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Vav1 Regulates Mesenchymal Stem Cell Differentiation Decision Between Adipocyte and Chondrocyte via Sirt1

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Abstract

Mesenchymal stem cells (MSCs) are multipotent stromal cells residing in the bone marrow. MSCs have the potential to differentiate to adipocytes, chondrocytes, and other types of cells. In this study, we investigated the molecular mechanism that controls MSC cell fate decisions for differentiation. We found that Vav1, a guanine nucleotide exchange factor for Rho GTPase, was highly expressed in MSCs. Interestingly, loss of Vav1 in MSCs led to spontaneous adipogenic but impaired chondrogenic differentiation, and accordingly Vav1 null mice displayed an increase in fat content and a decrease in cartilage. Conversely, ectopic expression of Vav1 in MSCs reversed this phenotype, and led to enhanced MSC differentiation into chondrocyte but retarded adipogenesis. Mechanistically, loss of Vav1 reduced the level of Sirt1, which was responsible for an increase of acetylated PPARγ. As acetylation activates PPARγ, it increased C/EBPα expression and promoted adipogenesis. On the other hand, loss of Vav1 resulted in an increase of acetylated Sox9, a target of Sirt1. As acetylation represses Sox9 activity, it led to a dramatic reduction of collagen 2α1, a key regulator in chondrocyte differentiation. Finally, we found that Vav1 regulates Sirt1 in MSCs through Creb. Together this study reveals a novel function of Vav1 in regulating MSC cell fate decisions for differentiation through Sirt1. Sirt1 deacetylates PPARγ and Sox9, two key mediators that control adipocyte and chondrocyte differentiation. The acetylation status of PPARγ and Sox9 has opposite effects on its activity, thereby controlling cell fate decision.

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AUTHOR CONTRIBUTIONS

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DISCLOSURE

The authors indicate no potential conflicts of interest.

Keywords

Vav1; MSC; Sirt1; Adipogenesis; Chondrogenesis

Introduction

Mesenchymal stem cells (MSCs) are a heterogeneous subset of stromal stem cells traditionally found in the bone marrow (BM). They are multipotent stroma cells and have the potential to differentiate into cells of the mesodermal lineage, such as adipocytes, chondrocytes, and osteocytes. Cell differentiation is controlled by transcription factors through induction of cell type specific gene expression. The activity of transcription factors is regulated at multiple levels including post-translational modifications such as protein acetylation. The acetylation levels of proteins are regulated by both acetylase and deacetylase. Sirt1 is a NAD-dependent deacetylase that removes acety group from proteins and thereby modulating protein activity. It was reported that Sirt1 directly deacetylates PPARγ and represses its activity in adipogenesis [1, 2]. Several studies have demonstrated important roles of Sirt1 in both adipocyte and chondrocyte differentiation [3–5].

The characteristics and molecular mechanisms underlying adipocyte differentiation have been extensively investigated. Among them, PPARγ and C/EBPα, two transcription factors, play critical roles [6–8]. PPARγ is a key regulator of adipocyte development both in vitro and in vivo. It is required for adipocyte differentiation [6] and in many cases sufficient to convert nonadipose cells to adipocyte-like cells [7, 9]. PPARγ and C/EBPα act sequentially and participate in the same pathway to control adipocyte differentiation. PPARγ regulates C/EBPα expression [6], and mice deficient in C/EBPα exhibit development defect in adipose tissues. Additionally, C/EBPα-deficient adipocytes accumulate less lipid, and they do not induce expression of endogenous PPARγ. These findings indicate that cross-regulation between C/EBPα and PPARγ is important in adipocyte differentiation and the maintenance of the differentiated state of the cells [7].

Sox9 is a high-mobility-group domain transcription factor that is expressed in chondrocytes and other tissues. Sox9 plays critical roles for chondrocyte differentiation [10–12]. Inactivation of Sox9 in chondrocytes at different stages of cell differentiation suggests that Sox9 is essential for the survival of chondrocytes so that they can progress to terminal differentiation [10, 12]. Moreover, mutations in Sox9 gene are associated with the human skeletal malformation syndrome, campomelic dysplasia, in which skeletal abnormalities can be attributed to the disruption of the chondrogenic differentiation program due to failure to express Sox9 target genes such as type-ll collagen gene, the major cartilage matrix protein [10, 11, 13].

Vav is a family of evolutionarily conserved proteins from nematodes to mammals. In mammals, the Vav family has three members, Vav1, 2, and 3. While Vav2 and 3 are more broadly expressed in various types of cells, Vav1 has been thought to be restricted in hematopoietic cells in normal development [14, 15]. Mice without Vav1 are viable, fertile and grossly normal, but with defective lymphocyte development [15, 16]. The Vav1 protein was originally identified as an oncogene capable of inducing NIH 3T3 cell transformation.

Subsequently it is identified as a guanine nucleotide exchange factors (GEFs) for small Rho GTPase [14, 15]. In addition, Vav 1 contains multiple SH2 and SH3 domains and plays important roles in many aspects of cellular signaling, coupling cell surface receptors to various effectors functions in regulating cell proliferation, survival, migration and differentiation [14, 15]. The transcription factor cAMP-responsive element binding protein (Creb) is a regulator of gene expression, and Vav1 is implicated in Creb activity and gene expression in T lymphocytes [17]. Interestingly, a study shows that Sirt1 levels are transcriptionally regulated by nutrient availability through Creb in epithelial cells [18].

In this study, we report a nonhematopoietic function of Vav1 in normal development. Vav1 is highly expressed in MSCs, and it regulates cell fate decisions for differentiation. Loss of Vav1 led to spontaneous MSC adipogenic differentiation and concurrent impairment of chondrogenic differentiation. Vav1 regulate Sirt1 through Creb, and Sirt1 deacetylates PPARγ and Sox9, two important transcription factors for adipocyte and chondrocyte differentiation, respectively. Since the acetylation status of these two transcription factors has opposite effects on their activity, it results in opposing functions in murine MSC adipogenic and chondrogenic differentiation.

Materials and Methods

Mouse Model

C57BL/6J mice were purchased from the Jackson Lab and Vav1 null mice in the C57/BL6 background were kindly provided by Dr. Victor Tybulewicz at the MRC National Institute for Medical Research, U.K. [16]. Vav1 null and wild-type (WT) mice were maintained in a pathogen-free facility at the National Cancer Institute (Frederick, MD) in accordance with Animal Care and Use Committee regulations. Sex and age matched mice were used in all the studies.

Total body fat was determined by NMR using the Burker Minispec (Woodlands, TX) in the Mouse Metabolic Phenotyping Center at Vanderbilt University.

The distal femoral from 4 months old WT and Vav1 null mice ($n = 5$) were fixed in 10% formalin for 48 hours, decalcified in formic acid for another 48 hours, dehydrated in ethanol water, infiltrated with xylenes, paraffin embedded, and sectioned. After deparaffinization, the section was stained with 0.1% safranin O.

MSC Preparation and Differentiation

BM cells were harvested from femurs and tibiae of 7 week-old female mice, followed by selection with CD45-microbeads (Miltenyl Biotec Inc., San Diego, CA, www.miltenyibiotec.com). CD45-cells were cultured in Mesencult Basal Medium plus 20% MSC stimulatory supplements (StemCell Technologies Inc., Vancouver, BC, www.stemcell.com) at 37°C with 3% 0_2 for 2 days, followed by replacing with fresh medium. The cells were expanded and passaged with changing half of the medium every 3 days thereafter.

MSC differentiation was performed according to manufacture's protocols (R&D system, www.rndsystems.com).

Human BM derived MSCs were purchased from Lonza (Cat. PT-2501, Walkersville, MD), and peripheral blood cell-derived MSCs were isolated from human blood. All of human MSCs were cultured in MSCGM Mesenchymal Stem Cell Growth Medium (Lonza, www.lonza.com).

Retroviral Vector Construction

A murine Vav1 retroviral vector, pMSCV-mCherry-Vav1, was purchased from Addgene (plasmid # 50044, www.addgene.org), which was transfected into Eco-phoenix cells to generate Vav1 retroviral vectors. The culture medium containing virus was collected, filtered, and concentrated using Retro-X Concentrator (Clontech, # 631455, www.clontech.com) 48 hours post transfection. MSCs were infected with the concentrated virus in the presence of polybrene at a concentration of 10 μg/ml for 72 hours prior to subsequent experiments.

Sirt1 inhibitor Ex527 purchased from EMD Millipore (Cat. 566322, www.emdmillipore.com) was dissolved in DMSO:PBS (1:1 pH 7.2) and used at a concentration of 10 μM for 48 hours.

