Single-cell analysis of cultured bone marrow stromal cells reveals high similarity to fibroblasts in situ

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Within the heterogenous pool of bone marrow stromal cells, mesenchymal stromal cells (MSCs) are of particular interest because of their hematopoiesis-supporting capacities, contribution to disease progression, therapy resistance, and leukemic initiation. Cultured bone marrow-derived stromal cells (cBMSCs) are used for in vitro modeling of hematopoiesis−stroma interactions, validation of disease mechanisms, and screening for therapeutic targets. Here, we place cBMSCs (mouse and human) in a bone marrow tissue context by systematically comparing the transcriptome of plastic-adherent cells on a single-cell level with in vivo counterparts. Cultured BMSCs encompass a rather homogenous cell population, independent of the isolation method used and, although still possessing hematopoiesis-supporting capacity, are distinct from freshly isolated MSCs and more akin to in vivo fibroblast populations. Informed by combined cell trajectories and pathway analyses, we illustrate that TGFb inhibition in vitro can preserve a more “MSC”-like phenotype

HIGHLIGHTS

- Cultured BM stromal cells are distinct from in vivo mesenchymal stromal cells.
- Cultured BM stromal cells are transcriptionally similar to in vivo fibroblasts.
- Cultured BM stromal cells retain plasticity ex vivo.
- Cultured BM stromal cells have hematopoiesis-support capacity, increased through TGFb inhibition.

Bone marrow stromal cells (BMSCs) are essential cells of the bone marrow (BM) [1,2]. Particularly mesenchymal stromal cells (MSCs), also referred to as CXCL12-abundant reticular (CAR) cells, have been reported to play key roles in both the homeostatic and malignant BM niches [1]. Classically, MSCs are defined by in vitro characteristics including trilineage differentiation [1,2] and typical surface marker expression and, thus, are often termed *progenitor stromal cells* [3]. Cultured bone marrow-derived stromal cells (cBMSCs) are relatively easy to propagate and are used widely for in vitro assays. However, previous transcriptional studies point toward an altered biological state in vitro and raise the question of how these cBMSCs relate to their in vivo stromal counterparts [4,5]. Here, we systematically compare in vivo BMSCs with in vitro cBMSCs on a single-cell level in both human and mouse to explore their biological role in greater detail.

METHODS

Methods are described in detail in the Supplementary Data (online only, available at www.exphem.org).

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USAS and BB contributed equally to this work. USAS and BB designed and carried out experiments, analyzed results, and wrote the article. IAMS, JSN, NBL, RL, AB, JP, HM, SN, and EB performed experiments, analyzed, interpreted data, and reviewed the article. IGC and RKS obtained funding, designed the study, performed experiments, analyzed data, and wrote the article. All authors provided critical analysis of the article.

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Animal Studies

All mouse studies were conducted according to protocols approved by the Central Animal Committee (Centrale Commissie Dierproeven [CCD], Netherlands) in accordance with legislation in The Netherlands (Approval No. AVD1010020173387).

Murine cBMSCs

For murine cBMSC isolation, bones of adult wild-type (WT) mice were crushed and digested as described previously under normoxic conditions [6]. For single-cell RNA sequencing (scRNA-seq), one Ptprca Pepcb/BoyCrl (B6.SJL) mouse and one Mx1-Cre^{+} mouse without activation of Cre recombinase were used. Samples were kept separate throughout and combined bioinformatically during downstream analysis. Cells were passaged every 3−5 days at approximately 80% confluence and sort-purified for viable/CD11b^{-} single cells to submit to scRNA-seq or flow cytometric analysis after 21 days at passage 3. For TGFb stimulation, cells were treated with 2.5 ng/μL human recombinant TGF-b1 (Invivogen, San Diego, CA). For TGFb inhibition, cells were treated with 10 μmol/L TGFb inhibitor SB431542 (StemCell Technologies, Vancouver, BC, Canada).

Human cBMSC Isolation

For human cBMSC isolation, bone marrow aspirates from a 57-year-old man with follicular lymphoma grade 2 without BM infiltration and digested femur head biopsy from a 66-year-old female orthopedic patient undergoing hip replacement surgery were washed, and whole BM was plated out. Cultured BMSCs were isolated by attachment selection and passaged every 5−7 days at approximately 80% confluence. At passage 3, the cells were used as input for scRNA-seq. All patient material was de-identified at inclusion (Ethics Approval Nos. MEC-2018-1445 and EK300-13).

