Inhibition of IRE1 signaling affects expression of a subset genes encoding for TNF-related factors and receptors and modifies their hypoxic regulation in U87 glioma cells

1 Introduction

The endoplasmic reticulum (ER) is the primary organelle able to activate a distinct cellular stress response, termed the Unfolded Protein Response (UPR) in which a moiety of factors (typically aggregates of misfolded proteins) trigger activation of a complex set of signaling pathways to incite resolution to the causative stress. Malignant tumors utilize the endoplasmic reticulum stress response to adapt to stressful environmental conditions [1-3]. Moreover, the circadian rhythms of cancers are controlled by endoplasmic reticulum-mediated mechanisms [4]. The rapid growth of solid tumors generates micro-environmental changes in regards to hypoxia, nutrient deprivation and acidosis thus inducing the formation of new blood vessels, cell proliferation and survival [2,3,5]
processes relying on activation of endoplasmic reticulum stress signalling pathways [2,3]. UPR is mediated by three interconnected, endoplasmic reticulum-resident sensors, IRE1 (inositol requiring enzyme-1) being the most evolutionary conserved sensor that responds to protein misfolding with a highly tuned program aiming to either resolve the stress or direct the cell towards apoptosis in the case rectification is not viable; thus making it a key regulator of life and death processes of the cell [1,6-9].

The IRE1 enzyme contains two distinct catalytic domains; a protein kinase and endoribonuclease. Kinase activity is responsible for the autophosphorylation of IRE1 and aids in endoribonuclease activity and controls the expression of the EREG gene [10]. The endoribonuclease aspect of IRE1 is involved in the degradation of a specific subset of mRNA and initiation of the cytosolic splicing of pre-XBP1 (X-box binding protein 1) mRNA, whose mature transcript encodes for a transcription factor that stimulates the expression of numerous UPR-specific genes [11-13]. Moreover, activation of the IRE1 branch of the endoplasmic reticulum stress response is intimately linked to apoptosis. Ablation of this sensor’s function by a dominant-negative construct of IRE1 (dnIRE1) has been shown to result in a significant anti-proliferative effect in glioma growth [2,10]. This is due to down-regulation of prevalent pro-angiogenic factors and up-regulation of anti-angiogenic genes, both in vitro and in the CAM (choorio-allantoic membrane) model, as well as in mice engrafted intracerebrally with U87 glioma cell clones [10,14,15]. However, the executive mechanisms of the exhibited anti-proliferative effects of dnIRE1 are not yet known. The possible involvement of tumor necrosis factor (TNF) and its receptors was made evidently pertinent through analysis of U87 glioma cells expressing the dominant-negative mutant of IRE1 [15].

The TRAIL (TNF-related apoptosis-inducing ligand) and the associated receptors, TNFRSF10A and TNFRSF10B, are members of a subset of the TNF receptor superfamily known as death receptors. The interaction between the TRAIL and these receptors initiates the extrinsic apoptotic pathway characterized by the recruitment of death domains, assembly of the death-inducing signaling complex (DISC), caspase activation and ultimately apoptosis. Conversely, the decoy receptors TRAILR3 and TRAILR4, which lack the pro-apoptotic death domain, function to dampen the apoptotic response by competing for TRAIL ligand.

To date, the overwhelming majority of studies on death receptors have explored the role of these receptors as initiators of apoptosis; however, sporadic reports also suggest that engagement of death receptors can lead to other outcomes such as cytokine and chemokine production, cell proliferation, migration and differentiation. Although transformed cells frequently express TRAIL, most do not undergo apoptosis upon the engagement of death receptors and significant effort has been devoted toward exploring the potential of sensitizing such cells to the pro-apoptotic effects of such receptors stimulation [16-20]. Moreover, the expression of death receptors is greatly elevated in many cancer types such as hepatocellular carcinoma, renal carcinoma and ovarian cancer thus suggesting the benefit of such tumors from the expression of these receptors [17]. It is becoming increasingly clear that engagement of death receptors, especially in the context of cancer, can lead to non-apoptotic outcomes that become subverted to the benefit of certain tumors [17].

