Inherent and Emergent Heterogeneity in Clonal Stem Cell Populations

[1,2]Brian D. Cosgrove
[1,2]Megan J. Farrell
[1]Margaret Dunagin
[1,2]Claire M. McLeod
[1,3]Allison J. Cote
[1]Arjun Raj, PhD
[1,2]Robert L. Mauck, PhD

[1]University of Pennsylvania, Department of Bioengineering, Philadelphia, PA, USA

[2]University of Pennsylvania, Department of Orthopaedic Surgery, Philadelphia, PA, USA

[3]University of Pennsylvania, Department of Cell and Molecular Biology, Philadelphia, PA, USA

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bcosg@seas.upenn.edu

Introduction

One limitation in the use of mesenchymal stem cells (MSCs) for tissue engineering is the heterogeneity inherent to these cell populations. Previous studies have characterized variability between clones with respect to differentiation potential for various lineages,[1] single cell mechanical properties,[2] and traction force generation.[3] Our previous work showed large variations between clones from the same donor (inter-clonal) and within individual clones (intra-clonal) with respect to traction force generation in clonal bMSC populations (passage 4, >28 days of culture).[4] The goal of this study was to better characterize when heterogeneity develops within primary MSC populations, using both biophysical (cell traction) and transcriptional assays (single molecule RNA FISH). By evaluating both non-selected MSC populations ("Mixed") derived from standard isolation protocols and donor-matched clonal populations in various culture conditions, we better defined the heterogeneity that is inherent to these populations. Further we showed how increased variability emerges during early clone isolation and with the addition of soluble factors.

Methods

Cell Culture and Clonal Isolation

Juvenile bovine MSCs were isolated from bone marrow as in Huang *et al*.[5] Multiple clonal populations from one donor were isolated using the trypsin spot method.[6]

Traction Force Microscopy (TFM)

5 kPa polyacrylamide hydrogels (ν=0.45) were prepared as in Aratyn-Schaus *et al*.[7] In short, 0.2μm-diameter fluorescent microspheres were mixed into PA at 1% v/v. Fibronectin (20 μg/mL) coating of gel surfaces was accomplished with 2 mg/mL sulfo-SANPAH. For early timepoint TFM studies, clones were acquired following 14d of culture in basal medium ("BM": DMEM & 10%FBS). Additional TFM studies evaluating cells cultured in chemically defined media supplemented with and without 10 ng/mL TGF-β3 ("CM− & CM+") were performed using passage 3 (p3) mixed MSC populations. TFM data analysis (PIV, FTTC, stack alignment) was performed using a freely available plugin suite for ImageJ created by Q. Tseng.[8] Cellular areas were measured in ImageJ.

Fluorescent In-Situ Hybridization (RNA FISH)

Probe hybridization was completed as detailed in Raj *et al*.[9] In short, multiple singly-labeled oligonucleotide probes (for aggrecan [AGC], GAPDH, and cartilage oligomeric matrix protein [COMP] gene sequences) were applied to fixed and permeabilized cells. Single mRNA molecules were quantified using a custom MATLAB script.[9] In these studies, p3 clonal and mixed populations were cultured in either CM− or CM+ for 7 days before FISH was performed. To query expression in fresh isolates, colonies were identified after 11 days of culture in BM. At this point, the media was changed to CM+ for four additional days before FISH analysis of these nascent colonies.

Statistics and Data Analysis

Mann-Whitney U tests adjusted for multiple comparisons were used to compare across TFM groups. Violin plots and kernel density data were generated using the ggplot2 library.

Results

Traction forces across two clones ("clone 1 & 2") and a donor matched mixed MSC population ("Mixed") were measured after 3d of culture (Figure 1). While there were no significant differences in cell area, TFM showed significant inter-clonal differences in traction stress generation, with intra-clonal standard deviations that were of similar magnitude to those within the Mixed population (c1: 50% of Mixed, c2: 115% of Mixed). Non-selected MSC populations (p3) were cultured for 16 hours with and without TGF-β3 (CM-/CM+) and then subjected to TFM. In these populations, the addition of TGF-β3 increased cellular contractility by ~2.7-fold, with the CM+ population exhibiting a much higher variability in traction stress (Figure 1C). Next, later passage (p3) mixed and clonal MSC populations were analyzed by RNA FISH. In the presence of TGF-β3, average COMP expression (across all groups) increased ~200-

