RNAi-mediated knockdown of E2F2 inhibits tumorigenicity of human glioblastoma cells

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Abstract. In a previous genome-wide expression profiling study, we identified E2F2 as a hyperexpressed gene in stem-like cells of distinct glioblastoma multiforme (GBM) specimens. Since the encoded E2F2 transcription factor has been implicated in both tumor suppression and tumor development, we conducted a functional study to investigate the pertinence of E2F2 to human gliomagenesis. E2F2 expression was knocked down by transfecting U87MG cells with plasmids carrying a specific silencing shRNA. Upon E2F2 silencing, in vitro cell proliferation was significantly reduced, as indicated by a time-course analysis of viable tumor cells. Anchorage-independent cell growth was also significantly inhibited after E2F2 silencing, based on cell colony formation in soft agar. Subcutaneous and orthotopic xenograft models of GBM in nude mice also indicated inhibition of tumor development in vivo, following E2F2 silencing. As expression of the E2F2 gene is associated with glioblastoma stem cells and is involved in the transformation of human astrocytes, the present findings suggest that E2F2 is involved in gliomagenesis and could be explored as a potential therapeutic target in malignant gliomas.

Introduction

Glioblastoma multiforme (GBM) is the most frequent, aggressive and lethal primary malignant tumor of the central nervous system in adults worldwide. No effective treatment for this highly aggressive and infiltrative tumor is available, and the median survival time is <16 months following initial diagnosis, with a five-year survival rate as low as 4.7% (1,2). High proportion of mitotically active cells displaying pleomorphic morphology, pseudopalisading necrosis associated with microvascular hyperplasia, and infiltrative cell growth towards the parenchyma are some of the main histopathological features of GBM. Such key features that are observed in the majority of GBM specimens can be recapitulated in orthotopic xenograft models by the intracerebral injection of patient-derived stem-like cells, which are considered to be responsible for gliomagenesis (3).

A previous genome-wide expression profiling study identified E2F2 as a hyperexpressed gene in CD133⁺ stem-like cells isolated from fresh GBM specimens. Furthermore, the frequency and levels of E2F2 expression correlated significantly with the malignancy of astrocytomas, being predominantly hyperexpressed in GBM (4). The E2F2 protein belongs to a large family of transcription factors regulating cell proliferation, cell division and cell differentiation. The E2F family has nine members, which have been divided into two subclasses (activators and repressors) based on their transcriptional properties and conserved structural features. E2F2 has a strong transcriptional activation domain and is able to interact with the tumor suppressor Rb (5). The Rb/E2F network regulates the expression of genes involved in cell cycle progression, DNA replication, checkpoint control, apoptosis, differentiation, DNA damage repair and development (6). Despite its well-known positive regulation of cell proliferation, the contribution of E2F2 to tumorigenesis is not so clear, since it has been reported to exert either pro-oncogenic or tumor suppression effects (7).

Since CD133⁺ GBM cells have originally been reported to have increased tumor initiating capability in vivo (3,8), a functional study was carried out to address the relevance of E2F2 to the tumorigenic properties of GBM, and its value as a therapeutic target for treatment of this highly aggressive brain tumor.

Materials and methods

Cell culture. The human glioblastoma cell line U87MG was kindly provided by Dr Suely K. N. Marie from the Laboratory of Medical Investigation (LIM15) at the University of São Paulo (São Paulo, Brazil). Cells were grown in Dulbecco’s modified Eagle’s Medium-low glucose (DMEM-LG; Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (all Life Technologies, Grand Forks, ND, USA).
Island, NY, USA), in a humidified atmosphere at 37°C with 5% CO₂.

