosyltransferase 1 (HPRT1) and relative expression levels compared with the sample of JMN with csh were shown. Data are shown as mean ± SEM of three different experiments. *, **$P < 0.0001$. (B) Production of periostin protein in the culture supernatants of shRNA-transfected JMN (csh. sh-1 or sh-2) was quantified by ELISA. Data are shown as mean ± SEM of three different experiments with triplicates. *, **$P < 0.0001$. (C) In MSTO-Mock, MSTO-CD26WT or MSTO-CD26/10Chi cells, mRNA expression of periostin was quantified by real-time RT-PCR. Each expression was normalized to HPRT1 and relative expression levels compared with the sample of MSTO-Mock were shown. Data are shown as mean ± SEM of three different experiments. *, **$P < 0.0001$. *NS* denotes 'not significant'. (D) Production of periostin protein in the culture supernatants of MSTO-Mock, MSTO-CD26WT or MSTO-CD26/10Chi cells was quantified by ELISA. Data are shown as mean ± SEM of three different experiments with triplicates. *, **$P < 0.0001$. *NS* denotes 'not significant'.

abrogate CD26-mediated costimulation in T cells [18]. CD10, as is the case with CD26, is a type II transmembrane glycoprotein with a relatively short cytoplasmic tail containing signal sequence that has an expected membrane topology similar to CD26 [19,20]. Cell surface CD26 expression levels were confirmed by flow cytometry (Supplementary Fig. S1B). As shown in Fig. 1C, periostin mRNA level was significantly increased in MSTO-CD26WT, compared to MSTO-CD26/10Chi or MSTO-Mock. In addition, using ELISA, we observed that protein expression level of periostin was significantly increased in MSTO-CD26WT, compared to MSTO-CD26/10Chi or MSTO-Mock (Fig. 1D). These observations indicate that periostin upregulation is associated with expression of a full-length CD26 molecule, but not with the CD26 extracellular region containing DPPIV activity, suggesting that the intracytoplasmic region of CD26 mediates upregulation of periostin in MPM cells.

3.2. CD26 expression leads to nuclear translocation of Twist1 via activation of Src

It has been previously shown that transcription of periostin is regulated by the bHLH transcription factor Twist1 as a downstream signaling event of c-Src [21,22]. For this purpose, we analyzed the expression levels of these signaling molecules in JMN cells in the presence of CD26-shRNAs. As shown in Fig. 2A, a significant decrease in p-Src level was observed in JMN cells in the presence of CD26-shRNAs (lanes 2 and 3), compared to those treated with control shRNA (lane 1). In addition, as shown in Fig. 2B, p-Src level was increased in MSTO-CD26WT (lane 2), compared to MSTO-Mock (lane 1) or MSTO-CD26/10Chi (lane 3). Moreover, as shown in Fig. 2C, a significant decrease in nuclear Twist1 level was observed in JMN cells in the presence of CD26-shRNAs (lanes 2 and 3 of second panel from the bottom), compared to those treated with control shRNA (lane 1 of second panel from the bottom). Furthermore, as shown in Fig. 2D, nuclear Twist1 level was clearly increased in MSTO-CD26WT (lane 2), compared to MSTO-Mock (lane 1) or MSTO-CD26/10Chi (lane 3), with Twist1 level being higher in the cytoplasmic fraction (top panel) than in the nuclear fraction (second panel from the bottom). These data suggest that the expression of CD26, particularly its intracytoplasmic region, mediates increased Twist1 level and activity through phosphorylation of Src, resulting in enhanced periostin expression.

