Brief Report

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CCL5/RANTES is important for inducing osteogenesis of human mesenchymal stem cells and is regulated by dexamethasone

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Summary **In this study, we examine the effect of chemokine (C-C motif) ligand 5 (CCL5)/Regulated on Activation Normal T cell Expressed and Secreted (RANTES), a pro-inflammatory cytokine on osteogenic differentiation of human mesenchymal stem cells (hMSCs). We found CCL5 expression was increased during osteogenic differentiation of hMSCs and CCL5 expression is dependent on the presence of dexamethasone. Knocking down endogenous CCL5 expression blocked osteogenesis, as revealed by decreasing alkaline phosphatase (ALP) activity and a reduction in the expression levels of ALP, bone sialoprotein (BSP), and osteopontin (OPN). Of note, the overexpression of CCL5 was sufficient to increase ALP expression and activity. Moreover, the down-regulation of chemokine (C-C motif) receptor 1 (CCR1), one of the CCL5 receptors, significantly decreased the osteogenesis of hMSCs. Interestingly, the down-regulation of CCR1, but not CCL5, was sufficient to affect the cell numbers during the process of osteogenesis. Our findings reveal that both CCL5 and CCR1 are required for osteogenesis of human MSCs, CCL5 is sufficient for the osteogenesis, and provide a novel link between dexamethasone and CCL5 in human osteogenesis.**

Keywords: CCL5, osteogenesis, mesenchymal stem cell, dexamethasone, CCR1

1. Introduction

Bone is a tissue with continuous turnover and is maintained and manipulated by two opposite processes. One is bone formation by osteoblasts originating from mesenchymal stem cells (MSCs), and the other is bone resorption by osteoclasts derived from macrophages. When the balance between bone formation and resorption is disrupted, bone diseases such as osteoporosis occur. Some signaling pathways related to inflammation have been suggested to be involved in osteoblast and osteoclast differentiation or formation (*1*). For example, chemokine (C-C motif) receptor 4 (CXCR4) deficiency impairs osteogenic differentiation of MSCs (*2*), while interleukin-1 (IL-1) promotes osteoclast formation. To explore the interplay between inflammation signaling and osteogenesis, we examined the roles of chemokine (C-C motif) ligand 5 (CCL5) function in bone formation.

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CCL5, also called Regulated on Activation Normal T cell Expressed and Secreted (RANTES), is a pro-inflammatory chemokine. CCL5 was reported to play important roles in chemo-attraction (*3*), recruitment of leukocytes to the sites of injury and inflammation, and carcinogenesis (*4-5*). In CCL5 deficient mice, the effect of loss of CCL5 is dependent upon different age. Only in 6-month-old mice, but not in young mice or 12-month-old mice, bone volume and bone formation rate (BFR) were observed to be decreased (*6*). In a mouse model, CCL5 had been shown to promote chemotaxis and survival of osteoblasts (*7*). The function of CCL5 in osteogenesis, particularly the effects of CCL5 in the process of MSC differentiate into osteoblast, has not been determined in human cells.

CCL5 can mediate signals through three receptors: chemokine (C-C motif) 1 (CCR1), chemokine (C-C motif) receptor 3 (CCR3), and chemokine (C-C motif) receptor 5 (CCR5) (*8*). The profile of chemokine receptors was analyzed by flow cytometry of human MSCs, and the results revealed that at least 70% of MSCs express CCR1, while none express CCR3 or CCR5 (*9*). Moreover, modulating the receptor activator of nuclear factor-κB ligand (RANKL)/RANK mediated interaction and osteoclast/osteoblast function in a mouse model (*10*), and osteopenia occur within CCR1-deficienct mice where they have fewer and thinner trabecular bones.

The regulatory mechanisms of CCL5 are unknown in osteogenesis of human MSCs. Dexamethasone, a glucocorticoid hormone, is well known for its function to modulate the osteoblast differentiation of human MSCs and augmenting alkaline phosphatase (ALP) activity. Low dosage of dexamethasone functions induced osteogenesis as inducer of osteogenesis, but high dosage of dexamethasone suppress osteogenesis. The relation between dexamethasone and CCL5 in the progress from hMSCs into osteo-committed cells has not been investigated.

In this study, we demonstrated that CCL5 was essential and sufficient for osteogenesis and also found the vital roles of CCR1 in the osteogenesis of human MSCs. Interestingly, we also found that CCL5 expression levels increased upon osteogenesis and were regulated by dexamethasone.