RESULTS AND DISCUSSION

We used a widely applied protocol to grow out cBMSCs from murine bone chips in 2-D culture [6]. Early-passage murine cBMSCs, capable of trilineage differentiation (Supplementary Figure E1A, online only, available at www.exphem.org), were submitted to scRNA-seq to investigate transcriptomic profiles and potential heterogeneity within the pool of cultured cells. Unsupervised clustering of two merged biologically independent cBMSC samples revealed a rather homogenous cell population (Figure 1A; Supplementary Figure E1B,C). Probing different clustering resolutions and differentially expressed genes between clusters, we found that differences between subclusters were subtle and derived mainly from gradients in cell cycle state, hypoxia signature, transforming growth factor β (TGFb) signaling, and extracellular matrix (ECM) protein production (Figure 1B; Supplementary Figure E1D), highlighting that cBMSCs seem to be rather uniform. Standard (surface) markers used to define stromal cell populations also did not demarcate distinct cell populations among the cultured cells on gene expression (Supplementary Figure E1D) or on the protein level (Figure 2E; Supplementary Figure E2D, online only, available at www.exphem.org).

To investigate the transcriptomic similarity of prospectively isolated BMSCs in more detail, we integrated the cBMSC data set with published murine in vivo scRNAseq data sets consisting of a multitude of stromal populations [2,7]. Integration of data sets revealed minimal overlap of cultured cells with the in vivo populations, but interestingly, cBMSCs associated very closely with fibroblast (FB) and fibroblast/chondrocyte progenitor clusters (Figures 1C and 2A). Bootstrap-based hierarchical clustering of pseudobulk cluster transcriptomes indicated a significant overlap of the FB cluster with the cBMSC cluster, while MSCs cluster separately (Supplementary Figure E1E). It is plausible that with the use of bone chips, FB-like cells are either enriched or selected for in vitro. To further investigate the transcriptional similarity to our cBMSC data set, we used CIBERSORT to quantify the cellular signatures of published bulk stromal populations (Figure 1D; Supplementary Figure E1I). Data sets from prospectively sorted stromal cell reporter lines such as Gli1, LepR, NG2, and Myh11 revealed considerable alignment within the MSC cluster. In contrast, sorted and in vitro propagated Gli1;tdTom^{+} cells aligned with the cBMSC signature, suggesting that their in vivo identity is changed ex vivo and that adherent culture supports development of a FB-like phenotype. As cBMSCs are often used experimentally to validate hematopoiesis−stromal interactions, we assessed the expression of hematopoiesis support factors (Figure 1E,F). Cultured BMSCs exhibit a lower aggregate expression of hematopoiesis support factors compared with freshly isolated MSCs, but a higher aggregate of expression compared with isolated FBs. Importantly, the potential hematopoiesis support is still preserved in an in vitro setting as illustrated by the routinely performed propagation of hematopoietic cells on top of cBMSCs—a protocol employed for the last 50 years [8,9]. However, it seems that cBMSCs are less specialized for this function than in vivo MSCs. MSCs produce a specific array of key chemokines (Supplementary Figure E1H), likely guided through the interactions with other cells in the BM that are absent in a vastly simplified in vitro BM setting. We, and others, have used cBMSCs to evaluate stromal contributions to fibrotic transformation. Cultured BMSCs exhibited high aggregate expression of a matrisome signature (Supplementary Figure E1G) [10], as well as collagens (Figure 1E), comparable to freshly isolated MSCs and FBs, indicating their matrix-depositing potential.

