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Nanog-induced dedifferentiation of p53-deficient mouse astrocytes into brain cancer stem-like cells

Jai-Hee Moon^a, Suhyun Kwon^a, Eun Kyoung Jun^a, Aeree Kim^b, Kwang Youn Whang^c, Hyunggee Kim^c, Sejong Oh^d, Byung Sun Yoon^{a,*}, Seungkwon You^{a,*}

^a Laboratory of Cell Function Regulation, College of Life Sciences and Biotechnology, Korea University, Seoul, Republic of Korea

^b Department of Pathology, College of Medicine, Korea University Guro Hospital, Seoul, Republic of Korea

^c Division of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul, Republic of Korea

^d Division of Animal Science, Chonnam National University, Gwangju, Republic of Korea

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ABSTRACT

Self-renewal, differentiation, and tumorigenicity characterize cancer stem cells (CSCs), which are rare and maintained by specific cell fate regulators. CSCs are isolated from glioblastoma multiforme (GBM) and may be responsible for the lethality of incurable brain tumors. Brain CSCs may arise from the transformation of undifferentiated, nestin-positive neural stem or progenitor cells and GFAP-expressing astrocytes. Here, we report a role of Nanog in the genesis of cancer stem-like cells. Using primary murine p53-knock-out astrocytes (p53^{-/-} astrocytes), we provide evidence that enforced Nanog expression can increase the cellular growth rate and transform phenotypes in vitro and in vivo. In addition, Nanog drives p53^{-/-} astrocytes toward a dedifferentiated, CSC-like phenotype with characteristic neural stem cell/progenitor marker expression, neurosphere formation, self-renewal activity, and tumor development. These findings suggest that Nanog promotes dedifferentiation of p53-deficient mouse astrocytes into cancer stem-like cells by changing the cell fate and transforming cell properties.

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1. Introduction

The stemness of stem cells is regulated by well-defined stem cell-specific transcription factors. Deregulation of such key regulators abolishes both self-renewal and multipotency, resulting in abnormal differentiation potential [1]. The molecular circuitry controlling stemness is active in certain tumors and plays a direct role in the genesis of a malignant phenotype, the presence of self-renewing stem-like cells [2]. Cellular phenotypes in many types of cancer can be organized into hierarchies from malignant cancer stem cells (CSCs) that have extensive proliferative potential, to differentiated cancer cells, which have limited proliferative potential. This CSC paradigm [3] has been well documented in the highly malignant brain cancer, primary glioblastoma multiforme (GBM) [4,5]. GBM growth and persistence depends on CSCs [5,6] with enhanced DNA damage repair programs that also induce recurrence and resist current chemo and radiotherapies [7]. Brain CSCs may arise from the transformation of undifferentiated, nestin-positive neural stem or progenitor cells, or GFAP-expressing astrocytes [8–13].

The homeodomain protein Nanog was initially discovered to be over-expressed in embryonic stem (ES) cells, and its over-expression

is sufficient to drive the self-renewal of undifferentiated ES cells [14,15]. Nanog is also required in the regulation of the cell fate of the pluripotent inner cell mass during embryonic development, the pluripotency of ES cells, and prevention of differentiation to primitive endoderm [16]. Furthermore, in reprogramming experiments, Nanog facilitated molecular reprogramming [17] and promoted the transfer of pluripotency after ES cell fusion [18]. Nanog is also required for embryo and germline development; Nanog deficiency results in a failure to maintain pluripotent stem cells and the inner cell mass being trapped in the pre-pluripotent, undetermined state [19–21], suggesting that Nanog has an important function in regulating stemness features [22].

