Epigenetic Modulation in Alzheimer's disease: Function of Hippocampal microRNAs

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Declaration

I herewith declare that I have prepared the dissertation 'Epigenetic modulation in Alzheimer's disease: function of hippocampal microRNAs' entirely by myself with no other aids or sources than quoted.

29.5.2015, Göttingen

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List of abbreviations

3' UTR	3' untranslated region
A-beta	Amyloid beta
ACC	Anterior cingulate cortex
AD	Alzheimer's disease
Ago2	Argonaute
ApoE4	Apolipoprotein E4
APP	Amyloid precursor proteins
APS	Ammonium persulfate
CA	Cornu ammonis
cAMP	Cyclic adenosine monophosphate
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CREB	cAMP response element-binding protein
CSF	Cerebrospianl fluid
DG	Dentate gyrus
DGCR8	DiGeorge Syndrome Critical Region 8
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicles
F	Fusion protein
FCS	Fetal Calf Serum
FD	Frontotemporal Dementia
FGFR1	Fibroblast growth factor receptor 1
FTLD-17	Frontotemporal Lobar Dementia linked to chromosome 17
G418	Geneticin
GTP	Guanosine-5'-triphosphate
H	Hemagglutinin protein
HM	Henry Molaison
HRP	Horseradish peroxidase conjugated antibodies
HT1080-Luc	Human fibrosarcoma cell line with luciferase activity
IEG	Immediate early genes
long-ncRNA	long non-coding RNAs
MAPK	Mitogen activated protein kinase
miRNA	Micro RNAs
miscRNA	miscellaneous RNAs
mRNA	Messenger RNA
MVBs	Multivesicular bodies
MVG	Measles virus glycoprotein
N2a	Mouse neuroblastoma cell line
NFkB	nuclear factor kappa-light-chain-enhancer of activated B cells
ncRNA	Non-coding RNAs
NGS	Next Generation Sequencing
NSC	Neural stem cells
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal component analyses
PD	Parkinson's Disease
Pen/Strep	Penicillin/Streptomycin
piwiRNA	Piwi-interacting RNA
precursor-miRNA	Pre-miRNA
pri-miRNA	Primary miRNA
PSEN	presenilin gene
qRT-PCR	Quantitative real time polymerase chain reaction

Ran	RAs-related Nuclear protein
RISC	RNA-induced silencing complex
RNAi	RNA interference
rRNA	Ribosomal RNA
RVG	Rabid virus glycoproteins
SDS	Sodium dodecyl sulphaste
siRNA	Small interfering RNAs
snoRNA	Small nucleolar RNA
snRNA	Small nuclear ribonucleic RNA
TEMED	N'N'N'-tetramethylethylene diamine
TOD	Tangle-only Dementia
tPA	Tissue plasminogen activator
TRBP	RNA-binding protein
tRNA	Transfer RNA
Zif268	Zinc-finger protein

Introduction

1.1 Memory deterioration with aging and Alzheimer's disease

Aging is a complex biological mechanism, which can be defined as the accumulation of processes that decline many physiological functions and simultaneously increase chance of mortality in animals as well as humans. Along with many other functions of the body, aging massively declines the ability to learn and remember. Neurodegeneration is the process of neuronal cell damage, which accelerate dramatically with aging and could lead to Alzheimer's disease (AD). Loss of neurons and disease manifestation are rather late events in the progression of AD when therapeutic interventions are no longer effective.

Diagnosis of AD in its asymptomatic phases provides the possibility of adequate therapeutic interventions. Hence, the needs for finding new and non-invasive methods for diagnosis of the pathology of AD are urgent. The following sections summarize the current knowledge about the molecular changes that occur in the brain and in biofluids during aging, with a focus on identifying novel and non-invasive diagnostic and therapeutic candidates.

1.1.1 Brain and memory organization

Learning and memory are two unique functions of the brain enabling the organism to acquire and retrieve an imagination of itself and its world. Learning can be described as encoding of the data while memory is the process of recalling the data. According to the time span that it can be stored, memory is subdivided into two major subtypes: short-term and long-term. 1) Short-term memory is the kind of memory that stores information temporarily, which can be recalled shortly thereafter. 2) Long-term memory requires retrieval of information from the past. This information could be from a few minutes ago or along time ago. Long-term memory itself can be divided into implicit and explicit memory. Explicit memory is dependent on consciousness for storage of information such as memory of an event or specific names. In contrast, implicit memory does not need consciousness and is present in non-mammalian vertebrates and even invertebrates. Implicit memory is also called non-declarative memory and refers to remembering skills such as walking or swimming. Explicit or declarative memory itself is divided into episodic and semantic memory (Tulvig, 1983). Episodic memory is about events, such as of places and time while semantic memory refers to the stored information about the world (Squire et al., 1993).

1.1.2 Memory storage regions in the brain

The hippocampus is a neuroanatomical structure that is part of the limbic system and is present in all mammals. It is perhaps the most studied part of the brain and it has been widley established that the hippocampus is responsible for memory, learning and spatial navigation functions. A large body of our knowledge about hippocampus and its significant role in short-term and long-term memory comes from Brenda Milner's pioneering studies on the patient Henry Molaison (known as HM, died at 2008) that suffered from severe epilepsy so that his medial temporal lobe was removed via surgery. Following surgery HM's brain was unable to convert short-term memory to new long-term memory, while he could remember older events that had occurred before the surgery very well. Milner has studied this patient for almost thirty years and thanks to her reports now we have a comprehensive knowledge about different types of memories and the role of hippocampus in memory (Scoville and Milner, 1957).

Hippocampus or hippocampal formation has subregions called dentate gyrus (DG) and cornu ammonis (CA). CA itself is divided into three subdivisions CA1, CA2, CA3. Among these subdivisions CA1 and CA3 have distinct functions. There are three important connectional pathways in the hippocampus namely the Schaffer-collateral pathway, the perforant pathway and the mossy fiber pathway. The Schaffer-collateral pathway refers to the path between CA1 and CA3. Axons of the perforant path convey sensory information from neurons in entorhinal cortex to the DG. Mossy fiber pathway refers to the DG unmyelinated axons that project to CA3. These pathways create hippocampal trisynaptic loop (Amarel et al., 2006).

The anterior cingulate cortex (ACC) is part of the limbic system and in humans is involved in attention that regulates emotion and cognition (Bush et al., 2000). One example for this function is error detection, which was first, described by the American psychologist John Ridley Stroop and subsequently is called Stroop effect (Stroop, 1935). The stroop effect is referred to a conflict-urging stimulus by reading color names, which are denoted in corresponding versus not corresponding colors. However it has been shown that ACC along with posterior cingulate cortex go under profound laminar loss in AD (Scheff and Price, 2001).

In rodents ACC plays an important role in long-term memory formation (Weible et al., 2012).

1.1.3 Biological and molecular basis of memory

Pioneering studies performed by Eric Kandel and his colleagues on *Aplysia* paved the way to have a clear understanding of memory formation and consolidation at the molecular level. These studies showed that short-term memory is formed upon the increase in the concentration of cyclic adenosine monophosphate (cAMP) in the sensory neurons, which initiates by a stimulus and neurotransmitter secretion. All these processes finally lead to increased concentration of glutamate receptor in the synaptic cleft (Brunelli et al., 1976). Also from vast amount of studies on long-term memory consolidation it came out that the formation of long-term memory requires new protein synthesis and subsequently new gene expression. An important protein expression, which is expressed and activated during long-term memory consolidation, is cAMP response element-binding protein (CREB). CREB activation occurs after persistent elevation in the amount of cAMP and mitogen activated protein kinase (MAPK) levels (Bacskai et al., 1993).

1.1.4 Genetic basis of memory formation

As mentioned above, long-term memory formation needs *de novo* protein synthesis: hence, immediate early genes (IEG) come into play. These genes get activated in response to the stimuli with cellular basis and start the transcription process for new protein synthesis. The majority of IEGs are transcription factors like c-Fos, c-Myc, c-Jun, Egr1 and zinc-finger protein Zif268 (Guzowski et al., 2002) Zif268 has a crucial role in consolidation and reconsolidation of different forms of explicit memories (Veyrac et al., 2014). There are also non-transcription factor genes like Arc and a tissue plasminogen activator (tPA) that get activated during long-term memory formation (Qian et al., 1993).

1.1.5 Impact of aging on brain gene expression

Aging is the process of getting older over time. Different biological changes are happening in an organism during aging, e.g. reduced ability to regenerate the damages or coping with stress, and consequently increased risk of diseases. The risk for cognitive diseases also drastically increases because brain tissue goes under the process of aging as well. Since the hippocampus has a significant role in memory consolidation, it is one of the first regions that will suffer in memory-associated diseases such as AD. However the major

changes happening in the hippocampus are not on the morphological level but at the gene expression level leading to some functional modifications, like changes in the synaptic properties of hippocampal cells (Burger, 2010).

1.1.6 Epigenetic mechanisms in Alzheimer's disease

The term 'epigenetic' was introduced to biology by Conrad Waddington. Nowadays epigenetic is defined as mitotically and meiotically heritable changes in gene expression without a change in the DNA sequence. DNA methylation, histone acetylation and RNA-mediated gene silencing are considered as major epigenetic modulations in eukaryotic cells. They play important roles in development and gene regulation. These mechanisms can also underlie cancer and neurodegeneration mechanisms (Klose and Bird, 2006; Jones and Baylin, 2002; Baylin and Ohm, 2006; Qureshi and Mehler, 2013).

Neurodegeneration in AD is associated with two abnormal protein aggregations: neurofibrillary tangles and the amyloid-beta (A-bet) plaques. Neurofibrillary tangles or insoluble aggregates of tau protein are one of the crucial hallmarks of AD and other neurodegenerative diseases grouped as taupathies, diseases like Niemann Pick disease (Auer et al., 1995), Frontotemporal Lobar Dementia linked to chromosome 17 (FTLD-17) (Mackenzie and Rademakers, 2007) and Tangle-only Dementia (TOD) (Yamada, 2003).

These amyloid plaques are the result of the β and γ secretases enzymatic action on amyloid precursor proteins (APP).

Besides aging, a combination of genetic and environmental factors have been considered as risk factors for sporadic form of AD. From genetic point of view, the disease can be caused by autosomal dominant mutations in the amyloid precursor protein (APP) or presenilin gene (PSEN). However these familiar forms account for only approximately 5% of the disease and the majority of AD patients suffer from the sporadic form with Apolipoprotein E (ApoE4) genotype. As previously mentioned another crucial risk factor for this disease etiology is the environment. Epigenetic mechanisms in combination with environmental factors regulate gene expression at the systems level. Epigenetic modifications are manifested at different levels of the gene expression machinery: modifications of histone proteins and DNA methylation exert a direct effect at gene-transcription levels while miRNAs influence posttranscriptional mechanisms. Aside from that, non-coding RNAs (ncRNA) play important

roles in hereditary changes of DNA-methylation in response to environmental factors. These molecules might contribute to the increased risk of sporadic AD that is reported among first grade relatives of AD patients (Malecova and Morris, 2010).

1.1.7 Non-coding RNAs

Non-coding RNAs are functional RNAs that are transcribed from DNA and as their name suggests do not encode proteins. Instead they have regulatory roles in gene expression. Non-coding RNAs include long and small non-coding RNAs. Long non-coding RNAs have over 200 nucleotides while small non-coding RNAs have usually less than 200 nucleotides. Long non-coding RNAs (long-ncRNA) have epigenetic regulatory roles in transcription, splicing and imprinting (Mercer et al. 2009). One well-known small non-coding RNAs is transfer RNA (tRNA), which is involved in protein synthesis by mediating amino acid transfer (Phizicky and Hopper 2010). Another small non-coding RNA is ribosomal RNA (rRNA) found in the ribosome and is involved in protein translation (Stults et al., 2008). Another small non-coding RNAs are small nucleolar RNA (snoRNA) which acts in the processing and modifications of other non-coding RNAs like rRNA (Holley and Topkara, 2011), small nuclear RNA (snRNA) which is involved in splicing, (Valadkhan, 2005), small interfering RNAs (siRNA) has a significant role in RNA interfering pathway and can splice mRNA in site-specific manner (Kawaji and Hayashizaki, 2008), Piwi-interacting RNA (piwiRNA) is so named because of its interaction with Piwi proteins and have post-transcriptional roles specially in germ line cells (Lin, 2007), and microRNAs (miRNA) which are very well known because of their important role in posttranscriptional gene expression modification in different organism life time, like development, growth and even diseases (Bartel 2004). In the present study the main concentration was on the miRNA profiling and expression, so the next parts deal with miRNA properties and biosynthesis.

1.1.8 MicroRNA

MicroRNA (miRNA) constitutes a class of endogenous non-coding small RNAs, which is single-stranded in its mature form and consists of 18-25 nucleotides and regulates gene expression. MiRNAs are found abundantly in plant and animal cells and hence they are well conserved through the evolution, it can be claimed that miRNAs are crucial components of the gene regulation system (Chen and Rajewsky, 2007). The function of miRNAs as gene expression regulators was described in early 2000 (Reinhart et al., 2000; Lagos-Quintana, 2001) although they were discovered few years earlier in *C. elegans* (Lee et al., 1993). Up to now, over 1800 different miRNAs have been identified in humans. The largest and most distinct number of all presently identified miRNAs is expressed in the brain, where they have been implicated in neuronal differentiation, development and synaptic function (Bartel, 2004, Motti et al., 2012). Interestingly one miRNA can regulate several different downstream targets, in line with that dysregulation of the neuronal miRNAs could have a deleterious effect on neuronal function and survival. Also many studies have shown that some miRNAs in AD brain are deregulated including miRNAs that target the expression of APP or APP processing secretases (Satoh, 2010, Cogswell et al., 2008; Herbert et al. 2008; Schonrock et al., 2010; Shioya et al., 2010; Smith et al., 2011).

1.1.9 Biogenesis of the miRNA

The canonical process of miRNA biogenesis is pictured in Fig. 1.1.1. However, there are also many miRNAs that are generated via alternative mechanisms (Miyoshi et al., 2010).
MiRNAs are either derived from intronic sequences, intergenic regions or encoded by clusters like a polycistronic transcript (Lagos-Quintana, 2001). MiRNA genes are transcribed by either RNA polymerase II or RNA polymerase III into primary miRNA transcripts (Lee et al., 2004; Cai et al., 2004; Borchert et al., 2006) primary miRNA (pri-miRNA), which is a hairpin structure, goes under nuclear processing. Nuclear processing depends on the function of the nuclear protein known as DiGeorge Syndrome Critical Region 8 (DGCR8). DGCR8 associates with the enzyme Drosha, a protein that cuts RNA to form the "Microprocessor" complex. In this complex, DGCR8 orients the catalytic RNase III domain of Drosha to liberate hairpins from pri-miRNAs by cleaving RNA about eleven nucleotides from the hairpin base (two helical RNA turns into the stem). The resulting product has a 3' hydroxyl and 5' phosphate with a two-nucleotide overhang at its 3’ end and is often termed as a precursor-miRNA (pre-miRNA). Pre-miRNAs, which still have the hairpin structure, are exported from the nucleus in a process involving the nucleocytoplasmic shuttler Exportin-5.

This protein recognizes a two-nucleotide overhang left by the RNase III enzyme Drosha at the 3' end of the pre-miRNA hairpin. Exportin-5-mediated transport to the cytoplasm is an energy-dependent process; therefore, it is associated with Guanosine-5'-triphosphate (GTP) and RAs-related Nuclear protein (Ran). In the cytoplasm, the pre-miRNA hairpin is cleaved to its mature size by Dicer (an RNase III enzyme). Dicer acts in association with the double-stranded RNA-binding protein (TRBP). The functional mature miRNA is then loaded along with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC) and then it guides RISC to silence target mRNAs by mRNA cleavage, deadenylation and translational inhibiton.

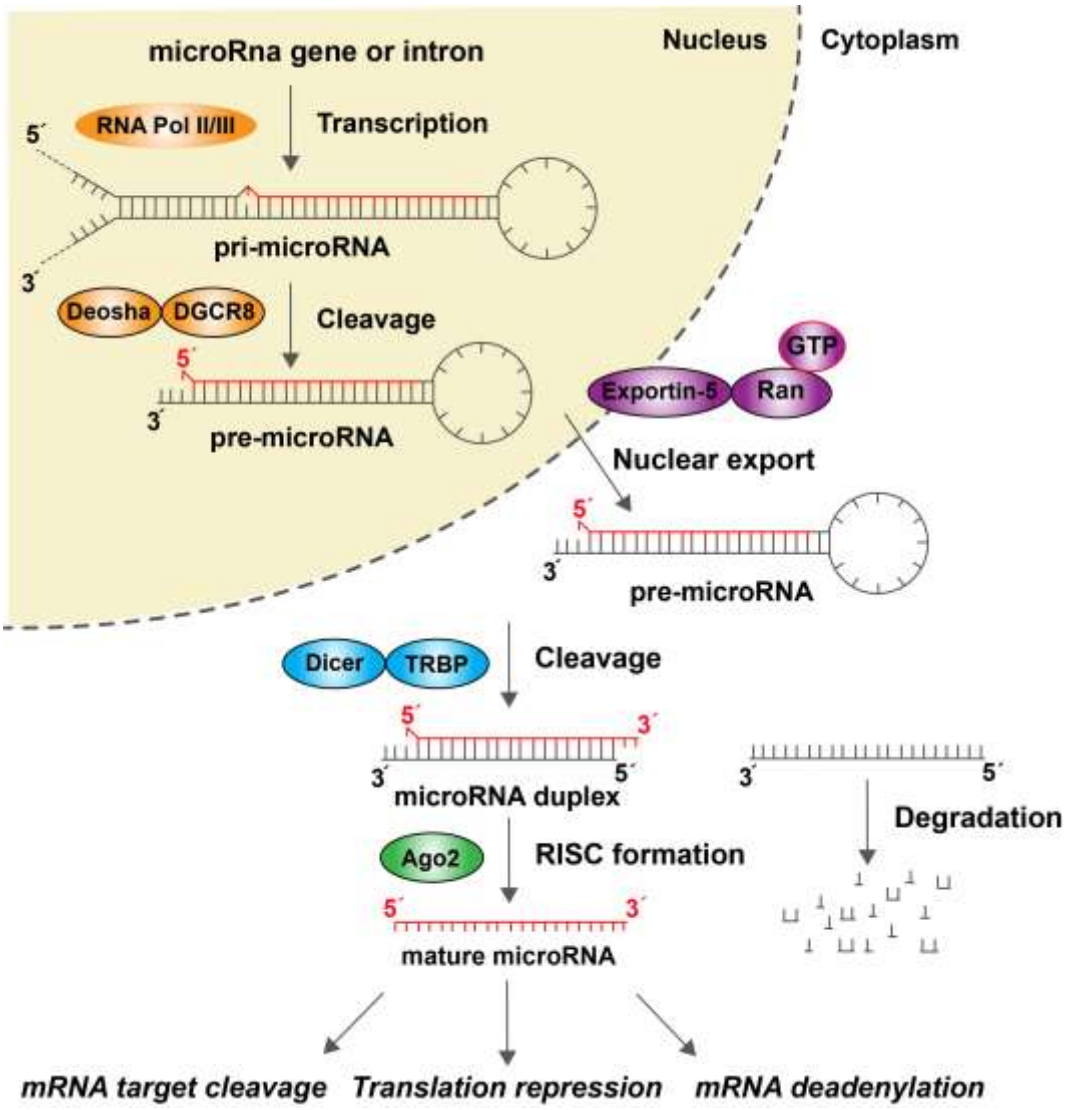

Fig. 1.1.1 Canonical pathway of biogenesis of microRNA. (adapted from: Winter et al., 2009: 229).

1.1.10 MiRNAs in the central nervous system

MiRNAs are key regulators of the posttranscriptional control of the gene. Since discovery of the miRNAs the role of the miRNAs in the central nervous system has been studied massively. Interestingly, the enrichment of miRNAs in different parts of adult brain shows distinct expression patterns, e.g. miR127, miR128, miR9 and let-7 members are highly expressed in the brain (Bak et al., 2008). It has also been revealed that different miRNAs are expressed in different neuronal stages like development, proliferation, differentiation and synaptogenesis. The role of miRNAs in neuronal development has been shown by Dicer ablation (Andersson et al., 2010). As Dicer is a key enzyme in the miRNA maturation process, targeting this enzyme can reveal the role of miRNAs at different stages of the neuronal development (Kawase-Koga et al., 2009). Besides neuronal development, neuronal proliferation and differentiation of the neural stem cells to adult neurons is regulated by miRNAs. The miRNAs studied so far include MiR9, miR124, miR134, miR137 and miR184 are examples of some studied miRNAs in this area (Zhao et al., 2009; Lagos-Quintana et al., 2002; Szulwach et al., 2010; Liu et al., 2010). Of note, miR137 and miR184 expression is controlled by epigenetic regulation linked to DNA methylation.

Synaptogenesis and neurite outgrowth are two critical processes that are crucial to memory formation and consolidation. The epigenetic role of two non-coding RNAs including miRNA 124 and piRNA-F has been already reported in the long-term memory consolidation (Landry et al., 2013). It has also been revealed that miR132 and miR212 play an important role in dendrite outgrowth and neurogenesis in the granule cells of DG in the adult hippocampus (Magill et al., 2010).

MiRNAs have also significant roles in dysfunction of the neuronal cells and neurodegeneration. MiRNAs mostly exert their role in neurodegenerative diseases by dys-regulating the disease-related proteins. MiR9 is downregulated in AD and targets three important proteins namely Fibroblast growth factor receptor 1 (FGFR1), nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) and Sirtunin1 (SIRT1) (Femminella et al., 2015). Down regulation of miR107 and miR29a/b leads to the increase at BACE1 level in AD patients (Wang et al., 2008; Hebert et al., 2008). MiR298 and miR328 show their effect by repressing BACE1 expression and A-beta accumulation (Boissonneault et al., 2009). MiR101 regulated repression of APP expression, which itself leads to A-beta accumulation (Vilardo et al., 2010). It has been shown that miR34c is expressed at high level in the hippocampus of the AD patients and AD mouse models (Zovoilis et al., 2011) the upregulation of miR34c in AD

is associated with SIRT1. Besides Amyloid beta expression regulations, miRNAs role in tau-associated mechanisms in AD has been studied. MiR146a is an example for this case upregulation of miR-146a has been reported in AD patients (Lukiw et al., 2008). MiRNAs are also involved in other neurodegenerative diseases like Frontotemporal Dementia (FD) and Parkinson's disease (PD) (Arrant and Roberson, 2014; Doxakis 2010).

1.1.11 MiRNA potential as biomarker

Biomarkers (abbreviation for biological markers) are usually defined as a measurable indicator of the pathophysiological or healthy state of an organism or pharmacological response to a treatment. Biomarkers or surrogate markers have been used to diagnose or prognose a disease, blood pressure, high temperature, antibodies, electrolyte levels and blood cell counts are known examples of biomarkers. These biomarkers are used to monitor and predict health states in individuals so that appropriate therapeutic intervention can be planned. Moreover a cell or a molecule can serve as a biomarker. In this study a biomolecule, hippocampal and peripheral blood mononuclear cells (PBMC) miRNA, is used, to explore biomarker for aging and consequently AD simply because aging is one of the highly important risk factors for sporadic form of AD. Referring to studies, miRNA deregulations, deficiencies or excesses have been associated with a variety of diseases including cancer, AD, metabolic diseases, and many others (Wiemer, 2007; Nelson and Keller, 2007; Krutzfeldt and Stoffel, 2006; Calin et al., 2002).

In different mechanisms such as mRNA degradation or protein synthesis inhibition miRNA molecules target mostly the 3'UTR (Barbato et al., 2009). The interesting fact is that the polymorphisms in the 3'UTR of the binding sites of the miRNAs can change the affinity of these molecules to the target (Wang et al., 2008). So downregulation of the genes occurs upon the upregulation of the miRNAs. There is an inverse correlation between miRNA and mRNA and protein. MiRNA abnormal expression has been shown in the pathways involved in the mechanisms of neurodegenerative diseases. On the other hand the complementarity between the miRNA and its target site on the mRNA is imperfect, so it is possible for each miRNA to regulate hundreds of potential targets (De Felice, 2014).

