Cell and Tissue Research
https://doi.org/10.1007/s00441-019-03130-7

REGULAR ARTICLE

Dose-dependent mechanism of Notch action in promoting osteogenic differentiation of mesenchymal stem cells

Daria Semenova[1,2,3] · Maria Bogdanova[1] · Aleksandra Kostina[1,3] · Alexey Golovkin[1] · Anna Kostareva[1] · Anna Malashicheva[1,2,3]

Received: 10 December 2018 / Accepted: 23 October 2019

Abstract

Osteogenic differentiation is a tightly regulated process realized by progenitor cell osteoblasts. Notch signaling pathway plays a critical role in skeletal development and bone remodeling. Controversial data exist regarding the role of Notch activation in promoting or preventing osteogenic differentiation. This study aims to investigate the effect of several Notch components and their dosage on osteogenic differentiation of mesenchymal stem cells of adipose tissue. Osteogenic differentiation was induced in the presence of either of Notch components (NICD, Jag1, Dll1, Dll4) dosed by lentiviral transduction. We show that osteogenic differentiation was increased by NICD and Jag1 transduction in a dose-dependent manner; however, a high dosage of both NICD and Jag1 decreased the efficiency of osteogenic differentiation. NICD dose-dependently increased activity of the CSL luciferase reporter but a high dosage of NICD caused a decrease in the activity of the reporter. A high dosage of both Notch components NICD and Jag1 induced apoptosis. In co-culture experiments where only half of the cells were transduced with either NICD or Jag1, only NICD increased osteogenic differentiation according to the dosage, while Jag1-transduced cells differentiated almost equally independently on dosage. In conclusion, activation of Notch promotes osteogenic differentiation in a tissue-specific dose-dependent manner; both NICD and Jag1 are able to increase osteogenic potential but at moderate doses only and a high dosage of Notch activation is detrimental to osteogenic differentiation. This result might be especially important when considering possibilities of using Notch activation to promote osteogenesis in clinical applications to bone repair.

Keywords Mesenchymal stem cells · Osteogenic differentiation · Notch

Introduction

Osteogenic differentiation is a tightly regulated process realized by specialized cells called osteoblasts. The activation factors for genes and cellular signaling pathways at the early stages of osteogenic differentiation both in normal condition and in pathological calcification remain the subject of active research. The interpretation of early mechanisms of triggering osteogenic differentiation is important as a potential possibility of controlling this differentiation for induction of osteogenesis, when bone formation is necessary, or for prevention in various pathologies associated with pathological calcification of tissues.

Notch (1 to 4) are single-pass transmembrane receptors that play a critical role in cell fate decisions (Andersson et al. 2011). Notch controls skeletal development and homeostasis, as well as osteoblast and osteoclast differentiation (Zanotti and Canalis 2016; Canalis 2018). Notch is activated by receptor-ligand interactions, resulting in the release of the Notch intracellular domain (NICD). In the canonical signaling pathway, NICD translocates to the nucleus, displacing transcriptional repressors and interacting with recombination signal binding protein for immunoglobulin κJ region (Rbpjκ) and with Mastermind-like (Maml) proteins to regulate transcription (Kovall et al. 2017). Classic targets of Notch canonical signaling include hairy enhancer of split (Hes)1, -5 and -7 and Hes related with YRPW motif (Hey)1, -2 and -L (Kovall et al. 2017).

Interest in the role of the Notch pathway in proosteogenic events during bone development and healing has grown

✉ Anna Malashicheva
malashicheva_ab@almazovcentre.ru; amalashicheva@gmail.com

[1] Almazov Federal Medical Research Centre, Akkuratova, 2, St. Petersburg 197341, Russia

[2] Saint-Petersburg State University, Universitetskaya nab., 7/9, St. Petersburg 199034, Russia

[3] Institute of Cytology, Russian Academy of Sciences, Tikhoretsky Ave., 4, St.Petersburg 194064, Russia

Published online: 28 November 2019

dramatically over the past decade. However, interpretation of various experimental models such as transgenic mouse models and cell lines of different origin and various modes of Notch activation via different ligands gave contradictory opinions concerning the role of Notch pathway in promoting or opposing osteogenic differentiation.

Deletion or mutation of any one or more of genes in Notch signaling pathway resulted in severe skeletal phenotypes in humans and in mouse models (Canalis 2018). Mutations in the Jagged-1 (Jag1) or Notch2 genes cause Alagille syndrome in humans, an autosomal dominant disorder with characteristic skeletal manifestations, including osteopenia and high prevalence of fractures. Dominant-positive Notch2-activating mutations cause Hajdu–Cheney syndrome, which also has a strong skeletal phenotype (Canalis 2018). In cell cultures, activation of Notch signaling was reported to either promote (Tezuka et al. 2002; Doi et al. 2006; Ugarte et al. 2009; Shimizu et al. 2011) or inhibit (Shindo et al. 2003; Bai et al. 2008; Hilton et al. 2008a, 2008b) osteoblast differentiation and mineralization. In murine preosteoblast cells, Notch repressed osteoblast maturation through the binding of NICD or the Notch target genes *HES1* and *HEY1* to *RUNX2* (Engin et al. 2008; Hilton et al. 2008a, 2008b; Salie et al. 2010). More recent works using cellular models showed Notch to promote osteogenic differentiation (Liu et al. 2016; Cao et al. 2017; Liao et al. 2017a, 2017b ; Cui et al. 2018). In transgenic mouse models, loss of Notch function resulted in radiodense or osteoporotic bones, while gain of Notch function was reported to have either osteoporotic or osteosclerotic phenotypes (Deregowski et al. 2006; Engin et al. 2008; Hilton et al. 2008a, 2008b; Zanotti et al. 2008; Zanotti and Canalis 2014; Canalis et al. 2016). Thus, controversial results still raise the question about the role of Notch osteogenic differentiation.

Notch is known for its tissue-specificity and also for fine-tuning of the signaling due to a lack of amplification step (Andersson et al. 2011; Yamamoto et al. 2014).

Gene-dosage sensitivity has been known for Notch in some experimental systems (Guruharsha et al. 2012) but to our knowledge has not been explored for osteogenic differentiation.

In this work, we sought to find how different Notch signaling components influence the osteogenic capacity of mesenchymal stem cells derived from adipose tissue (MSC) and if/how osteogenic potential is influenced by dosage of Notch. To test this, we used lentiviral transduction and altered the dosage of the used genes by adding different amounts of corresponding viruses to the MSC. We show that both NICD and Jag1 positively regulate the osteogenic potential of MSC in a dose-dependent manner, though a high dosage of either NICD or Jag1 could have a negative influence and prevent osteogenic differentiation of MSC. Our findings suggest that the fine-tuned dosage of Notch signaling is critical for osteogenic differentiation of mesenchymal stem cells.