GBMs and lower-grade gliomas exhibit a core ES-like stemness signature that includes the expression of Nanog, Oct4, Sox2, and Bmi1. GBM growth, the number of CSCs, and Nanog expression is regulated by HEDGEHOG–GLI signaling [23]. Nanog is essential for neural stem cell (NSC) and CSC clonogenicity, CD133+ stem cell behavior, and proliferation, as well as tumorigenicity in orthotopic xenografts [24,25]. In addition, the expression of Nanog in NIH3T3 cells promotes growth and a transformed phenotype [26]. Expression microarray analysis has indicated that a portion of the Nanog target genes in ES cells are activated in Nanog-expressed NIH3T3 cells [27], suggesting that Nanog possesses oncogenic potential, and part of the mechanism that supports Nanog-induced self-renewal may be related to cancer cell proliferation. In this study,

* Corresponding authors. Fax: +82 2 3290 3507.

E-mail addresses: biosun302@korea.ac.kr (B.S. Yoon), bioseung@korea.ac.kr (S. You).

we characterize an alteration in cell fate and dedifferentiation of p53-knockout mouse astrocytes into tumorigenic, CSC-like cell genesis induced by Nanog.

2. Materials and methods

2.1. Cell culture conditions

Mouse astrocyte cells were isolated from the cerebral cortices of 5-day-old p53-knockout mice as described previously, with minor modifications [8,13,28]. Briefly, p53^{-/-} astrocytes and human GBM cell lines were maintained in DMEM high glucose medium enriched with 10% fetal bovine serum (Hyclone), 1% penicillin and streptomycin (Gibco-BRL), and 2 mM L-glutamine (Gibco-BRL). Cell growth was measured by plating cells at a density of $1\text{--}2.5 \times 10^4$ cells per 6-well plate. Each day after plating, cells were counted using a hemocytometer. To determine the colony-forming capacity, cells were maintained for 7 or 14 days at a density of 100 cells per 6-well plate. The resulting colonies were stained with 0.01% crystal violet solution for enumeration [29]. For the anchorage-independent assay, 1×10^4 cells were seeded on 6-well plate with a bottom layer of 1.6% agarose in DMEM and a top layer of 0.7% agarose containing cell culture medium. BJ fibroblasts and 293T cells were grown as negative and positive cells as described above [8,13,28]. Only colonies greater than 0.2 mm in diameter were counted after 2 weeks of culture. Such colonies were visible without microscopy and typically contained 50–60 cells. For neurosphere formation, cells (1000/6-well plate) were maintained under NSC culture conditions (NSC medium; 1:1 ratio of DMEM/F12 supplemented with N2 (Invitrogen, Carlsbad, CA), B27 (Invitrogen), penicillin/streptomycin (Cambrex Bioscience, Walkersville, MD, USA), 10 ng/ml of bFGF (R&D Systems, Minneapolis, MN), and 10 ng/ml of EGF (R&D Systems)). As a reference cell line, mouse fetal NSCs were isolated from the brain subventricular zone of E13.5 embryos as described previously [29]. Cells were passaged by dissociation of the spheres into single cells by trituration through a fire-polished pipette. Floating cells were plated (1×10^5 /60-mm diameter plates; BD Bioscience) as suspensions in NSC medium and maintained in this medium, replacing the growth factor each day. For secondary sphere formation assays, single cells from the neurospheres were plated in 12-well plates at a density of 100 cells/well, and the number of single cell-derived spheres was counted after 7 days [29]. To assess the multipotency of cells from the neurospheres, cells were plated (2.5×10^4 cells/cm²) in 4-well plates (Nalgen Nunc International, USA) pre-coated with poly-L-ornithine and laminin (Sigma) as described previously [29]. For spontaneous differentiation, cells were plated in NSC culture medium without bFGF and EGF for 7 days. The immunocytochemically labeled cells were observed under fluorescence microscopes using Zeiss Axiovert as described. The cells were counterstained with DAPI (Sigma) to identify cell nuclei. At least three independent assays were performed in triplicate. Details of the materials and methods used in this study are provided as Supplementary data.

