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

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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-knockout 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.

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,

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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 1–2.5 $\times$ 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 $\times$ 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 $\times$ 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 $\times$ 10^4 cells/cm^2) 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, LNZ308, 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 $\times$ 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 $\times$ 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 $\times$ 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

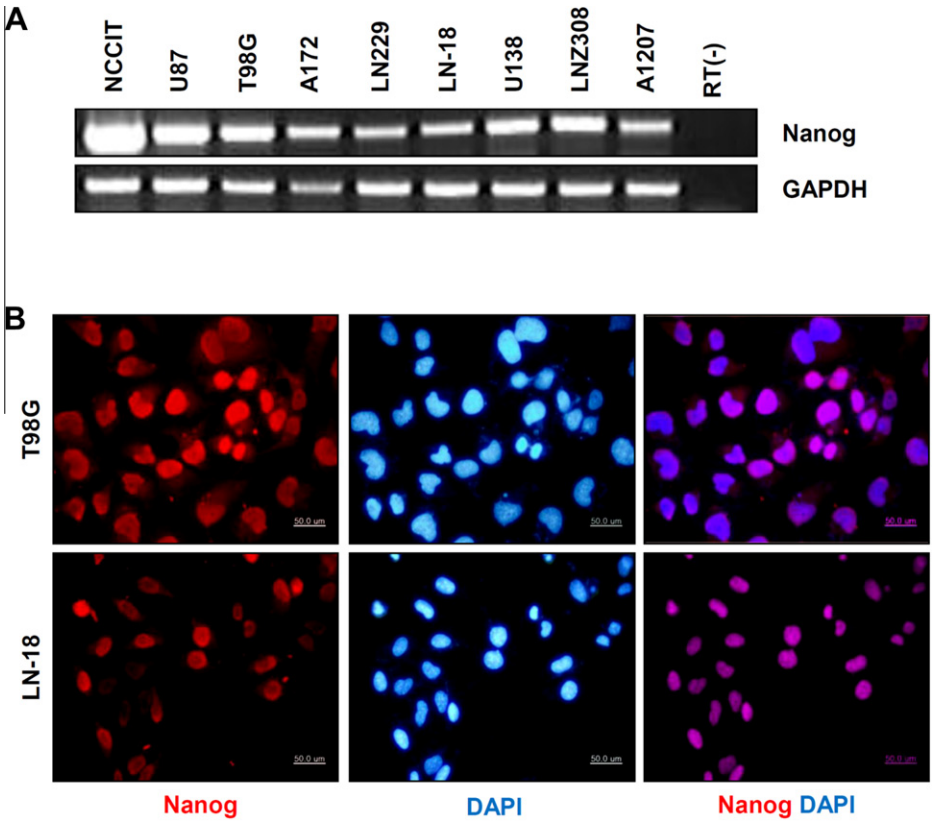

Fig. 1. Over-expression of Nanog in human GBM cell lines. (**A**) Nanog expression in human GBM cell lines and embryonic carcinoma (NCCIT) was determined by semi-quantitative PCR. GAPDH was used as a loading control. (**B**) Nanog expression determined by immunofluorescence.

PI3K/AKT inhibitor, whereas the number of neurospheres in the LY294002-treated cells decreased compared to DMSO-treated controls (9.6 ± 1.5 vs. 12.3 ± 1.2; Fig. 3D). In contrast to the LY294002-treated cells, in the presence of U0126 (MEK/ERK inhibitor) or both LY294002 and U0126, Nanog-transduced $p53^{-/-}$ astrocytes failed to form neurospheres. These results suggest that MEK/ERK signaling is a critical component in neurosphere formation in Nanog-transduced $p53^{-/-}$ astrocytes.

RT-PCR showed that serially passaged Nanog-transduced neurospheres expressed typical NSC marker genes (Fig. 3C). Scatter plot analysis of the global gene expression profiles demonstrated that Nanog-transduced neurospheres were 92% similar to NSCs (Fig. 3D). Differentiated cells from Nanog-transduced neurospheres expressed neuron and astrocyte markers, which was confirmed by immunocytochemistry (Fig. 3E). These results suggest that Nanog can dedifferentiate $p53^{-/-}$ astrocytes into stem-like cells with characteristic NSC markers, self-renewal, and multipotency.

