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Inhibition of VEGFR-2 Reverses Type 1 Diabetes in NOD Mice by Abrogating Insulitis and Restoring Islet Function

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The dysregulation of receptor tyrosine kinases (RTKs) in multiple cell types during chronic inflammation isindicative of their pathogenic role in autoimmune diseases. Among the many RTKs, vascular endothelial growth factor receptor (VEGFR) stands out for its multiple effects on immunity, vascularization, and cell migration. Herein, we examined whether VEGFR participated in the pathogenesis of type 1 diabetes (T1D) in nonobese diabetic (NOD) mice. We found that RTK inhibitors (RTKIs) and VEGF or VEGFR-2 antibodies reversed diabetes when administered at the onset of hyperglycemia. Increased VEGF expression promoted islet vascular remodeling in NOD mice, and inhibition of VEGFR activity with RTKIs abrogated the increase in islet vascularity, impairing T-cell migration into the islet and improving glucose control. Metabolic studies confirmed that RTKIs worked by preserving islet function, as treated mice had improved glucose tolerance without affecting insulin sensitivity. Finally, examination of human pancreata from patients with T1D revealed that VEGFR-2 was confined to the islet vascularity, which was increased in inflamed islets. Collectively, this work reveals a previously unappreciated role for VEGFR-2 signaling in the pathogenesis of T1D by controlling T-cell accessibility to the pancreatic islets and highlights a novel application of VEGFR-2 antagonists for the therapeutic treatment of T1D. Diabetes 62:2870–2878, 2013

In type 1 diabetes (T1D), genetic and environmental risk factors lead to immune dysregulation, provoking an autoimmune response directed toward insulin-producing β-cells of the islets of Langerhans. Previous investigations have estimated that β-cells or islets in nonobese diabetic (NOD) mice and humans are diminished to 10–30% of their initial mass (1,2), and the residual islets are largely dysfunctional when hyperglycemia is first detected (1,2). However, low levels of C-peptide can be detected in T1D patients as far out as 1–2 years postdiagnosis, indicating a window of opportunity for therapies that can restore or preserve islet mass and function (3).

Multitarget receptor tyrosine kinase inhibitors (RTKIs), such as sunitinib, were originally designed to target malignant tumors that express dysregulated tyrosine kinases, including platelet-derived growth factor (PDGF)-R, c-fms, or c-Kit. However, these inhibitors also target vascular endothelial growth factor (VEGF) receptors (VEGFRs), which are elevated in the parenchyma and tissue vasculature in many tumor microenvironments and during chronic inflammation. VEGF regulates vasculogenesis and angiogenesis largely through activation of VEGFR-2 (4). In addition to stimulating endothelial cell mitogenesis and cell migration, VEGF also has effects on a limited number of other cell types, including stimulation of monocyte/macrophage migration. Studies of transgenic mice lacking VEGFR-1 (5) or that express VEGF-1 with a “dead” kinase domain (6) reveal that VEGFR-1 functions as a negative regulator of vasculogenesis and angiogenesis. Similarly, VEGF-2 deficiency is embryonically lethal in mice but is attributed to a nonfunctional and underdeveloped vascular system (7). The phenotypes of VEGF-1 and VEGF-2-null mice indicate that, although VEGF-A has limited function through VEGFR-1, the vascular remodeling functions of VEGF-A are largely mediated through the activation of VEGFR-2.

Tyrosine kinase inhibitors (TKIs) have shown efficacy in mouse models of muscular dystrophy (8), multiple sclerosis (9), rheumatoid arthritis (10–12), and psoriasis (13). TKI can prevent and reverse diabetes in NOD mice (14–16). Imatinib, which predominantly targets c-abl and PDGF, reversed diabetes in NOD mice (14), but other RTKIs with distinct inhibitory profiles (e.g., sunitinib) were even more effective, suggesting that the precise constellations of TK targets were critical for maximum efficacy. In this regard, the VEGF-A/VEGFR-2 pathway, a key target of sunitinib, stands out as a key kinase regulating the pathogenesis of several of these inflammatory disorders (17–19). Intriguingly, VEGF serum levels are elevated in T1D patients compared with healthy controls and positively correlate with increased HbA1c levels (20).

