FTY720 Promotes Local Microvascular Network Formation and Regeneration of Cranial Bone Defects

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The calvarial bone microenvironment contains a unique progenitor niche that should be considered for therapeutic manipulation when designing regeneration strategies. Recently, our group demonstrated that cells isolated from the dura are multipotent and exhibit expansion potential and robust mineralization on biodegradable constructs *in vitro*. In this study, we evaluate the effectiveness of healing critical-sized cranial bone defects by enhancing microvascular network growth and host dura progenitor trafficking to the defect space pharmacologically by delivering drugs targeted to sphingosine 1-phosphate (S1P) receptors. We demonstrate that delivery of pharmacological agonists to (S1P) receptors S1P₁ and S1P₃ significantly increase bone ingrowth, total microvessel density, and smooth muscle cell investment on nascent microvessels within the defect space. Further, *in vitro* proliferation and migration studies suggest that selective activation of S1P₃ promotes recruitment and growth of osteoblastic progenitors from the meningeal dura mater.

Introduction

CRANIAL NEURAL CREST progenitors are a unique population of cells that originate during development by delaminating from the ectodermal epithelial sheet before neural tube closure. This distinct population of cells, known to be highly migratory and plastic, gives rise to a diverse range of tissues in the craniofacial region, including peripheral nerve, muscle, cartilage, frontal bones, and dura mater tissue—a meningeal layer between the brain and skull.^{1,2} Many studies have suggested that the underlying dura mater plays an important role in maintaining suture patency and controlling the rate and extent of overlying cranial bone growth.^{3,4} *In vitro* studies confirm premature fusion of bone plates and hence premature differentiation of progenitor cells within suture lines when calvarial tissue is cultured without the underlying dura intact.⁵ These results suggest that the spatial and temporal cues responsible for cranial bone morphogenesis are embedded in the dura mater, a concept referred to as dural imprinting. Thus, the proximity of dural tissue to the cranial vault and the role this tissue plays in controlling patterning and growth of cranial plates during development make this a unique microenvironmental niche in which to study osseous regeneration of intramembranous bone.

Interestingly, growing evidence supports the dura mater tissue as a significant contributor to cranial bone regenera-

tion. Physical blockades of the underlying dura mater have been shown to inhibit cranial re-ossification, and significant reductions in new bone formation have also been reported when the dura is completely removed from the defect site.⁶ Others have shown that osteoblasts cocultivated in the presence of dura mater show enhanced proliferation and mineralization.⁷ Our laboratory has shown that cells isolated from dura mater tissue differentiate along multiple tissue-specific pathways and adopt an osteogenic phenotype after medium induction. Interestingly, unlike many other mesenchymal progenitor populations, fate-mapping studies using WntCre/R26R transgenic reporter mice confirm the neural crest origin of dura mater tissue.⁸ Further, isolated dura mater cells are capable of robust expansion and significant matrix production across polymer constructs *in vitro*, highlighting the biocompatibility and thus potential for *in vivo* application with implantable biomaterials.⁹ These results suggest that therapies targeted to enhance progenitor cell recruitment from this unique cranial niche have significant potential in improving healing outcomes.

Craniofacial tissues are also highly vascularized, and success of regeneration relies heavily on incorporation of extensive vascular networks to transport chemotactic factors, nutrients, and oxygen within the tissue space. However, effective incorporation of mural support cells to stabilize newly formed blood vessels utilizing protein delivery strategies,

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including platelet-derived growth factor-BB, angiopoietin-1, and transforming growth factor- β , have been met with a variety of drawbacks.^{10–12} Alternatively, our laboratory has shown that sphingosine 1-phosphate (S1P), a small bioactive phospholipid, promotes both arteriogenic microvascular remodeling and bone regeneration *in vivo*.¹³ Specifically, local release of S1P from biodegradable poly-lactic-co-glycolic acid (PLGA) constructs significantly enhances luminal diameter expansion of arterioles after 3 days, as well as increases the number of proliferating smooth muscle cells on α -smooth muscle actin (α -SMA)-stained microvessels compared with unloaded controls. Further, S1P-treated cranial defects generate significantly greater amounts of new bone volume compared with control groups after 6 weeks of healing.

