

## $\alpha$ SMA Expression in Large Colonies of Colony-Forming Units-Fibroblast as an Early Predictor of Bone Marrow MSC Expandability

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Clinical applications of mesenchymal stromal cells (MSCs) require the manufacture of large cell lots, which involves multiple passages for cell expansion and sometimes genetic modification. MSCs from various sources, including bone marrow (BM), exhibit high donor-to-donor variability in their growth characteristics. This can lead to unpredictable manufacturing outcomes with respect to success or failure of individual lots. Early determination of lot success has the potential to reduce the cost and improve the efficiency of the MSC manufacturing process. However, methods that effectively predict lot growth potential early in the manufacturing process are currently lacking. Here we report that the growth potential of an MSC lot can be predicted a few days after BM plating based on  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) protein expression in large colony-forming unit-fibroblast (CFU-f) colonies. The proposed prediction method could be a useful tool to prospectively determine MSC lot success or failure.

**Key words:** Mesenchymal stromal cells (MSCs); Colony-forming units-fibroblast (CFU-f);  $\alpha$ -Smooth muscle actin ( $\alpha$ SMA); Assay

### INTRODUCTION

Mesenchymal stromal cells (MSCs) were first detected in bone marrow (BM) cultures as cells forming adherent clonal fibroblastic colonies and capable of undergoing more than 20 population doublings, hence their name: colony-forming units-fibroblast (CFU-f)<sup>1,2</sup>. The ability of these culture-expanded cells to differentiate into bone, cartilage, reticular, and adipose tissue and to transfer hematopoietic microenvironment<sup>3,4</sup>, along with their potent secretome<sup>5</sup> and immunomodulatory properties<sup>6</sup>, brought them to the frontier of cell therapeutic efforts. More than 350 clinical trials currently use expanded MSCs from BM or other sources as a potential treatment for various skeletal, degenerative, and immune disorders. Moreover, large cell lots of allogeneic MSCs can be manufactured for “off-the-shelf” use on a number of patients. The manufacturing of large BM-MSC lots, however, can be sometimes challenging due to the high variability in the growth potential of MSCs derived from different BM donors<sup>7–9</sup>. It would therefore be advantageous to have tools to predict MSC culturing outcome and terminate unsuccessful lots early in the manufacturing process.

CFU-f assay enumerates clonogenic adherent cells (CFU-f) within the BM preparation. At BM plating

densities ensuring linearity between numbers of plated cells and resulting colonies, colony number/ $10^5$  plated cells [colony-forming efficiency (CFE)] is dependent on the donor, the BM specimen harvesting method, the cell isolation steps (i.e., washing), and the culturing protocol<sup>10,11</sup>. In 7–10 days, the cultures contain colonies that vary greatly in size. CFU-fs represent a mixture of committed and intermediate progenitors and stem cells; they are typically distinguished on the basis of the potency of their colonies to undergo single, bilineage, or trilineage mesenchymal differentiation. It is generally believed that in culture, stem cells are more prolific than both committed and intermediate progenitors, but have longer lag period after BM explantation than committed progenitors<sup>12</sup>. In light of this, the following assays have been proposed as predictors of MSC growth potential: trilineage differentiation potential of colonies<sup>13,14</sup>, CFE after the first passage<sup>9</sup>, monitoring of proliferation and viability at early passages<sup>15</sup>, and using motility as a marker of multipotent (stem) cells<sup>16</sup>. However, these approaches require MSC passaging and are laborious, which makes them impractical as growth predictors for cell manufacturing.

