# The role of NOTCH receptors in the osteoblastogenic differentiation of mesenchymal C3H10T1/2 cell line.

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XIX CONGRESO de la SEBC
BOADILLA DEL MONTE (Madrid)
26, 27, 28 y 29 de octubre de 2021

## SUMMARY

**OBJECTIVE.** To analyse the role of NOTCH receptors on the osteoblastic differentiation of mesenchymal C3H10T1/2 cells.

**MATERIALS AND METHODS.** Cell populations stably overexpressing each of the four NOTCH receptors were generated by transfection with a plasmid. Expression levels of *Notch1-4* genes and its target genes, *Hes1* and *Hey1,* were assessed by RT-qPCR. The level of each NOTCH receptor in its overexpressing population was determined by Western blot. NOTCH activity was measured by luciferase assays. The osteoblastic differentiation capacity of each population was evaluated by the alkaline phosphatase staining method of induced cell cultures and the measurement of the expression levels of osteogenic differentiation markers in RNA samples obtained from differentiation assays.

**RESULTS.** The overexpression of a single NOTCH receptor produces an increase in the global NOTCH activity and modifies the expression of the other *Notch* and their target genes, *Hes1* and *Hey1*. The generated transfectants showed different levels of osteogenic differentiation in *in vitro* assays. Alkaline phosphatase staining was more intense in cells overexpressing NOTCH3 or NOTCH2 and less intense in cells overexpressing NOTCH1 or NOTCH4. Populations overexpressing NOTCH1, NOTCH2 or NOTCH3 exhibit an increase in mRNA expression of markers compared with the control. Cells overexpressing NOTCH4 only showed a significative increase in *Alpl* marker expression.

**CONCLUSIONS.** The expression of *Notch*, *Hes1* and *Hey1* is interrelated. The overexpression of the NOTCH1-3 receptors seems to have an inducing effect on the osteoblastic differentiation of C3H10T1/2 cells, while NOTCH4 overexpressing cells does not seem to modify the osteoblastic process.

## RESULTS AND CONCLUSIONS

### 1. C3H10T1/2 cells can stably overexpress NOTCH proteins

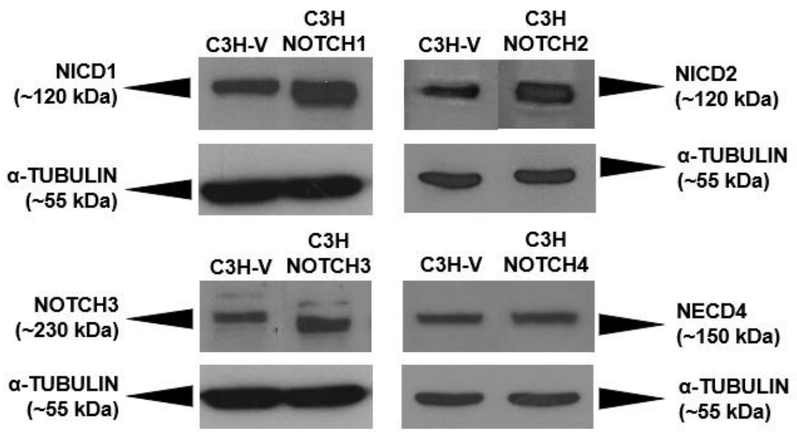


**Figure 1. NOTCH proteins expression levels in stable C3H10T1/2 transfectants of *Notch* genes.** Representative Western blot of NOTCH proteins expression levels in the stable transfectants of *Notch* genes compared with empty-vector control transfectants (C3H-V). Data were normalized with α-tubulin expression levels, used as a loading and sample quality control. NICD: NOTCH intracellular region. NECD: NOTCH extracellular region.

### 2. Stable overexpression of *Notch* genes in C3H10T1/2 cells increases NOTCH receptors-dependent transactivation levels

