miRNA-124-3p.1 Inhibits the Osteogenic and Odontogenic Differentiation of SCAPs via MACF1/smad7 Axis

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Research Article

Keywords: miRNA-124-3p.1, osteogenetic and odontogenetic differentiation, stem cells from apical papillae, MACF1

Posted Date: December 13th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1121374/v1

Abstract

Background: Previous research has indicated that altered expression of micro-RNAs (miRNAs) is in connection with differentiation of stem cells from apical papillae (SCAPs). We investigated the mechanisms that miR-124-3p.1 inhibited osteogenic and odontogenic differentiation of SCAPs.

Methods: SCAPs were isolated from dental apical papilla. MiR-124-3p.1 mimic and inhibitor were used for overexpression and knockdown assays. For overexpression and knockdown of microtubule actin cross-linking factor 1 (MACF1), lentivirus infection and siRNA transfection were performed. Luciferase reporter assay was performed to determine the relationship between miR-124-3p.1 and MACF1. The osteogenic and odontogenic differentiation potential was analyzed by alkaline phosphatase activity analysis (ALP), alizarin red S (ARS) staining, quantitative real time reverse-transcription polymerase chain reaction (qRT-PCR), western blot and immunofluorescence (IF) staining.

Results: We observed a time dependent decrease of miR-124-3p.1 level in mineralization induction of SCAPs. Further study found that miR-124-3p.1 exhibited an inhibitory effect on SCAPs osteo/odontogenic differentiation. Similarly, we found that the overexpression of miR-124-3p.1 dramatically inhibited MACF1 protein level in SCAPs and knockdown of miR-124-3p.1 significantly increased MACF1 protein level in SCAPs. Moreover, MACF1 was verified as the targeting of miR-124-3p.1. Meanwhile, the expression of MACF1 was related to smad7 nuclear translocation.

Conclusion: Collectively, diverse data demonstrated that miR-124-3p.1 is a regulator of MACF1/smad7, playing plays an important role in osteogenic and odontogenic differentiation of SCAPs via MACF1/smad7 axis.

Introduction

Pulpitis and periapical periodontitis are common oral diseases. Root canal treatment (RCT) is commonly used to treat these problems and relieve the pain of patients. But the traditional method is limited by the incomplete root formation with the open apex and short roots[1]. Treating immature permanent teeth with pulpitis and periapical periodontitis is a challenge in dental medicine[2]. The immature pulpless tooth with an open apex become easy to fracture, resulting in a higher incidence of tooth extraction [3]. Adult stem cells play a significant role in maintaining homeostasis of tissue and in the process of tissue repair and regeneration[4, 5]. In fact, adult mesenchymal stem cells (MSCs) are pluripotent adult stem cells that are isolated from a variety of tissues or organs[6]. Additionally, a recent report indicates that MSCs have chondrogenic, osteogenic, or adipogenic differentiation potential. Stem cells from the tooth mesoderm derived from neural crest cells may be sources for tissue regeneration, including stem cells from apical papilla (SCAPs)[7, 8]. SCAPs residing in the apical papilla play a critical role in tooth development and pulp regeneration in permanent teeth[1]. SCAPs were classified as pluripotent MSCs after a positive expression of CD146, CD90, CD73, STRO-1 and CD105 markers[8]. SCAPs t exhibit the properties of high proliferative potential, low immunogenicity, self-renewal capacity and multipotency of differentiation

such as osteogenetic, odontogenetic, dentinogenetic, adipogenetic and chondrogenic[9, 10]. In addition, considerable evidence indicates that after culture in mineral induction medium (MM) containing β-glycerophosphate, dexamethasone, and L-ascorbate-2-phosphate, SCAPs are found to express a variety of osteo/odontogenic markers, such as ALP, osterix (OSX), runt-related transcription factor 2 (RUNX2) and dentin sialophosphoprotein (DSPP) [11]. Previous studies have found DSPP, RUNX2, ALP and OSX to be important transcriptional regulators of osteogenic and odontogenic differentiation[12]. SCAPs were used to reconstruct the spinal cord injury in animal model[13]. Therefore, SCAPs have been increasingly employed to investigate regenerative medicine.

MiRNAs are a class of highly conserved small noncoding RNAs molecules that operates as genome main regulators and fine tuners via post-transcriptional gene silencing[14]. MiRNAs containing 19-22 nucleotides RNA molecules, always act as a negative regulator in the process of target genes expression in a sequence-specific manner by directly binding partially complementary sequences of target mRNAs in 3'UTRs leading to suppression of the expression of their target mRNAs and its corresponding protein in many biological processes [15, 16]. MiRNAs extensively distributed in the body[17]. Evidences showed that miRNAs are ubiquitous and potent regulators of almost all biological processes, including proliferation, cell differentiation, metabolism, tumorigenesis, apoptosis, and tissue development[18–20]. Emerging evidence also suggests that miRNAs are active regulators in the self-renewal ability and differentiation potential of stem cells by post-transcriptionally targeting factors implicated in stem cell maintenance[12, 21]. It is reported that increasing number of miRNAs exert a significant impact on apoptosis, proliferation, differentiation and bone resorption of osteoblasts[19, 22]. There are studies have demonstrated that miRNAs almost function in the whole process of osteogenic differentiation[23, 24]. Most of these miRNAs have been reported to promote or inhibit the formation and maturation of osteoblasts, and a few miRNAs are involved in the regulation of osteoblasts functions[25]. Recently, some studies have demonstrated the importance of miRNAs in regulating osteogenic differentiation[26]. For instance, miR-138 reduces ectopic bone formation *in vivo* through negatively regulating osteogenic differentiation of human MSCs[27]. MiR-124 also inhibits osteogenic differentiation of MSCs and *in vivo* bone formation[28]. Our team have demonstrated that miRNA *let7b* played the important role in the differentiation of SCAPs[29]. Previous studies reported that miR-124-3p.1 is involved in cancer development and has also been identified as a potential tumor suppressor in certain cancers[30–32]. Moreover, miR-124-3p.1 sensitizes carboplatin induced mitochondrial apoptosis by inhibiting CAV1 in ovarian cancer[33]. Recently, there are studies demonstrated that miR-124-3p.1 overexpression negatively regulated proliferation and osteogenic differentiation of human bone marrow mesenchymal stem cells (BMSCs)[34]. However, the role of miR-124-3p.1 in the differentiation of SCAPs are still unclear. The purpose of this study was to explore the effect and mechanism of miR-124-3p.1 on proliferation and osteo/odontogenic differentiation of SCAPs.

Materials And Methods

2.1 Cell culture

SCAPs were isolated from dental apical papilla tissue. Dental apical papillae were isolated and transferred into phosphate-buffered saline (PBS; Gibco) containing penicillin-streptomycin (Pen-Strep; Gibco) and trimmed to small pieces under sterile conditions. The samples containing stem cells were transferred into a flask containing α-modified Eagle's minimum essential medium (α-MEM; Gibco), 10% fetal bovine serum (FBS; BI) and 2% Pen-Strep.

2.2 Characterization of SCAPs

2.2.1 Immunofluorescence staining (IF)

SCAPs were cultured for 3 days. Briefly, cells were incubated with primary antibody STRO-1 (eBioscience) and Cy3-conjugated secondary antibodies. After counterstained with 4,6-diamidino-2-phenylindole (DAPI), cells were observed under a fluorescence microscope (Leica, Germany).

2.2.2 Flow Cytometry Analysis

For surface markers characterization, cells were stained with fluorescent monoclonal mouse anti-human antibodies containing CD34-PE, and CD45-PE, CD73-PE, CD29-APC, CD90-FITC, CD105-PerCP-Cy5.5^{TM} (BD Biosciences, USA). Fluorescence analysis was evaluated in flow cytometer (Thermo Fisher Scientific).

2.2.3 Colony Forming Assay

After 10 days of culture, cells were stained with 0.1% toluidine blue (Sigma Aldrich, MO, USA).

2.2.4 Chondrogenic Differentiation

SCAPs were cultured in chondrogenic medium for 1 month. After that, cells were immersed in paraformaldehyde for 48 hours, then imbedded in paraffin, and sliced into 5 μm sections. Then, cells were stained with Alcian blue staining for 30 min.

2.2.5 Osteogenic differentiation

Cells were seeded in 12-well plates. Afterward, mineral induction medium consisting ofβ-glycerophosphate10 mM, ascorbate-2-phosphate50 mM, and dexamethasone 100 nM (Sigma), 10% FBS and 1% Pen-Strep was added to the wells. The culture plates were incubated in humidified condition for 21 days.

2.2.6 Adipogenic Differentiation

SCAPs were cultured in adipogenic medium (Cyagen, China) for 28 days. After stained with Oil Red O reagent (Cyagen) for 5 min, lipid droplets were observed under microscope.

2.3 Transfection

The miR-24-3p oligos (inhibitor, mimic, and inhibitor NC and mimic NC) and control siRNA (NC), siRNA for MACF1(siMACF1) were designed by Ribio (Ribio CO., China). Cells were transfected with oligos and siRNAs via riboFECTTM CP following the manufacturer's protocols. Western blot and qRT-PCR were used to verify the interference efficiency. Lentiviral vectors overexpressing MACF1 were constructed and produced by Shanghai Genechem Company (Shanghai, China). SCAPs were inoculated overnight and infected with lentiviruses with polybrene.