$\alpha$ -Smooth muscle actin ( $\alpha$ SMA) is a contractile actin isoform, a hallmark of vascular smooth muscle cells and myofibroblasts that differentiate from many mesenchymal

cell types in response to tissue injury and repair; in the wound, it is stimulated by the increased stiffness of the substrate<sup>17</sup>. In cultured MSCs,  $\alpha$ SMA colocalizes with stress fibers developing during culturing<sup>18</sup>. Sorting passaged MSCs by cell size (small and large cell populations) concomitantly sorts them by expression of  $\alpha$ SMA (low and high, correspondingly) and by clonal and differentiation potential (high and low, correspondingly). Moreover, knockdown of  $\alpha$ SMA or plating cells onto a soft surface restores clonogenicity and differentiation potential<sup>19</sup>. Thus, to a certain degree,  $\alpha$ SMA expression in early MSCs could be regarded as an artifact of culturing these mechanosensitive cells on a stiff plastic surface<sup>20</sup>. While there is widespread use of plastic surfaces in manufacturing of MSCs, the variability among BM donors with respect to their MSC response to the stiff plastic culture surface has not been studied yet.

Here we describe a novel colorimetric colony-based assay that can be used to predict the growth rates of MSC lots later in the manufacturing culture process based on parameters measured only few days after BM plating. The  $\alpha$ SMA/lactate dehydrogenase (LDH)-based colony (ALC) assay quantifies both  $\alpha$ SMA expression and intracellular LDH activity in CFU-f colonies. We show that the output of the ALC assay on day 10 of BM culturing correlates well with the subsequent MSC lot growth up to passage 3.

## MATERIALS AND METHODS

### *BM Cell Preparation, Plating, and Culturing*

Undiluted BM aspirates from healthy individual human donors were purchased from Lonza (Walkersville, MD, USA) or AllCells (Alameda, CA, USA) and delivered overnight with cold packs. Lonza donors (samples D121–D128 and L1) were 21- to 25-year-old males, while AllCells donors (AC12–14) were 32- to 33-year-old males. The aspirate sample (1–3 ml) was diluted three times in MSC growth medium consisting of  $\alpha$  minimum essential medium ( $\alpha$ MEM; Mediatech, Manassas, VA, USA) supplemented with 10% fetal bovine serum (lot selected for MSC growth; HyClone, Logan, UT, USA), GlutaMAX (Invitrogen, Thermo Fisher Scientific; www.thermo-fisher.com), and penicillin/streptomycin (Mediatech) and centrifuged at  $200\times g$  for 8 min. The supernatant was carefully removed, and the pellet was resuspended to the prior volume of the diluted sample. White blood cell (WBC) counts were determined in aliquots after diluting them in Erythrocyte Lysis Reagent (Sigma-Aldrich, St. Louis, MO, USA). For the analysis of CFU-f colonies, washed BM cells were resuspended at  $6.6\times 10^4$  cells/ml and plated into 96-well black microplates with a clear bottom (100  $\mu$ l/well; Costar, Corning, NY, USA); two rows were left empty. This cell concentration resulted in growth of

colonies in less than 30% of wells, thus ensuring that there would be a low probability of having more than one colony per well. For mass culture plating, BM cells were resuspended and plated at about  $2\text{--}4\times 10^5$  WBC/cm<sup>2</sup> into a T75 flask. On day 3, the majority of the nonadherent cells were removed during medium change. Thereafter, medium was changed every 2–3 days. On day 10, microplates were used in the ALC assay, while mass cultures were cultured an additional 0–4 days before passaging. For passaging, cells were lifted with 0.25% trypsin/EDTA (Mediatech), counted using trypan blue (Sigma-Aldrich), and replated at about  $0.5\text{--}1\times 10^4$  cells/cm<sup>2</sup>. Subsequent passages (up to 3) were performed when cultures reached 70%–80%, which took 4–7 days. MSC mass culture growth rate at passage  $n$  ( $GR_n$ ) was calculated as follows:

$$GR_n = dH_0/dWBC \times dH_1/dP_1 \times \dots \times dH_n/dP_n$$

where  $dH_n$  is the cell density at harvest of passage  $n$ ,  $dP_n$  is the cell density at plating of passage  $n$ , and  $dWBC$  is the cell density at initial BM plating.

