SPECIFIC AIMS

Mesenchymal stem cell (MSC) therapies are starting to reshape regenerative medicine, with over 400 clinical trials underway [1]. Despite the promise of MSCs, their autologous transplantation in the elderly remains a challenge, due to the loss of functionality that occurs in MSCs during aging [2, 3], and progressive stem cell senescence with time in culture [4]. Although the ability to isolate and delineate functionally competent cells from patient-derived MSCs is critical, current approaches used for assessing the identity and quality of MSCs are *time-consuming, **terminal and destructive,*** and as such, cannot be used prior to cell transplantation. Therefore, rapid strategies are needed for quality control assays that can preserve MSC viability, and that can concentrate highly viable subpopulations of MSCs harvested from aged donors. Therefore, ***there is a critical unmet need for non-destructive profiling of MSC functional potency and viability, which will accelerate the isolation of cell subsets that can be used for transplantation***.

The major innovation in this R21 lies in creating a next-generation high-content image informatics (HCII) method that will be able to examine bright-field images of live cells in vitro, *and report on the quality of the cells for use in regenerative medicine, at the single-cell level* (Figure 1). Our lab previously advanced a high content imaging methodology that required staining to forecast the differentiation fate of MSCs. This work highlights the fact that cell attributes are encoded in microscopic features of cells and can be parsed computationally. The HCII method analyzed the texture of fluorescently stained intracellular proteins [5]. Despite its promise, the method is an endpoint assay, and thus cannot be implemented to assist in transplantation. Thus, we propose a new paradigm for non-destructive, live-cell analysis based on advanced HCII methods. The workflow involves creating an image dataset for "deep learning", comprised of paired brightfield and fluorescently labeled images of MSCs. The latter images are the basis of the sub-phenotype labels for the former images, which are used to train a custom-built convolutional neural network (CNN). Hence, the CNN learns how to classify cells based on bright-field images alone, enabling a live-cell characterization method. The hypothesis of this R21 will be focused on the important goal of elucidating "aging biology", by classifying cells from elderly donors for MSC identity, differentiation potential, proliferative capacity, and senescence.

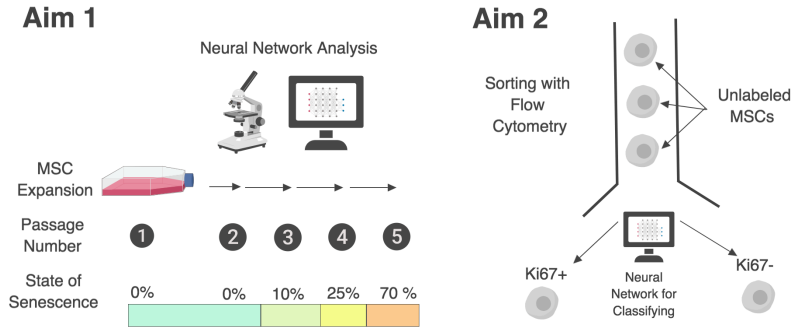

Figure 1: Quality control methods for MSCs are time-consuming and material intensive. HCII approaches can be advanced to develop a rapid, live-cell method for improved quality control and viable cell sorting. A neural network (NN) will be trained to evaluate properties of MSCs at relevant passage numbers using brightfield images (Aim 1). The population can also be enriched for traits for therapeutic applications to enhance performance. The MSC population will be analyzed with a custom imaging flow cytometry device and sorted to enrich MSCs of interest (Aim 2). The cellular marker for proliferation, Ki67, is shown as an example attribute of MSCs sorted by unlabeled, brightfield images alone. Sorting occurs after training with brightfield images that are labeled according to accompanying fluorescent images.

Beyond the ability to elucidate phenotypic differences, a key gap remains, namely the parallel ability to utilize this information to isolate stem cells with the most functional phenotypes prior to transplantation. With the onset of aging, senescent cells accumulate throughout different tissues in the body [6]; senescence in stem cells also impairs their proliferation and differentiation *in vitro* [7]. For this reason, we also propose to utilize a label-free HCII model to improve on the efficacy of MSCs obtained from elderly donors by "enriching" a less senescent cell population [8] (Figure 1). We have designed two specific aims first to characterize MSCs and then to generate highly enriched, functional MSC populations for therapeutic applications:

AIM 1: Can a convolutional neural network (CNN), based on live-cell, imaging-based assay, provide a multifactorial report on an MSC's overall phenotype and viability?

We will create a CNN to analyze brightfield images of MSCs, labeled during training by their fluorescent marker identity. *Outcome:* A high-throughput method will be developed to assess the quality and identity of MSCs, while preserving cell viability.

AIM 2: Will an MSC enriching platform, based on live-cell sorting with imaging flow cytometry, improve the regenerative potential of a donor cell population, enabling better precision medicine?