2. Materials and Methods

2.1. *Cell culture*

Human primary bone marrow (hMSCs) were obtained from Lonza (Basel, Switzerland) and cultured in MesenPRO RS media (Invitrogen, Carlsbad, CA, USA). For osteoblast differentiation, hMSCs were treated with low glucose DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), 0.1 μM dexamethasone (Sigma, St. Louis, MO, USA), 10 mM β-glycerophosphate (Sigma), and 0.05 mM L-ascorbic acid phosphate (Sigma). The differentiation media were replaced twice weekly during the process of osteogenesis. Recombinant CCL5 (Peprotech, Rocky Hill, NJ, USA) was given to promote hMSC differentiation as indicated.

2.2. *Lentivirus production and infection*

Lentivirus production was performed as described previously (*11*). The shRNA (small hairpin RNA) lentiviruses against CCL5 (shCCL5-1: TRCN0000058005, 5'- GTATTTCTACACCAGTGGCAA -3'; shCCL5-2: TRCN0000371627, 5'-CCTGCTGCTTTGCCTACATTG -3') and CCR1 (shCCR1-1: TRCN0000008184, 5'-CCCTGGTAGAAAGAAGATGAA -3'; shCCR1-2: TRCN0000273656, 5'-ATTCTGCTAAGACGACCAAAT-3') were purchased from the National RNAi Core Facility (Taipei, Taiwan). Cells were infected with the shRNA lentiviruses of CCL5, CCR1, red fluorescence protein (RFP) in the presence of 8 μg/mL protamine sulfate, and the media were changed to differentiation media at 24 h post-infection.

2.3. *Alamar Blue assays and alkaline phosphatase activity assays*

For measuring the relative cell number, cells were cultured in the presence of 10% Alamar Blue reagent (Bio-Rad, Oxford, UK) for 1 h and the absorbance was measured at wavelength of 570 nm/600 nm. The relative cell numbers were calculated. Alkaline phosphatase activity assays were performed as previously described (*11*). The absorbance was measured at OD 405 nm, and the fold change of ALP activity was normalized to corresponding cell numbers measured in the Alamar Blue assays.

2.4. *Quantitative real-time reverse transcription PCR (qRT-PCR)*

Total RNA was isolated by RNeasy Micro kit according to the manufacturer's instructions (Qiagen, Dusseldorf, Germany). Then RNA was treated with DNase I (Promega, Fitchburg, WI, USA), and used Superscript III (Invitrogen) to generate complementary DNA (cDNA). The amount of cDNA for each gene was analyzed by quantitative real-time reverse transcription PCR (qRT-PCR; ABI7900, Applied Biosystems, Carlsbad, CA, USA) with gene specific primers and SYBR GREEN 2x master mix (KAPA Biosystems, Wilmington, MA, USA). All results were normalized against the mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (GADPH). The sequences of qRT-PCR primers used were as follows: CCL5 forward, 5'-CGC TGT CAT CCT CAT TGC TA-3'; reverse, 5'-GAG CAC TTG CCA CTG GTG TA-3'; ALP forward, 5'-TGG AGC TTC AGA AGC TCA

ACA CCA-3'; reverse, 5'-ATC TCG TTG TCT GAG TAC CAG TCC-3'; BSP forward, 5'-GAG AAT ACC ACA CTT TCT GCT AC-3'; reverse, 5'-AAG TAG CTG TAC TCA TCT TCA TAG G-3'; OPN forward, 5'-GCC AAA ATA GAG CTG CCT TG-3'; reverse, 5'-GTC ATG GCT TTC GTT GGA CT-3'; DLX5 forward, 5'-GAG AAG GTT TCA GAA GAC TCA GTA-3'; reverse, 5'-CTA GAA CAG CAA AAC ACA GTA GTC-3'; GADPH forward, 5'-CAT CAC CAT CTT CCA GGA GC-3'; reverse, 5'-ATG CCA GTG AGC TTC CCG TTC-3'.

2.5. *Western blot*

Western blot was performed as previously described (*11*). Specific antibodies against Runx2 (Santa Cruz biotechnology, CA, USA), CCL5 (Santa Cruz biotechnology), β-actin (Sigma) were used. Densitometry was performed by Image J software, and the expressed of various proteins were all normalized against the loading level of actin.