### *ALC Assay*

In preliminary experiments, an assay that can simultaneously measure  $\alpha$ SMA in a colony and the colony's cell number was developed using passaged MSCs. Intracellular LDH activity, a surrogate for cell number, was colorimetrically measured in cells processed for immunodetection of  $\alpha$ SMA, that is, after light formalin fixation<sup>21</sup> [4% paraformaldehyde (PFA) for 20 min; Electron Microscopy Sciences, Hartfield, PA, USA], permeabilization [0.2% Triton X-100; 20 min; Sigma-Aldrich], and incubation with anti- $\alpha$ SMA antibody conjugated to horseradish peroxidase (HRP; Abcam, Cambridge, MA, USA). This residual LDH activity was found to be proportional to plated MSC numbers and/or numbers of cell nuclei quantified using a fluorescent nuclear dye Hoechst 33342 (Molecular Probes, Thermo Fisher Scientific) and image analysis by Cytation 5 multimode plate reader, objective 4 $\times$ , and Gen5 software (BioTek, Winooski, VT, USA).

Therefore, for the colonies' measurements, two colorimetric assay protocols—residual LDH activity and  $\alpha$ SMA protein expression detection—were incorporated into a single protocol performed on the plate with cultured adherent BM cells; for each assay, empty wells were designated for standards and controls. Wells designated for LDH standards and HRP controls were kept unused until indicated in the protocol below. On day 10 after BM plating, plates were washed with phosphate-buffered saline (PBS), fixed with 4% PFA for 20 min, and then the fixative was replaced with PBS. Meanwhile, surrogate  $\alpha$ SMA standards were prepared by serially diluting AffiniPure Donkey Anti-Mouse IgG (Jackson

Immunoresearch Laboratories, West Grove, PA, USA) in PBS and adsorbing the solutions in the designated wells for 1 h; these wells were then blocked with the MSC growth medium. All wells (except for those designated for LDH and  $\alpha$ SMA standards and HRP controls) were then washed once and incubated with 0.2% Triton X-100 in PBS for 20 min, followed by blocking in 0.5% normal donkey serum (Jackson Immunoresearch Laboratories) for 30 min. The wells (except for those designated for LDH standards and HRP controls) were then incubated with monoclonal anti- $\alpha$ SMA antibody conjugated to HRP (1:1,000) for 1 h and washed three times with PBS. The LDH assay was then performed. LDH standards were prepared in designated wells by serially diluting bovine LDH (Sigma-Aldrich), and then catalyst/dye (0.25 ml:11 ml) mixture from LDH Cytotoxicity Detection kit (Clontech Laboratories, Mountain View, CA, USA) was added to all wells with cultures and LDH standards. The signals were read at 490 nm with correction at 650 nm. After LDH detection, plates were washed once and prepared for HRP detection. To ensure that readings on all plates would be comparable across different experiments, an HRP control prepared from highly stabilized HRP (Sigma-Aldrich) was used on each plate. Wells containing cultures, surrogate  $\alpha$ SMA standards, and HRP controls were filled with the HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB; eBioscience, San Diego, CA, USA). The absorbance was measured at 370 nm with the correction at 492 nm. Both LDH and HRP signals were quantified using standard curves generated in SoftMax Pro (Molecular Devices, Sunnyvale, CA, USA); thus, LDH activity units were expressed in mU/ml, and  $\alpha$ SMA expression units represented the corresponding concentration of anti-mouse antibody in ng/ml.

#### *Data Processing for ALC Assay*

The processing of ALC assay data was conducted using a programmed Excel template (Microsoft, Redmond, WA, USA). HRP control data were used to make adjustments between different plates and experiments if needed. Then for each plate the background values for both measured parameters were calculated: for LDH, as the average of all wells without a colony + 1 standard deviation (SD), and for HRP, as the average of all wells without colonies. The background was subtracted from the corresponding dataset. This method enabled the elimination of wells with less than 50 cells from further calculations; at the same time, less stringent conditions for HRP signals prevented the exclusion of colonies with very low levels of  $\alpha$ SMA expression. The  $\alpha$ SMA expression was normalized to the corresponding LDH signal from the same well (a surrogate for  $\alpha$ SMA/cell). All colony data were sorted from high to low LDH levels, and the percentage of colonies with LDH  $\geq 0.4$  mU/ml ("large colonies")

was determined. This threshold typically categorized more than 15% colonies as large in the BM lots. In these large colonies, normalized  $\alpha$ SMA expression was averaged. Each BM lot was thus characterized by the average  $\alpha$ SMA level in cells of large colonies [termed here as  $Av(\alpha$ SMA/LDH)<sub>LC</sub>].