In line with this a reliable biomarker should have some criteria; miRNAs are to a high extent specific to each organ and diseased organ, so they have an optimal pathological sensitivity. Furthermore miRNA detection is rapid, robust, accurate and inexpensive. They are also

present in body fluids and that makes them a non-invasive or minimally invasive biomarker for pathological situations (Etheridge et al., 2011). Although the majority of miRNAs are found intracellular, remarkable number of miRNAs has been isolated from a variety of human body fluids, including plasma, urine, cerebrospinal fluid and blood serum (Weber et al., 2010; Turchinovich et al., 2011). There are ribonuclases in extracellular space and body fluids, which can degrade freely circulating RNAs, amazingly there are remarkable amount of RNAs in plasma, cerebrospinal fluid (CSF) and other extracellular space fluids so it seems that miRNAs are protected against RNase digestion. Interestingly, a certain subset of total circulating miRNAs was found to be exclusively transported within nanovesicles like exosomes while a distinct proportion of miRNAs was only present in the not-encapsulated Ago2-bound form (Arroyo et al., 2011). Aside from Ago2, existence of other RNA binding protein has been also confirmed (Wang et al., 2010). Another study also showed the existence of miRNA protein chaperones that could selectively protect miRNAs in the extracellular environment (Kosaka et al., 2010). In the present study the main focus was on isolation of small RNAs from solid tissue (brain) and PBMC (peripheral blood mononuclear cell), so the concerns about ribonuclases are not the case.

1.1.12 MiRNA detection and profiling approaches

Since miRNA discovery and revealing its role in gene expression regulation, techniques and methods, for miRNA detection and profiling, are progressing. Choosing the method for miRNA profiling depends highly on the aim of the project and also cost affords limitations. One of the major methods to detect miRNAs is quantitative real time polymerase chain reaction (qRT-PCR), which is an established method and has an acceptable sensitivity; miRNA microarray method is also a highly applied miRNA detection method with relatively lower costs in comparison to qRT-PCR but its specificity is not as good as qRT-PCR. Both methods have the disadvantage that they cannot identify novel miRNAs. Novel RNA sequencing methods like high-throughput next-generation sequencing (NGS) function highly sensitive and specific and these methods can detect novel miRNAs (Pitchard et al., 2012). This special advantage of NGS makes it a reliable method for biomarker discovery. However NGS needs bioinformatics experts to analyze the raw data from sequencer. Beside all the benefits, which NGS provides for miRNA profiling studies it has also some shortcomings for example it cannot be used for absolute quantification and its dependency on computational data analysis makes it very costly.

1.2 MiRNAs as therapeutic targets in Alzheimer's disease

Altered expression of certain miRNAs is reported in development of the neurodegenerative diseases. It has been shown that differentially expressed miRNAs like miR-29a/29b-1 and miR-9 target BACE1 mRNA in AD (Hebert et al., 2008 and Shioya et al., 2010). MiRNA 34c has been shown to be upregulated in the hippocampus of AD patients. In the corresponding mouse models (Zovoilis et al., 2011) this miRNA reduces Sirtuin1 protein level. Depletion of upregulated miRNA, which underlies the disease or pathological processes, could be a proper way to influence the disease progression. One important obstacle in targeting miRNAs is delivery of the therapeutics to the target cells. A pioneering study confirmed that exosomes contain different types of small RNAs (Valadi et al., 2007). Based on this study, experiments that focused to load the exosomes with siRNAs were initiated by Alvarez-Erviti et al. (2011). In this study to circumvent the immunogenicity, exosomes were purified from dendritic cells of the same animal and to target neural cells specifically rabid virus glycoproteins (RVG) were sorted in the exosomes. In the present study a system, which is based on sorting the measles virus glycoprotein (MVG) in the exosomes, was applied in order to pseudotype exosomes for neuronal cells. The following sections deal with the exosome biology and its properties for siRNA delivery.

1.2.1 Exosomes

Intercellular communication is a crucial hallmark of the eukaryotic cells. This communication can be either direct cell-cell communication or mediated by extracellular vesicles (EV). According to their biogenesis and function these vesicles can be divided into different groups, e.g. ectosomes, shedding vesicles, microparticles and microvesicles (Hess et al., 1999; Holme et al., 1994; György et al., 2011; Cocucci et al., 2009).

The term exosome also refers to a group of extracellular nanovesicles in eukaryotic cells. Pan and Johnstone described Exosomes for the first time as a means to dispose of unwanted material during reticulocyte maturation (Pan and Johnstone, 1983). Electron microscopic images of these nanovesicles show a distinct saucer-shape with the size of 30 to 100nm (van Niel et al., 2006). However this saucer-shape like appearance was later reported to be a consequence of sample preparation for electron microscopy (Raposo et al., 1996). Exosomes in reality have a completely rounded shape, which can be demonstrated by cryo-electron microscopy analysis, because preparation process for this kind of microscopy does not deform the original shape of the exosome (Conde-Vancells, 2008).

1.2.2 Biogenesis of the exosomes

Numerous studies have confirmed that exosomes originate from multivesicular bodies (MVBs). Exosomes get released into the extracellular milieu as a consequence of fusion of the MVB limiting membrane with the cell membrane (Fig.1.2.1). This distinguishes exosomes from ectosomes, which are produced by direct budding of the cell membrane (Valssov et al., 2012). Based on its protein composition an MVB can have two fates: it can either end up in the lysosome for degradation or merge with the cell membrane to release exosomes (Thery et al., 2006). The synthesis of the exosomes and sorting of cargo requires the function of endosomal sorting complex required for transport (ESCRT); this machinery is also needed for lysosome synthesis. The ESCRT is composed of four main protein complexes: ESCRT0, ESCRT1, ESCRT2, and ESCRT3 (Williams and Urbe, 2007; Hurley, 2008). ESCRT0, ESCRT1, ESCRT2 have important function in recognition and sorting the ubiquitinylated proteins for secretion via exosomes, while ESCRT3 is important in invagination of the cell membrane (Raiborg and Stenmark, 2009).

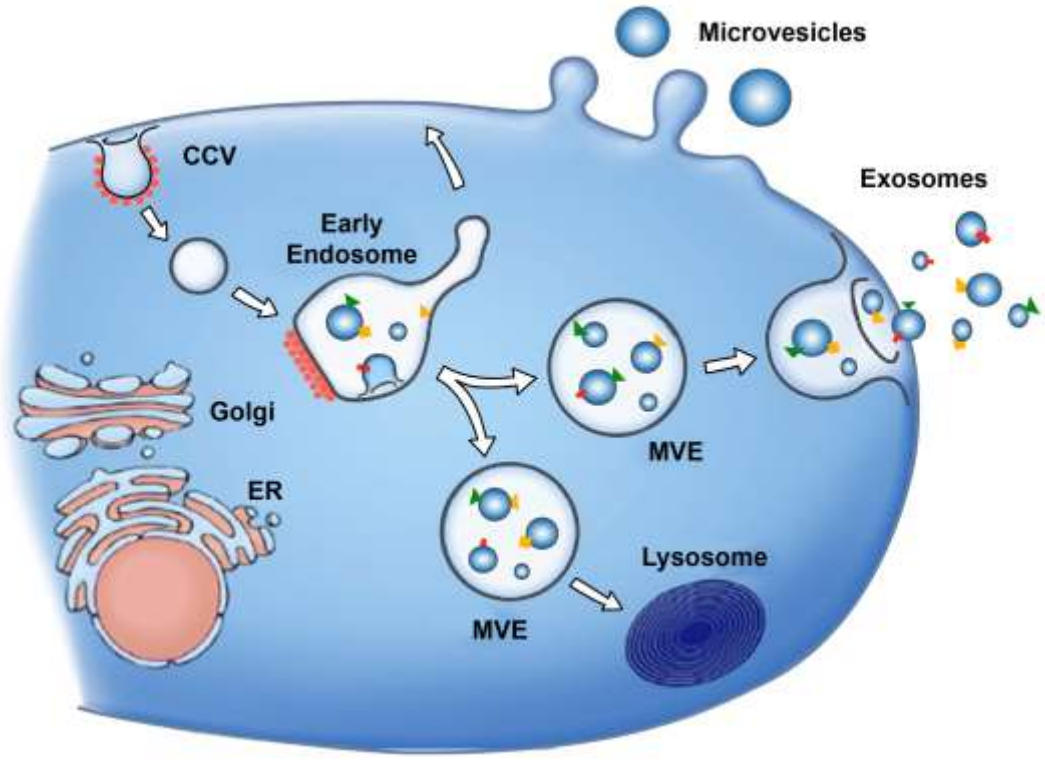

Fig. 1.2.1 Formation of MVE and release of exosomes in extracellular milieu (adapted from: Raposo and Stoorvogel, 2013: 375).

1.2.3 Composition of the exosomes

Beside the unique shape of the exosomes, their protein and lipid composition can also be regarded as exclusive properties. Proteins with MVB origins like Alix and Flotillin, heat shock proteins like hsc70 and hsc90, as well as integrins and tetraspanins e.g. CD63, CD9, CD81 and CD82 have been found in exosomes. Since exosomes have an endosomal origin another group of proteins that can be detected in them are fusion and membrane transport proteins e.g. Annexin and Flotillin. (Simons and Raposo, 2009).

The lipid compartment of the exosomes is similar to the plasma membrane of cells of their origin (Subra et al., 2007). A large proportion of the exosomal lipids are raft-lipids like ceramide, sphingolipids and glycerophospholipids (Subra et al., 2007; Trajkovic et al., 2008). The involvement of ceramide in biogenesis of exosomes has been confirmed since an ESCRT-independent pathway requires it (Trajkovic et al., 2008).

1.2.4 Function of the exosomes

Early studies, which led to the discovery of exosomes, described these vesicles as a carrier to dispose the superfluous cellular material from reticulocytes (Pan and Johnstone, 1983). Other roles have been proposed for exosomes in further studies e.g., Raposo et al. (1996) surveyed the role of exosomes in antigen presentation in pioneering studies. In this study they proved that exosomes work as MHCII presenters to T cells. In the nervous system as well, some functions for exosomes have been revealed, Bakhti et al. (2011) reported exosomal function in myelin formation. In this project the role of exosomes in material transportation between the cells was of paramount interest, hence the next sections deal with the function of exosomes in delivering the desired cargo.

1.2.5 Exosomes as delivery vehicles

Another feature of exosomes, which is highly compelling, is their role in intracellular communication. This intracellular transmission can include the transfer of pathogenic nervous system proteins like amyloid beta peptide (Rajendran et al., 2006) and alpha-synuclein (Emmanouilidou et al., 2010). These bioactive nanovesicles also transfer mRNA and miRNA between cells (Valadi et al., 2007). This special property of exosomes in mediating the cell contents has great benefits for biomarker research. Exosome-derived miRNAs and proteins has been studied as diagnostic biomarkers for prostate cancer, lung cancer and kidney injuries (Duijvesz et al., 2013; Yamashita et al., 2013; Zhou et al., 2006).

This function of exosomes is particularly useful because it can be exploited to deliver desirable cargoes to target cells. One interesting cargo could be a therapeutic agent. Many studies put their efforts to load exosomes with their desired cargo. Table 1.1 shows a list of these studies, which loaded various kinds of cargo into the exosomes, and specifies the method used for loading. The main trials have been done with electroporation and transfection methods. In electroporation an external electric field is applied to make the biological membrane permeable then a desired cargo, which could be a drug or a genetic piece like DNA or siRNA can be loaded to the cell. Transfection as word is a combination of trans and infection. Different genetic material can be entered to the cell with this method; siRNA constructs are among these different materials. It should be taken into account that in animal cells most of the time transfection involves transient pores in the cell membrane in order to uptake of the transfection material. Different methods are used for transfection: cell squeezing, calcium phosphate, electroporation, or most recently by mixing a cationic lipid with the material to produce liposomes, which fuse with the cell membrane and deliver their cargo inside.

Table 1.1 Recently published studies, which used exosomes as delivery vehicles.

Study	**Cargo**	**Method**
Alvarez-Erviti et al. (2011)	siRNA	Electroporation
Shtam et al. (2013)	siRNA	Electroporation
Wahlgren et al. (2012)	siRNA	Electroporation
Pan et al. (2012)	shRNA	Transfection of exosome producing cells
Chen et al. (2014)	miRNA	Transfection of exosome producing cells
Bryniarski et al. (2013	miRNA	Incubation of exosome with cargo
Zhang et al. (2010)	miRNA	Transfection of exosome producing cells
Katakowski et al. (2010)	miRNA	Transfection of exosome producing cells
Kosaka et al. (2012)	miRNA	Transfection of exosome producing cells
Pan et al. (2012)	miRNA	Transfection of exosome producing cells
Xin et al. (2012)	miRNA	Cell activation
Ohno et al. (2012)	miRNA	Transfection of exosome producing cells
Munoz et al. (2013)	miRNA	Transfection of exosome producing cells

In all the studies listed in the above table, interfering RNAs are adopted as a therapeutic agent. Some other studies have applied exosomes for other therapeutic cargoes, e.g. doxorubicin as chemotherapeutic in cancer treatment research (Tian et al., 2013; Jang et al., 2013) and curcumin as an anti-inflammatory mediator (Zhuang et al., 2011; Sun et al., 2010).

In the present study in order to use exosomes as reliable drug delivery vehicle for neuronal cells, the main concentration is to develop a method to produce exosomes, which can target neurons specifically. Pseudotyping of exosomes with rabid virus glycoproteins (RVG) was performed before by Alvarez-Erviti et al. (2011) to target glia and neurons. Anliker et al. (2010) introduced a method, which apply pseudotyped lentiviral vectors for specific gene transfer to different cell types, including neurons. This method is based on single chain antibodies, which recognize cell surface antigens. The process of pseudotyping is accomplished by using measles virus glycoproteins (MVG). This virus provides a promising strategy for specific cell entry by retargetable envelope MVG namely hemagglutinin protein (H), which is responsible for receptor recognition, and fusion protein (F) (Funke et al., 2008). Interestingly variants of the hemagglutinin protein (H) and the measles virus fusion protein (F) truncated at their cytoplasmic tails have shown efficient incorporation into lentiviral particles (Funke et al., 2009). In this project MVG H and F are used for exosome pseudotyping.

1.3 Project objectives

AD is the most frequent cause of dementia in elderly people. Due to increased life expectancies the number of people afflicted with this disease is believed to double in the near furture. Despite intensive research there is no effective therapy for AD. Clinical trials targeted towards amyloid pathology have all failed. It is believed that this is at least in part due to the fact that diagnosis is made too late, when causative treatments are ineffective. Hence, there is great need to identify biomarker for early diagnostic methods and therapeutic intervention. A central hypothesis of this thesis is that microRNAs provide a novel avenue to develop biomarker and therapeutic approaches for AD. Thus, this thesis had two central aims:

1. Detection of the circulating miRNAs as implications for age-related cognitive impairments and AD in body fluids like blood from aged versus young mice as a non-invasive method to search for miRNA signatures of aging and AD.

2. Since miRNAs can interfere at posttranscriptional level we sought to establish a therapeutic method which is based on RNA interfering. To conquer the blood brain barrier the exosomes were planned as delivery vehicles. To end up with a high efficiency method, in this project experiments were concentrated on neuronal targeting of the exosomes.

Materials and Methods

2.1 Materials

2.1.1 Animals

Four-month-old adult and eighteen-month-old adult male C57BL/6 wild-type mice were purchased from Janvier Rodent research models and associated service. All animals were housed in the animal facility of the European Neuroscience Institute, Göttingen with standard temperature and humidity and dark/light condition. They were fed and watered *ad libitum* with a standard laboratory mouse diet and cages were changed every ten days. All animal care and testing protocols were approved by the Veterinary Institute of the Lower Saxony State Office for Consumer Protection and Food Safety.

2.1.2 Antibodies

Primary antibodies, which were used, are listed in Table 2.1. Secondary horseradish peroxidase (HRP)-conjugated antibodies were purchased from Dianova (1: 2,000; Hamburg, Germany) and for western blot analysis, which were performed in Paul Ehrlich Institute, Langen the secondary antibodies conjugated with horseradish peroxidase were obtained from DakoCytomation (1:2,000; Hamburg, Germany).

Table 2.1 Primary antibodies used for western blot

Target	Host species	Application	Reference
Flotillin-2	Mouse	WB (1:1000)	BD Biosciences, Heidelberg, Germany
F (F431)	Rabbit	WB (1:1000)	Buchholz CJ, Laboratory, Paul Ehrlich Institute, Langen, Germany
H (606)	Rabbit	WB (1:2000)	Buchholz CJ, Laboratory, Paul Ehrlich Institute, Langen, Germany

2.1.3 Plasmids

Table 2.2 Plasmids donated from other laboratories

Plasmid	Reference
pCG_Hwt	Buchholz CJ, Paul Ehrlich Institute, Langen, Germany
pCG _Fwt	Buchholz CJ, Paul Ehrlich Institute, Langen, Germany
pCG _Hcd18	Buchholz CJ, Paul Ehrlich Institute, Langen, Germany
pCG _Fcd30	Buchholz CJ, Paul Ehrlich Institute, Langen, Germany
pCG _Hcd14	Buchholz CJ, Paul Ehrlich Institute, Langen, Germany
pCG _Fcd24	Buchholz CJ, Paul Ehrlich Institute, Langen, Germany

The plasmid cards are attached in appendice1-4.

2.1.4 Buffers and solutions

Table 2.3 Phosphate buffered saline (PBS) (for preparation of 1 Liter of 10x PBS).

Ingredient	Percent/concentration
NaCl	80.0g
KCl	2.0 g
Na_2HPO_4 (or 18.05 g Na_2HPO_4 _ $2H_2O$)	14.4g
KH_2PO4	2.4g

Table 2.4 CHAPS lysis buffer (The pH was adjusted to 8) (For preparation of 1 Liter 1x Lysis buffer).

Ingredient	Percent/concentration
3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), (^= 1%)	10 g
Tris (hydroxymethyl) aminomethane (Tris), (^=50 mM)	6.1 g
Ethylenediaminetetraacetic acid (EDTA) (^= 5 mM)	1.5 g

Table 2.5 Components of 400µl Electroporation buffer (The pH was adjusted to 7.2).

Ingredient	Percent/concentration
Potassium phosphate	1,15 mM
Potassium chloride	1, 25 mM
Optiprep	21%

Table 2.6 Protein loading buffer (The pH was adjusted to 6.8) Loading buffer 4x

Ingredient	Percent/concentration
Glycerol	10 %
Tris-HCl	50 mM
EDTA	2 mM
SDS	2 %
βmercaptoethanol	144 mM
Bromophenol blue	0.05%

2.1.5 Media and Sera

Table 2.7 Commercially available media, sera and additives.

Medium name	Reference
Dulbecco's Modified Eagle Medium (DMEM)	PAA Laboratories, Pasching, Austria
Fetal Calf Serum (FCS)	PAA Laboratories, Pasching, Austria
GlutaMAX™-I supplement	Invitrogen, Darmstadt, Germany
LB medium + LB agar plate	AppliChem (Darmstadt, Germany)
Opti-MEM + GlutaMAX™-I	Invitrogen, Darmstadt, Germany
Phosphate Buffered Saline (PBS) PAA	Laboratories, Pasching, Austria
Penicillin/Streptomycin (Pen/Strep) 100x	Invitrogen, Darmstadt, Germany
Geneticin (G418)	Sigma-Aldrich, Taufkirchen, Germany
Optiprep (Density Gradient Medium)	Axis-schield, Oslo, Norway

2.1.6 Commercial kits

Table 2.8 Commercial kits used in this study.

Kit	Application	Origin
Bardford protein assay	Exosome protein concentration measurement	BioRad
Xtra Midi Kit	DNA extraction	Machery-Nagel, Dueren, Germany
Luc-Screen® System	Luciferase activity measurement	Applied Biosystems, Bedford, MA, USA
RNeasy Protect animal Blood kit	RNA extraction from mice blood	Qiagen, Germany
TruSeq small RNA sample preparation kit	Small RNA library preparation	Illumina, San Diego, CA, USA

2.2 Methods

2.2.1 RNA Isolation

Mice were anesthetized with ketamine/xylazin diluted in PBS (per mouse 18µl Ketamin, 8,6 µl Xylazin diluted in 173,4 µl PBS). 500µl Blood was taken from heart using an Insulin syringe 1ml/ 40 I.U. and 12 gauge x 40 mm needle (BBraun, Meslungen, Germany) quickly and transferred to blood collection tubes from kit to avoid coagulation. RNA isolation from blood was performed with RNeasy Protect Animal kit (see table 2.8) according to the manufacturer protocol. For RNA isolation from brain regions first the brain was dissected. The dissection of ACC, DG, CA1 and CA3 regions was performed under a stereomicroscope (Motic) as described before (Hagihara et al., 2009). The RNA isolation from each region was done using TRI Reagent (Sigma-Aldrich Chemie GmbH, Munich, Germany). For RNA isolation the tissue was thoroughly homogenized in TRI Reagent. After that chloroform (AppliChem, Darmstadt, Germany) was added to the samples and was shaken vigorously and left at room temperature (RT) for 15 min. Then the samples were centrifuged at 12000xg, at 4°C for 15 min to acquire the three phases, the aqueous phase which is containing RNA was collected into a new 1.5 ml tube, mixed with Isopropanol (AppliChem, Darmstadt, Germany) and kept at -20°C for at least 14h. Afterwards the aqueous phase Isopropanol mixture was centrifuged for 30 min at 4C at 12000xg thereafter the supernatant was discarded and washed with 75% ethanol twice (12000xg for 5 minutes each wash step). Finally the pellet was dissolved in 15µl RNase free water. The exact amount of TRI reagent, chlorophorm, Isopropanol and ethanol per each tissue is listed in the table below:

Table 2.9 The exact volume of each solution for RNA isolation per brain tissue.

Brain tissues	TRI reagent	Chlorophorm	Isopropanol	Ethanol
ACC	400µl	80µl	200µl	500µl
DG	400µl	80µl	200µl	500µl
CA1	800µl	160µl	400µl	500µl
CA3	800µl	160µl	400µl	500µl

2.2.2 RNA quantity and quality

RNA from blood and brain tissue was quantified using a NanoDrop spectrophotometer (Thermoscientific, Peqlab). The exact quantity and quality measurement of the blood and brain RNA was performed with a 2100 Agilent Bioanalyzer microfluidics platform according to the manufacturer's instructions.

2.2.3 Library preparation

Small RNA libraries were prepared from total RNA using the TruSeq small RNA sample preparation kit (see table 2.8) according to the manufacturer's instructions. In all cases blood, ACC and hippocampal subregions 100 ng of total RNA was used as starting material.

2.2.4 Bioinformatics and statistical analysis

Small RNA detection was performed using Oasis (Capece et al., 2015) web tool for analysis of small RNAseq libraries. The following steps were performed by the Oasis pipeline. The raw Illumina reads were preprocessed to filter out reads of length smaller than 15 nucleotides and greater than 32 nucleotides. The filtered reads were mapped to the reference genome using STAR (Dobin et al., 2013) in non splice-junction-aware mode. The counts of the samples were summarized by MiRBase release v20 for miRNAs, piRNAbank V.2 for piwiRNAs and Ensembl for snRNAs, snoRNAs and rRNAs. It then predicts the novel miRNAs using miRDeep2 (Friedländer et al., 2012). All NGS data are publicly available in GEO database.

2.2.5 Statistical analysis of next-generation sequencing data

In order to perform the subsequent bioinformatics and statistical data analysis, Python (version 2.7.6) and R (version 2.7.6) environment were used. We normalized the read counts using standard quantile normalization method. We filtered out samples with library size < 100,000 reads. A threshold of minimum of 100 reads was used to filter out low abundant reads and outliers were also filtered out that were obtained from pairwise correlation (Pearson correlation coefficients) matrix. We then used customized python scripts for description, summarization and visualization of the data. Differential expression analysis was performed using DESeq2 (Love et al., 2014) package. Heatmaps and venn diagrams were generated using python's plotting library matplotlib (v1.4.3). Clustering of the pairwise correlation matrix and the differentially expressed miRNAs was performed using hierarchical clustering (SciPY version 0.15.1). The Ingenuity Pathway Analysis ((IPA, Qiagen) was used to analyze the list selected miRNA targets. We selected only experimentally verified genes and removed duplicates in Ingenuity Pathway Analysis (IPA).

