MOLECULAR INSIGHTS INTO THE EFFECTS OF CANCER AND CHEMOTHERAPY ON THE BRAIN

Anna Kovalchuk
Bachelor of Science, University of Lethbridge, 2014
Master of Science, University of Lethbridge, 2015

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Anna Kovalchuk

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Dr. Bryan Kolb Supervisor	Professor	Ph.D.
Dr. Robbin Gibb Co-Supervisor	Assoc. Professor	Ph.D.
Dr. Aru Narendran Thesis Examination Committee Member	Professor	M.D./Ph.D.
Dr. James Thomas Thesis Examination Committee Member	Professor	Ph.D.
Dr. Claudia Gonzalez Internal External Examiner	Assoc. Professor	Ph.D.
Dr. Quentin J Pittman External Examiner	Professor	Ph.D.
Dr. David Euston Chair, Thesis Examination Committee Member	Assoc. Professor	Ph.D.

ABSTRACT

An array of central nervous system complications, neurological deficits, and cognitive impairments come about and persist as a result of cancer and cancer treatments. These conditions are known as 'tumor brain' and 'chemo brain', respectively, and affect many cancer survivors. We proposed that the mechanisms behind tumor and chemo brain were epigenetic and associated with aberrant global gene expression and metabolome deregulation. We used mouse TumorGraft models of breast, lung, and pancreatic cancer, and sarcoma to profile the epigenetic, transcriptome, small RNAome, and metabolome changes in chemo-treated tumor-bearing mice, as compared to untreated tumor-bearing mice, and to control mice. We noted that tumor presence alone caused oxidative stress and affected global gene and small RNA expression, DNA methylation, and metabolic activity in the brain. Chemotherapy affected similar domains, at times seeming to build on tumor effects. The observed molecular changes strongly resembled those associated with neurodegenerative diseases and brain aging.

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LIST OF ABBREVIATIONS

4-HNE - 4-hydroxynonenal

5hmC – 5-hydroxymethylcytosine

5mC – 5-methyl-cytosine

8-Oxo-2'-deoxyguanosine

ANOVA - analysis of variance

APE1 - apurinic/apyrimidinic endonuclease

BDNF - brain-derived neurotrophic factor

BDNF - Brain-derived neurotrophic factor

BER – base excision repair

CNS – central nervous system

CARASIL - cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy

CFI-SAGES – Canada Foundation for Innovation – Southern Alberta Group for Epigenetics Studies

CMF - Cyclophosphamide-Methotrexate-Fluorouracil

CNS – central nervous system

CPP - cyclophosphomade

CRIZ - Crizotinib

DI-MS - direct flow injection/mass spectrometry

DNMT – DNA methyltransferase

ECL – enhanced chemiluminescence

DPC - Doxorubicin/Paclitaxel/Cyclophosphamide

ECM - extracellular matrix

EGF - epidermal growth factor

FDR - false discovery rates

GABA - gamma-aminobutyric acid

Gabrb1 - gamma-aminobutyric acid receptor subunit beta-1 gene

γH2AX – phosphorylated histone H2AX

Htra1 - high temperature requirement of a serine peptidase 1 gene

IP intra-peritoneal

IV intra-venous

MAPK - Mitogen-activated protein kinase

MBD – methyl-binding domain

MDS - multidimensional scaling

MeCP2 – methyl CpG binding protein 2

miRNA/miR – microRNA

mRNA – messenger RNA

MSEA - metabolite set enrichment analysis

MMC - mitomycin C

NPAS4 – neuronal PAS domain protein 4

Npr3 - natriuretic peptide receptor 3

NSCLC - non-small cell lung carcinoma

OGG1 - 8-Oxoguanine glycosylase

PAGE – polyacrylamide gel electrophoresis

PCR – polymerase chain reaction

PI3K – phosphatidylinositide 3-kinases

PO – per os (oral route)

PCA - principle component analysis

Pcsk1 - proprotein convertase subtilisin/kexin type 1

PDX – patient derived xenograft

PFC - prefrontal cortex

PI3K - phosphatidylinositol-3 kinase

Plekhf1 - Pleckstrin Homology and FYVE Domain Containing 1 gene

PR+BC - progesterone receptor positive breast cancer

RIN – RNA integrity number

RT-PCR – reverse transcription PCR

SEM – standard error of the mean

SMPDB - The Small Molecule Pathway Database

Srebf2 - the sterol regulatory element binding transcription factor 2 genes

TET - Ten-eleven translocation methylcytosine dioxygenase

TNBC - triple negative breast cancer

TOP - Topotecan

CHAPTER 1: INTRODUCTION

Malignant tumors – Facts and figures

Malignancies encompass a wide array of diseases where abnormal cells divide and grow in an uncontrolled fashion, invade nearby tissues, and further spread to distant tissues and organs through the blood and lymphatic systems. Malignant tumors can arise from different cell types. Malignancies of epithelial origin are generally referred to as cancers. Central nervous system malignancies involve the brain and spinal cord. Soft tissue tumors, known as sarcomas, arise from mesenchymal cells of the connective and support tissues, such as from cartilage, bone, and muscle. Leukemia is a blood malignancy originating from the bone marrow, and lymphoma and multiple myeloma are malignant immune system tumors. In common, as well as research terms, malignant tumors are often referred to as cancers.

Cancer is not a single disease, but rather a broad term applied to a variety of diseases involving the loss of cellular differentiation and abnormal growth of cells of epithelial origin, which have the potential to spread and invade other organs and body parts. Cancer has a multifactorial etiology, whereby genetic, environmental, medical, and lifestyle factors interact to give rise to malignancy. Cancer cells are different from normal cells and their phenotypes exhibit three notable characteristics: (i) immortalization, or indefinite proliferative life span; (ii) lack of response to conventional regulators of cell growth; and (iii) metastasis, the capacity to leave a tumor and invade distal tissues and organs. Phenotypically, malignant tumors have irregular edges and a high growth rate. Their cells have abnormal nuclei, and they invade adjacent tissue and metastasize locally and distally. All cancer cells are clonal–descendants of a single malignant cell; however,

they evolve overtime, acquiring a quicker and more aggressive growth form. This happens through a series of stepwise changes, each representing a new clonal population.

Despite the efforts of the research and healthcare communities, cancer remains a growing health concern. It continues to be one of the leading causes of death worldwide, claiming 8.8 million lives in 2015 alone. Currently, 1 out of every 6 deaths in the world is attributable to cancer.

The number of cancer cases continues to rise. According to the World Health Organization, there were approximately 14 million new cancer cases in 2012, and the global incidence of cancer is projected to increase by around 70% in the upcoming decades. According to the estimates of the American Cancer Society, by 2030, the world will see up to 21.7 million new cancer cases and 13 million cancer deaths (https://www.cancer.org/research/cancer-facts-statistics/global.html). Undeniably, the global economic impact of cancer is a growing burden; the economic cost of cancer in 2010 alone was estimated at approximately 1.16 trillion US dollars (http://www.who.int/mediacentre/factsheets/fs297/en/).

As in all developed countries, cancer is a serious public health issue in Canada and is responsible for 30.2% of all deaths nationwide. According to predictions of the Canadian Cancer Society, 1 in 2 Canadians will develop cancer in their lifetimes, and about 1 in 4 Canadians will succumb to it. It is estimated that 206,200 new cancer cases and 80,800 cancer deaths will occur 2017. Canadian males have a 45% lifetime probability and females have a 42% lifetime probability of developing cancer. Combined, four types of cancer—prostate, breast, lung, and colorectal—account for half of all diagnosed cases in Canada in 2016 (CCS, 2017).

In Alberta, nearly 1 in 2 people will develop cancer in the lifetime, and 1 in 4 will die of it (Shack, 2015). The latest Alberta Health Services (AHS) report predicts that 19,865 new cases and 6,653 cancer-related deaths will occur in Alberta in 2017. The most common cancers in Alberta include breast, lung, prostate, colorectal, and childhood cancer. Together, these make up 52% of all malignancies.

Breast cancer

Breast cancer continues to be the most prevalent malignancy in women worldwide. In Alberta, 1 in 8 females are expected to develop breast cancer, and 1 in 35 are projected to die from the disease. Breast cancer encompasses four key subtypes that are characterized by patterns of gene expression and regulation (Schnitt, 2010). These are:

1) **Luminal A** breast cancer: This cancer type entails the development of hormone-receptor positive (estrogen-receptor [ER] and/or progesterone-receptor [PR] positive) and HER2 receptor negative tumors. Luminal A tumors have low levels of Ki-67 protein, a marker of cellular proliferation that is detected only in cells undergoing mitotic cell division. These tumors are usually low-grade (tumor grade being a degree of cellular differentiation) and well differentiated; they grow slowly relative to other cancer types and therefore have the best prognosis of all other breast tumor types.

2) **Luminal B** breast cancer is also hormone-receptor positive (ER+ and/or PR+), and either HER2 positive or HER2 negative, but with elevated Ki-67 levels. Luminal B cancers have a faster growth rate and a slightly worse prognosis than Luminal A tumors.

3) **HER2-positive** breast cancer is characterized by increased levels of HER2 (ERBB2, *human epidermal growth factor receptor 2)* receptor. HER2+ tumors are

hormone-receptor negative (ER- and PR-). They grow much faster than Luminal A and B tumors and have a worse prognosis.

4) **Triple-negative/basal** breast cancer is both hormone-receptor and HER2 negative. It is most common in women with *BRCA1* gene mutations. It has the worst prognosis and is the most difficult type to treat.

Other cancer types

Lung cancer

Even though lung cancer rates started to level off and even decline since the mid-1980s, due in part to the rise of smoking cessation programs, it is still a leading cause of cancer-related death in Canada. In 2016, 28400 Canadians were predicted to contract lung cancer, and 20,800 were predicted to die from it (Canadian Cancer Society, 2017).

In Alberta, almost 1 in 13 males and 1 in 14 females will come down with invasive lung cancer in their lifetime, and close to 2,350 lung cancer cases are predicted to be diagnosed in 2017. What's more, the likelihood of getting lung cancer increases with age. About 85 percent of lung cancers are consequences of tobacco smoking, while 10–15% of cases happen in people who have never smoked (Shack L., 2015).

In 2015, lung cancer claimed 1.69 million deaths worldwide (Organization, 2017). The five-year survival for lung cancer in Alberta is approximately 17% (Shack L., 2015). Lung cancer is also a main contributor to the potential years of life lost (PYLL), whereby lung cancer is responsible for 25% of all cancer PYLL (Canadian Cancer Society, 2014). The two main types are small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC).

Pancreatic cancer

Pancreatic cancer is one of the deadliest cancer malignancies; it has a dismal prognosis, particularly in the case of a late-stage diagnosis. Each year approximately 4,800 Canadians are diagnosed with pancreatic cancer. Of those diagnosed, 75 % do not survive past a one year mark. In 2016, approximately 5,200 Canadians (2600 males and 2600 females) were predicted to get diagnosed with pancreatic cancer, and 4800 patients were predicted to die from it (Canadian Cancer Society, 2016a). In Alberta, the five-year survival ratio for pancreatic cancer is about 7% (Hatcher, 2012).

Sarcoma

Adult soft tissue sarcomas are rare and heterogeneous malignant tumors that arise from mesenchymal cells of connective and support tissues. They frequently develop in the extremities and in or around organs (Canadian Cancer Society, 2016b). The majority of sarcomas (80%) are derived from soft tissues, and 20% have bone tissue origin. Sarcomas readily metastasize and are very difficult to treat. The median survival of patients with sarcoma metastases is about 11-15 months (Services, 2017).

Cancer chemotherapy

Recent increases in cancer survival rates are largely attributable to early diagnostics and to the development of new, effective cancer therapy regimens. Proper cancer therapy regimens lead to high cure and survival rates and to a near normal life expectancy in early-stage cancer patients (Etzioni et al., 2003, Etzioni et al., 2013).

Chemotherapy is one of the main types of cancer treatment. It is based on the application of drugs that eradicate cancer cells (Hassan et al., 2010). In most cases,

chemotherapy is systemic, administered intravenously through the bloodstream to cells all over the body, thus affecting not only cancer cells, but also healthy cells.

Chemotherapy treatments can be classified as (i) neoadjuvant (pre-operative) chemotherapy, which is applied to reduce the size of a tumor prior to radiation therapy or surgical resection (Thompson and Moulder-Thompson, 2012); (ii) adjuvant therapy, in which chemotherapeutic agents are applied to eradicate residual cancer after other treatments (Anampa et al., 2015); and (iii) combination therapy, in which chemotherapeutic agents are applied to eradicate cancer either by itself or in conjunction with other therapies (Hassan et al., 2010). Depending on the patient's cancer type, stage, grade, underlying conditions, and other treatments used, chemotherapy can be applied to either cure or control cancer. It can also be used in a palliative setting to reduce tumor burden, lighten tumor pressure on organs, and thereby ease pain (DeVita et al., 2005).

Depending on the types of agents used, chemotherapy is classified as cytotoxic (non-targeted) or targeted. Targeted chemotherapy is designed to act upon certain molecules and pathways in tumor cells. Cytotoxic, non-targeted chemotherapy agents enter the blood stream and, on reaching cancer cells, hinder their ability to grow and divide, and induce apoptosis. Along with cancer cells, cytotoxic chemotherapy agents affect all dividing cells in the body (DeVita et al., 2005).

The most common cytotoxic chemotherapy agents include alkylating agents, anticancer anthracycline antibiotics, mitotic spindle inhibitors, antimetabolites, and topoisomerase inhibitors (American Cancer Society, 2015).

Alkylating agents attach a small alkyl group (CnH2n+1) to the seventh nitrogen atom of the guanine (N-7) base in DNA. Monoalkylating agents react only with one N-7

of guanine, whereas dialkylating agents can react with two different N-7 guanine residues on different DNA strands, thus giving rise to crosslinks. The most common alkylating agents include nitrogen mustards (cyclophosphamide, mustargen, melphalan, ifosfamide), nitrosoureas (carmustine, lomustine), and alkyl sulfonates (busulfan). Some of these agents (e.g., cyclophosphamide) must undergo activation to form an active substance and cause alkylation. DNA alkylation modifications prevent DNA replication and activate cell death (American Cancer Society, 2015).

One of the most common and successful anticancer agents, cyclophosphamide, is often applied in combination chemotherapy regimens for solid tumors, including breast cancer. Upon entering the organism and undergoing activation in the liver, it attaches the alkyl group to N-7 of guanine on DNA, thus causing the formation of intra- and inter-strand DNA crosslinks that block DNA replication (Emadi et al., 2009, American Cancer Society, 2015).

Procarbazine is another effective alkylating agent used to treat various forms of cancer, including breast cancer. Procarbazine's precise mode of cytotoxic action has not been clearly defined. However, there is evidence that the drug may act by inhibiting protein, RNA, and DNA synthesis. Autoxidation of procarbazine leads to the production of hydrogen peroxide and oxidative stress (https://www.drugbank.ca/drugs/DB01168).

Anthracyclines were originally derived from *Streptomyces* in the 1960s, and they are currently some of the most broadly used and effective antineoplastic agents (Weiss, 1992, Minotti et al., 2004). Of the anthracyclines, doxorubicin (or adriamycin) is a widely used chemotherapeutic agent with a broad activity spectrum. It is normally prescribed as part of combination therapy and is one of the most effective agents for treating breast,

lung, and ovarian cancer, and other solid tumors. Doxorubicin is frequently used in adjuvant and palliative breast cancer regimens (Dean-Colomb and Esteva, 2008).

Doxorubicin's mechanism of cytotoxic activity is based on its intercalating ability whereby it inserts itself between the DNA bases in GC-rich sequences. This causes interference with functions of topoisomerase 2α enzyme (Top2A), which is important for unwinding supercoiled DNA during transcription and DNA replication. When doxorubicin intercalates DNA and inhibits the progression of Top2A, it stabilizes the Top2A complex after the complex breaks DNA. Doing so prevents DNA from being re-sealed and leads to apoptosis induction. Doxorubicin also causes oxidative stress, which, in conjunction with the doxorubicin-induced inhibition of Top2A, causes DNA breaks (Fortune and Osheroff, 2000), (ACS, 2015).

Along with doxorubicin, there are several other types of **topoisomerase inhibitors**, which block the function of both topoisomerase type-I and type-II enzymes and thus affect cell division. Topoisomerase I inhibitors include camptothecin, whereas topoisomerase II inhibitors include doxorubicin (described above), as well as etoposides and mitoxantrone. These agents have been extensively used to destroy rapidly dividing cancers (American Cancer Society, 2015).

Mitotic spindle inhibitors include vinca alkaloids, originally derived from the Madagascar periwinkle plant, vinblastine and vincristine; and taxanes (paclitaxel), derived from Pacific yew. Vinca alkaloids and taxanes bind tubulin and inhibit the formation of spindle microtubules, thereby interfering with cell division and causing cell death (Wali et al., 2017), (American Cancer Society, 2015).

Antimetabolite chemotherapy agents—which include pyrimidine and purine analogues, as well as folic acid analogues—were among the first effective antineoplastic agents to be discovered. These molecules have structures that are highly similar to the bases of nucleic acids; ergo, they interfere with DNA and RNA synthesis. One of the most widely used of these agents, 5-flurouracil (5-FU), has a long history of successful application for the treatment many cancers, including breast, head and neck, adrenal, pancreatic, gastric, colon, rectal, esophageal, liver, bladder, and prostate (American Cancer Society, 2015).

Recent advances in molecular pharmacology have led to the development of many **targeted therapies**, including tyrosine kinase inhibitors (TKIs), the chemotherapy agents that inhibit protein tyrosine kinases (PTKs). TKIs were developed based on the analysis and mechanisms of cell signalling pathways and on the cell cycle regulation processes commonly affected in cancers. TKIs interact with and block various PTKs. As such, they constitute the first class of targeted chemotherapy drugs (Arora and Scholar, 2005).

The latest research innovations have led to the development of anti-neoplastic agents that can target molecules involved in cancer progression and can be customized to individual patient's tumors. These **new targeted therapies** include monoclonal antibodies (mabs), small molecule inhibitors (mibs), and immunotoxins (American Cancer Society, 2015).

Of these, Herceptin (trastuzumab) is a monoclonal antibody targetting HER2 that revolutionized the treatment of HER2 positive breast cancer (Dent et al., 2009). Multi-kinase inhibitor crizotinib (Wali et al., 2017) is one of the newer anti-cancer agents acting as an anaplastic lymphoma kinase (ALK) and c-ros oncogene 1 (ROS1) inhibitor.

Crizotinib also inhibits the c-Met/Hepatocyte growth factor receptor (HGFR) tyrosine kinase, which is involved in the development and growth of many cancers, including breast cancer (Cozzo et al., 2016, Jackson and Chester, 2015).

Cancer survivorship

While cancer incidence continues to rise, cancer is becoming less of a death sentence and more of a chronic condition. Although a cure is still not possible, many cancers can be controlled and managed for long periods of time owing to significant advances in the development of novel therapeutic modalities and improvements in cancer care. In Alberta, a recent report by Alberta Health Services highlights the fact that cancer mortality has decreased in the province. The five-year relative survival rate for breast cancer has improved from 74.5% in 1975 to 89% in 2016, and the 10- and 15-year survival rates for breast cancer have reached 83% and 78%, respectively (DeSantis et al., 2014, Miller et al., 2016).

Currently, many people live with and beyond cancer, bringing the problem of cancer survivorship to the forefront of healthcare. Cancer survivorship encompasses an array of physical, psychosocial, and economic issues, and focuses on efforts to increase the quality of life of cancer survivors.

Side effects of cancer chemotherapy

Chemotherapy agents are designed to target rapidly dividing cells, and affect both tumor cells and healthy cells. As a result, they can cause numerous debilitating side effects. Cycling and dividing cells are found in hair follicles, skin and mucous membranes, bone marrow, and the gastrointestinal tract. Consequently, cytotoxic chemotherapy causes skin and mucous membrane lesions and sores, hair loss,

dysfunction of the bone marrow, and gastro-intestinal syndromes (Group, 2002, Raji, 2005, Kayl and Meyers, 2006).

Neurotoxic chemotherapy side effects – chemo brain

Whereas traditional side effects of chemotherapy, such as hair loss, nausea, vomiting and other gastro-intestinal manifestations, bleeding, and low white blood cell counts, have been known for some time, concerns about central nervous system (CNS) toxicity-associated side effects were first described in the mid-1970s (Silberfarb, 1983), when Weiss and colleagues reported the neurotoxicity of cancer therapies (Weiss et al., 1974b, Weiss et al., 1974a).

Later, Silberfarb and colleagues outlined cognitive impairment in post-chemotherapy cancer patients (Silberfarb et al., 1980a, Silberfarb, 1983) and proposed "*chemotherapy as a possible source of behavioral change and emotional distress in cancer patients*" (Silberfarb et al., 1980b). These studies, coupled with patient concerns, gave rise to the analysis of the CNS toxicity of chemotherapy regimens. From the patients' perspective, fatigue and cognitive dysfunction constitute a significant quality-of-life issue that persists over time.

These initial studies, as well as the majority of all studies on the CNS effects of chemotherapy, were done in breast cancer cohorts. To draw attention to the cognitive symptoms they experienced, breast cancer survivors coined the term "chemo brain," which is now widely used to describe post-chemotherapy CNS side effects. In self reports of chemo brain, women described a decline in their short-term memory, concentration, speech and word-finding ability, mental fluency, and information processing, often saying that these impairments affected every aspect of their lives (Downie et al., 2006).

