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Influence of *HOXA9* expression in the PI3K-mediated oxidative stress in GBM

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"Everything that we see is a shadow cast by that which we do not see"

Martin Luther King, Jr.

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ABBREVIATIONS LIST

CNS – Central Nervous System

DMEM – Dulbecco's Modified Medium

DMSO – Dimethylsulfoxide

DNA – Deoxyribonucleic Acid

EGFR – Epidermal Growth Factor Receptor

ETC – Electron Transfer Chain

FBS – Fetal Bovine Serum

GBM – Glioblastoma

HOX – Homeobox

hTERT – human Telomerase Reverse Transcriptase

H_2O_2 – Hydrogen peroxide

H_2DCFDA – Dichlorodihydrofluorescein diacetate

LOH – Loss of heterozygosis

MGMT – *O^6-methylguanine methyltransferase*

MSCV – Murine Stem Cell Virus

PTEN – Phosphatase and Tensin homologue

PBS – Phosphate Buffered Saline

PDGFR – Platelet-Derived Growth Factor Receptor

PI3K – Phosphatidylinositol 3-Kinase

Rb – Retinoblastoma

RT-PCR – Reverse Transcriptase Polimerase Chain Reaction

ROS – Reactive oxygen species

SOD – Superoxide dismutase

TMZ – Temozolomide

WHO – World Health Organization

ABSTRACT

Reactive oxygen species (ROS) are important molecules that regulate several physiological processes, and excessive levels can contribute to tumorigenesis. In cancer, the PI3K/Akt pathway is associated with the increase of ROS; maybe to act as signaling messengers of other molecules that integrate this important pathway. One of the genes that are transcriptionally activated by PI3K/Akt is *HOXA9*. We consider that *HOXA9* could be contributing for ROS production in the PI3K/Akt pathway. To investigate this, intracellular ROS levels were measured in human GBM cell line (U87MG) and in immortalized human astrocytes (hTERT/E6/E7), both with *HOXA9* overexpression as well as in their control cells. Hydrogen peroxide was used as an exogenous source of ROS and its functional effects were studied by the assessment of cell viability. Since, cancer cells rely mostly on aerobic glycolysis to satisfy their energetic demands and to decrease ROS levels, it was also investigated the metabolic profile mediated by *HOXA9*. Ultimately, by submitting the previous cell lines and additionally the A172 cell line to a PI3K inhibitor, it was possible to evaluate the role of *HOXA9* regarding ROS production in the PI3K/Akt pathway. The results showed that *HOXA9* overexpression is associated with the increase of ROS production in the U87MG cell line. This increase in ROS production is parallel to a lower glycolytic profile present by the U87MG-*HOXA9* cell line, which produced less lactate comparing to the U87MG-MSCV control cell line. Also, ROS accumulation accompanied U87MG-*HOXA9* cell line resistance after challenges with hydrogen peroxide. Conversely, we also show that exposure of U87MG-*HOXA9* cells to PI-103, an inhibitor of the PI3K pathway, results in the inhibition of the pathway, decrease *HOXA9* expression, and enhance ROS levels. Thus, this study provides more understanding about the regulatory role of *HOXA9* for ROS production in the PI3K/Akt pathway, pointing this gene as a possible responsible for ROS generation in this pathway. Importantly, the resistance to oxidative stress mediated by *HOXA9* in GBM cells could be relevant to the understanding of therapy resistance, since ROS are associated with resistance to clinical alkylatant agents in glioma cells.

RESUMO

Espécies reativas de oxigénio (ERO) são moléculas importantes que regulam vários processos fisiológicos, e em excesso podem contribuir para a tumorigénese. No cancro, a via da PI3K/Akt está associada com o aumento de ERO; possivelmente com a função de atuarem como mensageiros na sinalização de outras moléculas que integram esta importante via. Um dos genes que é *transcricionalmente* ativado pela via da PI3K/Akt é o *HOXA9*. Neste trabalho, pretende-se avaliar a contribuição do *HOXA9* na produção de ERO na via da PI3K/Akt. Para isso, foram avaliados os níveis intracelulares de ERO em linhas celulares derivadas de células de glioblastoma (U87MG) e em astrócitos humanos imortalizados (hTERT/E6/E7), ambas com sobre-expressão de *HOXA9*, bem como em células controlo *HOXA9*-negativas. O peróxido de hidrogénio foi usado como uma fonte exógena de ERO, e os seus efeitos funcionais foram avaliados através da viabilidade celular. Uma vez que, as células tumorais dependem maioritariamente da glicólise aeróbica para satisfazerem as suas necessidades energéticas e para diminuírem os seus níveis de ERO, foi também investigado o perfil celular metabólico mediado pelo *HOXA9*. Por fim, ao submeter as linhas celulares anteriores e adicionalmente a linha A172 a um inibidor da via da PI3K foi possível estudar o papel do *HOXA9* no que concerne à produção de ERO nesta via. Os resultados obtidos mostraram que a sobre-expressão do *HOXA9* está associada a um aumento dos níveis de ERO na linha celular U87MG. Este aumento de ERO é paralelo a um perfil glicolítico mais reduzido apresentado pelas células da linha celular U87MG-*HOXA9,* que se caracteriza pela baixa produção de lactato comparativamente com a sua linha celular controlo U87MG-MSCV. Ainda, a acumulação de ERO foi acompanhada pela resistência da linha U87MG-*HOXA9* após tratamentos com peróxido de hidrogénio. Por outro lado, também foi demonstrado que a exposição das células U87MG-*HOXA9* ao PI-103, um inibidor da via PI3K, resultou na inibição da via, na diminuição da expressão do *HOXA9,* e no aumento dos níveis de ERO. Assim, este estudo fornece uma melhor compreensão sobre o papel do *HOXA9* na produção de ERO na via PI3K/Akt, sugerindo este gene como um possível responsável pela produção de ERO nesta via. Ainda, é de realçar que a resistência ao stress oxidativo mediado pelo *HOXA9* em células glioblastoma pode ser relevante para a compreensão da resistência à terapia, uma vez que as ERO estão associadas a uma resistência ao tratamento com agentes alquilantes em células de glioma.

INTRODUCTION

1 GLIOMAS: an overview

Tumors of the Central Nervous System (CNS) represent only about 2% of all human tumors[1]. However, they are an important cause of morbidity and mortality in both children and adults, being the first and the fourth leading cause of cancer-related death in children and in middle-aged adults, respectively[2]. The global annual incidence of primary brain tumors is about 5 to 10 cases per 100,000 persons. According to World Health Organization (WHO), Portugal stands as one of the countries with the highest incidence of this type of tumors, where 989 new cases of nervous system tumors were diagnosed in 2008, with 753 related deaths[3].

Associated with the CNS are several types of primary brain tumors, being gliomas the most comum[4, 5, 6], representing about 80% with a lower rate of survival[7]. Glioma designation arises from their cellular origin; they are glial-derived cells, including astrocytes, oligodendrocytes and ependymal cells[8, 9]. Depending on the cell type of origin, gliomas are divided into four major histological types: astrocytic (Table I), oligodendroglial, mixed oligoastrocytic tumors and ependymonas[8, 9]. According to the WHO classification system, gliomas can be classified into four grades of malignancy (I-IV)[10, 11, 12]: grade I (pilocytic astrocytoma), grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma) and grade IV (glioblastoma - GBM). Grade I gliomas correspond to those with a benign behavior and therefore are less aggressive, while grades II-IV correspond to malignant and more aggressive gliomas[8]. Among differential grading, GBM stands as the most prevalent, accounting for 60-70%[11,13], and deadly malignant glioma.

Table I. WHO classification of astrocytic tumors[8].

1 Astrocytic tumors	
1.1 Astrocytoma	WHO Grade II
1.1.1 Fibrilar	WHO Grade II
1.1.2 Protoplasmic	WHO Grade II
1.1.3 Gemistocytic	WHO Grade II
1.2 Anaplastic Astrocytoma	WHO Grade III
1.3 Glioblastoma	WHO Grade IV
1.3.1 Giant cells Glioblastoma	WHO Grade IV
1.3.2 Gliosarcoma	WHO Grade IV
1.4 Pilocytic Astrocytoma	WHO Grade I
1.5 Pleomorphic Xanthoastrocytoma	WHO Grade II
1.6 Subependymal giant cell astrocytoma	WHO Grade I

1.1 Glioblastoma

1.1.1 Etiological and molecular basis of Glioblastoma

Little is known about GBM etiology. Exposure to ionizing radiation, rare mutations of penetrant genes, and inherited susceptibility represent a reduced portion of some proposed risk factors[10, 14]. Nevertheless, during the last decade there have been significant advances in understanding the molecular profiles of GBMs. The malignant glioma arises from sequential accumulation of genetic aberrations and deregulation of growth factors signaling pathways[8, 15] (Figure 1). GBMs can be separated into two main types based on biological and genetic differences[8]: primary (*de novo*) and secondary GBMs. Primary GBMs typically occur in patients older than 50 years and are characterized by amplification and mutations in *Epidermal Growth Factor Receptor* (EGFR). The amplification of the *EGFR* gene in chromosome 7 occurs in about 40% of the GBM[10, 16], and appears to be a late event in the development of malignant astrocytomas, often followed by gene rearrangements which result in deletions of certain portions encoding the protein. The most frequent GBM molecular rearrangement involves the deletion of *EGFR* exons and it occurs in a large fraction of *de novo* GBMs, but it is not frequently observed in progressive (or secondary) tumors. Loss of heterozygosis (LOH) of chromosome 10q, deletion of the

p16 gene and deletion of *Phosphatase and Tensin homologue* (PTEN) are also changes that occur in primary GBMs. LOH of chromosome 10q includes the *PTEN* gene (chromosomal region 10q23.3), and is one of the most common genetic defects found in GBM, occurring in about 70-90% of tumors[17, 18,19]. There are evidences to suggest that the mutation of the *PTEN* gene is a marker for a poor prognosis in patients with gliomas[19]. Moreover, the secondary GBMs alterations occur in young patients, and develop from low-grade gliomas or anaplastic astrocytomas, progressing after a period of several years into GBM. These tumors, which are much less common than primary GBMs, are characterized by mutations in the *TP53*[8, 20, 21], overexpression of the *Platelet-derived growth factor receptor* (PDGFR) gene, alterations in *Rb* and p16 pathways, and LOH of chromosome 10q[8, 22]. *TP53* is an important tumor suppressor gene, and is mapped on chromosome 17p region, a region of frequent deletion in GBMs. In response to various stresses, this can trigger various biological functions at the level of the cell cycle, apoptosis, differentiation and angiogenesis[20, 21]. While *TP53* mutations are rare in primary GBMs (<10%), they are common in secondary GBMs (<65%), and in 90% of cases the mutation was already present in the first biopsy[20]. The main consequence of functional *TP53* mutations is the loss of specificity of DNA binding and transcriptional activity[23].

Despite their genetic differences, primary and secondary GBMs are morphologically indistinguishable and respond similarly to conventional therapy. However, they may react differently to molecular-targeted therapies[8]. Genetic alterations have been identified by being important in the development and proliferation of malignant characteristics that are associated with GBM. Some of these genetic aberrations appear to have prognostic significance. Nonetheless, knowledge of these provides clues for the identification of signaling pathways responsible for carcinogenesis, as well as potential therapeutic agents[15]. However, despite a huge progress in the genomic characterization of GBM, it still has no effective translation value.

