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Effect of CRABP2 on the proliferation and odontoblastic differentiation of hDPSCs

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Abstract: Cellular retinoic acid-binding protein 2 (CRABP2) has been detected in several organs during embryonic development. Recent studies have demonstrated that CRABP2 plays important roles in the retinoic acid, β-catenin and Notch signaling pathways, as well as in the interaction between epithelial and mesenchymal cells, which are important for human dental pulp stem cells (hDPSCs) and tooth development. In the present study, the expression of CRABP2 during mouse molar development and the role of CRABP2 in hDPSC odontoblastic differentiation were evaluated. CRABP2 was gradually decreased during the development of the first maxillary molar, which exhibited the same trend as the expression of CRABP2 during the odontoblastic induction of hDPSCs. CRABP2 knockdown inhibited the proliferative ability of hDPSCs, while it enhanced odontoblastic differentiation via promoting mineralization nodule formation and upregulating the activity of alkaline phosphatase and the expression of mineralization-related genes. The present study uncovered a novel function of CRABP2 in hDPSCs. Our data suggest that CRABP2 may act as a regulator during the proliferation and differentiation of hDPSCs.

Keywords: Dental Pulp; Retinoic Acid Binding Protein II, Cellular.

Introduction

Human dental pulp contains ecto-mesenchymal components with neural crest-derived cells; these undifferentiated mesenchymal cells play an important role in dentinogenesis.[1] Dentinogenesis is a progress involving the transformation of pre-existing mesenchymal stem cells into odontoblasts. Once exposed to the appropriate stimuli or certain inductive factors, dental pulp stem cells (DPSCs), as a type of mesenchymal cell, can create reparative dentin through cell proliferation and differentiation into odontoblasts.[2] Therefore, DPSCs are suitable for investigating odontoblastic differentiation *in vitro*. hDPSCs may be used to prevent and treat heritable and acquired loss of dentin, which may even be applied to treat alveolar bone loss in the clinical setting.[3,4] Understanding the key genetic and molecular events that regulate the odontoblastic differentiation of hDPSCs is crucial for clinical applications and for the approach to dentin and bone regeneration.

Cellular retinoic acid-binding proteins (CRABPs), which are expressed in numerous developing tissues[5] and mouse developing organs,[6] and are known to perform specific functions during morphogenesis,[7] comprise a well-characterized family of small proteins that specifically bind retinoic acid (RA). RA and its related molecules will specifically bind several distinct cytoplasmic proteins to stimulate the RA pathway, which is involved in a number of biological processes, including differentiation, proliferation and apoptosis.[8]

As a member of the CRABP family, CRABP2 is a low-molecular-mass (15 kDa) protein with a high affinity for RA. CRABP2 has been detected in the central nervous system, epidermis, proliferative zone of the retina, pectoral fins and branchial arches during zebrafish embryonic development.[9] CRABP2 is key to the normal development and maintenance of motor neurons in the spinal cord.[10] In addition, CRABP2 has been detected in the epithelial and mesenchymal cells within the hair follicle,[11] suggesting that it may participate in the transit molecular signaling between epithelial and mesenchymal cells. Furthermore, CRABP2 is involved in the RA, β-catenin and Notch signaling pathways, as well as in the interaction between epithelial and mesenchymal cells, all of which are reported to regulate tooth development.[12] Most importantly, CRABP2 regulates bone remodeling in murine degenerative joint disease models *in vivo* and osteogenic differentiation of myoblast cell lines (C2C12) *in vitro*,[13] which shares similar characteristics with odontoblastic differentiation. To the best of our knowledge, the expression and biological role of CRABP2 in hDPSCs have not been reported to date. Therefore, we hypothesized that CRABP2 may affect odontoblastic differentiation during tooth development and reparative dentin formation associated with deep carious lesions.

The aim of the present study was to investigate the expression of CRABP2 during maxillary molar development in mice, as well as the expression and role of CRABP2 during human dental pulp stem cell (hDPSC) proliferation and odontoblastic differentiation.

Materials and methods

The study protocol was approved by the Ethics Committee of Shanghai Engineering Research Center of Tooth Restoration and Regeneration.

Immunohistochemical analysis

Nine adult C57BL/6J mice (3 male and 6 female, aged 8 weeks) were mated overnight in a specific pathogen-free animal center. The day a vaginal plug was observed was designated as embryonic day 0.5 (E0.5). In the present study, we selected the embryos and postnatal (PN) mice at different tooth developmental stages (E13.5, E18.5, and PN6). All the animals were deeply anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg) prior to sample collection, and all the procedures and protocols used were in accordance with the Guidelines for Ethical Care of Experimental Animals. Samples were prepared for immunohistochemistry by fixing mandibles isolated at each stage in 4% paraformaldehyde overnight at 4˚C, followed by demineralization with 10% EDTA (pH 7.4) for 2 weeks at 4˚C. Following dehydration and embedding in paraffin, the samples were sectioned at a thickness of 5 μm. The sections were dipped in xylene to remove the paraffin and rehydrated using a graded alcohol series. The sections were incubated in 3% hydrogen peroxide for 10 min at room temperature to prevent endogenous peroxidase activity, then incubated in 0.01 M citrate for 10 min at 100˚C and cooled at room temperature for 20 min. The slides were subsequently blocked in 5% bovine serum albumin in phosphate-buffered saline (PBS). The slides were then incubated with primary antibody against CRABP2 (1:400, rabbit polyclonal antibody) overnight at 4˚C. A subset of slides were incubated with PBS as the negative control. The slides were washed with PBS and incubated with polymer helper and poly-HRP-anti-rabbit IgG (ZhongShan Golden Bridge Biotechnology Inc., Beijing, China) for 1 h at 37˚C. Following counterstaining with hematoxylin, the samples were visualized under a light microscope (Carl Zeiss AG, Oberkochen, Germany).

