Universidad de Navarra

Facultad de Ciencias

Transcriptional networks controlled by SOX2 in glioblastoma stem cells

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“Transcriptional networks controlled by SOX2 in glioblastoma stem cells”

Memoria presentada por Dª Arlet María Acanda de la Rocha para aspirar al grado de Doctor por la Universidad de Navarra

El presente trabajo ha sido realizado bajo mi dirección en el Departamento de Pediatría y autorizo su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, 5 de julio de 2016

Dra. Marta María Alonso Roldán **Dr. Salvador Martín Algarra**

Este trabajo ha sido realizado con la ayuda de una beca concedida por:
"**Amigos de la Universidad de Navarra**"

A mi ángel de la guarda, mi Tata

A Rafa, por la vida compartida

A mis padres, por tanto amor

Agradecimientos

"Aprendí pronto que al emigrar se pierden las muletas que han servido de sostén hasta entonces, hay que comenzar desde cero, porque el pasado se borra de un plumazo y a nadie le importa de dónde uno viene o qué ha hecho antes."

— Isabel Allende

"No se extraña un país, se extraña un barrio en todo caso, pero también lo extrañas si te mudas a 10 cuadras. El que se siente patriota, el que cree que pertenece a un país es un tarado mental, la patria es un invento. Tu país son tus amigos, y esos si se extrañan, pero se pasa..."

—Fragmento de la película argentina Martín (Hache), dirigida por Adolfo Aristarain y estrenada en 1997

Quiero comenzar mis agradecimientos con estas dos grandes frases que han marcado los últimos 5 años de mi vida. Todavía recuerdo como si fuera ayer el día en que me despedí de mi familia y de mis amigos, de mi barrio y de mi casa, para emprender rumbo a España detrás de un sueño. Hoy mi sueño se ha cumplido (por fin!!) y ha sido gracias a innumerables personas e instituciones, que no puedo dejar de mencionar en esta página.

Primero quiero agradecerle a la Universidad de Navarra por abrirme las puertas y admitirme en el Programa de Doctorado en Investigación Biomédica, con el apoyo de la Asociación de Amigos de la Universidad de Navarra (ADA) que me ha sostenido económicamente durante estos 4 años de doctorado. Quiero agradecer públicamente a todas las personas, empresas e instituciones que cada año donan dinero a ADA, para que personas como yo puedan beneficiarse de becas para investigación doctoral.

Dicho esto, no puedo pasar por alto mi agradecimiento a la Agencia de Cooperación Internacional para el Desarrollo (MAEC-AECID), por haberme concedido mi primera beca de estudios para cursar el máster en la Universidad de Cantabria.

La primera persona a la que quiero agradecer todos estos años es a mi directora de tesis, Marta, sin ti nada de esto hubiera sido posible. Gracias por haberme dado la oportunidad de trabajar en tu grupo y por haberme apoyado en todo. Gracias por tu dedicación, por tu confianza, por tus consejos y enseñanzas, por tus ánimos, por tu ejemplo, por tu amistad y cercanía y por haberme engrosado las alas para volar. Ya ves que no existen palabras

suficientes para agradecer todo lo que has hecho por mí durante todo este tiempo y lo que has significado en mi vida. He sido dichosa por tener la mejor tutora que alguien puede desear: GRACIAS, GRACIAS y GRACIAS!!!

Gracias a todos mis compañeros del grupo BTBT, los que ya no están y los de nueva incorporación: Marisol, Naiara, Patri, Bea, Marc, Javi y Enric. De todos me llevo lo mejor, gracias por compartir las risas, y haber sido siempre un punto de apoyo en este largo trayecto de 4 años. Hemos compartido desde la organización de carreras solidarias y competencias de pádel para recaudar fondos para nuestra investigación, hasta el estrés del laboratorio cuando los experimentos no salen como esperamos, y nos ponemos hombro con hombro a darnos ánimo. Gracias por haberme permitido crecer con todos vosotros y formar juntos un equipo. También tengo que mencionar a Fernando, Elena y Mario, con quienes compartimos laboratorio, poyatas e incluso a veces hasta pipetas (eso va por ti Helen!!); gracias por el clima jovial y de compañerismo que mantenemos en el labo, gracias por la interesante retroalimentación, por las nuevas ideas y los buenos deseos; y muy especialmente gracias a Elena por nuestras conversaciones de política, familia, hijos y viajes y por el apoyo y el cariño que siempre he sentido!

No puedo pasar sin mencionar antes muy especialmente a Marisol, el alma de nuestro grupo. Gracias por sacarme siempre una sonrisa cada día, por tus enseñanzas, por tu apoyo y tu inmensa generosidad. Gracias por las risas, las copas y también las lágrimas. Gracias por saber que puedo contar contigo, no hasta dos o hasta diez, sino que puedo contar contigo, y sobre todo, porque es lo más importante, gracias por tu amistad!

He tenido la oportunidad de conocer a muchas personas durante estos 4 años en el CIMA-UNAV, a las cuales les quiero agradecer porque de una manera u otra han ayudado a que este proyecto saliera adelante, dando ideas, tendiendo manos, compartiendo conocimientos, regalando sonrisas. Gracias a Carolina Matute por ayudarme a hacer magia con los tiempos, a "Lola" por ser tan cercana y ayudarme en todo, a las chicas de Morfología, a Ainhoa y Cristina en Imagen, a Eukene en el TEM, y a Elizabeth de Bioinformática y a Diego e Idoia de Citometría por vuestro apoyo y dedicación.

De estos 4 años me llevo también una gran amiga, Yolandita, la cual no puedo dejar de mencionar. Gracias Yoli por ser como una hermana, por acompañarme en los mejores y peores momentos, por compartir casa y comida, risas y enseñanzas, consejos y esperanzas; y porque aún en la distancia sigues estando muy cerca. Gracias por abrirme las puertas de Donosti y hacerme sentir como en casa cuando estoy contigo, ahora te toca a ti venir a Cuba!!!

A los chicos del departamento de Microbiología en la UNAV, en donde impartí prácticas como PIF durante los 4 años de mi doctorado. Gracias muy especialmente a Carlos por aceptarme desde el principio y confiar en mí, por tu apoyo y cariño, por tus locas propuestas

que siempre terminaban en hacer las clases mucho más interesantes y diferentes; a mis compis de prácticas: Ana, Miriam, Yadira, Andoni y Mario, por enseñarme todo lo que se de Microbiología, por vuestra paciencia y vuestro compañerismo, por las risas y por esos cafés detrás de cada clase para recargar las pilas. Gracias a todos vosotros disfruté mucho la experiencia de enseñar y de todos vosotros me llevo el mejor de los recuerdos!

Agradecer a mis antiguos compis de labo en Santander: David y María, por haberme acogido desde el primer día que llegué. Gracias chicos por seguir estando en mi vida a pesar del tiempo y la distancia, gracias por haberme apoyado siempre, aún en mis momentos más duros, gracias por formar piña conmigo y porque sé que el día de la defensa de esta tesis estaréis cruzando los dedos por mí. Ahora que ya acabé (no me lo creo!!) tenemos que organizar esa visita pendiente a Galicia para que María nos enseñe las bellezas de su tierra!! A donde sea que vaya, ya sabéis que tenéis vuestra casa también!

And of course I have to thanks (and I am writing this in English just for their comprehension) to the entire group of Dr. Laterra in Kennedy Krieger Institute (Baltimore, USA), where I stayed for 3 months learning new ideas and techniques and developing an important part of this project. I want to especially thank to Dr. Laterra for admitting me in your group from the very first email, for the warm welcome, for your dedication and interest to my project, for your enthusiasm and support during the 3 months, for helping me to ask the right questions and experimental directions and for let me learn from your intelligence and experience in the field of glioblastoma. Thanks for organizing the Christmas lunch with everyone to give me a proper send-off; I have to say that it was the best send-off I have ever had! I have to mention to the incredible Dr. Lal, the heart of the group; I want to thank you for leave me your benchtop space, for your wise advices, for make me feel part of the group and taking care of me in Baltimore, and for organizing the many happy hours on Fridays that we all enjoyed. I have to say that you touch my heart with your special samosas you brought to the happy hour of the last Friday I worked there: Thanks for everything! I also want to thank to Larry, "the man who knows everything", for let me be your friend, for put all your effort to show me the best places in Baltimore/Maryland, for organize gastronomic hangouts and of course for take me to the best place where you can eat the best Baked Alaska in Baltimore (thanks for that!!!), to Tobi for the positive connection, the interesting talks, the smiles and for teaching me to do the typical sound of Baltimoreans used for affirmative expressions (ujum!!), although I think I will need more practice, to Han Sun for the shared mirth, and to Yunqing Li, Shuli, Min Li and Mingyao for accepting me and making me feel so comfortable in the lab.

Pero, sin duda alguna la persona que más se implicó en este proyecto y a la que por supuesto le tengo que agradecer su compromiso desde el principio, es a Hernando. Gracias por acogerme tan amablemente en el labo, por interesarte desde el principio de mi estancia en este proyecto, por enseñarme las nuevas técnicas y formar equipo conmigo compartiendo

tus conocimientos y consejos, por hacerme sentir parte del labo y del grupo, por tus ánimos y tu positividad todo el tiempo. Gracias a tí guardo en mi memoria esta estancia como una experiencia maravillosa que sin duda volvería a repetir. Gracias por todo!!!!!!

A Nayara y Paul, por compartir vuestra casa conmigo durante los 3 meses que estuve en Baltimore. Gracias por hacerme parte de vuestros planes y hacerme sentir parte de la familia, por no dejarme tiempo libre para la morriña, por llenarme de sorpresas el día de mi cumple y festejarlo conmigo de la mejor manera: bailando! Por esas noches de charlas filosóficas y tardes de "gym" para liberar tensiones, por enseñarme lo mejor de Baltimore, por compartir amigos y tantos otros buenos momentos vividos, y por ese regalo sorpresa que me disteis de despedida que llevaré siempre conmigo como recuerdo de una etapa inmejorable…pero sobre todo gracias por regalarme vuestra amistad! Os espero en Pamplona!!!!

Y dicho esto, por supuesto no puedo dejar de agradecer a mi gran familia de Miami, no solo de sangre sino también adquirida, por ser siempre un pilar importante en mi vida. A mi querida Bertha, por la preocupación constante desde siempre, por los mimos y los consejos, y por su empeño constante en alimentar mi memoria con la historia de mi familia paterna; a mi prima Maité por cuidarme como una hija y quererme tanto, por darme su ejemplo, por su enorme generosidad y por enseñarme que los sueños están para cumplirlos (y que gracias a ella he cumplido muchos de ellos!!); a mi primo David por sus maravillosas ocurrencias y por enseñarme que el cielo es el límite; a mis queridos Vitico y Pepin, que me han sacado las mejores carcajadas de mi vida y que demuestran cada día lo valiosa que son las amistades; a mis primos Daniela, Alexandra y Francesco, por quererme desde el primer momento que nos conocimos, por decorarme la habitación donde viví 3 meses en Baltimore, por enseñarme los mejores sitios de Washington, y sobre todo por toda la energía positiva que siempre regalan; a mi primo Jose por dejarme conocerle y enseñarme que la familia no solo es con la que nacemos, sino también la que vamos descubriendo con la vida; a Carmen y Toño, por hacerme sentir en familia desde el primer día que nos conocimos; y a mis maravillosas primas adquiridas: Betty, Cecilia, las dos Luisas, Elizabeth, sin ustedes los encuentros de domingo en "el farito" no son iguales!

A todos mis amigos del parque de Barañain con los que comparto el mismo amor por los perretes, especialmente a Aurora, Isabel, Josetxu y Alberto. Gracias chicos por compartir durante estos 4 años paseos perrunos, aventuras, comidas, y buenos momentos; gracias por regalarme vuestra amistad! Y por supuesto a mi compañera inseparable de cuatro patas, a mi Malucita, por regalarme tanta felicidad. Desde que llegaste a mi vida iluminas cada día con tu amor incondicional, sin pedir nada a cambio, dejando tu generosidad en el aire. Gracias por llenarme la vida con tanto amor!!!

A todos mis amigos de Cuba, mi "cuadrilla" de toda la vida, que siempre han estado presente de muchas maneras, apoyándome desde lejos y llenándome de ánimos en este largo camino, especialmente a Dali, Chiri y Yula. Gracias por acompañarme siempre!!

Y finalmente, y sin que ello signifique menos importante (todo lo contrario!) a mi familia que sin ella no estuviese hoy aquí. A mi esposo Rafael, por ser mi compañero de vida, en las buenas y en las malas, en la enfermedad y en la salud, y en todo lo que venga por delante. Gracias por acompañarme en todas las aventuras, por ser el tronco sobre el cual encuentro descanso y paz, por ponerte de almohada para el cansancio de cada día, por ser camino y ser escala, por ser la esperanza de la estrella, por amarme desde la raíz hasta la flor, por dibujar mis días con color de aurora. Gracias por tanto amor y felicidad juntos. Gracias por estos increíbles 8 años compartidos y por demostrarme que los sueños compartidos contigo saben a gloria!

A mis padres, por darme las alas necesarias para perseguir mis sueños y hacerlos suyos, por estar tan cerca a pesar de la distancia, por ser mi punto de apoyo necesario para sostenerme durante todo este tiempo, por darme la vida y la felicidad, por regalarme tanto amor, por ser la luz del faro que guía mi camino; y por ser los mejores padres que alguien puede tener! Y por supuesto a mi ángel de la guarda, mi Tata, porque aunque hace 3 años volaste hacia ese arcoíris que está en el cielo, te siento cada día a mi lado, dándome tu mano como solíamos hacer, protegiéndome y dándome fuerzas para seguir adelante. Gracias por llenarme mi corazón cada vez que te extraño demasiado; pero sobre todo, gracias por regalarme la esperanza de saber que, al final de mi camino, estarás tú esperándome para volver a estar juntas. Hoy por fin ha llegado el momento que un día soñamos todos juntos, y solo me queda dar mi agradecimiento eterno porque sin ustedes nada de esto hubiera sido posible! A vosotros, os debo todo!!!

GRACIAS!!!!

"Gracias a la vida que me ha dado tanto
Me dio dos luceros que cuando los abro
Perfecto distingo lo negro del blanco
Y en el alto cielo su fondo estrellado
Y en las multitudes el hombre que yo amo.

Gracias a la vida que me ha dado tanto,
Me ha dado el sonido y el abecedario,
Con él las palabras que pienso y declaro
Madre amigo hermano y luz alumbrando,
La ruta del alma del que estoy amando..."

Violeta Parra, 1966.

"No es verdad que las personas paran de perseguir sueños porque se hacen viejos, se hacen viejos porque paran de perseguir sus sueños."

Gabriel García Márquez

Abbreviations

5-ALA: 5-aminolevulinic acid

Ago2: Argonaute-2

AIC: 5-aminoimidazole-4-carboxamide

ASCL1: achaete-scute family bHLH transcription factor 1

ATRX: thalassemia/mental retardation syndrome X-linked

BBB: blood-brain barrier

BCL2: B-cell lymphoma 2

B-CLL: B-cell chronic lymphocytic leukemia

Bmi1: proto-oncogene, polycomb ring finger

BP: biological process

CC: cellular compartment

cDNA: complementary DNA

CD133: prominin-1

CDKN2A: cyclin-dependent kinase inhibitor 2A

C. Elegans: Caenorhabditis elegans

CHI3L1/YKL40: chitinase 3 like 1

ChIP: chromatin immunoprecipitation

c-Myc: v-myc avian myelocytomatosis viral oncogene homolog

CNS: central nervous system

CRNDE: colorectal neoplasia differentially expressed

CSCs: cancer stem cells

CpG: cytosine and guanine islands

CT: X-ray computed tomography

D. Melanogaster: Drosophila melanogaster

DCP2: decapping mRNA 2

DEPC: Diethylpyrocarbonate

DGCR8: DGCR8 microprocessor complex subunit

DNA: Deoxyribonucleic acid

Dnmt3a: DNA (cytosine-5)-methyltransferase 3A

Dnmt3b: DNA (cytosine-5)-methyltransferase 3B

DSBs: double strand breaks

dsRNA: double strand RNA

EGF: epidermal growth factor

EGFR: epidermal-growth factor receptor

eIF4E: ukaryotic initiation factor 4E

ENCODE: Encyclopedia of DNA Elements

ESCs: embryonic stem cells

EXP5: Exportin 5

FAK: focal adhesion kinase

FBS: fetal bovine serum

FGF: fibroblast growth factor

FOXM1: forkhead box M1

G-CIMP: glioma-CpG island methylator phenotype

GFP: green fluorescence protein

GLI1: GLI family zinc finger 1

GLUT3: solute carrier family 2 member 3

GO: gene ontology

GSCs: glioma stem-like cells

Gy: gray

HDM2: human double minute 2

HEF1/NEDD9: neural precursor cell expressed, developmentally down-regulated 9

HGNC: HUGO Gene Nomenclature Committee

HMG: high mobility group

hnRNPA1: heterogeneous nuclear ribonucleoprotein A1

HRP: Horseradish perxidase

hTERT: telomerase reverse transcriptase

ID1: inhibitor of DNA binding 1

IDH1: isocitrate dehydrogenase 1

IGF-1R: insulin-like growth factor 1 (IGF-1) receptor

IHC: immunohistochemistry

IPA: Ingenuity Pathway Analysis

iPSC: induced pluripotent stem cell

lncRNAs: long non-coding RNA

MDM2: mouse double-minute 2 gene

MEG3: maternally expressed 3

MF: molecular function

MGMT: methyl guanine methyl transferase

miRNAs: MicroRNAs

MMP-9: Matrix metallopeptidase 9

MMR: mismatch repair mechanism

MRI: Magnetic resonance imaging

mRNA: messenger RNA

MTIC: 3-methyl-(triazen-1-yl) imidazole-4-carboxamide

MYOD1: myogenin and myoblast determination 1

NADPH: nicotinamide adenine dinucleotide phosphate

ncRNAs: non-coding RNAs

NF1: neurofibromin 1

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NSCs: neural stem cells

OCT4: octamer-binding transcription factor

Olig2: oligodendrocyte lineage transcription factor 2

ORFs: open reading frames

OS: overall survival

PACT: protein activator of interferon induced protein kinase EIF2AK2

PBS: phosphate-buffered saline

qPCR: polymerase chain reaction

PET: positron emission tomography

PDCD4: programmed cell death protein 4

PDGF: platelet-derived growth factor

PDGFRA: platelet derived growth factor receptor alpha

PI: phosphatidyl inositol

PI3K: phosphatidylinositol 3-kinase

PPAR: peroxisome proliferator-activated receptors

PRC2: Polycomb Repressive Complex 2

pre-miRNA: microRNA precursor

pri-miRNAs: primary miRNA

PTEN: phosphatase and tensin homolog gene

qCHIP: chromatin immunoprecipitation followed by qRT-PCR

RB1: retinoblastoma gene

RISC: RNA-induced silencing complex

RNA: Ribonucleic acid

qRT: real time

siRNAs: small interfering RNAs

SOX2: SRY (sex determining region Y)-box 2

SOX2-OT: SOX2 overlapping transcript

ssRNA: single strand RNA

STAT3: signal transducer and activator of transcription 3

TCGA: The Cancer Genome Atlas

TEM: transmission electron microscopy

TF: transcription factor

TGF-β: Transforming growth factor beta

TKR: tyrosine kinase receptor

TLDA: TaqMan® low density array

TMZ: temozolomide

TP53: tumor protein p53

TRBP: TAR RNA-binding protein

TUs: transcriptional units

UTR: untranslated region

VEGF: vascular endothelial growth factor A

WB: western blotting

WHO: World Health Organization

Xrn1: 5'-3' exoribonuclease 1

ZFX: zinc finger protein, X-linked

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Introduction

1. Glioblastoma

Glioblastoma is the most common and aggressive primary malignant brain tumor in adults. Approximately 4000 new cases are diagnosed each year with an incidence of about 3 to 7/100.000 habitants, although incidence rates vary significantly by histologic type, age at diagnosis, gender, race, and country. Glioblastoma constitutes 45,2% of all malignant central nervous system (CNS) tumors and 80% of all primary malignant CNS tumors (Ostrom et al., 2015) . The median age at diagnosis is 64 years, and it is more common in men than woman and two times more common in whites than blacks (Dolecek et al., 2012). Despite the advance in multiple treatments, prognosis for glioblastoma patients remains very poor, with a median survival of 12 to 15 months (Stupp et al., 2009).

Based on the clinical course of the disease, there are two major forms of glioblastoma: primary or *de novo*, which occurs spontaneously without any previous history of lower grade tumor, which represents approximately 90% of all glioblastomas and develops preferentially in elderly patients. The other tumor presentation is known as secondary glioblastoma and arises as the result of progression of an already diagnosed lower grade glioma and occurs in younger patients (Ohgaki and Kleihues, 2013). Primary and secondary glioblastomas are indistinguishable at histological level, although they harbor differences at the molecular level.

Primary glioblastomas have often amplified and mutated the epidermal-growth factor receptor (*EGFR*), amplification of mouse double-minute 2 gene (*MDM2*), mutation of phosphatase and tensin homolog gene (*PTEN*), loss of heterozygosity 10q, p16 deletions, homozygous deletions of *CDKN2A*, high frequency of telomerase reverse transcriptase (*hTERT*) promoter mutations and absence of the isocitrate dehydrogenase 1 gene (*IDH1*) mutation (Killela et al., 2013; Kleihues and Ohgaki, 1999).

Secondary glioblastomas are characterized by more prevalent *TP53* mutations, *IDH1* mutations, a thalassemia/mental retardation syndrome X-linked (*ATRX*), *MET* amplification and overexpression of *PDGFRA* (Ohgaki et al., 2004; Ohgaki and Kleihues, 2007; Ohgaki and Kleihues, 2009). Mutations in *EGFR* and *PDGFRA* lead to increased tyrosine kinase receptor (*TKR*) activity and consequently to activation of *RAS* and *PI3K* pathways (Louis, 2006). The inactivation of the retinoblastoma gene (*RB1*) and the increased activity of human double minute 2 (*HDM2*) is associated with the

progression of low-grade to high-grade glioma (Kleihues and Ohgaki, 1999; Louis, 2006).

Glioblastoma is commonly located in the supratentorial region (frontal, temporal, parietal, and occipital lobes), and is rarely seen in the cerebellum and in the spinal cord, with a difference in the behavior at these 2 locations (Adams et al., 2013; Engelhard et al., 2010). Cerebellar glioblastoma arising in younger patients, accounts for 0.4% to 3.4% of all glioblastomas and is less common in whites and smaller in size, compared with supratentorial gliomas (Adams et al., 2013; Babu et al., 2013; Jeswani et al., 2013).

1.1 Classification

The World Health Organization (WHO) classification system categorizes gliomas into 4 histological grades based on certain pathological features, such as nuclear atypia, mitotic activity, vascular proliferation, necrosis, proliferative potential and features of clinical course and treatment outcome (Louis et al., 2007). This classification system was employed for the first time in the 1920s when Bailey and Cushing categorized glial tumors by their similarity to known glial cell types: astrocytes, oligodendrocytes (Erridge et al., 2011; Pollo, 2011).

Infiltrating gliomas are graded as WHO II-IV; grade I are typically solid and non-infiltrative tumors such as pilocytic astrocytomas and subependymal giant cell astrocytomas. Astrocytoma (diffusely infiltrated into surrounding neural tissue), oligodendroglioma (occurs in the white matter and cortex of the cerebral hemisphere) and oligoastrocytoma (diffuse mixed tumor with mix glial background) are classified as Grade II and are more differentiated neoplasms that invariably progress to a higher-grade tumor with time. Anaplastic astrocytoma/oligodendroglioma correspond to Grade III and glioblastoma is classified as Grade IV (Louis et al., 2007). Nuclear atypia and mitotic activity are required criteria for grade III lesions, and the presence of necrosis or microvascular proliferation is required for the diagnosis of grade IV astrocytoma, glioblastoma (Miller and Perry, 2007).

Additionally a rare subtype of glioblastoma was added to the WHO classification, termed as “glioblastoma with oligodendroglioma component", defined as glioblastoma having areas that resemble anaplastic oligodendroglioma with hallmark features of glioblastoma, necrosis with or without microvascular proliferation (Louis et al., 2007). At the molecular level several gliomas subtypes can be distinguished according to their genomic profile: classical, mesenchymal, proneural and neural type (Cancer Genome

Atlas Research Network, 2008; Phillips et al., 2006; Verhaak et al., 2010). These newly defined subtypes carry specific genetic abnormalities, including discrete mutations of oncogenes or tumor suppressor genes and loss or gain of partial or entire chromosomes.

The classical subtype accounts for about 21% of all glioblastomas and the amplification of *EGFR* can be considered its hallmark, detected in 97% of tumors. Other particularly common alterations in this subtype comprise the loss of heterozygosity of 10q23 (that harbor the *PTEN* locus), amplification of chromosome 7, loss of chromosome 10, homozygous deletion at chromosome 9p21.3 (encodes for *p16INK4A* and p14 ARF) and the lack of *TP53* mutations. In addition, high expression of the NOTCH and Sonic Hedgehog signaling pathways is also detected.

The expression of mesenchymal histologic markers, like CHI3L1/YKL40, VEGF and CD44 are displayed in the mesenchymal subtype (Cancer Genome Atlas Research Network, 2008; Phillips et al., 2006; Verhaak et al., 2010). Deletion of 17q11.2, where the tumor suppressor gene *NF1* is located, commonly occurs in the mesenchymal subtype, being *NF1* mutation the most frequently detected.

The proneural subtype has a high expression of oligodendrocytic genes and accounts for 31% of all glioblastomas. This subtype is characterized by *TP53* mutations and *TP53* loss of heterozygosity. Approximately a 35% of all proneural types exhibits amplification at the locus 4q12, where *PDGFRA* gene is located, although this amplification can be found in all subtypes of glioblastoma. Mutations in *IDH1* gene are commonly found in this class and serve as diagnostic and prognostic markers (Verhaak et al., 2010). The proneural subtype also includes another group of tumors that express the glioma-CpG island methylator phenotype associated with younger patients and with more favorable outcomes (Noushmehr et al., 2010).

The neural subtype accounts for 16% of glioblastomas and is characterized by the expression of neuron markers. Chromosome 7 amplification associated with loss of chromosome 10 is commonly observed in the neural subtype (Verhaak et al., 2010).

With the advances on the sequence of the complete human genome and the availability of high-throughput genomic technologies, has become easier the comprehensive molecular characterization of human cancer genomes. Glioblastoma was the first tumor type to be analyzed by The Cancer Genome Atlas (TCGA), a government-funded project(RW.ERROR - Unable to find reference:742). In 2008 the TCGA showed the integrative analysis of DNA copy number, gene expression and DNA methylation aberrations of 206 glioblastoma tumor samples. Overall these

analyses identified a highly interconnected network of aberrations, including three major pathways: receptor tyrosine kinaeses (RTKs) signaling, and the p53/RB tumor suppressor pathways (Figure 1) (Cancer Genome Atlas Research Network, 2008; Parsons et al., 2008). By copy number data alone 66%, 70% and 59% of the samples harbored somatic alterations of the RB, TP53 and RTK pathways, respectively. Approximately 86% of the samples harbored at least one genetic event in the core RTK/PI3K pathway, 13% for PDGFRA and 4% for MET aberrations. Inactivation of the p53 pathway occurred in the form of ARF deletions (55%), amplifications of MDM2 (11%) and MDM4 (4%), in addition to mutations of p53 itself. Approximately 77% of samples harvored RB pathway alterations, being the most common event deletion of the CDKN2A/CDKN2B locus. Overall, this integrated analysis suggests a series of inter-related events that may impact the clinical response and outcome.

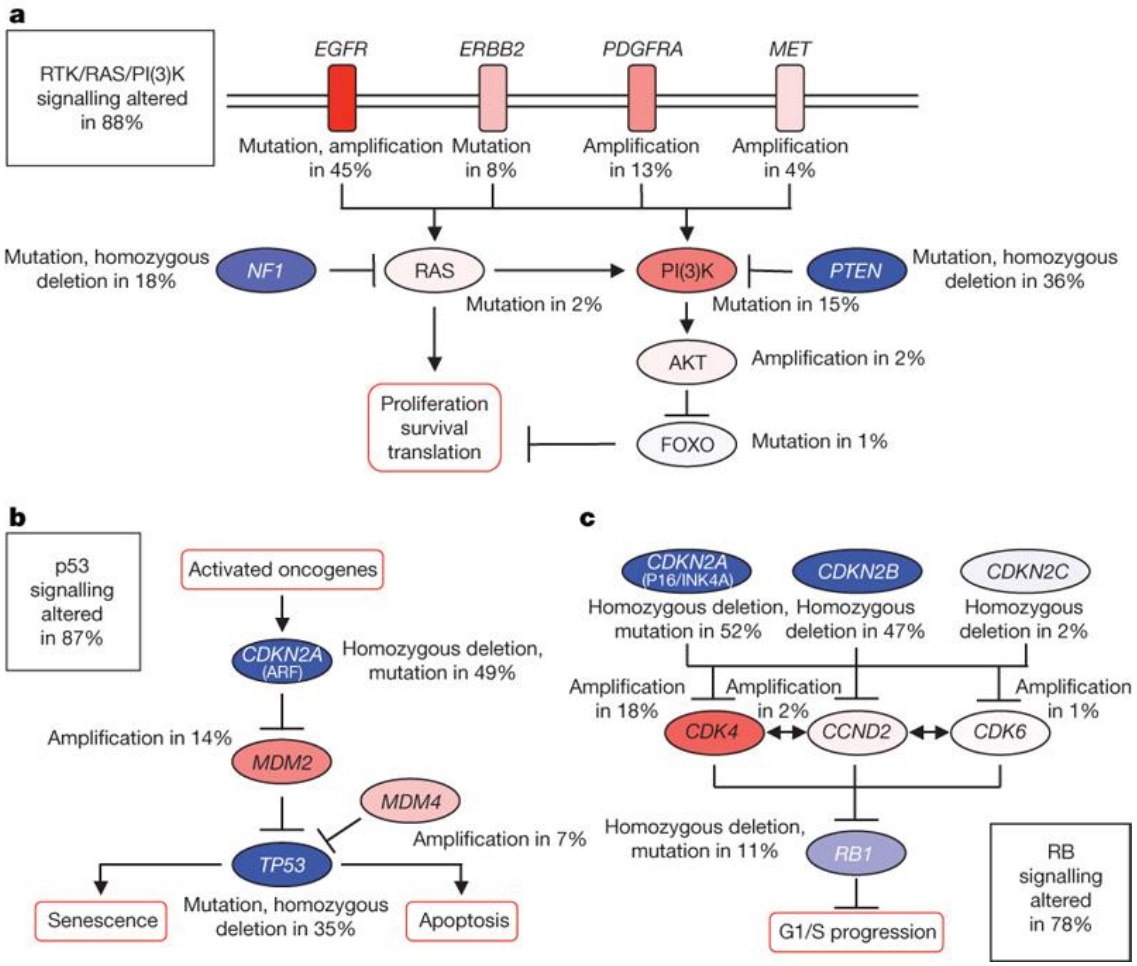


Figure 1. Frequent genetic alterations in three critical signalling pathways in glioblastoma. Primary sequence alterations and significant copy number changes for components of the (a) RTK/RAS/PI(3)K, (b) p53, (c) and RB signalling pathways are shown. Red indicates activating genetic alterations, with

frequently altered genes showing deeper shades of red. Conversely, blue indicates inactivating alterations, with darker shades corresponding to a higher percentage of alteration. For each altered component of a particular pathway, the nature of the alteration and the percentage of tumours affected are indicated. Boxes contain the final percentages of glioblastomas with alterations in at least one known component gene of the designated pathway. Adapted from (Cancer Genome Atlas Research Network, 2008).

Molecular research has led to the elucidation of the underlying genomic changes in the many pathological variants of glioblastoma and has shed light into the complex subtypes. Nevertheless, since there are not yet specific treatments based on the molecular characteristics, the histopathological characteristics of the tumor remain still the most recommended system to classify gliomas.

1.2 Clinical Presentation and Early Diagnosis

The most common sign of glioblastoma is headaches due to the localization and/or the increasing intracranial pressure, as the result of the clinical stage of the disease. Headaches are relatively frequent, present in about 50% of patients at diagnosis, but usually with a nonspecific pain pattern (Forsyth and Posner, 1993). Nevertheless, a progressive severity, unilateral localization, and new onset headache in elderly patients are some of the features that may distinguish a tumor-associated headache from a benign headache. Other common signs include nausea and/or vomiting, wide and/or rapid mood swings, memory loss, ataxia, lowered levels of alertness, dizziness, hearing loss, fatigue, weakness in one part of the body, reduced sensitivity to touch, difficulties in speech and/or swallowing, decreased levels of coordination, frequent syncope, hemiparesis, confusion/disorientation and visual field disturbances (Lakhan and Harle, 2009; Levine et al., 1987).

Cognitive difficulties and personality changes may also develop and are often mistaken for psychiatric disorders or dementia, particularly in elderly individuals. Language difficulties may be mistaken for confusion or delirium. Gait imbalance and incontinence may be present, usually in larger tumors with significant mass effect. Occasionally, the development of symptoms is rapid, mimicking a stroke. Seizures are manifested in about 20% to 40% of patients, and usually a focal onset is reported (Glantz et al., 2000). Due to these unspecific symptoms, glioblastoma is often misdiagnosed as infection, inflammatory proces or circulatory and immunological disease (Lakhan and Harle, 2009).

The diagnostic modality of choice is the magnetic resonance imaging (MRI). For patients unable to undergo MRI (individuals with pacemakers) a computed tomography (CT) scan is reserved (Bradley et al., 1984). Positron emission tomography (PET) allows integration of MRI and CT information with metabolic, pharmacokinetics and pharmacodynamics data (Demetriades et al., 2014) and is also used as a molecular neuroimaging technique, to support diagnosis.

Glioblastoma typically have central areas of necrosis surrounded by white matter edema. Nevertheless different syndromes may mimic malignant gliomas on neuroimaging, including brain abscess, sub-acute stroke, multiple sclerosis, and other inflammatory diseases (Omuro et al., 2006); so it becomes important to make an accurate diagnosis, integrating the patient's history, a physical exam, a neurological exam, the scans of the brain and a biopsy with pathological characterization.

1.3 Treatment of Glioblastoma and Therapeutic Resistance

The current standard of care for glioblastoma includes maximal surgical resection, radiation, and chemotherapy with temozolomide (TMZ) (Figure 2) (Stupp et al., 2009). It also includes symptomatic treatment of seizures, cerebral edema, infections, depression, cognitive dysfunction, fatigue and venous thromboembolism (Wen et al., 2006). Despite steady advances in new treatments to improve survival rates while preserving acceptable quality of life among patients, the overall survival (OS) of patients with glioblastoma has not improved over the last several decades, remaining at 12 to 18 months from diagnosis with <5 % of patients surviving 5 years after diagnosis (Ostrom et al., 2014; Stupp et al., 2005; Wen and Kesari, 2008).

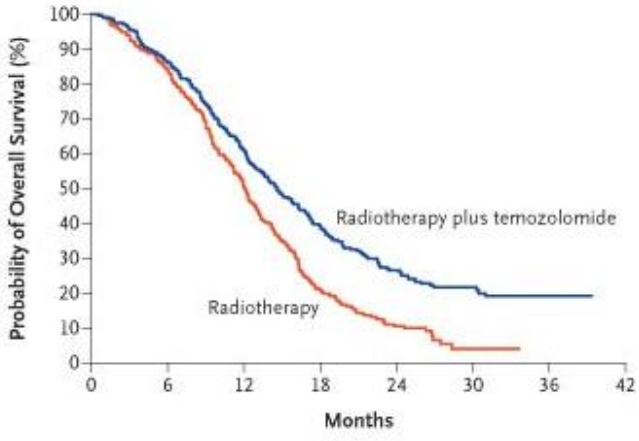


Figure 2. Kaplan–Meier Estimates of Overall Survival for glioblastoma patients. The hazard ratio for death among patients treated with radiotherapy plus temozolomide, as compared with those who received radiotherapy only, was 0.63 (95 percent confidence interval, 0.52 to 0.75; P<0.001). Adapted from (Stupp et al., 2005).

Surgical resection provides histological diagnosis, symptomatic relief and a reduction in tumor burden. Increase patient survival is associated with the degree of resection (Sanai and Berger, 2008), which is possible combining intraoperative magnetic resonance imaging with fluorescence-guided surgery with administration of 5-aminolevulinic acid (5-ALA) (Hauser et al., 2016). However, due to the infiltrative nature of glioblastoma and frequent proximity to critical neural structures, complete resection is often not feasible.

Fractionated localized radiation constitute one of the main therapeutic options against gliomas, being 60 Gy the standard final dose delivered in hypo fractionated regimens (Walker et al., 1979). Defining tumor margins has remained a challenge for surgery and radiation therapy, even though new imaging techniques allow a better demarcation of tumor borders. Tumor resistance to radiation emerges in part to a small subset of tumor cells with stemness properties, known as glioma stem-like cells (GSCs). Radiation dramatically increases GSC population in glioblastoma and minimally affects GSC tumorigenic potential (Bao et al., 2006a). Furthermore, GSCs are more resistant to radiation induced apoptosis via activation of DNA damage repair mechanisms throughout the activation of several DNA damage checkpoint proteins (Bao et al., 2006a).

The standard chemotherapeutic agent used in patients with glioblastoma is TMZ-, an oral cytotoxic DNA-alkylating agent from the imidazotetrazine family (Pletsas et al., 2013). TMZ was synthesized in the early 1980s (Stevens et al., 1984) and was approved in 1999 for recurrent glioblastoma based on the data of 2 phase II trials (Brada et al., 2001; Yung et al., 2000).

The mechanism of action of TMZ is based on DNA damage through the methylation of DNA. The methylation occurs at the O^6 and N^7 positions of guanine and the N^3 position of adenine, although the O^6 methylation accounts for 5% of the total lesions caused by TMZ in DNA, playing a critical role in the antitumor activity of the agent (Drablos et al., 2004). The methyl adducts formed causes a continuous cycle of DNA base mismatch repair mechanism (MMR) with eventual strand breaks, ultimately leading to cellular apoptosis (Fu et al., 2012).

Acquired chemoresistance to TMZ developed by tumor cells represents a major limitation to this therapy, as >90% of recurrent glioblastomas shows no response to a second cycle of chemotherapy (Oliva et al., 2010). Several mechanisms play critical roles in the chemoresistance to TMZ. The primary mechanism involves MGMT, which

is a small DNA enzyme repair protein acting to directly remove O^6-meG from the O^6-guanine position (Kitange et al., 2009). It acts as a suicide enzyme removing the alkyl group from the lesion in a stoichiometric reaction (Tubbs et al., 2007); and after alkylation this protein is degraded by the proteasome (Xu-Welliver and Pegg, 2002).

