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Intercellular Transfer of Microvesicles from Young Mesenchymal Stromal Cells Rejuvenates Aged Murine Hematopoietic Stem Cells

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ABSTRACT

Donor age is one of the major concerns in bone marrow transplantation, as the aged hematopoietic stem cells (HSCs) fail to engraft efficiently. Here, using murine system, we show that a brief interaction of aged HSCs with young mesenchymal stromal cells (MSCs) rejuvenates them and restores their functionality via inter-cellular transfer of microvesicles (MVs) containing autophagy-related mRNAs. Importantly, we show that MSCs gain activated AKT signaling as a function of aging. Activated AKT reduces the levels of autophagy-related mRNAs in their MVs, and partitions miR-17 and miR-34a into their exosomes, which upon transfer into HSCs downregulate their autophagy-inducing mRNAs. Our data identify previously unknown mechanisms operative in the niche-mediated aging of HSCs. Inhibition of AKT in aged MSCs increases the levels of autophagy-related mRNAs in their MVs and reduces the levels of miR-17 and miR-34a in their exosomes. Interestingly, transplantation experiments showed that the rejuvenating power of these "rescued" MVs is even better than that of the young MVs. We demonstrate that such ex vivo rejuvenation of aged HSCs could expand donor cohort and improve transplantation efficacy. STEM CELLS *2018;36:420–433*

SIGNIFICANCE STATEMENT

Hematopoietic stem cells (HSCs) of aged individuals are dysfunctional, limiting their applications in bone marrow transplantations. This study reports a novel finding that aged mesenchymal stromal cells (MSCs) possess activated AKT signaling, which affects the RNA profile of their vesicles. Intercellular transfer of these vesicles causes aging of HSCs. Importantly, vesicles collected from young MSCs or from aged MSCs treated with AKT inhibitors rejuvenate aged HSCs and restore their functionality. Such pretreatment of aged HSCs would improve the efficacy of autologous transplants and also expand donor cohort. These data have implications in other stem cell-based therapies as well.

INTRODUCTION

Donor age is one of the major concerns in bone marrow (BM) transplantation (BMT). Studies on murine system have demonstrated that aged marrow harbors increased pool of phenotypically defined hematopoietic stem cells (HSCs) exhibiting myeloid bias and having compromised competitive repopulating ability [1–3]. Aged HSCs also exhibit multiple epigenome and transcriptome changes [4]. DNA damage, replication stress, and ribosomal stress have been shown to cause aging of HSCs [5, 6].

Age-associated changes in human HSCs were similar to those observed in mouse HSCs, suggesting that hematopoietic aging is an evolutionarily conserved process [7]. In contrast to mouse model of aging, the frequency of non-obese diabetic/severe combined immunodeficient (NOD/SCID)/IL2 receptor gamma (IL2Rc) null (NSG) repopulating cells present in the BM of elderly subjects was not affected by age [8]. However, a retrospective study done in BMT patients showed age as the only donor trait associated with their overall and disease-free survival [9]. Similarly, in patients undergoing autologous stem cell transplant (SCT), a close correlation of aging with impaired long-term hematopoiesis regeneration was found [10]. This was also supported by the finding in patients undergoing reduced intensity allogenic SCT, that the younger donor age was associated with better outcome [11].

Since donor age is such an important concern in BMT, it might be argued that the upper limit of donor age may be lowered. However, patients having an older individual as the sole HLA-matched donor could be denied access to

this potentially life-saving treatment. To overcome this impediment, efforts are being made to rejuvenate aged HSCs to improve their performance [12–15].

This study reports a novel finding that a brief exposure of aged HSCs to young mesenchymal stromal cells (MSCs) rejuvenates them via intercellular transfer of microvesicles (MVs) containing "youth signals." We also demonstrate that intercellular transfer of aged exosomes carrying negative regulators of autophagy causes aging of HSCs. Our data are relevant in both allogenic as well as autologous transplantations involving older individuals as donors and recipients, respectively. Rejuvenation of aged HSCs before transplantation could expand donor cohort and also help older individuals undergoing autologous SCT.

Materials and Methods

Animals

C57BL/6J (CD45.2) and B6.SJL- Ptprc^{a} Pep3^{b}/BoyJ (CD45.1) mice purchased from Jackson Laboratory (Bar Harbor, ME) were housed and bred in the institutional experimental animal facility. In this study, 6–8 weeks (young) and 18–24 months (aged) old mice were used. All procedures were approved by the Institutional Animal Care and Use Committee (IEAC/2014/B-178-II) of National Centre for Cell Sciences, Pune, India.

Cell Culture

BM-derived Lin^{-} cells or sort-purified Lin^{-}Sca-1^{+}c-Kit^{+} (LSK) HSCs were either cocultured with MSCs or treated with extra-cellular vesicles (EVs), MVs, or exosomes isolated from conditioned media (CM) of MSCs for 36 hours. The output cells were subjected to phenotypic, functional, and molecular characterizations.

Hematopoietic Cell Transplantation

Cocultured cells (CD45.1) were infused into lethally irradiated recipients (CD45.2). For secondary transplantations, engrafted donor cells from the BM of primary recipients were sort-purified and infused into lethally irradiated secondary recipients. Peripheral blood (PB) chimerism and BM engraftment were assessed by flow cytometry at 16 weeks post-transplant in both primary and secondary transplantations.

Detailed methodology can be found in Supporting Information.

Results

Generation of Ageing-Specific Gene Signature

First, we determined the gene expression profile of aged (>18 months old) versus young (6–8 weeks old) LSK HSCs (hereafter referred to as HSCs) as a quantifiable molecular parameter to assess rejuvenation of aged HSCs. Sort-purified young and aged HSCs were subjected to quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analyses (Supporting Information Fig. S1A). Consistent with earlier reports [2, 3], aged HSCs showed a significantly higher expression of mRNAs of inflammatory markers, Cox2 and Selp, and myeloid commitment marker, Itga2b, and a significantly lower expression of various DNA-methylation-, DNA-repression-, DNA acetylation (Sirtuins), autophagy-related mRNAs, and lymphoid commitment marker, Il7r . Aged HSCs also showed higher levels of Akt-specific and lower levels of Foxo-3-specific mRNAs.

Brief Exposure to Young MSCs Rejuvenates Aged HSCs

Next, we examined whether a brief interaction of aged HSCs with young MSCs rejuvenates them. Lineage negative (Lin^{-}) cells (an HSC-enriched population) from aged or young BM cells were cocultured with young or aged MSCs, respectively, for 36 hours and the output cells were analyzed by flow cytometry (Supporting Information Fig. S1B). We found that aged Lin^{-} cells cocultured with young MSCs showed reduced proliferation, reduced pool of HSCs, increased frequency of CXCR4^{+} HSCs, reduced frequency of CD41^{+} myeloid-biased HSCs, higher frequency of apoptotic HSCs, reduced frequency of transforming growth factor β1R^{+} HSCs, and higher frequency of HSCs in G1 phase of cell cycle (Supporting Information Fig. S1C) when compared with the HSCs from coculture of aged Lin^{-} cells and aged MSCs (Fig. 1A–1F). Flow analyses of progenitors generated in coculture of aged Lin^{-} cells with young MSCs (Supporting Information Fig. S3A1) showed an increased frequency of common lymphoid progenitors (CLP; Supporting Information Fig. S3A2), resulting in a significant reduction in common myeloid progenitors: CLP ratio (Fig. 1G). On the other hand, young Lin^{-} cells cocultured with aged MSCs showed all characteristics of aged HSCs [3, 15–17] (Supporting Information Figs. S2A–S2G, S3A3).

HSCs sorted from cocultures of aged Lin^{-} cells and young and aged MSCs were subjected to qRT-PCR analyses. As shown in Figure 2A, the gene-signature of aged HSCs treated with young MSCs showed a significant reversal in the expression levels of most of the mRNAs examined. Importantly, autophagy-related genes, namely, Beclin-1, Atg7, Lc3a and Lc3b, and Sirt 1, 2, and 7 were significantly upregulated. Levels of Dnmt3a, Dnmt3b, Ezh2, and Sirt3 did not change. Expression of Akt was reduced and that of *Foxo3* was increased. Expression of Il7r was significantly upregulated, whereas that of *Itga2b* was downregulated, indicating reversal of myeloid bias.

When cocultured Lin^{-} cells were serially transplanted in irradiated mice (Fig. 2B), aged cells cocultured with young MSCs showed a significantly higher level of engraftment in both primary (Fig. 2C1, 2C2; Supporting Information Fig. S1D) as well as secondary transplantations (Fig. 2C3, 2C4) when compared with those cocultured with aged MSCs. Aged Lin^{-} cells cocultured with young MSCs produced significantly fewer myeloid cells (Fig. 2E1). Surprisingly, young cells cocultured with aged MSCs showed higher engraftment in both primary (Fig. 2D1, 2D2) as well as secondary transplantations (Fig. 2D3, 2D4) when compared with their counterparts cocultured with young MSCs. This could partly be due to an increased number of long term HSCs (LT HSCs) (Supporting Information Fig. S2A3, sixfolds higher) and reduced level of apoptosis (Supporting Information Fig. S2D, twofolds lower) in young HSCs after their coculture with aged MSCs. The molecular mechanism involved in this counter-intuitive observation remains to be formally examined. Young cells cocultured with aged MSCs did not show myeloid bias in PB of recipients (Fig. 2E2).

