CRISPRa of Novel Gene, ZNF865, Amplifies Cartilage Deposition in Engineered Discs

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INTRODUCTION: CRISPR-activation (CRISPRa) screens have led to our lab's discovery of a previously unknown function for a zinc finger protein, ZNF865. ZNF865 appears to regulate senescence, cell activity, and protein processing with the potential to be used as a novel cell engineering tool. Disc-like angle ply structures (DAPS) are tissue engineered total IVD replacements that have shown promise as therapeutic options to treat severe cases of degenerative disc disease. Here we are testing ZNF865's ability to amplify cell phenotype with targeted gene expression modulation using CRISPRa. Utilizing synergistic CRISPRa upregulation of aggrecan (ACAN), collagen-II (Col2), and ZNF865 in ASCs, we hypothesize that we can amplify cartilaginous ECM deposition in DAPS without the use of growth factors.

METHODS: ACAN/Col2-NTC and ZNF865 upregulated human adipose-derived stem cells (ASCs) were pellet cultured for 21-days after which pellets were harvested, fixed, and submitted for histology (n=5), or designated for biochemical analysis (n=10). ZNF865-edited ASCs were evaluated for increased rates of proliferation and RNA was isolated from ACAN/Col2-ZNF865 and ACAN/Col2-NTC-edited ASCs for RNA-sequencing. For DAPS seeding, ACAN/Col2-NTC and -ZNF865 edited cells were seeded onto electrospun polycaprolactone (PCL) annulus fibrosus and agarose nucleus pulposus scaffolds and cultured for 2.5 weeks prior to combining scaffolds and culturing for an additional 2.5 weeks (**Figure 1J**, n=6-9). After 5 total weeks of culture DAPS were harvested, fixed, and processed for histology. A one-way ANOVA with a Tukey's *post hoc* analysis was used to determine statistical significance.

RESULTS: ZNF865-edited ASCs show increased tissue (**Figure 1A**) and proteoglycan deposition (**Figure 1B**) in pellet culture (n=5). ZNF865-edited ASCs show increased rates of proliferation compared to control cells (**Figure 1C**, n=4), decreasing the overall doubling time of ASCs from 41-hours to 31-hours. ZNF865-edited pellets show significant increases in collagen deposition, 13.8µg compared to 6.4µg (**Figure 1D**), collagen retention, 35.8% compared to 22.3% (**Figure 1E**), sGAG deposition, 2.3µg compared to 1.0µg (**Figure 1F**), and sGAG retention, 22.6% compared to 13.7% (**Figure 1G**), compared to the -NTC (n=10). Hoechst analysis shows no significant difference in DNA content between pellets, with 5.2µg of DNA in ZNF865-edited pellets compared to 4.4µg in NTC-edited pellets (**Figure 1H**, n=10). Gene ontology analysis of RNAseq data shows ZNF865 highly regulates RNA and protein processing (**Figure 1I**). ZNF865-edited ASCs produced 9X and 3X more DAPS compared to the naïve control cells and ACAN/Col2-NTC cells, respectively (**Figure 1K**). ZNF865-edited DAPS showed increased deposition of collagen and proteoglycan deposition in NP and AF regions of DAPS (**Figure 1L/M**) and combination staining shows dramatically darker staining in ZNF865-edited ASCs compared to ACAN/Col2-NTC-edited ASCs (**Figure 1N/O**).

DISCUSSION: This study provides evidence for ZNF865 as a tool to amplify cell phenotype and improve tissue engineering strategies treating DDD. ZNF865 is a powerful regulator of protein processing and RNA transport that can be used to enhance cell and tissue engineering strategies treating DDD. By increasing proliferation rates of ASCs, we can decrease the expansion rate of ASCs and improve the functional maturation rate of DAPS. Further, ZNF865 enhances the protein processing and regenerative capacity of ASCs by increasing the protein processing rate and capacity in individual cells. The targeted multiplex upregulation of ACAN/Col2/ZNF865 significantly increases cartilaginous ECM deposition in pellet cultures and medium-sized DAPS compared to ACAN/Col2 upregulation and ASCs dosed with growth factors. Our results indicate that synergistic upregulation of ACAN/Col2/ZNF865 drastically increases cartilage deposition in DAPS without growth factors and showcases the potential of ZNF865 as a tool to amplify cell phenotype and ECM deposition.

SIGNIFICANCE/CLINICAL RELEVANCE: The identification and targeted upregulation of ZNF865 displays profound effects on cell phenotype and the continued development of ZNF865 as a cell and tissue engineering tool could dramatically improve strategies treating musculoskeletal disease.

