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Role of Notch expression in premature senescence of murine bone marrow stromal cells

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Abstract

The aim of the present study was to investigate the role of the Notch signaling pathway in premature senescence of murine bone marrow stromal cells *in vitro*. The intracellular domain of Notch 1 (ICN) was transfected into cultured murine bone marrow stromal cells by lipofectamine transfection. After three days, the proliferation of transfected cells was measured by MTT assay. Cell cycle distribution was analyzed by flow cytometry. Senescence-associated beta-galactosidase (SA-beta-gal) was measured, and the percentage of positive cells was evaluated by assessing 1000 cells in random fields of view. The expressions of p53 and $p21^{Cip1/Waf1}$ were analyzed by both RT-PCR and Western blot analysis. The results showed that activation of Notch signaling inhibited proliferation of murine bone marrow stromal cells with induction of G_1 arrest, increased the percentage of SA-beta-gal positive cells, and upregulated p53 and $p21^{Cip1/Waf1}$ mRNA and protein expression levels. Thus, the activated Notch signaling could induce premature senescence of bone marrow stromal cells through the p53-$p21^{Cip1/Waf1}$ pathway.

Keywords: Bone marrow stromal cells; Premature senescence; Notch signaling pathway

1. Introduction

Bone marrow stromal cells (MSCs) are important components of the hematopoietic inducible microenvironment (HIM). Previous studies have shown that MSCs constitutively secrete many cytokines that promote the homing or proliferation and differentiation of hematopoietic cells, and hence play important roles in promoting hematopoietic recovery [1].

Radiation injury is a major and refractory trauma caused by nuclear weapons and accidents, and is also a common complication in patients receiving radiation therapy. Although there is increasing evidence that radiation can alter the HIM, the mechanisms of radiation-induced damage to MSCs remain largely unknown.

Radiation can cause apoptosis, cell cycle arrest, or premature senescence by inducing DNA damage. However, the outcomes are different for different cell types [2]. Many studies have shown that MSCs have a very low sensitivity towards radiation-induced apoptosis, although there are more recent contradictory data reported [3]. Cell senescence is a relatively stable state. Senescent cells lose their proliferative potential and exit from the cell cycle with induction of permanent growth arrest. Thus, senescent cells remain alive but have some altered functions. Importantly, we have previously shown that ^{60}Co-γ irradiation inhibited the proliferation of MSCs by blocking them in the G_1 stage. These cells possessed the morphological features of premature senescence, and SA-β-gal activity, a biological marker

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for premature senescence cells, was also increased [4]. Thus, we proposed that premature senescence might be a key mechanism of radiation-induced damage to MSCs, although the mechanism of how radiation induces premature senescence of MSCs is unknown.

The Notch receptor is a single-span transmembrane receptor that is activated through direct contact with receptor-bound ligands. Four Notch receptors, termed Notch 1, 2, 3 and 4, respectively, have been identified. They were found being activated by the Delta 1-4 and Serrate/Jagged 1 and 2 ligands [5,6]. Notch ligand interaction results in proteolytic cleavage of the Notch receptor with release of the intracellular domain (ICN) [7], which, after translocation to the nucleus, interacts with the CSL (CBF1/RBP-Jκ, SuH and Lag-1) family of DNA binding proteins [8,9]. These nuclear proteins include C promoter binding factor (CBF) 1 or recombination signal binding protein of the Jκ Ig gene (RBP-Jκ) in mammals, suppressor of Hairless (Su(H)) in *Drosophila* and Lag-1 in *Caenorhabditis elegans* [10–12]. CSL transcription factors bind to the promoter region of the Enhancer of Split (E(spl)) or its mammalian homolog, the Hairy Enhancer of Split-1 (HES-1) and the HES-5 complex of genes, resulting in repression of transcription after recruitment of a corepressor complex [12]. Notch signaling modulates cell survival, death, proliferation and differentiation [13]. Constitutive expression of active ICN in targeted cells also results in an "activated" Notch phenotype [14,15]. Notch was recently reported to be involved in the process of kidney irradiation injury [16], and we have previously shown that the Notch signaling pathway is involved in radiation injury of MSCs. Further, there is evidence for the interaction between Notch and NF-kappa B [17]; and that NF-kappa B plays an important role in radiation injury of MSCs.

