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Introduction

BAG-1 (BCL-2-associated athanogene-1), the founding member of the BAG-family of co-chaperones, is a multifunctional protein which, by its ability to bind multiple partners, regulates gene transcription and molecular signalling crucial for cell proliferation, differentiation and apoptosis. Murine cells express two BAG-1 isoforms, BAG-1L (50 kDa) and BAG-1S (32 kDa), which are generated using alternate translation initiation sites in a single *Bag-1* mRNA transcript. Expression of *Bag-1* mRNA has been identified in several organs, with cartilaginous tissues showing highest expression in the developing mouse embryo¹. Furthermore, in long bones of postnatal mice, expression of BAG-1L and BAG-1S isoforms is detected in both chondrocytes and osteoblast-lineage cells².

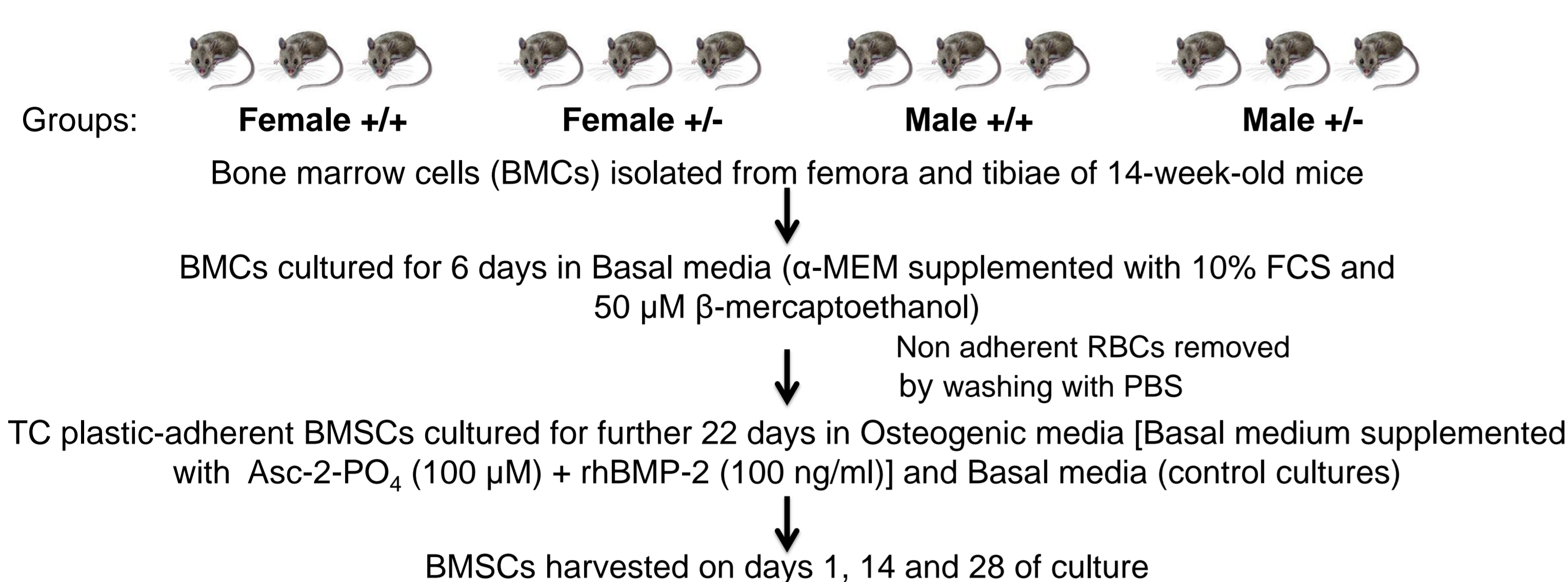
Significant apoptosis in the embryonic liver and brain, along with defective haematopoiesis and neuronal cell differentiation are the major causes of death in *Bag-1*^{-/-} (null) mice between E12.5 and E13.5 of gestation³, i.e. before the crucial stages of endochondral ossification characterised by vascular invasion of the mineralised hypertrophic cartilage matrix and deposition of bone by osteoblasts. Mice heterozygous for *Bag-1* (*Bag-1*^{+/-}) are viable, thereby allowing the analysis of the role of BAG-1 in bone development.

