

## Selective Activation of Sphingosine 1-Phosphate (S1P) Receptors Enhances Vessel Stabilization and Bone Regeneration in a Cranial Defect Model

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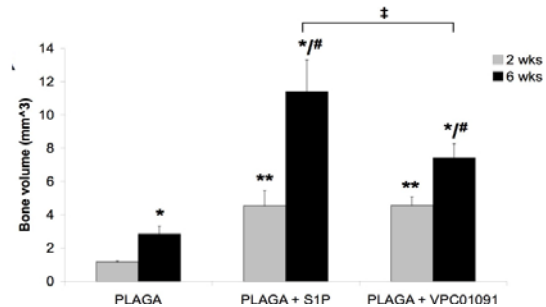
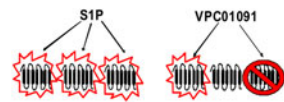
**Statement of Purpose:** Early stage treatment of many musculoskeletal diseases focus on addressing aberrant or disrupted vascular networks. Similarly, strategies to incorporate new vascular elements in large scale skeletal repair remain unanswered. Thus, we investigate the ability of sustained S1P delivery to simultaneously enhance *both* mineralization and microvascular remodeling within a critical-size osseous defect. With the advent of receptor specific agonists/antagonists, we use these tools to further elucidate the pathway through which these effects are mediated.

**Methods:** Thin polymeric films of 50:50 PLGA ( $M_w = 72.3\text{kDa}$ ) loaded with 1:400 S1P (wt./wt.) or 1:200 VPC01091 (wt./wt.) were fabricated using a solvent casting method. Microspheres of 85:15 PLGA ( $M_w = 100\text{kDa}$ )

were fabricated using a single emulsion method. To fabricate 3D scaffolds, 85:15 PLGA microspheres and 20 x 1mm 50:50 PLGA films loaded with either S1P or VPC01091 were mixed together, poured into molds measuring 8mm x 1 mm, and sintered at 75°C for 3 hours in the circular copper molds.

Adult male retired breeder rats (400-550g) were used in a critically sized 8mm circular defect. Following either 14 or 42 days healing, the animal was euthanized and the defect was excised and immediately placed into 10% formalin. First, samples were x-ray imaged for 6 sec at 12.5 kVp. Second, new bone healing was quantified utilizing a high resolution 45 kVp microCT imaging scan. Bone void volume, threshold (160), and scan parameters (support = 2, width = 1.2) were kept constant throughout the entire study. Finally, following *ex vivo* x-ray imaging and *ex vivo* microCT scanning, samples were decalcified, cut along the coronal plane at the midline of the defect and embedded in paraffin. Half the sections were stained with hematoxylin and eosin and half were stained with lectin-AlexaFluor488, an endothelial cell marker, and SMA- $\alpha$ -IA4, a smooth muscle cell marker. Total number of vessels per area of defect was counted per group using H&E stained sections. Number of vessels invested with smooth muscle cells was counted per group using SMA- $\alpha$ -IA4 staining.

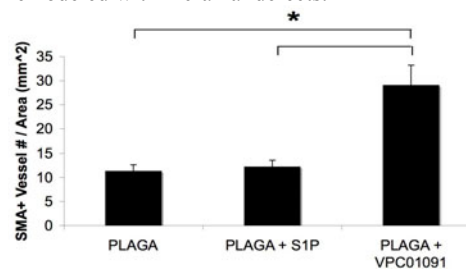
**Results:** After 6 wks, quantitative microCT analysis confirmed a significant increase in new bone volume compared with 2 wk data across all experimental groups. At 2 and 6 wks, both loaded groups had significantly greater amounts of bone volume compared with unloaded control. At 6 wks, the total amount of new bone formed was significantly greater with S1P delivery compared with that of VPC01091.



Quantitative bone volume amounts measured from microCT imaging results. \* Significant from respective 2 wks timepoint. \*\* Significant from PLAGA 2wks. # Significant from PLAGA 6 wks. ‡ Significant between groups.

In all groups, new bone volume observed was located predominantly around the perimeter of the defect along the dural side, as confirmed by qualitative microCT images and 2D x-ray imaging. H&E stained histological sections confirmed greater amounts of bone healing in loaded constructs compared with vehicle control. Although normalized H&E vessel data showed no significant differences in total vessel number across groups, immunostained sections revealed a significant increase in the number of SMA+ vessels in the VPC01091-loaded group compared with both S1P-loaded and vehicle control groups.

**Conclusions:** Localized release of S1P from 3D biodegradable scaffolds significantly enhances the formation of new bone within the defect area after both 2 and 6 wks compared to unloaded control. S1P-loaded constructs exhibited significantly greater amounts of new bone volume at 6 wks compared with VPC01091, suggesting other receptors, particularly S1P3, are at play. Histological analysis after 6 wks demonstrated significantly greater amounts of SMA+ vessels in VPC01091 constructs compared with S1P and vehicle controls. These results suggest that the S1P1 receptor may be the predominant pathway mediating vessel stabilization of networks remodeled within cranial defects.



SMA+ vessels counted from SMA+-Cy3 / lectin-488 immunostained sections, normalized by defect area. \* Significant between groups.