Flow Cytometry

Cells were stained with different fluorochrome conjugated anti-mouse antibodies against Sca-1, CD73, CD44, CD105, CD31, CD34, and CD45 (BioLegend, San Diego, CA). Acquisition was performed on LSRII instrument (Becton Dickinson, Franklin Lakes, NJ, www.biolegend.com) and data were analyzed using FlowJo software. Negative and single fluorochrome controls were performed to allow accurate compensation. The MSCs from WT or Vav1 null mice were incubated in 30 μg/ml BrdU for 48 hours. The cells were collected and fixed, BrdU staining were performed according to protocol of FITC BrdU Flow Kit (Cal# 559619, BD Bioscience, CA, www.bdbiosciences.com)

Quantitative Real Time PCR

Total RNA was purified from MSCs, followed by qRT-PCR analysis. Primer sets include:

GAPDH

Forward: 5′-CCCATGTTCGTCATGGGTGT-3′

Reverse: 5′-TGGTCATGAGTCCTTCCACGATA-3′

HP (Haptoglobin)

Forward: 5′-GAGAAACTCCCCGAATGTGA-3′

Reverse: 5′-ATCCATAGAGCCACCGATGA-3′

Leptin

Forward: 5′-GTGCGGATTCTTGTGGCTTT-3′

Reverse: 5′-GGAATGAAGTCCAAACCGGTG-3′

Adiponectin

Forward: 5′-CCCAAGGGAACTTGTGCAGGTTGGATG-3′

Reverse: 5′-GTTGGTATCATGGTAGAGAAGAAAGCC-3′

Col2α1

Forward: 5′-TATGGAAGCCCTCATCTTGC-3′

Reverse: 5′-TCACCTCTGGGTCCTTGTTC-3′

Aggrecan

Forward: 5′-TCGAGGACAGCGAGGCC-3′

Reverse: 5′-TCGAGGGTGTAGCGTGTAGAGA-3′

Coll10

Forward: 5′-AAACGCCCACAGGCATAAAG-3′

Reverse: 5′-CAACCCTGGCTCTCCTTGG-3′

SOX5

Forward: 5′-AGGCAGGAAATGCGACAGTAC-3′

Reverse: 5′-CTCGGAGGGCAGGTGAGG-3′

Mouse SOX6

Forward: 5′-TACCCACAGCTCCCCTGAAG-3′

Reverse: 5′-CTCACCTTCAGTGGCAAGAGC-3′

UCP1

Forward: 5′-GGTCGTGAAGGTCAGAAT-3′

Reverse: 5′-AGAGTTATAGCCACCACAG-3′

PGC-1α

Forward: 5′-AACAATAACAACAACAACCATAC-3′

Reverse: 5′-CTGAAGAGGCAAGAGACA-3′

Chemerin

Forward: 5′-TACAGGTGGCTCTGGAGGAGTTC-3′

Reverse: 5′-CTTCTCCCGTTTGGTTTGATTG-3′

Resistin

Forward: 5′-AAGAACCTTTCATTTCCCCTCCT-3′

Reverse: 5′-GTCCAGCAATTTAAGCCAATGTT-3′

Immunohistochemistry

Cultured cells were incubated with goat anti-mouse FABP4 polyclonal Ab (1:100, Cat. AF1443-SP, R&D system, U.S.) or sheep anti-mouse collagen II polyclonal Ab (1:100, Cat. AF3615-SP, R&D system, U.S.) or goat anti-mouse osteopontin polyclonal Ab (1:100, Cat. AF808, R&D system, U.S.), respectively. A Cy2-conjugated donkey anti-goat IgG or Cy2-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch, West Grove, PA, www.jackonimmuno.com) were used as the secondary antibodies.

Oil red 0 staining was performed following a published procedure [19]. Briefly, frozen tissue sections were prepared from fat after a standard cryostat procedure. Tissue section slides were stained with oil red 0 solution (0.5% in propylene glycol) in a 60°C oven for 10 minutes and placed in 85% propylene glycol for 1 minute.

Hematoxylin and eosin staining was analyzed and photographed using a Leica microscope equipped with Qimaging camera and ImageJ software.

DNA Extraction

Epididymal fat pads were harvested from age matched male WT and Vav1 null mice. Individual fat pads were weighed and digested, and DNA was extracted following the manufacturer's protocol (DNeasy Blood & Tissue Kit, Qiagen, Venlo, The Netherlands, www.qiagen.com). The DNA concentration was measured by NanoDrop spectrophotometer (ND1000; NanoDrop Technologies, Wilmington, DE, www.nanodrop.com).

Antibodies and Western Blotting Analysis

Twenty micrograms of cell lysate were analyzed on a denaturing 4%-15% gradient polyacrylamide gel (Biorad, 567–1081, www.bio-rad.com), transferred onto a PVDF membrane (Millipore, IPVH08100, www.merckmillipore.com) and incubated with various antibodies purchased from Cell Signaling Technology, which include anti-Vav1 (Cat. 4657), Sirt1 (Cat. 3931), Sirt1 (Cat. 8469S, for human), PPARγ (Cat. 2443), C/EBPα (Cat. 2295), Creb (Cat. 9197S), phospho-Creb (Cat. 9191S), and acetylated lysine (Cat. 9441). Antibody against Col2α1 was purchased from Santa Cruz Biotechnology (Cat. sc-52658). All antibodies were used at 1:500 dilution unless otherwise specified. Immune membranes were visualized using horseradish peroxidase-conjugated secondary antibodies (Cell signaling, Cat 7074 and 7076, www.cellsignal.com) and a chemiluminescent substrate (ECL system, GE Healthcare, www.gelifesciences.com).

Statistical Analysis

Results are expressed as the mean ± standard deviation. Comparisons between groups were performed using a two-tailed Student *t* test. *p*-value< .05 is considered statistical significant.

Results

Vav1 is Expressed in Murine MSCs and Negatively Regulates MSC Differentiation to Adipocyte

During analysis of Vav1 knockout mice, we noticed an increase of fat content in the mice. It led us to explore if Vav1 plays a role in MSC biology as these cells have the potential to differentiate into adipocytes. To test this hypothesis, we started by determining if Vav1 is expressed in MSCs. We isolated CD45-cells from the BM of WT and Vav1 null mice, and cultured them in Mesencult MSC Basal Medium to enrich and propagate MSCs. Cultured cells at the second passage at which approximately 90% of the cells expressed MSC markers (CD45−, CD31−, CD11b−, CD44+, CD29+ Sca1+) were harvested for evaluation of Vav1 expression by Western blot. Interestingly, high levels of Vav1 protein were detected in WT MSCs, but not in Vav1 null cells (Fig. 1A). This finding of Vav1 expression in MSCs is contrary to our current knowledge that Vav1 expression has been assumed to be restricted to hematopoietic cells in normal development.

Next, we characterized any potential role of Vav1 in MSC biology. CD45-cells isolated from the BM of WT and Vav null mice using magnetic cell sorting were cultured in Mesencult MSC Basal Medium for 10 days. We did not observe a significant difference in the number of MSC colony forming unit between WT and Vav1 null cells (Supporting Information Fig. 1A). To determine if Vav1 null MSCs could be passaged in vitro, the cells were cultured and passaged in Mesencult MSC Basal Medium. The cells between the first and second passage were analyzed for MSC cell surface marker expression by flow cytometry. There was no significant difference on CD45, CD34, CD31, CD44, CD73, and CD105 expression in MSCs between WT and Vav1 null mice (Supporting Information Fig. 1B–1D). However, there was a slight reduction of Sca1+ cells in the Vav1 null group compared to WT cells (Supporting Information Fig. 1D). Finally, we incubated MSCs with BrdU and cell proliferation was analyzed 48 hours later. We did not observe any significant difference between WT and Vav1 null MSCs (Supporting Information Fig. 1E). These findings suggest that Vav1 does not play a significant role in MSC proliferation, which led us to analyzing MSC differentiation.

While WT MSCs after passaging in Mesencult MSC Basal Medium showed homogenous population with a typical MSC morphology, while some of the Vav1 null cells showed a gradual morphological alteration under these conditions after repeated passaging in the MSC culture conditions. Oil red 0 staining revealed that some of the cells resemble those of adipocytes (upper panel Fig. 1B). The expression of Fabp4 (a marker for adipocyte) on those cells confirmed the results. Numerous Fabp4+ cells were observed in cultured Vav1 null cells but were largely absent in WT cells (bottom panel Fig. 1B). Enumeration of Fabp4+ cells in 10 randomly selected microscopic fields for each group confirmed a significant increase of adipogenesis in cultured Vav1 null MSC when compared to WT cells (Fig. 1C). Furthermore, we compared expression of additional adipocyte markers, haptoglobin (Hp), leptin and adiponectin, between the two groups using qPCR. The levels of mRNA for Hp, leptin, and adiponectin are 7.3, 5.9, and 3.1 times more in Vav1 null cells than those of WT

cells (Fig. 1D). These findings reveal a new role of Vav1 in MSC differentiation. Loss of Vav1 leads to spontaneous adipogenic differentiation during the expansion of MSCs.