We will test whether removing detrimental cells improves cell proliferation and differentiation. *Outcome*: An MSC enrichment method will be developed which will help rescue the "aged phenotype" and additional traits of cell functionality.

We aim to build a cost- and time-efficient platform that will integrate the assessment of these crucial MSC characteristics into a single HCII workflow. A single image will provide a measure of the fitness of the cells at a highly granular level for use in transplantation, for example.

SIGNIFICANCE

Barriers to Mesenchymal Stem Cell Quality Control: While MSCs show great promise in cellular regenerative medicine, clonal heterogeneity is seen as a major barrier to translating stem cell therapies clinically [9]. Reliable quality control and standardized manufacturing are required in stem cell production [10]. Furthermore, stem cells' performance and differentiation potential vary based on age, donor condition, and conditions of tissue collection and cell culture[11].

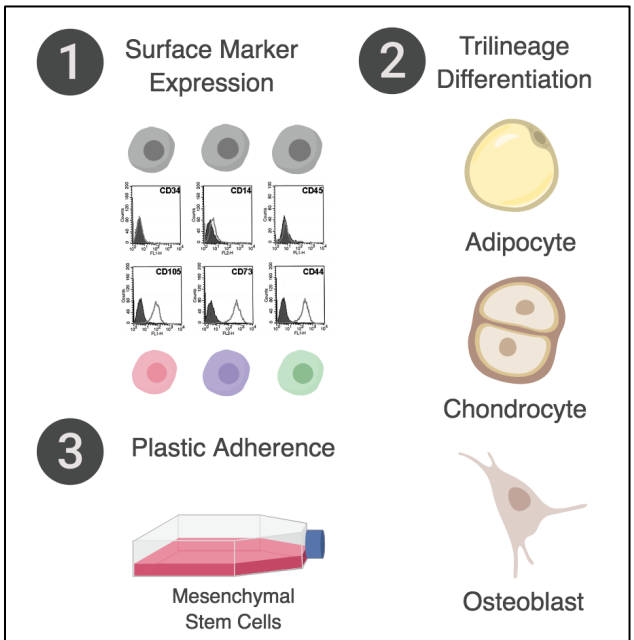

Figure 2: MSCs are validated by their (1) display of particular surface markers (color) or lack thereof (gray), (2) differentiation ability, and (3) plastic adherence.

Current quality control methods for MSCs use endpoint assays that are time-consuming and require expensive reagents. The typical panel to validate MSCs is multifaceted (Figure 2): it includes positive marker expression (CD105, CD73 and CD90); and negative marker expression (CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR) of specific surface antigens; plastic adherence under standard culture conditions; trilineage mesenchymal differentiation (into adipocytes, chondroblasts, and osteoblasts); [12]. Importantly, while these validation assays unmask the identity of the MSCs, they do not inform on their quality at a single cell level. This enables sorting a population of MSCs for desirable traits rather than assessing the quality of a batch of cells. After training with labels derived from accompanying fluorescence images, the CNN in this work will recognize the identity and classify the attributes of new MSCs from brightfield images alone (Figure 3).

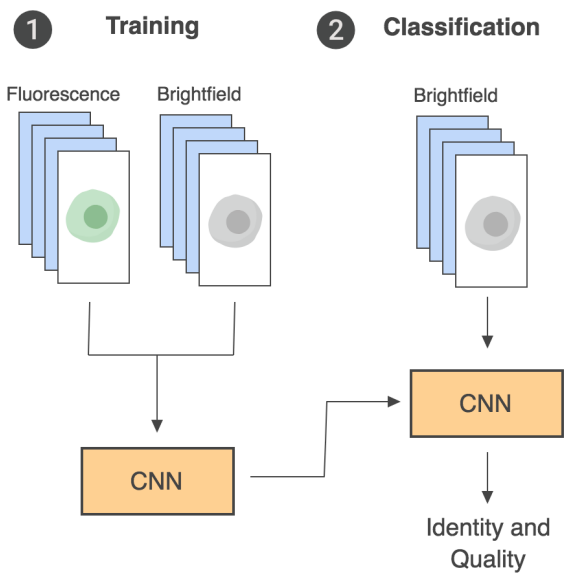

Figure 3: The workflow for training the CNN requires fluorescence images to detect attributes of MSCs, which are paired with the brightfield images as input to the CNN. Brightfield images alone are then classified by the trained CNN.

Enrichment from an Aged Population: Aging and senescence affect MSC traits, such as differentiation and proliferative capacity, yet, for clinical trial transplantation, large-scale expansion of MSCs is needed (one to two million MSCs per kilogram bodyweight) [13]. Undesirable medical outcomes of aged stem cell transplantation is due to their decreased therapeutic potential and regenerative capacity. This is problematic in intravenous delivery of MSCs, for instance, for even a small increase in passage number correlates negatively with patient response and survival [14]. It is therefore important to assess the age or senescence of MSCs before their therapeutic application.