Following intracerebral injection, the Nanog-transduced neurospheres (500 or 5×10^4 cells/mouse) and U87 (1×10^5 cells/mouse, positive control) formed brain tumors within 4–6 weeks, but no brain tumors formed after injecting the same number of vector-transduced $p53^{-/-}$ astrocytes (Fig. 4A). The tumors derived from Nanog-transduced neurospheres exhibited historical features of high cellularity and diffuse invasion, as well as Nanog expression (Fig. 4B). These results suggest that Nanog drives the dedifferentiation of $p53^{-/-}$ astrocytes to an immature NSC-like state with multipotent plasticity, oncogenic transformation, and classical GBM phenotypes.

4. Discussion

In this study, we found that the over-expression of Nanog in $p53^{-/-}$ astrocytes [27] increased oncogenic properties: growth rate, foci formation, anchorage independent growth, and tumor formation. These results confirmed the role of Nanog in regulating genes implicated in cell cycle control, cancer cell self-renewal, and tumorigenesis, as demonstrated in various studies on cancer and stem cells [24,25,31–36]. Nanog expression is enriched in CD133+ GBM stem cells, and interference with its function inhibits tumor development, which is associated with an inhibition of cell proliferation, clonal expansion, clonogenic tumor cell growth, growth of the tumor bulk, and stem cell expansion [24,34]. These results identified Nanog as an oncogene and suggest the involvement of Nanog in brain tumors.

Nanog is a critical component for the maintenance of pluripotency and its over-expression promotes ES cell self-renewal [14,15,20,22,37]. In addition, Nanog, in association with other reprogramming factors, has been shown to not only reprogram differentiated somatic cells into pluripotent stem cells [17], but also overcome reprogramming barriers and induce pluripotency [38]. Speculating that Nanog can not only provide stemness features to somatic cells, but also function in tumor development, is tempting. In the present study, we demonstrated that Nanog dedifferentiated $p53^{-/-}$ astrocytes into cells with stem-like and tumor promoting properties. Nanog-transduced neurospheres were expandable under typical NSC culture conditions [39], and serial subculturing resulted in secondary neurospheres. Moreover, these cells can differentiate into neurons and astrocytes, fulfilling stem cell criteria for self-renewal and multipotency. Brain tumor stem cells, which have NSC properties such as clonogenicity, neurosphere formation, and multipotency, were isolated from human GBM [5], but different interpretations about the origin of brain tumor stem cells have been proposed. Series of studies favor mitotically active, undifferentiated precursors to GFAP-expressing astrocytes as the most likely source of brain CSCs [9,11,12]. However, a combined loss of tumor suppressor genes