Thus, the aim of the present study was to investigate the role of Notch in premature senescence of murine MSCs *in vitro* by transfection of the exogenous ICN.

2. Materials and methods

2.1. Animals

Inbreeding line Balb/c mice (both males and females, 6–8 weeks old, 18–22 g) were provided by the Laboratory Animal Center of Tongji Medical College. The Institutional Animal Care and Use Committee of Huazhong University of Science and Technology, approved all experimental procedures.

2.2. Reagents and plasmid

Tetramethyl-azo-amytal-zole (MTT) and X-galactoside were purchased from Amersco (Solon, USA). Lipofectamine 2000 and anti-His-antibody were purchased from Invitrogen (Carlsbad, USA). Anti-p53 antibody, anti-$p21^{Cip1/Waf1}$ antibody, biotinylated goat anti-mouse secondary antibody and FITC labeled goat anti-mouse secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). A FACSort flow cytometer was from Becton Dickinson (San Jose, USA).

Plasmid pcDNA3.1C-ICN (denoted as ICN) was a gift from Professor Tom Kadesch (Department of Genetics, University of Pennsylvania School of Medicine, USA). ICN was generated by PCR and was cloned into the vector pcDNA3.1C at the XhoI or KpnI sites, which contains a consensus kazak start site and was cloned in-frame with the myc-his tags. The correct gene sequence contained in this plasmid was confirmed by gene sequencing analysis.

2.3. Isolation and culture of MSCs

MSCs were isolated from the long bones of the mice, and cultured at a density of 1×10^6 cells/ml in Iscove's Modified Dulbecco's medium (IMDM) supplemented with 2 M glutamine, 10% fetal calf serum, 100 U/ml PG and 100 U/ml streptomycin at 37 °C in a 5% CO_2 atmosphere. The culture medium was changed every 3–5 days. And the cells from the fourth generation onwards were used for experiments.

2.4. Detections of activation of Notch signaling pathway and premature senescence of MSCs

2.4.1. Transfection of ICN

The MSCs were inoculated onto 24- or 96-well plates, and ICN or empty plasmid transfection was performed as soon as the cell growth reached above 80% confluence. The transfection of ICN was performed using lipofectamine 2000 reagent according to the manufacturer's instruction. Three experimental groups were set including a non-transfected group, an empty plasmid transfected group, and an ICN-transfected group. All experiments were performed in triplicate.

2.4.2. ICN protein expression in MSCs detected by flow cytometry

The MSCs were harvested after 48 h transfection, washed twice in PBS, and fixed with 2% paraformaldehyde containing 0.1% Triton X-100 at 4 °C for 30 min. Next, normal goat serum was added and incubated at 4 °C for 30 min, and the supernatant then discarded. The diluted anti-His Ab (1:100) was added and incubated at 4 °C for 30 min. The MSCs were then washed twice in PBS. Next, the FITC-labeled secondary antibody (1:50) was added and incubated at 4 °C for 30 min, followed by two washes in PBS, then detected by flow cytometry.

2.4.3. Proliferation rate of MSCs measured by MTT assay

MSCs in 180 μl IMDM were inoculated onto 96-well plates. Four parallel rows of wells were set for each group. MTT assay was performed three days after transfection.

2.4.4. Cell cycle analysis of MSCs by flow cytometry

MSCs were collected three days after transfection. The cell cycle study was performed with a flow cytometer, and the data were analyzed with Multicycle software (Phoenix Flow Systems Inc., USA).

2.4.5. Detection of β galactoside (SA-β-gal) activity

MSCs were inoculated onto 24-well plates. Three days after transfection, the SA-β-gal activity was detected as previously described [18].

