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Placental Growth Factor-1 and -2 Induce Hyperplasia and Invasiveness of Primary Rheumatoid Synoviocytes

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Inflammation-mediated oncogenesis has been implicated in a variety of cancer types. Rheumatoid synovial tissues can be viewed as a tumor-like mass, consisting of hyperplastic fibroblast-like synoviocytes (FLSs). FLSs of rheumatoid arthritis (RA) patients have promigratory and invasive characteristics, which may be caused by chronic exposure to genotoxic stimuli, including hypoxia and growth factors. We tested whether a transformed phenotype of RA-FLSs is associated with placental growth factor (PlGF), a representative angiogenic growth factor induced by hypoxia. In this study, we identified *PlGF-1* and *PlGF-2* as the major *PlGF* isoforms in RA-FLSs. Global gene expression profiling revealed that cell proliferation, apoptosis, angiogenesis, and cell migration were mainly represented by differentially expressed genes in RA-FLSs transfected with small interfering RNA for *PlGF*. Indeed, *PlGF*-deficient RA-FLSs showed a decrease in cell proliferation, migration, and invasion, but an increase in apoptotic death in vitro. *PlGF* gene overexpression resulted in the opposite effects. Moreover, exogeneous PlGF-1 and PlGF-2 increased survival, migration, and invasiveness of RA-FLSs by binding their receptors, Flt-1 and neuropilin-1, and upregulating the expression of antiapoptotic molecules, pErk and Bcl2. Knockdown of *PlGF* transcripts reduced RA-FLS proliferation in a xenotransplantation model. Collectively, in addition to their role for neovascularization, PlGF-1 and -2 promote proliferation, survival, migration, and invasion of RA-FLSs in an autocrine and paracrine manner. These results demonstrated how primary cells of mesenchymal origin acquired an aggressive and transformed phenotype. PlGF and its receptors thus offer new targets for anti-FLS therapy. ***The Journal of Immunology*****, 2015, 194: 2513–2521.**

Rheumatoid arthritis (RA) is characterized by tumor-like expansion of the synovium, angiogenesis, and destruction of adjacent articular cartilage and bone (1, 2).Various cell populations, including innate immune cells, adaptive immune cells, endothelial cells, and fibroblast-like synoviocytes (FLSs), are activated in RA joints (3). FLSs in particular represent a major effector in the invasive pannus, directly participating in chronic inflammation and joint destruction (4). They produce high levels of matrix metalloproteinases, proinflammatory cytokines, such as IL-1 and IL-6 (3, 5), and angiogenic factors, including vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) (6, 7). Moreover, although RA-FLSs are primary cells, they proliferate abnormally and exhibit characteristics of metastatic cancer cells, represented by somatic mutations of H-Ras and p53 genes (8, 9). Besides abnormal proliferation, RA-FLSs also show invasiveness and excessive migratory capacity (10, 11), although the mechanisms involved are not fully understood. Despite the importance of FLS in RA pathogenesis, there have been no trials to specifically suppress FLS proliferation and invasiveness.

PlGF, originally identified in the placenta, is a member of the VEGF family (12, 13). The human *PlGF* gene encodes four different isoforms, that is, *PlGF-1* (131 aa), *PlGF-2* (152 aa), *PlGF-3* (203 aa), and *PlGF-4* (224 aa), as a result of alternative splicing, whereas mice only express *PlGF-2* (12, 13). Unlike VEGF, which binds to VEGFR-1 (Flt-1) and VEGFR-2, PlGF selectively binds VEGFR-1 and its coreceptors neuropilin (NP)-1 and -2 (12). PlGF has potent angiogenic activities via Flt-1, and it directly mediates chronic inflammation by stimulating leukocyte infiltration (14–16). Furthermore, PlGF regulates the migration and survival of myeloid and endothelial cells (12, 14, 17). PlGF is also expressed in various cell types, including hematopoietic cells, keratinocytes, and bronchial epithelial cells, and it plays a critical role in tumor growth, invasion, and metastasis in some types of cancer (12, 14).

Chronic inflammation has been implicated in tumorigenesis (18–21). Inflammation-mediated oncogenesis is found in a variety of cancers, including skin, lung, bladder, stomach, and liver cancer

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Abbreviations used in this article: DEG, differentially expressed gene; FLS, fibroblast-like synoviocyte; GOBP, gene ontology biological process; KEGG, Kyoto Encyclopedia of Genes and Genomes; NP, neuropilin; PlGF, placental growth factor; RA, rheumatoid arthritis; siRNA, small interfering RNA; SNP, sodium nitroprusside; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

(18). Although the exact mechanism is still unclear, some growth factors and proinflammatory cytokines play an important role. For example, mice deficient in TNF-α are resistant to skin carcinogenesis (19). Host-derived IL-1 is required for tumor invasiveness and angiogenesis. Constitutive expression of platelet-derived growth factor-BB can lead to malignant transformation of human cells (20). Sustained JNK1 activation enhances chemical hepatocarcinogenesis (21). Taken together, it appears that under pathological conditions, chronic exposure to proinflammatory cytokines and growth factors may convert host primary cells to a transformed phenotype.

PlGF, an angiogenic growth factor, is frequently found at high concentrations in RA joints (6). We postulated that a transformed and aggressive phenotype of primary RA-FLSs may be caused by chronic exposure to proinflammatory cytokines and growth factors, including PlGF. We first demonstrated that PlGF-1 and -2 were critical for proliferation and invasiveness of primary rheumatoid synoviocytes. *PlGF*-deficient RA-FLSs showed a decrease in cell proliferation, migration, and invasion, but an increase in apoptotic death. Conversely, exogeneous PlGF-1 and PlGF-2 increased survival, migration, and invasiveness of RA-FLSs by binding their receptors, Flt-1 and NP-1, and upregulated the expression of antiapoptotic molecules, pErk and Bcl2. Collectively, our data indicate that PlGF-1 and -2 promote proliferation, survival, migration, and invasion of primary rheumatoid cells, and thus could be a potential target for anti-FLS therapy.

Materials and Methods

Isolation and culture of synoviocytes

FLSs were prepared from the synovial tissues of RA patients as described previously (22) and incubated in DMEM supplemented with 10% FBS.

Microarray experiments

Total RNA was isolated from RA-FLSs 12 h after *PlGF* small interfering RNA (siRNA) or control siRNA treatment. RNA integrity was evaluated by a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). The RNA integrity number in all samples was >9.5. Following the standard Agilent protocols, RNA was reverse-transcribed, amplified, and then hybridized onto the Agilent SurePrint G3 human gene expression 8 × 60K v2 microarray containing 62,976 probes for 23,284 annotated genes. The data were deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64922) under accession no. GSE64922.

Overexpression of PlGF-2 gene in SV40-immortalized RA-FLSs

The *PlGF* plasmid DNA tagged with pCDNA3 (24 μg) was transfected into SV40-immortalized RA-FLSs by Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Three to four weeks after transfection, stable expression of *PlGF-2* in transfected cells was screened by ELISA, and bulk populations of cells harboring *PlGF* constructs tagged with pCDNA3 were selected. The cells were subsequently maintained in 0.5 mg/ml G418 (Life Technologies, Gaithersburg, MD).