**Transient E2F2 silencing.** U87MG cells were transfected with Sure Silencing™ shRNA plasmids (Super Array, SABiosciences, Frederick, MD, USA) designed to specifically knock down the expression of the E2F2 gene. After 24 h without FBS for synchronization, U87MG cells were seeded in six-well plates at a density of 10⁵ cells per well and incubated for 24 h. Cells were then transfected with non-specific DNA (negative, non-specific control; NS) or shRNA silencing E2F2 (shE2F2) (both SABiosciences, Frederick, MD, USA), using Lipofectamine™ RNAiMAX (Life Technologies) according to the manufacturer's instructions. The total plasmid concentration in each well was 0.5 µg. Positive control cells were treated with Lipofectamine RNAiMAX, identically to the other experimental groups, but received no plasmids. Twenty-four and 72 h after transfection, the glioblastoma cells displaying neomycin resistance were selected in medium containing 500 µg/ml G418 (Life Technologies) and harvested after 96 h of culture for in vitro and in vivo experiments.

**Quantification of gene expression by quantitative polymerase chain reaction (qPCR).** Total RNA was extracted using an RNeasy® mini kit (50) (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions, and quantified by measuring the absorbance at 260 nm (NanoDrop 2000 spectrophotometer, Thermo Fisher Scientific, Wilmington, DE, USA). The reverse transcription (RT) reaction was performed using 1 µg of total RNA with Superscript™ II Reverse Transcriptase enzyme (Life Technologies). Real-time RT-PCR was performed in a 7500 Real-time RT-PCR system (Life Technologies), by the SYBR® GreenER™ incorporation method (Power SYBR Green PCR Master Mix; Life Technologies). The cycling conditions were as follows: 95°C for 15 sec, followed by 50 cycles at 60°C for 30 sec, 95°C for 1 h and 55°C for 30 sec. All primer pairs were designed in different exons using Primer3 Input version 0.4.0 (http://gmdd.shgo.org/primer3/?seqid=47), and synthesized by Promega Corporation (Madison, WI, USA). The primer sequences were as follows: Forward, 5'-GGACAGGAATGGCCTC-3' and reverse, 5'-GTCCTTCGAGGAGGCTC-3' for E2F2; and forward, 5'-GGACAGGAATGGCCTC-3' and reverse, 5'-GTCCTTCGAGGAGCTC-3' for GAPDH.

**Cell proliferation assays.** U87MG cells were seeded on 96-well plates at an initial density of 5x10⁴ cells/well, and proliferation was measured 24, 48 and 72 h after plating by direct counting of viable cells in a Neubauer chamber with Trypan blue (1:1; Sigma-Aldrich, St. Louis, MO, USA). The number of viable cells was measured 24, 48 and 72 h after plating by direct counting (B) or the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay. Control, treated with Lipofectamine™ only; NS, non-specific control; shE2F2, E2F2 knockdown. *P<0.05.

Figure 1. (A) Effects of E2F2 knockdown on glioblastoma cell proliferation. E2F2 silencing levels 96 h post-transfection of U87MG cells. Total number of viable tumor cells after 24, 48, 72 and 96 h of cell culture accessed by (B) direct cell counting or (C) 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay. Control, treated with Lipofectamine™ only; NS, non-specific control; shE2F2, E2F2 knockdown. *P<0.05.

**In vivo tumorigenesis**

**Subcutaneous xenograft model.** U87MG glioblastoma cells (10⁶ cells/mouse) were suspended in DMEM-LG, injected subcutaneously into the right flank of BALB/c nude mice (male; 4-8 weeks old) obtained from the University of São Paulo, and allowed to grow for 50 days or until the tumor reached a volume of 2,500 mm³ (tumor weight, 100-200 mg). Animals (n=5 per group) were monitored daily and tumors were measured with a digital caliper rule twice a week. Tumor volume was estimated using the formula: Volume = (minor diameter² x major diameter)/2.

**Orthotopic glioblastoma xenograft model.** Adult BALB/c nude mice (~20 g) were anesthetized by intraperitoneal administration of ketamine (100 mg/kg)/xylazine (15 mg/kg) (both Syntec Brasil, Cotia, Brazil). Following sedation, mice were positioned in a stereotaxic frame. The scalp was sterilized with iodine and 70% ethanol and a median incision of ~1.0 cm was made. The cranial cavity was assessed by a right frontal hole using an electric mini-drill (Micromotor LB100; Beltec, Araraquara, Brazil). A total of 10⁴ U87MG cells (E2F2

