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SHORT COMMUNICATION

Expression of Osteogenic Factors in FasL-Deficient Calvarial Cells

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Summary

During bone development, FasL acts not only through the traditional apoptotic mechanism regulating the amount of bone-resorbing osteoclasts, but there is also growing evidence about its effect on cell differentiation. Expression of osteoblastic factors was followed in non-differentiated and differentiating primary calvarial cells obtained from FasL-deficient (gld) mice. The gld cells showed decreased expression of the key osteoblastic molecules osteocalcin (Ocn), osteopontin (Opn), and alkaline phosphatase (Alpl) in both groups. Notably, receptor activator of nuclear factor kappa-B ligand (Rankl) was unchanged in non-differentiated gld vs. wild type (wt) cells but decreased in differentiating gld cells. Osteoprotegerin (Opg) in the gld samples was increased in both groups. Opg vs. Rankl expression levels favored Opg in the case of non-differentiated cells but Rankl in differentiating ones. These results expand information on the involvement of FasL in non-apoptotic cell pathways related to osteoblastogenesis and consequently also osteoclastogenesis and pathologies such as osteoporosis.

Key words

FasL • Osteogenesis • Calvarial cells • Rankl • Opg

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Fas/FasL (CD95/CD178) is the key signalling triggering the extrinsic apoptotic pathway in frame of homeostasis and immune system surveillance [1]. More recently, attention has also expanded to emerging non-apoptotic functions of Fas/FasL and related apoptotic components and this also applies for bone cells [2-4].

In the apoptotic manner, FasL expressed by osteoblasts triggers cell death of osteoclasts upon binding to their Fas receptors and thus tunes bone remodelling [5]. Additionally, osteoblasts regulate osteoclastogenesis by the Rank/Rankl/Opg system (rev. in [6]).

FasL-deficient knock-out mice (gld mutation – generalized lymphoproliferative disorder) die prematurely due to autoimmune syndromes [7]. The gld phenotype includes changes in bone mass, whole body bone mineral density and trabecular bone volume [8]. Importantly, along with impact on apoptosis, Fas/FasL was shown to modulate osteoblast differentiation [2,8]. Higher levels of Opg produced by osteoblasts were emphasized in gld mice as demonstrated in the case of osteogenic regeneration after bone marrow ablation. Additionally, more Opg mRNA was detected in bone diaphyseal shafts and bone marrow stromal fibroblasts derived from gld mice. Notably, Rankl levels were found unchanged [8]. And in a later report, even Opg levels were reported equal in osteoblast progenitors/osteoblast cell cultures derived from the conditional gld and wt animals [5]. The different findings can be influenced by different approaches but also by the differentiation state of the osteoblasts [9].

Craniofacial bones, including calva, undergo intramembranous ossification which compared to the

endochondral one, typical for long bones, does not include cartilage inter-step. Cells isolated from calva can be differentiated into osteoblasts and are widely used to investigate osteoblastogenesis in primary cell cultures [10,11].

The aim of this work was to test if there is any difference in expression of Rankl/Opg in calvarial cells derived from the gld and wt mice when cultured in a non-differentiating vs. differentiating (osteogenic) medium. Additionally, expression of the major osteogenic factors, Ocn, Opn, Runt-related transcription factor 2 (Runx-2), Alpl, phosphate regulating gene with homologies to endopeptidases on the X chromosome (Phex) and sclerostin (Sost) was examined.