Collectively, these data demonstrate that a brief exposure of aged HSCs to young MSCs rejuvenates them.

Young EVs Contain "Youth Signals"

Taking clue from our previous study [18], we examined whether the rejuvenating factors are present in the EVs

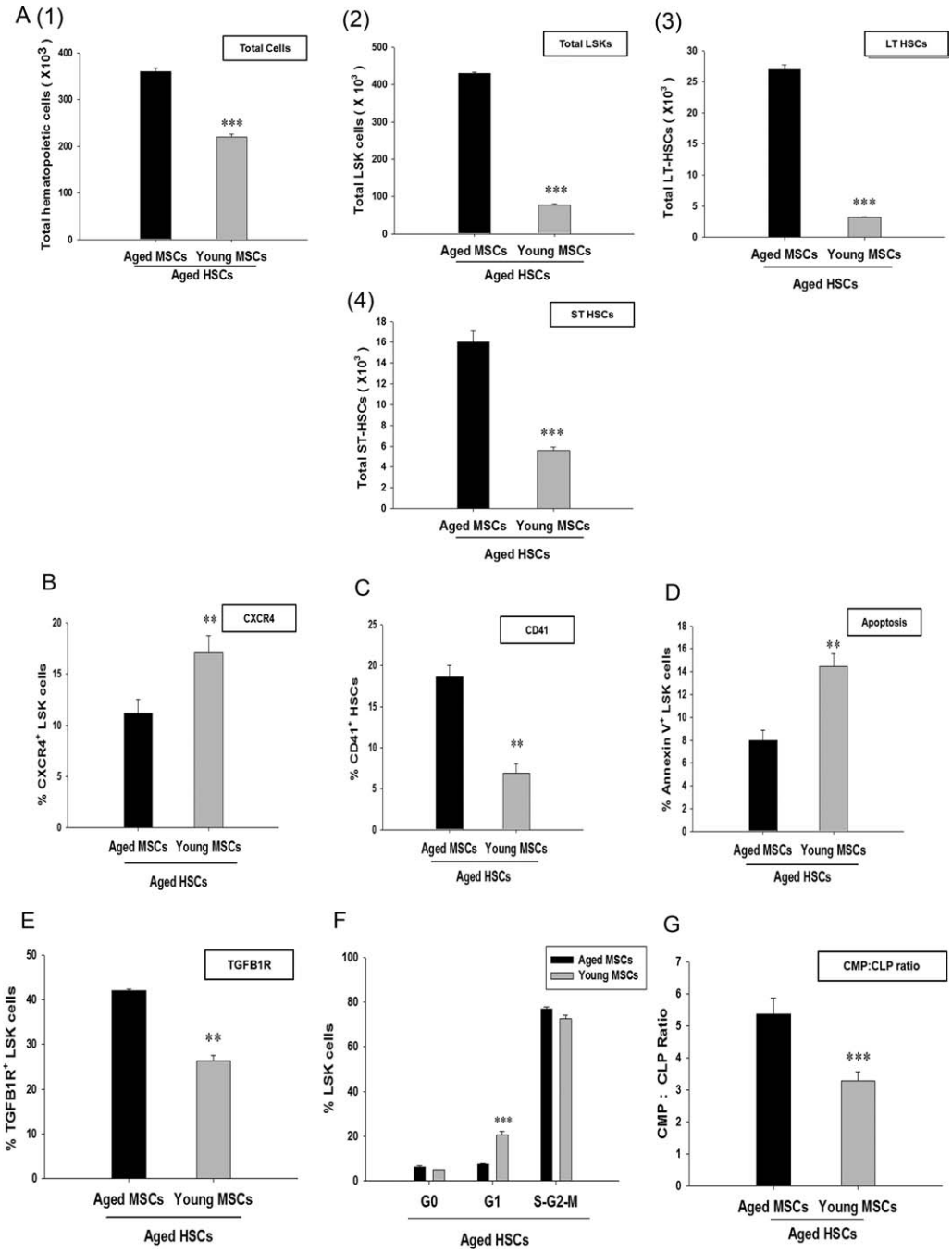

Figure 1. A brief exposure of aged hematopoietic stem cells (HSCs) to young mesenchymal stromal cells (MSCs) rejuvenates them. **(A):** Aged Lin$^-$ cells cocultured with young MSCs show **(A1)** reduced proliferation, **(A2–A4)** reduced pool of Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK)-, LT-, and ST-HSCs, **(B)** increased percentage of CXCR4$^+$ LSK HSCs, **(C)** reduced myeloid bias, **(D)** increase in apoptosis, **(E)** reduced percentage of transforming growth factor βR1$^+$LSK HSCs, **(F)** increase in G1 population, and **(G)** decrease in common myeloid progenitors (CMPs) to common lymphoid progenitors (CLP) (CMP:CLP) ratio. Data are represented as mean ± SEM. At least three replicates were kept in each experiment and each experiment was repeated thrice. **, $p < .01$; ***, $p < .001$. Abbreviations: CLP, common lymphoid progenitor; CMP, common myeloid progenitor; HSC, hematopoietic stem cells; LT, long term (LT) HSCs; LSK, Lin$^-$Sca-1$^+$c-Kit$^+$; MSC, mesenchymal stromal cell; TGFBR1, transforming growth factor βR1.

secreted by the MSCs. CM were subjected to ultra-centrifugation (100,000*g*) and the pellets containing EVs were suspended in sterile medium. Anti-CD63 antibody was used to characterize the isolated EVs by Western blotting (Supporting Information Fig. S4A). Sort-purified HSCs were incubated with these EVs for 36 hours and analyzed on a flow cytometer. We found that young EVs reduced the unwarranted proliferation of aged HSCs (Fig. 3A1, 3A2) suggesting that the rejuvenating factors are present in the EVs.

Aged Lin$^-$ cells treated with young and aged EVs (36 hours) were infused into irradiated recipients. We found that the aged Lin$^-$ cells treated with young EVs showed significantly higher levels