We next wondered whether primary human cBMSCs (hcBMSCs) capable of trilineage differentiation (Supplementary Figure E2A) exhibit a similar transcriptome signature. Human cultured BMSCs from two independent donors without bone marrow disease were isolated by classic attachment selection before scRNA-seq. Samples overlapped well and revealed a homogenous cell population with mainly cell cycle-driven differences between subclusters (Supplementary Figure E1J). We integrated hcBMSCs with recently published prospectively sorted human BM stroma data sets, consisting of nine individuals [7,11]. Interestingly, and in line with the murine data, we observed a clear association of hcBMSCs with prospectively sorted in vivo hFBs as the cells mapped to the same cluster (Figure 1G; top markers shown in Supplementary Figure E1K). With CIBERSORT deconvolution (Figure 1H), we found that freshly isolated CD271^{+}CD105^{+} hBMSCs were indeed enriched within the scRNA-seq hMSC cluster [4]. In contrast, prospectively sorted CD271^{+} cells [5] aligned mostly with the hMSC cluster and partially with the hFB/hcBMSC cluster, indicating that CD271 alone might not solely mark hMSCs in vivo. In parallel, the same CD271^{+} cells were sorted for in vitro culturing in the published study. On culturing, their signature aligned with the hFB/hcBMSC cluster and no longer with the hMSC cluster, irrespective of passage number. Strikingly, 3-D-cultured CD271^{+} cells retained a more pronounced hOLC/hMSC-like signature, while also exhibiting a hFB/hcBMSC signature (Figure 1H;