Knockdown of PlGF and PlGF receptor transcripts

RA-FLSs were transfected with *PlGF* siRNA, *Flt-1* siRNA, or *NP-1* siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) using Lipofectamine 2000 (22).

Real-time PCR

Total RNA was isolated from RA-FLSs using an RNeasy Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Real-time PCR was performed in the CFX96 real-time PCR system (Bio-Rad, Hercules, CA) using SYBR Premix (Bio-Rad). Transcript levels were calculated relative to controls, and relative fold inductions were calculated by using the $2^{-\Delta\Delta Ct}$ algorithm. The following primers were used for amplification (forward, reverse): E2F2, 5′-GGTGAGCTGAAGAACCTTGC-3′ and 5′-GCCCAGAGCTGCAGTTTAAT-3′; CDK1, 5′-TTTTCAGAGCTTTGGGCACT-3′ and 5′-AAATTCGTTTGGCTGGATCA-3′; CASP1, 5′-GCTTTCTGCTCTTCCACACC-3′ and 5′-CATCTGCGCTCTACCATCTG-3′; CASP7, 5′-GGCAAAGATTTTTGGCACTT-3′ and 5′-TGGTTAGGAATTAAGCAACCACA-3′; BCL2, 5′-TTTACCTTCCATGGCTCTTTTT-3′ and 5′-GGCAATGTGACTTTTTCCAA-3′; NANOS1, 5′-CAGTATTAATTTGGGCGGGTA-3′ and 5′-AAGGTGGAAAAGGCTAGTTGC-3′; KIT, 5′-ACTTCAGGGGCACTTCATTG-3′ and 5′-GACTCATGGGCTTGGGAATA-3′; CCL28, 5′-GGGAAACACGAAACATACGG-3′ and 5′-TTGGCTACATTTGCATACCG-3′; PlGF-1, 5′-CTGCGAATGCCGGCCTCT-3′ and 5′-GCACCTTTCCGGCTTCAT-3′; PlGF-2, 5′-CTGCGAATGCCGGCCTCT-3′ and 5′-TGTGGGTCTCTGCTTCTCTCT-3′; PlGF-3, 5′-GGCTGATGCTCCCTCCTT-3′ and 5′-GCACCTTTCCGGCTTCAT-3′; PlGF-4, 5′-GGCTGATGCTCCCTCCTT-3′ and 5′-TGTGGGTCTCTGCTTCTCTCT-3′; GAPDH, 5′-TGATGACATCAAGAAGGTGG-3′ and 5′-TTTCTTACTCCTTGGAGGCC-3′.

PlGF-1– and PlGF-2–conditioned media preparation

HEK-293 stable clones overexpressing human PlGF-1 or human PlGF-2 were obtained as previously described (23). A stable clone obtained after transfection of empty pCDNA3 vector was used as control. Serum-free–conditioned media were collected form semiconfluent cultured cells and concentrated on a Centricon (Sartorius) with a 10,000 Da molecular mass cutoff. The concentration of PlGF-1 and PlGF-2 was determined by sandwich ELISAs, as previously described (23).

ELISA for PlGF

The PlGF concentrations were also measured in the culture supernatants using an ELISA kit (R&D Systems, Minneapolis, MN).

Immunofluorescence staining

RA synovium was fixed with cold acetone for 10 min at −20°C and blocked with 1% BSA for 30 min. Tissue sections were then incubated with goat anti-PlGF Abs (1:100; Santa Cruz Biotechnology) plus mouse anti-CD55 Abs (1:200; Santa Cruz Biotechnology) or with goat anti-PlGF Abs (1:100; Santa Cruz Biotechnology) plus mouse anti-CD68 Abs (1:50; Santa Cruz Biotechnology) overnight at 4°C. Each slide was washed three times in PBS and incubated with Cy3-conjugated anti-goat IgG and Alexa Fluor 488–conjugated anti-mouse IgG (Invitrogen). After washing in PBS, the coverslips were mounted on glass slides with ProLong antifade solution (Invitrogen).

Cell proliferation assay

FLS proliferation rate after *PlGF* siRNA transfection was determined using a BrdU proliferation assay kit according to the manufacturer's instructions. Manual cell counts were performed by trypan blue exclusion to identify viable cells.

Determination of cell viability

The viability of FLSs was determined by an MTT assay as described previously (22).

Detection of apoptosis

FLS apoptosis was determined using the APOPercentage apoptosis assay kit (24). Digital images of APOPercentage dye-labeled cells, which appear bright pink against a white background under a light microscope, were used to quantify apoptotic cell numbers. FLS apoptosis levels were expressed as a pixel number.

Western blot analysis

FLSs were lysed in a lysis buffer, and insoluble material was removed by centrifugation at 14,000 rpm for 20 min at 4°C. Final protein concentrations were determined using the Bradford protein assay. Electrophoresis was performed using SDS-PAGE, and blots were transferred to nitrocellulose membranes. Membranes were incubated with Abs to PlGF (Santa Cruz Biotechnology), Bcl2 (Santa Cruz Biotechnology), Bax (Santa Cruz Biotechnology), pErk (Cell Signaling Technology, Beverly, MA), Erk (Cell Signaling Technology), pAkt (Cell Signaling Technology), Akt (Cell Signaling Technology), and β-actin (Sigma-Aldrich). Membranes were then visualized using an enhanced chemiluminescent technique.

Wound migration assay

The wound migration of RA-FLSs was measured as described previously (25). In brief, FLSs plated to confluence on a six-well plate were wounded with pipette tips and then treated with IL-1β or PlGF in DMEM supple-

mented with 1% FBS. After 12 h of incubation, FLS migration was quantified by counting the cells that had moved beyond a reference line. The migration of *PlGF*-deficient RA-FLSs was assessed in some experiments after 24 h of transfection with *PlGF* siRNA.

Matrigel invasion assay

The BD BioCoat Matrigel invasion chamber assay system (Becton Dickinson, Heidelberg, Germany) was used to evaluate FLS invasion, according to the manufacturer's instructions (25). Briefly, 24 h after transfection with siRNA, RA-FLSs were allowed to migrate in a Matrigel layer chamber for an additional 12 h in the presence of DMEM containing 10% FBS or 10 ng/ml IL-1β in DMEM supplemented with 1% FBS. In some experiments, medium supplemented with PlGF was used as an attractant in the lower chamber. The noninvading cells were subsequently removed by scrubbing with a cotton-tipped swab, and the cells on the lower surface of the membrane were stained with Diff-Quik stain (Baxter Diagnostics, McGaw Park, IL). For quantification, cells were manually counted in eight random fields.

Determination of FLS proliferation in vivo

RA-FLSs (5×10^5) were transfected with control or *PlGF* siRNA for 24 h and then mixed with 500 μl Matrigel (Becton Dickinson). The Matrigel containing RA-FLSs was injected s.c. into the abdomen of athymic nude mice (The Jackson Laboratory, Bar Harbor, ME), as previously described (26). After 7 d, the skin containing the Matrigel plugs was excised and snap-frozen. Sections were stained with anti-human HLA class I Ab and analyzed by confocal microscopy (Zeiss LSM 510; Carl Zeiss, Thornwood, NJ). The number of HLA class I^+ cells, indicating RA-FLSs, was determined by counting the number of positively stained cells.