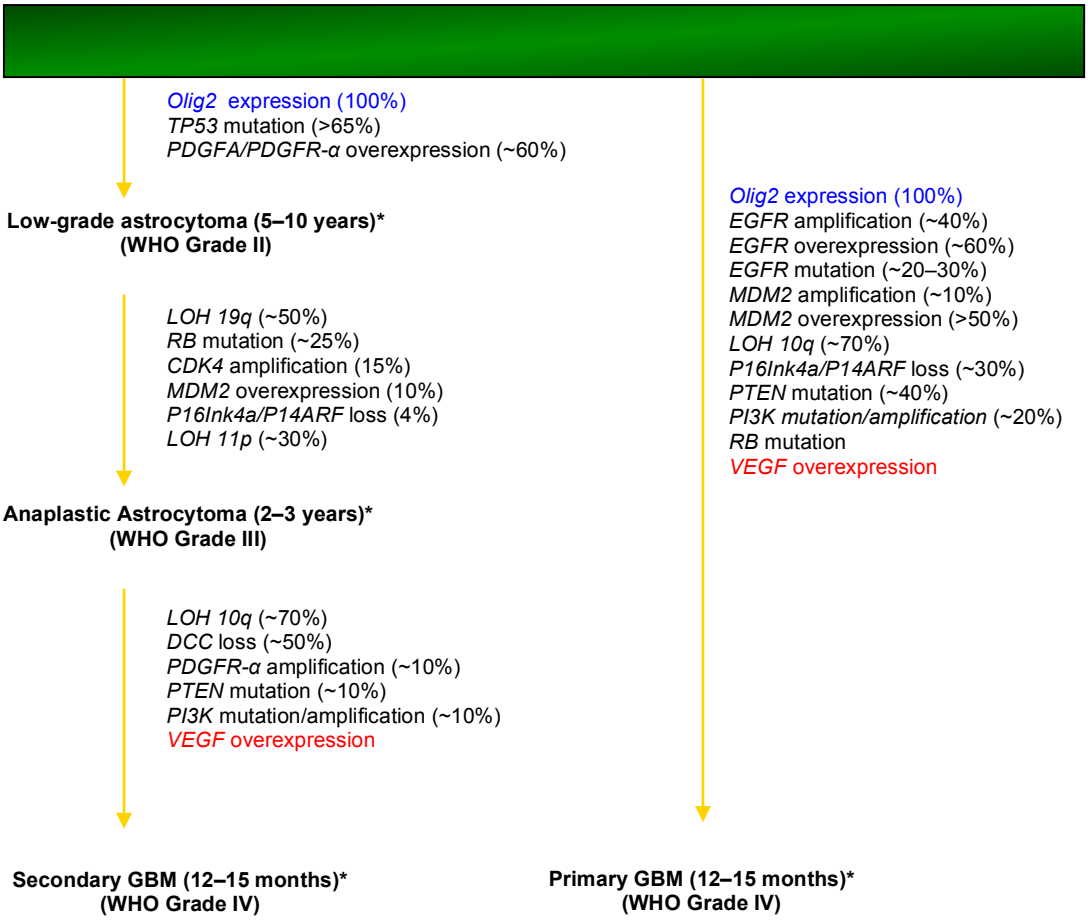

Figure 1. Summary of genetic and chromosomal alterations involved in the development of primary and secondary GBMs. The *transcription factor Olig2* (blue) and *vascular endothelial growth factor* (VEGF, red) are virtually expressed in all high-grade gliomas. The median survival (asterisk) is shown (Adapted from Kleihues P[8]).

1.1.2 Therapy and outcomes

Without doubt, GMB is the main responsible for overall shorter survival in CNS tumors. Despite clinical efforts, the prognosis of patients remains dismal, with a median survival after diagnosis of approximately 12-15 months[4]. Its highly infiltrative growth, very aggressive nature, relative resistance to radiotherapy and chemotherapy treatments[8], and relentless recurrence are the main features of GBM patient's poor survival. Behind these features are already known GBM biological hallmarks such as uncontrolled cellular proliferation, resistance to apoptosis,

genomic instability, invasion, angiogenesis, and tumor-initiating cells[24]. It is important to notice that their diffuse and infiltrative growth pattern becomes a clinical challenge by preventing complete surgical removal. Taken all these factors together there is an understanding of the unquestionable difficulty to improve the disease outcome. Actually, even with the current standard of care, which includes surgical resection safely feasible, followed by chemoradiotherapy with concurrent temozolomide (TMZ), and then adjuvant chemotherapy with TMZ, most of the patients die after two years[15]; reason why the standard treatment is still far from the ideal. Therefore, there is an urgent need to identify molecular markers; a better tumor classification and patients stratification into clinically-relevant prognostic subgroups would be a new framework to treat GBM. Already established GBM markers are: age at diagnosis[25, 26], Karnofsky Performance Status[26, 27, 28], and extent of surgical resection[28]. Although they are important clinical factors, they are not enough as GBM prognostic markers, opening the path for a challenging discovery of new biomarkers with significant impact in prognosis or in prediction of response to specific therapies. Currently, one of the promising prognostic biomarker for patients with GBM is the methylation status of *O^6-methylguanine methyltransferase* (MGMT) promotor[29]; its methylation is predictive of a response of GBM patients to treatment[30], being associated with prolonged survival in patients with GBM who receive alkylating chemotherapy.

1.1.3 *HOXA9*: a potential Glioblastoma biomarker

In an attempt to search for new prognostic biomarkers in GBM, recent studies highlight the importance of the *Homeobox (HOX)*-containing genes, particularly the *HOXA* cluster, reported to be aberrantly expressed in GBMs[31, 32, 33, 34]. *HOX* genes constitute a gene family that encode specific nuclear proteins (homeoproteins) that act as transcription factors[35, 36], which play an important role during normal development by regulating primary cellular processes, such as cell identity, cell division and differentiation[36]. In humans, *HOX* genes are known to have a unique genomic organization; they are distributed in four genomic clusters, each localized on a different chromosome (*HOXA* at 7p15.3, *HOXB* at 17p21.3, *HOXC* at 12q13.3 and *HOXD* at 2q31)[37]. These remarkable evolutionarily conserved genes have been the topic of various cancer-related studies due to its aberrant expression in many solid tumors[38, 39] and leukemia[40, 41, 42]. Among all *HOXA* genes, *HOXA9* was found to be overexpressed in myeloid leukemogenesis[40] and to be involved in the regulation of

leukemogenic pathways by potentially activating and repressing several oncogenes and tumor suppressor genes, respectively. Also, it was associated with poor prognosis in patients with acute myeloid leukemia[43]. *HOXA9* seems to play significant roles in other malignancies besides leukemia, such as ovarian[44, 45] and breast[46] cancer. In GBM, *HOXA9* emerged as the only aberrantly expressed *HOX* gene associated with poor patient outcome and shorter survival[32]. Additionally, *in vitro* studies showed that *HOXA9* has pro-proliferative and anti-apoptotic properties. Importantly, it was also reported that *HOXA9* transcriptional activation is regulated by phosphatidylinositol 3-kinase (PI3K) pathway through reversible epigenetic modifications[32]. Also, *HOXA9* expression was shown to be associated with shorter survival of pediatric high-grade gliomas[33]. Taken all together, it is no surprise the emergent interest in the study of *HOXA9* gene.

2 OXIDATIVE STRESS IN GLIOBLASTOMA

GBM are notorious by their aggressiveness and resistance to radiotherapy and chemotherapy[8]. A huge number of studies are developed in a tentative to understand and overcome GBM remarkable resistance to cell death[24]. Among several proposed molecules and altered pathways, oxidative stress, implicated in the etiology of cancer[47, 48, 49], plays a significant role in the development and resistance of gliomas[50].

2.1 Reactive oxygen species: definition and sources

Reactive oxygen species (ROS) is a collective term used to describe a heterogeneous group of oxygen (O_2)-derived free radicals, including superoxide anion ($O_2^{\bullet -}$), hydroxyl ($HO^{\bullet}$), peroxyl ($RO_2^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals, as well as non-radical species including molecules like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), oxygen (O_2) and peroxynitrite ($ONOO^-$)[51, 52,] also capable of generating free radicals. By definition, a free radical is a molecule, which contains one or more unpaired electrons in its outer orbital[53].

Although O_2 is a requirement of all aerobic organisms for their energy production, it is also a source of ROS[51]. In fact, most of the energy that aerobic cells need to live depends on oxidative phosphorylation[49] - a mitochondrial process that requires O_2 by which inevitably ROS are generated mainly due to the incomplete

reduction of O_2 by the electron transfer chain (ETC) system in the mitochondria[54, 55]; therefore, ROS are a normal byproduct of cellular metabolic processes. Mitochondria are considered the major source of intracellular ROS[56], where in normal conditions it is estimated that 1–2% of the O_2 consumed by mitochondria is converted to ROS[52, 56, 57]. However, this is not a mitochondrial exclusive process; other organelles, such as peroxisomes[58] and the endoplasmic reticulum[59], as well as external sources such as UV radiation, toxic chemicals and drugs[60], and the enzyme NADPH oxidase[61], can also contribute to ROS generation. ROS production begins with the "leaky" transfer of a single electron to O_2, mainly at ETC complex I and III[54, 55], resulting in its partial reduction and consequent generation of $O_2^{\bullet-}$. A wide of a variety of potential chemical reactions lies at $O_2^{\bullet-}$, setting it as the precursor to other major forms of ROS[62]. Although there are evidences that $O_2^{\bullet-}$ may enter the cytosol through specialized mitochondrial channels, such as the voltage-gated anion channel[63], due to its negative charge $O_2^{\bullet-}$ that does not diffuse freely across membranes and therefore needs to be converted into ROS membrane permeable molecules. Thus, $O_2^{\bullet-}$ can be converted to H_2O_2 and O_2 by the enzyme manganese superoxide dismutase (SOD)[62]. H_2O_2 can be further transformed either via Fenton reactions or by enzymatic catalysis to several highly oxidizing derivatives including $HO^{\bullet}$ radicals and HOCl[64, 65].

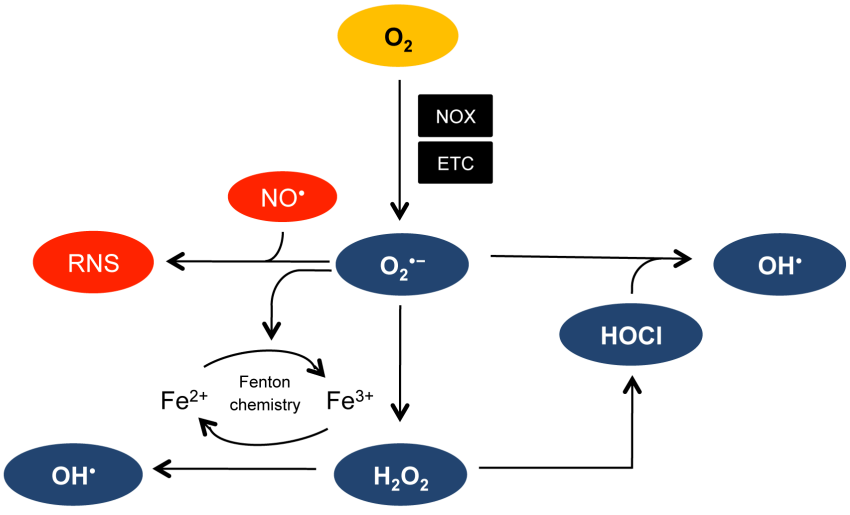

Figure 2. Schematic representation of physiologic ROS.