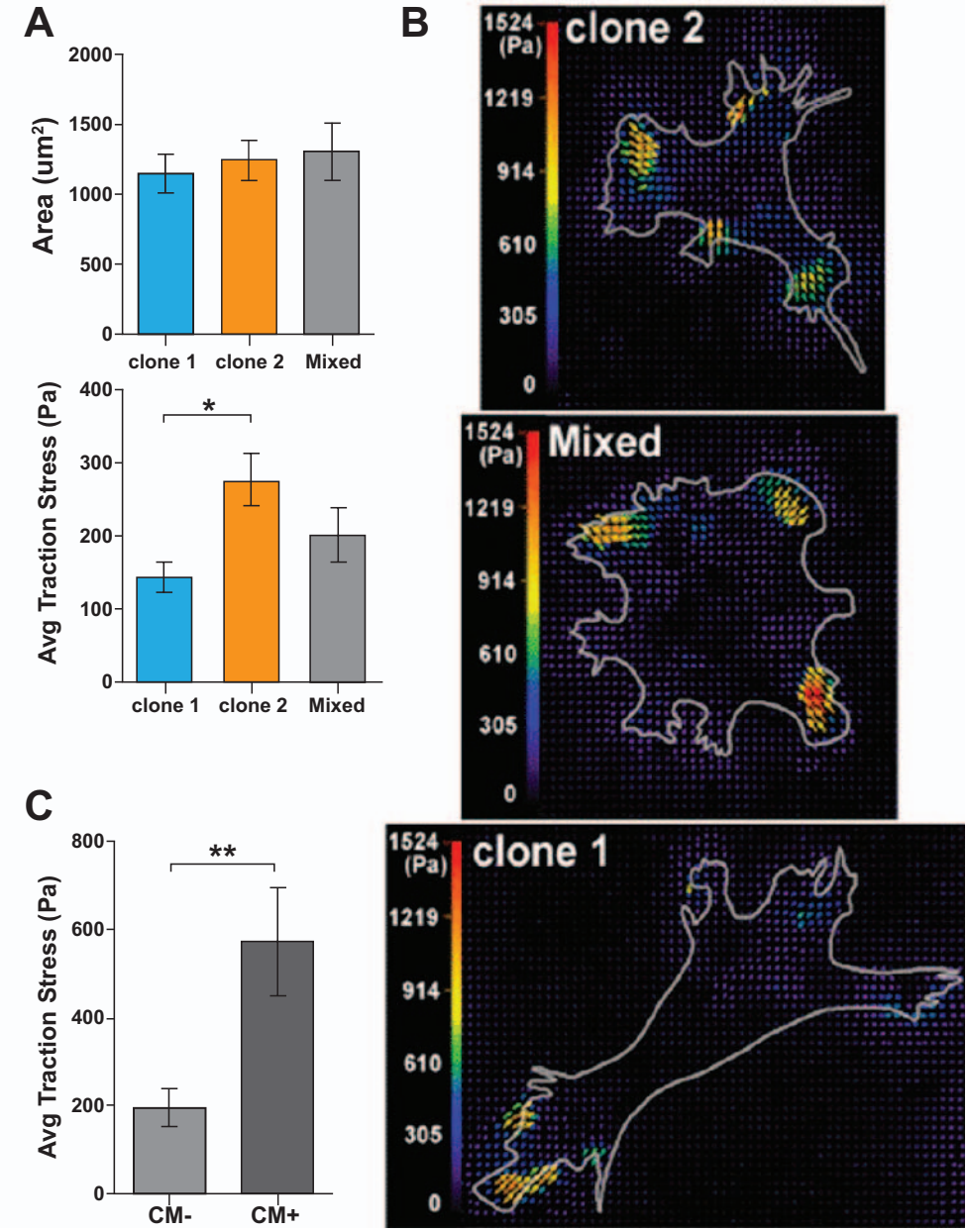

Figure 1. (A) Cellular area and average traction stress generation. n=13-19 cells per group. * denotes p<0.05. (B) Representative traction stress vector maps from Figure 1A. (C) Average traction stress generation of a late stage MSC population (p3) in CM− or CM+ n=21-23 cells per group. ** denotes p<0.01.

fold, while AGC expression increased by ~20-fold (Figure 2A/B). Interestingly, not every clone responded the same way (inter-clonal variability), and within the same clone, copy number showed a wide variability, in most cases more so than the variation seen in the mixed population. Finally, RNA FISH of very early passage colonies (i.e., those still in their initial colony) revealed that intra-clonal mRNA levels were heterogeneous with chondroinduction, before any passaging had occurred, and that this heterogeneity did not appear to depend on location within the colony (Figure 3).