MGMT expression is correlated with the methylation profile of the *MGMT* promoter. The density of the methylation in the CpG islands in the 5` region of MGMT promoter causes a lower rate of its mRNA transcription and lower levels of the protein (Bhakat and Mitra, 2003). MGMT gene silencing is ensured by hypermethylation of promoter CpG islands (Silber et al., 2012). Consequently in glioblastoma patients *MGMT* methylation status emerges as a potentially important molecular test to determine which patients will benefit and should receive TMZ (Weller et al., 2010). The *MGMT* promoter is typically reported methylated in 30–60% of glioblastomas (Havik et al., 2012). *MGMT* promoter methylation correlates with better outcome of patients (Christmann et al., 2010)(Malmstrom et al., 2012). Low MGMT expression and *MGMT* promoter methylation are both predictive markers for slower tumor progression in patients with glioblastoma (Hegi et al., 2005; Sonoda et al., 2010).

Unfortunately glioblastoma is characterized by resistance to all therapies and frequently recurs rapidly within months of aggressive treatment. Traditional therapies provide only palliative effects, probably because they target proliferating non-tumorigenic cells. Targeting the GSC compartment could provide therapeutics benefits for glioma patients. Understanding the molecular mechanisms governing the function of GSCs is crucial to develop targeted therapies against this recalcitrant population.

1.4 Glioblastoma Heterogeneity and Glioma Stem-like Cells

Heterogeneity defines both the clinical and the pathological features of glioblastoma (Aum et al., 2014; Morokoff et al., 2015). At the cellular level glioblastoma is composed by heterogeneous cell populations, among which the GSCs exhibits stemness properties because they share similarities with normal neural stem cells (NSCs) in terms of their gene expression signatures and cellular functions.

GSCs have been defined as slow-cycling tumor cells with enhanced self-renewal potential, ability to differentiate into different lineages, increased resistance to radio- and chemotherapy, and the ability to reconstitute the original tumor upon orthotopic implantation (Singh et al., 2004; Vescovi et al., 2006). GSCs also have the ability to

remain quiescent (Li and Bhatia, 2011); therefore, their persistence results in tumor re-initiation.

GSCs are thought to originate from either NSCs or from the de-differentiation of normal brain cells, such as astrocytes and oligodendrocytes (Safa et al., 2015; Schneider et al., 2016), although this de-differentiation remains controversial (Morokoff et al., 2015). In 2002, Ignatova and colleagues were the first to describe cells with stem cell properties within human cortical glial tumors (Ignatova et al., 2002). Singh and colleagues later reported the identification and purification of cells from primary human medulloblastoma and glioblastoma that had a marked capacity for proliferation, self-renewal, and differentiation, represent a minority of tumor cells characterized by expression of the cell surface marker CD133 (Singh et al., 2003).

Due to the lack of consensus criteria, Vescovi and colleagues established guidelines to identify the GSCs population (Vescovi et al., 2006):

1. Cancer-initiating ability upon orthotopic implantation (tumors should be a phenocopy of the tumor of origin);
2. Extensive self-renewal ability, demonstrated either ex vivo (by showing both sequential-clonogenic and population-kinetic analyses) or *in vivo* (by serial, orthotopic transplantation);
3. Karyotype or genetic alterations;
4. Aberrant differentiation properties;
5. Capacity to generate non-tumourigenic cells;
6. Multilineage differentiation ability.

The development of a rigorous definition is important to standardize and guide future work. Understanding the intrinsic properties that characterize GSCs and distinguish them from their normal counterparts is critical for the development of successful and more selective therapies.

1.4.1 Models of the Origin of Cancer

There has been intense discussion concerning the origin of cancer and cancer stem cells (CSCs). The first concept that emerged was the clonal evolution (or stochastic)

model, and in the last decades after the demonstration that cancers are heterogeneous masses containing a hierarchy of cells, a new hierarchical CSC model replace it (Figure 3).

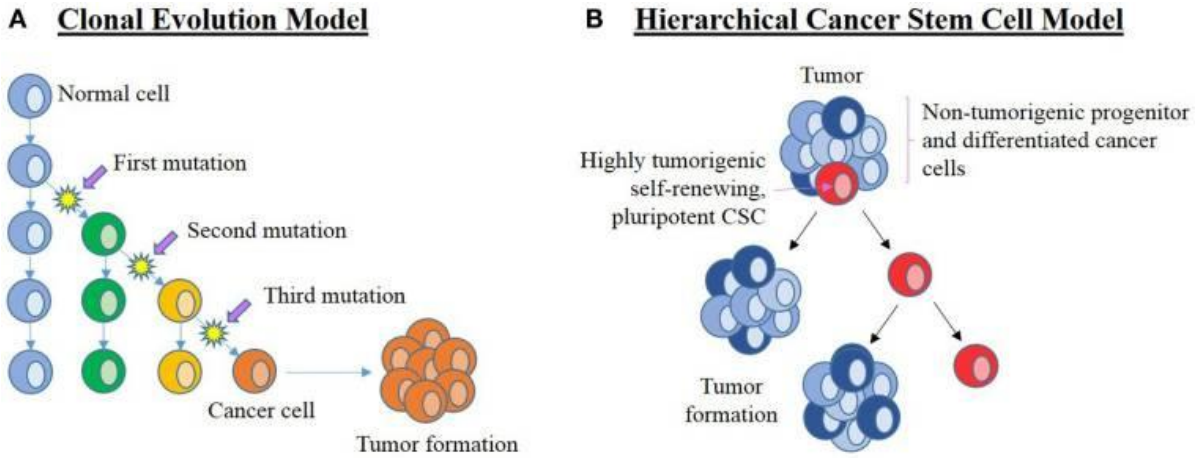


Figure 3. Models of cancer origin. (A) The clonal evolution model hypothesizes that a normal cell (blue) within the organism undergoes a series of mutations to form a cancer cell (orange) that clonally expands and form the bulk of the tumor. **(B)** The CSC hierarchical model proposes that the origin of cancer being CSCs (red) that are pluripotent and self-renewing. They are highly tumorigenic with the ability to establish new tumors. CSCs divide asymmetrically to form new CSCs and progenitor (dark blue) cells that in turn give rise to differentiated cancer cells (light blue) that form the bulk of the tumor. These downstream cancer cells are low or non-tumorigenic. Adapted from (Bradshaw et al., 2016).

The clonal evolution model of cancer proposes cumulative genetic mutations that occur over time in a normal cell, leading to the formation of a cancer cell that clonally expands to form identical copies, each with identical tumorigenic potential (Adams and Strasser, 2008; Salk et al., 2010), and if such changes confer a selective advantage, then this will allow individual clones of cancer cells to out-compete other clones (Shackleton et al., 2009). Propagation of this selected clone means that a substantial number of cells in the tumor are able to maintain tumor growth, so any effective treatment would require the elimination of all clonal cells.

On the other hand, the new hierarchical CSC theory postulates that tumors comprise a cellular hierarchy. From a hierarchical viewpoint, normal embryonic stem cells (ESCs) are the most primitive cells within a biological system, and are considered pluripotent with the capacity to differentiate into any cell type in a given organism (Chen and Zhong, 2011). Downstream from ESCs there are a group that includes NSCs (Gage, 2000), mesenchymal stem cells (Billing et al., 2016), endothelial progenitor cells (Rafii et al., 2013), and hematopoietic stem cells (Doulatov et al., 2013), with more

restricted lineage differentiation capacity (Gage, 2000). The multipotent NSCs further differentiate, giving rise to more downstream progenitor cells with reducing differentiation, mitotic, and self-renewal potential, ultimately forming the majority of the organism (Dietrich et al., 2008).

The CSC model proposes that the small CSC subpopulation drives tumor formation, growth, metastasis and resistance to therapeutic treatments (Clevers, 2011). CSCs demonstrate self-renewal ability maintaining the small cohort of these cells into the tumor and multilineage capacity, giving rise to the diverse progeny that constitutes the bulk of the tumor (Clarke et al., 2006). The hierarchy is dynamic with respect to cell type and is maintained by the balance between self-renewal and differentiation (Jackson and Alvarez-Buylla, 2008; Yu and Thomson, 2008). There is compelling evidence in support of its existence in hematological malignancies and in numerous solid epithelial types of cancer including glioblastoma and medulloblastoma (Visvader, 2011).

The stochastic theory is inconsistent with the identification of GSCs in different tumor types (Adams and Strasser, 2008). However the CSCs model and the clonal evolution model are not mutually exclusive in cancers that are hierarchically organized into epigenetically distinct populations of tumorigenic and non-tumorigenic cancer cells, subjected to evolve by clonal evolution (Barabe et al., 2007).

1.4.2. GSC Regulatory Mechanisms

GSCs are regulated by several mechanisms, which include intrinsic factors such as genetics, epigenetics, metabolic and extrinsic cues derived from niche factors, cellular microenvironment, and the host immune system (Figure 4).

Advances in genomic technologies have highlighted the significant degree of inter-tumoral heterogeneity present in glioblastoma, which is further translated to the transcriptional and epigenetic levels (Phillips et al., 2006; Verhaak et al., 2010), underscoring the complexity of the clonal evolution, clonal hierarchy and clonal diversity during glioblastoma formation.

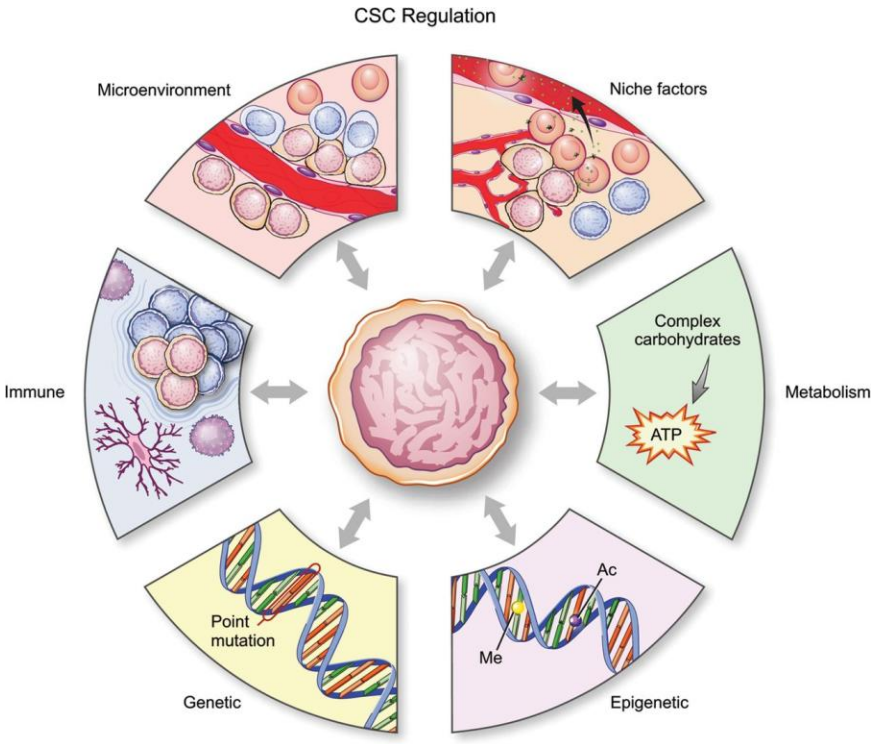


Figure 4. Regulation of CSCs. Cell-autonomous and external cues regulate the CSC state. Key intrinsic regulators include genetic, epigenetic, and metabolic factors, while extrinsic regulators include factors of the microenvironment, including niche factors and the immune system. Adapted from (Lathia et al., 2015).

Epigenetic maintenance of the GSC state is regulated at the level of transcriptional and chromatin regulation. Many transcription factors have been identified as important for GSCs identity, including c-MYC (Wang et al., 2008), STAT3 (Sherry et al., 2009), SOX2 (Gangemi et al., 2009), FOXM1 (Joshi et al., 2013), GLI1 (Clement et al., 2007), ASCL1 (Rheinbay et al., 2013), ZFX (Fang et al., 2014) and NANOG (Zbinden et al., 2010). These studies highlight the importance of understanding the dynamics of core transcription factors in maintaining stem cell state and the effect that these factors have on shaping the chromatin landscape of cells within the tumor hierarchy.

Other aberrant epigenetic mechanisms, such as DNA methylation, histone modifications, chromatin remodeling through histone methylation, and regulatory non-coding RNAs are currently recognized as relevant events in tumor formation (Caren et al., 2013; Heddleston et al., 2011). The epigenetic state of a cell is critically important in determining both the reprogramming and differentiation potential of a cell (Papp and Plath, 2013; Singh et al., 2009). DNA methylation involves the addition of methyl groups to cytosine residues, throughout DNA methyltransferases, such as Dnmt3a and

Dnmt3b (Jurkowska et al., 2011). High expression of Dnmt1 and Dnmt3b in glioblastoma has been associated with the hyper-methylation of tumor suppressor genes, regulating genomic stability and cell cycle progression and therefore influencing cell tumorigenicity (Rajendran et al., 2011). Moreover, deregulation of DNA methyltransferases has been associated with the tumor cell phenotype and stem cell compartment (Fanelli et al., 2008). The inhibition of these enzymes can induce differentiation of stem cells (Banerjee and Bacanamwo, 2010; Tsai et al., 2012).

Other studies have revealed that mutations in the IDH1 gene, which are associated with a proneural glioblastoma subgroup, are sufficient to establish the glioma-CpG island methylator phenotype (G-CIMP), leading to changes at the transcriptional profile and altering the differentiation state (Noushmehr et al., 2010; Turcan et al., 2012).

The polycomb family represents other epigenetic effectors involved in CNS development and cancer progression. In glioblastoma, the aberrant expression of polycomb genes inhibits differentiation, activates transformation, invasion-related genes and sustains GSCs self-renewal (Abdouh et al., 2009; Bruggeman et al., 2007; Suva et al., 2009). In conclusion, multiple changes in the DNA methylation pattern of gene promoters involved in cell cycle regulation, tumor suppression, DNA repair and genome integrity, as well as genes associated with regulation of tumor invasion and inhibition of apoptosis have been reported in glioblastoma (Horiguchi et al., 2003; Martinez et al., 2007; Mellai et al., 2013; Nakamura et al., 2001; Stone et al., 2004).

Hypoxia is a hallmark of glioblastoma microenvironment and plays an important role in maintaining stem-like phenotype and tumor growth and progression (Yang et al., 2012). GSCs are present in tumor microenvironments that limit nutrients, such as glucose and oxygen. Under such conditions, GSCs demonstrate plasticity in the metabolic pathways used in response to metabolic restrictions and may predominantly utilize aerobic glycolysis for energy production (Kathagen et al., 2013). Under hypoxic and acidic conditions, GSCs preferentially use the HIF-2α signaling, promoting the activation of stemness genes that contribute to maintenance of self-renewal, proliferation, and survival (Li et al., 2009c). In conditions of nutrient deprivation such as low glucose, GSCs out-compete neighboring non-stem tumor cells for glucose uptake through preferential up-regulation of the high-affinity GLUT3 transporter (Flavahan et al., 2013).

In the adult brain NSCs were shown to be concentrated around blood vessels prone to signaling cues, nutrients and evasion using nascent vasculature (Palmer et al., 2000).

Similar to NSCs, GSCs resides in specific anatomical and functional locations or niches (e.g. subventricular zone in the brain, perivascular niche, hypoxic niche) in direct contact with several cell types and with access to extracellular matrix, secreting factors that play a role in maintaining properties of stem cell self-renewal and proliferation and regulating supply of oxygen and nutrients (Gilbertson and Rich, 2007; Jandial et al., 2008). GSCs are located in areas of increased microvessels density, distributed along endothelial vascular tubes, suggesting that tumor vasculature generates specific niche microenvironment that promote formation and maintenance of GSCs (Calabrese et al., 2007; Tavazoie et al., 2008). Moreover GSCs can stimulate angiogenesis through secretion of pro-angiogenic growth factors and physical contributions to the vasculature (Bao et al., 2006b; Ricci-Vitiani et al., 2010). This is an evidence of the reciprocal relationship between GSCs and their micro-environmental niche, where GSCs not only receive signals from surrounding area but are also capable to stimulate signals that contributed to support and replenish the pool of GSCs. Furthermore, increasing number of endothelial cells or blood vessels promotes the expansion of GSC population and accelerates growth of the tumor (Calabrese et al., 2007). Indeed recent studies have identified that about 20-90% of endothelial cells in glioblastoma have the same genomic alteration often present in tumors cells (Wang et al., 2010b), supporting the idea that vascular endothelium may have a neoplastic origin (Ricci-Vitiani et al., 2010). Endothelial cells can also protect GSCs from radiation damage (Garcia-Barros et al., 2003; Paris et al., 2001), highlighting the protective role play by the micro-environmental niche. Therefore understanding the relationship between GSCs and their microenvironment is crucial for developing novel therapeutic strategies.

1.4.3 GSCs Markers

Gene expression profiles have shown similarities between GSCs and NSCs, supporting the idea that GSCs are malignant variants of NSCs (Jackson and Alvarez-Buylla, 2008). There are many common cellular markers and pathways between CSCs and NSCs. CD133 is the best-studied GSC biomarker and is often used experimentally to identify and enrich GSCs population. CD133 is also known as prominin-1, a cell surface glycoprotein associated with NSCs, and is expressed during embryonic development (Coskun et al., 2008; Pfenninger et al., 2007; Zacchigna et al., 2009).

Previous reports have demonstrated that tumor cells isolated from glioblastoma tumors, grew as neurospheres in serum-free medium (indicating self-renewal

capabilities) and recapitulate tumors phenotypically similar to glioblastoma being CD133-positive (CD133+) (Dirks, 2008; Lathia et al., 2011; Singh et al., 2003). In 2007 Beier and colleagues identified previously unknown CD133-negative (CD133-) GSCs in glioblastomas with similarly tumorigenic potential that CD133+ GSCs, a lower proliferation index and a signature of 117 differentially expressed genes (Beier et al., 2007), suggesting that CD133 may serve as GSC marker in only a subgroup of glioblastomas (Wang et al., 2010b). In any case, tumors arising from CD133+ cells recapitulated the histopathological phenotype of the parental tumors, suggesting that the CD133+ subpopulation is capable of giving rise to the full heterogeneous complement of cells present in the tumor (RW.ERROR - Unable to find reference:96).

Over the last decade several additional cell surface markers for GSCs have emerged, including A2B5 (Ogden et al., 2008), CD44 (Anido et al., 2010), CD171 (Bao et al., 2008), CD15 (Read et al., 2009), CD49f (integrin α6) (Lathia et al., 2010), used in traditional methods of GSCs enriching such as flow cytometry.

Many of the transcription factors (TFs) or structural proteins essential for normal NSCs function also mark GSCs, including SOX2 (Guo et al., 2011; Hemmati et al., 2003), NANOG (Ben-Porath et al., 2008), OLIG2 (Ligon et al., 2007), MYC (Kim et al., 2010), MUSACHI1 (Hemmati et al., 2003), BMI1 (Hemmati et al., 2003), NESTIN (Tunici et al., 2004), and ID1 (Anido et al., 2010).

Several methods other than expression markers have been used to enrich for GSCs, such as the neurosphere assay. Neurospheres are floating structures that can be obtained by the dissociation of embryonic or adult germinal tissue into individual cells and exposing the individual cells to growth factors in a non-adherent culture system (Reynolds et al., 1992). These heterogeneous spherical structures arise through the clonal expansion of a NSC. Theyadheres to its progeny to form a three-dimensionally organized sphere composed of NSCs along with more-differentiated lineage specific cells. Neurospheres can be used to evaluate NSC self-renewal by testing the capacity of cells isolated from a primary neurosphere to generate new spheres. This assay is often used to define NSC identity, since NSCs are characterized by their capability to generate new neurospheres with serial passaging, but fails to address cellular hierarchy and does not recapitulate the tumor microenvironment.

1.4.3.1 SOX2 and Glioblastoma

SOX2 belongs to the SOX (Sex-determining region Y (SRY)-box protein) family members, which are characterized by a conserved high mobility group (HMG) DNA-binding domain (Gubbay et al., 1990). There are, at least, 20 members divided into 8 groups (from A to H), based on their HMG sequence identity in humans (Schepers et al., 2002). Members within a group preserve higher than 80% identity in their HMG-domain and share other well-conserved regions (Bowles et al., 2000). In addition, they share biochemical properties, have overlapping expression patterns and perform synergistic or redundant functions. In contrast, members from different groups usually perform different functions.

SOX2 is located in the human chromosome 3q26.3 (Chr3q26.3) and is a single exon gene that encodes a member of the SoxB1 group (together with SOX1 and SOX3) required for the maintenance of the early embryo, before implantation (Avilion et al., 2003). SoxB1 group members are co-expressed in the neuroepithelium and show certain degree of functional redundancy in the developing CNS (Avilion et al., 2003). In particular, SOX2 is one of the four essential factors required for induced pluripotent stem (iPS) cell induction (Takahashi and Yamanaka, 2006) and its expression is conserved in humans (Sisodiya et al., 2006). It is widely expressed in the embryo, in particular in the developing CNS where its expression is initiated concomitantly to the acquisition of neural progenitor identity. High levels of SOX2 are indispensable for maintaining neural stem cell activity during embryogenesis and adult life (Bylund et al., 2003; Graham et al., 2003; Gubbay et al., 1990). In the adult, its expression is maintained in different populations of stem cells (Ellis et al., 2004; Fauquier et al., 2008; Taranova et al., 2006), acting intrinsically to confer stem cell properties(Pevny and Nicolis, 2010). Additionally SOX2 is not expressed in immature neurons, being down-regulated during differentiation. These findings emphasize the role played by SOX2 as a key factor in the control of stem cell fate and activity. In humans rare *SOX2* mutations cause anophtalmia, defective hippocampal development, cognitive defects and seizures (Fantes et al., 2003; Kelberman et al., 2006; Sisodiya et al., 2006).

SOX2 is highly expressed in several human cancers (Bass et al., 2009), including glioblastoma (Alonso et al., 2011; Annovazzi et al., 2011; Holmberg et al., 2011). Interestingly, the expression of SOX2 and other stem cell markers identify a subset of patients with a poorer clinical outcome highlighting its clinical relevance in glioblastoma and in several other neoplasms (Ben-Porath et al., 2008).

Functionally, SOX2 is enriched in human GSCs where it sustains stemness properties and maintenance of tumorigenicity (Gangemi et al., 2009; Ikushima et al., 2009). Indeed, siRNA-mediated downregulation of SOX2 in GSCs impaired proliferation and their ability to form tumors *in vivo* (Gangemi et al., 2009). SOX2 maintains GSC stemness using the same molecular targets as normal NSCs (Gangemi et al., 2009), supporting a hierarchical model of glioblastoma controlled by SOX2. Furthermore, elevated expression of SOX2 is essential but not sufficient for maintaining the self-renewal of GSCs (Alonso et al., 2011) indicating that other factors cooperate to activate stem cell-like properties. Further supporting this notion, recently Suva and colleagues identified a core set of neurodevelopmental TFs (POU3F2, SOX2, SALL2, and OLIG2) essential for glioblastoma propagation. These TFs are able to reprogram differentiated tumor cells into GSCs, recapitulating the epigenetic landscape and phenotype of native GSCs, highlighting that SOX2 among other three TFs is required for the maintenance of the tumor-forming capability of these cells (Suva et al., 2014). In addition, SOX2-driven malignant GSCs are highly invasive and have migratory characteristics (Alonso et al., 2011), mimicking those of NSCs (Cayre et al., 2009). Indeed, SOX2 depletion induced attenuated cell proliferation, caused by decreased levels of Cyclin D1 (Oppel et al., 2011), while the impaired invasive activity is mediated by inhibition of focal adhesion kinase (FAK) signaling and downstream proteins such as HEF1/NEDD9 and matrix metalloproteinases 1 and 2 (Oppel et al., 2011).

In the last years the mechanism of *SOX2* activation in glioblastoma has started to be unraveled. Our group identified *SOX2* gene amplification and promoter DNA hypomethylation in a set of glioblastoma patients as the leading mechanism responsible for SOX2 aberrant expression (Alonso et al., 2011). *SOX2* presents a high CpG density throughout the promoter that may poise the gene for repression upon differentiation (Mikkelsen et al., 2007), suggesting that *SOX2* promoter hypomethylation in glioblastoma might reflect a more primitive cellular state resembling the one found in neural stem cells (Mikkelsen et al., 2007).

SOX2 is also regulated transcriptionally and acts downstream relevant pathways in glioblastoma formation, such as TGF-β and PDGF, which regulates GSCs properties through SOX2 (Ikushima et al., 2009). In fact, transforming activity of PDGF in neural progenitors and PDGF-dependent tumors in mice triggered SOX2 expression (Appolloni et al., 2009). Moreover, SOX2 is activated at translational level by

eukaryotic initiation factor 4E (eIF4E) (Ge et al., 2010), showing a positive correlation between SOX2 and eIF4E in glioblastoma human samples. Together, all these results underscore the major role that SOX2 display in the malignant phenotype of glioblastoma.

2. MicroRNAs

MicroRNAs (miRNAs) are single strand RNA of ~22 nucleotides in length, that negatively regulate gene expression through base pairing with complementary mRNA sequences, resulting in translational repression or induction of the RNA degradation (Doench and Sharp, 2004) . MiRNAs are found in a wide variety of organisms, ranging from plants to worms to humans (Bartel, 2004; Lai, 2003).

2.1. Discovery

MiRNAs were discovered in the early nineties by Ambros, Lee and Feinbaum, while studying genes that control development in the nematode Caenorhabditis elegans (C. elegans) (Lee et al., 1993). They reported the surprising finding that mutations in lin-4, a gene with an important role in developmental control of C. elegans, did not code for any protein, but rather was transcribed in two small transcripts of 22nt and 61nt of length. These two small RNAs were complementary to multiple conserved sites of the 3`untranslated region (UTR) of lin-14, their target transcript, and were able to down-regulate the lin-14 protein levels (Lee et al., 1993). The decrease in lin-14 protein level without a decrease in mRNA level was dubbed translational repression (Wightman et al., 1991; Wightman et al., 1993). After a gap of 7 years Reinhart and coworkers found another small 21-nucleotide RNA called lin-7 that was complementary to 3`UTR regions of various genes (lin-14, lin-28, lin-41, lin-42 and daf-12), and was evolutionary conserved (Reinhart et al., 2000). This sparked the idea that gene regulation through small RNAs could be a much more widespread phenomenon than was appreciated at the time. Not long after the findings of the first miRNAs, a number of reports started to appear describing numerous conserved endogenous small RNA genes from different organisms, ranging from worms, to plants, to flies, to humans (Lagos-Quintana et al., 2003), suggesting that these molecules participate in essential processes (Pasquinelli et al., 2000). At present 1881 precursor molecules, which generate 2588 mature forms,

have been identified in the human genome, according to miRNA databases (miRBase) release 21 (Kozomara and Griffiths-Jones, 2014).

2.2 Mechanism of Action

The binding of mature miRNAs to their target mRNAs occurs through a specific miRNA region of about 6-8 nt in length often in the 5`end of the miRNA sequence (nucleotide 2 to 8). This region is termed the "seed region" and allows for each miRNAs to regulate the expression of genes, generally by binding to the mRNA 3` UTR region (Bartel, 2009). MiRNAs that share a similar seed region belong to the same miRNA family, and in general members of a miRNA family regulate related genes or are involved in the regulation of similar biological events.

Two kinds of mechanisms for miRNA action have been identified depending on the degree of complementarity between a miRNA and its target (Figure 7) (Hutvagner and Zamore, 2002). In the case the target site is perfectly complementary to the miRNA, the miRNA functions like a short interfering RNAs (siRNAs) and the target is sequence-specifically cleaved by miRISC. This is very rare in mammals but more frequent in plants (Pattanayak et al., 2005). Binding to partially complementary target sites leads to repression of translation or degradation of the target transcript, and mainly occurs in mammals (Krol et al., 2010). This degradation process involves the recruitment of deadenylase complexes such as the CCR4-NOT complex to the mRNA to remove or shorten the poly(A) tail, inducing the removal of the 5' cap of the mRNA, process known as de-capping, through the decapping enzyme DCP2. Decapped mRNAs are rapidly removed from the cell by 5' to 3' exonucleases such as XRN1 (Huntzinger and Izaurralde, 2011).

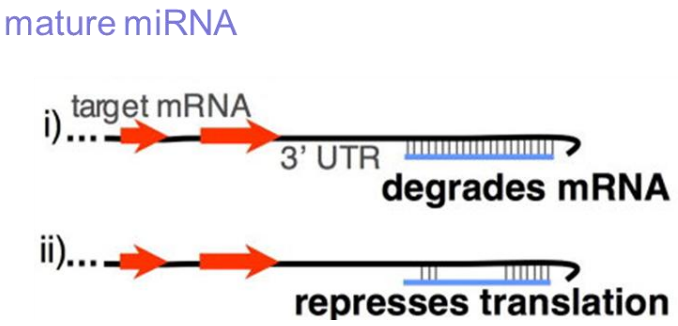


Figure 7. Mechanism of action of mature miRNA. The ~22-nt mature miRNA is excised from the pre-miRNA and hybridizes to its complementary site in the 3`UTR of target mRNA. i: When miRNA and

mRNA target sites are perfectly complementary, mRNA is degraded. ii: Imperfect complementarity leads to inhibition of protein translation. Adapted from (Kiefer, 2006).

2.3 Identification of MiRNA Target Genes

The identification and characterization of miRNA predicted targets is an important issue in miRNA research. Currently several bioinformatics tools have been developed for predicting miRNA target interactors, although is relatively difficult due to the involvement of several factors, such as the context of surrounding sequence in mRNA (Grimson et al., 2007), the number of target sites, the protection of target sites by RNA-binding proteins (Mazière and Enright, 2007), and the fact that miRNAs often bind to targets with incomplete complementarity, containing mismatches, gaps, and G:U base pairs at multiple positions (Brennecke et al., 2005; Hammell, 2010).

Based solely on the principles of miRNA-target recognition, on average, each miRNA can recognize about 100-200 potential target sites of the transcriptome (Brennecke et al., 2005; Brodersen and Voinnet, 2009; Krek et al., 2005), highlighting that one of the problems of these computational algorithms is the target over-prediction. Each of these *in silico* approaches utilizes a unique combination of criteria for miRNA target site prediction. Although the criteria most commonly employed are the sequence similarity between miRNA and target, especially continuous Watson-Crick base paring in 5` proximal half of the miRNA, the free energy and thermodynamics of miRNA-target mRNA duplex and the evolutionary conservation of the miRNA binding site in the 3` UTR of the target gene to eliminate false positives.

The computational tools most popularly used are listed in Table 1. In addition to this conventional approaches, other tools provide several other valuable features, including information on polymorphisms in miRNAs and target sequences, like Patrocles (Hiard et al., 2010), co-expression of miRNA and their targets, like miRGator (Cho et al., 2013), analysis of pathways regulated by specific miRNAs, like miRPath (Vlachos et al., 2012), and miRNA disease relationships, like miR2disease (Jiang et al., 2009). Further applications, such as miRWalk (Dweep et al., 2011) link the information across several independent prediction software tools and enable parallel analysis across multiple databases. A number of machine-learning algorithms, such as TargetBoost (Saetrom et al., 2005), TargetSpy (Sturm et al., 2010), MultiMiTar (Mitra and Bandyopadhyay, 2011), and NBmiRTar (Yousef et al., 2007) have also been developed, utilizing a database of validated interactions for prediction. However the dependence of

this machine learning algorithms on the quality of training data set of experimentally verified target genes represents a weakness.

Experimental identification of the genes regulated by specific miRNAs is essential to elucidate the biological functions of miRNAs. Microarray profiles are widely utilized approaches to study genome-wide changes in gene expression, establishing global effects. However the appropriate interpretation of the massive amount of data generated remains a challenge. The best approach for miRNA target identification is the experimental demonstration. This can be addressed through different analysis, such as luciferase assay, qRT-PCR, western blotting, and immunohistochemistry, which along with animal studies demonstrated the complexity of miRNA-regulated pathways in physiological conditions.

Table 1: **Summary of prediction techniques for miRNA target recognition**

Name	Characteristic	Link	Reference
Diana Micro-T	Target prediction made with miRNA or mRNA sequences as input, combining conserved and non-conserved miRNA recognition elements into a final prediction score	http://diana.cslab.ece.ntua.gr/microT/	(Maragkakis et al., 2009)
MicroInspector	Identification of potential miRNA binding sites in user-submitted sequences, searching against databases of known miRNA binding sites	http://bioinfo.uni-plovdiv.bg/microinspector/	(Rusinov et al., 2005)
miRanda	Optimizes sequence complementarity based on position-specific rules and interspecies conservation.	http://www.microrna.org	(Betel et al., 2008; John et al., 2004)
miRBase	Complete repository of miRNA sequences and targets	http://www.mirbase.org/	(Kozomara and Griffiths-Jones, 2014)
miRecords	Two main modules, experimentally validated targets, and integrated information across 11 independent prediction softwares	http://mirecords.umn.edu/miRecords/index.php	(Xiao et al., 2009)
miRTar	Identifies the biological functions and regulatory relationships between a group of known/putative miRNAs and protein coding genes.	http://mirtar.mbc.nctu.edu.tw/human/	(Hsu et al., 2011)
MiRTarBase	Information on experimentally verified miRNA targets by data mining and manually surveying literature related to functional studies on miRNAs	http://mirtarbase.mbc.nctu.edu.tw/	(Chou et al., 2016)
PicTar	Provides details about 3' UTR alignments with predicted sites, and	http://pictar.mdc-berlin.de	(Krek et al., 2005)

	links to various public databases.		
PITA	Secondary structure of the miRNA-mRNA hybrid for target gene prediction.	http://genie.weizmann.ac.il/pubs/mir07/index.html	(Kertesz et al., 2007)
RNA-hybrid	Determines the most favourable hybridization site between two sequences.	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid	(Kruger and Rehmsmeier, 2006; Rehmsmeier et al., 2004)
TarBase	Experimentally validated miRNA targets	http://diana.cslab.ece.ntua.gr/tarbase/	(Vergoulis et al., 2012)
TargetScan	Search for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA, in 5 vertebrates.	http://www.targetscan.org	(Lewis et al., 2003; Lewis et al., 2005)

2.4 Downregulation of miRNAs

In recent years several strategies have been developed to achieve miRNA loss-of-function, either by the introduction of antisense oligonucleotides (Krutzfeldt et al., 2005; Meister et al., 2004), or by overexpressing transgenic reporters that contain miRNA binding sites (Brown et al., 2007; Care et al., 2007; Ebert et al., 2007).

Oligonucleotide miRNA inhibitors are industrially available and are known as antago-miRs or anti-miRs, comprising small single-stranded RNA oligonucleotides with near perfect complementarity against a miRNA, modified to improve their stability and increase their efficacy (Elmen et al., 2008; Krutzfeldt et al., 2005).

The other strategy frequently used are the artificial miRNA sponges introduced for first time on 2007 (Ebert et al., 2007). Natural non-coding RNAs acting as miRNA sponges are present in plants (Franco-Zorrilla et al., 2007; Ivashuta et al., 2011), animals (Poliseno et al., 2010), and humans (Hansen et al., 2013; Wang et al., 2013e). These natural miRNA sponges serve as an endogenous regulatory mechanism to sequester sequence-specific miRNAs, regulating miRNA distribution on their mRNA targets. Artificial miRNA sponges are DNA constructs that produces artificially designed miRNA binding sites on the 3'UTR region of a non-toxic gene such as GFP. These binding sites are usually tandem repeats of identical sites designed to target either single specific miRNAs or miRNA family members sharing the same seed region. After transfection with a non-viral vector or transduction with a viral vector, miRNA sponges are expressed by mRNA transcription inside cells, impairing the binding availability of the miRNA for natural target mRNAs and inhibiting its posttranscriptional regulation activity. Although antago-miRs have advantages including of the simple synthesis and

diverse chemical modification to improve their stability (Lennox et al., 2013; Takahashi et al., 2013), miRNA sponges can achieve inducible/tissue specific and stable expression for *in vitro/vivo* use (Chen et al., 2014a).

A typical miRNA sponge vector contains an expression cassette with a miRNA binding sponge sequence inserted into the 3′ UTR of a reporter gene driven by promoters such as U6 or cytomegalovirus, which are among the strongest drivers of expression in mammalian systems (Ebert and Sharp, 2010).

Apart from antago-miRs and miRNA sponge technology, the other miRNA loss-of function strategies include the genetic knockout/inactivation (Wang et al., 2007). MiRNA gene knockout is the only convincing way for complete loss-of-function of a particular miRNA; however is a relatively difficult, time-consuming and costly procedure. Furthermore, since many miRNA genes reside within protein-coding genes, knockout of a miRNA gene would often adversely affect nearby protein-encoding genes (Kim et al., 2009).

As mentioned above each technology has pros and cons, however as a tool to manipulate miRNA function in cells, miRNA sponge technology exhibits the strongest potential.

2.5 MiRNAs in Cancer

MiRNAs are involved in the tumorigenesis of human malignancies. The earliest observation that provided a potential link between miRNA and tumor biology was the phenotype of lin-4 and let-7 loss-of-function mutations in C. elegans. These mutants caused larval stages repetitively and underwent extra cell divisions, implicating lin-4 and let-7 in the control of cell differentiation and proliferation (Lee et al., 1993; Reinhart et al., 2000). The most direct evidence linking miRNAs to cancer came from the genetic analysis of B-cell chronic lymphocytic leukemia (B-CLL). Calin and colleagues found a commonly deletion of the miR-15a/16-1 cluster in chromosome 13q14 in B-CLL patients (Calin et al., 2002).

Since then, thousands of tumor miRNA expression profiling studies have generated an expansive list of these non-coding genes that are differentially expressed in tumors versus normal tissue. MiRNAs exhibit oncogenic or tumor suppressing activities depending on their respective targets oncogenes or tumor suppressor genes, and are known as “oncomiRs” or “tumor suppressor miRs”. One of the clearest examples of miRNAs acting with tumor suppressor functions is provided by miR-15a/miR-16-1

which negatively regulate the expression of the anti-apoptotic factor BCL2, suggesting that their absence in B-CLL inhibit apoptosis by reactivation of BCL2 (Cimmino et al., 2005). On the other hand, the miR-17-92 cluster exhibits an oncogenic function since its expression along with cMYC accelerated the development of B-cell lymphoma in mouse models (He et al., 2005).

Different mechanisms have been shown to be responsible for altered miRNA expression in tumours: 1) the establishment of an abnormal cancer epigenome, mainly mediated by DNA hypermethylation, occurring at miRNAs regulatory regions (Lujambio et al., 2008), 2) allelic loss due to localization of miRNAs sequence inside or close to cancer associated genomic regions subjected to genomic instability (Lujambio et al., 2007; Makunin et al., 2007), 3) localization of chromosomal abnormalities or other types of genetic alterations at miRNAs genomic region, 4) abnormal transcriptional regulation of miRNA promoter by tumour suppressors or oncogenes, (O'Donnell et al., 2005; Xi et al., 2006), 5) abnormalities in miRNA processing (Lee et al., 2008). In addition, different mutations affecting the functional activity of miRNAs in gene silencing have been described. Polymorphisms and potential deaminations affect the seed sequence or the binding site of miRNA in the 3`UTR of its target, resulting in either abolished or weakened miRNAs/mRNA interaction or redirection of miRNA to a new wrong target, and have been detected in various cancers (Mishra et al., 2008).

