THE EFFECTS OF DIFFERENT TYPES OF CALORIE RESTRICTION ON MICRORNA PROFILE IN MOUSE BRAIN TISSUE

by
Ümit Özorhan

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University
2019

THE EFFECTS OF DIFFERENT TYPES OF CALORIE RESTRICTION ON MICRORNA PROFILE IN MOUSE BRAIN TISSUE

APPROVED BY:

Assoc. Prof. Dr. Soner Doğan .....
(Thesis Supervisor)
(Yeditepe University)

Assist. Prof. Dr. Bilge Güvenç Tuna .....
(Thesis Co-Supervisor)
(Yeditepe University)

Prof. Dr. Michelle Adams .....
(Bilkent University)

Prof. Dr. Ömer Faruk Bayrak .....
(Yeditepe University)

Assist. Prof. Dr. Yavuz Oktay .....
(Dokuz Eylül University)

DATE OF APPROVAL: ..../..../2019

ACKNOWLEDGEMENTS

I would like to thank my MSc supervisors Assoc. Prof. Dr. Soner DOĞAN and Asst. Prof. Dr. Bilge Güvenç TUNA for their continuous support and motivation during my MSc study. In addition to taking part in this research, working with their research groups provided me invaluable experiences and courage to work harder for further achievements. After graduation, I will emphatically work to create better future for everybody and I am always looking forward with this goal.

I would like to thank Prof. Dr. Ömer Faruk BAYRAK, Assist. Prof. Dr. Ayşegül Kuşkucu and Dr. Emre Can TÜYSÜZ for their continuous support to my thesis by permitting to use laboratory facilities and consumables and their invaluable information about microarray and micro RNA expression. In addition to that, without their and Assoc. Prof. Dr. Soner DOĞAN lead, this thesis will not be possible.

I would like to thank my love Hatice Kübra PARLAKTAŞ and my friends Barış DERGİN, Göktuğ KARABIYIK, Sezer AKGÖL, and Bilge Nisan UĞURSU for never leaving my side. Also, I sincerely thank to my lab mates, Burcu ÇİÇEKDAL, Nazlı ATAŞ, Cihan ERDOĞAN, other lab mates and interns worked with our research group during this time for their help, support, and endless cheerfulness.

Finally, I would like to thank my family for their lifetime support and I could not put all the effort to complete my bachelor degree without the endless support of my family. I am deeply thankful to my dear parents Yunus ÖZORHAN and Fatma ÖZORHAN, my brother Muratcan ÖZORHAN. They were always ready to give me advice anytime I need and encourage me in any of my decision to build my future. I could not overcome any difficulties I had encountered without their help and love behind me.

ABSTRACT

THE EFFECTS OF DIFFERENT TYPES OF CALORIE RESTRICTION ON MICRORNA PROFILE IN MOUSE BRAIN TISSUE

Ageing is a complex natural event characterized by deterioration of the physiological functions of the cell and organs. Ageing increases disease vulnerability such as cancer and neurodegenerative diseases, and age-related diseases are one of the leading cause of deaths in humans. However, the effects of ageing could be ameliorated by calorie restriction (CR) which is a noninvasive intervention methods against ageing and applied by limiting calorie intake by up to 40 percent without malnutrition. Studies have shown that CR increases longevity and reverses the effect of ageing. On the other hand, microRNAs (miRNA), small noncoding RNAs that regulate 30 percent of protein expression are reported to play important roles in ageing, age-related diseases and cancer. Although, there have been many studies in relation to the effects of CR and ageing the exact molecular mechanisms of this phenomenon is not clearly known. Therefore, the aim of the present thesis was 1) to study the effects of long term CR on miRNA profile in brain of aged mice and 2) to compare miRNA profiles in brain tissue of mice with and without mammary tumor development. For this purpose, 10 weeks old MMTV-TGFα mice were enrolled in AL, CCR (15 percent CR), or ICR (three weeks of AL, one week of 60 percent CR in a cyclic manner) groups. Brain tissues were collected at week 10 and 81/82 in healthy and MT developed mice and miRNA expressions were profiled. Lower MT incidence and higher survival rates were observed in the CCR group. Similarly, CCR application showed better results in brain protection and modulate additional protective effects by increasing expression of mmu-miR-713 compared to AL and ICR groups. In ageing, regulated expression of mmu-miR-184-3p and mmu-miR-351-5p by CR could exhibit anti-ageing effects and other miRNAs that were regulated by CCR also alleviate effects of ageing. In conclusion, CR could confer anti-ageing effects by regulating miRNA expression. In addition, MT development disturbs brain miRNA profile by regulating miRNAs that are important in neuronal survival and neuron interaction related pathways. In conclusion, protective effects CR in brain could be modulated by regulating miRNAs including mmu-miR-713 and other miRNAs.

ÖZET

FARKLI ŞEKİLLERDE UYGULANAN KALORİ KISITLAMASININ FARE BEYİN DOKUSUNDAKİ MİKRORNA PROFİLİ ÜZERİNE ETKİSİ

Yaşlanma, hücrenin ve organların fizyolojik fonksiyonların bozulması ile karakterize edilen karmaşık ve doğal bir olaydır. Yaşlanma kanser ve nörodejeneratif hastalıklar gibi hastalıklara hassasiyeti arttırmaktadır ve insanlarda önde gelen ölümlerin nedenlerinden biridir. Fakat, yaşlanmanın sebepleri kalori kısıtlaması (KK) ile hafifletilebilir. KK noninvaziv bir metot olarak yaşlanma ile mücadelede en etkili yöntemlerden biri olup kalori alımının yüzde 20 ile 40 arasında kısıtlanması ile uygulanır. Çalışmalar göstermiştir ki KK, yaşam süresini arttırıp, yaşlanmanın etkilerini tersine çevirmektedir. Diğer yandan, ikroRNA'lar (miRNA), protein ifadesinin yüzde 30'unu düzenleyen ve mRNA'nın yıkılmasına veya ifade baskısına sebep olan küçük, kodlama yapmayan RNA'lardır. miRNA'lar yaşlanmada, yaşlanma ile ilgili hastalıklarda ve kanserin düzenlenmesinde önemlidir. KK ve yaşlanmanın ilişkisine dair çok sayıda çalışma olmasına rağmen bu fenomenin moleküler mekanizmaları bilinmemektedir. Bu çalışmanın amacı 1) uzun dönem KK ve yaşlanmanın beyin miRNA profili üzerindeki etkilerini göstermek ve 2) meme tümörü geliştirmiş ve sağlıklı farenin beyin miRNA profili arasındaki farkı ortaya çıkarmaktır. Bu amaçla, 10 haftalık MMTV-TGFα fareler, AL, SKK (yüzde 15 kısıtlama) veya AKK (döngüsel olarak üç hafta AL, bir hafta yüzde 60 KK) gruplarından birine yerleştirildi. Sağlıklı ve meme tümörü geliştiren farelerde 10. ve 81/82. haftalarda beyin dokuları toplandı ve miRNA seviyeleri profilllendi. Bu çalışmada, KKK grubunda düşük meme tümörü insidansı ve daha yüksek sağ kalım oranları gözlendi. Bu sonuçlarla uyumlu olarak SKK, AL ve AKK gruplarına kıyasla beyinde mmu-miR-713'ün ifadesini arttırarak beyinde ek koruyucu etkileri düzenleyebilir. Yaşlanmada, mmu-miR-184-3p ve mmu-miR-351-5p'nin KK tarafından düzenlenmiş ifadesi, yaşlanma karşıtı etkiler gösterebilir. Ek olarak, meme tümörü gelişimi, nöronal sağkalım ve nöron etkileşimi ile ilişkili yolaklarda önemli olan miRNA'ları düzenleyerek beyin miRNA profilini etkileyebilir. Sonuç olarak, Beyin dokusunda KK'nın koruyucu etkileri, mmu-miR-713 ve diğer miRNAların ekspresyonunun düzenlenmesi yoluyla düzenleniyor olabilir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

°C	Degree centigrade
µl	Microliter
AD	Alzheimer's disease
Ago2	Argonaute 2
ALS	Amyotrophic lateral sclerosis
bHLH	Basic helix-loop-helix
BMI	Body mass index
CALERIE	Comprehensive assessment of long-term effects of reducing intake of energy
CCR	Chronic calorie restriction
CNS	Central nervous system
CR	Calorie restriction
CRS	Calorie restriction society
CSC	Cancer stem cell theory
CSF	Cerebrospinal fluid
CVD	Cardiovascular disease
DE	Differentially expressed
DG	Dentate gyrus
EGFR	Epidermal growth factor
ER	Estrogen receptor
g	Gravitational force
GEO	Gene expression omnibus
GO	Gene Ontology
h	Hour
HD	Huntington's disease
HDAC	Histone deacetylase
ICR	Intermittent calorie restriction
ICR-R	Intermittent calorie restriction-restriction
ICR-RF	Intermittent calorie restriction-refeed

IPC	Intermediate progenitor cells
KEGG	Kyoto encyclopedia of genes and genomes
miRNA	MicroRNA
mM	Millimolar
MMTV-TGFα	Mouse mammary tumor virus-transforming growth factor alpha
MS	Multiple sclerosis
MT	Mammary tumor
n	Number of samples
ng	Nanogram
NPC	Neural precursor cell
NSC	Neural stem cell
OncomiR	Oncogenic miRNA
PD	Parkinson’s disease
PR	Progesterone receptor
RGC	Radial glial cell
RGL	Radial glial like cells
RMS	Rostral migratory stream
ROS	Reactive oxygen species
RT	Room temperature
SD	Standard deviation
SGZ	Subgranular zone
SIRT	Sirtuins
SVZ	Subventricular zone
TAC	Transcriptome analysis console
TNBC	Triple negative breast cancer
TNM	Tumor, node, metastasis
TSmiR	Tumor suppressor miRNA
YÜDETAM	Yeditepe Üniversitesi Tıp Fakültesi Deneysel Araştırmalar Merkezi

1. INTRODUCTION

Ageing is a complex and natural process that occurs in most living organisms. This intraindividual event characterized by deterioration of the physiological functions of the cell and organisms due to progressive accumulation of changes over time that leads to loss of the physiological homeostasis and organismal integrity [1-5]. Calorie restriction (CR) is limiting calorie intake up to 40 percent without malnutrition. CR is one of the most effective methods for healthy ageing and to prevent age-related diseases including neurodegeneration by modifying metabolic processes, satiety, energy balance and also promotes cognitive function [6-9]. Chronic calorie restriction (CCR) and intermittent calorie restriction (ICR) are the most prevalent options for the limiting food intake. CCR consists of constant regulation of food and energy intake, while ICR consists of regulated food and energy intake in a certain period with the following *ad libitum* food intake period in a cyclic manner. Although protective effects of either type of CR has been reported in numerous studies, the exact molecular mechanism(s) of it remain(s) to be unknown [10-14]. In this process, epigenetic factors like miRNAs and/or gene methylation may play important roles [15, 16].

Cancer, which is one of the most important health problems in the world, is an uncontrollable, malignant growth of the body cells that could grow in any body organ [17]. In the USA, cancer is responsible for 22 percent of all deaths and the second leading cause of death in 2016. In 2019, more than 1.75 million new cancer cases are expected in the USA and approximately 270 thousands of these new cases are expected to be breast cancer which is the second most lethal cancer in women after lung cancer [18]. According to the Turkey Ministry of Health report in 2017, breast cancer is the most common cancer type among women [19]. Therefore, breast cancer diagnosis, prevention, and treatment are crucial steps to improve the health conditions of individuals. There are several risks factors for breast cancer development such as genetic factors, ageing, hormone levels, diet, obesity and alcohol consumption which could lead to variation in types of breast cancers. Although some of these risks factors, such as diet and obesity could be controlled by individuals' lifestyle, the other factors could be out of ones' control. In addition, there are various treatment methods in the fight against breast cancer. Even though there has been significant improvement in the fight against breast cancer, the complexity of biologic development of cancer and variation of individual response to new medication slow down progress of

scientists. Therefore, new and improved methods in every aspect are needed to improve the success in breast cancer therapy [20-23].

On the other hand, neurogenesis is an event of new neuron generation from progenitor cells, which plays a core role in development of Central Nervous System (CNS) throughout life [24]. Neurogenesis consists of developmental and adult neurogenesis [25]. Developmental neurogenesis occurs during embryonic development and completes at birth [26]. Adult neurogenesis occurs throughout life and new neurons are generated in the dentate gyrus (DG) of the hippocampus, the subventricular zone (SVZ) of the lateral ventricles [27, 28]. Studies have reported that adult neurogenesis has a key role in learning and memory, and impairment of adult neurogenesis could lead to neurodegenerative diseases such as Alzheimer disease and schizophrenia [29-33]. Moreover, several intrinsic and extrinsic factors including ageing, diet, exercise and also epigenetic factors such as miRNAs and methylation may affect this process [7, 9, 34, 35].

MicroRNAs (miRNAs), 18-25 nucleotide long are noncoding RNAs that regulate gene expression by blocking target mRNA activity by base-pairing at the 3'UTR region in eukaryotes. One-third of all human protein expression is thought to be regulated by miRNAs [36-40]. Regulation of gene expression via miRNAs is reported to be important in apoptosis, insulin synthesis, cell proliferation and differentiation, muscle development, neurogenesis, cancer and ageing [36, 41, 42]. On the other hand, various diet factors as such exercise, chemical additives, vitamins, selenium, fatty acids, and polyphenols that affect the level of miRNAs are related with ageing and development of ageing related diseases [41, 42].

In this thesis, the aim of the study was 1) to understand the effects of long term application of different types of CR on miRNA profile in brain of aged mice, 2) to compare miRNA profile in brain of mice with and without MT development in MMTV-TGF-alfa transgenic mouse model at 81/82 weeks of mouse age. Specifically, neurogenesis, neuroprotection, neurodegeneration and ageing related miRNAs and their target genes were analyzed.

1.1. AGEING

Ageing is a complex and natural process that occurs in most living organisms. This intraindividual event characterized by deterioration of the physiological functions of the cells tissues of the organisms due to progressive accumulation of changes over the time that leads to loss of the physiological homeostasis and organismal integrity [1-5]. The causes of ageing are classified under two theories: programmed ageing and damage related ageing. According to the programmed ageing theory, ageing occurs in programmed way according to a biological agenda with programmed molecular and physiological changes over time, on the other hand, damage related ageing theory implies that progressive accumulation of damage over the time causes ageing. Programmed ageing depends on intrinsic factors such as gene expression, epigenetic factors, hormones, and immune response [3, 43, 44] while damage related ageing depends on several factors such as DNA damage, mutations, waste accumulation, free radicals, mitochondrial mutations and autophagy [2, 3, 43]. In 2013, Lopez-Otin et al. combined these two theories to define common hallmarks of ageing in organisms [2]. These defined traits are genomic instability, epigenetic alterations, telomere attrition, loss of protein folding and proteolysis (proteostasis), deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication [2]. In this context, brain ageing is specifically characterized by accumulation of oxidative damage, impaired molecular waste disposal, dysregulated energy metabolism, aberrant neural network activity, dysregulated neuronal calcium homeostasis and inflammation [45]. As it is commonly accepted vulnerability for age-related diseases such as diabetes, cardiovascular diseases (CVD), cancer and neurodegenerative diseases, increases with ageing. Later, increase in vulnerability leads to common causes of deaths [2, 18, 46, 47]. In addition to that, Belikov has reported that mortality of age-related diseases increases exponentially with ageing [48]. It is also suggested that ageing itself is also one of the risk factors for age-related diseases, and prevention and delaying in the ageing could improve effects of age-related diseases. There are numerous factors which may play roles in healthy ageing and age-related disease such as diet, CR, regular exercise, and proper sleep cycle. These factors could be classified under lifestyle. Other factors like medical interventions including resveratrol, metformin, rapamycin have also shown to have important roles in the prevention of age-related diseases[5, 49-55]. For example, study conducted by Miller et al showed that rapamycin administration increases lifespan 10

percent in male and 18 percent in female mice [54]. Although, many studies have conducted to clarify the molecular mechanism(s) of ageing this complex phenomenon still needs to be elucidated.

1.2. CANCER

Cancer is an uncontrollable, malignant growth of cells that occurs and/or metastases in any body organ, which could result to death of individuals [17]. In the USA, cancer is the second leading cause of death and was responsible for approximately 600 thousand deaths in 2016 [18]. Moreover, 1.75 million new cancer cases are expected in the USA in 2019 [18]. According 2014 cancer statistics data for Turkish population, more than 160 thousand new cancer cases were reported and age adjusted total cancer incidence rate was 210.2 per 100 thousand people in 2014 [19]. Tumorigenesis is transformation of normal cells to cancer cells that undergoes in common key principles called as hallmarks of cancer in all cancer types. Hallmarks of cancer were defined as six common traits of cancer cells. These traits are sustained proliferative signaling, evaded growth suppressors, activated invasion and metastasis, enabled replicative immortality, induced angiogenesis and resistance to cell death [56]. In addition to the these hall marks listed earlier, two emerging hallmarks and two emerging traits were also introduced in to the list . These are deregulated cellular energetics, avoided immune destruction, instability and mutation of genome, and tumor promoting inflammation respectively [57]. Tumorigenesis (formation of cancer) is caused by several intrinsic and extrinsic risk factors. Intrinsic risk factors are genetic and epigenetic abnormalities, DNA damage, instabilities in DNA repair mechanisms, and hormonal abnormalities while, some of the extrinsic risk factors are irradiation, viral factors, tobacco, excessive alcohol consumption, diet, obesity, oxygen free radicals, and ageing [17, 18, 22, 23, 42, 58-60].

Cancer types were classified under five main types as their origin cell types. These types are named as carcinoma that have epithelial origin, sarcoma that have mesenchymal origin, leukemia and lymphoma that derived from blood cells, blastoma that derived from embryonic tissues and germ cells tumor that derived from pluripotent cells [17, 61-63]. Furthermore, cancer is divided into five stages to show cancer progression according to TNM (Tumor, Node, Metastasis) as 0, I, II, III and IV. Stage 0 represents early form of

carcinoma in the local area, stage I represents localized tumors, stage II represents early stage of locally spread and grown tumors, stage III represents late stage of locally spread and grown tumors and stage IV represents metastasized tumors [17, 21, 63]. After diagnosis and determination of cancer stage, several treatment methods are used in cancer therapy. The most common methods are chemotherapy, immunotherapy, radiation therapy, hormone therapy and surgery. However, these methods are serious consequences since they are either invasive or have serious side effects for the patients. For these reasons, several methods have been developed to cope with the side effects of these therapies such as targeted drug deliveries, adjuvant and neoadjuvant therapies [17, 20, 21]. Moreover, life style changing regimes, diets, exercises are also used as prevention and treatment methods in cancer to improve life quality of patients [17, 22, 60].

1.2.1. Breast Cancer

Breast cancer is one of the most prevalent cancer type in the world with being the second lethal cancer in women throughout the World [18, 19, 23]. It is estimated that in 2019, approximately 270 thousands of new breast cancer cases are expected in the USA (2). According to the recent data (2015 for USA, 2014 for Turkey), breast cancer incidence rates are 124.7 and 43 per 100 thousand people in the USA and Turkey, respectively [18, 19] while the mortality rate for it are 20.6 and 11.9 per 100 thousand in the US and Turkey respectively [18, 64].

Development of breast cancer is a result of numerous complex biological processes and controlled by several intrinsic and extrinsic risk factors. Intrinsic risk factors are genetic and epigenetic abnormalities, DNA damage, instabilities in DNA repair mechanisms, and hormonal abnormalities while the extrinsic risk factors are irradiation, viral factors, tobacco, alcohol consumption, diet, obesity, oxygen free radicals, and ageing [17, 18, 22, 23, 42, 58-60]. Specifically, mutations in the tumor suppressor genes and/or overexpression of oncogenes are common risk factors for the development of breast cancer. For example, mutations in BRCA1 and BRCA2 genes are responsible for five to ten percent of all breast cancers while TP53 mutations exists in 30 percent of all breast cancers patients. In addition, 10 to 20 fold increase in risk for breast cancer was reported in human with mutations in BRCA1, BRCA2 and TP53 genes which are involved in the regulation of cell cycle and

DNA double strand break repair mechanisms [20]. Another, common mutation seen in breast cancer is HER2 mutations. In this context, it was reported that overexpression of HER2 gene exists in 20 percent of primary breast cancers while EGFR genes exist more than 30 percent of invasive breast cancers [22]. Furthermore, mutations in ATM, BRIP1, CHEK2 and PALB2 genes were also reported to increase relative breast cancer risk by two to four fold. However, mutation of PTEN, RAS, RAD50, NBS1 genes and overexpression of c-MYC, CCND1 genes are also important in breast cancer development and progression [17, 20, 22, 23, 65, 66]. Moreover, epigenetic abnormalities like overexpression of miRNAs, methylation of histones and genes increase the risk of breast cancer [58, 67, 68]. Breast cancer diagnosed by various methods. For the diagnosis of breast cancer, mammography is the most common screening method and it is a gold standard for breast cancer diagnosis. Mammography obtains high resolution images of the breast using X-ray beams. However, new methods such as magnetic resonance imaging and molecular imaging are developing to improve diagnosis of breast cancer [22, 69].

Molecular classification of breast cancer can be classified under different molecular subtypes according to the their immunoprofile, genetic structure, response to chemotherapy, and other factors. The most common factors in the classification of breast cancer subtypes are estrogen receptor (ER), progesterone receptor (PR), epidermal growth factor receptor (EGFR), HER2, Ki67 and claudin levels [22, 70-73]. These subtypes are named as luminal a, luminal b, basal like, claudin low and HER2 positive. The detail information about the subtypes is given in Table 1.1. These molecular classification of breast cancer are important for understanding prognosis of disease and determining appropriate treatment for breast cancer [68, 71, 73-79].

Table 1.1. Molecular classification of breast cancer and prevalence in Turkey [76].

Classification	**Immunoprofile**	**Prevalence in Turkey**
Luminal A	ER+, PR+, HER2-, low/no KI67	62%
Luminal B	ER+, PR+, HER2+/-, high KI67	15%
Basal Like (TNBC)	ER-, PR-, HER2-, EGFR+, K5/14+, high KI67	15%
HER2 positive	ER-, PR-, HER2+, high KI67	8%
Claudin low	ER-, PR-, HER2-, low KI67, low Claudin-3/4/7, low E-Cadherin	No Data

Treatment strategies for breast cancer differ according to its molecular subtypes, histological grading and stages of breast cancer. Surgery is one of the most common methods in breast cancer treatment although it depends on histological grade and tumor stage. To support surgical treatment, patients may also receive chemotherapy, radiotherapy, adjuvant therapy to improve patients conditions. In chemotherapy application, breast cancer characteristics are important in determination of chemical drugs like paclitaxel, cisplatin, doxorubicin to improve conditions of patients. Moreover, hormone therapies are designed according to molecular markers of breast cancer. Estrogen inhibitors, antiestrogen drugs, aromatase inhibitors improves treatment ER, PR positive breast cancer subtypes with inhibiting/decreasing estrogen formation. HER2 inhibitors improve HER2 positive breast cancer subtypes. Furthermore, neoadjuvant, monoclonal, nanomedicine, radiation and targeted therapies are also used to treat patients [20-22, 80-82]. Although there are many treatment methods in the fight against cancer most of these methods have severe side effects. Therefore, the latest studies have been trying to find alternative and noninvasive methods to prevent and/or cure breast cancer. One of the most promising methods in this subject is the application of calorie restriction which has no known side effects.

1.3. NEURODEGENERATION

Neurodegeneration is a progressive process with functional and structural loss or death of neurons [83]. Neurodegeneration caused by several internal and external risk factors such as DNA damage, ageing, mitochondrial DNA mutations and oxidative stress, however, ageing is the most important risk factor in the neurodegeneration [83, 84]. Mechanisms of neurodegeneration varies between genetic mutations, protein misfolding, deficiencies in DNA repair and protein degradation mechanisms to altered glial function and inflammation [84-86]. Neurodegeneration in the nervous system could result in neurodegenerative diseases which are degenerative neurological disorders that could affect specific structures and functions of either central or peripheral nervous system. Today more than 100 subtypes of neurodegenerative disease are identified, however, most common ones are Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). However, each neurodegenerative disease is characterized by different traits [83, 84]. For example, AD is characterized by loss of neurons and axons in the cerebral cortex which results to progressive decline in the memory function, but PD is characterized

by loss of dopamine neurons in the substantia nigra which results to impairment in motor symptoms and tremor [87, 88].

Ageing is the main risk factor for all neurodegenerative diseases and it is characterized by various hallmarks [1, 89]. These hallmarks are impairment in cognition and memory via mitochondrial dysfunction, inflammatory response, oxidative stress, impaired lysosome and proteasome functions, proteotoxicity, dysregulation of neuronal calcium homeostasis, compromised adaptive cellular responses, aberrant neural network activity, impaired neurogenesis and dysregulated energy metabolism [45, 90]. In addition to the these hallmarks, ageing increases damage accumulation in genetic materials including proteins. Moreover, ageing negatively effects telomere maintenance and DNA repair mechanisms. On the other hand, reactive oxygen species (ROS), and accumulated oxidative damage with ageing could result in neurodegeneration with decreasing levels of antioxidants and activity of DNA repair systems [45, 85]. Thus, these characteristics of ageing increases vulnerability for the neurodegenerative diseases like AD and PD [2, 45, 47, 91].

1.4. NEUROGENESIS

Neurogenesis is generation of new neurons from neural stem cells and/or progenitor cells, which were formed in developmental stage and adulthood [24]. Neurogenesis divides into two main events: developmental neurogenesis and adult neurogenesis [25]. Developmental neurogenesis starts at embryonic development and completes after formation of central nervous system that completes at birth. During developmental neurogenesis, radial glial cells form new neurons which migrate to their locations which are determined by molecular and genetic cues [26, 92, 93]. After developmental stage, low levels of neurogenesis, known as adult neurogenesis occurs throughout life. In adult neurogenesis, new neurons are generated in the dentate gyrus of the hippocampus, the subventricular zone (SVZ) of the lateral ventricles [27, 28, 94]. Adult neurogenesis especially in the dentate gyrus of hippocampus has crucial roles in memory and learning. Moreover, deficiencies in adult neurogenesis play key roles in the development of neurodegenerative diseases including Alzheimer disease, and schizophrenia [29-33]. Furthermore, several intrinsic and extrinsic factors including ageing, diet, exercise and epigenetic factors such as miRNAs and DNA methylation were reported to affect neurogenesis [7, 9, 34, 35].

1.4.1. Adult Neurogenesis

Adult neurogenesis which occur throughout life is generation of new neurons in adult brain after embryonic development. Although in 1962, it was first reported by Joseph Altman in rat hippocampus, these findings were not generally accepted by researchers until 1999 [95]. In 1977, Michael Kaplan observed adult neurogenesis in vertebrates with electron microscopy [96, 97]. In 1999, Elizabeth Gould reported generation of new neurons in olfactory cortex and hippocampus of primates [98-100]. This research have proven that neurogenesis occurs in adult vertebrates. Today's scientific world, 700 new neurons are thought to be generated in adult hippocampus every day [101]. Findings have shown that adult neural stem cells are located in the spinal cord, midbrain and subventricular region of the cerebellum, however, adult neurogenesis only occurs in the subgranular zone (SGZ) in dentate gyrus of the hippocampus, the subventricular zone (SVZ) of the lateral ventricles. In addition to these regions of adult brain, several research were claimed to occurrence of adult neurogenesis in neocortex. However, integration of newly formed neurons into existing circuits only occur in SGZ and SVZ of adult brain [26, 98, 99, 102-104]. Recent studies have reported new neurons generated from adult neurogenesis in humans could be important in behavioral pattern separation, spatial navigation, adaptability to novel context, episodic and autobiographic memory, even forgetting [105].

Various research reported continuation of adult neurogenesis in humans throughout the life [106-108]. These findings were supported by the recent publication. For example, Boldrini et al. reported that adult neurogenesis is stable throughout life [109]. But, Sorrels et al, recently showed controversial results on adult neurogenesis which claimed that neurogenesis in human hippocampus rarely continues after childhood [110]. These controversial finding on adult neurogenesis reignited the debate of situation of adult neurogenesis in humans. In this context, Kempermann et al. published a review on this reignited debate to clarify situation of adult neurogenesis in humans and pointed out technical and experimental issues on adult neurogenesis. They indicated that Sorrels et al. research is not well suited to determine adult neurogenesis in humans because of their sample selection and preparation processes [105]. All these findings and debate on adult neurogenesis indicates that our understanding on adult neurogenesis is still limited and needs new and standardized research to clarify situation of adult neurogenesis in humans.

1.4.1.1. Subventricular Zone Neurogenesis

Adult neurogenesis could be divided into two sub groups which are subventricular zone and subgranular zone neurogenesis. Neurogenesis in the subventricular zone (SVZ) of lateral ventricles starts with primary neural stem cells. Neural stem cells (NSC) in the SVZ have similar characteristics with radial glial cells (RGC) and astrocytes, and they are located near to cerebrospinal fluid (CSF) and blood vessels. These NSC are named as radial glial like cells (RGL). Genesis of new neurons in the striatum and olfactory bulb starts with transition of RGL to B cells in the SVZ [26, 30]. B cells are intermediate progenitor cells (IPC) which divided into three subgroups; B1, B2 and B3. B1 cells are quiescent, dormant, inactive neural stem cells, however, B2 and B3 cells are actively proliferating stem cells but have limited proliferation capacity. B cells are enclosed by ciliated ependymal (E cells) cells and this structure is named as pinwheel rosetta structure [26, 30]. Number of these structures and their neurogenic potency decrease with ageing. B3 cells give rise to transit amplifying stem cells (C cells) which are progenitor proliferating stem cells that have faster cell cycle compared to B3 cells and divide asymmetrically [26, 30]. Asymmetrical divisions of C cells generate neural precursor cells (A cells) which migrate through rostral migratory stream (RMS) to the olfactory bulb and striatum. Migration of A cells in RMS facilitated by cell to cell interactions, neuregulin, glial cells and adhesion molecules as NCAM. A cells differentiate to interneurons as olfactory bulb granule and periglomerular cells [26, 94, 102-104]. B, C and A cells are classified with their expression of characteristics markers. For example, B and C cells express Nestin and Sox2 markers. Specifically, B1 cells express GFAP marker, and have constant Notch activity while, B2 cells express GFAP, BLBP markers and have oscillating Notch/Ascl1 activity. In addition, B3 cells do not express GFAP marker, however, have similar levels of BLBP marker and Notch/Ascl1 activity with B2 cells. On the other hand, C cells express EGF receptor (EGFR) and have oscillating Notch/Ascl1 activity beside Nestin and Sox2 markers. A cells express DCX and DLX2 markers and have constant Ascl1 activity [26, 27, 30, 94].

Studies have shown that several extrinsic and intrinsic factors regulate neurogenesis in the subventricular zone of lateral ventricles. For example, decreasing Notch signal and VCAM levels promotes cell proliferation and differentiation, however constant Notch signal and VCAM levels promotes quiescence state [26, 30]. Another study has reported that serotonin

signaling from serotonergic axons increases the activation of B1 cells and promotes proliferation [26, 30]. Likewise, acetyltransferase neurons and dopaminergic neurons promotes proliferation and neurogenesis. Endothelial cell interaction promotes Notch signaling and quiescence state in B cells. Similarly, EGF signaling represses Notch signaling and promotes proliferation in C cells. A cells secrete GABA neurotransmitter as negative feedback mechanism to attenuate C cell divisions, on the other hand B cells secrete diapezam binding inhibitory protein as a competitive GABA inhibitor to increase C cell divisions. Moreover, bloodborne factors like GMP11 could be important for proliferation of stem cells [26, 30, 111, 112]. Other internal and external factors have also shown to play important roles in the regulation of neurogenesis in the SVZ such as epigenetic modifications, ageing, stress and olfactory stimulus enrichment [34, 94, 113]. In this context, newly formed neurons in the olfactory bulb take role in olfactory function in rat, mice and other animals [26, 114]. In humans, almost no new neurons could be found in olfactory bulb, however, newly formed neurons migrate to striatum to take role in coordination of movement, learning and memory [26, 28, 107, 114].