#### *Cell Proliferation Assay and Immunofluorescence*

Proliferation assay was conducted on colonies using Click-iT Plus 5-ethynyl-2'-deoxyuridine (EDU) Alexa Fluor 594 Imaging Kit (Life Technologies, Frederick, MD, USA) according to the manufacturer's protocol. Cells were labeled with EDU for 5 h. After performing EDU detection, the colonies were blocked with 0.3% normal donkey serum and probed with fluorescein isothiocyanate (FITC)-conjugated anti- $\alpha$ SMA monoclonal antibody (Sigma-Aldrich), followed by washing. Nuclei were counterstained using Hoechst 33342. To quantify the percentage of EDU<sup>+</sup> nuclei, images of colonies were acquired using Cytation 5 under a 1.25 $\times$  objective and analyzed using Gen5 software. The colony data were then sorted in Excel. Colonies exhibiting highest and lowest % EDU (three to four colonies/group) were selected and acquired under 4 $\times$  magnification to visualize and quantify  $\alpha$ SMA mean fluorescence intensity using ImageJ as mean gray value with the subtraction of the corresponding background. For the analysis of  $\alpha$ SMA and EDU distribution in one colony, the image of a colony was digitally enlarged and  $\alpha$ SMA<sup>+</sup> areas were selected; then equal areas were selected in the  $\alpha$ SMA<sup>-</sup> region of a colony. Total and EDU<sup>+</sup> nuclei were manually counted in these areas.

