Contribution of 3q26-29 gene cluster to glioma invasion

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SUMMARY

Diffuse gliomas are the most common and lethal brain tumors. Cell invasion into the surrounding brain tissue is a hallmark feature of glioma. Understanding the mechanism of glioma invasion could lead to the discovery of novel therapeutic strategies to treat affected patients. Earlier gene expression analyses on human glioma biopsies showed that *SOX2* is associated with glioma invasiveness. The gene for transcription factor SOX2 localizes to 3q26.3 in the human genome amid oncogene *PIK3CA* and genes regulating mitochondrial fusion, *MFN1* and *OPA1*. Increasing evidence points to a role for 3q26-29 genes in tumor invasion. We hypothesized that SOX2 regulates the 3q26-29 candidate genes as effectors of glioma cell invasion.

We used SOX2 expressing human glioma cell lines, LN319 and U373 to test our hypothesis *in vitro*. Lentiviruses expressing shRNAs against *PIK3CA*, *MFN1*, *OPA1* or *SOX2* were used for genetic knockdown. Engineered cells were assayed for invasion and migration using Boyden chamber and wound healing assays, respectively. Chromatin immuno-precipitation and luciferase assays were used to demonstrate protein-DNA interactions and trans-activation of 3q26-29 genes by SOX2.

Our results show that cells downregulated for 3q26-29 genes exhibited enhanced invasion and migration, while sh*SOX2* and sh*PIK3CA* cells exhibited reduced proliferation rates compared to sh scramble controls. Furthermore, we show that SOX2 knockdown reduced gene and protein expression of *PIK3CA*, *MFN1* and *OPA1* except for *PIK3CA* at the protein level. Chromatin immuno-precipitation assays suggested that SOX2 binds to the upstream region of 3q26-29 gene promoters in the glioma cells.

Preliminary luciferase assays in HEK293 cells suggested that SOX2 trans-activates *PIK3CA* and *OPA1.* Preliminary immunofluorescence analysis showed that cells knocked-down for 3q26-29 genes demonstrated altered mitochondrial morphology compared to sh scramble controls. Overall, our results show that *SOX2*, *PIK3CA*, *MFN1* and *OPA1* contribute to glioma invasion and that SOX2 is a potential regulator of the 3q26-29 genes.

Abbreviations

ATP- Adenosine Tri Phosphate

CDKN – Cyclin Dependent Kinase

cDNA – complementary DNA

ChIP – Chromatin Immunoprecipitation

CNS – Central Nervous System

CSF – Cerebro Spinal Fluid

DNA – Deoxyribonucleic Acid

ECM – Extra Cellular Matrix

EGFR- Epidermal Growth Factor Receptor

EMT – Epithelial-Mesenchymal Transition

ETC – Electron Transport Chain

EU – European Union

FACS – Fluorescence Activated Cell Sorting

GBM – Glioblastoma Multiforme

GM – Gray Matter

GSC – Glioma Stem Cell

HEY1 – Hairy/Enhancer of split-related protein (with YRPW motif) 1

IDH – Isocitrate Dehydrogenase

IFU – Infectious Units (viral)

IMM – Inner Mitochondrial Membrane

IZ – Infiltration Zone

KD – Knockdown

LGG – Low-grade glioma

MFN1 – Mitofusin 1

MRI – Magnetic Resonance Imaging

mRNA – messenger RNA

mTOR – mammalian Target of Rapamycin

NICD- Notch Intracellular Domain

NSC – Neural Stem Cell

OMM – Outer Mitochondrial Membrane

OPA1 – Optic Atrophy 1

OXPHOS – Oxidation Phosphorylation

PI3K – Phosphoinositide 3 kinase

PIK3CA- PI3K catalytic subunit alpha

PTEN – Phosphatase and Tensin homolog

RB – Retinoblastoma

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

RSC – Respiratory Super Complex

RTK – Receptor Tyrosine Kinase

sh RNA – short hairpin RNA

SOX2 – Sex determining region Y-Box 2

TCA – Tri Carboxylic Acid

TCGA – The Cancer Genome Atlas

TGFβ – Transforming growth Factor Beta

TMZ – Temozolomide

TP53 – Tumor Protein 53

UV – Ultra-violet

VEGF – Vascular Endothelial Growth Factor

WHO – World Health Organization

WM – White Matter

1. INTRODUCTION

1.1. CANCER

Cancer is a leading cause of death from disease in the world. Recent estimates, made in the time window of 2009-14 and published by World Health Organization (WHO), reveal that nearly 14.1 million people worldwide are diagnosed with cancer annually and that an estimated 8.2 million die of the disease per year (WHO, 2014). In Switzerland alone, 37,000 people are diagnosed with cancer and nearly 16,000 of them die every year (Krebsliga, 2014).

Cancer incidence is growing exponentially and is predicted to increase by 70% in the next two decades. Such steep increase in cancer incidence, apart from demographic regional population and diet choice, is largely attributed to exposure to toxic environmental factors, and genetic susceptibility of an individual to the disease. Growing trends of cancer incidence and cancer-related morbidity dictates the need to focus on methods to improve cancer prevention and therapeutics.

1.1.1. Causes of cancer

Epidemiological studies show that many factors contribute to cancer development. Unhealthy diet, exposure to carcinogens such as high doses of ionizing radiation or UV, chemicals contained in tobacco smoke, and chronic infections caused by biological agents such as viruses or bacteria are well known inducers of cancer.

Genetic association studies revealed that a portion of population carries damaged or mutated copy of genes, which increases the risk of developing cancer. For instance, Li-Fraumeni syndrome is a rare hereditary cancer pre-disposition syndrome that is linked to germline mutations of *TP53*, a tumor suppressor that regulates cell cycle progression and induces suicide of damaged cells.

1.1.2. Sustained proliferation

Uncontrolled cellular proliferation is a fundamental process that contributes to tumor formation and progression (Feitelson et al., 2015; Hanahan D, 2011). Deregulated anti-proliferative signals and oncogenic mitotic stimuli promote sustained tumor cell proliferation. In neoplastic cells, constitutive proliferative signals can be activated by multiple ways: (i) unchecked constant activation of growth factor receptors through auto- and paracrine signaling in neoplastic cells, (ii) somatic mutations in mitogenic signaling determinants such as receptor tyrosine kinase (RTK), *B-raf*, and *PIK3CA* (iii) disruption of anti-proliferation signaling proteins such as Ras GTPases, PTEN phosphatases and mTOR kinases (iv) overexpression of telomerase (amplification of *TERT* and/or *TERC*, catalytic and RNA component of the reverse transcriptase enzyme respectively), that maintains sufficient telomere length for constant cell division and induce replicative immortality.

1.1.3. Malignant transformation

Stepwise accumulation of molecular changes eventually leads to cancer cell evolution towards a malignant state. Neoplastic molecular changes include loss of cell-to-cell contact inhibition resulting in increased cell proliferation and motility, enhanced production of growth factors that promote tumor vascularization (TGFβ and VEGF), loss of tumor suppressors (*TP53* and *RB*), and deregulated apoptotic and autophagic pathways. Malignant transformation (MT) is hence characterized by tumor cell invasion that most often leads to the formation of secondary tumor at distant organs within the body (metastasis), tumor angiogenesis, and resistance to therapeutic agents.

Excessive activation of proliferative signals in a hypoxic environment induces cell senescence and promotes necrosis (outbursts of bloated and enlarged neoplastic cells). Necrosis triggers pro-inflammatory responses, which in return enhances the secretion of pro-proliferative signals and growth factors in the tumor. Thus, necrosis and hypoxia contribute to the acceleration of tumor malignancy (Hanahan D, 2011). Moreover, enhanced secretion of immuno-suppressive proteins by tumor cells such as the growth factor TGFβ and immuno-suppressive acidic protein (IAP), disrupt the

function of host immune cells. This is one of the strategies that neoplastic cells use to escape detection by host immune cells and evade host immune surveillance.

Overall, genome instability and altered cellular metabolism contribute to neoplastic transformation. Thus, sustained cell proliferation, inflammation and evasion of host immunological responses, tumor cell invasion, angiogenesis, and resistance to therapy-induced apoptosis are features of tumor progression (Hanahan D, 2011).

1.1.4. Cancer prevention

Avoiding exposure to carcinogens including high-energy radiation, maintaining healthy diet, and early diagnosis are keys to cancer prevention. In some developed countries, cancer-related mortality rates have started to decline as a result of improved early cancer detection, existence of advanced cancer therapy centers, and reduced smoking (WHO, 2014).

1.1.5. Detection

Screening tests in patients without symptoms such as the pap smear test for cervical cancers and the mammography for breast cancers, allow early detection and greatly increase the success of cancer treatment. Preventive surgery in patients that are genetically susceptible to cancer for instance by prophylactic mastectomy in individuals with mutations in breast cancer-causing genes (*BRCA1* and *BRCA2*) greatly reduces the risk of developing breast cancer.

Magnetic resonance imaging (MRI) of body tissue is an effective means of tumor detection. Diagnostic surgery i.e. surgical resection of potential tumor tissue, followed by microscopic examination helps to detect the tumor and determine its malignancy index.

1.1.6. Conventional therapies

Currently, the majority of cancer therapies consist of surgical resection of tumor followed by adjuvant chemo- and radiotherapies. Chemotherapy uses chemical drugs while radiotherapy use high doses of ionizing radiation to kill proliferative cells.

Unfortunately, the primary cause of cancer-related morbidity in the patients is the relapse of therapy-resistant tumors.

1.1.7. Novel therapies

Growing knowledge on cancer progression shows that accumulation of genetic mutations triggers cancer. Identification of genetic mutations and gene signatures that define the cancer type has revolutionized the field of cancer research. These advances have led to the development of novel molecular therapies that target a specific molecular pathway. For instance, the kinase inhibitor erlotinib specifically targets epidermal growth factor receptor (EGFR) that shows a gain of function mutation and is highly expressed in many cancer types. Despite these efforts, most of the cancer types do not have a permanent cure.

1.2. GLIOMA – CLINICAL FACTS

1.2.1 The glial lineage

The human brain is made up of neurons and glial cells. Cells of glial lineage represent more than 80% of the brain cells. Glial cells secrete myelin that surrounds and insulates neurons, and protects them from cytotoxic injury. In addition, glial cells maintain homeostasis and are scavengers for dead neurons.

Three different types of glial cells are known in the central nervous system (CNS): (i) Oligodendrocytes, cells that produce myelin; (ii) Astrocytes, which are a heterogeneous group of cells involved in blood brain barrier (BBB) formation; (iii) Ependymal cells, that are involved in the production of cerebrospinal fluid (CSF).

Tumors that arise from cells of glial origin are called glioma. They comprise a broad group of tumors that represent almost 30% of all brain tumors including brain metastasis, and 80% of all malignant tumors of the central nervous system (CNS) (Alcantara Llaguno et al., 2009). Gliomas are among the most aggressive and the most lethal CNS tumors (Grier and Batchelor, 2006; Louis et al., 2007).

1.2.2. Histology

Based on the cellular morphology of gliomas, the tumors can be classified into:
(i) Oligodendrogliomas, tumors that primarily consist of neoplastic cells with oligodendroglial morphology; (ii) astrocytoma, tumors in which neoplastic cells show astrocytic morphology; (iii) mixed gliomas are those that display both above mentioned cellular morphologies; (iv) ependymomas that consist of neoplastic cells with ependymal morphology; (v) Glioblastoma multiforme (GBM) includes undifferentiated and a heterogenous population of neoplastic glial cells.

1.2.3. Glioma grading

WHO-based classification of glioma ranges from low grades (I and II) to high grades (III and IV) for the most malignant forms. Glial tumor grading is based on pathologic features such as mitotic index, necrosis, extent of differentiation, and vascular endothelial hyperplasia (Louis et al., 2007). Different grades of glioma are shown in Fig 1.

Grade I is applied to tumors that show low proliferative potential, and are often curable by surgical resection. Pilocytic astrocytomas that develop in children and young adults belong to this category.

Grade II is assigned to gliomas that are infiltrative in nature despite low proliferative capacity. They gradually progress to higher grades and tend to relapse after surgery. Diffuse astrocytomas, oligodendrogliomas, oligoastrocytomas belong to this grade.

Grade III gliomas have histological evidence of malignancy such as abnormal nuclei and high mitotic index. Anaplastic or poorly differentiated astrocytomas, oligodendrogliomas and oligoastrocytomas belong to this category.

Grade IV gliomas show high microvascular proliferative density, are mitotically active, necrosis-prone, and cytologically malignant with a high degree of cell heterogeneity. GBM is synonymous of grade IV glioma and is the most aggressive form of glioma.

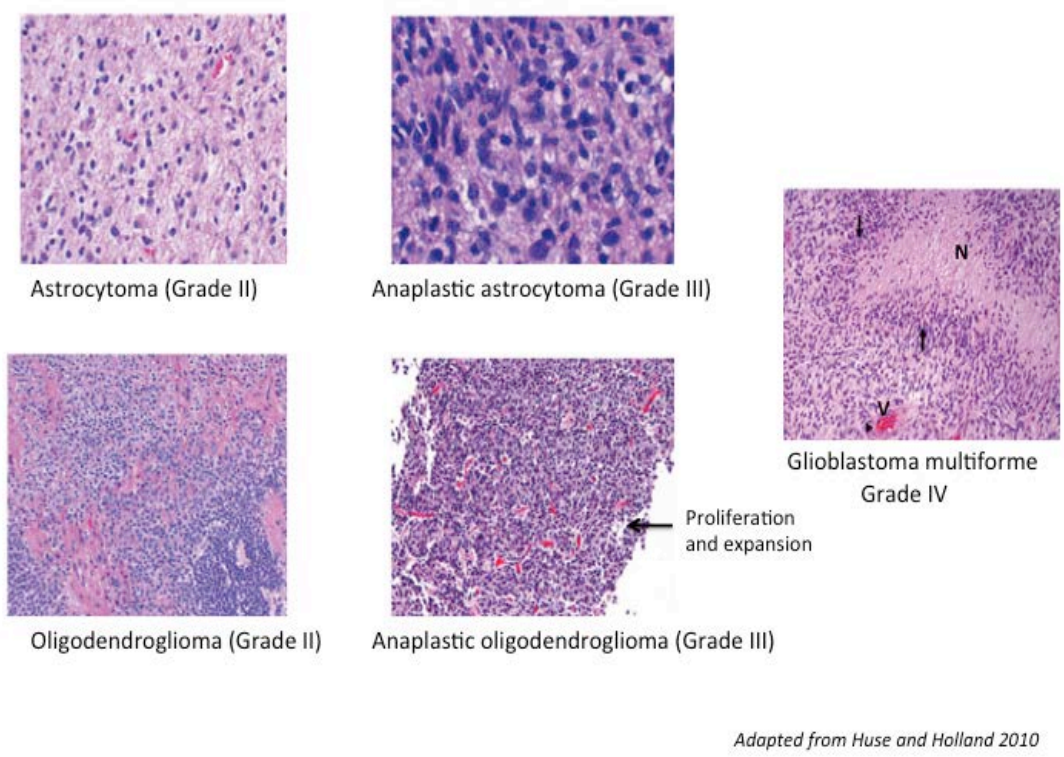


Fig 1. Schematic representation of histology based WHO grade (II-IV) diffuse glioma classification. Grade II gliomas undergo slow progression to anaplastic grade III lesions that subsequently gives rise to secondary GBM. Shown are hallmark features of GBM such as microvasculature proliferation (marked V and indicated by black arrowhead) and pallisading necrosis (marked N).

1.2.4. Incidence

In Switzerland, with an annual incidence of 3.55 cases per 100,000 patients, high-grade GBM are more frequent than low-grade glioma (LGG), with an incidence of 0.63 per 100,000 patients (Ohgaki, 2005).

In Switzerland, association studies of tumor histology with the sex of individual patients revealed that anaplastic oligoastrocytomas and GBM are more common in men: the male to female ratio being 3.3 and 1.34 respectively (Ohgaki, 2005). LGG is more common in younger patients between the age group of 20 and 40 years. GBM is common in individuals in the age group of 45 to 70 years.

1.2.5. Major risk factors

Exposure to high dose of ionizing radiation and inherited genetic associations are the only known risk factors for glioma (Braganza et al., 2012; Edick et al., 2005; Ohgaki, 2005). Hereditary associations represent only a small percentage (5%) of gliomas. These genetic associations include neurofibramatosis syndrome (*NF1-2* mutation), tuberous sclerosis, retinoblastoma (*RB* mutation), Li-Fraumeni syndrome (*TP53* mutation), Cowden syndrome (*PTEN* mutation), and Turcot's syndrome (mutations in *adenomatous polyposis coli* (*APC*)) (El-Zein et al., 2002).

1.2.6. Signs and symptoms

Symptoms of a brain tumor depend on the location of the tumor in the brain. The most common symptoms associated with gliomas are seizures, neurological deficits – behavior and personality changes, and increased intracranial pressure.

1.2.7. Etiology

Gliomas arise from glial precursor and neural stem cells (Swartling et al., 2012). Growing evidence suggests that genetic transformation of glial and neural precursor cells lead to *gliomagenesis* (Ilkanizadeh et al., 2014; Lei and Canoll, 2011; Sugiarto et al., 2011; Zong et al., 2012). This will be further discussed in section 1.4.5.

1.2.8. Patient survival

In addition to the age at the time of diagnosis, survival time for patients with glioma is chiefly influenced by the histology and the extent of surgical resection of the tumor. Patients with grade I glioma are most often cured by surgical resection of the tumor. Patients with LGG (especially oligodendrogliomas and oligoastrocytomas) can survive up to ≈ 20 years.

GBM that arise *de novo* are primary GBM, while those that arise due to progressive transformation of LGG are secondary GBM. Patients with primary GBM do not survive more than 24 months after diagnosis; however patients with secondary GBM

show longer median survival of over 4.7 months than primary GBM patients (Ohgaki et al., 2013).

1.2.9. Current therapies and treatment

Volume of tumor resected is of high prognostic value for patient survival (Barker et al., 1998; Hess, 1999; McGirt et al., 2008; Steiger et al., 2000). Therefore, surgical resection of the tumor followed by adjuvant chemo- and radiotherapy are opted to treat the disease (Stupp et al., 2005),

1.3. GLIOMA – BIOLOGICAL FEATURES

Somatic mutations found in glioma mostly target molecular pathways that govern cell proliferation, migration, invasion, angiogenesis, and survival. Consequently, the hallmarks of glioma are enhanced tumor proliferation, infiltrative behavior, tumor angiogenesis, and presence of therapy-resistant cells, along with altered cellular metabolism. The following sections of the thesis address each of these features in detail.

1.4. PROLIFERATION

1.4.1. Molecular genetics of GBM proliferation

1.4.1.1. Genes contributing to cell proliferation

Extracellular ligands such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF) play an important role in stimulating the growth and survival of neural and non-neural cells of the CNS. These growth factors bind to receptor tyrosine kinases (RTK) to transduce mitogenic signals in the cells. The genes for RTK such as *EGFR* and *PDGFR* are frequently altered in glioma.

Gain of function amplification of *EGF receptor (EGFR)* with deletion of exons 2 to 7, known as variant 3 (vIII *EGFR)* was first observed in glioma (Libermann et al., 1985). Focal amplifications of *EGFR* (locus 7p12) have been frequently found in primary GBM (36-57%) and very rarely in secondary GBM (0-8%) (Quezado et al.,

2005; Watanabe et al., 1996; Hegi et al., 2011; Ohgaki and Kleihues 2013). A gain of function mutation or amplification of *EGFR* leads to permanent activation of the PI3K/Akt pathway for continuous glioma cell proliferation (Ekstrand et al., 1992; Narita et al., 2002).

Gain of function mutations of PDGF receptor alpha (*PDGFRα*) (locus 4q12) is found in over 10% of GBM samples (The Cancer Genome Atlas Research Network, 2008). The deletion of exons 8 and 9 in *PDGFRα* gene are commonly observed events that result in constitutive activation of the receptor that induces tumor cell proliferation and survival (Clarke and Dirks, 2003).

Loss of function mutation and deletions in *Phosphatase and Tensin homolog (PTEN)* are frequent events observed in over 41% of GBM samples (Brennan et al., 2013). *PTEN* (locus 10q23.3) encodes for Ptdlns (4,5) P_3 phosphatase, an antagonist of Phosphoinositide 3-kinase (PI3K) enzymes. The tyrosine phosphatase is a negative regulator of PI3K/AKT signaling pathway therefore acts as a tumor suppressor.

Gain of function mutations in *PIK3CA* the gene encoding p110 catalytic subunit of PI3K enzyme, is observed in over 15% of GBM samples (Gallia et al., 2006; Samuels et al., 2004). The gene for PIK3CA (**P**hosphatidyl **i**nositol 4,5 bisphosphate **3 k**inase, **c**atalytic subunit **a**lpha) is located at 3q26 in the human genome. PI3K enzymes are heterodimeric lipid kinase that is composed of p110 catalytic and p85 regulatory sub units. PI3K enzymes are the mediators of the PI3K signaling pathway. The PI3K signaling pathway is activated by various RTK such as EGFR, PDGFR and MET. Once activated, the phosphatidylinositol 3,4,5 bisphosphate (PIP_3) acts as a second messenger to transduce the signal downstream and activate AKT and mTOR thus regulating numerous cellular functions such as cell growth, survival, transformation, motility, and apoptosis (Cantley, 2002; Volinia et al., 1994). Gain of function mutation in *PIK3CA* and loss of *PTEN*, therefore leads to permanent activation of PI3K pathway in glioma cells (Knobbe and Reifenberger, 2003; Ohgaki et al., 2004). The PI3K signaling pathway is one of the most frequently altered pathway in glioma(Fig. 2).

1.4.1.2. Genes regulating cell cycle progression

The Retinoblastoma (RB) pathway largely transduces growth inhibitory signals from extra-cellular sources and governs cell cycle progression that prevents replication of cells with damaged DNA. Members of E2F family of transcription factors, regulate genes involved in cell cycle progression and DNA replication (Macaluso et al., 2005). Under un-stimulated conditions, hypo-phosphorylated protein RB binds to E2F factors and prevents them from trans-activating genes required for cell cycle progression. Mitogenic stimulation activates cyclin D1 and dependent kinases CDK4 and CDK6, which in turn phosphorylates RB and relieves E2F factors. E2F factors then trans-activate genes required for early S phase entry and cell cycle progression (Aktas et al., 1997; Frolov and Dyson, 2004; Lavoie et al., 1996; Weinberg, 1995). Hence, RB acts as a checkpoint during G1-S transition of cell cycle. In gliomas, frequent mutations (7.6%) in *Rb1* gene (located on chromosome 13q14), amplification of *CDK4* located on chromosome 12q13-14, low-level amplifications *CDK6* located at 7q21-22, and homozygous deletion of *CDKN2A* at the locus 9p21 is the most frequent alteration of this pathway These mutations favor cell cycle progression (Costello et al., 1997; James et al., 1988; Jen et al., 1994; Merlo et al., 1995; Reifenberger et al., 1994; Schmidt et al., 1994).

1.4.1.3. Genes regulating cell survival

TP53 (tumor suppressor protein 53) encodes a transcription factor TP53 that trans-activates >2500 target genes. *TP53* is well characterized for its role in maintaining genome integrity and is known as “the guardian of the genome”. The TP53 pathway receives inputs from intra-cellular sources such as cellular stress and abnormal microenvironment (sub-optimal levels of nucleotide pools, growth promoting signals, or oxygenation) and induces apoptosis or cell cycle arrest in cells with unstable genome. Under normal conditions, TP53 remains inactive by binding to its negative repressor and TP53 ubiquitin ligase, mouse double minute homologue 2 (MDM2). During oncogenic stress the association between MDM2 and TP53 is disrupted by protein $p14^{ARF}$, encoded by the gene *CDKN2A*. This leads to TP53-mediated transcriptional activation of downstream target genes involved in apoptosis, for instance members of Bcl2 family. Thus, TP53 acts as a tumor suppressor by regulating programmed cell death in cells with unstable genome (Miyashita et al.,

1994; Pomerantz et al., 1998; Stott et al., 1998). In gliomas however, *TP53* (located on chromosome 17p) is mutated or lost in over 60% of LGG and in 28% of GBM samples, which provides survival advantage to glioma cells (Ohgaki and Kleihues, 2007; Brennan et al., 2013). Most of the cancer cells experience strong selective pressure to disable TP53 activity. In fact, *TP53* mutation is considered as an early event during glioma progression (Fridman and Lowe, 2003; Louis, 1994; Meyer-Puttlitz et al., 1997; Ohgaki and Kleihues, 2007). The chromosomal region 12q14-15 and 1q32 harboring *MDM2* and *MDM4* (*MDM2-related gene*) are found to be amplified in 4-10% of GBM, which strengthens TP53 repression and thus supports cells to escape TP53-mediated apoptosis (Gu et al., 2002; Schmidt et al., 1994; Shvarts et al., 1996).

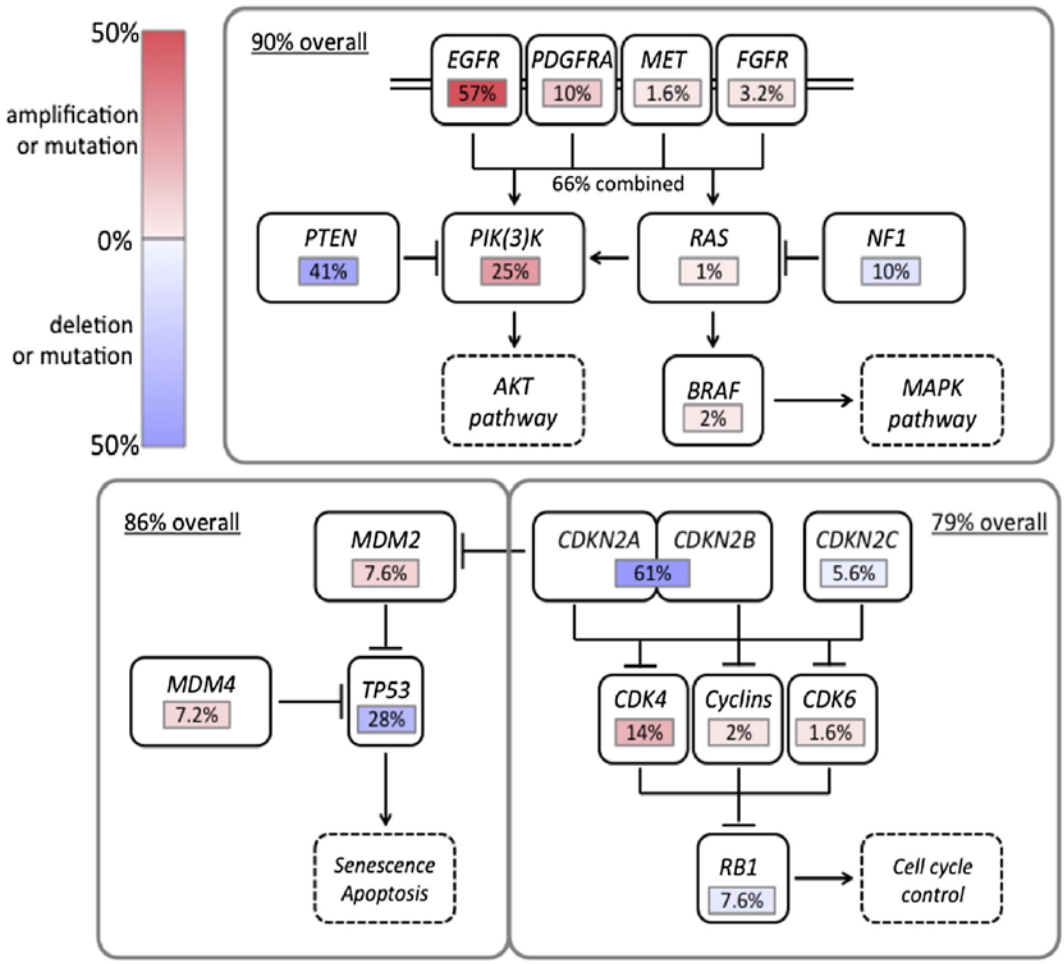
1.4.1.4. Major signaling pathways altered in GBM

GBM is the first cancer to be molecularly profiled by The Cancer Genome Atlas (TCGA) consortium. Most of the discovered genetic alterations hit 3 key signaling pathways. Deregulation of these three pathways is a core requirement for GBM pathogenesis (The Cancer Genome Atlas Research Network, 2008; Brennan et al., 2013).

(i) RTK/PI3K pathway – This pathway is altered in 90% of the GBM samples, and constitutive activation of PI3K/Akt pathway is triggered by loss of function mutation in *PTEN* and gain of function mutation in *PIK3CA* and RTKs such as in *EGFR, PDGFR and MET*. Constant activation of this pathway induces cancer cell proliferation and survival.

(ii) RB signaling pathway – This pathway is altered in over 79% of the GBM samples. Loss of cell cycle control by this pathway supports uncontrolled cell division in glioma cells.

(iii) TP53 pathway – This pathway is altered in 86% of GBM samples. Loss of *TP53* in glioma cells, promotes survival advantage by escape of senescence (The Cancer Genome Atlas Research Network, 2008). The schematic representation of targeted pathways is depicted in Fig. 2.



Brennan et al., 2013

Fig 2. Schematic representation of major pathways targeted by genetic alterations in GBM. *EGFR* amplifications (57%), *PTEN* loss (41%), *CDKN2A/2B* deletions (61%), loss of *TP53* (28%) are hallmark genetic alterations in GBM. These alterations target 3 major signaling pathways – PI3K (90%), RB pathway (79%) and TP53 pathway (86%). Frequencies of genetic alterations (glioma samples) are mentioned in percentage.

1.4.2. Gene expression profiles of GBM

1.4.2.1. Signaling pathways that govern neural/glia development

(i) Notch signaling pathway – Notch 1-4 belong to the family of trans-membrane receptor proteins. Notch signaling maintains tissue homeostasis and promotes neural stem cell growth and gliogenesis (Artavanis-Tsakonas, 1999; del Amo et al., 1993; Gridley, 1997; Scheer et al., 2001). Loss of Notch function induces differentiation of neural stem cells. Notch signaling pathway receives inputs from adjacent cells by membrane-bound ligands with DSL (Delta, Serrate and Lag2) domain such as Delta-like 1,4, Jagged 1-2 (Henderson et al., 1994; Tax et al., 1994). Binding of the ligands

to Notch receptors exposes Notch intracellular domain (NICD) to proteolytic cleavage. This releases NICD into cytosol. Translocation of NICD into the nucleus, assisted by RBPJ-κ/ MAML complex, results in trans-activation of *HES* and *HEY* families of transcription factors. HES and HEY transcription factors in turn, activate the downstream target genes and promote glioma progression (Androutsellis-Theotokis et al., 1996; Gaetani et al., 2010; Hulleman et al., 2009; Lino et al., 2010). Overexpression of Notch ligand *Dll1* and *Hash 1*, a genetic regulator of *Dll1,* are commonly found in LGG and secondary GBM with an exception of LGG with 1p19q co-deletion (Alqudah et al., 2013).

(ii) NF-kB pathway – NF-kB proteins are a family of transcription factors (RelA, RelB, c-Rel, NF-kB1 and NF-kB2). These are activated by pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1), and genotoxic stress factors such as oxidative stress and physical stress induced by exposure to UV or gamma irradiation. NF-kB pathway plays a central role in enabling cellular adaptation to environmental stress. It is well characterized for its role in neuro-protection and inducing inflammatory responses (Ghosh et al., 1994; Li and Verma 2002). NF-kB targets >150 genes in the genome and regulates cellular processes such as cell proliferation, angiogenesis, invasion, and apoptosis (Gastonguay et al., 2012, Brantley et al., 2001). Many mitogenic signaling pathways such as RAS/MAPK and PI3K pathways are known to activate NF-kB signaling (Fan et al., 2002; Mischel and Cloughesy, 2003). Under unstimulated conditions, NF-kB exists as dimers bound to IκB inhibitory proteins in the cytosol and remains inactive. Appropriate stimulus such as those afore mentioned, activates IKK complex (IκB kinase complex) that targets IκB proteins to phosphorylation induced proteosomal degradation. Nuclear translocation of NF-kB dimers, assisted by rapid degradation of IκB inhibitory proteins, allows trans-activation of downstream target genes involved in vital cellular processes. Gene expression of *NF-kB* is elevated in GBM (Ito et al., 1994, Wang et al., 2004). It has been shown that NF-kB activation correlates with poor response to radiation therapy and is associated with shorter survival in GBM patients (Bhat et al., 2013).

(iii) Sonic hedgehog (SHH) signaling pathway – SHH is a member of the hedgehog (HH) family of secreted proteins. HH proteins, produced in the cell undergo extensive post-translational modifications such as palmitoylation, are secreted into the extracellular space by neuronal cells via cellular filopodia. During neural development, gradient activity of SHH induces zonal polarization and contributes to

neural patterning (Ericson et al., 1997). The mitogen SHH often elicits paracrine signaling in adjacent cells and propagates canonical HH signaling through a receptor complex that consists of G-protein coupled receptor smoothened (SMO) and the transmembrane receptor Patched (PTC1). Under unstimulated conditions, PTC1 inhibits SMO action. In the presence of SHH, PTC1 binds to the ligand and relieves SMO. This results in SMO-mediated activation of Gli (Glioma- associated oncogene family members) family of transcription factors (Gli1, Gli2 and Gli3) that regulates the expression of multiple downstream target genes. Members of SHH pathway are known to contribute to various cellular processes through non canonical HH signaling for instance, activation of SHH inactivates the tumor suppressor *RB1* and causes overexpression of the cell cycle regulator N-myc, and PTC1 directly regulates cell cycle through interaction with cyclin B1, which acts as a G2/M checkpoint. The Gli transcription factors are also known to be trans-activated by other signaling pathways such as RTK/ PI3K signaling pathways, independent of SHH pathway (Stecca and Ruiz, 2010). It was recently demonstrated that cross-talk between SHH and PI3K/Akt pathways promote GBM progression. Loss of SHH or mutations in *PTC*, the SHH receptor, results in enhanced susceptibility to cerebellar brain tumors (medulloblastoma). *PTC* mRNA levels correlate with grade II and grade III glioma, suggesting a potential role for the SHH pathway in glioma development (Abdouh et al., 2009; Ehtesham et al., 2007; Filbin et al., 2013).

1.4.2.2. Molecular subtypes of GBM

Gene expression analysis of GBM tumors reflects the tumor heterogeneity and biological state of malignancy more accurately than histopathological grading. Transcriptomic profiling based gene signatures can be effectively used to classify and study GBM tumors (Godard et al., 2003; Nutt et al., 2003; Rickman et al., 2001).

Several reports classified GBM based on gene expression profiles. In 2003, Mischel et al. performed a global transcriptomic profiling of GBM and classified tumors into EGFR-positive, EGFR-negative, and a molecular subtype, which was predominantly characterized by overexpression of genes present in locus 12q13-15 and gene features of astrocytes and oligodendroglia (Mischel and Cloughesy, 2003). In 2010, Verhaak et al. integrated transcriptomic profiles together with gene copy number and mutation data, and globally classified GBM into 4 distinct transcriptional subclasses:

Classical subtype: Focal amplification of chromosome 7 (*EGFR*) paired with loss of chromosome 10 (*PTEN*) is the genetic signature of this subtype. These tumors show high-level amplification of *EGFR* and/or gain of function vIII *EGFR* mutation, and frequent homozygous deletion of *CDKN2A*. Tumors of this subtype show high gene expression for neural stem cell marker Nestin (*NES*), members of Notch (*NOTCH3*, *JAG1*) and SHH (*SMO*, *GAS1* and *Gli2*) signaling pathways.

Proneural subtype: Genetic alteration of 4q12 (*PDGFRα*) and point mutation in isocitrate dehydrogenase 1 (*IDH1*) that encodes a metabolic enzyme, and is the genetic signature that defines this subtype. Focal amplification of *PDGFRα* is the most frequently observed genetic alteration of 4q12 in this tumor subtype. Although rare, gain of function mutation and in frame deletion of *PDGFRα* is also documented in tumors of this subtype. Heterozygous loss (LOH) and loss of function mutation of *TP53* are frequent in this subtype. Tumors of proneural subtype show high mRNA levels for genes associated with oligodendrocytic development - *PDGFRα*, *NKX2-2* and *OLIG2* and developmental genes - *DCX, TCF4, SOX* members, and Notch members - *DLL3*, *ASCL1*. Most of the secondary GBM are part of this subtype.

Neural subtype: These tumor subtypes do not show a specific genetic signature. However, they show high gene expression for neuronal and astrocytic markers like neurofilament (*NEFL*), gamma-aminobutyric acid A receptor alpha 1 (*GABRA1*), Synaptotagmin 1 (*SYT1*), and potassium/chloride transporter (*SLC12A5*).

Mesenchymal subtype: The majority of mesenchymal GBM show focal hemizygous deletions of 17q11.2 (*Neurofibromatosis 1-NF1*) together with low mRNA levels of *NF1*. Loss of function *co-mutation* is frequently observed for *TP53* and *NF1* in this subtype. Mesenchymal GBM show high gene expression for astrocytic and mesenchymal markers such as *MERTK, CHI3L1*, *MET*, *and CD44*, members of TNF-NF-kB pathway such as *TRADD*, *RELB*, and *TNFRSF1α*, and those involved in inflammation.

1.4.3. Low-grade glioma

Genetic alterations discovered in GBM provide information about glioma progression. However, alterations that describe glioma initiation are yet to be discovered. LGG gradually progresses to secondary GBM, by sequential accumulation of mutations.

Therefore, LGG represents a better candidate to study early gliomagenesis. Known genetic alterations in LGG are discussed in the following section.

1.4.3.1. Genetic alterations in LGG

Mutation in isocitrate dehydrogenase 1 *(IDH1)* is most commonly observed in LGG. IDH are homodimeric metabolic enzymes (IDH1-4) that localize in cell cytosol and peroxisomes (IDH1), and in mitochondria (IDH2-4). IDH enzymes catalyze the conversion of isocitrate to α-ketoglutarate (αKG) during Krebs cycle (TCA cycle) in mitochondria. In 2008, point mutations in isocitrate dehydrogenase 1 (*IDH 1*) were identified in GBM (Parsons et al., 2008). Further studies showed that over 80% of LGG and secondary GBM harbor *IDH1* mutations compared to primary GBM (4-7%) (Ohgaki and Kleihues, 2013). Mutation in *IDH* (at Arg 132 for *IDH1* and very rarely for Arg140 and 172 for *IDH2*) alter tumor cell metabolism and induce genome-wide changes in the epigenetic profile of tumor cell. Mutation in *IDH 1* results in a novel enzyme function that catalyzes the conversion of α-KG to an oncometabolite 2-hydroxy-glutarate (2HG). 2HG induces the genome-wide methylation of cytosine-phosphate-guanine (CpG) islands found around promoter regions of genes (Zhu et al., 2011). In 2010, Noushmehr et al. performed promoter DNA methylation analysis and identified that a subset of proneural GBM showed hyper-methylation of a large number of loci, called *glioma CpG island methylator phenotype* (G-CIMP) (Christensen et al., 2011; Laffaire et al., 2011). Such epigenetic changes regulate mammalian gene expression primarily by gene silencing.

Promoter methylation of O^6- Methyl Guanine DNA Methyl Transferase (*MGMT*) is a common epigenetic alteration observed in 80% of LGG and 35-40% of malignant gliomas (Thon et al., 2012; Hegi et al., 2005). *MGMT* (locus 10q26) encodes for a DNA repair enzyme that removes alkyl adducts from O^6-alkyl guanine (O6-Al-G). For example, the enzyme prevents methylation of CpG islands. Alkylated O^6- Guanine leads to double-stranded DNA breakage and base mispairing. Loss of MGMT activity results in gene silencing and cell death due to genome instability. Unfortunately in glioma cells, presence of MGMT contributes to resistance against methylating chemotherapeutic agents such as temozolomide (TMZ), as MGMT repairs the lesions introduced by TMZ. Consequently, glioma patients with epigenetically silenced *MGMT* gene (*MGMT* promoter methylation) respond well to TMZ treatment

compared to glioma patients that do not have *MGMT* promoter methylation (Hegi et al., 2005).

Point mutation in *TP53* is a frequent event observed in over 60% of LGG and is most often found in astrocytoma (Ohgaki and Kleihues, 2007). Frequent point mutations in CpG sites from G:C to A:T results in loss of TP53 gene expression. TP53 is a tumor suppressor that maintains genome integrity (the cellular functions of TP53 is discussed in detail in section 1.4.1.3). Loss of function mutation in *TP53* is associated with unfavorable outcome in LGG (Ohgaki and Kleihues, 2007; Ständer et al., 2004). *TP53* mutations occur in over 65% of secondary GBM that arise from LGG, in contrast to primary GBM (28%) that arise *de novo* (Ohgaki et al., 2004).

Codeletion of 1p and 19q arms is observed in over 80% of oligodendroglioma. Genes located in this region such as *Notch 2* and *CCNL2* (a regulator of cell cycle progression) are known to contribute to oligodendroglioma initiation and progression (Ferrer-Luna et al., 2009; Mukasa et al., 2002; Tews et al., 2006). In fact, combined heterozygous allelic loss of 1p and 19q is a good prognostic factor in patients with oligodendroglioma (Zhao et al., 2014).

Point mutations in *Telomerase reverse transcriptase (TERT)* were found in over 50% of gliomas and in 78% of oligodendrogliomas (Killela et al., 2013). The reverse transcriptase TERT is responsible for maintaining telomere length and thus regulate age and ability of a cell to divide (Masutomi et al., 2005). Frequent activating point mutations (C250T and C228T) in the promoter of *TERT* contribute to elevated levels of telomerase activity that contributes to gliomagenesis (Killela et al., 2013). Interestingly mutations in telomere binding proteins, that participates in maintaining telomere length such as *alpha thalassemia/mental retardation syndrome X-linked* (*ATRX*) and *death-domain associated protein* (*DAXX*) are mutually exclusive with *TERT* mutation (Killela et al., 2013).

It has been demonstrated that gain of function mutation in *IDH*, *TERT,* loss of function mutation in *TP53* contribute to gliomagenesis (Cohen et al., 2013; Labussière et al., 2014; Moriya et al., 2014). As *IDH* mutations are present in over 80% of LGG irrespective of the histology of the tumor, it is postulated that *IDH* mutations occur early during gliomagenesis (Appin and Brat, 2015; Louis, 1994; Ohgaki and Kleihues, 2011; Watanabe et al., 2009; Yan et al., 2009).

1.4.3.2. Molecular subtypes of LGG

LGG are broadly classified into 3 categories based on identified genetic aberrations (Eckel-Passow et al., 2015; TCGA, 2015; Leu et al., 2013; Suzuki et al., 2015):

(i) *IDH*mut/*1p19q* LOH – LGG of this subtype show *IDH* mutation (*IDH*mut) and codeletion of *1p19q* arms (*1p19q* LOH). In addition, this subtype harbors mutations that contribute to glioma proliferation such as in *Capicua* (*CIC)- a* modulator of ErbB signaling pathway (locus 1p), *Far upstream element binding protein 1* (*FUBP1)*- an activator of *myc (locus 19q)*, *NOTCH1* and *TERT*. Patients with tumors of this subtype respond well to aggressive treatments and have high overall median survival of 8 years compared to other groups (TCGA 2015). Over 80% of *IDH* mut LGG also carry *MGMT* promoter methylation (*MGMT*met). In fact, patients with *IDH* mut/*1p19q* LOH/*MGMT*met LGG are also associated with higher overall survival time compared to *IDH* wt (Leu et al., 2013).

(ii) *IDH*mut – In addition to *IDH*mut, this LGG subtype also shows frequent coexistence of inactivating mutations in *TP53 (TP53*mut*)* and *ATRX*. Patients with this tumor subtype show median survival of 6.3 years (TCGA 2015). Consistently, *IDH*mut/*TP53* mut LGG that also show *MGMT*met (*IDH*mut/*TP53*/*MGMT*met) is associated with reduced overall survival time compared to *IDH*mut/*1p19q* LOH/ *MGMT* met but higher compared to *IDH* wt (Leu et al., 2013).

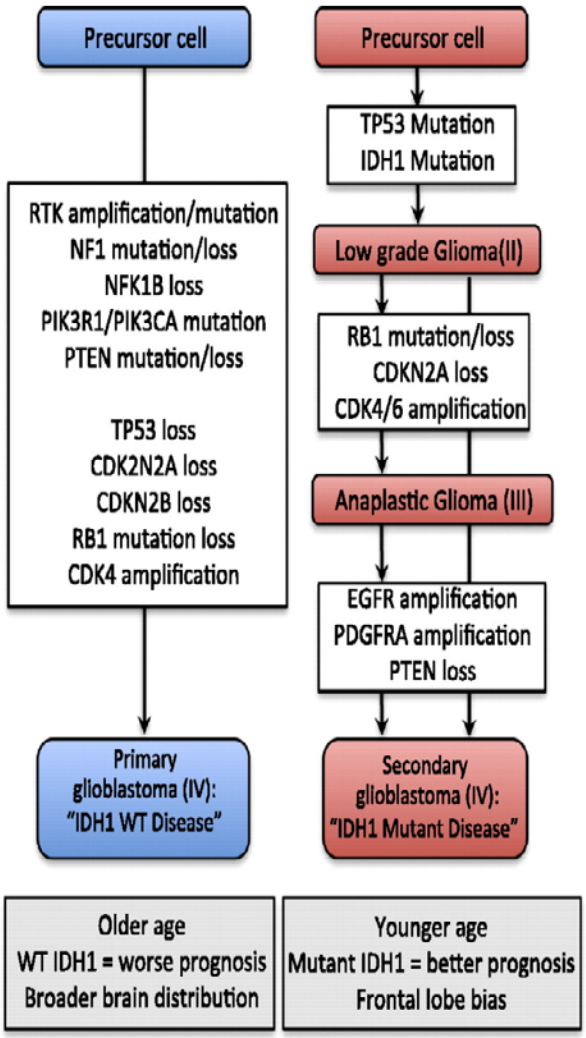
(iii) *IDH*wt – LGG of this subset express wild type *IDH* and harbor classical GBM subtype-like mutations and copy number variations (CNVs) for instance, gain of function mutation and amplification of *EGFR* and homozygous deletion of *CDKN2A*. The *IDH*wt subtype account for 20% of all the LGG and majority of the tumors are anaplastic astrocytomas. Patients with *IDH*wt LGG have the shortest survival with a median of 1.7 years compared to other afore mentioned LGG subtypes (TCGA 2015).

Overall, our current understanding of gliomagenesis is not yet sufficient to portray the complete picture of gliomagenesis. However, further understanding of sequential accumulation of mutations in glioma could give deeper insights into gliomagenesis.