The TNFRSF21 gene encodes for death receptor 6, which induces apoptosis through a new pathway, different from the that of type I and type II pathways [21]. B-cells lacking TNFRSF21 show increased proliferation, cell division and cell survival upon mitogenic stimulation (anti-CD40 and LPS); however, this gene is highly expressed in many tumor cell lines and tumor samples [17]. Therefore, the role of TNFRSF21 as an apoptosis-inducing receptor is less clear and is perhaps dependent upon cell type. The nuclear death receptor, TNFRSF10B, inhibits maturation of the microRNA let-7 and increases proliferation of tumor cells [22]. Recently, TNFRSF10B expression was shown to be essential in endoplasmic reticulum stress-mediated apoptosis in human lung cancer cells and regulated by the ubiquitin-proteasome system [23,24]. TNF receptor signaling is controlled by a set of adaptor molecules and has significant potential to exert pro-survival and protective roles in several diseases [25,26]; however, the mechanism of these receptor signal transductions is still poorly understood. Mitochondrial aminopeptidase P3 (APP3m) has been shown to be a new member of the TNF-TNFR2 signaling complex that exerts an anti-apoptotic function and induces activation of JNK1 and JNK2 [26].

The adaptor protein TRADD (TNFRSF1A-associated via death domain) contains a death domain and interacts with TNFRSF1A and along with CCN1 gene, has recently been shown to be down regulated by microRNA-30c-2-3p in breast cancer, leading to negative regulation of NF-κB signaling as well as cell cycle progression [27]. Of important significance in TNFα signaling is the cytokine, TNFSF7 (CD70), a ligand for TNFRSF27 capable of specifically activating death receptors and inducing proliferation, consequently and is significantly increased in some cancers [28,29].
The biological effects of TNFα are realized through the TNF receptor mediated induction of specific TNFα-induced proteins such as TNFAIP1 and TNFAIP3, which each possess different functionalities. By targeting the mRNA of TNFAIP1 miRNA-372 plays crucial roles in gastric tumor genesis by inducing TNFα and is thus up-regulated in gastric adenocarcinoma tissue as compared to normal gastric tissues [30]. Consequently, the overexpression of miR-373 in gastric cancer cells results in increased cell proliferation and reduced expression of TNFAIP1 in both mRNA and protein [30]. Moreover, the tumor suppressor RhoB interacts with TNFAIP1 to regulate apoptosis in HeLa cells via a SAPK/JNK-mediated signal transduction mechanism [31]. Additionally, TNFAIP3 is a zinc finger protein and ubiquitin-editing enzyme that has been shown to inhibit NF-κB activation as well as TNF-mediated apoptosis with its expression being increased in a number of solid human tumors [32]. TNFAIP3 itself is also a NF-κB dependent gene, that has been shown to also exert cell-type specific anti- or pro-apoptotic functions and suppresses TP53 protein levels [33].

The LITAF (lipopolysaccharide-induced TNFα factor), which also known as PIG7 (p53-induced gene 7 protein), is an important factor in the regulation of the TNFα expression by direct binding to the promoter region of this gene. Consequently, its altered expression is associated with cancer and obesity [34,35]. Based on the amalgamation of evidence presented, the aim in this study was to investigate the possible roles of a subset of genes encoding for TNF-related factors and receptors as they apply to the suppression of glioma cell proliferation via inhibition of the endoplasmic reticulum stress sensor, IRE1. By exploring hypoxic regulation, the hope is to elucidate the possible mechanistic roles of these genes in the development and progression of certain cancers and their contribution to the unfolded protein response.

2 Material and Methods

Cell lines. The glioma cell line U87 (HTB-14) was obtained from ATCC (USA) and grown in high glucose (4.5 g/l) Dulbecco’s modified Eagle’s minimum essential medium (DMEM; Gibco, Invitrogen, USA) supplemented with glutamine (2 mM), 10% fetal bovine serum (Equitech-Bio, Inc., USA), penicillin (100 units/ml; Gibco, USA) and streptomycin (0.1 mg/ml; Gibco) at 37°C in a 5% CO₂ incubator. In this work we used sublines of U87 glioma cells, which were described previously [10,15,36]. One subline was obtained by selection of stable transfected clones overexpressing empty vector (pcDNA3.1), which was used for creation of dominant-negative constructs of IRE1 (dnIRE1). This untreated subline of glioma cells was used as a control (control glioma cells) in the study of the effects of inhibition of IRE1, in regards to the expression of the TNF receptor superfamily members and TNFα-induced proteins of interest (Table 1). The second sub-line was obtained by the selection of stable transfected clones overexpressing dnIRE1, having suppression of both the protein kinase and endoribonuclease activities of IRE1 [15]. It has recently been shown that these cells have a low rate of proliferation and do not express spliced XBP1, a key transcription factor in IRE1 signaling, after induction of endoplasmic reticulum stress by tunicamycin [10,15].

Induction of Hypoxia. For creation of hypoxic conditions, the culture plates were exposed in a special incubator with 3 % oxygen, 5 % carbon dioxide, and 92 % nitrogen mix and exposed for 16 hrs.