Statistical analysis

For the analysis of gene expression data, $\log_2$ intensities in individual samples were first normalized using the quantile normalization method (27). An integrative statistical method previously described (28) was applied to the normalized intensities to identify differentially expressed genes (DEGs). Briefly, for each gene a Student *t* test and $\log_2$ median ratio test were applied, resulting in *T* values and $\log_2$ median ratios between RA-FLSs treated with *PlGF* and control siRNAs. Empirical null distributions for *T* values and $\log_2$ median ratios were estimated by applying the Gaussian kernel density estimation method (29) to *T* values and $\log_2$ median ratios obtained from 1000 random permutations of the samples. For each gene, adjusted *p* values of the two tests were calculated by two-tailed tests for the observed *T* value and $\log_2$ median ratio using the empirical distributions, and *p* values of the two tests were then combined to an overall *p* value by using Stouffer's method (30). The DEGs were identified as genes with overall p values < 0.05 and absolute $\log_2$ fold change $>$ the mean of the 2.5th and 97.5th percentiles of the empirical distribution for the $\log_2$ median ratio. Gene ontology biological process (GOBP) enrichment analysis of the DEGs was conducted using DAVID software (31). Cellular processes represented by the DEGs were identified as the GOBPs and Kyoto encyclopedia of genes and genomes (KEGG) pathways with $p < 0.1$ computed from DAVID.

Data from the in vivo and in vitro functional experiments were expressed as the mean ± SD or SEM (SEM). Comparisons of the numerical data between groups were performed by the paired or unpaired Mann–Whitney *U* test. A *p* value <0.05 was considered statistically significant.

Results

PlGF isoforms in FLS and their role in FLS biology

We first performed immunofluorescence staining of the synovium of RA patients to investigate the distribution and localization of PlGF in joint tissues. The cellular origins of the synovial tissues were examined by double staining for PlGF and CD55, a specific marker for FLSs (10). We found that PlGF-expressing cells in the lining layer were also positive for CD55, indicating that FLSs were the major source of PlGF production (Fig. 1A). However, as a control, $CD68^+$ cells, which indicate synovial macrophages, were not colocalized with $PlGF^+$ cells (Fig. 1A). Alternative splicing of the primary *PlGF* transcript generates four different isoforms of *PlGF*, that is, *PlGF-1, PlGF-2, PlGF-3*, and *PlGF-4* (12, 13). Among these, *PlGF-1* and *PlGF-2* are the major isoforms to mediate cell growth and migration (12, 14). We next analyzed the expression of *PlGF* isoforms in FLSs using real-time PCR. As a result, *PlGF-1* and *PlGF-2*, but not *PlGF-3* and *PlGF-4*, were predominantly expressed in RA-FLSs and OA-FLSs (Fig. 1B and data not shown) and were significantly increased on IL-1β stimulation. These data indicated that *PlGF-1* and *PlGF-2* were the major *PlGF* isoforms expressed in FLSs.

To understand functions of *PlGF* in RA-FLSs, we next performed gene expression profiling of RA-FLSs treated with *PlGF* siRNA and control siRNA. We identified 1732 DEGs (972 upregulated and 760 downregulated genes; Supplemental Table I) by comparing the gene expression profiles. The cellular processes in which the DEGs are mainly involved were determined by the functional enrichment analysis of GOBPs and KEGG pathways using DAVID software (31). The results showed that the DEGs were mainly involved in cellular processes related to 1) cell proliferation, 2) cell survival or apoptosis, and 3) cell migration (Fig. 1C, Supplemental Fig. 1, Supplemental Table II). Real-time PCR confirmed differential expression of the following eight representative genes related to these processes (Fig. 1D): *CDK1* and *E2F2* involving cell proliferation (32); *CASP1*, *CASP7*, and *BCL2* involving apoptosis; and *NANOS1*, *KIT*, and *CCL28* involving cell migration and invasion (33–35). These data indicate that *PlGF* may function as a regulator of proliferation, apoptosis, and migration of RA-FLSs.

PlGF regulation of FLS proliferation

RA-FLSs display similar proliferative properties to cancer cells, resulting in the formation of a hypertrophic synovial pannus (10). To validate the effect of PlGF on FLS biology suggested by gene expression profiling, we first investigated whether PlGF mediates the proliferation of RA-FLSs in vitro. As shown in Fig. 2A, TNF-α– or TGF-β–induced increases in BrdU incorporation were almost completely prevented by *PlGF* siRNA, but not by control siRNA. The number of RA-FLSs stimulated with TNF-α or TGF-β was also completely reduced by *PlGF* siRNA transfection (Fig. 2B). Alternatively, overexpression of the *PlGF-2* gene in SV40-immortalized RA-FLSs promoted cell proliferation under serum-free conditions (Fig. 2C, 2D). These data suggest that PlGF plays an essential role in FLS proliferation.

To test the PlGF involvement in FLS proliferation in vivo, we s.c. implanted Matrigel plugs containing RA-FLSs transfected with *PlGF* siRNA into immune-deficient mice in the presence of TGF-β. We have previously shown that TGF-β increases RA-FLS proliferation in the same in vivo model (26). In the present study, we first confirmed that the downregulatory effect of *PlGF* siRNA persisted to 7 d (Fig. 2E). Immunofluorescence staining revealed that the number of HLA class I^+ cells, indicating RA-FLSs, was significantly lower in the Matrigels with *PlGF* siRNA than in those with control siRNA (Fig. 2F), indicating that *PlGF* siRNA inhibited TGF-β–induced FLS proliferation in vivo. We also found that von Willebrand $factor^+$ cells, indicating neovascularization, were significantly reduced in *PlGF* siRNA–containing Matrigels than in control Matrigels (Fig. 2G), suggesting that suppression of FLS proliferation by *PlGF* siRNA reduces endothelial cell recruitment to the surroundings of synoviocytes in vivo.