In particular, the enrichment of MSC populations for non-senescent cells could improve clinical responses to autologous transplantation in the elderly. Briefly, senescent cells release chemical factors which disrupt the structure and function of tissue, affecting cell growth and migration, blood vessel formation, and differentiation [15]. The senescence-associated secretory phenotype (SASP) presented by senescent cells is physiologically detrimental, affecting tissue and organ

functionality, as well as organismal aging [16]. There are numerous senescent cells among elderly MSCs, and they display SASP, contributing to inflammation and diminishing MSC function [17]. As a result, autologous stem cells in the elderly can contribute to age-related degeneration [17]. Beyond degenerative potential, senescent cells also carry the risk of promoting cancer progression [15, 18]. In mouse xenografts, malignant progression of cancer cells that are either precancerous or established is promoted by senescent cells [15]. Also, SASP factors stimulate phenotypes that are connected with aggressive cancer cells, and other age-related pathologies can be driven by cellular senescence [15]. Thus, removing senescent cells could improve the long-term health of patients undergoing MSC therapies.

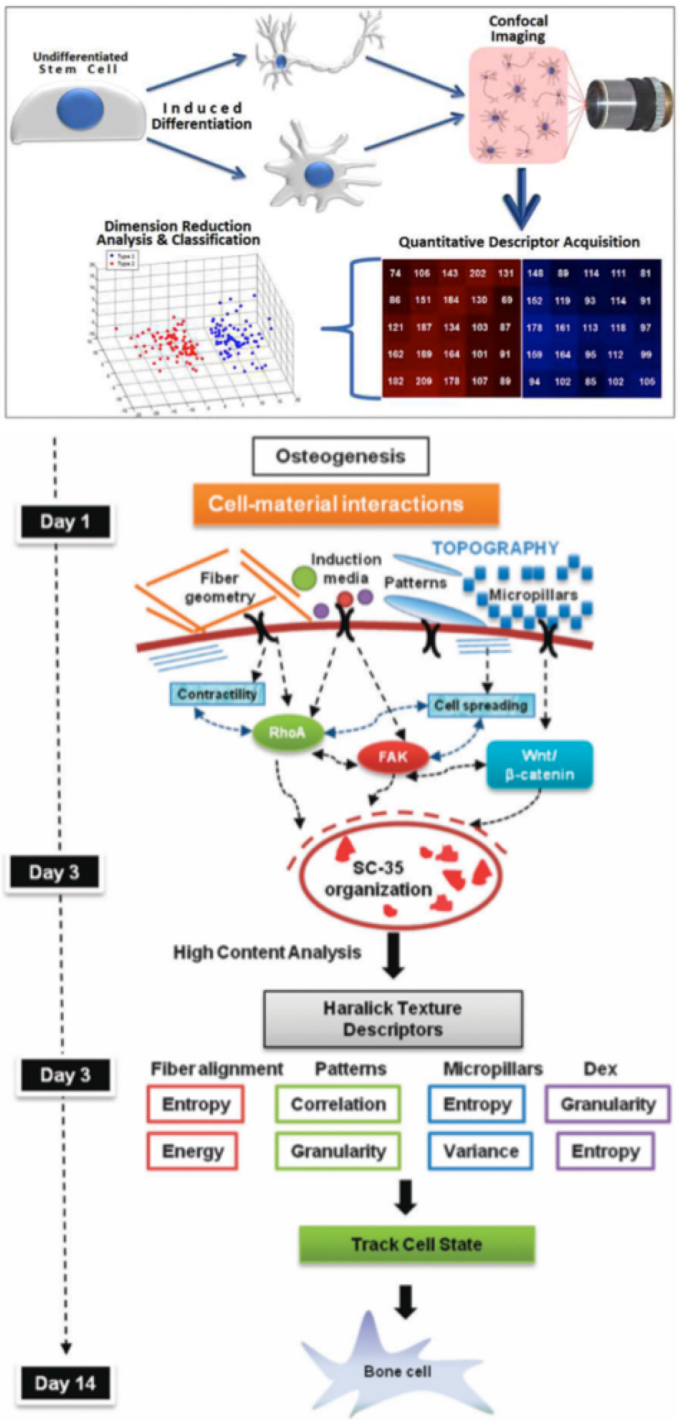

Figure 4: Confocal images of stem cells after their induced differentiation are analyzed for texture analysis to classify cells (top). Cell-material interactions reach the nucleus through protein interactions and impact the organization of nuclear proteins, which can be analyzed by HCIA (bottom).

