Epigenetics of paediatric glioma stem cells; focusing on exosomes, miRNAs and DNA methylation

Ágota Tűzesi

Department of Laboratory Medicine
Institute of Biomedicine
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2019

Cover illustration: designed by Ágota Tűzesi. Title: “Use your little grey cells mon ami" (Agatha Christie).

Epigenetics of paediatric glioma stem cells; focusing on exosomes, miRNAs and DNA methylation

© Ágota Tűzesi 2019
agota.tuzesi@gu.se

ISBN 978-91-7833-366-0 (PRINT)
ISBN 978-91-7833-367-7 (PDF)

Printed in Gothenburg, Sweden 2019
Printed by BrandFactory

“Nothing in life is to be feared, it is only to be understood”

Marie Curie

To my beloved Family and Friends

Epigenetics of paediatric glioma stem cells; focusing on exosomes, miRNAs and DNA methylation

Ágota Tűzesi

Department of Laboratory Medicine, Institute of Biomedicine
Sahlgrenska Academy, University of Gothenburg
Gothenburg, Sweden

ABSTRACT

Tumours in the central nervous system are accountable for the majority of cancer-related deaths in children. Glioblastoma multiforme, one of the deadliest of the central nervous system tumours, is partly driven by glioma stem cells. The generation and maintenance of these cells are orchestrated by complex genetic and epigenetic mechanisms.

This thesis investigates the role of two epigenetic players, miRNAs and DNA methylation, as well as the involvement of exosomes in paediatric glioma stem cells. The first study profiles the miRNA content of these cells and compares it to normal neural stem cells. Furthermore, the miRNA content of the exosomes secreted by glioma stem cells and its effect on normal stem cells is determined. The second study investigates how specific miRNAs are regulated and how they could potentially influence glioma stem cells' response to the chemotherapeutic agent temozolomide.

These studies provide new insights into the multifaceted epigenetic regulation of glioma stem cells. The gained knowledge could lead to a better understanding of the biological processes behind brain tumours.

Keywords: epigenetics, microRNA, DNA methylation, exosomes, glioblastoma, paediatric, glioma stem cells, neural stem cells, TMZ response.

ISBN 978-91-7833-366-0 (PRINT)
ISBN 978-91-7833-367-7 (PDF)

SAMMANFATTNING PÅ SVENSKA

De flesta cancerrelaterade dödsfall hos barn och ungdomar orsakas av tumörer i det centrala nervsystemet. Glioblastom är en av det centrala nervsystemets dödligaste cancerformer, och den drivs delvis av speciella gliomstamceller; celler som kan ge upphov till nya tumörceller. Gliomstamcellernas uppkomst och fortlevnad styrs av komplexa genetiska och epigenetiska mekanismer. Medan genetik studerar hur arvsmassan är uppbyggd är epigenetik den vetenskap som berör regleringen av arvsmassan, alltså de processer som styr när och var specifika gener ska uttryckas och bilda protein, trots samma DNA sekvens.

I den här avhandlingen undersöks rollen av två epigenetiska processer, miRNA och DNA metylering, liksom rollen av exosomer (små membranförsedda vesiklar som kan knoppas av från celler), i gliomstamceller från barn. Den första studien studerar miRNA-innehållet i gliomstamceller jämfört med friska neurala stamceller. Vidare bestäms innehållet av miRNA i de exosomer som utsöndras från gliomstamcellerna, och vilken effekt detta har på normala stamceller. I den andra studien undersöks hur specifika miRNA regleras och hur de potentiellt kan påverka gliomstamcellernas svar på cytostatikumet temozolomide.

Sammantaget ger dessa studier nya insikter i den mångfacetterade epigenetiska regleringen av gliomstamceller. Sådan kunskap kan leda till en bättre förståelse av de biologiska processer som ligger bakom hjärntumörer.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Tűzesi Á, Kling T, Wenger A, Lunavat TR, Jang SC, Rydenhag B, Lötvall J, Pollard SM, Danielsson A and Carén H.; Pediatric brain tumor cells release exosomes with a miRNA repertoire that differs from exosomes secreted by normal cells. *Oncotarget, 2017 Oct 6;8(52):90164-90175.*

II. Tűzesi Á, Wenger A, Magnusson M, Danielsson A, Kling T and Carén H. The role of miR-497-5p in mediating response to temozolomide in paediatric glioma stem cells. *Manuscript in preparation.*

CONTENT

Abbreviations IV
1. Introduction 1
1.1 **Genetics** 1
1.2 **Epigenetics** 2
1.2.1 Epigenetic mechanisms 2
1.2.2 DNA methylation 3
1.2.3 Micro RNAs 5
1.3 **Cancer genetics and epigenetics** 8
1.3.1 Cancer 8
1.3.2 Genetics and epigenetics of cancer 9
1.4 **Cancer stem cells** 9
1.4.1 Stem cells and cancer stem cells 9
1.4.2 Cell cycle and dormancy 10
1.4.3 Origins, genetics and epigenetics of CSC 12
1.4.4 Therapy and CSC 14
1.5 **Exosomes** 14
1.5.1 Exosomes biogenesis 14
1.5.2 Molecular composition of exosomes 16
1.5.3 Exosome release and up-take 17
1.5.4 Exosomes function 18
1.6 **Paediatric GBM** 20
1.6.1 Disease and epidemiology 20
1.6.2 Genetics and epigenetics of GBM 21
1.6.3 CSC role in GBM 24
1.6.4 Exosomes role in GBM 26
1.6.5 Therapy and treatment in GBM 28
2. Objectives 33
3. Materials and Methods 37
3.1. **Cells** 37
3.1.1 Patient materials 37
3.1.2 Cells and cell cultures 37

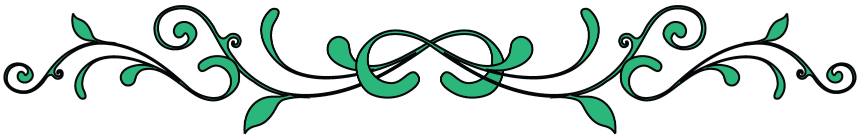
3.1.3 Drug treatments 38
3.1.4 Transfection with siRNA and CRISPR 38
3.2 **Exosomes** 39
3.2.1 Exosomes isolation 39
3.2.2 Exosomes characterization 40
3.2.3 Treatment of cells with exosomes 41
3.3 **Molecular biology methods** 41
3.3.1 RNA extraction, qRT-PCR for miRNA and gene expression studies 41
3.3.2 MiRNA array and TLDA cards (Study I)…………………..…………………………43
3.3.3 Immunocytochemistry…………………………………………………….…………43
3.3.4 DNA methylation profiling…………………………………………………………44
3.4 **Data analysis and interpretation**……………………… ……………………………44
3.4.1 Statistics and bioinformatics……………………………… ………………………44
3.4.2 Pathway analysis and network buildings……… …………………………………46
4. Results and Discussion……………………………………… ………………………49
5. Conclusions………………………………………… ………………………………59
6. Future Perspectives……………………………………………………………………63
7. Acknowledgements……………… ………………………………………………67
8. References…………………………………… ………………………………………73
Appendix…………………………………………… ……………………………………95

ABBREVIATIONS

A	Adenine
AB	Apoptotic body
Ago	Argonaute
BBB	Blood brain barrier
bMMRD	Biallelic mismatch repair deficiency
C	Cytosine
cDNA	Complementary DNA
CNS	Central nervous system
CNV	Copy number variation
CpG	Cytosine-guanine dinucleotide
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CSC	Cancer stem cell
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ESCRT	Endosomal sorting complexes required for transport
EV	Extracellular vesicle
Exo	Exosome
G	Guanine
GBM	Glioblastoma multiforme
GF	Growth factors
gRNA	Guide RNA
GSC	Glioma stem cell
HDAC	Histone deacetylase
hnRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2B1
ILV	Intra luminal vesicle
KEGG	Kyoto Encyclopedia of Genes and Genomes
LGG	Low grade gliomas
lncRNA	Long non-coding RNA
MBD	Methyl-CpG-binding domain
MGMT	O-6-methylguanine-DNA methyltransferase
miRNA	MicroRNA
mRNA	Messenger RNA

MSC	Mesenchymal stem cell
mtDNA	Mitochondrial DNA
MV	Microvesicle
MVB	Multivesicular body
NOS	Not otherwise specified
NSC	Neural stem cell
NTA	Nanoparticle tracking analysis
PCR	Polymerase chain reaction
pHGG	Paediatric high grade glioma
qRT-PCR	Quantitative reverse transcriptase PCR
RBP	RNA binding protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RTK	Receptor tyrosine kinases
SC	Stem cell
siRNA	Small interfering RNA
SNARE	Soluble N-ethylmale-imide-sensitive factor-attachment protein receptor
T	Thymine
TCGA	The Cancer Genome Atlas
TEM	Transmission electron microscopy
TET	Ten-eleven translocation
TLDA	TaqMan low density arrays
TMZ	Temozolomide
tRNA	Transfer RNA
U	Uracil
UTR	Untranslated region
WHO	World health organization
YBX1	Y-box protein 1

INTRODUCTION



1

"One never notices what has been done;
one can only see what remains to be done."
Marie Curie

1.1 Genetics

The hereditary material of an organism is encoded in the most fascinating and elegant macromolecule, deoxyribonucleic acid (DNA), located inside the nucleus of each cell. Prior to the discovery of the structure and principal functions of the DNA molecule by Watson and Crick in the fifties [1], few theories existed on the hereditary mechanism. The presence of hereditary material that passed between generations was first described hundred years before by Gregor Mendel [2].

Genes are traditionally defined as specific sequences of DNA that code for different bio-macromolecules, proteins with diverse cellular functions [3]. The term “gene” was first mentioned in 1905 by Wilhelm Johannsen who also coined the term “genotype” as “the sum total of all the genes in a gamete or in a zygote” [4]. A genome consists of all the genetic material of an organism, and the field studying this is called genomics. Currently, 19,000 protein coding genes are known [5], while the rest of the genome consists of so called ‘non-coding DNA’ such as introns, retrotransposons, and regions that encode non-coding ribonucleic acid (RNA) [6, 7].

The DNA molecule is made up by four nucleotide bases: adenine (A), cytosine (C), guanine (G), and thymine (T) paired in a double helix. These almost two metre long strings of sequences are folded and wrapped around the four core histone proteins: H2A, H2B, H3, and H4 [8, 9]. Further packing of this structure will result in higher order chromatin and finally give rise to the most condensed state, chromosomes, only visible at cell division (illustrated in Figure 1).

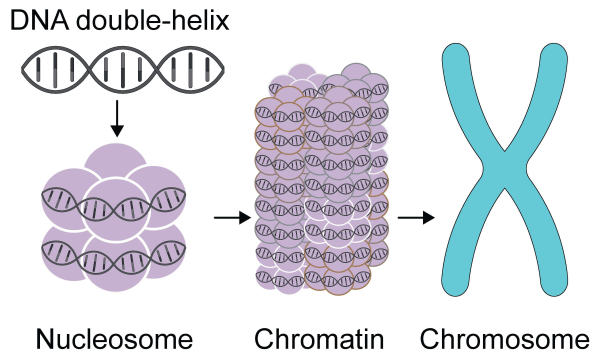


***Figure 1. Chromatin organisation**. The DNA double helix is wrapped around histones in a structure named nucleosome. Further packing will result in a more compact form termed chromatin. The final and most condensed state is the chromosome.*

Transcription is the step in which the DNA is copied into RNA which later can be translated into chains of amino acid residues producing functional proteins [10]. Genes can also code for RNA molecules that never give rise to proteins, but are functional themselves, often with regulating roles. The process of synthesising functional molecules from genes is termed gene expression. RNA molecules, similar to DNA, are composed of four nucleotide bases: A, G, C, and uracil (U). However, RNA is often single stranded and does not have the complex secondary structure of DNA.

1.2. Epigenetics

1.2.1. Epigenetic mechanisms

The term 'epigenetics' was coined by Conrad Hal Waddington in 1946 to describe how genes interact with the environment, sometimes also changing the characteristics of the organism [11]. A more contemporary definition of epigenetics refers to changes in characteristics or gene expression that do not involve alterations in the DNA sequence [12]. Numerous epigenetic modifications exist, however the most studied ones are DNA methylation and histone modifications [13, 14]. While DNA methylation most often occurs through the addition of a methyl group to a cytosine residue in the 5th position, modifications can also occur on the N-terminal of the histone

tails; such modifications are acetylation, methylation, phosphorylation, sumoylation, ubiquitination, and ADP ribosylation [9, 14]. Currently, other epigenetic modifications such as posttranscriptional modifications are also gaining interest [14, 15]. These include modifications at the RNA level [16], as well as the involvement of non-coding RNAs in regulation of gene expression [17, 18].

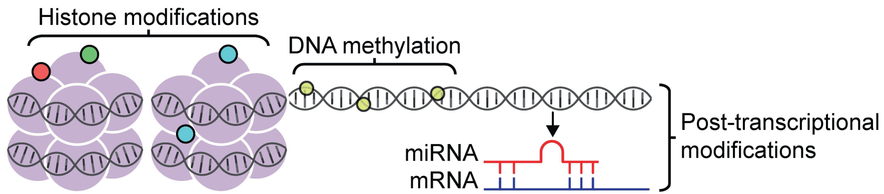


Figure 2. Epigenetic modifications*. Red, green, and blue circles indicate histone tail modifications, yellow circles indicate DNA methylation. Other epigenetic modifications such as posttranscriptional modifications are performed for example through miRNA silencing that will lead to gene expression changes.*

1.2.2. DNA methylation

DNA methylation occurs mainly on a cytosine that is followed by a guanine (CpG dinucleotide) through the addition of a methyl group by enzymes termed DNA methyltransferases (DNMTs) [14, 19, 20]. In this process, the DNMTs use S-adenosyl methionine as the methyl donor.

There are approximately 28,000 CpG islands in the human genome [21]. They often have regulatory roles, and at least half of them can be found in promoter regions of genes [14, 22], while others are located in gene bodies [14, 23]. In general, CpG island methylation in promoter regions has been linked to transcriptional repression [19, 24, 25] (Figure 3) through physically hindering the binding of proteins required for transcription to the DNA sequence.

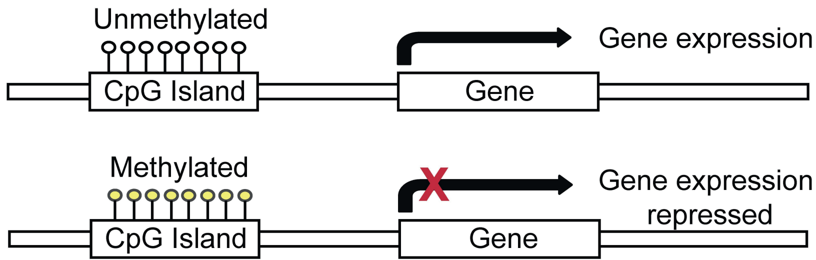


***Figure 3. DNA methylation and gene expression**. Gene expression may be regulated through the presence or absence of DNA methylation at promoter regions. Adapted from [26].*

Methylated DNA can also be bound by methyl-CpG-binding domain proteins (MBDs) [27, 28]. Those will engage further chromatin and histone modifying proteins, thus causing a compact form of chromatin that is not accessible for transcription. This type of chromatin is called heterochromatin.

DNA methylation in the gene body can be found in highly transcribed genes, and this is conserved between plants and animals [29]. DNA methylation in the gene body is known to prevent aberrant transcription initiation [30].

Methyl groups can also be removed from the DNA, in a manner that can be either passive or active. Passive DNA demethylation is a result of improper re-establishment of methylation marks after DNA replication, while active demethylation is catalysed by different enzymes [31, 32]. Some of these enzymes are known as ten-eleven translocation enzymes (TETs) which can oxidize the methyl group, thus giving rise to 5-hydroxymethylcytosine [33].

DNA methylation plays an important role in embryonic development [34, 35]. Experimentally induced mutations in DNA methyltransferase genes have been shown to decrease the levels of DNA methylation in mouse embryonic stem cells without having an effect on the viability or proliferation of the cells. However, *in vivo* experiments showed abnormal development or death in embryos [35]. DNA methylation is also involved in cellular differentiation [36]. Experimental evidence has

identified DNMT1 as responsible for the maintenance of DNA methylation patterns during cell replication [35, 37]. The role of DNA methylation in cancer has been investigated since the early 1980's, when decreased methylation was detected in tumour tissues from patients with colorectal adenocarcinoma and small cell carcinoma of the lung compared to normal tissue [38]. Since then, many tumour suppressor genes have been found to be methylated in their promoters in different tumours [25], for example *CDKN2A* in head and neck carcinoma, gliomas, breast, prostate, and renal cancer [39, 40], and *BRCA1* in breast carcinoma [41].

1.2.3. MicroRNAs

A considerable part of the human genome consists of genes that are not coding for proteins, but for RNAs with regulatory roles.

These RNAs are termed non-coding RNAs and are very diverse, but often have significant roles in cellular processes by regulating gene expression, translation, RNA splicing, and DNA replication [42]. They are important for proper cell functioning and have been found to be dysregulated in different diseases [43]. Based on their size, two main categories exist: long non-coding RNAs (lncRNAs) and short non-coding RNAs.

MicroRNAs (miRNAs) are part of the short non-coding RNA group (they are approximately 22 nucleotides long in their mature form). They act as gene expression regulators through their complementary sequences to mRNAs, mostly in the 3'UTR and less commonly in the 5'UTR region of the target RNA [44]. One miRNA can have several hundred of target mRNAs; even when the sequences are only partially complementary, recognition is possible. However, most often there is a perfect complementarity between the miRNA "seed" sequence and the target mRNA sequence. The miRNA seed sequence is a region found between the second and seventh nucleotide on the 5' end of the mature miRNA [45, 46].

MiRNA genes are located in the introns or exons of other protein coding genes and are transcribed in parallel with those, but can also

be located between coding regions (intergenic miRNAs) [47]. The miRNA genes are most often transcribed by RNA Polymerase II, and less commonly by RNA Polymerase III, in a long (several hundreds of nucleotides) stem-loop shape termed pri-miRNA. This double stranded hairpin shape undergoes processing by the Microprocessor complex where it is recognized by the nuclear protein DGCR8, which then associates with Drosha that cuts the RNA. This will result in a pre-miRNA form that is exported from the nucleus through Exportin-5 into the cytoplasm where it is further processed by the RNase III enzyme Dicer. The processing by Dicer will result in the removal of the loop which joins the 3' and 5' arms, resulting in an imperfect miRNA duplex. Only one of the strands is incorporated into the so-called RISC complex. This complex has Argonaute proteins (Ago) as its catalytic centre, where the mature miRNA and its target mRNA will interact [48] (Figure 4).

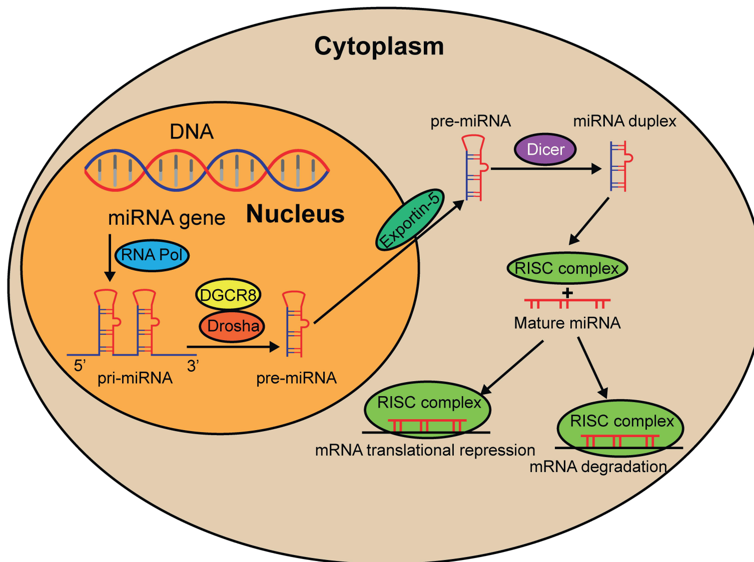


Figure 4. MiRNAs biogenesis and function. *MiRNA genes are transcribed by RNA Pol in pri-miRNAs that are further processed into pre-miRNAs. These are transported from the nucleus to cytoplasm and further processed by Dicer. Only one strand is incorporated in RISC complex where mature miRNAs interact with their target mRNA leading to translational repression or degradation of the target RNA. Adapted from [49].*

There are approximately 2,600 different types of mature miRNAs in the human body. The miRNAs are named through a system where the first part specifies the organism (hsa if human) and the next part reveals if it is a mature miRNA (miR). Next follows a sequence of numbers that are specific for each miRNA, indicating the order in which they were named (and most likely discovered). In addition, the miRNA can be assigned either 3p or 5p mainly depending on from which precursor it is originating. MiRNAs with identical mature sequences, but with distinct precursor sequences, contain a letter (a, b, c etc.) after the miRNA number as described in miRBase nomenclature guide [50-52]. MiRNA clusters are formed by miRNAs that are less than 3,000 nucleotides away from each other, while miRNA families consist of miRNAs with identical ancestors in the phylogenetic tree and have similar biological functions [53].

