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Molecules and Cells

The Neural Stem Cell Fate Determinant TLX Promotes Tumorigenesis and Genesis of Cells Resembling Glioma Stem Cells

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A growing body of evidence indicates that deregulation of stem cell fate determinants is a hallmark of many types of malignancies. The neural stem cell fate determinant TLX plays a pivotal role in neurogenesis in the adult brain by maintaining neural stem cells. Here, we report a tumorigenic role of TLX in brain tumor initiation and progression. Increased TLX expression was observed in a number of glioma cells and glioma stem cells, and correlated with poor survival of patients with gliomas. Ectopic expression of TLX in the U87MG glioma cell line and Ink4a/Arf-deficient mouse astrocytes (Ink4a/Arf$^{-/-}$ astrocytes) induced cell proliferation with a concomitant increase in cyclin D expression, and accelerated foci formation in soft agar and tumor formation in *in vivo* transplantation assays. Furthermore, overexpression of TLX in Ink4a/Arf$^{-/-}$ astrocytes inhibited cell migration and invasion and promoted neurosphere formation and Nestin expression, which are hallmark characteristics of glioma stem cells, under stem cell culture conditions. Our results indicate that TLX is involved in glioma stem cell genesis and represents a potential therapeutic target for this type of malignancy.

INTRODUCTION

Glioblastoma multiforme (GBM; a WHO grade IV astrocytoma) is the most prevalent and aggressive type of human brain malignancies (Kleihues and Cavenee, 2000). Despite advanced and intensive radiotherapy and chemotherapy regimens, this malignancy remains incurable with an estimated median survival of less than 1 year. GBMs are comprised of heterogeneous cells, and accumulating evidence indicates that they possess a population of tumor-initiating cells resembling stem cells that are capable of self-renewal and differentiation (Singh et al., 2004). These cancer stem cells (CSCs) demonstrate a gene expression profile similar to that of embryonic and adult stem cells (Ben-Porath et al., 2008). Since self-renewal and differentiation in stem cells is predominantly regulated by a number of stem cell fate determinants such as Notch, Wnt, Hedgehog, PTEN, and TLX (Androutsellis-Theotokis et al., 2006; Goodrich et al., 1997; Groszer et al., 2001; Korinek et al., 1998; Shi et al., 2004), we reasoned that deregulation of such determinants should give rise to organ-specific CSCs (Garraway and Sellers, 2006). Indeed, Notch, Wnt, Hedgehog, and PTEN are commonly deregulated in many types of malignancies and are recognized as major factors driving CSC genesis (Hill and Wu, 2009; Koch and Radtke, 2007; Taipale and Beachy, 2001).

TLX, an orphan nuclear receptor, is predominantly expressed in embryonic and adult forebrains, and is a crucial regulator of neurogenesis through the regulation of neural stem cell self-renewal and maintenance (Qu et al., 2010; Shi et al., 2004; Zhao et al., 2009). However, its role in brain malignancy is not well understood. In the present study, we report that TLX is overexpressed in various glioma cell lines and glioma stem cells, and that its expression in glioma patients is correlated with poor prognosis. Ectopic expression of TLX in U87MG cells and Ink4a/Arf$^{-/-}$ astrocytes accelerates cell proliferation and tumorigenesis, and promotes neurosphere formation and expression of neural stem cell marker.

MATERIALS AND METHODS

Cell culture

U87MG glioma cells and Ink4a/Arf$^{-/-}$ astrocytes were maintained in DMEM high glucose medium enriched with 10% fetal bovine serum (Gibco BRL), 1% penicillin and streptomycin (Gibco BRL), and 2 mM L-glutamine (Gibco BRL). To determine cell growth rates, cells were plated in 6-well plates at a density of 10^4 cells/well, and cell numbers were counted every other day up to 6 days using a hemacytometer. For cell cycle analysis, cells were stained with propidium iodide and their DNA content was analyzed using a FACScan (BD Biosciences). The percentage of cells in each cell cycle stage was determined using a Cell Quest software program (BD Biosciences). For the neu-

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rosphere formation assay, Ink4a/Arf$^{-/-}$ astrocytes were maintained in N2 medium (Invitrogen) enriched with EGF (20 ng/ml), FGF (20 ng/ml), 1X B27, and 1% penicillin and streptomycin (Gibco BRL) for 10 days (Jeon et al., 2008).

