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RANKL expression in myeloma cells is regulated by a network involving RANKL promoter methylation, DNMT1, microRNA and TNFα in the microenvironment

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ABSTRACT

We studied the regulation of *RANKL* expression in myeloma by promoter DNA methylation. Methylation-specific polymerase chain reaction showed complete methylation of *RANKL* promoter in WL-2 myeloma cells but partial methylation in eight other lines. 5-AzadC treatment of WL-2 cells led to demethylation and re-expression of *RANKL*. Transwell and contact co-culture of WL-2 cells with normal bone marrow-derived mesenchymal stromal cells (BMSCs) resulted in comparable repression of DNA methyltransferase-1 (*DNMT1*) and re-expression of *RANKL* in WL-2 cells. Moreover, treatment of WL-2 cells with TNFα led to repression of *DNMT1* and re-expression of *RANKL* in association with upregulation of miR-140-3p and miR-126, which are partially offset by addition of anti-TNFα antibody to transwell-coculture of WL2 with BMSC. Taken together, our results showed that TNFα in the marrow microenvironment led to *RANKL* demethylation and re-expression in myeloma cells through *DNMT1* repression and upregulation of miR-126-3p and miR-140, both known to repress *DNMT1* translation.

1. Introduction

Multiple myeloma (MM) is characterized by clonal proliferation of malignant plasma cells in the bone marrow, associated with osteolytic bone lesions. Previous studies showed that the receptor for activation of the nuclear factor-κB ligand (*RANKL*) is essential for inducing osteoclast differentiation and activity [1]. Moreover, clinical studies have shown that *RANKL* level was significantly increased in the serum and bone marrow (BM) plasma of MM patients [2,3]. Early studies suggested that *RANKL* is principally expressed by bone marrow mesenchymal stromal cells (BMSCs) and osteoblasts. However, recent studies indicated that *RANKL* could be secreted by myeloma cells, and *RANKL* secreted by myeloma cells could induce osteoclast formation, similar to *RANKL* derived from BMSCs or osteoblasts [4,5]. Furthermore, *RANKL* expression level in myeloma cells is significantly correlated with the extent of osteolytic myeloma bone disease [3,6,7]. Therefore, *RANKL* in the BM microenvironment plays a pivotal role in osteolytic myeloma bone disease.

In MM, the BM microenvironment plays an essential role in myeloma plasma cell proliferation, migration, survival and drug resistance [8]. Moreover, it has been suggested that the BM microenvironment stimulated *RANKL* expression from BMSCs when co-cultured with myeloma cells [9,10]. However, whether the microenvironment is involved in the expression of *RANKL* in myeloma cells remains unclear.

Promoter DNA methylation is characterized by the addition of a methyl group to the carbon 5 position of cytosine ring in promoter-associated CpG islands, which is associated with gene silencing [11]. However, DNA methylation is dynamic and reversible. Moreover, recent studies indicated that DNA methylation is involved in the regulation of *RANKL* expression [12]. Herein, we study whether the microenvironment may influence the *RANKL* expression in MM cells, and thereafter the mechanisms involved.

2. Material and methods

2.1. Cell culture

2.1.1. Human BMSCs

Bone marrow mononuclear cells from healthy bone marrow transplantation donors were obtained with written informed consent under approval of Institutional Review Board (The Hong Kong University and Hong Kong West Cluster Hospitals). Normal BMSCs were

Abbreviations: MM, Multiple myeloma; RANKL, receptor for activation of the nuclear factor-κB ligand; BM, bone marrow; BMSCs, bone marrow mesenchymal stromal cells

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harvested using standard Ficoll-Hypaque density sedimentation methods then expanded and characterized in vitro according to our previous published method [10].

2.1.2. MM cell lines

Culture conditions of WL-2 and 8 other myeloma cell lines are described in the supplemental material.

2.1.3. Co-culture of MM and BMSCs

A serial co-culture experiment was used, 2×10^6/well WL-2 cells were placed in a transwell insert (0.4 μm pore size; Coring Costar, Cambridge, MA) to confluent BMSCs (2×10^5/well), or added directly to BMSCs. To separate the MM and BMSCs in contact co-culture, co-cultures were harvested from myeloma cells using a positive immunoselection with anti-CD138 mAb (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) at the end of culture period [13,14].