The most often described function of miRNAs is the repression of their target genes, however up-regulation of genes also occurs [54]. Repression can be achieved either by inhibiting the translation of the target gene to protein or by direct degradation of the mRNA [55].

MiRNAs are involved in the regulation of vast cellular processes. They have an important role in embryogenesis [56, 57]; for example miR-430 that is involved in zebrafish brain morphogenesis [58]. MiRNAs can also regulate cell differentiation and cell fate [59]. Up-regulation of the miR-290-295 cluster was detected in murine embryonal stem cells [60] while the miR-302, miR-17, and miR-106a clusters are highly expressed in human embryonal stem cells [61]. MiRNA expression can be dysregulated in different types of cancers such as for example the members of the miR-17~92 cluster, which are considered to have oncogenic functions and are up-regulated in leukaemia, lymphoma, and glioma [62, 63]. Furthermore, miRNAs can have a role in regulation of DNA methylation by targeting components of the DNA methylation machinery. For example, miRNAs from the miR-290-295 cluster target DNMT genes in mouse embryonic stem cells [64]. The expression of miRNAs can also be silenced by DNA methylation in the corresponding genomic sequence, which has been described for example for miR-34b-3p and -5p in neuroblastoma cell lines [65].

Another posttranscriptional regulator of gene expression is small interfering RNA (siRNA). This is a class of small RNAs (20-25 base pairs), similar to miRNAs in structure and function. SiRNAs are broadly used in gene silencing studies, since they easily can be introduced into cells and have high target specificity through their full complementarity to the mRNA [66].

1.3. Cancer genetics and epigenetics

1.3.1. Cancer

The concept of cancer covers a group of diseases that are the most common sources of death caused by health conditions. Cancer involves abnormal cell growth that has the potential to spread to different parts of the body in a way that will affect the normal functioning of the organism, ultimately resulting in death. There are more than 100 types of cancers that can affect humans and these can arise in any parts of the body [67, 68].

Nowadays, there are several treatment options for different types of cancers, and the survival rates are much better than in previous decades. However, a successful treatment highly depends on the type of cancer and the time of diagnosis. Despite the great advances in the field of cancer treatment, still full elimination of cancer, a good quality of life during and after treatment, and a long survival time are hard to achieve. Most treatment regimens involve surgical removal of the tumour, treatment with chemotherapeutical drugs, and ionizing radiation. New treatments have been introduced or are investigated, such as the use of immunotherapies and epigenetic drugs for specific diagnoses [69-71].

Cancer can affect all age categories; however some types of cancer increase in frequency with age. Breast and prostate cancer are among the most common cancers in adults while in children cancers in the blood, brain, and lymph nodes are the most common [68, 72]. Certain cancers are due to genetic aberrations and epigenetic modifications caused by environmental factors, such as an unhealthy life style (smoking, dietary habits, and lack of physical activities) [73] or

exposure to damaging factors (chemicals, radiation and infections). The main cause of lung cancer is attributed to smoking [74] while dietary habits have been associated with the occurrence of gastro-intestinal cancers [75]. Some cancer forms have been linked to the effects of hormones (insulin-like growth factors) [76] or associated with autoimmune diseases (celiac disease and Crohn's disease) [77, 78]. A smaller part of cancers can have hereditary origins [79, 80].

1.3.2. Genetics and epigenetics of cancer

The presence of mutated genes in cancer cells is common. Two main groups of genes are specifically mutated in cancer cells; oncogenes and tumour suppressors. Proto-oncogenes can be activated into oncogenes by mutations, amplifications, and translocations which can promote transformation of cells [81-83]. The role of tumour suppressors is to inhibit abnormal cell proliferation thereby protecting cells from cancer transformation. However, loss of function of tumour suppressor genes can result in malignant changes [84]. *MYC*, *ERBB2*, *BRAF*, *KRAS*, and *EGFR* are some of the most well-known oncogenes while *RB*, *PTEN*, and *TP53* are considered tumour suppressors [85].

In the classical view of cancer development, genetic alterations have been considered the driving forces. In the modern view, it is known that beside genetic modifications, also epigenetic alterations play a major role in cancer formation, progression, and even relapse through their effect on gene expression regulation [86]. Such epigenetic modifications are DNA methylation, histone modifications, and posttranscriptional modifications [87, 88].

1.4. Cancer stem cells

1.4.1. Stem cells and cancer stem cells

Cancer stem cells (CSCs) have common features with stem cells (SCs) [89]. Both SCs and CSCs have the ability to differentiate into multiple types of cells and also to divide and maintain stemness (self-renewal) [90]. The regulation of self-renewability in both SCs and CSCs involves signalling pathways such as BMI-1, Notch, MAPK,

Sonic Hedgehog, and Wnt. Furthermore, they have the capacity for increased life span through extended telomerase activity, stimulation of angiogenesis, and the ability to secrete growth factors [91].

CSCs have been identified in several tumour forms, including leukaemia [92], brain [93], breast [94], colon [95], and melanoma [96].

For the identification of CSCs, several different surface markers are used such as for example CD24 for ovarian cancer stem cells [97], $CD33^+$ and $CD38^-$ for acute myeloid leukaemia [92], while for brain CSCs CD44 and CD133 are the most commonly used [91, 98].

In case of the high-grade brain tumour Glioblastoma Multiforme (GBM) the CSCs can be called glioma stem cells (GSCs). Isolation of the GSC population can be done by flow cytometry where cells are sorted by the surface antigen CD133 [98]. However, the validity of using CD133 as a universal marker for GSCs has been questioned [99, 100] and other approaches include enriching the GSCs by culturing the cells under stem cell conditions [101, 102]. Other commonly used experiments for validating GSC features are to test the cells' neurosphere formation abilities [103] and their tumour initiating properties in animal models [102, 104].

CSCs have specific properties that normal SCs do not exhibit. CSCs are considered to be the driving forces behind many tumours due to their tumour initiating properties, as well as due to their abilities of indefinite self-renewal, migration, and aberrant differentiation [91]. They also play a major role in cancer relapses as a result of their ability to escape traditional treatments [98].

1.4.2. Cell cycle and dormancy

The ability of CSCs to resist treatments might be due to that these cells, as in general all SCs, have a slower proliferation rate than rapidly dividing cancer cells [90]. Several studies have identified a stage in cancer progression where cells stop dividing but survive in a so-called dormant (quiescence) state when the environmental conditions are not beneficial for proliferation [105]. Cells that enter this

quiescence state are found in cell cycle arrest in the G0-G1 phase. The cell cycle in human cells consists of three phases; interphase, mitosis and cytokinesis. The interphase, when the cell is preparing for division by taking up nutrients, can be divided further into three phases: Gap 1 (G1) when the cell grows in size, the S phase when the DNA replication takes place, and Gap 2 (G2) when the cell grows further preparing for mitosis. During the G1 phase the cell has the option for three routes: to continue the cell cycle by entering the S phase, to stop the cell cycle by entering the G0 phase for differentiation, or to undergo cell cycle arrest in the G1 phase that will lead to either entering the G0 phase or re-entering the cell cycle. The mitotic phase (M phase) is a short but complex time in the cell cycle that consists of nuclear division. This phase is followed by cytokinesis, in which the cell division is finalised by the division of nuclei, cytoplasm, organelles, and cell membrane resulting in two daughter cells that are genetically identical to each other and to their parental cell (reviewed in [106]). The main phases of the cell cycle are showed in Figure 5.

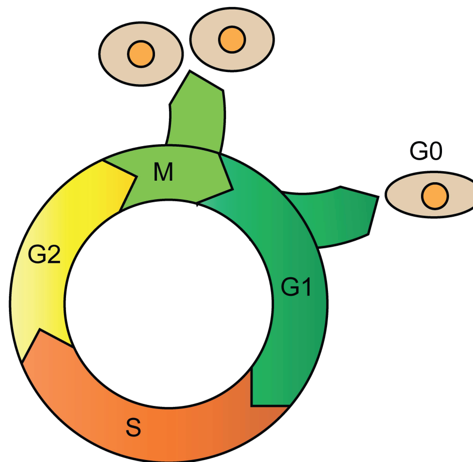


***Figure 5. Cell cycle phases**. In G1 cells grow in size and can enter either G0 phase or continue with S phase when DNA replication takes place. During G2 phase cells grow further preparing for mitosis: M phase, resulting in two daughter cells.*

The cell cycle is regulated strictly by different molecules (cyclins and cyclin-dependent kinases) to ensure proper cell division. During the cell cycle phases, several checkpoint control mechanisms ensure proper cell cycle progression. When a dysregulation occurs and remains uncorrected, this could lead to tumour formation for example through uncontrollable cell division. During increased cell proliferation, dysregulation of several cell cycle genes were detected in many different types of cancers [107]. Furthermore, typical gene expression changes were described in case of cancer cells that stop the cell cycle and enter into a dormant state [108]. Some of these genes were identified in slow proliferating tumours and related to the S phase of the cell cycle, such as *CDT1* and *PCNA* [109, 110], while other genes such as *TGFB2* and *THBS1* were found to have a higher expression in dormant tumour cells than in fast proliferating cancer cells [108].

Common traits exist between dormant tumour cells and CSCs, however very few studies investigated if CSCs can enter a dormant state [111]. These studies suggest that CSC niches can be formed also by a heterogeneous subpopulation, including a quiescent fraction [112, 113]. This raises the question of the existence of fast and slower proliferating cells in the CSC niche.

1.4.3. Origins, genetics, and epigenetics of CSCs

Several hypotheses exist that try to explain the origins of CSCs.

The so called “tumour hierarchy” hypothesis suggests that a tumour niche is built up by heterogeneous cells, which all might have the same or very similar mutations, but present different phenotypes [114]. Simply described, this niche is formed by cancer cells and CSCs. According to this hypothesis (also known as the CSC model) the growth of a tumour and the disease progression are due to a small population of cells from the tumour niche, known as CSCs [89, 115]. This is possible since CSCs are capable of symmetric and asymmetric division while cancer cells divide symmetrically. Asymmetric division of CSCs will result in a cancer cell and a new CSC that can continue further with symmetric or asymmetric division (Figure 6).

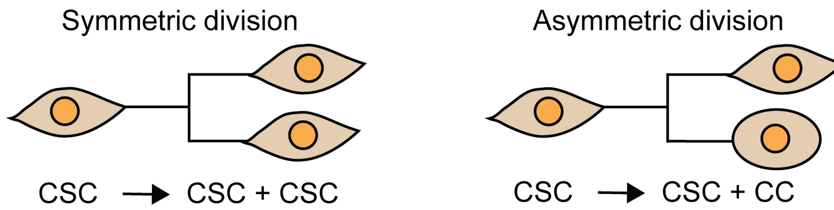


Figure 6. CSC division *can be symmetric resulting in CSCs, and asymmetric resulting in one CSC and one cancer cell (CC). Adapted from [116].*

Mathematical simulations showed that the fast proliferating cancer cells are at the periphery of a tumour niche and spatially inhibit the CSCs from the tumour periphery to the quiescence tumour part [116]. When the cancer cells from the outer periphery exhaust their proliferation potential, the CSCs from the core of the tumour can re-enter a faster proliferation state and through asymmetrical division again repeating the previous dynamics or through symmetrical division produce new CSCs. These new CSCs can, through migration, form a spatially new tumour population as part of the "self-metastatic tumour progression" mechanism [116, 117].

Another hypothesis claims that the occurrence of CSCs is due to mutations in the stem cell niche acquired during development that is later shared through cell division [118]. It was found that astrocyte-like neural stem cells (NSCs) with low level driver mutations, found in the subventricular zone, can migrate to different regions of the brain and induce high-grade gliomas [119].

An alternative theory associates CSCs with adult stem cells since these cells have a higher cell division rate and a long life span, features favourable for the accumulation of mutations leading to cancer occurrence [120]. It was shown that the risk of developing cancer during a life time is strongly correlated with the number of cell divisions. In tissues most of the cells are differentiated and have a short lifespan, which probably makes them unable to form tumours. However SCs have the capability of self-renewal and through this to maintain the tissue structure [121].

The de-differentiation theory claims that cells that acquire mutations could gain the ability to undergo a change that will lead them back to a stem-like state. This theory is supported by experimental evidence, for example the study in which oncogenes was found to induce dedifferentiation of neurons and astrocytes leading to tumour formation in mice [122].

The generation and maintenance of CSCs are orchestrated by different epigenetic changes. Several pathways with roles in self-renewal and differentiation of CSCs are affected by epigenetic mechanisms, such as the Sonic Hedgehog, Notch, Wnt/β-catenin, and TGF-β/BMP signalling pathways. One example is Wnt signalling activation by several transcription factors whose expression is regulated by their promoter H3K27me3 pattern in GSCs [123]. MiRNAs are also players in CSCs, for example let-7 that has low expression in breast CSCs and increase with differentiation. *In vivo* experiments showed that let-7 reduced tumour formation and metastasis, suggesting its role in self-renewal of CSCs [124].

1.4.4. Therapy and CSCs

Traditional cancer therapy involves the use of chemotherapeutical drugs with the aim to decrease cancer cell proliferation. Since CSCs are considered the driving force behind many tumours due to their exceptional therapy escaping features, several studies are investigating the possibilities of developing new types of drugs that will lead to more efficient therapies. As epigenetic mechanisms play a major role in CSC biology, one group of new therapies aim to target different epigenetic players. The most studied epigenetic inhibitors are designed for HDACs and DNMTs [70, 125].

1.5. Exosomes

1.5.1. Exosomes biogenesis

Cells release different types and sizes of extracellular vesicles (EVs) into their environment. The EVs, based on their size or release mode, can be classified into three main categories: apoptotic bodies (ABs), microvesicles (MVs), and exosomes. The ABs are the biggest in size,

with a diameter of 1,000-5,000 nm. They are released by cells that undergo apoptosis. The MVs have a size of 100-1,000 nm in diameter, and they are shed from the plasma membrane. The exosomes are the smallest extracellular vesicles with a size of 30-100 nm in diameter. They have an endocytic origin [126].

Exosome formation starts by invagination of the cell membrane. This process will result in the formation of endosomes. The early endosomes mature into late endosomes. The inward budding of the endosomal membrane will form intra luminal vesicles (ILVs). Due to their morphological features, they are often named multivesicular bodies (MVBs) [127, 128]. The MVBs can fuse either with lysosomes for degradation, or with the plasma membrane of the cell to secrete the vesicles to the extracellular environment. The main steps of exosome biogenesis are graphically presented in Figure 7.

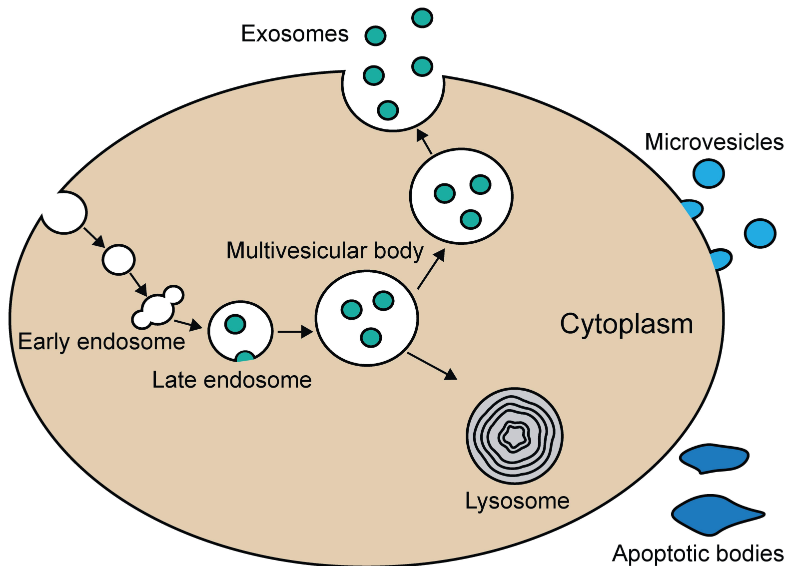


Figure 7. Exosomes biogenesis *starts with the invagination of the cell membrane, forming endosomes. The early endosomes mature into late endosomes which will lead to the formation of MVBs. The MVBs can fuse either with lysosomes and will be degraded or with the plasma membrane to release the vesicles to the extracellular environment. These released vesicles are termed: exosomes. Adapted from [128].*

1.5.2. Molecular composition of exosomes

The first observation and description of the existence of small extracellular vesicles occurred in the 1980's [129]; however, they have gained more attention in the last decade since the discovery of their molecular content [130]. All types of extracellular vesicles have a complex molecular content, which differ between the different types of vesicles [131]. EVs contain various sorts of proteins, such as annexins and tetraspanins, which also can be used as markers for EVs.

During exosome formation, the endosome membrane in the ILV is enriched in tetraspanins such as CD9 and CD63 [127, 132]. The presence of endosomal sorting complexes (ESCRTs) required for transport is an important step in the exosome forming process [133] and their lack can lead to reduced exosome release [134, 135]. Also, ESCRTs are essential for protein sorting in these processes [136]. Exosomes are enriched in several diverse molecules such as lipids, lipid rafts, adhesion molecules, signal transduction molecules, immune regulator molecules, heat-shock proteins, and cytoskeletal proteins [137, 138]. Some studies have described the presence of mitochondrial DNA (mtDNA) in exosomes released by cells such as astrocytes and glioblastoma cells [139]. Fragmented double-stranded DNA has also been found in exosomes [140], or attached to the exosome surface [141]. The presence of diverse RNA species in exosomes has been widely described in several studies; however little is known about how and why these RNAs are packed into the exosomes.

Exosomes have a rich non-coding RNA repertoire alongside mRNAs [130, 131, 142]. Beside miRNAs, exosomes also contain long non-coding RNAs, transfer RNA (tRNA), vault RNA, Y RNA, siRNA, circular RNA, and mitochondrial RNA. The enrichment of certain miRNAs and other RNA species in exosomes lead to the assumption of the existence of cargo sorting mechanisms. This would presume that sorting of specific RNAs into exosomes is actively regulated. Such a mechanism was described in a study where a Dicer deletion was found to lead to reduced miRNA content in exosomes compared to in cells [143]. Changes in miRNA and target gene levels in the cell can

also influence the sorting of RNA into exosomes [143]. Mechanisms and molecules that have been associated with RNA sorting mechanisms into exosomes include: a “zipcode-like” 25 nucleotide long sequence in the 3’UTR of the mRNAs enriched in EVs [144], KRAS-MEK signalling controlling Ago2 sorting to exosomes [145], and RNA binding proteins (RBPs) [146]. The RBPs identified to have a role in the sorting of small non-coding RNAs into exosomes are: the Y-box protein 1 (YBX1) [147, 148], and the heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) [149]. HnRNPA2B1 was found to be sumoylated in exosomes, a post-translational modification that controls protein binding to miRNAs by recognizing specific miRNA sequences termed “exo-motifs” [149]. The enrichment of exo-motifs containing miRNAs in exosomes was also found in Study I presented in this thesis [150].

1.5.3. Exosome release and up-take

When cells release extracellular vesicles, these end up in the extracellular environment, and they can reach other parts of the body through the circulating body fluids.

Exosome release by cells takes place through the fusion of MVBs with the plasma membrane, a mechanism that involves a variety of proteins [151]. MVB transport and docking to the plasma membrane is cortactin (which is an actin binding protein) dependent, and the presence or absence of this protein can increase or decrease exosome release [152]. Among the proteins associated with exosomal release are Rab GTPases [153, 154] and other small GTPases, SNARE proteins [155], and many more.

EVs can be taken up by a variety of other cells in their nearest environment or by cells from distant body parts. In the beginning, most of these vesicles were considered as waste, but were later identified to have a role in cell to cell communication [156]. When the EVs are taken up by other cells, they can affect the receiver cells through their molecular content [130]. Exosomes dock at the plasma membrane of the cells where, based on their surface adhesion molecules (integrins and tetraspanins), the up-take fate is decided [156]. Apparently, the

up-take can also be dependent on the exosome size [157]. Exosome up-take can occur through two main processes: either the vesicle fuses with the plasma membrane, or it is taken up by the cell through endocytosis. Vesicles internalised through endocytosis will fuse with the membrane of an endocytic compartment or will be delivered to lysosomes for degradation. Through both up-take processes the vesicle content will be delivered into the cytosol or to the membrane of the receiver cell [156]. Exosome up-take by cells can be confirmed with fluorescence/confocal microscopy or flow cytometry methods where the vesicles are stained with dyes; PKH67, a fluorescent lipid membrane dye, being one of the many used for EV detection [158].

1.5.4. Exosomes function

The most frequently described function of EVs, including exosomes, is their role in cell to cell communication through their molecular content. These vesicles are implicated in the maintenance of normal physiological processes and can be involved in pathological processes.

EVs can mediate immune modulation with immune activating or immunosuppressive effects [159]. Mature dendritic cells can release exosomes that activate T cells by binding to their receptors and induce an adaptive immune response [160]. One study also described that exosomes released from dendritic cells could eradicate tumours in a mouse model [161], while vesicles isolated from serum of oral cancer patients induced apoptosis of activated T lymphocytes [162] pointing towards an immunosuppressive role of these vesicles.