Aim

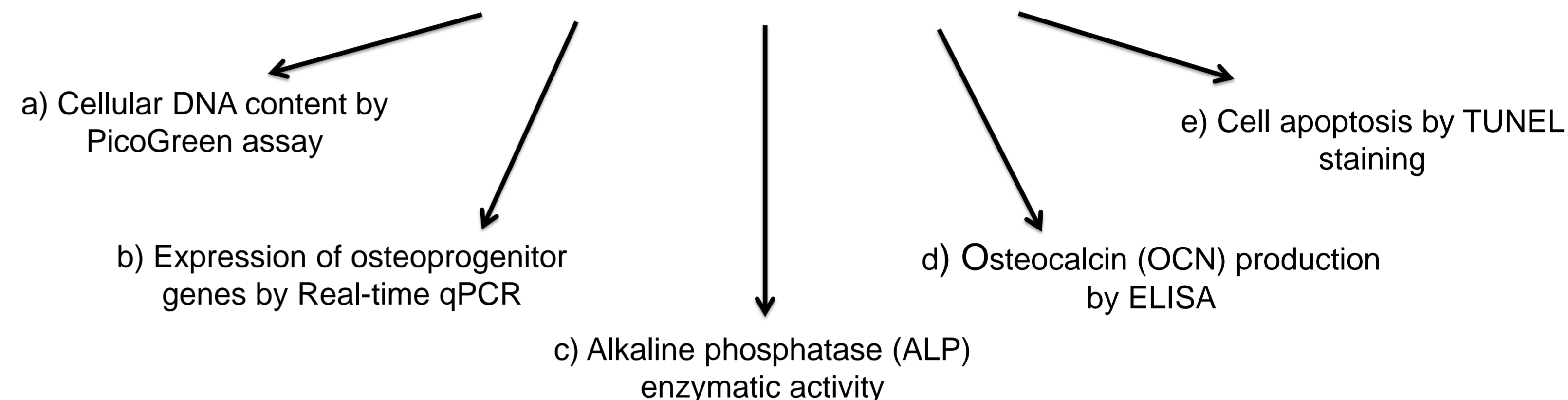
The study aims to elucidate the function of BAG-1 in osteoblast development by examining differences in osteogenic differentiation of bone marrow stromal cells (BMSCs) from *Bag-1* heterozygous (+/-) and wild-type (+/+) mice.

Methods

1. Isolation and culture of murine BMSCs



2. Analysis of BMSC cultures for



Statistical analyses were performed using one way ANOVA with a post Tuckey test and results were considered to be significantly different if $P \leq 0.05$ (probability of occurrence by random chance alone was less than 5%).

Results

1. BMSC cultures of *Bag-1*^{+/-} female mice were characterised by significantly higher cell numbers and increased rate of cell proliferation in osteogenic culture conditions