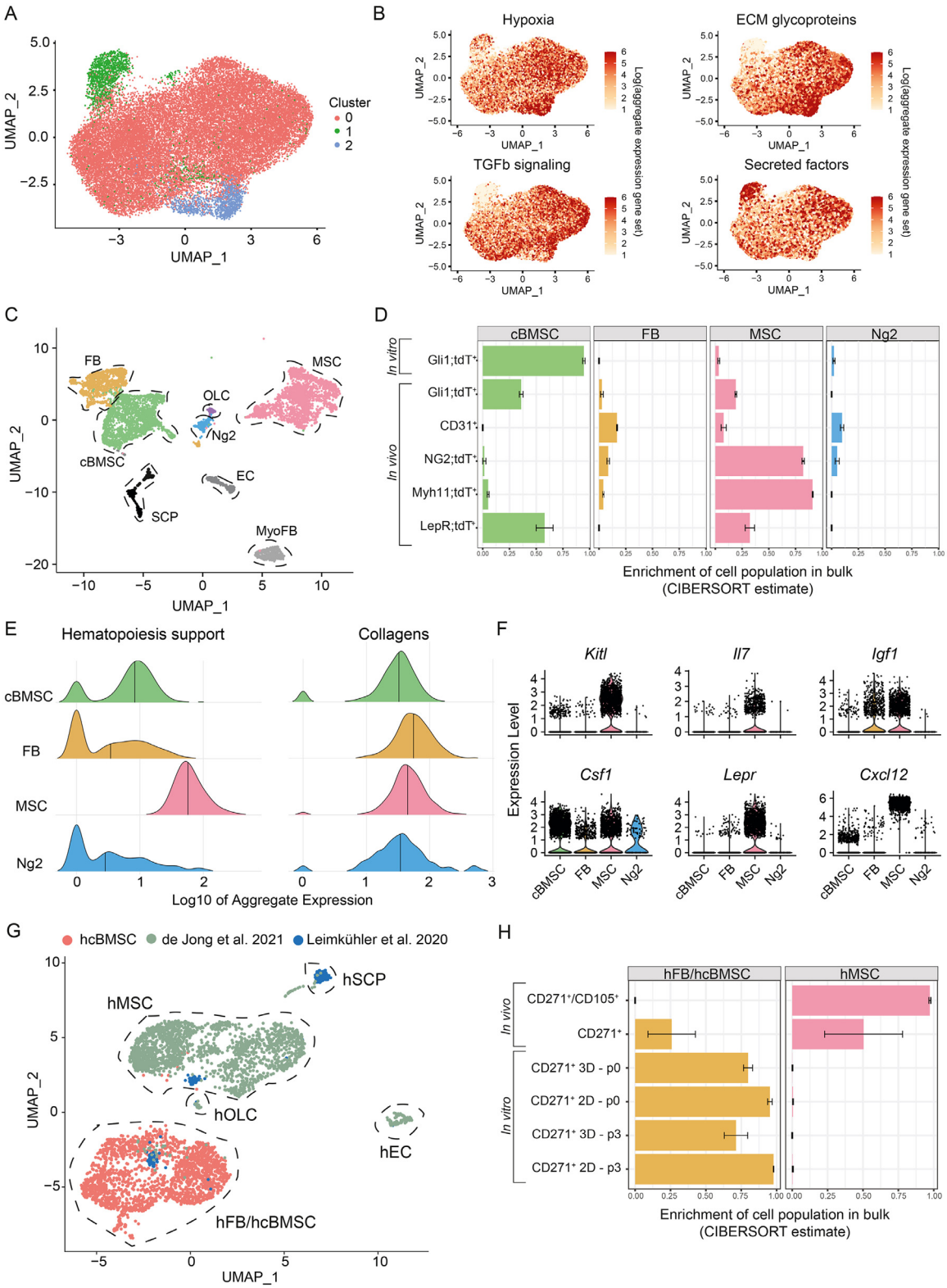

Figure 1 Murine and human cultured bone marrow stromal cells are homogenous and are closely related to putative in vivo fibroblasts (see also Supplementary Figure E1, online only). **(A)** UMAP of two merged cBMSC samples at passage 3, $n = 27{,}306$ cells. **(B)** UMAP visualization of cBMSC cells featuring aggregated gene set expression of the Hallmark gene sets "Hypoxia" and "TGF_beta_-signaling" and the NABA Matrisome gene sets "ECM_Glycoproteins" and "Secreted Factors." **(C)** UMAP visualization of integrated

Supplementary Figure E1L). Given the interesting recent observation that 2-D-cultured hcBMSCs are perhaps functionally different from 3-D hcBMSC sphere cultures [12], it could be postulated that 3-D culture reduces the selection pressure on certain cell types. In summary, our analyses indicate that regardless of the isolation method (i.e., growing out from digested bone chips, adherence selection from human aspirate or digested femur head biopsy, or prospective sorting of MSC-like cells into culture), propagated "putative MSCs" in vitro resemble BM-resident fibroblasts. Although a pure FB population in the BM is still a matter of debate, multiple publications have provided convincing evidence that these stromal cells indeed reside within the BM and not just in the periosteum [2,13].

We used trajectory prediction methods to visualize directionality of differentiation within cBMSCs and in vivo stromal cells (Figure 2A,B). NG2+ MSCs were placed at the apex of the differentiation trajectory as has been described previously [2], with a differentiation path toward MSCs. Cultured BMSCs were positioned between the Ng2+ MSCs and FB clusters. Interestingly, cBMSCs exhibited two directionality patterns, toward FBs and toward Ng2+ MSCs, indicative of a possible intermediate cellular state (Figure 2B). Top genes exhibiting dynamic splicing behavior in the cBMSC cluster include *Col5a2, S100a6, CD44,* and *Csf1*. *Col5a2* and *CD44* are highly expressed in the FB/cBMSC branch and increased in the unspliced/spliced ratio in the cBMSC cluster (Supplementary Figure E2B). Interestingly, *CD44* has been described as a marker of inflammatory stromal cells [11] and cancer-associated fibroblasts [14]. *Csf1,* on the other hand, is increased in unspliced/spliced ratio in the cBMSC cluster and highly expressed in the Ng2/MSC branch (Supplementary Figure E2B). *Csf1*, encoding for macrophage colony-stimulating factor 1, is an important cytokine secreted by osteoblasts, concerting paracrine activation of osteoclastogenesis [15] and playing a role in hematopoiesis regulation [16]. To evaluate more globally which signaling pathways drive cellular identity, we employed the pathway footprinting tool PROGENy [17]. Cultured BMSCs exhibited increased activation of PI3K, MAPK, and TGFb signaling, while MSCs had upregulation of estrogen and JAK−STAT signaling (Figure 2C,D). We thus hypothesized that TGFb signaling was a factor responsible for facilitating the enrichment of FB-like cells in culture. To test this, we digested murine bone chips as previously described [6], but kept cells under constant TGFb inhibition or TGFb stimulation (Supplementary Figure E2C). Cells from all culturing conditions retained surface marker expression that is generally considered "MSC-like" (Figure 2E; Supplementary Figure E2D), highlighting that these commonly used surface markers are insufficient to distinguish between MSC- and FB-like cells in vitro [18]. As hypothesized, cells under constant TGFb inhibition exhibited a trend of increased expression of hematopoiesis-supporting genes (*Cxcl12, Kitl, Lpl*), more Ng2− surface marker expression (CD200, CD63) indicating a more immature phenotype, and higher expression of "MSC" markers (VCAM1, PDGFRA/B, LAMP1) (Figure 2E). CD61 expression was markedly reduced in TGFb inhibitor-treated cells, in line with the previous observation that CD61 is directly responsive to TGFb and promotes mesenchymal cell differentiation [19]. TGFb inhibition in stromal cells increased survival and quiescence of co-cultured ckit-enriched hematopoietic stem and progenitor cells (Supplementary Figure E2E−G), indicating superior hematopoiesis support compared with cBMSCs cultured in the absence of TGFb inhibition. Thus, our findings indicate that cBMSCs still retain plasticity in vitro, which can be preserved by TGFb inhibition to ensure a more "MSC"-like phenotype of cultured cells.