In this study, we determined whether VEGFR-2 might be involved in the pathogenesis of T1D and tested the therapeuetic efficacy of VEGFR-2 inhibition in the NOD mouse model of T1D. We report that inhibition of VEGFR-2 by RTKIs or blocking antibodies rapidly reversed diabetes and maintained euglycemia with continued drug administration. Reversal of diabetes was attributed to an abrogation of vascular remodeling in the pancreatic islets, which impairs T-cell trafficking and the severity of insulitis, ultimately improving glucose tolerance. Histological analysis of human and mouse pancreata revealed a positive correlation between the severity of insulitis and islet vascularity, implicating inflammation as a major driving force in the vascular
remodeling observed in the islets. Collectively, our findings suggest that VEGF/VEGFR-2 signaling serves a critical gatekeeper function by controlling essential remodeling of the vasculature that is necessary for T cells to gain access to tissues.

RESEARCH DESIGN AND METHODS

Animals. Female NOD mice were purchased from Taconic. NOD.GREAT mice were derived in our laboratory (21). All mice were housed in a pathogen-free facility at the University of California, San Francisco. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

Compositional changes in treatments. Sunitinib (22), SU-9518 (23), and PF-337210 (24), provided by Pfizer, were resuspended in methylcellulose (MC) at 10 mg/mL. Inhibitory profiles and structure data are provided in Supplementary Tables 1 and 2. Female NOD mice with new-onset diabetes were treated with RTKI or MC only immediately on the day blood glucose levels were >250 but not >400 mg/dL. In some experiments, treatment was initiated 3 weeks after the onset of hyperglycemia (long-term diabetic mice). Diabetes reversal is defined as decline in mice of blood glucose levels to <250 mg/dL. NOD mice with new-onset diabetes were treated intraperitoneally three times per week for 3 weeks with 800 µg of DC101 (anti-VEGFR-2 antibody; BioXCell). Diabetic NOD mice were also treated with weekly intraperitoneal injection of 1 mg anti-PDGF-BB, anti-PDGF-DD mAb (provided by Pfizer), anti-VEGFRβ (eBioscience), or an isotype-matched, monoclonal rat IgG1 antibody. At the end of the dosing period, mice were killed and pancreases were dissected. Half the pancreas was embedded in optimum cutting temperature (OCT) compound and snap-frozen in liquid nitrogen; the remaining half was fixed in formalin and then embedded in paraffin for subsequent histological analysis.

Assessment of diabetes and insulitis. Blood glucose levels were measured with a LifeScan glucose meter (OneTouch Ultra). Severity of insulitis was performed as previously described (25) on hematoxylin and eosin-stained sections separated by a 100-µm distance.

Flow cytometry analysis. Single-cell suspensions were prepared from the lymph nodes and purified islets using standard procedures, followed by Fc receptor blocking with anti-CD16/32 (clone 2.4G2) prior to staining. Single-cell suspensions were stained with antibodies against the following cell surface antigens: CD4 and Thy1.2 or Thy1.1 (Biolgend); CD25L, CD8, CD11c, CD11b, CD25, CD44, and CD69 (BD Biosciences); and DAPI (Invitrogen Life Technologies) to assess cell viability. Analysis was performed on live cells (DAPI−) on a BD LSRII flow cytometer with FACSDiva software (BD Pharmingen).

Postacquisition analysis was performed with Flowjo software version 9.1.

Histological staining of pancreatic tissue. Frozen sections of human and mouse pancreas were analyzed using standard immunofluorescence staining procedures. The following antibodies were used: guinea pig anti-insulin (DAKO), mouse anti-human or mouse VEGF-2 (R&D Systems), mouse anti-human CD31 and CD45 (Biolgend), and rat anti-mouse CD31 and CD45 (Southern Biotech). Sections were subsequently washed and incubated with the species-appropriate, Alexa-conjugated secondary antibodies (Invitrogen). Sections were stained with DAPI for 5 min to stain nuclei and mounted with FluorSave reagent (Calbiochem).

Metabolic studies. Intraperitoneal insulin (IPITT) and glucose tolerance testing (IPGTT) were performed on prediabetic NOD mice as described previously (26). In brief, NOD mice were treated with MC or PF-337210 (5–10 mg/kg) by gavage for 3 weeks, and IPITT or IPGTT were performed weekly. For IPITT, mice were intraperitoneally injected with regular human insulin (Novolin E) at 1.0 unit/kg after a 10-h fast. For IPGTT, mice were intraperitoneally injected with sterile 30% glucose solution (Sigma-Aldrich) at 1.5 g/kg after a 17-h fast. Blood glucose levels were measured at indicated times postfeeding after insulin or glucose administration. Insulin was measured by insulin ELISA (Excocell).

