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DLK1, an inhibitory non-canonical ligand of NOTCH receptors, inhibits osteoblastic differentiation of pre-osteoblastic MC3T3-E1 cell line

González-Gómez, M.J.[1], *Rodríguez-Cano, M.* [2], *Resuela-González, J.L.*[2], *Nueda, M.L.* [2], *Laborda, J.*[2], *and Baladrón, V* [3].

Laboratory of Biochemistry and Molecular Biology, Department of Inorganic and Organic Chemistry and Biochemistry, Higher Technical School of Agricultural and Forestry Engineering [1]/ Pharmacy School [2]/ Medical School [3]/ Regional Biomedical Research Center (CRIB)/ Biomedicine Unit, UCLM/CSIC. Albacete, Spain.

SUMMARY

OBJECTIVE. To analyze the effects of DLK1 protein expression, an inhibitor of NOTCH receptor signaling, on the osteogenic differentiation of MC3T3-E1 pre-osteoblastic cells, which lack endogenous *Dlk1* expression.

MATERIALS AND METHODS. Pools of cells stably overexpressing DLK1 protein were generated by plasmid transfection. *Dlk1* expression levels were analyzed by RT-qPCR and DLK1 protein levels by Western Blot and they were referred to those of cells transfected with an empty plasmid vector. We performed osteogenic differentiation assays by treatment with culture medium supplemented with 10 mM β-glycerophosphate and 50 µg/ml ascorbic acid. The differentiation capacity of the DLK1-overexpressing cells was assessed by alkaline phosphatase staining and by the analysis of osteogenic differentiation markers by RT-qPCR. These assays were also performed using DAPT, a pharmacological inhibitor of NOTCH receptors.

RESULTS. Cells overexpressing *Dlk1* showed a lower rate of osteogenic differentiation with respect to control cells, as shown by the lower degree of alkaline phosphatase staining and lower mRNA expression of some osteogenic markers, such as *Alp*, *Col1A1*, *Runx2* and Osteocalcin. The NOTCH receptor inhibitor, DAPT, also caused the decrease of both alkaline phosphatase staining and osteogenic markers in MC3T3-E1 cells treated with the osteogenic inducers.

CONCLUSIONS. The results obtained indicate that *Dlk1* overexpression or treatment with DAPT exert an inhibitory effect on the osteoblastic differentiation of MC3T3-E1 cells. This indicates that NOTCH signaling is required for osteogenesis of these cells and that DLK1 could exert its inhibitory effect on this process through inhibition of NOTCH signaling.

RESULTS AND CONCLUSIONS

1. MC3T3-E1 PRE-OSTEOBLASTIC CELLS CAN STABLY OVEREXPRESS DLK1 mRNA and PROTEIN

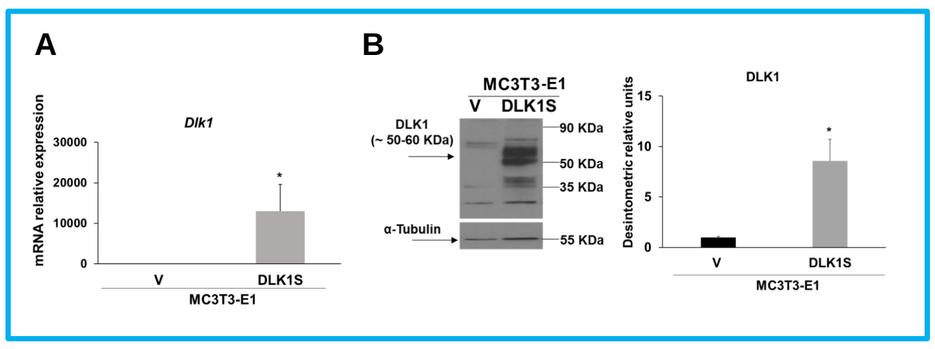

Figure 1. Overexpression of DLK1 mRNA and protein in pre-osteoblastic MC3T3-E1 cells. MC3T3-E1 cells, lacking endogenous *Dlk1* expression, were stably transfected with the empty vector (V), or *Dlk1* expression plasmid (DLK1S). **A)** RT-qPCR analysis of the relative mRNA expression levels in stable DLK1 transfectant pools. **B)** Representative Western blot assay and densitometric analysis that show the relative protein levels in stable DLK1 transfectant pools. Data from qRT-PCR assays were normalized to P0 mRNA expression levels. Data in Western blot assays were normalized with tubulin expression levels, used as a loading control. The fold activation or inhibition was measured as relative to that of cells transfected with the empty vector, which was set arbitrarily at 1. Data are shown as the mean ± SD of at least three independent assays. Student T-Test was performed relative to the empty vector sample : * p<0,05.