Materials and methods

Cell culture and differentiation

The study was performed according to the Helsinki declaration and approval was obtained from the local Ethics Committees in Almazov National Medical Research Centre. Written informed consent was obtained from all subjects prior to fat tissue biopsy. Adipose tissue-derived mesenchymal stem cells (MSC) were collected from healthy donors as previously described (Malashicheva et al. 2015); cultured for three to six passages. The cells were maintained in tissue culture grade uncoated Petri dishes (Corning, USA) in alpha-MEM medium (PANECO, Russia) supplemented with 10% fetal calf serum (Hyclone, USA), 50 U/mL penicillin and 50 μg/mL streptomycin (Invitrogen, USA) at 37 °C and 5% CO_2. MSC immunophenotype was verified with flow cytometer GuavaEasyCyte8 (Millipore, USA) using CD19, CD34, CD45, CD73, CD90 and CD105 monoclonal antibodies (BD, USA) as previously described (Dmitrieva et al. 2015).

Osteogenic differentiation was induced by the addition to the culture medium of 50 μM ascorbic acid, 0.1 μM dexamethasone and 10 mM beta-glycerophosphate (Sigma, USA). The culture media was changed twice a week. The differentiation was considered terminal after 21–22 days of induction.

Calcium deposits were demonstrated by Alizarin Red staining. Cells were washed with PBS, fixed in 70% ethanol for 60 min, washed twice with distilled water and stained using Alizarin Red solution (Sigma). The images of calcium phosphate deposition were analyzed for the ratio of differentiated and undifferentiated cell areas with MosaiX software (Carl Zeiss microsystems, Germany).

For co-culture experiments, half of the required amount of MSC were seeded and transduced by lentiviral particles. The next day, the other part of MSC, without any genetical modifications, was seeded on the cell monolayer.

Genetic constructs and lentivirus production

Lentiviral packaging plasmids were a generous gift from Prof. Didier Trono (École Polytechnique Fédérale de Lausanne, Switzerland). pGa981-6 plasmid was a gift from Prof. Urban Lendahl (Karolinska Institutet, Sweden). The 12XCSL-Luciferase was cloned at the AscI and SpeI restriction sites of pLVTHM-T7-cm. Lentiviral production was performed as described previously (Malashicheva et al. 2007). In brief, 100-mm dishes of subconfluent 293T cells were co-transfected with 15 μg pLVTHM-T7-NICD, 5.27 μg pMD2.G and 9.73 μg pCMV-dR8.74psPAX2 packaging by the calcium-phosphate method. The following day, the medium was changed to the fresh one and the cells were incubated

for 24 h to obtain high-titer virus production. Produced lentivirus was concentrated from the supernatant by ultracentrifugation, resuspended in 1% BSA/PBS and frozen in aliquots at − 80 °C. The virus titer was defined by GFP-expressing virus; the efficiency of MCS transduction was 85–90% by GFP.

The viruses bearing Notch intracellular domain (NICD), Dll1, Dll4 and Jag1 were described previously (Kostina et al. 2016).

qPCR

RNA from cultured cells was isolated using ExtractRNA (Eurogen, Russia). Total RNA (1 μg) was reverse transcribed with MMLV RT kit (Eurogen, Russia). Real-time PCR was performed in the ABI 7500 system (Applied Biosystems, USA) with the following cycling conditions: 10 min at 95 °C, 15 s at 95 °C and 1 min at 60 °C for 40 cycles with a final 4 °C hold, using specific forward and reverse primers for target genes. Corresponding gene expression level was normalized to *GAPDH* from the same samples. The primer sequences are available upon request. The data were analyzed via 7500 Software v2.0.6 and the Ct values were extracted for each gene. A melting curve analysis was performed to verify that the product consisted of a single amplicon. The thresholds and baselines were set according to the manufacturer's instructions (SABiosciences, Qiagen, USA). The data were analyzed using software supplied by Qiagen (http://www.sabiosciences.com/pcr/arrayanalysis.php). The fold change in gene expression (compared to control samples) was calculated using the ΔΔCt method. The gene was considered as differentially expressed if it had an absolute intensity fold-change more than 1.5 compared to the control sample ($P < 0.1$). dChip 2008 software (Cheng Li Lab, Boston, MA) was used to screen for differentially expressed genes and perform hierarchical clustering (Li and Wong 2001). All experiments were performed with three biological replicates, i.e., three independent experimental procedures.

Detection of apoptosis/necrosis

On day 4 of differentiation, MSC were removed from the surface of the plate using trypsin. The cells were suspended in Annexin binding buffer (Biolegend) and stained using AnnexinV-PE (Biolegend) according to the manufacturer's recommendations. Flow cytometry was performed on GuavaEasyCyte8 with detection of Annexin V positive (apoptosis) cells in relative count in percent. At least 10,000 cells were analyzed per each sample. All analyses were performed in triplicates.

Promoter activity assay

To estimate Notch activity, we transfected cells with lentiviral 12XCSL-Luciferase reporter construct and measured CSL activity. Cell lysis was performed using the Luciferase Assay System (Promega) according to the manufacturer's recommendations. Luciferase activity was measured with Synergy2 (BioTek, USA). Samples were normalized by protein content using Pierce BCA Protein Assay Kit (Thermo Scientific).

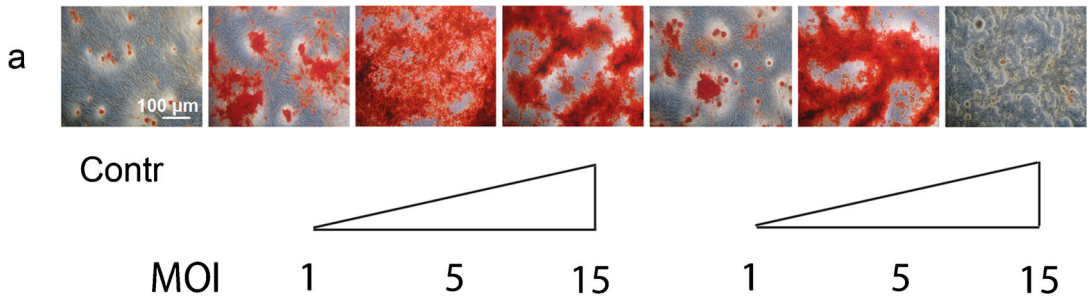
Results

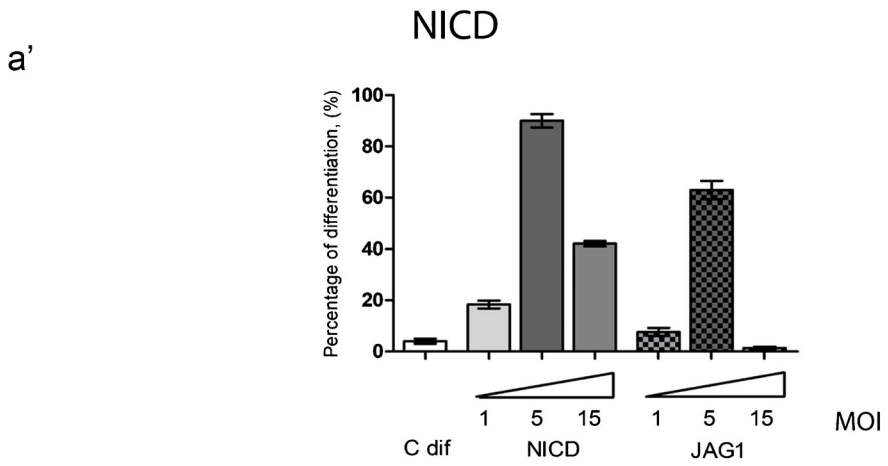
Influence of different components of Notch signaling on osteogenic potential of MSC