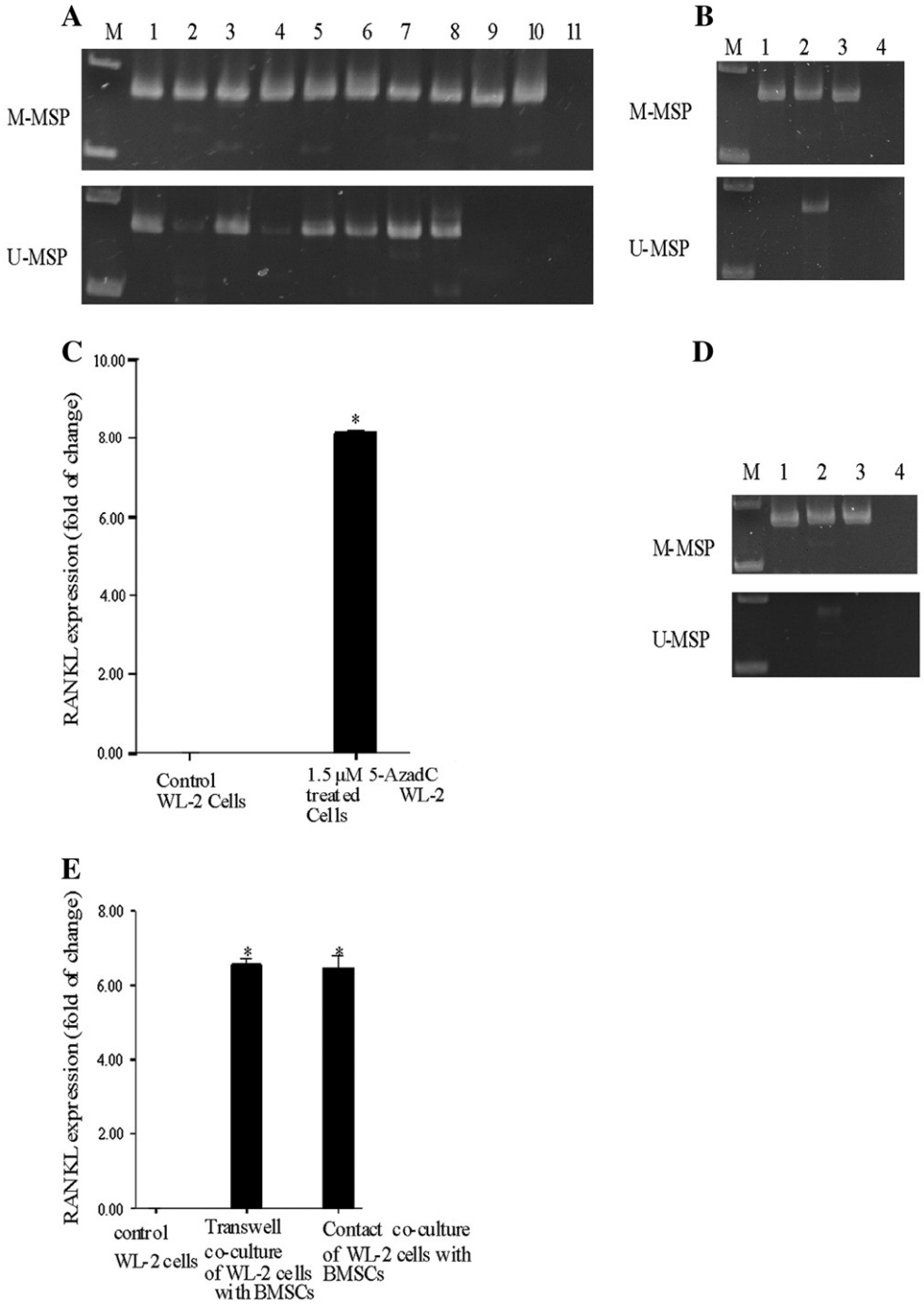

Fig. 1. Promoter methylation and expression of *RANKL*. (A) M-/U-MSP shows that the WL-2 myeloma cells (lane 9) and methylated control [PC] (lane 10) are totally methylated, while JJN-3 (lane 1), KMS-12-PE (lane 2), MOLP-8 (lane 3), NCI-H929 (lane 4), OCL-MY5 (lane 5), OPM-2 (lane 6), RPMI-8226 (lane 7), U266 (lane 8) are partially methylated. Lane 11 is negative (no template) control. (B) M-/U-MSP analysis of *RANKL* promoter methylation status in WL-2 cells (lane 1), and 1.5 μm 5-AzadC treatment resulted in demethylation of *RANKL* promoter in WL-2 cells (lane 2). Methylated control (lane 3) and negative control (lane 4). (C) qRT-PCR analysis of the *RANKL* expression in WL-2 cells. 5-AzadC treatment led to re-expression of *RANKL* expression in WL-2 cells. (D) Transwell co-culture of WL-2 cells and BMSCs resulted in demethylation of *RANKL* promoter in WL-2 cells. M: marker; lane 1: WL-2 cells; lane 2: transwell co-culture of WL-2 cells and BMSCs; lane 3: positive control; lane 4: negative control (reagent blank). (E) *RANKL* expression in WL-2 cells after transwell or contact co-culture with BMSCs.

To determine the influence of TNFα on WL-2 cells, different concentrations of recombinant human TNFα (Sigma, St. Louis, Mo.) were added to the culture medium of WL-2 cells.

To verify the effect of TNFα, WL-2 cells were pretreated for 20 min in the presence of anti-TNFα monoclonal antibody (1 μg/ml, R&D Systems, Minneapolis, MN) or anti-immunoglobulin G (IgG) control, and these antibodies were maintained throughout the transwell co-culture period.

3. Methylation-specific polymerase chain reaction and quantitative bisulfite pyrosequencing

DNA was extracted from JJN-3, KMS-12-PE, MOLP-8, NCI-H929, OCL-MY5, OPM-2, RPMI-8226, U266 and WL-2 myeloma cell lines by standard method. Bisulfite conversion was performed using EpiTect Bisulfite Kit (QIAGEN). Primers and condition of MSP and pyrosequencing are detailed in supplementary material.