Since loss of Vav1 in MSCs led to increased adipocyte differentiation, we performed a complimentary experiment to determine if overexpression of Vav1 could block the differentiation. WT MSCs at the second passage were infected with a retroviral vector either for Vav1 or vector control, respectively. Elevated Vav1 expression was confirmed by qPCR (Fig. 2A). These MSCs were then cultured in an adipogenic differentiation medium for adipogenic induction for 2 weeks. Under this condition, vector control infected MSCs acquire Fabp4 expression and differentiate to adipocytes. In contrast, ectopic expression of Vav1 significantly inhibited MSC differentiation to Fabp4+ adipocytes (Fig. 2B, 2C). Analysis of additional adipocyte markers including Hp, leptin, and adiponectin after induction of cell differentiation confirmed that ectopic expression of Vav1 in MSCs significantly inhibited MSC adipogenesis in vitro (Fig. 2D). These results together show a negative role for Vav1 in MSC to adipocyte differentiation.

Finally, we evaluated the body weight and the body fat content in 12 weeks old female WT and Vav1 null mice. We observed a slight but significant increase in the body weight in Vav1 null mice compared to WT mice (Fig. 3A). Importantly, there was approximately 30% more total body fat measured by NMR using Bruker Minispec in Vav1 null mice than WT mice (Fig. 3B). Leptin is a hormone made by fat cells and its level increases in obese conditions. Consistently, there was a significant elevation of serum leptin concentration in Vav1 null mice compared to WT mice (Fig. 3C). Moreover, histological evaluation of subcutaneous and abdominal visceral fat from the mice confirmed an increase of fat content in Vav1 null mice compared to WT mice (Fig. 3D). We further quantitated the numbers of adipocytes by measuring the DNA content in epididymal fat pads. DNA content per fat pad was significantly higher in Vav1 null mice than WT mice (Fig. 3E). After analysis of cell diameter of adipocytes, we found a clear increase of cell size in white adipocytic tissues (WAT) from Vav1 null mice compared to those from WT mice (Fig. 3F). These data support a negative role of Vav1 in adipogenesis, which is consistent with our observation that loss of Vav1 leads to spontaneous MSC differentiation to adipocytes.

Next, we evaluated the role of Vav1 on white versus brown fat by measuring specific markers. The levels of UCP1 mRNA were significantly lower in brown adipocytic tissues harvested from Vav1 KO mice than the one from WT mice. Although there is a decrease in PGC-1α mRNA, it did not reach statistical significance (Fig. 3G). On the other hand, Chemerin and Resistin mRNA expression were distinctly increased in epididymal WAT from Vav1 KO mice compared with WT mice (Fig. 3G).

Vav1 Positively Regulates MSC Differentiation to Chondrocyte

In addition to adipocytes, MSCs also give rise to chondrocytes. Therefore, we evaluated if loss of Vav1 in MSCs affects chondrocyte differentiation. MSCs at passage 2 isolated from the BM of WT and Vav1 null mice, at which time Vav1 null cells largely maintained MSC morphology, were cultured in chondrogenic-induction medium for 3 weeks to induce cell differentiation. Chondrogenic differentiation was evaluated by expression of Collagen II, a marker for chondrocytes, using IHC. Contrary to the negative role of Vav1 in MSC

adipogenesis, loss of Vav1 inhibited MSC differentiation to chondrocytes (Fig. 4A, 4B). Furthermore, we collected the cells after induction of cell differentiation and compared mRNA expression for various chondrocyte Vav1 null MSCs showed a significantly reduced ability to differentiate into chondrocytes markers, including collagen 2a1 (Col2α1), Aggrecan, Sox5, Sox6, and Coll10 between the two groups using qPCR. There was a consistent and significant reduction of these chondrocyte markers in Vav1 null cells compared with those of WT cells (Fig. 4C). Finally, we harvested the distal femoral bone from 4 months old WT and Vav1 null mice and subjected to safranin O staining. There was a clear reduction of cartilage in Vav1 null mice compared to WT mice (Fig. 4D). These findings together suggest a positive role for Vav1 in MSC chondrogenic differentiation.

Due to the fact that loss of Vav1 in MSCs inhibited chondrocyte differentiation, we performed a complimentary experiment to determine if overexpression of Vav1 could increase the differentiation. WT MSCs were infected with a retroviral vector either for Vav1 or vector control, respectively, followed by induction of chondrocyte differentiation for only 10 days, which normally takes 3 weeks. A small percentage of vector infected WT MSCs acquired Collagen II expression. Notably, the majority of Vav1 vector infected WT MSCs acquired Collagen II expression under these conditions (Fig. 5A, 5B). Further analysis of chondrocyte markers confirmed a significant increase of mRNA levels for Col2α1, Aggrecan, Sox5, Sox6, and Coll10 in Vav1 overexpression cells (Fig. 5C). These data together reveal a positive role for Vav1 in controlling MSC differentiation to chondrocyte.

Vav1 Regulates MSC Differentiation Switch Between Adipocyte and Chondrocyte via Creb/Sirt1 Mediated Deacetylation of Transcription Factors

Above findings reveal opposing roles for Vav1 in regulating MSC differentiation to adipocytes and chondrocytes. Cell differentiation is largely controlled by transcription factors that direct cell type specific gene expression. Among them, C/EBPα is a key and positive regulator of adipogenesis. Therefore, we evaluated C/EBPα protein expression between WT and Vav1 null MSCs. Consistent with the positive role of C/EBPα in adipogenesis and our observation that loss of Vav1 enhanced MSC to adipocyte differentiation, we found more C/EBPα proteins in Vav1 null MSCs than WT cells (Fig. 6A).

Based on the negative role of Vav1 in MSC adipocyte differentiation, we speculated that the levels of Vav1 should decrease during normal adipogenic differentiation. To test this, we cultured WT MSCs in adipogenic induction medium to induce cell differentiation. Cells were harvested and evaluated for protein expression at multiple time points. Vav1 was strongly expressed in MSCs, but its levels gradually decreased during adipocyte differentiation and largely absent in fully differentiated cells. Interestingly, the decrease of Vav1 correlated with an increase of C/EBPα proteins during MSC adipogenesis (Fig. 6B). These results raise a possibility that Vav1 inhibits MSC adipocyte differentiation through suppression of C/EBPα Because PPARγ and C/EBPα act sequentially to control adipocyte differentiation and PPARγ regulates C/EBPα expression [6], we therefore evaluated the effects of Vav1 on PPARγ by comparing the levels of PPARγ . WT and Vav1 null MSCs between the second to third passage. Although loss of Vav1 in MSCs had no effect on the

PPARγ protein levels, but it resulted in a clear increase of acetylated PPARγ compared to WT MSCs (Fig. 6C), indicative of activated PPARγ that is consistent with increased C/EBPα observed in these cells.

Moreover, we also investigated the mechanism by which Vav1 positively regulates chondrocyte differentiation. MSCs were cultured in chondrogenic induction medium for 3 weeks, followed by harvesting the cells and Western blot analysis for Col2α1, a marker for chondrocytes, in cell lysates. As expected, there was less of Col2α1 in Vav1 null cells than WT cells (Fig. 6D), which correlated with a retarded chondrocyte differentiation in Vav1 null MSCs. Since the transcription factor Sox9 binds to the promoter of Col2α1 and activates its expression [10, 11, 13], we then evaluated the levels of Sox9 using the same protein samples. Interestingly, we found that loss of Vav1 led to an increase in acetylated Sox9 (Fig. 6E). These data provide molecular evidence explaining retarded chondrocyte differentiation in Vav1 null cells as acetylation represses Sox9 activity. As a result, it inhibited Col2α1 expression and chondrogenic cell differentiation. Additionally, we analyzed expression of Vav1 during MSC chondrocyte differentiation. There was a decrease of Vav1 at the initial stage of induction of differentiation, but its level remained throughout the differentiation (Fig. 6F).

Above findings point out a potential role of Vav1 in regulating protein acetylation of PPARγ and Sox9, thereby regulating their activity. The levels of protein acetylation are controlled by both acetylase and deacetylase. Sirt1, a deacetylase, is known to target both PPARγ and Sox9. Sirt1 levels or activity is responsible for the elevation of Col2α1 expression through Sox9 in chondrocytes [4]. This led us to examine if Vav1 regulates Sirt1 expression by comparing Sirt1 levels in WT and Vav1 null MSCs at passage 2. We found the levels of Sirt1 were lower in Vav1 null than WT cells (Fig. 6A). Moreover, induction of WT MSC differentiation to adipocyte correlated with a reduction of both Vav1 and Sirt1 (Fig. 6B). This raises a possibility that Sirt1 is downstream of Vav1 and mediates transcription factor activity in MSC differentiation. To test this hypothesis, we treated WT MSCs with Ex527, a cell-permeable, and selective inhibitor for Sirt1 [20]. Inhibition of Sirt1 activity increased the levels of acetylated PPARγ and Sox9 (Fig. 6G), as well as increased the levels of C/EBPα but decreased the levels of Col2α1 in these cells (Fig. 6H). These results are in line with the transcription factor activity as acetylation of PPARγ and Sox9 activates and represses these two transcription factors, respectively, thus resulting in an increase of C/EBPo; a target of PPARγ, and a decrease of Col2α1, a target of Sox9, transcription factors.