Cell Culture and Differentiation

The primary cultured hDPSCs were isolated from healthy human premolars following extraction for orthodontic treatment (age, 12-14 years), with informed consent obtained prior to extraction, and were cultured DMEM (Gibco-BRL, Grand Island, USA) supplemented with 20% fetal bovine serum (FBS; Gibco-BRL Life Technologies, Paisley, UK) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin, Gibco-BRL, USA) in a humidified atmosphere of 5% CO_2 at 37˚C. The isolation of hDPSCs was performed as previously described[14]. When primary cells reached 80% confluence, the cells were digested into single-cell suspensions to collect cell clones by limiting dilution in 96-well plates. The cells from these clones were characterized as hDPSCs, which were expanded for the experiments.

For odontoblastic induction experiments, the cells were cultured in DMEM supplemented with 10% FBS, antibiotics, 50 μg/ml ascorbic acid (Sigma, St. Louis, MO, USA), 10 mmol/l sodium β-glycerophosphate (Sigma), and 10 nmol/l dexamethasone (Sigma).

CRABP2 lentivirus transfection

For the lentivirus transfection, shRNAs against human CRABP2 (shCRABP2) were constructed and provided by GeneChem, China. hDPSCs were seeded in 6-cm plates to reach a ~50% confluence and were then transfected according to the manufacturer's recommendations. Subsequently, 8 μg/ml puromycin was added to positively select the transfected cells for 1 week at 37˚C, 5% CO_2. Stably transfected cells (termed as hDPSC/control or hDPSC/shcrab) were selected and used for further experiments. The transfected cells were verified by fluorescent microscopy, RT-PCR and western blots.

Cell proliferation assay

The proliferation of CRABP2 on hDPSCs was measured by Cell Counting Kit-8 (CCK-8; Dojindo Kagaku Co, Kumamoto, Japan) according to the manufacturer's protocol. Briefly, the hDPSC/wt, hDPSC/control or hDPSC/shcrab cells were seeded at a density of $5x10^3$ cells/well in four 96-well plates (Corning Inc, Corning, USA) for 1, 3, 5 and 7 days. After being cultured overnight, fresh culture medium was added and changed every 3 days. At different culture timepoints, absorbance was measured using a microplate reader at 450 nm to determine the number of viable cells in each well. The well without cells was used as a blank control. Cell numbers were recorded as the mean ± standard deviation (SD) of the absorbance for five wells from each group.

Alizarin Red Staining and Alkaline Phosphatase (ALP) Staining

The hDPSC/wt, hDPSC/control or hDPSC/shcrab cells were incubated in odontoblastic induction medium for 14 days. Mineralized nodules were assessed following alizarin red staining. Briefly, the cells in six-well plates were cultured in 1% alizarin red (pH 4.3) for 30 min at 37˚C following fixation in 4% paraformaldehyde. For ALP staining, the plates were harvested on day 7. The ALP color development kit (Beyotime Institute of Biotechnology, China) was used according to the manufacturer's protocol. All the cells were washed thrice with distilled water and then observed under a phase-contrast microscope.

ALPase Activity Assay

The hDPSC/wt, hDPSC/control or hDPSC/shcrab cells were incubated in odontoblastic induction medium for 0, 7 and 14 days. ALPase activity was determined using cell lysates, using p-nitrophenylphosphate as a substrate, as in our previous study. A protein extraction kit (Pierce, Rockford, USA) was used to collect the cell lysates. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce). ALPase activity (U/mg) was defined as the release of 1 mol p-nitrophenol per mg total cellular protein.

RNA isolation and semi-quantitative RT-PCR analysis

Total RNA from the hDPSC/wt, hDPSC/control or hDPSC/shcrab cells was isolated following odontoblastic induction for 0, 7 and 14 days with TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. cDNA was synthesized using a PrimeScript RT reagent kit with gDNA Eraser (Takara Bio, Shiga, Japan). The odontoblast-related markers dentin sialophosphoprotein (DSPP), dentin matrix acidic phosphoprotein-1 (DMP-1) and alkaline