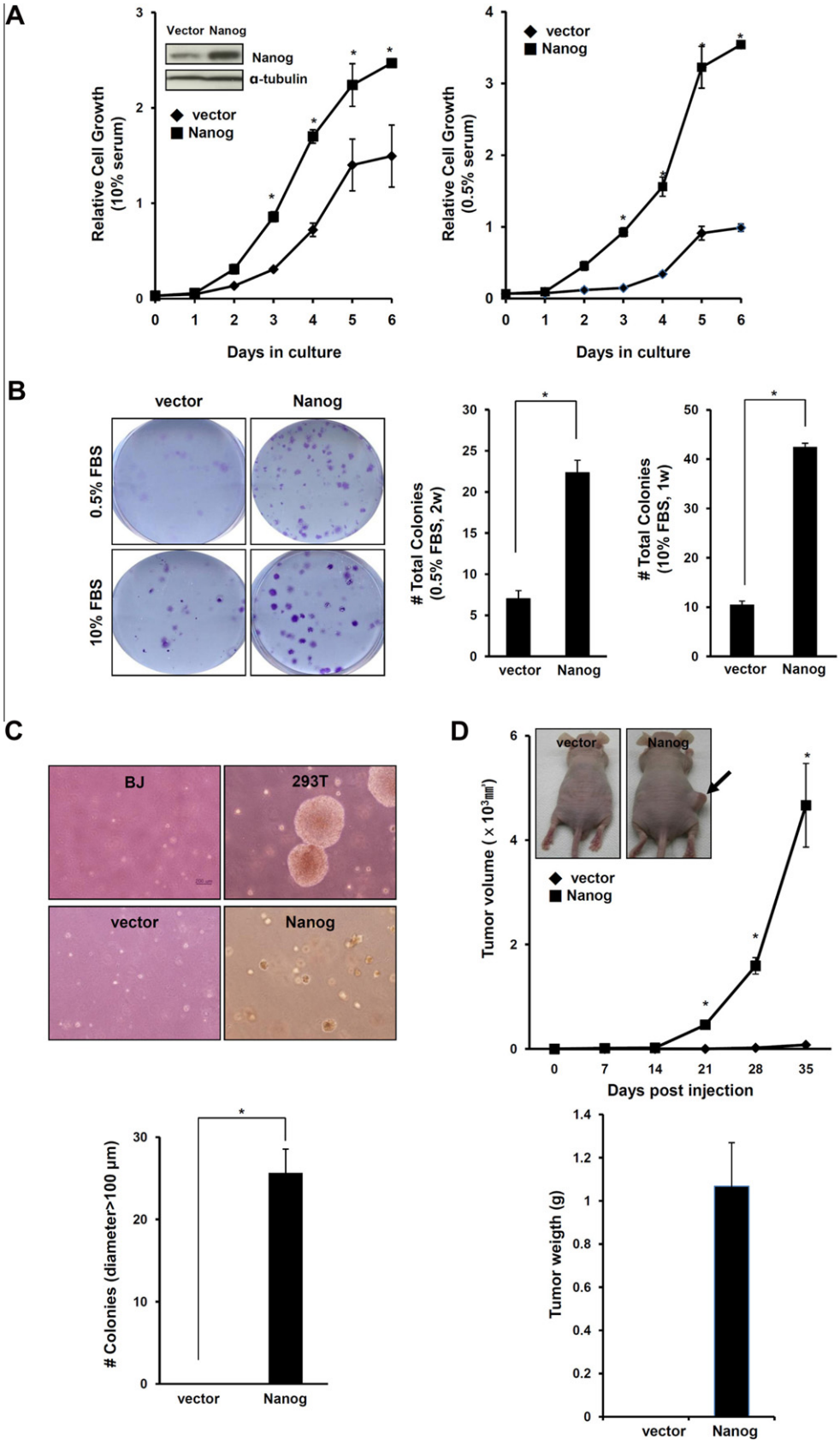

Fig. 2. Over-expression of Nanog in p53$^{-/-}$ astrocytes is responsible for an elevated growth rate and tumorigenicity. (A) In vitro growth rate of vector and Nanog-transduced p53$^{-/-}$ astrocytes (left, 10% serum; right, 0.5% serum). Nanog expression determined by Western blot analysis (inset). $^{*}p < 0.05$. (B) Low density seeding assay of vector and Nanog-transduced p53$^{-/-}$ astrocytes. $^{*}p < 0.05$. (C) Soft-agar colony-forming assay of vector and Nanog-transduced p53$^{-/-}$ astrocytes. $^{*}p < 0.05$. (D) Tumor growth rate (upper panel) in nude mice injected with vector and Nanog-transduced p53$^{-/-}$ astrocytes. $^{*}p < 0.05$. Tumor weight (lower panel) of nude mice injected subcutaneously with vector and Nanog-transduced p53$^{-/-}$ astrocytes ($n = 6$). $^{*}p < 0.05$.