2.4 Cell Counting Kit-8 Assay (CCK-8)

SCAPs were respectively transfected with miR-124-3p.1 inhibitor, mimic, inhibitor NC and mimic NC for 48 h. Next, cells were seeded on 96-well plates and cultured for 0, 1, 3, 5 and 7 d. Afterward, 10 μL CCK-8 (Dojindo, Japan) and 90 μL α-MEM were added to each well for 2 h. The optical densities (OD) were measured at 450 nm.

2.5 5-Ethynyl-2′-Deoxyuridine (EdU) assay

The EdU DNA Proliferation Detection kit (Ribo Biotechnology, China) was used to detect cell proliferation ratio. After culture, cells were fixed and administrated with EdU labeling solution. Cells were photographed under inverted fluorescence microscope (Leica, Germany).

2.6 Alizarin red staining

After washed and fixed, SCAPs were stained with ARS solution (Sigma, USA). The red sediments of calcium deposition were observed under microscope. For quantitative analysis, mineralized nodules were dissolved by 10% cetylpyridinium chloride (CPC; Sigma, USA). Then OD value was measured at 562 nm.

2.7 Alkaline phosphatase activity

Cells were harvested and permeated in Triton X-100. After centrifugation, the supernatant transferred into fresh 1.5 ml tubes with relative working solution and then were incubated. The OD value was measured at 520 nm.

2.8 ALP staining

After washed and fixed, cells were stained using the BCIP/NBT ALP staining kit (Beyotime, China). Cells were observed with microscope.

2.9 RNA extraction and quantitative real- time polymerase chain reaction (qRT-PCR)

RNA isolation was performed using the TRIzol™ (Invitrogen) method. The cDNAs were synthesized and used for PCR. qRT-PCR reactions were performed by SYBR Green method. GAPDH and U6 were used as internal controls. Bulge-Loop miRNA qPCR Primer kit (RiboBio) was used for measuring miRNA-124.3p.1 expression. Primers in this study are listed in Table 1.

2.10 Western blotting

RIPA buffer was used to extract proteins with protease inhibitor cocktail. After PAGE, the proteins were blotted onto PVDF membranes. Membranes were blocked, incubated with specific primary antibody and next day with the secondary HRP-conjugated antibody

2.11 Immunofluorescence staining

Cells were fixed, permeabilized. Then, cells were blocked and incubated with primary antibodies, fluorescent dye-labeled designated secondary antibody and DAPI above

2.12 Luciferase Assay

The potential binding site of MACF1-wt and mutant sequence MACF1-mut was synthesized into pmiR-GLO (Promega, Madison, WI, USA). SCAPs were co-transfected with the MACF1-wt or MACF11-mut reporter gene plasmid and miR-124-3p.1 mimic. The activities were measured by a Promega luciferase assay (Promega, USA) were normalized against the activity of the Renilla luciferase gene.

2.13 Co-immunoprecipitation (Co-IP) assay

After cells lysed and centrifuged, supernatants were incubated with the anti-MACF1 antibody in rotation. Next day, the supernatants were mixed with protein G plus A agarose (Beyotime, China) at in rotation. Then, the immunocomplexes were washed. Beads complexes were resuspended in 2 × loading buffer.

2.14 Animal procedures

SCAPs with miR-124-3p.1 mimic and miR-124-3p.1 mimic NC induced in MM for 7 days were harvested for the *in vivo* study. About 6.0×10^6 SCAPs were mixed with Bio-Oss Collagen scaffolds (Geistlich, Germany). And then the mixtures were implanted into the dorsal surface of BALB/c homozygous 5-week-old nude mice. Two months later, the implants were harvested and were detected under micro-CT analysis. After that, implants were decalcified and embedded with paraffin. Paraffin sections were stained with hematoxylin and eosin (H&E) staining and Masson's trichrome staining.

2.15 Statistical analysis

All data are expressed as the mean ± SD and were analyzed by one-way analysis of variance (ANOVA). $p<0.05$ was considered statistically significant.

Results

3.1 Characterization of SCAPs

Primary SCAPs were cultured after 3 days (Figure 1A above). SCAPs were displayed long spindle shape at passage 3 (Figure 1A below). IF staining showed SCAPs were stained positively for MSCs surface

molecule STRO-1 (Figure 1B). SCAPs positively expressed the mesenchymal stem cell markers CD73, CD90, CD29 and CD105, but negatively expressed the hematopoietic cell markers CD45, CD34 (Figure 1C). Colony-forming assays were conducted to detect the colony forming efficiency. The result showed single colony-forming unit of SCAPs (Figure 1D). The results of staining showed that SCAPs had the potential of differentiation into chondrocytes osteoblasts and adipocytes (Figure 1E, F, G).

3.2 miR-124-3p.1 was downregulated and MACF1 was upregulated during mineralization induction of SCAPs

The expression of miR-124-3p.1 was markedly downregulated in SCAPs after 7days of mineralization induction, but the MACF1 expression showed an inverse trend (Figure. 2A, B). To detect the effect of miR-124-3p.1 on differentiation, SCAPs were transfected with miR-124-3p.1 mimic, mimic NC, inhibitor, inhibitor NC. Transfection efficacy in SCAPs was verified by qRT-PCR. The miR-124-3p.1 level was dramatically increased in miR-124.3p.1 mimic, whereas decreased in miR-124.3p.1 inhibitor group, compared with relative NC groups (Figure 2C, D). CCK-8 assay and EdU staining were conducted to detect the effect of miR-124-3p.1 on the proliferation of SCAPs. The result of CCK-8 showed that knockdown of miR-124-3p.1 showed no significant influence on the proliferation of SCAPs (Figure 2E). There was also no significant difference between miR-124-3p.1 mimic NC and miR-124-3p.1 mimic groups (Figure 2F). EdU assay showed that there was no significant difference on the DNA synthesis in miR-124-3p.1 inhibitor NC group and miR-124-3p.1 inhibitor group (Figure 2G, I). Moreover, miR-124-3p.1 overexpression has no significant influence on the proliferation of SCAPs (Figure 2H, J).

3.3 Effect Of Mir-124-3p.1 On Differentiation Of Scaps

ALP staining and ALP activity assay showed increased ALP activity after the knockdown of miR-124-3p.1 but demonstrated decreased ALP activity after overexpression of miR-124-3p.1 (Figure 3A, B). Similarly, ARS staining showed that miR-124-3p.1 knockdown promoted mineralized nodules formation in SCAPs, whereas the formation of mineralized nodules was negatively mediated by upregulation of miR-124-3p.1 in SCAPs (Figure 3C, D). In addition, qRT-PCR analysis showed that the expression of DSPP, RUNX2, ALP and OSX mRNA had all showed a significant increase in miR-124-3p.1 knockdown group compared to control group, while the expression of DSPP, RUNX2, ALP and OSX mRNA in miR-124-3p.1 overexpression group was dramatically decreased (Figure 3E). Western blot analysis showed that miR-124-3p.1 knockdown enhanced the odontogenic and osteogenic differentiation related proteins (DSPP, RUNX2, ALP and OSX). Overexpression of miR-124-3p.1 in SCAPs could significantly decrease the expression of odontogenic and osteogenic differentiation markers (DSPP, RUNX2, ALP and OSX) (Figure 3F, G). Immunofluorescence staining indicated that miR-124-3p.1 knockdown significantly up-regulated the levels of DSPP, RUNX2, ALP in SCAPs (Figure 3H, J, L). Conversely, miR-124-3p.1 overexpression remarkably down-regulated the expressions of DSPP, RUNX2, ALP (Figure 3I, K, M). SCAPs stably expressing miR-124-3p.1 mimic and miR-124-3p.1 mimic NC loaded on Bio-Oss Collagen scaffolds were

implanted in immunocompromised mice subcutaneously (Figure 4A). MicroCT imaging showed BV/TV in miR-124-3p.1 mimic group was less than miR-124-3p.1 mimic group (Figure 4B). Masson and H&E staining showed less bone-like structures formed in in the presence of mimics for miR-124-3p.1 in SCAPs than the control group (Figure. 4C, D). These results indicate that miR-124-3p.1 negatively regulated osteogenic and odontogenic differentiation of SCAPs.

3.4 Macf1 Was The Target Of Mir-124-3p.1

Subsequently, the potential binding sequences between miR-124-3p.1 and MACF1 were predicted by miRanda (Figure 5A). Dual-Luciferase reporter gene assay showed that co-transfection of miR-124-3p.1 mimic and MACF1 3'-UTR WT could remarkably decrease the Luciferase activity (Figure 5B). Hence, the binding relationship of MACF1 to miR-124-3p.1 was verified. The overexpression of miR-124a-3p.1 in SCAPs significantly upregulated the mRNA and protein levels of MACF1. However, miR-124a-3p.1 knockdown could up-regulate its expression level (Figure 5C, D, E). Immunofluorescence staining showed the same trend above (Figure 5F). These results provided strong evidence that MACF1 was the target of miR-124-3p.1.