There is a pronounced need to detect MSC changes due to aging and senescence by non-invasive imaging technologies that can analyze cells at any stage of processing, including upon receipt from the donor to after *ex vivo* expansion for transplantation. One current approach for MSC enrichment is based on negative selection – it is not selecting the quality of MSCs – and requires an expensive reagent. Further, a recently developed microfluidic device for enrichment yields only 73% recovery [19]. Again, these approaches only sort based on identity while the proposed technology enriches based on quality.

Enrichment Based on Senescence and Beyond: Replicative senescence is a continuous process that begins from the start of growth and alters the phenotype, differentiation potential, and gene expression patterns of MSCs [20]. Expanding MSCs long-term in culture causes larger cell size, decreased differentiation potential, and cell cycle arrest at senescence [10]. The quality control approach proposed herein is based on training a convolutional neural network (CNN) with at least thousands of fluorescent and brightfield images of single cells. The gradual, continuous morphological alteration of MSCs during expansion is foundational to the hypothesis that a CNN can learn to detect senescence by brightfield images alone and along a continuum [20]. We envision the analysis of MSCs to encompass additional traits, such as differentiation potential and identity. The workflow for teaching the neural network to recognize additional traits, such as differentiation capacity, mirrors that of senescence except using different markers for training.

INNOVATION

Continuously evaluating the quality of MSCs during their expansion with current methods is impractical due to cell loss in assays, in addition to the time, cost, and material commitment involved. Also, some applications, such as cell sorting based on the knowledge of each MSCs' particular set of qualities, are currently impossible. The proposed technology would detect not only senescence but also confirm the identity of the MSCs and their quality

(proliferation and differentiation potential) at any point in the MSC preparation process. This high-throughput assay will enable further applications, such as the enrichment of higher quality MSCs to meet the particular needs of each patient.

This approach represents a major advance over current technologies thus saving time, cost, and materials. Conventional quality control assays of MSCs (SA-β-Gal, differentiation and proliferation assays, flow cytometry) are laborsome and time- and material-intensive. This technology will rapidly assess the identity of MSCs at any point in the cell preparation process with no loss of MSCs or significant added cost. It will also provide measures of each cell's quality while maintaining their viability. The successful development of this technology could lead to its implementation towards analyzing other stem cell types.

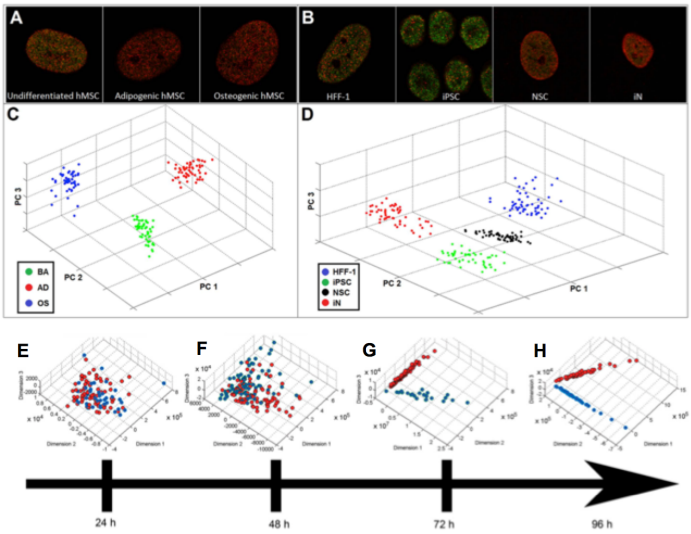

Figure 5: Nuclei of seven different distinct cellular phenotypes were immunocytochemically labeled for H3K4me3 (green) and H3K27me3 (red) and imaged with a LSCM G-STED (A and B). Quantitative texture descriptors of H3K4K27me3 organizational expression were extracted from these images and subsequently analyzed via PCA, which reveals the ability to clearly classify each different cellular phenotype from each other based on H3K4K27me3 textural expression with 100% sensitivity and specificity (C and D). On nontreated glass, the cytoskeletal based segmentation process of hMSCs treated with adipogenic or osteogenic induction becomes progressively more pronounced with culture time (E–H).

Next-Generation High-Content Image Informatics: Previously, our lab extensively researched high-content image informatics (HCII) to forecast MSC state based on textural descriptors calculated from the cytoskeletal and nuclear arrangement of proteins in terminal assays (Figures 4 and 5). Our lab's enhanced *in silico* toolbox will provide for:

a) Label-Free Classification: As stated, long-term culture of MSCs results in predictable morphological changes — the cells become larger and the cytoplasm more granular [20]. These changes not only occur upon senescence but are continuous alterations in MSC culture [20]. This platform technology, while rooted in established neural network technology, is innovative in evaluating multiple properties of every cell in a single bright-field image. Brightfield images have already been used as input to predict lineage choice in primary hematopoietic progenitors using a deep neural network [21], but they have not been used to evaluate numerous properties of MSCs with one image. There is promise in using bright-field images to classify the extent of senescence and other properties of MSCs, and this work would be the first demonstration of its capability with stem cells for regenerative medicine.