2.2 Dual role of reactive oxygen species

It is well known that ROS play a dual role by acting as both key components of many normal physiological processes[66] and as a deleterious cellular stimulus[67]. This double-edged sword has major implications in the cell path, and its modulation is dependent on the levels of ROS produced in the cell[49, 68, 69]. On the one hand, appropriate low levels of ROS participate in the maintenance of cellular homeostasis. On the other hand, overproduction of ROS, as well as a defective antioxidant system, leads to the accumulation of oxidative stress, potentiating the onset of oxidative-related diseases. The damaging effect of ROS is due to its capacity to interact with all biological macromolecules, such as lipids, proteins and nucleic acids, by modifying both their structure and function, ultimately oxidizing and damaging them[68]. Its no surprise that cells have developed ways to control the continuous flux of ROS and prevent oxidative stress. These systems are well studied non-enzymatic and enzymatic antioxidizing agents, namely: glutathione[70], SOD[71], catalase[72] and peroxiredoxin[73]. The antioxidant process is a result of an interplay of the antioxidants components; for example, SOD present both in the cytosol and in mitochondria, converts superoxide into H_2O_2[61], which is further detoxified into H_2O and O_2 by catalase[72] or peroxidases, such as the glutathione peroxidase, which is the main redox agent of the cell[70]. In addition, other molecules such as cytochrome C have an important antioxidant function by acting as a powerful O_2^- scavenger in the intermembrane space of mitochondria[49]. However, as is evident, more work is required to better understand the role of ROS in tumor progression.

2.3 Reactive oxygen species, cancer and PI3K pathway

Given the broad range of ROS sources and their highly reactive nature, it is not surprising that ROS have been implicated in a number of disease like neurodegenerative[75, 76, 77], cardiovascular diseases[78, 79], and cancer[49, 80, 81].

It is known that one of the main features of cancer cells is their extremely high level of ROS[82, 83], giving them a persistent pro-oxidative state, which ultimately can lead to intrinsic oxidative stress. How do these cells survive under such elevated baseline ROS levels, and even benefit from them, has been a key question that was only recently answered.

Cancer cells have developed mechanisms to protect themselves from intrinsic oxidative stress, by negatively regulating ROS through the increase

expression of the antioxidant enzymes[67], and activation of redox-sensitive transcription factors, including NFκB, HIF-1, and c-jun[84, 85, 86, 87], which may contribute to cancer cell adaptation to persistent oxidative stress. Higher levels of antioxidant enzymes are present in malignant tissues compared with the normal tissue[88, 89]. Thus, it seems that the increase of antioxidant defenses are acquired by cancer cells to escape severe oxidative damage and to survive in the presence of intrinsic oxidative stress.

Since cancer cells can produce large amounts of ROS, it has been reported that ROS may have a role in cancer initiation, promotion, progression[49, 90], and even at therapy resistance[91, 92, 93]. Increased ROS levels contribute to malignancy by persistent promotion of cell survival and proliferation, whether by exerting an oncogenic stimulation or inhibiting apoptosis[91]. Doubtless is that ROS can contribute to oncogene activation, or act as their downstream effectors[94]. Not only, enhancing the rate of mutations, which leads to DNA damage and chromosomal instability contributes to cancer progression[95], but also cytoskeletal rearrangements, regulation of signaling pathways and transcriptional activities favors the metastatic process through cell migration, invasion, and metastization[96, 97]. The activation of several oncogenes, such as the c-myc[98], increases generation of ROS and consequently DNA damage, interfering with the p53-mediated DNA damage response[99], enabling cells with damaged genomes to enter the cycle and accelerate tumor progression via genetic instability[100]. Also, metalloproteinases -3, -10, and -13, which are involved in the remodeling of extracellular matrix, seem to be upregulated by prolonged H_2O_2-induced oxidative stress[101]. In addition, the transformation of hematopoietic cells by the oncogenic tyrosine kinase BCR-ABL is associated with a chronic increase in intracellular ROS level of mitochondrial origin[102, 103, 104].

Other than acting at the transcriptional level, ROS can also act at the signal-transduction level to exert pro-survival functions, by activating several pathways, including the Phosphatidylinositol 3-kinase (PI3K)/Akt pathway[105, 106, 107, 108]. The PI3K family is constituted by lipid kinases that phosphorylate the 3'-hydroxyl group of phosphoinositides. Within the 3 classes of PI3K[109, 110], the class IA PI3K is the cancer-related[109]. PI3K is one of the principal components of the PI3K/Akt signaling pathway, and its activation initiates a signal transduction pathway that promotes cancer cell growth, metabolism, and survival[110, 111, 112]. Akt, a serine-threonine kinase directly activated in response to PI3K[110], is a crucial downstream effector of PI3K in tumorigenesis. Due to its well recognized vital importance in cancer cells survival, PI3K inhibitors are reaching the clinical field. The activation of this pathway has been negatively linked to several type of tumors, where its constitutively activation is

correlated with poor prognosis[113, 114] and therapy resistance[115, 116]. PI3K is activated by a wide range of upstream signals, including ROS acting as second messengers[105, 106, 107, 73]. In acute lymphoblastic leukemia, PI3K pathway activation by IL-7 is dependent of ROS upregulation[108]. Additionally, signaling pathway itself contributes to ROS production in order to sustain its own activity. Also, the elevated ROS levels of BCR-ABL-expressing cells in chronic myelogenous leukemia contributes to the pathway activation, either through the inhibition of PP1α[103], conferring a higher resistance to apoptosis, or by activation of pathway members such as Akt and GSK3β[102]. Indirect activation of the pathway can be regulated by ROS; ROS can inactivate PTEN[105, 106, 117], which is a negative regulator of the pathway, driving the cells to their pro-survival functions. Therefore, these strong evidences establish that PI3K/Akt/mTOR may be redox regulated.

2.4 Oxidative Stress in Glioblastoma: a commitment to resistance

As mention before, oxidative stress has been implicated in GBM radio and chemoresistance[50]; however, the role of ROS in these processes remains unclear. Until now, oxidative stress-indissociable ROS production ROS production and alterations in the expression of antioxidant enzymes have been implicated with cancer cells survival and resistance to anticancer treatment. ROS production by enzymes such as NADPH oxidase Nox4, a major non-mitochondrial source of ROS[61], is associated with the proliferation[118] and resistance of glioma cell to apoptosis[119]. The NADPH oxidase Nox4 overexpression in GBM can regulate apoptosis evasion through the cascade of antiapoptotic proteins, such as the PI3K/Akt pathway, the mTOR, and the NF-κB.[120] Contributing to this resistance phenomena are the antioxidant enzymes such as, peroxiredoxin II[121], catalase and glutathione peroxidase 1[122], where their overexpression regulate the oxidative stress in GBM cells, by mediating resistance to ROS-mediated cell death. This studies show that inhibition of these enzymes sensitizes glioma cells to oxidative stress, presenting them as potential therapeutic targets. Overall, the search for genetic alterations in pro-oxidant enzymes and the knowledge about the antioxidant enzyme status of GBM may be useful tools for overcome ROS inducing resistance in GBM.

AIMS

General Aim

Emerging studies report that the PI3K pathway may be redox-regulated, whether by being activated or contributing to sustain intracellular ROS levels in cancer. Since *HOXA9* activation is PI3K pathway-dependent, we therefore hypothesized that *HOXA9* could be one of the effector proteins for ROS upregulation in this pathway. So, the overall aim of this thesis is to understand how the PI3K pathway-associated generation of ROS may be dependent on *HOXA9*.

Specific Aims

To meet the main purpose of this thesis, our specific aims are to:

- Evaluate the influence of *HOXA9* in ROS production in GBM cells.
- Investigate the influence of *HOXA9* in the response of GBM cells to ROS stress.
- Clarify if *HOXA9*-mediated ROS production is independent of PI3K pathway.

MATERIALS AND METHODS

1 Cell Culture

The *in vitro* studies comprised the use of three cell lines: the U87MG, the A172 (both human GBM-derived cells) and the hTERT/E6/E7 (human astrocytes immortalized with the human telomerase reverse transcriptase and with the human papillomavirus oncogenes E6 and E7). Both U87MG and the putative GBM precursor cell line hTERT/E6/E7 were previously established[7] by having a stable overexpression of *HOXA9* as a result of a retroviral infection with the murine stem cell virus (MSCV) encoding *HOXA9* (MSCV-*HOXA9*) or a control vector (MSCV-Control). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, from Gibco - Invitrogen®) supplemented with 10% fetal bovine serum (FBS, from Biochrom AG) and 1% penicillin/streptomycin (100U/ml penicillin, 100 ug/ml streptomycin, from Gibco - Invitrogen®). *HOXA9* overexpressing cell lines and their counterparts were additionally supplemented with 500 µg/ml G418 (Sigma-Aldrich), an antibiotic that selects cells containing the neomycine resistance gene present in the retroviral construct. The cells were maintained at 37ºC in an incubator with 5% CO_2.

2 Induction of Oxidative Stress with H_2O_2

For all oxidative stress measurements, 1 x 10^6 cells were plated in cell culture flasks with 75 cm² surface area and allowed to adhere overnight. Exposure with either 1500 µM of H_20_2 or DMEM medium supplemented with 10% FBS (untreated control) was carried out from 15 to 30 minutes and from 15 minutes to 4 hours in hTERT/E6/E7-*HOXA9* and U87MG-*HOXA9* as well as in their respective control cells, respectively.

3 Reactive Oxygen Species Quantification

Intracellular ROS generation was determined by measuring the levels of H_2O_2 produced in the cells by flow cytometry after staining cells with cell permeable 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, from Molecular Probes - Life Technologies). After incorporation into cells, H_2DCFDA is readily cleaved by

esterases and oxidized by ROS to form fluorescent 2', 7'-dichlorofluorescein (DCF). Thus, DCF fluorescence estimates ROS generation in living cells. Briefly, the cells were washed twice with PBS and then 2,5 x 10^7 cells were incubated with 5 μM H_2DCFDA at 37ºC for 25 minutes in the dark. After incubation, cells were washed and ressuspended in PBS and analyzed for ROS generation using LSRII flow cytometer (BD Biosciences). For all the experiments, approximately 10,000 events were evaluated for each sample. ROS levels were calculated by measuring the mean fluorescence using FlowJo software (Tree Star, Ashland).

4 Cell Viability Assays

Metabolic activity was measured using CellTiter 96® AQueous One Solution Reagent (Promega Corporation) a (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) compound. The measurement of cell metabolic activity is based on the activity of cell dehydrogenases and their ability to cleave tetrazolium salts, forming a colored formazan product. Briefly, 4x10^3 cells per well were cultured in 96-well culture plates followed by H_2O_2 exposure for the indicated concentrations and desired time, according to experimental purpose. At every time point, 10μl per well of MTS was added and incubated at 37ºC for 2 hours. The absorbance was measured on Infinite 200 NanoQuant microplate reader (Tecan) at 490 nm. Absolute absorbance was normalized to the absorbance of untreated cells in each plate and expressed as a percentage of the control value.

