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Interactions between cadherin-11 and platelet-derived growth factor receptor-alpha signaling link cell adhesion and proliferation

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ABSTRACT

Cadherins are homophilic cell-to-cell adhesion molecules that help cells respond to environmental changes. Newly formed cadherin junctions are associated with increased cell phosphorylation, but the pathways driving this signaling response are largely unknown. Since cadherins have no intrinsic signaling activity, this phosphorylation must occur through interactions with other signaling molecules. We previously reported that cadherin-11 engagement activates joint synovial fibroblasts, promoting inflammatory and degradative pathways important in rheumatoid arthritis (RA) pathogenesis. Our objective in this study was to discover interacting partners that mediate cadherin-11 signaling. Protein array screening showed that cadherin-11 extracellular binding domains linked to an Fc domain (cad11Fc) induced platelet-derived growth factor (PDGFR)-α phosphorylation in synovial fibroblasts and glioblastoma cells. PDGFRs are growth factor receptor tyrosine kinases that promote cell proliferation, survival, and migration in mesodermally derived cells. Increased PDGFR activity is implicated in RA pathology and associates with poor prognosis in several cancers, including sarcoma and glioblastoma. PDGFRα activation by cadherin-11 signaling promoted fibroblast proliferation, a signaling pathway independent from cadherin-11-stimulated IL-6 or matrix metalloproteinase (MMP)-3 release. PDGFRα phosphorylation mediated most of the cad11Fc-induced phosphatidyl-3-kinase (PI3K)/Akt activation, but only part of the mitogen-activated protein kinase (MAPK) response. PDGFRα-dependent signaling did not require cell cadherin-11 expression. Rather, cad11Fc immunoprecipitated PDGFRα, indicating a direct interaction between cadherin-11 and PDGFRα extracellular domains. This study is the first to report an interaction between cadherin-11 and PDGFRα and adds to our growing understanding that cadherin-growth factor receptor interactions help balance the interplay between tissue growth and adhesion.

1. Introduction

Cadherin-11 is a homophilic cell-to-cell membrane adhesion molecule normally expressed by mesenchymal cells such osteoblasts, neurons, and fibroblasts, including joint synovial fibroblasts [1] and lymph node fibroblastic reticular cells [2]. Cadherin-11 contributes to normal tissue function, as demonstrated by reduced fear response [3], bone density [4], and synovial lining cell number [5] in cadherin-11 knockout mice. Cadherin-11 also contributes to pathologic processes. Increased cadherin-11 expression is associated with tumor epithelial-to-mesenchymal transition and metastasis [6–8], while loss of cadherin-11 function reduces inflammation and fibrosis in mouse rheumatoid arthritis, interstitial lung disease, and systemic sclerosis models [5,9,10].

Cadherins are not simply passive adhesion molecules. Like integrins, cadherins signal changes in cell microenvironment, specifically in relationships between cells [11]. We previously showed that cadherin-11

Abbreviations: Cad11Fc, cadherin-11 extracellular binding domains linked to an Fc domain; DMEM, Dulbecco's Modified Eagle's Medium; E-cadherin, epithelial cadherin; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; FGFR, fibroblast growth factor receptor; HPRT, hypoxanthine-guanine phosphoribosyltransferase; MMP, matrix metalloproteinase; MAPK, mitogen activated protein kinase; N-cadherin, neural cadherin; NF-kB, nuclear factor-kB; PI3K, phosphatidyl-3-kinase; PDGFR, platelet-derived growth factor receptor; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RA, rheumatoid arthritis; RTK, receptor tyrosine kinase; TNF, tumor necrosis factor; VEGFR, vascular endothelial growth factor receptor; VE-cadherin, vascular endothelial cadherin

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engagement on synovial fibroblasts activated PI3K/Akt, MAPK, and nuclear factor-kB (NF-kB) signaling pathways, increasing IL-6, chemokine, and MMP expression [12,13]. Since cadherin-11 is not a kinase, these pathways must be activated through interactions with other signaling molecules.

One type of cadherin signaling interaction involves growth factor receptor tyrosine kinases (RTKs), linking changes in cell adhesion with pathways that control cell growth and migration [14]. Interactions between cadherins and growth factor RTKs act to regulate each other's function. For example, endothelial cell vascular endothelial growth factor receptor (VEGFR) stimulation reduces vascular endothelial cadherin (VE-cadherin) junctions [15], promoting vascular permeability. In contrast, accumulation of VE-cadherin junctions dampens VEGFR signaling [16,17], restoring vessel homeostasis. Additional reports link epithelial cadherin (E-cadherin)/epidermal growth factor receptor (EGFR) in adenocarcinomas [18–20], neural cadherin (N-cadherin)/platelet-derived growth factor-β (PDGFRβ) in sarcoma [21], N-cadherin/fibroblast growth factor receptor (FGFR) in breast cancer [22,23], and cadherin-11/FGFR in neurons [24]. Our initial objective was to assess if growth factor RTKs contributed to cadherin-11 signaling in fibroblasts. Using a screening array, we discovered a previously unrecognized interaction between cadherin-11 and PDGFRα.

PDGFRs increase mesenchymal cell migration and proliferation, are critical for normal tissue development, and contribute to several diseases, including cancer, fibrosis, and rheumatoid arthritis [25–31]. Two PDGFRs, PDGFRα and PDGFRβ, homo- or heterodimerize upon ligand binding, leading to autophosphorylation and activation of common signaling pathways, including MAPKs, PI3K/Akt, janus kinases (JAK)/signal transducer and activator of transcription (STAT), Src, and phospholipase C. Although PDGFRα and PDGFRβ signaling activates largely overlapping pathways, a transgenic PDGFR linking the β ligand to the α signaling domains failed to fully rescue perinatal lethality in PDGFRβ null mice, directly demonstrating unique receptor subtype signaling functions [32]. Furthermore, specific PDGFRα or PDGFRβ stimulation in the same cell type resulted in distinct gene and micro RNA transcriptional profiles, further supporting signaling differences between these two receptors [33–35].

In this study, we present a PDGFRα-specific signaling pathway involving the cell-to-cell adhesion molecule cadherin-11. We show that a soluble protein expressing the cadherin-11 extracellular binding domains (cad11Fc) interacts with PDGFRα to stimulate cell proliferation, even in the presence of strong PDGFRβ co-expression. This PDGFRα activation does not depend on cell surface cadherin-11 expression or PDGFRα ligands, indicating that cadherin-11 binding domains directly activate PDGFRα. This interaction provides further evidence that PDGFRα and PDGFRβ signaling are not redundant and adds to the evidence that cross talk between cadherin and RTKs regulates important cellular process such as growth and survival.