b) Enrichment of MSCs from Aged Donors: The composition of a mixture of cells from elderly donors is altered by aging and contains some senescent cells [17]. It is possible to harvest cells presenting a young phenotype from such donors using flow cytometry and expanded to generate large amounts of high-quality MSCs to potentially treat age-related diseases [17]. We propose expanding upon this recent study by enriching MSCs for desirable properties without labeling the cells. This will lead to a high-throughput approach of sorting with imaging flow cytometry. The innovative paradigm that will emerge from this proposal will provide for the rapid, real-time evaluation of therapeutic properties of transplantable MSCs; and the patient-centric enrichment of MSCs for autologous transplant on a case-by case basis.

RESEARCH APPROACH

AIM 1: Can a convolutional neural network (CNN), based on live-cell, imaging-based assay, provide a multifactorial report on an MSC's overall phenotype and viability?

In this R21 we will design a new live-cell imaging-based assay, which will use brightfield microscopy to discern between positive and negative expression of MSC markers at the single-cell level.

RATIONALE: The current quality control approach for MSCs requires expensive reagents and lengthy, endpoint assays to identify the cells. The proposed technology will expedite analysis of MSCs and provide a readout of their quality beyond identity.

PRELIMINARY RESULTS:

Design of a highly accurate convolutional neural network: We have designed a CNN that is highly accurate and able to distinguish between images of similar mother and daughter cells of different passages. Based on our previous work analyzing actin textural features to identify MSC fate identity [22], we tested the hypothesis that cellular organizational features change as MSCs age *in vitro* by analyzing their actin cytoskeleton as they were serially passaged. Starting at passage 6 (P6), cells were plated on glass-bottom well plates, fixed, stained with phalloidin Alexa Fluor 647, and imaged on a Zeiss LSM 780 confocal microscope. Although we have not been able to distinguish between the passages by eye, our CNN can classify between pairs of P6 through P12 samples with an average accuracy of over 90%. Thus, the CNN is able to classify cellular images for *in vitro* aging with high accuracy (Figure 7, top)). While this study involved fluorescent labeling, it shows the promise of classification by morphological attributes.

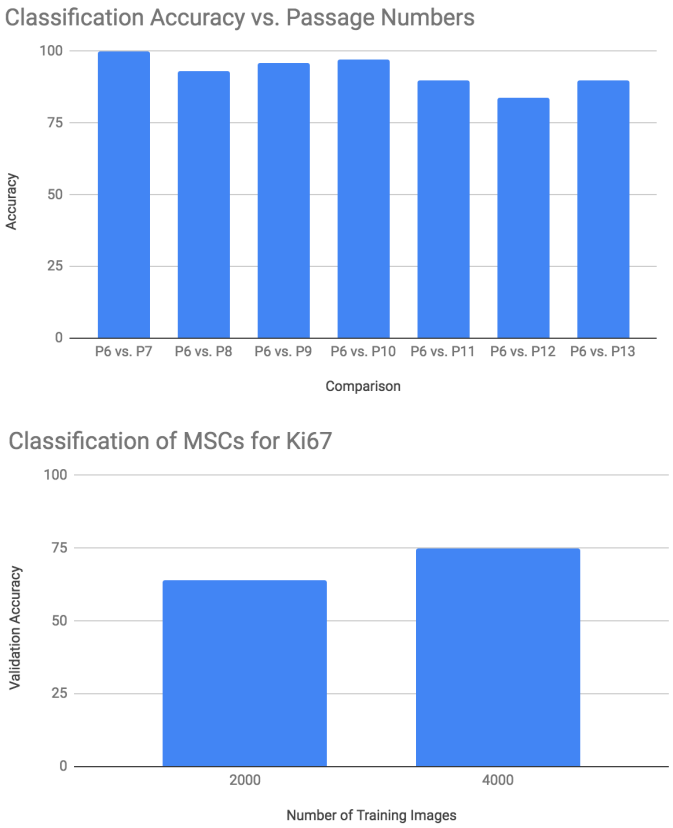

Figure 7: Classification accuracy by *in vitro* age and for proliferation. The CNN was able to parse between passage 6 (P6) and higher passages with greater than 90% accuracy on average (top). By doubling the sample size, the CNN was able to detect Ki67+ MSCs with an accuracy of 75% compared to 64%

Accurately classifying MSCs based on brightfield images: Although we have previously successfully classified MSCs with HCII using images of fluorescently stained cells, our previous methods were not optimized for analyzing bright-field images and they were destructive assays. To test the applicability of our new CNN for bright-field images, we have analyzed a population of MSCs for Ki67+ cells (Figure 7, bottom). We used fluorescent images to determine whether each cell positively or negatively expressed Ki67; the corresponding bright-field images were fed into our CNN to learn how to classify each marker. So far, our CNN has been able to classify for Ki67 using only bright-field images with approximately 75% accuracy. We expect that this accuracy will continue to improve with larger data sets as the accuracy improved to 75% from 64% by doubling the size of the training set. This shows that our CNN is indeed able to categorize MSCs based on bright-field images.