Mice homozygous for the gld mutation (B6Smn.C3-Faslgld/J) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the Medical University of Vienna, Institute of Biomedical Research under specific-pathogen-free (SPF) conditions. Mice were subjected to treatment according to the approved protocol GZ BMWFW-66.009/0359-V/3b/2018. The calvarial bones of 3-day-old mice were digested with 0.1 % collagenase and 0.2 % dispase II (both Sigma, St. Louis, MO) in α-modified Eagle's medium (αMEM) for 15 min at 37 °C with stirring. After discarding the initial digest, this process was repeated five more times. Calvarial cells from these digests were collected by centrifugation and expanded in a proliferation medium consisting of MEM alpha (Gibco, Waltham, MA, USA), 10 % fetal bovine serum (Sigma-Aldrich, Burlington, MA, USA) and 1 % penicillin/streptomycin (Sigma-Aldrich) at 37 °C and 5 % CO_2 for 3 days (group ND). For the next 3 days, the medium was enriched with 50 mg/ml ascorbate and 4 mM β-glycerophosphate (group 3D). Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Germany) and cDNA was synthesized using the GB Elite Reverse Transcription Kit (Generi Biotech, Czech Republic). The TaqMan Gene Expression Assay (Thermo Fisher Scientific) was applied for detecting the gene expression of Alpl (Mm00475834_m1), Bglap (Ocn, Mm03413826_mH), Opg (Tnfrs11b, Mm00435454_m1), Phex (Mm00448119_m1), Rankl (Tnfsf11, Mm00441906_m1), Runx2 (Mm00501584_m1), Sost (Mm00470479_m1) and Spp1 (Opn, Mm00436767_m1), qPCR was performed in 10 µl of a final reaction mixture containing the one-step GB Ideal PCR Master Mix (Generi Biotech) using Light-Cycler 96 (Roche, Switzerland). Real-time PCR expression levels were calculated using the ΔΔCT method and the results were analyzed using a 2-tailed *t*-test. Reactions were performed in triplicate for each sample.

Calvarial bone belongs to the intramembranous bones where osteoblasts differentiate directly from mesenchymal precursors and thus, the process committing the cells into their osteogenic fate resembles *in vivo* and is easy to follow. We took advantage of the system to compare expression levels of osteoblast-related molecules in gld and control wt calvarial cells under non-differentiating conditions and after commitment into osteogenic differentiation. The process was monitored by increasing levels of Ocn, Opn and Alpl (Fig. 1A-C, respectively) along with Runx2 (almost equal expression, Fig. 1D), Rankl (strongly increased expression, Fig. 1E) and Opg (increased expression, Fig. 1F) in the control group.

The first set of results comparing gld and wt cells provided data to Rankl and Opg expression levels and the Rankl/Opg ratio. While prior to differentiation, the wt and gld cells displayed the same Rankl expression (Fig. 2A), the Opg levels (Fig. 2C) differed showing a higher amount of the transcripts in gld samples (1.98-fold wt level). Notably, after initiation of differentiation, Rankl expression (Fig. 2B) significantly decreased in the gld cells (0.06-fold of the samples). Opg kept its elevated level (Fig. 1D) in the gld cells also after initiation of differentiation (1.81-fold of the wt). Increased levels of Opg in gld mice were reported earlier [2,8] despite the fact that they were not supported by later observation [5] where Rankl was also unchanged. Our results support the hypothesis that Rankl and Opg expression depends on the differentiation status of the isolated cells which may also be associated with their origin (long bone marrow, calvarial cells). While in the Rankl/Opg ratio in non-differentiated wt cells Opg was favored (Fig. 1G), after 3 days of differentiation the expression of Rankl was significantly higher than that of Opg (Fig. 1H). Apparently, upon differentiation into osteoblastic fate, the Rankl/Opg ratio in gld mice favors bone formation/protection by inhibition of osteoclasto-genesis [12]. The Rankl/Opg ratio is critical not only for bone homeostasis and disorders [13] but also pathologies related to other organs [14].

The observation in non-differentiated and differentiating calvarial cells is in agreement with a previous study in gld mice showing an age-dependent bone phenotype [15]. In this case, a difference in expression of Sost in gld vs. wt mandibular bone was the