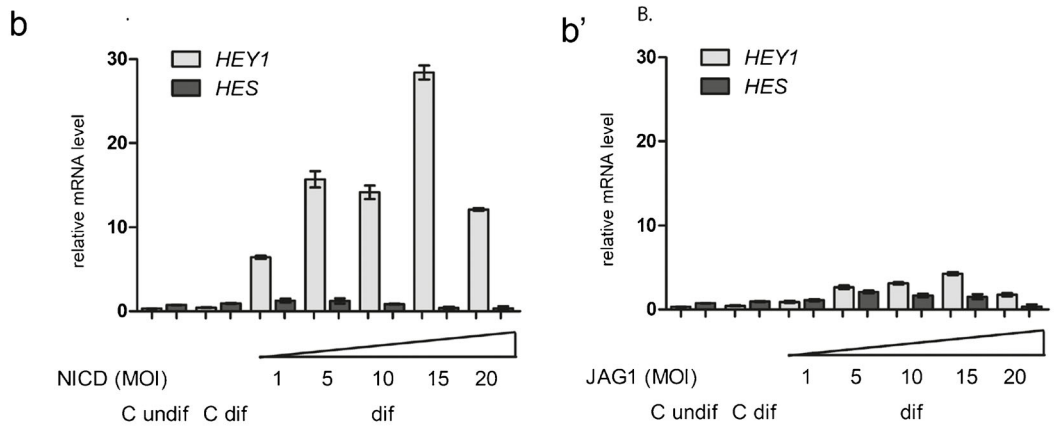
To analyze how various Notch components influence the osteogenic differentiation potential of mesenchymal stem cells (MSC), we used the viruses bearing one of the following Notch ligands; Dll1, Dll4, Jag1 and Notch intracellular domain (NICD) (Kostina et al. 2016). We hypothesized that different dosages of the ligands and NICD could influence osteogenic differentiation of MSC. To test this hypothesis, we transduced MSC with different amounts of corresponding viruses, induced osteogenic differentiation on the following day and analyzed Alizarin staining after 21 days of differentiation (Fig. 1a). Dll1 and Dll4 have not influenced osteogenic potential of MSC (data not shown), whereas NICD and Jag1 both increased Alizarin staining of the cultures at the dosage of 1 and 5 moi (multiplicity of infection) of the viruses; however, the higher dosage of 15 moi inhibited osteogenic differentiation for both NICD and Jag1 (Fig. 1a).

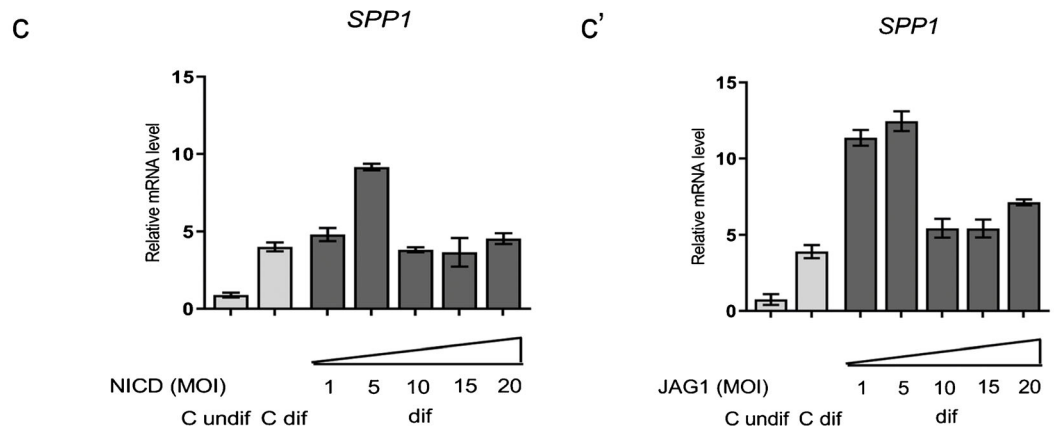
Consequently, we analyzed the activation of Notch target genes *HEY1* and *HES1* in response to the induction of osteogenic differentiation in the presence of different amounts of NICD and Jag1 (Fig. 1b). We observed dose-dependent activation of both *HEY1* and *HES1* transcription in both NICD and Jag1 stimulated cultures. NICD activated a high level of *HEY1* transcription, whereas Jag1 activated only a moderate transcription of *HEY1*. *HES1* transcription was also moderately activated by both NICD and Jag1. The level of activation of both Notch targets was dependent on the dosage of NICD and Jag1. Remarkably, high doses of NICD or Jag1 (20 moi) caused a decrease in expression of both targets.

Next, we analyzed expression of *SPP1* (bone sialoprotein I also known as osteopontin *OPN*), a gene associated with osteoblastic lineage (Merry et al. 1993). Correspondingly, expression of *SPP1* was elevated at the induction of osteogenic differentiation of MSC (Fig. 1c). Transcription of *SPP1* was dose-dependently elevated by NICD and Jag1 at 1 and 5 moi and decreased at a further elevation of NICD and Jag1 dosage.


a
100 µm
Contr
MOI
1
5
15
1
5
15


a'
NICD
JAG1
Percentage of differentiation, (%)
100
80
60
40
20
0
1
5
15
1
5
15
MOI
C dif
NICD
JAG1


b
HEY1
HES
relative mRNA level
30
20
10
0
NICD (MOI)
1
5
10
15
20
C undif
C dif
dif
b'
B.
HEY1
HES
relative mRNA level
30
20
10
0
JAG1 (MOI)
1
5
10
15
20
C undif
C dif
dif


c
SPP1
Relative mRNA level
15
10
5
0
NICD (MOI)
1
5
10
15
20
C undif
C dif
dif
c'
SPP1
Relative mRNA level
15
10
5
0
JAG1 (MOI)
1
5
10
15
20
C undif
C dif
dif

◀ **Fig. 1** NICD and Jag1 influence osteogenic differentiation in a dose-dependent manner. Mesenchymal stem cells (MSC) were transduced with different amounts of lentiviruses bearing either Notch intracellular domain (NICD) or Jag1 and then osteogenic differentiation was induced. Different amounts of viruses correspond to 1, 5 and 15 multiplicity of infection units (MOI). Alizarin red staining of MSC cultures 21 days after the induction of osteogenic differentiation (**a**). Histogram represents estimation of differentiated areas by MosaiX software (**a'**). Expression of Notch target genes *HEY1* and *HES1* in MSC differentiating in the presence of different dosages of NICD (**b**) and Jag1 (**b'**). Expression of proosteogenic gene *SPP1* in MSC differentiating in the presence of different dosages of NICD (**c**) and Jag1 (**c'**). Undif: undifferentiated cells, dif: the cells induced to osteogenic differentiation

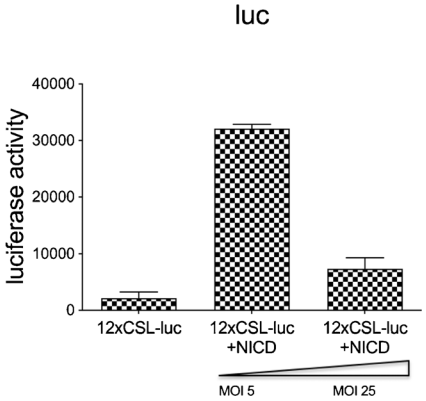


Fig. 2 NICD influences CSL-dependent transcription in a dose-dependent manner. Mesenchymal stem cells (MSC) were co-transduced with different amounts of lentivirus bearing Notch intracellular domain (NICD) and 12xCSL-Luciferase. Different amounts of NICD virus correspond to 5 and 25 multiplicity of infection units (MOI). CSL activity was analyzed using Luciferase Assay System

The data shown here suggest that both NICD and Jag1 are capable of promoting osteogenic differentiation of MSC in a dose-dependent manner most probably through dose-dependent activation of transcription of downstream targets. However, a high dosage of Notch activation causes inhibition of high levels of osteogenic differentiation.