EVs play an important role in the communication between brain cells as was demonstrated in a study where exosomes released by oligodendrocytes enhanced neuronal stress tolerance in neurons that had taken up these vesicles [163]. Furthermore, these exosomes promoted neuronal survival in a cerebral ischemia model, during oxygen-glucose deprivation [163].

EVs are also associated with many different diseases, such as for example liver disease [164], neurodegenerative diseases [165], and

cancer [166]. In cancers, EVs can manipulate the tumour environment and facilitate metastasis [167, 168]. This was shown for gastric cancer, where mesenchymal stem cell (MSC) exosomes promoted cell growth and migration [169], and in glioblastoma cells where these vesicles promoted cell proliferation [142].

Extracellular vesicles can be detected in body fluids such as blood, serum [170], cerebrospinal fluid [171], urine [172], saliva [173], breast milk [174], seminal fluid [175], nasal lavage [176], and amniotic fluid [177]. The detection of EVs from body fluids can due to their molecular content be used in medicine as biomarkers of different diseases such as for example cancer [178]. Several studies suggested that these EVs are mirroring the molecular content of their source cell [142, 179] and therefore could be used as biomarkers that show a glimpse of the ongoing malfunctions of malignant cells. Their utility in disease monitoring and their prognostic value have been demonstrated; for example, exosomal miR-301a was found to be up-regulated in the serum of GBM patients compared to in healthy controls. Furthermore, expression of this miRNA decreased after surgery and increased again during relapse in the serum of GBM patients, proving its usefulness in monitoring the disease [180].

Another medical advantage of these small vesicles is the possibility to use them for delivering therapeutic molecules. It has been shown that treatment with exosomes carrying a cargo of chemotherapeutics was more efficient and had fewer side effects than the common way of administrating therapeutic agents [181]. Due to their small size, exosomes can also get through the blood brain barrier (BBB) [182], an advantage in brain tumour therapy. Furthermore, EVs have the potential to be used in immunotherapy for the treatment of different types of cancers. A clinical study was performed with exosomes derived from dendritic cells, which were pulsed with MAGE3 peptides and used for the immunization of stage III/IV melanoma patients with promising results [183]. The use of exosomes as delivery vectors of siRNAs were also shown [184], as well as their capability in delivering miRNAs. Evidence for this comes from a study where the most abundant miRNA in brain, miR-124 [185], was delivered by exosomes and promoted neurogenesis after ischemia in mice [186].

1.6. Paediatric GBM

1.6.1. Disease and epidemiology

GBM is a high grade brain tumour, with very poor survival outcome. GBMs can be either primary or secondary tumours. Secondary GBMs arise from lower grade gliomas and they show molecular differences from primary GBMs [187]. GBM is the most common tumour of the central nervous system (CNS) in adults. In paediatric patients the incidence is lower than in adult patients; however due to their aggressive clinical behaviour, they cause significant mortality and morbidity among children with brain tumours [188].

The incidence of GBM is approximatively 3 in 100,000 people per year (in the US), more frequent in males than females [189]. It can occur at any time during life, though the incidence increases with age [190]. In children the incidence of high-grade brain tumours is 0.85 in 100,000 people of which 3-15 % are GBM [188, 190]. Glioblastomas in children can result from a background of cancer predisposition syndromes as for example Li Fraumeni Syndrome or be caused by biallelic mismatch repair deficiency (bMMRD), however most of the time the tumour is sporadic [191, 192].

Traditional classification of brain tumours has been done based on histopathological analyses. For example, in the 2007 World Health Organization (WHO) Classification of Tumours of the Central Nervous System, all tumours with an astrocytic phenotype where grouped together even if they showed different clinical features [193]. Nowadays the molecular based classifications are gaining more focus, since they can offer a more precise diagnosis. The newest classification of central nervous system tumours by WHO (from 2016) incorporates, in addition to the histopathological methods, also molecular specifications [194]. Based on this classification, glioblastomas are divided into IDH wild type and IDH mutant categories with a third group termed glioblastomas NOS (not otherwise specified) for tumours without a full IDH status evaluation.

Most paediatric brain tumours are supratentorial but they can arise also in the cerebellum, brainstem, ventricles, spinal cord, suprasellar region, or cranial nerves. At least half of the CNS tumours in children are gliomas; most often low grade gliomas (LGG) such as pilocytic astrocytomas and embryonal tumours. Medulloblastomas are the most common embryonal tumours. Paediatric high grade gliomas (pHGG) are mostly glioblastoma (WHO grade IV tumours), but can also be diffuse midline glioma and anaplastic astrocytoma [195]. The pHGG most often arise in the cerebral hemispheres, but can also originate in the thalamus, brainstem, cerebellum, or spinal cord [195].

1.6.2. Genetics and epigenetics of GBM

In GBM several signalling pathways are altered, the receptor tyrosine kinase (RTK) pathway being one of those. RTKs bind growth factors (GFs) and the epidermal growth factor receptor (*EGFR*) is frequently mutated or amplified in GBM [196]. EGFR signalling has important functions in brain cell proliferation, differentiation and survival [197]. The most common mutation of this receptor is EGFRvIII which results in constant activation of this signalling [198]. Aberrations in the Ras/MAPK pathway can cause abnormal cell proliferation and invasion, and is also commonly altered in GBM, either by mutations in *RAS* or by activation through EGFR [199]. Activated Ras can lead to MAPK activation and also affect another pathway, the PI3K/PTEN/Akt/mTOR pathway. Here, growth factors with their receptors, such as for example EGFR, activate PI3K, which will further activate Akt leading to the activation of mTOR which is involved in cell growth [200]. *PTEN* is a tumour suppressor that can inhibit this pathway; however *PTEN* is frequently mutated or deleted in GBM [198]. The p53 pathway is frequently mutated, in primary GBMs less frequently than in secondary GBMs [200]. Another deregulated pathway in GBM is the tumour suppressor pRB pathway, where pRB has a crucial role in inhibiting cell cycle progression [200].

Genetic alterations can lead to aberrant signalling pathways and in GBM these alterations are frequent. The Cancer Genome Atlas Project has catalogued genomic aberrations in GBM [198] and based on genetic profiles GBM can be divided into four groups: 1) the

classical type characterised by multiple copies of *EGFR*, 2) the pro-neural type with mutations in *TP53*, *PDGFRA*, and *IDH1*, 3) the mesenchymal type with mutations in the *NF1* gene, and 4) the neural type which shows features of normal cells [201].

Several studies also revealed heterogeneity among GBM as well as diversity between those that arise in children and adults [202]. According to a study by Paugh et al. from 2010, pHGG have minimal copy number changes compared to those in adults and do not have *IDH1* hotspot mutations. Furthermore, pHGG exhibit *PDGFRA* amplification and more frequent gain of chromosome arm 1q than tumours in adult patients. The gain of chromosome arm 7q and loss of 10q is more frequent in adult than pHGG [203]. Another study showed that mutations in the genes coding for H3.3 are very specific to and frequent in GBM that arise in children and young adolescence [204].

Incorporation of epigenetic profiles has further divided tumours into six subgroups: IDH, K27, G34, RTK I, mesenchymal subtype, and RTK II (with their main characteristics presented in Table 1) [202]. These six epigenetic subgroups are also delimited based on their genome-wide DNA methylation profile [202]. Based on the DNA methylation profile, the IDH subgroup showed genome-wide hypermethylation and in contrast to this, the G34 subgroup exhibited global hypomethylation [202].

***Table 1. Epigenetic and genetic subgroups of GBM** with their main characteristics. Adapted from [202].*

Epigenetic subgroup	Mutations	CNV	Gene Expression TCGA	Age groups (age range)
IDH	IDH1, TP53		Proneural	Younger adults 13-71
K27	$H3F3A^{mut}K27$, TP53		Proneural	Childhood 5-23
G34	$H3F3A^{mut}G34$, TP53		Mixed	Adolescent 9-42
RTK I		Amplification: PDFRA Deletion: CDKN2A	Proneural	Paediatric/Adult 8-74
Mesenchymal		low	Mesenchymal	Paediatric/Adult 2-85
RTKII		Amplification: EGFR Deletion: CDKN2A	Classical	Older adult 36-81

In GBM, *MGMT* methylation status is well studied. This gene encodes a DNA repair enzyme, O6-methylguanine methyltransferase, which is responsible for removing alkyl groups from the guanine O-6 position. In many GBM tumours, the promoter region of this gene is hypermethylated leading to silencing of the *MGMT* gene [205]. Studies have shown that GBM patients with hypermethylated *MGMT* promoters responded better to chemotherapy using temozolomide (TMZ) and had better outcome than patients where the *MGMT* is expressed [206, 207]. *MGMT* methylation is therefore used as a prognostic marker for therapy response especially in GBM patients older than 60 years of age [206, 208].

An important part of tumour epigenetics is deregulation of miRNA expression. The majority of deregulated miRNAs are overexpressed in GBM compared to normal brain tissue [209]. Deregulated expression of miRNAs can result from deletions or amplifications at the genomic level as has been described for miR-25, miR-26, miR-495, miR-1286, and miR-4484 [210]. Furthermore, expression of several miRNAs such as miR-124, miR-148a, miR17, miR-30c, miR200a, miR217, and miR-

265-5p has been shown to be regulated by their DNA sequence methylation status in adult GSCs [211].

The miRNAs can have different roles in GBM biology. They can be involved in tumour suppression, such as for example miR-34a that suppresses tumour growth by targeting *Notch* [212], while let-7a silences *K-ras* and reduces malignancy [213]. Proliferation of GBM cells can be influenced by the down-regulation of tumour suppressor miRNAs such as miR-491, miR-218, miR-219-5p, and many others [214-216]. OncomiRs are a group of miRNAs with tumour growth promoting features, and they are known to be overexpressed in GBMs. The oncomiRs miR-21, miR-23a, and miR-26a have been demonstrated to down-regulate *PTEN* in glioma cell lines [217, 218]. In pHGG tissue several miRNA clusters were found to be up-regulated compared to in adult glioma or normal samples. Among these were the oncogenic miR-17~92 cluster members miR-195 and miR-497 [63].

MiRNAs are also involved in chemo and radiotherapy response, such as for example miR-132 [219], miR-20a [220], and miR-497 [221]. Furthermore, miRNAs with accurate prognostic value have been identified: miR-7, miR-124a, miR-129, miR-139, miR-218 (which were downregulated), miR-15b and miR-21(which were up-regulated) [222].

1.6.3. CSC role in GBM

GBM tumours are built up by a heterogeneous cell population and the cell of origin is still controversial. Some studies claim that astrocytes [223] or oligodendrocyte precursor cells [224, 225] that undergo malignant transformation could be cells of origins. The presence of CSCs in brain tumours was described for the first time by Singh et al. in 2004, when they succeeded to isolate a cell population that expressed CD133 and presented with stem cell properties including the ability to initiate tumours *in vivo* [98]. Since then, several studies have investigated and confirmed the presence of CSCs in brain tumours with evidence of these cells' role in tumourigenesis and in tumour progression. The presence of CSCs in paediatric brain

tumours, including medulloblastomas and gliomas, has also been described [226].

GSCs share common features with normal NSCs such as the expression of certain cell surface markers, transcription factors, and structural proteins. GSCs frequently show expression of SOX2 [226] OLIG2 [227], NANOG [228], MYC [229], MUSASH-1, BMI-1 [226], the neural progenitor/stem cell marker Nestin [230], and the epithelial-mesenchymal transition (EMT) marker Vimentin [231]. A property of CSCs is that they should be able to respond to differentiation cues (which can be either withdrawal of growth factors, or addition of serum or bone morphogenic proteins) [101]. The differentiated GSCs show decreased expression of NSC markers and increased expression of the neuronal markers GFAP, MAP2 or TUJ1 [101, 102].

CSCs show a higher resistance to traditional treatments than the other more rapidly cycling cells of the tumour [232, 233]. GSCs are considered to be the main players in treatment resistance and relapse of GBM [232]. The majority of conventional chemotherapies are designed to target fast proliferating cancer cells. However, CSCs are slower cycling cells [90] and therefore have the capability to escape these treatments and lead to relapse as shown in the graphical representation in figure 8.

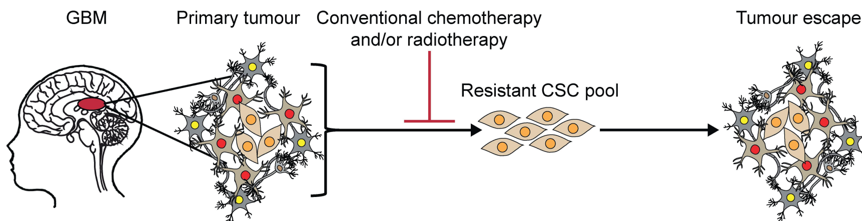


Figure 8. GSC involvement in conventional chemotherapy/radiotherapy response. *These cells are resistant to many of conventional chemo- and radiotherapies, leading to relapse.*

Several signalling pathways are also involved in the GSC response mechanism to conventional treatments. In a study, Bao et al. showed

that GSCs that express CD133 can activate DNA damage checkpoints, as well as repair radiation induced DNA damage [233].

1.6.4. Exosomes role in GBM

Glioma cells, like many other cells, secrete EVs such as exosomes. The first report on exosomes secreted by GBM cells was published by Skog et al. who showed that these vesicles, through their RNA and protein content, promote tumour growth, and could furthermore be used as diagnostic biomarkers [142]. In that study, 4,700 transcripts were exclusively found in vesicles but not in the originating cells, suggesting a selective sorting process into the exosomes. The most abundant genes in the exosomes were shown to be involved in biological processes such as cell proliferation, cell migration, and angiogenesis. The study also provided experimental evidence that RNA delivered by glioma exosomes were translated into proteins [142].

An important step during invasion is angiogenesis, to ensure access to nutrients. Studies have shown that GBM exosomes contain angiogenic factors such as miRNAs, mRNAs, proteins, and extracellular proteases necessary for migration, differentiation, proliferation, and progression [234-236].

A detailed characterisation of the RNA repertoire in the extracellular vesicles secreted by GSC identified small RNA enrichments as miRNAs [237]. Furthermore, tRNAs, Y RNA, and fragmented mRNAs were detected in these vesicles. Very little is known about the average copy number of the different RNA species per vesicles and this study points to a very low number of transcripts that might influence the studies aimed to investigate the functional effect of these vesicles [237]. One study describes an exosomal miRNA signature specific for phenotypically diverse subpopulations of GSCs [238]. A direct visualisation of extracellular vesicles released by glioma cells and their up-take by microglia and macrophages in mouse brain supports these vesicles' functional effect on their environment through their molecular content delivery [239]. The functional effect of the exosomal content was also described in a study where glioma extracellular vesicles

delivered and induced gene expression changes in the receiver endothelial cells [240].

Since these small vesicles have the property to mirror their originating cells, several studies have investigated the use of extracellular vesicles in medical diagnostics and prognostics [241, 242]. Among the exosomal content, DNA [243], miRNA [244] and other small non-coding RNAs [170], mRNA [142, 245], and protein [246, 247] have been used. *EGFRvIII* in exosomes secreted by glioma cells was detected in serum from glioma patients but not from healthy donors [142]. MiR-21, a key player in GBM [248], also has diagnostic value; it was found to have higher expression in exosomes found in blood and cerebrospinal fluid from GBM patients than in the ones from healthy subjects [142, 249]. Exosomes were found to be good tools in the prediction of drug response in GBM patients through the *MGMT* gene levels detectable in these vesicles [250].

The involvement of EVs such as exosomes in GBM resistance to treatments as well as their use as a treatment delivery system has been investigated by several studies [251, 252]. A study with U87 glioma cells showed that exogenous miR-124 delivered by MSC exosomes decreased proliferation and migration of glioma cells and also enhanced sensitivity to TMZ [253]. Another study showed similar results and pointed out the possible use of MSC for producing exosomes with miR-124 as treatment for GBM [254]. The mRNA levels found in GSC exosomes accurately reflected the cells profile of TMZ resistance related gene expression, such as *MGMT*, *TGM2*, and *NESTIN* [255], suggesting these vesicles possible use as biomarkers. Exosomal miR-221 was found to be involved in glioma progression and TMZ resistance by targeting *DNM3* based on a study with glioma cell lines [256]. Exosomes with *PTPRZ1–MET* fusion oncogenes, derived from GBM cells, induced EMT in human astrocytes and contributed to TMZ resistance, cell migration, invasion, angiogenesis, and neurosphere formation [257].

Despite the extensive amount of studies on GBM exosomes, scant information exists on these vesicles' role in paediatric gliomas, especially those secreted by GSCs.

1.6.5. Therapy and treatment in GBM

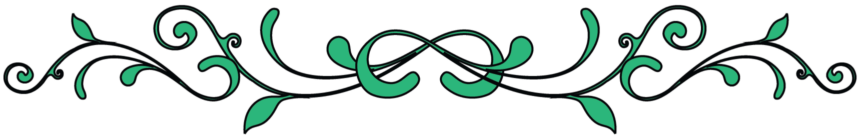
Maximal safe surgical resection of the tumour is important, however due to the infiltrating behaviour of GBM cells, all tumour cells cannot be removed. Concomitant and adjuvant treatment with TMZ and radiotherapy is also part of the treatment [258]. TMZ is the most often used chemotherapeutic agent against GBM. TMZ can alkylate/methylate DNA, which may cause DNA damage. This will lead to cell cycle arrest in the G2/M phase, ultimately resulting in apoptosis [259]. However, the DNA damage can also be repaired by an enzyme encoded by the *MGMT* gene, therefore patients with no or low expression of *MGMT* benefit from TMZ treatment [206]. In parallel with the daily TMZ treatment, patients also receive external beam radiation therapy [260]. Nevertheless, the tumour will eventually regrow; re-operation is not always an option, and new chemotherapy and irradiation can have toxicity concerns [258]. The use of chemotherapeutical agents and irradiation in paediatric patients is applied with consideration to the possible negative late effects on the developing brain [261]. The use of radiotherapy is recommended mostly in children above the age of three to avoid severe adverse neurocognitive effects [188]. In adult patients the use of chemotherapy is an integral part of GBM treatment. In paediatric patients, similar management is used. The efficacy of TMZ for GBM treatment was demonstrated in adult patients [260] but not in children [262]. TMZ treatment showed a better response in adult patients with methylated *MGMT* and since in pHGG *MGMT* promoter methylation is less frequent, this might be a reason for the lower response [263]. Present studies are investigating possibilities how to sensitise paediatric gliomas to TMZ, one of them being through therapeutic inactivation of *MGMT* [264].

Several new treatment options are investigated, such as the use of immunotherapy, targeted therapies, and epigenetic drugs. Targeted therapies target tumour growth factor receptors, cell cycle regulation, angiogenesis, antitumour immune response, and several pathways involved in tumour biology. Some of these treatments are used in pHGG management, such as Bevacizumab which is an angiogenesis inhibitor against VEGFA [263]. The two most well-known classes of

epigenetic drugs are HDAC inhibitors and those that target DNMTs. Over the years, many clinical and preclinical studies have investigated the effect of epigenetic drugs in different types of cancers. In brain tumours, some of these epigenetic drugs are or have been investigated in clinical trials, also as part of a combination therapy. Valproic acid, Vorinostat, Belinostat, and Romidepsin are among those [125].

These diverse approaches and several clinical trials in the field of GBM indicate the possibility in the future to find more efficient therapy for these patients.

OBJECTIVES



2

"Don't let anyone rob you of your imagination, your creativity, or your curiosity."

Mae Jemison

The overall aim of this thesis was to gain more insights into the epigenetic mechanisms and their role in GSCs derived from paediatric patients, for a better understanding of the biological processes in brain tumours. This knowledge provides a foundation for more efficient treatment designs for paediatric GBM patients in the future.

Specific aims:

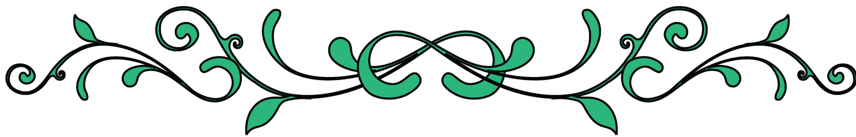
Study I

- Investigate the miRNA repertoire of GSCs and NSCs.
- Explore the miRNA content of the exosomes released by these cells.
- Study the GSC exosomes' effect through their miRNA content on NSCs.

Study II

- Explore the epigenetic interplay between miRNA expression and their DNA methylation in GSCs.
- Investigate the role of miR-497-5p in mediating treatment response against TMZ in GSCs.

MATERIALS AND METHODS



3

"Just remember, there's a right way and a wrong way to do everything and the wrong way is to keep trying to make everybody else do it the right way."

Harry Morgan – M*A*S*H

3.1 Cells

3.1.1 Patient materials

For the studies included in this thesis patient-derived primary cell lines established from the high-grade paediatric gliomas were used [102]. Regional ethical approval was obtained for the studies (Dnr 604-12).

3.1.2. Cells and cell cultures

Patient-derived primary cell lines have the great advantage that they maintain the originating tumour features in comparison with commercial cell lines which have acquired mutations and chromosomal aberrations during a long culturing time. The cell lines were enriched for stem cells by growing the cells in stem cell media DMEM-F12 supplemented with B27 (Gibco), N2 (Gibco), EGF (20 ng/ml, Peprotech) and in some cases FGF-2 (20 ng/ml). To gain adherent cultures, the culturing flasks/plates were coated with laminin (Sigma or BioLamina).