2.2.6 Cell culture and exosomes

All cell culture work was performed according to security level S1 safety rules and was done under sterile conditions. Cell culture and exosome isolation work were performed in the laboratory of Prof. Anja Schneider, at the Max Planck Institute for Experimental Medicine, Göttingen. Murine Neuroblastoma cell line (N2a) was used for cell culture and exosome isolation and Human fibrosarcoma cell line with luciferase activity (HT1080Luc) was used as the reporter cell line. Freezing, thawing, growth and maintenance of N2a cell line are explained below:

2.2.7 Freezing of cells

For long-term storage of cell lines, N2a cells were frozen at -160°C liquid Nitrogen. For freezing, as the first step the cells were detached or trypsinised with 2 ml of 0.05% trypsin-EDTA (Gibco). The trypsinization reaction was then stopped with 10 ml of Dubelco's Modified Eagle's Medium (DMEM) (see table 2.7) and cell suspension was centrifuged subsequently after centrifugation of the cell suspension for 5 min at 900xg the pellet was resuspended in 0.5 mL DMEM, 0.5 mL of 2x freezing medium (40 % FCS, 20 % DMSO in DMEM) was added, the suspension was mixed gently and transferred into a Nalgene® Sterile Cryogenic Vial (Thermo Fisher Scientific, Roskilde, Denmark). Cell vials were transferred to a Nalgene® Cryo freezing container (Thermo Fisher Scientific, Roskilde, Denmark), which was filled with Isopropanol and allowed slow freezing at a temperature-dropping rate of -1C/min in an -80°C freezer. For permanent storage, cells were stored at -160°C liquid nitrogen.

2.2.8 Thawing of cells

To thaw the cells, a cryogenic vial was removed from liquid nitrogen and immediately incubated in a 37°C water bath. Rapidly after the cell suspension became liquid, 10 mL of pre-warmed fresh growth medium was added and the suspension was centrifuged for 5 min at 900xg. The cell pellet was resuspended in fresh pre-warmed growth medium and plated on a 10 cm petri dish or a 75 cm cell culture flask.

2.2.9 Growth and maintenance of cells

Cells were grown at 37°C and 5% CO2 in humidified incubators. Cells were grown in

general growth medium. General growth medium is composed of: 1mL Penicillin/Streptomycin, 5000 U/5000µg, 1mL GlutaMAX™-I supplement, 200 mM (see table 2.7) 10 mL Fetal Calf Serum (FCS, see table 2.7) in Dulbecco's Modified Eagle Medium (see table 2.7) with 4.5 g/L glucose. HT1080Luc cell line was kindly donated from Paul Ehrlich Institute, Langen and was used as luciferase reporter cell line. The medium which was used for this cell line was the same as N2a cells but instead of Penicillin/Streptomycin antibiotic 1mg/mL Gentecin (G418) (see table 2.7) antibiotic was added to the DMEM. G418 is commonly used in laboratory research to select genetically engineered cells. In this case it was used to select the marker gene, which is responsible for luciferase activity in HT1080Luc cells, which express luciferase activity. PBS was used for washing the cells; PBS was either purchased from PAA (see table 2.7) or prepared manually.

To obtain 1x PBS, 10xPBS was diluted 10 times with bi-distilled H_2O. The pH value was adjusted to 7.2-7.4 (see table 2.3)

2.2.10 Transformation of E. coli

pCGHwt and pCGFwt and their mutant variants plasmids were kindly donated from Prof. Christian Buchholz, Paul Ehrlich Institute, Langen, Germany (see table 2.2 and Plasmid maps in appendices 1-4) For amplification of plasmid DNA constructs in bacteria, the chemocompetent E. coli strand Subcloning Efficiency DH5alpha' (Invitrogen, Carlsbad, CA, USA) was used. For transformation, 50 µL of competent cells were thawed on ice for 5 min. thereafter, 0,5 µg of the desired plasmid for example pCGHwt and pCGFwt or other variants were added to the cells, the suspension was mixed and incubated on ice for 20 min followed by heat-shock at 42°C for 42 sec. After recovery on ice for 2 min, 500 µL of LB medium (25 g LB in 1 L H2O, autoclaved) was added and the cells were incubated for 1 h at 37C under shaking. For single transformants selection, the culture was spread on LB plates (LB-Agar 40 g per 1 L H20, autoclaved) supplemented with the appropriate antibiotics (100 µg/ml ampicillin) and incubated at 37C for 14- 20 h.

2.2.11 Plasmid DNA isolation from E. coli

To amplify plasmid DNA from transformed E. coli at a medium-scale, a single colony was picked from the LB plate using an autoclaved tip and transferred to 100 mL of antibiotic supplemented LB medium. Bacteria were incubated for 10-16 h at 37°C under constant shaking. Thereafter, cells were harvested by centrifugation for 10 min at 3,000 rpm and 4°C and plasmid DNA was isolated and purified with the NucleoBond Xtra Midi Kit (see table

2.8) according to the manufacturer's instructions. Extracted DNA was dissolved in 350 µl TE buffer from the kit.

2.2.12 DNA concentration measurement

DNA concentration was determined by measuring the optical density at 260 nm (A260), samples were measured on Nanodrop spectrophptometer (Thermoscientific, Peqlab).

2.2.13 Transfection of plasmids

Two Plasmid DNAs (see Plasmid maps in appendices 1-4) were cotransfected into murine N2a cell lines using Mirus Bio TransIT®-LT1 Transfection Reagent (Mirus Bio LLC, Madison, WI, USA). Before transfection, the N2a cells were grown to a confluence of 60% in a 10cm dish culture. 27µl transfection reagent and 4,5µg of each plasmid DNA were added to 18µl Opti-MEM (see table 2.7), mixed gently and incubated at room temperature for 25-45 min. After incubation, the mixture was added to the cells in a drop-wise manner, the cell culture dish was shaken gently and the cells were incubated under culture conditions for 16-24 h.

2.2.14 Exosome collection and preparation

Exosome collection was accomplished by changing from growth medium to collecting medium, which is free from FCS. Exosome isolation was performed according to a protocol based on Thery et al. (2006), which is considered as the golden standard method to isolate exosomes from cell culture medium.

The cell culture supernatant was subjected to serial centrifugation at 3500xg for 10 minutes, 4500xg for 10 minutes (two times) and 10,000xg for 30 minutes. Finally the supernatant was subjected to ultracentrifugation at 100,000xg to obtain the exosome pellet and the exosome-free supernatant. Exosome pellet was resuspended in electroporation buffer. To confirm the presence of expressed proteins in N2a cells, after cell culture supernatant collection these cells were harvested. For protein analysis, corresponding cells of a 10 cm petri dish were scraped in 400 µl of CHAPS lysis buffer (see table 2.4) and centrifuged for 10 min at 45,00xg and 4°C. Protein from cell lysates were resuspended in loading buffer (see table 2.6) and kept at -80°C to be subjected later to western blotting.

2.2.15 Determination of the exosome protein concentration

The protein concentration of exosomes was determined using Bradford Protein Assay (BioRad, Germany) according to the manufacturer's instructions. This assay is based on the Coomassie blue dye binding to proteins i.e. the higher the protein concentration the darker is the color of the test sample. Coomassie absorption is at 595 nm; protein concentration of the test samples is determined by comparing to that of a series of protein standards, which in this study was Bovine Serum Albumin (BSA). The absorbance of the reaction was measured at 595 nm using a 96-well plate reader (MRXTc Revelation, Dynex Technologies).

2.2.16 Electroporation

Two different amounts of exosomes and siRNA were used for the experiment. The first step was conducted with 3µg of exosomes (from transfected and not-transfected cells). These exosomes were electroporated with 3µg of GL3siRNA (Qiagen, Hilden, Germany). For the next step of the experiment 10µg of exosomes (from transfected and not-transfected cells) were electroporated with 10µg of GL3siRNA. In both steps for electroporation the resuspended exosomes in 100µl electroporation buffer (see table 2.5) were electroporated in a 4 mm cuvette (Gene Pulser/MicroPulser, BioRad, Germany). The cuvettes were put in the shock pod cuvette chamber of Gene Pulser Xcel electroporation device (BioRad, Germany). For electroporation the Exponential program was used at 400 mV and 125µF capacitance (pulse time 10–15 ms).

The electroporated exosomes were added drop wise to the HT1080Luc cells, which have been before cultivated in 24well plates and were incubated at 37°C for 48h. As the standard control for transfection efficiency, the GL3siRNA was mixed with OptiMEM (see table 2.7) and oligofectamine, the transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer transfection protocol and added to the cells. As negative control only oligofectamine was added to the cells.

2.2.17 Luminometer analysis

RNA interference (RNAi) responses of luciferase in luciferase-expressing cells were measured with Luc-Screen® System kit (see table 2.8) 48h after the HT1080 Luc cells were

treated with GL3 siRNA electroprated exosomes. Buffer1 (contains buffered luciferase reaction and cell lysis reagents) and buffer2 (contains luciferin substrate) were equilibrated at room temperature and then 200µl of buffer1 was added to 400µl medium from each well, buffer2 was added in 5 minutes. The luminescence was measured on a luminometer. Data analyses for luminescence intensity were performed with Excel and Prism 6 softwares.

2.2.18 Western blot analysis

In the case that exosomes were intended to be subjected to western blotting the exosome pellet was resuspended in 20µl loading buffer (see table 2.6).

Exosome isolation was performed from non-transfected and transfected cells. For confirming the exosome isolation quality the western blot was performed. Flotillin-2 antibody was used as positive marker for exosomes.

2.2.19 SDS-PAGE

Sodium dodecyl sulfate ployacrylamide gel electrophoresis (SDS-PAGE) was performed using the Bio-Rad Mini-PROTEAN 3 electrophoresis system (Bio-Rad Laboratories GmbH, Munich, Germany) for proteins separation based on the molecular weight after denaturation. Preparation of two-layered polyacrylamide gels was conducted in the Bio-Rad Mini-PROTEAN 3 casting system. Composition for the upper stacking gel (2 ml) and the lower resolving gel (5 ml) is listed below:

Table 2.10 Stacking gel used for electrophoresis

Ingredient	Percent/concentration
Acrylamide/bis-acrylamide (29:1) solution	4%
Tris-HCl pH 6.8	125 mM
SDS	0.1%
Ammonium persulfate (APS)	0.05%
N'N'N'-tetramethylethylene diamine (TEMED)	0.005% (v/v)

For loading on the gel, exosome and cell lysate samples were prepared as follows: Exosome pellets were resuspended in 20 µl of denaturing protein-loading buffer (see table 2.6). Before loading on the gel, the samples were boiled at 95°C for 5 min. The gel was run in running tris-glycine electrophoresis buffer (25 mM tris, 192 mM glycine, 0.1% SDS) for 90 min at 100 V.

Loading of the marker PageRuler® Plus Prestained Protein Ladder (Fermentas, St. Leon-Rot, Germany) enabled estimation of molecular weights of the analyzed proteins.

Table 2.11 Resolving gel (10 or 12 %) used for electrophoresis

Ingredient	**Percent/concentration**
Acrylamide/bis-acrylamide (29:1) solution	10 or 12%
Tris-HCl pH 6.8	325 mM
SDS	0.1%
APS	0.05%
TEMED	0.005% (v/v)

2.2.20 Western blotting

After electrophoresis separation, proteins were subjected to Western blotting by using the Bio-Rad Mini-Protein System according to the manufacturer's instruction. In the procedure, proteins were transferred from SDS-polyacrylamide gels onto a Whatman ® Protran Nitrocellulose Transfer Membrane (Whatman GmbH, Dassel, Germany) by application of 100 V for 55 min at room temperature in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol).

Following transfer of proteins, the nitrocellulose membrane was incubated in 4% nonfat dried milk (Sigma-Aldrich Chemie, Munich, Germany) in PBS for 30 min at room temperature to avoid nonspecific binding of immunoglobulins. The membrane was then incubated with primary antibody in PBST (0.1% Tween-20 in PBS) (dilutions according to Table 2.1) for at least 18 h at 4°C or 1h at room temperature followed by 3 washing steps of 10 min in PBST at room temperature. Subsequently, the membrane was incubated with horseradish peroxidase-(HRP) conjugated secondary antibody (1:1,000 in PBST) for 1-2 h at room temperature and again washed 3x with PBST each washing step 10 min.

HRP-antibody binding was visualized by chemiluminescence using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientifc Inc., Rockford, IL, USA). The emitted light signal was captured on X-ray films (CL-XPosure™ Film, Thermo Fisher Scientific, Rockford, IL, USA), which were scanned with a conventional scanner.

Results

3.1 Molecular changes in brain aging

3.1.1 Small RNAome of memory subregions in adult mouse brain

In order to have an overview of the small RNAome of memory subregions in the adult brain, four subregions that are crucial in memory and cognitive function namely anterior cingulate cortex (ACC), dentate gyrus (DG), cornu ammonis (CA1) and (CA3) from 4-month-old mice (n=9) were subjected to small RNA sequencing. As mentioned before ACC is responsible for long-term memory consolidation and formation in rodents (Weibele et al., 2012). Other three hippocampal subregions represent the short-term memory related areas. Our analysis revealed that microRNA (miRNA) is the biggest proportion of small RNAs in adult mice brain, small nucleolar RNAs (snoRNA) is the second abundant small RNAs and piwi-interacted RNA (piwiRNA) is the third most abundant small non-coding RNAs, read amounts of other small non-coding RNAs like ribosomal RNA (rRNA) and small nuclear RNA (snRNA) are extremely low (Fig. 3.1.1).

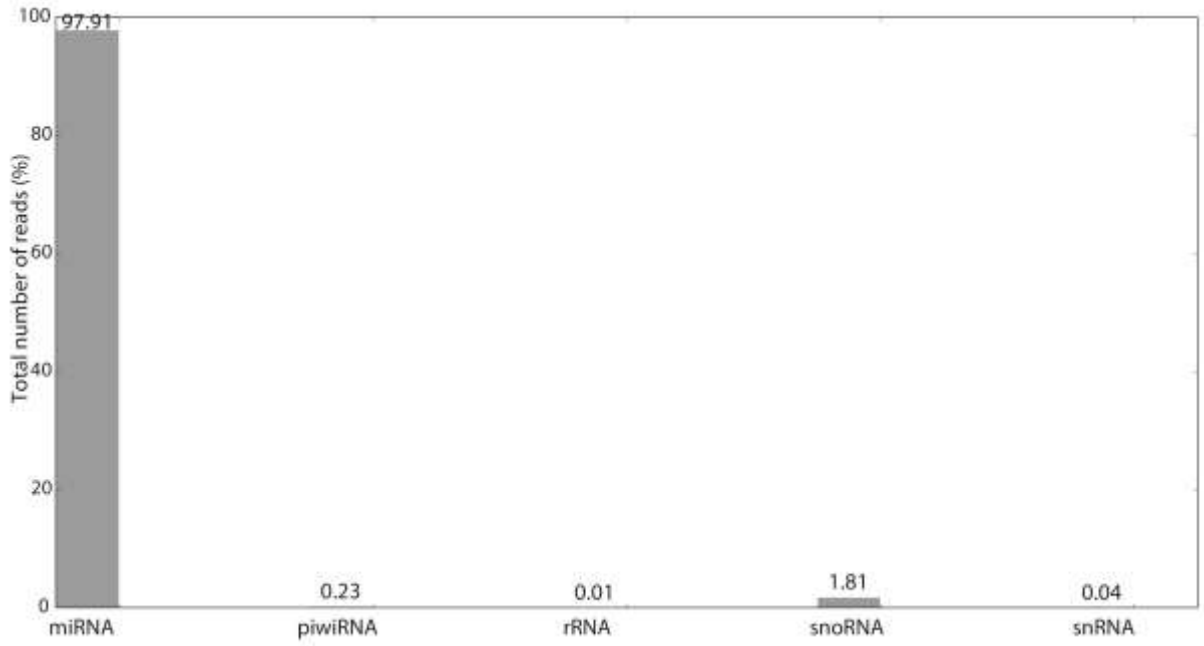

Fig. 3.1.1 Distribution frequency of mapped small RNA class reads combined for all brain regions. The bar graph shows that miRNAs are the most abundant class of small RNAs in memory subregions of young mice. SnoRNA population is the second most abundant small RNAs.

Further analyses were concentrated on the miRNAs. We detected 205, 214, 200, 214 miRNAs in the CA1, CA3, DG and ACC of these mice respectively (Fig. 3. 1. 2A, facing page). Interestingly, 172 miRNAs are commonly expressed in all brain regions of young mice (Fig 3.1.2B facing page). Barplot in Fig 3.1.2C (facing page) displays the most frequent mapped miRNAs. MiRNAs with the expression level smaller than around 1.50% are shown as rest. As shown in Fig. 3.1.1, miR127-3p is the most frequent miRNA in brain subregions. Other highly expressed miRNAs are: 92b, 125a, 22, 92a, 99b, 128, 30d, 434, 191, 30a, 29a and 204 (Fig. 3.1.2C). In order to understand the association of these commonly expressed miRNAs with biological pathways, we used the IPA (ingenuity pathway analysis tool) for functional pathways identification taking into account only confirmed target genes.

Since ingenuity software ranks the cancer-related canonical pathways as the highest expressed canonical pathways, the top biological functions were linked to cancer mechanism, cell death and survival (Fig. 3.1. 2C). However the analyses revealed a number of highly enriched pathways that are linked to cell growth, tumor suppression, development and neurogenesis function such as cyclins cell cycle, PTEN (Phosphate and tensin homolog) signaling and Aryl hydrocarbon receptor (Fig 3.1.2D).

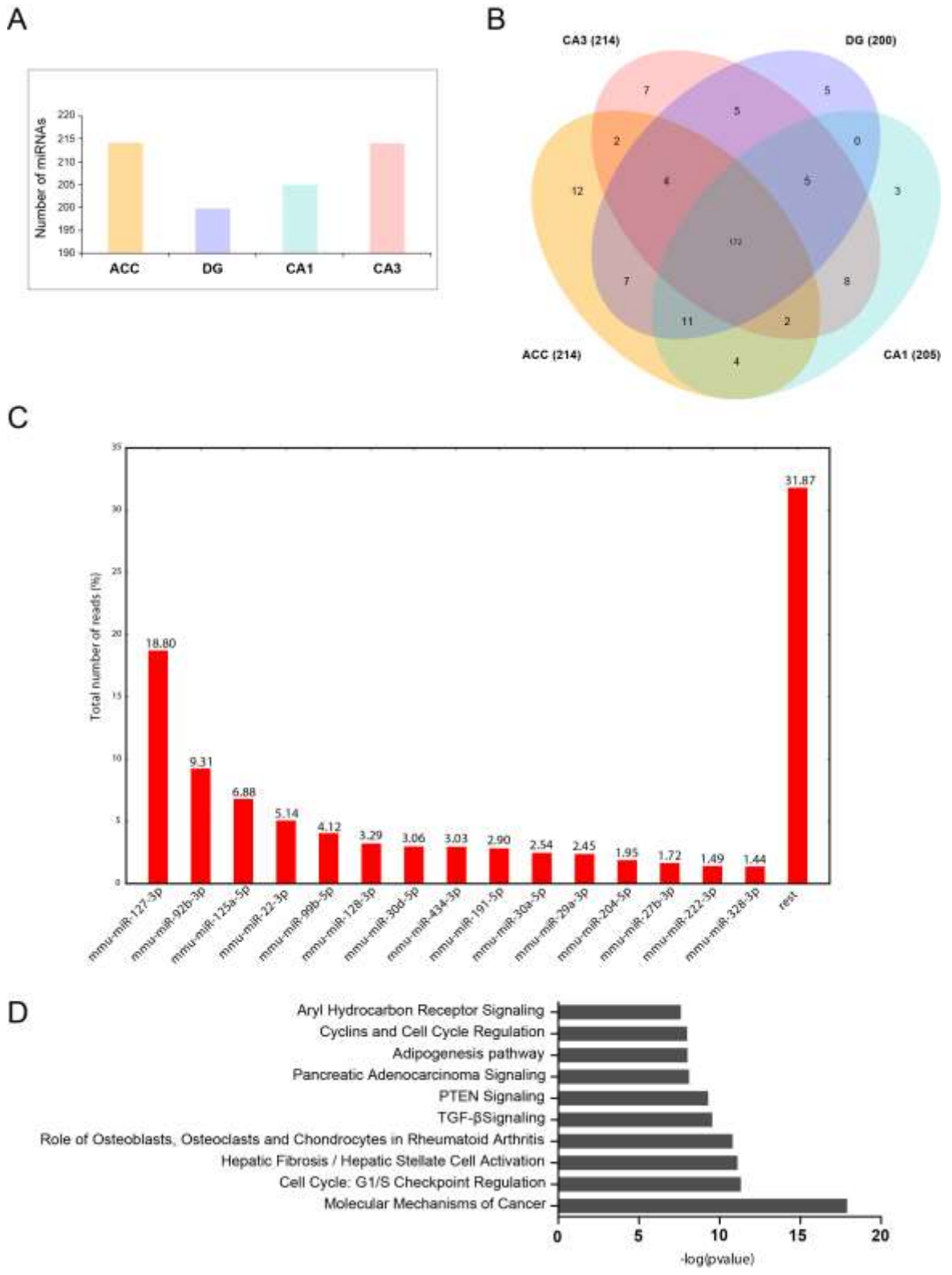

Fig. 3.1.2 Number of expressed miRNAs in different brain subregions of young mice (A) Number of unique and overlapping miRNAs in brain subregions (B) Most frequently expressed miRNAs in brain subregions of young mice (C) Top 10 canonical pathways regulated by highly expressed miRNAs (D).

Aside from biological pathways that are correlated with neuronal function and develepoment, pathways that don't have direct correlation with the function of neurons like hepatic fibrosis, role of osteoblasts and chondrocytes in rheumatoid arthritis, pancreatic adenocarcinoma signaling and adipogenesis are also present.

Albeit the pathway analysis has to be interpreted with care, the data indicates that the four investigated brain subregions share a core miRNAome signature that controls key molecular pathways linked to brain cell development, homeostasis and plasticity.

Since our analysis was so far based on the presence or absence of a given miRNA, we decided to test if miRNAs present in the four brain subregions differ significantly regarding absolute expression level, which may point to distinct function of the investigated brain subregions. Hierarchical clustering analysis using Pearson correlation indicated that the four different brain subregions showed distinguishable expression profiles, which was particularly obvious for the DG (Fig. 3.1.3). Nevertheless, the correlation between all brain regions was still high.

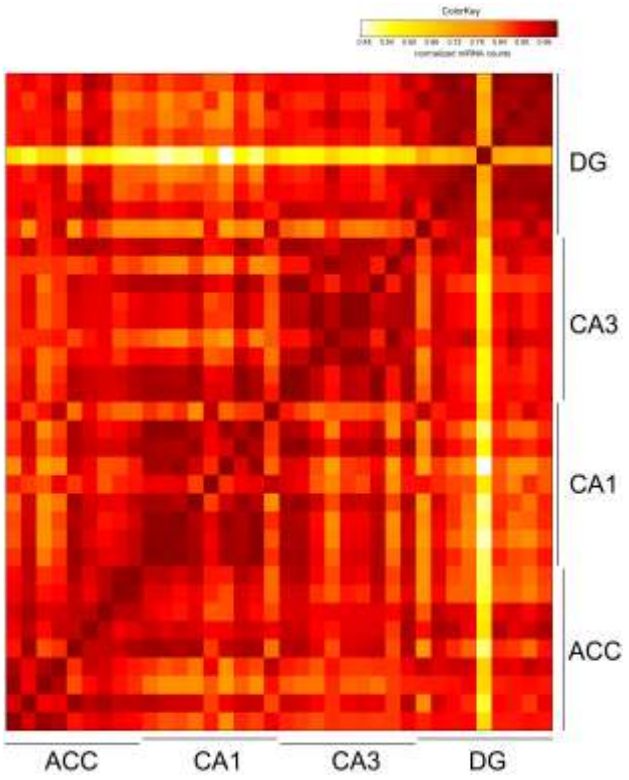

Fig. 3.1.3 Correlation (Pearson) matrix of miRNAome expression patterns between different brain subregions in young mice. It shows that DG has a most distinguishable expression profile. Samples with lower correlation are represented by the shades of yellow and samples with higher correlation are represented by the shades of orange to red.

3.1.2 MiRNA profile of memory subregions differs in young and old mice

Many studies show role for selected miRNAs in neuronal plasticity, memory function and the pathogenesis of cognitive diseases such as AD. To better understand the dynamics of the brain miRNAome during cognitive function and diseases, the small non-coding RNAome in young and old mice in ACC, DG, CA1 and CA3 (brain subregions) were compared. The laboratory of Prof. Fischer has established that 16- month old mice shown impared memory function, when compared to young 3-momth old mice (Peleg, 2010). Fig 3.1.4 shows unpublished data that was generated in the laboratory by another PhD student (Pooja Rao), showing impared learning in the Morris Water Maze test, a commonly used paradigm to analyze spatial memory in rodents.