In this work, we examine more closely the role of specific S1P receptors in mediating neovascularization and osteogenesis by locally delivering pharmacological agonists and antagonists of S1P receptors. Prior evidence supporting the positive role of S1P₁ and S1P₃ in maintaining vascular cell proliferative/migratory phenotypes^{14,15} guided our choice to evaluate the ability of two S1P receptor-targeted compounds, FTY720 (S1P₁, S1P₃ agonist) and VPC01091 (S1P₁ agonist, S1P₃ antagonist), to enhance neovascularization, smooth muscle cell recruitment, and new bone formation within a cranial defect model. This new study demonstrates that by using receptor-selective compounds we can accurately amplify the therapeutic effects of endogenous S1P and subsequently improve both microvascular remodeling and osseous tissue growth *in vivo* to regenerate and restore tissue function. Further, *in vitro* delivery of FTY720 significantly amplifies the proliferative and migratory effects of S1P signaling in dural progenitor cells. Collectively, we believe that these S1P receptor-specific pharmacological agents represent attractive therapeutics for bone repair strategies, given their ability to act on a diverse set of cell types within the wound bed.

Materials and Methods

Materials

Polymer constructs using 50:50 and 85:15 PLGA (molecular weight [Mw] = 72.3 and 123.6 kDa, respectively) were purchased from Lakeshore Biomaterials (Birmingham, AL). S1P (Mw = 379.5 Da) and FTY720 (Mw = 307.5 Da) were purchased from Cayman Chemical (Ann Arbor, MI). The prodrug compound VPC01091 (Mw = 303.3 Da) and its phosphorylated analog, VPC01211, (Mw = 383.5 Da) were synthesized in the laboratories of coauthors Dr. Macdonald and Dr. Lynch.

Fabrication and implantation of loaded scaffolds in cranial defects

Thin polymeric films (1 mm diameter and 0.5 mm height) of 50:50 PLGA were loaded with (1) 1:400 S1P:PLGA (wt./wt.), (2) 1:200 VPC01091:PLGA (wt./wt.), or (3) 1:200 FTY720:PLGA (wt./wt.) using a solvent casting method as previously described.¹³ Separately, unloaded microspheres (average diameter 50–300 μ m) of 85:15 PLGA were fabricated using a single emulsion method as previously described.¹⁶ For each cranial defect scaffold, 20 thin films from each experimental group (unloaded, S1P-loaded, VPC01091-loaded, and FTY720-loaded) were mixed with a volume of

unloaded microspheres and placed into individual circular copper molds shaped to match the defect space (8 mm diameter and 1 mm height). Subsequently, all microsphere–film scaffolds were sintered at 75°C for 3 h in the circular molds. Scaffolds were implanted into adult male rat retired breeders (400–550 g, wild-type Sprague Dawley; Harlan Laboratories, Indianapolis, IN) as previously described.¹³ All surgeries were performed according to a protocol approved by the Institutional Animal Care Committee at the University of Virginia.

Microcomputed tomography imaging analysis of cranial defect (in vivo and ex vivo)

New bone healing within the defect area was followed over a 42-day time course. *In vivo* microcomputed tomography (CT) scans were used to assess new bone formation within the defect space at 0, 2, 4, and 6 weeks. Animals were anesthetized by intraperitoneal injection and were imaged for 14 min utilizing a low-resolution 45 kVp scan to generate qualitative microCT images of bone healing. At end time points, animals were sacrificed, and *ex vivo* scans of each specimen were obtained utilizing a high-resolution 45 kVp scan to generate quantitative values of new bone volume formed. After reconstruction of the two-dimensional slices, an appropriate threshold range matching the original grayscale images was chosen. Contour lines were drawn around the defect area to appropriately select a circular defect void volume of 8 $\times$ 1 mm, taking care to exclude neighboring native bone. Three-dimensional images were created from two-dimensional slices, and the bone volume within the selected circular defect was calculated using the three-dimensional evaluation program. Bone void volume, threshold (160), and scan parameters (support = 2, width = 1.2) were kept constant throughout the entire study.

Histological sectioning and staining of cranial tissue

After *ex vivo* microCT scanning, each sample was placed into a histology cassette, labeled, and decalcified using Richard Scientific Decalcifying Solution (Kalamazoo, MI) for 5 days at room temperature on a rotating rocker. After decalcification, samples were dehydrated overnight. Samples were then cut along the coronal plane at the midline of the defect and embedded in paraffin. Each 7 μ m section was mounted onto individual slides. Sphingolipid sections were stained with hematoxylin and eosin (H&E), biotin rat anti-mouse CD45 clone 30-F11 primary antibody (1:50, pan-leukocyte marker; BD Biosciences, San Jose, CA), and streptavidin AlexaFluor-488 secondary (1:200), or costained with lectin-AlexaFluor-488 (1:200, an endothelial marker; Sigma, St. Louis, MO) and SMA- α -IA4 (1:500, a smooth muscle cell marker; Sigma).