2.4.6. Detection of p53 and $p21^{Cip1/Waf1}$ mRNA expression in MSCs by RT-PCR

MSCs were collected three days after transfection, and *p53* and $p21^{Cip1/Waf1}$ mRNA expressions were detected by RT-PCR. The sequences of primers used were CAC GTACTCTCCTCCCCTCAA (sense) and GGCTCATAA GGTACCACCACG (anti-sense) for *p53*; AATCCTGG TGATGTCCGACC (sense) and AAAGTTCCACCGT TCTCGG (anti-sense) for $p21^{Cip1/Waf1}$; and ACCACAGT CCATGCCATCAC (sense) and TCCACCACCCTGTTG CTGTA (anti-sense) for *GAPDH*. The PCR conditions for *p53* and $p21^{Cip1/Waf1}$ were 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 60 °C for 40 s and 72 °C for 50 s; and an extension at 72 °C for 7 min. The PCR conditions for *GAPDH* were 94 °C for 5 min; 30 cycles of 94 °C for 15 s, 58 °C for 45 s and 72 °C for 1 min; and an extension at 72 °C for 7 min. The amplified PCR products were electrophoresed on agarose gels, and the fragments were analyzed using the MUVB-20 gel analysis system (Ultralum Inc., USA). The absorbance (A) value of the gene band/*GAPDH* band was taken as the relative amount of the target gene.

2.4.7. p53 and $p21^{Cip1/Waf1}$ protein expression in MSCs detected by Western blot hybridization

The proteins from cell lysates of MSCs were separated by SDS–polyacrylamide gel electrophoresis and transferred onto the PVDF membranes (Millipore, USA). The blots were incubated with the anti-p53 antibody or the anti-$p21^{Cip1/Waf1}$ antibody (1:100 dilution in a blocking solution) at 4 °C overnight, then washed with a solution containing 5% milk, 20 mM Tris–HCl (pH 7.5), 500 mM NaCl, 0.05% Tween 20 (TBS-T). After incubation with a secondary antibody (1:500 dilution in a blocking solution, 3 h at room temperature), positive signal bands were detected by the addition of nitroblue tetrazolium (NBT)-5-bromo-4-chloro-3-indolylphosphate (BCIP, Sigma, USA). The blots were scanned using Quantity One 4.4.1 software (Bio-Rad Technical Service Department, USA).

2.5. Statistical analysis

All experiments were conducted in triplicate. The results are presented as mean ± SD. All data were processed with SPSS10.0 program (SPSS Inc., USA), and statistical differences were determined by ANOVA followed by the *q* test.

3. Results

3.1. ICN expression rate of MSCs at 48 h post-transfection

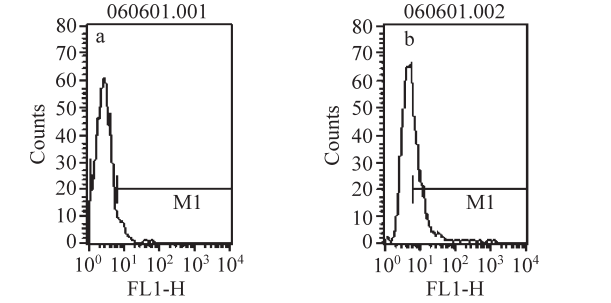
MSCs, which are $CD11b^-$, $CD11c^-$, $CD45R/B220^-$, $PDCA\text{-}1^-$ and $Sca\text{-}1^+$, and are capable of undergoing osteogenic, adipogenic and myogenic differentiation, were transfected with ICN-containing plasmid. After 48 h, the ICN expression rate was 33.81 ± 4.82% as detected by flow cytometry (Fig. 1).

3.2. Constitutive activity of Notch signaling inhibited proliferation of MSCs

MTT assays showed that the cell proliferation of the ICN-transfected group was significantly inhibited when compared with the control group ($p < 0.01$; Fig. 2).

3.3. Constitutive activity of Notch signaling induced arrested G_0/G_1 phases in MSCs

Cell cycle distributions of MSCs were analyzed by flow cytometry. In the non-transfected group, the cells at the



Marker	% Gated	Mean	Geo Mean
All	100.00	3.04	2.71
M1	4.66	8.75	8.32

Marker	% Gated	Mean	Geo Mean
All	100.00	7.07	5.26
M1	30.70	13.66	9.68

Fig. 1. ICN expression rate detected with FCM. (a) Non-transfected group; (b) ICN-transfected group.

