HEB silencing induces anti-proliferative effects on U87MG cells cultured as neurospheres and monolayers

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Abstract. Glioblastoma multiforme (GBM) is a lethal tumor and novel strategies are required to overcome resistance. Transcription factor 12 (*HEB)* has been associated with neural and stem cell proliferation, is overexpressed in certain tumor types and is induced in irradiated U87MG cells. The present study aimed to determine whether *HEB* knockdown, with or without irradiation, may sensitize GBM cells. U87MG GBM and ACBRI-371 primary human astrocytes were cultured in monolayers or neurospheres. Cell proliferation and death, cell cycle and sub-G1 detection, and cluster of differentiation (CD) 133 immunofluorescence were analyzed by flow cytometry, whereas HEB protein expression was analyzed by immunocytochemistry and western blotting. Greater HEB protein expression was observed in U87MG neurospheres compared with ACBRI-371, and the two cell lines exhibited nuclear HEB expression. *HEB* silencing in cells grown in monolayers induced a significant reduction in proliferation and decreased the proportion of cells in G0/G1 phase. In addition, *HEB* silencing reduced (two-fold) the number of neurospheres compared with control scrambled (SCR) cells. *HEB* silencing combined with irradiation reduced U87MG cell proliferation when cultured in monolayers and reduced neurosphere cell number compared with the SCR irradiated group; however, not significantly. Differentiation of U87MG cells from neurospheres was reduced in *HEB*-silenced cells, whereas in irradiated cells the proportion of $CD133^+$ cells was similar in *HEB*-silenced cells compared with the SCR control. These results suggest that *HEB* may contribute to the proliferation and maintenance of GBM cells. However, only limited effects were exerted by irradiation in *HEB*-silenced cells. *HEB* may be a potential target to decrease proliferation in U87MG GBM cells, grown as monolayers or neurospheres, and may provide important information for the development of novel strategies for cancer therapy.

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Introduction

Glioblastoma multiforme (GBM) is the most common type of malignant brain tumor in adults, with 3.6 cases per 100,000 patients diagnosed annually in Europe (1). Median survival for patients with GBM is 12-15 months following diagnosis, despite surgical resection, chemo- and radiotherapy (2).

Genetic heterogeneity is characteristic of GBM (3). Among the high mutational background, mutations in *TP53* have been detected in 31% of cases of primary GBM (4). *TP53* status affects transcriptional profiles in tumor cell lines (5,6), including GBM cells exposed to irradiation (7,8).

The existence of cancer stem cells (CSCs) in tumors may be associated with chemo- and radioresistance (9-11). In brain tumors, cluster of differentiation (CD) 133 expression is considered a marker of stem cells (12,13) and is increased in GBM cell lines grown as spheres, compared with adherent cells (14). There is evidence that GBM tumors may originate from CSCs that are present in neurospheres, and these cells often reflect the histopathological features of the tumor, indicating their suitability to reproduce the cellular heterogeneity of human GBM (13). Transcription factor 12 (*TCF12*; *HEB*) is a transcription factor (TF) involved in the proliferation of neuronal stem cells via the maintenance of their undifferentiated state during embryonic and adult neurogenesis (15).

Novel therapies are required to improve overall survival, and therapies targeting molecules involved in proliferation, survival and invasiveness of GBM cells have been evaluated (16). Increased cell death was reported using LY294002 (a phosphoinositide 3-kinase inhibitor) in association with cisplatin (17), and using methoxyamine (a base excision repair blocker) (18) or apurinic apyrimidinic endonuclease redox effector factor-1 silencing (19) in combination with temozolomide in GBM cell lines.

TFs have been suggested as potential therapeutic targets for cancer treatment (20), and they have previously been evaluated in prostate (21) and breast cancer (22). Our laboratory has identified certain TFs associated with genes modulated

in GBM compared with healthy brain samples (unpublished data), following treatment with cisplatin associated with LY294002 (17), and in response to gamma-rays, where *HEB* was associated with upregulated genes in the U87MG cell line (23).

HEB is a member of the helix-loop-helix protein family (24), and together with *TCF3* (*E12*, *E47*) and *TCF4* (*E2-2*) is known as an E-protein with DNA binding properties (25). *HEB* functions include T-cell development (26,27), myogenesis (28,29), dedifferentiation of renal tubular epithelial cells (30), and proliferation of neural and progenitor cells (15). Recently, dysregulated *HEB* expression has been reported in leukemia (31,32), oligodendroglioma (33) and colorectal cancer (CRC) (34,35). In glioma, *HEB* was demonstrated to be highly expressed (36).