In sphingolipid-loaded sections, total numbers of vessels per area of defect were counted per group (unloaded, S1P loaded, VPC01091 loaded, and FTY720-loaded) using H&E-stained sections at 10 $\times$ magnification. Additionally, defect sections were divided into subregions to compare spatial distributions of total vessel numbers. Center and edge subregions were compared by dividing each section into three areas (two edges and one center). Dural and periosteal subregions were compared by dividing each section into two areas (top = periosteal; bottom = dural). The number of α -SMA-positive vessels per area was also counted for each group (unloaded, S1P-loaded, VPC01091-loaded, and FTY720-loaded) after

SMA- α -IA4 staining. Additionally, defect sections were divided into similar subregions and spatial distributions of SMA⁺ vessels were quantified.

Dura stem cells expression of S1P receptors

Total RNA extraction and cDNA synthesis from dural stem cell (DSC) progenitors was performed using a Qiagen mini-kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Specific primer sequences were amplified using a Biorad iCycler (Biorad, Hercules, CA). Each 50 μ L reaction mixture contained iQ SYBR Green Supermix (Biorad), the cDNA, and both forward and reverse primers for each gene. Manufacturer-suggested melting temperatures were used for each primer. 18s rRNA was used as the housekeeping gene. After amplification using the iCycler, samples were run out on a 2% agarose gel along with a standard, stained with ethylene bromide, and imaged using an Alpha Innotech imaging system (San Leandro, CA).

In vitro DSC progenitor response to exogenous S1P

A small amount of acidified dimethyl sulfoxide (DMSO; 95% DMSO/5% 1N HCl) was added to lyophilized S1P and VPC01211 separately until each final concentration was 20 mM. The phosphorylated analog to FTY720 could not be obtained and therefore was not included in this set of experiments. Solutions were further diluted to 10 and 0.01 μ M in a low serum medium (1% fetal bovine serum [FBS]). A vehicle control stock solution of 95% DMSO/5% HCL was made from 1 mL of 0.2% fatty-acid-free bovine serum albumin (FAF-BSA) in phosphate-buffered saline and 19 mL of acidified DMSO. To assess DSC progenitor proliferation in response to exogenous S1P, cells were seeded 13,000 cells/cm² and allowed to attach overnight in the basal medium containing 10% FBS. After 18 h of incubation, the existing medium was exchanged for a low serum medium (1% FBS). Cell monolayers were serum starved for 24 h. After incubation in low serum conditions, cell monolayers were incubated an additional 24 h in experimental medium groups (vehicle FAF-BSA/DMSO control, 10% FBS, S1P-containing, or VPC01211-containing) followed by proliferation assessment using Promega's CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega BioSciences, Madison, WI).

To assess DSC progenitor migration in response to exogenous S1P, a 96-well migration assay kit was purchased from Neuroprobe (Gaithersburg, MD) (area = 25 mm²; pore size = 8 μ m). A cell suspension of DSC progenitors at 13,000 cells/cm² was seeded in the upper chamber containing a low serum medium (1% FBS). The lower chamber was filled with a medium from each experimental group (vehicle FAF-BSA/DMSO control, 10% FBS, S1P-containing, or VPC01211-containing). After 4 h of incubation at 37°C, a cotton tip applicator removed nonmigrated cells from the top side of the membrane. The porous membrane was then incubated in 3% paraformaldehyde for 30 min at 4°C, stained with 0.2% crystal violet for 15 min, and solubilized using a 50/50 mixture of 0.1M NaH₂PO₄, pH 4.5, and ethanol. Cell migration in response to S1P was expressed in terms of absorbance values.

Statistics

Results are presented as mean $\pm$ standard error of mean. Statistical analysis of bone volume, vessel length density

calculations, and vessel number per area was performed using a General Linear Model analysis of variance, followed by Tukey's Test for pairwise comparisons. Significance was asserted at $p < 0.05$.

