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Functional impact of alternative splicing on the transcriptomic landscape and fate of multipotent skeletal stem cells and osteosarcoma

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Functional impact of alternative splicing on the transcriptomic landscape and fate of multipotent skeletal stem cells and osteosarcoma

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Background

Skeletal Stem Cells (**SSCs**) are the progenitors of the skeletal system, giving rise to **bone**, **cartilage**, and **stroma**.[1,2] These populations are defined by their functional output both in-vitro and in-vivo, their ability to self-proliferate, as well as their molecular profile (Fig. 1).

Insights into aging and cancer of the skeletal system have revealed fundamental **changes to the transcriptomic landscape** of SSCs throughout life that govern their ability to function as healthy stem cells.[3] Thus, we explore the significance of **alternative splicing** (**AS**) in the skeletal system.

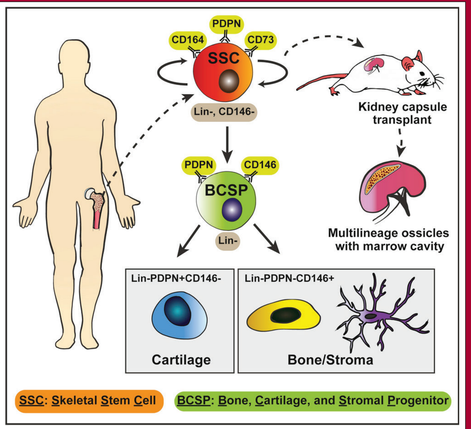

Figure 1: ***Prospective isolation of human SSCs.*** SSCs can be extracted from human bone and sorted using the above surface marker profile by fluorescence-assisted flow cytometry (FACS). These cells have been shown to reconstitute the skeletal niche in mice and display differentiation potential towards bone, cartilage, and stromal cells.

In humans, **>90% of protein-coding genes** undergo post-translational AS.[4] Through rearrangement of functional domains prior to translation, AS allows a single gene to encode a variety of proteins that may function in varying degrees of similarity or differ entirely in their activity. Recent advances in our understanding of **human SSCs** (**hSSCs**) and the skeletal niche have demonstrated the **need to examine AS** as it relates to **skeletal development**, **aging**, **loss of skeletal regenerative capacity** and **skewing of hSSCs** towards non-skeletogenic lineage fates. Moreover, mounting evidence that the progression of **osteosarcoma** corresponds with aberrations in the AS machinery, producing cellular phenotypes that result in malignant tumors of the bone.

Objectives

In the present study, we aimed to characterize the relationship between AS and maintenance of hSSCs, the skeletal niche, and osteosarcoma by identifying **RNA-binding proteins** (**RBPs**) involved in cell maintenance and differentiation. We evaluate the effect of **inhibiting these proteins** on cell viability and differentiation potential.

Methods

Bones are provided by our partners in Stanford department of Surgery. hSSCs are extracted from human bone and acute fracture-driven callus formation and mechanically digested (Fig 2A).

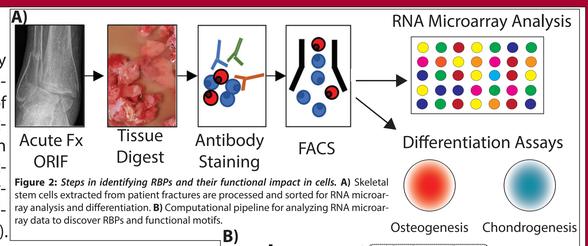

Figure 2: ***Steps in identifying RBPs and their functional impact in cells.*** **A)** Skeletal stem cells extracted from patient fractures are processed and sorted for RNA microarray analysis and differentiation. **B)** Computational pipeline for analyzing RNA microarray data to discover RBPs and functional motifs.

Cells are filtered and stained for surface markers (**PDPN+; CD164+; CD73+; Lin-; CD146-**) and sorted by FACS. hSSCs are lysed and processed for RNA microarray analysis or plated for in-vitro assays. Osteosarcoma cells U2OS and SAOS2 were obtained from ATCC.

Large-scale RNA-seq data is processed using replicate **Multivariate Analysis of Transcript Splicing** (**rMATS**) statistical model and its latest software implementation rMATS-turbo to **discover and quantify alternative splicing**.[5,6] We developed a computational approach to prioritize RBPs that might orchestrate AS using time series data over a 12-day time-course of **induced osteogenesis** cell culture (Fig 2B).

Results

Among the 26,000 genes detected using our computational pipeline, we discovered **eleven candidate RNA binding motifs** from **nine RBPs** in or near correlated events of transcriptional splicing (Fig. 3A).