To further establish the role of Sirt1 in Vav1-mediated adipogenesis, we performed rescue experiments by ectopic expression of Sirt1 in Vav1 null MSCs using lentiviral vectors. Overexpression of Sirt1 in Vav1 null MSCs resulted in a reduction of acetylated PPARγ (Fig. 6I), as well as a reduction in adipocyte differentiation as measured by oil red 0 staining (Fig. 6K). Taken together, these findings imply that Vav1 controls MSC differentiation by regulating PPARγ and Sox9 acetylation via Sirt1.

To validate the role of Vav1 in protein acetylation via Sirt1, we further performed complementary experiments by ectopic expression of Vav1 in WT MSCs. Consistent with knockdown studies, overexpression of Vav1 demonstrated reduced level of C/EBPα and the

increased levels of Sirt1and Col2α1 in MSCs (Fig. 7A), which correlated with a decrease of the acetylation levels of both PPARγ and Sox9 (Fig. 7B). These data are in agreement with the findings that overexpression of Vav1 inhibited MSC to adipocyte differentiation but promoted MSC to chondrocyte differentiation. Importantly, we treated Vav1 overexpressing MSCs with Ex527, a Sirt1 inhibitor. Neutralization of Sirt1 activity reversed the repression of Vav1 on C/EBPα expression and induction of Vav1 on Col2α1 in MSCs (Fig. 7C).

Above findings reveal that Vav1 regulates MSC differentiation through Sirt1, which led us to explore the mechanism by which Vav1 regulates Sirt1 expression in these cells. Based on the findings that Vav1 plays a role in Creb activation in T cells and Creb regulates Sirt1 expression in epithelial cells [17, 18], we reasoned that Vav1 regulates Sirt1 expression through the Creb transcription factor. To test this hypothesis, we evaluated the levels of Creb and phospho-Creb in MSCs. We found that loss of Vav1 has no effect on total Creb protein levels, but it resulted in a significant reduction of phospho-Creb (Fig. 7D). Furthermore, ectopic expression of Vav1 in WT MSCs led to an increase of phospho-Creb in MSCs (Fig. 7E). As expected, knockdown of Creb using shRNA in WT MSCs led to a reduction of Sirt1 (Fig. 7F). These results imply that Vav1 regulates Sirt1 expression through Creb.

To generalize the findings from murine MSCs and mouse models, we evaluated the role of Vav1 in human MSCs. Both BM derived human MSCs as well as MSCs isolated from human peripheral blood were cultured in MSCGM Mesenchymal Stem Cell Growth Medium. Western blotting results demonstrated that Vav1 protein was expressed in these human MSCs (Fig. 7G). In addition, partial knockdown of Vav1 in these cells resulted in a reduction of Sirt1 (Fig. 7G). These data implicate a similar function of Vav1 in MSC differentiation in human cells.

Discussion

MSCs are adult stem cells traditionally found in the BM. They are multi-potential stem cells and can differentiate into adipocytes, chondrocytes, and osteocytes. What regulates the cell fate decisions in MSC differentiation is an important question and has important implication in MSC based therapy. In this study, we report a novel role for Vav1 in MSC fate decisions in cell differentiation. Vav1 is highly expressed in MSCs, and its expression decreases during MSC differentiation to adipocyte. Consistently, genetic deletion of Vav1 in mice leads to spontaneous or accelerated MSC to adipocyte differentiation in vitro and increased body fat content in vivo. Surprisingly, loss of Vav1 inhibits MSC to chondrocyte differentiation, suggesting opposing roles for Vav1 in MSC to adipocyte versus chondrocyte differentiation. Conversely, ectopic expression of Vav1 in MSCs reverses the phenotype. These data collectively reveal a role for Vav1 in regulating cell fate decisions during MSC differentiation (Fig. 7H).

The Vav family proteins have three members in mammals. While Vav2 and 3 are widely expressed in various types of cells, Vav1 has been thought to be restricted in hematopoietic cells in normal development. Genetic deletion of Vav1 in mice leads to defective lymphocyte development [15, 16]. Accordingly, the Vav1 promoter driven Cre mice have been commonly used for specific gene deletion in hematopoietic cells. However in the current

study, we report that Vav1 is highly expressed in MSCs, a nonhematopoietic lineage cells. This finding expands our knowledge of Vav1 in biology. It also raises a concern regarding the specificity when using Vav1-Cre mice for gene deletion in hematopoietic cells.

The levels of Vav1 decrease during MSC adipogenic differentiation, which correlates with a reduction of Sirt1. Consistently, loss of Vav1 reduces and over expression of Vav1 increases the levels of Sirt1, which suggests a positive role for Vav1 in regulating Sirt1 expression in MSCs. Loss of Vav1 leads to spontaneous or accelerated MSC to adipocyte differentiation in vitro and adipogenesis in vivo. This observation in Vav1 null conditions is consistent with the function of Sirt1 in adipogenesis [1–3]. Thus, our data suggest that Vav1 regulates MSC adipogenic differentiation likely through Sirt1. In addition to functioning as a GEF protein for small RhoGTPase, Vav1 contains SH2 and SH3 domains and functions as an important signal transducer with a pivotal role in hematopoietic cell activation, cell growth, and differentiation [14].

Sirt1 is a NAD1-dependent deacetylase, which deacetylates various proteins including PPARγ, a transcription factor important for adipocyte differentiation [1, 2]. Sirt1 deacetylates PPARγ on Lys268 and Lys293 that are required to recruit the brown adipose tissue coactivator Prdm16 to PPARγ, leading to selective induction of brown fat genes and repression of white adipose tissue genes. An acetylation-defective PPARγ mutant induces a brown phenotype in white adipocytes, whereas an acetylated mimetic fails to induce “brown” genes but retains the ability to activate “white” genes [1]. In addition, Sirt1 protein binds to and represses genes controlled by PPARγ, including genes mediating fat storage. Sirt1 represses PPARγ by docking with its cofactors NCoR and SMRT. Repression of PPARγ by Sirt1 is also evident in 3T3-L1 adipocytes, where overexpression of Sirt1 attenuates adipogenesis, and knockdown of Sirt1 enhances it [2]. Therefore, Sirt1 regulates adipogenesis as well as fat content in adipocytes by regulating the transcriptional activity of PPAR and subsequent activation of C/EBPα [6]. In this study, we show that deletion of Vav1 in MSCs reduces Sirt1, which in turn leads to increased acetylation status of PPARγ and subsequent increase of C/EBPα in adipogenesis.

Sirt1 also plays a role in chondrogenesis. Elevation of Sirt1 protein levels or activity in chondrocytes leads to a dramatic increase in cartilage-specific gene expression, whereas a reduction in Sirt1 levels or activity significantly lowered the cartilage gene expression [4]. The functions of Sirt1 in chondrogenesis are likely mediated through Sox9 as Sirt1 associates with Sox9, modulates its acetylation status, and enhances transcription activity [4]. Sox9 is a key regulator that is required for multiple steps in chondrogenesis. It governs differentiation stage-specific gene expression in chondrocytes via direct concomitant transactivation and repression [21]. Mutations in Sox9 genes lead to the skeletal malformation syndrome campomelic dysplasia [22, 23]. In this study, we reveal a positive role of Vav1 in MSC to chondrocyte differentiation. Loss of Vav1 reduces the levels of Sirt1, which contributes to increased levels of acetylated Sox9 in MSCs. As acetylation represses Sox9, thereby inhibits chondrocyte specific gene expression and chondrogenic differentiation. Furthermore, it was reported that Sox9 downregulation is required for adipocyte differentiation. Sox9 directly binds to the promoter regions of C/EBPβ and C/EBPδ to suppress their promoter activity, preventing adipocyte differentiation [24]. It is

conceivable that loss of Vav1 in MSCs results in activation of PPARγ and inactivation of Sox9, which together promotes adipogenesis.

The important roles of Sirt1 in MSC differentiation have been reported [3–5]. Our findings demonstrate that Vav1 regulates MSC differentiation through Sirt1. Further investigation reveals that Vav1 regulates Sirt1 expression through the Creb transcription factor. Loss of Vav1 led to a reduction of phospho-Creb, suggesting Vav1 regulates Creb activation. Conversely overexpression of Vav1 increased Creb activity as measured by protein phosphorylation. Knockdown of Creb resulted in a reduction of Creb in MSCs. These findings are in agreement with the report that Vav1 regulates Creb activity in T cells and Creb regulates Sirt1 expression in epithelial cells [17, 18].

