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Targeting phosphatase-dependent proteoglycan switch for rheumatoid arthritis therapy

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Abstract

Despite the availability of several therapies for rheumatoid arthritis (RA) that target the immune system, a large number of RA patients fail to achieve remission. Joint-lining cells, called fibroblast-like synoviocytes (FLS), become activated during RA and mediate joint inflammation and destruction of cartilage and bone. We identify RPTP σ , a transmembrane tyrosine phosphatase, as a therapeutic target for FLS-directed therapy. RPTP σ is reciprocally regulated by interactions with chondroitin sulfate or heparan sulfate containing extracellular proteoglycans in a mechanism called the proteoglycan switch. We show that the proteoglycan switch regulates FLS function.

Incubation of FLS with a proteoglycan-binding RPTP σ decoy protein inhibited cell invasiveness

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Author contributions: K.M.D., S.M.S., C.S., M.N.D.S., W.B.K., E.C., C.H.C., N.M., R.L.-B., and D.L.B. contributed to acquisition of data. K.M.D., S.M.S., M.C., H.A.A., T.M., J.D.E., M.L.T., G.S.F., A.R.A., and N.B. contributed to study conception and design. K.M.D., S.M.S., C.S., M.N.D.S., W.B.K., B.B., C.F., M.C., R.L.S., T.M., J.D.E., M.L.T., G.S.F., A.R.A., and N.B. contributed to analysis and interpretation of data.

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Data and materials availability: LJI holds a pending patent "PTPRS and proteoglycans in autoimmune disease," PCT/US2013/051723, with S.M.S. and N.B. named as inventors.

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and attachment to cartilage by disrupting a constitutive interaction between RPTP σ and the heparan sulfate proteoglycan syndecan-4. RPTP σ mediated the effect of proteoglycans on FLS signaling by regulating the phosphorylation and cytoskeletal localization of ezrin. Furthermore, administration of the RPTP σ decoy protein ameliorated in vivo human FLS invasiveness and arthritis severity in the K/BxN serum transfer model of RA. Our data demonstrate that FLS are regulated by an RPTP σ -dependent proteoglycan switch in vivo, which can be targeted for RA therapy. We envision that therapies targeting the proteoglycan switch or its intracellular pathway in FLS could be effective as a monotherapy or in combination with currently available immune-targeted agents to improve control of disease activity in RA patients.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease that ultimately results in joint destruction, and although there has been much recent progress in therapies for RA, many patients still fail to achieve remission. There are currently no treatments in the clinic that target fibroblast-like synoviocytes (FLS), which are key orchestrators in RA pathogenesis. FLS are specialized synovial lining cells that secrete synovial fluid and extracellular matrix (ECM) and provide structure to the joint. In RA, FLS mediate joint destruction by invading cartilage and promoting inflammation and bone erosion (1).

The behavior of FLS is regulated by several intracellular pathways involving protein tyrosine phosphorylation (2). Although protein tyrosine phosphatases (PTPs) are important regulators of signaling, they remain uncharacterized in FLS. We previously reported that several PTPs are highly expressed in FLS (3). In the attempt to ascertain if any of these PTPs regulate FLS pathogenic behavior in RA, we assessed FLS from arthritic mice and identified a transmembrane PTP belonging to the R2A subclass (4), called RPTP σ (gene *Ptprs*), as a highly expressed phosphatase in arthritic FLS.

In neurons, engagement of RPTP σ N-terminal extracellular immunoglobulin-like domains 1 and 2 (Ig1&2) by heparan sulfate (HS) or chondroitin sulfate (CS) glycosaminoglycan (GAG) moieties of various proteoglycans (PGs) controls axonal extension (5, 6). The interaction of RPTP σ Ig1&2 with HS-containing PG induces RPTP σ oligomerization and functional inactivation, which promotes axonal extension. On the other hand, CS-containing PG can compete with HS-containing PG for binding to the same RPTP σ Ig1&2 domains, de-clustering RPTP σ and inhibiting axonal extension (6). This mechanism has been termed the “PG switch” (6), and it mediates inhibition of axonal growth through CS-rich repair tissue after spinal cord injuries. Inhibition of RPTP σ through in vivo short hairpin RNA or a cell-permeable peptide able to induce phosphatase oligomerization can induce neuronal regeneration in models of spinal cord contusion (7, 8). It is unclear whether the PG switch depends on RPTP σ phosphatase activity and what intracellular signaling pathways mediate its actions. It is also unknown whether the PG switch exists in tissues outside the central nervous system.

The joint is composed of highly PG-rich tissue, and CS is the predominant GAG in cartilage (9). On the other hand, HS-containing PGs are primarily located on cell surfaces where they mediate interaction between cells and surrounding ECM. In FLS, the HS PG syndecan-4 is

required for the attachment of FLS to cartilage (10), an important pathogenic FLS behavior (1). However, the regulation of FLS intracellular signaling by PG has not been addressed.

Here, we found that RPTP σ expression is up-regulated during arthritis progression, and that the PG switch is active in FLS. Manipulation of the PG switch using exogenous CS or recombinant PG-binding decoy RPTP σ protein inhibited FLS invasion, migration, and attachment to cartilage. We identified the intracellular mechanism of action of the PG switch as the dephosphorylation of the RPTP σ substrate ezrin, which disrupts its cytoskeletal localization during migration. Finally, we show that RPTP σ Ig1&2 treatment has therapeutic potential for arthritis in vivo by inhibiting attachment of FLS to cartilage and decreasing disease severity.

RESULTS

RPTP σ is selectively expressed on FLS in arthritic synovium

Several PTPs are highly expressed in FLS (3). In the attempt to identify if any of these PTPs regulate the pathologic behavior of FLS in RA, we assessed fluorescence activated cell sorting–isolated FLS from mice subjected to passive transfer of K/BxN serum (11)—an FLS-dependent model of RA (1)—and discovered that a transmembrane PTP called RPTP σ (gene *Ptprs*) was induced during arthritis progression (Fig. 1A and table S1). In comparison, RPTP σ expression was minimal in synovial macrophages from the same mice (Fig. 1B and table S1), supporting previous reports that RPTP σ is almost absent in immune cells (12). To determine what induces RPTP σ expression during chronic arthritis, we considered the formation of the FLS-rich pannus during chronic arthritis (13). As shown in Fig. 1C, pannus formation is much more pronounced during chronic K/BxN arthritis compared to acute K/BxN arthritis. We therefore hypothesized that pannus formation in chronic arthritis could increase *Ptprs* expression due to increased cell density of FLS, as was previously described in 3T3 cells (14). Figure 1D and table S1 shows that *Ptprs* expression increases with increasing cell density of cultured FLS, with an average ~2.5-fold increase in expression in densely crowded cells compared to subconfluent cells. Thus, RPTP σ is a transmembrane PTP highly expressed in FLS and is induced in the arthritic synovial lining.

The RPTP σ -dependent PG switch is functional in FLS and regulates FLS invasiveness and migration

We reported that RPTP σ is highly expressed in human RA FLS (3). Further investigations in FLS from osteoarthritis (OA) patients showed that RPTP σ is similarly highly expressed in OA FLS (fig. S1 and table S2). Because RPTP σ is induced during arthritis and PGs are one of the main components of the joint ECM, we sought to determine whether the RPTP σ -dependent PG switch exists in FLS and if it regulates FLS pathogenic behavior. Serum-induced FLS invasiveness through Matrigel-coated Transwell chambers (Fig. 1E) and migration through uncoated Transwell chambers (Fig. 1F) were decreased in the presence of exogenous cartilage-derived CS, and these phenomena were dependent on RPTP σ expression. On the other hand, in contrast to exogenous CS, treatment with exogenous HS did not affect FLS migration (Fig. 1G). Because CS competes with HS for binding to RPTP σ Ig1&2 domains, we hypothesized that the effect of CS on FLS behavior is due to

disruption of a constitutive interaction between RPTP σ and cell surface HS. Removal of HS, by either heparinase III digestion or knockdown of *Ext1*, a crucial component of the HS biosynthetic pathway, from FLS was sufficient to induce RPTP σ -dependent inhibition of serum-induced cell migration (Fig. 1H and fig. S2). These results demonstrate that the PG switch exists in mouse FLS, and we then wished to determine whether this occurs in human FLS. Indeed, treatment of primary FLS from RA patients (RA FLS) with CS inhibited migration in an RPTP σ -dependent manner (Fig. 1I and fig. S3).