The following six GSC lines were used: GU-pBT-7, GU-pBT-10, GU-pBT-15, GU-pBT-19, GU-pBT-23, and GU-pBT-28. All cell lines were characterised for stemness in a previous study by stem cell markers expression, differentiation properties, and tumour initiating potential *in vivo* [102, 104]. Furthermore, DNA mutations, CNV and DNA methylation patterns were described for these cell lines [102]. All samples are *IDH1* and *IDH2* wild type.

For the authentication of the established cell lines short tandem repeat (STR) profiling was used.

The NSC lines used in these studies; NS-1, NS-4, and NS-5 have previously been described and characterised [265].

Cell culture media was changed every 4th or 5th day and all cell cultures were confirmed negative for mycoplasma contamination.

For Study I media was collected from each cell line for exosome isolation, as described below.

For functional experiments 5-7,000 cells/well in 96-well plates or $4x10^4$-$1x10^5$/well in 24-well plates was used.

3.1.3. Drug treatments

In Study II cells were treated with TMZ (Selleckchem, S1237) to investigate GSC response to the most frequently used chemotherapeutical drug in GBM treatment. TMZ cytotoxic effect results from the addition of methyl groups on the DNA nucleotides, specifically to adenosines on O^3 and guanines on N^7 and O^6 sites. During DNA replication the added methyl group on the guanines O^6 sites will result in the insertion of a thymine instead cytosine and this will lead to cell death [259]. For these experiments, 600 µM TMZ was added to the cells every day, for 3 days followed by immunocytochemical analysis to evaluate the effect on number of cells, viability and proliferation.

To study if miRNA expression was dependent on DNA methylation status in Study II, the cells were treated with Decitabine (Selleckchem, S1200). Decitabine, also known as 5-aza-2'-deoxycytidine, is a cytidine analogue which is incorporated into the DNA during DNA synthesis and by this interrupts the interaction with DNMTs. This will lead to DNMT degradation resulting in the lack of methylation of cytosine [266]. Cells were treated with 500 µM Decitabine every day for four days followed by gene expression analysis to investigate if the expression of the miRNA of interest was affected by the DNA methylation changes.

3.1.4. Transfection with siRNA and CRISPR

In Study II synthetic siRNA inhibitors were used for the silencing and synthetic mimics for overexpression of the studied miRNAs. Transfection with 4D-Nucleofector System was used to deliver the synthetic siRNAs to the cells. All control samples were transfected with negative control siRNAs, to distinguish between specific activity given by inhibitor/mimic and background effects. The negative control mimics/inhibitors used have minimal sequence identity with human miRNAs. $1x10^5$ cells were transfected and seeded. At 24 h post

transfection the cell culture media was changed or cells were collected for RNA extraction.

Since the expression of miR-497-5p was very high in GSCs and the silencing with siRNA does not have a lasting effect necessary for functional studies, a more long-term method was used; knock-out using CRISPR constructs. Guide RNA (gRNA) was designed with the Benchling web site (https://benchling.com/crispr), using a scoring system developed by Doench et al. [267]. For the transfection of the cells, a ribonucleoprotein (RNP) complex was prepared according to the manufacturer's instructions. These RNP complexes were transfected into the cells through electroporation with the 4D Nucleofection System (Lonza). Controls were transfected with a non-targeting crRNA. The effect of knock-out was investigated based on the miR-497-5p expression at 72 hours post transfection. For the knock-out functional effect, the cells were seeded in a 96 well plate and treated with TMZ for 3 days followed by immunocytochemistry studies.

3.2 Exosomes

3.2.1 Exosomes isolation

In Study I, exosomes isolated from cell culture media were used for miRNA profiling and for functional studies.

For this purpose, the golden standard exosome isolation method was used; differential centrifugation. This is the most widely used method for EVs isolation from cell culture media [268]. The method principle is to use several numbers of centrifugation steps with different speed and time. The aim is to pellet the diverse sized/density particles from the media, starting with a shorter and slower centrifugation speed that will pellet the cell contaminations. The pellet from this step is eliminated and the supernatant will be centrifuged at a higher sped to eliminate apoptotic bodies, followed by ultracentrifugation with Ti70 fixed-angel rotor (Optima L-90 K Beckman Coulter). Ultracentrifugation is necessary to pellet smaller vesicles. A shorter time of 30 minutes and a slower speed of 28,000 x g (19,400 rpm) are

used to pellet microvesicles. A filtration step with a 0.22 μm filter is included followed by a final step of ultracentrifugation at a higher speed and longer time to isolate the smallest EVs; the exosomes. Based on previous studies, the time and speed of ultracentrifugation can influence the exosomal RNA yield [269]; therefore a two hour at 118,000 x g (40,000 rpm) was used for this step. The used isolation steps are represented in Figure 9. The exosome pellets were re-suspended in PBS and kept at -80°C for further processing.

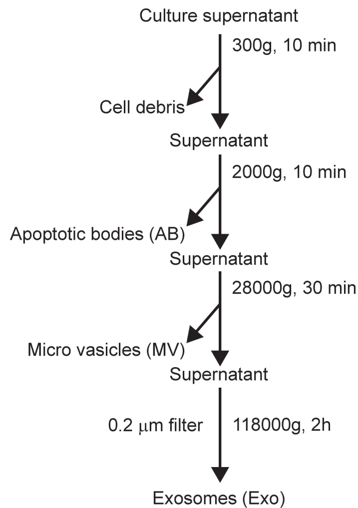


***Figure 9. Exosome isolation steps** where the cell culture media first is centrifuged at a slower speed to pellet cell debris, followed by a higher speed to pellet ABs. The next step of centrifugation will result in pelleting MVs, followed by the supernatant filtration and centrifugation at a higher speed during 2h. The resulting pellet contains the exosomes.*

3.2.2 Exosomes characterisation

In order to characterise the isolated vesicles three different methods were used which confirmed exosome features.

One of the methods to characterise the isolated vesicles as exosomes is to show that these vesicles contain the most common exosomal proteins. This can be achieved with Western blot analysis with antibodies against the most common exosome markers, CD81 and

CD9. In parallel, the presence/absence of Calnexin, an endoplasmic reticulum marker was investigated. Calnexin can be detected in cell samples and other EVs but not commonly in exosomes. Protein extracted from these vesicles was used for the Western blot analysis.

A second characterisation of the isolated vesicles was based on their size. For this a nanoparticle tracking analysis device termed Zeta View (Particle Matrix, Germany) was used. The principle of this method is based on the Brownian motion of the particles, in this case the vesicles. The different sized vesicles have different diffusion movements in the re-suspended liquid and based on this the hydrodynamic diameter of each vesicle is determined.

A third characterisation method for the isolated vesicle was based on their shape and size. For this transmission electron microscopy (TEM) was used that is capable to visualise these small vesicles.

3.2.3 Treatment of cells with exosomes

To study the effect of exosomes secreted by GSCs on NSCs (Study I), these vesicles were isolated with differential centrifugation as previously described, and added to NSCs. The exosome concentration was measured with BCA Protein Assay (ThermoFisher) on Wallac 1420 multilabel counter (Perkin Elmer). A total of 30 µg/ml GSC exosomes were added every day for 8 days to NSCs, control samples were treated in the same way but with NSC-derived exosomes. At the end of the experiments exosome treated cells were collected for RNA isolation.

3.3 Molecular biology methods

3.3.1 RNA extraction, qRT-PCR for miRNA and gene expression studies

In Study I the RNA extraction from cells and exosomes was done with QIAzol (Qiagen) according to the manufacturer's protocol, with the addition of Glycogen (Invitrogen) to increase the RNA yield. The RNA extracted from cells was treated with TURBO DNase (Invitrogen) and

enriched in small RNA fraction with RNA Clean & Concentrator™-5 (Zymo Research).

In Study II total RNA extraction was performed with Direct-zol™ RNA MiniPrep, (R2052, Zymo research) according to the manufacturer's protocol.

RNA was quantified with Qubit RNA HS Assay Kit (Invitrogen) in both studies.

The isolated RNA from exosomes and cells (Study I) were used for miRNA array and the array data validation with quantitative real-time PCR (qRT-PCR). Furthermore, the RNA isolated from cells treated with exosomes, were used for TaqMan Low Density Array (TLDA) cards. The detailed descriptions of the two types of arrays are presented in the following subchapter. For the qRT-PCR (used for the miRNA array results validation) the miRCURY LNA™ Universal RT microRNA PCR, Starter Kit with validated primer sets (Exiqon, Denmark) was used and ABI 7500 FAST Real-Time PCR System. For the reverse transcription 20ng of total RNA from exosomes or small RNA enriched from cell samples were used. UniSp6 RNA Spike-in template was added to each sample and used for normalization.

In Study II for the relative quantification of miRNAs the TaqMan Single Tube Assays were used and ABI 7500 FAST Real-Time PCR System. First, cDNA was synthetized with specific RT primers and TaqMan Small RNA TaqMan™ MicroRNA Reverse Transcription Kit followed by qPCR performed with TaqMan™ Universal Master Mix II, with UNG according to the manufacturer's protocol (Thermofisher). For normalisation the miR-92-a was used.

For gene expression analysis (Study II), cDNA synthesis was performed with SuperScript™ III Reverse Transcriptase (Invitrogen) followed by qPCR performed by using GoTaq qPCR Master Mix (Promega). For ΔCT calculations *GAPDH* was used as a reference gene.

3.3.2 MicroRNA array and TLDA cards (Study I)

To characterise and quantify the miRNA content of the exosomes and their originating cells, a platform with several benefits compared to others found on the market was used, the 3D-Gene Human miRNA Oligo chip ver.21 (Toray Industries). This array detects as many as 2,565 miRNA transcripts. Another benefit of this array is the low amount of RNA input that is required (100ng). The RNA is directly hybridized to beads and the intensity of each miRNA is measured and analysed with the 3D-Gene Scanner 3000 (Toray), according to the manufacturer's instructions. The data was normalized using miRNA "spots" with background subtracted data.

For the functional study of the exosomes' effect on NSCs, TLDA cards were used. These cards were custom made, examining 192 genes in duplicates. The genes were selected based on that they were either predicted or experimentally validated target genes of the GSC exosomal miRNAs or have roles in cell cycle, stemness, differentiation, glioma genesis, or neurogenesis. For cDNA synthesis the High- Capacity RNA-to-cDNA Kit (Applied Biosystems) was used. In total, 10-15ng of cDNA was loaded per port on TaqMan Custom Arrays (Applied Biosystems) and run on a Viia7 Real-Time PCR system (Applied Biosystems) according to the manufacturer's instructions. *GAPDH* was used as reference gene for ΔCT calculations.

3.3.3 Immunocytochemistry

In Study I for the exosomes up-take by cells the PKH67 Green Fluorescent Cell Linker for General Cell Membrane Labelling (Sigma-Aldrich) was used to stain the GSC secreted exosomes that were added to NSCs. The cells were stained with mouse monoclonal Nestin (R&D MAB1259), 1:500 and DAPI was used as a nuclear counterstain. Confocal imaging (LSM 700 Carl Zeiss microscope) was used for the visualisation of the exosome up-take by cells.

In study II for the assessment of the cells response towards TMZ with or without the miR-497-5p or miR-195-5p overexpression/silencing the

LIVE/DEAD™ Cell Imaging Kit (488/570; Thermofisher) and Hoechst (1:2000; Thermofisher) was used. In case of cell proliferation studies EdU staining (Thermofisher) and DAPI as a nuclear counterstain, according to the manufacturer's instructions were used. Image acquisition of the cells was performed with Operetta High-Content Imaging System and the data processing with Harmony software (Perkin Elmer).

3.3.4 DNA methylation profiling

In Study II the used DNA methylation data set previously was generated [102] using the Infinium Human Methylation 450K BeadChips (Illumina). This array identifies the methylation levels of more than 450,000 CpG sites. These CpG sites are distributed across the genome and cover most coding and non-coding genes and also regulatory regions such as enhancers. The CpG sites are further annotated to different genomic features such as CpG islands, shores, shelves and open-sea. Important to note is that the bisulfite-modification of DNA which is performed prior to hybridisation to the arrays, do not distinguish between 5-methylcytosine and 5-hydroxymethylcytosine. Hence, the CpG sites identified as differentially methylated could have either of these methylation forms.

3.4 Data analysis and interpretation

3.4.1 Statistics and bioinformatics

In Study I for the validation of miRNA array with qRT-PCR, Pearson correlation between the miRNAs relative quantities was used and standard error was calculated.

The microarray data in Study I was mainly analysed with GenEx analysis software (MultiD Analyses). Differentially expressed miRNAs between cell and exosome samples were identified with R/package *limma*. Hierarchical clustering was applied to all miRNAs for the visualisation of differentially expressed miRNAs, using Euclidean distance metric and average linkage. In order to elucidate why some miRNAs had higher intensities in exosomes than in cells, similarities in

their sequences were searched for. The online application, Improbizer (https://users.soe.ucsc.edu/~kent/improbizer/improbizer.html) was used. All miRNAs included on the array were used as background. Clustering of the miRNA sequences was done with ClustalW, default settings, using the R package *msa* and visualized with the R package *ape*.

For the TLDA analysis (Study I), ΔCT was calculated using *GAPDH* as reference gene. Hierarchical clustering was performed using Euclidean distance metric and average linkage. The ΔΔCT-values were calculated between non-treated and GSC exosome treated, between NSC exosome and GSC exosome treated cells to estimate Gaussian distribution. This was used to determine differentially expressed genes based on the mean ± 1.96*(standard deviation).

In Study II the genomic position of the miRNAs detected by the microarray were mapped to the genomic position of the CpG sites included on the 450K methylation arrays (data obtained from previous studies). In this analysis all sites located at most 1,600 base pairs (bp) upstream or downstream of the DNA sequences coding for the miRNAs were included. The focus of this data analysis was the relationship between methylation and miRNA expression, therefore only miRNAs that had at least one associated measured CpG were included, and those miRNAs that were detected in all cell samples. Pearson (pairwise) correlations between the expression of each miRNA and the methylation levels of all the adjacent CpG sites were calculated. High correlations created by one single outlier sample were excluded, as well as high correlations from pairs where the maximum difference in beta-value between two samples was below 0.2.

For Study II, public DNA methylation data sets were downloaded from NCBI GEO database, accession numbers: GSE36278 and GSE52556. In the two datasets the total number of samples was (after removal of irrelevant samples) 78. From this 51 were paediatric GBM and 27 were control tissue (13 foetal and 14 paediatric). The purpose of the analysis of these data sets was to explore the miR-497-5p and

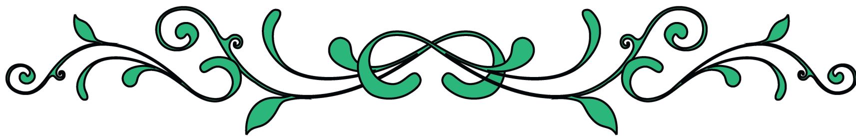
miR-195-5p DNA methylation patterns in a bigger sample cohort, than in the used cell lines. For the identification of the differences in Beta level for the two CpG sites between the two groups (normal and cancerous) two-sample t-test was applied.

All experiments in Study II were performed in at least technical triplicates. Most of the experiments, as in case of the miRNAs expression, target genes expression, the miRNAs effect on cell number and response to TMZ were performed in biological triplicates, each with three technical replicates. To establish statistically significant differences the t-Test: Two-Sample Assuming Equal Variances was applied and p values <0.05 were considered statistically significant.

3.4.2 Pathway analysis and network buildings

In order to gain more knowledge of the miRNAs role, in Study I pathways enriched with miRNA target genes based on Kyoto Encyclopedia of Genes and Genomes (KEGG pathways) were explored with the use of the DIANA TOOLS mirPath v3 software. The micro-T-CDS algorithm predicts miRNA targets in CDS or 3'-UTR regions while Tarbase uses validated miRNA targets [270]. Based on these two tools offered by the DIANA web tool, target networks were constructed for miRNAs and target genes with Cytoscape (Version 3.1.1) for a better visualisation of miRNA and target genes interaction [271]. Furthermore, for the target genes involvement in biological processes, the DAVID web tool was used with the GO BP_ALL settings [272].

RESULTS AND DISCUSSION



4

"I was taught that the way of progress was neither swift nor easy."
Marie Curie

Study I: Exploring the miRNA content of GSC exosomes and their role in inducing gene expression changes in NSCs

Several studies have pointed out the differences on a molecular level between GBM in children and in adults [203, 204]. Exosomes have gained much attention especially in the last decade, since the discovery of their molecular content and their involvement in cell to cell communication [130]. However, extracellular vesicles from paediatric GSCs have not been investigated. Since GSCs are considered to be one of the driving forces behind GBM, it is essential to investigate these cells' genetics and epigenetics. Therefore, in this study the miRNA content of GSCs isolated from paediatric patients was investigated. Furthermore, the miRNA content of the exosomes released by these GSCs was explored in detail. Finally, the GSC exosomes and their miRNAs effect on NSC gene expression were studied.

Six different primary paediatric glioma stem cell lines were used in this study. As controls three neural stem cell lines were used. Characterisation of the EVs isolated from the cell culture media based on three different methods confirmed their identity as exosomes, shown in Figure 10.

The investigation of miRNA expression in GSCs in comparison with NSCs led to the identification of several differentially expressed miRNAs. Some of these differentially expressed miRNAs were found to be up-regulated in GSCs compared to NSCs. One miRNA upregulated in GSCs was miR-497-5p, which has previously been associated with brain tumours [63, 273]. High expression of this miRNA in the U87 glioma cell line was associated with TMZ resistance and was also described to have a higher expression in pHGG than adult high grade gliomas or normal tissue [63, 274].

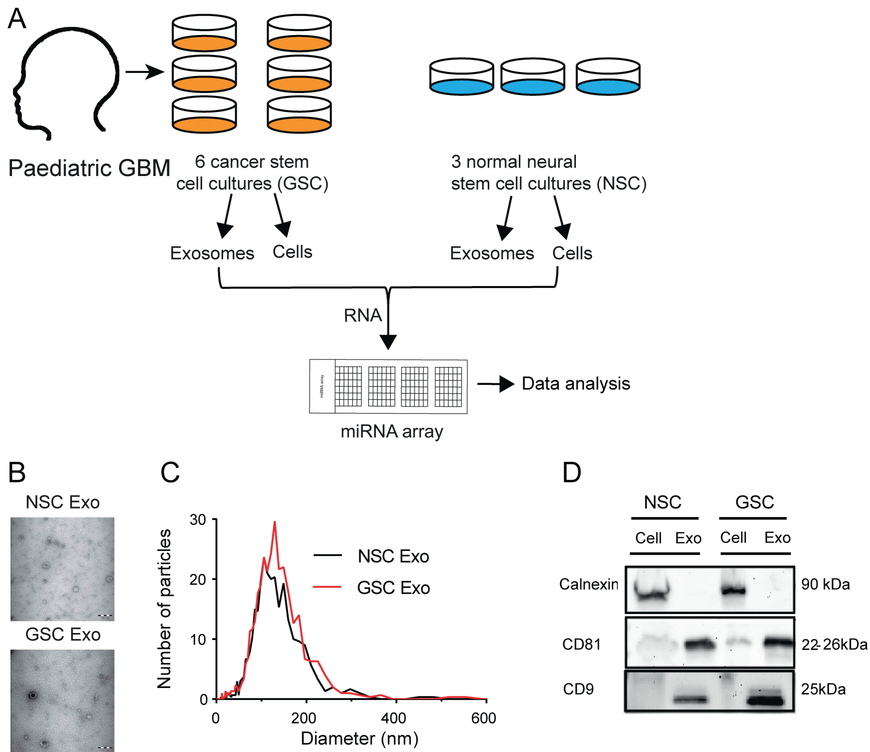


Figure 10. Experimental set-up and exosome characterisation. *A) Six GSC and three NSC lines were used for RNA extraction and exosome isolation from their cell culture media. The RNA extracted from cells and exosomes was used for miRNA array to identify differentially expressed miRNAs. B) Exosome characterisation with TEM. C) Exosome characterization by size. D) Exosome characterization by surface markers.*

Based on their expression level (intensity), the miRNAs clustered the cell and exosome samples in two different groups. Several miRNAs were found to be expressed just in the cell samples while others were expressed only in the exosome samples.

Forty-two differentially expressed miRNAs were identified between GSC and NSC exosomes. Some of them, for example miR-1290 and miR-1246, both associated with neural differentiation [275], and were up-regulated in GSC exosomes compared to NSC exosomes. These

miRNAs have not been described previously in the context of GSC exosomes.

MiRNAs identified to be differentially expressed in exosomes originating from GSCs compared to those secreted by NSCs, were found to be enriched in cancer related KEGG pathways.

The identification of a higher number of differentially expressed miRNAs in exosomes than in cells was an interesting finding that led to further exploration of this phenomenon. Most of these miRNAs were up-regulated in exosomes compared to cells (GSC exosomes: 147 and NSC exosomes: 100 miRNAs). In order to understand why some miRNAs have a higher expression in exosomes than in their originating cells, investigations of the miRNAs sequences were done. Two often repeating sequences were detected in the miRNAs that had higher expression in exosomes than in cells. One of these repeating patterns in the miRNAs sequence was previously described and termed exo motif (GGAG) [149], while the newly identified sequence was GGGGC (Figure 11).