From the 1990s and onwards, a number of studies analyzed the neurocognitive side effects of chemotherapy and uncovered changes in motor skills, cognition, attention and concentration, verbal and visual working memory, and processing speed (reviewed in (Vardy and Tannock, 2007, Ahles and Saykin, 2007, Ahles, 2012). By now, in breast cancer alone, more than 60 studies have analyzed and found various extents of association between chemotherapy and cognitive impairments (Wefel and Schagen, 2012). A study by Wieneke and Dienst showed that 75% of breast cancer survivors treated with cyclophosphamide, methotrexate, and 5-FU (CMF regimen) suffered cognitive impairment, with concentration and memory as the main domains affected. Similarly, Brezden and colleagues confirmed substantial cognitive impairment in breast cancer chemotherapy patients post-CMF treatment (Brezden et al., 2000). At present, 21 large-scale breast cancer population studies reported significant evidence of post-treatment cognitive changes (reviewed in (Ahles, 2012)). On average, reports show cognitive impairment in 15%–25% of subjects (Ahles et al., 2012), although some analyses revealed percentages as high as 61% (Wefel et al., 2010) and 75% (Wieneke and Dienst, 1995).

Chemo brain manifestations can be persistent, with approximately 35% of patients reporting CNS side effects for months to years after treatment cessation. Ahles and colleagues analyzed the data of breast cancer and lymphoma survivors, and reported that chemotherapy-induced CNS side effects persisted for five years in 39% of patients who received chemotherapy (Ahles and Saykin, 2002). A multicentre, prospective longitudinal study found that breast cancer patients reported substantially more cognitive impairments up to 6 months after treatment, compared with age-matched non-cancer control subjects

(Janelsins et al., 2016). Furthermore, reports by the International Cognitive Workshop suggest that cancer treatment-related cognitive side effects can last for as long as 5 to 10 years after treatment completion (Mitchell and Turton, 2011b, Vardy et al., 2008a).

Aside from its incidence after breast cancer treatment, chemo brain has been reported to manifest after chemotherapy in lung, lymphoma, and gastrointestinal cancers, among others (reviewed in (Olson et al., 2016, Vardy and Tannock, 2007, Ahles, 2012)). Given all reports and studies outlining its occurrence, chemo brain is now accepted as an adverse side effect of chemotherapy.

In spite of this, it remains unclear which cognitive domains are most influenced and susceptible to chemotherapy's effects. This may be due to the multifactorial nature of the neuropsychological analyses and tests used in clinical studies (O'Farrell et al., 2013). O'Farrel and colleagues confirmed four key cognitive factors that were altered in chemo brain cases; these included working memory, visual and verbal memory, and processing speed. This finding coincided well with patients' self-reporting (O'Farrell et al., 2013). However, other studies showed only a weak correlation between self-reported cognitive impairments and performance in neurocognitive tasks (Castellon and Ganz, 2009). This dichotomy suggests that tests for neurocognitive tasks may not always be reflective of patients' routine performance in their day-to-day lives. To show better correlations, an in-depth strategy that integrates self-reporting, neurocognitive testing, and mechanistic analyses of the molecular and cellular bases of the observed changes is needed. Moreover, the initial studies should dissect the molecular mechanisms of chemo brain, as later those can be used to guide neuroanatomical, neurocognitive,and behavioral analyses.

Cancer-associated cognitive change – the tumor brain

Recent longitudinal studies suggest that a degree of cognitive impairment may be present in some patients prior to receiving chemotherapy, and that this impairment worsens with chemotherapy (Vardy and Tannock, 2007). Furthermore, a thorough pre-treatment assessment of patients revealed that, compared with their age groups, 20%–30% of breast cancer patients exhibited decreased cognitive performance prior to treatments, and such manifestations were not linked to depression, anxiety, or fatigue (Ahles et al., 2008, Wefel et al., 2004). Later, Hurria and colleagues proposed the definition of "*cancer and cancer treatment-associated cognitive change*" (Hurria et al., 2007a), or, as we recently termed it, 'tumor brain'. The underlying molecular mechanisms of tumor brain remain unexplored.

Mechanism of chemo brain and tumor brain

An increasing amount of data has shown that chemotherapy imposes toxic effects on the CNS (Kaiser et al., 2014). As discovered in animal studies, chemotherapy exposure triggers oxidative stress and apoptosis, activates microglia, causes neuroinflammation and dysregulates myelination, suppresses neuronal proliferation and differentiation, affects levels of DNA methylation and hydroxymethylation, and alters histone modification and chromatin remodeling, causing aberrant gene expression of neurotrophic and neurogenic proteins in the brain (Christie et al., 2012, Seigers and Fardell, 2011, Seigers et al., 2015, Seigers et al., 2016, Seigers et al., 2010a, Seigers et al., 2008, Seigers et al., 2013, Seigers et al., 2010b, Briones and Woods, 2014, Kovalchuk et al., 2016c). These molecular changes account for the alterations in neurogenesis and exert a negative impact on memory and learning (Christie et al., 2012).

The frequency and timing of chemo brain and its persistence suggest that the origins of chemo brain may be epigenetic and associated with aberrant global gene expression patterns (Wang et al., 2015b).

One of our recent studies confirmed this notion. We analyzed epigenetic and gene expression changes in the hippocampus and prefrontal cortex of mice 24 h and three weeks after treatment with cytotoxic chemotherapy agents mitomycin C (MMC) and cyclophosphomade (CPP), which were previously reported to cause chemo brain. We found that MMC and CPP treatments led to persistent drug-, sex-, and brain region-specific changes in global gene expression profiles. We also observed that these chemotherapy agents caused changes in the global levels of DNA methylation and DNA hydroxymethylation, and led to increased levels of oxidative DNA damage (Kovalchuk et al., 2016c).

Unlike chemo brain, the mechanisms of tumor brain have not been analyzed at all. In human studies, Ahles and Saykin suggested the possible roles of cancer-induced inflammatory response, as well as the potential contribution of genetic factors that underlie breast cancer formation, as potential mechanisms of tumor brain, albeit none of these mechanisms have been experimentally validated (Ahles and Saykin, 2007, Ahles, 2012). Nevertheless, based on the timing, frequency, and persistence of the cancer-associated cognitive impairment of tumor brain, it may well be associated with epigenetic and gene expression changes. An in-depth analysis of epigenetic mechanisms of tumor brain and chemo brain is essential for understanding this neurological complication, and for the development of robust diagnostic and treatment strategies.

Epigenetics

Brain development is genetically predetermined; it is a stepwise and highly coordinated program that is executed via precise gene activity. While each cell of an organism carries the same amount and sequence of DNA, cells and tissues vary greatly in their structure and function. These structural and functional differences result from differential gene expression in various cell types. Gene expression underlies and determines organismal development, growth, functioning, and aging, as well as environmental interactions.

Epigenetic mechanisms set and maintain meiotically and mitotically heritable and stable patterns of gene expression and regulation. These occur without changing the DNA sequence. Epigenetic regulation controls gene expression, chromatin structure, and genome functioning through processes that include DNA methylation, histone modifications, chromatin remodeling, and noncoding RNAs (Sandoval and Esteller, 2012, Jaenisch and Bird, 2003).

DNA methylation

Cytosine DNA methylation (or DNA methylation) is the covalent modification of DNA, whereby a methyl group (CH_3-) from S-adenosyl-L-methionine is added to the carbon 5 position of cytosine, yielding 5-methylcytosine (5mC). DNA methylation was the first epigenetic alteration identified. This process is crucial for the proper functioning of normal cells and tissues. In normal cells, DNA methylation governs the regulation of cell-type and tissue-specific gene expression, the silencing of parasitic and highly repetitive sequences, X-chromosome inactivation, the correct organization of active and

inactive chromatin, and genomic imprinting (Esteller and Herman, 2002, Jaenisch and Bird, 2003) .

In mammalian somatic cells, including brain cells, DNA methylation takes place mainly in the context of CpG dinucleotides (where a cytosine is followed by a guanine), which occur throughout the genome and are highly methylated to 70%-90% (Pogribny and Beland, 2012). These CpGs lie outside regulatory regions. In contrast, some CpGs are grouped in clusters called "CpG islands". They are located within the regulatory promoter regions of many genes and are usually unmethylated (Esteller and Herman, 2002, Pogribny and Beland, 2012). Promoter regions may gain or lose DNA methylation and thus become hyper- or hypo-methylated, leading to decreased or increased gene expression. While CpG methylation is central to the proper functioning of normal cells and tissues, non-CpG methylation is rare in mammals and has only been found in embryonic stem cells.

DNA methylation is carried out by DNA methyltransferase enzymes (DNMT1, DNMT3a, and DNMT3b), which work together to create and maintain methylation patterns. DNMT1 is involved in the maintenance of DNA methylation patterns after DNA replication, while DNMT3a and DNMT3b are *de novo* methyltransferases that target unmethylated and hemimethylated sites and establish new methylation patterns.

DNA hypermethylation is the gain of methylation at sites that are unmethylated under normal conditions, whereas DNA hypomethylation is the loss of methylation at sites that are methylated under normal conditions. Deregulation of the levels or activity of DNA methyltransferases may lead to altered methylation patterns (Pogribny and Beland, 2012).

DNA methylation regulates gene expression. Hypermethylated gene promoters result in an "off" state of gene expression, while those unmethylated or undermethylated are deemed "on" (Jaenisch and Bird 2003). The association of DNA methylation with the repression of gene expression is mediated by methyl CpG-binding domain (MBD) proteins, which selectively bind to and interact with methylated DNA. They then recruit additional proteins capable of modifying histones, conducting chromatin remodeling, and yielding compact and genetically-inactive heterochromatin. The presence of CH3- groups may deter transcription factors from interacting with DNA, thus exerting an inhibitory effect on gene expression. Overall, DNA methylation is a known cause of inactive chromatin states and repressed gene expression, while the loss of DNA methylation correlates with elevated gene expression (Kovalchuk and Kovalchuk, 2012).

DNMTs and MBDs are expressed and active throughout the developing brain. They support neuronal survival and plasticity, and assist in the regulation of learning and memory (Klose and Bird, 2006, Ooi et al., 2007, Mehler, 2008). The importance of DNA methylation extends far beyond the development of the nervous system. DNA methylation is implicated in many physiological neural functions, such as synaptic plasticity, the proper function of adult CNS neurons (Feng et al., 2010), and CNS repair mechanisms. In addition, it mediates responses to environmental stressors, such as exposure to ionizing radiation and chemotherapy agents (Kovalchuk et al., 2016c, Kovalchuk et al., 2016a, Kovalchuk et al., 2016b, Iskandar et al., 2010). For a long time, DNA methylation changes were thought to be permanent modifications that arose as a result of failure to maintain methylation patterns during and after cell division. However, it has become apparent that DNA methylation and DNA demethylation are dynamic

processes, especially in the brain. In addition, DNA demethylation governs tissue-specific differentiation (Kovalchuk and Kovalchuk, 2012).

Recent studies have found a second type of cytosine modification in mammalian DNA, 5-hydroxymethylcytosine (5-hmC). It was reported that 5-hmC is an oxidative derivative of 5-methylcytosine (5-mC) (Globisch et al., 2010), and a principal component of the DNA demethylation process. The existence of DNA demethylation was doubted until the discovery of DNA hydroxymethylation. The current model of DNA demethylation is as follows: in a set of reactions, TET (Ten-Eleven-Translocation) proteins oxidize 5-mC to 5-hmC, which can then be further modified, yielding unmethylated cytosine and thereby demethylating DNA (Guo et al., 2011, Wu and Zhang, 2011).

In the genome, 5-hmC appears primarily within gene regions, including untranslated regions and exons. In contrast, its levels are depleted in introns and intergenic regions (reviewed in (Sherwani and Khan, 2015). The existence of 5-hmC was documented in various mammalian tissues and cell types, supporting its potential role in the maintenance of DNA methylation and demethylation balance, and in regulation of gene expression (Globisch et al., 2010). Brain tissues exhibit high levels of 5-hmC (Wen and Tang, 2014); 5-hmC-mediated epigenetic changes are important for neurodevelopment and play a role in various neurological diseases (Chen et al., 2014), (Szulwach et al., 2011). Several studies established the importance of DNA hydroxymethylation in Alzheimer's disease, Huntington's disease, Parkinson's disease, malignant gliomas, autism, and other neurological and psychiatric diseases (Sherwani and Khan, 2015). Furthermore, exposure to environmental agents such as ascorbic acid,

phenobarbital, diethylstilbestrol, and hydroquinone altered levels of cellular 5-hmC and TET proteins (reviewed in (Dao et al., 2014). DNA methylation levels are affected by radiation exposure (Koturbash et al., 2016). Our recent studies showed that exposure to chemotherapy agent mitomycin C affected the levels of both 5-mC and 5-hmC in the murine brain and led to decreased global DNA methylation and increased DNA hydroxymethylation in the prefrontal cortex tissues of female mice (Kovalchuk et al., 2016c).

Histone modifications

DNA methylation is closely connected with the other components of chromatin structure, primarily, with histone modifications. The basic structure of chromatin is the nucleosome, where a strand of DNA is wrapped around an octamer of histone proteins (a tetramer of H3-H4 histone proteins with a H2A-H2B dimer situated on either side). Histones are small basic proteins that have a high affinity for DNA based on their positive charges and DNA's negative charge. The linker histone, H1, locks this structure and participates in the formation of higher-order chromatin packaging. In chromatic packaging, the small histone H1 is positioned outside the octamer and helps stabilize the structure. Octamers and DNA further fold to form a solenoid-like structure, which folds more to create radial loops. This happens with the help of non-histone scaffolding proteins. In the end, each chromosome is a small unit. Therefore, chromatin is very flexible; the tightness of the interaction between histones and DNA may change, allowing for the formation of loose, genetically active chromatin (euchromatin) and tightly-packaged, genetically inactive heterochromatin (Kovalchuk and Kovalchuk, 2012). The connections between DNA and histone proteins within the nucleosome core and higher

levels of chromatin packaging allow them to organize DNA, and therefore control gene expression, genome organization, and genome stability.

Histone proteins are composed of a high proportion of positively charged amino acids, such as lysine and arginine, giving them a positive charge and therefore the ability to interact electrostatically with negatively charged DNA. Histone proteins, especially their lysine and arginine-rich N-terminal tails, are often subject to chemical modifications that affect their interactions with the DNA strand, thus influencing chromatin packaging and gene expression. These alterations include acetylation, deacetylation, methylation, phosphorylation, SUMOylation, and ubiquitination (Jenuwein and Allis, 2001, McGowan et al., 2008, Kovalchuk and Kovalchuk, 2012).

Histone modification patterns, termed the 'histone code,' are cell and tissue-specific, and any histone modification changes can result in altered chromatin structure and gene activation or silencing (Jenuwein and Allis, 2001, Kovalchuk and Kovalchuk, 2012). Histone acetylation is linked to increased transcriptional activity, while histone deacetylation is linked with repression of transcription. Dynamic histone modifications and changes in chromatin structure occur not only during brain development, but also in mature neurons (Tsankova et al., 2007, Renthal and Nestler, 2008, Kovalchuk and Kovalchuk, 2012), whereby they help control expression of important genes.

Small RNAs

Small non-coding RNAs also partake in epigenetic control. Since the studies of Nobel Laureates, Fire and Mello, who first identified RNA-induced gene silencing in animals in 1998, knowledge of non-coding RNA-mediated regulation of gene expression has grown significantly. Around 98% of transcribed genomic DNA gives rise to non-

coding RNAs (Yu, 2008), including several groups of small RNAs, such as microRNA (miRNA), small interfering RNA (siRNA), and piwi-interacting RNA (piRNA) (Ghildiyal and Zamore, 2009). These small RNAs are characterized by their small size (20-32 nucleotides), origins and production, interactions with members of the Argonaute family of proteins, and their roles in controlling gene expression (Ghildiyal and Zamore, 2009).

The most studied are miRNAs, small (22-24 nucleotides long) single-stranded RNA molecules that regulate gene expression by altering mRNA stability and translational initiation. miRNAs are potent and universal regulators, and each miRNA can regulate thousands of different mRNA targets (Nelson et al., 2008). Similarly, each gene can be regulated by numerous individual miRNAs (Kovalchuk and Kovalchuk, 2012).

Several hundred miRNAs are expressed at specific times during mammalian brain development (Nugent BM, 2015). These miRNAs can lead to the downregulation of gene expression through their interactions with complementary mRNA sequences, which can result in the degradation of mRNA or interfere with translation (Nugent BM, 2015, Filipowicz et al., 2008). Because a single miRNA can regulate hundreds of mRNAs, miRNAs are critical for controlling the brain's gene networks. Furthermore, miRNAs can alter gene expression through interactions with other epigenetic mechanisms. For example, they can direct DNA methylation, alter post-translational modifications of chromatin, and even regulate translation in neuronal polyribosomes (Nugent BM, 2015). While miRNAs are involved in several cellular processes, their expression can be regulated by other epigenetic changes, such as DNA methylation, histone modifications, and chromatin structure (Saito and Jones, 2006). The expression of miRNAs is cell- and

tissue-specific, and they are abundant in the brain (Kosik, 2006). In the CNS, the expression of many miRNAs is regulated in a spatial and temporal manner in the CNS, suggesting that their regulation may be important in brain development and function (Barbato et al., 2009, Nugent BM, 2015), as well as in the brain's responses to various environmental factors and stressors (Koturbash et al., 2011a).

Metabolomics

The metabolome is the sum of all small molecular weight metabolites present within cells, tissues, or the entire organism (Johnson et al., 2016, Vasilopoulou et al., 2016). Metabolomics is one of the recent additions to the '-omics' group of biology technologies, which focuses on the profiling and analysis of the entirety of metabolites in cells and tissues (Bino et al., 2004). It has become an excellent diagnostic and prognostic tool. It helps uncover and explain disease mechanisms, characterize new potential drug targets, and develop novel treatments. It is also useful for explaining drug interactions and for monitoring treatment progress (Wishart, 2016). Metabolomics has become increasingly important in neuroscience, whereby metabolomics analyses are used to uncover the mechanisms of CNS disorders (Gonzalez-Riano et al., 2016).

The hippocampus and prefrontal cortex – main chemo brain targets

Hippocampus

The hippocampus is a bilateral limbic structure with a tube-like appearance that is positioned within the anterior medial region of the temporal lobe. It is comprised of two gyri, the Ammon's horn (Cornu Ammonis or "CA") and the dentate gyrus (DG), each of which contains distinctive types of cells. The cells of the Ammon's horn are pyramidal neurons. The Ammon's horn is subdivided into four regions: CA1, CA2, CA3, and CA4.

Along with molecular and polymorphic layers, the dentate gyrus contains the most prominent layer of stellate granule cells (Kolb and Whishaw, 2015, Dalley R, 2008).

The DG is extensively connected to the CA, resulting in virtually every pyramidal cell being connected with almost every granule cell. In case of structural hippocampal damage, this arrangement allows the DG to maintain some hippocampal functions (Kolb and Whishaw, 2015).

There are massive connections from the hippocampus to other parts of the brain through several neuronal pathways. Of those, the perforant pathway, termed so because it “perforates” the hippocampus, links the hippocampus to the entorhinal cortex, which in turn has connections with most neocortical regions. The arch-fringe (fimbria/fornix) pathway arches along the edge of the hippocampus and links it with the anterior thalamus, prefrontal cortex, hypothalamus, and the basal ganglia. Hence, as suggested by Kolb and Whishaw (2015), the hippocampus may serve as “a way station” between the posterior neocortex on one end, and the frontal cortex, basal ganglia, and brainstem on the other. Within the hippocampus itself, input from the neocortex proceeds to the DG and further projects to the Ammon’s horn (Kolb and Whishaw, 2015, Dalley R, 2008).

The hippocampus is one of the sites of adult neurogenesis, which takes place within the subgranular zone of the DG. This layer contains several types of cells, of which the most prominent are the neural stem cells, as well as the astrocytes, endothelial cells, and blood vessels that come together to create a nurturing environment for neuroblast proliferation, migration, and differentiation (Kolb and Whishaw, 2015).

The hippocampus regulates several vital cognitive processes, such as spatial navigation and memory processing. It is also involved in the consolidation and storage of

long-term memory, including declarative memory. The hippocampus is extremely vulnerable to stress, especially to the lack of oxygen, whereby even a short period of oxygen deprivation can result in damage (Kolb and Whishaw, 2014). Several studies have reported that the hippocampus is a known target of chemo brain (Andres et al., 2014, Apple et al., 2017, Dietrich et al., 2015, Seigers et al., 2010a, Seigers et al., 2008, Seigers et al., 2010b).

Prefrontal cortex

The frontal lobe controls behaviour in time and space. The prefrontal cortex (PFC), as originally studied by Rose and Wolsey in 1948, is found in all terrestrial mammals. It is a large area of the frontal lobe, anterior to the motor, premotor, and cingulate cortex. The PFC receives projections from the thalamus and controls key executive functions such as planning, strategizing, emotional behaviours, and behavioural inhibition. It also governs temporal and working memory, and is responsible for abstract thinking and behavioural regulation (Kolb and Whishaw, 2015).