3.5 Silencing of MACF1 inhibited the odontogenic and osteogenic differentiation of SCAPs, overexpression of MACF1 promoted the odontogenic and osteogenic differentiation of SCAPs

To explore the potential influence of MACF1 on the osteogenic and odontogenic differentiation of SCAPs, MACF1 knockdown and MACF1 overexpression were established. qRT-PCR showed that a significant decrease of MACF1 expression in siMACF1 group and an obvious increase in the MACF1 overexpression group compared to relative control groups (Figure 6A). Therefore, western blot analysis confirmed the protein expression level of MACF1 was significantly downregulated in siMACF1 group and was dramatically upregulated in MACF-1-over group (Figure 6B, C). ALP staining and ALP activity assay showed that ALP activity in siMACF1 group was clearly decreased and ALP activity were obviously enhanced in the MACF1 overexpression group (Figure 6D, E). ARS staining and relevant CPC quantitative analysis showed matrix mineralization was dramatically downregulated in siMACF1 group and a contrary trend was detected in MACF-1 overexpression group (Figure 6F, G). Besides, qRT-PCR and western blot showed that deletion of MACF1 downregulated the expression of DSPP, RUNX2, ALP and OSX. MACF1 overexpression increased gene and protein expression associated with osteo/odontogenetic differentiation (DSPP, RUNX2, ALP and OSX) (Figure 6H, I, J). These results indicated that MACF1 downregulation is negatively associated with the osteogenic and odontogenic differentiation in SCAPs. But MACF1 overexpression promoted the osteogenic and odontogenic differentiation of SCAPs *in vitro.*

3.6 Downregulation of miR-124-3p.1 enhanced odontogenic and osteogenic differentiation via inhibiting MACF1 expression

To investigate the mechanism of miR-124-3p.1 in regulating odontogenic and osteogenic differentiation, SCAPs were transfected with miR-124-3p.1 inhibitor NC, miR-124-3p.1 inhibitor or miR-124-3p.1 inhibitor+ NC, miR-124-3p.1 inhibitor+ siMACF1, respectively. The result of qRT-PCR demonstrated that miR-124-3p.1 knockdown could upregulate the expression of MACF1, while siMACF1 rescued the above changes induced by miR-124-3p.1 inhibitor, causing the decreased level of MACF1 (Figure 7A). In consonance with the results of qRT-PCR, western blot and immunofluorescence staining showed that upregulation of MACF1 induced by miR-124-3p.1 inhibitor could be negatively regulated by knockdown of MACF1 (Figure 7B, C, D). Analogously, the relative expression levels of DSPP, RUNX2, ALP and OSX were significantly up-regulated after miR-124-3p.1 inhibitor. However, the levels of these above genes were markedly inhibited by MACF1 knockdown (Figure 7E). In addition, western blot showed that downregulation of miR-124-3p.1 remarkably promoted the expression of DSPP, RUNX2, ALP and OSX. Meanwhile, the expression of DSPP, RUNX2, ALP and OSX was reversed by co-transfection of miR-124-3p.1 inhibitor and siMACF1 (Figure 7F, G). Immunofluorescence staining further verified the above results (Figure 7H, I, J). Our data suggested that MACF1 repression was able to partially inhibited the positive effect of miR-124-3p.1 downregulation on osteogenic and odontogenic differentiation.

3.8 Overexpression of miR-124-3p.1 inhibited odontogenic and osteogenic differentiation via mediating MACF1 expression

To further explore the mechanism of miR-124-3p.1 in regulating odontogenic and osteogenic differentiation, miR-124-3p.1 mimic and MACF1-over lentivirus were transfected, respectively. The result of qRT-PCR showed that MACF1 was markedly decreased by overexpression of miR-124-3p.1, which could be reversed by MACF1 overexpression (Figure 8A). Western blot and immunofluorescence staining showed that downregulation of MACF1 induced by miR-124-3p.1 mimic could be significantly reversed by overexpressing MACF1 (Figure 8B, C, D). Moreover, MACF1 overexpression also reversed the variation of the relative expression levels of DSPP, RUNX2, ALP and OSX inhibited by the miR-124-3p.1 mimic (Figure 8E). Western blot showed that upregulation of miR-124-3p.1 also decreased the expression of DSPP, RUNX2, ALP and OSX. Meanwhile, the expression of DSPP, RUNX2, ALP and OSX was reversed by co-transfection of miR-124-3p.1 mimic and MACF1-over lentivirus (Figure 8F, G). Immunofluorescence staining further verified the above results (Figure 8H, I, J). Our data suggested that MACF1 overexpression was able to partially enhance the inhibitory effect of miR-124-3p.1 upregulation on osteogenic and odontogenic differentiation.

3.9 Macf1 Interacts With Smad7 In Scaps

To further explore the mechanism that MACF1 regulates SCAPs' differentiation, western blot and CO-IP were conducted. The result of western blot showed that MACF1 knockdown not only significantly decreased expression of smad7 in the cytoplasm, but also inhibited the expression of smad7 in the nucleus (Figure 9A, C). Next, we found that smad7 were increased in SCAPs as compared with NC-over cells, the enhancement was especially obvious in the nucleus (Figure 9B, D). We found that smad7 were co-immunoprecipitated with MACF1 using the antibody recognizing MACF1 (anti-MACF1). Co-IP result showed that smad7 was detectable in the anti-MACF1 immunoprecipitated products, indicating that MACF1 can interact with smad7 in SCAPs. Moreover, MACF1 is related to smad7 nuclear translocation (Figure 9E). The mechanism diagram in this study was showed in Figure 9F.

Discussion

Multiple studies have demonstrated that dental apical papillae directly contribute toward the formation of the tooth roots[35]. Furthermore, SCAPs are an effective cell resource for tissue regeneration[36]. In the present study, we found that miR-124-3p.1 inhibited the osteo-/odontogenic differentiation potential of SCAPs. Importantly, we found that miR-124-3p.1 played an inhibitory role in the differentiation of SCAPs by binding to the 3'-UTR of MACF1, further regulation of smad7 entry into the nucleus.

Recent evidence indicates that miRNAs play important role in osteoblast differentiation and bone formation[37]. Previous studies have demonstrated that miRNAs act as important regulators for the stemness of oral mesenchymal stem cells[37, 38]. Recently, the critical role of miR-124-3p.1 in osteogenic differentiation of human BMSCs has been previously demonstrated[34]. We, therefore, assume that miR-124-3p.1 can inhibit osteogenic and odontogenic differentiation of SCAPs through the MACF1/smad7 axis. Our study showed that overexpression of miR-124-3p.1 decreased the odonto/osteogenic differentiation of SCAPs while miR-124-3p.1 inhibition elevated osteogenic and odontogenic differentiation. The function and molecular mechanisms of miR-124-3p.1 in osteogenic and odontogenic differentiation was remains unclear. According to bioinformatics prediction (TargetScan V7.2), we discovered that MACF1 was a predicted target gene of miR-124-3p.1. As a member of the spectraplakin family of cytoskeletal crosslinking proteins, MACF1 is widely expressed in different tissues[39]. Accumulating evidence indicates that MACF1 is a critical factor in modulating actin and microtubule cytoskeletal networks and regulating cytoskeletal distribution, cell migration, cell survival and cell differentiation[40]. It was reported that MACF1 correlates with various physiological and pathological processes[41]. MACF1 has a significant effect on osteogenesis the differentiation of primary osteoblasts[42]. MACF1 is an important regulator in various signal transduction and cellular processes[43]. There are reports showed that downregulation of MACF1 suppressed the differentiation of osteoblastic cell line[44, 45]. Taken together, the evidence illustrated that MACF1 can regulate the differentiation of osteoblast via Wnt signaling[46]. Moreover, smad7 is identified as a new downstream

target of MACF1 and MACF1 promotes bone formation by facilitating smad7 nuclear translocation[47]. Previously, smad7 acts as an inhibitor to inhibit bone formation by a negative feedback way[48]. However, recent studies have emphasized the positive role of smad7 in stem cells such as osteoblasts and myoblasts[49, 50]. It is also reported that nucleus smad7 can enhance osteogenic differentiation potential[47, 49].

Conclusion

In summary, we found that miR-124-3p.1 negatively regulated the osteogenic and odontogenic differentiation potential by negatively regulating MACF1/smad7 axis in SCAPs. Additional studies are required to further confirm whether miR-124-3p.1 and MACF1 may enhance odontogenesis of SCAPs *in vivo*. Further studies are also needed to confirm the relationship between smad7 and differentiation of SCAPs, which will provide a theoretical basis for treating dental diseases.

Declarations

Acknowledgements

Not applicable. We would like to give our sincere appreciation to the reviewers for their helpful comments on this article.

Conflict of interest

Authors declare no conflicts of interest.

Authors Contributions

LN conceived and designed the experiments, collected and assembled data, and wrote the manuscript. LZH, YM and GYC performed data analysis and interpretation. WYQ, WJT and YCT collected and analyzed data. XT and FL reviewed the manuscript. YJH conceived and designed the study, provided financial support and study material, performed the data analysis and interpretation, and approved the final version of the manuscript. All authors read and approved the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (grant numbers: 81900962 and 82170940).

Availability of data and materials

Datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Studies were carried out in accordance with the Declaration of Helsinki and got the approval of the Ethical Committee of Nanjing Medical University.

Consent for publication

Not applicable.

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Tables

Table 1. Primer sequences for qRT-PCR analysis of gene expression.