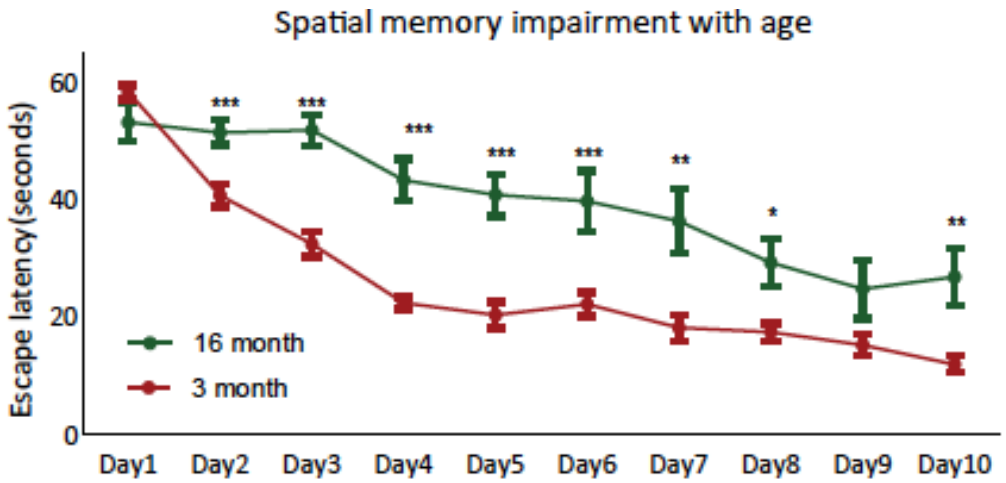

Fig. 3.1.4 Morris water maze experiment. Graphs show the escape latency of young (3-month) and old mice (16-month) throughout the water maze training. Old mice show significantly enhanced escape latency when compared to young mice, which is indicative of impaired spatial memory formation. Error bars indicate SEM. (*P < 0.05; **P < 0.01; ***P < 0.001) (Courtesy Pooja Rao)

Analyses on small RNAome in old mice revealed that like young animals the major proportion of the small RNAome in the old animals are miRNAs and second most abundant small RNAs are snoRNAs (Fig. 3.1.5). In order to have an overview of the miRNA population in old animals in chosen brain subregions, we looked at number of expressed miRNA numbers in these subregions. The bar graph shows the number of miRNAs in ACC, DG, CA1 and CA3 (Fig. 3.1.6A, facing page). 191 miRNAs in ACC and 192 miRNAs in DG were detected respectively; while in CA1 and CA3 the number of detected miRNAs are 186

and177. In total 155 commonly expressed miRNAs are detected (Fig. 3.1.6B, facing page). Bar plot in Fig. 3.1.6C (facing page) shows most abundant miRNAs. Like the young group, miR127-3p is the most abundant miRNA in brain subregions with 24.68% abundance out of all detected miRNAs in old mice. Other highly expressed miRNAs are: 92b, 125a, 191, 99b, 22, 434,27b, 128 and 204 (Fig. 3.1.6C, facing page).

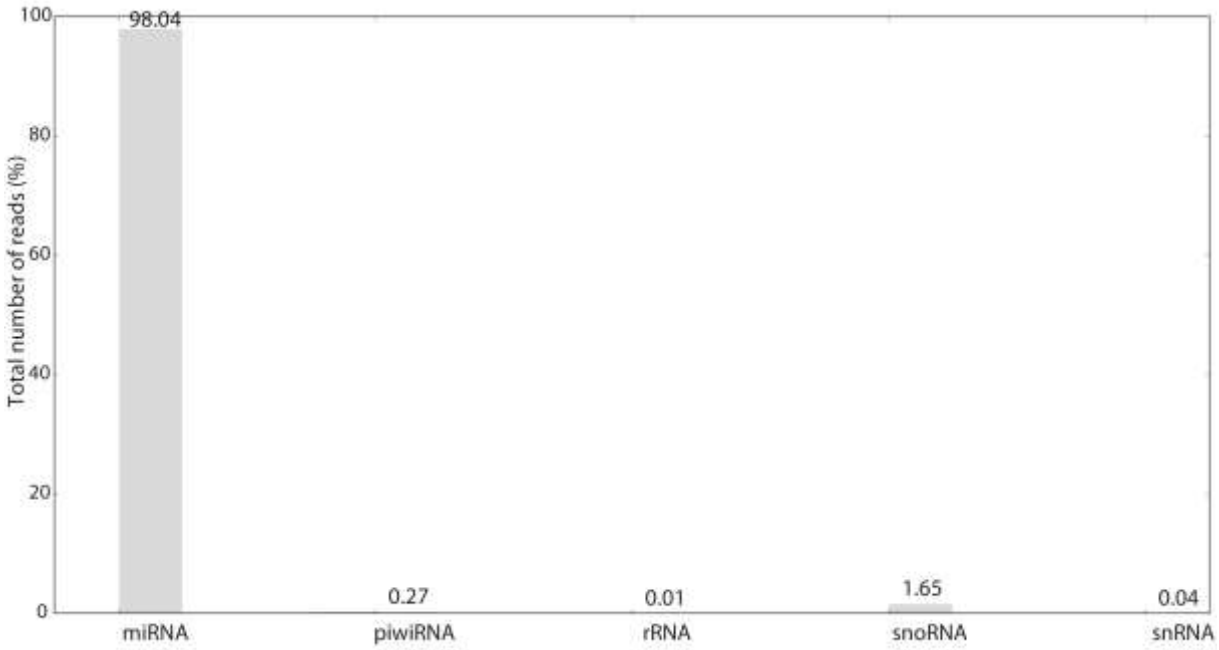

Fig. 3.1.5 Distribution frequency of mapped small RNA class reads combined for all brain regions. The bar graph shows that miRNAs are the most abundant class of small RNAs in memory subregions of aged mice. SnoRNA population is the second most abundant small RNAs.

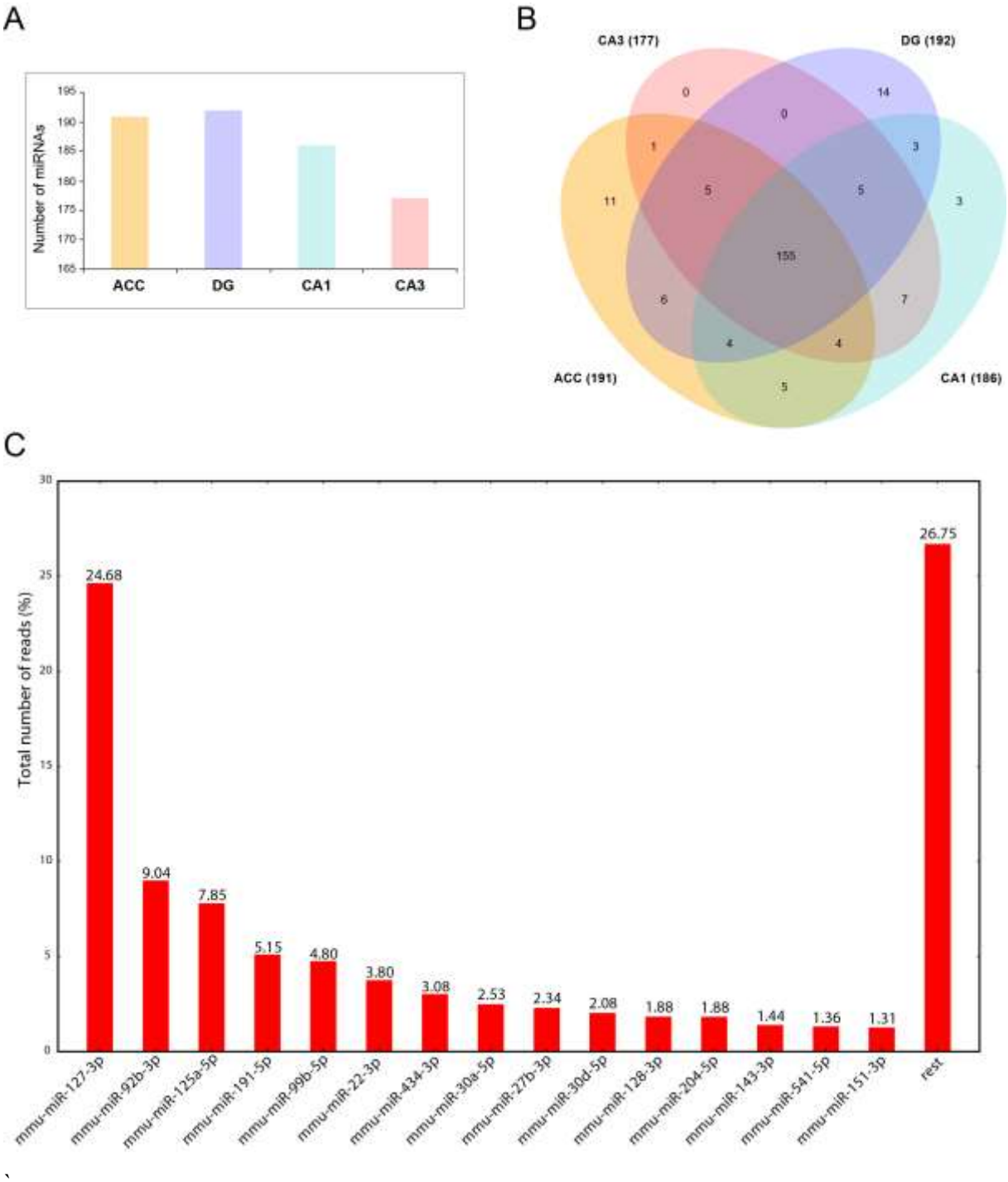

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Fig.3.1.6 Number of expressed miRNAs in different brain subregions of old mice (A) Number of unique and overlapping miRNAs in brain subregions (B) Most frequently expressed miRNAs in brain subregions of old mice (C).

The data analyses so far were based on screening the miRNAome in young and old animals according to their uniquely mapped reads. To understand the difference between miRNAome of memory subregions in young and old mice and to trace the influence of aging on the miRNA pool of the memory subregions, we performed differential expression analyses (padj < 0,05; log2 fold change <> 0,5; basemean >= 100).

In all investigated brain subregions, we found remarkable number of miRNAs that were differentially expressed when comparing the young to old brain (Fig. 3.1.7 A-D, facing page) some of them with fold change greater than 20, which are almost exceptional for the regulation of miRNAs. We found 120 differentially expressed miRNAs (padj < 0,05; log2 fold change <> 0,5; basemean >= 100) in ACC, 86 miRNAs in CA1, 166 in CA3 and 104 in DG (Fig 3.1.8, facing page). This data suggests that while the four investigated brain subregions share a substantial miRNAome signature, the aging process affects these brain regions in a distinct manner. The number of differentially expressed miRNAs is not the same in all subregions. CA1 with 86 has the lowest amount of differentially expressed miRNAs while its adjacent subregion CA3 has the largest number of differentially expressed miRNA.

We then looked at miRNAome expression patterns in aged mice by performing a hierarchical clustering analysis on the correlation (Pearson) matrix of brain subregions, which showed a clear separation of these four subregions (Fig. 3.1.9). ACC and DG display more significant changes when comparing to CA1 and CA3.

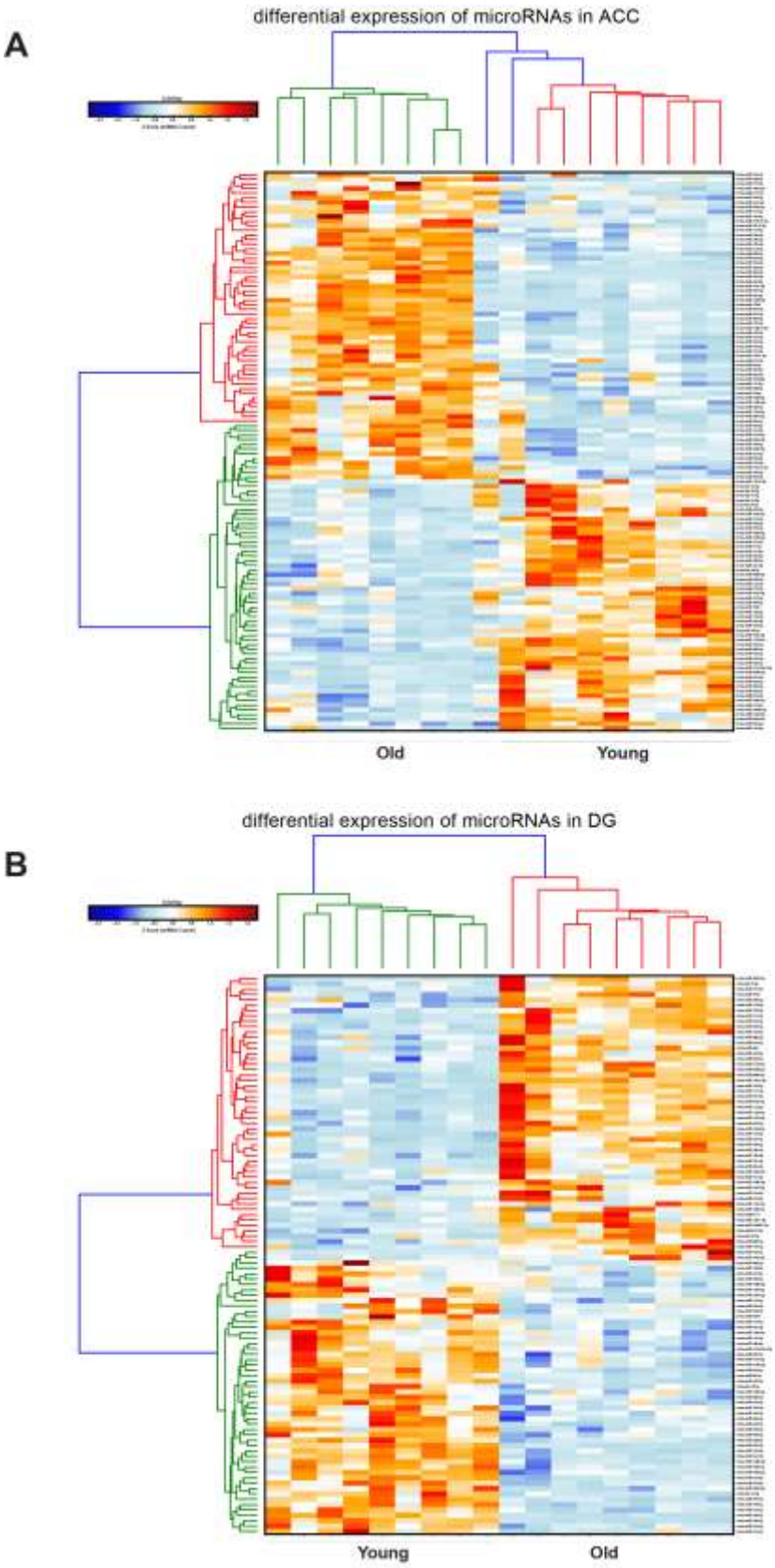

Fig. 3.1.7 (A-B) Heat maps show the Euclidean distances between the differentially expressed miRNAs in old over young animals. (A) and (B) show the clustration of the miRNAs in the ACC and DG respectively.

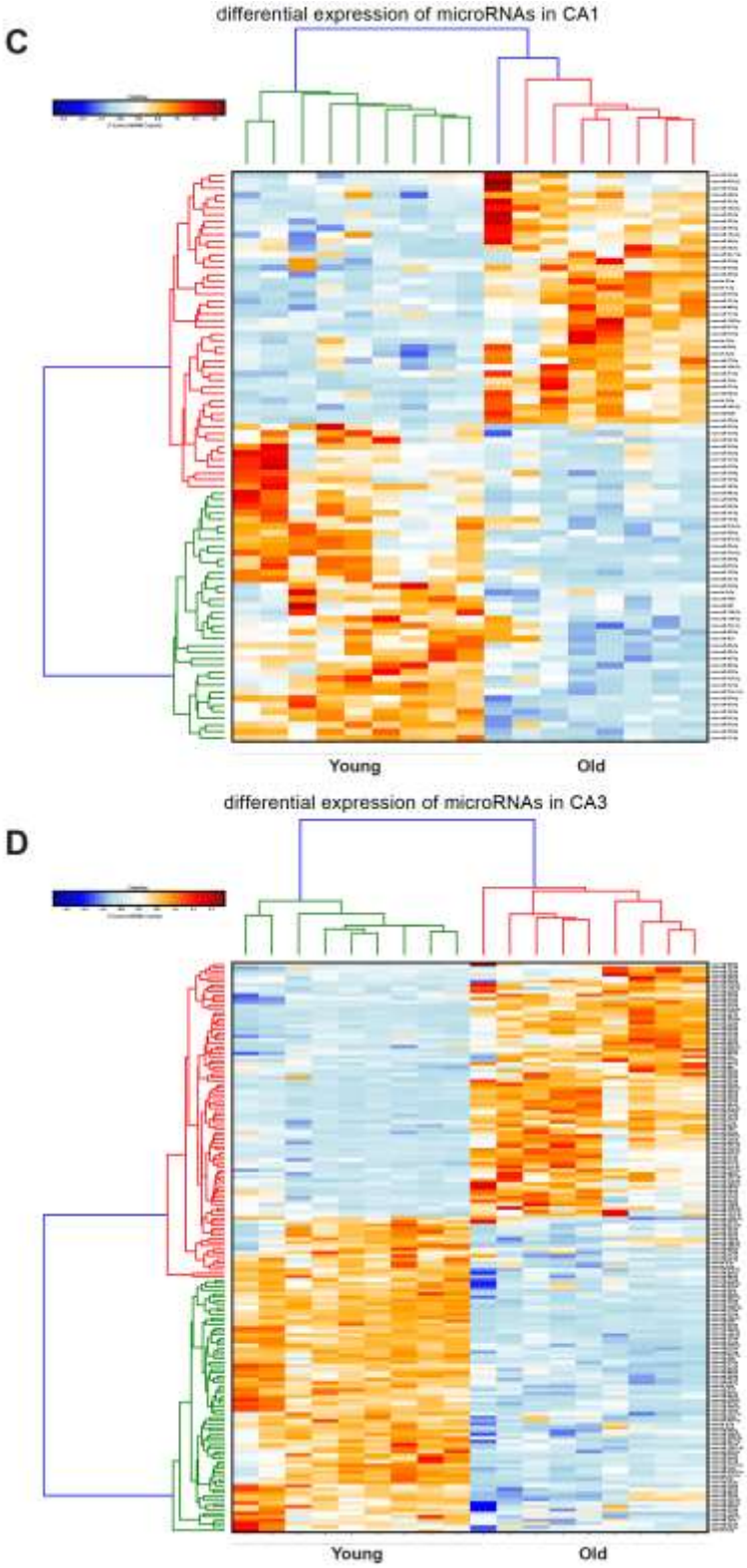

Fig. 3.1.7 (C-D) Heat maps show the Euclidean distances between the differentially expressed miRNAs in old over young animals. (C) and (D) heat maps show the clustration of the miRNAs in the CA1and CA3 respectively. As it is pictured in the key color, colors in red region show higher and colors in blue region show lower expression.

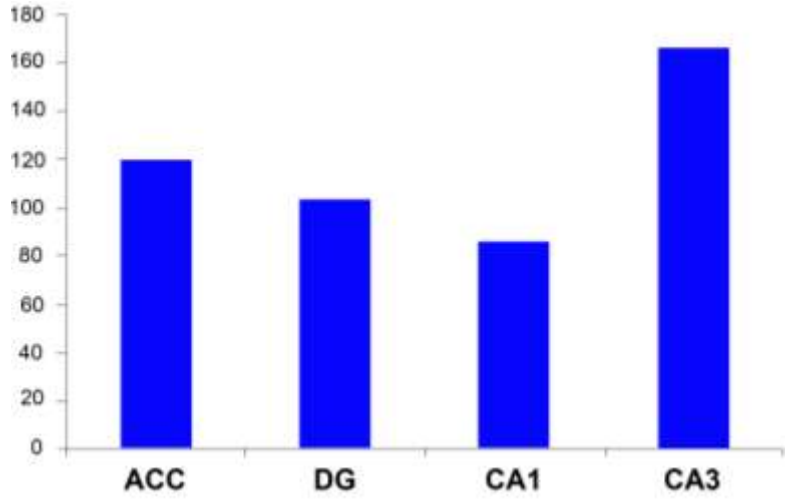

Fig. 3.1.8 Number of the differentially expressed miRNAs in the brain subregions of young and old mice.

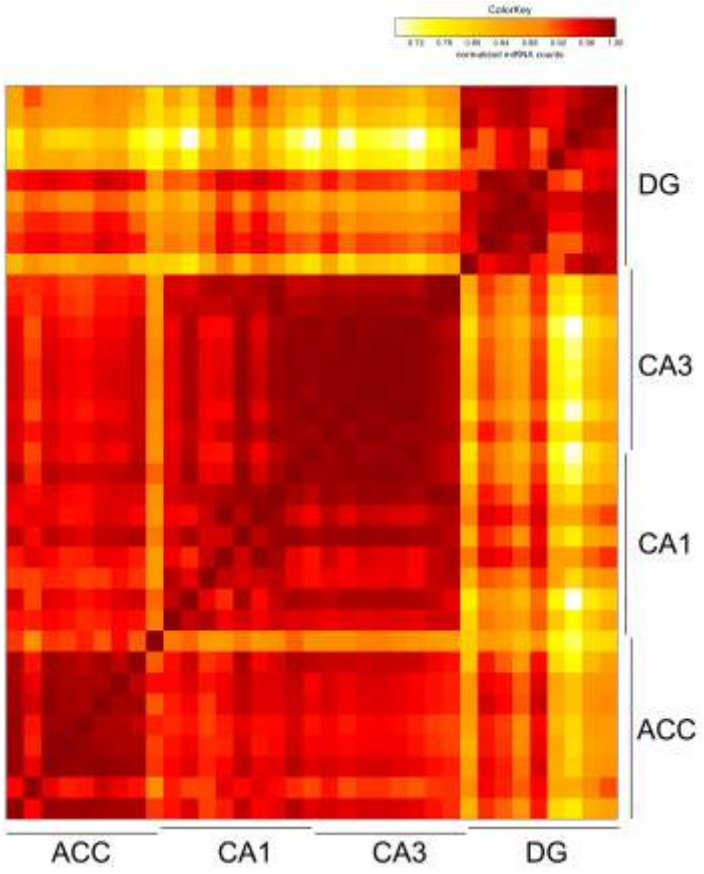

Fig. 3.1.9 Correlation (Pearson) matrix of miRNAome expression patterns between different brain subregions in aged mice. Samples with lower correlation are represented by the shades of yellow and samples with higher correlation are represented by the shades of orange to red.

Differential expression analyses of miRNAs for each subregion gave us a closer look at miRNA deregulation along aging. Of note, deregulation pattern of the miRNAs differs from region to region, for example one miRNA that is upregulated in ACC might be downregulated in CA1 and vice versa. To have a better understanding of the effect of aging on the miRNAome of brain memory subregions, we looked at the common upregulated miRNAs in brain subregions and also the common downregulated miRNAs along aging in the brain. We found four commonly upregulated miRNAs in brain subregions upon aging. These miRNAs are: miR-10a-5p, miR-191-5p, miR-411-3p and miR-541-5p (Fig.3.1.10A, facing page). Pathway analyses for these commonly upregulated miRNAs revealed that these miRNAs are associated with immune system coordination and activation, pathways like communication between innate and adaptive immune system, TREM1 (Triggering Receptor Expressed on Myeloid cells 1) signaling which is correlated with inflammatory responses in the cell, dendritic cell maturation that is crucial in antigen processing and Interleukines production pathways (Fig.3.1.10B, facing page).

Venn diagram in (Fig.3.1.11A) shows that there are six commonly downregulated miRNAs in all investigated brain subregions. These miRNAs are: miR-137-3p, miR-298-5p, miR-29c-3p, miR-504-5p, miR-543-3p and miR-708-3p. Pathway analyses for these commonly downregulated miRNAs showed that few of these pathways are associated with inflammation and immune system activity like dendritic cell maturation and cyclin and cell cycle regulation (Fig.3.1.11B). In total, upregulated and downregulated miRNAs along aging in these subregions are correlated with cell proliferation and immune system activity.