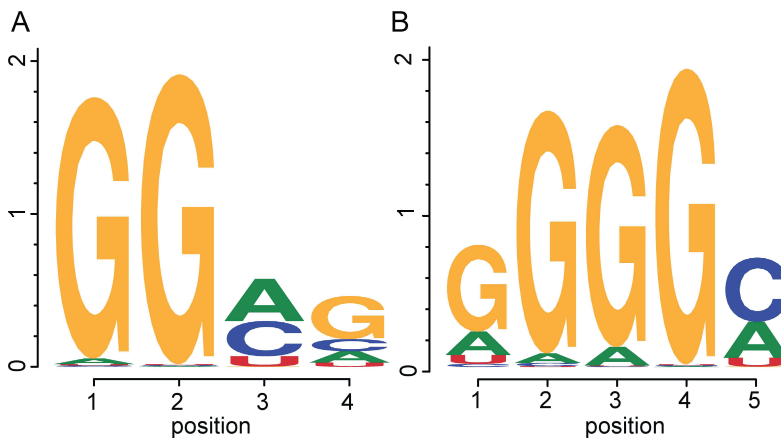


Figure 11. Exo motifs. *A) The previously known exo-motif and B) the newly detected exo-motif.*

The Villarroya-Beltri et al. study on exo motifs suggested that these repeating sequences might play a role in miRNA sorting into exosomes [149]. The exact mechanism of RNA sorting into exosomes

is yet unknown, however more studies have provided evidence that support the existence of such a mechanism [144, 146, 147]. The Study I results sustain these miRNA sorting mechanisms hypothesis.

Some miRNAs with these exo-motifs seem to be present both in cancer and normal samples, while some are specific to either group of samples. However, some miRNAs with these exo-motifs were identified as differentially expressed just in the GSC exosomes but not in the case of normal samples. We termed this group of miRNAs 'glioma exosome specific' and most of them were up-regulated in the exosomes compared to their originating cells. One could speculate that the preferential sorting of the miRNAs into exosomes in cancer cells is influenced by malfunctioning cellular mechanisms, resulting in the secretion of exosomes with a miRNA content that could play a role in the cellular niche tumour transformation.

Building on the hypothesis that the exosomal content could influence surrounding cells, the GSC exosomes effect on NSC gene expression was studied. Twenty-three genes were down-regulated while four up-regulated in the samples treated with GSC exosomes compared to the control samples. Some of the down-regulated genes were tumour suppressor genes (*PTEN, TET3*) or genes with a role in stemness, differentiation, or cell fate (*NOTCH1*, *NOTCH2*, *JAG1*, *DLL1* and *GFAP*) [276, 277].

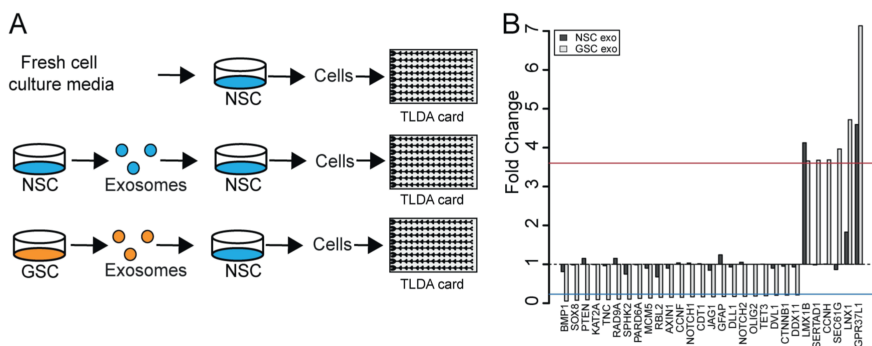


***Figure 12. GSC exosomes effect on NSCs**. A) Experimental set-up and B) the GSC exosomes effect on NSC gene expression.*

These findings suggest that the GSC exosomes, through their miRNA content, are capable of inducing gene expression changes in NSCs confirming these small vesicles' possible role in tumour biology.

Study II: Epigenetic interplays and their involvement in mediating response to chemotherapy in GSCs

GSCs have an important role in GBM development, maintenance and resistance to chemotherapeutical agents such as TMZ. Epigenetic modifications are imperative for all cells, including GSCs. However, the knowledge on the role of epigenetic mechanisms and their potential involvement in GSCs resistance to chemotherapy is scant. Thus, in this study DNA methylation and its role in regulating miRNAs expression were investigated.

DNA methylation array and miRNA array data from two previous studies [102, 150] of six paediatric GSC and three NSC cultures was used to identify correlations between the miRNA expression and their DNA methylation. The experimental set-up is shown in figure 13.

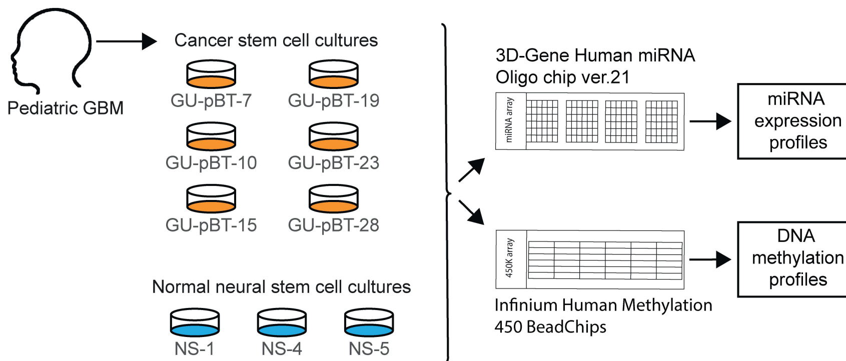


Figure 13. Experimental set-up *for study II. MiRNA and DNA methylation data was used from six GSC and three NSC lines to identify correlations between miRNA expression and their DNA sequence methylation.*

Several miRNAs expression correlated with their DNA sequence methylation; negatively or positively. The top miRNA that had a negative correlation was miR-497-5p, while miR-195-5p from the

same chromosomal region was also found to negatively correlate with its DNA sequence methylation. These two miRNAs were identified in Study I as up-regulated in GSCs compared to NSCs. These results suggest that the miR-497-5p and miR-195-5p high expression in GSCs could be a result of the lack of DNA methylation. To further support this finding, we used publically available data to obtain a larger sample cohort. These data sets confirmed the differential DNA methylation of two CpG sites for miR-497-5p and miR-195-5p between normal brain and tumour tissue. Additionally, our experiments with the DNA methyltransferase inhibitor decitabine confirmed that miR-497-5p but not miR-195-5p expression, at least indirectly is regulated by its DNA methylation pattern.

Previous studies using traditional glioma cell lines such as U87 have investigated the role of miR-497-5p in TMZ resistance [221, 274]. They showed that the high expression of this miRNA decreased cells' sensitivity to TMZ by inhibiting apoptosis [221, 274]. However, another study showed that the low expression of miR-497-5p in glioma patients is associated with poor prognosis [278]. The miR-497-5p role in paediatric GSCs remained unexplored. In several different tumour types, high expression of miR-497-5p was associated with decreased cell proliferation, therefore it was considered to have tumour suppressive properties [279, 280]. These contradictory observations from previous studies [278-280] and the findings from the Study I led to more detailed investigations of the miR-497-5p and miR-195-5p role in GSC response to TMZ. Hence, these miRNAs were overexpressed in NSCs and GSCs followed by TMZ treatment for 3 days. Immunocytochemistry analysis revealed a decreased cell number and cell proliferation when miR-497-5p was overexpressed, but not for miR-195-5p overexpression. The TMZ treatment resulted in a higher cell number in the samples with overexpressed miR-497-5p than in the control samples or those with miR-195-5p overexpressed. This suggests the miR-497-5p overexpression, but not miR-195-5p overexpression lead to a decreased cell number but also to a lower TMZ response both in NSCs and GSCs (graphical representation in Figure 14). The lower response to TMZ by the cells with high miR-497-5p could be due to the slower cell proliferation induced by this

miRNA. TMZ affects especially fast proliferating cells by inducing cell cycle arrest in G2/M phase which lead to apoptosis [259].

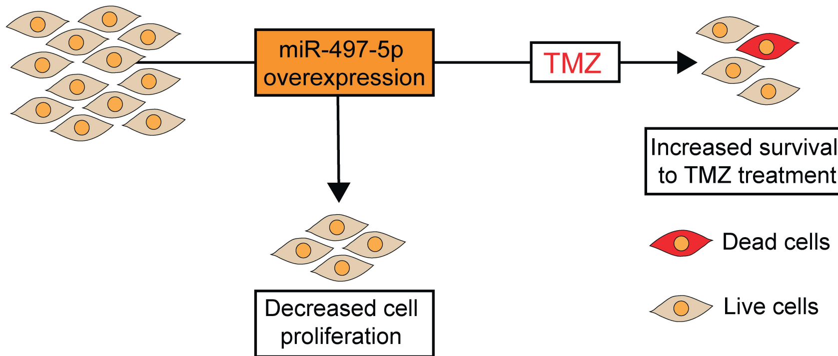


Figure 14. MiR-497-5p expression and its link to GSCs response towards TMZ. *The overexpression of miR-497-5p in GSCs and NSCs lead to decreased cell proliferation and lower sensitivity to TMZ.*

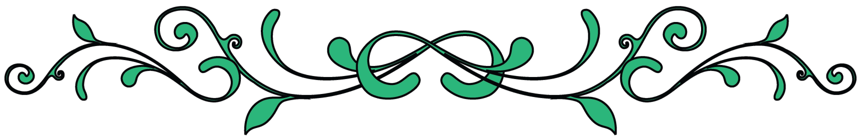
The miR-497-5p has a very high expression in GSCs and the siRNA silencing is a transient effect. Therefore, a CRISPR knock-out system was used for the miR-497-5p silencing in GSCs used in the functional part of the study. The miR-497-5p knockout resulted in the opposite effect as the miRNA overexpression; the cell number increased and effect of TMZ increased compared to control cells that had been transfected with negative guide RNA.

In order to gain more knowledge about the mechanisms by which miR-497-5p influences cell proliferation miRNA target genes were investigated. The *KCNN4* is a direct target gene of miR-497-5p with a role in cell proliferation, while *SP9* is a transcription factor [281, 282]. Both genes' expression decreased when the miRNA of interest was overexpressed in NSCs. Also, the expression of the genes increased when the miR-497-5p was silenced in GSCs. Furthermore, the investigation of the mechanism behind the reduced cell proliferation rate caused by miR-497-5p overexpression led to the investigation of S phase related genes expression. The miR-497-5p overexpression resulted in decreased gene expression of three genes involved in DNA replication; *PCNA*, *MCM2* and *CDT1* [109, 110]. Previous

studies have shown that these genes have higher expression in faster proliferating cells. Their downregulation resulting from miR-497-5p overexpression suggest this miRNA is affecting cell proliferation through S phase related genes. Several genes have previously been described to have differential expression in fast growing and dormant tumour cells [108, 283]. Therefore, several genes characteristic for dormant tumour cells were investigated after the miR-497-5p overexpression in NSCs or silencing in GSCs. The expression of the studied genes (*TGF-β2*, *THSBS-1*, *EphA5* and *IGFBP-5)* was affected accordingly by the miR-497-5p overexpression and silencing, respectively, in NSCs and GSCs. This indicates that miR-497-5p does not affect only genes related to cell proliferation but also genes related to dormant tumour cells.

These results suggest that miR-497-5p has a role in cell proliferation; affects genes found to be upregulated in dormant tumour cells and can be linked to TMZ response.

CONCLUSIONS



5

"I hadn't been aware that there were doors closed to me until I started knocking on them."

Gertrude B. Elion

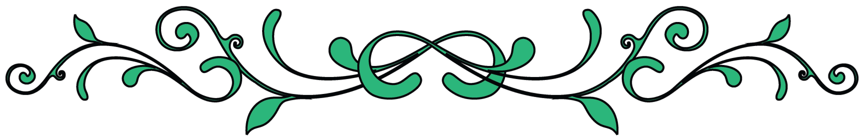
Based on the findings from Study I the following conclusions can be drawn:

- The miRNA profiles differ between NSCs and GSCs as do the exosomal miRNA content of these cells.
- The number of differentially expressed miRNAs is higher between exosomes than between the cellular content of NSCs and GSCs.
- MiRNAs up-regulated in exosomes compared to their originating cells often contain two repeating sequences, termed exo-motifs: GGAG and GGGGC
- The GSC exosomes can induce gene expression changes in receiver NSCs.

From Study II the following can be concluded:

- The miR-497-5p expression is indirectly regulated by DNA methylation.
- Overexpression of miR-497-5p, but not miR-195-5p, results in a reduction of cell number in GSCs and NSCs.
- MiR-497-5p, but not miR-195-5p, overexpression results in a lower response to TMZ in NSCs and GSCs.
- The miR-497-5p induces expression changes in S phase and genes previously described as dormancy-related.

FUTURE PERSPECTIVES



6

"It is the brain, the little grey cells on which one must rely. One must seek the truth within - not without."
Agatha Christie

Developing more efficient treatment strategies are vital, as high-grade gliomas are incurable. To achieve this, a deep knowledge of the driving mechanisms in paediatric GBM is important. CSCs have a significant role in GBM and the generation and maintenance of these cells are orchestrated by different epigenetic mechanisms.

The first study in this thesis brings a detailed profiling of the miRNA content of GSC exosomes, which has not previously been investigated in CSC from paediatric GBM. This shed light on one of the possible communication channels of the GSCs. The exosome content of other cells from the tumour niche have not been investigated, which future studies need to address in order to advance our understanding of the cell to cell communication in paediatric GBM. Furthermore, the GSC secreted exosomes miRNA profile from Study I could serve as a base to identify biomarkers from patient's blood for diagnostic and prognostic purposes.

Elucidating the reason why GSCs are secreting exosomes with higher expression of the miRNAs miR-1290 and miR-1246 (both associated with neural differentiation) than NSCs would be an interesting topic to investigate. One could speculate that this is due to a mechanism by which GSCs try to maintain their stemness. Deciphering the exact mechanism of miRNA sorting into exosomes has been investigated previously and also in Study I in this thesis, however its complexity requires more in depth studies [147, 149, 150]. The understanding of these mechanisms could have an important role in understanding why some cells are packing up certain miRNAs into exosomes and releasing them, especially if this could be a strategy by which cancer cells are assuring their invasion success.

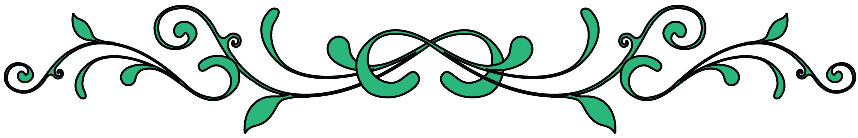
The induced gene expression changes in NSCs by the GSC exosomes found in Study I are an example of a functional effect that might take place in the niche of the brain cells. Further investigations, such as the effect of GSC exosomes on differentiated glioma cells and vice versa would be an interesting follow-up study. Also, the validation of these results in animal models could lead to more knowledge on the role of these vesicles in paediatric GBM biology.

The TMZ efficiency in the treatment of children with GBM is lower than in adults [262]. This has been linked to the fact that in most of the paediatric GBM, the *MGMT* gene promoter is unmethylated. The development of new strategies to sensitise these cells to TMZ are ongoing [263]. The second study in this thesis showed that the higher expression of miR-497-5p in GSCs compared to NSCs could be due to the unmethylated adjacent CpG sites. A question raised based on this finding is if restoring methylation would sensitise these cells to TMZ. Study II showed that miR-497-5p expression change influenced not just the proliferation and response to TMZ but also affected the expression of genes that are upregulated in dormant tumour cells. These results raise further questions on what is more ideal; GSCs with high miR-497-5p that will result in a lower proliferation but worse TMZ response or GSCs with low miR-497-5p, higher cell proliferation and better response to TMZ. MiR-497-5p was found in a previous study to have higher expression in GSCs than in differentiated cells [284], but the expression change cause and role is unknown. The evaluation of miR-497-5p expression and its DNA methylation in differentiated paediatric glioma cells would be an interesting next step. Also, the study of miR-497-5p overexpression and silencing in differentiated cells and its effect on TMZ response could be complementary to the present study. Targeting this miRNA might contribute to a more efficient TMZ response in paediatric GBM.

The two studies of the thesis contribute at some extent to the deciphering of epigenetic mechanisms and exosomes involvement in GSC biology. However more knowledge on these mechanisms is required to better understand paediatric GBM towards a more efficient therapy design in the future.

.

ACKNOWLEDGEMENTS



7

“The Answer to the Great Question...Of Life, the Universe and Everything... Is... Forty-two.”
Douglas Adams

"Science is the greatest of all adventure stories, one that's been unfolding for thousands of years as we have sought to understand ourselves and our surroundings" (Brian Greene). Now, that I arrived to the end of my PhD journey I would like to thank all those people who were part in some ways of this extraordinary adventure:

Helena Carén, my main supervisor to whom I wish to express my sincere gratitude for giving me this opportunity to work on the most fascinating PhD topic. I would like to thank for guiding me through my PhD and for all the support throughout these years as well as for your enormous patience with me. Thank you for all your help from lab work to writing and scientific discussions.

Anna Danielsson, my co-supervisor who always tried to make sure that I feel good during these challenging years. Thank you for your kind and caring guiding as well as for your help in the lab and in scientific discussions.

Teresia Kling, my co-supervisor without whom it wouldn't be possible to have all those bioinformatics data analysis in my studies. Thank you for all your help, and the practical and realistic advices.

Magnus Tisell, my co-supervisor whose dedication for his work I truly admire.

Hadi Valadi, my mentor who introduced me to the fantastic world of exosomes. Thank you for believing in me and giving me a chance when I need it the most. Thank you for all your help, guidance and friendship during these years. Your scientific fascination and curiosity is really inspiring!

I want to thank all group members for your great help, expertise in the lab (and outside the lab), for the scientific and non-scientific discussions and fun times: **Susanna** (your energy is remarkable and your good mood is contagious ☺), **Anna** (your sense for small details is amazing and I really enjoyed the late evening chats), **Sandra** (you are always pleasant and encouraging; I miss our rides on bus number 25), **Maja** (I am glad that you are back in the group and I am grateful

me to DIANA), **Mia** (your fantastic way of thinking and sense of humour always surprised me), **Kristell** (is inspiring how energetic and brave you are, mon très bonne amie), **Pavle** (just one question: is statistics or party time?), **Elizabeth** (I appreciate your kindness and dedication), **Katja** (I really enjoyed your presentations), **Agnes** (always happy and curios) and **Ariel** (happy to have you in the lab).

I would like to thank all former lab members for your help, for the fun times and I do miss you all: **Charlotte, Camilla, Patricia, Kirstine, Tanvir, Angelo, Susanne, Sofie and Elin.**

I feel really lucky to sit in the “Awesome people office” and I would like to thank all the office members for your help in work and for the fun times ☺: **Karin** (you make all days feel like they are sunny), **Elin** (for the best medical advice on how to survive challenging moments during PhD), **Junchi** (a good friend with a fantastic personality), **Ellen** (always eager to help), **Gustav** (for sharing and not taking over all the office desk ☺), **Mamen** (I enjoyed all our chats and laughs), **Carmen** (la mia principessa preferita and the most social one) and **Melita** (I agree with your opinion on liquorish candies).

The highlight of my every day was to have lunch with the “Funny lunch group” and for that I would like to thank for all the members of these cool group: **Jana** (smart, direct and good friend), **Agnieszka** (unexpected jokes and remarks), **Dorota** (always chill and happy), **Hanna** (very calm but with surprising funny comments), **Toshima** (irresistible laugh) and **Gautam** (surprising stories every day). Thank you for taking me in your fun group and making me smile even in the hardest moments ☺.

I would like to thank the “Masters of exosomes” from the Krefting Research Center for the help with the exosome work and the wonderful times: **Jan Lötvall** and **Cecilia** (for your valuable scientific and practical help), **Aleksander** (always being modest, kind and helpful), **Su Chul** (fun and knowledgeable), and **Taral** (made me feel welcomed in the lab).

I would like to thank co-authors for the scientific help: **Bertil Rydenhag, Steven Pollard**, **Virginia Claudio** and **Georg Kuhn.**

Great thanks for **Anne Uv** and **Ulrika Lantz Carlsson** for the help in the administrative maze of PhD studies.

Big thanks for all the Cancer Center members: **Shawn** (for all the help getting around with PhD things, aloha), **Anna Linder** (great weekend chats), **Junrui** (you are fun), **Maryam** (always kind), **Parmida** (I miss the "are you there" chats) and **Malin Hagberg** (for your kindness and help). Great thanks for corridor and elevator chats (even help): **Therese**, **Sohelia**, **Kristell**, **Murali**, **Mohamed**, **Daniel**, **Karoline**, **Stefan**, **Anna**, **Paul**, **Emma**, **Tobias** and **Elin**.

Thank you former Cancer Center colleagues for the help and good company: **Susann** (so very kind person), **Thomas** (being my un-official qPCR supervisor) and **Pinar** (I miss you).