Target gene	Sequences (5¢-3¢)	Product size (bp)
RUNX2	Forward, TCTTAGAACAAATTCTGCCCTTT	136
	Reverse, TGCTTTGGTCTTGAAATCACA	
OSX	Forward, CCTCCTCAGCTCACCTTCTC	148
	Reverse, GTTGGGAGCCCAAATAGAAA	
ALP	Forward, GACCTCCTCGGAAGACACTC	137
	Reverse, TGAAGGGCTTCTTGTCTGTG	
DSPP	Forward, ATATTGAGGGCTGGAATGGGGA	136
	Reverse, TTTGTGGCTCCAGCATTGTCA	
GAPDH	Forward, GAAGGTGAAGGTCGGAGTC	
	Reverse, GAGATGGTGATGGGATTTC	225

Figures

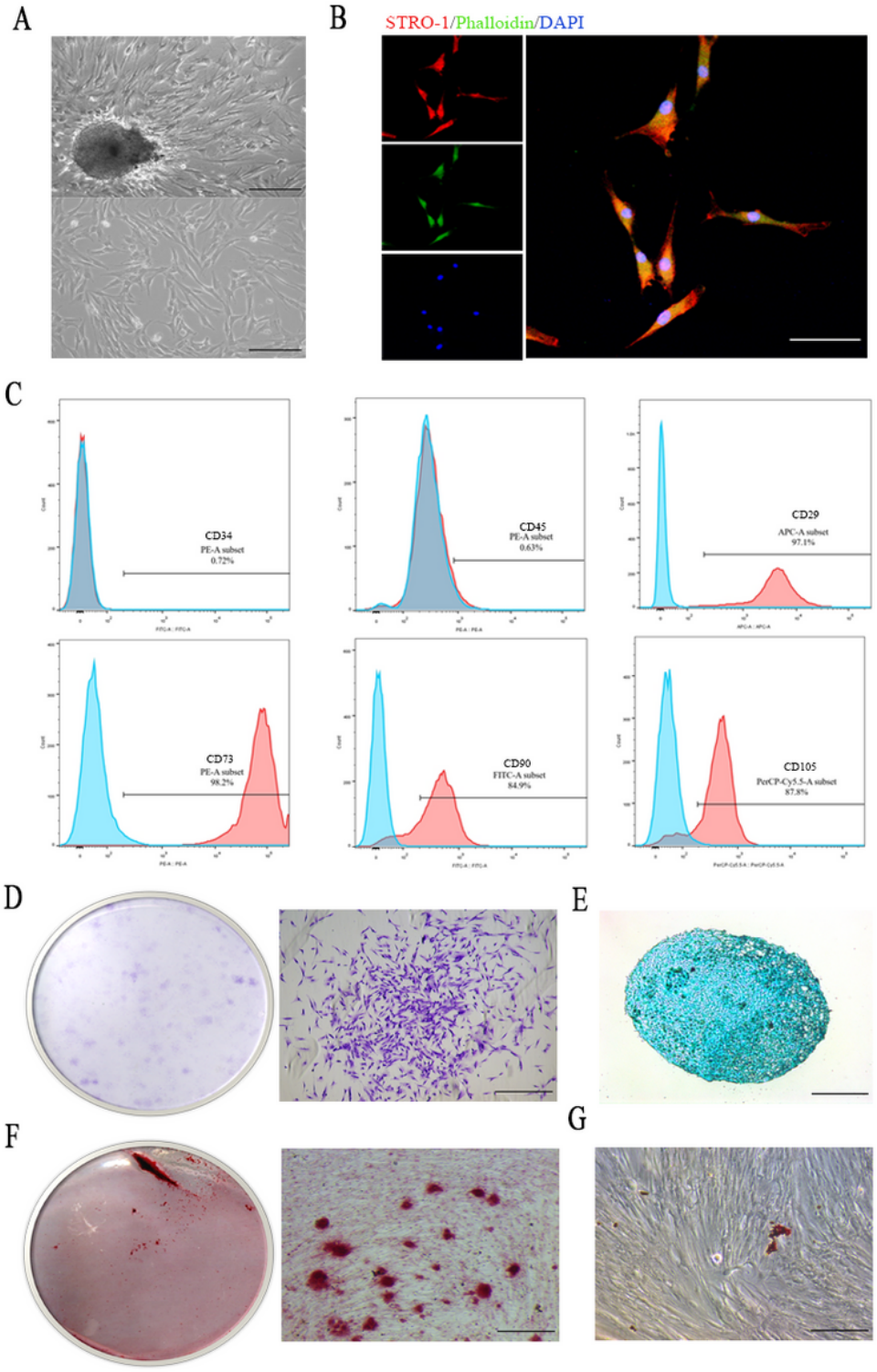

Figure 1

Characterization of SCAPs A. Primary cultured SCAPs (Above, scale bar= 200 μm); SCAPs at passage 3 (Below, scale bar= 200 μm). B. Immunofluorescence staining showed that SCAPs expressed STRO-1 (scale bar= 200 μm). C. SCAPs expressed high levels of the mesenchymal stem cell marker CD73, CD90, CD29 and CD105, but expressed low levels of the hematopoietic cell marker CD34, CD45. D. Colony forming units of hPDLSCs after cultivation (Scale bar= 200 μm). E. SCAPs had the potential of

differentiation into chondrocytes (Scale bar= 200 μm). F. SCAPs had the potential of differentiation into osteoblasts (Scale bar= 200 μm). G. SCAPs had the potential of differentiation into adipocytes (Scale bar= 200 μm).

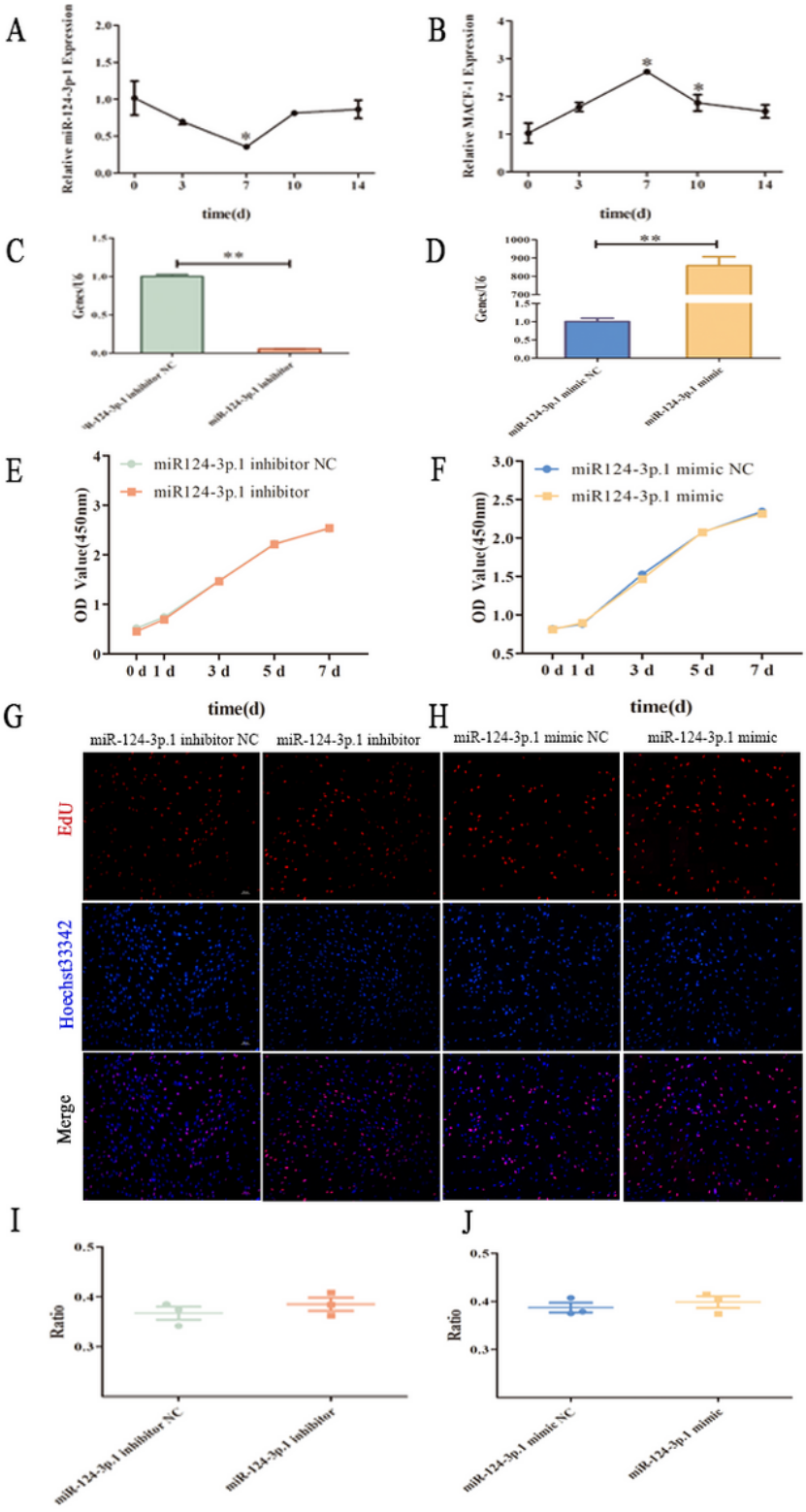

Figure 2

Effect of miR-124-3p.1 on the proliferation in SCAPs A. miR-124-3p.1 expression profile throughout differentiation induction (* p<0.05, ** p<0.01). B. MACF1 expression profile throughout differentiation

induction (* p<0.05, ** p<0.01). C. The transfection effects of miR-124-3p.1 inhibitors were confirmed by qRT-PCR (* p<0.05, ** p<0.01). D. The transfection effects of miR-124-3p.1 mimics were confirmed by qRT-PCR (* p<0.05, ** p<0.01). E. Cell proliferation detected using CCK-8 assay in miR-124-3p.1 inhibitor NC, miR-124-3p.1 inhibitor groups (p>0.05). F. Cell proliferation detected using CCK-8 assay in miR-124-3p.1 mimic NC, miR-124-3p.1 mimic groups (p>0.05). G. Proliferation ability assay of miR-124-3p.1 inhibitor NC, miR-124-3p.1 inhibitor groups, EdU (red) marked proliferating cells and Hoechst (blue) labeled total cell nuclei, and the red and blue images were merged to purple ones. H. Proliferation ability assay of miR-124-3p.1 mimic NC, miR-124-3p.1 mimic groups. (I) The relative quantification of EdU-positive cells from G (p>0.05). (J) The relative quantification of EdU-positive cells from H (p>0.05).