Decoy RPTP σ Ig1&2 competes for FLS surface HS and regulates FLS invasiveness, migration, and attachment to cartilage

To test if disrupting the interaction between RPTP σ and HS replicates the effect of CS on FLS migration, we incubated FLS with various concentrations of PG-binding RPTP σ Ig1&2 (herein called “Ig1&2”) recombinant fragment during migration (6). Ig1&2 inhibited FLS migration starting at a 20 nM concentration, which is compatible with the K_D (dissociation constant) \approx 10 to 20 nM between the Ig1&2 domain and HS (6). Increasing the concentration of Ig1&2 beyond 20 nM did not further inhibit FLS migration (Fig. 2A). We then used 20 nM Ig1&2 to determine its effect on FLS migration and invasiveness. Treatment of FLS with Ig1&2 inhibited migration of FLS in response to serum and platelet-derived growth factor (PDGF), a prominent growth factor in the synovium of RA patients (Fig. 2, B and C), as well as invasiveness of FLS (Fig. 2D), in an RPTP σ -dependent manner. Furthermore, Ig1&2 K, a non-GAG binding mutant form of Ig1&2, in which lysine residues K67, K68, K70, and K71 have been mutated to alanine residues (6), was not able to affect migration of FLS (Fig. 2E), confirming that the effect of Ig1&2 is through its binding to HS. Ig1&2 treatment did not affect FLS survival (fig. S4 and table S3) or response to cytokine stimulation (fig. S5 and table S4); therefore, we determined that the effect of Ig1&2 is selective for migration and invasiveness of FLS. Treatment with Ig1&2 together with heparinase III or CS did not lead to additional inhibition of FLS migration (Fig. 2, F and G). We conclude that constitutive interaction between RPTP σ and HS-containing PG on the cell surface promotes PDGF-induced FLS invasiveness and migration. This interaction can be abrogated by decoy Ig1&2 or by CS, which effectively competes with endogenous HS for binding to the Ig1&2 domains of RPTP σ . Ig1&2 was also effective in human FLS, because treatment of RA FLS with Ig1&2 also caused inhibition of migration in Transwell assays (Fig. 2H and table S5) as well as in scratch wound assays (Fig. 2I).

Attachment of RA FLS to cartilage correlates with cartilage damage in RA (1) and is dependent on surface HS expression on FLS as well as damage and subsequent PG exposure on cartilage upon treatment with inflammatory cytokines such as interleukin-1 β (IL-1 β) (10). Ig1&2 reduced mouse FLS attachment to ECM (fig. S6 and table S6) and significantly diminished the ability of RA FLS (Fig. 3A) and mouse FLS (Fig. 3B and table S7) to attach to IL-1 β -pretreated cartilage. This effect of Ig1&2 on FLS cartilage attachment was dependent on RPTP σ expression (Fig. 3B). RPTP σ -deficient FLS displayed normal attachment to cartilage (Fig. 3B), suggesting that RPTP σ does not simply act as an adhesion molecule for cartilage-expressed PG. We confirmed that removal of surface HS from FLS by heparinase III digestion was sufficient to inhibit attachment to cartilage (10), whereas treatment with heparinase III and Ig1&2 did not lead to additive inhibition of cartilage

attachment (Fig. 3C and table S7). We conclude that the interaction of RPTP σ with FLS cell surface HS modulates intracellular signaling to promote FLS attachment to cartilage.

Regulation of FLS migration by RPTP σ Ig1&2 is dependent on syndecan-4

We next sought to determine which HS-containing PG on FLS is responsible for the effect of RPTP σ Ig1&2. We assessed the expression of cell surface HS PG syndecan (*SDC*) and glypican (*GPC*) family members in human RA FLS and found *SDC2*, *SDC3*, *SDC4*, and *GPC1* to be the most highly expressed (Fig. 4A and table S8). We then designed cell-permeable ASO to knock down expression of these HS PG and identified an ASO able to knock down *SDC2* and *GPC1* (*SDC2/GPC1* ASO) and a second ASO able to knock down *SDC4* (*SDC4* ASO; fig. S7 and table S9). Ig1&2 was able to inhibit the migration of RA FLS treated with control or *SDC2/GPC1* ASO, but not of cells treated with the *SDC4* ASO (Fig. 4B). We also assessed the effect of Ig1&2 on the migration of FLS from *Sdc3* and *Sdc4* knockout (KO) mice. Ig1&2 was able to inhibit migration of *Sdc3* KO FLS (Fig. 4C), but not *Sdc4* KO FLS (Fig. 4D), suggesting that syndecan-4 is the HS PG that physiologically regulates RPTP σ in FLS. This conclusion is in line with the known role of syndecan-4 as a promoter of FLS cartilage attachment (10).

We next performed immunoprecipitation studies to confirm that RPTP σ indeed interacts with syndecan-4. Figure 4E shows that syndecan-4 coprecipitated with RPTP σ when both proteins were overexpressed in human embryonic kidney (HEK) 293T cells. Further, to determine whether Ig1&2 interacts with syndecan-4, we immunoprecipitated overexpressed syndecan-4 from HEK293T cells and found that it was able to pull down purified recombinant Ig1&2 (Fig. 4F). We also studied the colocalization between Ig1&2 and endogenous syndecan-4 by incubating recombinant Ig1&2 with ankle sections from mice with chronic K/BxN arthritis and performing immunofluorescence microscopy. As shown in Fig. 4G, Ig1&2 colocalizes with syndecan-4 at the interface between pannus and cartilage. These results strongly support a model where RPTP σ , as well as the PG-binding decoy receptor fragment Ig1&2, interacts with syndecan-4.

RPTP σ catalytic activity is necessary for the PG switch

To assess whether the mechanism of action of the PG switch in FLS requires RPTP σ phosphatase activity, we reconstituted *Ptprs* KO FLS with full-length wild-type RPTP σ or an inactive C1548S/C1839S mutant (C/S) (fig. S8) and assessed the effect of Ig1&2 on FLS migration. Expression of wild-type, but not phosphatase-dead (C/S), RPTP σ restored sensitivity of *Ptprs* KO FLS to Ig1&2, providing the first direct evidence that RPTP σ catalytic activity is necessary for the PG switch (Fig. 5A).

Ezrin is a substrate of RPTP σ in FLS, and its phosphorylation and cytoskeletal localization is regulated by the PG switch

We next endeavored to identify the substrate dephosphorylated by RPTP σ in response to the PG switch. We prioritized cytoskeleton-associated proteins whose tyrosine phosphorylation promotes cell adhesion and migration, and thus examined the effect of Ig1&2 on phosphorylation of ezrin, focal adhesion kinase (FAK), paxillin, and p130Cas. We discovered that phosphorylation of ezrin was decreased in PDGF-stimulated FLS treated

with Ig1&2 (Fig. 5B and table S10), whereas phosphorylation of FAK, paxillin, and p130Cas was unaffected (fig. S9). A recent report also identified ezrin as an RPTP σ substrate in colon epithelial cells (15). To confirm that RPTP σ directly interacts with ezrin in FLS, we performed a substrate-trapping assay using an RPTP σ D1516A mutant catalytic domain (D/A) and found that it precipitated ezrin from RA FLS lysates (Fig. 5C).

We next hypothesized that because ezrin is a substrate of RPTP σ , tyrosine phosphorylation of ezrin was necessary for the effect of Ig1&2 on FLS migration. Mouse FLS were transfected with wild-type ezrin or phosphomimetic ezrin mutants, which contain tyrosine-to-glutamic acid mutations on residues 146 and 353, which are the mouse homologues of the ezrin tyrosines targeted by human RPTP σ in colonic epithelial cells (15). Phosphomimetic mutants represent constitutively phosphorylated tyrosine residues that are insensitive to dephosphorylation. Figure 5D shows that the migration of FLS overexpressing phosphomimetic ezrin mutants with mutations at residues 146 and 353 was unaffected by Ig1&2 treatment. This demonstrates that the ability of Ig1&2 to reduce migration of mouse FLS is dependent on ezrin tyrosine dephosphorylation.

Tyrosine phosphorylation of ezrin regulates cell migration through its association with actin (16). In PDGF-stimulated migrating cells, ezrin colocalized with the actin cytoskeleton (fig. S10) and displayed accumulation at the leading edge (Fig. 5E). In contrast, upon Ig1&2 treatment, ezrin displayed reduced colocalization with the actin cytoskeleton and no enrichment at the leading migration edge (Fig. 5E and fig. S10). This effect was dependent on RPTP σ catalytic activity, because *Ptprs* KO FLS reconstituted with wild-type, but not phosphatase-dead (C/S), RPTP σ displayed disruption of ezrin localization upon Ig1&2 treatment (Fig. 5F).

We conclude that triggering the PG switch results in RPTP σ -mediated dephosphorylation of ezrin, which disrupts ezrin localization at the cytoskeleton during migration. Our data support previous reports that ezrin is necessary for RA FLS invasiveness (17, 18).