High dosage of Notch decreases CSL-dependent transcription

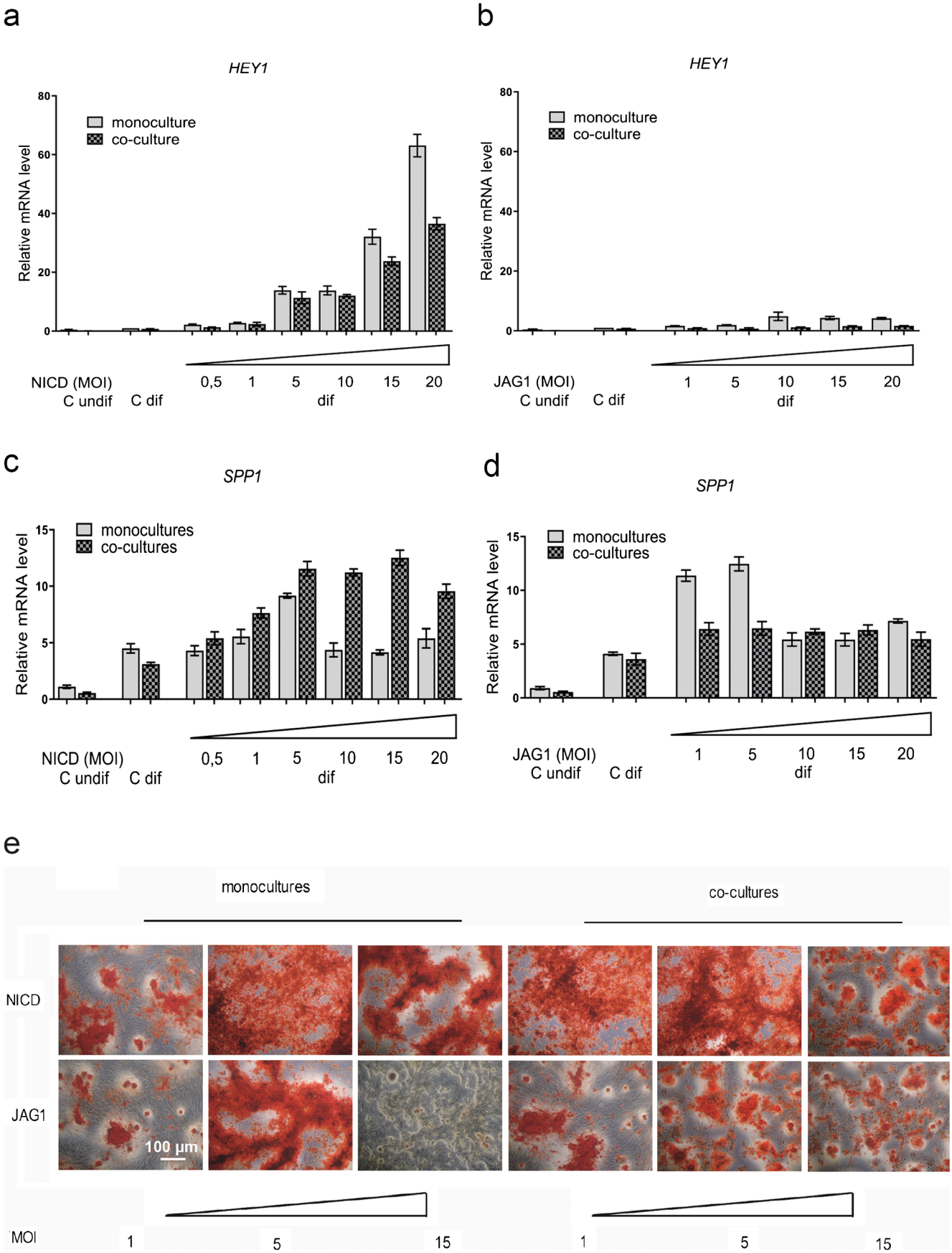
A high dosage of Notch activation caused a decrease in expression of both Notch targets and inhibition of high levels of osteogenic differentiation. To verify how activated Notch operates in MSC at the CSL level, we co-transduced cells with different amounts of lentivirus bearing NICD and 12xCSL-Luciferase and measured CSL luciferase activity using Luciferase Assay System. Elevation of NICD caused an increase of CSL activity and further elevation of NICD dosage caused a decrease of CSL activity (Fig. 2). Thus, it seems that NICD-induced transcription depends on a fine-tuned dosage and high doses of Notch activation could inhibit transcription of Notch target genes.

Intercellular communications contribute to the level of osteogenic differentiation

We noticed a more moderate activation of *HEY1* transcription, when we transduced MSC with various dosages of Jag1 compared to NICD. We hypothesized that the lateral regulation known for Notch signaling (Sjöqvist and Andersson 2017) could be involved in osteogenic induction. Lateral regulation is especially important in intercellular communication and tissue-specific differentiation decisions. Therefore, to elucidate how intercellular communication between MSC contributes to osteogenic differentiation, we compared NICD and Jag1 induced target gene activation in monoculture and in co-culture. In the first type of experiment, we directly introduced NICD and Jag1 bearing viruses in different dosages (direct induction) and induced osteogenic differentiation; for the induction in co-culture, we first transduced corresponding viruses into half of the MSC, then added the second half of the MSC and then induced osteogenic differentiation (see "Materials and methods"). Then, we analyzed the expression of Notch target gene *HEY1* and osteogenic gene *SPP1* and stained differentiated cells for Alizarin (Fig. 3). NICD activated *HEY1* in a dose-dependent manner in monoculture and to a lesser extent in co-culture (Fig. 3a). However, the level of proosteogenic *SPP1* dropped at a high dosage of NICD (Fig. 1c), and correspondingly, the efficiency of differentiation decreased at a high dosage of NICD in both monoculture and co-culture (Fig. 3e). Thus, both NICD in monoculture and co-culture acts in a similar dose-dependent manner and a high dosage of NICD inhibits osteogenic differentiation.

Next, we analyzed if Jag1 had the same mode of action. In both monoculture and co-culture, Jag1 induced moderate dose-dependent activation of *HEY1* transcription (Fig. 3b). Jag1 caused expression of proosteogenic *SPP1* in a dose-dependent manner with its decrease at high concentrations of Jag1 in monoculture but not in co-culture (Fig. 3d). In co-culture, the expression level of *SPP1* was the same at all the concentrations of Jag1 and osteogenic potential estimated by Alizarin staining remained more or less the same regardless the dosage of Jag1 (Fig. 3e).

