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PhD Thesis:

IDENTIFICATION AND CHARACTERIZATION OF THE ROLE PLAYED BY THE NEURONAL NCX2 IN GLIOBLASTOMA PROGRESSION AND MALIGNITY

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Abstract

Glioblastoma multiforme (GBM) is one of the most malignant primary brain tumors with an aggressive phenotype and poor lines of evidence in genetic or environmental risk factors. In addition, glioblastoma patients show an ineffective treatment and a very short survival rate. For these reasons, there is a great demand of novel pharmacological targets for new effective strategies of treatment.

A common feature of all GBM sub-types is the alteration in the activity of pathways controlling the expression of several transcription factors involved in the up- or down-regulation of pro-oncogenes or anti-oncogenes, respectively. Among the downregulated genes there is *Slc8a2* encoding for the brain Sodium Calcium Exchanger 2 (NCX2). Recent data suggest that this antiporter can represent a possible anti-oncogene for GBM since it is silenced in all glioma stages.

To explore the genetic and epigenetic mechanisms leading to NCX2 downregulation, we identified, cloned and analyzed both rat and human *slc8a2* promoters in two cell lines PC12 and U87 expressing high or low level of this antiporter, respectively. In addition, we selected several transcription factors (TFs) able to modify both rat and human promoter activity of NCX2 in PC12 and U87 cell lines. However, TFs were able to increase mRNA expression of endogenous NCX2 only in PC12 cells. Interestingly, pharmacological inhibition of EGF pathway at different points restored NCX2 mRNA expression levels and increased its promoter activity in U87 cells. Moreover, transfection of NFkB, a downstream transcription factor of EGF pathway, downregulated NCX2 expression even in the presence of an inhibitor of EGF pathway. In addition, the blockage of this receptor-dependent cascade, or the stably transfection of NCX2, or the other isoform NCX1, hampered cell growth of U87 cells.

In a prospective therapeutic approach, we also analyzed the effect of two compounds, namely neurounina-1 and CN-PYB2, on the vitality and cell growth of U87 cell line. Neurounina-1 is a stimulator of both NCX1 and NCX2 activity, whereas CN-PYB2 is a selective stimulator of NCX1 activity. Results showed that these compounds hampered in a concentration- and time-dependent manner cell growth of U87 cell line, whereas they were ineffective in other cell lines, including BHK, SH-SH5Y and PC12.

Altogether, these data suggest that glioblastoma silences NCX2 by an EGF pathway via NFkB, and the increase of NCX expression via EGF-pathway inhibition, or the increase of NCX activity, slows-down glioblastoma cell growth and thus might exert a tumor suppressor effect.

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List of Abbreviation

ChiP	Chromatin Immunoprecipitation assay
CNS	Central Nervous System
CRE	cAMP Response Elements
CREB	CRE Binding factor
DNA	Deoxyribonucleic acid
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HIF-1	Hypoxia-inducible Factor 1
NCX	Sodium/Calcium Exchanger
NK-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
PCR	Polymerase Chain Reaction
PKA	Protein Kinase A
PTEN	Phosphatase and Tensin homolog
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
TMS	Transmembrane Segments
TSS	Transcription Starting Site
UTR	Untranslated Region

Chapter 1: Introduction

1.1 Brain Tumors

Tumors localized in central nervous system can be of either primary or secondary origin. In the first case tumor directly arises in the brain whereas in the secondary origin it metastasizes from a lesion outside the cranial cavity. Primary brain tumors are subdivided into low (grade I-II) and high grade (grade III-IV) tumors according to the World Health Organization (WHO) grading system. This grading is based on histological features with increasing grade correlating to enhanced aggressiveness and poorer patient prognosis (Louis et al. 2016). Brain tumors are quite rare accounting for about 1.6% of all cancers (AIOM AIRTUM 2017 data) accounting of 23,000 new cases in United States (Nabors et al. 2013) and 6,300 new cases in Italy (Registro Tumori 2019). However, incidence of primary brain tumors has been increasing over the past 30 years, especially in elderly persons (Maher and McKee 2003). It has been estimated that malignant tumors affecting CNS are responsible of 14,080 deaths per year in United States and approximately 4,100 in Italy (data of 2016, from Registro Tumori 2019).

Among primary intracranial tumors, gliomas account about 40% of the cases and 80% of all malignant brain tumors, thus they represent the most frequent and malignant tumors affecting CNS (Ostrom et al. 2014). These tumors develop from the glial cells and include some sub-types:

- Pilocytic astrocytomas (grade I) represent about 5% of gliomas with high incidence in the pediatric age. They are often located in the cerebellum and surgery is curative in most cases.

- Diffuse astrocytomas (grade II) derive from astrocytes, represent about 15% of all gliomas, are poorly circumscribed and invasive, and most gradually evolve into higher-grade astrocytomas. The average age of patients affected is 30-40 years old.
- Anaplastic astrocytomas (grade III) mainly consist of immature cells and affect people between 30 and 50. Over time they tend to turn into more aggressive gliomas that diffusely infiltrate surrounding tissues and the contralateral brain producing considerable edema and mass effect.
- Oligodendrogliomas (grade II or III) derive from the oligodendrocytes, comprising about 5% of brain tumors. They include tumors of varying degrees and aggressiveness. They usually develop slowly and generally, are responsive to therapies. Low-grade oligodendrogliomas appear well demarcated, occasionally contain calcifications and have specific molecular genetic alterations that distinguish them from other types of gliomas.
- Mixed gliomas (grade II or III) are made up of a mixed and different population of glial cells (astrocytes and oligodendrocytes) with a highly variable evolution. They occur more frequently in middle-aged patients.
- Cranial primitive neuroectodermal tumors are embryonal neoplasms at different degrees of differentiation. They can be classified by their location as infratentorial (medulloblastomas) and supratentorial (cerebral neuroblastoma, pineoblastoma, or esthesioneuroblastoma). They are invasive, rapidly growing and have also the tendency to disseminate through the cerebrospinal fluid.
- Medulloblastomas (grade IV) originate in the cerebellum from primitive and poorly differentiated cells but can also spread to other areas of the brain. They arise frequent in childhood or adolescence.

- Meningiomas (grade I to III) originate from meninges, in the membranes that surround and protect the brain. They represent about 30% of central nervous system malignancies. They are more frequent in women and affect elderly people.
- Ependymomas (grade II) originate from the ependymal cells that perform the function of lining the brain "channels" where the liquor that protects the brain and spinal cord is stored. There are the 2.3% of intracranial tumors. They usually occur in children in the first ten years of life.
- Hemangioblastomas (grade I) are very rare, usually benign and slow-growing tumors. They originate in the cells of the blood vessels. Sporadic and familial forms are observed often associated with von Hippel Lindau (VHL) disease.
- Germinoma (grade I or II) are rare tumors and originate from germ cells. They represent about 3% of primitive brain tumors, are typical of teenage males and are more common in Asian populations.
- Neuromas are benign tumors that mainly affect the acoustic nerve and the trigeminal nerve. They originate from the Schwann cells that cover nerve fibers.
- Craniopharyngiomas are benign tumors that derive from embryonic residues. They account for 0.7% of population with two peaks on the base of the age. In particular, in children from 6-16 years account for 5-13% of children's brain tumors whereas craniopharyngiomas occurs mainly between 50-70 years in adult patients.
- Primitive lymphomas (grade III) arise from lymphocytes and are limited to the central nervous system. They are particularly malignant and common in immunosuppressed individuals. They represent 3.1% of all primary brain tumors; in the past decades, the incidence is slowly increasing.

- Glioblastomas (grade IV) represent about 15% of all brain tumors; they are composed of poorly differentiated cells. They are the most malignant form of gliomas and mainly affect people between 45 and 75 years of age (Fig. 1).

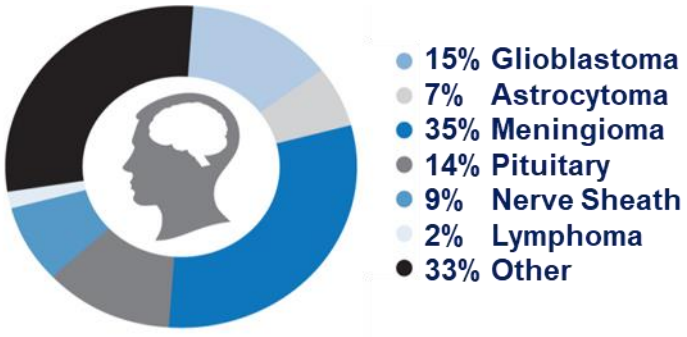

Fig. 1 Frequency of tumors affecting central nervous system.

1.2 Glioblastoma

Glioblastoma multiforme (GBM) represents about 45% of all glioma subtype, 15% of all brain tumor with a global frequency among all tumors of only 3.17 cases per 100,000 in the Western countries (Walsh et al. 2016). Unfortunately, GBM also represents the most devastating tumor and constitutes the most common malignant primary brain tumor (Louis et al. 2016). In addition, this tumor is associated with an extremely aggressive clinical course since only 0.05% to 4.7% of patients survive 5 years from the time of diagnosis with a median survival time of 12-15 months (Stupp et al. 2005, Ostrom et al. 2014). The principal causes determining the poor prognosis are the late stage of diagnosis and the lack of effective therapies (Louis et al. 2016). Numerous targeted therapies and several molecular targeted drugs aimed to reduce the blood supply and/or to inhibit tumor growth have already progressed into the clinical setting. These strategies include interference with oncogenic cancer cell signaling or angiogenic tumor endothelial cell signaling. However, results of clinical trials have been largely disappointing, demonstrating lack of efficiency and resistance development in glioblastoma multiforme patients (Sathornsumetee and Rich 2008, Van Meir et al. 2010) despite recent advances in cancer therapeutics, neurological surgery, and the development of innovative targeted therapies.

1.2.1 Epidemiology, symptoms and diagnosis

Glioblastoma multiforme can occur at any age, but typically affects adults, with a peak between 45 and 70 years and an increasing incidence until aged 85 and above. The median age at diagnosis is 64 years with only 1% diagnosed glioblastoma patients younger than 20 years, whereas more than 80% are patients older than 55 years (CBTRUS 2008). Males are more commonly affected by GBM, with a male/female ratio of 1.5:1 (CBTRUS 2011). Females, in addition to the lower incidence, display better survival rates (Matteoni et al. 2020). The reason of the prevalence of GBM in males is considered linked not only to the presence and effect of sex hormones, but mainly to cell intrinsic sex-dependent dissimilarities, related to the different genetic context between cells carrying the XX and XY karyotypes (Matteoni et al. 2020). For example, a major role has been attributed to the tumor suppressor gene RB1 and its protein product pRB, its inactivation is related to a sexual dimorphism in astrocyte transformation, where glial cells of male origin appear more inclined to undergo transformation than those of female origin (Sun et al. 2014). In fact, Ostrom *et al*, showed evidence of significant biomolecular differences between sexes demonstrating a key role of EGFR in male gliomas and GBMs, whereas TERT appears involved mainly in female patients by genome-wide association studies combined via a meta-analysis (Ostrom et al. 2019).

GBM mainly causes symptoms that are secondary to the intracranial pressure. Indeed, the most common symptoms include headache, nausea vomiting, and papilledema. Other frequent symptoms can include non-specific neurological symptoms, such as focal neurological deficits, personality changes, confusion, memory loss, and tension headaches and epileptic seizure present in one third of patients (Wen and Kesari 2008, Louis et al. 2016).

Diagnosis of malignant glioma could be detected by magnetic resonance imaging (MRI) or computed tomography scanning (TC) of the brain. It could appear as a heterogeneously enhancing mass with surrounding edema. Glioblastoma multiforme could be enriched by irregular enhancement and extensive peritumoral edema. However, diagnosis should be histopathologically confirmed with surgical debulking of the tumor mass (Wen and Kesari 2008).

Regarding the identification of the causes determining the onset of the pathology, ionizing radiation is the only unequivocal environmental risk factor that has been identified (Bondy et al. 2008). Other suggested risk factors, including head injury, dietary risk factors, and exposure to electromagnetic fields remain inconclusive (Wrensch et al. 2002, Fisher et al. 2007). Nevertheless, there is an increasing amount of observational clinical studies that tries to associate the exposure to occupational risk factors, including the exposure of vinyl chloride, phenolic based compounds and aromatic hydrocarbons with the risk to develop GBM. Genetic predisposition has been observed only in 5-10% of glioma cases (Fisher et al. 2007), that, considering the infrequence of GBM, only account for few cases (Bondy et al. 2008) (Fig. 2).

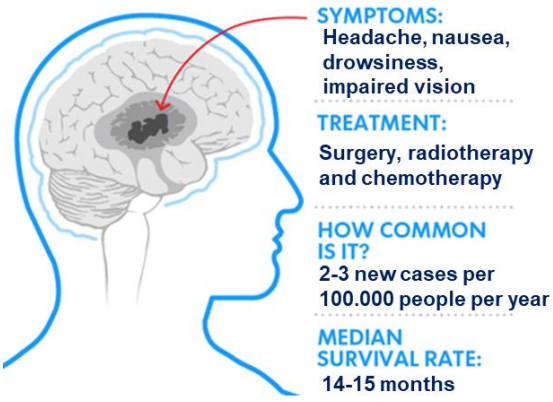

Fig. 2 Summary of symptoms, treatment and epidemiology of glioblastoma.

1.2.2 Pathological Features

Glioblastomas occur most often in the subcortical white matter of the cerebral hemispheres. The most frequently affected sites are the temporal lobe (31%), the parietal lobe (24%), the frontal lobe (23%) and the occipital lobe (16%). The neoplasm often extends through infiltration to the adjacent cortex, the basal ganglia and therefore to the contralateral hemisphere. The tumor occupies the same position in the two hemispheres and has a "butterfly" appearance. Bilateral supratentorial extension requires rapid growth along the myelinated structures, particularly through the corpus callosum and along the fornix towards the temporal lobes (Louis et al. 2016) (Fig. 3). The boundaries of the neoplastic mass are blurred everywhere and poorly delineated and having a high degree of regional heterogeneity. The complexion is grayish, but abundant variegations can be found, caused by necrosis or rougly recent bleeding. Highly proliferating cancer cells are usually found in the peripheral, hypercellular zone of the tumor, whereas the central tumor area mainly consists of necrotic tissue, comprising up to 80% of the total tumor mass. Histopathologically, the lesions typically exhibit cellular hyperplasia in peripheral zones harboring cancer cells with atypical nuclei, cellular pleomorphism, high mitotic activity, as well as pseudopalisading necrotic areas and microvascular proliferation that distinguish them from the gliomas of lower grades (Hambardzumyan and Bergers 2015). A major obstacle in glioblastoma treatment is represented by infiltrating tumor cells dispersed within the normal brain tissue in characteristically distinctive patterns known as Scherer’s structures, resembling threads of a spider’s web, which are responsible of the tumor recurrence as they escape surgical resection and high-dose radiotherapy (Cuddapah et al. 2014). In addition, the conditions of pro-tumoral inflammation and/or EMT in the extracellular facilitate tumor invasion through the nerve

tracts and blood vessels to invade nearby tissue (Cuddapah et al. 2014, Noroxe et al. 2016). Tumor cell invasion requires the detachment from the primary tumor mass, adhesion to extracellular matrix (ECM), degradation and remodeling of ECM, and cell motility through brain parenchyma (Onishi et al. 2011). Infiltration of GBM cells involves adhesion molecules as integrins that allow cell to adhere to ECM proteins such as laminin, collagens, matrigel and fibronectin (Juliano and Haskill 1993, Nakada et al. 2007). Focal adhesion kinase (FAK) has been proposed as a key component of integrin-mediated signal transduction pathways, leading to enhanced GBM cell adhesion, proliferation and migration (Zagzag et al. 2000). In addition, GBM expresses elevated levels of matrix metalloproteases (MMPs), in particular MMP9 and MMP2 that degrade and remodel ECM proteins, favoring cell invasion (Konnecke and Bechmann 2013).

Another pathological feature of GBM, that deserves attention, is the vascularization. Indeed, glioblastoma multiforme is one of the most vascularized human tumors because of three different processes of vascularization: vessel co-option, vasculogenesis and angiogenesis (Louis et al. 2016). In particular, vessel co-option is a non-angiogenic process where pre-existing vessels within the normal brain tissue migrate from perivascular zone to the periphery of the tumor stimulated by signaling molecules secreted by circulating bone-marrow derived cells (Jain et al. 2007). Angiogenesis is the physiological forming of normal vessels into the tumor area, mediated by endothelial cell proliferation and migration concurrently with the remodeling of the perivascular extracellular matrix (Fischer et al. 2005). Angiogenesis occurs when proangiogenic growth factors were secreted either by cancer cells or stromal cells of the tumor. Vascular endothelial growth factor-A (VEGF-A) is the major proangiogenic growth factor, it was strongly stimulated by hypoxia, inducing vascular permeability and consequent endothelial cell proliferation and migration. Besides VEGF-A, fibroblastic growth

factor (FGF), the angiopoietins (Ang 1 and Ang 2) and platelet-derived growth factor (PDGF) are important proangiogenic growth factors (Fischer et al. 2005, Jain et al. 2007). The neo-formed tumor vessels are phenotypically different from physiological vessels: they appear tortuous, disorganized, with a significantly larger diameter than vessels of the normal brain and present pericyte coverage, and expression of basement membrane proteins (Jain et al. 2007). The tumor vessels are also highly leading to accumulation of fluid and plasma proteins in the tumor and surrounding brain tissue contributing to an increase in interstitial pressure and brain edema and intracerebral pressure (Jain et al. 2007). In addition, the heterogeneous perfusion of the tumor leads hypoxia and acidosis, reducing sensitivity of the cancer cells to radiation therapy (Fukumura and Jain 2007).

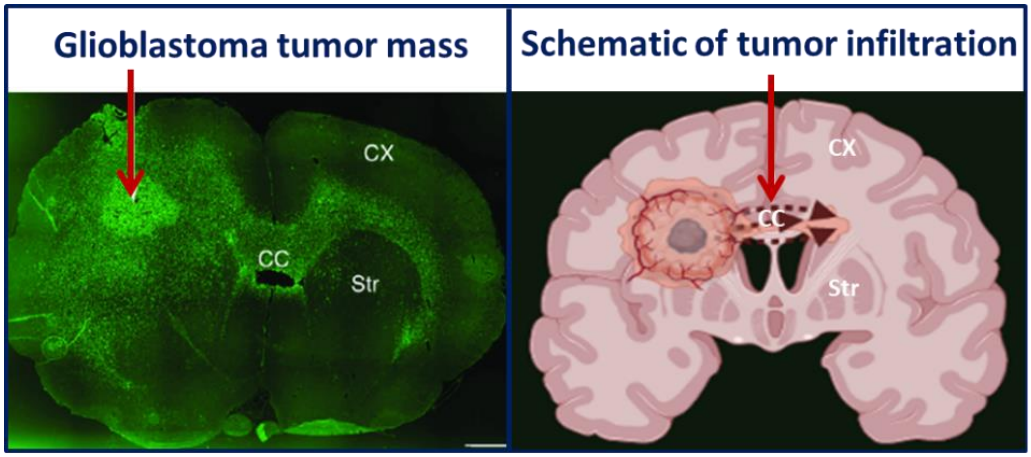

Fig. 3 . (Left) Glioblastoma mass (red arrow) and infiltration to the contralateral hemisphere through the corpus callosum (CC) to the contralateral white matter located between striatum (Str) and cortex (CX). (Right) Schematic of tumor infiltration (red arrow).

1.2.3 Etiology

The cellular origin of GBM is still unclear and needs further investigations. Current hypothesis theorizes that GMB can arise from a multistep process involving sequential and cumulative genetic alterations resulting from intrinsic and environmental factors (Omuro and DeAngelis 2013). In particular, recent research suggests that GMB may stem from malignant transformation of neural stem cells or related progenitor cells (Stiles and Rowitch 2008). Accordingly, cells with stem cell-like properties have been isolated from tumor. These cells represent only a small fraction of the tumor, but exhibit an extensive proliferative potential, self-renewal, and multipotency and when implanted into immunocompromised mice, they can originate tumors mimicking the phenotype all the different cell types of parent tumor (Das et al. 2008). In addition, the presence of these stem-like cells might be responsible for the pharmacological resistance. The main surface markers characterizing neural stem cells are CD133 (Zheng et al. 2008), and high expression of genes such as SOX2, NESTIN, OCT4, KLF4, NOTCH1, and GFAP that is associated with stem/progenitor cells in glioblastoma. SALL1, POU3F2 (OCT7), OLIG2 and SOX2 are the four master transcription factors associated with glioma stemness and tumor progression and underlie therapeutic resistance (Patel et al. 2014). Genome-wide association studies have identified a few susceptibility variants such as 20q13.33 (RTEL), 5p15.33 (TERT), 9p21.3 (CDKN2BAS), 7p11.2 (EGFR), 8q24.21 (CCDC26), and 11q23.3 (PHLDB1), but these genes are only weakly associated with glioma, possibly reflecting multiple molecular subsets (Rajaraman et al. 2012). Experimentally, these cells can be modeled to grow as spheres *in vitro* (similar to neural stem cells) and have been inferred the capacity to initiate tumors and recapitulate histology of the initial tumor in animal models (Zheng et al. 2008, Chen et al. 2012).

1.2.4 Sub-Classes

Glioblastoma classification includes a spectrum of biologically distinct tumors having different age of onset, tumor location and prognosis. Glioblastoma can develop from a diffuse astrocytoma (grade II) or from an anaplastic astrocytoma (grade III) and in this case it is called "secondary glioblastoma", but more frequently it occurs *de novo*, without any evidence of previous neoplasia as "primary glioblastoma" (Suryadinata et al. 2010, Barnum and O'Connell 2014). Secondary glioblastoma typically affects younger patients with a mean age of 45, while primary lesions develop in older patients with a mean age of 62 years at diagnosis (Foster et al. 2010). However, since these two sub-classes of glioblastoma show a common phenotype, they can be identified only based on of genetic abnormalities. Indeed, primary glioblastoma is characterized by the overexpression and mutation of the epidermal growth factor receptor (EGFR), loss of heterozygosity (LOH) of chromosome 10q, mutation of the phosphatase and tensin homology (PTEN) gene, and deletion of the p16 gene. By contrast, secondary glioblastoma is identified by mutations in the p53 gene, LOH of chromosome 10q, and abnormalities in the pathway regulating the tumor suppressor RB (Fig. 4). In addition, glioblastomas can be clustered into four main subtypes with a prognostic value (Phillips et al. 2006): proneural, neural, classical, and mesenchymal on the base of DNA and RNA profiling of bulk tumors and on aberrations in EGFR, NF1, PDGFRA and IDH1 genes (Verhaak et al. 2010, Brennan et al. 2013). The neural subtype is characterized by the increase of expression of bone morphogenetic protein 4 (BMP4), doublecortin (DCX), p16INK4a, and inhibitor of differentiation 2 (ID2). Enrichment of oligodendrocyte lineage transcription factor 2 (OLIG2), NK2 homeobox 2 (NKX2 2), Notch1, and Notch3 is typical of the proneural and classical subtypes. Classical subtype is also characterized by an increase in EGFR expression,

a deletion of CDKN2 A gene, and a lack of TP53 mutations. On the other hand, the mesenchymal subtype is enriched for mutation and/or loss of NF1. Proneural subtype is characterized by alterations of PDGFRA and point mutation in IDH1, and neural subtype is typified by the expression of neuronal markers such as NEFL, GABRA1, SYT1 and SLC12A5 (Lee et al. 2018) (Fig. 5).