phosphatase (ALP) were analyzed. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize RNA expression. The sequences of the specific primers designed in this study were as follows: DSPP (forward: 5′-TCG GTT ACC GGT TGA CAT GG-3′, reverse: 5′-TCA CAA GGG AGA AGG GAA TGG-3′); DMP1 (forward: 5′-CCC TTG GAG AGC AGT GAG TC-3′, reverse: 5′-CTC CTT TTC CTG TGC TCC TG-3′); ALP (forward: 5′-CCA CAA GCC CGT GAC AGA-3′, reverse: 5′-GCG GCA GAC TTT GGT TTC-3′); CRABP2 (forward: 5′-GAG ACC CTG TAA GAG TTT GG-3′ , reverse: 5′- AAC GTC ATC TGC TGT CAT T-3′); and GAPDH (forward: 5′-TGG GTG TGA ACC ATG AGA AGT-3′, reverse: 5′-TGA GTC CTT CCA CGA TAC CAA-3′). Real-time PCR was performed with HieffTM qPCR SYBR® Green Master Mix in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Amplification and detection were performed under the following conditions: initial denaturation for 5 min at 95˚C at the holding stage; 40 cycles of 10 sec at 95˚C, 30 sec at 60˚C at the cycling stage; and 15 sec at 95˚C, 1 min at 60˚C, and 15 sec at 60˚C at the melt curve stage. Relative gene expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method. The mean Ct value of the target gene was normalized to its averaged Ct values of GAPDH to obtain a ΔCt value, which was then normalized to control samples to obtain a ΔΔCt value. Each measurement was performed in triplicate. The gene expression ratio was shown as the mean ± SD from three independent experiments.

Western blot analysis

The same cell lysates were used in this experiment as ALPase activity. Equal amounts of protein were separated and then transferred onto nitrocellulose membranes (Millipore Corporation, Billerica, USA). The primary antibodies [rabbit anti-mouse CRABP2 (1:1000), rabbit anti-mouse DMP1 (1:1000), rabbit anti-mouse DSPP (1:500) and rabbit anti-mouse β-actin (1:5000)] were hybridized with the membranes. After washing, the membranes were incubated with a secondary goat anti-rabbit IRDye680 antibody (1:10000). After the final wash, the membranes were visualized using the Odyssey LI-CDR system.

Statistical analysis

Experiments were performed in triplicate, and data are presented as mean ± SD. Data were evaluated by one-way ANOVA using the SPSS software, version 10.0 (SPSS, Chicago, IL, USA). P-values < 0.05 were considered to indicate statistically significant differences.

Results

Location of CRABP2 protein during mouse tooth development

Immunohistochemical analysis detected the expression of CRABP2 in the front sections of mouse first maxillary molar at the predefined experimental time points. The results demonstrated that CRABP2 was highly expressed in the dental epithelium at E13.5, while CRABP2 decreased from E13.5 to PN6 during dentin development (Figure 1).

Expression of CRABP2 during odontoblastic differentiation of hDPSCs

The mRNA and protein levels of DMP1, DSPP, ALP and CRABP2 were measured by real-time PCR and western blot analysis following odontoblastic induction of hDPSCs (Figure 2). The mRNA and protein levels of DMP-1, DSPP and ALP increased during the induction (Figures 2A-C, F), and ALPase activity exhibited the same trend (Figure 2E). However, the mRNA and protein levels of CRABP2 were downregulated during the same process (Figures 2D and F).

CRABP2 knockdown in hDPSCs

hDPSCs were transfected with a lentivirus encoding green fluorescent protein (GFP) *in vitro*. The results demonstrated that GFP was expressed in almost all cells after positive selection of the transfected cells using puromycin (Sigma) for 1 week (Figure 3A). The mRNA and protein expression of CRABP2 were notably lower in hDPSC/shcrab compared with the hDPSC/control cells (Figures 3B and C). These results demonstrated that CRABP2 was stably knocked down in the hDPSC/shcrab group.