Thus, Jag1 dose-dependent action in promoting osteogenic differentiation is different in monoculture and co-culture. In contrast to Jag1 action in monoculture, intercellular communications somehow are able to regulate a high dosage of Jag1 in co-culture and support osteogenic differentiation at the same level.


a
HEY1
monoculture
co-culture
Relative mRNA level
NICD (MOI)
C undif
C dif
0,5
1
5
10
15
20
dif
b
HEY1
monoculture
co-culture
Relative mRNA level
JAG1 (MOI)
C undif
C dif
1
5
10
15
20
dif
c
SPP1
monocultures
co-cultures
Relative mRNA level
NICD (MOI)
C undif
C dif
0,5
1
5
10
15
20
dif
d
SPP1
monocultures
co-cultures
Relative mRNA level
JAG1 (MOI)
C undif
C dif
1
5
10
15
20
dif
e
monocultures
co-cultures
NICD
JAG1
100 µm
MOI
1
5
15
1
5
15

◀ **Fig. 3** NICD and Jag1 act differently in monoculture and co-culture. MSC were seeded at a density of 80 × 10^3/cm^2 and corresponding lentiviral concentrates were added immediately to the culture medium; after 24 h, osteogenic differentiation was induced. For co-culture experiments, the cells were seeded at a density of 40 × 10^3/cm^2, corresponding lentiviral concentrates were added immediately to the culture medium; after 24 h, the cells at a density 40 × 10^3/cm^2 were added to the same culture well and after 24 h, osteogenic differentiation was induced. Different amounts of viruses correspond to 1, 5, 10, 15 and 20 multiplicity of infection units (MOI). Expression of Notch target genes *HEY1* in MSC differentiating in the presence of different dosages of NICD in monoculture and co-culture (**a**). Expression of Notch target genes *HEY1* in MSC differentiating in the presence of different dosages of Jag1 in monoculture and co-culture (**b**). Expression of proosteogenic gene *SPP1* in MSC differentiating in the presence of different dosages of NICD in monoculture and co-culture (**c**). Expression of proosteogenic gene *SPP1* in MSC differentiating in the presence of different dosages of Jag1 in monoculture and co-culture (**d**). Alizarin red staining of MSC cultures 21 days after the induction of osteogenic differentiation (**e**). Undif: undifferentiated cells, dif: the cells induced to osteogenic differentiation

Induction of Notch genes in monoculture and co-culture

To elucidate how Notch genes are induced by NICD in monoculture and co-culture, we introduced different doses of NICD to MSC in monoculture and co-culture correspondingly, induced osteogenic differentiation and analyzed expression levels of *NOTCH1*, *NOTCH2*, *NOTCH3*, *NOTCH4*, *DLL1*, *DLL4* and *JAG1* (Fig. 4) 3 days after the induction of differentiation. The amount of NICD corresponding to 10 moi applied in co-culture activated substantially elevated transcription of *NOTCH1*, *NOTCH3*, *NOTCH4*, *DLL1* and *DLL4*. Transcription of *JAG1* was activated in a dose-dependent manner in both co-culture and mono-culture. Along with the data presented in the previous sections about the influence of NICD dosage on osteogenic differentiation, our results suggest that "an optimal" NICD dosage could be used to elevate the efficiency of osteogenic differentiation and this "optimal dosage" is achieved through a concerted action of several Notch components.

High dosages of NICD and Jag1 cause apoptosis of MSC

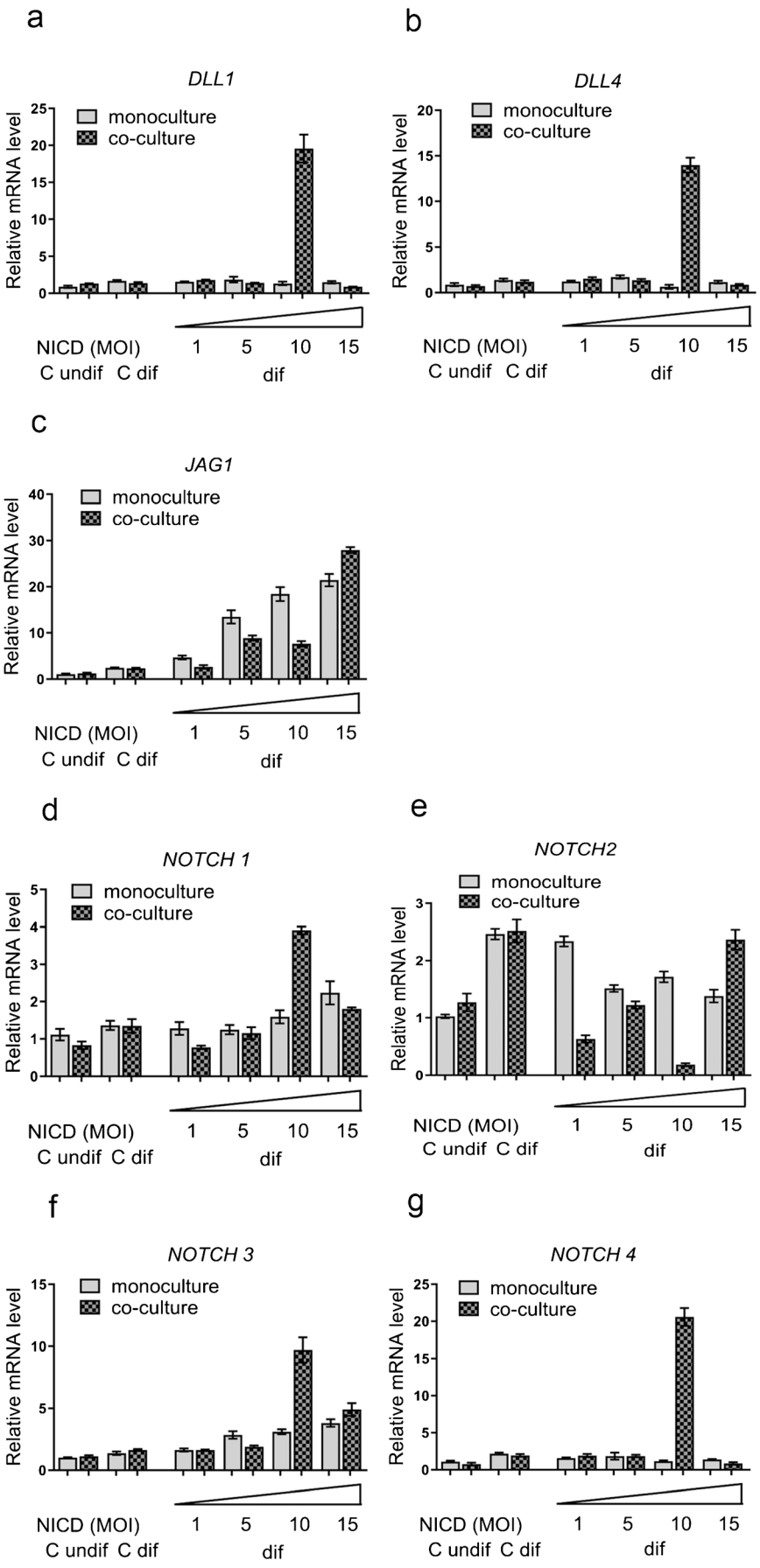
Both NICD and Jag1 promoted osteogenic differentiation of MSC in a dose-dependent manner in monoculture; however, at a high dosage of both NICD and Jag1, we observed a decrease in MSC differentiation. We transduced MSC with various dosages of NICD and Jag1 and used GFP-bearing virus as a control to lentiviral infection cytotoxicity. Three days after transduction, we verified the level of apoptosis by Annexin V labeling using flow cytometry (Fig. 5). In undifferentiated cells, GFP-bearing virus caused a slight induction of apoptosis in MSC close to background. A similar level of apoptosis was detected when NICD-bearing virus was added to undifferentiated MSC. However, a high dosage of Jag1 corresponding to 10 moi induced visible elevation of apoptotic cell percentage (up to 10%). MSC induced to osteogenic differentiation were more sensible to apoptotic induction by NICD and even more sensible to Jag1 in a dose-dependent manner. The data suggest that a high dosage of both NICD and to a greater extent of Jag1 induces apoptosis in differentiating MSC thus preventing further differentiation, which corresponds to the data obtained by staining with Alizarin red (Fig. 1 a).