2. Materials and methods

2.1. Ethics statement

Human primary synovial fibroblasts were derived from de-identified, discarded surgical tissues. The Brigham and Women's Hospital and the University of Washington Institutional Review Boards approved all human research in this study.

2.2. Cells

Human osteoarthritis (OA) and RA synovial fibroblasts were isolated as previously described [36] from de-identified tissues discarded after synovectomy or joint replacement surgery and used experimentally between passages 5 and 10. Patient diagnosis was confirmed prior to de-identification. Available donor demographic information is given in Supplemental Table 1. RA synovial fibroblasts were used unless otherwise noted. U118-MG glioblastoma cells were provided by the American Type Culture Collection (ATCC). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)-based fibroblast media [13] supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products). Prior to stimulation, cells were serum-starved overnight in fibroblast media supplemented with 1% FBS, unless otherwise indicated.

2.3. Reagents

These reagents were commercially purchased: *R&D Systems* — Proteome Profiler Human Phospho-RTK Array (proteins assayed listed in Suppl. Table 2), ELISAs (total and phospho-PDGFRs, IL-6, pro-MMP-1, MMP-3), human cytokine and growth factors (PDGF-AA, PDGF-BB, PDGF-DD, tumor necrosis factor (TNF)-α); *Sigma-Aldrich* — human IgG_1 isotype control, anti-β-actin antibody (AC-15); *Thermo Fisher Scientific* — alamarBlue®, Dharmafect 3; *Tocris* — axitinib; *EMD Millipore* — U01261, SP600125, SB203580, LY294002, SC-514; *Cell Signaling* — antibodies against phosphorylated (p)Akt (9271), Akt (9272), pJNK (4668), JNK (9252), pERK1/2 (4370), ERK1/2 (4695), p-p38 kinase (4115); *Santa Cruz Biotechnologies* — antibodies against PDGFRα (C-20) and PDGFRβ (11H4). Human cad11Fc and EcadFc was purified from the culture media of stable HEK293 transfectants as previously described [13,37] by protein G columns (BioXCell). All Fc proteins tested low for endotoxin contamination before use.

2.4. Proliferation assays

^{3}H-thymidine assay: Serum-starved fibroblasts were stimulated overnight and then pulsed for 6 h with ^{3}H-methyl-thymidine. Thymidine incorporation was measured using a beta scintillation counter after harvesting cell contents onto glass fiber filters. *AlamarBlue® assay*: AlamarBlue® (10% volume) was added to fibroblast cell culture media for 1 h at 37 °C. The conversion of non-fluorescent resazurin to fluorescent resorufin by cell metabolic activity was measured spectrophotometrically. *Cell Count Assay*: Serum-starved fibroblasts were stimulated for 3 days. Cells were removed from tissue culture plates by trypsinization. The cell number was counted on a hemacytomer using trypan blue to exclude dead cells.

2.5. siRNA/shRNA silencing

Pooled siRNA against PDGFRα and PDGFRβ were purchased from Santa Cruz Biotechnology. Control (5′ CAACAAGAUGAAGAGCACCA AUU 3′) and cadherin-11 (5′ CCACUUUCCAACCAGCCAAUUUU 3′) were synthesized by Thermo Fisher Scientific; sequences was validated in previous fibroblast studies [13,38]. Primary human fibroblasts were transfected overnight with siRNA using the lipid reagent Dharmafect 3, rested for 24 h and then serum-starved overnight before additional analysis. Silencing efficiency was confirmed by western analysis, flow cytometry staining, or quantitative reverse transcription polymerase chain reaction (qRT-PCR), as indicated. Lentiviral short-hairpin (sh) RNA silencing of cadherin-11 was performed as previously described [13].

2.6. Cell lysates, western blots and immunoprecipitation

Cell lysates for PDGFR ELISAs and signaling molecule analysis were made using the following buffer: 20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, and a mixture of protease inhibitors (EDTA-free, Roche Applied Science). Lysates were analyzed by ELISA per manufacturer's instructions or electrophoresed though polyacrylamide-SDS gels and transferred to Immobilon-P membranes (Bio-Rad, Hercules, CA) for western analysis. For cadherin-Fc immunoprecipitation, cad11Fc or EcadFc were cross-linked with the bi-functional sulfo-SBED

biotin cross-linker (Thermo Fisher Scientific) and purified using a spin desalting column. U118-MG cells were incubated for 15 min at 37 °C with cross-linked cadherin-Fc and then UV-cross-linked to interacting proteins on ice for 10 min. Cell lysates were made with the following buffer: 20 mM Tris pH 7.4, 150 mM NaCl, 0.5% deoxycholine, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, and protease inhibitors (EDTA-free, Roche Applied Science). Cadherin-Fc proteins were removed from cell lysates using protein G-agarose beads (GE Lifesciences) and electrophoresed and transferred as above. All transferred protein membranes were blocked in phosphate-buffered saline containing 0.05% Tween supplemented with 5% BSA and incubated with indicated antibodies. After washing, antibody binding was detected chemiluminescently using horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG.

2.7. Flow cytometry

Cells were removed from plates by gentle trypsinization with 0.02% trypsin (Worthington Biochemical) in HBS Ca (20 mM Hepes, 137 mM NaCl, 3 mM KCl, 1 mM CaCl, pH 7.4) for 3–4 min at 37 °C to minimize cadherin-11 proteolysis. Cadherin-11 surface expression was analyzed by flow cytometry (CytoFlex, Beckman Coulter) using ZombieAqua Live/Dead stain (BioLegend) and anti-cadherin-11 23C6 primary antibody as previously described [13]. Data was analyzed using Kaluza software.

2.8. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total cellular RNA was extracted from equivalent numbers of cultured RA synovial fibroblasts using RNeasy Micro columns (Qiagen, Valencia, CA) and reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen) per manufacturer's instructions. Equivalent volumes of cDNA were analyzed by qPCR using Brilliant SYBR® Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA) using the following primers (Integrated DNA Technologies, Coralville, IA): *PDGFA* Forward 5′-AGGATTCTTTGGACACCAGC-3′ Reverse 5′-ATGGC TGCCGTACTCATTCTCCACA-3′; *PDGFB* Forward 5′-ATGATCTCCAACG CCTGC-3′ Reverse 5′-TCAGCAATGGTCAGGGAAC-3′; *PDGFC* Forward 5′-TCTCCTGGTTAAACGCTGTG-3′ Reverse 5′-CCGGTCTTTGGTCTCAA CTG-3′; *PDGFD* Forward 5′-GAAATTGTGGCTGTGGAACTG-3′ Reverse 5′-GGATGTCAACTAGAGCCATGG-3′; *PDGFRA* Forward 5′-GCACGCCG CTTCCTGATATT-3′ Reverse 5′-CTCCGTGATGATGTTTGAGACA-3′; *PDGFRB* Forward 5′-AGACACGGGAGATACTTTTGC-3′ Reverse 5′-AGT TCCTCGGCATCATTA GGG-3′; *hypoxanthine-guanine phosphoribosyltransferase (HPRT)*: Forward 5′-GGGCTATAAATTCTTTGCTGAC-3′ Reverse 5′-CTGGTCATTACAATAGCTCTTCAG-3′.