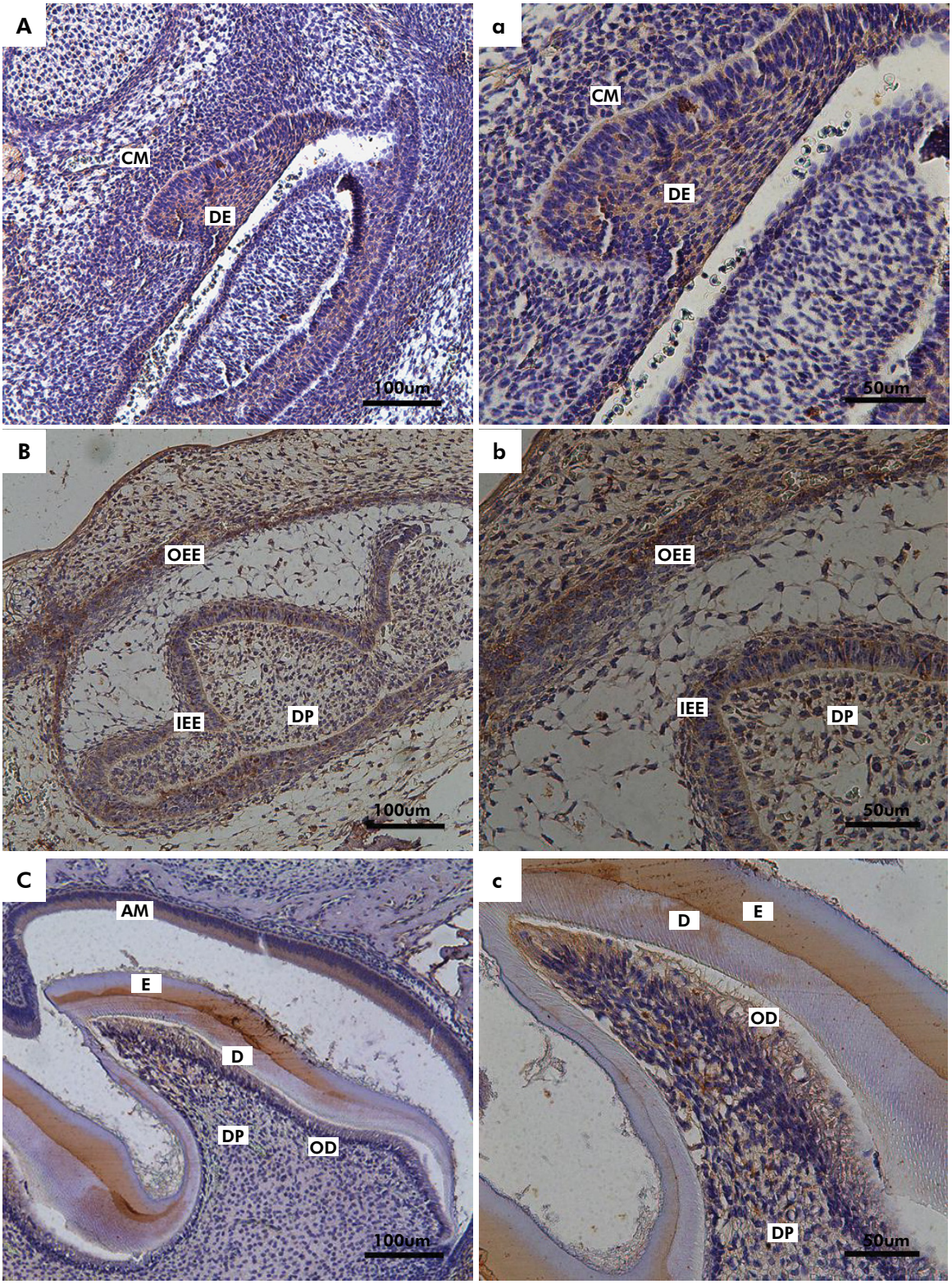

Figure 1. CRABP2 protein expression in the first maxillary molar in mice was detected by immunohistochemical analysis. A and a: CRABP2 was strongly expressed in the dental epithelium during the early bud stage (E13.5). Positive immunolabelling was observed in the basal dental epithelial cells (DE) and surrounding mesenchymal cells (MC). B and b: Positive immunolabelling was also observed in the inner enamel epithelium (IEE), outer enamel epithelium (OEE) and dental pulp (DP) cells (E18.5). C and c: During PN6, CRABP2 was poorly expressed in the odontoblasts (OD) and just several dental DP cells near the OD. (A, B and C: x200; a, b and c: x400).