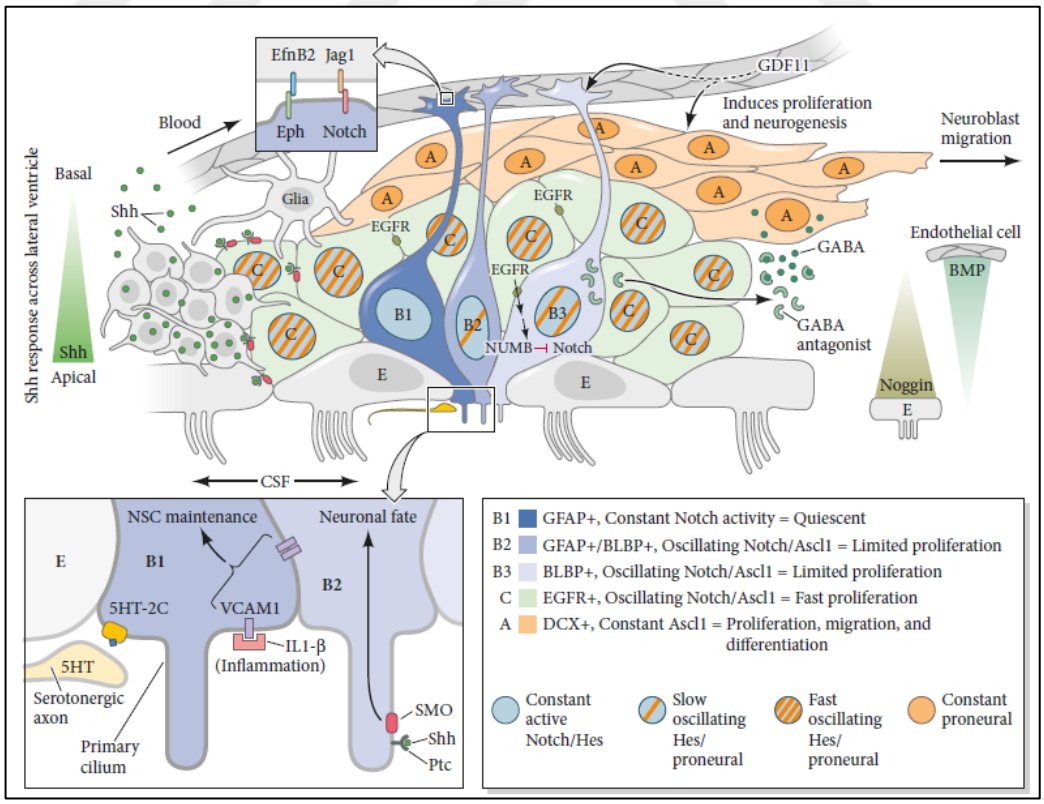
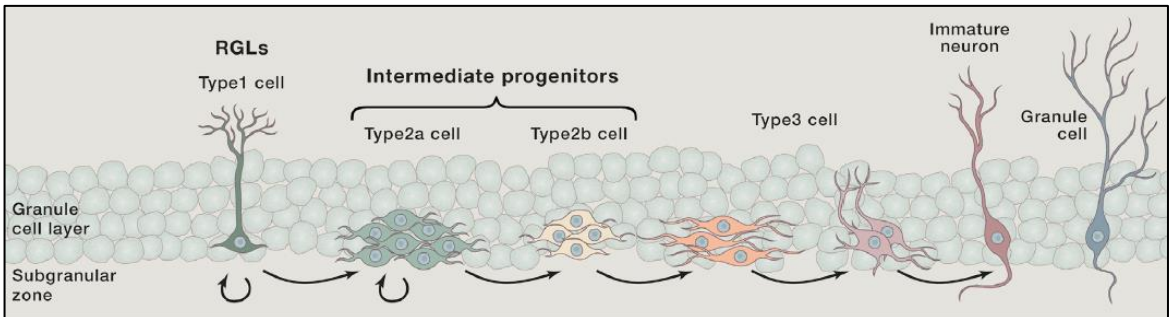


Figure 1.1. Subventricular neurogenesis in the adult brain [26].

1.4.1.2. Subgranular Zone Neurogenesis

Neurogenesis in the subgranular zone in the dentate gyrus of the hippocampus starts with primary neural stem cells. Neural stem cells (NSC) in the SGZ like NSC in the SVZ have similar characteristics with radial glial cells (RGC) and astrocytes, named as radial glial like cells (RGL). Genesis of new neurons in the dentate gyrus starts with the transition of RGL to type I cells in the SGZ. Type I cells reside in the basal level of the granule cell layer near to blood vessels. Type I cells give rise to transit amplifying progenitor cells also called as Type II cells which are divided into two subgroups as Type IIa and IIb. Later type IIa cells are differentiated into glial cells while type IIb cells are differentiated into neuroblasts also called as Type III cells. Finally, Type III cells are differentiated into granule cells which are translocated to the granule cell layer [115, 116]. Type I, II and III cells are classified with their expression of characteristics markers. For example, Spot14, REST, Nestin and Sox2 proteins are commonly expressed both in Type I and Type II cells. Specifically, GFAP, Hes1/5, TLX, and FoxO3 proteins are expressed only in Type I cells which also have constant Notch signaling activity. On the other hand, Type IIa cells express Hes1/5, TLX, FoxO3 markers as well as Ascl1, Neurog and Tbr2 while, Type IIb cells express Ascl1, Neurog and Tbr2 markers. However, Type III cells express Prox1, NeuN, Sox11, CREB, Klf9, NeuroD1 and DCX markers [30, 111, 117]. Studies have reported that several extrinsic and intrinsic factors regulate neurogenesis in the subgranular zone of hippocampus region [112, 118]. Both Type I cell activation and Type II cell proliferation are shown to be regulated by Wnt, Shh, Sox2, Notch, BMP signaling, and inflammatory cytokines including BDNF, TNFα and IL6 [117, 119]. In addition, regulation of these cells by the growth factors like EGF, FGF2, IGF2, VEGF and neurotransmitters released from GABAergic axons, and serotonergic neurons. On the other hand, Type III cell differentiation and migration are regulated by NeuroD1, Prox1, SoxC, MBD1, BDNF, Wnt, Cdk5, Disc1, glutamate, CREB signaling and neurotransmitters like norepinephrine, GABA, dopamine, semaphorin/plexin. [30, 112, 117-120]. Other internal and external factors also have important roles in the regulation of neurogenesis in the SGZ such as epigenetic modifications, stress, ageing, voluntary exercise, diet and environmental enrichment [118, 121-123]. Newly formed neurons in the dentate gyrus of the hippocampus could take role in hippocampal functions like learning, memory, pattern separation and appetite regulation [89, 118, 124]. However, exact role and mechanism of hippocampal neurogenesis and role of epigenetic factors

remains to be elucidated. Therefore, in this thesis, role of epigenetic factors on brain neurogenesis were analyzed to assess importance of epigenetic factors in neurogenesis.



	Quiescence	Proliferation/ Activation	Fate specification	Morphogenesis and differentiation	Maturation
Signals	BMP, Notch, GABA	Canonical Wnt, IGF, Shh, FGF-2, EGF, Dopamine	Canonical Wnt, NT-3	Wnt/PCP, Notch, BDNF, Reelin, GABA	Glutamate, NRG2, Neuronal activity
Transcription factors	Hes5, FoxO, REST	Sox2, Tlx	Tbr2, Ascl1/Mash1, NeuroD1, Prox1	NeuroD1,Prox1, CREB, AP-1?	CREB
Epigenetic regulation		Gadd45b, LSD1	Fmrp, Mbd1	Gadd45b, MeCP2	MeCP2, HDAC2

Figure 1.2. Subgranular neurogenesis in the adult brain [119].

1.4.2. Neurogenesis and Ageing

Ageing is characterized by deterioration of cellular and physical functions and loss of the physiological homeostasis and organismal integrity [1, 2]. It is widely accepted that adult neurogenesis is gradually decreased with ageing. Specifically, proliferation of neural stem cells and RGL cells was shown to be decreased with ageing [89, 108, 123]. In this context, in 2018, data from two independent research groups which was published in highly prestigious journals showed controversial outcomes of ageing in human adult neurogenesis [110, 125]. In March 2018, Sorrels et al, published that neurogenesis in human hippocampus decreased to undetectable levels after childhood [110]. In the same study they also reported -that SGZ neurogenesis rarely continues in the aged adults (between 70-79 years old) [110]. On the other hand, in April 2018, Boldrini et al. showed that adult hippocampal neurogenesis levels were stable throughout life, however, quiescent neural stem cell pool, angiogenesis and neural plasticity was decreased with ageing [109]. These controversial findings on the adult neurogenesis in the humans indicate that molecular mechanisms, pathways and neurogenesis itself remain to be elucidated.

Many studies have conducted in order to understand the molecular mechanisms of the neurogenesis and ageing. In this context, studies have reported that ageing regulates adult neurogenesis by variety of factors and/or mechanisms such as bloodborne growth factors, hormones, inflammatory cytokines, local signals and neurotransmitters [89]. For example, heterochronic parabiosis studies show that blood factors of young mice such as GDF11, IGF1 increased neurogenesis in the old age mice [126-128]. In another study, decrease in neurogenesis in dentate gyrus of the hippocampus with ageing was reported to be linked with memory impairments [89]. In addition, age-related increase of glucocorticoid protein levels in brain tissue and their receptor levels in dentate gyrus were decreased in adult neurogenesis [129]. Neuroprotective properties of ghrelin growth hormone, secreted from stomach, have been shown in mice. Ghrelin promotes neurogenesis in the hippocampus and is abundant in young mice whereas ghrelin levels decrease with ageing [130]. A research conducted by Sonntag WE et al. in rats, showed that FGF2, IGF1 and VEGF like neurogenesis promoting growth factors are significantly lower in the old rats compared to young rats [131]. Other than growth factors, bloodborne molecules could affect neurogenesis because blood brain barrier permeability changes with ageing [132, 133]. CCL11 chemokine and β2 microglobulin increases with ageing in blood plasma of mice and could result in reduction of neurogenesis in dentate gyrus [134, 135]. Ageing also effects neurogenesis via changing local factors. FGF2 growth factor secreted from supportive astrocytes are important in maturation of neurons, however, ageing decreases secretion of FGF2 growth factor from supportive astrocytes [132]. Notch signaling, and WNT signaling decrease with ageing and these negatively regulated factors decrease neurogenesis [136, 137]. Local factors from microglia such as cytokines and growth factors could also affect neurogenesis in adult brain. Microglia activation is increased with ageing and inflammation. Activated microglia release pro-inflammatory cytokine as a response to inflammation and ageing. Secreted cytokines from microglia like TNFα, IL1β, and IL6 could inhibit differentiation of newly formed neurons. Inhibition of microglia activation via neuroimmunoregulatory protein FKN in old rats increase neurogenesis. Moreover, in young rats blocking FKN receptor reduce neurogenesis in SGZ [133, 138]. In SVZ, IFN-I signaling increases, IFN-II signaling decreases in the choroid plexus with age and this could result in decreased neurogenesis [138]. Neurotransmitter levels in the hippocampus decrease with ageing. Research conducted in mice and rat hippocampus showed that decreases in the GABA, Acetylcholine and dopamine decreases neurogenesis via decreasing proliferation, neuronal

survival, differentiation and increasing apoptosis [133]. Moreover, during ageing, telomere shortening occurs in the neural precursor cells because of decreasing telomerase activity and this shortening decrease neurogenesis in aged animals [132, 133]. Ageing also increases neural precursor cells senescence with reducing activity of BMI1 epigenetic regulator in NPC, and Wnt signaling in DG [133]. Furthermore, tumor suppressive genes like $p16^{Ink4a}$ and $p21^{Cip1}$ increase with ageing and decrease neurogenesis in aged individuals [138]. All these factors show that ageing is an important regulator on adult neurogenesis.

1.5. CALORIE RESTRICTION

Calorie restriction (CR) is an noninvasive method for healthy ageing by limiting calorie intake by 20 to 40 percent without malnutrition to modify metabolic processes, satiety, and energy balance. Therefore, calorie restriction is important in the prevention of age-related diseases including neurodegeneration, cancer and also promotes cognitive function [8, 139-142]. Most prevalent options of calorie restriction are chronic calorie restriction (CCR) and intermittent calorie restriction (ICR) for the limiting food intake. Chronic calorie restriction (CCR) is constant regulation of food and energy intake, while intermittent calorie restriction (ICR) is regulation of food and energy intake in a certain period with the following *ad libitum* food intake period. Positive effects of CR on longevity and ageing were first shown in rats at 1935 [143]. Although implication of CR on humans is a complex process, research show that low intake of calories have positive effects on ageing and age-related diseases in humans. People who consumed less calories than their normal diet, showed lower rates of cancer and other chronic diseases compared to normal fed humans [142, 144-146]. Protective effects of either type of CR has been reported in numerous studies [10-14]. Human clinical trials on application of CR showed that CR improved body weight, body mass index (BMI) and other metabolic conditions. Biosphere 2 experiment reported that 6 month application of 30 percent CR reduced BMI, body weight, blood pressure, total serum cholesterol, leukocyte count, fasting glucose, triglyceride counts. Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy (CALERIE) experiment showed that 25 percent of CR decreased CVD risk, reduced DNA damage, insulin resistance, and preserved Calcium intake, improvement on body temp, blood pressure, BMI, hormone and cholesterol levels. Moreover, Calorie Restriction Society (CRS) also showed similar results under 30 percent of CR application [6, 142, 146-148]. Application of CR decreases

insulin, IGF-1, leptin, leptin/adiponectin ratio, cytokines, PAI-1, p53, VEGF levels, growth factor signaling, cytokines, glycolysis, glutaminolysis, inflammation, ROS production, BMI, body fat, inflammatory and vascular perturbations. Thus, application of CR increases ghrelin, SIRT1, HDAC1, hTERT, FoxO, adiponectin levels, ketogenesis, lipolysis, autophagy and apoptosis [125, 142, 149-154]. These changes promote healthy ageing, prevent age-related diseases and other body functions [142, 144, 145]. However, the exact mechanisms of CR remains unknown. In this process, epigenetic factors like miRNAs and/or gene methylation may play important roles [15, 16].

1.5.1. Calorie Restriction and Ageing

Ageing is known as deterioration of physiological and structural abilities of individuals in molecular and physiological level with age [2]. Calorie restriction is limiting calorie intake without malnutrition and known as most effective noninvasive methods to delay ageing and ageing related diseases. CR ameliorates effects of ageing by inhibiting cell proliferation, glycolysis, inflammation, increasing autophagy, antioxidant expression and improving mitochondrial physiology [6]. In brain, CR improves nutrient sensing, cognitive function, redox status, and inhibits neurodegeneration, neuroinflammation. CR ameliorates ageing induced decrease in brain volume, dendritic spines, grey matter, neuronal network activity, calcium homeostasis and stimulates neurogenesis and mitochondrial biogenesis. Moreover, CR upregulates autophagy, DNA repair, neurotransmitter, neurotrophic factor signaling, and decreases ROS levels, oxidative stress, inflammation [8, 9, 45]. CR inhibits cell proliferation and glycolysis by blocking IGF1R/mTOR and inflammation by inhibiting NFkB dependent pathways [6, 148, 155]. Furthermore, CR increases autophagy by activating FoxO, Ghrelin signaling antioxidant expression by FoxO and NRF2 dependent pathways, induces mitochondrial activation by activating SIRT/AMPK pathway [6, 9]. Positive effects of CR on ageing intervention originate by different and complimentary pathways which are important to alleviate effects of ageing.

1.5.2. Calorie Restriction and Cancer

Calorie restriction is limiting calorie intake without malnutrition and creates negative energy balance, resulting in decreased body weight and prevents age-related diseases like cancer [125]. The first study conducted to show effect of CR on cancer published in 1909 and indicates that CR inhibits tumor growth in tumor transplanted in mice [156]. Recent studies also showed similar results of CR slowed down tumor growth [157, 158]. Effects of CR on cancer varies with percentage of restriction, period, when did CR application started, strain or species that CR was applied to [149]. According to the research which combined 82 published papers, calorie restricted rodents took 29 percent less calories and showed 42 percent decrease in tumor incidence. However, most of the tumor incidence reduction (49.6 to 62.2 percent) was obtained in 21 to 58 percent CR application which shows that percentage of restriction is important to increase effects of CR on cancer [149, 159]. Calorie restriction reduces growth factor signaling, vascular perturbations, production of ROS and inflammation. These reduced levels decreases risk and progression of cancer. Moreover, CR could activate longevity pathways via activating sirtuins and AMPK signaling and inhibiting IGF-1/AKT and mTORC1 signaling to limit cancer progression [142, 150]. With recent advancements in cancer treatment, application of CR are being used with cancer therapy and improves therapy efficacy and decreases side effects. CR reduces side effects and cell toxicity in normal cells, reduces growth factors, inflammation in circulation, increase tumor clearance and anti-mitotic agent sensitivity in tumor cells and enhance drug delivery in tumor microenvironment. However, ICR is considered as a more suitable application of CR for cancer therapy [125, 151, 154]. CR application is a powerful method to limit progression of cancer and improve patients health conditions with appropriate treatment.

1.5.3. Calorie Restriction and Neuroprotection

Neuroprotection is a protection of neurons by preserving function or structure of neurons and limiting neural loss caused by neurodegeneration, neurodegenerative diseases and other factors [160]. Neuroprotection could protect neurons from neurodegeneration via inhibiting apoptosis, autophagy, excitotoxicity signals, stress inducing signals [161, 162]. However, exact mechanism of neurodegeneration, neurodegenerative diseases and neuroprotection are

not clear. Exercise, CR and diet which are associated with neuroprotection could improve and postpone the effects of neurodegeneration and neurodegenerative diseases [163-166]. Epigenetic mechanisms such as miRNAs also have important roles in both neurodegeneration and neuroprotection however underlying mechanisms of epigenetic factors remain to be elucidated [167-169].

Calorie restriction is limiting calorie intake of individuals without malnutrition [125]. CR is well known method for increasing longevity and protects against ageing in individuals. CR ameliorate effects of ageing and neurodegenerative diseases [6, 155, 170]. CR could preserve motor performance, improve memory function and increase neurogenesis [16, 171]. Furthermore, CR could delay iron accumulation in brain, attenuate amyloid deposition on AD models, reduces the risk of AD, PD, HD, multiple sclerosis (MS), [16, 162, 166, 172]. Neuroprotective effects of CR depend on various pathways such as autophagy, neurogenesis, inflammation, plasticity in brain [7, 173]. CR promotes adult neurogenesis, neuronal survival via BDNF and HSP70 [91, 162, 173, 174]. CR regulates activity of histone deacetylase (HDAC) protein sirtuin-1 (SIRT1) which is correlated with ageing. SIRT1 regulates CR mediated neuroprotection with NFkB, PGC-1α, SIRT3, FoxO, NRF2. These factors increase antioxidant activity, mitochondrial activity, decrease inflammation, oxidative damage, neuronal cell death, protein aggregation and prevents apoptosis [7, 91, 175-177]. In addition to these factors, CR protects against neurodegeneration with other factors. CR application increases levels of ghrelin in plasma which increases neuronal survival of substantia nigra dopamine neurons. Survival of dopamine neurons in the substantia nigra is important in neuroprotection of PD [178]. CR increases expression of liver Fgf21 that reduces neurofibrillary tangle formation in AD by inducing AMPK/mTOR pathway in brain [166]. In addition to these factors, epigenetic factors like microRNAs and histone modifications are also important in CR mediated neuroprotection [7, 16, 171].

1.5.4. Calorie Restriction and Neurogenesis

Adult neurogenesis is generation of new neurons in adult brain after embryonic development and occur throughout life [24]. Adult neurogenesis moderately decreases with age, however, CR which is known as one of the most effective methods against ageing could alleviate the effects of ageing on neurogenesis [6, 7, 89]. Calorie restriction induce neurogenesis by

promoting neural stem cell survival, neural differentiation and neural protection [122, 179, 180]. Calorie restriction increases BDNF, NT3, Hsp70, GRP78, IFNγ and CREB levels in brain [7, 122, 170, 179-181]. Increased BDNF and NT3 levels in hippocampus promote stem cell proliferation, survival and differentiation [7, 122, 170, 180, 181]. Increasing Hsp70 and GRP78 protein levels in brain protects neurons from factors like oxidative damage [122, 179]. Upregulated IFNγ expression has been shown in the rat hippocampus which promotes neuronal differentiation of NSC and axonal outgrowth [180]. *İn vitro* research indicates that CR activated CREB expression enhances adult hippocampal neural stem cell self-renewal capacity [181]. In addition to these, increased circulating ghrelin levels by CR could promote adult hippocampal neurogenesis by increasing neuronal survival [182]. These research show that CR improve neurogenesis in the adult brain by different pathways.

1.6. MicroRNAs

MicroRNAs (miRNAs) are small noncoding RNAs of 18-25 nucleotide length that regulate 30 percent of protein expression at the post-transcriptional level. miRNAs regulate protein expression through binding to 3'UTR region of mRNAs by mRNA degradation and translation repression via 2-8 nucleotide long seed region [37-39]. According to the miRBase miRNA database, mice (*Mus musculus*) have 1237 precursors, 1926 mature miRNAs, meanwhile, humans (*Homo sapiens*) have 1917 precursors, 2654 mature miRNAs [183]. miRNAs are transcript from DNA by RNA polymerase II in nucleus. After transcription, this precursor miRNA is named as pri-miRNA and cleaved by DROSHA/DGCR8 complex to form pri-miRNA. Pri-miRNA is exported to cytoplasm by exportin-5 and further cleaved by Dicer/TRBP to form miRNA duplex. miRNA duplex forms RNA induced silencing complex with Argonaute 2 (Ago2) and DICER. Passenger strand of miRNA duplex is separated and degraded. After removal of passenger strand, other strand is named as mature miRNA and regulates protein expression via mRNA cleavage, translational repression and miRNA deadenylation [26, 184]. Regulation of protein expression via miRNAs is important in physiological functions as development, cell proliferation and differentiation, apoptosis, cholesterol metabolism, stress response and insulin synthesis [41, 42]. On the other hand, various diet factors such as exercise, CR, chemical additives, vitamins, selenium, fatty acids and polyphenols affect level of miRNAs that are related with ageing and development of ageing related diseases [41, 42].

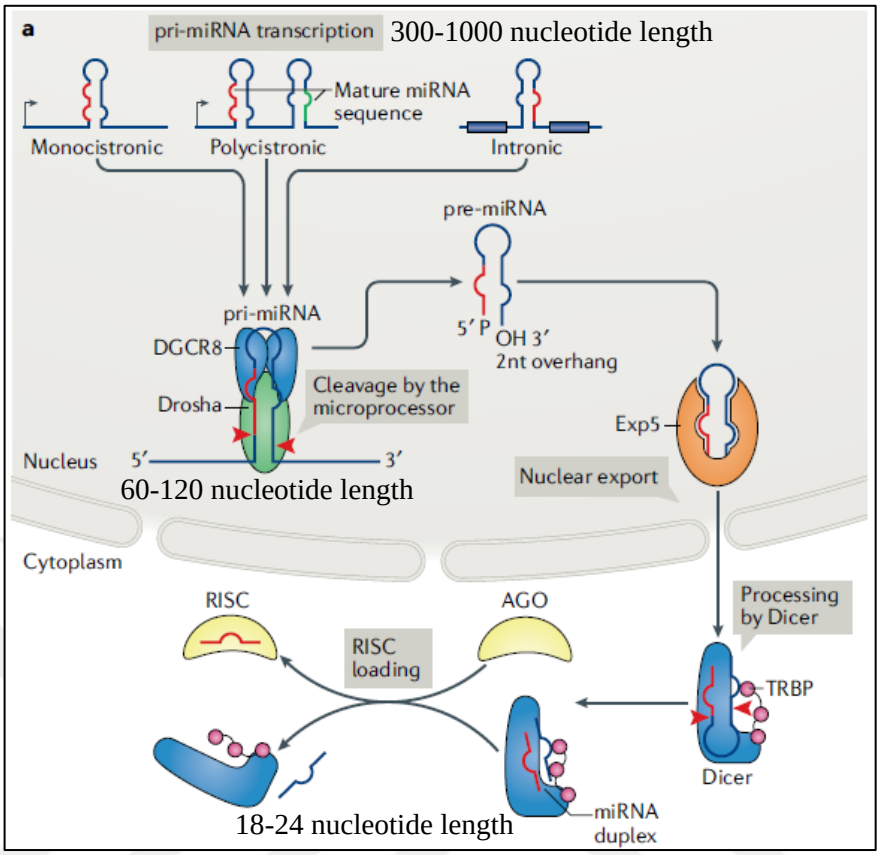


Figure 1.3. Schematic representation of miRNA biogenesis [184].

1.6.1. miRNAs, Ageing and Brain

Ageing is a natural process by deterioration of the cellular and physical functions [1, 2]. miRNAs which is small noncoding RNAs could be important in regulation of ageing and ageing related processes [5]. Ageing is characterized by hallmarks and miRNAs could regulate metabolic hallmarks of ageing and other factors. miRNAs could deregulate nutrient sensing, mitochondrial dysfunction, oxidative stress, telomere shortening, cellular senescence, stem cell exhaustion, impaired DNA repair, altered protein homeostasis, altered gene expression and altered intercellular communication by inflammation [5, 185-187]. However, miRNA profiles and regulated pathways change depend on tissue and organs [187]. miRNAs deregulate nutrient sensing by affecting growth hormone, insulin/IGF1 signaling, mTOR signaling, AMPK and sirtuins signaling [5, 185]. miRNAs also important in mitochondrial dysfunction and metabolic ageing. Deregulation of mitochondria related

miRNAs could induce mitochondrial dysfunction. [5, 185, 186]. Moreover, miRNAs regulate ageing related metabolic inflammation, oxidative stress, telomere length, DNA repair pathways, cellular senescence, protein homeostasis, stem cell exhaustion, and gene expression control [185, 186].

Table 1.2. miRNAs related with hallmarks of ageing [5, 185-187].

Ageing Hallmarks	**miRNAs**
Regulation of Nutrient Sensing	let-7, miR-1, miR-15b, miR-17, miR-19/a, miR-20/a, miR-21, miR-29, miR-34a, miR-93, miR-106a, miR-126, miR-134, miR-142-3p, miR-143/145, miR-182, miR-195, miR-206, miR-217, miR-221, miR-222, miR-223, miR-320, miR-451, miR-470, miR-496, miR-519, miR-681, miR-669, miR-766, miR-4495
Deregulation of Mitochondria	let-7, miR-1, miR-15b, miR-19b, miR-20a, miR-34a/b/c, miR-101a, miR-106a, miR-133b, miR-145, miR-146a, miR-181a, miR-210, miR-221, miR-335, miR-376a, miR-378/378*, miR-486-5p, miR-494, miR-542-5p, miR-1973, miR-4495
Metabolic Inflammation	miR-10a, , miR-15a, miR-16, miR-17, miR-18a/b, miR-19a, miR-20a/b, miR-21, miR-34a, miR-93, miR-106a/b, miR-126, miR-139b, miR-146/a/b, miR-155, miR-181a, miR-206, miR-222, miR-223, miR-376c, miR-411
Regulation of Telomeres	miR-23a, miR-29a-3p, miR-30a-5p, miR-34a-5p, miR-34a, miR-138, miR-155, miR-512-5p
Regulation of Oxidative Stress	miR-20a, miR-50, miR-51, miR-58, miR-84, miR-128, miR-193b, miR-200c, miR-329, miR-455, miR-499
Regulation of DNA Repair	miR-21, miR-24, miR-34a, miR-215, miR-315
Regulation of Cellular Senescence	miR-24, miR-26a, miR-29, miR-34a, miR-43a, miR-106, miR-125b, miR192, miR-194, miR-215, miR-290, miR-504 and miR-17-92 Family
Regulation of Protein Homeostasis	miR-1, miR-26, miR-34a, miR-301, miR-320
Regulation of Stem Cell Exhaustion	let-7, miR-17, miR-290, miR-295, miR-302, miR-369, miR-371
Gene Expression Control	miR-1, miR-7, miR-9, miR-15a, miR-29, miR-34a, miR-124, miR-143, miR-144, miR-340

In brain, miRNAs regulate ageing by regulating ageing hallmarks and lifespan, synaptic plasticity and cognition, neuroprotection and neurodegeneration [185, 188]. miRNAs regulates lifespan by regulating longevity and neuronal survival, neuroprotection by regulating neural differentiation and other factors and important in synaptic plasticity and cognition and progression and development of neurodegenerative diseases [186, 188-191]. Moreover to these, miRNAs could regulate neurogenesis in the brain by regulating proneural genes, signaling pathways and transcription factors [121, 192, 193]. miRNAs could regulate

activation, proliferation, cell fate specification, differentiation, migration and synaptic integration in adult neurogenesis. Expression of let-7b, miR-9, miR-34a, miR-145 inhibits and miR-137, miR-25b promotes activation and proliferation of neural stem cells. miR-26b, miR-124 and miR-125b expression promotes fate specification of NPCs. Let-7b, miR-9, miR-145 expression promotes and miR-34a, miR-137, miR-184 inhibits neuroblast differentiation. miR-379/410 family miRNAs promotes and miR-9, miR-134 inhibits migration of neuroblasts. Moreover to that, several miRNAs regulate synaptic integration, axonal and dendritic growth, synaptogenesis in newly generated neurons. miR-29a/b, miR-34a, miR-137 expressions regulate neuronal apoptosis [192, 193]. These research show that miRNAs are important in regulation of ageing especially in brains. miRNA expression regulates the factors important in neurogenesis, neuroprotection and neurodegeneration, however, our understanding on miRNAs and their effects are limited.

Table 1.3. miRNAs related with brain ageing [185, 186, 188-191].

Brain Ageing	**miRNAs**
Longevity and Neuronal Survival	miR-29, miR-34, and miR-144
Neuronal Differentiation	let-7f, miR-1, miR-132, miR-137, miR-430
Synaptic Plasticity and Cognition	miR-25, miR-34c, miR-101, miR128b, miR-132, miR-134, miR-138, miR-144, miR-182, miR-185, miR-204, miR-283, miR-501
Neurodegenerative Diseases	miR-1a, miR-9, miR-16, miR-16-2-3p, miR-22-5p, miR-26a, miR-26a-2-3p, miR-27a-3p, miR-29a, miR-30a, miR-155, miR-320, miR-331-5p, miR-450b-5p, miR-505, miR-626 and miR-1826

1.6.2. miRNAs and Cancer

Cancer is an uncontrollable, malignant growth of cells that could occur and/or metastases in any body organ, which could result to death of individuals [17]. Epigenetic factors like methylation and miRNAs are important in development and regulation of cancer [67]. miRNAs are small noncoding RNAs and regulate 30 percent of protein expression through binding to 3’UTR region of mRNAs by mRNA degradation and translation repression [38, 39]. In cancer, microRNA expression become aberrant and this aberrant expression could

promote cancer progress. This aberrant expression of miRNAs could support hallmarks of cancer such as genomic instability, tumor promoting inflammation, cell death resistance, evading immune surveillance by altered expression levels [194, 195]. In cancer, general miRNA expression decreases, however, some miRNA levels increase with cancer [196]. These aberrant expression of miRNAs could regulate oncogenic and tumor suppressive protein expressions. Oncogenic miRNAs or oncomiRs regulate expression of tumor suppressor genes. On the other hand, tumor suppressor miRNAs or TSmiRs regulate expression of oncogenes [197, 198]. OncomiRs are important in initiation, progression and metastasis in cancer and generally upregulated with cancer [197]. OncomiRs deregulates their target genes that in tumor suppressor pathways such as Myc, RAS, Notch, BRAF and Shh pathways [198]. In addition to the OncomiRs, miRNAs promote metastasis known as metastamiRs [199]. On the other hand, TSmiRs are important in protection against tumor initiation, development and progression, and expression levels downregulate in cancer progression [197, 199]. TSmiRs deregulates their target genes in oncogenic pathways such as TP53, PTEN, TGFβ and Wnt pathways [198, 199]. However, functions of these oncomiRs and TSmiRs could change depending on the tissue and organ. Moreover, even miRNAs could be both oncomiRs and TSmiRs at the same time in a tissue because miRNA could target more than 100 mRNAs. For example, miR-122, miR-155, miR-214, and miR-30b/d shows oncomiR and TSmiR properties simultaneously [198, 200]. In breast cancer, miR-9, miR-10b, miR-17/20 cluster, miR-155, miR-196, miR-21, miR-210, miR-221 and miR-222 function as oncomiRs, furthermore, let-7, miR-7, miR-17-5p, miR-30a, miR-34a, miR-200 and miR-205 function as TSmiRs [197].

Table 1.4. miRNAs related with cancer in humans [199].

Cancer Related miRNAs	miRNAs
OncomiRs	miR-10b, miR-21, miR-23b, miR-27a, miR-100, miR-125b, miR-132, miR-155, miR-182, miR-214, miR-221, miR-222, miR-296, miR-301, miR-372, miR-373, miR-375, miR-378, miR-519a, miR-675, miR-1908
MetastamiRs	miR-9, miR-10b, miR-15b, miR-19a/b, miR-20a, miR-21, miR-96, miR-105, miR-122, miR-135b, miR-181a, miR-182, miR-183, miR-200s, miR-214, and miR-296-3p/5p
TSmiRs	let-7, miR-1, miR-7, miR-9, miR-15a, miR-16, miR-16-1, miR-18a, miR-25, miR-27a, miR-29a/b1/b2/c, miR-30b, miR-31, miR-33a/b, miR-34a/b/c, miR-101-3p, miR-122a, miR-124, miR-125a, miR-128, miR-133a/b, miR-135a, miR-137, miR-143, miR-145, miR-146a/b, miR148a/b, miR-149, miR-182, miR-193b, miR-198, miR-204, miR-205, miR-206, miR-214, miR-218, miR-296-5p, miR-302, miR-302b, miR-335, miR-383, miR-449, miR-493, miR-504, miR520c, miR-545, miR-596

1.6.3. miRNAs and Calorie Restriction

Diet is one of the most important factors in the regulation of miRNA expression and different types of diets such as Western diet and Calorie restriction (CR) could change the miRNA function in cells [37, 201]. CR delays ageing and could reverse the effects of ageing with changing expression levels of miRNAs. Several research shows that application of CR increases longevity and delays ageing by reversing expression of ageing related miRNAs in blood, liver, brain of mice, rat and rhesus monkeys [16, 202-207]. Research conducted by Noyan et al. indicated that even one week application of 30 percent CR in male C57BL/6J mice has protective effects on cardiac system of ischemia model mice with decreasing expression levels of miR-92, miR-21 and increasing expression levels of miR-27, miR-29, miR-208, miR-214 [208]. Studies performed in rats and mice show that 30 percent calorie restriction have inhibitory effects on miRNAs and decrease apoptosis, tumor progression, metastasis, and also have suppressive effects on colon carcinogenesis [207, 209-211]. Orom et al. showed that 6 months of 30 percent chronic CR application on C57BL/6J mice in breast tissue induces expression of tumor suppressor miR-203 and this increased miRNA by CR could be important in ageing and longevity [207]. Jin et al. reported that 30 percent chronic

CR application in breast cancer and breast tissue of female Balb/c mice decrease expression of miR-17, miR-20a and miR-92 and metastatic potential of triple negative breast cancer via regulating miRNA expression [209]. Olivo-Marston et al. indicated that in 20 week of 30 percent chronic CR applied male FVB mice colon tissue and tumor, CR decreased size of colon tumor. Furthermore, CR application showed suppressive effects on colon tumorigenesis by decreasing level of miR-155 and increasing level of miR-150, miR-351 and miR-34c in colon tumor and other biological pathways [211]. Green et al. reported that more than one year of 30 percent CCR applied C57BL/6J mice liver samples, CR decreased expression of miR-107, miR212-3p and miR-34a and increased longevity and lifespan by regulating chromatin related gene targeting miRNAs [204]. Makwana et al. showed that 2 years of 30 percent CCR application on female C57BL/6J mice in liver samples increased expression of miR-125a-5p and downregulated miR-125a-5p target genes Stat3, Casp2 and Stard13 expression. Furthermore, ageing decreased expression of miR-125a-5p in liver and upregulated target genes. CR could mediate anti-ageing properties via miR-125a-5p regulation [206]. Mori et al. reported that up to 14 week of 40 percent CCR application on C57BL/6J mice increased expression of let-7e, miR-24, miR-30c, miR-99b and miR-181d in adipose tissue. CR application reversed age-related decrease of these miRNAs and restored expression levels of miRNAs [212]. Dhahbi et al. indicated that up to 26 months of 40-45 percent CR application in B6C3F1 long live mice model antagonized ageing related increase of circulating miRNAs in serum [213]. Khanna et al. reported that 2 years of 40 percent CCR was applied to Bcl-2 overexpressing C57BL/6J mice. This CR application decreased expression of miR-34a, miR-30e and miR-181a* in age dependent manner at brain, furthermore, Bcl-2 levels were high and stable in CR application compared to AL group. CR could mediate neuronal survival via these regulation of miRNAs in long lived CR applied mice [15].