Results

S1P₁/S1P₃ synergism enhances bone remodeling in cranial defect

Previously, we reported that local delivery of S1P encapsulated in PLAGA scaffolds significantly enhanced new bone formation in a critical-sized rat cranial defect model compared with unloaded controls.¹³ Based on expanded window chamber studies, we hypothesized that selective activation of two receptors (S1P₁ and S1P₃) that are most abundantly expressed on DSC progenitors and known to stimulate both endothelial cell and smooth muscle cell proliferation and migration, new osseous growth, and vessel remodeling would be significantly enhanced in the cranial defect space. Specifically, PLAGA constructs were loaded with S1P, FTY720 (S1P₁ and S1P₃ agonist), or VPC01091 (S1P₁ agonist and S1P₃ antagonist) (Fig. 1) and implanted in a critical-sized defect to assess new bone formation at 2 and 6 weeks. Quantitative microCT analysis revealed similar amounts of new bone volume generated in FTY720-stimulated defects compared with S1P-induced groups, where both were significantly greater than unloaded controls at 6 weeks (Fig. 2A). Alternatively, both qualitative and quantitative microCT analysis revealed that new bone volume generated in VPC01091-stimulated defects was not significantly different from unloaded controls and, in fact, was significantly less compared with FTY720- or S1P-treated defects (Fig. 2A, B).

FIG. 1. Selective activation of S1P receptor subtypes by pharmacological compounds. Here, S1P activates all three receptors on vascular endothelial and smooth muscle cells (S1P₁, S1P₂, and S1P₃), whereas FTY720 is a selective S1P₁ and S1P₃ agonist, and VPC01091 is an S1P₁ agonist and S1P₃ antagonist. S1P, sphingosine 1-phosphate. Color images available online at www.liebertonline.com/ten.

FIG. 2. Evaluation of new bone volume generated in critical-sized rat cranial defects. (A) Qualitative microCT imaging scan of scaffold preimplantation. (B) Quantitative microCT evaluation of new bone volume generated in a critical-sized rat cranial defect 2 and 6 weeks postimplantation. (C) Three-dimensional reconstructed microCT renderings of healing within critical-sized rat cranial defect 6 weeks postimplantation. *Significant between groups, where $p < 0.05$. CT, computed tomography. Color images available online at www.liebertonline.com/ten.

S1P₁/S1P₃ synergism enhances microvascular remodeling in cranial defect

After 6 weeks of implantation, immunohistochemical staining revealed many microvessel lumens surrounding newly formed bone islands (Fig. 3A). These cranial tissue

sections were used to quantify total vessel numbers within the defect space after 6 weeks of healing. FTY720-stimulated tissues showed approximately a twofold increase in vessel number compared with all other groups (Fig. 3B). Further, vessel counts of immunostained montaged sections revealed no significant differences in SMA⁺ vessels between control and S1P-stimulated groups (Fig. 3C). However, FTY720 stimulation yielded the greatest number of SMA⁺ vessels per tissue area. Interestingly, total vessel counts and SMA⁺ vessel counts for FTY720-stimulated tissues are statistically similar, suggesting that FTY720 stimulated both new vessel growth and vessel maintenance over the course of 6 weeks. In contrast, VPC01091-treated defects showed significantly decreased numbers of both total vessels and smooth muscle cells (SMC)-invested vessels, compared with FTY720 treatment.

Montaged histological sections were divided into subregions of interest to understand how different compounds may affect spatial distribution of vessels within the defect space. First, defects were divided equally into dural and periosteal regions by a horizontal bisector (Fig. 4A, B). Both total vessels in H&E-stained sections and SMA⁺ vessels in immunostained sections were counted. Total vessels counts are distributed evenly between periosteal and dural regions in control, S1P-stimulated, and VPC01091-stimulated groups. Total vessel density significantly increased in periosteal regions of FTY720-stimulated tissues compared with all other experimental groups. Periosteal regions showed greater amounts of SMA⁺ vessels compared with dural regions across all experimental groups. FTY720-induced tissues showed the greatest difference in SMA⁺ counts between periosteal and dural regions, followed by VPC01091 groups.