To better understand how miRNAs function in neoplastic cells, a large wealth of data has been generated. Altered miRNA expression has been reported in all tumors investigated to date, including brain tumors. Interestingly, miRNAs are particularly abundant in the brain. At least 60% of known microRNA species are detected in the adult brain (Bak et al., 2008), so many of which are drastically regulated during embryonic brain development (Miska et al., 2004). MiRNAs exhibit cell type and subcellular specific brain. For example upon neural lineage specification miR-9, miR-124, and miR-128 are selectively expressed in neurons, and play pivotal roles in neuronal development and synaptic plasticity. In contrast other miRNAs species are preferentially expressed in glial lineages controlling normal glia cell proliferation and differentiation (Dugas et al., 2010; Lau et al., 2008; Zhao et al., 2010). Besides cell type-specificity, particular miRNAs demonstrate unique patterns of regional and subcellular brain localization. Regarding regional localization specificity, miR-218, miR-221, miR-222, miR-26a, miR-128a/b, miR-138, and let-7c are preferentially

enriched in the hippocampus, while miR-195, miR-497, and miR-30b are found to be enriched in the cerebellum (Bak et al., 2008).

2.5.1 MiRNAs in Glioblastoma

In glioblastoma the number of studies covering the miRNA expression and functional characterization has grown and miRNA signatures are redefining glioblastoma classification, differentiating between the different grades and stages, providing key regulatory links to disrupted signaling. This has led to a more in depth understanding about glioblastoma pathology (Kim et al., 2011).

One of the first studies that examined global miRNA expression covered 245 miRNAs in glioblastoma tissue and cell lines and found a strong upregulation of miR-221 in glioblastoma while miR-128, miR-181a, miR-181b, and miR-181c were downregulated (Ciafre et al., 2005). Since then multiples profiling studies have been performed using glioblastoma tissues and cell lines. The most extensively investigated miRNA is miR-21, which is consistently reported to be overexpressed in glioblastoma in a grade-specific manner (Chan et al., 2005; Conti et al., 2009; Gabriely et al., 2008; Lakomy et al., 2011; Li et al., 2011; Malzkorn et al., 2010; Papagiannakopoulos et al., 2008; Zhou et al., 2010a). At least for glioma, miR-21 appears to be the major anti-apoptotic and pro-survival factor that is linked to shorter progression-free survival (Chao et al., 2013; Lakomy et al., 2011; Quintavalle et al., 2013a).

MiRNAs could also be used as biomarkers for brain tumors, for example, Nass and collaborators showed that miR-92b and miR-9/9* are significantly overexpressed in primary brain tumors samples but not in metastatic brain tumor samples, suggesting that these miRNAs represent a valid biomarker to discriminate between primary brain tumors and brain metastasis (Nass et al., 2009). Moreover miRNAs can be used to distinguish between different types of brain tumors; for example Rao and colleagues identified a signature of miRNAs that can differentiate secondary glioblastoma from primary glioblastoma and from anaplastic astrocytoma, which are likely to lead to a rapid and accurate molecular diagnostic test in the future (Rao et al., 2010).

A number of well characterized microRNA expression patterns and functional studies have been carried out to date in glioblastoma. Across all the profiling studies the most consistently up-regulated miRNAs are miR-21, miR-10b, miR-155, miR-210 and miR-221; while the most consistently down-regulated are miR-128, miR-330, miR-124, miR-149, miR-153, miR-154, miR-181(a, b, c), miR-323 and miR-328. These

prominent miRNAs can be used as potential biomarkers and/or therapeutic targets as they act as oncomiRs or tumor suppressor-miRs, directly involved in the development and growth of glioblastoma (Novakova et al., 2009). Several studies have described the role of different miRNAs in core signaling pathways in glioblastomas. MiR-21 is one of the components of the EGFR signaling pathway (Zhou et al., 2010a) and miR-7 directly inhibited EGFR expression (Kefas et al., 2008); miR-26a and miR-451 regulates the PI3K/AKT signaling pathway (Huse et al., 2009; Nan et al., 2010); miR-221/222 enhance glioma malignant phenotype via activation of the AKT signaling pathway (Zhang et al., 2010b); miR-21 controlled p53, TGF-β and mitochondrial apoptotic networks (Papagiannakopoulos et al., 2008), down-regulate the expression of PDCD4 (Chen et al., 2008) and activate caspase 9 and 3 (Zhou et al., 2010b), affecting the migratory and invasive abilities in glioblastoma cells; miR-326 and miR-34a are associated with Notch signaling pathway in glioblastomas (Kefas et al., 2009; Li et al., 2009a); miR-218 inactivate NF-κB/MMP-9 signaling (Song et al., 2010), among other examples.

2.5.1.1 hsa-miR-301a-3p

MiR-301a plays an important role in various biological and pathological processes, including cellular development, proliferation, migration, differentiation and apoptosis (Liang et al., 2015; Ma et al., 2014a; Wang et al., 2013c; Zhang et al., 2014c). MiR-301a has been linked to several neoplasms, including colorectal cancer (Fang et al., 2015), laryngeal squamous cell carcinoma (Lu et al., 2015), breast cancer (Ma et al., 2014a), gastric cancer (Wang et al., 2013c), ovarian carcinoma (Cui et al., 2011), small cell lung cancers (Miko et al., 2009), hepatocellular carcinoma (Jiang et al., 2008), and pancreatic cancer (Lee et al., 2007), where contributes to NF-κB activation (Lu et al., 2011). Panguluri and colleagues reported that miR-301a was down-regulated in diabetic heart and modulated Kv4.2 by directly binding its 3'-UTR (Panguluri et al., 2013), and contributing to IL-6-induced insulin resistance by direct regulation of PTEN expression (Dou et al., 2015). In prostate cancer miR-301a function as an oncogene by directly targeting the p63 tumor suppressor gene (Nam et al., 2016) acting as a potential marker for metastasis in these patients (Damodaran et al., 2016). MiR-301a form an important axis along with b-NDRG2 modulating autophagy and viability under hypoxia (Guo et al., 2016).

In an elegant study Boyer and colleagues demonstrated that SOX2, NANOG and OCT4 co-occupied the promoter region of miR-301a, suggesting that this miRNA could be an important component of the transcriptional regulatory circuitry in human embryonic stem cells (Boyer et al., 2005). Moreover miR-301a has been previously described as one of the miRNAs up-regulated in GSCs (Lavon et al., 2010) although its role in glioblastoma is unclear.

2.5.1.2 hsa-miR-425-5p

MiR-425 is involved in different biological processes, such as angiogenesis (Gao et al., 2016), chemoresistance (Zhang et al., 2016), radioresistance (Moskwa et al., 2014), invasion and metastasis (Zhang et al., 2015c). Although its molecular mechanism is not very well understood. Despite the limited number of studies, miR-425-5p has been reported to be deregulated in several tumors, like cervical cancer (Gao et al., 2016), colorectal cancer (Zhang et al., 2016), osteosarcoma (Li et al., 2015a), gastric cancer (Peng et al., 2014), lung squamous cell carcinoma (Wang et al., 2015a), renal cell carcinoma (Ge et al., 2015) and human triple negative breast carcinoma cells (Ahir et al., 2016), where also has been shown its association with miR-191 forming a cluster (Di Leva et al., 2013). Regarding glioblastoma a recent work has described its implication on radioresistance through the up-regulation of cell-cycle checkpoint response (Moskwa et al., 2014). Nevertheless, the precise role of miR-425 and its clinical significance in glioblastoma remains poorly investigated making it an interesting candidate.

2.6 MiRNAs and GSCs

Many groups have highlighted the key role of miRNAs in self-renewal, differentiation and neural stem cell development (Christensen and Schratt, 2009; Gao, 2008; Hatfield and Ruohola-Baker, 2008). Increasing evidence shows that the expression profile of miRNAs in stem cells resemble those of cancer cells (Papagiannakopoulos and Kosik, 2008).

To date many groups have investigated the role of microRNAs in GSCs, casting a specific miRNA signature, for example miR-124 and miR-137 induce differentiation of GSCs (Silber et al., 2008); miR-451, miR-125b, miR-29b and miR-125a are significantly down-regulated in GSCs (Cortez et al., 2010; Gal et al., 2008; Shi et al.,

2010) suggesting a potential role for these microRNAs in regulatory signaling pathways related to maintenance of stem cell properties and self-renewal of cancer cells.

3. Long Non-Coding RNAs

The revolution in secuencing technology has generated a vast amount of information regarding the human genomic landscape, and has highlighted the fact that the coding regions comprise only a tiny fraction of the human DNA. Precisely around the 98% of all transcriptional output in humans is non-coding RNA (Lander et al., 2001). Ten years later, the ENCODE (Encyclopedia of DNA Elements) study reported the existence of over 9,640 lncRNA loci in the human genome, roughly half the number of protein-coding genes (Djebali et al., 2012). These studies have changed our view of the mammalian genome and underlined the importance of understanding non-coding transcripts.

Non-coding transcripts are further divided into housekeeping non-coding RNAs (ncRNAs) and regulatory ncRNAs. Housekeeping ncRNAs, which are usually considered constitutive, include ribosomal, transfer, small nuclear and small nucleolar RNAs. Regulatory ncRNAs are generally divided into two classes based on nucleotide length. Those less than 200 nucleotides are short/small ncRNAs, including miRNAs, and those greater than 200 bases are known as long non-coding RNAs (lncRNAs) (Nagano and Fraser, 2011).

3.1 Identification and Definition of lncRNAs

Unfortunately at present there is not a clear definition of lncRNAs. The latest definition proposed by HUGO Gene Nomenclature Committee (HGNC) describes lncRNAs as spliced, capped and polyadenylated RNAs (Wright and Bruford, 2011) with more than 200 nucleotides that are not predicted to be translated to a functional protein product (Kung et al., 2013). This distinction, while somewhat arbitrary and based on technical aspects of RNA isolation methods, serves to distinguish lncRNAs from miRNAs and other small RNAs. They typically do not possess functional open reading frames (ORFs). However, this distinction is blurred by the discovery of bifunctional RNAs that can have both protein-coding and coding-independent functions (Chooniedass-Kothari et al., 2004; Warden et al., 2008), raising the possibility that many protein-coding genes may also have non-coding functions.

It has been reported that lncRNAs have an unusual exonic structure, but exhibit standard canonical splice site signals and alternative splicing (Derrien et al., 2012). Many lncRNAs are characterized by 'K4–K36' domains, which consist of histone 3 Lys 4 trimethylation (H3K4me3) at the promoter followed by histone 3 Lys 36 trimethylation (H3K36me3) across its actively transcribed region (Cabili et al., 2011; Guttman et al., 2009), which marks the promoters of genes actively transcribed. There is substantial evidence indicating that lncRNAs, just like mRNAs, are transcribed by RNA polymerase II and usually contain canonical polyadenylation signals, even though some lncRNAs are likely to be transcribed by polymerase III (Pagano et al., 2007).

LncRNAs can be classified using a canonical classification (Gibb et al., 2011; Ponting et al., 2009), by which can be grouped into five biotypes according to their proximity to protein-coding genes: sense (overlap coding mRNAs on the same strand), antisense, bidirectional, intronic (in the intron of a protein-coding gene) and intergenic (in intergenic regions located between annotated protein-coding or noncoding genes) (Figure 8).

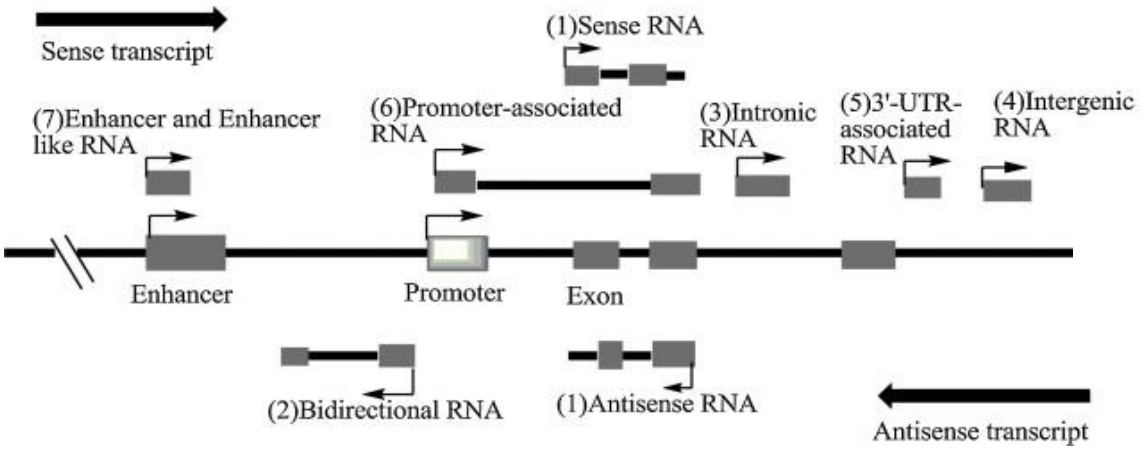


Figure 8. Schematic diagram illustrating the localization of lncRNAs. LncRNA are divided into three large groups. (1) Sense or antisense RNAs (when the lncRNA overlaps one or more exons of another transcript on the same or opposite strand respectively; (2) bidirectional RNAs (when the expression of lncRNA and a neighboring coding transcript on the opposite strand is initiated in close genomic proximity); (3) intronic RNAs, when the lncRNA is derived from an intron of a second transcript; (4) intergenic RNAs, when the lncRNA is localized between two genes, also called large intergenic RNAs, (lincRNAs); (5) 3′-UTR associated RNAs (lncRNAs derived from 3′-untranslated regions of protein-coding transcript, also named uaRNAs) (Mercer et al., 2011); (6) promoter associated RNAs (lncRNAs transcribed from promoter domains of protein-coding genes) (Hung et al., 2011); (7) enhancers or enhancer-like lncRNAs (lncRNAs transcribed from enhancer domains and expressed coordinately with, activity-dependent genes, or lncRNAs exhibiting enhancer activity) (Ørom et al., 2010). Adapted from (Wu et al., 2013a).

3.2 Conservation and Cellular Localization of lncRNAs

LncRNAs are present in large numbers in the mammalian genome (Carninci et al., 2005; ENCODE Project Consortium et al., 2007). Many lncRNAs are scarcely expressed (Derrien et al., 2012), posing a challenge in terms of exploration of lncRNA functions. Several lncRNAs are ultra-conserved in DNA sequence, approximately 3% of lncRNAs appear to have originated more than 300 million years ago and can be found in organisms ranging from Xenopus and chicken to man (Necsulea et al., 2014). In addition, lncRNAs exhibits conserved biological function but low sequence conservation (Brockdorff et al., 1992; Lin et al., 2014; Ulitsky et al., 2011), suggesting that RNA molecules need less sequence conservation to retain their function compared to proteins. Conversely, there is high sequence conservation of lncRNA promoters, which is even higher than that of protein-coding gene promoters (Carninci et al., 2005), suggesting that regulation of lncRNA expression is important.

LncRNAs can be found in many tissues, although the brain and the central nervous system appear to have the highest diversity of expressed lncRNAs (Ravasi et al., 2006). In addition, they tend to be enriched in the nucleus (Cheng et al., 2005; Kapranov et al., 2007), although some lncRNAs localize to the cytosol and actually they associate with ribosomes (van Heesch et al., 2014).

3.3 Functions of lncRNAs

LncRNAs have a broad spectrum of functions involved in almost every aspect of the biological process, from chromatin structure to the protein level. Although the detailed functions of lncRNAs are not yet clearly defined, lncRNAs play different roles in cell physiology. They can function as signals for integrating temporal, spatial, developmental and stimulus-specific cellular information; as decoys with the ability to sequester a range of RNA and protein molecules, thereby inhibiting their functions; as guides for genomic site-specific and more widespread recruitment of transcriptional and epigenetic regulatory factors; and as scaffolds for macromolecular assemblies with varied functions (Figure 9) (Wilusz et al., 2009). Even more, lncRNAs can interact with other three kinds of biomolecules- DNA, RNA and proteins, forming binary even ternary interaction complexes.

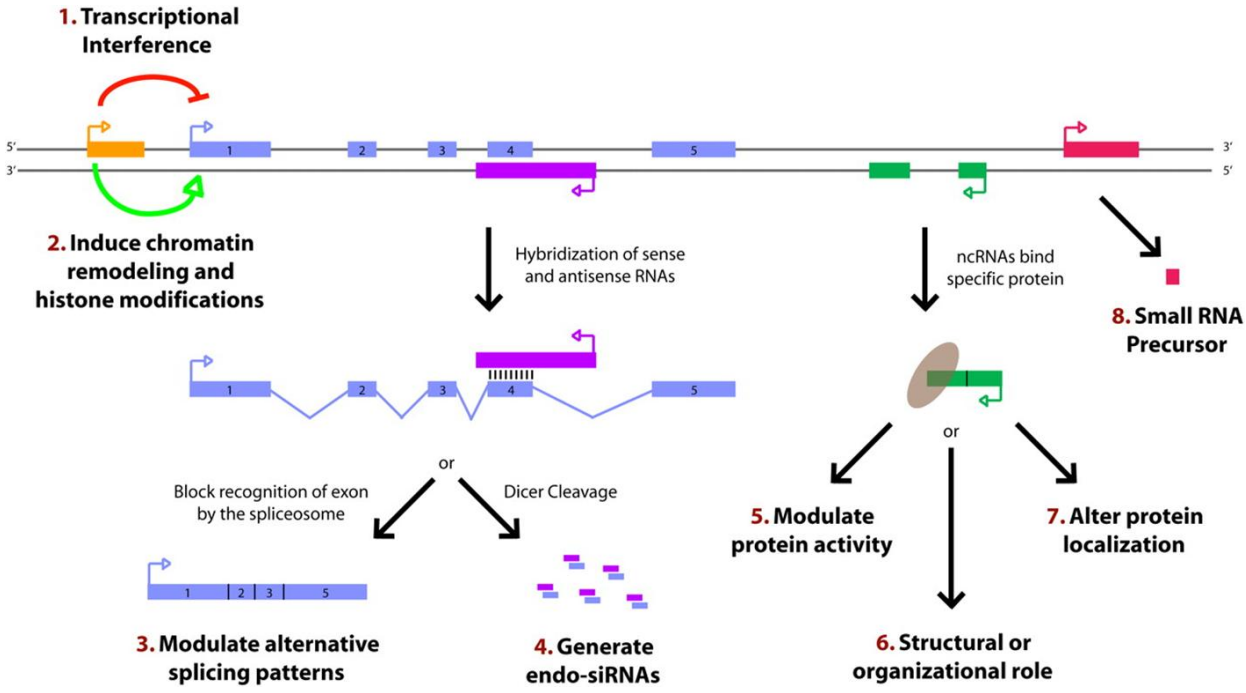


Figure 9. Main functions of lncRNAs. Transcription from an upstream noncoding promoter (orange) can negatively (1) or positively (2) affect expression of the downstream gene (blue) by inhibiting RNA polymerase II recruitment or inducing chromatin remodeling, respectively. (3) An antisense transcript (purple) is able to hybridize to the overlapping sense transcript (blue) and block recognition of the splice sites by the spliceosome, thus resulting in an alternatively spliced transcript. (4) Alternatively, hybridization of the sense and antisense transcripts can allow DICER to generate endogenous siRNAs. By binding to specific protein partners, a noncoding transcript (green) can modulate the activity of the protein (5), serve as a structural component that allows a larger RNA–protein complex to form (6), or alter where the protein localizes in the cell (7). (8) LncRNAs (pink) can be processed to yield small RNAs, such as miRNAs, and other less well-characterized classes of small transcripts. Adapted from (Wilusz et al., 2009).

In the nucleus, the main roles of lncRNAs are associated with the regulation of gene and genome activity on various levels. LncRNAs can influence chromatin modifications and chromatin structure through several processes, such as modifications of histones by the interaction with polycomb repressive complexes (da Rocha et al., 2014; Plath et al., 2003); modulation of DNA methylation (Arab et al., 2014; Di Ruscio et al., 2013); regulation of chromatin remodeling complexes altering the nucleosome spacing (Prensner et al., 2013). Even more lncRNAs interact with transcription factors regulating differentiation process (Jiang et al., 2015; Kurian et al., 2015).

LncRNAs primarily found in the cytosol are thought to be involved in gene regulation on the posttranscriptional level. Several lncRNAs have been shown to influence splicing patterns of either specific genes or globally by interacting with splicing factors (Tripathi et al., 2010). Besides, lncRNAs may control nuclear/cytoplasmic shuttling of mRNA, affecting their availability to the translation

machinery and the resulting protein levels (Gong and Maquat, 2011; Mourtada-Maarabouni and Williams, 2013), or conversely stabilizing mRNAs (Matsui et al., 2008).

3.4 LncRNAs in Glioblastoma

In addition to their activity in normal physiological processes, just as other molecules, lncRNAs are also linked to human diseases, including a variety of human cancers and human genetic disorders (Figure 10) (Brunner et al., 2012; Gibb et al., 2011).

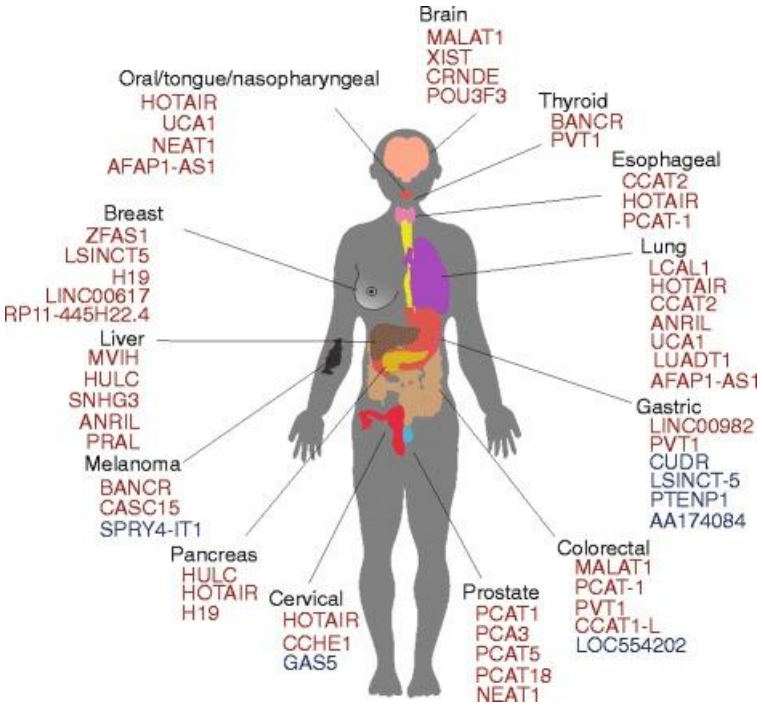


Figure 10. LncRNAs associated in cancer. The color represents either up-regulated (red) or down-regulated (blue) compared to normal tissues. Adapted from (Bartonicek et al., 2016).

Given that numerous lncRNAs are involved in a wide range of CNS pathophysiology, such as neural differentiation (Mercer et al., 2010), brain development (Sauvageau et al., 2013) and neural cell fate (Amaral et al., 2009); they are also key regulators in brain cancers, including glioblastoma (Han et al., 2012). Information on the central role of lncRNAs in gliomagenesis has only become to emerge during the past few years. LncRNAs appear to be exceptionally important in all different aspects of glioma pathophysiology, from malignant transformation to tumor recurrence, and also in disease prognosis (Figure 11).

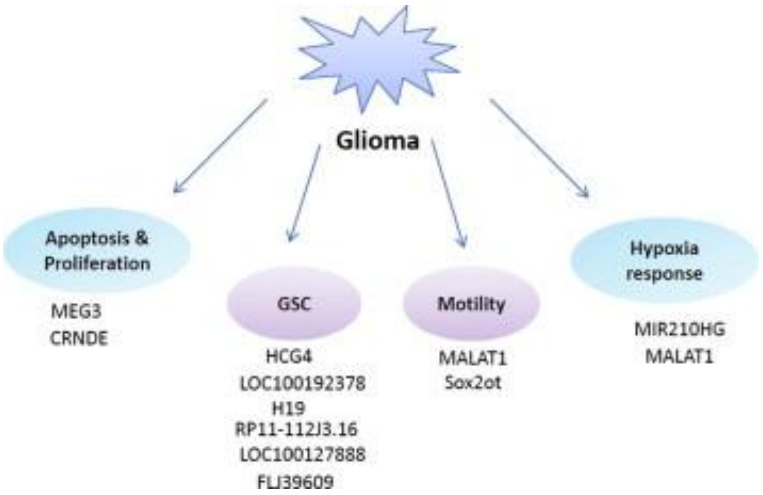


Figure 11. Effects of lncRNAs in regulating glioma biological processes. LncRNAs are implicated in various biological processes of glioma, although the detailed molecular mechanisms remain unclear.. Adapted from (Zhang and Leung, 2014).

LncRNAs are involved in cell proliferation, apoptosis and invasion (Gordon et al., 2010; Shi et al., 2014b; Wang et al., 2012a), and are potentially implicated in determining glioma development through interaction with different molecules and through diverse signaling pathways; for example MEG3 controls proliferation via interacting with p53 and MDM2 protein (Benetatos et al., 2011); CRNDE regulates glioma cell growth via mTOR signaling (Wang et al., 2015b); and ASLNC22381 and ASLNC20819 promote proliferation through the IGF-1R signaling pathway (Trojan et al., 2003). However, the mechanisms through which lncRNAs regulate signaling pathways remain largely unknown.

It has been proposed that dysregulation of lncRNAs are significantly associated with glioma pathogenesis (Yao et al., 2015), through their transcriptional regulation by TFs. Bio-computational analyses have demonstrated abundant TF binding sites in lncRNA promoter regions (Ma et al., 2014b; Yang et al., 2013; Zhang et al., 2015a) . Moreover, TFs could bind directly to lncRNAs and regulate their expressions, for example the lncRNA HOTAIR is a direct target of c-MYC, by which HOTAIR is activated and can drive tumor progression (Ma et al., 2014b).

Differential expressions of lncRNAs between normal and different grades of gliomas offer significant promises of using lncRNA signatures in glioma diagnosis and prognostication (Ma et al., 2015; Vital et al., 2010; Zhang et al., 2012c). In glioma recurrence lncRNAs also participates, for example, the PPAR signaling pathway was found to be the most significant pathway through which glioma-associated lncRNAs may act (Han et al., 2012). Furthermore, profiling studies have used lncRNAs to

establish a molecular sub-classification based on lncRNAs expression with important clinical implications, defining three lncRNAs signature subgroups: astrocytic tumor with high EGFR amplification, neuronal-type tumor and oligodendrocytic tumor enriched with IDH1 mutation and 1p19q co-deletion (Li et al., 2014a). Some lncRNAs have also associated with glioma patient survival (Ma et al., 2015; Zhang et al., 2013).

3.4.1 ANRIL

ANRIL (officially known as CDKN2B antisense RNA 1) was originally identified in the familial melanoma patients (Pasmant et al., 2007). Since its identification, accumulating studies have showed that ANRIL is deregulated in a number of malignancies such as gastric, breast, lung and bladder cancer (Iranpour et al., 2016; Naemura et al., 2015; Zhang et al., 2014a; Zhu et al., 2015). ANRIL was originally identified from familial melanoma patients with a large of germline deletion in the INK4B-ARF-INK4A gene cluster (officially known as CDKN2B, CDKN2AIP and CDKN2A), which was located a 42-kb stretch on the chromosome 9p21 (Gil and Peters, 2006; Kia et al., 2008; Ozenne et al., 2010; Pasmant et al., 2007). This gene locus is transcriptionally silenced or homozygously deleted in a lot of tumors with a frequency of about 40 % exhibiting one of the most diversify genes in human tumors (Gil and Peters, 2006; Kia et al., 2008). The INK4B-ARF-INK4A locus is governed by PRCs and ANRIL is involved in suppressing this locus (Kheradmand Kia et al., 2009; Yap et al., 2010).

Overexpression of ANRIL is linked to poor prognosis in prostate and gastric cancer (Kotake et al., 2011; Yap et al., 2010; Yu and Thomson, 2008), and have been associated with glioma and basal cell carcinoma, discovered based on the known risk loci established through genotyping of cancer patients (Bennett and Swayze, 2010; Guttman et al., 2011). However, the detail molecular mechanism of ANRIL remains to be studied.

3.4.2 SOX2OT

The SOX2 overlapping transcript, known as SOX2OT, is a multi-exon lncRNA which harbors *SOX2* gene in its intronic region and is transcribed in the same orientation as *SOX2* (Fantes et al., 2003). While little is known about the exact role of SOX2OT, recent studies have demonstrated a positive role for it in the regulation of *SOX2* gene in human stem cells (Amaral et al., 2009; Shahryari et al., 2014).

The lncRNA SOX2OT is co-upregulated with master regulators of pluripotency, *SOX2* and *OCT4,* in tumor samples of esophageal squamous cell carcinoma, which suggested a potential part for it in tumorigenesis of esophagus (Shahryari et al., 2014). Overexpression of both SOX2OT and SOX2 has been reported in human primary lung cancer tissues, in comparison with the corresponding non-tumor samples, where they act as a novel prognostic indicators (Hou et al., 2014). Furthermore, SOX2OT plays regulatory function in cell cycle progression; being associated with carcinogenesis in different human tumors, such as breast (Askarian-Amiri et al., 2014), and esophagus (Shahryari et al., 2014) cancers. Current evidences indicate a functional association between SOX2OT and SOX2 in tumorigenesis, cellular differentiation, and pluripotency, although more remains to be investigated on the mechanisms underlying this regulation (Shahryari et al., 2015).

3.5 LncRNAs in NSCs and GSCs

The functional role of lncRNAs in GSCs have been demonstrated in a comparative analysis of microarray data, where different patterns of lncRNAs expression, such as H19, XIST and MIAT in undifferentiated tumor cells were found (Bao et al., 2006a; Cheng et al., 2010; Galli et al., 2004). In an exploratory analysis, a set of 35 lncRNAs were differentially expressed between progenitor and mature states, showing that at least one lncRNA can interact with the neurogenesis repressor complex REST/NRSF (neural restrictive silencer factor) to regulate neuronal gene expression (Ng et al., 2012). Another refined study monitored induced pluripotent stem cells (iPSC)-NSC differentiation, and revealed a gradual increase in the expression of different lncRNAs as NSCs differentiate (Hjelm et al., 2013). Another report using adult NSCs in mice has further confirmed that lncRNAs increase as cells differentiate (Ramos et al., 2013), indicating that lncRNAs are important regulators of pluripotency and neurogenesis, and exert important roles in human brain development.

Recent global analysis showed that cancer transcriptome is more complex than previously anticipated. Dysregulated expression of lncRNAs has potential pervasive roles as drivers of human cancers. Therefore, understanding the precise molecular mechanisms of lncRNAs to the various biological processes will be a critical step in exploring new strategies in future cancer therapy.

Hypothesis and Objectives

Hypothesis

SOX2 is highly expressed in the GSC compartment, where it controls stemness properties and maintenance of tumorigenicity; however its mechanism of action in the context of the GSCs is poorly understood. We hypothesized that SOX2 plays a prominent role in driving the growth, treatment resistance and recurrence of glioblastoma, through the orchestration of different transcriptional pathways. The elucidation of the transcriptome and the molecular pathways involved in the generation and maintenance of GSCs is critical to understand the molecular underpinnings of glioblastoma malignancy and could allow the identification of relevant and novel therapeutic targets.

In order to address our hypothesis we propose the following aims.

Aims

1. To identify the gene-coding landscape controlled by SOX2 in GSCs
2. To uncover the non-coding landscape controlled by SOX2 in GSCs
3. To study the functional role of key miRNAs controlled by SOX2 in GSCs *in vitro* and *in vivo*.

Materials and Methods

1. Cell lines and culture conditions

We worked with different GSC lines in order to have a representative number of cell lines. Some of them were available at our group, other were available at Dr. Laterra´s group, where I made a short-term stay performing some of the experiments of this thesis.

The adult neurosphere lines GSC-11 and GSC-23, a kind gift of Dr. Lang at UT MD Anderson Cancer Center, were established from acute cell dissociation of human glioblastoma surgical specimens and maintained in our lab. The neurosphere lines GBM1A and GBM1B were originally derived and characterized by Vescovi and co-workers (Galli et al., 2004) and available at Dr. Laterra`s lab. All neurosphere cell lines were cultured and maintained in serum-free medium containing Dulbecco's modified Eagle's medium/nutrient mixture F12 (1:1, vol/vol) (Thermo Fisher Scientific Inc, Waltham, MA) supplemented with 10% of Penicillin/Streptomycin (Lonza, Verviers, Belgium) B27 10x (Thermo Fisher Scientific Inc, Waltham, MA) and 20 ng/ml of both EGF and FGF (Sigma-Aldrich, St Louis, MO) according to the procedures described by Galli (Galli et al., 2014).

The human glioblastoma xenograft line Mayo 39, was originally obtained from the Mayo Clinic (Rochester, MN) and maintained at Dr. Laterra`s group as attached cell line. The established glioma cell lines U87 MG, A172 and the human embryonic kidney 293FT (HEK293FT) cell lines were obtained from the ATCC and were grown attached. All attached cell cultures were maintained in Dulbecco's modified Eagle/F12 medium (1:1, vol/vol) and supplemented with 10% FBS (Fetal Bovine Serum, Thermo Fisher Scientific Inc, Waltham, MA). Finally all cell lines were grown at 37°C in a humidified incubator with 5% CO_2. All cell lines are routinely fingerprinted to ensure authenticity.

2. Patient samples and ethic statement

Glioblastoma tissues were obtained from surgical procedures performed at the Department of Neurosurgery of the University Hospital of Navarra. Tissue samples were resected during surgery and immediately frozen in liquid nitrogen. We collected 30 human glioblastoma specimens. Written informed consent was obtained from all subjects, specifying that sample collection was for experiment purposes, and approved by the Ethics Committee of of the University Hospital of Navarra (Ref. 123/2014).

3. Transient transfection of cells

To inhibit SOX2 expression, transient transfection assays were performed using commercially available, specific siRNA against human SOX2 (si-SOX2, si13295) and a non-targeting control siRNA (si-Scramble) (Ambion) in four independent experiments. The siRNA transfections were performed according to the manufacturer's instructions using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The cells were then cultured for 72 h after transfection and subjected to different analysis.

To down-regulate miR-301a expression, GSC-23 cells were transfected with an anti-miR inhibitor miR-301a (anti-miR-301a) (MH10978, Ambion), or a scrambled sequence miRNA (anti-miR-Sc) (AM17010, Ambion) to a final concentration of 100nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's recomendations. Cells were harvested 72h post-transfection and subjected to different analysis.

4. Lentivirus generation

Lentiviral vectors are potent gene delivery vehicles that enable stable expression of transgenes in both dividing and post-mitotic cells. Lentivirus are capable of infecting a wide range of cell types and tissues *in vitro* and *in vivo*, without toxicity or immune responses (Blomer et al., 1997; Kafri et al., 1997; Miyoshi et al., 1997; Naldini et al., 1996), integrate stably into the host genome, and result in long term expression of the transgene. The safety of the lentiviral vectors has been further improved with the generation of self-inactivating vectors and a minimal packaging system (Miyoshi et al., 1997; Zufferey et al., 1998).

For the production of lentiviral particles, we used a 2nd-generation lentiviral system according to Addgene instructions, using three components: 1) the lentiviral transfer vector containing the insert of interest, in this case we used LentimiRa-Off-hsa-miR-425-5p vector to inhibit the expression of miR-425-5p or pLenti-III-mir-GFP as a positive control empty vector (Applied Biological Materials Inc, Richmond, BC, Canada), 2) one packaging vector which contain all necessary viral structure proteins, psPAX2 (Addgene, Cambridge, MA), and 3) an envelope vector expressing Vesicular Stomatitis Virus (VSV) glycoprotein (G), using the pMD2.G plasmid (Addgene, Cambridge, MA). The plasmid map of LentimiRa-Off-hsa-miR-425-5p vector is shown in Figure 12. Co-transfection of the lentiviral packaging/envelope plasmids and transfer

vector into the HEK239FT packaging cell line, allows efficient production of lentiviral supernatant. The lentivirus miRNA inhibitor used enables a long-term potent inhibition of miR-425-5p without repeated transfections. Figure 12 outlined the process of the production of infectious lentivirus and cell infection.

First, 1 × 10^6 HEK293FT cells were seeded on 10 cm plates and 24h later were transfected with 2 µg of PAX2 packaging plasmid, 1 µg of pMD2.G envelope plasmid, and 3 µg of lentiviral vector using 18 µl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The lentiviral particles in supernatant were collected at 48–72 h and used to infect cells.

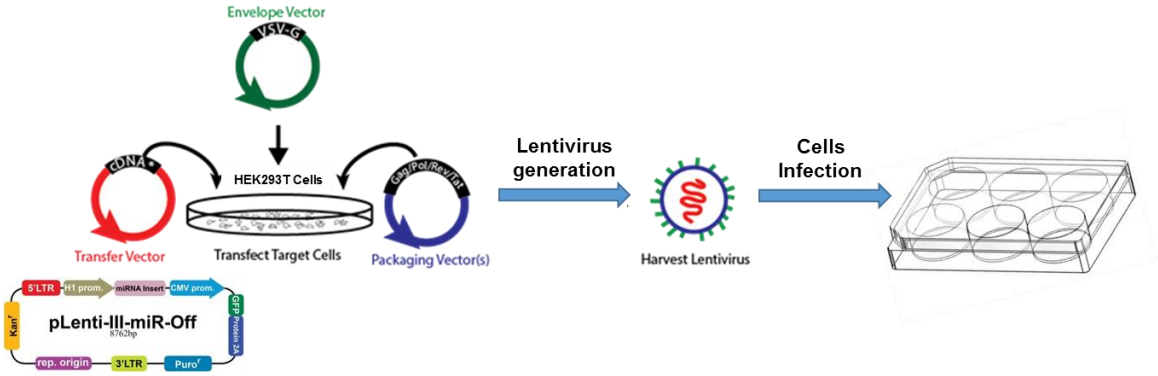


Figure 12. Scheme of the production of 2nd-generation lentiviral system and cell infection, adapted from (Addgene Lentiviral guide).

5. Lentiviral transduction

GSC-23, GSC-11, GBM1A, GBM1B, A172, U87 MG and HEK293T cells were transduced with lentiviral particles. Briefly, a total of 1.5 × 10^4 cells were seeded in a 6-well cell culture plate and infected with lentiviral medium containing lentiviral particles and polybrene (1 µg/mL), supplemented with appropriate medium. GFP expression was assessed using an inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

6. Forced differentiation of neurospheres

Forced differentiation was performed according to the method of Galli and co-workers (Galli et al., 2004) with some modifications. Briefly, the neurosphere cells were plated onto Matrigel in FGF-containing neurosphere medium (no EGF) for 2 days and subsequently grown in 1% FBS without EGF/FGF for 5 days.

7. Neurosphere size assessment

To assess neurosphere size, cells were dissociated into single cells and cultured in ultra-low attachment flasks (2.5×10^4 cells/ml). After 10 days, neurospheres were embedded in 1% agarose and stained with 0.1% Wright stain solution for 1–2 h at 37 °C. Cells were washed four times with phosphate-buffered saline (PBS) and incubated at 4 °C overnight (in PBS) before quantification. Spheres larger than 100 µm were quantified using computer-assisted image analysis (MCID™ Analysis Software).