In addition to CR and miRNA interaction in mice experiments, effects of CR on miRNA expression are researched on other animal models such as rats and rhesus monkeys. Devlin et al. indicated that up to 12 weeks of 30 percent CCR application on Sprague-Dawley rats in mammary tissue and expression of tumor progression related miR-200a and overall tumor size decreased and increased tumor free survival [210]. Lee et al. showed that 4 weeks of 40 percent CCR application on Sprague-Dawley rats showed decreased exosomal miR-500-3p and miR-770-3p expression in serum, however, these miRNAs were upregulated by ageing.

These miRNAs could be important in ageing progression and longevity effect of CR [202]. Csiszar et al showed that 2 years of 40 percent CR application in male F344xBN ageing model rats decreased expression of miR-144 that targeting Nrf2 in cerebromicrovascular endothelial cells. CR application preserves young phenotype with retaining anti-inflammatory, anti-oxidative, and pro-angiogenic effects [214]. Wood et al. reported that in the brain of up to 26 months 45 percent CR applied male BN rats, application of CR decreases expression of miR-98-3p that important in regulation of histone deacetylase and histone acetyltransferase activity. Wood et al. claims that CR increase longevity and promote neuroprotection via histone deacetylase/histone acetyltransferase homeostasis [16]. Schneider et al reported that in the plasma of 17 years of 30 percent CCR applied rhesus monkeys, CR application changed miR-125a-5p, miR-130a-5p, and miR-143-5p levels compared to control group. CR regulated decrease in miR-125a-5p were positively and negatively correlated with adiposity and insulin sensitivity respectively. CR also changed insulin and growth signaling related miRNAs in plasma of rhesus monkeys to alleviate ageing [203]. Mercken et al. reported that in the skeletal muscle of CCR applied rhesus monkeys, CR applications ameliorate age induced increase of miR-144 and miR-451 and increases miR-181b levels compared to ageing. CR could reverse ageing phenotype by changing miRNA levels [215]. These research showed that CR application regulates miRNA expression in various tissues. Even short term CR applications improve effects of ageing and age-related diseases with miRNAs and other factors [208].

1.7. AIM OF THE STUDY

In this thesis, miRNA profiles of brain tissue samples taken from mice which were enrolled to different types of calorie restriction were determined. Specifically, neurogenesis, neuroprotection and neurodegeneration related miRNAs and their target genes were analyzed. The main objective of this study is to understand the effects of long term application of different types of CR on miRNA profile in brain of aged mice to asses effects of CR on brain and to understand antiaging properties of CR in brain miRNA profile. In addition, compare miRNA profile in brain tissue of mice with and without MT development to determine the effects of MT development on brain in MMTV-TGFα mice at 81/82 weeks of age. Our hypothesis is CR application increases Neuroprotection in brain with regulating miRNA expression, and ameliorates effects of ageing.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Equipment

Table 2.1. Equipment used in miRNA microarray experiments.

#	**Brand**	**Item**	**Catalog No**
1	ThermoFisher	FinnPipette Micropipettes (1000 µl, 200 µl, 20 µl, 10 µl, 2 µl)	4642010, 30, 60, 80, 90
2	Axygen	Micropipette tips 1000 µl, 200 µl,10 µl	T1000, T200, T300
3	ISOLAB	Sterile PCR Tubes 0.2 ml	123.01.002
4	ISOLAB	Sterile Microcentrifuge Tubes 2 ml, 1.5 ml	078.03.023, 022
5	ISOLAB	Sterile Falcon Tubes 50 ml, 15 ml	078.02.002, 004
6	SSI BIO	Sterile Falcon Tubes 5 ml	1410-09
7	NextAdvance	Sterile Zirconium Oxide Beads 0.5 mm	ZROB05-RNA
8	ISOLAB	Polystrene Sterile Petri Dishes	081.02.091
9	MYMED	Sterile Scalpel Tip 10	TIB-00401
10	Parafilm M	Parafilm	05801001

2.1.2. Instruments

Table 2.2. Instruments used in miRNA microarray experiments.

#	Brand	Item	Catalog No
1	NextAdvance	Bullet Blender Storm	BBY24M
2	Beckman Coulter	Microfuge 16	392244
3	ThermoFisher	NanoDrop 2000	ND-2000
4	Cleaver Scientific	Vortexer	CSLVORTEX
5	Haier Biomedical	Ultra-Freezer -86^{o}C	DW86L628
6	ESCO	Laminar Flow Cabinet	Class II BSC
7	ThermoFisher	GeneAtlas Hybridization Station	00-0375
8	ThermoFisher	GeneAtlas Imaging Station	00-0376
9	ThermoFisher	GeneAtlas Fluidics Station	00-0377
10	ELGA	Purelab OptionQ	DV16
11	Vibra	Analytical Balance	HT-124
12	BioRad	CFX96 Touch RT PCR	1855196

2.1.3. Chemicals

Table 2.3. Chemicals used in miRNA microarray experiments.

#	Brand	Item	Catalog Nu
1	Sigma	Phosphate Buffer Saline Tablets	P4417
2	Tekkim	Ethanol 96%	TK.200650.05001
3	Merck	TRIS Buffer, 1.0 M, pH 8.0	648314

2.1.4. Kits

Table 2.4. Kits used in miRNA microarray experiments.

#	Brand	Item	Catalog Nu
1	Zymo	Direct-zol MiniPrep with TRI Reagent	R2053
2	ThermoFisher	FlashTag Biotin HSR RNA Labelling Kit	901911
3	ThermoFisher	GeneAtlas Hybridization, Wash and Stain Kit for miRNA Array Strips	902134
4	ThermoFisher	GeneChip Eukaryotic Hybridization Control Kit	900454
5	ThermoFisher	GeneChip miRNA 4.1 Array Strip	902404

2.2. METHODS

2.2.1. Animals

In this study, Dr. Margot Cleary from Hormel Institute Medical Research Center, University of Minnesota kindly donated MMTV-TGFα (C57BL/6) mice strain to form a breeding colony at YUDETAM. Donated MMTV-TGFα positive male mice were bred with TGFα negative female mice (C57BL/6) to obtain heterozygote MMTV-TGFα female offspring.

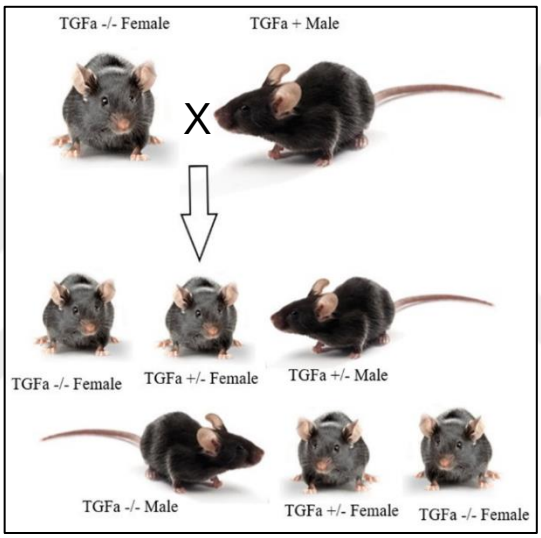


Figure 2.1. Schematic representation of breeding process for offspring.

MMTV-TGFα mice are recognized transgenic mice that are prone to develop breast cancer after post-menopause and development of breast cancer in these mice have similar characteristics with human breast cancer development [216]. MMTV-TGF-α mice overexpress human EGFR/ErbB cascade member TGF-α that have critical role in development of breast cancer [217, 218]. All MMTV-TGFα mice had free access to water and were accommodated in conditioned environment at room temperature (20-25°C) and 12h light/dark cycle. All processes that applied on animals under the guidelines of Ministry of Forestry and Water Affairs and approved by Yeditepe University Animal Care and Use Committee. Health status of all animals were supervised as daily basis by researchers and weekly basis by a veterinarian.

2.2.2. Study Design

Female MMTV-TGFα mice were assigned in 3 different CR diets as random when mice were 10 weeks old. These CR groups are *ad libitum* (AL), chronic and intermittent calorie restriction (CCR and ICR respectively). Pellet feed which used to fed mice were supplied with Altromin TPF1414 from Kobay A.Ş. (Ankara, Turkey). All groups as explained in previous section had free access to water. Food access of MMTV-TGFα mice throughout the study was determined according to the diet regimens. Mice that assigned to AL group had free access to food and mean food consumption per mice calculated from food consumption of AL mice in age-dependent manner, however, CCR and ICR groups were 15 percent restricted compared to AL in a monthly cyclic manner. Compared to the AL group, 15 percent daily CR applied to mice in CCR group and these mice were daily consumed 85 percent of food consumed by AL equivalent. Furthermore, in ICR group, 60 percent CR applied for one week, after followed by three week of AL period. This feeding regimen was applied mice until they were sacrificed at designated age points. ICR group were divided into two group according to feeding periods. ICR group mice sacrificed at after three week of AL period were referred as ICR-refeeding (ICR-RF) and sacrificed at one week of 60 percent CR referred as ICR-restriction (ICR-R). Animals were sacrificed after overnight fasting at designated age: 10 (baseline) and 81 (end of refeed period), 82 weeks (end of restriction period) of age. Food consumption of animals were recorded daily. AL and ICR-R groups were fed at first day of the each week, and CCR and ICR-RF groups were fed predetermined amounts as daily basis. Body weights of animals were also recorded at first day of the each week and health status of animals were controlled as daily basis. For AL and CCR groups, size, weight, psychological and other conditions of mice did not differ at week 81 and 82, thus samples obtained at these weeks were combined as one timepoint (week 81/82). Whole brain samples were collected and one half of fresh tissues were immediately frozen via placing into dry ice and stored at -86°C and other half were collected into 10 percent formaldehyde to send pathology for further investigation of mice pathological status. According to the pathology results, samples used as mammary tumor developed mice brain samples have two grade II and one grade III carcinoma and the other samples are healthy brain tissues.

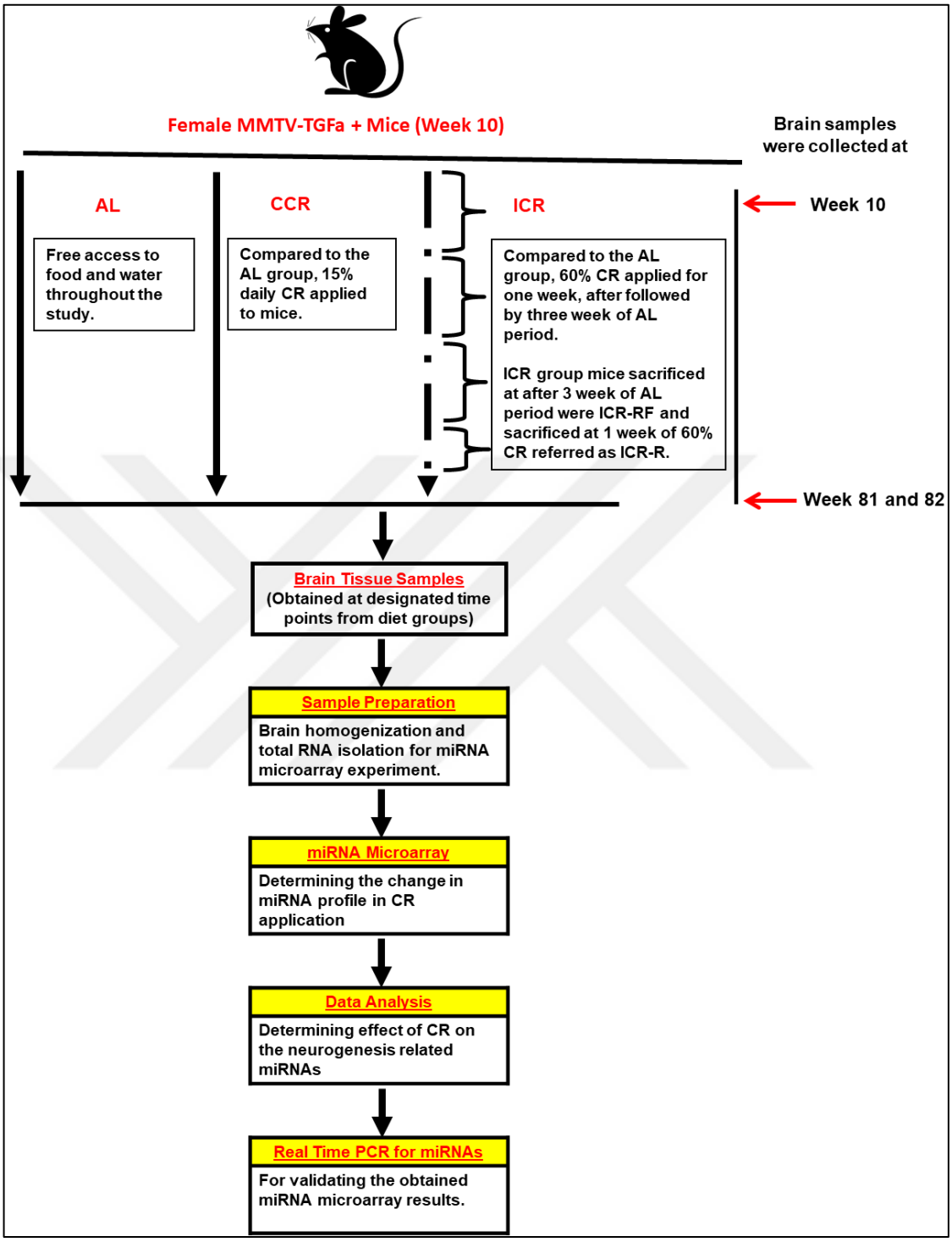


Figure 2.2. Schematic representation of experimental design.

2.2.3. Sample Preparation

Three brain samples from each group and three MT developed mice brain samples were taken out from ultra-freezer and placed onto ice and weight of the brain samples were measured. Then, samples were cut into long, thin strips and placed into 1.5 ml microcentrifuge tubes. Samples were washed (pipette up and down) three times with 600 µl of clean PBS to remove hair, blood, residues and debris. One spoon (0.1 gauge) of sterile 0.5 mm zirconium beads were added onto samples. PBS buffer that is twice the weight of the sample were added into each microcentrifuge tubes, however, if volume was exceeded 300 µl, excess amount were added after homogenization and vortexed to obtain homogeneous mixture. Tubes were tightly closed and sealed with parafilm and placed into the Bullet Blender. Samples were homogenized at speed eight for three minutes. After the run, samples were visually inspected and centrifuged at max RPM (14,000-20,000) at 4^{o}C for 10 min. to remove beads. Beads were removed from samples by transferring pellet and supernatant to another tube. Samples were mixed and 50 µl of samples were transferred to new 1.5 ml microcentrifuge tubes for RNA isolation. Stock of samples were stored at -86°C for other experiments.

Table 2.5. Number of brain samples were collected at determined weeks and diets.

Diet	**Week 10**	**Week 81/82**
AL	3	3
CCR		3
ICR-R		3
ICR-RF		3
MT developed mice brain	-	3
Number of Mice	**3**	**12**

2.2.4. Determination of miRNA Profile in Microarray

Affymetrix GeneChip miRNA 4.1 Array strips were used to profile miRNAs in whole brain samples. This array strip includes 30,424 mature miRNA probes from 203 organisms

according to miRBase v20 (www.mirbase.org). This probe includes 1,908 mature miRNAs and 1255 pre-miRNAs for mouse.

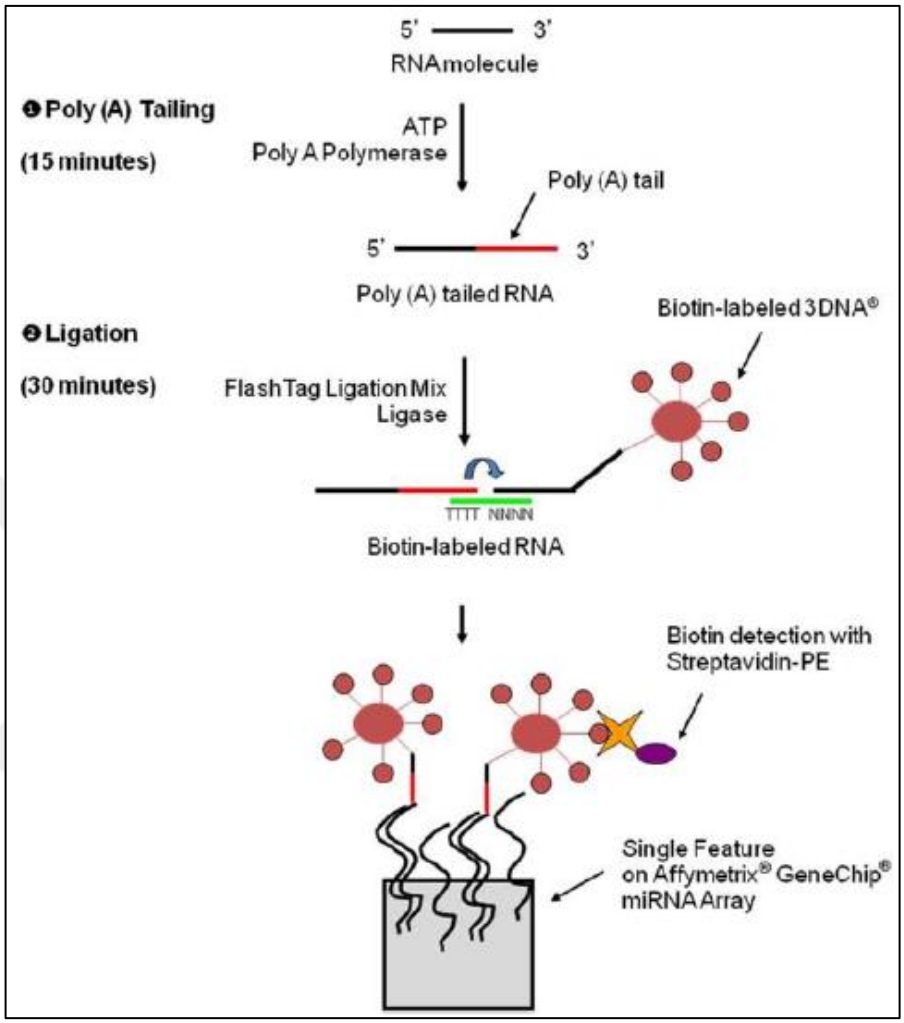


Figure 2.3. Schematic representation of miRNA microarray procedure.

2.2.4.1. RNA Isolation, Quantitation and Adjusting

After homogenization, 50 µl of samples were transferred to new 1.5 ml microcentrifuge tubes. 600 µl of TRI Reagent were added into samples and vigorously vortexed for one minute. Mixtures were incubated in ice for 5 minutes and centrifuged at room temperature (RT), 16,000g for one minute. Supernatant were transferred into new microcentrifuge tubes and 600 µl of 96 percent ethanol were added into mixture and vortexed. 700 µl of this mix

transferred into the Zymo Direct-zol MiniPrep Total RNA isolation columns and collection tubes are centrifuged at RT, 16,000g for one minute and flow-through was discarded. This step was repeated until whole mixture were passed through columns. Then, 400 µl RNA wash buffer were added into columns and centrifuged at RT, 16,000g for one minute and flow-through was discarded. Columns were treated with DNase I with mixture of six U/µl DNase I and 75 µl DNA Digestion Buffer. This mixture were directly added into matrix of columns and columns were incubated at RT for 15 minutes. After DNase I treatment, 400 µl Direct-zol RNA PreWash Buffer were added into columns and centrifuged at RT, 16,000g for one minute and flow-through was discarded. This step was repeated once. 700 µl RNA Wash Buffer were added into columns and centrifuged at RT, 16,000g for two minutes and flow-through was discarded. Columns were aerated to completely remove wash buffer. Columns were placed into 1.5 ml microcentrifuge tubes and 35 µl of RNase/DNase-free pure or distilled water were added into columns and incubated for one minute at RT. Incubated columns were centrifuged at RT, 16,000g for two minutes and RNA concentrations were determined with a NanoDrop 2000 spectrophotometer and integrity of total RNA controlled by one percent agarose gel electrophoresis. Isolated RNA samples were stored at -86°C and 500 ng total RNA from samples (n=3 for each group) which show 1.9-2.1 A260/280 nm absorbance ratio were prepared in PCR tubes and kept on ice for the next step. For the preparation of poly(A)tailing, HSR ligation and array strip hybridization, 10X reaction buffer, 25 mM $MnCl_2$, 5X FlashTag Biotin HSR ligation mix, HSR stop solution, nuclease free water, TRIS buffer, DMSO and 27,5 percent formamide were thawed at RT before use, ATP mix and RNA spike control oligos were thawed on ice, and PAP enzyme and T4 DNA Ligase were removed from -20°C just before use.

2.2.4.2. Poly(A) Tailing

Volume of 500 ng RNA containing samples were adjusted to eight µl with nuclease free water. ATP mix was diluted in 1 mM TRIS (1:500). Poly (A) tailing master mix are prepared in a nuclease free tube according to the following table 2.6 for each sample with 10 percent error margin.

Table 2.6. Poly(A) tailing master mix for one reaction.

#	Material	Volume
1	10X Reaction Buffer	1.5 µl
2	25 mM MnCl2	1.5 µl
3	Diluted ATP Mix	1.0 µl
4	PAP Enzyme	1.0 µl

Two µl RNA Spike Control Oligos was added into each sample and samples are transferred to the ice. Then, five µl of master mix were added into the 10 µl RNA/Spike Control Oligos mix to reach volume of 15 µl. Samples were mixed gently without vortexing and then microfuged. Samples were incubated in a 37°C for 15 minutes in thermal cycler and samples were taken after temperature reaches 4°C . Unused, Diluted ATP mix was discarded.

2.2.4.3. FlashTag Biotin HSR Ligation

After poly(A) tailing samples were labeled using FlashTag Biotin HSR RNA Labelling Kit (Applied Biosystems, Cat No: 901911). After poly(A) tail addition, samples were mixed gently (not vortex) and microfuged. Four µl 5X FlashTag Biotin HSR ligation mix were added to each sample. T4 DNA Ligase were taken from freezer and microfuged. Following that, two µl of T4 DNA Ligase were added to each sample. Samples were mixed gently (do not vortex) and microfuged. Then, incubated in a 25°C (RT) for 30 minutes. During this 30 minutes incubation, GeneChip miRNA 4.1 Array Strips were taken from 4°C storage-room to equilibrate with room temperature. Hybridization master mix were prepared according to the table 2.7 with 10 percent margin of error. Before preparing hybridization control, 20X Eukaryotic Hybridization Controls (bioB, bioC, bioD, cre from GeneChip Eukaryotic Hybridization Control Kit) were heated for five minutes at 65°C in thermal cycler. Meanwhile, GeneAtlas Hybridization Station was turned on and temperature was set 48°C. After the 30 minutes incubation period, 2.5 µl HSR Stop Solution was added into samples. Then, two µl of the biotin-labeled sample was discarded for each sample and total volume must be 21.5 µl per sample. 103.5 µl Hybridization Master Mix were added into samples and total volume for each sample was reached to 125 µl.

Table 2.7. Hybridization master mix for one reaction.

#	**Material**	**Volume**	**Final Conc.**
1	2X Hybridization Mix	62.5 µl	1X
2	27.5% Formamide	18.2 µl	4%
3	DMSO	12.1 µl	9.7%
4	20X Hybridization Controls	6.3 µl	1X
5	Control Oligo B2, 3nM	2.1 µl	50 pM
6	Nuclease Free Water	2.3 µl	
Σ	**Total Volume**	**103.5 µl**	

2.2.4.4. Array Strip Hybridization

After labelling samples, these prepared mixtures were denaturized at 95°C for five minutes, followed by 45°C for five minutes in thermal cycler. Then, 120 µl of sample and hybridization master mix were applied to the middle of the appropriate wells of hybridization trays of GeneChip miRNA 4.1 Array Strips. Array strips were taken from protective cover and carefully placed into the hybridization tray that containing sample mixes to avoid bubble formation between hybridization tray and array strip. Hybridization tray with the array strip were placed into a clamp inside the Hybridization Station and strips were incubated 18-22 hours. one hour before the end of the incubation, Affymetrix array strip box, GeneAtlas Hybridization, Wash and Stain Kit for miRNA Array Strips, and GeneAtlas Wash Buffers were taken from 4°C storage-room to equilibrate to room temperature. Wash and stain reagents are prepared 15 minutes before running the fluidics station. Reagents were added to fluidics station tray according to the following figure. Fluidics station and Imaging station were turned on before experiment. After that, these trays were placed into fluidics station. GeneAtlas Instrument Control Software 2.0 was opened and in hybridization section, clicked to the “add strip”. Barcode on array strips were scanned with barcode scanner. Sample file name and sample names were written and clicked to the “add”. Then, clicked on start and stop button respectively. After these hybridization step which actually done without computer program, clicked on “go to fluidics”. In fluidics section, clicked to the “add strip” and barcode on array strips were scanned with barcode scanner. Clicked to the “add”. Tray was placed on fluidics station and clicked to the “start”. At the end of this process, imaging section was opened and clicked to the “add strip”. Barcode on array strips were scanned with barcode scanner. Clicked to the “add” and clicked to the “open imaging station”. Imaging

tray with array strips were placed on imaging station. Then, clicked to the “close imaging station”. Imaging process were started after clicking to the “start”. After imaging step, quality of results were checked in quality control section, and results that obtained as .cel files were transferred into Transcriptome Analysis Console (TAC) 4.0.1.

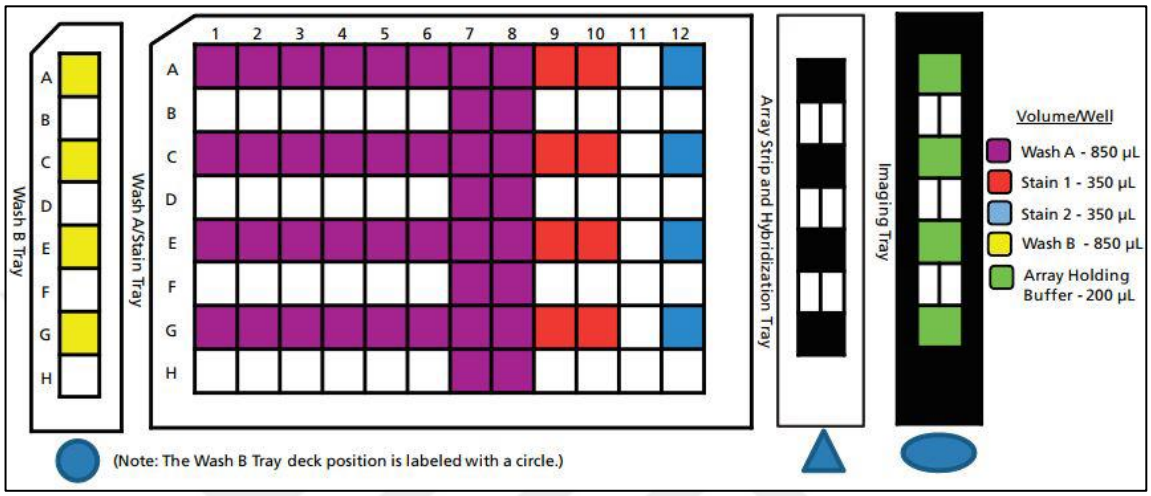


Figure 2.4. Wash and stain tray map for Fluidics station.

2.2.4.5. Data Analysis

Results that transferred into TAC 4.0.1 program were tagged with identifiers according to their diet and ages. Then, array type, analysis type and summarization were chosen as miRNA-4_1, Expression (Gene) and RMA+DABG (Mouse Only) respectively. Comparison configuration was selected as default, which contains gene-level fold change >2, gene level $p<0.05$, FDR false, eBayes ANOVA and DABG<0.05. Comparison criteria were selected and analysis were performed. After analysis, differentially expressed (DE) miRNAs that compared between diet and age were exported into excel with name of miRNAs, accession numbers, fold change and p values. Validated targets of DE miRNAs were determined using multiMiR R package using Tarbase, miRTarbase and miRecords database [219]. Predicted targets of DE miRNAs were determined in Diana microT CDS 5, Targetscan Mouse 7.2 and miRDB 6.0. In Diana microT CDS 5, targets were sorted according to the miTG score [220]. In miRDB 6.0, targets were sorted according to the target score, and in Targetscan Mouse 7.2, targets were sorted according to the their cumulative weighted context++ score [221,

222]. However, in tumor developed mice brain and healthy mice brain comparison, top 20 percent of targets from each databases that sorted according to their scores were compared and concurrent ones were considered as potential targets of miRNAs. When miRNA targets were determined, targets of DE miRNAs that determined in comparisons were transferred into Cytoscape plugin Cluego 2.5.4 and Cluepedia 1.5.4 to perform gene ontology (GO) analysis [223, 224]. In GO analysis, ClueGo functional analysis were selected and *Mus Musculus* (10090) marker lists were loaded. Targets of DE miRNAs were loaded and GO BiologicalProcess-EBI-UniProt-GOA, CellularComponent-EBI-Uniprot-GOA, ImmuneSystem Process-EBI-Uniprot-GOA, and MolecularFunction-EBI-Uniprot-GOA ontologies were selected to determine GO results. Before starting analysis, only pathways that had p values <0.05 was selected to show in results. Minimum GO Tree Interval and maximum GO Tree Interval were determined as three and eight respectively, and minimum number of genes in a cluster and percentages of genes selected as three and four percent. Kappa scores were determined as 0.4, as a statistics Enrichment/Depletion (Two-sided hypergeometric test) were used with Bonferroni step down p value correction. As a reference set, selected ontologies reference set was used, GO terms were grouped and leading group term was determined according to highest significance. As a figure layout, yFiles Organic Layout were used. Data were obtained and brain related target genes of DE miRNAs in GO were determined. ClueGO functional enrichment analysis were performed using GO BiologicalProcess-EBI-UniProt-GOA, CellularComponent-EBI-Uniprot-GOA, ImmuneSystem Process-EBI-Uniprot-GOA, and MolecularFunction-EBI-Uniprot-GOA ontologies, KEGG, Proteome pathway and Wikipathways databases. KEGG analysis of targets of DE miRNAs were performed using DAVID 6.8 [225]. Targets of DE miRNAs were loaded and OFFICIAL_GENE_SYMBOL was selected as identifier. List type was selected as gene list and species were selected as *Mus Musculus*. Then, Functional annotation tool was selected, and pathway was determined as KEGG_PATHWAY. Annotations were observed as functional annotation clustering, chart and table with default settings. Data were obtained and neurogenesis related target genes of DE miRNAs in KEGG pathway were determined. Functional enrichment analysis of predicted target genes were performed using default settings of DAVID 6.8. Enrichment score higher than two considered as significantly enriched in common groups. Morpheus matrix visualization software were used to visualize differentially expressed miRNAs in comparisons and form K means cluster [226]. Venny

venn diagram maker software used to create venn diagram between DE miRNAs and their targets in comparisons [227].

2.2.5. Validation of miRNAs

cDNA synthesis was performed using 100 ng total RNA and preamplified with miRCURY LNA RT Kit (Qiagen, Cat No:339340) according to the manufacturer protocol. Three µl of preamplified cDNA template for each replicate were loaded into 96-well plate. 10 µl of qRT-PCR reaction was performed as duplicates with miRCURY LNA Sybr Green PCR Kit (Qiagen, Cat No:339347) with manufacturer protocol in BioRad CFX96 Touch Real-Time PCR Detection System. mmu-miR-713 (MIMAT0003504) primer designed using miRprimer2 and best primer pair selected [228]. Obtained results were normalized via ΔCt method using Qiagen 5S rRNA (YP00203906) as housekeeping. Expression data were obtained with BioRad CFX Maestro software.

