Plant-derived zein scaffold provides a local source of glutamine for bone repair.

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ABSTRACT INTRODUCTION: More than 500,000 bone grafting procedures are performed each year to treat bone defects, trauma and non-unions. Autogenous bone graft, from the iliac crest graft, remains the ideal choice because it has the three essential bone formation elements, which are osteogenic activity, osteoinductive and osteoconductive properties. However, harvesting autogenous bone is associated with clinical morbidity including pain, prolonged hospitalization, increased infection risk, and surgical complications (i.e., fracture, hematoma, neuroma etc.). Moreover, the quantity of available graft is often suboptimal requiring augmentation with allograft. Bone grafting using allograft is associated with several complications including lack of graft incorporation, delayed union and increased risk of infection. For these and other reasons, an impetus exists to develop alternative technologies that can replicate the performance of iliac crest autograft while eliminating associated morbidity and complications. Here, we have investigated the utility of corn-derived zein as a biomaterial scaffold for bone regeneration. Zein has shown promise as a biomaterial for bone repair, having low immunogenicity, antibacterial properties, and biodegradation. Importantly, zein is rich in glutamine (21-26%) which can become available by degradation of zein via collagenase and other proteolytic enzymes. Glutamine is essential to support skeletal stem cell (SSC) proliferation and osteoblast differentiation during bone homeostasis. Moreover, glutamine is essential for periosteal SSC survival and bone healing in a critical size defect model. Thus, we hypothesized scaffolds made of zein will provide a local source of glutamine to enhance SSC proliferation and osteoblast differentiation and ultimately improve bone healing.

METHODS: Scaffolds were fabricated using 30% (w/w) zein in 80/20 ethanol/water solutions. Solutions were electrospun using standard conditions and crosslinked with 10% trimethylolpropane triglycidyl ether (TMPGE). To determine the effects of zein on bone healing, we generated a 0.7mm cortical defect injury 1.5 mm below the growth plate in 2-month-old mice using a sterile 22G syringe needle. The defect was then loaded with the zein scaffold and subsequently analyzed by x-ray, µCT and histology 3-, 7-, 14-, 21- and 28-days post injury (DPI). *LeprCre;Rosa26$^{tdTomato/+}$* mice were used in which the SSC and their progeny express tdTomato. SSC were directly visualized using confocal microscopy. To test if the zein scaffold increases glutamine availability, we used *LeprCre;Rosa26$^{tdTomato/+}$;Gls$^{fl/fl}$* mice in which the SSC and their progeny are unable to metabolize glutamine due to deletion of glutaminase (GLS). Immunostaining with antibodies recognizing OCN (osteoblasts), Endomucin (vascular endothelial cells), RFP (SSCs and progeny), or F4/80 (macrophage) were used to identify and quantify cells in the zein scaffold. Osteoclasts were quantified using TRAP staining.

RESULTS: The zein scaffold supported rapid ingrowth of SSC as determined by the presence of tdTomato positive cells within the material beginning 3 days post injury (DPI) (Fig.1). Notably, tdTomato positive SSC were enriched on the perimeter of the material and were lacking from the center (Fig.1). By 7DPI, tdTomato positive and negative cells were found throughout the material. Immunofluorescent analyses identified both monocyte and macrophage lineage cells within the tdTomato negative population. EMCN positive endothelial cells, OCN positive osteoblasts and TRAP-stained osteoclasts were identified primarily on the material surface beginning at 7DPI (Fig 2). Consistent with the presence of osteoclasts, we observed an almost complete degradation of the zein material between 14- and 28DPI in wild type mice. Concomitant with this rapid degradation, the zein material was progressively mineralized between 14- and 28DPI as determined by both µCT and picrosirius red staining (Fig.3). *LeprCre;Rosa26$^{tdTomato/+}$;Gls$^{fl/fl}$* mice had significantly less mineralization the zein material at 21DPI (percent bone volume/material volume = 12.9±6.7 vs 7.3±4.1 for wild type and *LeprCre;Gls$^{fl/fl}$* mice respectively, p=0.016 paired *t* test) in. Consistent with decreased bone formation, scaffolds placed in *LeprCre;Gls$^{fl/fl}$* had significantly fewer osteoblasts per material surface compared to the *LeprCre;Gls$^{fl/+}$* (wild type) littermates.

