Original Article

P27 deletion enhances hematopoiesis by paracrine action of IL22 secreted from bone marrow mesenchymal stem cells

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Abstract: Previous studies have reported that p27 deletion stimulates the proliferation of bone marrow mesenchymal stem cells (BM-MSCs) and their differentiation into osteoblasts, it also increases bone marrow hematopoietic progenitor cells (HPCs). However, it is unknown whether the enhanced hematopoiesis induced by p27 deficiency was associated with releasing hematopoietic stem cell (HSC) and HPC supporting factors by BM-MSCs. To answer this question, we cultured the BM-MSCs from wild-type (WT) or p27 knockout (KO) mice, analyzed their proliferation, apoptosis and osteogenesis and harvested their conditioned medium (CM); We also cultured the bone marrow cells (BMCs) with normal medium or CM from WT or KO BM-MSCs and analyzed changes of HSCs and HPCs and colony forming cells (CFCs). Our results showed that the proliferation and osteogenic differentiation of BM-MSCs were increased significantly and their apoptosis was reduced significantly in p27 deficient mice. Simultaneously, we demonstrated that the CM from p27 deficient BM-MSCs stimulated the expansion of HSCs/HPCs more dramatically than that from WT BM-MSCs. Five 2-fold up-regulated proteins were identified in the CM from p27 deficient BM-MSCs by protein chip assays, including interleukin-22 (IL-22), transforming growth factor-β type I receptor, tumor necrosis factor-related Apoptosis-inducing ligands, VE-cadherin and vascular endothelial growth factor B. We confirmed that expression of IL22 at both mRNA and protein levels were up-regulated significantly in p27 deficient BM-MSCs. The treatment of IL22 at both mRNA and protein levels were up-regulated significantly in p27 deficient BM-MSCs. The treatment of IL22 increased the percentages of HSCs and HPCs in BMC cultures and the number of CFCs in the colony formation assay, whereas the increased HSC/HPC expansion induced by the CM derived from p27 deficient BM-MSCs was blocked by the addition of anti-IL22 antibody in a dose dependent manner. We also found that the percentages of IL22R1, Stat3 and p-Stat3-S727 positive HSCs and HPCs were increased significantly in p27 deficient BMCs. Our findings in this study indicate that p27 deficiency stimulates HSC/HPC expansion by increasing secretion of IL22 by BM-MSCs and activating IL22-Stat3 signaling in HSCs and HPCs.

Keywords: p27 deficiency, bone marrow mesenchymal stem cells, hematopoietic stem cells, hematopoietic progenitor cells, IL22-Stat3 signaling

Introduction

p27Kip1 (hereinafter referred to as p27) is a 27 KD protein that binds to the cyclin E/cyclin-dependent kinase 2 (CDK2) complex and then inhibits the activity of the complex [1]. The human p27 (CDKN1B) gene is located on chromosome 12p13 [2]. This protein has significant sequence homology with the other two members of the Cip-Kip CDK inhibitor family, p21Cip1 and p57Kip2 [3]. To gain insight into the role of p27, the p27 gene knockout (p27−/−) mice were constructed and analyzed in 1996. The p27−/− mice displayed multiple organ hyperplasia, retinal dysplasia and pituitary tumors [4]. Hematopoietic progenitor cells (HPCs) are significantly increased in the bone marrow and spleen of p27−/− mice [5]. These hematologic phenotypes are similar to those of patients with myeloproliferative neoplasms, although red blood cells,
neutrophils, monocytes, and platelets counts in the peripheral blood of p27−/− mice were normal [5].

Previous study has been reported that p27 deletion can stimulate the proliferation of bone marrow mesenchymal stem cells (BM-MSCs) and their differentiation into osteoblasts [6]. Recently, we also demonstrated that p27 deficiency increased bone mass with increasing numbers of osteoblasts, total fibroblast colony forming units (CFU-f) and alkaline phosphatase (ALP)-positive CFU-f, indicating p27 deletion stimulates osteogenesis of BM-MSCs and osteoblastic bone formation [7]. However, it is unknown whether p27 deficiency improved hematopoietic microenvironment by increasing BM-MSCs and osteoblasts.