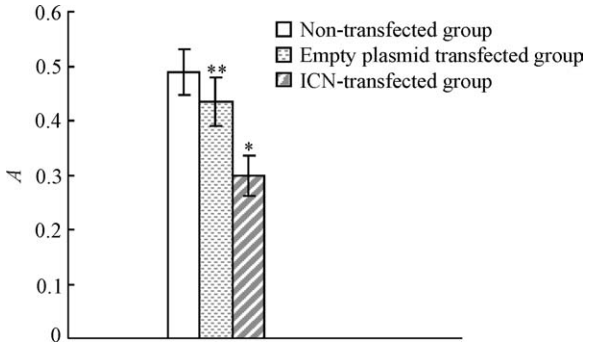


Fig. 2. Effect of active Notch 1 on growth of murine bone marrow stromal cells as measured by MTT assay. $^*p < 0.01$ *vs.* non-transfected group and empty plasmid transfected group, respectively. $^{**}p > 0.05$ *vs.* non-transfected group.

G_0/G_1 phase, the G_2/M phase and the S phase accounted for $70.37 \pm 4.96\%$, $12.37 \pm 3.35\%$ and $17.26 \pm 1.61\%$, respectively. In the empty plasmid transfected group, the cells at the G_0/G_1 phase, the G_2/M phase and the S phase accounted for $71.79 \pm 2.15\%$, $12.45 \pm 4.09\%$ and $15.75 \pm 2.69\%$, respectively. In the ICN-transfected group, the cells at the G_0/G_1 phase, the G_2/M phase and the S phase accounted for $88.86 \pm 2.95\%$, $4.86 \pm 2.16\%$ and $5.95 \pm 0.26\%$, respectively. There was a significant difference in the percentage of G_0/G_1 phase cells and proliferation indexes ($S + G_2/M$) between the ICN-transfected group and the non-transfected group or the empty plasmid transfected group ($p < 0.01$), but not between the non-transfected group and the empty plasmid transfected group, suggesting that constitutive activity of Notch signaling inhibited proliferation of MSCs by blocking the cells at the G_1 phase (Fig. 3).

3.4. Influence of the Notch signaling pathway on the senescence of MSCs

SA-β-gal activation of MSCs was detected by immunocytochemistry post-transfection. The SA-β-gal positive-staining cell population of the ICN-transfected group was higher than that in the control group ($p < 0.01$) (Figs. 4 and 5).

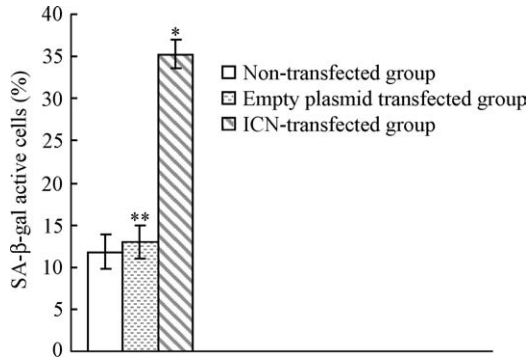


Fig. 5. Percentage of SA-β-gal active murine bone marrow stromal cells. $^{*}p < 0.01$ *vs.* non-transfected group and empty plasmid transfected group, respectively. $^{**}p > 0.05$ *vs.* non-transfected group.

3.5. p53 and $p21^{cip1/waf1}$ expression measured by RT-PCR and Western blot analysis

To determine whether Notch signaling pathway activation induced senescence of MSCs via the p53-$p21^{cip1/waf1}$ pathway, p53 and $p21^{cip1/waf1}$ expressions were measured by RT-PCR and Western blot analysis. p53 and $p21^{cip1/waf1}$ expressions in the ICN-transfected group were higher than those in both the non-transfected group and the empty plasmid transfected group ($p < 0.01$; Fig. 6, Table 1). There was