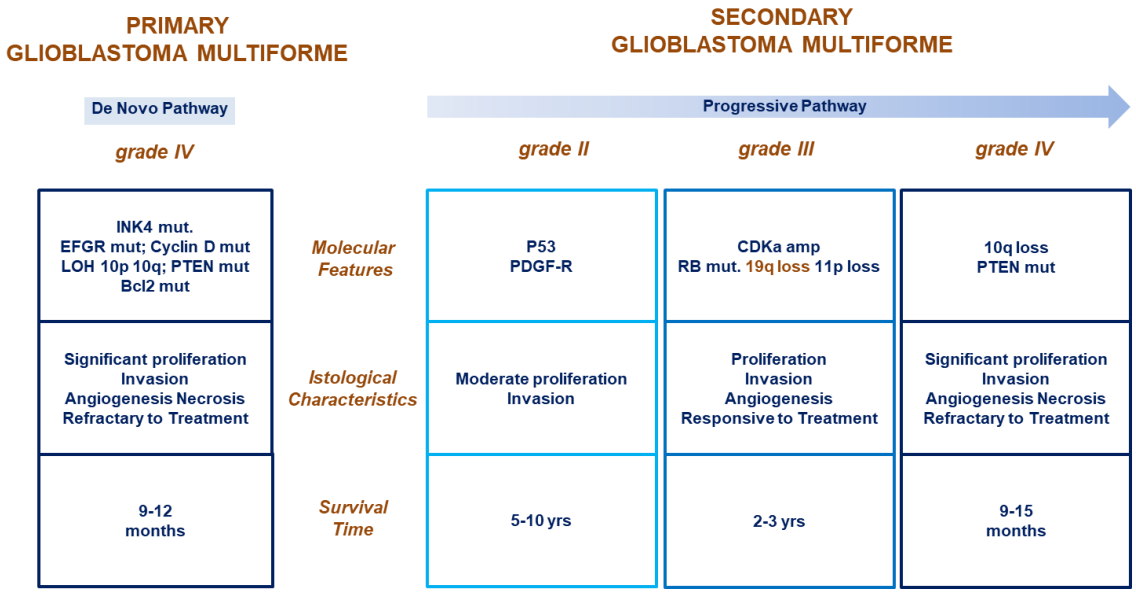

Fig. 4 Summary of criteria for glioma sub-classes classification and the averages of survival time

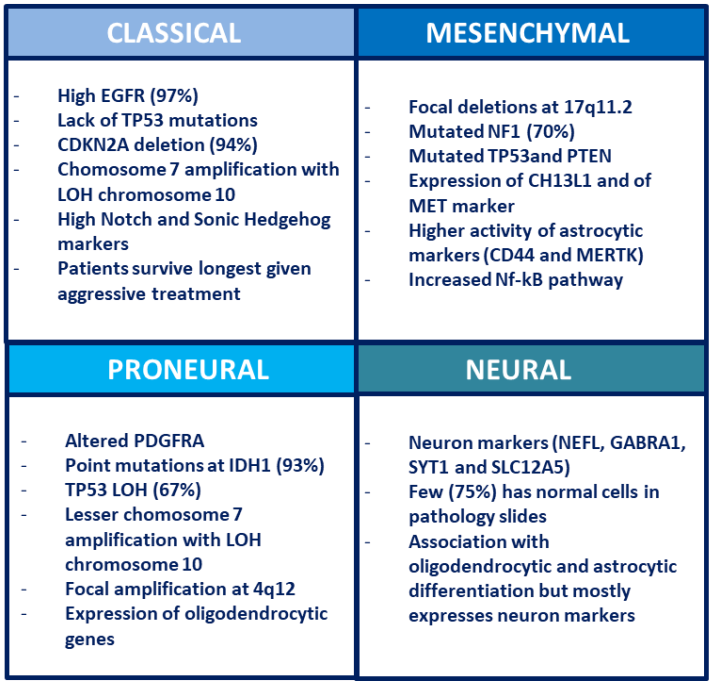

Fig. 5. Classification of glioblastoma subtypes

1.2.5 Role of Ca^{2+} in progression and invasion

Ion channels are multimeric proteins located in the plasma membrane of cells, rapidly interfering with the electrical potential across the cell membrane (Guan et al. 2018). The flow of ions through ion channels can regulate several cellular functions in response to different stimuli, including cell cycle checkpoints (Molenaar 2011) and thus the progression, migration, and angiogenesis of tumor cells (House et al. 2015, Nelson et al. 2015, Li et al. 2016). Indeed, expression or functioning of ion channels are frequently modified in glioblastoma (Polisetty et al. 2012) where they have a great influence on cell morphology, invasiveness and proliferative capacity (Li et al. 2016).

Among intracellular ions, Ca^{2+} is the most abundant second messenger and regulates gene transcription, cell proliferation, migration and death. For these reasons, Ca^{2+} homeostasis is finely regulated and maintained at very low levels in cytosol ($\pm 10^{-7}$ mol/L), low levels in intracellular organelles ($\pm 10^{-5}$ mol/L), and high levels in the extracellular space ($\pm 10^{-3}$ mol/L). Intracellular Ca^{2+} homeostasis is maintained by Ca^{2+}-permeable ion channels, receptors, Ca^{2+} pumps and Na^{+}/Ca^{2+} exchangers (NCX) (Alexander et al. 2013) located in the plasma membrane and membranes of intracellular organelles (Verkhratsky et al. 1998, Blaustein et al. 2002). The intracellular Ca^{2+} oscillations can generate marked signals to activate downstream signaling cascade providing efficient means to transmit intracellular biological information. Accumulating evidence suggests that Ca^{2+} might be an important positive regulator of tumorigenesis because it is involved in quiescence, maintenance, apoptosis, proliferation and migration in glioblastoma cells (Sforna et al. 2014). In particular, the deregulation of the normal Ca^{2+} signaling elements contributes to the progression of the disease (Boscia et al. 2016). The loss of normal Ca^{2+} homeostasis and regulation can favorite

uncontrolled growth and dysregulation in metabolic events resulting in the tumor growth (Rimessi et al. 2015). Ca^{2+} is released by intercellular stores by inositol 1,4,5- trisphosphate receptor (IP3R) (Li et al. 2018) and enters through stromal interacting molecular 1 (STIM1) and Ca^{2+} channel Orai1 (Potier and Trebak 2008, Kang et al. 2010). In particular, Ca^{2+} entry through Orai1 and STIM1 is crucial to sustain cell proliferation in both rat C6 and human U251 glioma cells (Liu et al. 2011). In addition, glioblastoma cells express high levels of Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptors. Glutamate promotes migration by activation of Ca^{2+}-permeable AMPA receptors acting as an autocrine and paracrine signal in GBM (Lyons et al. 2007). Moreover, fluctuations in Ca^{2+} concentration induce calmodulin-dependent protein kinase II (CaMKII) to activate Chloride Channel 3 protein (ClC-3), which begins GBM invasion (Molenaar 2011). In fact, concentrations of calmodulin (CaM) are significantly correlated with the invasive capacity of invadopodia formation in GBM. Invadopodia promotes extracellular matrix degradation and cell motility, thus facilitating their migration through the tissue microenvironment (Li et al. 2018).

Transient receptor potential (TRP) channels are located in the plasma membrane, where they act as the driving force for Ca^{2+} entry (Nilius and Owsianik 2011). Interestingly, TRP channels are significantly overexpressed in GBM cells as compared to low-grade gliomas; including transient receptor potential melastatin subfamily member-2 (TRPM2), TRPM3, TRPM7, TRPM8, transient receptor potential canonical channel protein-1 (TRPC1), TRPC6, transient receptor potential vanilloid subfamily member-1 (TRPV1), and TRPV2 (Bomben and Sontheimer 2010, Stock et al. 2012, Leng et al. 2015). The TRPC family, are activated directly by diacylglycerol (DAG) or indirectly through IP3-mediated Ca^{2+} release (Bomben and Sontheimer 2010). TRPC1 is involved in cell migration, indeed, if stimulated by

epidermal growth factor (EGF), leads to the activation of the p38/MAPK/JNK signaling pathway (Bomben et al. 2011). Instead, TRPV1 via activating transcription factor-3 (ATF3) (Stock et al. 2012) mediates an endoplasmic reticulum stress regulating glioma cell death (Amantini et al. 2007). Ca^{2+} flux is modulated also by T-type low voltage-gated calcium channels (VGCC) and promote proliferation of Glioblastoma Stem Cell (GSC) cell line while their inhibition suppresses the tumor growth and increased tumor cell apoptosis disrupting Akt-signaling, through changings in Ca^{2+} intercellular concentration (Zhang et al. 2012). In a therapeutic point of view, the manipulation of Ca^{2+} signaling could represent a strategy to overcome the checkpoints and could be use as tools to reprogram cancer cells (Fig. 6).

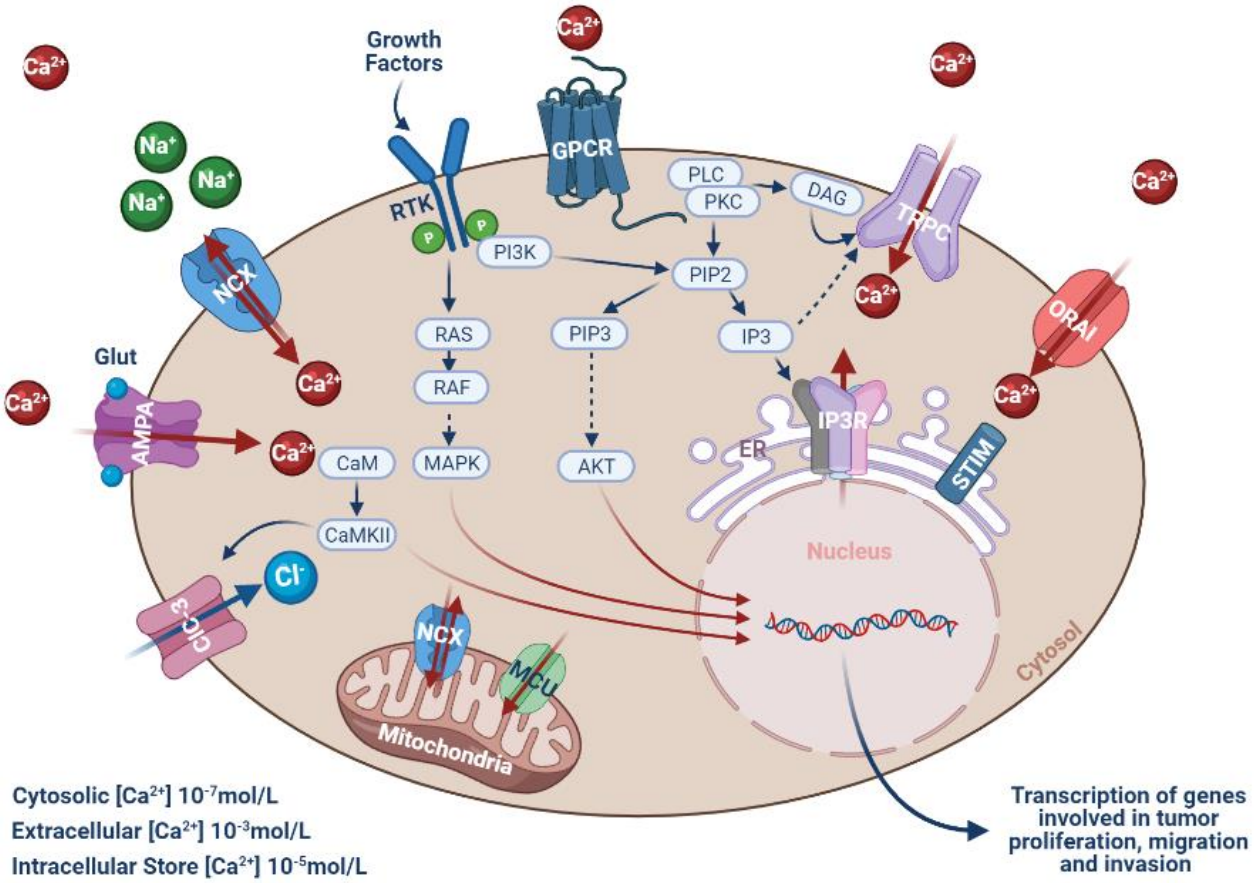

Fig. 6. Cartoon summarizing intracellular Ca^{2+} signaling and pathways activated from growth factors by means of receptors tyrosine kinase (RTK) in glioblastoma. Continuous lines indicate direct activation; dashed lines indicate indirect activation.

1.2.6 Molecular biomarkers involved in the diagnosis

We distinguish two types of alterations occurring in GBM: mutations that activate oncogenes and mutations that deactivate specific onco-suppressor genes. Among the most frequent oncogenes that are activated in GBM there are: (i) genes involved in Epidermal Growth Factor (EGF) transductional pathway; (ii) Mouse Double Minute 2 (MDM2) oncoprotein that promotes cell survival and cell cycle progression by inhibiting the tumor suppressor gene TP53; (iii) Platelet-Derived Growth Factor (PDGF) transductional pathway. As regard the deactivation of oncosuppressor genes, the most frequent are: (i) Deleted in Colorectal Cancer (DCC) tumor suppressor gene; (ii) p16, a tumor suppressor gene and tumor antigen; (iii) TP53, another tumor antigen; (iv) Phosphatase and TENsin homolog (PTEN), a tumor suppressor that controls cell growth, proliferation and survival; (v) RetinoBlastoma (RB) tumor suppressor gene. Aberration in the epigenetic regulation mechanisms also determine the inactivation of critical genes and are important mechanisms in the evolution of neoplasms contributing to tumorigenesis. Among the epigenetic mechanisms have been observed in gliomas there is DNA hypermethylation, histone modifications, nucleosome rearrangement and dysregulation of the expression of non-coding RNA. For instance, methylation of the PTEN promoter is an early event in the evolution of gliomas inducing their progression to glioblastoma. On the other hand, the most relevant of the known epigenetic changes in GBM refers to mutation in methylguanine-DNA methyltransferase O-6 (MGMT), a DNA repair enzyme that catalyzes the removal of alkyl groups added to the O-6 position of the guanine by environment or drugs. Hence, cells that express high levels of MGMT are considered more resistant to alkylating agents as temozolomide (TMZ). Another target of the epigenetic regulatory mechanism in glioblastoma is represented by isocitrate dehydrogenase (IDH1 and

IDH2), key enzymes involved in cellular energy metabolism. IDH1 mutations were identified in a large fraction of young patients and in most patients with secondary glioblastomas, associated with significantly better overall survival (Parsons et al. 2008). The mutations of IDH1 and IDH2 determine the loss of the normal catalytic activity in the oxidative decarboxylation of the isocitrate in alpha-ketoglutarate (α-KG) and NADPH and, on the other hand, the gain of a new function: the ability to catalyze the NADPH-dependent reduction of α-KG reduction in 2-hydroxyglutarate (2-HG) promoting tumor angiogenesis through the stabilization of the protein HIF-1α. Furthermore, the inhibition of histone demethylases by 2-HG, together with the reduction of α-KG, would contribute to the alteration of the methylation patterns with the variation of the expression of oncogenes, tumor suppressor genes and other key components of metabolic pathways and signal transduction, indeed IDH mutation is associated with an aberrant DNA and histone methylation profile that leads to widespread hypermethylation of CpG islands, termed 'glioma-CpG island methylator phenotype' (Noushmehr et al. 2010). Glioblastoma molecular biomarkers such as IDH1/2 mutations, 1p/19q co-deletion, MGMT promoter methylation, and EGFRvIII amplification could be useful in routine clinical practice. In addition, basing on IDH mutations, 1p/19q co-deletion and TERT promoter mutations, and TP53, EGFR, or PTEN gliomas could be classified into five groups for the refinement of glioma diagnosis (Eckel-Passow et al. 2015). Novel techniques for the analysis of DNA methylation and mutational profiling by next generation sequencing will lead to defining better biomarkers to improve treatment response in patients.

1.2.6.1 Markers of loss of cell cycle control

Cell cycle is under control of cyclin/cyclin-dependent kinases (CDK) complexes, which act as master regulators of cell cycle progression by phosphorylating downstream substrates. Cyclin D-CDK4/6, cyclin E-CDK2, cyclin B–CDK1 and cyclin A–CDK1/2 are critical for cell cycle progression through G1/S transition, S phase and G2/M transition (Suryadinata et al. 2010) and their activities are coordinate by cyclin-dependent kinase inhibitors (CDKIs) by binding to CDKs (Barnum and O'Connell 2014). In addition, several signaling pathways have been involved in the regulation of cell cycle progression, including the retinoblastoma pathway (RB) and p53 pathways. In particular, RB prevents cell progression into S phase when associated with the transcription factor E2F. Following stimulation, activated cyclin D-CDK4/6 phosphorylates and inactivates RB, causing the release of E2F and the activation of genes involved in the G1/S transition (Foster et al. 2010). Alterations in the RB signaling occur in about 80% of GBM and are implicated in the progression of astrocytoma. Dysregulations include genetic loss of RB, CDK4/6 amplification and deletion of *CDKN2A* (Cancer Genome Atlas Research 2008). The p53 pathway is involved in cell cycle arrest, apoptosis, senescence and DNA damage repair (Vazquez et al. 2008). In quiescent cells p53 is stabilized by binding to p14 and degraded by murine double minute 2 (MDM2). In response to DNA damage, p53 is activated and acts as a transcription factor promoting the cycle progression in G1 phase through its effector p21 (Abbas and Dutta 2009). Moreover, p53 mutations, such as inactivation of cell cycle inhibitor p14 and amplification of MDM2 (Mao et al. 2012), are founded in primary GBM with a low frequency of 28-35%, and loss of p53 is frequently observed in the progression of secondary GBM (Ohgaki and Kleihues 2007, Brennan et al. 2013). These alterations impair cell cycle progression by affecting both G1/S

and G2/M transition (Agarwal et al. 1995) and altered p53 expression affects the response of GBM cells to TMZ (Hirose et al. 2001, Blough et al. 2011).

1.2.6.2 Growth factors and kinases in GBM

GBM expresses a large number of growth factors and their corresponding membrane receptor kinases, causing the activation of mitogenic signaling pathways triggering intracellular signals responsible for cell growth, proliferation, and survival upon the binding to transmembrane receptors (Carrasco-Garcia et al. 2014). GBM is enriched in a variety of growth factors, including IGF-1, FGF-2, EGF, PDGF, VEGF and others (Witsch et al. 2010). Three key growth factors are important for GBM proliferation: (i) EGF is a potent mitogen factor whose primary function is to stimulate DNA synthesis and cell division of GBM via EGF/EGFR signaling pathway (Lund-Johansen et al. 1990). In addition, EGF is related to cell invasion and angiogenesis in GBM (Dunn et al. 2000, Zheng et al. 2013) and is essential for the maintenance of neural stem cells (NSC) as well as glioma stem cells (GSC), encouraging the self-renewal capacity (Soeda et al. 2008); (ii) Fibroblast growth factor family (FGFs) plays crucial roles in cell proliferation, differentiation, migration and angiogenesis (Yun et al. 2010), thus alterations in its FGFR signaling pathways are responsible for the malignant progression of glioblastoma (Stefanik et al. 1991, Morrison et al. 1994). In addition, FGF-2 is also involved in the process of angiogenesis, by cooperation with VEGF (Dunn et al. 2000, Cuevas et al. 2011), in the proliferation of endothelial cells, and it is considered essential for self-renewal and maintenance GSC pool (Lee et al. 2006); (iii) Insulin-like growth factor (IGF) axis is made up of ligands IGF-1 and IGF-2, transmembrane receptors IGF-1R and IGF-2R, and binding proteins IGFBP1-6. IGF-1 is an anti-apoptotic factor involved in the

control of cell proliferation, differentiation and apoptosis and it is highly expressed in glioblastoma (Sandberg-Nordqvist et al. 1993). Furthermore, IGF-1 can also regulate GBM cell invasion by mediating cytokine secretions (Ho et al. 2017).

1.2.6.3 Receptor tyrosine kinase

Receptor tyrosine kinases (RTK) are transmembrane glycoproteins with an amino-terminal extracellular ligand-binding domain and a cytosolic carboxy-terminal domain with tyrosine kinase enzymatic activity (Carrasco-Garcia et al. 2014). The RTK activation leads to receptor homo/hetero-dimerization and the juxtaposition of tyrosine kinase domain, promoting the trans-phosphorylation of tyrosine residues to which cytoplasmic proteins such as PI3K, phospholipase C, growth factor receptor-binding protein, or the kinase Src can bind (Hubbard and Miller 2007). Alterations of RTK through protein overexpression, genetic amplification or mutations are essential component of oncogenic pathways (Nakada et al. 2011). Epidermal growth factor receptor EGFR, also known as ERBB1 or HER1, is a transmembrane protein belonging to ERBB family of RTKs. Alterations in EGFR activation are frequent and occur in about 57% of primary GBM patients (Brennan et al. 2013). EGFR upregulation can occur with an increase in EGFR gene expression and/or protein, or with a constitutively activation of oncogenic mutations (Huang et al. 2009). The most common type of EGFR mutations is the variant III EGFR deletion mutant (EGFRvIII) and it is expressed in about half of GBMs with EGFR amplification (Gan et al. 2009). The mutation generates a truncated form of EGFR constitutively activated and autophosphorylated, this alteration contributes to dysregulation of downstream pathways involved in amplified oncogenic effects, including increased

proliferation, angiogenesis, invasion and resistant to therapy (Gan et al. 2009, Montano et al. 2011).

EGFR dysregulation is associated with poor prognosis and decreased survival time in GBM patients (Shinojima et al. 2003) and as a hallmark of high-grade GBM, alterations of EGFR are rare in low-grade gliomas (Hatanpaa et al. 2010). In addition, some lines of evidence show that EGFR can translocate to the nucleus and interact with genes transcription modulating radio- and chemo-resistance (Brand et al. 2011). Insulin-like growth factor-1 receptor (IGF-1R) system is modulated by IGFBP, leading to signal transduction via PI3K/AKT pathway or MAPK pathway (Denley et al. 2005), so the overexpression of IGF-1R leads to the disruption of these pathways and subsequently increasing of growth, migration, angiogenesis and survival of malignant cells and tumorigenesis in GBM (Denduluri et al. 2015). In addition, overexpression of IGF-1R was associated with resistance to TMZ in GBM patients (Maris et al. 2015). Besides, the inhibition of IGF-1R exerts a direct effect on tumor cell proliferation and on tumor vascularization (Zamykal et al. 2015). Furthermore, IGF-1R was responsible for serum-induced activation of ERK1/2 *in vitro*, highlighting the mitogenic effect of IGF-1R signaling (Wang et al. 2013).