PlGF control of FLS survival and apoptosis

PlGF can promote survival of macrophages, endothelial cells, and cancer cells (12, 14, 17). We next investigated whether PlGF regulates FLS survival. As shown in Fig. 3A, *PlGF* knockdown significantly decreased the viability of RA-FLSs in the presence of sodium nitroprusside (SNP), an apoptosis inducer. On phase-contrast microscopy, FLSs transfected with *PlGF* siRNA be-

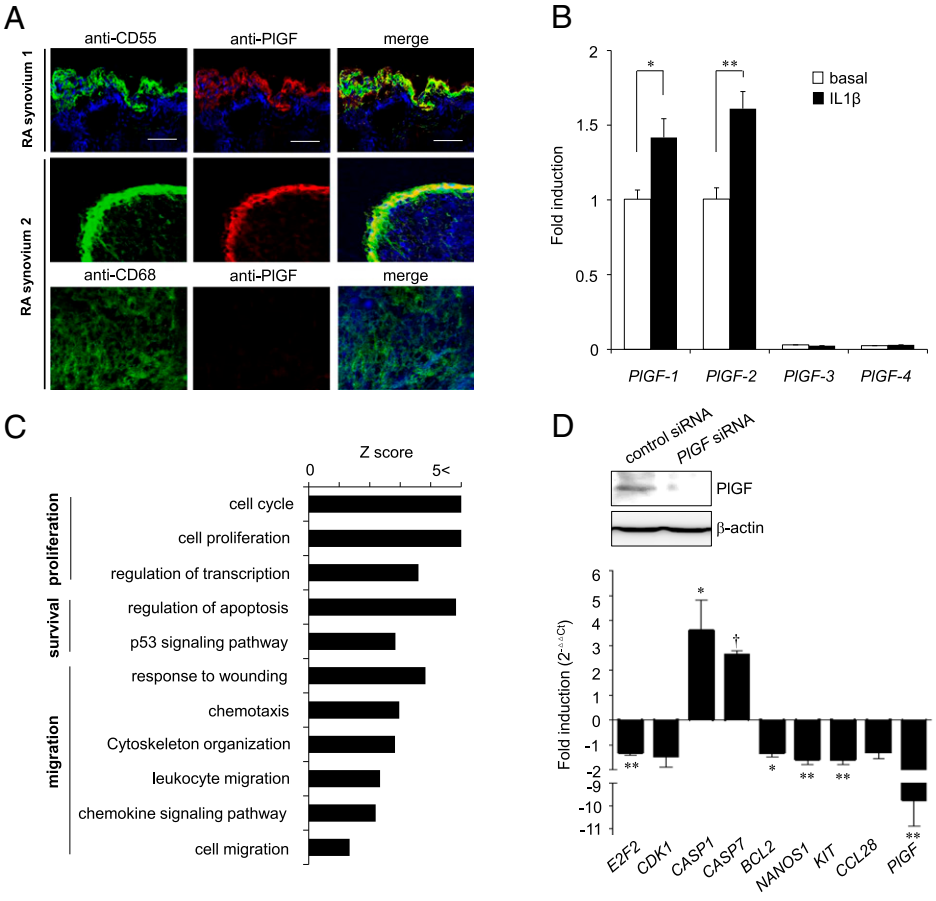

FIGURE 1. PlGF isoform in RA-FLSs and its target gene signatures. (**A**) Double immunofluorescence staining of PlGF and CD55 in synovial tissues of RA patient using anti-PlGF and anti-CD55 Abs. Anti-CD68 Abs for synovial macrophages were used as a control. Sections were subsequently stained with Cy3-conjugated anti-mouse IgG (red) for anti-PlGF Ab and Alexa Fluor 488–conjugated anti-goat IgG (green) for anti-CD55 Ab (or CD68 Ab), respectively. Colocalization of CD55 and PlGF is visualized in yellow on the merged images. The cell nucleus was stained with DAPI. Scale bars, 100 μm. (**B**) Quantitative real-time PCR assays for isoforms of *PlGF* in RA-FLSs in the presence or absence of IL-1β (1 ng/ml). Data are the means ± SEM of five different RA-FLSs. $*p < 0.05$, $**p < 0.01$ versus unstimulated cells. (**C**) Functional enrichment analysis for the DEGs in RA-FLSs treated with PlGF siRNA, as compared with control (scrambled) siRNA. GOBPs and KEGG pathways representing the DEGs were mainly involved in 1) cell proliferation, 2) cell survival or apoptosis, and 3) cell migration. The bars indicate *Z* score = $N^{-1}(1 - P)$. (**D**) Quantitative real-time PCR assay for the eight representative DEGs to validate the result of functional enrichment analysis, which was assessed 12 h after transfection of RA-FLSs with *PlGF* siRNA or control siRNA (*lower panel*). A decrease in PlGF expression was confirmed in RA-FLSs by Western blot analysis after 24 h of transfection (*upper panel*). Data in the bar graphs are the means and SD of three independent experiments. One-sample *t* test was applied to the fold induction. $*p < 0.1$, $**p < 0.05$, $^{\dagger}p < 0.01$ versus RA-FLSs treated with control siRNA.

came spherical, shrunken, and detached from the bottom of the culture plate, whereas control siRNA-transfected cells retained a bipolar appearance. Furthermore, *PlGF* knockdown rendered RA-FLSs more susceptible to serum starvation or SNP-induced apoptosis, as determined by the APOPercentage apoptosis assay (Fig. 3B). Conversely, stable overexpression of *PlGF-2* gene inhibited the SNP-induced death of SV40-immortalized RA-FLSs, as assessed by the MTT assay (Fig. 3C). Starvation or SNP-induced FLS apoptosis was also blocked by stable overexpression of the *PlGF-2* gene (Fig. 3D).

The regulation of Bcl2 family members is important to cell apoptosis and survival (36), which is triggered by serum starvation and SNP treatment (22). Additionally, the activation of pErk maintains mitochondrial integrity via upregulation of Bcl2 expression or via the inhibition of proapoptotic Bcl2 family (22, 36). Based on our finding on PlGF inhibition of FLS apoptotic death, we determined whether PlGF regulates the expression of pErk and Bcl2/Bax in RA-FLSs. As shown in Fig. 3E, downregulation of *PlGF* transcripts in RA-FLSs decreased pErk and Bcl2 expression but increased Bax expression as compared with the control siRNA transfection. FLSs stably overexpressing the *PlGF-2* gene showed the opposite results, indicating that PlGF controls pErk/Bcl2/Bax expression in RA-FLSs. Moreover, the protective effect of *PlGF-2* overexpression on SNP-induced apoptosis was cancelled by treating RA-FLSs with the PD98059 Erk inhibitor (Fig. 3F). Overall, the present findings, together with previous reports (22), suggest that PlGF regulates FLS survival through a mitochondrial apoptotic pathway involving Bcl2, Bax, and pErk.

PlGF-dependent FLS survival is mediated by Flt-1 and NP-1

PlGF is primarily derived from FLSs and is detected at high levels in the synovial fluids of RA patients (6). Thus, we conducted an experiment to determine the effects of soluble PlGF on FLS survival. The result showed that conditioned media obtained from HEK-293 clones overexpressing human *PlGF-1* or human *PlGF-2* significantly blocked SNP- or starvation-induced apoptosis of RA-FLSs; however, the addition of anti-PlGF Abs to conditioned media restored the apoptotic effect evaluated by the APOPercentage assay (Fig. 4A, 4B). SNP-induced FLS death, determined by the MTT assay, was also inhibited by conditioned media