The phosphorylation of β -catenin, which is a known substrate of RPTP σ in other tissues (19) and regulates migration of other cell types (20), was unaffected by Ig1&2 treatment of FLS (fig. S11). Cadherin-11, a critical FLS migration regulator (21, 22), did not mediate the effect of the PG switch, and its functions were unaffected by incubation of FLS with Ig&2 (fig. S11). Treatment with Ig1&2 did not alter PDGF receptor (PDGFR) expression or activation of Akt or mitogen-activated protein kinases (MAPKs) downstream of the PDGFR (fig. S12), suggesting that the PG switch selectively modulates the ezrin pathway in PDGF-stimulated FLS.

Decoy RPTP σ Ig1&2 is active in vivo and decreases arthritis severity

We next examined whether the PG switch regulates FLS behavior in vivo. We assessed the effect of Ig1&2 on arthritis development and severity in mice injected with K/BxN serum. In this model, FLS have a demonstrated pathogenic role (1). Treatment of mice with Ig1&2 in parallel to arthritis induction resulted in decreased arthritis severity compared to control-treated mice as assessed by clinical score and ankle thickness (Fig. 6A and table S11), intravital joint inflammation (Fig. 6B and table S11), microcomputed tomography

(microCT) of bone (Fig. 6C), and histological analysis of cartilage damage and bone erosion (Fig. 6D and table S11). As expected, Ig1&2 had no effect on arthritis severity of *Ptprs* KO mice (Fig. 6, E and F, and table S11), which developed disease similar to wild-type littermates (fig. S13 and table S12). Ig1&2 treatment also reversed severity of established arthritis (Fig. 6, G and H, and table S11). Together, these results show that interference with the PG switch in vivo has an RPTP σ -dependent therapeutic effect on arthritis.

Decoy RPTP σ Ig1&2 reduces FLS attachment to cartilage in vivo

FLS critically mediate cartilage damage in the K/BxN model (1). In line with our observation that Ig1&2 inhibits FLS migration and attachment to cartilage ex vivo (Fig. 3, A and B), the reduced cartilage damage in Ig1&2-treated mice (Figs. 6D and 7A, red insets) correlated with the reduced FLS crawling across the surface of cartilage in arthritic joints (Fig. 7A, yellow insets). To further confirm that Ig1&2 inhibits the ability of FLS to damage cartilage in vivo, we used the severe combined immunodeficiency (SCID) model of FLS cartilage coengraftment (23). Ig1&2 administration markedly decreased RA FLS invasion of cartilage in this model (Fig. 7B).

DISCUSSION

A major unmet medical need in RA is that despite the availability of several disease-modifying agents, a substantial fraction of patients fails to achieve remission. A proposed solution to this problem is the development of therapies that target FLS, a nonimmunological joint-resident cell type that drives RA pathogenesis (1). FLS-associated molecules that could be targeted for adjuvant therapy for RA are highly sought after; however, so far, only one such candidate, the transmembrane adhesion molecule cadherin-11, has been identified (1). We identified another therapeutic target for FLS-directed therapy in the R2A subclass PG-binding RPTP σ tyrosine phosphatase, an enzyme that has been reported to be almost absent in immune cells (12). Here, we show that the expression of RPTP σ is induced in FLS during arthritis, and that the RPTP σ -mediated PG switch selectively regulates FLS pathogenic behaviors. We also developed an approach to pharmacologically manipulate the phosphatase through its extracellular domain, and we show that targeting RPTP σ ameliorates established arthritis in mice.

Two additional members of the R2A subclass of PTPs, RPTP and RPTP-LAR, can also bind PG (6, 24); however, their expression is minimal in RA FLS (3) and undetectable in FLS from arthritic mice (fig. S14 and table S13). Along with the ineffectiveness of the PG switch in FLS lacking expression of RPTP σ from mice and RA patients, we conclude that the PG switch is specifically mediated in FLS by RPTP σ .

Dissociation of RPTP σ from surface HS-containing PG using CS or with Ig1&2, a recombinant HS-binding decoy fragment of RPTP σ , is sufficient to “flip” the PG switch to the “on” state, leading to functional activation of the phosphatase and inhibition of migration, invasion, and cartilage attachment. Our data provide mechanistic support to previously proposed models where CS releases RPTP σ from a constitutive interaction with HS-containing PG on neuronal surfaces, thus declustering and functionally activating the enzyme (5, 6, 25, 26). However, a notable difference in the physiology of the PG switch

between neurons and FLS is that addition of exogenous HS does not enhance FLS migration and invasion. A possible explanation is that in the absence of CS, most RPTP σ is bound to HS on the surface of FLS and kept in a functionally inactive state (Fig. 8).

In addition to cell migration and invasion, another pathogenic FLS phenotype that is prominently regulated by the PG switch is cartilage attachment. It is known that in FLS, the HS PG syndecan-4 is required for the attachment of FLS to cartilage (10). Our study also points to syndecan-4 as the main HS PG responsible for the physiological regulation of RPTP σ function in FLS. Thus, we speculate that syndecan-4 on the surface of FLS promotes cartilage attachment through oligomerization and inactivation of RPTP σ . The fact that inactivation of RPTP σ per se does not reduce the ability of FLS to attach to cartilage rules out that RPTP σ simply acts as a cartilage adhesion molecule and supports our hypothesis that the PG switch regulates cell adhesiveness through RPTP σ -mediated regulation of intracellular signaling. Further experimentation is necessary to elucidate the downstream molecular mediators of FLS adhesion that are regulated by the PG switch.

The intracellular mechanism behind the PG switch has not yet been addressed. We show that the switch is dependent on the phosphatase activity of RPTP σ and that in FLS, RPTP σ couples ECM composition with intracellular signaling through PG switch-regulated dephosphorylation of ezrin (Fig. 8). Ezrin is a cytoskeleton-associated protein that is involved in invasiveness of RA FLS (18). Tyrosine phosphorylation of ezrin has been shown to regulate the migration and invasion of various cell types through the modulation of its localization and association with actin (16). It remains to be elucidated whether the functional activation of RPTP σ that occurs as a result of flipping the PG switch on the surface of FLS depends on catalytic activation of the phosphatase, increased access of RPTP σ to ezrin after monomerization, or on additional unknown molecular mechanism(s).

We find that flipping the PG switch in vivo with Ig1&2 decreases arthritis severity and cartilage damage in the K/BxN serum transfer model of arthritis. Administration of Ig1&2 was also able to reverse established arthritis in a model of chronic K/BxN serum transfer arthritis. Lack of efficacy of Ig1&2 in *Ptprs* KO mice provides strong evidence that Ig1&2 exerts its antiarthritic effect by targeting RPTP σ . Evidence of reduced cartilage damage and FLS crawling onto cartilage and decreased invasion of RA FLS into cartilage implants in mice treated with Ig1&2 correlates well with the above-mentioned in vitro evidence and strongly points to an FLS-specific effect of Ig1&2 in vivo.

Arthritis that develops in the passive K/BxN model depends on innate immunity (11) and FLS (1). Although an effect of Ig1&2 on innate immune cells cannot be entirely ruled out, it appears to be unlikely based on the fact that RPTP σ is minimally or not expressed in synovial macrophages (Fig. 1B) and reportedly in other immune cells (12). Also, incubation with Ig1&2 had no effect on macrophage activation (fig. S15 and table S14).

Because of the known RA-protecting action of spinal cord lesions/denervation in humans and in the K/BxN model (27), the established role of RPTP σ as an inhibitor of axonal growth raises the question whether Ig1&2 decreases severity of arthritis in mice by reducing pro-arthritis neural input. Indeed, Ig1&2 is unlikely to penetrate the blood-brain barrier after

parenteral injection but could still affect peripheral neuroautonomic or sensory fibers. However, inhibition of sympathetic or parasympathetic input does not affect arthritis severity in the K/BxN model (fig. S16 and table S15) (27). Deficiency of the transient receptor potential vanilloid cation channel (*Trpv1* KO), a proinflammatory receptor on sensory nerve fibers (28), does not affect disease severity in this model as well (27), and arthritic *Trpv1* KO mice were sensitive to Ig1&2 treatment (fig. S16 and table S15). Because arthritis protection by denervation in the K/BxN model is mediated by alteration in endothelial permeability (27), we also assessed whether administration of Ig1&2 reduces early vascular permeabilization caused by injection of K/BxN serum; however, we did not see any difference between Ig1&2-treated and untreated mice (fig. S17 and table S16). We conclude that it is unlikely that the arthritis protection by Ig1&2 in the K/BxN model is directly or indirectly mediated by actions at the central or peripheral nervous system level or on vascular permeability.

There are interesting parallels between the phenotype of mice treated with Ig1&2 and that of mice that are deficient in, or treated with inhibitors of, cadherin-11, an FLS-selective adhesion molecule and a proposed drug target for therapy of RA (1). Lack or inactivation of cadherin-11 similarly impairs FLS migration and invasiveness *ex vivo*, and prevents and reverses arthritis in the K/BxN serum transfer model. *Cadherin-11* (*Cdh11*) KO mice display a similar reduction in cartilage damage, which correlates with reduced crawling of FLS onto cartilage (1). We show that the pathways controlled by cadherin-11 and the PG switch in FLS are nonoverlapping. Overall, the finding that the manipulation of two independent pathways controlling FLS migration and adhesion results in a similar reduction in severity of arthritis supports the idea that these are key phenotypes underlying the pathogenic action of FLS in RA. Recent findings that migration and adhesion pathways are hotspots of epigenetic modification in FLS from human RA patients lend further support to this model (29).