Since the cytoplasmic tail of CD26 consists of only 6 amino acids in length without any conserved kinase or protein-binding domain, it is unlikely that such a short cytoplasmic domain proximal to the membrane interacts directly with Src. On the other hand, lipid raft clustering plays a crucial role in CD26-mediated costimulatory signaling or activation of Src kinase [16,18,23]. We thus analyzed the molecular clustering induced by lipid raft aggregation in MPM cells. As shown in Fig. 2E, increased levels of CD26, p-Src and total Src molecules in lipid raft fractions were observed in

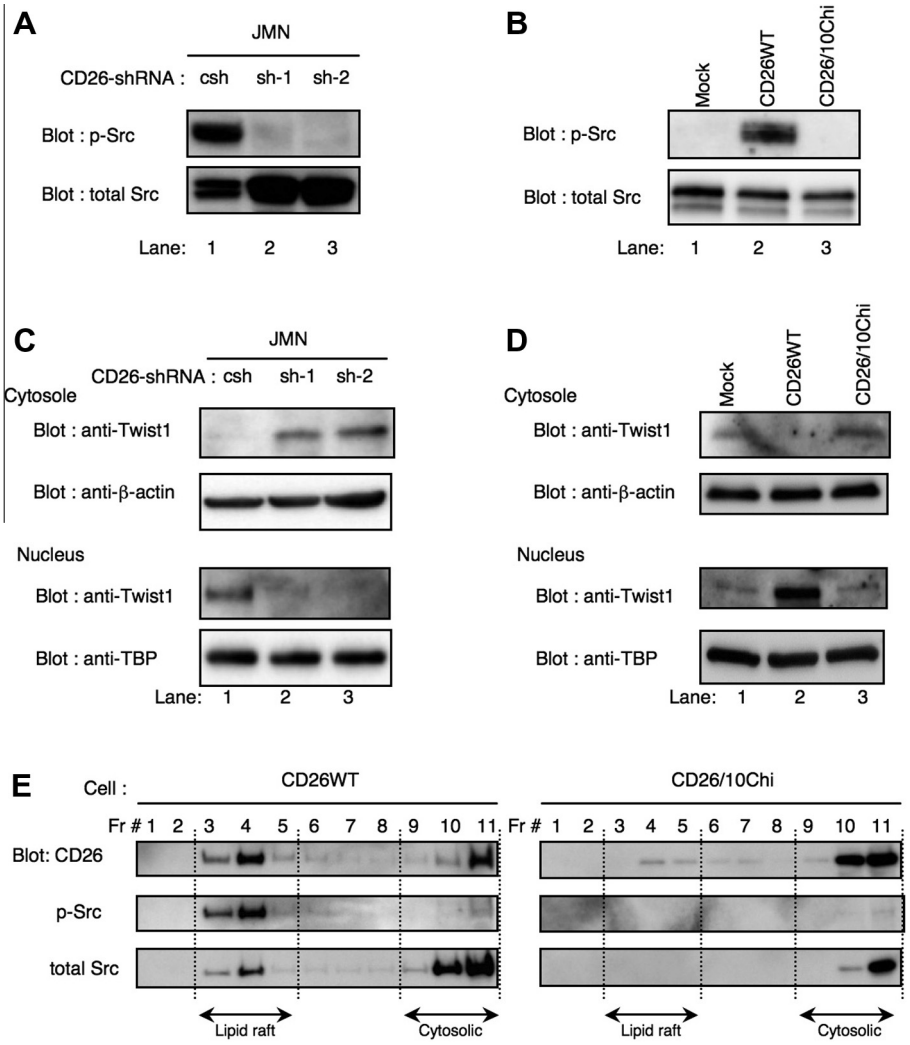

Fig. 2. Nuclear translocation of Twist1 via phosphorylation of Src. (A) Total lysates (each, 10 μg) from JMN transfected with two different CD26-shRNAs (sh-1 or sh-2) or control shRNA (csh) and immunoblotted using anti-phospho Src (p-Src) mAb followed by stripping and reprobing with anti-Src mAb (total Src). Similar results were obtained in three independent experiments. (B) Total lysates from each cell type were resolved by SDS–PAGE, and immnoblotted by the same method as conducted in (A). Similar results were obtained in three independent experiments. (C) Cytosolic (upper two panels) and nuclear extracts (lower two panels) (each, 10 μg) from JMN transfected with two different CD26-shRNAs (sh-1 or sh-2) or control shRNA (csh) were resolved by SDS–PAGE, and immnoblotted using anti-Twist1 mAb, followed by stripping and reprobing with anti-β-actin mAb or anti-TBP (TATA-binding protein) mAb, which were used as a quality control marker indicating equal amounts in the experiments. Similar results were obtained in three independent experiments. (D) Cytosolic (upper two panels) and nuclear extracts (lower two panels) from each cell type were resolved by SDS–PAGE, and immnoblotted by the same method as conducted in (C). Similar results were obtained in three independent experiments. (E) Lipid raft or cytosolic fractions of MSTO-CD26WT (left panels) or CD26/10Chi (right panels) cells were prepared by sucrose gradient ultracentrifugation. The distribution of CD26, phosphorylated Src (p-Src), and total Src was determined by immunoblotting with respective antibodies. Fraction number (Fr#) 3–5 or 9–11 contains lipid raft or cytosolic fractions, respectively. Similar results were obtained in three independent experiments.