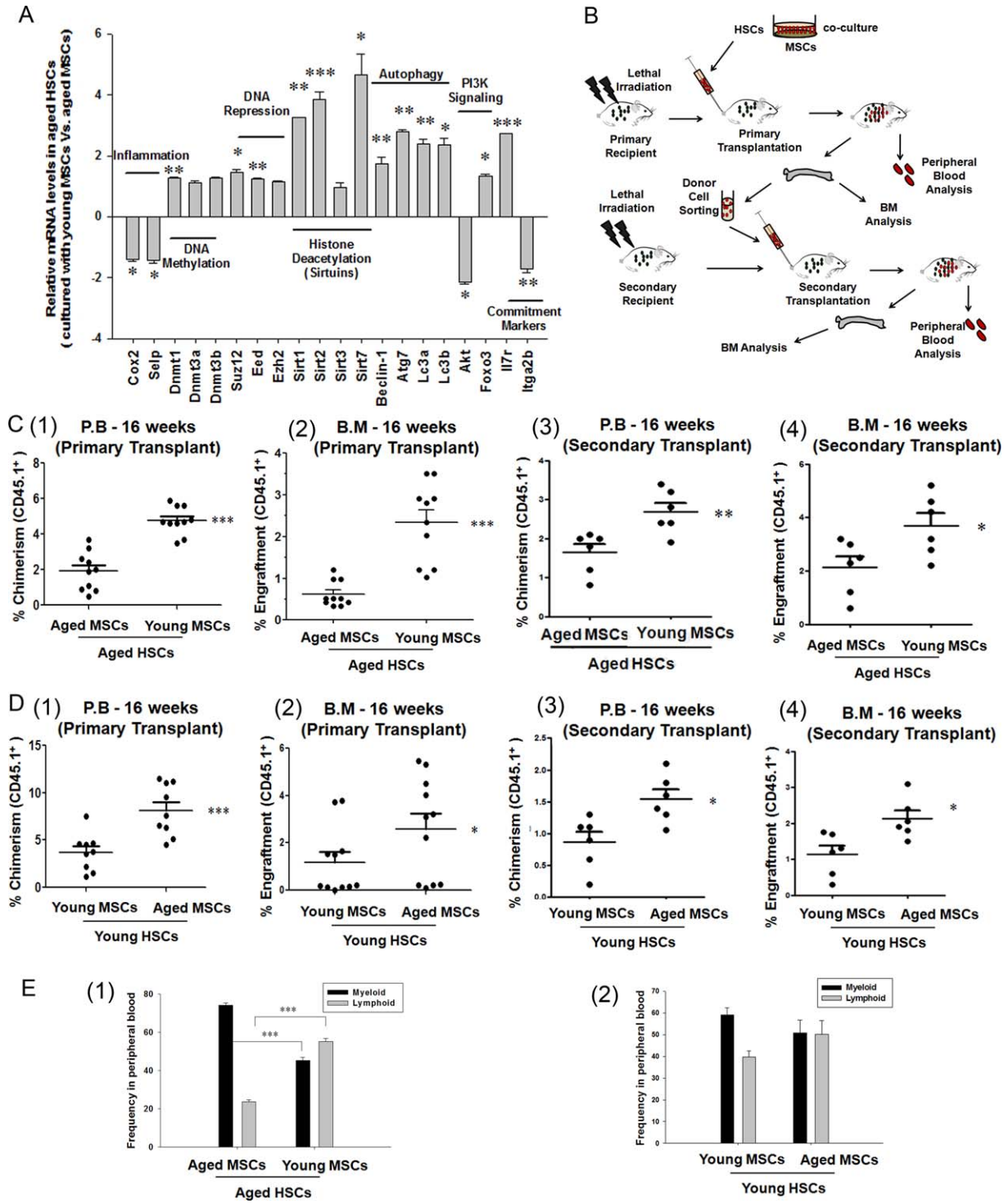

Figure 2. Young mesenchymal stromal cells (MSCs) rejuvenate aged hematopoietic stem cells (HSCs) and boost their long-term engraftment capacity. **(A):** $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK)-HSCs sort-purified from coculture of aged Lin^{-} cells and young MSCs show significant reversal of their aging-specific gene expression profile when compared with their counterparts cocultured with aged MSCs. Three replicates were kept in each experiment and the experiment was repeated thrice. **(B):** Schematic representation of transplantation experiments. **(C1, C2):** Aged Lin^{-} cells cocultured with young MSCs establish significantly increased levels of peripheral blood (PB) chimerism (C1) and bone marrow (BM) engraftment (C2) in irradiated recipients at 16 weeks post-transplant. **(C3, C4):** Sort-purified engrafted aged HSCs from BM of primary recipients receiving aged cells cocultured with young MSCs establish significantly increased levels of PB chimerism (C3) and BM engraftment (C4) in irradiated secondary recipients at 16 weeks post-secondary-transplant. Aged MSCs enhance long-term engraftment capacity of young HSCs. **(D1, D2):** Young Lin^{-} cells cocultured with aged MSCs show increase in PB chimerism (D1) and BM engraftment (D2) in irradiated recipients at 16 weeks post-transplant. **(D3, D4):** Similar enhancement is also seen in secondary transplants as well. **(E):** Young MSCs reduce in vivo myeloid bias of aged HSCs **(E1)**, but aged MSCs do not increase in vivo myeloid bias of young HSCs **(E2)**. Each group contained 6–10 mice. Data are represented as mean ± SEM. Experiment was repeated twice with similar results. * $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: BM, bone marrow; HSC, hematopoietic stem cells; MSC, mesenchymal stromal cell; PB, peripheral blood; PI3K, phosphoinositide-3-kinase.

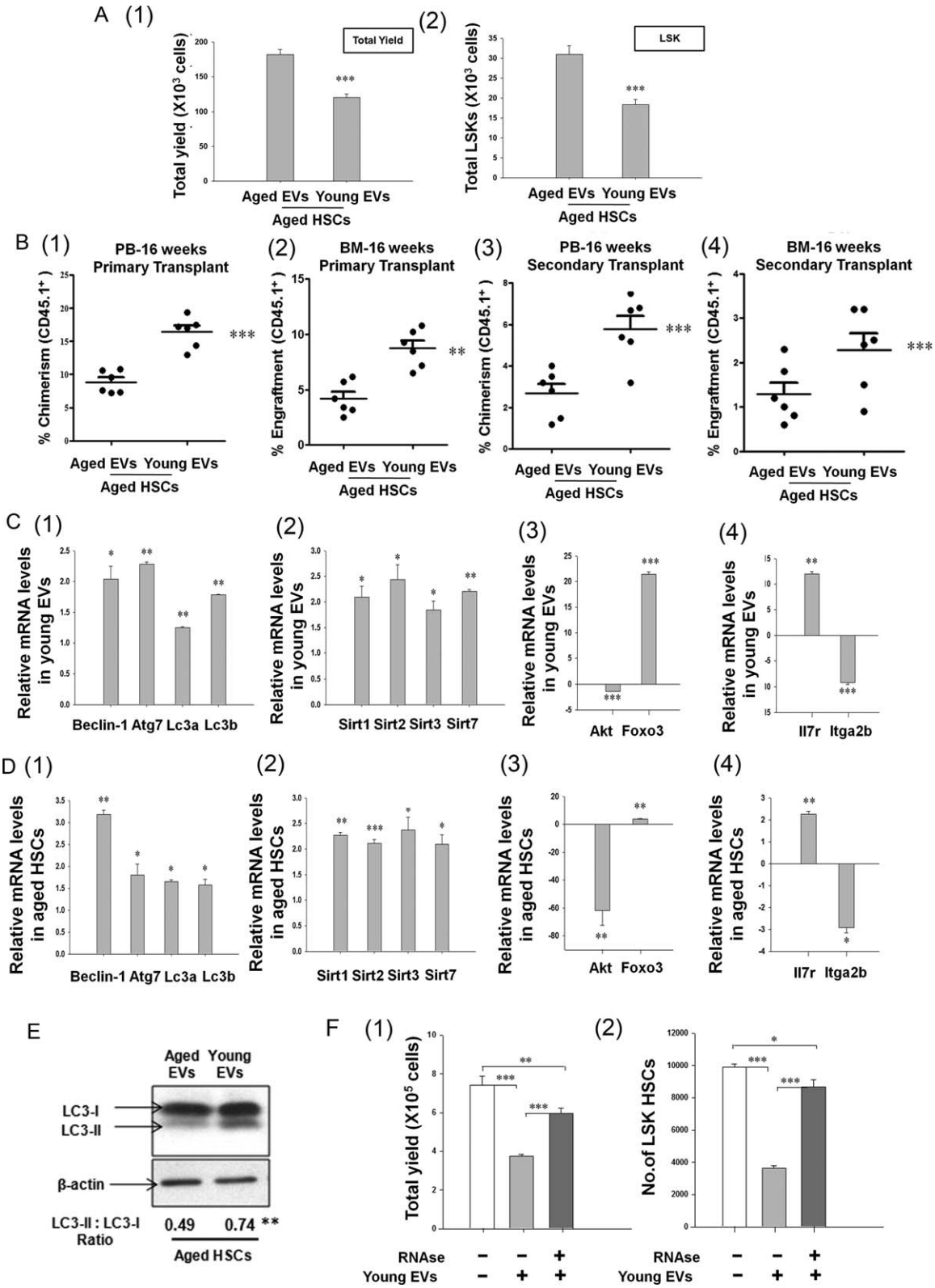

Figure 3. Rejuvenating factors are present in extra-cellular vesicles (EVs). **(A):** Aged Lin^- cells incubated with EVs collected from young mesenchymal stromal cells (MSCs) show reduction in overall proliferation **(A1)** and $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) hematopoietic stem cell (HSC) output **(A2)**. At least three replicates were kept in each experiment and each experiment was repeated thrice. Young EVs boost the engraftment potential of aged HSCs. **(B):** Aged Lin^- cells incubated with young EVs show significant increase in peripheral blood chimerism and bone marrow engraftment at 16 weeks post-transplant in both, primary **(B1, B2)** as well as secondary **(B3, B4)** transplantations when compared with their counterparts incubated with aged EVs. Six mice were kept in each group. Experiment was repeated twice. **(C1–C4):** Comparative gene expression in young versus aged EVs is depicted. **(D1–D4)** Comparative gene expression in aged HSCs incubated with young EVs versus those incubated with aged EVs is shown. **(E):** Western blot analysis of LC3 protein in aged HSCs treated with aged and young EVs for 36 hours. **(F):** RNAse treated young EVs significantly lose their ability to control the excessive proliferation of aged HSCs. Numbers of total cells **(F1)** and LSK HSCs **(F2)** are graphically illustrated. Data are represented as mean ± SEM. At least three replicates were kept in each experiment and each experiment was repeated thrice. * $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: BM, bone marrow; EV, extra-cellular vesicles; HSC, hematopoietic stem cell; LSK, $Lin^-Sca\text{-}1^+c\text{-}Kit^+$; PB, peripheral blood.

of engraftment when compared with those treated with aged EVs in both primary and secondary transplants (Fig. 3B1–3B4), confirming the functional rejuvenation of aged HSCs by young EVs.

Taken together, these data demonstrate that EVs of young MSCs contain "youth signals" or rejuvenating factors.

