HHS Public Access

Author manuscript

Cancer Gene Ther. Author manuscript; available in PMC 2011 March 01.

Published in final edited form as:

Cancer Gene Ther. 2010 September ; 17(9): 664–674. doi:10.1038/cgt.2010.31.

Embryonic stem cell (ESC)-mediated transgene delivery induces growth suppression, apoptosis, radiosensitization, and overcomes temozolomide resistance in malignant gliomas

Isabelle M. Germano, **Luni Emdad**, **Zulekha A. Qadeer**, and **Mahmud Uzzaman**

Department of Neurosurgery, Mount Sinai School of Medicine, New York, NY 10029

Abstract

High-grade gliomas are among the most lethal of all cancers. Despite considerable advances in multi-modality treatment, including surgery, radiotherapy, and chemotherapy, the overall prognosis for patients with this disease remains dismal. Currently available treatments necessitate the development of more effective tumor-selective therapies. The use of gene therapy for malignant gliomas is promising as it allows *in situ* delivery and selectively targets brain tumor cells while sparing the adjacent normal brain tissue. Viral vectors to deliver pro-apoptotic genes to malignant glioma cells have been investigated. Although tangible results on patients' survival remains to be further documented, significant advances in therapeutic gene transfer strategies have been made. Recently, cell-based gene delivery has been sought as an alternative method. In this paper, we report the pro-apoptotic effects of embryonic stem cell (ESC)-mediated *mda-7/IL-24* delivery to malignant glioma cell lines. Our data show that these are similar to those observed using a viral vector. Additionally, acknowledging the heterogeneity of malignant glioma cells and their signaling pathways, we assessed the effects of conventional treatment for high grade gliomas, IR and TMZ, when combined with ESC-mediated transgene delivery. This combination resulted in synergistic effects on tumor cell death. The mechanisms involved in this beneficial effect included activation of both apoptosis and autophagy. Our *in vitro* data supports the concept that ESC-mediated gene delivery might offer therapeutic advantages over standard approaches to malignant gliomas. Our results corroborate the theory that combined treatments exploiting different signaling pathways are needed to succeed in the treatment of malignant gliomas.

Keywords

Embryonic stem cell; apoptosis; autophagy; malignant gliomas; gene delivery

Corresponding author: Isabelle M. Germano, MD, FACS, Professor Neurosurgery, Neurology, Oncological Sciences, Department of Neurosurgery, Mount Sinai School of Medicine, 5 East 98th Street-Box 1136, New York (NY) 10029, Phone 212-241-9638, Fax 212-831-3324, isabelle.germano@mountsinai.org.

Conflict of interest: The authors declare no conflict of interest.

INTRODUCTION

Primary malignant brain tumors are relatively uncommon and yet they are one of the most lethal forms of cancer. Glioblastoma multiforme (GBM) is the most common and aggressive type of primary brain tumor.1 In children, it is the second most common malignancy following leukemia and represents the leading cause of cancer death in children under the age of 15.2 In adults, GBMs account for approximately 50% of primary brain tumors.3 Aggressive multimodality treatments with surgery, radiation, and chemotherapy have led to some improvement in prognosis for patients with glioblastoma and high-grade gliomas. Recent evidence-based Class IIb clinical data supports the concept that aggressive surgical resection of GBM has a positive impact on survival.4 However, the 5 year survival rate for patients with GBM is less than 5% and a median survival rate of less than 12 months.1 The futility of present treatments in combating this disease is in part due to their inability to address the highly invasive nature of these neoplasms. Malignant cells intersperse themselves with normal brain parenchyma and typically give rise to tumor recurrence in close proximity of the operated surgical site.5 The strategy of targeting these tumor cells while sparing the normal cells may prove to be critical for the success of any potential therapeutics.

Gene therapy strategies currently in clinical trials use viral vectors to deliver therapeutic transgenes directly to normal and tumor cells within the central nervous system (CNS). To date, however, none of these trials has shown a significant clinical benefit.6 This can be attributed in part to the theoretical and practical limitations of viral vectors, including limited diffusion into brain parenchyma, poor transfection of some cell types, antigenicity, and insertional mutagenesis with the potential to cause new tumors.7 The use of genetically modified cells to deliver gene therapy to the CNS may avoid some of these limitations.8

Embryonic stem cells (ESC) are totipotent cells obtained from the inner cell mass of the blastocyst stage embryo.9 They have unlimited proliferative capacity. ESC-derived astrocytes have potential advantages over other types of genetically engineered neural cells, such as neuronal progenitor cells (NPCs). First, astrocytes are fully differentiated unlike pluripotent NPCs that could theoretically differentiate into functional neurons and interfere with existing neural circuits. Second, unlike NPCs, ESCs can be permanently genetically modified using homologous recombination rather than potentially hazardous or transiently expressed viral vectors. Finally, ESCs have unlimited proliferative capacity; if stocks of undifferentiated human ESC of various HLA types are established in the future, an unlimited supply of immunologically matched cells for transplantation would be available. We have shown that we can differentiate ESC in a pure astrocytic population 9 and these maintain migratory capacity.10 Additionally, we have shown that we can engineer ESC-derived astrocytes to express a pro-apoptotic gene under tight control of a *tet-on* promoter.

In this study, we show that we can engineer ESC-derived astrocytes to conditionally express *mda-7/IL-24*, a pro-apotptotic gene shown to have selective effects on cancer cells while sparing the normal tissue.11–14 We also demonstrate that ESC-mediated gene delivery causes apoptosis in a variety of malignant glioma cell lines, independent of p53 status. Furthermore, our *in vitro* experiments show that ESC-mediated *mda-7/IL-24* delivery causes

radiosensitization and overcomes temozolomide resistance. These effects seem to be mediated by interference with signaling pathways of both apoptosis and autophagy.

MATERIALS AND METHODS

Cell lines and cultures

Human malignant glioma cells—Six cell lines were used for these *in vitro* experiments, U87, U251, U343, U373, A172, and T98G. Cells were cultured in standard condition. In brief, human malignant glioma cell lines U87, A172, and U343 were obtained from the Neurosurgery Tissue Bank (San Francisco, CA) and T98G, U251, and U373 cell lines from the American Type Culture Collection (ATCC) (Rockville, MD). Cells were cultured in Dulbecco's modified essential medium (DMEM, Cellgro, CA) supplemented with 10% FCS at 37°C incubator supplemented with 5% CO_2. *Mouse ESCs*. These were passaged and maintained according to previously published protocols.9 Mouse Ainv-18 ESCs (gift of Michael Kyba) constitutively express a Dox-binding-transcriptional activator fusion protein and include a Dox-responsive promoter upstream of a Lox cloning site. Genes cloned into the Lox site using Cre-assisted recombination are then expressed when the cells are exposed to Dox (Sigma, St Louis, MO). ESCs were directed to differentiate into astrocytes using protocols recently developed in our lab.9, 10

Engineering of ESC-derived astrocytes conditionally expressing *mda-7/IL-24*

A cDNA containing the entire coding sequence of *mda*-7/IL-24 (Dr. Paul B. Fisher, Virginia Commonwealth University, Richmond, VA) was cloned into the pLox vector as previously described.3 *mda*-7-pLox plasmid DNA and Cre recombinase-expressing helper plasmid DNA were prepared using a column purification system (Qiagen, Valencia, CA) and electroporated into appropriately prepared Ainv-18 mouse ES cells. Electroporated Ainv-18 cells were plated onto drug-resistant embryonic feeder cells, and recombinant clones were selected with increasing concentrations of G418. Individual G418-resistant colonies were selected and expanded. Insertion of transgene into the Lox cloning site was confirmed using PCR from a genomic DNA template. Forward Primer: 5′-CTC GAG GCC GCC ACC ATG AAT TTT CAA CAG- 3′; Reverse: 5′-TCT AGA TTC AGA GCT TGT AGA ATT TCT GCA -3′; conditions: 30 cycles, 2 min 98°C denature; 20 sec 95°C; denature; 20 sec 55°C annealing; 60 sec 68°C extension; 10′ min 68°C elongation.