Apart from the commonly deregulated miRNAs in these brain subregions, we were able to find miRNAs, which are specifically deregulated in each memory subregion. Table 3.1 shows the list of miRNAs that are downregulated or upregulated specifically in each memory subregion. CA1 shows the lowest number of exclusively deregulated miRNAs and CA3 has the largest number of excusively upregulated and downregulated miRNAs.

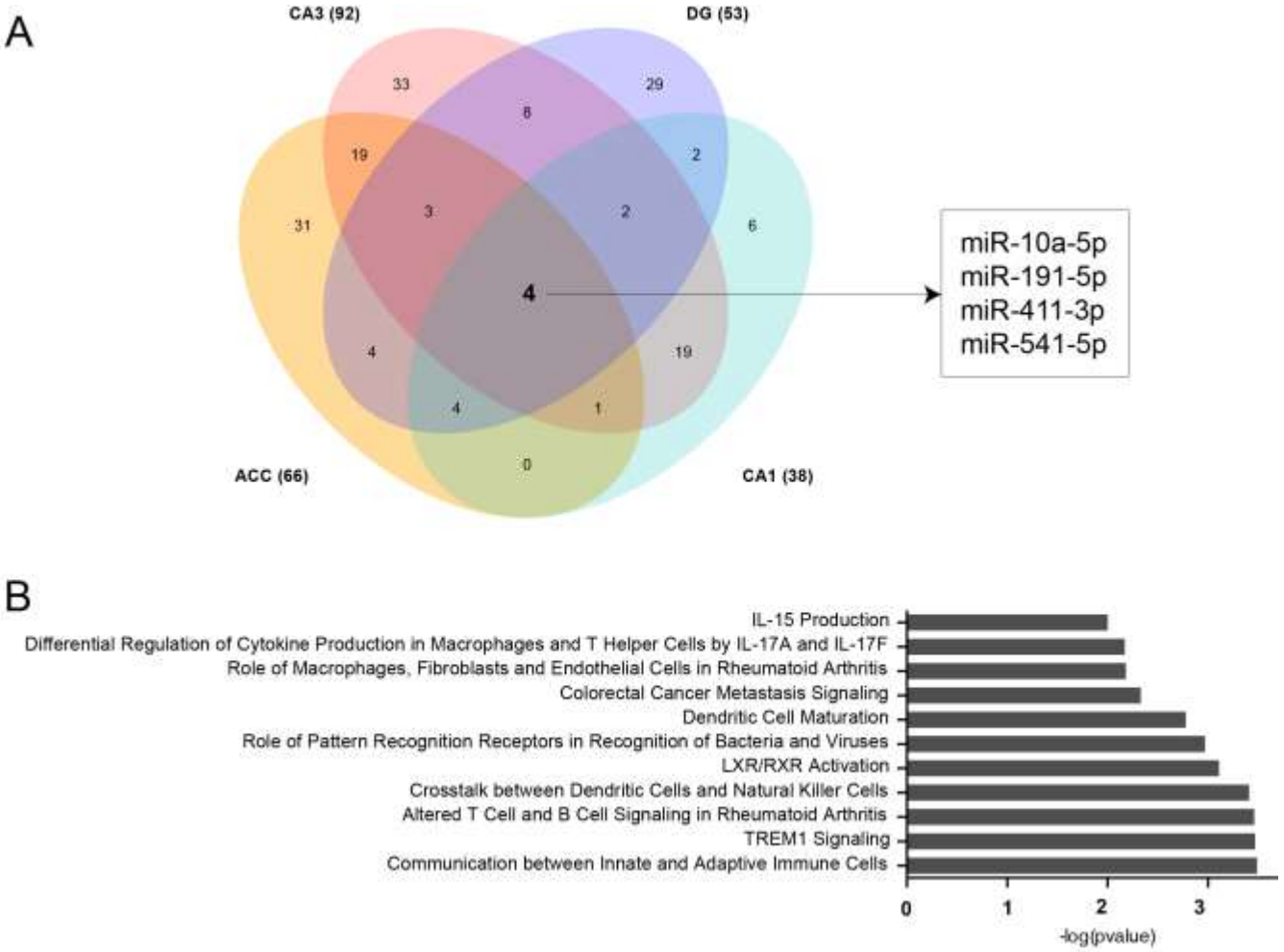

Fig. 3.1.10 Venn diagram shows commonly upregulated miRNAs in brain subregions upon aging (A) Shows top 10 canonical pathways that are associated with the four commonly upregulated miRNAs in brain subregions (B).

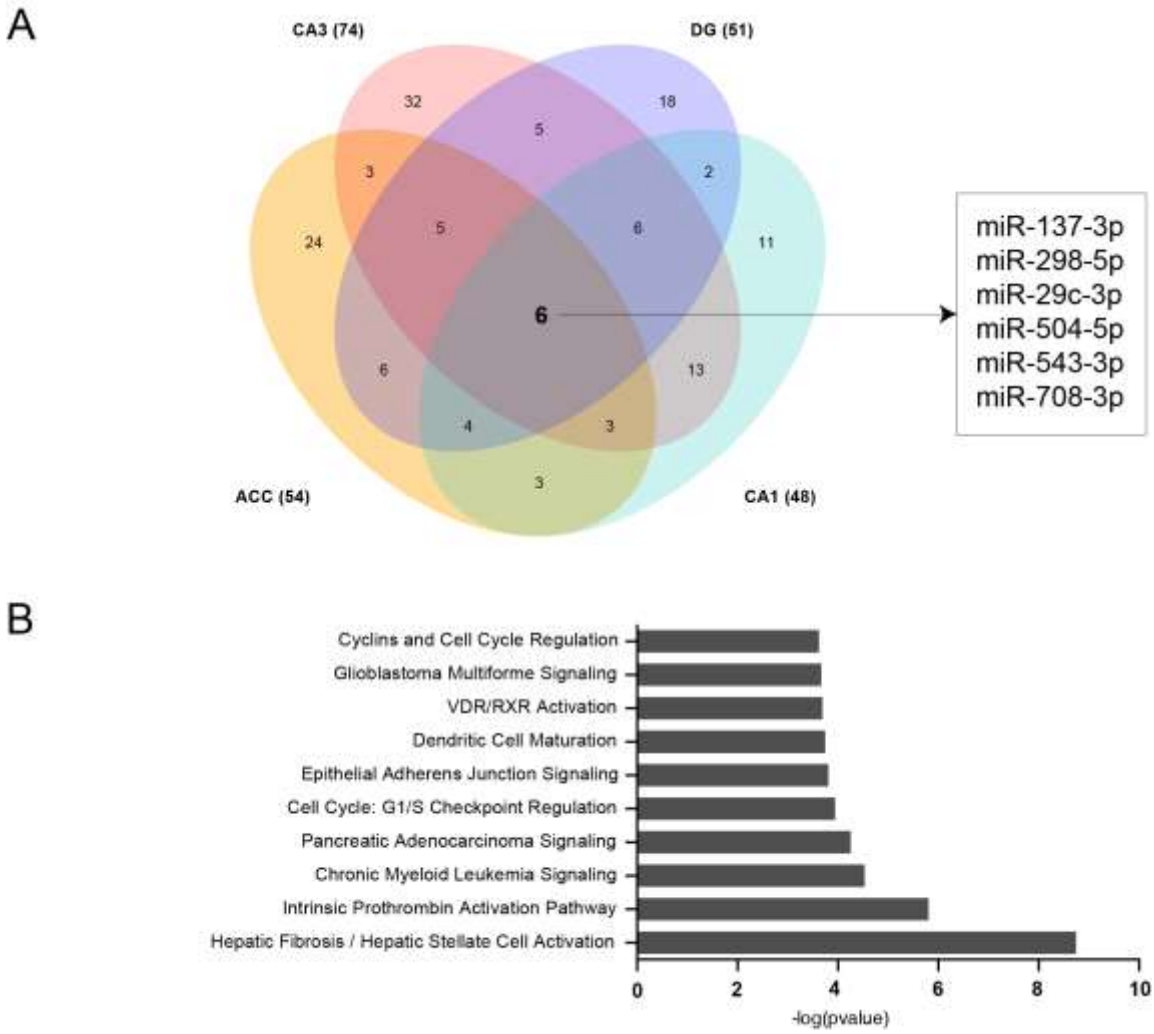

Fig. 3.1.11 Venn diagram shows the commonly downregulated miRNAs in all brain subregions upon aging (A) Top 10 canonical pathways that are associated with the 6 commonly downregulated miRNAs in all brain subregions (B).

Table 3.1 List of miRNAs that are upregulated and downregulated along aging specifically in each memory subregion.

	Upregulated along aging			Downregulated along aging		
ACC	miR-125b-2-3p	miR-3061-3p	miR-431-3p	let-7a-5p	miR-139-5p	miR-412-5p
	miR-125b-5p	miR-326-3p	miR-434-3p	let-7c-5p	miR-1839-5p	miR-495-3p
	miR-127-5p	miR-330-5p	miR-434-5p	let-7d-5p	miR-1843a-3p	miR-598-3p
	miR-1298-3p	miR-338-3p	miR-540-3p	let-7e-5p	miR-185-5p	miR-6540-5p
	miR-132-3p	miR-346-5p	miR-582-3p	let-7f-5p	miR-221-3p	miR-673-5p
	miR-140-3p	miR-370-3p	miR-652-3p	let-7g-5p	miR-3068-3p	miR-6944-3p
	miR-149-5p	miR-381-3p	miR-666-5p	miR-1224-5p	miR-320-3p	miR-7080-3p
	miR-181c-3p	miR-383-5p	miR-676-3p	miR-128-3p	miR-322-3p	miR-7224-3p
	miR-1981-5p	miR-410-3p	miR-7019-3p			
	miR-24-2-5p	miR-423-3p	miR-7046-3p			
			miR-92a-3p			
DG	let-7i-3p	miR-3102-3p	miR-486-5p	miR-101b-3p	miR-324-3p	
	miR-1247-5p	miR-325-3p	miR-543-5p	miR-126a-5p	miR-328-3p	
	miR-128-2-5p	miR-340-3p	miR-664-3p	miR-1306-5p	miR-345-3p	
	miR-132-5p	miR-344d-3-5p	miR-6948-3p	miR-148a-3p	miR-434-3p	
	miR-145a-5p	miR-345-5p	miR-6989-3p	miR-148b-3p	miR-483-3p	
	miR-148a-5p	miR-3535	miR-7044-3p	miR-1964-3p	miR-5099	
	miR-1839-3p	miR-361-5p	miR-7220-5p	miR-21a-5p	miR-92a-3p	
	miR-212-3p	miR-369-5p	miR-8111	miR-26b-5p	miR-98-3p	
	miR-212-5p	miR-376b-5p	miR-874-3p	miR-296-5p	miR-99a-3p	
	mmiR-23b-3p	miR-484				
CA1	let-7d-5p			miR-125b-5p	miR-221-5p	
	miR-185-5p			miR-1298-5p	miR-341-3p	
	miR-195a-5p			miR-140-3p	miR-377-3p	
	miR-335-3p			miR-181a-1-3p	miR-381-3p	
	miR-384-5p			miR-181c-5p	miR-409-5p	
	miR-877-3p				miR-758-3p	
CA3	let-7b-3p	miR-1843a-5p	miR-488-3p	miR-129-1-3p	miR-23b-3p	miR-423-3p
	let-7f-5p	miR-1843b-3p	miR-598-3p	miR-130b-5p	miR-24-2-5p	miR-434-5p
	let-7i-5p	miR-1843b-5p	miR-664-5p	miR-132-5p	miR-27a-3p	miR-487b-3p
	miR-106b-3p	miR-1981-3p	miR-667-3p	miR-138-1-3p	miR-322-5p	miR-582-5p
	miR-10b-5p	miR-3057-5p	miR-671-3p	miR-151-5p	miR-338-3p	miR-708-5p
	miR-125b-1-3p	miR-3078-5p	miR-673-5p	miR-187-3p	miR-340-3p	miR-744-3p
	miR-139-3p	miR-3099-3p	miR-7068-3p	miR-190a-5p	miR-345-5p	miR-770-5p
	miR-148a-3p	miR-323-3p	miR-8114	miR-212-3p	miR-34b-3p	miR-7a-1-3p
	miR-148b-3p	miR-3475-3p	miR-873a-5p	miR-219a-5p	miR-374b-5p	miR-7a-2-3p
	miR-152-3p	miR-369-3p	miR-877-5p	miR-222-3p	miR-376a-5p	miR-874-5p
	miR-184-3p	miR-411-5p	miR-92b-3p	miR-23a-3p	miR-376b-3p	

3.1.3 Blood small RNAome changes along aging

An additional focus of this project so far was to find out the aging-induced changes at epigenome level in memory and learning subregions of the brain in mice. Since searching for miRNA biomarkers in the biofluids is becoming more important in neurodegenerative diseases research field, an interesting approach was to survey aging influence on blood small RNAome as a non-invasive method. Furthermore, it was also of paramount importance to seek if the aging-induced changes in brain could also be detected in blood. Blood was also taken from the same mice that their brain subregions were subjected to RNA isolation and NGS. Similar to brain subregions, the biggest population of small RNAome in blood is also miRNAs but second most abundant small RNAs are piwiRNAs (Fig. 3.1.12). Other small non-coding RNAs like snoRNA, rRNA and sRNA are extremely low.

The highly abundant miRNAs in blood samples is different from highly abundant miRNAs brain samples; miR-451a, miR-92a, and miR-191 are amongst the most expressed miRNAs in blood (Fig. 3.1.13). However there are some common miRNAs between blood and brain highly expressed miRNAs, these miRNAs are: miR-191, miR-22, miR-30a, miR-30d and miR-151. To find out the importance of these differences and similarities more detailed analyses were performed.

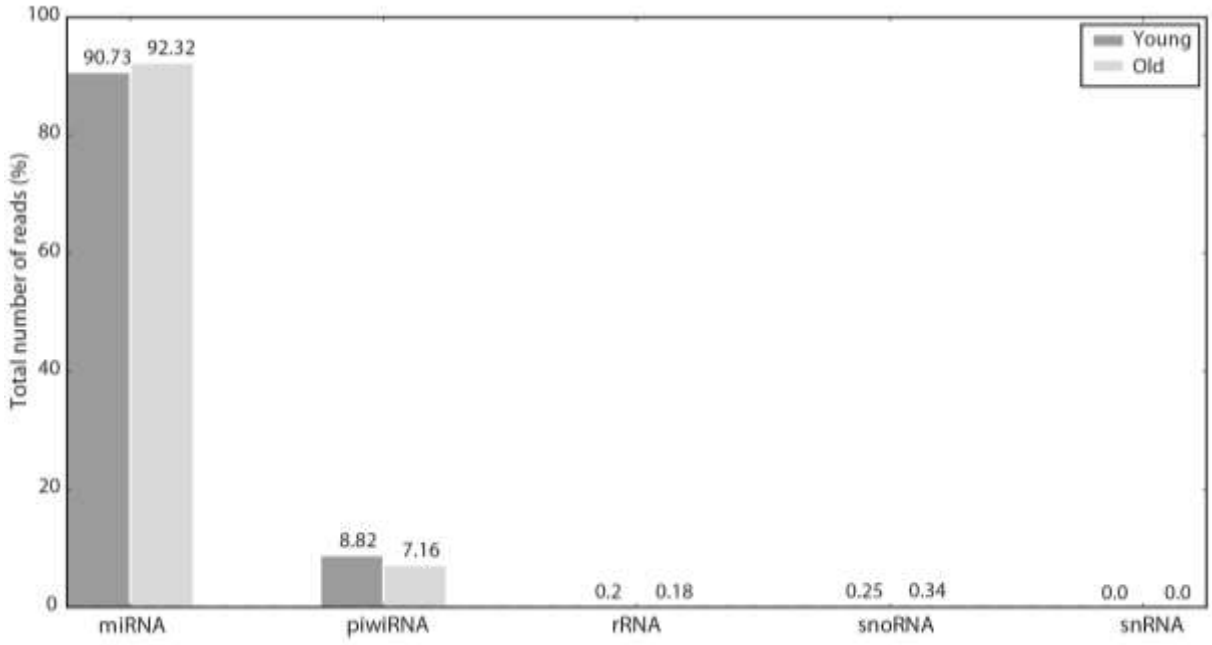

Fig. 3.1.12 The bar graph shows that miRNA are the most abundant class of small RNAs in blood of young and aged mice. PiwiRNAs with 8.82%in young and 7.16% in old animals are the second most abundant small RNA.

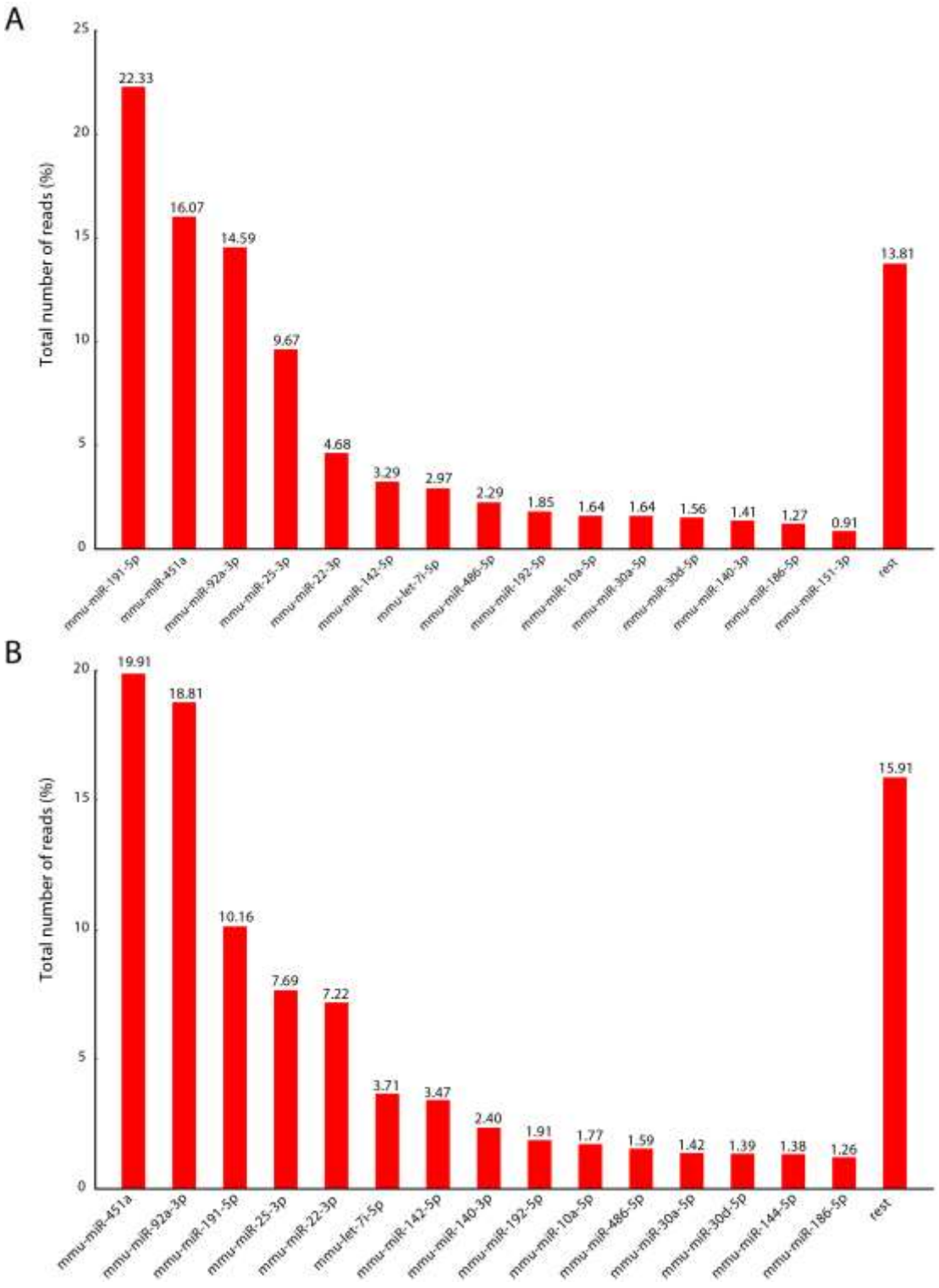

Fig. 3.1.13 Blood miRNA population by frequency of uniquely mapped reads in young (A) and in old mice (B).

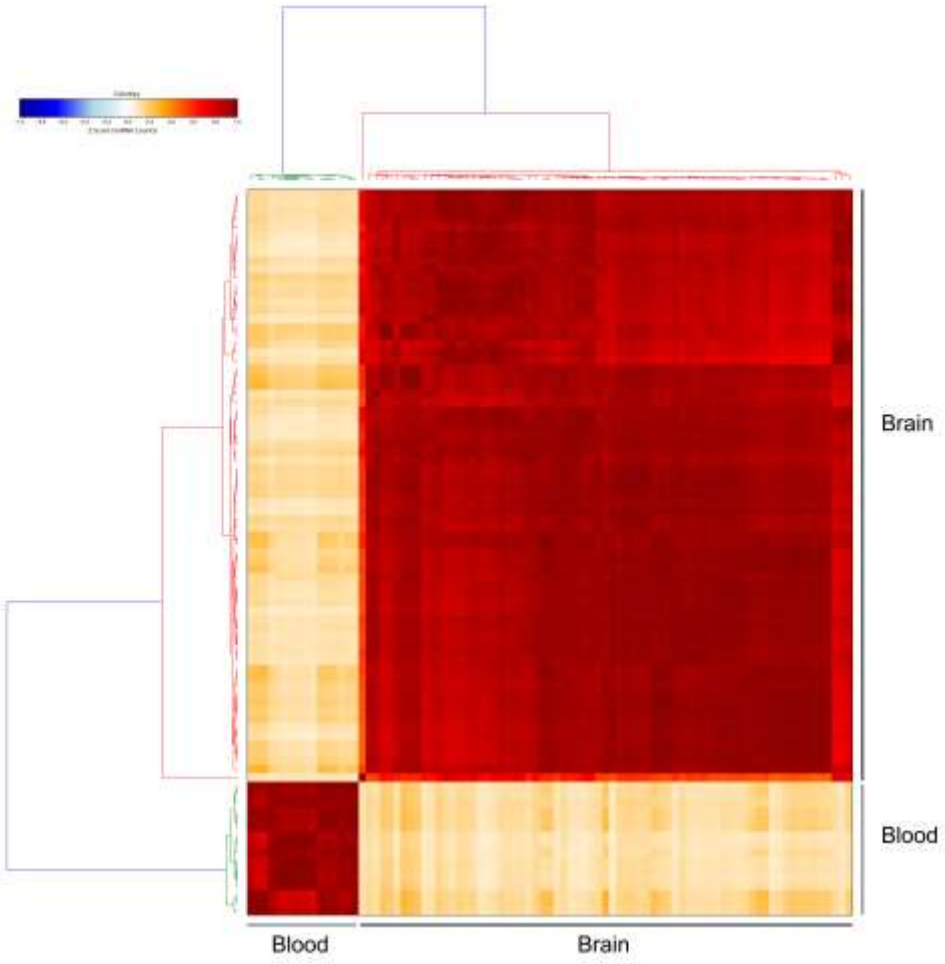

Fig. 3.1.14 Heat map shows the correlation between miRNAs in the brain and blood in both young and old group. Clustering pattern shows brain miRNAome is different from blood miRNAome.

Further clustering analysis shows that the blood miRNAome in young and old mice is substantially different from the same animals' brain miRNAome (Fig 3.1.14). While this is expected, an interesting question here is to see if there is an overlap between the aging brain and blood miRNAome. To this end we first compared the blood miRNAome in young and old mice (Fig 3.1.15).