The nomenclature and functionality of stromal cell populations within the BM are still a matter of discussion. Here, we add granularity to the subject matter by providing insights into cBMSCs at a higher resolution using single-cell data. Although 2-D cBMSCs still express hematopoiesis-supporting genes and have a certain extent of plasticity in vitro, they are less MSC-like than previously thought. Our data highlight the fact that BMSCs in vitro are distinct from in vivo stromal populations and that this finding needs to be considered when interpreting in vitro assays and disease modeling. TGFb inhibition can be applied as a rather simple tool to preserve an "MSC-like" phenotype if needed for the downstream analysis.

Declaration of Competing Interest

The authors declare no conflict of interests.

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murine cBMSCs and stromal populations from the Baccin et al. [2] and Leimkühler et al. [7] data sets. Supplementary Figure E1F illustrates top cluster markers. **(D)** CIBERSORT enrichment of the gene expression signatures of cBMSCs, FBs, MSCs, and Ng2+ cells in previously published transcriptomes of relevant stromal populations. CD31+ cells serve as a control and are aligned neither with cBMSC nor MSC signatures but with endothelial cells (see also Figure S1I). Error bars represent the means. **(E)** Density ridge plot of aggregated expression of NABA Collagens geneset and Hematopoiesis support geneset of integrated object shown in 1C. **(F)** Violin plots of single-cell expression distributions of hematopoiesis support genes in clusters cBMSC, FB, MSC, Ng2+ MSC, each dot corresponding to an individual cell. **(G)** UMAP visualization of integrated human data sets. Human cultured BMSCs combined with published bone marrow stromal niche data [7,11]. S1K shows cluster markers. **(H)** CIBERSORT enrichment of the gene expression signatures of hMSCs and hFBs/hBMSCs in previously published transcriptomes of prospectively sorted cells. $CD271^+$/$CD105^+$ denotes prospectively sorted $CD45^-CD235a^-CD31^-CD271^+CD105^+$ cells [4], directly used for bulk RNA sequencing. $CD271^+$ denotes prospectively sorted $Lin^-CD45^-CD31^-CD71^-CD235a^-/CD271^+$ cells, that were either directly sorted for microarray analysis (in vivo), or sorted into 2-D or 3-D culture (in vitro) and sequenced at either passage 0 or passage 3 [5]. Error bars represent the means (see also Supplementary Figure E1L, online only). *EC*=endothelial cells; *FB*=fibroblast; *cBMSC*=cultured bone marrow stromal cells; *hcBMSC*=human cultured bone marrow stromal cells; *hEC*=human endothelial cells; *hFB*=human fibroblasts; *hMSC*=human mesenchymal cells; *MSC*=mesenchymal stromal cells; *hOLC*=human osteolineage cells, hSCP=human Schwann cell progenitors; *MyoFB*=myofibroblasts; *Ng2*=Ng2+ MSCs; *UMAP*=Uniform Manifold Approximation and Projection.

