CRISPR Regulation of Noggin for Growth-Factor-Free Vertebral Endplate Tissue Engineering

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INTRODUCTION: Spatially and temporally controlling cell behavior is a continuing challenge for tissue engineering composites, including a tissue-engineered total intervertebral disc replacement. CRISPR regulation provides an opportunity not only to enhance cell differentiation and matrix deposition absent exogenous stimuli but also to spatially pattern cells with distinct phenotypes for the creation of composite tissues [1]. Implantations of composite tissue-engineered discs in a large animal model demonstrated that acellular vertebral endplate analogs failed to adequately integrate with the adjacent vertebral bodies [2] and may greatly benefit from these CRISPR interventions. CRISPR downregulation of noggin in adipose-derived human mesenchymal stromal cells (hMSCs) resulted in increased mineralized tissue formation both in two- and three-dimensional cultures [1]. ZNF865 has also been identified as a powerful regulator of cellular senescence, cell cycle progression, and protein processing [3] and has previously been shown to enhance chondrogenesis when multiplexed with aggrecan and collagen II upregulation [4]. This marks the first time Noggin downregulation and ZNF865 upregulation have been co-transduced, and we hypothesize that their co-expression will enhance osteogenesis and calcium deposition overall in tissue engineered vertebral endplates.

METHODS: Adipose-derived hMSCs were either naïve or transduced with lentivirus containing the CRISPRi dCas9-KRAB system with guide RNAs. Guide RNAS targeted Noggin downregulation alone or Noggin downregulation in conjunction with ZNF865 upregulation using an EF1α or EFS promoter for ZNF865. Transduced cells were plated in monolayer and given basal, chondrogenic, or osteogenic media for 3 weeks, as previously described [4]. On day 22, wells were stained with 2% alizarin red. Red calcium staining was quantified using a plate reader with absorbance set to 405 nm, and wells were subsequently imaged at 4X on a brightfield microscope. The most osteogenic cell types from this screen will be selected for tissue engineering vertebral endplate replacements and used for future comparisons to naïve hMSCs deployed in a rat subcutaneous model of semi-orthotopic bone formation [5], as approved by IACUC. Salt-leached PCL scaffolds 5 mm in diameter and 5 mm in height were fabricated and hydroxyapatite (HA)-coated using an established biomimetic method [6] and seeded with naïve hMSCs. Seeded scaffolds were precultured in chondrogenic media supplemented with TGF-β3 for 1 day or 7 weeks and subsequently press fit into rings of trabecular bovine bone (10 mm diameter x 5 mm height) harvested from the tibias of 1 month old animals. Bone rings were divided into six experimental groups and filled with (1) nothing (negative control), (2) bone chips (positive control), (3) acellular HA-coated scaffolds (HA), (4) HA-coated scaffolds + 1 day hMSCs (HA+MSCs 1D), or (5) HA-coated scaffolds + 7 week hMSCs (HA+MSCs 7W). Animals were euthanized 8 weeks post-implantation, at which point bone ring+implant constructs were fixed in 10% formalin, microCT scanned, and processed for histology. Paraffin sections were taken from the middle of each construct and stained with Hematoxylin/Eosin, Mallory-Heidenhain trichrome, and red/green/blue (RGB) [7]. Mineralizing area and proteoglycan deposition was quantified within each scaffold via color thresholding of the Mallory-Heidehnain and RGB stains, respectively.

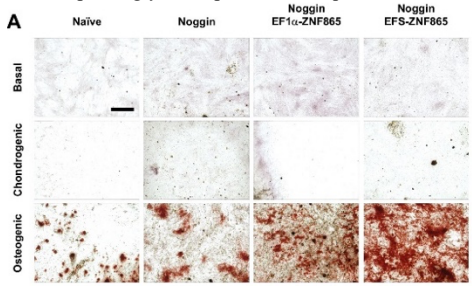

Figure 1. CRISPR Osteogenesis Screen. Alizarin red (A) staining (scale = 1 mm) and (B) quantification of naïve or CRISPR-edited hMSCs given basal, chondrogenic, or osteogenic media over 3 weeks.