3. Results

3.1. Nanog is expressed in human GBM cell lines and can drive transformation of murine p53^{-/-} astrocytes

Given the biological significance of Nanog in the regulation of stem cell self-renewal and differentiation, we hypothesized that Nanog over-expression might cooperate with tumor suppressor genes to affect transformation and endow stem cell-like renewal activity. In particular, we focused on the expression and functional

activity of Nanog in GBM given its prominent roles in governing NSC fate decisions [24,25] and promoting the growth of and transformed phenotype in NIH3T3 cells [26,27]. Nanog mRNA and protein were expressed in human glioma cell lines (U87MG, T98G, A172, LN229, LN18, U138, LN2308, and A1207) and an embryonic carcinoma cell line (NCCIT) (Fig. 1A and B). These findings and previous reports [24–27] prompted us to test whether Nanog over-expression is relevant to the genesis and maintenance of GBM and can drive the malignant transformation of p53^{-/-} astrocytes.

p53^{-/-} Astrocytes were transduced with virus encoding Nanog or a control vector, and stable clones were selected using puromycin. First, we first investigated the effect of Nanog expression on the proliferation of the p53^{-/-} astrocytes. Consistent with other reports [26,27], the over-expression of Nanog enhanced the proliferation of p53^{-/-} astrocytes compared to controls in both 10% and 0.5% serum (Fig. 2A). Ectopic expression of Nanog induces transformation, as shown by increased foci and colony formation under soft agar culture conditions [27]. To assess whether the increased foci formation by the p53^{-/-} astrocytes was mediated by Nanog, the cells were plated at low density (1×10^3 cells per 6-well plate) and then maintained for 1 or 2 weeks in high (10%) or low (0.5%) serum, respectively. Cells over-expressing Nanog formed abundant and larger foci compared to vector control (Fig. 2B).

Next, we performed a colony formation assay under soft agar culture conditions. Cells (1×10^3 per 6-well plate) were resuspended in medium containing 0.7% agar and layered on medium containing 1.6% agar, maintained for 2 weeks, and then the number and size of colonies analyzed. Nanog transduced p53^{-/-} astrocytes and 293T cells exhibited colony formation, but no colony formation was detected in the mock-transduced p53^{-/-} astrocytes and BJ cells, using human fibroblasts as a negative control (Fig. 2C). Though the number of colonies produced by Nanog-expressing cells was comparable to 293T cells, the colony size was smaller in Nanog-expressing cells compared to 293T cells.

To further characterize the functional activities of Nanog, we performed an in vivo transformation assay. Nanog-expressing p53^{-/-} astrocytes (1×10^6 cells), but not mock-transduced p53^{-/-} astrocytes, injected subcutaneously into nude mice resulted in tumor formation, which was observed within 2 weeks, with a significant increase in mass thereafter (Fig. 2D). Overall, these findings suggest that Nanog has oncogenic transformation activity in p53^{-/-} astrocytes.

3.2. Nanog drives the dedifferentiation of p53^{-/-} astrocytes into cancer stem-like cells

Unlimited self-renewal, multipotent differentiation, and cancer initiation in vivo are the defined properties of brain CSCs [30]. Recently, Nanog was demonstrated to be highly expressed in NSCs and GBM and to modulate the proliferation and self-renewal of stem cells [24,25]. We and others have demonstrated an oncogenic function of Nanog in NIH3T3 cells and p53^{-/-} astrocytes, prompting an assessment of whether Nanog directly enables the alteration of cell fate to cancer stem-like cells. To this end, mock- or Nanog-transduced p53^{-/-} astrocytes were subjected to NSC culture conditions (N2B27 + EGF + bFGF) [28]. Compared to mock-transduced cells, Nanog-transduced cells shrunk to form small spheres that became hyperplastic, forming large and floating neurosphere masses (Fig. 3A and B). Nanog-transduced neurospheres showed self-renewal activity, forming secondary neurospheres, and they were morphologically indistinguishable from NSCs under NSC culture conditions (Fig. 3A and B). Nanog-transduced neurospheres also possessed actively proliferating nestin-, Sox2-, CD133-, and Nanog-positive cells (Fig. 3C). Nanog-transduced p53^{-/-} astrocytes exhibited neurosphere formation in the presence of LY294002, a

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