Young EVs Harbor Significantly Higher Levels of mRNAs Involved in Autophagy and Lineage Commitment

As rejuvenation of aged HSCs by young MSCs was associated with upregulation of various autophagy-related mRNAs in them (Fig. 2A), we determined whether these mRNAs get packaged in the EVs. qRT-PCR analyses of EVs showed that young EVs harbor significantly higher levels of autophagy-related mRNAs, namely, Beclin-1, Atg7, Lc3a, and Lc3b, when compared with the aged EVs (Fig. 3C1). Young EVs also contained higher levels of Sirt 1, 2, 3, and 7 (Fig. 3C2) and Cxcr4 mRNAs (Supporting Information Fig. S4E1). Sirtuins regulate autophagy process and also play an important role in HSC functionality [19]. Young EVs contained lower levels of Akt, but significantly higher levels of Foxo3 (Fig. 3C3). Foxo3 is known to direct the protective autophagy program in HSCs [20]. Interestingly, young EVs contained significantly higher levels Il7r and significantly lower levels of Itga2b, when compared with aged EVs (Fig. 3C4). This difference was not due to the different amounts of EVs secreted, as the protein content of EVs collected from identical number of young and aged MSCs was equal (Supporting Information Fig. S4B).

To determine whether these mRNAs get transferred from young EVs to aged HSCs, sort-purified aged HSCs were incubated with young and aged EVs and subjected to qRT-PCR analyses. The aged HSCs incubated with young EVs showed a significantly elevated expression of Beclin1, Atg7, Lc3a, Lc3b, Sirt-1, 2, 3, and 7 (Fig. 3D1, 3D2), and Cxcr4 (Supporting Information Fig. S4E2), when compared with their counterparts incubated with aged EVs. Levels of Akt were reduced and that of Foxo3 were significantly increased (Fig. 3D3). Interestingly, aged HSCs incubated with young EVs showed increased levels of Il7r and decreased levels of Itga2b (Fig. 3D4), suggesting that perhaps niche regulates the lineage commitment of HSCs via EV-mediated transfer of lineage-specific mRNAs into them.

Aged HSCs treated with young and aged EVs were subjected to Western blot analyses. We found that aged HSCs incubated with young EVs showed increased LC3II:LC3I ratio (Fig. 3E), indicating the increased autophagy flux in them [21].

To determine the nature of the rejuvenation principles present in the EVs, aged LSK HSCs were cultured with young EVs treated or not with RNAse. RNAse treated young EVs showed significantly reduced ability to restrict the excessive proliferation of aged HSCs incubated with them (Fig. 3F1, 3F2). Moreover, aged HSCs incubated with RNAse-treated young EVs showed significantly reduced expression of Sirtuin-1 and other autophagy-specific mRNAs when compared with those incubated with untreated young EVs (Fig. 4A1–4A5, last two bars). These data demonstrated that the rejuvenating principle present in the EVs is RNA in nature. Interestingly, aged HSCs incubated with RNAse-treated aged EVs showed significantly higher expression of Sirt1 and other autophagy-related mRNAs when compared with those incubated with untreated aged EVs (Fig. 4A1−4A5, first two bars), suggesting that aged EVs transfer some inhibitory RNA entities to the HSCs, and their degradation by RNAse protected the "pre-existing" mRNAs in them

To confirm that the increase in autophagy-related mRNAs seen in the aged HSCs incubated with young EVs involved intercellular transfer mechanism, sort-purified aged HSCs were incubated with young EVs in the presence of Actinomycin D (Sigma-Aldrich, Saint Louis, MI) (ActD; 5 μg/ml) and subjected to qRT-PCR analyses. Untreated and ActD-treated aged HSCs were used as controls. Treatment of the aged HSCs with ActD alone resulted in suppression of Beclin-1, Atg-7, Lc3a, and Lc3b, completely (Fig. 4B1–4B4, second bars). However, aged HSCs incubated with young EVs in the presence of ActD showed significantly higher levels of these mRNAs (Fig. 4B1–4B4, third bars) when compared with those treated with ActD alone. Last bar in Figure 4B1–4B4 shows mRNA levels in young EVs. These results confirmed that the enhanced mRNA levels seen in the aged HSCs incubated with young EVs was due to the direct transfer of autophagy-related mRNAs from the young EVs.

Collectively, these data suggest that EV-mediated transfer of autophagy- and lineage commitment-related mRNAs could be one of the mechanisms involved in the rejuvenation of aged HSCs by the young MSCs.

MVs Carry "Youth Signals" While Exosomes Harbor Inhibitory Moieties

EVs were separated into MVs and exosomes by differential ultra-centrifugation [22]. Isolated MVs and exosomes showed typical size and morphology (Supporting Information Fig. S4C1–S4C4, S4D1, S4D2). Independent effect of young EVs, MVs, and exosomes on the expression of autophagy-inducing mRNAs in sort-purified aged HSCs was examined. Surprisingly, we found that aged HSCs incubated with young MVs showed even higher levels of these mRNAs when compared with those incubated with young EVs (Supporting Information Fig. S5A1–S5A4; second and third bars), whereas those incubated with young exosomes showed downregulation of Beclin1 and Atg7 (Supporting Information Fig. S5A1, S5A2). Levels of Lc3a and Lc3b were not affected significantly (Supporting Information Fig. S5A3, S5A4).

When sort-purified young HSCs were incubated with aged exosomes, we found that levels of Bec-1, Atg7, Lc3a, and Sirt-1 were downregulated in a dose-dependent fashion by the aged exosomes (Supporting Information Fig. S5B1–S5B3, S5B5). Effect on Lc3b was seen only at the highest concentration of exosomes applied (Supporting Information Fig. S5B4). Treatment of exosomes with RNAse before their incubation with HSCs abolished this inhibitory effect, confirming the presence of negative regulatory RNAs in them (Supporting Information Fig. S5B1–S5B5, last bars).

When young MVs and exosomes were subjected to qRT-PCR analyses, we found that all the autophagy-inducing mRNAs present in the young EVs could be solely detected in the MVs and not in the exosomes (Supporting Information Fig. S5C1–S5C4). RNAseq analyses (Supporting Information Excel File 1 and 2) confirmed that young MVs contain higher levels of autophagy-related mRNAs, namely, Beclin-1, Atg7, Lc3a, Lc3b, Sirt 2, and Foxo3, when compared with aged MVs (Fig. 5C).

To rule out the possibility that some unknown factor(s) present in young MVs triggered the accumulation of endogenous mRNAs or slowed down their decay and also to confirm the translation of transferred mRNAs in HSCs, murine BM MSC line M2–10B4 (M2) was genetically modified to stably