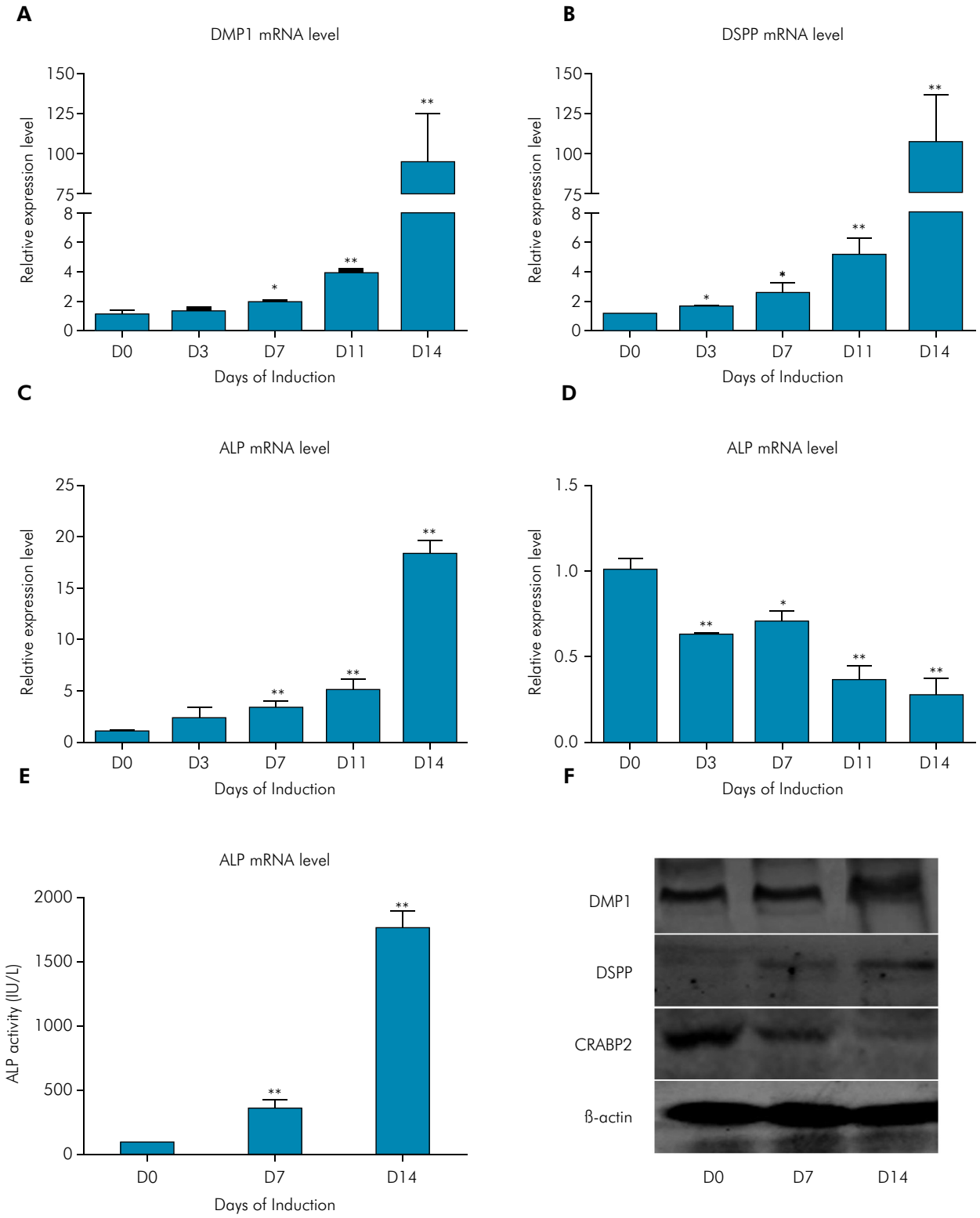

Figure 2. Expression of CRABP2 during odontoblastic differentiation of hDPSCs. A, B and C: The mRNA level of DMP1, DSPP and ALP, respectively, was upregulated during the induction. D: The mRNA level of CRABP2 was downregulated. E: The ALP activity was increased during the same process. F: Western blotting results of DMP1, DSPP and CRABP2 during the induction, which exhibited the same trend as mRNA. The data represent the mean ± SD of at least 3 independent experiments.