2.6. *Statistical analyses*

All statistical data are presented as the mean ± standard deviation (S.D.) of at least three biological replicates. Statistically significant differences were assessed by Student's unpaired two-tailed *t*-test. *p*-values < 0.05 were considered to represent significant differences.

3. Results and Discussion

3.1. *CCL5 plays an essential role in the osteogenic differentiation of human MSCs*

CCL5 is a chemokine that is well known for playing roles in inflammation, cancer progression, and wound healing. The interplay between inflammation and bone remodeling has drawn a lot of attention recently. It has been established that some chemokines and chemokine receptors are involved in cell fate determination or affect the cell numbers of osteoblasts or osteoclasts. Since whether CCL5 expression will be increased upon osteogenic differentiation has never been explored, we analyzed the CCL5 expression levels at different time points upon osteogenic differentiation. In these assays, human primary bone marrow MSCs were incubated with osteogenic differentiation medium consisting of dexamethasone, L-ascorbic acid, and β-glycerophosphate, and the samples were collected at different time points. As shown in Figure 1A, the expression of CCL5 was increased gradually during the process of osteogenic differentiation and the induction folds at day 7 and 10 were 1.7- and 5-fold, respectively (Figure 1A). In addition, the protein expression of CCL5 expression was also increased (Figure 1B). Because of the expression levels of CCL5 increased significantly upon osteogenic induction, we performed

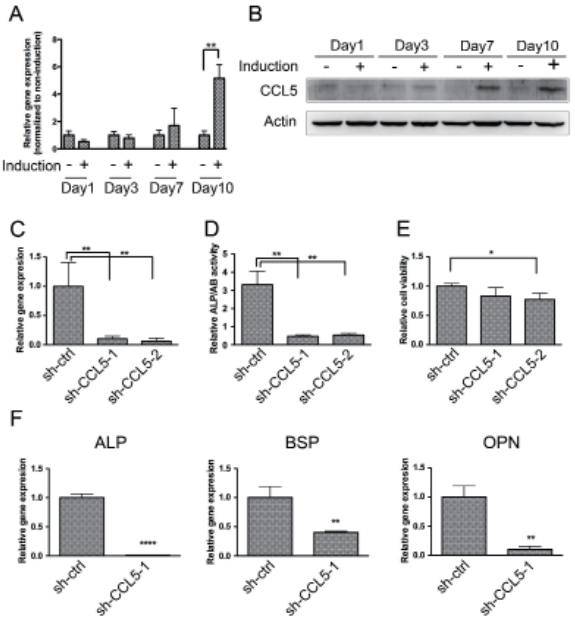

Figure 1. CCL5 plays an essential role in osteogenesis. (A) Expression of CCL5 increased upon osteogenic differentiation of human mesenchymal stem cells in a time dependent manner. hMSCs were cultured in osteogenic induction medium for 1, 3, 7 and 10 days. CCL5 mRNA levels were increased around day 7 and 10 after osteo-induction. CCL5 mRNA gene expression in cells were examined by quantitative-reverse transcription PCR (qRT-PCR) and the relative expression levels were normalized against the expression levels of glyceraldehyde 3-phosphate dehydrogenase (GADPH) and hMSCs without induction. **(B)** The protein expression of CCL5 was increased during osteogenesis. Cell lysates were collected from different time-point of hMSCs during osteogenesis and the protein expression amounts were determined by Western blot analysis. Actin was loading control. **(C)** shRNAs of CCL5 downregulated the expression of CCL5. hMSCs were infected with lentiviruses of two independent shRNAs targeting CCL5 (shCCL5-1 or shCCL5-2), or control shRNA targeting RFP (sh-ctrl), respectively. The multiplicity of infection was 10. Various hMSCs were independently induced into osteogenic differentiation for 7 days. The CCL5 mRNA expression was examined by quantitative-reverse transcription PCR (qRT-PCR) and the relative expression level was normalized against the expression levels of glyceraldehyde 3-phosphate dehydrogenase (GADPH) and the cells infected with sh-ctrl. **(D)** The inhibition of CCL5 expression hampered the ALP activity upon osteo-induction. ALP activities were measured and normalized against the relative cell number after cells were infected with shRNAs against CCL5 in the osteogenesis (ALP/AB ratio). **(E)** The relative cell number was not changed upon CCL5 knockdown during the process of osteogenesis. The relative cell number upon CCL5 knockdown during the process of osteogenesis was measured by Alamar blue assay. **(F)** The blockage of CCL5 expression downregulated the mRNA expression levels of osteogenic markers ALP, BSP, and OPN. The RNA expression of osteogenic markers ALP, BSP, and OPN were measured by qRT-PCR. Biological triplicates were performed in all experiments. Results are shown as the mean ± S.D. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.*p*-values were the results of applying the Student's *t*-test.