2.2.6. Statistical Analysis

Microarray results were normalized and quality controlled with miRNA QC tool of Affymetrix Expression Console 1.4 software and results were analyzed using fold change as two and p-value smaller than 0.05 and ebayes ANOVA with the Affymetrix Transcriptome Analysis Console 4.0.1 software. As a summarization method, mouse only RMA-DABG with default TAC 4.0.1 preferences were used. Targets of DE miRNAs were transferred into Cytoscape plugin Cluego 2.5.4 and Cluepedia 1.5.4 and Gene Ontology (GO) analysis were performed with Enrichment/Depletion (Two-sided hypergeometric test) with Bonferroni step down p value correction which were accessed at May 2019. For KEGG analysis DAVID 6.8 were used which were accessed at May 2019. Quantitative Real Time PCR expression data were analyzed ΔCt method with Excel 2019 and GraphPad Prism 7 using one way ANOVA and Tukey post-hoc test. Outliers were removed using ROUT method with 2 percent Q value. P value smaller than 0.05 accepted as significant. Graphics were represented as mean±SEM.

3. RESULTS

To measure the effects of different types of CR on the miRNA profile of brain tissue, a total of 1237 pre-miRNAs (precursors) and 1926 mature miRNAs were analyzed on mice brains. Of these precursors and mature miRNAs, 55 of them were differentially expressed at different diet applications and ageing combinedly. Average expression (log2) of these differentially expressed miRNAs in all comparisons were given in five subgrouped K means cluster at Fig 3.1. High, middle and low expressions marked with red, black and green respectively.

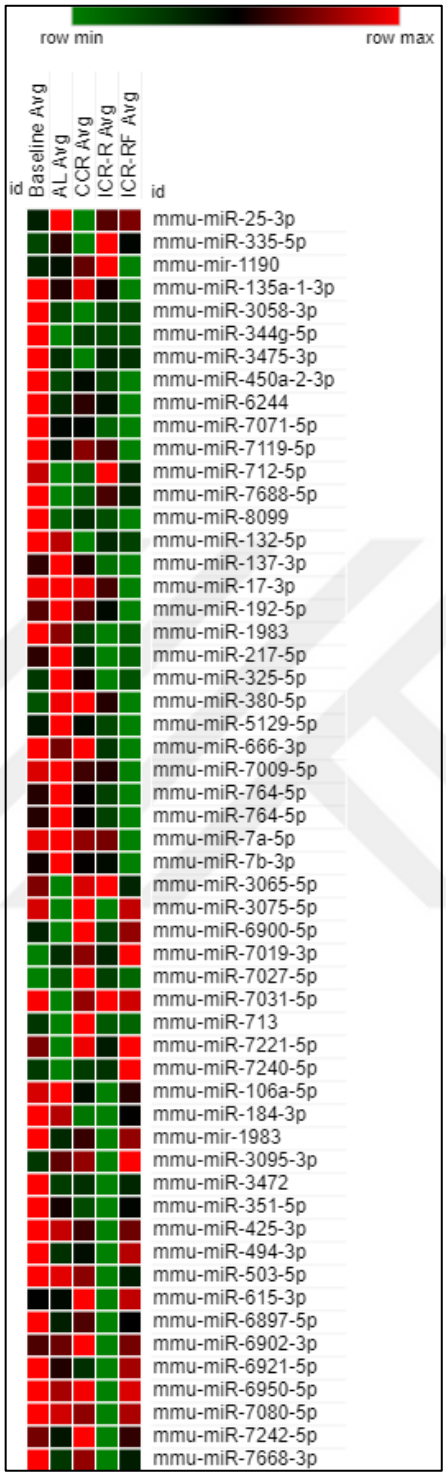


Figure 3.1. K means hierarchical cluster of DE miRNAs with average expression (log2) of each group. K means clustering performed with 5 main divisions. High, middle and low expressions marked with red, black and green respectively.

3.1. THE EFFECTS OF CR ON MICRORNA PROFILE IN BRAIN

In different CR applications, 28 miRNAs and two precursors were differentially expressed in comparisons. Compared to AL group, four miRNAs were upregulated and two miRNAs were downregulated in CCR. In ICR-R, two miRNAs were upregulated and two miRNAs were downregulated. In ICR-RF group, three miRNAs were upregulated and nine miRNAs were downregulated. Compared to CCR group, one miRNA was upregulated and seven miRNAs and one precursor were downregulated in ICR-R. In ICR-RF, two miRNAs were downregulated compared to CCR application. Compared to ICR-R, one miRNA and one precursor were upregulated and one miRNAs and one precursor were downregulated in ICR-RF diet. These number of upregulated and downregulated miRNAs in each comparison was given at table 3.1. Names of DE miRNAs and expression levels compared to different diets were given in Fig 3.2. Lowest and highest fold change in comparisons are -3.84 and 4.33 and represented with blue and red respectively.

Table 3.1. Numbers of differentially expressed miRNAs among CR groups.

	Compared to	**CCR**		**ICR-R**		**ICR-RF**		
Week 81/82	**AL**	4 ↑	2 ↓	2 ↑	2 ↓	3 ↑	9 ↓	miRNAs
		-	-	-	-	-	-	Precursors
	CCR	-	-	1 ↑	7 ↓	-	2 ↓	miRNAs
		-	-	-	1 ↓	-	-	Precursors
	ICR-R	-	-	-	-	1 ↑	1 ↓	miRNAs
		-	-	-	-	1 ↑	1 ↓	Precursors

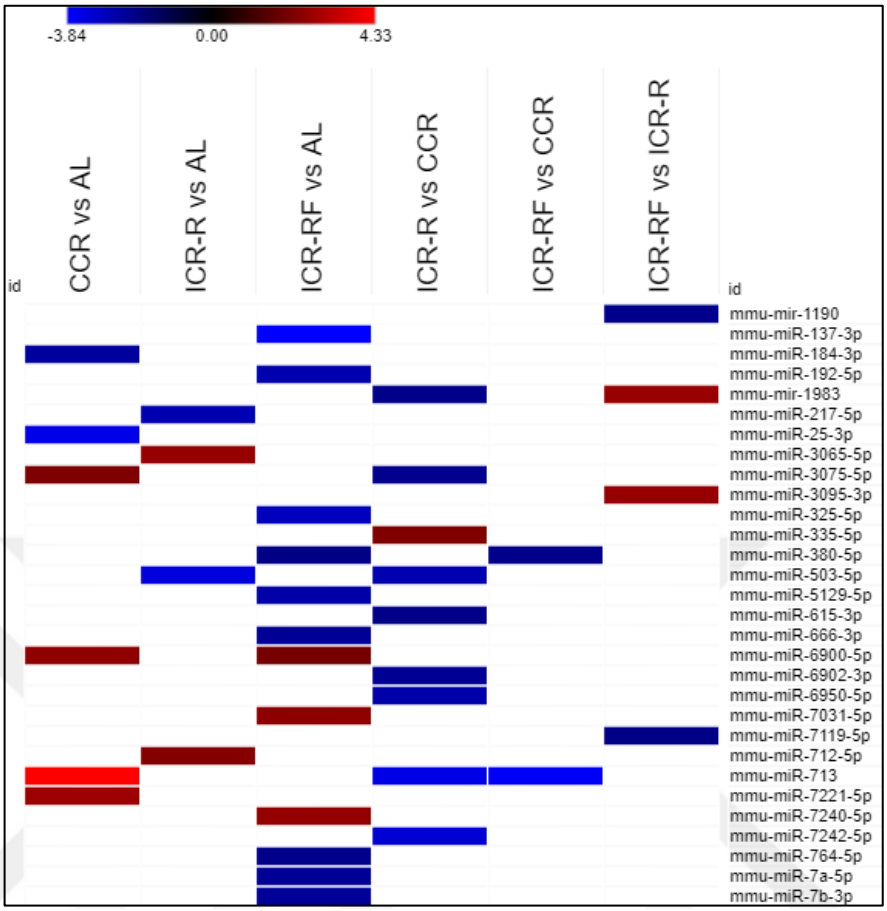


Figure 3.2. miRNAs differentially expressed among CR groups. miRNAs are listed alphabetically. Lowest and highest fold change are -3.84 and 4.33 and represented with blue and red respectively.

Common miRNAs in comparisons were determined in Fig 3.3A and B. Compared to AL, there was no common miRNAs found between CCR, ICR-R and ICR-RF, however, mmu-miR-6900-5p was common between CCR and ICR-RF compared to AL. mmu-miR-6900-5p levels were significantly lower in the AL group compared to CCR and ICR-RF. Compared to CCR diet, mmu-miR-713 was common between AL, ICR-R, and ICR-RF. Level of mmu-miR-713 was significantly higher in CCR group compared to AL, ICR-R and ICR-RF. Moreover, mmu-miR-3075-5p was common between AL and ICR-R group compared to CCR. Expression level of mmu-miR-3075-5p was significantly higher in CCR diet applied mice brain samples. Compared to ICR-R diet, there was no common miRNAs between AL, CCR and ICR-RF, however, mmu-miR-503-5p was common between AL and CCR compared to ICR-R. Expression of mmu-miR-503-5p was significantly lower in ICR-

R diet compared to AL and CCR. Furthermore, mmu-mir-1983 was common between CCR and ICR-RF compared to ICR-R and its expression was significantly lower in ICR-R. Compared to ICR-RF, only mmu-miR-380-5p was common between AL and CCR and expression level was significantly lower in ICR-RF.

3.1.1. Targets of miRNAs

In different CR applications, 28 miRNAs and two precursors were differentially expressed in all comparisons. Validated targets of these differentially expressed miRNA were determined using Tarbase, miRTarbase and miRecords. Compared to AL group, total of 56 validated targets were determined in CCR group. In ICR-R, total of 223 validated targets were determined compared to AL. In ICR-RF, total of 103 validated targets were determined compared to AL. Compared to CCR, total of 68 validated targets were determined in ICR-R. In ICR-RF, 31 validated targets were determined compared to CCR. Compared to ICR-R group, total of 17 validated targets were determined in ICR-RF.

Predicted targets of these differentially expressed miRNA were determined using Diana microT CDS 5.0, Targetscan mouse 7.2 and miRDB 6.0. Concurrent targets from each databases were considered as predicted targets of DE miRNAs. Compared to AL group, total of 1036 predicted targets were determined and 324 of them were upregulated by downregulation of miRNAs and 757 of them were downregulated by upregulation of miRNAs compared to AL. 45 of these predicted targets were common in upregulated and downregulated predicted targets. In ICR-R, total of 1173 predicted targets were determined and 211 of them were upregulated and 994 of them were downregulated compared to AL. In ICR-R group, 32 of these predicted targets were common in upregulated and downregulated predicted targets in comparison with AL group. In ICR-RF, total of 1738 predicted targets were determined and 1210 of them were upregulated and 625 of them were downregulated compared to AL. In ICR-RF group, 97 of these predicted targets were common in upregulated and downregulated predicted targets in comparison with AL group. Compared to CCR, total of 1010 predicted targets were determined and 930 of them were upregulated and 84 of them were downregulated in ICR-R. Four of these predicted targets were common in upregulated and downregulated predicted targets in ICR-R group compared to CCR. In ICR-RF, 289 predicted targets were determined and all of them upregulated compared to

CCR. Compared to ICR-R group, total of 257 predicted targets were determined and 930 of them were upregulated and 84 of them were downregulated in ICR-RF. Six of these predicted targets were common in upregulated and downregulated predicted targets in ICR-RF group compared to ICR-R. Number of predicted targets of upregulated and downregulated miRNAs in each comparison are given at table 3.2.

Table 3.2. Numbers of predicted targets of DE miRNAs among CR groups.

	Compared to	**CCR**			**ICR-R**			**ICR-RF**		
		Up	**Down**	**Common**	**Up**	**Down**	**Common**	**Up**	**Down**	**Common**
Week 81/82	**AL**	324	757	45	211	994	32	1210	625	97
	CCR	-	-	-	930	84	4	289	-	-
	ICR-R	-	-	-	-	-	-	133	130	6

Common predicted targets in comparisons were determined in Fig 3.3C and D. Compared to AL, 87 of predicted targets were common between CCR, ICR-R and ICR-RF. 75 of predicted targets were common between CCR and ICR-R. 389 of predicted targets were common between CCR and ICR-RF. 192 of predicted targets were common between ICR-R and ICR-RF. Compared to CCR, 236 of predicted targets were common between AL, ICR-R and ICR-RF. 240 of predicted targets were common between AL and ICR-R. Three of predicted targets were common between AL and ICR-RF.

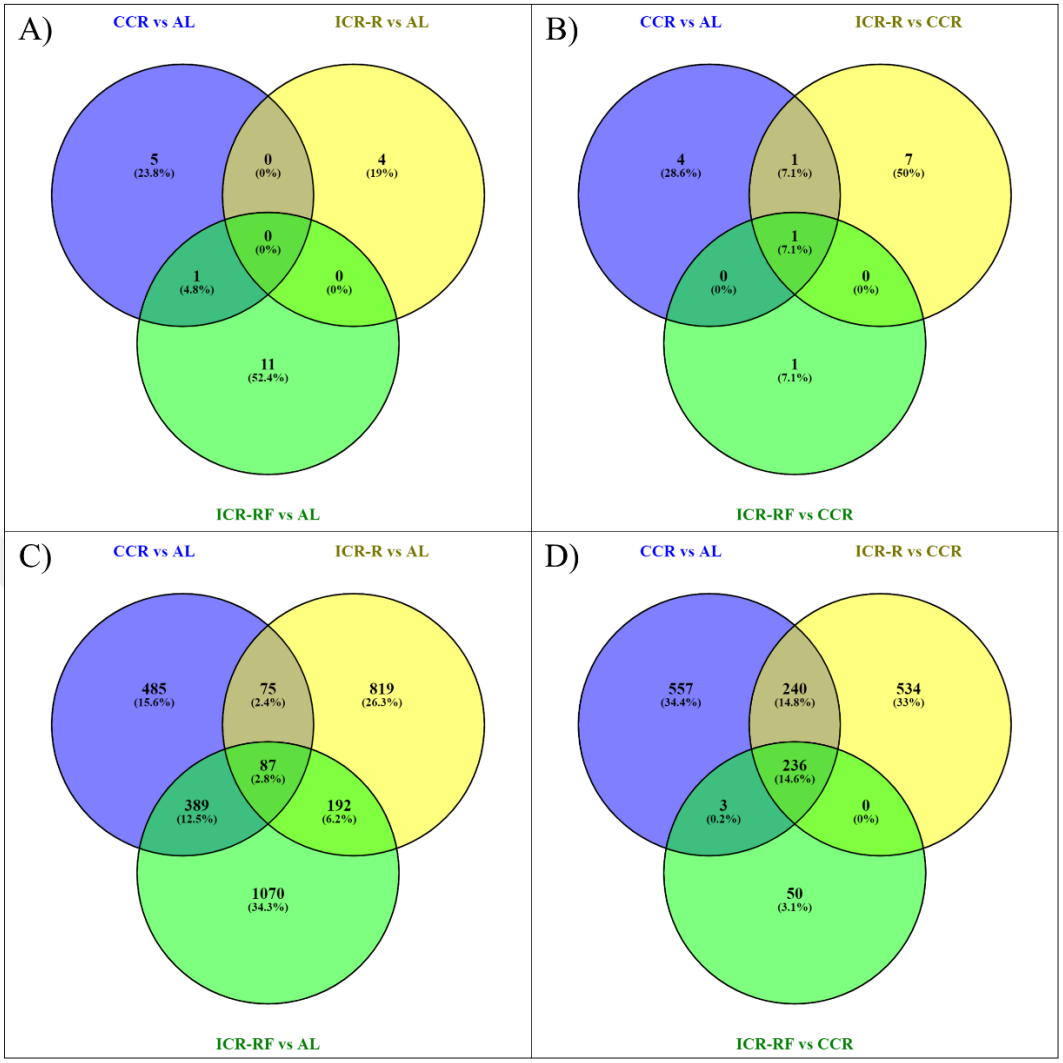


Figure 3.3. Venn schematics of miRNAs and their predicted targets compared to AL and CCR among CR groups. A and C are miRNAs and their targets compared to AL respectively. B and D are miRNAs and their targets compared to CCR respectively.

3.1.2. Gene Ontology of Predicted Targets

Application of different types of CR on mice changed several GO terms in different CR applied mice. In gene ontology analysis, gene ontology terms were grouped under biological processes, immune system process, cellular component and molecular function. Compared to AL diet, 271 GO terms were significantly enriched in CCR group, 420 GO terms were significantly enriched in ICR-R group and 568 GO terms were enriched in ICR-RF group. Of these GO terms, 217 of them were common in CCR, ICR-R and ICR-RF group compared

to AL which includes terms like nervous system development, neurogenesis and generation of neurons. Compared to CCR diet, 227 GO terms were significantly enriched in ICR-R group and 13 GO terms were significantly enriched in ICR-RF group. Of these GO terms, presynaptic membrane term were common in AL, ICR-R and ICR-RF group compared to CCR. Top brain related GO terms were given with their GO ids, sources, levels, percentage of associated genes and number of predicted genes that found in GO term in table 3.3.

Table 3.3. Gene Ontology of predicted targets of DE miRNAs among CR groups.

CCR vs AL						
GO ID	**GO Term**	**GO Source**	**Bonferroni P Val.**	**GO Levels**	**% Assoc. Genes**	**Nr. Genes**
GO:0007399	Nervous system development	GO_BP	1.36E-14	[4, 5]	8.29	222.00
GO:0022008	Neurogenesis	GO_BP	1.77E-11	[4, 5, 6]	8.55	170.00
GO:0048699	Generation of neurons	GO_BP	2.01E-11	[5, 6, 7]	8.72	161.00
GO:0030182	Neuron differentiation	GO_BP	5.21E-10	[4, 6, 7, 8]	8.71	145.00
GO:0048666	Neuron development	GO_BP	2.27E-09	[4, 5, 7, 8, 9]	9.10	124.00
GO:0050767	Regulation of neurogenesis	GO_BP	4.64E-09	[5, 6, 7, 8]	9.69	104.00
GO:0045664	Regulation of neuron differentiation	GO_BP	1.56E-08	[5, 6, 7, 8, 9]	10.17	89.00
GO:0043005	Neuron projection	GO_CC	3.81E-08	[3, 4, 5]	8.29	143.00
GO:0031175	Neuron projection development	GO_BP	4.76E-08	[5, 6, 8, 9, 10]	9.11	110.00
GO:0097458	Neuron part	GO_CC	5.45E-08	[2, 3]	7.77	173.00

ICR-R vs AL						
GOID	**GO Term**	**GO Source**	**Bonferroni P Val.**	**GO Levels**	**% Assoc. Genes**	**Nr. Genes**
GO:0007399	Nervous system development	GO_BP	7.61E-17	[4, 5]	9.37	251.00
GO:0022008	Neurogenesis	GO_BP	1.17E-10	[4, 5, 6]	9.25	184.00
GO:0048699	Generation of neurons	GO_BP	1.28E-10	[5, 6, 7]	9.42	174.00
GO:0050767	Regulation of neurogenesis	GO_BP	1.94E-10	[5, 6, 7, 8]	10.90	117.00
GO:0097458	Neuron part	GO_CC	2.20E-10	[2, 3]	8.94	199.00
GO:0030182	Neuron differentiation	GO_BP	1.77E-08	[4, 6, 7, 8]	9.25	154.00
GO:0007417	Central nervous system development	GO_BP	2.43E-08	[4, 5, 6]	10.18	117.00
GO:0048667	Cell morphogenesis involved in neuron differentiation	GO_BP	2.83E-08	[5, 6, 7, 8, 9, 10]	11.88	81.00
GO:0098793	Presynapse	GO_CC	8.44E-08	[2, 3, 4]	11.84	78.00
GO:0050808	Synapse organization	GO_BP	1.52E-07	[3]	12.62	66.00

ICR-RF vs AL						
GOID	**GO Term**	**GO Source**	**Bonferroni P Val.**	**GO Levels**	**% Assoc. Genes**	**Nr. Genes**
GO:0007399	Nervous system development	GO_BP	6.54E-24	[4, 5]	13.59	364.00
GO:0097458	Neuron part	GO_CC	1.29E-20	[2, 3]	13.83	308.00
GO:0048699	Generation of neurons	GO_BP	5.43E-20	[5, 6, 7]	14.46	267.00
GO:0022008	Neurogenesis	GO_BP	7.66E-20	[4, 5, 6]	14.13	281.00
GO:0048666	Neuron development	GO_BP	2.74E-17	[4, 5, 7, 8, 9]	15.19	207.00
GO:0043005	Neuron projection	GO_CC	4.22E-17	[3, 4, 5]	14.21	245.00
GO:0030182	Neuron differentiation	GO_BP	3.58E-16	[4, 6, 7, 8]	14.18	236.00
GO:0097060	Synaptic membrane	GO_CC	4.47E-14	[2, 3, 4, 5, 6, 7]	18.87	107.00
GO:0051960	Regulation of nervous system development	GO_BP	6.85E-14	[4, 5, 6]	14.98	179.00
GO:0031175	Neuron projection development	GO_BP	1.69E-13	[5, 6, 8, 9, 10]	14.83	179.00

ICR-R vs CCR						
GO ID	**GO Term**	**GO Source**	**Bonferroni P Val.**	**GO Levels**	**% Assoc. Genes**	**Nr. Genes**
GO:0007399	Nervous system development	GO_BP	8.17E-06	[4, 5]	6.91	185.00
GO:0036477	Somatodendritic compartment	GO_CC	1.25E-05	[3, 4]	8.25	100.00
GO:0007417	Central nervous system development	GO_BP	1.09E-04	[4, 5, 6]	8.09	93.00
GO:0097458	Neuron part	GO_CC	2.35E-04	[2, 3]	6.92	154.00
GO:0097060	Synaptic membrane	GO_CC	3.38E-04	[2, 3, 4, 5, 6, 7]	9.70	55.00
GO:0045664	Regulation of neuron differentiation	GO_BP	5.85E-04	[5, 6, 7, 8, 9]	8.46	74.00
GO:0030425	Dendrite	GO_CC	6.65E-04	[4, 5, 6, 7]	8.60	71.00
GO:0050767	Regulation of neurogenesis	GO_BP	6.83E-04	[5, 6, 7, 8]	8.01	86.00
GO:0043025	Neuronal cell body	GO_CC	7.37E-04	[3, 4, 5]	8.53	71.00
GO:0050770	Regulation of axonogenesis	GO_BP	7.63E-04	[6, 7, 8, 9, 10, 11, 12, 13]	12.95	29.00

ICR-RF vs CCR						
GO ID	**GO Term**	**GO Source**	**Bonferroni P Val.**	**GO Levels**	**% Assoc. Genes**	**Nr. Genes**
GO:0042734	presynaptic membrane	GO_CC	7.78E-03	[3, 4, 5, 6, 7, 8]	5.15	12.00
GO:0021954	Central nervous system neuron development	GO_BP	1.55E-02	[5, 6, 7, 8, 9, 10]	7.02	8.00
GO:0098889	Intrinsic component of presynaptic membrane	GO_CC	1.67E-02	[3, 4, 5, 6, 7, 8, 9]	6.12	9.00
GO:0021537	Telencephalon development	GO_BP	2.44E-02	[3, 4, 6, 7, 8, 9]	4.05	14.00
GO:0050954	Sensory perception of mechanical stimulus	GO_BP	2.69E-02	[5]	5.21	10.00
GO:0099240	Intrinsic component of synaptic membrane	GO_CC	3.05E-02	[2, 3, 4, 5, 6, 7, 8]	4.41	12.00
GO:0043195	Terminal bouton	GO_CC	3.07E-02	[3, 4, 5, 6, 7, 8, 9, 10]	7.29	7.00
GO:0048787	Presynaptic active zone membrane	GO_CC	3.96E-02	[3, 4, 5, 6, 7, 8, 9]	10.64	5.00
GO:0021953	Central nervous system neuron differentiation	GO_BP	3.96E-02	[5, 6, 7, 8, 9]	4.56	11.00

ICR-RF vs ICR-R						
GO ID	**GO Term**	**GO Source**	**Bonferroni P Val.**	**GO Levels**	**% Assoc. Genes**	**Nr. Genes**
GO:0032591	Dendritic spine membrane	GO_CC	3.28E-02	[3, 4, 5, 6, 7, 8, 9, 10]	18.75	3.00

3.1.3. Pathway Analysis of Predicted Targets

Application of different types of CR on mice changed KEGG terms in different CR applied mice. KEGG analysis of predicted targets of DE miRNAs were revealed several pathways in each comparison. Compared to AL diet, five KEGG pathways were significantly enriched according to Bonferroni p value correction in CCR group including glutamatergic synapse and circadian entrainment. In ICR-R, six KEGG pathways were significantly enriched including signaling pathways regulating pluripotency of stem cells and axon guidance. In ICR-RF, 16 KEGG pathways were significantly enriched including mTOR and AMPK signaling pathways compared to AL diet. Compared to CCR, Jak-STAT and sphingolipid signaling pathways were significantly enriched in ICR-R. In ICR-RF, dopaminergic and glutamatergic synapse pathways were significantly enriched in ICR-R compared to ICR-RF, however, no significantly enriched pathways were found in ICR-RF compared to ICR-R. Top KEGG pathways were given with their term ids, number of predicted genes that found in KEGG pathway, percentage of associated genes and genes in table 3.4.

Table 3.4. KEGG pathway analysis of predicted targets of DE miRNAs among CR groups.

CCR vs AL					
Term ID	**KEGG Pathway**	**Count**	**%**	**Bonferroni P Val.**	**Genes**
mmu05205	Proteoglycans in cancer	26	2.51	8.90E-04	CAV1, GRB2, PPP1R12C, RDX, SDC2, SRC, PDPK1, TIAM1, ITGAV, FASL, CAMK2B, MSN, PIK3R1, PIK3CG, FZD8, BRAF, PRKCG, DDX5, ARHGEF12, FZD7, ITPR1, EIF4B, FZD10, ITGA5, MAPK14, COL1A2
mmu04724	Glutamatergic synapse	17	1.64	1.19E-02	ADCY3, GNAI3, SLC38A2, ADCY7, PRKCG, GRIA3, GRIA4, KCNJ3, ITPR1, GRM4, SLC1A2, SLC17A6, GNAQ, GRIA2, DLG4, PPP3CA, PLA2G4C
mmu04713	Circadian entrainment	15	1.45	2.61E-02	ADCY3, GNAI3, ADCY7, CACNA1I, PRKCG, GRIA3, GRIA4, KCNJ3, ITPR1, FOS, GNAQ, GRIA2, GUCY1A2, PER2, CAMK2B
mmu04921	Oxytocin signaling pathway	19	1.83	2.92E-02	ADCY3, GNAI3, ADCY7, PPP1R12C, PRKCG, KCNJ2, CACNA2D2, KCNJ3, SRC, ITPR1, FOS, GNAQ, CAMK4, GUCY1A2, EEF2K, CAMK2B, PRKAA2, PPP3CA, PLA2G4C
mmu04510	Focal adhesion	23	2.22	3.30E-02	COL4A4, PIK3CG, IBSP, CAV1, BRAF, PDGFA, PGF, GRB2, PPP1R12C, ITGA10, PRKCG, SRC, CHAD, PDPK1, ITGA5, ITGAV, GSK3B, COL27A1, ITGA8, COL1A2, RAP1B, CRK, PIK3R1

ICR-R vs AL					
Term ID	**KEGG Pathway**	**Count**	**%**	**Bonferroni P Val.**	**Genes**
mmu05205	Proteoglycans in cancer	30	2.56	9.04E-04	TFAP4, ERBB4, WNT3A, PDCD4, TIMP3, KRAS, WNT3, PIK3CA, PIK3R3, CAMK2A, PIK3R1, TWIST1, PRKCA, PIK3CB, ROCK2, MET, ESR1, IGF1, FZD5, PPP1CB, FZD7, ITPR2, KDR, FZD6, PTPN11, CTSL, CCND1, VEGFA, HBEGF, WNT7A
mmu04550	Signaling pathways regulating pluripotency of stem cells	22	1.88	7.85E-03	BMI1, PIK3CB, WNT3A, IGF1, REST, FZD5, MEIS1, ZIC3, FZD7, ACVR1C, FZD6, WNT3, KRAS, RIF1, PIK3CA, AXIN2, PIK3R3, ZFHX3, WNT7A, PIK3R1, KAT6A, BMPR1A
mmu04360	Axon guidance	21	1.79	9.20E-03	ABLIM1, PLXNA2, ROCK2, MET, PPP3R1, NTNG1, SLIT1, EPHA2, EPHA4, EPHA7, KRAS, PAK2, ROBO1, SEMA7A, SEMA3E, SEMA4C, SEMA3D, PPP3CA, SEMA3A, SEMA4D, RASA1
mmu04960	Aldosterone-regulated sodium reabsorption	11	0.94	1.49E-02	PRKCA, ATP1B1, KRAS, ATP1B3, PIK3CB, NR3C2, IGF1, PIK3CA, PIK3R3, INSR, PIK3R1
mmu04310	Wnt signaling pathway	21	1.79	3.28E-02	CER1, PRKCA, AF366264, NKD1, ROCK2, WNT3A, BTRC, PPP3R1, FZD5, FZD7, FZD6, CCND1, WNT3, CCND2, PPP3CA, AXIN2, PLCB1, FOSL1, CAMK2A, WNT7A, FBXW11
mmu04015	Rap1 signaling pathway	27	2.30	4.70E-02	PRKCA, FGF5, FGF7, TLN2, PIK3CB, CSF1, MET, IGF1, FGF13, KIT, RALGDS, EPHA2, DOCK4, KDR, KRAS, GNAQ, CNR1, P2RY1, VEGFA, PIK3CA, RAP1B, PLCB1, RAPGEF2, PIK3R3, CRK, INSR, PIK3R1

ICR-RF vs AL					
Term ID	**KEGG Pathway**	**Count**	**%**	**Bonferroni P Val.**	**Genes**
mmu04012	ErbB signaling pathway	24	1.38	5.98E-05	PRKCA, NRG3, ERBB4, BRAF, PIK3CD, ELK1, RAF1, PRKCG, RPS6KB1, SRC, PAK6, CBLB, CDKN1B, EREG, NCK1, GSK3B, CAMK2B, SHC1, PIK3R5, PIK3R3, CRK, ABL2, PIK3R1, AKT2
mmu04015	Rap1 signaling pathway	41	2.36	9.14E-05	FGF7, ADCY2, TLN2, EFNA3, FGF16, FGF11, LPAR4, CTNND1, KIT, SRC, PFN2, TEK, RAPGEF6, PIK3R5, THBS1, RAPGEF2, PLCB1, PIK3R3, INSR, PIK3R1, AKT2, PRKCA, MAGI2, BRAF, GRIN1, PIK3CD, SIPA1L2, RAF1, IGF1, PRKCG, KITL, PRKD1, VEGFC, MAPK14, EFNA5, RAP1B, PARD6G, CRK, PRKD3, CALM1, LCP2
mmu04150	mTOR signaling pathway	19	1.09	1.24E-04	PRKCA, BRAF, PIK3CD, IGF1, PRKCG, RPS6KB1, RICTOR, RRAGD, DDIT4, EIF4B, PDPK1, TSC1, ULK2, PRKAA1, PIK3R5, MLST8, PIK3R3, PIK3R1, AKT2
mmu04151	PI3K-Akt signaling pathway	56	3.22	2.63E-04	FGF7, PPP2R5A, EFNA3, PPP2R5C, FGF16, FGF11, LPAR4, RPS6KB1, CHAD, PDPK1, CREB3L2, MLST8, INSR, AKT2, SYK, PPP2R1B, PRKCA, SGK1, PIK3CD, PKN2, DDIT4, VEGFC, G6PC, CCND3, GNB1, CHRM1, EFNA5, PHLPP2, MCL1, COL3A1, GNG11, COL2A1, KIT, PPP2CA, TEK, PIK3R5, PRKAA1, PIK3R3, THBS1, PPP2R2D, PIK3R1, COL4A4, TNXB, RAF1, IGF1, ITGA4, KITL, EIF4B, ITGA9, HSP90B1, CDKN1B, TSC1, ITGA5, GSK3B, IKBKG, YWHAQ
mmu04910	Insulin signaling pathway	30	1.73	4.55E-04	HK2, MKNK1, RHOQ, PDE3B, ELK1, RPS6KB1, PRKAR2B, PDPK1, HK3, PRKAA1, SHC1, PIK3R5, PIK3R3, INSR, PIK3R1, AKT2, IRS4, BRAF, PIK3CD, PRKAB1, RAF1, SOCS4, PPARGC1A, PPP1CB, CBLB, G6PC, TSC1, GSK3B, CRK, CALM1
mmu04810	Regulation of actin cytoskeleton	39	2.24	5.98E-04	GNA13, FGD1, ENAH, FGF7, FGF16, SSH2, WASF2, FGF11, ABI2, ARHGAP35, ARPC5, PXN, IQGAP1, SRC, MYL9, PAK6, PFN2, ARPC2, PIK3R5, MSN, PIK3R3, PIK3R1, APC, BRAF, ARHGEF7, LIMK1, ARHGEF6, PIK3CD, RAF1, ITGA4, ARHGEF12, VAV2, PPP1CB, ITGA9, ITGA5, CHRM1, CYFIP2, CRK, PIP4K2C
mmu05100	Bacterial invasion of epithelial cells	21	1.21	6.22E-04	CAV1, CLTA, SEPT3, WASF2, PIK3CD, ARPC5, CLTC, CD2AP, SRC, ELMO2, PXN, CBLB, ARPC2, ITGA5, SHC1, PIK3R5, PIK3R3, CRK, DNM1, SEPT8, PIK3R1
mmu04510	Focal adhesion	37	2.13	2.02E-03	CAV1, TLN2, COL3A1, ELK1, ARHGAP35, COL2A1, PXN, SRC, MYL9, CHAD, PAK6, PDPK1, PIK3R5, SHC1, THBS1, PIK3R3, PIK3R1, AKT2, PRKCA, COL4A4, TNXB, BRAF, PIK3CD, RAF1, IGF1, PRKCG, ITGA4, VAV2, CAPN2, PPP1CB, ITGA9, VEGFC, CCND3, ITGA5, GSK3B, RAP1B, CRK
mmu04152	AMPK signaling pathway	26	1.50	6.85E-03	PPP2R1B, IRS4, RAB2A, PFKFB4, PPP2R5A, PPP2R5C, PIK3CD, PRKAB1, IGF1, RPS6KB1, PPARGC1A, LEP, PDPK1, G6PC, TSC1, PPP2CA, RAB11B, CREB3L2, EEF2K, PIK3R5, PRKAA1, PIK3R3, INSR, PPP2R2D, PIK3R1, AKT2
mmu04713	Circadian entrainment	22	1.27	7.15E-03	PRKCA, ADCY2, GRIN1, CACNA1I, PRKCG, GNG11, GRIA4, KCNJ3, FOS, GRIA2, GRIA1, GNB1, RYR3, PER2, GUCY1A2, CACNA1G, CACNA1H, CAMK2B, PLCB1, RASD1, CACNA1D, CALM1

ICR-R vs CCR					
Term ID	**KEGG Pathway**	**Count**	**%**	**Bonferroni P Val.**	**Genes**
mmu04630	Jak-STAT signaling pathway	20	1.98	1.34E-02	PIK3CG, IL2RA, IL23R, IL5, OSMR, IL6ST, GRB2, IL7, STAT5A, LIFR, IL7R, IL22, IFNAR2, CCND1, IL23A, PRLR, CCND2, PRL, PIK3R1, GHR
mmu04071	Sphingolipid signaling pathway	18	1.78	1.86E-02	PPP2R1B, PIK3CG, ADORA3, GNAI3, SPHK2, ROCK1, GNAI2, SGPP1, CERS5, CERS4, GAB2, CERS1, BCL2, MAPK14, ABCC1, PLCB1, PIK3R1, OPRD1

3.1.4. Functional Enrichment Analysis of Common Predicted Targets

Various miRNAs and their predicted targets are common in each diet group. As indicated in Fig 3.3C,D, some predicted targets of miRNAs are common between comparisons even when there was no common miRNA present. In different CR applications, functional enrichment of predicted targets were performed in common predicted targets of CCR and ICR-R compared to AL to determine effects of CR application. Functional enrichment analysis of common predicted targets in CCR and ICR-R compared to AL revealed that these common predicted targets were important in transcription regulation and dendritic synapses. In addition to that, to assess difference of CCR application over AL, ICR-R and ICR-RF, common predicted targets of AL, ICR-R and ICR-RF compared to CCR were functionally enriched in DAVID 6.8. Functional enrichment revealed that predicted targets changed with CCR compared to AL, ICR-R and ICR-RF effected transcription regulation and nervous system development. These functionally enriched terms were given at table 3.5. In addition to DAVID 6.8 functional enrichment, predicted target genes were functionally enriched in ClueGO. ClueGO enrichment on common predicted targets of CCR and ICR-R group compared to AL were revealed that five different significantly enriched groups including regulation of dendritic spine development and Alzheimer disease related terms and pathways. ClueGO enrichment on common predicted targets of AL, ICR-R and ICR-RF groups compared to CCR revealed that there are 17 different significantly enriched groups including dopaminergic synapse, regulation of dendrite development and Alzheimer disease related terms and pathways.