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 5.01 or Microsoft Office Excel 2007. Statistical comparisons between two groups were performed using an unpaired two-tailed Student t test or a nonparametric Mann-Whitney test. Comparisons between multiple groups were performed by one-way or two-way ANOVA, followed by a post hoc Bonferroni test to determine significance of differences between two groups. Values of P ≤ 0.05 were considered significant.

RESULTS

Inhibition of VEGF-R2 reverses T1D in NOD mice. To delineate the specific contributions of PDGFR and VEGFR activity in the reversal of diabetes after RTKI treatment (14), we treated diabetic NOD mice at the onset of diabetes with oral doses of SU-9518 (40 mg/kg) and PF-337210 (10 mg/kg), RTKIs with varying affinity for VEGFRs (Supplementary Table 1). SU-9518, a strong PDGFR inhibitor with weak affinity for VEGFR-2, was ineffective at reversing hyperglycemia (Fig. 1A), suggesting a lack of efficacy in compounds that ineffectively block VEGFR activity. However, daily dosing of diabetic mice with the potent VEGFR inhibitor PF-337210 (5 mg/kg) reversed diabetes and reduced blood glucose levels in all mice by the first week of treatment (Fig. 1A and B). Despite 10 weeks of continuous dosing, all mice became diabetic within 3–5 weeks after drug administration was discontinued (Fig. 1C). All RTKI-mediated reversal of diabetes was dependent on viable islet mass, as we found no drug efficacy when treating mice that had no or very few remaining islets by dosing with streptozotocin or using long-standing diabetic mice (Supplementary Fig. 1A and B).

We tested whether an anti–VEGFR-2 blocking antibody, DC101, could also reverse diabetes (27–29). DC101 efficiently reversed diabetes in all mice treated for 3 weeks (Fig. 1D and E). Moreover, indirect blockade of VEGF-R2 activity through the use of a neutralizing anti–VEGF-A antibody (clone r84) (30) reversed diabetes in 70% of mice (Supplementary Fig. 1C). However, treatment of diabetic NOD mice with blocking antibodies against PDGFRβ-specific ligands (PDGF-BB and PDGF-DD) or PDGFRβ did not reverse diabetes (Fig. 1F). Although these data are in contrast with those made previously that soluble PDGFRβ receptor can reverse disease (14), this antagonist can also block elements of the VEGF pathway (31). Taken together, these data suggest that VEGFR-2 is a central target in the RTKI-mediated reversal of diabetes and reveal a previously unappreciated role for VEGF-R2 signaling in the pathogenesis of T1D.

VEGF-R2 expression in islets is confined to the vasculature of NOD mice with new-onset diabetes. We next examined which cell types in the pancreas expressed VEGFR-2 to identify the key cellular target of RTKIs. Using immunofluorescence staining for CD45, a pan leukocyte marker, and VEGFR-2, we found that leukocytes did not express VEGFR-2 (Supplementary Fig. 2A). Flow cytometric analysis of CD11b+CD8+ and naive or activated CD4+ immune cells isolated from the auxiliary or pancreatic lymph nodes of new-onset diabetic NOD mice confirmed our histological findings (Supplementary Fig. 2B). We also found that insulin-producing β-cells or CK19+ duct cells do not express VEGFR-2 (Supplementary Fig. 3). However, examination of the islet vasculature using immunofluorescence for CD31 (Fig. 2A and Supplementary Fig. 3E and F) and VEGFR-2 (Fig. 2B and Supplementary Fig. 3C and D) showed that CD31+ vessels distributed within the islets express significant levels of VEGFR-2 (overlay, Fig. 2C and Supplementary Fig. 3G and H). Quantitative PCR revealed that VEGFR-2 levels were not differentially regulated across the conditions we tested (Fig. 2D). Together, these results suggested that RTKI-mediated reversal of diabetes likely was not due to a direct effect on leukocytes or insulin-producing β-cells but, rather, occurred through a direct action on endothelial cells.