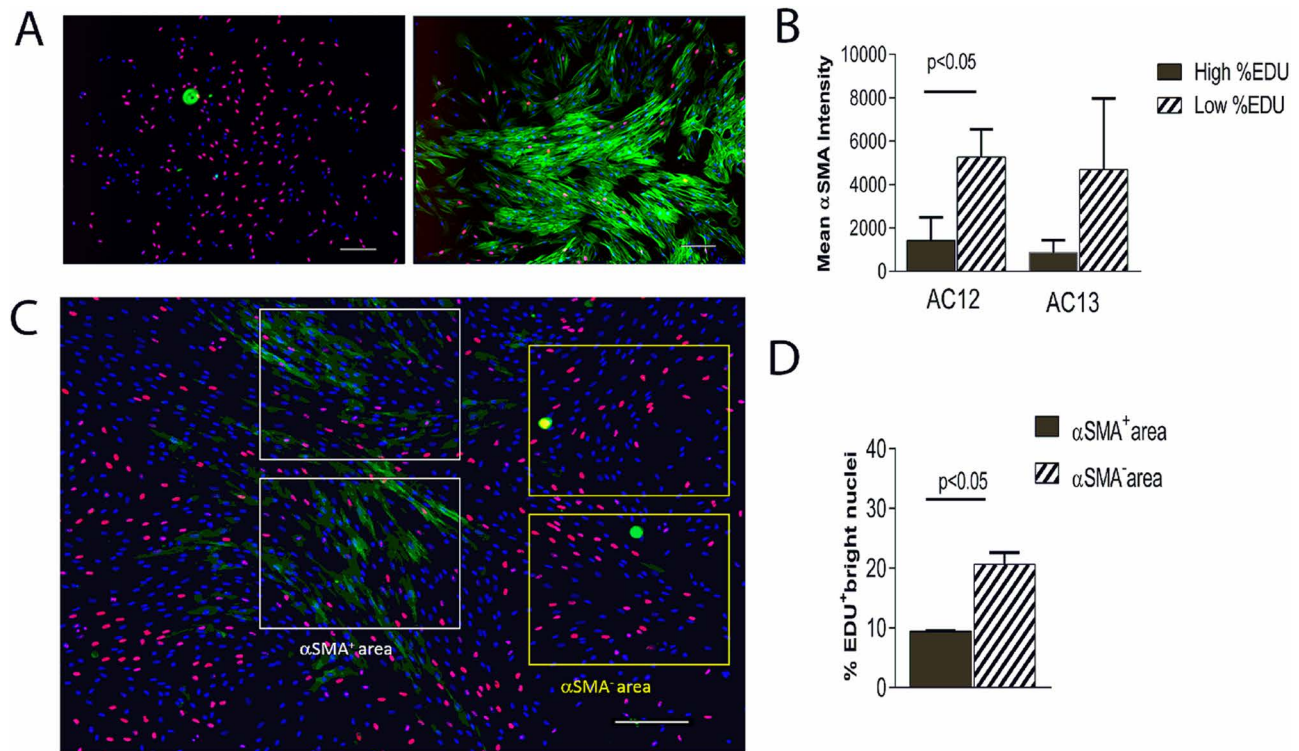
#### *Statistics*

Statistical analysis (paired or unpaired *t*-test and linear regression analysis) and graphing were done using GraphPad Prism 6 (San Diego, CA, USA). A value of  $p < 0.05$  was considered statistically significant.

## **RESULTS**

### *Correlation Between Cell Proliferation and $\alpha$ SMA Expression in Day 10 Colonies*

In an MSC lot, highly proliferative colonies (high % EDU incorporation) exhibited lower  $\alpha$ SMA expression than did colonies with low % EDU (Fig. 1A). In two lots, colonies with the highest % EDU and the lowest % EDU exhibited correspondingly low and high mean fluorescence intensity for  $\alpha$ SMA, and the effect was either statistically significant or a strong trend (Fig. 1B). There was a negative correlation between the level of  $\alpha$ SMA expression and the percent of nuclei with bright EDU staining (Fig. 1C and D).  $\alpha$ SMA<sup>+</sup>, but not  $\alpha$ SMA<sup>-</sup>, areas also contained nuclei with very low levels of EDU in addition to brightly stained ones. Taken together, these observations indicated that  $\alpha$ SMA expression appeared negatively



**Figure 1.** Correlation between  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) expression and cell proliferation in mesenchymal stromal cell (MSC) colonies. Colonies were labeled with 5-ethynyl-2'-deoxyuridine (EDU) for 5 h prior to fixation, and EDU (red) and  $\alpha$ SMA (green) were detected; nuclei were stained with Hoechst dye (blue). (A)  $\alpha$ SMA expression in representative large colonies with high % EDU (left) and low % EDU (right). (B) In two lots, four colonies with highest % EDU (AC12: EDU >39% and AC13: EDU >28%) and four colonies with lowest % EDU (AC12: EDU <18% and AC13: EDU <15%) were labeled using fluorescein isothiocyanate (FITC)-conjugated anti- $\alpha$ SMA; the mean FITC intensities of colonies in the high and low % EDU groups were compared. (C) A highly proliferative colony expressing some  $\alpha$ SMA is shown; in areas designated as  $\alpha$ SMA<sup>+</sup> and  $\alpha$ SMA<sup>-</sup> (white and yellow rectangles, respectively), percent bright EDU<sup>+</sup> nuclei was compared (D). Scale bars: 200  $\mu$ m. Error bars represent SD between two rectangles.

correlated with both the colony's overall proliferation rate and the proliferation status within the colony.

