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## Insulin-like Growth Factor-1 Binding Proteins Alter Osteoclastogenesis

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### Introduction:

Aging is accompanied by a shift in bone marrow (BM) cell fate and greater osteoclastogenesis [1]. The production of IGF-1 and the binding proteins (BP) that comprise the IGF-1 complexes are also affected by aging [2,3]. To determine the auto/paracrine relationship between these constituents and osteoclastogenesis in BM we evaluated the osteoclastogenic potential of marrow-derived mesenchymal stromal cells (MSCs) from mice with IGF-1 deficiency due to knockdown of IGF-1 production by the liver or knockout of the binding proteins (BP) that stabilize IGF-1.

### Methods:

We employed 10-16 week old, male, liver-specific IGF-1 deficient (LID), IGFBP-3 knockout (BP3KO) and acid-labile subunit knockout (ALSKO) mice and assessed BM cell differentiation *in vitro* and BM gene expression *in vivo* (n=5 mice per strain). All mouse strains were backcrossed to a C57BL/6/J background. IACUC approval was obtained.

BM cells were flushed from femurs immediately after sacrifice and collected in  $\alpha$ -MEM culture. Cells were then either cultured or sorted (FACS). For sorting cells were preincubated with rat anti-mouse CD16/CD32 (BD Biosciences) at 4°C to block Fc receptors. Cells were then incubated with fluorescently labeled antibodies: R-Phycoerythrin (PE)-c-Fms/CSF-1R (Santa Cruz Biotechnology) and PE-Cy7-conjugated rat anti-mouse CD45R/B220 (BD Biosciences). The cells were washed and then resuspended in staining buffer (Streptavidin-FITC, BD Biosciences). The cells were incubated for 20 min, washed, and resuspended in PBS containing 1% paraformaldehyde. Cell acquisition was performed in a Becton Dickinson FACScan, and a minimum of 10,000 events was acquired for each test. Data was analyzed with FlowJo software (v7.2).

Osteoblastogenesis was performed with MSCs cultured in  $\alpha$ MEM supplemented with 10% heat-inactivated FBS with  $\beta$ -glycerolphosphate and ascorbic acid. Cellular alkaline phosphatase activity was determined using a para-nitrophenol phosphate based colorimetric assay with staining from a kit (Sigma-Aldrich's).

Osteoclastogenesis was determined by culturing BM cells in FBS-supplemented  $\alpha$ MEM medium. Nonadherent cells were re-plated in  $\alpha$ MEM medium (10% FBS with RANKL and M-CSF) in an incubator for 4 - 7 d. Cells were then fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity, and TRAP+ cells containing  $\geq 3$  nuclei were counted as mature osteoclasts (OC). IGF-1, ALS, or IGFBP-3 were added in separate rescue experiments.

For co-culture experiments, primary osteoblast (OB) cultures were established as above from each mouse strain. Cells were harvested after 14 d and re-seeded in  $\alpha$ MEM. Non-adherent cells from Control mice only were co-cultured with each OB culture for 7 d. Co-cultures were then fixed, stained and TRAP+ cells counted.

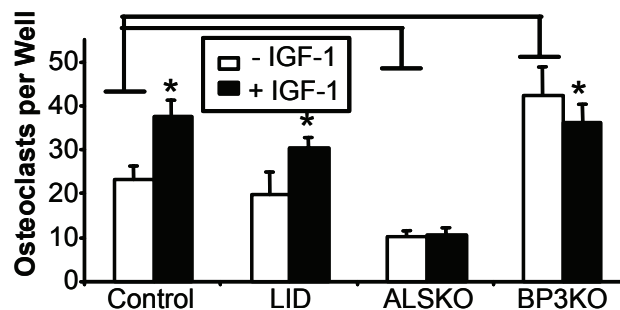
BM cells were isolated and cultured as above for 18 h. Non-adherent cells were re-plated on BD BioCoat,™ Osteologic™ calcium hydroxyapatite (HA)-coated discs (BD Biosciences) in osteoclastogenic conditions for 7 d as above. On day 8, a pit assay was performed by quantifying the resorption areas on each disc after Von Kossa staining.

Differences between groups were evaluated by 2-way ANOVA with Fisher PLSD post-hoc testing. Results are expressed as means  $\pm$  SE.

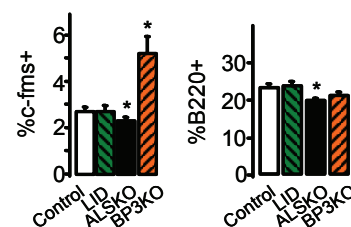
### Results:

ALSKO and BP3KO mouse cells exhibited an increase in alkaline phosphatase activity in culture. Despite this indication of enhanced early osteoblastogenesis we found that ALSKO mice had a significant decrease in the number of OCs formed *in vitro* (Fig. 1) and reduced c-fms+ and B220+ BM cells *in vivo* (Fig. 2). Likewise, in co-culture, OBs from ALSKO mice failed to induce OC formation (Fig. 3). In contrast, BP3KO mice exhibited an increase in the number of OCs in culture (Fig. 1), and an increase in c-fms+ BM cells *in vivo* (Fig. 2). The pit assays for OC formation and function on HA confirmed our results for culture on plastic.

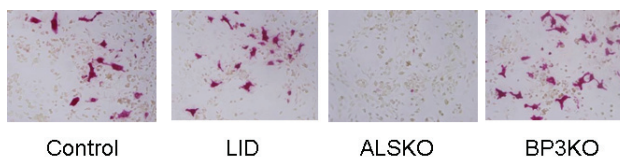
The defect in OC formation for primary ALSKO culture was rescued by reintroduction of ALS protein and not by addition of IGF-1 (Fig. 1).



**Figure 1.** TRAP staining of OC cultures derived from non-adherent BM cells of mutant mice revealed that both ALS and IGFBP-3 gene inactivation affect OC formation. However, attempts at rescue by addition of IGF-1 only affected BP3KO.



**Figure 2.** FACS revealed c-fms, a receptor critical for osteoclastogenesis, to be increased and decreased in BP3KO and ALSKO, respectively. ALSKO mice exhibited a significantly lower fraction of B220+ cells which express RANKL.



**Figure 3.** TRAP stained co-cultures. Only in experiments including OBs derived from ALSKO was there failure to support osteoclastogenesis of non-adherent cells derived from Control mice.

### Discussion:

Our results indicate that impairment of IGF-1 complex formation in BM alters cell fate by modifying osteoclastogenesis. Importantly, the determination of BM cellular populations that express the mouse colony stimulating factor-1 (M-CSF1) receptor (i.e., c-fms), which is a critical factor for osteoclastogenesis, and B220+ cells, pre-pro-B cells, which express membrane-bound and secreted RANKL, suggest an *in vivo* role for both ALS and IGFBP-3.

The inability of IGF-1 to rescue osteoclastogenesis in ALSKO combined with the data indicating that the *als* gene need not be active for OC formation to be rescued with ALS protein suggest a direct role for ALS in determining cell fate in the BM micro-environment.

Our study further reinforces the tenet that there is a strong interaction among hematopoietic and mesenchymal stem cells in BM, and raises the possibility that the IGF-1 complex constituents IGFBP-3 and ALS play a more active role in the assignment of cell fate in BM than previously thought.

### References:

[1] Meunier *et al.*, CORR 1971;80:147. [2] Kasukawa *et al.*, Curr Pharm Des 2004;10:2577. [3] Niu and Rosen, Gene 2005;361:38.

### Acknowledgements:

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