Cell viability was also measured using trypan blue exclusion assay (Invitrogen, Life Technologies), where non-viable (dead) cells with compromised membranes are permeable to the blue staining. Cells were cultured in 6-well culture plates with an initial density of 6 x 10^4 cells per well. After 24 hours of cell plating, 1500 μM of H_2O_2 was added in 2 ml of DMEM supplemented with 10% FBS and treatment was executed for 4, 8, 24, 48, 72 and 96 hours. At each experimental time point, the viable cells were counted in duplicate, using a Neubauer chamber, under the IX51 inverted microscope (Olympus). The total number of viable cells was calculated using the following:

$$\text{Total cells} = \text{mean of viable cells} \times \text{dilution factor} \times \frac{1000}{0,1} \text{ cells/ml}$$

5 Glucose Consumption and Lactate Production

To measure glucose consumption and lactate production, 5 x 10^4 cells were cultured in 24-well plates with overnight adherence. Every 24 hours during 4 days, the media was collected and frozen until the time for glucose uptake and lactate production quantification. Extracellular lactate quantification was determined using a commercial assay kit Lactate Enzymatic colorimetric LO-POD (Spinreact) and following the manufacturers' instructions. Briefly, lactate production was calculated by measuring the absorbance (490 – 570 nm) of lactate oxidized by lactate oxidase (LO) to pyruvate and H_2O_2, which under the influence of peroxidase (POD), 4-aminophenazone and 4-chlorophenol form a red quinone compound. The intensity of the color formed is proportional to the lactate concentration in the sample. Glucose quantification was performed using an enzymatic colorimetric assay Glucose god-pap (Roche Diagnostic) based on the conversion of glucose into gluconate by glucose oxidase (GOD). The H_2O_2 produced in the reaction is degraded by POD and gives a colored product phenol and 4-aminoantipyrine, which is measurable using Trinder indicator reaction at 505 nm. The increase in absorbance correlates with the glucose concentration of the sample. All glucose and lactate quantifications were normalized to cell biomass by sulphorodamine B (SRB) assay (Sigma-Aldrich), where the adherent cells were fixed with 10% trichloroacetic acid for 1 h at 4°C, rinsed with water, air-dried and stained with 0.4% SRB for 30 min at 37°C. The bound dye was solubilized with 10 mM Tris (100 µl per well) and the absorbance measured at 540 nm.

6 Treatment of Cell Lines with PI-103

The U87MG-*HOXA9*, hTERT/E6/E7-*HOXA9* and their respective control cells, as well as the A172 cell line were exposed for 24 hours to 1, 2 and 6 µM of the PI3K inhibitor PI-103 (Selleck Chemicals) or DMSO vehicle as control, respectively.

7 Western Blot

The cell lines under study were scrapped off and washed twice in cold PBS, followed by protein extraction. Thirty ug of total protein extract was resolved in 10%

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto a nitrocellulose membrane (Amersham™ - GE Healthcare Life Sciences) and probed with antibodies raised against Akt and phospho-Akt (p-Akt Ser 473) (1:500; Cell Signaling). The secondary antibody was goat anti-mouse IgG-HRP (1:5000; Santa Cruz Biotechnologies). Each membrane was re-probed with antibody against α-tubulin (1:100; Santa Cruz Biotechnologies) for protein loading control. Detailed Western Blot protocol is described in Appendix I.

8 Quantitative Real-Time PCR

The cell lines were lysed with TRIzol (Life Technologies, Inc) and the isolated RNA was reverse transcribed using Reverse Transcriptase (Invitrogen); both total RNA extraction and cDNA generation protocols are described at appendix I**.** *HOXA9* was amplified by qPCR using Kapa™ SYBR ® FAST qPCR Master Mix Kit (Kapabiosystems) in parallel with *hGUS* to use as a control gene for quantification. Primers used for quantitative real-time PCR were as follows: HOXA9, *5′-GGAGATGCTCACTTCGATGA-3′* (sense) and *5′ ATACCCAAAGAATGGCCAAG-3′* (antisense); and hGUS, *5′-AGGAGGAGGGCAGAATCATCA-3′* (sense) and *5′-CTCGATTGGATGGCAGTAGCT-3′* (antisense). The relative quantification of gene expression was determined by the equations: $\Delta Ct = (Ct_{HOXA9} - Ct_{hGUS})$ followed by $(2^{-\Delta Ct})*100$ (expressed as percentage). qPCR reactions were performed in triplicate.

9 Statistical Analysis

To evaluate the trypan blue viability assay's, glucose uptake and lactate production differences, repeated two-way ANOVA measures were used to compare each pair of curves along the time. For the rest experimental analyses statistical significance was assessed by using the t-test Student's. The results are expressed as mean ± standard deviation (S.D.), and analyzed by GraphPad Prism 7.6 (GraphPad Software). All experiments were conducted in triplicate and repeated 3 times. Statistical significant differences were considered when $P < 0,05$.

RESULTS

1 *HOXA9* upregulates intracellular ROS in U87MG GBM cells

Elevated ROS levels are demonstrated to be associated with cancer[58]. As previously reported, *HOXA9* is important for GBM cell viability *in vitro*, and since ROS is related to cancer cell survival, we investigated what would be the influence of *HOXA9* in ROS production in both GBM-derived cell line and its putative precursors. To this purpose, cell lines with frequent confirmation of *HOXA9* overexpression by RT-PCR (Appendix I and II) were cultured for 15 and 30 minutes for hTERT/E6/E7 cell line, and for 15 minutes and 24 hours for U87MG, with or without H_2O_2 treatment. Then, intracellular ROS production was evaluated by flow cytometry using H_2DCFDA. We observed that in basal conditions, U87MG GBM cells that overexpressed *HOXA9* had a significantly higher accumulation of intracellular ROS (Figure 1A), comparing with their control cell line (U87MG-MSCV). Curiously, the ROS levels detected in hTERT/E6/E7 cell line overexpressing *HOXA9* were significantly lower despite the expression of *HOXA9* (Figure 1B).

Furthermore, we aimed to see what would be the effect of exposure of these cells to an exogenous source of oxidative stress using a ROS molecule, H_2O_2. We found that treatment with 1500 µM of H_2O_2 for the same time points caused an increase of ROS in all cell lines (hTERT/E6/E7±*HOXA9* and U87MG±*HOXA9*); however, different profiles were observed within each cell line. On one hand, the U87MG-*HOXA9* had an increase of ROS levels of approximately 2- and 1,5 –folds for 15 minutes and 24 hours of H_2O_2 exposure, respectively (Figure 1C). This ROS upregulation by *HOXA9* overexpressing GBM cells was significantly higher than the control cell line, suggesting that *HOXA9* may have an influence in intracellular ROS production. On the other hand, although both hTERT/E6/E7 cell lines did increase the ROS levels upon H_2O_2 exposure, it was clear that hTERT/E6/E7-*HOXA9* cell line produced significantly lower levels of ROS comparing to its counterpart (figure 1D). These results suggest that *HOXA9* do not have a pronounced effect in terms of ROS production in the astrocytic lineage.

Overall, these data indicate that treatment with pro-oxidants cause significant increases in intracellular levels of ROS in both cell lines. However, ROS production by U87MG-*HOXA9* was significantly higher compared to the U87MG-MSCV cells; on the contrary, it was markedly lower for hTERT/E6/E7-*HOXA9* in relation to its control cell line.

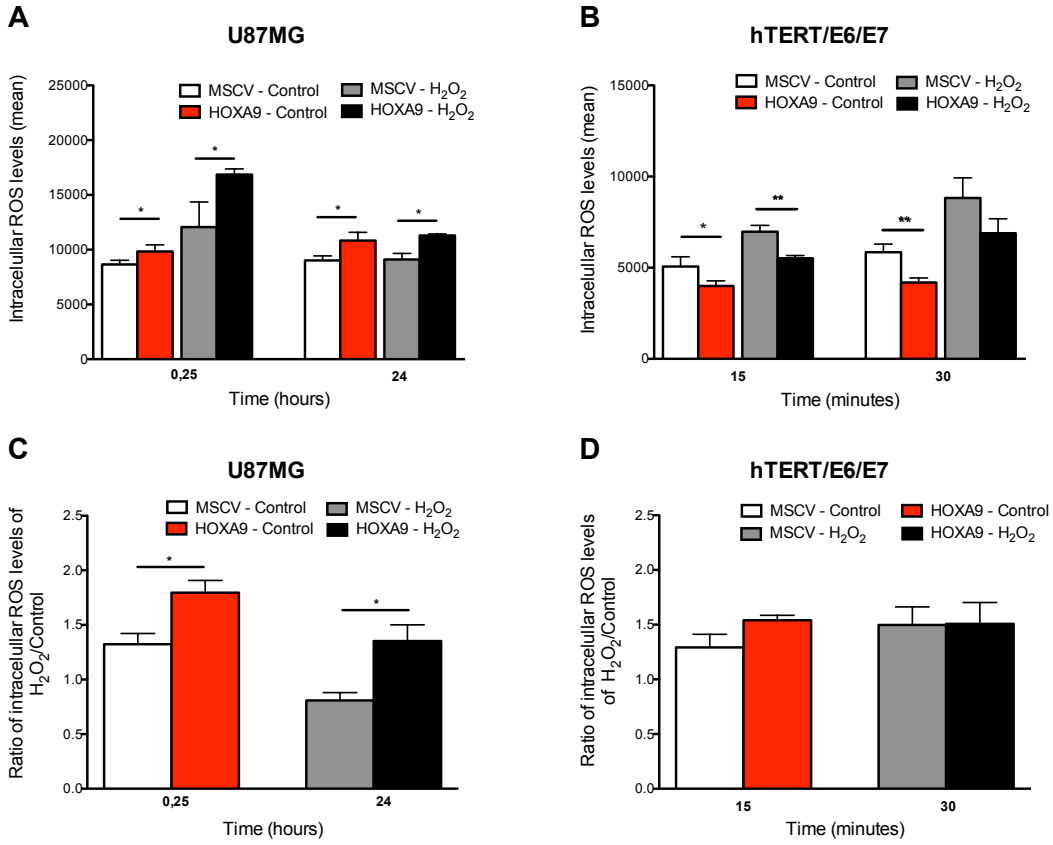

Figure 3. *HOXA9* overexpression enhances ROS production in U87MG GBM cells. ROS production was estimated by oxidation of the fluorescent probe, H_2DCFDA in (A) U87MG and (B) hTER/E6/E6 cell lines after indicated time of treatment with vehicle (control) or H_2O_2 (1500 μM). Ratio of intracellular ROS production between H_2O_2 and control conditions was determined for (C) U87MG and (D) hTERT/E6/E7 cell lines. **P* < 0,05; ***P* < 0,01.

2 *HOXA9* mediates a metabolic shift in U87MG GBM cells

The high production of ROS by U87MG-*HOXA9* cells may reflect a lower glycolytic rate. To test this hypothesis, we investigated the metabolism of each cell line by measuring the glucose uptake and lactate production. By these assays, it is possible to see that both cell lines, the U87MG (Figure 2A) and hTERT/E6/E7 (Figure 2B), presented higher glucose consumption in *HOXA9*-overexpressing cells, when compared to the control MSCV-cells. However, only the hTERT/E6/E7 cell lines presented statistical significant difference over time (P= 0,0187), while the U87MG cell lines only showed statistical significant difference in the 4th day (P= 0,0015). When looking to the lactate production, it is clear that there is a difference between cell lines; the U87MG-*HOXA9* cell line produces less lactate (Figure 2C) than its *HOXA9*-negative counterpart, and therefore is less glycolytic. On day 4, the cells from the U87MG-MSCV line were not completely attached to the plate surface, which affected SRB assay, and, ultimately decreased lactate production. In this sense, it was not possible to obtain statistical significant differences between the U87MG-*HOXA9* and U87MG-MSCV. However, statistically significance was reached in the 3th day (P= 0,0076). On the contrary, the lactate production by the hTERT/E6/E7-*HOXA9* cell line was higher (Figure 2D) comparing to their control cells along the time (P= 0,0013).