#### Correlating ALC Assay Data on Day 10 With the Subsequent Mass Culture Growth

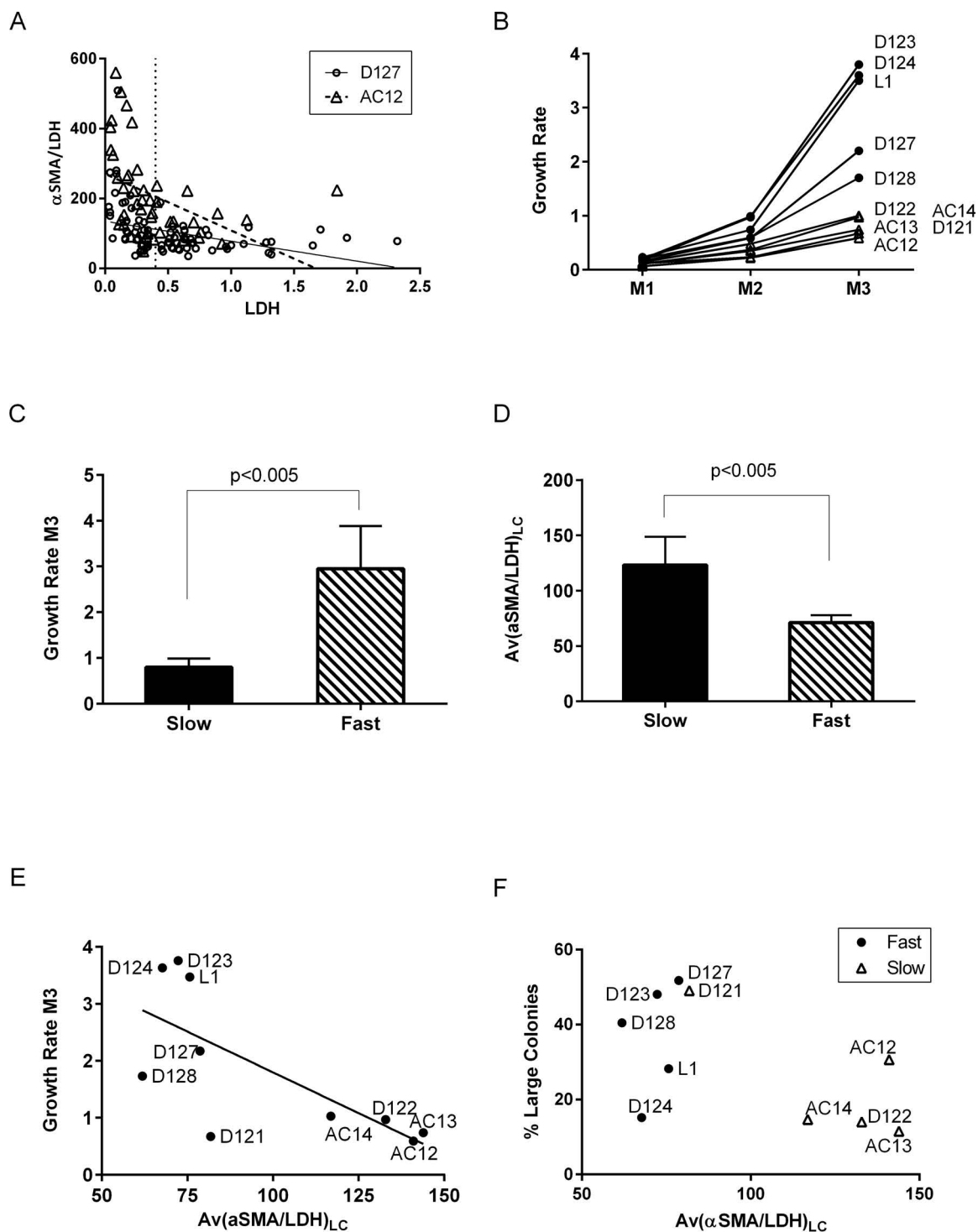
On day 10, LDH values (colony size) negatively correlated with  $\alpha$ SMA/LDH values (surrogate for  $\alpha$ SMA/cell) in 9 out of 10 tested BM-MSC lots (34–89 colonies/lot;  $p < 0.05$ ), and the distribution pattern of colonies on the  $\alpha$ SMA/LDH versus LDH plot was found to be characteristic for individual BM lots (Fig. 2A). Because of the exponential character of cell growth, the contribution of large colonies in mass culture population is substantially greater than that of small colonies. Therefore, we arbitrarily defined large colonies as those with LDH  $\geq 0.4$  mU/ml (i.e., colonies with approximately 2,000 cells, based on nuclei quantification). ALC assay output allowed determining percentage of large colonies, their averaged  $\alpha$ SMA/LDH, and CFE and subsequently testing the correlation of these parameters with the corresponding mass culture growth.