2.9. Statistics

Statistical comparisons were analyzed with GraphPad Prism software (v. 6.07). Box and whisker plots show the following information: box, 25–75% percentile; error bars, minimum to maximum; horizontal line, median; and dot, mean. All other error bars represent standard deviation of the mean. Two-way comparison significance was determined by unpaired or paired Student's *t*-test, as indicated in the figure legend. Three-way significance comparison was determined by one-way ANOVA. p values < 0.05 were considered significant. Protein band pixel densities were analyzed by Image J/FIJI.

3. Results

3.1. PDGFRα interacts with cadherin-11 to stimulate synovial fibroblast proliferation

We reported that synovial fibroblasts produced IL-6, chemokines, and MMPs after exposure to a soluble protein linking the human chimeric cadherin-11 extracellular binding domain to the human IgG_1 Fc domain (cad11Fc) [12,13]. This production was mediated by MAPK and nuclear factor-κB (NF-κB) activation and required engagement of cell cadherin-11. Since cadherin-11 has no direct kinase activity, we next searched for signaling molecules that interact with cadherins and discovered growing evidence showing cadherin growth factor RTK co-regulation. Therefore, we screened for changes in human synovial fibroblast RTK phosphorylation after stimulation with cad11Fc using a western-blot protein array (Fig. 1A, Suppl. Table 1). This array showed that cad11Fc activated PDGFRs, especially PDGFRα. This phosphorylation was confirmed by ELISA (Fig. 1B–C). Compared to a matched human isotype control antibody, cad11Fc strongly activated PDGFRα, but not PDGFRβ, as shown in a representative experiment (Fig. 1B) and in pooled data from several different donors (Fig. 1C). Expected ligand-induced PDGFR phosphorylation patterns were demonstrated as a control (dark bars Fig. 1B, Suppl. Fig. 1A). The PDGFRα-specific ligand PDGF-AA induced only PDGFRα phosphorylation, the broadly active PDGF-BB ligand induced both PDGFRα and PDGFRβ phosphorylation, and TNF-α induced no PDGFR phosphorylation (Fig. 1B). Equal lysate loading was confirmed by measuring total PDGFRα or PDGFRβ (gray bars).

We also assessed for other interactions between PDGFRs and cadherin-11. Prolonged silencing of cadherin-11 using lentiviral shRNA as previously described [13] resulted in a consistent loss of PDGFRα protein expression by western analysis (Fig. 1D, representative blots Suppl. Fig. 1B). Some loss of PDGFRβ expression was also noted, but the effect was inconsistent. In addition, activation of PDGFRα or PDGFRβ differentially affected cadherin-11 surface expression, as measured by flow cytometry (Fig. 1E, representative staining Suppl. Fig. 1C). We found that PDGFRα homodimer stimulation (by PDGF-AA) did not affect cadherin-11 surface expression (mean fluorescence intensity (MFI)). In contrast, stimulation with the PDGFRβ-selective ligand, PDGF-DD, resulted in loss of cadherin-11 surface expression (see Suppl. Fig. 1A for ligand activation pattern). This difference was not due to differences in total phosphorylation levels, cadherin-11 expression was not affected by stimulation with PDGF-BB, which broadly induces high levels of phosphorylation of both receptors (Suppl. Fig. 1A). These differences were also not due to any ligand-specific loss of cell viability (Suppl. Fig. 1D). Taken together, these results show further evidence of a differential interaction between cadherin-11 and PDGFRα, compared to PDGFRβ.

PDGFRs strongly stimulate mesenchymal cell proliferation and migration [39], but their reported effects on cytokine and chemokine production are modest [40]. Therefore, we next compared the functional consequences of independently stimulating PDGFRα and cad11Fc (Fig. 2). As previously reported, cad11Fc induced fibroblast release of both IL-6 and MMP-3 (Fig. 2A–B). In contrast, PDGFRα-specific stimulation with PDGF-AA induced little IL-6 and MMP-3 release, but did markedly increase fibroblast proliferation (Fig. 2D–F). Cad11Fc also stimulated synovial fibroblast proliferation, as measured by cell metabolic activity (Fig. 2C, Supplemental Fig. 2A), ^{3}H-thymidine incorporation (Supplemental Fig. 2B) and direct cells counts (Supplemental Fig. 2C).

To test if cadherin-11 induced proliferation through PDGFRα, PDGFRs were blocked by a small molecule inhibitor (Fig. 3A) or siRNA silencing (Fig. 3B–C). Axitinib, a multi-tyrosine kinase inhibitor with activity against PDGFRs, inhibited both cad11Fc- and PDGF-BB-stimulated proliferation, but did not block TNF-α-induced proliferation (Fig. 3A). Silencing fibroblast PDGFR with siRNAs demonstrated that cad11Fc acted through PDGFRα, as proliferation was completely blocked in cells lacking PDGFRα, but not PDGFRβ, expression (Fig. 3B). Taken together, these results show that cadherin-11 regulates cell proliferation through PDGFRα activation and that PDGFRα activation contributes to, but does not solely explain, the effects of cadherin-11 signaling.