loss-of-function experiments using a shRNA lentivirus technology to evaluate the functional roles of CCL5. The knockdown efficiencies of CCL5 shRNAs in hMSCs were examined. Human MSCs were infected with two independent shRNAs, sh-CCL5-1 and sh-CCL5-2, the expression levels of CCL5 were reduced by ~90% compared with control cells (Figure 1C). The

activity of osteogenic marker, alkaline phosphatase (ALP) during the osteogenic differentiation of MSCs was dramatically decreased within CCL5-knockdown hMSCs (Figure 1D) without concomitant changes in cell number (Figure 1E). In addition, knockdown of CCL5 also repressed the expression levels of three osteoblastic markers, such as ALP, bone sialoprotein (BSP), and osteopontin (OPN) (Figure 1F). These observations indicate that CCL5 is required for the osteoblast differentiation from human MSCs into osteoblastic-committed cells.

3.2. *CCL5 promotes the osteogenesis of human MSCs*

Although we have discovered the essential role of CCL5 in the osteogenesis, the effect of exogenous CCL5 in the osteogenesis of any MSC has not been reported before. We stimulated hMSCs with human recombinant CCL5 during differentiation to investigate whether exogenous CCL5 can further promote the osteogenesis of MSCs. ALP activity was increased in CCL5 supplemented osteogenic MSCs on 7 days compared to solvent control in a dose-dependent manner (Figure 2A). This outcome does not due to any alteration of cell number of hMSCs since the cell number was not affected by CCL5 treatment (Figure 2B). Consistently, the expression levels of three osteogenic markers, ALP, distal-less homeobox 5 (DLX5), and BSP were up-regulated by CCL5 during the osteogenesis of hMSCs (Figure 2C). Additionally, the protein expression of Runt-related transcription factor 2 (RUNX2) was significantly increased in CCL5-treated osteogenic differentiation of hMSCs (Figure 2D). Our experiments provide the first evidence that CCL5 is sufficient to trigger the osteogenesis of MSCs. Taken together, these data indicate besides the known functions of CCL5 in cancer, cardiovascular disease and inflammation, we found CCL5 is essential and sufficient to promote the progression of osteogenesis of human primary MSCs.