The outcome of mass culturing the set of 10 MSC lots was studied during three passages (Fig. 2B). The

lots could be categorized as either slow or fast growing based on their cumulative growth rates (GRs) at passage 3 (GR  $\leq 1$  or  $>1$ , respectively); the difference in GRs between two groups was statistically significant (Fig. 2C).

The three lots that were produced from BM aspirates obtained at AllCells and, coincidentally, from older donors (see Materials and Methods) were all in the slow-growing group; however, this group also contained two lots produced from younger donors from BM aspirates purchased at Lonza, indicating that the donor's age is not a good predictor of MSC growth.

MSC growth data were compared to CFE, percent of large colonies, and  $Av(\alpha$ SMA/LDH)<sub>LC</sub>. Slow-growing lots had significantly higher  $Av(\alpha$ SMA/LDH)<sub>LC</sub> than did fast-growing lots (Fig. 2D). Interestingly, this was true irrespective of how large colonies were defined; however, if defined as those with LDH  $\geq 0.4$ , the statistical difference between  $Av(\alpha$ SMA/LDH)<sub>LC</sub> of the two groups was the greatest ( $p < 0.005$  and  $R^2 = 0.71$ ). There was no correlation between CFE or percentage of large colonies and mass culture growth.



**Figure 2.** Correlation of ALC assay (day 10) and mass culture growth (passage 3) data in 10 BM lots. (A) Normalized  $\alpha\text{SMA}$  [ $\alpha\text{SMA}$ /lactate dehydrogenase (LDH)] plotted against colony size (LDH) for each colony (dot) for two representative lots: AC12 (triangles) and D127 (circles). Linear regression lines are shown. Vertical dotted line shows the arbitrary threshold for large colonies. (B) Mass culture growth rate curves from three passages (M1–M3) for 10 lots. MSC lots with growth rate above 1 were categorized as fast (black circles), and the others as slow (clear triangles). (C, D) Comparison of fast and slow MSC groups: (C) mass culture growth rates on passage 3 and (D) average  $\alpha\text{SMA/LDH}$  in large colonies ( $\text{LDH} \geq 0.4$ ). (E) Correlation between average  $\alpha\text{SMA/LDH}$  in large colonies and growth rate on passage 3; the line shows linear regression for 10 lots; nonzero slope,  $p < 0.02$  and  $R^2 = 0.5138$ . Note that D121 is an outlier; its exclusion gives  $p < 0.01$  and  $R^2 = 0.6846$ . (F) ALC data plot: percentage of large colonies versus average  $\alpha\text{SMA/LDH}$  in them. Note clear separation of lots with the subsequent fast and slow growth.

There was a significant linear correlation between mass culture growth rate and  $Av(\alpha\text{SMA/LDH})_{LC}$  (slope nonzero with  $p < 0.02$ ,  $R^2 = 0.514$ ) (Fig. 2E); removing one outlier (D121) made the correlation stronger. Plotting percentage of large colonies against  $Av(\alpha\text{SMA/LDH})_{LC}$  (Fig. 2F) separated all data points into two groups, one consisted of four lots that all exhibited slow growth and another consisted of all five fast-growing lots plus D121, a slow-growing lot. The culturing data on this lot suggested that it was unintentionally overgrown at passage 2, which could explain why this lot grew slower than was predicted by the ALC assay. Acknowledging the small sample size, the existing data suggested that an  $Av(\alpha\text{SMA/LDH})_{LC} > 100$  predicted subsequent slow growth of mass culture with 100% positive and 80% negative predictive values and 100% specificity and 83% sensitivity.