In rodents, the PFC is subdivided into the medial PFC and the orbital frontal cortex, each of which has subregions. It receives dopaminergic inputs from the ventral tegmental area and projects to the nucleus accumbens. Most importantly, it projects to the other regions of the limbic system, such as the hippocampus and amygdala. The PFC undergoes prolonged development and is extensively interconnected with other cortical, subcortical, and brainstem sites (Kolb et al., 2012). The human dorsal lateral PFC, which is roughly equivalent to the medial PFC of rodents, is linked to brain regions involved in attention, cognition, and action, whereas the orbital PFC is connected to regions involved with emotion. As such, the PFC directs the control of cognitive actions, guiding the other

regions of the brain to direct the flow of activities necessary to accomplish a certain task (Miller and Cohen, 2001). Recent studies show that PFC is significantly affected by cancer treatments such as radiation therapy and cytotoxic chemotherapy and is a target of chemo brain (Kovalchuk et al., 2016a, Kovalchuk et al., 2016b, Kovalchuk et al., 2016c).

Study Rationale

Chemotherapy causes numerous side effects, including central nervous system (CNS) toxicity (Soffietti et al., 2014, Ahles et al., 2012). Recent research shows that some chemotherapy agents may even be more toxic to healthy brain cells than to the cancer cells they are designed to treat (Han et al., 2008), and many studies have provided evidence of the occurrence of chemotherapy-induced cognitive dysfunction (Kaiser et al., 2014, Moore, 2014, Vardy et al., 2008b). The side effects of chemotherapy treatment influence the cognitive domains of executive function, attention, processing speed, and memory (Seigers and Fardell, 2011, Seigers et al., 2015, Seigers et al., 2010a, Seigers et al., 2008, Seigers et al., 2009, Seigers et al., 2013, Seigers et al., 2010b, Christie et al., 2012, Joly et al., 2011), and cause a condition called 'chemo brain'(Mitchell and Turton, 2011a). Some of the proposed mechanisms behind chemo brain include increased oxidative stress, chronic inflammation, inhibition of neuronal proliferation, induction of apoptosis, alterations in brain blood flow, disruption of the blood-brain barrier, and white matter dysfunction (Raffa, 2011, Lyons et al., 2011, Briones and Woods, 2014, Briones et al., 2015, Christie et al., 2012, Han et al., 2008, Joshi et al., 2010, Seigers and Fardell, 2011). Recent cell line and animal model-based studies, including one of our own (Kovalchuk et al., 2016c), have shown that chemotherapy exposure can cause oxidative stress and oxidative DNA damage. It can also inhibit neuronal proliferation and

differentiation, increase apoptosis, alter levels of histone modification and chromatin remodelling, and lead to aberrant gene expression in the brains of exposed animals. These changes were associated with altered neurogenesis and deficits in learning and memory processes (Mustafa et al., 2008, Briones and Woods, 2014, Christie et al., 2012).

Accumulating evidence suggests that cognitive impairments manifest long before chemotherapy, even prior to cancer diagnosis, implying that the growth of the malignant non-CNS tumors exerts negative effects on the brain. This cancer-induced cognitive impairment can be referred to as 'tumor brain'. While the molecular and cellular mechanisms of chemo brain and tumor brain require more investigation, the frequency and timing of their occurrence and persistence suggest that both may be epigenetically regulated and associated with abnormal global gene expression patterns. Therefore, an in-depth analysis of global gene expression may provide solid understanding of the molecular mechanisms behind chemo brain and tumor brain.

Moreover, all available data on chemo brain were based on models in which healthy animals were treated with chemotherapy drugs. These models lack one important biological component: the presence of a tumor. In order to gain a complete understanding of the molecular mechanisms and pathways affected by tumor brain and chemo brain, the effects of tumor growth and chemotherapy in tumor-bearing animals must be analysed.

Patient tumor-derived xenograft (PDX) models provide excellent tools to model and study organismal effects of cancers, including the CNS side effects. One of the most advanced approaches is the Champions TumorGraft® technology that effectively replicates human tumors in immunodeficient mice. Champions Oncology uses PDX mouse models in a unique precision medicine approach aimed to determine

chemotherapy sensitivity of tumors and ensure chemotherapy success for each individual patient. In the precision approach protocol, a piece of individual's tumor is biopsied or surgically resected and then implanted in mice. In the Champions TumorGraft® PDX modelsm tumors are implanted together with the microenvironment and uphold the histopathological architecture and molecular characteristics of tumors. Therefore, Champions Oncology TumorGraft® PDX models very closely resemble patients' tumors, reaching 94 percent genetic correlation between a PDX and an original tumor. After implantation into TumorGraft mice, tumor PDX models are propagated. Following propagation, Champions Oncology scientists, in collaboration with treating physicians, decide which chemotherapy agents to test on each individual patient's PDX model set. Mice are treated using classical clinical chemotherapy protocols and usual routes, with doses calculated per body weight. Living tumor's responses to chemotherapy regimens are established and reported to physicians, thus personalizing each patient's chemotherapy treatment and increase the treatment effectiveness. Based on each individual patient's tumor type, chemotherapy treatments may include various single agents or combinations of chemotherapy modalities (Bertotti et al., 2011, DeRose et al., 2011, Hidalgo et al., 2011, Morelli et al., 2012, Stebbing et al., 2014). The Champions TumorGraft® PDX model testing approach is very robust, leading to highly effective tumor control and durable remissions (Hidalgo et al., 2011, Izumchenko et al., 2016, Stebbing et al., 2014, CO, 2017).

Theory and Hypotheses

An in-depth understanding of the mechanisms of cancer and cancer treatment-related cognitive impairments, tumor brain and chemo brain, is essential for the future development of effective prevention and mitigation strategies. Based on previous data, we propose an epigenetic theory of chemo brain and tumor brain in which the mechanisms behind the neurotoxic side effects of cancer and chemotherapy are epigenetically regulated, include alterations in the global transcriptome and the small RNAome, and are paralleled by changes in DNA methylation and the metabolome.

This study will concentrate on the hippocampus and the prefrontal cortex (PFC), as based on their roles in memory and learning and executive functions, as well as on previous data suggesting that both of these brain regions are affected in chemo brain (Kovalchuk et al., 2016c).

We propose the following hypotheses:

(1) Tumor brain will manifest itself in tumor-bearing mice, whereby tumor presence will affect molecular networks in the brain.

(2) Molecular changes will be more pronounced in tumor-bearing animals treated with chemotherapy than in untreated ones.

(3) Tumor brain and chemo brain will affect the transcriptome, epigenome, and metabolome of the PFC and hippocampus.

The following objectives were formulated to test the hypotheses:

Objective 1: To analyse gene expression and DNA methylation changes in the PFC of chemotherapy treated and untreated tumor-bearing animals, as compared to controls.

Objective 2: To analyse the role of microRNAome deregulation in the PFC of chemotherapy treated and untreated tumor-bearing animals.

Objective 3: To analyse genetic molecular mechanisms altered in the hippocampus of untreated tumor-bearing animals.

Objective 4: To analyse the effects of non-CNS tumor growth on the brain metabolome of untreated tumor-bearing animals.

Main findings

In Chapter 1, we review the latest information available on cancer incidence, modern cancer therapies, and the effects of cancer and cancer chemotherapies on the brain. We discuss the incidence and mechanisms of chemo brain and tumor brain, as well as the role of gene expression and epigenetic changes in the central nervous system (CNS) effects of non-CNS tumors and chemotherapy.

Chapter 2 explains how the growth of malignant non-CNS tumors causes molecular alterations in the PFC. The main findings presented in the chapter are as follows: (i) The growth of malignant non-CNS tumors negatively affected the PFC. (ii) Patient-derived xenograft (PDX) mice carrying triple negative breast cancer (TNBC) and progesterone-positive breast cancer (PR+BC) PDXs exhibited altered gene expression in the PFC. (iii) The growth of TNBC and PR+BC tumors caused oxidative stress and aberrant DNA methylation. (iv) Chemotherapy treatments did not have any additional synergistic effects on the analyzed processes. (v) The molecular changes observed in this study embody known signs of neurodegeneration and brain aging.

Chapter 3 presents an in-depth analysis of microRNAome changes in the PFC tissues of chemotherapy treated and untreated mouse TumorGraft™ models with triple negative and progesterone receptor positive breast cancer. Our key findings are as follows: (i) While miRNAome changes related to tumor growth were observed in the PFC tissues of both breast cancer groups, they were more pronounced in PR+BC animals than in TNBC ones. (ii) Both tumor growth and chemotherapy treatments led to the upregulation of the miR-200 family and the miR-183/96/182 cluster, as compared to controls. (iii) miRNA changes were seen in parallel with decreased levels of the miRNA

target, namely, the brain-derived neurotrophic factor (BDNF), which is an important regulator of neural development, survival, growth, differentiation, and plasticity. (iv) The molecular changes observed were similar to those seen in neurodegenerative diseases and aging.

Chapter 4 discusses tumor brain's manifestations in the hippocampus. This is the first analysis of the molecular mechanisms of tumor brain's manifestations in the hippocampal tissues of TumorGraft mice. This chapter reports that the growth of malignant non-CNS tumors has an impact on molecular processes in the murine hippocampus. The major findings of our study are as follows: (i) The growth of TNBC and PR+BC tumors significantly altered gene expression in the murine hippocampus. (ii) TNBC tumor growth caused oxidative stress that manifested in significantly elevated levels of 4-HNE. (iii) Tumor growth negatively affected the levels of neuronal transcription regulator NPAS4 and its target genes, among them BDNF, a member of the neurotrophin family of growth factors. (iv) Tumor growth was associated with a significant downregulation of PCNA, AKT 1, and ERK1/2, proteins that are important for controlling neuronal proliferation and survival. (v) Finally, the observed molecular changes strongly resembled those associated with neurodegenerative diseases and brain aging.

Chapter 5 presents data on how the growth of malignant non-CNS tumors affects the brain metabolome. We showed that the growth of pancreatic cancer, sarcoma, and lung cancer xenografts significantly affected the metabolic pathways in the brain. Tumor brain manifested as aberrant activity of protein synthesis pathways, sphingolipid

pathways, and several other pathways that were previously shown to be affected in aging and neurodegenerative diseases.

Overall, the experimental results summarized in this thesis provide solid evidence showing that the growth of malignant breast, lung, and pancreatic cancer, as well as sarcoma, negatively impacts the brain. While some common patterns were observed between different tumor groups, several changes were persistent and specific to the type of tumor and affected brain region. All the observed molecular alterations strongly resembled those seen in neurodegenerative disorders and brain aging.

Chapter 6 presents a brief discussion of the key findings, limitations, and conclusions of our research. It also offers an outlook that can be useful for future research efforts for further dissecting the mechanisms of chemo and tumor brain, and for developing diagnostic and prognostic biomarkers and mitigation strategies.

CHAPTER 2: CHEMO BRAIN OR TUMOR BRAIN - THAT IS THE QUESTION: THE PRESENCE OF EXTRACRANIAL TUMORS PROFOUNDLY AFFECTS MOLECULAR PROCESSES IN THE PREFRONTAL CORTEX OF TUMORGRAFT MICE

Chapter 2 has been accepted for publication in its entirety:

Kovalchuk, A., Ilnytskyy, Y., Rodriguez-Juarez, R., Shpyleva,S., Melnyk, S., Pogribny, I., Katz, A., Sidransky, D., Kovalchuk., O. Kolb, B. Chemo brain or tumor brain - that is the question: the presence of extracranial tumors profoundly affects molecular processes in the prefrontal cortex of TumorGraft mice. *AGING* (in press).

Abstract

Chemotherapy agents are more toxic to healthy brain cells than to cancer cells, and a wide array of central nervous system complications occur and persist as a result of systemic cancer treatments. This condition is known as chemo brain, and affects 25-75% of adult cancer survivors. Cognitive impairments occur even before treatment, and hence may be referred to as cancer associated cognitive changes, or tumor brain. There is much yet to be learned about the mechanisms of chemo brain, and the mechanisms of tumor brain have remain unexplored. The frequency and timing of chemo brain occurrence and persistence strongly suggest it may be epigenetic in nature and associated with altered gene expression. To gain a full understanding of tumor brain and chemo brain, we used TumorGraftTM models wherein part of a patient's tumor is removed and grafted into immune-deficient mice. This is the first study showing that malignant non-central nervous system tumor growth causes profound molecular alterations in the brain. We showed that mice harbouring triple negative or progesterone positive breast cancer TumorGrafts exhibited altered gene expression, decreased levels of DNA methylation, increased levels of DNA hydroxymethylation, and oxidative stress in the prefrontal cortex. Interestingly, chemotherapy did not have any additional synergistic effects on the analyzed processes. The molecular changes observed in this study are known signs of neurodegeneration and brain aging. This study shows links between tumor brain and brain aging, and provides an important roadmap for future large-scale analysis of the molecular and cellular mechanisms of tumor brain.

Introduction

Breast cancer is one of the most commonly diagnosed cancers in the world, and it is the most common cause of cancer-related deaths in women. According to the Canadian Cancer Society in 2015, breast cancer accounted for 26% of all new cancer cases in Canadian women. Most breast cancer patients undergo chemotherapy treatments, and the development of new chemotherapy regimens resulted in significant improvement of patient outcomes and increased survival. Despite the undisputed benefits, chemotherapy causes an array of side effects, such as myelosuppression, nausea, vomiting, infections, and bleeding. Additionally, many patients experience profound psychosocial effects that decrease their quality of life, including fatigue, cognitive dysfunction, and other signs of central nervous system (CNS) toxicity post-chemotherapy (Ahles, 2012). Thus, chemotherapy-induced cognitive changes have become an increasing concern among cancer survivors. Survivors have coined the terms *chemo fog* or *chemo brain* to explain their symptoms (Kaiser et al., 2014). While the initial reports of chemo brain go back to the 1970s and the mid-1980s, the problem started to gain attention only in the '90s. Since then, numerous longitudinal and cross-sectional studies have reported the existence of chemo brain and its severity. Among these, the vast majority of work was conducted in breast cancer cohorts. It has been found that chemotherapy-induced CNS side effects, or chemo brain, affects almost half of all breast cancer survivors and impacts the cognitive domains of attention, memory, psychomotor speed, and executive function. Current research shows that chemotherapy agents are more toxic to healthy brain cells than to cancer cells, and debilitating chemo brain manifestations affect patients for as long as five to ten years

after treatment completion (Ahles et al., 2012, Ahles and Saykin, 2007, Ahles et al., 2008, Andreotti et al., 2016).

To prevent and mitigate chemo brain side effects, it is important to understand the underlying neural mechanisms that are altered by chemotherapy agents. At present, mechanistic data on chemo brain are scarce. While some molecular mechanisms underlying chemo brain have been assessed in clinical studies, analyses are difficult to conduct because of large inter-patient variability, different treatment protocols, disease statuses, and co-morbidities (Kaiser et al., 2014). Thus, recent chemo brain research has employed cell lines as well as rodent models in which healthy animals are treated with chemotherapy drugs. In these animal models, chemotherapy caused oxidative stress, inhibited neuronal proliferation and differentiation, induced apoptosis, and altered levels of histone modification and chromatin remodeling, leading to the aberrant levels of neurotrophic and neurogenic proteins (Mustafa et al., 2008, Joshi et al., 2010). These molecular changes were associated with altered neurogenesis and deficits in learning and memory processes (Mustafa et al., 2008, Briones and Woods, 2011, Christie et al., 2012).

Interestingly, recent data based on thorough pre-treatment assessments have revealed that 20–30% of breast cancer patients exhibit reduced cognitive performance prior to chemotherapy treatment, and this cognitive impairment was not related to surgery, fatigue, depression, or anxiety associated with their breast cancer diagnoses and treatments. Instead, these symptoms were correlated with the presence of the malignant tumor. While earlier studies suggested that cognitive impairment was due to chemotherapy, recent evidence of pre-treatment cognitive deficits led to a new term,

"*cancer and cancer treatment-associated cognitive change"*(Ahles, 2012). This phenomenon may thus be referred to as *tumor brain*.

While the molecular and cellular mechanisms of chemo brain are poorly investigated, and the mechanisms of tumor brain have not even been looked at, the frequency, timing, and persistent nature of these phenomena strongly suggest they may be epigenetic. Epigenetic changes are meiotically heritable and mitotically stable alterations that regulate gene expression and genome stability; they include DNA methylation and hydroxymethylation, histone modification, and non-coding RNA regulation (Wang et al., 2015a). Epigenetic changes underlie aberrant global gene expression patterns, and they are critical to neurogenesis and CNS development and functioning (Jobe et al., 2012). Furthermore, chemotherapy drugs may alter epigenetic homeostasis and gene expression (Csoka and Szyf, 2009). Epigenetic changes that underlie aberrant gene expression patterns have been well-documented in breast cancer literature. Recently, we analysed the molecular mechanisms of chemo brain (Kovalchuk et al., 2016c) by investigating the gene expression profiles in the prefrontal cortex (PFC) and hippocampus (HPC) of mice 3 weeks after treatment with the cytotoxic chemotherapy agents mitomycin C (MMC) and cyclophosphamide (CPP)(Kovalchuk et al., 2016c). We showed that chemotherapy altered gene expression profiles in the PFC and HPC tissues; the changes were most prominent in the PFC tissues of females 3 weeks after MMC treatment. MMC exposure led to oxidative stress, decreased global DNA methylation and increased DNA hydroxymethylation in the PFC tissues of females. This opened new avenues for the analysis of epigenetic mechanisms of chemo brain (Kovalchuk et al., 2016c).

Nonetheless, all data on chemo brain, including ours, stem from models in which healthy animals are treated with chemotherapy drugs. These models lack one important biological component—the presence of a tumor. To gain a complete understanding of the molecular mechanisms and pathways affected in tumor brain and chemo brain, we use TumorGraft™ models whereby tumor tissue is engrafted into immune-deficient animals (Garralda et al., 2014, Stebbing et al., 2014). TumorGraft technology is used as a cutting-edge personalized approach to cancer therapy. It preserves the characteristics of the live tumor, creating a replica that is identical to the tumor in the patient's body (DeRose et al., 2011). Our study is the first to show that non-CNS malignant tumor growth causes profound molecular alterations in the prefrontal cortex, a key regulatory region that is involved in executive functions, such as working memory, decision-making, planning, judgment, social behavior, as well as abstract thinking.

Results

Breast cancer growth affects global gene expression in prefrontal cortex tissue of tumor-bearing mice

Analysis of differential gene expression: Global transcriptome reflects all expressed mutational and non-mutational changes, and hence it is one of the best representations of molecular processes in cells and tissues. Global transcriptomic profiling constitutes an excellent tool to dissect underlying mechanisms of various diseases and conditions, as well as treatment responses. To gain a complete understanding of the effects of non-CNS tumors and chemotherapy on the brain, we used the Illumina next generation sequencing platform to perform an in-depth transcriptome analysis of thePFC tissue of TumorGraft

mice with either triple negative breast cancer (TNBC) or progesterone receptor positive breast cancer (PR+).

The differential gene expression analysis revealed notable changes in PFC tissues of TNBC and PR+ animals (Fig. 2.1A). Hierarchical clustering of the gene expression data showed that each experimental group could be distinguished by its gene expression profile. Furthermore, the principal component analysis based on the entire transcriptome dataset showed good clustering for each group and clear differences between gene expression profiles in the PFC tissues of intact, TNBC and PR+BC TumorGraft mice (Figure 2.2).

In the PFC tissues of TNBC animals, 171 genes were upregulated, and 124, downregulated, as compared to intact mice. In the PFC tissues of PR+BC PDX mice, 548 genes were upregulated, and 109 were downregulated as compared to intact animals. Amongst those, 85 were upregulated, and 60 were downregulated in the PFC tissues of both TNBC and PR+BC PDX mice as compared to intact animals (the adjusted p-value was <0.05; the fold change was 1.5) (Fig. 2.1A) Commonly upregulated genes included bone morphogenetic protein (*BMP*) and collagen genes, laminins, and histocompatibility loci (Fig. 2.1B). Laminin1-2 and BMP4 upregulation was also confirmed on the protein level in the PFC tissues of PR+BC mice, but not in TNBC mice (Fig. 2.1C).

To gain further insight into the functional significance of the observed transcriptome changes, we conducted an in-depth pathway analysis, during which we performed a functional annotation of differentially expressed genes using the Pathview/KEGG and DAVID bioinformatics platforms. One-directional pathway analysis revealed multiple differentially affected pathways. Amongst those, 46 pathways were

upregulated in the PFC tissues of the PR+BC animals and 63 pathways – in the TNBC animals. Of those, 38 pathways were common to both tumor groups and included pathways involved in graft−versus−host disease, natural killer cell-mediated cytotoxicity, oxidative phosphorylation, as well as other pathways implicated in the inflammation and immune responses. In addition, one pathway was downregulated in the PFC tissues of TNBC animals, and 7 pathways in the PR+BC harboring animals. The neuroactive ligand pathway was common for both aforementioned groups (Fig. 2.1 D).

Oxidative damage in the PFC tissues of tumor-bearing mice

We noted alterations in the oxidative phosphorylation pathways in the PFC tissues of PDX mice. Oxidative stress is a hallmark of cancer. Previous studies, including our own, have shown increased oxidative stress in chemo brain (Wang et al., 2015a, Joshi et al., 2005). With this in mind, we analyzed the levels of 8-oxo-2'-deoxyguanosine (8-oxodG) in genomic DNA from the PFC tissues of TNBC- and PR+BC PDX–harboring animals. The 8-oxodG is one of the predominant and best-studied markers of oxidative DNA damage. It is formed by the action of reactive oxygen species (Dizdaroglu and Jaruga, 2012). The growth of a non-CNS TNBC tumor caused a strong and statistically significant (p=0.0472) increase in the 8-oxodG levels in the PFC tissues of TumorGraft animals (Fig. 2.3A). However, PR+BC tumor growth did not cause any significant increase in the 8-oxodG levels in the PFC of TumorGraft mice. We also determined levels of 8-oxoguanine glycosylase (OGG1) and apurinic/apyrimidinic endonuclease 1 (APE1), the base excision repair proteins that partake in the repair of oxidative DNA damage and constitute well-accepted markers of oxidative DNA damage (Shpyleva et al., 2014, Powell et al., 2005). Western immunoblotting revealed a statistically significant

reduction in the levels of OGG1 (p=0.0039) and APE1 (p=0.033) in the PFC tissues of PR+BC mice but not of TNBC mice (Fig. 2.3 B).