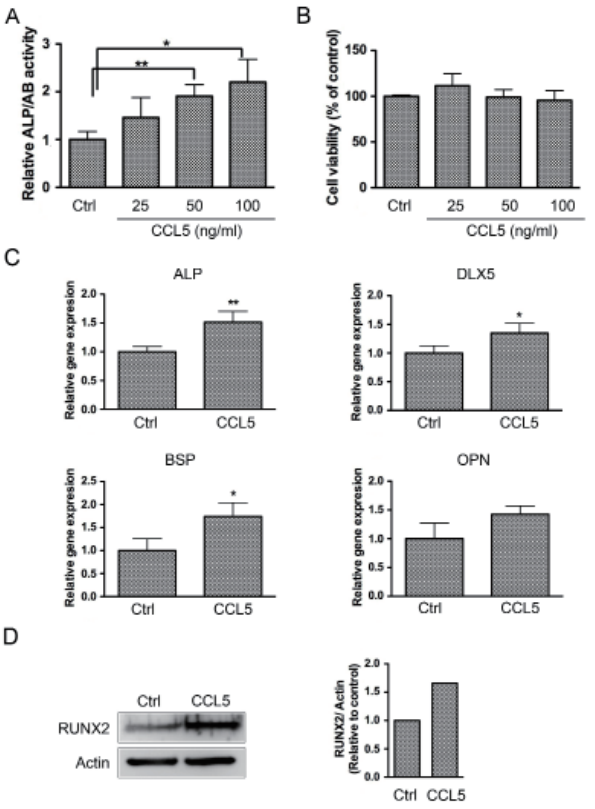

Figure 2. CCL5 promotes osteogenesis in human MSCs. hMSCs were treated with solvent control (ctrl) or different doses of CCL5 upon osteo-induction for 7 days. **(A)** ALP activity increased upon CCL5 treatment. ALP activities were measured and normalized against the relative cell number (ALP/AB ratio) after cells were treated with CCL5 in the osteogenic induction medium. **(B)** The relative cell number was not affected by CCL5 treatment during the process of osteogenesis. Alamar blue activity was measured. **(C)** The expression profile of osteogenic-related markers were measured in hMSCs treated by CCL5 during osteogenesis. Cells were incubated in the osteogenic differentiation medium with CCL5 (100 ng/mL) and RNA was collected. Relative expression levels of mRNA were determined by qRT-PCR, which were normalized against the expression levels of GAPDH and solvent control (ctrl). **(D)** The protein level of Runx2 was increased in MSCs treated by CCL5 during osteogenesis. (Left panel) hMSCs were treated with CCL5 and the RUNX2 expression amounts were determined by Western blot analysis. Actin was loading control. (Right panel) The induction fold of RUNX2 protein normalized with actin. Biological triplicates were performed in all experiments. Results are shown as the mean ± S.D. *, $p < 0.05$; **, $p < 0.01$; NS, non significant. *p*-values were the results of applying the Student's *t*-test.

3.3. *CCL5 receptor CCR1 is critical for the osteogenic differentiation of hMSCs*

CCR1, one of the CCL5 receptors, was the only receptor abundantly expressed in human MSCs (*9*). Since CCL5 plays an important role in osteogenesis (Figures 1 and 2), then we further examine whether CCR1 was required for osteogenesis of hMSCs. CCR1 was knocked down in hMSCs by the infection with shRNA lentiviruses. We found that ALP activity decreased significantly in CCR1 knockdown hMSCs (Figure 3A). Of note, the cell number was also dramatically reduced when hMSCs were infected with shRNAs against CCR1 (Figure 3B). These data suggest that CCR1 is both critical for the osteogenesis of hMSCs and maintain the cell numbers from hMSCs differentiate into osteo-committed cells. In contrast, CCL5 knockdown did not reduce the cell numbers (Figure 2). To explain why CCR1 but not CCL5 knockdown affects the cell numbers during the process of osteogenesis, one reason might be because CCR1 signaling can be activated by several different chemokines in addition to CCL5, such as chemokine (C-C motif) ligand 4 (CCL4), chemokine (C-C motif) ligand 6 (CCL6), chemokine (C-C motif) ligand 14 (CCL14), chemokine (C-C motif) ligand 15 (CCL15), chemokine (C-C motif) ligand 16 (CCL16) and chemokine (C-C motif) ligand 23 (CCL23) (12-15). There might have other chemokines in addition to CCL5 secreted by hMSCs upon osteogenic induction or present in the

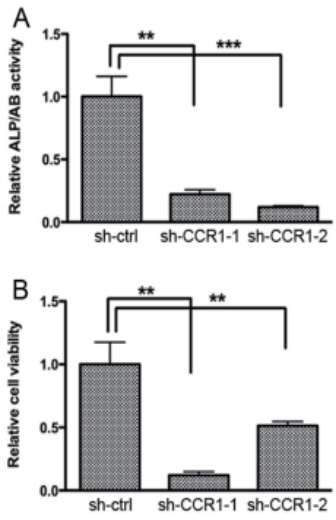

Figure 3. The knockdown of CCR1 expression inhibited osteogenic differentiation of hMSCs and decreased the relative cell numbers. hMSCs were infected with lentiviruses of two independent shRNAs targeting CCR1 (shCCR1-1 or shCCR1-2) or control shRNA targeting RFP (sh-ctrl) respectively. hMSCs were induced into osteogenic differentiation for 7 days. The multiplicity of infection was 10. **(A)** The downregulation of CCR1 blocked osteogenesis as revealed by the downregulation of ALP activity. ALP activities were measured and normalized against the relative cell number (ALP/AB ratio). **(B)** The knockdown of CCR1 downregulated the relative cell numbers during the process of osteogenesis. Alamar blue activity assays were performed to measure relative cell numbers. Biological triplicates were performed in all experiments. Results are shown as the mean ± S.D. **, $p < 0.01$; ***, $p < 0.001$. *p*-values were the results of applying the Student's *t*-test.