Discussion

Since each MSC clone arises from the division of an initially adherent parent cell, it has been assumed that clones should represent a distinct subpopulation and be more homogeneous than non-selected MSCs acquired using traditional methods. Surprisingly, extensive biophysical heterogeneity exists both between clones and within clones. RNA FISH of initial p0 colonies revealed that this intra-clonal heterogeneity exists at very early stages of colony formation, suggesting that MSC heterogeneity emerges very early. When MSC populations were cultured for longer time periods, and induced with TGF-β3, MSC heterogeneity was further amplified. After only 16 hours of induction, MSC populations (normal and clonal) induced with TGF-β3 exhibited more variability in traction stress than those without TGF-β3 exposure. Interestingly, mRNA levels of cartilage markers (COMP/AGC) within a clone did not always correlate (clone D). Nearly every clone exhibited a much

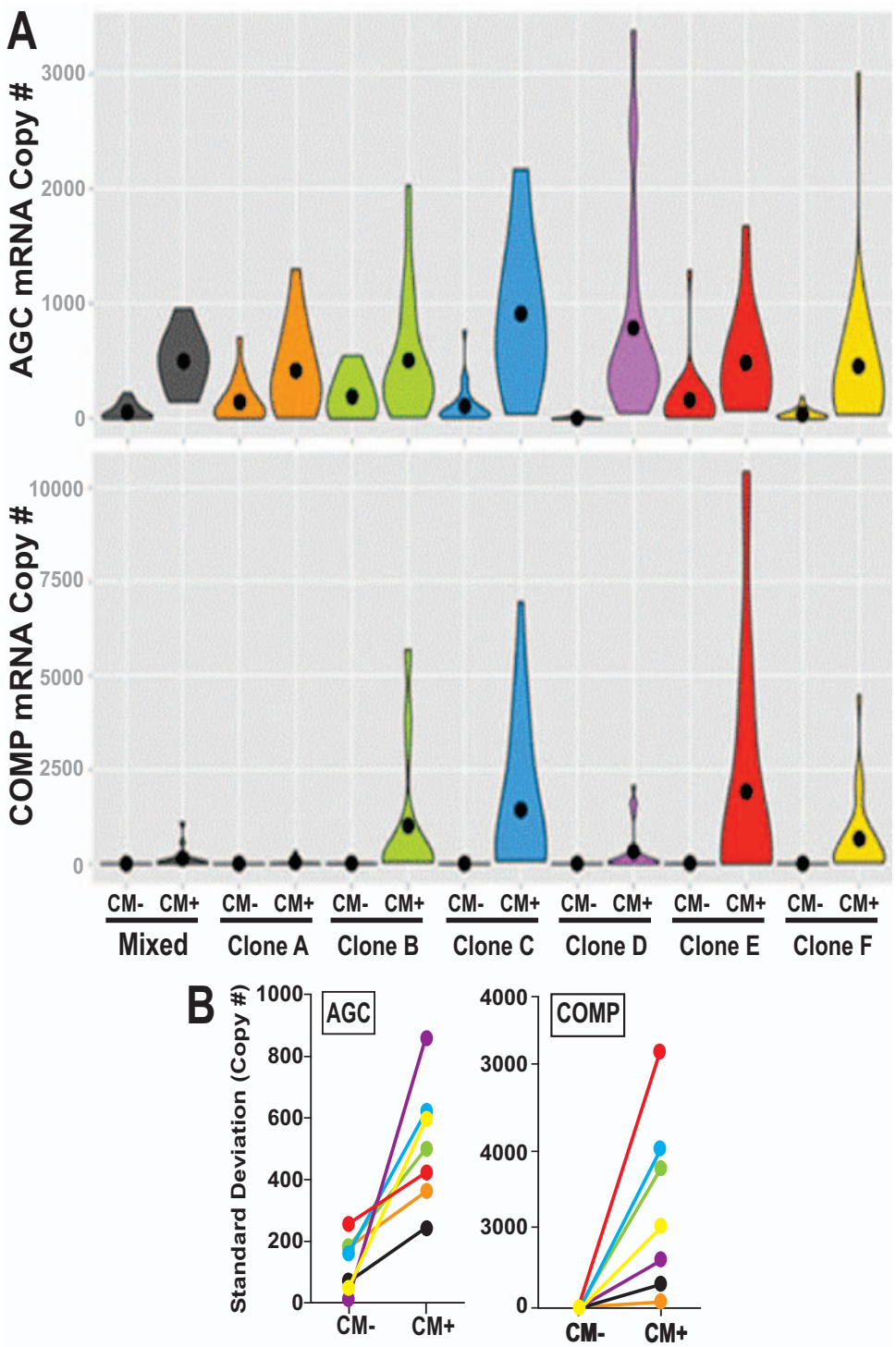

Figure 2. (A) Violin plots of mRNA copy numbers (for each group: y-axis = range of data, x-axis = normalized probability density function of data, black dot = group mean). n=16-31 cells per group. (B) Standard deviation values for each group from Figure 2A.