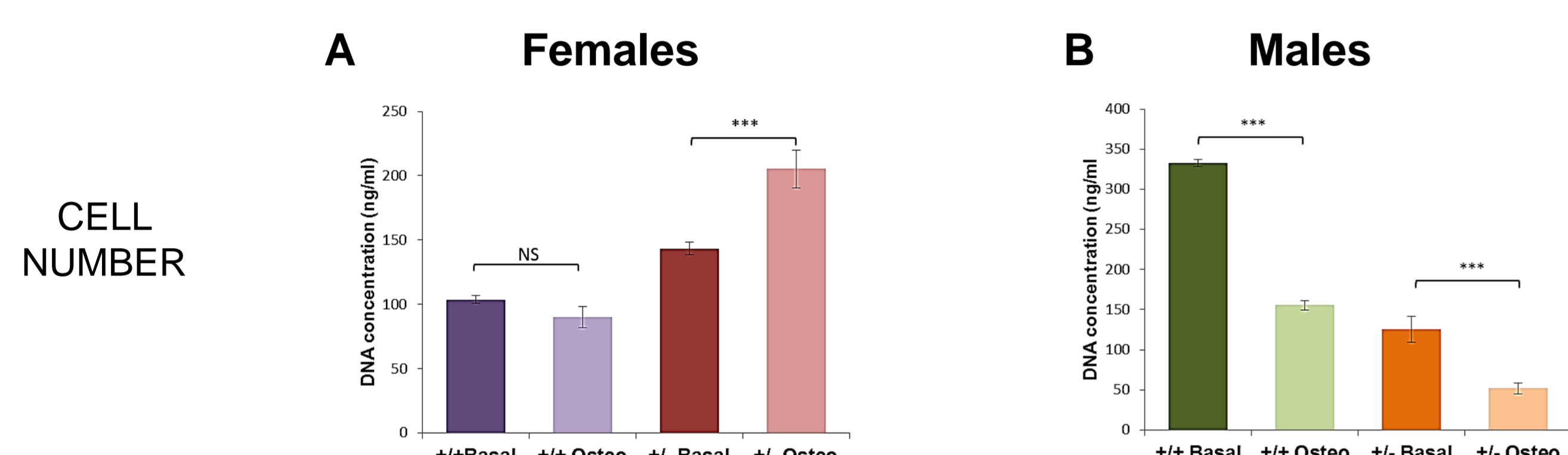


Figure 1. DNA content of day 28 cultures of BMSCs of female (A) and male (B) *Bag-1*^{+/-} and *Bag-1*^{+/+} mice. Results expressed as mean \pm SD, $n = 3$ mice in each group, *** $P < 0.001$.

- Cell numbers (reflected by the DNA content) were significantly higher in day 28 BMSC cultures of female *Bag-1*^{+/-} mice in osteogenic media compared to basal media (Fig. 1A).
- Cell numbers in day 28 osteogenic cultures of BMSCs of male mice were significantly lower compared to control cultures (Fig. 1B).

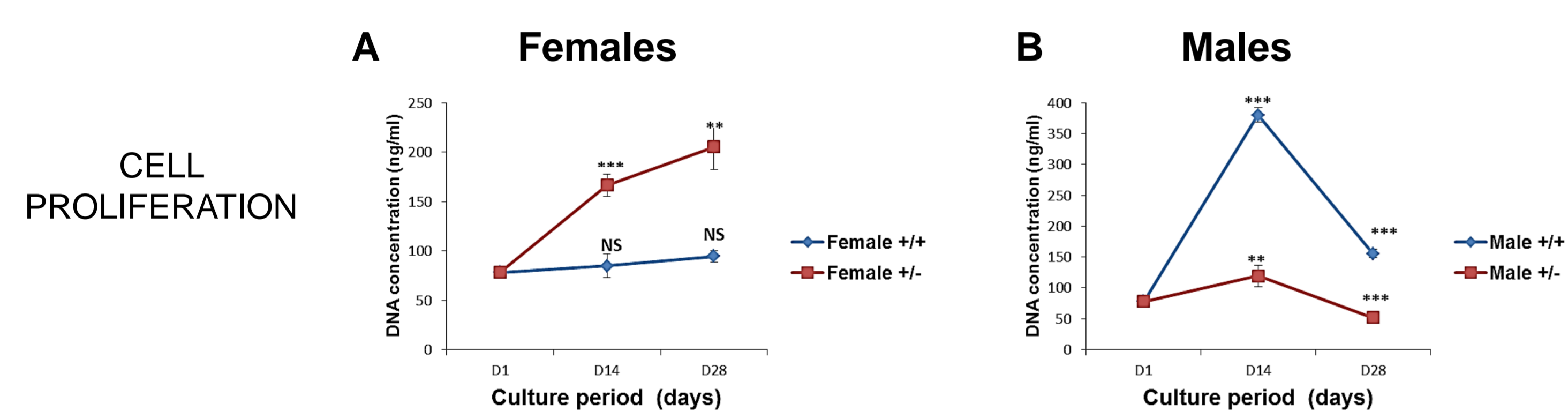


Figure 2. Cell proliferation profiles over the course of 28-day osteogenic cultures of BMSCs of female (A) and male (B) *Bag-1*^{+/-} and *Bag-1*^{+/+} mice. For statistical analyses, results were compared between days 1 and 14 of culture, and days 14 and 28 of culture. Results expressed as mean \pm SD, $n = 3$ mice in each group, *** $P < 0.001$, ** $P < 0.01$.