BM-MSCs have self-renewal and multi-differentiation potential, including differentiation into chondrocytes, osteoblasts and adipocytes [8]. Different offspring of BM-MSCs can secrete hematopoietic stem cell (HSC) supporting factors, such as C-X-C motif chemokines ligand 12 (CXCL12), angiopoietin, stem cell factor, etc., to form the important component and regulator of hematopoietic microenvironment [8]. Mounting evidence now suggests that the interaction between hematopoietic cells and the bone marrow microenvironment may play a key role in hematopoietic development [9, 10]. However, whether p27 deficient BM-MSCs participated in such enhanced hematopoiesis, and the potential molecular mechanisms involved has not been known so far.

To investigate whether the enhanced hematopoiesis induced by p27 deficiency was associated with releasing HSC/HPC supporting factors by BM-MSCs, we examined the effect of p27 deficiency on proliferation, apoptosis and osteogenic differentiation of BM-MSCs; the effect of the conditioned medium (CM) from p27 deficient BM-MSCs on the expansion of HSCs/HPCs. Furthermore, we identified paracrine factors released from p27 deficient BM-MSCs and assessed the action and mechanism of one of those factors in stimulating HSC/HPC expansion.

**Materials and methods**

**Mice and genotyping**

The p27−/− mice used in this study (C57BL/6J hybrid background, purchased from Jackson Lab, USA) [5] were fertile and were mated to produce wild-type (WT) and p27−/− (KO) mice [20]. In this study, 6-week-old littermates of WT and p27−/− mice were used for the following experimental studies. All animal experiments were performed in accordance with the approval of the Institutional Animal Care and Use Committee.

**Bone marrow mesenchymal stem cell (BM-MSC) cultures**

Tibiae and femurs of 6-week-old WT and p27−/− mice were removed under aseptic conditions, and bone marrow cells (BMCs) were flushed out with basic culture medium, which was α-MEM with 10% fetal bovine serum (FBS), 2 mM L-glutamine (GIBCO) and 1% antibiotics (GIBCO) as described previously [21]. Cells were dispersed by repeated pipetting, and a single-cell suspension was achieved by forcefully expelling the cells through a 22-gauge syringe needle. Total BMCs were seeded at 5 × 10^5 cells/cm^2 and subcultured when they reached a confluency of 90%. The adherent BM-MSCs were digested and passaged to the third generation for more experiments.

For osteoblast differentiation, BM-MSCs were cultured with osteogenic induction medium containing 10% fetal bovine serum, 2 mM L-glutamine and 1% antibiotic α-MEM, 50 μg/ml ascorbic acid (Sigma), 10 mM β-glycerophosphate (Sigma) and 10 nM dexamethasone (Sigma). After 14 days, resulting cells were stained cytochemically for alkaline phosphatase (ALP) and the numbers of ALP-positive CFU-F (CFU-fap) were counted by computer-assist image analyses [21]. Then cultured plates were de-stained with borate buffer (10 mM, pH 8.8) and re-stained with borate buffer containing 1% methylene blue (w/v), and then the numbers of methylene blue positive total CFU-f were counted by computer-assist image analyses [21].

For cell passage cultures, the adherent cells were digested and passaged to the third generations for more experiments. The proliferation of the BM-MSCs were examined by bromodeoxyuridine (BrdU) incorporation and flow cytometry analysis. Briefly, the BM-MSC cultures were changed with basic culture medium in the presence of 10 μM BrdU. After another 6 hours culture, the cells were collected by digestion and resuspended in FACS staining buf-
P27 deletion enhances hematopoiesis by paracrine action of IL22

Table 1. Sequences of primers employed for Real time RT-PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>S/AS</th>
<th>Sequence</th>
<th>Tm (°C)</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL22</td>
<td>S</td>
<td>5'-CTGCCTGCTCTCATTGCCCT-3'</td>
<td>59</td>
<td>678</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>5'-CAAGTCTACCTGTTGCTCCAT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>S</td>
<td>5'-GTCGTCCTGAGCAGTTGTTG-3'</td>
<td>55</td>
<td>508</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>5'-ATGAGCCCCTCCACAGG-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Real-time RT-PCR primers used with their name, orientation (S, sense; AS, antisense), sequence, annealing temperature (Tm), and length of amplicon (bp).