The RBPs CPEB2, KHDRBS3, and PCBP3 demonstrated **increased expression** during the osteogenic differentiation time course (Fig 3B). **Knockdown** of KHDRBS3 and CPEB2 by small interfering RNA during osteogenesis **inhibited formation of bone** in-vitro (data not shown).

Evaluation of GEXC microarray data demonstrated differential expression of the RBPs **SF3B6** and **SF3B1** -- subunits of the human U2 spliceosome complex -- between hSSCs and osteosarcoma cells (Fig. 4A). Similarly expression of these RBPs **increases with age** in mouse tissues (Fig 4B).

Inhibition of SF3B6 and SF3B1 by small molecule inhibitors **prevents the formation of bone** in-vitro and **reduces cell viability** by up to roughly 30% (Fig. 5A). Proliferation and differentiation to cartilage is also **inhibited** in-vitro with treatment of SF3B6 and SF3B1 inhibitors for both male and female hSSCs (Fig. 5B).

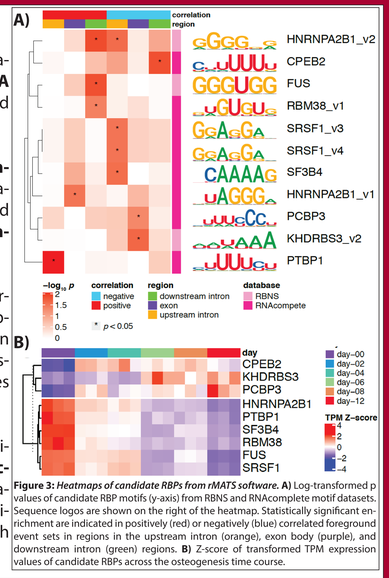

Figure 3: ***Heatmaps of candidate RBPs from rMATS software.*** **A)** Log-transformed p values of candidate RBP motifs (y-axis) from RBNS and RNAcomplete motif datasets. Sequence logos are shown on the right of the heatmap. Statistically significant enrichment are indicated in positively (red) or negatively (blue) correlated foreground event sets in regions in the upstream intron (orange), exon body (purple), and downstream intron (green) regions. **B)** Z-score of transformed TPM expression values of candidate RBPs across the osteogenesis time course.

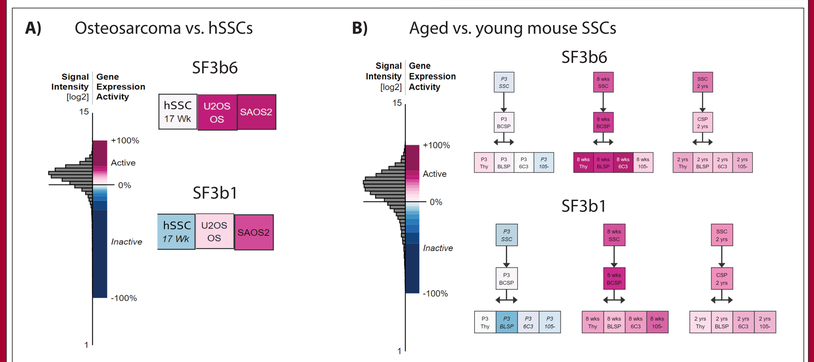

Figure 4: ***Gene expression commons microarray data from human and mouse SSCs and osteosarcoma cells.*** **A)** Comparison of SF3B6 and SF3B1 expression between 17 week old hSSCs and osteosarcoma cell lines U2OS and SAOS2 shows elevated expression of these RBPs in osteosarcoma but lower in basal hSSCs. **B)** Age comparison of SF3B6 and SF3B1 expression between mouse SSCs aged P3, 8 weeks, and 2 years apart shows low expression at P3, increased expression at 8 weeks, then tapered expression at 2 years.

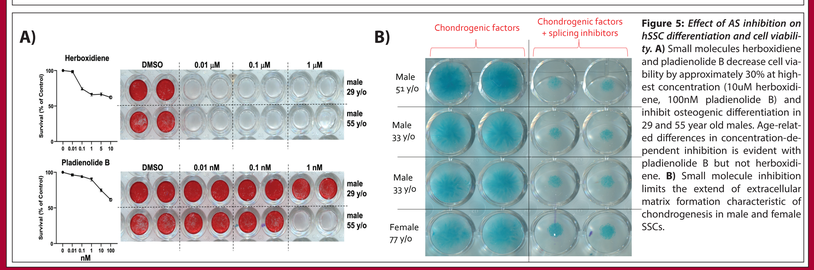

Figure 5: ***Effect of AS inhibition on hSSC differentiation and cell viability.*** **A)** Small molecules herboxidiene and pladienolide B decrease cell viability by approximately 30% at highest concentration (10uM herboxidiene, 100nM pladienolide B) and inhibit osteogenic differentiation in 29 and 55 year old males. Age-related differences in concentration-dependent inhibition is evident with pladienolide B but not herboxidiene. **B)** Small molecule inhibition limits the extend of extracellular matrix formation characteristic of chondrogenesis in male and female SSCs.