Table 3.5. Functional enrichment analysis of common predicted targets among CR groups.

Common between CCR and ICR-R compared to AL						
Annotation Cluster 1	**Enrichment Score: 4.24**					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
UP_KEYWORDS	Transcription regulation	21	28.00	7.97E-07	1.18E-04	3.53
UP_KEYWORDS	Transcription	21	28.00	1.33E-06	1.97E-04	3.42
UP_KEYWORDS	Nucleus	34	45.33	1.40E-06	2.07E-04	2.27
GOTERM_BP_DIRECT	GO:0006351~transcription, DNA-templated	21	28.00	1.23E-05	6.88E-03	2.92
GOTERM_MF_DIRECT	GO:0000977~RNA polymerase II regulatory region sequence-specific DNA binding	8	10.67	2.64E-05	5.31E-03	9.02
UP_KEYWORDS	Activator	11	14.67	3.41E-05	5.03E-03	5.33
GOTERM_MF_DIRECT	GO:0003700~transcription factor activity, sequence-specific DNA binding	14	18.67	3.63E-05	7.30E-03	3.95
GOTERM_BP_DIRECT	GO:0045893~positive regulation of transcription, DNA-templated	11	14.67	5.48E-05	3.02E-02	5.00
GOTERM_BP_DIRECT	GO:0006355~regulation of transcription, DNA-templated	22	29.33	5.93E-05	3.26E-02	2.53
GOTERM_MF_DIRECT	GO:0000978~RNA polymerase II core promoter proximal region sequence-specific DNA binding	9	12.00	8.33E-05	1.67E-02	6.25
GOTERM_CC_DIRECT	GO:0005634~nucleus	38	50.67	8.52E-05	1.36E-02	1.75
GOTERM_MF_DIRECT	GO:0001077~transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	8	10.67	9.36E-05	1.87E-02	7.38
GOTERM_BP_DIRECT	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	13	17.33	3.02E-04	1.56E-01	3.42
UP_KEYWORDS	DNA-binding	15	20.00	6.07E-04	8.59E-02	2.83
GOTERM_MF_DIRECT	GO:0003677~DNA binding	16	21.33	5.04E-03	6.40E-01	2.16
GOTERM_MF_DIRECT	GO:0043565~sequence-specific DNA binding	8	10.67	1.24E-02	9.19E-01	3.15

Annotation Cluster 2	**Enrichment Score: 2.05**					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
GOTERM_CC_DIRECT	GO:0043198~dendritic shaft	4	5.33	1.34E-03	1.94E-01	18.16
GOTERM_CC_DIRECT	GO:0043197~dendritic spine	4	5.33	1.58E-02	9.24E-01	7.48
GOTERM_CC_DIRECT	GO:0045202~synapse	6	8.00	3.39E-02	9.96E-01	3.29

Common between AL, ICR-R, ICR-RF compared to CCR						
Annotation Cluster 1	**Enrichment Score: 4.61**					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
UP_KEYWORDS	Transcription	48	20.34	8.46E-09	2.12E-06	2.48
UP_KEYWORDS	Transcription regulation	47	19.92	8.93E-09	2.24E-06	2.51
GOTERM_BP_DIRECT	GO:0006351~transcription, DNA-templated	49	20.76	3.43E-07	4.31E-04	2.16
UP_KEYWORDS	Nucleus	78	33.05	2.52E-06	6.31E-04	1.65
GOTERM_BP_DIRECT	GO:0006355~regulation of transcription, DNA-templated	49	20.76	6.18E-05	7.49E-02	1.78
GOTERM_MF_DIRECT	GO:0003700~transcription factor activity, sequence-specific DNA binding	24	10.17	2.06E-04	8.06E-02	2.36
GOTERM_CC_DIRECT	GO:0005634~nucleus	95	40.25	2.37E-04	6.51E-02	1.37
UP_KEYWORDS	Activator	17	7.20	8.43E-04	1.91E-01	2.62
GOTERM_BP_DIRECT	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	25	10.59	8.99E-04	6.78E-01	2.08
UP_KEYWORDS	DNA-binding	31	13.14	1.23E-03	2.65E-01	1.86
GOTERM_MF_DIRECT	GO:0003677~DNA binding	33	13.98	1.15E-02	9.91E-01	1.55

Annotation Cluster 2	**Enrichment Score: 2.21**					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
GOTERM_MF_DIRECT	GO:0008046~axon guidance receptor activity	4	1.69	7.97E-05	3.21E-02	43.40
KEGG_PATHWAY	mmu04360:Axon guidance	8	3.39	6.52E-04	1.19E-01	5.36
UP_KEYWORDS	Developmental protein	21	8.90	2.98E-03	5.28E-01	2.07
UP_KEYWORDS	Neurogenesis	9	3.81	4.36E-03	6.66E-01	3.50
UP_KEYWORDS	Differentiation	15	6.36	7.65E-03	8.54E-01	2.23
GOTERM_BP_DIRECT	GO:0007275~multicellular organism development	22	9.32	1.23E-02	1.00E+00	1.77
GOTERM_BP_DIRECT	GO:0007399~nervous system development	11	4.66	1.60E-02	1.00E+00	2.42
GOTERM_BP_DIRECT	GO:0030154~cell differentiation	17	7.20	2.62E-02	1.00E+00	1.81
GOTERM_BP_DIRECT	GO:0007411~axon guidance	3	1.27	5.36E-01	1.00E+00	1.67

3.2. THE EFFECTS OF AGEING ON MICRORNA PROFILE IN BRAIN

In ageing, 29 miRNAs and one precursor were differentially expressed in all comparisons. miRNA expression of AL, CCR, ICR-R and ICR-RF diet were compared to baseline (week 10). Compared to baseline (week 10), three miRNAs and one precursor were downregulated in AL at week 81/82. In CCR at week 81/82, two miRNAs were upregulated and five miRNAs were downregulated. In ICR-R at week 81/82, 13 miRNAs and one precursor were downregulated. In ICR-RF at weeks 81/82, one miRNA was upregulated and 10 miRNAs were downregulated. These number of upregulated and downregulated miRNAs in each comparison was given at table 3.6. Names of DE miRNAs and their expression levels compared to different diets were given in Fig 3.4. Lowest and highest fold change are -3.48 and 2.58 and represented with blue and red respectively.

Table 3.6. Numbers of differentially expressed miRNAs with ageing.

Compared to	**AL**		**CCR**		**ICR-R**		**ICR-RF**		
Baseline	-	3 ↓	2 ↑	5 ↓	-	13 ↓	1 ↑	10 ↓	miRNAs
	-	1 ↓	-	-	-	1 ↓	-	-	Precursors

Common miRNAs in comparisons were determined in Fig 3.5A. Compared to baseline (week 10), there was no common miRNAs found between AL, CCR, ICR-R and ICR-RF, however, mmu-mir-1983 precursor miRNA was common between AL and ICR-R compared to baseline and its expression level was significantly lower in AL and ICR-R compared to baseline. Furthermore, mmu-miR-184-3p and mmu-miR-351-5p were common between CCR and ICR-R compared to baseline. mmu-miR-184-3p and mmu-miR-351-5p levels were significantly lower in CCR and ICR-R compared to baseline. Moreover, mmu-miR-7019-3p was common between CCR and ICR-RF and its expression level was significantly higher in CCR and ICR-R compared to baseline. Lastly, mmu-miR-1983 and mmu-miR-7071-5p were common in ICR-R and ICR-RF compared to baseline and their expression levels were significantly lower in ICR-R and ICR-RF compared to baseline.

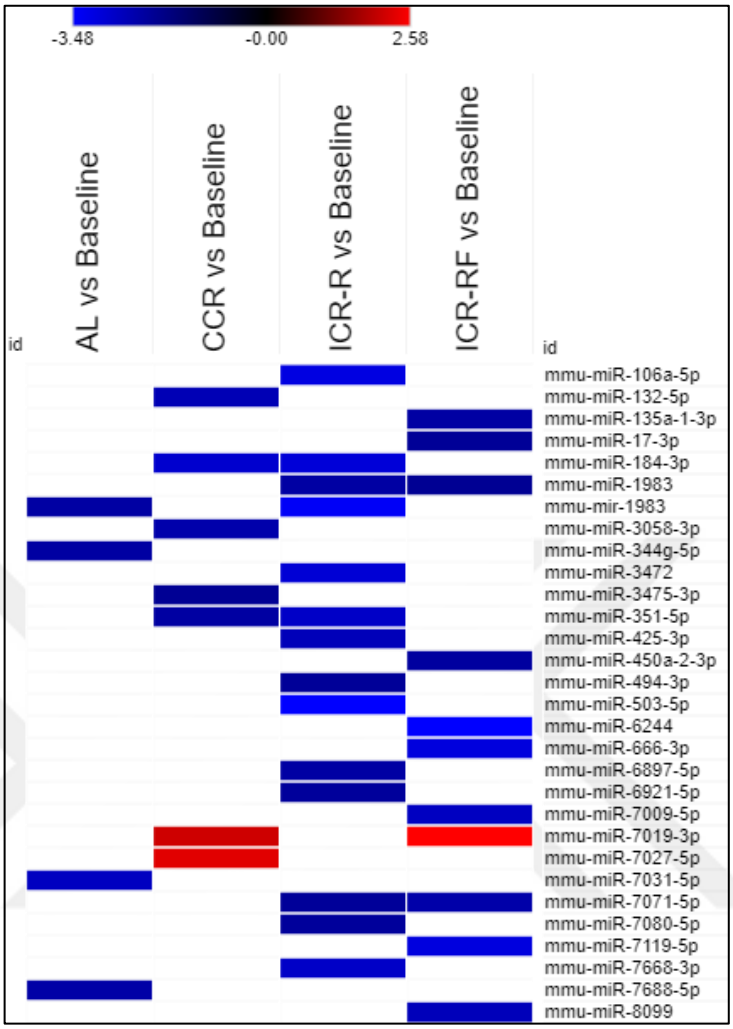


Figure 3.4. miRNAs differentially expressed with ageing. Lowest and highest fold change are -3.48 and 2.58 and represented with blue and red respectively.

3.2.1. Targets of miRNAs

In ageing, 29 miRNAs and one precursor were differentially expressed in all comparisons. miRNA expression of AL, CCR, ICR-R and ICR-RF diet were compared to baseline (week 10). Validated targets of these differentially expressed miRNA were determined using Tarbase, miRTarbase and miRecords. Compared to baseline (week 10), total of 2 validated targets were determined in AL. In CCR, total of 53 validated targets were compared to baseline. In ICR-R, total of 148 validated targets were determined compared to baseline. In ICR-RF, total of 31 predicted targets were compared to baseline.

Predicted targets of these differentially expressed miRNA were determined using Diana microT CDS 5.0, Targetscan mouse 7.2 and miRDB 6.0. Compared to baseline (week 10), total of 426 predicted targets were determined and all of them were upregulated by downregulation of miRNAs in AL. In CCR, total of 1495 predicted targets were determined and 864 of them were upregulated and 698 of them were downregulated compared to baseline. 67 of these predicted targets were common in upregulated and downregulated predicted targets in comparison with baseline. In ICR-R, total of 1521 predicted targets were determined and all of them were upregulated compared to baseline. In ICR-RF, total of 873 predicted targets were determined and 740 of them were upregulated and 149 of them were downregulated compared to baseline. 16 of these predicted targets were common in upregulated and downregulated predicted targets in comparison with baseline. Number of predicted targets of upregulated and downregulated miRNAs in each comparison was given at table 3.7.

Table 3.7. Numbers of predicted targets of miRNAs with ageing.

Compared to	**AL**			**CCR**			**ICR-R**			**ICR-RF**		
	Up	**Down**	**Common**	**Up**	**Down**	**Common**	**Up**	**Down**	**Common**	**Up**	**Down**	**Common**
Baseline	426	-	-	864	698	67	1521	-	-	740	149	16

Common predicted targets in comparisons were given in Fig 3.5B. Compared to baseline (week 10), 11 of predicted targets were common between AL, CCR, ICR-R and ICR-RF. 33 of predicted targets were common between AL, CCR and ICR-R. 11 of predicted targets were common between AL, CCR and ICR-RF. 12 of predicted targets were common between AL, ICR-R and ICR-RF. 85 of predicted targets were common between CCR, ICR-R and ICR-RF. 38 of predicted targets were common between AL and CCR. 37 of predicted targets were common between AL and ICR-R. 21 of predicted targets were common between AL and ICR-RF. 394 of predicted targets were common between CCR and ICR-R. 158 of predicted targets were common between CCR and ICR-RF. 132 of predicted targets were common between ICR-R and ICR-RF.

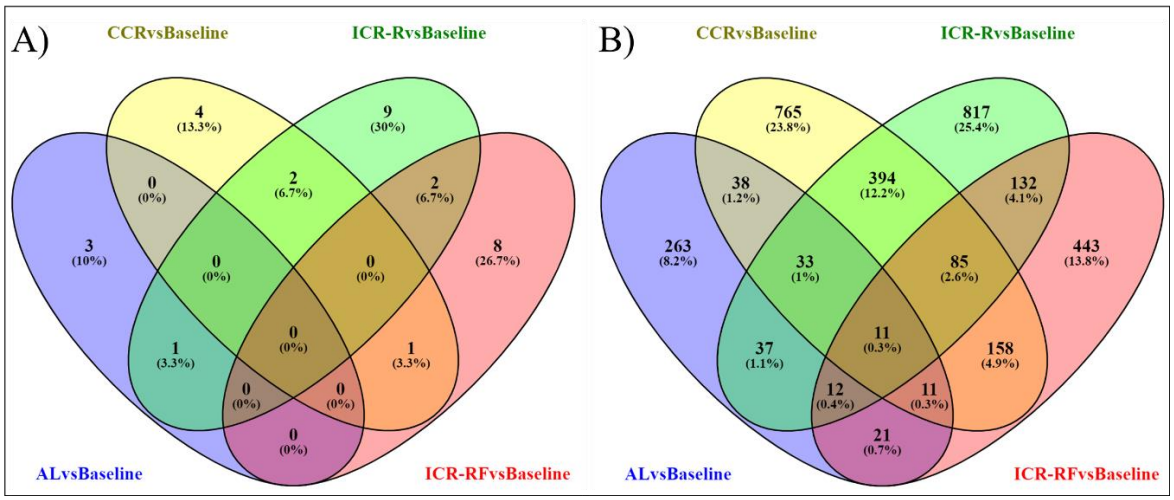


Figure 3.5. Venn schematics of miRNAs and their predicted targets compared to baseline in ageing. A and B are miRNAs and their targets compared to baseline respectively.

3.2.2. Gene Ontology of Predicted Targets

Application of different types of CR on mice changed several GO terms in different CR applied mice with ageing. miRNA expression of AL, CCR, ICR-R and ICR-RF diet were compared to baseline (week 10). In gene ontology analysis, gene ontology terms were grouped under biological processes, immune system process, cellular component and molecular function. Gene ontology analysis of predicted targets of DE miRNAs in comparisons revealed several terms in each comparison. Compared to baseline, 20 GO terms were significantly enriched in AL group, 296 GO terms were significantly enriched in CCR group, 552 GO terms were significantly enriched in ICR-R group and 218 GO terms were significantly enriched in ICR-RF group at week 81/82. Of these GO terms, cell morphogenesis, cell morphogenesis involved in differentiation, and plasma membrane bounded cell projection morphogenesis terms were common in AL, CCR, ICR-R and ICR-RF group compared to baseline. Moreover, nine GO terms were common in AL, CCR, and ICR-R group including neuron development and regulation of neuron differentiation. 157 GO terms were common in CCR and ICR groups including nervous system development and neurogenesis. There was no common term between AL and ICR compared to week 10. Top brain related GO terms were given with their GO ids, sources, levels, percentage of associated genes and number of predicted genes that found in GO term in table 3.8.

Table 3.8. Gene ontology of predicted targets with ageing.

AL vs Baseline						
GO ID	**GO Term**	**GO Source**	**Bonferroni P Val.**	**GO Levels**	**% Assoc. Genes**	**Nr. Genes**
GO:0048666	Neuron development	GO_BP	9.54E-06	[4, 5, 7, 8, 9]	4.18	57.00
GO:0050770	Regulation of axonogenesis	GO_BP	1.06E-04	[6, 7, 8, 9, 10, 11, 12, 13]	8.04	18.00
GO:0031175	Neuron projection development	GO_BP	1.07E-04	[5, 6, 8, 9, 10]	4.14	50.00
GO:0061564	Axon development	GO_BP	1.50E-04	[6, 7, 9, 10, 11]	5.35	30.00
GO:0007409	Axonogenesis	GO_BP	6.47E-04	[6, 7, 8, 9, 10, 11, 12]	5.30	27.00
GO:0048667	Cell morphogenesis involved in neuron differentiation	GO_BP	1.39E-03	[5, 6, 7, 8, 9, 10]	4.69	32.00
GO:0045664	Regulation of neuron differentiation	GO_BP	2.08E-03	[5, 6, 7, 8, 9]	4.23	37.00
GO:0010975	Regulation of neuron projection development	GO_BP	3.20E-03	[6, 7, 8, 9, 10, 11]	4.53	31.00
GO:0050772	Positive regulation of axonogenesis	GO_BP	2.01E-02	[5, 6, 7, 8, 9, 10, 11, 12, 13, 14]	8.70	10.00

CCR vs Baseline						
GO ID	**GO Term**	**GO Source**	**Bonferroni P Val.**	**GO Levels**	**% Assoc. Genes**	**Nr. Genes**
GO:0007399	Nervous system development	GO_BP	4.22E-09	[4, 5]	10.19	273.00
GO:0097458	Neuron part	GO_CC	1.33E-08	[2, 3]	10.51	234.00
GO:0036477	Somatodendritic compartment	GO_CC	3.47E-06	[3, 4]	11.39	138.00
GO:0043005	Neuron projection	GO_CC	5.39E-06	[3, 4, 5]	10.50	181.00
GO:0022008	Neurogenesis	GO_BP	5.46E-06	[4, 5, 6]	10.21	203.00
GO:0030424	Axon	GO_CC	6.36E-06	[4, 5, 6]	12.30	106.00
GO:0048699	Generation of neurons	GO_BP	2.96E-05	[5, 6, 7]	10.18	188.00
GO:0007417	Central nervous system development	GO_BP	5.30E-05	[4, 5, 6]	11.14	128.00
GO:0014069	Postsynaptic density	GO_CC	1.03E-04	[3, 4, 5]	14.00	64.00
GO:0050767	Regulation of neurogenesis	GO_BP	2.22E-04	[5, 6, 7, 8]	11.09	119.00

ICR-R vs Baseline						
GO ID	**GO Term**	**GO Source**	**Bonferroni P Val.**	**GO Levels**	**% Assoc. Genes**	**Nr. Genes**
GO:0022008	Neurogenesis	GO_BP	4.65E-20	[4, 5, 6]	12.82	255.00
GO:0007399	Nervous system development	GO_BP	5.47E-20	[4, 5]	11.84	317.00
GO:0048699	Generation of neurons	GO_BP	4.85E-19	[5, 6, 7]	12.94	239.00
GO:0030182	Neuron differentiation	GO_BP	2.48E-16	[4, 6, 7, 8]	12.86	214.00
GO:0048666	Neuron development	GO_BP	4.96E-15	[4, 5, 7, 8, 9]	13.35	182.00
GO:0050767	Regulation of neurogenesis	GO_BP	2.40E-14	[5, 6, 7, 8]	14.17	152.00
GO:0097458	Neuron part	GO_CC	6.26E-14	[2, 3]	11.54	257.00
GO:0031175	Neuron projection development	GO_BP	1.26E-13	[5, 6, 8, 9, 10]	13.50	163.00
GO:0048667	Cell morphogenesis involved in neuron differentiation	GO_BP	2.52E-13	[5, 6, 7, 8, 9, 10]	15.98	109.00
GO:0051960	Regulation of nervous system development	GO_BP	1.19E-12	[4, 5, 6]	13.31	159.00

ICR-RF vs Baseline						
GO ID	**GO Term**	**GO Source**	**Bonferroni P Val.**	**GO Levels**	**% Assoc. Genes**	**Nr. Genes**
GO:0097458	Neuron part	GO_CC	4.52E-13	[2, 3]	7.50	167.00
GO:0043005	Neuron projection	GO_CC	1.38E-08	[3, 4, 5]	7.37	127.00
GO:0030424	Axon	GO_CC	1.07E-05	[4, 5, 6]	8.24	71.00
GO:0007399	Nervous system development	GO_BP	2.98E-05	[4, 5]	6.09	163.00
GO:0022008	Neurogenesis	GO_BP	4.25E-04	[4, 5, 6]	6.28	125.00
GO:0048699	Generation of neurons	GO_BP	6.33E-04	[5, 6, 7]	6.33	117.00
GO:0098793	Presynapse	GO_CC	7.86E-04	[2, 3, 4]	8.19	54.00
GO:0001505	Regulation of neurotransmitter levels	GO_BP	4.18E-03	[3]	9.13	38.00
GO:0043009	Chordate embryonic development	GO_BP	4.52E-03	[5, 6]	7.33	63.00
GO:0098888	Extrinsic component of presynaptic membrane	GO_CC	4.78E-03	[3, 4, 5, 6, 7, 8, 9]	50.00	6.00

3.2.3. Pathway Analysis of Predicted Targets

In KEGG pathway analysis, KEGG analysis of predicted targets of DE miRNAs revealed several pathways in each comparison. Application of different types of CR on mice changed KEGG terms in different CR applied mice. Compared to baseline, there were no significantly enriched pathways could be found in AL, CCR, ICR-RF at week 81/82, however, 19 KEGG pathway terms were significantly enriched according to Bonferroni p value correction in CCR group including axon guidance and neurotrophin signaling pathway compared to baseline. Top 10 KEGG pathways were given with their term ids, number of predicted genes that are found in KEGG pathway, percentage of associated genes and genes in table 3.9.

Table 3.9. KEGG pathway analysis of predicted targets with ageing.

ICR-R vs Baseline					
Term ID	**KEGG Pathway**	**Count**	**%**	**Bonferroni P Val.**	**Genes**
mmu04010	MAPK signaling pathway	47	3.09	4.21E-07	FGF7, PDGFB, MRAS, FGF17, MKNK2, CACNB1, PPP3R1, GNG12, SRF, MAP3K5, KRAS, MAP3K2, MAP3K1, SOS1, JUND, FGF1, TRAF6, NFATC3, MAP2K7, AKT3, RASA2, TAOK1, TGFBR2, PTPRR, MAPK11, CACNG2, RPS6KA5, RPS6KA6, DUSP2, RPS6KA4, RPS6KA1, RASGRF2, MAPK12, PDGFRA, MAPK8IP2, RAP1A, MAPK9, RAP1B, MAP3K14, CACNA1C, MAP3K13, CRK, DUSP8, MAP3K12, DUSP7, CACNA1B, DUSP6
mmu04144	Endocytosis	41	2.70	6.98E-04	CLTA, CLTB, RAB5B, RAB5C, CYTH1, TSG101, ASAP1, VPS37B, ARF6, EEA1, ARF5, AMPH, DAB2, GBF1, WIPF2, VPS4B, RAB11A, NEDD4L, WIPF1, TRAF6, IQSEC1, IQSEC2, STAMBP, GIT1, PARD6B, KIF5A, KIF5C, TGFBR2, SMAD3, LDLRAP1, EPS15, RAB11FIP5, ACAP3, PSD, IST1, RAB22A, PDGFRA, SMURF1, RAB10, VPS26A, RAB11FIP1
mmu04360	Axon guidance	26	1.71	8.73E-04	PLXNA1, PPP3R1, LRRC4C, EPHB4, PAK7, KRAS, SEMA7A, SEMA3C, UNC5C, NFATC3, ROCK1, ROCK2, EFNB1, NTNG1, DPYSL5, NTN3, EPHA4, EPHA7, SEMA4G, SEMA4F, CFL2, SRGAP3, SEMA4C, SEMA4B, SEMA4D, SRGAP1
mmu04550	Signaling pathways regulating pluripotency of stem cells	27	1.78	9.64E-04	IL6ST, WNT3A, BMPR2, REST, MEIS1, LIF, WNT1, KRAS, PCGF6, AKT3, PIK3R1, APC, TBX3, OTX1, SMAD5, LIFR, SMAD3, NEUROG1, MAPK11, FZD2, STAT3, MAPK12, WNT9B, WNT11, ZFHX3, WNT7A, BMPR1A
mmu04722	Neurotrophin signaling pathway	24	1.58	3.46E-03	MAPK11, RPS6KA5, RPS6KA6, MAP3K5, PDPK1, KRAS, RPS6KA1, MAPK12, PRDM4, MAP3K1, SOS1, BCL2, GAB1, RAP1A, MAPK9, RAP1B, TRAF6, MAP2K7, CRK, FRS2, PIK3R1, AKT3, CALM1, SHC4
mmu04151	PI3K-Akt signaling pathway	48	3.16	4.14E-03	PHLPP1, CRTC2, FGF7, PHLPP2, MCL1, PDGFB, FGF17, LPAR4, ITGA10, GNG12, IL7R, PTEN, CCNE1, PDPK1, LAMB3, KRAS, ITGB8, BCL2, SOS1, PPP2CA, PDGFC, PRKAA2, FGF1, THBS2, THBS3, AKT3, PIK3R1, GHR, RBL2, CREB1, PKN2, CDK6, BCL2L11, IL6RA, OSM, ITGA9, CCND1, CDKN1A, CDKN1B, YWHAH, LAMA3, CHRM2, CCND2, ITGA8, VEGFA, PDGFRA, IFNA12, PPP2R2A
mmu04068	FoxO signaling pathway	25	1.64	5.53E-03	USP7, RBL2, TGFBR2, SMAD3, MAPK11, IL7R, PTEN, SIRT1, CCNG2, BCL2L11, STAT3, CCNB1, PDPK1, CCND1, CDKN1A, KRAS, CDKN1B, MAPK12, SLC2A4, CCND2, SOS1, MAPK9, PRKAA2, AKT3, PIK3R1
mmu05218	Melanoma	17	1.12	7.44E-03	E2F1, E2F2, E2F3, FGF7, PDGFB, FGF17, CDK6, RB1, PTEN, CDKN1A, CCND1, KRAS, PDGFRA, PDGFC, FGF1, AKT3, PIK3R1
mmu04710	Circadian rhythm	11	0.72	9.10E-03	NPAS2, BTRC, CREB1, RORB, RORA, PRKAA2, BHLHE40, ARNTL, BHLHE41, CLOCK, CUL1
mmu05215	Prostate cancer	19	1.25	9.66E-03	E2F1, E2F2, E2F3, PDGFB, CREB1, RB1, PTEN, CCNE1, CDKN1A, CCND1, PDPK1, KRAS, CDKN1B, SOS1, BCL2, PDGFRA, PDGFC, AKT3, PIK3R1

3.2.4. Functional Enrichment Analysis of Common Predicted Targets

In ageing, various miRNAs and their predicted targets are common in each group. Name of these common miRNAs in comparisons and number of their targets were given. As indicated in Fig 3.5B, some predicted targets of miRNAs are common between comparisons even when there was no common miRNA present. In ageing, functional enrichment of predicted targets were performed in common predicted targets of CCR and ICR-R compared to baseline to determine the effects of CR application. Functional enrichment analysis of common predicted targets in CCR and ICR-R compared to baseline revealed that these common predicted targets were important in transcription regulation, zinc finger ion interaction, cell cycle, cell division, differentiation and chromatin regulation. These functionally enriched terms were given at table 3.10. In addition to DAVID 6.8 functional enrichment, predicted target genes were functionally enriched in ClueGO. ClueGO enrichment on common predicted targets of CCR and ICR-R group compared to baseline revealed that there are 25 different significantly enriched groups including MAPK signaling, insulin and cytokine signaling related terms and pathways.

Table 3.10. Functional enrichment analysis of common predicted targets in ageing.