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knocked down and control) were suspended in 5 µl of DMEM low glucose without FBS and inoculated with a high-precision microsyringe (701RN; Hamilton Company, Reno, NV, USA) into the striatum, 0.9 mm in front of the bregma, 2.5 mm laterally to the right and 3.0 mm ventrally, at a 0.5 µl/min rate. At the end of cell injection, the needle was retained in the incision for 5 min and slowly removed to prevent the cell suspension from flowing back. The scalp was closed with 2-0 silk suture and the animals were housed under standard controlled conditions (7:00am to 7:00pm light/dark cycle; 20-22˚C; 45-55% humidity) with food and water ad libitum. Histological analysis was performed 30 days post-intracranial implantation of tumor cells. All efforts were made to minimize animal suffering as proposed by the International Ethical Guideline for Biomedical Research (CIOMS/OMS, 1985). The study was approved by the ethics committee for animal research of the University of São Paulo (CEUA protocol no. 132/2011).

**Histological analysis.** Brain samples were frozen in cold isopentane solution (Sigma-Aldrich) at -25˚C, and then sectioned at 20 µm on a cryostat. Coronal histological sections of the tumor xenograft and surrounding brain area were mounted on silanized microscope slides (StarFrost®, Knittel-Gläser, Braunschweig, Germany), and stained with hematoxylin and eosin. Microscope images were captured by an ExwaveHAD Color video digital camera (Sony Corporation, Tokyo, Japan) attached to a Nikon Eclipse E600 microscope (Nikon, Corporation, Tokyo, Japan), using the WinAVI Video Capture software (WinAVI, Eden Prairie, MN, USA).

**Statistical Analysis.** All experiments were performed in triplicate and three independent experiments were performed. Data were analyzed by one way analysis of variance with Bonferroni as the post hoc test, using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference. Data are presented as the mean ± standard deviation.

**Results**

*E2F2 knockdown inhibits glioblastoma cell proliferation in vitro.* Specific knockdown of *E2F2* expression was previously confirmed in U87MG cells, reaching a silencing level of ~60% after 96 h of transfection with shRNA. Under standard growth conditions in vitro, the total number of viable tumor cells was significantly lower after 48, 72 and 96 h of *E2F2* knockdown compared with that in the NS control group (P=0.0044, P=0.0007 and P=0.0035, respectively). Similar results were observed by the MTT assay, based on the activity of mitochondrial succinate dehydrogenase.
dehydrogenase, which indicated significantly lower numbers of viable tumor cells after 72 h and 96 h of E2F2 knockdown, compared with the controls (P<0.0001 for the two time points) (Fig. 1). The absorbance levels acquired from cells subjected to E2F2 knockdown were virtually unchanged over the time course examined (24-96 h), suggesting inhibition of cell proliferation.

Anchorage-independent cell growth is a valuable indicator of tumorigenic capability, since it is associated with neoplastic transformation and metastatic potential. In agreement with the previous cell viability experiments, the efficiency of U87MG cells to generate tumor cell colonies by anchorage-independent growth in a semi-solid medium was significantly reduced by knocking down E2F2. Both the total amount of colonies (≥100 µm) and the average size of the colonies were significantly lower when assaying U87MG cells subjected to E2F2 knockdown, compared with those of the control cells (P=0.0081 and P=0.0076, respectively) (Fig. 2).

E2F2 knockdown inhibits gliomagenesis in xenograft models. In order to test whether E2F2 knockdown would affect in vivo tumorigenesis, two xenograft models of human GBM were employed. Tumors derived from the subcutaneous injection of U87MG cells in nude mice were measurable ~30 days following injection, reaching volumes usually higher than 1,000 mm³ in the subsequent 20 days of in vivo growth. In a period of 50 days post-cell injection, although transient, the E2F2 knockdown in U87MG cells resulted in significantly smaller and significantly lighter tumors than those resulting from control cells (P=0.04) (Fig. 3A). Mice bearing orthotopic U87MG tumors also revealed differences in brain tumor development due to E2F2 knockdown. In agreement with the previous subcutaneous xenograft model, brain tumors derived from the stereotaxic intracerebral injection of U87MG cells with E2F2 knockdown were somewhat smaller than tumors derived from control cells, 30 days following injection in nude mice (Fig. 3B).