Next, we tested the hypothesis that receptor activity may be controlled by regulated expression of VEGF-A in the islets of new-onset diabetic NOD mice by immunohistochernistry and found increased VEGF-A expression in the islets (Fig. 2E). Similarly, we found increased VEGF-A staining in islets of prediabetic mice (Fig. 2F), whereas a low immunoreactivity for VEGF-A was detected in β-cells of islets from NOD-RagKO mice that are devoid of inflammation (Fig. 2G). Western analysis of whole-islet...
homogenates substantiated these histological observations (Fig. 2H), suggesting that increased expression of VEGF-A is linked to inflammation rather than a secondary response to hyperglycemia.

We performed coimmunofluorescence for VEGF-A, immune cell markers, and insulin to determine the identity of the VEGF-A–expressing cells in the inflamed pancreas. We found that insulin+ β-cells expressed detectable levels of VEGF-A (Fig. 2I–K, red arrows) as well as multiple immune cell subsets, including CD4+ (Fig. 2Iii, white arrows), CD8+ (Fig. 2Iii, white arrows), and CD11c+ (Fig. 2Kii, white arrows) cells. Collectively, these results indicate that VEGFR activity in endothelial cells is regulated by a multicellular source of VEGF that is increased in inflamed islets and highlight a role for VEGF-A–expressing cell and islet endothelium interactions in the pathogenesis of T1D.

RTKIs prevent increases in islet vascular density associated with insulitis. We next tested the hypothesis that the elevated expression of VEGF-A induces islet vascular remodeling, subsequently enhancing leukocyte migration and insulitis. Immunofluorescent staining of purified islets from NOD mice with new-onset diabetes revealed a more extensive and branched vascular network with an increased number of dilated vessels, compared with islets isolated at an age (4 weeks) preceding invasive insulitis (Fig. 3A and B). Islets of 18-week-old NOD mice were stained with anti-CD31 and anti-Thy1.2 antibodies to visualize the islet endothelium and infiltrating T cells, respectively. Linear regression analysis revealed a positive correlation between the CD31+ and Thy1.2+ islet area, indicating that increases in islet vascular area were partly attributed to increased insulitis (Fig. 3C). Moreover, the CD31+ islet area was increased threefold in islets isolated from 18-week-old NOD mice compared with islets isolated at 4 weeks of age (Fig. 3D). Treatment of NOD mice with new-onset diabetes with PF-337210 significantly decreased islet vascular density compared with control mice (Fig. 3E).

RTKI-mediated inhibition of VEGFR signaling impairs T-cell trafficking. We next examined the effects of VEGFR inhibition on T-cell migration. To minimize variability in the absolute number and frequency of T cells isolated from purified islets of prediabetic NOD mice treated with PF-337210 (data not shown), we took advantage of an islet antigen–specific T-cell transgenic model (BDC2.5 T cells congenically marked with Thy1.1+) to synchronize the migration and tracking of T cells into the islet in an in vivo migration assay (32). A 2-week pretreatment of NOD-RagKO mice with PF-337210 resulted in a significant reduction in the proportion of islets infiltrated with Thy1.1+ T cells 48 h after transfer (Fig. 4A). The enumeration of Thy1.1+ T cells by immunofluorescence revealed that T cells in islets were reduced by twofold in

**FIG. 1. Inhibition of VEGFR-2 reverses diabetes at onset.** A and B: NOD mice with new-onset diabetes (blood glucose levels ranging between 250 and 400 mg/dL) were treated daily (Monday–Friday) with SU-9518 (40 mg/kg), PF-337210 (PF; 10 mg/kg), or MC. C: Diabetic mice were treated with PF (5 mg/kg, n = 5) or MC (n = 10) for 10 weeks (Monday–Friday). D and E: Diabetic mice were treated with monoclonal antibodies specific for VEGFR-2 or a rat IgG1 isotype control (800 μg/mouse). F: Diabetic mice were treated with antibodies against PDGFRβ ligands (PDGF-BB or PDGF-DD) or PDGFRβ (1 mg/mouse). Five to ten mice were treated per group.
PF-337210–treated animals compared with control-treated mice (Fig. 4B–D, red). The impaired T-cell migration was associated with a reduced vascularity in NOD-RagKO mice that received activated BDC2.5 T cells and were treated with PF-337210 (Fig. 4E).