Global DNA methylation and level of DNA methyltransferases and methyl-CpG-binding protein MeCP2 in the PFC tissues of tumor-bearing mice

Several studies, including our own, have suggested that aberrant DNA methylation may occur because of oxidative DNA damage (Shpyleva et al., 2014). Aberrant DNA methylation is also associated with altered gene expression patterns (Liyanage et al., 2014, Valinluck and Sowers, 2007). We analyzed and compared the status of global DNA methylation in the PFC tissues of TNBC and PR+BC PDX mice. Both 5-hydroxymethyl-cytosine (5-hmC) and 5-methyl-cytosine (5-mC) have recently emerged as important epigenetic markers. In order to get a complete account of global DNA methylation, we determined the levels of 5-mC and 5-hmC, as well as the ratio between them, in the genomic PFC DNA of intact and tumor-bearing animals. We found a statistically significant decrease in 5-mC levels in the global DNA of PFC tissues of TNBC-bearing animals (p=0.014), as well as a trend toward a decrease (90% confidence level, p=0.078) in the PFC tissues of PR+BC PDX animals as compared to intact controls. While the levels of 5-mC were reduced, the levels of 5-hmC were significantly increased in the PFC tissues of TNBC- and PR+BC-bearing mice (p=0.0017 and p=0.0009, respectively) as compared to intact controls. The ratio between 5-hmC and 5-mC was also changed in the PDX animals and was significantly increased in both TNBC and PR+BC PDX animals as compared to controls (Fig. 2.4).

Having observed altered DNA methylation, we then determined the levels of DNA methyltransferases (DNMT1, DNMT3A and DNMT3B*)*, as their altered levels may be

associated with changes observed in 5-mC and 5-hmC levels. We noted that levels of DNMT1 were significantly (p=0.028) reduced in the PFC tissue of the TNBC PDX mice, but increased – in the PFC of the PR+BC mice (p=0.028). The levels of DNMT3A were decreased in the PFC tissues of PR+BC PDX animals (p=0.021), and unchanged in the PFC of TNBC mice. The levels of DNMT3B were unchanged in the PFC of PR+BC tissues, but upregulated in TNBC-bearing mice as compared to intact controls (p=0.021). At the same time, the levels of methyl-CpG-binding protein MeCP2 were significantly elevated (p=0.005) in the PFC tissues of PR+BC animals, and unaffected in those of TNBC mice as compared to intact control animals (Fig. 2.5).

Effects of chemotherapy treatments on the molecular processes in the PFC tissues of tumor-bearing animals

In order to establish whether or not chemotherapy treatments would further exacerbate tumor-induced molecular epigenetic changes in the PFC tissues of tumor-bearing animals, we analyzed the levels of genomic DNA methylation and oxidative stress marker 8-oxod-G in the PFC tissues of tumor-bearing and chemotherapy treated animals. The analyzed chemotherapy regimens constitute one of the treatments of choice for the TNBC and PR+BC tumors. Interestingly, chemotherapy treatments did not act in synergy with the 5-mC, 5-hmC and 8-oxo-dG changes induced tumor presence alone (Fig.2.6).

Chemotherapy treatments also affected the levels of DNMT1 and DNMT3A in the PFC tissues of tumor-bearing animals. There, doxorubicin-pactitaxel-cyclophosphamide (DPC) treatment of TNBC-bearing mice led to a statistically significant (p=0.024, as compared to intact controls) decrease in the levels of DNMT1 in the PFC tissues, and to

an insignificant trend towards a decrease in DNMT3A. While the presence of PR+BC caused an increase in the DNMT1 levels in the PFC tissues of PDX animals as compared to controls, crizotinib chemotherapy led to a significant, albeit small, decrease in the DNMT1 levels. Furthermore, topotecan and crizotinib chemotherapies led to significant (p=0.021 and p=0.004, respectively, as compared to intact animals) decreases in the levels of DNMT3A. Moreover, crizotinib treatment of PR+BC mice furthered the decrease of DNMT3a levels in the PFC tissues as compared to untreated PR+BC mice (p=0.0058) (Fig. 2.7A). Interestingly, chemotherapy treatments (DPC for TNBC and crizotinib and topotecan for PR+BC) strongly affected OGG1 levels, causing statistically significant decreases in the levels of this DNA repair protein (Fig. 2.7B).

Discussion

CNS toxicity is one of the major quality-of-life issues that cancer survivors face. Nevertheless, there is a lot to learn about the mechanisms of chemo brain (Ahles, 2012, Vardy and Tannock, 2007). Along with chemo brain, studies have emerged reporting notable cognitive changes and memory deficits prior to chemotherapy or other cancer treatments, the 'tumor brain' phenomenon (Ahles, 2012, Vardy and Tannock, 2007, Ahles and Saykin, 2007).

This is the first study showing that non-CNS malignant tumor growth causes profound molecular alterations in the brain. Our key findings were that: the growth of malignant non-CNS tumors profoundly affected the brain and exerted a negative influence on the PFC; PDX mice carrying TNBC and PR+BCX PDXs exhibited altered gene expression in the PFC; the growth of TNBC and PR+BC tumors caused oxidative

stress and aberrant DNA methylation in the PFC tissues of PDX mice; and chemotherapy treatments did not have any additional synergistic effects on the analyzed processes.

We observed profound changes in the global gene expression in the prefrontal cortex of PDX-carrying mice. We found the upregulation of laminin, bone morphogenic protein and collagen genes. Laminin and collagen are important components of the blood-brain barrier, and their expression is increased after a stroke (Fernandez-Lopez et al., 2012). The laminin matrix is important for neuronal survival.(Chen et al., 2003) The downregulation of laminins was reported to inhibit glioma invasion, metastasis, and angiogenesis (Nagato et al., 2005, Ljubimova et al., 2006). Bone morphogenic proteins are crucial for the development of both the central and peripheral nervous systems in vertebrates, regulating neural stem cell fate and maturation (Liu and Niswander, 2005, Bond et al., 2012). Their roles and regulation in chemo brain and tumor brain need to be further elucidated, especially given the fact that many of the BMP genes are epigenetically regulated via DNA methylation (Du et al., 2014). Our initial analysis suggested that non-CNS tumor growth led to demethylation of BMP4 promoter in the PFC tissues of tumor-bearing animals as compared to intact ones (Fig.2.2). Role of DNA methylation in BMP4 regulation in tumor brain and chemo brain needs to be further substantiated in the large-scale studies. Here, we noted the downregulation of the neurotrophic factors pathway, which was previously reported to be downregulated by chemotherapy agents and radiation (Kovalchuk et al., 2016b, Kovalchuk et al., 2016c), and may therefore constitute biomarkers of brain toxicity. In the future, to gain a full understanding of pathways and processes affected in tumor brain and chemo brain, it would be important to conduct a detailed analysis of the brain signalome and entire

interactome, using novel platforms such as OncoFinder and iPANDA (Buzdin et al., 2014, Ozerov et al., 2016).

An analysis of the mechanisms of tumor brain and chemo brain showed that the growth of TNBC tumors caused oxidative stress in the PFC tissues of PDX mice, which was evidenced by an increase in 8-oxodG levels. Interestingly, PR+BC tumor growth did not cause any significant increase in 8-oxodG levels in the PFC of TumorGraft mice. The 8-oxodG molecule is formed by the action of reactive oxygen species and is a key marker of oxidative DNA damage (Dizdaroglu and Jaruga, 2012, Dizdaroglu et al., 2002). We have previously shown that animal exposure to the chemotherapy agents mitomycin C and cyclophospamide caused an accumulation of 8-oxo-dG in PFC tissues(Kovalchuk et al., 2016c).

Increased levels of oxidative stress and accumulation of 8-oxodG have been reported in brain tumors (Iida et al., 2001) during neurodegeneration (Basu et al., 2015), ischemia (Akpinar et al., 2016b), Alzheimer’s disease, amyotrophic lateral sclerosis, Huntington's and Parkinson's diseases, autism, and other pathologies (Islam, 2017, Yui et al., 2016, Shpyleva et al., 2014). Elevated levels of 8-oxo-dG caused neurodegeneration (Sheng et al., 2012), and oxidative stress and oxidative DNA damage have been reported to be hallmarks of brain aging (Lacoste et al., 2016, Heemann et al., 2017).

The presence of 8-oxodG is highly toxic, and cells have an efficient repair mechanism to eliminate oxidative stress by-products via the action of the base excision repair protein OGG1. Alterations in cellular OGG1 levels constitute yet another marker of oxidative DNA damage (Shpyleva et al., 2014, Rusyn et al., 2004, Rusyn et al., 2005). Here, we found a significant reduction in the levels of OGG1 in the PFC tissues of

PR+BC mice and a trend towards a reduction of OGG1 in the PFC tissues of TNBC-harboring mice. OGG1 is a glycosylase involved in the initial steps of recognition and removal of 8-oxodG (Nishimura, 2002, Klungland and Bjelland, 2007), and the success of removal of this highly mutagenic and cytotoxic DNA lesion heavily depends on proper OGG1 function. *OGG1* is important for brain development and function, maintenance of neuronal connectivity, and protection against oxidative DNA damage and apoptosis (Liu et al., 2011a, Wong et al., 2008, Larsen et al., 2006). Loss or decrease in OGG1 levels and increase of 8-oxodG in the genome have been reported in cancer, neurodegenerative diseases, autism, and metabolic diseases (Lovell and Markesbery, 2007, Nakabeppu et al., 2007, Shpyleva et al., 2014, Kinnersley et al., 2014, Klungland and Bjelland, 2007, Nohmi et al., 2005, Osorio et al., 2014, Sampath et al., 2012), as well as in brain aging (Cardozo-Pelaez et al., 2012, Liu et al., 2011a, Swain and Rao, 2012).

Oxidative stress was previously reported to be associated with aberrant DNA methylation patterns (Shpyleva et al., 2014). Another key finding of our study is the decrease in 5-mC, and parallel increases in 5-hmC and the 5-hmC/5-mC ratio in the PFC tissues of tumor-bearing animals as compared to controls. For the first time, we showed that the growth of TNBC and PR+BC tumors caused a profound and significant reduction of 5-mC and an increase in the levels of 5-hmC levels in the PFC tissues of PDX mice. The ratio of 5-hmC/5-mC also increased.

DNA methylation is important for the maintenance of genome stability and gene expression (Tirado-Magallanes et al., 2016, Grigorenko et al., 2016). It regulates a wide array of cellular processes and is vital for brain development and functioning (Grigorenko et al., 2016). Altered DNA methylation has been reported in numerous neurological

diseases and conditions (Kato and Iwamoto, 2014), and global DNA hypomethylation in the brain has been reported to occur upon radiation exposure (Impey et al., 2016) and chemotherapy treatments (Kovalchuk et al., 2016c). DNA hydroxymethylation is a recently discovered epigenetic modification (Rudenko and Tsai, 2014, Grigorenko et al., 2016), and 5-hmC is crucial for brain development. It is significantly increased in neurons, whereby hydroxymethylation accounts for up to 40% of all modified CG dinucleotides in the prefrontal cortex (Kinde et al., 2015, Grigorenko et al., 2016). In conjunction with DNA methylation, hydroxymethylation regulates tissue-specific gene expression patterns (Ponnaluri et al., 2016). Altered hydroxymethylation levels have been reported to occur in autism (James et al., 2014, Shpyleva et al., 2014), Alzheimer's disease (Shu et al., 2016), intracerebral hemorrhage (Tang et al., 2016), and other conditions. Hydroxymethylation levels are affected by proton exposure (Impey et al., 2016). Recent studies showed increases in hydroxymethylation during aging, suggesting that hydroxymethylation might play a role in age-related neurodegeneration (Ellison et al., 2016, Jakovcevski and Akbarian, 2012).

DNA methylation is established and regulated by DNA methyltransferases (Rudenko and Tsai, 2014). We have shown that the growth of malignant non-CNS tumors caused changes in the levels of DNA methyltransferases. These changes can be viewed as protective or compensatory, aimed to restore 5mC losses. Alternatively, decreased levels of DNMTs may be causatively associated with the lessened levels of 5mC. Aberrant levels of DNMTs have been reported to occur in many neurological and psychiatric conditions. They have also been shown to occur upon exposure to radiation and toxic chemicals (Kovalchuk et al., 2016c, Silasi et al., 2004). The mechanisms of

their aberrant expression and the importance of DNMTs in tumor brain and chemo brain should be analyzed in the future.

Similarly, the mechanisms of 5mC loss and 5hmC gain and their functional consequences should be analyzed. Moreover, we studied the global levels of DNA methylation and hydroxymethylation that reflected the net gain or loss across the genome in PFC tissue. The precise locus specificity of the observed changes must be investigated in the future. An analysis of the mechanisms of DNA methylation loss may shed light on potential ways to prevent or mitigate tumor brain and chemo brain. Several studies have shown that alterations in DNA mechanisms led to a reversal of drug resistance in cell line models of breast cancer (Luzhna and Kovalchuk, 2010).

The observed loss of DNA methylation may be linked to altered gene expression and genome stability. As such, it would be prudent to link gene expression changes with locus-specific alterations in DNA methylation, as this would allow the establishment of mechanistic links between the two phenomena in the context of tumor brain and chemo brain. Epigenetic marks are tissue-specific, but for diagnostic purposes, the analysis of patient brain tissue is, not possible. Several studies have investigated epigenetic patterns in the brain, blood, and saliva. They reported a high correlation between blood and brain DNA methylation patterns (Horvath et al., 2012, Tylee et al., 2013, Davies et al., 2012). Furthermore, DNA methylation patterns in saliva correlated strongly with DNA methylation patterns in the brain (Smith et al., 2015). Therefore, it would be critical to analyze molecular epigenetic changes in the blood of PDX animals and correlate those with brain changes to establish possible mechanisms and the relationship between the

two. Blood-based liquid biopsy markers may, therefore, help establish a timeline of changes in tumor brain and chemo brain, as well as for clinically significant biomarkers.

Outlook

Both chemo brain and tumor brain were first reported in breast cancer; hence, we focused on breast-cancer PDX models. In this study, we analyzed mice that carried PDXs of T4 TNBC and T4 PR+BC tumors. In the future, it would be important to analyze tumor brain as a function of breast tumor type, stage, and grade. Clinical evidence shows that chemo brain occurs in other malignancies, including hematological malignancies, sarcoma, colon, and other cancers (Kaiser et al., 2014, Olson et al., 2016). It would be prudent to analyze tumor brain in the PDX models of these other cancers. Some changes may be tumor-specific, but some may be common for all tumor brain manifestations.

Changes seen in this study were observed after three weeks of treatment and 3-4 months of tumor propagation. Because of this, it is not possible to pinpoint when the changes occurred, which changes were primary, and which were secondary. It would be important to analyze changes as a function of time. Additionally, we focused on the analysis of molecular changes in the PFC tissues of TumorGraft animals. The PFC has been associated with the execution of functions such as planning, decision-making, behavioural inhibition, and working memory, to name a few (Faw, 2003). In our earlier study we noted that cytotoxic chemotherapy profoundly affected the PFC (Kovalchuk et al., 2016c). Divided into the medial PFC and orbital prefrontal cortex in rodents, the PFC receives dopaminergic inputs from the ventral tegmental area and connects with virtually all regions of the forebrain. Stress and psychoactive drugs both profoundly alter neuronal morphology in the subregions of the PFC (Muhammad et al., 2012). In a follow-up study

we will examine neuronal morphology in the PFCs of intact, tumor-bearing, and chemotherapy-treated TumorGraft mice.

It would also be important to extend tumor brain studies to other brain regions. Of those, the hippocampus is one of the main sites of adult neurogenesis. In adult mammals, neurogenesis occurs primarily in two germinal zones: the subgranular zone (SGZ) of the DG and the subventricular zone (SVZ)(Drew and Hen, 2007). Chemotherapy is known to affect neurogenesis. In the future, it would be essential to determine molecular manifestation of tumor brain and chemo brain in the hippocampus, and to analyze cell migration, cell number, and the number of newborn neurons in the dentate gyrus. It would also be crucial to correlate molecular and cellular changes with behavioral repercussions. These may serve as foundations for development of novel strategies for prevention and mitigation of both tumor brain and chemo brain.

While we noticed that the growth of malignant non-CNS tumors caused profound molecular changes in the PFC tissues of TumorGraft mice, chemotherapy-induced changes were rather modest, and no synergistic or additive affects were noted. This is an intriguing and unexpected finding, which, to our mind, may be due to the effectiveness of chemotherapy in reducing tumor growth. Tumor growth caused significant molecular changes in PFC tissue. All of the used chemotherapy regimens caused significant reduction in tumor volume. If tumor growth is an important culprit in tumor and chemo brain, lack of an additive effect of chemotherapy may be explained, at least in part, by tumor volume reduction. In the future, it would be important to analyze tumor brain and chemo brain as a function of chemotherapy effectiveness.

Additionally, in our earlier study we reported that cytotoxic chemotherapy agents mitomycin C and cyclophosphamide affected DNA methylation and caused oxidative stress, and that chemotherapy-induced effects were similar to aging-related processes. Moreover, recent clinical analysis suggested a link between brain aging and cancer treatments (Kesler, 2014). The molecular changes observed in this tumor brain study – altered gene expression, oxidative damage, reduced OGG1levels, and altered levels of DNA methylation and hydroxymethylation – are known signs of neurodegeneration and brain aging (Fig. 2.8) (Irier and Jin, 2012, van den Hove et al., 2012, Swain and Rao, 2012, Romanucci and Della Salda, 2015). The present study suggests links between tumor brain and brain aging, and provides an important roadmap for future analysis. Also, this study lays a foundation for the large-scale analysis of the molecular and cellular mechanisms of tumor brain.

Materials and methods

Animal model

Brain tissues of TumorGraft mice were provided by Champions Oncology, Inc. (Baltimore, MD). Patients from the United States diagnosed with triple negative breast cancer (TNBC) and progesterone positive breast cancer (PR+BC) had their tumors engrafted to generate a personalized TumorGraft patient-derived xenograft (PDX) mouse model. In this model, a fresh specimen of the patient's tumor is removed during surgery and fragments of the tumor measuring approximately 4 mm^3, containing both malignant cells and supportive stromal components, are implanted subcutaneously into the flanks of 6-week-old immunodeficient female mice and propagated as previously described (female *nu*/*nu* athymic mice; Harlan Laboratories, Indianapolis, IND) (Bertotti et al.,

2011, DeRose et al., 2011, Hidalgo et al., 2011, Morelli et al., 2012, Stebbing et al., 2014). Patients provided informed consent documents that followed all federal regulatory requirements and covered the use of tumor material for research purposes. Animal treatments were conducted in accordance with the Institutional Animal Care and Use Committee protocols. Upon propagation, when TumorGrafts reached a volume greater than 200 mm^3, the animals were divided into groups of 3 to 4, and dosing of drugs or drug combinations was implemented according to the individual physician's choice and in consultation with the specific patient, based on the well-established Champions Oncology protocols. Starting volumes varied between different TumorGraft models because of the individual doubling time. TNBC PDX-bearing TumorGraft animals were treated with Doxorubicin/Paclitaxel/Cyclophosphamide (n=4 treated and 4 untreated), and PR+BC animals with Topotecan (PR+BC/TOP) or Crizotinib (PR+BC/CRIZ) (n=3 treated and untreated). Intact animals of the same strain (n=3) served as control. Champions Oncology conducted chemotherapy treatments, and all chemotherapy agents were formulated according to manufacturer's specifications. Doxorubicin (3mg/kg IV)/Paclitaxel (20mg/kg IV)/Cyclophosphamide (100mg/kg IP), and Topotecan (1mg/kg IP) treatments were carried out according to a Q7Dx3 schedule (every 7 days for 3 doses). Crizotinib treatments (40mg/kg PO) were conducted based on the QDx21 schedule (one dose every day for 21 days). Animals were treated for 21 days.

To monitor chemotherapy effects, tumor dimensions were measured twice weekly and tumor volume was calculated as described (Stebbing et al., 2014). In both cases, chemotherapy applications resulted in successful reduction of tumor growth (data not shown). Upon completion of treatments animals were euthanized, and the brains were

removed from the skulls and immediately flash-frozen in liquid nitrogen and stored in -80C for molecular analysis.

Gene Expression Analysis

The prefrontal cortex (PFC) tissues of three–four animals per group were used for the analysis of gene expression profiles. RNA was extracted from PFC tissue using TRIzol® Reagent (Invitrogen, Carlsbad, CA), further purified using an RNeasy kit (Qiagen), and quantified using Nanodrop2000c (ThermoScientific). Afterwards, RNA integrity and concentration were established using 2100 BioAnalyzer (Agilent). Sequencing libraries were prepared using Illumina's TruSeq RNA library preparation kits, and global gene expression profiles were determined using the Next 500 Illumina deep-sequencing platform at the University of Lethbridge Facility. Statistical comparisons between the control and exposed groups within each tissue type were performed using the DESeq Bioconductor package (version 1.8.3) and the baySeq Bioconductor package (version 1.10.0). Clustering of the samples was assessed with multidimensional scaling (MDS) plots built using the plotMDS function from the edgeR Bioconductor package. MA plots showing the relationship between the average level of expression and the log2 fold change were created for each of the comparisons. The MA-plot is a plot of the distribution of the red/green intensity ratio ("M") plotted by the average intensity ("A"). Features with a false discovery rate (FDR) < 0.1 (10% false positive rate) were considered differentially expressed between conditions.