As regard the major pathways activated by RTKs in GBM there are PI3K-AKT-mTOR and MAPK/RAS-RAF-ERK1/2 signaling pathways (Fig. 7). As aforementioned, PI3K/AKT/mTOR signaling pathway regulates cell cycle, cell survival and cell growth (Porta et al. 2014). This pathway starts with the phosphorylation of RTK that, in turn, activates the phosphoinositide 3-kinases (PI_3K) (Cantley 2002) followed by the phosphorylation of phosphatidylinositol 4, 5-bisphosphate (PIP_2) and the generation of phosphatidylinositol 3,4,5-triphosphate (PIP_3). The main function of PIP_3 is the recruitment of serine/threonine kinase 3′-phosphoinositide-dependent kinase 1 (PDK1) that phosphorylates the protein kinase

B (PKB) known as AKT via its activation loop at threonine308 as well as by DNA-dependent protein kinase or mammalian target of rapamycin (mTOR) at serine473 by to plasma membrane (Sarbassov et al. 2005). AKT promotes cell survival, its targets are apoptosis-related genes such as Bad and caspase 9 (Song et al. 2005) and proteins involved in the cell cycle as cyclin D1, cyclin E and p21 (Mullany et al. 2007). Dysregulation of PI3K/AKT signaling pathways has been observed in 90% of GBM and plays a significant role in driving gliomagenesis (Dunn et al. 2012). In particular, genetic loss, mutation or epigenetic inactivation of phosphatase and tension homolog deleted on chromosome ten (PTEN) protein can be found in about 50% of GBM patients (Koul 2008, Dunn et al. 2012). In fact, the loss of PTEN, whose role is to dephosphorylate PIP_3 and thus to inactivate PI3K/AKT signaling pathway (Maehama and Dixon 1998), leads to constitutive activation of this pathway and sustains tumorigenesis in GBM (Song et al. 2012). Irradiation can induce AKT activation which modulates radio-resistance in GBM (Li et al. 2009), therefore, AKT is a potential target to increase radiation sensitivity in GBM (Narayan et al. 2013, Mehta et al. 2015).

Another RTK pathway is represented by mitogen-activated protein kinase (MAPK) that is activated by ligands binding to the receptor, and that contains Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains like SHC. In particular, SHC allows the release of GDP and binding of GTP on RAS by recruiting growth factor receptor-bound protein 2 (Grb2) and guanine exchange factor Son of Sevenless (SOS). RAS activated enables the translocation of serine/threonine kinase RAF to plasma membrane where is phosphorylated and activated triggering the phosphorylation of ERK through MEK. Finally, ERK translocates to the nucleus to interact with transcription factors involved in cell proliferation, differentiation, cell cycle progression, protein synthesis and migration (Chang and Karin 2001, Pearson et al. 2001). Neurofibromin is the product of tumor suppressor gene neurofibromatosis type I (*NF*-

1) and functions as a negative regulator of RAS (Yunoue et al. 2003). MAPK signaling pathway is also responsible for the activation of Nuclear factor KappaB (NFκB), a cytokine-regulated transcription factor, that plays an important role in the regulation of the transcription of target genes responsible for cell survival and inflammation (Ghosh et al. 1998). Five NFκB family members are discovered in mammalians: RelA (p65), RelB, c-Rel, p50/p105 (NFκB1) and p52/p100 (NFκB2) (Tieri et al. 2012). The activation of NFκB, is trigged by the phosphorylation of IκB kinase complex (IKK) leading to the degradation of the inhibitory protein; NFκB translocates to the nucleus, where it binds genomic DNA and regulates the expression of specific genes (Sheppard et al. 1999). MAPK signaling pathway is altered in about 88% of GBM. Mutations consist in RTK overexpression, activation of ligands via sustained autocrine or paracrine, *B-RAF* gene mutation or *NF-1* gene deletion (Brennan et al. 2013) leading to with increased radiation resistance in patients with GBM (Pelloski et al. 2006). MAPKs contain three members: extracellular signal-regulated kinase1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 MAPK each of which is activated via different stimuli and initiate individual MAPK cascades (Dhillon et al. 2007). Enhanced ERK1/2 activity has been found in tumor tissue of GBM patients, suggesting that ERK1/2 activation contributes to gliomagenesis (Bhaskara et al. 2005). Furthermore, 50% of GBM tissues with ERK1/2 expression had EGFR amplification (Lopez-Gines et al. 2008). It is also demonstrated that ERK1/2 activity is under PDGFRA control contributing to growth of GBM (Chen et al. 2014). JNKs are stress-activated protein kinases belonging to MAPK family (Kyriakis et al. 1994). The three JKNs isoforms (JNK1, JNK2 and JNK3) are activated by phosphorylation of tyrosine and threonine residues in a reaction catalyzed by the dual-specificity kinase MKK4 and MKK7 (Bogoyevitch and Kobe 2006). JNK is an oncogene and its signaling pathway is critical for cell proliferation, survival and inflammation in cancers, including GBM

(Antonyak et al. 2002). Indeed, the constitutive activation of JNK contributes to enhanced transformation and resistance to apoptosis in GBM (Wu et al. 1999, Cui et al. 2006) and it is involved in radiation-induced apoptosis (Chen et al. 1996).

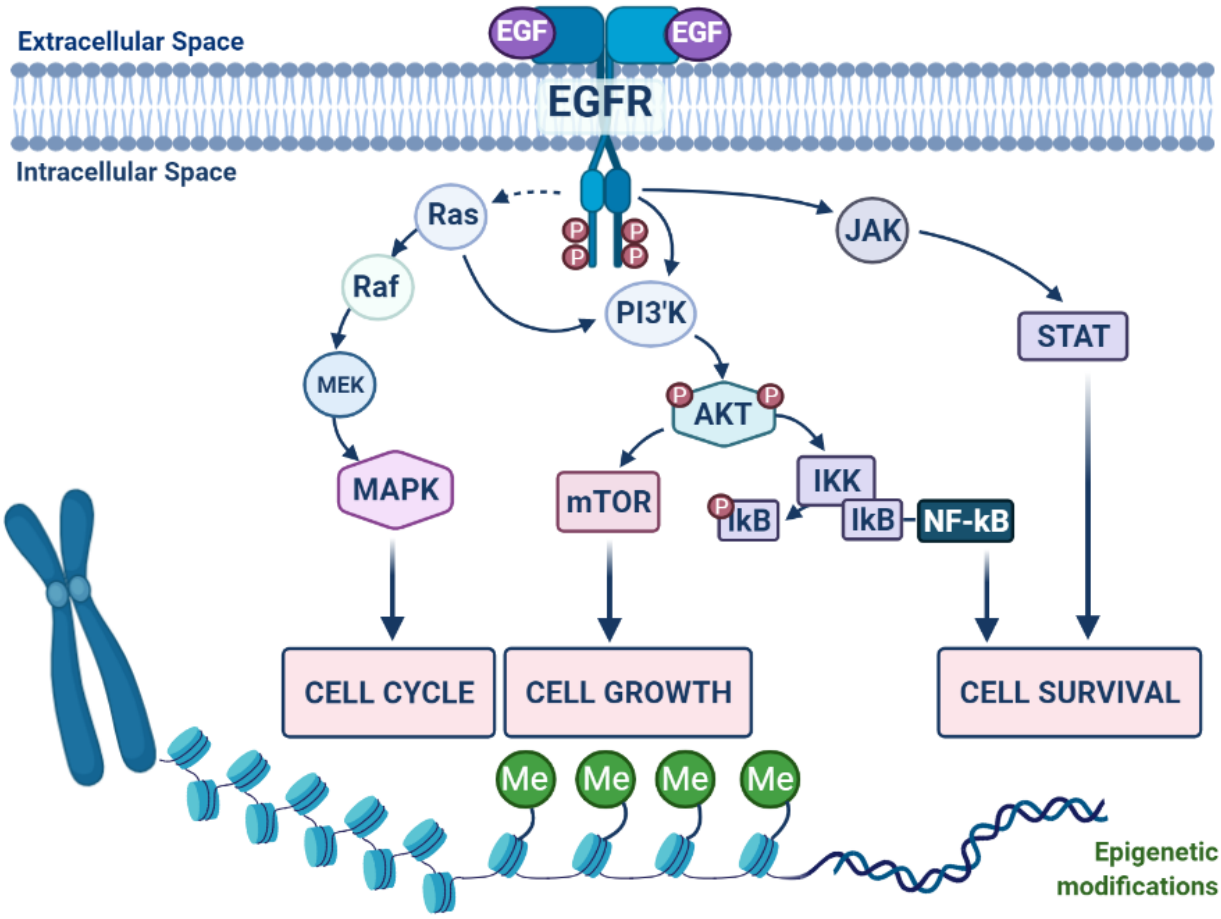

Fig. 7. Cartoon representing EGFR pathway in glioblastoma.

1.3 Sodium Calcium Exchanger and Ca^{2+}

It is becoming evident that Ca^{2+} channels/transporters/pumps are involved in a wide range of cancers and their expression has been shown to be altered in carcinogenesis. Indeed, tumors remodel their Ca^{2+} signaling network to proliferate at high rate and to increase cell motility and invasion, or to have neovascularization, in this regard, Ca^{2+} ATPases (PMCA and SERCA), Ca^{2+} channels (voltage-gated and TRP family), and also the purinergic P2X receptors represented interesting pharmacological target for several studies in oncology field (Monteith et al. 2007). Among the proteins controlling the intracellular calcium levels, the Na^{+}/Ca^{2+} exchanger (NCX) is an important bidirectional plasma membrane transporter driven by electrochemical gradient (Baker et al. 1969) widely distributed in mammalian cells (Quednau et al. 1997). The primary function of NCX is to couple the extrusion of one Ca^{2+} ion from the cytosol with the influx of three Na^{+} ions (forward mode or Ca^{2+}-efflux mode) by using the electrochemical gradient (Annunziato et al. 2004). However, NCX could revert its mode of operation, promoting Ca^{2+} influx and Na^{+} efflux (reverse mode o Ca^{2+}-influx mode), causing an increase in $[Ca^{2+}]_i$ when the electrochemical gradient is reversed (Annunziato et al. 2004). Notably, NCX is essential for the maintenance of Ca^{2+} homoeostasis in both cardiovascular and neurological tissues under physiological and pathological conditions. NCX belongs to a multigene family comprising three isoforms, named NCX1, NCX2, and NCX3, encoded by three different genes, *slc8a1*, *slc8a2*, and *slc8a3*, respectively, which are differentially expressed in mammalians. NCX1 is ubiquitously distributed in the body (Quednau et al. 1997), whereas NCX2 is mainly expressed in the brain (Jeon et al. 2003) and NCX3 is expressed exclusively in brain, immune system, and skeletal muscles (Quednau et al. 1997). All these antiporter isoforms share the same topology with 10 hydrophobic transmembrane segments and a large intracellular loop (Ren and Philipson 2013) (Fig. 8) but

differ for regulating mechanisms (Annunziato et al. 2004). Among these isoforms, NCX2 was proposed as an anti-oncogene for GBM (Qu et al. 2010).

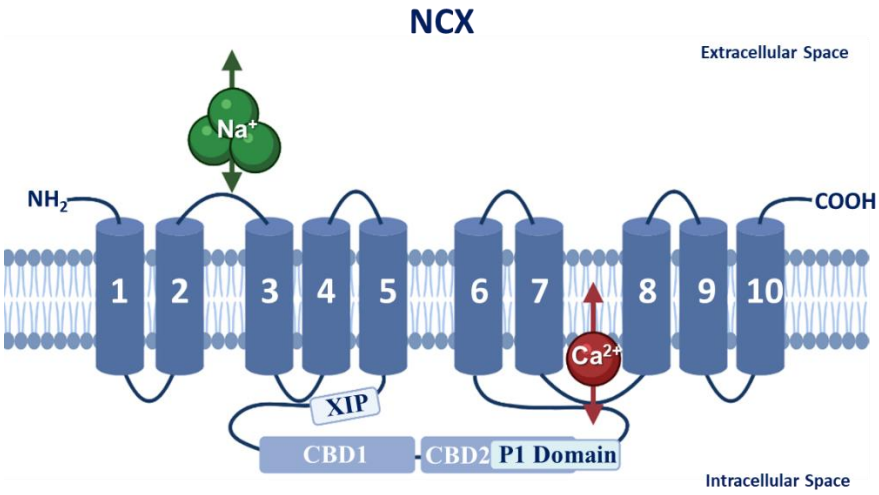

Fig. 8. Current NCX topology

1.3.1 NCX2 in neurological disorders

Dysregulation of the Na^+/Ca^{2+} homeostasis is a common feature of glial and neuronal cells in neurodegenerative disorders (Annunziato et al. 2020). Indeed, NCX was found to be involved in several neuronal diseases including stroke (Pignataro et al. 2004, Jeon et al. 2008, Molinaro et al. 2008, Molinaro et al. 2016), hypoxia ischemic encephalopathy (HIE) (Cerullo et al. 2018), multiple sclerosis (MS) (Boscia et al. 2012, Casamassa et al. 2016), amyotrophic lateral sclerosis (ALS) (Anzilotti et al. 2018), spinal muscular atrophy (SMA) (Valsecchi et al. 2020) and Alzheimer's disease (AD) (Pannaccione et al. 2012) (Fig. 9). In particular, NCX2 is deregulated in AD (Sokolow et al. 2011, Moriguchi et al. 2018), Parkinson's disease (PD) (Wood-Kaczmar et al. 2013, Sirabella et al. 2018), SMA (Valsecchi et al. 2020) and

stroke (Jeon et al. 2008, Molinaro et al. 2013, Molinaro et al. 2013). AD is the most common form of dementia and is characterized by alterations in amyloid-beta (Aβ) metabolism and synaptic dysfunction in aging brain (Khachaturian 1987). The Ca^{2+} hypothesis of AD proposes that the amyloidogenic pathway contributes to the remodeling of Ca^{2+} signaling responsible for cognitive deficits (Mattson et al. 1993). In this regard, all NCX isoforms co-localize with Aβ in the parietal cortex of AD patients (Sokolow et al. 2011). In addition, NCX3 levels are significantly reduced resulting in increased of NCX2 expression through a compensatory mechanism in AD terminals as shown by quantitative flow cytometry. In particular, the accumulation of NCX2 might also result from a blockade of retrograde axonal transport leading to Aß accumulation in AD neurons. PD is a neurodegenerative progressive disorder that affects the motor system and in particular the dopaminergic neurons in the substantia nigra. PD is also associated with the presence of proteinaceous inclusions, Lewy bodies and neurites, in surviving neurons. There are several genes mutated in PD: α-synuclein, leucine-rich repeat kinase 2 (LRRK2), DJ-1, ATP13A2, parkin and PTEN-induced kinase 1 (PINK1). Several studies showed that in the absence of PINK1, NCX activity was severely impaired in mitochondria (Gandhi et al. 2009), demonstrating the presence of a NCX regulation PINK1-mediated (Wood-Kaczmar et al. 2013). In particular, PINK1 interacts with NCX2 and NCX3 to protect dopaminergic neurons from degeneration in Parkinson's disease. Moreover, NCX2 seems to be involved also in the progression of SMA (Valsecchi et al. 2020), a severe neuromuscular disease characterized by alterations of the survival motor neuron gene, which results in progressive degeneration of motor neurons. The regulation of the exchanger expression, mediated by mir-206, exerts a protective effect on motor neurons of the facial nuclei in SMA. In fact, NCX2 is downregulated during the first weeks of age in WT animals, whereas the exchanger expression is increased during development, in SMA

mice. These findings suggest that NCX2 upregulation may be linked to the progression of SMA. During stroke there is an accumulation of $[Na^+]_i$ caused by inhibition of Na^+/K^+ ATPase activity due to a decreased of ATP production; under this pathological condition NCX is forced to operate in the reverse mode reducing Na^+-dependent cell swelling in the first phase of anoxia (Annunziato et al. 2004) and thus reducing the infarct volume and the neuronal loss in a transient focal cerebral ischemia (Jeon et al. 2008). In line with these data, a heterocyclic compound that stimulates NCX1/NCX2 activity, named neurounina-1, displays a neuroprotective effect in stroke (Molinaro et al. 2013).

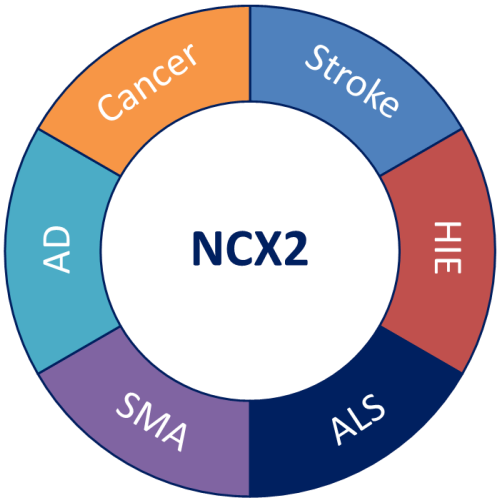

Fig. 9. Neurological and neurodegenerative disorders in which NCX2 plays a role. HIE: Hypoxia ischemic encephalopathy; ALS: Amyotrophic lateral sclerosis; SMA: Sclerosis muscular atrophy; AD: Alzheimer's disease.

1.3.2 NCX in tumors

NCX is involved in several tumors with distinct roles. For instance, SEA0400, a potent and unspecific inhibitory compound for NCX, prevents sodium nitroprusside-dependent apoptosis in human neuroblastoma cells (SH-SY5Y), suggesting that Ca^{2+} influx through the reverse mode of NCX contributes to the NO-induced cytotoxicity (Nashida et al. 2011, Rodrigues et al. 2019). In addition, another NCX inhibitor, KB-R7943 increases cisplatin-induced apoptosis only in the cisplatin-resistant ovary carcinoma cells without affecting the susceptibility of sensitive cells (Pelzl et al. 2015). Moreover, the natural steroid saponin increases intracellular calcium levels through the inhibition of the reverse mode activity of NCX, causing cytotoxicity in the leukemic cell lines OSW-1 (Garcia-Prieto et al. 2013). On the other hand, NCX1 protein expression is significantly higher in human esophageal squamous carcinoma cells compared to normal esophageal epithelial cell line, and for this reason this antiporter isoform was proposed as a biomarker of esophageal cancer. Accordingly, the treatment with NNK, a tobacco-specific nitrosamine that stimulates the reverse mode of NCX1 activity, leads to an increase in tumor invasion and proliferation (Wen et al. 2016, Ding et al. 2020). Similarly, NCX is also overexpressed in metastatic human melanoma cells where it operates in reverse mode (Sennoune et al. 2015). By contrast, the blockage of NCX1 in the forward activity suppresses glioblastoma, whereas the inhibition of its activity selectively in the reverse mode does not affect tumor growth (Hu et al. 2019). Moreover, SKF 96365, a TRPC channel blocker, suppresses proliferation of glioblastoma cells by enhancing NCX1 activity in the reverse mode and thus increasing intracellular calcium concentration (Song et al. 2014). In contrast to NCX1, much less information is available on the role played by NCX2 and NCX3 in carcinogenesis. In particular, NCX2 is

almost silenced in grades 2, 3, and 4 of gliomas (Kong et al. 2006, Paugh et al. 2010). In addition, *slc8a2* gene, encoding for NCX2, is highly methylated in glioblastoma tissues as compared to normal tissues (Qu et al. 2010), and it is known that methylation is one the epigenetic mechanism that prevents gene expression and plays a key role in the transcriptional silencing of this gene in gliomas (Qu et al. 2017). Based on this observation, it has been hypothesized that *slc8a2* may be a possible tumor suppressor gene, and thus an important gene for glioma development.

1.4 Treatment of glioblastoma

In the treatment of GBM we distinguish supportive therapies from curative therapies. Supportive treatment aims to relieve symptoms and improve the patient's neurological functions. The primary support agents are antiepileptic drugs and corticosteroids. Antiepileptic drugs are administered to approximately 25% of patients who have had epileptic seizures on presentation of the disease. Phenytoin (300-400 mg/d) is the most used drug, but carbamazepine (600-1,000 mg/d), phenobarbital (90-150 mg/d) and valproic acid (750-1,500 mg/d) are equally effective. Corticosteroid drugs reduce peritumoral edema, decreasing both neoplasm mass effect and intracranial pressure. This treatment has an immediate effect, headache relief and an improvement of the "lateralizing" signs. Dexamethasone represents the first choice among corticosteroids due to its minimal mineralocorticoid activity. The starting dose is around 16 mg/d. This quantity can be increased or decreased until the minimum dose necessary to keep neurological symptoms under control. However, prolonged use of corticosteroids is associated with hypertension, diabetes mellitus, non-ketotic hyperosmolar hyperglycemic state (life-threatening disease), myopathy, weight gain,

insomnia and osteoporosis. For these reasons, once the curative treatment has started steroid dose should be gradually reduced "as quickly as possible". Brain tumor healing therapies essentially include surgery, radiation therapy and chemotherapy. The surgical approach must be chosen carefully to balance the requirement to remove the maximum possible of the tumor mass, and to preserve vital structures to minimize risks of postoperative neurological deficit. Radiation therapy, which is normally carried out after the surgery, concerns the part of the brain affected by the intervention as well as a slight external margin, and has the purpose of damaging the DNA of any cancer cells left after the operation and escaped to the surgeon because they are not visible under the microscope (as they have infiltrated around distant from the operation area). Standard protocol of postoperative radiotherapy provides by partial-field external beam irradiation, targeting brain tissue at a 2-3 cm margin around the operative cavity (Wen and Kesari 2008), it is to deliver a total dose of 60Gy in 2Gy per fraction using external-beam radiation. Radiation therapy relies on generation of free radicals in oxygen-rich environments that, in turn, damage DNA in mitotically active cells. Cancer cells are less capable of dealing with DNA damage and eventually decrease in mitotic activity or die. This therapeutic approach typically used with anti-neoplastic chemotherapy. However, GBM occurs again in 90% of cases, the radio-resistance is mediated by both intrinsic and extrinsic factors (Ramirez et al. 2013). The DNA damage repair and the accelerated tumor repopulation are caused by radiation-induced microenvironment changes which provide suitable conditions for tumor survival (Barker et al. 2015, Kelley et al. 2016).

Average survival for GBM patients is 12-15 months after diagnosis. Less than 30% of the 27,000 patients diagnosed with GBM each year will survive beyond 2 years (Schneider et al. 2010, Teodorczyk and Martin-Villalba 2010). In 1980, the post-resection median survival of GBM patients was six months. If chemotherapy and radiation were included at the time the

median survival increased to approximately twelve months (Salcman 1980). Decades of focused research have improved GBM characterization and diagnosis. However, current median survival for GBM after aggressive treatment is a modest 12-15 months (Georges et al. 2014). Thirty years of research only increased patient survival approximately of 3 months. Current pharmacological approaches of GBM could be classified in conventional, targeted, immune, and supporting therapy.

1.4.1 Conventional treatment

Classical GBM treatment includes aggressive surgical resection followed by radiation and treatment with the alkylating agent, temozolomide (Hegi et al. 2005, Schneider et al. 2010, Sanai et al. 2011, Hart et al. 2013). However, in some cases, GBM may reside in critical regions that preclude resection. Regardless of completeness of resection, infiltrative cells always remain following surgical cytoreduction leading to recurrence (Di et al. 2010). Controlling and/or targeting infiltrative tumor cells may improve the extent of surgical resection and improve patient survival.