- In osteogenic media, BMSCs of *Bag-1*^{+/-} female mice continued to proliferate steadily over 28 days (Fig. 2A).
- Under osteogenic conditions, BMSCs of wild type and *Bag-1*^{+/-} male mice proliferated significantly between days 1 and 14 of culture, while cell proliferation decreased significantly between days 14 and 28 of culture (Fig. 2B).

2. BMSC cultures of *Bag-1*^{+/-} female mice exhibited reduced osteogenic differentiation potential in response to BMP-2

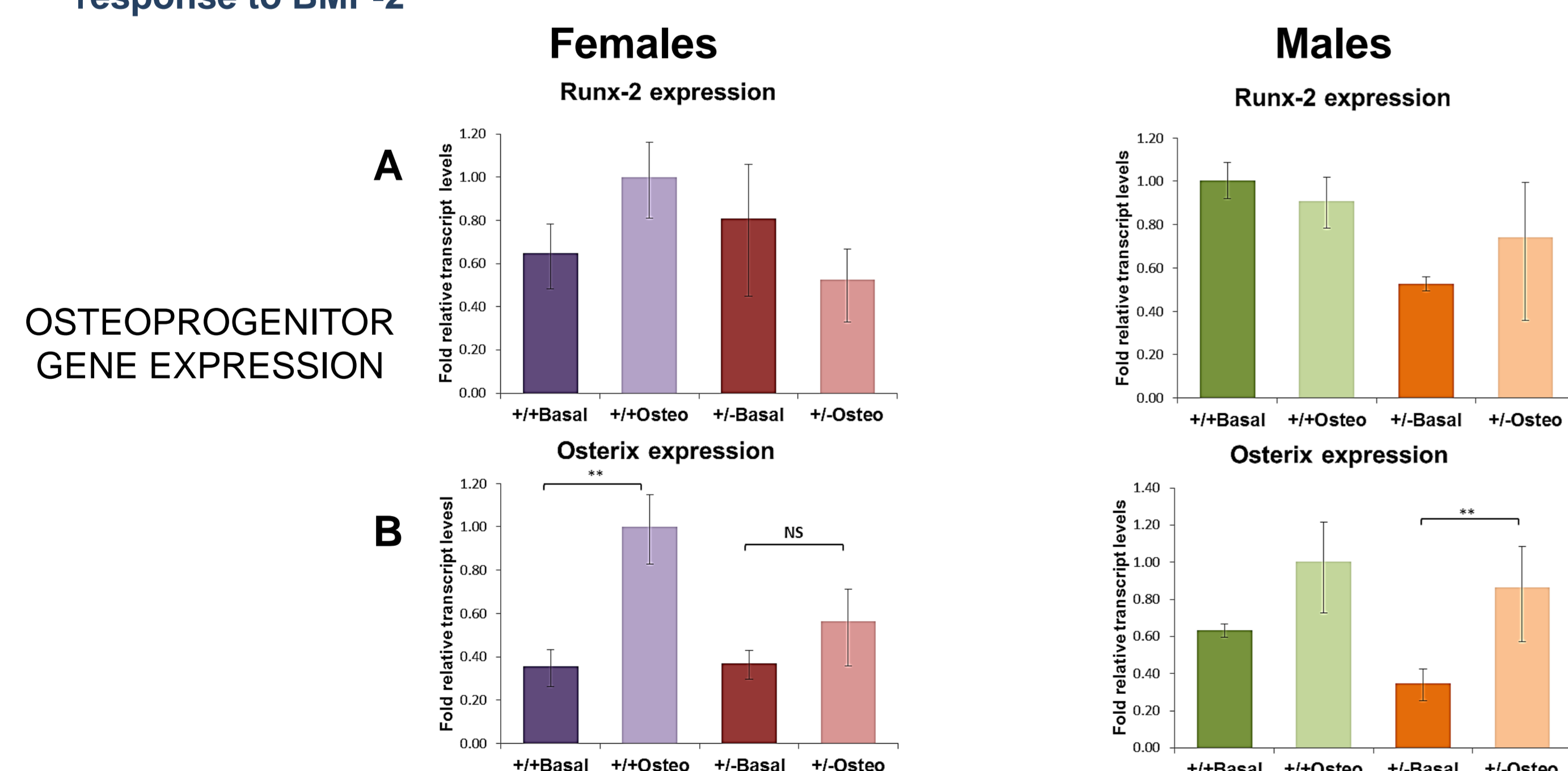


Figure 3. Analysis of expression of osteoprogenitor genes, *Runx-2* and *Osterix*, in day 28 cultures of BMSCs by Real-time qPCR. Bar graphs illustrate fold changes in relative mRNA transcript levels normalised to the endogenous reference (β Actin). The range of gene expression is indicated by $2^{-(\Delta\Delta C_T + SD)}$ and $2^{-(\Delta\Delta C_T - SD)}$, where SD is the standard deviation of the $\Delta\Delta C_T$ value, $n = 3$ mice per group, ** $P < 0.01$.