The joint is a PG-rich environment, yet little is known about how PGs affect intracellular signaling in joint-lining cells. Our study shows that RPTP σ mediates the action of extracellular PG on FLS functions through the PG switch (5, 6). The role of this mechanism in joint physiology remains to be fully understood. Because FLS do not attach well to intact cartilage [consisting primarily of CS PG (9, 30)], and cartilage PG loss is necessary for FLS attachment (10, 23), we speculate that RPTP σ is one of the mediators of physiological inhibition of FLS attachment and invasion of cartilage in healthy joints. Therefore, we hypothesize that treatment of arthritic joints, where CS-mediated inhibition might be reduced following cartilage damage (31, 32), with Ig1&2 restores a physiological safety mechanism that deters FLS from damaging the joint. However, the observation that inactivation of RPTP σ in mice does not lead to joint pathology and does not influence K/BxN serum-induced arthritis suggests that additional mechanisms, perhaps mediated by expression of CS receptors NgRs (33) on FLS, can compensate for the loss of RPTP σ to avoid cartilage damage by FLS in physiological or pathological conditions.

It has been proposed that agents that selectively target FLS in RA without causing significant immune suppression could be combined with current disease-modifying antirheumatic drugs (DMARDs) to improve disease control and remission rates (2). In

addition, recent studies of RA synovial pathology suggest that disease might be driven by FLS activation more in some patients than others, suggesting that FLS-targeted interventions could be effective as a monotherapy in the appropriate subset of patients (34).

Cadherin-11 is currently the only validated target for FLS-directed therapy for RA, and anti-cadherin-11 antibodies are in preclinical development as first-in-class agents for combination therapies for RA (2). Similar to anti-cadherin-11 antibodies, Ig1&2 was able to reverse established disease in an FLS-dependent model of RA. Also, like cadherin-11, RPTP σ displays minimal or no expression in immune cells (Fig. 1B and fig. S15) (12), suggesting that drugging RPTP σ is unlikely to result in immune suppression. Therefore, we propose that RPTP σ Ig1&2 or other biologics or small molecules able to selectively disrupt the interaction between RPTP σ and HS on the surface of FLS are attractive candidates for development of FLS-directed therapies to complement existing DMARDs for RA. The absolute conservation between human and mouse RPTP σ Ig1&2 increases confidence in the translational potential of our proposed approach for therapy for RA.

There are a number of limitations of our study. For example, the molecular events that lead to triggering of ezrin dephosphorylation by RPTP σ after activation of the extracellular PG switch remain to be addressed. Because syndecan-4 is known to oligomerize and trigger intracellular signaling (35), it is possible that syndecan-4-mediated signaling also contributes to the signal transduction of the PG switch in FLS. Additional limitations will need to be addressed before translation of our findings into the clinic is attempted. These include the need for preclinical studies characterizing the pharmacology of Ig1&2 and its efficacy in combination with available DMARDs in models of RA. Possible effects of Ig1&2 on immune cells and on other organs and systems will also need to be quantified in appropriate models to assess the therapeutic potential of Ig1&2 and its suitability for combination with other therapeutic agents.

MATERIALS AND METHODS

Study design

The objective of this study was to demonstrate that the RPTP σ -mediated PG switch regulates invasiveness and migration of FLS, and that it can be manipulated *in vivo* using a decoy RPTP σ Ig1&2 fragment to decrease cartilage destruction and arthritis in a mouse model. This was achieved through *ex vivo* FLS experiments and *in vivo* studies of arthritis development in Ig1&2-treated mice. Mice genetically manipulated to lack RPTP σ were used to demonstrate target specificity. For *ex vivo* experiments, FLS lines were acquired from multiple different human patients or from multiple littermate pairs of mice. For *in vivo* experiments, mice were randomly assigned to different treatments and blinding was achieved by scoring mice before reading their ear tag. Experimental endpoints for the acute K/BxN model of arthritis were determined to be at least 12 days after serum injection, when disease resolution is achieved; for the chronic K/BxN model, the endpoint was determined to be at least 10 days after disease plateau. Clinical endpoints were determined by Institutional Animal Care and Use Committee-approved protocols (#AP140-NB4-0610) at the La Jolla Institute for Allergy and Immunology (LJI) and were never reached throughout the experiments. The number of replicate measurements and mice is stated in each figure

legend. Sample sizes were determined using pilot studies and power calculations on preliminary data to achieve statistical power of 0.90 ($\alpha = 0.05$).

Statistical analysis

For comparison of treatment groups, Mann-Whitney and unpaired *t* tests were performed where appropriate as reported in the figure legends. All statistical analyses were performed using GraphPad Prism software (version 6.0). Statistical significance was achieved when $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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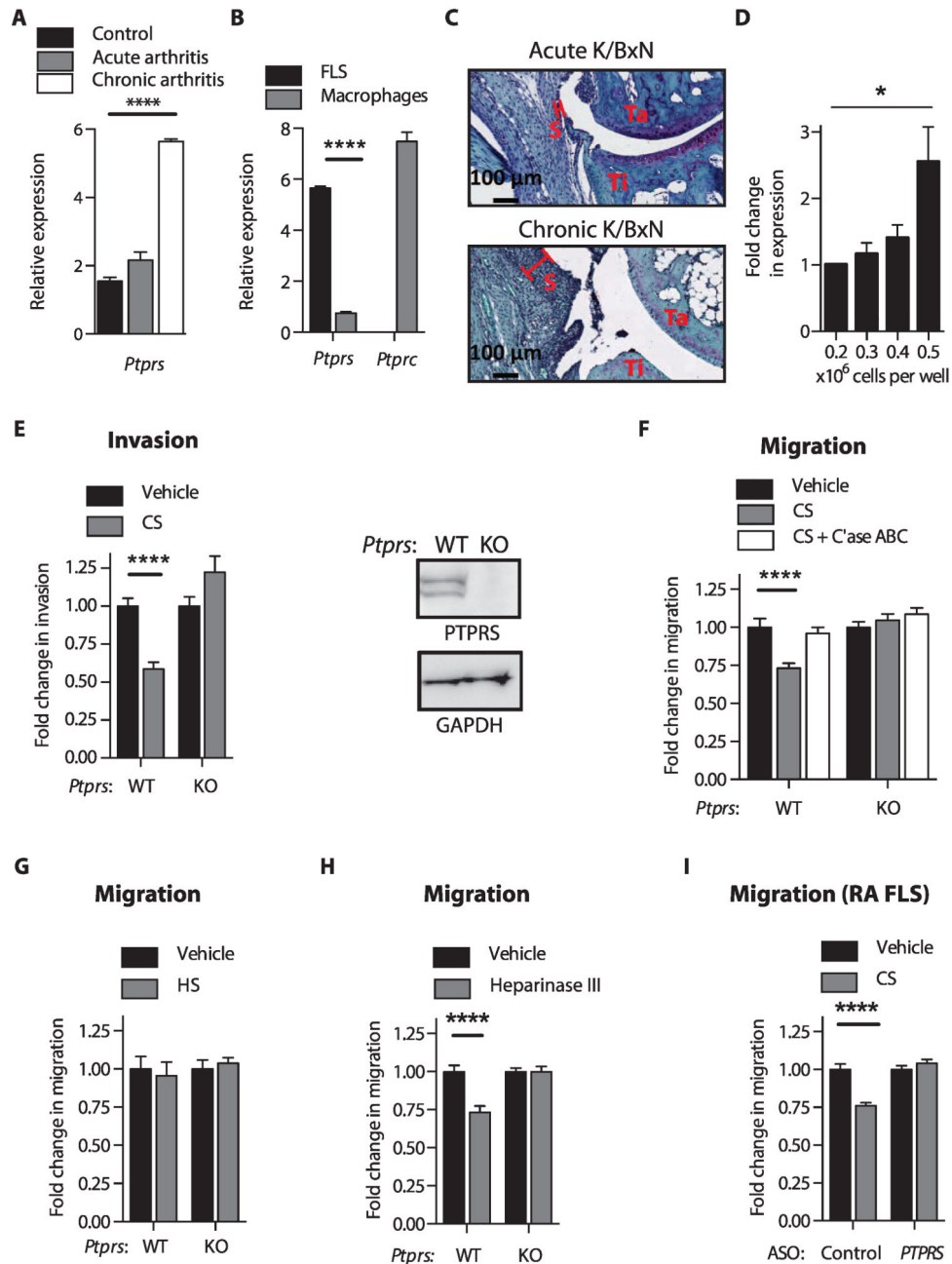