Overall, the metabolism of the cell lines under study is in agreement with the reported metabolic program of cancer cells by Otto Warburg. However, some are more glycolytic than others. The lower glycolytic profile of U87MG-*HOXA9* is suggestive of an increased oxidative phosphorylation to satisfy its energetic demands, which is consistent with our previous data regarding ROS upregulation by U87MG GBM *HOXA9*-overexpressing cells.

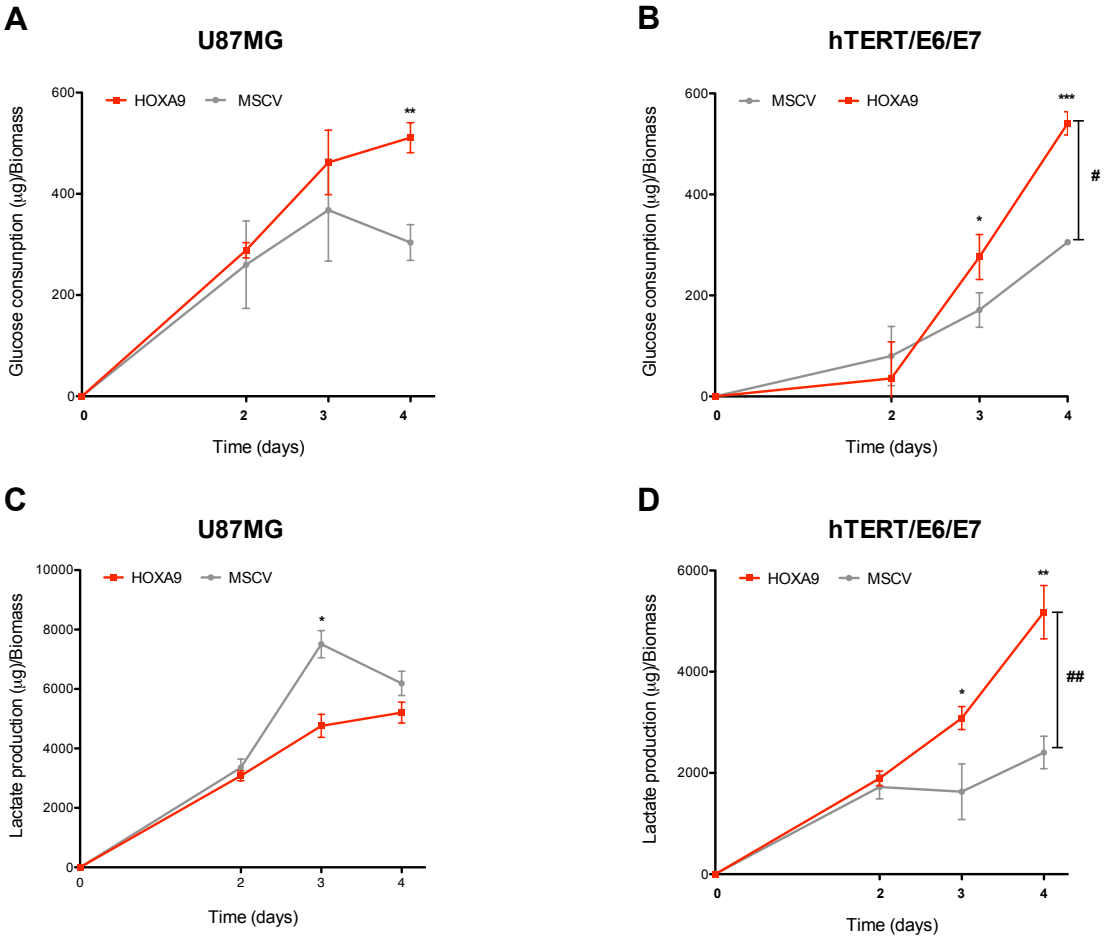

Figure 4. *HOXA9* overexpression is associated with reduced glycolytic status in U87MG GBM cells. Quantification of (A) glucose uptake and (C) lactate production in U87MG cell lines. (B) Glucose uptake and (D) lactate production in hTERT/E6/E7 cell lines. $^{\#}$, $^{*}P < 0,05$; $^{\#\#}$, $^{**}P < 0,01$.

3 *HOXA9* influences the viability of U87MG GBM cells after oxidative stress

Knowing that ROS could either be exerting beneficial or compromising effects on cancer cells, we investigated how *HOXA9*-mediated upregulation of ROS may affect cellular response, specifically cell viability. A range of H_2O_2 concentrations (500, 1500 and 3000 µM) were used to treat cells for 24 hours and evaluate their response under such oxidative stress.

By the MTS assay, it is clear that both cell lines, the U87MG and hTERT/E6/E7 (Figure 3A and B, respectively) were not tolerant to this extremely

oxidative stress driven by H_2O_2, resulting in an accentuated decrease on cellular metabolic activity. However, the U87MG-*HOXA9* cell line (Figure 3A) remarkably maintained its normal metabolic activity until the H_2O_2 concentration of 1500 µM, from which began to decrease cell metabolic viability. This result evidenciate that *HOXA9* mediates cell resistance to oxidative stress in GBM-derived cells.

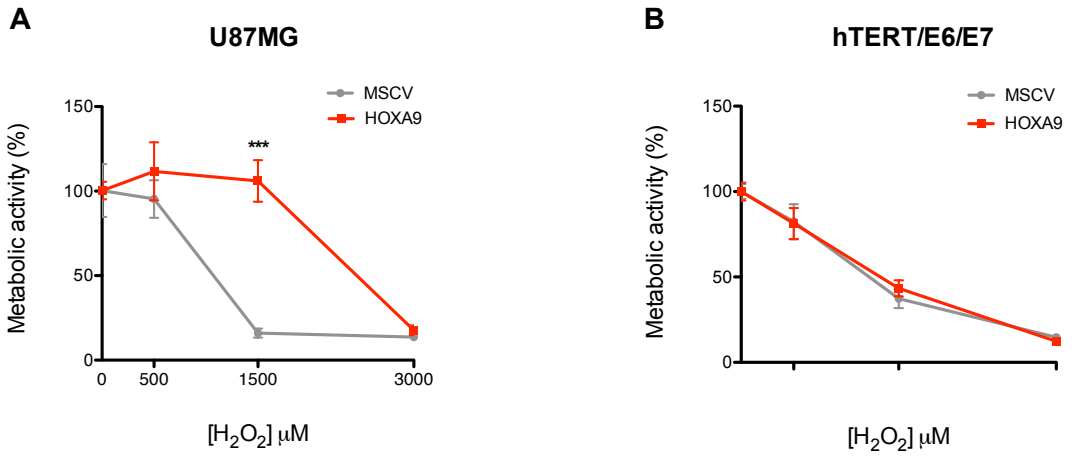

Figure 5. Viability of *HOXA9* overexpressing cells is H_2O_2 dose-dependent. Determination of cellular metabolic viability by MTS assay performed in GBM-derived cell line (A) U87MG and in (B) astrocytic cell line hTERT/E6/E7. Both lines were treated with or without H_2O_2 for 24 hours. ****P* = 0,0002.

This result really escalated the interest to evaluate the response of both cell lines to the same H_2O_2 concentration along the time. To assess this, cells were treated with 1500 µM of H_2O_2 for 4, 8, 24, 48, 72 and 96 hours. The trypan blue assay results show that in basal conditions, both U87MG-*HOXA9* (Figure 4A) and hTERT/E6/E7-*HOXA9* (Figure 4B) cell lines presented a significant increase in cell viability (P= 0,0170 and P < 0,0001, respectively), when compared to the control MSCV-cells. Under H_2O_2 treatment, only GBM cells with *HOXA9*-overexpression (Figure 4A) demonstrated higher viability comparing to their counterpart (P= 0,0045); while the hTERT/E6/E7 cell line (Figure 4B) did not present differences between *HOXA9*-overexpressing cells and their control cells, being their viability extremely affected along the time. A similar reduction in cell viability after 4 and 8 hours of exposure to H_2O_2 was observed in both cell lines. However, U87MG-*HOXA9* cells presented a notably recovery of their cell viability along the time, compared to the control MSCV-cells. This result indicates that *HOXA9* may play an important role in oxidative stress resistance in GBM-derived cells.

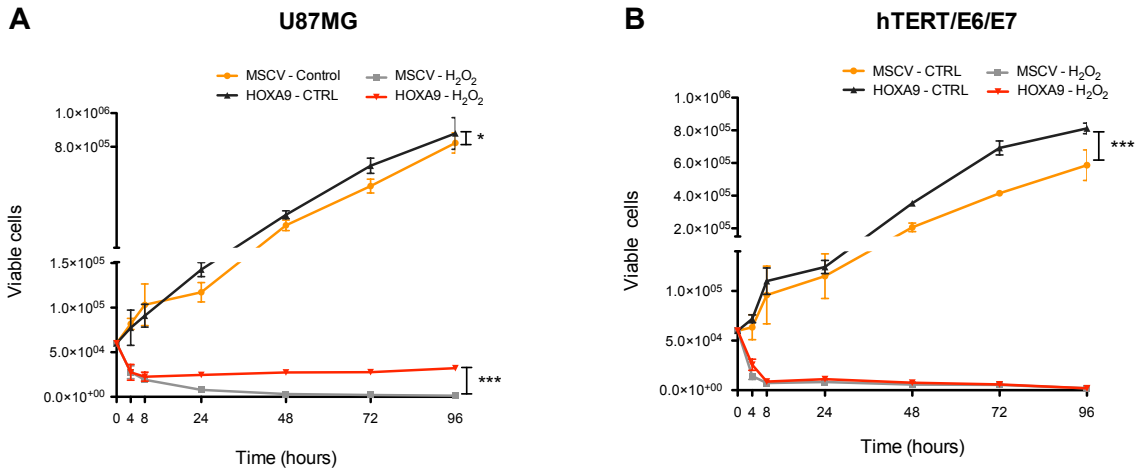

Figure 6. *HOXA9* overexpression confers resistance to oxidative stress in U87MG GBM cells. Trypan blue cell viability assay was executed in (A) U87MG and (B) hTERT/E6/E7 cell lines. Cell lines were treated with or without H_2O_2 for the indicated time. Effect of H_2O_2 compared with untreated cells: **P* < 0,05; ****P* < 0,001.

Similar results were observed in a different approach to analyze cell viability using the MTS assay. In the same experimental conditions as used in the previous assay, the ratio of cell metabolic viability between H_2O_2-treated and untreated cells was outlined for both cell lines. For the first 4 hours of H_2O_2 exposure, in the U87MG cell line (Figure 5A) it is noted that although there was a decrease of 40% and 70% for *HOXA9* overexpressing cells and their *HOXA9*-negative controls, respectively, *HOXA9* expression seems to mediate a metabolic viability recovery until the end of the experiment. Also, the *HOXA9*-overexpressing cells demonstrate a statistical significant higher metabolic activity than the MSCV-Control cells across the time ($P \leq 0.0001$ for 4, 8, 24 and 72 hours, and P= 0,0003, P= 0,0043 for 48 and 96 hours, respectively). When comparing the metabolic activity of the hTERT/E6/E7 (Figure 5B) it significantly decreased its metabolic activity down to 70% - 75%, with absence of a visible recover along the time. In addition, the *HOXA9*-overexpressing cells demonstrate a significant lower metabolic activity than the MSCV-Control cells (P= 0,0011, P= 0,0101, and P=0,0080 for 4, 8 and 24 hours, respectively). At 96 hours it was a statistically significant inversion of this behavior (P = 0,0249).