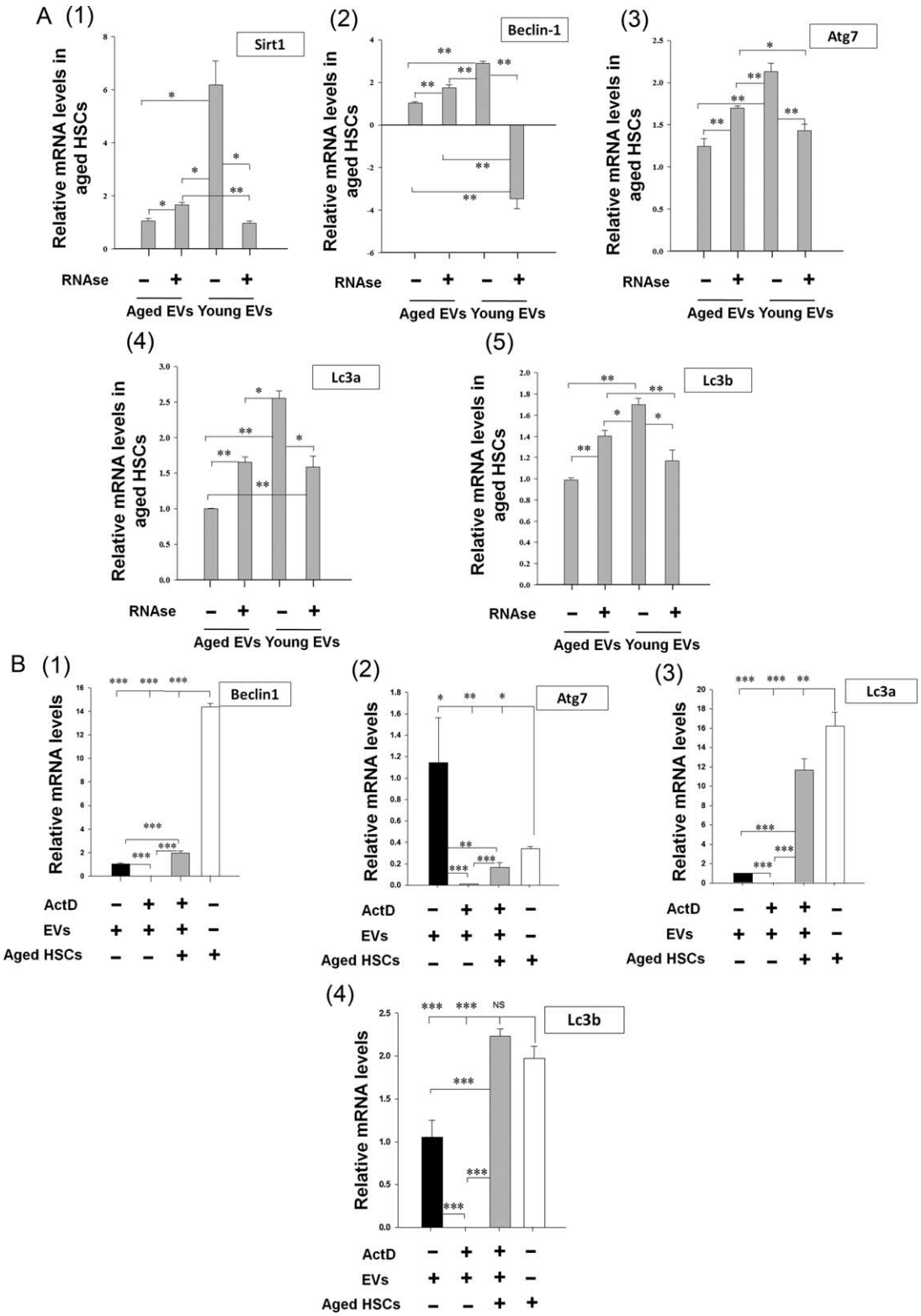

Figure 4. Rejuvenating principle in young extra-cellular vesicles (EVs) is RNA is nature. **(A1–A5):** Effect of RNAse-treated aged and young EVs on levels of various autophagy-related mRNAs in the aged hematopoietic stem cells (HSCs) is depicted. **(B1–B4):** Sort-purified aged $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ HSCs incubated with 5 μg/ml Actinomycin D (ActD) alone (second bar), or with young EVs in the presence (third bar) or absence (first bar, control) of ActD were subjected to quantitative reverse transcriptase polymerase chain reaction analyses to quantify autophagy-related mRNAs in them. Gapdh was used as a calibrator. ($n = 3$). Last bar shows the levels in young EVs. * $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: EV, extra-cellular vesicles; HSC, hematopoietic stem cell.

express LC3 green fluorescent protein (GFP) (M2-LC3GFP) as large amounts of LC3-specific mRNAs were found to be transferred from young EVs to aged HSCs (Fig. 4B3, 4B4). Sort-purified HSCs were incubated with MVs isolated from M2 and M2-LC3GFP. We found that the HSCs incubated with MVs isolated from M2-LC3GFP cells, but not with MVs isolated from M2 cells, were GFP positive (Fig. 5A). GFP expression was abolished in the presence of Puromycin (1 μg/ml) confirming that LC3GFP-specific mRNA, and not the protein, was getting intercellularly transferred (Fig. 5A). Consistent with these data, M2-LC3GFP cells, their MVs, and the HSCs incubated with these MVs showed the presence of fusion mRNA when examined by qRT-PCR (Fig. 5B).

Collectively, these results show that MVs contain "youth signals," whereas exosomes carry negative regulators of autophagy.

Pharmacological Inhibition of AKT Favorably Alters the mRNA Profile of Aged MVs

Based on our earlier work [18], we speculated that perhaps aged MSCs possess activated AKT signaling and this could be the reason behind the loss of functionality in the aged HSCs. To examine this possibility, we subjected young and aged MSCs to Western blot analysis. We found that the aged MSCs showed a significant increase in the levels of p-AKT (both Ser473 and Thr308) and native AKT when compared with the young MSCs (Fig. 5D).

To examine whether inhibition of AKT in MSCs favorably alters the mRNA profile of their MVs, young and aged MSCs were treated with LY294002 (LY-10 μM, Cell Signaling Technology, Danvers, MA) for 48 hours before collection of their MVs. MVs from untreated aged MSCs were used as controls. These MVs were then subjected to qRT-PCR analyses. We found that the levels of Beclin-1, Atg7, Lc3a, Lc3b, and Sirt 1, 2, 3, and 7 increased significantly in MVs isolated from LY treated aged MSCs (aged-LY MVs, Fig. 5E1, 5E2). Surprisingly, MVs isolated from LY treated young MSCs (young-LY MVs) showed significantly reduced levels of these mRNAs, suggesting that a complete inhibition of AKT results in a loss of these mRNAs in them (Fig. 5E3, 5E4).

Analysis of the LY-treated aged MSCs also showed significantly higher levels of Beclin-1, Atg7, Lc3a, Lc3b, and Sirt 1, 2, 3, and 7 (Fig. 5F1, 5F2). Consistent with the affected mRNA profile of their MVs, the LY-treated young MSCs showed decreased levels of Atg7, Lc3b, Sirt2, and Sirt3 (Fig. 5F3, 5F4). Levels of Sirt1 and 7 were not affected by LY treatment of young MSCs (Fig. 5F4), but, surprisingly, levels of *Beclin1* and *Lc3a* were upregulated (Fig. 5F3). The decrease in the levels of *Beclin1* and *Lc3a* in the young-LY MVs despite their increase in their parent MSCs suggests that perhaps a basal level of activated AKT is necessary for their partitioning into the MVs.

Since pharmacological compounds like LY may have off-target effects, we used clones of M2 stably expressing constitutively activated Akt-1(M2-Akt) and Akt-1-specific shRNA (M2-shAkt) [18]. We found that levels of autophagy-related mRNAs- Beclin1, Atg7, Lc3a, and Lc3b along with Sirt 1, 2, 3, and 7 were significantly lower in MVs isolated from M2-Akt and significantly higher in MVs isolated from M2-shAkt when compared with those isolated from parental M2 cells (Supporting Information Fig. S6A, S6B). Similar pattern was also seen in the respective parent cells, except for the Sirt 2, which showed higher levels in M2-Akt cells (Supporting Information Fig. S6C, S6D). These data confirm that the observed effect was due to inhibition of AKT activity by LY.

"Rescued" Aged MVs Rejuvenate Aged HSCs

Aged HSCs were incubated with aged/young MVs or aged/young-LY MVs and their mRNA profiles were compared. We found that the aged HSCs incubated with aged-LY MVs showed a significantly higher expression of Beclin-1, Atg7, Lc3a, Lc3b, and Sirt 1, when compared with their respective controls (Fig. 6A1–6A5; second and third bars). Aged-LY MVs transferred significantly higher amounts of Beclin1, Lc3a, and Lc3b to the aged HSCs when compared with young MVs (Fig. 6A1, 6A3, 6A4; third and fourth bars), but the amounts of Atg7 and Sirt1 were comparable in these two sets (Fig. 6A2, 6A5). Treatment of young MSCs with LY reduced their rejuvenating capacity, as evidenced by a significantly lower expression of these mRNAs in the aged HSCs treated young-LY MVs (Fig. 6A1–6A5; last two bars). The first bar shows mRNA levels in untreated aged HSCs.

Confocal microscopy analysis of the aged HSCs incubated with aged-LY MVs showed a significantly higher number of LC3-puncta per cell when compared with those incubated with aged MVs (Fig. 6B1, 6B2). The mean fluorescence intensity (MFI) of LC3 in these HSCs was also significantly higher (Fig. 6B3). These data confirm that inhibition of AKT in aged MSCs leads to formation of MVs having salutary effects on aged HSCs. Addition of Bafilomycin-A (200 nM) significantly boosted the number of LC3-puncta per cell and also the intensity of LC3 signal, confirming the autophagy flux (Fig. 6B1–6B3).

"Rescued" MVs Boost the Functionality of Aged HSCs

Aged HSCs incubated with young, aged, or aged-LY MVs were transplanted in irradiated recipients. As expected, aged HSCs incubated with young MVs showed better PB and BM reconstitution when compared with those incubated with aged MVs. Interestingly, aged HSCs incubated with aged-LY MVs exhibited significantly higher PB chimerism and BM engraftment in the recipient mice in both primary (Fig. 6C1, 6C2) and secondary (Fig. 6C3, 6C4) transplantations when compared with those incubated with young MVs.

In consensus, these data clearly showed that inhibition of AKT signaling in aged MSCs restores their rejuvenating capacity by improving the mRNA profile of their MVs.

Identification of Negative Regulators of Autophagy Present in the Exosomes

Exosomes are known to harbor miRNAs [23]. MiR-17 inhibits Beclin-1 and Atg-7 expression [24, 25], and miR-34a inhibits *Sirt-1* expression [26]. Therefore, taking a candidate approach we quantified the expression of these two miRNAs in young and aged exosomes and also in their respective MSCs. We found that aged exosomes contained significantly higher levels of both miRNAs when compared with young exosomes (Fig. 7A1). Aged MSCs showed a significantly higher expression of miR34a, but, surprisingly, a significantly lower expression of miR-17 when compared with their young counterparts (Fig. 7A2). These data show that aging decreases the expression of miR-17 in the MSCs, but increases the partitioning of both miR-17 and miR-34a into their exosomes (Fig. 7A3).