1.4.4. Distinct pathways for glioma initiation

In 1940, Hans-Joachim Scherer, a german neuropathologist was the first to suggest that based on biologic and clinical point of view, primary and secondary GBM can be distinguished. Although the view was clinically recognized, Scherrer's distinction remained conceptual as the two lesions are histopathologically undistinguishable (Homma et al., 2006; Lee et al., 2013; Ohgaki and Kleihues, 2007; Ohgaki, 2005; Peiffer and Kleihues, 1999).

In 1996, Ohgaki et al. reported that inactivating mutation in *TP53* is more frequent in secondary GBM (65%) than in primary GBM (28%). Subsequent studies made similar observations, for instance focal amplifications in *EGFR* are more common in primary rather than in secondary GBM (Fujisawa et al., 2000; Ohgaki and Kleihues, 2007;Ohgaki et al., 2004; Watanabe et al., 1996). In 2009, Nobusawa et al. identified point mutations in *IDH1* as a molecular marker for secondary GBM (Nobusawa et al., 2009). Subsequently, in 2010, Noushmehr et al. performed epigenetic profiling of all GBM samples and showed that over 93% of the proneural GBM harbored *IDH1* mutations and mostly are secondary GBM. The authors further showed that the *IDH* mutant secondary GBM showed a distinct glioma-CpG island methylator phenotype called *G-CIMP* (Noushmehr et al., 2010). Thus, all these observations show that primary and secondary GBM are genetically distinct. This led to postulate that two distinct pathways for gliomagenesis exist (Fig. 3).



Dunn et al., 2012

Fig. 3. Schematic representation of glioma progression: Primary and secondary GBM are genetically distinct suggesting two pathways for gliomagenesis. The scheme represents the known genetic alterations in chronological order of disease progression. Primary GBM arise *de novo* and are characterized by alterations in PI3K pathway, *p53*, and *RB* pathways. Primary GBM occur in older individuals and have worse prognosis for survival (12-14 months). Secondary GBM occur by gradual progression of LGG to GBM. Secondary GBM are characterized by mutations in *IDH*, *TP53*. *IDH* mutations in secondary GBM and LGG are associated with a favorable patient outcome.

1.4.5. Cellular origin for gliomagenesis

It is postulated that GBM contains a sub-population of cells that exhibit stem-like behavior (Altaner, 2008; Cho et al., 2013; Lathia et al., 2015; Persano et al., 2013). These so called glioma stem-like cells exhibit tumorigenic properties. Currently, these cells are identified using a cocktail of markers such as prominin

(CD133/PROM1), stage specific embryonic antigen 1(SSEA), integrin α6, CD44, and SOX2 (Suvà et al., 2014).

Histologically, oligoastrogliomas offer deeper insight into the origin of gliomas. Gliomas of this subtype contain neoplastic cells with astrocytic and oligodendroglial morphology, suggesting that oligodendrocyte precursor cells (OPCs) and neural stem cells (NSCs) are potential candidates for gliomagenesis. In fact, using lineage-tracing experiments such as mosaic analysis with double markers (MADM) on a syngenic glioma mouse model, Liu et al. showed that orthotopic implantation of OPCs genetically inactivated for tumor suppressor genes *TP53* and *NF1*, results in sporadic GBM formation (Liu et al., 2011). Swartling et al. showed that orthotopic implantation of NSCs genetically altered to stably express high levels of *N-myc*, into the mouse forebrain results in formation of diffuse gliomas (Swartling et al., 2012).

Overall, growing evidence supports that genetically altered NSC or OPC could be the cellular origin of glioma (Alderton, 2011; Lei and Canoll, 2011; Galvao et al., 2014; Lindberg et al., 2014; Monje et al., 2011).

1.5. MALIGNANT TRANSFORMATION

1.5.1. Glioma invasion

Glioma cells invade deep into the brain tissue by the time of its detection and surgical resection (Matsukado, et al. 1961). This shows the extent of infiltrative behavior of glioma. Advanced stages of glioma present multifocal lesions with higher incidence of tumor relapse (Silbergeld et al 1999; Barnard and Geddes, 1987; Gaspar et al., 1992; Giese et al., 2001). Over 90% of gliomas relapse within 2-3 cm from the site of surgical resection. Tumor relapse is the primary reason for tumor related morbidity in glioma patients (Burger et al., 1983; Gaspar et al., 1992). Extent of surgical tumor resection remains one of the strongest prognostic factors for glioma patient survival (Nitta and Sato, 1995).

1.5.1.1. Gliomas invade but rarely metastasize

Malignant tumors have one common denominator; they are highly invasive and often metastasize. *Metastasis* occurs in two steps: (i) Tumor cells migrate from their

primary site of origin in to distant organs that are capable of homing them through navigators such as blood vessel system (ii) Once reached, then proliferate and locally infiltrate the organ resulting in the formation of a secondary tumor at the new site – *metastasis* (Chambers et al., 2002).

Unlike other solid tumors, gliomas very rarely (0.4%-2%) metastasize outside the brain. Many explanations have been proposed towards this behavior of gliomas. First, it is possible that individuals with GBM do not survive long enough for extra-cranial metastasis to occur (Stupp et al., 2005 (radiotherapy), Stupp et al., 2005 (chemotherapy)). Second, extra-cranial tissues may not contain the right microenvironment to support glioma cell survival. Third, glioma cells are unable to intravasate the BBB in order to enter into systemic circulation and metastasize outside the brain. There have been no clear demonstrations so far, to confirm these hypotheses (Beauchesne, 2011; Boelke et al., 2013).

1.5.1.2. Modes of glioma invasion

Glioma are known to invade along the long tracts of nerve fibers or white matter tracts (Bernstein et al., 1990; Giese et al., 1996; Yamada et al., 1994). Recently, it was shown that glioma participate in tumor angiogenesis and could migrate along the tumor vessels (Bao et al., 2006; El Hallani et al., 2014; Ricci-Vitiani et al., 2010). Brain parenchyma, perivascular structures, and blood vessel membranes are composed of diverse extracellular matrix (ECM) molecules and represent potential channels through which glioma cells can invade. The three known paths along which glioma cells invade and migrate are discussed below:

1.5.1.2.1. Through brain parenchyma

Glioma cells have long known to invade along the nerve fibers and white matter tracts. The extracellular matrix of brain parenchyma is made of proteoglycans such as lectican family members (eg. BEHAB/brevican, a known inducer of glioma cell motilty), and their binding partners hyaluronans, tenascins (TNC, TNW) (Bonneh-Barkay and Wiley, 2009; Gary et al., 1998). Many of these ECM molecules are secreted by astrocytes and oligodendrocytes such as TNC. Cell bodies of astrocytes and oligodendrocytes among others, in the brain parenchyma contribute to enhanced physical resistance for glioma cell migration. The glioma cells in response to such stiff ECM secrete proteins and matrix-metallo-proteases (MMP) that help to digest and remodel ECM, thus facilitating their infiltration through the brain parenchyma.

1.5.1.2.2. Along the blood vessels

BBB is composed of tight junctions between astrocytic end feet, endothelial cells and vascular pericytes called *neurovascular units*. These structures ensure proper flow of blood and availability of nutrients to brain tissues. Glioma cells are often found to crowd around the perivascular spaces in the tumor (Anand Cuddapah et al., 2014; Rutka et al., 1998). Close association of glioma cells to the vascular network helps them to derive maximum nutrition directly from the blood stream.

Many research studies using xenograft mouse and rat models of glioma, demonstrated that majority of glioma cells migrate along the blood vessels by disrupting the BBB and invade the surrounding unaffected brain tissue (Farin et al., 2006; Zagzag et al., 2000; Watkins et al., 2014). In 2014, Cudappah et al. postulated that invading glioma cells surround blood vessels and compete with astrocytic endfeet processes for physical interaction with vascular endothelial cells. In the process they lift up the astrocytic processes and degrade the underlying basement membrane by producing ECM proteins and proteases. In response, astrocytes become reactive and withdraw their end feet processes, thus disrupting the neurovascular unit (Anand Cuddapah et al., 2014). Glioma cells hence invade along the blood vessels by breaking the BBB (Fig. 4A).

1.5.1.2.3. Along the subarachnoid-perivascular space

Large blood vessels have considerably larger perivascular spaces called the *Virchow Robin space*, which is continuous with subarachnoid space. This space is separated from the brain parenchyma by the basement membrane, created by astrocytes along their end feet. Cerebrospinal fluid (CSF) is present in this space and this is regarded as the lymphatic system of the brain. This space consists of collagens type- I, III, and IV, fibronectin, laminins, and other proteoglycans. This perivascular space offers the path of least resistance for glioma migration (Giangaspero and Burger, 1983, Jones T G et al., 1981). It is thought that the convection of CSF towards the brain surface and other subarachnoid space helps in directional glioma migration. (Jones et al., 1981, Anand Cuddapah et al., 2014).

1.5.1.3. Mechanistic overview of glioma invasion

Glioma cells are highly sensitive to ECM stimulus that causes cell detachment and triggers cell migration. Cancer cells that disseminate from the primary site can exhibit mesenchymal or amoeboid migration (Friedl and Wolf, 2003). Gliomas exhibit mesenchymal invasion where the cells become polarized and secrete MMP and ECM molecules. Characteristic features of a migrating glioma cell are cell polarization, protrusion, and formation of focal adhesion complexes (explained in detail in the following section 1.5.1.4) at the leading edges that enable dynamic actin polymerization.

The trans-differentiation of epithelial cells to motile mesenchymal cells, a process known as epithelial-mesenchymal transition (EMT), is commonly observed during embryogenesis, organogenesis, wound healing, and tissue remodeling. EMT is well described in many epithelial cancer types as an important mediator of cancer invasion and metastasis (Eckert et al., 2011; Huber et al., 2005; Thiery, 2002; Tsai et al., 2012).

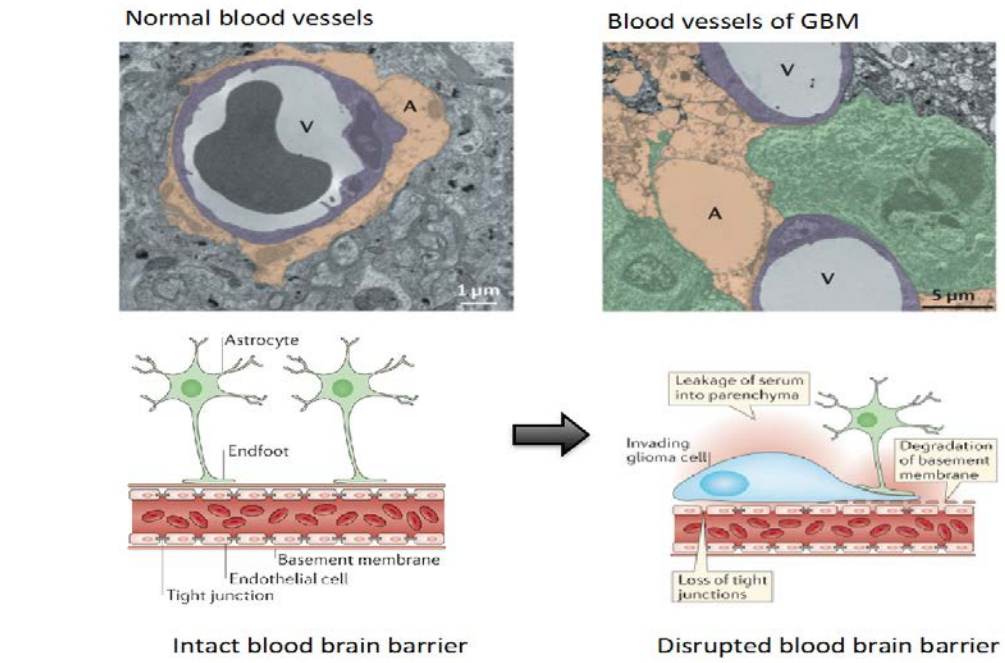
This phenomenon is characterized by transition of epithelial cells to polarized mesenchymal cells that leads to loss of adherent cell-cell junctions, gain of migratory/invasive phenotype, and elevated resistance to apoptosis. This trans-differentiation is tightly controlled by key transcription factors such as SNAIL, Zinc-finger E box (Zeb), and other basic helix loop helix factors such as myoD (Cannito et al., 2008; Nagaharu et al., 2011; Peinado et al., 2003). TGFβ directly regulates the expression of these transcription factors and plays an important role in inducing EMT (Peinado et al., 2003).

Many reports have shown the existence of a EMT-like phenomenon in glioma (Anand Cuddapah et al., 2014; Soda et al., 2011; Zhao et al., 2010). Thus, hinting that EMT might be at play to during glioma invasion. Overall, glioma invasion can be described in 4 stages:

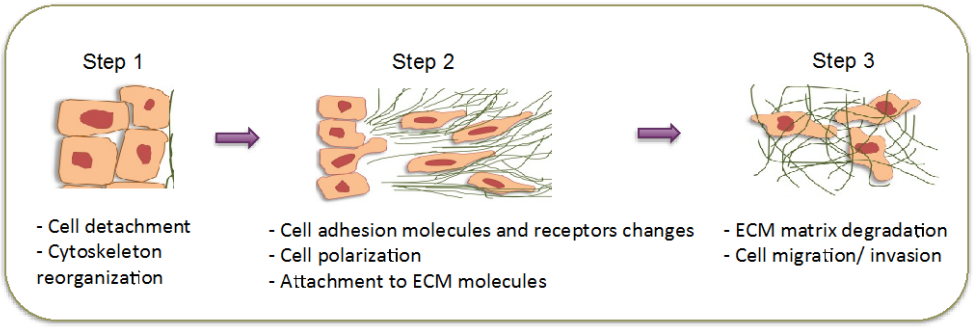
a. Non-polarized epithelial-like cells detach from their primary site of origin. b. They undergo massive cytoskeletal reorganization, and develop new cell adhesion molecules and receptor repertoires that enable them to attach to the extracellular matrix (ECM). This results in cell polarization. c. The polarized cells then degrade and remodel the ECM with the help of proteases (MMP2, MMP 9) secreted into the

ECM, d. Glioma cells thus invade along the degraded ECM structures (Nakada et al., 2007; Teodorczyk and Martin-Villalba, 2010; Wolf, 2003) (Fig. 4B).

A



B



Adapted from Cudappah et al.,2014, Nakada et al.,2007, Tim Demuth et al.,2002

Fig 4. Glioma invasion along the blood vessel structures: A. *Top*: Electron micrographs of intact blood vessel showing normal astrocytic (labeled A) end feet coverage and that of a disrupted blood vessel with glioma cells (green) covering most of the blood vessels (labeled V) in GBM patient derived cell lines. *Bottom*: Schematic representation of intact and disrupted BBB. Glioma cells displace astrocytic endfeet and migrate/invade along the blood vessels. B. Glioma invasion is effected in 3 steps. Step 1. Cells detach from their primary site of origin and undergo massive cytoskeletal reorganization. Step 2. They develop new repertoire of cell adhesion molecules and receptors and attach to ECM. During this phase cells become polarized. Step 3. They secrete various ECM ligands and proteases that degrade and remodel the ECM, and then effect invasion.

1.5.1.4. Molecular determinants of glioma invasion

1.5.1.4.1. Rho GTPases

Rho family members are small GTPases that belong to the Ras super family, and are known to play a crucial role in actin cytoskeletal remodeling. The small GTPases comprises more than 20 members. Among them, RhoA, Rac1, and Cdc42 have been well-studied for their role in cell invasion and migration (Bouzahzah et al., 2001; Keely et al., 1997). RhoA regulates the dynamic formation of stress fibers and adhesion complexes. Rac1 mediates the formation of lamellipodia at the leading edge of a migrating cell. Cdc42 promotes directional movement by regulating polarization and filipodia formation in the cell (Nobes and Hall, 1999). Interestingly, in glioma cells inhibition of RhoA results in Rac1 compensated cell migration (Salhia et al., 2005; Tran et al., 2006).

1.5.1.4.2. Integrins and members of focal adhesion complex

Integrins are trans-membrane heterodimer receptors consisting of an α- and a β subunit. Integrins primarily provide structural support to the cells by enabling them to attach to ECM molecules such as fibronectin and laminin. So far, over 18α and 8β subunits have been discovered and their heterodimeric combinations determine the ligand affinity. In addition to providing structural support, integrins also regulate anchorage-dependent cell growth by controlling ionic exchanges such as Ca^{++} influx and the Na^{+}/K^{+} antiporter (Burridge and Chrzanowska-Wodnicka, 1996). Clustering of integrins on the leading edge of migrating cell results in the formation of focal adhesion contacts that serve as a link between ECM and cellular actin cytoskeleton. This serves as a docking site for focal adhesion kinases (FAK). Aggregated integrins, cytoskeletal proteins such as vinculin, and focal adhesion kinases (FAK) called *focal adhesion complex* facilitates dynamic cytoskeletal reorganization and establishes cell polarity. Formation of focal adhesion complex is required for glioma cell migration (D'Abaco and Kaye, 2007). The expression of αVβ1, αVβ5 integrins is shown to correlate with glioma invasiveness (Belot et al., 2001; Paulus et al., 1996).

1.5.1.4.3. ECM molecules and proteases

Tenacin-C (TNC) is a glycoprotein secreted by glioma cells into their extracellular stroma. The ECM molecule is strongly linked to glioma malignancy and associated with poor prognosis in GBM patients (Bourdon et al., 1983; Leins et al., 2003). In fact, TNC is trans-activated by the Notch signaling pathway to induce glioma invasion (Sivasankaran et al., 2009). Laminin is a major component of the vascular basement membrane and a potent stimulator of glioma migration (Goldbrunner et al., 1996). Other ECM molecules such as vitronectin is an inhibitor of glioma cell migration when tested *in vitro* (Giese and Westphal, 1996). MMP belong to the family of Zn^{2+}dependent proteases that are responsible for ECM degradation and maintenance. Glioma cells secrete MMPs such as MMP2, MMP9, whose expression correlate with glioma invasiveness and poor prognosis (Badiga et al., 2011; Lu et al., 2012; Sun et al., 2013).

1.5.1.4.4.Growth factor receptors

The members of RTK family of growth factor receptors are important contributors to glioma cell proliferation, survival, invasion, and angiogenesis. For instance, the hepatocyte growth factor (HGF) receptor, *MET* (located at 7q) is frequently amplified and its expression correlates with tumor invasion and progression (Abounader and Laterra 2005; Koochekpour et al., 1997; Lamszus et al., 1998; Kong et al., 2009). Upon binding to HGF, the MET receptor undergoes phosphorylation and activates the Ras/MAPK and Akt signaling pathways. HGF/MET signaling is important for maintaining tissue homeostasis under normal physiological conditions. Similarly, VEGF is a chemotactic inducer of tumor cell invasion and mesenchymal transition and transduces signals through a complex system of RTK VEGF (VEGFR 1-4). It has been demonstrated that the pro-invasive ligand VEGF contributes to glioma invasion by activating downstream signals upon binding to MET/VEGFR2 complex (Nicola McCarthy 2012). Similarly, binding of EGF to EGFR promotes cell proliferation, invasion, and survival of glioma (Lund-Johansen et al., 1990). It was shown that *EGFRvIII* (one of the most common gain of function mutations in EGFR in GBM) promotes glioma cell invasion through protein kinase A (PKA) dependent activation of Rac1. Thus, contributing to cell cytoskeleton reorganization in addition to activating the PI3K pathway (Feng et al., 2014; Lund-Johansen et al., 1990; Micallef et al., 2009).

1.5.1.4.5. Epithelial-Mesenchymal Transition related markers

Emerging literature supports the role of EMT inducers in glioma invasion. The transcription factor ZEB1 is an inducer of EMT and contributes to aggressive glioma behavior (invasion, migration and therapy resistance). Recently, markers associated with EMT such as Twist, Emx, and Snail have been shown to also contribute to glioma invasion (Bhat et al., 2011; Elias et al., 2005; Savary et al., 2013; Zhao et al., 2012).

1.5.2. Glioma angiogenesis

Tumor angiogenesis is the principal source of nutrition for the growing tumor and is required to remove metabolic wastes and carbon dioxide from the tumor tissue. As gliomas progress to higher grades of malignancy, they become highly vascularized eventually forming abnormal leaky blood vessels, observed in GBM. Enhanced tumor vascularization is hence one of the biological features of GBM.

Angiogenesis is the process of formation of new blood vessels from existing ones (Folkman, 1971; Kerbel, 2008). In adults, we commonly observe transient phases of angiogenesis for instance, during wound healing. These phases are tightly controlled by fine balance between pro- and antiangiogenic factors. Blood vessel formation from the pre-existing vessels is the primary mechanism of brain vascularization observed during both brain development and in brain cancer (Kurz et al., 2004, 2001; Plate et al., 1994; Risau, 1997).

1.5.2.1. Types of tumor angiogenesis

During embryonic development blood vessels initially arise from endothelial precursor cells called angioblasts. These cells are recruited and subjected to differentiation in response to cues from its local microenvironment. Followed by the *de novo* initiation of angiogenesis from precursor cells, activated endothelial cells respond to gradients of pro-angiogenic factors by sprouting.

Although, tumor-associated angiogenesis classically involves endothelial sprouting, many different modes of angiogenesis have been documented during tumor angiogenesis. These modes of angiogenesis are listed below:

Vasculogenesis is the process where angioblasts from the host are recruited by pro-angiogenic factors, such as VEGF. This process leads to *de novo* initiation of blood vessel formation, and is primarily observed during embryonic development. During tumor angiogenesis, tumor cells secrete VEGF to attract and recruit endothelial precursor cells from perivascular niches in to the tumor to form tube like structures (Patan, 2004).

Sprouting angiogenesis is defined as the growth of a new blood vessel from a pre-existing one, by means of endothelial proliferation (Hillen and Griffioen, 2007). This is the classical form of angiogenesis observed most commonly during embryonic development, wound healing, reproduction and tumor-associated angiogenesis. This process is also observed during tumor angiogenesis particularly in GBM (Ausprunk and Folkman, 1977).

Intussusceptive angiogenesis is the process where a new vessel arises by splitting of pre-existing blood vessel. This type of angiogenesis is often considered to be fast and robust where microvascular growth can take place within hours because cell proliferation is not essential under these circumstances. This type of angiogenesis is observed during lung development (Djonov et al., 2000).

Vessel co-option is the process where tumor cells invade through the normal vascular tissue and become part of the existing vasculature (Leenders et al., 2002).

Vasculogenic mimicry is defined as the process where tumor cells replace endothelial cells and contribute to formation of vascular lumen. The consequence of such mimicry is the formation of abnormal and leaky blood vessels in the tumor (Folberg et al., 2000).

Trans-differentiation is the process where cancer-stem like cells (CSCs) are recruited from tumor to form new blood vessels. CSCs differentiate towards mesenchymal phenotype in the presence of angiogenic factors and have been shown to participate in GBM neovascularization (Scully et al., 2012).

Lymphangiogenesis is the form of angiogenesis that is observed in lymphatic system. Unlike the conventional vascular network formations, the lymphatic vascular

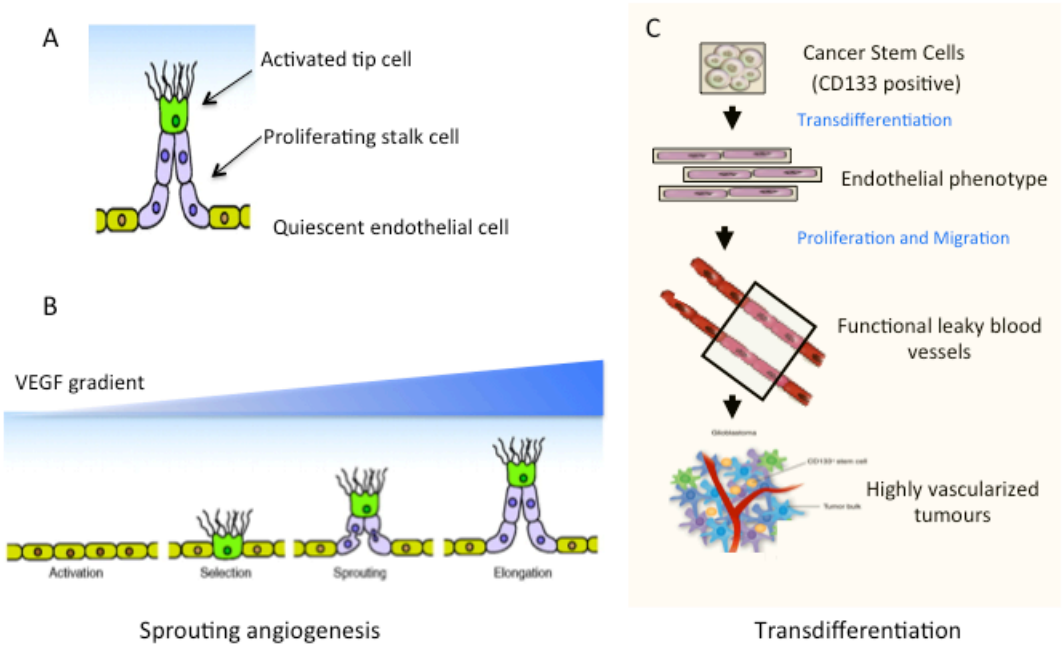
system collects and drains blood from each organ for downstream detoxification and then returns the blood into circuitry. In tumors, lymph angiogenesis is thought to be one of the primary gateways for cells to metastasize into different organs. Many of the vascular endothelial growth factor receptors (VEGFR) family members and PDGF have been associated with this type of angiogenesis (Stacker, 2009).

1.5.2.2. Mechanistic overview

Pro-angiogenic stimuli activate endothelial cells to form vascular sprout. Tip cells with numerous filopodia, showing enhanced migratory behavior are present in the leading front of a sprout. Tip cells are required to create new connections between different vascular sprouts. In the vascular sprout, beneath the tip cells are endothelial stalk cells that are characterized by fewer filopodia and high proliferative ability. Stalk cells are required to create adherent and tight junctions, and form the initial vascular lumen (Dejana et al., 2009; Gerhardt et al., 2003; Phng and Gerhardt, 2009) (Fig. 5A,B).

VEGF is secreted by tumor-associated endothelial cells and promotes GBM angiogenesis. The pro-angiogenic ligand present in ECM and vascular niches binds to endothelial VEGF receptor (VEGFR) and activates downstream signals that results in enhanced the migratory behavior of tip cells. Activation of VEGFR signaling cascade in tip cells induces the expression of notch ligand, Dll4. Secreted Dll4 activates paracrine Notch signaling in adjacent cells leading to endothelial proliferation, the stalk cell phenotype (De Bock et al., 2013; Dejana et al., 2009; Gerhardt et al., 2003; Phng and Gerhardt, 2009; Harjes et al., 2012) (Fig. 5A,B).

The work from 4 independent groups showed that glioma cells grow along the blood vessels by recruiting GSCs for tumor angiogenesis without triggering an angiogenic response. Trans-differentiation of GSCs to endothelial-like cells to participate in tumor angiogenesis, indicates the extent of GSC plasticity (Donnem et al., 2013; Folberg et al., 2000; Holash et al., 1999; Ricci-Vitiani et al., 2010) (Fig. 5C). In 2010, Hallani et al. showed that glioma cells directly participate in blood vessel formation by the process of vasculogenic mimcry (El Hallani et al., 2010). This is schematically represented in Figure 5.



Adapted from Geudens and Gerhardt 2011, Vitiani et al.,2011, Stupp and Hegi 2007

Fig. 5. Schematic representation of glioma angiogenesis processes observed. A. Representative image of an active sprout - during angiogenesis in response to pro-angiogenic stimulus (VEGF) endothelial cells are activated to form a sprout. Migrating tip cells are present at the leading front of a sprout, followed by proliferating stalk cells and quiescent endothelial cells. B. The process of sprouting angiogenesis in response to endothelial activation by pro-angiogenic factors is graphically shown. C. Glioma stem-like cells trans-differentiate towards endothelial-like cells during glioma angiogenesis. The process is known to contribute to the formation of abnormal leaky blood vessels.

1.5.2.3. Molecular players

Tumor cells respond to microenvironmental cues and recruit endothelial cells during angiogenesis. When pro-angiogenic factors like VEGF and angiopoietins (1-4), exceed the anti-angiogenic factors like endostatin and angiostatin, tumors experience an angiogenic switch that favors glioma angiogenesis (Bergers and Benjamin, 2003; Wicki and Christofori, 2008). In GBM, VEGF is highly upregulated and secreted by the tumor cells (Plate et al., 1994; Shweiki et al., 1992). During GBM growth, tumor proliferation exceeds the rate of tumor angiogenesis creating a hypoxic microenvironment inside the tumor. Hypoxia induces and enhances the secretion of VEGF, by activating the expression of hypoxia-inducible transcription factors (HIF1 and 2) (Heddlestone et al., 2010; Li et al., 2009).

Tie2, an endothelial tyrosine kinase receptor for angiopoietins, is constitutively expressed in adult vasculature and is important for homeostasis of mature vasculature and pericyte coverage. Homozygous deletion of *Tie2* in mouse model results in severe vascular defects (Dumont et al., 1994). In GBM, Tie2/Angiopoietin signaling pathway is upregulated and is known to promote tumor angiogenesis (Stratmann et al., 1998). Endothelial cells also express the trans-membrane notch receptors 1 and 4. The ligand Dll4 plays an important role in inducing angiogenesis (Ribatti D and Crivellato E, 2012). Notch together with VEGF regulates stalk cell fate. High expression of Dll4 has been observed in glioma that support tumor vascularization (Jubb et al., 2012). Blocking Dll4-Notch signaling results in hyper-nonfunctional vascularization that results in tumor regression (Kuhnert et al., 2011).

1.5.3. Therapies and therapy resistance

1.5.3.1. Current therapies

Surgical resection coupled with ionizing radiation (radiation therapy) followed by chemotherapy with temozolomide (TMZ) is the current standard of care for glioma patients. TMZ is an alkylating agent that methylates DNA, most commonly at the N^7 and O^6 positions of guanine and O^3 position of adenine residues. Failure of DNA repair mechanisms to replace the methylated residues leads to DNA base mispairing induced breakage therefore cytotoxicity (Friedman et al., 2000b). Overall, treatment with TMZ improves median patient survival time by 2.5 months (Stupp et al., 2005).

1.5.3.2. Therapy resistance

Relapse of tumors that are insensitive to therapy-induced apoptosis, is the primary reason for morbidity among GBM patients (Wen, 2008). Infiltrative glioma cells and chemotherapy-resistant GSC are known to contribute to tumor relapse (Bao et al., 2006; Bleau et al., 2009; Chen et al., 2012; Rich and Bao, 2007). In addition, the limited efficacy of chemotherapeutic drug (because of its low diffusion limit) also contributes to therapy resistance (Kesari et al., 2011; Reardon et al., 2011).

Studies on molecular mechanisms of tumor resistance suggest that therapy resistance can be classified into *intrinsic* or *innate* and *acquired* or *adaptive*

resistance. Pre-existing evident genetic aberrations that could contribute to therapy resistance observed during the first course of treatment are called *intrinsic* or *innate* resistance, for instance, radio-resistance in GBM with *TP53* loss (Shu et al., 1998). Development of resistance to therapy, observed during the course of treatment and relapse of therapy-resistant tumor are called *acquired* or *adaptive* resistance for instance, resistance to Bevacizumab in GBM (Piao et al., 2013). The field of therapy resistance is relatively new and molecular mechanisms for therapy resistance in GBM are currently being understood. Known mechanisms and molecular players in therapy resistance of GBM cells are detailed in following sections.

1.5.3.2.1. MGMT mediated therapy resistance

TMZ treatment introduces methyl groups on nucleotides including O^6-Methyl Guanine (O^6-Me-G). MGMT, a DNA repair enzyme, repairs this lesion by its DNA repair activity, thus reverting the effects of TMZ (Friedman et al., 2000a, 2000b; Park et al., 2010). Subsequently, *MGMT* expression inversely correlates to sensitivity towards TMZ treatment. Thus, *MGMT* expressing GBM do not respond to TMZ treatment. In fact, *MGMT* promoter methylation observed in 80% of LGG and in 40% of GBM, which results in reduced *MGMT* expression, predicts favorable response towards TMZ treatment in glioma patients (Gerson, 2002; Hegi et al., 2004; Sato et al., 2012). Although, *MGMT* mediated therapy resistance could represent an innate mechanism, it is yet to be demonstrated.

1.5.3.2.2. Resistance against Bevacizumab

VEGF-A is a key proangiogenic factor for GBM angiogenesis and associated cerebral edema. Bevacizumab is a EU approved human monoclonal antibody against VEGF-A. Initial administration of Bevacizumab in GBM patients, improves progression-free survival by 6 months (Moen, 2010). Unfortunately, patients develop resistance to bevacizumab and experience tumor relapse (Lu et al., 2013; Iwamoto et al., 2009).

At molecular level, it is proposed that Bevacizumab induces vessel normalization. Perivascular pericytes line the normal blood vessels, in contrast to tumor blood vessels with compromised BBB. Administration of Bevacizumab reduces the number of immature blood vessels found in tumor blood vessels and increases pericyte

coverage in the tumor vasculature, a process known as *vessel normalization*. This results in initial tumor regression. However, relapsed GBM with normalized vasculature do not respond to subsequent Bevacizumab administration (Arjaans et al., 2013).

1.5.3.2.3. Known players in therapy resistance

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that integrates growth signals and metabolic requirements in a cell. Therefore, the kinase is a master regulator of cell proliferation, survival, and metabolism. Over 90% of GBM samples show oncogenic PI3K signaling. Thus, mTOR emerged as a prime therapeutic target to treat GBM patients. However, patients experience relapse of mTOR-resistant GBM. Recently, Tanaka et al. using xenograft glioma mouse model and human GBM patient samples, showed that treatment with mTOR inhibitors alter cancer cell metabolism. The authors showed that compensatory glutamine metabolism contribute to GBM resistance to mTOR (Tanaka et al., 2015).

***Deregulated apoptotic pathway*s** contribute to cancer development (Adams and Cory, 2007; Evan and Littlewood, 1998; Lowe et al., 2004; Ziegler et al.,2008). The process of programmed cell death (PCD) or apoptosis is vital for maintaining a pool of healthy cells, required for physiological growth and tissue homeostasis. PCD can be activated by *extrinsic* stimuli such as activation of TNFα receptors or by *intrinsic* factors such as cellular stress due to exposure of radiation or DNA damage induced by therapeutic targets, that trigger mitochondria mediated PCD. Both the intrinsic and extrinsic pathways for apoptosis trigger the caspase cascade to induce cell death.

Bcl2 family of apoptotic proteins control and regulate mitochondria dependent cell death (*intrinsic pathway*). The family is composed of pro-apoptotic proteins (Bcl10, Bak, Bax, Bim, Bad, etc.) and anti-apoptotic proteins (Bcl2, Bcl-X, Bcl-w, etc.). Over 25 genes for the Bcl2 family members have been identified. Activation of pro-apoptotic proteins, Bax and Bak, located on the outer mitochondrial membrane (OMM), results in mitochondrial membrane permeabilization and subsequently releases cytochrome c (cyt c) from inner mitochondrial membrane (IMM). Release of cytochrome c activates the caspase cascade and induces cell death. In GBM, anti-apoptotic proteins are upregulated while pro-apoptotic proteins are downregulated (Ruano et al., 2008; Stegh et al., 2007). Mitochondria mediated apoptotic pathway is

associated with acquired GBM resistance to RTK-targeted therapies (Ziegler et al.,2008). In fact, overexpression of *OPA1*, a regulator of cytochrome c release, blocks caspase induced apoptosis in gliomas and contributes to tumor resistance in many cancers (Arnoult et al., 2005; H.-Y. Fang et al., 2011; Frezza et al., 2006; X. Zhao et al., 2013).

The transcription factor ***SOX2*** is a regulator of stemness in embryonic and neural stem cells. Recent studies on breast cancer and medulloblastoma cells show that SOX2 expressing CSCs are able to resist anti-mitotic drugs and repopulate the tumor, contributing to tumor relapse (Piva et al., 2014; Vanner et al., 2014). Molecular mechanisms regulating the contribution of SOX2 towards therapy resistance is yet to be elucidated.

1.6. METABOLIC PERSPECTIVE ON GLIOMA CELLS

Otto Warburg received the Nobel Prize in 1931, for his seminal observations that cancer cells exhibit altered cell metabolism. He noted that cancer cells show high consumption of glucose, the majority of which was converted into lactate even in the presence of oxygen, compared to normal cells (Koppenol et al., 2011). This phenomenon is known as the "*Warburg effect*". Warburg hypothesized that this altered metabolism was specific to cancer cells due to mitochondrial defects. However in recent times, it has become clear that not only cancer cells, but also healthy proliferating cells (cells with high energy and metabolic demand) reprogram their metabolism to undergo aerobic glycolysis, the reason for which, is still debated (Lunt and Vander Heiden, 2011).

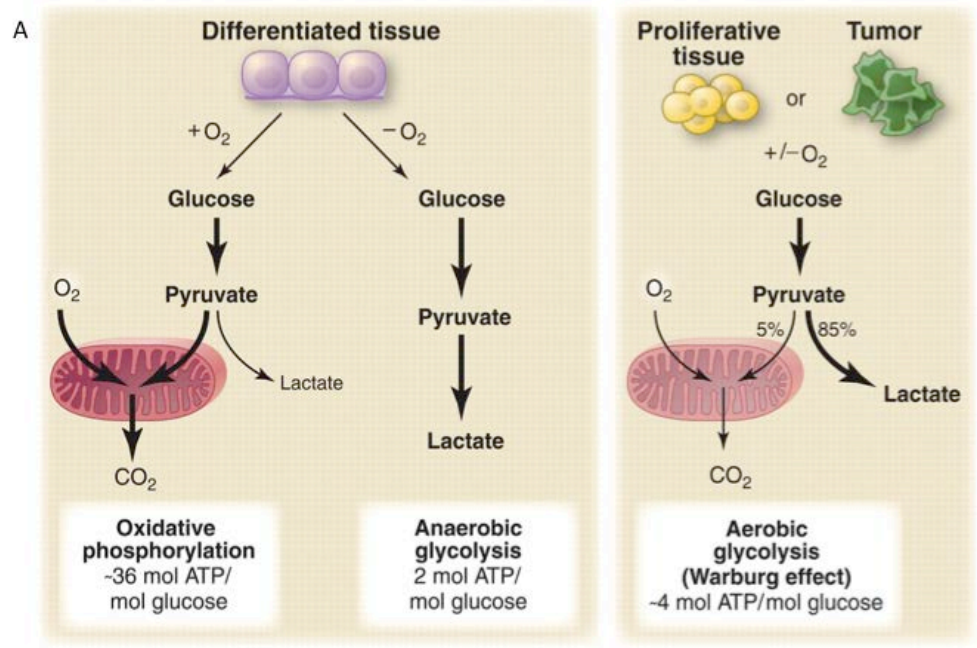
1.6.1. Cellular respiration

1.6.1.1. Glycolysis and oxidative phosphorylation

Generating cellular energy in the form of ATP through mitochondria, in the presence of oxygen is termed as *aerobic respiration*. Cellular glucose is metabolized to pyruvate by the process of glycolysis in the cell cytosol. In the presence of oxygen, pyruvate enters mitochondria and undergoes further oxidation via the tri-carboxylic acid cycle (TCA), also known as the Krebs cycle. In mitochondria, NADH, the by-product of TCA cycle further undergoes oxidation via electron transport chain (ETC) and adenosine tri-phosphate (ATP) is synthesized as the final outcome. This process is known as *oxidative phosphorylation* (OXPHOS). Glucose metabolism in the presence of oxygen is highly efficient and generates 36 mol of ATP per mol of glucose consumed (Fig. 6A *Left*) (Berg et al., 2006; Porter and Brand, 1995).

1.6.1.2. Aerobic glycolysis

Glucose metabolism can occur both in the presence and in the absence of oxygen. Cancer or proliferating cells with high metabolic demand undergo an inefficient glucose metabolism known as *aerobic glycolysis*, even in the presence of oxygen. Glucose is metabolized to pyruvate via glycolysis and during reduced oxygen levels and increased metabolic needs, pyruvate is then converted to lactate in the cell cytosol, releasing 4 mol of ATP per mol of glucose (Fig. 6A *Right*) (Postovit et al., 2002; Zu and Guppy, 2004).



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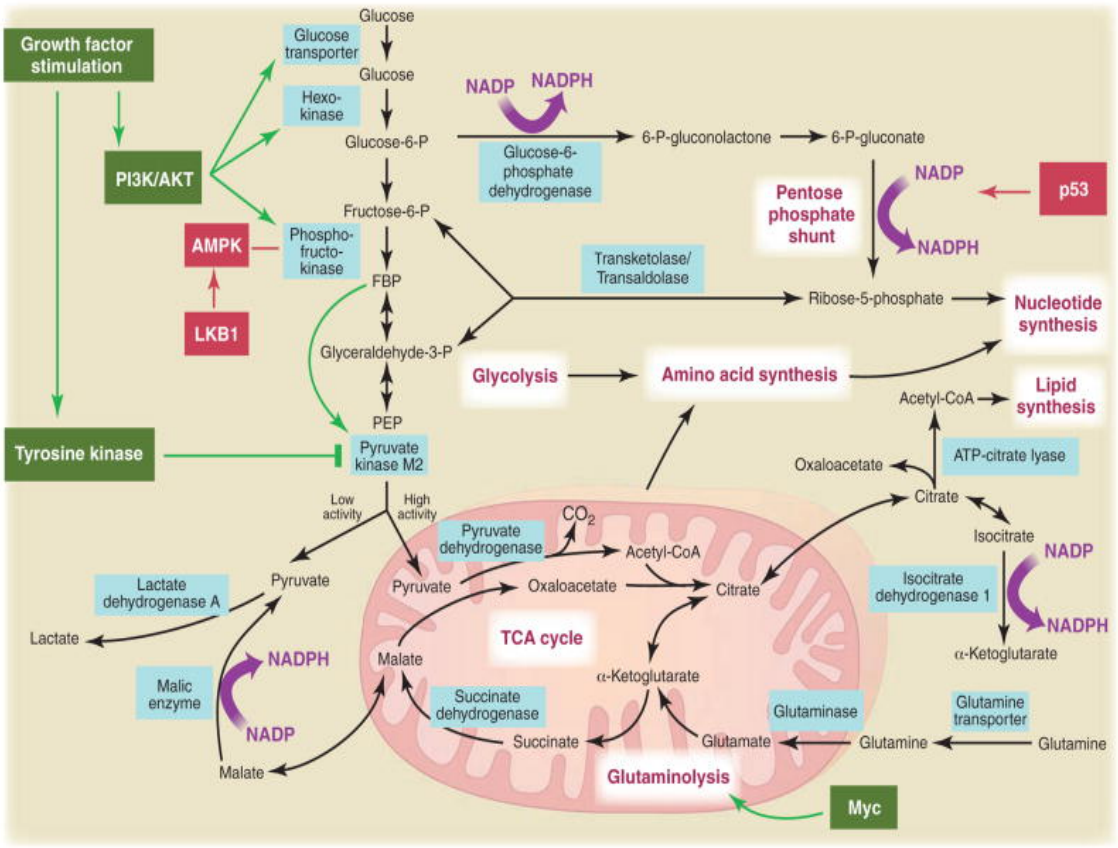


Heiden et al., 2009

Fig 6. Cellular respiration: A. Schematic representation of the differences in cellular respiration between nonproliferating (differentiated) and proliferating tissue including tumor cells: Glucose consumed by the cell is metabolized to pyruvate in the cell cytosol via glycolysis. *Left*: In the presence of oxygen, pyruvate is oxidized to CO_2 during OXPHOS in mitochondria. Because oxygen is the final electron acceptor in the ETC, presence of oxygen is crucial for mitochondrial respiration. Oxidative phosphorylation results in the generation of 36 mol of ATP per mol of glucose consumed. *Right*: Low levels of oxygen or increased metabolic demand in the cell results in the reduction of pyruvate to lactate in the cytosol. Cancer cells convert over 85% of the pyruvate into lactate even in the presence of oxygen, (aerobic glycolysis). Aerobic glycolysis results in minimal generation of ATP compared to oxidative phosphorylation. B. Global overview of active metabolic pathways in a proliferating cell: Glycolytic intermediates are precursor substrates for the biosynthesis of nucleotide via the pentose phosphate pathway (PPP) and amino acid biosynthesis. Increased production of lactate in the cells creates an acidic environment that alters the glutaminolysis pathway, thus contributes to lipid synthesis. Enhanced glycolysis thus can cater cellular metabolic demand. These metabolic pathways are directly under the control of growth factor signaling pathways, tumor suppressors and oncogenes. The activation of mitogenic PI3K signaling enhances glycolytic influx by directly controls key enzymes involved in glycolysis pathway such as Glucose transporter, Hexokinase and Phosphofructokinase. The tyrosine kinase activation inhibits the enzyme pyruvate kinase M2, thus making the glycolytic intermdiates available for biosynthesis of macromolecules. Presence of TP53, a tumor suppressor decreases the influx of glycolytic intermediates into PPP pathway, thus reduces proliferation in response to stress. The tumor suppressors are shown in red, and oncogenes are in green and key metabolic enzymes are shown in blue.

Enhanced glycolysis and lipid biosynthesis is characteristic feature of cells with high metabolic demand. Studies show that intermediates of glycolysis and TCA cycle are precursors for many macromolecular biosynthetic pathways (Fig. 6B). For instance, glucose along with many other amino acids are the precursors for pentose phosphate pathway (PPP), the metabolic pathway that generates nucleic acids; glycolytic intermediates are precursor substrates for fatty acids and non-essential amino acids biosynthesis (Bauer et al., 2005; Deberardinis et al., 2008.; Hatzivassiliou et al., 2005; Kuhajda et al., 1994; Pizer et al., 1996). Thus, suggesting that enhanced aerobic glycolysis in cancer or proliferating cells cater to metabolic needs of the cell.

Metabolic reprogramming in cancer cells is caused by activation of certain oncogenes such as *PIK3CA*, inactivation of tumor suppressors such as *TP53*, and sustained aerobic glycolysis (Ward and Thompson 2012; Babbar and Sheikh, 2013; Dang, 2012; Hanahan and Weinberg, 2011; Maroon et al., 2015; Seyfried et al., 2011; Solaini et al., 2010). Known inducers of metabolic reprograming in glioma are briefly discussed below:

(i) Cancer cells adapt to hypoxic conditions by stabilizing hypoxia-inducing factors (HIF1 and HIF2). Stabilization of HIF factors occurs not only during hypoxic conditions but also during inactivating mutations in tumor suppressors. The HIF factors regulate numerous genes involved in glycolytic pathways (Kucharzewska et al., 2015; Lu et al., 2002; Xiang et al., 2011; Zhou et al., 2011). Stabilization of HIF factors thereby triggers the glycolytic switch.

