Autocrine and paracrine Notch receptor 3 signaling in the myeloma niche stimulates tumor growth and bone destruction

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INTRODUCTION

Multiple myeloma causes an accumulation and growth of malignant plasma cells in the bone marrow, which causes increased bone resorption and reduced bone formation. This leads to the formation of osteolytic lesions, which causes bone destruction in MM patients (1). The localization of MM cells in the bone marrow causes direct contact between tumor cells and non-tumor cells, which leads to increased cell proliferation and survival (2). Communication between MM cells and cells in the bone/BM environment promotes Notch signaling. Notch activation promotes MM cell proliferation by upregulating Notch signaling (1). Previous research suggests that bidirectional Notch signaling, with MM cells and osteocytes, further increases MM cell growth and proliferation, acting specifically through Notch receptor 3. Notch receptor 3 is upregulated in MM and osteocyte interactions, which leads to increased cell proliferation in MM cells (1). In this study, we hypothesize that Notch receptor 3 signaling in multiple myeloma cells mediates the communication with neighboring multiple myeloma cells (autocrine) and with osteocytes (paracrine) and contributes to tumor progression and bone destruction.

AIMS

1. To characterize in vitro the effects of genetic deletion of NR3 in MM cells on proliferation, gene expression, and osteoclastogenic potential.
2. To examine in vitro whether genetic inhibiting of NR3 signaling in MM cells prevents the stimulation of MM cell proliferation induced by direct cell-to-cell contact with osteocytes.
3. To determine in vivo the effects of NR3 knockdown in MM cells on tumor burden and MM-induced bone disease.

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METHODS

Cells. Murine 5TGM1 MM cells were transduced with lentiviral particles containing shRNA-scramble (control) or shRNAs targeting NR3. Three different shRNA-NR3s were analyzed. The cells were selected with puromycin and 5TGM1 cells transduced with shRNA-NR3 clone 1 exhibited a 50-60% decrease in NR3 mRNA expression and was selected for follow-up studies.

Cell Culture. 5TGM1 MM cells and osteocytes were maintained as previously described (1).

Cell proliferation. MM cells were plated on 96 well plates with a density of 60,000 cells per well. Cells were incubated with MTT solution and SDS + HCl solution was added 4 hours later. The plate was incubated overnight for 18h. The absorbance was measured at 570nm on a plate reader for each experiment after 24h, 48h, and 72h.

Osteocyte:MM cell co-cultures. Cell-to-cell co-culture experiments were established as reported before (1).

Gene expression. mRNA gene expression was performed as previously described (1).

Western Blot. Western Blots were performed as previously described (3) using primary antibodies for Gapdh (1:1000), Notch receptor 1, 2, 3 (1:1000), and Notch receptor 4 (1:500), diluted in 4% BSA solution. The goat anti-rabbit secondary antibody (1:5000), conjugated to horseradish peroxidase, was diluted in 5% milk.

Osteoclast formation. Osteoclast formation experiments were established as shown before (4).

Animals. Immunocompetent 6-7 week old C57BL/KaLwRijHsd (RADL) female and male mice with early MM disease were used. The mice were divided in three groups and injected intratibially with saline, 10^5 5TGM1 shRNA-Scramble MM cells, or 10^5 5TGM1 shRNA NR3 MM cells. Tumor burden was analyzed at 5 weeks by ELISA and osteolytic lesions were counted by X-Ray. 3D reconstruction of the injected tibia was performed by MicroCT.

Statistical Analysis. Statistical analysis was performed using SigmaPlot. All the results are presented as mean ± (SD).

CONCLUSIONS

- **Autocrine NR3 signaling between MM cells stimulates their proliferation and increases their osteoclastogenic potential.**
- **Paracrine NR3 signaling mediates the activation of Notch signaling and stimulation of myeloma proliferation induced by osteocytes.**

FIGURE 1: NR3 knock down decreases Notch in MM cells

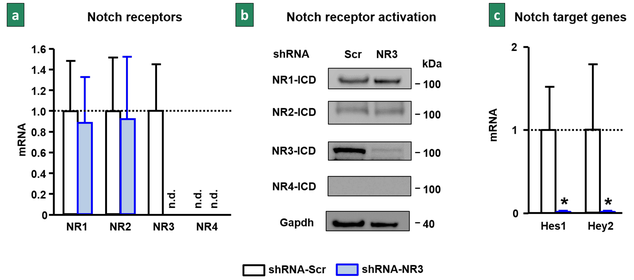

Figure 1: a) mRNA expression analysis shows NR3 was not detected in NR3 knockdown MM cells, while NR1 and NR2 were unchanged and NR4 was undetected. b) Western blot shows an 80% decrease in NR3 activation (NICD) in NR3-knockdown MM cells compared to controls. There were no changes in the activation of NR1, NR2, or NR4. c) Notch target genes Hes 1 and Hey 2 were downregulated in NR3 knockdown MM cells compared to control cells. Representative experiments out of 3 are shown. n=4/group, *p<0.05 vs Scr by t-test, n.d.=not detected.