Chemotherapy aims to damage the DNA organization of cancer cells, possibly left after surgery and escaped radiation therapy. If the chemotherapy manages to unhinge this DNA, the cancer cell passes into the "programmed death" phase apoptosis. The main chemotherapeutic agent used for treatment of glioblastoma multiforme is temozolomide, an oral alkylating agent with a reasonable penetration of the normal blood-brain barrier (Stupp et al. 2007). Temozolomide was introduced in 1999 and increases GBM patient survival approximately of 3 months. This cytotoxic agent, once converted to its active form in the alkaline tumor environment, damages cells by methylating DNA guanine bases at the N7, N3,

and O-6 positions. TMZ's cytotoxic effects are mediated by its methylation at the O6 position of guanine (O6-MeG). This methylation causes a persistent mismatch of O6-MeG to thymine rather than cystine that, in turn, prevents DNA strand elongation, futile cycling of DNA repair mechanisms that lead to cessation of cell replication and death. However, some tumor cells can acquire resistance by expressing MGMT, an enzyme that can efficiently repair O6-MeG, and thus can resist to TMZ treatment (Zhang et al. 2012, Hart et al. 2013). For these reasons chemotherapy has limited benefits for glioblastoma patients. In clinical trials, the use of nitrosureas does not significantly increase the average survival. Treatment in combined radiotherapy-temozolomide was on average well tolerated and with minimal additional toxicity, so that this protocol became the therapeutic standard of choice for all new glioblastoma patients. Common unwanted long-term effects include toxicity to adjacent brain structures and cognitive deficits and epilepsy due to neuronal damage (Wen and Kesari 2008). Despite the limited initial successes of the therapies, practically all glioblastomas recur, the reason is both the late stage of the disease at diagnosis, and the inability of available therapy to efficiently eradicate all glioblastoma cells (Van Meir et al. 2010). GBM recurs after irradiation of tumor margins at varying depth (Wen and Kesari 2008). Although irradiating the sub-ventricular zone appears to have a prognostic benefit for GBM patients (Ramirez et al. 2013), it does not cure these patients, this can occur for two reasons: in one case, GBM-Initiating Cells (GICs) already present in the parenchyma of the brain restart the tumor after a dormant period (horizontal recurrence) or in the second, dormant cells in the sub-ventricular zone re-migrate to the tumor bed area, where they create the recurrent tumor (vertical recurrence). In addition, infiltrative tumor cells are often less responsive to TMZ because of (i) a decreased mitotic activity, and (ii) distance from the alkaline tumor environment (Wolf et al. 2011, Zhang et al. 2012). Therefore, agents specifically targeting infiltrative cells may

be required in addition to standard chemotherapeutics. Once infiltrative cells migrate away from vasculature their oxygen access diminishes and they switch from aerobic to anaerobic metabolism, a change known as the Warburg effect. Indeed, this altered environment and metabolism may attenuate the effects of chemotherapy and radiation on infiltrative tumor cells (Wolf et al. 2010, Wolf et al. 2011, Mattox et al. 2012). Controlling GBM infiltration may improve patient outcomes by increasing the efficacy of current GBM therapies.

Furthermore, advances in molecular neuropathology have shown promise for better diagnosis of the tumors, and therapeutics in development. Large-scale genomic and epigenomic studies has provided deeper insights into molecular pathology of gliomas and identified novel targets for therapy such as the mutant IDH1 enzyme or therapy immune-based such as dendritic cell vaccines and checkpoint inhibitor drugs, developed to block tumor cells blunting of the immune response (Stupp et al. 2007, Wen and Brandes 2009, Barker et al. 2015, Kelley et al. 2016).

1.4.2 Targeted Cancer Therapy

More than 100 years ago, Paul Ehrlich supposed the original “magic bullet theory”, according to which cancer cells can be specifically targeted by directing drugs to molecular targets exclusively found in malignant cells (Strebhardt and Ullrich 2008). Consequently, the conducted research has focused on two different strategies: targeting drugs directly to the cancer cells, through specific interaction (Gabizon et al. 2006, Sathornsumetee and Rich 2008), or targeting drugs to the tumor vasculature (Chan et al. 2008, Chi et al. 2009, Siemann and Horsman 2009), exploiting the starvation of the cancer cells.

Drugs directly targeting cancer cells can be divided into two main groups: the first group, known as molecular targeted drugs, aims at interfering with intracellular signaling pathways, overactivated in glioblastoma (Cavaliere et al. 2007, Van Meir et al. 2010) by binding directly to a target molecule to inhibit its functions. The second group of drugs targeting cancer cells provides for use various types of drug carriers able to prevent the interaction of the drug with healthy cells, by modification of the drug carrier with a ligand or antibody directs towards a specific cancer cell surface molecule (Lammers et al. 2008). Examples of these strategies include different types of EGFR inhibitors, such as cetuximab, a monoclonal anti-body, and the EGFR tyrosine kinase inhibitors gefitinib and erlotinib. BBB acts like a barrier especially for the large monoclonal antibodies, whereas the tyrosine kinase inhibitors have an advantage due to their small molecular weight that allows them to easier penetrate in the tumor mass (Van Meir et al. 2010). Targeted protein toxins are bacterial toxins mutated in their binding domain conjugated to a targeting molecule and are the only tumor-directed drug carriers currently in use in clinical trials for glioblastoma multiforme, delivered locally into the tumor through an infusion of the drug through catheters inserted in the brain. Examples of protein toxins in clinical trials for glioblastoma multiforme are Tf-CRM107 (Weaver and Laske 2003), a modified diphtheria toxin conjugated to transferrin and pseudomonas exotoxin conjugated to an interleukin: interleukin-4 PE38KDEL and interleukin-13 PE38QQR (Husain and Puri 2003, Kawakami et al. 2003). Another type of drug carrier suggested are targeted liposomes thanks to the large variety of drugs that can be encapsulated, an example is the chemotherapeutic doxorubicin (Drummond et al. 1999). Indeed, as the available preclinical data demonstrate superior antitumor activity of formulations of liposomal doxorubicin as compared to unencapsulated drug (Drummond et al. 1999, Mamot et al. 2005, Gupta and Torchilin 2007, Madhankumar et al. 2009).

Another anti-neoplastic strategy is to inhibit angiogenesis and thus to deprive cancer cells of oxygen and nutrients. Furthermore, targeting the tumor vasculature has some advantages compared to direct targeting of the cancer cell because blood-borne drugs have easy access to target molecules expressed by tumor endothelial (Alessi et al. 2004, Hinnen and Eskens 2007) and vascular disruption has a significant amplification effect, since a single blood vessel might supply a lot of cancer cells (Ahlskog et al. 2006, Hinnen and Eskens 2007).

The most studied strategy targeting to the tumor vasculature is represented by angiogenesis inhibitors. These drugs interfere with the formation of new blood vessels directed towards secreted proangiogenic growth factors, their cell surface receptors, or other molecules (Chan et al. 2008, Chi et al. 2009). Another drug class inhibiting blood supply to the tumor is the vascular disrupting agents (VDAs) (Tozer et al. 2005, Hinnen and Eskens 2007, Siemann and Horsman 2009), they can be divided into two main groups: small-molecule that take advantage of epithelial structural and molecular abnormalities and ligand-based VDAs that bind their target through antibody- or peptide-conjugated drug carriers (Thorpe 2004, Siemann and Horsman 2009). Bevacizumab is a monoclonal antibody that binds VEGF-A, preventing it from interacting with VEGFR-2 to exert its proangiogenic effects (Norden et al. 2008). In addition, several tyrosine kinase inhibitors have gone into clinical trials as the pan-VEGFR tyrosine kinase inhibitor cediranib with additional activity against PDGF receptor (PDGFR) and c-Kit (Wedge et al. 2005). PDGFR tyrosine kinase inhibitors tandutinib and dasatinib, and the αvβ3/αvβ5 integrin inhibitor cilengitide (Norden et al. 2008, Chi et al. 2009) displayed an antitumor activity in glioblastoma multiforme, when administered as monotherapy and in combination with chemotherapy (Mathieu et al. 2008, Wen and Kesari 2008). However, the clinical use of antiangiogenic therapy is complicated by fast development of tumor resistance because of due to activation of alternative angiogenic

signaling pathways, indeed, cells submitted to an antiangiogenic therapy have a more invasive phenotype (Norden et al. 2008). Flavone acetic acid and its derivative 5,6-dimethylxanthenone-acetic acid are the most studied small-molecule VDAs (Hinnen and Eskens 2007), they act on of the release of tumor necrosis factor α (TNF-α) and other cytokines from the tumor endothelial cells (Baguley and Ching 2002). Other ligand-targeted VDAs examined, in other experimental *in vivo* cancer models, include fusion proteins, immunotoxins, drug-conjugates and immunoliposomes targeted to different *de novo* expressed membrane proteins on the tumor endothelial cells vasculature, like vascular cell adhesion molecule-1 (VCAM-1) (Ran et al. 1998, Dienst et al. 2005, Gosk et al. 2008), integrins aminopeptidase N (Pastorino et al. 2003, Pastorino et al. 2006, Garde et al. 2007), endoglin (Seon et al. 1997, Matsuno et al. 1999, Takahashi et al. 2001), VEGFR-2 (Arora et al. 1999, Wild et al. 2000, Veenendaal et al. 2002, Mohamedali et al. 2005), and extracellular matrix proteins, such as ED-B domain of the glycoprotein fibronectin (Nilsson et al. 2001, Carnemolla et al. 2002, Halin et al. 2002, Marty et al. 2002, Borsi et al. 2003). Despite VDAs have shown little effect as monotherapy (Siemann and Horsman 2009), the combined VDAs with radiotherapy or chemotherapy enhanced antitumor activity in *experimental in vivo* models of glioblastoma multiforme (Thorpe 2004, Siemann and Horsman 2009).

Chapter 2: Objectives and aims

The overall objective of this thesis is to examine the mechanisms of silencing of *slc8a2* gene encoding for the Na^+/Ca^{2+} exchanger 2 (NCX2) protein, a negative biomarker of glioblastoma.

Specific aims include:

1) Identification and characterization of the human promoter for *slc8a2* gene
2) Identification of the transcriptional factors involved in the transcriptional repression of NCX2 in glioblastoma.
3) Identification and characterization of the pathways involved in NCX2 silencing
4) Evaluate the role of NCX2 expression in glioblastoma vitality.

Chapter 3: Materials and Methods

3.1 Cell Cultures

Baby hamster kidney (BHK) cell lines were grown on plastic dishes in a mix of Dulbecco's modified Eagle's medium (DMEM; Gibco) and Ham's F-12 medium (1:1) (Life Technologies, San Giuliano Milanese, Italy) supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma-Aldrich, St. Louis, MO).

Pheochromocytoma cell line (PC12) was maintained in culture in complete RPMI (500 ml RPMI (Invitrogen, Italy) 100 µg/ml penicillin 100 µg/ml streptomycin 4 mM L-glutamine 10% of decomplemented fetal bovine serum, 5% of horse serum). When confluence reached 80%, cells were washed with PBS (0.2 g/l KCl, 8.0 g/l NaCl, 0.2 g/l KH2PO4, 1.44 g/l Na_2HPO_4, up to 500 ml of ddH_2O), detached from plates using Versene solution (0.2 mg/ml EDTA disodium salt in PBS) and a new sub-colony was established.

Neuroblastoma SH-SY5Y and Glioblastoma U87-MG cells were maintained in culture in complete DMEM medium (500 ml DMEM, 100 µg/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine. 10% of decomplemented fetal bovine serum), washed with PBS and detached from the plate using trypsin-EDTA solution.

All cell lines were incubated in a humidified incubator at 37 ºC and 5 % CO_2. Cells in their early passage were detached and counted. Then, 2 million cells were suspended in 1 ml of freezing solution (90% growth medium, 10% DMSO) and put in 1 ml cryogenic vials and stored at -80 ºC. For the thawing procedure, cells were rapidly thawed in a water bath at 37 ºC and transferred to a 15 ml tube with 7 ml of complete growth media. Then, cells were spun at 1,200 rpm (250 g) for 5 min, the media was removed, and cells were suspended in the culture plate.

3.2 DNA cloning in promoterless luciferase reporter vector and site-direct mutagenesis

Genomic DNA containing NCX2 promoter was cloned in the pSC-b vector by using StrataClone Blunt PCR cloning kit (Agilent) by using the manufacturer protocol as illustrated in Fig. 10. Briefly, PCR products were ligated with the StrataCLONE Blunt Vector Mix and added to an aliquot of StrataCLONE SOLO ultracompetent cells. Bacterial cells were incubated in ice for 30 minutes, treated with a thermic shock (42°C for 30 seconds, 2 minutes in ice), resuspended in 300 µl of LB medium and then incubated for 1 hour at 37°C to express the antibiotic resistance of the internalized vector. Finally, cells were plated on LB-agar plates with ampicillin (50 µm/ml) and incubated overnight at 37°C. Single colonies were incubated in 5 ml of LB medium with ampicillin (50 µg/ml) overnight at 37°C. DNA plasmid was extracted from bacteria cultures with PureYield™ Plasmid Maxi- and Mini-prep System (Promega, Italy). Positive vectors were identified by using enzymatic digestion followed by DNA sequencing (Microgem, Italy). pSC-B-promoter and pGL3-Basic plasmids (Promega) were digested with the appropriate restriction enzymes. Fragments of the samples were electrophoretically separated on an agarose gel and the corresponding bands were extracted with StrataPrep DNA Gel Extraction Kit (Agilent) according to with the manufacturer protocol. Promoter fragments and pGL3 fragment were ligated with T4 ligase enzyme and transformed into Subcloning Efficiency™ DH5α™ competent cells (Thermofisher, USA). Positive colonies were incubated in 400 ml of LB medium and plasmids were extracted from bacterial cells with PureYield™ Plasmid Maxi-Prep Kit (Promega).

All mutagenesis experiments were performed by using QuikChange Lightning Site-Direct Mutagenesis Kit (Agilent) according to manufacturing protocol. PCR products were incubated with Dpn I restriction enzyme for 5 minutes at 37°C. Subsequently, the transformation of Dpn I-treated samples was performed into 45 ul XL10-Gold bacterial cells according to the above-mentioned protocol.

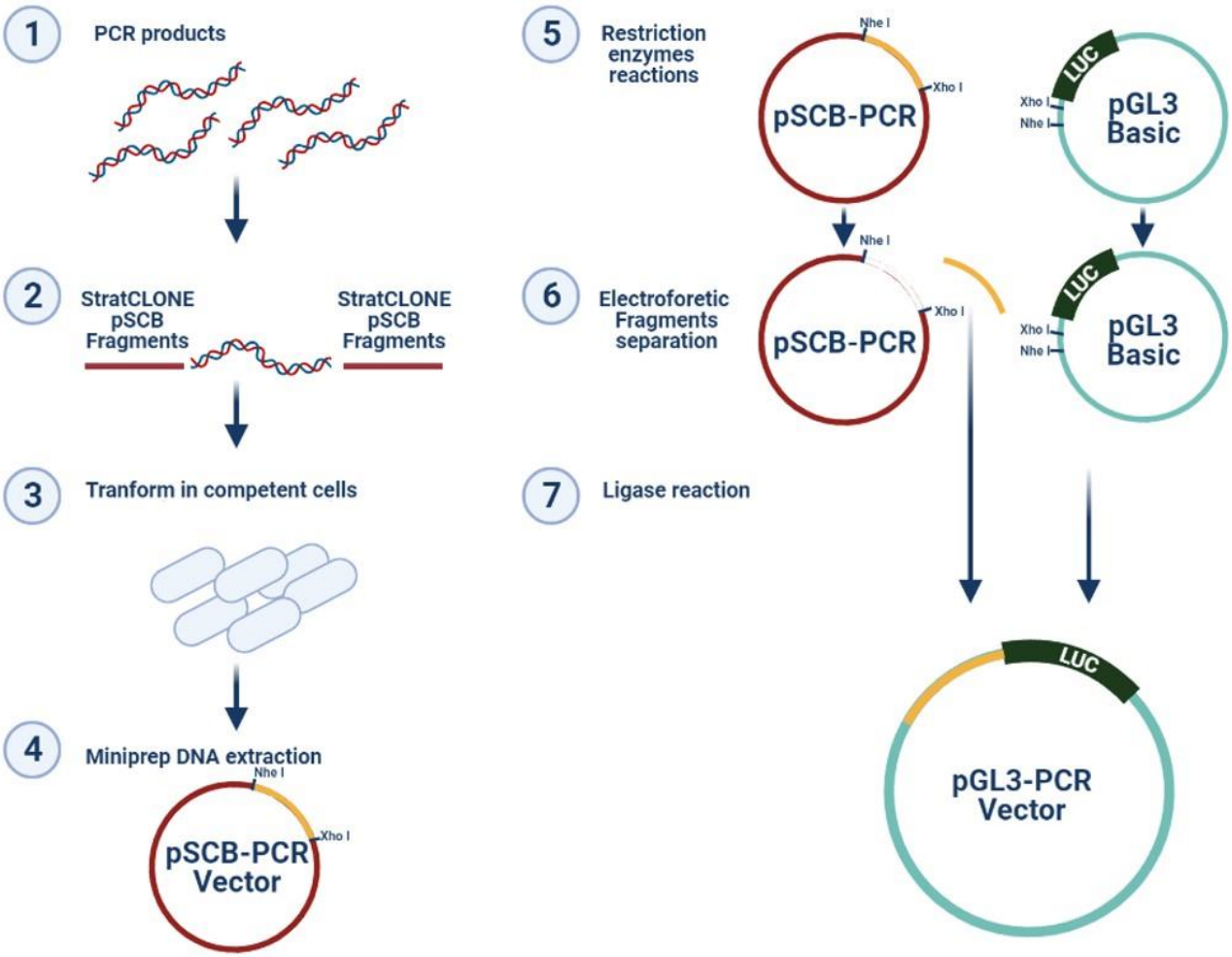

Fig. 10. Schematic representation of insertion of PCR product in pGL3-basic vector.

3.3 Retrotranscription of RNA and real-time PCR

Total RNA was extracted from cells using TRIzol® (Thermofisher) reagent according to Maniatis protocol (Maniatis et al. 1982). Two µg of total RNA were treated with DNase I (Sigma) and retrotranscribed by High-Capacity cDNA Reverse Transcription Kits (Life Technologies) according to the manufacturing protocol.

Quantitative real-time PCR was carried out using 1/20 of the cDNAs with Fast SYBR Green60 Master Mix (Applied Biosystems). Samples were amplified simultaneously in triplicate (7500 fast real-time PCR system, Applied Biosystems, Italy). qPCR data was collected using ABI Prism 7000 SDS software (Applied Biosystems). Differences in mRNA content between groups were calculated by using HPRT mRNA as the internal control. Data were analyzed with Pfaffl method (Pfaffl 2001).

3.4 DNA Transfections and Gene Reporter luciferase Assay

All transfections were performed with Lipofectamine 2000 (Life technologies). For the luciferase experiments, cells were plated in a 12-well plate at a concentration at planting time of 40,000 cells/well. After 24 h, cells were transfected with 1 µg of total plasmid DNA and 4 µl of Lipofectamine 2000 according to manufacturer protocol. For the promoter individuation experiments 900 ng and 100 ng of pGL3- promoter and pRL-TK plasmids were used, respectively. 720 ng of pGL3- Promoter, 80ng of pRL-TK and 200ng of TF-cDNA expression plasmid were used in the analysis of the promoter regulation. For the transfection of transcription factor cDNAs, cells were plated in 60 mm plate with a concentration of 150,000 cells/plate at plating time and transfected with 5 µg of plasmid DNA with 10 µl of Lipofectamine 2000. For all the experiments, during transfection cell medium was substituted

with reduced serum medium Opti-mem (Gibco) for 5 h. Afterwards, cells were incubated for 48 h with the culture medium until the experiments.

Luciferase assay was performed with Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer protocol. Cells were collected from the plate using provided lysis buffer, treated with two subsequent frosthawk cycles (-80 °C for 40 minutes, 37 °C for 2 minutes) and centrifuged (12,000 g for 20 minutes at 4 °C). Bioluminescence measurement of Pontius luciferase was performed with Glomax® 20/20 luminometer by adding LAR substrate buffer to a protein sample. Next, Renilla luciferase luminescence was measured adding the Stop&GLOW buffer to inhibit Photinus luciferase and providing a correct substrate to Renilla luciferase. Photinus luminescence was normalized with Renilla luminescence and expressed as “Relative Luciferase Activity”.

3.5 DNA bisulfite conversion and sequencing

In silico prediction of bisulfite-treated DNA sequence was performed with Bisulfite Primer Seeker tool (Zymoresearch) and primers were designed to amplify PCR products of 200-500bp. Bisulfite reaction was performed using EpiJET Bisulfite Conversion Kit (Thermofisher Scientific) according to the manufacturer protocol. Converted genomic DNA was amplified by PCR reaction using Hot Start Taq DNA polymerase (Thermo Scientific). PCR products were electrophoretically separated on 1% agarose gel. Bands were extracted from the gel, purified and singly cloned in the pSC-B vector as described previously. pSC-B-fragment vectors were sequenced (Microgem, Italy).

3.6 Cell count and vitality

Viable cells were measured using CCK8 assay. Briefly, cells were plated into 96-well plates at a density of 1 × 103 cells per well and incubated overnight in a 5% CO_2 atmosphere at 37 °C before exposure to drugs. Then, cells were treated with or without the indicated concentrations of drugs for the indicated time. Subsequently, CCK8 reagents were added to each well and cells were incubated for another 1 h at 37 °C. The absorbance of optical density at 450 nm was determined using FLUOstar Omega (MBG LABTECH) at 450 nm.

3.7 In Silico Analysis

Human intergenic region between *kptn* and *slc8a2* genes was analyzed by comparing the putative transcription binding sites with those obtained from the orthologous region in rat genome. We obtained the putative transcription binding sites by using Genomatrix MatInspector (http://www.genomatix.de) and Jaspar (http://jaspar.genereg.net) websites with a threshold of 0.80 for both matrix similarity and core similarity scores. Afterwards, we selected those binding sites that are conserved among the species for the relative position and that present possible cofactors in their proximity.

3.8 Polymerase chain reaction

PrimeSTAR GXL DNA Polymerase (Takara, cod. R050A) was used to obtain all PCR products for cloning experiments, according to the manufacturer protocol.

Primers used to amplify candidate promoter regions were designed according to the melting temperature reported by Oligo-Calculator analysis tool, version 3.27 (http://biotools.nubic.northwestern.edu).

Primers for site-directed mutagenesis were designed with a Tm >78°C calculated according to manufacturer protocol (Agilent technologies, Italy). Briefly, we used the following formula: Tm =81.5 + 0.41(%GC) – (675/N) - % of Mismatch where N is the primer length and %GC and % of mismatch are whole numbers. Primers were provided from Eurofins Genomics (Munich, Germany) in lyophilized form and eluted to obtain a stock concentration of 100 mM stored at -20 °C and thaw in ice before use.

3.9 Drug treatment

Cells were plated into 60mm-well plates at a density of $1 \times 120{,}000$ cells per dish and incubated overnight in a 5% CO_2 atmosphere at 37 °C before exposure to drugs. Cells were treated with IKK inhibitor BMS-345541, Erlotinib hydrochloride, Temozolomide, 5-AZA-dC or LY294002 hydrochloride (Sigma-Aldrich) for 6h, 24h, 48h and 72h before RNA extraction and purification.

Cells were plated into 60mm-well plates at a density of $1 \times 120{,}000$ cells per dish and incubated overnight in a 5% CO_2 atmosphere at 37 °C before exposure to drugs.