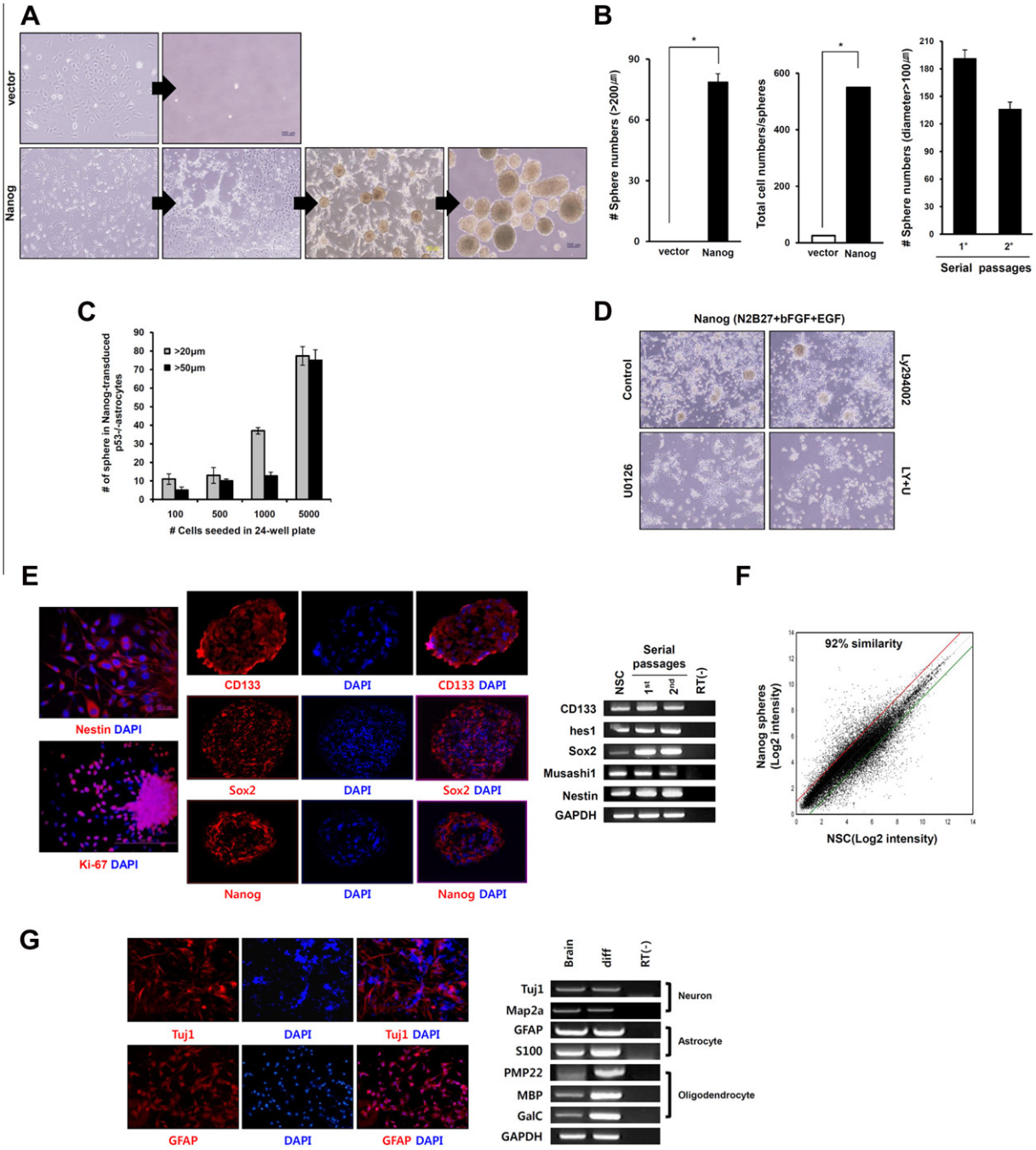

Fig. 3. Nanog-induced conversion of p53$^{-/-}$ astrocytes to neural stem-like cells. (A) Phase contrast images of vector-and Nanog-transduced p53$^{-/-}$ astrocytes cultured in proliferation medium and NSC medium. Nanog-transduced p53$^{-/-}$ astrocytes cultured in NSC medium for 7–10 days rapidly changed morphology, resulting in bipolar cells and neurospheres. (B) Number of neurospheres (>200 μm) generated from vector and Left, Nanog-transduced p53$^{-/-}$ astrocytes grown in neural stem cell culture for 7 days. Middle, Number of total cells generated from vector and Nanog-transduced p53$^{-/-}$ astrocytes grown in neural stem cell culture for 7 days. Right, Neurosphere formation during serial passaging of Nanog-transduced p53$^{-/-}$ astrocytes grown in neural stem cell culture for 7 days. The number of spheres per well was also assessed. The data are means ± SE of three independent experiments performed in triplicate. $^{*}p < 0.05$. (C) Total neurosphere numbers (>20 and >60 μm in size) for Nanog-transduced p53$^{-/-}$ astrocytes grown at different densities (100, 500, 1000, and 5000 cells per 24-well plate) and NSC culture conditions. (D) MEK and AKT signaling pathways in Nanog-transduced p53$^{-/-}$ astrocytes are responsible for neurosphere formation. Cell morphology of Nanog-transduced p53$^{-/-}$ astrocytes grown in neural stem cell culture with or without (control) LY294002, U0126, or LY294002 + U0126 (LY + U) for 7 days. (E) Immunofluorescence images of nestin and Ki67 (left panel) in Nanog-transduced p53$^{-/-}$ astrocytes grown in neural stem cell culture. Representative immunofluorescence images showing CD133, Sox2, and Nanog in the cryo-microdissected neurospheres derived from Nanog-transduced p53$^{-/-}$ astrocytes (middle panel). The expression of neural stem