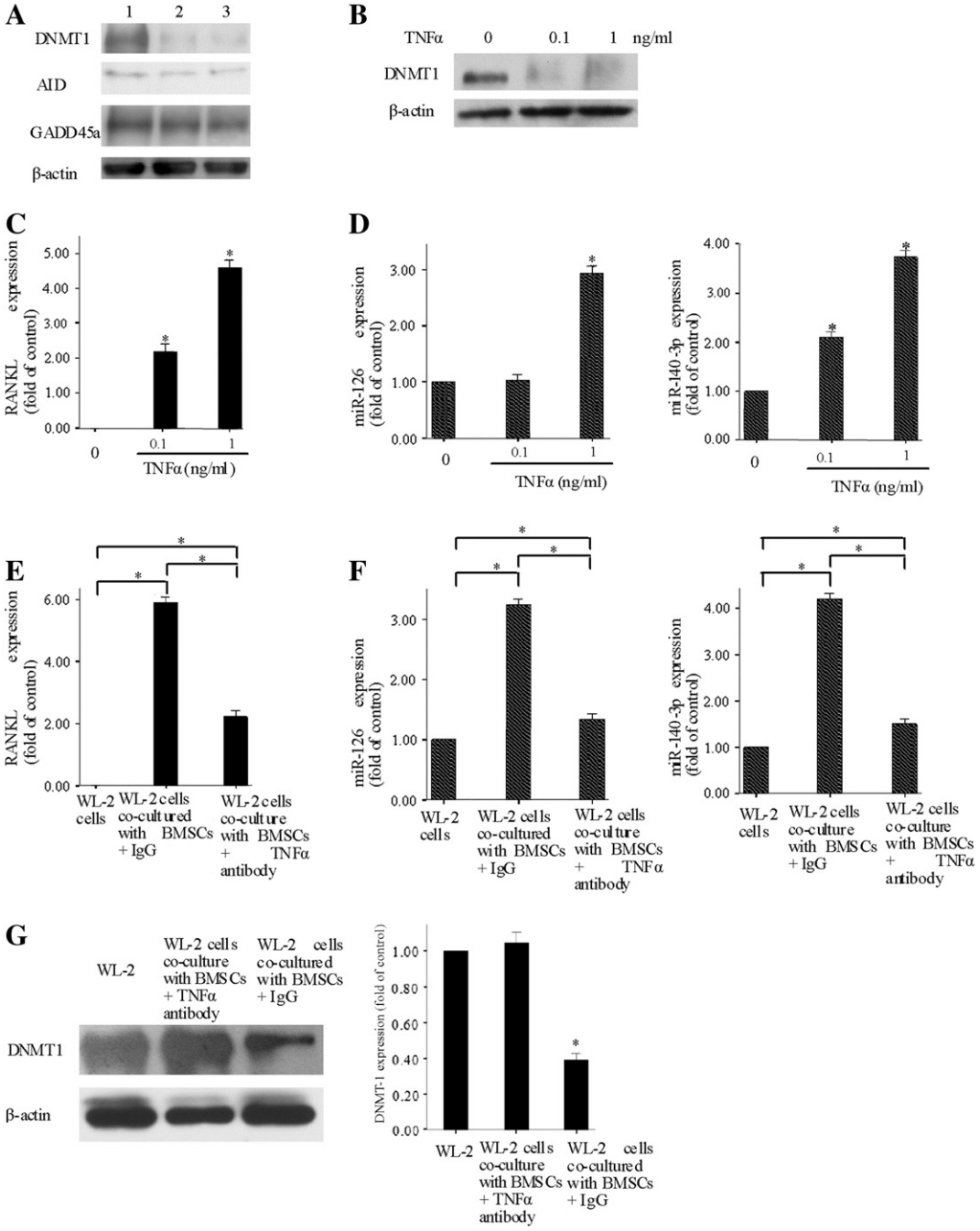

Fig. 2. Mechanism study of *RANKL* promoter demethylation in WL-2 after co-culture with BMSCs. (A) Expression of molecules implicated in DNA demethylation including *DNMT1*, AID and GADD45a in WL-2 cells (lane 1), WL-2 cells transwell co-cultured with BMSCs (lane 2) and WL-2 cells in contact co-culture with BMSCs (lane 3). (B) *DNMT1* expression was determined using Western blot after treatment of WL-2 cells with varying concentrations of recombinant TNFα protein for 6 days. (C) *RANKL* expression in WL-2 cells after treatment with recombinant TNFα protein with indicated concentration was determined by qRT-PCR. (D) miR-126 and miR-140-3p expression in WL-2 cells after treatment with recombinant TNFα protein using stem-loop qRT-PCR analysis. (E) *RANKL* expression in WL-2 cells after transwell co-culture with BMSCs in the presence of anti-TNFα monoclonal antibody or IgG. (F) miR-126 and miR-140-3p expression in WL-2 cells after transwell co-culture with BMSCs in the presence of anti-TNFα monoclonal antibody or IgG. (G) *DNMT1* expression in WL-2 cells after transwell co-culture with BMSCs in the presence of anti-TNFα monoclonal antibody or IgG, representative Western blot result is shown.

3.1. 5-Aza-2′-deoxycytidine (5-AzadC) treatment

WL-2 cells were seeded in six-well plates at a density of 1×10^6 cells/ml and cultured with 1.5 μM of 5-AzadC (Sigma-Aldrich, St. Louis, MO) for 6 days.

