SIRT6 Promotes Osteogenic Differentiation of Adipose-Derived Mesenchymal Stem Cells Through Activating Notch Signaling via Antagonizing DNMT1

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Abstract

Background: Adipose-derived stem cells (ADSCs) are increasingly accepted as one of ideal seed cells for regenerative medicine for its potential to differentiate into multiple cell types, including osteogenic lineages. Sirtuin proteins 6 (SIRT6) is a nicotinamide adenine dinucleotide (NAD)-dependent deacetylase and plays important roles in a variety of biological processes, including cell differentiation.

Methods: Alkaline phosphatase (ALP) activity, ALP staining, and Alizarin Red Staining was performed to explore the roles of SIRT6 in the osteogenic differentiation of ADSCs. Western blot , RT-qPCR,Luciferase reporter assay and Co-Immunoprecipitation assay were applied to confirm the relationship between of Sirt6, DNA methyltransferases (DNMTs) and NOTCHs.

Results: SIRT6 leads to increased alkaline phosphatase (ALP) activity, enhanced mineralization and upregulated expression of osteogenic-related genes of human adipose-derived mesenchymal stem cells (hADSCs) in vitro and in vivo. Further mechanistic studies showed that SIRT6 regulated osteogenic differentiation of hADSCs depending on its deacetylase activity. SIRT6 selectively prevents abnormal DNA methylation of NOTCH1, NOTCH2 in hADSCs by antagonizing DNMT1. DNMT1 expression was suppressed in SIRT6 overexpression hADSCs, and knockdown partially rescued abnormal DNA methylation of NOTCH1 and NOTCH2, leading to the increased capable of osteogenic differentiation.

Conculsions: SIRT6 promotes the osteogenic differentiation of hADSCs.The SIRT6 protein suppresses DNMT level via physical interaction with the DNMT1 protein, deacetylating and destabilizing DNMT1 protein,leading the activation of NOTCH1 and NOTCH2.

Introduction

Adipose-derived stem cells (ADSCs) are a type of mesenchymal stem cells and being increasingly accepted as one of ideal seed cells for regenerative medicine for its potential to differentiate into multiple cell types, including adipogenic, osteogenic, and chondrogenic lineages[1, 2, 3]. Thanks to their osteogenic capacity and easy to acquire and culture, hADSCs are considered as a suitable cellular source in the regeneration of bone loss and fractures[4, 5]. However, the microenvironment such as inflammation, age, gender can impair the multiplex differentiation potential of hADSCs and the exact underlying mechanisms are still unknown.

Sirtuin proteins 6 (SIRT6) is a NAD+-dependent protein deacetylase that involved in several important biological processes including genomic stability, transcriptional silencing and DNA repair[6, 7]. SIRT6 has high affinity to chromatin and universally deacetylates the histone H3 lysine 9 (H3K9), and H3 lysine 56 (H3K56) in a NAD+-dependent manner[8]. The deacetylation of telomeric H3K9 by SIRT6 is necessary for the function of telomeric chromatin and structures stability[9]. SIRT6 is a key factor for the embryonic stem cells development, SIRT6 inactivating mutation results in perinatal lethality and severe congenital anomalies[10]. C-terminal binding protein interacting protein, an important mediator in DNA end resection is

was deacetylated by SIRT6[11]. Recent studies imply SIR6 is associated with stem cell regulation. SIRT6 deacetylates the H3K56ac and H3K9ac of Oct4, Sox2 and Nanog at the promoter regions, and in turn control embryonic stem cell differentiation[12]. SIRT6 deficiency leads the histone hyperacetylation at the imprinting control region of long non-coding RNA H19, the activation of H19 delays neuronal differentiation[13]. These studies suggest roles of SIRT6 in the regulation of stem cell differentiation.

The modification of histone proteins and DNA impacts the chromatin structure and in turn directs transcription of cells[14]. Particularly, methylation in DNA notably impacts the expression and stability of certain genome[15]. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) at the 5mC of CpG dinucleotides. DNMT1, DNMT3a, DNMT3b and DNMT3l are responsible for de novo DNA methylation[16]. Among them, DNMT1 often methylates newly replicated DNA and is regarded as a maintenance enzyme. DNA methylation is necessary for fundamental biological processes, including embryonic development, gene regulation, developmental potential of stem cells, genomic imprinting and so on [17, 18, 19].

In this study, we investigated the effect of SIRT6 on the osteogenic differentiation of hADSCs and found SIRT6 overexpression promoted osteogenesis of hADSCs. The increase in osteogenic differentiation of hADSCs by SIRT6 could be impaired by DNMT1 or DAPT, a NOTCH inhibitor. We further explore the mechanism and confirmed SIRT6 increased the transcription of NOTCH1 and NOTCH2, while DNMT1 decreased their expression level. SIRT6 protein antagonizes DNMT1 by modifying the histone acetylation status of the DNMT1 promoter. These results suggest that SIRT6 orchestrates DNA methylation patterns of NOTCH1 and NOTCH2 to promote the osteogenic differentiation of hADSCs.

Methods

Cell lines and reagents

Human adipose-derived stem cells (hADSCs) were isolated and purified from fresh human adipose tissues donated from healthy adults less than 40 years of age. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) containing 10% fetal bovine serum and cultured at 37 °C in a humidified atmosphere of 5% CO2. Osteogenic induction medium was purchased from SALILA (SALILA, Guangzhou,China). The medium was changed every 2 days. To inhibit DNA methylation, hADSCs were treated by 5 μmol/L 5-aza-2'-deoxycytidine (DAC, Sigma Aldrich, Munich, Germany). To inhibit NOTCH signaling, cells were treated with 2 μM DAPT(Selleck), protein deacetylation was inhibited by 20 μM OSS_128167 (Selleck). For the cycloheximide (CHX)-chase assay, cells were treated with 100 μg/mL CHX (Sigma-Aldrich) for the indicated hours in the absence or presence of 5 μg/ml actinomycin D (Sigma-Aldrich) and western blot analysis was performed.

Western blot

hADSCs were lysed on ice for 15 mins using RIPA lysis buffer (BeyoTime, China) supplemented with protease inhibitors cocktail (Roche). The protein concentration of the lysate was then measured using the BCA Protein Assay Kit (Beyotime, China) according to the manufacturer's protocol. Protein sample were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, USA), which were blocked with 5% fat-free milk and then incubated with specific primary antibodies overnight at 4 °C. An anti-rabbit-HRP (horseradish peroxidase) secondary antibody was added, and the staining was visualized using an enhanced chemiluminescence detection system (Millipore, Billerica, USA). The primary antibodies used in this study were as follows: SIRT6, RUNX2, Sp7, COL1A1, NOTCH1, NOTCH2, NOTCH3, NOTCH4, Jag1, HEY1(Abcam, UK), HA, Flag, GAPDH (Beyotime, China) DNMT1,Acetylated Lysine (Ac-K) (Cell Signaling Technology).

Quantitative Reverse Transcription (RT)-PCR

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesized using a Reverse Transcription Kit (Promega, Madison, WI). cDNA was applied for quantitative PCR reactions to determine the expression of specific genes with SYBR Green Real-Time PCR Master Mix Kit (Invitrogen) according to the manufacturer’s instructions. GAPDH mRNA was applied as endogenous controls. Primer sequences were shown in Supplementary Table 1.