Conclusion

This study demonstrates for the first time that Vav1 is expressed in both murine and human MSCs and regulates cell differentiation likely through Sirt1. Since Sirt1 deacetylates PPARg and Sox9 and the acetylation status of these two transcription factors has opposite function on its activity, it provides a molecular mechanism by which Vav1 controls MSC fate decisions in differentiation between adipocyte and chondrocyte.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance Statement

This study investigates the mechanism that controls mesenchymal stem cell (MSC) cell fate decisions for differentiation, a fundamental question that has important implications in MSC based therapy. We show that Vav1 is highly expressed in MSCs and regulates MSC differentiation through the Creb/Sirt1 pathway. Sirt1 deacetylates PPARγ and Sox9, two key mediators for adipogenesis and chondrogenesis. Since the acetylation status of PPARγ and Sox9 has opposite effects on its activity, thereby regulating MSC differentiation decision. This study reveals a novel function of Vav1 in nonhematopoietic lineage cells in normal development.

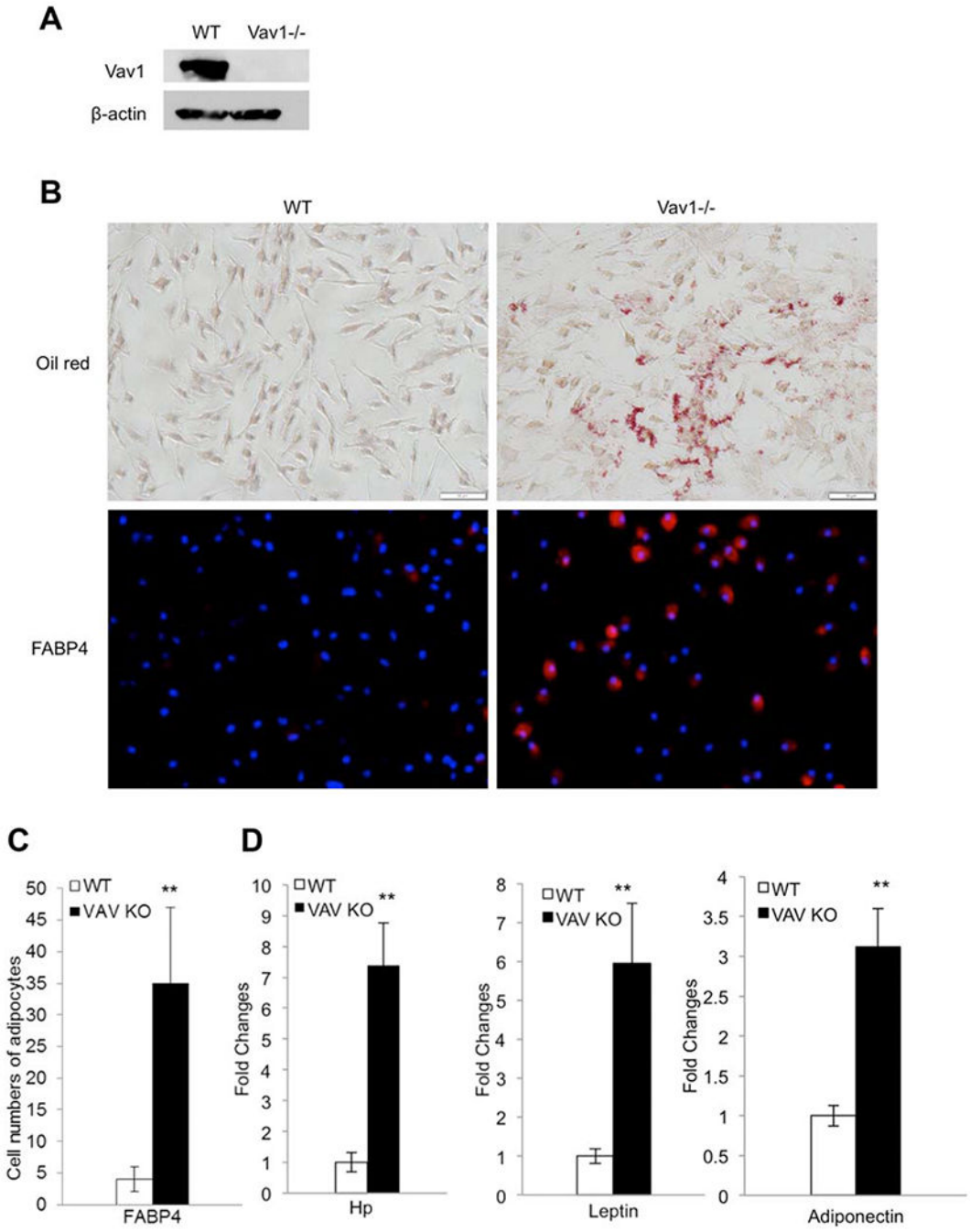

Figure 1.

Vav1 is expressed in murine MSCs and that negatively regulates MSC differentiation to adipocytes. **(A)**: Cultured MSCs at the second passage from WT and Vav1 null bone marrow were analyzed for Vav1 expression by Western blot. **(B)**: Oil red O staining was performed on the fourth passage MSCs cultured in Mesencult basal medium. FABP4 expression in the cells above was analyzed by immunofluorescent staining. Representative images were shown (bar = 50 μm). **(C)**: FABP4+ cells were enumerated in 10 randomly selected microscopic fields from cultures in each group. **(D)**: MSCs at the fourth passage were

harvested and the mRNA levels of Hp, leptin, and adiponectin were analyzed by qPCR. MSCs were pooled from three mice in each experiment, and each experiment was repeated twice. **, $p < .01$. Abbreviations: Hp, haptoglobin; MSCs, mesenchymal stem cells; WT, wild type.

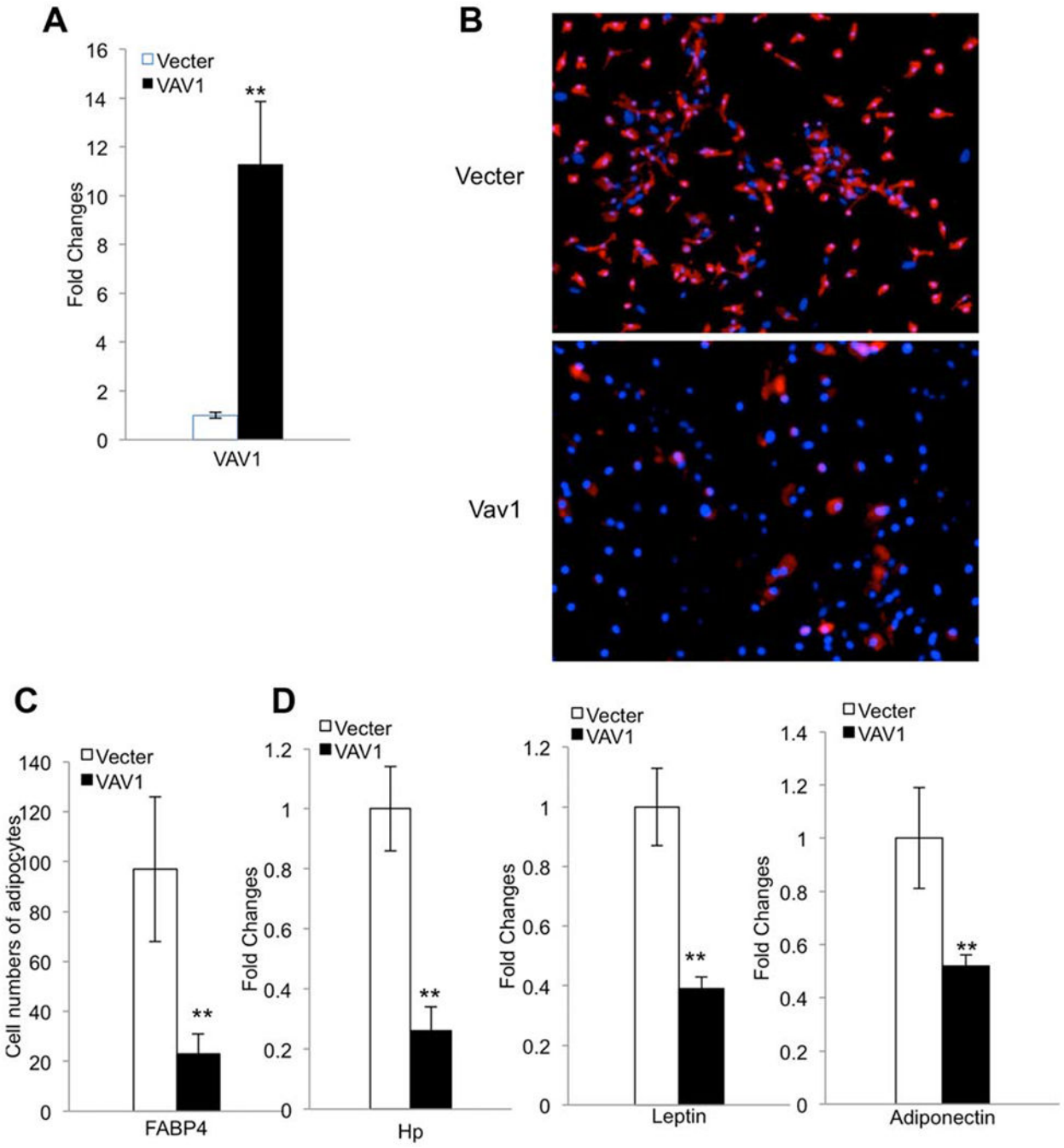

Figure 2.