Great thanks I owe to the people and their partners from "Botan", it was always fun to spend time with you guys: **Ylva, Otilia, Somnath, Triranta**, **Ema and Srdjan.**

I would like to thank the "Transylvanian gang" for the moral support: **Marika** néni (to remember the taste of home), **Péter** (to help me fit in this new environment) and **Helén** (you are a great friend and your strength is inspiring). Köszönöm szépen! Thanks **Bee** (my SFI friend) for being there for me from the beginning.

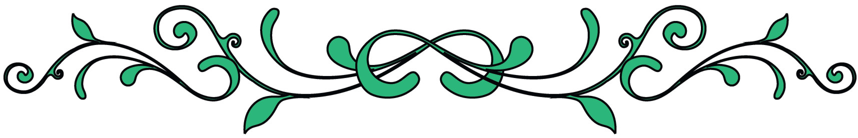
I would like to express my greatest thank for my loving family who always supported me in all my crazy adventures: my father who taught me too choose a field in my carrier that always will fascinate me, my mother who sparked in me the fascination for the world of molecular biology by handing me the first book that I ever read on genetics, my brother on whom I can always count and whom I admire. Nincsenek szavak amivel köszönetemet ki tudnám fejezni az egy életen át tartó szülői gond-viselésetekért és a testvéri szeretetért. Nektek köszönhetem, hogy álmaim valóra váltak és azt az életet élem amire

mindig is vágytam. Szívből köszönök szépen mindent, ti vagytok a legcsodálatosabbak és nagyon szeretlek!

I wish to thank for all the support of **Herdean** family. You made me feel that I am part of your family. Vă mulțumesc pentru suportul vostru și toată dragostea cu care m-ați primit în familia voastră. Vă mulțumesc din suflet pentru tot.

Last, but not least I would like to thank for my greatest support in everything, my better half, my fantastic husband: **Andrei**. Ești cel mai minunat om pe care l-am întâlnit vreodată și mă simt cea mai norocoasă femeie din lume că te am lângă mine, că ești soțul meu. Îți mulțumesc din tot sufletul și toată inima pentru ajutorul tău cu teza, doctoratul și totul în viață.

REFERENCES



8

"...when you have eliminated the impossible, whatever remains, however improbable, must be the truth?”

Sherlock Holmes

1. Watson JD and Crick FH. Molecular structure of nucleic acids. Nature. 1953; 171(4356):737-738.
2. Gayon J. From Mendel to epigenetics: History of genetics. Comptes rendus biologies. 2016; 339(7-8):225-230.
3. Pearson H. Genetics: what is a gene? Nature. 2006; 441(7092):398-401.
4. Johannsen W. The genotype conception of heredity. The American Naturalist. 1911; 45(531):129-159.
5. Ezkurdia I, Juan D, Rodriguez JM, Frankish A, Diekhans M, Harrow J, Vazquez J, Valencia A and Tress ML. Multiple evidence strands suggest that there may be as few as 19,000 human protein-coding genes. Human molecular genetics. 2014; 23(22):5866-5878.
6. An integrated encyclopedia of DNA elements in the human genome. Nature. 2012; 489(7414):57-74.
7. Derrien T, Johnson R, Bussotti G, Tanzer A, Djebali S, Tilgner H, Guernec G, Martin D, Merkel A, Knowles DG, Lagarde J, Veeravalli L, Ruan X, Ruan Y, Lassmann T, Carninci P, et al. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. Genome research. 2012; 22(9):1775-1789.
8. Kornberg RD. Chromatin structure: a repeating unit of histones and DNA. Science (New York, NY). 1974; 184(4139):868-871.
9. Felsenfeld G and Groudine M. Controlling the double helix. Nature. 2003; 421(6921):448-453.
10. Crick FH. On protein synthesis. Symposia of the Society for Experimental Biology. 1958; 12:138-163.
11. Waddington CH. (2015). How animals develop: Routledge).
12. Goldberg AD, Allis CD and Bernstein E. Epigenetics: a landscape takes shape. Cell. 2007; 128(4):635-638.
13. Esteller M. Epigenetics in evolution and disease. The Lancet. 2008; 372:S90-S96.
14. Portela A and Esteller M. Epigenetic modifications and human disease. Nature biotechnology. 2010; 28(10):1057-1068.
15. Handy DE, Castro R and Loscalzo J. Epigenetic modifications: basic mechanisms and role in cardiovascular disease. Circulation. 2011; 123(19):2145-2156.
16. Fu Y, Dominissini D, Rechavi G and He C. Gene expression regulation mediated through reversible m(6)A RNA methylation. Nature reviews Genetics. 2014; 15(5):293-306.
17. Lee RC, Feinbaum RL and Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993; 75(5):843-854.
18. Buchan JR and Parker R. Molecular biology. The two faces of miRNA. Science (New York, NY). 2007; 318(5858):1877-1878.
19. Herman JG and Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. The New England journal of medicine. 2003; 349(21):2042-2054.

20. Esteller M. Epigenetics in cancer. The New England journal of medicine. 2008; 358(11):1148-1159.
21. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, Harris K, Heaford A, Howland J, Kann L, et al. Initial sequencing and analysis of the human genome. Nature. 2001; 409(6822):860-921.
22. Ioshikhes IP and Zhang MQ. Large-scale human promoter mapping using CpG islands. Nature genetics. 2000; 26(1):61-63.
23. Hellman A and Chess A. Gene body-specific methylation on the active X chromosome. Science (New York, NY). 2007; 315(5815):1141-1143.
24. Bird A. DNA methylation patterns and epigenetic memory. Genes & development. 2002; 16(1):6-21.
25. Herman JG. Hypermethylation of tumor suppressor genes in cancer. Seminars in cancer biology. 1999; 9(5):359-367.
26. Regents UoC. http://missinglink.ucsf.edu/lm/genes_and_genomes/methylation.html.
27. Esteller M. Epigenetic gene silencing in cancer: the DNA hypermethylome. Human molecular genetics. 2007; 16 Spec No 1:R50-59.
28. Lopez-Serra L and Esteller M. Proteins that bind methylated DNA and human cancer: reading the wrong words. British journal of cancer. 2008; 98(12):1881-1885.
29. Zemach A, McDaniel IE, Silva P and Zilberman D. Genome-wide evolutionary analysis of eukaryotic DNA methylation. Science (New York, NY). 2010; 328(5980):916-919.
30. Neri F, Rapelli S, Krepelova A, Incarnato D, Parlato C, Basile G, Maldotti M, Anselmi F and Oliviero S. Intragenic DNA methylation prevents spurious transcription initiation. Nature. 2017; 543(7643):72-77.
31. Inoue A and Zhang Y. Replication-dependent loss of 5-hydroxymethylcytosine in mouse preimplantation embryos. Science (New York, NY). 2011; 334(6053):194.
32. Bayraktar G and Kreutz MR. The Role of Activity-Dependent DNA Demethylation in the Adult Brain and in Neurological Disorders. Frontiers in molecular neuroscience. 2018; 11:169.
33. Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC and Zhang Y. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature. 2010; 466(7310):1129-1133.
34. Geiman TM and Muegge K. DNA methylation in early development. Molecular reproduction and development. 2010; 77(2):105-113.
35. Li E, Bestor TH and Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell. 1992; 69(6):915-926.
36. Fouse SD, Shen Y, Pellegrini M, Cole S, Meissner A, Van Neste L, Jaenisch R and Fan G. Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation. Cell stem cell. 2008; 2(2):160-169.
37. Khavari DA, Sen GL and Rinn JL. DNA methylation and epigenetic control of cellular differentiation. Cell cycle (Georgetown, Tex). 2010; 9(19):3880-3883.

38. Feinberg AP and Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature. 1983; 301(5895):89-92.
39. Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB and Sidransky D. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nature medicine. 1995; 1(7):686-692.
40. Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, Sidransky D and Baylin SB. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer research. 1995; 55(20):4525-4530.
41. Catteau A, Harris WH, Xu CF and Solomon E. Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics. Oncogene. 1999; 18(11):1957-1965.
42. Cech TR and Steitz JA. The noncoding RNA revolution-trashing old rules to forge new ones. Cell. 2014; 157(1):77-94.
43. Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nature reviews Genetics. 2009; 10(10):704-714.
44. Lytle JR, Yario TA and Steitz JA. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104(23):9667-9672.
45. Lewis BP, Burge CB and Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005; 120(1):15-20.
46. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009; 136(2):215-233.
47. Georgakilas G, Perdikopanis N and Hatzigeorgiou AG. Identifying Pri-miRNA Transcription Start Sites. Methods in molecular biology (Clifton, NJ). 2018; 1823:11-31.
48. Ketting RF. microRNA Biogenesis and Function : An overview. Advances in experimental medicine and biology. 2011; 700:1-14.
49. Spadaro PA and Bredy TW. Emerging role of non-coding RNA in neural plasticity, cognitive function, and neuropsychiatric disorders. Frontiers in genetics. 2012; 3:132.
50. Issler O and Chen A. Determining the role of microRNAs in psychiatric disorders. Nature reviews Neuroscience. 2015; 16(4):201-212.
51. Ambros V, Bartel B, Bartel DP, Burge CB, Carrington JC, Chen X, Dreyfuss G, Eddy SR, Griffiths-Jones S, Marshall M, Matzke M, Ruvkun G and Tuschl T. A uniform system for microRNA annotation. RNA (New York, NY). 2003; 9(3):277-279.
52. miRBase. http://www.mirbase.org/help/nomenclature.shtml.
53. Altuvia Y, Landgraf P, Lithwick G, Elefant N, Pfeffer S, Aravin A, Brownstein MJ, Tuschl T and Margalit H. Clustering and conservation patterns of human microRNAs. Nucleic acids research. 2005; 33(8):2697-2706.

54. Vasudevan S, Tong Y and Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. Science (New York, NY). 2007; 318(5858):1931-1934.
55. Ha M and Kim VN. Regulation of microRNA biogenesis. Nature reviews Molecular cell biology. 2014; 15(8):509-524.
56. Laurent LC. MicroRNAs in embryonic stem cells and early embryonic development. Journal of cellular and molecular medicine. 2008; 12(6a):2181-2188.
57. Gross N, Kropp J and Khatib H. MicroRNA Signaling in Embryo Development. Biology. 2017; 6(3).
58. Giraldez AJ, Cinalli RM, Glasner ME, Enright AJ, Thomson JM, Baskerville S, Hammond SM, Bartel DP and Schier AF. MicroRNAs regulate brain morphogenesis in zebrafish. Science (New York, NY). 2005; 308(5723):833-838.
59. Ivey KN and Srivastava D. MicroRNAs as regulators of differentiation and cell fate decisions. Cell stem cell. 2010; 7(1):36-41.
60. Marson A, Levine SS, Cole MF, Frampton GM, Brambrink T, Johnstone S, Guenther MG, Johnston WK, Wernig M, Newman J, Calabrese JM, Dennis LM, Volkert TL, Gupta S, Love J, Hannett N, et al. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell. 2008; 134(3):521-533.
61. Laurent LC, Chen J, Ulitsky I, Mueller FJ, Lu C, Shamir R, Fan JB and Loring JF. Comprehensive microRNA profiling reveals a unique human embryonic stem cell signature dominated by a single seed sequence. Stem cells (Dayton, Ohio). 2008; 26(6):1506-1516.
62. Garzon R and Croce CM. MicroRNAs in normal and malignant hematopoiesis. Current opinion in hematology. 2008; 15(4):352-358.
63. Miele E, Buttarelli FR, Arcella A, Begalli F, Garg N, Silvano M, Po A, Baldi C, Carissimo G and Antonelli M. High-throughput microRNA profiling of pediatric high-grade gliomas. Neuro-oncology. 2013:not215.
64. Sinkkonen L, Hugenschmidt T, Berninger P, Gaidatzis D, Mohn F, Artus-Revel CG, Zavolan M, Svoboda P and Filipowicz W. MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. Nature structural & molecular biology. 2008; 15(3):259-267.
65. Parodi F, Carosio R, Ragusa M, Di Pietro C, Maugeri M, Barbagallo D, Sallustio F, Allemanni G, Pistillo MP, Casciano I, Forlani A, Schena FP, Purrello M, Romani M and Banelli B. Epigenetic dysregulation in neuroblastoma: A tale of miRNAs and DNA methylation. Biochimica et biophysica acta. 2016; 1859(12):1502-1514.
66. Lam JK, Chow MY, Zhang Y and Leung SW. siRNA Versus miRNA as Therapeutics for Gene Silencing. Molecular therapy Nucleic acids. 2015; 4:e252.
67. Sudhakar A. History of Cancer, Ancient and Modern Treatment Methods. Journal of cancer science & therapy. 2009; 1(2):1-4.
68. Hassanpour SH and Dehghani M. Review of cancer from perspective of molecular. Journal of Cancer Research and Practice. 2017; 4(4):127-129.

69. Romani M, Pistillo MP and Banelli B. Epigenetic Targeting of Glioblastoma. Frontiers in oncology. 2018; 8:448.
70. Yoo CB and Jones PA. Epigenetic therapy of cancer: past, present and future. Nature reviews Drug discovery. 2006; 5(1):37-50.
71. Lim M, Xia Y, Bettegowda C and Weller M. Current state of immunotherapy for glioblastoma. Nature reviews Clinical oncology. 2018; 15(7):422-442.
72. Siegel RL, Miller KD and Jemal A. Cancer statistics, 2016. CA: a cancer journal for clinicians. 2016; 66(1):7-30.
73. Kushi LH, Doyle C, McCullough M, Rock CL, Demark-Wahnefried W, Bandera EV, Gapstur S, Patel AV, Andrews K and Gansler T. American Cancer Society Guidelines on nutrition and physical activity for cancer prevention: reducing the risk of cancer with healthy food choices and physical activity. CA: a cancer journal for clinicians. 2012; 62(1):30-67.
74. O'Keeffe LM, Taylor G, Huxley RR, Mitchell P, Woodward M and Peters SAE. Smoking as a risk factor for lung cancer in women and men: a systematic review and meta-analysis. BMJ open. 2018; 8(10):e021611.
75. Thomson CA, LeWinn K, Newton TR, Alberts DS and Martinez ME. Nutrition and diet in the development of gastrointestinal cancer. Current oncology reports. 2003; 5(3):192-202.
76. LeRoith D and Roberts CT, Jr. The insulin-like growth factor system and cancer. Cancer letters. 2003; 195(2):127-137.
77. Askling J, Linet M, Gridley G, Halstensen TS, Ekstrom K and Ekbom A. Cancer incidence in a population-based cohort of individuals hospitalized with celiac disease or dermatitis herpetiformis. Gastroenterology. 2002; 123(5):1428-1435.
78. Canavan C, Abrams KR and Mayberry J. Meta-analysis: colorectal and small bowel cancer risk in patients with Crohn's disease. Alimentary pharmacology & therapeutics. 2006; 23(8):1097-1104.
79. Apostolou P and Fostira F. Hereditary breast cancer: the era of new susceptibility genes. BioMed research international. 2013; 2013:747318.
80. Zhen JT, Syed J, Nguyen KA, Leapman MS, Agarwal N, Brierley K, Llor X, Hofstatter E and Shuch B. Genetic testing for hereditary prostate cancer: Current status and limitations. Cancer. 2018; 124(15):3105-3117.
81. Huang PH, Xu AM and White FM. Oncogenic EGFR signaling networks in glioma. Science signaling. 2009; 2(87):re6.
82. Laigle-Donadey F, Crinière E, Benouaich A, Lesueur E, Mokhtari K, Hoang-Xuan K and Sanson M. Loss of 22q chromosome is related to glioma progression and loss of 10q. Journal of neuro-oncology. 2006; 76(3):265-268.
83. Liu L and Li X. IDH gene mutation in glioma. Cancer Translational Medicine. 2018; 4(5):129.
84. Grander D. How do mutated oncogenes and tumor suppressor genes cause cancer? Medical oncology (Northwood, London, England). 1998; 15(1):20-26.
85. Lee EY and Muller WJ. Oncogenes and tumor suppressor genes. Cold Spring Harbor perspectives in biology. 2010; 2(10):a003236.

86. Kim TY, Zhong S, Fields CR, Kim JH and Robertson KD. Epigenomic profiling reveals novel and frequent targets of aberrant DNA methylation-mediated silencing in malignant glioma. Cancer research. 2006; 66(15):7490-7501.
87. Rasime K. Epigenetics of glioblastoma multiforme. Journal of Clinical Research & Bioethics. 2015; 6(3):1.
88. Peng Y and Croce CM. The role of MicroRNAs in human cancer. Signal transduction and targeted therapy. 2016; 1:15004.
89. Reya T, Morrison SJ, Clarke MF and Weissman IL. Stem cells, cancer, and cancer stem cells. nature. 2001; 414(6859):105-111.
90. Li L and Neaves WB. Normal stem cells and cancer stem cells: the niche matters. Cancer research. 2006; 66(9):4553-4557.
91. Rahman M, Jamil HM, Akhtar N, Rahman K, Islam R and Asaduzzaman S. Stem cell and cancer stem cell: a tale of two cells. Progress in Stem Cell. 2016; 3(02):97-108.
92. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA and Dick JE. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. 1994; 367(6464):645-648.
93. Galli R, Binda E, Orfanelli U, Cipelletti B, Gritti A, De Vitis S, Fiocco R, Foroni C, Dimeco F and Vescovi A. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer research. 2004; 64(19):7011-7021.
94. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ and Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100(7):3983-3988.
95. O'Brien CA, Pollett A, Gallinger S and Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature. 2007; 445(7123):106-110.
96. Schatton T, Murphy GF, Frank NY, Yamaura K, Waaga-Gasser AM, Gasser M, Zhan Q, Jordan S, Duncan LM, Weishaupt C, Fuhlbrigge RC, Kupper TS, Sayegh MH and Frank MH. Identification of cells initiating human melanomas. Nature. 2008; 451(7176):345-349.
97. Gao MQ, Choi YP, Kang S, Youn JH and Cho NH. CD24+ cells from hierarchically organized ovarian cancer are enriched in cancer stem cells. Oncogene. 2010; 29(18):2672-2680.
98. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD and Dirks PB. Identification of human brain tumour initiating cells. nature. 2004; 432(7015):396-401.
99. Beier D, Hau P, Proescholdt M, Lohmeier A, Wischhusen J, Oefner PJ, Aigner L, Brawanski A, Bogdahn U and Beier CP. CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. Cancer research. 2007; 67(9):4010-4015.

100. Brescia P, Ortensi B, Fornasari L, Levi D, Broggi G and Pelicci G. CD133 is essential for glioblastoma stem cell maintenance. Stem cells (Dayton, Ohio). 2013; 31(5):857-869.
101. Pollard SM, Yoshikawa K, Clarke ID, Danovi D, Stricker S, Russell R, Bayani J, Head R, Lee M, Bernstein M, Squire JA, Smith A and Dirks P. Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. Cell stem cell. 2009; 4(6):568-580.
102. Wenger A, Larsson S, Danielsson A, Elbaek KJ, Kettunen P, Tisell M, Sabel M, Lannering B, Nordborg C, Schepke E and Caren H. Stem cell cultures derived from pediatric brain tumors accurately model the originating tumors. Oncotarget. 2017; 8(12):18626-18639.
103. Kondo T, Setoguchi T and Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101(3):781-786.
104. Larsson S, Wenger A, Dosa S, Sabel M, Kling T and Caren H. Cell line-based xenograft mouse model of paediatric glioma stem cells mirrors the clinical course of the patient. Carcinogenesis. 2018; 39(10):1304-1309.
105. Gao XL, Zhang M, Tang YL and Liang XH. Cancer cell dormancy: mechanisms and implications of cancer recurrence and metastasis. OncoTargets and therapy. 2017; 10:5219-5228.
106. Harashima H, Dissmeyer N and Schnittger A. Cell cycle control across the eukaryotic kingdom. Trends in cell biology. 2013; 23(7):345-356.
107. Stewart ZA, Westfall MD and Pietenpol JA. Cell-cycle dysregulation and anticancer therapy. Trends in pharmacological sciences. 2003; 24(3):139-145.
108. Almog N, Ma L, Raychowdhury R, Schwager C, Erber R, Short S, Hlatky L, Vajkoczy P, Huber PE, Folkman J and Abdollahi A. Transcriptional switch of dormant tumors to fast-growing angiogenic phenotype. Cancer research. 2009; 69(3):836-844.
109. Pozo PN and Cook JG. Regulation and Function of Cdt1; A Key Factor in Cell Proliferation and Genome Stability. Genes. 2016; 8(1).
110. Choe KN and Moldovan GL. Forging Ahead through Darkness: PCNA, Still the Principal Conductor at the Replication Fork. Molecular cell. 2017; 65(3):380-392.
111. Schillert A, Trumpp A and Sprick MR. Label retaining cells in cancer--the dormant root of evil? Cancer letters. 2013; 341(1):73-79.
112. Sertil AR. (2014). Hypoxia and Tumor Dormancy: Can the Two Tango? Tumor Dormancy, Quiescence, and Senescence, Vol 3: Springer), pp. 13-24.
113. Hermitte F, Brunet de la Grange P, Belloc F, Praloran V and Ivanovic Z. Very low O2 concentration (0.1%) favors G0 return of dividing CD34+ cells. Stem cells (Dayton, Ohio). 2006; 24(1):65-73.
114. Rich JN. Cancer stem cells: understanding tumor hierarchy and heterogeneity. Medicine. 2016; 95(1 Suppl 1):S2-7.
115. Shackleton M, Quintana E, Fearon ER and Morrison SJ. Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell. 2009; 138(5):822-829.
116. Enderling H. Cancer stem cells and tumor dormancy. Advances in experimental medicine and biology. 2013; 734:55-71.