HEB is involved in neural and stem cell proliferation, is overexpressed in certain tumor types, and is associated with upregulated genes in irradiated U87MG cells. Therefore, the present study aimed to test the hypothesis that *HEB* knockdown may sensitize GBM cells by affecting cell proliferation, cell death and cell cycle kinetics, as well as affecting the formation of neurospheres and differentiation in irradiated GBM cells. In addition, the effects of *HEB* silencing combined with irradiation were evaluated.

Materials and methods

Cell lines and monolayer cultures. The U87MG human GBM cell line, [American Type Culture Collection (ATCC)® HTB-14™] was obtained from the ATCC (Manassas, VA, USA) and the ACBRI-371 human primary astrocyte cell line was obtained from Cell Systems Corporation (Kirkland, WA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) Ham's F-10 (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, Brazil) and 1% penicillin (10.000 units) /streptomycin (10 mg) (Sigma-Aldrich; Merck Millipore). Cells were incubated at 37˚C in an atmosphere containing 5% CO_2 until they reached semi-confluence, when they were used for experiments. ACBRI-371 was used to compare HEB protein expression by western blotting and immunocytochemistry.

Neurosphere cell culture. U87MG and ACBRI-371 cells were initially cultured in monolayers, dissociated with Accutase® (EMD Millipore, Billerica, MA, USA), and plated in neurosphere culture medium, containing DMEM/F12 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 20 ng/ml basic fibroblast growth factor (PeproTech EC Ltd., London, UK), 20 ng/ml epidermal growth factor (PeproTech EC Ltd.), 10 ng/ml leukemia inhibitory factor (Merck Millipore), 20 μl/ml B27 supplement (1:50; Thermo Fisher Scientific, Inc.) and 1% penicillin (10.000 units)/streptomycin (10 mg). The neurosphere medium was replaced every 2-3 days. This protocol was based on previous studies of neurosphere culture (12,14,37). For neurosphere formation, ACBRI-371 cells were cultured for 14 days, whereas for U87MG cells, the culture time was 3 days for western blotting and 7 days for neurosphere assays. The neurospheres derived from ACBRI-371 were used for western blotting experiments.

Western blotting. Cells were lysed in ProteoJET™ Mammalian Cell Lysis reagent (Fermentas; Thermo Fisher Scientific, Inc.), supplemented with Halt™ Protease Inhibitor Cocktail kit (Thermo Fisher Scientific, Inc.). Protein concentrations were determined using a Bicinchoninic Acid Protein Assay (Thermo Fisher Scientific Inc.), according to the manufacturer's protocol. Proteins (30 μg) were separated by electrophoresis on a NuPAGE 4-12% Bis-Tris gel (Invitrogen; Thermo Fisher Scientific, Inc.) and were transferred onto a polyvinylidene difluoride membrane (Invitrogen; Thermo Fisher Scientific, Inc.) using the XCell SureLock™ Mini-Cell system (Invitrogen; Thermo Fisher Scientific, Inc.).

Membranes were incubated in blocking buffer (WesternBreeze Chromogenic kit; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature prior to the addition of a rabbit primary antibody recognizing HEB (1:1,000; catalog no. sc-357; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), followed by incubation with an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (catalog no. WB7105; WesternBreeze Chromogenic kit) for 30 min at room temperature. A rabbit anti-β-actin antibody (ACTB; 1:1,000; catalog no. 4967; Cell Signaling Technology, Inc., Danvers, MA, USA) served as an endogenous control. Protein bands were visualized using the Western Breeze Chromogenic kit. Densitometric analysis of protein bands was performed using Gel Pro Analyzer software version 4.0 (www.gelanalyzer.com/download.html); HEB expression was calculated relative to ACTB.

Immunocytochemistry. To assess HEB expression in monolayer cultures, 50,000 cells were seeded in 6-well plates containing one coverslip/well. After 24 h, cells were fixed with 3% paraformaldehyde and 2% sucrose in phosphate-buffered saline (PBS) and were permeabilized with 0.5% Triton X-100. Cells were incubated with the primary antibody [anti-HEB; 1:100 in PBS/2% bovine serum albumin (BSA)] for 30 min at 37˚C, and subsequently with the secondary antibody [Alexa Fluor 488-conjugated anti-rabbit immunoglobulin (Ig)G; 1:100 in PBS/2%BSA; catalog no. A21441; Thermo Fisher Scientific, Inc.] for 30 min at 37˚C. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 90 mM in PBS; Sigma-Aldrich; Merck Millipore) for 10 min. Coverslips were then mounted using Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA), sealed and stored at 4˚C in the dark until analysis. Subsequently, the slides were analyzed under a fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with filters for DAPI and Alexa Fluor 488. Images were captured at magnification, x20.