Gene transduction

A full length human *TLX* cDNA fragment was amplified by RT-PCR with human TLX-specific primer sets and cloned into pcDNA3.1/V5-His A plasmid (Invitrogen). The TLX-V5-His fragment was subsequently re-cloned into pWZL-blast retroviral vector. Cells were infected with retrovirus produced from the PT67 amphotropic packaging cell line (Clontech) transfected with retroviral vectors (pWZL-TLX-blast). Cells were plated at a density of 10^6 cells/10-cm dish 24 h before infection and transduced by refeeding with pre-filtered (0.45 μm) retroviral supernatant containing 6 μg/ml polybrene (Sigma). This step was repeated twice without polybrene. Twelve hours after the final infection, cells were subjected to antibiotic selection with blasticidin for 7-14 days.

Soft-agar and subcutaneous tumorigenicity assays

To examine anchorage-independent growth, U87MG glioma cells and Ink4a/Arf$^{-/-}$ astrocytes were cultured in 6-well soft-agar dishes (0.7% top agar and 1.6% bottom agar) for 2 weeks. For transplantation experiments, cells were washed twice in PBS, resuspended in 100 μl PBS, and 10^6 cells were transplanted subcutaneously into nude mice (BALB/c nu/nu). Subcutaneous tumors were grossly visible at the site of injection after 13 days. All mouse experiments were approved by the Animal Care Committee at the College of Life Sciences and Biotechnology, Korea University, and were performed in accordance with government and institutional guidelines and regulations.

RNA analysis

For reverse transcriptase polymerase chain reaction (RT-PCR), total RNA was isolated from cells using TRIzol (Gibco BRL) according to the manufacturer's instructions. DNase I-treated RNA (3 μg) was converted to cDNA with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. A 1-μl aliquot of the RT reaction was used to amplify human *TLX* and *GAPDH* fragments. All RT-PCR amplifications were verified to be in the linear range. Information regarding PCR parameters used in RT-PCR will be provided upon request.

Protein analysis

Whole cell extracts were prepared using RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 50 mM Tris pH 7.4) containing 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM NaF, 1 mM Na_3VO_4, and protease inhibitor (Roche). Protein in the extracts was quantitated using the Bradford assay reagent (Bio-Rad) as recommended by the manufacturer, and 50-100 μg protein was separated by a 4-12% gradient or 10% SDS-PAGE NuPAGE gel (Invitrogen) and transferred to a PVDF membrane (Millipore). Membranes were blocked with 5% non-fat milk and incubated with antibodies specific for V5 (Invitrogen) to detect TLX-V5 fusion protein, cyclin D1 (H-295) (sc-753, Santa Cruz Biotechnology), cyclin E (c-19) (sc-198, Santa Cruz Biotechnology), cyclin A (c-19) (sc-596, Santa Cruz Biotechnology), and α-tubulin (Sigma). Membranes were incubated with horseradish peroxidase-conjugated anti-IgG secondary antibody (Pierce) and visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Immunofluorescence

Cells grown in 12-well plates (Falcon) were fixed with paraformaldehyde for 20 min at room temperature. After two washes with PBS, cells were blocked with PBS supplemented with 0.1% bovine serum albumin (Sigma), goat or donkey serum, and 0.3% Triton X 100 (Fluka) for 40 min to reduce nonspecific binding. Cells were then incubated with antibodies against cyclin D1 and Nestin (MAB353, Chemicon) for 12 h at 4°C. After two washes with PBS, cells were incubated with a fluorescein-conjugated secondary antibody (Pierce). Nuclei were stained with DAPI (1 μg/ml) for 5 min, and cells were mounted with Vectashield mounting medium (Vector). Fluorescence images were obtained using a confocal microscope (Zeiss).

***In vitro* cell migration and invasion assays**

For *in vitro* cell migration assays, cells were grown on 60-mm culture plates to a confluent monolayer. A scratch was introduced by scraping the monolayer with a p200 pipette tip and images were obtained at the indicated time-points using an inverted microscope (Olympus). For the *in vitro* invasion assay, basement membrane matrix (Matrigel, BD Biosciences) was diluted to achieve a final protein concentration of 1 mg/ml in cold serum-free cell culture media. The upper chambers of 24-well transwells (Corning Costar) were coated with diluted Matrigel (100 μl) and incubated at 37°C for at least 4-5 h to allow gelling. Cells were harvested by trypsin/EDTA, washed three times with serum-free cell culture media, and resuspended in serum-free media at a density of 3×10^5 cells/ml. Cell suspension (100 μl) was plated onto the Matrigel in the upper chamber and the lower chamber of the transwell was filled with 700 μl culture media. After incubation at 37°C for 48 h, the transwell was removed from the 24-well plates and cells that had invaded into the lower chamber were stained with crystal violet and counted under a light microscope.