The impairment in T-cell trafficking was not restricted to islets, as we noted reduced spleen mass, total splenocyte counts, and numbers of transferred Thy1.1+ T cells in the spleen (Supplementary Fig. 4A–C). However, the number of PBMCs or Thy1.1+ T cells in the blood was not affected by PF-337210 administration (Supplementary Fig. 4D and E), suggesting that VEGFR inhibition did not lead to T-cell toxicity or altered proliferation that could account for the reduced number of transferred BDC2.5 T cells in the tissues of PF-337210–treated mice. The expression of T-cell activation markers on transferred T cells in the blood and spleen was not affected by PF-337210 (Supplementary Fig. 5). Taken together, these data are consistent with our observations that leukocytes in NOD mice express insignificant levels of VEGFR-2 and confirm that PF-337210 does not directly affect T cells.

**VEGFR-2 inhibition reduces insulitis and preserves islet function.** We next examined whether RTKI or the anti-VEGFR antibody (DC101) led to reduced insulitis and improved islet function in NOD mice. Examination of islet infiltration after drug administration showed that the percentage of severely infiltrated islets (score = 3) in diabetic mice treated for 3 weeks with RTKI or DC101 was significantly reduced compared with vehicle-treated (MC) mice (Fig. 5A–E). Furthermore, the decrease in severely infiltrated islets was accompanied by an increased proportion of immunologically spared islets (score = 0) and islets with noninvasive insulitis (score = 1). Similar to the transfer setting, inhibition of VEGFR-2 with PF-337210 had no effect...
RTKI RESTORES ISLET FUNCTION BY REDUCING INSULITIS

FIG. 3. VEGFR inhibition prevents the increases in islet vascularity associated with insulinoma. Representative micrographs of purified islets from 4-week-old (A) and new-onset (B) NOD females that were subjected to immunofluorescent staining with anti-CD31 (teal). C: Linear regression analysis shows a positive correlation between CD31+ and Thy1.2+ islet area in islets isolated from prediabetic mice. The micrograph depicts the cumulative data from 67 purified islets analyzed from five mice. D: The islet CD31+ area was increased in purified islets of 18-week-old, prediabetic mice relative to islets from 4-week-old NOD mice. E: A 5-day dosing period of mice with new-onset diabetes with PF-337210 (PF; 20 mg/kg) prevented further increases in islet vascular density. MC, 28 islets analyzed from two mice; PF-337210 (PF), 45 islets analyzed from three mice.

DISCUSSION

Herein, we demonstrate that VEGFR-2 is a central target in the RTKI-mediated reversal of diabetes in NOD mice. The pathogenesis of TID requires infiltration of immune cells into the pancreatic islets, and this is dependent upon VEGF-mediated activation of VEGFR-2 within the islet endothelium. Inhibition of VEGFR-2 signaling impairs T-cell migration and reduces insulitis, restoring islet function and glucose control. Histological studies of human pancreata indicate that a similar pathogenic process occurs in T1D patients and suggests that activation of the VEGF/VEGFR-2 signaling axis is a key molecular event linking vascular remodeling and insulitis, processes that contribute to loss of glucose control and onset of hyperglycemia.
VEGFR-2 is a master regulator of angiogenesis and vascular permeability and contributes to the establishment of chronic inflammation in various pathological settings (35–37). We show that VEGFR-2 inhibitors abrogate increases in islet vascular density and subsequently impede the development of insulitis. Similar observations have been reported in streptozotocin-induced diabetes (38) and other inflammatory settings in which inhibition of VEGFR-2 signaling impairs vascular remodeling and reduces inflammation (19,28,39,40). In addition to promoting increased vascularity, VEGF-A contributes to inflammation and leukocyte recruitment by inducing the expression of adhesion molecules and chemokines by endothelial cells (19,41–43). Our findings support the conclusion that reduced insulitis is due to decreased islet vascularity. However, there is also the possibility that VEGFR-2 antagonists repress the expression of molecules that promote leukocyte trafficking/homing to the islet vasculature. Future studies will require the characterization of potential molecular changes in activated islet endothelial cells to fully understand the role of islet vascular remodeling in the pathogenesis of T1D.