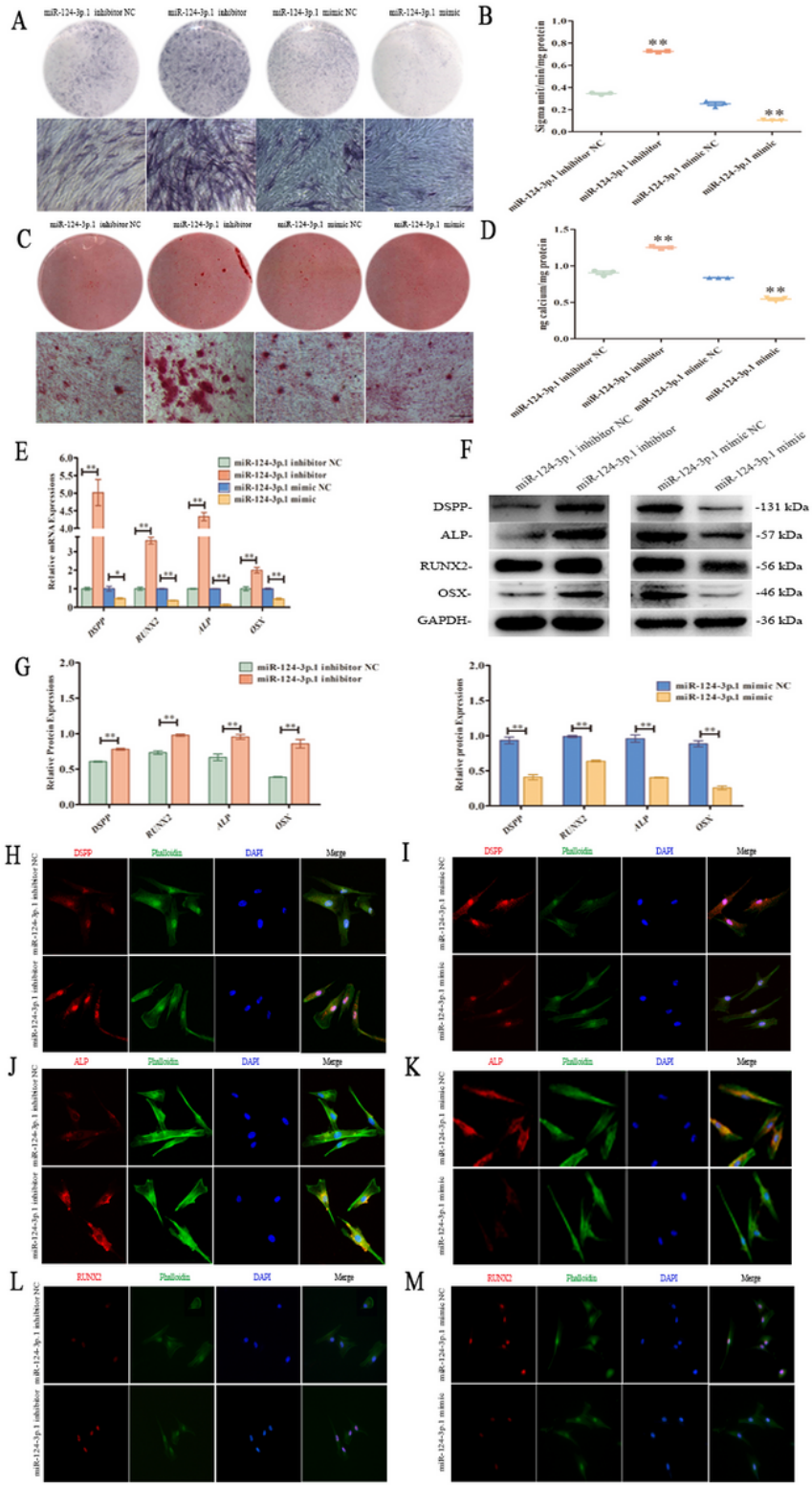

Figure 3

Effects of miR-124-3p.1 on the osteogenic and odontogenic differentiation in SCAPs A. Images of ALP staining in the miR-124-3p.1 inhibitor NC, miR-124-3p.1 inhibitor groups, miR-124-3p.1 mimic NC, miR-124-3p.1 mimic groups (Scale bar= 200 μm). B. ALP activity assay was conducted in miR-124-3p.1 inhibitor NC, miR-124-3p.1 inhibitor groups, miR-124-3p.1 mimic NC, miR-124-3p.1 mimic groups (* $p<0.05$, ** $p<0.01$). C. ARS staining on day 14 as indicated treatment (Scale bar= 200 μm). D. CPC

analysis of miR-124-3p.1 inhibitor NC, miR-124-3p.1 inhibitor groups, miR-124-3p.1 mimic NC, miR-124-3p.1 mimic groups (* p<0.05, ** p<0.01). E. The expressions of DSPP, RUNX2, OSX and ALP were determined by qRT-PCR analysis (* p<0.05, ** p<0.01). F. The expressions of DSPP, RUNX2, OSX and ALP were determined by western blot assay. G. Relative quantitative analysis of western blot analyses in D (* p<0.05, ** p<0.01). H, I. Immunofluorescence staining of DSPP (Scale bar= 50 μm). J, K. Immunofluorescence staining of ALP (Scale bar= 50 μm). L, M. Immunofluorescence staining of RUNX2 (Scale bar= 50 μm).