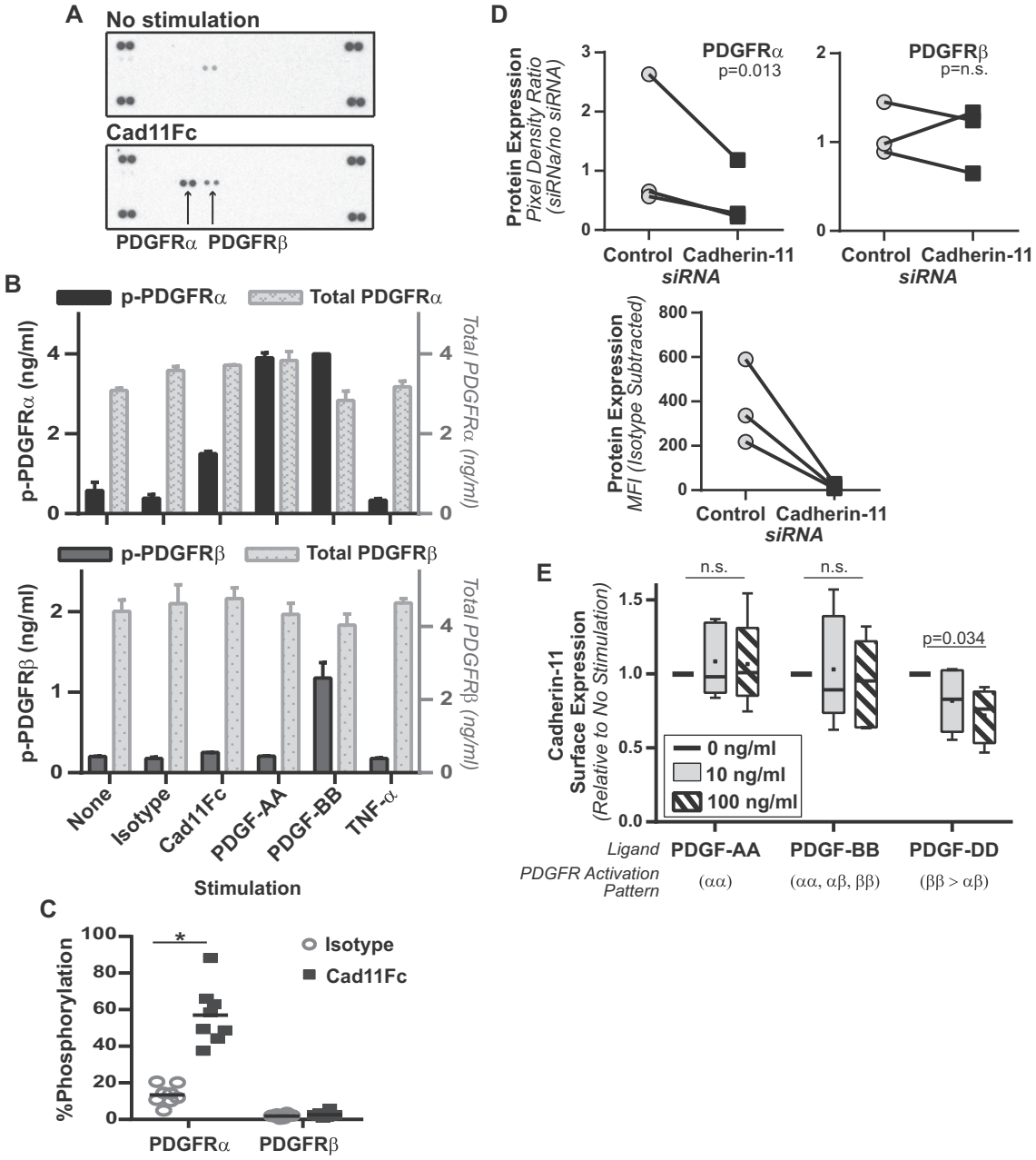

Fig. 1. Synovial fibroblast PDGFRα activated by cadherin-11. (**A**) RTK phosphorylation was compared in cell lysates made from serum-starved human synovial fibroblasts stimulated with or without cad11Fc for 10 min using a western blot array. (**B–C**) PDGFR phosphorylation after cad11Fc stimulation was confirmed using ELISA. (**B**) A representative experiment is shown demonstrating both phosphorylated (left y-axis) and total (right y-axis) PDGFR expression in serum-starved fibroblasts stimulated for 5 min with media alone, 40 μg/ml human IgG_1 isotype control, 40 μg/ml cad11Fc, 50 ng/ml PDGF-AA, 50 ng/ml PDGF-BB, or 10 ng/ml TNF-α. (**C**) Mean PDGFRα or PDGFRβ phosphorylation induced by cad11Fc was calculated as a percent of total PDGFRα or PDGFRβ receptor expression ($n = 8$ experiments with five RA fibroblast lines). Comparison cad11Fc v. Control: $*p < 0.0001$, paired student *t*-test. (**D**) Synovial fibroblasts were infected with control or cadherin-11-specific shRNA-containing lentivirus. Loss of cadherin-11 expression was confirmed by flow cytometry, subtracting isotype control MFI from cadherin-11 MFI. Fibroblasts were then starved in serum-free media for 2 days before measuring PDGFRα or PDGFRβ protein expression in cell lysates by western blot ($n = 3$ RA fibroblast lines; statistics, paired student *t*-test; n.s., not significant). (**E**) Serum-starved synovial fibroblasts were treated over night with the indicated ligands before measuring cadherin-11 surface expression by flow cytometry. Data is presented as the fold-change of ligand-treated cells over unstimulated cell ($n = 5$ (2 RA and 3 OA lines); statistics one-way ANOVA).

3.2. PDGFRα contributes to PI3K/Akt and MAPK signaling downstream of cadherin-11

To better understand how cadherin-11 interacts with PDGFRα, signaling molecules downstream of this interaction were investigated. Since cad11Fc activates multiple pathways, including MAPKs, PI3K/Akt, and NF-κB [12,13], we used small molecule enzymes inhibitors to focus on pathways that contribute to cad11Fc-stimulated proliferation (Fig. 4A). PI3K/Akt (LY2949922), ERK (U0126), and JNK (SP600125) inhibitors all blocked basal proliferation and proliferation induced by cad11Fc and PDGF-BB, pointing to a central role for these molecules in synovial fibroblast proliferation. The function of the MAPK p38 was more complex. Although the p38 inhibitor (SB203580) did not change basal proliferation, it increased proliferation in cad11Fc-stimulated cells, suggesting that p38 negatively regulates proliferation, at least after some stimuli. NF-κB inhibition did not affect fibroblast