Discussion

We have shown here that activation of Notch by either NICD or Jag1 increases osteogenic differentiation capacity of mesenchymal stem cells in a dose-dependent manner but high doses of activation of the signaling cause a decrease in osteogenic differentiation. We also showed the differences between a dose-dependent response of the cells to Jag1 or NICD activation when Jag1 and NICD activation is applied in co-culture with only half of the cells bearing the exogenous Notch signal.

The dose-dependent manner of Notch has been described in some cellular contexts. Depending on the level of activation, Notch induced opposite responses in the same cell type (Guentchev and McKay 2006; Kristoffersen et al. 2013). To our knowledge, there has been no description of dose-dependent action of Notch for osteogenic lineages.

The question about the role of Notch in supporting osteogenic differentiation still remains unresolved and controversial. There is a considerable piece of data supporting its promoting role in osteogenic differentiation, while at the same time, some data support the opposite view. Several in vivo and in vitro models are employed to explore the role of Notch in osteogenic differentiation. Mouse models for the study of Notch signaling include Notch1-4 null knockout mice. Global inactivation of Notch1,2 leads to early embryonic lethality and the role of Notch3,4 in the skeleton has not been described (Canalis 2018). Conditional activation of NICD of either Notch1 or Notch2 was detrimental to normal bone formation (Deregowski et al. 2006; Zanotti and Canalis 2014; Canalis et al. 2016). This view was supported by in vitro experiments: stable Notch activation in C2C12 myoblastic cells, ST-2 stromal cells and osteoblastic MC-3T3 cells suppressed osteoblastic differentiation (Nofziger et al. 1999; Sciaudone et al. 2003; Deregowski et al. 2006). At the same time, NICD activation in MC3T3-E1, C3H10T1/2 lines and human MSC stimulated osteogenic differentiation (Tezuka et al. 2002; Ugarte et al. 2009). Thus, both in vivo and in vitro activation and inactivation studies give contradictory signals. However, another kind of experiment such as analysis of Notch genes activation in the course of osteogenic


a
DLL1
monoculture
co-culture
Relative mRNA level
25
20
15
10
5
0
NICD (MOI)
C undif
C dif
1
5
10
15
dif
b
DLL4
monoculture
co-culture
Relative mRNA level
20
15
10
5
0
NICD (MOI)
C undif
C dif
1
5
10
15
dif
c
JAG1
monoculture
co-culture
Relative mRNA level
40
30
20
10
0
NICD (MOI)
C undif
C dif
1
5
10
15
dif
d
NOTCH 1
monoculture
co-culture
Relative mRNA level
5
4
3
2
1
0
NICD (MOI)
C undif
C dif
1
5
10
15
dif
e
NOTCH2
monoculture
co-culture
Relative mRNA level
3
2
1
0
NICD (MOI)
C undif
C dif
1
5
10
15
dif
f
NOTCH 3
monoculture
co-culture
Relative mRNA level
15
10
5
0
NICD (MOI)
C undif
C dif
1
5
10
15
dif
g
NOTCH 4
monoculture
co-culture
Relative mRNA level
25
20
15
10
5
0
NICD (MOI)
C undif
C dif
1
5
10
15
dif

◀ **Fig. 4** Expression of Notch component genes in MSC transduced with different doses of lentiviruses bearing NICD in co-culture and monoculture. Different amounts of viruses correspond to 1, 5, 10 and 15 multiplicity of infection units (MOI). NICD-bearing viruses were added to either monocultures or co-cultures of MSC (see figure legend of Fig. 2). Undif: undifferentiated cells, dif: the cells induced to osteogenic differentiation

differentiation revealed that Notch genes are activated during the differentiating process with a definite timing of activation/inactivation (Ongaro et al. 2016; Urbanek et al. 2017; Bagheri et al. 2018), which supports the promoting role of Notch in osteogenic differentiation. A recent work proposed a two-phase action model of Notch signaling in osteogenesis, where activation of Notch signaling in early stages of osteoblast differentiation results in proliferation of immature preosteoblast lineage cells and activation in late stages promotes differentiation of osteoblasts into osteocytes (Ji et al. 2017). It has been shown recently that the inhibitory or promoting role of Notch could also be regulated by microRNAs (Liao et al. 2017a, 2017b). However, none of these works considered the signal dosage as a possible reason of contradictions.

Notch-signaling is extremely dose sensitive due to the lack of a signal amplification step or utilization of secondary messengers to transmit the signal from the cell surface to the nucleus (Yamamoto et al. 2014). By altering the amount of ligands and receptors expressed in a cell, numerous scenarios of Notch activation patterns can be generated. We suggest that the initial level of Notch signal could be critical for the cell fate decision in relation to osteogenic differentiation. How this tight regulation is controlled at a molecular level is the subject of future studies.

We observed the differences in activating effects of NICD and Jag1 when used in co-culture experiments. It seemed that Jag1 had more power to regulate the strength and toxicity of the signal when used in co-culture. This finding is well in line with the recently described importance of Jag1 ligand in osteoblast lineage cells directly altering bone geometry and bone mass (Youngstrom et al. 2016) and also with a phenomenon that delivery of Jag1 using clinically applicable collagen sponges significantly enhances bone healing without excess ectopic bone (Youngstrom et al. 2017). A known clinical problem in bone regeneration is that the methods based on overproduction of bone tissue by bone morphogenetic proteins (BMPs) could include potentially fatal ectopic bone and malignant transformation. We in the present study and the group of Hankenson in their recent paper show that fine-tuning dose of Notch ligands will be an important aspect of promoting a physiologically appropriate bone response (Youngstrom et al. 2017). Unlike BMP2, Jag1 appears to promote a bone-prone phenotype only among cell types that are predisposed to form bone, such as post-injury mesenchyme: enhancing the safety profile bone (Youngstrom et al. 2017). Together, the data suggest that Jag1 treatment in co-culture could be more therapeutically applicable. Long-term safety studies of Notch-activating therapies in bone will be required to prove this suggestion.