The functional annotations of differentially expressed genes were performed using David, GO (Gene Ontology) Elite, and GO-TermFinder (Boyle et al., 2004). Pathways were visualized using Pathview/KEGG and DAVID bioinformatics platforms DAVID

Bioinformatics Resources 6.7 KEGG Pathway platforms (Huang da et al., 2009b, Huang da et al., 2009a, Huang et al., 2007).

Analysis of 8-oxo-7-hydrodeoxyguanosine, 5-methylcytosine, and 5-hydroxymethylcytosine in DNA

DNA was extracted from PFC tissues using the Qiagen DNeasy Kit. The levels of 8-oxodG, 5mC, and 5hmC in the DNA of mouse PFC tissues were measured by liquid chromatography combined with electrospray tandem mass spectrometry (LC-MS/MS) as previously described (Shpyleva et al., 2014, James et al., 2013, Kovalchuk et al., 2016c).

Analysis of BMP4 promoter methylation

BMP4 promoter methylation was analyzed using the EpiTect Methyl II DNA Restriction Kit and the EpiTect Methyl II PCR Primer Assay for Mouse Bmp4 (CpG Island 103407) (SABiosciences/ Qiagen, Toronto, Ontario) following manufactuurer's instructions.

Western immunoblotting

Western immunoblotting was conducted as previously described (Silasi et al., 2004, Kovalchuk et al., 2016b, Kovalchuk et al., 2016c). In brief, around 50 mg of PFC tissues were sonicated in ice-cold 1% SDS and immediately boiled. Protein concentrations were ascertained using the Bradford assay (BioRad, Hercules, CA). Equal amounts of protein (10-30 μg) were separated by SDS-PAGE into slab gels of 10-15% polyacrylamide and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Baie d'Urfé, Quebec). The membranes were incubated with primary antibodies against APE1, OGG1, DNMT1, DNMT3A, MeCP2, BMP4, DNMT3B, Laminin 1-2 (1:1000, Abcam), and actin (1:2000, Abcam) overnight at 4° C. Primary antibody binding was detected

using horseradish peroxidase-conjugated secondary antibodies and the Enhanced Chemiluminescence Plus System (Amersham Biosciences, Baie d'Urfé, Quebec). Chemiluminescence was detected using a FluorChem HD2 camera with FluorChem software (Cell Biosciences). The membranes were stained with Coomassie blue (BioRad, Hercules, CA) to confirm equal protein loading. Signals were quantified using NIH Image J64 software and normalised relative to actin or Coomassie staining.

Statistical analyses

Statistical analysis (Student's t-test) was performed using the Microsoft Excel software package.

Figures

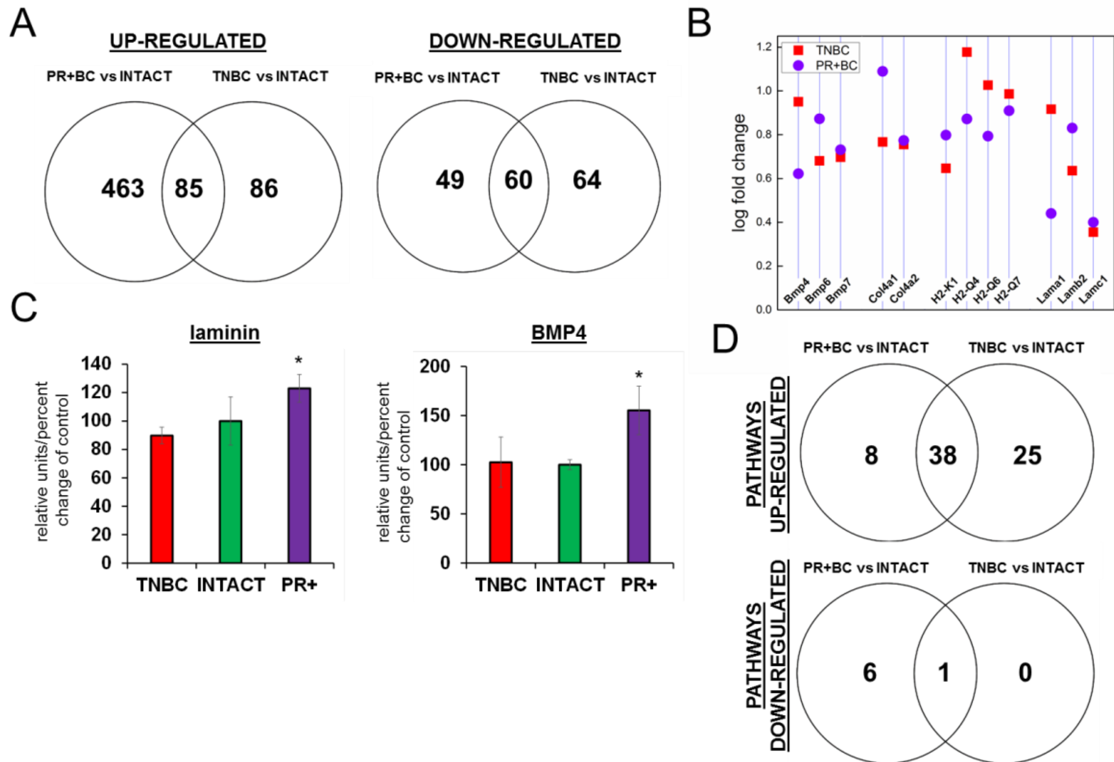

Figure 2.1: Next generation sequencing-based analysis of gene expression in the PFC tissues of intact and TNBC and PR+BC-bearing TumorGraft mice. (A) Venn diagram showing genes that were significantly different between TNBC and PR+BC mice, as compared to intact controls; (B) Fold changes in the levels of expression of several selected genes. Genes were selected based on their fold expression change and potential functional significance; (C) Western immunoblotting analysis of laminin and BMP4 proteins in the PFC tissues of TNBC and PR+BC mice; data are shown as relative units/percent change from control. Due to size difference the same membrane was used for both proteins. * $p<0.05$, Student's *t*-test; (D) Summary of molecular pathways that were altered in the PFCs of TNBC and PR+BC mice as compared to intact controls. The Pathview/KEGG analysis was used to determine differentially affected pathways.

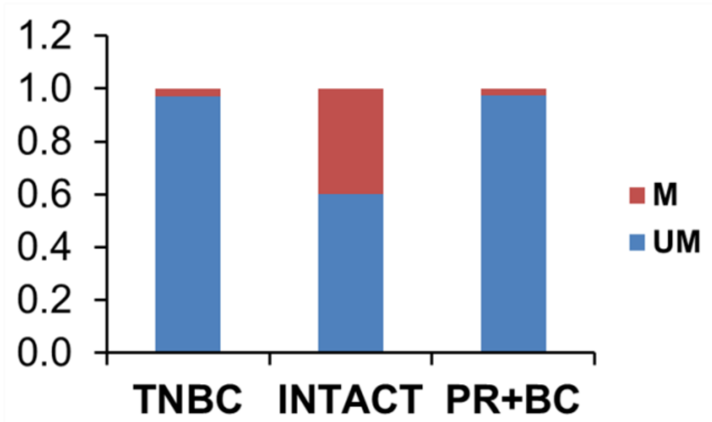

Figure 2.2: Levels of BMP 4 promoter methylation. UM-unmethylated, M-methylated; n=3 for intact and TNBC animals, and 4 for PR+BC animals.

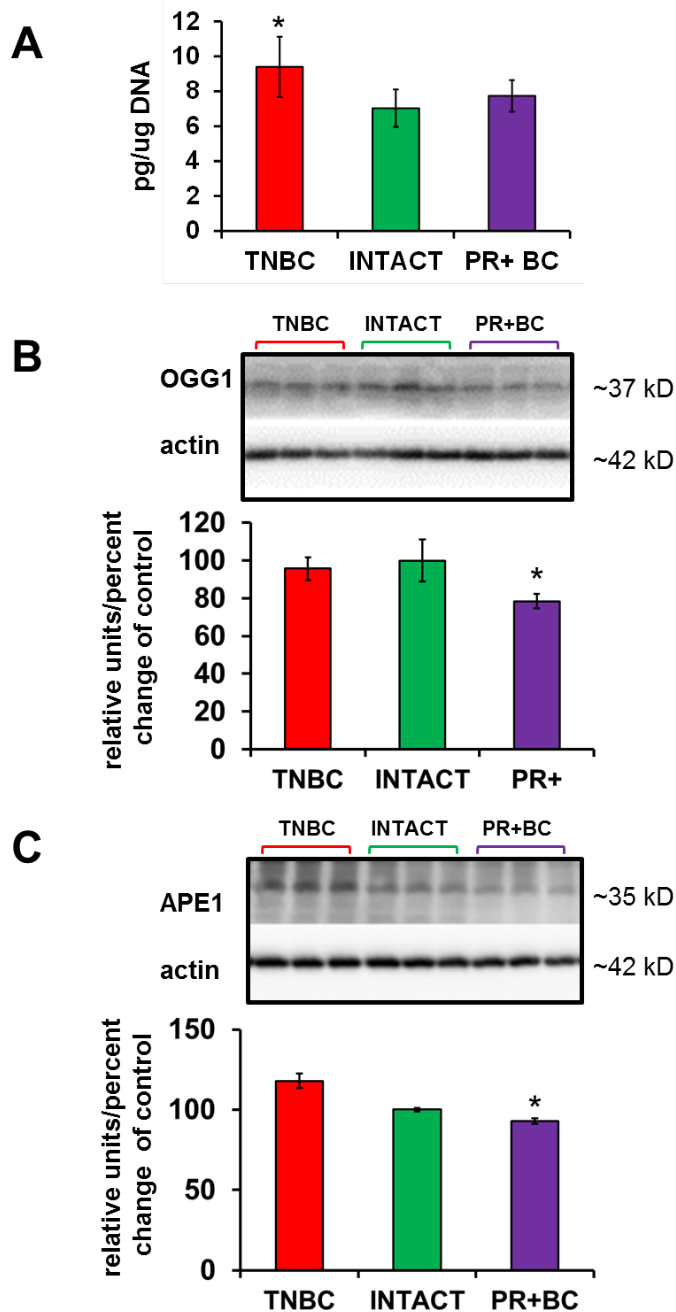

Figure 2.3: Oxidative DNA damage in PFC tissues of intact and TNBC and PR+BC-bearing TumorGraft mice. (A) Levels of 8-oxo-7-hydrodeoxyguanosine (8-oxodG) in genomic DNA isolated from PFC tissues (mean ± SD, n=3 for intact and TNBC animals, and 4 for PR+BC animals); (B) Western immunoblotting analysis of the base excision repair protein OGG1; data are shown as relative units/percent change of control. Each line represents an individual animal, with 3 animal samples/group. Due to protein size differences and scarcity of tissue, membranes were re-used several times. * - significantly different from control mice, $p<0.05$, Student's *t*-test.

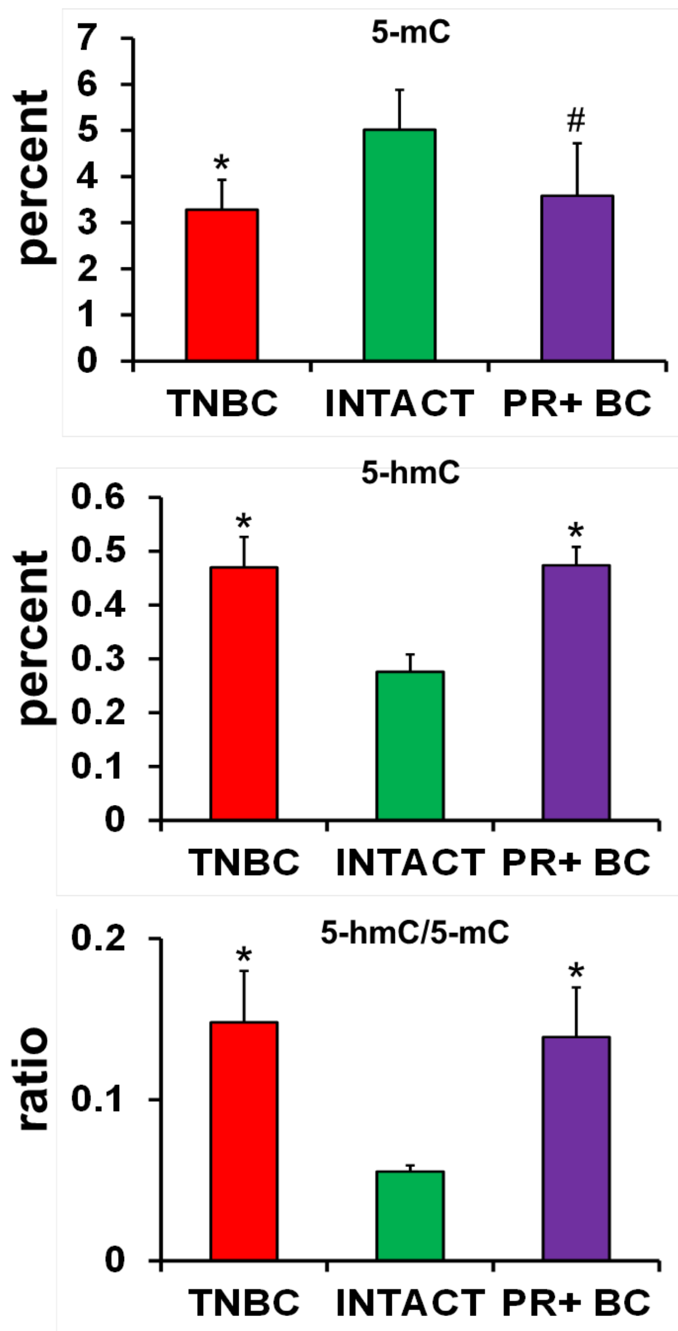

Figure 2.4: Levels of 5-mC and 5-hmC and ratio of 5-hmC/5-mC in the genomic DNA of PFC tissues of intact and TNBC and PR+BC-bearing TumorGraft mice. N=3 for intact and TNBC animals, and 4 for PR+BC animals. * $p<0.05$, # $p<0.10$, Student's *t*-test.

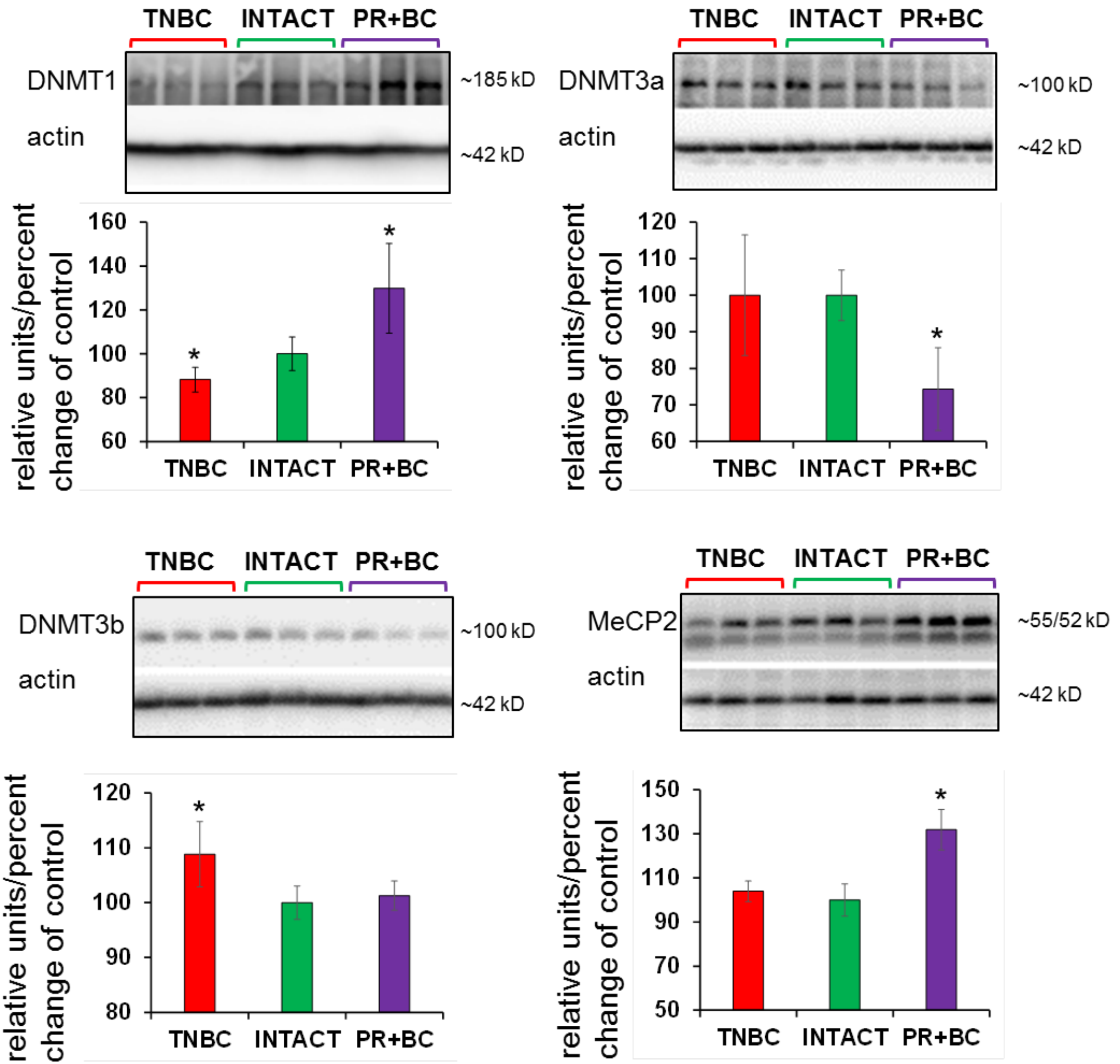

Figure 2.5: Levels of DNA methyltransferases DNMT1 and DNMT3a, and methyl-CpG binding protein MeCP2, in in the PFC tissues of intact and TNBC and PR+BC-bearing TumorGraft mice. Data are shown as relative units/percent change of control. Each line represents an individual animal sample, with 3 samples/group. Due to protein size differences and scarcity of tissue, membranes were re-used several times. Significantly different from control mice - * p<0.05, # p<0.10, Student's *t*-test.

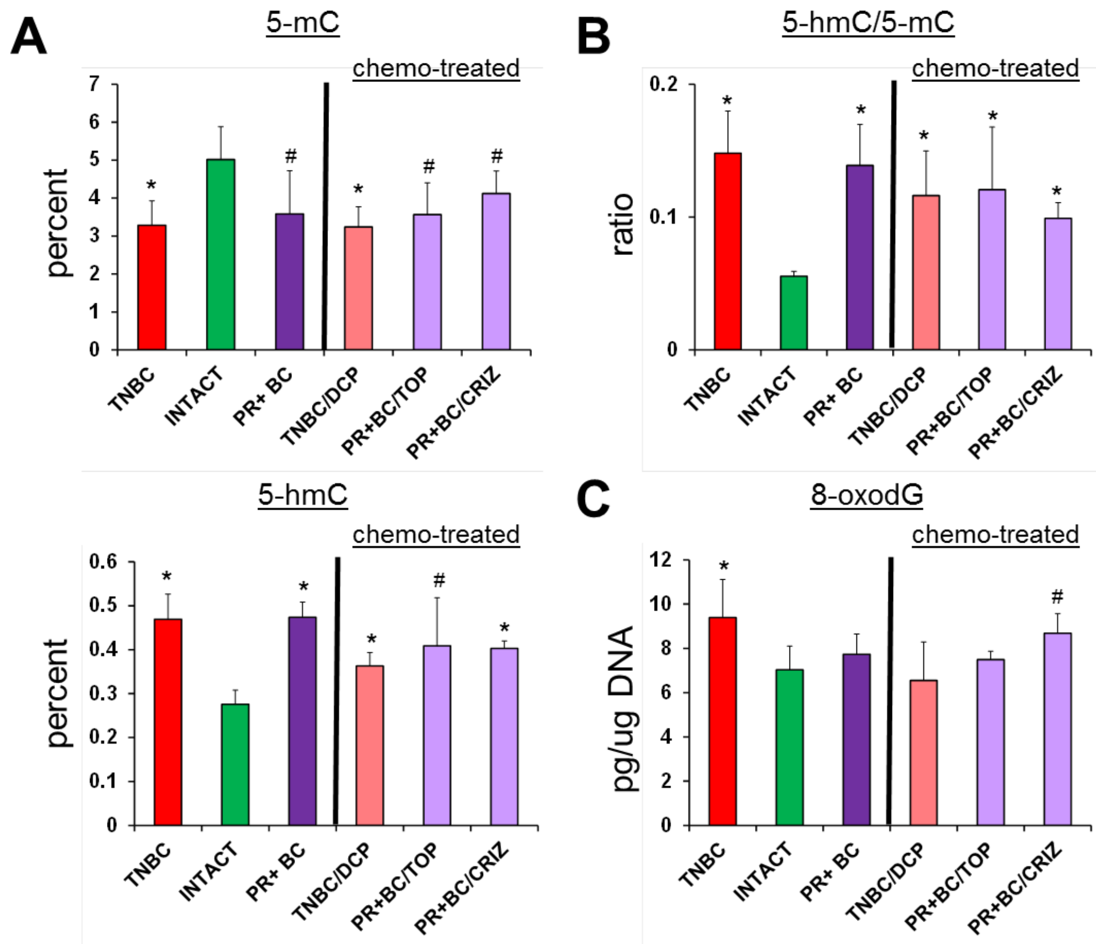

Figure 2.6: Tumor vs. chemo brain – levels of 8-oxodG, 5-mC and 5-hmC, and ratio of 5-hmC/5-mC in genomic DNA isolated from the PFC tissues of TNBC and PR+BC-bearing chemotherapy treated and untreated TumorGraft mice. N=3 for intact and TNBC animals, and 4 for PR+BC animals. Significantly different from intact control mice -* $p<0.05$, # $p<0.10$, Student's *t*-test.