Cell migration and transwell experiments

To assess the migratory capacity of ESC-derived astrocytes conditionally expressing *mda-7/IL-24*, transwell experiments were performed according to published protocols.15 For these experiments, ESC-derived astrocytes conditionally expressing *mda-7/IL-24* [cells 10^5] were placed in the upper well (6.5 mm; Corning Inc, Corning, NY) of transwell plates with porous polycarbonate membrane (8 μm pores) coated with 0.1% gelatin. Glioma cells U87 (5×10^4 cells/well) were placed in the lower wells and incubated in DMEM +10% serum. At 24 and 48 hrs after plating, the migration of ESC-derived astrocytes was determined by fixing the membrane, staining the cells using the Hema3 staining kit (Fisher Diagnostics, Pittsburgh, PA), directly counting the number of migrated cells in 10 high-power fields, and calculating the mean. Experiments were done in triplicates.

RT-PCR, immunocytochemistry, ELISA, FACS for detection of transgene expression

Reverse transcriptase-Polymerase chain reaction (RT-PCR) analysis—Gene expression was analyzed by RT-PCR. Total RNA was extracted and RT-PCR performed as previously described.9 In brief, ESC-derived astrocytes conditionally expressing *mda-7/IL-24* were cultured for 24 hours in the presence or absence of 1μg/ml Dox and harvested for RT-PCR. Total cellular RNA was purified using the RNeasy kit (Qiagen, Valencia, CA), with on-column DNAse treatment. All RNA preparations were spectrophotometrically quantified and examined for degradation using gel electrophoresis prior to reverse transcription. Reverse transcription was performed on 1 μg of total RNA using the Omniscript Reverse Transcriptase Kit (Promega, Madison, WI) and PDN6 random hexamer primers (Pharmacia, Piscataway, NJ) in a total volume of 20 μL. A total volume of PCR mixture was 12.5 μL each contained 0.5 μL cDNA template. All PCR amplifications were carried out for 30 cycles. PCR products were examined on 1.8% agarose gels and photographed using an Eagle Eye II imager (Stratagene, La Jolla, CA).
Immunocytochemistry. Differentiated cells were grown on glass cover slips (Fisher Scientific, Hampton, NH) coated with poly D-lysine [Sigma, St. Louis, MO]. For immunofluorescent labeling, cells were washed with PBS, fixed in 4% paraformaldehyde (PFA) in PBS for 30 minutes at room temperature (rT), washed, pre-incubated in 10% normal goat serum (Sigma, St. Louis, MO) for 30 minutes at rT, then in primary antibody for 1 hour at rT. Primary antibodies and dilutions are as follows: monoclonal anti-GFAP 1:200 (Sigma, St. Louis, MO) and polyclonal anti-*mda*-7/IL-24 (Dr. Paul B Fisher, VCU, Richmond, VA) 1:200. Secondary antibodies and dilutions are as follows: Alexa 594 anti rabbit (1:400) and Alexa 594 (1:250) anti mouse antibodies (Chemicon International, Temecula, CA). Cells were then washed and mounted in Vectashield with added DAPI (Vector Laboratories, Burlingame, CA), and examined under fluorescent microscopy using the Openlab imaging system (Improvision, Lexington, MA). Omission of the primary antibody served as negative control.

Enzyme-Linked ImmunoSorbent Assay (ELISA)—Ad.*mda*-7 used for this experiment was kindly provided by Dr. Paul B Fisher (VCU, Richmond, VA). The virus was aliquoted and kept in −80°C until the experimental use. U87 cells were plated in 6 well plate and next day infected with different MOIs of Ad.*vec* and Ad.*mda-7*. Twenty-four hours after infection conditioned media was collected and stored in -80°C until experimental use. ESC-derived astrocytes conditionally expressing *mda-7/IL-24* were plated [2×10^5] in 0.1% gelatin coated 12 well plates and cultured in IMDM, 15% serum replacement (SR). Twenty-four hours after induction with Dox, the supernatants were collected and the MDA-7/IL-24 content was determined with an ELISA test kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's protocol. The amounts of MDA-7/IL-24 were quantified on the basis of a standard curve after optical density was measured at 450 nm with a reference of 550 nm on an ELISA reader (Dynex Technologies, Chantilly, VA). The mean values at each time point were then used directly for the analysis reported.

Fluorescence activated cell sorting (FACS)—ESC-derived astrocytes conditionally expressing *mda*-7/IL-24 growing in monolayer were trypsinized and then washed with medium containing FCS. Cells were then centrifuged, resuspended in wash buffer (PBS,

10% FCS, and 0.01% NaN3), and counted. For flow cytometry, cells sequentially labeled with primary (monoclonal anti-GFAP (Sigma, St. Louis, MO) and human IL-24 (GeneHunter Corp, Nashville TN) antibodies and appropriate secondary antibodies were counted with a FACS scanner (Calibur; Becton Dickinson, San Jose, CA) and further analyzed with Cell Quest software package (BD Biosciences). A gating technique, with appropriate measurement of forward and side scatter, was used to exclude dead cells and cell aggregates from the sorting, and 5000 to 10,000 cells were counted per analysis.

Exposure to ionizing radiation (IR), temozolomide (TMZ), and clonogenic assays

Exposure to IR and clonogenic assays—Human malignant glioma cells were exposed to IR for a total dose of 6 Gy using ^{137}Cs-irradiater^{-}Model E-0103, (U.S. Nuclear Corp., Burbank, CA) at a dose rate of 3.312 Gy/min, as previously described.16 To determine the intrinsic cellular radiosensitivity, clonogenic assays were used following established methods.17 Exponentially growing cells were radiated as above described, then immediately counted, diluted, and plated on 60mm culture dishes. After incubation for 14 days, the plates were stained with cresyl violet (Sigma, St. Louis, MO) for colony counting. Colonies with more than 50 cells were scored, and plating efficiency was determined. To determine the effects of concurrent transgenic *mda-7/IL-24*, and/or TMZ, glioma cells were exposed to the chosen treatment(s) for 72 hours after IR and then plated as above.

Exposure to TMZ—Temozolomide was supplied by Schering- Plough [Kelinworth, NJ] and dissolved in DMSO (dimethyle sulfoxide, SIGMA, St Louis, MO) in 50mM stock solution. TMZ was used at a concentration of [100 μmol/L], as previously decribed 18.

Detection, quantification, and inhibition of apoptosis and autophagy

Apoptosis—For detection of *in vivo* apoptosis, cells were grown on poly-D-lysine coated cover slips and apoptosis signal was detected using ApopTag plus fluorescein in situ apoptosis detection kit (Chemicon International, Temecula, CA), indirect TUNEL method. All procedures were performed according to the kit protocol. After experiments, cells were mounted on slides with Vectashield mounting medium (Vectashield, Burlingame, CA) and images were taken with a Leica fluorescence microscope (Leica, Bannockburn, IL). FACS analysis was used to quantify apoptotic and dead cells, following labeling with Annexin V-Phycoceruthium (PE) to detect early apoptotic cells and vital dye 7-Amino-Actinomycin (7AAD) to detect late apoptotic and necrotic cells (BD Biosciences, San Diego, CA).19 Gating with appropriate measurement of forward and side scatter was used to exclude dead cells and cell aggregates from the sorting; 5, 000 to 10,000 cells were counted per analysis. Flow cytometry (Calibur, Becton Dickinson, San Jose, CA) data were further analysed using the Cell Quest software package (BD Bioscences, San Jose, CA). *Autophagy*. Autophagy is the process of sequestrating cytoplasmic proteins into the lytic component and characterized by the formation of acidic vesicular organelles (AVO). To detect and quantify the AVO in *mda*-7/IL-24, IR and TMZ-treated cells, we performed the vital staining with acridine orange, as previously described.18 Three days after treatment, cells were stained with acridine orange at a final concentration of 1 μg/ml for a period of 15 min, removed from the plate with trypsin-EDTA, and collected in phenol red-free growth medium. Green (510–530 nm) and red (>650 nm) fluorescence emission from 10^4 cells illuminated with blue (488 nm)

excitation light was measured with a FACSCalibur from Becton Dickinson using CellQuest soft-ware. *Inhibition of apoptosis and autophagy*. To inhibit autophagy, 1.0 mM 3-methyl adenine (MA) was added next day after addition of TMZ. To inhibit JNK pathways cells were pre-treated with 5μM of JNK-inhibitor, SP600125, before exposure to IR.