Lentivirus, siRNAs and shRNAs transfection

The lentivirus particles for overexpression of knocking down genes were purchased from GeneChem (GeneChem, Shanghai, China). hADSCs cells in 24-well plates were infected with the lentiviral vectors fixed with 10 mg/ml polybrene (GeneChem) in DMEM (Invitrogen, USA). Stable clones were selected using 0.5 μg/ml puromycin. Short hairpin RNAs (Supplementary Table 2) were synthesized by RiboBio(Guangzhou, China). hADSCs cells in 6-well plates were treated with 50nM siRNAs or 4 μg shRNAs using Lipofectamine 3000 reagent (Invitrogen) according to the manuscript and then harvested for assays.

ALP staining and activity

ALP staining was performed in cells seeded in 24-well plates. After treatment with osteogenic differentiation medium for 7 days, the cells were fixed in 70% ethanol and incubated with buffer a staining solution containing 0.1% naphthol AS-TR phosphate and 2% fast violet B (Sigma-Aldrich, St Louis, MO, USA) for 1 h at room temperature. The ALP activity was calculated quantitatively using a kit (Cell Biolab, San Diego, CA, USA) according to the manufacturer’s protocol

Alizarin red staining

hADSCs were incubated with osteogenic red S (pH 4.2, Sigma) for 10 min. For the quantitative assessment of the mineralization, the mineralized bone nodules were eluted by 10% (w/v) cetylpyridinium chloride (Sigma-Aldrich) for 1 h and determined by measuring the absorbance at 562 nm.

Masson’s trichrome staining

In vivo transplantation was previously described[20]. In brief, 4 weeks old NOD mice were purchased from the Provincial Animal Center (Guangdong, China). The osteogenic differentiation-induced hADSCs were loaded onto 5 mg hydroxyapatite/tricalcium phosphate (HA-TCP; Sigma, St Louis, MO) and subcutaneously inoculated into the right dorsal region of mice (5 mice per group). 4 weeks later, the mice were subjected to surgical procedures as previously described [20]. The research proposal on animal experiments was approval by the local Ethics Committee for Animal Study. All procedures were approved by the Animal Care Committee of Southern Medical University. The samples were collected, fixed with 4% paraformaldehyde, and decalcified in 10% EDTA (pH 6.0) for 7 days. Paraffin sections were prepared and stained with hematoxylin and eosin, Masson's Trichrome stain (Sigma) according to the manufacturers' protocols. The research proposal on animal experiments was approved by the local Ethics Committee for Animal Study. All procedures were approved by the Animal Care Committee of Southern Medical University.

Luciferase reporter assay

The 2 kb region upstream of the transcription starting site of NOTCH1, NOTCH 2 and DNMT1 were synthesized and cloned into upstream of the luciferase gene in the pmirGLO luciferase vector (GeneChem, Shanghai, China). hADSCs and 293T cells were treated with the indicated Transfection particles using Lipofectamine 2000 (Invitrogen). Promoter activity was measured using the luciferase assay kit (Promega) and normalized to the Firefly and Renilla luciferase activities 48 h after transfection.

Co-Immunoprecipitation

NP-40-containing lysis buffer supplied with protease inhibitor cocktail was used to lysed cells (Roche), the immunoprecipitated complexes were recovered with ChIP grade antibodies against acetylated lysine (Ac-K) (Cell Signaling Technology), HA epitope(Beyotime, Shanghai,China), Flag epitope (Beyotime, Shanghai, China), rabbit immunoglobulin (Ig)G control antibodies (Sigma-Aldrich), which were incubated with protein A/G Sepharose beads (Santa Cruz, USA) first, and then rinsed with wash buffer. The eluted immune complexes were denatured and subjected to western blot assay.

Statistical Analysis

The data were presented as the mean± SD. All data were analyzed by one- or two-way ANOVA with Bonferroni post hoc tests. SPSS 18.0 was used to perform all analyses. Statistical significance was defined as $p< 0.05$.

Results

SIRT6 promoted osteogenic differentiation of hADSCs both in vitro and in vivo

In order to explore the role of SIRT6 in the osteogenic differentiation, hADSCs were cultured with osteogenic inductive conditions. hADSCs sustained capacity for osteogenic differentiation, as indicated by gradually increased expression of osteogenic markers Runx2, SP7 and ALP. And the SIRT6 expression was increased after osteogenic induction (Fig. 1A and 1B). Then, we constructed stable SIRT6 - overexpression or knockdown hADSCs, confirmed by RT-qPCR and Western blot (Fig. 1C and 1D). Alizarin red S (ARS) staining, ALP staining and activity assays revealed that upregulated SIRT6 promoted mineralized bone matrix formation and ALP activity in hADSCs, SIRT6 knockdown inhibited osteogenic differentiation in hADSCs (Fig. 1E-G). To further evaluate the osteogenic function of SIRT6 in hADSCs in vivo. SIRT6 overexpression hADSCs was administered with osteogenic differentiation for 2 weeks and implanted into immunocompromised mice subcutaneously using HA/ TCP as a carrier. hADSCs with SIRT overexpression exhibited significantly increased capacities to generate new bone as shown by Masson's Trichrome stain(Fig. 1H). These results strongly suggest that SIRT6 promoted the osteogenesis of hADSCs both in vitro and in vivo.

NOTCH signaling contributed to SIRT6-mediated promotion of osteogenic differentiation in hADSCs

NOTCH signaling has been reported to regulate bone mesenchymal cell differentiation[21]. We hypothesized NOTCH signaling was regulated by SIRT6 in the process of osteogenic differentiation in hADSCs. To test this hypothesis, we detected Notch family members expression in SIRT-overexpressed or silenced hADSCs, The results showed that NOTCH1 and NOTCH2 were increased by SIRT6 overexpression and decreased by SIRT6 knockdown both at mRNA and protein levels (Fig. 2A and 2B). Then, we overexpressed NOTCH1 and NOTCH2 in hADSCs and found osteogenic markers Runx2,SP7 and ALP were increased consequently (Fig. 2C-2F). Mineralized bone matrix formation and ALP activity in hADSCs were also elevated in NOTCH1 and NOTCH2 overexpressed hADSCs as shown by Alizarin red S staining, ALP staining and activity assays (Fig. 2G-2L).

To identify the role of NOTCH signaling in SIRT6-mediated promotion of osteogenic differentiation, NOTCH signaling inhibitor, DAPT, was applied in SIRT6-overexpressed hADSCs. DAPT treatment reversed the increased expression of NOTCH1, NOTCH2, JAG1, HEY1 by SIRT6 (Fig. 3A and 3B). In addition, DAPT partially restrained SIRT6-induced osteogenic differentiation capacity, as indicated by mineralized nodule formation and ALP activity (Fig. 3C-3E). In vivo experiment, DAPT also impaired the promoting effect of

SIRT6 on hADSCs' function in mineralized nodule formation, as illustrated by Masson's Trichrome stain (Fig. 3F). To further confirm the role of NOTCH signaling in the regulation of hADSCs osteogenic differentiation, SIRT6-overexpression hADSCs were transfected with NOTCH1 or NOTCH2 shRNAs. Alizarin red S staining, ALP staining and activity assays demonstrated that silencing NOTCH1 or NOTCH2 could abolish the increased capacity of osteogenic differentiation by SIRT6(Fig. 3G-3I). These data indicate that NOTCH signaling played an important role in the SIRT6- induced promotion of osteogenic differentiation in hADSCs.