(ii) Mutations in *IDH1,* key metabolic enzyme involved in the conversion of isocitrate to α Ketoglutarate (α-KG) in TCA cycle, causes gain of a novel enzyme function that converts isocitrate into 2 hydroxy glutarate (2HG). This alters mitochondrial respiration in the cells. Over 80% of the LGGs are mutated for *IDH1,* which results in dysfunctional oxidative phosphorylation in gliomas (Mustafa et al., 2014; Wolf et al., 2010 ; Zhang et al., 2013).

(iii) Constitutively active RTK receptor genes *EGFR*, *PDGFR* or amplification of *PIK3CA,* induce tumor proliferation. Activation of PI3K/Akt pathway targets mTOR, a major downstream cell metabolism regulator that regulates key genes involved in

glycolysis. Activation of mTOR, a cell metabolism regulator, triggers the glycolytic switch (Beckner et al., 2005; Elstrom et al., 2004; Tanaka et al., 2015).

(iv) Myc is an important regulator of growth factors that controls cell proliferation. Myc directly regulates metabolic enzymes involved in glutamine uptake. Myc is overexpressed in GBM thereby contributes to Warburg effect in glioma cells (Annibali et al., 2014).

(iv) TP53, also known as the guardian of the genome, is an important negative regulator of cell proliferation. During cellular stress, TP53 decreases the influx of glycolytic intermediates into metabolic pathways such as in PPP thereby reducing cells ability to proliferate. Thus, loss of *TP53* results in constant influx of glycolytic intermediates that are precursor substrates to many macro-molecular biosynthesis. Loss of function mutation in *TP53* is a frequent event in GBM (Annibali et al., 2014).

1.7. MITOCHONDRIA

Mitochondria are small, semi-autonomous double membrane organelles that cater to bioenergetic needs in the cell. Therefore, they are also known as the powerhouse of cell. These organelles are hub to many biosynthetic pathways and play a crucial role in regulating cell metabolism (Harbauer et al., 2014; Kornmann and Walter, 2010; Scheffler, 2007). The functional importance of mitochondrial membranes and the matrix within are detailed below:

The outer mitochondrial membrane (OMM) is lipid bilayer similar to cellular plasma membrane. It contains large number of integral membrane proteins called porins that allow the transport of molecules between cellular cytosol and mitochondria. In addition, the OMM hosts a range of enzymes including monoamine oxidase, NADH-cytochrome c reductase that participate in fatty acid biosynthesis, oxidation of epinephrine (neurotransmitter), and degradation of tryptophan. The OMM can attach to the endoplasmic reticulum (ER). This helps in maintaining mitochondria-ER calcium signaling, as well as in lipid transfer between the two organelles. The inner mitochondrial membrane (IMM) is compartmentalized into folded junctions called the cristae. The IMM hosts a range of enzymes including the respiratory super complexes (RSC) (I-IV) and the ATP synthase. Therefore, IMM is the primary site for mitochondrial respiration (OXPHOS). Mitochondrial matrix enclosed by IMM contains

mitochondrial genome and hosts TCA or Krebs cycle. In addition, the matrix often remains filled with enzymes that are required for TCA cycle and fatty acid oxidation. Because of their key role in regulating cell metabolism and survival, they control major cellular functions including cell growth, invasion, differentiation, and death (Agorreta et al., 2014; Ahn and Metallo, 2015; Antico Arciuch et al., 2013).

1.7.1. Mitochondrial dynamics

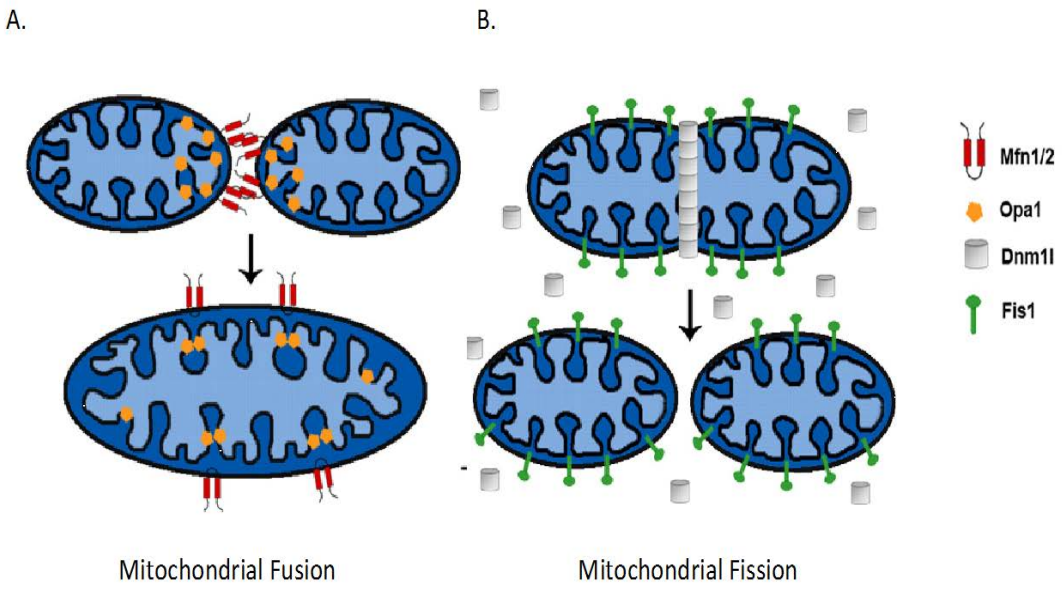
Mitochondrial organelles are highly dynamic in nature. They undergo permanent cycles of fusion and fission. Fission serves to generate new mitochondria, through a process called *mitochondrial biogenesis*, and to eliminate dysfunctional mitochondria by the process termed *mitophagy* (Chen and Chan, 2009; Twig et al., 2008).

Mitochondria contain their own genome that is highly susceptible to mutations. Mitochondrial fusion and fission cycles allow efficient mixing of mitochondrial DNA content, and are required for quality control of the organelles in order to maintain *mitochondrial genome integrity* (Ni et al., 2015). Mitochondrial dynamics are tightly regulated by fusion-fission machinery. Disruption of fission-fusion balance alters cell metabolism (Babbar and Sheikh, 2013; Boland et al., 2013).

1.7.1.1. Molecular machinery

The subtle balance between fission and fusion cycles is tightly controlled by large GTPases in the dynamin family that are evolutionarily conserved. The fission and fusion processes involve the division and fusion of mitochondrial membranes. GTPases mitofusins (MFN1 and MFN2) and optic atrophy 1 (OPA1) are principle regulators of mitochondrial fusion. Mitofusins help the organelles to tether and fuse their OMM while OPA1 regulates the fusion of IMM. In addition, OPA1 governs mitochondrial cristae remodeling and helps to maintain the membrane potential gradient required to carry out the mitochondrial respiration in the organelles (Frezza et al., 2006; Olichon et al., 2003). In the cristae junctions, OPA1 holds cytochrome c, an important initiator of mitochondrial apoptotic pathway. The release of cytochrome c triggers the caspase cascade causing cell death (Cipolat et al., 2006.; Olichon et al., 2003; Patten et al., 2014).

Stimuli like decreased energy status; cellular stress and apoptosis activate mitochondrial fission. A number of fission factors such as Mid 49, 51 and Mitofusin fission factor (MFF) together with Fission factor 1 (Fis1) recruit Dynamin-related protein 1 (DRP1) or Dynamin like protein 1 (Dnm1) from the cytosol to sites on ER that are in contact with OMM (Frank et al., 2001; Smirnova et al., 2001; Youle et al., 2012). Recruited DRP1 then forms ring like clusters around the mitochondrial membranes and helps to pinch off the fused organelles by constriction resulting in mitochondrial fission (Fig. 7) (Westermann, 2012).



Mandemakers et al., 2007

Fig 7. Simple schematic overview of mitochondrial fusion-fission components: A. Mitochondrial fusion – the Mitofusins (MFN1 and MFN2) regulate the fusion of OMM. The Optic Atrophy 1 (OPA1) located on the IMM and regulates the fusion of IMM. B. Mitochondrial fission – Dynamin related Protein (DRP1) or Dnm1l is recruited to ER, that interacts directly or indirectly with Fission factor 1 (FIS1). The DRP1 forms a coating surrounding the elongated mitochondria and helps to constrict and pinch off the organelles. Core proteins involved in the mitochondrial involved in fission-fusion process are shown in the schematic diagram.

1.7.2. Mitochondrial dynamics and cell invasion

Mitochondrial fission and fusion play important roles in cellular processes such as maintaining calcium homeostasis, generation and distribution of ATP, generation of ROS, and cell metabolism. Thus, mitochondria represent the central metabolic

organelle, as they coordinate cellular metabolism and cell response to a given stimuli (Muliyil and Narasimha, 2014; Park et al., 2012; Xie et al., 2015).

1.7.2.1. Increased levels of ROS contribute to tumor cell invasion

During OXPHOS, electrons are delivered through mitochondrial respiration super complexes (RSC) that establish a proton gradient, which facilitates ATP synthesis. However, during ETC, electrons escape from the RSC, especially from complex I and III and reacts with oxygen to form superoxide radicals (ROS). Under physiological conditions appropriate levels of ROS are required to ensure redox balance and proliferation signals in the cell (Pelicano et al., 2003). Enhanced ROS production primarily from the mitochondria is one of the key features observed in cancer cells (Boland et al., 2013). This is mainly attributed to inefficient electron transport during the mitochondrial respiration, enhanced metabolic demand in the cells, reduced ROS scavenging and altered mitochondrial dynamics (Schumacker 2006; Hamanaka and Chandel 2010; Sena and Chandel 2012). The accumulation of ROS causes intracellular damages like DNA damage due to oxidation, inactivation of enzymes such as PTEN, protein oxidation. In fact, increased accumulation of ROS promotes mitochondrial fission and triggers the release of cytochrome c from IMM (Yu et al., 2005). Several studies suggest that enhanced ROS production in Hela, glioma, mouse embryonic fibroblasts, cortical neuronal cultures promotes cell invasion, while beyond the tolerable limits induces autophagy in the cell (De Vos et al., 2005; Liot et al., 2009; Pletjushkina et al., 2006; Westermann, 2012; Kolli-Bouhafs et al., 2014). In fact, in 2014, using multiple breast cancer cell lines such as 4T1,B16F10 and MDA-MB231, and xenograft mouse model, LeBleu et al. demonstrated that mitochondrial ATP is required for breast cancer cell invasion (LeBleu et al., 2014). Overall, these observations suggest that accumulation of ROS due to imbalance of mitochondrial dynamics or defects in mitochondrial respiration contributes to cancer cell invasion (Boland et al., 2013).

1.7.2.2. Metabolic enzymes contribute to glioma invasion

In 2005, Yu et al. demonstrated *in vitro* using rat liver cells and myoblasts that high glycolytic influx in these cells resulted in enhanced ROS production and promoted mitochondrial fission in the cells (Yu et al., 2005). In fact, Glucose-6-phosphate (G6P), a key enzyme of glycolysis pathway that is over-expressed in GBM,

contributes to glioma invasion (Abbadi et al., 2014). Lactate dehydrogenase A (LDH-A) converts pyruvate to lactate during aerobic glycolysis. Baumann et al. showed that lactate induces glioma cells to secrete TGFβ2 that enhances glioma migration. Phoshoglycerate dehydrogenase (PHGDH) is a key enzyme that utilizes the intermediates from glycolysis pathway for serine biosynthesis, and is highly expressed in astrocytic tumors and more aggressive cancer types (Liu et al., 2013). Inhibition of this enzyme down-regulates expression of *VEGF*, *MMP2* and reduces overall aggressiveness of glioma cells by reducing proliferation and invasion. Thus, increased expressions of metabolic enzymes contribute to glioma invasion (Baumann et al., 2009, Abbadi et al., 2014, Liu et al., 2013).

1.8. PRELIMINARY OBSERVATIONS

Astrocytomas, oligoastrocytomas, GBM, and to some extent oligodendrogliomas show diffused infiltration irrespective of their WHO grade (Louis et al., 2007). In order to study the infiltrative behavior and identify genes involved in glioma invasion, glioma biopsies were classified based on their invasive ability and subjected to transcriptomic analysis.

Measuring thickness of the infiltration zone (IZ) surrounding the tumor mass, visualized using magnetic resonance imaging (MRI) can help assess the infiltrative nature of glioma. Gliomas were classified based on their infiltration grade (IG): IG-1 (0-3 mm), IG-2 (3-10 mm), and IG-3 (>10 mm). Transcriptomic analyses on glioma biopsies (n=20), showed that mRNA levels of *BEHAB*, a known inducer of glioma invasion, *SOX2* and *HEY1* correlate with increasing IG (unpublished data). This association suggested a potential role for *SOX2* and *HEY1* in glioma invasion (Fig. 8).

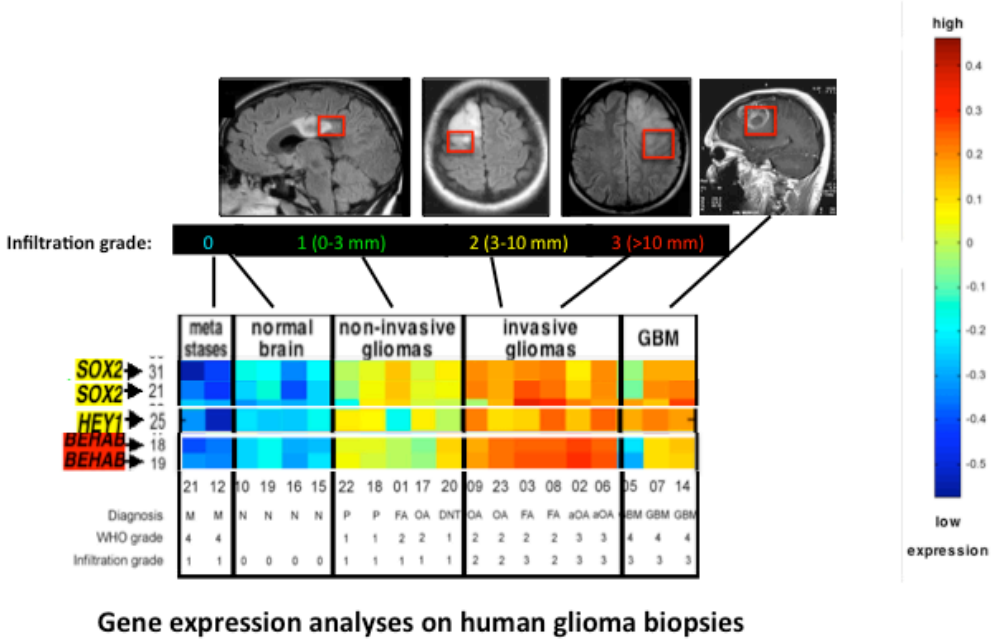


Fig 8. Glioma classification based on their infiltration grades (IG): *Top*: MRI allows classification of tumors based on the thickness of their infiltration zone (IZ). Based on the IZ (the distance between tumor periphery and core) gliomas were classified into IG I (0-3 mm), IG II (3-10mm) and IG III (10mm). *Bottom*: Gene expression analyses on glioma biopsies suggest that *BEHAB*, *SOX2* and *HEY1* mRNA levels correlate with high infiltration grades. BEHAB/Brevican is an ECM molecule and a known inducer of glioma invasion and is used as a positive control to validate the analyses. Gene expression analysis was performed on 20 glioma samples in total and is an unpublished work of Prof Dr med Luigi Mariani.

1.9. SOX2

SOX2 (**S**ex determining region of Y chromosome- related High Mobility Group (HMG) b**ox 2**) is a transcription factor that regulates cell fate determination and differentiation. SOX2 is expressed in the embryo and is required to maintain embryonic cell stemness. In neural progenitors, expression of *SOX2* prevents neuronal differentiation and maintains neural cell stemness (Graham et al., 2003; Heavner et al., 2014). The 32 KDa transcription factor has numerous effector genes and is a master regulator of many vital cellular functions such as cell survival and cell differentiation (Agathocleous et al., 2009; Ding et al., 2012; Hawkins et al., 2014).

In developmental mouse model, genetic ablation of *SOX2* is embryonic lethal. Conditional knockout of *SOX2* at various developmental stages results in defective stem cell proliferation, neural crest cell migration, failed wound healing, and brain abnormalities (Ferri et al., 2004; Pevny and Nicolis, 2010; Que et al., 2009; Zenteno et al. 2005). Aberrant SOX2 expression is associated with many developmental disorders like progressive hearing loss, developmental malformations of the eye, and is also observed during cancer development (Hever et al., 2006; Rodriguez-Pinilla et al., 2007).

1.9.1. Oncogenic role of *SOX2*

Gain of *SOX2* function is oncogenic in many epithelial cancer types (Bass et al., 2009; Justilien et al., 2014; Watanabe et al., 2014). Focal amplification of *SOX2* locus (3q26.3) is frequently observed in GBM and in many other solid cancers such as squamous and small cell lung carcinoma (23-27%), esophagus (15%), and breast cancer (8-10%). Consistent with its crucial role in stem cell proliferation, *SOX2* gene amplification is strongly associated to tumorigenesis (Chen et al., 2014).

Recently, it was shown that SOX2 expressing cancer stem-like cells are the founding population of tumor relapse after treatment with anti-mitotic drugs and contributes to therapy-induced chemoresistance in breast cancer, medulloblastoma, and squamous cell carcinoma (Boumahdi et al., 2014; Piva et al., 2014; Vanner et al., 2014).

1.9.2. SOX2 contributes to tumor cell invasion

Literature suggests conflicting roles for SOX2 in cancer cell migration, including in the field of glioma, as evidence support overexpression as well as reduced SOX2 mRNA levels, contribute to cancer cell invasion and migration (Annovazzi et al., 2011; Feng et al., 2013; Stolzenburg et al., 2012). Overall, SOX2 expression is correlated with tumor cell motility (Girouard et al., 2011). Although the involvement of SOX2 is clearly depicted in cancer invasion, the molecular mechanism remains elusive.

1.10. HEY1

HEY1 (Hairy/Enhancer of Split Related with YRPW motif 1) belongs to basic-helix-loop-helix-orange (bHLH) family of transcription factors. Members of the family of Hairy/Enhancer of split (HES 1-7 and HEY-1,2 and L) are transcriptional repressors. The HES and HEY family of bHLH transcription factors are direct transcriptional targets of Notch signaling pathway. *HEY1* is overexpressed in GBM and its expression in GBM correlates with tumor grade and progression.

The role of *HEY1* and its contribution to glioma invasion is discussed in detail in Chapter II of this thesis.

1.11. AIM OF THE THESIS

My PhD project has been devoted to understand the molecular mechanisms involved in glioma invasion. Our preliminary observations on human glioma biopsies showed that *SOX2* and *HEY1* mRNA levels correlate with glioma invasiveness.

Thus the aims of my project have been:

1. Chapter I: The gene for *SOX2* localizes to 3q26.3 in the human genome, a region frequently amplified in glioma. The locus 3q26-29 contains key glioma oncogene *PIK3CA*, and mitochondrial dynamics regulators *MFN1* and *OPA1*. Therefore, our aim was to investigate (i) whether 3q26-29 genes participate in glioma invasion and (ii) whether SOX2 is a regulator of this gene cluster.

2. Chapter II: To explore the role of *HEY1*, an effector of notch signaling pathway, in glioma invasion.

CHAPTER I

Contribution of 3q26-29 gene cluster to glioma invasion

2.1. 3q26-29 gene cluster

The gene for SOX2 localizes to 3q26.3 in the human genome, in close proximity to the glioma oncogene *PIK3CA* (2.1Mb upstream of *SOX2* gene), and the genes that regulate mitochondrial fusion *MFN1* (2Mb upstream) and *OPA1* (12Mb downstream) (Fig. 9).

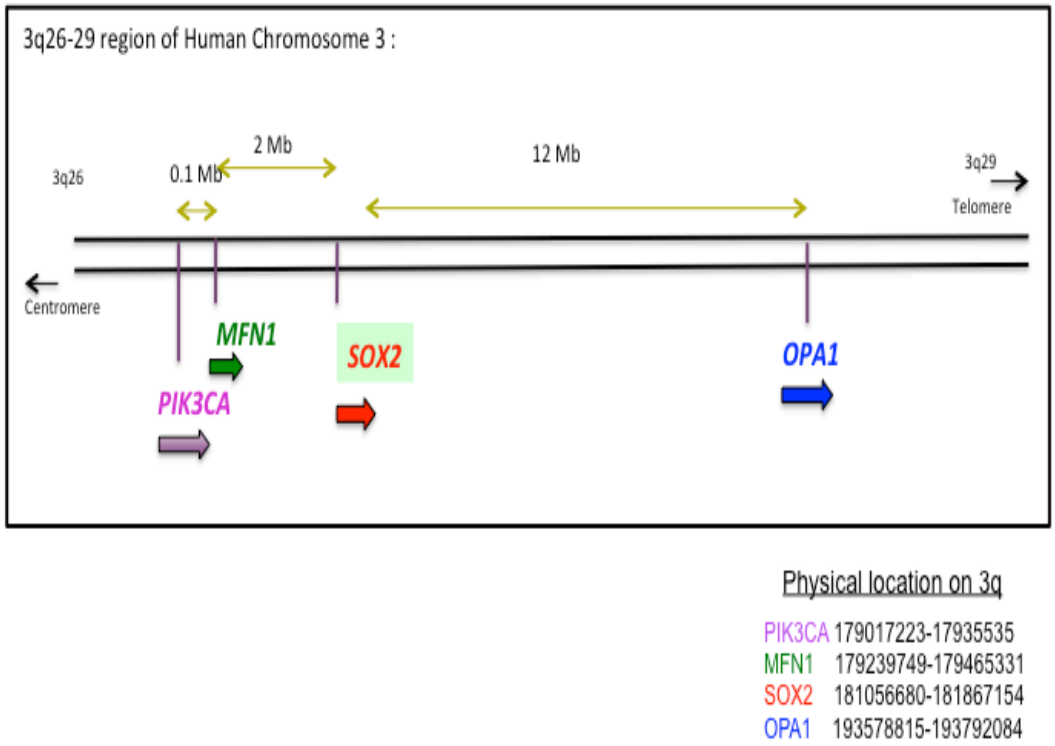


Fig 9. Schematic representation of strategic localization of *SOX2*: *SOX2* colocalizes with *PIK3CA*, gene for key signaling component of RTK/PI3K pathway, *MFN1* and *OPA1*, regulators of the mitochondrial fusion process.

2.2. PIK3CA

2.2.1. PIK3CA during development

During glial cell development, PI3K signaling pathway is activated by brain derived neurotrophic factors (BDNF), glial cell derived neurotrophic factor (GDNF), and insulin-like growth factors (IGF). The PI3K signaling pathway promotes the production of myelin in oligodendrocytes and schwann cells (Kristjan et al., 2005). The PI3K-Akt signaling pathway mediates cell proliferation and survival by regulating glucose metabolism in the cells (Danielsen et al., 2014).

2.2.2. Oncogenic role of *PIK3CA*

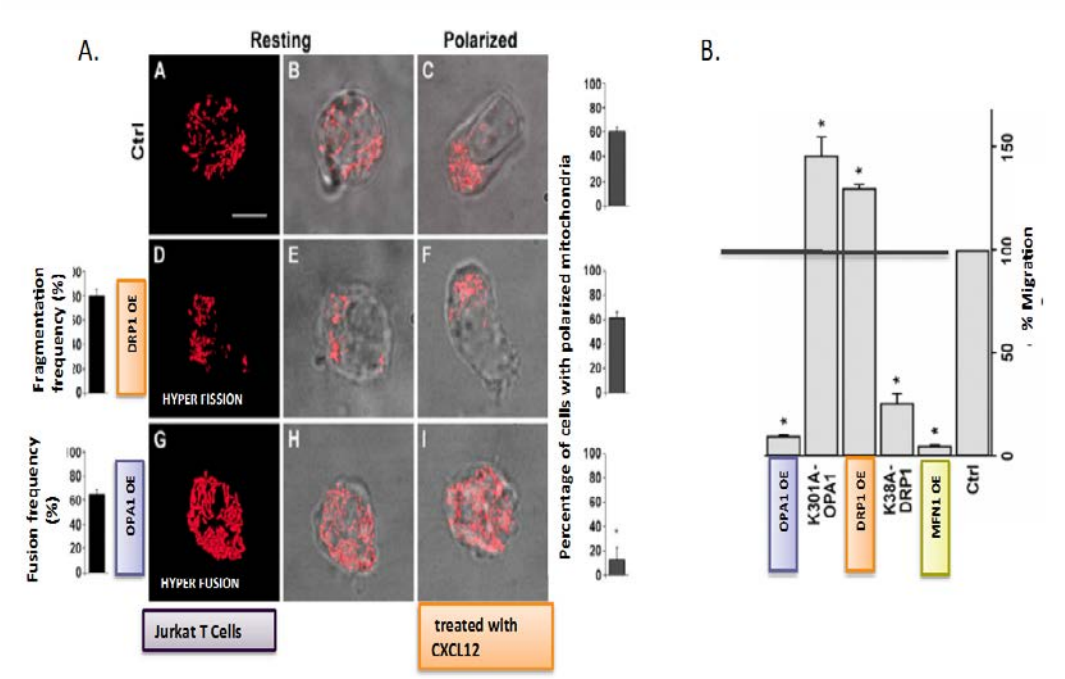
PIK3CA is a well-characterized oncogene that is amplified in many cancer types (Katso et al., 2001; Shayesteh et al., 1999). In gliomas, *PIK3CA* is mutated and amplified in over 14-17% of the samples (The Cancer Genome Atlas Research Network, 2008). *PIK3CA* often harbors somatic missense activating mutations that lead to permanent activation of the PI3K signaling pathway (Gallia et al., 2006; Samuels et al., 2004). Loss of *PIK3CA* reduces glioma cell proliferation (Weber et al., 2011). Activating *PIK3CA* mutation via the Akt – mTOR signaling pathway induces cell proliferation and is associated with colorectal cancer cell invasion (Samuels 2005).

2.2.3. PIK3CA contributes to tumor cell invasion

PIK3CA, like *SOX2*, is associated with cancer cell invasion. However, the role of *PIK3CA* is not well elucidated. In 2005, Samuels et al. showed that in colorectal cancers, permanent *PIK3CA* activation leads to enhanced cancer cell motility which was inhibited by LY294002, a general PI3K inhibitor. However in 2015, Caino et al. showed that, glioma cells and prostate cancer cells treated with GDC-0941, PIK3CA inhibitor, became highly invasive. The mechanism behind such activation or inhibition of cell motility is yet to be explored (Caino et al., 2015; Samuels et al., 2005).

2.3. MFN1 AND OPA1

MFN1 and OPA1 are core regulators of mitochondrial fusion (detailed in section 1.7.1). Therefore, they govern mitochondrial dynamics. In migrating lymphocytes, mitochondria localize between the nucleus and the trailing edge of the migrating cells. Disrupting the functions of MFN1 and OPA1 alters the migrating capacity of the cells (Campello et al., 2006). In epithelial cells, down-regulation of *OPA1* resulted in increased mitochondrial fission and increased cell migration velocities during chemokine-induced epithelial cell migration. As a consequence of knockdown mitochondrial morphology is permanently altered and random cell motility is induced (Fig. 10) (Campello et al., 2006; Desai et al., 2013). Many studies show that the fission-fusion balance is frequently disturbed in cancer cells. This perturbation is associated with tumor cell motility (Lebleu et al., 2014; J. Zhao et al., 2013).



Adapted from Campello et al., 2006

Fig 10. Mitochondrial fusion abolishes lymphocyte migration: A. Upon chemotactic stimulation with chemokine, CXCL12, DRP1 overexpression (OE) mediated mitochondrial fission promotes cell polarization in Jurkat T cells, whereas OPA1-OE mediated mitochondrial fusion abolishes cell polarization ability in the cells B. Summary of migration assay showing enhanced mitochondrial fission (DRP1 OE) in Jurkat T cell promotes cell migration which was abolished in cells exhibiting enhanced mitochondrial fusion (OPA1 OE).

2.4. GENOMIC ALTERATIONS AT 3q26-29 LOCUS IN GLIOMA

2.4.1. Glioma samples from TCGA repository

Comprehensive analysis of the GBM genome, based on copy number analyses (CNA) on 543 GBM samples, reveal significant focal amplification at 3q26 and deletion at the 3q29 locus (Brennan et al., 2013). Similar patterns are observed in LGG (upon CNA on 717 LGG samples). Although the focal amplification at 3q26 and deletion at 3q29 were significant, they are less pronounced as compared to GBM (Suzuki et al., 2015) (Fig. 11).

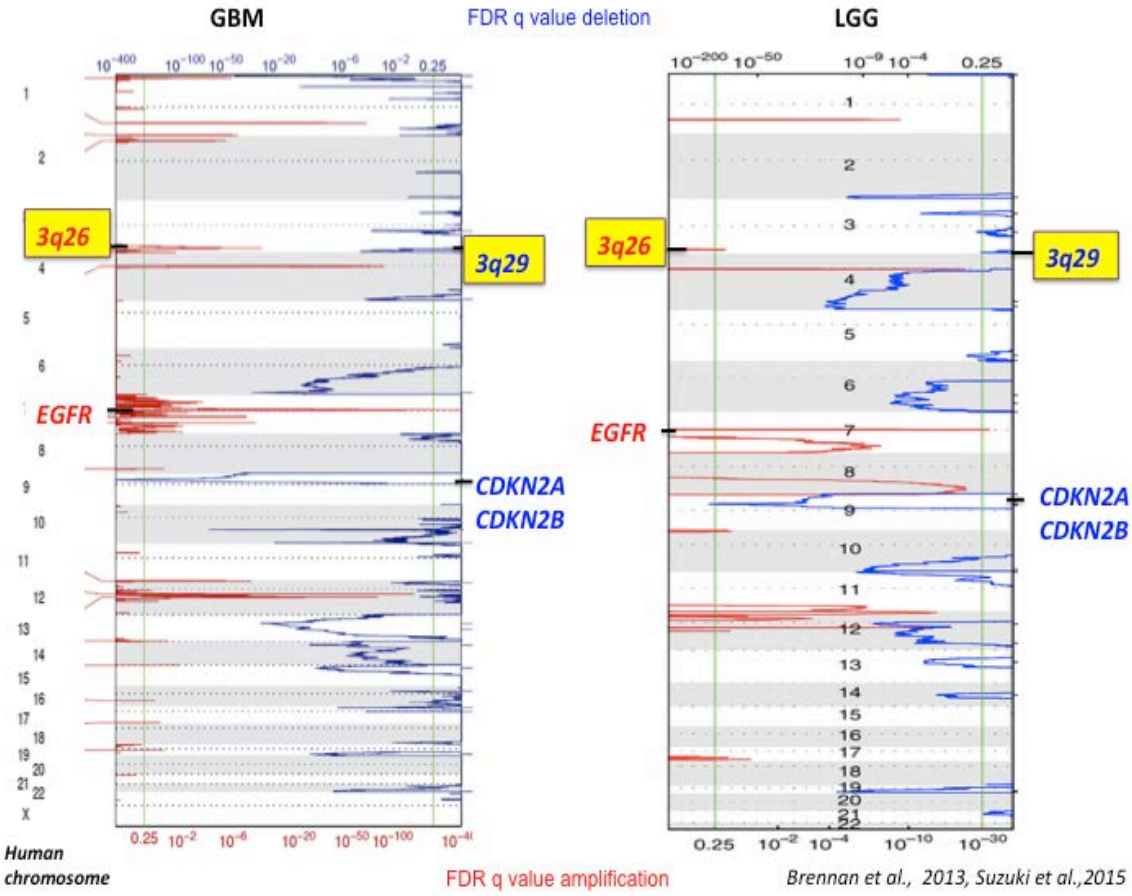


Fig 11. 3q26-29 genetic alterations in glioma: Somatic landscapes showing genetic alterations in gliomas, highlighted are 3q26 (amplification), 3q29 (deletion), EGFR (amplification), and CDKN2A/2B (deletion). A. Schematic representation of recurrent copy number aberrations in 543 samples in GBM B. Schematic representation of recurrent copy number aberrations in 717 samples in LGG (Grade II-III). Statistically significant focally amplified (red) and deleted (blue) regions are shown in the plot.

2.4.2. Glioma biopsies from BTB tumor bank

A total of 68 GBM and 32 LGG are used for copy number analysis (CNA) of 3q26-29 genes from BTB-Lab tumor bank. CNA show that focal amplification of *SOX2* (3q26.3) is observed in 35% of GBM and in 17% of LGG samples. Both GBM and LGG show homozygous deletion of *OPA1* (29% in GBM and 36% in LGG). A small population of 2-8% shows amplification of *PIK3CA*, *MFN1* or *OPA1,* while none was observed in LGG. In GBM and LGG, over 7% and 17% samples harbor *SOX2* deletion. These copy number variations (CNVs) in 3q26-29 genes are significant compared to well characterized somatic alterations such as *EGFR* amplifications (53%) and *CDKN2A* (33%) deletions in GBM (Fig. 12). In summary, glioma samples show significant genetic alterations at 3q26-29 loci. Focal amplification of *SOX2* (3q26.3) and homozygous deletion of *OPA1* (3q29) are the most frequent genetic aberrations observed in this region.

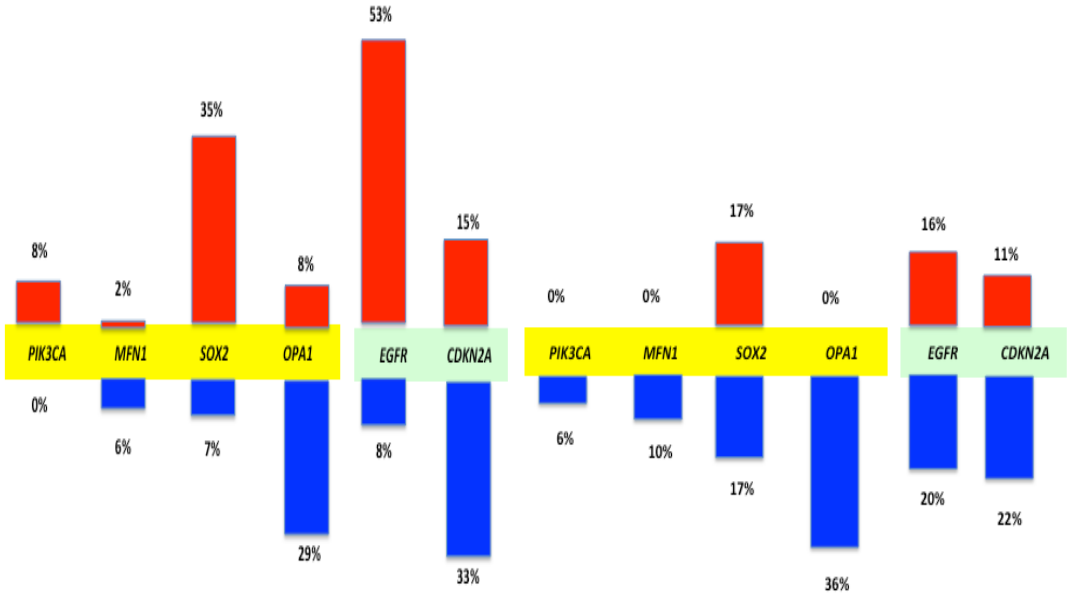


Fig 12. 3q26-29 genetic alterations in glioma samples (BTB tumor bank): Red bars indicate amplifications and blue bars indicate deletions. *Left*. Copy number analysis on GBM samples (n=68). The significance of these alterations in 3q26-29 genes can be estimated compared to frequency of *EGFR* amplifications and *CDKN2A* deletions (major somatic alterations known in GBM) in our samples. *Right*. Copy number analysis on LGG samples (n=32) significant alterations in 3q26-29 genes. (Experiment was performed by Cristobal Tostado and data analysis by PD Dr Jean-Louis Boulay).

2.5. CHAPTER I – WORKING HYPOTHESIS

Given that the hallmark feature of gliomas is their infiltrative nature, which results in tumor malignancy (inducing angiogenesis, therapy resistance and tumor relapse), understanding the molecular mechanisms governing glioma invasion will help in the development of novel strategies targeting glioma progression. Invasive gliomas display high mRNA levels of the gene for the transcription factor *SOX2*. SOX2 localizes to 3q26 in the human genome along with *PIK3CA*, *MFN1* and *OPA1*, a region that is significantly altered in gliomas. These genes (are known to) contribute to cancer cell invasion.

Therefore we hypothesized that:

1. 3q26-29 genes participate in glioma invasion

2. SOX2 regulates the 3q26-29 gene cluster and contributes to the invasive phenotype of the cells

3. CHAPTER I - RESULTS

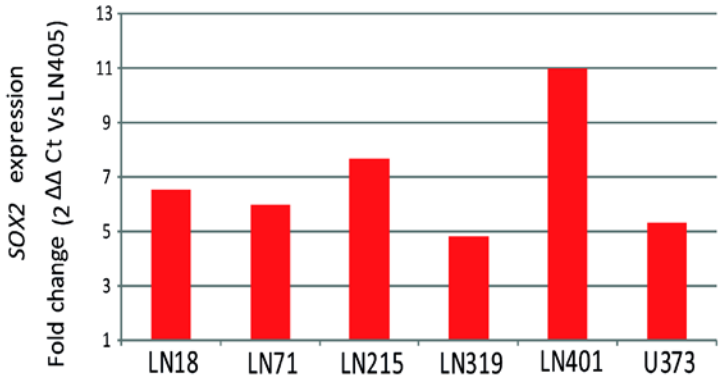
3.1. Selection of SOX2-expressing cell lines

3.1.1. SOX2 expression and localization in glioma cell lines

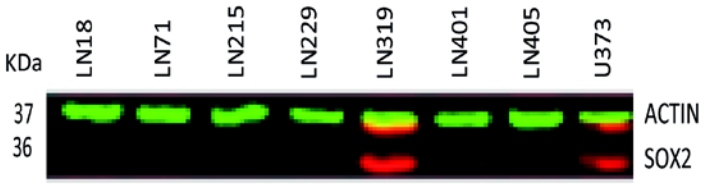
Our preliminary observations suggested that SOX2 was overexpressed in invasive gliomas (Fig. 13). In order to investigate the role of SOX2 in glioma invasion, we selected glioma cell lines based on SOX2 expression levels. We first analyzed *SOX2* mRNA levels in seven glioma cell lines by qRT-PCR. Results showed that LN405 cell line had the lowest *SOX2* mRNA levels; hence, data from other cell lines were normalized using LN405 levels as a baseline. U373, LN319, LN215 and LN401 expressed over 5-, 7- and 11- fold higher *SOX2* mRNA levels compared to LN405, respectively (Fig. 13A). We next performed Western blot analysis in order to estimate SOX2 protein levels. U373 and LN319 were the only SOX2 protein expressors among the cell lines studied (Western blot was performed by Alexandra Gerber) (Fig. 13B).

Nuclear localization of SOX2 indicates that the transcription factor is functionally active (Baltus et al., 2009). Immunofluorescence staining of glioma cells for SOX2 and examination by confocal microscopy revealed that LN319 cells had a higher number of cells exhibiting nuclear localization rather than cytoplasmic. U373 cells showed both nuclear and cytoplasmic SOX2 localization, whereas in LN215 cells SOX2 was predominantly in the cytosol (Fig. 13C).

A mRNA levels



B Protein levels



C Subcellular SOX2 localization

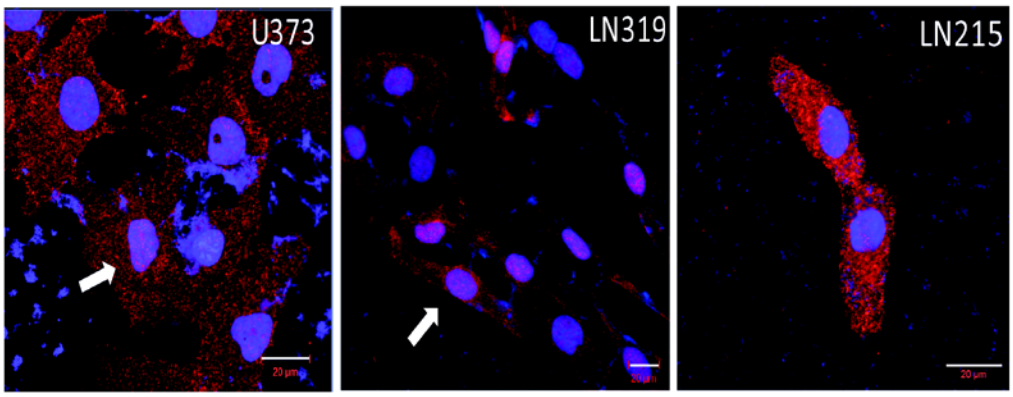


Fig 13. Selection of glioma cell lines for further experiments : A. qRT-PCR analysis for *SOX2* expression in 6 glioma cell lines compared to LN405 (the lowest *SOX2* expressing glioma cells). B. Western blot for SOX2 protein expression in the cells. C. Immunofluorescence on glioma cells (U373, LN319 and LN215) showing SOX2 (red) localization. Arrows (white) show nuclear localization of SOX2. Scale bars represent 20µm.

3.1.2. Expression of mitochondrial fusion proteins in glioma cell lines

We next performed Western blot analysis to study the expression of mitochondrial fusion proteins in glioma cells. Mitofusins (*MFN1, MFN2*) and Optic Atrophy 1 (*OPA1)* are regulators of mitochondrial fusion. Our results showed that glioma cell lines have variable expression levels of mitochondrial fusion proteins compared to gray matter (GM) and white matter (WM) controls (Fig. 14). This Western blot was performed at the Institute of Pathology at the University Hospital of Basel, Switzerland under the guidance of Dr Donato D'Alonso.

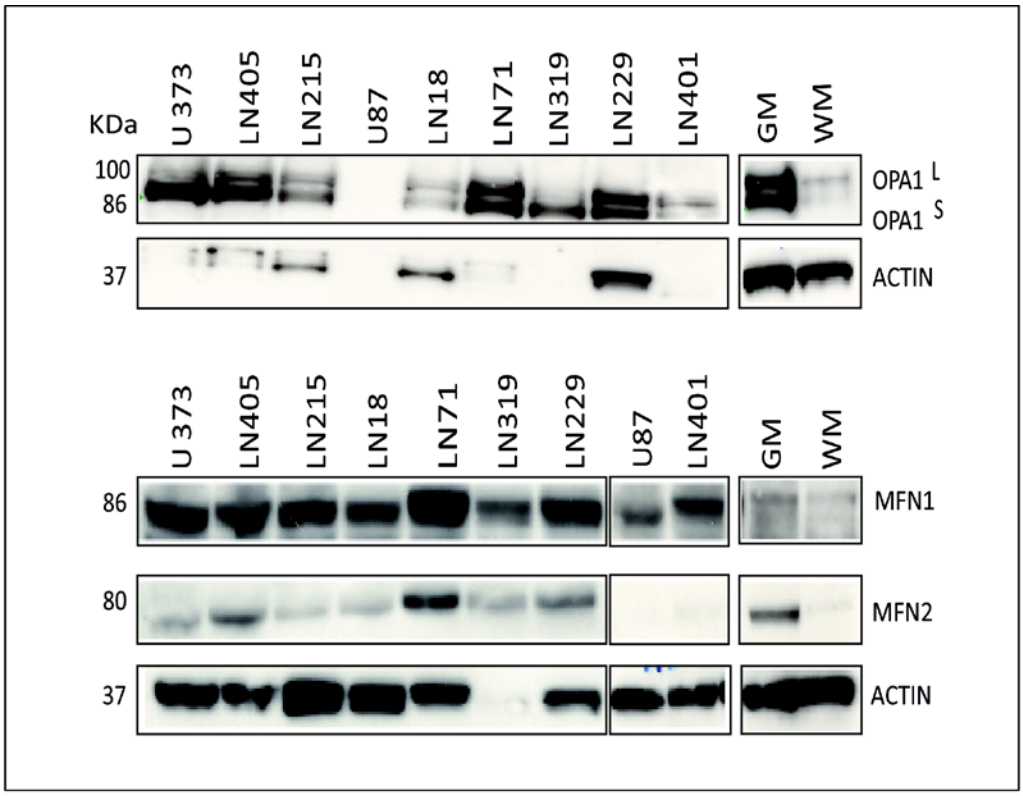


Fig 14. Selection of glioma cell lines for futher experiments : Western blot analysis of mitochondrial fusion proteins *Top.* Optic atrophy 1 long and short isoforms ($OPA1^{L}$, $OPA1^{S}$) compared to gray matter (GM) and white matter (WM) of non-neoplastic brain tissue control, *Bottom.* Mitofusins (MFN1 and MFN2) in glioma cells.

Based on SOX2 expression (mRNA and protein) and protein levels of MFN1 and OPA1, we selected LN319 and U373 glioma cell lines for further experiments.

3.2. Genetic inactivation of 3q26-29 genes

In order to study the function of these genes in both selected cell lines, 3q26-29 genes were individually knocked-down (KD) using lentiviruses expressing short hairpin RNA (shRNA) against *SOX2*, *MFN1*, *OPA1* and *PIK3CA*, respectively. We used $5X10^4$ infectious units (IFU) of virus. Glioma cells transduced with lentiviruses expressing GFP were used to measure transduction efficiency. The percentage of GFP positive cells was estimated by fluorescent activated cell sorting (FACS) in both cell lines to be over 80% in LN319 and 60% in U373, respectively (Fig. 15 and 16). Puromycin was used for long-term selection of KD cells.