Gene silencing by small interfering (si) RNA. U87MG cells ($1x10^6$) were seeded in 25 cm^2 culture flasks containing 3 ml DMEM Ham's F-10 supplemented with 10% FBS without antibiotics. Cells were incubated until they reached 60-80% confluence (18-24 h). Specific oligomers for *HEB* siRNA were used (catalog no. sc-35552; Santa Cruz Biotechnology, Inc.), which includes three sequences of 20-25 nucleotides for *HEB* silencing. Oligomer transfections into U87MG cells were performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were transfected for 6 h, followed by a 24 h

incubation and irradiation. Cells were then analyzed for cell death, proliferation and cell cycle kinetics (at 24, 48 and 72 h following irradiation). To assess the efficiency of transfection, $1x10^6$ cells were seeded and harvested after 72 h for western blotting. The scrambled sequence (SCR; catalog no. sc-37007; Santa Cruz Biotechnology, Inc.) served as a negative control and was used at an identical concentration to *HEB* siRNA. For neurosphere culture, 24 h following transfection in monolayers, 500 cells were seeded in 96-well plates in triplicate, in 200 μl specific medium for neurosphere formation, as described earlier. Cells were irradiated or sham-irradiated 24 h later and cultured for 6 days at 37˚C and 5% CO_2, at which point they were harvested for neurosphere counting.

Cell irradiation. U87MG cells cultured as monolayers or neurospheres were irradiated or sham-irradiated 24 h following transfection with *HEB* siRNA or SCR. Cells were irradiated with 4 Gy of gamma-rays (^{60}Co source; dose rate, 0.5 Gy/min; Gammatron S-80; Siemens AG, Munich, Germany).

Proliferation, cell death and cell cycle analyses. Following *HEB* knockdown by siRNA, U87MG cells were incubated for 24 h, irradiated with 4 Gy, and harvested at 24, 48 and 72 h subsequent to irradiation. Cells were detached with Accutase® followed by measurements of cell viability and cell death, using the Guava ViaCount kit (Merck Millipore), according to the manufacturer's protocol. Samples were analyzed using the Guava cytometer EasyCyte Mini system (Merck Millipore), and GuavaCytoSoft software version 4.2.1 (Merck Millipore); ≥1,000 events were analyzed for each sample.

Following the removal of an aliquot of cells for the proliferation assay, the remaining cells were fixed in 70% ethanol and frozen at -20˚C for ≥24 h, prior to cell cycle analysis. Cells were incubated with Guava Cell Cycle reagent (Merck Millipore), according to the manufacturer's protocol, and analyzed using the Guava cytometer EasyCyte Mini system and GuavaCytoSoft software version 4.2.1; ≥5,000 events were analyzed for each sample.

Neurosphere formation, determination of cell number and cell death. Neurospheres >60 microns were counted using a Nikon inverted microscope (TS100; Nikon Corporation, Tokyo, Japan) at magnification, x100. The following day, the neurospheres were dissociated with Accutase® in order to determine cell number and death using the Guava ViaCount kit as described in the previous section.

Detection of CD133 by immunofluorescence. Neurospheres formed from U87MG adherent cells were dissociated 7 days following treatment. Cells were re-incubated in cellular differentiation medium (DMEM/F12 containing 10% FBS and 1% penicillin/streptomycin) and harvested following an additional period of 8 days. Cells were detached using Accutase®, washed in 1X PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. Subsequent to washing in 1X PBS, cells were incubated in blocking solution (0.5% BSA in PBS), followed by incubation with a rabbit primary anti-CD133 antibody (1:100 in PBS/2%BSA; catalog no. 3663; Cell Signaling Technology, Inc.). Cells were subsequently incubated with an Alexa Fluor 488-conjugated anti-rabbit IgG secondary antibody (1:100 in PBS/2%BSA). The samples were analyzed using the Guava cytometer EasyCyte Mini system and GuavaCytoSoft software version 4.2.1.

The presence of CD133 protein is characteristic of certain types of tumor stem cells and is associated with their differentiation capacity. Therefore, neurospheres cultured for 8 days in neurosphere culture medium were used as a positive control.

Statistical analysis. In general, at least three independent experiments were performed, except for HEB protein expression analysis by western blotting and immunofluorescence, for which one experiment was conducted. The results were analyzed by Student's t-test if less than three groups were compared, or by one-way analysis of variance followed by Holm-Sidak pairwise multiple comparison test if more than two groups were compared. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using SigmaStat for Windows software version 3.5 (Systat Software, Inc., San Jose, CA, USA), and the graphs were plotted in Microsoft Excel version 2010 (Microsoft Corporation, Redmond, WA, USA). Data are expressed as the mean ± standard deviation.