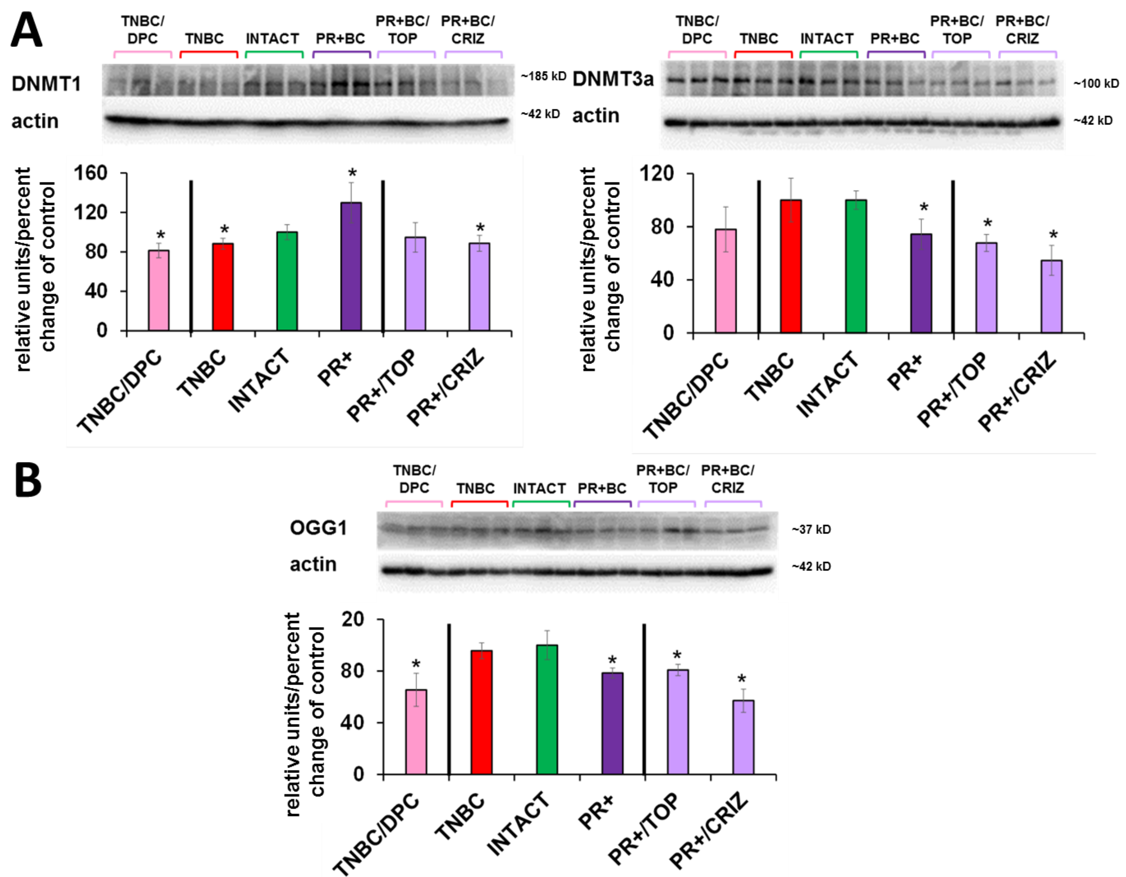

Figure 2.7: Levels of DNA methyltransferases DNMT1 and DNMT3a, methyl-CpG binding protein MeCP2, and oxidative damage repair protein OGG1, in in the PFC tissues of intact and TNBC and PR+BC-bearing chemotherapy treated and untreated TumorGraft mice. (A) DNA methyltransferases and MeCP2 protein; (B) OGG1 protein. Data are shown as relative units/percent change of control. Each line represents an individual animal sample, with 3 samples/group. Data from chemotherapy-treated animals are shown along with intact controls and tumor-bearing untreated animals (also see Fig. 2B and 4). Due to protein size differences and scarcity of tissue, membranes were re-used several times. Significantly different from control mice -* $p<0.05$, # $p<0.10$, Student's *t*-test.

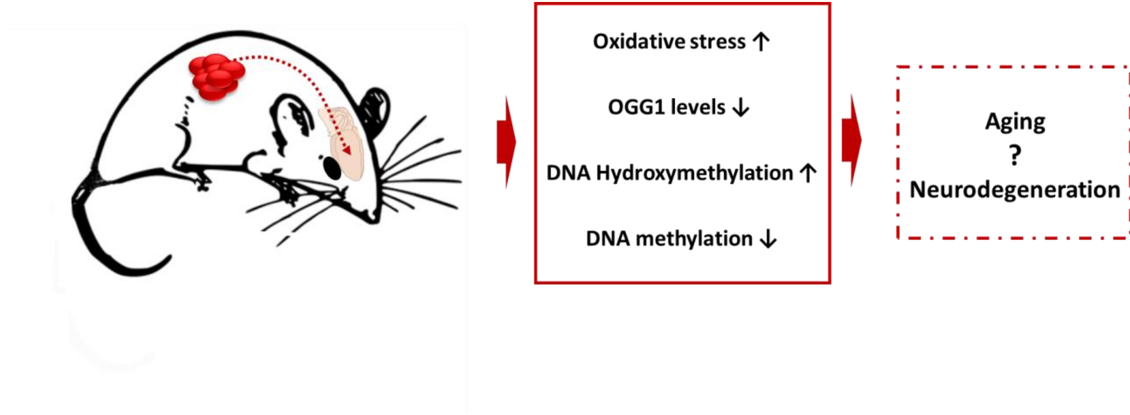

Figure 2.8: Tumor brain-induced changes may be connected to the aging and neurodegeneration - model scheme.

CHAPTER 3: GROWTH OF MALIGNANT EXTRACRANIAL TUMORS ALTERS MICRORNAOME IN THE PREFRONTAL CORTEX OF TUMORGRAFT MICE

Chapter 3 has been accepted for publication in its entirety:

Kovalchuk, A., Yaroslav Ilnytskyy, Y., Rodriguez-Juarez, R., Katz, A., Sidransky, D., Kolb, B., Kovalchuk, O. Growth of malignant extracranial tumors alters microRNAome in the prefrontal cortex of TumorGraft mice. *Oncotarget* (in press).

Abstract

A wide array of central nervous system complications, neurological deficits, and cognitive impairments occur and persist as a result of systemic cancer and cancer treatments. This condition is known as **chemo brain** and it affects over half of cancer survivors. Recent studies reported that cognitive impairments manifest before chemotherapy and are much broader than chemo brain alone, thereby adding in **tumor brain** as a component. The molecular mechanisms of chemo brain are under-investigated, and the mechanisms of tumor brain have not been analyzed at all. The frequency and timing, as well as the long-term persistence, of chemo brain and tumor brain suggest they may be epigenetic in nature. MicroRNAs, small, single-stranded non-coding RNAs, constitute an important part of the cellular epigenome and are potent regulators of gene expression. miRNAs are crucial for brain development and function, and are affected by a variety of different stresses, diseases and conditions. However, nothing is known about the effects of extracranial tumor growth or chemotherapy agents on the brain microRNAome.

We used the well-established TumorGraft ™ mouse models of triple negative (TNBC) and progesterone receptor positive (PR+BC) breast cancer, and profiled global microRNAome changes in tumor-bearing mice upon chemotherapy, as compared to untreated tumor-bearing mice and intact mice. Our analysis focused on the prefrontal cortex (PFC), based on its roles in memory, learning, and executive functions, and on published data showing the PFC is a target in chemo brain.

This is the first study showing that tumor presence alone significantly impacted the small RNAome of PFC tissues. Both tumor growth and chemotherapy treatment affected

the small RNAome and altered levels of miRNAs, piRNAs, tRNAs, tRNA fragments and other molecules involved in post-transcriptional regulation of gene expression. Amongst those, miRNA changes were the most pronounced, involving several miRNA families, such as the miR-200 family and miR-183/96/182 cluster; both were deregulated in tumor-bearing and chemotherapy-treated animals. We saw that miRNA deregulation was associated with altered levels of brain-derived neurotrophic factor (BDNF), which plays an important role in cognition and memory and is one of the known miRNA targets. BDNF downregulation has been associated with an array of neurological conditions and could be one of the mechanisms underlying tumor brain and chemo brain. In the future our study could serve as a roadmap for further analysis of cancer and chemotherapy's neural side effects, and differentially expressed miRNAs should be explored as potential tumor brain and chemo brain biomarkers.

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Introduction

Initial reports on the cognitive changes associated with cancer chemotherapies appeared in the late 1970s to the mid-1980s, but received scientific attention starting only in the mid-1990s. Several studies reported significant cognitive changes in cancer survivors (Andreotti et al., 2016). These changes included problems with concentration, learning and memory, and executive functions. Central nervous system (CNS) toxicity manifestations had major negative effects on patient's quality of life. Most of the chemotherapy-induced cognitive deficits were reported in breast cancer patients, in which deficits affected up to 50% of survivors and lasted for more than 10 years (Ahles et al., 2002). These effects were so significant that breast cancer survivors even coined a term for them—"chemo fog" or "chemo brain" (Kaiser et al., 2014, Vardy and Tannock, 2007). The latter term is now widely accepted and used to describe the CNS toxicity of chemotherapy (reviewed in (Ahles, 2012, Kaiser et al., 2014)). A large-scale study following 200 breast cancer survivors for 21 years post chemotherapy reported on the long-term persistence of changes in executive functioning, verbal memory, and processing speed; likewise, they saw reductions of grey matter volume, and changes in white matter microstructural integrity (Koppelmans et al., 2012a, Koppelmans et al., 2014, Koppelmans et al., 2012b). While the majority of chemo brain reports come from the analysis of breast cancer survivor cohorts, chemo brain has also been reported in lymphoma, leukemia, lung, gastrointestinal, and other cancers, and has been accepted as a form of general post-chemotherapy CNS toxicity (Vardy and Tannock, 2007, Olson et al., 2016).

The clinical analysis of chemo brain's molecular mechanisms is complicated due to varying treatment protocols, significant inter-patient variability (as a result of co-morbidities), and various other factors (Yang et al., 2012, Wefel et al., 2011, Myers, 2010). Hence, the majority of chemo brain mechanistic studies used cell lines and rodent models (reviewed in (Seigers and Fardell, 2011, Seigers et al., 2015)), to determine that chemotherapy caused oxidative stress, suppressed neuronal proliferation and differentiation, induced apoptosis, affected the levels of histone modification and chromatin remodeling, caused the aberrant expression of genes, and altered the levels of neurotrophic and neurogenic proteins (Myers et al., 2008, Mustafa et al., 2008, Joshi et al., 2010). These molecular changes were associated with altered neurogenesis and deficits in learning and memory processes (Mustafa et al., 2008, Briones and Woods, 2011, Christie et al., 2012, Myers, 2012).

Recent studies reported that cognitive impairments manifest before chemotherapy in 20%–30% of breast cancer patients and that these phenomena might be much broader than chemo brain alone. They may instead constitute both cancer and cancer treatment-associated cognitive changes (Ahles, 2012), thereby adding in the tumor brain component. While these studies provided significant insights into chemo brain, much remains to be learned about its molecular and cellular mechanisms. The molecular mechanisms of tumor brain has not been analyzed at all.

The frequency and timing, as well as the long-term persistence, of chemo brain and tumor brain suggest they are epigenetic in nature. Epigenetic changes encompass meiotically heritable and mitotically stable alterations that regulate gene expression and genome stability; these include DNA methylation, histone modification, and non-coding

RNA regulation (Wang et al., 2015a). Among small non-coding RNAs, microRNAs (miRNAs) are the most studied. MiRNA loci are transcribed by polymerase II, which gives rise to primary miRNA transcripts (pri-miRNAs). Portions of pri-miRNAs form hairpin structures that are cleaved and released by the action of ribonuclease Drosha, producing precursor miRNAs (pre-miRNAs). Next, pre-miRNAs are exported to the cytoplasm with the help of the Exportin 5 system, where the ribonuclease Dicer cleaves them, yielding mature single-stranded miRNA. Together with RNA-induced silencing complex (RISC) proteins such as Ago2, miRNAs interact with their associated mRNAs, thereby regulating the production of proteins. MiRNAs are crucial for brain development, neuronal differentiation, and axon regeneration following injury (Radhakrishnan and Alwin Prem Anand, 2016, Miska et al., 2004, Sempere et al., 2004),(Li et al., 2016). They regulate neuronal plasticity, and are likewise involved in the regulation of learning and memory (Wang et al., 2012, Bredy et al., 2011, Saab and Mansuy, 2014). Interestingly, miRNA deregulation was reported in autism (Hicks and Middleton, 2016), Alzheimer's and Parkinson's diseases, traumatic brain injury, stroke, amyotrophic lateral sclerosis, schizophrenia, and many other diseases and conditions (Karthikeyan et al., 2016, Guedes et al., 2013, Meydan et al., 2016, Koch et al., 2013, Luoni and Riva, 2016, Lukiw, 2007). Dicer and RISC proteins are important for brain development (Howng et al., 2015, Ristori et al., 2015, Saurat et al., 2013), and alterations in the expression of miRNA-processing machinery and RISC members are associated with brain diseases and conditions (Hebert et al., 2010, Fiorenza and Barco, 2016, Jafari et al., 2015, Santarelli et al., 2011). Furthermore, miRNA-based therapies may provide novel approaches for the treatment of neurodegenerative diseases (Pereira et al., 2016), and miRNA profiles

constitute essential diagnostic and prognostic biomarkers of diseases and conditions (Martinez and Peplow, 2016, Cosin-Tomas et al., 2016, Zeng et al., 2015). Various stresses and exposures affect brain microRNAome (Koturbash et al., 2011a, Dasdag et al., 2015, Deng et al., 2014). However, nothing is known about the effects of extracranial tumor growth or chemotherapy agents on the brain microRNAome.

Analyzing these phenomena in tumor-bearing animals is needed to gain a full mechanistic understanding of both chemo brain and tumor brain. In a previous study, we analyzed gene expression and DNA methylation changes in the PFC tissues of triple negative breast cancer (TNBC) and progesterone positive breast cancer (PR+BC) mice. We noted that tumor growth caused changes in gene expression (in submission). We used mouse TumorGraft ™ models of untreated TNBC and PR+BC, as well as TNBC TumorGraft animals treated with Doxorubicin/ Cyclophosphamide/ Paclitaxel (TNBC/DCP) and PR+BC animals treated with Topotecan (PR+BC/TOP) or Crizotinib (PR+BC/CRIZ) to analyze the roles of miRNAs in tumor brain and chemo brain.

Here we report that extracranial malignant tumor growth had a profound effect on the microRNAome of the prefrontal cortex of experimental animals and caused changes in the levels of the miRNA processing machinery protein Ago2. Chemo and tumor brain-induced miRNA changes involved several miRNA families, such as the miR-200 family and miR-183/96/182 cluster, which were deregulated in PR+BC tumor-bearing and chemotherapy-treated animals. MiRNA deregulation was associated with altered levels of BDNF (brain-derived neurotrophic factor), a miRNA target that plays a key role in cognition and memory. Furthermore, deregulated miRNAs may serve as biomarkers of tumor brain and chemo brain.

Results

Descriptive statistics of the next generation sequencing (NGS)

The NGS approach offers excellent technological opportunities to capture the entire repertoire of small RNAs and conduct a comprehensive analysis of the small RNAome (Liu et al., 2011b).A total of 3853355, 3061761, 5573723, 4534609, 4291129, and 3808590 mappable reads were detected from the intact control, TNBC, PR+BC, TNBC/DCP, PR+BC/CRIZ, and PR+BC/TOP animal samples, respectively. These were mapped to various classes of non-coding RNAs (miRNAs, piRNAs, snoRNAs, snRNAs, rRNAs, and tRNAs) (Table 3.1). MicroRNAs constituted the largest part of the small RNA pool, reaching 81.7, 77.8, 79.8, 77.9, 72.4, and 82.1% of all small RNA molecules detected in intact controls, TNBC, PR+BC, TNBC/DCP, PR+BC/CRIZ, and PR+BC/TOP samples, respectively (Table 3.1).

Differential expression of miRNAs in the PFC tissues of tumor-bearing treated and untreated animals

We identified all classes of differentially expressed (DE) small RNAs with a fold change > 2.0 and a false discovery rate cut-off of 0.05. Initial unsupervised hierarchical clustering was performed using all DE small RNAs. We noted a distinct separation between intact control PFC tissues and PFC tissues of tumor-bearing animals (Fig. 3.1A, Fig. 3.2). Next, we focused on DE miRNA analysis (fold change > 2.0 and false discovery rate cut-off of 0.05) and unsupervised hierarchical clustering. Similar to the whole of the small RNAs, we noted a miRNA level based separation of the intact controls from the samples of tumor-bearing treated and untreated animals, proving that generated miRNA signatures discriminate between sample types (Fig. 3.1B).

While there was an apparent separation between the small RNA and miRNAome profiles of the PFC tissues of the intact control and TNBC- and PR+BC-bearing animals, profiles of the PFC samples of tumor-bearing treated animals differed only from those of intact controls and not from the tumor-bearing untreated animals. Amongst samples, TNBC samples clustered better than the PR+BC samples. These differences may depend upon tumour transplantation, positioning and growth in each individual animal and host responses.

Furthermore, sample clustering revealed they are primarily distinguished by the relative expression of a common set of miRNAs (Fig. 3.1B). To gain further insight into the differentially expressed miRNAs and their potential roles in chemo brain and tumor brain, we proceeded to analyze and compare miRNAs that were differentially expressed in various experimental groups.

NGS data revealed that the presence of a malignant extracranial tumor alone had a major effect on the miRNAome. We found that 5 miRNAs were up-regulated in the PFC tissues of TNBC bearing mice, as compared to intact controls. In the PFC tissues of PR+BC bearing mice, 33 miRNAs were up-regulated, and 4 miRNAs were down-regulated. Among those, miR-191-5p was up-regulated in both TNBC and PR+BC tumor-bearing groups (Fig. 3.3).

Treatment of TNBC animals with the DCP regimen led to the upregulation of 18 and downregulation of 2 miRNAs in the PFC tissues of tumor-bearing treated animals. Two miRNAs (miR-191-5p and miR-100-5p) were up-regulated in TNBC and TNBC/DCP animals, as compared to intact controls (Fig. 3.4). Interestingly, no

differential miRNA expression was detected between TNBC tumor-bearing treated and untreated animals.

The growth of PR+BC and subsequent chemotherapy treatments also affected the miRNAome profiles of PFC tissues of TumorGraft mice. There, treatment of PR+BC animals with crizotinib led to the upregulation of 13 microRNAs in the PFC tissues of experimental animals, as compared to intact controls. Topotecan treatment led to the upregulation of 37 and downregulation of 7 miRNAs (Fig. 3.5).

We noted a commonality between all three groups (PR+BC, PR+BC/CRIZ, and PR+BC/TOP): miR 200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429), miR-183/96/182 cluster, miR-30d-5p, and miR-191-5p were up-regulated, as compared to intact controls. Moreover, miRNAs of the let-7 family were up-regulated in the PFC tissues of PR+BC untreated and crizotinib-treated animals as compared to intact controls. MiR-409-3p and miR-22-3p were down-regulated in the PFC tissues of the PR+BC untreated and crizotinib-treated PR+BC animals. Similar to the TNBC TumorGraft mice, no differential miRNA expression was identified between PR+BC tumor-bearing treated and PR+BC tumor-bearing untreated mice.

Altered levels of BDNF in the PCF tissues of tumor-bearing treated and untreated animals

MiR-191-5p was up-regulated in all animal groups. Interestingly, miR-191 was recently reported to be a regulator of brain-derived neurotrophic factor (BDNF) (Varendi et al., 2014). BDNF is a member of the nerve growth factor family and one of the fundamental factors regulating neuronal growth, maturation and survival in the brain and spinal cord (Hohn et al., 1990). Among the other miRNAs, the miR-183/96/182 family

and miR-10b target BDNF as well (Varendi et al., 2014, Li et al., 2015). With this in mind, using western immunoblotting, we analyzed the levels of BDNF in the PFC tissues of intact controls, and TNBC and PR+BC tumor-bearing treated and untreated mice.