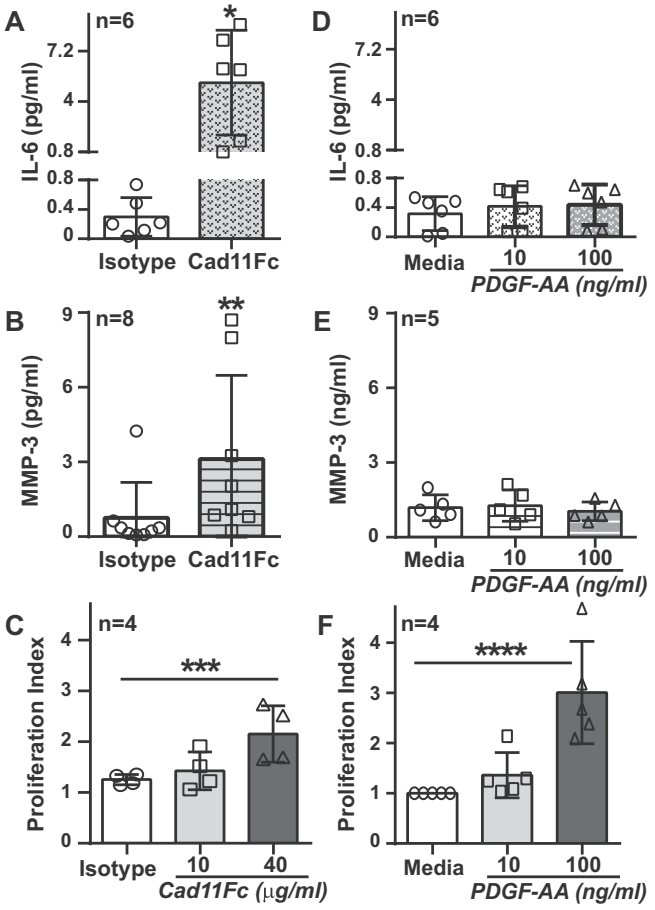

Fig. 2. Comparison of synovial fibroblast activation by cad11Fc and PDGF-AA. Serum-starved synovial fibroblasts stimulated with 10 μg/ml (**C**) or 40 μg/ml cad11Fc (**A–C**) or 10–100 ng/ml PDGF-AA (**D–F**) were compared to isotype control or media alone, respectively. IL-6 (**A**, **D**) and MMP-3 (**B**, **E**) release was measured by ELISA. Proliferation (**C**, **F**) was measured by alamarBlue® assay after a 3 or 4 day stimulation. Data presented is pooled from the indicated number (n) of experiments with at least three fibroblast lines. Comparison of unstimulated (media alone/isotype) to stimulated (cad11Fc/PDGF-AA): $^{*}p < 0.013$ and $^{**}p < 0.049$, paired t-test; $^{***}p = 0.044$ and $^{****}p < 0.01$, one-way ANOVA.

proliferation, although it did block MMP-1 production as expected (Supplemental Fig. 3).

To determine if cad11Fc-induced MAPK and PI3K/Akt phosphorylation required PDGFRα, the ability of PDGFRα silencing to block that phosphorylation was assessed. As seen in both a representative experiment (Fig. 4B) and by average fold change in pixel density (Fig. 4C), PDGFRα silencing strongly reduced Akt phosphorylation (mean inhibition 84.6% ± 11.4%), indicating that most Akt activation by cad11Fc depends on PDGFRα. In contrast, the effect of PDGFRα silencing on MAPK (ERK, JNK, p38) activation was more variable. MAPK signaling inhibition after PDGFRα silencing was roughly half that of Akt (%inhibition: ERK, 57 ± 50%; JNK, 31 ± 33%; p38: 44 ± 12%). In contrast, PDGFRβ silencing had little effect on either PI3K/Akt or MAPK phosphorylation. These results indicate that the interaction between cadherin-11 and PDGFRα promotes cell proliferation mainly through PI3K/Akt phosphorylation, with lesser contributions from JNK and ERK activation.

3.3. Cadherin-11 interacts with PDGFRα through its extracellular binding domains

We then examined how cadherin-11 interacts with PDGFRα, expecting that PDGFRα phosphorylation was dependent on cell cadherin-11, as was previously reported for cad11Fc-stimulated IL-6 and MMP expression [12,13]. Our hypothesis was that cad11Fc clustered both cell cadherin-11 and associated PDGFRα molecules, promoting PDGFRα autophosphorylation. Therefore, we silenced cadherin-11 expression on fibroblasts using siRNA. Similar to cadherin-11 shRNA silencing (Fig. 1D), siRNA silencing did result in a 32 ± 22% loss of PDGFRα as measured by ELISA ($n = 4$). However, after correcting for this loss by calculating the percentage of total PDGFRα phosphorylated, silencing cell cadherin-11 did not prevent cad11Fc-induced PDGFRα phosphorylation (Fig. 5A). This data suggested that clustering of cell surface cadherin-11 was not driving PDGFRα activation.

We next investigated if cad11Fc promoted PDGFRα activation by facilitating the autocrine loading of PDGF ligands. PDGF gene expression analysis showed that synovial fibroblasts produce the PDGFRα-specific ligands, *PDGFA* and *PDGFC*, especially *PDGFC* (Fig. 5B). In contrast, they express almost none of the ligands that independently activate PDGFRβ, *PDGFB* and *PDGFD*. Therefore, *PDGFA* or *PDGFC* expression was silenced before treatment with cad11Fc (silencing efficacy confirmed by qRT-PCR: *PDGFA* silencing 66%; *PDGFC* silencing 84%). No decrease in PDGFRα phosphorylation was observed in the ligand-silenced cells compared to controls, suggesting that cad11Fc does not work by facilitating ligand binding to PDGFRα (Fig. 5C).

