Delivery of Slit3 Peptide via a Novel Composite Bone Wound Healing Patch Improved Osseointegration and Cranial Bone Defect Repair

Xiaojie Xing[1], Min Hao[2], Jessica Ritz[1], Jiang Chen[1], Younan Xia[2], Xinping Zhang[1*]
1 Center for Musculoskeletal Research, University of Rochester, School of Medicine and Dentistry, Rochester, NY 14642, USA
2 School of Chemistry & Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332, USA
*Xinping_Zhang@urmc.rochester.edu

INTRODUCTION: Cranioplasty is a common neurosurgical procedure performed to reconstruct the missing cranial bone segments to protect cerebral structures and to restore fluid flow of the injured brain. While autografts remain the clinical standard, the use of allografts and synthetic substitutes is gaining favor due to advantages such as reduced surgery time, potential for customized fitting, and elimination of donor site complications. However, due to the limited osteogenic and angiogenic activities of the skull bone, along with the compromised regenerative environment at the site of healing, both allograft and synthetic materials exhibit poor osseointegration compared to autograft. To enhance healing and osseointegration, a series of novel angiogenic factors and bone stimulating growth factors are being developed and incorporated into various grafting materials to enhance vascularization and promote bone tissue formation. Slit family proteins are secreted repulsive axon guidance molecules that bind to Robo receptors. Prior studies show that Slit3 regulates endothelial cell proliferation and migration. In addition to its role in angiogenesis, recent studies demonstrate that Slit3 stimulates osteoblast migration and bone formation *in vivo*. Slit3 secreted from osteoblasts controls type H vessel formation and is further required for fracture healing in mice. The goal of our current study was to evaluate the therapeutic potential of Slit3 overexpression via adenovirus-mediated transduction, and to further assess the beneficial effects of delivery of an active peptide of Slit3 through a novel composite bone wound healing patch, designed to be applied to the wound healing site like a bandage, for restoration of the lost bone and improvement of cranial defect repair following cranioplasty.

METHODS: *Adenovirus-mediated transduction of Slit3 into BMSCs and in vivo ectopic bone formation assay.* Adenovirus encoding full length human Slit3 gene (Ad-hSlit3) was made and used to transduce BMSCs isolated from B6 mice at 100 MOI. The transduction efficiency and production of hSlit3 protein were confirmed via RT-PCR, western blot analyses and endothelial cell migration assays. The transduced cells were loaded into collagen gel and implanted subcutaneously into the bilateral dorsal region of Rag1-/-; AplnCreERT2; Ai14 mice, which allowed visualization of vessel formation using multiphoton microscopy. The implants were harvested at 4 weeks post-surgery and analyzed by microCT, multiphoton microcopy and histology. Ad-hSlit3 virus was further transduced into ADTC5 cells to examine osteogenic differentiation via RT-PCR analysis. *Delivery of Slit3 peptide via bone wound healing patches.* Bone-wound healing patch consisting of nanowire hydroxyapatite (HA) sheet fabricated through sodium oleate-calcium chloride solvothermal method, and adhesive hydrogel made from 10% (w/w) gelatin and 1% (w/w) EDC-NHS on ultrathin polycaprolactone (PCL) film was prepared before surgery. Slit3 peptide (GenScript) at a dose of 500 ng/ml was loaded into adhesive hydrogel for local delivery. *Cranial defect model in mice.* A 2 mm circular defect was created on the parietal bone of Col 1(2.3) GFP mice, which mark osteoblasts with GFP. The defect was repaired using the composite bone healing patch loaded with or without Slit3 peptide. To fit the defect, the HA nanowire sheet was cut to 2 mm size using a biopsy punch and subsequently affixed to the PCL backing film with adhesive hydrogel. The composite bone healing patch was then trimmed and adhered to the soft tissue around bone defect on the mouse skull. The defect repair was examined at week 5 post-surgery for routine MicroCT and histologic analyses. Macro-indentation was performed in selected groups to examine the mechanical properties of the repair tissue at week 5 post-surgery.

RESULTS: *Overexpression of hSlit3 in BMSCs led to increased ectopic bone nodule formation in vivo.* The ad-hSlit3-mediated overexpression of hSlit3 was confirmed by western blots using cell lysates and supernatants from 293T cells (Fig. 1A). The supernatant collected from Slit3-transduced cells promoted sprouting and migration of HUVEC cells following 24-hour treatment (Fig. 1B). To determine the effects of Slit3 on bone regeneration and bone tissue vascularization, BMSCs transduced by Ad-hSlit3 and Ad-CMV were implanted subcutaneously into Rag1-/-; AplnCreERT2; Ai14 mice. Compared to Ad-CMV transduced cells, BMSCs overexpressing hSlit3 showed 3.5-fold more bone formation at 4 weeks post-implantation (Fig.1D&G, n=4, $p<0.05$), inducing significant more AplnCreER+ vessels within newly formed bone (Fig. 1E&H). Further histologic analyses confirmed significant bone formation within the implants (Fig. 1F). To determine whether overexpression of hSlit3 had any effects on osteogenesis, we examined the effects of Slit3 on differentiation of ATDC5 cells following adenovirus-mediated transduction. As shown (Fig. 1C), hSlit3 overexpression significantly increased OSX, OCN, Col2a1 expression and enhanced BMP-2 and 4 expressions. *Delivery of Slit3 peptides via bone wound healing patch led to improved osseointegration in cranial defect repair.* To determine the effects of Slit3 as an angiogenic and osteogenic factor for bone repair and reconstruction, the bone wound healing patch was loaded with a Slit3 LRRD2 peptide via adhesive hydrogel and used to repair a cranial bone defect created in mice. As shown in MicroCT scanning, on day 3, the thin composite bone patch generated weak mineral signals from HA nanowire (Fig. 1I). By week 5, the implants generated highly mineralized tissue that effectively filled the defect voids (Fig. 1J&O). Bone mineral density measurements demonstrated that the newly formed mineralized tissue exhibited comparable density to native bone (Fig. 1Q). Macro-indentation analysis revealed that the mechanical properties of the mineralized tissue were similar, though not identical, to those of the surrounding bone (not shown). Compared with other groups, the patches containing Slit3 peptide showed better integration with the host bone, as evidenced by MicroCT 3D imaging and measurement of the osseointegrated surface area around the defects (Fig. 1P). Histologic and fluorescence imaging analyses further confirmed mineralized scaffolds and increased new bone formation in defects treated with Slit3 peptide containing patches (Fig. 1K&L&R), with increased presence of the Col 1 (2.3) GFP+ osteoblasts within and on top of the scaffold (Fig. 1M&N).