Bone marrow cell condition cultures

Conditioned medium (CM) was consist of 50% basic culture medium and 50% of supernatants which were harvested from BM-MSC cultures derived from WT or p27<sup>-/-</sup> mice (hereinafter reported as WT-CM or KO-CM). BMCS were derived from WT mice were cultured with WT-CM or KO-CM for 4 days and then collected for stem cell examination by flow cytometry analysis or colony forming cells (CFCs) assay.

IL22 condition culture medium included normal medium (hereinafter reported as NM, consist of 100% basic culture medium) with different concentration (0 µg/L, 50 µg/L and 100 µg/L) of recombinant mouse IL22 (R & D Systems, Inc., USA) and KO-CM with different concentration (0 µg/ml, 1 µg/ml, 2 µg/ml and 4 µg/ml) of polyclonal rabbit anti-IL22 antibody (Millipore, USA). WT BMCs were cultured in these medium for 4 days and then collected for stem cell examination by flow cytometry analysis or CFC assay.

Flow cytometry analysis

For stem cell analysis, BMCs were stained with PE-conjugated anti-Sca1 (BioLegend), PE-Cy5.5-conjugated anti-c-Kit (eBioscience), and Alexa Fluor 488-conjugated mouse lineage mixture antibodies (Invitrogen). The hematopoietic stem cells (HSCs) were defined as Sca-1<sup>-/+</sup>c-Kit<sup>-/+</sup>Lin<sup>-</sup> and the hematopoietic progenitor cells (HPCs) as Sca-1<sup>-/+</sup>c-Kit<sup>-/-</sup>Lin<sup>-</sup>. To further analyze stem cell surface molecule expression, BMCs were incubated with polyclonal rabbit anti-interleukin 22 receptor A1 (Millipore). To analyze intracellular signaling molecules, BMCs were fixed in periodate-lysine-formaldehyde (PLP) solution and permeabilized with 0.1% Triton X-100. After washing twice with PBS, BMCs were incubated with 2 signal transduction and transcription activator 3 (Stat3) antibodies, respectively, which were polyclonal rabbit anti-Stat3 (Bioworld Technology, USA) and polyclonal rabbit anti-p-Stat3 (Ser727, Bioworld Technology, USA) antibodies. Finally, cells were stained with DyLight 649 donkey anti-rabbit IgG (Biolegend). All analyses were performed on a FACSCalibur (BD Biosciences) as previously described [11].

Colonies-forming cell (CFC) assays for mouse MSCs

CFC assays were performed following MethoCult<sup>®</sup> GF M3434 (StemCell technology) product specification and the colony numbers of the CFCs were counted by computer-assist image analyses.

Protein chip analysis

Supernatants from WT or p27<sup>-/-</sup> mouse BM-MSC cultures were collected for protein microarray (RayBio® Label-based Mouse Antibody Array) containing 308 proteins to detect protein expression. Microarray experiments were performed by KangChen Biotech (Kang Chen, Shanghai, China).

RNA isolation and real-time RT-PCR

RNA was isolated from BM-MSCs using TRIzol reagent (Invitrogen) according to the manufacturer's protocol [21]. Sample mRNA levels were quantified by real-time RT-PCR and calculated as ratios to GAPDH mRNA as previously described [22]. The PCR primers are shown in Table 1.

Western blot

Proteins were extracted from BM-MSCs of WT or p27<sup>-/-</sup> mice and protein quantitation was per-
formed by a kit (Bio-Rad, Mississauga, Ontario, Canada). 30 μg of the protein sample was fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was performed using antibodies against p27 (Santa Cruz Biotechnology, USA), IL22 (Millipore, USA) and β-actin (BioWorld Technology, USA) as previously described [22]. The bands were visualized using enhanced chemiluminescence (ECL, Amersham) and quantified by Scion Image Beta 4.02 (Scion Corporation, Bethesda, MD, USA).

Statistical analysis

Data from analysis are presented as mean ± s.e.m. Statistical comparisons were performed by use of a Student’s t-test or a two-way ANOVA, with P < 0.05 considered to be significant.