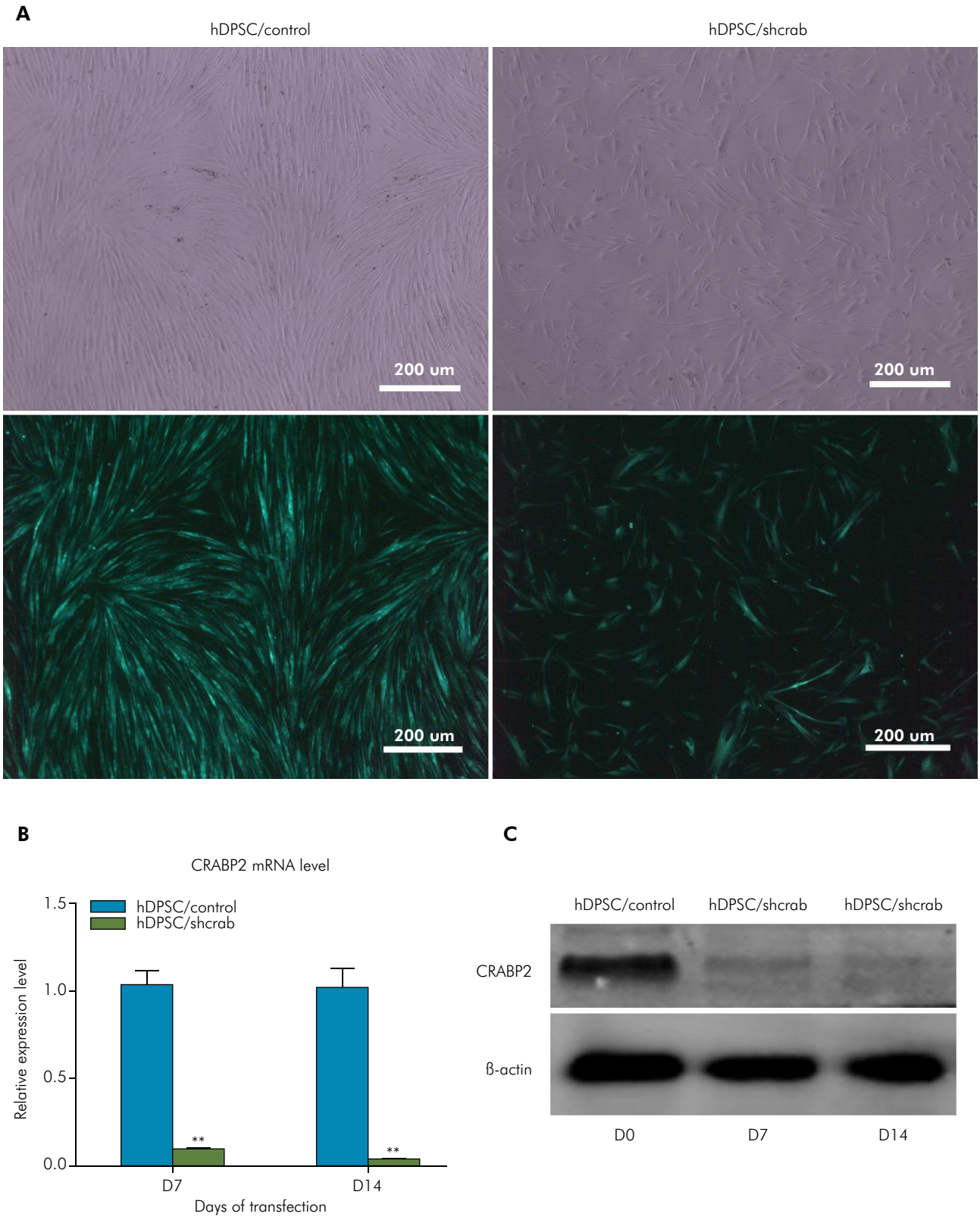

Figure 3. Stable knockdown of CRABP2 in hDPSCs. A: hDPSCs transfected with lentiviral vector *in vitro* exhibited GFP expression following transfection for 1 week and selection (x200). B and C: mRNA and protein levels of CRABP2 following hDPSC transfection with lentiviral vector *in vitro* for 7 and 14 days, respectively.

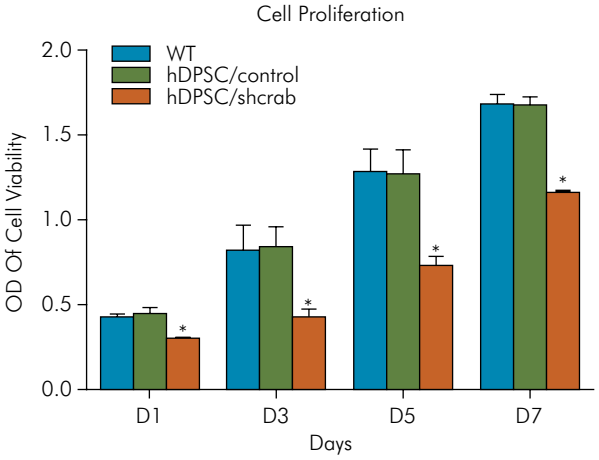

Figure 4. CRABP2 promotes the proliferation of hDPSCs. The CCK-8 assay indicated that the cell viability of the hDPSC/shcrab group was significantly lower compared with the hDPSC/control and wt groups ($P<0.05$) on days 1, 3, 5, and 7. Data represent the mean ± SD of at least 3 independent experiments.

CRABP2 knockdown inhibited proliferation of hDPSCs

The CCK-8 assay results indicated that the optical density value of the hDPSC/shcrab group was significantly lower compared with the hDPSC/control group ($p < 0.05$) on days 1, 3, 5 and 7 (Figure 4). These results suggested that CRABP2 knockdown inhibited the proliferation of hDPSCs *in vitro*.

CRABP2 knockdown promoted the odontoblastic differentiation of hDPSCs

Mineralization-related genes, ALPase activity and mineralization assay were measured to directly address the function of CRABP2 on hDPSCs during odontoblastic differentiation. CRABP2 knockdown upregulated the mRNA levels of DSPP, DMP-1 and ALP ($p < 0.05$; Figures 5A-D). Both DMP-1 and DSP protein levels in the CRABP2 knockdown groups were higher than in the control groups (Figure 5E). A significantly higher level of ALP staining was observed in the hDPSC/shcrab group on day 7 compared with in the hDPSC/wt and hDPSC/control groups (Figure 6A). The mineralized nodules formed in the hDPSC/shcrab group on day 14 exhibited the same trend as the ALP staining in the three groups (Figure 6B). These results indicated that CRABP2 knockdown promoted the odontoblastic differentiation of hDPSCs.

Discussion

Tooth development, morphogenesis and postnatal maintenance are regulated by non-protein factors, including the hormone-like RA.[15] RA homeostasis is crucial for normal embryonic development, whereas its deficiency or excess are associated with congenital malformations. The RA signaling pathway can regulate tooth progenitors by controlling the growth of the neural crest and manipulating tooth development. The RA signaling pathway plays an important role in the diversification of teeth in Cyprinids.[16] Blocking RA signaling results in an abnormal phenotype, whereby the epidermis fails to differentiate,[17] which may include tooth developmental malformations.

CRABP2 belongs to a family of small cytosolic lipid-binding proteins, which are specific carriers for RA,[6,18] facilitating the transcriptional activities of the RA signaling pathway by translocating RA from the cytoplasm into the nucleus to form a complex with the nuclear RA receptor. CRABP2 has diverse functions, such as directing RA to catabolism, delivering RA to specific nuclear receptors, and generating non-canonical actions.[19] CRABP2 is a major factor in promoting robustness in hindbrain development.[20] CRABP2 is also involved in skeletal muscle development and myogenic transformation.[6] It was recently proven that CRABP2 expresses and modulates mouse embryonic stem cell differentiation in a number of organs.[21] To the best of our knowledge, this study is the first to analyze the functions of CRABP2 during the proliferation and odontoblastic differentiation of hDPSCs.

