Multivalent Sonic Hedgehog-Hyaluronic Acid Conjugates for Enhanced Neovascularization During Diabetic Wounding Healing

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Statement of Purpose: Limited vascularization, caused by an inadequate migration of microvascular endothelial cells into the wound bed, plays a crucial role in the etiology of diabetic ulcers. Thus, promoting neo-vascularization is a critical step to improving material transport and ensuring rapid wound resolution. Sonic hedgehog (Shh) is an important growth and differentiation factor that up-regulates multiple angiogenic signaling pathways, and exogenous delivery of Shh can improve the rate and quality of diabetic would closure[1]. However, poor control over growth factor localization and bioactivity during drug delivery has limited its use as a wound healing clinical treatment. We have previously developed multivalent protein-polymer bioconjugates to enhance the potency and stability of growth factors following targeted in vivo administration[2]. This method also allows for stoichiometric control over the conjugation of proteins to a soluble biopolymer, such as hyaluronic acid (HyA). In this work, we utilize multivalent conjugation to increase the bioactivity of Shh and promote neovascularization in diabetic wounds relative to unconjugated Shh at identical molar ratios.

Methods: Exploiting carboimiide and maleimide chemistry, we conjugated a cysteine modified variant of recombinant Shh to long-chain HyA polymers. The poly-valency of the HyA conjugates was verified by size exclusion chromatography with multiangle-light scattering (SEC-MALS). We validated the sensitivity of fibroblasts to Shh valency by measuring their Shh pathway activation using NIH/3T3s transduced with luciferase for Gli-1 transcriptions, which occurs downstream of Shh pathway activation. Finally, to demonstrate the effectiveness of mvShh conjugates to improve diabetic wound healing *in vivo*, we generated excisional dermal wounds on the dorsum of db/db mice and treated the wounds with mvShh conjugates via methylcellulose carrier. Tissue samples were subsequently harvested after 4 and 7 days for analysis with histochemistry.

Results: We synthesized soluble clusters of Shh conjugated to single-chains of HyA at the following defined ratios of Shh to HyA: 1:1, 3.5:1, 7:1, 14:1, and 22:1. We also confirmed that multivalent conjugation yields greater protein bioactivity *in vitro*, demonstrated in fibroblasts by increased luciferase signal in response to mvShh relative to soluble Shh **(Figure 1a)**. We next determined that high-valency Shh conjugates (i.e. 14:1 / Shh:HyA) promote more rapid migration of CD31+ endothelial cells into the wound bed (**Figure 2b**), drastically increasing the density of vascular structures during early phases of wound healing. Thus, diabetic wounds treated with mvShh exhibited improved healing on the basis of overall wound appearance and closer rate.

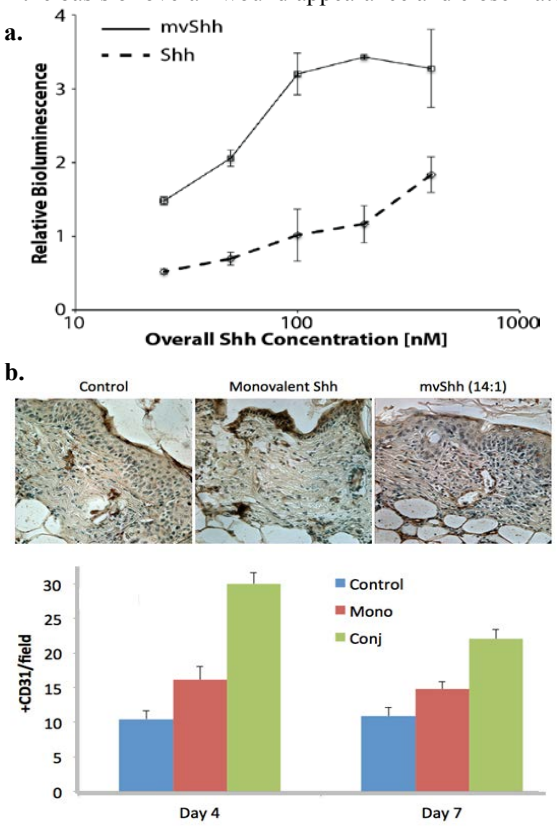

Figure 1. a. *mvShh induced higher bioluminescence response than soluble Shh in luciferase-transduced fibroblasts, indicating increased bioactivity of Shh upon HyA-conjugation*. **b**. *mvShh promoted more rapid migration of CD31+ endothelial cells into the wound bed by a factor of 3 relative to negative controls, yielding increased neovascularization.*

Conclusions: We demonstrated that conjugation of Shh to HyA increases bioactivity on a per-molecule basis *in vitro* and accelerated neovascularization *in vivo.* Thus, our findings suggest that our method of multivalent conjugation Shh to soluble HyA biopolymers may be an enabling approach for novel therapeutics for diabetic wound healing. Patients should ideally require less frequent administration of mvShh. Furthermore, multivalent conjugation technology can be applied to other growth factors and treatments with the potential to overcome many current clinical limitations.

References: 1. Luo JD. *Am J Physiol Endocrinol Metab*. 2009;297:525-531.

2. Wall ST. *Bioconjug Chem*. 2008;19:806-812.