3.1.1. Quantitative PCR

RNA was isolated, reverse transcribed for quantitative real-time PCR (qRT-PCR) of *RANKL*, miR126 and miR140-3p. *GAPDH* and *SNORD48* were used as reference for *RANKL* and microRNA using the $2^{-\Delta\Delta Ct}$ method.

3.1.2. Western blot

Western blot was carried out as previously described using anti-*DNMT1*, -GADD45a, -AID and β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) [15].

4. Results and discussion

A previous study showed that methylation of the CpG island located between −260 bp to +615 of the TSS correlated with *RANKL* expression [12], and hence studied herein. MSP showed complete methylation of *RANKL* in WL-2 cells and positive control, and partial methylation in JJN-3, KMS-12-PE, MOLP-8, NCI-H929, OCL-MY5, OPM-2, RPMI-8226 and U266. (Fig. 1A, MSP primer target on −48 to 107 bp). Furthermore, complete methylation of *RANKL* promoter in WL-2 cells was associated with undetectable *RANKL* expression determined by qRT-PCR (Fig. 1C). Incubation of WL-2 cells with 1.5 μM of 5-AzadC resulted in demethylation of *RANKL* promoter as evidenced by emergence of the U-MSP signal (Fig. 1B), which was associated with re-expression of *RANKL* as evidenced by qRT-PCR (Fig. 1C). Therefore, WL-2 cells were utilized to explore the mechanisms of promoter DNA methylation regulating expression of *RANKL* in myeloma.