MSTO-CD26WT (left panels), while in MSTO-CD26/10Chi, most CD26/10Chi and Src molecules were located in the cytosolic fractions, and phosphorylated Src was not observed (right panels). These results suggest that the cytoplasmic region of CD26 plays a pivotal role in the clustering of Src molecules in lipid rafts, hence providing a platform for downstream signaling leading to nuclear translocation of Twist1 in CD26-expressing MPM cells.

3.3. Src inhibitor decreases production of periostin

As shown above, since Src kinase appears to play an important role in the upregulation of periostin expression, we next examined whether this process is affected by Src inhibition. As shown in Fig. 3A, in the presence of the Src inhibitor PP2 (lane 2 of panels a or b), Twist1 nuclear translocation (second panels from the bottom) was clearly decreased in JMN or MSTO-CD26WT, compared to treatment with control solvent (lane 1 of panels a or b). Moreover, a significant decrease in periostin mRNA expression in JMN or MSTO-CD26WT treated with PP2 was observed (Fig. 3B). Similarly, a significant decrease in secretion of periostin in JMN or MSTO-CD26WT treated with PP2 was observed (Fig. 3C). These observations strongly suggest that expression of a full-length CD26 in MPM cells activates Src/Twist signaling, promoting subsequent upregulation of periostin expression and secretion.

3.4. Periostin enhances migration and invasion of MPM cells

We recently demonstrated that CD26–α5β1 integrin complex enhances cell migration and invasion in CD26-expressing MPM cells [11]. Since periostin plays a pivotal role in cell migration and invasion via its interaction with integrin and ECM [24], we next examined whether periostin enhances cell motility in MPM cells. As shown in Fig. 4A, a significant increase in migration or invasion was observed in MSTO-CD26WT (white bars of panels a