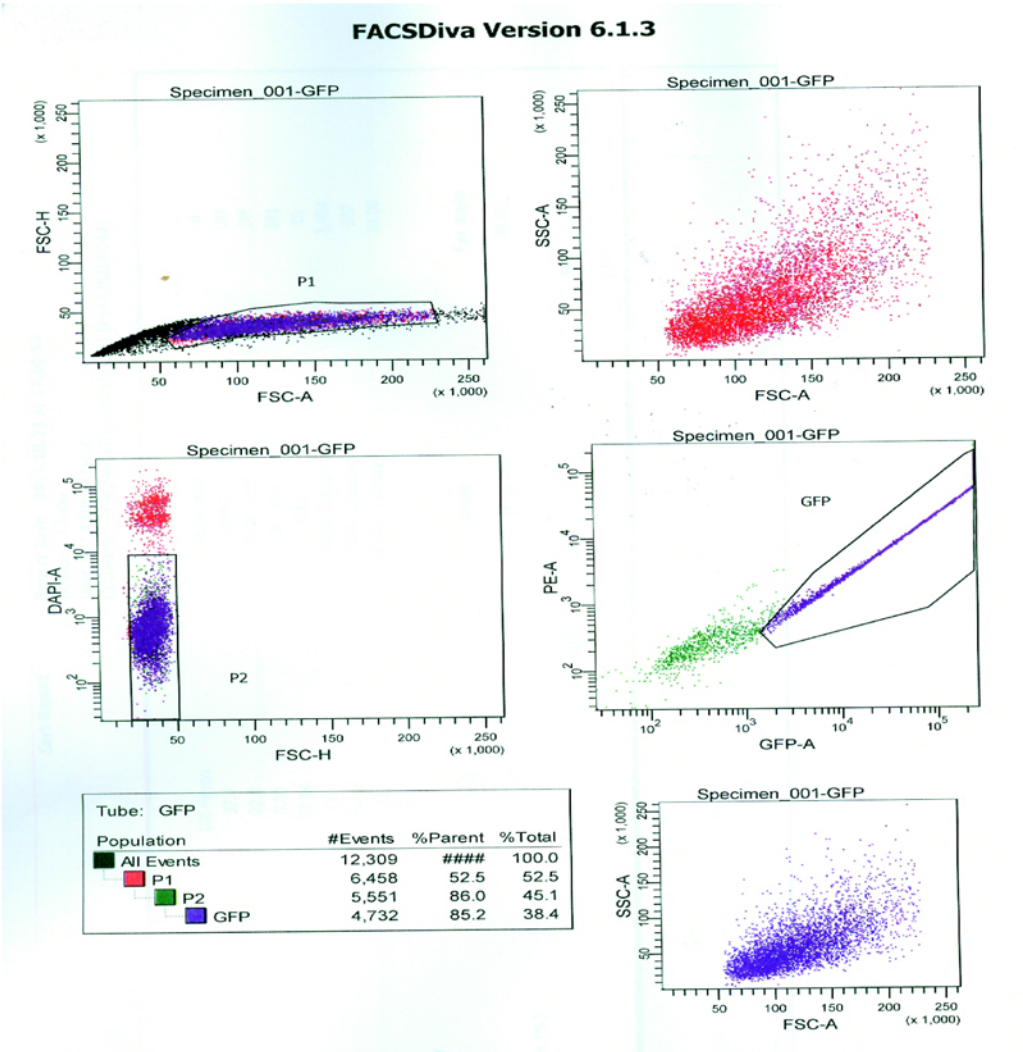


Fig 15. Estimation of lentiviral transduction efficiency in LN319 cells : LN319 glioma cells were transduced with GFP expressing lentiviruses. Transduction efficiency was measured using Fluorescence activated cell sorting (FACS). Side scatter - area (SSC A) vs Forward scatter area (FSC A) , FSC width (W) vs H (height) , SSC width (A) vs height (H) were used to gate single cells. Viable cells were gated with DAPI stained population and GFP positive cells were measured among the viable cells. Among viable, single cells percentage of GFP positive cells was estimated, as shown in table.

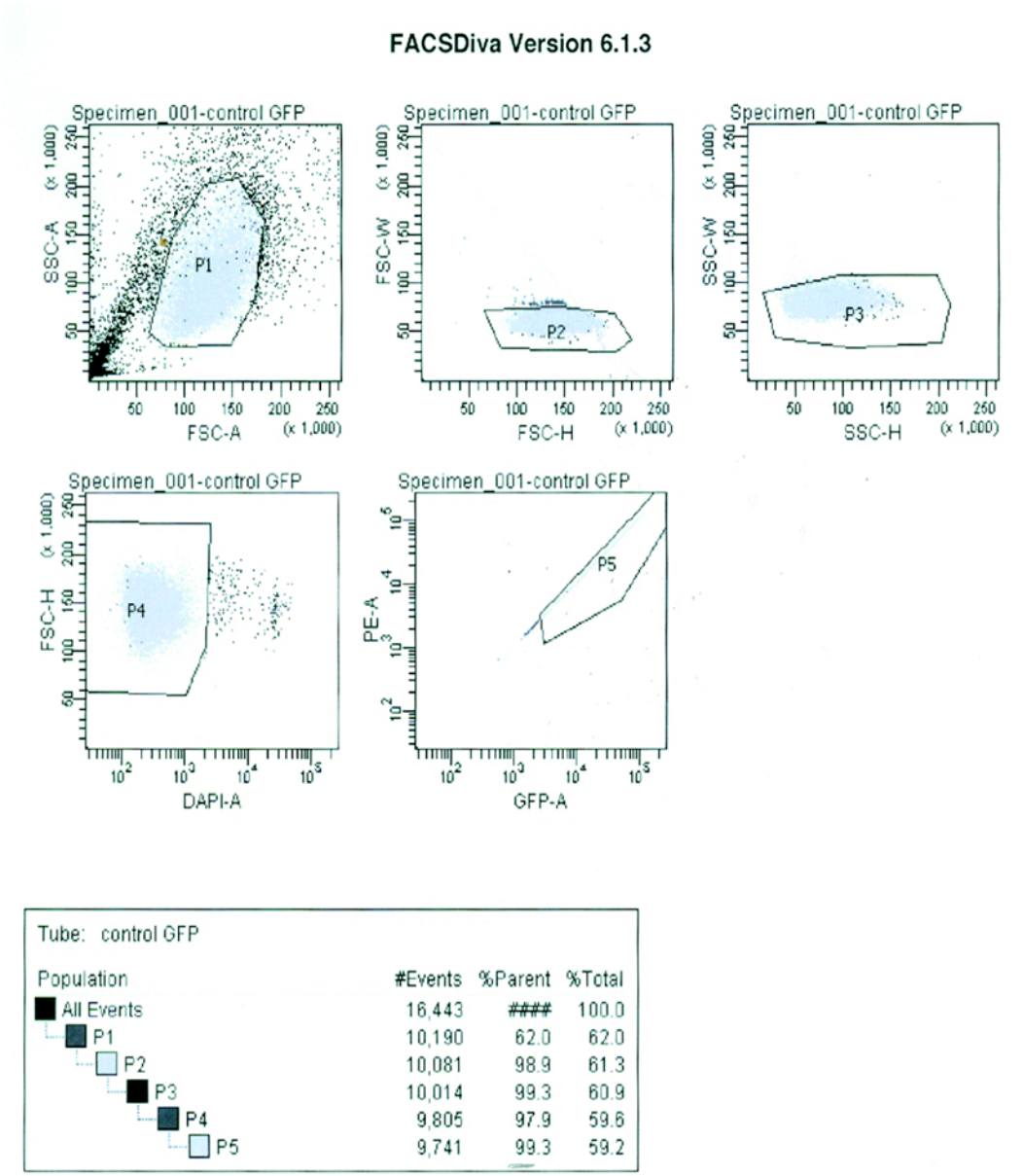


Fig 16. Estimation of lentiviral transduction efficiency in U373 cells : U373 glioma cells were transduced with GFP expressing lentiviruses. Transduction efficiency was measured using Fluorescence activated cell sorting (FACS). Side scatter - area (SSC A) vs Forward scatter area (FSC A) , FSC width (W) vs H (height) , SSC width (A) vs height (H) were used to gate single cells. Viable cell population was estimated by gating for DAPI , and GFP positive cells were measured among the viable cells. Among viable, single cells P5 was the estimated percentage of GFP positive cells, as shown in table.

In LN319 cells, we achieved over 6-, 8-, 23- and 5-fold decrease in mRNA levels of *SOX2*, *MFN1*, *OPA1* and *PIK3CA, r*espectively (Fig. 17A *Top*) and in U373 cells, decrease of 11-, 6-, 1.5- and 2-fold, respectively (Fig. 17B *Top*). KD of 3q26-29

genes were confirmed at the protein level using Western blot (Fig. 17A and 17B *Bottom*). The cells KD for the 3q26-29 genes will henceforth be designated as sh *SOX2,* sh *MFN1,* sh *OPA1* and sh *PIK3CA*.

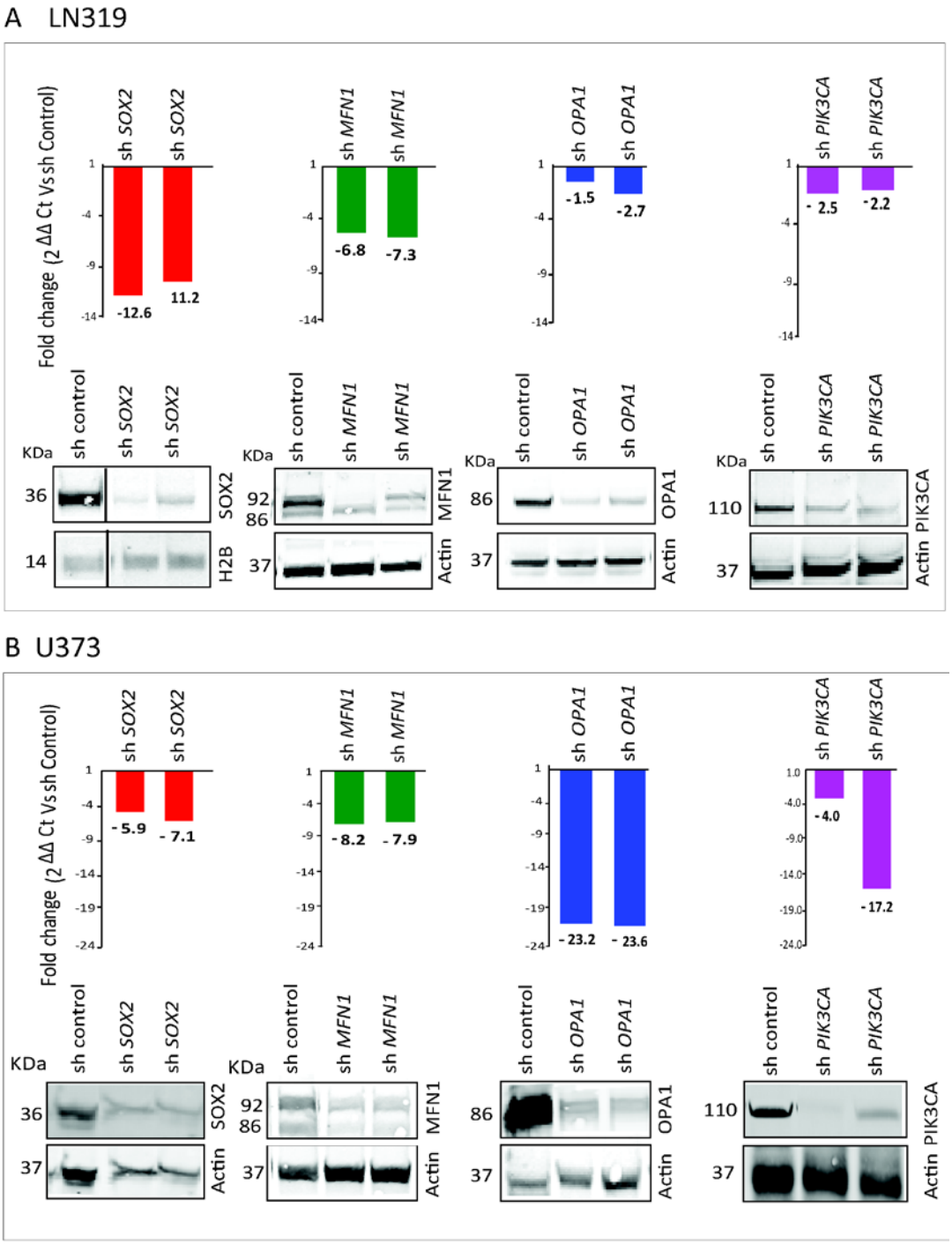


Fig 17. Knockdown of 3q26-29 genes in LN319 and U373 : Lentivirus expressing shRNAs against 3q26-29 genes were used for knockdown in glioma cells. A and B. *Top*. qRT-PCR analysis for gene expression. *Bottom*. Western blot analysis for the candidate genes respectively.

3.3. 3q26-29 gene inactivation alters cell motility

To assess the invasive potential of KD cells we performed matrigel-coated Boyden chamber invasion assay over 16 hours. Invasive cells were stained and counted.

Overall, we observed that LN319 were less invasive compared to U373 cells. The sh*SOX2* cells exhibited enhanced invasive phenotype compared to the sh scrambled control cells in the two cell lines. We observed over 2.5-fold and 3-fold increase in invasive cell numbers for LN319 and U373 respectively, compared to sh scrambled control cells (Fig. 18 *Top*).

Similarly, gene inactivation of the other three 3q26-29 genes (*MFN1*, *OPA1* and *PIK3CA*) resulted in over 2-fold increase in invasive cell numbers in LN319 cells compared to sh scramble controls (Fig. 18 *Bottom*). On the other hand, while sh*MFN1* cells in U373 displayed increased relative invasiveness, sh*OPA1* cells in U373 did not result in a significant increase in invasion (Fig. 18 *Bottom).*

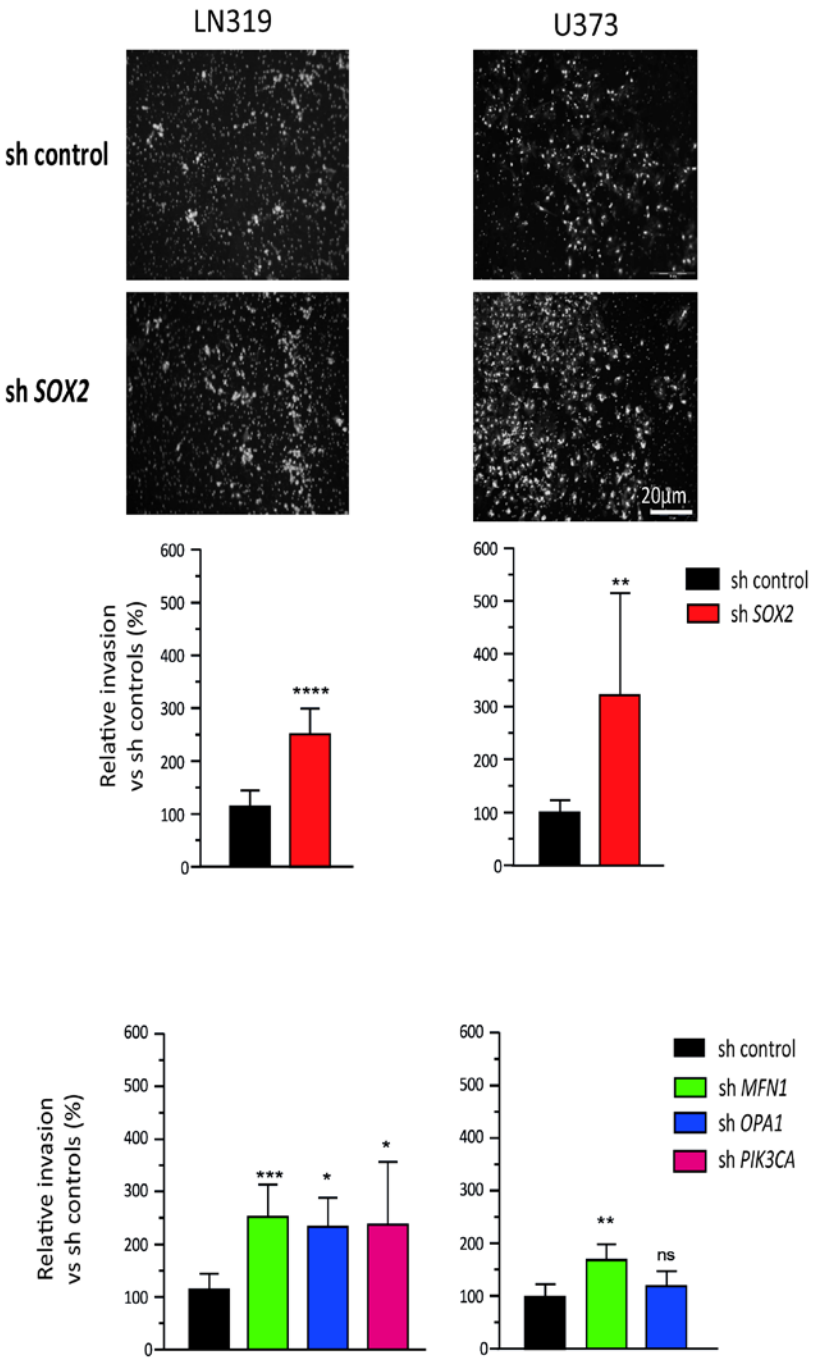


Fig 18. Knockdown of 3q26-29 candidate genes induces invasive phenotype in glioma cells : *Top*. Representative images of Boyden chamber assays on *SOX2* knockdown cells in LN319 and U373. Five microscopic fields per condition were selected to assess invasiveness of the cells vs sh-controls. *Bottom*. Quantification summary of Boyden chamber assays on knockdown cells. All assays were done in triplicates and at least 3 independent experiments in both cell lines. Unpaired two-tailed t tests were performed to determine the statistical significance. $p<0.01$ (**) and $p<0.005$ (***) and ns, non significant. Error bars represent standard deviation (mean±S.D).

In order to assess the migratory phenotype in KD cells, we performed wound-healing assays. Cells were allowed to migrate into scratch wounds created using 200µl pipette tips. The assays were carried out over 24 hours on a Cell IQ analyzer. The

percentage of wounded area closed at 24 hours was used as the final readout for the assay.

We observed that LN319 cells migrated slower than U373 cells. KD of *SOX2* resulted in an enhanced migratory phenotype in the two cell lines. We observed that sh*SOX2* cells covered 1.5-fold more area during the assay compared to sh scrambled control LN319 and U373 cells (Fig.19 *Top*.).

Similarly, gene inactivation of *MFN1*, *OPA1* resulted in an enhanced migratory phenotype in the two cell lines (Fig. 19 *Bottom*). We observed that KD of these genes in LN319 cells resulted in 1.5-fold more area coverage compared to sh scrambled control cells. Although we observed that sh*PIK3CA* cells covered over 1.5-fold more wound area, the results were not statistically significant. While, in U373, consistent with our previous observations, we found that sh*OPA1* cells showed over 1.5-fold more wound area coverage compared to sh control cells. The wound closure by sh*MFN1* cells was however, not significantly different compared to sh scrambled control cells.

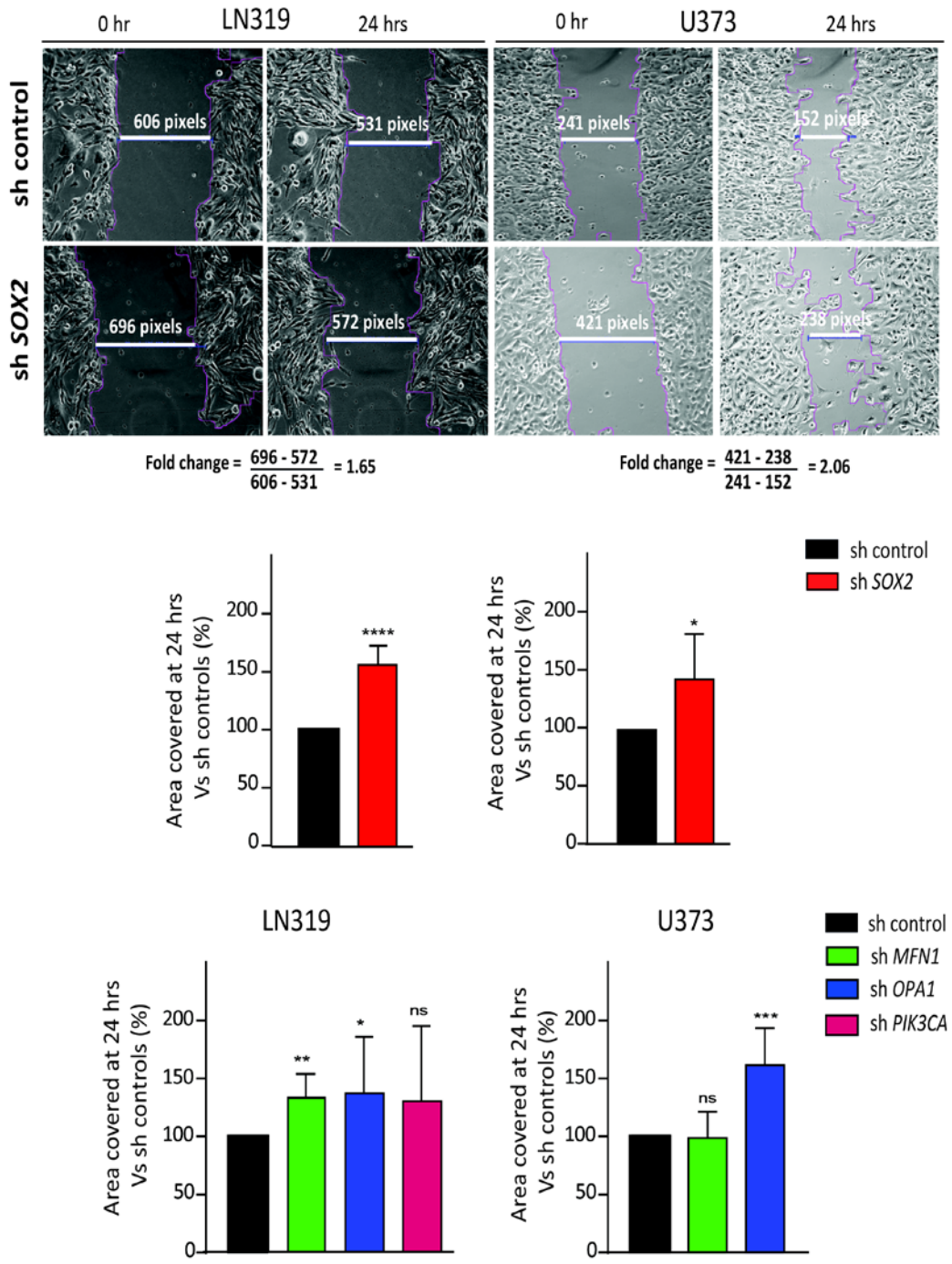


Fig 19. Knockdown of 3q26-29 candidate genes induces migratory phenotype in glioma cells : *Top*. Representative images of wound healing assays performed over 24 hours sh*SOX2* cells LN319 and U373. *Bottom*. Percentage of wound closure area vs sh controls at 24 hours were quantified to assess migratory ability of the cells. All assays were done in six replicates and at least 3 independent experiments in both cell lines. Unpaired two-tailed t tests were performed to determine statistical significance. $p<0.05$(*), $p<0.01$ (**) and $p<0.005$ (***) and ns, non significant. Error bars represent standard deviation (mean± S.D).

3.4. *SOX2* and *PIK3CA* KDs reduce glioma proliferation

Although *SOX2* and *PIK3CA* are oncogenes and are known inducers of tumor proliferation, the KD effect of *MFN1* and *OPA1* on cell proliferation remains to be determined in glioma cells (Santini et al., 2014, Meyer et al., 2013, Hagey and Muhr 2014, Samuels et al., 2005). We therefore investigated the effect of *MFN1* and *OPA1* KD in our glioma cell lines using proliferation assays. Cells were seeded with sparse density and imaged over 6 days using the Cell IQ image analyzer. Relative proliferative index was calculated with respective to time (t=0) for each condition. These assays were performed in the laboratory of Prof Dr Primo Schär at DBM Mattenstrasse, University of Basel.

We observed that LN319 cells proliferated slower compared to U373 cells. In LN319 and U373 cell lines, sh*SOX2* and sh*PIK3CA* cells showed strong reduction in proliferation, compared to the respective sh scramble controls (Fig. 20 *Top)*.

Consistent with the expected role of OPA1, sh*OPA1* cells in LN319 showed reduced proliferation rate. In contrast sh*OPA1* and sh*MFN1* cells, in U373 showed significantly enhanced glioma proliferation (Fig. 20 *Bottom*).

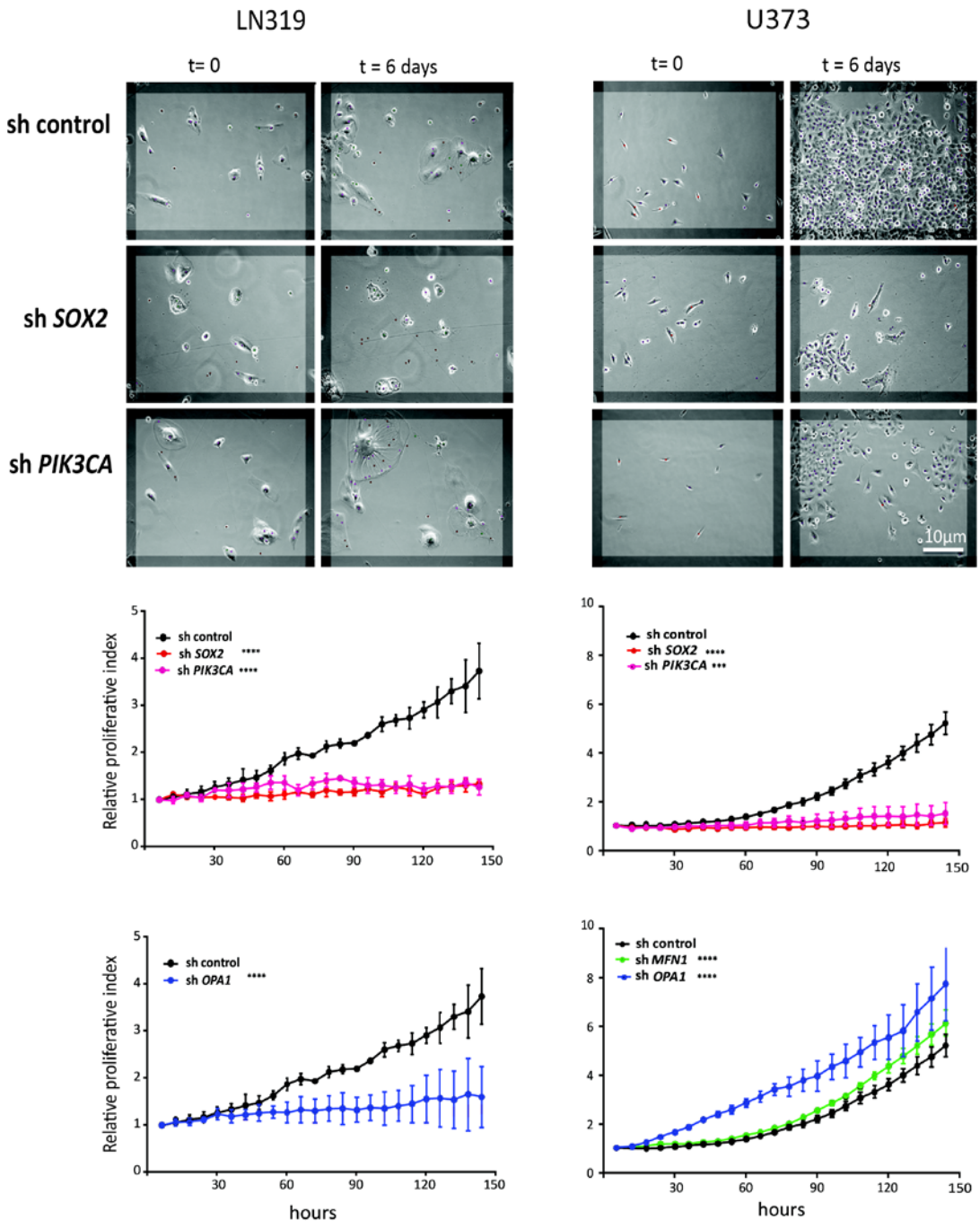


Fig 20. Glioma cells knockdown for 3q26-29 genes affect cell proliferation in LN319 and U373 : *Top*. Representative images of proliferation assays performed cells knockdown for SOX2 in LN319 and U373 cells. *Bottom*. Relative proliferative index from t=0 to t=6 days was quantified in order to assess proliferative ability of the knockdown cells. Quantification summary of relative proliferative index in cells knockdown for *OPA1* in LN319 and *OPA1* and *MFN1* in U373 cells. All assays were performed in six replicates and at least 3 independent experiments were summarized for quantification. Paired t tests were performed to determine the statistical significance. p<0.005(***),p<0.001(****). Error bars represent standard deviation (mean± S.D).

3.5. KDs of *3q26-29 genes* induce mitochondrial fragmentation

KD of mitochondrial fusion genes (*MFN1*, *MFN2*, *OPA1*) induces mitochondrial fragmentation, which is a prerequisite for cancer cell invasion (Campello et al., 2006, Chen et al., 2003, Zhang et al., 2011). We therefore investigated mitochondrial morphology in the KD cells. To study live mitochondrial dynamics, cells were stained with mito-tracker green that labels mitochondria and Hoechst that labels the nucleus.

This analysis reveals that sh scrambled control cells in both cell lines as estimated by eye, exhibited elongated mitochondria. In contrast, sh*OPA1* cells exhibited 100% mitochondrial fission (mitochondria existed as small and round organelles), whereas KD of *MFN1* resulted in partial mitochondrial fission (around 60% of mitochondria existed as individual organelles, while the rest remained fused). KD of *PIK3CA* and *SOX2* had a phenotype reminiscent of mitochondrial fission, as they appeared to be similar to sh*MFN1* cells in LN319 and U373 cells (Fig. 21). We are currently quantifying our results to determine the extent of fission in sh*SOX2* and sh *PIK3CA* cells.

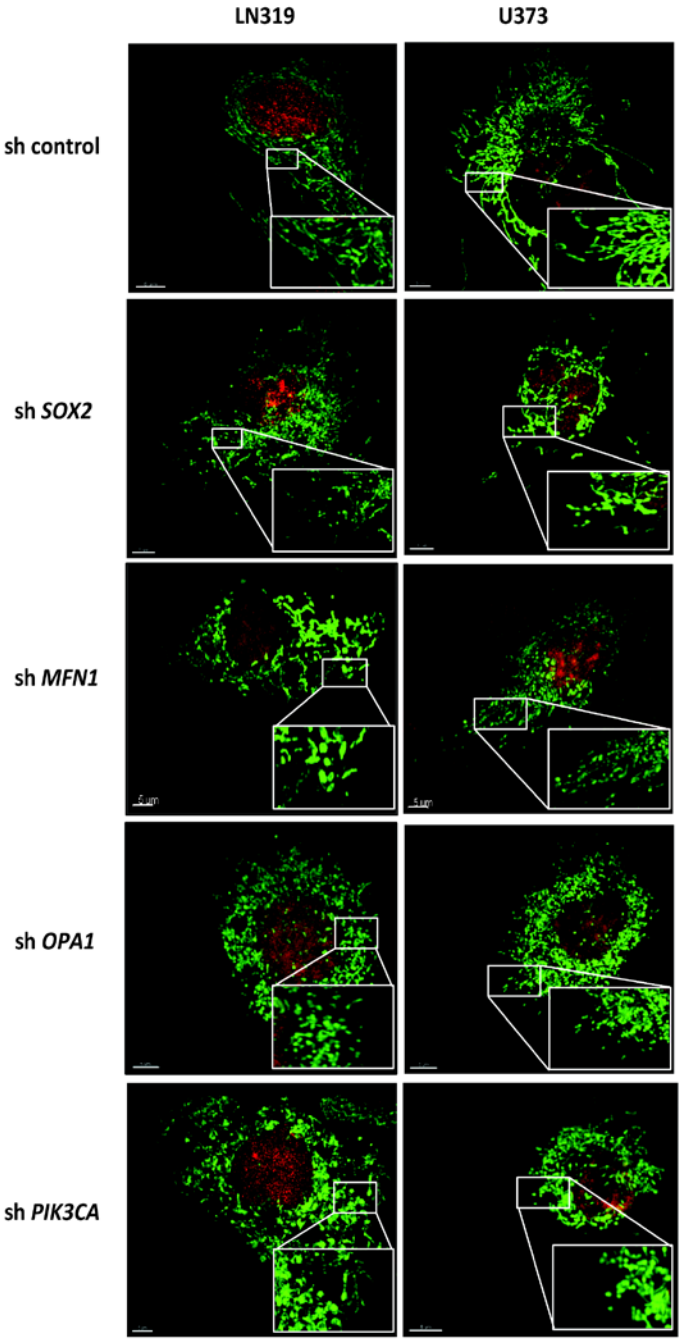


Fig 21. Confocal images of 3q26-29 genes showing altered mitochondrial morphology in glioma cells : Maximum intensity projections of confocal images of mito-tracker green labelled glioma cells treated with lentiviruses expressing shRNA against candidate genes listed above. Nucleus is labelled with Hoechst. Scale bar represents 5µm.

3.6. SOX2 is a potential activator of 3q26-29 gene promoter

3.6.1. KD of *SOX2* reduces expression of 3q26-29 genes

Since SOX2 is a transcription factor, we hypothesized that SOX2 could trans-activate the 3q26-29 genes through regional regulation. It has been observed in many cases that genes co-localized together could regulate each other.

qRT-PCR analysis on up to 10 individual colonies of sh*SOX2* cells in LN319 and U373 glioma cells were performed. KD of S*OX2* reduced mRNA levels for *MFN1*, *OPA1* and *PIK3CA* between 1.5- to 3- fold in both cell lines (Fig. 22A). Protein levels quantified using Western blot, were consistent with corresponding mRNA levels for *OPA1* and *MFN1* in both cell lines (Fig. 22B). However, reduction in PIK3CA protein level was inconclusive (data not shown).

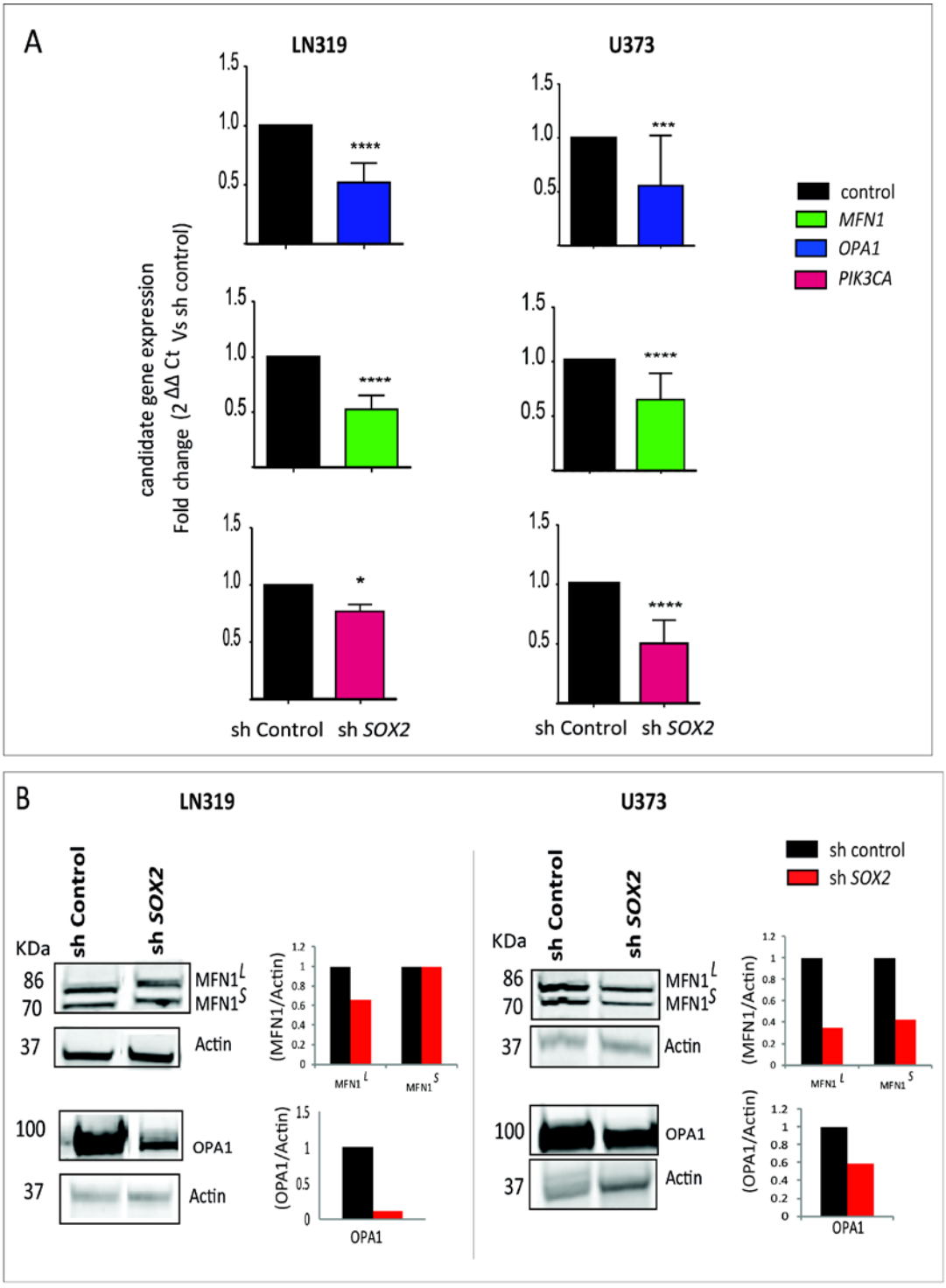


Fig 22. SOX2 knock down reduces the expression of 3q26-29 candidate genes : A RT-qPCR mediated mRNA expression analysis on upto 10 individual clones of sh*SOX2* cells in LN319 and U373 cells respectively. Paired t tests were performed to determine statistical significance. p<0.05 (*),p<0.005 (***) and p<0.001 (****). B. Western blots showing reduced protein expression for MFN1 isoforms (L - long, S - short) and OPA1 in sh*SOX2* cells in LN319 and U373 along with quantification of the relative intensity ratios (MFN1/Actin) and (OPA1/Actin) respectively.

3.6.2. Potential SOX2 binding sites on upstream region of 3q26-29 genes

Since KD of SOX2 reduced expression of PIK3CA, MFN1 and OPA1 in the two cell lines, we therefore looked for potential SOX2 binding sites on the upstream region of 3q26-29 genes. Fig. 23A. is a schematic representation of the candidate genes co-localized on 3q26-29 locus in the human genome.

The SOX2 binding motif was obtained from JASPAR database (Fig. 23B). Conserved SOX2 binding motifs between mouse and human on up to 2000 bp upstream of 3q26-29 genes were searched for. Potential SOX2 binding sites were found in the promoters of *SOX2* (8 sites), *PIK3CA* (1 site), *MFN1* (1 site) and *OPA1* (2 sites) (Fig. 23C). The panel on the right in Fig. 23C is a schematic representation of the identified binding sites with respect to distance from the transcription start site and orientation of the binding.

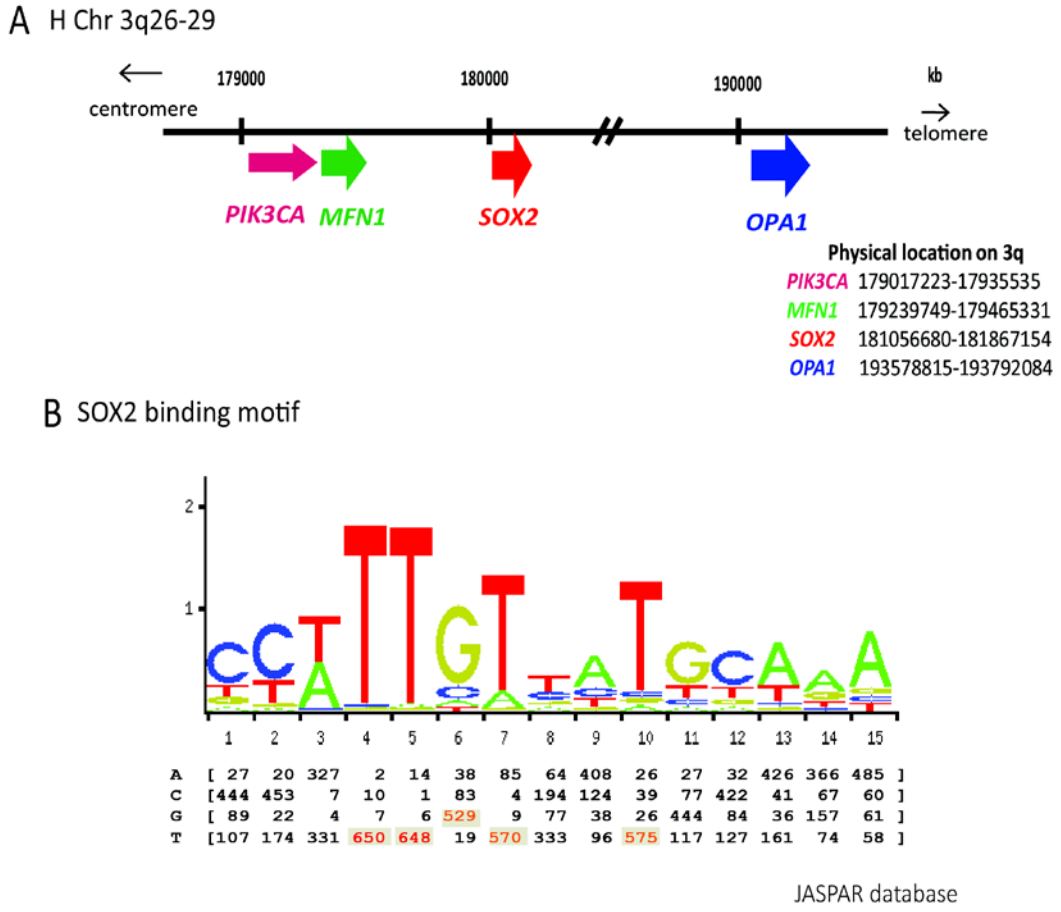


Fig 23. Colocalization of candidate genes at 3q26 and potential SOX2 binding sites on candidate gene promoters: A. Schematic representation of genes (*PIK3CA*, *MFN1, SOX2* and *OPA1*) localized at chromosome 3q26-29 in human. B.Consensus SOX2 binding site obtained from JASPAR database. The matrix below shows the occurence of each nucleotide at the given position.C. Conserved nucleotides are highlighted in black and bold (font). SOX2 binding sites are highlighted in red and gray. A schematic representation of the identified binding site on candidate gene promoters is displayed adjacent.

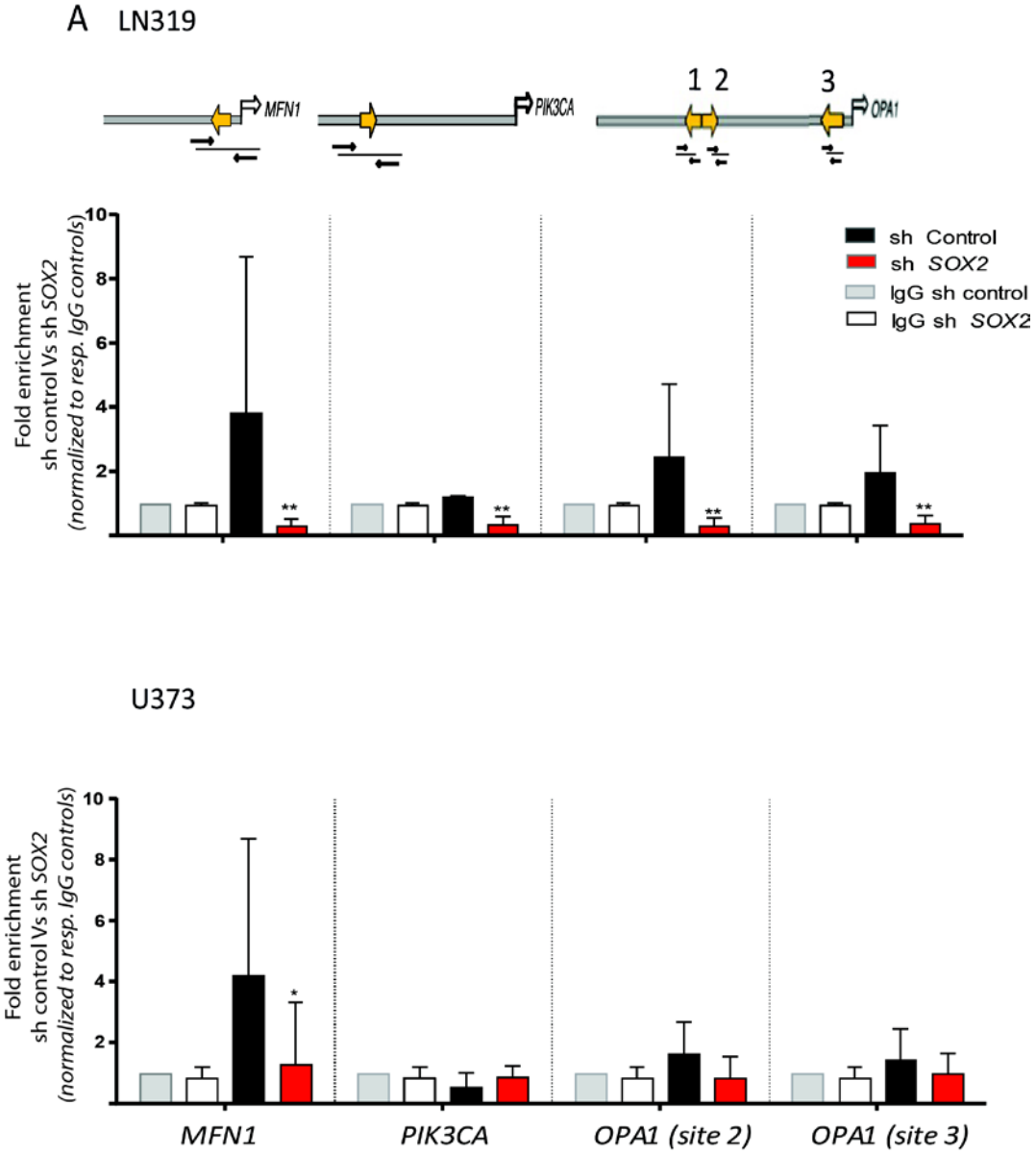
3.6.3. SOX2 binds upstream of 3q26-29 genes in glioma cells

In order to test whether SOX2 binds to the upstream region of 3q26-29 genes (detailed in section 3.6.1), we performed chromatin immuno-precipitation (ChIP) assays on genomic DNA fragments containing the potential binding sites. Immuno-precipitations (IP) were performed on sh scramble control and sh*SOX2* cells using anti-SOX2 and goat anti-IgG antibodies (to estimate nonspecific binding of DNA to beads).