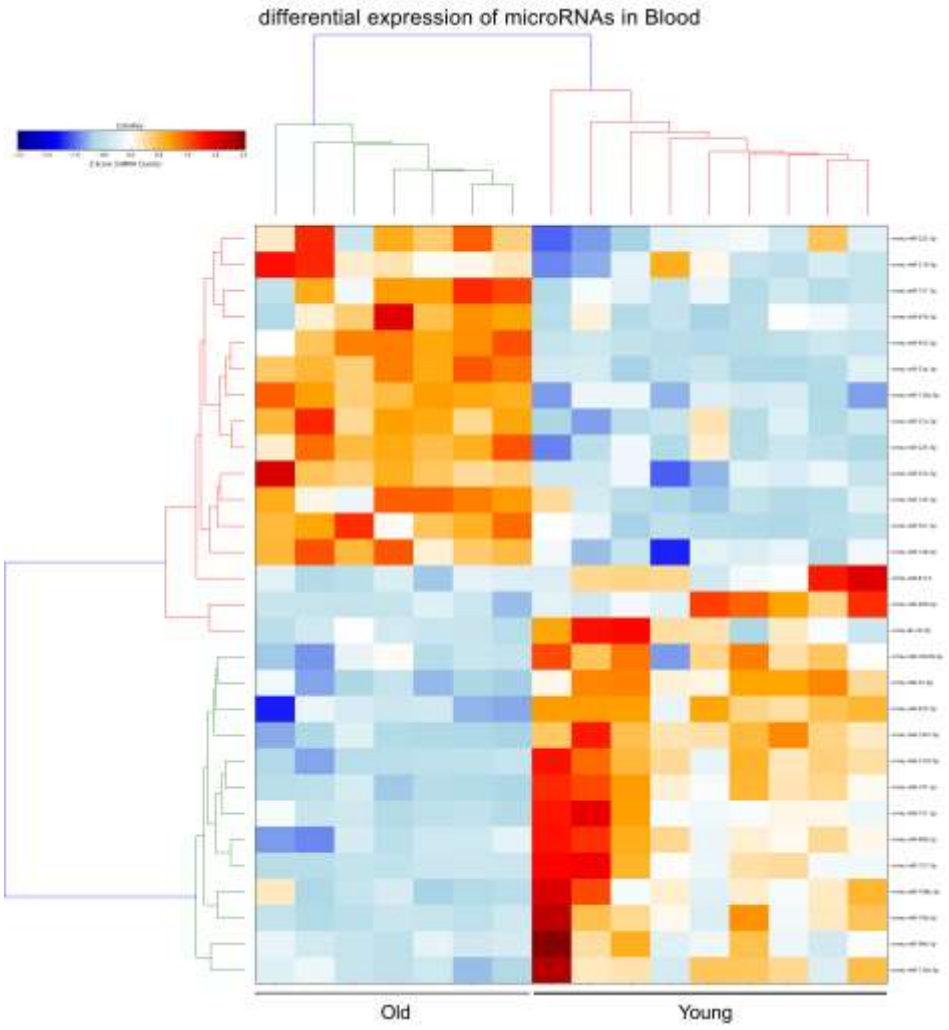

Fig. 3.1.15 Heat map shows the differential miRNA expression changes in blood of old mice.

In order to have a more precise look to blood miRNAome overlapping with memory-associated miRNAome changes, we looked specifically to commonly upregulated and downregulated miRNAs for each brain subregion and blood. The miRNAs that were commonly upregulated in ACC and blood shows 15% similarity (Fig. 3.1.16A) miR-143-3p, miR-151-5p, miR-351-5p, miR-423-3p, miR-676-3p are the common upregulated miRNAs in ACC and blood. The same comparison for DG shows a 15% of similarity (Fig. 3.1.16B) miR-151-5p and miR-23a-3p are the common upregulated miRNAs in DG and blood. CA1 has only one commonly upregulated miRNAs as in blood which is correspondent to 7,6% of similarity, miR-23a-3p is the common upregulated miRNA in CA1 and blood (Fig. 3.1.16C). Comparison between commonly upregulated miRNAs in CA3 and blood shows that 15% of miRNAs have overlapping, miR-143-3p and miR-351-5p are the two commonly upregulated miRNAs in blood and CA3 (Fig. 3.1.16D).

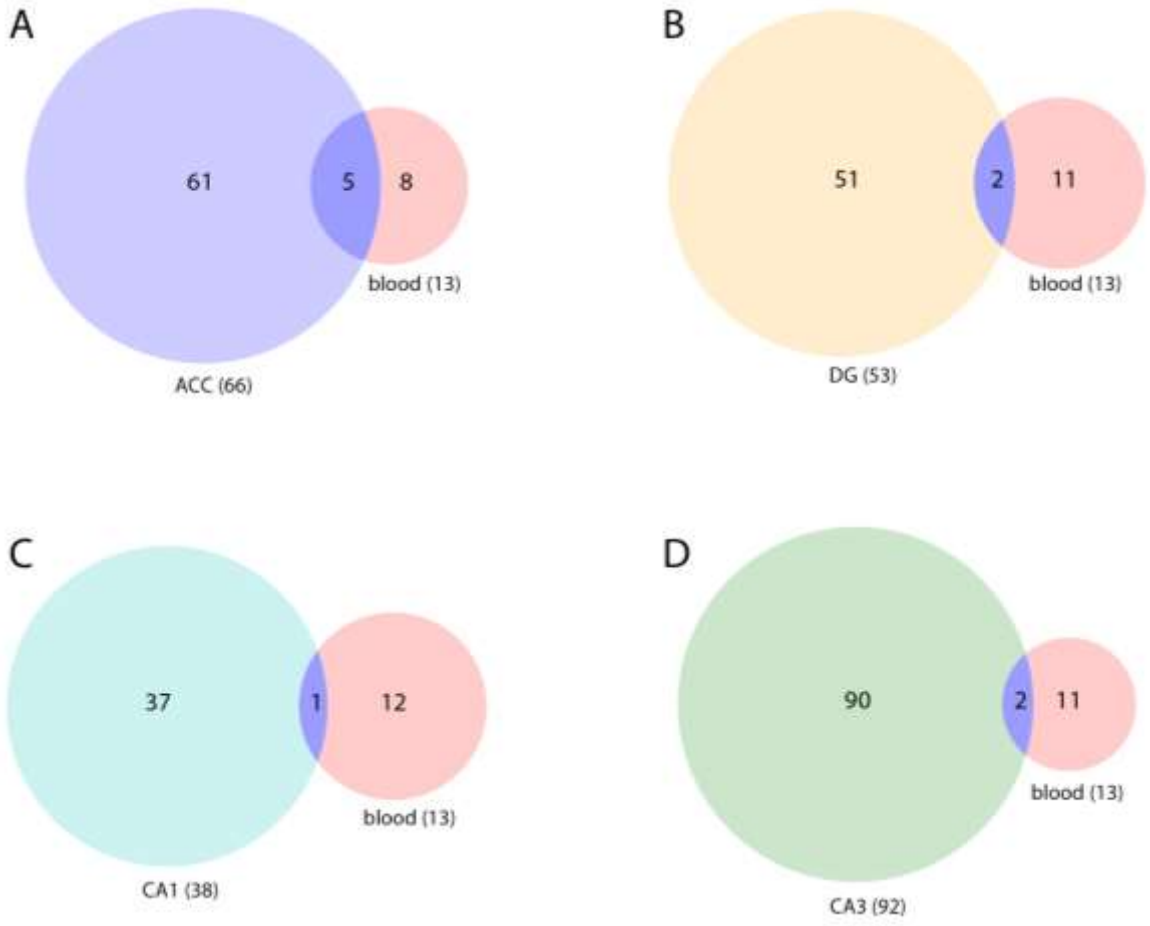

Fig. 3.1.16 Venn diagrams show the number of unique and overlapping upregulated miRNAs between the separate brain regions and blood in the old mice.

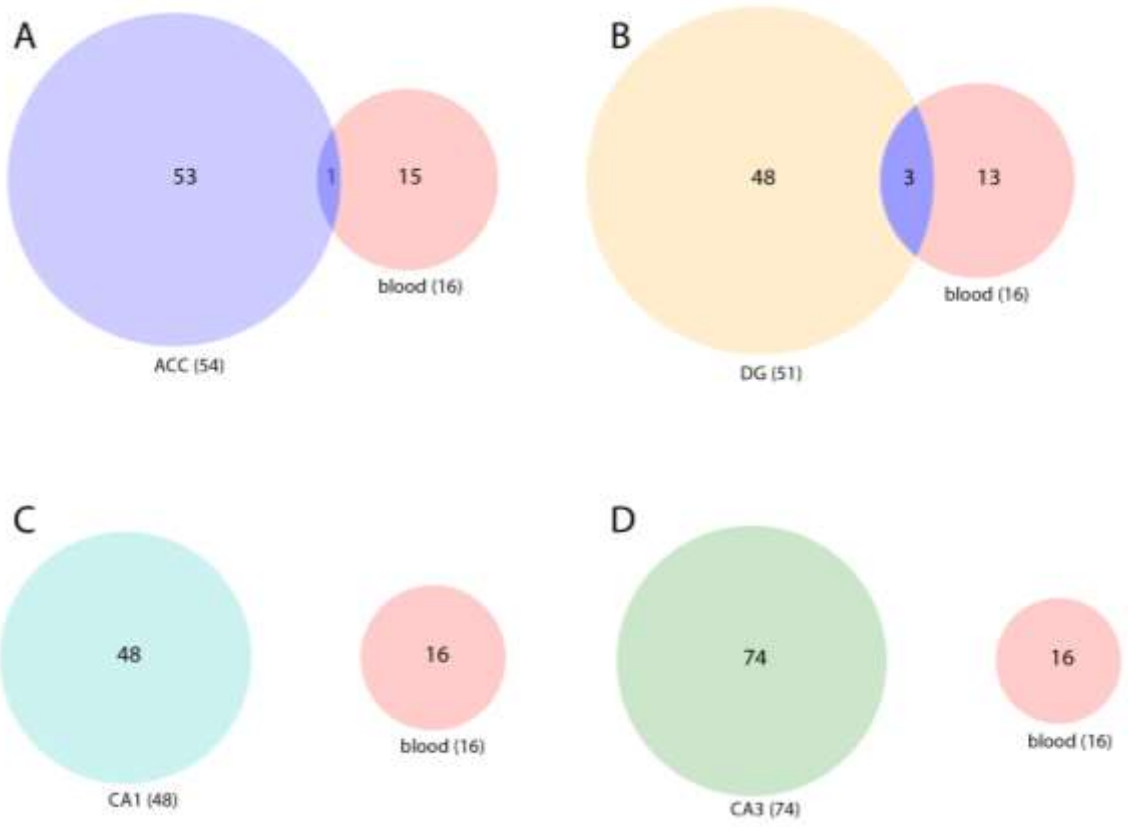

Fig. 3.1.17 Venn diagrams show the number of unique and overlapping downregulated miRNAs between the separate brain regions and blood in the old mice.

As it is pictured in Venn diagrams in Fig 3.1.17 ACC and DG have the common downregulated miRNAs with blood (Fig. 3.1.17A –B) let-7d-3p is the ACC signature in blood while let-7d-3p, miR-126a-5p, miR-26b-5p are DG signatures which can be detected in blood. CA1 and CA3 don't show any commonly downregulated miRNAs with blood.

Rather then assuming that a potential blood miRNAome signature of the aging brain reflects only selected brain subregions, it can also be hypothesized that age-associated changes across various brain regions are – at least partially - reflected in blood. Thus, we treated all miRNA changes observed in the aging brain as one signature. When comparing this signature to the blood miRNAome, nearly 83% of the changing miRNAs seen in blood are also observed in the brain. Performing a hypergeometric test indicated that this finding is highly relevant (< 7.712e-05). We detected 29 differentially expressed miRNAs (log2 fold change > 0,3; padj 0,05 and basemean of 25 counts, excluding two samples as outliers). The below listed miRNAs are the 24 differentially expressed miRNAs in the old brain and blood: let-7d-3p, miR-106b-3p, miR-10b-5p, miR-126a-5p, miR-127-3p, miR-128-3p, miR-143-3p, miR-151-

3p, miR-151-5p, miR-1843b-3p, miR-191-5p, miR-222-3p, miR-23a-3p, miR-25-3p, miR-26b-5p, miR-27a-3p, miR-351-5p, miR-423-3p, miR-425-5p, miR-486-5p, miR-532-5p, miR-676-3, miR-8114 and miR-99a-5p (Fig. 3.1.18A)

Among these 24 deregulated miRNAs 9 miRNAs have the same deregulation pattern, which is around 31% of the deregulated miRNAs in blood. The 6 common upregulated miRNAs are: miR-143-3p, miR-151-5p, miR-23a-3p, miR-351-5p, miR-423-3p, miR-676-3p (Fig. 3.1.18B). The 3 common downregulated miRNAs are: let-7d-3p, miR-126a-5p, miR-26b-5p (Fig. 3.1.18C). Pathway analyses for commonly downregulated and commonly upregulated miRNAs in brain and blood shows that these miRNAs are linked to tumor prevention pathway, cell apoptosis and cell antiproliferation mechanisms like p53, cell apoptosis signaling and TGF-beta respectively (Transforming growth factor beta) signaling (Fig.3.1.19 A and B). Although the canonical pathways that are regulated by commonly upregulated and downregulated miRNAs are different, these pathways show a high level of unity in terms of mechanisms that they regulate.

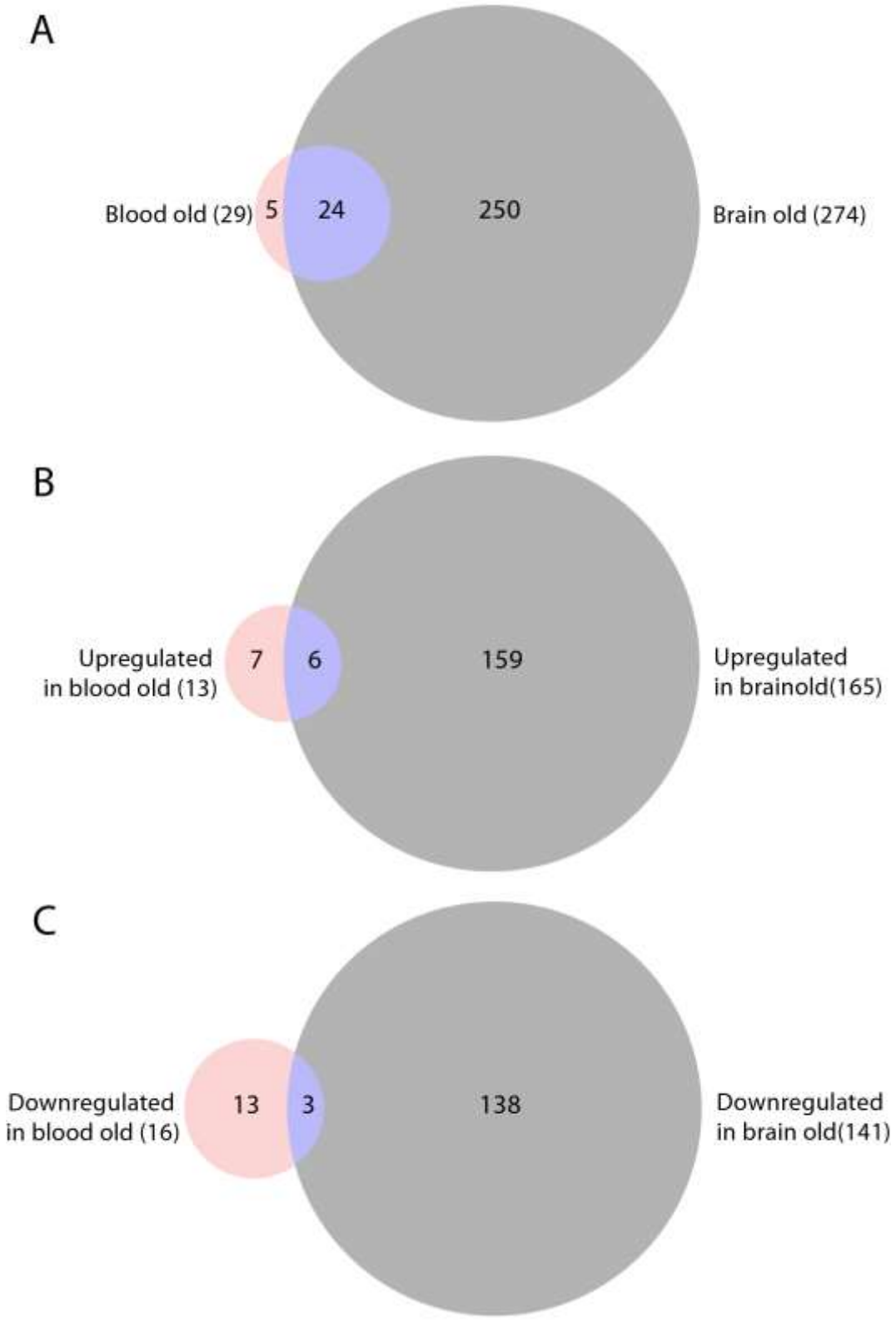

Fig. 3.1.18 Venn diagrams show the number of unique and overlapping upregulated and down regulated miRNAs between the whole brain subregions and blood in the old mice.

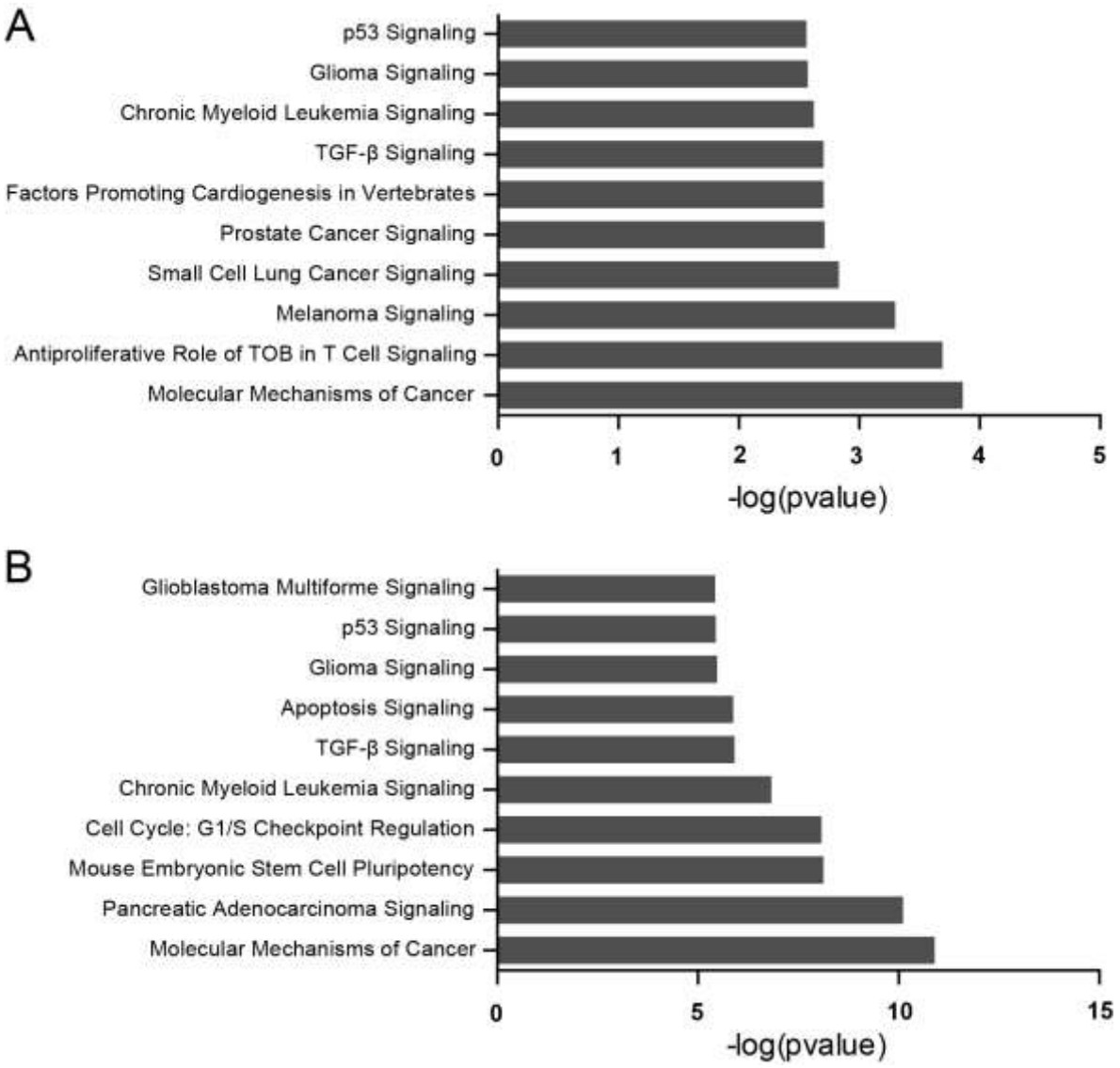

Fig. 3.1.19 Top 10 canonical pathways that are linked to commonly downregulated (A) and commonly upregulated miRNAs (B) in brain regions and blood.

3.1.4 Other small RNAs in brain and blood

Data analyses show that other small RNAs are also present in the brain and blood as well. The population of these non-miRNA small RNAs is different in brain and blood. SnoRNA is found as the second most abundant small RNA in young and old brain (1.65% and 1.81% respectively (Fig 3.1.1 and Fig. 3.1.5)). Fig (3.1.20 A-D) shows the heat map for ACC, DG, CA1 and CA3 for differentially expressed snoRNAs. As it is obvious the abundance of snoRNAs is not the same in all four brain subregions. CA1 shows the less number of differentially expressed snoRNAs. SnoRNAs that are highly abundant in all brain subregion in young and old mice are: Scarna 3a, Snord 85, Snord 99, Snord 104, Snorna 36b, Mir 1839, Snord 12, Snord 57 and Scarna 3b. Different nomenclature for snoRNAs arises from their found sites in the cells, Scarna (small Cajal-body specific RNAs) is found with cajal bodies that are subnuclear complexes in the cells. Mir 1839 has been named as a miRNA because of its similarity with miRNAs in terms of function.

In blood samples, piwiRNA is the second most abundant smallRNA class in both young and old mice with 8.82% and 7.16% respectively (Fig. 3.1.12). DQ695413 is the most abundant piwiRNA in young and old group and other piwiRNAs are expressed or detected at a very low level (Fig. 3.1.21). According to piwiRNA bank DQ695413 is the accession number for mmu-piR-016327, which is present in chromosome Y and 22 base pairs in size. Interestingly piwiRNA is the largest class of non-coding RNAs in eukaryotic cells and found mostley in germ line cells. Unlike snoRNAs that show some similarities with miRNAs in terms of biogenesis and function, piwiRNAs biogenesis and function is distinctive from miRNAs.

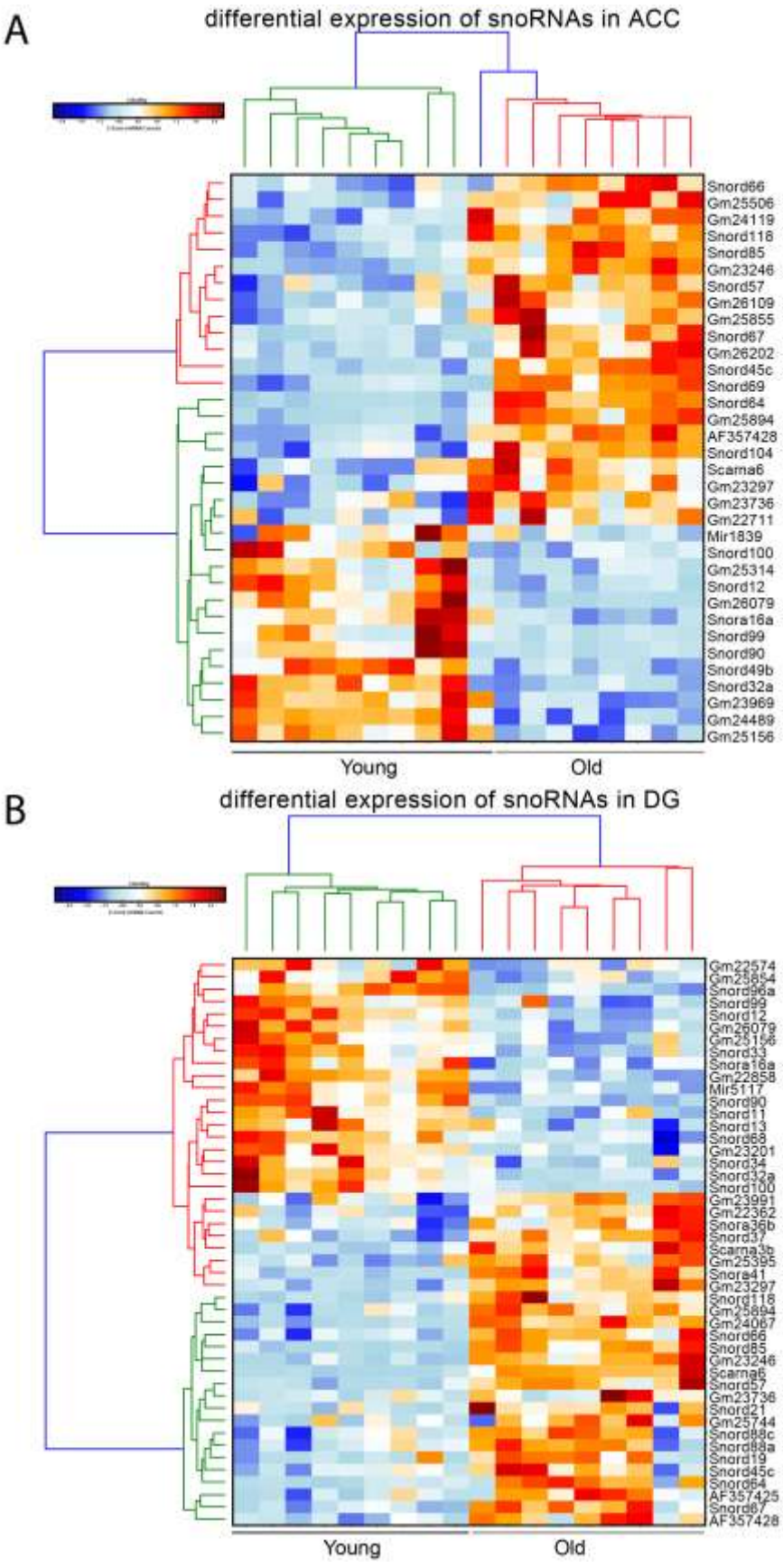

Fig. 3.1.20 (A-B) Heat map shows the Euclidean distances between the differentially expressed snoRNAs in ACC (A), DG (B) from old over young mice.