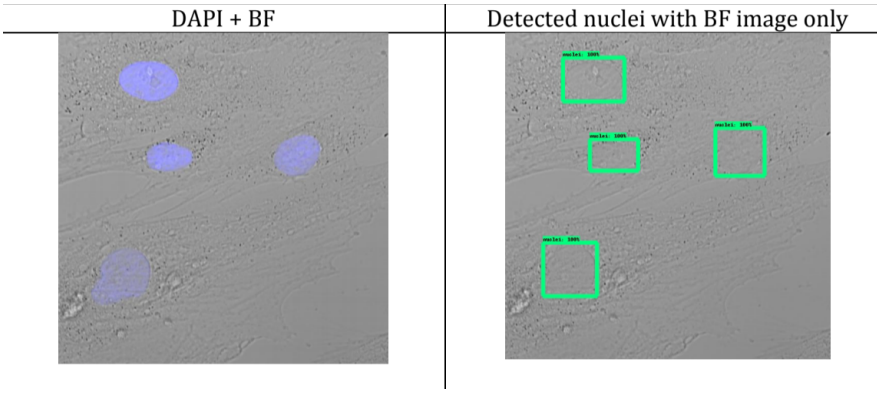

Figure 8: Nuclei detection with trained neural network. Nuclei shown by staining with DAPI (left) are detected in the bright-field image alone by a trained neural network(right).

Automated nuclei detection in bright-field images: The neural network classifying the MSCs will be trained on a consistent region of the cell – the nucleus. Neural networks are powerful at object detection, and we trained an additional neural network to detect nuclei in brightfield images. Of the nuclei positioned fully within the image borders, approximately 95% were successfully identified (Figure 8). This work will enable a seemless workflow from image to nuclei to classification.

The following steps will be used to accomplish AIM 1.

EXPERIMENTAL DESIGNS AND METHODS

Creation of a "Deep Learning" Image Dataset: First we will create an image dataset for the MSC markers which have been established by the International Society for Cellular Therapy (ISCT), a panel of ten markers (CD34, CD45, CD11b, CD14, CD79 α, CD19 α, HLA CII, CD73, CD90, and CD1050) (Figure 6) [12]. The creation of a deep learning dataset for machine learning requires thousands of images, which will be acquired on a Zeiss LSM 780 confocal microscope. During imaging we will acquire brightfield images of MSCs, and fluorescent images of the same cells, which have been stained with antibodies for the MSC markers. Fluorescent images will be used to pre-classify the training images into "ground truth" MSC identities (i.e. cells with positive and negative marker expression). The corresponding bright-field images will then be used to train an HCII algorithm to distinguish between the positive and negative marker expression classes.

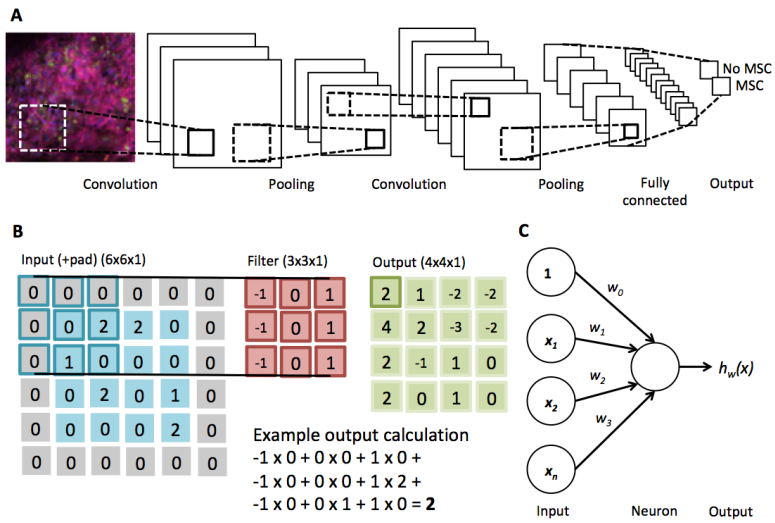

Figure 9: Example of CNN architecture and operations. A typical CNN (A) consists of layers of convolution and pooling, two operations to identify patterns in the picture. In convolution, a filter is applied pixel-by-pixel producing an output (B). The CNN's final flattened layer is sent to a layer of neurons (C) to calculate the output, or the class. Each neuron (C) in the fully connected layer receives weighted inputs and a bias (an added constant), and the output is this sum applied to a function known as an activation function.