DNMT1 suppressed osteogenic differentiation in hADSCs by inducing hypermethylation of the NOTCH1 and NOTCH2

Epigenetic regulation, such as DNA methylation, is essential for the self-renewal and differentiation of stem cells[22, 23]. DNA methylation of CpG dinucleotides is regulated by DNMTs, including DNMT1, Dnmt3a and Dnmt3b and DNMT3l[24]. We examined whether DNA methylation was involved in osteogenic differentiation of hADSCs. ShRNAs targeting DNMT1, Dnmt3a and Dnmt3b and DNMT3l were transfected into hADSCs and the capacity of osteogenic differentiation were assessed. We found DNMT1, rather than Dnmt3a and Dnmt3b or DNMT3l, was verified as a potential regulator in osteogenic differentiation of hADSCs(Fig. 4A and 4B). When overexpressing DNMTs, only DNMT1 significantly decreased osteogenic markers and impair the capacity of osteogenic differentiation in hADSCs as confirmed by Alizarin red S staining, ALP staining and activity assays (Fig. 4C-4E). Otherwise, DNMT1 silencing increased the mineralized nodule formation and ALP activity in vitro(Fig. 4F-4H).

To gain insights into weather the hypermethylation of the NOTCH1 promoters was regulated by DNMT1.The expression of NOTCH1, NOTCH2 JAG1,HEY1 were remarkably increased in DNMT1 silenced hADSCs at both transcription and protein levels(Fig. 5A and 5B). In contrast, DNMT1 overexpression suppressed the NOTCH signal (Fig. 5C and 5D). Moreover, when Cotransfected with NOTCH1 or NOTCH2 overexpression plasmid in DNMT1-overexpressed hADSCs partially rescued the DNMT1 induced impairment of osteogenic differentiation hADSCs, as indicated by expression of Runx2, SP7 and the mineralized nodule formation capacity and ALP activity (Fig. 5E-5H). These data demonstrate that DNMT1 promoted osteogenic differentiation of hADSCs by inducing the hypermethylation of the NOTCH1and NOTCH2.

SIRT6 suppressed the DNMT1 transcription and deacetylated the DNMT1 protein via physical interactions

Based on the above results, we hypothesized that DNMT1 regulated by the SIRT6 protein was responsible for the inhibition of NOTCH signaling in osteogenic differentiation of hADSCs. Consistent with the above result, DNMT1 overexpression could abolish the SIRT6-induced increase of NOTCH1, NOTCH2, JAG and HEY1(Fig. 6A). In order to assesse whether SIRT1 could suppress DNMT1 transcription, a luciferase vector containing the upstream of the transcription starting site of DNMT1 was constructed. We confirmed that SIRT6 overexpression suppressed the DNMT1 promoter activity, in contrast, SIRT6 knockdown increased the luciferase activity (Fig. 6B and 6C). As reported, the SIRT1 protein physically

interacted with DNMT3l and regulates its activity by protein deacetylation. In this study, we assessed the potential physical interactions between SIRT6 and DNMT1 proteins. Co-Immunoprecipitation assay was performed in hADSCs and 293T cells with flag-tagged SIRT6 proteins. Endogenous DNMT1 proteins were identified in the SIRT6 protein complex (Figure. 6D and 6E).

To identify whether SIRT6 could deacetylate DNMT1, we examined the acetylation status of the DNMT1 protein in hADSCs via immunoprecipitation assays. The results demonstrated that an amount of DNMT1 protein was acetylated in hADSCs, and acetylated DNMT1 was found at a remarkably lower level in SIRT6 overexpressed hADSCs (Fig. 6F and 6G). These results suggest that DNMT1 protein could be acetylated by SIRT6 in hADSCs.

Next, we explored the biological significance of the acetylation status in DNMT1, SIRT6 overexpression hADSCs were administrated with OSS_128167, a chemical inhibitor of SIRT6. OSS_128167 treatment increased the protein and mRNA level of DNMT1 (Fig. 6H-6J). Then, we performed a cycloheximide (CHX) chasing assay to check the stability of the DNMT1 protein with or without OSS_128167 treatment. As shown in Fig. 6K, blocking the SIRT6 deacetylase activity dramatically increased the stability of DNMT1. In addition, the DNMT1 protein stability was weaker in SIT6 overexpression hADSCs cells than in control cells (Fig. 6L). These results indicate that SIRT6 regulated DNMT1 protein stability via a posttranslational modification mechanism.

Discussion

SIRT6 has been identified as an NAD[1]-dependent deacetylase and is crucial for genome stability, telomere integrity, and life span[7, 25]. In the present study, we demonstrated that SIRT6 expression was increased after osteogenic induction of hADSCs, SIRT6 overexpression significantly increased the expression of osteogenic markers and promoted mineralized bone matrix formation and ALP activity. The effects of SIRT6 overexpression on osteogenic differentiation could be offset by NOTCH1/2 shRNAs or DAPT. The expression of NOTCH1 and NOTCH2 was epigenetic regulated by DNMT1. DNMT1 impaired the capacity of osteogenic differentiation in hADSCs as confirmed by Alizarin red S staining, ALP staining and activity assays, which could be rescued by SIRT6 overexpression as well as NOTCH1 and NOTCH1 plasmid transfection. SIRT6 promoted osteogenic differentiation via preventing abnormal DNA methylation of NOTCH1 and NOTCH2 by antagonizing DNMT1.

Altered DNA methylation of the NOTCH family protein has been observed in stem cells[26 27]. Consistent with these reports, we identified that NOTCH1 and NOTCH2 were regulated by DNMT1. But we were unable to explain why DNMT1 selectively hypermethylated NOTCH family members. DNA methylation of these two genes resulted in increased ALP activity, enhanced mineralization and elevated expression level of osteogenic-related genes. Inhibition of NOTCH signaling via silencing JAG1 caused an impairment of osteogenic differentiation[28]. Tetrahedral DNA nanostructures, could dramatically enhance the proliferation and osteogenic differentiation of dental pulp stem cells by upregulating the NOTCH signaling[29]. In this article, overexpression of NOTCH1 and NOTCH2 promoted the osteogenic

differentiation of hADSCs and DNMT1 rather than DNMT3a, DNMT3b or DNMT3L could restrain this effect. Hence we speculated that the specificity of SIRT6 targets could be determined by their association with the DNMT1 protein.

In our study, the SIRT6 protein deacetylated and destabilized DNMT1 protein via physically interaction. Peng et al. reported DNMT3l was a substrate of the SIRT1 protein[30], And DNMT3l expression and the protein stability were regulated by SIRT1[31]. Here, we identified that SIRT6 protein strongly interacted with DNMT1. DNMT1 overexpression significantly supressed NOTCH1 and NOTCH2 and impaired the osteogenic differentiation. This suggested that DNMT1, a crucial target of SIRT6, involved in regulation of DNA methylation of NOTCH1 and NOTCH2 in hADSCs. Thus, SIRT6 might affect distinct downstream targets via DNMT1, which regulated the DNA methylation status of this gene. Here, we revealed that DNMT1 was a substrate for SIRT6 deacetylase in hADSCs and identification of the proteins directly modified by SIRT6 would be helpful to understand the biological significance of the SIRT6-DNMT1 complex in the multi-directional differentiations of hADSCs.

Conclusions

In our study, we identified the regulation of osteogenic potential of hADSCs composed of SIRT6/DNMT1/NOTCHs axis. SIRT6 increased the levels of NOTCH1 and NOTCH2 via acetylated DNMT1,which could hypermethylated NOTCH1 and NOTCH2. Our study exhibited the promotion of SIRT6 in osteogenic differentiations and the potential role in the treatment of osteogenesis disorders.