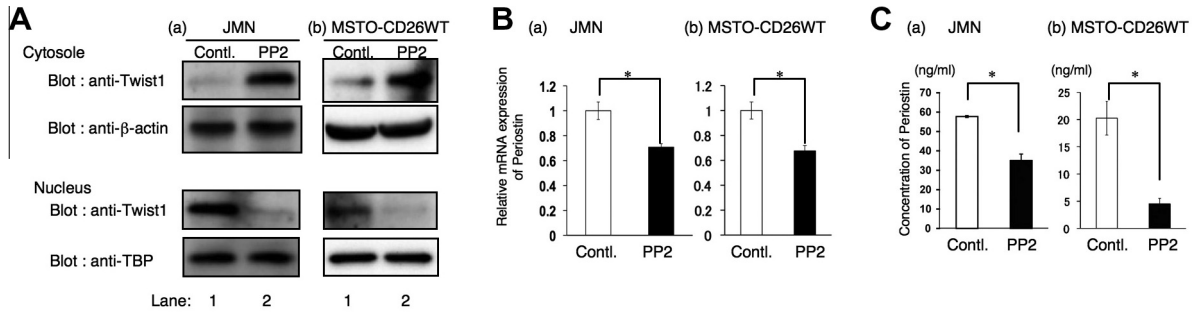

Fig. 3. Inhibition of Twist1 nuclear translocation and decreased production of periostin following treatment with Src inhibitor PP2. (A) JMN (*panel a*) or MSTO-CD26WT (*panel b*) cells were treated with Src inhibitor PP2 (1 μM) or DMSO as control solvent (Contl.) for 30 min. Cytosolic (upper two panels) and nuclear extracts (lower two panels) (each, 10 μg) from each cell type were resolved by SDS–PAGE, and immnoblotted using anti-Twist1 mAb, followed by stripping and reprobing with anti-β-actin mAb or anti-TBP mAb, which were used as a quality control marker indicating equal amounts in the experiments. Similar results were obtained in three independent experiments. (B) JMN (*panel a*) or MSTO-CD26WT (*panel b*) cells were cultured for 24 h in the presence of Src inhibitor PP2 (1 μM) or DMSO as control solvent (Contl.). mRNA expression of periostin was quantified by real-time RT-PCR. Each expression was normalized to HPRT1 and relative expression levels compared with the sample of each control solvent were shown. Data are shown as mean ± SEM of three different experiments. $^{*}P < 0.0001$. (C) JMN (*panel a*) or MSTO-CD26WT (*panel b*) cells were cultured by the same method conducted as in (B). The culture supernatants were harvested, and concentration of periostin was quantified by ELISA. Data are shown as mean ± SEM of three different experiments with triplicates. $^{*}P < 0.0001$.

or b) in which periostin was upregulated (Fig. 1C and D), compared to MSTO-Mock or MSTO-CD26/10Chi (black or gray bars, respectively). On the other hand, in the presence of siRNAs against periostin, a significant decrease in migration or invasion was observed in JMN and MSTO-CD26WT, compared to those treated with control siRNA (Fig. 4B and C). Data demonstrating knockdown of periostin by siRNAs, and the lack of effect on CD26 expression levels, are shown in Supplementary Fig. S2. In contrast, a significant increase in migration or invasion was observed in MSTO-Mock (Fig. 4D) and MSTO-CD26/10Chi (Fig. 4E) in the presence of periostin-containing culture medium (white bars), which was not observed in the presence of medium lacking periostin (black bars of Fig. 4D and E). These observations hence suggest that cell migration and invasion are enhanced by the presence of exogenous periostin. Taken together with the above data, our findings strongly indicate that migration and invasion of MPM cells are enhanced by periostin produced through CD26-regulated Src/Twist1 signaling.

4. Discussion

In the present study, we show that CD26-expressing MPM cells exhibit enhanced production of periostin via activation of Src/Twist1 signaling. Moreover, the presence of periostin in the culture medium leads to increased cell migration and invasion of MPM cells.

We recently demonstrated that CD9 suppressed cell invasion and migration by inhibiting the formation of CD26–α5β1 integrin complex with an associated decrease in phosphorylation of β1 integrin-related proteins such as focal adhesion kinase (FAK) and Crk-associated substrate lymphocyte type (Cas-L/HEF1/NEDD9) [11]. While these finding indicate that CD26 regulates ECM-associated tumor cell behavior, the exact molecular events in tumor biology involving ECM interaction that are associated with the cytoplasmic region of CD26 remain to be elucidated. In the present study, we demonstrate that the CD26 cytoplasmic region plays a crucial role in *in vitro* migration and invasion of MPM cells through its linkage to periostin production mediated by Src/Twist1 signaling.