Designing an HCII that can Identify MSC Markers in a Population: We will code a custom CNN (example in Figure 9A) consisting of convolution and pooling operations (Figure 9B) before a fully connected layer (Figure 9A and C) that will be able to discern between positive and negative marker expression in the panel of ten MSC markers. Our CNN will be designed to operate on bright-field images, which will allow the CNN to classify unlabeled and unaltered cells. To ensure accuracy and prevent overfitting in our CNN modeling, a separate group of images that the CNN has not been trained on will be used to validate the accuracy of our method [23]. In order to analyze single-cells, a neural network will be trained to perform object detection to recognize nuclei in the bright-field images.

Validation of HCII Accuracy in vitro: To validate how well our CNN works, we will obtain bone marrow or adipose tissue isolates from multiple donors, and isolate MSCs by plastic adherence [24, 25]. We will then inspect the MSC marker expression of the cells with flow cytometry, and compare the flow cytometry results with our HCII results. For flow cytometry, cells will be analyzed on a Beckman Coulter Gallios Flow Cytometer.

Examination of HCII Prediction of Cell Quality: We will explore whether the HCII classification outcomes of an elderly patient's MSCs can predict how well those MSCs can proliferate and differentiate. MSCs will be obtained from multiple elderly donors (age 60+) and multiple young donors (age ≤25). The MSCs from different donors will be imaged and analyzed with HCII. We will assay donor's cells for osteogenesis using an alkaline phosphatase (ALP) assay [26]; adipogenesis using Oil red O staining [27]; and chondrogenesis using a DMMB assay [28]. Proliferation will be assayed by computationally counting the total number of cells in each condition.

Expansion of in-silico MSC Marker Panel: To produce a more comprehensive cell quality assay, we will expand the panel of markers that our HCII examines with more recently identified MSC markers. These will include: more recent positive MSC markers (e.g. CD271, CD200) [29-31]; differentiation potential markers (CD106,

CD146, CD49f, SSEA-3) [32, 33]; proliferation markers (CD106, BIRC5, CDK1) [32, 34]; and senescence markers (GATA4, p16INK4A, Ki-67, pH2A.X) [6, 35, 36].

Expected Outcomes and Alternative Approaches: The successful outcome of the proposed plan will be the creation of a novel assay for validating the identity and quality of MSCs, which will be able to classify unlabeled live cells. This HCII platform will be cost- and time-efficient, and it will ultimately enable investigators to predict systematically how viable an elderly donor's MSCs will be for autologous MSC therapy. In the unlikely event that our proposed CNN architecture cannot classify cells adequately based on bright-field images even after fine-tuning of the network's parameters, we will attempt other existing AI architectures, such as recurrent neural networks (RNNs), which requires a series of images.

AIM 2: Will an MSC enriching platform, based on live-cell sorting with imaging flow cytometry, improve the regenerative potential of a donor cell population, enabling better precision medicine?

RATIONALE: In Aim 1, the ability to assess a population of MSCs on their identity and quality on a single cell basis is established. Aim 2 encompasses building a microfluidic device to sort MSCs cell-by-cell for downstream application in regenerative medicine.

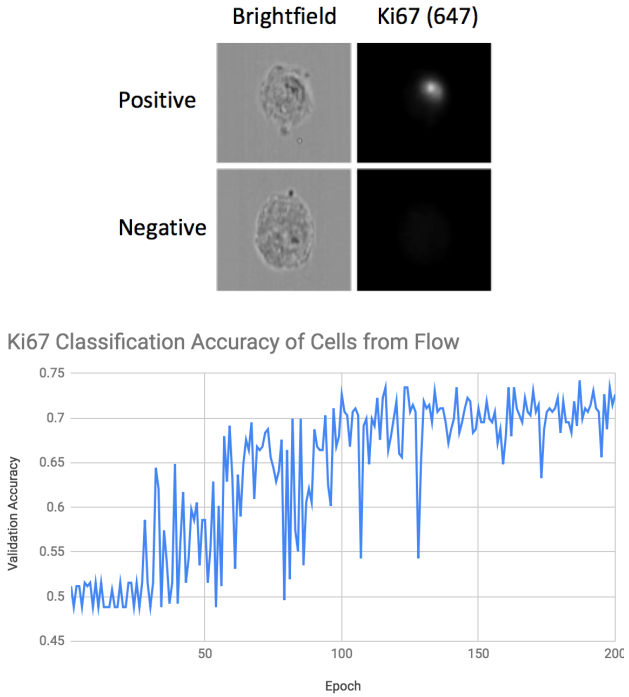

Figure 9: Training accuracy of our CNN over each epoch in classifying cells in brightfield images as positive or negative for Ki67 as determined by fluorescence imaging in flow

PRELIMINARY RESULTS:

Classification of MSCs for Proliferation with Ki67 and Imaging Flow Cytometry: MSCs were stained for Ki67, and images were captured with an imaging flow cytometer (Figure 9, top). With a small sample size of hundreds of images, the neural network was able to classify MSCs as positive or negative for Ki67 by brightfield alone at approximately 75% accuracy (Figure 9, bottom).