RESULTS AND DISCUSSION

TLX is upregulated in human glioma cells and glioma stem cells and is associated with poor prognosis

Of the seven glioma cell lines analyzed, we found that TLX was upregulated in A172, A1207, LN18, LN229, and T98G cells compared with normal human fibroblasts (BJ) (Fig. 1A). We next compared relative *TLX* mRNA levels in normal human astrocytes (NHA) and six human glioma stem cells (Soeda et al., 2008) and showed significant elevation of *TLX* expression in two glioma stem cells (XO2 and #436) compared with NHA cells (Fig. 1B). To assess whether high TLX levels correlate with poor survival in patients with gliomas, we classified glioma patients (n = 297) according to their *TLX* mRNA levels using the REMBRANDT (Repository of Molecular Brain Neoplasia Data) database of the National Cancer Institute (http://caintegrator-info.nci.nih.gov/rembrandt) into a high TLX expression group (n = 60; ≥ 2-fold higher TLX compared with the intermediate TLX expression group) and a low TLX expression group (n = 72; ≤ 2-fold lower TLX compared with the intermediate TLX expression group). As shown in Fig. 1C, the high TLX expression group was associated with significantly worse survival compared with the intermediate and low TLX expression groups. This result suggests that TLX might be a potential diagnostic marker for glioma patients with dismal prognosis.

TLX accelerates cell proliferation and transformation in U87MG glioma cells

To understand the tumorigenic role of TLX, we infected a retrovirus expressing TLX-V5 fusion protein into U87MG glioma

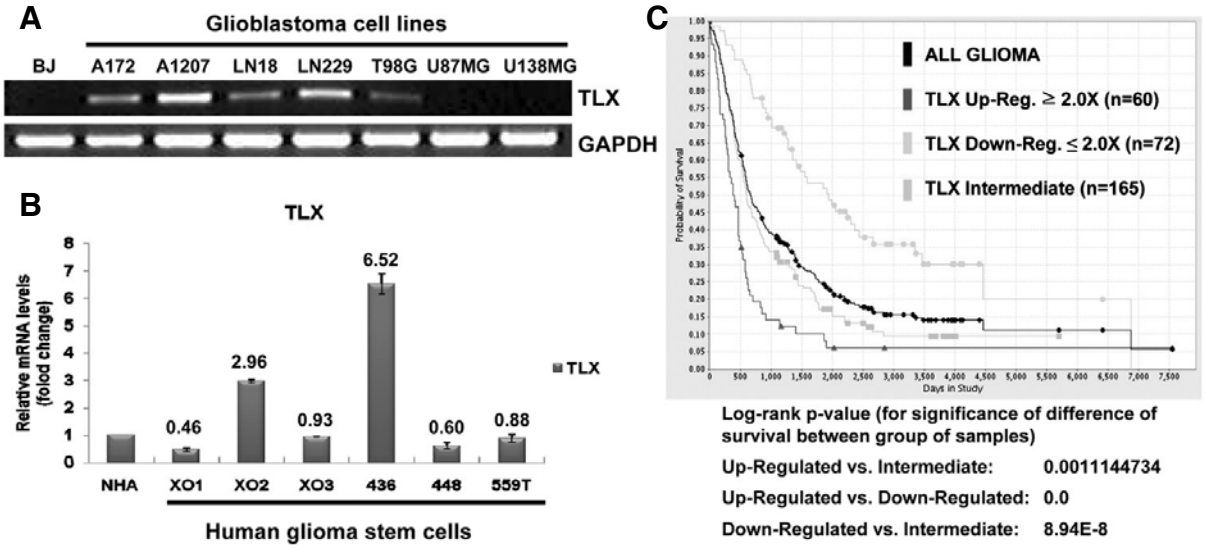

Fig. 1. TLX is upregulated in human glioma cells and glioma stem cells, and is associated with poor prognosis in glioma patients. (A) Increased *TLX* mRNA levels in human glioma cell lines. Human BJ fibroblast cells were used as a control. (B) Increased *TLX* mRNA levels in glioma stem cells derived from patients with gliomas. (C) High *TLX* mRNA level correlates with poor survival in patients with gliomas (data taken from REMBRANDT database).