Proliferation assay. The proliferative rate of control glioma cells and ERN1 knockdown cells treated by hypoxia and TNFRSF21 siRNA (small interfering RNA) was measured by counting the cells in triplicates after 2 days of growth using a cell counter (Coultronics, Margency, France).

Apoptosis assay. Apoptotic cells were detected by flow cytometry using the Annexin V-FITC Apoptosis Detection kit from Sigma-Aldrich (Saint Louis, U.S.A.). Cells were incubated with annexin V-FITC and propidium iodide at room temperature and analyzed by flow cytometry after a 10 minute incubation period.

Downregulation of TNFRSF21 by small interfering RNA. Small interfering RNA against human TNFRSF21 (DR6) and control siRNA, received from Santa Cruz Biotechnology (Dallas, U.S.A.), were used to downregulate TNFRSF21 gene in control and dnIRE1 U87 glioma cells. Transfection was performed for 48 hours using lipofectamine RNAiMAX (Invitrogen, Carlsbad, U.S.A.) in accordance with the manufacturer’s protocol, with siRNA at a final concentration of 100 nM in six-well plates.

Isolation of RNA. Total RNA was extracted from both glioma and normal human astrocyte cells using Trizol reagent according to the manufacturer’s protocol (Invitrogen, USA) as described previously [46].

Reverse transcription and qPCR analysis. QuantiTect Reverse Transcription Kit (QIAGEN, Germany) was used for cDNA synthesis according to the manufacturer’s protocol. Expression of TNFRSF21, TNFRSF10B, TNFRSF10D, TNFRSF11B, TNFRSF1A, LITAF, TNFAIP1, TNFAIP3, TNFSF7, TRADD, and ACTB mRNA was measured in U87 glioma cells by real-time quantitative polymerase chain reaction using Mx 3000P QPCR (Stratagene, USA) and Absolute qPCR SYBRGreen Mix (Thermo Fisher Scientific,
ABgene House, UK). Polymerase chain reaction was performed in triplicate using specific primers, which were received from Sigma-Aldrich (Table 1).

Analysis of quantitative PCR was performed using a special Differential Expression Calculator. Quantitative expression of TNFRSF21, TNFRSF10B, TNFRSF11B, TNFRSF1A, TNFAIP1, TNFAIP3, LITAF, TNFSF7, and TRADD genes were normalized to beta-actin and presented as a percent of the control (100%).

Statistical analysis was performed using OriginPro 7.5 software. All values were expressed as mean ± SEM from triplicate measurements performed in 4 independent experiments. Comparison of two means was performed by the use of two-tailed Student’s t-test as described previously [37]. P < 0.05 was considered significant in all cases.

### Results

#### 3.1 Hypoxia affects the expression of TNFRSF21, TNFRSF10B, TNFRSF11B, TNFRSF1A, TRADD, TNFSF7, TNFAIP1, TNFAIP3, and LITAF genes in glioma cells preferentially in an IRE1-dependent manner

Expression of tumor necrosis receptor superfamily genes were studied by quantitative PCR. In order too test the effect of IRE1 on expression levels of the TNF-related factors and receptors of interest in relation to the control of apoptosis and cell proliferation, we used U87 glioma cell sub-lines constitutively expressing the dominant-negative mutant of IRE1, dnIRE1, lacking both endoribonuclease and kinase activities [10,15].