2. OVEREXPRESSION OF DLK1 PROTEIN INHIBITS THE OSTEOBLASTIC DIFFERENTIATION OF MC3T3-E1 CELLS

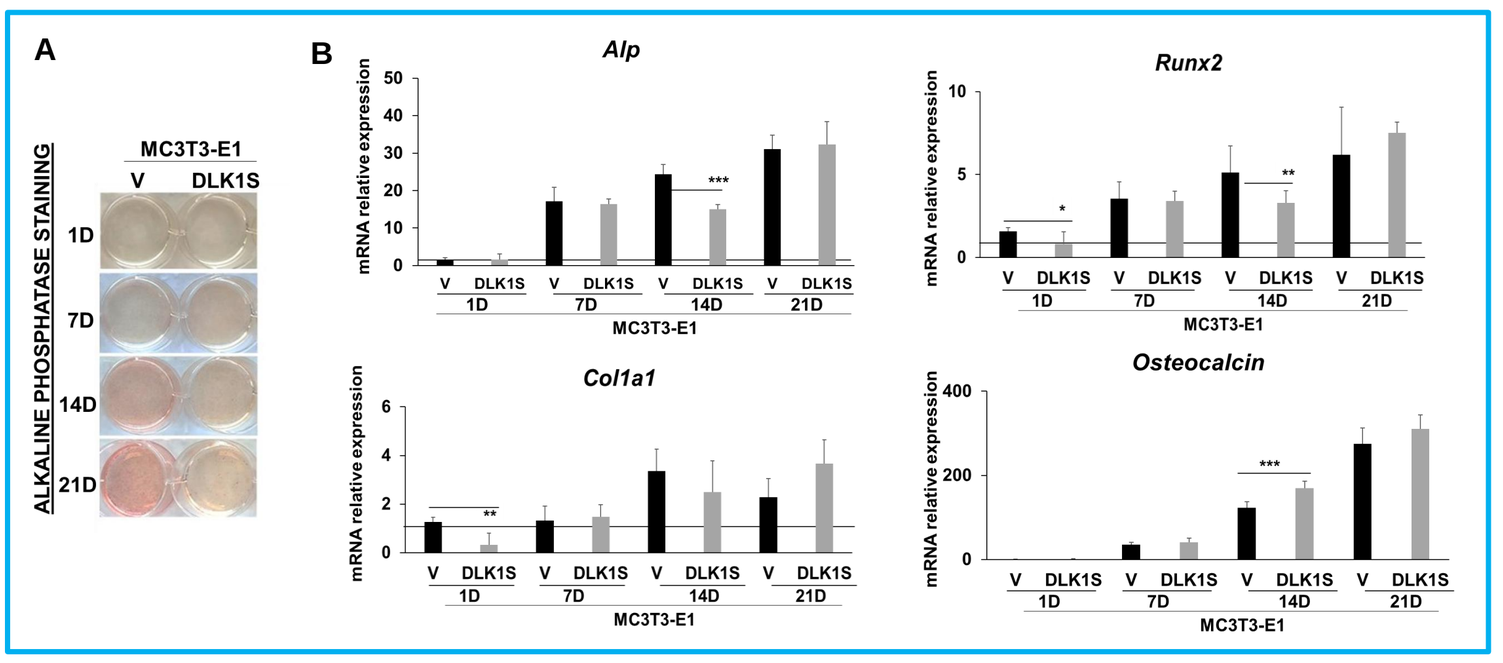

Figure 2. Effect of DLK1 protein overexpression on osteogenic differentiation of MC3T3-E1 cells. **A)** Representative images of alkaline phosphatase staining levels of MC3T3-E1 cells stably transfected with the empty-vector (V) or *Dlk1* expression plasmid (DLK1S) during the osteoblastic differentiation process, at the indicated days (D). **B)** Relative mRNA expression levels of four osteogenic markers [fosfatase alcaline (*Alp*), Collagen Type I Alpha 1 Chain (*Col1a1*), *Runx2*, and Osteocalcin] during the osteogenic differentiation process of MC3T3-E1 cells stably transfected with the empty vector (V) or a *Dlk1* expression plasmid (DLK1S), at the indicated days (D). Data from qRT-PCR assays were normalized to P0 mRNA expression levels. The fold activation or inhibition was calculated relative to empty-vector cells without osteogenic treatment on day 1, which was set arbitrarily at 1. The mean ± SD of at least three independent assays is shown. Student T-Test were performed relative to samples indicated. ** p<0,01; *** p<0,001.