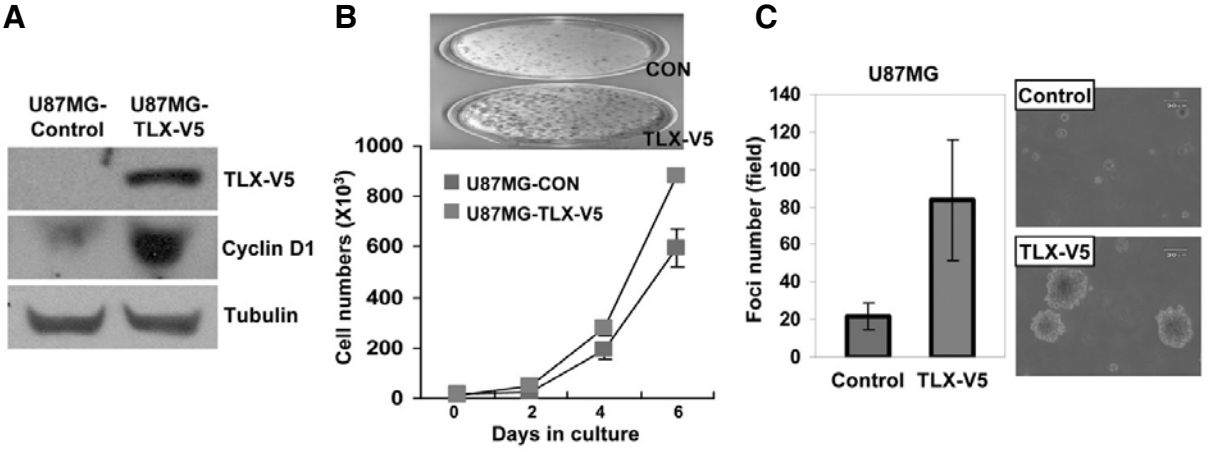

Fig. 2. Accelerated cell proliferation and transforming capacity in TLX-overexpressing U87MG glioma cells. (A) Expression of TLX-V5 fusion protein and cyclin D1 in TLX-overexpressing U87MG glioma cells. Tubulin was used as a loading control. (B) Increased cell proliferation in TLX-overexpressing U87MG glioma cells. (C) Accelerated foci formation of TLX-overexpressing U87MG glioma cells in soft-agar culture conditions.

cells that express a barely detectable level of endogenous TLX. Since aggressive tumor cells show accelerated cell proliferation that is mainly executed by alteration of positive cell cycle regulators such as cyclins and cyclin-dependent kinases, we investigated the expression of cyclin D, which is predominantly elevated in many types of malignancies (Diehl, 2002) and is known to be regulated by TLX (Li et al., 2008; Miyawaki et al., 2004). As shown in Fig. 2A, ectopic expression of TLX in U87MG glioma cells led to a marked increase in cyclin D1 expression. We also found that TLX promoted cell proliferation as determined by counting cell number and a low-density seeding assay (Fig. 2B). In addition, TLX significantly induced anchorage-independent growth of U87MG glioma cells as evidenced by an increase in the size and number of foci in soft-agar culture conditions (Fig. 2C). These results indicate that TLX accelerates cell proliferation and transformation with concomitant elevation of cyclin D1 expression.

TLX induces cell proliferation and tumorigenesis in Ink4a/Arf$^{-/-}$ mouse astrocytes

A loss of Ink4a/Arf is one of the most frequently observed genetic alterations in human GBMs (Cancer Genome Atlas Research Network, 2008). Ink4a/Arf$^{-/-}$ mouse astrocytes are non-tumorigenic immortalized cells, but can easily be converted into transformed cells by introducing a number of oncogenic factors, such as a constitutively active epidermal growth factor receptor mutant (EGFRvIII; Bachoo et al., 2002) or cell fate determinants, such as inhibitor of differentiation 4 (Id4; Jeon et al., 2008). To address the direct role of TLX in tumorigenesis, we infected Ink4a/Arf$^{-/-}$ mouse astrocytes with a retrovirus expressing TLX-V5 fusion protein and measured the expression of cell cycle positive regulators in these TLX-overexpressing Ink4a/Arf$^{-/-}$ cells.