Ectopic expression of Vav1 in MSCs inhibited adipogenic differentiation. **(A)**: WT MSCs were infected with a lentiviral vector for Vav1 (Vav1) or a vector control (vector), respectively. The levels of Vav1 were determined by qPCR. **(B)**: The cells were cultured in an adipogenic differentiation medium for 14 days, followed by immuno-fluorescent staining for FABP4. Representative images were shown. 100x mag. **(C)**: The numbers of FABP4+ cells were counted in 10 randomly selected fields under microscopy. **(D)**: The mRNA levels of Hp, leptin, and adiponectin in MSCs was analyzed by qPCR. MSCs were pooled from

three mice in each experiment, and each experiment was repeated twice. **, $p < .01$. Abbreviations: Hp, haptoglobin; MSCs, mesenchymal stem cells; WT, wild type.

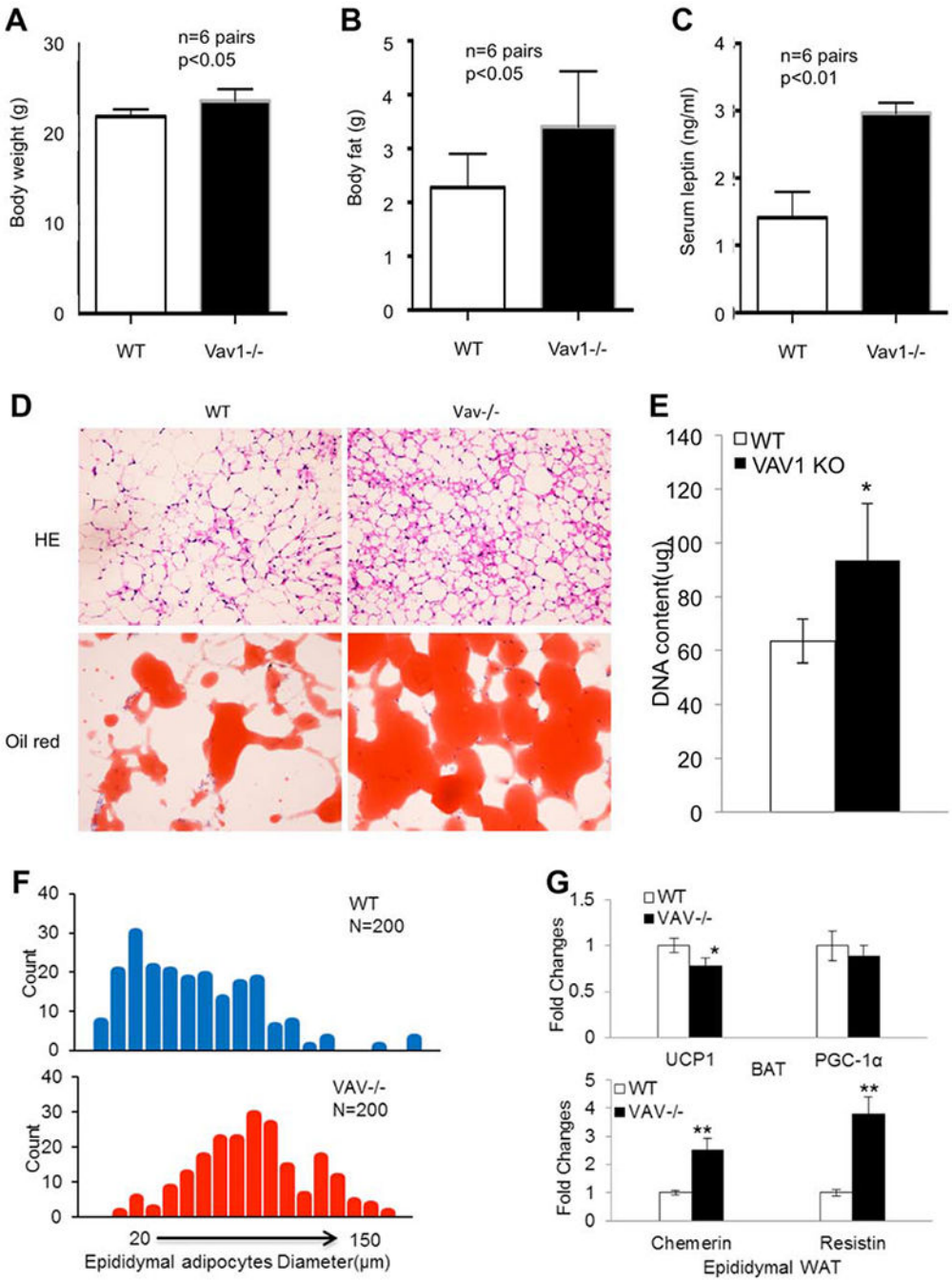

Figure 3.

Genetic deletion of Vav1 in mice resulted in increased body fat content. **(A)**: The body weight of 12 weeks old female mice was measured. **(B)**: Total body fat was determined by NMR using the Burker Minispec. **(C)**: Serum leptin levels were measured by ELISA. **(D)**: Subcutaneous and abdominal visceral fat was harvested and processed for HE staining and oil red staining. Representative images were shown. 100x mag. **(E)**: The numbers of adipocytes were determined by measuring the DNA content in epididymal fat pads. **(F)**: The distribution of diameter of adipocytes in the epididymal fat pads of 12 weeks old female WT

and Vav1 KO mice. **(G)**: qPCR analysis for UCP1 and PGC-1α in BAT; and chemerin and Resistin in epididymal WAT between WT and Vav1 null mice. Values represent mean ± SD. *, $p < .05$; **, $p < .01$. Abbreviations: BAT, brown adipocytic tissue; HE, hematoxylin and eosin; WAT, white adipocytic tissue; WT, wild type.

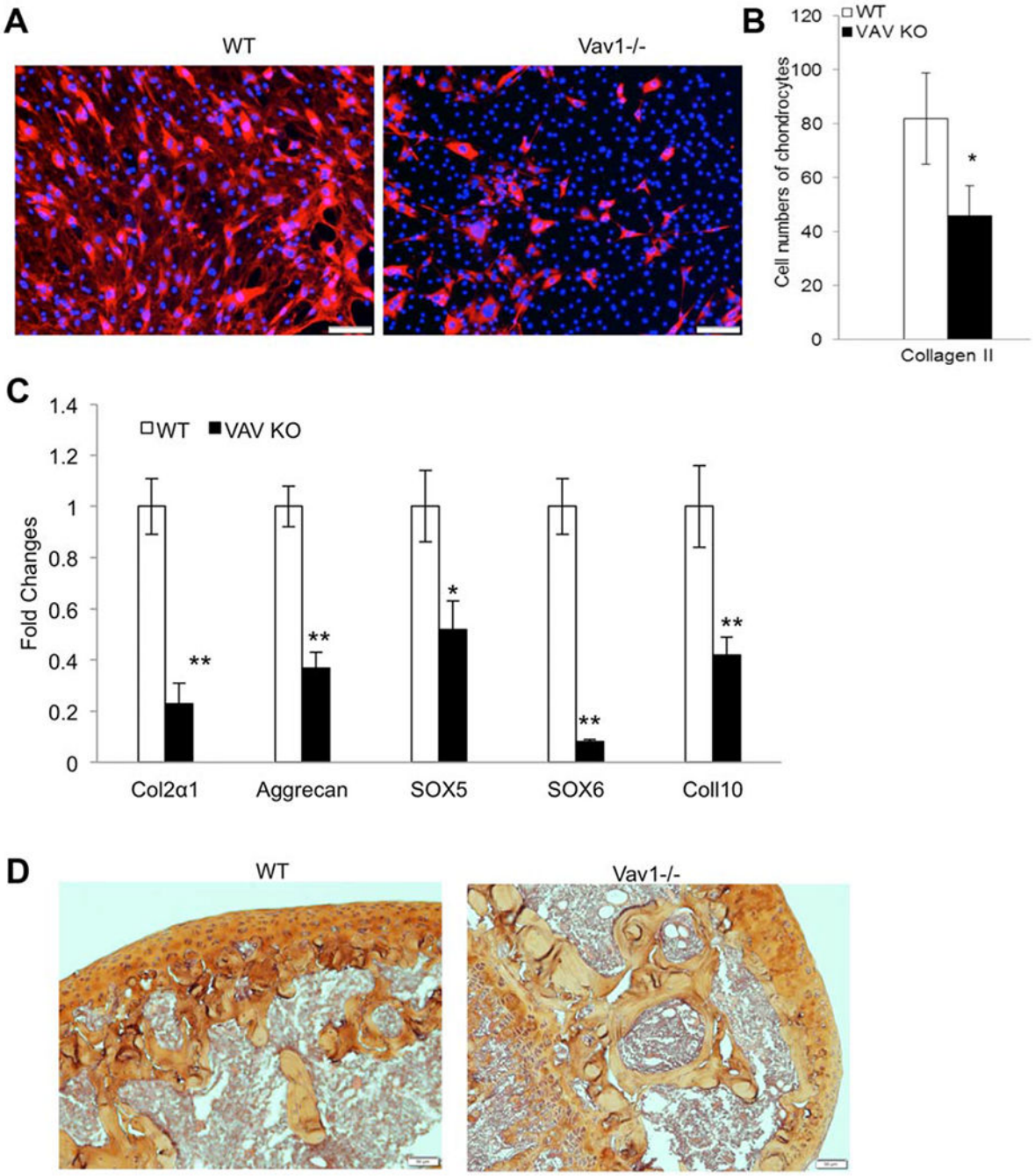

Figure 4.