3. OVEREXPRESSION OF DLK1 PROTEIN IN MC3T3-E1 CELLS INCREASES THE EXPRESSION OF *Notch1* gene BUT INHIBITS THE ACTIVATION OF THE NOTCH1 INTRACELLULAR DOMAIN (NICD1)

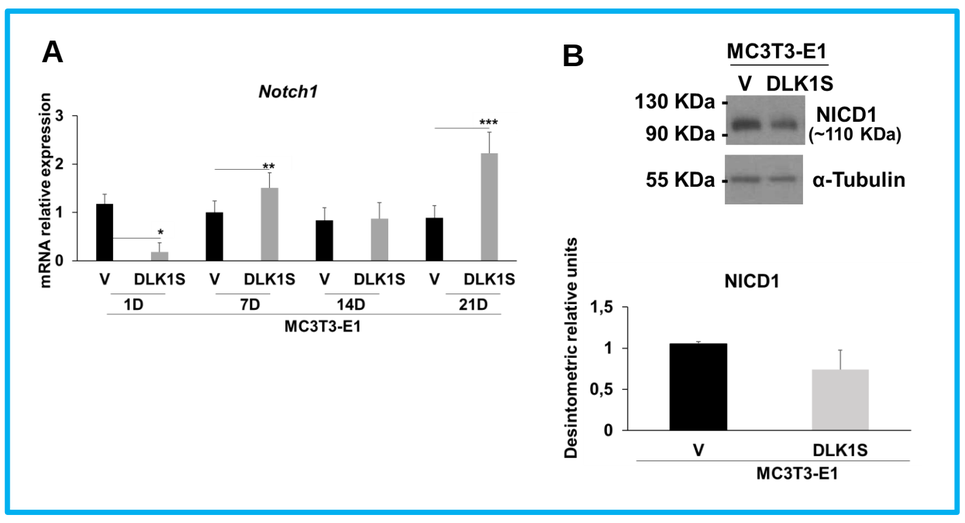

Figure 3. Analysis of the expression levels of *Notch1* mRNA and the active intracellular domain of NOTCH1 receptor (NICD1) in MC3T3-E1 cells that stably overexpress DLK1 protein. A) Expression pattern of *Noth1* gene during the osteogenic differentiation process at indicated days (D). The relative mRNA expression levels of *Notch1* gene in the empty-vector (V) or the *Dlk1* expression plasmid (DLK1S) stable transfectant pools was analyzed by RT-qPCR . Data from RT-qPCR assays were previously normalized to P0 mRNA expression levels**. B)** Representative Western blot assay and densitometric analysis of active NICD1 expression levels in the empty-vector (V) or the *Dlk1* expression plasmid (DLK1S) stable transfectant pools. Data in Western blot assays were normalized with tubulin expression levels, used as a loading control. The fold activation or inhibition was measured as relative to that of cells transfected with the empty vector, which was set arbitrarily at 1. Data are shown as the mean ± SD of at least three independent assays. Student T-Test were performed relative to empty-vector samples. * p<0,05; ** p<0,01; *** p<0,001.