3.10 Statistical Analysis

All data are presented as the mean ± standard error of the mean (SEM) using GraphPad Prism 8 (GraphPad for Science, San Diego, CA). Statistical significance was calculated by student's t-test or by one-way ANOVA with Turkey post-test unless otherwise mentioned in the figure legends. p values < 0.05 were assumed as statistically significant for all the tests.

Chapter 4: Results

4.1 Inhibition of EGF-R pathway reduces the mRNA expression of MMP2 but increases NCX2 in glioblastoma cells in a NFkB-dependent manner

All tested compounds inhibiting EGF-R pathway at different levels showed an increase in NCX2 mRNA expression and a decrease in the transcription of the EGF-R downstream gene, MMP2, in U87 cell line. In particular, 10 μM of the EGF-Receptor inhibitor, Erlotinib, decreased the expression MMP-2 at 48h and 72h of incubation (Fig. 11) and increased NCX2 mRNA expression at 72h (Fig. 12). Similarly, 25 μM of the phosphoinositide 3-kinases (PI3Ks) inhibitor, LY294002, an upstream activator of NFkB in glioblastoma, caused an increase in NCX2 mRNA expression levels at 72h (Fig. 13). Moreover, 10 μM of the IKK-inhibitor, BMS-345541, a more direct inactivator of NFkB, caused a reduction of the mRNA expression of NFkB (Fig. 14) and its target gene MMP2 at 24h, 48h and 72h of treatment (Fig. 15), whereas increased NCX2 mRNA levels at 48h and 72h (Fig. 16). In addition, transfection of both p50 and p65 subunits (NFkB) displayed an increased in mRNA expression of p65 (Fig. 17), its target gene MMP2 and a transcription downregulation of the endogenous NCX2 gene (Fig. 18) in U87 cell lines.

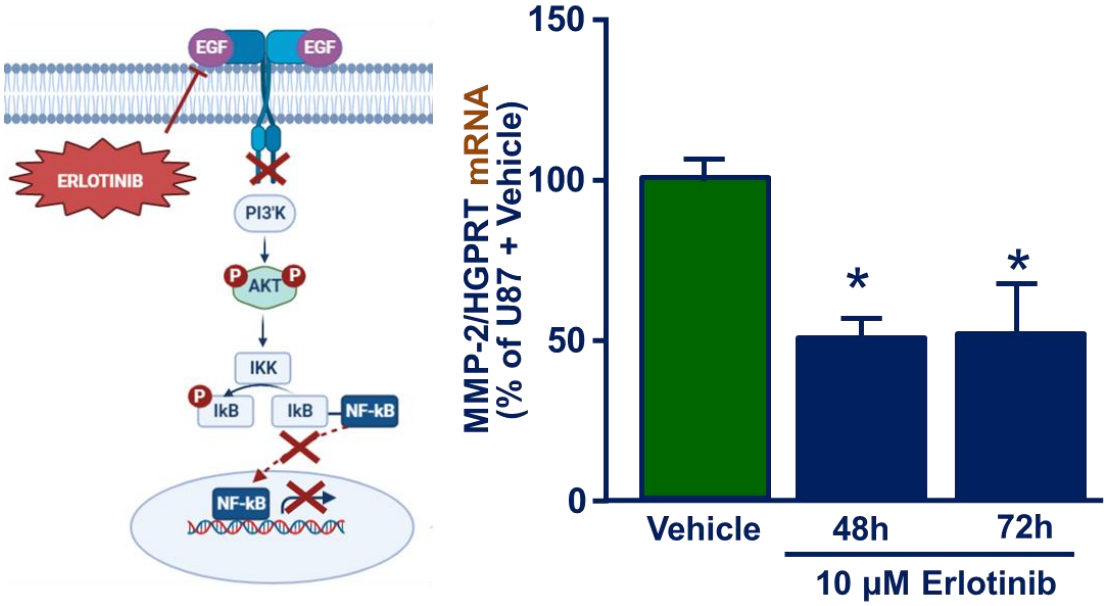

*Fig. 11. mRNA expression levels of MMP2, normalized for HGPRT and expressed as percentage of vehicle, at 48h or 72h of 10 µM Erlotinib incubation. *, p<0.05 vs vehicle (n=8 of two independent sessions; ANOVA followed by Turkey's post-hoc test).*

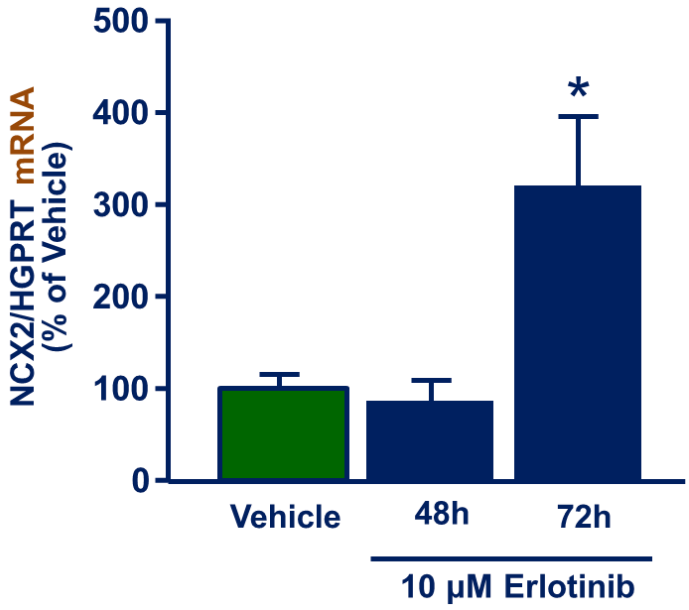

*Fig. 12. mRNA expression levels of NCX2, normalized for HGPRT and expressed as percentage of vehicle, at 48h or 72h of 10 µM Erlotinib incubation. *, p<0.05 vs vehicle (n=8 of two independent sessions; ANOVA followed by Turkey's post-hoc test).*

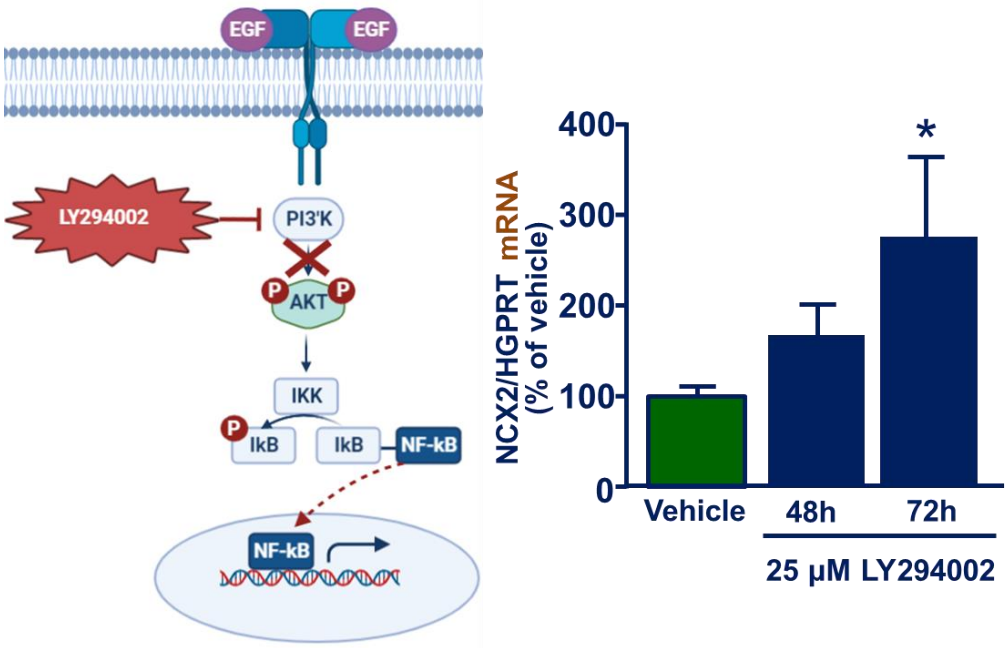

*Fig. 13. mRNA expression levels of NCX2, normalized for HGPRT and expressed as percentage of vehicle, at 48h or 72h of incubation with 25 µM LY294002 in U87 cells. *, p<0.05 vs vehicle (n=10 of three independent sessions; ANOVA followed by Turkey's post-hoc test).*

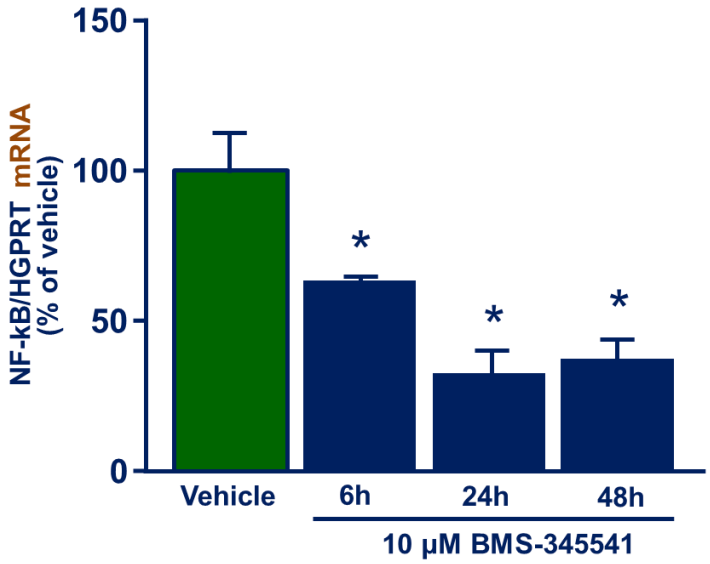

*Fig. 14. mRNA expression levels of p65 (NFkB1), normalized for HGPRT and expressed as percentage of vehicle, at 6h, 24h, or 48h of incubation with 10 µM BMS-345541 in U87 cells. *, p<0.05 vs vehicle (n=4 of one session; ANOVA followed by Turkey's post-hoc test).*

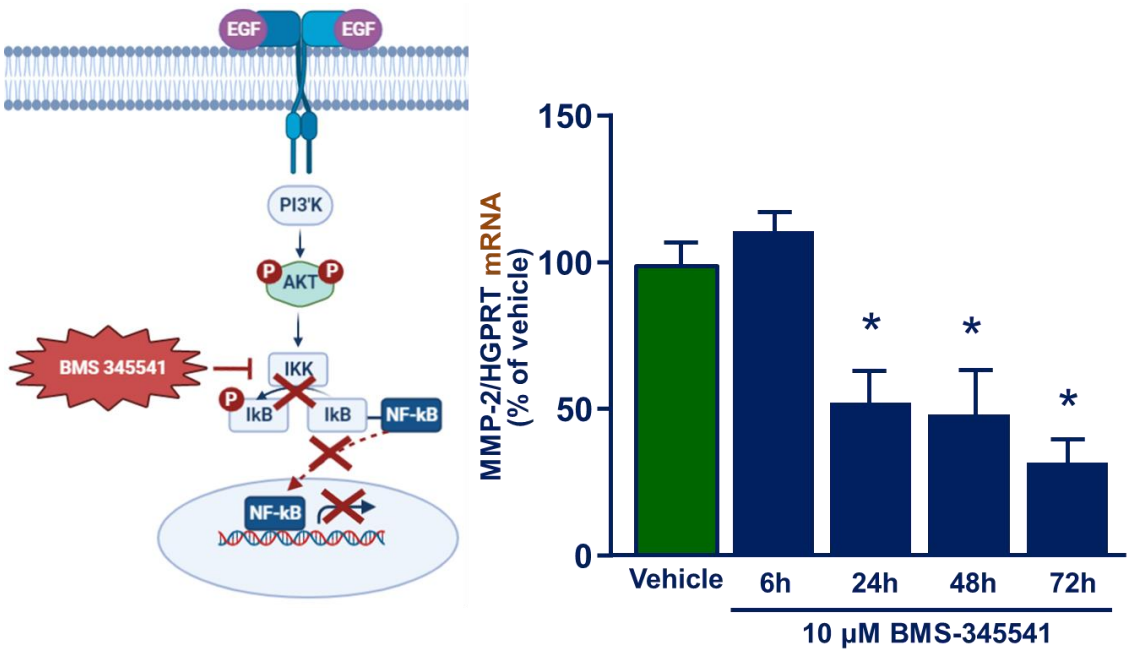

*Fig. 15. mRNA expression of MMP2, normalized for HGPRT and expressed as percentage of vehicle, at 6h, 24h, 48h or 72h of incubation with 10 µM BMS-345541 in U87 cells. *, p<0.05 vs vehicle (n=10 of three independent sessions; ANOVA followed by Turkey's post-hoc test).*

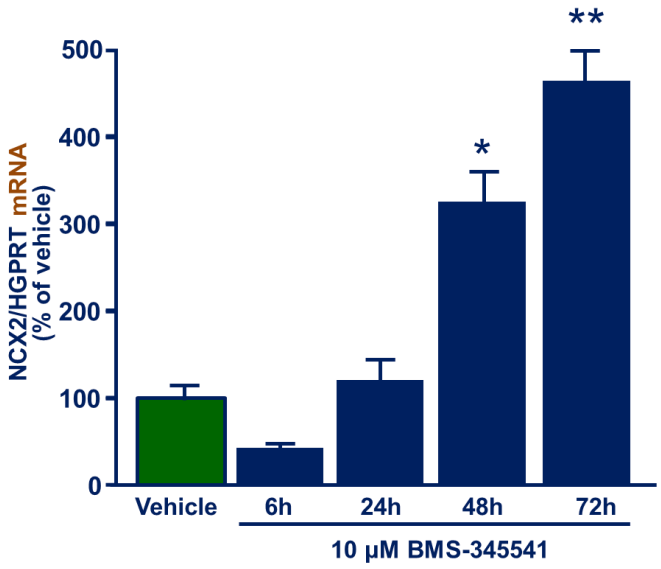

*Fig. 16. mRNA expression of NCX2, normalized for HGPRT and expressed as percentage of vehicle, at 6h, 24h, 48h or 72h of incubation with 10 µM BMS-345541 in U87 cells. *, p<0.05 vs vehicle (n=9 of 4 independent sessions; ANOVA followed by Turkey's post-hoc test).*

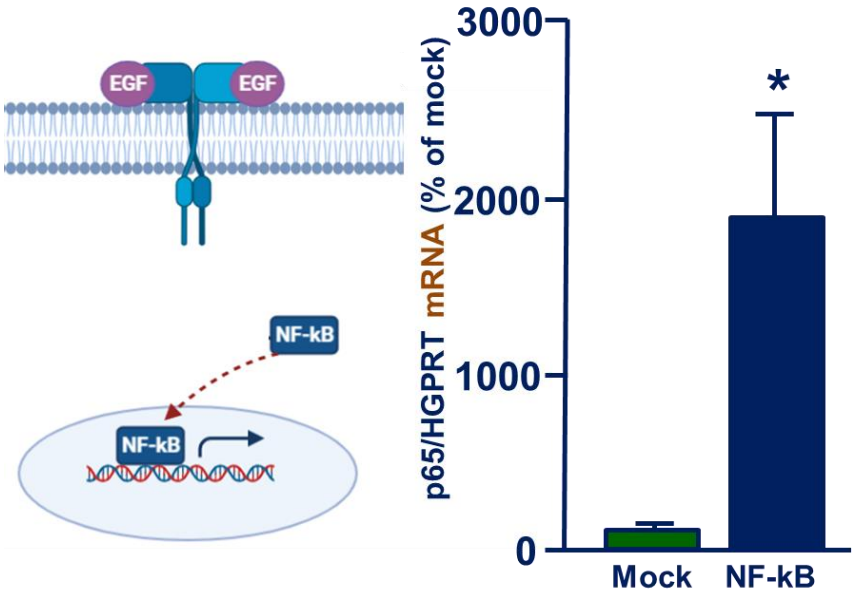

*Fig. 17. mRNA expression levels of p65, normalized for HGPRT and expressed as percentage of mock, at 48h after the transfection of both p65 and p50 (NF-kB) plasmids in U87 cell line. *, p<0.05 vs mock (n=13 of 4 independent sessions; Student' t-test).*

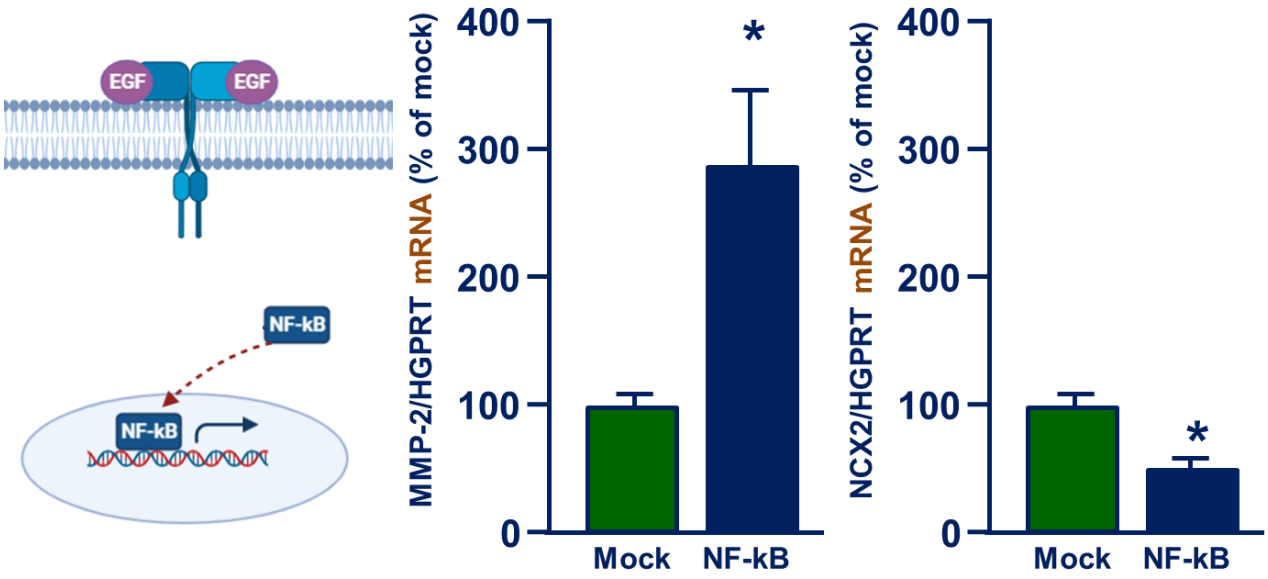

*Fig. 18. MMP2 and NCX2 mRNA expression levels, normalized for HGPRT and expressed as percentage of mock, at 48h after NF-kB transfection in U87 cells. *, p<0.05 vs mock (n=10 of 4 independent sessions; Student' t-test).*

4.2 Identification and characterizing the human NCX2 promoter in U87 cell line

BHK, PC12, U87 and SHSY cell lines were analyzed for the expression of NCX2 under basal conditions. Results showed that PC12 and SHSY cell lines express both mRNA (Fig. 20) and protein (Fig. 19) of NCX2, whereas BHK displayed undetectable signals of NCX2. Notably, a slight signal was obtained in U87 cell line (Fig. 19 and Fig. 20).

Based on the rat promoter sequence of NCX2, recently found by our research group in PC12 cells, we identified a putative promoter region between *kptn* and *slc8a2* genes in the human glioblastoma cell line U87 (Fig. 21). *In silico* analysis revealed several putative and conserved binding sites for transcription factors with a homology sequence above 75% on both human and rat NCX2 promoters (Fig. 21 and Fig. 22).

Rat (S3) and human (HP) NCX2 promoters were cloned in the promoterless luciferase plasmid pGL3b obtaining pGL3b-S3 and pGL3b-HP, respectively. Transfection of either, pGL3b-S3, or pGL3b-HP, plasmid increased luciferase activity in both PC12 and SHSY cell lines, whereas a small but significant luciferase activity was found in U87 cell line as compared with respective control group transfected with an empty pGL3b plasmid (Fig. 23 and Fig. 24). Both rat and human NCX2 promoters displayed a similar pattern of expression in PC12, U87, and SHSY cell lines (Fig. 23 and Fig. 24).

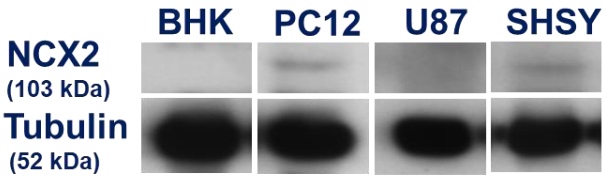

Fig. 19. Representative Western blot of NCX2 and tubulin in whole protein extracts of BHK, PC12, U87, and SHSY cell lines.

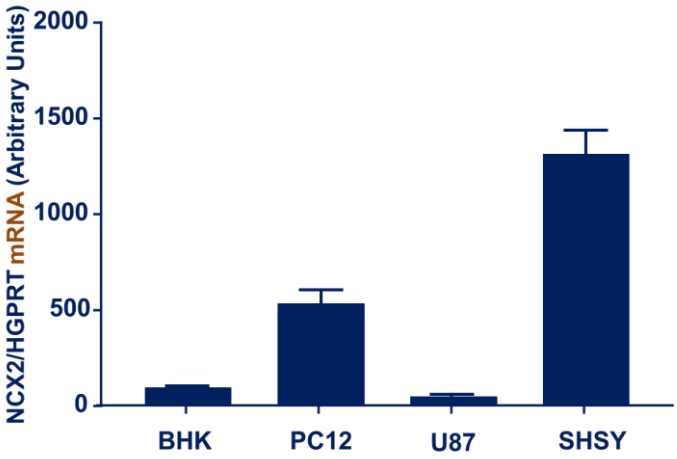

Fig. 20. Expression level of NCX2 mRNA, normalized for HGPRT signal and expressed as arbitrary units, in BHK, PC12, U87, and SHSY cell lines.