Results

HEB protein expression. HEB protein expression was assessed in U87MG and ACBRI-371 cells in adherent monolayer cultures in the absence of radiation treatment. Using immunofluorescence, nuclear localization of HEB protein in U87MG (Fig. 1A) and ACBRI-371 (Fig. 1B) was observed.

Western blotting revealed a relative HEB expression of 1.35±0.16 and 1.31±0.01 (neurospheres) in U87MG cells, and 1.14±0.24 and 0.76±0.11 (neurospheres) in ACBRI-371 cells (Fig. 1C and D). No significant differences were observed between each cell line and their respective neurospheres. Although U87MG cells have greater HEB protein expression levels compared with astrocytes, the difference was not significant. Notably, U87MG neurospheres have significantly greater HEB protein expression levels compared with ACBRI-371 neurospheres ($P=0.001$). *HEB* silencing resulted in a 97.8% reduction in HEB protein expression levels 72 h following transfection (Fig. 1E and F). These results indicated that HEB protein is a potential target for therapeutic strategies based on molecular inhibition.

Effects of HEB silencing on U87MG monolayer cells. To determine whether *HEB* knockdown affects the proliferation and cell death of U87MG cells, cell cultures were irradiated or sham-irradiated 24 h following transfection with *HEB* siRNA; cells were analyzed 24, 48 and 72 h later. Inhibiting *HEB* decreased cell proliferation at all time points, compared with the SCR group (Fig. 2A). This decrease was significant at 72 h (siHEB, 2.0±0.4; SCR, 3.4±0.8; $P=0.008$). In addition, *HEB* silencing in combination with irradiation reduced U87MG cell doubling (0.9±0.1) compared with the non-irradiated siHEB group (2.0±0.4; $P=0.013$) at 72 h. An increase in cell death was detected in irradiated compared with non-irradiated siHEB cells ($P=0.007$); however, this increase was proportional to SCR irradiated cells ($P=0.005$), at 48 h (Fig. 2B).