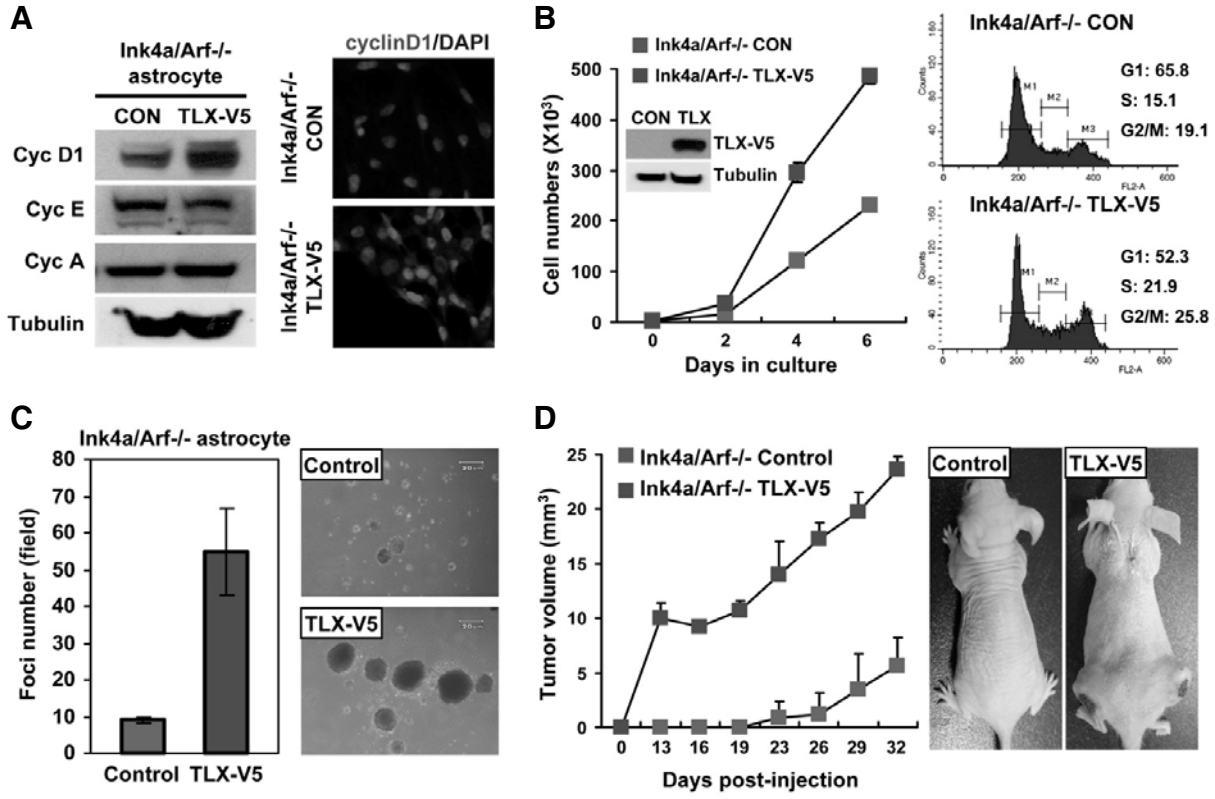

Fig. 3. Increased cell proliferation and tumorigenesis in TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes. (A) Increased expression levels of cyclin D1, but not cyclin E and A, in TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes. Representative immunofluorescence images show cyclin D1 as red and nuclear DAPI staining as blue. (B) Increased cell proliferation in TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes (left graph). The proportion of cells in S and G2/M phases was increased in the TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes (right graphs). (C) Accelerated foci formation of TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes in soft-agar culture conditions. (D) Elevated tumor formation of TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes in nude mice (n = 6).