cell markers in the neurospheres derived from Nanog-transduced p53$^{-/-}$ astrocytes was determined by RT-PCR. (F) Scatter plots of the global gene expression in NSCs and neurospheres derived from Nanog-transduced p53$^{-/-}$ astrocytes. (G) Neurospheres derived from Nanog-transduced p53$^{-/-}$ astrocytes exhibit multipotency similar to NSCs, which give rise to cells expressing neuronal and glial markers. After 14 days in the differentiation medium, cultures were fixed and stained with antibodies specific for the neuron and glial markers. Expression of the neuronal and glial markers in the differentiated cells was determined by RT-PCR. Brain was used as a positive control.

and oncogenes activation has been shown to promote the generation of brain cancer stem-like cells from mature astrocytes [8,13,28]. These results suggest that enforced expression of Nanog can drive astrocytes into a cancer stem-like cell state through a process similar to somatic cell reprogramming and the cellular origin of CSCs.

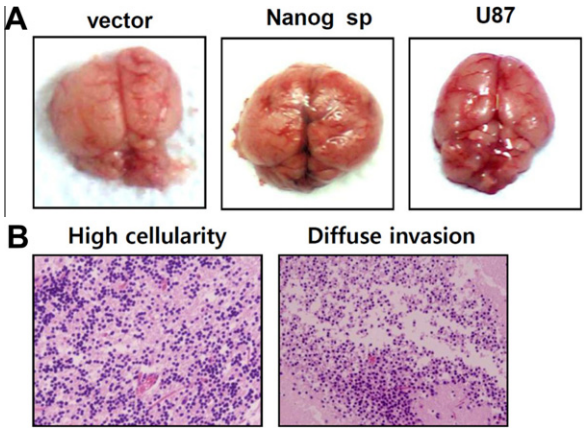

Fig. 4. Gliomagenesis and in vivo differentiation of Nanog-transduced p53$^{-/-}$ astrocytes after intracerebral implantation. (A) Whole brains of nude mice injected with 500 and 50,000 vector-transduced p53$^{-/-}$ astrocytes (no tumor), Nanog-transduced p53$^{-/-}$ astrocytes, and human GBM cells (U87) (positive control) were visualized. No tumors were observed in a nude mouse injected with vector-transduced p53$^{-/-}$ astrocytes. (B) Development of a glioma after intracerebral implantation with Nanog-transduced p53$^{-/-}$ astrocytes was visualized by H/E staining. Nanog-transduced p53$^{-/-}$ astrocyte-derived gliomas show high cellularity and diffusive invasion.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.07.070.

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