Groups studied—Autophagy and apoptosis experiments were carried out in human malignant glioma cell lines [U87, U251, U343, U373, A172, and T98G] 48 hours after exposure to treatment. Non-engineered ESC-derived astrocytes were used as controls. Commercially available IL24 (50 ng/ml) [R&D systems, Minneapolis, MN] was tested on the same human glioma cell lines.

Cell viability assay

Effects of autophagy and apoptosis inhibition on cell viability were measured by 3-*(4,5-Dimethylthiazol-2-Yl)*-2,5-Diphenyltetrazolium Bromide (MTT) cell viability assay. Cells were seeded in 96-well tissue culture plates (1.0×10^3 cells per well) and treated the next day as described in the figure legends. At the indicated time points, the medium was removed, and fresh medium containing 0.5 mg/ml MTT was added to each well. The cells were incubated at 37°C for 4 h and then an equal volume of solubilization solution (0.01 N HCl in 10% SDS) was added to each well and mixed thoroughly. The optical density from the plates was read on an Elisa plate reader at 570 nm.

Western Blot Analysis

Briefly, for western blotting experiments, cells were harvested in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS in PBS) containing protease inhibitor cocktail (Protease Arrest TM; Calbiochem, San Diego, CA), 50 mM NaF and 1 mM Na3VO4, homozinized and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was used as total cell lysate. Protein lysates (30–50 μg) were denatured in 2% SDS, 10 mM dithiothreitol, 60 mM Tris-HCl (pH 6.8), and 0.1% bromphenol blue, and loaded onto a 12% polyacrylamide/SDS gel (BIO-RAD). The separated proteins were then transferred by electroblot (80 mA, 2 h) onto a nitrocellulose membrane (BIO-RAD). The membrane was blocked for 1 hour at rT in TBS-T (0.1 M Tris-HCl (pH 7.6), 1.37 M NaCl, 0.1% Tween 20) containing 5% nonfat dry milk and incubated overnight at 4°C in TBS-T containing the primary antibody. The membrane was washed in TBS-T, incubated with the secondary antibody conjugated to horseradish peroxides for 1 hour at rT, and then washed in TBS-T. The ECL non-radioactive detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK) was utilized to detect the antibody-protein complexes by exposure of the membrane to an X-ray film. X-ray films were scanned and bands were densitometrically evaluated by Image J software (NIH).

Following primary antibodies were used: Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb (1:1000), Total SAPK/JNK (1:1000) (Cell Signaling Technology, Danvers, MA), mouse monoclonal GADD153 (1:500), mouse monoclonal Bcl-xL (1:500) and rabbit polyclonal Bax (1:1000) (SantaCruz Biotechnology, Santa Cruz, CA), and rabbit polyclonal caspase-3 (1:200) (Chemicon, Temecula, CA).

Statistical Analysis

All studies were repeated three times using triplicate samples for cell counting analysis. The data are expressed as mean ± S.D and were considered significant when $P<0.05$. The statistical significance of the experimental results was evaluated using Student's t-test. Comparison of the effects of various treatments was done using one-way ANOVA. For synergistic analysis, a modified Student's t-test was used.

RESULTS

Engineered Embryonic Stem Cell-derived astrocytes conditionally express *mda-7/IL-24* and maintain "homing"

To overcome the limitations of Ad-mediated gene transfer for the treatment of malignant gliomas, we engineered ESC-derived astrocytes to conditionally express *mda-7/IL-24*. Figure 1A shows the schematic diagram of the DNA insertional cassettes. Undifferentiated Ainv-*mda-7/IL-24* ESCs were then differentiated into astrocytes.9 Eight days after plating, >85% of the ESC-derived cells were differentiated and GFAP positive, confirming their astrocytic nature (Figure 1B, C). ESC-derived astrocytes maintained "homing" characteristics typical of stem cells, with 37% migration rate at 48 hours (Figure 1D) and 80% at 4 days (data not shown).

Semi quantitative RT-PCR (Figure 2A) and immunohistochemistry (Figure 2B) performed after Dox induction showed expression of *mda-7/IL-24* by ESC-derived astrocytes. Quantification by FACS showed conditional expression of *mda-7/IL-24* in >80% of ESC-derived astrocytes, and minimal "leaky" expression in the absence of Dox (Figure 2C).

To quantify MDA-7/IL-24 secretion by ESC-derived astrocytes after Dox induction we used ELISA and compared it with the MDA-7/IL-24 level secreted by U87 cells infected with different MOIs of Ad.*mda*-7. As shown in Figure 2D, MDA-7/IL-24 secreted by ESC-derived astrocytes conditionally expressing *mda*-7/IL-24 is comparable to the level secreted by U87 cells infected with Ad.*mda*-7 (25 pfu).

ESC-derived astrocytes conditionally expressing *mda-7/IL-24* induce apoptosis in human malignant glioma cells independent of p53 status

To asses the pro-apoptotic effects of transgenic *mda-7/IL-24* transfer; we used six human glioma cell lines (Figure 3A). These were co-cultured with ESC-derived astrocytes conditionally expressing *mda-7/IL-24* after Dox induction. Among the cell line tested A172, U343, and U87 are wild type p53 (*p53*wt); T98, U251, and U373 are mutant p53 (*p53*mt). In four of the six cell lines, a significant increase in apoptotic rate was found at 24 hours after co-culture. The ESC-derived astrocytes mediated *mda-7/IL-24* pro-apoptotic effects was not correlated to the *p53* status, as previously reported with Ad-mediated transfer.11 A similar apoptotic rate was observed in U87 cells (Figure 3B) treated with either Ad.*mda*-7 (25 pfu) or ESC-derived astroytes mediated *mda*-7/IL-24 which is in agreement with the level of MDA-7/IL-24 detected by ELISA (Figure 2D). Commercially available IL24 tested on the same human glioma cell lines, did not induce any significant cell death. Non-engineered

ESC-derived astrocytes, used as controls, did not show cell death when exposed to ESC-derived astrocytes conditionally expressing *mda-7/IL24.*

ESC-mediated *mda-7/IL-24* delivery induces growth suppression, radiosensitization, and overcomes temozolomide resistance in malignant glioma cells

To assess the effects of ESC-mediated transgene delivery of *mda-7/IL-24* on malignant glioma cells in combination with conventional treatment for GBM, we performed clonogenic assays. For these experiments, we used two cell lines partially resistant to TMZ: U87 (*p53*wt) and T98 (*p53*mt) (Figure 3C, D). Cells were exposed to transgene delivery alone or in combination with TMZ and/or IR. Exposure to each individual treatment resulted in a decrease of the survival fraction ranging from 35% to 48% for U87 cells and 19% to 35% for T98G cells, respectively. Exposure to all three treatments resulted in significant decrease of survival fraction compared to each treatment alone or two combined in both glioma cell lines tested (Anova, $p<.05$). This was not seen in control cells (non-engineered ESC-derived astrocytes). When considering only two treatments combined, *mda7/IL-24* plus IR resulted in the strongest decrease in survival fraction. The addition of *mda7/IL-24* to TMZ resulted in a significant decrease in survival fraction compared to TMZ alone ($p<.05$) in both cell lines.

Both apoptosis and autophagy signaling pathways are activated by ESC-mediated *mda-7/IL-24* delivery combined with IR and TMZ

To study the mechanism(s) responsible for the significant decrease in survival fraction of human malignant glioma cells when the IR, TMZ, and transgene *mda-7/IL-24* were used, we quantified apoptosis, autophagy, and their inhibition.