Common between CCR, ICR-R compared to Baseline						
Annotation Cluster 1	**Enrichment Score: 6.09**					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
UP_KEYWORDS	Transcription regulation	74	17.33	2.89E-10	7.33E-08	2.18
UP_KEYWORDS	Transcription	75	17.56	5.04E-10	1.28E-07	2.14
UP_KEYWORDS	Nucleus	135	31.62	1.09E-08	2.77E-06	1.58
GOTERM_BP_DIRECT	GO:0006351~transcription, DNA-templated	76	17.80	2.90E-08	5.05E-05	1.92
GOTERM_BP_DIRECT	GO:0006355~regulation of transcription, DNA-templated	80	18.74	3.26E-06	5.67E-03	1.67
GOTERM_CC_DIRECT	GO:0005634~nucleus	161	37.70	7.51E-05	2.70E-02	1.30
GOTERM_MF_DIRECT	GO:0003677~DNA binding	64	14.99	9.02E-05	4.66E-02	1.63
UP_KEYWORDS	DNA-binding	51	11.94	2.97E-04	7.26E-02	1.69
GOTERM_MF_DIRECT	GO:0003700~transcription factor activity, sequence-specific DNA binding	35	8.20	5.66E-04	2.59E-01	1.87

Annotation Cluster 2	**Enrichment Score: 5.69**					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
UP_KEYWORDS	Zinc-finger	62	14.52	4.61E-08	1.17E-05	2.10
UP_KEYWORDS	Zinc	74	17.33	1.83E-07	4.65E-05	1.87
UP_KEYWORDS	Metal-binding	100	23.42	3.51E-06	8.91E-04	1.56
GOTERM_MF_DIRECT	GO:0008270~zinc ion binding	46	10.77	9.22E-06	4.86E-03	2.02
GOTERM_MF_DIRECT	GO:0046872~metal ion binding	101	23.65	1.25E-04	6.42E-02	1.42

Annotation Cluster 3	**Enrichment Score: 2.24**					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
UP_KEYWORDS	Cell cycle	28	6.56	6.11E-05	1.54E-02	2.38
GOTERM_BP_DIRECT	GO:0007049~cell cycle	25	5.85	2.61E-03	9.89E-01	1.94
UP_KEYWORDS	Cell division	16	3.75	4.84E-03	7.09E-01	2.28
GOTERM_BP_DIRECT	GO:0051301~cell division	16	3.75	1.25E-02	1.00E+00	2.04
UP_KEYWORDS	Mitosis	10	2.34	5.62E-02	1.00E+00	2.05
GOTERM_BP_DIRECT	GO:0007067~mitotic nuclear division	11	2.58	6.65E-02	1.00E+00	1.89

Annotation Cluster 4	Enrichment Score: 2.09					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
UP_KEYWORDS	Developmental protein	34	7.96	8.63E-04	1.97E-01	1.85
GOTERM_BP_DIRECT	GO:0007275~multicellular organism development	36	8.43	3.26E-03	9.97E-01	1.67
UP_KEYWORDS	Differentiation	22	5.15	1.08E-02	9.37E-01	1.81
GOTERM_BP_DIRECT	GO:0030154~cell differentiation	22	5.15	1.43E-01	1.00E+00	1.35

Annotation Cluster 5	Enrichment Score: 2.05					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
INTERPRO	IPR011011:Zinc finger, FYVE/PHD-type	11	2.58	4.10E-04	2.69E-01	4.04
INTERPRO	IPR019786:Zinc finger, PHD-type, conserved site	7	1.64	1.72E-03	7.33E-01	5.47
UP_SEQ_FEATURE	zinc finger region:PHD-type	6	1.41	2.21E-03	9.41E-01	6.47
INTERPRO	IPR001965:Zinc finger, PHD-type	8	1.87	2.30E-03	8.29E-01	4.39
INTERPRO	IPR019787:Zinc finger, PHD-finger	7	1.64	4.38E-03	9.65E-01	4.54
SMART	SM00249:PHD	8	1.87	6.54E-03	7.33E-01	3.61
UP_KEYWORDS	Chromatin regulator	10	2.34	6.90E-02	1.00E+00	1.97
GOTERM_BP_DIRECT	GO:0016569~covalent chromatin modification	9	2.11	1.95E-01	1.00E+00	1.61
GOTERM_MF_DIRECT	GO:0035064~methylated histone binding	3	0.70	2.62E-01	1.00E+00	3.01

3.3. THE EFFECTS OF MAMMARY TUMOR DEVELOPMENT ON MIRNA PROFILE IN MICE BRAIN

To measure the effects of mammary tumor on the miRNA profile of brain tissues, a total of 1237 pre-miRNAs (precursors) and 1926 mature miRNAs were analyzed on mice brains. Of these precursors and mature miRNAs, 102 of them were differentially expressed. In these 102 precursors and mature miRNAs, 20 mature miRNAs were upregulated and five precursors and 77 mature miRNAs were downregulated in MT developed mice brains compared to healthy brains. Expression (log2) of these differentially expressed miRNAs MT developed mice brain compared to healthy mice brain were given in hierarchical cluster at Fig 3.6. High, middle and low expressions marked with red, black and green respectively.

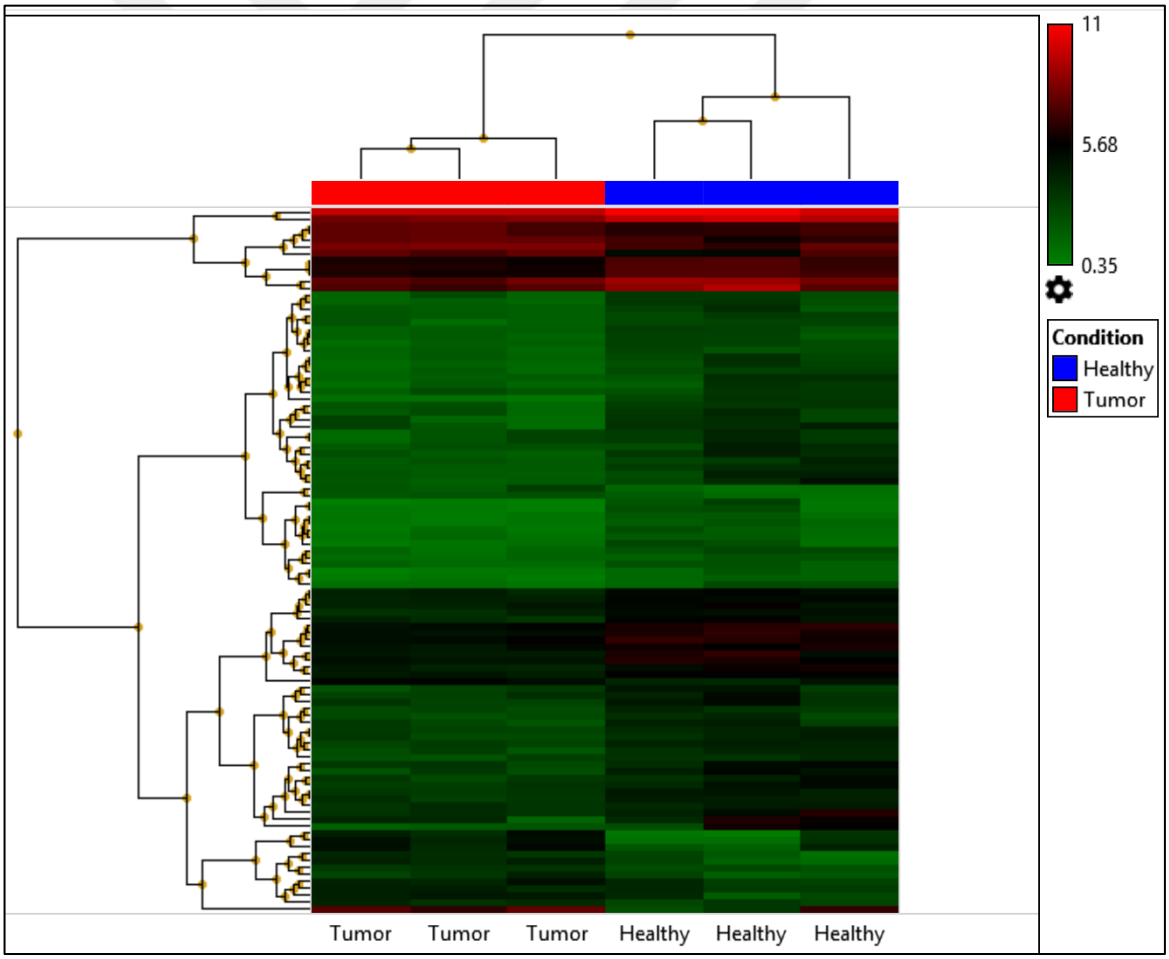


Figure 3.6. Hierarchical cluster of DE miRNAs in mice brain with and without MT. Lowest and highest fold changes represented with green and red respectively.

3.3.1. Targets of miRNAs

In comparison of mammary tumor developed mice and healthy mice brains, 97 mature miRNAs and five precursors were differentially expressed in all comparisons. Validated targets of these differentially expressed miRNA were determined using Tarbase, miRTarbase and miRecords. Total of 1387 validated targets were determined in MT developed mice brain samples compared to healthy mice brain samples. Predicted targets of these miRNAs were determined through combining Diana microT CDS 5.0, Targetscan mouse 7.2 and miRDB 6.0. Concurrent ones in the top 20 percent of from each databases were considered as potential targets of miRNAs. Total of 1298 predicted targets were determined and 945 of them were upregulated by downregulation of miRNAs and 416 of them were downregulated by upregulation of miRNAs. 63 of these predicted targets were common in upregulated and downregulated predicted targets. Between these 1387 validated and 1298 predicted targets, 165 of these targets were common in both validated and predicted targets.

3.3.2. Gene Ontology of Predicted Targets

Several GO terms were changed in mammary tumor developed mice brain compared to healthy mice brain. Compared to healthy mice brain, 556 GO terms were significantly enriched in mammary tumor developed mice brain. Of these GO terms, positive regulation of biological process and metabolic process, system and multicellular organismal development like GO terms were significantly enriched in mammary tumor developed mice brain compared to health brain. Top brain related GO terms like nervous system development were given in table 3.11.

Table 3.11. Gene ontology of predicted targets in MT developed mice brain samples compared to healthy brain samples.

Mammary Tumor Developed Mice Brain vs Healthy Mice Brain						
GO ID	**GO Term**	**GO Source**	**Bonferroni P Val.**	**GO Levels**	**% Assoc. Genes**	**Nr. Genes**
GO:0097458	Neuron part	GO_CC	8.16E-26	[2, 3]	11.81	263.00
GO:0007399	Nervous system development	GO_BP	2.10E-24	[4, 5]	11.02	295.00
GO:0048699	Generation of neurons	GO_BP	1.02E-21	[5, 6, 7]	11.97	221.00
GO:0022008	Neurogenesis	GO_BP	1.33E-20	[4, 5, 6]	11.56	230.00
GO:0030182	Neuron differentiation	GO_BP	2.22E-19	[4, 6, 7, 8]	12.02	200.00
GO:0051960	Regulation of nervous system development	GO_BP	7.72E-18	[4, 5, 6]	13.05	156.00
GO:0043005	Neuron projection	GO_CC	1.20E-17	[3, 4, 5]	11.60	200.00
GO:0050767	Regulation of neurogenesis	GO_BP	7.51E-17	[5, 6, 7, 8]	13.33	143.00
GO:0045664	Regulation of neuron differentiation	GO_BP	2.86E-15	[5, 6, 7, 8, 9]	13.83	121.00
GO:0048666	Neuron development	GO_BP	7.91E-12	[4, 5, 7, 8, 9]	11.30	154.00

3.3.3. Pathway Analysis of Predicted Targets

Mammary tumor developed mice brain changed different KEGG pathways compared to healthy mice brain. 14 pathways were significantly enriched according to Bonferroni p value correction including focal adhesion, axon guidance and MAPK signaling pathway. Top KEGG pathways were given with their term ids, number of predicted genes that found in KEGG pathway, percentage of associated genes and genes in table 3.12.

Table 3.12. KEGG pathway analysis of predicted targets in MT developed mice brain samples compared to healthy brain samples.

Mammary Tumor Developed Mice Brain vs Healthy Mice Brain					
Term ID	**KEGG Pathway**	**Count**	**%**	**Bonferroni P Val.**	**Genes**
mmu04510	Focal adhesion	38	2.93	7.95E-07	GRB2, PGF, PDGFA, TLN2, PPP1R12C, PXN, VCL, CHAD, MYL9, RAC2, PAK3, SOS1, ITGAV, RAC1, ILK, SOS2, PARVG, BRAF, ROCK1, PIK3CB, MYLK4, PIK3CD, RAF1, IGF1, PRKCG, COL5A3, COL5A1, COL4A5, ITGA9, LAMA3, ITGA5, GSK3B, JUN, VEGFA, RAP1A, RAP1B, COL1A1, PARVB
mmu05211	Renal cell carcinoma	20	1.54	3.82E-06	BRAF, EPAS1, PIK3CB, GRB2, PIK3CD, RAF1, EGLN1, FLCN, TGFB2, NRAS, HIF1A, PAK3, JUN, SOS1, VEGFA, SOS2, RAC1, SLC2A1, RAP1A, RAP1B
mmu04010	MAPK signaling pathway	40	3.08	1.67E-05	FGFR2, FGF7, FGF9, GRB2, PDGFA, FGF14, MAP4K1, CACNB4, TGFB2, BDNF, RAC2, MAP3K2, SOS1, MAP3K1, RAC1, SOS2, PRKACB, RAPGEF2, NFATC3, RASA1, BRAF, TAOK1, TGFBR1, MAP2K4, TAOK3, RAF1, PRKCG, CACNA2D2, RPS6KA5, NRAS, DUSP3, JUN, MAPK8IP2, RAP1A, RAP1B, STMN1, MAPK8IP1, GADD45A, MAP3K12, MAP3K11
mmu05205	Proteoglycans in cancer	35	2.70	1.90E-05	WNT5A, TFAP4, GRB2, PPP1R12C, RPS6KB1, PDCD4, TIMP3, HOXD10, PXN, TGFB2, CTTN, SOS1, ITGAV, RAC1, SOS2, NUDT16L1, MSN, PRKACB, FRS2, ROCK1, BRAF, PIK3CB, PIK3CD, RAF1, IGF1, IGF2, PRKCG, FZD4, STAT3, NRAS, HIF1A, ITGA5, VEGFA, COL1A1, SLC9A1
mmu04360	Axon guidance	26	2.00	6.20E-05	PLXNA4, NRP1, RAC2, ROBO1, SEMA3G, CXCR4, PAK3, SEMA3E, RAC1, NFATC3, RASA1, ROCK1, LIMK2, EFNB3, DPYSL5, NTNG1, EPHA2, NRAS, EPHA4, SEMA6A, EPHA7, SEMA4G, EPHA8, GSK3B, CFL2, EFNA5
mmu04810	Regulation of actin cytoskeleton	35	2.70	6.28E-05	FGFR2, ENAH, FGF7, FGF9, PDGFA, FGF14, PPP1R12C, IQGAP3, ARPC5, PXN, MYL9, VCL, PFN2, RAC2, ARPC2, PAK3, SOS1, ITGAV, RAC1, SOS2, MSN, LIMK2, ROCK1, BRAF, PIK3CB, PIK3CD, MYLK4, RAF1, ITGA9, NRAS, ITGA5, CHRM1, CFL2, CYFIP2, SLC9A1
mmu04014	Ras signaling pathway	36	2.77	1.23E-04	FGFR2, FGF7, FGF9, RAB5C, PGF, PDGFA, FGF14, GRB2, ARF6, BCL2L1, RASAL2, RAC2, GRIN2B, PAK3, SOS1, RAC1, SOS2, PRKACB, GNG4, GNG5, RASA1, RALBP1, PIK3CB, GRIN1, PIK3CD, IGF1, RAF1, PRKCG, EPHA2, NRAS, VEGFA, RAP1A, EFNA5, RAP1B, NGFR, ABL2
mmu05200	Pathways in cancer	51	3.93	1.83E-04	ADCY3, FGF7, FGF9, PDGFA, FGF14, PGF, MITF, ADCY6, TGFB2, CCNE2, EDNRB, CXCR4, SLC2A1, PRKACB, GNG4, GNG5, BRAF, ROCK1, PIK3CB, RALBP1, PIK3CD, PRKCG, RB1, HIF1A, JUN, VEGFA, FGFR2, WNT5A, GRB2, EGLN1, BCL2L1, ZBTB16, TCF7L2, RAC2, ITGAV, SOS1, RAC1, SOS2, RUNX1, TRAF3, BMP2, EPAS1, TGFBR1, IGF1, RAF1, FZD4, STAT3, COL4A5, NRAS, LAMA3, GSK3B
mmu04151	PI3K-Akt signaling pathway	46	3.54	4.80E-04	FGFR2, YWHAZ, FGF7, FGF9, GRB2, PGF, PDGFA, FGF14, PPP2R5C, RPS6KB1, BCL2L1, CHAD, CCNE2, ITGAV, SOS1, PPP2CA, RAC1, PPP2CB, SOS2, CREB3L1, GNG4, GNG5, PIK3CB, PIK3CD, YWHAB, RAF1, IGF1, COL5A3, YWHAE, BCL2L11, EPHA2, COL5A1, COL4A5, ITGA9, NRAS, YWHAG, LAMA3, ITGA5, CHRM1, GSK3B, VEGFA, RHEB, EFNA5, COL1A1, NGFR, PPP2R2A
mmu04015	Rap1 signaling pathway	32	2.47	1.76E-03	FGFR2, ADCY3, FGF7, FGF9, PGF, PDGFA, FGF14, TLN2, ADCY6, CTNND1, PFN2, GRIN2B, RAC2, CNR1, RAC1, RAPGEF2, BRAF, PIK3CB, GRIN1, PIK3CD, SIPA1L2, IGF1, RAF1, PRKCG, EPHA2, NRAS, VEGFA, RAP1A, EFNA5, RAP1B, NGFR, LCP2
mmu04024	cAMP signaling pathway	30	2.31	2.55E-03	ADCY3, ATP1B2, ADCYAP1R1, ATP1B4, ADCY6, VIPR2, MYL9, ATP2B1, BDNF, GRIN2B, RAC2, RAC1, PDE4B, CREB3L1, PRKACB, HTR1F, ROCK1, BRAF, PIK3CB, GRIN1, PIK3CD, ATP1A3, RAF1, JUN, CHRM1, RAP1A, RYR2, RAP1B, FSHB, SLC9A1

mmu04919	Thyroid hormone signaling pathway	20	1.54	1.59E-02	THRB, ATP1B2, PIK3CB, PIK3CD, ATP1B4, ATP1A3, RAF1, PRKCG, MED12L, NRAS, SLC16A2, NCOA1, HIF1A, ITGAV, GSK3B, SLC2A1, RHEB, PRKACB, SLC9A1, MED1
mmu05221	Acute myeloid leukemia	13	1.00	3.13E-02	NRAS, BRAF, GRB2, PIK3CB, SOS1, SOS2, PIK3CD, RAF1, RPS6KB1, ZBTB16, RUNX1, TCF7L2, STAT3
mmu04722	Neurotrophin signaling pathway	20	1.54	4.01E-02	BRAF, PIK3CB, GRB2, PIK3CD, RAF1, YWHAE, RPS6KA5, NRAS, BDNF, JUN, MAP3K1, GSK3B, SOS1, SOS2, RAC1, PSEN2, RAP1A, RAP1B, NGFR, FRS2

3.3.4. Functional Enrichment Analysis of Predicted Targets

Mammary tumor developed mice brains have different miRNA profile than the healthy mice brains. Several GO terms and KEGG pathways including proteoglycans in cancer were significantly enriched in MT developed mice brains compared to healthy ones. After determining the GO terms and KEGG pathways, functional enrichment analysis were performed to obtain combinatorial results in seven default parameters of DAVID 6.8. In MT developed mice brains compared to healthy brains, transcription regulation, cell junction, multicellular organism development, zinc-ion binding, protein kinases, and synapses terms were enriched. Top three enrichment results were given at table 3.13. In addition to KEGG pathway analysis, predicted target genes were functionally enriched in ClueGO. ClueGO enrichment on predicted targets of MT regulated miRNAs revealed that there are 105 different significantly enriched groups including neurogenesis, metabolic process, brain development related terms and pathways.

Table 3.13. Functional enrichment analysis of predicted targets in MT developed mice brain samples compared to healthy brain samples.

Mammary Tumor Developed Mice Brain vs Healthy Mice Brain						
Annotation Cluster 1	**Enrichment Score: 12.05**					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
UP_KEYWORDS	Nucleus	397	30.59	4.48E-21	1.68E-18	1.53
UP_KEYWORDS	Transcription regulation	187	14.41	6.66E-16	2.50E-13	1.82
UP_KEYWORDS	DNA-binding	169	13.02	6.61E-15	2.50E-12	1.84
UP_KEYWORDS	Transcription	188	14.48	7.91E-15	2.96E-12	1.77
GOTERM_MF_DIRECT	GO:0003677~DNA binding	203	15.64	2.38E-14	2.64E-11	1.69
GOTERM_BP_DIRECT	GO:0006351~transcription, DNA-templated	192	14.79	1.67E-11	7.01E-08	1.60
GOTERM_BP_DIRECT	GO:0006355~regulation of transcription, DNA-templated	219	16.87	1.11E-10	4.65E-07	1.51
GOTERM_MF_DIRECT	GO:0043565~sequence-specific DNA binding	74	5.70	1.24E-06	1.38E-03	1.80
GOTERM_MF_DIRECT	GO:0003700~transcription factor activity, sequence-specific DNA binding	89	6.86	3.89E-05	4.23E-02	1.55

Annotation Cluster 2	**Enrichment Score: 10.84**					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
UP_KEYWORDS	Cell junction	87	6.70	7.00E-13	2.62E-10	2.30
GOTERM_CC_DIRECT	GO:0045202~synapse	74	5.70	1.15E-11	7.46E-09	2.36
UP_KEYWORDS	Synapse	56	4.31	1.86E-11	6.98E-09	2.74
GOTERM_CC_DIRECT	GO:0030054~cell junction	90	6.93	2.99E-10	1.94E-07	2.02

Annotation Cluster 3	**Enrichment Score: 6.91**					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
UP_KEYWORDS	Developmental protein	104	8.01	1.11E-09	4.15E-07	1.86
GOTERM_BP_DIRECT	GO:0007275~multicellular organism development	111	8.55	3.57E-08	1.50E-04	1.70
UP_KEYWORDS	Differentiation	63	4.85	4.63E-05	1.72E-02	1.70

3.4. FUNCTIONAL ENRICHMENT ANALYSIS OF SELECTED MIRNAS FOR FURTHER ANALYSIS

In ageing and different CR applications, combinedly 53 miRNAs and two precursors were differentially expressed. Of these 53 mature miRNA, 28 miRNAs and two precursors were differentially expressed in different CR applications and 29 miRNAs and one precursor were differentially expressed in ageing. In the current study, our lab show that, MT incidence and MT weight of mice were lower, survival percent was higher in the CCR group compared to AL and ICR (manuscript in progress, $p>0.05$). For that reason we focused on CCR regulated miRNAs. mmu-miR-713 expression was significantly higher in CCR group compared to AL, ICR-R and ICR-RF at week 81/82 and had 233 predicted targets. This miRNA expression was not statistically different compared to baseline and level of this miRNA was higher in the CCR and baseline group compared to others, also level of mmu-miR-713 was lower in the tumor developed mice brain compared to healthy mice brain. In addition to that, mmu-miR-7221-5p level in CCR applied mice brain samples was significantly higher than AL group at week 81/82 and had 93 predicted targets. This miRNA expression was not statistically different compared to baseline and level of this miRNA was higher in the CCR, ICR-RF and baseline group compared to others, also level of mmu-miR-713 was lower in the tumor developed mice brain compared to healthy mice brain, however, level of this miRNA in ICR-RF group was not statistically significant compared AL group. Moreover, mmu-miR-132-5p, mmu-miR-3058-3p, mmu-miR-3475-3p and mmu-miR-7027-5p were only differentially expressed in CCR compared to baseline in ageing. mmu-miR-132-5p, mmu-miR-3058-3p, mmu-miR-3475-3p and mmu-miR-7027-5p have two, 250, 286 and 567 had predicted targets respectively.

mmu-miR-713, mmu-miR-7221-5p, mmu-miR-3058-3p, mmu-miR-3475-3p, and mmu-miR-7027-5p were selected for further analysis because of their expression levels in CCR and their number of predicted targets. In functional enrichment analysis, neuron, transcription, Golgi and other factors were enriched at these CCR effected miRNAs. In mmu-7221-5p, transcription regulation, synapse, circadian rhythms were functionally enriched. In mmu-miR-3058-3p predicted targets, dendritic synapse formation, zinc finger ion interaction, cell junction and synapse, protein kinase activity, transcription and chromatin regulator were functionally enriched. Synapse, cell junction, Golgi apparatus,

transmembrane region, glycosylation terms were functionally enriched in predicted targets of mmu-miR-3475-3p. In mmu-miR-7027-5p, transcription regulation, zinc finger ion interaction, sequence-specific DNA binding, proteolysis, hippocampus development, RNA mediated gene silencing terms were enriched.

Transcription regulation, Golgi apparatus, neurogenesis, endoplasmic reticulum, fibronectin functions were functionally enriched in predicted targets of mmu-miR-713. Top three enrichment results for mmu-miR-713 were given at table 3.14. In addition to DAVID 6.8 functional enrichment on mmu-miR-713, predicted target genes of mmu-miR-713 were functionally enriched in ClueGO. ClueGO enrichment on mmu-miR-713 revealed that regulation of axonogenesis, dopaminergic synapse, prolactin signaling, presynaptic membrane and several other pathways and terms were significantly enriched.

Table 3.14. Functional enrichment analysis of predicted targets of selected miRNAs.

mmu-miR-713						
Annotation Cluster 1	**Enrichment Score: 4.43**					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
UP_KEYWORDS	Transcription	47	20.17	1.61E-08	4.02E-06	2.46
UP_KEYWORDS	Transcription regulation	46	19.74	1.72E-08	4.31E-06	2.49
GOTERM_BP_DIRECT	GO:0006351~transcription, DNA-templated	48	20.60	5.63E-07	7.04E-04	2.14
UP_KEYWORDS	Nucleus	77	33.05	2.94E-06	7.35E-04	1.65
GOTERM_CC_DIRECT	GO:0005654~nucleoplasm	42	18.03	5.72E-05	1.59E-02	1.91
GOTERM_BP_DIRECT	GO:0006355~regulation of transcription, DNA-templated	48	20.60	8.81E-05	1.04E-01	1.77
GOTERM_MF_DIRECT	GO:0003700~transcription factor activity, sequence-specific DNA binding	24	10.30	1.64E-04	6.46E-02	2.39
GOTERM_CC_DIRECT	GO:0005634~nucleus	93	39.91	3.81E-04	1.01E-01	1.36
GOTERM_BP_DIRECT	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	25	10.73	7.38E-04	6.03E-01	2.11
UP_KEYWORDS	Activator	16	6.87	2.02E-03	3.97E-01	2.50
UP_KEYWORDS	DNA-binding	30	12.88	2.02E-03	3.97E-01	1.82
GOTERM_MF_DIRECT	GO:0003677~DNA binding	32	13.73	1.59E-02	9.99E-01	1.53

Annotation Cluster 2	**Enrichment Score: 1.97**					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
UP_KEYWORDS	Golgi apparatus	17	7.30	4.84E-03	7.02E-01	2.20
GOTERM_CC_DIRECT	GO:0005794~Golgi apparatus	23	9.87	1.58E-02	9.88E-01	1.70
GOTERM_CC_DIRECT	GO:0000139~Golgi membrane	11	4.72	1.65E-02	9.90E-01	2.41

Annotation Cluster 3	Enrichment Score: 1.85					
Category	**Term**	**Count**	**%**	**P Value**	**Bonferroni**	**Fold Enrichment**
GOTERM_MF_DIRECT	GO:0008046~axon guidance receptor activity	4	1.72	7.62E-05	3.05E-02	44.06
KEGG_PATHWAY	mmu04360:Axon guidance	7	3.00	3.30E-03	4.73E-01	4.74
UP_KEYWORDS	Developmental protein	20	8.58	5.70E-03	7.60E-01	1.99
UP_KEYWORDS	Neurogenesis	8	3.43	1.39E-02	9.70E-01	3.15
GOTERM_BP_DIRECT	GO:0007275~multicellular organism development	21	9.01	2.02E-02	1.00E+00	1.72
UP_KEYWORDS	Differentiation	13	5.58	3.42E-02	1.00E+00	1.96
GOTERM_BP_DIRECT	GO:0007399~nervous system development	10	4.29	3.59E-02	1.00E+00	2.23
GOTERM_BP_DIRECT	GO:0030154~cell differentiation	15	6.44	8.03E-02	1.00E+00	1.62
GOTERM_BP_DIRECT	GO:0007411~axon guidance	3	1.29	5.29E-01	1.00E+00	1.69

3.5. VALIDATION OF MMU-MIR-713

mmu-miR-713 was selected for miRNA validation because their functional enrichment results showed that the target genes of these miRNAs were related with genesis of neurons. Levels of mmu-miR-713 was higher in CCR compared to baseline at week 10, and AL, ICR-R, ICR-RF at week 81/82. RT-PCR results were validated that mmu-miR-713 levels were higher in the CCR group. Validation results of mmu-miR-713 were given at Fig 3.7.

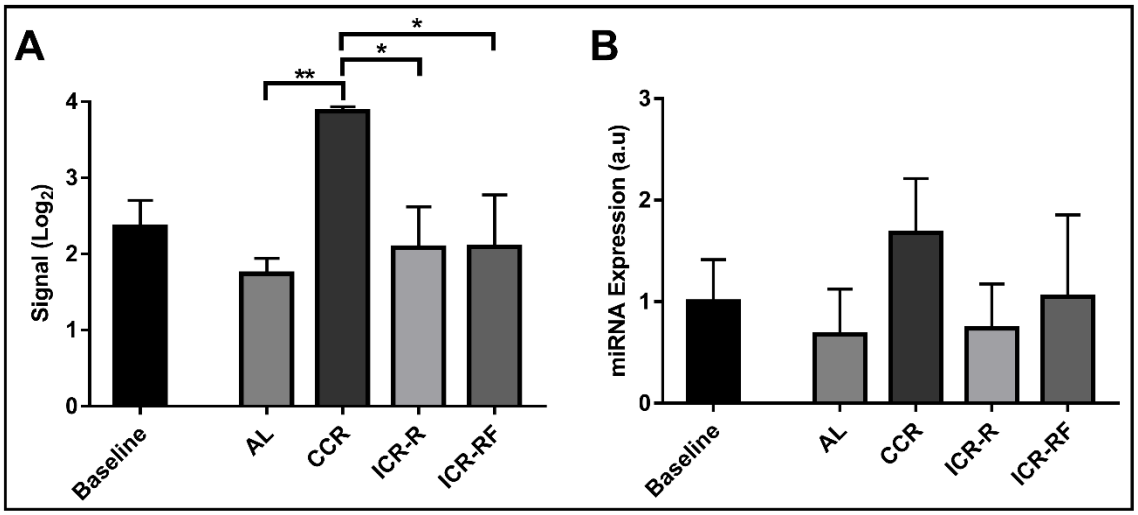


Figure 3.7. Validation of miRNA array result for mmu-miR-713 level using RT-PCR. Panel A shows mmu-miR-713 level using miRNA array (A, n=3) and Panel B shows RT-PCR results (B, n=3-5) at 10 and 81/82 weeks of mouse age.

4. DISCUSSION

Ageing is a complex and natural event which characterized by deterioration of the physiological functions of the cell and organisms. Ageing is a result of progressive damage accumulation and programmed internal factors that could result to organismal and cellular death by deteriorating homeostasis and organismal integrity [2-5, 43]. With ageing, genomic instability, epigenetic alterations, telomere attrition, loss of protein folding and proteolysis (proteostasis), deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication like inflammation occurs in cells and body [2]. Ageing induces changes, age-related disease vulnerability increases [2, 46, 47]. However, effects of ageing could be ameliorated by changing lifestyle and using medical intervention methods which could increase lifespan, longevity, and reverse effects of ageing [5, 50, 53, 55]. One of the most effective intervention methods that could ameliorate and reverse effects of ageing known as calorie restriction. CR is noninvasive method and is applied by limiting calorie intake by 20 to 40 percent without malnutrition to modify metabolic processes, satiety, and energy balance. This negative energy balance increase longevity, lifespan, reverses effect of ageing and is important in the prevention of age-related diseases including neurodegeneration, cancer and also promotes cognitive function by inhibiting cell proliferation, glycolysis, inflammation, increasing autophagy, antioxidant expression and improving mitochondrial physiology in ageing [6, 8, 139, 140].

In brain, ageing is the most common and main risk factor for neurodegeneration and neurodegenerative diseases [1, 89]. In addition to common hallmarks of ageing, brain ageing occurs with accumulation of oxidative damage, impaired molecular waste disposal, aberrant neural network activity and dysregulated neuronal calcium homeostasis [45]. Neurodegeneration is progressive loss of function and structure of neurons eventually could lead to death of neurons [83]. Neurodegeneration in the nervous system could result to neurodegenerative diseases. Neurodegenerative diseases are degenerative neurological disorders that could affect specific structures and functions of either central or peripheral nervous system such as AD, PD, HD, and ALS [83, 84]. Moreover, ageing in the brain decreases adult neurogenesis which is generation of new neurons in SVZ of lateral ventricles and SGZ of hippocampus and occur throughout life [123]. Moderate decrease of neurogenesis by ageing is modulated by decreasing proliferation of neural stem cells and

radial glial like cells via several factors and mechanisms such as bloodborne growth factors, hormones, inflammatory factors, local signals and neurotransmitters [89, 108, 123]. CR could alleviate and ameliorate effects of ageing by improving ageing hallmarks, neuroprotection, inducing neurogenesis, improve memory functions, [8, 9, 171]. CR protects brain against neurodegeneration by reducing the risk of AD, PD , HD and MS via regulating calcium homeostasis, autophagy, inflammation, plasticity and neurogenesis [7, 91, 173]. Calorie restriction induces neurogenesis by promoting neural stem cell survival, neural differentiation and neural protection. CR induced neurogenesis helps neuroprotection and improves memory functions [122, 179, 180]. In the effects of ageing and CR on body and brain could be regulated by epigenetic factors such as miRNAs [5, 16].

Cancer is uncontrollable, malignant growth of cells that could occur and/or metastases in any body organ, which is the second leading cause of death [17, 18]. More than 160 thousand of new cases were diagnosed in Turkey at 2014 and breast cancer is the leading cancer in women [19]. Breast cancer is a type of carcinoma that develops from breast tissue [17]. There are several risks factors for breast cancer such as ageing, diet, obesity which could cause a diverse variety of breast cancers [17, 22]. Normal cell to cancer cell transformation is defined with sustained proliferative signaling, evaded growth suppressors, activated invasion and metastasis, enabled replicative immortality, induced angiogenesis and resistance to cell death, deregulated cellular energetics, avoided immune destruction, instability and mutation of genome, and tumor promoting inflammation [56, 57]. In addition to these hallmarks, epigenetic abnormalities like overexpression of miRNAs, methylation of histones and genes increase the risk of breast cancer [58, 67, 68].