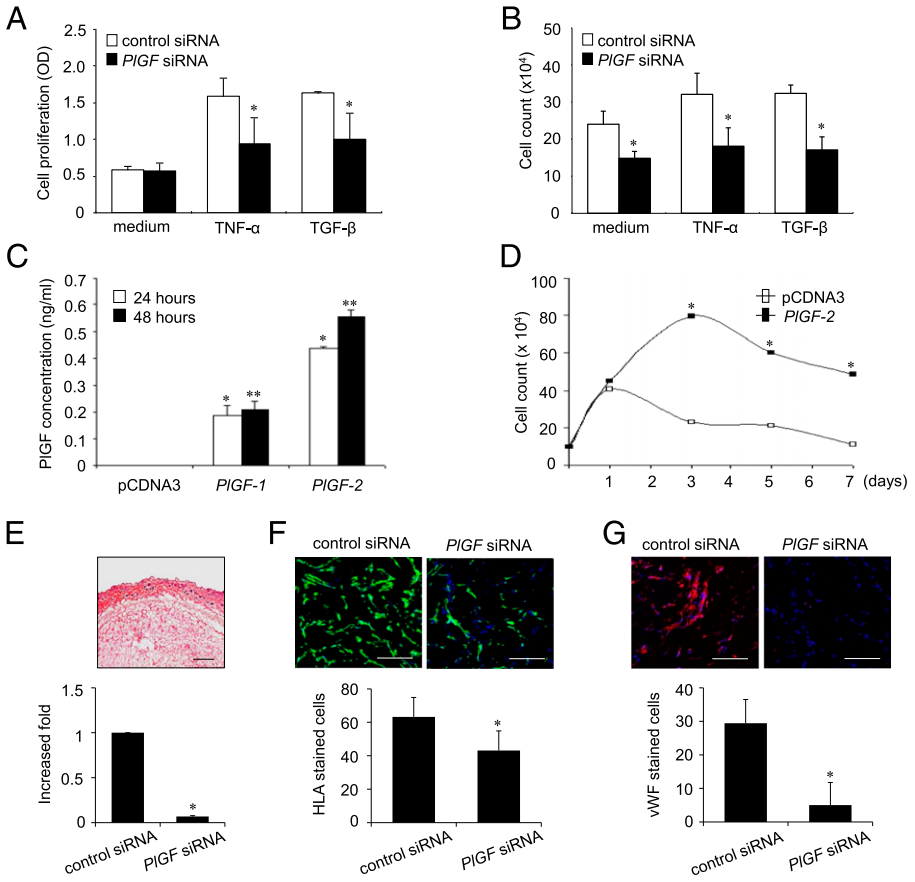

FIGURE 2. Effect of PlGF on FLS proliferation. (**A** and **B**) FLS proliferative responses to TNF-α and TGF-β. Twenty-four hours after transfection with *PlGF* siRNA, RA-FLSs were treated with TGF-β (10 ng/ml) or TNF-α (10 ng/ml) for 72 h. The FLS proliferation rate was assessed by the BrdU incorporation assay (A) and trypan blue exclusion assay (B). Results are the means ± SD of three independent experiments performed in triplicate. $*p < 0.05$ versus control (scrambled) siRNA-transfected cells. (**C**) PlGF production by SV40-immortalized RA-FLSs transfected with the pCDNA3-*hPlGF-1,* pCDNA3-*hPlGF-2*, or pCDNA3 vector only. PlGF concentrations in the culture supernatant were determined by ELISA. Data are the means ± SD of five independent experiments. $*p < 0.05$, $**p < 0.01$ versus vector-transfected cells. (**D**) Proliferation of SV40-immortalized RA-FLSs transfected with pCDNA3-*hPlGF-2* gene versus pCDNA3 vector. The cells (1×10^5) were cultured in serum-free media for 7 d. The number of cells was manually counted every 2 d. $*p < 0.05$ versus vector-transfected cells. (**E**) H&E staining of Matrigels containing RA-FLSs implanted in immunodeficient mice (*upper panel*). Scale bar, 500 μm. Prior to implantation, the expression of PlGF was determined in RA-FLSs by real-time PCR assay 7 d after transfection of *PlGF* siRNA or control siRNA (*lower panel*). $*p < 0.05$ versus control siRNA-transfected cells. (**F**) Effect of *PlGF* siRNA on FLS proliferation in vivo. Matrigels containing *PlGF* siRNA-transfected RA-FLSs plus TGF-β (50 ng/ml) were implanted into SCID mice. After 7 d, RA-FLSs in the Matrigels were identified by immunofluorescence labeling for HLA class I Ag. Representative photographs of HLA^+ cells are shown in green. Cells were manually counted under a magnification of ×200. Scale bars, 100 μm. Values are the means ± SD of four mice per group. $*p < 0.05$ versus control siRNA-transfected cells. (**G**) Infiltrating mouse endothelial cells were stained using mouse anti-vWF Ab in the same Matrigels used for HLA staining. The cells positive for vWF are shown in red. Scale bars, 100 μm. $*p < 0.05$ versus control siRNA-transfected cells.

containing PlGF-1 or PlGF-2 (Fig. 4C), indicating that soluble PlGF promotes FLS survival. Moreover, after treatment of conditioned media for PlGF-1 plus PlGF-2, pErk expression in RA-FLSs rapidly increased, as early as 10 min and remained high up to 12 h (Fig. 4D). In parallel, the expression of antiapoptotic Bcl2 increased, whereas proapoptotic Bax decreased 12 h after PlGF-1 plus PlGF-2 stimulation (Fig. 4D). Considering that FLSs secrete PlGF by themselves, our data suggest that secreted PlGF-1 and PlGF-2 promote the survival of RA-FLSs in an autocrine/paracrine manner.

We have demonstrated that RA-FLSs express Flt-1 and NP-1, but rarely KDR, on their surface (22). Additionally, PlGF selectively binds Flt-1 and NP-1 (12, 14). Thus, we wanted to determine whether PlGF by binding its receptors protects from FLS apoptosis. To this end, we conducted a blocking experiment using siRNA for *Flt-1* or for *NP-1*. As shown in Fig. 4E, conditioned media for PlGF-1 plus PlGF-2 reduced SNP-induced FLS apoptosis, which was partially restored by the transfection of *Flt-1* siRNA or *NP-1* siRNA. Of note, cotransfection of *Flt-1* siRNA and *NP-1* siRNA almost completely abrogated the PlGF protection from FLS apoptosis, implying that both receptors are required for PlGF-1– and PlGF-2–dependent FLS survival.