Figure 4A and B shows that the apoptotic rate is significantly increased when IR was added to *mda-7/IL-24* or TMZ. However, a synergistic effect was noted only when IR was combined to *mda-7/IL-24* (modified Student's t-test $p<.05$). The combination of all three treatments resulted in further enhanced apoptotic effects. Autophagy was significantly increased by TMZ, as previously reported by our group,18 and also by *mda-7/IL-24*, although to a lesser extent (Figure 4C).

To investigate the signaling pathways involved in decreased human glioma cells survival after exposure to each treatment alone or in combination we performed Western blot analysis (Figure 5A, B). A synergistic increase in CCAAT/enhancer binding protein homologous transcription factor (CHOP/GADD153) was found when all three treatments were used concomitantly compared to each treatment alone or two in combinations. On the other hand, the decrease in BcL-xL and increase in caspase 3 and Bak/Bax were moderately enhanced. Combination of all three treatments also resulted in a modest activation of phosphorylated- c-Jun N-terminal kinase (JNK) compared to only 2 treatments combined. These results suggest that CHOP/GADD153 serves as the common final pathway to apoptosis and autophagy.

To confirm that the decrease in survival fraction is caused by combined activation of autophagy and apoptosis, we inhibited these processes by using 3MA, a non-specific

inhibitor of autophagy, and SP600125, an inhibitor of JNK activation, respectively.20 Quantification of surviving glioma cells was done by MTT. The use of SP600125 resulted in a significant increase in cell viability after exposure to *mda-7/IL-24* and IR. This was not observed with the other treatments. 3MA resulted in a significant increase in cell viability after exposure to TMZ and IR only (Figure 5C, D).

These results taken together with the clonogenic assays, suggest that the combination of IR, TMZ, and *mda-7/IL-24* has a synergistic effect on malignant glioma cells death by activating CHOP/GADD153 which results in activation of both apoptosis and autophagy, as shown in a schematic diagram (Figure 6).

DISCUSSION

Malignant gliomas remain a therapeutic challenge.21 Substantial progress in gene delivery by viral vectors has being accomplished. Yet, clinical significance for patients with malignant gliomas is still far from being tangible. Therefore, cell-based therapy is being investigated as an alternative to *in situ* gene delivery. Recent *in vitro* and *in vivo* data support the concept that *mda-7/IL-24* causes selective tumor cell death while sparing normal cells and make this gene a good candidate for malignant astrocytoma therapy.11 Recent work focused on “targeted” therapy22 in conjunction with “standard therapy”, such as TMZ and IR23;seem to show excellent clinical results.24 Strategies aimed at targeting multiple mechanisms responsible for cell death are scientifically promising as GBM cells are very heterogeneous. In this study, we have made a few new *in vitro* observations that might have important impact on therapeutic applications for malignant gliomas.

We have published that we can generate astrocytes form ESC engineered to carry pro-apoptotic genes with conditional expression controlled by administration of Dox.25 In this paper, we first confirm that we can engineer ESC-derived astrocytes conditionally expressing *mda-7/IL-24* tightly regulated by Dox administration. We then investigate the effects of ESC-mediated *mda-7/IL-24* delivery to malignant glioma cells. First, we compared the pro-apoptotic effects of this gene when delivered by Ad and by ESC-derived astrocytes. Our results show that the transgene effects are similar using each of these two delivery methods. To the best of our knowledge, this is the first time that protein expression and pro-apoptotic effects after transgene delivery have been semi-quantitatively compared using viral and cell vectors.

Malignant gliomas and in particular glioblastoma are among the most chemoresistant tumors.26 Current literature supports the concept that combined IR and TMZ, an alkylating oral chemotherapeutic agent, improves outcome 27 and represents the standard of care for patients with high grade gliomas.23 Yet, the average survival using IR and TMZ remains dismal, with a two-year survival of 26.5%.27, 28 In this study, we combined the use of IR and TMZ with ESC-based transgene delivery of *mda-7/IL-24.* Our colony survival experiments showed that the combination of these 3 agents resulted in significant decrease of the survival fraction compared to each treatment alone or two in combinations. It is important to note that when we looked at the apoptotic rate the combination of the 3 treatments did not result in synergistic effects. Therefore, we explored autophagy as a

possible alternative explanation for programmed cell death. Data from our laboratory and others had previously shown that TMZ and *mda-7/IL-24* induce cell death by autophagy.18, 29 Semi-quantitative analysis of autophagy showed that additive effects, but not synergistic were obtained when using the 3 treatments together. These results taken together with the clonogenic assay suggested that the significant increase in tumor cell death seen when ESC-mediated *mda-7/IL-24* delivery is combined to IR and TMZ is based on activation of both apoptosis and autophagy.

To elucidate the mechanism(s) at the basis of the decreased survival observed, we investigated the unfolded protein responses (UPR) changes occurring after “endoplasmic reticulum (ER) stress”.30, 31 It has been reported that IR,32 TMZ,33 and *mda-7/IL-24* 20 induce ER stress. In particular, it has been reported that as a result of ER stress induction, all 3 modalities cause activation of the JNK by phosphorylation to P-JNK.30, 34 Our study corroborates the evidence that each treatment activated JNK, IR having the strongest effect. When TMZ or transgenic *mda-7/IL-24* was combined with IR, further enhanced effects were noted. It is interesting to note, however, that when all 3 modalities were combined, there was no additive increase of P-JNK. On the other hand, a synergistic increase in expression of CHOP/GADD153 was seen when all 3 modalities are used. Recent work supports the evidence that the CCAAT/enhancer binding protein homologous transcription factor (CHOP/GADD153) is one of the critical executioners of the pro-apoptotic arm of the UPR. 35 In our study, we showed that there is a synergistic increase expression of CHOP/GADD153 when all 3 modalities are used. Based on this finding, we suggest that CHOP/GADD153 should be considered the “common pathway” for both autophagy and apoptosis in programmed cell death, as summarized in our schematic overview in Figure 6.

The potential detrimental effects of ESC are discussed in the literature. Experimental allotransplantation of undifferentiated ESC into the brain or other tissues results in teratoma formation in recipient animals.36–40 Tranplantation of differetnaited ESC prevents teratoma formation in some experimental studies41, 42 but not in others.38, 39 In our animal studies, we have not seen teratoma formation.10 On the other hand, the uses of normal adult glial progenitor cells have the capacity to give rise to malignant gliomas,43 hindering their use as vectors. Due to the magnitude of these potential side effects, additional studies using both ESC-derived astrocytes and NPC-derived cells are necessary before these cells can be considered suitable for a clinical trial.

CONCLUSIONS

In summary, in this study we made a few new observations that can positively impact future treatment of patients with malignant gliomas. We showed that ESC-mediated pro-apoptotic gene delivery for human malignant glioma has similar biological effects as Adviral-mediated. Additionally, our study supports the concept that the pro-apoptotic effects of transgene delivery is augmented when combined with conventional therapy currently used for malignant gliomas, i.e. IR and TMZ. Furthermore, our work shows that the activation of autophagy and apoptosis is at the basis of the decreased malignant glioma cell survival when the 3 treatments are combined. Finally, we suggest that CHOP/GADD 153 serves a common final pathway for both apoptosis and autophagy. This observation has the potential for

providing a new “target” for malignant astrocytoma. Additional *in vitro* studies using NIH-approved human ESC are currently ongoing in our laboratory to validate the concept that this strategy could be used in humans.

Acknowledgments

Dr. Paul B Fisher (Virginia Commonwealth University, Richmond, VA) generously provided *mda*-7/IL-24 gene and Ad.*mda*-7. Temozolomide was supplied by Schering- Plough (Kelinworth, NJ). Dr. Gordon Keller was instrumental in guidance for the ESC differentiation into astrocytes previously published by the same Authors and used in this work.

Grant support: The present research was supported by the National Institute of Health and the National Cancer Institute NIH/NCI R01 CA129489-01A1.