In LN319, we observed the IP of 4-chromatin fragments containing the predicted binding sites. This IP was reduced in sh*SOX2* cells. IP of chromatin fragments in LN319 sh*SOX2* cells, showed 4-fold decrease in amount of fragments containing *MFN1*, a 2-fold decrease in the amount of fragments containing OPA1 (site 2 or 3), and a 2-fold decrease in chromatin fragments *PIK3CA* upstream regions carrying the potential binding sites compared to LN319 sh scrambled control cells (Fig. 24A *Top*). As we observed qRT-PCR signal (with IP'd chromatin fragments) only for SOX2 binding sites 2 and 3 found in the upstream region of *OPA1*, these two sites have been analyzed with this assay.

In U373 sh*SOX2* cells, we observed a 2.6-fold decrease in the amount of DNA precipitated when analyzed for the predicted *MFN1* promoter site. Although we observed a decrease in the chromatin DNA IP of sh *SOX2* when analyzed for the two regions containing the potential binding sites in *OPA1*, the observed results were not statistically significant, similar to that observed for *PIK3CA* (Fig. 24A *Bottom*).

Thus our results show physical binding of SOX2 upstream of 3q26-29 genes in LN319. Although not statistically significant, ChIP data from U373 cells supports the observations made in LN319 cells.


A LN319
MFN1
PIK3CA
1 2
3
OPA1
Fold enrichment
sh control Vs sh SOX2
(normalized to resp. IgG controls)
sh Control
sh SOX2
IgG sh control
IgG sh SOX2
U373
MFN1
PIK3CA
OPA1 (site 2)
OPA1 (site 3)

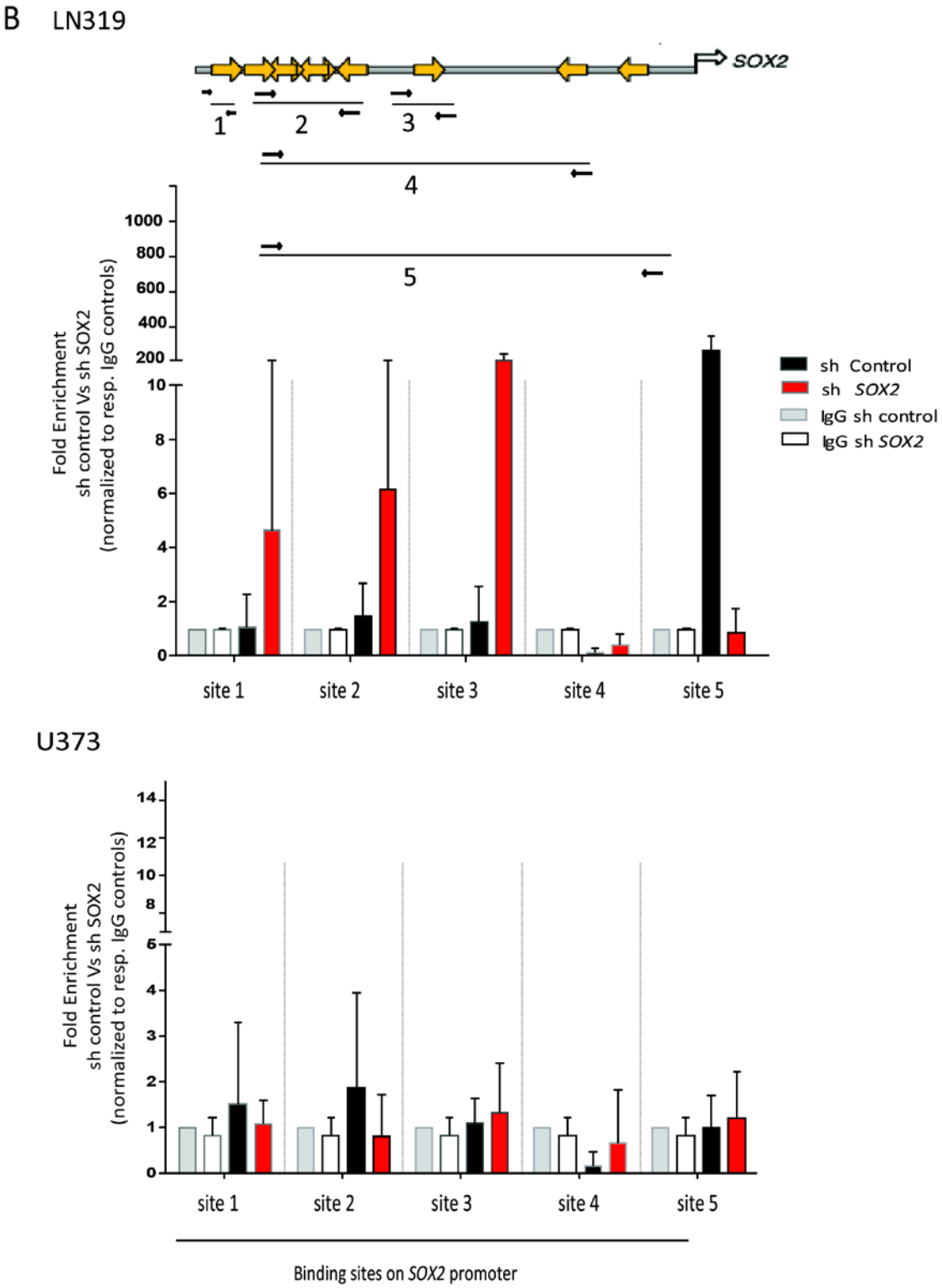


Fig 24. SOX2 binds to predicted the binding sites on upstream region of 3q26-29 genes in glioma cells : Chromatin Immunoprecipitation analysis A. on upstream region of *PIK3CA*, *MFN1* and *OPA1* and B. of *SOX2* genes. a.*Top*.in LN319 cells *Bottom*. in U373 cells. Paired t tests were performed to determine statistical significance. p<0.05 (*), p<0.01 (**) and ns (non significant). Error bars indicate SEM (mean±SEM). As we observed qRT PCR signal only for SOX2 binding sites 2 and 3 in the upstream region of *OPA1*, they have been analyzed for the ChIP assay.

We also predicted multiple SOX2 binding sites on the *SOX2* promoter. In order to evaluate whether SOX2 binds to its own promoter, we performed the ChIP analysis with chromatin DNA IP samples in sh control and sh *SOX2* cells in both cell lines. Primers were designed to amplify five regions upstream of *SOX2*, containing potential SOX2 binding sites, as indicated in Fig. 24B.

In LN319 sh*SOX2* cells, we observed over 200-fold decrease in chromatin DNA IP when analyzed for region 5 (which contained most of the predicted binding sites) compared to sh control cells; whereas in U373 sh*SOX2* cells, we observed over 1.5-fold and 2.5-fold decrease in chromatin DNA IP when analyzed for region 1 and 2 (Fig. 24B). Although these results suggested that SOX2 could bind to its own promoter, these results were not statistically significant.

3.6.4. SOX2 trans-activates 3q26-29 genes – preliminary results

To test whether the association of SOX2 to genetic elements in the 3q26-29 promoter region results in gene transcription activation, we are using a quantitative reporter gene assay based on luciferase activity. We constructed luciferase plasmids containing the putative promoter region of the 3q26-29 genes in front of the luciferase reporter gene. Since glioma cells are transfected with low efficiency, we used human embryonic kidney cells, HEK293.

3.6.4.1. Experimental settings

To maximize SOX2 expression, we transfected HEK293 cells with increasing amounts of flag-tagged SOX2 expression vector pMSCV-SOX2 (1,10,100 ng). After 48 hours post transfection, proteins were extracted from the cells and Western blot analysis was performed on the lysates. SOX2 protein levels were higher in pMSCV-SOX2 treated cells compared to mock transfected or parental control cells (Fig. 25A). We observed that transfection of HEK293 cells with over 10ng of pMSCV-SOX2 vector did not show further increase in SOX2 expression. Therefore, these conditions were used for further transfection experiments. These experiments were performed under the guidance of Dr. Lionel Tintignac (from the lab of Prof. Michael Sinnreich, DBM, University of Basel).

3.6.4.2. Activation of *PIK3CA* promoter by SOX2

In order to optimize the *PIK3CA*-Luc (*PIK3CA* promoter with Luc reporter gene) concentration for further experiments, we transfected increasing amounts of *PIK3CA*-Luc (1,10,100 ng) in mock-treated as well as in SOX2 over-expressing HEK293 cells (10 ng pMSCV-SOX2). In this experiment, we observed that SOX2 overexpression resulted in 2-fold higher *PIK3CA* promoter activity compared to mock-treated (Fig. 25B *Left*).

To test whether SOX2 overexpression can increase *PIK3CA* promoter activity, we transfected increasing amounts of pMSCV-SOX2 (1,10,100 ng) while keeping the *PIK3CA*-Luc vector concentration constant (10ng). Consistent with previous observations, a 2-fold increase in the *PIK3CA* promoter activity was observed (Fig. 25B *Right*).

Thus, these experiments support the observation of a functional-physical association of SOX2 on *PIK3CA* upstream of that transcription initiation site (Fig. 25B *Right*). Whether the SOX2 binding site we suspect is indeed a cis-acting element of the *PIK3CA* promoter will be further tested using promoter constructs mutated at potential SOX2 binding sites.

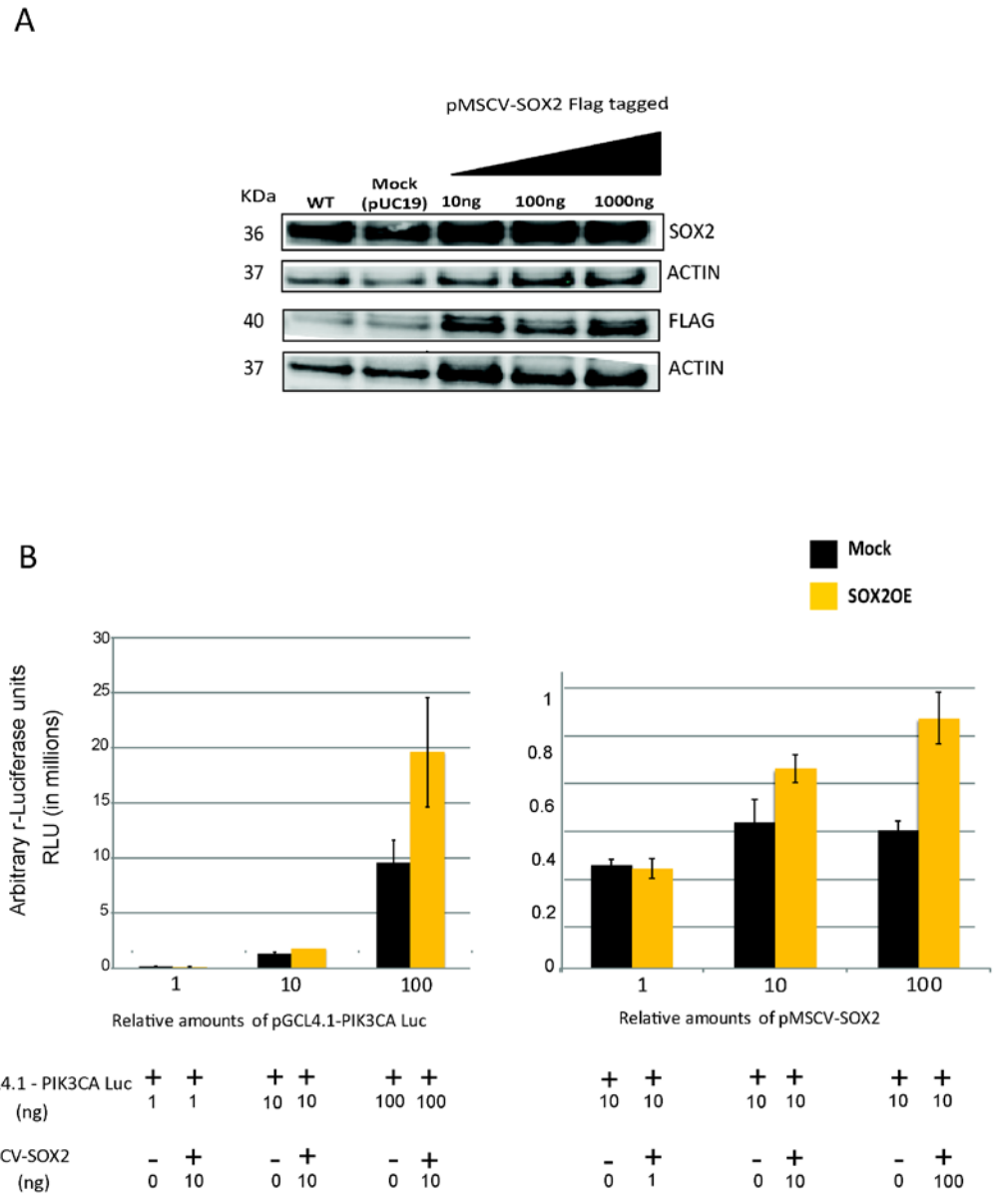
3.6.4.3. SOX2 trans-activates *OPA1* promoter

In order to optimize the OPA1-Luc (OPA1 promoter vector with Luc promoter gene) concentration for further assays, we transfected increasing amounts of OPA1-Luc (1,10,100 ng) in HEK293 cells in mock-treated as well as SOX2 over-expressing HEK293 cells (10 ng pMSCV-SOX2). SOX2 overexpression resulted in 7-fold higher *OPA1* promoter activity compared to mock-treated (Fig. 25C *Top*).

We next transfected HEK293 cells with increasing amounts of pMSCV-SOX2 (1,10,100 ng) while keeping OPA1-Luc constant (10ng). Unfortunately, we did not observe differences in *OPA1* promoter activity between mock-treated and SOX2 overexpressing HEK293 cells. However quantification of OPA1 protein in HEK293 cell lysates using Western blot of cell lysates suggested that OPA1 protein

expression was 2 fold higher compared to the mock-treaed controls (Fig. 25C *Bottom*).

Hence, overall preliminary results from luciferase assay suggest that the physical association of SOX2 on *PIK3CA* and *OPA1* promoters could potentially result in functional activation of these promoters (Fig. 25B *Right*).



C

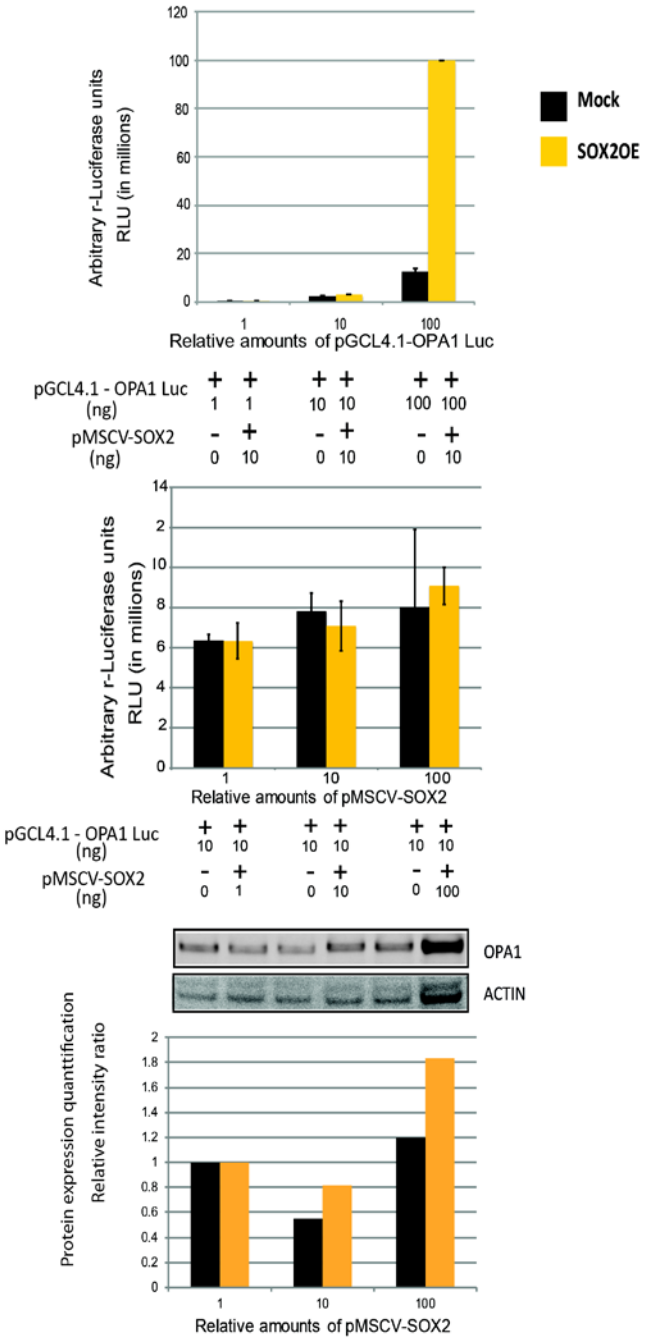


Fig 25. SOX2 activates *PIK3CA* and *OPA1* promoters in HEK293 cells: A. Western blots of HEK293 cells transfected with pMSCV-Flag tagged SOX2. SOX2 overexpression in HEK293 cells was confirmed using anti SOX2 and anti Flag antibodies. B. Luciferase assay on HEK293 cells transfected with increasing amounts of pGCL4.1-PIK3CA Luc promoter vector and pMSCV-SOX2 expression vector respectively. We observed over 2 fold increase in promoter activity in both cases. C. Luciferase assay on HEK293 cells transfected with pGCL4.1- OPA1 Luc promoter and pMSCV-SOX2 expression vector respectively. We observed over 7 fold increase OPA1 promoter activity in the former and over 1.5 fold in latter, quantified by western blot. Results are summary of triplicates from 1 experiment. Error bars indicate SD (mean ± SD).

4. CHAPTER I - DISCUSSION

Invasiveness of gliomas contributes to the notorious nature of the tumor. The infiltrative nature of glioma cells is one of the primary causes of tumor relapse after surgery (Gaspar et al., 1992; Giangaspero and Burger, 1983). Thus, understanding molecular mechanisms that govern glioma cell invasion will help in the development of novel therapeutics for glioma treatment.

Earlier gene expression analyses on glioma biopsies suggested that *SOX2* mRNA was overexpressed in invasive gliomas. *SOX2* is an oncogene, and an inducer of glioma proliferation (X. Fang et al., 2011; Gangemi et al., 2009). *SOX2* mRNA expression levels correlate with tumor malignancy (Berezovsky et al., 2014; Li et al., 2014; Neumann et al., 2011). The gene for SOX2 co-localizes with the oncogene *PIK3CA* and the mitochondrial fusion regulators *MFN1* and *OPA1*, at 3q26-29 in the human genome. The region 3q26-29 is subjected to frequent genetic alterations in GBM and LGG (The Cancer Genome Atlas Research Network, 2008; TCGA, 2015;). Mitochondria are dynamic organelles that undergo constant fission-fusion cycles, a process that regulate cell proliferation, invasion and survival. Mitochondrial fission is required to establish cell polarity (Campello et al., 2006; Desai et al., 2013). Based on our observations and the importance of mitochondrial dynamics, we hypothesized that 3q26-29 genes contribute to glioma invasion. We used two human glioma cell lines to test this hypothesis.

Our results show that the 3q26-29 genes contribute to glioma invasion. Further, we have accumulated arguments suggesting that SOX2 regionally regulates *OPA1*, *MFN1* and *PIK3CA* to induce glioma invasion.

4.1. The use of glioma cell lines

Although qRT-PCR analysis showed that most glioma cell lines exhibit high *SOX2* mRNA levels, Western blot analysis revealed that expression of SOX2 protein was observed only in LN319 and U373 cells, showing that there is not necessarily a correlation between mRNA and protein levels of SOX2. Nuclear localization, indicative of functionally active SOX2, was observed in LN319 and U373 cells. Therefore, LN319 and U373 were chosen as appropriate glioma cell lines for further investigation (Table 1).

Overall, cellular assays revealed that U373 cells exhibited higher invasive (2.5-fold), migratory (≈2-fold) and proliferative (≈3-fold) behavior compared to LN319 cells. During migration assays, we observed that U373 cells exhibited polarized phenotype i.e. individual cells were migrating alone, as opposed to LN319 cells that displayed collective amoeboid migration. Thus, U373 cells were more aggressive than LN319 cells.

Despite quantitative differences in migratory, invasive and proliferative abilities between the two cell lines, our results from all experiments consistently show that KD of 3q26-29 genes in both cell lines resulted in a similar phenotype (Tables 2 and 3). We found that KD of 3q26-29 genes (*SOX2*, *MFN1*, *OPA1* and *PIK3CA*) resulted in enhanced invasive and migratory behavior in both cell lines. In LN319 cells, KD of 3q26-29 genes resulted in a 2 to 2.5-fold increase in cell invasiveness. Similarly in U373 cells we observed 3.5-fold increase in invasiveness of sh*SOX2* cells, and 1.5-fold increase in case of the sh*OPA1* cells compared to sh scramble controls. A similar trend in migratory behavior was observed in both cell lines. We found that in LN319 and U373 cells KD of 3q26-29 genes resulted in over 1.5 times increased wound area closure compared to sh scramble controls.

In U373 cells, KD of *MFN1* did not induce a significant increase in migration. It could be possible that *MFN2* compensated for the KD. In U373 cells, although KD of *OPA1* resulted in 2-fold enhanced invasiveness, and KD of *PIK3CA* resulted in enhanced migratory phenotype, we found that these observations were not statistically significant. As LN319 cells were slower in cell migration and invasion compared to U373, it could be possible that altered cellular dynamics contribute to such variations. It is also possible that 24 hours were not sufficient to assess their motility in LN319, as their proliferation rate was observed to be over 3 days. Hence experiments could be performed over 48 or more hours to reduce such variations.

	LN319				U373			
	SOX2	***MFN1***	***OPA1***	***PIK3CA***	***SOX2***	***MFN1***	***OPA1***	***PIK3CA***
Gene/protein Expression in the cell lines	+++	+	+	*To do*	++	++	++	*To do*

Table 1. Expression of 3q26-29-genes between LN319 and U373 glioma cell lines

	KD of LN319				KD of U373			
	sh SOX2	***sh MFN1***	***sh OPA1***	***sh PIK3CA***	***sh SOX2***	***sh MFN1***	***sh OPA1***	***sh PIK3CA***
Invasion ***vs sh control***	++	++	++	++	+++	++	+ ns	*To do*
Migration ***vs sh control***	+	+	+	+ ns	+	ns	+	*To do*
Proliferation ***vs sh control***	**---**	*To do*	**---**	**---**	**---**	++	++	**---**
Mitochondrial fragmentation ***vs sh control***	++	++	+++	++	++	+	+++	++

Table 2. Comparison of KD effect of 3q26-29 genes between LN319 and U373

	LN319 sh *SOX2*				U373 sh *SOX2*			
Gene expression	***SOX2***	***MFN1***	***OPA1***	***PIK3CA***	***SOX2***	***MFN1***	***OPA1***	***PIK3CA***
mRNA levels in sh *SOX2* cells	**---**	**-**	**--**	**--**	**---**	**-**	**--**	**--**
protein levels in sh *SOX2 cells*	NA	**-**	**--**	*To do*	NA	**--**	**--**	*To do*
ChIP assay (IP) of DNA region containing putative binding sites for 3q26-29 genes	NA	**--**	**--**	**--**	NA	**-**	**-** ns	ns

Table 3. Comparison of KD effect of sh*SOX2* on 3q26-29 genes between LN319 and U373: The tables above represent differences in expression levels of 3q26-29 genes between two cell lines (**+++** very high; **++** high; **+** slight increase. **---** very low, **--** low, **-** slight decrease, ns, non significant; NA not applicable, all symbol representations are compared to respective controls).

In order to evaluate the proliferative ability of the glioma cells, we imaged cells over 6 days and counted the cells. As expected, given the role of SOX2 and PIK3CA in cell proliferation, KD of these genes consistently displayed reduced proliferation rates compared to sh scrambled control cells. In addition, based on the expected role of mitochondrial fusion genes in inducing cell proliferation, we expected that sh*MFN1* and sh*OPA1* cells would display reduced proliferation rates compared to sh scrambled control cells. Consistently, we found that in LN319 cells, sh*MFN1* and sh*OPA1* cells displayed reduced proliferative abilities. In contrast, in U373 cells sh*MFN1* and sh*OPA1* cells exhibited significantly higher proliferative rates.

It could be possible that U373 cells display metabolic adaptation in response to loss of OPA1 and MFN1 and switch to mitochondria-independent cellular growth. This hypothesis could be explored by testing active metabolic pathways and for the state of glycolysis in the cells (discussed in detail in section 4 of this chapter). In addition, other proliferation assays such as EdU (E-Ethynyl-2-deoxyuridine) incorporation could be performed to confirm the observed results and to evaluate whether cell cycle progression is indeed altered.

In LN319 cells, physical association studies reveal that SOX2 binds to the predicted region upstream of 3q26-29 gene promoters. Although data from U373 cells were not statistically significant, they support the observations made in LN319 cells. Subsequently, preliminary reporter gene assays performed so far suggest that SOX2 trans-activates *PIK3CA* and *OPA1* gene promoters in HEK293 cells.

4.2. Immediate perspectives of the project

4.2.1. Completion of invasion and proliferation assays

In order to further understand the role of 3q26-29 genes in glioma invasion, migration, and proliferation, Boyden chamber and wound healing assays on sh*PIK3CA* cells in U373, and proliferation assays on sh*MFN1* cells in LN319 need to be performed.

4.2.2. Completion of luciferase assays in HEK293 cells

We observed that SOX2 binds to the predicted region upstream of 3q26-29 genes. To further show that the physical association of SOX2 results in functional activation of 3q26-29 genes, reporter gene assays need to be performed.

We have recently set up dual luciferase reporter assay in our laboratory. This assay system relies on two reporter genes *Renilla* and *firefly luciferase*, to quantify promoter activity. In the presence of substrate, enzymes such as *Renilla* and *firefly luciferase* oxidize the substrate to release energy in the form of photons, which can be captured by a luminometer. The assay will assess the activity of *PIK3CA*, *MFN1*, *SOX2,* and *OPA1* promoters in response to SOX2 overexpression in HEK293 cells. Promoter regions up to 2000 bp upstream of transcription start site of 3q26-29 genes have been cloned into the pGCL4.1 basic luciferase vector (for vector map and strategy refer to Materials and Methods). Loss of luciferase gene activation compared to WT promoter sequence would demonstrate that suspected SOX2 binding sites are indeed used for gene promoter activation. Therefore, promoter constructs mutated at predicted SOX2 binding sites are currently being generated by site directed mutagenesis.

4.2.3. Mitochondrial morphology

Mitochondrial morphology

Ablation of mitochondrial fusion proteins (MFN1, MFN2 or OPA1) enhances mitochondria fission (Campello et al., 2006; Chen et al., 2003; Suen et al., 2008). Mitochondrial fission enhances cell motility. Therefore, genetic regulation of *MFN1* and *OPA1* by SOX2 could induce mitochondrial fragmentation. In order to reproduce the KD phenotype of *MFN1* and *OPA1* and to study mitochondrial morphology of KD of SOX2 in live glioma cells, 3D-time lapse imaging of cells stained for mitochondria was opted for (Marín-García et al., 2013; Mitra and Lippincott-Schwartz, 2010). Mito-tracker green, a membrane potential insensitive dye was used to stain mitochondria. Mitochondrial morphology of cells KD for *MFN1* and *OPA1* are being used as phenotype controls to estimate the extent of mitochondrial fragmentation in sh*SOX2* cells. We plan to image at least 20 cells per condition. So far, we have analyzed over 4 cells per condition. We are currently quantifying our results.

We are collaborating with the electron microscope (EM) facility (at the Biocenter imaging facility of University of Basel) to image the mitochondria. Although this approach enables us to study of mitochondrial shape, it is biased by altered cell shape due to suspension in culture medium before the fixation, as opposed to studying live cell dynamics.

Caino et al. recently showed that infiltration of mitochondria to the lamellipodia at the leading front in an actively migrating cell could be visualized by staining mitochondria (Caino et al., 2015). Thus, tracking dynamic infiltration of migrating mitochondrial organelles towards the lamellipodia in sh*SOX2* cells compared to sh controls could help determine altered mitochondrial dynamics in *SOX2* depleted cells. To do so, we will use mito-tracker green to stain the mitochondria in living cells and perform wound-healing assays using the Cell-IQ imaging system.

Apoptosis and membrane potential

Flow cytometry could be used to quantify independent apoptosis-associated events in KD cells for LN319 and U373 glioma cells. To determine the alterations in membrane potential and apoptosis in the cells, we could use two established cell death assays: $DiOC_6(3)$ to measure alterations of the mitochondrial membrane potential Δy_m (Kluck et al., 1997; Kroemer et al., 1998; Scaffidi et al., 1998), and terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick-end labeling (TUNEL), to quantify apoptotic DNA fragmentation (Gorczyca et al., 1993).

4.2.4. Mitochondrial respiration

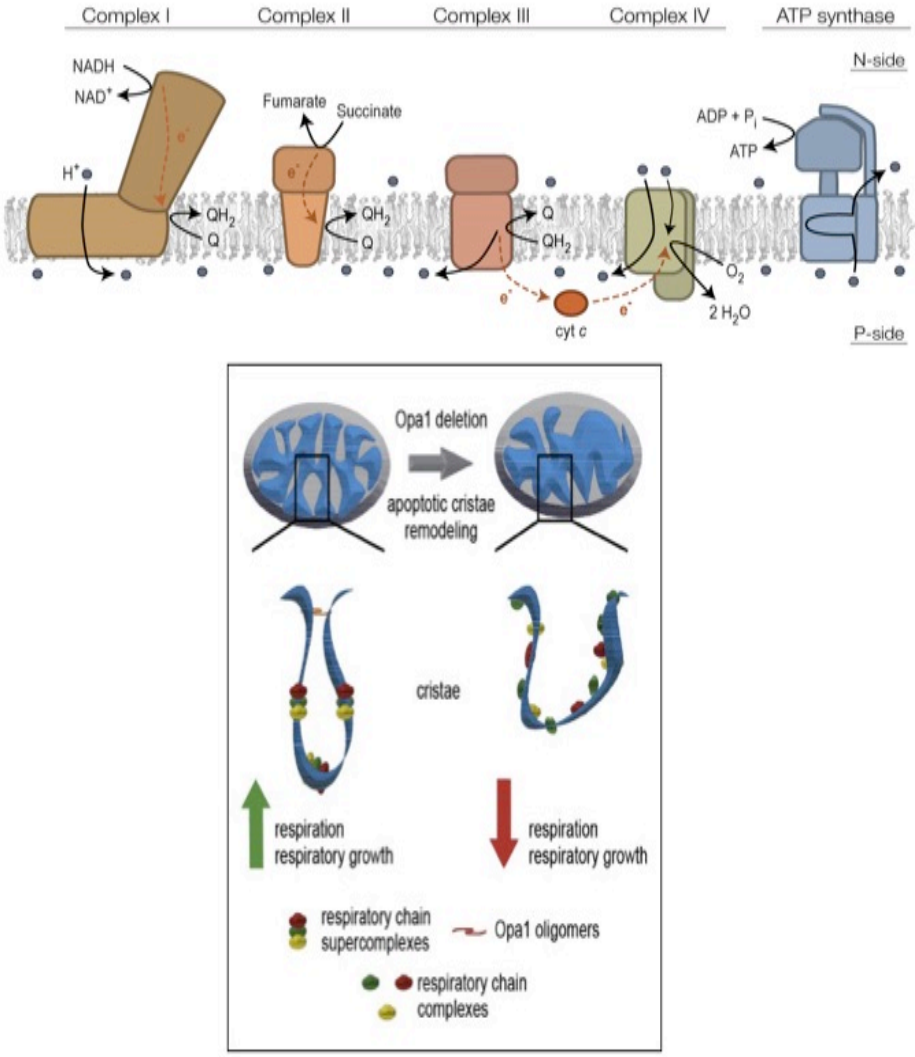
Mitochondrial respiration involves the oxidation of NADH (a reduced form of nicotinamide adenine dinucleotide), which is a byproduct of Krebs cycle (TCA cycle) that finally generates energy in the form of ATP. During mitochondrial respiration, the respiratory super complexes (RSC) (I-IV) are assembled in the cristae at the mitochondrial inner membrane (IMM) to establish a proton gradient that further oxidizes NADH to generate ATP through the electron transport chain (ETC). This process is also described as oxidative phosphorylation (OXPHOS) (Fig. 26.*Top*).

Recent evidence suggests that reduced mitochondrial respiration and bioenergetics affects cell invasion (Caino et al., 2015; Kastl et al., 2014; J. Zhao et al., 2013; Zhou et al., 2014). Indeed, increased levels of reactive oxygen species (ROS), a byproduct of OXPHOS is observed in cancer cells and promotes migration and invasion (Ishikawa et al., 2008; Luanpitpong et al., 2010; Pelicano et al., 2009).

In fact, loss of mitochondrial ATP generation reduces cancer cell migration (Hao et al., 2010; J. Zhao et al., 2013). In 2014, LeBleu et al. used orthotopic breast cancer mouse model and cell lines to show that invasive cancer cells favored mitochondrial respiration and increased ATP generation to invade and metastasize (LeBleu et al., 2014). Thus, cancer cell invasion depends on ATP generated during mitochondrial respiration.

OPA1 regulates cristae remodeling, the site where respiratory super complexes (I-IV) assemble (Fig. 26.*Bottom*). Thus, short-term loss of OPA1 reduces the mitochondrial respiration rate (Cogliati et al., 2013; Kushnareva et al., 2013). If SOX2 plays a role in glioma invasion by genetically activating *OPA1,* mitochondrial respiration rate could be altered.

In order to confirm the source of energy for invasion and migration in KD cells, we will evaluate functional mitochondrial measurements such as basal respiration and ATP production using a Seahorse bioanalyzer.



Adapted from website of Stockholm University, and Cogliati et al., 2013

Fig 26. OPA1 regulated cristae shape controls respiratory chain super complex assembly and stability: *Top***.** Respiratory chain super complex (RCS) I-IV and ATP synthase machinery. The RCS complex oxidizes NADH, a byproduct of Krebs cycle, to ultimately generate ATP by establishing a proton gradient across these complexes, also known as electron transport chain. The process of oxidation of NADH and synthesis of ATP from the energy generated is OXPHOS. *Bottom*. RCS complexes are found to be present on mitochondrial inner membrane called cristae. The cristae shape determines the assembly and stability of the complex, thus regulates mitochondrial respiration. OPA1 is the regulator of cristae shape depletion of OPA1 thus reduces mitochondrial respiration.

4.2.5. SOX2 overexpression in glioma cells

Our observations have shown that KD of *SOX2* enhances glioma invasion. SOX2 rescue experiments could be performed in order to show that the observed phenotype is KD specific. Ectopic expression of SOX2 can be performed by transient transfection of SOX2 expression vector (plasmid DNA) or by transducing viral particles for efficient and long-term expression. It has been noted that glioma cells are difficult to transfected with plasmid DNAs, hence, we chose to prepare viruses that overexpressed SOX2 to reach maximum transduction efficiency.

We generated lentiviruses that could overexpress SOX2 in glioma cells. However, qRT-PCR analysis on transduced cells showed that SOX2 overexpression could not be achieved upon transduction of produced viruses in HEK293 cells (Fig. 27A). We tested lentiviral particles custom made by a biotech company (Genecoepia). Similar to the previously observed results, qRT-PCR analysis on treated glioma cells compared to control scramble treated cells showed that SOX2 could not be overexpressed in glioma cells (Fig. 27B). Glioma cells are high expressors of SOX2 (Annovazzi L, Mellai M, Caldera V, Valente G, 2011). Hence, it could be possible that beyond the threshold limits for SOX2 expression in glioma cells, we might saturate the system.

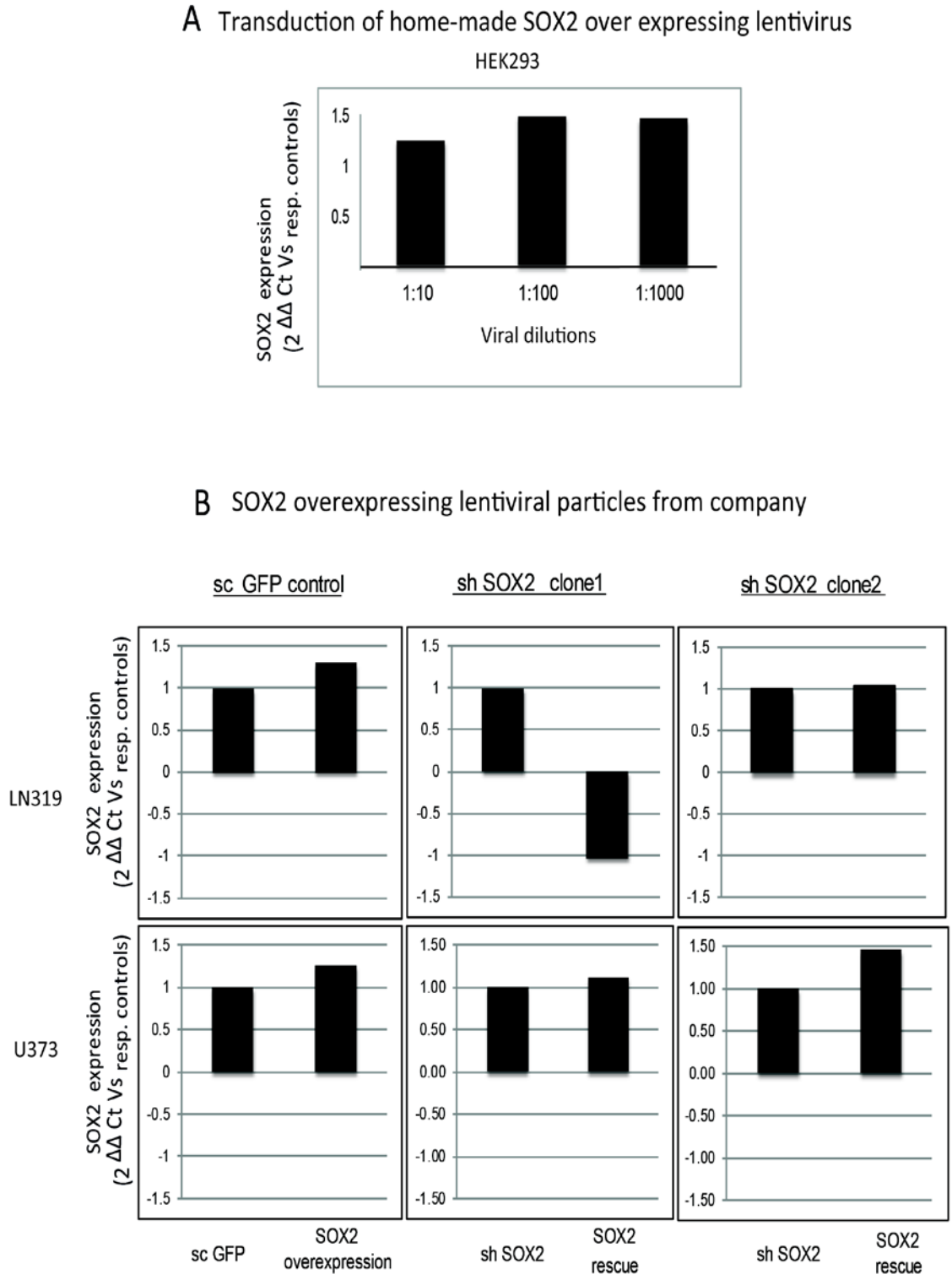


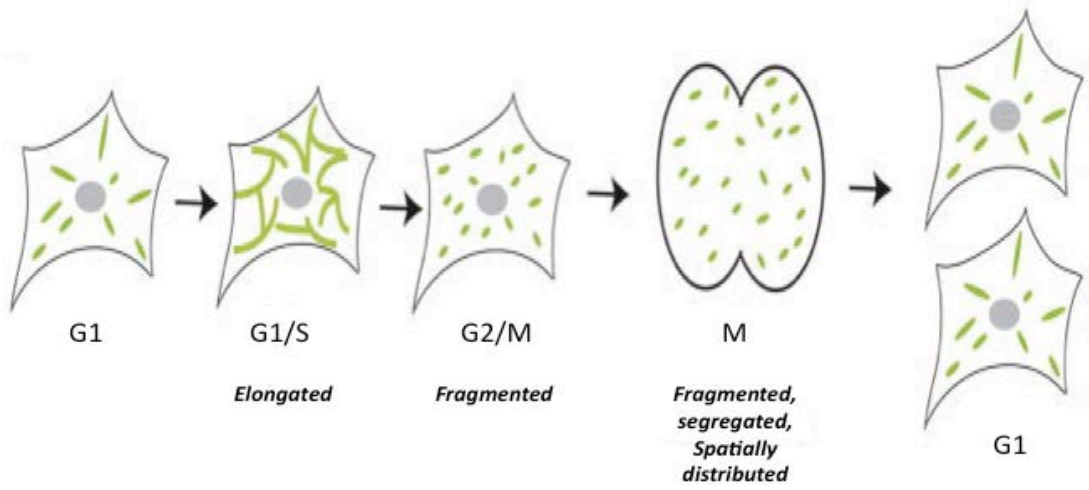
Fig 27. SOX2 overexpression in HEK293 and glioma cells : A. qRT-PCR mediated mRNA expression analysis of SOX2 in HEK293 cells. Home made lentiviruses overexpressing SOX2 were transduced in HEK293 cells. B. qRT-PCR analysis of company made lentiviruses that overexpress SOX2 in LN319 and U373 cells compared to scrambled GFP treated controls. SOX2 knockdown cells were also transduced with the lentiviruses. However, SOX2 expression was not significantly altered in the transduced cells.

4.3. Suppression of mitochondrial fusion increases glioma invasion

Mitochondrial fission and fusion cycles are maintained by subtle balance between the two processes (Chen et al., 2003, Stojanovski et al., 2004, Ishihara et al., 2009, Wakabayashi et al., 2009, Waterham et al., 2007). KD of mitochondrial fusion genes *MFN1* and *OPA1* enhances mitochondrial fission in cells (Chen et al., 2003, Campello et al., 2006, Desai et al., 2013). Mitochondrial fission regulates cell invasion, as mitochondria are required to establish polarity in the cells, a prerequisite for cell migration (refer to introduction section 1.7.2). We showed that KD of *MFN1* and *OPA1* in LN319 and U373 glioma cells resulted in enhanced invasion and migration and mitochondrial fission.

4.4. *MFN1* and *OPA1* contribute to cell proliferation

During the process of cell division, cells enter the M (mitotic) phase during which cells actively undergo mitosis and division of cytoplasm (*cytokinesis*). G1 (Gap1) phase is when cells are metabolically active and grow without replicating their DNA, and in S (synthesis) phase, DNA replication occurs. Following the phase of DNA synthesis, cells enter G2 (Gap2) phase during when cells double their masses and protein synthesis takes place as the cells prepare to re-enter into the M phase to form new daughter cells, unless stopped by cell cycle regulators (Cooper and Hausman, 2007). Mitochondrial morphology coordinates with cell cycle progression. It has been observed that mitochondria fuse and become elongated during G1-S transition to support the metabolic requirements of the cycling cell (Mishra and Chan, 2014). Mitochondria remain fragmented as individual organelles through G2-M phase and equally split among the daughter cells (Antico Arciuch et al., 2013; Mishra and Chan, 2014). The schematic representation of the coordination of mitochondrial dynamics with cell cycle progression is depicted in the Figure 28.



Mishra and Chan, 2014

Fig 28. Mitochondrial dynamics during cell cycle progression: Mitochondrial dynamics coordinate with cell cycle and promote equal segregation of mitochondria during cell division. At G1 stage mitochondria show various shapes, during the G1/S transition, mitochondria fuse and become elongated presumably to support the metabolic need of the cell. In contrast, at G2 and M phases, mitochondria undergo fission and form numerous individual organelles that are spatially distributed throughout the cell soma thus promoting equal segregation of the mitochondria to newly formed daughter cells at M phase.

Cell proliferation is regulated by mitochondrial cristae remodeling, which allows cellular adaptations to meet the metabolic need (Merkwirth and Langer, 2009; Patten et al., 2014). OPA1, chief regulator of cristae shape, plays an important role in cell proliferation (Cogliati et al., 2013; Frezza et al., 2006; Garedew et al., 2010; Patten et al., 2014; Scorrano, 2007). Consistent with the proposed role for OPA1, we observed that in LN319 sh*OPA1* cells, proliferation was reduced.

Interestingly in U373 cells, depletion of *OPA1* and *MFN1* resulted in a significant increase of proliferation rate. Two plausible arguments could be put forth for the differences observed between the two cell lines. First, the two human glioma cell lines exhibit different degrees of invasiveness and cell proliferation rates. Hence, their responses towards depletion of 3q26-29 genes could be different (Discussion section: *Table 1*). Second, short-term *OPA1* depletion affects the cristae shape and remodeling dynamics, and therefore reduces cell proliferation (Cogliati et al., 2013). However, long-term depletion of *OPA1* and *MFN1* also reduces mitochondrial biogenesis. Hence, it could be possible that long-term depletion of OPA1 in U373 cells could favor mitochondria-independent cell growth potentially supported by

enhanced glycolysis. Metabolic assays could be performed to investigate whether long term *OPA1* depletion could result in increased rates of macromolecular biosynthesis in U373 cells.

4.5. *SOX2* and *PIK3CA* induce glioma proliferation

SOX2 and *PIK3CA* are oncogenic in glioma and many other solid cancers including breast, pancreatic cancers, small cell lung adenocarcinomas or squamous cell carcinomas (Boumahdi et al., 2014; Chou et al., 2013; Samuels et al., 2005; Bader et al., 2006). Supporting the known role and functions of *SOX2* and *PIK3CA* in inducing tumor cell proliferation, our results show that KD cells consistently reduced glioma proliferation.

4.6. Increased glioma cell motility upon KD of *SOX2 and PIK3CA*

Our initial observations based on gene expression analysis on human glioma biopsies showed that *SOX2* mRNA was highly expressed in invasive gliomas. Thus, we hypothesized that *SOX2* contributes to glioma invasion. *PIK3CA* is a component required for a functional PI3K/Akt signaling pathway, which is associated with tumor malignancy (Samuels et al., 2005). In fact, constitutively active PI3K-Akt-mTOR pathway also promotes cancer cell invasion (Shukla et al., 2007). Hence these evidences suggested that *SOX2* and *PIK3CA* overexpression induce cancer cell invasion.

Surprisingly, we observed that KD of *SOX2* and *PIK3CA* resulted in enhanced invasive and migratory phenotype in LN319 and U373 cells. Similar observations reported by Caino et al. showed that *in vitro* treatment of prostate cancer (PC3), adenocarcinoma (A549), and glioblastoma (LN229) cell lines with PI3K inhibitors (AZD6482, GCD0941 and BKM120) targeting PI3K/Akt signaling pathway, enhanced cancer cell invasion by mitochondrial relocalization to cortical cytoskeleton (cytoplasmic cytoskeleton beneath the plasma membrane) (Caino et al., 2015).