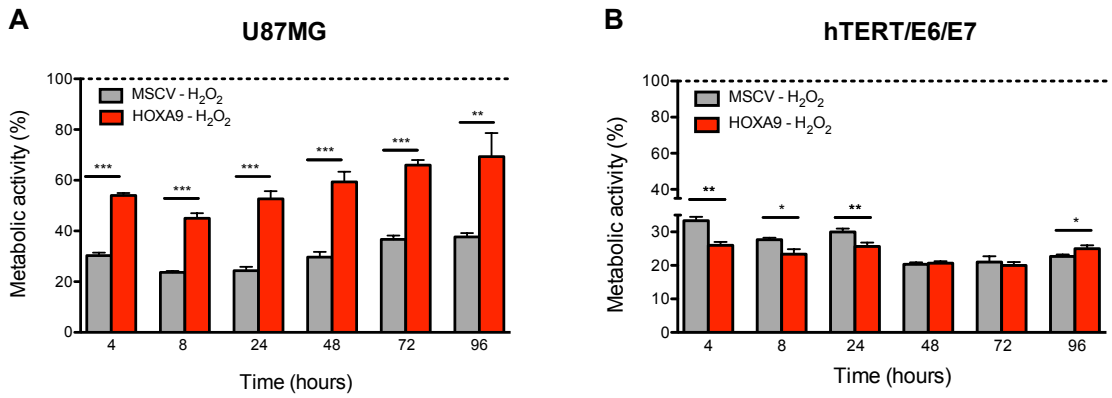

Figure 7. *HOXA9* overexpression influences cellular metabolic viability. Metabolic viability of (A) U87MG and (B) hTERT/E6/E7 cell lines was determined by the MTS assay. Cell lines were treated with or without H_2O_2 for the indicated time. Effect of H_2O_2 compared with untreated cells: **P* < 0,05; ***P* < 0,01; ****P* < 0.0001.

4 Inhibition of PI3K pathway does not impair ROS production in *HOXA9* overexpressing cells.

To further explore the consequence of inhibition of PI3K pathway regarding ROS production in our cell models, an inhibitor of this pathway, PI-103, was used for 24 hours with different concentrations according to the cell line. Because Akt is a downstream effector of PI3K pathway, contributing for the activation of several molecules, we first determined the status of p-Akt and total Akt by immunobloting the cell lysates against these proteins. In U87MG and hTERT/E6/E7 cell lines, treatment with 1 µM and 2 µM of PI-103, respectively, decreased phosphorylation of Akt (figure 8 A and B, respectively). This result is indicative of a partial inactivation of PI3K/Akt signaling pathway. Additional studies using a different cell line, the A172, which is reported to express *HOXA9* endogenously[32] showed a completely inhibition of PI3K/Akt pathway (Figure 8C).

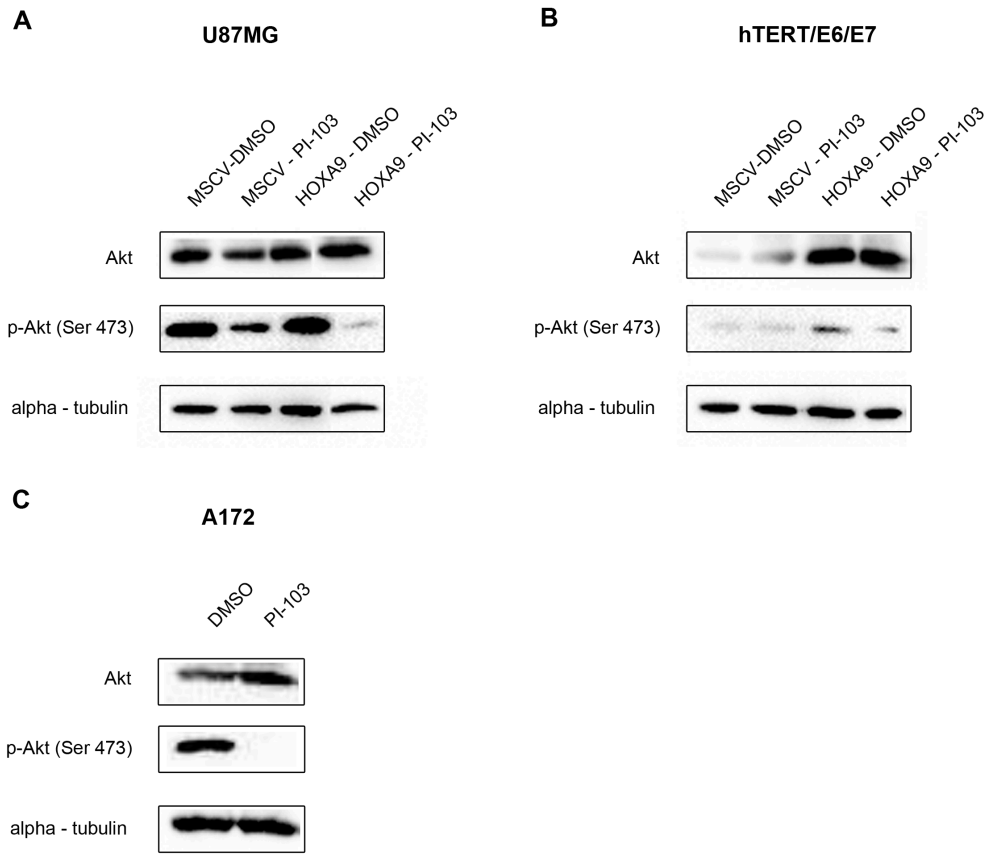

Figure 8. PI-103 suppresses PI3K pathway through Akt phosphorylation. Western blot analyses of (A) U87MG, (B) hTERT/E6/E7 and (C) A172 cell lines. All cell lines were treated with control (DMSO) or with PI-103 for 24 hours.

Since, the activation of PI3K/Akt signaling pathway is crucial for *HOXA9* activation in GBM cells[32], the expression of *HOXA9* upon inhibition of p-Akt by PI-103 was evaluated by qRT-PCR. The results show that inhibition of p-Akt significantly reduced the levels of *HOXA9* expression in all cell lines (Figure 9A, B and C), even those that exogenously overexpress *HOXA9*. The inhibitory effect of PI-103 in *HOXA9* expression reached statistical significance in all the three cell lines, with P = (0,0031; 0,0101; 0,0085) for U87MG-*HOXA9*, hTERT/E6/E7-*HOXA9*, and A172, respectively.

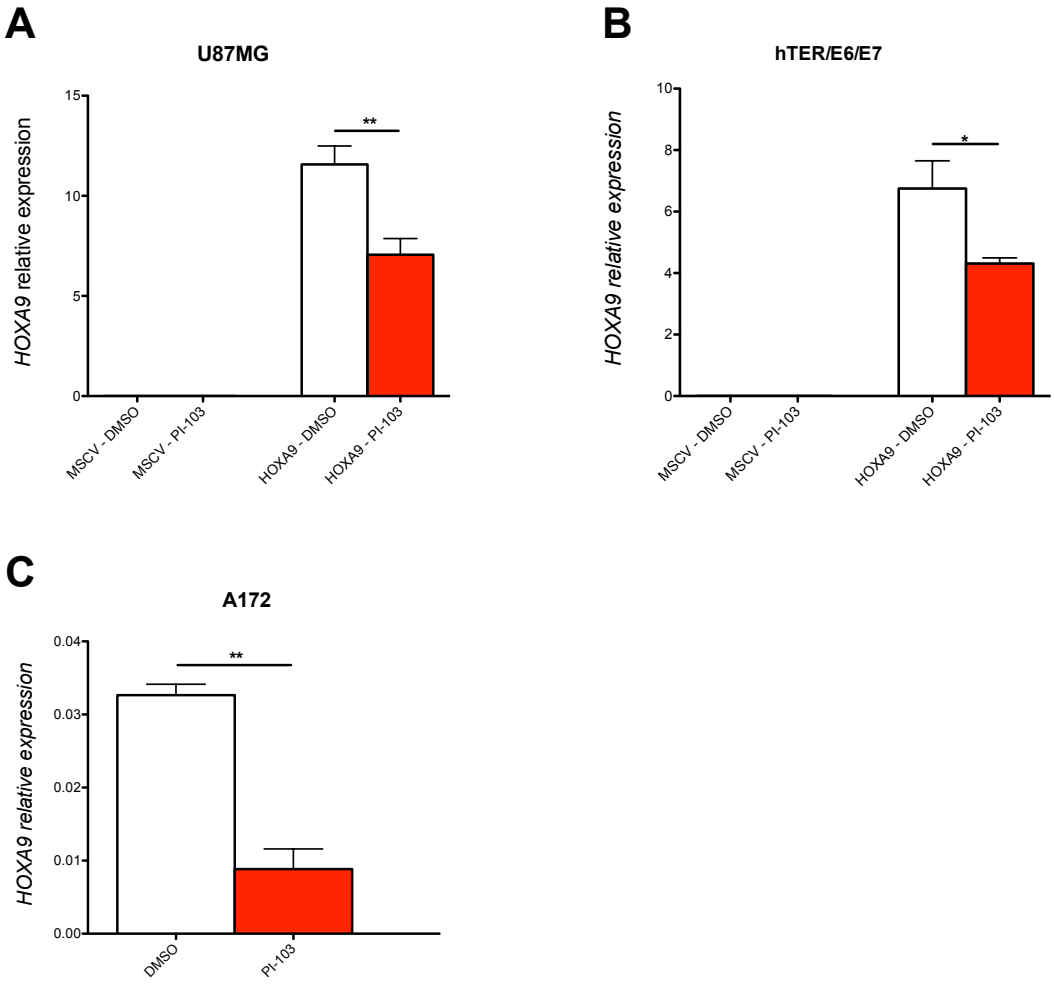

Figure 9. Suppression of PI3K activity reduces *HOXA9* expression. The effect of PI-103 on expression levels of *HOXA9* was determined by qRT-PCR for the (A) U87MG, (B) hTERT/E6/E7, and (C) A172. Effect of PI-103 compared with DMSO: **P* < 0.05; ***P* < 0.01.

Regardless the *HOXA9* expression have an endogenous or exogenous origin, the inhibition of PI3K/Akt pathway reduced its expression. Knowing this, it was investigated if it would affect ROS production. For this, the same experimental conditions for each cell line were kept, and intracellular ROS levels were measured in all the cell lines. Firstly, using the A172 cell line in which its known that *HOXA9* expression relies on PI3K/Akt pathway activation, it was showed that in the deprivation of *HOXA9* activation through PI3K/Akt signaling inhibition it impaired ROS production (Figure 10C, P = 0,0309). In the other cell lines with exogenous *HOXA9* expression it was observed that the inhibition of PI3K/Akt signaling did significantly increase the levels of ROS (P= 0,0376) in the U87MG-*HOXA9* cells

(Figure 10A), standing these cells with a major ROS upregulatory effect. On the contrary, the U87MG-MSCV (Figure 10A) and the hTERT/E6/E7-*HOXA9* (Figure 10B) cell lines did not show alterations in ROS levels after PI-103 treatment. It was also noted the significant ROS decrease in the hTERT/E6/E7-MSCV cell line (P = 0,0093).