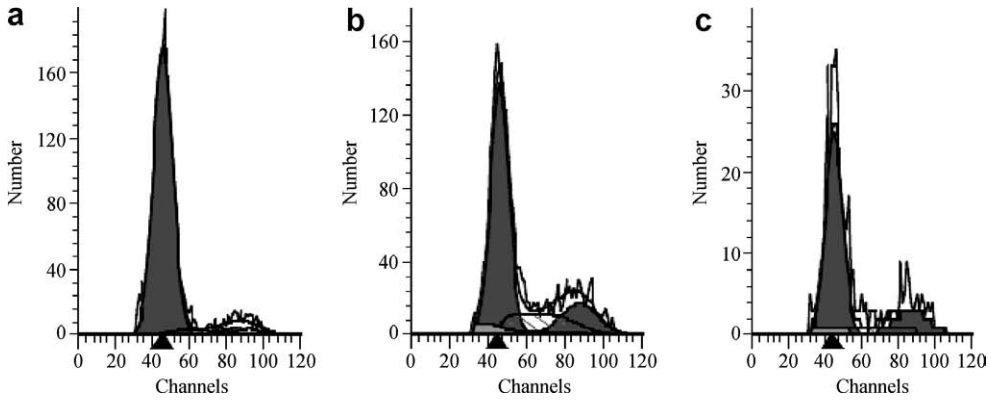


Fig. 3. Cell cycle distribution of murine bone marrow stromal cells detected with FCM. (a) Non-transfected group; (b) empty plasmid transfected group; (c) ICN-transfected group.

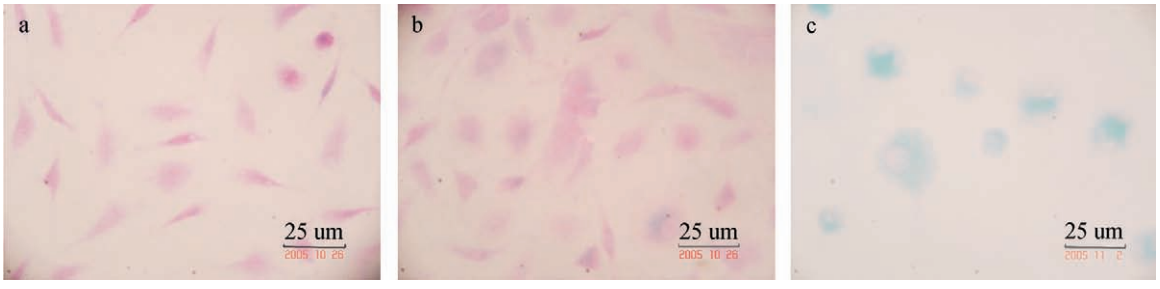


Fig. 4. SA-β-gal activity of murine bone marrow stromal cells post-transfection. (a) Non-transfected group; (b) empty plasmid transfected group; (c) ICN-transfected group.