Results

Effect of p27 deletion on proliferation, apoptosis and differentiation of bone marrow mesenchymal stem cells

To investigate the effect of p27 deletion on proliferation, apoptosis and differentiation of bone marrow mesenchymal stem cells (BM-MSCs), 5-bromodeoxyuridine (BrdU) incorporation assays, propidium iodide (PI) staining and flow cytometry analysis, and fibroblast colony forming units (CFU-f) assays were performed in BM-MSCs from 6-week-old wild type (WT) and p27⁻/⁻ mice. The results showed that the percentage of BrdU positive cells was significantly increased (Figure 1A and 1B); G1 phase cells were decreased, G2 and S phase cells were increased, and apoptotic cells were reduced (Figure 1C); The number of total CFU-f and alkaline phosphatase (ALP)-positive CFU-f (CFU-fap) were increased significantly (Figure 1D-F) in BM-MSC cultures from p27⁻/⁻ mice compared with those from WT mice. These results demonstrated that p27 deletion could stimulate the proliferation and osteogenic differentiation and inhibit the apoptosis of BM-MSCs.

Effect of the conditioned medium from p27 deficient BM-MSCs on the expansion of HSCs/HPCs

To assess whether the conditioned medium (CM) from p27 deficient BM-MSCs stimulated the expansion of HSCs/HPCs, bone marrow cells (BMCs) from WT mice were cultured with the normal medium (NM) or the CM from WT BM-MSC cultures (WT-CM) or from p27 deficient BM-MSC cultures (KO-CM). After 4 days of culture, the resulting cells were analyzed using flow cytometry for HSCs/HPCs and colony forming cell (CFC) assays. Results from flow cytometry showed that HSC (sca-1⁺ckit⁻Lin⁻) and HPC (sca-1⁺ckit⁻Lin⁺) fractions were significantly increased in the cultures with WT-CM or KO-CM compared with those with NM, and more dramatically in cultures with KO-CM compared with those with WT-CM (Figure 2A-C). Results from CFC assays showed that the numbers of CFCs were also significantly increased in the cultures with WT-CM or KO-CM compared with those with NM, and more dramatically in cultures with KO-CM compared with those with WT-CM (Figure 2D and 2E). These results support that p27 deletion can stimulate the expansion of HSCs/HPCs by increasing paracrine factors released from BM-MSCs.

Identify paracrine factors released from p27 deficient BM-MSCs

To identify paracrine factors released from p27 deficient BM-MSCs, differences in protein expression profiles between WT-CM and KO-CM were analyzed using protein chip assay. Compared to WT-CM, there were five 2-fold up-regulated protein spots and twenty-seven 2-fold down-regulated protein spots in KO-CM (Figure 3A and 3B). The five up-regulated protein spots were interleukin 22 (IL22), transforming growth factor beta type I receptor (TGF-βRI), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), vascular endothelial-cadherin (VE-Cadherin) and vascular endothelial growth factor B (VEGF-B). There have been reports of the role of TGF-βRI, TRAIL, VE-cadherin and VEGF-B in regulating hematopoietic function, however, the role of IL22 in regulating hematopoietic function is unknown. Thus, in this study we examined changes of IL22 expression at mRNA and protein levels in WT and p27 deficient BM-MSCs. Results demonstrated that both mRNA and protein expression levels were up-regulated significantly in p27 deficient BM-MSCs compared to WT BM-MSCs (Figure 3C-E).

Role of IL22 in mediating p27 deletion-induced HSC/HPC expansion

To determine whether IL22 mediated p27 deletion-induced HSC/HPC expansion, BMCs derived from WT mice were cultured with 0, 50 or
P27 deletion enhances hematopoiesis by paracrine action of IL22

100 µg/L IL22 or with KO-CM containing 0, 1, 2, 4 µg/ml anti-IL22 antibody. After 4 days of culture, the resulting cells were analyzed using flow cytometry for HSCs/HPCs and colony forming cell (CFC) assays. Results from flow cytometry showed that both HSC and HPC fractions were significantly increased in the cultures with 100 µg/L IL22 (Figure 4A and 4B), whereas they were reduced significantly in cultures with KO-CM in an anti-IL22 antibody dose