Micro RNAs are small noncoding RNAs that regulate 30 percent of protein expression with binding 3'UTR region of mRNAs and leads to mRNA degradation and translation repression [37, 188]. miRNAs transcript by RNA polymerase II, cleaved by DROSHA/DGCR8, exported to cytoplasm by exportin-5, further cleaved by Dicer/TRBP, cleaved miRNA duplex forms RNA induced silencing complex with Ago2 and DICER, and regulates protein expression via mRNA cleavage, translational repression and miRNA deadenylation [26, 184]. miRNAs regulates protein expression of different pathways such as development, cell proliferation and differentiation, apoptosis [41, 42]. Ageing, CR, cancer, and other factors could regulate miRNA expression [15, 188, 195]. Ageing induced change in the miRNA expression levels could regulate nutrient sensing, mitochondrial dysfunction, oxidative

stress, telomere shortening, cellular senescence, stem cell exhaustion, DNA repair, protein homeostasis, gene expression and inflammation by regulating several pathways such as insulin/IGF1 signaling, mTOR signaling, AMPK and sirtuins signaling [5, 185-187]. Moreover, ageing induced change in the miRNA expression levels could regulate lifespan, synaptic plasticity and cognition, neuroprotection and neurodegeneration by regulating longevity and neuronal survival, neural differentiation, proneural genes, signaling pathways and transcription factors [186, 188-191]. miRNAs also regulate activation, proliferation, fate specification, differentiation, migration, synaptic integration of neurogenesis [192, 193]. Calorie restriction is an another factor in regulation of miRNA expression. CR induced miRNA expressions could alleviate the effects of ageing with changing expression levels of miRNAs by increasing longevity, lifespan, neuronal survival, neuroprotection, tumor free survival, decreasing apoptosis, tumor progression, metastasis [15, 16, 202-211]. CR mediated miRNA expression shows neuroprotective effects by increasing longevity, lifespan, neuronal survival via promoting anti-oxidative, anti-inflammatory and pro-angiogenic factors [15, 16, 214].

In this study, we researched the effects of long term CR (CCR and ICR) and ageing on brain miRNA profile. In addition to this, we also examined the difference between healthy brain and mammary tumor developed mice brain to assess effect of tumor development in brain. Brain samples were homogenized and miRNA profiles evaluated using miRNA microarray. Total of 1237 pre-miRNAs (precursors) and 1926 mature miRNAs were analyzed on mice brains and differentially expressed miRNAs were determined. Target genes of DE miRNAs were predicted with combination of three miRNA databases. Predicted targets of DE miRNAs were used to determine miRNA regulated GO terms and KEGG pathways. Furthermore, common effects of both CCR and ICR on GO terms and KEGG pathways were researched using common predicted targets in CCR and ICR. Then, five miRNA which DE in CCR compared to other diets were selected for functional enrichment. mmu-miR-713 was further enriched by other pathways and level of mmu-miR-713 was controlled with RT-PCR for validation of results.

Calorie restriction is a well-known method for ameliorating effects of ageing, increasing longevity, and preventing age-related diseases [6]. miRNA expression in the brain could be regulated by ageing and have roles in ageing process [186, 188]. CR application could regulate ageing process by regulating miRNA expression [16]. We profiled the miRNA

levels in CCR and ICR application compared to AL at week 81/82 and several GO terms including neurogenesis, nervous system development, and generation of neurons were common in CR applications. CCR and ICR (ICR-R and ICR-RF) diet regulated proteoglycans, focal adhesion and several other pathways. DE miRNAs in AL and ICR diets compared to CCR were also determined because CCR conferred better protective results in tumor development in our current study. Only presynaptic membrane GO term was common between diets and several KEGG pathways were significantly changed compared to CCR diet. Moreover, research indicated that both chronic and intermittent application of CR retain their anti-ageing effects [10-12]. To assess the role of CCR and ICR on brain miRNA profile, we functionally enriched common predicted targets in CCR and ICR-R compared to AL. Even though there were no common miRNA found, 75 predicted targets were common between ICR-R and CCR because of the multiregulative role of miRNAs [188]. Functional enrichment of these common predicted targets of DE miRNAs in CCR and ICR-R revealed that transcription regulation, dendritic spine development, axonal transport, Alzheimer disease and miRNA regulated gene silencing pathways/terms were significantly enriched. Protective effects of both CR applications could be regulated by these same genes and pathways in brain. In addition to that we also examined the effect of CCR on brain miRNA profile by functionally enriching common predicted targets of AL and ICR compared to CCR. One miRNA was common between AL and ICR compared to CCR, furthermore, 233 of 236 of common predicted targets derived from mmu-miR-713. Transcription regulation, neurogenesis, dopaminergic synapse, cyclase activity, presynaptic membrane and several other pathways/terms were significantly enriched. CCR could elaborate their additional protective activity via these pathways and terms by regulating mmu-miR-713 activity.

In addition to effects of CR on brain miRNA profile at week 81/82, we compared miRNA levels of AL, CCR, ICR (both ICR-R and ICR-RF) to baseline (week 10). General miRNA levels were decreased at week 81/82 compared to baseline which is coherent with literature, however, two miRNAs were upregulated in CCR and one miRNA was upregulated in ICR-R [229]. mmu-miR-7019 was common between CCR and ICR-R. Furthermore, mmu-miR-184-3p and mmu-miR-351-5p were common between CCR and ICR-R and lower compared to ICR-R. 11 predicted targets of DE miRNAs in AL, CCR, ICR-R and ICR-RF was determined and eight of them including Shank2, Bak1, Hif1an were upregulated by downregulation of miRNAs which are important in apoptosis and spine morphogenesis [230,

231]. GO analysis revealed that several brain related terms including neurogenesis, axonogenesis were significantly changed with ageing. Three GO terms were common between AL, CCR and ICR including morphogenesis. 84 GO terms were common between CCR and ICR-R including cognition and memory. It is known that CR application could improve memory and cognition [8]. However, in KEGG pathway analysis, only predicted targets of DE miRNAs in ICR-RF group compared to baseline was showed significantly changed pathways including axon guidance, MAPK, PI3K-Akt signaling pathways. As mentioned above, to asses role of CCR and ICR on brain miRNA profile, we functionally enriched common predicted targets in CCR and ICR-R compared to AL. 359 of 394 common predicted targets were from mmu-miR-184-3p and mmu-miR-351-5p. Functional enrichment of these common predicted targets in CCR and ICR-R compared to baseline was revealed that transcription regulation, cell cycle, differentiation, chromatin regulator, axonogenesis, cytokine, VEGF, MAPK, insulin signaling and several other pathways/terms were significantly enriched. VEGF, insulin signaling were upregulated in CCR and ICR-R by downregulation of mmu-miR-184-3p and mmu-miR-351-5p. It is shown that CR could positively regulate life span and neuroprotection by regulating chromatin and histone related genes via miRNAs in liver and brain [16, 204]. Also, activity of VEGF and insulin signaling pathways were decreased with ageing and CR regulated increase by miRNAs could be important for longevity and neural survival [5, 8, 10, 131]. CCR and ICR-R could elaborate their anti-ageing effects by downregulating mmu-miR-184-3p and mmu-miR-351-5p.

Effects of CR on brain were further analyzed to reveal interaction of CR on mRNA profile and relation with mRNA and miRNA expression, Gene Expression Omnibus (GEO) datasets were researched and only three research were performed brain samples. Two of them were performed in mice hippocampus and one of them performed in mice neocortex [232-234]. All these researches were performed using CCR application. According to the research performed by Barger et al. in 2017, 25 percent CCR application through 8^{th} to 14^{th} weeks and 40 percent CCR application through 14^{th} to 22^{nd} weeks in C57BL/6 mice were not changed mRNA levels in mice neocortex compared to control group [232]. In addition to that, research conducted by Schafer et al. in 2015 applied 30 percent CCR to mice which started at 2.5^{th} months till 15^{th} months however calorie intakes were restricted by reducing carbohydrate intake. This research showed that 30 percent CCR application were regulated calcium signaling and axonal guidance signaling related genes and suppressed age dependent

transcriptional changes by regulating transcription factors. In addition to that, CCR were increased neuroprotective factors, heat shock proteins and improved learning and memory related genes. According to this research, CCR application ameliorate and suppress hippocampal ageing especially in CA1 sector [234]. Research conducted by Wahl et al. in 2018 showed that 20 percent CCR application performed 3rd months to 15th months were positively regulated longevity, dendrite morphogenesis, synapse functioning and neuronal development related gene expressions with upregulating 237 genes and downregulating 238 genes compared to control diet. In addition to that nutrient sensing pathways were positively regulated in CCR with increasing SIRT1 expression and decreasing mTOR activation compared to control diet. Moreover, dendritic spine density in the dentate gyrus were increased and cognitive and behavioral functions were improved by CCR application [233]. These researched showed that hippocampal gene expression levels were changed with CCR application and neurogenesis and neuroprotection related gene expressions were positively regulated by CCR. These results are in line with our research and indicates that CR application regulates gene expression by regulating miRNAs and ameliorates effects of ageing by regulating miRNA and mRNA profile in brain especially in hippocampal region. In addition to that CR application increase dendrite formation and neuronal survival.

Difference between MT developed mice brain and healthy brain miRNA profile at week 81/82 also determined using microarray. 20 miRNAs were upregulated and 77 miRNAs were downregulated in MT developed mice brain. Mammary tumor grades of samples were II and III which means tumor cells were rapidly dividing but not migrating. Gene ontology analysis was performed on predicted targets of these DE miRNAs and GO analysis revealed that several brain related terms including neurogenesis and neuron differentiation were significantly enriched. Also, 14 different KEGG pathways including focal adhesion, MAPK signaling pathway and cancer pathways were significantly effected in MT developed mice brain compared to healthy one at week 81/82. ClueGO functional enrichment show that neurogenesis, MAPK signaling, neuron-neuron interaction and several other pathways/terms were significantly enriched. Neurogenesis, focal adhesion, MAPK signaling and other factors are important for brain function [30, 118, 235, 236]. This indicates that tumor development in mammary tissue could affect brain via regulating miRNA levels in brain and could regulate neuronal survival, neuron interaction and neurogenesis. This effect of MT on

brain could be conducted via brain adipocyte crosstalk and other bloodborne factors which are regulated by tumor development [11, 12, 14, 237].

Moreover, we found that CCR have showed lower MT incidence and MT weight of mice percent survival was higher in the CCR group compared to AL and ICR ($p>0.05$). Because of improved effects of CCR on animal health and longevity we focused to unveil the additional positive effects of CR in brain miRNA levels. Several miRNAs including mmu-miR-713, mmu-miR-7221-5p, mmu-miR-132-5p, mmu-miR-3058-3p were significantly expressed in CCR compared to other groups. Functional enrichment analysis on these miRNAs showed that neuron transcription, transcription regulation, synapse formation, hippocampus development and other pathways and terms were significantly enriched. However, mmu-miR-713 which is significantly higher in CCR compared to both AL and ICR groups at week 81/82 showed enriched neurogenesis, transcription regulation, fibronectin functions in DAVID 6.8. Furthermore, additional ClueGO enrichment also showed regulation of axonogenesis, dopaminergic synapse, prolactin signaling, presynaptic membrane and several other significantly enriched pathways and terms. mmu-miR-713 levels were significantly higher in CCR group compared to AL and ICR, moreover, expression levels were similar in the baseline (week 10). In addition to that in MT developed mice brain samples levels of mmu-miR-713 was lower ($p>0.05$). These result show that CCR could persist additional anti-ageing and longevity effect by persisting and increasing mmu-miR-713 levels in brain by regulating neurogenesis and several other pathways. Research conducted by Hsieh et al. in 2015 shows that mmu-miR-713 expression levels were increased in serum samples of male diet induced obesity mouse model compared to control mouse [238]. In addition to that Koch et al. published a paper in 2012 which indicated that loss of emerin protein expression in mouse myogenic progenitors were upregulated mmu-miR-713 expression 1.92 fold in myogenic progenitor cells compared to control cells could cause disruption in myogenic signaling [239]. Moreover, research performed by Xu et al. in 2012 showed that oxidative stress in primary mouse hippocampal neuron culture were downregulated expression of mmu-miR-713 significantly and indicated that these downregulated miRNAs could be important in neurodegenerative diseases like AD [240]. These research show that roles of mmu-miR-713 in brain tissue and the other tissues were not researched in depth. However, our research showed that mmu-miR-713 levels could be important in neuroprotection by regulating neurogenesis and other pathways.

5. CONCLUSION

In this study, we focused on three main objectives. Firstly we researched the effects of long term CR on brain miRNA profile compared to old AL fed mice. CR application confer protective results compared to AL although there was no statistical difference. CR and ICR-R confer protective effects in ageing via regulating miRNAs that are important in transcription regulation, dendritic and axonal pathways. In addition to that CCR showed better protective results compared to ICR by upregulating mmu-miR-713 activity. Increased mmu-miR-713 levels effected neurogenesis, neuronal survival and axon guidance related terms and pathways, that could be important in healthy ageing and reverse effects of ageing.

Secondly, we analyzed difference between brain miRNA profiles of AL, CCR and ICR at week 81/82 compared to baseline to assess longevity effects of CR which is connected with CR regulated miRNA application. miR-184-3p and mmu-miR-351-5p were common between CCR and ICR-R compared to baseline and predicted targets of these miRNAs and other common targets revealed that chromatin and histone regulation terms and pathways were significantly enriched. CR confer anti-ageing effects by regulating miRNAs which are important in chromatin and histone regulation related terms and pathways [16, 204].

Lastly, difference between mammary tumor developed mice and healthy mice brain miRNA profile was analyzed to assess epigenetic effect of MT development on brain. Several miRNAs were changed including mmu-miR-200 family in MT developed mice brain which affects regulating focal adhesion, neurogenesis and several other pathways. MT development in breast tissue could affect brain functions by regulating miRNAs and this interaction could be regulated via brain adipocyte crosstalk and other bloodborne factors [11, 237].

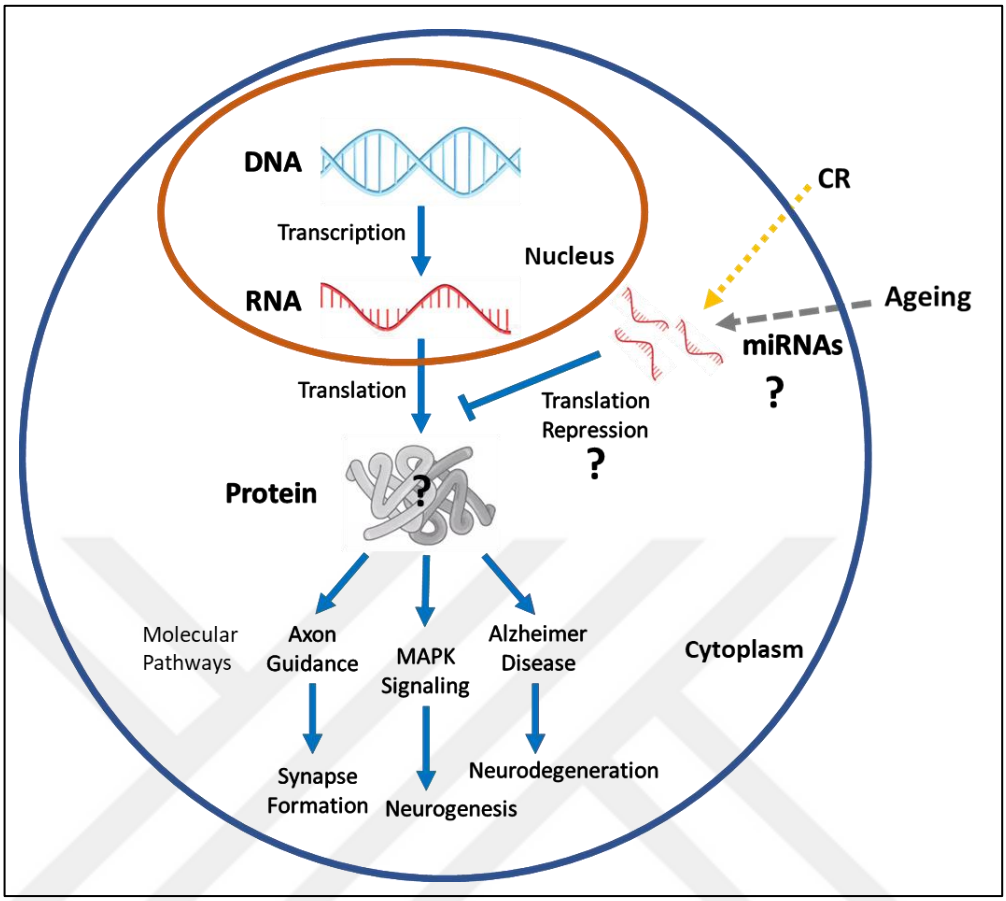


Figure 5.1. Summary of effects of ageing and CR on brain.

As indicated in Fig 5.1, ageing regulates miRNA expression which repress translation of proteins important in neurogenesis, synapse formation and neurodegeneration related terms. Ageing negatively regulates synapse formation, MAPK signaling and positively regulates neurodegenerative disease related pathways like AD that could lead to decrease in synapse formation and neurogenesis and increase in neurodegeneration. However, CR application (both CCR and ICR-R) ameliorates effects of ageing by positively regulating synapse formation, MAPK signaling and negatively regulating neurodegenerative disease related pathways like AD that could lead to increase in synapse formation and neurogenesis and decrease in neurodegeneration. However, miRNAs related to these processes and their effects on translation as well as proteins in these processes must be revealed to unveil underlying epigenetic mechanisms of ageing and CR by regulating miRNA expression.

In conclusion, protective effects CR in brain could be modulated by miRNAs, and CCR showed better results in protection compared to ICR. CCR could modulate additional

protective effects by increasing expression of mmu-miR-713 compared to AL and ICR groups. In ageing, regulated expression of mmu-miR-184-3p and mmu-miR-351-5p by CR could exhibit anti-ageing effects and other miRNAs that were regulated by CCR could also alleviate the effects of ageing. CR could alleviate effects of ageing by regulating miRNAs. In MT developed mice brain, MT development disturbs brain miRNA profile which could regulate neuronal survival and neuron interaction related terms and pathways. However, further research are needed to assess the effects of CR regulated miRNAs in ageing brain and effects of mammary tumor on brain.

REFERENCES

1. Harman D. The aging process. *Proceedings of the National Academy of Sciences of the United States of America*. 1981;78(11):7124-8.

2. Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell*. 2013;153(6):1194-217.

3. Rebelo-Marques A, De Sousa Lages A, Andrade R, Ribeiro CF, Mota-Pinto A, Carrilho F, et al. Aging hallmarks: The benefits of physical exercise. *Frontiers in Endocrinology*. 2018;9:258.

4. Barbosa MC, Grosso RA, Fader CM. Hallmarks of aging: An autophagic perspective. *Frontiers in Endocrinology*. 2018;9:790.

5. Victoria B, Nunez Lopez YO, Masternak MM. Micrornas and the metabolic hallmarks of aging. *Molecular and Cellular Endocrinology*. 2017;455:131-47.

6. Lopez-Lluch G, Navas P. Calorie restriction as an intervention in ageing. *Journal of Physiology*. 2016;594(8):2043-60.

7. Pani G. Neuroprotective effects of dietary restriction: Evidence and mechanisms. *Seminars in Cell and Developmental Biology*. 2015;40:106-14.

8. Hadem IKH, Majaw T, Kharbuli B, Sharma R. Beneficial effects of dietary restriction in aging brain. *Journal of Chemical Neuroanatomy*. 2019;95:123-33.

9. Morgan AH, Andrews ZB, Davies JS. Less is more: Caloric regulation of neurogenesis and adult brain function. *Journal of Neuroendocrinology*. 2017;29(10):89-102.

10. Dogan S, Johannsen AC, Grande JP, Cleary MP. Effects of intermittent and chronic calorie restriction on mammalian target of rapamycin (mtor) and igf-i signaling pathways in mammary fat pad tissues and mammary tumors. *Nutrition and Cancer*. 2011;63(3):389-401.

11. Dogan S, Ray A, Cleary MP. The influence of different calorie restriction protocols on serum pro-inflammatory cytokines, adipokines and igf-i levels in female c57bl6 mice: Short term and long term diet effects. *Meta Gene*. 2017;12:22-32.

12. Dogan S, Rogozina OP, Lokshin AE, Grande JP, Cleary MP. Effects of chronic vs. Intermittent calorie restriction on mammary tumor incidence and serum adiponectin and leptin levels in mmtv-tgf-alpha mice at different ages. *Oncology Letters*. 2010;1(1):167-76.

13. Omeroglu Ulu Z, Ulu S, Dogan S, Guvenc Tuna B, Ozdemir Ozgenturk N. Transcriptome analysis of the thymus in short-term calorie-restricted mice using rna-seq. *International Journal of Genomics*. 2018;2018(1):7647980.

14. Tuna BG, Atalay PB, Altunbek M, Kalkan BM, Dogan S. Effects of chronic and intermittent calorie restriction on adropin levels in breast cancer. *Nutrition and Cancer*. 2017;69(7):1003-10.

15. Khanna A, Muthusamy S, Liang R, Sarojini H, Wang E. Gain of survival signaling by down-regulation of three key mirnas in brain of calorie-restricted mice. *Aging (Albany NY)*. 2011;3(3):223-36.

16. Wood SH, van Dam S, Craig T, Tacutu R, O'Toole A, Merry BJ, et al. Transcriptome analysis in calorie-restricted rats implicates epigenetic and post-translational mechanisms in neuroprotection and aging. *Genome Biology*. 2015;16:285.

17. W. RR. *Cancer biology*. Fourth ed. New York: Oxford University Press; 2007.

18. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA: A Cancer Journal for Clinicians*. 2019;69(1):7-34.

19. Hacikamiloglu E, Gultekin M, Boztas G, Dundar S, Utku ES, Kavak-Ergun A, et al. Türkiye kanser istatistikleri. Ankara: T.C. Sağlık Bakanlığı, Türkiye Halk Sağlığı Kurumu; 2017.

20. Majeed W, Aslam B, Javed I, Khaliq T, Muhammad F, Ali A, et al. Breast cancer: Major risk factors and recent developments in treatment. *Asian Pacific Journal of Cancer Prevention*. 2014;15(8):3353-8.

21. Akram M, Iqbal M, Daniyal M, Khan AU. Awareness and current knowledge of breast cancer. *Biological Research*. 2017;50:33.

22. Sun YS, Zhao Z, Yang ZN, Xu F, Lu HJ, Zhu ZY, et al. Risk factors and preventions of breast cancer. *International Journal of Biological Sciences*. 2017;13(11):1387-97.

23. Ban KA, Godellas CV. Epidemiology of breast cancer. *Surgical Oncology Clinics of North America*. 2014;23(3):409-22.

24. Steiner E, Tata M, Frisen J. A fresh look at adult neurogenesis. *Nature Medicine*. 2019;25(4):542-3.

25. Berg DA, Su Y, Jimenez-Cyrus D, Patel A, Huang N, Morizet D, et al. A common embryonic origin of stem cells drives developmental and adult neurogenesis. *Cell*. 2019;177(3):654-68.

26. Gilbert SF, Barresi MJF. *Developmental biology*. Eleventh ed. Sunderland, Massachusetts: Oxford University Press; 2017.

27. Ming GL, Song H. Adult neurogenesis in the mammalian brain: Significant answers and significant questions. *Neuron*. 2011;70(4):687-702.

28. Ernst A, Frisen J. Adult neurogenesis in humans- common and unique traits in mammals. *PLoS Biology*. 2015;13(1):1002045.

29. Kempermann G, Wiskott L, Gage FH. Functional significance of adult neurogenesis. *Current Opinion in Neurobiology*. 2004;14(2):186-91.

30. Braun SM, Jessberger S. Adult neurogenesis: Mechanisms and functional significance. *Development*. 2014;141(10):1983-6.

31. Neves G, Cooke SF, Bliss TV. Synaptic plasticity, memory and the hippocampus: A neural network approach to causality. *Nature Reviews: Neuroscience*. 2008;9(1):65-75.

32. Moreno-Jimenez EP, Flor-Garcia M, Terreros-Roncal J, Rabano A, Cafini F, Pallas-Bazarra N, et al. Adult hippocampal neurogenesis is abundant in neurologically healthy subjects and drops sharply in patients with alzheimer's disease. *Nature Medicine*. 2019;25(4):554-60.

33. Liu H, Song N. Molecular mechanism of adult neurogenesis and its association with human brain diseases. *Journal of Central Nervous System Disease*. 2016;8:5-11.

34. Yao B, Christian KM, He C, Jin P, Ming GL, Song H. Epigenetic mechanisms in neurogenesis. *Nature Reviews: Neuroscience*. 2016;17(9):537-49.

35. Delgado-Morales R, Agis-Balboa RC, Esteller M, Berdasco M. Epigenetic mechanisms during ageing and neurogenesis as novel therapeutic avenues in human brain disorders. *Clinical Epigenetics*. 2017;9:67.

36. Lin S, Gregory RI. Microrna biogenesis pathways in cancer. *Nature Reviews: Cancer*. 2015;15(6):321-33.

37. Cui J, Zhou B, Ross SA, Zempleni J. Nutrition, micrornas, and human health. *Advances in Nutrition*. 2017;8(1):105-12.

38. Croce CM, Calin GA. Mirnas, cancer, and stem cell division. *Cell*. 2005;122(1):6-7.

39. Iorio MV, Casalini P, Piovan C, Braccioli L, Tagliabue E. Breast cancer and micrornas: Therapeutic impact. *Breast*. 2011;20:63-70.

40. Macfarlane LA, Murphy PR. Microrna: Biogenesis, function and role in cancer. *Current Genomics*. 2010;11(7):537-61.

41. Parasramka MA, Ho E, Williams DE, Dashwood RH. Micrornas, diet, and cancer: New mechanistic insights on the epigenetic actions of phytochemicals. *Molecular Carcinogenesis*. 2012;51(3):213-30.

42. Ross SA, Davis CD. Microrna, nutrition, and cancer prevention. *Advances in Nutrition*. 2011;2(6):472-85.

43. Jin K. Modern biological theories of aging. *Aging and Disease*. 2010;1(2):72-4.

44. Mitteldorf J. Can aging be programmed? *Biochemistry (Moscow)*. 2018;83(12):1524-33.

45. Mattson MP, Arumugam TV. Hallmarks of brain aging: Adaptive and pathological modification by metabolic states. *Cell Metabolism*. 2018;27(6):1176-99.

46. Mitchell SJ, Scheibye-Knudsen M, Longo DL, de Cabo R. Animal models of aging research: Implications for human aging and age-related diseases. *Annual Review of Animal Biosciences*. 2015;3:283-303.

47. Titorenko VI. Molecular and cellular mechanisms of aging and age-related disorders. *International Journal of Molecular Sciences*. 2018;19(7):2049.

48. Belikov AV. Age-related diseases as vicious cycles. *Ageing Research Reviews*. 2019;49:11-26.

49. Pryor R, Cabreiro F. Repurposing metformin: An old drug with new tricks in its binding pockets. *Biochemical Journal*. 2015;471(3):307-22.

50. Cui M, Yu H, Wang J, Gao J, Li J. Chronic caloric restriction and exercise improve metabolic conditions of dietary-induced obese mice in autophagy correlated manner without involving ampk. *Journal of Diabetes Research*. 2013;2013:852754.

51. Woods JA, Wilund KR, Martin SA, Kistler BM. Exercise, inflammation and aging. *Aging and Disease*. 2012;3(1):130-40.

52. Wei M, Fabrizio P, Hu J, Ge H, Cheng C, Li L, et al. Life span extension by calorie restriction depends on rim15 and transcription factors downstream of ras/pka, tor, and sch9. *PLoS Genetics*. 2008;4(1):139-50.

53. Stankiewicz AJ, McGowan EM, Yu L, Zhdanova IV. Impaired sleep, circadian rhythms and neurogenesis in diet-induced premature aging. *International Journal of Molecular Sciences*. 2017;18(11):2243.

54. Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de Cabo R, et al. Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice. *Journals of Gerontology. Series A: Biological Sciences and Medical Sciences*. 2011;66(2):191-201.

55. Lam YY, Peterson CM, Ravussin E. Resveratrol vs. Calorie restriction: Data from rodents to humans. *Experimental Gerontology*. 2013;48(10):1018-24.

56. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100(1):57-70.

57. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. *Cell*. 2011;144(5):646-74.

58. Duru N, Gernapudi R, Eades G, Eckert R, Zhou Q. Epigenetic regulation of mirnas and breast cancer stem cells. *Current Pharmacology Reports*. 2015;1(3):161-9.

59. Ferguson LR, Chen H, Collins AR, Connell M, Damia G, Dasgupta S, et al. Genomic instability in human cancer: Molecular insights and opportunities for therapeutic attack and prevention through diet and nutrition. *Seminars in Cancer Biology*. 2015;35:5-24.

60. Anand P, Kunnumakkara AB, Sundaram C, Harikumar KB, Tharakan ST, Lai OS, et al. Cancer is a preventable disease that requires major lifestyle changes. *Pharmaceutical Research*. 2008;25(9):2097-116.

61. Yang J, Ren Z, Du X, Hao M, Zhou W. The role of mesenchymal stem/progenitor cells in sarcoma: Update and dispute. *Stem Cell Investigation*. 2014:1(10);18.

62. Ulbright TM. Germ cell tumors of the gonads: A selective review emphasizing problems in differential diagnosis, newly appreciated, and controversial issues. *Modern Pathology : an Official Journal of the United States and Canadian Academy of Pathology, Inc*. 2005;18(2):61-79.

63. Alberts B, Johnson AD, Lewis J, Morgan D, Raff M, Roberts K, et al. *Molecular biology of the cell*. Sixth ed. New York: Garland Science; 2014.

64. Dogan N, Toprak D. Female breast cancer mortality rates in turkey. *Asian Pacific Journal of Cancer Prevention*. 2014;15(18):7569-73.

65. Stratton MR, Rahman N. The emerging landscape of breast cancer susceptibility. *Nature Genetics*. 2008;40(1):17-22.

66. Varna M, Bousquet G, Plassa LF, Bertheau P, Janin A. Tp53 status and response to treatment in breast cancers. *Journal of Biomedicine and Biotechnology*. 2011;2011:284584.

67. Basse C, Arock M. The increasing roles of epigenetics in breast cancer: Implications for pathogenicity, biomarkers, prevention and treatment. *International Journal of Cancer*. 2015;137(12):2785-94.

68. Tao Z, Shi A, Lu C, Song T, Zhang Z, Zhao J. Breast cancer: Epidemiology and etiology. *Cell Biochemistry and Biophysics*. 2015;72(2):333-8.

69. Drukteinis JS, Mooney BP, Flowers CI, Gatenby RA. Beyond mammography: New frontiers in breast cancer screening. *The American Journal of Medicine*. 2013;126(6):472-9.

70. Dai X, Xiang L, Li T, Bai Z. Cancer hallmarks, biomarkers and breast cancer molecular subtypes. *Journal of Cancer*. 2016;7(10):1281-94.

71. Dai X, Li T, Bai Z, Yang Y, Liu X, Zhan J, et al. Breast cancer intrinsic subtype classification, clinical use and future trends. *American Journal of Cancer Research*. 2015;5(10):2929-43.

72. Lehmann BD, Pietenpol JA. Identification and use of biomarkers in treatment strategies for triple-negative breast cancer subtypes. *Journal of Pathology*. 2014;232(2):142-50.

73. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. *Nature*. 2000;406(6797):747-52.

74. Cancer Genome Atlas N. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012;490(7418):61-70.

75. Malhotra GK, Zhao X, Band H, Band V. Histological, molecular and functional subtypes of breast cancers. *Cancer Biology and Therapy*. 2010;10(10):955-60.

76. Ozmen V. Breast cancer in turkey: Clinical and histopathological characteristics (analysis of 13.240 patients). *The Journal of Breast Health*. 2014;10(2):98-105.

77. Eliyatkin N, Yalcin E, Zengel B, Aktas S, Vardar E. Molecular classification of breast carcinoma: From traditional, old-fashioned way to a new age, and a new way. *The Journal of Breast Health*. 2015;11(2):59-66.

78. Weigelt B, Geyer FC, Reis-Filho JS. Histological types of breast cancer: How special are they? *Molecular Oncology*. 2010;4(3):192-208.

79. Cleary MP, Grossmann ME. Minireview: Obesity and breast cancer: The estrogen connection. *Endocrinology*. 2009;150(6):2537-42.

80. Miller SM, Goulet DR, Johnson GL. Targeting the breast cancer kinome. *Journal of Cellular Physiology*. 2017;232(1):53-60.

81. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ, et al. Strategies for subtypes--dealing with the diversity of breast cancer: Highlights of the st. Gallen international expert consensus on the primary therapy of early breast cancer 2011. *Annals of Oncology*. 2011;22(8):1736-47.

82. Ito Y, Iwase T, Hatake K. Eradication of breast cancer cells in patients with distant metastasis: The finishing touches? *Breast Cancer*. 2012;19(3):206-11.

83. Przedborski S, Vila M, Jackson-Lewis V. Neurodegeneration: What is it and where are we? *Journal of Clinical Investigation*. 2003;111(1):3-10.

84. Katsnelson A, De Strooper B, Zoghbi HY. Neurodegeneration: From cellular concepts to clinical applications. *Science Translational Medicine*. 2016;8(364).