### Table 1. Characteristics of studied TNF-related genes and primers used for qPCR

| Gene symbol | Gene name | Primer's sequence | Primers position | GenBank accession number |
|-------------|-----------|-------------------|-------------------|-------------------------|
| **TNFRSF21** (DR6) | Tumor necrosis factor receptor superfamily, member 21 (Death receptor 6) | F: 5'-tgattgtgcttttcctgctg<br>R: 5'-ctcaactggcattgcaaagaa | 1491–1510<br>1759–1740 | NM_014452 |
| **TNFRSF10B** (TRAIL-R2; DRS) | Tumor necrosis factor receptor superfamily, member 10B (TNF-related apoptosis-inducing ligand receptor 2; Death receptor 5) | F: 5'-tgcagccgtagtcttgattg<br>R: 5'-tcctggacttccatttcctg | 953–972<br>1171–1152 | NM_003842 |
| **TNFRSF10D** (TRAIL-R4) | Tumor necrosis factor receptor superfamily, member 10D (TNF-related apoptosis-inducing ligand receptor 4) | F: 5'-caggaaatccaaggtcagga<br>R: 5'-agcctgcctcatcttcttca | 969–988<br>1233–1214 | NM_003840 |
| **TNFRSF11B** | Tumor necrosis factor receptor superfamily, member 11B | F: 5'-tgctcagttcttgtctctc<br>R: 5'-ttctgtgtcagttctgtgctc | 819–838<br>1067–1048 | NM_002546 |
| **TNFRSF1A** | Tumor necrosis factor receptor superfamily, member 1A | F: 5'-tgtgctcctccacagcttgag<br>R: 5'-gacgaaagctcgggctttgg | 884–903<br>1126–1107 | NM_001065 |
| **TNFAIP1** | Tumor necrosis factor alpha-induced protein 1 (endothelial) | F: 5'-ggtctgtgtaatagttgcag<br>R: 5'-gggagacgtgtcaaagcag | 793–812<br>1020–1001 | NM_021137 |
| **TNFAIP3** | Tumor necrosis factor alpha-induced protein 3 | F: 5'-caagggagaacagacacacag<br>R: 5'-aaagggggggaatggaacc | 712–731<br>1002–983 | NM_006290 |
| **CD70** (TNFSF7) | CD70 Molecule (tumor necrosis factor ligand superfamily member 7) | F: 5'-ctgcctttgatctggctgctc<br>R: 5'-cattgcagcgggggdctgctg | 212–231<br>374–355 | NM_001252 |
| **LITAF** (PIG7) | Lipopolysaccharide-induced TNF factor (p53-induced gene 7 protein) | F: 5'-gatcgtgctcttgctgcgtctc<br>R: 5'-tgaaagcggcgggaagggtgg | 539–558<br>785–766 | NM_004862 |
| **TRADD** | TNFRSF1A-associated via death domain | F: 5'-tgcagatgctgaagatccac<br>R: 5'-gctcagccagttcttcatcc | 256–275<br>543–524 | NM_003789 |
| **ACTB** | beta-actin | F: 5'-ggacactgcaggaagagatgg<br>R: 5'-agcactgtctgtggctgacag | 747–766<br>980–961 | NM_001101 |
Figure 1 and Table 2 demonstrate that inhibition of IRE1 gene function in U87 glioma cells by dnIRE1 leads to the up-regulation of TNFRSF21 mRNA (7.5 fold) as well as TNFRSF11B (4.0 fold) and TNFRSF10D (3.9 fold). At the same time, TNFRSF10B gene expression is down-regulated (2.3 fold) in glioma cells without IRE1 gene function (Figure 1 and Table 2). As shown in Figure 1, no significant changes are observed in the expression level of TNFRSF1A gene.

Thereafter, we tested the impact of IRE1 inhibition on the modulation of expression levels of TRADD (TNFRSF1A-associated via death domain), TNFSF7 (TNF ligand superfamily member 7), LITAF (lipopolysaccharide-induced TNF factor), and two TNFα-induced proteins (TNFAIP1 and TNFAIP3). As shown in Figure 2 and Table 2, inhibition of IRE1 by dnIRE1 has a suppressive effect on the expression of TRADD, TNFSF7, and TNFAIP3 genes (down 2.5, 4.35, and 3.2 fold); however, in dnIRE1 glioma cells, devoid of both endonuclease and kinase activities, the expression of TNFAIP1 and LITAF genes is increased in 1.42 and 1.66 fold, correspondingly.

In conclusion, we have demonstrated that the IRE1 signaling enzyme participates in the fine-tuning of mRNA levels of a subset of TNF receptors and TNF related factor genes, which are important for control of apoptosis and proliferation such as: TNFα-induced proteins, TNF receptor superfamily members, TNFRSF1A-associated protein TRADD, TNF receptor ligand superfamily 7

![Figure 1](image-url)

**Figure 1.** Expression of TNFRSF21 (tumor necrosis factor receptor superfamily, member 21), TNFRSF10B, TNFRSF11B, TNFRSF10D, and TNFRSF1A mRNA in glioma cell line U87 (Vector) and its sublines with a deficiency of both protein kinase and endoribonuclease of the signaling enzyme IRE1 (dnIRE1) measured by qPCR. mRNA expressions values were normalized to beta-actin mRNA expression and presented as percent of control (vector, 100 %); mean ± SEM; n = 4; * - P < 0.001 versus control.