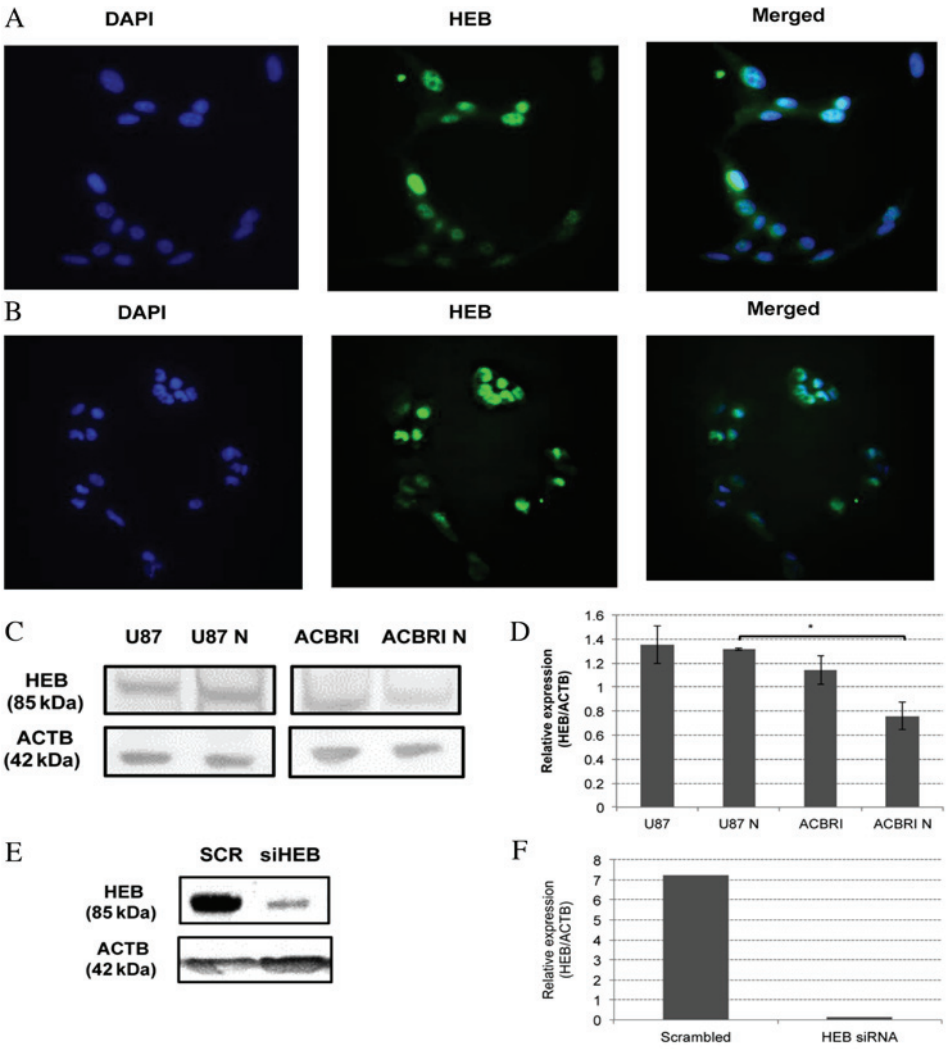

Figure 1. HEB expression and silencing. HEB protein expression was visualized by immunocytochemistry in (A) U87MG and (B) ACBRI-371 cell lines. HEB was localized to the nuclei of the two cell lines. Magnification, x20. (C) HEB expression was analyzed by western blotting in U87MG monolayers and neurospheres and ACBRI-371 astrocyte monolayers and neurospheres. (D) Relative protein expression levels of HEB calculated by densitometric analysis relative to ACTB. HEB protein expression levels were increased in U87MG neurospheres compared with ACBRI-371 neurospheres. $^{*}P<0.05$. (E) HEB protein expression in U87MG cells transfected with 25 nM HEB siRNA or SCR, analyzed 72 h following transfection. (F) HEB protein expression was decreased by 97.8% in cells transfected with HEB siRNA compared with SCR. HEB, transcription factor 12; U87, U87MG monolayers; U87N, U87MG neurospheres; ACBRI, ACBRI-371 monolayers; ACBRI N, ACBRI-371 neurospheres; ACTB, β-actin; siRNA, small interfering RNA; SCR, scrambled sequence.

Cell cycle kinetics and sub-G1 cells were analyzed by flow cytometry (Fig. 2C). A significantly decreased proportion of *HEB*-silenced cells were in G0/G1 phase (P=0.042) and a non-significantly increased proportion was detected in the sub-G1 fraction at 24 h compared with the SCR group. Irradiation of *HEB* siRNA-transfected cells further decreased the percentage of cells in G0/G1 phase (P=0.001) and non-significantly increased the percentage of sub-G1 cells at 72 h compared with sham-irradiated *HEB*-silenced cells. In addition, a reduction in the proportion of G0/G1 cells (P=0.01) and an increase in the sub-G1 population (P=0.007) were observed in the irradiated SCR group at 72 h compared with the non-irradiated SCR group.

Effects of HEB silencing on neurosphere formation. *HEB* silencing significantly reduced the neurosphere number compared with the SCR group (P=0.002; Fig. 3A); however, no significant differences were observed in the number of cells (Fig. 3B) or the percentage of cell death (Fig. 3C). Irradiation significantly decreased cell number (P=0.006) and increased cell death (P=0.005) independent of *HEB* silencing. The irradiated *HEB*-silenced group demonstrated a non-significantly decreased number of neurosphere cells compared with the irradiated SCR group, but without a concurrent decrease in neurosphere number, which was however reduced in the SCR irradiated group compared with the respective control (P=0.003).

A high percentage of neurospheres cultured for 8 days expressed CD133 (44.1±4.1%; Fig. 3D). Neurospheres dissociated and cultured under differentiation conditions demonstrated a significant reduction (P<0.0001) in the percentage of $CD133^{+}$ cells (SCR transfected group, 8.9±6.4%). These results confirm the presence of $CD133^{+}$ stem cells in neurospheres, and indicate that the differentiation medium reduced the proportion of $CD133^{+}$ cells, which may be directly correlated with the differentiation of stem cells.