Periostin is a secreted cell adhesion protein of approximately 90 kDa, which shares a homology with the insect cell adhesion molecule fasciclin I (FAS1) [25]. The N-terminal region regulates cellular functions by binding to integrins at the plasma membrane of the cells through cell adhesion domain, FAS1 [25]. The C-terminal region of the protein regulates cell–matrix organization and interaction by binding such ECM proteins as collagen, fibronectin, tenascin C, and acid mucopolysaccharides, such as heparin and periostin itself [26], leading to increased tumor cell motility [24]. In the clinical setting, periostin upregulation has been reported for many cancer types including MPM, and may potentially be a tumor-enhancing factor [25]. Previous work also suggested that periostin expression in MPM cells may be an independent prognostic factor for overall survival [27]. Periostin was shown to be not only a marker of epithelial–mesenchymal transition (EMT), but to be itself an inductor of this phenomenon [28]. The association of EMT with periostin produced by CD26-expressing MPM cells will be investigated in future studies.

Since the cytoplasmic tail of CD26 is only 6 amino acids in length without any conserved kinase or protein-binding domains, it is conceivable that CD26 may exert its effect on Src activation in an indirect manner. Meanwhile, it is recognized that lipid raft platforms may activate or inhibit many signaling pathways, including CD26-mediated costimulation in human normal T cells [16,18]. These platforms may be of utmost relevance for the integration of complex signaling processes such as those involved with cell migration, as lipid rafts may favor protein interaction in specific cell locations. In fact, we showed that Src was clustered in lipid rafts along with the CD26 molecules. Moreover, the short cytoplasmic tail preceding the transmembrane domain of CD26 is essential for locking CD26 molecules in the lipid bilayer [29]. Accordingly, we postulate that Src/Twist1 activation occurs through lipid raft partitioning and/or clustering by CD26 molecules.

In conclusion, CD26-associated upregulation of periostin in MPM cells results from nuclear translocation of Twist1 transcription factor as downstream signaling events of CD26-mediated activation of Src. Importantly, increased secretion of periostin leads to enhanced migratory and invasive activity of MPM cells. By providing further insights into the molecular mechanisms of MPM biology, our current findings may lead to future development of novel therapeutic strategies for MPM.

Acknowledgments

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The authors declare no competing financial interests.