**Table 2.** Summary of changes in the expression of tumor necrosis factor receptor superfamily genes at different tested conditions

| Tested condition | Gene                | dnIRE1-cells versus control cells | Effect of hypoxia in control cells | Effect of hypoxia in dnIRE1-cells |
|------------------|---------------------|-----------------------------------|------------------------------------|-----------------------------------|
| TNFRSF21         | Up 7.5 fold         | Down 8.3 fold                     | No changes                         | Down 1.8 fold                     |
| TNFRSF10B        | Down 2.3 fold       | No changes                        | Up 1.39 fold                       |                                   |
| TNFRSF11B        | Up 4.0 fold         | Down 1.23 fold                    | Up 1.41 fold                       | Down 2.4 fold                     |
| TNFRSF10D        | Up 3.9 fold         | Up 1.41 fold                      | Up 1.47 fold                       |                                   |
| TRADD            | Down 2.5 fold       | Up 1.31 fold                      | Up 1.62 fold                       |                                   |
| TNFSF7           | Down 4.35 fold      | No changes                        | Down 1.53 fold                     |                                   |
| TNFAIP1          | Up 1.42 fold        | Up 1.42 fold                      | Up 1.46 fold                       |                                   |
| TNFAIP3          | Down 3.2 fold       | Up 7.4 fold                       | Up 7.4 fold                        |                                   |
| LITAF            | Up 1.66 fold        | Down 1.70 fold                    | No changes                         |                                   |
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Figure 2. Expression of LITAF/PIG7 (lipopolysaccharide-induced TNF factor), TNFAIP1 (TNFα induced protein 1), TNFAIP3, TRADD (TNFRSF1A-associated via death domain), and TNFSF7 (TNFSF ligand 7) mRNA in glioma cell line U87 (Vector) and its sublines with a deficiency of both protein kinase and endoribonuclease of the signaling enzyme IRE1 (dnIRE1) measured by qPCR. mRNA expressions values were normalized to beta-actin mRNA expression and presented as percent of control (vector, 100%); mean ± SEM; n = 4; * - P < 0.001 versus control; ** - P < 0.01 versus control.

3.2 Hypoxia regulates expression of most TNF-related genes in IRE1 dependent manner in U87 glioma cells

To determine if hypoxia regulates the genes tested above through the IRE1 branch of ERS response, we investigated the effect of hypoxic conditions (3% oxygen – 16 hrs) on the mRNA expression levels of different members of TNF-related proteins as well as LITAF, which can mediate the TNFα expression and is induced by tumor suppressor p53.

Inhibition of IRE1 introduces sensitivity of this gene expression to hypoxia (up 1.39 fold) (Figure 3B and Table 2). These results demonstrate that TNFRSF10B gene is resistant to hypoxia, but inhibition of the IRE1 leads to up-regulation. As shown in Figure 3C and Table 2, hypoxia slightly down-regulates the expression of TNFRSF11B mRNA in control glioma cells (1.23 fold), but the effect of hypoxia is more significant (down 2.4 fold) in glioma cells without IRE1 activity. At the same time, hypoxia up-regulates TNFRSF10D gene expression in both types of glioma cells in similar ways: 1.41 and 1.47 fold in control and IRE1 knockdown glioma cells, respectively (Figure 3D and Table 2). Hypoxic regulation of the TNFRSF1A gene is similar to that of the TNFRSF10B gene which does not change significantly in control glioma cells and is up-regulated (1.47 fold) in cells overexpressing dnIRE1 (Figure 3E and Table 2). It was also shown that TRADD, an adaptor protein that interacts with TNFRSF1A and mediates programmed cell death signaling, is up-regulated in both types of glioma cells upon the induction of hypoxia, but inhibition of IRE1 enhances the effect of hypoxia on gene expression: up 1.31 fold in control glioma cells and up 1.62 fold in cells with dnIRE1 (Figure 3F and Table 2).

Furthermore, we studied hypoxic regulation of TNFSF7 gene. As shown in Figure 3G and Table 2, TNFSF7 gene expression is down-regulated by hypoxia in glioma cells overexpressing dnIRE1 (1.53 fold), but does not change significantly in control glioma cells.
Inhibition IRE-1 and hypoxia affects expression of TNF-related genes

Investigation of the expression level of TNFα-induced proteins demonstrates that hypoxia up-regulates both TNFAIP1 and TNFAIP3 mRNA in control glioma cells and that inhibition of IRE1 does not significantly change the effect of hypoxia on both of these gene expressions, but that the TNFAIP3 gene is more sensitive to hypoxic treatment (Figure 4A, 4B and Table 2). Thus, treatment of control glioma cells by hypoxia induces expression of TNFAIP1 and TNFAIP3 mRNA (1.42 and 7.4 fold, correspondingly) and in cells harboring dnIRE1 by similar ways (1.46 and 7.4 fold, correspondingly); although, it is interesting to note that the expression of LITAF, which can mediate the TNFα expression and is implicated in the TP53-induced apoptotic pathway, is down-regulated (1.70 fold) by hypoxia in control glioma cells (Figure 4C and Table 2). However, inhibition of IRE1 eliminates the
effect of hypoxia on \textit{LITAF} gene expression. As shown in Figure 4D, the level of HIF1α protein in control glioma cells significantly increased upon the induction of hypoxic conditions, clearly demonstrating the hypoxic state of the cells exposed.