Loss of Vav1 in MSCs retarded chondrocyte differentiation. **(A)**: MSCs at passage 2 were cultured in chondrogenic differentiation medium for 21 days, followed by immuno fluorescent staining for Collagen II (bar= 100 μm). Representative images were shown. **(B)**: The numbers of Collagen II positive cells were counted in 10 randomly selected microscopic fields. **(C)**: The levels of mRNA for Col2α1, aggrecan, Sox5, Sox6, and Coll10 were analyzed by qPCR. **(D)**: The sections of distal femoral from 4 months old WT and Vav1 null mice ($n = 5$) were stained with 0.1% safranin O. Representative images were shown. All

other experiments were repeated at least twice. Values represent mean ± SD, *, $p < .05$; **, $p < .01$. Abbreviations: MSCs, mesenchymal stem cells; WT, wild type.

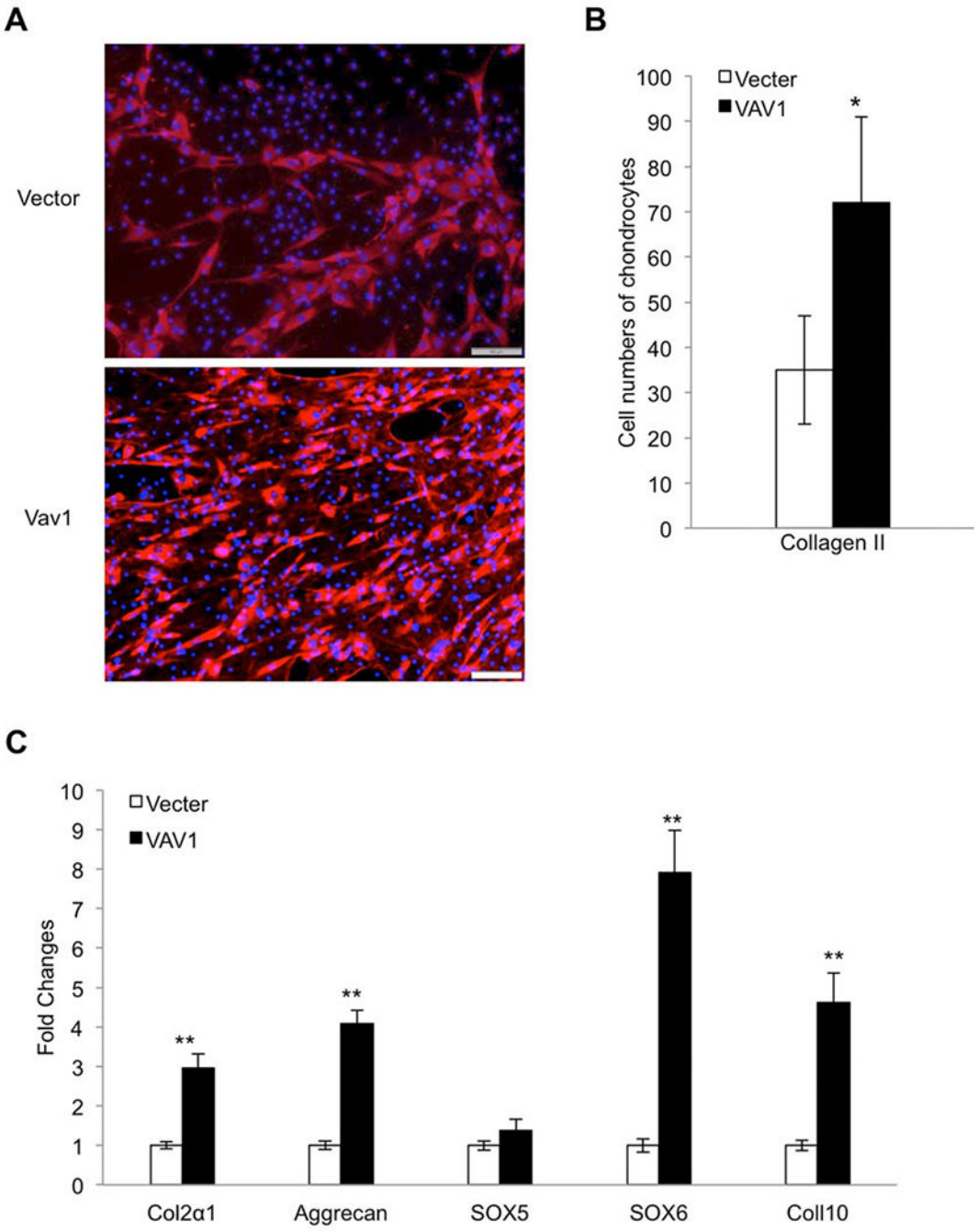

Figure 5.

Ectopic expression of Vav1 promoted MSC chondrogenic differentiation. **(A)**: Wild type MSCs were infected with a lentiviral vector for Vav1 (Vav1) or vector control (Vector), followed by culturing the cells in chondrogenic induction medium for 10 days. The cells were immuno-stained for Collagen II (bar = 100 μm). Representative images were shown. **(B)**: The numbers of Collagen II + cells were counted in 10 randomly selected microscopic fields. **(C)**: The levels of mRNA for Col2α1 and Aggrecan, Sox5, Sox6, and Coll10 were

analyzed by qPCR. Each experiment was done in triplicate and repeated twice. *, $p < .05$; **, $p < .01$. Abbreviations: MSCs, mesenchymal stem cells.