8. Cell viability assay

To assess the effect of the inhibition of miR-425-5p expression in cell viability we used the Cell-Titer 96 One Solution Aqueous Proliferation kit (Promega) following manufacturer's instructions. This is a colorimetric method for determining the number of viable cells in proliferation. This assay contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. The MTS tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells.

Neurosphere cells were seed at a density of $5x10^3$ cells per well in a 96-well plate. After either 3 or 5 days, MTS reagent was added to cells, incubated during 2h at 37ºC and then the absorbance was measured at a wavelength of 490nm in a Sunrise microplate reader with Magellan Software (Tecan, Mannedorf, Switzerland).

9. Cell cycle analysis

To analyze the cell cycle progression after miR-425-5p inhibition we used flow cytometry. GSC-23 cells were collected after 7 days of infection with miR-425-5p lentivirus, washed with PBS 1x, fixed by adding ice-cold 70% ethanol and store at 4°C for at least 30 min. Then cells were washed with PBS and stained with10 µg/ml propidium iodide (Roche), 100µg/ml RNase (Sigma-Aldrich) in PBS, and incubated at 37°C for 30 min in the dark. The percentage of cells in the different phases of the cell

cycle was measured with a FACS Calibur instrument (Becton-Dickinson, Franklin Lakes, NJ, USA) and analyzed with FlowJo (Ashland, OR, USA) software.

10. Migration and invasion assays

The Transwell migration assay is a commonly used test to study the migratory/invasive response of cells to chemical cues. This assay consists of a chamber with a membrane which separates the wells of a multiwell plate into top and bottom compartments. Cells were seeded into the top compartment and the chemo attractant solution is placed in the bottom compartment. After incubation, counting the cells in the bottom compartment allows quantification of migration induced by chemo attractants. For invasion assay the pores of the membrane were covered with a gel composed of extracellular matrix, known as Matrigel. By placing the cells on one side of the gel and a chemo-attractant on the other side of the gel, invasion was determined by counting those cells detected in the lower side of the compartment, having invaded towards the higher concentration of chemoattractant (Figure 13).

In our study, 1×10^4 cells were plated in the top chamber with the non-coated membrane (24-well insert; pore size, 8 µm; BD Biosciences). For invasion assays, 1×10^5 cells were plated in the top chamber with a Matrigel-coated membrane (24-well insert; pore size, 8 µm; BD Biosciences). In both assays, cells were plated in serum-free medium without growth factors, and medium supplemented with 2% fetal bovine serum was used as a chemo attractant in the lower chamber. The cells were then incubated for 12h for invasion or 6h for migration at 37°C. Cells that did not migrate or invade through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were stained with crystal violet and counted under a conventional microscope at 200X magnification (Leica Microsystems, Wetzlar, Germany).

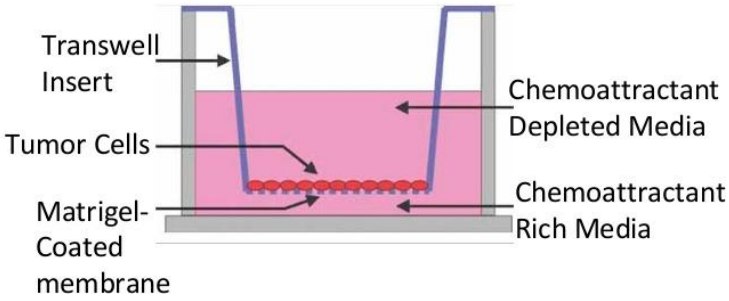


Figure 13. Schematic representation of Transwell invasion assay.

11. Caspase 3/7 activity

The activation of Caspase 3/7 was assessed using the Caspase-Glo 3/7 assay kit (Promega Corp, Wisconsin, USA). Caspase 3 and 7 detection is based on coupled reactions. In the first reaction, the cleavage of the target tetrapeptide sequence DEVD by active caspase 3 and/or 7 takes place. This cleavage produces aminoluciferin, which is further used as the substrate for the luciferin enzyme in the second reaction, to finally produce a light signal that could be measured in a luminometer.

Briefly, 1.5×10^4 cells were seeded in quadriplicate into 96-well plate after 1 day of infection and 50 µL of Caspase-Glo 3/7 reagent was added in quadruplicate the days of measure (7 and 10 days after infection) and incubated during 1h at 37ºC. Bioluminiscent fluorescence was detected using spectrofluorometer (SpectraMAX Gemini XS, Molecular devices). Luminescence was proportional to the amount of caspase activity present, so the proportional fluorescence intensity between infected and non-infected cells allowed the determination of the changes in caspase activity.

12. Annexin V assay

For apoptosis determination we used APC-Annexin V (BD Pharmingen™, San Jose, CA) staining and SYTOX™ Blue (Invitrogen, Carlsbad, CA) for determination of viability. In apoptotic cells, phosphatidyl serine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. Annexin V labeled with a fluorophore or biotin can identify apoptotic cells by binding to PS exposed on the outer leaflet of the membrane. SYTOX™ Blue dead cell stain is a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes but will not cross uncompromised cell membranes. After brief incubation with SYTOX™ Blue stain, the nucleic acids of dead cells fluoresce bright blue when excited with 405 nm violet laser light. Briefly, 1.5×10^5 GSC-23 cells were seeded into 6-well plate and after 7 days of transduction cells were washed and resuspended in 1x Binding Buffer. APC Annexin V and SYTOX Blue were added to cells and incubated during 15 min in the dark and analyzed by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA).

Viable cells are both APC-Annexin V and SYTOX Blue negative, while cells that are in early apoptosis were APC-Annexin V positive and SYTOX Blue negative and

cells that were in late apoptosis or already dead are both APC-Annexin V and SYTOX Blue positive.

13. Transmission electron microscopy

To perform the ultrastructural analysis of cell morphology after inhibition of miR-425-5p expression we used the transmission electron microscopy (TEM). TEM is a high resolution microscopy which allows enough magnification to visualized cell morphology. GSC-23 cells were collected after 7 days of infection with miR-425-5p lentivirus and fixed with a solution containing 4% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4 during 1h at 4ºC. Then cells were centrifuged and treated with 0.25M of sacarose in 0.1M cacodylate buffer, postfixed with 1% buffered osmium tetroxide for 1h at 4ºC and stained in block with 1% Millipore-filtered uranyl acetate. The samples were dehydrated with increasing concentrations of ethanol, infiltrated and embedded in LX-112 medium. Then, cells were polymerized in an oven at 60ºC for 2 days. Ultrathin section (65nm) were cut in a Leica Ultracut microtome, stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a Jeol-1210 transmission electron microscope (Jeol Ltd., Herts, UK) in the Department of Histology and Pathology of The University of Navarra.

14. Array Studies

14.1 Microarray expression analysis

To identify genome-wide SOX2-regulated coding and non-coding transcripts, we subjected samples to an Agilent Array. Total RNA was isolated from scrambled and SOX2-siRNA GSC-11 cells using Trizol extraction and quantified using Nanodrop 1000 (Thermo Fisher Scientific). RNA was purified by the QIAGEN RNAeasy mini kit (QIAGEN) according to the manufacturer´s protocol. One-color Cy3 RNA labeling, array hybridization to Agilent SurePrint G3 8 × 60 K Human Gene Expression Arrays (Agilent Technologies), data collection, and analysis were performed at the Department of Biostatistic (Fundación para la Investigación Médica Aplicada, CIMA, Pamplona, Spain). The microarray data from this study have been submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE79302.

14.2 TaqMan Low Density microRNA Array (TLDA)

Total RNA from scrambled and SOX2-knock down GSC-11 cells was reverse transcribed into cDNA using Taqman miRNA Reverse Transcription Kit (Applied Biosystems) and loaded onto a TLDA Human miRNAs panel containing 384 wells according to manufacturer's protocol (Applied Biosystems). Quantitative miRNAs expression data were normalized with U6b housekeeping gene and quantified using ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA).

14.3. Functional group analysis

In this study we applied Gene Ontology (GO) analysis to find the primary function of the differential expression of mRNAs regulated by SOX2, using online software DAVID (Database for Annotation, Visualization and Integrated Discovery, http://david.abcc.ncifcrf.gov/). GO analysis can organized genes into hierarchical categories (Gene Ontology Consortium, 2006). To identify the significant pathway of the differential genes participating we performed gene regulatory network analysis using Ingenuity Pathway Analysis (IPA) software (http://www.ingenuity.com), which can integrate gene-expression data with other molecular databases to facilitate the development of new and more complete pathway maps. Fisher's exact test was used to select the significant GO categories. The threshold of significance was defined by P value with a cut-off set in 0.05.

15. *In silico* prediction of binding sites

SOX2 binding sites 2 kb upstream of the miR-301a-3p and miR-425-5p translation start site were identified using the PROMO (Messeguer et al., 2002) algorithm using search term 'SOX2' (Transcription factor identifier: T01836), which is specific for SOX2.

16. Predicted target gene analysis

In the present study miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/), miRDB (http://mirdb.org/miRDB/) and PicTar (http://pictar.mdc-berlin.de/) were used to predict target genes for miR-301a-3p and miR-425-5p. We considered as target genes only genes that were predicted by all three software programs.

17. Total Nucleic Acids Methods

17.1 RNA extraction

For RNA isolation, cells were incubated with Trizol reagent (Life Technologies, California, USA). Trizol is a monophasic lysis solution which recovers total RNA and inactivates RNAse enzymes. Chloroform addition separates the RNA from the DNA, maintaining the RNA exclusively in the aqueous phase. After phase separation, RNA is precipited by the addition of isopropanol and then followed by drying with the subsequent ethanol step. Finally RNA is hydrated with free RNAse water (Sigma) (Green and Sambrook, 2012). RNA samples were quantified using Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) and then stored at -80ºC until further used.

17.2 Reverse Transcription

cDNA (complementary DNA) is the total RNA copy translated to DNA. For gene expression analysis we obtained cDNA by reverse transcriptase reaction (retro-transcription) using total RNA as the template. The primers are known as random hexamers, which allow the copy of total RNA in the same proportion and this characteristic allows the evaluation of initial RNA (Green and Sambrook, 2012). cDNA was obtained by standard retro-transcription, using the MultiScrib Reverse Transcriptase kit (Thermo Fisher Scientific). For the procedure, the following mixture was prepared:

Total RNA (extracted from cells)	1µg
MultiScrib Reverse Transcriptase	1 µL
10x RT Buffer	2 µL
dNTPs (2.5mM each)	1 µL
Random deoxynucleotide hexamers (60 ng/µL)	1 µL
RNAsa Inhibitor (1U/uL)	1 µL
DEPC water	Up to 20 µL

Sample reaction was incubated at 25ºC for 10 minutes, followed by a 2 hours step at 37ºC in the thermal cycler GeneAmp PCR System 2700 (Applied Biosystems), and cDNA was stored at -20ºC until further evaluation.

For miRNA analysis, reverse transcriptions were performed using TaqMan® miRNA Reverse Transcription Kit (Life Technologies) and specific RT Taqman primer for each miRNA, according to the manufacturer's instructions.

17.3 Quantitative real-time PCR

Real Time (RT) Polymerase Chain Reaction (PCR) allows monitoring the PCR in a real time. In our study, we used the Taqman Master Mix No Amperase (Applied Biosystems) for miRNA and lncRNA expression analysis and the SYBR-Green Master Mix (Applied Biosystems) for gene expression analysis. SYBR-Green dye is a cyanine molecule that intercalates with the DNA by non-specifically binding. The complex DNA-dye emits green light (497nm). We analyzed relative levels of several genes and lncRNAs, listed in the following table and normalized against human GAPDH levels. MiRNA levels were normalized against human U6b housekeeping RNA.

To guarantee the specificity of the reaction, all pairs of primers were tested using the melting curve analysis and afterwards PCR products were visualized in agar gels. RT-PCR was monitored using ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). Expression levels were calculated using the ddCt method (Livak and Schmittgen, 2001). P-values were calculated by analysis of variance using Microsoft Excel.

Primers for lncRNA detection and quantification were designed at Universal Probe Library Assay Design Center (http://www.roche-applied-science.com/). Each full sequence of the selected candidate lncRNAs was searched by UCSC Genome Browser Home and inputted into the Roche Applied Science: Universal Probe Library System-Assay Design Center (https://www.roche-applied-science.com) to design primers for the RT-PCR. All primer sequences are listed in Appendix 1.

17.4 Chromatin immunoprecipitation

SOX2 binding sites 2kb upstream of the miR-425-5p translation start site were identified using the PROMO algorithm (Messeguer et al., 2002), using search term 'SOX2' (transcription factor identifier: T01836) which is specific for SOX2. Chromatin

immunoprecipitation was performed using the MAGnify Chromatin Immunoprecipitation System (Life Technologies Corporation). Briefly, DNA from GBM1A neurosphere over-expressing SOX2 was crosslinked using formaldehyde and chromatin was isolated and fragmented by sonication. DNA fragments were incubated with specific antibodies, ChIP formulated, against human SOX2 (Cell Signaling) or IgG (Life Technologies) overnight at 4 °C. Precipitation of DNA fragments complexed with SOX2 at the miR-425-5p promoter was quantified using qRT–PCR. We select a specific region of miR-425-5p promoter region (Region 1) and design a pair of primers covering multiple SOX2 potential binding sites. Primers targeting promoter regions lacking SOX2 binding sites were used as a negative control. Primer sequences for Region 1 were: Forward primer: 5`- CCTGCCCCACGGATCTAA-3` and Reverse primer: 5`-AGCAGGGGACGAAATCCAA-3`.

18. Protein Methods

18.1 Protein isolation

For protein extraction, cells were washed twice with PBS and then lysed during 30 min at 4ºC in lysis buffer (PBS 1% Triton X100) supplemented with one tablet protease inhibitor cocktail (Roche, Basilea, Switzerland) per 5ml of lysis buffer. Samples were centrifuged during 15 min at 10 000G at 4ºC on a benchtop centrifuge (Eppendorf). Supernatants were collected and transferred to a clean tube and store at -80ºC.

18.2 Protein quantification

Protein concentration was determined by a colorimetric assay. We used the Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratory, West Berkeley, CA). We performed a BSA (bovine serum albumin, Sigma-Aldrich, St Louis, MO) standard curve. Protein samples were added in duplicates and mixed with protein assay diluted 1/5. Absorbance at 550 nm was measured in a Sunrise Microplate Reader, using Magellan Software (Tecan, Mannerdorf, Switzerland).

18.3 Immunoblotting Assay

Immunoblotting (Western Blotting, WB) is a rapid and sensitive assay for the detection and characterization of proteins, based on the inherent specificity in antigen-antibody recognition. The technique uses three elements to accomplish this task: (1)

solubilization and electrophoretic separation of proteins by size, (2) transfer and irreversible binding to nitrocellulose, PVDF, or nylon, and (3) marking target protein using a proper primary and secondary antibody to visualize. The immunoblotting technique is useful in identifying specific antigens recognized by polyclonal or monoclonal antibodies and is highly sensitive (1 ng of antigen can be detected). The membrane is then detected using the label antibody, usually with an enzyme such as Horseradish Peroxidase (HRP), which is conjugated with a secondary antibody that specifically binds to the primary one, and the signal is captured on a X-ray film. Time of exposition is variable, intense bands appears within few seconds, whereas weak bands need 30 min at least to be developed (Green and Sambrook, 2012).

Western blot uses two different types of agarose gel: stacking and separating gel. The higher, stacking gel is slightly acidic (pH 6.8) and has a lower acrylamide concentration making a porous gel, which separates protein poorly but allows them to form thin, sharply defined bands. The lower gel, known as the resolving gel, is basic (pH 8.8), and has a higher polyacrylamide content, making the gel's pores narrower. Protein is thus separated by their size, as the smaller proteins to travel more easily and hence rapidly, than larger proteins.

In this case, proteins were separated according to their size mixing 25-40µg of samples with commercial loading buffer NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA). For reducing conditions, 10% β-mercaptoethanol was also added. The mixed was heated for 5 min at 95ºC, in order to denature the higher order structure while retaining sulfide bridges, snap-cooled to avoid protein refolding, and loaded into the gel.

Electrophoresis was carried out during 90 min at 120V in running buffer (50mM MOPS, 50mM Trizma-base, 0.1% SDS and 1mM EDTA, pH 7,7). After electrophoresis, gels were carefully placed in cuvettes, and rinse with ultrapure water. Then proteins were transferred electrophoretically during 2 to 3 hours at 100V from the gel into a nitrocellulose membrane (0,45 µm pore size). In order to verify the correct amount of protein loaded, membranes were stained with the removable stain Ponceau S (Sigma-Aldrich, St Louis, MO).

Nitrocellulose membrane (Bio-Rad Laboratories, West Berkeley, CA) was blocked using 5% nonfat dried Milk diluted in PBS 0,1% during 1 hour to avoid non-specific union of antibodies. The proteins immobilized in the membrane were incubated with a

primary specific polyclonal or monoclonal antibody at 4ºC overnight, with the subsequent secondary HRP-conjugated secondary antibody that specifically binds to the primary antibody.

To detect the signal, membrane was incubated with Amersham Enhanced Chemiluminiscence (Perkin Elmer Waltham, MA). The X-ray film confrontation with the membrane was performed in darkness, using an automated Curix 60 device (AGFA, Mortsel, Belgium) to reveal the X-ray film.

Table 2. Antibodies used in Western Blot

Antibody	Dilution	Company	Reference
α-SOX2	1/1000	Cell Signaling Technology, Danvers, MA	2748 (Rabbit)
α-Caspase 3	1/1000	Cell Signaling Technology, Danvers, MA	9668 (Mouse)
α-Cleaved Caspase 3	1/500	Cell Signaling Technology, Danvers, MA	9661 (Rabbit)
α-PARP1	1/1000	Cell Signaling Technology, Danvers, MA	9542 (Rabbit)
α-p62	1/1000	Sigma-Aldrich, St Louis, MO	P0067 (Rabbit)
α-E2F1	1/1000	Santa Cruz Biotechnology, Santa Cruz, CA	SC-251 (Mouse)
α-CDK4	1/1000	Cell Signaling Technology, Danvers, MA	9868 (Rabbit)
α-CDK6	1/1000	Cell Signaling Technology, Danvers, MA	9868 (Rabbit)
α-Phospho-Cyclin D1	1/1000	Cell Signaling Technology, Danvers, MA	3300 (Rabbit)
α-AKT(pan) C67E7	1/1000	Cell Signaling Technology, Danvers, MA	4691 (Rabbit)
α-Phospho-AKT (Ser473)	1/1000	Cell Signaling Technology, Danvers, MA	4060 (Rabbit)
α-H2AX	1/1000	Cell Signaling Technology, Danvers, MA	9718 (Rabbit)
α-Tubulin	1/1000	Sigma-Aldrich, St Louis, MO	T40206 (Mouse)
α-GRB2	1/1000	BD Transduction Laboratories™, San Jose, CA	610112 (Mouse)
α-GAPDH	1/1000	Abcam plc, Cambridge, UK	Ab9485 (Rabbit)
α-Mouse IgG HRP	1/5000	Sigma-Aldrich, St Louis, MO	A9044
α-Rabbit IgG HRP	1/5000	Cell Signaling Technology, Danvers, MA	7074

19. Animal Methods

19.1 Intracranial animal model

All the animal experimental protocols were reviewed and approved by the Comitee of Bioethics of the Government of Navarra and the Institutional Animal Care Department. In this work we used the guide-screw animal orthotopic brain tumor model described by Lal and colleagues (Figure 14) (Lal et al., 2000). Briefly, it consists in two different surgeries where the animal is anesthetized by intraperitoneal injection with ketamine (Imalgene)/xylazine (Rompun 2%, Bayer) solution (200 mg ketamine and 20 mg xylazine in 17 ml of saline) at a dosage of 0,15 mg/10g body weight. The first procedure consists in the implantation of 2.6-mm guide screw at 2,5 mm lateral and 1 mm anterior to the bregma. The second intervention is performed one week after bolt implantation and involves the cell injection using an infusion pump. We used the GSC-11 cell line, grown as neurospheres (500 000 cells/mouse) diluted in 5µL of free medium. Cells were implanted through the screw using a Hamilton syringe (Fisher Scientific). In order to avoid a reflux, an infusion pump (Harvard Apparatus, Holliston, MA) was used to control the flow ratio using 15 µL for 1 hour.

Athymic nude female mice were obtained from Harlan Laboratories (Barcelona, SP) and maintained at The Center for the Applied Medical Research (CIMA, Pamplona, SP) in specific pathogen-free conditions and feed standard laboratory chow. Experiments were performed using 6-8 weeks old mice and were sacrificed when symptoms; including neurological deficit (hemiparesis, seizures), unresponsiveness to stimuli, hunched posturing and weight loss.

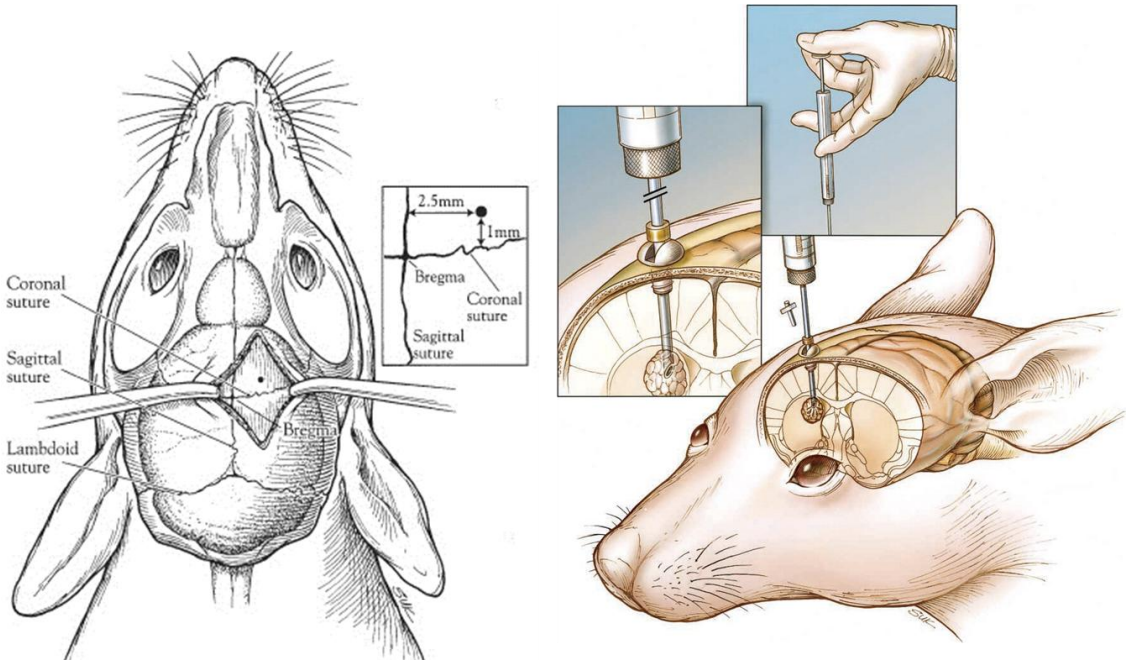


Figure 14. Artist`s drawing showing the method of implanting the guide screw and the injection of cells, by (Lal et al., 2000). Left: The skin is incised and the coordinates for the point of screw implantation are visualized on the skull. This point is directly over the caudate nucleus. Right: Three-dimensional and cross-sectional view of an engrafted tumor being treated with an intratumoral injection by using the Hamilton syringe. The screw fixes the anterolateral point of injection and the depth is determined by the cuff in the Hamilton syringe.

19.2 Immunohistochemical analysis

Immunohistochemistry (IHC) is a wide-used biological technique that combines anatomy, physiology, immunology and biochemistry. Developed from the antigen-antibody binding reaction, immunohistochemistry can be considered as a method that visualizes distribution and localization of specific antigen or cellular components in tissue sections. It comprises three major components: 1) primary antibody binds to specific antigen, 2) the antibody-antigen complex is formed by incubation with a secondary, enzyme-conjugated, antibody and 3) the enzyme catalyzes the substrate generating colored deposits at the sites of antibody-antigen binding.

In this study, brains extracted from treated mice were fixed in formaldehyde for 48 hours, at that point they were transferred to ethanol, and then embedded in paraffin blocks and sectioned (5 µm) (Morphology Core facility, CIMA). For histological analysis, slides were stained with Hematoxylin and Eosin. For IHC, sections were deparaffinized, hydrated and incubated for 10 min with 3.3% H_2O_2 in water to block endogenous peroxidase. Standard protocols for antigen retrieval were employed.

Samples were incubated with primary antibodies at 4°C overnight. Images were captured with a fluorescent microscope (Zeiss AXIO Imager Z1) equipped with Imaging System V.5.0 software (MetaSystem GmbH, Altlussehem, Germany).

20. Statistical analysis

Experimental data are represented as the mean ± standard deviation of three biologic replicates and were compared using Student's t-test. Significant P-values are indicated with asterisks as follows: *P < 0.05, **P < 0.01, ***P < 0.001. Survival rate was performed using Kaplan-Meier method. For statistical analysis the log rank was used. The statistical program used for the analysis is the GraphPad Prism Software (GraphPad Software, San Diego, CA).

Results

1. Transcriptomic signature regulated by SOX2

Since SOX2 is a key driver in the maintenance of the GSCs phenotype and therefore in the perpetuation of this devastating tumor, we down-regulated the expression levels of this gene in the human glioblastoma stem cell line GSC-11. We performed four independent experiments, using a siRNA specific for SOX2 (Figure 15A). The efficiency of SOX2 knockdown was assessed by real-time PCR and western blot analysis (Figure 15B) and was also confirmed in our array results. Microarray data identified a total of 2048 differentially expressed coding transcripts and 261 non-coding transcripts (B value >0) (Figure 15A).

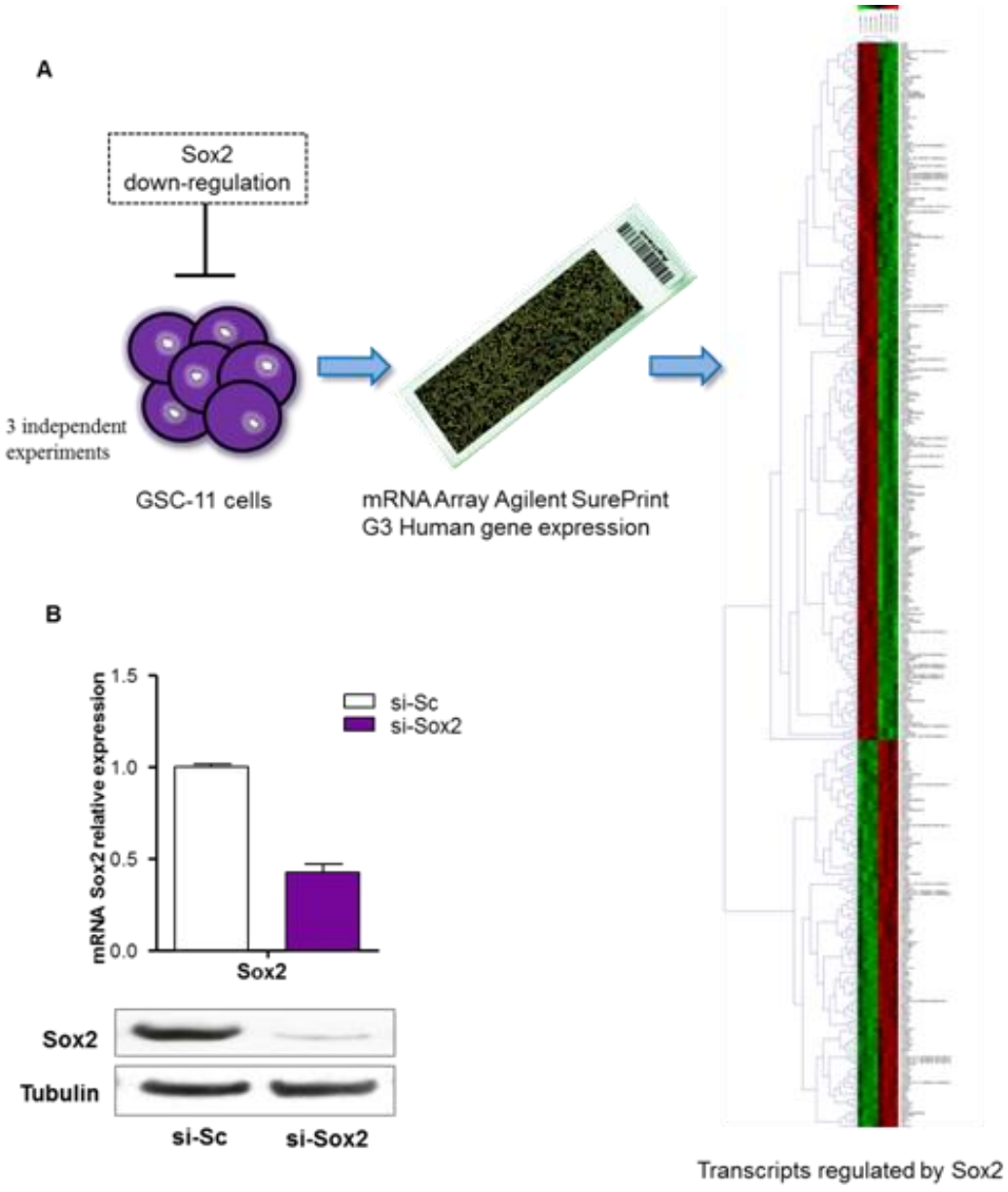


Figure 15 Transcripts regulated by SOX2. (A) Schematic representation of the research design employed to uncover the SOX2 transcriptome in GSC-11 cells. (B) qRT-PCR and western blot confirmation of SOX2 inhibition in GSC-11 cells after 72h of si-SOX2 or si-Scramble (si-Sc) transfection. SOX2 relative mRNA levels are presented as $2^{-\Delta\Delta}$Ct standardized with their constitutive

gene GAPDH. Each bar represents the mean ± SD. For western blot Tubulin was used as housekeeping control and shown as a representative blot of four independent experiments. For space reason we have omitted the list of candidate transcripts regulated by SOX2.

1.1 SOX2 controls a wide spectrum of protein-coding genes and pathways in GSCs

To further narrow the coding transcripts data, we set a cut-off of 1 logarithmic fold change difference and B>0 between SOX2 knockdown and scrambled GSC-11 cells, identifying 35 up-regulated and 100 down-regulated genes. These results suggest that SOX2 act primarily as a transcriptional activator. In Table 3 we showed the top-10 up or down-regulated protein coding-genes. We selected the top 5 candidates of each group for further validation by qRT-PCR in the GSC-11 and GSC-23 cell lines. We confirmed the observed microarray expression changes in 5 out of 5 down-regulated coding-genes in GSC-11 cells and in 4 out of 5 in GSC-23 cells, including SOX2 (Figure16A). Regarding the up-regulated coding-genes in GSC-11 and GSC-23 we confirmed one out of 5 and 5 out of 5 in GSC-11 and GSC-23 respectively (Figure16B), partially validating our microarray results.

Table 3: The top 10 up- and down-regulated protein-coding genes in SOX2-down-regulated GSC-11 cells, organized by logFC.

GeneName	logFC	P.Value	B
PLP1	2,443	4,73E-05	2,488
COL2A1	2,122	2,18E-05	3,281
ATP8B1	1,954	1,50E-06	5,943
PPP1R1B	1,925	7,27E-06	4,393
CMTM5	1,702	1,12E-04	1,586
ELMO1	1,66	5,97E-05	2,246
ITIH5L	1,555	6,67E-05	2,133
IGFBP5	1,522	1,82E-06	5,759
SCARNA9	1,498	7,10E-05	2,067
SCARNA17	1,435	4,46E-06	4,879
GALNT14	-3,006	0,000299	0,569
F11R	-2,824	1,13E-07	8,308
SYT4	-2,664	2,61E-06	5,405
SLC18A1	-2,335	4,01E-06	4,984
ITLN2	-2,329	3,57E-07	7,288
RASEF	-2,294	9,38E-07	6,39
GADD45G	-2,231	3,55E-08	9,277
CYP26A1	-2,056	7,66E-10	11,938
KRTAP21-1	-2,028	0,00021	0,94
PNLIPRP2	-1,992	0,000495	0,042

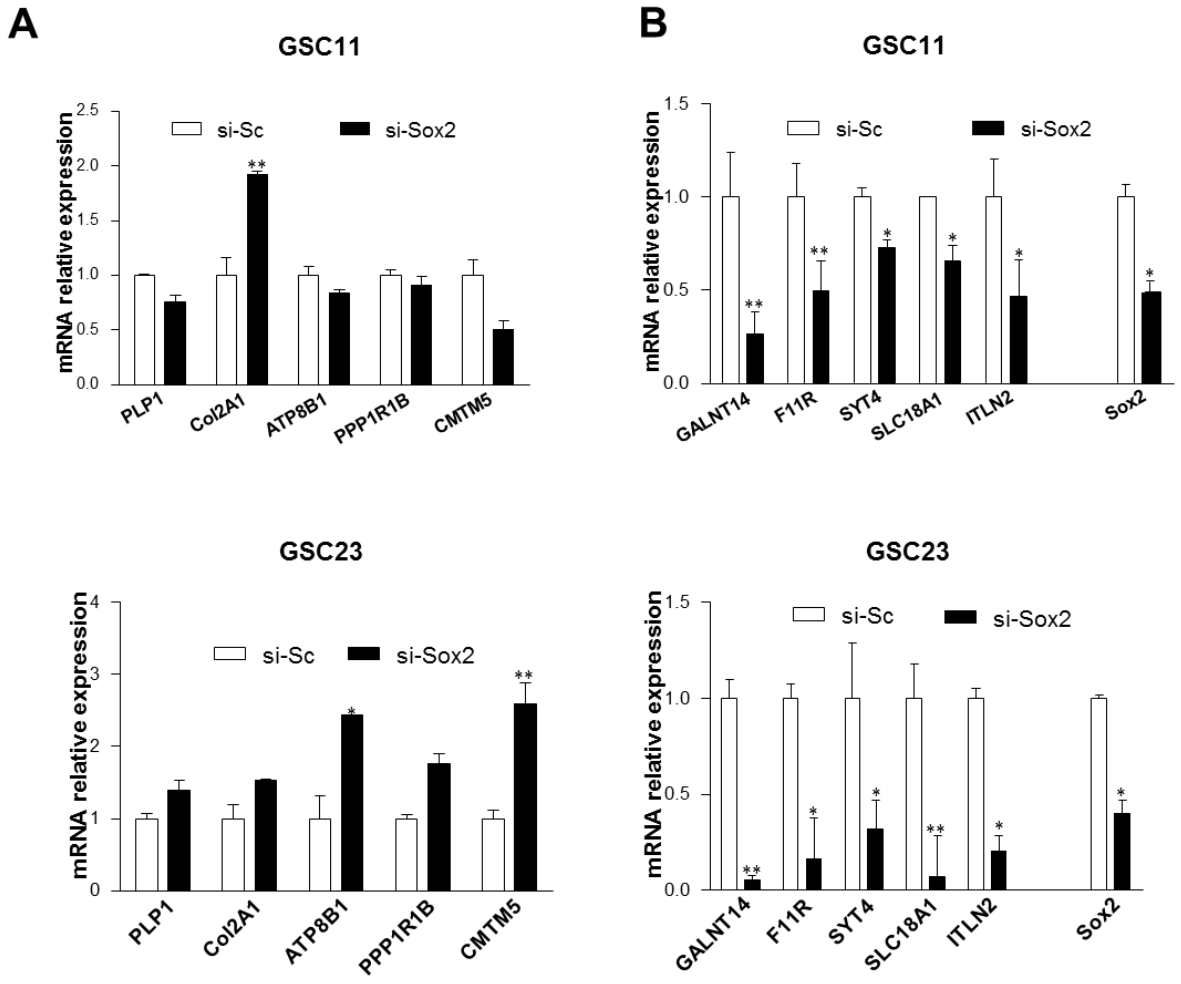


Figure 16 Analysis by qRT-PCR of the top 5 (A) up- and (B) down-regulated coding transcripts in SOX2 silenced GSC-11 and GSC-23 cells. Total RNA was extracted after 72h of si-Scrbl or si-SOX2 transfection in GSC-11 and GSC-23 cells. Values were normalized to GAPDH. Each bar represents the mean ± SD of three independent experiments.

To understand the significance of differential gene expression in the context of SOX2 dowregulation, we performed bioinformatics analysis related to Gene Ontology (GO) Classification and pathway analysis. GO classifications using the DAVID web tool and pathway analysis using Ingenuity Pathway Analysis (IPA) were performed. For these analyses, gene lists were classified based upon decreased (logFC < -1) or increased (logFC > 1) expression relative to control and analyzed altogether as a single list.

Enrichment analysis of GO categories including biological process (BP), molecular function (MF), and cellular component (CC) were obtained using DAVID web tool (Figure 17). We observed the highest enrichment in the categories related to "cell

adhesion", "biological adhesion", "cell-cell signaling", "extracellular region" and "calcium ion binding".

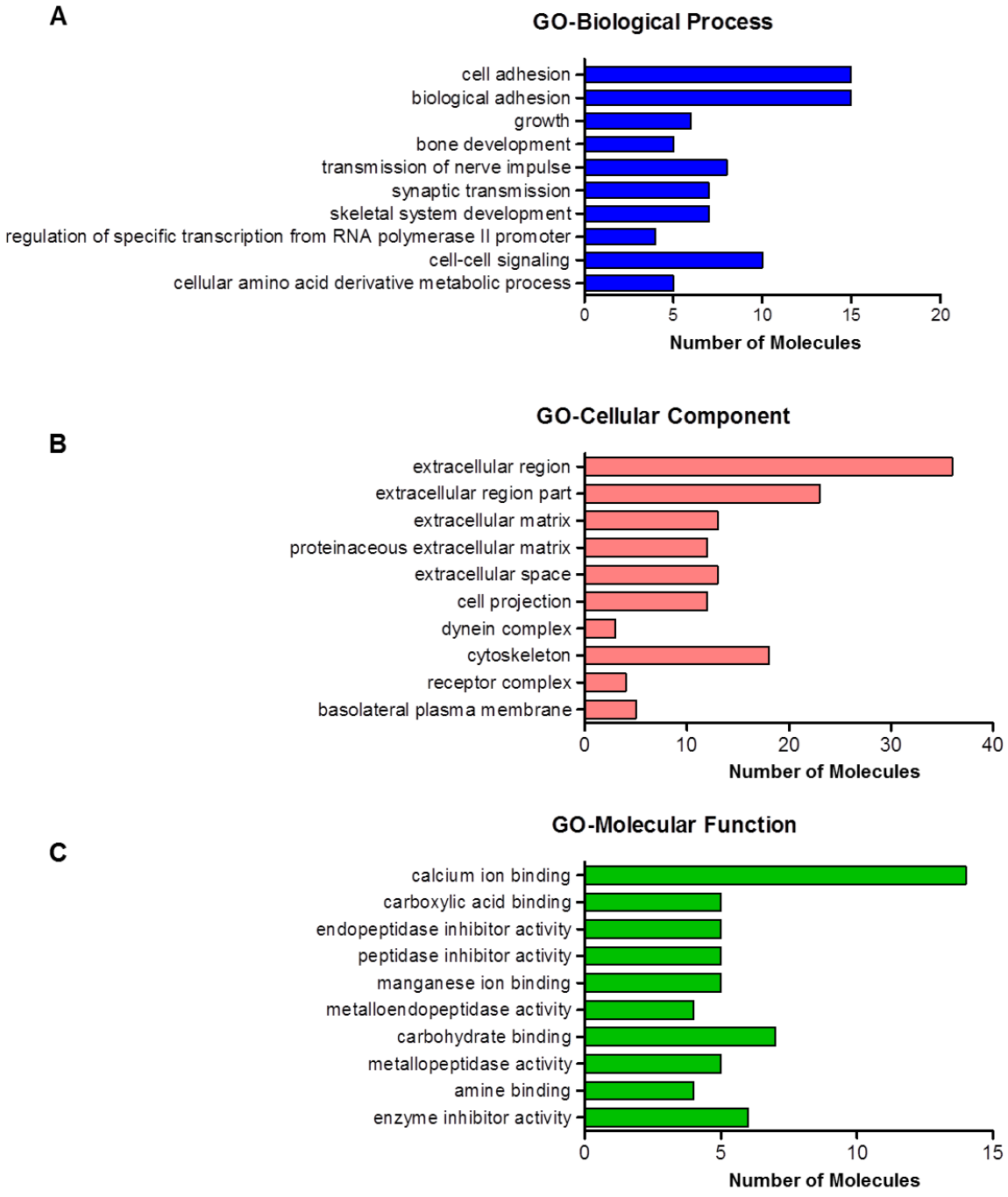


Figure. 17. Analysis of the top-10 GO Biological Processes of protein-coding genes regulated by SOX2 in GSC-11 cells. Bar chart represents classification of GO Biological Processes (A), Cellular Component (B) or Molecular Function (C), as determined by DAVID web tool. Bars represent the number of genes in the specified category, organized by p-value.