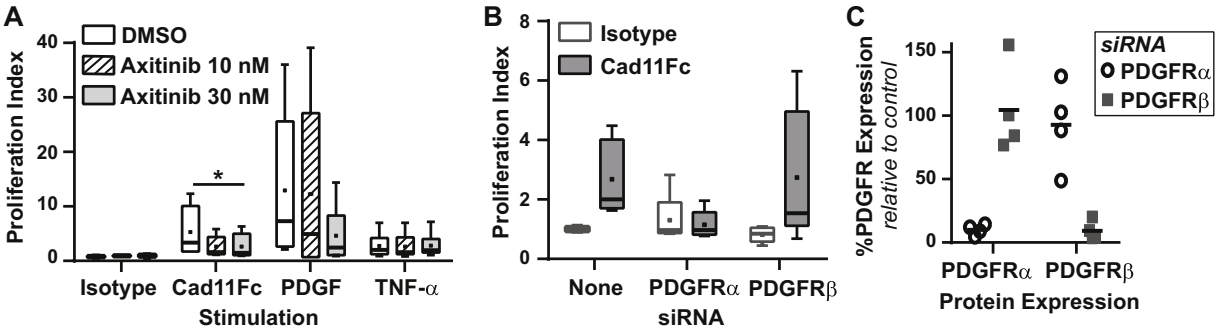

Fig. 3. Cadherin-11 regulates proliferation through PDGFRα activation. (**A**) Synovial fibroblasts were pretreated with the multi-kinase inhibitor axitinib or vehicle control for 1 h before stimulation by indicated methods. Proliferation was measured one day later by ^{3}H-thymidine incorporation ($n = 6$ experiments, three RA fibroblast lines). Comparison DMSO to axitinib-treated: $^{*}p = 0.042$, repeated measured one-way ANOVA. (**B**) PDGFRα and PDGFRβ gene expression was silenced by siRNA transfection before measuring proliferation after cad11Fc stimulation using ^{3}H-thymidine incorporation ($n = 6$ experiments, five **RA** fibroblast lines). Comparison isotype to cad11Fc: **No siRNA $p = 0.028$; PDGFRα siRNA $p = 0.83$; ***PDGFRβ siRNA $p = 0.014$, paired t-test. (**C**) PDGFRα or PDGFRβ surface expression was measured by flow cytometry in fibroblasts transfected with the indicated siRNA and percent expression of each receptor compared to control was calculated ($n = 4$ RA fibroblasts).

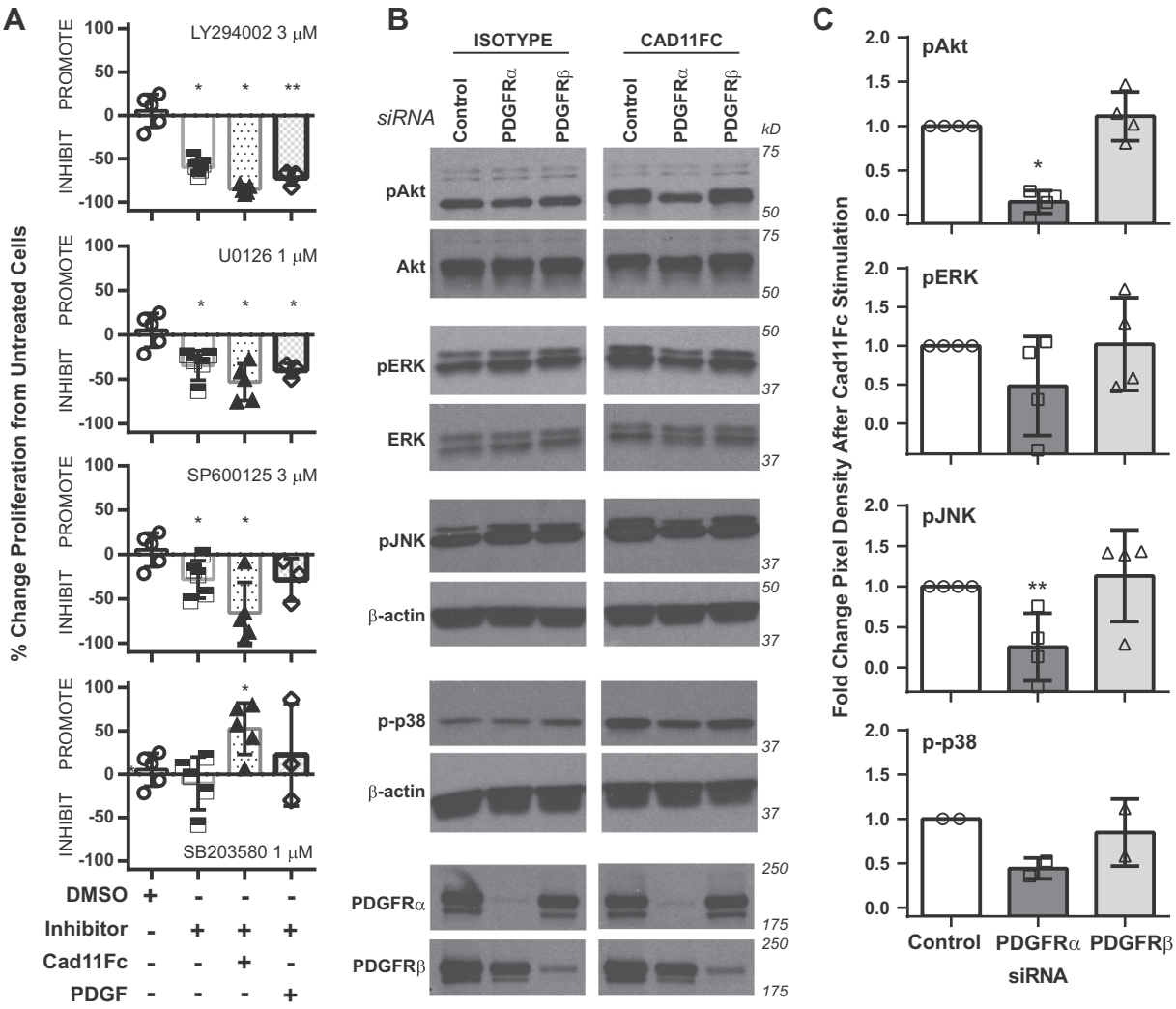

Fig. 4. PDGFRα mediates downstream Akt and MAPK activation by cadherin-11 signaling. (**A**) Serum-starved synovial fibroblasts were pretreated with the indicated inhibitors or vehicle control (DMSO) for 1 h and then incubated overnight with or without 20 μg/ml cad11Fc or 50 ng/ml PDGF-BB. Proliferation was measured by ^{3}H-thymidine incorporation. The results are expressed as percent change in proliferation from baseline (media alone). The data summarizes five experiments with four RA fibroblast lines. Comparison to DMSO control: $*p \leq 0.017$ and $**p = 0.025$, paired *t*-test. (**B–C**) Synovial fibroblast PDGFRα or PDGFRβ expression was silenced by siRNA transfection. Lysates generated after a 15-minute stimulation with cad11Fc or isotype control antibody were analyzed for the indicated signaling molecules or loading controls. (**B**) A representative western blot experiment demonstrates changes in Akt, ERK, JNK, and p38 phosphorylation after cad11Fc stimulation in control, PDGFRα, and PDGFRβ siRNA silenced fibroblasts. Isotype and Cad11Fc lanes are from the same gels but are separated because non-relevant lanes were removed. (**C**) Pixel density for phosphorylation Akt and MAPKs bands was measured using Image J. Fold change in pixel density after cad11Fc stimulation was calculated by comparing the increase in pixel density in PDGFRα or PDGFRβ silenced cells with that in control siRNA treated cells $((\text{Experimental}_{\text{cad11Fc}} - \text{Experimental}_{\text{isotype}}) / (\text{Control}_{\text{cad11Fc}} - \text{Control}_{\text{isotype}}))$, where experimental values used include control, PDGFRα, and PDGFRβ siRNA pixel density). Data summarizes four independent experiments for pAkt, pERK, and pJNK and two independent experiments for p-p38. Comparison control to PDGFRα siRNA: $*p = 0.0009$ and $**p = 0.038$, paired *t*-test.