117. Enderling H, Hlatky L and Hahnfeldt P. Migration rules: tumours are conglomerates of self-metastases. British journal of cancer. 2009; 100(12):1917-1925.
118. Wang Y, Yang J, Zheng H, Tomasek GJ, Zhang P, McKeever PE, Lee EY and Zhu Y. Expression of mutant p53 proteins implicates a lineage relationship between neural stem cells and malignant astrocytic glioma in a murine model. Cancer cell. 2009; 15(6):514-526.
119. Lee JH, Lee JE, Kahng JY, Kim SH, Park JS, Yoon SJ, Um JY, Kim WK, Lee JK, Park J, Kim EH, Lee JH, Lee JH, Chung WS, Ju YS, Park SH, et al. Human glioblastoma arises from subventricular zone cells with low-level driver mutations. Nature. 2018; 560(7717):243-247.
120. Lopez-Lazaro M. Stem cell division theory of cancer. Cell cycle (Georgetown, Tex). 2015; 14(16):2547-2548.
121. Tomasetti C and Vogelstein B. Cancer etiology. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. Science (New York, NY). 2015; 347(6217):78-81.
122. Friedmann-Morvinski D, Bushong EA, Ke E, Soda Y, Marumoto T, Singer O, Ellisman MH and Verma IM. Dedifferentiation of neurons and astrocytes by oncogenes can induce gliomas in mice. Science (New York, NY). 2012; 338(6110):1080-1084.
123. Rheinbay E, Suva ML, Gillespie SM, Wakimoto H, Patel AP, Shahid M, Oksuz O, Rabkin SD, Martuza RL, Rivera MN, Louis DN, Kasif S, Chi AS and Bernstein BE. An aberrant transcription factor network essential for Wnt signaling and stem cell maintenance in glioblastoma. Cell reports. 2013; 3(5):1567-1579.
124. Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, Huang Y, Hu X, Su F, Lieberman J and Song E. let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell. 2007; 131(6):1109-1123.
125. Lee DH, Ryu HW, Won HR and Kwon SH. Advances in epigenetic glioblastoma therapy. Oncotarget. 2017; 8(11):18577-18589.
126. Gyorgy B, Szabo TG, Pasztoi M, Pal Z, Misjak P, Aradi B, Laszlo V, Pallinger E, Pap E, Kittel A, Nagy G, Falus A and Buzas EI. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cellular and molecular life sciences : CMLS. 2011; 68(16):2667-2688.
127. Abels ER and Breakefield XO. Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. Cellular and molecular neurobiology. 2016; 36(3):301-312.
128. Colombo M, Raposo G and Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annual review of cell and developmental biology. 2014; 30:255-289.
129. Johnstone RM, Adam M, Hammond JR, Orr L and Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). The Journal of biological chemistry. 1987; 262(19):9412-9420.

130. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ and Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nature cell biology. 2007; 9(6):654-659.
131. Lunavat TR, Cheng L, Kim DK, Bhadury J, Jang SC, Lasser C, Sharples RA, Lopez MD, Nilsson J, Gho YS, Hill AF and Lotvall J. Small RNA deep sequencing discriminates subsets of extracellular vesicles released by melanoma cells--Evidence of unique microRNA cargos. RNA biology. 2015; 12(8):810-823.
132. Pols MS and Klumperman J. Trafficking and function of the tetraspanin CD63. Experimental cell research. 2009; 315(9):1584-1592.
133. Williams RL and Urbe S. The emerging shape of the ESCRT machinery. Nature reviews Molecular cell biology. 2007; 8(5):355-368.
134. Tamai K, Tanaka N, Nakano T, Kakazu E, Kondo Y, Inoue J, Shiina M, Fukushima K, Hoshino T, Sano K, Ueno Y, Shimosegawa T and Sugamura K. Exosome secretion of dendritic cells is regulated by Hrs, an ESCRT-0 protein. Biochemical and biophysical research communications. 2010; 399(3):384-390.
135. Baietti MF, Zhang Z, Mortier E, Melchior A, Degeest G, Geeraerts A, Ivarsson Y, Depoortere F, Coomans C, Vermeiren E, Zimmermann P and David G. Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. Nature cell biology. 2012; 14(7):677-685.
136. Raiborg C, Rusten TE and Stenmark H. Protein sorting into multivesicular endosomes. Current opinion in cell biology. 2003; 15(4):446-455.
137. Mathivanan S, Ji H and Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. Journal of proteomics. 2010; 73(10):1907-1920.
138. Mathivanan S and Simpson RJ. ExoCarta: A compendium of exosomal proteins and RNA. Proteomics. 2009; 9(21):4997-5000.
139. Guescini M, Genedani S, Stocchi V and Agnati LF. Astrocytes and Glioblastoma cells release exosomes carrying mtDNA. Journal of neural transmission (Vienna, Austria : 1996). 2010; 117(1):1-4.
140. Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, Zheng Y, Hoshino A, Brazier H, Xiang J, Williams C, Rodriguez-Barrueco R, Silva JM, Zhang W, Hearn S, Elemento O, et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. Cell research. 2014; 24(6):766-769.
141. Nemeth A, Orgovan N, Sodar BW, Osteikoetxea X, Paloczi K, Szabo-Taylor KE, Vukman KV, Kittel A, Turiak L, Wiener Z, Toth S, Drahos L, Vekey K, Horvath R and Buzas EI. Antibiotic-induced release of small extracellular vesicles (exosomes) with surface-associated DNA. Scientific reports. 2017; 7(1):8202.
142. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT, Jr., Carter BS, Krichevsky AM and Breakefield XO. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nature cell biology. 2008; 10(12):1470-1476.
143. Squadrito ML, Baer C, Burdet F, Maderna C, Gilfillan GD, Lyle R, Ibberson M and De Palma M. Endogenous RNAs modulate microRNA sorting to exosomes and transfer to acceptor cells. Cell reports. 2014; 8(5):1432-1446.

144. Bolukbasi MF, Mizrak A, Ozdener GB, Madlener S, Strobel T, Erkan EP, Fan JB, Breakefield XO and Saydam O. miR-1289 and "Zipcode"-like Sequence Enrich mRNAs in Microvesicles. Molecular therapy Nucleic acids. 2012; 1:e10.
145. McKenzie AJ, Hoshino D, Hong NH, Cha DJ, Franklin JL, Coffey RJ, Patton JG and Weaver AM. KRAS-MEK Signaling Controls Ago2 Sorting into Exosomes. Cell reports. 2016; 15(5):978-987.
146. Statello L, Maugeri M, Garre E, Nawaz M, Wahlgren J, Papadimitriou A, Lundqvist C, Lindfors L, Collen A, Sunnerhagen P, Ragusa M, Purrello M, Di Pietro C, Tigue N and Valadi H. Identification of RNA-binding proteins in exosomes capable of interacting with different types of RNA: RBP-facilitated transport of RNAs into exosomes. PloS one. 2018; 13(4):e0195969.
147. Shurtleff MJ, Temoche-Diaz MM, Karfilis KV, Ri S and Schekman R. Y-box protein 1 is required to sort microRNAs into exosomes in cells and in a cell-free reaction. eLife. 2016; 5.
148. Shurtleff MJ, Yao J, Qin Y, Nottingham RM, Temoche-Diaz MM, Schekman R and Lambowitz AM. Broad role for YBX1 in defining the small noncoding RNA composition of exosomes. Proceedings of the National Academy of Sciences of the United States of America. 2017; 114(43):E8987-e8995.
149. Villarroya-Beltri C, Gutierrez-Vazquez C, Sanchez-Cabo F, Perez-Hernandez D, Vazquez J, Martin-Cofreces N, Martinez-Herrera DJ, Pascual-Montano A, Mittelbrunn M and Sanchez-Madrid F. Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. Nature communications. 2013; 4:2980.
150. Tuzesi A, Kling T, Wenger A, Lunavat TR, Jang SC, Rydenhag B, Lotvall J, Pollard SM, Danielsson A and Caren H. Pediatric brain tumor cells release exosomes with a miRNA repertoire that differs from exosomes secreted by normal cells. Oncotarget. 2017; 8(52):90164-90175.
151. Hessvik NP and Llorente A. Current knowledge on exosome biogenesis and release. Cellular and molecular life sciences : CMLS. 2018; 75(2):193-208.
152. Sinha S, Hoshino D, Hong NH, Kirkbride KC, Grega-Larson NE, Seiki M, Tyska MJ and Weaver AM. Cortactin promotes exosome secretion by controlling branched actin dynamics. The Journal of cell biology. 2016; 214(2):197-213.
153. Stenmark H. Rab GTPases as coordinators of vesicle traffic. Nature reviews Molecular cell biology. 2009; 10(8):513-525.
154. Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A, Moita CF, Schauer K, Hume AN, Freitas RP, Goud B, Benaroch P, Hacohen N, Fukuda M, Desnos C, Seabra MC, et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. Nature cell biology. 2010; 12(1):19-30; sup pp 11-13.
155. Pfeffer SR. Unsolved mysteries in membrane traffic. Annual review of biochemistry. 2007; 76:629-645.
156. Raposo G and Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. The Journal of cell biology. 2013; 200(4):373-383.

157. Caponnetto F, Manini I, Skrap M, Palmai-Pallag T, Di Loreto C, Beltrami AP, Cesselli D and Ferrari E. Size-dependent cellular uptake of exosomes. Nanomedicine : nanotechnology, biology, and medicine. 2017; 13(3):1011-1020.
158. Fitzner D, Schnaars M, van Rossum D, Krishnamoorthy G, Dibaj P, Bakhti M, Regen T, Hanisch UK and Simons M. Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. Journal of cell science. 2011; 124(Pt 3):447-458.
159. Isola AL and Chen S. Exosomes: The Messengers of Health and Disease. Current neuropharmacology. 2017; 15(1):157-165.
160. Corrado C, Raimondo S, Chiesi A, Ciccia F, De Leo G and Alessandro R. Exosomes as intercellular signaling organelles involved in health and disease: basic science and clinical applications. International journal of molecular sciences. 2013; 14(3):5338-5366.
161. Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, Ricciardi-Castagnoli P, Raposo G and Amigorena S. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. Nature medicine. 1998; 4(5):594-600.
162. Kim JW, Wieckowski E, Taylor DD, Reichert TE, Watkins S and Whiteside TL. Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes. Clinical cancer research : an official journal of the American Association for Cancer Research. 2005; 11(3):1010-1020.
163. Frohlich D, Kuo WP, Fruhbeis C, Sun JJ, Zehendner CM, Luhmann HJ, Pinto S, Toedling J, Trotter J and Kramer-Albers EM. Multifaceted effects of oligodendroglial exosomes on neurons: impact on neuronal firing rate, signal transduction and gene regulation. Philosophical transactions of the Royal Society of London Series B, Biological sciences. 2014; 369(1652).
164. Masyuk AI, Masyuk TV and Larusso NF. Exosomes in the pathogenesis, diagnostics and therapeutics of liver diseases. Journal of hepatology. 2013; 59(3):621-625.
165. Vella LJ, Sharples RA, Nisbet RM, Cappai R and Hill AF. The role of exosomes in the processing of proteins associated with neurodegenerative diseases. European biophysics journal : EBJ. 2008; 37(3):323-332.
166. Hannafon BN and Ding WQ. Intercellular communication by exosome-derived microRNAs in cancer. International journal of molecular sciences. 2013; 14(7):14240-14269.
167. Hood JL, San RS and Wickline SA. Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis. Cancer research. 2011; 71(11):3792-3801.
168. Hood JL, Pan H, Lanza GM and Wickline SA. Paracrine induction of endothelium by tumor exosomes. Laboratory investigation; a journal of technical methods and pathology. 2009; 89(11):1317-1328.
169. Gu H, Ji R, Zhang X, Wang M, Zhu W, Qian H, Chen Y, Jiang P and Xu W. Exosomes derived from human mesenchymal stem cells promote gastric cancer

cell growth and migration via the activation of the Akt pathway. Molecular medicine reports. 2016; 14(4):3452-3458.

170. Manterola L, Guruceaga E, Gallego Perez-Larraya J, Gonzalez-Huarriz M, Jauregui P, Tejada S, Diez-Valle R, Segura V, Sampron N, Barrena C, Ruiz I, Agirre A, Ayuso A, Rodriguez J, Gonzalez A, Xipell E, et al. A small noncoding RNA signature found in exosomes of GBM patient serum as a diagnostic tool. Neuro Oncol. 2014; 16(4):520-527.
171. Otake K, Kamiguchi H and Hirozane Y. Identification of biomarkers for amyotrophic lateral sclerosis by comprehensive analysis of exosomal mRNAs in human cerebrospinal fluid. BMC medical genomics. 2019; 12(1):7.
172. Overbye A, Skotland T, Koehler CJ, Thiede B, Seierstad T, Berge V, Sandvig K and Llorente A. Identification of prostate cancer biomarkers in urinary exosomes. Oncotarget. 2015; 6(30):30357-30376.
173. Machida T, Tomofuji T, Maruyama T, Yoneda T, Ekuni D, Azuma T, Miyai H, Mizuno H, Kato H, Tsutsumi K, Uchida D, Takaki A, Okada H and Morita M. miR1246 and miR4644 in salivary exosome as potential biomarkers for pancreatobiliary tract cancer. Oncology reports. 2016; 36(4):2375-2381.
174. Naslund TI, Paquin-Proulx D, Paredes PT, Vallhov H, Sandberg JK and Gabrielsson S. Exosomes from breast milk inhibit HIV-1 infection of dendritic cells and subsequent viral transfer to CD4+ T cells. AIDS (London, England). 2014; 28(2):171-180.
175. Madison MN, Roller RJ and Okeoma CM. Human semen contains exosomes with potent anti-HIV-1 activity. Retrovirology. 2014; 11:102.
176. Lasser C, O'Neil SE, Ekerljung L, Ekstrom K, Sjostrand M and Lotvall J. RNA-containing exosomes in human nasal secretions. American journal of rhinology & allergy. 2011; 25(2):89-93.
177. Ebert B and Rai AJ. Isolation and Characterization of Amniotic Fluid-Derived Extracellular Vesicles for Biomarker Discovery. Methods in molecular biology (Clifton, NJ). 2019; 1885:287-294.
178. Properzi F, Logozzi M and Fais S. Exosomes: the future of biomarkers in medicine. Biomarkers in medicine. 2013; 7(5):769-778.
179. Taylor DD and Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecologic oncology. 2008; 110(1):13-21.
180. Lan F, Qing Q, Pan Q, Hu M, Yu H and Yue X. Serum exosomal miR-301a as a potential diagnostic and prognostic biomarker for human glioma. Cellular oncology (Dordrecht). 2018; 41(1):25-33.
181. Tian Y, Li S, Song J, Ji T, Zhu M, Anderson GJ, Wei J and Nie G. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. Biomaterials. 2014; 35(7):2383-2390.
182. Yang T, Martin P, Fogarty B, Brown A, Schurman K, Phipps R, Yin VP, Lockman P and Bai S. Exosome delivered anticancer drugs across the blood-brain barrier for brain cancer therapy in Danio rerio. Pharmaceutical research. 2015; 32(6):2003-2014.

183. Escudier B, Dorval T, Chaput N, Andre F, Caby MP, Novault S, Flament C, Leboulaire C, Borg C, Amigorena S, Boccaccio C, Bonnerot C, Dhellin O, Movassagh M, Piperno S, Robert C, et al. Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of thefirst phase I clinical trial. Journal of translational medicine. 2005; 3(1):10.
184. Wahlgren J, De LKT, Brisslert M, Vaziri Sani F, Telemo E, Sunnerhagen P and Valadi H. Plasma exosomes can deliver exogenous short interfering RNA to monocytes and lymphocytes. Nucleic acids research. 2012; 40(17):e130.
185. Sun Y, Luo ZM, Guo XM, Su DF and Liu X. An updated role of microRNA-124 in central nervous system disorders: a review. Frontiers in cellular neuroscience. 2015; 9:193.
186. Yang J, Zhang X, Chen X, Wang L and Yang G. Exosome Mediated Delivery of miR-124 Promotes Neurogenesis after Ischemia. Molecular therapy Nucleic acids. 2017; 7:278-287.
187. Mansouri A, Karamchandani J and Das S. (2017). Molecular Genetics of Secondary Glioblastoma. In: De Vleeschouwer S, ed. Glioblastoma. (Brisbane (AU): Codon Publications Copyright: The Authors.).
188. Das KK and Kumar R. (2017). Pediatric Glioblastoma. In: De Vleeschouwer S, ed. Glioblastoma. (Brisbane (AU): Codon Publications Copyright: The Authors.).
189. Tamimi AF and Juweid M. (2017). Epidemiology and Outcome of Glioblastoma. In: De Vleeschouwer S, ed. Glioblastoma. (Brisbane (AU): Codon Publications Copyright: The Authors.).
190. Ostrom QT, Gittleman H, Farah P, Ondracek A, Chen Y, Wolinsky Y, Stroup NE, Kruchko C and Barnholtz-Sloan JS. CBTRUS statistical report: Primary brain and central nervous system tumors diagnosed in the United States in 2006-2010. Neuro Oncol. 2013; 15 Suppl 2:ii1-56.
191. Guerreiro Stucklin AS, Ramaswamy V, Daniels C and Taylor MD. Review of molecular classification and treatment implications of pediatric brain tumors. Current opinion in pediatrics. 2018; 30(1):3-9.
192. Zhang J, Walsh MF, Wu G, Edmonson MN, Gruber TA, Easton J, Hedges D, Ma X, Zhou X, Yergeau DA, Wilkinson MR, Vadodaria B, Chen X, McGee RB, Hines-Dowell S, Nuccio R, et al. Germline Mutations in Predisposition Genes in Pediatric Cancer. The New England journal of medicine. 2015; 373(24):2336-2346.
193. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW and Kleihues P. The 2007 WHO classification of tumours of the central nervous system. Acta neuropathologica. 2007; 114(2):97-109.
194. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, Ohgaki H, Wiestler OD, Kleihues P and Ellison DW. The 2016 World Health Organization classification of tumors of the central nervous system: A summary. Acta neuropathologica. 2015:1-18.
195. Chiang JC and Ellison DW. Molecular pathology of paediatric central nervous system tumours. The Journal of pathology. 2017; 241(2):159-172.
196. Humphrey PA, Wong AJ, Vogelstein B, Zalutsky MR, Fuller GN, Archer GE, Friedman HS, Kwatra MM, Bigner SH and Bigner DD. Anti-synthetic peptide

antibody reacting at the fusion junction of deletion-mutant epidermal growth factor receptors in human glioblastoma. Proceedings of the National Academy of Sciences of the United States of America. 1990; 87(11):4207-4211.
197. Nicholas MK, Lukas RV, Jafri NF, Faoro L and Salgia R. Epidermal growth factor receptor - mediated signal transduction in the development and therapy of gliomas. Clinical cancer research : an official journal of the American Association for Cancer Research. 2006; 12(24):7261-7270.
198. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature. 2008; 455(7216):1061-1068.
199. Rajasekhar VK, Viale A, Socci ND, Wiedmann M, Hu X and Holland EC. Oncogenic Ras and Akt signaling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes. Molecular cell. 2003; 12(4):889-901.
200. Mao H, Lebrun DG, Yang J, Zhu VF and Li M. Deregulated signaling pathways in glioblastoma multiforme: molecular mechanisms and therapeutic targets. Cancer investigation. 2012; 30(1):48-56.
201. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding L, Golub T, Mesirov JP, Alexe G, Lawrence M, O'Kelly M, Tamayo P, Weir BA, Gabriel S, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer cell. 2010; 17(1):98-110.
202. Sturm D, Witt H, Hovestadt V, Khuong-Quang DA, Jones DT, Konermann C, Pfaff E, Tonjes M, Sill M, Bender S, Kool M, Zapatka M, Becker N, Zucknick M, Hielscher T, Liu XY, et al. Hotspot mutations in H3F3A and IDH1 define distinct epigenetic and biological subgroups of glioblastoma. Cancer cell. 2012; 22(4):425-437.
203. Paugh BS, Qu C, Jones C, Liu Z, Adamowicz-Brice M, Zhang J, Bax DA, Coyle B, Barrow J, Hargrave D, Lowe J, Gajjar A, Zhao W, Broniscer A, Ellison DW, Grundy RG, et al. Integrated molecular genetic profiling of pediatric high-grade gliomas reveals key differences with the adult disease. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2010; 28(18):3061-3068.
204. Schwartzentruber J, Korshunov A, Liu XY, Jones DT, Pfaff E, Jacob K, Sturm D, Fontebasso AM, Quang DA, Tonjes M, Hovestadt V, Albrecht S, Kool M, Nantel A, Konermann C, Lindroth A, et al. Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. Nature. 2012; 482(7384):226-231.
205. Silber JR, Mueller BA, Ewers TG and Berger MS. Comparison of O6-methylguanine-DNA methyltransferase activity in brain tumors and adjacent normal brain. Cancer research. 1993; 53(14):3416-3420.
206. Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, Baylin SB and Herman JG. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. The New England journal of medicine. 2000; 343(19):1350-1354.

