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Bmi-1 regulates self-renewal, proliferation and senescence of human fetal neural stem cells in vitro

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ABSTRACT

Knockout and knockdown studies have shown that the polycomb gene Bmi-1 is important for mouse postnatal and prenatal neural stem cells (NSCs) self-renewal and proliferation. Different downstream targets of Bmi-1 gene have been identified in mouse, including Ink4a/Arf locus in adult NSCs and p21 gene in embryonic NSCs. However, little is known regarding the role of Bmi-1 in human NSCs. Here, using lentiviral-delivered shRNA knockdown and over-expression techniques, we examined whether Bmi-1 is required for the self-renewal and proliferation of human fetal NSCs (hfNSCs) in vitro. Our results showed that shRNA-mediated Bmi-1 reduction profoundly impaired hfNSCs self-renewal and proliferation, whereas Bmi-1 over-expression promoted hfNSCs self-renewal capacity. Interestingly, different from mouse embryonic NSCs, Bmi-1 repressed Ink4a/Arf locus instead of p21 gene in human fetal NSCs. Moreover, Bmi-1 knockdown induced obvious senescence phenotype in hfNSCs. Further studies on the Bmi-1 pathways would help to understand the molecular mechanisms underlying hfNSCs self-renewal and human brain development.

Neural stem cells (NSCs) proliferation is active and extensive during prenatal cerebral development and is then confined to the subventricular zone (SVZ) and hippocampal dentate gyrus through adulthood [9,15–17,19,20]. As development proceeds, NSCs self-renewal mechanisms are likely to change as stem cell population and proliferation significantly reduced to low levels. Various types of genes and pathways are involved in regulating this process [29].

Bmi-1 gene, a polycomb gene family transcriptional repressor and a proto-oncogene, is required for maintenance of self-renewal and proliferation in mouse NSCs [1,10,31–33]. First, Bmi-1 knockout (Bmi-1−/−) mouse shows progressive postnatal growth retardation and Bmi-1 depletion causes significant defects in NSCs, strongly reducing SVZ neurosphere forming frequency by 80% at postnatal stages [22,33]. Moreover, in mouse embryonic NSCs, Bmi-1 knockdown leads to severe self-renewal and proliferation reduction and presents increasingly Bmi-1 dependence as embryonic stage proceeds [7]. Finally, diversified human brain tumors including medulloblastoma and glioblastoma are detected with high-level Bmi-1 expression [2,6,8,21,24].

Interestingly, different Bmi-1 downstream pathways have been found in mouse NSCs that were isolated from different stages of development or different regions of nervous system [7,23]. The downstream target Ink4a/Arf locus has been firstly identified in mouse embryonic fibroblasts (MEFs) and then confirmed in mouse postnatal NSCs and hematopoietic stem cells (HSCs) [14,22]. The Ink4a/Arf locus encodes two types of cyclin-dependent kinase (CDK) inhibitors: $p16^{Ink4a}$ and $p19^{Arf}$ (human homologue is $p14^{ARF}$). $P16^{Ink4a}$ induces the retinoblastoma protein (pRB) hypophosphorylation by inhibiting the activity of cyclin-dependent kinases cdk4 and cdk6, thereby triggering cell cycle arrest and senescence. $P19^{Arf}$ binds mouse double minute 2 (MDM2) to prevent the degradation of p53, thus resulting in p53-mediated cell cycle arrest and apoptosis [12,26,28,34]. Deletion of $p16^{Ink4a}$ or $p19^{Arf}$ can partially rescues the self-renewal capacity in Bmi-1−/− postnatal NSCs [22]. On the other hand, comparing to mouse NSCs at postnatal stages, cyclin-dependent kinase inhibitor p21 has been verified as a Bmi-1 downstream target in mouse NSCs at embryonic stages. P21 prevents pRB phosphorylation through inhibiting cyclin E and cdk4, thus repressing cell cycle progression [7].

Such discrepancy exists between mouse adult and embryonic NSCs and whether human NSCs are the same is unclear. To our knowledge, although Bmi-1 has been extensively studied in mouse models and human cancer (stem) cells, little is known regarding Bmi-1 gene regulation in human normal NSCs. Therefore, we performed lentiviral-mediated RNA interference and over-expression experiments to investigate whether Bmi-1 regulates human fetal NSCs (hfNSCs) growth and survival and what the downstream targets are.