Abbreviations

ADSCs, Adipose-derived stem cells; ALP, Alkaline phosphatase; SIRT6, Sirtuin proteins 6; DNMTs, DNA methyltransferases; ARS, Alizarin red S; CHX, cycloheximide

Declarations

Fundings

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Author contributions

BO JIAJUN CHEN, YAN HE and QINGSONG YE conceived the research idea and designed the whole experimental plan. JUN CHEN, QIN WANG and XIANG SUN isolated and characterized the adipose-derived mesenchymal stem cells. BO JIA, JIUSONG HAN, SHIJIAN XIANG performed the animal study. BO JIA and QINGSONG YE analyzed the in vitro and in vivo results and discussed the findings with YAN HE, BO JIA

wrote the initial draft of the manuscript. YAN HE and QINGSONG YE revised and finalized the manuscript. FERNANDO GUASTALDI provide constructive suggestion of the work and proofread the language of the manuscript.

Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

Ethics approval

The research protocols associated with the experimental mice were approved by the Ethics Committee of the Southern Medical University.

Disclosure of Potential Conflicts of Interest

The authors declared no conflicts of interest.

References

1. Bianco P, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2008, **2**(4)**:** 313-319.
2. Worthley DL, Churchill M, Compton JT, Tailor Y, Rao M, Si Y*, et al.* Gremlin 1 identifies a skeletal stem cell with bone, cartilage, and reticular stromal potential. *Cell* 2015, **160**(1-2)**:** 269-284.
3. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J Cell Physiol* 2007, **213**(2)**:** 341-347.
4. Schubert T, Lafont S, Beaurin G, Grisay G, Behets C, Gianello P*, et al.* Critical size bone defect reconstruction by an autologous 3D osteogenic-like tissue derived from differentiated adipose MSCs. *Biomaterials* 2013, **34**(18)**:** 4428-4438.
5. Heilmeier U, Hackl M, Skalicky S, Weilner S, Schroeder F, Vierlinger K*, et al.* Serum miRNA Signatures Are Indicative of Skeletal Fractures in Postmenopausal Women With and Without Type 2 Diabetes and Influence Osteogenic and Adipogenic Differentiation of Adipose Tissue-Derived Mesenchymal Stem Cells In Vitro. *J Bone Miner Res* 2016, **31**(12)**:** 2173-2192.
6. Kanfi Y, Naiman S, Amir G, Peshti V, Zinman G, Nahum L*, et al.* The sirtuin SIRT6 regulates lifespan in male mice. *Nature* 2012, **483**(7388)**:** 218-221.
7. Mostoslavsky R, Chua KF, Lombard DB, Pang WW, Fischer MR, Gellon L*, et al.* Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. *Cell* 2006, **124**(2)**:** 315-329.

8. Kawahara TL, Michishita E, Adler AS, Damian M, Berber E, Lin M*, et al.* SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. *Cell* 2009, **136**(1)**:** 62-74.
9. Michishita E, McCord RA, Berber E, Kioi M, Padilla-Nash H, Damian M*, et al.* SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. *Nature* 2008, **452**(7186)**:** 492-496.
10. Ferrer CM, Alders M, Postma AV, Park S, Klein MA, Cetinbas M*, et al.* An inactivating mutation in the histone deacetylase SIRT6 causes human perinatal lethality. *Genes Dev* 2018, **32**(5-6)**:** 373-388.
11. Kaidi A, Weinert BT, Choudhary C, Jackson SP. Human SIRT6 promotes DNA end resection through CtIP deacetylation. *Science* 2010, **329**(5997)**:** 1348-1353.
12. Etchegaray JP, Chavez L, Huang Y, Ross KN, Choi J, Martinez-Pastor B*, et al.* The histone deacetylase SIRT6 controls embryonic stem cell fate via TET-mediated production of 5-hydroxymethylcytosine. *Nat Cell Biol* 2015, **17**(5)**:** 545-557.
13. Zhang W, Wan H, Feng G, Qu J, Wang J, Jing Y*, et al.* SIRT6 deficiency results in developmental retardation in cynomolgus monkeys. *Nature* 2018, **560**(7720)**:** 661-665.
14. Steffen PA, Ringrose L. What are memories made of? How Polycomb and Trithorax proteins mediate epigenetic memory. *Nat Rev Mol Cell Biol* 2014, **15**(5)**:** 340-356.
15. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* 2003, **33 Suppl:** 245-254.
16. Cedar H, Bergman Y. Linking DNA methylation and histone modification: patterns and paradigms. *Nat Rev Genet* 2009, **10**(5)**:** 295-304.
17. Hayashi K, Surani MA. Resetting the epigenome beyond pluripotency in the germline. *Cell Stem Cell* 2009, **4**(6)**:** 493-498.
18. Lee JT, Bartolomei MS. X-inactivation, imprinting, and long noncoding RNAs in health and disease. *Cell* 2013, **152**(6)**:** 1308-1323.
19. Reik W, Walter J. Genomic imprinting: parental influence on the genome. *Nat Rev Genet* 2001, **2**(1)**:** 21-32.
20. Jia B, Qiu X, Chen J, Sun X, Zheng X, Zhao J*, et al.* A feed-forward regulatory network lncPCAT1/miR-106a-5p/E2F5 regulates the osteogenic differentiation of periodontal ligament stem cells. *J Cell Physiol* 2019, **234**(11)**:** 19523-19538.
21. Dong Y, Jesse AM, Kohn A, Gunnell LM, Honjo T, Zuscik MJ*, et al.* RBPjkappa-dependent Notch signaling regulates mesenchymal progenitor cell proliferation and differentiation during skeletal development. *Development* 2010, **137**(9)**:** 1461-1471.
22. Challen GA, Sun D, Jeong M, Luo M, Jelinek J, Berg JS*, et al.* Dnmt3a is essential for hematopoietic stem cell differentiation. *Nat Genet* 2011, **44**(1)**:** 23-31.
23. Tsai CC, Su PF, Huang YF, Yew TL, Hung SC. Oct4 and Nanog directly regulate Dnmt1 to maintain self-renewal and undifferentiated state in mesenchymal stem cells. *Mol Cell* 2012, **47**(2)**:** 169-182.

24. Denis H, Ndlovu MN, Fuks F. Regulation of mammalian DNA methyltransferases: a route to new mechanisms. *EMBO Rep* 2011, **12**(7)**:** 647-656.
25. Michishita E, Park JY, Burneskis JM, Barrett JC, Horikawa I. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Mol Biol Cell* 2005, **16**(10)**:** 4623-4635.
26. Yan J, Tie G, Wang S, Tutto A, DeMarco N, Khair L*, et al.* Diabetes impairs wound healing by Dnmt1-dependent dysregulation of hematopoietic stem cells differentiation towards macrophages. *Nat Commun* 2018, **9**(1)**:** 33.
27. Liu S, Liu D, Chen C, Hamamura K, Moshaverinia A, Yang R*, et al.* MSC Transplantation Improves Osteopenia via Epigenetic Regulation of Notch Signaling in Lupus. *Cell Metab* 2015, **22**(4)**:** 606-618.
28. Qu X, Chen Z, Fan D, Sun C, Zeng Y, Guo Z*, et al.* MiR-199b-5p inhibits osteogenic differentiation in ligamentum flavum cells by targeting JAG1 and modulating the Notch signalling pathway. *J Cell Mol Med* 2017, **21**(6)**:** 1159-1170.
29. Zhou M, Liu NX, Shi SR, Li Y, Zhang Q, Ma QQ*, et al.* Effect of tetrahedral DNA nanostructures on proliferation and osteo/odontogenic differentiation of dental pulp stem cells via activation of the notch signaling pathway. *Nanomedicine* 2018, **14**(4)**:** 1227-1236.
30. Peng L, Yuan Z, Ling H, Fukasawa K, Robertson K, Olashaw N*, et al.* SIRT1 deacetylates the DNA methyltransferase 1 (DNMT1) protein and alters its activities. *Mol Cell Biol* 2011, **31**(23)**:** 4720-4734.
31. Heo J, Lim J, Lee S, Jeong J, Kang H, Kim Y*, et al.* Sirt1 Regulates DNA Methylation and Differentiation Potential of Embryonic Stem Cells by Antagonizing Dnmt3l. *Cell Rep* 2017, **18**(8)**:** 1930-1945.

