Machine Learning Image Classification of Cell State Morphologies to Understand Mechanisms in the Effects of Salubrinal and ISRIB on Aging and Differentiation

Samiha Mahin[1], Hanzhi Zhang[1], Alexander Brown[1], Andrew Brack, Brian Feeley[1,2]
[1]University of California San Francisco Department of Orthopaedic Surgery, [2]San Francisco Veterans Affairs Health Care System
samihamahin@berkeley.edu

DISCLOSURES: Andrew Brack (ARPA-H)

INTRODUCTION: Skeletal muscle regeneration relies on the coordinated activity of muscle stem cells (MuSCs) and fibro/adipogenic progenitors (FAPs). MuSCs drive myogenic repair, whereas dysregulated FAPs contribute to fibrosis, a major barrier to functional recovery following injury and during aging-related muscle decline. Building on our previous single-cell sequencing data, we reveal a dual role of the integrated stress response (ISR): activation rejuvenates aged MuSC metabolism, while inhibition limits FAP-mediated fibrogenesis. We hypothesize that applying machine learning binary classification algorithms can robustly distinguish ISR-mediated effects on MuSC and FAP morphology and organelle features, thereby validating our methodology as a means to capture functionally relevant changes in rejuvenation capacity and fibrotic potential.These insights advance our understanding of regenerative mechanisms and may inform therapeutic strategies to restore muscle function and reduce fibrosis in age-related and injury-induced muscle disorders.

METHODS: MuSCs were isolated from adult (4 month) and aged (22 month) mice by Floursescent Activated Cell Sorting and plated in extracellular matrix coated plate in growth media with Salubrinal003 or DMSO added and kept for an 18 hour activation period (**Fig 1A**). FAPs were isolated from adult mice and seeded in extracellular matrix plates for 24 hours. The treatments DMSO, ISRIB or fibrogenic differentiation factor TGFβ1 were then added for 24 hours and stained (**Fig 2A**). Both cell types followed the same staining, imaging, and data analysis protocols. The following stains were used live mitochondrial stain (PhenoVue 641), nucleic stain (PhenoVue Hoecsht 3342), cytoplasmic stain (PhenoVue 568-Phalloidin), and endplasmic reticulum (ER) stain (PhenoVue 488-Conaclavin A). Imaging followed with Opera Phenix High Content Screening and individual cell organelle morphology data (i.e. standard area, roundness, width, length, texture) was extracted using Cell Harmony software. Plates were normalized using pyctominer's normalize function with parameter of standardize. Using scikit-learn's binary classification models, classifiers were trained on 75% and tested on 25% of the dataset. Models were evaluated on their ability to accurately classify MuSCs as adult or aged, and FAPs as differentiated or non-differentiated. Models were ranked by AUC scores and top three models were then applied to treated cells (Sal003 or ISRIB) to classify MuSCs as adult or aged, and FAPs as differentiated or non-differentiated. Key features driving classification were compared between control and treatment groups. All protocols have been approved by IACUC.

RESULTS: Cell distribution between treatment and control groups were roughly equal for both MuSCs and FAPs (**Fig 1B & Fig 2B**). For MuSCs, the top three binary classifiers with the highest AUC scores were Random Forest, XGBoost, and MLP (**Fig 1C**). Since an AUC of 0.5 indicates random classification, the observed scores of 0.752, 0.741, and 0.732 suggest that these models can distinguish adult from aged cells with approximately 70% accuracy, indicating they capture morphological features associated with cell age.When given aged cells treated with Sal to top performing classifiers, the models had categorized 60-75% of cells as adult rather than aged (**Fig 1D**). Feature importance analysis revealed the key characteristics used for classification, "Nucleus intensity mean" and "Actin intensity mean" consistently ranked as top features across all models (**Fig 1E**). Nucleus intensity mean is the average intensity given off by a cell's nucleus, a high value can be indicative of higher DNA content which occur in later cell cycle phases. Actin intensity mean is the average intensity given off by a cell's F-actin structures in the cytoskeleton, a low value can be indicative of a compromised cell structure. For FAPs, the top three binary classifiers with the highest AUC scores were Logistic Regression Model (0.942), XGBoost (0.940), and Random Forest (0.937) (**Fig 2C**), indicating strong performance in distinguishing differentiated from non-differentiated cells. When applied to ISRIB- and TGFβ1-treated cells, the models classified over 50% as non-differentiated (**Fig 2D**), suggesting that ISRIB shifts a significant portion of the population toward a phenotypically less differentiated state. Feature importance analysis identified "Actin intensity sum" and "Mito intensity sum" as key contributors, with ISRIB treatment appearing to restore these features toward levels seen in non-differentiated FAPs (**Fig 2E**).