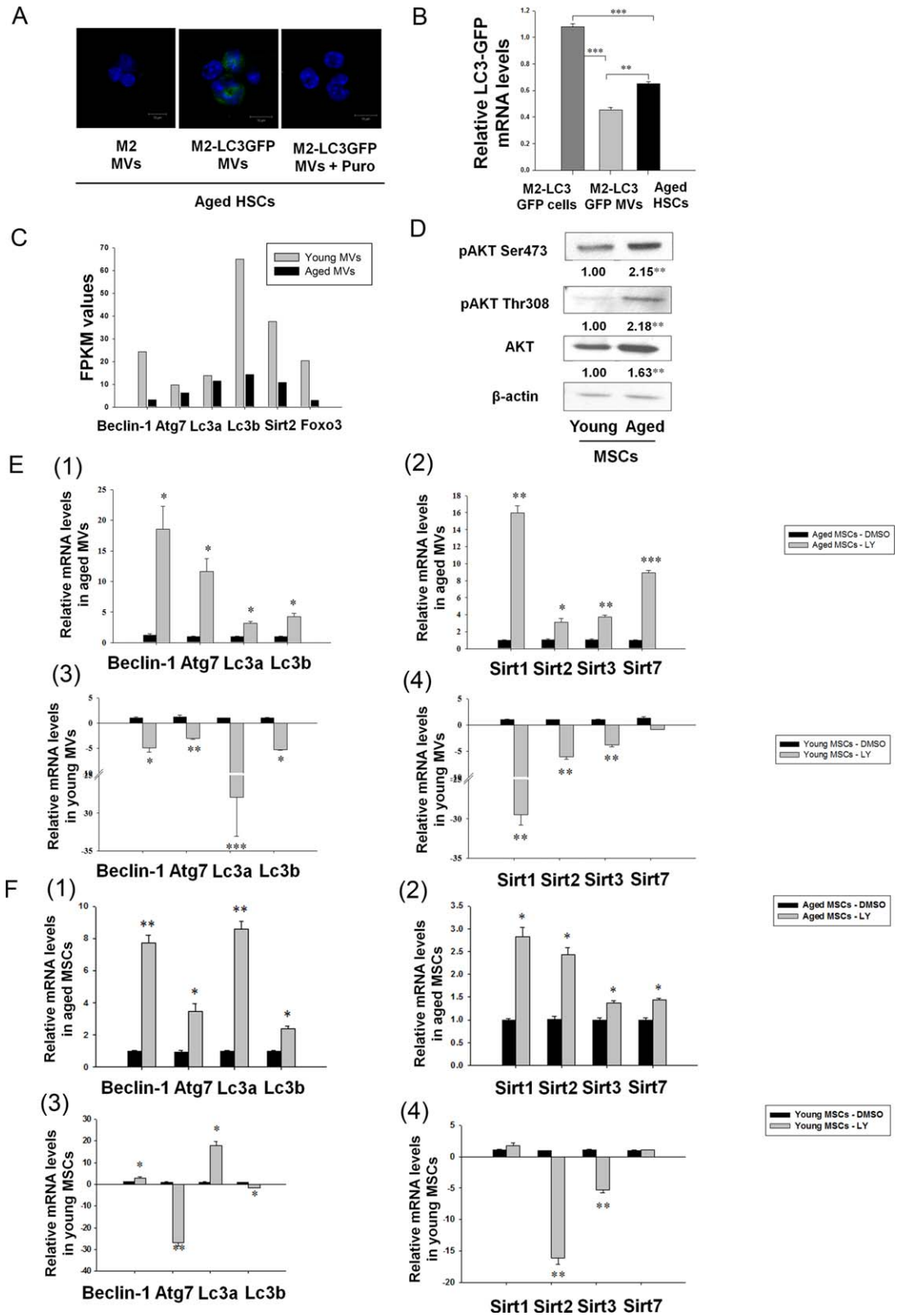

Figure 5. Intercellular transfer of autophagy-related mRNAs from mesenchymal stromal cells (MSCs) to aged hematopoietic stem cells (HSCs). **(A):** Representative images showing LC3 green fluorescent protein (GFP) in the aged HSCs incubated with M2-LC3GFP cells with or without Puromycin (1 μg/ml). Nuclei are demarcated by 4′,6-diamidino-2-phenylindole. Scale bar = 10 μm. **(B):** Relative expression of LC3GFP fusion mRNA in M2-LC3GFP cells, microvesicles (MVs) collected from them and aged HSCs incubated with these MVs is graphically illustrated. **(C):** Representative RNAseq data show higher expression (FPKM values) of autophagy-related mRNAs in young MVs compared with aged MVs. **(D):** Representative blot of young and aged MSCs is shown. Blot was probed sequentially with antibodies to phosphorylated and native forms of AKT followed by β-actin. Fold difference in band intensity was determined by Image J analysis. A pharmacological inhibition of AKT in aged MSCs rescues the mRNA profile of their MVs. **(E):** Aged and young MSCs were treated with LY294002 (LY, 10 μM) or equivalent amounts of dimethyl sulfoxide for 48 hours and their MVs were collected. These MVs were subjected to quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analyses. Inhibition of AKT in aged MSCs restores the expression of various autophagy-related mRNAs in their MVs **(E1, E2)**, whereas treatment of young MSCs with LY significantly reduces the autophagy-related mRNAs in their MVs **(E3, E4)**. **(F):** qRT-PCR analyses of LY-treated aged **(F1, F2)** and young MSCs **(F3, F4)** are depicted. Data are represented as mean ± SEM. At least three replicates were kept in each experiment and each experiment was repeated thrice. *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: FPKM, fragments per kilo base of transcripts per million mapped reads; GFP, green fluorescent protein; HSC, hematopoietic stem cells; LC3, microtubule-associated protein 1A/1B light chain 3; MSC, mesenchymal stromal cell; MV, microvesicle.