## DISCUSSION

The goal of this study was to develop a method to predict MSC lot failure early in the BM culturing process, preferably before the first passage. Commercial manufacture defines failure as production of an insufficient number of cells after a defined number of passages using a standardized culture process. We hypothesized that the growth properties of an MSC lot can be predicted on the basis of the characteristics of CFU-f colonies, specifically on  $\alpha\text{SMA}$  expression levels in large colonies. By using a simple colorimetric assay, we determined the average  $\alpha\text{SMA}$  level in cells of large colonies on day 10 after BM plating and showed that this parameter is negatively correlated with the subsequent growth rate of MSC lot up to passage 3.

Scoring MSC lots in part based on large colonies can be justified because of the major contribution of large, but not small, colonies to the resulting cell population. Although we defined large colonies arbitrarily, by intracellular LDH levels corresponding to more than approximately 2,000 cells/colony, the percentage of large colonies likely reflects the proportion of stem cells in the adherent cell population of the donor BM lot. Indeed, several studies have shown that large colonies of CFU-f are formed by MSCs (i.e., tripotent), in contrast to smaller colonies that are believed to be formed by more differentiated progenitors<sup>13,22,23</sup>.

Our MSC growth-predictive model is based on  $\alpha\text{SMA}$  expression in large colonies. Strong  $\alpha\text{SMA}$  expression is known to be a hallmark of large, flat, stress fiber-abundant, nonproliferating senescent MSCs appearing with passaging<sup>24</sup>. A recent study using passaged MSCs demonstrated a direct correlation of  $\alpha\text{SMA}$  expression with the substrate stiffness and reduced clonogenicity and showed  $\alpha\text{SMA}$  regulation by yes-associated protein/transcriptional coactivator with PDZ-binding motif (YAP/TAZ) pathway<sup>19</sup>. Our study shows that  $\alpha\text{SMA}$  is detectable before the first passage, at the CFU-f colony formation stage, that

is, on day 10 or even earlier after BM plating. We find a donor-to-donor variability in  $\alpha\text{SMA}$  expression levels in colonies and establish a correlation of  $\alpha\text{SMA}$  levels in large colonies with future growth rates of MSC lots.

The fact that the percentage of large colonies was not a predictor of mass culture growth relates to the fact that the proliferation of large colonies at a given time does not predict lot growth, though it generally inversely correlates with the colony's level of  $\alpha\text{SMA}$ . In addition to the culture substrate stiffness,  $\alpha\text{SMA}$  expression responds to specific culture conditions, such as medium change frequency (i.e., the removal of factors secreted by growing MSC population and contaminating nonadherent BM cells). Indeed, expression of  $\alpha\text{SMA}$  can be potently induced by transforming growth factor- $\beta$  (TGF- $\beta$ ) and inhibited by platelet-derived growth factor-BB (PDGF-BB)<sup>25</sup>, both factors known to be secreted by MSCs. The influence of the microenvironment on MSCs' ability to proliferate is also evident from a correlation between expression of  $\alpha\text{SMA}$  and proliferation in different areas of the same colonies. This observation is in agreement with the notion of a reciprocity between the inner and the outer region of a colony in the expression of extracellular matrix (ECM) proteins and cell cycle-related proteins<sup>26</sup>. Thus, it is reasonable to suggest that the adaptation of MSCs from an individual donor to given culture conditions is a complex process determined by the functioning of various pathways including those regulating  $\alpha\text{SMA}$  expression, the production of certain growth factors (by MSCs and contaminating cells), and the deposition of ECM. It appears that the average  $\alpha\text{SMA}$  expression level in cells of large colonies could be a reflection of various genetic and external factors shaping MSC growth potential in rigidly defined manufacturing conditions. Identification of pathways and specific factors regulating the adaptation of MSCs to in vitro culture conditions is a matter of future studies. The assay described here can help to exclude lots with low growth potential and could be especially effective if lot exclusion criteria are adjusted for specific manufacturing conditions that may differ from those described here. In conclusion, the ALC assay assesses  $\alpha\text{SMA}$  in large CFU-f colonies a few days after plating of BM. It provides a simple and easily standardized measurement that predicts the subsequent growth of MSCs. It could be readily incorporated as a screen to increase efficiency and reduce cost in manufacturing large-scale batches of MSCs for clinical use.

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