DISCUSSION: Model classification results (**Fig 1D**) indicate that Sal treatment restores the morphology of aged MuSCs toward an adult-like state. This shift is primarily driven by changes in nuclear and cytoskeletal features, which the models identified as key determinants for classification. Specifically, Sal-treated aged cells exhibit increased nuclear intensity compared to untreated aged cells, approaching levels observed in adult cells—suggesting enhanced cell cycle activity. This morphological finding aligns with previous transcriptomic data showing upregulation of CCND1, a marker of cell cycle progression, in Sal-treated aged cells. Furthermore, Sal-treated aged cells display both higher average nuclear DNA content and greater overall DNA intensity than untreated aged cells, indicating a heightened metabolic and proliferative state. This is supported by elevated expression of Nduf genes involved in mitochondrial respiration. Cytoskeletal features also appear to be restored or even enhanced beyond adult levels, consistent with gene ontology analysis showing increased expression of genes related to cytoskeleton organization in Sal-treated aged cells compared to controls. For FAPs, classification models predicted that a majority of ISRIB-treated cells were non-differentiated, despite co-treatment with TGFβ1 (**Fig 2D**). This suggests that ISRIB alters cell morphology to resemble a non-differentiated state. Feature analysis indicates this reversion due to restoration of cytoskeleton organization and mitochondrial function.

SIGNIFICANCE: Using binary classification of cell painting morphological and organelle-level features, we demonstrate that machine learning can detect treatment-induced shifts in cell state, such as Sal-treated aged cells classified as adult and ISRIB-treated differentiated cells classified as non-differentiated. Interpreting these classifier decisions reveals compound effects that are otherwise difficult to detect by manual inspection **(Fig 1F & Fig 2F)** and by linking morphological profiling to functional outcomes, this approach provides a powerful platform to identify therapeutic targets for restoring muscle regeneration and preventing fibrosis in aging-related and injury-induced muscle disorders.

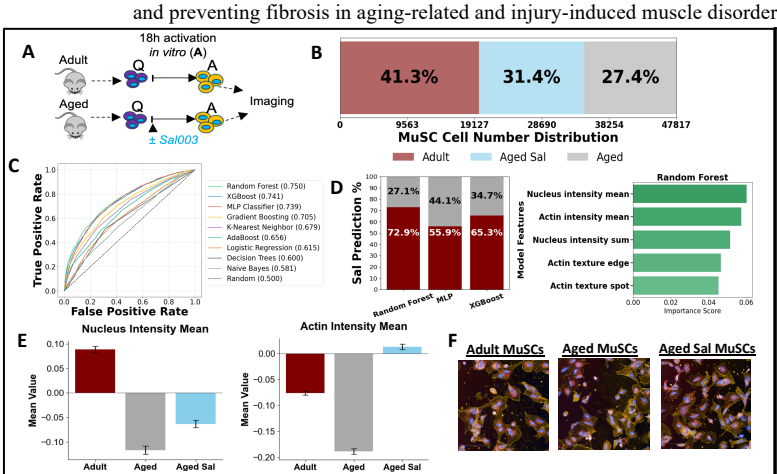

Figure 1. Cell Painting of Aged Sal MuSCs show a rejuvenation to adult-like state through restoration of Nucleus and Actin Behavior (A) Experimental workflow of seeding and dosing (B) MuSC cell numbers show even distribution between groups (C) AUC Score testing show binary classifiers perform better than random (D) Classfication of Sal treated aged cells as adult and top classifiying features from top performing classifier (E) Top two features show rejuvenation to adult levels (F) Cell painting images of stains are difficult to discern manually.

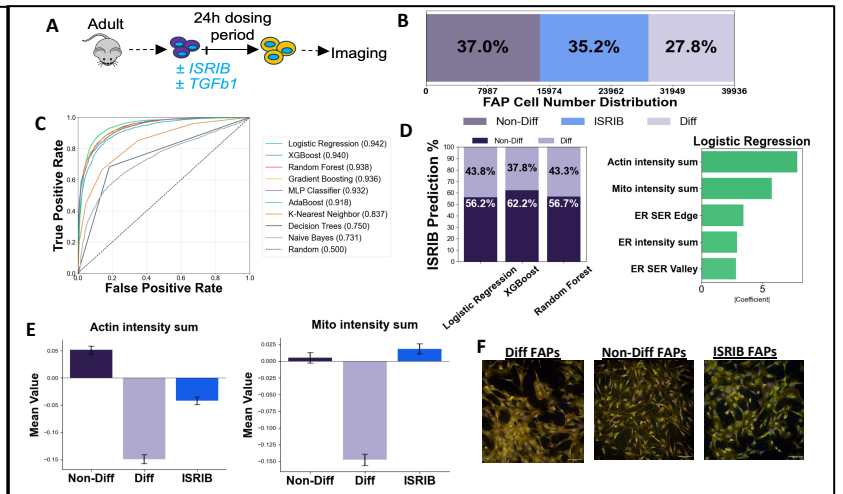

Figure 2. Cell Painting of ISRIB FAPs show a return to non-differentiated state through restoration of Actin and Mitochondrial Behavior (A) Experimental workflow of seeding and dosing (B) FAP cell numbers show even distribution between groups (C) AUC Score testing show binary classifiers perform better than random (D) Classfication of ISRIB treated differentiated cells as non-differentiated and top classifiying features from top performing classifier (E) Top two features show return to undifferentiated levels (F) Cell painting images of stains are difficult to discern by manually.