We used IPA analysis to uncover the canonical pathways regulated by SOX2 in GSCs. Our results showed 13 pathways significantly altered (Table 4). Most of them

related with amino-acid metabolism pathways, such as "histamine biosynthesis". This is consistent with the fact thathistamine represents an important regulator of numerous physiological processes including neurotransmission in the central nervous system (CNS) (Nuutinen and Panula, 2010); "L-cysteine degradation process" where cystathionine γ-lyase (CTH) activity has been related with glioblastoma treatment (Chen et al., 2015) and "serotonin receptor signaling pathway" being serotonin an important neurotransmitter in the CNS during neuronal development (Dinan, 1996). Other enriched pathways were "hematopoiesis from multipotent stem cells", where KITLG has been reported to regulate neoplastic processes such as growth and invasion (Yang et al., 2014b); apoptosis (Carson et al., 1994) and cell adhesion (Flanagan et al., 1991). Role of JAK2 in Hormone-like Cytokine Signaling stood out because GHR (growth hormone receptor) and IRS1 (insulin receptor substrate 1) has been linked with glioma progression (Lea et al., 2015; Minchenko et al., 2013). A well-characterized pathway frequently altered in tumors is the NOTCH signaling cascade, which was also enriched in our analysis. The NOTCH pathway is a conserved intercellular signaling route that has been implicated in different developmental processes. Interestingly, NOTCH pathway is deregulated in human glioblastoma and plays a key role in maintaining the growth, the undifferentiated state of glioma cells and tumorigenesis (Kanamori et al., 2007); (Fan et al., 2010; Gilbert et al., 2010; Wang et al., 2010a). The integrated analysis of SOX2 enriched canonical pathways revealed the link between this transcription factor and multiple cellular processes such as amino-acid metabolism and intercellular signaling cascades, including the NOTCH pathway.

Table 4: List of top-13 canonical pathways identified by IPA software, organized by the negative logarithm of p-values (Fisher Test), calculated by IPA ([-Log (0.05) =1.3]).

Pathway	-log(p-value)	Ratio	Molecules
Glycine Betaine Degradation	2,63E+00	2,50E-01	DMGDH,PIPOX
Hepatic Stellate Cell Activation	2,38E+00	4,42E-02	LY96,COL2A1,COL22A1 ,IGFBP5,COL28A1
Histamine Biosynthesis	2,03E+00	1,00E+00	HDC
L-cysteine Degradation II	2,03E+00	1,00E+00	CTH
Triacylglycerol Degradation	2,02E+00	1,25E-01	PNLIPRP2,CES1
Retinol Biosynthesis	2,02E+00	1,25E-01	PNLIPRP2,CES1

Hematopoiesis from Multipotent Stem Cells	1,73E+00	5,00E-01	KITLG
Cysteine Biosynthesis/Homocysteine Degradation	1,73E+00	5,00E-01	CTH
Role of JAK2 in Hormone-like Cytokine Signaling	1,61E+00	7,69E-02	GHR,IRS1
Serotonin Receptor Signaling	1,58E+00	7,41E-02	SLC18A1,HTR1D
Phenylethylamine Degradation I	1,56E+00	3,33E-01	AOC3
NOTCH Signaling	1,35E+00	5,56E-02	HES5,HEY1
Lysine Degradation V	1,34E+00	2,00E-01	PIPOX

The IPA analysis also showed the most relevant biological functions and diseases in our data set. The most significant bio-functions altered following SOX2 down-modulation are shown in Table 5. The set of SOX2-associated genes were assigned mainly to the following networks: "cancer", "organismal injury and abnormalities", "cellular movement", "tissue morphology", "cellular development" and "hematopoiesis". Interestingly, most of these networks involved very well-known functions of SOX2 such as morphology determination (Zhou et al., 2014), development (Ferri et al., 2013) and cellular proliferation and migration in glioma (Alonso et al., 2011). Figure 18 shows the most relevant selection of bio-function categories: disease and disorders, molecular and cellular functions and physiological system development and function, obtained by using IPA software and organized by p-value.

Table 5: **The top ten significant Bio-Functions altered following SOX2 silencing in the GSC-11 cell line.** The p-value range indicates the p-values of the various pathways and processes belonging to that category. The number of targets indicates the total number of genes associated with the functional category.

Category	p-value	Number of Targets
Dermatological Diseases and Conditions	$1,63 \times 10^{-08}$ - $9,35 \times 10^{-03}$	74
Cancer	$2,93 \times 10^{-08}$ - $9,35 \times 10^{-03}$	93
Organismal Injury and Abnormalities	$2,93 \times 10^{-08}$ - $9,35 \times 10^{-03}$	100
Cellular Movement	$3,98 \times 10^{-06}$ - $9,35 \times 10^{-03}$	41
Connective Tissue Development and Function	$1,71 \times 10^{-05}$ - $9,35 \times 10^{-03}$	23
Tissue Morphology	$1,71 \times 10^{-05}$ - $9,35 \times 10^{-03}$	39

Reproductive System Disease	$2,52 \times 10^{-05}$ - $9,35 \times 10^{-03}$	16
Cellular Development	$2,72 \times 10^{-05}$ - $9,35 \times 10^{-03}$	51
Hematological System Development and Function	$2,72 \times 10^{-05}$ - $9,35 \times 10^{-03}$	31
Hematopoiesis	$2,72 \times 10^{-05}$ - $9,35 \times 10^{-03}$	9

These results established a signature of protein coding-genes regulated by SOX2 in GSCs with biological functions relevant to glioblastoma growth and maintenance of its malignant phenotype. The tight overlap between the existing literature and our enrichment analysis highlights the robustness of our results and predicts that this approach will be an excellent discovery platform to identify novel SOX2 targets.

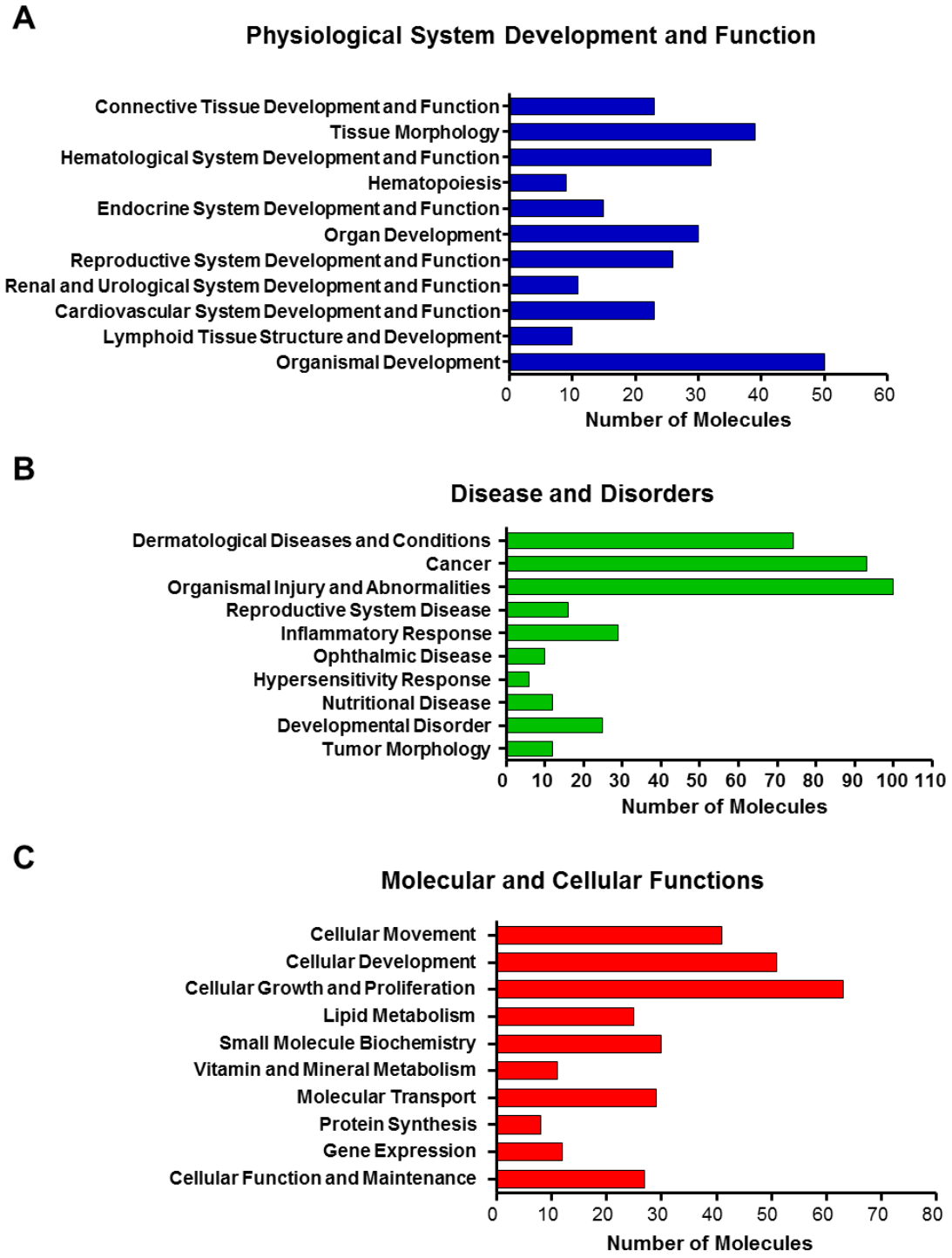


Figure. 18. 10-Top Bio Functions categories altered following SOX2 inhibition. The categories listed are (A) Physiological System Development and Function, (B) Molecular and cellular Functions and (C) Disease and Disorders, identified using IPA software. Bars represent the number of genes in the specified category, organized by p-value.

1.2 SOX2–regulated non-coding RNAs in GSCs

Reprogramming transcription factors, including SOX2, have been shown to regulate both coding and non-coding RNAs (Dinger et al., 2008). LncRNAs are

emerging as key regulators of biological processes and disease (Dey et al., 2014) therefore, seems reasonable to hypothesize that SOX2 will regulate this class of genes as well. The strength of our data-sets allowed us to identify potential non-coding transcripts differentially expressed (B value > 0) regulated by SOX2 in GSCs. After biotype distribution analysis we identified protein coding RNAs (44% for up-regulated and 41% for down-regulated), while the rest were classified as different types of non-coding transcripts. Out of the total number of transcripts differentially expressed we identify 80 up-regulated and 181 down-regulated and we classified them as intergenic RNAs, antisense, processed transcripts, transcripts derived from pseudogenes and unassigned transcripts (Figure 19). The transcripts classified as "others" correspond to transcripts derived from miRNAs, rRNAs, sense-overlaping and sense intronic transcripts. The lncRNA annotation was performed with the Bioconductor package ChIPpeakAnno (Zhu et al., 2010) and using Gencode v19 as reference (Harrow et al., 2012). The gene type corresponding to the gene that overlaps with the lincRNA locus was assigned to each lincRNA. Table 9 shows the top 25 non-coding transcripts regulated by SOX2 in GSCs.

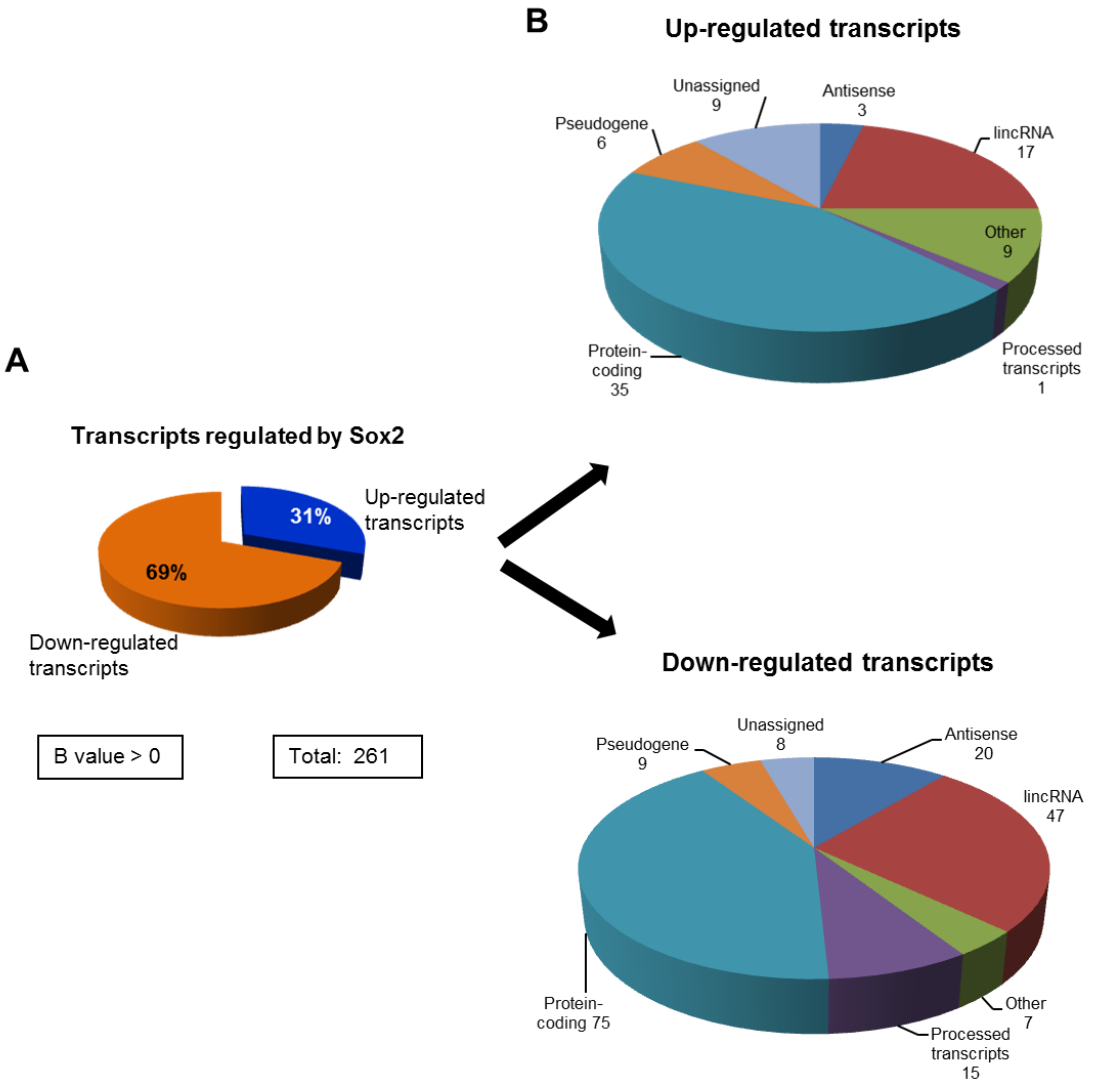


Figure 19. SOX2 regulated non-coding transcripts. (A) A total of 261 transcripts were found differentially expressed (B > 0), including 80 upregulated and 181 downregulated transcripts. **(B)** Biotype distribution of the differentially expressed transcripts following SOX2 down-modulation in GSC-11 cells.

We selected the top four differentially expressed lncRNAs (Appendix 2) that presented chromatin marks and high abundance in brain using GRCh37/hg19 assembly in UCSC Genome Browser. We validated the expression of these lncRNAs using qRT-PCR in GSC-11 cells, comparing SOX2-siRNA versus si-scrambled control. Our data indicated that the expression of chr19:28,281,401-28,284,848 (TCONS_00027256) was significantly down-regulated (p value = 0,018), while chr11:121899032-121899389 (TCONS_00020142) was significantly up-regulated (p value = 0.042) after SOX2 inhibition (Figure 20A). These results were consistent with the microarray data (Figure 20B).

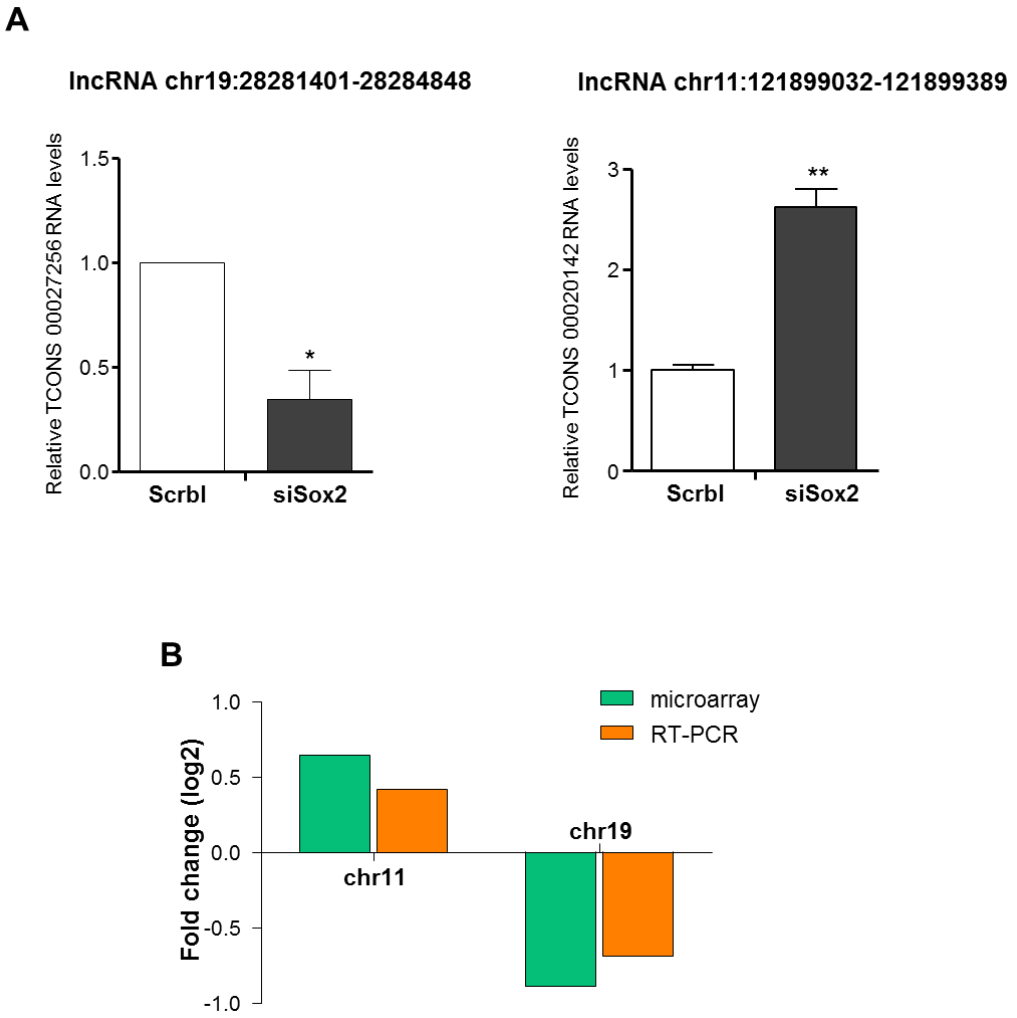


Figure 20. Validation of two lncRNAs regulated by SOX2 in GSC-11 cells. (A) The expression of the transcripts located in chr11:121899032-121899389 (TCONS_00020142) and chr19:28,281,401-28,284,848 (TCONS_00027256) were assessed. In both cases GSC-11 cells were transfected with siRNA control or siRNA against SOX2 and three days later RNA was extracted and subject to RT-PCR. Values were normalized to GAPDH (mean ± SD of three replicates). (B) Comparison between microarray and qRT-PCR results. The height of each column in this graph represents the log-transformed mean fold change in the expression of lncRNA between Scramble and siSOX2 transfected cell line.

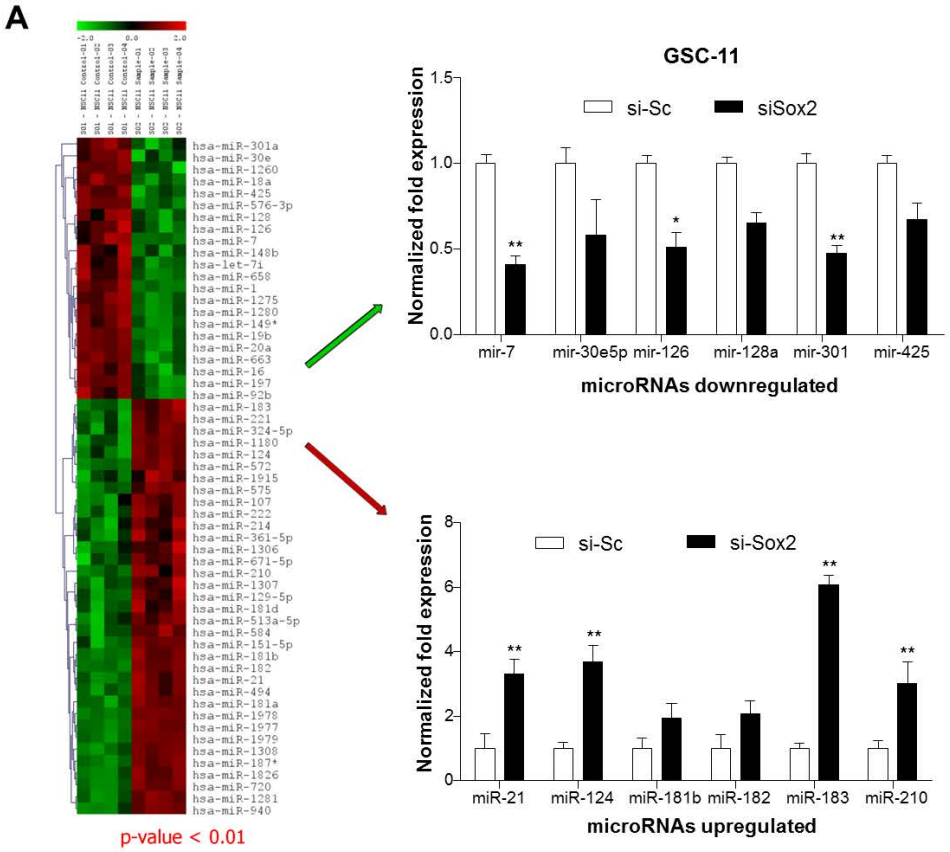
Altogether, these results identified and confirmed the non-coding transcript profile controlled by SOX2 in GSCs. Characterizing the functional relevance of these lncRNAs will undoubtedly impact in our understanding of the glioblastoma biology.

2. Differentially expressed miRNAs regulated by SOX2 in GSCs

To identify the set of miRNAs regulated by SOX2 in GSCs, we down-regulated the expression of SOX2 in GSC-11 cell line in four independent experiments, using a SOX2 specific siRNA and subjected to a miRNA array analysis. The efficiency of SOX2 down-regulation was assessed by real-time PCR and western blot (Figure 15B).

After the hierarchical clustering analysis we identified 21 down-regulated and 35 up-regulated differentially expressed miRNAs ($p < 0.01$) in GSC-11 cell line (Figure 21). Interestingly many of the deregulated miRNAs found in our data set are among the several well studied miRNAs in glioblastoma (Yang et al., 2015b) and moreover are among a previously reported set of deregulated miRNAs in GSCs (Lavon et al., 2010).

To validate our results we selected 6 miRNAs from each group based in their P value and their role as described in the literature. Their expression was analyzed using qRT-PCR in GSC-11, GSC-23 and LN-229 cell lines, comparing SOX2-siRNA versus si-scrambled control. The expression levels of miRNAs were normalized to the U6 RNA level. We confirmed the observed microarray expression changes in all the down-regulated miRNAs (miR-7, miR-30e5p, miR128, miR-126, miR-301 and miR-425) and up-regulated miRNAs (miR-21, miR-124, miR-181b, miR-182, miR-183 and miR-210) after SOX2 down-regulation in 3 different cell lines (Figure 21). The robustness of our results suggests that this approach is an excellent platform to identify miRNAs controlled by SOX2 in GSCs.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hsa-miR-30e
hsa-miR-1260
hsa-miR-18a
hsa-miR-425
hsa-miR-576-3p
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hsa-miR-126
hsa-miR-7
hsa-miR-148b
hsa-let-7i
hsa-miR-658
hsa-miR-1
hsa-miR-1275
hsa-miR-1280
hsa-miR-149*
hsa-miR-19b
hsa-miR-20a
hsa-miR-663
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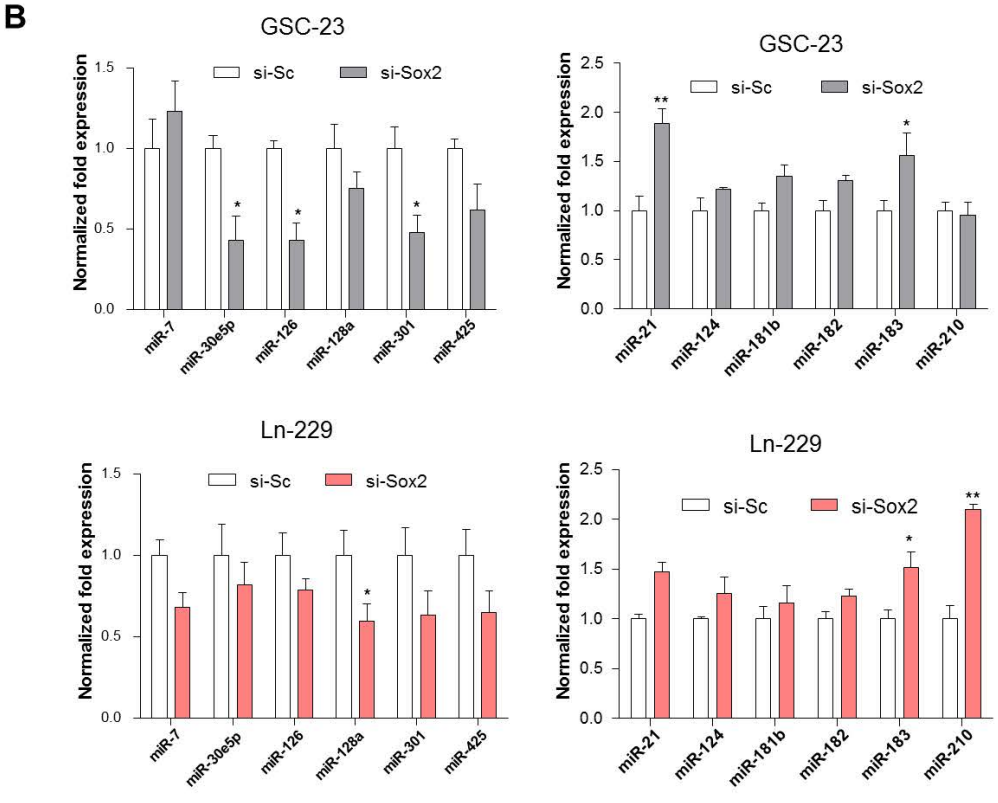
hsa-miR-584
hsa-miR-151-5p
hsa-miR-181b
hsa-miR-182
hsa-miR-21
hsa-miR-494
hsa-miR-181a
hsa-miR-1978
hsa-miR-1977
hsa-miR-1979
hsa-miR-1308
hsa-miR-187*
hsa-miR-1826
hsa-miR-720
hsa-miR-1281
hsa-miR-940
p-value < 0.01
GSC-11
si-Sc
siSox2
Normalized fold expression
mir-7
mir-30e5p
mir-126
mir-128a
mir-301
mir-425
microRNAs downregulated
si-Sc
si-Sox2
Normalized fold expression
miR-21
miR-124
miR-181b
miR-182
miR-183
miR-210
microRNAs upregulated


B
GSC-23
si-Sc
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miR-7
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miR-126
miR-128a
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miR-425
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si-Sc
si-Sox2
Normalized fold expression
miR-21
miR-124
miR-181b
miR-182
miR-183
miR-210
Ln-229
si-Sc
si-Sox2
Normalized fold expression
miR-7
miR-30e5p
miR-126
miR-128a
miR-301
miR-425
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si-Sc
si-Sox2
Normalized fold expression
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miR-124
miR-181b
miR-182
miR-183
miR-210

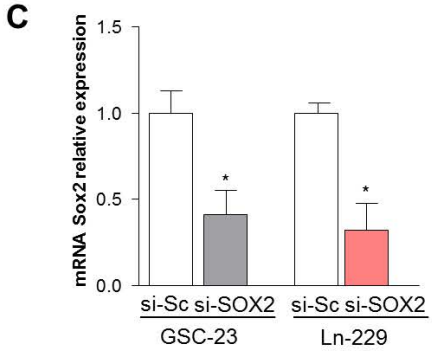

C
mRNA Sox2 relative expression
si-Sc si-SOX2
si-Sc si-SOX2
GSC-23
Ln-229

Figure 21. Analysis of miRNAs regulated by SOX2 in GSCs. (A) GSC-11 cells were transfected with si-Scrambled or si-SOX2. Total RNA was extracted 72h later and subjected to a miRNA array analysis (TLDAs). We selected 6 up-regulated and down-regulated miRNAs based in their p value and their putative role as described in the literature. We validated their expression in GSC-11 cells. Quantification of the relative miRNA expressions were performed using specific Taqman expression assays. RNU6B was used an internal control. To determine relative miRNA expression we used the comparative threshold cycle method. Each bar represents the mean ± SD. (B) GSC-23 and Ln-229 were transfected with si-Scrambled or si-SOX2. Total RNA was extracted 72h after transfection and subjected to qRT-PCR analysis. Quantification of the relative miRNA expression was performed using specific Taqman expression assay. RNU6B was used an internal control. To determine relative miRNA expression we used the comparative threshold cycle method. (C) qRT-PCR confirmation of SOX2 inhibition in GSC-23 and LN-229 cells after 72h of si-SOX2 or si-Sc transfection Each bar represents the mean ± SD of three independent experiments.

2.1 miR-301a-3p regulates migration/invasion properties of GSCs

miR-301a-3p was one of the miRNAs significantly downregulated in our study. Based on the previous report of Boyer and colleagues, where they described the occupancy of the promoter region of miR-301a by SOX2, among other TFs (Boyer et al., 2005), and the fact that miR-301a has been previously described to be up-regulated in GSCs (Lavon et al., 2010) we decided to focused on this miRNA to further study its potential role in GSCs biology.

We first examined the expression of miR-301a-3p by qRT-PCR in 30 glioblastoma tissue samples, compared with a normal human brain reference RNA sample. As shown in Figure 22A, the expression of miR-301a-3p was significantly overexpressed in all glioblastoma samples when compared with its expression in normal brain. We also showed that the expression of SOX2 and miR-301a-3p showed a significant positive correlation (R2= 0.8579; P<0.0001) in the same set of glioblastoma tissues as assessed by qRT-PCR (Figure 22B).

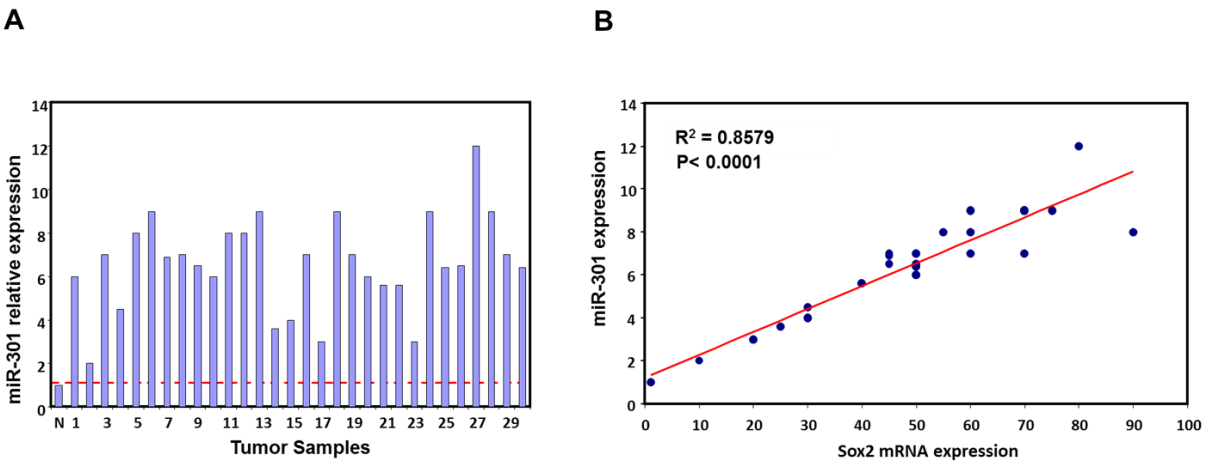
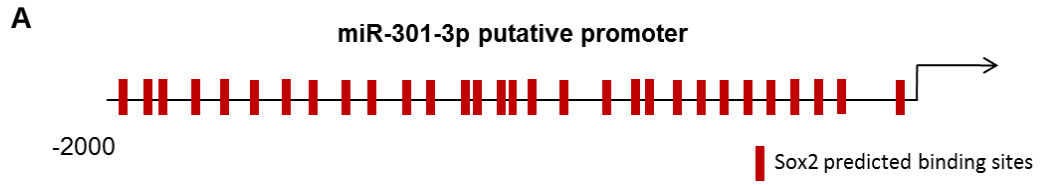


Figure 22. MiR-301a expression correlates positively with SOX2 expression in glioblastoma tissues. (A) Assessment of miR-301a-3p expression in 30 glioblastoma tissues where we previously demonstrated SOX2 overexpression. Total RNA was extracted from paraffin-embedded tissues using the RecoverAll Total Nucleic Acid Isolation Kit. The expression of miR-301a-3p was profiled using a qRT-PCR and values were normalized to U6b compared to RNA brain reference. To determine relative miRNA expression we used the comparative threshold cycle method. Each bar represents the mean ± SD. (B) Correlation between SOX2 mRNA expression and miR-301a-3p expression in 30 glioblastoma samples. Scatter plot and lowess line illustrated the correlation between SOX2 and miR-301 expression levels.

In silico analysis of the miR-301a-3p 5′ promoter from the translation start sites identified several potential binding sites for SOX2 (Figure 23A), suggesting that SOX2 can directly activate miR-301a-3p expression, supported by the study of Boyer and colleagues (Boyer et al., 2005). We therefore hypothesized that miR-301a-3p could function as a sort of onco-miRNA, inhibiting tumor suppressor target genes.

To identify the target genes of miR-301a-3p we employed three different bioinformatic programs (miRDB, miRTarBase and PicTar) in base on the differentially combination of criteria used to predict target genes and the availability of predicted miR-301a-3p target genes. We select only the genes identified by all the three approaches. As Figure 23B illustrates, a total of 45 predicted target genes was obtained.



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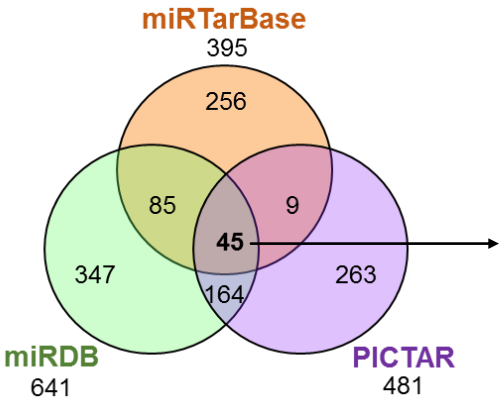

miR-301a-3p predicted target genes

ACBD5	ENPP5	MBNL1	RUNX3
ACSL4	ERBB2IP	MEOX2	SMOC1
ACVR1	ESR1	MID1IP1	SOX4
ARHGAP1	FBXO28	NIPA1	SPG20
ARHGAP12	HABP4	NRBF2	STARD13
ATP6V1B2	HBP1	PAPD4	UBE2D2
BLCAP	IER3IP1	PPP6R3	USP32
BTBD3	IRF1	PRKAA1	VPS37A
CFL2	LONRF1	PTPRG	WHSC1L1
CUL3	MAP3K9	RAB34	ZBTB4
DPYSL2	MB21D2	RPS6KA5	ZFYVE9
			ZNF800

C

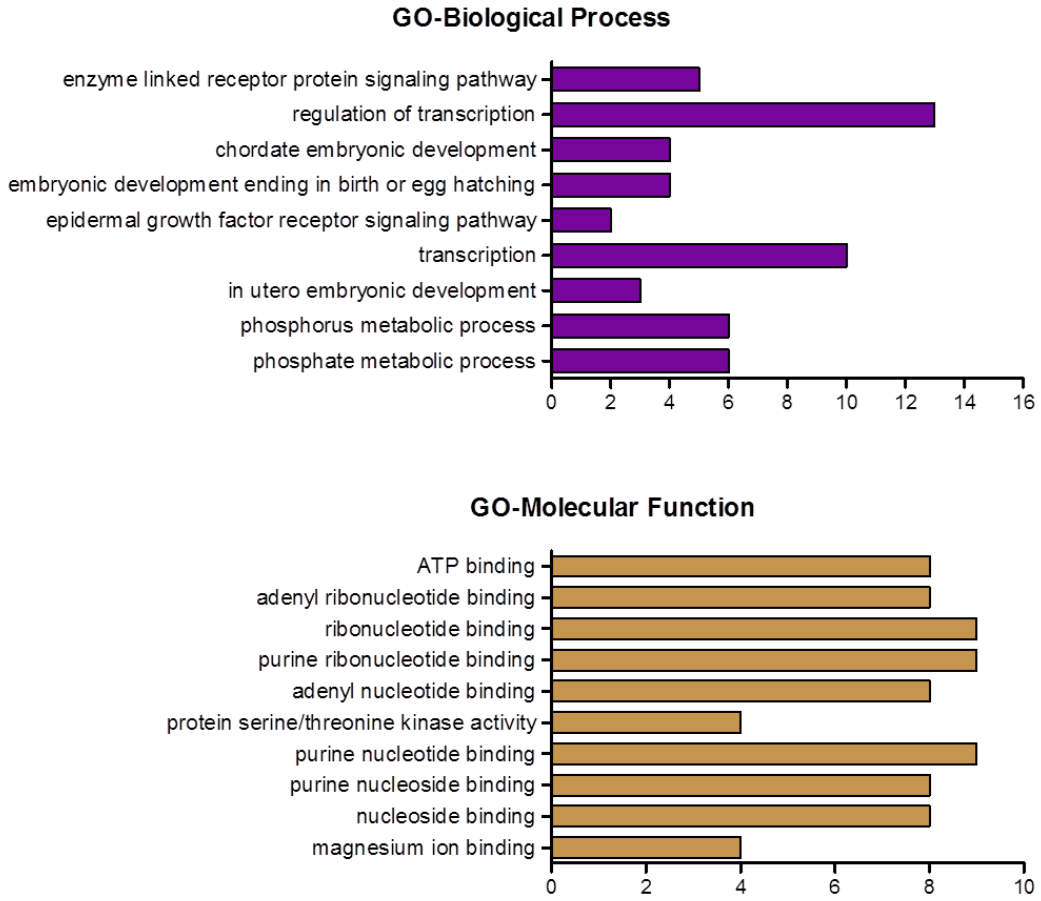

Figure 23. Analysis of the putative promoter region of miR-301a-3p. (A) Analysis *in silico* of the miR-301a-3p putative promoter region using a distance of 2kB from the transcription start site (TSS).A total of 30 binding sites for SOX2 were found using PROMO software. (B) Identification of predicted target genes of miR-301a-3p using 3 different bioinformatic programs (miRDB, miRTarBase and PicTar). A total of 45 common predicted genes were found. (C) Gene Ontology (GO) Biological Process and Molecular Function categories were determined for the 45 predicted target genes, using DAVID web tool. Bars represent the number of genes in the specified category, organized by p-value.