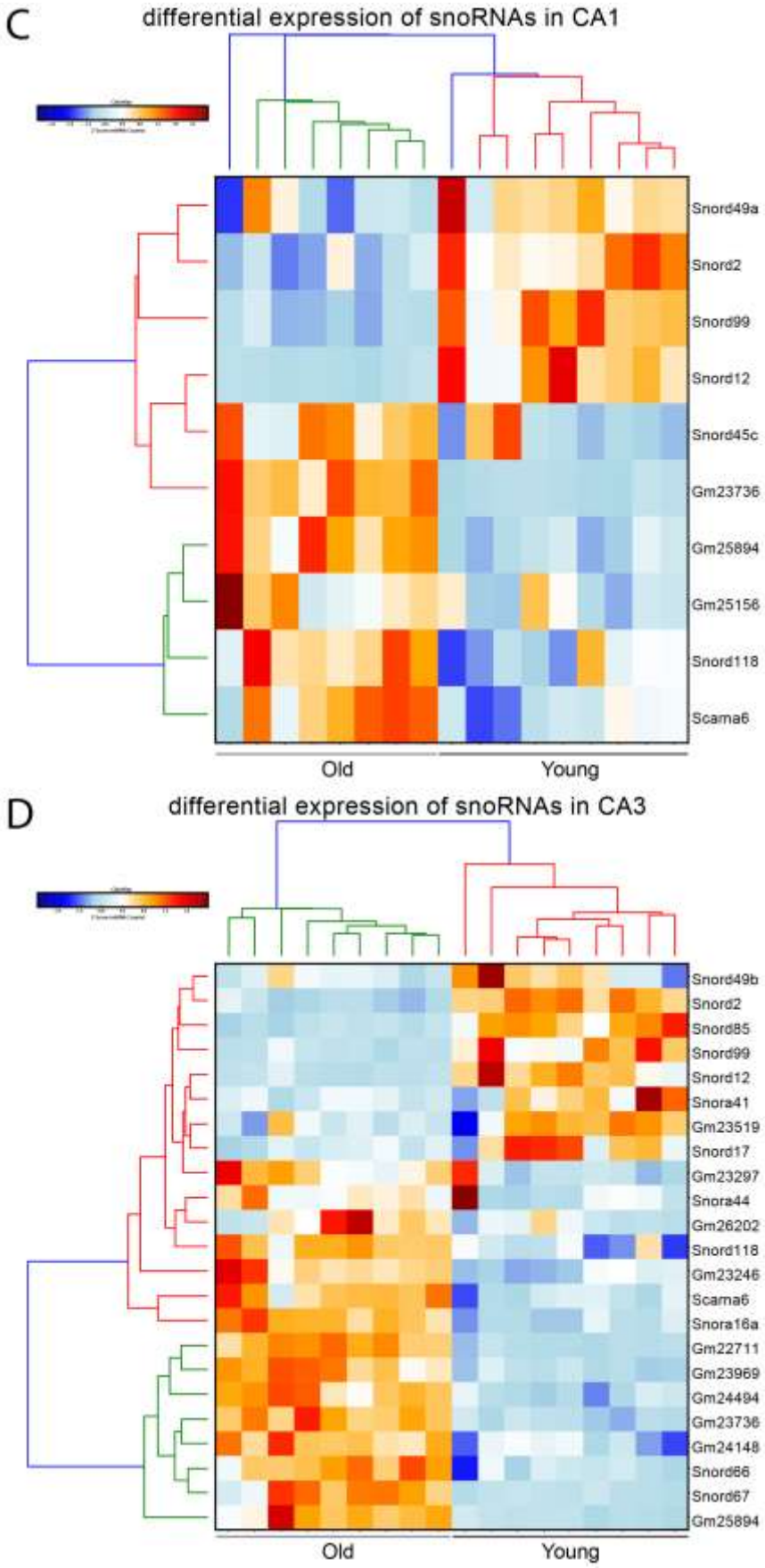

Fig. 3.1.20 (C-D) Heat map shows the Euclidean distances between the differentially expressed snoRNAs in CA1 (C) and CA3 (D) from old over young mice.

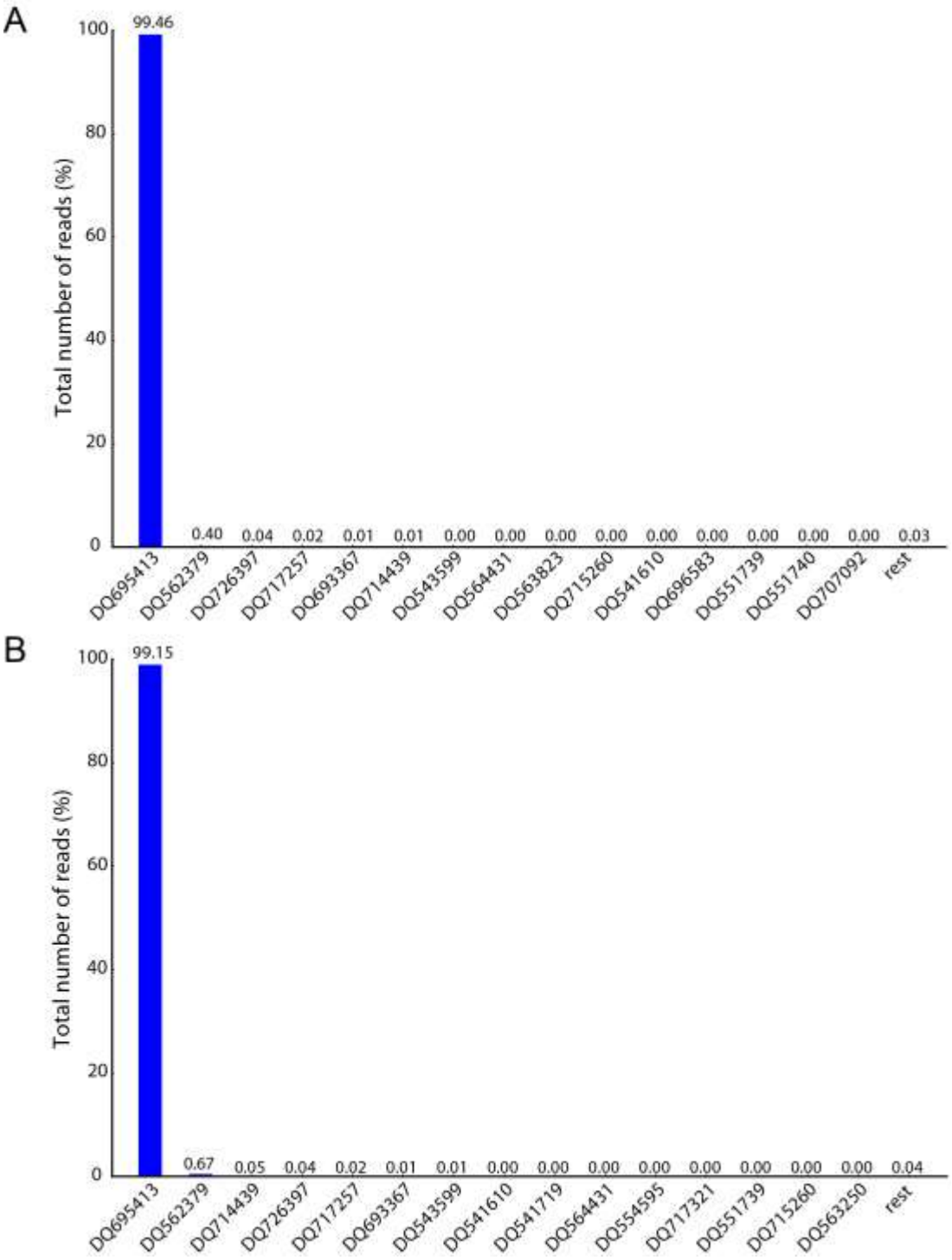

Fig. 3.1.21 PiwiRNA population by frequency of uniquely mapped reads in blood samples from young (A) and old mice (B).

3.2 MiRNAs as therapeutic targets in Alzheimer's disease

Pioneering studies have shown that deregulation of miRNAs plays a significant role in pathological mechanisms. Therefore one interesting approach is to suppress the expression of disease-related miRNAs. In order to circumvent the obstacles in miRNA delivery process, different methods have been used and proposed. In the present study the main focus was to use exosomes as delivery vehicles for miRNA inhibitory molecules. To increase the efficiency of delivery by exosomes they should be modified specifically for the target cells, which in our case are neuronal cells. The following sections show the results of applying measles virus glycoprotein (MVG) pseudotyping system in order to producing specific exosomes, which target only neurons.

3.2.1 Measles virus glycoproteins can be sorted in the exosomes

Exosomes were prepared from the cell culture medium of the N2a cells cotransfected with plasmids encoding the wild type and mutant variants of the MVG H and F. As explained before the aim of plasmid transfection was to obtain modified exosome with more efficient delivery capability for neuronal cells. N2a cell lysates were used for protein extraction, which served as a positive control for plasmid transfection. All exosome preparations were positive for the exosomal marker protein Flotillin-2 (Fig. 3.2.1). Additionally, the presence of the wild and mutant forms of the glycoprotein H and F was shown in all exosome preparations, suggesting that these proteins are highly enriched in exosomes (Fig. 3.2.1). All three variants of the H protein differing in their size were detected with the same antibody (H 606). For the F proteins (wild type and two mutants) F431 antibody was used, according to the western blot signals Hdc18 and Fdc30 were chosen for pseudotyping the exosomes (facing page).

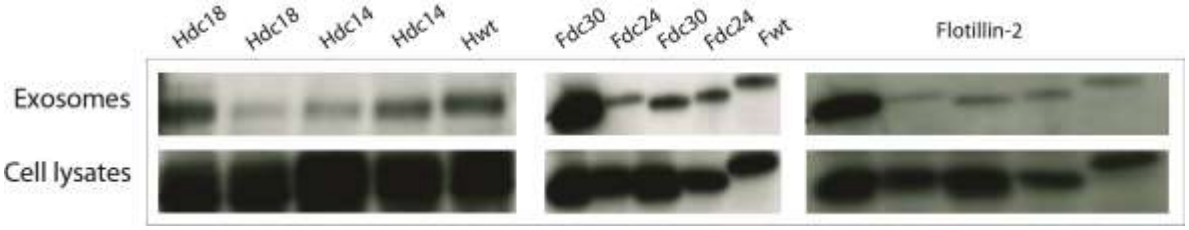

Fig. 3.2.1 Exemplary picture of the western blot analysis of the protein preparations from the exosomes from the cell culture medium and N2a cell lysates. The western blot shows the presence of measles virus H and F proteins, strong signals of Flotillin-2 serve as a quality check for the successful exosome extraction.

3.2.2 Modification of the exosomes with the measles virus glycoproteins did not change efficiency of the cargo delivery

Exosomes prepared from the culture medium of transfected (see section 2.3.1) and not transfected cells were first electroporated with Luciferase GL3 specific siRNA and then added to HT1080Luc cells. Standard siRNA transfection using oligofectamine reagent was performed to check specificity and efficiency of the chosen siRNA. As shown in Fig. 3.2.2 and 3.2.3, both modified and unmodified exosomes were able to deliver siRNA to cells but without any difference. This experiment was performed with two different amounts of exosomes and siRNAs (Fig. 3.2.2 and 3.2.3). The knock down effect of 3µg exosomes and 3µg siRNAs is not significant while knock down effect of 10µg exosomes and 10µg siRNAs seems to be significant. Of note, knockdown effect of siRNA from the exosomes is much milder than in complex with oligofectamine, although statistically significant compared to control (facing page). However this project was not continued since the preliminary results were not convincing and for improving the results the available technology was not efficient (see part 4.2.2 in discussion)

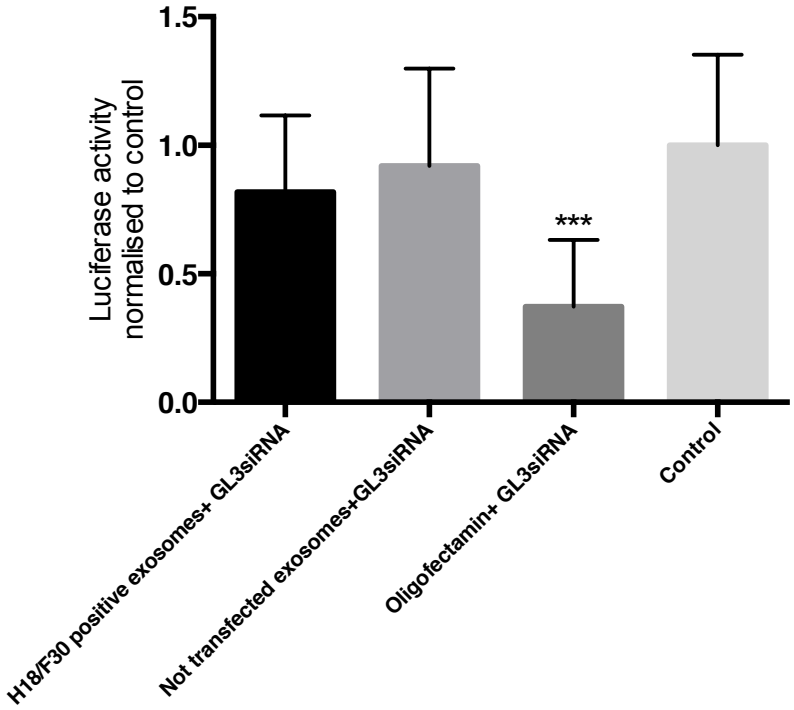

Fig.3.2.2 Results of the luminometer analyses of exosomal delivery of 3 µg GL3 siRNA. 3 µg exosomes carrying the measles virus glycoproteins deliver siRNA to the target cells as efficient as exosomes without modifications. Error bars show standard deviation.

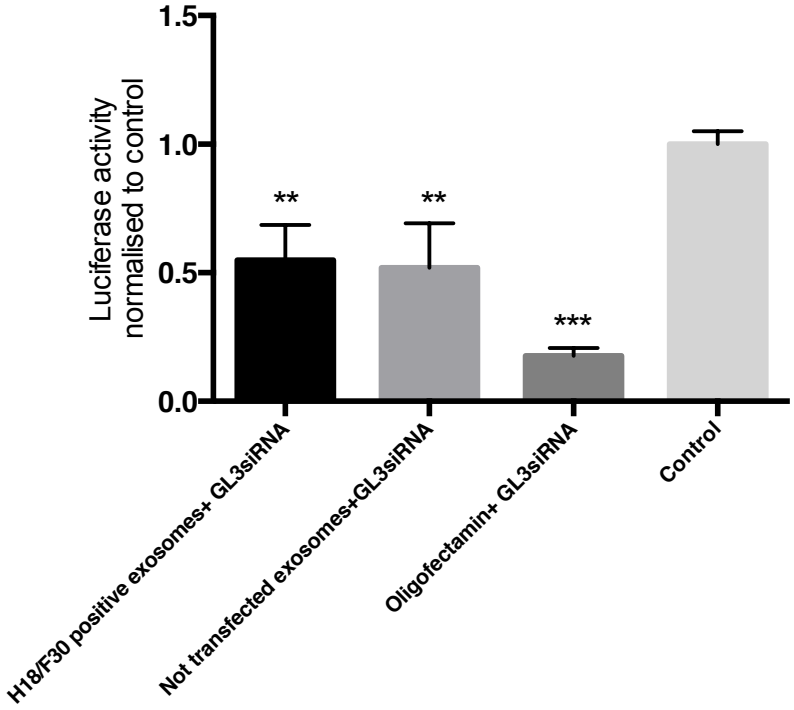

Fig.3.2.3 Results of the luminometer analyses of exosomal delivery of 10µg GL3 siRNA. 10µg exosomes carrying the measles virus glycoproteins deliver siRNA to the target cells as efficient as exosomes without modifications. Error bars show standard deviation.

Discussion

4.1 Impact of aging on the brain

4.1.1 Small RNA expression in memory subregions changes with aging

Neurodegeneration manifestations are obvious at the late stages of the AD when no cure is effective. Considering aging as the major risk factor for AD and exploring the changes at molecular level could be a promising approach to figure out the underlying biological abnormalities that increase the chance of AD in late elderly. So far the knowledge about molecular mechanisms of human AD are based on the post-mortem observations. Studies that could trace the changes in the aging brain and simultaneously seek for malignancies along aging are not feasible in human. In this study we used young (4-month-old) and old mice with 18 months of age that corresponds to late middle age in human and performed high throughput next generation sequencing (NGS) to detect the changes at small RNA level along aging. The results show that aging causes a remarkable change in the small RNA profile of the brain subregions that are intimately linked to learning, memory and cognitive functions. Previous studies were performed in mice, primates and also post mortem tissues from humans to study the influence of aging on the gene expression regulation patterns and pathways in the brain (Lee et al., 2000; Jiang et al., 2001; Fraser et al., 2005; Lu et al., 2004). However these studies did not put their focus on the regulation of the affected pathways during aging in the brain. To include the role of miRNAs as a regulatory molecule of the aging-related pathways a similar study to ours elucidated the role of miRNAs in the aging-associated pathways in the mouse brain (Inukai et al., 2012). In this study using Solexa technology they could show that many miRNAs including several novel miRNAs are differentially expressed upon aging and the pathways like insulin signaling pathway that are regulated by novel miRNAs along aging have significant roles in the process of aging.

However in this study they used the whole brain and the main emphasis was to find out the novel miRNAs that are expressed during aging, while in our study we focused mainly on the expression pattern of the miRNAs and other non-coding small RNAs in each memory associated subregion of the brain along aging.

4.1.2 MiRNAome changes along aging in memory subregions distinctly

Along with other biological changes in the process of aging the miRNA profile and its expression pattern also goes under changes. The alteration in miRNAome can be considered as an important factor because this biomolecule has significant regulatory role in crucial

biological pathways. Furthermore, the regulatory role of miRNAs in the aging network has been confirmed before (Chen et al., 2010)

The results in this study show that the largest proportion of the small RNAs in brain subregions is miRNA and snoRNA is the second most abundant small non-coding RNA and other small non-coding RNAs like piwiRNA and rRNA are present in a very low range (Fig.3.1.1 and Fig.3.1.5). One reasoning behind this could be Truseq small RNA kit that was used for preparing the small RNA library. Although Truseq small RNA is designed for high throughput sequencing of small RNAs it has an affinity for miRNAs that are generated by Dicer processing and it is because of the modified adaptor targeting. However there is no gold standard method for miRNA expression profiling and each method has its biases that should be taken into consideration. According to our results aging induces remarkable changes in the miRNA profile. Since the only difference between the groups of animals was age, therefore it can be claimed that aging causes this profile change in the miRNAome of the brain. A remarkable point that came out from the results of this study was that the changes in miRNA profile and expression was specific to each subregion. Aside from this specific differential expression, we could show that miR-127 and miR-128 and members of the let-7 group are among the commonly expressed miRNAs in all of young and old brain subregions, the presence of these miRNAs in brain tissues was shown before (Bak et al., 2008, Babak et al., 2004; Barad et al., 2004). Of note the presence of region-specific miRNAs is of paramount importance as it can suggest that each region has its own specific regulatory miRNAs, which can regulate region-specific functions. MiRNAs regulate protein expression at posttranscriptional level and a tremendous number of proteins, which are regulated by miRNAs, are key players of the biological pathways. Therefore difference between miRNA species of the memory subregions would suggest the presence of different biological pathways.

According to our results after miRNA snoRNA is the most abundant small non-coding RNA in the brain subregions (Fig. 3.1.1 and Fig.3.1.5). Although the frequency of this class of small RNA in young and old mice is under 2%, Euclidean distance analyses could be performed for all subregions (Fig. 3.1.20). Presence of snoRNAs in central nervous system has been shown before and the role of some specific snoRNAs in learning and memory has also been studied (Rogelj et al., 2003). In this study they could show that hippocampal snoRNAs regulate higher cognitive functions of the brain. Role of snoRNAs in diseases has been first observed in cancer. It has been shown that differentially expressed snoRNAs and snoRNAs with mutations are crucial in development of lung cancer and prostate cancer

respectively (Liao et al., 2010; Dong et al., 2008). So far Prader-Willi syndrome is the most-studied neurological disorder that is associated with mutations in snoRNAs (Sahoo et al., 2008) and there has been no specific role for snoRNAs in AD. However the significant role of snoRNA in gene expression flexibility can suggest potentials for this class of small non-coding RNAs in neuropathological processes.

4.1.3 Aging-associated inflammatory and immune system activation pathways

Diverse molecular pathways and biochemical mechanisms control the process of aging. In our study the role of miRNAs in regulating aging-associated pathways were analyzed by IPA (Ingenuity software). The results show that miRNAs that are differentially expressed upon aging control the immune system activity-related pathways (Fig. 3.1.10 and Fig.3.1.11). The deregulated activation of immune system indicates the over expression of inflammation mechanisms.

However the mechanisms related to aging is not similar in all species since life span in different species is not the same, mechanisms like oxidative stress and telomerase shortening are species-specific (Kim 2007). Beside specific aging-associated pathways there are also common pathways, which are happening in all species in old ages mechanisms like inflammation (Franceschi et al., 2000). In line with this it should be taken into account that computational analyses that are used for miRNA target prediction provide helpful tool for miRNA research but it should be noted that there is no prediction algorithm that can capture all of the biological aspects of the miRNAs.

4.1.4 MiRNA as a predictive biomarker

A good and reliable molecular biomarker should be detectable, robust and specific. The choice of the biomarker could have different reasoning behind. One decisive factor for choosing the biomarker is the nature of the disease. There are some diseases that usually begin with no symptom like AD and in this case biomarkers help to identify the individuals with high risk for the disease (Craig-Schapiro et al., 2009). In our study we focused on aging as a risk factor for AD and sought for the differentially expressed miRNAs in aged animals to find any similarities between the miRNA profile of the aged animals and miRNA profile in AD. Our results show that miRNA expression in brain tissue is changing massively in aged mice (Fig. 3.1.7). Among deregulated miRNAs miR34c is downregulated in hippocampal subregions while in AD disease mouse model and patients this miRNA is upregulated

(Zovoilis et al., 2011). This observation can suggest that in healthy aged animals in contrast to AD mouse model miR34c is not upregulated. According to our results there are also some miRNAs that are highly upregulated in one subregion and exactly the same miRNA is downregulated in another subregion. Among these differentially deregulated miRNAs miR-212 was particularly interesting since this miRNA was only upregulated in DG of the old mice and downregulated in other memory subregions. This finding is in accordance with previous studies on DG that showed this miRNA is highly expressed in granular cells of the DG (Magill et al., 2010). One possible explanation for the upregulation of this miRNA in DG upon aging could be the compensatory role of this miRNA for producing more newborn neurons to replenish the deleterious effect of the aging-associated processes. In total our results can lead to this point that miRNA expression profile is an indicator for aging in brain memory subregions.