Figures

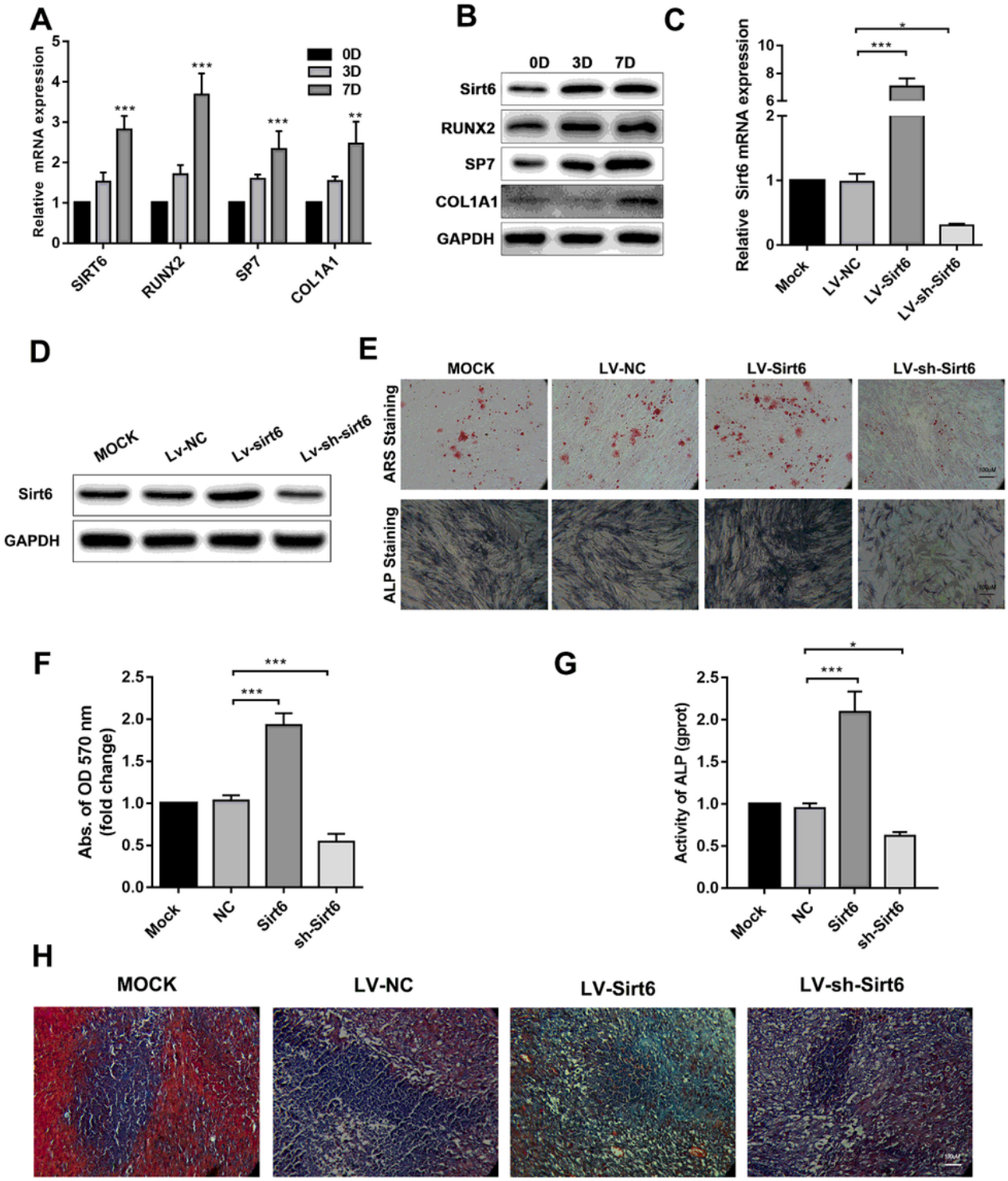

Figure 1

SITR6 promoted osteogenic differentiation of hADSCs. (A and B) Relative expression of SIRT6, RUNX2, SP7 and COL1A1 during osteogenic differentiation of hADSCs at day 0, 3, 7, determined by RT-qPCR and Western blot. (C) RT-qPCR analysis of SIRT6 in overexpression or knockdown hADSCs. (D) Western blot analysis of SIRT6 in overexpression or knockdown hADSCs. (E) Images of Alizarin red staining and ALP staining in SIRT6 overexpressed or silenced hADSCs. Cells were cultured in osteogenic differentiation for

14 days. Alizarin red staining was quantified by spectrophotometry (F) Activity of ALP (G) (normalized to the NC groups). (H) SIRT6 promoted the osteogenesis of hADSCs in vivo. hADSCs loaded on HA-TCP were transplanted into the dorsal region of nude mice for 4 weeks. Then, the samples were removed and measured by Masson's trichrome staining. Data were shown as means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