Fig. 1. RPTP σ -dependent PG switch controls FLS invasiveness and migration

(A) *Ptprs* expression in freshly sorted FLS from joints of nonarthritic mice or mice with K/BxN serum–induced arthritis. (B) *Ptprs* and *Ptprc* expression in freshly sorted FLS and synovial macrophages from mice with chronic K/BxN arthritis. (A and B) Mean \pm SEM of expression relative to *Polr2a*; **** P < 0.0001, unpaired t test and Welch's correction. (C) Pathology of safranin O–stained ankles from mice on day 14 of acute, or day 24 of chronic, K/BxN arthritis. Representative of three ankles per group; S, synovial lining; Ta, talus; Ti, tibia. (D) *Ptprs* expression in mouse FLS cultured at 0.2 \times 10⁶ (subconfluent) to 0.5 \times 10⁶ (maximum density) cells per well of a six-well plate. Mean \pm SEM of expression relative to

Polr2a ($n = 3$; $*P = 0.0418$, two-tailed unpaired t test). **(E)** (Left) Invasion of wild-type (WT) or *Ptprs* KO FLS through Matrigel in response to 5% fetal bovine serum (FBS) in the presence or absence of CS (100 $\mu\text{g}/\text{ml}$). (Right) Western blot of RPTP σ expression in *Ptprs* WT and KO FLS. **(F)** WT or *Ptprs* KO FLS migrated through Transwells in response to 5% FBS in the presence or absence of CS with or without chondroitinase (C'ase) ABC treatment. **(G and H)** Transwell migration of WT or *Ptprs* KO FLS in response to 5% FBS **(G)** in the presence or absence of HS (100 $\mu\text{g}/\text{ml}$) or **(H)** with or without pretreatment with 10 mU of heparinase III. **(I)** RA FLS were treated for 7 days with cell-permeable antisense oligonucleotides (ASO) to knock down *PTPRS* or control ASO and allowed to migrate through Transwells in response to 5% FBS in the presence of CS (100 $\mu\text{g}/\text{ml}$) or vehicle. **(E to I)** Mean \pm SEM fold change of invasion or migration relative to the vehicle-treated cells from the same experiment is shown. Data are from three independent experiments ($n = 45$ fields; $****P < 0.0001$, Mann-Whitney).

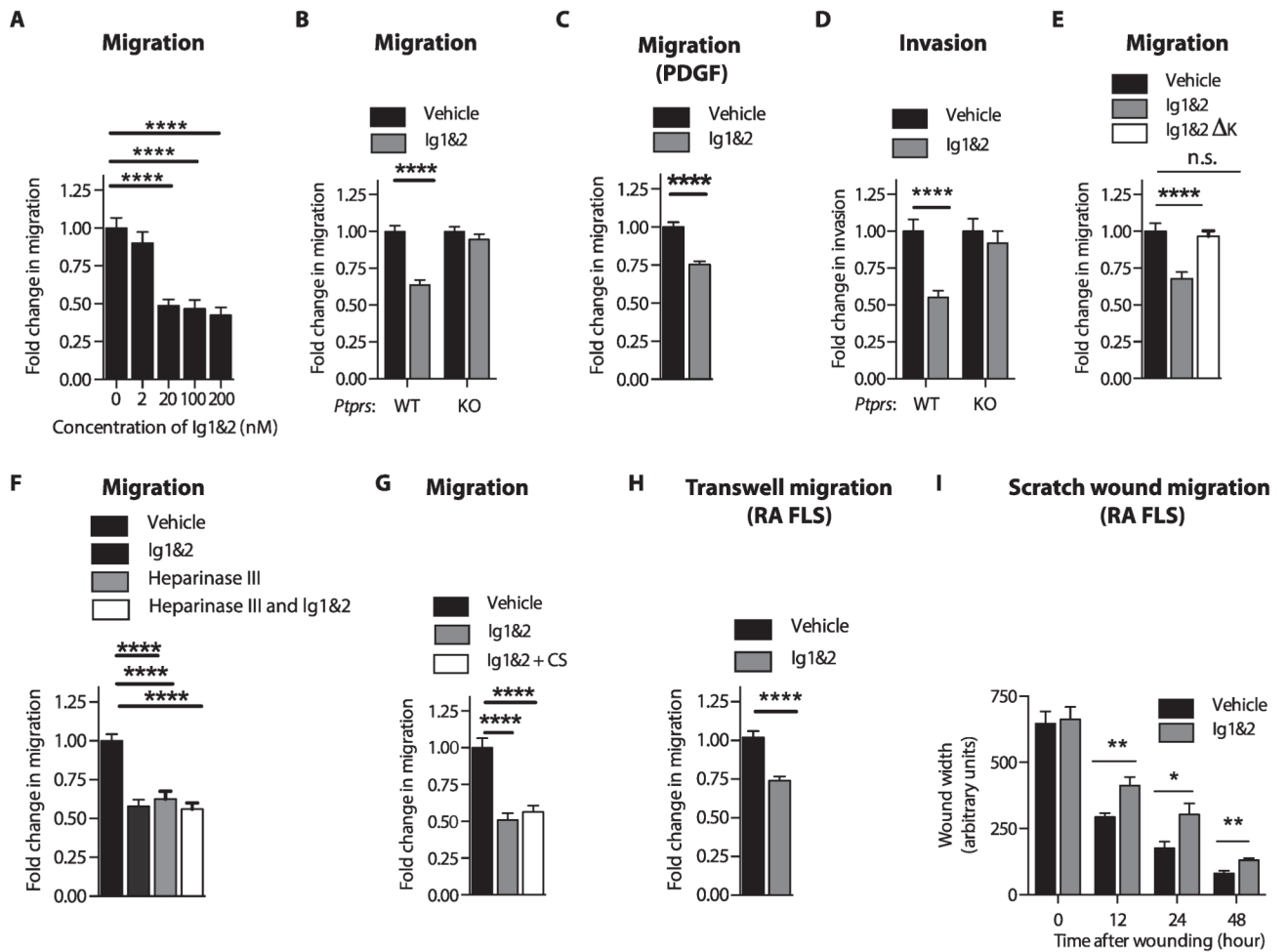


Fig. 2. RPTP σ Ig1&2 inhibits FLS migration and invasiveness in an RPTP σ -dependent manner (A) FLS migrated through Transwells in response to 5% FBS in the presence of the indicated concentrations of Ig1&2. (B and C) Migration of WT (B and C) or *Ptprs* KO FLS (B) through Transwells in response to (B) 5% FBS or (C) PDGF-BB (50 ng/ml) in the presence of 20 nM Ig1&2 or vehicle. (D) Invasion of WT or *Ptprs* KO FLS through Matrigel in response to 5% FBS in the presence of 20 nM Ig1&2 or vehicle. (E) Migration of FLS through Transwells in response to 5% FBS in the presence of vehicle or 20 nM Ig1&2 or non-GAG binding Ig1&2 K mutant. n.s., not significant. (F and G) FLS migrated through Transwells in response to 5% FBS in the presence of (F) vehicle or 20 nM Ig1&2, with or without 10 μ M of heparinase III pretreatment, or (G) vehicle, 20 nM Ig1&2, CS (100 μ g/ml), or both Ig1&2 and CS. (H) RA FLS migrated through Transwells in response to 5% FBS in the presence of vehicle or 20 nM Ig1&2. (I) RA FLS monolayers were serum-starved before scratch-wounding and stimulated with 10% FBS in the presence of 20 nM Ig1&2 or vehicle. Wound width was measured at three points at the indicated times. (A to H) Mean \pm SEM fold change of invasion or migration relative to vehicle-treated cells. Data are from three independent experiments ($n = 45$ fields; **** $P < 0.0001$, Mann-Whitney). (I) Mean \pm SEM wound width from two independent experiments ($n = 6$ fields; 12 hours, ** $P = 0.0087$; 24 hours, * $P = 0.0411$; 48 hours, ** $P = 0.0043$; Mann-Whitney).