larger range of mRNA copy number than the donor-matched parent MSC population.This suggests that the traditional idea of the heterogeneity in a mixed MSC population being the sum of its clonal parts may not be entirely correct. Rather, our data suggests a more complicated scenario in which both emergent and inherent heterogeneity prevails in MSC populations. Further studies will examine how this variability can be reduced during the expansion process and work towards a better understanding of how such heterogeneity influences MSC differentiation.

Significance

Cartilage tissue engineering approaches have been hampered by MSC heterogeneity, but characterizations of this heterogeneity have not clearly established at what point this heterogeneity arises. Results from this study suggest

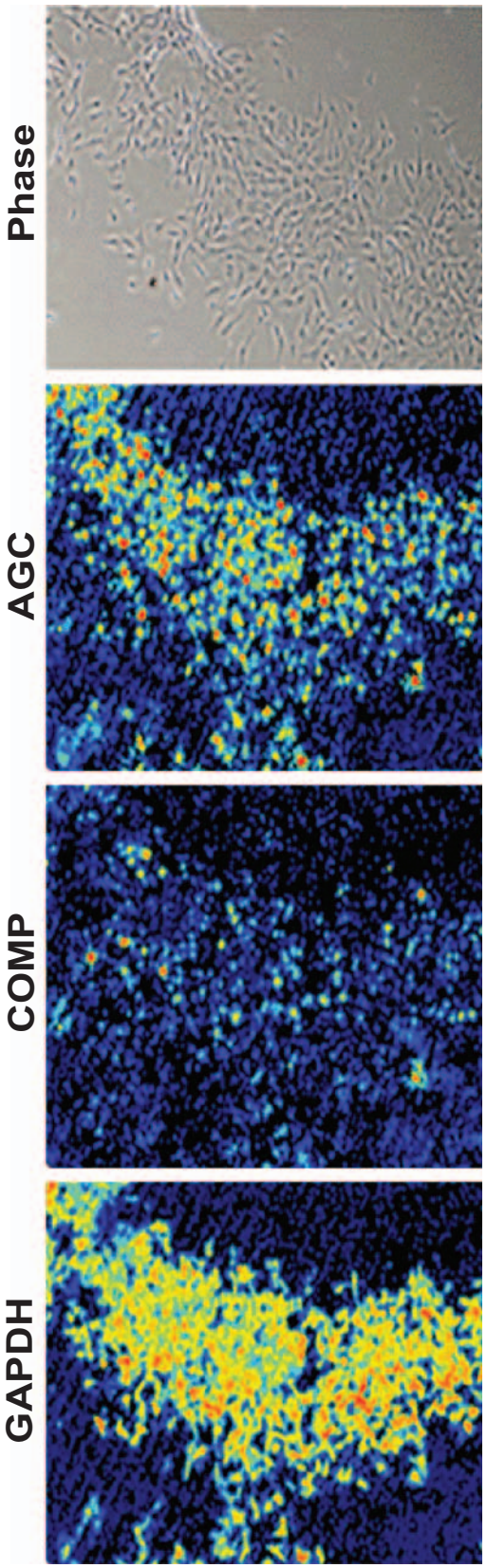

Figure 3. RNA FISH cluster scans of an initial passage (p0) MSC colony. Nucleus-centered pseudocolor dots represent the number of mRNA molecules within a single cell (relative scale is from: dark blue=no copies, to red=most copies).

that heterogeneity emerges early in the derivation of MSC clonal populations, exists both within and between clonal populations of MSCs, and persists throughout later passages.

Acknowledgments

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