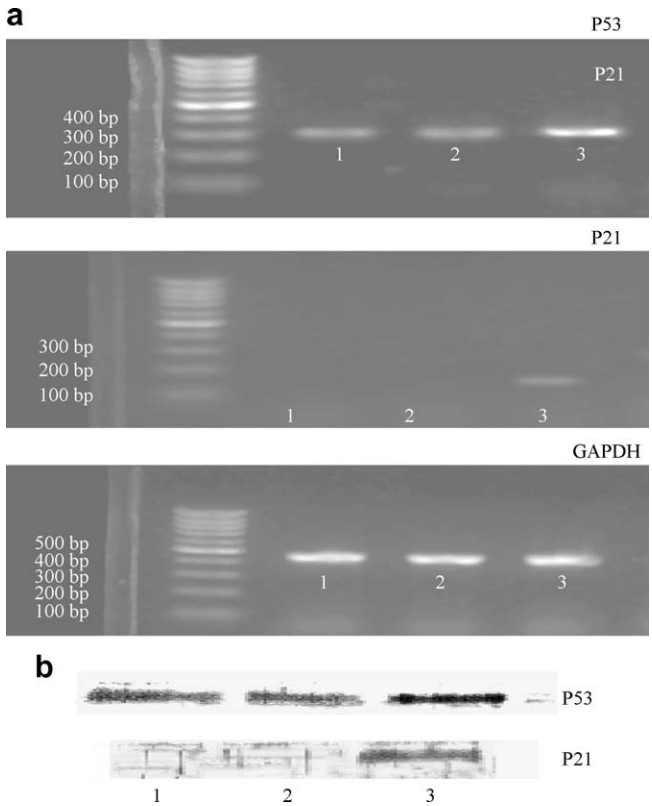


Fig. 6. Expressions of p53 and $p21^{cip1/waf1}$ in murine bone marrow stromal cells post-transfection. (a) RT-PCR; (b) Western blot. 1, Non-transfected group; 2, empty plasmid transfected group; 3, ICN-transfected group.

Table 1
p53 expression in murine bone marrow stromal cells post-transfection (mean ± SD; $n = 3$).

Group	RT-PCR	Western blot
Non-transfected group	0.298 ± 0.015	162.00 ± 5.44
Empty plasmid transfected group	$0.302 \pm 0.008^{**}$	$162.40 \pm 3.77^{**}$
ICN-transfected group	$0.844 \pm 0.057^{*}$	$184.85 \pm 2.86^{*}$

* $p < 0.01$ *vs.* non-transfected group and empty plasmid transfected group.
** $p > 0.05$, *vs.* non-transfected group.

no difference between the non-transfected group and the empty plasmid transfected group.

4. Discussion

The Notch signaling pathway regulates cellular differentiation in a variety of tissue types throughout the life of multicellular organisms. Notch signaling has been shown to affect a wide range of cellular functions including proliferation, apoptosis and differentiation. Notch proteins belong to a highly conserved family of transmembrane receptors involved in intercellular signalings that regulate cell fate. Notch interacts with ligands presented on neighboring cells, triggering a two-step proteolytic cleavage of the receptor which releases its C-terminal intracellular region (ICN). Translocation of ICN to the nucleus results in association with DNA-binding transcriptional repressor recombination signal binding protein-Jκ (RBP-Jκ), converting it from a transcriptional repressor into a transcriptional activator. The $Notch^{IC}$/RBP-Jκ complex transactivates various transcriptional factors and affects downstream target genes that mediate cell differentiation, proliferation and apoptosis [19]. We have previously found that the Notch signaling pathway is involved in radiation injury of MSCs.

Senescence is a kind of relatively stable state post cell detachment from the cell cycle, and is a state where cells are irreversibly deprived of reproductive activity. Senescence is categorized into two groups: internal factor-induced cleavage stop (termed replicative senescence) and external factor-induced primary cell permanent, irreversible proliferation stasis (termed premature senescence). Radiation induces cellular DNA damage, and different cell types may respond differently, including apoptosis, cell cycle arrest, or senescence. Our previous studies indicated that senescence may occur in MSCs post-irradiation, implicating senescence as a major mechanism leading to injury of MSCs.

Numerous studies have implicated two major pathways in the induction of premature senescence. The p53-$p21^{Cip1/Waf1}$ or $p19^{Arf}$-Mdm2-p53-$p21^{Cip1/Waf1}$ pathway is triggered by DNA damage, while the $p16^{Ink4a}$-Rb pathway is activated by the Ras-mitogen-activated protein kinase cascade. Activation of either pathway is sufficient to induce senescence. However, extensive cross-talk exists at multiple levels between these two pathways, and they frequently work in concert to induce premature senescence. We have previously found increased expressions of p53 and $p21^{cip1/waf1}$ in MSCs exposed to radiation, but no changes in $p16^{Ink4a}$ and $p19^{Arf}$. Therefore, we propose that the p53-$p21^{cip1/waf1}$ pathway is a critical mediator of radiation-induced senescence of MSCs.

To study the role of the Notch signaling pathway in radiation-induced senescence of MSCs, we transferred the Notch 1 activated form (ICN) into the cultured murine MSCs, and found that Notch signaling pathway activation led to arrest of proliferation of murine MSCs, with the cell cycle arrested in G_1. Morphological features of senescence cells and increased SA-β-gal activity were also evident, indicating that Notch signaling pathway activation plays an important role in senescence of MSCs. This type of effect may be associated with increased p53 and $p21^{cip1/waf1}$ expressions. These data are not in agreement with the study by Sciaudone et al. [20], who reported that Notch signaling pathway activation promoted proliferation of MSCs. This difference may come from the different choice of experimental model [21], and the different dose of gene transferred into the cells [22].