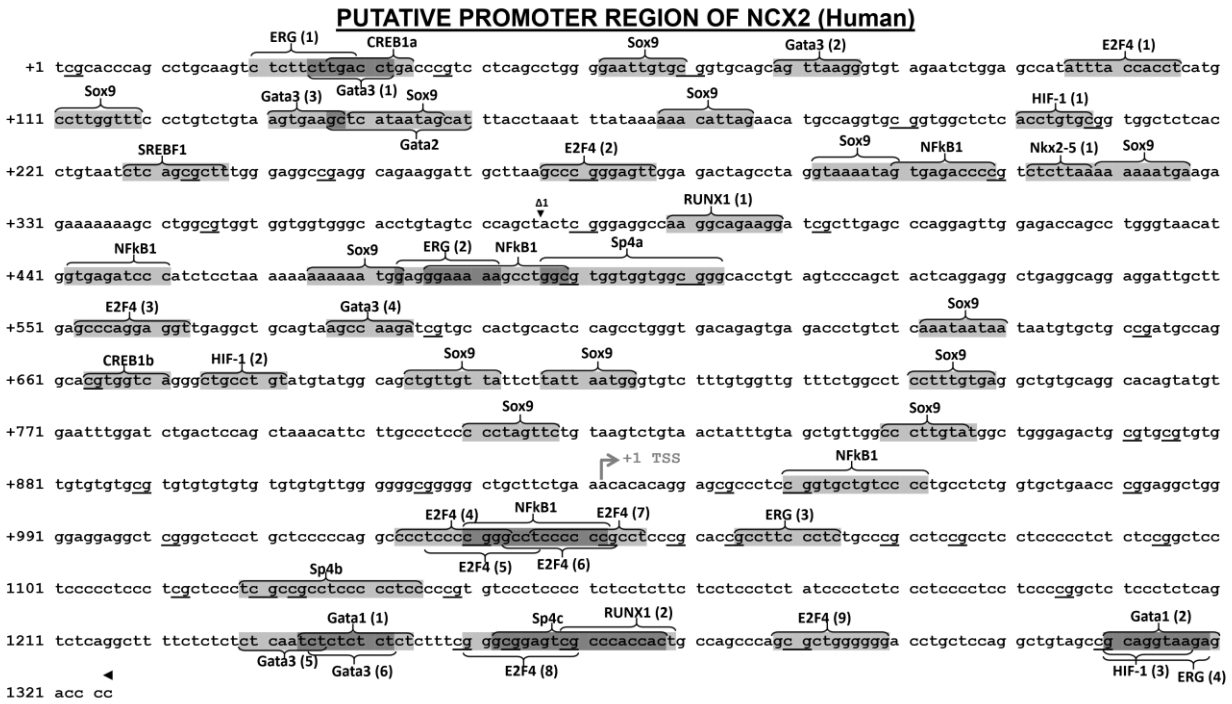

Fig. 21. Sequence of the putative human NCX2 promoter identified and cloned in pGL3basic plasmid (pGL3b-HP).

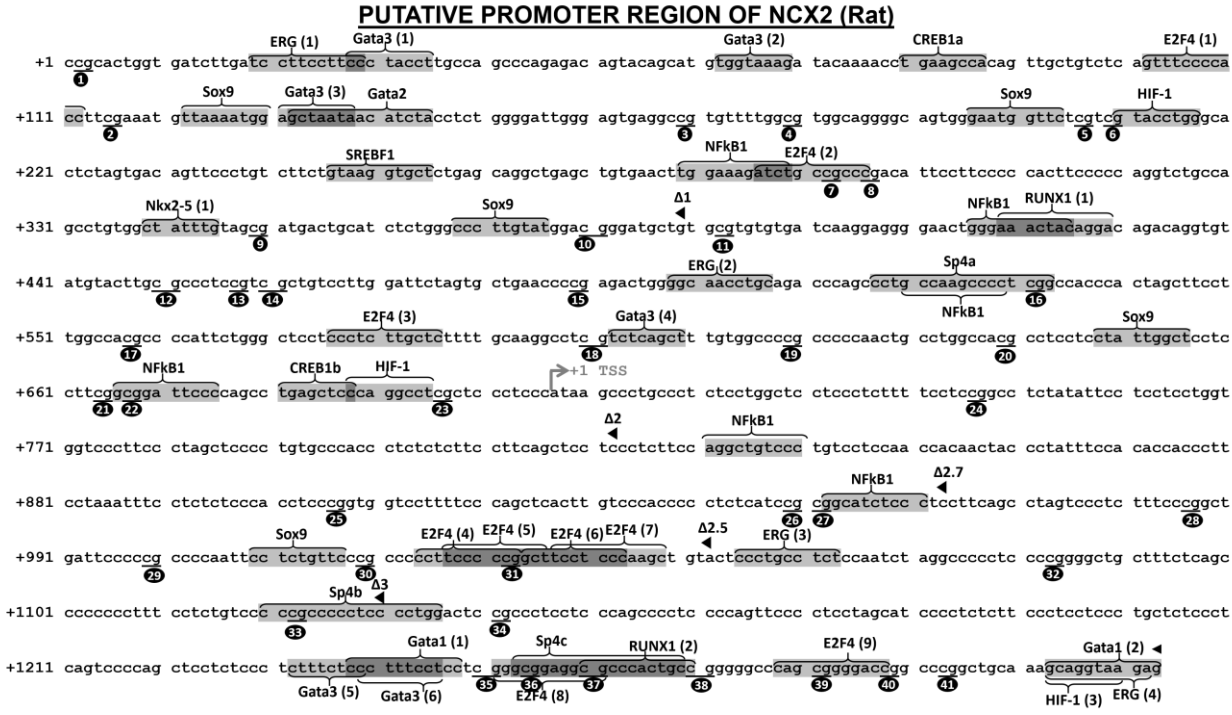

Fig. 22. Sequence of the putative rat NCX2 promoter identified and cloned in pGL3basic plasmid (pGL3b-S3).

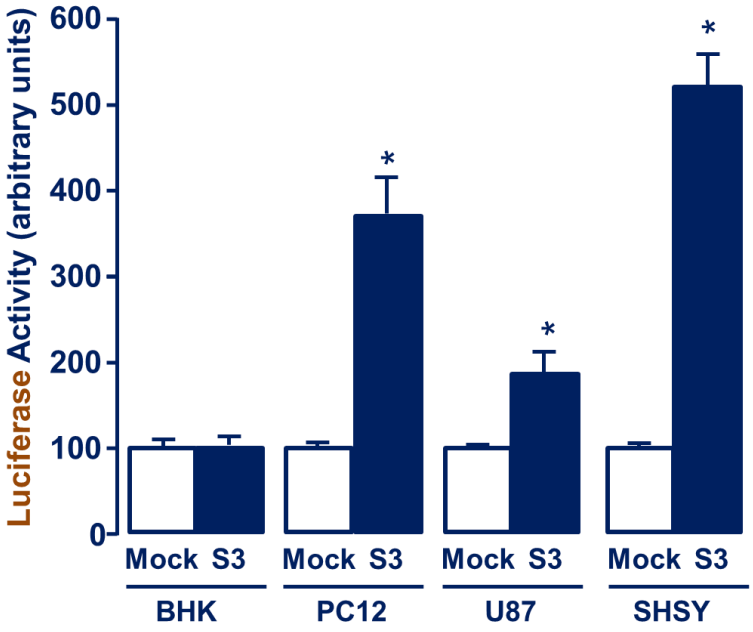

*Fig. 23. Luciferase activity of the promoterless (mock) and rat NCX2 promoter (pGL3b-S3) luciferase plasmids in BHK, PC12, U87, or SHSY cell lines. *, p<0.05 vs respective mock (n=33 of 10 independent sessions; Student' t-test).*

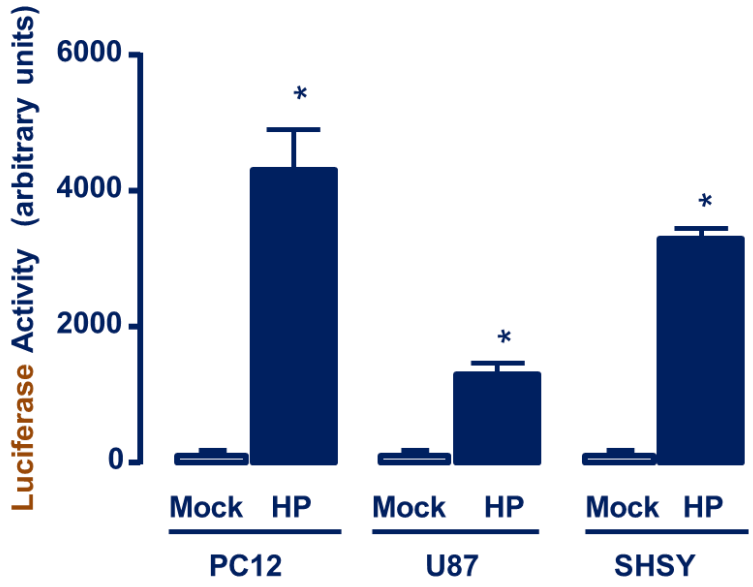

*Fig. 24. Luciferase activity of the promoterless (mock) and human NCX2 promoter (pGL3b-S3) plasmids, expressed as arbitrary units, in PC12, U87, or SHSY cell lines. *, p<0.05 vs respective mock (n=9 of three independent sessions; Student' t-test).*

Transfection of selected TFs, revealed that CREB and GATA2 increased, whereas NF-kB transfection decreased, the human NCX2 promoter activity in U87 cells (Fig. 25). In addition, 10 μM of the EGFR inhibitor, Erlotinib, increased the luciferase activity of pGL3b-HP at 72h of incubation (Fig. 26). Notably, transfection of NFkB1 reverted the Erlotinib-dependent enhanced luciferase activity of the human NCX2 promoter (Fig. 26). By contrast, Sp1 and Sp4 did not displayed effects on the luciferase activity driven by the human NCX2 promoter, although *in silico* analysis revealed that this sequence contains several conserved putative binding sites for both TFs (Fig. 21). On the other hand, transfection of either CREB, Sp1, Sp4, GATA2 or NF-kB increased rat NCX2 promoter activity in PC12 cells as measured by luciferase assay (Fig. 27).

Interestingly, to deeper investigate potential differences between two species we tested rat promoter in human cells and *vice versa*. Transfection of CREB, NF-kB or Gata2, but not Sp1 and Sp4, enhanced rat NCX2 promoter activity in U87 cell line (Fig. 28), whereas the transfection of GATA2, but not CREB, Sp1, Sp4 or NF-kB, exercised a stimulatory effect on the human promoter activity in PC12 cells (Fig. 29).

To further investigate the molecular determinant of TFs on human NCX2 promoter we used the deletion mutant pGL3b-HPΔ1-376 (pGL3B-HPΔ1) carrying the luciferase reporter gene. Trasfection of either CREB, Gata2 or NF-kB1 failed to modify the activity of the mutant promoter pGL3B-HPΔ1 lacking the region ranging between 1-376 (Fig. 30).

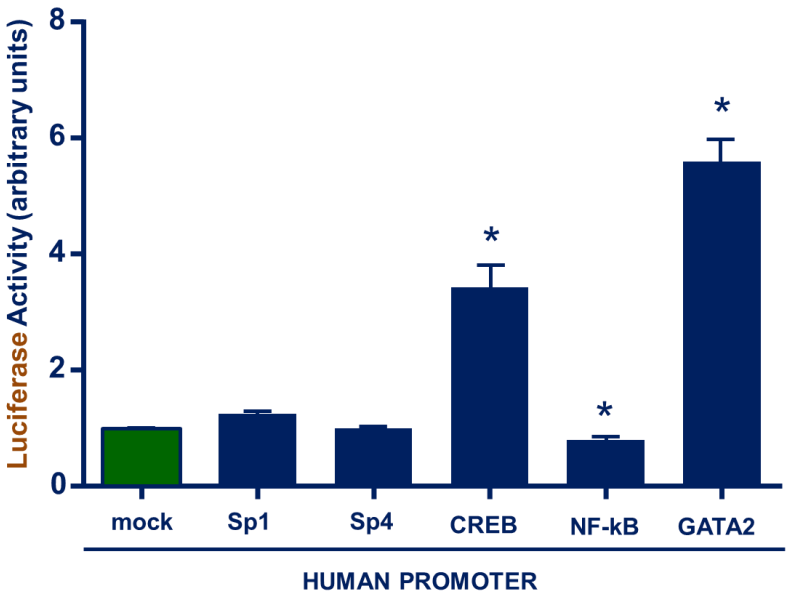

*Fig. 25. Luciferase activity of the human NCX2 promoter (pGL3b-HP), expressed as arbitrary units, at 48h after the transfection of pN3 (mock), Sp1, Sp4, CREB, NF-kB and Gata2 in U87 cell line. *, p<0.05 vs mock (n=16 of 6 independent sessions; ANOVA followed by Turkey's post-hoc test).*

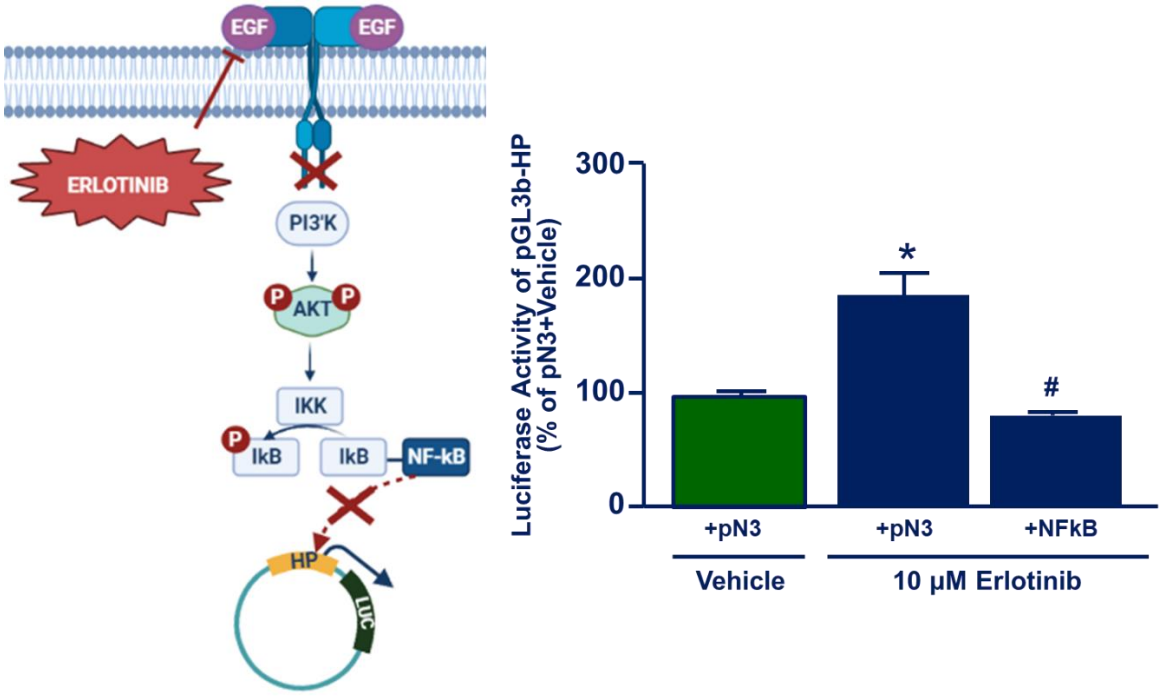

*Fig. 26. Luciferase activity of the human NCX2 promoter, expressed as percentage of pN3+vehicle, at 72h after incubation with 10 µM Erlotinib and transfection of mock or NFkB. *, p<0.05 vs pN3+Vehicle, #, p<0.05 vs pN3+ 10 µM Erlotinib (n=9 in three independent sessions; ANOVA followed by Turkey's post-hoc test).*

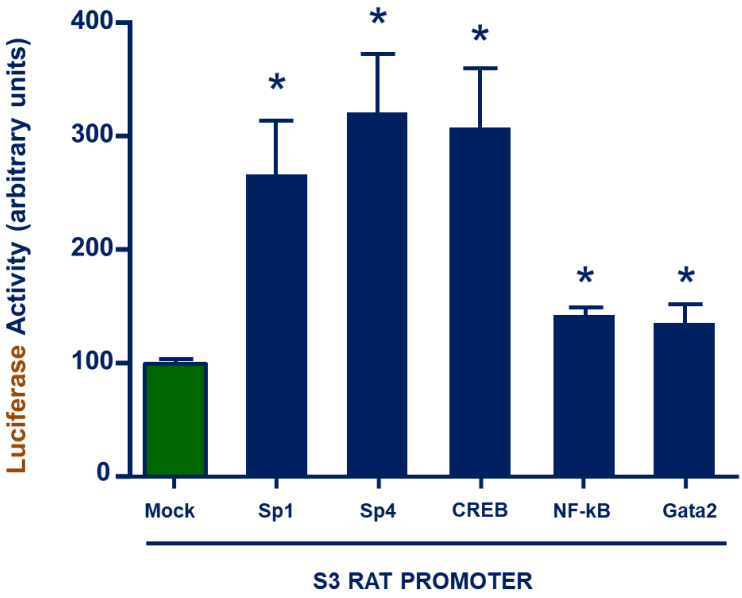

*Fig. 27. Luciferase activity of the rat NCX2 promoter (pGL3b-S3), expressed as percentage of mock, at 48h after the transfection of pN3 (mock), Sp1, Sp4, CREB, NF-kB and Gata2 in PC12 cell line. *, p<0.05 vs mock (n=12 in 4 independent sessions; ANOVA followed by Turkey's post-hoc test)*

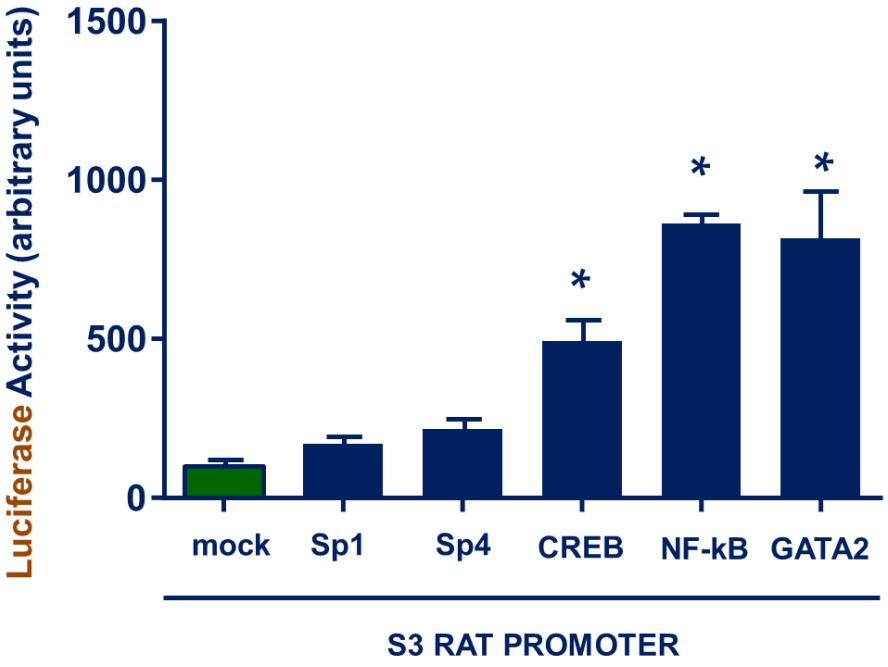

*Fig. 28. Luciferase activity of the rat NCX2 promoter (pGL3b-S3), expressed as percentage of mock, at 48h after the transfection of pN3 (mock), Sp1, Sp4, CREB, NF-kB and Gata2 in U87 cell line. *, p<0.05 vs mock (n=17 in 6 independent sessions; ANOVA followed by Turkey's test).*

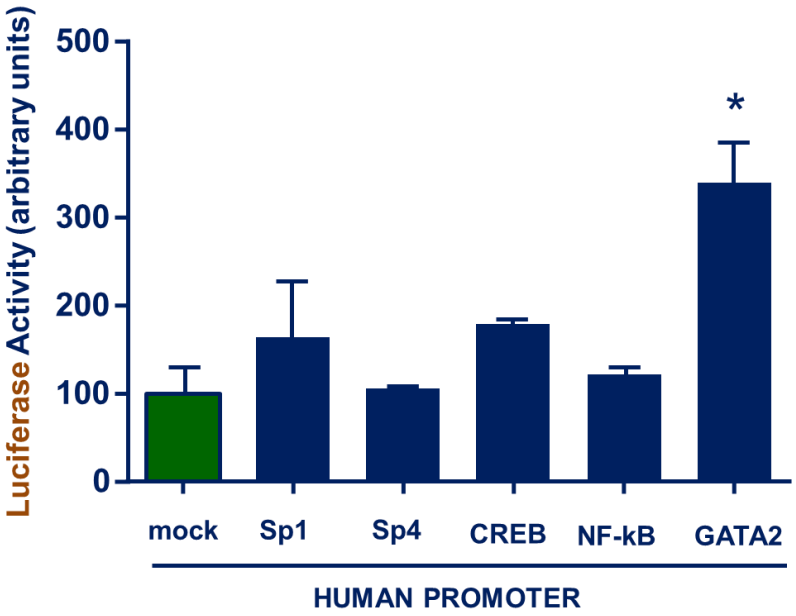

*Fig. 29. Luciferase activity of the human NCX2 promoter (pGL3b-HP), expressed as percentage of mock, at 48h after the transfection of pN3 (mock), Sp1, Sp4, CREB, NF-kB and Gata2 in PC12 cell line. *, p<0.05 vs mock (n=3 in one session; ANOVA followed by Turkey's test).*

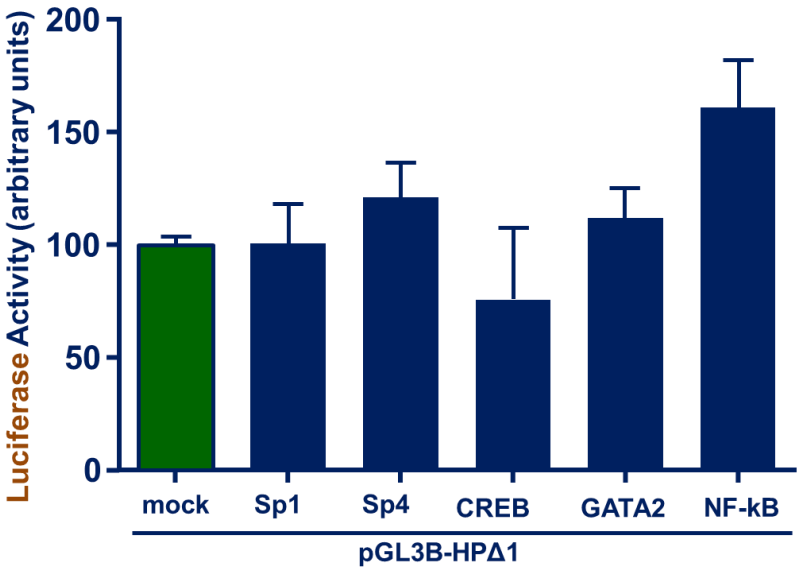

Fig. 30. Luciferase activity of the deletion mutant of the human NCX2 promoter (pGL3-HPΔ1), expressed as percentage of mock, at 48h after the transfection of Sp1, Sp4, CREB, GATA2 and NFkB in U87 cell line. (n=3 in one session; ANOVA followed by Turkey's test).

4.3 TFs regulating the transcription of the endogenous NCX2 gene in PC12 and U87 cell lines

All transfected plasmids significantly increased the transcription of its respective TF in both PC12 and U87 cells. In addition, transfection of either CREB, Sp1 or Sp4 also enhanced the transcription of the endogenous NCX2 gene in PC12 cells (Fig. 31). On the other hand, Sp1 (Fig. 32), Sp4 (Fig. 33), CREB (Fig. 34) and Gata2 (Fig. 36) failed to upregulate the expression of the endogenous NCX2 gene in U87 cells. By contrast, NFkB1 displayed an inhibitory effect on the transcription of the endogenous NCX2 gene in U87 (Fig. 35) and a stimulatory effect on the transcription of NFkB1-target gene, MMP2.