In the present study, CRABP2 was highly expressed in odontoblasts and DPCs on E13.5, and gradually decreased during dentin formation (Figure 1). During the induction of hDPSCs, ALP expression and ALPase activity increased. DMP1 and DSPP were upregulated during the same procedure, while the mRNA and protein levels of CRABP2 were downregulated during the induction. These results suggested that CRABP2 maybe play a key role in regulating dentin development and repair.

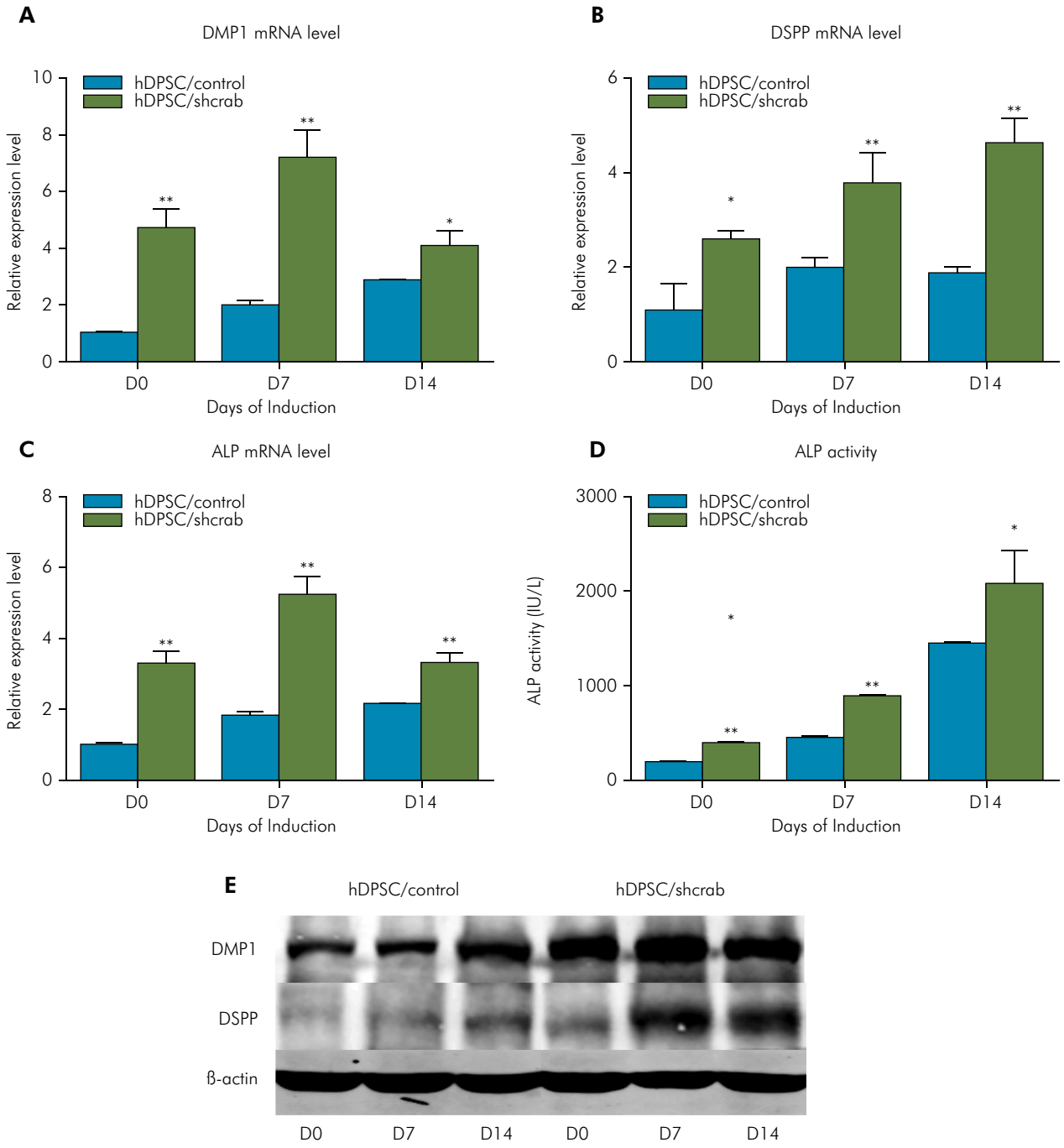

Figure 5. Inhibitory effect of CRABP2 on the odontoblastic differentiation of hDPSCs. A, B and C: mRNA level of DMP1, DSPP and ALP, respectively, following CRABP2 knockdown in hDPSCs during odontoblastic differentiation. D: ALP activity following CRABP2 knockdown. E: Protein levels of DMP1 and DSPP by western blot analysis. The data represent the mean ± SD of at least 3 independent experiments.