3.3 Hypoxia affects cell death and the rate of proliferation of U87 glioma cells in IRE1 dependent manner and TNFRSF21 mRNA knockdown by siRNA modifies the effect of hypoxia on cell death

IRE1 knockdown strongly up-regulated the expression of the \textit{TNFRSF21} gene that encodes for death receptor 6 and participates in the control of cell proliferation, cell
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survival and apoptosis; however, the role of TNFRSF21 as an apoptosis-inducing receptor is not clear yet. As shown in Figure 5A, IRE1 knockdown by dnIRE1 decreases the rate of cell proliferation in U87 glioma cell under normoxic condition (down 2 fold) and hypoxia does not significantly change the effect of IRE1 knockdown. Furthermore, TNFRSF21 mRNA knockdown by siRNA does not change the effect of IRE1 knockdown on cell proliferation under this experimental condition (Figure 5A). Hypoxia enhances the rate of proliferation in a slight and statistically significant ($P < 0.05$) manner in control U87 glioma cells (1.21 fold) and the knockdown of TNFRSF21 mRNA by siRNA does not change this effect (Figure 5A).

By detecting apoptotic cells via flow cytometry using Annexin V-FITC, it is evident that IRE1 knockdown by dnIRE1 increases the number of apoptotic U87 glioma cells in normoxic condition (up 1.45 fold) but upon
Figure 5. Effect of hypoxia (3 % oxygen – 16h) on cell proliferation (A), cell death (B), and the expression levels of TNFRSF21 (C) in control glioma cells (Vector) and cells with a deficiency of IRE1 (dnIRE1) treated by control siRNA and TNFRSF21 siRNA. mRNA expressions values were normalized to beta-actin mRNA expression and presented as percent of normoxia (control glioma cells transfected by vector, 100 %); mean ± SEM; n = 7.
hypothesis this increase is augmented (up 1.79 fold) (Figure 5B). This effect of the inhibition of IRE1 enzyme function on apoptotic cell death is eliminated by siRNA-mediated TNFRSF21 mRNA knockdown (Figure 5B). Furthermore, TNFRSF21 mRNA knockdown does not change apoptotic cell death in control glioma cells both in normoxic and hypoxic conditions, but suppresses apoptosis in cells overexpressing dnIRE1: down 1.35 fold and down 1.65 fold in glioma cell exposure under normoxic and hypoxic conditions, respectively (Figure 5B).

We also studied the effect of siRNA-mediated TNFRSF21 mRNA knockdown on the expression levels of TNFRSF21 mRNA (Figure 5C). Therefore, the expression levels of TNFRSF21 mRNA in control U87 glioma cells was decreased 3.70 fold in normoxic conditions and in 3.66 under hypoxia. Similar changes are founded in glioma cells with a deficiency of IRE1 (dnIRE1): down 3.70 fold upon normoxic conditions versus 3.24 fold in glioma cells treated by hypoxia (Figure 5C). Moreover, knockdown of TNFRSF21 does not significantly change hypoxic regulation of TNFRSF21 gene both in control glioma cells and cells without functional activity of IRE1.

In conclusion, hypoxia affects the expression of most of the genes in this study in a manner that is gene specific. Moreover, inhibition of the IRE1 signaling enzyme modifies hypoxic regulation of all genes studied, with the exception of TNFRSF10B and TNFAIP3. These results demonstrate that gene expression of this TNF receptor superfamily is responsive to hypoxia preferentially through the IRE1 signaling pathway of the endoplasmic reticulum stress response, but the mechanisms of its activation or deactivation are variable.