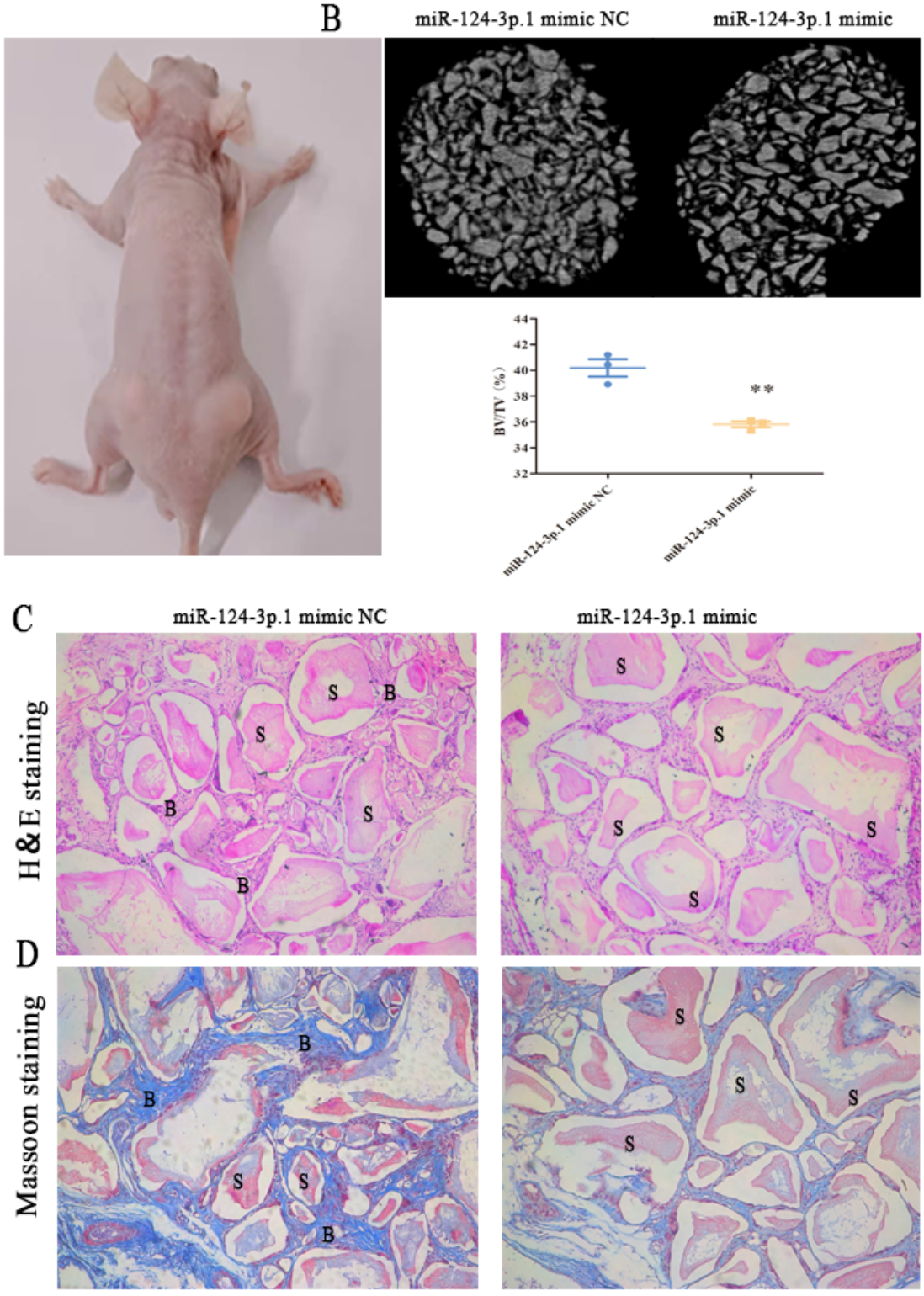

Figure 4

miR-124-3p.1 enhanced the osteo/dentinogenesis of SCAPs in vivo A. SCAPs with miR-124-3p.1 overexpression and control group were transplanted subcutaneously into nude mice. B. The results of micro-CT analyses in miR-124-3p.1 mimic group and miR-124-3p.1 mimic NC groups. C. H&E staining of osteocalcin in miR-124-3p.1 mimic group and miR-124-3p.1 mimic NC groups. D. Masson staining of osteocalcin in miR-124-3p.1 mimic group and miR-124-3p.1 mimic NC groups.

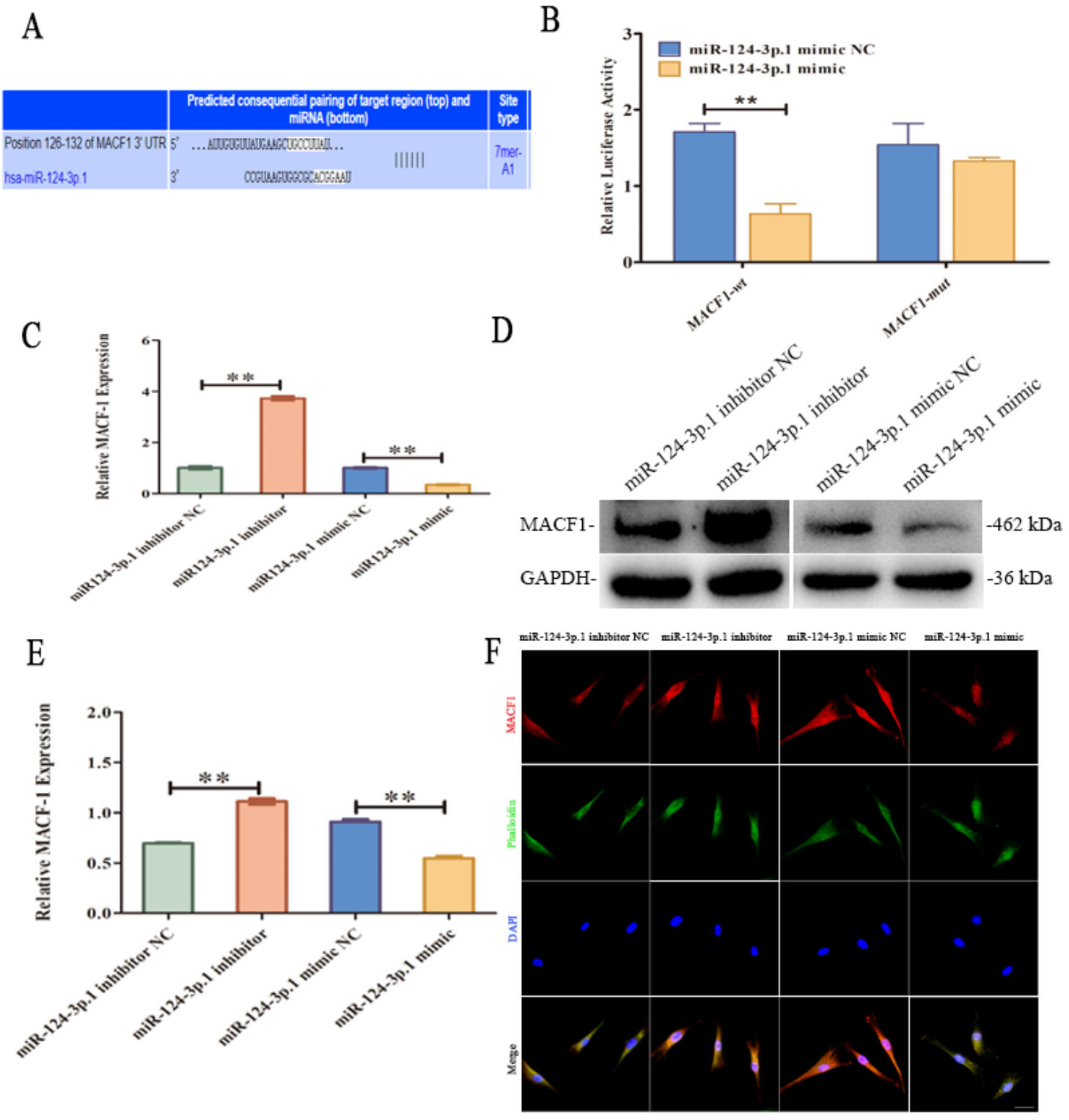

Figure 5

MACF1 is a direct target of miR-124-3p.1 A. Sequences of miR-124-3p.1 and predicted binding sites in the 3'- UTR of MACF1. B. 293T cells were transfected with either pGV272 luciferase vector containing a fragment of the MACF1 3'- UTR harboring a binding site for miR-124-3p.1, or the corresponding Mut constructs. Ectopic expression of miR-124-3p.1 led to a significant decrease of the reporter luciferase activity with the WT 3'- UTR but not that of the Mut reporter (* $p<0.05$, ** $p<0.01$). C. SCAPs were transfected with miR-124-3p.1 inhibitor NC, miR-124-3p.1 inhibitor, miR-124-3p.1 mimic NC and miR-124-3p.1 mimic. After transfection for 48 hours, the mRNA level of MACF1 was measured by qRT-PCR (* $p<0.05$, ** $p<0.01$). D. Western blot analysis of protein expression of MACF1 and the internal control GAPDH in miR-124-3p.1 inhibitor NC, miR-124-3p.1 inhibitor, miR-124-3p.1mimic NC and miR-124-3p.1 mimic groups. E. The quantification analysis of band intensities (* $p<0.05$, ** $p<0.01$). F. Immunofluorescence staining of MACF1 (Scale bar= 50 μm).