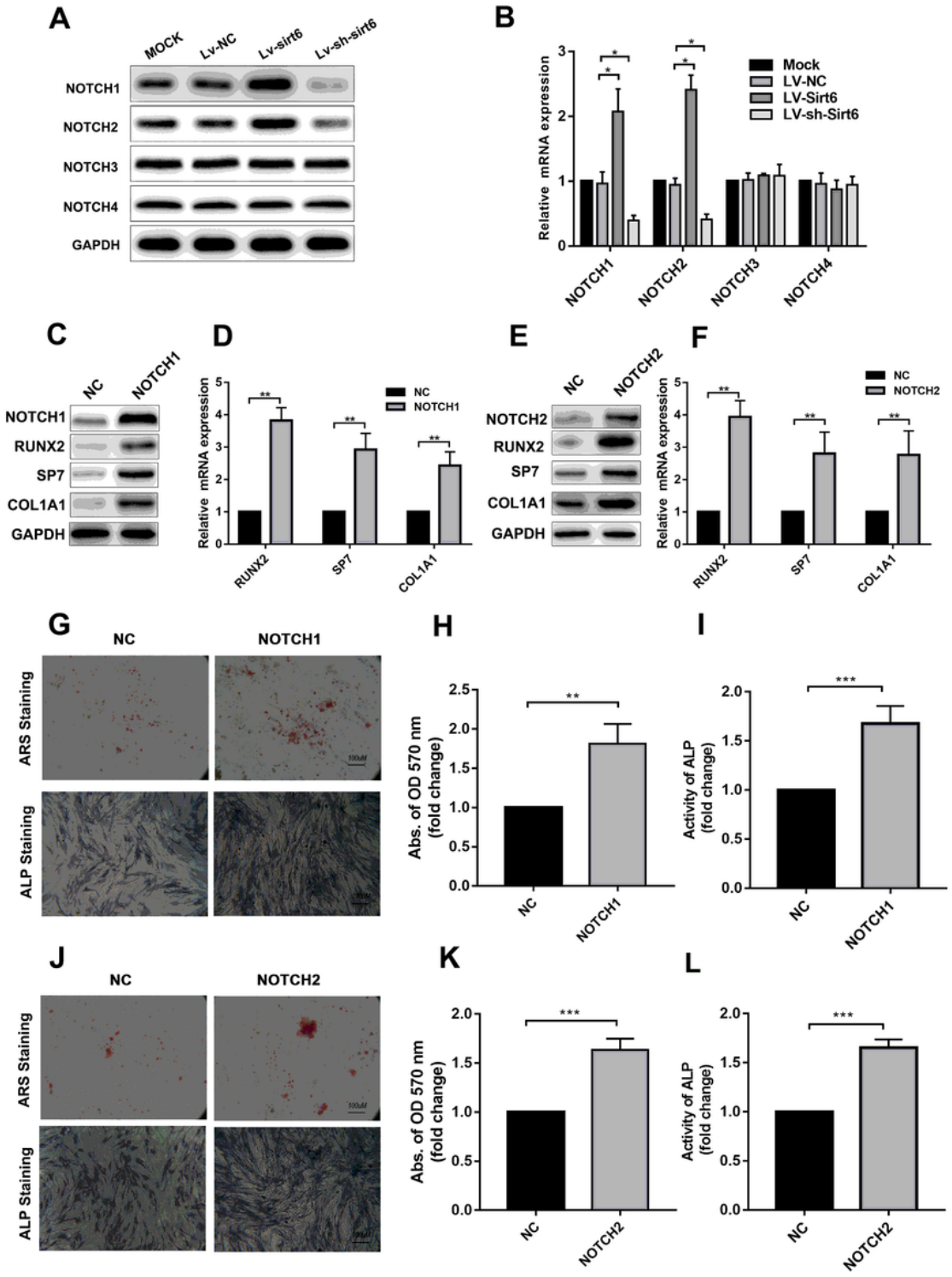

Figure 2

(A and B) Western blot and RT-qPCR showed the expression of NOTCH1, NOTCH2 was increased by SIRT6 overexpression and decreased by SIRT6 knockdown. (C and D) High expression of NOTCH1,RUNX2, SP7 and COL1A1 in NOTCH1 overexpressed hADSCs, was determined by Western blot and RT-qPCR.(E and F) Expression of NOTCH1,RUNX2, SP7 and COL1A1 in NOTCH2 overexpressed hADSCs, determined by Western blot and RT-qPCR.(G-I) Images of Alizarin red staining and ALP staining in NOTCH1 overexpressed hADSCs. Histograms show ALP activity and quantification of Alizarin red staining by spectrophotometry. (J-L) Images of Alizarin red staining and ALP staining in NOTCH2 overexpressed hADSCs. Histograms show ALP activity and quantification of Alizarin red staining by spectrophotometry. Data were shown as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

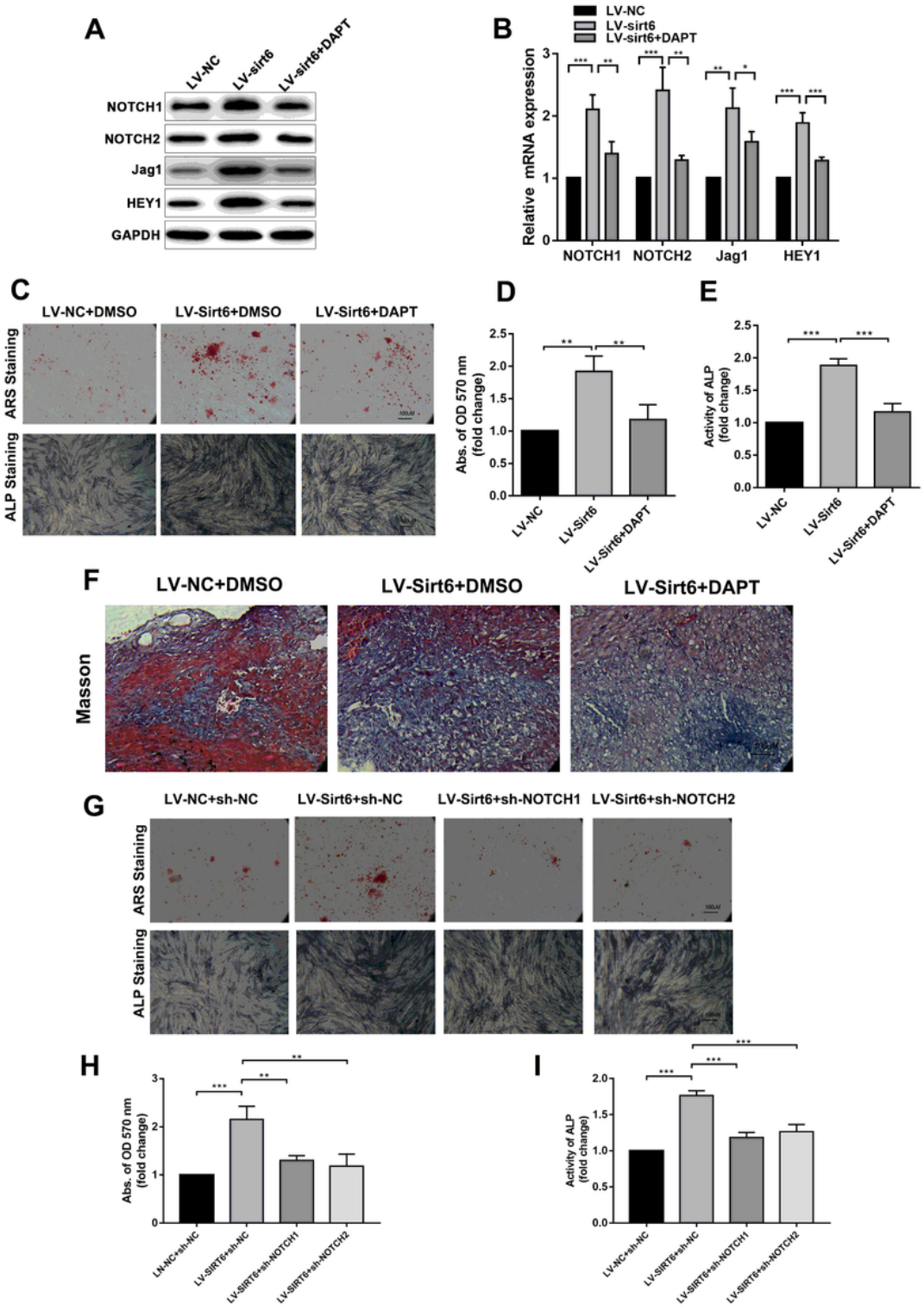

Figure 3

(A and B) SIRT6 overexpressed hADSCs were administered with DAPT, expression of NOTCH1,NOTCH2, JAG1,HEY1 were determined by Western blot and RT-qPCR. (C-E) SIRT6 overexpressed hADSCs were administered with DAPT and osteogenic differentiation medium for 14 days, Alizarin red staining and ALP staining showed mineralized nodule formation. ALP activity and quantification of Alizarin red staining by spectrophotometry were determined. (F) SIRT6 overexpressed hADSCs loaded on HA-TCP

were transplanted into the dorsal region of nude mice, the mice were intraperitoneal injected with DAPT for 4 weeks. Then, the removed samples were measured by Masson's trichrome staining. (G-I) SIRT6 overexpressed hADSCs were transfected with NOTCH1 or NOTCH2 shRNAs and cultured in osteogenic differentiation medium for 14 days, Alizarin red staining and ALP staining showed mineralized nodule formation. ALP activity and quantification of Alizarin red staining by spectrophotometry were determined.Data are shown as means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