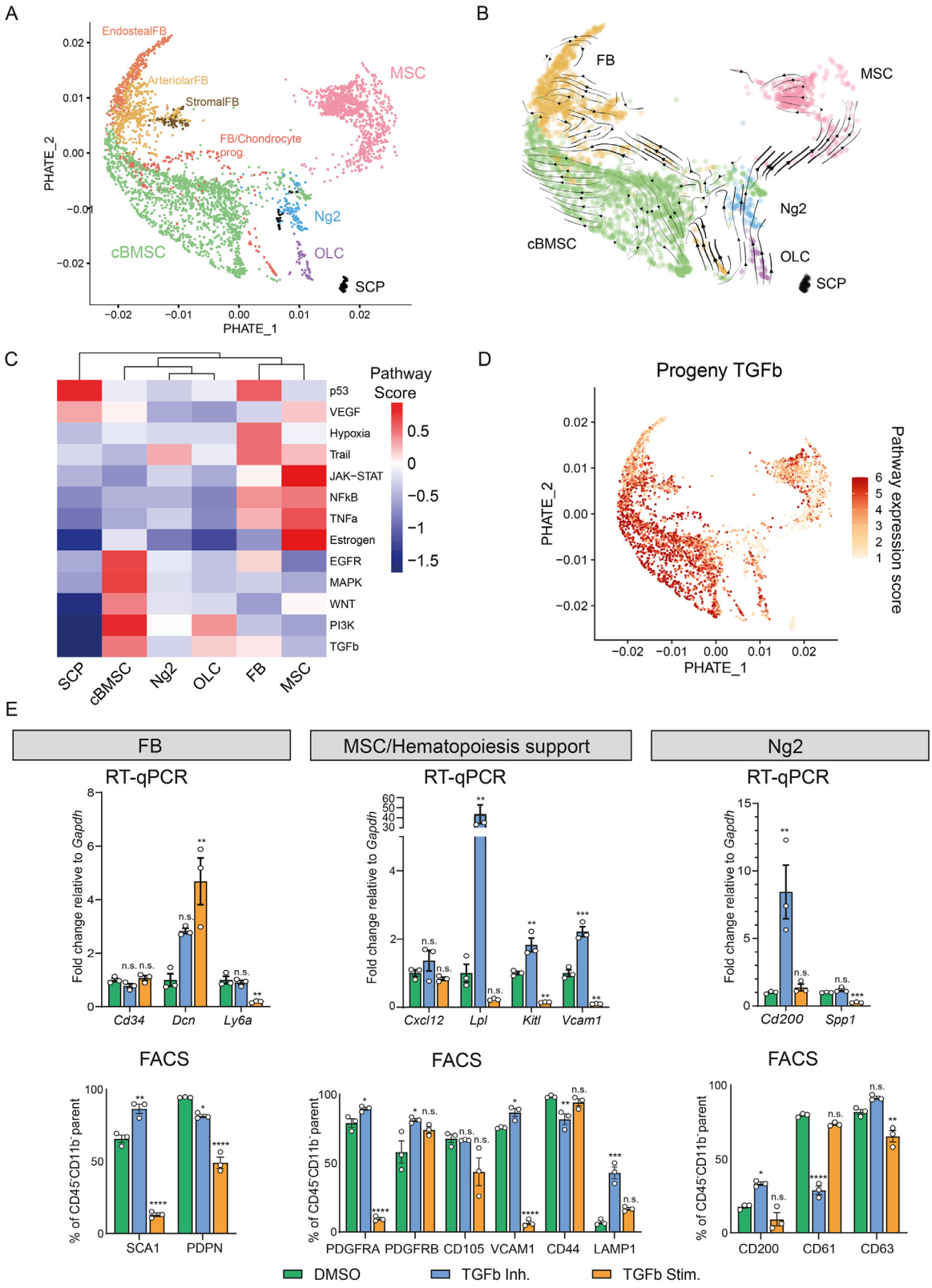

Figure 2 Cultured bone marrow stromal cells retain plasticity and are altered by TGFb signaling (see also Figure E2, online only). **(A)** Dimensionality reduction PHATE projection of murine integrated data set as in Figure 1C. **(B)** RNA velocity projected on PHATE dimensionality reduction of murine integrated data set. **(C)** Heatmap of pathway response signature scores for 14 pathways per cell identity cluster using the method PROGENy (Pathway Responsive Genes for Activity Inference). **(D)** PHATE visualization of clustered

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.exphem.2022.03.010.

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cells featuring pathway score expression of PROGENy gene signature TGFb. **(E)** RT-qPCR and FACS of cBMSCs with TGFb inhibition or stimulation or control (DMSO). Bars for RT-qPCR represent mean fold change of respective genes relative to *Gapdh.* Bars for FACS represent mean frequencies of live/$CD45^+$/$CD11b^-$ cells positive for respective surface marker (individual data points represent $n = 3$ biological replicates, error bars = SEM). One-way analysis of variance performed per gene/surface marker with multiple comparisons; significance shown compared with dimethyl sulfoxide control; *n.s.*=not significant, $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p < 0.0001$. *DMSO*=dimethyl sulfoxide; *FACS*=fluorescence-activate cell sorting; *PHATE*=potential of heat-diffusion for affinity-based trajectory embedding; *RT-qPCR*=reverse transcription quantitative polymerase chain reaction; *TGFb*=transforming growth factor β.