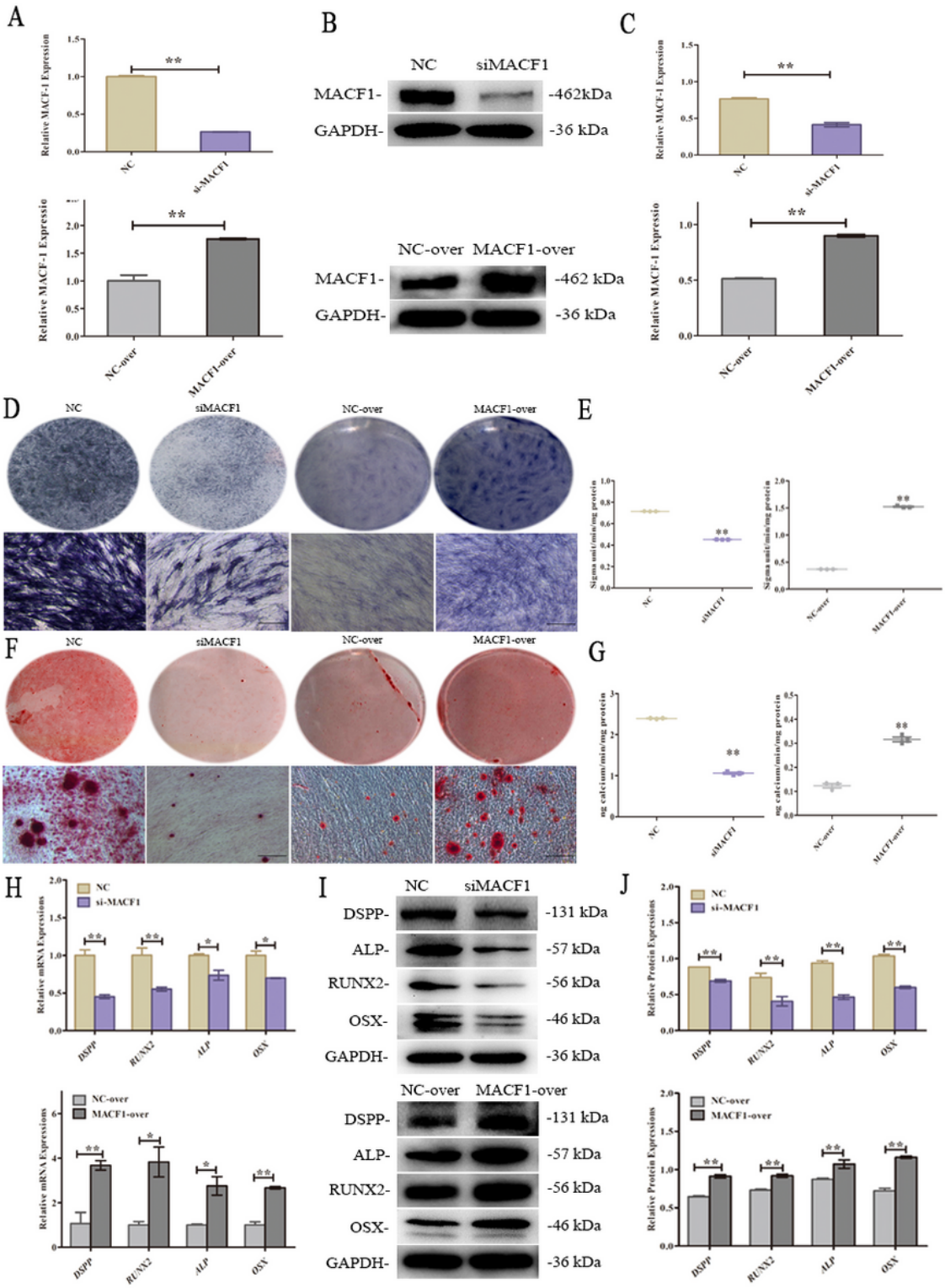

Figure 6

Knockdown of MACF1 inhibited the osteogenic and odontogenic differentiation of SCAPs, Overexpression of MACF1 promoted the odontogenic and osteogenic differentiation of SCAPs A. qRT-PCR analysis of the expression levels of MACF1 in NC, siMACF1, NC-over and MACF1-over groups (* p<0.05, ** p<0.01). B. Western blot analysis of the protein expression levels of MACF1 in NC, siMACF1, NC-over and MACF1-over groups. C. Quantification analysis of protein band in B (* p<0.05, ** p<0.01). D. Images of

ALP staining in NC, siMACF1, NC-over and MACF1-over groups (Scale bar= 200 μm). E. ALP activity analysis in NC, siMACF1, NC-over and MACF1-over groups (* p<0.05, ** p<0.01). F. The mineral nodes of SCAPs were visualized by Alizarin red staining (Scale bar= 200 μm). G. Calcium quantitative analysis of NC, siMACF1, NC-over and MACF1-over groups. H. qRT-PCR analysis of DSPP, RUNX2, ALP and OSX mRNA expression in SCAPs (* p<0.05, ** p<0.01). I. Western blot analysis of DSPP, RUNX2, ALP and OSX protein expression in SCAPs. J. The quantification analysis of band intensities (* p<0.05, ** p<0.01).

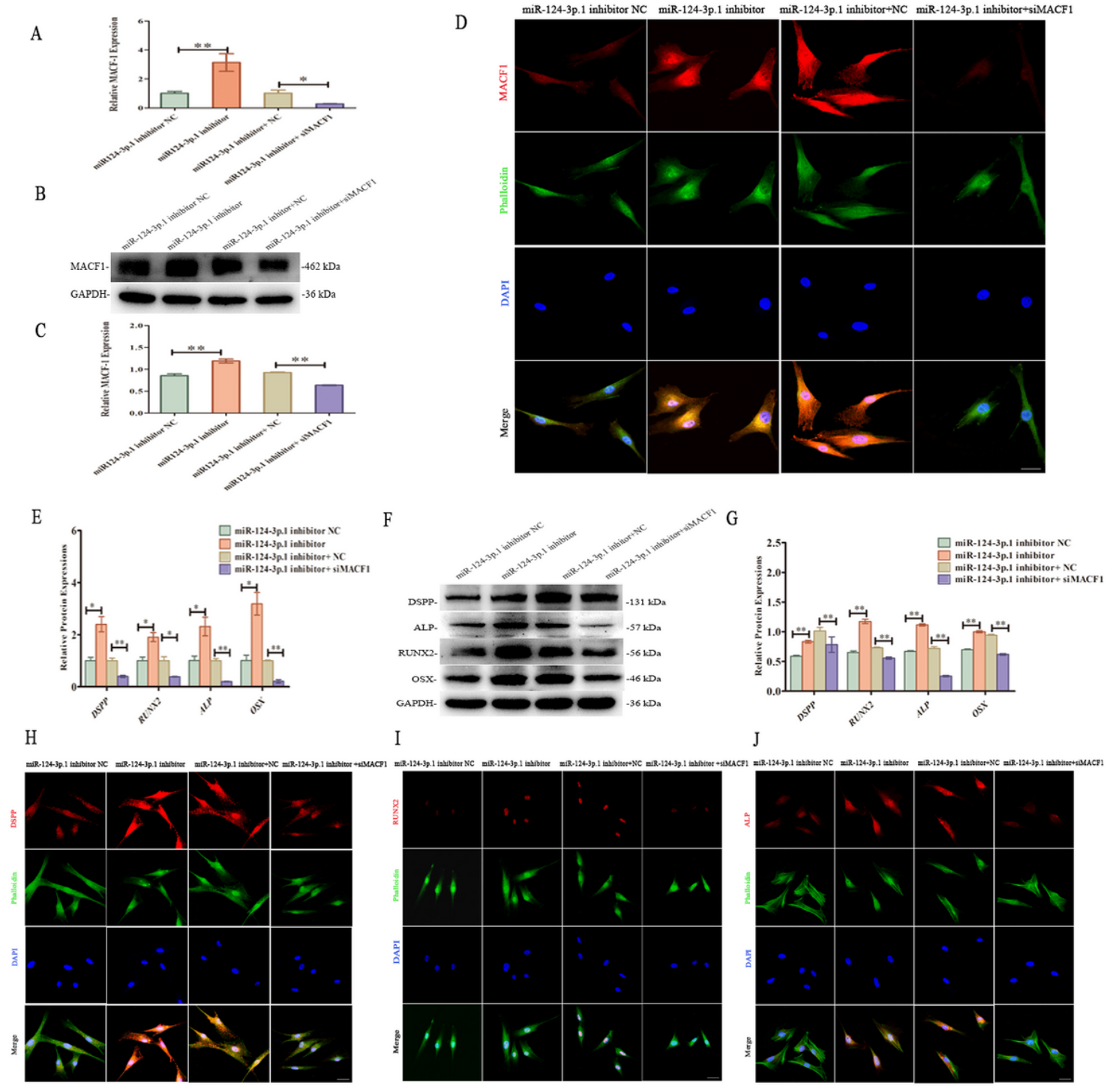

Figure 7

Suppression of miR-124-3p.1 contributed to osteogenic differentiation of SCAPs via mediating MACF1 A. qRT-PCR analysis of MACF1 in miR-124-3p.1 inhibitor NC, miR-124-3p.1 inhibitor, NC/miR-124-3p.1 inhibitor and siMACF1/miR-124-3p.1 inhibitor groups (* $p<0.05$, ** $p<0.01$). B. Western blot analysis for MACF1 in miR-124-3p.1 inhibitor NC, miR-124-3p.1 inhibitor, NC/miR-124-3p.1 inhibitor and siMACF1/miR-124-3p.1 inhibitor groups. C. Relative quantitative analysis of western blot analysis for MACF1 (* $p<0.05$, ** $p<0.01$). D. Immunofluorescence analysis showed the expression of the protein expression of MACF1 (Scale bar= 50 μm). E. The mRNA levels of DSPP, RUNX2, ALP and OSX in miR-124-3p.1 inhibitor NC, miR-124-3p.1 inhibitor, NC/miR-124-3p.1 inhibitor and siMACF1/miR-124-3p.1 inhibitor groups (Scale bar= 50 μm). F. Western blot analyses of DSPP, RUNX2, ALP and OSX in miR-124-3p.1 inhibitor NC, miR-124-3p.1 inhibitor, NC/miR-124-3p.1 inhibitor and siMACF1/miR-124-3p.1 inhibitor groups (Scale bar= 50 μm). G. Relative quantitative analysis of western blot analyses (* $p<0.05$, ** $p<0.01$). H. Immunofluorescence staining of DSPP (Scale bar= 50 μm). I. Immunofluorescence staining of RUNX2 (Scale bar= 50 μm). J. Immunofluorescence staining of ALP (Scale bar= 50 μm).