CRABP2 has been shown to directly bind with HuR to enhance the stability of Apaf-1, which leads to the suppression of cell proliferation.[22] Moreover, CRABP2 regulated the cell cycle during myoblast differentiation of the C2C12 cell line.[6] To further elucidate the functions of CRABP2 in the proliferation

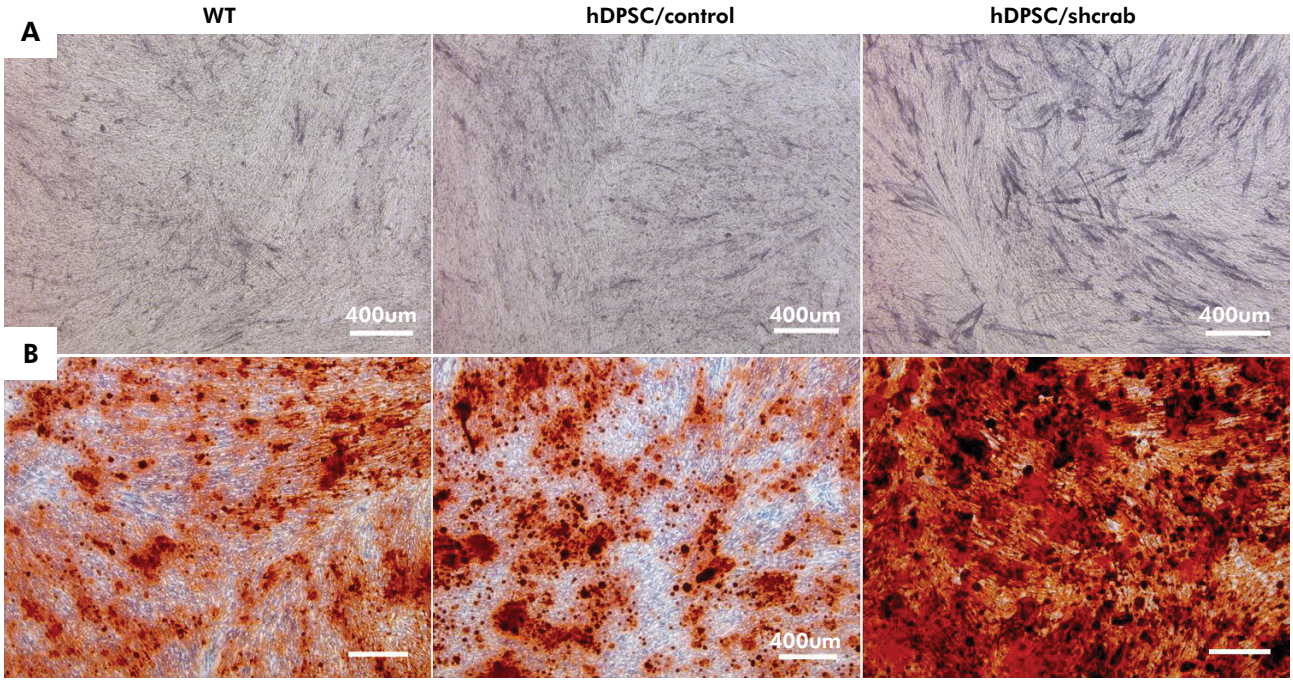

Figure 6. ALP staining and mineralized nodule formation. A: ALP staining was significantly higher in the hDPSC/shcrab group on day 7 compared with the wt and hDPSC/control groups. B: The number of mineralized nodules formed in the hDPSC/shcrab group was significantly higher compared with that in the wt and hDPSC/control groups (A and B: phase-contrast microscopy, x50).

of hDPSCs *in vitro*, hDPSCs were cultured as previously described.[14] Lentiviral vectors provided efficient gene knockdown and transfected hDPSCs *in vitro* (Figure 3). The results of the CCK-8 assay demonstrated that CRABP2 knockdown inhibited hDPSC proliferation (Figure 4), which exhibited the same trend as in skeletal myotubes,[6] osteoblasts[23] and malignant glioma cells.[24]

CRABP2 regulates osteogenic differentiation via LIMK to remodel cell morphology *in vitro*[13]. CRABP2 was also reported to regulate bone remodeling in murine degenerative joint disease models *in vivo*,[24] and to be modulated by the transcription factors MyoD and Sp1 to promote myoblast differentiation in C2C12 cells.[6] During early-stage mouse embryonic development, CRABP2 mRNA was detected in several organs, indicating that CRABP2 may play a vital role during embryonic development.[6] In the present study, the mRNA and protein levels of the odontoblastic differentiation markers DMP1, DSPP and ALP were upregulated in the hDPSC/shcrab group (Figure 5). ALP activity, ALP staining and mineralization assays were also performed to confirm the results. ALP activity and ALP staining were increased, which was consistent with the ALP mRNA level in the hDPSC/shcrab group (Figure 6A). A mineralization assay demonstrated that CRABP2 knockdown increased the mineralization potential of hDPSCs (Figure 6B). Taken together, all these results suggest that CRABP2 downregulation promoted odontoblastic differentiation of hDPSCs, which may regulate dentin formation during tooth development.

FABP5, another group of RA-binding proteins, as critical intracellular partitioning factors of RA between the nuclear receptors RAR and PPARbeta/delta, may exert opposite (anti-survival or pro-survival) effects.[25] The RA signaling pathway plays an important role in tooth development, which depends on the expression ratio of CRABP2/FABP5 in hDPSCs. Therefore, the detailed molecular mechanisms underlying the role of CRABP2 in hDPSC differentiation remain to be further investigated.

Conclusions

In summary, the present study demonstrated that CRABP2 downregulation inhibited proliferation, and increased mineralization and expression of mineralization-associated genes and proteins in hDPSCs.

Acknowledgements

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