Abbreviations

Ad.*mda*-7	adenovirus-mediated *mda*-7
CHOP/GADD153	CCAAT/enhancer binding protein homologous transcription factor
Dox	doxycyclin
ER	endoplasmic reticulum
ESC	embryonic stem cells
IR	ionizing radiation
JNK	c-Jun N-terminal kinase
***mda*-7/IL-24**	melanoma differentiation associated gene-7/Interleukin-24
PFU	plaques-forming units
TMZ	temozolomide
UPR	unfolded protein response

References

1. CBTRUS. Statistical Report: Primary Brain Tumors in the United States, 1998–2002. Chicago (IL): Central Brain Tumor Registry of the United States; 2005–2006.
2. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. CA Cancer J Clin. 2003; 53:5–26. [PubMed: 12568441]
3. Benveniste R, Germano IM. Evaluation of factors predicting accurate resection of high-grade gliomas using frameless image-guided stereotactic guidance. Neurosurg Focus. 2003; 14(2):E5. [PubMed: 15727426]
4. Stummer W, Reulen HJ, Meinel T, Pichlmeier U, Schumacher W, Tonn JC, et al. Extent of Resection and Survival in Glioblastoma Multiforme: Identification of and Adjustment for Bias. Neurosurgery. 2008; 62:564–76. [PubMed: 18425006]
5. Chiocca EA. Gene therapy: A primer for neurosurgeons. Neurosurgery. 2003; 53:364–73. [PubMed: 12925253]
6. Germano IM, Binello E. Gene therapy as an adjuvant treatment for malignant gliomas: from bench to bedside. J Neurooncol. 2009; 93:79–87. [PubMed: 19430884]
7. Kyritsis AP, Sioka C, Rao JS. Viruses, gene therapy and stem cells for the treatment of human glioma. Cancer Gene Ther. 2009; 16:741–52. [PubMed: 19644531]

8. Germano IM, Uzzaman M, Keller G. Gene delivery by embryonic stem cells for malignant gliomas. Cancer Biol Ther. 2008; 7:81–7. [PubMed: 18347419]
9. Benveniste RJ, Keller G, Germano IM. Embryonic stem cell-derived astrocytes expressing drug-inducible transgenes: differentiation and transplantation into the mouse brain. J Neurosurg. 2005; 103:115–23. [PubMed: 16121982]
10. Uzzaman M, Benveniste RJ, Keller G, Germano IM. Embryonic stem cell-derived astrocytes: a novel gene therapy vector for brain tumors. Neurosurg Focus. 2005; 19(3):E6. [PubMed: 16190605]
11. Fisher P. Is *mda-7*/IL-24 a "Magic Bullet" for Cancer? Cancer Res. 2005; 65:10128–38. [PubMed: 16287994]
12. Ekmekcioglu S, Mumm JB, Udtha M, Chada S, Grimm EA. Killing of human melanoma cells induced by activation of class I interferon-regulated signaling pathways via MDA-7/IL-24. Cytokine. 2008; 43:34–44. [PubMed: 18511292]
13. Sauane M, Su ZZ, Gupta P, Lebedeva IV, Dent P, Sarkar D, et al. Autocrine regulation of *mda-7*/IL-24 mediates cancer-specific apoptosis. Proc Natl Acad Sci USA. 2008; 105:9763–8. [PubMed: 18599461]
14. Yacoub A, Park MA, Gupta P, Rahmani M, Zhang G, Hamed H, et al. Caspase-, cathepsin-, and PERK-dependent regulation of MDA-7/IL-24-induced cell killing in primary human glioma cells. Mol Cancer Ther. 2008; 7:297–313. [PubMed: 18281515]
15. Nakamizo A, Marini F, Amano T, Khan A, Studeny M, Gumin J, et al. Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. Cancer Res. 2005; 65(8):3307–18. [PubMed: 15833864]
16. Yao KC, Komata T, Kondo Y, Kanzawa T, Kondo S, Germano IM. Molecular response of human glioblastoma multiforme cells to ionizing radiation: cell cycle arrest, modulation of the expression of cyclin-dependent kinase inhibitors, and autophagy. J Neurosurg. 2003; 98:378–84. [PubMed: 12593626]
17. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells *in vitro*. Nat Protoc. 2006; 1:2315–9. [PubMed: 17406473]
18. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ. 2004; 11:448–57. [PubMed: 14713959]
19. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Meth. 1995; 184:39–51.
20. Su ZZ, Lebedeva IV, Sarkar D, Emdad L, Gupta P, Kitada S, et al. Ionizing radiation enhances therapeutic activity of mda-7/IL-24: overcoming radiation- and *mda-7*/IL-24-resistance in prostate cancer cells overexpressing the anti-apoptotic proteins *bcl-x*$_L$ or *bcl-2*. Oncogene. 2006; 25:2339–48. [PubMed: 16331261]
21. Wen P, Kesari S. Malignant Gliomas in Adults. N Engl J Med. 2008; 359:492–507. [PubMed: 18669428]
22. Fiveash JB, Gillespie GY, Oliver PG, Zhou T, Belenky ML, Buchsbaum DJ. Enhancement of glioma radiotherapy and chemotherapy response with targeted antibody therapy against death receptor 5. Int J Radiat Oncol Biol Phys. 2008; 71:507–16. [PubMed: 18474311]
23. Mason WP, Maestro RD, Eisenstat D, Forsyth P, Fulton D, Laperriere N, et al. Canadian recommendations for the treatment of glioblastoma multiforme. Curr Oncol. 2008; 14:110–7. [PubMed: 17593983]
24. Hutterer M, Gunsilius E, Stockhammer G. Molecular therapies for malignant gliomas. Wien Med Wochensch. 2006; 156:351–63.
25. Germano IM, Uzzaman M, Benveniste RJ, Zaurova M, Keller G. Apoptosis in human glioblastoma cells produced using embryonic stem cell-derived astrocytes expressing tumor necrosis factor-related apoptosis-inducing ligand. J Neurosurg. 2006; 105:88–95. [PubMed: 16871882]
26. Giles, GG.; Gonzales, MF. Epidemiology of brain tumors and factors in prognosis. Edinburgh: Churchill Livingston; 1995. p. 47-69.

27. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med. 2005; 352:987–96. [PubMed: 15758009]

28. Hegi ME, Liu L, Herman JG, Stupp R, Wick W, Weller M, et al. Correlation of O^6-Methylguanine Methyltransferase (MGMT) Promoter Methylation with Clinical Outcomes in Glioblastoma and Clinical Strategies to Modulate MGMT Activity. J Clin Oncol. 2008; 26:4189–99. [PubMed: 18757334]

29. Park MA, Yacoub A, Sarkar D, Emdad L, Rahmani M, Spiegel S, et al. PERK-dependent regulation of MDA-7/IL-24-induced autophagy in primary human glioma cells. Autophagy. 2008; 4:513–5. [PubMed: 18299661]

30. Kim I, Xu W, Reed JC. Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. Nat Rev Drug Discov. 2008; 7:1013–30. [PubMed: 19043451]

31. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol. 2007; 8:519–29. [PubMed: 17565364]

32. Lisbona F, Rojas-Rivera D, Thielen P, Zamorano S, Todd D, Martinon F, et al. BAX Inhibitor-1 is a Negative Regulator of the ER Stress Sensor IRE1α. Mol Cell. 2009; 33:679–91. [PubMed: 19328063]

33. Chakravarti A, Erkkinen MG, Nestler U, Stupp R, Mehta M, Aldape K, et al. Temozolomide-Mediated Radiation Enhancement in Glioblastoma: A Report on Underlying Mechanisms. Clin Cancer Res. 2006; 12:4738–46. [PubMed: 16899625]

34. Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, et al. Autophagy is Activated for Cell Survival after Endoplasmic Reticulum Stress. Mol Cell Biol. 2006; 26:9220–31. [PubMed: 17030611]

35. Pyrko P, Schonthal AH, Hofman FM, Chen TC, Lee AS. The Unfolded Protein Response Regulartor GRP78/BiP as a Novel Target for Increasing Chemosensitivity in Malignant Gliomas. Cancer Res. 2007; 67:9809–16. [PubMed: 17942911]