Next, defects were divided into central and edge regions by two vertical bisectors (Fig. 4C, D). Counting total vessels in H&E stained sections, vessel densities were evenly divided between central and edge regions of the defect across all experimental groups. FTY720-stimulated groups showed significantly greater numbers of vessels in center and edge regions compared with controls. Counts of immunostained sections revealed greater numbers of SMA⁺ vessels in edge regions compared with center regions in control, S1P-induced, and VPC01091-induced tissues. This trend was significant in VPC01091 groups. In contrast, FTY720-induced samples show an even distribution of SMA⁺ vessels across central and edge regions of the defect space. These results imply that FTY720 delivery stimulated greater vessel formation within periosteal regions of the defect space, suggesting greater penetration of mature vessel networks throughout the defect space.

S1P₁/S1P₃ synergism reduces inflammatory cell trafficking to defect area

Inflammatory cells are known to traffic to areas of wounding to aid in vascularization and overall wound healing. Thus, we assessed the presence of inflammatory cells in the defect space using CD45, a marker to all hematopoietic cells except erythrocytes and platelets. Studies have shown that S1P prevents monocyte adhesion to the endothelium via the S1P₁ receptor, thereby limiting its extravasation into the tissue space.¹⁷ Thus, we hypothesized that treatment with S1P, FTY720, and VPC01091 would diminish the amount of the CD45⁺ cells in the defect space compared with controls.

FIG. 3. Evaluation of vessel remodeling within critical-sized rat cranial defects. (A) Top row: immunostained cranial defect sections show SMA⁺ vessel lumens (red) within defect space. Bottom row: matching sections stained for hematoxylin and eosin show newly remodeled bone and surrounding vasculature. Scale bar = 50 μ m. (B) Quantification of total vessel density and (C) SMA⁺ vessels counted within critical-sized rat cranial defect 6 weeks postimplantation. *Significant between groups, where $p < 0.05$. SMA, smooth muscle actin. Color images available online at www.liebertonline.com/ten.

Figure 5A–D shows representative images of CD45⁺/SMA⁺ staining in the cranial defect. S1P- and FTY720-treated tissues reveal a decreased presence of inflammatory cells compared with control or VPC01091-treated tissues, suggesting a role for S1P₃ in modulating inflammatory cell recruitment in peripheral regenerating tissue spaces.

S1P enhances proliferation and migration of dural progenitors in vitro

Utilizing the rat cranial defect model to confirm FTY720 as a potential candidate for enhancing vessel remodeling, receptor selective activation was also evaluated *in vitro* to determine effects on proliferation and migration of dural

progenitors. First, we investigated the S1P receptor profile of DSC progenitors. Qualitative results revealed that primary DSCs (passage 3) express receptors S1P₁ and S1P₃ and possibly S1P₄. In low passage unstimulated primary DSCs, S1P₃ has the highest relative expression level (Fig. 6A). Subsequent *in vitro* studies were conducted to assess DSC proliferation and migration in response to exogenous S1P. As a prodrug, VPC01091 requires internalization, phosphorylation, subsequent exocytosis, and then autocrine signaling. Therefore, for *in vitro* studies, VPC01211, the phosphorylated analog to VPC01091, was used. After 24 h of incubation with S1P, DSCs had significantly increased proliferation compared with 10% serum control, vehicle control, and VPC01211 (Fig. 6B). After 4 h of incubation with S1P, a

FIG. 4. Quantifying vessel distribution throughout cranial defect space after 6 weeks of healing. (A, B) Comparing vessel distribution between dural and periosteal regions. (C, D) Comparing vessel distribution between edge and center regions. (A, C) Total number of vessels counted using hematoxylin and eosin-stained sections. (B, D) SMA⁺ number of vessels counted using immunostained sections. *In (A), significant to all groups. In (B–D), significant between noted groups. Significance was asserted at $p < 0.05$.

significantly greater number of DSCs had migrated across the porous membrane compared with 10% serum control, vehicle control, and VPC01211 (Fig. 6C). Collectively, these results suggest that the S1P₃ receptor pathway is critical for S1P-enhanced DSC proliferation and migration.

Discussion

During cranial skeletogenesis, onset of vascularization precedes intramembranous ossification of mesenchymal cells.¹⁸ Blood vessels initiate this process by invading a pool of mesenchymal tissue, resulting in direct differentiation of progenitor cells toward newly woven bone. Regulation between capillary invasion and osteoblast maturation is supported by observations demonstrating the physical proximity of osteoblasts hovering at the tips of sprouting capillary beds.¹⁹ Further, microvessels have been shown to accelerate bone formation even before flow has been established,²⁰ and others

have suggested that the preceding vascular network may serve as a scaffold for subsequent osteoblast differentiation.^{21,22} Therefore, incorporating strategies to improve vascular cell recruitment may also increase native progenitor residence time within the defect space and improve overall cranial regeneration.