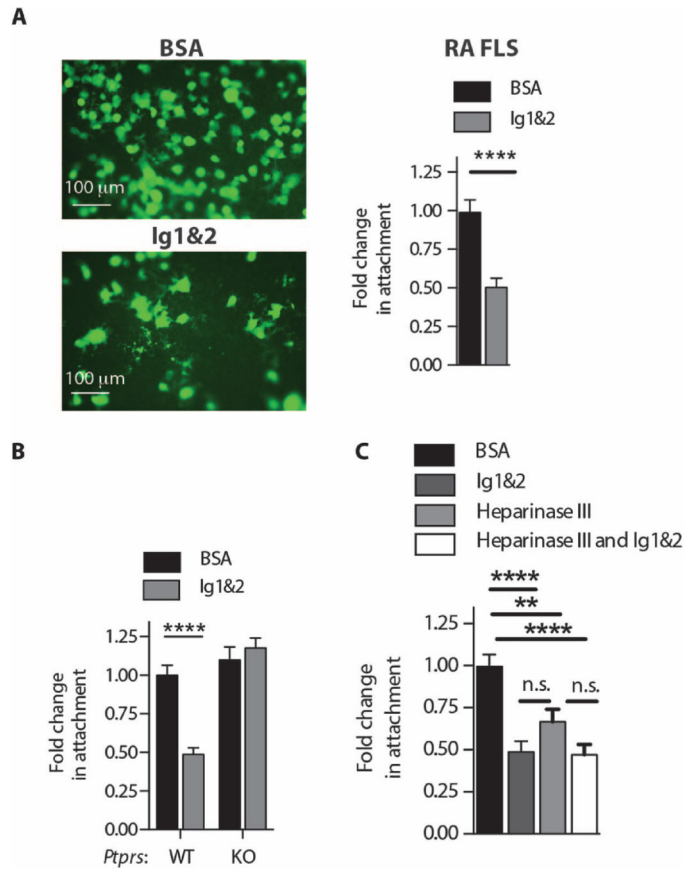


Fig. 3. RPTP σ Ig1&2 decreases FLS attachment to cartilage in vitro in an RPTP σ -dependent manner

(A) RA FLS were allowed to attach to cartilage explants in the presence of 20 nM bovine serum albumin (BSA) or Ig1&2. Representative images (left) and quantification of RA FLS (right) attached to cartilage explants in vitro in the presence of 20 nM Ig1&2 or BSA ($n = 20$ fields; **** $P < 0.0001$, Mann-Whitney). (B) Quantification of *Ptpσ* WT or KO FLS attached to cartilage explants in vitro in the presence of 20 nM Ig1&2 or BSA ($n = 15$ fields; **** $P < 0.0001$, Mann-Whitney). (C) Quantification of *Ptpσ* WT FLS attached to cartilage explants in vitro in the presence of 20 nM Ig1&2 or BSA with or without pretreatment with heparinase III (10 mU/ml) ($n = 15$ fields; ** $P = 0.0033$, **** $P < 0.0001$, Mann-Whitney). (A to C) Mean \pm SEM of fold change of number of cells attached to cartilage per 100 \times field relative to the BSA-treated cells from the same experiment is shown. Data are from three independent experiments.

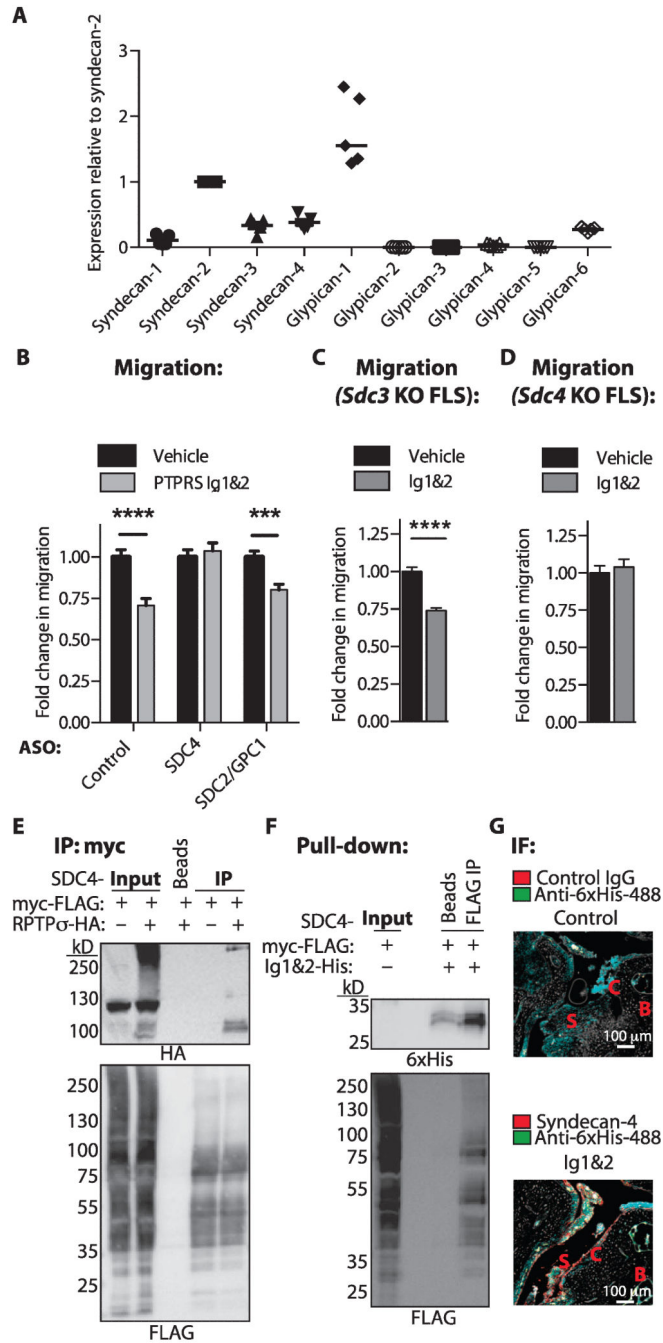


Fig. 4. Regulation of FLS migration by RPTPσ Ig1&2 is dependent on syndecan-4
 (A) mRNA expression of syndecan and glypican family members in RA FLS. Mean ± SEM of expression normalized to *POL2RA* house-keeping gene and relative to *SDC2*; *n* = 5 RA lines. (B) RA FLS were treated with cell-permeable ASO to knock down *SDC2/GPC1* or *SDC4* or control ASO and allowed to migrate through Transwells in response to 5% FBS in the presence of 20 nM Ig1&2 or vehicle. Mean ± SEM of the fold change of migration relative to that of the same cells treated with vehicle. Data are representative of three independent experiments (*n* = 45 fields; ****P* = 0.0002, *****P* < 0.0001, Mann-Whitney).

(C and D) *Sdc3* (C) or *Sdc4* (D) KO FLS were allowed to migrate through Transwells for 4 hours in response to 5% FBS in the presence of vehicle or 20 nM Ig&2. Mean \pm SEM fold change of migration relative to that of the same cells treated with vehicle. Data are representative of two independent experiments in triplicate ($n = 30$ fields; **** $P < 0.0001$, Mann-Whitney). (E) Syndecan-4 was coexpressed with empty vector or RPTP σ in HEK293T cells. Representative Western blot of input and immunoprecipitation of syndecan-4 probed with anti-hemagglutinin (HA) and anti-FLAG antibodies ($n = 3$). (F) Syndecan-4 was expressed in HEK293T cells, immunoprecipitated, and then incubated with Ig1&2. Representative Western blot of input and pull-down of Ig1&2 probed with anti-6 \times His (Ig1&2) and anti-FLAG antibodies (representative of $n = 2$ independent experiments). (G) Ankle sections of mice with chronic arthritis were incubated with purified Ig1&2 for 2 hours and then stained for syndecan-4 and 6 \times His (Ig1&2) and imaged by confocal microscopy. Representative image of three ankles from three individual mice; S, synovial infiltrate; B, bone; C, cartilage.