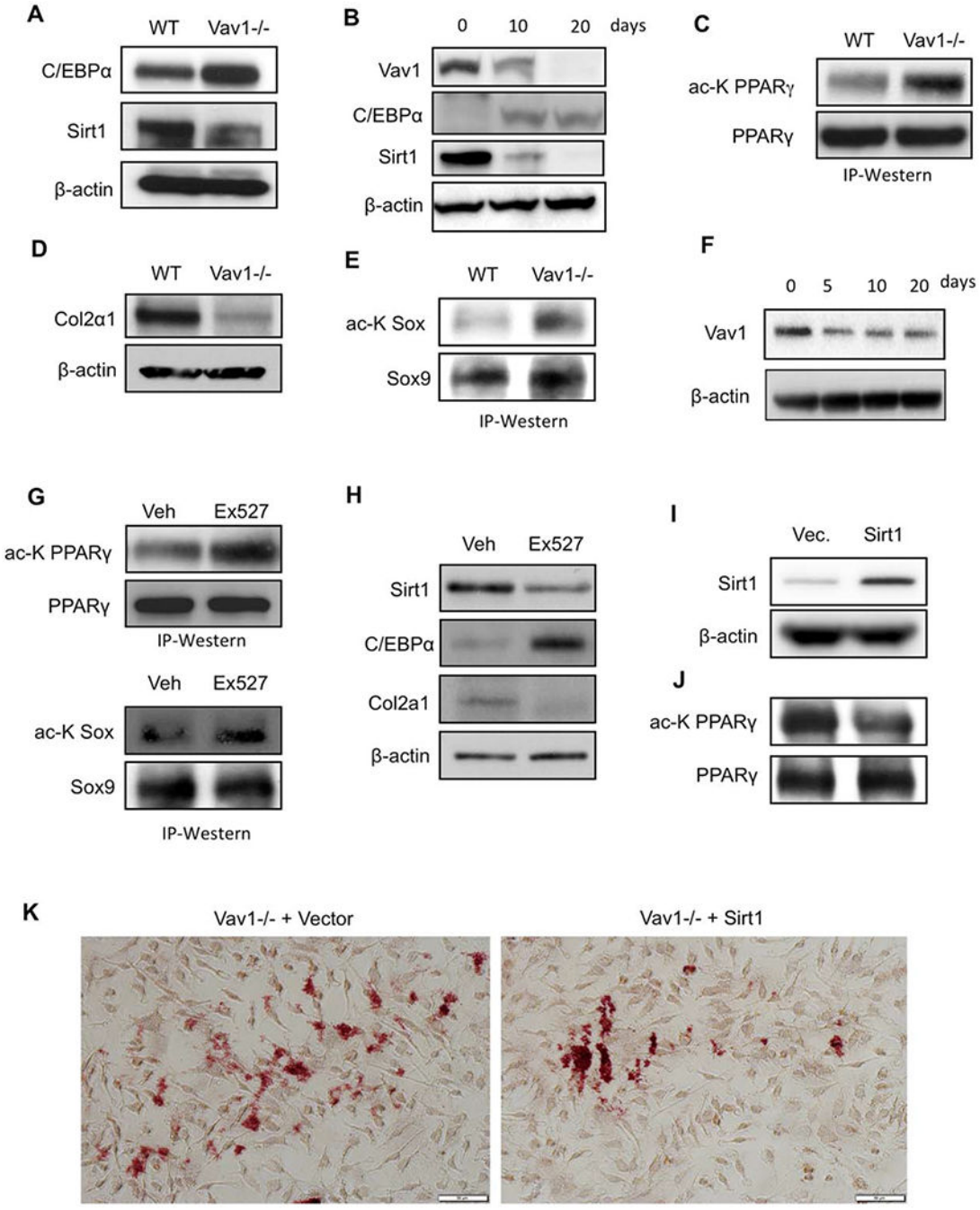

Figure 6.

Vav1 regulates MSC differentiation through Sirt1. **(A)**: MSCs at the second to third passage were harvested and evaluated for Sirt1 and C/EBPα expression by Western blot. **(B)**: WT MSCs were cultured in adipocyte differentiation induction medium. Cells were harvested at indicated time points and subjected to Western blot for Vav1, Sirt1, and C/EBPα **(C)**: Cell lysates of MSCs at the second to third passage were immunoprecipitated with a PPARγ antibody, followed by Western blot for total PPARγ and acetylated PPARγ. **(D)**: Second passage MSCs from WT or Vav1 null mice were cultured in chondrocyte culture medium for

3 weeks. Cell lysates were analyzed for Col2α1 expression by Western blot. **(E)**: Cell lysates of MSCs after chondrocyte differentiation induction were immunoprecipitated with a Sox9 antibody, followed by Western blot for total Sox9 and acetylated Sox9. **(F)**: WT MSCs were cultured in chondrocyte differentiation induction medium. Cells were harvested at indicated time points and subjected to Western blot for Vav1. **(G)**: WT MSCs were cultured in the presence of Ex527 at 10 μM for 48 hours, followed by the evaluation for total PPARγ and Sox9 proteins as well as acetylated PPARγ and Sox9 proteins. **(H)**: The levels of Sirt1, C/EBPα, and Col2α1 were evaluated after incubation with Ex527. (**I, J,** and **K)**: Vav1 null MSCs were infected with lentiviral vectors for Sirt1 or vector control for 48 hours. The cell lysates were subjected to Western blot analysis for Sirt1, acetylated PPARγ, and total PPARγ. Oil red O staining was performed on cultured cells (bar = 50 μm). Abbreviations: MSCs, mesenchymal stem cells; WT, wild type.

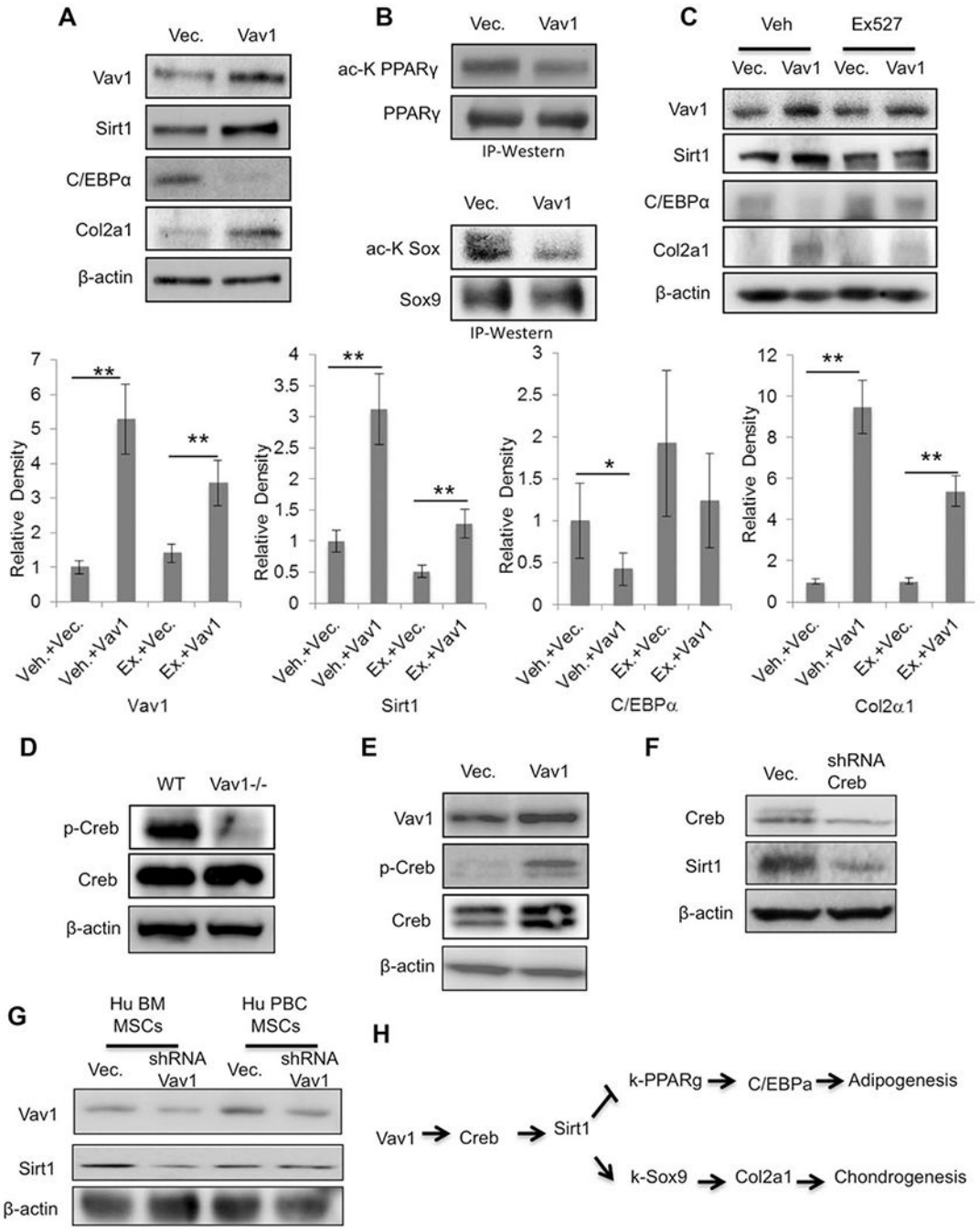

Figure 7.

Vav1 regulates MSC differentiation through Creb-mediated Sirt1 expression. **(A** and **B)**: WT MSCs were infected with lentiviral vectors for Vav1 or vector controls for 48 hours. The cell lysates were subjected to Western blot analysis for Vav1, Sirt1, C/EBPα, and Col2α1, as well as IP-Western for total PPARγ and acetylated PPARμ, and total Sox9 and acetylated Sox9. **(C)**: WT MSCs were infected with lentiviral vectors for Vav1 or vector controls for 48 hours in the absence or presence of Ex527 at l0 μM. The cell lysates were subjected to Western blot analysis for Vav1, Sirt1, C/EBPα, and Col2α1. The protein levels were

quantitated by measuring the intensity of Western blot. Each experiment was repeated at least twice. *, $p < .05$; **, $p < .01$. **(D)**: Cell lysates from second passage MSCs of WT or Vav1 null mice were subjected to Western blot analysis for phosphor-Creb and total Creb protein. **(E)**: WT MSCs were infected with lentiviral vectors for Vav1 or vector controls for 48 hours. The cell lysates were subjected to Western blot analysis for Vav1, phosphor-Creb, and total Creb protein. **(F)**: WT MSCs were infected with shRNA for Creb or vector controls for 48 hours. The cell lysates were subjected to Western blot analysis for Sirt1 and total Creb protein. **(G)**: The protein levels of Vav1 and Sirt1 were evaluated from both human bone marrow and peripheral blood cell derived MSCs, which were infected with either shRNA for Vav1 or vector controls and cultured in MSCGM Mesenchymal Stem Cell Growth Medium for 48 hours. **(H)**: A diagram of putative signaling pathway, by which Vav1 acts as a central regulator via Creb/Sirt1 to control the MSC commitment towards adipocytes versus chondrocytes. Abbreviations: BM, bone marrow; MSCs, mesenchymal stem cells; PBC, peripheral blood cell; WT, wild type.