36. Asano T, Ageyama N, Takeuchi K, Momoeda M, Kitano Y, Sasaki K, et al. Engraftment and tumor formation after allogeneic in utero transplantation of primate embryonic stem cells. Transplantation. 2003; 76:1061–1067. [PubMed: 14557753]

37. Bjorklund LM, Sanchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, et al. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. Proc Natl Acad Sci USA. 2002; 99:2344–2349. [PubMed: 11782534]

38. Erdo F, Buhrle C, Blunk J, Hoehn M, Xia Y, Fleischmann B, et al. Host-dependent tumorigenesis of embryonic stem cell transplantation in experimental stroke. J Cereb Blood Flow Metab. 2003; 23:780–785. [PubMed: 12843782]

39. Harkany T, Andang M, Kingma HJ, Gorcs TJ, Holmgren CD, Zilberter Y, et al. Region-specific generation of functional neurons from naive embryonic stem cells in adult brain. J Neurochem. 2004; 88:1229–1239. [PubMed: 15009679]

40. Wakitani S, Takaoka K, Hattori T, Miyazawa N, Iwanaga T, Takeda S, et al. Embryonic stem cells injected into the mouse knee joint form teratomas and subsequently destroy the joint. Rheumatology. 2003; 42:162–165. [PubMed: 12509630]

41. Barberi T, Klivenyi P, Calingasan NY, Lee H, Kawamata H, Loonam K, et al. Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. Nat Biotechnol. 2003; 21:1200–1207. [PubMed: 14502203]

42. Chiba S, Ikeda R, Kurokawa MS, Yoshikawa H, Takeno M, Nagafuchi H, et al. Anatomical and functional recovery by embryonic stem cell-derived neural tissue of a mouse model of brain damage. J Neurol Sci. 2004; 219:107–117. [PubMed: 15050446]

43. Assanah M, Lochhead R, Ogden A, Bruce J, Goldman J, Canoll P. Glial progenitors in adult white matter are driven to form malignant gliomas by platelet-derived growth factor-expressing retroviruses. J Neurosci. 2006; 26:6781–90. [PubMed: 16793885]

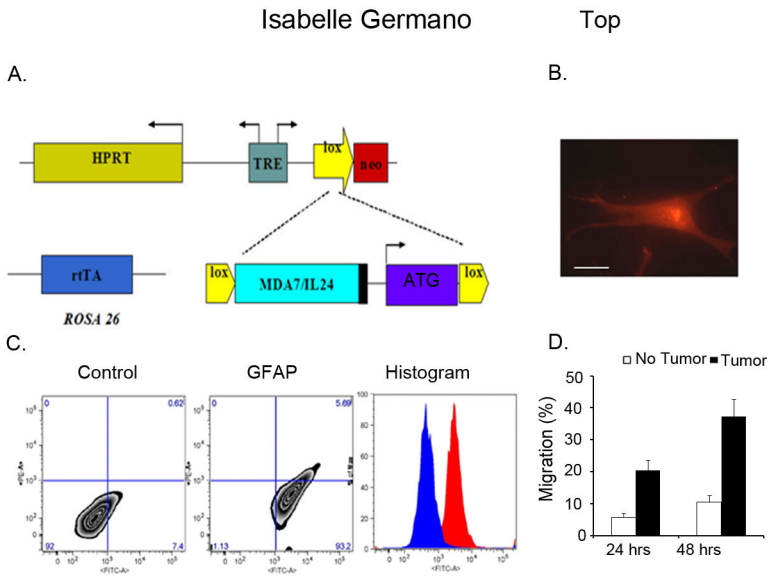


Figure 1. ESC-derived cells are astrocytes and maintain "homing" capacity

A) Schematic representation of integrated expression DNA cassettes. The rtTA is integrated into the constitutive ROSA 26 locus on chromosome 6. Cre-mediated recombination of targeting vectors into the homing site on the X chromosome restores resistance to the antibiotic G418 (neo), therefore facilitating efficient isolation of transgenic cells. HPRT= Hypoxanthine phosphoribosyltransferase; TRE= *tet*-responsive element; neo=neomycin; rtTA: reverse *tet* transactivator; ATG= methionine initiation codon. **B**) Microphotograph of ESC-derived cell conditionally expressing *mda-7/IL-24* after GFAP immunofluorescence labeling. **C**) FACS analysis of ESC-derived cell conditionally expressing *mda-7/IL-24* after isotype control (left panel, control) and after GFAP labeling (middle panel, GFAP). As shown in the histogram (right panel) >85% cells were GFAP-positive (light area on histogram shows shift after GFAP labeling). **D**) Bar graph representation of transwell experiments with ESC-derived astrocytes in the upper well and human malignant glioma cells in the bottom well showing 37% migration rate 48 hours after plating in the presence of tumor (U87 human malignant glioma cells); 80% of astrocytes had migrated by day 4 (data not shown).

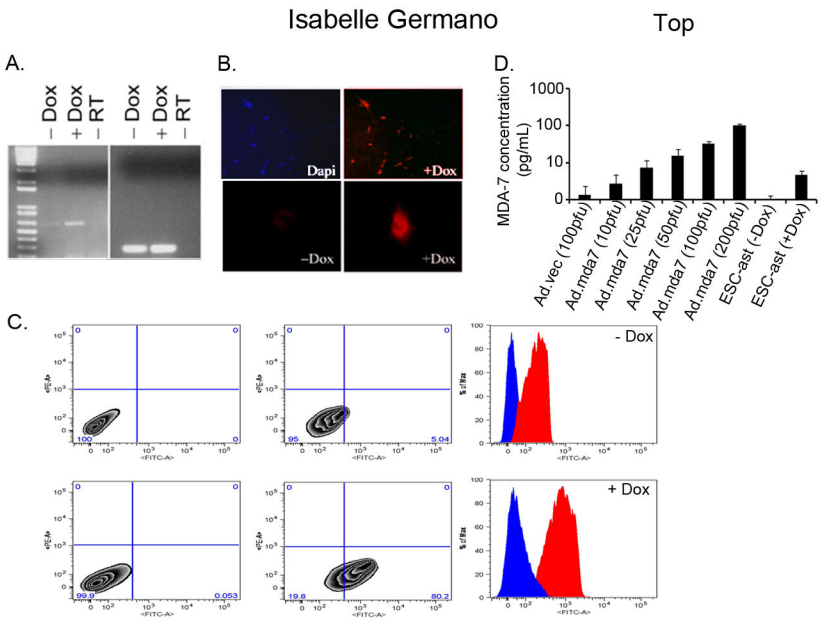


Figure 2. ESC-derived astrocytes conditionally express *mda-7/IL-24*

A) RT-PCR analysis showing *mda-7/IL-24* expression 24 hrs after Dox induction (+Dox). Absence of Dox (−Dox) and reverse transcriptase (RT) used as negative controls. Actin (*right panel*) used as positive control. **B**) Immunofluorescence microphotographs of ESC-derived astrocytes conditionally expressing *mda-7/IL-24* after labeling with *mda-7/IL-24* antibody in absence (−Dox, left lower panel) and presence (+Dox, right upper [10×] and lower [40×]) of Dox. *mda-7/IL-24* was detected in the cell cytoplasm after Dox induction. Antibody visualization: Alexa 594. **C)** FACS analysis of ESC-derived astrocytes conditionally expressing *mda-7/IL-24* showing *mda-7/IL-24* expression in the absence (−Dox, upper row) and presence (+Dox, lower row) of Dox. Cells are labeled with isotype control (left panels) and MDA-7 antibody (middle panels). Right panels shows quantification of expression with isotype control (black) and after MDA-7 antibody (gray). Data show that ≥ 80% of cells expressed *mda-7/IL-24* after Dox induction. Minimal expression is noted in absence of Dox. **D**) Protein quantification by ELISA of U87 cells infected with different MOIs of Ad. *mda-7* and ESC-derived astrocytes conditionally expressing *mda*-7/IL-24. Data shown are mean±SD of three independent experiments.

