Targeting Notch Signaling to Enhance 3D-printed Bone Scaffold Vascularization and Callus Formation

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Introduction: Rapid angiogenesis and local vascular penetration are essential to deliver cells and growth factors that promote bone callus formation in large bone defect repair surgery. Although multiple cell signaling pathways are involved in regulation of vessel formation, increasing evidence shows that Jagged1 (JAG1)-mediated Notch signaling plays a crucial role in angiogenesis during development. However, it is unknown whether JAG1-mediated Notch activation could be used as a therapeutic target to enhance vascularization and bone formation during large bone defect repair. Here we undertook a study to determine if Notch JAG1 protein could be utilized to enhance angiogenesis and bone formation when coated to 3D-printed scaffolds in a mouse femur bone defect model.
Methods: First we performed *in vitro* experiments to test whether Notch activation could be used to enhance tubulogenesis in Endothelial stromal cell (ESC) cultures. ESCs were exposed to either lgG (a control protein) or JAG1 for a period of three days and then plated on a dish coated with Matrigel for tube formation. 3D-printed biodegradable Polycaprolactone (PCL) scaffolds with similar biomechanical properties to real bone were coated with JAG1 protein or lgG. Tetramethylbenzidine (TMB) assay was performed to monitor the controlled release of JAG1 from PCL scaffolds. Finally, a mouse femur bone defect model was used to test the in vivo repair ability of JAG1 coated PCL scaffolds. H&E staining and immunostaining were used to monitor angiogenesis and bone callus formation.
Results: Our results showed in Figure 1 demonstrated that ESCs exposed to JAG1 in cultures resulted in a measurable increase of capillary tube formation with thicker tubes and more connections. TMB assay showed the JAG1 protein cross-linked on the surface of PCL scaffold could hold on to and slowly release JAG1 protein over 11 days and possibly even weeks. Histological assessment of transplanted PCL scaffolds showed bone callus formation surrounding the JAG1-coated scaffolds in the bone defect mouse model was significantly increased when compared to lgG-coated scaffolds at 8 weeks after surgery (Figure 2). More importantly, an enhanced expression of angiogenic markers CD31 and vWF was observed in the callus adjunct to JAG1-coated scaffolds at 2 weeks after surgery (Figure 2), suggesting that JAG1 induced rapid ESC angiogenic differentiation and local vascularization in bone callus.
Discussion: Angiogenesis is a critical onset step for bone tissue repair. Increased angiogenesis often leads to rapid tissue regeneration. Knockout JAG1 in mice to inhibit Notch signaling shows severe vascular defects and embryonic lethality. In contrast, tumor cells expressing JAG1 are correlated with worse prognosis and greater tumor neovascularization suggesting an important role of Notch signaling pathway in angiogenesis. Data from this study clearly showed that JAG1-mediated Notch activation significantly enhanced tube formation in ESC cultures confirming that JAG1 is a strong inducer for angiogenesis. More importantly, expressions of angiogenic markers vWF and CD31 were significantly increased in bone callus adjunct to JAG1-coated PCL scaffold in mice when compared to lgG-coated scaffold.
Significance/Clinical Relevance: Taken together, our results support the idea that JAG1 protein-coated PCL scaffold could be used as a novel bone substitute for rapid bone defect repair by enhancing ESC angiogenic differentiation/vascularization and subsequent bone callus formation**.**

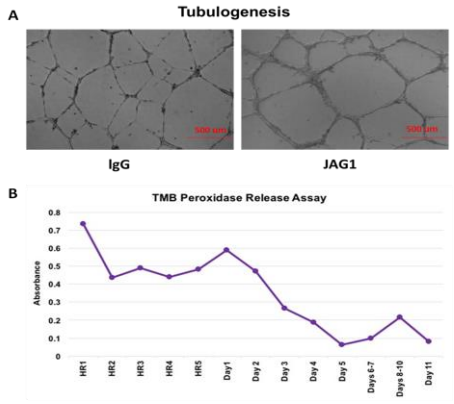

Figure 1: (A) JAG1-induced tubulogenesis in cell cultures. Bright field image showed JAG1 (10 mg/ml) protein enhances tubulogenesis in Endothelial stromal cell (ESC) culture. ESCs were exposed to either lgG or JAG1 for a period of three days and then plated on a dish coated with matrigel. Scale bar represents 50 µm. **(B) Crosslink coated JAG1 can be hold on to scaffold and slowly released for more than 11 days.** JAG1-coated 3D printed bone was soaked in 2ml of PBS for one hour before being removed and added to a second vial. This was repeated for five hours before switching to removal every day. By Day 6 the bone was allowed to soak for two days and on Day 8 soaked for 3 days.

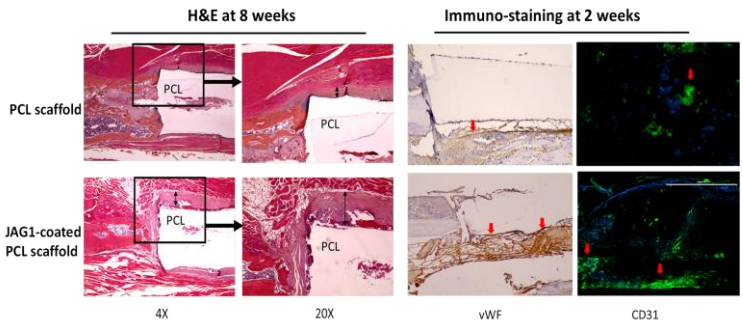

Figure 2: JAG1-coated 3D printed scaffold significantly enhanced vessel formation at 2 weeks and subsequent bone formation at 8 weeks post transplantation.
The left panels are representative 4X and 20X H&E images of bone callus surrounding PCL scaffold at 8 weeks post transplantation. Bone callus thickness is marked with black double head black arrows.
The right panels are representative images of immunostaining and immunofluorescence. Bone callus and PCL scaffold were harvested at 2 weeks post transplantation and stained using specific antibodies for angiogenesis markers vWF and CD31. Positive area is marked with red arrows.