FIGURE 2: NR3 inhibition decreases the proliferation and osteoclastogenic potential of MM cells

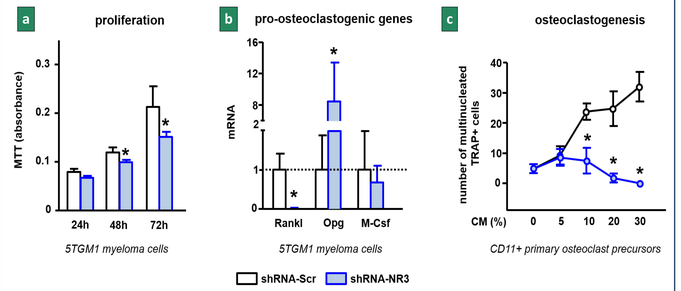

Figure 2: a) MTT assays show a decrease in proliferation in NR3 knockdown cells compared to control cells after 48h and 72h of culture. b) Rankl mRNA expression was 95% decreased and Opg mRNA expression increased 6 fold in NR3 knockdown MM cells compared to control cells. c) Unlike conditioned media (CM) from control cells, CM from NR3 knockdown MM cells did not increase the number of TRAP+ osteoclasts. Representative experiments out of 3 are shown. n=4-6/group, *p<0.05 vs Scr by t-test.

FIGURE 3: NR3 knock down partially prevents the increase in Notch activation & myeloma proliferation induced by osteocytes

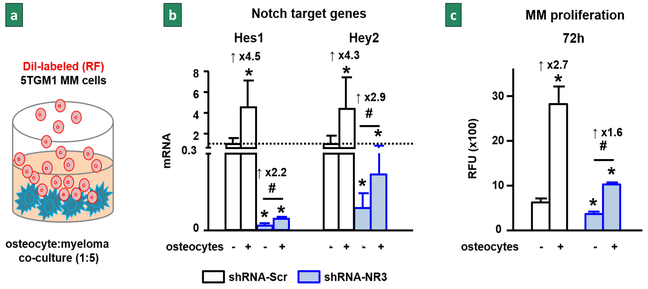

Figure 3: a) Osteocytes and DiI labeled MM cells were co-culture in a 1:5 (OT:MM) fashion. b) The expression on Notch target genes is increased in MM cells by co-culture with osteocytes. Osteocytes also activated Notch signaling in NR3 knockdown MM cells, by these effect was 50% decreased compare to that found in control MM cells. c) Osteocyte ncreased cell proliferation in control and NR3 knockdown MM cells, but the magnitude of this effects was decreased in NR3 knockdown cells.. Representative experiments out of 3 are shown. *p<0.05 vs Scr vehicle by 2-way ANOVA. #p<0.05 vs NR3 vehicle by t-test. n=6/group.

FIGURE 4: Mice injected with NR3 knockdown myeloma cells have less tumor growth and osteolysis

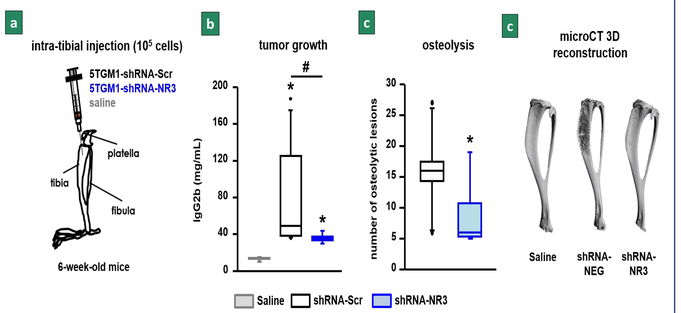

Figure 4: a) Control and NR3 knock down cells were injected in the tiabia of RADL mice. b) Tumor growth decreased in mice injected with NR3 knockdown MM cells compared to control cells. *p<0.05 vs saline by ANOVA on Ranks (Dunn's), # p<0.05 vs Scr by T-test. c) Osteolytic lesion number was lower in mice injected with NR3 knockdown MM cells compared to control cells. *p<0.05 vs Scr by t-test. d) Representative 3D microCT reconstruction images. n=8-12/group.