4 Discussion

This study has demonstrated that inhibition of both the endoribonuclease and kinase activities of the IRE1 signaling enzyme in U87 glioma cells causes a strong increase (more than 8 fold) in the levels of TNFRSF21 mRNA, which is known as death receptor 6 (Figure 1 and Table 2). Thus, the changes observed in the above gene, which is related to TNF-directed apoptosis, correlates well with slower cell proliferation in cells harboring dnIRE1, attesting to the fact that endoplasmic reticulum stress is a necessary component of malignant tumor growth and cell survival [2,3,6,10,38]. New data suggests that TNFRSF21 induces apoptosis through a unique mitochondria-dependent pathway through Bax protein mediated interactions that are different from the type I and type II pathways [21]. It is interesting to note that B-cells lacking TNFRSF21 show increased rates of cell proliferation and survival in the instance of mitogenic stimulation [17]. At the same time, the expression of this gene is increased in some tumors and tumor cell lines [17]. Therefore, the role of TNFRSF21 as a cell death receptor is less clear and perhaps cell type dependent.

Another death receptor, TNFRSF10B, is a receptor for the TNF-related apoptosis-inducing ligand, TRAILR2, and can initiate an extrinsic apoptotic pathway characterized by the recruitment of death domains, assembly of the death-inducing signaling complex (DISC), caspase activation and ultimately apoptosis. At the same time, we have shown in this study that TNFRSF10B gene expression is down-regulated when IRE1 is inhibited by moderating its enzymatic signaling capabilities (Figure 1 and Table 2). Thus, the changes observed in the TNFRSF10B gene, as an initiator of apoptosis, do not correlate with slower cell proliferation in cells harboring dnIRE1. It is possible that TNFRSF10B facilitates only endoplasmic reticulum stress-mediated apoptosis and inhibition of IRE1 signaling causes down-regulation of this gene expression; correlating to recent data stating that DDIT3 and KAT2A proteins regulate TNFRSF10B expression in endoplasmic reticulum stress-mediated apoptosis in human lung cancer cells [23]. Recently, it was shown that TNFRSF10B inhibits maturation of the microRNA let-7 and thus increases proliferation of pancreatic cancer cell lines and other tumor cells [22]. Moreover, the expression of TNFRSF family receptors is greatly elevated in many cancer types, suggesting that tumors benefit from the expression of these receptors [17]. To date, the majority of studies on these receptors have explored their roles as initiators of apoptosis; however, sporadic reports also suggest that these receptors can lead to other outcomes such as cytokine and chemokine production, cell proliferation and cell migration. Indeed, although transformed cells frequently express TRAIL, most do not undergo apoptosis upon engagement of these receptors and significant efforts have been devoted to elucidating how to sensitize such cells towards the pro-apoptotic effects of death receptor stimulation [17-20].

At the same time, we have observed significant up-regulation of TNFRSF10D in glioma cells through inhibition of IRE1 (Figure 1 and Table 2). It lacks the pro-apoptotic death domain, could not induce apoptosis and has been shown to play an inhibitory role in TRAIL-induced cell apoptosis [124]. Therefore, the induction of TNFRSF10D gene in U87 glioma cells via inhibition of IRE1 correlates with down-regulation of TNFRSF10B. Similar negative correlation was observed in breast cancer cells treated by short-hairpin RNA for suppression of adenine
nucleotide translocase-2 (ANT2) [39]. Suppression of ANT2 restores susceptibility of breast cancer cells to TRAIL-induced apoptosis by activating JNK and up-regulating the expression of death receptors 4 and 5 (TNFRSF10A and TNFRSF10B) and down-regulating the TNFRSF10D [39]. Interestingly, the silencing of TNFRSF10D is related to melanomagenesis [40,41] making it is possible that TNFRSF10D and TNFRSF10B are related to regulation of endoplasmic reticulum stress-mediated apoptosis. Moreover, modulation of CHOP-dependent expression of TNFRSF10B by Nelfinavir sensitizes glioblastoma multiforme cells to tumor necrosis factor-related apoptosis-inducing ligand [42]. Therefore, a better understanding of the mechanisms underlying TNFRSF10D as well as TNFRSF10B is required.

We have also demonstrated that expression of TRADD (TNFRSF1A-associated via death domain) is significantly down-regulated in glioma cells when IRE1 function is inhibited (Table 2 and Figure 2). This protein is an adaptor molecule that interacts with TNFRSF1A and participates in programmed cell death signaling. Our results are consistent with recent data that shows [27] that knock down of TRADD gene expression by an antisense oligonucleotide results in a decrease of TRADD protein by 60%, coinciding with an increase of apoptotic cell death of up to 30% and that microRNA-30c-2-3p down-regulates TRADD and CCNE1 in breast cancer leading to negative regulation of cell cycle progression. Thus, this decrease of TRADD expression may contribute to the suppression of cell proliferation and glioma growth by increased cell death and negative regulation of cell cycle progression. Of great significance in TNFα signaling is the cytokine TNFSF7, a ligand for TNFRSF27, which can specifically activate TRAIL receptors and induce proliferation [28,29]. Its expression is significantly increased in renal cell carcinoma as compared to normal kidney tissue; data which confirms our results. We have shown that TNFSF7 is strongly down-regulated in glioma cells when IRE1 is inhibited (Table 2 and Figure 2) and, thus, down-regulation of this gene expression should suppress cell proliferation and tumor growth.