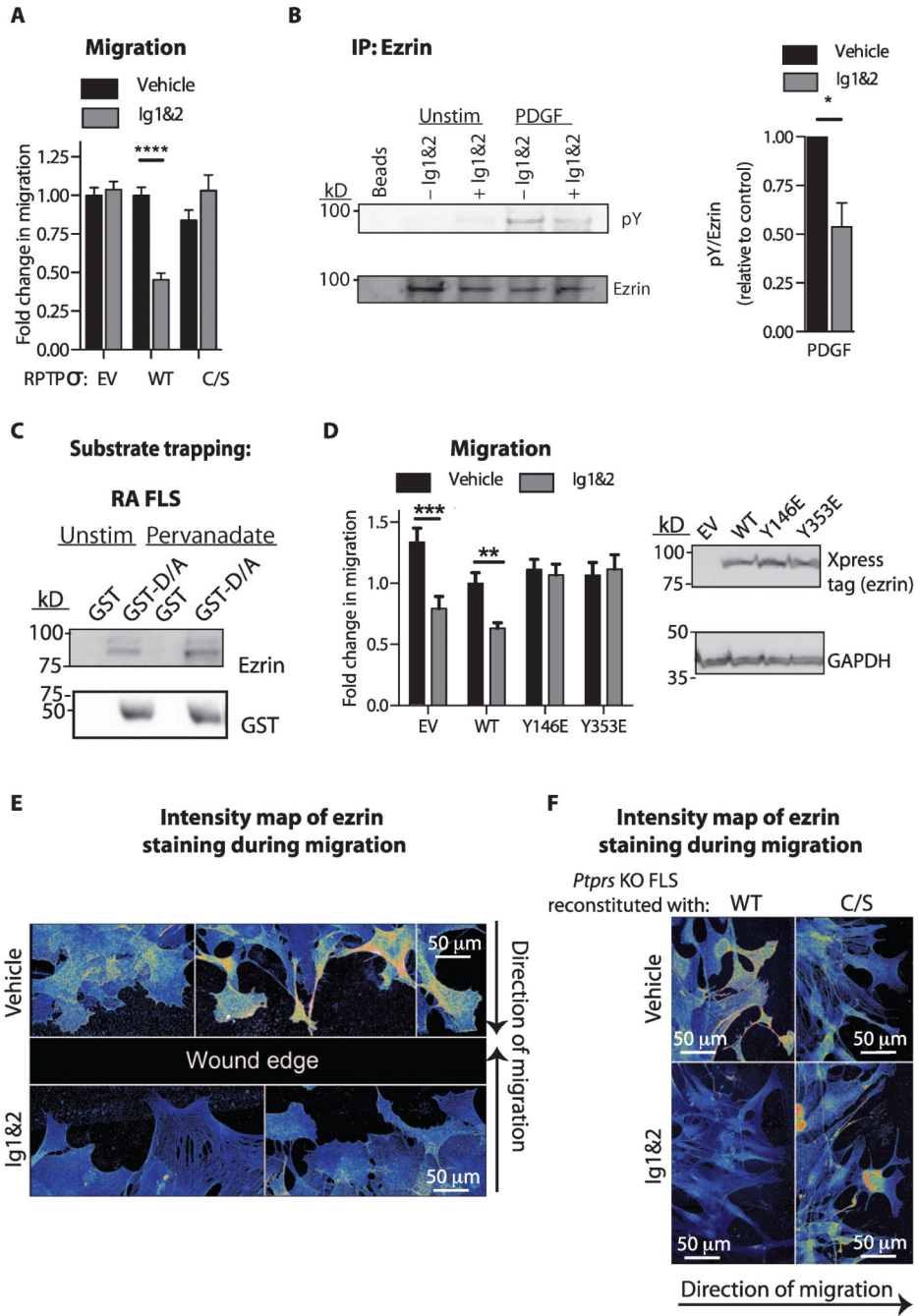


Fig. 5. Ezzrin is regulated by the PG switch through RPTPσ-mediated dephosphorylation (A) Transwell migration in response to 5% FBS of *Ptprs* KO FLS expressing WT or C/S RPTPσ or empty vector (EV) and treated with vehicle or Ig1&2. Mean ± SEM fold change of migration relative to vehicle-treated EV FLS from the same experiment ($n = 45$ fields; **** $P < 0.0001$, Mann-Whitney). Data are from three independent experiments. (B) FLS were unstimulated or stimulated with PDGF (50 ng/ml) for 15 min in the presence of 20 nM Ig1&2 or vehicle, and ezrin was immunoprecipitated from cell lysates. Representative Western blot (left) and densitometric analysis (right) of PDGF-induced ezrin

phosphorylation from three independent experiments. Mean \pm SEM ($n = 3$; $*P = 0.0138$, one-tailed unpaired t test). (C) Western blot of lysates from unstimulated or pervanadate-stimulated RA FLS incubated with glutathione S -transferase (GST) or GST-RPTP σ D1516A substrate-trapping mutant. (D) Transwell migration in response to 5% FBS of mouse FLS expressing WT or phosphomimetic Y146E or Y353E ezrin mutants or EV and treated with vehicle or Ig1&2. (Left) Mean \pm SEM fold change of migration relative to the vehicle-treated EV sample from the same experiment ($n = 30$ fields; $****P < 0.0001$, Mann-Whitney). (Right) Representative Western blot of lysates from FLS transfected with EV or WT or phosphomimetic mutant ezrin and immunoblotted for Xpress tag or glyceraldehyde phosphate dehydrogenase (GAPDH) (loading control). Data are from three independent experiments. (E and F) Monolayers of (E) WT FLS or (F) *Ptprs* KO FLS expressing WT or C/S RPTP σ were scratch-wounded and stimulated with PDGF for 8 hours in the presence of vehicle or 20 nM Ig1&2. Pseudocolored map of intensity of ezrin staining in cells migrating toward the wound edge (blue, low; red, high). Representative fields are from three independent experiments.

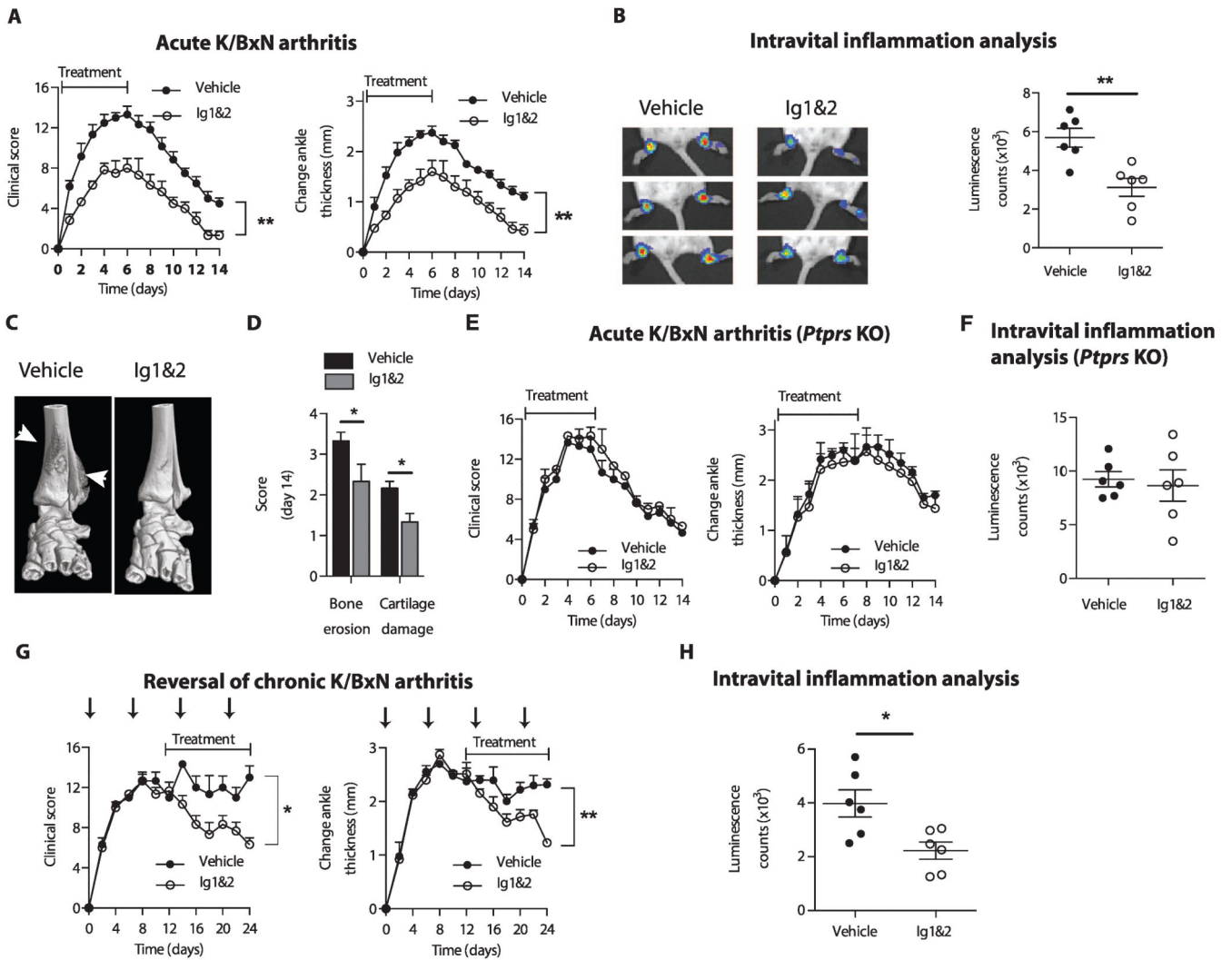


Fig. 6. RPTP σ Ig1&2 administration decreases arthritis severity in an RPTP σ -dependent manner

(A to D) BALB/c mice were induced with acute K/BxN arthritis and treated with vehicle or 0.5 mg of Ig1&2 intravenously daily (days 0 to 6). (A) Clinical score (left) and change in ankle thickness (right) ($n = 6$; $**P = 0.0022$, Mann-Whitney). (B) Ankle luminescence (left) and luminescence counts (right) of mice treated with inflammation probe on day 4 of arthritis ($n = 6$; $**P = 0.0043$, Mann-Whitney). (C) Three-dimensional microCT of ankles on day 14. White arrows indicate bone erosion or reactive bone deposition. (D) Histopathological score of bone erosion and cartilage damage of ankle sections from mice in (A) on day 14 ($n = 6$; bone erosion $*P = 0.0299$, cartilage damage $*P = 0.0056$, two-tailed unpaired t test). (E and F) *Ptpσ* KO mice were induced with arthritis as in (A). (E) Clinical score (left) and change in ankle thickness (right); $n = 3$ per group. (F) Mice from (E) were injected with RediJect Inflammation Probe on day 4 of arthritis induction. Mean \pm SEM of luminescence in each ankle joint measured. $n = 3$ mice per treatment. (G and H) BALB/c mice were induced with chronic K/BxN arthritis (serum administration shown by arrows) and treated daily with vehicle or 0.5 mg of Ig1&2 intravenously (days 12 to 24). (G) Clinical

score and change in ankle thickness ($n = 3$; $*P = 0.0135$, $**P = 0.0058$, two-tailed unpaired t test). (H) Mice were injected with RediJect Inflammation Probe on day 20 of arthritis, and luminescence in each ankle joint was measured. Graphs represent mean \pm SEM ($n = 6$; $*P = 0.0260$, Mann-Whitney).