RESULTS: Noggin-downregulated/ZNF865 upregulated cells, regardless of ZNF865 promoter, produced significantly more calcium than naïve hMSCs (Figure 1A-B) in osteogenic media. None of the cell types tested produced calcium when given basal or chondrogenic media. When implanted in vivo (Figure 2A), naïve hMSCs precultured for 7 weeks deposited observable volumes of new mineral in all eight HA scaffolds, whereas naïve hMSCs precultured for 1 day deposited observable volumes of new mineral only in the best performing HA scaffolds (Figure 2B). Cells secreted areas of mineralizing matrix in all HA groups (Figure 3B, dark purple staining), although mineralizing matrix was diffuse on acellular HA scaffolds. Mineralized matrix (pink staining) was found in 28.5% of acellular HA scaffolds, 62.5% of 1D HA scaffolds, and 75% of 7W HA scaffolds. This increase in mineralized matrix production within precellularized implants may be attributed to the increased number of cells found in these groups, particularly in the 7W group (Figure 2D). The 7W group was the only experimental group to experience significant increases in overall mineral volume when compared to the negative control (Figure 2E). However, the level of mineral deposition was heterogeneous among 7W samples, and more consistent in 1D samples (Figure 2F).

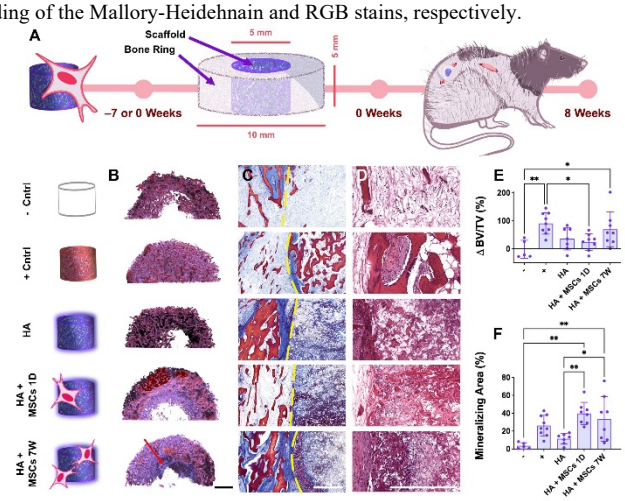

Figure 2. In Vivo Subcutaneous Screen of Naïve Human MSCs. (A) Study design. After 8 weeks in vivo (n = 5-9): (B) MicroCT 3D renderings of bone rings and their implants in which each construct is cut in half; newly deposited bone appears light purple and is indicated with red arrows in the implant space (scale = 2.0 mm). (C) Mallory-Heidenhain (left: bone ring; right: implant; boundary demarcated by yellow dashed line; scale = 0.5 mm) and (D) Hematoxylin/Eosin staining at the bone ring-implant boundary (scale = 0.5 mm). (E) Percent change in BV/TV of implants plus their bone rings between 0 and 8 weeks. (F) Quantification of mineralizing bone area within each implant from Mallory-Heidenhain-stained sections at 8 weeks.

DISCUSSION: ZNF865 upregulation with Noggin downregulation enhanced hMSC osteogenesis in vitro. Although the results were heterogeneous, 24 hours of preculture in chondrogenic media was insufficient to induce significant deposition of mineralized bone from naïve hMSCs in vivo over 8 weeks. Pre-culture of naïve hMSCs on HA PCL scaffolds for 7 weeks contributed to mineralized bone formation in vivo in all implants tested, even the lowest performing samples. Previous work using bovine MSCs showed that this HA coating drove osteogenesis in vitro via substrate stiffness, as measured by microindentation, alone [6]. This work indicates that in addition to the HA-coated substrates, increased MSC preculture duration improves eventual osteogenic differentiation, even though these cell-seeded scaffolds were cultured in chondrogenic media. Future work will involve characterization of matrix deposition and cell morphology in precultured scaffolds immediately before in vivo implantation, as well as additional assays on in vivo samples to verify the level of hMSC proliferation, osteogenic differentiation, and vessel ingrowth. We will then determine if Noggin-downregulated/ZNF865 upregulated cells can further enhance and accelerate in vivo bone formation in HA PCL endplate scaffolds.

SIGNIFICANCE: Tissue engineering of biomimetic vertebral endplates better enables potential biomaterial therapies for end-stage intervertebral disc degeneration and provides an opportunity to simulate composite tissues in vitro for the study of disc-cartilage-bone crosstalk in a highly controllable manner.

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