Figure 1. p27 deletion stimulates the proliferation and osteogenic differentiation and inhibit the apoptosis of BM-MSCs. (A) Representative graphs of flow cytometry analysis for BrdU, and (B) the percentage of BrdU positive BM-MSCs from WT and p27⁻/⁻ (KO) mice. (C) Representative diagram for cell cycle of BM-MSCs from WT and KO mice analyzed by PI staining and flow cytometry, and (D) the percentages of cells at apoptosis, G0, S and G2 stages. (E) Representative images of CFU-f stained with methylene blue (up penner) or cytochemically for ALP (CFU-fap, bottom penner) and (F) the numbers of CFU-f and CFU-fap/dish. *P < 0.05, **P < 0.01, ***P < 0.001, compared with WT mice.
P27 deletion enhances hematopoiesis by paracrine action of IL22

Results from CFC assays showed that the numbers of CFCs were significantly increased in the cultures with 100 µg/L IL22, and more dramatically in cultures with KO-CM, but were reduced significantly in the cultures with KO-CM containing 2 µg/ml anti-IL22 antibody compared with those with KO-CM along (Figure 4E and 4F). These results demonstrated that IL22 mediated p27 deletion-induced HSC/HPC expansion.

Role of IL22-Stat3 signaling in mediated p27 deletion-induced HSC/HPC expansion

To determine whether IL22-Stat3 signaling mediated p27 deletion-induced HSC/HPC expansion, we examined changes of IL22 receptor A1 (IL22RA1), Stat-3 and p-Stat3-S727 expression levels in HSCs/HPCs derived from WT and p27 deficient mice. Results showed that the percentages of IL22RA1⁺, Stat3⁺ and p-Stat3-S727⁺ HSCs and HPCs were significantly
increased in the BMCs from p27 deficient mice compared to those from WT mice (Figure 5A-F). These results support that IL22-Stat3 signaling mediated p27 deletion-induced HSC/HPC expansion.

### Discussion

Our and other studies have reported that p27 deletion stimulates the proliferation of BM-MSCs and their differentiation into osteoblasts.
P27 deletion enhances hematopoiesis by paracrine action of IL22

[6, 7], it also increases bone marrow HPCs [5]. However, it is unknown whether the enhanced hematopoiesis induced by p27 deficiency was associated with releasing HSC/HPC supporting factors by BM-MSCs. In this study, we not only confirmed that the proliferation and osteogenic differentiation of BM-MSCs were increased significantly in p27 deficient mice, but also found that the apoptosis of BM-MSCs was reduced significantly in p27 deficient mice. Simultaneously, we demonstrated that the CM from p27 deficient BM-MSCs stimulated the

Figure 4. IL22 mediated p27 deletion-induced HSC/HPC expansion. The percentages of (A) HSCs and (B) HPCs in WT BMC cultures with 0, 50 or 100 μg/L IL22. *P < 0.05, compared with control cultures. The percentages of (C) HSCs and (D) HPCs in WT BMC cultures with KO-CM containing 0, 1, 2, 3, 4 μg/ml anti-IL22 antibody. *P < 0.05, **P < 0.01, ***P < 0.001, compared with control cultures only with KO-CM. (E) Representative images for CFCs from WT BMC cultures with NM or IL22 or KO-CM or KO-CM+IL22 antibody. (F) The number of CFCs. *P < 0.05, **P < 0.01, #P < 0.05, ###P < 0.001, compared with KO-CM cultures.
P27 deletion enhances hematopoiesis by paracrine action of IL22


In order to identify paracrine factors released from p27 deficient BM-MSCs, differences in protein expression profiles between WT-CM and KO-CM were analyzed using protein chip assay. Results revealed that five 2-fold up-regulated protein spots were detected in KO-CM, including IL22, TGF-βRI, TRAIL, VE-cadherin and VEGF-B. The roles of later 4 molecules in regulating hematopoiesis have been reported.