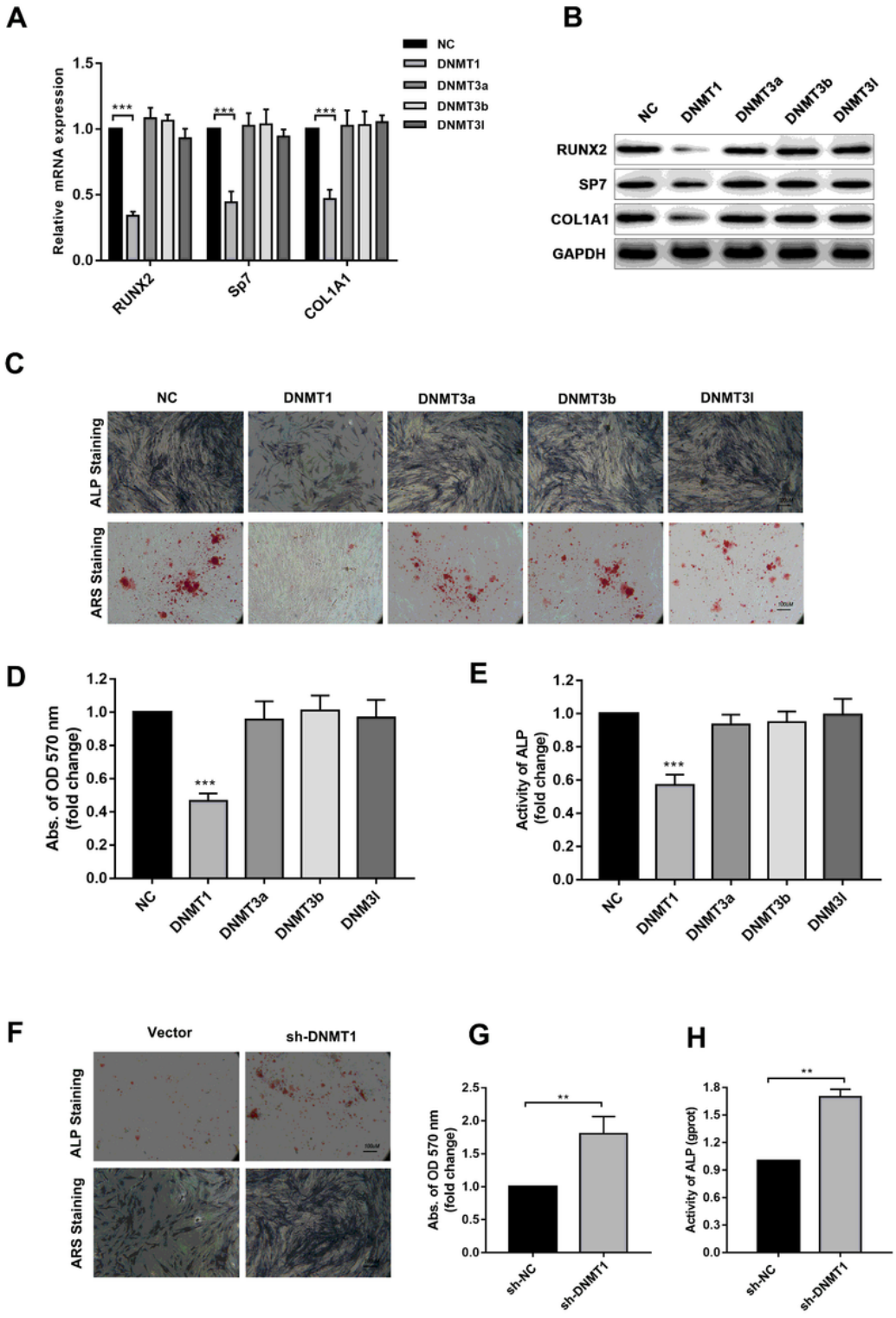

Figure 4

(A and B) Expression of RUNX2, SP7 and COL1A1 4 in DNMT1, DNMT3a, DNMT3b, DNMT3l overexpressed hADSCs were determined by Western blot and RT-qPCR. (C-E) hADSCs were transfected with DNMTs plasmid and cultured in osteogenic differentiation medium for 14 days, Alizarin red staining and ALP staining showed mineralized nodule formation, ALP activity and quantification of Alizarin red staining by spectrophotometry were determined. (F-I) DNMT1 silenced hADSCs were cultured in osteogenic differentiation medium for 14 days, Alizarin red staining and ALP staining showed mineralized nodule formation, ALP activity and quantification of Alizarin red staining by spectrophotometry were determined.Data are shown as means ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