Interestingly, *PDGFC* silencing did modestly decrease PDGFRα phosphorylation in unstimulated cells (~20% reduction), suggesting that this ligand is important for basal synovial fibroblast proliferation and/or survival.

These results led to an alternative hypothesis, mainly that cad11Fc interacted directly with PDGFRα to promote its activation. This hypothesis is supported by other reports of ligand-independent PDGFR activation, including activation of the growth factor RTKs, EGFR and FGFR2, by E-cadherin and N-cadherin extracellular domains, respectively [19,23,41–44]. Therefore, the ability of cad11Fc to bind directly to PDGFRα was tested by immunoprecipitation using the human glioblastoma cancer cell line U-118MG. Glioma tumors represent another cell type where cadherin-11 PDGFRα interactions may be particularly relevant, as cadherin-11 and PDGFRs expression are linked to malignant transformation [28,45]. Switching to this cancer cell line provided sufficient cell quantities for an immunoprecipitation-based analysis, as primary human fibroblasts are both slow growing and senesce after several passages.

First, the ability of cad11Fc to stimulate PDGFRα or PDGFRβ phosphorylation was tested in U-118MG cells (Fig. 5D). Similar levels of U-118 MG and synovial fibroblasts PDGFRα phosphorylation were detected after cad11Fc incubation, while no PDGFRβ phosphorylation was seen in either cell type. As an additional control, cells were also stimulated with an E-cadherin chimeric protein (EcadFc). E-cadherin, a marker of epithelial cells, is not typically expressed on mesodermally-derived tissues such as fibroblasts or neurons and, therefore, should have no biologic activity in these assays. Indeed, EcadFc treatment of fibroblasts or U-118MG did not induce either PDGFRα or PDGFRβ activation. Next, we assessed for an interaction between cad11Fc and PDGFRα at the cell surface. U-118MG cells were incubated with

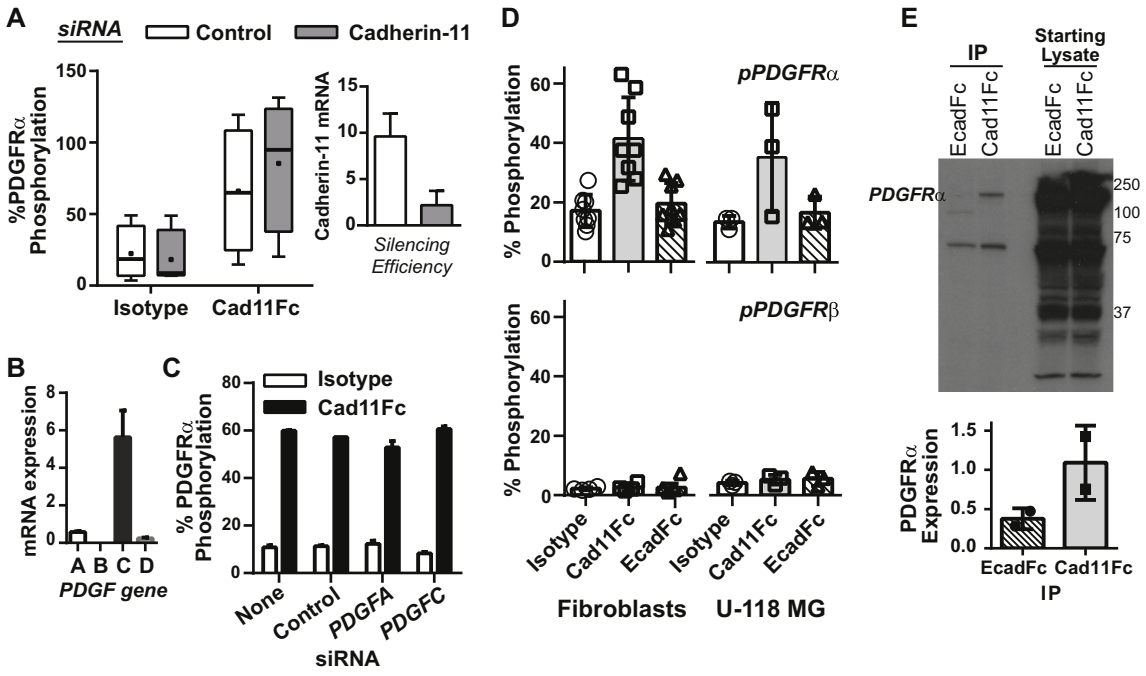

Fig. 5. Physical association between cad11Fc and PDGFRα does not require PDGF ligands or cell cadherin-11. (**A**) Serum-starved RA fibroblasts transfected with control or cadherin-11 siRNA were stimulated with cad11Fc or isotype control antibody for 15 min before cell lysis. Total and phosphorylated PDGFRα expression was measured by ELISA. PDGFRα phosphorylation was expressed as a percentage of isotype-stimulated control siRNA sample ($n = 4$). Cadherin-11 silencing efficiency was assessed by qRT-PCR, with gene expression normalized to the housekeeping gene HPRT. (**B**) PDGF ligand gene expression was determined in synovial fibroblasts (representative > 3 experiments). (**C**) Serum-starved synovial fibroblasts transfected with control, PDGF-A or PDGF-C siRNA were stimulated for 10 min with cad11Fc. PDGFRα phosphorylation was determined by ELISA (representative of 2 experiments). Efficiency of siRNA silencing relative to control siRNA confirmed by qRT-PCR (average silencing: *PDGFA* 66%; *PDGFC* 84%). (**D**) Synovial fibroblasts or U-118 MG glioblastoma cells were stimulated with human isotype control antibody, cad11Fc, or EcadFc for 10 min (representative of 3 experiments). Total and phosphorylated PDGFRα and PDGFRβ expression was determined by ELISA. The amount of phosphorylated PDGFR was expressed as a percentage of total cell PDGFRα or PDGFRβ. (**E**) U-118 MG surface proteins interacting with cad11Fc or EcadFc were cross-linked together and immunoprecipitated with protein G agarose beads. Starting lysates and immunoprecipitated proteins were analyzed for PDGFRα expression. The amount of PDGFRα immunoprecipitated by EcadFc or cad11Fc was expressed by dividing the pixel density of immunoprecipitated PDGFRα by the total amount of starting Fc protein ($n = 2$).

cad11Fc or EcadFc covalently attached to a crosslinking reagent that captures interacting proteins after UV irradiation. Association of PDGFRα with immunoprecipitated cad11Fc or EcadFc was detected by western analysis (Fig. 5E). PDGFRα was preferentially immunoprecipitated by cad11Fc, supporting an interaction between the extracellular domains of these two molecules. In summary, these data suggest that heterotypic interactions between cadherin-11 and PDGFRα may be important across many cell types.