4. THE γ-SECRETASE COMPLEX INHIBITOR DAPT REDUCES MC3T3-E1 OSTEOBLASTIC DIFFERENTIATION LEVELS

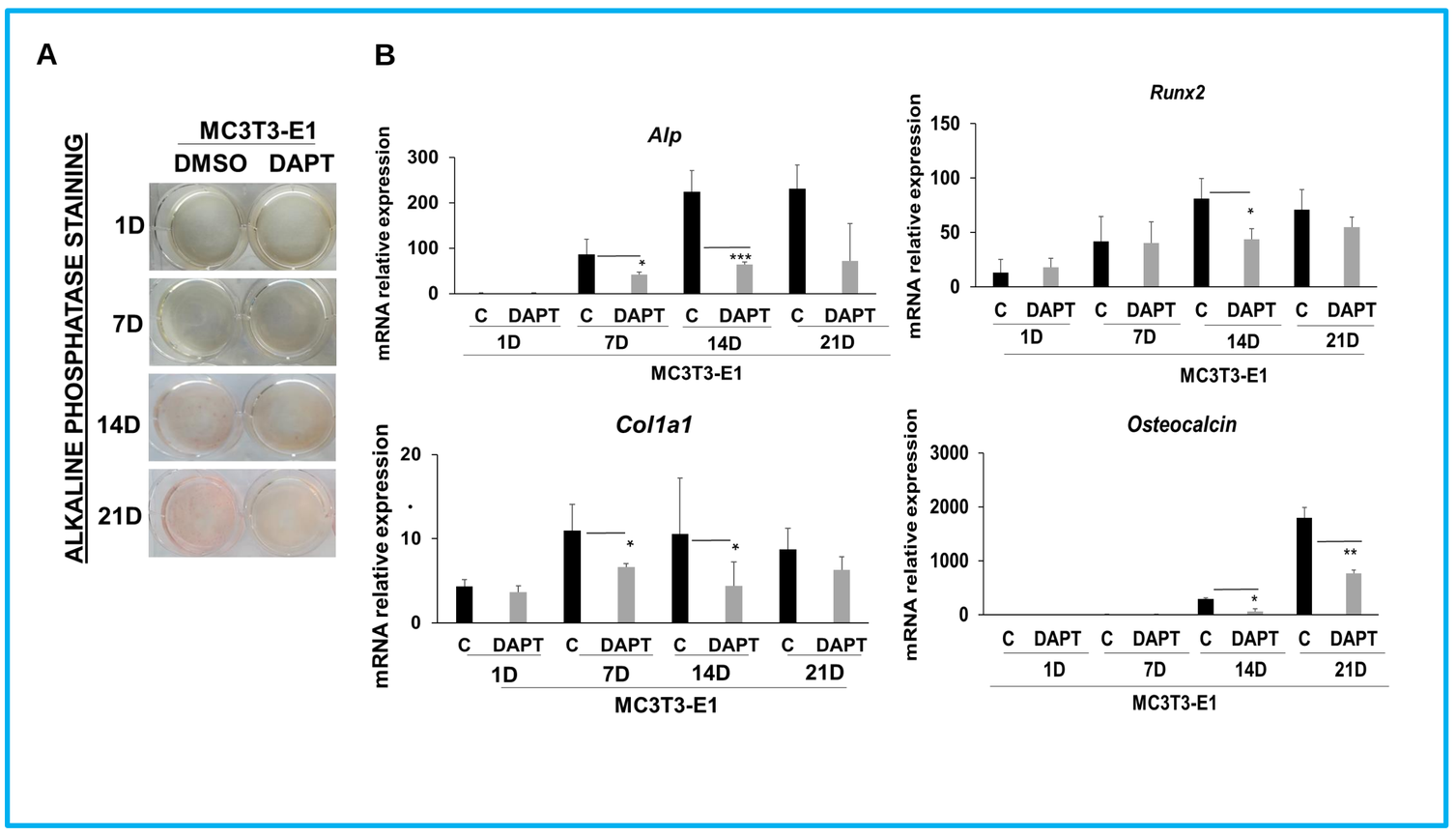

Figure 4. Effect of DAPT, a γ-secretase complex inhibitor, on osteogenic differentiation of MC3T3-E1 cells. A) Representative images of alkaline phosphatase staining levels of MC3T3-E1 cells treated with DMSO (used as a control) or DAPT during the osteoblastic differentiation process at the indicated days (D). **B)** Relative mRNA expression levels of four osteogenic markers [(fosfatase alcaline (*Alp*), Collagen Type I Alpha 1 Chain (*Col1a1*), *Runx2*, and Osteocalcin (*Ocn*)] during the osteogenic differentiation process of MC3T3-E1 cells treated with DMSO (C) or DAPT, at the indicated days (D). Data from qRT-PCR assays were normalized to P0 mRNA expression levels. The fold activation or inhibition was calculated relative to cells treated with DMSO (C) on day 1, which was set arbitrarily at 1. The mean ± SD of at least three independent assays is shown. Student T-Test were performed relative to samples indicated. * p<0,05; ** p<0,01; *** p<0,001.

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Castilla-La Mancha

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