- No statistically significant differences in *Runx-2* expression were observed between day 28 cultures of BMSCs in basal and osteogenic media (Fig. 3A).
- Expression of *Osterix* was upregulated in day 28 osteogenic cultures of BMSCs compared to control cultures; the differences in *Osterix* expression between basal and osteogenic culture conditions, however, were statistically significant in wild-type female and *Bag-1*^{+/-} male mice (Fig. 3B).

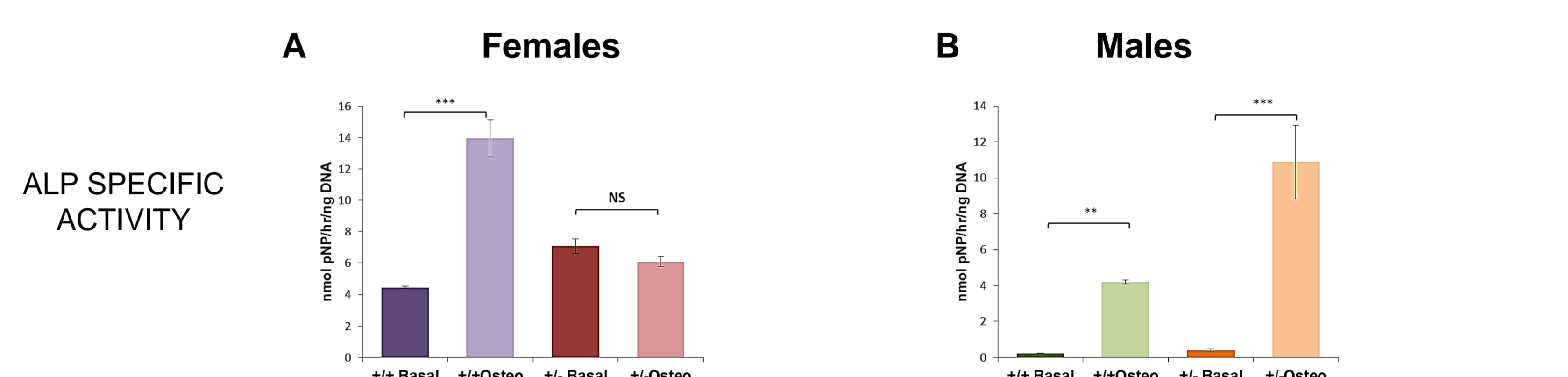


Figure 4. Intracellular ALP enzyme activity (early osteoblast marker) was measured in day 28 cultures of BMSCs. Enzyme activity was normalised to the DNA content of the culture and ALP specific activity was calculated as nmol of pNP (*p*-nitrophenol) released per hour per ng DNA. Results were expressed as mean \pm SD, $n = 3$ mice in each group, *** $P < 0.001$, ** $P < 0.01$.