In 2011, Oppel et al. demonstrated that KD of *SOX2* in U343 glioma cells in a xenografted mouse model resulted in invasive tumors (Oppel et al., 2011). Molecular mechanisms that contribute to the enhanced invasiveness in *SOX2* KD cells remain elusive. However, based on our results and literature evidence, we propose that the

molecular mechanism involving SOX2-OPA1 signaling pathway could provide arguments towards the documented counterintuitive observations (For further discussion refer to 4.9.1 of this chapter).

In addition, higher *SOX2* mRNA levels observed during gene expression analysis on invasive glioma biopsies could be due to contamination of proliferating neoplastic cells present in tumor core rather than infiltrative neoplastic cells at the tumor periphery.

Overall, to test the hypothesis that *SOX2* and *OPA1* overexpression can influence glioma invasion and migration, we could ectopically induce SOX2 expression or use a glioma mouse model that can conditionally overexpress SOX2 and OPA1, and assay the tumor cells for invasion, migration and proliferation phenotype.

4.7. SOX2 is a potential genetic regulator of 3q26-29 genes

Since SOX2 is a transcription factor, we hypothesized that SOX2 could regulate transcription of the 3q26-29 genes. As predicted, we observed decreased mRNA and protein levels of 3q26-29 genes, except for *PIK3CA* at the protein level. *PIK3CA* expression is regulated by many factors such as transcription factors NFκB and TP53. Hence, reduction of mRNA levels by SOX2 could have triggered compensatory mechanism to maintain *PIK3CA* expression levels (Astanehe et al., 2008; Yang et al., 2008).

In order to further study the genetic regulation of 3q26-29 genes by SOX2, we identified conserved potential SOX2 binding sites on upstream regions of the 3q26-29 genes. Physical association studies by ChIP showed that in LN319 cells, SOX2 binds to the predicted region on the promoters of *PIK3CA*, *MFN1* and *OPA1*. Although in U373 cells we did observed similar trends in U373 cells, statistical significance could not be determined. Depending on their sequences, the predicted SOX2 binding sites may have distinct affinities for SOX2, and are therefore sensitive to differences in SOX2 levels. Consistent with association studies, preliminary luciferase assays indicate that SOX2 trans-activates *PIK3CA* and *OPA1* genes.

4.8. Correlation between clinical data and *in vitro* observations

4.8.1. Clinical data

4.8.1.1. Glioma samples from TCGA

In 2013, Verhaak et al. from the TCGA consortium performed copy number analysis on 543 human GBM samples and showed that the region 3q26 is subjected to frequent amplification while 3q29 is frequently deleted. Following this observation, in 2015, Suzuki et al. showed similar patterns of 3q26 amplification and 3q29 deletion in LGG.

4.8.1.2. Copy number analysis of glioma biopsies from BTB tumor bank

Copy number analysis (CNA) for 3q26-29 genes on 68 freshly resected human GBM samples from BTB tumor bank showed that *SOX2* (3q26.3) is the most frequently amplified gene (35%) while *OPA1* (3q29) is frequently deleted (29%). The frequency of these events can be compared to *EGFR* amplification (53%) and *CDKN2A* deletion (33%), which are well-characterized genetic alterations in GBM. Amplifications of *MFN1*, *PIK3CA,* and *OPA1* were observed only in 2%, 8%, and 8% of the GBM samples, respectively, while deletions of *MFN1* and *SOX2* were also observed in 6% and 7% of samples respectively.

CNA on 32 LGG samples showed that *SOX2* was amplified (17%) and frequently deleted (17%) while *OPA1* deletions were most frequent (36%). We also observed that *PIK3CA* and *MFN1* were deleted in 6% and 10% of the LGG samples.

Our observations are consistent with the CNA from TCGA data that 3q26 is amplified and 3q29 is deleted. We found that *SOX2*, located on 3q26.3 is frequently amplified and *OPA1*, located on 3q29, is frequently deleted in gliomas.

4.8.1.3. Mutual exclusivity

Key genetic alterations in GBM are mutually exclusive when they target a common signaling pathway (The Cancer Genome Atlas Research Network, 2008). Our observations show that frequent *SOX2* amplifications and *OPA1* deletions appear to be mutually exclusive.

4.8.2. Correlation with *in vitro* experiments

4.8.2.1. *OPA1* deletions

Based on CNA from BTB samples, we found that *OPA1* deletion is one of the frequent events observed in GBM (29%) and in LGG (36%). Consistently, our *in vitro* experiments show that KD of *OPA1* results in enhanced glioma invasion. Thus, we provide initial arguments that *OPA1* deletion could contribute to tumor aggressiveness. MRI data of glioma patients with *OPA1* deletion could be used to analyze tumor volume and assess the infiltration grade of glioma. These data together with our *in vitro* observations could help us understand whether *OPA1* deletion affect tumor progression in glioma patients.

4.8.2.2. *SOX2* amplifications

SOX2 amplification is one of the major events (35%) observed in GBM samples. Our *in vitro* results provide arguments that SOX2 is a potential genetic regulator of *OPA1*. It has been shown that, in glioma genetic alterations in genes targeting the same pathway are often mutually exclusive (TCGA 2008). Indeed our copy number analysis (CNA) suggests that SOX2 amplification and OPA1 deletion appear to be mutually exclusive. Gene signature analysis could be performed on glioma samples with focal *SOX2* amplification and homozygous *OPA1* deletion to study the correlation with tumor malignancy/aggressiveness. Although the phenotype (cell motility) of SOX2 overexpressing glioma cells is yet to be determined experimentally, evidence from literature show that SOX2 overexpression is capable of inducing invasive phenotype in glioma cells (Berezovsky et al., 2014; Alonso et al., 2011).

Also, our *in vitro* observations show that loss of *SOX2* enhances glioma invasion. MRI data of glioma patients with *SOX2* deletion (7% of GBM and 17% of LGG samples) could be used to analyze tumor volume and assess the infiltration grade of these glioma. These data together with our *in vitro* observations could help us understand whether *SOX2* deletion alters infiltrative capacity of human glioma cells during tumor progression.

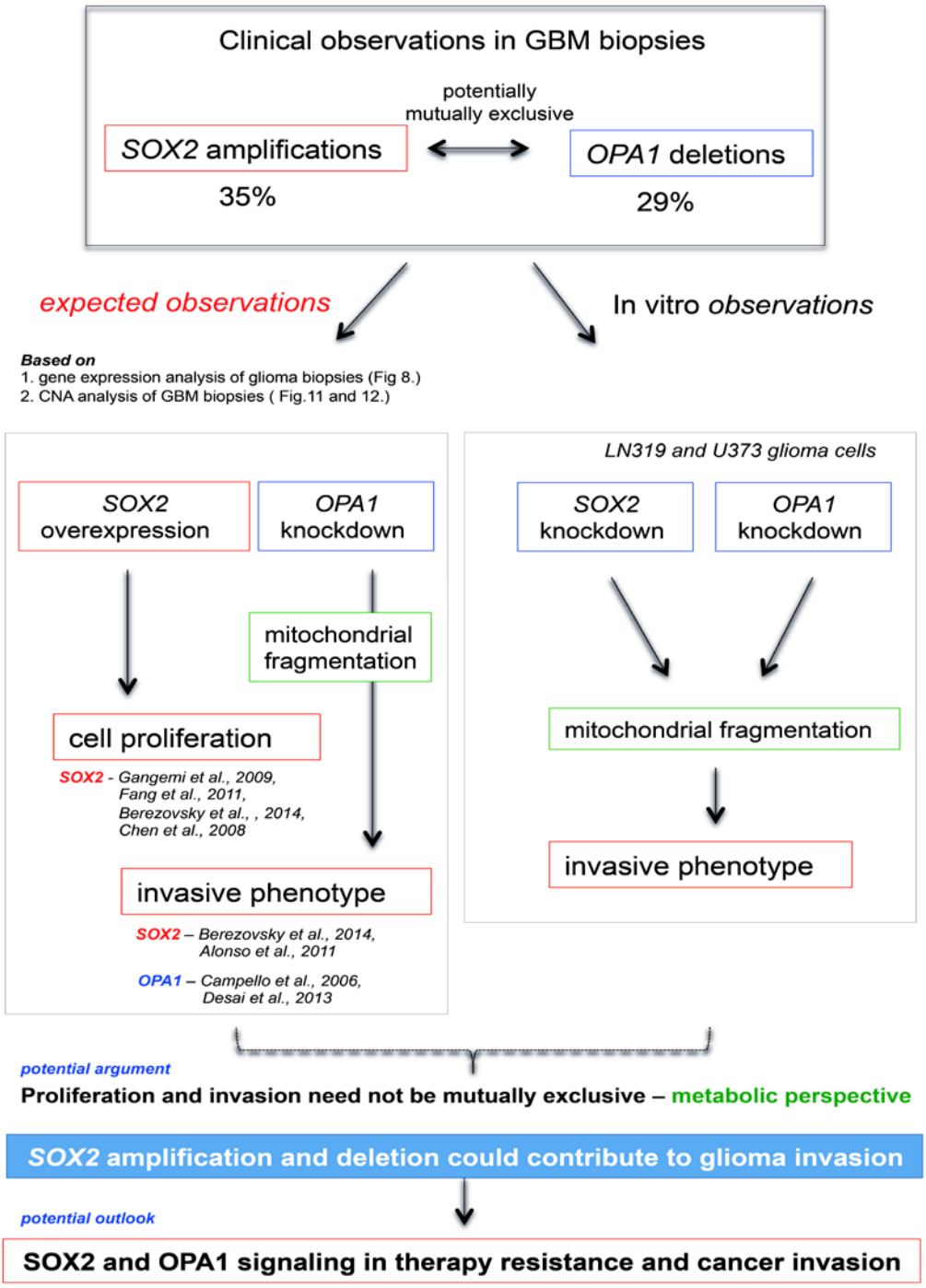


Fig 29. Correlation of clinical data with observed *in vitro* results: Clinical observations show that *SOX2* is frequently amplified whereas *OPA1* is deleted in glioma. Based on clinical observations we expected that *SOX2* overexpression and *OPA1* deletion could contribute to glioma invasion. Surprisingly, we observed that SOX2 down regulation enhanced, while OPA1 depletion as expected contributed to glioma invasion. Based on the metabolic perspective, we provide arguments that support the notion that proliferation and invasion need not be mutually exclusive and hence hypothesize that in glioma cells *SOX2* amplification and deletion both contribute to invasion. However, studies on glioma cells overexpressing *SOX2* and *OPA1* should to be done in order to assess the invasive potential of the cells and to confirm the above hypothesis. Red box indicates amplification/overexpression and blue box indicates deletion/ downregulation.

4. 9. GLOBAL PROJECT OUTLOOK

4.9.1. SOX2-OPA1 signaling pathway

4.9.1.1. Invasion and proliferation are not always mutually exclusive

Cell proliferation is a phase during which cells experience greater energy and metabolic needs. During periods of high metabolic needs mitochondria fuse and forms elongated tube-like structures (Escobar-Henriques and Anton, 2013). Elongated mitochondrial structures increase membrane potential, thus maintaining it across the entire cell (Westermann, 2012). This helps to maintain ATP supply throughout the cell and also triggers glycolysis that helps in the biosynthesis of many macromolecules (Mitra, 2013; Rehman et al., 2012).

In contrast to proliferation, cell invasion and migration are events with low metabolic demands (Chernoivanenko et al., 2014; Deberardinis et al., 2008; Desai et al., 2013; Schulze and Harris, 2012; Vander Heiden et al., 2009). Many studies have shown that blocking the local energy supply by inhibiting mitochondrial OXPHOS using oligomycin A inhibits cancer cell invasion. This indicates that ATP production through mitochondrial respiration is an important source of local energy (Hao, 2010, Ma, 2013, Zhao, 2014). Mitochondria fission facilitates improved mitochondrial respiration and dynamic re-localization of the organelles and thus caters to local energy needs (Campello et al., 2006; Desai et al., 2013; Hollenbeck and Saxton, 2005).

Invasion and proliferation are both manifestations of a tumor growing towards malignancy. In fact, it has been observed that cells at any given time point can switch between proliferative and invasive phenotype, while also displaying the two phenotypes simultaneously (Fortin Ensign et al., 2013; Gao et al., 2005; Liu et al., 2013; Liuq et al., 2014). Such studies have provided evidence that suggest that cell proliferation and invasion are not necessarily mutually exclusive.

4.9.1.2. Proliferation and therapy resistance

SOX2 overexpression is associated with tumor malignancy. SOX2 expression induces tumor proliferation in breast, colorectal, gastric, GBM, hepatocellular carcinoma and ovarian cancer cells (Weina and Utikal, 2014).

In 2014, Piva et al. demonstrated using *in vitro* as well as *in vivo* experiments that SOX2 overexpressing breast cancer cells were resistant to tamoxifen, an oestrogen receptor antagonist. Vanner et al., using a syngenic mouse model of medulloblastoma showed that SOX2 positive *quiescent* cells resisted antimitotic drugs (cytarabine and vismodegib). And Boumahdi et al., using a syngenic mouse model of skin-squamous cell carcinoma (SCC), showed that SOX2 positive stem-cells exhibited higher mRNA levels of genes involved in therapy resistance and cell survival (*Chek 2*, *Itga3*, *Aurka/b*, *mgmt*, *Abcb1*). In addition, Rothenberg et al. showed *in vitro* and *in vivo* using a xenograft mouse model for lung cancer, that treatment of cells with erlotinib, a kinase inhibitor of EGFR, induced SOX2 expression. Recent reports showed that SOX2 positive cancer stem cells are required for tumor relapse and that SOX2 regulates mitochondria induced apoptosis and contribute to therapy resistance in cancer stem cells (Boumahdi et al., 2014; Piva et al., 2014; Vanner et al., 2014).

Similarly, in 2012, Fang et al. demonstrated *in vitro* using lung adenocarcinoma cells that *OPA1* overexpression induces therapeutic resistance against cisplatin. In 2013, Zhang et al. showed that treatment of hepatocellular carcinoma (HCC) *in vivo* with sorafenib, a kinase inhibitor of VEGFR and PDGFR, induced apoptosis by blocking OPA1, thereby targeting mitochondria-induced autophagy. Unfortunately, HCC patients treated with sorafenib, developed resistance against this drug over time. The potential molecular mechanism behind the development of therapy resistance is yet to be elucidated (H.-Y. Fang et al., 2011; Rudalska et al., 2014; Viscomi et al., 2015; Zhai and Sun, 2013; X. Zhao et al., 2013). In SCC and pulmonary carcinoid tumors, chemotherapy resistance is shown to be dependent on loss of mitochondria mediated apoptosis (Gandhi et al., 2009; Thomas and Gustafsson, 2013).

Little literature evidence is currently available that could demonstrate the role of OPA1 in inducing tumor proliferation. However, given the role of OPA1 in promoting

therapy resistance, it is not difficult to conceive that overexpression of *OPA1* might contribute to enhanced tumor proliferation.

4.9.1.3. Cancer cell invasion and migration

In HeLa a cervical cancer cells overexpression of OPA1 beyond threshold limits of the cell induces mitochondrial fragmentation and perinuclear clustering of mitochondrial organelles promoting cancer cell invasion (Cipolat et al., 2006; Griparic et al., 2004; Olichon et al., 2002). Concurrently, many reports show that *SOX2* overexpression induces cancer cell invasion and migration. Lou et al. demonstrated, using ovarian cancer cell lines *in vitro,* that SOX2 genetically targets *Fibronectin 1* (*FN1*) to promote cancer cell invasion (Lou et al., 2013). Sun et al. showed using a xenograft mouse model of HCC, that SOX2 overexpression correlated with enhanced metastatic ability in the tumors (Zhai and Sun, 2013). Alonso et al. demonstrated using *in vitro* experiments in brain tumor stem cells and glioma cell lines, that SOX2 overexpression resulted in enhanced invasive and migratory properties (Alonso, 2011).

These observations demonstrate that overexpression of *SOX2* or *OPA1* could potentially contribute to cancer cell invasion. Although the role for overexpression of *SOX2* and *OPA1* in cancer cell invasion is demonstrated, molecular mechanisms behind such observations remain to be explored (Fig. 29).

4.9.1.4. Potential perspective

Together with our observations and evidence from literature, it can be proposed that SOX2-OPA1 overexpression could promote tumor invasion as well as therapy resistance, thus contributing to tumor aggressiveness. Therefore, we could hypothesize that enhanced SOX2-OPA1 signaling contributes to glioma invasion. Invasion, migration, proliferation, and apoptosis assays could be performed on SOX2 overexpressing human glioma cell lines or on mouse glioma cells, to test the above hypothesis.

4.9.2. Mitochondrial biogenesis and mitophagy

Mitochondrial biogenesis is the process of formation of new mitochondria in the cells. The process includes replication of the mitochondrial genome and serves to maintain mitochondrial mass in the cells. Mitochondrial biogenesis is very tightly associated with mitochondrial fission and fusion cycles. Given such close associations between the two processes it would be essential to understand if KD of *SOX2* in glioma cells alters mitochondrial biogenesis. qRT-PCR experiments using primers targeting mtDNA could be performed in order to estimate alterations in mitochondrial biogenesis.

Mitophagy is the process of removing of defective mitochondria from the mitochondrial pool in a cell, thus helps to maintain the mitochondrial genome integrity. Several studies show that loss of mitochondrial membrane potential induces autophagic machinery (Tolkovsky, 2009). Mitochondrial fission plays an important role in depolarizing the mitochondrial membrane, thus fission is known to promote mitophagy (Dagda et al., 2009; Martínez-Fábregas et al., 2014). In response to membrane depolarization, PTEN induced kinase 1 (PINK1) accumulates at the Outer mitochondrial membrane and phosphorylates MFN2, which in turn acts as receptor for the E3 ubiquitin ligase, Parkin. Parkin, ubiquitinylates the dysfunctional mitochondria. These ubiquitinylated mitochondria are further recognized by autophagosome-specific proteins such as microtubule-associated protein 1 light chain 3 (LC3) and delivered to lysosome for degradation. In addition to the PINK/PARKIN pathway, hypoxia inducible genes BNIP3 and NIX have also been implicated in mitophagy by associating with apoptotic proteins Bcl-2 and Bcl-X_L (Zhang et al., 2008).

Since OPA1 is a key mediator of apoptosis, it would be worth investigating whether sh *SOX2* and sh*OPA1* glioma cells affect mitophagy. Western blot analysis mediated estimation of LC3 II/I ratio could be performed to estimate autophagy/ mitophagy in the system.

4.9.3. Orthotopic glioma mouse model

In vitro assays on 2D cell culture model represent a simple system to visualize and study a phenotype and to dissect a genetic mechanism. However, because of the

simplicity of the system, the model does not take into account the complex biological interactions during tumor progression. Critical aspects of tumor growth including tumor proliferation and primary metabolic responses are shown to be altered in *in vitro* culture conditions compared to *in vivo* tumor progression (Gillet et al., 2011; Lukk et al., 2010). In addition, our experiments to overexpress SOX2 have not been successful in glioma cell lines. Using a mouse model of glioma, conditional *SOX2* overexpression and deletion experiments can be performed. Thus, mouse models could provide insights into tumor progression during SOX2 overexpression or depletion conditions, and the associated metabolic changes.

The xenograft mouse model, in which human glioma cell lines are orthotopically introduced into the mouse's brain, is the most commonly studied model system, is the most common mouse model. However, recent evidence shows that because of the difference in host micro-environment (in species variation), the infiltrative ability of cells is altered (Stylli et al., 2015). Another well-characterized genetic mouse model is the *TP53-/-PDGF*+/+ model (Weiss, 2003). In addition to insights into tumor growth *in vivo* conditions, mouse models can provide further insights into the behavior of 3q26-29 genes upon treatment with chemotherapeutic agents.

5. CHAPTER I - CONCLUSION AND SIGNIFICANCE

The aim of this work was to uncover a novel molecular mechanism that regulates glioma invasion. Oncogenes *SOX2* and *PIK3CA*, and mitochondrial fusion genes *MFN1* and *OPA1* co-localize to 3q26-29 in the human genome, a region subjected to frequent genetic alterations in gliomas. Our study explored the role of 3q26-29 genes in glioma invasion *in vitro*.

We showed that individual genetic inactivation of 3q26-29 genes lead to alteration of invasion, migration and proliferation. Given their respective function in cell physiology, we suspected a cascade that regulates glioma progression.

Our work provides arguments to support a molecular model that SOX2 contributes to glioma invasion and proliferation by regional trans-activation. This model proposes that SOX2 could act as a potential sensor that regulates both glioma cell invasion and proliferation as shown in the model depicted in Fig. 30.

Thus, we provide novel insights into the molecular mechanism of SOX2-driven mitochondria-dependent glioma invasion, through regional regulation of *OPA1* and *MFN1* by SOX2. This work highlights that therapeutic targeting of *SOX2* and *PIK3CA* (oncogenes) in glioma should be carefully addressed, as we show that KD of the genes induces glioma cell invasion. Altogether, the finding of a novel oncogenic molecular pathway provides tool for developing future target therapies to control glioma progression.

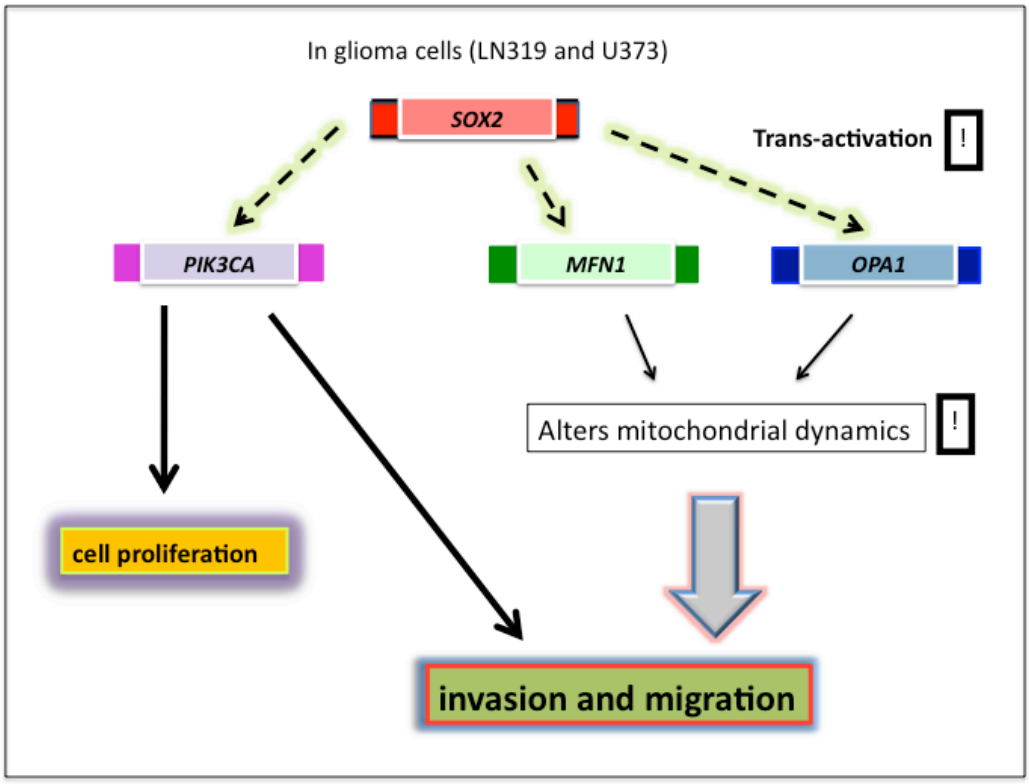


Fig 30. Molecular model based on our observations in glioma cells: Schematic summary of our observations in glioma cell lines LN319 and U373. KD of *SOX2*, *MFN1*, *OPA1* or *PIK3CA* in glioma cells results in enhanced invasiveness and migratory behavior. Glioma cells KD for *SOX2* or *PIK3CA* reduce tumor cell proliferation. Our results suggest that SOX2 exerts regional trans-activation of 3q26-29 genes in order to contribute to glioma invasion. Filled-solid black arrows represent completed experiments, whereas dotted arrows and exclamatory mark inside the box show preliminary observations currently under validation.

CHAPTER II

SOX2-HEY1 signaling in glioma invasion

6. CHAPTER II – INTRODUCTION – SOX2-HEY1 SIGNALING

The notch signaling pathway is an evolutionarily conserved pathway that plays a central embryonic role in development and organogenesis by regulating cell fate, survival, and apoptosis. Notch effectors *HEY1* and *HEY2* are involved in embryonic vascular development and promote neural stem cell proliferation (Fischer et al., 2004).

6.1. Notch signaling pathway in glioma invasion

The notch signaling pathway allows cell-to-cell communication by juxtacrine signaling between neighboring cells. Binding of Notch ligands to their receptors result in cleavage of the cytosolic part of trans-membrane Notch receptors to release the Notch intracellular domain (NICD) into the cytoplasm. NICD binds to RBPJ-κ/MAML complex and is translocated into the nucleus, where it induces expression of Notch target genes such as members of hairy/enhancer of split (HES and HEY) in humans.

Malignant gliomas express high levels of *HEY1* mRNA and the expression levels correlate with poor prognosis (Gaetani et al., 2010; Hulleman et al., 2009). In a xenograft model, the notch signaling pathway contributes to mammary breast cancer cells invasion, osteosarcoma invasion, and metastasis. Activation of Notch signaling pathway *in vitro* induced the expression of Snail and subsequent-induction of epithelial-mesenchymal transition (EMT) (Zavadil et al., 2009; Leong et al., 2007). In 2009, Sivasankaran et al. demonstrated that TNC, an ECM component and a diagnostic marker for glioma progression, is regulated by Notch signaling to facilitate glioma invasion (Sivasankaran et al., 2009).

6.2. Notch regulates expression of *SOX2*

SRY-related HMG box 2 (SOX2) is transcription factor that is primarily known for its role in regulating cell stemness. SOX2 targets many genes involved in cell fate determination, growth, survival and apoptosis. Hence, it is considered to be a master regulator of cellular processes. In neural progenitor cells SOX2 is expressed at different stages of CNS development. SOX2 transcription is promoted by Notch signaling pathway (Dabdoub, 2008.; Neves et al., 2012; Pan et al., 2013). Members of SOX transcription factors are thought to inhibit bHLH-mediated neuronal

differentiation, thus maintaining neural precursor cells in the progenitor state, while bHLH proteins inhibit the expression and activation of SOX members in order to induce cellular differentiation (Dabdoub et al., 2008). Although it is known that SOX2 is regulated by Notch signaling pathway during development, such regulations are yet to be demonstrated in cancer cells.

6.3. Aim of the project

Based on our observations that invasive cells show higher levels of mRNA expression for *HEY1* and *SOX2*, we aimed to understand the role SOX2 and HEY1 in glioma invasion.

7. CHAPTER II - RESULTS

7.1. Expression of *SOX2* and *HEY1* in glioma biopsies

Our preliminary results suggested that *HEY1* is highly expressed in invasive gliomas. In order to investigate whether *HEY1* also correlated with tumor progression and to select glioma cell lines for further experiments, we studied the mRNA expression patterns in glioma cells lines and WHO-classified glioma biopsies.

qRT-PCR mediated mRNA expression analysis of over 30 glioma biopsies classified according to WHO grade, showed that *SOX2* and *HEY1* expression indeed correlated with tumor progression. We also observed that *NOTCH1* mRNA expression levels also correlated with increasing glioma malignancy. Our results support the observations made by others that *SOX2* and *HEY1* gene expression correlates with tumor progression (Fig. 31A).

7.2. Selection of *SOX2* and *HEY1* expressing glioma cell lines

To investigate the role of SOX2 and HEY1 in glioma invasion, we selected glioma cell lines based on mRNA levels for both *SOX2* and *HEY1* and SOX2 protein expression in the glioma cells. LN405 showed least mRNA levels for *SOX2* and *HEY1* among the cells, hence data from other cell lines were normalized using LN405 as a baseline. Most of the cell lines analyzed, showed over 20 fold higher

HEY1 expression levels compared to LN405, except LN71 and LN319 which showed over 5 fold increase in *HEY1* expression compared to LN405. *SOX2* mRNA levels were higher in LN319 and U373 cells (Fig. 31B). We thus selected LN319 and U373 cells for further experiments.

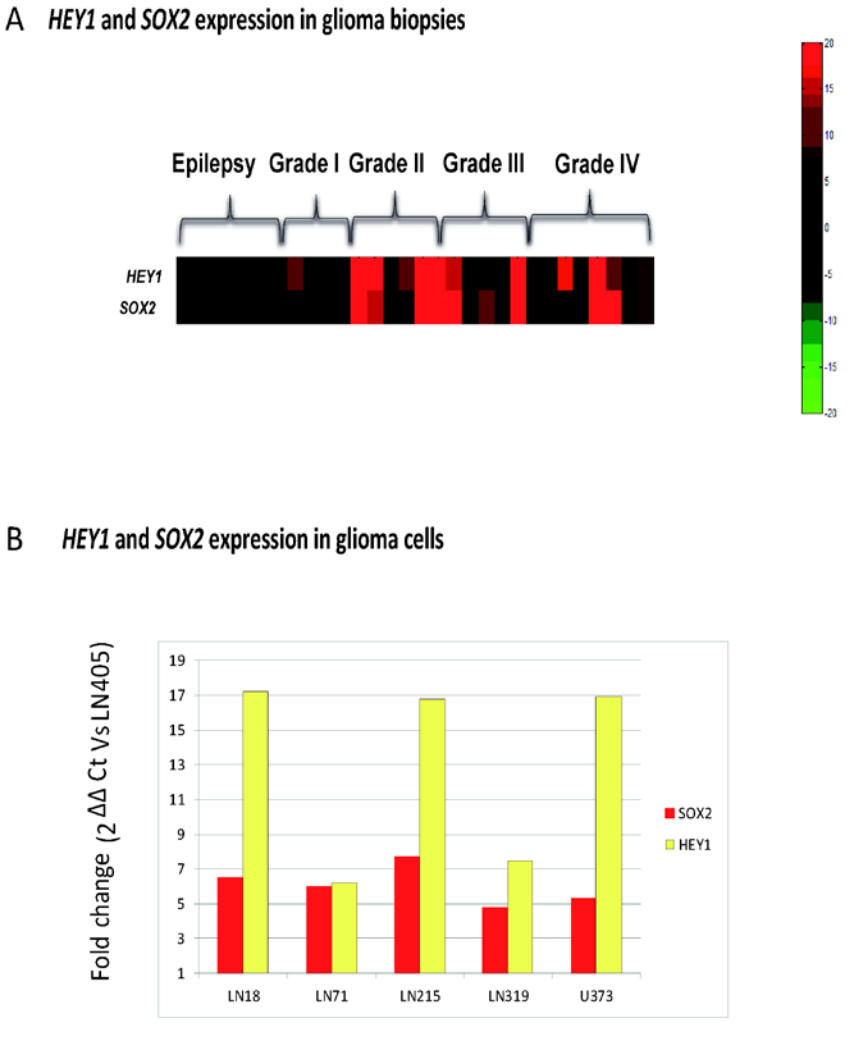


Fig 31. Gene expression analysis of *SOX2* and *HEY1* in glioma : qRT-PCR mediated mRNA expression analysis of *HEY1* and *SOX2* in A. glioma biopsies classified based on WHO grades (n=30). Data is normalized to GAPDH of universal human reference. B. Six human glioma cell lines. Data is normalized to LN405,the lowest *SOX2* and *HEY1* expressor.

7.3. Genetic inactivation of *SOX2* and *HEY1* alter glioma invasion

SOX2 and *HEY1* genes were KD using lentiviruses expressing shRNAs again *SOX2* and *HEY1* in LN319 and U373 cells. We confirmed the KD for SOX2 using qRT-PCR as well as western blot (refer to thesis part 1 result section Fig.13). KD of *HEY1* was confirmed using qRT-PCR in U373 and LN319 (Fig. 32A).

In order to study if KD of *SOX2* and *HEY1* could alter invasive ability in glioma cells, we performed Boyden chamber assays over 16 hours. Invasive cells were stained and counted as a read out for the experiment. We observed that KD of SOX2 resulted in enhanced glioma invasion in both cell lines (refer Fig 18. Chapter 1 results for detailed analysis). Consistent with the known role for HEY1, in LN319 and U373 we observed that sh*HEY1* cells showed 40% and 50% reduction in glioma invasion compared to sh controls, respectively. The results thus show that KD of *SOX2* and *HEY1* had opposite effects on glioma invasion (Fig. 32B).

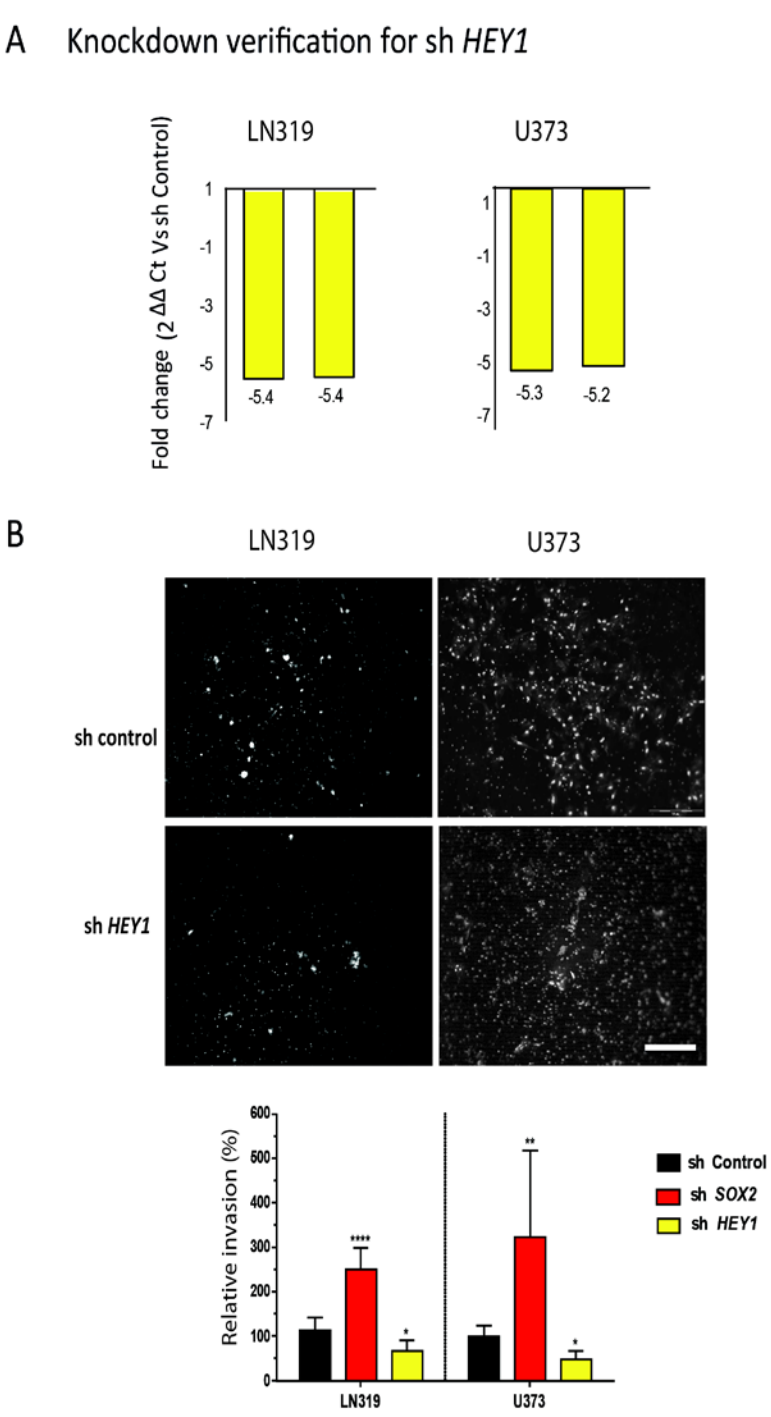


Fig 32. *HEY1* and *SOX2* contribute to glioma invasion in glioma cells (U373 and LN319) : A. qRT-PCR mediated KD verification sh *HEY1 cells* in LN319 and U373. B. *Top*. Representative images from Boyden chamber invasion assays for sh *HEY1* cells in LN319 and U373. The KD and invasion assays for sh *HEY1* and sh*SOX2* cells were performed together with those of sh cells KD for 3q26-29 genes. KD verification and Invasion assay results for sh *SOX2* cells in both cell lines are shown in results section I-Fig15-17 and 18, respectively.Scale bar represents 20µm. *Bottom*. Quantification of relative invasion (vs sh controls) was done on triplicates from at least 3 independent experiments in both cell lines. Five microscopic fields were used to quantify invasiveness in cells. Unpaired two-tailed t test was used to determine statistical significance of the data.p<0.05(*),p<0.01 (**) and p<0.001 (****) Error bars represent SD.

7.4. Gene expression analysis of *SOX2* and *HEY1* in KD cells

To define possible downstream targets of *SOX2* and *HEY1*, we performed genome-wide expression analysis on sh*SOX2* and sh*HEY1* compared to sh control cells in U373. Microarray analyses show that in U373 cells, KD of SOX2 enhances gene expression of members of TGF β signaling pathway and EMT, while KD of *HEY1* reduces the expression of members involved in Notch signaling pathway but increases the expression of members of the TGF β signaling pathway. It should be noted that the experiments were performed on sh*SOX2* cells that had decrease of 1.4-fold for *SOX2* mRNA levels, while sh*HEY1* had over 3.4-fold decreased *HEY1* mRNA levels compared to sh scramble controls respectively (Fig. 33). Data that had $p<0.05$ were considered for the analysis.

In order to achieve better KD efficiency for *SOX2* and *HEY1*, we tested new sh*SOX2* and sh*HEY1* cells (from aliquots of frozen transduced cells, refer materials and methods for detailed explanation) for U373 that had better downregulation for SOX2 using qRT-PCR analysis. We found that in sh*SOX2* and sh*HEY1* cells with KD of over 4-fold and 5-fold, respectively (KD efficiency shown Fig. 34), gene expressions for *SOX2* and *HEY1* were altered in both KD cells. We observed that sh*SOX2* cells had reduced mRNA levels for *HEY1* by over 6-8 folds, whereas, sh*HEY1* cells showed enhanced mRNA levels for *SOX2* by over 20 folds in U373 cells.

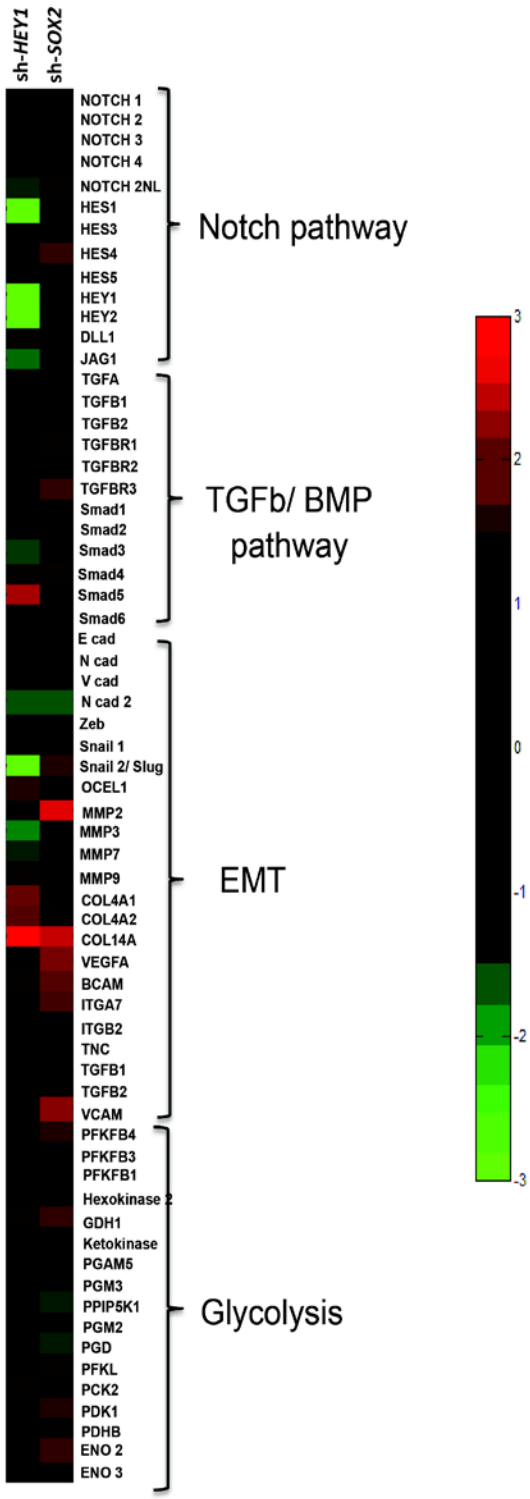


Fig 33. Gene expression analysis on sh *SOX2* and sh*HEY1* in U373 : Microarray based gene expression analysis in sh *HEY1* and sh *SOX2* in U373 glioma cells compared to sh controls and genes with p<0.05 were used for the analysis. Red represents overexpression, green represents underexpression and black represents no difference in expression.

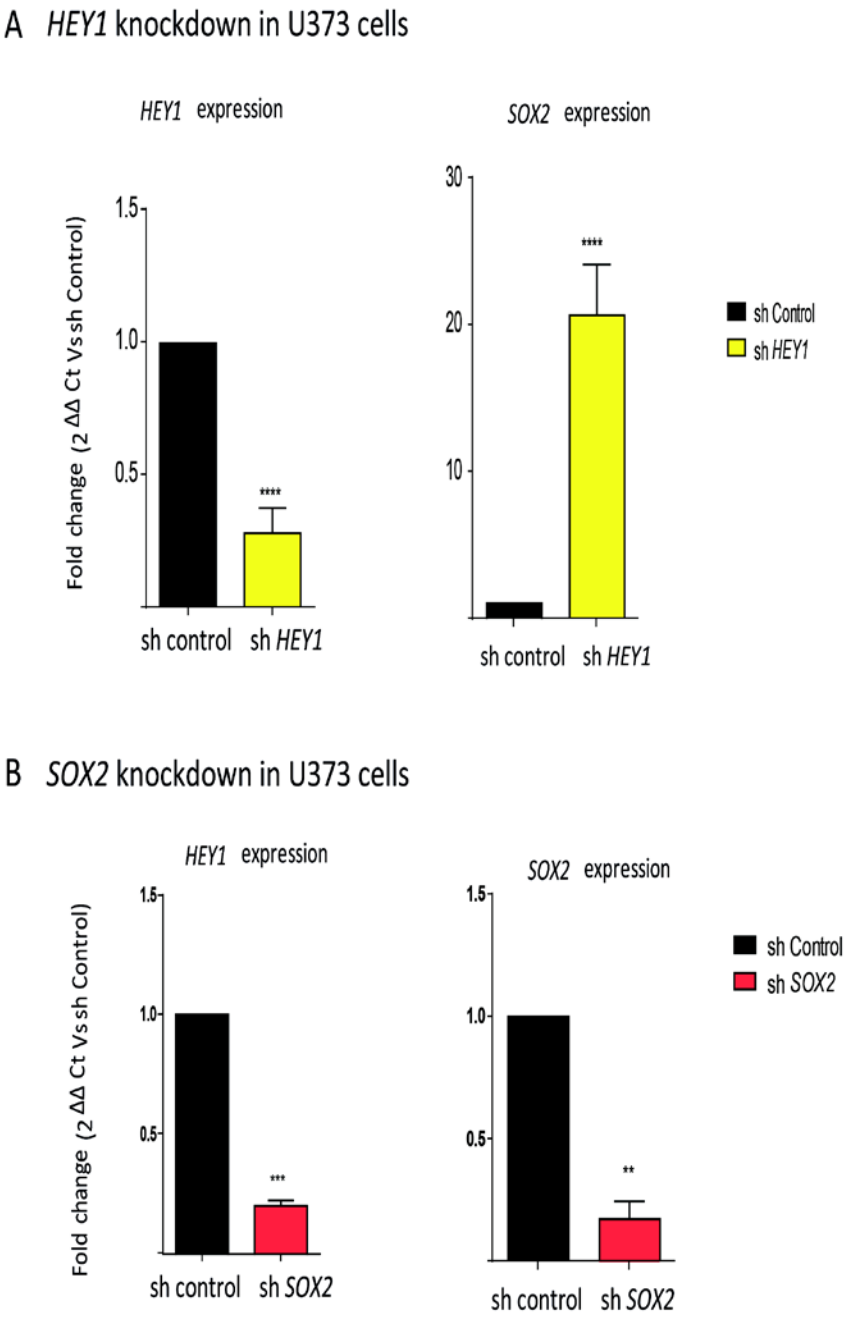


Fig 34. Effect of *SOX2* and *HEY1* knockdown in U373 glioma cells : A. qRT -PCR mediated mRNA expression analysis of glioma cells knockdown for *HEY1*. *Left*. Lentiviral mediated knockdown of *HEY1* results in over 4 fold decrease in *HEY1* mRNA expression. *Right*. U373 cells knockdown for *HEY1* upregulates *SOX2* mRNA expression levels (over 20 folds). B. qRT -PCR mediated mRNA expression analysis of glioma cells knockdown for *SOX2*. *Left*. Lentiviral mediated knockdown of *SOX2* results in over 5 fold decrease in *SOX2* mRNA expression. *Right*. U373 cells knockdown for *SOX2* show reduced *HEY1* mRNA levels (over 6 fold). Quantification of relative mRNA expression levels was performed on at least 3 individual clones that was knockdown for *SOX2* or *HEY1*. Paired t tests were used to estimate statistical significance. $p<0.01$ (**), $p<0.005$ (***), $p<0.001$ (****). Error bars represent SD.

8. CHAPTER II - DISCUSSION

Overall our mRNA expression analysis shows that *HEY1* mRNA levels correlate with glioma malignancy particularly with tumor invasiveness. We confirmed the KD for the genes using qRT-PCR. In contrast to *SOX2*, we show that sh*HEY1* cells display reduced invasion phenotype in glioma. Gene expression analysis on sh*SOX2* and sh *HEY1* cells in U373 suggested that members of TGFβ signaling pathway and EMT were upregulated in sh*SOX2* cells and members of Notch and EMT were downregulated in the sh *HEY1* cells. Although these are preliminary data from one cell line (U373), these observations should be validated using qRT-PCR and confirmed in another cell line or glioma model.