Our results suggest that there may be distinct molecular and functional properties of islet endothelial cells in a pathogenic state versus those in homeostasis, rendering them more sensitive to RTKI treatment. We found that the islet vascularity showed obvious morphological changes indicative of pathological angiogenesis, which is induced by elevated expression of VEGF-A (36). Although Akirav et al. (44) previously reported that VEGF-A is increased in the inflamed islets of prediabetic NOD mice, they found that islet vascularity decreases with the progression of diabetes. The discrepancies between these studies may reflect a difference in the assays used to measure islet vascularity.

A pathogenic role for VEGF-A is supported by the observation that the overexpression of VEGF-A in β-cells led to increased islet vascularity, inflammation, and disruption in islet architecture, resulting in impaired glucose homeostasis and insulin secretion during a high-fat diet (45). However, a complete loss of VEGF signaling in islets is also detrimental. For instance, β-cell–specific ablation of VEGF-A resulted in reduced islet vascularity and endothelial cell fenestration, culminating in impaired insulin secretion and glucose intolerance. However, in the setting of autoimmune diabetes, this potential impairment in function is outweighed by the protection offered by preventing diabetogenic leukocytes from entering the islet and destroying β-cells. Collectively, our data and the altered islet function after perturbations in β-cell–specific VEGF-A expression indicate that VEGF-A functions in a narrow physiological range to maintain islet homeostasis and function.

We propose a model in which reversal of diabetes is delineated in three distinct stages, including 1) acute reversal, 2) maintenance of euglycemia, and 3) long-term remission of diabetes in the absence of continued drug dosing. The acute reversal and maintenance of euglycemia of diabetes is dependent on the inhibition of VEGFR-2. However, the lack of complete diabetes reversal in mice treated with anti–VEGF-A antibody indicates that other VEGFRs may also mediate the islet vascular remodeling. In this regard, inhibition of VEGFR-3 was shown to reduce insulitis in NOD mice (38). Alternatively, it is very likely that small molecule antagonists, such as the RTKIs used in this study, are able to penetrate the target tissue more effectively than mAbs such as r84. We cannot exclude the effect of RTKIs on other physiological systems that control glucose metabolism (e.g., liver or adipose tissue). For instance, RTKI treatment also enhances insulin-independent glucose metabolism in peripheral tissues. In this regard, recent studies have shown that JAK-STAT inhibitors increased peroxisome proliferator–activated receptor-γ (PPAR-γ), a critical regulator of fatty acid storage and glucose metabolism, expression in adipocytes and immune cells (46).

The discrepancy between the lack of reversal of diabetes with the PDGFR antagonists used in this study and the previously reported reversal using a soluble PDGFRβ-Ig fusion protein (14) may be explained by functional differences between the reagents used to inhibit PDGFR. Kielly and colleagues (31) recently demonstrated that PDGFR is able to functionally bind VEGF ligand, leading to PDGFR activation. Therefore, it is possible that the acute reversal of diabetes after soluble PDGFRβ-Ig may be attributed to the binding and neutralization of VEGF ligands in addition to PDGF ligands. The studies using Gleevec suggest that c-Abl also participates in reversal of hyperglycemia and long-term remission, but through a distinct mechanism that likely involves the inhibition of proapoptotic pathways in β-cells (47) and altered PT-cell immunity (48), leading to islet mass preservation. Similarly, the current study suggests that RTKIs function by preserving islet mass. Although we cannot rule out the possibility of β-cell regeneration, previous studies have shown that the most likely effect of drugs on the islets is to reverse the metabolic...
stress caused by the immune response, leading to recovery of the insulin production by the remaining β-cells (49,50).

In summary, our findings that VEGFR-specific RTKIs and blocking anti–VEGFR-2 antibodies reverse diabetes by preserving islet function suggest that therapeutic modalities aimed at preventing islet vasculature activation may provide an alternative treatment strategy for T1D.

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S.A.V. designed and performed research, analyzed data, and wrote the manuscript. J.L., S.K., B.K., G.L.S., and C.W. performed research. B.C. assisted in designing the in vivo migration assay used in this study. M.A.A. designed research. R.A.B. and N.P. contributed data as well as new reagents/analytic tools. R.H.A. designed research and contributed data as well as new reagents/analytic tools. J.A.B. designed research, analyzed data, and wrote the manuscript. J.A.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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