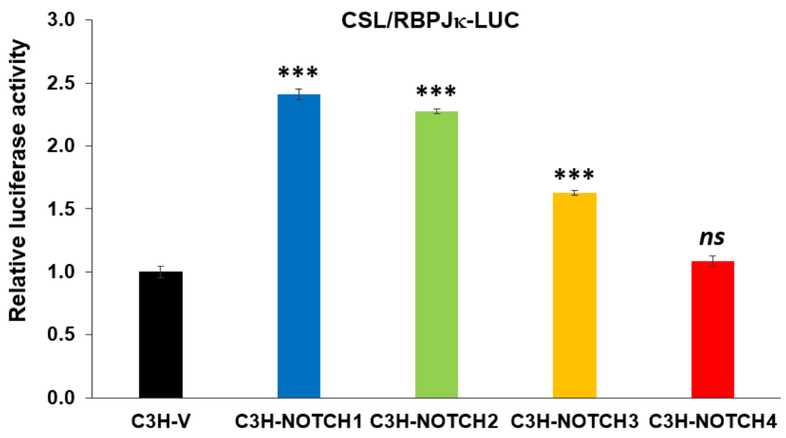


**Figure 2. Transcriptional activation of NOTCH receptors in stable C3H10T1/2 transfectants that overexpress NOTCH receptors.** Relative levels of NOTCH receptors-dependent transcriptional activation (CSL/RBP-Jk-LUC) were determined by luciferase assays in C3H-V, C3H-NOTCH1, C3H-NOTCH2, C3H-NOTCH3, and C3H-NOTCH4. The relative levels of luciferase activity were normalized to the renilla values in each sample. The fold activation was calculated relative to those obtained in C3H-V cells, which were arbitrarily set to 1. Data are shown as the mean ± SE of at least three assays performed in triplicate. The statistical significance is indicated by Student's t-test (*** $p \leq 0.001$). Statistically non-significant results are indicated by ns.

### 3. Stable overexpression of NOTCH receptors in C3H10T1/2 cells modulates *Hes1* and *Hey1* expression levels

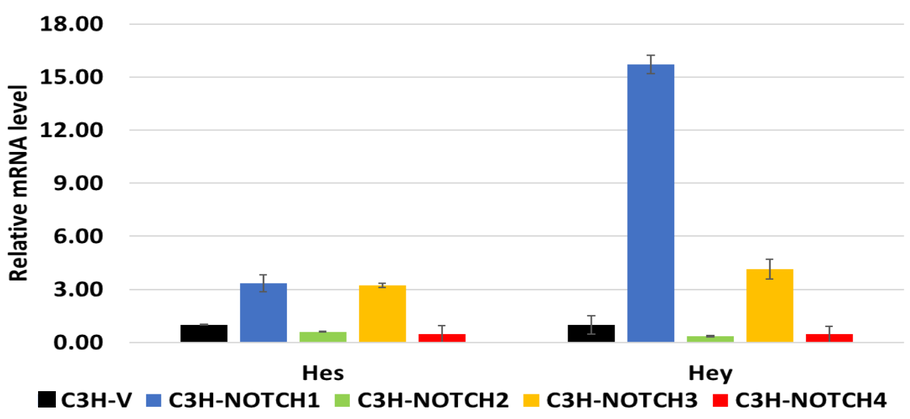


**Figure 3. Endogenous expression levels of *Hes1* and *Hey1* genes, two targets of *N*OTCH receptors, in stable C3H10T1/2 transfectants that overexpress NOTCH receptors.** qRT-PCR analysis of the relative mRNA expression levels of *Hes1* and *Hey1* in each of the C3H10T1/2 stable transfectants (C3H-NOTCH1, C3H-NOTCH2, C3H-NOTCH3 and C3H-NOTCH4). Data were normalized to *Rplp0* mRNA expression levels. The fold activation or inhibition was calculated relative to that obtained in cells transfected with the empty vector (C3H-V)), arbitrarily set at 1. Data are shown as the mean ± SD of of three RT-qPCR technical replicas.