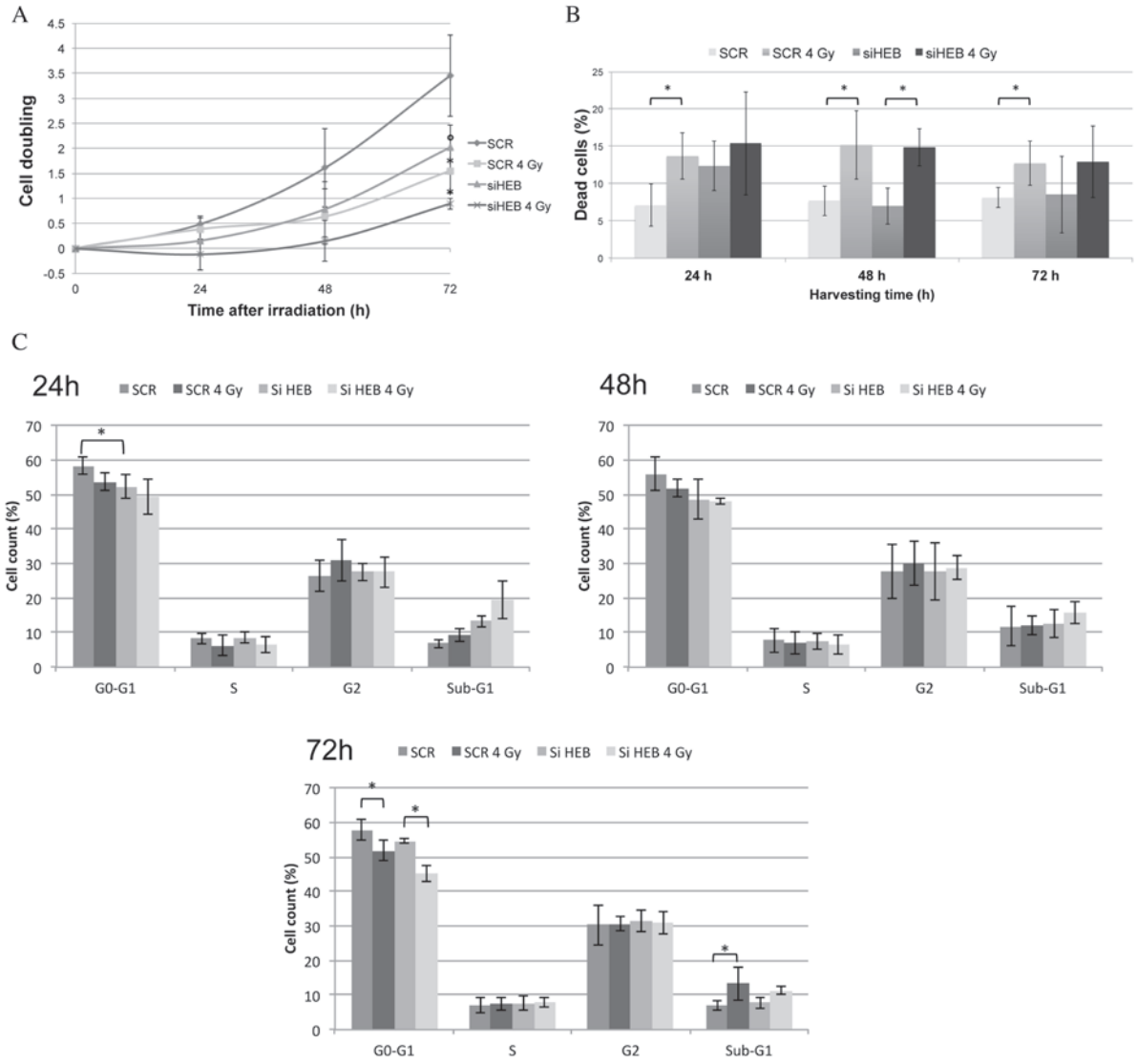

Figure 2. Biological effects of *HEB* silencing. U87MG cells transfected with *HEB* siRNA or SCR were analyzed at 24, 48 and 72 h following irradiation or sham-irradiation. (A) Cell proliferation curves based on cell doubling. The zero time refers to irradiation (doubling value=zero). *HEB* siRNA and irradiation reduced proliferation of cells. *P<0.05, SCR vs. SCR 4 Gy and siHEB vs. siHEB 4 Gy; °P<0.05, SCR vs. siHEB. (B) Percentage of dead cells in culture. The proportion of dead cells was increased by irradiation. (C) Cell cycle analysis. *HEB* siRNA decreased the percentage of cells in G0/G1 phase at 24 h, compared with the SCR group. *P<0.05. HEB, transcription factor 12; siRNA, small interfering RNA; SCR, scrambled sequence.

Irradiation did not affect the $CD133^+$ population in *HEB*-silenced cells, whereas a greater percentage of irradiated SCR transfected cells were $CD133^+$ compared with non-irradiated SCR cells. A greater proportion of siHEB-transfected cells were $CD133^+$ (21.6±5.5%; P=0.0010) compared with the SCR group; however, this remained almost 3 times lower than cells cultured under neurosphere growing conditions (P<0.0001). These results indicate that irradiation or *HEB* silencing inhibits or delays GBM cell differentiation; however when combined, the effect is not additive or synergistic.

Discussion

The present study investigated the effects of *HEB* knockdown on the proliferation of GBM cells, and whether *HEB*-silenced cells may be sensitized to irradiation. HEB protein expression was analyzed in the nuclei of U87MG and ACBRI-371 monolayers and neurosphere cells. Although significant differences were not observed between the two cell lines, U87MG cells demonstrated greater HEB protein expression levels compared with astrocytes when cultured in monolayers. Consistent with these results, *HEB* has been revealed to be transcriptionally induced in oligodendroglioma and astrocytic glioma compared with healthy brain tissue (36). A previous study of 120 patients with CRC associated *HEB* overexpression with metastasis and poorer survival, using microarray data and validation (34). In addition, the same study observed high levels of HEB in certain CRC cell lines.