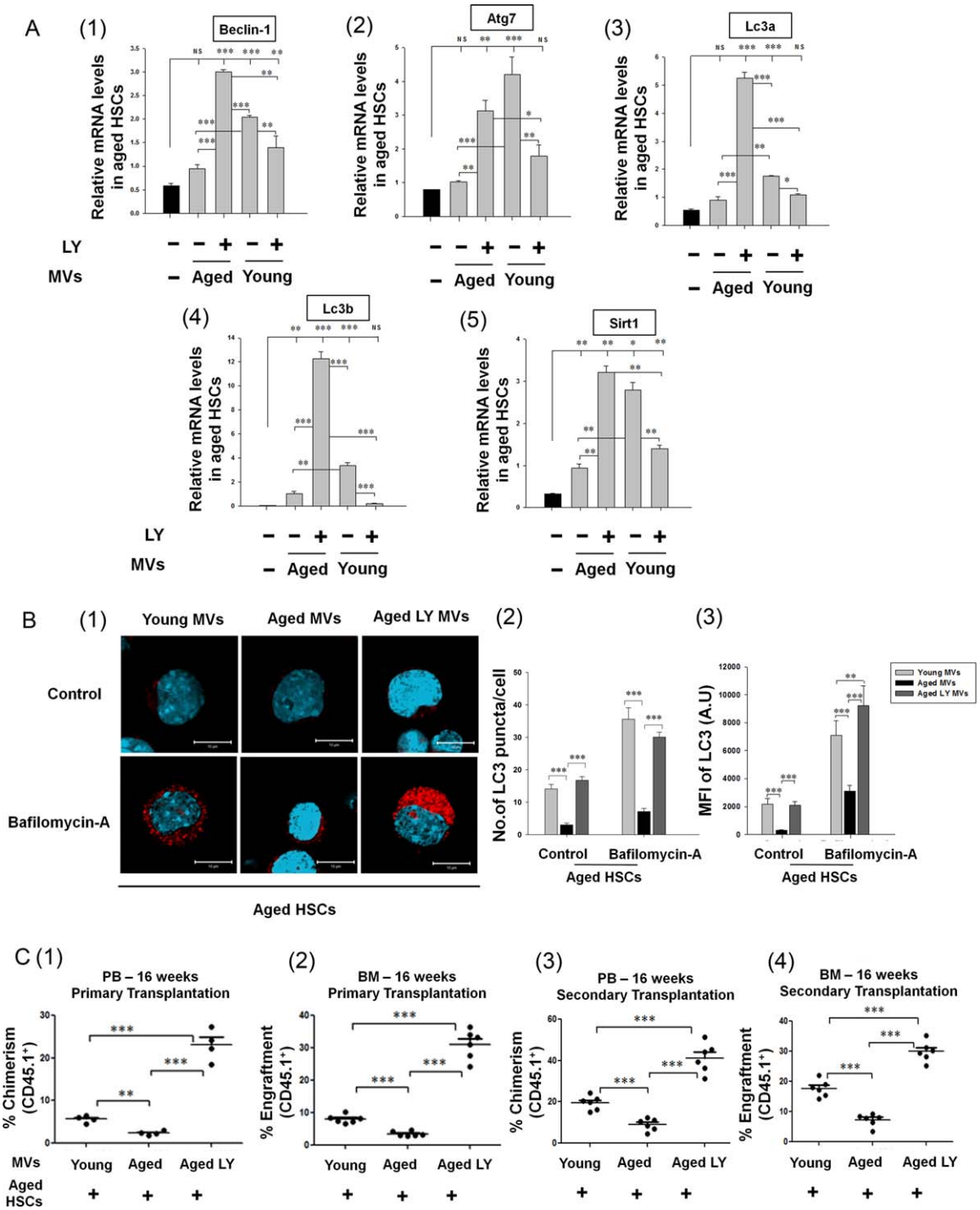

Figure 6. A pharmacological inhibition of AKT favorably alters the mRNA profile of aged microvesicles (MVs) and rejuvenates them. **(A1–A5):** Aged and young mesenchymal stromal cells (MSCs) were treated with LY294002 (LY, 10 μM) or equivalent amounts of dimethyl sulfoxide (DMSO) for 48 hours and their MVs were collected. quantitative reverse transcriptase polymerase chain reaction analyses of sort-purified aged hematopoietic stem cells (HSCs) incubated with MVs collected from LY- or DMSO-treated aged and young MSCs are shown. First bar shows the levels in untreated aged HSCs. **(B):** Aged HSCs were incubated with MVs isolated from young, aged and LY treated aged MSCs in presence or absence of Bafilomycin-A (200 nM) and subjected to confocal microscopy to quantify LC3 (red) puncta. The panel shows the representative single cell images of each group. 4′,6-Diamidino-2-phenylindole was used to demarcate nuclei. Scale bar = 10 μm **(B1)**. Average number of LC3 puncta per cell in each set is graphically illustrated **(B2)**. Aged HSCs incubated with young MVs show significantly higher LC3 MFI when compared with those incubated with aged MVs. Aged HSCs incubated with MVs from LY treated aged MSCs show restoration of LC3 MFI **(B3)**. At least 6 non-overlapping fields (having 8–10 cells) per set were analyzed. At least three replicates were kept in each experiment and each experiment was repeated thrice. "Rescued" MVs boost the functionality of aged HSCs. **(C):** Aged HSCs treated with young, aged and aged-LY MVs were transplanted into irradiated recipients. The dot plot represents peripheral blood-chimerism and bone marrow engraftment levels in primary **(C1, C2)** and secondary **(C3, C4)** recipient mice at 16 weeks post-transplant. Each group contained 4–6 mice. Data are represented as mean ± SEM. Experiment was repeated twice with similar results. * $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: HSC, hematopoietic stem cells; LY, LY294002; MV, microvesicle; PB, peripheral blood.

AKT Regulates the Expression of miR-17 and Controls the Partitioning of miR-17 and miR-34a into Exosomes

To examine whether AKT regulates partitioning of miR-17 and miR-34a into the exosomes of MSCs, the levels of miR-17 and miR-34a were quantified in the exosomes isolated from LY-treated and untreated aged MSCs. We found that inhibition of AKT signaling in aged MSCs significantly increased the levels of both mi-RNAs in them, (Fig. 7B2), but significantly decreased their levels in the exosomes (Fig. 7B1), showing that AKT positively regulates the partitioning of the mi-RNAs in the exosomes of MSCs (Fig. 7B3).

Similarly, young MSCs treated with SC79 (Tocris Bioscience, Bristol, UK), a known activator of AKT [27] showed significantly reduced levels of miR-17 and increased levels of miR-34a in their exosomes (Fig. 7C1). Activation of AKT resulted in decrease in the expression of both mi-RNAs in young MSCs (Fig. 7C2). As miR-17 is known to control the "stemness" of stromal cells [28], these data suggest that activation of AKT in the MSCs leads to the loss of their "stemness". Importantly, activation of AKT in young MSCs significantly increased the partitioning of miR-17 and miR-34a into their exosomes (Fig. 7C3).

The role of AKT in partitioning of miR-17 and miR-34a in exosomes was confirmed using M2, M2-Akt, and M2-shAkt system [18]. Consistent with the results obtained with LY and SC79, a significant increase in partitioning of these two miRNAs in M2-Akt exosomes and a significant decrease in M2-shAkt exosomes when compared with M2 exosomes was seen (Supporting Information Fig. S6E).

These data demonstrate that aging-mediated activation of AKT negatively regulates the expression of miR-17 in the MSCs and positively regulates the partitioning of miR-17 and miR-34a into their exosomes.

AKT-Mediated Sorting of miR-17 and miR-34a into Exosomes

Argonaute2 (Ago2) is known to play a vital role in sorting of miRNAs into exosomes, but Ago2-independent mechanisms are also known to be involved in the process [29]. Hence, we examined whether Ago2 was involved in the increased partitioning of miR-17 and miR-34a into the exosomes of aged MSCs. The levels of Ago2-specific mRNA were similar in young and aged MSCs (Fig. 7D). The LY-treated aged MSCs showed a significant, but marginally decreased levels of cellular Ago2 in them (Fig. 7E1, 7E2). Young exosomes had significantly higher level of Ago-2 in them when compared with the aged ones, but interestingly, aged-LY exosomes had even higher levels of Ago2 in them (Fig. 7E1, 7E2), showing that partitioning of Ago2 into exosomes is negatively regulated by AKT. These data suggest that sorting of these miRNAs into exosomes is an AKT-mediated, but Ago-2-independent process. Further studies are needed to elucidate these Ago2-independent mechanisms involved in the process.

Discussion

Donor age has been shown to be an important factor affecting the outcome of clinical transplantations, and therefore, physicians usually prefer younger donors, if given a choice. However, when only one HLA-matched older donor is available such preference cannot be availed. Similar situation is also present in the autologous settings involving older patients. Rejuvenation of the aged HSCs by their ex vivo manipulations would be the only solution to such problem. Here, for the first time, we show that it is possible to rejuvenate aged HSCs via their brief interaction with young MSCs or young MVs. This study is perhaps the first report showing that niche-mediated rejuvenation of HSCs is mediated via intercellular transfer of MVs from young MSCs to the aged HSCs. Application of the MSCs as well as MVs in clinical BMT/SCT might be logistically straightforward, as they can be cryopreserved as "ready-to-use" reagents.

Use of pharmacological compounds to rejuvenate aged stem cells in general, and aged HSCs in particular, is being pursued to gain clinical advantage [12, 14, 15, 30]. However, most pharmacological compounds could show off-target effects and they also regulate diverse processes and pathways. Therefore, use of clinical grade "cellular products" in manipulating HSCs, although expensive, would be a safer approach than the direct application of pharmacological tools on them.

EVs comprising of MVs and exosomes have emerged as a form of intercellular communication with important roles in several physiological processes and diseases [31]. Recently, the EVs from HSC-supportive fetal liver-derived stromal cells have been shown to specifically target and support hematopoietic stem and progenitor cells by preventing apoptosis, confirming their vital role in regulation of HSPCs by niche cells [32]. This study reports a very novel finding that the active rejuvenating principle present in young MVs comprises of autophagy- and lineage-commitment-related mRNAs. This study further shows that aged MSCs and their MVs have significantly reduced expression of these important mRNAs and this could be one of the mechanisms involved in niche-mediated aging of HSCs. It would be interesting to examine this aspect in other systems as well.

Reduced autophagy is associated with aging, whereas stimulation of autophagy is speculated to have anti-aging effects [33, 34]. Aged HSCs having high autophagy levels are known to preserve their regenerative capacity [35]. This study provides a direct evidence for this hypothesis. This study demonstrates that young MSCs transfer autophagy-initiating mRNAs to the aged HSCs via inter-cellular transfer of MVs, leading to their rejuvenation. ATG-7 is a critical component of autophagy pathway and has been shown to be essential for the maintenance of human $CD34^+$ HSCs [36]. This study shows that young MSCs and their MVs transfer Atg7 to aged HSCs. Sirtuin 1 is required for initiation of autophagy [37]. Resveratrol- and Calorie Restriction (CR)-mediated increase in SIRT1 expression is known to reduce aging and prolong life-span [13]. We have demonstrated that young MSCs also transfer Sirt1 to aged HSCs, thereby mimicking the effect of Resveratrol treatment and CR.

FOXO3a has been linked to longevity in multiple population studies [38]. FOXO3a is known to stimulate autophagy in primary mouse renal cells by trans-activation of *bnip3*, which codes for a potent autophagy inducer [39]. Similarly, FOXO3a inhibition or depletion prevents autophagy induction by starvation in vivo in the mouse muscle [40], confirming a strong link between transcription factors of the FOXO family and autophagy. We found that the aged HSCs treated with young MVs show high levels of FOXO3. In the light of these reports, our data clearly demonstrate that direct transfer of MVs containing autophagy-inducing mRNAs seems to be one of the important mechanisms involved in rejuvenation of aged HSCs by young MSCs.