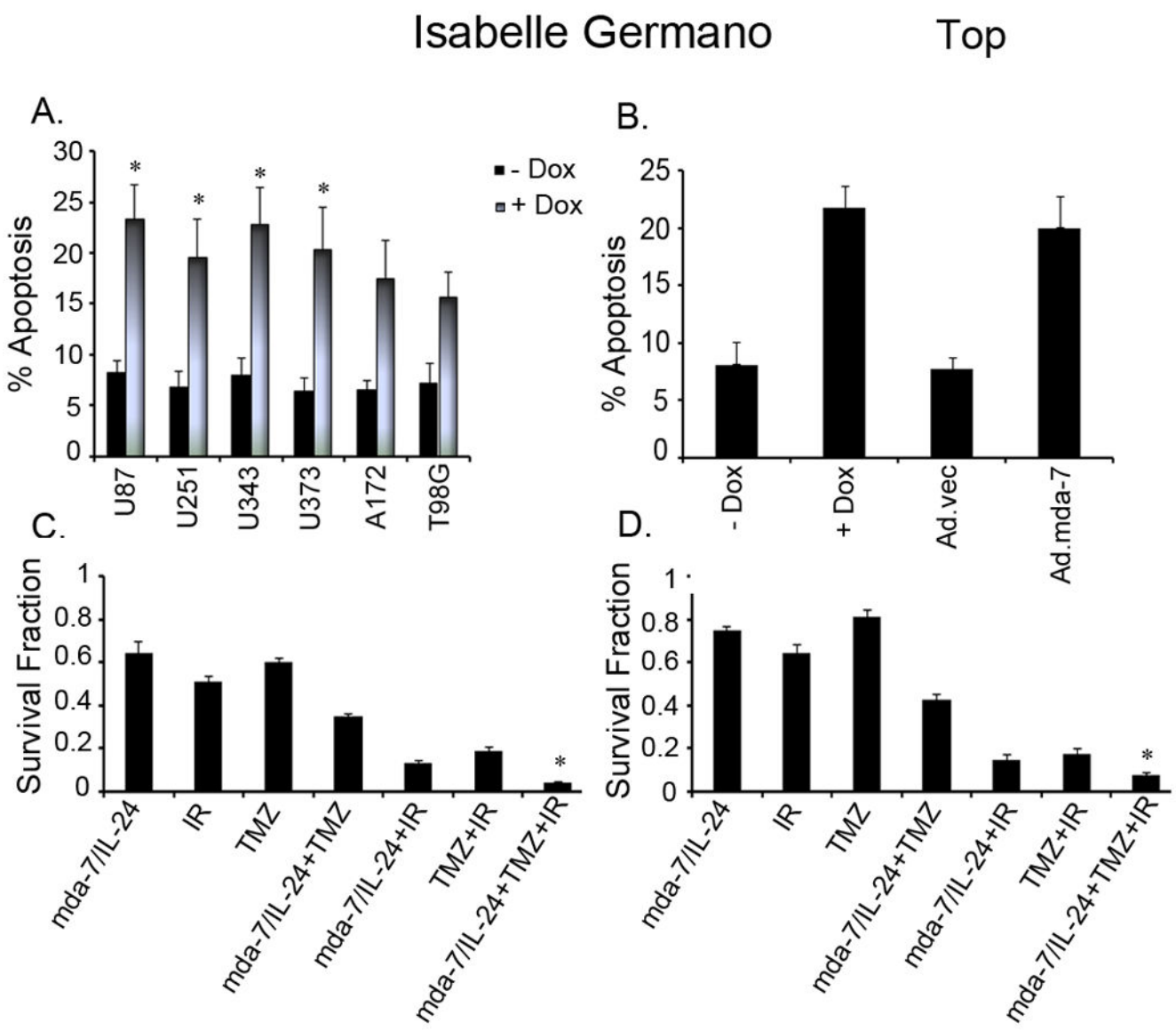
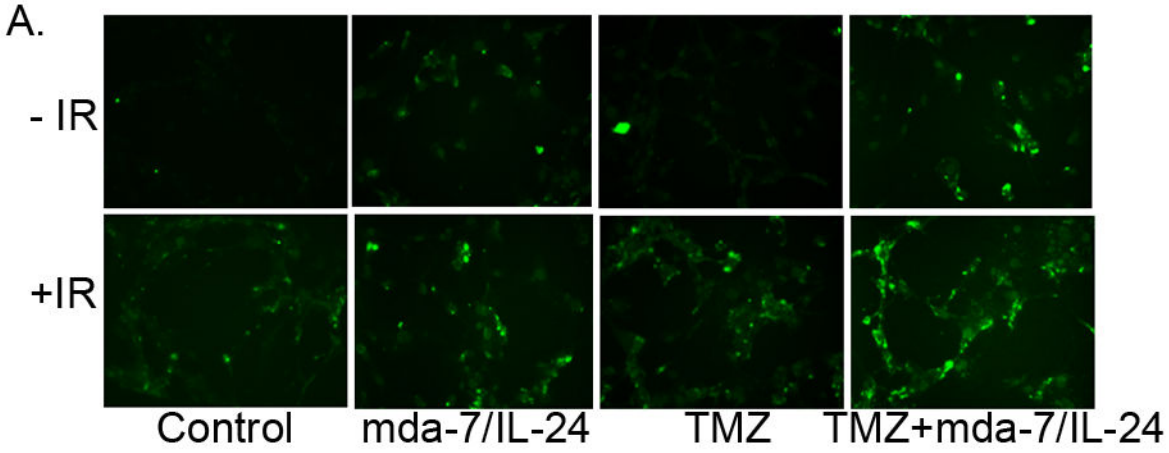


Figure 3. ESC-derived astrocytes conditionally expressing *mda-7/IL-24* induce p53-independent apoptosis, growth suppression, radiosensitization, and overcomes temozolomide resistance in malignant gliomas

A) Bar graph showing significant increase of apoptotic rate in 4/6 human glioma cells lines after co-culture with ESC-mediated mda-7/IL-24 in the presence of Dox for 24 hours. Tumor cells: ESC-astrocytes ratio is 1:1. Pro-apoptotic effects are p53-independent. Dox alone did not have any pro-apoptotic effects (data not shown). Apoptosis was measured as described in Material and Methods using FACS. *$p<0.05$ Student's-t test compared to −Dox. **B)** Bar graph showing similar apoptotic rate when using Ad.*mda-7* and ESC-derived astrocytes mediated *mda-7/IL-24.* Data shown in U87 glioma cell line. **C)** Clonogenic experiments in U87 (p53wt) and **D)** T98G (p53mt) cells after exposure to ESC-mediated *mda-7/IL-24*, IR, or TMZ alone or in combination. Exposure to all three treatments resulted

in significant decrease of survival fraction compared to each treatment alone or two combined (Anova $p<.05$).

Isabelle Germano Top



B.