In the present study, *HEB* silencing was confirmed by western blotting 72 h following transfection, and reached 97.8% inhibition compared with the SCR group. *HEB* silencing induced a significant reduction in cell proliferation at 72 h, and a decrease of cells in the G0/G1 phase at 24 h.

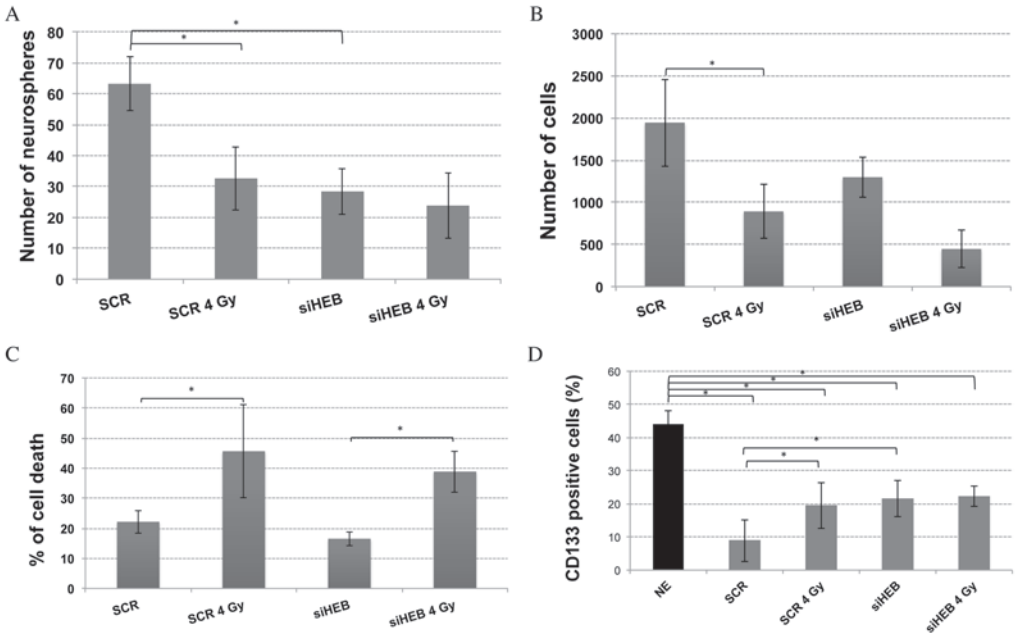

Figure 3. Effects of *HEB* silencing on neurospheres. U87MG neurospheres were transfected with SCR or *HEB* siRNA and irradiated or sham-irradiated 24 h following transfection. (A) Neurosphere number formed from 500 cells, at 6 days post-irradiation. The neurosphere number was decreased in irradiated SCR cells, and irradiated and non-irradiated siHEB cells, compared with non-irradiated SCR cells. (B) Number of cells present in neurospheres, at 7 days post-irradiation. The number of cells within neurospheres was decreased by irradiation. (C) Percentage of dead cells, at 7 days post-irradiation. The proportion of dead cells was increased by irradiation. (D) Percentage of $CD133^+$ cells, measured following 8 days culture in differentiation medium, initiated 7 days following irradiation, and compared with cells maintained in neurosphere formation medium. The proportion of $CD133^+$ cells was decreased in all differentiation medium-cultured cells compared with neurosphere medium-cultured cells, and further decreased in SCR non-irradiated cells. $^*P<0.05$. HEB, transcription factor 12; siRNA, small interfering RNA; SCR, scrambled sequence; CD133, cluster of differentiation 133.

This transient effect of *HEB* silencing at 24 h may be due to the effect of different quantities of HEB protein at this time, as the inhibition increased over time, at least at 48 and 72 h following transfection. HEB protein may act via threshold levels, as for the E2 factor family of transcription factors, in which one threshold is associated with apoptosis, and a lower threshold is associated with proliferation (38).

A previous study has reported that *HEB* silencing in CRC cell lines resulted in antitumor effects, via reduced migration, invasion and metastasis, through greater cell-cell contact and gap-junction activity, and via increased E-cadherin, connexin 26 and connexin 43, but decreased fibronectin levels (34). Clinically, *HEB* mRNA overexpression has been correlated with E-cadherin mRNA downregulation in tumor tissues (34). Cadherins are integral membrane proteins that mediate calcium-dependent cell-cell adhesion, and they may be involved in the development and maintenance of tissues, and the invasion and metastasis of malignant tumors (39). Therefore, cadherins are considered as potential targets to reduce chemo- and radioresistance (40). Our previous study revealed an upregulation of three cadherins (*CDH8*, *CDH13* and *CD93*) and one integrin *(ITGA5)* in mutated *TP53* GBM cell lines compared with wild-type cells (6).