4. Discussion

Although cadherins do not have any intrinsic signaling activity, cadherin junctions contain several types of receptor and non-receptor kinases and phosphatases [46]. Therefore, we screened for signaling molecules that interact with cadherin-11 and discovered that cadherin-11 signaling is mediated, in part, through an interaction with PDGFRα on both RA and OA fibroblasts, in keeping with robust PDGFR expression seen transcriptionally on human fibroblasts freshly isolated from synovial tissue [47]. This interaction was also detected in a glioblastoma cell line, indicating this interaction may be relevant in diverse cellular contexts. We also found that cadherin-11 silencing preferentially decreased PDGFRα protein expression and PDGFRα signaling preferentially preserved cadherin-11 surface expression, providing further evidence of biologic cross-talk between these two molecules.

PDGFRα activation divides cadherin-11 signaling into PDGFRα-dependent and -independent pathways. PDGFRα-dependent signaling stimulates robust cell proliferation *via* PI3K/Akt and MAPK phosphorylation. PDGFRα phosphorylation by cad11Fc accounts for nearly all the Pi3K/Akt activation but only part of the MAPK activation. These results suggest a potential signaling mechanism that co-regulates cell adhesion and cell growth. In contrast, PDGFRα-independent cadherin-11 signaling triggers more inflammatory fibroblast behaviors, such as IL-6, chemokine, and MMP release. What signaling partners contribute to this pathway is not yet known. However, proteins that interact at the cadherin cytoplasmic domains are potential candidates, including beta-catenin, p120 catenin, small G proteins, Src-family kinases, or other growth factor receptors [11,14,46].

The role of cell cadherin-11 also distinguishes how cad11Fc interacts with PDGFRα-independent and -dependent signaling pathways. We previously reported that cad11Fc stimulates IL-6 and MMP-3 expression by binding to cell cadherin-11, mimicking cadherin-11 adhesive junctions. This requirement for cell cadherin-11 is consistent with the enrichment of tyrosine-phosphorylated proteins detected at newly formed cadherin adherens junctions [46]. However, to our initial surprise, cad11Fc induced PDGFRα phosphorylation independently of cell cadherin-11 expression. In fact, cad11Fc immunoprecipitated PDGFRα from cell lysates, indicating that these proteins interact through the extracellular domains.

A direct interaction between cadherin-11 and PDGFRα extracellular domains is consistent with other reported interactions between cadherins and RTKs, including interactions between E-cadherin and EGFR family members [19,42,48] and N-cadherin and FGFR1 [23,24]. Interestingly, another extracellular interaction between cadherin-11 and the FGFR1 was reported to promote axonal extension in neurons [24]. Since synovial fibroblasts express FGFRs, including FGFR1, it was a

little surprising that cad11Fc did not induce FGFR activation in our RTK screening array (Fig. 1A). The preference for an interaction with PDGFRα over FGFRs in synovial fibroblasts is not understood, but may reflect their relative expression levels, location in specific membrane microdomains, or associations with other regulatory molecules, further highlighting the complexity of growth factor RTK cadherin interactions.

Chimeric cadherin Fc proteins are frequently used to investigate cadherin function *in vitro*, as these constructs greatly facilitate isolation of cadherin binding domains. However, it is challenging to extrapolate what role cadherin signaling plays *in vivo* from these assays, in part because cadherin Fc proteins may mimic either membrane or soluble cadherin binding. One possibility is that cad11Fc models the effect of shed soluble cadherin-11 ectodomains. Although cadherin adhesion classically involves homotypic binding of membrane cadherins, soluble cadherin extracellular binding domains may be released by cell sheddases. We have reported that soluble cadherin-11 is present in osteoarthritis and RA synovial fluid, indicating that this cleavage pathway is active inside the joint [38]. Another possibility is that cad11Fc mimics *trans* binding between membrane cadherin-11 on one cell with PDGFRα on an adjacent cell. In this case, cadherin-11 may cluster PDGFRα, favoring ligand-independent autophosphorylation. *Trans* cadherin RTK interactions may be particularly favored when there is a mismatch in cadherin expression between adjacent cells, limiting formation of homotypic adhesions. One place this type of mismatch may be relevant is in stromal-tumor interactions, where marked cadherin-11 upregulation on cancer cells undergoing epithelial-to-mesenchymal may activate cancer associated fibroblasts, promoting tumor spread.

This study also provides new insights into PDGFR regulation. PDGFRα and PDGFRβ drive different types of pathology in atherosclerotic and fibrosis disease models, despite frequent co-expression and highly redundant signaling functions. These functional differences point to unique regulatory mechanisms activating each receptor. Although much of this regulation is through restricted ligand expression, this report demonstrates a cadherin-dependent, ligand-independent mechanism that only activates PDGFRα, consistent with a limited number of studies reporting PDGFR activation by other receptors, including dopamine D4 and tissue factor receptors [41,43]. This ligand-independent activation may provide additional pathways to augment PDGFR responses in a tissue and/or cell-specific manner. For example, in the joint, injury or induction of autoimmune arthritis is accompanied by rapid synovial fibroblast proliferation and increased expression of cadherin-11 and PDGFRs [31,49–53].

5. Conclusions

In this study, we report a newly discovered interaction between cadherin-11 and PDGFRα that promotes PDGFRα activation in synovial fibroblasts and other cells. Our data adds to a growing appreciation that cadherins and growth factor RTKs interactions help coordinate the changes in cell adhesion, growth, and migration necessary for tissue responses in health and disease.

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Credit author statement

Bhanupriya Madarampalli: conceptualization, investigation, writing-review & editing. **Gerald F.M. Watts**: methodology, investigation, writing-review & editing. **Paul M. Panipinto**: investigation, writing-review & editing. **Hung Nguyen**: methodology, resources, writing — review & editing. **Michael B. Brenner**: writing — review & editing, supervision, funding acquisition. **Erika H. Noss**: conceptualization, methodology, formal analysis, investigation, writing — original draft, supervision, funding acquisition.

Competing interests

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Transparency document

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