Effects of PlGF on FLS migration and invasion

Invasiveness and increased migration are unique features of RA-FLSs in addition to abnormal proliferation and apoptotic resistance (10, 11). PlGF increases cell migration of some types of cancer cells and myelomonocytic cells (12, 14). Finally, we tested whether PlGF controls FLS migration and invasion. As shown in Fig. 5A, under nonlethal time conditions (after 12 h), knockdown of *PlGF* transcripts suppressed the wound migration of RA-FLSs stimulated with 10% FBS. The IL-1β–induced increase in FLS migration was also mitigated by *PlGF* siRNA. Furthermore, 10% FBS- or IL-1β–stimulated invasion of RA-FLSs in a Matrigel chamber was significantly blocked by transfection with *PlGF* siRNA (Fig. 5B). Conversely, conditioned media containing PlGF-1

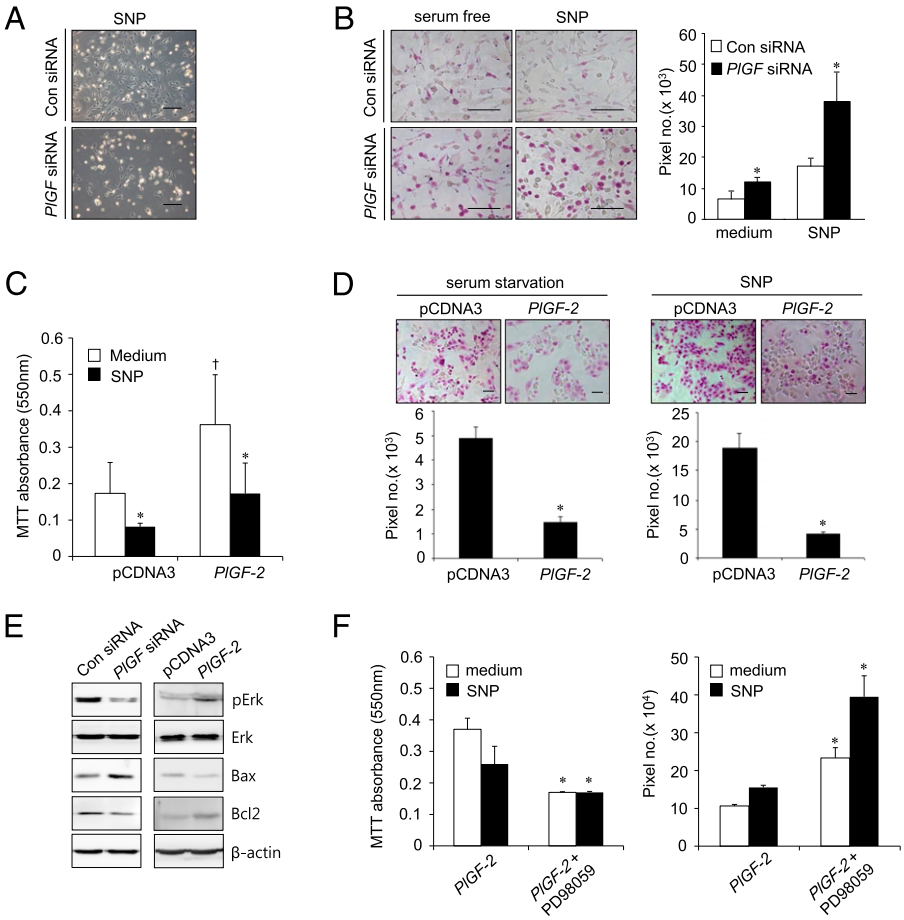

FIGURE 3. Effect of PlGF on FLS survival. (**A**) Twelve hours after treatment of the cells with SNP (1 mM), photographs of RA-FLSs transfected with *PlGF* siRNA or control siRNA were taken under phase-contrast microscopy. Scale bars, 100 μm. (**B**) Increase in FLS apoptosis by *PlGF* siRNA. FLS apoptosis was induced by treatment with 1 mM SNP for 3 h or by serum starvation for 12 h. The degree of apoptosis was assessed by the APOPercentage apoptosis assay, a colorimetric method, and is expressed as pixel numbers. Apoptotic cells appear bright pink. Results are the means ± SD of three independent experiments performed in triplicate. Scale bars, 100 μm. $*p < 0.05$ versus control siRNA-transfected cells. (**C**) SV40-immortalized RA-FLSs transfected with the pCDNA3-*hPlGF-2* gene or pCDNA3 vector only were treated with 2.5 mM SNP for 24 h. Cell viability was determined by the MTT assay. Data are expressed as the means ± SD of six independent experiments in triplicate. $*p < 0.05$ versus untreated cells, $^{\dagger}p < 0.05$ versus vector only. (**D**) The apoptosis of SV40-immortalized RA-FLSs harboring the pCDNA3-*hPlGF-2* gene or pCDNA3 vector only was induced by serum starvation for 24 h or by treating cells with 1 mM SNP for 24 h. The degree of apoptosis was assessed by the APOPercentage assay. Data show means ± SD of three independent experiments in triplicate. Scale bars, 100 μm. $*p < 0.05$ versus vector only. (**E**) Expressions of pErk, total Erk, Bax, and Bcl-2 in RA-FLSs, as determined by Western blot analysis. *PlGF* gene in RA-FLSs was downregulated by *PlGF* siRNA for 24 h (*left panel*) or upregulated by stable transfection of pCDNA3-*hPlGF-2* gene (*right panel*). Con siRNA, control siRNA. Data are representatives of three independent experiments with similar results. (**F**) Effect of Erk inhibitor on apoptotic death of RA-FLSs. The *hPlGF-2* overexpressed SV40-immortalized FLSs were incubated for 24 h with SNP (2.5 mM) in the absence or presence of PD98059 (20 μM), an Erk inhibitor. The viability and survival of cells were determined by the MTT (*left panel*) and APOPercentage assay (*right panel*), respectively. Data show means ± SD of three independent experiments in triplicate. $*p < 0.05$ versus *hPlGF-2*–overexpressed FLSs.

or PlGF-2 markedly increased FLS migration and invasion as comparable to 10% FBS (Fig. 5C, 5D). These data showed that PlGF was a positive regulator for migration and invasion of RA-FLSs. Additionally, similar to FLS survival, the exogenous PlGF-1– or PlGF-2–induced increase in FLS invasion was blocked by *Flt-1* siRNA and/or *NP-1* siRNA (Fig. 5E), indicating that both Flt-1 and NP-1 were also required for PlGF-induced FLS invasion. Taken together, these data suggest that PlGF promotes FLS invasion via a receptor-coupling event.

Discussion

PlGF-1 and *PlGF-2* isoforms may play different biochemical functions in endothelial cells (12, 14). Interestingly, increased PlGF-1 and PlGF-2 are of prognostic value in cancer patients (12, 14). PlGF is primarily secreted by FLSs in RA synovial tissue (6), and its production can be induced by hypoxia (14, 37), cytokine stimulation (14), and a cell-to-cell contact with mesenchymal stem cells (14). Because RA joints are in a hypoxic condition, our recent findings on involvement of HIF-1α in the upregulation of *PlGF* expression through chromatin remodeling is particularly relevant to RA (37). In the present study, we identified that *PlGF-1* and *PlGF-2* are the major *PlGF* isoforms in FLSs. Global gene expression profiling revealed that a variety of pathologic processes, including cell proliferation, apoptosis, angiogenesis, cytokine production, and cell migration, were represented by the DEGs in RA-FLSs deficient of *PlGF* transcripts. These results are indicative that PlGF is crucial to RA pathogenesis via diverse pathologic processes.