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We firstly isolated human fetal cortical NSCs from spontaneous aborted fetuses (8–12 weeks). The study protocol was approved by Institutional Review Committee (IRB) of Xuanwu Hospital of Capital Medical University. Written informed consent was obtained from patients. Three independent cell lines were obtained in this study. Cerebral cortices tissue was gently dissociated and immediately dissected into small pieces (about 1 mm^3), and the minced tissue was triturated gently with Pasteur pipet in 15 ml centrifuge tube (Corning) and was pelleted by gravity for several minutes, then the supernatant was filtrated with 50-μm cell strainer and the cells were plated onto 24-well plate (Corning) in NSC medium (NSCM) consisting of DMEM-F12 (Gibco), 20 ng/ml bFGF (R&D), 20 ng/ml EGF (R&D), 1% BSA (Gibco), 2% B27 (Gibco) and 1% penicillin–streptomycin (Gibco). Culture dishes were incubated at 37 °C in 5% CO_2 atmosphere and the primary neurospheres formed within 10–14 days. The spheres were dissociated into single cells by accutase (PAA) for further passages. For neurosphere forming frequency assay, single cells were plated at a density of 2000 per well with 200 μl culture medium in 96-well culture dish (Corning) and incubated for 7 days, the numbers of neurospheres were counted on day 7.

For BrdU detection, neurospheres were plated onto poly-D-lysine (Sigma) coated 24-well plate in NSCM. After 2 days, 10 μM BrdU (Sigma) was added to the newly changed culture medium and cells were incubated for another 24 h. The numbers of BrdU-positive cells were analyzed by immunocytochemistry. Briefly, NSCs were fixed with 4% paraformaldehyde (PFA) for 10 min, then treated with 2N HCl for 20 min at room temperature (RT), washed 3 times with PBS and blocked for 1 h at RT with horse serum solution (PBS containing 5% horse serum, 0.1% BSA, 0.3% Triton-X-100). Primary mouse anti-BrdU antibody (1:200; Roche) was added in PBS containing 0.1% BSA, 0.3% Triton-X-100 overnight at 4 °C, followed by secondary CY2-conjugated goat anti-mouse IgG (1:200, Jackson ImmunoResearch) for 2 h at RT. Then, cells were stained with DAPI (1:1000; Sigma) for 10 min at RT and washed with PBS for 3 times. Fluorescent images of monolayer cells were captured using Leica DMI 4000B. Numbers of BrdU-positive cells were counted and divided by total $DAPI^+$ nuclei (BrdU/DAPI). The numbers of total cells ($DAPI^+$ nuclei) and BrdU-positive cells were counted on more than 10 non-overlapping fields in each well. For each treatment, four different wells were analyzed, and the experiments were repeated more than 3 times.

Lentiviral-based Bmi-1 shRNA vectors were synthesized by Shanghai GeneChem Inc (Shanghai, China). An oligonucleotide was synthesized that consisted of a sequence-specific 19 nucleotide stretch designed to target the Bmi-1 ORF (AAGGAGGAGGTGAATGATAAA) or the Bmi-1 3′UTR (AGAATTGGTTTCTTGGAAA and CGGAAAGAATATGCATAGA) followed by the loop sequence (TTCAAGAGA) and finally the reverse complement of the targeting sequence. These sequences are downstream of human H1 promotor using AgeI/EcoRI cloning sites. The over-expression vector (pGC-FU-Bmi-1) was constructed by driving Bmi-1 open reading frame (ORF)-eGFP expression by ubiquitin promoter and eGFP for visualization in cells. For viral transduction, shRNA lentiviral vectors at a multiplicity of infection (moi) of 2.5–5 and over-expression vector (pGC-FU-Bmi-1) at a moi of 1, 2.5, 5 were added to dissociated cortical NSCs just after plating. For rescue experiments, cells were cotransduced with pGC-FU-Bmi-1 and Bmi-1 shRNA vector at equal moi of 5. Bmi-1 mRNA and protein levels were measured at 48 h and 72 h posttranduction.