Building upon previous studies demonstrating S1P-enhanced vessel remodeling and new data suggesting that selective receptor activation can enhance S1P-mediated blood flow recovery,²³ we observed significantly greater vessel density and SMC staining in FTY720-treated cranial tissue compared with S1P groups. Specifically, FTY720-treated tissue sections reveal many α -SMA-positive vessel lumens surrounding new bony islands within the defect space, reminiscent of *de novo* skull formation in the embryo. Further, antagonism of S1P₃ via VPC01091 resulted in a significant decrease in vascular density compared with FTY720. Collectively, these studies, which manipulate activation of

FIG. 5. Cranial defect tissue (A–D) stained for CD45⁺ (green) and SMA⁺ cells (red) to qualitatively assess local changes in inflammatory cell recruitment. In cranial tissue after 42 days, (A) control poly-lactic-co-glycolic acid; (B) VPC01092; (C) S1P; and (D) FTY720, where scale bar = 50 μ m. Color images available online at www.liebertonline.com/ten.

specific receptor subsets, suggest that S1P₃ activation may play a supporting role in FTY720's robust vessel remodeling response compared with S1P₁ signaling alone. Poor smooth muscle cell investment and significantly lower vessel density were observed in untreated controls.

Unlike the significant vascular remodeling events observed in FTY720-treated calvarial tissues compared against S1P-treated tissues, total volumes of osseous generation were similar between S1P and FTY720 groups. These observations may be a function of indiscriminate nonspecific activation of various S1P receptors. For example, osteoclastic precursor cells, also present within the wound bed, have been shown to express S1P₁ and migrate toward SEW2871, a potent S1P₁ agonist *in vivo*.²⁴ Further, others have demonstrated that treatment with FTY720 relieves osteoporosis in mice by reducing the population size of osteoclastic cells. In contrast, primary calvarial cells have only been shown to express receptors S1P₂, S1P₅, and S1P₁²⁵ and therefore are most likely not a primary responder to S1P₃ activation by agonist FTY720. Interestingly, Walter *et al.* demonstrated enhanced bone marrow cell homing and blood flow recovery in FTY720-stimulated hind limb ischemia models, where hind limbs with S1P₃^{-/-} osteoprogenitors failed to revascularize FTY720-stimulated tissue.²³ Here, we confirm expression of both S1P₁ and S1P₃ in DSC osteoprogenitor populations, where FTY720-enhanced DSC proliferation and migration is

attenuated when selectively inhibiting S1P₃. Collectively, this evidence suggests a unique balancing act of bone homeostasis where various ratios of S1P₁ and S1P₃ signaling determine mobilization of osteoprogenitors and osteoclast precursors from local niches to participate in cranial remodeling. Therefore, cell-type-targeted S1P signaling may create even more potent tissue remodeling responses.

Selective receptor activation aside, one argument for the substantial increase in arteriogenic responses to FTY720 is prolonged bioactivity *in vivo*. A multitude of sphingolipid metabolic pathways result in rapid terminal degradation of S1P *in vivo*. Therefore, it may be challenging to maximize the therapeutic effect of S1P *in vivo* due to its reported short half-life (15 min).²⁶ Alternatively, receptor-specific compounds have been developed that are not susceptible to degradation via S1P lyase. Instead, these compounds circulate between phosphorylated and unphosphorylated states, thus extending *in vivo* half-lives (approximately 4 days) by evading rapid enzymatic degradation. Therefore, substantial increases in arteriogenic remodeling via FTY720-stimulation could result from both selective receptor activation and prolonged *in vivo* half-lives, notwithstanding the possibility of altered half-lives after PLGA encapsulation. Interestingly, similar amounts of bone volume were generated between FTY720 and S1P-stimulated groups at 6 weeks. These results suggest that although S1P is susceptible to rapid degradation *in vivo*,

FIG. 6. Primer sequences used to detect the expression profile of S1P receptors in primary isolated rat dural stem cells (DSCs) (A). Results show that expanded DSC populations express receptors S1P₃, S1P₁, and S1P₄ in order of decreasing abundance. Quantitative assessment of *in vitro* DSC proliferation (B) and migration (C) in response to exogenous S1P and VPC01211 in low serum medium conditions. *Significant between groups, where $p < 0.05$.

long-term therapeutic effects induced by S1P signaling are still achieved. One explanation for this observation may be that in contrast to soluble bolus injections, our method of delivery from PLAGA films allows for slow sustained release—a mechanism by which short compound half-lives may be overcome with continuous release of signal.