207. Hegi ME, Liu L, Herman JG, Stupp R, Wick W, Weller M, Mehta MP and Gilbert MR. Correlation of O6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008; 26(25):4189-4199.
208. Malmstrom A, Gronberg BH, Marosi C, Stupp R, Frappaz D, Schultz H, Abacioglu U, Tavelin B, Lhermitte B, Hegi ME, Rosell J and Henriksson R. Temozolomide versus standard 6-week radiotherapy versus hypofractionated radiotherapy in patients older than 60 years with glioblastoma: the Nordic randomised, phase 3 trial. The Lancet Oncology. 2012; 13(9):916-926.
209. Moller HG, Rasmussen AP, Andersen HH, Johnsen KB, Henriksen M and Duroux M. A systematic review of microRNA in glioblastoma multiforme: micro-modulators in the mesenchymal mode of migration and invasion. Molecular neurobiology. 2013; 47(1):131-144.
210. Nawaz Z, Patil V, Thinagararjan S, Rao SA, Hegde AS, Arivazhagan A, Santosh V and Somasundaram K. Impact of somatic copy number alterations on the glioblastoma miRNome: miR-4484 is a genomically deleted tumour suppressor. Molecular oncology. 2017; 11(8):927-944.
211. Lopez-Bertoni H, Lal B, Li A, Caplan M, Guerrero-Cazares H, Eberhart CG, Quinones-Hinojosa A, Glas M, Scheffler B, Laterra J and Li Y. DNMT-dependent suppression of microRNA regulates the induction of GBM tumor-propagating phenotype by Oct4 and Sox2. Oncogene. 2015; 34(30):3994-4004.
212. Li Y, Guessous F, Zhang Y, Dipierro C, Kefas B, Johnson E, Marcinkiewicz L, Jiang J, Yang Y, Schmittgen TD, Lopes B, Schiff D, Purow B and Abounader R. MicroRNA-34a inhibits glioblastoma growth by targeting multiple oncogenes. Cancer research. 2009; 69(19):7569-7576.
213. Wang XR, Luo H, Li HL, Cao L, Wang XF, Yan W, Wang YY, Zhang JX, Jiang T, Kang CS, Liu N and You YP. Overexpressed let-7a inhibits glioma cell malignancy by directly targeting K-ras, independently of PTEN. Neuro Oncol. 2013; 15(11):1491-1501.
214. Mathew LK, Skuli N, Mucaj V, Lee SS, Zinn PO, Sathyan P, Imtiyaz HZ, Zhang Z, Davuluri RV, Rao S, Venneti S, Lal P, Lathia JD, Rich JN, Keith B, Minn AJ, et al. miR-218 opposes a critical RTK-HIF pathway in mesenchymal glioblastoma. Proceedings of the National Academy of Sciences of the United States of America. 2014; 111(1):291-296.
215. Rao SA, Arimappamagan A, Pandey P, Santosh V, Hegde AS, Chandramouli BA and Somasundaram K. miR-219-5p inhibits receptor tyrosine kinase pathway by targeting EGFR in glioblastoma. PloS one. 2013; 8(5):e63164.
216. Li X, Liu Y, Granberg KJ, Wang Q, Moore LM, Ji P, Gumin J, Sulman EP, Calin GA, Haapasalo H, Nykter M, Shmulevich I, Fuller GN, Lang FF and Zhang W. Two mature products of MIR-491 coordinate to suppress key cancer hallmarks in glioblastoma. Oncogene. 2015; 34(13):1619-1628.
217. Zhou X, Ren Y, Moore L, Mei M, You Y, Xu P, Wang B, Wang G, Jia Z, Pu P, Zhang W and Kang C. Downregulation of miR-21 inhibits EGFR pathway and suppresses the growth of human glioblastoma cells independent of PTEN status.

Laboratory investigation; a journal of technical methods and pathology. 2010; 90(2):144-155.

218. Huse JT, Brennan C, Hambardzumyan D, Wee B, Pena J, Rouhanifard SH, Sohn-Lee C, le Sage C, Agami R, Tuschl T and Holland EC. The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo. Genes & development. 2009; 23(11):1327-1337.
219. Cheng ZX, Yin WB and Wang ZY. MicroRNA-132 induces temozolomide resistance and promotes the formation of cancer stem cell phenotypes by targeting tumor suppressor candidate 3 in glioblastoma. International journal of molecular medicine. 2017; 40(5):1307-1314.
220. Wei J, Qi X, Zhan Q, Zhou D, Yan Q, Wang Y, Mo L, Wan Y, Xie D, Xie J and Yang S. miR-20a mediates temozolomide-resistance in glioblastoma cells via negatively regulating LRIG1 expression. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2015; 71:112-118.
221. Zhu D, Tu M, Zeng B, Cai L, Zheng W, Su Z and Yu Z. Up-regulation of miR-497 confers resistance to temozolomide in human glioma cells by targeting mTOR/Bcl-2. Cancer medicine. 2017; 6(2):452-462.
222. Chen W, Yu Q, Chen B, Lu X and Li Q. The prognostic value of a seven-microRNA classifier as a novel biomarker for the prediction and detection of recurrence in glioma patients. Oncotarget. 2016; 7(33):53392-53413.
223. Bachoo RM, Maher EA, Ligon KL, Sharpless NE, Chan SS, You MJ, Tang Y, DeFrances J, Stover E, Weissleder R, Rowitch DH, Louis DN and DePinho RA. Epidermal growth factor receptor and Ink4a/Arf: convergent mechanisms governing terminal differentiation and transformation along the neural stem cell to astrocyte axis. Cancer cell. 2002; 1(3):269-277.
224. Lindberg N, Kastemar M, Olofsson T, Smits A and Uhrbom L. Oligodendrocyte progenitor cells can act as cell of origin for experimental glioma. Oncogene. 2009; 28(23):2266-2275.
225. Liu C, Sage JC, Miller MR, Verhaak RG, Hippenmeyer S, Vogel H, Foreman O, Bronson RT, Nishiyama A, Luo L and Zong H. Mosaic analysis with double markers reveals tumor cell of origin in glioma. Cell. 2011; 146(2):209-221.
226. Hemmati HD, Nakano I, Lazareff JA, Masterman-Smith M, Geschwind DH, Bronner-Fraser M and Kornblum HI. Cancerous stem cells can arise from pediatric brain tumors. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100(25):15178-15183.
227. Suva ML, Rheinbay E, Gillespie SM, Patel AP, Wakimoto H, Rabkin SD, Riggi N, Chi AS, Cahill DP, Nahed BV, Curry WT, Martuza RL, Rivera MN, Rossetti N, Kasif S, Beik S, et al. Reconstructing and reprogramming the tumor-propagating potential of glioblastoma stem-like cells. Cell. 2014; 157(3):580-594.
228. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A and Weinberg RA. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nature genetics. 2008; 40(5):499-507.
229. Kim J, Woo AJ, Chu J, Snow JW, Fujiwara Y, Kim CG, Cantor AB and Orkin SH. A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. Cell. 2010; 143(2):313-324.

230. Jin X, Jin X, Jung JE, Beck S and Kim H. Cell surface Nestin is a biomarker for glioma stem cells. Biochemical and biophysical research communications. 2013; 433(4):496-501.
231. Satelli A and Li S. Vimentin in cancer and its potential as a molecular target for cancer therapy. Cellular and molecular life sciences : CMLS. 2011; 68(18):3033-3046.
232. Chen J, Li Y, Yu TS, McKay RM, Burns DK, Kernie SG and Parada LF. A restricted cell population propagates glioblastoma growth after chemotherapy. Nature. 2012; 488(7412):522-526.
233. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD and Rich JN. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature. 2006; 444(7120):756-760.
234. Quezada C, Torres A, Niechi I, Uribe D, Contreras-Duarte S, Toledo F, San Martin R, Gutierrez J and Sobrevia L. Role of extracellular vesicles in glioma progression. Molecular aspects of medicine. 2018; 60:38-51.
235. Gourlay J, Morokoff AP, Luwor RB, Zhu HJ, Kaye AH and Stylli SS. The emergent role of exosomes in glioma. Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia. 2017; 35:13-23.
236. Kucharzewska P, Christianson HC, Welch JE, Svensson KJ, Fredlund E, Ringner M, Morgelin M, Bourseau-Guilmain E, Bengzon J and Belting M. Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110(18):7312-7317.
237. Wei Z, Batagov AO, Schinelli S, Wang J, Wang Y, El Fatimy R, Rabinovsky R, Balaj L, Chen CC, Hochberg F, Carter B, Breakefield XO and Krichevsky AM. Coding and noncoding landscape of extracellular RNA released by human glioma stem cells. Nature communications. 2017; 8(1):1145.
238. Godlewski J, Ferrer-Luna R, Rooj AK, Mineo M, Ricklefs F, Takeda YS, Nowicki MO, Salinska E, Nakano I, Lee H, Weissleder R, Beroukhim R, Chiocca EA and Bronisz A. MicroRNA Signatures and Molecular Subtypes of Glioblastoma: The Role of Extracellular Transfer. Stem cell reports. 2017; 8(6):1497-1505.
239. van der Vos KE, Abels ER, Zhang X, Lai C, Carrizosa E, Oakley D, Prabhakar S, Mardini O, Crommentuijn MH and Skog J. Directly visualized glioblastoma-derived extracellular vesicles transfer RNA to microglia/macrophages in the brain. Neuro-oncology. 2016; 18(1):58-69.
240. Li CC, Eaton SA, Young PE, Lee M, Shuttleworth R, Humphreys DT, Grau GE, Combes V, Bebawy M, Gong J, Brammah S, Buckland ME and Suter CM. Glioma microvesicles carry selectively packaged coding and non-coding RNAs which alter gene expression in recipient cells. RNA biology. 2013; 10(8):1333-1344.
241. Andre-Gregoire G and Gavard J. Spitting out the demons: Extracellular vesicles in glioblastoma. Cell adhesion & migration. 2017; 11(2):164-172.

242. Santiago-Dieppa DR, Steinberg J, Gonda D, Cheung VJ, Carter BS and Chen CC. Extracellular vesicles as a platform for 'liquid biopsy' in glioblastoma patients. Expert review of molecular diagnostics. 2014; 14(7):819-825.
243. Garcia-Romero N, Carrion-Navarro J, Esteban-Rubio S, Lazaro-Ibanez E, Peris-Celda M, Alonso MM, Guzman-De-Villoria J, Fernandez-Carballal C, de Mendivil AO, Garcia-Duque S, Escobedo-Lucea C, Prat-Acin R, Belda-Iniesta C and Ayuso-Sacido A. DNA sequences within glioma-derived extracellular vesicles can cross the intact blood-brain barrier and be detected in peripheral blood of patients. Oncotarget. 2017; 8(1):1416-1428.
244. Saadatpour L, Fadaee E, Fadaei S, Nassiri Mansour R, Mohammadi M, Mousavi SM, Goodarzi M, Verdi J and Mirzaei H. Glioblastoma: exosome and microRNA as novel diagnosis biomarkers. Cancer gene therapy. 2016; 23(12):415-418.
245. Noerholm M, Balaj L, Limperg T, Salehi A, Zhu LD, Hochberg FH, Breakefield XO, Carter BS and Skog J. RNA expression patterns in serum microvesicles from patients with glioblastoma multiforme and controls. BMC cancer. 2012; 12:22.
246. Jayaram S, Gupta MK, Polisetty RV, Cho WC and Sirdeshmukh R. Towards developing biomarkers for glioblastoma multiforme: a proteomics view. Expert review of proteomics. 2014; 11(5):621-639.
247. Garnier D, Jabado N and Rak J. Extracellular vesicles as prospective carriers of oncogenic protein signatures in adult and paediatric brain tumours. Proteomics. 2013; 13(10-11):1595-1607.
248. Masoudi MS, Mehrabian E and Mirzaei H. MiR-21: A key player in glioblastoma pathogenesis. Journal of cellular biochemistry. 2018; 119(2):1285-1290.
249. Shi R, Wang PY, Li XY, Chen JX, Li Y, Zhang XZ, Zhang CG, Jiang T, Li WB, Ding W and Cheng SJ. Exosomal levels of miRNA-21 from cerebrospinal fluids associated with poor prognosis and tumor recurrence of glioma patients. Oncotarget. 2015; 6(29):26971-26981.
250. Shao H, Chung J, Lee K, Balaj L, Min C, Carter BS, Hochberg FH, Breakefield XO, Lee H and Weissleder R. Chip-based analysis of exosomal mRNA mediating drug resistance in glioblastoma. Nature communications. 2015; 6:6999.
251. Katakowski M and Chopp M. Exosomes as Tools to Suppress Primary Brain Tumor. Cellular and molecular neurobiology. 2016; 36(3):343-352.
252. Rufino-Ramos D, Albuquerque PR, Carmona V, Perfeito R, Nobre RJ and Pereira de Almeida L. Extracellular vesicles: Novel promising delivery systems for therapy of brain diseases. Journal of controlled release : official journal of the Controlled Release Society. 2017; 262:247-258.
253. Sharif S, Ghahremani MH and Soleimani M. Delivery of Exogenous miR-124 to Glioblastoma Multiform Cells by Wharton's Jelly Mesenchymal Stem Cells Decreases Cell Proliferation and Migration, and Confers Chemosensitivity. Stem cell reviews. 2018; 14(2):236-246.
254. Lang FM, Hossain A, Gumin J, Momin EN, Shimizu Y, Ledbetter D, Shahar T, Yamashita S, Parker Kerrigan B, Fueyo J, Sawaya R and Lang FF.

Mesenchymal stem cells as natural biofactories for exosomes carrying miR-124a in the treatment of gliomas. Neuro Oncol. 2018; 20(3):380-390.

255. Garnier D, Meehan B, Kislinger T, Daniel P, Sinha A, Abdulkarim B, Nakano I and Rak J. Divergent evolution of temozolomide resistance in glioblastoma stem cells is reflected in extracellular vesicles and coupled with radiosensitization. Neuro Oncol. 2018; 20(2):236-248.
256. Yang JK, Yang JP, Tong J, Jing SY, Fan B, Wang F, Sun GZ and Jiao BH. Exosomal miR-221 targets DNM3 to induce tumor progression and temozolomide resistance in glioma. J Neurooncol. 2017; 131(2):255-265.
257. Zeng AL, Yan W, Liu YW, Wang Z, Hu Q, Nie E, Zhou X, Li R, Wang XF, Jiang T and You YP. Tumour exosomes from cells harbouring PTPRZ1-MET fusion contribute to a malignant phenotype and temozolomide chemoresistance in glioblastoma. Oncogene. 2017; 36(38):5369-5381.
258. Davis ME. Glioblastoma: Overview of Disease and Treatment. Clinical journal of oncology nursing. 2016; 20(5 Suppl):S2-8.
259. Lee SY. Temozolomide resistance in glioblastoma multiforme. Genes & diseases. 2016; 3(3):198-210.
260. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. The New England journal of medicine. 2005; 352(10):987-996.
261. Sposto R, Ertel IJ, Jenkin RD, Boesel CP, Venes JL, Ortega JA, Evans AE, Wara W and Hammond D. The effectiveness of chemotherapy for treatment of high grade astrocytoma in children: results of a randomized trial. A report from the Childrens Cancer Study Group. J Neurooncol. 1989; 7(2):165-177.
262. Cohen KJ, Pollack IF, Zhou T, Buxton A, Holmes EJ, Burger PC, Brat DJ, Rosenblum MK, Hamilton RL, Lavey RS and Heideman RL. Temozolomide in the treatment of high-grade gliomas in children: a report from the Children's Oncology Group. Neuro Oncol. 2011; 13(3):317-323.
263. Vanan MI and Eisenstat DD. Management of high-grade gliomas in the pediatric patient: Past, present, and future. Neuro-oncology practice. 2014; 1(4):145-157.
264. Zhang D, Liu X, Fan C and Chen J. Novel drugs in pediatric gliomas. Oncology letters. 2017; 13(5):2881-2885.
265. Sun Y, Pollard S, Conti L, Toselli M, Biella G, Parkin G, Willatt L, Falk A, Cattaneo E and Smith A. Long-term tripotent differentiation capacity of human neural stem (NS) cells in adherent culture. Molecular and cellular neurosciences. 2008; 38(2):245-258.
266. Gnyszka A, Jastrzebski Z and Flis S. DNA methyltransferase inhibitors and their emerging role in epigenetic therapy of cancer. Anticancer research. 2013; 33(8):2989-2996.
267. Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, Smith I, Tothova Z, Wilen C, Orchard R, Virgin HW, Listgarten J and Root DE. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. Nature biotechnology. 2016; 34(2):184-191.

268. Thery C, Amigorena S, Raposo G and Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Current protocols in cell biology. 2006; Chapter 3:Unit 3.22.
269. Cvjetkovic A, Lotvall J and Lasser C. The influence of rotor type and centrifugation time on the yield and purity of extracellular vesicles. Journal of extracellular vesicles. 2014; 3.
270. Vlachos IS, Zagganas K, Paraskevopoulou MD, Georgakilas G, Karagkouni D, Vergoulis T, Dalamagas T and Hatzigeorgiou AG. DIANA-miRPath v3. 0: deciphering microRNA function with experimental support. Nucleic acids research. 2015:gkv403.
271. Kohl M, Wiese S and Warscheid B. Cytoscape: software for visualization and analysis of biological networks. Data Mining in Proteomics: From Standards to Applications. 2011:291-303.
272. Huang da W, Sherman BT and Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols. 2009; 4(1):44-57.
273. Yang C, Wang C, Chen X, Chen S, Zhang Y, Zhi F, Wang J, Li L, Zhou X, Li N, Pan H, Zhang J, Zen K, Zhang CY and Zhang C. Identification of seven serum microRNAs from a genome-wide serum microRNA expression profile as potential noninvasive biomarkers for malignant astrocytomas. International journal of cancer. 2013; 132(1):116-127.
274. Lan J, Xue Y, Chen H, Zhao S, Wu Z, Fang J, Han C and Lou M. Hypoxia-induced miR-497 decreases glioma cell sensitivity to TMZ by inhibiting apoptosis. FEBS letters. 2014; 588(18):3333-3339.
275. Yelamanchili SV, Morsey B, Harrison EB, Rennard DA, Emanuel K, Thapa I, Bastola DR and Fox HS. The evolutionary young miR-1290 favors mitotic exit and differentiation of human neural progenitors through altering the cell cycle proteins. Cell death & disease. 2014; 5:e982.
276. Endersby R and Baker SJ. PTEN signaling in brain: neuropathology and tumorigenesis. Oncogene. 2008; 27(41):5416-5430.
277. Cui Q, Yang S, Ye P, Tian E, Sun G, Zhou J, Sun G, Liu X, Chen C, Murai K, Zhao C, Azizian KT, Yang L, Warden C, Wu X, D'Apuzzo M, et al. Downregulation of TLX induces TET3 expression and inhibits glioblastoma stem cell self-renewal and tumorigenesis. Nature communications. 2016; 7:10637.
278. Feng F, Kuai D, Wang H, Li T, Miao W, Liu Y and Fan Y. Reduced expression of microRNA-497 is associated with greater angiogenesis and poor prognosis in human gliomas. Human pathology. 2016; 58:47-53.
279. Liu J, Li Y, Zou Y, Zhang J, An J, Guo J, Ma M and Dai D. MicroRNA-497 acts as a tumor suppressor in gastric cancer and is downregulated by DNA methylation. Oncology reports. 2017; 38(1):497-505.
280. Ruan WD, Wang P, Feng S, Xue Y and Zhang B. MicroRNA-497 inhibits cell proliferation, migration, and invasion by targeting AMOT in human osteosarcoma cells. OncoTargets and therapy. 2016; 9:303-313.
281. Chen Y, Kuang D, Zhao X, Chen D, Wang X, Yang Q, Wan J, Zhu Y, Wang Y, Zhang S, Wang Y, Tang Q, Masuzawa M, Wang G and Duan Y. miR-497-5p

inhibits cell proliferation and invasion by targeting KCa3.1 in angiosarcoma. Oncotarget. 2016; 7(36):58148-58161.

282. Safe S and Abdelrahim M. Sp transcription factor family and its role in cancer. European journal of cancer (Oxford, England : 1990). 2005; 41(16):2438-2448.
283. Adamski V, Hempelmann A, Fluh C, Lucius R, Synowitz M, Hattermann K and Held-Feindt J. Dormant glioblastoma cells acquire stem cell characteristics and are differentially affected by Temozolomide and AT101 treatment. Oncotarget. 2017; 8(64):108064-108078.
284. Sana J, Busek P, Fadrus P, Besse A, Radova L, Vecera M, Reguli S, Stollinova Sromova L, Hilser M, Lipina R, Lakomy R, Kren L, Smrcka M, Sedo A and Slaby O. Identification of microRNAs differentially expressed in glioblastoma stem-like cells and their association with patient survival. Scientific reports. 2018; 8(1):2836.