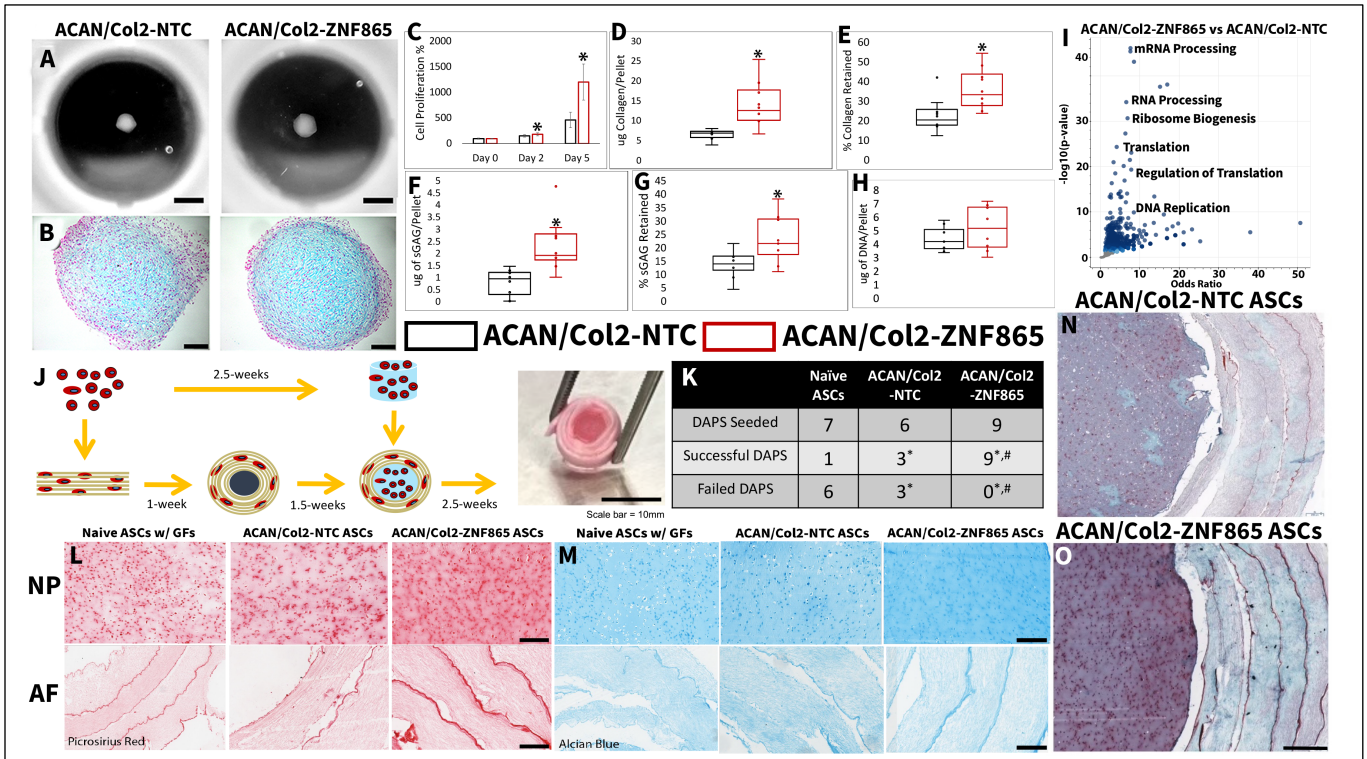

Figure 1: (A) gross pellet morphology (scale bar=1mm) and (B) proteoglycan deposition between groups (scale bar=100µm). (C) ZNF865-edited ASCs show increased rates of proliferation compared to -NTC ASCs (*=p<0.05, n=4). Biochemical analysis shows significant increases in (D) collagen deposition, (E) collagen retention, (F) proteoglycan deposition, (G) proteoglycan retention, and (H) no significant difference in DNA content (*=p<0.05, n=10). (I) GO Biological Process shows the top processes affected due to ZNF865 upregulation. (J) Schematic of the seeding and culture process of DAPS over 5-weeks. (K) ZNF865-edited ASCs seeded 9 DAPS with all 9 DAPS being successful compared to only 3 and 1 successful DAPS with the other groups (*,#=p<0.05, *=compared to naïve ASCs, #=compared to ACAN/Col2-NTC). (L) Pircosirius red and (M) Alcian blue staining shows collagen and proteoglycan deposition in NP and AF regions of DAPS, respectively (scale bar=200µm). A combination picrosirius red and Alcian blue stain shows the differences in collagen and proteoglycan deposition in (N) ACAN/Col2-NTC-edited ASCs compared to (O) ACAN/Col2-ZNF865-edited ASCs (scale bar=500µm).