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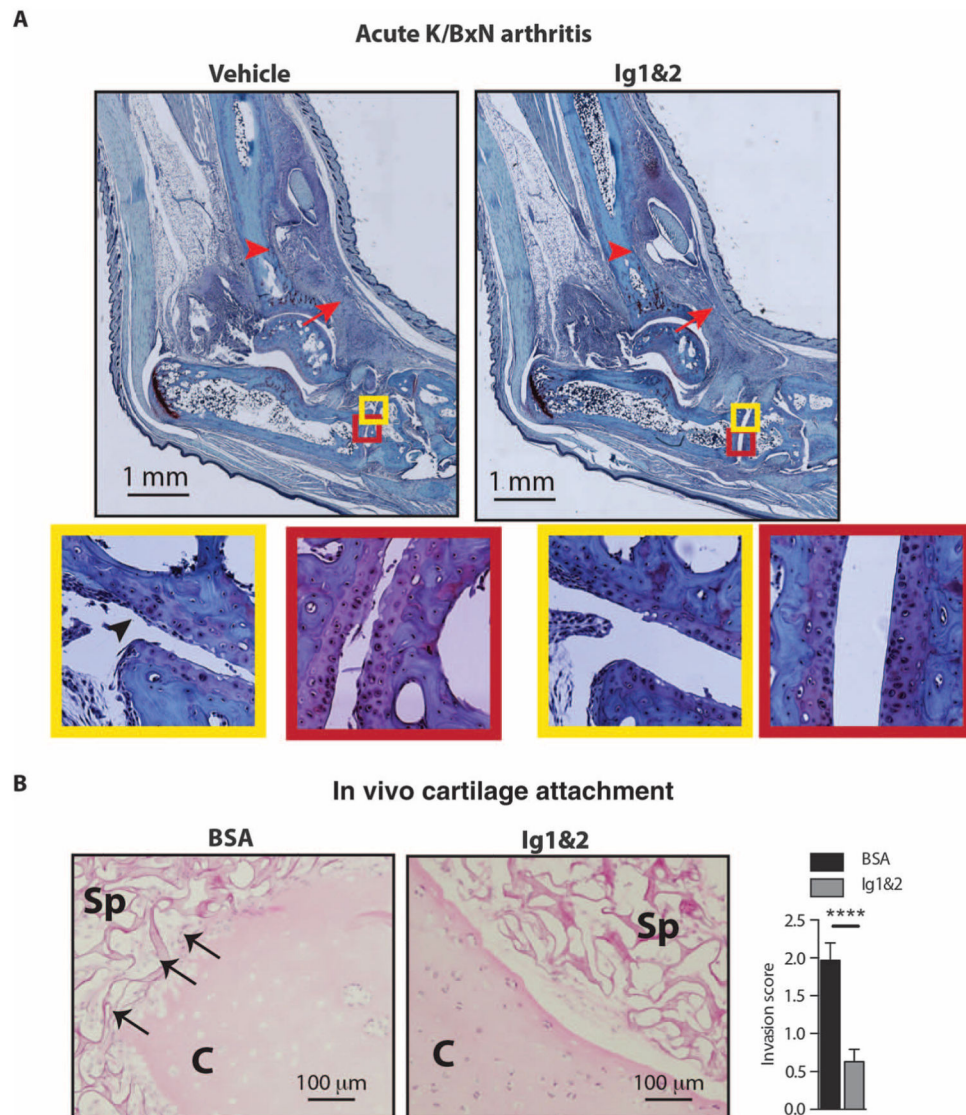


Fig. 7. RPTP σ Ig1&2 decreases FLS attachment to cartilage in vivo
(A) BALB/c mice were induced with acute K/BxN arthritis and treated with vehicle or 0.5 mg of Ig1&2 intravenously daily (days 0 to 6). Pathology of safranin O–stained ankle sections from mice on day 14 of arthritis. Arrow indicates inflammation; arrowhead indicates bone erosion. FLS crawling over cartilage (arrow) (yellow insets) and reduced chondrocyte layer (red insets) in (left) vehicle-treated versus (right) Ig1&2-treated mice. Cartilage PG content (red insets) assessed by safranin O staining. **(B)** SCID mice were implanted subcutaneously with cartilage explants and RA FLS in surgical sponge and treated with BSA or Ig1&2 intravenously for 35 days. (Left) Representative images showing cartilage (C) and sponge (Sp). Arrows indicate FLS attaching to and invading cartilage. (Right) Mean \pm SEM invasion scores; $n = 3$ mice per group ($n = 30$ fields; **** $P < 0.0001$, Mann-Whitney).

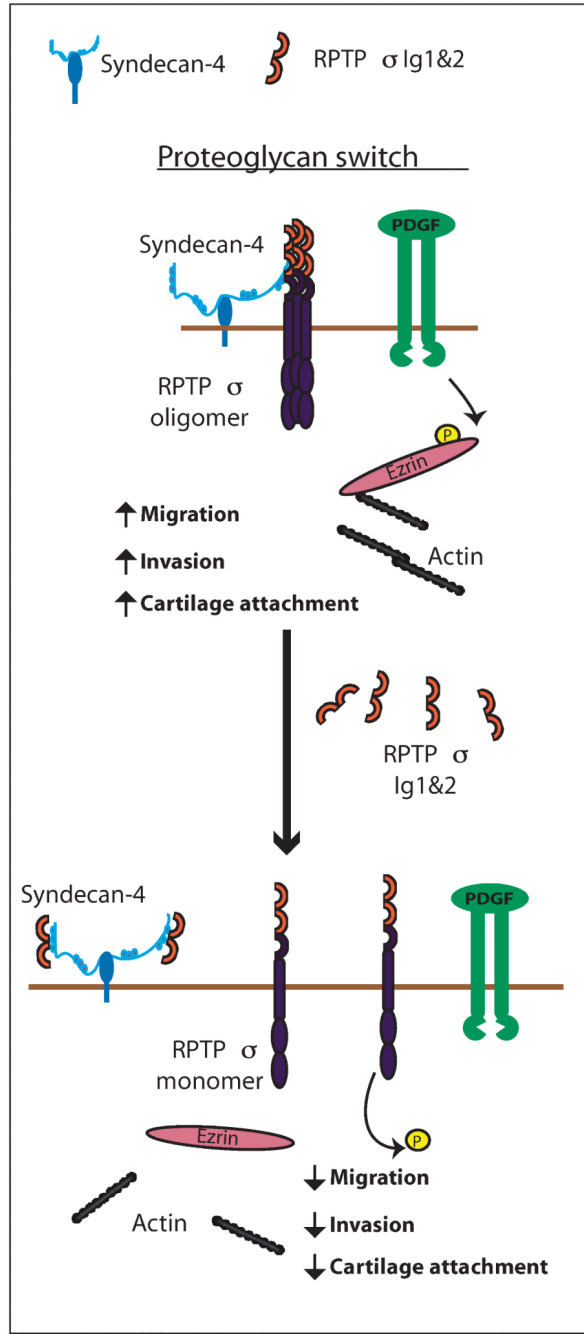


Fig. 8. Working model for the RPTPσ-dependent PG switch in FLS

RPTPσ interacts with the HS PG syndecan-4 on the surface of FLS and is maintained in an inactive oligomeric state. Tyrosine phosphorylation of ezrin downstream of the PDGFR promotes its localization to the actin cytoskeleton, enabling cell migration and invasion. Disruption of the RPTPσ-HS interaction by the HS-binding decoy RPTPσ Ig1&2 fragment displaces RPTPσ from HS. This leads to dephosphorylation of ezrin and disassembly of

ezrin from the actin cytoskeleton, decreasing FLS migration, invasion, and attachment to cartilage.

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