The mechanisms by which normal cells acquire a transformed phenotype have been an important issue in the pathogenesis of human diseases. The uniquely transformed phenotype of FLSs in

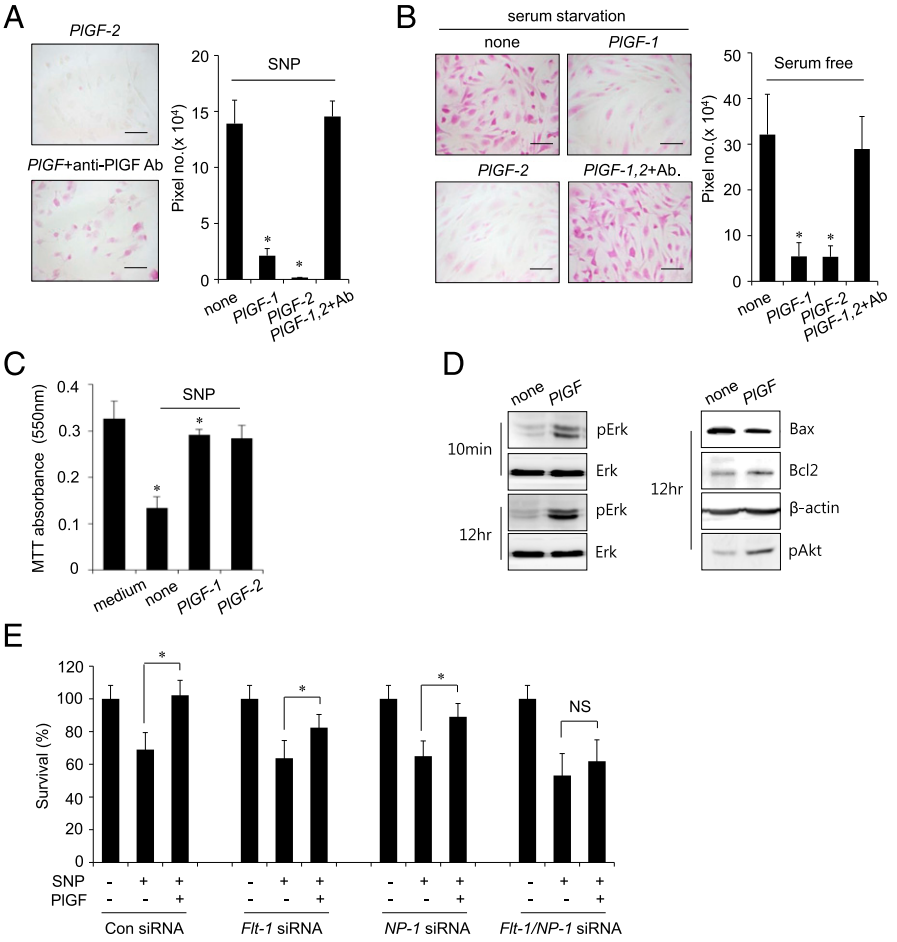

FIGURE 4. Exogenous PlGF increases FLS survival via its receptors, Flt-1 and NP-1. (**A**–**C**) Protection of RA-FLSs from apoptotic death by exogenous PlGF. RA-FLSs were treated with conditioned media containing PlGF-1 (500 ng/ml) or PlGF-2 (500 ng/ml) for 3 h. FLS apoptosis was induced by treating SNP (1 mM) for 6 h (A) or by serum starvation for 12 h (B) in the absence or presence of anti-PlGF Abs (100 μg/ml). FLS apoptosis and viability were determined by the APOPercentage (A and B) and MTT assay (C), respectively. Data show means ± SD of three independent experiments in duplicate. Scale bars, 50 μm. *$p < 0.05$ versus SNP or serum starvation only. (**D**) RA-FLSs were stimulated with conditioned media for PlGF-1 (100 ng/ml) plus PlGF-2 (100 ng/ml) for 10 min or 12 h. The expressions of pErk, Bax, Bcl2, and pAkt were determined by Western blot analysis. Data are representatives of three independent experiments with similar results. (**E**) Dependency of PlGF-induced survival on Flt-1 and NP-1 12 h after transfection of siRNA for *Flt-1*, *NP-1*, or both. The RA-FLSs were treated with SNP (1 mM) for 12 h in the absence or presence of conditioned media for PlGF-1 (100 ng/ml) plus PlGF-2 (100 ng/ml). Cell viability was determined by the MTT assay. Data show means ± SD of three independent experiments in triplicate. *$p < 0.05$ versus SNP only.

RA has been associated with a genotoxic environment containing reactive oxygen species, growth factors, and proinflammatory cytokines (10). We clearly showed that PlGF is a critical regulator of FLS proliferation by using *PlGF*-deficient and overexpressing systems. Of note, TNF-α– or TGF-β–induced increases in FLS proliferation were nearly completely blocked by *PlGF* siRNA. We also confirmed that PlGF plays a role in TGF-β–stimulated FLS proliferation in an in vivo xenotransplantation model. Overall, our results suggest that PlGF mediates TNF-α– or TGF-β–induced FLS proliferation, thus providing an explanation for the proliferative phenotype of primary rheumatoid cells.

Angiogenesis has been considered to be a critical step in the initiation and progression of chronic arthritis (2). It is upregulated by many angiogenic factors, such as VEGF and PlGF. Indeed, PlGF may directly stimulate vessel growth by directly promoting the growth, migration, and survival of endothelial cells and also by increasing the recruitment of smooth muscle cells and supporting the proliferation of fibroblasts (12, 14). In the present study, knockdown of the *PlGF* transcript in RA-FLSs significantly impeded the recruitment of endothelial cells to Matrigel implants engrafted into immunodeficient mice. These data, together with previous reports (6), suggest that PlGF-mediated FLS proliferation increases production of angiogenic factors, including VEGF and PlGF, by FLS themselves, and that this indirectly contributes to neovascularization and pannus formation.

RA-associated changes in FLSs from normal to aggressive behavior may be attributable to the upregulation of antiapoptotic genes (10, 36). RA-FLSs abundantly express several antiapoptotic proteins, including the FLICE inhibitory protein and Bcl2 (10, 36), both of which protect against death receptor– or mitochondria-dependent apoptotic pathways (36). We showed in the present study that PlGF was essential to FLS survival. *PlGF* knockdown resulted in increased apoptotic death of FLSs with Bcl2 downregulation and Bax upregulation, whereas *PlGF* overexpression showed the opposite results. Additionally, *PlGF* siRNA decreased pErk activity, whereas *PlGF* overexpression enhanced it. Because pErk is a well-known upstream regulator of Bcl2 (22, 36), and its inhibitor (PD98059) restored SNP-induced apoptosis in *PlGF-2*–overexpressed RA-FLSs (Fig. 3F), PlGF promotes FLS survival by regulating Bcl2/Bax expression possibly through the activation of pErk. Taken together, our data suggest that chronic exposure of FLSs to PlGF within the joints protects RA-FLSs from apoptosis, thereby maintaining a unique hyperplastic phenotype.

PlGF activation of Flt-1 induces the Erk pathways (38). Because RA-FLSs express Flt-1 and NP-1 on their surface (22), it is possible that secreted PlGF activates FLSs themselves in a paracrine/autocrine manner by binding to Flt-1 and/or NP-1. However, the direct role of the PlGF–Flt-1/NP-1 axis in synoviocyte pathology, particularly the proliferative and invasive properties of RA-FLSs, remains to be determined. The present study demonstrated that both Flt-1 and NP-1 in RA-FLSs were required for signal transduction of PlGF. Exogenous PlGF-1 and -2 rapidly triggered pErk activity, increased Bcl2 over Bax expression, and prevented FLS apoptosis. In contrast, PlGF-induced survival advantage was cancelled by the addition of anti-PlGF Ab or siRNAs for both *Flt-1* and *NP-1*. These findings indicate that the interaction of PlGF with Flt-1/NP-1 enhances the survival of RA-FLSs in an autocrine/paracrine manner. Our results offer a new possibility for PlGF receptors as potential targets for controlling FLS hyperplasia.