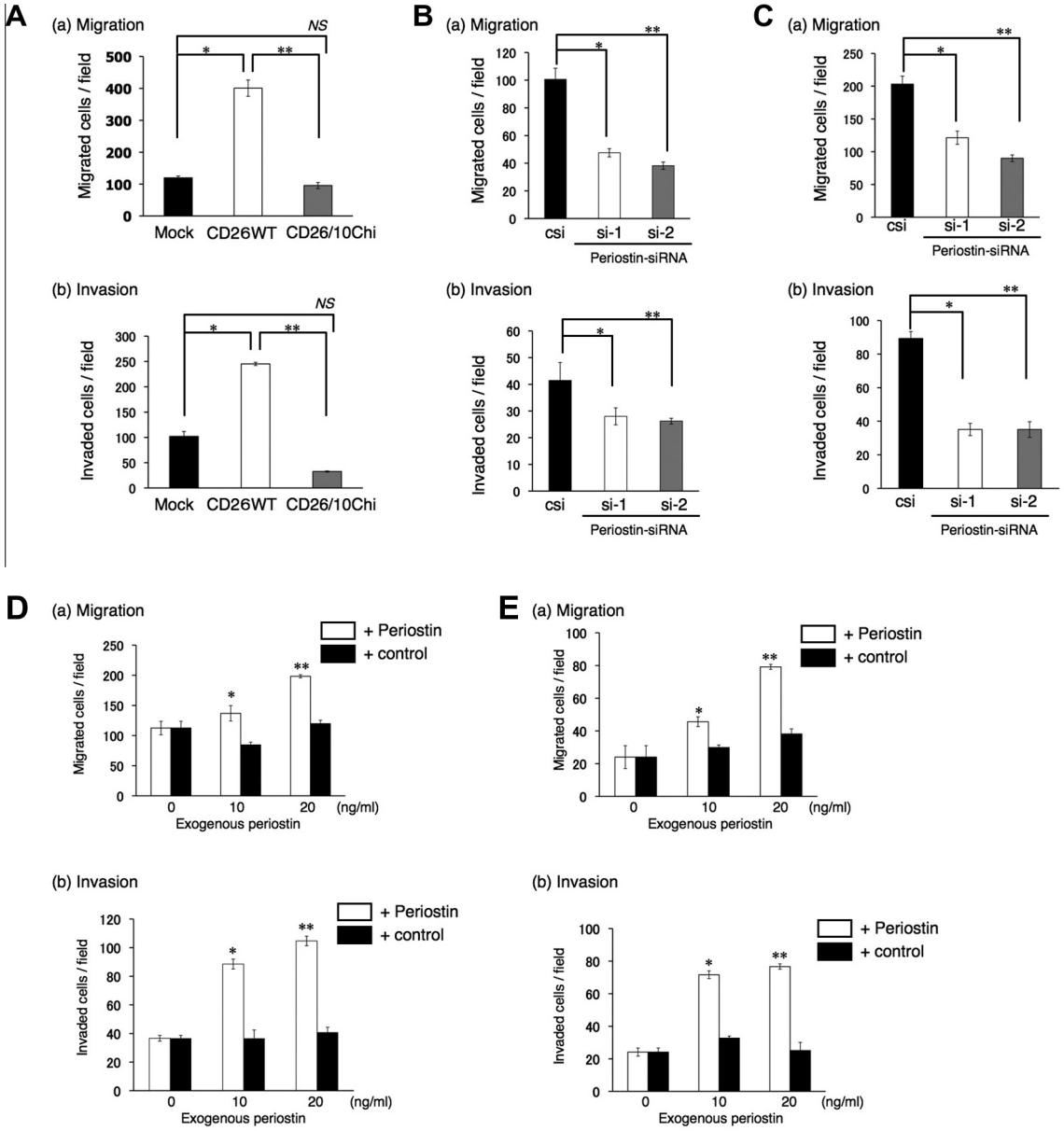

Fig. 4. Periostin enhances migration and invasion of MPM cells. (A) MSTO-Mock, CD26WT or CD26/10Chi cells were seeded on top of an uncoated filter (*panel a*) or Matrigel-coated (*panel b*) chamber inserts. The number of cells that migrated or invaded through the insert filter in the lower chamber was counted. The mean number of cells per field was determined from 5 fields per filter. Data are shown as mean ± SEM of three independent experiments with triplicates. *, **$P < 0.0001$. *NS* denotes 'not significant'. (B and C) MSTO-CD26WT (B) or JMN (C) cells were transfected with two different Periostin-siRNAs (si-1 or si-2) or control siRNA (csi). After 24 h of transfection, cells were subjected to migration (*panels a*) or invasion (*panels b*) assay by the same method conducted as in (A). *, **$P < 0.0001$. (D and E) MSTO-Mock (D) or CD26/10Chi (E) cells were seeded on top of an uncoated filter (*panels a*) or Matrigel-coated (*panels b*) chamber inserts with periostin- or control solvent-medium (white or black bars, respectively) at indicated concentrations in the lower cell. The mean number of cells per field was determined from 5 fields per filter. Data are shown as mean ± SEM of three independent experiments with triplicates. *, **$P < 0.0001$ *vs.* 0 ng/ml of periostin or corresponding control solvent. Both the white and black bars at 0 ng/ml of periostin or control solvent were plotted using the same data.

E.K, and K.O. contributed to the conception and design of the study, or acquisition of data, H.Y., R.H., S.I., T.O., and T.Y. contributed to analysis and interpretation of data, K.O. and C.M. designed the research, interpreted the data and wrote the paper, N.H.D. interpreted the data, assisted with the paper, and proofread the manuscript. All authors showed final approval of the version to be submitted.

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.2014.04.037.

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