We propose that extreme sensitivity of Notch signaling to the dosage defines that even slight perturbations of the strength of the signaling could cause detrimental effects on tissue differentiation state. Thus, considering the dosage of the signaling is a very important issue.

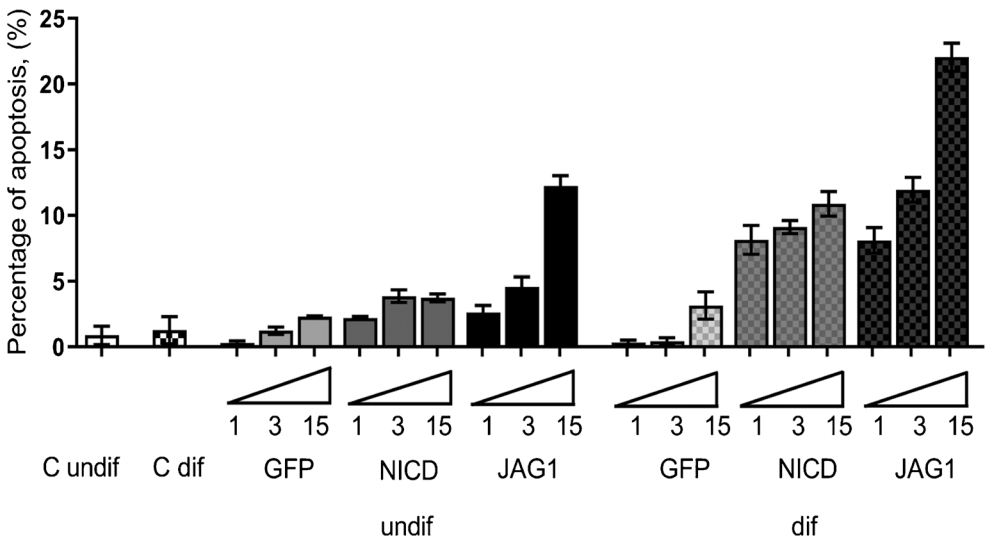


Fig. 5 High doses of NICD and Jag1 induce apoptosis in MSC induced to osteogenic differentiation. Mesenchymal stem cells (MSC) were transduced with different amounts of lentiviruses bearing either Notch intracellular domain (NICD) or Jag1 and then osteogenic differentiation was induced for 3 days where indicated. Undifferentiated cells were also harvested after 3 days of culture. Different amounts of viruses correspond to 1, 3 and 15 multiplicity of infection units (MOI). GFP-bearing virus was used as a control. Annexin V labeling was analyzed by flow cytometry. Undif: undifferentiated cells, dif: the cells induced to osteogenic differentiation

Funding This work was supported by the grant of Russian Science Foundation 18-14-00152.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

References

Andersson ER, Sandberg R, Lendahl U (2011) Notch signaling: simplicity in design, versatility in function. Development **138**(17):3593–3612

Bagheri L, Pellati A, Rizzo P, Aquila G, Massari L, De Mattei M, Ongaro A (2018) Notch pathway is active during osteogenic differentiation of human bone marrow mesenchymal stem cells induced by pulsed electromagnetic fields. J Tissue Eng Regen Med **12**(2):304–315

Bai S, Kopan R, Zou W, Hilton MJ, Ong C-t, Long F, Ross FP, Teitelbaum SL (2008) NOTCH1 regulates osteoclastogenesis directly in osteoclast precursors and indirectly via osteoblast lineage cells. J Biol Chem **283**(10):6509–6518

Canalis E (2018) Notch in skeletal physiology and disease. Osteoporos Int 29:2611–2621

Canalis E, Bridgewater D, Schilling L, Zanotti S (2016) Canonical Notch activation in osteocytes causes osteopetrosis. Am J Physiol Endocrinol Metab **310**(2):E171–E182

Cao J, Wei Y, Lian J, Yang L, Zhang X, Xie J, Liu Q, Luo J, He B, Tang M (2017) Notch signaling pathway promotes osteogenic differentiation of mesenchymal stem cells by enhancing BMP9/Smad signaling. Int J Mol Med **40**(2):378–388

Cui J, Zhang W, Huang E, Wang J, Liao J, Li R, Yu X, Zhao C, Zeng Z, Shu Y, Zhang R, Yan S, Lei J, Yang C, Wu K, Wu Y, Huang S, Ji X, Li A, Gong C, Yuan C, Zhang L, Liu W, Huang B, Feng Y, An L, Zhang B, Dai Z, Shen Y, Luo W, Wang X, Huang A, Luu HH, Reid RR, Wolf JM, Thinakaran G, Lee MJ, He TC (2018) BMP9-induced osteoblastic differentiation requires functional Notch signaling in mesenchymal stem cells. Lab Investig 99:58–71

Deregowski V, Gazzerro E, Priest L, Rydziel S, Canalis E (2006) Notch 1 overexpression inhibits osteoblastogenesis by suppressing Wnt/β-catenin but not bone morphogenetic protein signaling. J Biol Chem **281**(10):6203–6210

Dmitrieva RI, Revittser AV, Klukina MA, Sviryaev YV, Korostovtseva LS, Kostareva AA, Zaritskey AY, Shlyakhto EV (2015) Functional properties of bone marrow derived multipotent mesenchymal stromal cells are altered in heart failure patients, and could be corrected by adjustment of expansion strategies. Aging (Albany NY) **7**(1):14

Doi H, Iso T, Sato H, Yamazaki M, Matsui H, Tanaka T, Manabe I, Arai M, Nagai R, Kurabayashi M (2006) Jagged1-selective notch signaling induces smooth muscle differentiation via a RBP-Jκ-dependent pathway. J Biol Chem **281**(39):28555–28564

Engin F, Yao Z, Yang T, Zhou G, Bertin T, Jiang MM, Chen Y, Wang L, Zheng H, Sutton RE (2008) Dimorphic effects of Notch signaling in bone homeostasis. Nat Med **14**(3):299

Guentchev M, McKay RD (2006) Notch controls proliferation and differentiation of stem cells in a dose-dependent manner. Eur J Neurosci **23**(9):2289–2296

Guruharsha KG, Kankel MW, Artavanis-Tsakonas S (2012) The Notch signalling system: recent insights into the complexity of a conserved pathway. Nat Rev Genet **13**(9):654–666

Hilton MJ, Tu X, Wu X, Bai S, Zhao H, Kobayashi T, Kronenberg HM, Teitelbaum SL, Ross FP, Kopan R (2008a) Notch signaling maintains bone marrow mesenchymal progenitors by suppressing osteoblast differentiation. Nat Med **14**(3):306

Hilton MJ, Tu X, Wu X, Bai S, Zhao H, Kobayashi T, Kronenberg HM, Teitelbaum SL, Ross FP, Kopan R, Long F (2008b) Notch signaling maintains bone marrow mesenchymal progenitors by suppressing osteoblast differentiation. Nat Med **14**(3):306–314

Ji Y, Ke Y, Gao S (2017) Intermittent activation of notch signaling promotes bone formation. Am J Transl Res **9**(6):2933–2944