Several of these target genes have been reported to be tumor suppressor genes in different tumor types (Table 6), reinforcing our hypothesis that miR-301a-3p can act as an onco-miRNA in glioblastoma.

Table 6. MiR-301a-3p predicted target genes previously reported to function as tumor suppressor genes.

Symbol	Name	Tumor type in which the gene act as tumor suppressor	Reference
BLCAP	bladder cancer associated protein	cervical carcinoma	(Zuo et al., 2006)
CUL3	cullin 3	lung cancer	(Dorr et al., 2015)
ESR1	estrogen receptor 1	hepatocellular carcinoma	(Escamilla-Powers et al.,

			2010; Hishida et al., 2013)
HBP1	HMG-box transcription factor 1	breast cancer	(Escamilla-Powers et al., 2010)
IRF1	interferon regulatory factor 1	breast cancer, gastric adenocarcinoma	(Bouker et al., 2005; Gao et al., 2011; Rettino and Clarke, 2013)
MEOX2	mesenchyme homeobox 2	mammary carcinoma, hepatocarcinoma	(Valcourt et al., 2007)
PTPRG	protein tyrosine phosphatase, receptor type G	nasopharyngeal carcinoma	(Cheung et al., 2015)
RUNX3	runt-related transcription factor 3	human neoplasia	(Chen et al., 2016b)
SOX4	SRY (sex determining region Y)-box 4	glioblastoma	(Zhang et al., 2014b)
STARD13	StAR-related lipid transfer (START) domain containing 13	breast cancer	(Hanna et al., 2014)
WHSC1L1	Wolf-Hirschhorn syndrome candidate 1-like 1	breast cancer	(Zhou et al., 2010c)
ZBTB4	zinc finger and BTB domain containing 4	breast cancer	(Kim et al., 2012)

To understand the biological process where these predicted target genes were involved, we performed bioinformatics analysis related to GO Classification. Using the DAVID web tool, we obtained enrichment analysis of GO categories including biological process, molecular function and cellular component (Figure 23C). We observed the highest enrichment in the categories related to "regulation of transcription", "transcription", "ribonucleotide binding", "purine ribonucleotide binding" and "purine nucleotide binding".

To elucidate the role of miR-301a-3p in the development of glioblastoma and more precisely in GSCs maintenance, we used GSC-11 cells to conduct further functional studies. We transfected cells with an anti-miR-301a-3p or an anti-miR-scrambled and evaluated the effect of loss of function on the proliferation, migration and invasion of the GSCs. The efficiency of miR301a-3p down-regulation was confirmed by qRT-PCR (Figure 24A). We found that miR-301a-3p inhibition did not affect the proliferation rate of GSC-23 cells when compared with the proliferation of scrambled control, measured by MTS assay (Figure 24B). Of importance, the transwell assay revealed a significant decreased in the GSC-11 cell capacity to invade after miR-301a-3p inhibition (Figure 24C), suggesting that miR-301a-3p has a functional relevance in the invasive properties of GSCs.

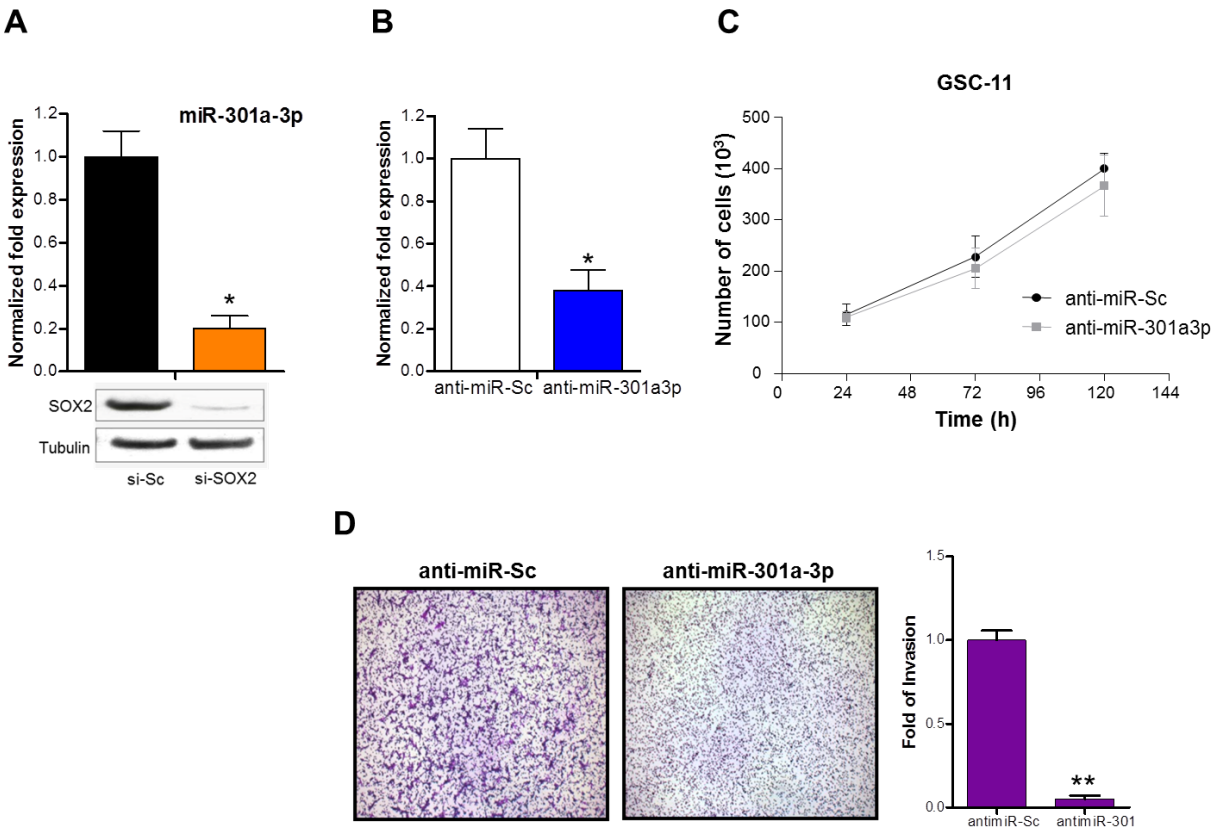

Figure 24. Inhibition of miR-301a-30 inhibits invasionnes of GSC-23 cells. (A) Validation of miR-301a-3p as a downstream target of SOX2. Total RNA and proteins were extracted after 72h of si-Sc or si-SOX2 transfection in GSC-11 cells and subjected to qRT-PCR and Western blot analysis. Quantification of the relative miR-301a-3p expression was performed using specific Taqman expression assay. RNU6B was used an internal control. To determine relative miRNA expression we used the comparative threshold cycle method. Each bar represents the mean ± SD in three independent experiments. SOX2 expression at protein level was measured using western blot. (B) Quantification of miR-301a-3p expression levels by qRT-PCR in GSC-11 cells transfected the anti-miR-301a or anti-miR-Sc. Expression was performed using Taqman gene expression assay. (C) Proliferation assay of GSC-11 cells transfected with anti-miR-301a or anti-miR-Sc, measured by MTS assay at indicated time points. (D). Assessment of invasion in GSC-11 cells after 12h of transfection with anti-miR301 and anti-miR-Sc, following the conditions described in materials and methods. Representative images of three independent experiments.

To expand our knowledge regarding the role of miR-301a-3p in the invasion capacities of GSCs, we examined a set of molecular markers involved in tumoral migration and invasion using miR-301a-3p silenced GSC-23 cells. We analyzed the expression of EphA, RAC, RHO, ROCK1, ROCK2, VEGF, TGFB, MMP9, MMP2 and NOTCH1-3 genes using qRT-PCR and normalized the expression to GAPDH levels. After miR-301a-3p inhibition, the expression levels of all the genes assayed was significantly down-modulated (Figure 25), suggesting that miR-301a-3p regulates the expression of numerous molecular makers required for the mobility, migration and invasion of GSCs.

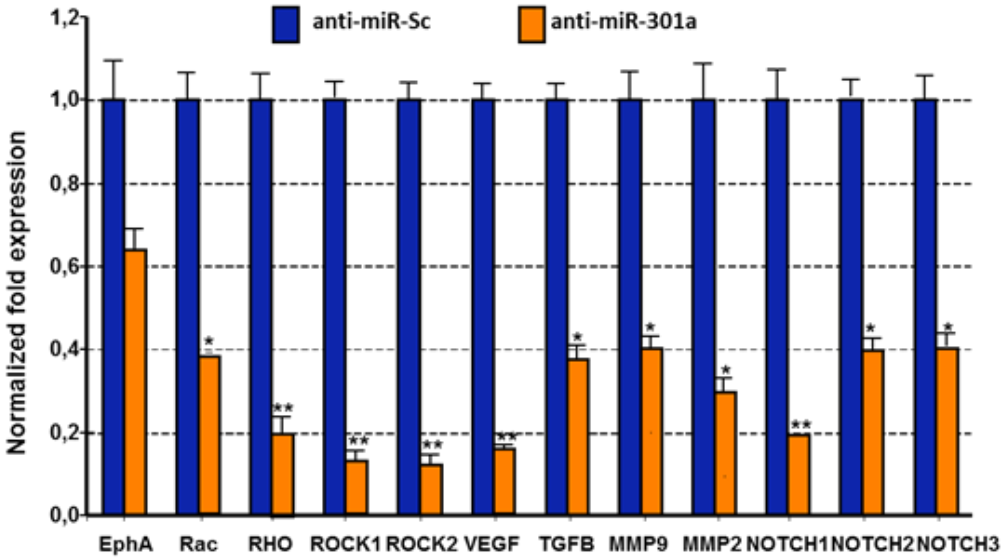

Figure 25. Analysis of invasion markers in miR301-silenced GSC-11 cells. Total RNA was extracted after 72h of anti-miR-Sc or anti-miR-301a transfections in GSC-11 cells. Quantification of the expression of the indicated genes was performed using qRT-PCR. Values are normalized to GAPDH and represents the mean ± SD of three independent experiments.

Overall these results showed the miRNA signature controlled by SOX2 in GSCs, where miR301a-3p is one of the validated SOX2 miRNA targets and that could act as an onco-miR regulating migration/invasion property in GSCs.

3. Elucidation of SOX2-miRNA targets combining different experimental approaches

To provide robustness to the SOX2-regulated-miRNAs profile in GSCs, we confronted our miRNA array results to another set of miRNAs data generated by Dr. Laterra`s group (John´s Hopkins, Baltimore, USA). In their case, the experimental setting was slightly different and they overexpressed SOX2 in the GSC cell line GBM1A. The experiment was performed in triplicates using a specific SOX2 lentivirus. RNA was extracted and subjected to a miScript miRNA PCR Array Human Brain Cancer (MIHS-108Z). The efficiency of SOX2 over-expression was assessed by RT-PCR and Western Blot (Lopez-Bertoni et al., 2015). Using this approach, they identified 12 down-modulated and 52 up-regulated differentially expressed miRNAs ($p < 0.01$) in GBM1A cells. Interrogation of our two differentially expressed miRNA candidate lists yielded 7 common miRNAs that were regulated by SOX2 in the same direction: miR-7, miR-16, miR-18a, miR-20a, miR-128a, miR-128b and miR-425-5p (Figure 26).

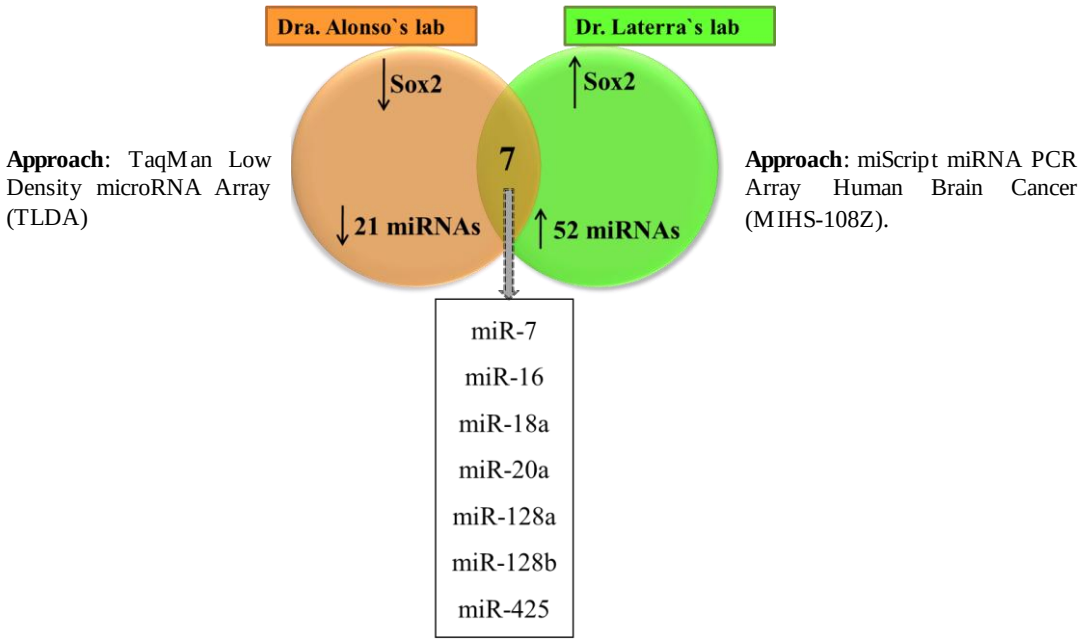

Figure 26. Schematic representation of the common miRNAs candidates obtained, by two different approaches. In our lab, SOX2 expression was down-regulated using a SOX2 specific siRNA in NSC-11 cells. In the other lab, the expression of SOX2 was over-expressed in GBM1A cells, using a lentiviral vector coding for mCitrine-SOX2 and then selected by flow cytometry, in the group of Dr. Laterra. In both cases RNA was extracted and subjected to 2 different miRNAs arrays, obtaining a list of miRNA candidates regulated by SOX2.

To validate these candidates we analyzed their expression by qRT–PCR using RNA isolated from different cell lines where SOX2 was over-expressed or down-modulated (Figure 27). As a proof of concept, we overexpressed SOX2 in HEK293FT. This cell line is easy to grow and maintain in culture, and is amenable for transfection with high efficiency. We also used the glioblastoma cell line A172 to up-regulate SOX2 expression. The expression of SOX2 was confirmed by Western Blot in both cell lines (Figure 27A). As we expected, we observed an increased in the expression of all miRNA candidates following SOX2 over-expression in both cell lines (Figure 27A). Conversely, to down-modulate SOX2 expression, we forced the differentiation in GBM1A and GBM1B in a physiological context. For that purpose we plated neurosphere cells in FGF-neurosphere medium without EGF for 2 days, and then we changed the medium adding 1% FBS without EGF/FGF for 5 days. The expression of differentiation markers (such as GFAP, astrocytic and O4, oligo) increased. On the other hand, the expression of SOX2 was significantly reduced (Figure 27B). In summary, we observed that in response to forced differentiation induces by growth

factor withdrawal and serum, miRNA candidates expression was significantly down-modulated.

Taken together, we confirmed that these 7 miRNAs are regulated by SOX2 in GSCs cells, reinforcing the robustness of the approach used.

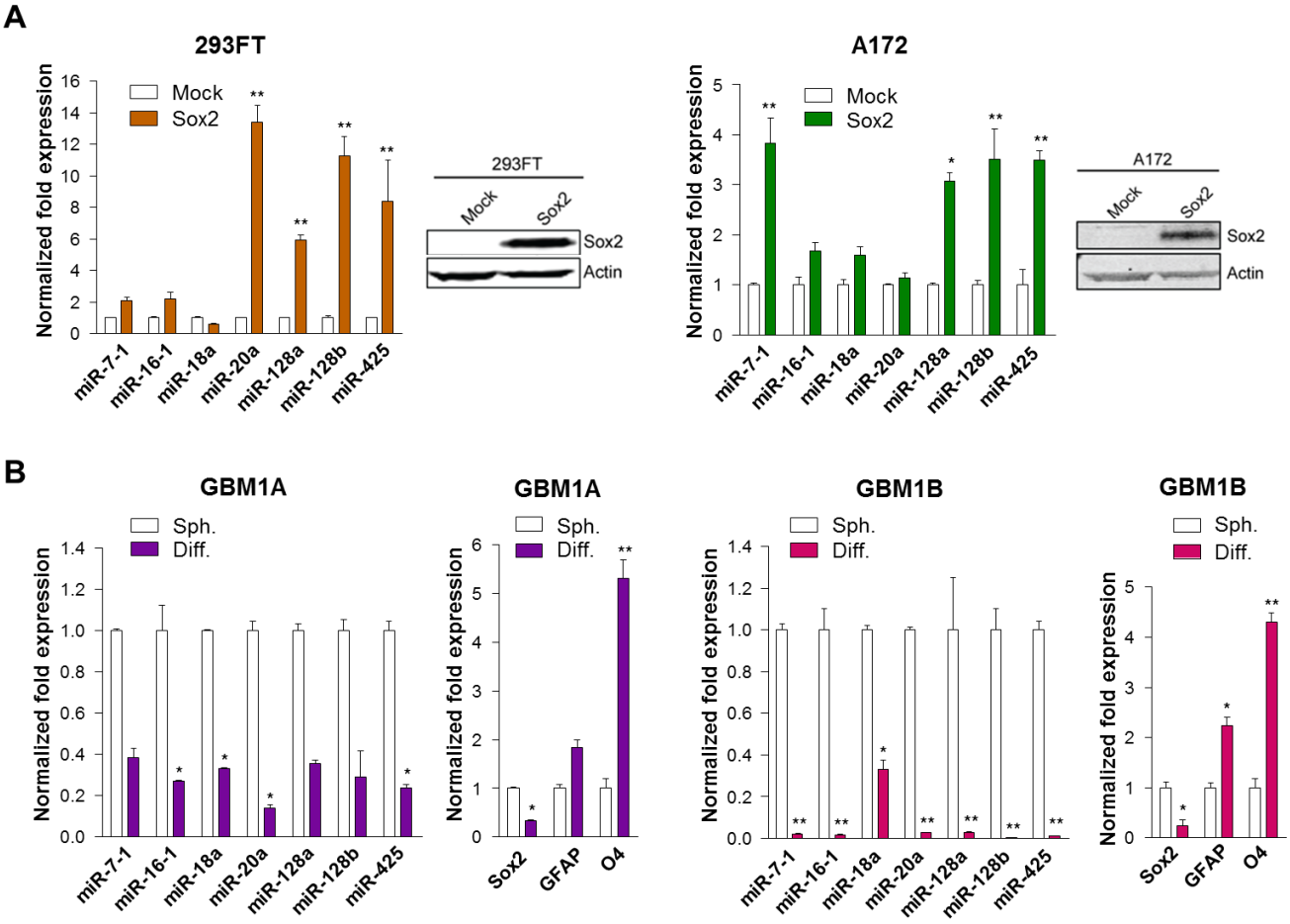

Figure 27. Analysis of the expression of common miRNAs candidates in different cell lines. (A) qRT-PCR analysis showing miRNA expression in A172 and HEK293FT cells after SOX2 over-expression using a lentiviral vector coding for mCitrine-SOX2 or control vector (mock). RNA and total protein was extracted 72 hours after transduction and qRT-PCR and western blot analysis was performed. Quantification of the relative expression of miRNAs was performed using specific Taqman expression assay. RNU6B was used an internal control. To determine relative miRNA expressions we used the comparative threshold cycle method. Each bar represents the mean ± SD. SOX2 expression at protein level was confirmed using western blot. (B) GBM1A and GBM1B cells were subjected to forced differentiation protocol. Cells were plated in FGF-neurosphere medium without EGF for 2 days, and then medium was changed by adding 1% FBS without EGF/FGF for 5 days. RNA was extracted and qRT-PCR analysis was performed. Quantification of the relative expression of miRNAs was performed using specific Taqman expression assay and the quantification of the expression of the indicated genes was performed using Sybr Green gene expression assays specific for each gene. GAPDH and RNU6B were used as internal control for qRT-PCR analysis. To determine relative gene expression we used the comparative threshold cycle method. Each bar represents the mean ± SD of three independent experiments.

Based on these data (Figure 27) and in our previous results obtained in GSC-11, GSC-23 and LNN229 cells (Figure 21), miR-425-5p emerged as one of the top miRNA regulated by SOX2 and we decided to further investigate the relation between SOX2 and this miRNA.

4. SOX2 transcriptionally activates miR-425-5p

We set to elucidate whether SOX2 could directly bind and thus transcriptionally control the expression of miR-425-5p. Therefore first, we performed *in silico* analysis of the miR-425-5p promoter region at 5` from the translation start site to investigate whether the promoter region of miR-425-5p had binding sites for SOX2, using the PROMO web tool (Messeguer et al., 2002). With this approach, we identified multiple potential binding sites for SOX2 (Figure 30A). Next, we over-expressed SOX2 in GBM1B cells using a lentiviral vector coding for mCitrine-SOX2 and we carried out a chromatin immunoprecipitation assay followed by qRT-PCR (qChIP). We designed primers for the region marked with blue arrows and that we designated as Region 1(Rg1) (Figure 30A), where there is a greater concentration of SOX2 binding motifs sites. We observed that SOX2 was bound to the analyzed predicted sites in the putative miR-425-5p promoter (Figure 30B). In fact the Rg1 assayed was significantly enriched (25% ± 4) when compared with the control. We cannot rule out that SOX2 could bind to other sites in the miRNA.

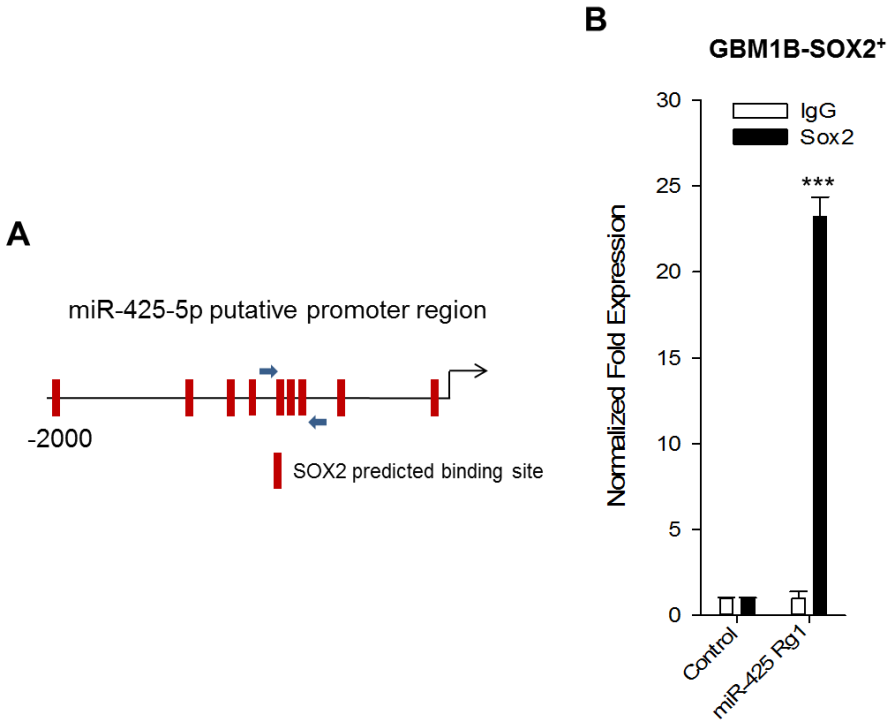

Figure 30. SOX2 binds miR-425-5p promoter and induces miR-425-5p expression. (A) The putative promoter region of miR-425-5p has multiple SOX2 binding sites (red rectangles) predicted by PROMO search tool. Blue arrows indicate the region (Rg 1) for which primer were designed for PCR analyses. (B) DNA purified from chromatin immunoprecipitation was analyzed by qRT-PCR using primer pairs designed to amplify fragments containing SOX2 binding sites and primers targeting promoter region lacking SOX2 binding sites, using SOX2 over-expressed GBM1B cells.

5. Functional role of miR-425-5p in glioblastoma

Since SOX2 can directly bind and activate miR-425-5p expression, we hypothesized that in turn this miRNA acts as an onco-miRNA, inhibiting the expression of tumor suppressor target genes (Figure 31). So with this hypothesis in mind, we focused on inhibit the expression of this potentially onco-miR, to subsequently enhance the expression of tumor suppressor target genes that could negatively regulate stemness and oncogenic properties of GSCs cells.

Figure 31. Inhibition of miR-425-5p expression can enhance the expression of tumor suppressor target genes interfering ultimately with the GSC phenotype.

To evaluate the biological effects of miR-425-5p in GSCs, we used a lentivirus expressing either a scrambled RNA sequence marked with a reporter GFP gene (GFP-sponge) or an antisense RNA oligo designed to target miR-425-5p (miR-425-5p sponge), and we transduced HEK293FT cells and GSC-11 and GSC-23 cells. MiR-425-5p sponge efficiently decreased pre-miR-425-5p levels in all cell lines, as measured by qRT–PCR (Figure 32), demonstrating that the generation of sponges is a good approach to inhibit miRNA expression.

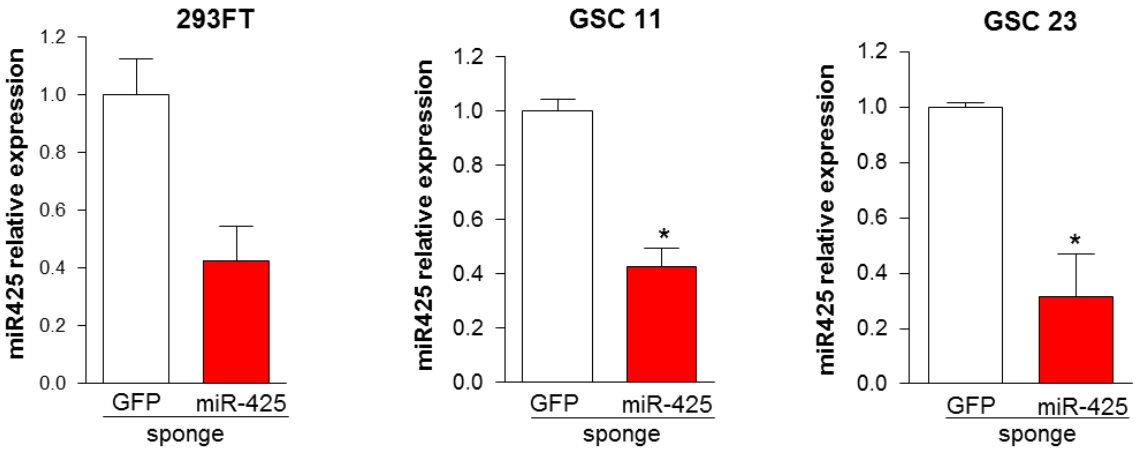

Figure 32. Efficiency of miR-425-5p inhibition using a specific sponge. HEK293FT, GSC-11and GSC-23 cells were transduced with GFP-sponge or miR-425-5p sponge. Total RNA was extracted 72 hours after transduction. Analysis by qRT-PCR was performed. Quantification of the relative expression of miR-425-5p was performed using specific Taqman expression assay. RNU6B was used an internal control. To determine miR-425-5p relative expression we used the comparative threshold cycle method. Each bar represents the mean ± SD of three independent experiments.

5.1. miR-425-5p affects neurosphere formation

Next, we wanted to evaluate the functional role of miR-425-5p in the GSC phenotype. Interestingly, we observed that inhibition of endogenous miR-425-5p decreased neurosphere formation as evidenced by a significant reduction in the number and size of neurospheres (Figure 33A-B). We could observed that downregulation of miR-425-5p overtime resulted in the disruption of neurospheres, finally leading to cell death. In addition, inhibition of miR-425-5p affected negatively cell proliferation (Figure 33C). Cells treated with the scramble-sponge showed a three fold increase proliferation than those treated with the miR-425-5p sponge.

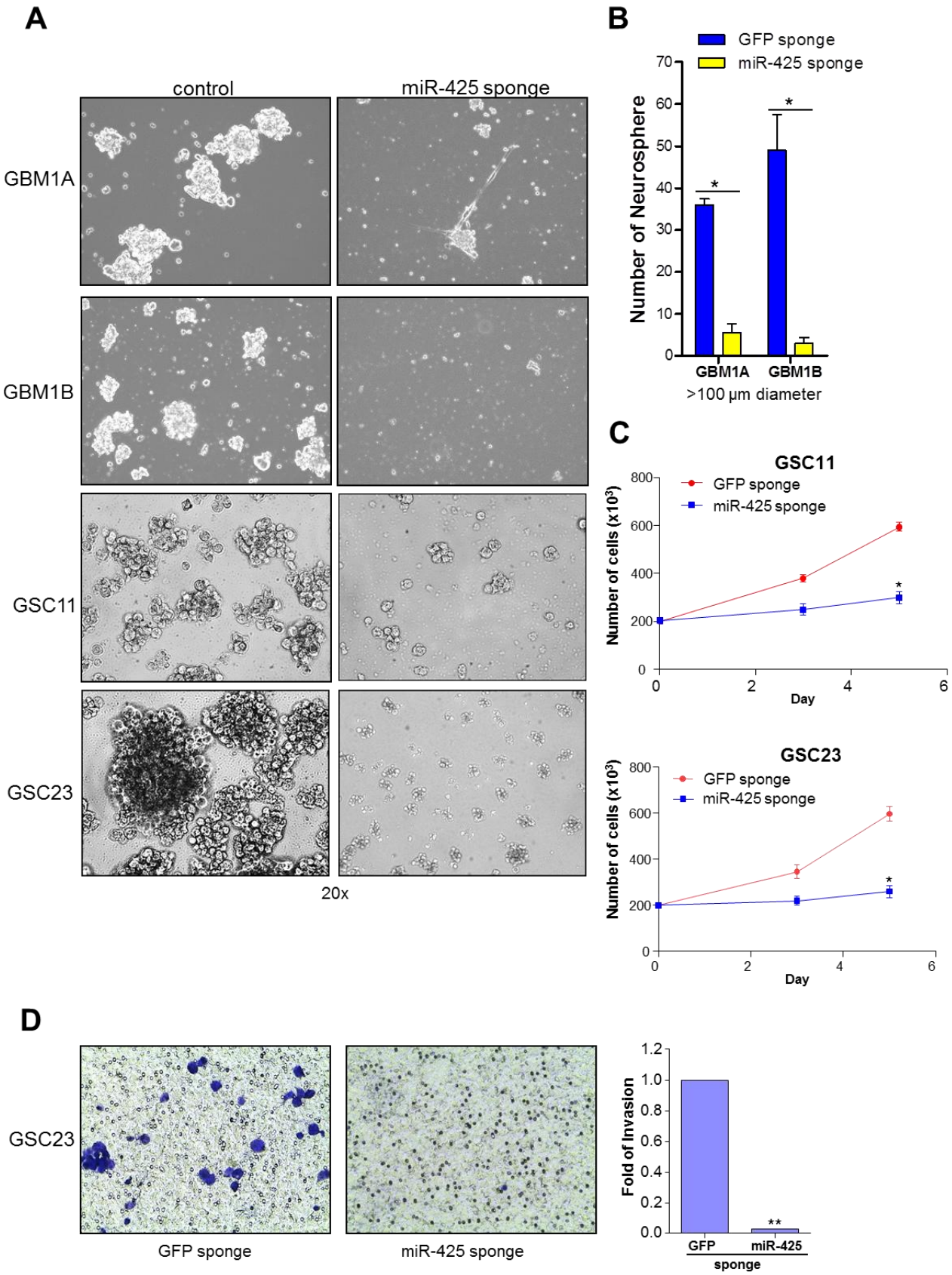

Figure 33. Inhibition of miR-425-5p affects neurosphere formation, cell proliferation and invasion properties of GSCs. (A) Morphology of GBM1A, GBM1B, GSC-11 and GSC-23 cells 7 days after transduction with GFP-sponge or miR-425-5p-sponge. (B) Equal numbers of GBM1A and GBM1B cells

transduced with GFP-sponge or miR-425-sponge were cultured in neuropshere medium for 1 week and spheres >100 µm in diameter were quantified. (C) Equal numbers of GSC-23 and GSC-11 cells transduced with GFP-sponge or miR-425-sponge were dissociated into single-cell suspensions and cultured in neuropshere medium. Cells were counted at the indicated intervals using Neubauer chamber. (D) Equal numbers of GSC-23 cells transduced with GFP-sponge or miR-425-sponge were dissociated into single-cell suspensions and seeded in the top of Matrigel coat chamber using Transwell invasion assay. After 12h invasion index was assessed.

We also evaluated the impact of inhibiting miR-425-5p on the capacity of GSC-23 cell line to invade. Inhibition of miR-425-5p completely abolished the invasion capacity of GSC-23 cells (Figure 33D). These data support our hypothesis that miR-425-5p acts as an oncogene in GSCs.

5.2. miR-425-5p alters cell cycle progression

To investigate the mechanism underlying the miR-425-5p mediated cell proliferation suppression, we analyzed cell cycle progression by flow cytometry. Inhibition of endogenous miR-425-5p promoted a G1/S arrest in GSC-23 cells transduced with GFP sponge or miR-425-5p sponge (Figure 34A).The percentage of G0 phase cells in GSC-23-miR-425-sponge was increased (13,9%) compared with those of control GFP-sponge cells (3%), and no significant change was observed in S phase or G2/M phase (Figure 34B). These results indicated that miR-425-5p down-regulation inhibited GSC-23 proliferation by inducing cell arrest in the G0/G1 phase.

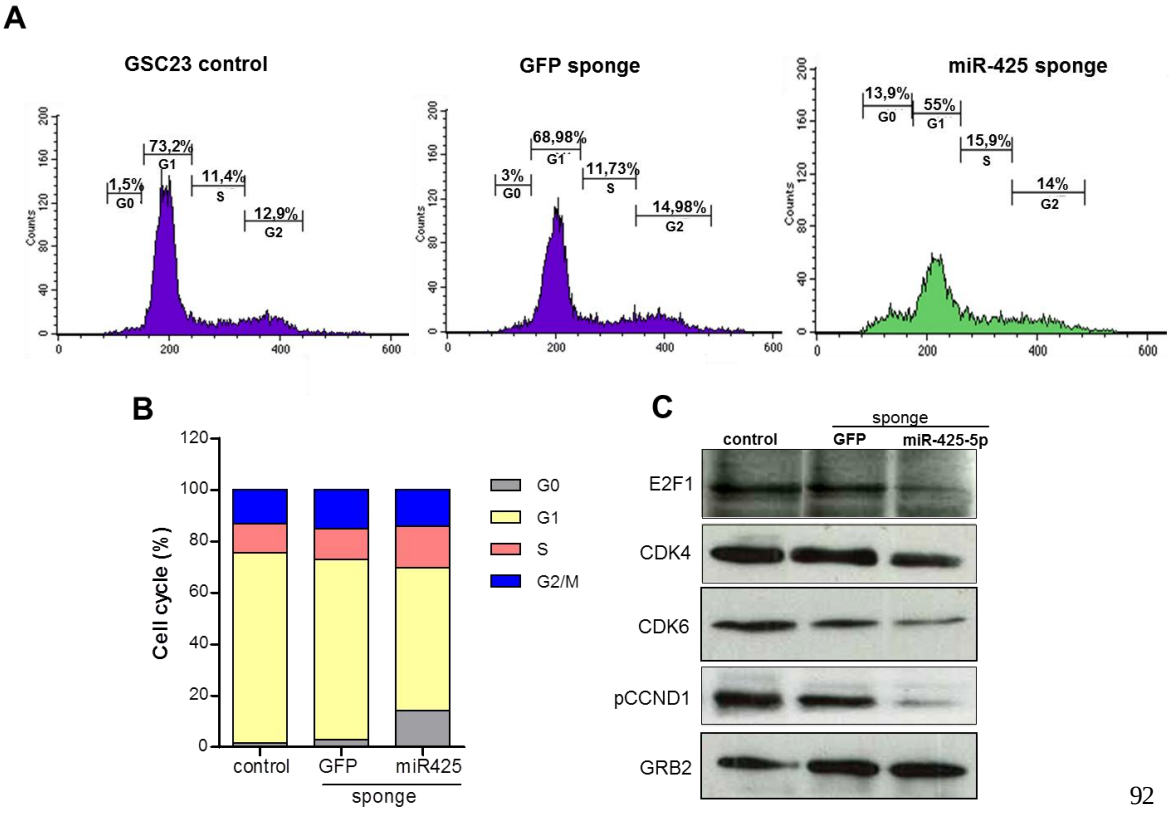

Figure 34. Inhibition of miR-425-5p caused a G0/G1 arrest in cell cycle of GSCs. (A) Equal numbers of GSC-23 cells transduced with GFP sponge or miR-425 sponge were cultured in neuropshere medium for 1 week and were analyzed using flow cytometer. (C) GSC-23 neurospheres were transduced with lentivirus expressing either GFP sponge or miR-425 sponge and total protein was extracted after 7 days. Western Blot showed decreased expression of cell cycle proteins CDK-4, CDK-6, phospho-CCND1 and E2F1. Normalization was performed with GRB2.