BDNF levels were significantly decreased in the PFC tissues of PR+BC TumorGraft animals (p=0.00006) and in PR+BC/TOP and PR+BC/CRIZ (p=0.0192 and p=0.0050, respectively), as compared to intact controls. Moreover, levels of BDNF in the PFC tissues of PR+BC/CRIZ were also significantly decreased, as compared to untreated PR+BC animals (p=0.0011). In the untreated TNBC cohort, BDNF levels exhibited a trend to decrease (90% confidence level). No significant change was observed in the TNBC/DPC animals, albeit a down-regulation trend was noted (p<0.10) (Fig. 3.6).

MiRNA processing machinery in the PCF tissues of tumor-bearing treated and untreated animals

To determine the possible mechanisms of aberrant miRNAome changes, we analyzed the levels of Dicer and Ago2 proteins – members of small RNA processing machinery. While no statistically significant changes were noted in the levels of Dicer, levels of Ago2 were significantly up-regulated in the PFC tissues of untreated PR+BC TumorGraft animals (p=0.0010) and in PR+BC/TOP and PR+BC/CRIZ (p=0.00005 and p=0.0020, respectively) (Fig. 3.7)

Discussion

This is the first in-depth analysis of microRNAome profiles in PFC tissues of chemotherapy treated and untreated mouse TumorGraft^{TM} models of triple negative and progesterone receptor positive breast cancer. We saw that the growth of TNBC and PR+BC tumors affects the miRNAome.

While a large body of evidence has accumulated on the incidence of cancer and cancer treatment–related cognitive changes (tumor brain and chemo brain), there is still a lot to learn about their molecular mechanisms. As per the timing and long-term persistence of tumor brain and chemo brain, these phenomena might be epigenetic in nature and based on altered gene expression patterns affecting brain function.

Epigenetic changes governing alterations in gene expression include DNA methylation, histone modification, and small RNA-associated silencing. Amongst the latter, miRNAs (evolutionarily conserved, small, single-stranded RNA molecules that operate as negative gene regulators) are of special interest and significance. MiRNAs are important in brain development and in all CNS functions and processes. They regulate cellular proliferation, differentiation, repair and death, and take part in inflammatory responses. Aberrant expression of miRNAs underlies a wide array of neurological and psychiatric diseases, such as Alzheimer's, Parkinson's, and Huntington's, stroke, traumatic brain injury, amyotrophic lateral sclerosis, autism, schizophrenia, and brain tumors (Kim et al., 2007).

Even so, nothing is known about the role of miRNAs in the brain's response to the combination of malignant non-CNS tumor growth and subsequent chemotherapy. This is the first study to analyze miRNA changes in tumor brain and chemo brain. We showed that TNBC and PR+BC tumor growth and presence significantly altered the miRNAome in the PFC tissues of TumorGraft animals. The PFC is a key regulatory region that receives input from all other cortical areas, and serves to coordinate executive function, motor, cognitive, and social behaviors, attention, and working memory (Lara and Wallis, 2015, Kolb et al., 2012).

While tumor growth-related miRNAome changes were observed in the PFC tissues of both breast cancer groups, they were more pronounced in the PFC tissues of PR+BC-bearing animals than in TNBC-bearing ones. The reasons behind these changes and their molecular mechanisms, along with potential cellular changes and behavioral repercussions need to be further studied and analyzed, and may depend upon the biology of tumors as well as chemotherapy responsiveness. Chemotherapy treatment of TNBC and PR+BC-bearing animals led to further alterations in the miRNAome, and amongst differentially expressed miRNAs, miR-191-5p was up-regulated in all experimental groups (tumor-bearing treated and untreated groups, as compared to intact controls). While this miRNA has not been extensively researched, some studies have reported that miR-191-5p was up-regulated in major depressive disorders and down-regulated in Alzheimer's disease (AD)(Mendes-Silva et al., 2016). This miRNA is commonly down-regulated in the blood and plasma of AD patients and is considered to be a circulating AD biomarker (Kumar and Reddy, 2016). Overall, miRNA expression patterns in the blood and brain do not always fully correlate. Therefore, it would be interesting to analyse the levels of miR-191-5p in the plasma and whole blood of tumor brain and chemo brain animals. This would help to better discern miR-191-5p's roles in tumor brain and chemo brain as a potential molecular driver and biomarker of the two phenomena. Likewise, it would help determine if there exists any long-distance signalling between the tumor and the brain that involves miR-191-5p or any other mRNAs. New studies reported the role of miR-191-5p in systemic inflammatory response syndrome (Caserta et al., 2016). Based on that, a potential next step is analyzing the links, if any, between miR-191-5p

expression and inflammation in chemo brain and tumor brain – especially in light of evidence revealing the role of inflammation in chemo brain (Cheung et al., 2015).

Along with miR-191-5p, miR-100-5p was upregulated in TNBC animals and TNBC/DCP animals, as compared to intact controls. Recently, miR-100-5p was shown to be an upregulated circulating marker of Huntington's disease (Diez-Planelles et al., 2016). Furthermore, this miRNA was said to be involved in the amyloid β-induced pathologies (Ye et al., 2015), whereby, along with miR-99b-5p, it affected neuronal survival by targeting PI3K/Akt/mTOR. In Alzheimer's disease (AD), miR-100-5p was downregulated in the early-middle stages,, but upregulated at the late stages of the disease. Activation of PI3K/Akt signaling promotes neuron survival. Downregulation of miR-100-5p at the early stages of AD may protect neurons from amyloid β-induced apoptosis, while such protection can be lost at later stages when miR-100-5p gets upregulated (Luo et al., 2014). More studies are needed to dissect the roles of miR-100-5p and PI3K/Akt/mTOR in tumor brain and chemo brain and how they relate to the changes induced by TNBC tumor growth and DCP treatment.

DCP treatment caused upregulation of the let-7 family miRNAs – let-7a, 7b, 7g, 7i – in the PFC tissues of TBNC mice, as compared to intact controls. These family members were also upregulated in the PFC tissues of PR+BC-untreated (let-7a, 7b, 7c, 7g, 7i) and PR+BC/topotecan-treated mice (let 7a, 7b, 7c, 7d, 7e, 7g, 7i). The let-7 family is highly conserved in both invertebrates and vertebrates, and was the second such miRNA family to be identified and characterized (Reinhart et al., 2000). Since the different let-7 family members have similar or even identical seed sequences, they likely have overlapping sets of target mRNAs. The let-7 family is one of the well-known tumor-

suppressor miRNA families (Esquela-Kerscher et al., 2008, Kumar et al., 2008) that target RAS (Johnson et al., 2005, Masliah-Planchon et al., 2016), a cellular oncogene. Many tumors exhibit profound downregulation of let-7 (Boyerinas et al., 2010), and the overexpression of let-7 strongly suppresses tumor cell growth. Let-7 levels are usually low in undifferentiated cells. In lung cancer, low let-7 levels are correlated with poor survival (Karube et al., 2005).

The let-7 family is involved in neural development and neuronal differentiation (Sempere et al., 2004, Aranha et al., 2010). In the brain, let-7 levels have been shown to increase after cerebral ischemia/reperfusion injury (Jeyaseelan et al., 2008, Wang et al., 2016b), whereas let-7 suppression inhibited apoptosis and inflammatory responses and caused an overall neuroprotective effect upon cerebral ischemia/reperfusion injury(Wang et al., 2016b). In a rat model of a middle cerebral artery occlusion and subsequent reperfusion injury, let-7 family members were upregulated at the 48-hour reperfusion time point (Jeyaseelan et al., 2008, Di et al., 2014). In *C. elegancs,* several neurons have shown increased levels of *let-7* as they age, whereby let-7 upregulation contributed to the decline of aging neurons' regeneration potential (Ivakhnitskaia et al., 2016). Let-7 microRNAs was downregulated in radiation-exposed neural granule cell progenitors, as well as in medulloblastoma (Tanno et al., 2016). Several other studies have shown radiation-induced downregulation of let-7 family members in cells and tissues, and this downregulation was associated with the altered expression of the DNA damage mediator protein p53 (Saleh et al., 2011, Weidhaas et al., 2007). The let-7 family is also involved in glioma, where the upregulation of let-7b inhibited proliferation, migration and invasion in glioma cell lines. Furthermore, increased levels of let-7b reduced the stemness of

glioma stem-like cells (Song et al., 2016). Overall, upregulation of the let-7 family may viewed as a positive and protective event in the context of cancer and brain metastasis, whereby it acts as a tumor-suppressor and blocks proliferation. Contrarily, in the context of some brain diseases and damage, it may have negative effects as well, as glial proliferation is important in repairing tissue damage. In the future, more studies are needed to dissect the cellular, tissue-specific, and behavioral repercussions of let-7 upregulation in tumor brain and chemo brain.

The most pronounced miRNAome changes were observed in the PR+BC group. PR+BC tumor growth and subsequent chemotherapy treatment with topotecan and crizotinib had profound effects on the miRNAome of TumorGraft animals' PFC tissue. Both tumor growth and chemotherapy treatment led to the upregulation of the miR-200 family, as compared to intact animals. We observed the upregulation of eight miRNAs that make up a related miR-200 family (miR-200a, 200b, 200c, miR-141, miR-429) and a miR-183/96/182 cluster. These miRNAs are often co-transcribed and referred to as the miR-200/182 cluster. It was shown the entire miR-200/182 cluster is upregulated in acute herpes simplex virus 1 encephalitis (Majer et al., 2017).

The miR-200 family is important for the proper balance between neuronal proliferation and differentiation during development (Pandey et al., 2015). Its aberrant expression has been associated with various types of malignant tumors (Zuberi et al., 2015), and was correlated with drug resistance and patients' overall survival(Senfter et al., 2016). A recent study analyzed and compared the miRNA profiles of gastric adenocarcinomas and brain metastatic carcinomas and identified the upregulation of, among others, the miR-200 family members miR-141-3p and miR-200b-3p in the brain

metastatic samples (Korpal et al., 2008). Therefore, deregulation of the miR-200 family could be involved in brain metastasis via the Zeb/miR-200 family feedback loop. Overall, much remains to be discovered about the miR-200 family's roles in various diseases and conditions, including cancer and cancer treatment-associated CNS toxicity.The miR-183/96/182 cluster upregulated in the PR+BC tumor-bearing treated and untreated mice was implicated in hepatocellular carcinoma, breast cancer, and glioma (Leung et al., 2015, Tang et al., 2013, Li et al., 2014). It has also been linked with light-induced retinal injury and was shown to target brain-derived neurotrophic factor (BDNF)(Li et al., 2015). BDNF is also a target of miR-191 that was upregulated in all experimental animal groups, as compared to controls (Varendi et al., 2014).

The negative correlation between the levels of miRNAs that target BDNF and the levels of BDNF protein constitutes an interesting and important finding. We noted that BDNF levels were strongly downregulated in PR+BC tumor-bearing treated and untreated animals; however, TNBC treated and untreated animals exhibited only a trend. BDNF, along with the nerve growth factors neurotrophin-3 and neurotrophin-4/5, is a member of the neurotrophin family (Baydyuk and Xu, 2014). BDNF is a key regulator of neural development, survival, growth, differentiation and plasticity (Bondar and Merkulova, 2016, Mitre et al., 2017) because of its involvement in controlling the expression of pro-survival and anti-apoptosis genes (Baydyuk and Xu, 2014). Furthermore, BDNF is known to modulate synaptic function and plasticity, and is involved in learning and memory (Baydyuk and Xu, 2014). Deletion of the BDNF gene causes dendritic degeneration and neuronal loss, and decreased BDNF levels are associated with cognitive impairments in patients with Parkinson's disease (Wang et al.,

2016a), Alzheimer's disease (Song et al., 2015), depression, and many other neurological and psychiatric disorders. A missense mutation in BDNF was shown to alter cognitive performance post-traumatic brain injury (Narayanan et al., 2016). As one of the main neurotrophic factors, BDNF constitutes a promising remedy for reducing neuronal injury after cerebral ischemia(Harris et al., 2016) and for functional recovery in ALS (Shruthi et al., 2017).

It was reported that depression, which might be a manifestation of tumor brain and chemo brain in cancer patients, is often associated with reduced BDNF serum levels (Wu et al., 2016, Jehn et al., 2015). Low levels of BDNF in cancer patients were connected with depression and poor prognosis(Wu et al., 2016). However, an animal model-based study of doxorubicin and cyclophosphamide chemotherapy-induced chemo brain showed that behavioral deficits (anxiety and spatial cognition impairments) were paralleled by decreased neurogenesis and lowered serum BDNF levels without alterations to BDNF, mRNA, or protein levels in the brain (Kitamura et al., 2015). In our study, we saw significant downregulation of BDNF in PR+BC tumor-bearing treated and untreated mice and a trend towards downregulation in tumor-bearing TNBC animals. Hence, BDNF downregulation may be due to the presence of non-CNS malignant tumors, rather than chemotherapy treatment itself. In context of previous studies, BDNF down-regulation may have negative consequences for both brain and behaviour. Going forward, we would seek to examine the mechanisms of tumor and chemotherapy-induced effects on BDNF levels in conjunction with miRNA. And, in addition, analyze the mechanisms regulating miRNA expression in the brains of tumor-bearing treated and untreated animals, as well as the neuroanatomical and behavioral repercussions of tumor and chemo brain-

associated BDNF downregulation. Furthermore, it would be important to establish and compare the roles of tumor growth and chemotherapy in BDNF regulation, and analyze BDNF regulation as a function of malignant tumor load. As such, successful chemotherapy leads to a reduced tumor load, which by itself may affect BDNF levels.

While we did not see any changes in the Dicer levels, we noted a significant upregulation in the levels of Ago2 in the PFC tissues of PR+BC tumor-bearing treated and untreated animals. Elevated Ago2 levels may, in turn, contribute to the more profound changes in miRNA expression observed in the PR+BC groups. Upregulation of the Ago2, protein that partakes in miRNA production and execution of miRNA-mediated gene silencing, was implicated in the regulation of cocaine addiction and anorexia (Mercader et al., 2012). Moreover, increased levels of Ago2 conveyed very poor prognosis in glioma (Feng et al., 2014). The mechanisms of Ago2 upregulation and its repercussions in tumor brain and chemo brain need to be further studied.

Here we focused on tumor brain and chemo brain as induced by the growth of TNBC and PR+BC tumors, both stage IV. In the future, it would be prudent to look at the miRNAome as a function of tumor stage and grade, as well as to analyse miRNAome deregulation caused by other tumor types. Additionally, we focused our attentions on the PFC; however, previous studies have suggested chemo brain also manifests in the hippocampus (Kitamura et al., 2015). Therefore, it would be interesting to analyze the brain region specificity of chemo brain and tumor brain and to correlate those findings with the roles of miRNAs.

In sum, growth of malignant TNBC and PR+BC tumors altered the miRNAome of tumor bearing treated and untreated animals' PFC. The observed changes may have

opposing functional consequences – some positive and protective and some, negative and deleterious. Among altered miRNAs, miR-183/96/182 cluster and miR-191-5p both target and downregulate BDNF. Here their increased expression was paralleled by a decrease in BDNF. Low BDNF levels have been shown to decrease neuronal survival, growth, differentiation and plasticity, and were reported in a wide array of neurological diseases and conditions. Meanwhile, miR-22 is a well-studied neuroprotective molecule (Ma et al., 2016) and its observed downregulation may have detrimental consequences for cells. The miR-200 family was associated with brain metastases (Korpal et al., 2008), and miR-30d-5p was implicated in medulloblastoma development (Lu et al., 2009). Furthermore, Ago2 protein upregulation is a very negative glioma prognostic factor (Feng et al., 2014) (Fig. 3.8).

Some of the observed miRNA changes could be associated with cellular senescence and aging. Amongst these, oxidative stress-induced upregulation of the miR-200 family caused senescence (Magenta et al., 2011)., and stress-induced cellular senescence was also promoted by the miR-183/96/182 cluster (Li et al., 2009). MiR-191-5p is one of the biomarkers of aging-associated Alzheimer's disease. These miRNAs therefore ought to be explored as potential drivers of aging processes. On the other hand, the let-7 family includes many potent senescence-inhibiting and anti-age miRNAs (Toledano, 2013). Overall, there may be complex interplay between miRNA-mediated pro-and anti-senescence pathways and processes in the PFC tissues of tumor-bearing animals. The precise nature, regulation, and organismal and behavioral repercussions of altered expression in these miRNA families and clusters needs to be examined (Fig. 3.8).

For a bigger picture of tumor brain and chemo brain's mechanisms, we would proceed with identifying molecular pathways that were aberrantly expressed and regulated by miRNAs As such, it would be prudent to analyse the mechanisms, timing, and potential feedback loops between miRNA and mRNA regulation and expression, and integrate transcriptome, small RNAome, as well as methylome. More studies are needed to dissect the effects of miRNAs on signalling pathways and to discern the interplay between the various signalling pathways underlying tumor brain and chemo brain in the context of the entire signalome and interactome, as well as their functional outcomes. We focused on miRNAs, molecules that constitute the largest proportion of differentially regulated small RNAs. In the future, we would seek to analyze the roles of other small RNA molecules, such as tRNAs, snoRNAs, snRNA and others. These could prove to be important tumor brain and chemo brain biomarkers. In summary, this study is the first to show miRNAome deregulation in tumor brain and chemo brain, and may serve as a roadmap for further analysis of cancer and chemotherapy's neural side effects. In the future, in order to gain a full understanding of tumor brain and chemo brain, molecular and cellular data need to be put in context of neuronal structure analysis and behavioral changes.

Materials and methods

Animal model

We used the well-established TumorGraft technology developed by Champions Oncology, Inc. (Baltimore, MD); the frozen brain tissues of TumorGraft mice carrying TNBC and PR+BC patient-derived xenografts (PDX) were provided by Champions Oncology, Inc. Patients diagnosed with TNBC and PR+BC had their tumors engrafted

into mice to generate personalized TumorGraft mouse models for precision oncology approaches. The patients provided their informed consent, which covered the use of tumor material for research purposes. TumorGrafts were generated, as previously described (Bertotti et al., 2011, DeRose et al., 2011, Hidalgo et al., 2011, Morelli et al., 2012, Stebbing et al., 2014). In brief, a fresh specimen of the tumor is obtained during surgery, and small fragments of the tumor containing malignant cells and supportive stromal components are inserted subcutaneously into the flanks of six-week-old immunodeficient female mice (female *nu*/*nu* athymic mice; Harlan Laboratories, Indianapolis, IND) and further propagated as earlier described. Animal experiments were approved by Institutional Animal Care and Use Committee protocols. Following tumor propagation, when TumorGrafts grew larger than 200 mm^3, the animals were divided into groups of three to five. Drug dosage and drug combinations were applied consistent with individual physicians' recommendations and in consultation with the patient, as per standard champions Oncology procedures. The starting volumes differed between individual TumorGraft models because of the varying doubling time involved. As per protocol, TNBC PDX-bearing TumorGraft animals were treated with Doxorubicin (3mg/kg IV)/Paclitaxel (20mg/kg IV)/Cyclophosphamide (100mg/kg IP) every 7 days for 3 doses (TNBC/DPC) (n=4 treated and 4 untreated), and PR+BC animals with Topotecan (1mg/kg IP) every 7 days for 3 doses (PR+BC/TOP) or Crizotinib (40mg/kg PO) one dose every day for 21 days (PR+BC/CRIZ) (n=3 treated and untreated). Animals were treated for 21 days.

Intact animals of the same strain (no tumour, no treatment, n=3) served as baseline controls. The aforementioned chemotherapy agents were prepared according to the

manufacturer's specifications, and Champions Oncology ran all chemotherapy treatments. Tumor growth and animal conditions were strictly monitored; tumor dimensions were regularly measured and tumor volumes were calculated as previously described (Stebbing et al., 2014). In both cases, chemotherapy applications resulted in successful tumor suppression and reduction of tumor growth (data not shown). Upon completion of the treatment, animals were humanely sacrificed; the brains were removed and immediately frozen in liquid nitrogen and stored in −80 °C for molecular analysis. The tissues were split to accommodate DNA, RNA, and protein analysis.

Total RNA isolation, small RNA sequencing, and analysis

Small RNA sequencing was conducted using Illumina next generation sequencing technology as previously described (Krishnan et al., 2015). In brief, total RNA from the PFC tissues was isolated using the RecoverAll Total Nucleic Acid Isolation Kit (Life Technologies). RNA quality and quantity were analyzed with Bioanalyzer 2100 and RNA Nano Chips (Agilent Technologies). Small RNAs were sequenced using TruSeq Small RNA Sequencing Kit (Illumina), TruSeq SR Cluster Kit v5-CS-GA (Illumina) and TruSeq SBS Kit v5-GA (Illumina) according to manufacturer's instructions. All the samples were sequenced on the Illumina Next 500 sequencer using the 36-cycle single-end protocol. Base calling and demultiplexing were done using CASAVA 1.8.2 with default settings, followed by trimming of adapters using Cutadapt version 1.8.dev0 (https://cutadapt.readthedocs.io/en/stable/). The quality of the sequenced reads after adapter trimming was assessed using FASTQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The bowtie v.1.1.2 version(Langmead et al., 2009) was used to map the reads to the reference mouse genome

(UCSC mm10 genome assembly). Trimmed reads were sequentially mapped to various small classes (miRNAs, snRNA, rRNA, snoRNA, tRNAs) and genomic features (repeats, exons and introns), reads that did not map to any of small RNAs or other genomic features were considered unclassified. Further analysis focused on miRNA group. To detect differentially expressed small RNAs. Raw counts of unique tags were loaded in R. Normalization and the detection of differentially expressed tags was done using DESeq2 Bioconductor package (Love et al., 2014). Multiple comparisons adjustment was done using the Benjamini-Hochberg procedure.