Discussion

Our gene expression analysis and rMATS modeling demonstrate the prevalence of AS in hSSCs and osteosarcoma. The **importance of AS** in hSSC maintenance and differentiation is demonstrated by a **lack of characteristic lineage differentiation** of SSCs and by decrease in cell number and viability.

We show osteogenic-related RBP expression increasing during osteogenesis, and knockdown of these RBPs prevents formation of bone-forming lineages from SSCs. These results indicate a **potential target** for bone deficiency disorders such as osteoporosis, osteoarthritis, and age-related bone degeneration.

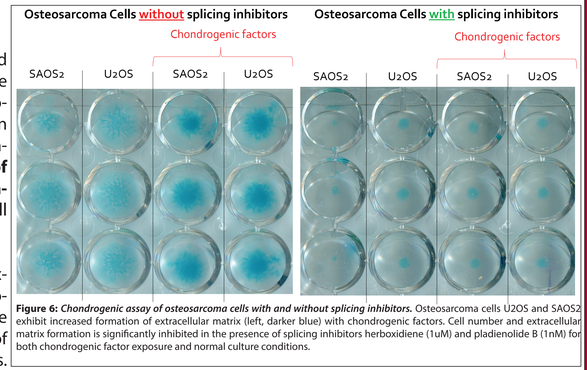

Figure 6: ***Chondrogenic assay of osteosarcoma cells with and without splicing inhibitors.*** Osteosarcoma cells U2OS and SAOS2 exhibit increased formation of extracellular matrix (left, darker blue) with chondrogenic factors. Cell number and extracellular matrix formation is significantly inhibited in the presence of splicing inhibitors herboxidiene (1uM) and pladienolide B (1nM) for both chondrogenic factor exposure and normal culture conditions.

Inhibition of AS may also be a potential treatment of **osteosarcoma** as demonstrated by chondrogenic differentiation assay (Fig. 6). Both U2OS and SAOS2 cell lines showed **inhibited** extracellular matrix formation and diminished cell viability using small-molecule inhibition of AS. These results indicate the potential therapeutic targets for treatment of intractable bone cancers such as osteosarcoma.

The importance of AS in hSSCs is made clear by our study, but more work must be done to further reveal how the transcriptional landscape of hSSCs changes during various cell stages. Critically, **age differences** in the expression of RBPs are observed and may play a larger role in the features of skeletal aging, such as skewed lineage differentiation and limits to hSSC proliferation. Significant **sex differences were not observed** during our studies, but more patient samples must be analyzed to determine whether sexual dimorphisms of the hSSC transcriptional landscape occur.

Future Directions

We aim to further investigate the **transcriptional targets** of RBPs involved in hSSC differentiation, and to determine whether overexpression of these RBPs may instigate greater hSSC differentiation/proliferation.

We plan for **in-vivo analysis** of RBP expression using rMATS through renal capsule transplantation, and will assess RNA expression at various time points (Fig.7). Knockdown or overexpression of RBPs in engrafted SSCs may provide insight into the potential to **upregulate bone and cartilage** formation in the mouse renal capsule with translation applications to human autologous therapy.

We plan to investigate the potential therapeutic capacity of AS inhibition in **osteosarcoma** by local versus systemic administration of small molecules in osteosarcoma-engrafted mice. Such results may offer an avenue for treatment of intractable bone diseases and skeletal degeneration due to over-abundance of bone-resorbing cells.

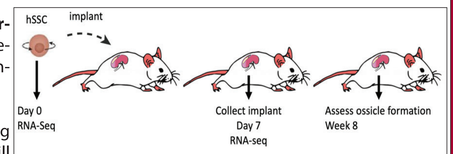

Figure 1: ***Plan for in-vivo engraftment of hSSCs onto the mouse kidney capsule for RNA-seq analysis.*** hSSCs will be collected and analysed for RNA expression at day 0, 7, and week 8 post-engraftment timepoints for evaluation of RBP expression and motif enrichment.

Acknowledgments

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