Bone wound healing is a continuum of remodeling processes involving hemostasis, inflammation, clearance, and repair. Both the function and remodeling of microvasculature is critical not only for oxygen and nutrient transport but also for initiation of ossification and recruitment of mesenchymal stem cells. Further, because angiogenesis is a critical component in the continuum of bone healing, it is not surprising that S1P-induced formation of mature vessel networks does promote bone healing. However, our experiments comparing various combinations of S1P receptor targeting suggest that other critical components of the bone healing cascade may be differentially regulated in an S1P-receptor-specific manner. To reinforce this point, we isolated resident DSCs and showed both migration and proliferation patterns can be significantly manipulated by selective S1P receptor signaling too. In conclusion, blood vessel growth is centrally important in cranial bone healing, but balancing other processes such as progenitor recruitment, immune cell trafficking, and osteoclastic resorption is also important. In this study, observed trends of significant *in vivo* microvas-

cular remodeling and osseous regeneration lend substantial credibility to the utilization of S1P-targeted compounds in the development of future therapeutic tissue remodeling strategies.

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Disclosure Statement

No competing financial interests exist.

References

1. LeDouarin, N.M., and Dupin, E. Multipotentiality of the neural crest. *Curr Opin Genet Dev* 13, 529, 2003.

2. Sieber-Blum, M., Grim, M., Hu, Y.F., and Szeder, V. Pluripotent neural crest stem cells in the adult hair follicle. *Dev Dyn* **231**, 258, 2004.
3. Opperman, L.A., Passarelli, R.W., Nolen, A.A., Gampper, T.J., Lin, K.Y.K., and Ogle, R.C. Dura mater secretes soluble heparin-binding factors required for cranial suture morphogenesis. *In Vitro Cell Dev Biol* **32**, 627, 1996.
4. Opperman, L.A., Chhabra, A., Cho, R.W., and Ogle, R.C. Cranial suture obliteration is induced by removal of transforming growth factor (TGF)- β 3 activity and prevented by removal of TGF- β 2 activity from fetal rat calvaria *in vitro*. *J Craniofac Genet Dev Biol* **19**, 164, 1999.
5. Opperman, L.A., Passarelli, R.W., Morgan, E.P., Reintjes, M., and Ogle, R.C. Cranial sutures require tissue interactions with dura mater to resist osseous obliteration *in vitro*. *J Bone Miner Res* **10**, 1978, 1995.
6. Reid, C.A., McCarthy, J.G., and Kolber, A.B. A study of regeneration in parietal bone defects in rabbits. *Plast Reconstr Surg* **67**, 591, 1981.
7. Spector, J.A., Greenwald, J.A., Warren, S.M., Bouletreau, P.J., Detch, R.C., Fagenholz, P.J., Crisera, F.E., and Longaker, M.T. Dura mater biology: autocrine and paracrine effects of fibroblast growth factor 2. *Plast Reconstr Surg* **109**, 645, 2002.
8. Gagan, J.R., Tholpady, S.S., and Ogle, R.C. Cellular dynamics and tissue interactions of the dura mater during head development. *Birth Defects Res C Embryo Today* **81**, 297, 2007.
9. Petrie, C.E., Tholpady, S.S., Ogle, R.C., and Botchwey, E.A. Proliferative capacity and osteogenic potential of novel dura mater stem cells on poly-lactic-co-glycolic acid. *J Biomed Mater Res A* **85**, 61, 2008.
10. Jain, R.K. Molecular regulation of vessel maturation. *Nat Med* **9**, 685, 2003.
11. Henry, T.D., Annex, B.H., McKendall, G.R., Azrin, M.A., Lopez, J.J., Giordano, F.J., Shah, P.K., Willerson, J.T., Benza, R.L., Berman, D.S., Gibson, C.M., Bajamonde, A., Rundle, A.C., Fine, J., and McCluskey, E.R.; and VIVA Investigators. The VIVA trial: vascular endothelial growth factor in ischemia for vascular angiogenesis. *Circulation* **107**, 1359, 2003.