Discussion

Despite the conserved functions in cell cycle regulation, development and tissue maintenance, E2F transcription factors may affect tumorigenic processes in different ways due to the fact that each member displays individual mechanisms of action and may control the expression of other family members through a complex feedback regulation (9). The function of E2F2 is less characterized relative to other members of the E2F family, and its involvement in tumorigenesis remains a matter of debate, since evidence of both tumor suppression and pro-oncogenic activities have been reported (5).

It has been shown in mice that deficiency in E2F2 caused by gene targeting (E2F2−/−) significantly increased the population of self-reactive peripheral T cells, causing symptoms similar to severe autoimmunity. Such increment in self-reactive T cells was demonstrated to be due to increased cell proliferation rates without evidence of differential resistance to apoptosis (10). More recently, however, overexpression of E2F2 was reported to induce p53-mediated apoptosis of mouse retina neurons lacking Rb and p107, independent of other activating E2Fs (11).

In a conditional bitransgenic mouse model of Myc-induced T-cell lymphomagenesis, Opavsky et al (12) demonstrated that inactivation of E2F2 (either E2F2−/− or E2F2−/−), but not of E2F1 or E2F3, significantly accelerated tumor onset and progression, indicating an haploinsufficient tumor suppressor function for E2F2 in T cells. Similar results were obtained with MMTV-Myc
transgenic mice, in which E2F2 knockout delayed latency and reduced the incidence of Myc-driven mammary tumors (13).

By contrast, in neuroblastomas, E2F2 was shown to positively regulate MYCN transcription and thought to be required for full activity of MYCN expression in aggressive neuroblastomas usually associated with poor prognosis (14). Stable overexpression of E2F2 in fibroblasts indeed revealed a strong oncogenic capacity for this E2F member (15). Transgenic mice have also supported a pro-oncogenic role for E2F2. In an Eμ-pp-E2F2 mouse model, overexpression of E2F2 induced mild hyperplasia of the thymus in young mice and subsequent development of thymomas (16). Notably, overexpression of E2F2 was predominantly found in cortical thymic epithelial cells, which are highly proliferative cells involved in T-cell development and regeneration capacity of the thymus (17). In E2F2 knockout mice, loss of this transcription factor resulted in cell cycle arrest in hematopoietic progenitors (18) and increased DNA double-strand breaks in erythroblasts (19).

Regarding human cancers, in addition to the abovementioned study in neuroblastomas, E2F2 has been shown to be under control of the AP-1 transcription factor in breast cancer cells, where it positively regulates cell proliferation (20). Accordingly, high E2F2 expression was recently reported to be associated with poor survival of breast cancer patients (21). In prostate cancer cells, E2F2 expression was reported to be inhibited by let-7a (22) and miR-31 (23) microRNAs, resulting in suppression of tumorigenesis in a nude mice ectopic xenograft model.

In GBM, E2F2 was identified as one of the hyper-expressed genes in CD133+ tumor cells, compared with their counterparts, and its expression correlated with malignancy grade (4). Such subcellular population of GBM had been reported to have neural stem cells characteristics and enhanced in vivo tumor initiation capability (8). Studies also isolated and characterized CD133+ stem-like cells in different GBM cell lines, including U87MG (24), establishing a useful experimental model to study cancer stem cell biology.

The effects of E2F2 knockdown in U87MG cells verified in the present study by in vitro and in vivo models of tumorigenesis are consistent with a pro-tumorigenic activity of E2F2 in GBM. In agreement with this notion, a recent study demonstrated that overexpression of the microRNA miR-125b inhibits proliferation of CD133+ GBM cells in vitro, by targeting E2F2 transcripts, and that such effect on in vitro proliferation is rescued by overexpression of E2F2 (25).

Overall, these concordant findings suggest that E2F2 is an important transcription factor regulating the tumor-initiating capability of human GBM cells. Inhibitors of E2F2 expression may therefore be considered as candidates for drug development to locally treat GBM, a highly malignant and devastating tumor of the central nervous system.

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