As shown in Fig. 3A, ectopic expression of TLX in Ink4a/ Arf$^{-/-}$ astrocytes resulted in an overt increase in expression of cyclin D1, but not cyclin E and cyclin A, as analyzed by western blot and immunofluorescence assays. We also found that TLX promotes cell proliferation as determined by counting cell number and FACS analysis (Fig. 3B). In addition, TLX significantly induced anchorage-independent growth of Ink4a/Arf$^{-/-}$ astrocytes as evidenced by increased size and number of foci in soft-agar culture conditions (Fig. 3C), and markedly stimulated tumor formation of Ink4a/Arf$^{-/-}$ astrocytes transplanted into nude mice (Fig. 3D). Taken together, our results clearly indicate that TLX stimulates both acceleration of tumor progression and initiation of tumorigenesis.

TLX induces glioma stem cell characteristics in Ink4a/Arf$^{-/-}$ mouse astrocytes

Neural stem cells are a subset of undifferentiated precursors that retain the capacity for proliferation and self-renewal and give rise to differentiated cells, such as neuronal and glial cells. These features of neural stem cells are tightly regulated by a variety of stem cell fate determinants, such as Notch, BMI1, and PTEN. Interestingly, a number of stem cell fate determinants are deregulated in many types of malignancies, and their deregulation is sufficient to give rise to cancer cells that retain stem cell characteristics, known as cancer stem cells (Bruggeman et al., 2007; Jeon et al., 2008; Zheng et al., 2008). Since TLX plays an essential regulatory role in the maintenance of adult neural stem cells in the undifferentiated and self-renewable state (Qu et al., 2010; Shi et al., 2004; Zhao et al., 2009), we wondered whether Ink4a/Arf$^{-/-}$ astrocytes that ectopically express TLX retain neural stem cell features. We examined the ability of control and TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes to form neurospheres by growing them in neural stem cell culture conditions (N2 medium supplemented with EGF and bFGF). As shown in Fig. 4A, TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes (high-density seeding condition; 6,000 cells per 6-well plate or 6.24 cells per square millimeter; Lee et al., 2008) were able to form neurosphere-like foci more effectively than control-Ink4a/Arf$^{-/-}$ astrocytes in the presence of EGF+bFGF, or either EGF or bFGF alone. To rule out the possibility that the formation of neurosphere-like foci was due to cell aggregation in the high-density cell culture condition, we incubated control and TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes in a low-density seeding condition (4,000 cells per 24-well plate or one cell per square millimeter; Jeon et al., 2008) and found that TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes also gave rise to neurospheres that expressed the neural stem cell marker Nestin under low-density conditions (Fig. 4B). These results indicate that ectopic expression of TLX causes the committed Ink4a/Arf$^{-/-}$ astrocytes to acquire neural stem cell-like properties, ultimately leading to glioma stem cell genesis.

Accumulating evidence suggests that many types of malignancies involve at least two different classes of cancer stem cells: migrating cancer stem cells and stationary cancer stem