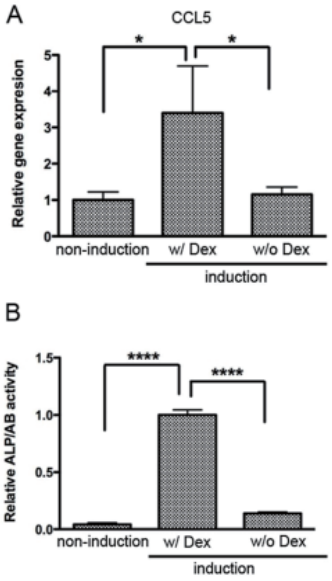

Figure 4. The expression of CCL5 was regulated by dexamethasone. (A) The induction of CCL5 expression upon osteogenic differentiation was dependent upon the presence of dexamethasone (Dex). hMSCs were cultured in expanded or osteogenic induction medium which contained with dexamethasone (w/Dex) or without dexamethasone (w/o Dex) for 7 days. The mRNA level of CCL5 was assessed by quantitative-reverse transcription PCR (qRT-PCR). The relative expression levels of CCL5 were normalized against the expression levels of glyceraldehyde 3-phosphate dehydrogenase (GADPH) and hMSC without induction. **(B)** ALP activity decreased in the osteo-induction medium without dexamethasone. ALP activities were measured and normalized against the relative cell number (ALP/AB ratio) after cells were incubated in osteo-induction medium with or without dexamethasone. Biological triplicates were performed in all experiments. Results are shown as the mean ± SD.Results are shown as the mean ± S.D. *, $p < 0.05$; ****, $p < 0.0001$. *p*-values were the results of applying the Student's *t*-test.

fetal calf serum that play a role in maintaining cell numbers during the process of osteogenesis. Thus only knockdown of CCR1 but not CCL5 is sufficient to affect the cell numbers during the process of osteogenesis.

3.4. *The expression of CCL5 is regulated by dexamethasone*

The mechanism of regulation of CCL5 expression in osteogenesis is also unknown. Dexamethasone is a glucocorticoid hormone that is well known for its role to induce osteoblastic differentiation in human MSCs when applied it at low dose (*16*). To identify an upstream regulator of CCL5, next, we checked whether dexamethasone would affect the expression of CCL5 in hMSCs during osteogenesis. We examined the expression levels of CCL5 in the osteogenic differentiation medium with or without dexamethasone. We found that the induction of CCL5 was blocked in the absence of dexamethasone (Figure 4A). Without dexamethasone, human MSCs could not differentiate into osteo-lineage cells, which is revealed by the decrease of ALP activity (Figure 4B). This observation suggests that dexamethasone is one of the important upstream regulators of CCL5 during osteogenesis. However, our finding is the opposite of previous reports that showed dexamethasone inhibits CCL5 expression in mouse calvarial osteoblasts (*7*). One possible explanation is that there is a difference between mouse and human cells in osteoblast induction. The other hypothesis is these experimental observations were simply made at different differentiation stages, that CCL5 was only induced by dexamethasone early when hMSC differentiate into pre-osteoblast, but this induction does not occur during the late stages of pre-osteoblast differentiate into osteoblast. Although the role of dexamethasone is well-known for promoting osteogenesis in human MSCs (*16*); the effect of dexamethasone in osteogenesis is controversial *in vivo*. Dexamethasone might promote the osteoporosis (*17*), but also shown beneficial effects in bone healing (*18*). Moreover, dexamethasone was reported induce Runx2 expression by FHL2/β-catenin-mediated transcriptional activation and upregulation of TAZ and MKP1(*19*). In this paper, we found that Runx2 was increased by CCL5 treatment. Therefore, dexamethasone may activate the CCL5/CCR1 pathway signal and mediate Runx2 signal (*19*).

In summary, these results demonstrate that CCL5 and its receptor CCR1 are essential for osteogenesis of hMSCs, and that CCL5 expression is regulated

by dexamethasone. Moreover, CCL5 can promote osteogenesis as revealed by increasing ALP activity and elevating ALP, DLX5, and BSP gene expression. CCL5 has been shown to associate with STAT3 in an autocrine-loop in breast cancer cells (*20*), and STAT3 was also shown to be involved in osteogenesis (*21*). Therefore, our future studies will examine whether there is an association between STAT3 and CCL5 that is crucial for osteogenesis of hMSCs.

Acknowledgements

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