In summary, the present study suggests that Notch signaling pathway activation can lead to senescence of MSCs, most likely via upregulation of p53 and $p21^{cip1/waf1}$ protein expressions. Interruption of the Notch signaling pathway may be a potential target for prevention and treatment of

the bone marrow hemopoietic microenvironment damage induced by radiation.

Acknowledgements

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References

[1] Deryugina EI, Muller-sieburg CE. Stromal cells in long-term cultures: keys to the elucidation of hematopoietic development? Crit Rev Immunol 1993;13:115–50.
[2] Meek DW. The p53 response to DNA damage. DNA Repair (Amst) 2004;3:1049–56.
[3] Grande T, Bueren JA. Involvement of the bone marrow stroma in the residual hematopoietic induced by irradiation of adult and young mice. Exp Hematol 1994;22:1283–7.
[4] Li CH, Tzeng SL, Cheng YW, et al. Chloramphenicol-induced mitochondrial stress increases p21 expression and prevents cell apoptosis through a p21-dependent pathway. J Biol Chem 2005;280:26193–9.
[5] Aster JC, Xu L, Karnell FG, et al. Essential roles for ankyrin repeat and transactivation domains in induction of T-cell leukemia by notch1. Mol Cell Biol 2000;20:7505–15.
[6] Bresnick EH, Chu J, Christensen HM, et al. Linking Notch signaling, chromatin remodeling, and T-cell leukemogenesis. J Cell Biochem 2000;35(Suppl.):46–53.
[7] Yanagawa S, Lee JS, Kakimi K, et al. Identification of Notch1 as a frequent target for provirus insertional mutagenesis in T-cell lymphomas induced by leukemogenic mutants of mouse mammary tumor virus. J Virol 2000;74:9786–91.
[8] Schroeter EH, Kisslinger JA, Kopan R. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature 1998;393:382–6.
[9] Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science 1999;284:770–6.
[10] Kopan R, Schroeter EH, Weintraub H, et al. Signal transduction by activated Notch: importance of proteolytic processing and its regulation by the extracellular domain. Proc Natl Acad Sci USA 1996;93:1683–8.
[11] Weinmaster G. Notch signal transduction: a real rip and more. Curr Opin Genet Dev 2000;10:363–9.
[12] Kao HY, Ordentlich P, Koyano-Nakagawa N, et al. A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. Genes Dev 1998;12:2269–77.
[13] Osborne B, Miele L. Notch and the immune system. Immunity 1999;11:653–63.
[14] Bigas A, Martin DI, Milner LA. Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines. Mol Cell Biol 1998;18:2324–33.
[15] Milner LA, Bigas A, Kopan R, et al. Inhibition of granulocytic differentiation by mNotch1. Proc Natl Acad Sci USA 1996;93:13014–9.
[16] Kruse JJ, te Poele JA, Velds A, et al. Identification of differentially expressed genes in mouse kidney after irradiation using microarray analysis. Radiat Res 2004;161:28–38.
[17] Cheng P, Zlobin A, Volgina V, et al. Notch 1 impairs osteoblastic cell differentiation. J Immunol 2001;167:4458–67.
[18] Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 1995;92:9363–7.
[19] Maillard I, Fang T, Pear WS. Regulation of lymphoid development, differentiation, and function by the Notch pathway. Annu Rev Immunol 2005;23:945–74.
[20] Sciaudone M, Gazzerro E, Priest L, et al. Notch1 impairs osteoblastic cell differentiation. Endocrinology 2003;144:5631–9.
[21] Nefedova Y, Cheng P, Alsina M, et al. Involvement of Notch-1 signaling in bone marrow stroma-mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines. Blood 2004;103:3503–10.
[22] Guentchev M, Mckay RD. Notch controls proliferation and differentiation of stem cells in a dose-dependent manner. Eur J Neurosci 2006;23:2289–96.