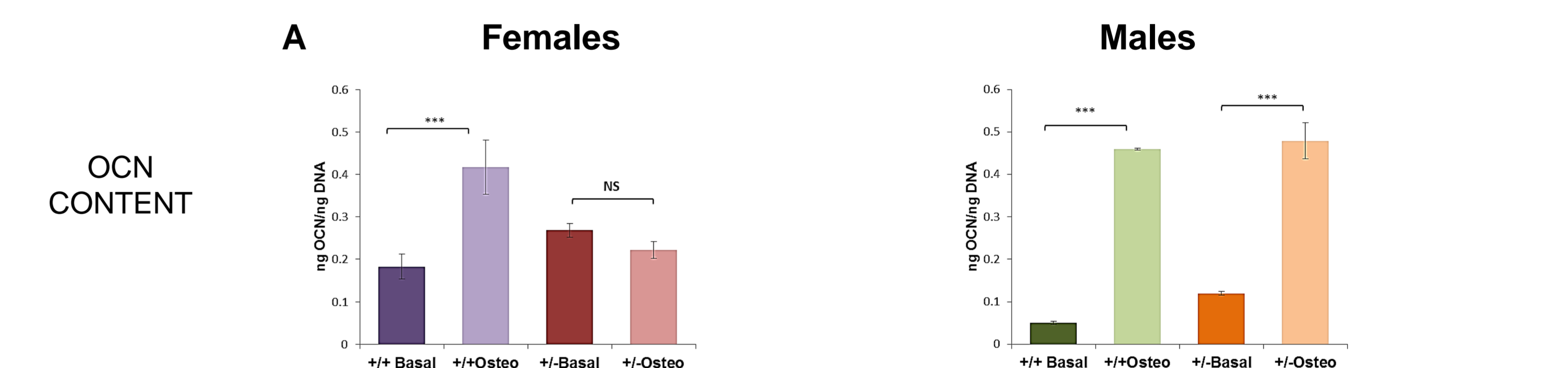


Figure 5. Intracellular concentration of OCN (mature osteoblast marker) was measured in day 28 cultures of BMSCs. Concentration of OCN was normalised to the DNA content of the cells. Results were expressed as mean \pm SD, $n = 3$ mice in each group, *** $P < 0.001$.

- In wild-type female mice, specific activity of ALP and concentration of OCN were significantly higher in day 28 BMSC cultures in osteogenic media compared to basal media. In contrast, in *Bag-1* heterozygous female mice, no significant differences in ALP specific activity and OCN content were observed between day 28 BMSC cultures in basal and osteogenic media (Fig. 4A, Fig. 5A).
- In day 28 cultures of BMSCs of both wild-type and *Bag-1* heterozygous male mice, ALP specific activity and OCN concentration were significantly higher in osteogenic conditions compared to basal conditions (Fig. 4B, Fig. 5B).