Kostina AS, Uspensky VE, Irtyuga OB, Ignatieva EV, Freylikhman O, Gavriliuk ND, Moiseeva OM, Zhuk S, Tomilin A, Kostareva AA, Malashicheva AB (2016) Notch-dependent EMT is attenuated in patients with aortic aneurysm and bicuspid aortic valve. Biochim Biophys Acta (BBA) - Mol Basis Dis **1862**(4):733–740

Kovall RA, Gebelein B, Sprinzak D, Kopan R (2017) The canonical Notch signaling pathway: structural and biochemical insights into shape, sugar, and force. Dev Cell **41**(3):228–241

Kristoffersen K, Villingshøj M, Poulsen HS, Stockhausen M-T (2013) Level of Notch activation determines the effect on growth and stem cell-like features in glioblastoma multiforme neurosphere cultures. Cancer Biol Ther **14**(7):625–637

Liao J, Wei Q, Zou Y, Fan J, Song D, Cui J, Zhang W, Zhu Y, Ma C, Hu X, Qu X, Chen L, Yu X, Zhang Z, Wang C, Zhao C, Zeng Z, Zhang R, Yan S, Wu T, Wu X, Shu Y, Lei J, Li Y, Luu HH, Lee MJ, Reid RR, Ameer GA, Wolf JM, He TC, Huang W (2017a) Notch Signaling Augments BMP9-Induced Bone Formation by Promoting the Osteogenesis-Angiogenesis Coupling Process in Mesenchymal Stem Cells (MSCs). Cell Physiol Biochem **41**(5):1905–1923

Liao J, Yu X, Hu X, Fan J, Wang J, Zhang Z, Zhao C, Zeng Z, Shu Y, Zhang R, Yan S, Li Y, Zhang W, Cui J, Ma C, Li L, Yu Y, Wu T, Wu X, Lei J, Wang J, Yang C, Wu K, Wu Y, Tang J, He BC, Deng ZL, Luu HH, Haydon RC, Reid RR, Lee MJ, Wolf JM, Huang W, He TC (2017b) lncRNA H19 mediates BMP9-induced osteogenic differentiation of mesenchymal stem cells (MSCs) through Notch signaling. Oncotarget **8**(32):53581–53601

Liu P, Ping Y, Ma M, Zhang D, Liu C, Zaidi S, Gao S, Ji Y, Lou F, Yu F, Lu P, Stachnik A, Bai M, Wei C, Zhang L, Wang K, Chen R, New MI, Rowe DW, Yuen T, Sun L, Zaidi M (2016) Anabolic actions of Notch on mature bone. Proc Natl Acad Sci U S A **113**(15):E2152–E2161

Malashicheva A, Kanzler B, Tolkunova E, Trono D, Tomilin A (2007) Lentivirus as a tool for lineage-specific gene manipulations. Genesis **45**(7):456–459

Malashicheva A, Bogdanova M, Zabirnyk A, Smolina N, Ignatieva E, Freilikhman O, Fedorov A, Dmitrieva R, Sjöberg G, Sejersen T (2015) Various lamin A/C mutations alter expression profile of mesenchymal stem cells in mutation specific manner. Mol Genet Metab **115**(2):118–127

Merry K, Dodds R, Littlewood A, Gowen M (1993) Expression of osteopontin mRNA by osteoclasts and osteoblasts in modelling adult human bone. J Cell Sci **104**(**Pt 4**):1013–1020

Nofziger D, Miyamoto A, Lyons KM, Weinmaster G (1999) Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. Development **126**(8):1689–1702

Ongaro A, Pellati A, Bagheri L, Rizzo P, Caliceti C, Massari L, De Mattei M (2016) Characterization of Notch Signaling During Osteogenic Differentiation in Human Osteosarcoma Cell Line MG63. J Cell Physiol **231**(12):2652–2663

Salie R, Kneissel M, Vukevic M, Zamurovic N, Kramer I, Evans G, Gerwin N, Mueller M, Kinzel B, Susa M (2010) Ubiquitous

overexpression of Hey1 transcription factor leads to osteopenia and chondrocyte hypertrophy in bone. Bone **46**(3):680–694

Sciaudone M, Gazzerro E, Priest L, Delany AM, Canalis E (2003) Notch 1 impairs osteoblastic cell differentiation. Endocrinology **144**(12): 5631–5639

Shimizu T, Tanaka T, Iso T, Matsui H, Ooyama Y, Kawai-Kowase K, Arai M, Kurabayashi M (2011) Notch signaling pathway enhances bone morphogenetic protein 2 (BMP2) responsiveness of Msx2 gene to induce osteogenic differentiation and mineralization of vascular smooth muscle cells. J Biol Chem **286**(21):19138–19148

Shindo K, Kawashima N, Sakamoto K, Yamaguchi A, Umezawa A, Takagi M, Katsube K, Suda H (2003) Osteogenic differentiation of the mesenchymal progenitor cells, Kusa is suppressed by Notch signaling. Exp Cell Res **290**(2):370–380

Sjöqvist M, Andersson ER (2017) Do as I say, Not (ch) as I do: lateral control of cell fate. Dev Biol 447:58–70

Tezuka KI, Yasuda M, Watanabe N, Morimura N, Kuroda K, Miyatani S, Hozumi N (2002) Stimulation of osteoblastic cell differentiation by Notch. J Bone Miner Res **17**(2):231–239

Ugarte F, Ryser M, Thieme S, Fierro FA, Navratiel K, Bornhauser M, Brenner S (2009) Notch signaling enhances osteogenic differentiation while inhibiting adipogenesis in primary human bone marrow stromal cells. Exp Hematol **37**(7):867–875.e861

Urbanek K, Lesiak M, Krakowian D, Koryciak-Komarska H, Likus W, Czekaj P, Kusz D, Sieron AL (2017) Notch signaling pathway and gene expression profiles during early in vitro differentiation of liver-derived mesenchymal stromal cells to osteoblasts. Lab Investig **97**(10):1225–1234

Yamamoto S, Schulze KL, Bellen HJ (2014) Introduction to Notch signaling. Methods Mols Biol **1187**:1–14

Youngstrom DW, Dishowitz MI, Bales CB, Carr E, Mutyaba PL, Kozloff KM, Shitaye H, Hankenson KD, Loomes KM (2016) Jagged1 expression by osteoblast-lineage cells regulates trabecular bone mass and periosteal expansion in mice. Bone **91**:64–74

Youngstrom DW, Senos R, Zondervan RL, Brodeur JD, Lints AR, Young DR, Mitchell TL, Moore ME, Myers MH, Tseng WJ, Loomes KM, Hankenson KD (2017) Intraoperative delivery of the Notch ligand Jagged-1 regenerates appendicular and craniofacial bone defects. NPJ Regen Med **2**:32

Zanotti S, Canalis E (2014) Notch1 and Notch2 expression in osteoblast precursors regulates femoral microarchitecture. Bone **62**:22–28

Zanotti S, Canalis E (2016) Notch signaling and the skeleton. Endocr Rev **37**(3):223–253

Zanotti S, Smerdel-Ramoya A, Stadmeyer L, Durant D, Radtke F, Canalis E (2008) Notch inhibits osteoblast differentiation and causes osteopenia. Endocrinology **149**(8):3890–3899