### 4. Stable overexpression of *Notch* genes in C3H10T1/2 cells increases alkaline phosphatase activity

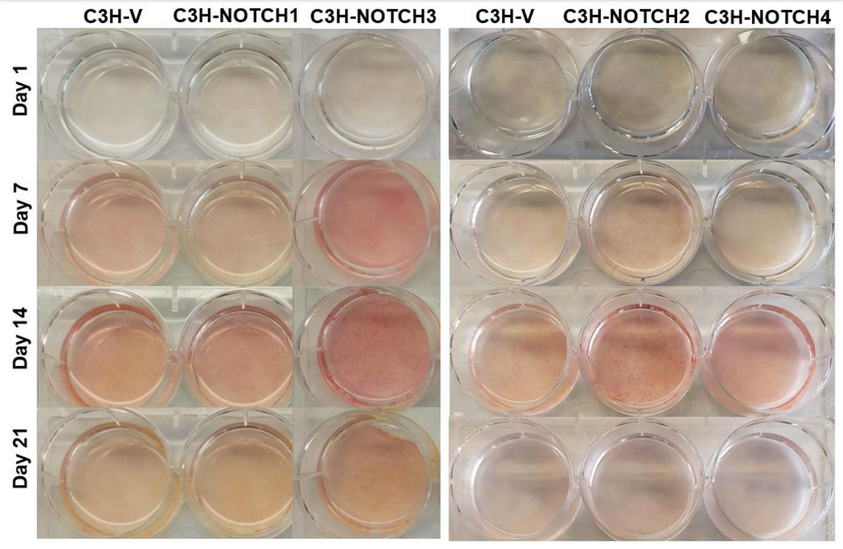


**Figure 4. Effect of stable overexpression of NOTCH receptors on the osteogenic potential of mesenchymal C3H10T1/2 cells by alkaline phosphatase staining.** Representative images of cell cultures after alkaline phosphatase staining of C3H10T1/2 stable transfectants of *Notch* genes, at the indicated days after the induction of the osteogenic differentiation.

### 5. Stable overexpression of NOTCH1-3 receptors in C3H10T1/2 cells enhances osteoblastogenesis

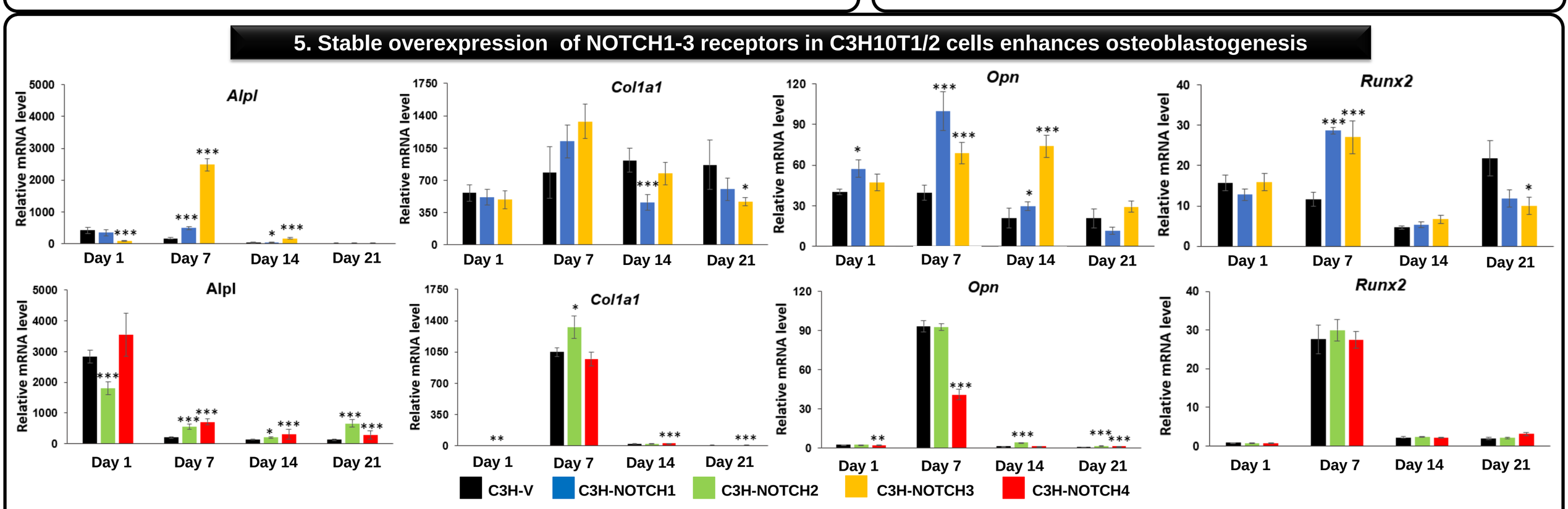


**Figure 5. Relative mRNA expression levels of osteogenic markers in differentiated C3H10T1/2 stable transfectants that overexpress NOTCH receptors.** qRT-PCR analysis of the relative mRNA expression levels of *Alpl*, *Opn*, *Col1a1* and *Runx2* osteogenic markers in the stable transfectants of *Notch* genes, at the indicated days after the induction of the osteogenic differentiation. Data were normalized to *Rplp0* mRNA expression levels. The fold activation or inhibition was calculated in relation to that obtained on day 1 in undifferentiated empty-vector cells (C3H-V), arbitrarily set at 1. Data are shown as the mean ± SD of at least three independent assays. Student t-Test: * (P<0,05); ** (P<0,01); *** (P<0,001).

FEDER Fondo Europeo de Desarrollo Regional
Castilla-La Mancha

**This work was supported by funds from the Ministry of Education, Culture and Sports of the Regional Government of Castilla-La Mancha (Spain), SBPLY/17/180501/000316, supported by the "Fondo Europeo de Desarrollo Regional" (FEDER).**