85. Maynard S, Fang EF, Scheibye-Knudsen M, Croteau DL, Bohr VA. DNA damage, DNA repair, aging, and neurodegeneration. *Cold Spring Harbor Perspectives in Medicine*. 2015;5(10).

86. Jeppesen DK, Bohr VA, Stevnsner T. DNA repair deficiency in neurodegeneration. *Progress in Neurobiology*. 2011;94(2):166-200.

87. Sterling NW, Lichtenstein M, Lee EY, Lewis MM, Evans A, Eslinger PJ, et al. Higher plasma ldl-cholesterol is associated with preserved executive and fine motor functions in parkinson's disease. *Aging and Disease*. 2016;7(3):237-45.

88. Tang H, Ma M, Wu Y, Deng MF, Hu F, Almansoub H, et al. Activation of mt2 receptor ameliorates dendritic abnormalities in alzheimer's disease via c/ebpalpha/mir-125b pathway. *Aging Cell*. 2019;18(2).

89. Seib DR, Martin-Villalba A. Neurogenesis in the normal ageing hippocampus: A mini-review. *Gerontology*. 2015;61(4):327-35.

90. Chakrabarti S, Mohanakumar KP. Aging and neurodegeneration: A tangle of models and mechanisms. *Aging and Disease*. 2016;7(2):111-3.

91. Calabrese V, Cornelius C, Mancuso C, Pennisi G, Calafato S, Bellia F, et al. Cellular stress response: A novel target for chemoprevention and nutritional neuroprotection in

aging, neurodegenerative disorders and longevity. *Neurochemical Research*. 2008;33(12):2444-71.

92. Lui JH, Hansen DV, Kriegstein AR. Development and evolution of the human neocortex. *Cell*. 2011;146(1):18-36.

93. Rakic P. Evolution of the neocortex: A perspective from developmental biology. *Nature Reviews: Neuroscience*. 2009;10(10):724-35.

94. Lim DA, Alvarez-Buylla A. The adult ventricular-subventricular zone (v-svz) and olfactory bulb (ob) neurogenesis. *Cold Spring Harbor Perspectives in Biology*. 2016;8(5).

95. Altman J. Are new neurons formed in the brains of adult mammals? *Science*. 1962;135(3509):1127-8.

96. Kaplan MS. Neurogenesis in the 3-month-old rat visual cortex. *The Journal of Comparative Neurology*. 1981;195(2):323-38.

97. Kaplan MS, Hinds JW. Neurogenesis in the adult rat: Electron microscopic analysis of light radioautographs. *Science*. 1977;197(4308):1092-4.

98. Gould E, Reeves AJ, Graziano MS, Gross CG. Neurogenesis in the neocortex of adult primates. *Science*. 1999;286(5439):548-52.

99. Gould E, Tanapat P, Hastings NB, Shors TJ. Neurogenesis in adulthood: A possible role in learning. *Trends in Cognitive Sciences*. 1999;3(5):186-92.

100. Gould E, Reeves AJ, Fallah M, Tanapat P, Gross CG, Fuchs E. Hippocampal neurogenesis in adult old world primates. *Proceedings of the National Academy of Sciences of the United States of America*. 1999;96(9):5263-7.

101. Bergmann O, Liebl J, Bernard S, Alkass K, Yeung MS, Steier P, et al. The age of olfactory bulb neurons in humans. *Neuron*. 2012;74(4):634-9.

102. Squire LR, Berg D, Bloom FE, Lac SD, Ghosh A, Spitzer NC. *Fundamental neuroscience*. Fourth ed. Waltham, Massachusetts: Elsevier; 2012.

103. Purves D, Augustine GJ, Fitzpatrick D, Hall WC, LaMantia AS, Mooney RD, et al. *Neuroscience*. Sixth ed. Sunderland, Massachusetts: Oxford University Press; 2018.

104. Bear MF, Connors BW, Paradiso MA. *Neuroscience: Exploring the brain*. Fourth ed. Pennsylvania: Lippincott Williams and Wilkins; 2016.

105. Kempermann G, Gage FH, Aigner L, Song H, Curtis MA, Thuret S, et al. Human adult neurogenesis: Evidence and remaining questions. *Cell Stem Cell*. 2018;23(1):25-30.

106. Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, et al. Neurogenesis in the adult human hippocampus. *Nature Medicine*. 1998;4(11):1313-7.

107. Ernst A, Alkass K, Bernard S, Salehpour M, Perl S, Tisdale J, et al. Neurogenesis in the striatum of the adult human brain. *Cell*. 2014;156(5):1072-83.

108. Spalding KL, Bergmann O, Alkass K, Bernard S, Salehpour M, Huttner HB, et al. Dynamics of hippocampal neurogenesis in adult humans. *Cell*. 2013;153(6):1219-27.

109. Boldrini M, Fulmore CA, Tartt AN, Simeon LR, Pavlova I, Poposka V, et al. Human hippocampal neurogenesis persists throughout aging. *Cell Stem Cell*. 2018;22(4):589-99.

110. Sorrells SF, Paredes MF, Cebrian-Silla A, Sandoval K, Qi D, Kelley KW, et al. Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. *Nature*. 2018;555(7696):377-81.

111. Zhang J, Jiao J. Molecular biomarkers for embryonic and adult neural stem cell and neurogenesis. *BioMed Research International*. 2015;2015:727542.

112. Urban N, Guillemot F. Neurogenesis in the embryonic and adult brain: Same regulators, different roles. *Frontiers in Cellular Neuroscience*. 2014;8:396.

113. Kurtenbach S, Ding W, Goss GM, Hare JM, Goldstein BJ, Shehadeh LA. Differential expression of micrornas among cell populations in the regenerating adult mouse olfactory epithelium. *PloS One*. 2017;12(11):0187576.

114. Bergmann O, Spalding KL, Frisen J. Adult neurogenesis in humans. *Cold Spring Harbor Perspectives in Biology*. 2015;7(7):018994.

115. Lazarov O, Hollands C. Hippocampal neurogenesis: Learning to remember. *Progress in Neurobiology*. 2016;138-140:1-18.

116. Kempermann G, Song H, Gage FH. Neurogenesis in the adult hippocampus. *Cold Spring Harbor Perspectives in Biology*. 2015;7(9):0188012.

117. Toda T, Gage FH. Review: Adult neurogenesis contributes to hippocampal plasticity. *Cell and Tissue Research*. 2018;373(3):693-709.

118. Toda T, Parylak SL, Linker SB, Gage FH. The role of adult hippocampal neurogenesis in brain health and disease. *Molecular Psychiatry*. 2019;24(1):67-87.

119. Goncalves JT, Schafer ST, Gage FH. Adult neurogenesis in the hippocampus: From stem cells to behavior. *Cell*. 2016;167(4):897-914.

120. Aimone JB, Li Y, Lee SW, Clemenson GD, Deng W, Gage FH. Regulation and function of adult neurogenesis: From genes to cognition. *Physiological Reviews*. 2014;94(4):991-1026.

121. Wakabayashi T, Hidaka R, Fujimaki S, Asashima M, Kuwabara T. Micrornas and epigenetics in adult neurogenesis. *Advances in Genetics*. 2014;86:27-44.

122. Levenson CW, Rich NJ. Eat less, live longer? New insights into the role of caloric restriction in the brain. *Nutrition Reviews*. 2007;65(9):412-5.

123. Apple DM, Solano-Fonseca R, Kokovay E. Neurogenesis in the aging brain. *Biochemical Pharmacology*. 2017;141:77-85.

124. Oppenheim RW. Adult hippocampal neurogenesis in mammals (and humans): The death of a central dogma in neuroscience and its replacement by a new dogma. *Developmental Neurobiology*. 2019;79(3):268-80.

125. Brandhorst S, Longo VD. Fasting and caloric restriction in cancer prevention and treatment. *Recent Results in Cancer Research*. 2016;207:241-66.

126. Conboy IM, Rando TA. Heterochronic parabiosis for the study of the effects of aging on stem cells and their niches. *Cell Cycle*. 2012;11(12):2260-7.

127. Isaev NK, Stelmashook EV, Genrikhs EE. Neurogenesis and brain aging. *Reviews in the Neurosciences*. 2019;30(6):573-80.

128. Gonzalez-Armenta JL, Mahapatra G, Allison Amick K, Li N, Lu B, Molina A. Heterochronic parabiosis: Old blood attenuates mitochondrial bioenergetics of young mice. *Innovation in Aging*. 2018;2(1):558-67.

129. Veena J, Rao BS, Srikumar BN. Regulation of adult neurogenesis in the hippocampus by stress, acetylcholine and dopamine. *Journal of Natural Science, Biology, and Medicine*. 2011;2(1):26-37.

130. Cahill S, Tuplin E, Holahan MR. Circannual changes in stress and feeding hormones and their effect on food-seeking behaviors. *Frontiers in Neuroscience*. 2013;7:140-8.

131. Shetty AK, Hattiangady B, Shetty GA. Stem/progenitor cell proliferation factors fgf-2, igf-1, and vegf exhibit early decline during the course of aging in the hippocampus: Role of astrocytes. *Glia*. 2005;51(3):173-86.

132. Lee SW, Clemenson GD, Gage FH. New neurons in an aged brain. *Behavioural Brain Research*. 2012;227(2):497-507.

133. Poulose SM, Miller MG, Scott T, Shukitt-Hale B. Nutritional factors affecting adult neurogenesis and cognitive function. *Advances in Nutrition*. 2017;8(6):804-11.

134. Villeda SA, Luo J, Mosher KI, Zou B, Britschgi M, Bieri G, et al. The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Nature*. 2011;477(7362):90-4.

135. Smith LK, He Y, Park JS, Bieri G, Snethlage CE, Lin K, et al. Beta2-microglobulin is a systemic pro-aging factor that impairs cognitive function and neurogenesis. *Nature Medicine*. 2015;21(8):932-7.

136. Chen M, Do H. Wnt signaling in neurogenesis during aging and physical activity. *Brain Sciences*. 2012;2(4):745-68.

137. Lugert S, Basak O, Knuckles P, Haussler U, Fabel K, Gotz M, et al. Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging. *Cell Stem Cell*. 2010;6(5):445-56.

138. Lupo G, Gioia R, Nisi PS, Biagioni S, Cacci E. Molecular mechanisms of neurogenic aging in the adult mouse subventricular zone. *Journal of Experimental Neuroscience*. 2019;13.

139. Morgan AH, Andrews ZB, Davies JS. Less is more: Caloric regulation of neurogenesis and adult brain function. *J Neuroendocrinol*. 2017;29(10):89-102

140. Pani G. Neuroprotective effects of dietary restriction: Evidence and mechanisms. *Semin Cell Dev Biol*. 2015;40:106-14.

141. Taormina G, Mirisola MG. Calorie restriction in mammals and simple model organisms. *BioMed Research International*. 2014;2014:308690.

142. Hursting SD, Dunlap SM, Ford NA, Hursting MJ, Lashinger LM. Calorie restriction and cancer prevention: A mechanistic perspective. *Cancer and Metabolism*. 2013;1(1):10.

143. McCay CM, Crowell MF, Maynard LA. The effect of retarded growth upon the length of life span and upon the ultimate body size. 1935. *Nutrition*. 1989;5(3):155-72.

144. Kagawa Y. Impact of westernization on the nutrition of japanese: Changes in physique, cancer, longevity and centenarians. *Preventive Medicine*. 1978;7(2):205-17.

145. Michels KB, Ekbom A. Caloric restriction and incidence of breast cancer. *JAMA*. 2004;291(10):1226-30.

146. Most J, Tosti V, Redman LM, Fontana L. Calorie restriction in humans: An update. *Ageing Research Reviews*. 2017;39:36-45.

147. Meydani M, Das S, Band M, Epstein S, Roberts S. The effect of caloric restriction and glycemic load on measures of oxidative stress and antioxidants in humans: Results from the calerie trial of human caloric restriction. *The Journal of Nutrition, Health and Aging*. 2011;15(6):456-60.

148. Al-Regaiey KA. The effects of calorie restriction on aging: A brief review. *European Review for Medical and Pharmacological Sciences*. 2016;20(11):2468-73.

149. Longo VD, Fontana L. Calorie restriction and cancer prevention: Metabolic and molecular mechanisms. *Trends in Pharmacological Sciences*. 2010;31(2):89-98.

150. Meynet O, Ricci JE. Caloric restriction and cancer: Molecular mechanisms and clinical implications. *Trends in Molecular Medicine*. 2014;20(8):419-27.

151. O'Flanagan CH, Smith LA, McDonell SB, Hursting SD. When less may be more: Calorie restriction and response to cancer therapy. *BMC Medicine*. 2017;15(1):106-12.

152. Martin SL, Hardy TM, Tollefsbol TO. Medicinal chemistry of the epigenetic diet and caloric restriction. *Current Medicinal Chemistry*. 2013;20(32):4050-9.

153. Golbidi S, Daiber A, Korac B, Li H, Essop MF, Laher I. Health benefits of fasting and caloric restriction. *Current Diabetes Reports*. 2017;17(12):123-30.

154. Kopeina GS, Senichkin VV, Zhivotovsky B. Caloric restriction - a promising anti-cancer approach: From molecular mechanisms to clinical trials. *Biochimica et Biophysica Acta - Reviews on Cancer*. 2017;1867(1):29-41.

155. Apple DM, Mahesula S, Fonseca RS, Zhu C, Kokovay E. Calorie restriction protects neural stem cells from age-related deficits in the subventricular zone. *Aging*. 2019;11(1):115-126.

156. Moreschi C. Beziehung zwischen ernahrung und tumorwachstum. *Z Immunitatsforsch*. 1909;2:651-75.

157. Harvey AE, Lashinger LM, Hays D, Harrison LM, Lewis K, Fischer SM, et al. Calorie restriction decreases murine and human pancreatic tumor cell growth, nuclear factor-kappab activation, and inflammation-related gene expression in an insulin-like growth factor-1-dependent manner. *PLoS One*. 2014;9(5):94151.

158. Hursting SD, Perkins SN, Phang JM, Barrett JC. Diet and cancer prevention studies in p53-deficient mice. *The Journal of Nutrition*. 2001;131(11):3092-4.

159. Albanes D. Total calories, body weight, and tumor incidence in mice. *Cancer Research*. 1987;47(8):1987-92.

160. Casson RJ, Chidlow G, Ebneter A, Wood JP, Crowston J, Goldberg I. Translational neuroprotection research in glaucoma: A review of definitions and principles. *Clinical and Experimental Ophthalmology*. 2012;40(4):350-7.

161. Hartman AL. Neuroprotection in metabolism-based therapy. *Epilepsy Research*. 2012;100(3):286-94.

162. Qiu G, Spangler EL, Wan R, Miller M, Mattson MP, So KF, et al. Neuroprotection provided by dietary restriction in rats is further enhanced by reducing glucocortocoids. *Neurobiology of Aging*. 2012;33(10):2398-410.

163. Archer T. Physical exercise alleviates debilities of normal aging and alzheimer's disease. *Acta Neurologica Scandinavica*. 2011;123(4):221-38.

164. Patel NV, Gordon MN, Connor KE, Good RA, Engelman RW, Mason J, et al. Caloric restriction attenuates abeta-deposition in alzheimer transgenic models. *Neurobiology of Aging*. 2005;26(7):995-1000.

165. Camandola S, Mattson MP. Brain metabolism in health, aging, and neurodegeneration. *EMBO Journal*. 2017;36(11):1474-92.

166. Ruhlmann C, Wolk T, Blumel T, Stahn L, Vollmar B, Kuhla A. Long-term caloric restriction in apoe-deficient mice results in neuroprotection via fgf21-induced ampk/mtor pathway. *Aging*. 2016;8(11):2777-89.

167. Samandari-Bahraseman MR, Jahanshahi M, Asadi Barbariha S, Elyasi L. Altered micro-rna regulation and neuroprotection activity of eremostachys labiosiformis in alzheimer's disease model. *Annals of Neurosciences*. 2019;25(3):160-5.

168. Her LS, Mao SH, Chang CY, Cheng PH, Chang YF, Yang HI, et al. Mir-196a enhances neuronal morphology through suppressing ranbp10 to provide neuroprotection in huntington's disease. *Theranostics*. 2017;7(9):2452-62.

169. Saugstad JA. Micrornas as effectors of brain function with roles in ischemia and injury, neuroprotection, and neurodegeneration. *Journal of Cerebral Blood Flow and Metabolism*. 2010;30(9):1564-76.

170. Arslan-Ergul A, Ozdemir AT, Adams MM. Aging, neurogenesis, and caloric restriction in different model organisms. *Aging and Disease*. 2013;4(4):221-32.

171. Hadad N, Unnikrishnan A, Jackson JA, Masser DR, Otalora L, Stanford DR, et al. Caloric restriction mitigates age-associated hippocampal differential cg and non-cg methylation. *Neurobiology of Aging*. 2018;67:53-66.

172. Kafami L, Raza M, Razavi A, Mirshafiey A, Movahedian M, Khorramizadeh MR. Intermittent feeding attenuates clinical course of experimental autoimmune encephalomyelitis in c57bl/6 mice. *Avicenna Journal of Medical Biotechnology*. 2010;2(1):47-52.

173. Jeong JH, Yu KS, Bak DH, Lee JH, Lee NS, Jeong YG, et al. Intermittent fasting is neuroprotective in focal cerebral ischemia by minimizing autophagic flux disturbance and inhibiting apoptosis. *Experimental and Therapeutic Medicine*. 2016;12(5):3021-8.

174. Rothman SM, Griffioen KJ, Wan R, Mattson MP. Brain-derived neurotrophic factor as a regulator of systemic and brain energy metabolism and cardiovascular health. *Annals of the New York Academy of Sciences*. 2012;1264:49-63.

175. Lettieri Barbato D, Baldelli S, Pagliei B, Aquilano K, Ciriolo MR. Caloric restriction and the nutrient-sensing pgc-1alpha in mitochondrial homeostasis: New perspectives in neurodegeneration. *International Journal of Cell Biology*. 2012;2012:759583.

176. Kincaid B, Bossy-Wetzel E. Forever young: Sirt3 a shield against mitochondrial meltdown, aging, and neurodegeneration. *Frontiers in Aging Neuroscience*. 2013;5:48.

177. Vasconcelos AR, Dos Santos NB, Scavone C, Munhoz CD. Nrf2/are pathway modulation by dietary energy regulation in neurological disorders. *Frontiers in Pharmacology*. 2019;10:33.

178. Bayliss JA, Lemus MB, Stark R, Santos VV, Thompson A, Rees DJ, et al. Ghrelin-ampk signaling mediates the neuroprotective effects of calorie restriction in parkinson's disease. *Journal of Neuroscience*. 2016;36(10):3049-63.

179. Tripathi A. New cellular and molecular approaches to ageing brain. *Annals of Neurosciences*. 2012;19(4):177-82.

180. Park JH, Glass Z, Sayed K, Michurina TV, Lazutkin A, Mineyeva O, et al. Calorie restriction alleviates the age-related decrease in neural progenitor cell division in the aging brain. *European Journal of Neuroscience*. 2013;37(12):1987-93.

181. Cavallucci V, Fidaleo M, Pani G. Neural stem cells and nutrients: Poised between quiescence and exhaustion. *Trends in Endocrinology and Metabolism*. 2016;27(11):756-69.

182. Kim Y, Kim S, Kim C, Sato T, Kojima M, Park S. Ghrelin is required for dietary restriction-induced enhancement of hippocampal neurogenesis: Lessons from ghrelin knockout mice. *Endocrine Journal*. 2015;62(3):269-75.

183. Kozomara A, Birgaoanu M, Griffiths-Jones S. Mirbase: From microrna sequences to function. *Nucleic Acids Research*. 2018;47(1):155-62.

184. Treiber T, Treiber N, Meister G. Regulation of microrna biogenesis and its crosstalk with other cellular pathways. *Nature Reviews. Molecular Cell Biology*. 2019;20(1):5-20.

185. Sarkar SN, Russell AE, Engler-Chiurazzi EB, Porter KN, Simpkins JW. Micrornas and the genetic nexus of brain aging, neuroinflammation, neurodegeneration, and brain trauma. *Aging and Disease*. 2019;10(2):329-52.

186. Reddy PH, Williams J, Smith F, Bhatti JS, Kumar S, Vijayan M, et al. Micrornas, aging, cellular senescence, and alzheimer's disease. *Progress in Molecular Biology and Translational Science*. 2017;146:127-71.

187. Smith-Vikos T, Slack FJ. Micrornas and their roles in aging. *Journal of Cell Science*. 2012;125(1):7-17.

188. Danka Mohammed CP, Park JS, Nam HG, Kim K. Micrornas in brain aging. *Mechanisms of Ageing and Development*. 2017;168:3-9.

189. Rege SD, Geetha T, Pondugula SR, Zizza CA, Wernette CM, Babu JR. Noncoding rnas in neurodegenerative diseases. *ISRN Neurology*. 2013;2013:375852.

190. Szafranski K, Abraham KJ, Mekhail K. Non-coding rna in neural function, disease, and aging. *Frontiers in Genetics*. 2015;6:87-94.

191. Earls LR, Westmoreland JJ, Zakharenko SS. Non-coding rna regulation of synaptic plasticity and memory: Implications for aging. *Ageing Research Reviews*. 2014;17:34-42.

192. Nampoothiri SS, Rajanikant GK. Decoding the ubiquitous role of micrornas in neurogenesis. *Molecular Neurobiology*. 2017;54(3):2003-11.

193. Bielefeld P, Mooney C, Henshall DC, Fitzsimons CP. Mirna-mediated regulation of adult hippocampal neurogenesis; implications for epilepsy. *Brain Plasticity (Amsterdam, Netherlands)*. 2017;3(1):43-59.

194. Shah MY, Ferrajoli A, Sood AK, Lopez-Berestein G, Calin GA. Microrna therapeutics in cancer - an emerging concept. *EBioMedicine*. 2016;12:34-42.

195. Goh JN, Loo SY, Datta A, Siveen KS, Yap WN, Cai W, et al. Micrornas in breast cancer: Regulatory roles governing the hallmarks of cancer. *Biological Reviews of the Cambridge Philosophical Society*. 2016;91(2):409-28.

196. Jansson MD, Lund AH. Microrna and cancer. *Molecular Oncology*. 2012;6(6):590-610.

197. Hemmatzadeh M, Mohammadi H, Jadidi-Niaragh F, Asghari F, Yousefi M. The role of oncomirs in the pathogenesis and treatment of breast cancer. *Biomedicine and Pharmacotherapy*. 2016;78:129-39.

198. Barbato S, Solaini G, Fabbri M. Micrornas in oncogenesis and tumor suppression. *International Review of Cell and Molecular Biology*. 2017;333:229-68.

199. Gambari R, Brognara E, Spandidos DA, Fabbri E. Targeting oncomirnas and mimicking tumor suppressor mirnas: Nuew trends in the development of mirna therapeutic strategies in oncology (review). *International Journal of Oncology*. 2016;49(1):5-32.

200. Svoronos AA, Engelman DM, Slack FJ. Oncomir or tumor suppressor? The duplicity of micrornas in cancer. *Cancer Research*. 2016;76(13):3666-70.

201. Zhu H, Dougherty U, Robinson V, Mustafi R, Pekow J, Kupfer S, et al. Egfr signals downregulate tumor suppressors mir-143 and mir-145 in western diet-promoted murine colon cancer: Role of g1 regulators. *Molecular Cancer Research*. 2011;9(7):960-75.

202. Lee EK, Jeong HO, Bang EJ, Kim CH, Mun JY, Noh S, et al. The involvement of serum exosomal mir-500-3p and mir-770-3p in aging: Modulation by calorie restriction. *Oncotarget*. 2018;9(5):5578-87.

203. Schneider A, Dhahbi JM, Atamna H, Clark JP, Colman RJ, Anderson RM. Caloric restriction impacts plasma micrornas in rhesus monkeys. *Aging Cell*. 2017;16(5):1200-3.

204. Green CD, Huang Y, Dou X, Yang L, Liu Y, Han JJ. Impact of dietary interventions on noncoding rna networks and mrnas encoding chromatin-related factors. *Cell Reports*. 2017;18(12):2957-68.

205. Victoria B, Dhahbi JM, Nunez Lopez YO, Spinel L, Atamna H, Spindler SR, et al. Circulating microrna signature of genotype-by-age interactions in the long-lived ames dwarf mouse. *Aging Cell*. 2015;14(6):1055-66.

206. Makwana K, Patel SA, Velingkaar N, Ebron JS, Shukla GC, Kondratov R. Aging and calorie restriction regulate the expression of mir-125a-5p and its target genes stat3, casp2 and stard13. *Aging*. 2017;9(7):1825-43.

207. Orom UA, Lim MK, Savage JE, Jin L, Saleh AD, Lisanti MP, et al. Microrna-203 regulates caveolin-1 in breast tissue during caloric restriction. *Cell Cycle*. 2012;11(7):1291-5.

208. Noyan H, El-Mounayri O, Isserlin R, Arab S, Momen A, Cheng HS, et al. Cardioprotective signature of short-term caloric restriction. *PloS One*. 2015;10(6):0130658.

209. Jin L, Lim M, Zhao S, Sano Y, Simone BA, Savage JE, et al. The metastatic potential of triple-negative breast cancer is decreased via caloric restriction-mediated reduction of the mir-17~92 cluster. *Breast Cancer Research and Treatment*. 2014;146(1):41-50.

210. Devlin KL, Sanford T, Harrison LM, LeBourgeois P, Lashinger LM, Mambo E, et al. Stage-specific micrornas and their role in the anticancer effects of calorie restriction in a rat model of er-positive luminal breast cancer. *PloS One*. 2016;11(7):0159686.

211. Olivo-Marston SE, Hursting SD, Perkins SN, Schetter A, Khan M, Croce C, et al. Effects of calorie restriction and diet-induced obesity on murine colon carcinogenesis, growth and inflammatory factors, and microrna expression. *PLoS One*. 2014;9(4):96765.

212. Mori MA, Raghavan P, Thomou T, Boucher J, Robida-Stubbs S, Macotela Y, et al. Role of microrna processing in adipose tissue in stress defense and longevity. *Cell Metabolism*. 2012;16(3):336-47.

213. Dhahbi JM, Spindler SR, Atamna H, Yamakawa A, Guerrero N, Boffelli D, et al. Deep sequencing identifies circulating mouse mirnas that are functionally implicated in manifestations of aging and responsive to calorie restriction. *Aging*. 2013;5(2):130-41.

214. Csiszar A, Gautam T, Sosnowska D, Tarantini S, Banki E, Tucsek Z, et al. Caloric restriction confers persistent anti-oxidative, pro-angiogenic, and anti-inflammatory effects and promotes anti-aging mirna expression profile in cerebromicrovascular endothelial cells of aged rats. *American Journal of Physiology Heart and Circulatory Physiology*. 2014;307(3):292-306.

215. Mercken EM, Majounie E, Ding J, Guo R, Kim J, Bernier M, et al. Age-associated mirna alterations in skeletal muscle from rhesus monkeys reversed by caloric restriction. *Aging*. 2013;5(9):692-703.

216. Matsui Y, Halter SA, Holt JT, Hogan BL, Coffey RJ. Development of mammary hyperplasia and neoplasia in mmtv-tgf alpha transgenic mice. *Cell*. 1990;61(6):1147-55.

217. Lundy J, Schuss A, Stanick D, McCormack ES, Kramer S, Sorvillo JM. Expression of neu protein, epidermal growth factor receptor, and transforming growth factor alpha in breast cancer. Correlation with clinicopathologic parameters. *American Journal of Pathology*. 1991;138(6):1527-34.

218. Murray PA, Barrett-Lee P, Travers M, Luqmani Y, Powles T, Coombes RC. The prognostic significance of transforming growth factors in human breast cancer. *British Journal of Cancer*. 1993;67(6):1408-12.

219. Ru Y, Kechris KJ, Tabakoff B, Hoffman P, Radcliffe RA, Bowler R, et al. The multimir r package and database: Integration of microrna-target interactions along with their disease and drug associations. *Nucleic Acids Research*. 2014;42(17):133-40.

220. Paraskevopoulou MD, Georgakilas G, Kostoulas N, Vlachos IS, Vergoulis T, Reczko M, et al. Diana-microt web server v5.0: Service integration into mirna functional analysis workflows. *Nucleic Acids Res*. 2013;41:169-73.

221. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microrna target sites in mammalian mrnas. *Elife*. 2015;4.

222. Liu W, Wang X. Prediction of functional microrna targets by integrative modeling of microrna binding and target expression data. *Genome Biology*. 2019;20(1):18-25.

223. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Research*. 2003;13(11):2498-504.

224. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, et al. Cluego: A cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics*. 2009;25(8):1091-3.

225. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using david bioinformatics resources. *Nature Protocols*. 2009;4(1):44-57.

226. Morpheus: Bioinformatics tool [cited 2019 24 April]. Available from: https://software.broadinstitute.org/morpheus/.

227. Oliveros JC. Venny. An interactive tool for comparing lists with venn's diagrams. 2007 [cited 2019 24 April]. Available from: http://bioinfogp.cnb.csic.es/tools/venny/.

228. Busk PK. A tool for design of primers for microrna-specific quantitative rt-qpcr. *BMC Bioinformatics*. 2014;15:29-38.

229. Inukai S, de Lencastre A, Turner M, Slack F. Novel micrornas differentially expressed during aging in the mouse brain. *PloS One*. 2012;7(7).

230. Micheli F, Palermo R, Talora C, Ferretti E, Vacca A, Napolitano M. Regulation of proapoptotic proteins bak1 and p53 by mir-125b in an experimental model of alzheimer's disease: Protective role of 17beta-estradiol. *Neuroscience Letters*. 2016;629:234-40.

231. Vinuesa A, Bentivegna M, Calfa G, Filipello F, Pomilio C, Bonaventura MM, et al. Early exposure to a high-fat diet impacts on hippocampal plasticity: Implication of microglia-derived exosome-like extracellular vesicles. *Molecular Neurobiology*. 2019;56(7):5075-94.

232. Barger JL, Vann JM, Cray NL, Pugh TD, Mastaloudis A, Hester SN, et al. Identification of tissue-specific transcriptional markers of caloric restriction in the mouse and their use to evaluate caloric restriction mimetics. *Aging Cell*. 2017;16(4):750-60.

233. Wahl D, Solon-Biet SM, Wang QP, Wali JA, Pulpitel T, Clark X, et al. Comparing the effects of low-protein and high-carbohydrate diets and caloric restriction on brain aging in mice. *Cell Reports*. 2018;25(8):2234-43.

234. Schafer MJ, Dolgalev I, Alldred MJ, Heguy A, Ginsberg SD. Calorie restriction suppresses age-dependent hippocampal transcriptional signatures. *PloS One*. 2015;10(7).

235. He Y, She H, Zhang T, Xu H, Cheng L, Yepes M, et al. P38 mapk inhibits autophagy and promotes microglial inflammatory responses by phosphorylating ulk1. *The Journal of Cell Biology*. 2018;217(1):315-28.

236. Caltagarone J, Jing Z, Bowser R. Focal adhesions regulate abeta signaling and cell death in alzheimer's disease. *Biochimica et Biophysica Acta*. 2007;1772(4):438-45.

237. Schulz C, Paulus K, Lehnert H. Adipocyte-brain: Crosstalk. *Results and Problems in Cell Differentiation*. 2010;52:189-201.

238. Hsieh C-H, Rau C-S, Wu S-C, Yang JC-S, Wu Y-C, Lu T-H, et al. Weight-reduction through a low-fat diet causes differential expression of circulating micrornas in obese c57bl/6 mice. *BMC Genomics*. 2015;16(1).

239. Koch AJ, Holaska JM. Loss of emerin alters myogenic signaling and mirna expression in mouse myogenic progenitors. *PloS One*. 2012;7(5).

240. Xu S, Zhang R, Niu J, Cui D, Xie B, Zhang B, et al. Oxidative stress mediated-alterations of the microrna expression profile in mouse hippocampal neurons. *International Journal of Molecular Sciences*. 2012;13(12):16945-60.

APPENDIX A: ETHICAL APPROVAL FORM

YEDİTEPE ÜNİVERSİTESİ

T.C. YEDİTEPE ÜNİVERSİTESİ, DENEY HAYVANLARI ETİK KURULU
(YÜDHEK)

ETİK KURUL KARARI

Toplantı Tarihi	Karar No	İlgi	Proje Yürütücüsü
27.03.2014	390	21.03.2014 Tarihli Yazı	Yrd.Doç.Dr. Soner DOĞAN

"MMTV-TGF- Farelerinde Meme Tümörü Gelişiminin Aralıklı Kalori Kısıtlaması ile Önlenmesinde Adipokinlerin Epigenetik Modifikasyonunun ve mikroRNA'ların Rollerinin Belirlenmesi." adlı bilimsel çalışma etik kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna oy birliğiyle karar verilmiştir.

Etik Onay Geçerlilik Süresi: 3 Yıl

GÖREVİ	ADI SOYADI	İMZA
Başkan	Prof. Dr. M. Ece GENÇ	
Başkan Yardımcısı	Prof. Dr. Erdem YEŞİLADA	
Raportör	Prof. Dr. Işıl Aksan KURNAZ	
Üye	Prof. Dr. Bayram YILMAZ	KATILMADI
Üye	Prof. Dr. Başar ATALAY	KATILMADI
Üye	Yard.Doç.Dr.Soner DOĞAN	KATILMADI
Üye	Yard. Doç. Dr. Ediz DENİZ	
Üye	Doç. Dr. C. Narter YEŞİLDAĞLAR	KATILMADI
Üye	Sumru KİRAZCI	