Total RNAs were extracted from cells using trizol reagent (Invitrogen) according to the user manual. Total RNA was then treated with RQ1 RNase-free DNase I (Promega) to eliminate genomic DNA contamination. Reverse transcription of purified RNA was performed using oligo(dT) priming and superscript II reverse transcriptase (Invitrogen). The quantification of all gene transcripts was performed with an Opticon II system (MJ research, MA, USA) using the SYBR Green I real-time PCR kit (Takara, Japan). All expression values were normalized against GAPDH. All amplifications were done in duplicate, and at least three technical and three biological replicates were performed. Real-time PCR primer sequences are provided in supplementary data.

Western blotting was performed to analysis the protein expression. Nuclear and cytoplasmic extracts were separately prepared and protein concentration was calculated using the BCA assay (Pierce). Nuclear protein was tested with Bmi-1 antibody (R&D) and cytoplasmic protein was tested with p16 antibody (BD) and GAPDH antibody (Bethyl Laboratories). Equal amounts of protein were subjected to SDS-PAGE, transferred to PVDF membrane and probed with respective antibodies.

Senescence-associated β-Galactosidase staining was detected histochemically at pH 6 as described previously [5]. Briefly, neurospheres were seeded onto 24-well culture dish coated with poly-L-lysine (Sigma) and cultured for 2 days to let the neurospheres fully extend into a single layer of cells, then fixed in PBS containing 1% formaldehyde, 0.4% glutaraldehyde, and 0.02% igepal. After three PBS washes, cells were incubated with the X-gal solution overnight (1 mg/ml X-gal; 5 mM $K_3Fe(CN)_6$; 5 mM $K_4Fe(CN)_6$; 1 mM $MgCl_2$, in PBS; pH 6.0) (Sigma). Cells images were captured using Nikon TS100.

Quantitative data were expressed as the means ± standard deviation (SD). Data were analyzed by one-way ANOVA followed by Bonferroni post hoc test for multiple comparisons or Student's *t*-test. $P < 0.05$ was considered statistical significance.

In this study, we used hfNSCs lines that are all within 20 passages from original derivation. Identification of NSCs has been proved (Supplementary Fig. 1). It was observed that levels of Bmi-1 expression at different stages of cell culture had no statistical difference (Supplementary Fig. 2A). In addition, the cell karyotypes continued to be normal in long term culture under current culture conditions (data not shown). To specifically knockdown Bmi-1 expression level, three independent designed lentiviral-based shRNA vectors targeting Bmi-1 gene were used in this study (Supplementary Fig. 2B). Another vector expressing only eGFP was defined as GFP vector. Results showed that Bmi-1 transcript and protein levels were rapidly reduced in shRNA groups (cells transduced with shRNA vectors) comparing with GFP control group (cells transduced with GFP vector) (Supplementary Fig. 2C and D).

To determine whether Bmi-1 regulates the self-renewal and proliferation of hfNSCs, we examined the effect of Bmi-1 deficiency on both characteristics of the stem cells in vitro. Comparing with control (nontransduced cells) and GFP control, Bmi-1-deficient cells exhibited distinct reduction in the frequency of neurosphere formation (6.6 ± 0.8% to 1.2 ± 0.2%), clone diameter (28.9 ± 5.2 μm to 23.6 ± 3.9 μm) (Fig. 1A–D) and BrdU incorporation rate (44.4 ± 9.3% to 14.1 ± 6.7%) (Fig. 1E–J), which all indicated that hfNSCs self-renewal and proliferation capacities were significantly impaired by reduced Bmi-1 expression. Three shRNA vectors resulted in similar phenotypes, suggesting that Bmi-1 gene knockdown is efficient and specific (Fig. 2A–C). Furthermore, apparent senescence phenotype, blue staining by classical β-Galactosidase assay, appeared in Bmi-1 knockdown cells whereas few or no blue staining in GFP control cells (Fig. 1K and L). Therefore, Bmi-1 knockdown profoundly impaired hfNSCs self-renewal and proliferation as well as resulted in hfNSCs senescence phenotype.

To upregulate Bmi-1 expression, a lentiviral vector expressing the Bmi-1 open reading frame (ORF)-eGFP fusion protein that enforces Bmi-1 expression (pGC-FU-Bmi-1) was used (Supplementary Fig. 2B). Different Bmi-1 over-expression levels were checked by transducing cells with various multiplicity of infection (moi) of pGC-FU-Bmi-1 vector. Bmi-1 expression increased approximately 8-fold, 30-fold and 60-fold while trans-