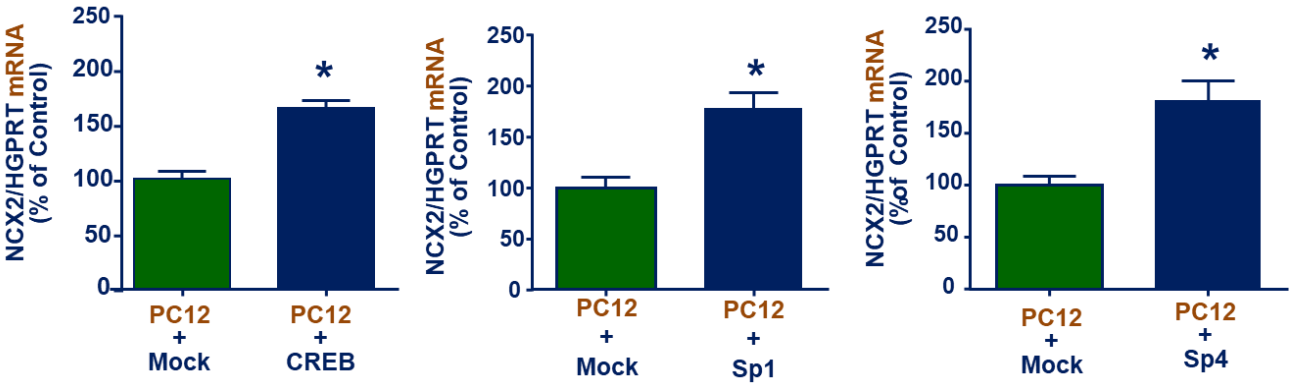

*Fig. 31. Expression level of NCX2 mRNA, normalized for HGPRT signal and expressed as percentage of mock, at 48h after the transfection of CREB, Sp1 or Sp4 in PC12 cell line. *, $p<0.05$ vs respective mock (n=9 in three independent sessions; Student' t-test).*

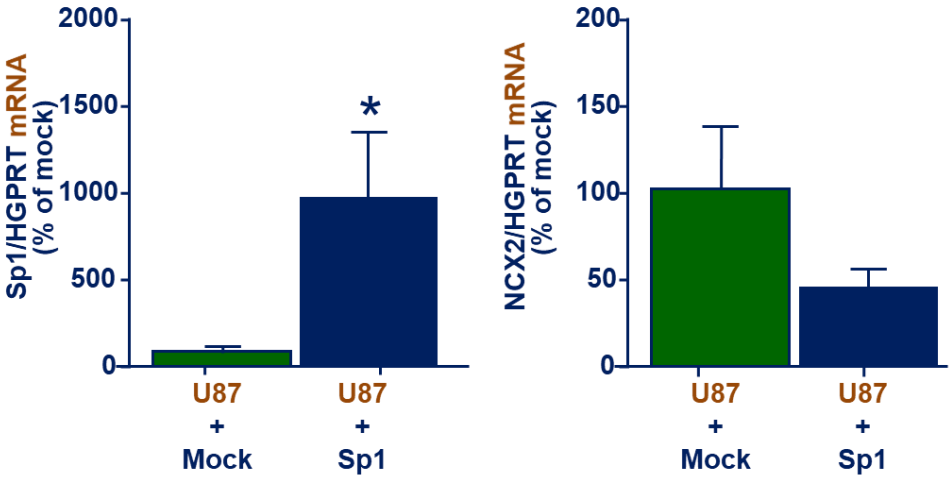

*Fig. 32. Expression levels of Sp1 (on the left) and NCX2 (on the right) mRNA, normalized for HGPRT signal and expressed as percentage of mock, at 48h after the transfection of Sp1 in U87 cell line. *, p<0.05 vs respective mock (n=4 in one session; Student' t-test).*

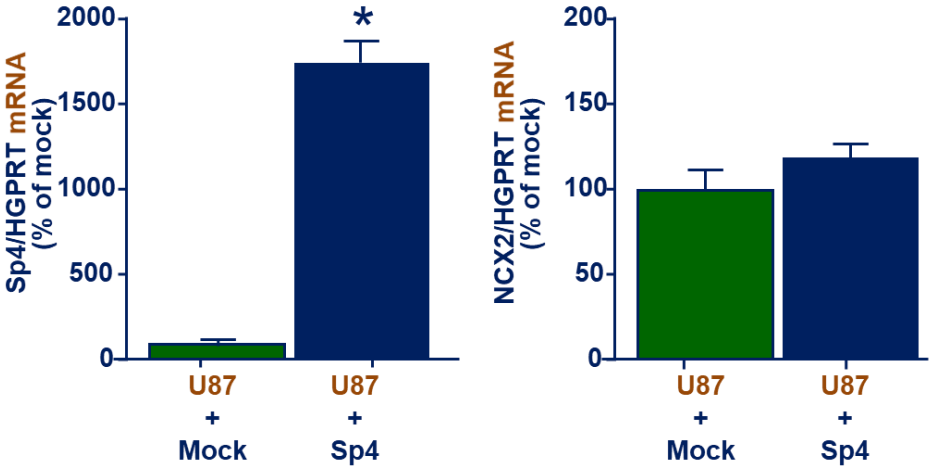

*Fig. 33. Expression levels of Sp4 (on the left) and NCX2 (on the right) mRNA, normalized for HGPRT signal and expressed as percentage of mock, at 48h after the transfection of Sp4 in U87 cell line. *, p<0.05 vs respective mock (n=4 in one session; Student' t-test).*

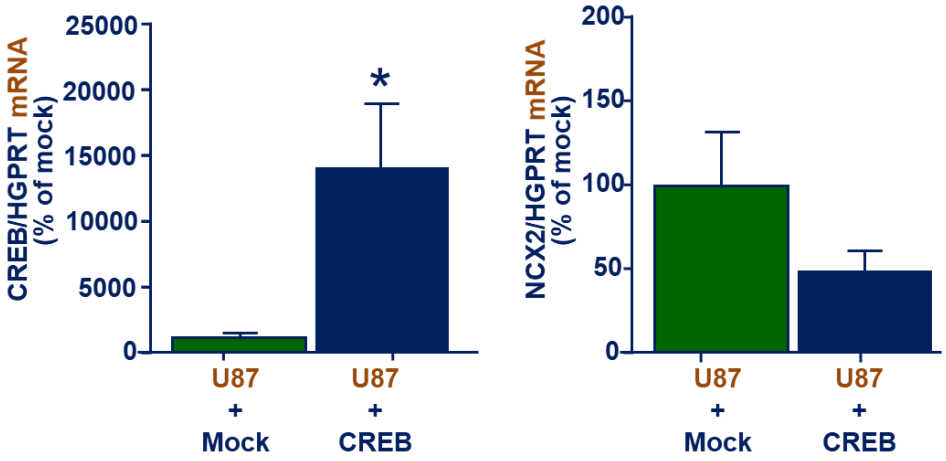

*Fig. 34. Expression levels of CREB (on the left) and NCX2 (on the right) mRNA, normalized for HGPRT signal and expressed as percentage of mock, at 48h after the transfection of CREB in U87 cell line. *, p<0.05 vs respective mock (n=4 in one session; Student' t-test).*

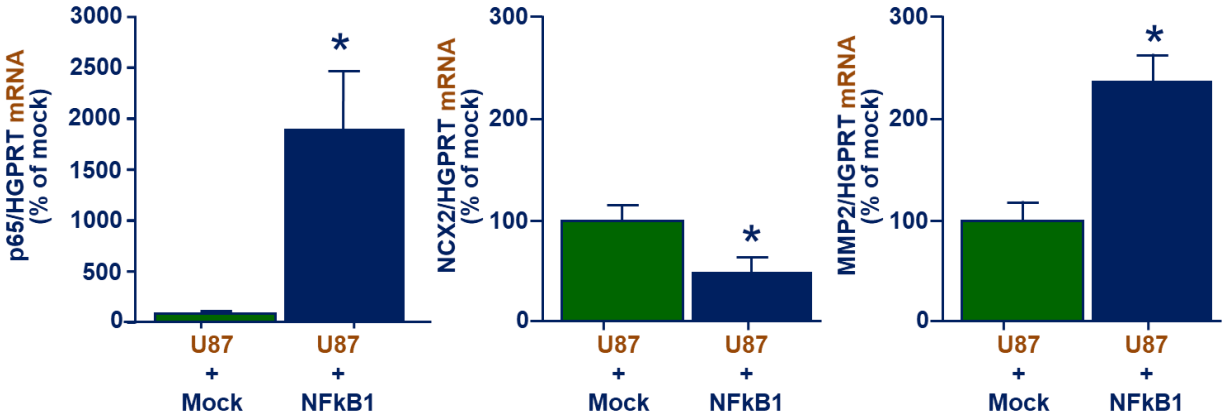

*Fig. 35. Expression levels of p65 (on the left), NCX2 (in the middle) and MMP2 (on the right) mRNA, normalized for HGPRT signal and expressed as percentage of mock, at 48h after the transfection of NFkB1 in U87 cell line. *, p<0.05 vs respective mock (n=13 in 4 independent sessions; Student' t-test).*

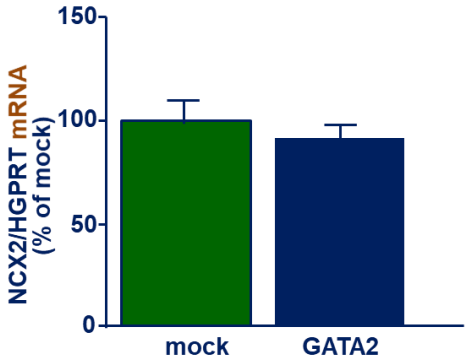

Fig. 36. mRNA expression levels of NCX2, normalized for HGPRT and expressed as percentage of mock, at 48h after GATA2 transfection in U87 cell line. (n=4 in one session; Student' t-test).

4.4 Epigenetic Analysis of human NCX2 promoter in U87 and SHSY cells

We analyzed the methylation status of the human NCX2 promoter in U87 cells, expressing low levels of *Slc8a2* gene, and the human SHSY-5Y cells, expressing higher levels of the antiporter. In particular, bisulfite assay showed several differences in the methylation status between genomic DNA of U87 and SHSY-5Y cells (Fig. 37). In particular, the region corresponding to the CREB binding site (Fig. 38) is completely methylated in SHSY-5Y cells whereas it is quietly methylated in U87 cells. On the other hand, regions corresponding to the Sp1/Sp4 did not show differences in the methylation status (Fig. 40 and Fig. 41). In addition, among TFs modulating NCX2 promoter activity the enhancer GATA2 appeared downregulated, whereas the transcriptional NCX2-repressor NFkB1 appeared upregulated, in U87 as compared to SHSY-5Y (Fig. 39).

More important, 5 μM of the demethylating agent 5-aza-2′-deoxycytidine (AZA) significantly increased the expression of endogenous NCX2 mRNA at 72h and 96h as compared to vehicle in U87 cell line (Fig. 42).

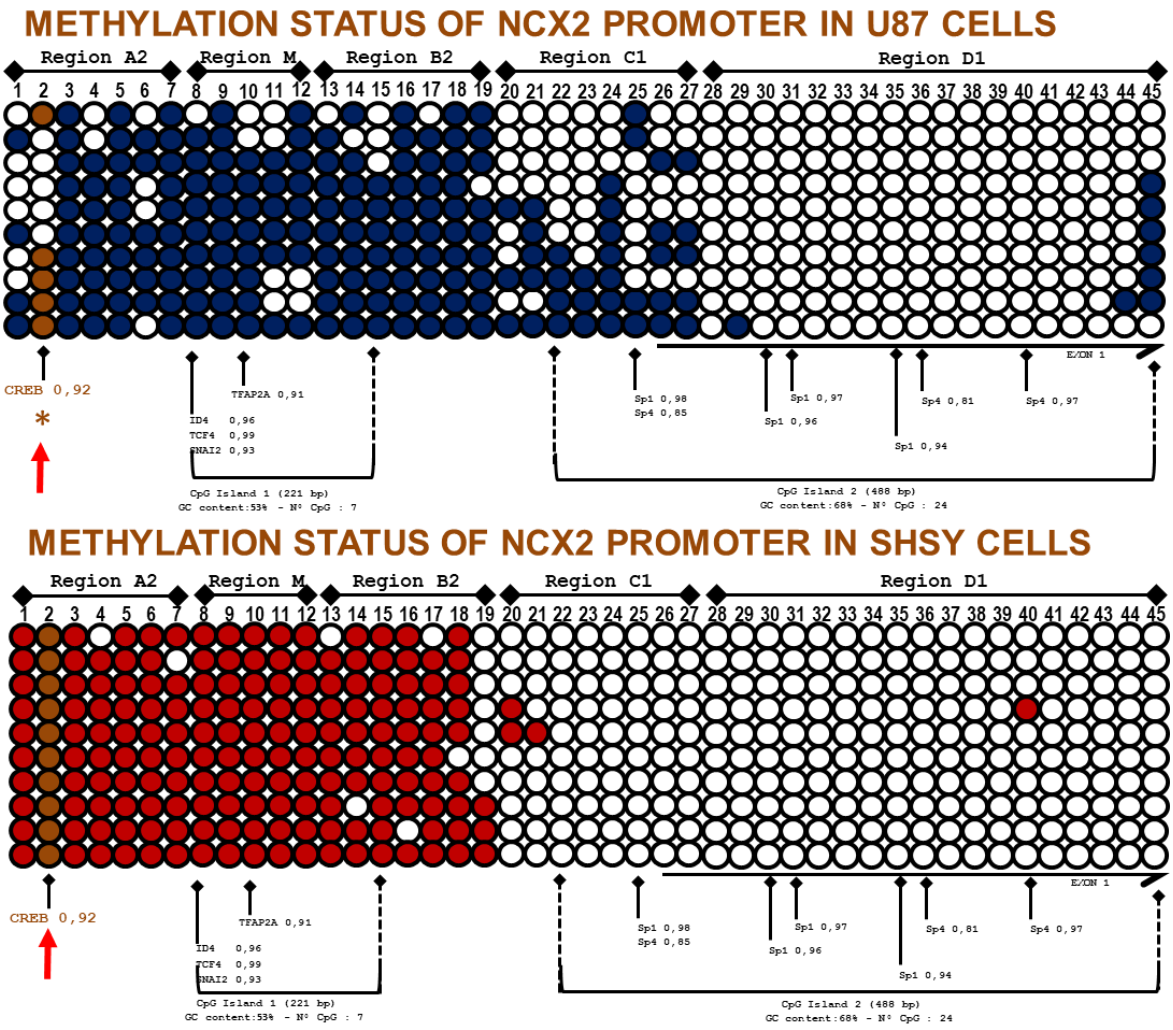

Fig. 37. Methylation status of 10 sequences of the human NCX2 promoter in U87 (up) or SHSY-5Y (below) cells. Filled circle corresponds to a methylated CG sequence, open circle corresponds to an unmethylated CG sequence. Arrow shows the CG site on CREB binding motif.

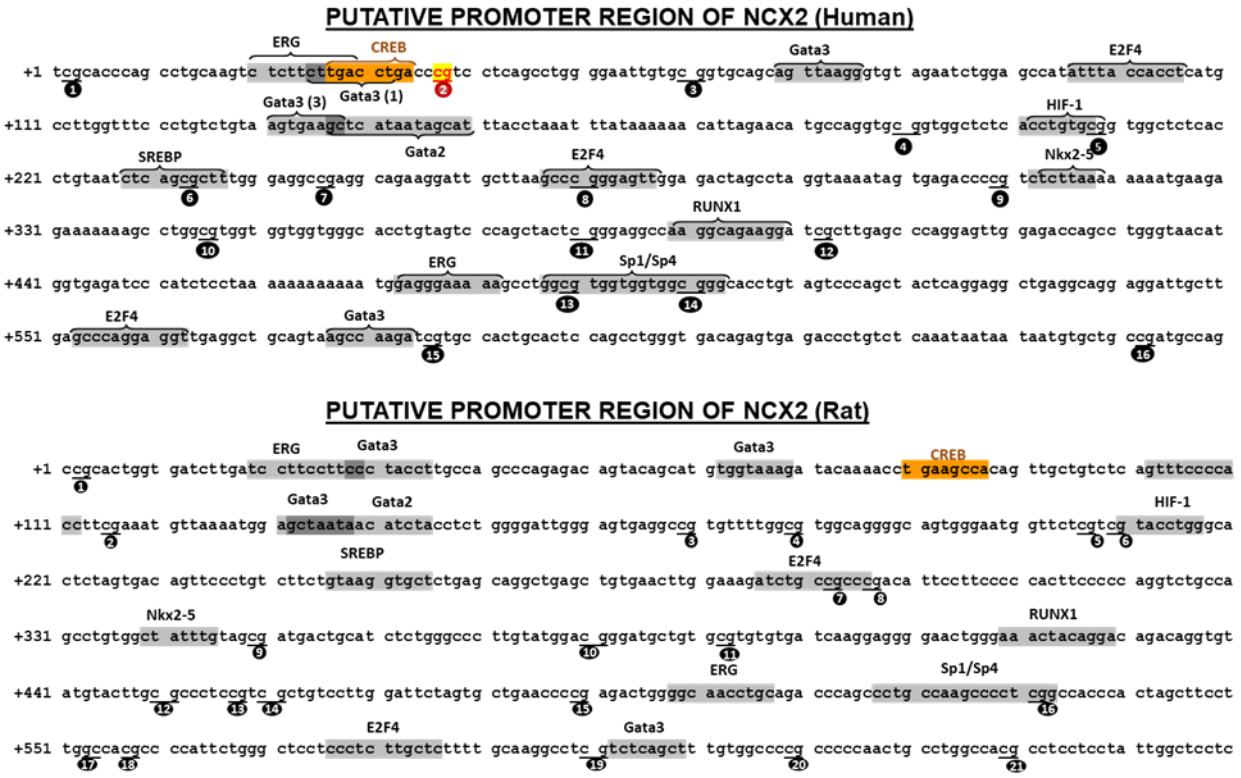

Fig. 38. Sequences of the first part of the human and rat NCX2 promoter. The putative CREB binding site (highlighted in orange) overlaps to the second CG methylation site, whereas it does not occur in the rat orthologous sequence.

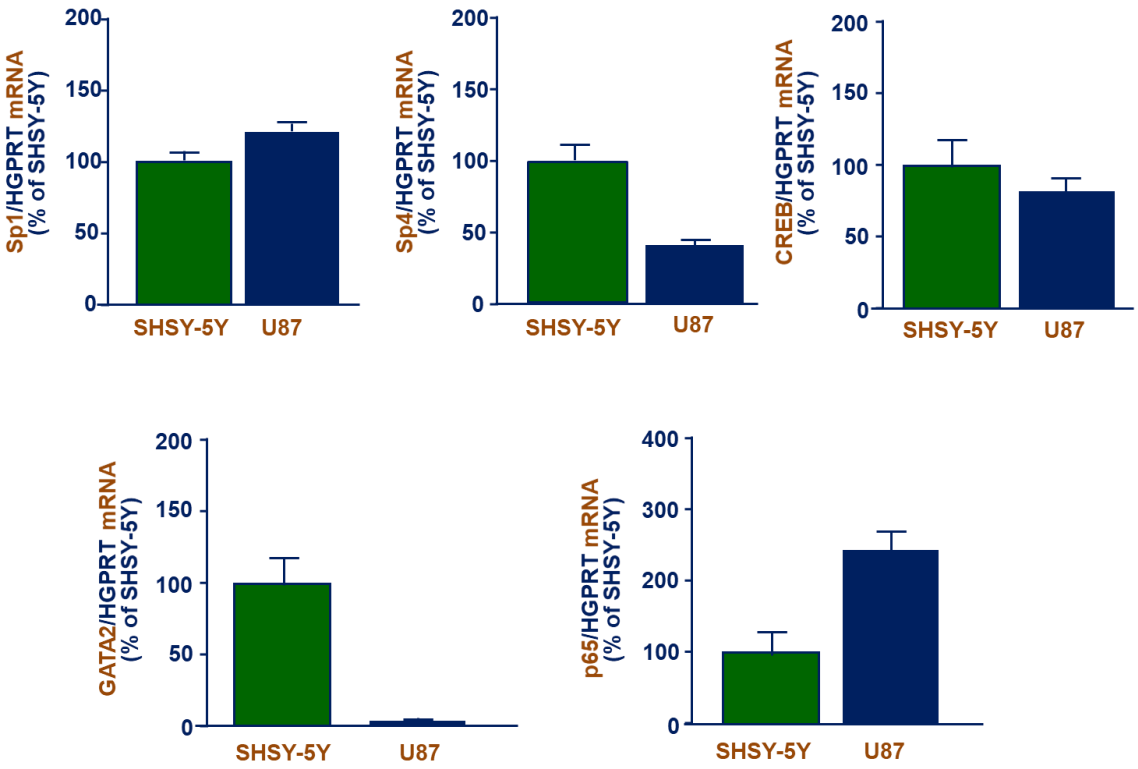

Fig. 39. Relative expression of Sp1, Sp4, CREB, Gata2, and p65 mRNA in human cell lines SHSY-5Y and U87. (n=4 in one session).

PUTATIVE PROMOTER REGION OF NCX2 (Human)

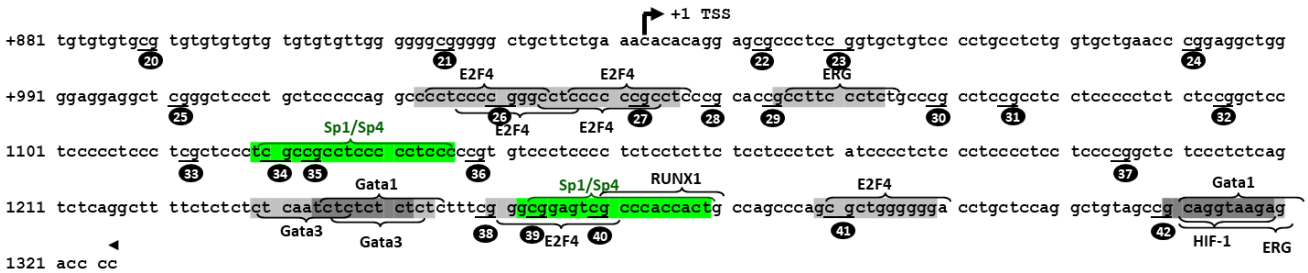

Fig. 40. Sequences of the first part of the human NCX2 promoter. The putative Sp1/Sp4 binding sites (highlighted in green) overlaps to the CG methylation sites 34, 35, 38, 39, and 40.