We investigated the status of the cell cycle proteins CDK4/CDK6, cyclin-D1 (CCND1) and E2F1 (Figure 34C). Inhibition of miR-425-5p decreased the expression of proteins involved in G1 phase transition, explaining the arrest during cell cycle in GSC-23 cells.

5.3. Inhibition of miR-425-5p did not induce autophagic cell death

Besides inducing cell cycle arrest, the inhibition of miR-425-5p resulted in an increased in cell death as shown by the increase of cells in G0 phase. Therefore, next we set to characterize the type of cell death induced.

We first examined the expression of the autophagy-related biochemical marker p62 and the conversion of LC3I to LC3II in GSC-23 cells transduced with miR-425 sponge compared with GFP-sponge and normal neurospheres (Figure 35). We observed that there is no conversion from LC3I to LC3II. Moreover, p62 protein levels remained unchanged in miR-425-5p knock-down GSC-23 cells. These data clearly indicated that the cell death mechanism induced after miR-425-5p inhibition in GSCs is not due to an autophagic process.

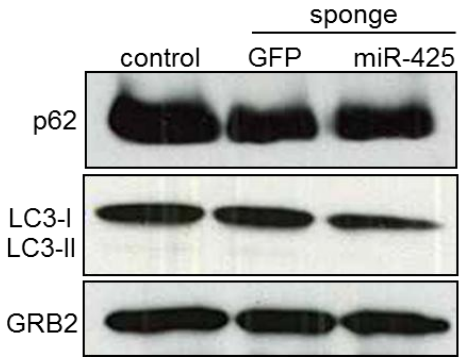

Figure 35. Inhibition of miR-425-5p does not induced autophagy cell death in GSCs. Equal numbers of GSC-23 cells transduced with GFP-sponge or miR-425-sponge and normal GSC neurospheres were cultured in appropriate neuropshere medium. Cells were collected 7 days later and total protein was extracted. Western blot analysis was performed using antibodies against p62, LC3 and GRB2. GRB2 was used as loading control protein. Shown is a representative western blot of three independent experiments.

5.4. Inhibition of miR-425-5p promotes DNA damage and activation of apoptosis

Since we ruled out the induction of autophagy, next we interrogated whether the inhibition of miR-425-5p can induce apoptosis preceded by DNA damage. Inability to repair double strand breaks (DSBs) at DNA level represent a lethal type of DNA damage. To determine if inhibition of miR-425-5p in GSC-23 cells promote lethal DNA damage, we assessed the expression of P-H2A.X, which is a DSB marker (Kuo and Yang, 2008). As Figure 36A shows, we observed an increased in the levels of P-H2A.X after miR-425-5p inhibition, compared with GFP-sponge cells and normal neurosphere GSC-23 cells. This result illustrates that inhibition of miR-425-5p caused DNA damage in GSC-23 cells, while the transduction with the GFP-sponge does not cause any DNA damage to cells, indicating that this effect is due to the inhibition of miR-425-5p expression per se, and not to the plasmid used.

We also assessed APC-AnnexinV/SYTOX Blue staining by flow cytometer (Figure 36D) and caspase 3/7 activity in GSC-23 cells transduced with miR-425 sponge compared with GFP-sponge and normal neurospheres (Figure 36C). As shown in Figure 36D, inhibition of miR-425-5p in GSC-23 cells produced a stronger pro-apoptotic effect as compared with that of the control (44,5% vs. 22,5 %). MiR-425-5p sponge cells displayed a significant 2 fold increase in Caspase-3/7 activity in GSC-23 cells (Figure 36B). Consistent with these results, the full form of Caspase 3 was decreased, assessed by western blot (Figure 36C). The PI3K-AKT pathway is a signal transduction pathway that promotes survival and growth in response to different stimuli (Dudek et al., 1997). Therefore, we also assessed the expression level of AKT protein and its phosphorylated active form (Figure 36C). We observed a significant decrease in p-AKT expression after miR-425-5p inhibition in GSC-23 cells. Overall these results indicated that inhibition of miR-425-5p activates apoptosis process and suppress survival pathways in GSCs.

To further confirm a possible apoptotic cell death mechanism we assessed the morphological changes produced in GSC-23 cells transduced with miR-425-5p sponge compared with GFP-sponge and normal neurospheres using transmission electron microscopy (Figure 36E). Apoptosis is characterized by chromatin condensation, internucleosomal degradation of the DNA, cell shrinkage and disassembly into membrane-enclosed vesicles as a consequence of caspase activation (Cotter et al., 1996). Images from TEM showed a marked condensation of the nuclear chromatin and disruptionof the plasmatic membrane after miR-425-5p inhibition in GSC-23 cells.

Normal neurospheres and cells transduced with GFP sponge did not display these characteristics, illustrating the morphology of normal cells.

Taken together these results support an apoptotic mechanism of action for miR-425-5p in GSCs.

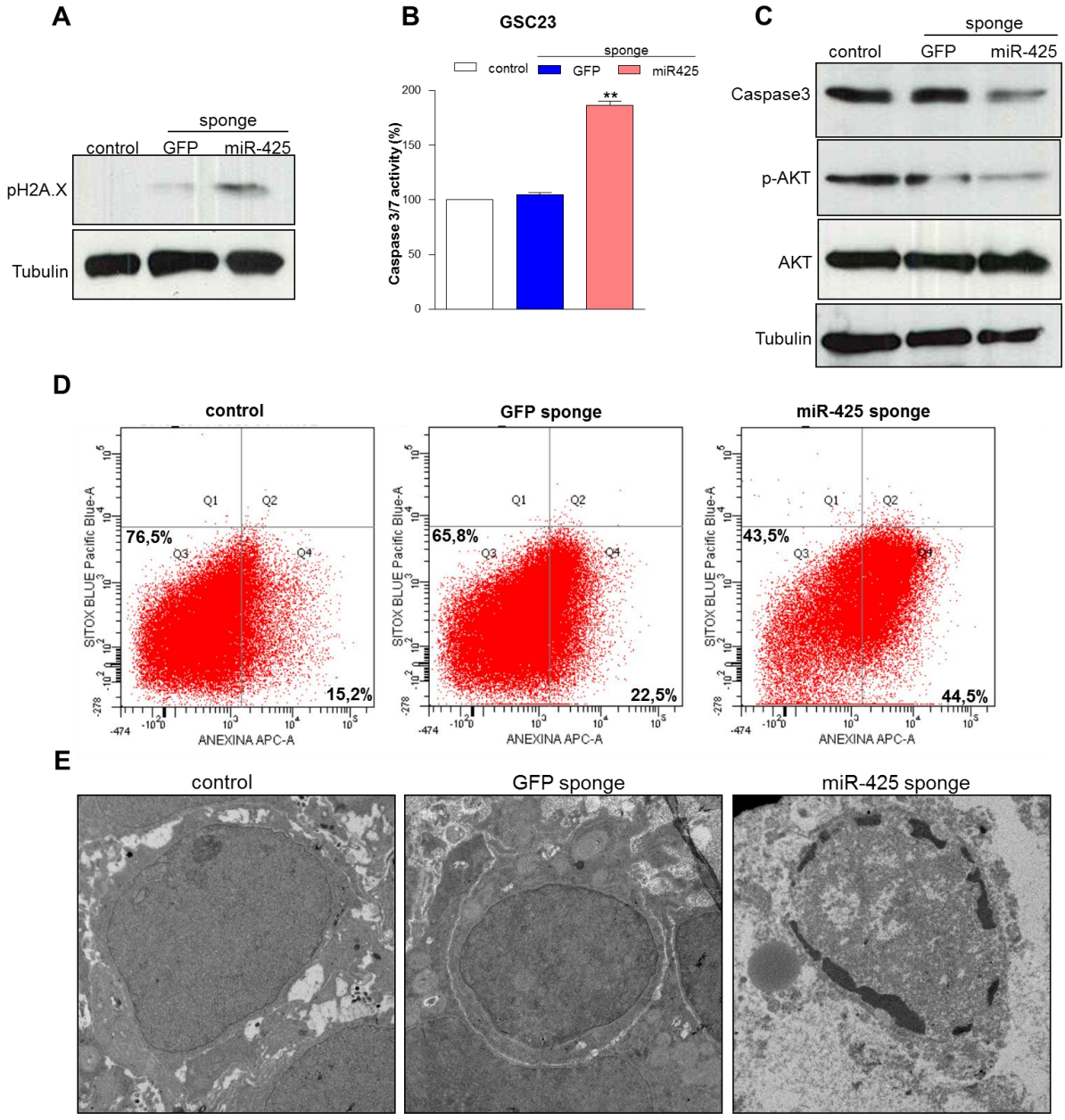

Figure 36. Inhibition of miR-425-5p promotes apoptosis in GSCs. (A) Western blotting analysis of full phosphorylated H2A.X protein. Equal numbers of GSC-23 cells transduced with GFP sponge or miR-425 sponge and normal GSC neurospheres were cultured in neuropshere medium. Cells were collected 7 days later and total protein was extracted. Tubulin was used as control protein. Shown is a representative western blot of three independent experiments. (B) Assessment of Caspase 3/7 activity. Equal numbers of GSC-23 cells transduced with GFP sponge or miR-425 sponge and normal neurospheres were cultured in neuropshere medium for 1 week ans subjected to analysis. (C) Western

blotting analysis of full caspase 3, phosphorylated AKT, and AKT was assessed. Equal numbers of GSC-23 cells transduced with GFP sponge or miR-425 sponge and normal GSC neurospheres were collected 7 days later and total protein was extracted.. Tubulin was used as control protein. Shown is a representative western blot of three independent experiments. (D) APC-AnnexinV and SYTOX Blue double staining analysis of cell apoptosis of GSC-23 cells. Equal numbers of GSC-23 cells transduced with GFP sponge or miR-425 sponge and normal GSC neurospheres were cultured in appropriate neuropshere medium and collected . 7 days later for analysis. (E) Representative images of TEM using GSC-23 cells transduced with GFP sponge or miR-425-sponge and control neurospheres. Cells were collected after 7 days. (Magnification 1000x).

5.5. miR-425-5p exerts a significant anti-tumoral effect *in vivo*

To determine the effect on tumorigenicity of miR-425-5p, we conducted *in vivo* experiment. GSC-11 cells were transduced with miR-425 sponge or GFP sponge lentivirus and we also used the parental GSC-11 cell line. After 2 days of transduction 500 000 viable cells were injected intracranially in nude mice (8 mouse/group) as previously described (Lal et al., 2000). Mice were distributed in 3 different groups; one group of mice injected with normal NSC-11 cells, other group of mice injected with cells transfected with GFP sponge and the last group of mice injected with miR-425 sponge transfected NSC-11 cells. The infusion pump used allows cell injection in up to 6 mice. We allocated mice from the 3 different groups in each injection batch, to ensure that cells injected were in the same conditions during the implantation process.

Survival curves indicated that inhibition of miR-425-5p expression exerted a significant increase in the overall median survival time compared with GFP sponge group and the control groups (Figure 37A). However, mice injected with GFP sponge transduced cells, have an overall median survival time greater that normal group, suggesting that GFP has a toxic effect to cells per se. The median survival of mice injected with GFP-sponge cells were 51 days, 65 days for mice injected with miR-425 sponge cells, and 35 days for mice injected with normal GSC-11 cells (Figure 37A). Pathological analysis of the brain of treated mice revealed a very aggressive tumor that infiltrated the brain parenchyma and invaded the contralateral hemisphere in the three groups, confirming cells engraftment.

All together, these results show that the inhibition of miR-425-5p resulted in a significant survival benefit in an aggressive glioblastoma model *in vivo*, highlighting the possibility of study miR-425-5p as an alternative therapeutic strategy for glioblastoma.

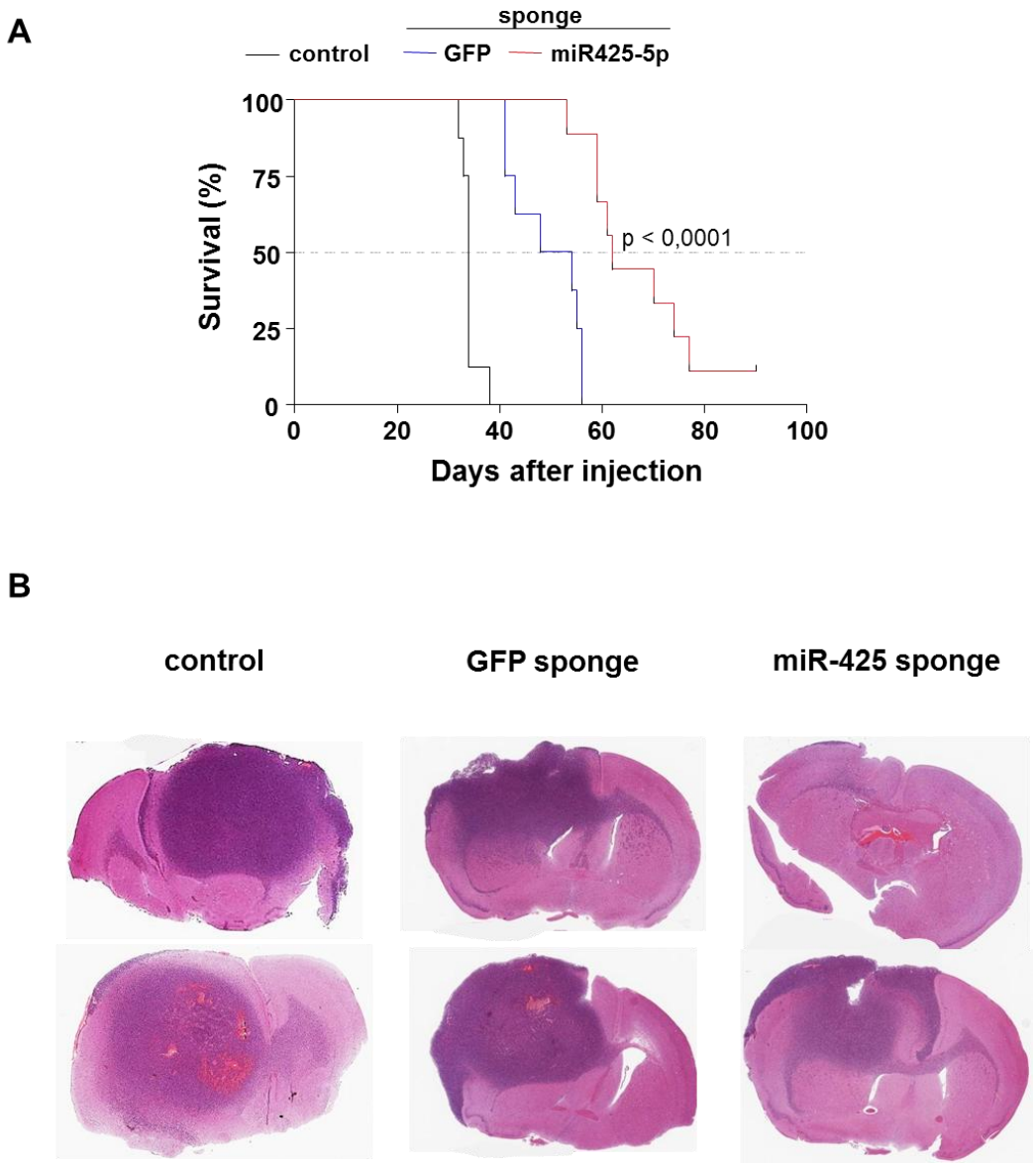

Figure 37. Inhibition of miR-425-5p results in a significant antitumor effect in vivo (A) Kaplan–Meier survival curve analysis for overall survival in nude mice after intracranial injection of GSC-11 tumor cells that were transduced with miR-425-5p-sponge versus GFP-sponge controls. MiR-425-5p inhibition results in a marked increase in median survival time relative to that in the control group (51 days and 65 days, respectively; $P = 0.0002$). P values were determined using a log-rank test; the values represent a comparison of survival rates associated with the different treated cells injected to the mice. (B) Hematoxylin and Eosin staining of 2 representative brains of the different groups of animals.

5.6. miR-425 expression is significantly increased in glioblastoma

We then asked if the expression of miR-425-5p could be over-expressed in glioblastoma patients. To this end first, we evaluated the expression levels of miR-425-5p in 15 samples. Interestingly, miR-425-5p expression was increased in all the samples assessed when compared to normal brain reference RNA (Figure 38A). SOX2

expression was also increased in the same samples (Figure 38B). A simple regression analyses showed that SOX2 significantly correlated with miR-425-5p expression (R^2 = 0.7, P<0.01) in these glioblastoma samples (Figure 38C).

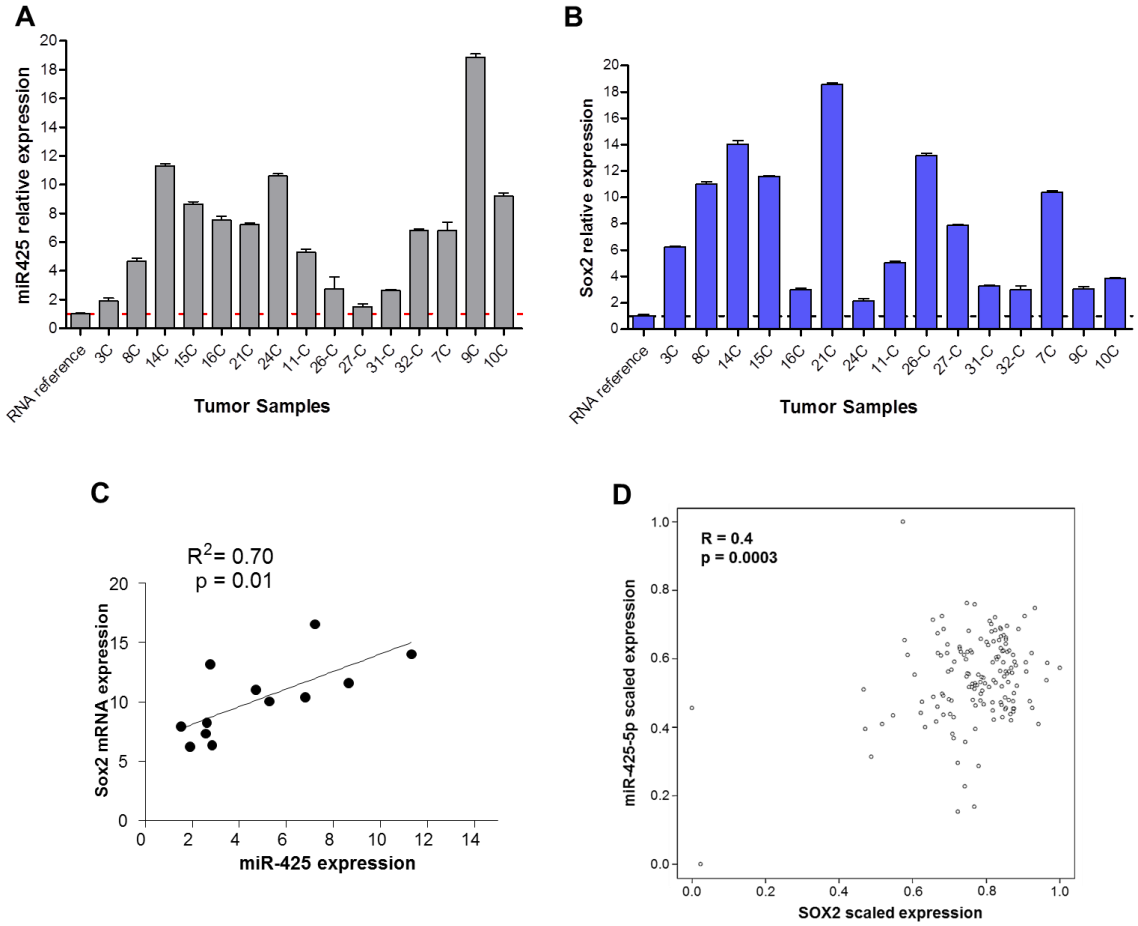

Figure 38. Assessment of miR-425-5p expression in clinical samples. (A) Evaluation of the expression of miR-425-5p in 15 glioblastoma samples. Total RNA was extracted and qRT-PCR analysis was performed compared to normal brain reference RNA commercially available. Quantification of the relative expression of miR-425-5p was performed using specific Taqman expression assay. RNU6B was used an internal control. To determine miR-425-5p relative expression we used the comparative threshold cycle method. Each bar represents the mean ± SD. **(B)** Assessment of SOX2 expression levels in 15 glioblastoma samples.Total RNA was extracted and qRT-PCR analysis was performed using specific Sybr Green gene expression assay. GAPDH was used an internal control. To determine SOX2 relative gene expression we used the comparative threshold cycle method. Each bar represents the mean ± SD. **(C)** Correlation between SOX2 mRNA expression and miR-425-5p expression in 15 glioblastoma samples. Scatter plot and lowess line ilustrate the correlation between SOX2 and miR-301 expression levels. **(D)** Pearson correlation between SOX2 mRNA expression and miR-425-5p expression in 166 glioblastoma samples publically available at TCGA portal, with a p value of 0.0003.

To further validate these results, we analyzed data available at TCGA. We used a set of 166 glioblastoma samples, from which there are data from RNA-seq and a

miRNA-array approaches. We again interrogated these data to elucidate whether in this bigger set there is also a correlation between the expression of SOX2 and miR-425-5p. Again, we observed a significant positive correlation between this two genes (R= 0.4; p= 0.0003), giving robustness to our previous results. These results highlight the fact that miR-425-5p represents a direct target of SOX2 and that its expression is regulated by this transcription factor.

5.7. Target genes of miR-425-5p

Finally we decided to identify the target genes of miR-425-5p. For that purpose we use three different bioinformatic programs (miRDB, miRTarBase and PicTar) based on on the differentially combination of criteria used to predict target genes and the availability of predicted miR-425-5p target genes. Weselected only the genes identified by all the three approaches. This analysis yield a list of 5 candidate genes: BEX4, FOXJ3, NRAS, Rab31 and SLC16A1 (Figure 39).

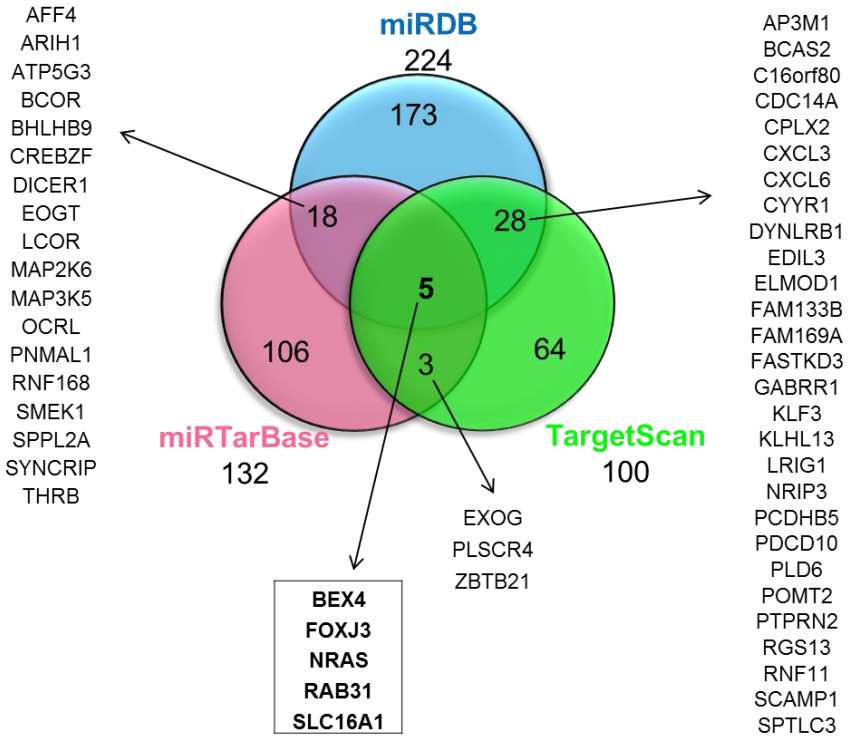

Figure 39. Predicted target genes for hsa-miR-425-5p. Search of predicted target genes using 3 different bioinformatic programs: miRDB, miRTarBase and TargetScan. The 5 candidates predicted by the 3 different approaches are listed in an underline box.

We next assessed the expression of the 5 predicted target genes in different cell lines to validate the bioinformatic prediction. We over-expressed SOX2 in HEK293FT and in the glioblastoma cell line A172. Levels of SOX2 expression in these cell lines was confirmed by Western Blot. As we expected, we observed a marked decreased in the predicted miR-425-5p target genes in both cell lines after SOX2 over-expression (Figure 40A). Conversely, we used the human glioblastoma xenograft line Mayo39 to down-regulate SOX2 expression, through forced differentiation, as previously described. Differentiation markers (GFAP and O4) were over-expressed and SOX2 expression was inhibited after differentiation process, confirmed by qRT-PCR (Figure 40B). We confirmed that the expression of the predicted miR-425-5p target genes was increased in response to the inhibition of SOX2 in a physiological context. Based on our results, FOXJ3 and NRAS were the most consistently regulated target genes of miR-425-5p. Further experiments are required to establish the functional relevance of these targets on miR-425-5p functions in GSCs.

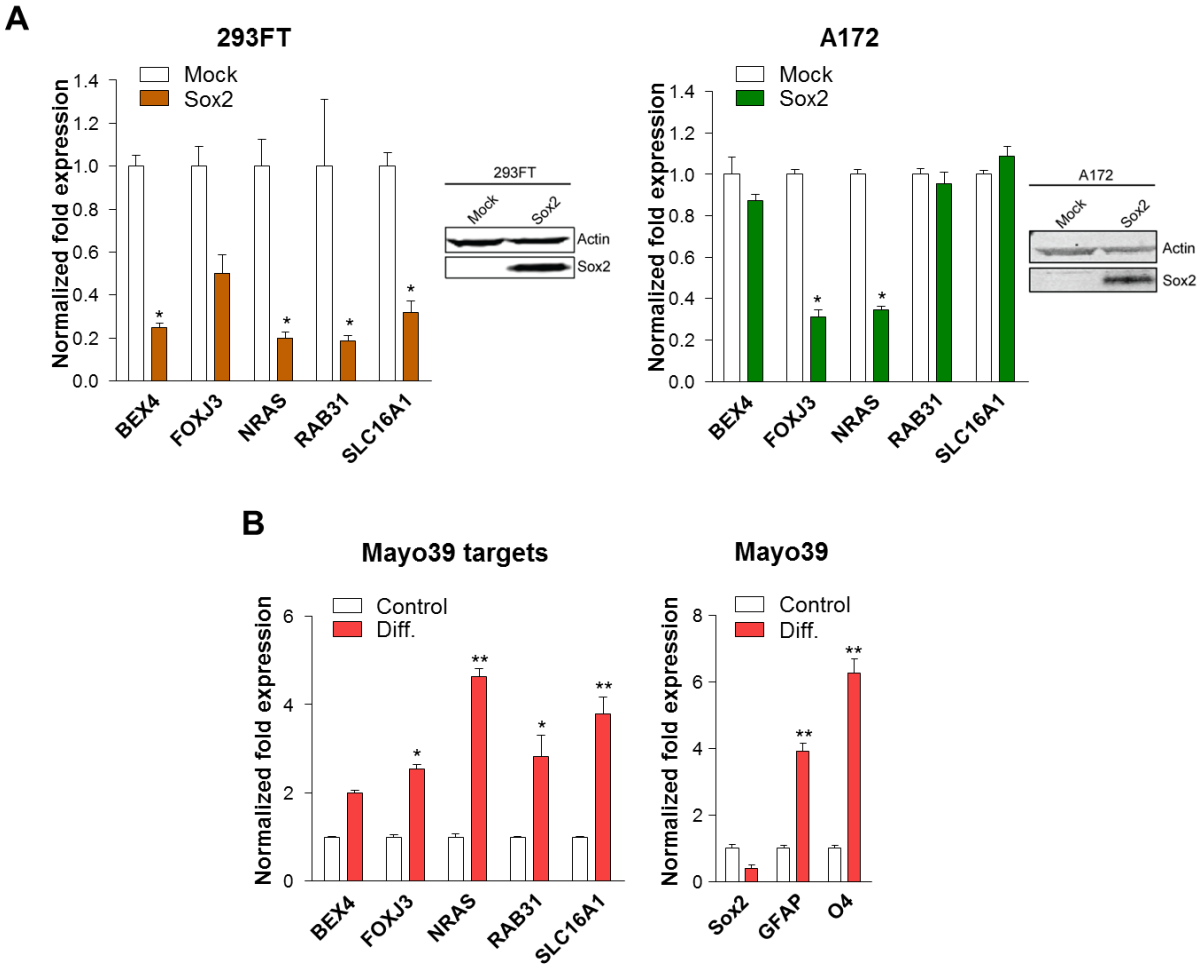

Figure 40. Validation of predicted target genes of miR-425-5p following Sox2 up-regulation. (A) A172 and HEK293FT cells were transduced using a lentiviral vector coding for mCitrine-SOX2 or

control vector (mock). RNA and total protein were extracted 72 hours after transduction. Quantification of the expression of the indicated genes was performed using Sybr Green gene expression assays specific for each gene. GAPDH was used an internal control for qRT-PCR analysis. To determine relative gene expression the comparative threshold cycle method was used. Each bar represents the mean ± SD. SOX2 over-expression at protein level was confirmed using western blot. (B) Mayo39 cells were subjected to forced differentiation protocol. Cells were plated in FGF-neurosphere medium without EGF for 2 days, 1% FBS was added without EGF/FGF for 5 days. RNA was extracted after forced differentiation process was completed and qRT-PCR analysis was performed. Quantification of the gene expression levels was performed using Sybr Green gene expression assays specific for each gene. GAPDH was used an internal control for qRT-PCR analysis. To determine relative gene expression we used the comparative threshold cycle method. Each bar represents the mean ± SD of three independent experiments.

We also evaluate the expression of this predicted target genes after miR-425-5p inhibition in HEK293FT cells and in GSC-11 and GSC-23 neurospheres. We transduced these cell lines with GFP-sponge or miR-425-5p sponge. As expected, we observed that the expression of the predicted miR-425-5p target genes was significantly increased in response to inhibition of miR-425-5p (Figure 41). Based on our results, FOXJ3 and NRAS were the most consistently regulated target genes of miR-425-5p. However, further experiments are required to establish the functional relevance of these targets on miR-425-5p functions in GSCs.

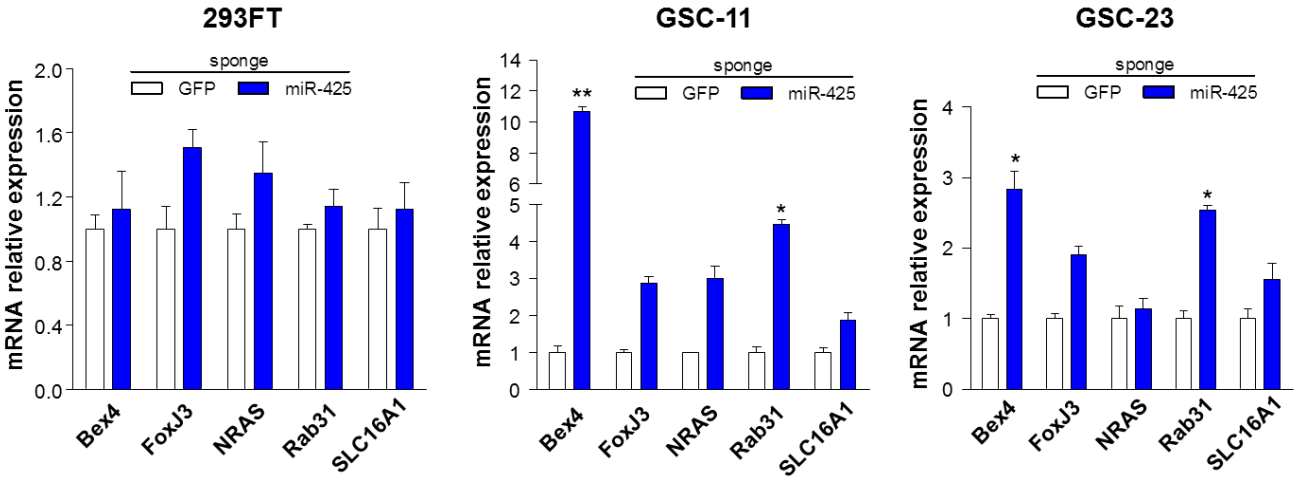

Figure 41. Analysis of the expression of predicted miR-425-5p target genes following miR-425-5p inhibition. HEK293FT, GSC-11 and GSC-23 neurospheres were transduced with GFP sponge or miR-425-5p sponge. RNA was extracted 72 hours after transduction and qRT-PCR analysis was performed. Quantification of the expression of the indicated genes was performed using Sybr Green gene expression assays specific for each gene. GAPDH was used an internal control. To determine relative gene expression levels the comparative threshold cycle method was used. Each bar represents the mean ± SD of three independent experiments.

Taken together, these results demonstrate that miR-425-5p is a direct target of SOX2 in GSCs and acts as an oncogene controlling glioblastoma pathogenesis.

Discussion

Glioblastoma is the most aggressive cerebral tumor in humans and is inherently associated with a dismal prognosis. At the cellular level, glioblastoma is made up of a heterogeneous population of tumor cells, where GSCs play essential functions. The GSC compartment is a constitutively dynamic cell population, characterized for its unlimited capacity of self-renewal and with differentiation capacity, and multipotentcy , leading the tumor-propagation and contributing to therapeutic resistance and tumor recurrence.

SOX2 is commonly overexpressed in GSCs, suggesting that is functionally involved in the generation and maintenance of the glioblastoma stem-like phenotype.

Therefore, elucidating the specific transcriptional networks controlled by SOX2, could find clues regarding stemness maintenance and proliferation of GSCs with the potential goal to uncover potential therapeutic targets (Chew et al., 2005). In this work we provided a comprehensive view of the genome wide SOX2 regulated transcripts in GSCs, illustrating a complex scenario where SOX2 is the central player that orchestrates key molecular pathways in glioblastoma.

1. Coding transcriptome and pathways regulated by SOX2 are involved in stem-like phenotype.

We applied state-of-the art microarray technology to identify the global SOX2 coding and non-coding RNA transcriptome in GSC cells. It is interesting to note that among the down-regulated genes following SOX2 knockdown, F11R has been shown to be overexpressed in glioblastoma cells (Alvarado et al., 2016; Lathia et al., 2014). F11R is necessary and sufficient for GSC maintenance and self-renewal and of clinical significance is associated with increased malignancy and poor patient prognosis (Alvarado et al., 2016; Lathia et al., 2014). CYP26A1 is another gene controlled by SOX2 found to be down-regulated in our microarray data. This gene has been reported to be necessary for proper differentiation of embryonic stem cells into neurons (Langton and Gudas, 2008). These previous reports reinforced the hypothesis that SOX2 controls genes associated with stemness properties in GSCs.

Another candidate IGFBP5, over-expressed in our array data, significantly correlated with glioma histologic grade, suggesting a role in glioma progression (Wang et al., 2006). On the other hand, we found several interesting over-expressed candidates controlled by SOX2; for example, PPP1R1B is a well-known striatal projection neuron signature marker (Arber et al., 2015). The fact that its expression increases following

SOX2 inhibition is in line with its role in neuronal differentiation. Overall these results highlight the link between SOX2 and GSCs biology.

The gene-set enrichment analysis shows that SOX2 is involved in the regulation of "cell adhesion", "biological adhesion", "cell-cell signaling", and "calcium ion binding" pathways, under covering key putative functions regulated by SOX2 (Dietrich et al., 2010; Inoue et al., 2010; Liebelt et al., 2016; Reddy et al., 2011).

The canonical pathways regulated by SOX2 related to amino-acid metabolism were among the most dysregulated, illustrating that SOX2 expression is critical for maintaining metabolic homeostasis in the GSC population, playing an important role in different tumor microenvironment conditions, such as hypoxic stress conditions (Kucharzewska et al., 2015). Other enriched pathway altered in our analysis was the NOTCH pathway, where HES5 and HEY1 had the most significantly down-modulated expression. HES5 is a marker of neural multipotent progenitors with stem cell properties (Basak and Taylor, 2007) where it sustains the proliferative state of progenitors inhibiting their differentiation into neurons (Ross et al., 2003). On the other hand, HEY1 has been linked to a subset of molecules directly associated with hypoxia in glioblastoma tumors (Irshad et al., 2015); and might be used as a marker to distinguish glioblastoma patients with a relative good prognosis (negative HEY1 expression) (Gaetani et al., 2010). Furthermore, HEY1 is up-regulated in glioma samples with a significant correlation with tumor grade; moreover, functionally its down-regulation results in a lower proliferation rate (Hulleman et al., 2009), suggesting a role in the progression of glioblastoma.

Thus, the canonical pathways more significantly altered after SOX2 inhibition are those related with intracellular signaling cascades and amino-acid metabolism pathways associated with tumor propagation.

Consistent with the current knowledge regarding SOX2 biological functions, our data-set is enriched with genes involved in morphology determination, development and cellular proliferation and migration.

Taken together, our microarray results showed a strong agreement with published reports regarding SOX2 role, underscoring the validity of our approach and the robustness for de novo discovery of SOX2 targets.

2. LncRNA landscape regulated by SOX2

One of the most exciting aspects of this study involved the identification of lncRNAs reulated by SOX2. In this work we showed and classified the lncRNA landscape regulated by SOX2 in GSCs. To our knowledge, this is the first study that evaluates the differentially expression of lncRNAs in GSCs controlled by SOX2. Even though one previous study determined the differentially expressed lncRNAs between glioblastoma and normal brain tissues, showing 654 lncRNAs upregulated and 654 down-regulated (Han et al., 2012).

We found 80 up- and 181 down-regulated lncRNAs controlled by SOX2 in GSCs. We validated the expression of the 2 top differentially expressed lncRNAs candidates in GSC-11 cells. These validated transcripts are non-annotated lncRNAs and are not described in the literature; however their TSS is marked by histone modifications associated with active chromatin, suggesting that are novel lncRNAs expressed under our experimental conditions. Mechanistic and functional studies need to be performed in order to unravel their role in the context of GSCs.

Among the transcripts regulated by SOX2 we found interesting candidates, such as SOX2OT and ANRIL; although more experiments are needed to elucidate its possible role in GSCs biology. SOX2OT is a lncRNA which harbors SOX2 gene in its intronic region and is transcribed in the same orientation as SOX2 (Fantes et al., 2003). Several studies have demonstrated a role of SOX2OT in the regulation of SOX2 gene in human stem cells (Amaral et al., 2009; Shahryari et al., 2014) although little is known about the exact role of this non-coding RNA. SOX2OT has been associated with carcinogenesis. For example, in breast cancer is involved in the induction and/or maintenance of SOX2 expression (Askarian-Amiri et al., 2014). In esophageal squamous cell carcinoma SOX2 is involved in tumor initiation and in the regulation of the pluripotent state of stem cells (Shahryari et al., 2014). In lung cancer is associated withthe maintenance of cell proliferation (Hou et al., 2014). Published data suggest the mediation of lncRNA SOX2OT in pluripotency and tumorigenesis events, probably through regulation of SOX2 expression (Shahryari et al., 2015). These data together with our own results suggest a possible role of SOX2OT in GSCs function during the malignant progression of glioblastoma.