It is interesting to note that inhibition of IRE1 via overexpression of dnIRE1 up-regulates the expression of the LITAF gene, an important factor in the regulation of TNFa expression by directly binding to the promoter region (Table 2 and Figure 2). These results correlate with our previous data that indicate that the inhibition of IRE1 up-regulates TP53 expression [38]. Therefore, an increased expression of LITAF gene can induce TNFa and TNFα-mediated apoptosis. Additionally, this data relates well with changes in the expression profile of TNFα-induced genes (increased expression of TNFAIP1 and decreased expression of TNFAIP3) in glioma cells harboring dnIRE1 (Table 2 and Figure 2). Therefore, TNFAIP1 is a pro-apoptotic protein which is decreased in cancer cells and regulates apoptosis through interaction with tumor suppressor RhoB [50]. At the same time, TNFAIP3 can also exert cell-type specific anti- or pro-apoptotic functions [51,52] data that relates well with the rate of cell proliferation seen in cells harboring dnIRE1 [10,15,38]. Endoplasmic reticulum stress is also responsible for mediating both apoptosis and autophagy by inducing cyclosporine A in malignant glioma cells via the mTOR/p70S6K1 pathway [43].

In this work we have studied the hypoxic regulation of a subset of TNF-related genes to clarify their relationship with IRE1-mediated endoplasmic reticulum stress. We have shown that hypoxia affects the expression of key TNF-related genes in directions opposite to that which induce IRE1 inhibition (Table 2); thus, hypoxia, as an important factor in tumor growth, it suppresses the expression of LITAF gene, is an important regulator of TNFa and TNFRSF21 production, it is possibly a positive regulator of apoptosis by up-regulating TNFAIP3 and TRADD genes aiding in its participation in programmed cell death signaling and it inhibits TNF-mediated apoptosis [21,33,34]. Consequently, these changes in the expression levels of LITAF, TNFRSF21, TNFAIP3, and TRADD genes all potentially contribute to increased cell survival under hypoxia. Concurrently, inhibition of IRE1 leads to a strong up-regulation of TNFRSF21 and LITAF genes and down-regulation of TRADD and TNFAIP3 genes (Table 2), which is possibly contributory to increasing cell death in these populations (Figure 5B). Moreover, inhibition of IRE1 suppresses the effect of hypoxia on TNFRSF21 gene expression and protects the LITAF gene from hypoxic regulation.

Thus, hypoxia affects the expression of most studied genes in a gene-specific manner and the hypoxic regulation of some genes depends on IRE1 function. For most of the genes studied, hypoxia had the opposite effect of IRE1 inhibition on the expression of these genes. Consequently, hypoxic regulation of TNFRSF11B, TRADD, TNFAIP3, and LITAF genes has pro-proliferative qualities. Recently, we showed that inhibition of IRE1 strongly suppresses the expression of the EPAS1 gene in glioma cells, which controls a specific group of hypoxia-responsive genes [38]. It is possible that the suppressive effect of IRE1 inhibition on hypoxic regulation of some
genes, particularly TNFRSF21 and LITAF, is determined by decreased expression of EPAS1 gene. Moreover, there are several regulatory mechanisms of gene expression which determine a gene-specific character of hypoxic regulation as well as its responsibility in regards to IRE1 signaling [44-46].

Our results demonstrate that almost all of the genes studied are both responsive to endoplasmic reticulum stress and hypoxia and are potentially contributory to regulation of apoptosis and cell proliferation, but the mechanisms of activation or suppression of expression through IRE1 inhibition and hypoxia are different and warrant further investigation.

In conclusion, inhibiting IRE1 affects the expression of a subset of TNF-related factors and receptors, all of which have a relation to the control of programmed cell death and proliferation and potentially contribute to retarded cell proliferation. Hypoxic regulation of most of the studied genes had pro-proliferative effects, but the TNFRSF21 gene was not, because siRNA-mediated knockdown of TNFRSF21 did not change the effect of hypoxia on proliferation. Thus, the changes observed in the studied factors and receptors correlate well with slower cell proliferation in cells harboring dnIRE1, attesting to the fact that targeting the unfolded protein response is viable, perspective approach in the development of cancer therapeutics [2, 3, 6,11,47,48].

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