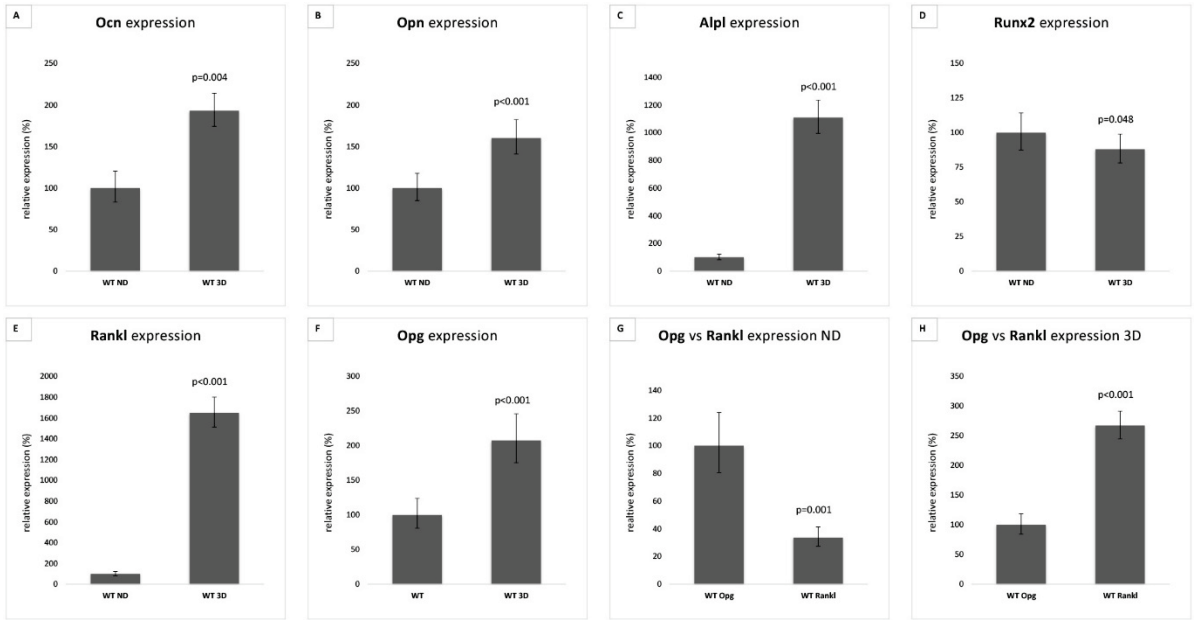

Fig. 1. Expression changes of Ocn, Opn, Alpl, Runx2, Rankl and Opg during 3 days of differentiation (3D). Expression in the non-differentiated (ND) cells was set to 100 %. (**A-F**). Expression ratio of Opg and Rankl in non-differentiated (**G**) and 3 days differentiated cells (**H**). Expression of Opg was set to 100 %. The results are shown in %, indicating the mean ± standard deviation of three replicates.