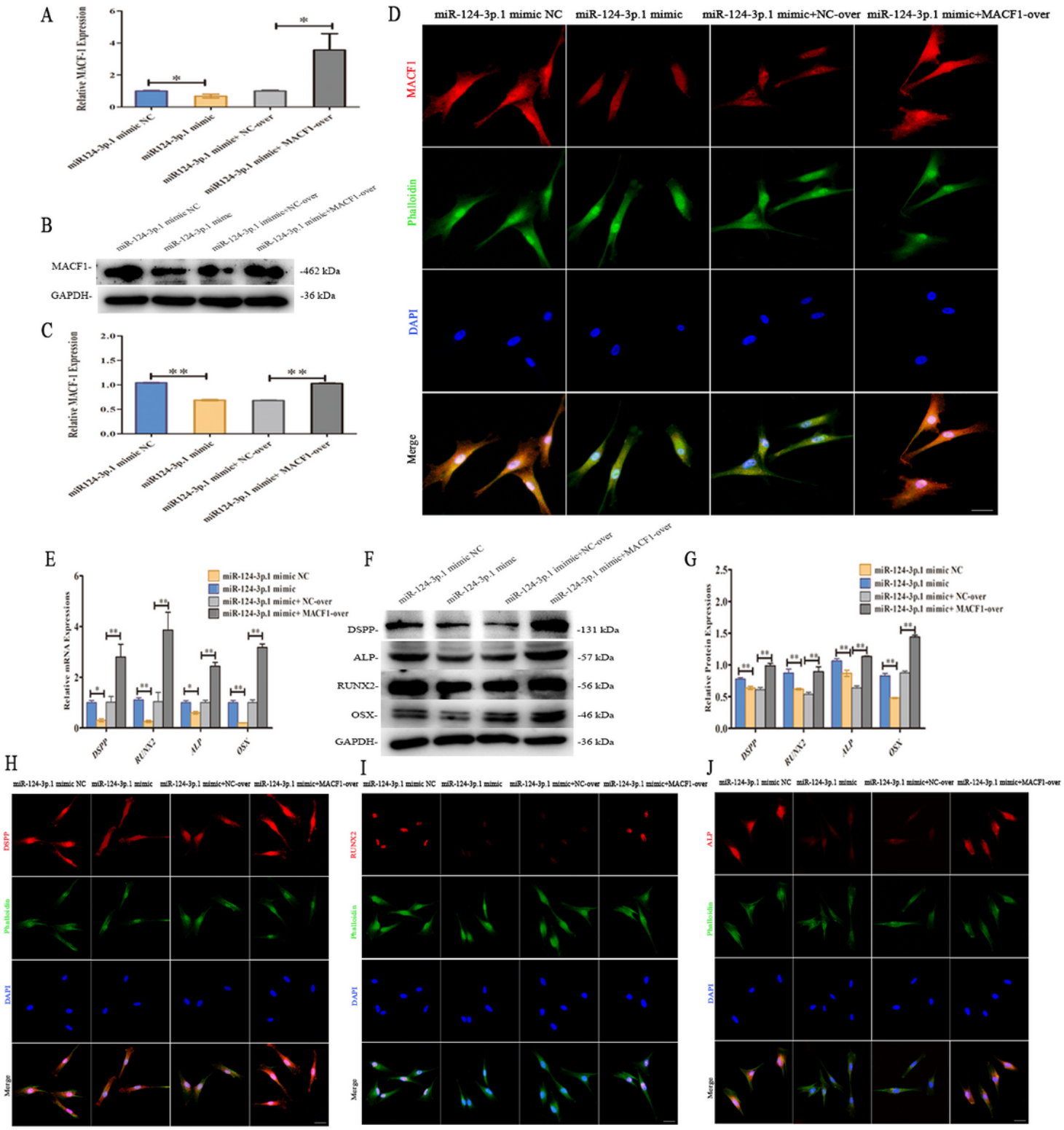

Figure 8

Overexpression of miR-124-3p.1 inhibited odontogenic and osteogenic differentiation via mediating MACF1 expression A. qRT-PCR analysis of MACF1 in miR-124-3p.1 mimic NC, miR-124-3p.1 mimic, NC-over/miR-124-3p.1 mimic and MACF1-over/miR-124-3p.1 mimic groups (* p<0.05, ** p<0.01). B. Western blot analysis for MACF1 in miR-124-3p.1 mimic NC, miR-124-3p.1 mimic, NC-over/miR-124-3p.1 mimic and MACF1-over/miR-124-3p.1 mimic groups. C. Relative quantitative analysis of western blot analysis

for MACF1 in B (* $p<0.05$, ** $p<0.01$). D. Immunofluorescence analysis showed the expression of the protein expression of MACF1 (Scale bar= 50 μm). E. The mRNA levels of DSPP, RUNX2, ALP and OSX in miR-124-3p.1 mimic NC, miR-124-3p.1 mimic, NC-over/miR-124-3p.1 mimic and MACF1-over/miR-124-3p.1 mimic groups (Scale bar= 50 μm). F. Western blot analyses of DSPP, RUNX2, ALP and OSX in miR-124-3p.1 mimic NC, miR-124-3p.1 mimic, NC-over/miR-124-3p.1 mimic and MACF1-over/miR-124-3p.1 mimic groups (Scale bar=50 μm). G. Relative quantitative analysis of western blot analyses (* $p<0.05$, ** $p<0.01$). H. Immunofluorescence staining of DSPP (Scale bar= 50 μm). I. Immunofluorescence staining of RUNX2 (Scale bar= 50 μm). J. Immunofluorescence staining of ALP (Scale bar= 50 μm).

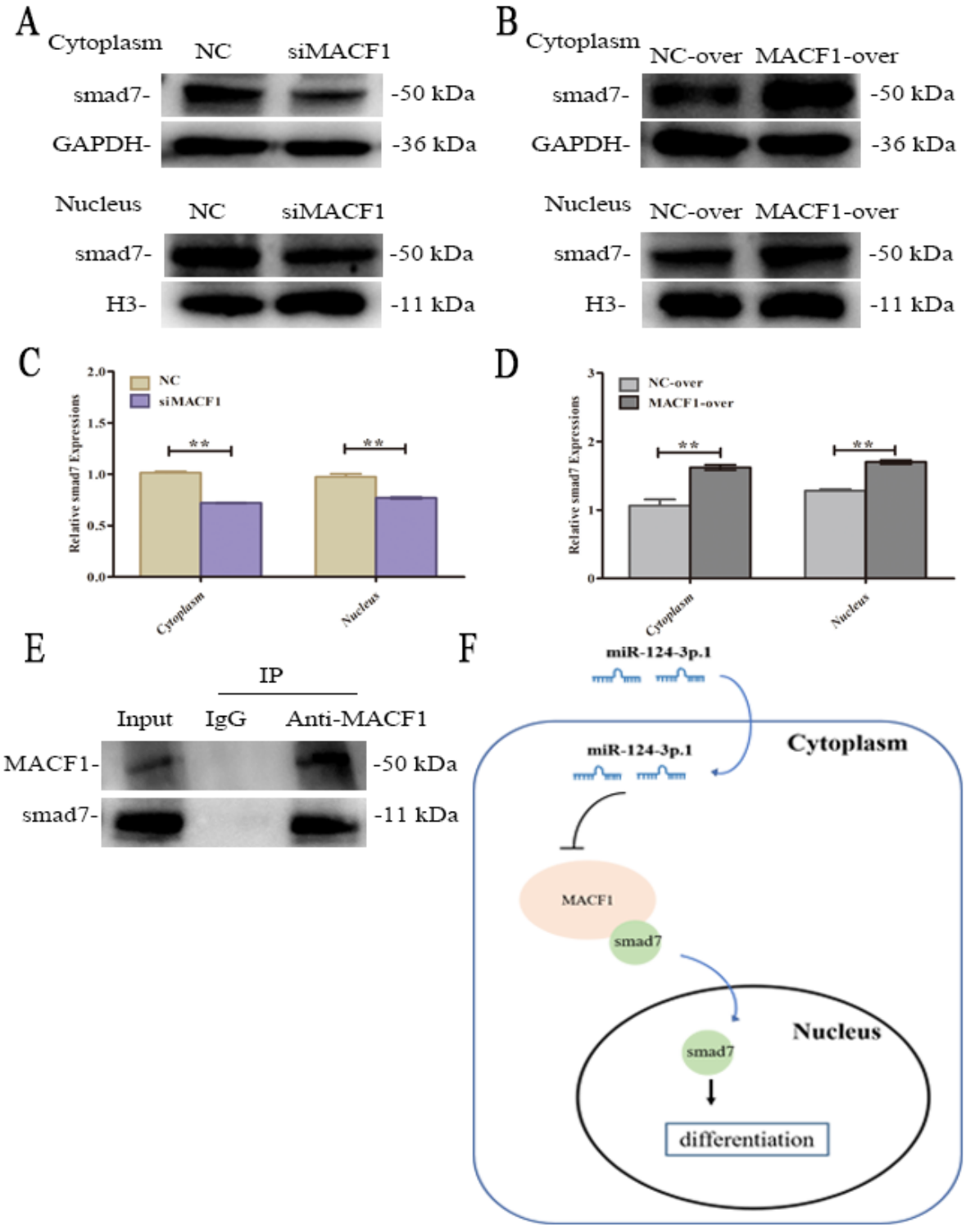

Figure 9

MACF1 interacts with smad7 in SCAPs A. Western blot analysis for smad7 in cytoplasm and nucleus in NC, siMACF1 groups. B, Western blot analysis for smad7 in cytoplasm and nucleus in NC-over, MACF1-over groups. C. Relative quantitative analysis of western blot analysis for smad7 in A (* $p<0.05$, ** $p<0.01$). D. Relative quantitative analysis of western blot analysis for smad7 in B (* $p<0.05$, ** $p<0.01$). E. Co-IP assay showing interaction of MACF1 and smad7 in SCAPs. F. A mechanism diagram.