Using a transwell co-culture system with WL-2 cells and BMSCs, we investigated whether the microenvironment affected *RANKL* promoter methylation and expression in WL-2 cells. After 6 days of transwell co-culture, partial demethylation of *RANKL* promoter occurred (Fig. 1D), accompanied by upregulation of *RANKL* expression (Fig. 1E). To see if contact co-culture might lead to additional upregulation of *RANKL*, WL-2 cells were cultured with BMSCs in a contact co-culture manner, which yielded *RANKL* expression comparable to that of transwell co-culture (Fig. 1E). These results suggested that soluble factors in the microenvironment were primarily involved in *RANKL* promoter demethylation and expression in WL-2 cells, which was not enhanced by cell adhesion through contact co-culture.

DNA demethylation may reverse epigenetic silencing by promoter DNA methylation. Recent studies revealed that demethylation of methylated CpG dinucleotides can be achieved by a passive or active process [16]. Passive DNA demethylation is mediated by inhibition of *DNMT1*, and hence unmethylated alleles are generated during cellular replication. On the other hand, active DNA demethylation of methylated CpG dinucleotides is catalyzed by several enzymes [17] including GADD45a, activation-induced deaminase (AID), ten–eleven translocation (TET) and elongator complex protein 3 (ELP3). To explore the mechanism of demethylation of the *RANKL* promoter in WL-2 cells, we determined the passive and active demethylation related protein expression after the transwell and contact co-culture of WL-2 with BMSCs. Our results showed that levels of GADD45a and AID remained unaltered after transwell or contact co-culture (Fig. 2A). By contrast, *DNMT1* expression was significantly reduced in WL-2 cells after the transwell co-culture with BMSCs for 6 days. However, contact co-culture of WL-2 cells and BMSCs did not yield additional *DNMT1* downregulation compared with transwell co-culture (Fig. 2A). The expression of TET-1 isn't detected in WL-2 cells and BMSCs (data not shown), and since ELP3 has been shown to lead to active demethylation of zygotic paternal genome rather than gene-specific demethylation [18], ELP3 was not examined in the present experiment.

The effect of transwell co-culture of WL-2 cells and BMSCs resulting in downregulation of *DNMT1* in WL-2 cells suggested a paracrine cytokine mechanism. Of the cytokines secreted by myeloma BMSCs, a literature review showed that TNFα has been reported to be involved in DNA demethylation [19]. To verify the role of TNFα in *RANKL* demethylation and re-expression in myeloma cells, WL-2 myeloma cells were treated with recombinant TNFα. A previous study showed that the median concentration of TNFα in the BM plasma of myeloma patients was about 0.35 ng/ml, and the supernatant of myeloma cells co-cultured with BMSCs ranged from 0.118 to 0.152 ng/ml [20]. Therefore, WL-2 cells were cultured with recombinant TNFα protein at concentrations between 0.1 and 1.0 ng/ml, which led to downregulation of *DNMT1* expression (Fig. 2B) and upregulation of *RANKL* expression (Fig. 2C) in WL-2 cells after 6 day treatment.

On the other hand, miR-140-3p and miR-126 [21,22], which are regulated by TNFα, have been shown by luciferase assays to directly target and repress *DNMT1* translation [23,24]. Consistent with these data, we showed that miR-140-3p and miR-126 expression was upregulated by treatment of WL-2 cells with recombinant TNFα (Fig. 2D). As previous studies have shown secretion of TNFα in the supernatant of transwell-coculture of myeloma cells with BMSCs [19], anti-TNFα antibody was added to the transwell co-culture of WL-2 with BMSCs, which partially abrogated the co-culture-induced *RANKL* expression (Fig. 2E) and upregulation of miR-140-3p and miR-126 (Fig. 2F), and offset *DNMT1* downregulation (Fig. 2G) in WL-2 cells. Furthermore, our pyrosequencing results showed the anti-TNFα antibody could attenuate the co-culture induced demethylation in WL-2 cells (Fig. S1). Taken together, the above results testified to the role of TNFα in the induction of demethylation and re-expression of *RANKL* in myeloma cells via upregulation of miR-126 and -140-3p, which may lead to translational inhibition, and hence downregulation, of *DNMT1*.

In conclusion, in the BM microenvironment, *RANKL* expression in myeloma cells is mediated by *RANKL* promoter demethylation induced by downregulation of *DNMT1*, which is associated with upregulation of miR-126 and -140-3p. TNFα plays a pivotal role in this paracrine mechanism.

Authorship contributions

CSC and LQY designed the study and analyzed the data. LQY conducted the experiments and helped in sample collection and clinical data retrieval. GCC and KLF helped in data analyses. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.05.010.

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