3. No differences in cell apoptosis were observed between the different groups of BMSC cultures

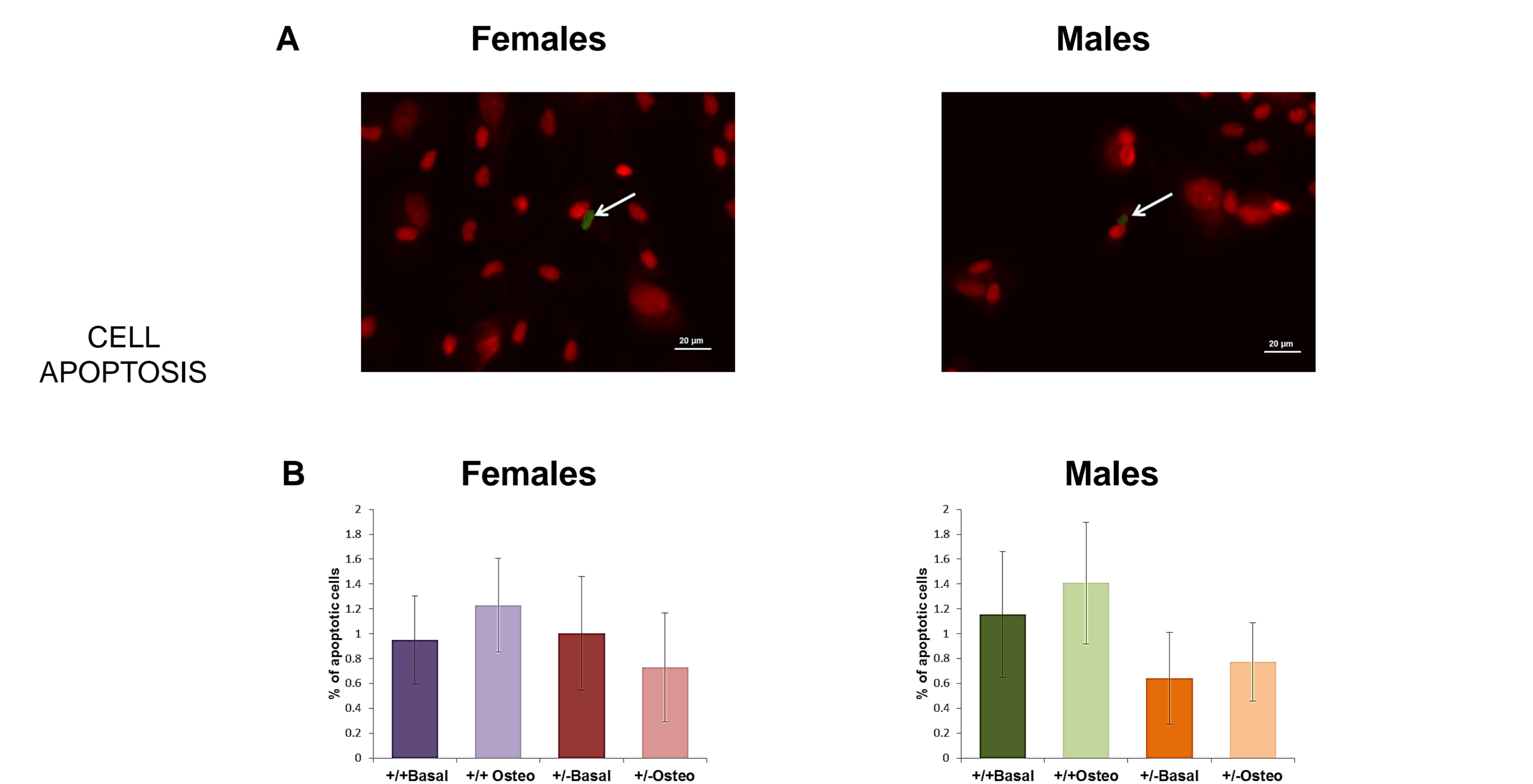


Figure 6. Representative images of day 28 cultures of BMSCs showing PI stained cell nuclei and TUNEL positive apoptotic cells indicated by arrows (A). Percentage of TUNEL positive apoptotic cells in day 28 BMSC cultures (B). To quantify the results of TUNEL staining, PI stained nucleated cells and TUNEL +ve apoptotic cells were counted in 5 different fields and the number of apoptotic cells was determined as % of total cell count.

- BMSC cultures exhibited low incident of apoptosis.
- No statistically significant differences in cell apoptosis were observed between the different groups of BMSC cultures (Fig. 6B).

Conclusions

In female mice heterozygous for *Bag-1*, proliferation of BMSCs was enhanced at the expense of osteogenic differentiation. These studies indicate an important role for BAG-1 in osteoblast development and the need to understand the role of interacting factors modulating gender differences.

References

1. Crocoll A *et al.* (2002) *Mech Dev*; 111: 149-52; 2. Tare R *et al.* (2008) *Bone*; 42: 113-28; 3. Gotz R *et al.* (2005) *Nat Neurosci*; 8: 1169-78

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The authors have no conflict of interest