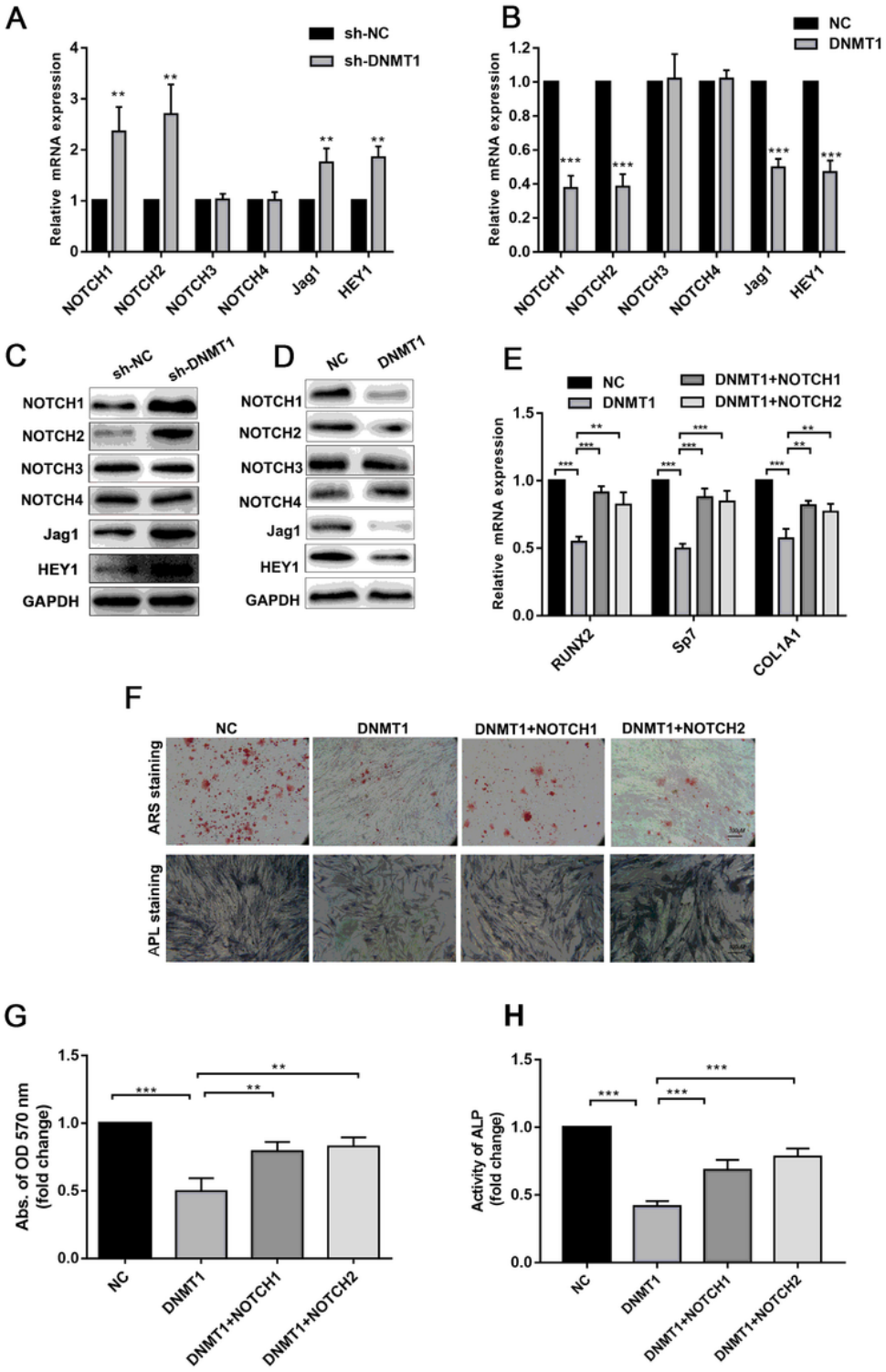

Figure 5

(A-B) hADSCs were transfected with DNMT1 shRNA plasmid, expression of NOTCH1,NOTCH2, NOTCH2, NOTCH3,NOTCH4,JAG1,HEY1,were determined by Western blot and RT-qPCR.C-DhADSCs were transfected with DNMT1 overexpression plasmid, expression of NOTCH1,NOTCH2, NOTCH2, NOTCH3,NOTCH4,JAG1,HEY1,were determined by Western blot and RT-qPCR. (E) DNMT1 overexpressed hADSCs were transfected with NOTCH1 or NOTCH2 overexpression plasmids, expression of RUNX2, SP7

and COL1A1 were determined by Western blot and RT-qPCR. (F-H) DNMT1 stably overexpressed hADSCs were transfected with NOTCH1 and NOTCH2. After differentiation medium for 14 days, Alizarin red staining and ALP staining showed mineralized nodule formation, ALP activity and quantification of Alizarin red staining by spectrophotometry were determined. Data are shown as means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

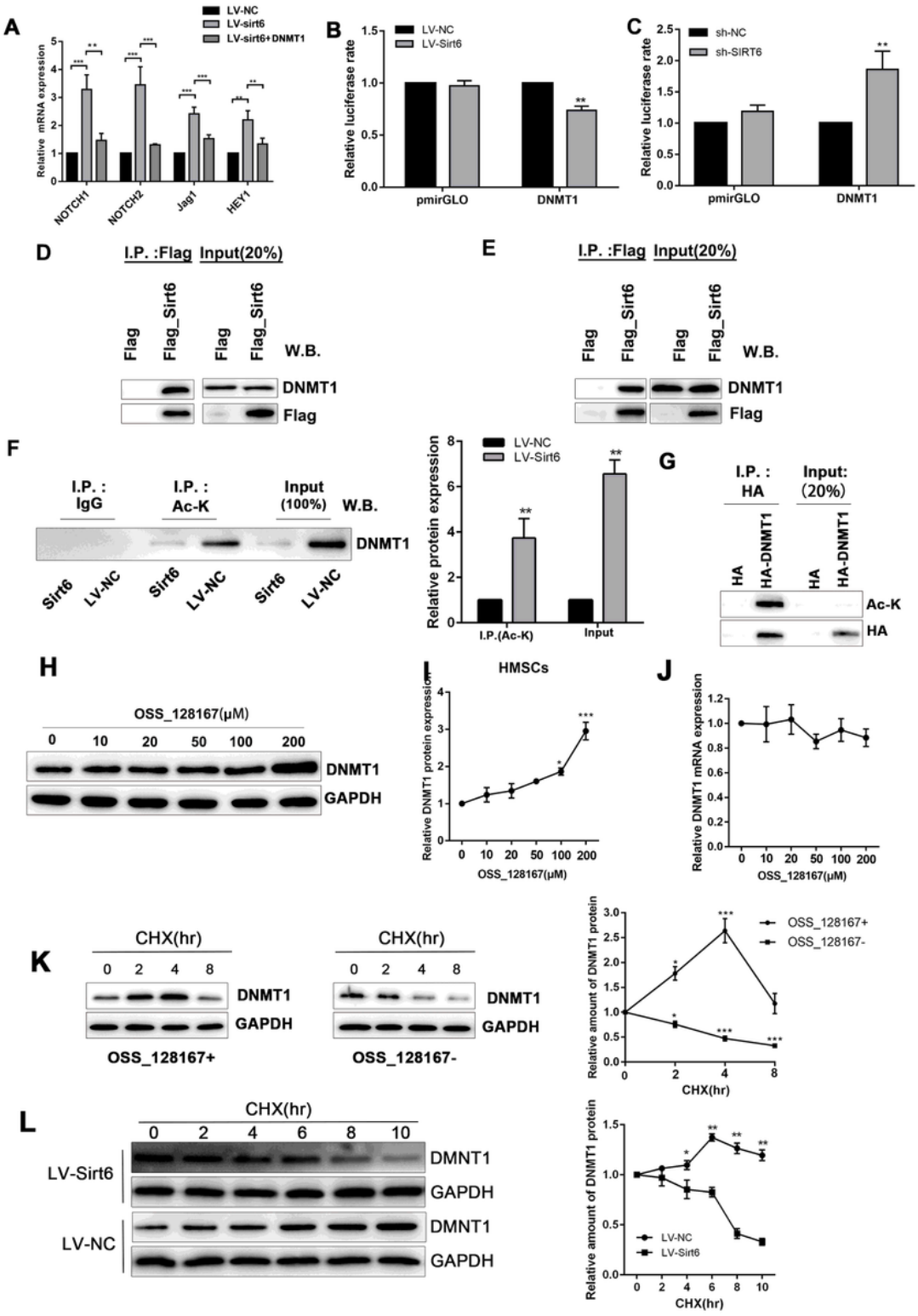

Figure 6

(A) SIRT6 overexpressed hADSCs were transfected with DNMT1 plasmid, the expression of NOTCH1, NOTCH2, NOTCH2, JAG1, HEY1 were determined by RT-qPCR. (B and C) Promoter activity was assessed for DNMT1 in SIRT6 overexpressed of silenced hADSCs cells (n = 3).D and EImmunoprecipitation (IP) analysis was performed in Flag-SIRT1-overexpressing hADSCs and 293T cells. A stable empty vector transfected cell line was established as control group. (F) IP analysis was performed with an acetylated lysine (Ac-K) antibody using flag-SIRT6-overexpressing hADSCs nuclear extracts. DNMT1 proteins levels in the Ac-K IP is shown as mean ± SEM. (G) HA-tagged Dnmt1 proteins were immunoprecipitated (IP) using an HA antibody. Acetylated proteins in the IP products were detected by western blot (H and I) The change of DNMT1 protein expression are shown during treatment of OSS_128167. (J)The change of DNMT1 mRNA expression is shown during treatment of OSS_128167. (K) DNMT1 proteins stability in hADSCs in the present or absent of OSS_128167 was measured using a cycloheximide (CHX)-chase assay (100 mg/mL CHX) for the indicated hours. (L) Stability of the Dnmt1 proteins in SIRT6 overexpressed or control hADSCs was measured using a cycloheximide (CHX)- chase assay involving 100 mg/mL CHX for the indicated hours. Data are shown as means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

Supplementary Files

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