Anyhow using miRNAs as diagnostic, prognostic or predictive biomarkers or surrogate endpoint of a disease is still in its infancy. There is still a long way of research ahead in order to overcome the shortcomings of this field of biomarker research.

4.1.5 Choice of biofluid for biomarker studies

Studies in the area of neurodegenerative diseases favor CSF as the most reliable body fluid in terms of biomarker research, simply because it bathes the brain. However there are considerable complications with using CSF as the source of biomarker. CSF collection is an invasive method and different factors can cause variability in the CSF samples. However other body fluids like urine, blood serum or even tears (Weber et al., 2010) have been used as biomarker sources for diseases. Kumar et al. (2013) published a list of plasma-based miRNAs as AD signature. In this study we used blood as the source of circulating miRNA since among above-mentioned body fluids so far blood offers the most non-invasive analysis tool.

4.1.6 Blood indicates changes in the aging brain

It was previously shown that blood is sensitive to changes in the central nervous system and is contributed to gene expression patterns (Scherzer et al., 2007). Our results show that small RNAome of young and old animals are distinctive and like brain samples miRNA is the largest proportion of the small RNAome in the blood samples of young and old animals and piwiRNA is coming up as the second abundant small RNA (Fig. 3.1.12). This shows that

small RNAome population in brain and blood are different. A previous study revealed that the piwiRNA proportion of blood in comparison to this in plasma is larger than in plasma (Spornraft et al., 2015). However we put the main focus on comparison between miRNAs of blood and brain.

Although correlation analyses between brain and blood show that there is a considerable discrepancy between miRNAome in blood and brain we could detect a convincing rate of similarity between miRNAome of blood and brain subregions that were differentially expressed along aging.

Of note the number of miRNAs in blood is very low in comparison to brain tissues. One reason for that could be the amount of blood that is subjected to RNA isolation. The amount of blood that is used for the whole RNA isolation and NGS process is on one hand a small proportion of the whole blood of the animal and on the other hand it is highly diluted in comparison to solid brain tissues. It might be that if the whole blood could be used for NGS we could observe a larger amount of miRNAs and consequently more common miRNAs between brain and blood. However numerous studies have used blood as biomarker source. Blood-based proteins have been studied as reliable biomarkers for AD (Doecke et al., 2012; Tan et al., 2012). Another study on AD biomarkers proposed a blood-based 12-miRNA signature (Leidinger et al., 2013). A novel aspect of our study in comparison to the similar studies was that we compared the miRNAome of blood with four subregion of the brain that are closely related to memory and cognition. These comparisons show that miRNA deregulation in each region is to a great extent detectable in blood (Fig. 3.1.16 and Fig. 3.1.17) and when we take all brain subregions as a whole and compare it to blood the number of similarly deregulated miRNAs is noticeable.

In this study we could detect one similar signature miRNA (miR-26b) in blood as it was reported in Leidinger et al. (2013) study for AD. Interestingly the regulation pattern of this miRNA is downregulated upon aging, which is similar to the regulation pattern of this miRNA in AD patients. However how the aging- induced miRNAs in blood can be related to AD-induced miRNAs in blood is a matter of debate.

Aside from all benefits that blood provides for biomarker research it should also be taken into consideration that blood is a heterogeneous fluid with different cellular and molecular components that can cause contamination in terms of biomarker study.

4.2 MiRNA as therapeutic target

4.2.1 Neuronal targeting of the exosomes

Role of deregulated miRNAs in pathological processes makes this biomolecule as a proper target for therapy. In this study we were planning to apply siRNA as inhibitors for miRNAs. Since the target miRNA of our study was shown to be upregulated in neuronal cells therefore in order to delivering the inhibitory siRNA to the neuronal cells exosomes were used as cargo vehicles.

Using exosome as drug carrier is becoming more popular in neuropathological area since it can cross the blood brain barrier. Numerous studies in the field of exosomes have immense impact on our understanding of possible applications and potentials of the exosome in this regard. In this project we investigated the possibility of pseudotyping of exosomes with measles virus glycoproteins (MVG). A similar study to ours used other virus glycoproteins namely rabid virus glycoprotein (RVG) to specify the exosomes for the target cells (Alvarez-Erviti et al., 2011). This specification made the exosomes specific for neural cells while our approach was to target only neurons and with this approach we used MVG. Our results show that MVG variants can be sorted in the exosomes. As control we used Flotillin to confirm the accuracy of exosome preparation. Using virus glycoproteins offers a pragmatic tool for adopting exosomes for target cells but on the other hand it can function as the immune system suppressor or stimulator and consequentially have some undesired effects on the organism. However applying exosomes as drug carriers is an emerging research field in contrast liposomes that are synthetic phospholipid vesicles are in use as drug carriers since several years. Exosomes offer some benefits in comparison to liposomes and on the other hand liposomes have some properties which exosomes are lacking them. One smart strategy could be to combine beneficial features of liposomes and exosomes to develop an efficient drug delivery system (van der Meel et al., 2014)

4.2.2 Loading the exosomes with desired cargo

Exosomes are endogenous nanovesicles that transfer different kinds of molecules between the cells. This natural potentiality of the exosomes as carriers can be utilized in pharmaceutical research. There are different methods to load the exosomes with desired cargos. In the present study electroporation was used in order to load the exosomes with siRNAs. As it is shown in luciferase assay results by using relatively high amount of exosomes and siRNAs an adequate delivery effect can be achieved. Anyway there was no difference between the delivery efficiency of the native exosomes and neuronal specified

exosomes. The electroporated exosomes in both cases (native and neuronal specified) showed less luciferase activity suppression in comparison to oligofectamine method.

This fact and similarity between delivery efficacies of both exosome variants raises these questions:

1) Do the exosomes after electroporation retain their shape?
2) Does electroporation load the exosomes with siRNA?

The study that was done by Kooijmans et al. (2013) revealed that electroporation doesn't load the exosomes with siRNAs. Electroporation of siRNAs and exosomes results into aggregation of siRNAs. This can be a persuasive explanation for the failure of our electroporation trails. Furthermore electroporation can cause the aggregation of the exosomes but this may be solved by optimization of the electroporation factors (Hood et al., 2013). Other methods that have been used or proposed in this regard have been mentioned before in Table 1.1 as it is obvious electroporation is ranked as the most applied method to load the exosomes with cargoes, incubation, transfection and cell activation are listed as the other methods in this regard. Although none of the above-mentioned tools offer an absolutely effective method to load the exosomes this area of research is highly promising. Further research and technological advances are required to fulfill the exosome application as a reliable and effective drug carrier.

Conclusion

5.1 Aging-associated changes in miRNA expression profile

The first part of this study was performed to identify the changes at molecular level that occur in association with aging. The aged-induced expression changes in miRNAome of blood was also observed and compared with change pattern in brain to figure out the overlapping. Based on the results we can conclude that:

- MiRNA expression level goes under profound changes along aging.
- The fold change in miRNA expression levels can be detected precisely in each subregion of the memory in the aging mouse brain.

5.2 Exosomal delivery of miRNA inhibitory molecules

The second part of this study was conducted to examine the exosome potential as proper delivery vehicle for targeting neurons exclusively. The results from this study can be concluded in this way:

- Non-exosomal glycoproteins can be sorted in the exosomes.
- Loading exosomes with desired cargoes requires more research and technical advances.

Summary

6. Summary

MicroRNAs (miRNA) are small non-coding RNAs that play a critical role in the regulation of gene expression programs and thus key mediators of cellular homeostasis. There is increasing evidence that deregulation of miRNAs contribute to neurodegenerative diseases and provide in turn novel opportunities to define biomarker signatures and develop novel therapeutic approaches. To develop effective methods for the delivery of therapeutic miRNA to neurons and to define miRNA changes during memory decline is this of utmost importance. In this project I addressed these important questions and could show that the use of modified exosomes might be a suitable approach to deliver miRNA into the brain. In addition I employed next-generation sequencing (NGS) to profile the miRNAome of 4 different brain regions in young and cognitively impaired old mice. I also examined aging-induced changes in blood from the same mice. My observation suggests that aging is associated with a remarkable deregulation of the brain and blood miRNA profile. Target prediction analyses revealed that the age-associated miRNA signature reflects inflammation-associated mechanisms. The data presents to most comprehensive analysis of the age-associated brain miRNAome and forms the basis for further mechanistic studies.

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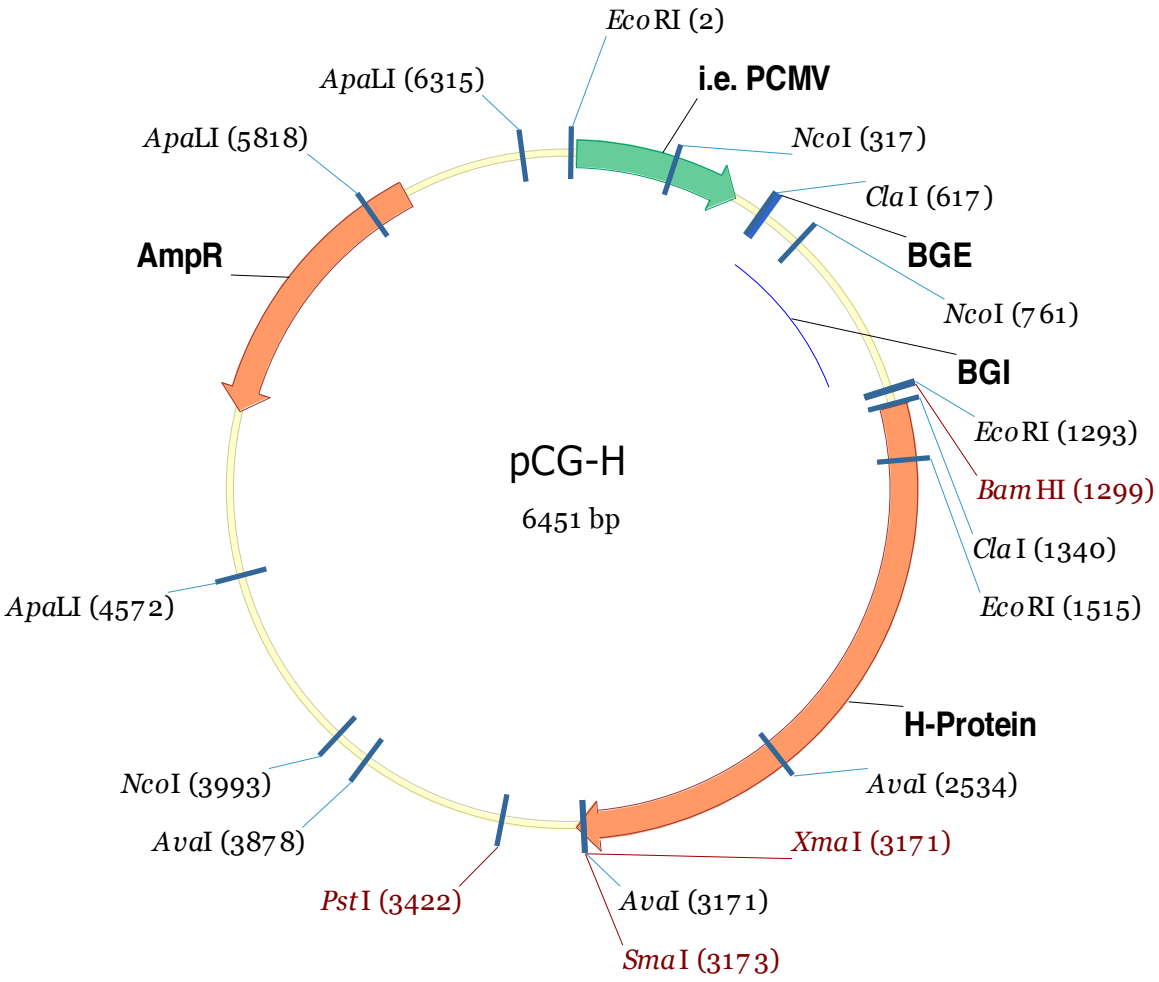
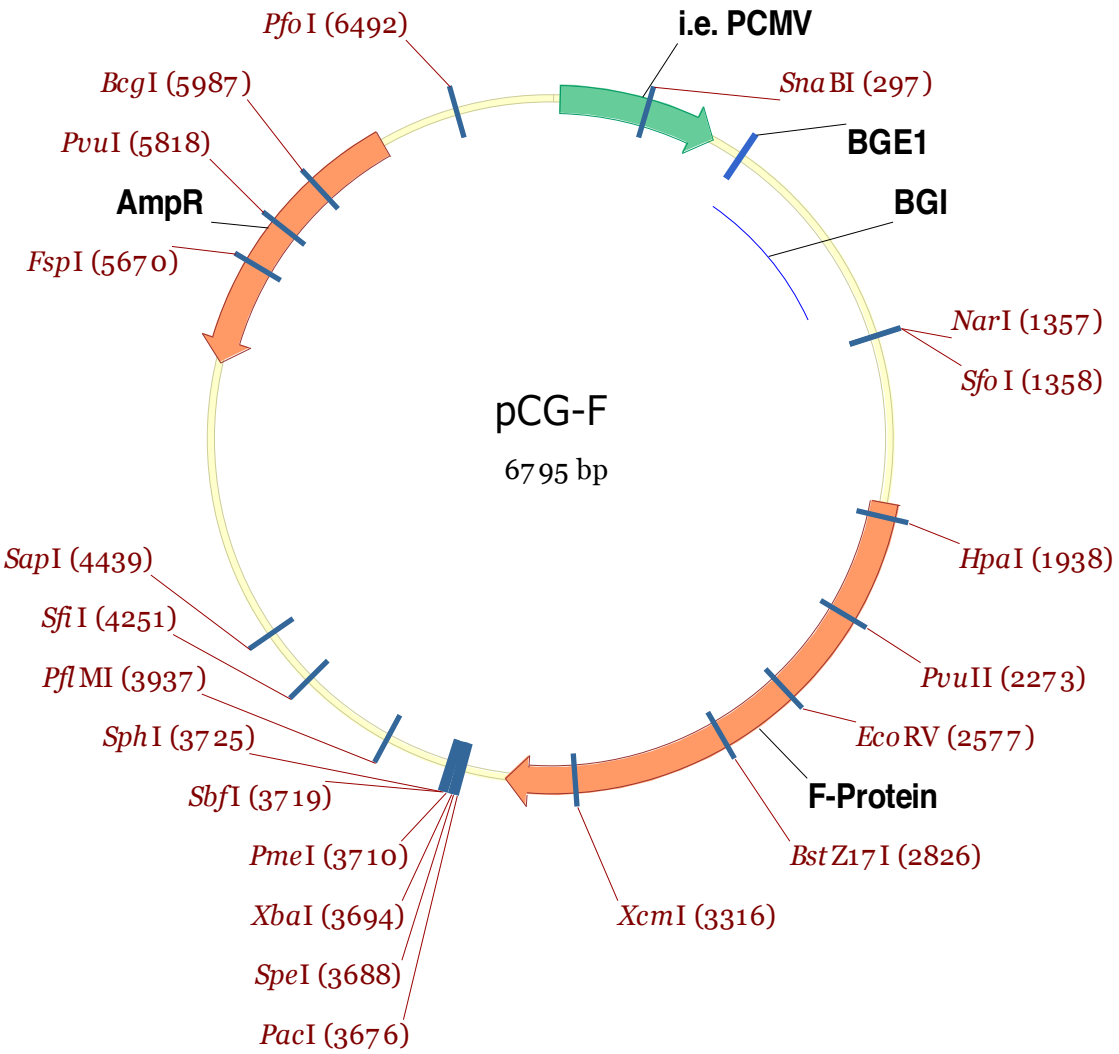
Appendices

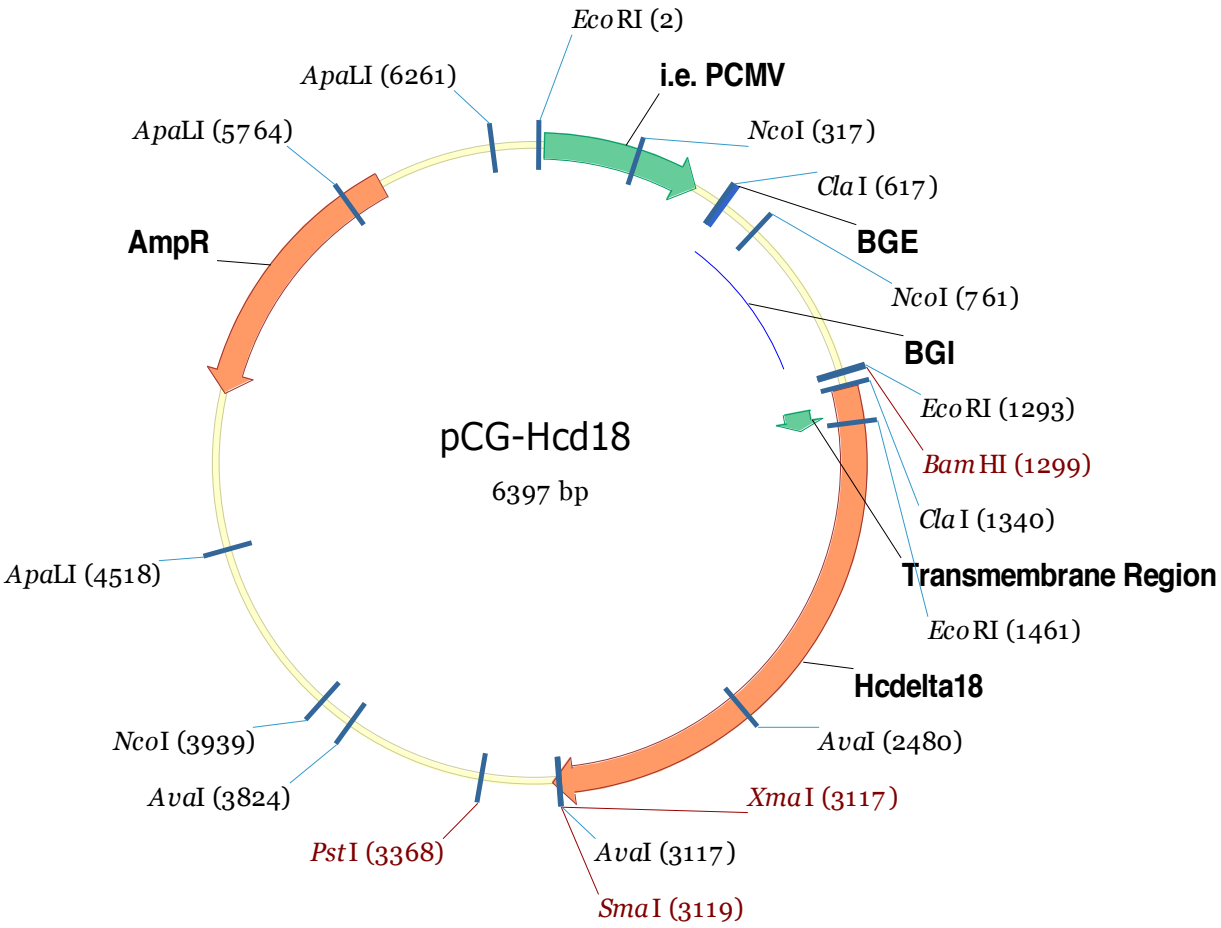
Appendix 1. pCG-H plasmid map



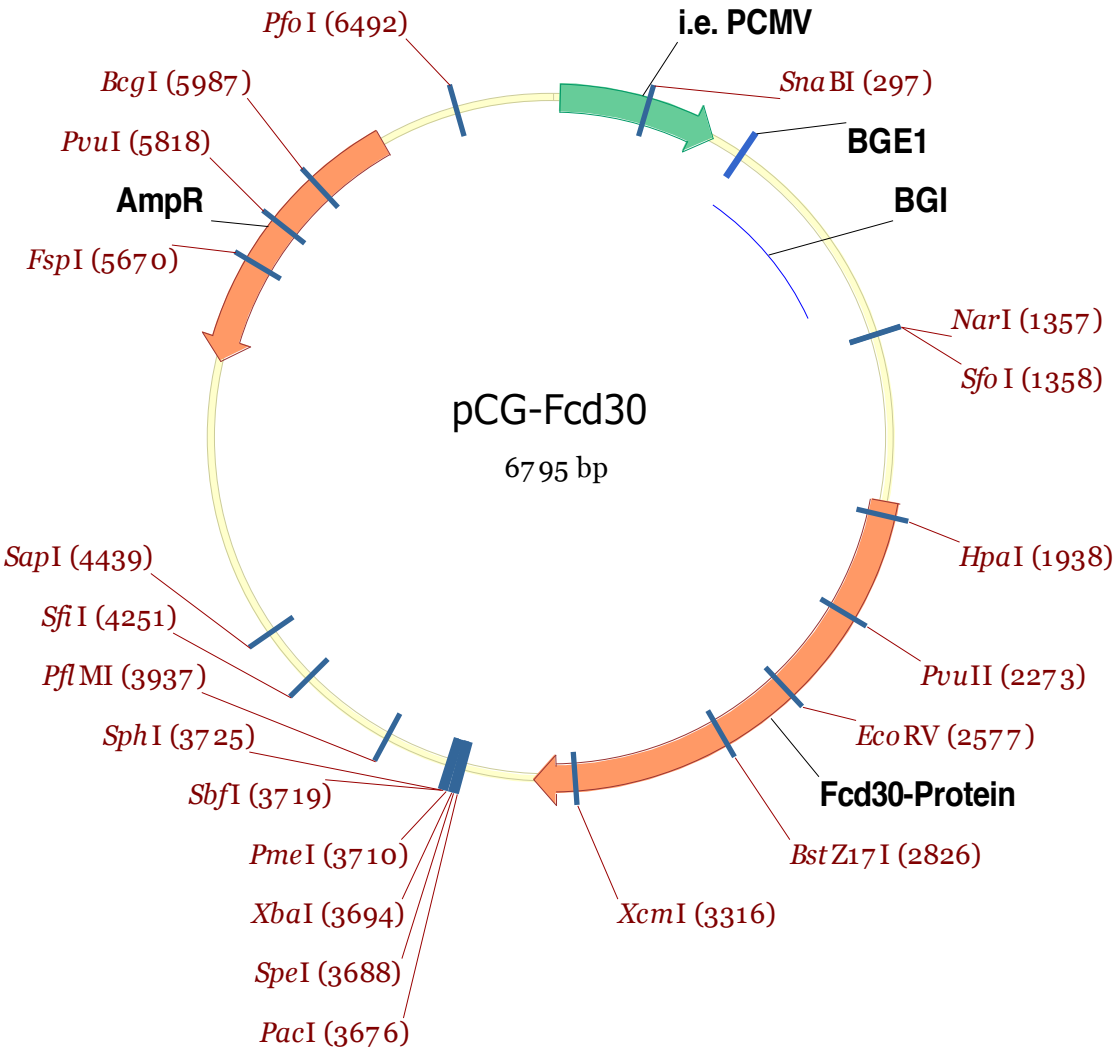
Appendix 2. pCG-F plasmid map



Appendix 3. pCG-Hcd18 plasmid map



Appendix 4. pCG-Fcd30 plasmid map



Curriculum Vitae

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EDUCATIONS

- PhD student in program CMPB (Center for Molecular Physiology of the Brain). University of Göttingen, DZNE Institute. Title of PhD thesis: "Epigenetic modulation in Alzheimer's disease: function of hippocampal miRNAs", supervisor: Prof. Dr. Andre Fischer. Since 1.12.2011.
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- B.Sc. in Biology, Alzahra University, Tehran, Iran, 2002-2006.

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PUBLICATIONS-PRESENTATIONS

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- Boroomandi, M & Fischer, A. 2014 miRNA profiles in the brain and blood as biomarker for aging-related cognitive disorders. Poster presentation in The Aging Brain summer school, Göttingen, Germany, September 2014
- Boroomandi, M. & Hofmann, M. H. 2011. A comparative neuroanatomical study of hypothalamus in Cichlids and other teleosts. Poster presentation in 8th World Congress of IBRO, Florence, Italy, July 2011.
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