EXPERIMENTAL DESIGN AND METHODS

We will evaluate whether it is possible to improve on the overall proliferation and differentiation of elderly patients' MSCs by sorting for MSCs with favorable attributes using our custom-built imaging flow cytometer. For example, proliferative and non-senescent cells will be enriched from an MSC population to yield a collection of high quality cells. The sorted population with desirable characteristics will be evaluated by *in vitro* assays for their quality, such as differentiation and proliferation capacity. The following steps will be used to accomplish AIM 2.

Imaging Flow Cytometer: The imaging flow cytometer we propose to construct will build upon the device recently described by Goda, *et al.* [37]. The cytometer will be able to acquire brightfield and fluorescent images for training and subsequent sorting of MSCs in real time.

Evaluation of Improvements in Proliferation and Differentiation: We will compare MSC populations enriched on the microfluidic device to control groups that still have senescent cells, and quantify the amount of differentiation and proliferation in each group. We will specifically assay for osteogenesis using an alkaline phosphatase (ALP) assay [26]; adipogenesis using Oil red O [27]; and chondrogenesis using a DMMB assay [28]. Proliferation will be assayed by digitally enumerating (by software and microscopy) the total number of cells in each condition.

Integration of HCII into the Cell Enrichment Protocol: Once it has been established that selective enrichment of the MSC population improves cell outcomes with one cell marker (senescence, GATA4), we will then integrate our HCII methodology to further screen for other markers. We will enrich cell populations for:

established negative MSC markers (CD34, CD45, CD11b, CD14, CD79 α, CD19 α, HLA CII) [38-40]; established positive MSC markers (CD73, CD90, CD1050) [38, 39]; additional newer positive MSC markers (e.g. CD271, CD200) [29-31]; differentiation potential markers (CD106, CD146, CD49f, SSEA-3) [32, 33]; proliferation markers (CD106, BIRC5, CDK1) [32, 34]; and other senescence markers (p16INK4A, Ki-67, pH2A.X) [35, 36]. Thus, the technology with be able to enrich a population of cells on the basis of identity and quality.

Induction of Senescence in vitro*:* In order to generate a population of cells with senescence to train our neural network, we will induce senescence in MSCs *in vitro* by inducing DNA double-stranded breaks in the cells with etoposide [41]. Cells will be treated with a concentration curve at first to determine the optimal dose for inducing senescence and retaining maximum viability. MSCs will be treated for 24 hours, and be allowed to recover and senesce for 4 days. Senescence will be assayed for by looking at SA-β-Gal, as well as GATA4, which has been shown to be a good marker for cellular senescence [6]. In addition, late passage MSCs and MSCs from older donors will also be examined for training and detection of senescence.

Implementation of a Reversible Live-Staining Senescence Protocol: The following staining approach will allow us to assess the downstreatm effect of enriching the MSC population for markers of interest. We will employ Streptolysin O, a bacterial toxin which forms temporary pores in the cellular membranes [42], in order to fluorescently labelled cells with our panel of antibodies. Duplicate control populations will be used to assay whether SLO affects MSC proliferation and differentiation.

Expected Outcomes and Alternative Approaches: The anticipated successful outcome will be improved MSC differentiation and proliferation in cells obtained from elderly patients, which will validate selective cell enrichment as a key therapeutic strategy for future MSC therapies. In the event that targeting GATA4 positive cells does not adequately improve MSC outcomes, we will try a different senescence marker (e.g. p16INK4A). We could also try a combination of senescent markers, or exclude cells that do not express a newer positive marker, such as CD271.

Rigor and Reproducibility: The experimental sample sizes for our *in vitro* studies will be determined via *a priori* power analysis, providing at least 80% power with 5% chance of Type I error. Full experimental details will be provided upon publication of data, to ensure independent reproducibility by other investigators.

Statistical Analysis: In all of the experiments, a sufficient number of experimental data points will be examined to avoid stochastic responses during experimental manipulations. Parametric tests such as Student's *t*-tests or two-way ANOVA plus Fisher's post hoc *t*-test will be used to analyze data.

SUMMARY AND FUTURE OUTLOOK: In summary, we will create a live-cell assay for determining the identity and viability of MSCs obtained from aged donors. We will validate whether it is possible to recover the regenerative potential of MSCs isolated from elderly patients by enriching the cell population, improving their efficiency for therapeutic applications. The future direction of this research will be to apply the approaches of screening and enrichment into a high throughput tool useful for other types of stem cells.

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