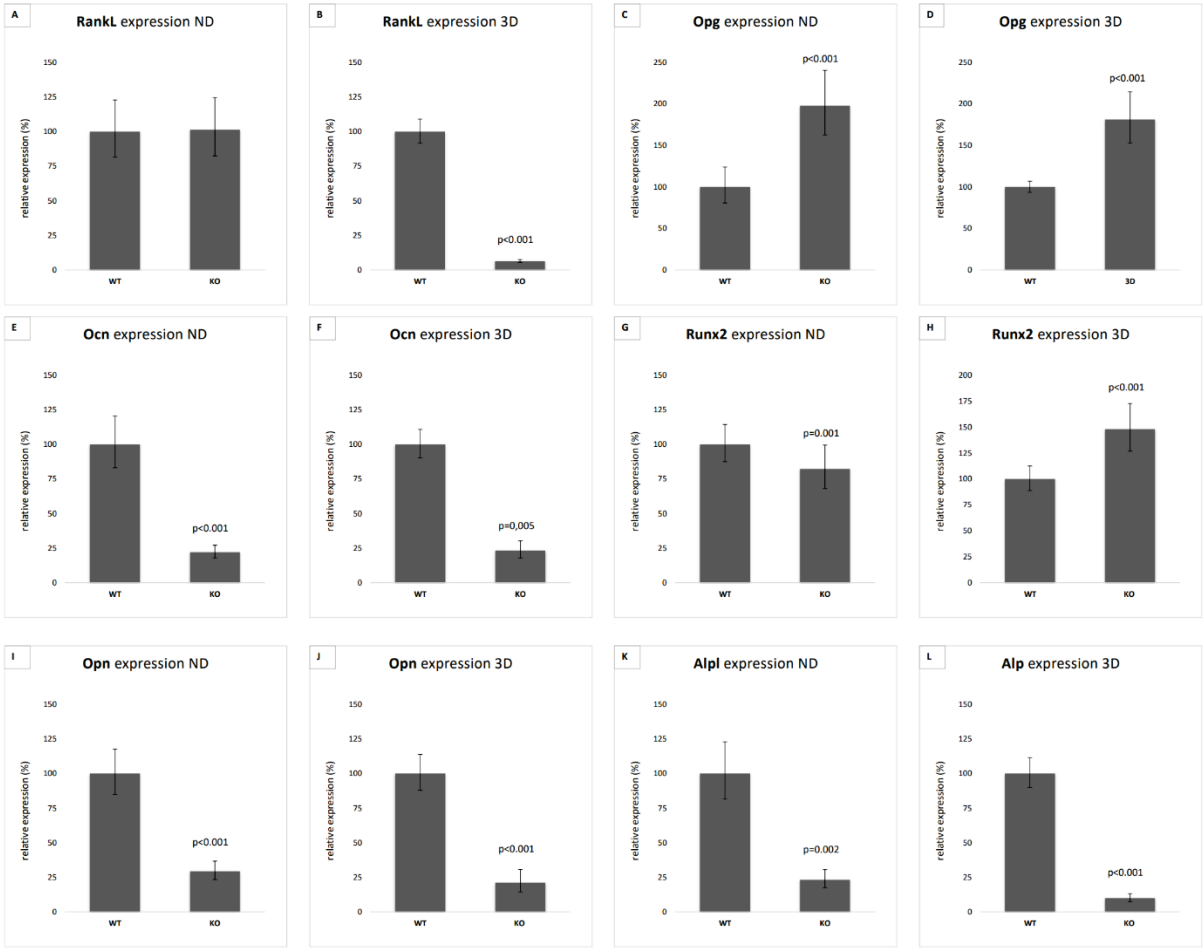

Fig. 2. Expression changes of Rankl, Opg, Ocn, Runx2, Opn and Alpl in gld (KO) cells compared to wild type (WT) cells. Non-differentiated cells (ND) (**A, C, E, G, I, K**), 3 days differentiated cells (3D) (**B, D, F, H, J, L**). Expressions of genes of interest in WT cells were set to 100 %. The results are shown in %, indicating the mean ± standard deviation of three replicates.

background for a hypothesis that osteoblasts/osteocytes ratio may be responsible for differences in prenatal and postnatal gld phenotype. Since osteocytes differentiate from osteoblasts, this is evidence related to the stage-dependent findings. Thus, the modulations at the level of osteoblasts can be a prerequisite of later, osteocyte-related findings.

The differentiation stage specific impact of FasL deficiency was also apparent in the case of other key factors such as Runx2 (Fig. 2G) which was detected as decreased (0.82-fold of the wt levels) in non-differentiating calvarial cells but increased in gld cells (1.48-fold of the wt levels) after induction of differentiation (Fig. 2H). The latter case corresponds to that observed in the FasL stimulated bone marrow osteoblastic colonies where FasL decreased expression of Runx2 after 7 days of culture but non-differentiated precursors were not evaluated [2]. Notably, in the case of Opn and Alpl, the effect of FasL deficiency was apparent not only in differentiated stage, but already in undifferentiated cells and both these markers were downregulated in cells of the gld mice. (Fig. 2I-L). The same situation was with Ocn (Fig. 2E and F), but in the case of undifferentiated cells, the expression of this molecule was very low. Anyway, all findings contribute to the growing evidence that the function of Fas/FasL signalling is important not only for osteoblasts-induced osteoclasts apoptosis [5] but also in non-apoptotic processes impacting osteoblastic differentiation. Additionally, the findings were found dependent on the differentiation status of osteoblastic cells which may later also impact osteoblast-osteocyte transition. The most recent reports support this evidence such as in the case of Sost expression in IDG-SW3 cells impacted by FasL [16] or bone healing of the alveolar socket in gld mice [17]. However, the apoptotic vs. non-apoptotic pathways mediated by Fas/FasL are likely to be more complicated, also due to interactions of the component within other pathways. For example, Opg induces apoptosis of osteoclasts *via* the Fas/FasL pathway [18].

In such case, the increased levels of Opg in gld calvarial cells (both, non- and differentiated) along with decreased Rankl can be a parallel mechanism to decrease osteoclastogenesis and thus contribute to the adult gld phenotype. The presented findings pointing to the stage-dependent impact of FasL deficiency on osteoblastic differentiation contributes to an explanation of the molecular background of the bone phenotype in gld mice but also some still controversial observations. Osteoblast-osteoclast communication is essential for bone formation, remodelling and functions and Rank/Rankl/Opg and Fas/FasL signalling are two major systems regulating osteoclasts by osteoblasts (reviewed e.g. in [19]). Notably, both of the pathways are considered in therapies such as osteoporotic ones [20,21] driving the basic research towards applications.

Conflict of Interest

There is no conflict of interest.

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