DISCUSSION: Slit3 is a key coupling factor for osteogenesis and angiogenesis during long bone development and potentially in repair. Both bulk and scRNAseq transcriptomic analyses as well as our own spatial transcriptomic analyses show that Slit3 expression is increased during bone healing, primarily in osteogenic cells. The mechanism underlying the effects of Slit3 on bone formation remain unclear, although studies suggest that Slit3 is involved in neovessel sprouting and may stimulate Type H vessel formation. Utilizing BMSC-based adenovirus-mediated bone formation assay, our data demonstrate direct induction of bone and vessel formation *in vivo*, an effect that may involve enhanced expression of BMP-2 and 4. Utilizing a novel bone healing patch as a delivery vehicle, we further showed that activation of Slit 3 signaling could be beneficial for osseointegration between donor grafts and host bone. Slit 3 peptide could be used in bone healing scaffolds for enhanced osseointegration and improved bone repair.

SIGNIFICANCE/CLINICAL RELEVANCE: The novel bone wound healing patches with the capacity to deliver growth factors and Slit3 peptide show strong promise for clinical translation. By combining structural support with bioactive signaling, the bone wound healing patches offer a strategy to accelerate and improve the repair and reconstruction of the missing bone segments in cranioplasty.

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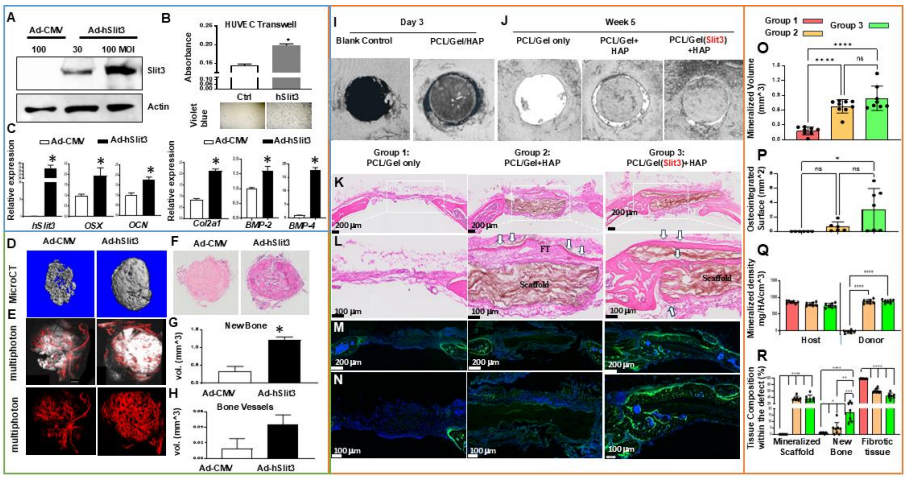

Fig.1. (A) Western blots confirm the expression of hSlit3 protein in lysates of 293T cells. (B) Supernatant from Ad-hSlit3 transduced cells enhanced HUVEC cell transwell migration. (C) Overexpression of hSlit3 had a positive effect on osteogenic differentiation of ATDC5 cells, increasing the expression of BMP2 and 4. (D) MicroCT images of bone nodules in BMSC implants. (E) Multiphoton microscopy images show bone and AplnCreER+ vessel formation in BMSC implants. vessels: red; SHG/bone: white. (F) Histologic H&E shows bone formation within implants. (G&H) quantification of total bone and vessels in implants. n=4 * $p<0.05$. (I) MicroCT images of the indicated defects at day 3. (J) MicroCT images of the indicated defects at week 5. (K&L) H&E histology at low and higher magnification show bone (arrows) and mineralized scaffold at the defect. (M&N) Fluorescence images show GFP+ osteoblasts at the defect. MicroCT analyses show mineralized volume (O), osteo-integrated surface area (P), and mineral density in implants and host bone (Q). Histomorphometric analyses show composition of bone, mineralized scaffold and fibrotic tissue formation in the defects (R). n=8-10, * indicates $p<0.05$. ** indicated $p<0.01$, **** indicate $p<0.0001$.