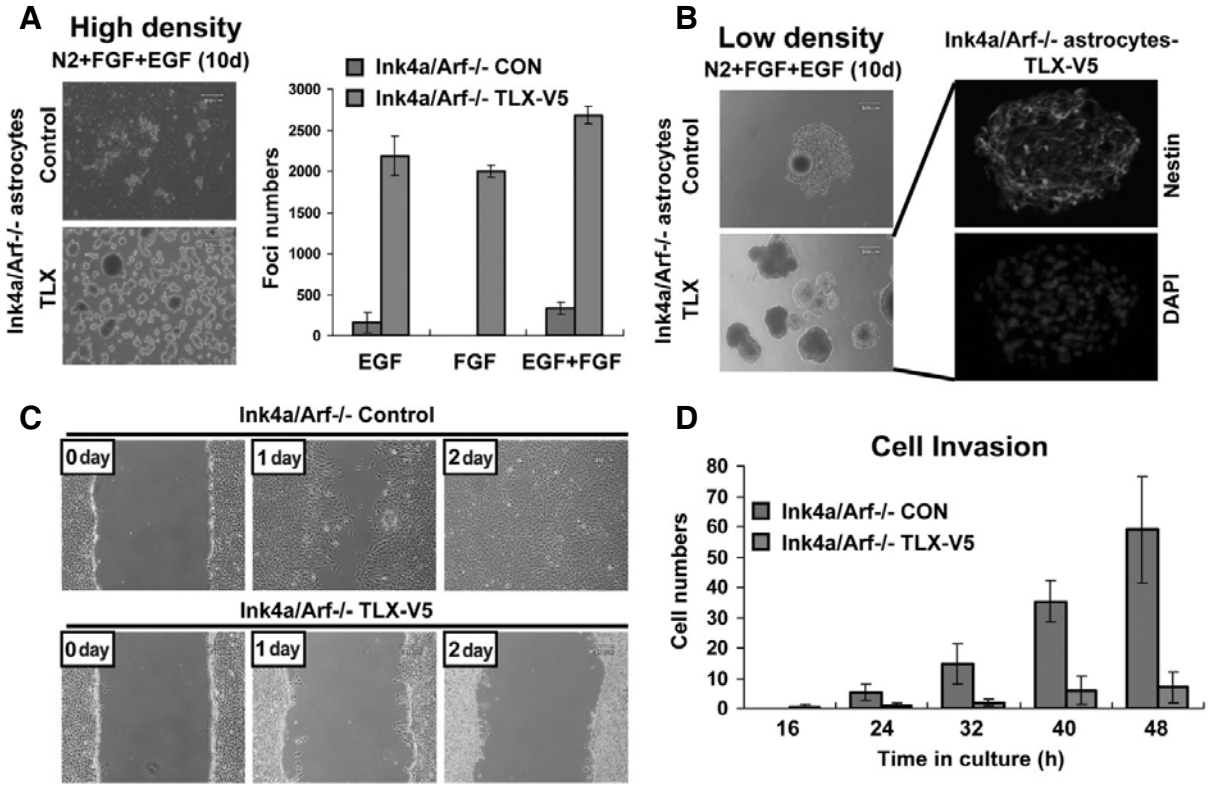

Fig. 4. Acquisition of glioma stem cell characteristics in TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes. (A) Increased neurosphere-like foci formation of TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes in high-density seeding and neural stem cell culture conditions (6,000 cells per 6-well plate or 6.24 cells per square millimeter). (B) Neurosphere formation of TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes in low-density seeding and neural stem cell culture conditions (4,000 cells per 24-well plate or one cell per square millimeter). Representative immunofluorescence images showing expression of Nestin (a neural stem cell marker) in a neurosphere derived from TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes. Nuclei were stained with DAPI. (C) Decreased cell migration of TLX-overexpressing Ink4a/ Arf$^{-/-}$ astrocytes in the *in vitro* wound-healing assay. (D) Decreased cell invasion of TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes in the *in vitro* Matrigel-coated transwell system.

cells. Moreover, it has been proposed that progression to a fully malignant cancer requires parallel progression of both primary and disseminated tumor cells (Brabletz et al., 2005; Klein, 2009). Therefore, we determined whether ectopic expression of TLX in the Ink4a/Arf$^{-/-}$ astrocytes induces a change in cell migration and invasion properties. As shown in Figs. 4C and 4D, *in vitro* cell migration and invasion, measured by wound-healing and Matrigel-coated transwell assays respectively, was dramatically inhibited in the TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes compared with control cells. It is plausible that TLX might be associated with generation of hyper-proliferative 'stationary' glioma stem cells and rapid growth of primary glioblastomas.

It is noteworthy that our result showing decreased migration and invasion of TLX-overexpressing Ink4a/Arf$^{-/-}$ astrocytes *in vitro* is different with a recent report that overexpression of TLX *in vivo* leads to migration of brain tumor stem cells in the perivascular niche (Liu et al., 2010). Such discrepancy might be attributed to *in vitro vs. in vivo* experimental condition and different genetic background. First, since cell migration and invasion is differentially regulated by different growth factor signaling, tumor-stromal cell interaction, and hypoxia (Polyak and Weinberg, 2009), an increased invasion of TLX-overexpressing neural progenitor cells *in vivo* should be regulated by a variety of non-cell-autonomous events triggered by tumor and host tissue environment. Second, cell migration and invasion would be differentially regulated by a cell-autonomous feature, such as a loss of Ink4a/Arf tumor suppressor in our study and loss of p53 tumor suppressor in the previous study (Liu et al., 2010). Therefore, given that a diffusive invasion of glioma cells is one of the general histopathological hallmarks of most aggressive glioblastoma, and is considered a major cause for tumor recurrence after surgical resection and subsequent chemo- and radio-therapies, the molecular and cellular mechanism regulating TLX-driven invasiveness should be addressed in future.

Taken together, our findings indicate that TLX, one of the major drivers of glioma stem cell genesis, might be a valuable diagnostic marker and potential therapeutic target for patients with incurable malignant gliomas.

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