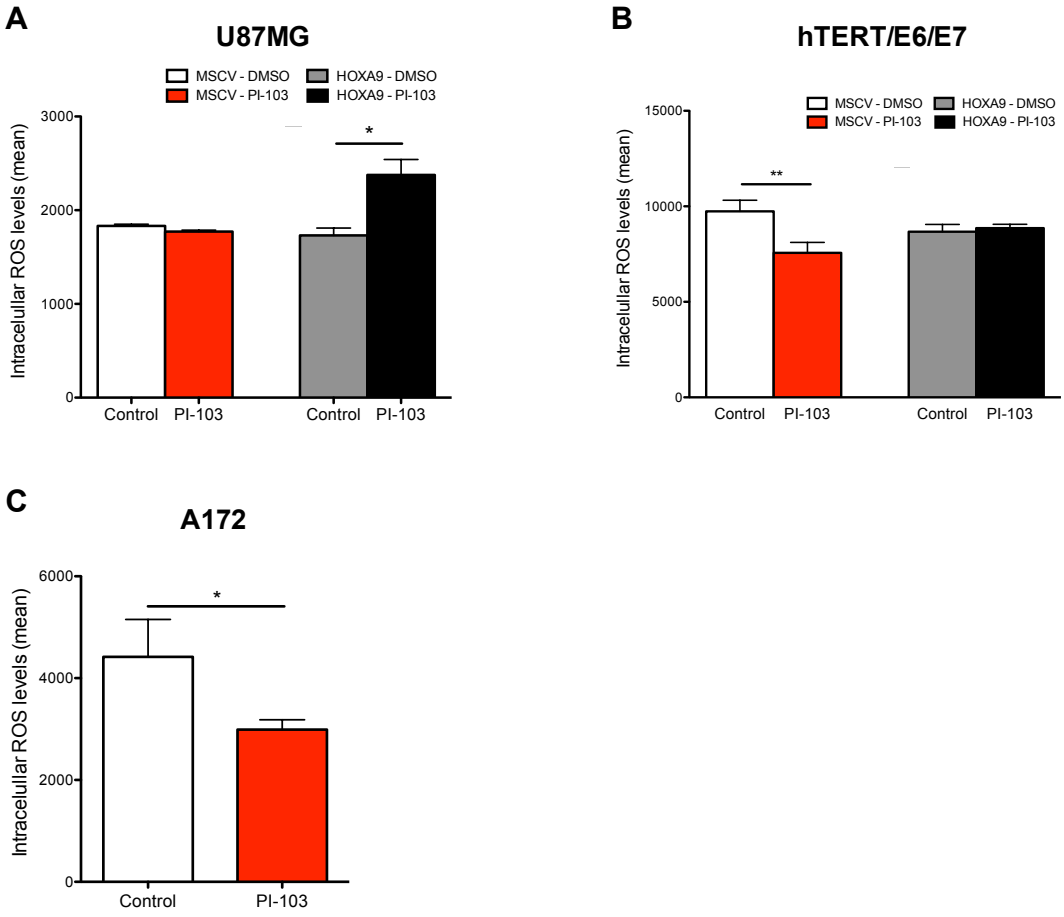

Figure 10. Exogenous *HOXA9* expression in U87MG GBM cells increases ROS production after PI-103 treatment. Total ROS production was measured in (A) U87MG, (B) hTER/E6/E7, and (C) A172 cell lines after PI-103 exposure. **P* < 0,05; ***P* < 0,01.

DISCUSSION

Due to the urge necessity to find biomarkers with a prognostic value for patients facing GBM disease, recent studies raised interested in the study of *HOX* genes, already shown to be abnormally expressed in GBM[31, 32, 33]. From all *HOX* genes, some from the cluster HOXA, especially *HOXA9* were reported to be overexpressed in GBMs[32]. Latter studies demonstrated that *HOXA9* aberrantly expression in GBM primary tumors is associated with shorter overall and progression-free survival for GBM patients[32]. It was also reported that the transcriptional activation of *HOXA9* in GBM is PI3K-dependent[32]. Given the extensively reported importance of PI3K pathway in cancer survival, recent studies associate this pathway with ROS production[105 - 108], indicating that it could be redox regulated. With the intent to deliver more understanding about *HOXA9* role in GBM, this work aimed to evaluate the effect of *HOXA9* regarding ROS production in GBMs.

In the U87MG GBM cells, it was found that cells with *HOXA9* overexpression produced ROS up to levels that were higher than the control cells lacking *HOXA9* expression. Cancer cells are well known to be under intrinsic oxidative stress[82, 83], which depending on the levels could act as a stimuli for promotion of proliferation, migration and invasion[49, 90]. In a previous study, *HOXA9* was established as a novel prognostic factor with negative value[32], most probably due to its capacity to enhance proliferation and evade apoptosis, where ROS can be contributing to this tumorigenic phenotype, by its elevated levels. To be true, this relation was found to be possible only in U87MG-*HOXA9* cells; their putative precursors, the human immortalized astrocytes, did not present an elevation of ROS generation even in the presence of *HOXA9*, which levels were intriguingly lower compared to the control cells. This result is indicative that *HOXA9* and the way it mediates the production of ROS is dependent of the cell lines and their inherent aggressiveness. Additionally to these findings it was important to assess the metabolism features of these cells lines. One of the reasons by which cancer cells rely their energetic demands on enhanced aerobic glycolysis, also known as Warburg effect[123], is to limit ROS levels[124] that can be harmful for the cells. This metabolic hallmark of cancer cells was extended to the current study, where the enhanced aerobic glycolysis presented by human immortalized astrocytes with *HOXA9* overexpression strongly supported the principles of Warburg effect; a higher lactate production that as been described to act as a ROS scavenger[125] is associated with a lower production of ROS. This analysis

also linked the high rates of ROS production in GBM-derived cells with *HOXA9* overexpression with their lower glycolytic status, suggesting a preference for the oxidative metabolic pathway. These evidences are indicative that *HOXA9* may be regulating cell metabolism in GBM cells towards mitochondrial phosphorylation in order to upregulate ROS production to achieve a major goal: continuous stimulation of pro-survival factor within survival pathways, like PI3K. Submitting the cells to oxidative stress by using an exogenous source of ROS, H_2O_2, was the next rational step with the purpose to look at their intracellular ROS behavior. As expected, both cell lines, with or without *HOXA9* expression presented an increase of ROS production after challenges with H_2O_2. The apparent non-alteration of ROS levels upon oxidative stress stimuli in both cell lines of the astrocytic lineage model is suggestive that these cells may have an anti-oxidant system widely activated. However, the main message is that *HOXA9* upregulates ROS production in GBM-derived cells. The *HOXA9* capacity to upregulate ROS in GBM cells, was found to affect cell viability; however, these cells present a resistance profile, which make them capable of recovering, even the presence of such oxidative stress. So, these evidences suggest that *HOXA9* overexpression confers resistance to oxidative stress in GBM cell line, and as previous mention this ROS are the way by which HOXA9 promotes cell proliferation and evasion to apoptosis. Is to discover the mechanism by which these cells resist to such oxidative stress. The literature often reports that cancer cells have higher levels of antioxidant enzymes[67] which balance ROS levels to levels that are tolerated by the cells, and that level vary from cell type to cancer type and aggressiveness. Opposite observations were made in the hTER/E6/E7 cell line, where *HOXA9* did not seem to play an important role in H_2O_2 resistance, which is probably the reason why this cells do not produce high levels of ROS due to the inability to tolerate excessive oxidative stress.

As previously described, in leukemia PI3K was found be responsible for elevated levels of ROS [102, 103, 105, 108]. Therefore, the final part of the project focused on investigating the effect of the inhibition of PI3K pathway on ROS production and ultimately, to unveil if *HOXA9*-mediated ROS production is independent of PI3K pathway. PI-103, a dual inhibitor of PI3K (upstream of Akt) and mTOR (downstream of Akt) has been reported to inhibit human glioma cells[126, 127]. Contrarily to reported in leukemia[108], PI-103 treatment and subsequent acceptable inhibition of PI3K signaling did not decrease ROS levels in U87MG-*HOXA9* cells; on the contrary, it increased ROS levels when compared to the control DMSO treatment group. The same was not valid for the U87MG-MSCV cell line, where PI-103 did not alter ROS production.

This result showed that ROS production by U87MG cell line seems to be independent of PI3K pathway, suggesting that *HOXA9* may have an outstanding role in this process. Further studies were needed to elucidate the *HOXA9* contribution for ROS generation in the pathway. To dissect this hypothesis, in the other way around, was used the A172 cell line with endogenous expression of *HOXA9*, proven to be dependent on PI3K/Akt signaling pathway for its transcriptional activation[32]. As theoretically expected, inhibition of the PI3K/Akt pathway decreased *HOXA9* expression and intracellular ROS. The results from the qRT-PCR for the cells with *HOXA9* overexpression revealed that *HOXA9,* even though exogenously expressed, it is somehow regulated by the PI3K or by PI-103; where after PI-103 treatment the *HOXA9* expression levels significantly decreased in both *HOXA9* overexpressing cell lines. A reasonable explanation for the reduction of *HOXA9* expression upon inhibition of PI3K/Akt pathway is that this pathway could be regulating *HOXA9* expression. By the fact that *HOXA9* expression was retroviral-induced, it does not exclude that its overexpression can not benefit from the PI3K regulatory activity. Actually, since this pathway regulates several other signaling molecules, it is reasonable to think that they could be afecting *HOXA9* expression trhough activating transcription factors that regulates *HOXA9* inside the retroviral construct. Another hypothesis that can deliver more understanding about this results, is the unknown effect of PI-103 on *HOXA9* expression. Apart from specific inhibition of PI3K isoforms but also of the phosphoinositide kinase family of proteins, there is not known the effect of PI-103 on other molecules; probably, PI-103 can interact with *HOXA9* by inhibiting it. At this point, its not possible to confirm the *HOXA9* role mediating ROS production in GBM cell lines independently of the PI3K pathway; however, these preliminary results suggest that *HOXA9* can be regulating ROS independently of PI3K/Akt signaling pathway, but dependently of cancer cell aggressiveness. Importantly, is the fact that *HOXA9* is implicated in ROS production in GBM. To discover are exact role of ROS mediated by HOXA9 in GBM.

CONCLUSIONS

This work reports the first study about the contribution of *HOXA9* in redox regulation of GBM disease and the contribution of PI3K pathway for ROS production in GBM. Establishing a rational in all the data from this thesis, it possible to conclude that the studied U87MG GBM cell line with *HOXA9* overexpression upregulated intracellular ROS, in basal and in oxidative stress conditions. This elevated oxidative stress translated into a moderated decrease of cell viability, but with the twist that these cells show a resistance phenotype by recovering their viability over the time. Not only, these cells are capable to resist to oxidative stress but also, under it they continuously presented elevated ROS levels. Maybe to promote cell proliferation through ROS production, *HOXA9* mediates a metabolic shift in GBM cells from aerobic glycolysis towards mitochondrial oxidative phosphorylation. We strengthened the role of *HOXA9* in ROS production, dependently or independently of PI3K pathway, in GBM context. We also deliver some additional information about the fact that *HOXA9* effects can be dependent on the cell context; the conjecture of characteristic malignancy and aggressiveness of the tumor cells may enhance *HOXA9* effect in ROS production. This opens new challenging opportunities to explore the relevance of ROS upregulation by *HOXA9* in the GBM resistance to therapy.

FUTURE DIRECTIONS

The results presented in this work favor future approaches to elucidate the role of *HOXA9* in ROS production in GBM.

Given the PI-103 results regarding *HOXA9* expression, it is essential to complement the data obtained by a direct targeting of *HOXA9*, other then using a chemical inhibition. Therefore, the silencing of *HOXA9* by siRNA will be performed in the A172 cell line to validate the contribution of *HOXA9* in ROS production, where we anticipate that silencing of *HOXA9* will decrease ROS production, and to assess the hypothesis whether *HOXA9* is the key mediator of ROS production in the PI3K pathway. Also, to known the impact of ROS production by *HOXA9* overexpression in GBM cells, we intent to treat cell lines with an antioxidant, for instance, glutathione or N-acetyl-cysteine, and assess the effects of *HOXA9*-mediated ROS effects on cell survival and proliferation.