Treatment	- IR	+ IR
Control	3.6 ± 2	18.3 ± 3
mda-7/IL-24	20.3 ± 4	45 ± 4.5
TMZ	26 ± 3.4	43 ± 5.3
TMZ+mda-7/IL-24	42 ± 3.6	58 ± 6.5

Apoptosis

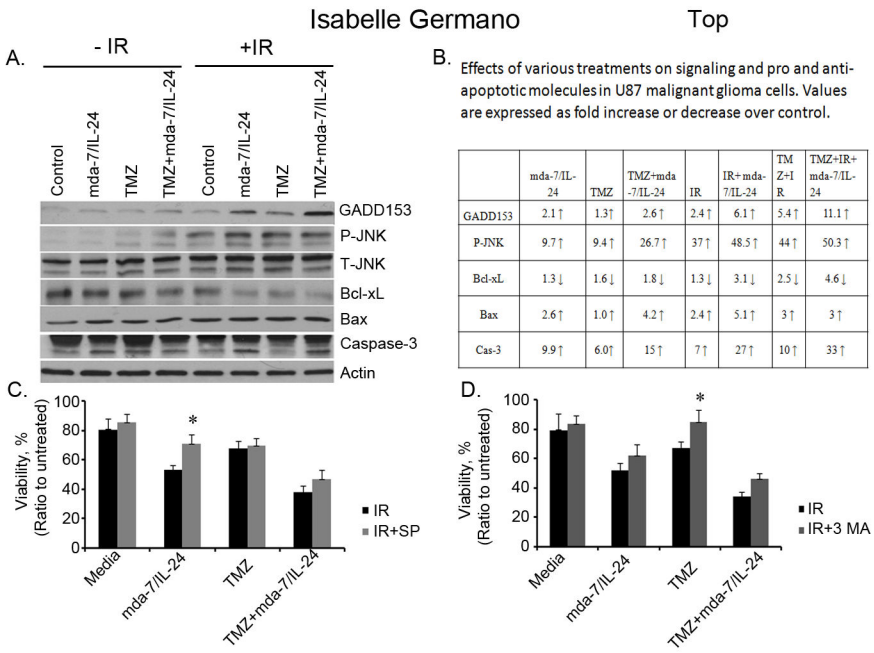
C.

Treatment	- IR	+ IR
Control	3.1 ± 2	5.4 ± 1
mda-7/IL-24	11.1 ± 4	16 ± 4
TMZ	23 ± 5	29 ± 4.2
TMZ+mda-7/IL-24	28 ± 3	33 ± 4.8

Autophagy

Figure 4. ESC-mediated transgene *mda-7/IL-24* delivery combined with IR and TMZ induces apoptosis and autophagy

A) Fluorescent microphotographs of U87 malignant glioma cells after TUNEL labeling. Numerous TUNEL-positive cells (green) are seen when IR is used (+IR, lower row). Combination of all three treatments resulted in a greater number of TUNEL-positive cells. Summary table of FACS analysis of apoptotic (**B**) and autophagy (**C**) rates in U87 cells after ESC-mediated *mda-7/IL-24* alone or in combination with IR and/or TMZ. The combination of all three treatments enhanced the apoptotic rate. Autophagy was significantly increased compared to control and radiation alone group by TMZ, and by *mda-7/IL-24* to a lesser extent (Student's t-test $p<.05$).



	mda-7/IL-24	TMZ	TMZ+mda-7/IL-24	IR	IR+mda-7/IL-24	TMZ+IR	TMZ+IR+mda-7/IL-24
GADD153	2.1↑	1.3↑	2.6↑	2.4↑	6.1↑	5.4↑	11.1↑
P-JNK	9.7↑	9.4↑	26.7↑	37↑	48.5↑	44↑	50.3↑
Bcl-xL	1.3↓	1.6↓	1.8↓	1.3↓	3.1↓	2.5↓	4.6↓
Bax	2.6↑	1.0↑	4.2↑	2.4↑	5.1↑	3↑	3↑
Cas-3	9.9↑	6.0↑	15↑	7↑	27↑	10↑	33↑

Figure 5. ESC-mediated transgene *mda-7/IL-24* combined with IR and TMZ results in over expression of CHOP/GADD153 and P-JNK and downregulates Bcl-xL

A) Pro- and anti-apoptotic protein analysis by Western blot in U87 glioma cells after ESC-mediated *mda-7/IL-24* alone or in combination with IR and/or TMZ 48 hours after treatment and **B)** quantification summary of Western blot by densitometry. A synergistic increase in CHOP/GADD153 was found when all three treatments were used concomitantly compared to each treatment alone or two in combinations. Enhanced effects were noted on decreased expression of Bcl-xL, and increase of caspase 3, Bax, and P-JNK following combination treatments compared to each treatment alone or two in combinations. Experiments were repeated three times. **C and D)** Bar graph representation of cells viability assessed by MTT assay after inhibition of JNK phosphorylation by SP600125 **(C)** or autophagy by 3MA **(D).** Inhibition resulted in significant protection (*p<.05) after exposure to *mda-7/IL-24* and *IR* **(C)** and *IR* and TMZ **(D).** Experiments done in triplicate. Data expressed as mean ±SD.

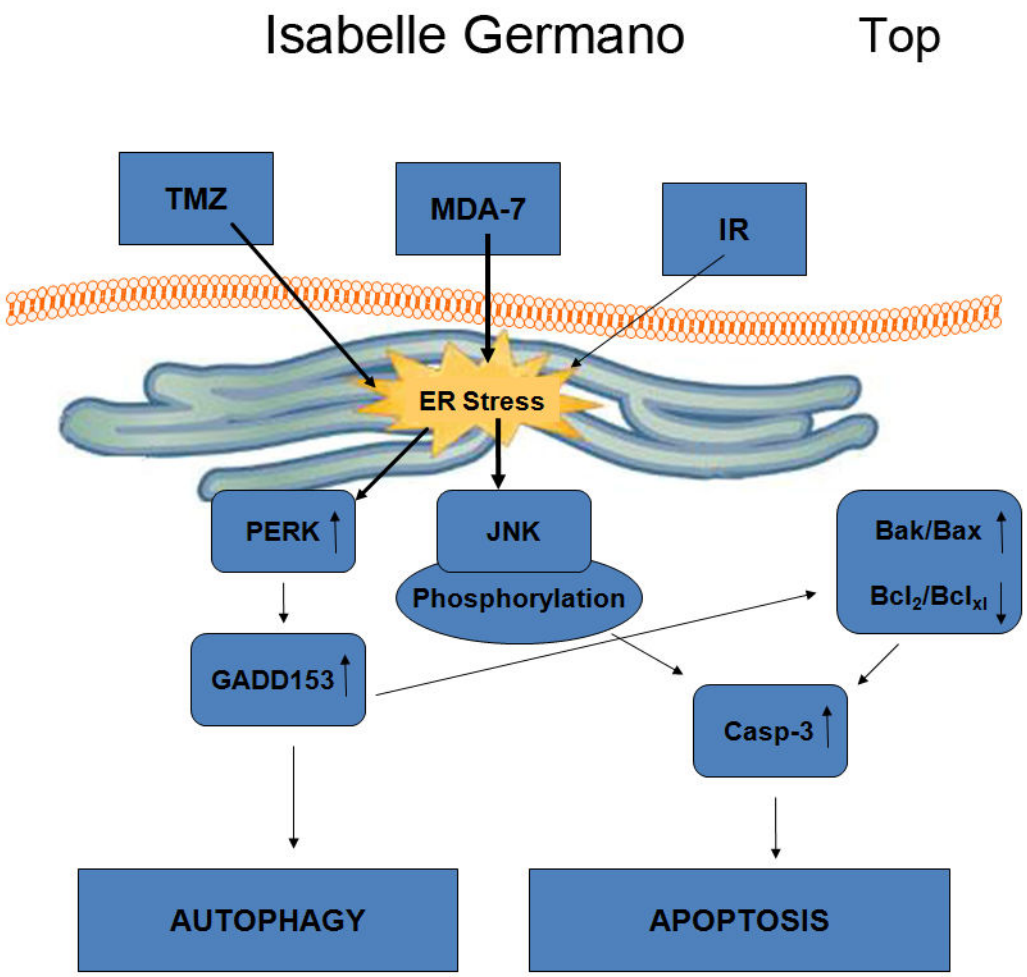


Figure 6. Schematic simplified overview of CHOP/GADD153 proposed as the "common pathway" to autophagy and apoptosis

IR, ESC-mediated transgene *mda-7/IL-24* and TMZ cause endothelial reticulum (ER) stress. 31, 35 This results in JNK phosphorylation which promotes over-expression of CHOP/GADD153. Over-expression of CHOP/GADD153 results in induction of the pro-apoptotic proteins Bak/Bax and decreases the anti-apoptotic proteins BcL-2/Bcl-xL with downstream casapase 3 activation, resulting in apoptosis. Independently, increased expression of CHOP/GADD153 results in increased autophagy.