12. Barnard, G.C., McCool, J.D., Wood, D.W., and Gerngross, T.U. Integrated recombinant protein expression and purification platform base on *Ralstonia eutropha*. *Appl Environ Microbiol* **71**, 5735, 2005.
13. Sefcik, L.S., Petrie Aronin, C.E., Wiegand, K.A., and Botchwey, E.A. Sustained release of sphingosine 1-phosphate for therapeutic arteriogenesis and bone tissue engineering. *Biomaterials* **29**, 2869, 2008.
14. Wamhoff, B.R., Lynch, K.R., Macdonald, T.L., and Owens, G.K. Sphingosine 1-phosphate receptor subtypes differentially regulate smooth muscle cell phenotype. *Arterioscler Thromb Vasc Biol* **28**, 1454, 2008.
15. Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C.X., Hobson, J.P., Rosenfeldt, H.M., Nava, V.E., Chae, S.S., Lee, M.J., Liu, C.H., Hla, T., Spiegel, S., and Proia, R.L. Edg-1, the G protein-coupled receptor for sphingosine 1-phosphate, is essential for vascular maturation. *J Clin Invest* **106**, 951, 2000.
16. Petrie Aronin, C.E., Sadik, K.W., Lay, A.L., Rion, D.B., Tholpady, S.S., Ogle, R.C., and Botchwey, E.A. Comparative effects of scaffold pore size, pore volume, and total void volume on cranial bone healing patterns using microsphere-based scaffolds. *J Biomed Mater Res A* **89**, 632, 2009.
17. Bolick, D.T., Srinivasan, S., Kim, K.W., Hatley, M.E., Clemens, J.J., Whetzel, A., Ferger, N., Macdonald, T.L., Davis, M.D., Tsao, P.S., Lynch, K.R., and Hedrick, C.C. Sphingosine-1-phosphate prevents tumor necrosis factor- α -mediated monocyte adhesion to aortic endothelium in mice. *Arterioscler Thromb Vasc Biol* **25**, 976, 2005.
18. Collins-Osdoby, P. Role of vascular endothelial cells in bone biology. *J Cell Biochem* **55**, 304, 1994.
19. Streeten, E.A., and Brandt, M. Biology of bone endothelial cells. *Bone Miner* **10**, 85, 1990.
20. Trueta, J., and Little, K. The vascular contribution to osteogenesis. II. Studies with the electron microscope. *J Bone Joint Surg Br* **42B**, 367, 1960.
21. Caplan, A., Syftestad, G., and Osdoby, R. The development of embryonic bone and cartilage in tissue culture. *Clin Orthop Relat Res* **174**, 243, 1983.
22. Drushel, R.F., Pechak, D.G., and Caplan, A.I. The anatomy, ultrastructure and fluid dynamics of the developing vasculature of the embryonic chick wing bud. *Cell Differ* **16**, 13, 1985.
23. Walter, D.H., Rochwalsky, U., Reinhold, J., Seeger, F., Aicher, A., Urbich, C., Spyridopoulos, I., Chun, J., Brinkmann, V., Keul, P., Levkau, B., Zeiher, A.M., Dimmeler, S., and Haendeler, J. Sphingosine 1-phosphate stimulates the functional capacity of progenitor cells by activation of the CXCR₄-dependent signaling pathway via the S1P₃ receptor. *Arterioscler Thromb Vasc Biol* **27**, 275, 2007.
24. Ishii, M., Egen, J.G., Klauschen, F., Meier-Schellersheim, M., Saeki, Y., Vacher, J., Proia, R.L., and Germain, R.N. Sphingosine 1-phosphate mobilizes osteoclast precursors and regulates bone homeostasis. *Nature* **458**, 524, 2009.
25. Grey, A., Xu, X., Hill, B., Watson, M., Callon, K., Reid, I.R., and Cornish, J. Osteoblastic cells express phospholipids receptors and phosphatases and proliferate in response to sphingosine 1-phosphate. *Calcif Tissue Int* **74**, 542, 2004.
26. Peng, X., Hassoun, P.M., Sammani, S., McVerry, B.J., Burne, M.J., Rabb, H., Pearse, D., Tudor, R.M., and Garcia, J.G. Protective effects of sphingosine 1-phosphate in murine endotoxin-induced inflammatory lung injury. *Am J Respir Crit Care Med* **164**, 1245, 2004.

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