HSC niche has a major impact on the declining stem cell function in elderly individuals, but the mechanism involved in

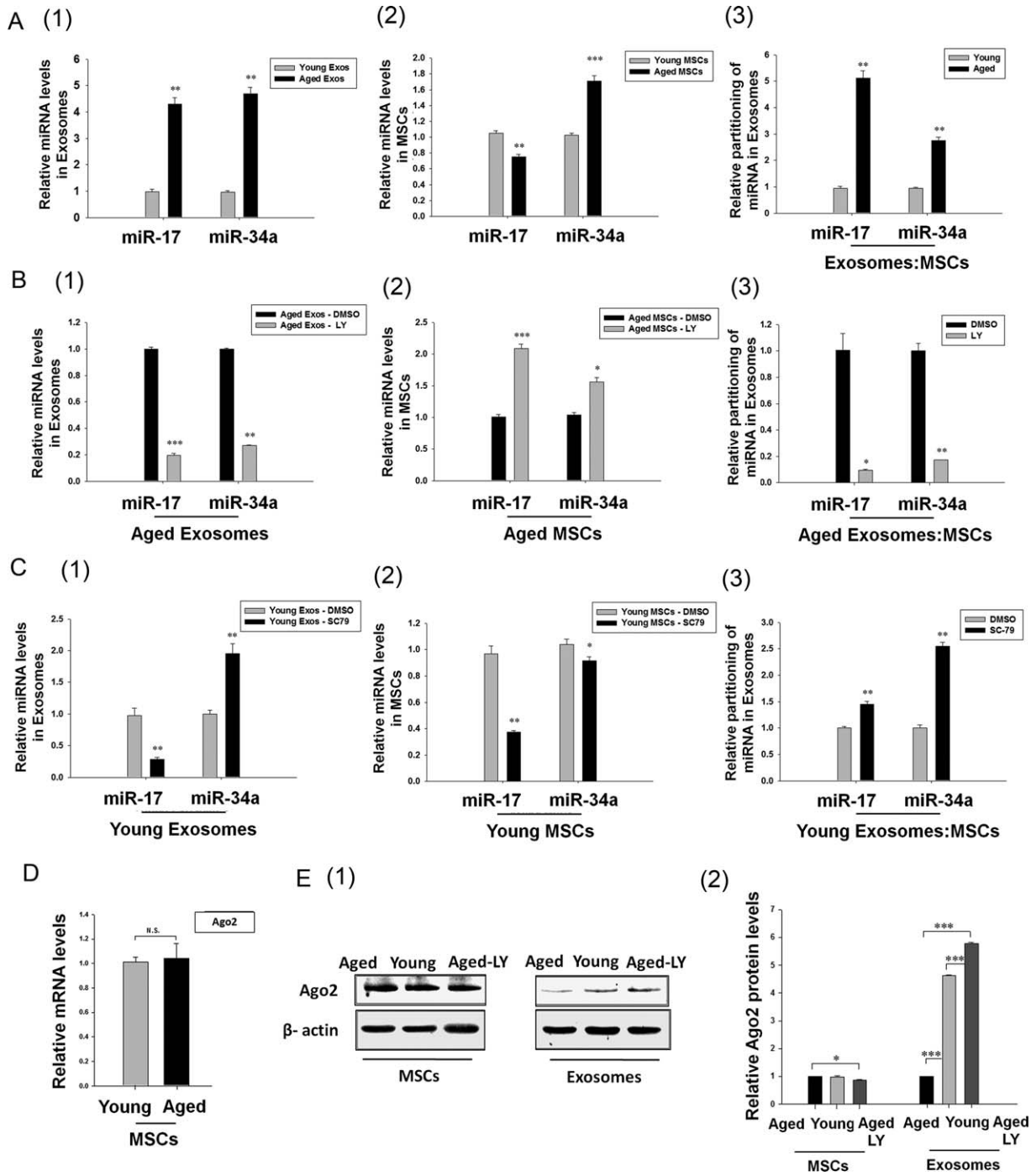

Figure 7. AKT regulates partitioning of miR-17 and miR-34a into exosomes. **(A):** Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analyses of miR-17 and miR-34a in exosomes **(A1)** and mesenchymal stromal cells (MSCs) **(A2)**. Significantly higher amounts of miR-17 and miR-34a get partitioned in aged exosomes when compared with young exosomes **(A3). (B):** Inhibition of AKT reduces the levels of miR-17 and miR-34a in aged exosomes **(B1)**, but increases their levels in aged MSCs **(B2)**. Treatment of aged MSCs with LY294002 (LY) significantly reduces partitioning of miR17 and miR-34a in their exosomes **(B3)**. **(C):** Treatment of young MSCs with SC79 (20 μM) increases the levels of miR-17 and miR34a in their exosomes **(C1)**, but reduces their levels in them **(C2)**. Treatment of young MSCs with SC79 increases the partitioning of miR-17 and miR-34a in their exosomes **(C3)**. **(D):** qRT-PCR analysis of Ago2 mRNA in young and aged MSCs. **(E):** Western blot analysis of aged, young, and LY treated aged MSCs and their respective exosomes to quantify Ago2 protein levels in them **(E1)**. Densitometric analysis of the data shown in panel E1 is graphically illustrated **(E2)**. Data are represented as mean ± SEM. At least three replicates were kept in each experiment and each experiment was repeated thrice. *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: DMSO, dimethyl sulfoxide; LY, LY294002; MSC, mesenchymal stromal cell.

the process was not known. AKT signaling is known to modulate aging of MSCs in vitro [41–43]. This study demonstrates that MSCs gain activated AKT as a function of aging in vivo. This not only leads to a significant reduction in autophagy-related mRNAs in their MVs but also increases the partitioning of miR-17 and miR-34a in their exosomes in an Ago2-

independent manner. Intercellular transfer of these exosomes suppresses the autophagy process in the HSCs, thereby causing their aging. Interestingly, these miRNAs were also found to reside in young exosomes, although at much lower concentrations, suggesting that perhaps in young MSCs a critical balance in positive and negative regulators is maintained. Importantly, we showed that pharmacological inhibition of AKT rejuvenates aged MSCs by favorably altering the mRNA profile of their MVs and also reducing the partitioning of miR-17 and miR-34a into their exosomes. Presence of activated AKT in the aged MSCs and decreased levels of Ago2 in their exosomes, and rescue of Ago2 levels in the LY treated aged MSCs, suggest that activation of AKT perhaps decreases Ago2-dependent and increases Ago2-independent sorting of miRNAs into exosomes. This aspect needs to be studied in details. Importantly, the MVs from pharmacologically modulated aged MSCs are as effective as young MVs, and thus, could be safely used in autologous settings. Inhibition of AKT has been shown to reduce in vitro aging of MSCs [41], but the effect of such "rescued MSCs" on HSCs was not examined.

Myeloid bias of HSCs has been considered as a hallmark of their aging. This has been attributed to an accumulation of myeloid-biased HSCs in the aged marrow [7, 15]. In heterochronic transplantation settings, microenvironment-mediated myeloid skewing has been demonstrated [16]. This study reports a novel finding that myeloid bias of aged HSCs could also be a non-cell-autonomous process involving intercellular communication mechanisms. We demonstrate that aged MVs contain higher levels of Itga2b, which is a myeloid commitment marker, whereas young MVs contain higher levels of IL7r, which is a lymphoid commitment marker. Importantly, this study shows that partitioning of these mRNAs depends upon the levels of activated AKT in the stromal cells. Thus, a continuous transfer of aged MVs containing Itga2b to the HSCs could impose a myeloid-bias in them, and this coupled with their low levels of apoptosis, could lead to the accumulation of aged HSCs in the marrow. Our data strongly suggest that the lineage bias of HSCs could be dictated by the mRNA profile of the MVs transferred to them, which in turn depends on the signaling mechanisms prevailing in the stromal cells [18]. This aspect needs further investigation. Nonetheless, our findings have certainly added a new dimension to the existing academic debate.

MSCs are increasingly being used in several regenerative medicine protocols. This study shows that the mRNA profile of MVs secreted by MSCs deteriorates with age, and also that their exosomes acquire negative regulators of autophagy. It is thus conceivable that the regenerative capacity of the MSCs sourced from older individuals could have been compromised. Our data suggest that a pharmacological inhibition of AKT could improve their therapeutic effects.

Studies carried out in allogenic, age-mismatched setting [44, 45] show that post-transplant, the epigenetic age of the transplanted donor cells matches with the donor age and not with the recipients' age. These data suggest that in vitro rejuvenation of aged donor HSCs before their transplantation may be a better approach. However, whether a stable rejuvenation is attained under allogenic setting remains to be examined. Nonetheless, our findings suggest that in vitro rejuvenation the aged HSCs would expand the repertoire of donors available for clinical BMT as well as for other regenerative therapies.

Conclusion

In summary, we demonstrate that young MSCs rejuvenate aged HSCs via intercellular transfer of MVs harboring autophagy- and lineage-commitment-related mRNAs. We further show that ageing-mediated activation of AKT in MSCs leads to increased partitioning of miR-17 and miR-34a into their exosomes, which upon transfer to HSCs cause their aging via down-regulation of autophagy-related genes. Importantly, we show that it is possible to rejuvenate aged MSCs by pharmacological means and thereby increase their therapeutic potential.

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Author Contributions

R.K.: conception and design, collection and assembly of data, data analysis and data interpretation, manuscript writing and editing; M.B., S.G., and S.J.: collection of data; L.L.: manuscript writing and editing; V.P.K.: conception and design, financial support, administrative support, collection and assembly of data, data analysis and interpretation, manuscript writing and editing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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