Regarding the lower lactate production and therefore a less use of glycolysis by U87MG-*HOXA9* GBM cell line, which probably reflects a high oxidative phosphorylation rate associated with the increase of ROS, further studies are needed to confirm the preference of *HOXA9* positive cells for the oxidative pathway. To assess this, it will be evaluated the expression at the protein level of key glycolytic and oxidative phosphorylation proteins, such as lactate dehydrogenase, pyruvate dehydrogenase, pyruvate dehydrogenase kinase, and proteins from the mitochondrial ETC. From these studies, it will be also evaluated if mitochondrial ETC proteins, mainly from the complex I, act as a source of ROS production mediated by *HOXA9* overexpression. Additionally, a chemical inhibition of this complex I by rotenone will allow to confirm the relationship between mitochondrial ROS mediated by *HOXA9* expression.

Although *HOXA9* overexpressing GBM cells appear to upregulate ROS, it is unclear the physiologic functions of these ROS: whether they act as pro-proliferative or pro-apoptotic stimulus. To assess this, it will be evaluated the expression status of pro-apoptotic proteins such as BAX, caspase – 3, – 7 and – 9, and anti-apoptotic proteins like Bcl-2 and PARP. Independently of the conclusions about ROS functions, it is necessary to discover the reasons behind the resistance mediated by *HOXA9* in GBM cells. The most probable hypothesis is that such resistance could be

due to the antioxidant system, which limit deleterious levels of oxidative stress. To bridge this gap it will investigate the antioxidant enzymes, such as SOD, glutathione and peroxiredoxin that are expected to be protecting cells from excessive ROS-associated damage that could lead to cell death. Maybe, *HOXA9* is regulating the antioxidant system in order to act when ROS levels are exceeded until beneficial ROS levels to these cells are restored. If valid, this could represent other studies in order to assess the therapeutic benefit of the inhibition of antioxidant enzymes.

Since chemoresistance is a severe limitation to TMZ therapy and that TMZ methylates nuclear DNA and induces apoptotic cell death through unbalanced ROS production, it will be evaluate the potential involvement of *HOXA9* in the response of GBM to ROS-inducing agents, particularly TMZ.

It is expected that together, the proposed complementary studies will greatly extend insights of the redox regulation mediated by *HOXA9* and its impact in GBM.

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APPENDIXES

APPENDIX I

WESTERN BLOT

Preparation of cell lysates and protein quantification

After removing the cells from the flasks by using a scrapper, cells where washed twice in cold PBS by two consecutive centrifugations at 900 rpm for 5 minutes at 4ºC, with subsequent PBS discard. Then cell lysates were prepared by solubilizing cells in ice-cold lysis buffer (to 100 µl of lysis buffer 1x (50 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA) was added 1 µl of 100 mM PMSF, 3 µl of proteinase inhibitor 25 x and 1 µl of Nonidet®P40) at approximately 0,5 ml per $5x10^7$ cells/60mm dish/75cm^2 flask. During the 1 hour incubation, the cell suspension was vortexed 3 times, followed by centrifugation at 13,000 rpm for 10 minutes at 4ºC. The supernatant, which contains the cell lysate, was transferred to a new tube and the pellet was discarded.

The total protein concentration of the samples was measured by using the Bradford Reagent (Bio-Rad). In polystyrene cuvettes were added 1 µl lysates samples plus 200 µl Bradford Reagent and 800 µl of ultra-pure water and incubation was allowed for 5 minutes at RT. After blank the spectrophotometer with the lysis buffer (1 µl lysates samples plus 200 µl Bradford Reagent and 800 µl of ultra-pure water) at 595 nm, the samples absorbance was read. The protein concentration of the sample is determined by comparison with the values from a standard curve of absorbance. An equal volume of 4X Laemmli buffer was added to all samples (30 µg of protein). Prior to protein separation by electrophoresis, the samples were boiled for 5 minutes.

SDS-PAGE

After the Western Blot system was prepared with confirmation that the system was watertight, the preparation of the gels, resolving and stacking gel was carried

out. Immediately after of gel preparation was added 50 µl of APS and 5 µl of TEMED to the 5 ml of resolving gel (10%), and the final mixture was placed inside of the glass frame. Water was added on top of the resolving mixture and discarded when the mixture reaches full polymerization. Next, the 20 µl of APS and 2 µl of TEMED to the 2 ml of stacking gel (4%), and the mixture was placed on the top of the resolving gel. The desired combs were placed on the top of the stacking gel. After complete polymerization, the structure that contains the gel was transferred to the running tank covered by the cold running buffer. Samples and 5 µl of protein ladder (Novex® Sharp Protein Standard, from Invitrogen) were loaded into the wells of the gel, followed by the initial electrophoresis run at 80v until the samples reached the resolving gel (approximately 20 minutes). Than, the voltage was increased to 100v until the bromophenol blue in the samples reaches the bottom of the gel (approximately 1 hour).

Semi-Dry Transfer

After protein separation, the gel content was transferred to a nitrocellulose membrane. The transfer materials were stacked in the following order: 3 Whatman papers (8x6cm) + membrane (8x6cm) + gel + 3 Whatman papers (8x6cm) on top of the semi-dry transfer system; all pre-wet in cold transfer buffer for 5 minutes. The proteins were transferred for 1 hour at 12v. After transfer, the membrane was stained with 0.1% Ponceau S for 1-2 minutes to visualize protein bands. Ponceau S was removed with cold PBS until the bands disappear.

Protein blotting

Unspecific protein receptors where blocked in 5% non-fat dry milk dissolved in PBS-Tween 20 (0.1%). Incubation was performed for 1 hour with agitation. Afterwards, the membrane was incubated overnight with the primary antibody at 4ºC (2.5 % milk in PBS-T for total Akt and 2.5 % milk in TBS-T for p-Akt). After incubation, the blocking solution was removed from the membrane by washing 3 times for 10 minutes with agitation in 1% milk in PBS/TBS-T, followed by the incubation with secondary antibody anti-mouse (2.5% milk in PBS/TBS-T) for 1 hour at RT. Additional 3 washes with PBS/TBS-T with agitation was executed, changing the wash buffer every 10 minutes.

The membrane was exposed to 400 µl of detection solution SuperSignal ®West Femto or Pico (Thermo Scientific), and antibodies were then visualized using

the Chemidoc XRS software (Bio-Rad). The same procedure was performed to the control protein, α-tubulin.

RNA ISOLATION FROM CELL CULTURES (TRIZOL)

Homogenization

Pellet cells by centrifugation at 900 rpm at 4ºC for 5 minutes with 2 subsequent wash in cold PBS. Per each 5-10 x 10^6 of cells was added 1 ml of the TRIZOL Reagent and allowed cell lysis by repetitive pipetting (pipet up and down several times.). The total volume was transferred to a 1,5 ml tube.

Phase Separation

In order to achieve complete dissociation of the nucleoprotein complexes, the samples were vortexed for 1 minute followed by incubated for 5 minutes at room temperature (RT). A volume of 0,2 ml of chloroform was added (0,2 ml of chloroform per 1 ml of TRIZOL Reagent), and the samples were hand shaken vigorously until a turbidity of the color pink was observed (approximately 15 seconds). After incubation of 5 minutes at RT, the samples were centrifuged at 13,000 rpm for 15 minutes at 4ºC. The centrifugation allowed the mixture to separate into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase, where it was transferred to a fresh tube, and stored at 4ºC.

RNA precipitation

The RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol (0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization). Precipitation was carried out during 10 minutes incubation at RT. Next, the samples were centrifuged at 13,000 rpm for 10 minutes at 4ºC. (The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube). The supernatant was completely discarded.

RNA wash

The RNA pellet was washed once by adding 1 ml of 75% ethanol (1 ml of 75% ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization). The samples were mixed by vortexing (10 seconds) and centrifuged at 8,600 rpm for 5 minutes at 4ºC. The supernatant was removed.

RNA quantification

At the end of procedure, it was ensured that the RNA pellet was air-dried for 10 minutes in an open tube in order not to affect its solubility. Finally, RNA was dissolved in 20-50 µl RNase-free water (dependent on RNA pellet) by passing the solution a few times through a pipette tip. RNA quantification for measuring its concentration and purity was carried out in NanoDrop ND-1000 spectrophotometer (Invitrogen).

cDNA SYNTHESIS

The cDNA synthesis from cell lines' RNA was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). To 10 µl of reverse transcription master mix (2 µl of 10× RT buffer, 0,8 µl of 25× dNTP mix (100 mM), 2 µl of 10× RT random primers, 1 µl MultiScribe™ reverse transcriptase and 4,2 µl of nuclease-free H_2O) was added 10 µl of RNA sample (2 µg of total RNA per 20 µL reaction). A total of 20 µl final volume was obtained, which was submitted to posterior synthesis. The thermal cycler conditions were as follows: primer extension at 25ºC for 10 minutes, cDNA synthesis at 37ºC for 120 minutes, reaction ending at 85ºC for 5 minutes and cooled at 4ºC. After synthesis, the 20 µl of cDNA were stored at -20ºC.

***HOXA9* RT-PCR**

For technical control of *HOXA9* overexpression in the retroviral infected cell lines, its expression was assess by RT-PCR. For 1 µl of cDNA sample a mix of 2.5 µl of AmpliTaq Gold Buffer 10 x, 1.5 µl of MgCl2 at 25 mM, 0.5 µl of dNTP mix at 10 mM, 1µl of HOXA9 primers (20 mM) (forward 5' – GCCCGTGCAGCTTCCAGTCC – 3' and reverse 5' – GAGCGCGCATGAAGCCAGTTG – 3'), 0.2 µl of Amplitaq Gold

enzyme and, water to a final volume of 25 µl was added. Samples were amplified in a thermal cycler with the following conditions: 4 minutes at 95ºC, 35 cycles of (30 seconds at 94ºC, 30 seconds at 61ºC, 30 seconds at 72ºC), and 8 minutes at 72ºC. Finally, RT-PCR products were resolved 2% of agarose gel, and the bands quantified using Alphaimager software (Alpha Innotech Corporation).

APPENDIX II

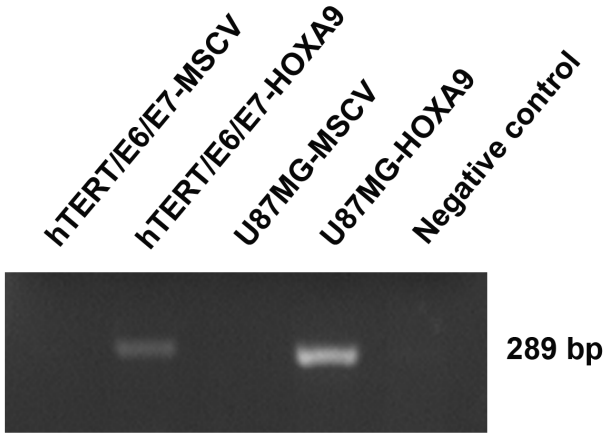

Figure 11 - RT-PCR control for *HOXA9* expression in engineered hTERT/E6/E7 and U87MG cell lines. Both hTERT/E6/E7-HOXA9 and U87MG-HOXA9 cell lines show HOXA9 expression (289 bp band), while their respective control cells, hTERT/E6/E7-MSCV and U87MG-MSCV, do not show any visible band. Water was included for the negative control.