DISCUSSION: Here we present data showing electrospun fibrous zein scaffolds support SSC ingrowth and mineralization. Our data indicates zein likely provides a local source of glutamine to support SSC proliferation and osteoblast differentiation. Moreover, as we have recently discovered a critical role for glutamine metabolism in osteoclast differentiation, zein-derived glutamine may also enhance osteoclastogenesis. This likely enhances degradation of the zein scaffold to increase local glutamine concentrations and further promote SSC invasion, osteoblastogenesis and bone formation.

SIGNIFICANCE: These data support the feasibility of fibrous zein scaffolds for the repair of bone defects. The ability of the fibrous zein scaffold to provide a local source of glutamine represents a novel strategy to increase the abundance of essential metabolites to enhance bone formation and repair.

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IMAGES:

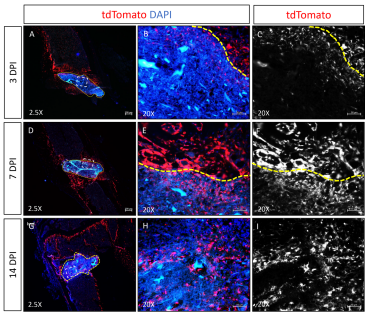

Figure 1: Zein supports SSC ingrowth *in vivo*. (A) Live RFP fluorescence showing of tdTomato positive cells invading the Zein scaffold at 3- (A-C), 7- (D-F), and 14DPI (G-I) shown at 2.5X (A,D,G) or 20X (B-C, E-F, H-I). Yellow square in (A,D,G) shows position of 20X image. Note: Zein scaffold has autofluorescence in the DAPI channel. Yellow dotted line highlights the edge of the material. Images representative of n=4 independent experiments.

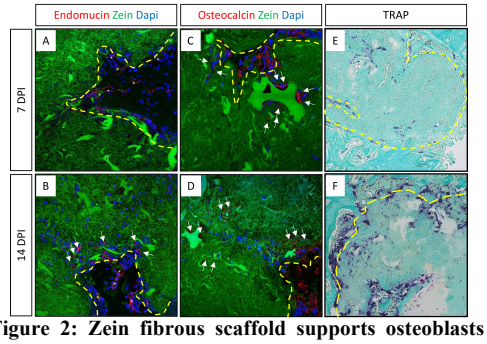

Figure 2: Zein fibrous scaffold supports osteoblasts and osteoclasts *in vivo*. Representative immunofluorescence staining at 7- or 14-DPI showing endomucin positive endothelial cells (A-B) or osteocalcin positive osteoblasts (C-D). Zein autoflourescence shown in green. Yellow dotted line denotes edge of material. Arrows highlight positive cells within the material. TRAP staining (E-F) demonstrates the osteoclasts on the material surface increases between 7- and 14-DPI. Images representative of n=3 independent experiments.

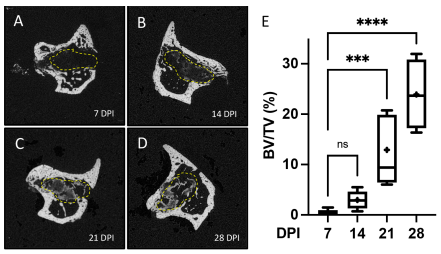

Figure 3: Zein fibrous scaffold support mineralization *in vivo*. Representative µCT images (A-D) through the injury site of wild type male mice. (E) Quantification of the µCT data showing rapid increase in bone volume per total material volume (BV/TV) between 14- and 28-DPI (N≥6). Dashed lines denote zein scaffold. *** p<0.0005, **** p≤0.00005 by ANOVA, ns – not significant. Similar results were observed in female mice.