In the present study, the effects of *HEB* silencing combined with irradiation were analyzed. Combined treatment reduced the proliferative capacity (doubling levels) of U87MG cells when cultured in monolayers; however, cell death was not increased compared with the SCR irradiated group. It is known that radiation exposure may not be manifested for several cell divisions (41). It is therefore possible that the decrease in proliferation due to HEB silencing may palliate the effects of irradiation in siHEB cells.

Our previous study identified differentially expressed genes in irradiated vs. sham-irradiated GBM cell lines, obtained by microarray (7). By performing *in silico* bioinformatics analysis, TFs associated with those differentially expressed genes were identified; among these TFs, *HEB* expression was greater in irradiated U87MG cells compared with controls (23). Genes controlled by *HEB* include neurofibromin 1 (*NF1),* a disintegrin and metalloproteinase with thrombospondin type and distal-less homeobox 6 (associated with cell proliferation), and G protein-coupled receptor 68, mannan binding lectin serine peptidase 2 and *NF1* (involved in wound healing); these were upregulated in U87MG cells following irradiation. These results are compatible with those obtained in the present study with *HEB* silencing, in which U87MG proliferation was decreased following irradiation.

The present study revealed greater HEB protein expression levels in U87MG neurospheres compared with astrocyte neurospheres. The number of neurospheres formed from *HEB*-silenced cells was significantly reduced compared with the SCR group, whereas there was a non-significant reduction in the number of cells present within siHEB neurospheres compared with the SCR group. The reduction of HEB protein expression may alter the maintenance of U87MG stem cells, possibly via a decrease in differentiation, thus decreasing the number of neurospheres. HEB expression in GBM stem cells may have similar proliferative roles in neural stem cells and progenitor cells (15), supporting the relevance of this TF in GBM stem cell maintenance.

In the present study, irradiation did not decrease the number of siHEB neurospheres; however, it did increase cell death in *HEB*-silenced cells, and a similar effect was observed in the irradiated SCR group. Although the number of neurospheres was unaffected by irradiation, there was a non-significant reduction in the number of cells within irradiated *HEB*-silenced neurospheres, compared with the non-irradiated siHEB group; in addition, the number of cells was decreased non-significantly compared with irradiated SCR cells. These results indicated that siHEB may have a minor influence on the radioresistance of U87MG GBM cells.

CD133 expression was evaluated 8 days following the dissociation of neurospheres and was greater in siHEB compared with SCR cells, indicating a possible decrease in cell differentiation. These results are in accordance with the literature, as HEB has been associated with differentiation in diverse cells, including oligodendrocytes (42). Although *HEB* appears to be important in GBM neurosphere maintenance/formation, siHEB cells exhibited a decrease in differentiation potential when cells were submitted to a differentiation stimulus.

Promoting the differentiation of CSCs, thus reducing tumor growth, is a novel approach to cancer therapy (43). Previous studies have described how CG500354 (44), a short hairpin RNA for ubiquitin ligases (45) and all-trans retinoic acid (46), may induce stem cell differentiation in GBM. However, in these studies treatments were performed in neurosphere formation medium, and stem cell and differentiation markers were analyzed days following treatment, whereas in the present study, CD133 expression was assessed following dissociation of neurospheres 7 days subsequent to treatment, and a further 8 days of culture. The high proportion of $CD133^+$ cells in the siHEB-silenced group compared with the SCR group suggested that reduced differentiation may be a side-effect of *HEB* silencing. Although under the differentiation therapy approach, it is desirable to increase differentiation of tumor stem cells, the primary aim is to reduce tumor proliferation, which was observed in siHEB cells. These findings indicated that *HEB* is a potential target in GBM treatment. Regarding irradiated cells, the proportion of $CD133^+$ cells was greater in the *HEB*-silenced group compared with the SCR group, independent of irradiation, indicating that *HEB* silencing, irradiation or combination therapy caused similar effects on the differentiation capacity of cells.

In conclusion, the results of the present study demonstrated that *HEB* may be involved in GBM cell proliferation, as *HEB* silencing reduced proliferation in cells cultured as monolayers or neurospheres. Furthermore, the results suggested a potential role for *HEB* in the maintenance of GBM stem cells, as *HEB* silencing affected the differentiation capacity of cells. However, only limited effects were exerted by irradiation, primarily on neurosphere cell number. *HEB* may be a potential target to decrease proliferation in U87MG GBM cells, grown as monolayers or neurospheres, and may provide important information for the development of novel strategies for cancer therapy.

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