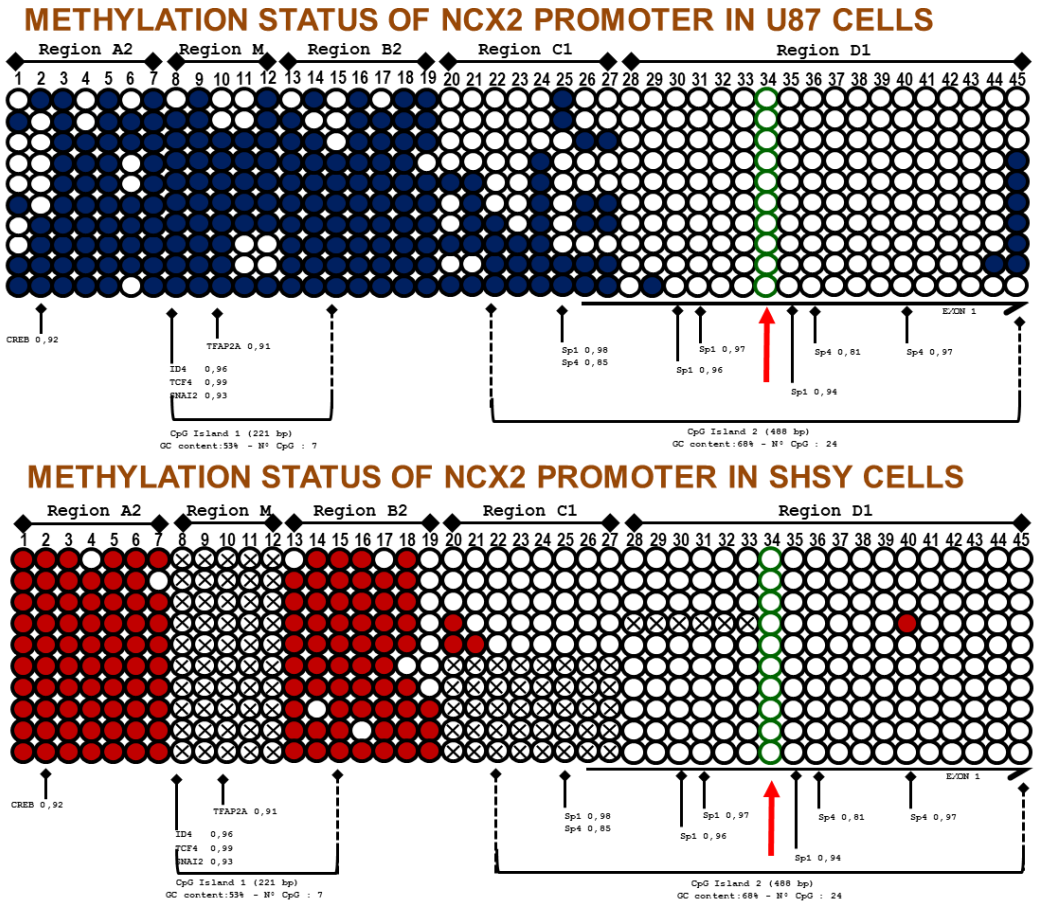

Fig. 41. Methylation status of 10 sequences of the human NCX2 promoter in U87 (up) or SHSY-5Y (below) cells. Filled circle corresponds to a methylated CG sequence, open circle corresponds to an unmethylated CG sequence. Arrow shows the CG site 34 on Sp1/Sp4 binding motif.

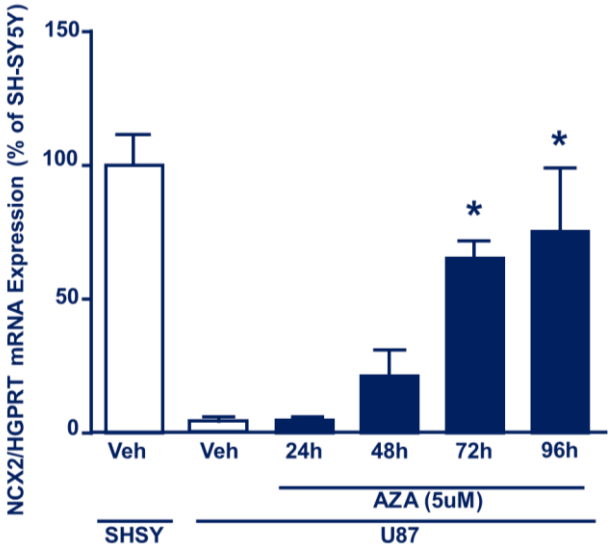

*Fig. 42. Expression level of NCX2 mRNA, normalized for HGPRT signal and expressed as percentage of non-treated SHSY-5Y, at 24h, 48h, 72h and 96h of 5 μM 5-aza-2'-deoxycytidine treatment in U87 cell line. *, $p<0.05$ vs U87+Vehicle.*

4.5 Drugs enhancing NCX2 expression or activity inhibit U87 cell growth

The expression levels of the three antiporter isoforms were preliminary evaluated by a quantitative PCR in U87 cells as compared to SHSY-5Y cell line. In particular, according with literature, NCX2 and NCX3 showed a very low levels of mRNA in U87 cell line, while NCX1 appeared less downregulated as compared with SHSY-5Y cells (Fig. 43). In addition, both neuronunina-1 and CN-PYB2 compounds were tested to increase NCX activity in forward and reverse modes of operations in U87 cells as measured by single cell path-clamp electrophysiology (Fig. 44).

Ten micromolar of the NCX2-upregulator, BMS-345541, decreases U87 cell growth at 48h, 72h, and 96h (Fig. 45). Moreover, incubation of either neurounina-1 or CN-PYB2 hampered U87 cell growth and increased cell death in a concentration-dependent manner with an IC50 of 100 μM for neurounina1 and 300μM for CN-PYB2, whereas were ineffective in PC12, BHK and SHSY cell lines (Fig. 46). In addition, transfection of either NCX1 or NCX2 increased the inhibitory effects of 10 nM neurounina-1 on U87 cell growth (Fig. 48).

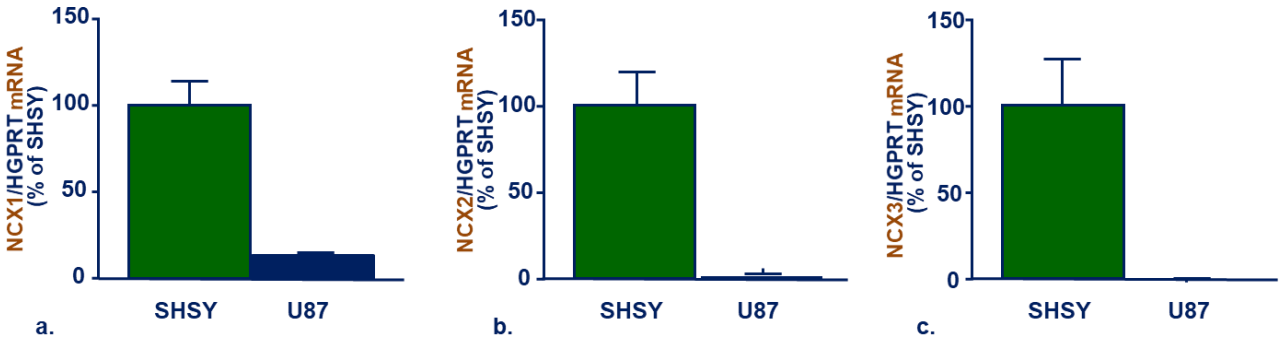

Fig. 43. Expression level of NCX1(a.), NCX2 (b.) and NCX3 (c.) mRNA, normalized for HGPRT signal and expressed as percentage of SHSY-5Y, in U87 and SHSY-5Y cell lines under basal conditions. (n=4 in one session).

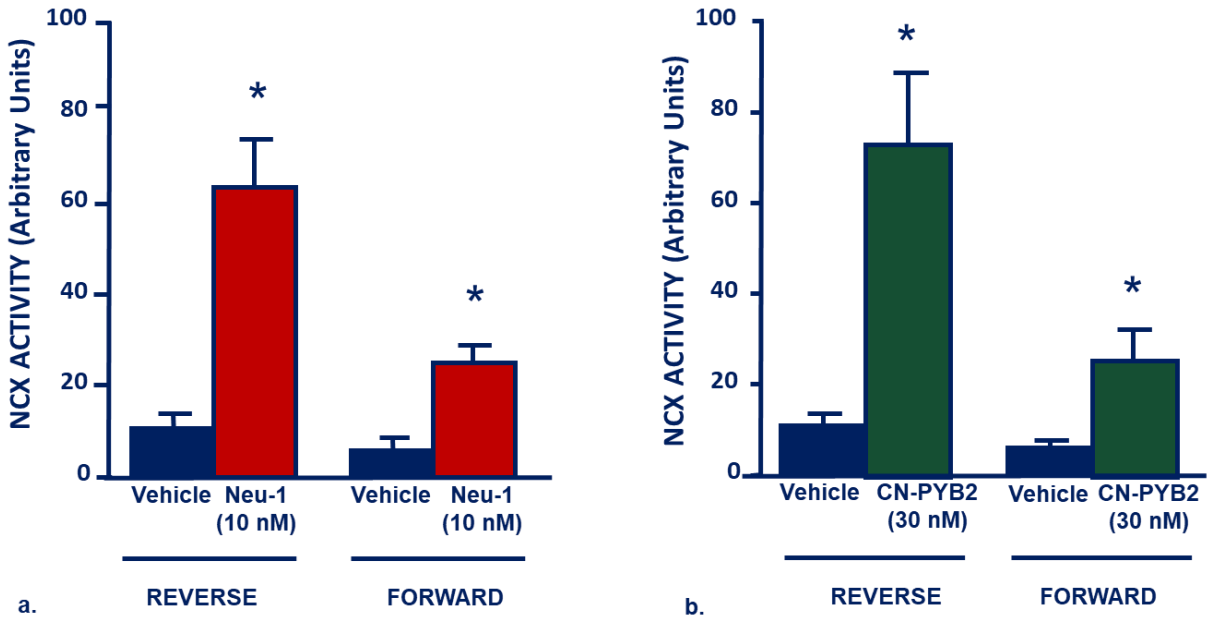

*Fig. 44. Neurounina-1(a.) and CN-PYB2 (b.) effect on NCX activity by electrophysiology experiments in U87 cell lines expressed as arbitrary units. *, p<0.05 vs respective Vehicle. (n=4 in two independent sessions; Student' t-test).*

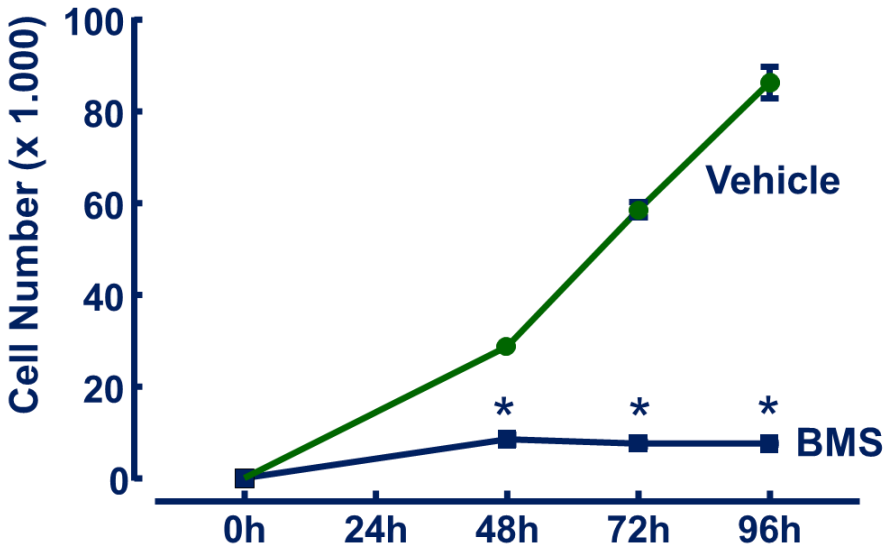

*Fig. 45. Cell growth of U87 over the time in the presence or in the absence of 10 μM BMS. *, p<0.05 vs vehicle (two-way ANOVA followed by Turkey's post-hoc test).*

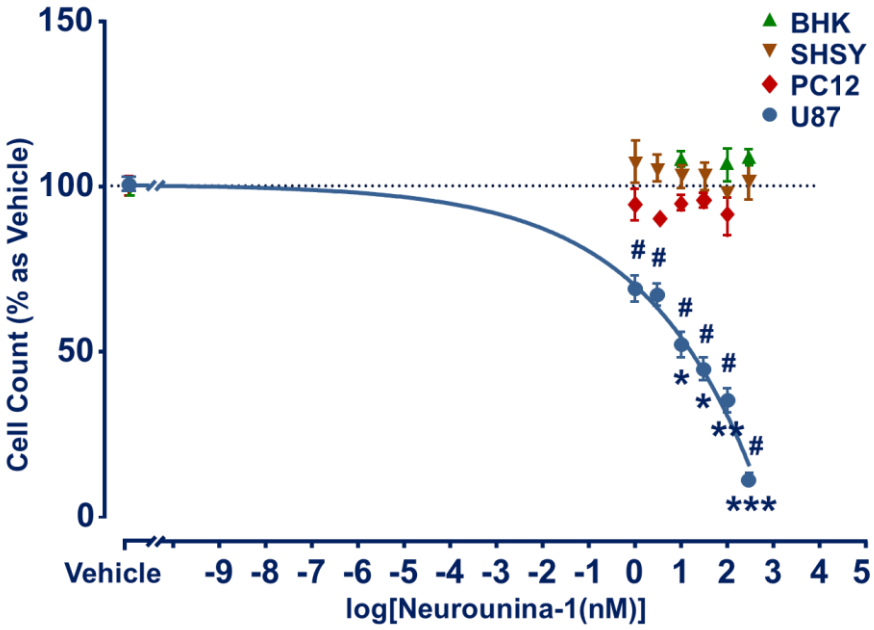

*Fig. 46. Neurounina-1incubation effect on cell growth of BHK, SHSY-5Y, PC12 and U87 cell lines. #, p<0.05 vs other cell lines; *, p<0.05 vs 1nM; **, p<0.05 vs 3nM; ***, p<0.05 vs 30nM. (two-way ANOVA followed by Turkey's post-hoc test).*

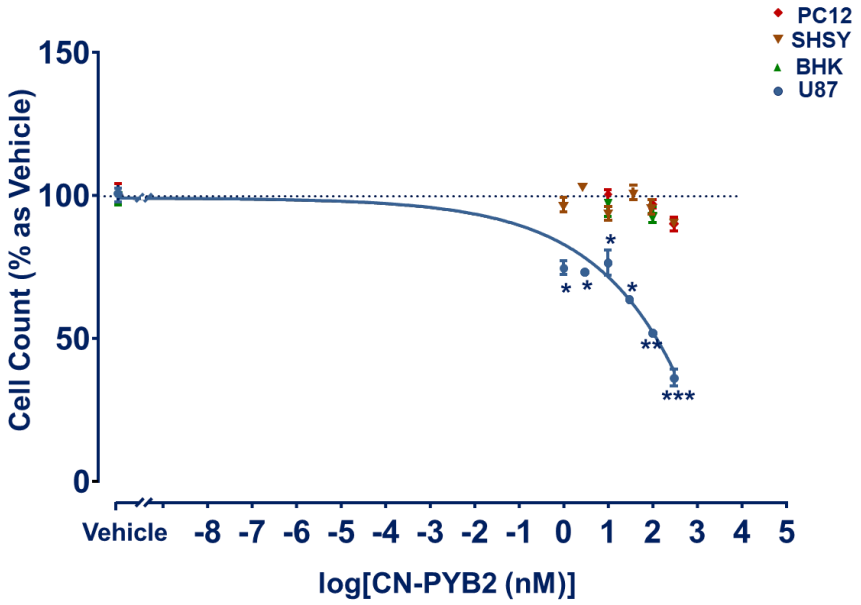

*Fig. 47. CN-PYB2 incubation effect on cell growth of BHK, SHSY-5Y, PC12 and U87 cell lines. #, p<0.05 vs other cell lines; *, p<0.05 vs 30nM; **, p<0.05 vs 100nM. (two-way ANOVA followed by Turkey's post-hoc test).*

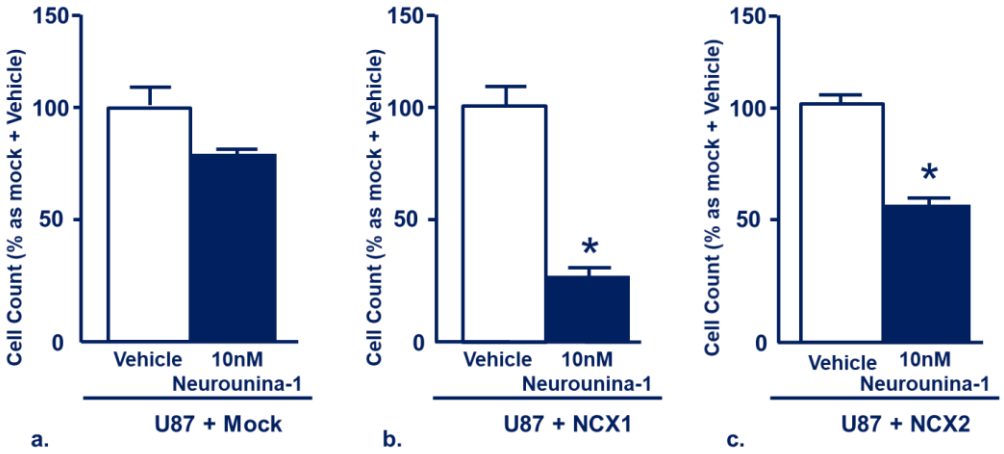

*Fig. 48. Effect of neurounina-1 incubation on cell growth of U87 cell lines transiently transfected with Mock (a.); NCX1 (b.) or NCX2 (c.). *, p<0.05 vs respective Vehicle (n=6 of two independent sessions; Student's t-test).*

Chapter 5: Discussion

Data presented in this thesis reported the characterization of the human NCX2 promoter gene and the molecular determinants of its silencing in glioblastoma cells. More important, results obtained could represent the basis for new therapeutic strategies aimed to increase NCX2 expression and/or activity in glioblastoma to slow-down tumor cell growth.

The main finding of this thesis was the identification of NF-kB1 as the EGFR downstream transcription factor responsible for NCX2 silencing in glioblastoma. In fact, NFkB1 was able to downregulate the transcription of the endogenous NCX2 gene whereas its inhibition displayed an increase in the endogenous NCX2 transcription. Furthermore, inhibition of EGFR pathway at different levels also resulted in an increase of the endogenous NCX2 mRNA expression. However, this drug-related increase of NCX2 expression was ineffective after the transfection of the downstream TF of EGFR pathway, NFkB1.

To deeper investigate the mechanisms underlying NCX2 silencing in glioblastoma, the human NCX2 promoter was identified in the intergenic DNA region between *kptn* and *slc8a2* genes and cloned in a luciferase reporter plasmid, named pGL3b-HP. Notably, this promoter construct displayed a pattern of luciferase activity comparable to that observed in rat NCX2 promoter and, more important, comparable to the respective levels of mRNA expression of NCX2 in BHK, PC12, U87, and SHSY. These results reinforced the hypothesis that pGL3b-HP contains some of the main regulatory mechanisms present in the endogenous NCX2 promoter. In particular, pGL3b-HP was not active in rodent BHK cells that do not endogenously express NCX2 but displayed low activity in cells expressing minimal amount of NCX2 as U87, and showed high activity in cells endogenously expressing high levels of NCX2 as SHSY5Y and PC12. Notably, these regulatory properties seemed to be not affected

by specie differences under control conditions. More important, the EGFR inhibitor, erlotinib, displayed to enhance the pGL3b-HP luciferase activity as it occurred for the transcription of the endogenous gene. These data further reinforce the reliability of the cloned promoter and the hypothesis that EGF pathway is involved in the transcriptional downregulation of NCX2 in glioblastoma cells. Moreover, the transfection of the downstream effector of EGF-R pathway, NFkB1, reversed the drug-related enhancement of pGL3b-HP activity, thus showing that this TF prevented the effects of EGF-R pathway inhibition and thus it may represent the main determinant for NCX2 gene silencing in glioblastoma. In fact, *in silico* analysis of the promoter sequence revealed several conserved putative binding sites for several transcription factors, including NFkB1. Interestingly, among these conserved TFs, only NFkB1 was able to inhibit both pGL3b-HP luciferase activity and the endogenous NCX2 transcription in U87 cells. By contrast, CREB and GATA2 were able to enhance pGL3b-HP but failed to increase the endogenous NCX2 transcription. Notably, either CREB or GATA2 enhanced the transcription of the endogenous NCX2 gene in PC12 cells. On the other hand, glioblastoma cells did not show relevant levels of GATA2 under basal conditions, and possibly lacks cofactors needed for NCX2 gene upregulation. Altogether, these data suggested an upstream mechanism of gene silencing of NCX2 in glioblastoma cells. These results appear of interest, since, CREB activity has been positively correlated with glioblastoma proliferation (Daniel et al. 2014, Daniel et al. 2018), whereas NCX2 was negatively correlated (Qu et al. 2017). Since the obtained results, it is conceivable to hypothesize that the methylation status of the CREB binding site on the human NCX2 promoter could be responsible for its refractoriness from CREB transfection/activity in U87 cell line. In addition, human and rat NCX2 promoters display differences in the localization of the CREB binding site and in the overlapping with an CG sequence, and this might explain why there are several differences in the sensitivity to

CREB between these two sequences. In support of this hypothesis, *slc8a2* gene, encoding for NCX2, is highly methylated in glioblastoma tissues as compared with normal tissues (Qu et al. 2010) as it occurs in the transcriptional repression of antioncogenes in several types of gliomas (Qu et al. 2017). Furthermore, the demethylating agent 5-aza-2′-deoxycytidine (AZA) caused an increase in the expression of endogenous NCX2 mRNA in U87 cell line. These results further support the idea that there is an epigenetic regulation mechanism responsible for shutting down NCX2 gene and further demonstrates that antineoplastic drugs increase NCX2 gene expression.

Another aspect deserving attention is that the transfection of either CREB, GATA2 or NFkB1 did not affect the activity of the deletion mutant, pGL3b-HPΔ1-376, lacking the region containing these putative binding sites, thus showing that these TFs exert their effect by a direct binding on the first part of NCX2 promoter. Interestingly, NFkB1 failed to affect pGL3b-HP luciferase activity in PC12 cells suggesting that these cells were provided with a different regulatory transcriptional mechanism as compared with U87 cells. Consistently, NFkB1 stimulated the luciferase activity of the ortholog rat NCX2 promoter in U87 cells.

Another aspect that deserves attention is that drugs directly or indirectly increasing NCX2 expression/activity showed to slow-down glioblastoma cell growth. In particular, neurounina-1 or CN-PYB2, stimulating both NCX1 and NCX2 or selectively stimulating NCX1, respectively, largely increased the total amount of NCX activity in U87 cell line, as measured by patch-clamp technique in whole cell configuration, and specifically decreased cell growth of U87 in a concentration-dependent manner with an IC_{50} of 100 μM for neurounina-1 and 300 μM for CN-PYB2. Similar results were obtained with the transient overexpression of either NCX1 or NCX2 in U87 cells. Moreover, BMS-345541 inhibited NFkB expression/activity, increased NCX2 expression/activity and blocked cell growth. According

with these results Qu *et al* found that U87 cells stable transfected with NCX2 display a reduced rate of tumor growth in mice (Qu et al. 2010). For these reasons, it is conceivable to hypothesize that NFkB, a pivotal player for glioblastoma proliferation and invasion (Li et al. 2007, Gray et al. 2014), exerts part of its activity also by silencing NCX2 gene.

Altogether, these results suggest that there are some molecular determinants on NCX2 promoter headed by NFkB1 and EGFR pathway that, by genetic and epigenetic mechanisms, prevent NCX2 expression in glioblastoma. In addition, NCX2 might represent an oncosuppressor gene whose increased expression/activity might be a useful pharmacological strategy to hamper cell growth and invasiveness of glioblastoma. Of course, additional experiments should be performed to evaluate if this approach could represent a valuable strategy to treat glioblastoma *in vivo*. In this perspective, the effects of drugs overexpressing NCX2 by inhibiting EGFR-NFkB1 pathway will be tested in xenograft models of glioblastoma by using zebrafish and/or mice models.

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