TGF-β binds to its type I (TGF-βRI) and type II (TGF-βRII) receptors to form a heteromeric cell surface complex. Upon ligand binding, TGF-βRII recruits and activates TGF-βRI, which in turn phosphorylates downstream targets [12]. TGF-β may be the most effective endogenous negative regulator of hematopoiesis. Destruction of the TGF-β signaling pathway may play a role in the development of leukemia [13]. Deletion of the TGF-βRI gene abolishes the growth regulation of TGF-β in cutaneous T-cell lymphoma [14]. TRAIL has previously been shown to be involved in red blood cell maturation. In fact, TRAIL acts as a negative regulator of adult erythropoiesis, selectively reducing the

Figure 5. IL22-Stat3 signaling mediated p27 deletion-induced HSC/HPC expansion. The percentages of IL22RA1 in (A) HSCs and (B) HPCs in BMCs from WT and p27 KO mice. The percentages of Stat3 in (C) HSCs and (D) HPCs in BMCs from WT and p27 KO mice. The percentages of p-Stat3 (S727) in (E) HSCs and (F) HPCs in BMCs from WT and p27 KO mice. *P < 0.05, compared with WT mice.
number of red blood cells in liquid culture and reducing the number and size of red blood cell colonies in semi-solid assays [15]. In addition, TRAIL inhibits the production of mature erythroblasts in liquid cultures [16]. The use of transgenic mouse models has demonstrated that most, if not all, adult bone marrow HSCs are derived from the expression of VE-cadherin ancestors [17]. VEGF has previously been shown to stimulate postnatal hematopoiesis by recruitment of HSCs [18]. However, how the increased these molecules released by p27-deficient BM-MSCs regulates hematopoiesis is under investigation. To date, the role of IL22 in regulating hematopoiesis has not been reported. Thus, we focused on IL22 in this study to assess its action in regulating hematopoiesis.

IL22 is a member of the IL10 cytokine family. The human IL22 gene is located on chromosome 12q15 [19]. The IL22 protein has 79% homology between mouse and human [20]. IL22 has been detected in many tissues including brain, thymus, spleen, heart, lung, gastrointestinal tract, liver, pancreas, kidney, skin, synovial tissue, adipose tissue, breast and eye [21-23]. Serum IL22 levels in peripheral blood of mice and human is extremely low under stress conditions [24]. Many reports have shown that IL22 is mainly produced by lymphoid lineage cells [20, 25]. Some studies have also described some non-lymphoid sources of IL22, including macrophages [26], neutrophils [27], and fibroblasts [28]. Results from our study demonstrated for the first time that BM-MSCs not only expressed IL22, but also secreted it and both were increased significantly in p27 deficient BM-MSCs. Furthermore, we also found that the treatment of IL22 increased the percentages of HSCs and HPCs in BMC cultures and the number of CFCs in the colony formation assay, suggesting that IL22 can stimulate the expansion of HSCs/HPCs. Whereas the increased HSC/HPC expansion induced by the CM derived from p27 deficient BM-MSCs was blocked by the addition of anti-IL22 antibody in a dose dependent manner, indicating that IL22 mediated p27 deletion-induced HSC/HPC expansion.

In summary, results from current study demonstrated that deletion of p27 could stimulate the proliferation and osteogenic differentiation of BM-MSCs and induced their apoptosis. Deletion of p27 also stimulated the expansion of HSCs/HPCs by increasing paracrine factors released from BM-MSCs. Five 2-fold up-regulated proteins were identified in the CM from p27 deficient BM-MSCs by protein chip assays, including IL-22, TGF-βRI, TRAIL, VE-cadherin and VEGF-B. The expression of IL22 at both mRNA and protein levels were up-regulated significantly in p27 deficient BM-MSCs. The treatment of IL22 increased the percentages of HSCs and HPCs in BMC cultures and the number of CFCs in the colony formation assay, whereas the increased HSC/HPC expansion induced by the CM derived from p27 deficient BM-MSCs was blocked by the addition of anti-IL22 antibody in a dose dependent manner. The percentages of IL22R1, Stat3 and p-Stat3-
S727 positive HSCs and HPCs were increased significantly in p27 deficient BMCs. Our findings in this study indicate that p27 deficiency stimulates HSC/HPC expansion by increasing secretion of IL22 by BM-MSCs and activating IL22-Stat3 signaling in HSCs and HPCs (Figure 6).

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Disclosure of conflict of interest

None.

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P27 deletion enhances hematopoiesis by paracrine action of IL22


P27 deletion enhances hematopoiesis by paracrine action of IL22

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