On the other hand, ANRIL is a bona-fide SOX2 transcriptional target suppressing the *INK4b/ARF/INK4a tumor suppressor* locus (Pasmant et al., 2007). ANRIL is involved in the recruitment of Polycomb Repressive Complex 2 (PRC2) for gene

silencing (Kotake et al., 2011). Interestingly, PRC2 is a key mediator of glioma stem-like cell plasticity, which is required for the adaptation of glioblastoma cells to their microenvironment (Natsume et al., 2013). These results suggest that ANRIL may play an important role in GSCs maintenance in glioblastoma. However further functional and mechanistic studies will be necessary to elucidate the precise role of SOX2OT, ANRIL and other lncRNA candidates in the tumorigenicity of glioblastoma.

3. SOX2 controls miRNAs in GSCs

In our study we also profile the SOX2-regulated miRNAome in GSCs. It is interesting to note that most of the miRNAs found in our array data have been consistently found as dysregulated in glioblastoma in other independent studies (Appendix 3). highlighting the robustness of this approach and emphasizing the possibility to use it as a platform to identify novel SOX2-miRNAs targets.

We analyzed the expression of the top 6 up- and down-regulated miRNAs in 3 different glioblastoma cell lines, LN-229, GSC-11 and GSC-23 cell, following SOX2 inhibition and validated all of them, which again reinforce the strength of our array platform.

4. miR-301a is over-expressed in glioblastoma and regulates migration/invasion properties of GSCs.

We first focused on the study of miR-301a-3p as an interesting candidate to be studied in glioblastoma. *In silico* analysis revealed the promoter region of miR-301a-3p has multiple binding sites for *SOX2*, supporting the notion that miR-301a-3p is one of the direct targets of SOX2, and therefore could act as an onco-miR in GSCs. In fact, SOX2 together with OCT-4 and NANOG co-occupy the promoter of miR-301 in pluripotent stem cells (Boyer et al., 2005).

Gene-set enrichment analysis of the predicted target genes shows that miR-301a-3p is involved in "transcription", "regulation of transcription" and "purine ribonucleotide binding" process, illustrating the relevance of miR-301a in key functions of SOX2 as a transcription factor.

By means of *in vitro* experiments we showed that the inhibition of miR-301a-3p does not affect proliferation properties of GSC-23 cells, however remarkably inhibits the migration/invasion ability of these cells. Moreover, the down-modulation of miR-

301a-3p in GSC-23 cells inhibits the expression of a large set of genes involved in motility/invasion tumoral process. For example EphA belongs to the family of receptor tyrosine kinases involved in tumor invasion and cytoskeleton rearrangement (Kandouz, 2012) and might function to promote malignant progression of glioblastoma (Day et al., 2014). RHO GTPases have important roles in regulating cytoskeletal dynamics (Ridley, 2006) and participate in tumor vascularization through the regulation of the induction of HIF1a (Turcotte et al., 2003). ROCK is involved in fundamental cellular functions such as cell contraction, cell migration and cytokinesis (Amano et al., 2010). One of the most important factors involved in the angiogenesis of glioma is the VEGF growth factor (Kargiotis et al., 2006), promoting also cell migration and survival in endothelial cells (Rahman et al., 2010). TGF-β also play important roles in regulation of cell differentiation, extracellular matrix remodeling, angiogenesis and cell adhesion (Blobe et al., 2000; Govinden and Bhoola, 2003); and is a well-known mediator of glioma progression (Kjellman et al., 2000). MMPs members modulate cell-cell communication and promote tumor progression (McCawley and Matrisian, 2001; Stetler-Stevenson, 1996). The NOTCH receptors (NOTCH1-3) are highly express in brain tumors and are implicated in glioblastoma cell growth (Chen et al., 2010b). Figure 42B summarizes the effect of inhibition of miR-301a-3p in GSCs.

Overall these results suggests that miR-301a-3p may function as an onco-miR contributing to tumoral progression of glioblastoma, reinforcing the idea that miR-301a-3p acts as an onco-miR in different tumor types (Liang et al., 2015; Lu et al., 2015; Xia et al., 2015). Nevertheless, more experiments are needed to address the functional and mechanistic implications of miR-301a in the biology of glioblastoma.

5. MiR-425-5p is a direct target of SOX2

We showed that *SOX2* directly binds to the promoter region of miR-425-5p promoting its activation. This evidence confirmed that miR-425-5p is a functional target of SOX2 in GSCs, remarking its possible role as an onco-miR. Taking this into consideration, we decided to focus on enhancing the expression of the predicted miR-425-5p tumor suppressor target genes, by means of inhibition of miR-425-5p expression, to negatively regulate the stem-cell driving genes expression. Using this approach, we intended to enhance the effect of tumor suppressor genes to attenuate GSC phenotype in glioblastoma, ultimately causing cell death.

6. Functional role of miR-425-5p in glioblastoma pathogenesis

6.1. miR-425 affects neurosphere formation and cell cycle progression

We inhibited miR-425-5p expression using a 2nd generation lentiviral vector that expressed an antisense RNA oligo designed to target miR-425-5p, to ensure the long-term inhibition of miR-425-5p expression in the experiments performed. Our results demonstrate the efficacy and feasibility of the miR-425-5p knock-down using this system in GSCs.

The down-regulation of miR-425-5p promotes a significant inhibitory effect on neurosphere formation, observing a significant reduction in the number and size of neurospheres. In addition, upon miR-425-5p knock-down, we also observed a significant reduction in cell proliferation and in cell invasion properties in GSCs. Since invasion capacity of GSCs was measured at 12h after miR-425 down-regulation, we can rule out that cell death was the direct cause of the decrease in the invasiveness of these cells. These results are consistent with the findings of a recently published study (Zhang et al., 2015c), showing the inhibition of cell proliferation and invasion in gastric cancer cells following miR-425-5p inhibition, and underscore the role of this miRNA as an oncogene in glioblastoma.

Furthermore, the inhibition of miR-425-5p expression also promoted a significant G0/G1 arrest in cell cycle, downmodulating the expression of cell cycle related proteins, which also explains the decrease in cell proliferation. Notably this effect is not observed in GFP sponge cells, which confirms that is not due to the plasmid used or infection process, but the effect of miR inhibition per se.

6.2. Inhibition of miR-425-5p promotes apoptotic cell death

Inhibition of miR-425-5p resulted in cell death. We observed that inhibition of miR-425-5p triggers apoptosis mechanism and suppression of survival machinery in GSCs. These results are in agreement with an elegant study by Ahir and collegues where they treated triple negative breast cancer carcinoma cells with tailored-CuO-nanowire decorated with folic acid (CuO-Nw-FA). They observed that treatment of the breast cancer cells with CuO-Nw-FA favoured down-regulation of miR-425 levels leading to induction of apoptosis and reduction of metastasis, through up-regulation of PTEN pathway, promoting inhibition of PI3/AKT pathway and cyclin-dependent kinases (Ahir et al., 2016). In another study, miR-425 emerged as a miRNA associated with radioresistance in glioblastoma through activation of the cell-cycle checkpoint

response (Moskwa et al., 2014). Moreover, the authors observed in the data sets from the TCGA that theses miRNA were expressed in patients with TGF-β signaling elevated, which in turn has been associated with a glioma stem cell signature (Penuelas et al., 2009; Seoane, 2009).

In accordance with these results, the analysis of AnnexinV/SYTOX Blue double staining demonstrated a marked increase in early apoptotic cells following miR-425-5p compared with cells treated with GFP lentivirus and normal control cells (Figure 36D). Notably, as phosphatidylserine is normally present on the inner leaflet of the membrane, any disruption of the membrane can lead to Annexin V positivity. We observed that neurosphere disaggregation using Acutase lead to membrane permeabilization, promoting false Annexin V positivity in GSCs. This fact made us to use another viability cell surface marker (Sytox Blue) to accurately discriminate between apoptotic and non-apoptotic cells.

Moreover, TEM images showed the characteristic changes in apoptotic cells, such as cell shrinkage, chromatin condensation, and nuclear fragmentation (Kerr et al., 1972), after miR-425-5p inhibition in GSCs compared with control cells (Figure 36E).

Taking the previous published reports in mind and our own results, we hypothesized that decreased miR-425-5p levels can promotes PTEN up-regulation, suppressing in turn AKT mediated pathway and inducing the expression of p27, which negatively regulates cyclin-dependent kinases and promotes cell cycle arrest, and thus cumulatively causes cell death via apoptosis (Figure 42A).

Overall, these results demonstrated the activation of apoptotic mechanism after inhibition of miR-425-5p in GSCs and reinforcing our hypothesis that miR-425-5p act as an onco-miR.

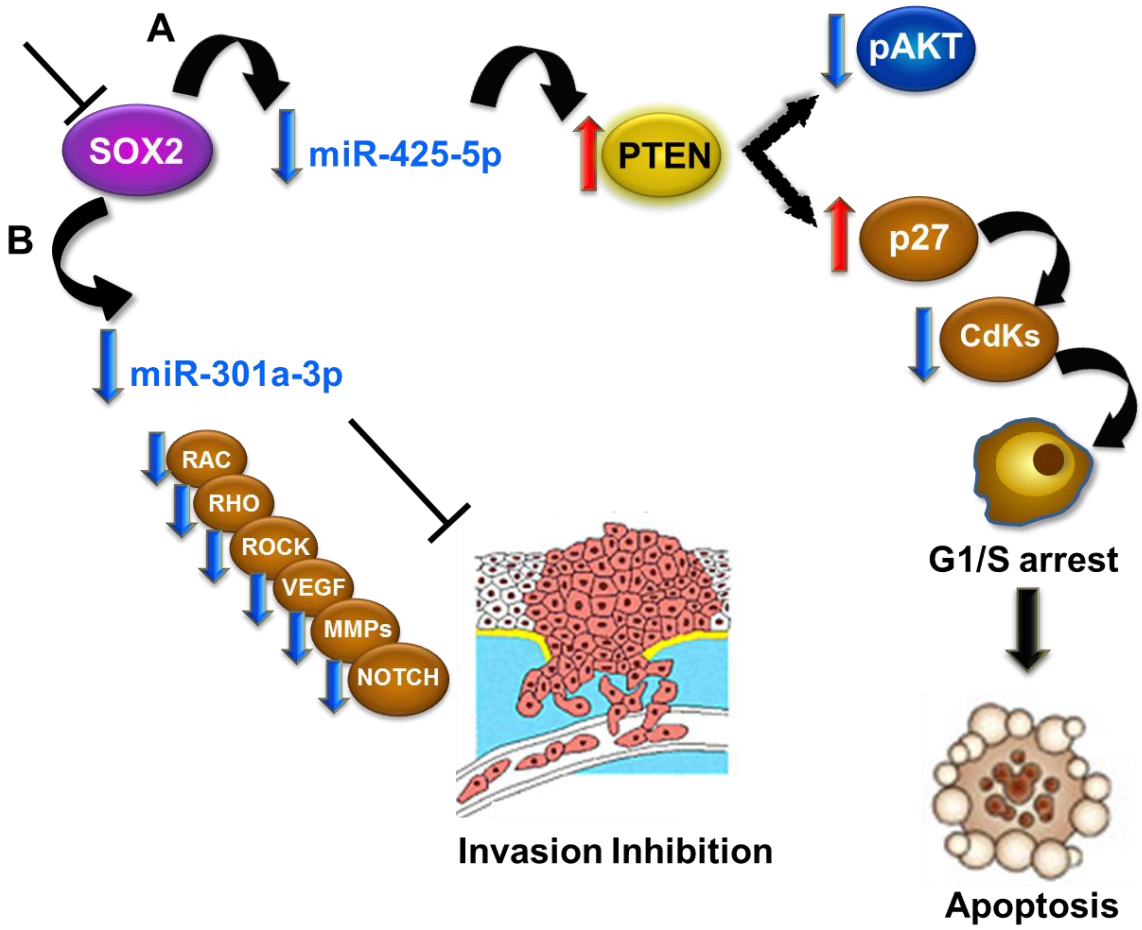

Figure 42. Schematic diagram depicting our model regarding the molecular mechanism induced following SOX2 inhibition. Following SOX2 inhibition we observed (A) decreased levels of miR-425-5p, which in turn activates PTEN levels. PTEN activation further leads the decreased levels of PI3/AKT pathway and the increased levels of p27, a known inhibitor of cyclin-dependant-kinases which causes G0/G1 arrest and thus cumulative causes apoptosis cell death. (B) Reduced levels of miR-301a-3p following SOX2 inhibition, promote a decrease in GSCs invasion capacity, down-modulating the expression of numerous tumoral invasion genes.

6.3. MiR-425-5p inhibition results in a significant anti-tumoral effect *in vivo*

Inhibition of miR-425-5p exerts a potent anti-tumor effect in nude mice, increasing the overall median survival time. This result allows us to suggest that miR-425-5p could be used into promising anticancer therapies either alone or in combination with current targeted therapies.

Although one of the greatest challenges with miRNA-based therapeutics is finding effective delivery systems, some exciting strategies are beginning to appear. Convection-enhanced delivery accompanied by imaging monitoring (Laske et al., 1997; Mut et al., 2008); modified peptides to transfer across the blood-brain barrier (BBB), carrying miRNAs/anti-miRNAs into glioblastoma cells (Oh et al., 2016; Song et al.,

2015); vectors based on modified adeno-associated viruses usefulness for prolonged high expression and tissue-specific tropism (Xie et al., 2015); modified nanoparticles which can cross the BBB accumulating within intracerebral gliomas an delivering the cargo (Kouri et al., 2015), and mesenchymal stem cells, which can deliver synthetic miRNAs to glioma cells through gap junction-dependent and independent mechanisms (Lee et al., 2013; Munoz et al., 2013), are among the effective delivery approaches for miRNA-based therapies currently in use.

Taking advantage of the ability of miRNAs to target multiple genes/pathways, and based on progress in delivery miRNA systems and in our own results, we propose that miR-425-5p is an interesting candidates to be further used for miRNA-therapy in glioblastoma. Taking into account that miR-425-5p plays an important role in GSCs biology, we can use it for example in combination with conventional chemotherapy, sensitizing glioblastoma cells to chemo drugs, based on previous reports (Zhang et al., 2016).

6.4. Glioblastoma expressed high levels of miR-425-5p expression

The analysis of 15 glioblastoma clinical samples revealed that expression levels of miR-425-5p are increased at the mRNA level, and that has a significant positive correlation with SOX2 expression (Figure 38C). MiR-425-5p is over-expressed (~80 percentile) in majority of human glioblastoma, based on analysis of TCGA database (Moskwa et al., 2014). These data confirmed that miR-425-5p is a direct target of SOX2 in glioblastoma. Interestingly, in the set of 166 glioblastoma specimens from TCGA was also found a significantly positive correlation between SOX2 and miR-425-5p. Regarding this data, the correlation coefficient obtained in this analysis is positive but low (R^2=0.4), however is statistically significant (p=0.0003). These results demonstrated that miR-425-5p is over-expressed in all glioblastoma subtypes, which leads us to think that does not have any impact in overall median survival time of glioblastoma patients. This reinforced the idea of the usefulness of miR-425-5p for target therapies leading to promising future clinical trials.

7. MiR-425-5p predicted target genes

We generated a robust miRNA candidate list regulated by SOX2 in GSCs, combining two different arrays approaches. Of all the candidates, miR-425-5p emerged

as the most consistently regulated miRNA by SOX2, after being validated in different GSCs and human glioblastoma cell lines, where SOX2 was over- or down-regulated.

We identified the 5 most consistently predicted target genes of miR-425-5p. We analyzed their expressions in different glioblastoma cell lines, validating the bioinformatic prediction. Based on our results, we found that FoxJ3 and NRAS emerged as the miR-425-5p top regulated genes. Interestingly, FOXJ3 is known to be a key transcription factor of mitochondrial biogenesis and was identified to regulate the adult skeletal muscle fiber type identity (Alexander et al., 2010; Landgren and Carlsson, 2004); however is not very well studied,. Therefore, without further experimentation is unclear the role that could play in the context of glioma.

Another, candidate target gene is NRAS, a member of the RAS oncogene family involved in MAPKs and PI3K/AKT signaling pathways, and various studies have demonstrated recurrent aberrant activation in glioblastoma (Knobbe et al., 2004). This result is puzzling since downregulation of either SOX2 or miR-425 results in upregulation of this oncogene. NRAS is known to have an oncogenic role in melanoma were activating mutations of this gene renders a subset of tumors with a more aggressive profile associated with poor outcome (Vu and Aplin, 2016)

These results uncover the complexity that intrinsically harbors the different signaling pathways and the possible crosstalk that inherently could be taking placed. It is clear, that more experiments are needed to understand which the targets of miR-425-5p are and how it exerts its function in GSCs.

In summary our study integrates for the first time the coding and non-coding transcriptome controlled by SOX2 in GSCs, defining miR-301 and miR-425-5p as novel oncomiRs in GSCs and gaining new insights about the molecular circuitries governing glioblastoma biology (Figure 43).

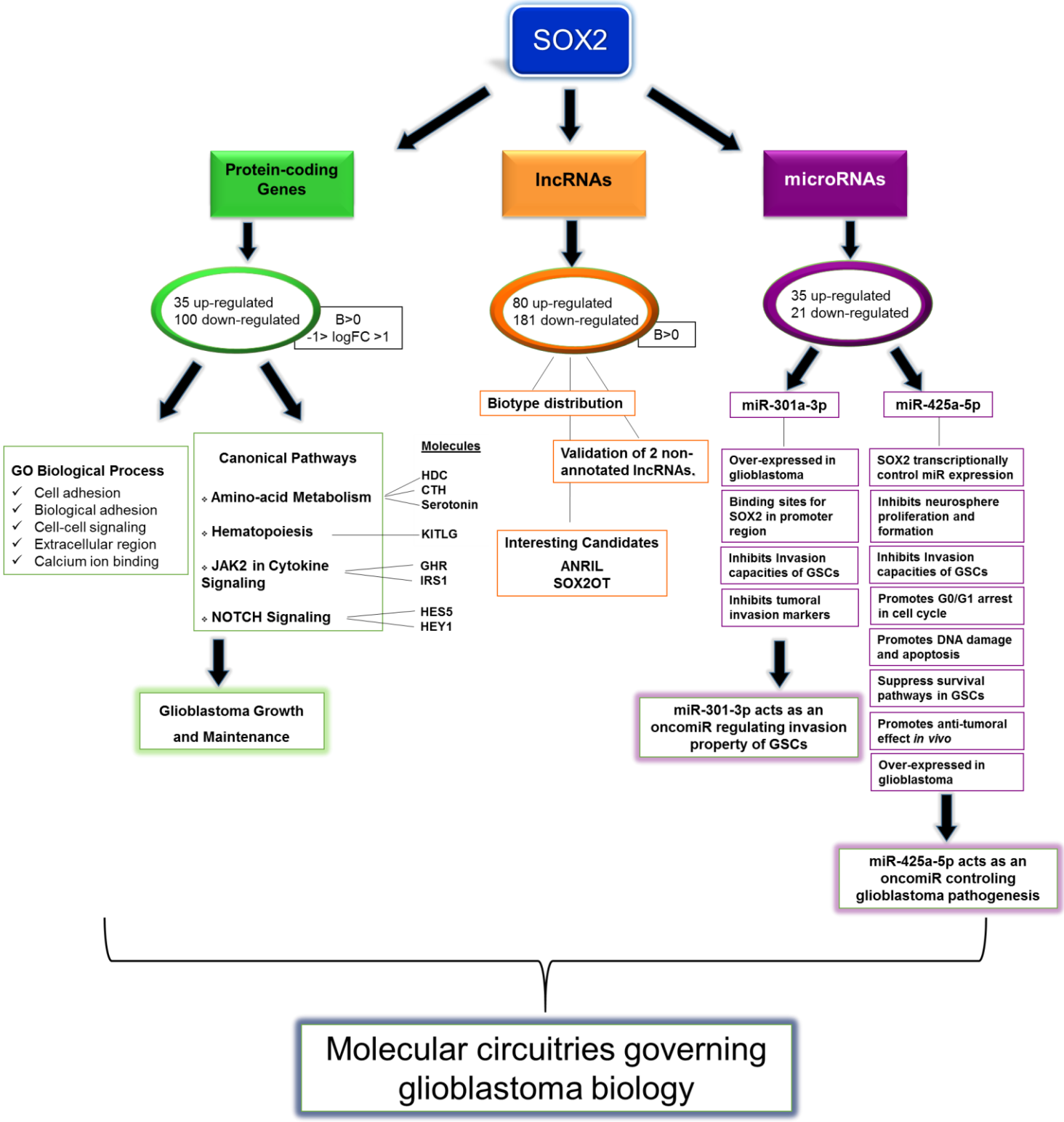

Figure 43. Summary of the results obtained in this work. SOX2 regulates the coding- and non-coding transcriptome in GSCs, orchestrating numerous molecules and pathways governing glioblastoma biology.

Conclusions

1. SOX2 regulates a wide spectrum of protein-coding genes, which are related to different biological process including cell adhesion and cell-cell signaling, and are involved in canonical pathways, related with intracellular signaling cascades and amino-acid metabolism pathways associated with GSC propagation. These evidence underscore the pleiotropic functions of SOX2 as a transcriptional factor.

2. SOX2 regulates different types of non-coding RNAs differentially expressed in GSCs, including miRNAs and LncRNAs amongst other.

3. SOX2 regulates miR-301a-3p. This miRNA is over-expressed in glioblastoma tissues, positively correlates with SOX2 expression and participate in the invasive properties of GSCs, acting as an onco-miR.

4. SOX2 controls miR-425-5p in glioblastoma. This miRNA is significantly overexpressed in glioblastoma tissue. We demonstrated that SOX2 activates its expression by directly binding miR-425-5p promoter.

5. Inhibition of miR-425-5p affects neurosphere formation, cell proliferation, cell invasion and promotes cell cycle arrest in GSCs, pointing to its role as an onco-miR regulated by SOX2.

6. Downregulation of miR-425-5p promotes cell death through inhibition of cell survival pathways and activation of the apoptotic machinery. Altogether, this leads to cell death *in vitro* and to a significant increase in overall median survival time of mice bearing orthotopic glioma xenografts.

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Appendix

Appendix 1

Primers used for lncRNAs detection

lncRNA position	TCONs	Forward Primer	Reverse Primer
chr19:28281401- 28284848	TCONS_00027256	GCCCAAAGTTTGATTTCTCG	CGAGGTCTAACCCAGGTGTG
chr11:121899032-121899389	TCONS_00020142	GCTGAGCCTTCCATGAAAAT	GTGCAAATCACTCCAGTCACA

Primers used for gene detection

Gene	Forward Primer (5' to 3')	Reverse Primer (3' to 5')
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
SOX2	AGCTCGCAGACCTACATGAA	CCGGGGAGATACATGCTGAT
PLP1	ACCTATGCCCTGACCGTTG	TGCTGGGGAAGGCAATAGACT
COL2A1	TGGACGCCATGAAGGTTTTCT	TGGGAGCCAGATTGTCATCTC
ATP8B1	ACGACATTTGACGAGGATTCTC	GGTTTTGTTCTGGTTCAACAGC
PPP1R1B	CAAGTCGAAGAGACCCAACCC	GCCTGGTTCTCATTCAAATTGCT
CMTM5	GGAGGACCACATCCGCTAGAT	CCAGGGAGTGGAAGCAGAT
GALNT14	CACTGCTGGTGTATTGCACG	CGGATCAGATGCGTAGGGG
F11R	GTGCCTACTCGGGCTTTTCTT	GTCACCCGGTCCTCATAGGAA
SYT4	ATGGGATACCCTACACCCAAAT	TCCCGAGAGAGGAATTAGAACTT
SLC18A1	GTGGTGGTATTCGTCGCTTTG	CCGAGGTGCAGAGAAGAGT
ITLN2	GCAGGGCAACAAAGCAGACTA	CAGGGCGCTGTTTCTCCAA
FoxJ3	GGAGAGCAGCCTAACGTCTAT	TGGCATAACTGTATGGAGGTTTC
NRAS	ATGACTGAGTACAAACTGGTGGT	CATGTATTGGTCTCTCATGGCAC
BEX4	AAAGAGGAACTAGCGGCAAAC	CCAAATGGCGGGATTCTTCTTC
RAB31	GGGGTTGGGAAATCAAGCATC	GCCAATGAATGAAACCGTTCCT
SLC16A1	AGTAGTTATGGGAAGAGTCAGCA	GTCGGGCTACCATGTCAACA
LIMS2	GCACCGGCACTATGAGAAGAA	ACGGGCTTCATGTCGAACTC
ACIN1	CCTTAACTCGACGTTCCATTAGC	TGGCCTAAAGTGAAAGGACGG

MPRIP	CGCAGGCAAAACCCATTTATG	CCGTGCTCGTAAAGGATGAAG
RHOA	GGAAAGCAGGTAGAGTTGGCT	GGCTGTCGATGGAAAAACACAT
UBQLN4	ATTCGGGTCACCGTCAAGAC	GCCTTAAACCTCCGGGAGATTT

Appendix 2.

List of the top non-coding transcripts upregulated or downregulated by SOX2, organized by B value.

Probe	GeneName	Classification	logFC	B
A_19_P00320471	lincRNA:chr9:2535671-2536375_R	antisense	-1,28	11,75
A_19_P00315804	lincRNA:chr9:2530903-2539456_R	antisense	-1,15	10,77
A_19_P00320469	lincRNA:chr9:2535671-2536375_R	antisense	-1,27	9,70
A_19_P00811613	lincRNA:chr9:2452800-2552025_R	antisense	-1,17	7,94
A_33_P3397743	LOC100128088	pseudogene	-1,83	7,42
A_19_P00321203	lincRNA:chr6:72126155-72129954_R	lincRNA	-0,92	7,33
A_19_P00322118	lincRNA:chr2:39745746-39826668_F	antisense	-0,86	6,90
A_23_P3552	LOC730092	pseudogene	-0,65	6,50
A_19_P00322220	lincRNA:chr20:37055062-37063887_R	processed_transcript	-0,69	6,28
A_33_P3392460	LOC100128077	processed_transcript	-1,47	6,18
A_19_P00322149	lincRNA:chr6:72126142-72129923_R	lincRNA	-0,91	6,16
A_19_P00317793	lincRNA:chr20:37055062-37063916_R	processed_transcript	-0,68	6,10
A_19_P00808846	lincRNA:chr21:17992729-18010729_F	lincRNA	-0,64	6,09
A_19_P00318304	lincRNA:chr20:37050986-37063998_R	processed_transcript	-0,67	5,94
A_19_P00316341	lincRNA:chr7:130600800-130606702_F	lincRNA	-0,86	5,93
A_19_P00316985	lincRNA:chr6:72126162-72129969_R	lincRNA	-0,91	5,93
A_19_P00322967	lincRNA:chr20:37050934-37057222_R	processed_transcript	-0,69	5,54
A_19_P00802098	lincRNA:chr2:3579550-3585150_R	lincRNA	-0,58	5,15
A_24_P756289	SOX2OT	other	-0,86	5,08
A_33_P3613516	LOC254057	antisense	-1,10	4,94
A_19_P00318174	lincRNA:chr2:3579840-3584422_R	lincRNA	-0,73	4,52
A_33_P3287710	chr10:79,686,570-79,689,583	unassigned	-0,67	4,46
A_33_P3405043	LOC100133264	unassigned	-0,72	4,43
A_32_P88349	LOC730256	pseudogene	-0,48	4,36
A_33_P3705884	chr19:28,281,401-28,284,848	lincRNA	-0,89	4,27
A_19_P00321044	lincRNA:chr16:50682543-50683160_F	lincRNA	1,04	6,09
A_19_P00315647	lincRNA:chr11:121899032-121899389_R	other	0,65	5,63
A_32_P63013	LOC283174	unassigned	1,32	5,08
A_32_P47157	LOC92973	unassigned	0,74	4,95
A_19_P00317484	lincRNA:chr3:112315643-112316945_R	lincRNA	0,55	4,11
A_19_P00809440	lincRNA:chr11:133765815-133774297_R	other	1,23	4,06
A_33_P3789382	chr10:65,224,989-65,226,322	antisense	0,57	3,98
A_19_P00321420	lincRNA:chr11:133766329-133767054_R	unassigned	1,34	3,74
A_19_P00332120	lincRNA:chr3:156455706-156471081_R	lincRNA	0,59	3,57
A_19_P00320101	lincRNA:chr11:133767609-133771496_R	other	1,06	3,51
A_19_P00812924	lincRNA:chr11:121895965-121904065_R	other	0,53	3,46
A_19_P00326763	lincRNA:chr3:112308735-112318605_R	lincRNA	0,48	2,73
A_33_P3753757	LOC158402	other	0,53	2,46
A_33_P3393679	LOC645323	lincRNA	0,47	2,24
A_19_P00315649	lincRNA:chr11:121899032-121899389_R	other	0,55	2,23
A_19_P00809838	lincRNA:chrX:100247844-100257469_R	unassigned	0,79	2,20
A_19_P00331576	lincRNA:chr3:114043485-114052926_F	unassigned	0,36	2,00
A_33_P3259557	LOC440104	pseudogene	0,45	1,67
A_19_P00319347	lincRNA:chr2:168149680-168414843_F	lincRNA	0,77	1,67
A_19_P00320212	lincRNA:chr9:114795825-114797203_R	other	0,41	1,63
A_24_P93703	LOC440104	pseudogene	0,40	1,50
A_19_P00318878	lincRNA:chr1:247350513-247352101_R	lincRNA	0,38	1,44
A_19_P00316010	lincRNA:chr17:67547498-67549996_F	lincRNA	0,57	1,43
A_24_P349207	ENST00000380727	pseudogene	0,29	1,31
A_19_P00802064	lincRNA:chr8:2522118-2527693_R	lincRNA	0,38	1,04

Appendix 3

Deregulated miRNAs previously reported in glioblastoma. Positive sign (+) represents induction and negative sign (-) represents inhibition of the indicated process.

miRNA	Functions	Targets	Expression pattern	Reference
miR-1	Radioresistance(+)	TGF-β	up	(Moskwa et al., 2014)
miR-7	Viability(-), Invasion(-)	EGFR, IRS-1/2	down	(Kefas et al., 2008)
	Migration(-), Invasion(-)	FAK	down	(Wu et al., 2011)
	Proliferation(-), Migration(-), Invasion(-), Apoptosis(+)	EGFR	-	(Wang et al., 2013d)
	Cell growth(-), Cell cycle arrest(+)	PI3K, Raf-1	down	(Liu et al., 2014)
	Angiogenesis(-), Cell viability(-), Tube formation(-), Sprouting(-), Migration(-), Tumor growth(-)	OGT	-	(Babae et al., 2014)
miR-16	Proliferation(-),Migration(-), Invasion(-)	Zyxin	down	(Li et al., 2013b)
	Invasion(-), Adhesion(-), Cell cycle(-),IL-6, IL-8, TGFβ production(-)	-	up	(Wang et al., 2014a)
	Invassion(-), Apoptosis(+), Glioma growth(-)	BCL2, NFκB	down	(Yang et al., 2014c)
	Proliferation(-), Migration(-), Extension and tubule formation(-)	Bmi-1	down	(Chen et al., 2016a)
miR-18a	Proliferation(-), Migration(-), Invasion(-), Cell cycle arrest(+),	CTGF	up	(Fox et al., 2013)

	Apoptosis(+)			
	Clonal proliferation in vitro(+),Ttumorigenicity *in vivo*(+)	DLL3	-	(Turchi et al., 2013)
	Proliferation(-), Migration(-), Invasion(-), cell cycle arrest(+), Apoptosis(+)	Neogenin	up	(Song et al., 2014)
miR-19b	-	PTEN	up	(Jia et al., 2013)
miR-20a	Invasion(+)	TIMP-2	up	(Wang et al., 2015c)
	TMZ-resistance(+)	LRIG1	up	(Wei et al., 2015)
	Apoptosis(+)	LIRG1	-	(Zhou et al., 2015)
miR-21	Cell growth(-), Apoptosis(+), Cell cycle arrest(+)	HNRPK, TAp63	up	(Papagiannakopoulos et al., 2008)
	Migration(-), Invasion(-), Glioma Cell Motility(-)	MMPs	up	(Gabriely et al., 2008)
	Chemoresistance(+)	LRRFIP1	up	(Li et al., 2009b)
	Apoptosis(+), Cell-cycle progression(-)	PTEN independent	up	(Zhou et al., 2010a)
	Proliferation(-), Apoptosis(+), Colony formation(-), Tumor formation(-)	Pdcd4	up	(Gaur et al., 2011)
	Cell growth(-), Tumor growth(-)	hTERT	up	(Wang et al., 2012b)

	Radiation resistance(+), Apoptosis(+), Cell cycle arrest(+)	PDCD4, hMSH2	up	(RW.ERROR - Unable to find reference:924)
	Proliferation(+), Chemosensitivity(-)	FOXO1	up	(Lei et al., 2014)
	Proliferation(-), Tumor formation(-)	IGFBP3	up	(Yang et al., 2014a)
	Proliferation(-), Apoptosis(+)	FASLG	up	(Shang et al., 2015)
	Proliferation(-)	MPS1	up	(Maachani et al., 2015)
	Number of viable cells(-), cell cycle arrest(+)	PTEN, Casp3	up	(Ananta et al., 2015)
miR-30e	Invasion(+)	IκBα	up	(Jiang et al., 2012)
	Invasion(+)	CBL-B	up	(Kwak et al., 2015)
miR-92b	growth (-), Apoptosis(+),invasion (-)	NLK	up	(Wang et al., 2013b)
	Viability(-) tumor growth(-)	Smad3	up	(Wu et al., 2013b)
	Proliferation(+) Overall survival(-)	DKK3	up	(Li et al., 2013a)
miR-107	Proliferation(-), cell cycle arrest(+)	CDK6, Notch-2	down	(Chen et al., 2013b)
	Migration(-), Invasion(-)	Notch2	down	(Chen et al., 2013a)
	Proliferation(-), Apoptosis(+)	SALL4	down	(He et al., 2013)

	Proliferation, Migration and Tube formation ability of human brain microvascular endothelial cells(-)	VEGF	down	(Chen et al., 2016c)
miR-124	Neuronal differentiation(+), Cell cycle arrest(+), Proliferation(-)	CDK-6	down	(Silber et al., 2008)
	Proliferation(-), G1/S transition(-),Invasion(-)	PPP1R13L	down	(Zhao et al., 2013)
	Immunosuppression(-), Tumor growth(-)	STAT3	down	(Wei et al., 2013)
	Proliferation(-)	SOS1	down	(Lv and Yang, 2013)
	Radiosensitivity(+)	CDK4	down	(Deng et al., 2013)
	Angiogenesis(-), Glioma growth(-)	LAMB1	down	(Chen et al., 2014b)
	Tumor cell survival under stressful microenvironments(+)	TEAD1, MAPK14/p38α, SERP1	down	(Mucaj et al., 2015)
	Migration(-), Invasion(-)	Capn4	down	(Cai et al., 2016)
miR-128	Cell proliferation in vitro(-) and glioma growth *in vivo*(-), glioma self-renewal(-)	Bmi-1	down	(Godlewski et al., 2008)
	Glioma-initiating neural stem cells growth(-), neuronal differentiation(+)	RTK	-	(Papagiannakopoulos et al., 2012)
	Radiosensitivity(+)	SUZ12	down	(Peruzzi et al., 2013)
	Cell cycle(+), proliferation(+) and invasion(+)	SP1	-	(Dong et al., 2014)

	Invasión(-)	Rap1B	down	(She et al., 2014)
miR-129-5p	Proliferation(-)	PDGFRa, Foxp1	down	(Tian et al., 2015)
	Proliferation(-), Cell cycle arrest(+)	IGF2BP3, MAPK1, CDK6	up	(Kouhkan et al., 2016)
miR-149	Proliferation(-), Invasion(-), Cycle arrest(+)	AKT1	down	(Pan et al., 2012)
	Invasion(-),	Rap1B	down	(She et al., 2014)
miR-181a	Radiosensitivity treatment(+)	Bcl-2	down	(Chen et al., 2010a)
miR-181b	Apoptosis(+), Invasion(-), Growth(-)	-	down	(Shi et al., 2008)
	neurosphere formation(-), Priliferation(-), Chemoresistance(-)	-	down	(Li et al., 2010)
	Proliferation(-), Migration(-), Invasion(-), Tumorigenesis(-)	IGF-1R	down	(Shi et al., 2013)
	Temozolomide Sensitivity(+)	MEK1	-	(Wang et al., 2013a)
	Teniposide Sensitivity(+)	MDM2	down	(Sun et al., 2014)
miR-181d	TMZ response	MGMT		(Zhang et al., 2012b)
miR-182	Apoptosis(+), Chemotherapeutic susceptibility(+) Differentiation of GSCs(+), Tumor cell proliferation (-)	Bcl2L12	down	(Kouri et al., 2015)
miR-183	HIF-1α leveles(+)	IDH2	up	(Tanaka et al., 2013)
	Apoptosis(+)	FGF9	-	(Tang et al., 2013)

miR-197	Proliferation(-), Migration(-) Invasion(-)	FUS1	down	(Xin et al., 2015)
miR-210	progression-free survival(-)	-	up	(Lai et al., 2014)
	Chemoresistance(-)	P4HB	up	(Lee et al., 2015)
	Proliferation(+), Apoptosis(-),	ROD1	up	(Zhang et al., 2015b)
miR-214	Proliferation(-)	UBC9	-	(Zhao et al., 2012)
	Overall survival(-)	UBC9	down	(Wang et al., 2014b)
MiR-221 /MiR-222	Apoptosis(+), Tumor growth(-)	PUMA	up	(Zhang et al., 2010a)
	Migration(+), Cell growth(+)	PTPμ	up	(Quintavalle et al., 2012)
	Apoptosis(+), Chemosensitivity(+)	-	-	(Chen et al., 2012)
	Proliferation(-), Invasion(-), Apoptosis(+)	Cx43		(Hao et al., 2012)
	Invasion(+)	-	up	(Zhang et al., 2012a)
	DNA damage(+), Cell death(+)	MGMT	up	(Quintavalle et al., 2013b)
	Radiosensitivity(+), DNA damage repair(+)	Akt	-	(Li et al., 2014b)
	cell survival(+), BCNU resistance(+), Apoptosis(-)	PI3-K/PTEN/Akt	up	(Xie et al., 2014)
	Proliferation(+), Cell cycle(+), Apoptosis(-), Migration(-),	TIMP2	up	(Yang et al., 2015a)

	Invasion(+),			
	Proliferation(-), Migration(-), Invasion(-)	SEMA3B	up	(Cai et al., 2015)
miR-425	Radioresistance(+)	TGF-β	up	(Moskwa et al., 2014)
miR-494	Invasion(-), Proliferation(-), Apoptosis(+)	PTEN	up	(Li et al., 2015b)
miR-663	Proliferation(-), Invasion(-)	PIK3CD	down	(Shi et al., 2014a)
	Proliferation(-), Invasion(-)	CXCR4	-	(Shi et al., 2015)
miR-1275	Tumor proliferation(-), Oligodendroglial lineage proteins(+)	CLDN11	down	(Katsushima et al., 2012)