8.1. *SOX2-HEY1* signaling loop in U373 cells

Our results suggested that KD of *SOX2* reduced *HEY1*, while KD of *HEY1* enhanced *SOX2* mRNA expression levels. This suggested that *SOX2* and *HEY1* could act in a loop in which *HEY1* could potentially repress SOX transcription factors and SOX2 could induce HEY1 expression (Dabdoub et al., 2008). The genetic regulation of *SOX2* and *HEY1* on each other should be explored using chromatin immuno-precipitation and reporter gene assays.

8.2. Notch – TGFβ pathway interplay in EMT

EMT is a process that plays an important role during cancer cell invasion. EMT is characterized by loss of cell adhesion contacts, dynamic reorganization of the cytoskeleton, gain of cell polarity, and cell spreading. TGFβ signaling pathway is a well-known inducer of EMT in cancer cells. Zavadil et al. using *in vitro* kinetic experiments and primary cultures from wild type and smad 3 (activator of TGF β signaling pathway) knockout mice, showed that *HEY1* is under transcriptional control of TGFβ signaling pathway, thus showing that HEY1 mediates the crosstalk between Notch and TGFβ signaling pathway during EMT (Zavadil et al., 2004).

9. CHAPTER II - CONCLUSION AND OUTLOOK

Overall, our results provide arguments that *HEY1* contributes to glioma invasion. Preliminary results suggest that SOX2 and HEY1 could act in a loop to contribute to glioma invasion. Although these results should be validated with another model system, our data provide molecular basis to explore novel molecular mechanisms involved in glioma invasion.

MATERIALS AND METHODS

Cell culture and reagents

Human glioma cell lines U373, LN319, LN18, LN71, LN215, LN229, LN319, LN401, LN405, U87 and Human embryonic kidney 293 (HEK293) cells were obtained from either University Hospital of Lausanne or ATCC. U373 and LN319 cells were cultured in Dulbecco's Modified Eagle Medium with high glucose (DMEM, D6429, Sigma-Aldrich) with 5% heat inactivated fetal bovine serum (non USA origin, F7524, Sigma-Aldrich), 1% Pen-Strep (P4333, Sigma-Aldrich). HEK293 was cultured in DMEM Glutamax (GIBCO) supplemented with 1% Sodium pyruvate solution (S8636, Sigma-Aldrich) and 10% heat inactivated fetal bovine serum (non USA origin, F7524, Sigma-Aldrich). Cells were incubated at 37°C in 5% CO_2 in a humidified atmosphere.

Invasion assay

Boyden chamber assays were carried out using the matrigel coated CytoSelect ™ 24-well cell invasion assay kits (Cat No. CBA-100-C,Cell Biolabs, Inc.), according to the manufacturers's instructions with minor modifications. Cells were serum starved overnight and $5X10^4$cells were seeded on the upper chamber. The cells were allowed to invade over 24 hours at 37^0C. The medium was then removed from the chambers and non-invading cells were scraped off the top of the membrane using Phosphate buffer saline (PBS) soaked cotton swab. The invaded cells (in the bottom side of the membrane) were fixed with 4% Para Formaldehyde (PFA). The cell nuclei were then stained with 4',6- diamidino-2 phenylindole (DAPI) and counted over five microscopic fields per well (at 10X magnification). Assays were in done in triplicates.

Migration assay

Wound healing assays were carried on 24 well plates. $5X10^4$cells were seeded overnight prior to the experiment. Scratches (~120-140 µm) were made using the 100µl pipette tips. The cells were imaged using the Cell-IQ machine (Chipman Technologies) with 30-minute interval over 24 hours. The cell-free area was analyzed as a measure of cell migration, using automated Cell Analyzer™ software. Assays were in done in triplicates. The assays were performed in Prof. Primo Schär's laboratory at Mattenstrasse, Department of Biomedicine, University of Basel. For quantification purposes, we used the following meta-data (provided by the Cell IQ

manufacturer) spatial resolution in X (µm/pixel) = 0.702 and the spatial resolution in Y (µm/pixel) = 0.702.

Cell proliferation assay

Cell proliferation assays were performed in 24 well plates. 3000 cells per well were seeded and imaged over a period of 6 days with 6 hours interval between successive images, using the Cell-IQ machine (Chipman Technologies). Cells were counted using the software Cell Analyzer ™ (Chipman Technologies). Assays were done in triplicates and statistical significance was estimated. The assays were performed in Prof. Primo Schär's laboratory at Mattenstrasse, Department of Biomedicine, University of Basel.

Lentivirus mediated stable KD

For stable transductions recombinant lenti-virus particles expressing short hairpin (sh) RNA against *SOX2* (sc-38408-V, Santa Cruz Biotechnology, Inc.), HEY1 (sc-37914-V, Santa Cruz Biotechnology, Inc.), *MFN1* (sc-43927-V, Santa Cruz Biotechnology, Inc), *OPA1* (sc-106808-V, Santa Cruz Biotechnology, Inc.) and *PIK3CA* (sc-39127-V, Santa Cruz Biotechnology, Inc.) were used according to manufacturer's instructions to stably KD of genes in U373 and LN319 glioma cell lines. Transduced cells were maintained in DMEM (D6429, Sigma-Aldrich) with the supplements mentioned in the previous section (cell culture and reagents). Long-term selection was applied to KD cells with 5µg/ml of puromycin (P8833, Sigma-Aldrich). After 1 day of selection, cells were used for experiments. Many aliquots were made and frozen for future experiments. RT-qPCR and Western blots were performed to validate cells for KD of candidate genes in the cell lines.

Lentiviral transduction efficiency was measured using FACS sorting for GFP upon transduction of GFP expressing lentiviruses in glioma cells. The experimental setting for FACS sorting is displayed in Fig 35.

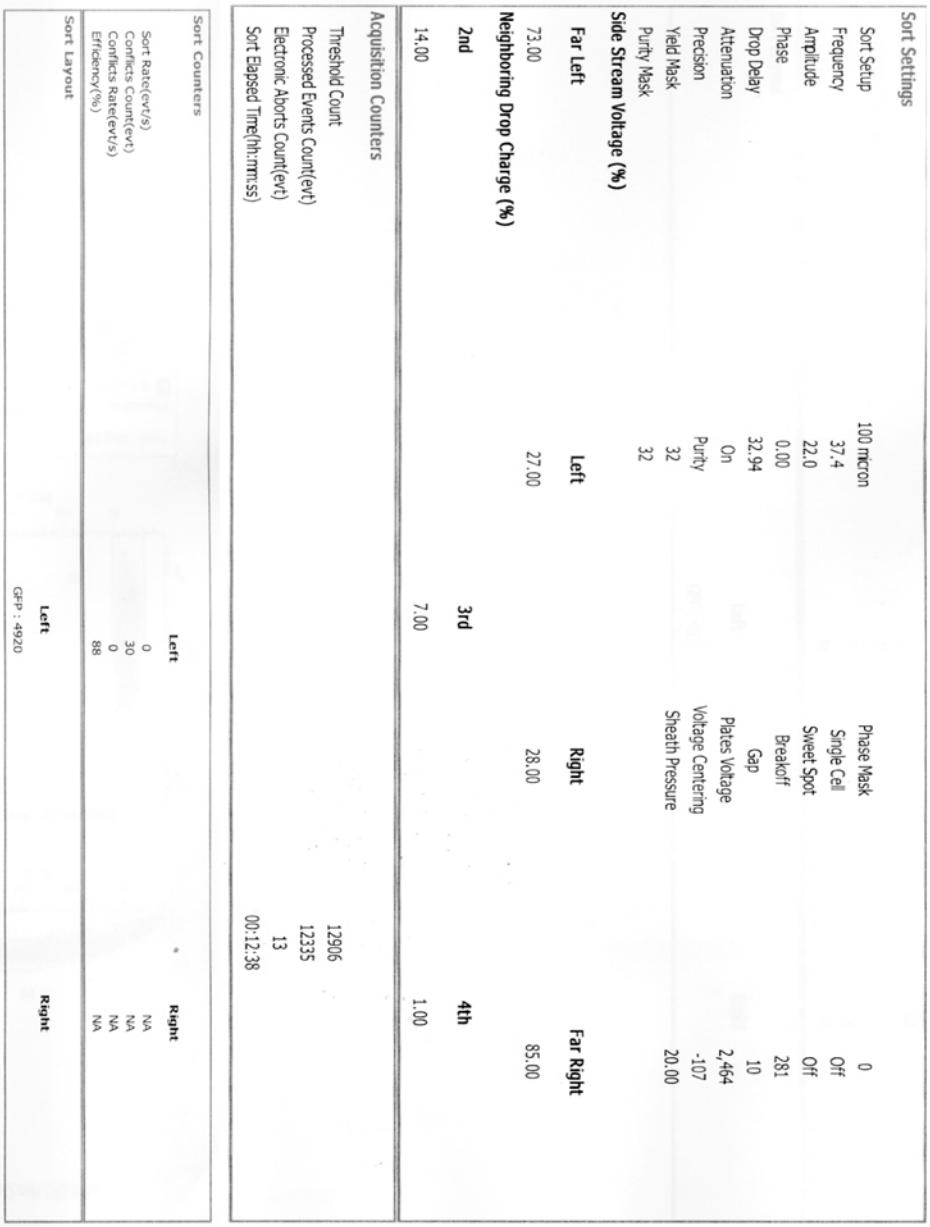


Fig 35. Experimental settings for FACS analysis: FACS Aria II was used to measure percentage of GFP positive cells in GFP-expressing-lentivirus transduced glioma cells.

Chromatin immunoprecipitation (ChIP assay)

General chromatin immunoprecipitation (ChIP) method described by Carey et al 2013, was followed with minor modifications. 50-100 million cells were used per experimental condition. Cells were treated with 1% formaldehyde for 15 mn for DNA cross-link at room temperature and then quenched with 0.125 M glycine for 5 mn. After wash with PBS, samples were homogenized in lysis buffer (5mM PIPES (pH 8.0), 0.5% NP40, 85mM KCl) containing protease inhibitors (Sigma-Aldrich) at 4°C.

Isolated chromatin was sheared with pulse sonicator for 8 cycles of 15 s ON/15 s OFF on ice. Average DNA fragment size in the range of 300–400 bp was confirmed by agarose gel electrophoresis. For immuno-precipitation (IP), 100 µg of chromatin was diluted to 750 µl with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl, pH 8.1). ~1% of the diluted sample was saved to serve as input controls. Before sample sonication, dyna beads protein A/G (Cat No. 10001D, Life Technologies) were incubated with anti-SOX2 antibody (ab59776, Abcam), anti-H3 antibody (ab1791, Abcam) or IgG (12-170, Millipore), at a concentration of 10µg antibody per 100µg chromatin. Chromatin-antibody mix was incubated overnight at 4°C under constant rotation in blocking solution (0.5% BSA in PBS). Following wash and re suspension of the antibody-bead conjugates, chromatin samples were added in antibody-bead mixtures and incubated for 16 h under constant rotation at 4°C. After immuno-precipitation, the beads were washed at least four times (10 mn each) with high salt wash buffer (50 mM HEPES-KOH, pH 7.6, 500 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.1% deoxycholate, 1% Triton X-100), one time with TE buffer (10 mM Tris-HCl, pH 8.1, 1 mM EDTA), and then eluted with 200 µl elution buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS). The eluate was incubated at 65°C overnight (o/n) to reverse the cross-link. The input DNA was diluted in elution buffer to a final volume of 200 µl and processed for cross-link reversal. Samples were then digested sequentially with 0.2 µg/ml RNase A for 2 h at 37°C and with 20 µg of proteinase K for 30 mn at 55°C. DNA was extracted by phenol/chloroform/isoamyl alcohol and subjected to quantitative PCR (IQ^{TM} Green Supermix, Cat No. 1708880, Bio-Rad) with primers specific to the predicted SOX2 binding sites on promoter region of *PIK3CA, SOX2, MFN1* and *OPA1* gene.

The quantitative real-time PCR data were analyzed by comparative *Ct* method. To control for variation between ChIP fractions, a Δ*Ct* value was calculated according to the following formula: $\Delta Ct_{\text{(Normalized ChIP)}} = Ct_{\text{(ChIP)}} - (Ct_{\text{Input}} - \text{Log}_2 (\text{fraction of the input saved})^{-1})$. Further, to estimate signal vs background, $\Delta\Delta Ct = \Delta Ct_{\text{(Normalized ChIP)}} / Ct_{\text{(IgG)}}$. Data are presented as fold enrichment above background for each IP sample: fold change = $2^{-(\Delta\Delta Ct)}$.

Primer pairs (PP) used for ChIP assay

PP1	SOX2 FP:	GACAGTAACAGGCTAGGGAG
	SOX2 RP:	GTATCTACCAGCCACGTTCC
PP2	SOX2 FP:	TTATAAAAAAGAAATGGCATCAGG
	SOX2 RP:	TAGAGGAGGATGAGATGGG

PP3 SOX2 FP: CACAATGGAAATCTACGGG
SOX2 RP: CGGCACTGTATGGAGGTG
PP4 SOX2 FP: TCGGGACTGTGAGAAGG
SOX2 RP: TGGAAACAGCCAGTGCAG
PP5 SOX2 FP: TCATTTAAGTACCCTGCACC
SOX2 RP: AGCCTGCCAGCCACTGAG
MFN1_ChIP_FW: TACTTGGACACTTTGAGGGG
MFN1_ChIP_RP: CGCCATTTTCCTTAAGGG
OPA1_ChIP_FP_1: TATTTTCTAAAATAACAAGG
OPA1_ChIP_RP_1: GCAGGTCCAGAGAGCAAT
OPA1_ChIP_FP_2: ATTGCTCTCTGGACCTGC
OPA1_ChIP_RP_2: GTCAGAATCATAAGTCTGGACC
OPA1_ChIP_FP_3: GTAGTTGAAGGAGTGCTC
OPA1_ChIP_RP_3: GTTGAAATGGTCCTCGTC
PIK3CA_ChIP_FP: CCTCCTCAGCTCTTACCC
PIK3CA_ChIP_RP: GGTCTCTTTTTCCGCTCACA

Affymetrix Gene Chip analysis

Microarray experiment was performed at the Life Sciences Training Facility (LSTF), Biozentrum, University of Basel. Total RNAs (200 ng) were used to synthesize target cRNA using the WT expression kit (Ambion). cRNAs (10 µg) were used to generate ~8.0 µg (±0.1 µg) of cDNAs. The WT Terminal Labelling Kit (Affymetrix) was used to fragment the cDNAs. The hybridization cocktail (85 µl) containing fragmented and biotin-labelled cDNAs (25 ng/µl) was transferred into Affymetrix GeneChip MoGene-1_0-st-v1 cassettes, which were incubated at 45°C inside a hybridization oven by rotating them at 60 rpm for 17 hours. Then the GeneChip arrays were washed and developed using the Hybridization Wash and Stain Kit in a Fluidics Station 450. The GeneChip arrays were read using the GeneChip Scanner 3000 7G and DAT image files were generated using the GeneChip Command Console (Affymetrix).

The Partek Genomic Suite 6.5 was employed to further validate the GeneChip results. Three completely independent samples for the cell line U373 (sh control, sh SOX2 and sh HEY1) were used to determine significant differences in expression levels. All Affymetrix CEL files were normalized using the RMA method and all data were *log2* transformed. To identify differentially expressed genes, two-way ANOVA analysis based on the 'method of moments' was used. Differentially expressed genes were further filtered using a *P*-value threshold of 0.05 or less.

Generation of promoter constructs and cloning into expression vector

Genomic DNA was isolated from HEK293 cells using the Qiagen DNeasy Blood and Tissue kit (Cat No. 69504, Qiagen) according to manufacturer's instructions. The promoters of *SOX2, MFN1, OPA1 and PIK3CA* were amplified from genomic DNA isolated from HEK293 cells. *MFN1*, *SOX2*, *PIK3CA* promoter fragments were amplified by generating BglII-HindIII over hangs. *OPA1* promoter fragments were generated with BamHI-NotI overhangs. All the promoter fragments were amplified using Expand long template dNTP pack 175U (Cat. No. 04829034001, Roche) and PCRs were performed according to manufacturer's instructions. Amplified promoter fragments were then cloned into pGCL4.1 basic expression plasmid (Promega) (kindly provided by Prof. G Christofori). PGCL4.1-Luc-SOX2, pGCL4.1-LucMFN1, pGCL4.1-Luc PIK3CA were constructed by inserting the respective BglII-HindIII promoter fragments into the BglII-HindIII site of the pGCL4.1 expression vector. pGCL4.1-Luc OPA1 vector was constructed by inserting the BamHI-NotI promoter fragment into BamHI-NotI site of the pGCL4.1 expression vector. Restriction enzymes BamHI (Cat No. R3136, NEB), NotI (Cat No. R3189, NEB), BglII (Cat No. 10348767001, Sigma-Aldrich) and HindIII (Cat No. 10656321001, Sigma-Aldrich) were used for cloning. Site directed mutagenesis is currently being performed. Primers used to amplify promoter regions and site directed mutagenesis are mentioned below.

1. Primers used for cloning promoter region

SOX2 FP: GGAAGATCTACGTGGCTGGTAGATACTATT

RP: GCGGAAGCTTTCCATATGTGACGGGGGCTGT

- Promoter was cloned into pGCL4.1 basic Luciferase expression vector by creating BglII- HindIII

MFN1 FP: GGAAGATCTGGAAGATCTCATGATCCAAACTGCTGG

RP: GGGAAGCTTGCCTCATCTTCCCGAG

- Promoter was cloned into pGCL4.1 basic Luciferase expression vector by creating BglII- HindIII

OPA1 FP: CGGCGCTAGCAGTAGAGACAGGGTTTCACC

RP: GGCTCGAGGGCGCATGGACTTCCGCAAG

- Promoter was cloned into pGCL4.1 basic Luciferase expression vector by creating NheI- XhoI

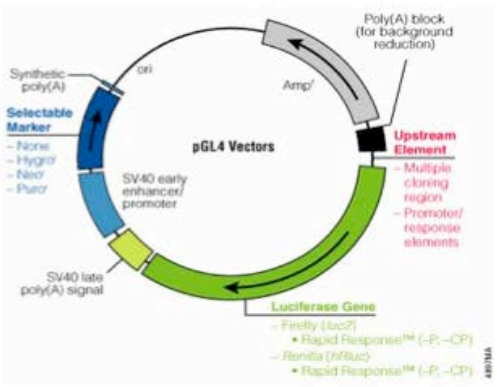
PIK3CA FP： GGCAGATCTCCTAGCACTCTGGGAAGTCG

RP： CGCAAGCTTACTTCTCGCTCCCTCTCCTC

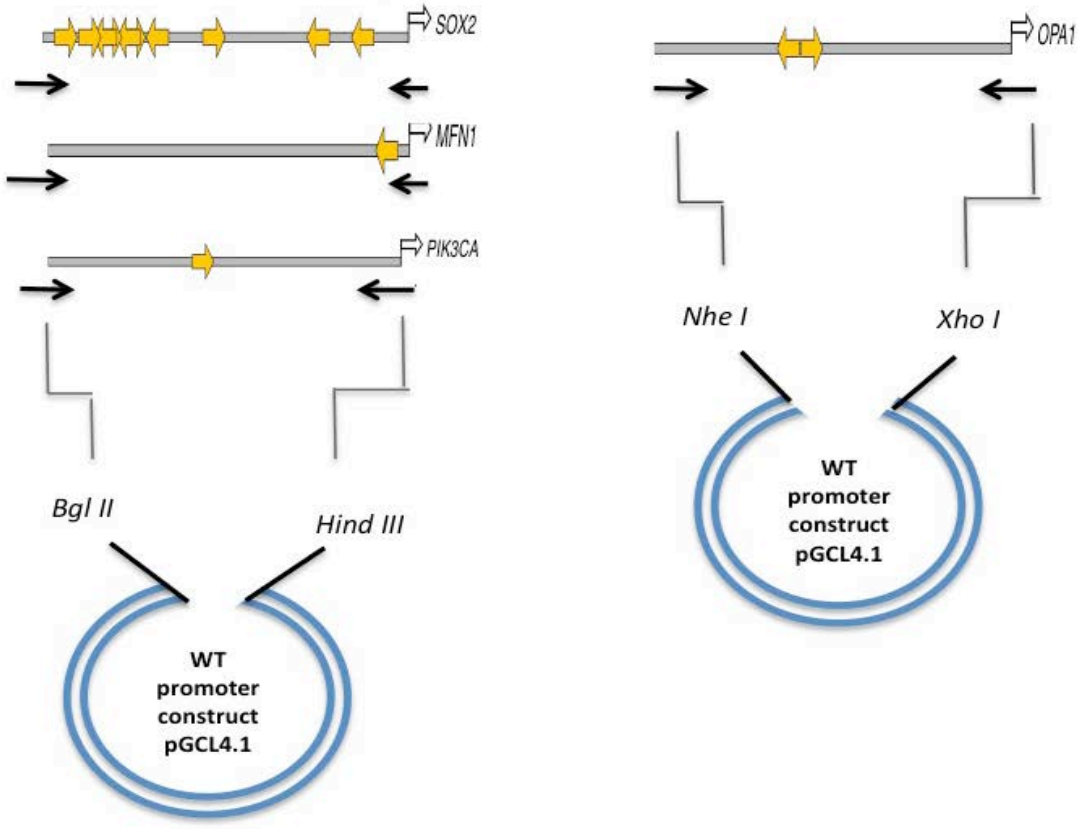
Promoter was cloned into pGCL4.1 basic Luciferase expression vector by creating BglII- HindIII

2. Cloning strategy for 3q26-29 promoter regions

A pGCL4.1 basic luciferase vector map



B Promoter cloning strategy



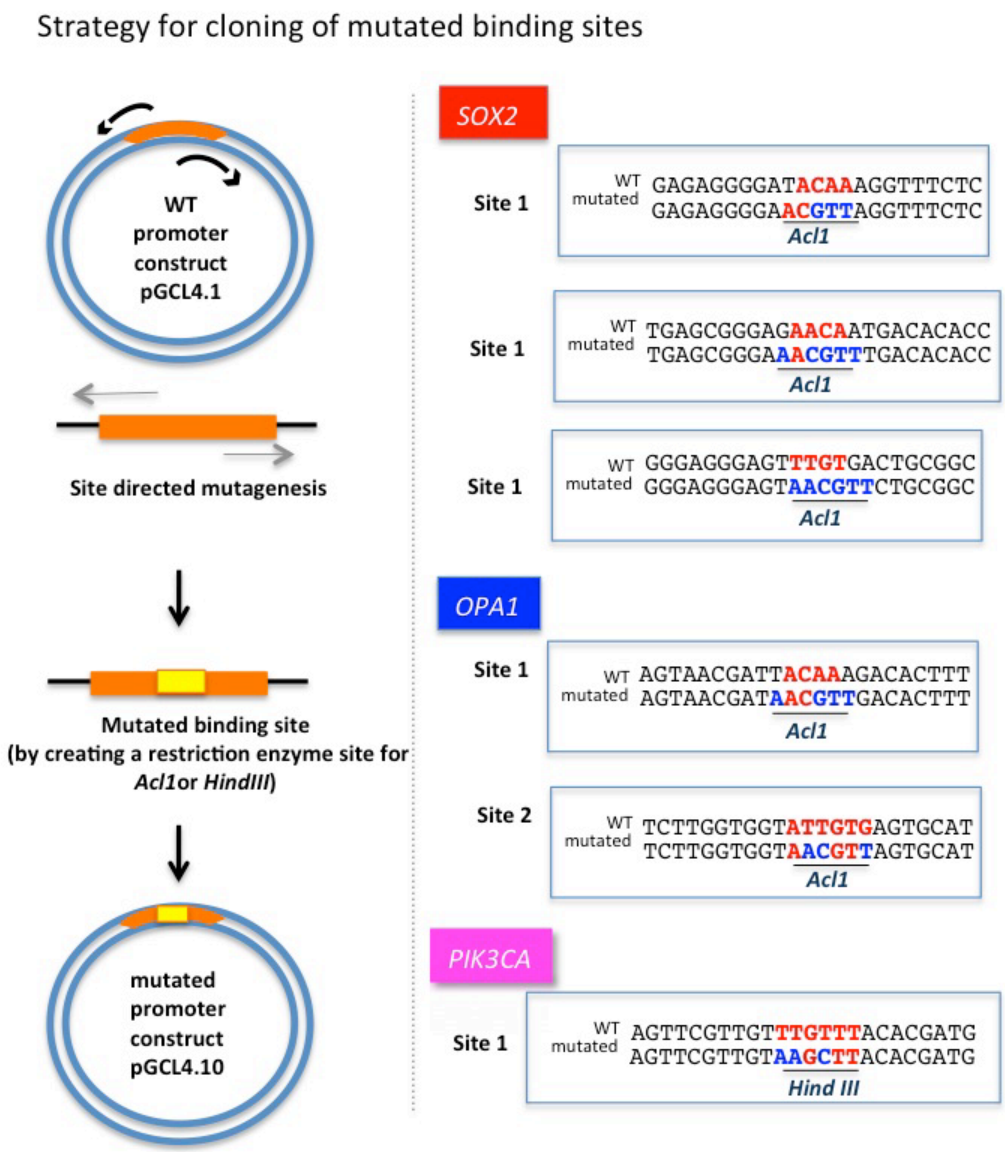
3. Primers used for site directed mutagenesis

Primer pairs were generated around the predicted SOX2 binding sites in promoter region of 3q26-29 genes. Mutated nucleotides are highlighted in red. Restriction enzyme sites formed on sdm constructs are underlined.

SOX2 site 1_FW: GCGAGAGGGGAACGTTAGGTTTCTCAGTGG
1_RP: CCACTGAGAAACCTAACGTTCCCCTCTCGC
SOX2 site 2_FW: GATGAGCGGGAAACGTTTGACACACCAACTC
2_RP: GAGTTGGTGTGTCAAAGTTCTCCCGCTCATC
SOX2 site 3_FW: GCGTGGGAGGGAGTAACGTTCTGCGGCCCA
3_RP: TGGGCCGCAGAACGTTACTCCCTCCCACGC

OPA1 site 1FW: GGAAGTAACGATTACTTAGACACTTTTTGTAC
1RP: GTACAAAAAGTGTCTAAGTAATCGTTACTTCC
OPA1 site 2FW: TACTCTTGGTGGTAAAGTGAGTGCATGGGTC
2RP: GACCCATGCACTCACTTTACCACCAAGAGTAG
OPA1 site 3FW: AGGCTCACTGGCCACTTACAATAGGGAGCTGG
3RP: CCAGCTCCCTATTGTAAGTGGCCAGTGAGCCT
PIK3CA site FW: GAAGAGTTCGTTGTAAGCTTACACGATGTG
RP: CACATCGTGTAAGCTTACAACGAACTCTTC

4. Cloning strategy for sdm of predicted binding sites in 3q26-29 promoters



Luciferase assays

Human Embryonic Kidney (HEK) 293 cells were used for Dual luciferase assay. 75,000 cells per well were seeded in 12 well plates. JET PEI™ transfection reagent (Cat No. 101-01N*, Polypus-transfection) was used to transfect HEK293 cells with luciferase reporter vector along with Renilla vector (0.1µg) for estimation of background. Cell lysates were extracted after 48 hours of incubation and analyzed using the Promega Dual Luciferase Assay kit (DLR™ Cat No. E1980, Promega) and

luciferase activity was measured using TECAN™ (TECAN Infinite 1000, Tecan group).

Generation of SOX2 overexpressing Lentiviruses

1. Cloning

The cDNA of *SOX2* was amplified from mRNA that was isolated from HEK293 cells using the Qiagen RNeasy kit (Cat No. 74104, Qiagen). The cDNA was amplified by generating BamHI-HindIII over hangs using Expand long template dNTP pack 175U (Cat. No. 04829034001, Roche) and PCRs was performed according to manufacturer's instructions. Amplified cDNA fragment were then cloned into pHAGE-Lentivirus vector system (Invitrogen, and was kindly provided by Prof. Yves Barde). Restriction enzymes BamHI (Cat No. R3136, NEB), NotI (Cat No. R3189, NEB), were used for cloning.

Primers used for SOX2 cDNA amplification:

FW:
GCGGCCGCGCCACCATGGAACAAAAACTTATTTCTGAAGAAGATCTGTACAACATGATGGAGACGGAG

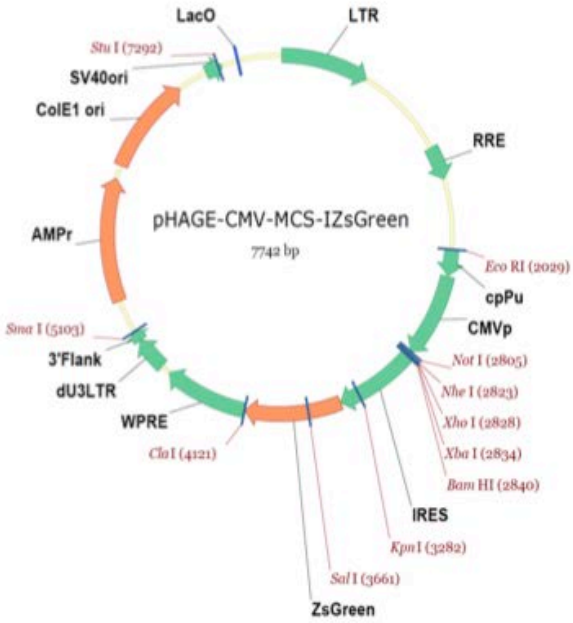
RW: GGATCCTCACATGTGTGAGAGGGGCAG

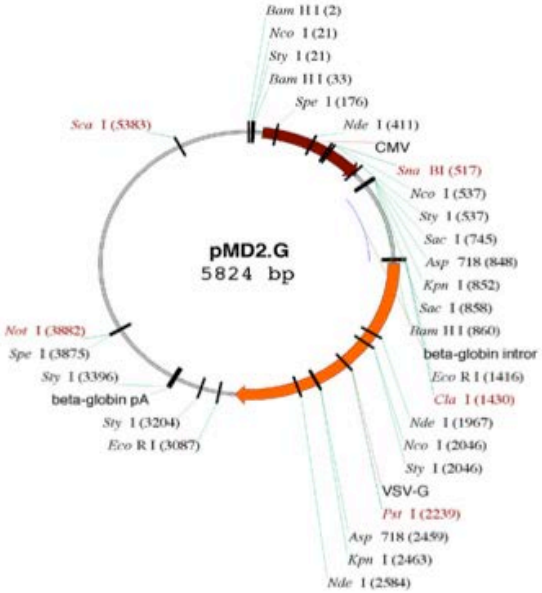
2. Lentiviral packaging and transduction

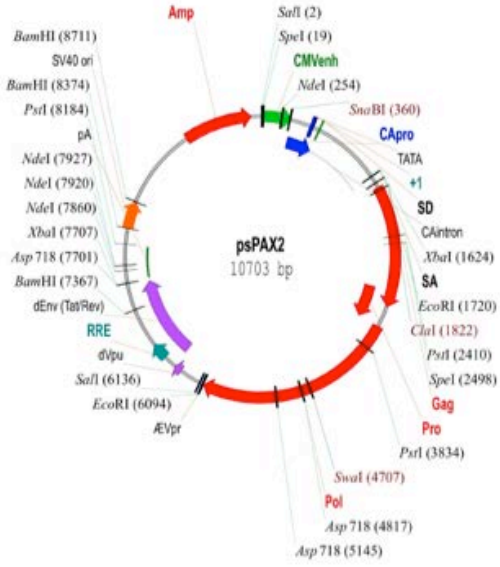
HEK-293T cells were seeded at 40% density on 10 cm culture dishes. After 24 hours, the packaging plasmid (psPAX2), the plasmid coding for the envelope proteins (pMD2-VSV-G) and Lentivirus vector (pHAGE-PGK-MCS-IRES-ZsGreen-W-scrambled, pHAGE-PGK-MCS-IRES-ZsGreen-W containing cDNA insert of SOX2 and pHAGE-PGK-MCS-IRES-ZsGreen-W containing cDNA insert of HEY1) that carries the gene of interest were transfected on HEK-293 cells using Pei transfection reagent (Cat No. 115-010, Polypus Transfection). The transfection mix contained a total of 8.2 µg of pHAGE- Lentivirus vector system/ 10-cm dish – 4 µg Lenti- SOX2/ Lenti-HEY1 vector, 3 µg PAX2, 1.2-µg pMD2-VSV-G.

After 48 hours of the transfection the supernatant was collected and centrifuged at 3000 rpm for 15 minutes. The supernatant was passed through a 0.45µm PVDF membrane filter. The viral supernatant was spun 3000 rpm for 90 min. The pellet was suspended in 100µl DMEM and were stored in -80°C. The supernatant was titrated on HEK293 cells to estimate the viral transduction efficiency.

Lentiviral vector system used for SOX2 overexpression







RT-qPCR

Total cellular RNA was extracted from subconfluent cells using Trizol reagent (Cat No. 15596-026, Thermo Fischer scientific) and RNeasy Qiagen kit (Cat No. 74104, Qiagen). RT-qPCR was performed with 300ng of RNA using iScript cDNA synthesis kit (Cat No. 170-8890, Bio-Rad) according to manufacturer's instructions. The qPCR from the cDNA was performed using Ssofast Evagreen supermix kit (Cat No. 172-5200, Bio-Rad) using the CFX90 qPCR thermocycler (Bio-Rad). The data obtained were quantified using ΔΔCt method and results were expressed as fold change ($2^{-\Delta\Delta Ct}$) compared to control samples and GAPDH (housekeeping gene).

Primers for cDNA synthesis were purchased from Qiagen

Hs_GAPDH_1_SG QuantiTect Primer Assay (QT00079247)

Hs_SOX2_1_SG QuantiTect Primer Assay (QT00237601)

Hs_MFN1_1_SG QuantiTect Primer Assay (QT00077966)

Hs_OPA1_1_SG QuantiTect Primer Assay (QT00085519)

Hs_PIK3CA_1_SG QuantiTect Primer Assay (QT00014861)

Hs_HEY1_1_SG QuantiTect Primer Assay (QT00035644)

Western blot analysis and antibodies

Total cellular lysates were obtained using the NE-PER nuclear and cytoplasmic extraction kit (Cat No. 78833, Thermofischer Scientific). Protein concentration was measured using Bicinchoninic acid (BCA) assay (Cat No. 23225, Pierce™ Protein assay kit, Thermofischer Scientific). Proteins were denatured in Laemmli sample buffer (1:1) and were resolved on 4-20% and 8-16% bis-tris gels (Novex Page, Invitrogen). Western blots were performed using anti-SOX2 (MAB4343, Millipore), anti-MFN1 (sc50330, Santa Cruz), anti-OPA1 (BD Bioscience), anti-actin (sc1615, Santa Cruz), anti-PIK3CA (Proteintech) antibody at 1:1000 dilution. All the blots were blocked in 3% Top-block solution and Li-cor Odyssey secondary antibodies were used to detect the bands in the Li-cor Odyssey machine.

Immunofluorescence imaging

In order to study mitochondrial dynamics, cells were co-stained with 12nM mito-tracker green and 1mM Hoechst dye for 30 minutes and were washed with PBS. Spinning disk confocal microscope (Perkin Elmer), available at the Imaging core facility (IMCF), in Biocenter, University of Basel, was used for mitochondrial imaging. In order to study mitochondrial dynamics, 5000 cells were seeded on borosilicate 1.5mm glass chambers (Cat No. 155411,Lab-Tek II 8 well, Thermofischer Scientific). Cells were stained with mito-tracker green and Hoechst. Live cell imaging was performed at 37^{o}C and 5% CO_2 conditions. Cells were imaged up to 7 minutes and 12 images per minute were recorded. All confocal images were subjected to standard data processing tools and were done according to the guidelines provided by the IMCF.

Immunocytochemical stainings were performed using anti-SOX2 antibody and DAPI. The cultured cells were fixed with PFA for 20 minutes and then were permeabilized with PBS containing 0.01% triton X-100. The permeabilized cells were incubated with primary antibody overnight and over 1.5 hours with secondary antibody. Cells were washed and stained for DAPI. The stained cells were then mounted on a glass slide for imaging. Laser scanning confocal microscopes were used from the imaging core facility of the Department of Biomedicine (DBM).

List of primary antibodies

Antibody	Cat. No	Company	Species/ Isotype IgG	Epitope (mAb/pAb)	Dilution	Experiment
anti-MFN1	sc-50330	Santacruz	Rabbit	pAb	1:1000	WB
anti-OPA1	612606	BD Lifescience	Mouse	mAb	1:1000	WB
anti-Actin	Sc-1615	Santacruz	Goat	pAb	1:1000	WB
anti-PIK3CA	20583-1-AP	Protein tech	Rabbit	pAb	1:1000	WB
anti-H3	Ab1791	Abcam	Rabbit	pAb	10µg	ChIP
anti-H2B	Ab 1790	Abcam	Rabbit	pAb	1:1000	WB
anti-SOX2	Ab 59776	Abcam	Rabbit	pAb	10µg	ChIP
normal IgG	12-370	Millipore	Rabbit	pAb	10µg	ChIP
anti-SOX2	mAB4343	Millipore	Mouse	mAb	1:1000	IF/WB
DAPI	D9542	Sigma-Aldrich	-	-	1:10,000	IF
Hoechst	H1398	Thermofischer Scientific	-	-	10nM	Live- Imaging
Mitotracker green	M7514	Thermofischer Scientific	-	-	10nM	Live- Imaging

List of secondary antibodies

Secondary Antibody	Cat. No	Company	Fluorescent conjugate	Dilution	Experiment
Goat anti- mouse	926-32210	Odyssey	IR-Dye* 800CW	1:10000	WB
Donkey anti-goat	925-32214	Odyssey	IR-Dye* 800CW	1:10000	WB
Goat anti-rabbit	A21109	Invitrogen	Alexa- 680	1:10000	WB
Goat anti-mouse	A21057	Invitrogen	Alexa- 680	1:10000	WB
Donkey anti-mouse	A21057	Invitrogen	Alexa- 555	1:10000	Confocal imaging
Goat anti-mouse	115-165-164	Jackson immunoresearch	Cy3	1:10000	Confocal imaging

Statistical Analyses

All data are presented as mean ± SEM or mean ± SD as mentioned. Student's t test, Mann-Whitney test were used where appropriate using graph pad prism v6.

3q26-29 genes promoter sequences

Human and mouse promoter sequences upto 2000 bp upstream of transcription start site of 3q26-29 genes are aligned using CLUSTAL W (1.83) multiple sequence alignment. Potential SOX2 binding sites are highlighted in yellow (List of potential binding sites for SOX2 are shown in Result section Fig. 23 of Chapter I).

1. *SOX2* promoter region

```
CLUSTAL W (1.83) multiple sequence alignment

hSOX2           AAGAACTAAAACAAGCCATAACTTGAGAGAAAAAGGAGAACCTTCGGGGG
mSOX2           A---------------------------------GGA----------GGG
                *                                 ***          ***

hSOX2           GCAGGAAGGTTGATTGGAAATAACTTAAGGAAAGTCTGCAGAATTCTTTT
mSOX2           GCCGC-----------------------GAGAG----------------
                ** *                       ** **

hSOX2           TTTTACAACTTTTCTGAGTTTCCAGTGGGTATATTTAGTGTGAGTTTGAC
mSOX2           ----------------------------TA--TT------------G--
```

```
                                        **  **           *

hSOX2           AGTAACAGGCTAGGGAGGGCAGAGATTGGAGAAATTGGGGGTCGGGGGAG
mSOX2           -----CA-----GG-----------------------------------
                     **     **

hSOX2           TGATTATGGGAAGAAGGTTAGTAAGGAACAAAACAATGCACCGTTTTGTA
mSOX2           ---------GAAA----------------AA------------------A
                         ***                 **                  *

hSOX2           AAGATAATAAATGGAACGTGGCTGGTAGATACTATTCAGTACATTTTCTT
mSOX2           AAGA--------------------------------------------
                ****

hSOX2           AGGGTGAGTAAGGGTAGACCAGGGGAGGAGGGGGCGGAGAGAGTGTTACA
mSOX2           ---------A----------------------------AG---------
                         *                             **

hSOX2           GAAGAAAGAAAATAAGTAACCCTGATGGTTTAAGCCCTTTAT--------
mSOX2           AAAGAAAGAAAATAAGTAACCCTGATGGTTTAAGCTCTTTATTTAAAAAA
                 ********************************** ******

hSOX2           AAAAAAGAAATGGCATCAGGTTTTTTTTTCTTTATTCCCCCCCACCCCAC
mSOX2           AAAAAAAAAATGGCATCAGGACTTTCTTCCTTCTCT--------CCCC-T
                ****** *************  *** ** ***   *        ****

hSOX2           CCTTTGTAGTCAAGTGCATTTTAGCCACAAAGATCCCAACAAGAGAGTGG
mSOX2           CTTTTGTACCCAAGTGCATTTTAGCCACAAAGGTTCCAACAAGAGAGTGG
                * ******  ********************** * ***************

hSOX2           AAGGAAACTTAGACGAGGCTTTGTTTGACTCCGTGTAGCGACAACAAGAG
mSOX2           AAGGAAACTTAGACGGGGCTTTGTTTGACTCCGTGTAGCGACAACAAGAG
                *************** ***********************************

hSOX2           AAACAAAACTACCTATTTGTAACGGACGTGCTGCCATTGCCCTCCGCA--
mSOX2           AAACAAAACTACCTATTTGTAACGGACGTGGGGCTGTGGCTCTGGGCTTT
                ******************************  **  * ** **  **

hSOX2           TTGAGCGCCTACCTATTGAAATCTTTACGTCGGGACAATGGGAGAGCGGC
mSOX2           TTGAGCGCCTGCCGATTGAAATCTTTACGTCTGGACAATGGGAGATCGGC
                ********** ** ***************** ************* ****

hSOX2           TAAAATTACCCTCTTGGGTCCTGGGCGGGCAAGATTCCTGAGCCCCTACC
mSOX2           TAAAATTACCCTCTCGGGTCCTGGGCAGGCAAGATTCTTGAACCCCTACC
                ************** *********** ********** *** ********

hSOX2           CCCGCCCCCATCTCATCCTCCTCTAACCCGGGCCTTGCTGGGCTCCCCCT
mSOX2           CCCGCCCCCAGCCCATTCTCCCACAGCCTGGG-CTTGCTTGGTGCCGGCT
                ********** * *** ****   * ** *** ****** **  **  **

hSOX2           TCCCCAGTCCCGGCCGCCTTCTCCCAGTGTGCGCTGCCTGCACCTGTGCC
mSOX2           CCTCGGGTTCGGCGGGCCTTCTCTCC---------GCCTGCACCTGCACC
                 * *  ** * *   ******** *          ***********  **

hSOX2           TGGAGAGCATCGACCCCGCCTCCCAGGCCTTGAGCCCCTTTGCGGCGCAG
mSOX2           TCTGGACC-CCG--CGCGCTTCCCAGGCCC------------CGGCGC--
                *   ** *  **  * *** *********             ******

hSOX2           CCCCAGCCTTGCGCGGCCTGGGCTTTGCGGCCACCACACAATGGAAATCTAC
mSOX2           -------CT--CTCGGCCGGGCCTTCGTGACTACCACCGTGCCTGTCAGC
                       **  * ***** ** *** * * * *****  **    **  *

hSOX2           GGGGAAAATGCCAGGGCTGGTTCTGCTGGAGTCCTGGGAACTCTGCGTGG
mSOX2           AGGGGCAATGCC--GGCTG-CGCTGCCCCA-TCCTGAGAACGCTGCGTGG
                 ***  ******  *****   ****   * ***** **** ********

hSOX2           GAGGGAGTTTGTGACTGCGGCCCAAAAGCCACCTCCATACAGTGCCGTGG
mSOX2           GAGGGAGTTTGTGAGCGCAGCCCAAATGCCACCTCCGCGCCTCGCGGTGG
                **************  ** ******* *********   *   ** ****

hSOX2           GATGCCAGGAAGTTGAAATCACCCTCC-CCCATCGCCTGCACTTTTGAGC
mSOX2           GATGCCAGGAAGTTGC-GTGACCCTCCCCCCATCCCCTGCACTCGGGAGC
                ***************   * ******* ****** ********   ****

hSOX2           GCCCTTCCGTCTGTGTCTT--T-CCCCAGCCCCCATTTGAAAGCCGCACG
mSOX2           TTCTTTCCGTTGATGCTTTCGTCCCCAAGGTTTCCTCTGAAACTGCCACC
                  * ******   **  **  * *** **    * * *****    ***
```

```
hSOX2           ACCGAAACCCTTCTTACGGGGAGGCATGGGA-TGGGAATGGGGAGTGGGG
mSOX2           ACTAAAAATCCTCTTGTGT-CAGGGTTGGGAGTTAGAA---AGAGCGGGA
                **  ***  * ****  *   ***  ***** *  ***    *** ***

hSOX2           GCAGACAGTAGAAGCATCCCCTTTGCTACGGTTGAATGAAGACAGTCTAG
mSOX2           GCAG--AGTTCCAGCTTTGCCTTTGCACCCTTTGGATGG-GGCAGGTTGG
                ****  ***   *** *  *******  *  *** ***  * ***  * *

hSOX2           TGGGAGATGTGGCT-GGGGCTAAGAGGAAGAGCTGCAGTTTCCTGGGCCA
mSOX2           TGGAGTAGGGAATTAGGGGTTGAGGACACGTGCTGCGGTTCCTTGAGCCA
                ***   * *    * **** * **   * * ***** *** * ** ****

hSOX2           AAGAGCTGAGTTGGACAGGGAGATGGCAGCTTACCAAGGCCTGCTGGT-T
mSOX2           C--ACCTGAGTCGGCCTAGGAAAAGGCTGGG-AACAAGGCCTGGTCCTAG
                   * ****** ** *  *** * *** *   * ********* *  *

hSOX2           CTCAGCTCTAGAGTCTGCCTTATGGTCCGAGCAGGATTTATTTTTAAGAA
mSOX2           CTCTGCTCTACAGTCAGCTACATGGGCAGA---GGACTCGTGTTTGGGAA
                *** ****** **** **   **** * **   *** *  * ***  ***

hSOX2           CAGAGCAAGTTACGTGGAAGCAAGGAAGGTTTTGAGGACAGAGGTTTGGG
mSOX2           CCGAGCCGATGACACGGAAGCCAGGAGGGCGCTGAGGCCCGAGGCTTGGG
                * ****   * **  ****** **** **   ***** * **** *****

hSOX2           TCTCCTAACTTCTAGTCGGGACTGTGAGAAG--GGCGTGAGAGAGTGTTG
mSOX2           TC---TAACTTCTCGTCTGTACGGTGAGAAGAGGGGGTGAGTGGGTGCTG
                **   ******** *** * ** ********  ** ***** * *** **

hSOX2           GCACCTGTAAGGTAAGAGAGGAGAGCGGAAGAGCGCAGTACGGGAGCGGC
mSOX2           GCGAC---AAGGT------------TGGAAGAG----------GGGCTGC
                **  *   *****             *******          * ** **

hSOX2           ACCAGAGGGGCTGGAGTTGGGGGGGAGTGCTGTGGATGAGCGGGAGAACA
mSOX2           GACAGAG---------------CGCAGTGCCGCGGATGAGCGCAGAAACA
                  *****                * ***** * *********    ****

hSOX2           ATGACACACCAACTCCTGCACTGGCTGTTTCCAGAAATACGAGTTGGACA
mSOX2           ATGGCACACCACCTCCGGCTCTGCCAGCTTCCTGAAATACTAGTTGGACA
                *** ******* **** ** *** * * **** ******* *********

hSOX2           GCCGCCCTGAGCCACCCACTGTGCCCTGCCCCACCCCCGCACCTTAGCTG
mSOX2           GTCGCCCTGAACCACCCA-TGGGCCTTGCCCCACCCTGGCCCC--A---G
                * ******** ******* ** *** **********  ** **  *   *

hSOX2           CTTCCCGCGTCCCATCCTCATTTAAGTACCCTGCACCAAAAAGTAAATCA
mSOX2           CTTCCCGCGCCCCATCCACCCTTATGTATCC-------AAGAGAGAGCCA
                ********* ******* *  *** *** **       ** **  *  **

hSOX2           ATATTAAGTTTAAAGAAAA-AAAAACCCACGTAGTCTTAGTGCTGTTTAC
mSOX2           ATATTCCGTAGCATGGGGGAAAGGAGC--TGTCGTCTTGGTGCTGTTTAC
                *****  **   * *     **  * *   ** ***** ***********

hSOX2           CCACTTCCTTCGAAAAGGCGTGTGGTGTGACCTGTTGCTGCG--------
mSOX2           CCACTTCCTTCGAACAGGCGTGCGCCGTGACCTGTTGCTGAAAACGGGGG
                ************** ******* *  ***************

hSOX2           ------AGAGGGGATACAAAGGTTTCTCAGTGGCTGGCAGGCTGGCTCTG
mSOX2           GCGGGGGGGGGGGATACAAAGGTTCCCCAGCGGCCGGCTG-CGGGC----
                       * *************** * *** *** *** * * ***

hSOX2           GGAGCCTCCTCCCCCTCCTCGCCTGCCCCCTCCTCCCCCGGCCTCCCCCG
mSOX2           ----CCGCCTCCCCCGCGCGGTTCG-GGGCA----CAGCGCTCTGCTGGG
                    ** ******** *   *   *    *     *  **  ** *   *

hSOX2           CGCGGC-CGGCGGCGCGGGAGGCCCCGCCCCCTTTCATGCAAAACCCGGC
mSOX2           CTCGGCTCGGCGGCGCGGCAGGCCCCGCCCCCTTTCATGCAAAACCCTCT
                * **** ********** ****************************

hSOX2           AGCGAGGCTGGGCTCGAGTGGAGGAGCCGCCGCGCGCTGATTGGTCGCTA
mSOX2           GGCGAGGCTGGGCTCGGGCGCAGGAGCCGGCGCTCGCTGATTGGCCGCCG
                 *************** * * ******** *** ********** ***

hSOX2           GAAACCCATTTATTCCCTGACAGCCCCCGTCACATATGGATGGTTGTCTA
mSOX2           GAAACCCATTTATTCCCTGACAGCCCCCATCACA--TGGATGGTTGTCTA
                **************************** *****  **************
```

```
hSOX2           TTAACTTGTTCAAAAAAGTATCAGGAGTTGTCAAGGCA------------
mSOX2           TTAACTTGTTCAAAAAAGTATCAGGAGTTGTCAAGGCAGAGAAGAGAGTG
                ************************************

hSOX2           --------------------------------------------------
mSOX2           TTTGCAAAAAGGGAAAAGTACTTTGCTGCCTCTTTAAGACTAGGGCTGGG

hSOX2           --------------------------------------------------
mSOX2           AGAAAGAAGAGGAGAGAGAAAGAAAGGAGAGAAGTTTGGAGCCCGAGGCT

hSOX2           --------------------------------------------------
mSOX2           TAAGCCTTTCCAAAAACTAATCACAACAATCGCGGCGGCCCGAGGAGGAG

hSOX2           --------------------------------------------------
mSOX2           AGCGCCTGTTTTTTCATCCCAATTGCACTTCGCCCGTCTCGAGCTCCGCT

hSOX2           --------------------------------------------------
mSOX2           TCCCCCCAACTATTCTCCGCCAGATCTCCGCGCAGGGCCGTGCACGCCGA

hSOX2           --------------------------------------------------
mSOX2           GGCCCCCGCCCGCGGCCCCTGCATCCCGGCCCCCGAGCGCGGCCCCCACA

hSOX2           --------------G-----------------------------AG
mSOX2           GTCCCGGCCGGGCCGAGGGTTGGCGGCCGCCGGCGGGCCGCGCCCGC
```