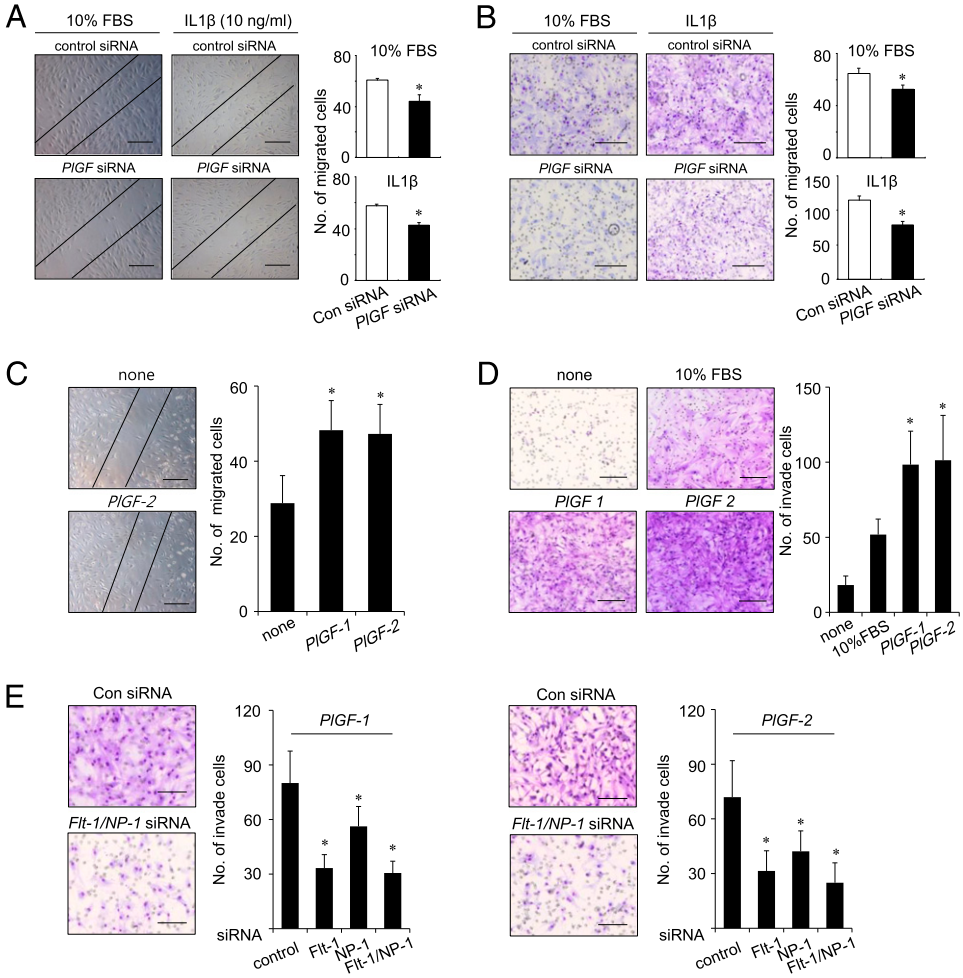

FIGURE 5. Exogenous PlGF increases FLS migration and invasion via its receptors, Flt-1 and NP-1. (**A**) Effects of *PlGF* siRNA on wound migration of RA-FLSs. After 12 h of transfection with *PlGF* siRNA or control siRNA (Con siRNA), RA-FLSs were incubated in DMEM containing 10% FBS or IL-1β (10 ng/ml) plus 1% FBS for 12 h. Cells migrating beyond the reference line were photographed and counted. Data show the means ± SD of three independent experiments. Scale bars, 200 μm. $*p < 0.05$. (**B**) RA-FLS invasion in a Matrigel chamber, as determined at 12 h after transfection of *PlGF* siRNA versus control siRNA. Invaded cells were stained violet using Diff-Quik kit. Results are the means ± SD of three independent experiments. Scale bars, 200 μm. $*p < 0.05$ versus control siRNA (Con siRNA). (**C** and **D**) Increases in wound migration and invasion of FLSs by exogenous PlGF. Under serum-free conditions, RA-FLSs were incubated with conditioned media containing PlGF-1 (100 ng/ml) or PlGF-2 (100 ng/ml) for 12 h. The migrated cells in a wound area (C) and invaded cells in a Matrigel chamber (D) were manually counted. FBS (10%) was used as a positive control. Data show means ± SD of three independent experiments in duplicate. Scale bars, 200 μm. $*p < 0.05$ versus serum-free media. (**E**) Reduction of RA-FLS invasion by *Flt-1* or *NP-1* siRNA. After 12 h of transfection with *Flt-1, NP-1*, or both, RA-FLSs were incubated in conditioned media for PlGF-1 (100 ng/ml) or PlGF-2 (100 ng/ml) 12 h. Results are the means ± SD of three independent experiments. Scale bars, 100 μm. $*p < 0.05$ versus control siRNA (Con siRNA)–transfected cells.

RA-FLSs actively participate in cartilage/bone destruction by releasing matrix degrading enzymes, such as matrix metalloproteinase. They can be detached from hyperplastic synoviums and then migrate to and invade adjacent structures without the help of other immune cells (10, 11). RA-FLSs can spread disease by migrating from affected to distant unaffected joints in immune-deficient mice (11). In this regard, antimigratory agents targeting RA-FLSs may thus be of therapeutic benefit. For example, treatment targeting cadherin-11, which is selectively expressed on FLSs, prevents arthritis in mouse models (39). In the present study, we found that *PlGF* siRNA hampered media or IL-1β–stimulated migration and invasion of RA-FLSs. Conversely, the ligation of PlGF-1 (or PlGF-2) to its receptors (Flt1-1/NP-1) promoted such processes, suggesting that the PlGF–Flt-1/NP-1 axis is essential for RA-FLSs to maintain a promigratory and invasive phenotype.

RA synovial tissues can be viewed as a tumor-like mass consisting of hyperplastic FLSs and surrounding inflammatory cells (1, 4). Given that current therapeutic agents targeting T cells, B cells, and cytokines show a limited success, RA-FLSs are an attractive target to achieve complete remission. In this study, we demonstrated that PlGF-1 and -2 were positive regulators for abnormal proliferation, apoptotic resistance, excessive migration, and invasion of RA-FLSs beyond their role for neovascularization. These results provided evidence on how primary cells of mesenchymal origin acquire an aggressive and transformed phenotype. The blockade of PlGF and its receptors could be a novel strategy to target the deleterious functions of RA-FLSs.

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Disclosures

The authors have no financial conflicts of interest.

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