2. *MFN1* promoter region

```
CLUSTAL W (1.83) multiple sequence alignment

hMFN1           CACACGCCTCATGATCCAA-ACTGCTGGGATTACAGGCGTGAGCCATCGC
mMFN1           TAGGTAC----TGACCTGTCTTTGCTGTGCTTT---GCAT-AGTATGTGC
                 *    *    *** *      ***** * **    ** * **     **

hMFN1           ACCCGGCAAGATTAATCTTTTCGTTAAACATGTAATAGAAAGTTACCCCG
mMFN1           ACACG-TGTGAGTACATTAGCCGCTAGAGAT-CAACAGTGAGCAACGTTC
                ** **    ** **   *   ** ** * **  ** **  **  **

hMFN1           AAAATCA-TCTAGACCTGGCTAGTTTTGAATATGTGGGAAACTTAGCCTA
mMFN1           ACTATCAGTCTCCACCT------------TATTTTGGAAAC--AGTCTT
                *  **** ***  ****             *** * ******  ** **

hMFN1           TTCGAGTTTTCTATTTTTTCTCGGTCTAATTTTGGTATTTGATGTTTTCC
mMFN1           T-C---TTTTTTGTTTTTTGTTTGTCAAGACAGGGTTTCT-CTGTGTA-G
                * *   **** * ****** *  *** *     *** * *  *** *

hMFN1           CAGATTTTATACTTCATCT-AAGCTTTTCAATTTGTTGGCATTTAT-ATA
mMFN1           CACTGGCTGTCCTGGAATTCACTCTGTAGACCAGGCTGGCCTCGAACTCA
                **     * * **  *  * *  ** *  *    * **** *  *    *

hMFN1           TATATCTATAGTTATTCT-CCCCTTAATTTGTGTCCTTTCATTTTTTATG
mMFN1           GAAATCCT-CCTGCCTCTGCCTCCCAAGTGCTGG--GTTCAAAG-GTGTG
                 * ***     *   *** ** *  ** *  **    ****     * **

hMFN1           TTCTTGAACAGATTTGGCAGTGATACATCAAATTGATAATTTCTTTCAAA
mMFN1           TGC--CACCATGCCTGGCAA--GTCTTTCAAAGAACTCAGCTCACTGA--
                * *   * **    *****    *   *****    * *  **  * *

hMFN1           TAACGTTAGTTTTTGTTAATATCTATGTTGGTTTTAGCGTTTATATTTTA
mMFN1           T-TTGCTAGCCTGGCCAGCCAGCTCCAGAGATTTCCCCACCCCCAGCATT
                *   * ***  *        * **     * ***   *      *   *

hMFN1           ATTCTTTTCCTTCTTTGTGTTTACTCTCCTGGCTTTTTGGTAATATGTGT
mMFN1           ATTGTTTTCCCCATGAGTGGCTAGAGATCTGGATCC-AGGTC-TTTATG-
                *** ******   *  ***  **     **** *    ***  * * **

hMFN1           GTTCCA-CACACACACAAAAGCAATGGATTAAATTTCTTATTACTTACTA
mMFN1           ---CCATCACAGCAAGAACTTTACTGCTTGAGCCTTCTCCCCAG----TC
```

```
                  *** ****   * **    * **  * *   ****    *     *

hMFN1          TATTAAACATTTA--ATTACCGTGCTATAATTTATAGCCTGTAATATGTC
mMFN1          TCTTAAGTACTGATCCTTATTG-GC-----TATATACCCTGGAAATAGGC
               * ****  * * *   ***  * **     * **** **** **   * *

hMFN1          ACTCTTGCTTTAAAATGCAGTAATTATATTAAATATAATTATTAAGTACA
mMFN1          AGTTCAAGGCCAGCCTGG--TCTATGGAGTGAGTTCCAGGGCTACACAGA
               * *        *   **   *   *  * * * *   *    **   * *

hMFN1          GAA-------CTAATAAAAGGATACCATCATGATTTTTAAAATTCAAACA
mMFN1          GAAACCCTGTCTCA-AAAAGAAAAA---AATCATTTTAAAAAATCTGGCC
               ***       ** * ***** * *     ** ***** **** **   *

hMFN1          GAGTAAATGTTCCCTTTGAGACTACCTATGCTCAGAACTGA--TGGATTA
mMFN1          TGGGAGGCAAAGGCATTGGATCTCTGTGAGTCCAAGGCTAACTTGTTCTA
                 * *        * ***   **   *  *  **   ** *  **   **

hMFN1          GCTGGCACGACCCAG-ACCGATAAACTGGCTCATCTGACCTAGTGTCCCC
mMFN1          CATAGTGAGTTCCAGGACCCA-GGACTGTCT-ATATAA---AATGACACT
                 * *   *  **** *** *   **** ** ** * *   * ** * *

hMFN1          CAACCCAGGAACTGACTCGTGCAGAAAGACAGCTTCCACTCCCTGCGATT
mMFN1          GTCTCCAAAAAACAACCCA---AAACAAAAATCTTCAAATTTAGGCAGAG
                   ***  **   ** *    * * * * * **** * *    **

hMFN1          TCATCCCTGACCAATCAGCACTCCTG-GCTCAGTGGCCTCCCCCAACCCA
mMFN1          TGAGAGC--ACAGATCTGAATTCTAGCACTTGGGAGGCTGTGACAGCGGG
               * *   *  **  *** * * **  *  **  *  * **    ** *

hMFN1          CCAAATTGTCCTTAAAAATTCTG--CTCCCCAGTGCTGCTCGGGGAGACT
mMFN1          TTTGTGAGTTCTTTTTGTTGTTGTTTTGTTTTGTTTTGTTTTTTGTTGTT
                      ** ***     *  **   *     **  ** *    *    *

hMFN1          GATTTCAGTAATAATAAAACTCCAGTCTCCCATACAGCC-GGCTTTGCGT
mMFN1          GTT-TGTTTGTTTG-------TTTGTTTTTTTTGGAGACAGGGTTTCTCT
               * * *   *  *           ** *    *  ** * ** ***   *

hMFN1          GACTAAGTCT--TTAT-TTGCAATCCCCCTGTCTTGTATCAGTTCTGGCT
mMFN1          GTATAGCTCTGGTTGTCTTGGAA-CTCACT---TTGT---AGATCAGGCT
               *  **  ***  ** * *** ** * * **   ****   ** ** ****

hMFN1          AGTCAGCGGGCAAGGTGAAGGCTTTCCTTGGGCGGTTACCATCCGA-AGC
mMFN1          GGCC-----TCAAACTCAGAAATCTGCCTGT-C-TCTGCCTCTCGAGTGC
                * *      ***  * *    * * * **  *   * **   ***  **

hMFN1          TTTAATTTTAAGTATA--ATGGCAAGCTGTTGGAAAGCTTTTTTTTTTTT
mMFN1          TGGGATTAAAGGTGTGTGCCACCACGCCCAGCTCATGCTTGTTTTTTTAA
               *   ***  * ** *        ** **       * **** *******

hMFN1          TTTTTTTTTTTTTTTTTTTTTTTTGAGATGGAGTCTGGCTCTGTC---GC
mMFN1          CTCATCCATTTTAGCACTGTCCTTCCTATG-TGTCTTGGACAGTTTTTCT
                *  *   ****     * *  **   ***  **** *  * **

hMFN1          CCAGGC---TGGAGT-GCAGTGGCGCGATCTCGGCTCACTGTAACCTCCG
mMFN1          CCAGATATTCCCAGTAACAGTC--CTGAAATGACCTGA--GAAATGACAT
               ****        ***  ****     **  *   ** *  * **   *

hMFN1          CCTC-CCGGGTTCAAGCAATTCTCTGCCTC-AGGCTCCTGAGTAGCTGGA
mMFN1          CCTTTCAGAAAACACATACCTCTTTGGCTCCACCTTGCTCATTTTTCTTC
               ***  * *    **   *  *** ** *** *   * ** * *

hMFN1          ACTACAGGCGCCCGCCACCACCACGCCCGGCTA--CTTCTTGTATTTTTA
mMFN1          ACTCTATCCTCTTGTCCTGATCTCCCCCCCCCCCCCATAATAGCAGTCATA
               ***  *  * *  * *   * * * ***  *     *  * * * *  **

hMFN1          GT-AGAGACGG--GCGTTCACCGTGT-TAGCCAGGATG-CTCCCGATCTC
mMFN1          GTCTGAAATGATCTCATTCACTGTATTTATCCAACTTTTTTATCCAACTT
               **  ** * *    * ***** ** * ** ***   *   *  * * **

hMFN1          CTGACCTTG-TGATCCGCCCGCCTCG---GCCTCCCAAAGTGCTGGG-A-
mMFN1          TTATTTTTATTTATCCAACTTTTGCAGGTGCCCATCAATGTTTTAAGCAC
                *    **  * ****  *     *    ***   *** **  *  * *

hMFN1          -TTACAGGCGTGAGCCACCCCGCCCGGCCTGGAAAGCTTTTTAAGTTCGG
mMFN1          TTTACAC-CCTAACCCATTT-ACTT-TCCCA--CAATTCATGAAGTAGGC
                *****  * * * ***     *    **     *  *  * ****  *
```

```
hMFN1           AAA-GTAATACGATCTGGATTTCTTTTCTTTTATTTATTTAT------TT
mMFN1           ATTTTTAGTATTTTATAGATGAGGTAGCTGCCATTCATATGGATAAAGGG
                *    ** **   * * ***    *  **   *** ** *

hMFN1           ATTTAT-TTGAAAAAATGTCTCGCTCTCTTGCCCAGGCTGGAGTG-CA-G
mMFN1           GCTTCCCTGGCAAAAGCATCTCATTTGCTAACTCTGTAAAGAACCTCTCA
                  **   * * ****   ****  *  **  * * *    **    *

hMFN1           TGGCACGATAAATCACAGTTCGCT-GCAGCCCCGGGCCCAAACAATCCCC
mMFN1           GGACACATGCTGTGAAACCTCTCTTGGAAGGTGGAACATGTAGAGTCCTT
                 * ***      * * *  ** ** * *     *  *    * * ***

hMFN1           CCGCCTCAGTCTCCGAAGTAGCTGGGACCACAGGTGTGTGCCACCACGCC
mMFN1           GGGCTTCACCCTC-GATCAATC---AATCAATGGT--TTAACAAGATTCC
                  ** ***  *** **   * *    * **  ***   *  **  *  **

hMFN1           TCGCTAAGTTTTTAAAATTTTTTTAATTTTTCATTTTTTAATTTTGTAGA
mMFN1           CAGGTGATTCC-TAACAAAGTT-TAAATCATGAACAATTAATTTTCTTGA
                  * * * *   *** *   ** *** *  * *    ******** * **

hMFN1           G----AC---GGGGAGGAGGAAGAGGGTCGGGGGCGGGGGGGGGGGCGCC
mMFN1           GTAATACCTCTTACAGTAAGTAGACACACACAGCTCACTCCAGTAACCCC
                *    **       ** * * ***    *   *         *   * **

hMFN1           ----TCCCTATGTTGCCCAGCCTAGTCTCGAACTCCTGGGCTTAAGAGAT
mMFN1           AGAAAACCAATCAAGACCAGAGCAATTGGAAAAGTCAAAGTTTTGACGAC
                      ** **   * ****   * *    **   *   * **    **

hMFN1           A--TCCCACCCCTTCGGTCTCCCAAAGTGCT--GGGATTATAGG---CGT
mMFN1           CCCCCCCCCCCCCCCCCGCATGATAAACATCTATGTGGTTTTGGGGTACTT
                    *** ****  * *  *   ***   **  * * ** * **   * *

hMFN1           CAGCCA--CCACCATCAT-------TGGAAGCTAGCTAAGCCTAGGCTGG
mMFN1           CTCCCCCTACACCCTCAGATAACCATGGACATCTTTTCACACTTGGCGCC
                *  **    **** ***        ****       * *  ** ***

hMFN1           GCAGATACTTGGACACTTTGAGGGGAGAAGGGAGGGGAAGAAGTTGGAAG
mMFN1           TTTGAGCTTTGAAAACTCTGAAGGGAAAAACCAAGGGGAGGAGTTGGAAG
                   **   *** * *** *** **** **   * *** ** *********

hMFN1           GGGGAACGGAAAACCACTCTCCTG---GTAGCAGTCTCTGCCCACAACAA
mMFN1           GTGGAACAGAAAACAGCCCAGTGGTTAATAAGAACTCCTGCCCAAAACAA
                * ***** ******  * *    *    **  *    ******* *****

hMFN1           ACCTTGCCTTCTGCCCTTAAGGAAAATGGCGCCTGGCATAGATGCGCTTC
mMFN1           ACCTCGCCTTCAGCCCTGAAGGAAAATGGCGCCCGCAATATATGCAGTTC
                **** ****** ***** *************** *  *** ****  ***

hMFN1           CGGCCACGGAGCCAAACAGAACAGAGGAGGCAAGAGGCCGGAAGTGACCG
mMFN1           TGGTCTCGAAGCGTCAGCCAATAGGAGATCCGAAGCGCCGGAAGTAGCCG
                 ** * ** ***   *   ** **  **  * *   *********  ***

hMFN1           CCCTTTGCCACTCCCCCTGCCTCCTCTCCGCCTTTAACTTCTCGGGAAGA
mMFN1           CCCCTAGCC---------------------------------------
                *** * ***

hMFN1           TGAGGCAGTTTGGCATCTGTGGCCGAGTTGCTGTTGCCGGGTGATAGTTG
mMFN1           -----------------------------------C-------------
                                                   *

hMFN1           GAGCGGAGACT
mMFN1           --------CTC
```

3. *OPA1* promoter region

```
CLUSTAL W (1.83) multiple sequence alignment

hOPA1           ACCTCTGCCTCCTGGGTTCAAGCAATTTTTCTGCCTCAGCCTCCGAGTAG
mOPA1           AGCAAAA-GTCCAACATCATTTCACACTGTGTAGCC-AAACAAAAAAACT
                * *      ***    *     **   * * *  *  *  *    *

hOPA1           CTGGCTAATTTTTTTATTTTTAGTAGAGACAGGGTTTCACCATATTGGCC
mOPA1           CTGGCCTGAATTTGAACTAGTGGTGGTGGTTTGATTTCATAA---CAGTC
                *****     ***  * *  * ** * *    * *****  *     * *
```

```
hOPA1           AGGCTGGT-CTCAAACTCCTGACCTCGTGATCT-GCCTTCCTCGGCCTCC
mOPA1           TAACTAATTTTCCTATTCAG--CATTATAAGTAGATGTTCCTCAATGTAC
                   **  *  **  * **    * *  * *       ******    * *

hOPA1           -CAAAGTGCTG-GGATTACAGGCATAAGCCGCCATGCCCAGCCCAAATGT
mOPA1           ATAGCGTGAAGTGGATGTCAT--ATAAAC--CCATTA-TAACTTGAA---
                  *  ***  * ****  **   **** *  ****    * *   **

hOPA1           AGTGTTATTTTTAAAACACTGCATAGGCCAA---------CACTGTCTAA
mOPA1           AGTCTCACTTGAGGCACACC-CATATCCCAAACTTCCTACCTCAGTCTAG
                *** * * **     ****  ****  ****         * * *****

hOPA1           ACC--AAAC--AAAACAGGTCTGTG--GGCTGAATTTG--GTCCAATGGT
mOPA1           CCCTCTAACCTTAAACATGCTTACAACAGATACATTAGCAGTCAAGTGGC
                 **   ***   ***** *  *      * *  *** *  *** * ***

hOPA1           TGCAGTTTAT----TATACAACAGTTTGCCCTTTTTTCATGGCAACACTA
mOPA1           C-TATTTTATATGGGATTGAGTAAATCACCCTTATTAGATAAT-ATATTA
                   * *****     **  *  *  *  ***** **  **    * * **

hOPA1           TGTATCTATGGGAGATCATTCCTTATTAGTAC-GGATGTA--------AT
mOPA1           AATATAGAAAACAGGCTGGTTGAAACAAGTATTGGATGCATGGCTTCTAT
                  ***  *    **     *    *  ****  ***** *        **

hOPA1           TTATGTGTCTAGTTCCTAT-ACATGGACATT--T---ATACTGATTCCAA
mOPA1           TGAATTTTCTTCTTCACATTTCTTTGACAGCATTGTGAAGTTAAAACAAA
                * *  * ***  ***  **  * * ****    *   *   * *  * **

hOPA1           TCTT-TTCCCATTT--CATACAGTGCAGAAATAAACATCATCATGCATAC
mOPA1           ACAAAAACCTAGATTAAAGCCATTTCAAATTAGGGCAATCTATTTCAGAT
                 *     ** *  *   *  ** * ** *      **   *  * ** *

hOPA1           ATCTTTGCATACATGGGAA-T--GATTCCACAGGATAAATGCCTAGA---
mOPA1           ATTATTCTCTATTTGGATACTGAGAGCTCATAGGTTTTGTGACTAGTGTG
                **  **   **  ***  * *  **   ** *** *   ** ****

hOPA1           AGTAGAACT-GCAAAACAAG-GGCTTCATA-AAACAT--ATGATAACCAT
mOPA1           AATGTAATTCACTTAATTAGCTACTATCTAGAGACTTTCATGCTAACTCT
                * *  ** *  *  **  **   **   ** * ** *  *** ****  *

hOPA1           ATATA------AATGGTGATGGTG---GTTAGTTTCTCCTTTTTTTATGG
mOPA1           ATTTTGCTATCTAATACAATGGTAGACATAAGTTACACATAGTCCCAGCC
                ** *        *     *****     * **** * * *  *   *

hOPA1           TTAAAGTC---TTTTATCCC-----------------TAATTTTTCTTTT
mOPA1           TAGAAGTGGAATTATAAAGCAAGGGCTTTGTAAACTGCAAAGACACATT-
                *  ****    ** **   *                    **     * **

hOPA1           TTGTTGTTGTTGTTAAAGTCTCTTGGTTTCTCTTATTTTATTTTCTAA-A
mOPA1           -ACACATGGAAGCAGCCGTCACTTACTTCTCCCTTTTGTGTTTTCCAACA
                      * *  *     *** ***  **   * * ** * ***** ** *

hOPA1           ATAACAAGGAAGTAACGATTACAAAGACACTTTTTGTACAACCCTACAAT
mOPA1           AGAATTAGGAAGCAACAATAACAAATGCATTTTTTATTCAATACTAGAAA
                * **  ****** *** ** *****  ** ***** * ***  *** **

hOPA1           --CCCAGG----AA---GATCTACAG-----TTTGCACAGGTTGG----T
mOPA1           AAGTTGGGGTTTAAACTGGTTTCTAGTTTGGTTTATACTGGTTAGGCTTT
                      **    **   * * *  **     ***  ** **** *    *

hOPA1           ACAGCAAATTGCTCTCTGGACCTGCCATTGAAACAGTCACCACAATAAAT
mOPA1           ATAGAAGGTTTCTATCTACAGCTATCATTGAGACAGTCATCACAGTAAAT
                * ** *  ** ** ***  * **  ****** ******* **** *****

hOPA1           AAAGAAAATTCTAAGAGTCCTGCATTTGGGAAGTTTACTATCA-ATGGCT
mOPA1           AT-----ATTCCTCAAGTCCTGCAATCAGAACGTTTACTGTCAGACAGTT
                *      ****    ********* *  * * ******* *** *  * *

hOPA1           AGGTCT--ACTCTTGGTGGTATTGTGAGTGCATGGGTCCAGACTTATGAT
mOPA1           GCGTTTATATACTTGCCACTATTGTGACTAGATGGATTCTGATTTATGGT
                  ** *  *  ****    ******** *  **** * * ** ***** *

hOPA1           TCTGACAATTAAGGGAAAAGGAATTGTCTTT--ATGCT-------CTGTG
mOPA1           TTTGATAATTAGATAAAAAGCAATTTATTTGGGGTGCTGGTGGTGGTGTG
                * *** *****    ***** ****   **    ****        ****
```

```
hOPA1           TATTT-GTGTATGTGTGTGGCAT-GGGTGGGGGGCGGGTAGTGGGGA--G
mOPA1           TATTTCATGTGCGTGTGTGAGAGAGAGAGACTGACAGAGACAGGGGAAGG
                *****  ***  *******  *  * * *   * * *  *  *****  *

hOPA1           GAG----GTGAAAGG------TCCCTGAAGTAAAGAGGATGCACAGGACC
mOPA1           GAGACGAGTGAAAGGAATGTATCCTTGAAGTGAA-ATAAAGTACAAGACT
                ***    ********      *** ****** ** *  * * *** ***

hOPA1           CTTTTATTTTAGTC------CCTAAAAATTGGCTTCATAACCGTTCACAG
mOPA1           ATCTGATTTTTTCCAATAACACCATTAATCGACTTCACAATCTTTCACTG
                 * * *****   *       * *  *** * ***** ** * ***** *

hOPA1           GCCACATCCCTTTTACGGGCCC-TGGTTTCCTTACAAAACATAAAAGGTT
mOPA1           GCAATATTCCTGTTTTGAGTCCCTGTTCTCTTTA-AAAAAATACAAAAAT
                ** * ** *** **  * * ** ** * ** *** **** *** **   *

hOPA1           TGTGTTGGAAGGGGAAAGGAGTATTTAAAGTGATGGAGG-AGAGGAGCTC
mOPA1           G--GTTGAAGATGGAGAAGA-TACTTGGATTGATACAAATAATTTAGATT
                   **** *   *** * ** ** **  * ****  *   *    ** *

hOPA1           AAGATGGCTGAGATCTGGGCCTGTCCAACATTGAGAAATTTGGGAGGGGA
mOPA1           ATTAAGGAGAAAATGTA-GCCAGA--AAAAGGAAGGAACTCAAAAGGCCC
                *  * **   * ** *  *** *   ** *   ** ** *    ***

hOPA1           G--CCATCAAAGAAGCCTGGGAGCAGCAGTTCCAGGGAAAAAGGAGAATG
mOPA1           AAAACCTAACTGGAACTTTTGAATATTAAATAA---TAGTTGGGAGAAGC
                    * * *  * * * *  **  *  *  *      *    ******

hOPA1           TGATGGCCAGAGAGCCAAAAGAAAAAGTA-GTTGAAGGAGTGCTCAGCAC
mOPA1           AGGTAGCTAG-GAGACTAGACATCAACTACGTCCGAAGAGGGGGTTGA---
                 * * ** ** *** * * * *  ** ** **   * *** * *

hOPA1           TAGGCATCTGAACTGAATGCTGTGGCAGGCTCACTGGCCACAAACAATAG
mOPA1           TAGGGA----------GGAGTG-GGGAGGG--ATAGGTTACAAGGAGAAG
                **** *             ** ** ***   *  **  ****  *  **

hOPA1           GGAGCTGG---TGGAGGCCTTGACGAGGACCATTTCAACAAACTGGTGGG
mOPA1           GAAGAATGAATCTGACGTCTTGAAAATCAAAA-GAAAACTAAGAAGAGAA
                * **   *     ** * *****  *  *  *    *** **   * *

hOPA1           CTTA------AAATCCGGAAGAAACAGTTGAACAAATCATTTTGACGCCT
mOPA1           CTTAGGCAGATACTACT-AAGGAGCCATTACTGGAG--A-GATGGCGCTG
                ****       * * *  *** * *  **     *   *   ** ***

hOPA1           TTTATAAACCA-CACAAG-CTTATTCCAAACCCGTTACTGGCCTAACTGA
mOPA1           TTAAGAGACTTTGACAAGGGCCATTTCAATAAAATGGGTGCCATTA-AAA
                ** * * **    *****    *** ***     *   ** * * *   *

hOPA1           TTTAAGTCCCTTTCCCATCTGATCCTCAGAGATTCTAAGGGACTTAGCCT
mOPA1           TTCAGGTGA--AAGCCAACAAAG---GGGGGAATG-AAAAAACAAAACAA
                ** * **       *** *  *      * ** *  **   **  * *

hOPA1           ATCCATGACTCTT-CGTCCTGCTTCTCACCTCCCATGATTGCCCTAACGA
mOPA1           AACAAAAACATTTGTGTCTTA-ATAAAACTGTATGTGTTTATCCCAAACC
                * * *  **  **  *** *   *   **      ** **  ** **

hOPA1           TGTGAAAGTGCTTTCAAACAAAGATGCC-CAAGAAAGAAGGTAGGCAAAT
mOPA1           T---AC--AGCTTA--------AATGTCAGAAGTCACTTTCCAATCAGAT
                *   *    ****          *** *  ***  *      *  ** **

hOPA1           GTGCAAGCATTAGTTTGTAGTACGCTATTACTGTAT-TTCACCTTGCACT
mOPA1           CTCGGA-CTTTCTAAGGTGTCTCGC--ATACTCGATCTTCTGGCTATATG
                 *   * * **     **    ***   ****  ** ***    *  *

hOPA1           CTCTAGTTTCCTTCGTGCTCCCTCAATATCCAACTCTTAATAAATTCATG
mOPA1           ATTCAGCTACGAAAATGATCTTTAAA-A--GA--TTTCCTTAAATTGGCT
                 *  ** * *     ** **  * ** *   *  * *   ******

hOPA1           GCTCCCGGTGAGCATTCATCAATTCTCATTCCACGCCTTTAGCCCTTCCC
mOPA1           GCTCCTAATAAACATTCGTCCAGTG-GATTTCCTGCACCCAGCCCGCCCA
                *****   * * ***** ** * *   *** *  **    *****  **

hOPA1           GTTCCCGCCCAACTCTCGCTCCCTCCCCTGGCCAAATCTCTAACCTGCAA
mOPA1           GTTCATTTTCAAAATTCCTACACTTTGAGGGCGGAGCCAAGAA---G-AG
                ****     ***   **   * **     ***  *  *   **   * *

hOPA1           GGCTAATTCCGAATTCCAAATCGGAAGCGGGCCCCGTGAGAGGCG-ATGG
```

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mOPA1           GAGCACTACACAGGTCCCGCCCAGTGGC--TCCCCGCGCA-TGCGCATGT
                *   * * *  *  ***    * *  **   ***** *    *** ***

hOPA1           ATTGCTCCAGTCCGTTCCCGACGCACTGTGCGCATGCGCTGGTCCTCCGC
mOPA1           C-ATCCGCGGCCCGTTTGT---GCAGCCTGCCTA-GCGT---TCCT--GT
                    *  * * *****      ***   ***  * ***    ****  *

hOPA1           GGACCGTTC
mOPA1           G----GGTG
                *    * *
```

4. *PIK3CA* promoter region

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hPIK3CA         ACATTATCAACCGTGAATTGAATAGTAAATGCTACTCCTGTACCAATGAA
mPIK3CA         GTTTTCTCGTTTTTAAGAGGAGCC----TTGGAAGGCATGTGTGTATGG-
                   ** **     * *   **        **  *  * ***    ***

hPIK3CA         TGGTGTCATGCATTCAAGTACCAGGTATGGCTTTTTCTGCTATG-ACACA
mPIK3CA         --GGGCTGGGGACTTGAGCACT-GGTACA--TTGTCCAGATGTGGTCAGA
                  * *    * * *  ** **  ****    ** * * * * **  ** *

hPIK3CA         CAACTTCTTAGGGGCAGATAATCACATAACAAAAAACATATTATGTAATT
mPIK3CA         GGACAGCTCTGGG--AACTGGTTCCCTGTGAGACCAGAGAT--TGAACTC
                  **  **  ***  *  *  *  * *   * *  * * * **  ** * *

hPIK3CA         AGCATTTTCTTATTAAAAAATAAATTTTAGGCTGGGCATGTCGGCTGAAG
mPIK3CA         AG-GTCATCTGATT-----------TCCATGGCAAGCACTTTTACCCATG
                **  *  *** ***           *  * *    ***  *   *  * *

hPIK3CA         TCTGTAATCCCAACACTTTGGGAGGCCGAGGTAGA-TGAATCTCTTGAGC
mPIK3CA         --AGCCATCTCAACAACT-GT-AAGCGTGCATATTTTAAAGGTTTTAATG
                   *  *** *****  * *  * **     **   * **  * ** *

hPIK3CA         CCAGGAGTTGGAGACCAGTCTGGGCAACGAAGCAAGACC-CTGTCTTTAC
mPIK3CA         GCAT--CTTCCAAATGAGTCC----------CCAAGACTGCTGTCATCT-
                 **    **  * *  ****           ******  ***** *

hPIK3CA         AAAAAATAAAACATTTTTAAAAATCACAAATAGGCCAGGTGCGGTGGCTT
mPIK3CA         AAAATATTATAAGTATTTA---ATC---------CGAAGTGGGGTGACAC
                **** ** * *  * ****   ***         * * *** **** *

hPIK3CA         ACGCCTGTAATCCTAGCACTCTGGGAAGTCGAGGGGGGTGGATCACCTGA
mPIK3CA         AGAGAGTTAGTCGTAGGTCCTTCAATA-TTTAAAACAGTCCATCTCAAGA
                *      ** ** ***  *  *    * *  *     **  *** *  **

hPIK3CA         AGTCAGAAGTTTGAGACCAGCCTGACCAACATGGTGAAACCCCGTCTCTA
mPIK3CA         ATATTAAGAATTGGC-ACAGCTTAATCACCATGCT--------------T
                *     *   ***    **** * * ** **** *

hPIK3CA         CTAAGAAATACAAAAATTAGCTGGCATGTTGGCTGGTGCCTGTAATCCCA
mPIK3CA         C-AAGGAA---AATAATT-GATGATATTTTGTTATGTAACAATATACTTT
                * *** **   ** **** * **  ** ***    **  *  **  *

hPIK3CA         GCTACTCGGGAGGCTGAGGCAGAAGAATCGCTTGAACCCGGGAGGCAGAG
mPIK3CA         AAAA---ACCAAATTGGGGGCTAAGAGTGGCACCAGCCTT-----TAGTC
                   *      *   ** **   **** * **   * **        **

hPIK3CA         GCTGCAGTGAGCCGAGACTGCACCGCTGAACTCCAGCCTGGGCTACAGAG
mPIK3CA         CCAGCACTTGG--GAG---GCACAG------GCTAGCCTGATCTACAGAG
                 * *** *  *  ***   **** *       * ******  ********

hPIK3CA         TGAGACTCTGGCTCAAAAAAATAATAATAATAAATAAATAATAAATTTTA
mPIK3CA         TAAGTTCCAGG-GCATCCAG--GCTACCCAGAAAGAAGCCCTGTCCTGAA
                * **   * **  **   *     **   * *** **    *    *  *

hPIK3CA         TAAGGAAAAATATCCCTAGTATATTTTTCCCCACCAAGGTCTACCTATTA
mPIK3CA         AAAGCAAAAA---CCAAAACAAATGA---GGCAGAGAGGTGGATTTGC--
                 *** *****   **  *  * **       **   ****  *  *

hPIK3CA         ATATCTCATATAACTCTGTGCT--TGAAACACAATTTGGGATATATTGCC
mPIK3CA         --AATTAAGAACACTTGGTGCTCTTGAA-GA--GGCCCAGAGTTCACGTC
                  *  * * *  ***  *****  ****  *        **  *   * *

hPIK3CA         TTAAAATTTACTTTGACTAGCAATTCAGTGTTCCTTTTTTAAAAAAATCT
mPIK3CA         CTAGCATTCACAT---CAGGCAGTTCA-------------CAGGCATCT
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                 **  *** ** *   *  *** ****              *   ****

hPIK3CA          GTACTCTGGAGTAACAGTGTTCTAAAACTGTTGAAGAACATTGGTTCGAG
mPIK3CA          GTAAC-TTCAGCTATACCTGTGTGAAT---TTGATGCTCTTTGGCCTCTG
                 ***   *  **  * *    * * **    **** *  * ****     *

hPIK3CA          AAAAACATTTAGAAACACAAACCCCTGGAATGTGAGATGAAAATCCGAGC
mPIK3CA          -----CAGTTACATATGCACAC--ATGCAA-----AAATAGAATACC-TT
                      ** *** * *  ** **   ** **      *  * *** *

hPIK3CA          TAAAGGGAGAAAAAGGACGAAAGAAAGAAAACACAGAAAAGAAAGAAATA
mPIK3CA          TAAAA------ACAGAATGGAAG-------CCACTTATAACCCCAACAGT
                 ****       * ** * * ***        ***  * **     * *

hPIK3CA          CAGTCAAGTGAAACTACGACCACAAAGAGGAACAGATCCATCTCATTTA-
mPIK3CA          CAG-CAGGTGGAATTAGAAGCAT----CGGAACA--TCAAGCAAGGCTAG
                 *** ** *** ** **  * **      ******  ** * *     **

hPIK3CA          CCATTGGGGTATGGACGGACGTGCGGTGTTCGAGAAGACTGTTATTGGTC
mPIK3CA          TCTTTGTTATAT---CGAAAATTCGAGGCCAGGCTGGACCGTCTT-AGAC
                  * ***   ***   ** *  * **  *   *    *** **  *  * *

hPIK3CA          AGAGTTTTGCTCTAGAGGACAAGTAGGACTGTAA-CATCTCTAGGATGGG
mPIK3CA          CCTTTCTCAAACAAAACCCCAGAGTGGTCTAAAAGTATCACTTAGACAA-
                     * *    * * *   **    ** **  **  *** **  **

hPIK3CA          TCACTTCCCAAGCCCTCTATTACTTAAAAATTCAAGGAGGAACCGATGCT
mPIK3CA          --ACAACCC-AGCCCCCTACCAACAGAA-----AGGAAGTGACAGGGACT
                   **  *** ***** ***  *    **     * * **  ** *   **

hPIK3CA          GGAGTACTTGTATCTCAGACTTCTAATCACTGC-TCCTACGCTTTTCCAA
mPIK3CA          AGAGAAATGG-CTTTCAGT----TAAG-AATGCATACTGCTCTTTCGGAG
                  *** * * *  * ****     ***  * *** * ** * ****   *

hPIK3CA          TATTACAACAAAAGACCAGTAGGGGGAGAAAAACGCACAGTACCGAACCC
mPIK3CA          -----GAACAGAAT-TTATT------TCTATCACGCACATCAGGCACTCC
                       **** **    * *         *  *******  *   *  **

hPIK3CA          TTATCAGTAGTAATCTCAAAAGTCAACAGATTGATTTACTCTCAAGCAAA
mPIK3CA          CTGCTGCCTGTAACTCCAG--CTC--CAGGGAGATCTGTTCCC------T
                  *       ****   **    **  ***   *** *  ** *

hPIK3CA          CAGACTTCTAAGGTACGCAGCACCAAGACACTACCTTGAATCAAATCTAT
mPIK3CA          CTGTCCTCTACAGGCGCCTGCACTCACA--------TGCACACATTCATT
                 * * * ****  *    * ****  * *        ** *   * **  *

hPIK3CA          AGCCTATATGACATTTCTGAAGTCTCTGTTGGCATTACGCGAAAAATCCC
mPIK3CA          AAGATTTAAGATAGAGCCGG---GCGTGGTGGCG-CACGCCTTTAATCCC
                 *   * ** ** *   * *       ** ****   ****    ******

hPIK3CA          CCACGTCTTCTGAATAGTTAGAATTGAATCCTACAAGCTGCTTCGAATCA
mPIK3CA          --A-GTACTCGGG--AGGCAGAG---------GCAGGCGGATT----TCT
                   * **  ** *   **  ***           ** ** * **    **

hPIK3CA          GAATTCGATTTAAAAAAAAAAATGAGGGCATAGCAAAAGGTCTCCACGAA
mPIK3CA          GAG-----------------TCTAAGGGCAGGCC---TGGTCT--ACAAA
                 **                  * ******   *    *****  ** **

hPIK3CA          GTGAGTCAAAGGACTGCAGAGGGCTGTGACAGTGCATTCCGCCTTCGGGA
mPIK3CA          GTGAGTTCCAGGACAGC-CAGGGCTAC-ACAGAGAAACCCTGTCTCGAAA
                 ******   ***** **  ******   **** * *  **    ***  *

hPIK3CA          -TGGTATACAACTTAAACCATGTCGGCAGAAGAACGCACAGCAACGCTTT
mPIK3CA          AAAACAAACAAA-CAAACAAAGAT---TTAAGATGGAA-----AGACT--
                      * ****   **** * *       ****  * *     *  **

hPIK3CA          GTAAAAAGCATTCTTTCTATTATAGAATCCATAACCACGCTGGTTAGCCA
mPIK3CA          ----AAGCCATTTAATTTCACAT-GACTGCATA----TGCTGGGTGAAGA
                     **  ****   * *   ** ** * ****     ***** *    *

hPIK3CA          CTGACAGCGGCGGTTAGCCACCGCACCTCCTCTCACCCCCGAACTAATCT
mPIK3CA          --GAAAGAAGGAG-GAGTATCTG-GGCTGTATTTACACTTAAATAAGTCA
                   ** **  *  *  **   * *   **    * ** *   **  * **

hPIK3CA          CGTTTCCTCTATGGGTGTAAAAGTGAAATAACCCACTTGCTCCCAATATT
mPIK3CA          GGA-CCCGTTAC----CTAAAA-------------------------GA
                  *   **  **      *****
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hPIK3CA         CCTTTCTATATCTCTACCCCAGCTCGCCTGCTGCTCGTAGAAACAAATAT
mPIK3CA         TC-TGCCATGTCTGTGGCA-TCCTCAAATGATAGTAACAGTGGCAGAAGC
                 * * * ** *** *  *    ***   ** *  *   **   ** *

hPIK3CA         ACTACACGTACG-CTGTCCTAGGATGACACAACACCCTCACTACTGCAGA
mPIK3CA         ----CATCTTCGTGTGTGCCAGG------CAA-GTGCTCACTACTCCAT-
                    **  * **  *** * ***      ***    ********* **

hPIK3CA         AGACGGATCATTAAACAAACGTCAGAAGAGCAGCCCCAACTGTACATAAA
mPIK3CA         ---CCCATCCCTAACCT---GTT-----GGCTGCTGCTACTTGAGACATG
                   *  ***  *** *    **       ** **  * ***  * * *

hPIK3CA         CTTCGGGCGGAAAAGCAAGACGCAGGCGCAGTAGCACATATTGTTACCCT
mPIK3CA         CTT---GC-TAAGTGTTCCAGGCAAGCCTTGAACC------TGCAAA--T
                ***   **  **  *    * *** **   * * *      **  *   *

hPIK3CA         ATTTGCCCACTCCCTGCTCCTCCTCGCCTCAATTTCGCTTCCGCTTCTTT
mPIK3CA         AAAT-CCCCCTGCCTCAGCCAAA-----GGAGCTGAGGTTGCAGGCC--T
                *  * *** ** ***   **          *  *  * ** *    *  *

hPIK3CA         GCG-CATCTGCTTCCGGGGGATTGTAGGCTCTGCCCCTCC-TCAGCTCTT
mPIK3CA         GCACCACCAAGCCTGGGGAGATGGTCATTTCTGGTTGAGTTACAGC--TT
                **  ** *       *** *** **    ****         ****  **

hPIK3CA         ACCCTCTTCTGCCGGAGGAGGGGGGGGCCGAGGGGGTGGGGAAGAGTTC
mPIK3CA         GCTTT-TTCATTC------------TTCCCAT-----------TGCTT
                 *  * ***   *              ** *             * *

hPIK3CA         GTTGTTTTGTTTACACGATGTGAGCGGAAAAAGAGACCAATAAAGTTTATT
mPIK3CA         GTT-TTTGTG-AGACCCA--GCTTAGAAAACCTATTCAATATTTTTAAAT
                *** *****  * **     *    *****      *****   ** * *

hPIK3CA         CTGGAAACAAAAGGAAAAAAAAACAGGGGCGACGGAGAAAGGAGTCGGGG
mPIK3CA         TTT-CATTAAATCTAATTAAATGCAGGTTATTTTTCTACTGTCTTCTGTA
                 *   *  ***   **  ***  ****          *  *   ** *

hPIK3CA         GCGGGGG-CGTGTGGCGGGGGCTAGCGAGGAG-AGGGAGCGAGAAGTAGA
mPIK3CA         ATAAAATCTGTGTA-----GCCTAGTTTAGAGCAACCATCTAGA-TTAAA
                          ****      * ****    *** *   * * ***  ** *

hPIK3CA         AAGCGGCAGTTCCGGTGCCGCCGCTGCGGCCGCTGAGGTGTCGGGCTGCT
mPIK3CA         ACACATAAGTT-----ATTACTGCT-------CTGATTAGTCAGACAACA
                *  *   ****         * ***       ****   *** * *  *

hPIK3CA         GCTGCCGCGGCCGCTGGGACTGGGGCTGGGGCCGCCGGCGAGGCAGGGCT
mPIK3CA         A--GTTGTGACCATTTGCACTTC--TCAGTGCTGTCGTC-----AGTGT-
                   *  * * **  * * ***       * ** * ** *     ** *

hPIK3CA         CGGGCCCGGCC-GGGCAGCTCCGGAGCGGCGGGGGAGAGGGGCCGGGAGG
mPIK3CA         -GTGATCAGCAATTGCTTCTCTCTA--------------------ACAGG
                 * *  * **    **  ***   *                      ***

hPIK3CA         CGGGGGCCGTGCCGCCCGCTCTCCTCTCCCTCGGCGCCGCCGCCGCCGCC
mPIK3CA         TGG------T-------TTTCTTCTTTTGTTCA-----------------
                 **      *         *** ** *   **

hPIK3CA         CGCGGGGCTGGGACCCGATGCGGTTAGAGCCGCGGAGCCTGGAA
mPIK3CA         ----GGGC----AC-T-----GTTT--------GGAGACAGTCG
                    ****    **       * **        **** * *
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