Western Immunoblotting

Western immunoblotting was conducted as previously described (Silasi et al., 2004, Kovalchuk et al., 2016a, Kovalchuk et al., 2016b, Kovalchuk et al., 2016c). In brief, around 50 mg of PFC tissues were sonicated in ice-cold 1% SDS and immediately boiled. Protein concentrations were determined using the Bradford assay (BioRad, Hercules, CA). Equal amounts of protein (10-30 µg) were separated by SDS-PAGE into slab gels of 10-15% polyacrylamide and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Baie d'Urfé, Quebec). Eight membranes were prepared. Due to scarce amount of tissues, membranes were re-used and re-probed to allow for analysis of miRNA machinery and targets (this study), as well as epigenetic regulators (Kovalchuk et al., 2017, submitted). The membranes were incubated with primary antibodies against BDNF, Dicer and Ago2 (1:1000, Abcam), and actin (1:2000, Abcam) overnight at 4° C. Primary antibody binding was detected using horseradish peroxidase-conjugated secondary antibodies and the Enhanced Chemiluminescence Plus System (Amersham Biosciences, Baie d'Urfé, Quebec). Chemiluminescence was detected using a FluorChem

HD2 camera with FluorChem software (Cell Biosciences). The membranes were stained with Coomassie blue (BioRad, Hercules, CA) to confirm equal protein loading. Signals were quantified using NIH Image J64 software and normalised relative to actin or Coomassie staining.

Statistical analyses

Statistical analysis (Student's *t*-test) was performed using the Microsoft Excel software package.

Figures

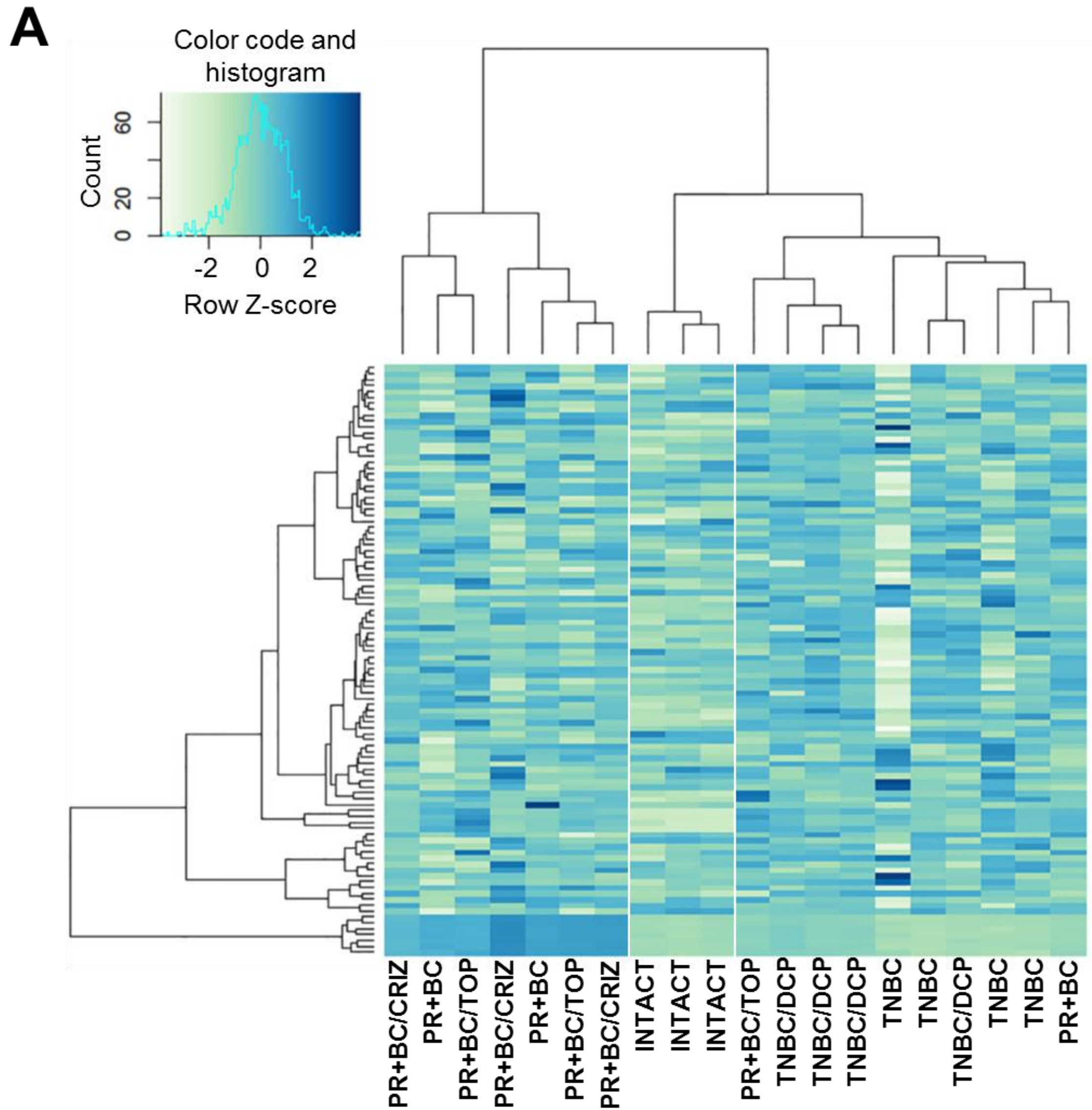
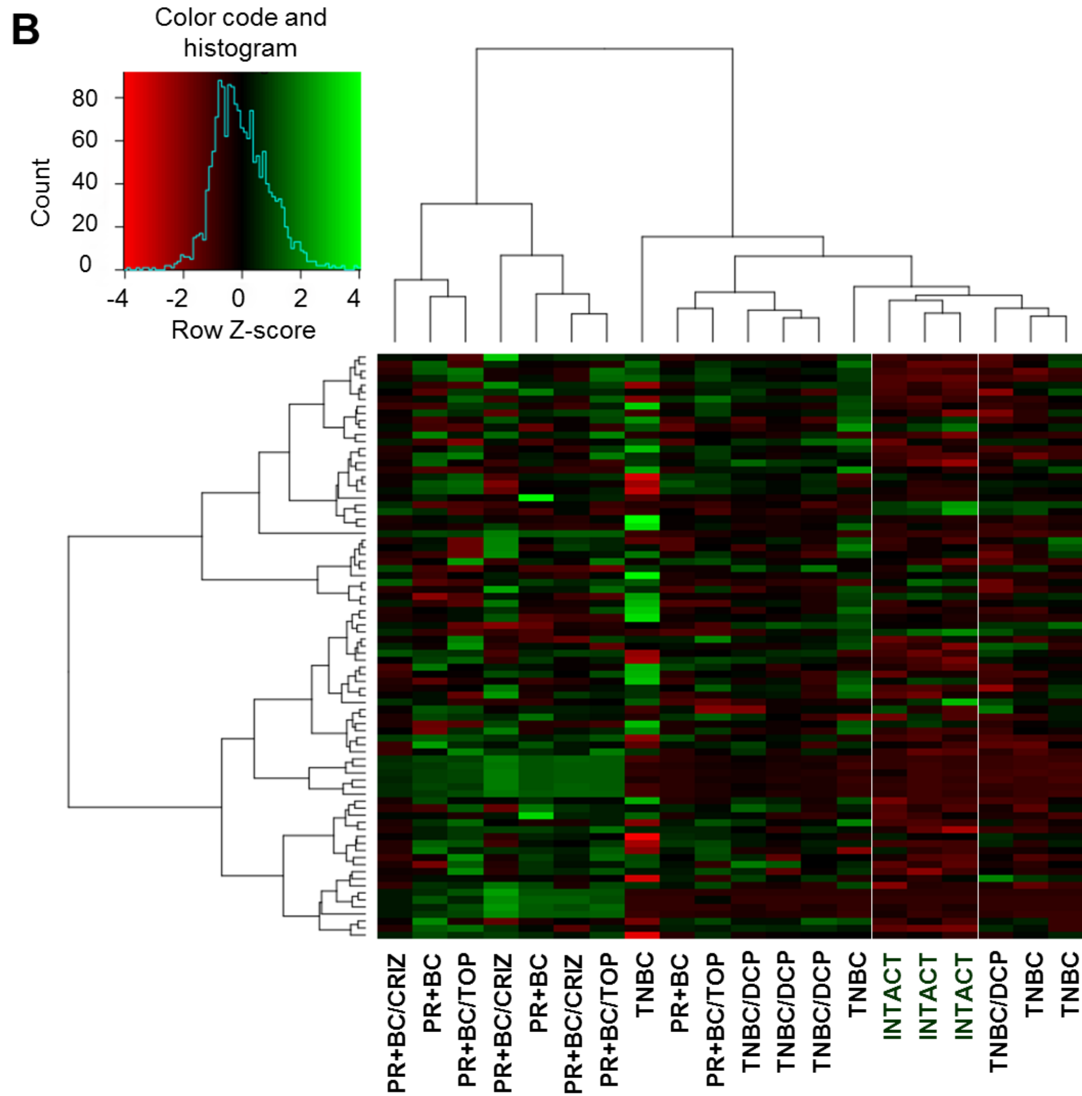


Figure 3.1: Next generation sequencing-based analysis of small RNA expression in the PFC tissues of intact, TNBC and PR+BC-bearing chemotherapy-treated and untreated TumorGraft mice.

A. Hierarchical clustering of all samples based on the entire small RNA profile. Clustering of individual animal samples (columns) based on expression of small RNAs (rows). The Z-score constitutes the number of standard deviations an observation is from the mean.



B. Hierarchical clustering of all samples based on the microRNAome profile.
Clustering of individual animal samples (<u>columns</u>) based on expression of miRNAs (<u>rows</u>). The Z-score constitutes the number of standard deviations an observation is from the mean.

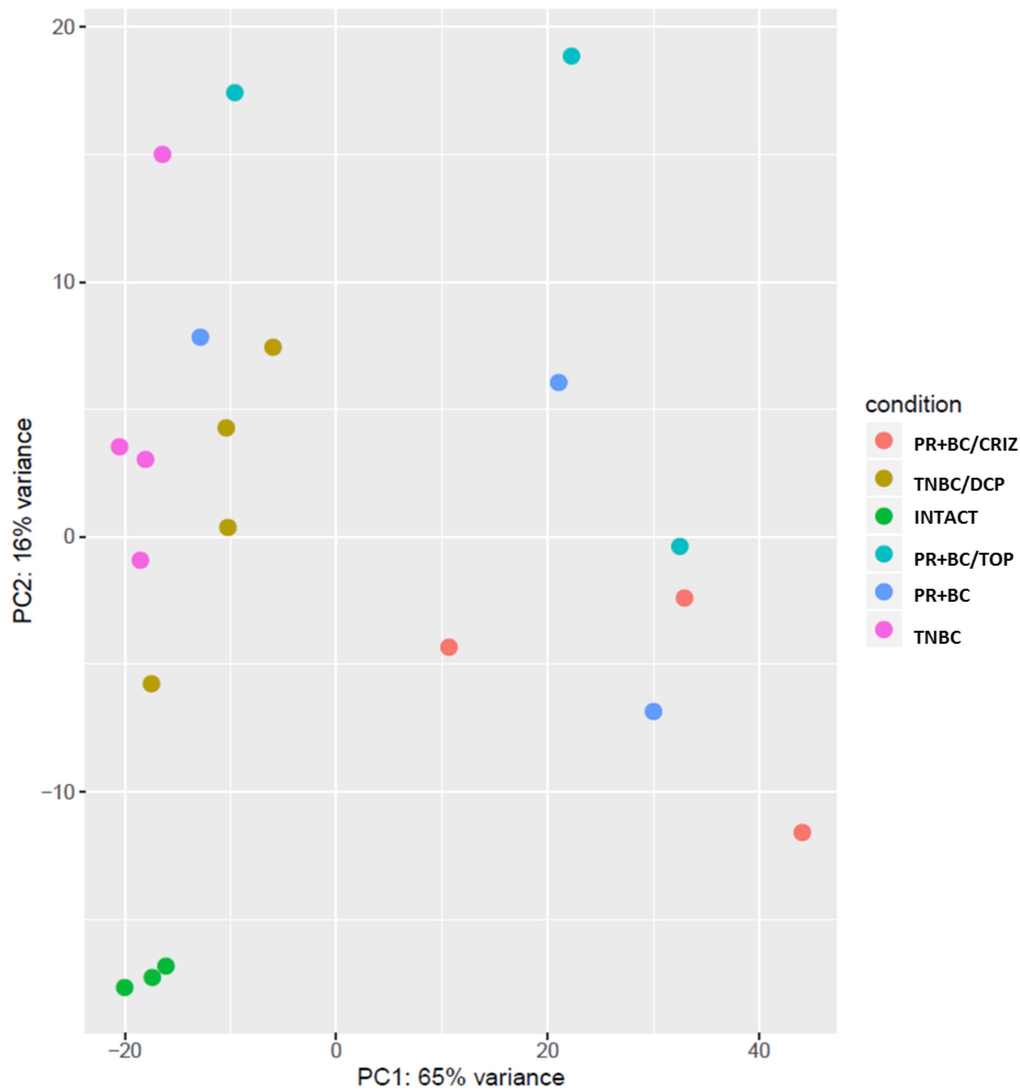


Figure 3.2: Principle component analysis (PCA) for clustering of the small RNA expression data – the PCA plot based on the expression of all of the detectable small RNAs. PCs reflect % variance.

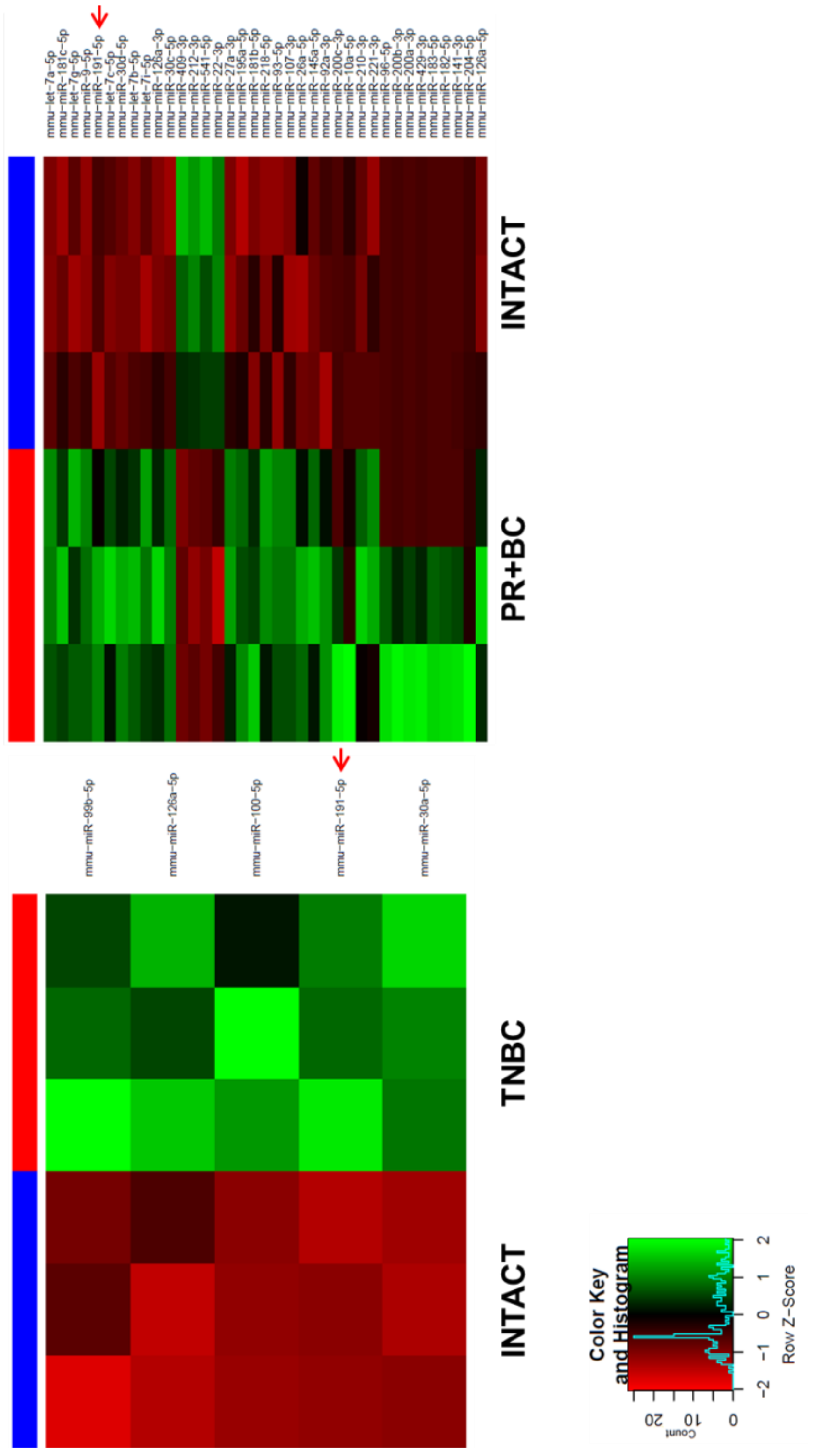


Figure 3.3: Heatmaps of microRNAs differentially expressed in the PFC tissues of the TNBC and PR+BC tumor bearing animals as compared to intact controls. Red arrow indicates miRNAs commonly regulated in both groups. Intact animal group -blue bar; treated group –red bar.

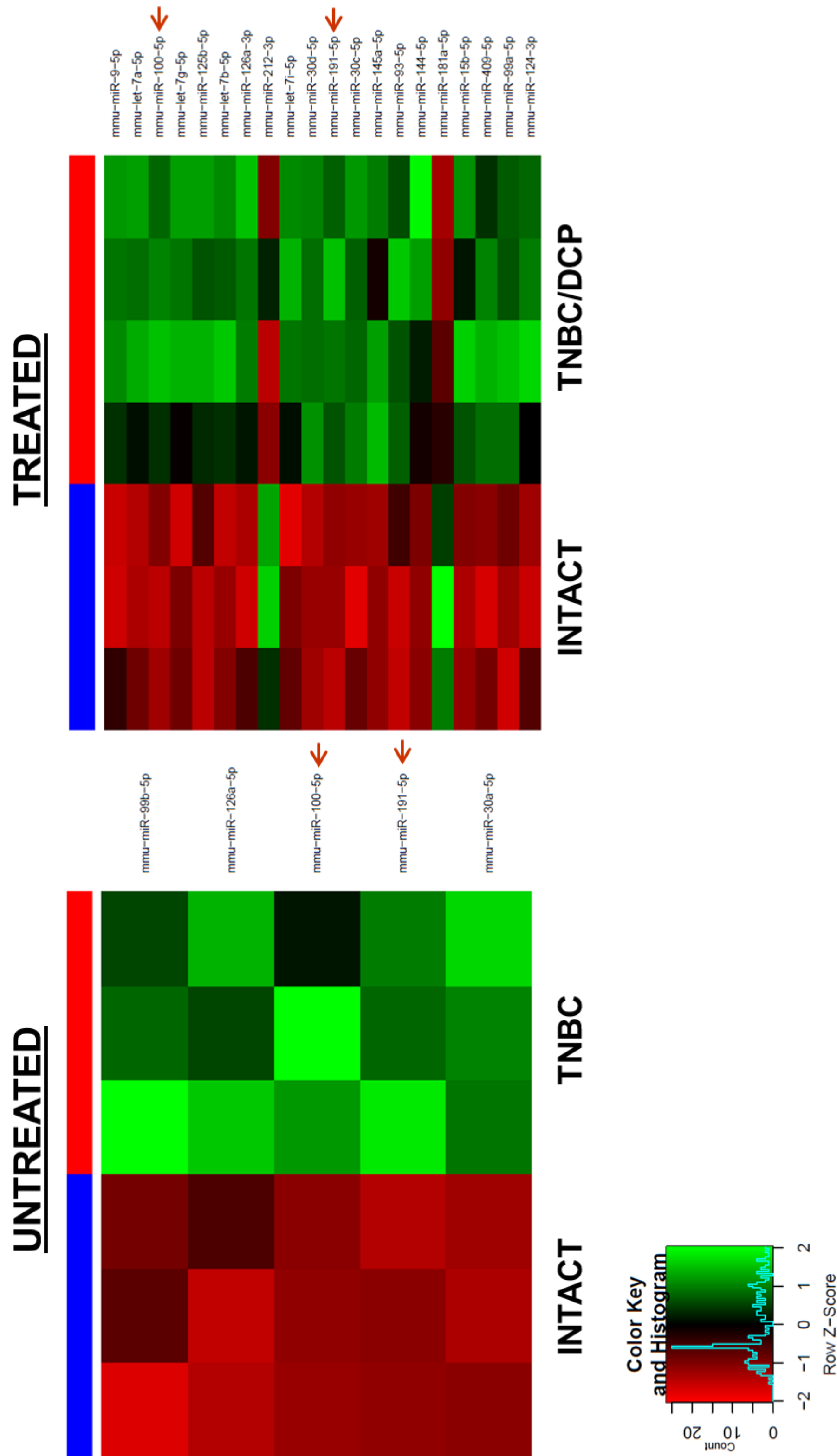


Figure 3.4: Heatmaps of microRNAs differentially expressed in the PFC tissues of the TNBC untreated and TBNC/DCP mice as compared to intact controls. Red arrows indicate miRNAs commonly regulated in both groups.

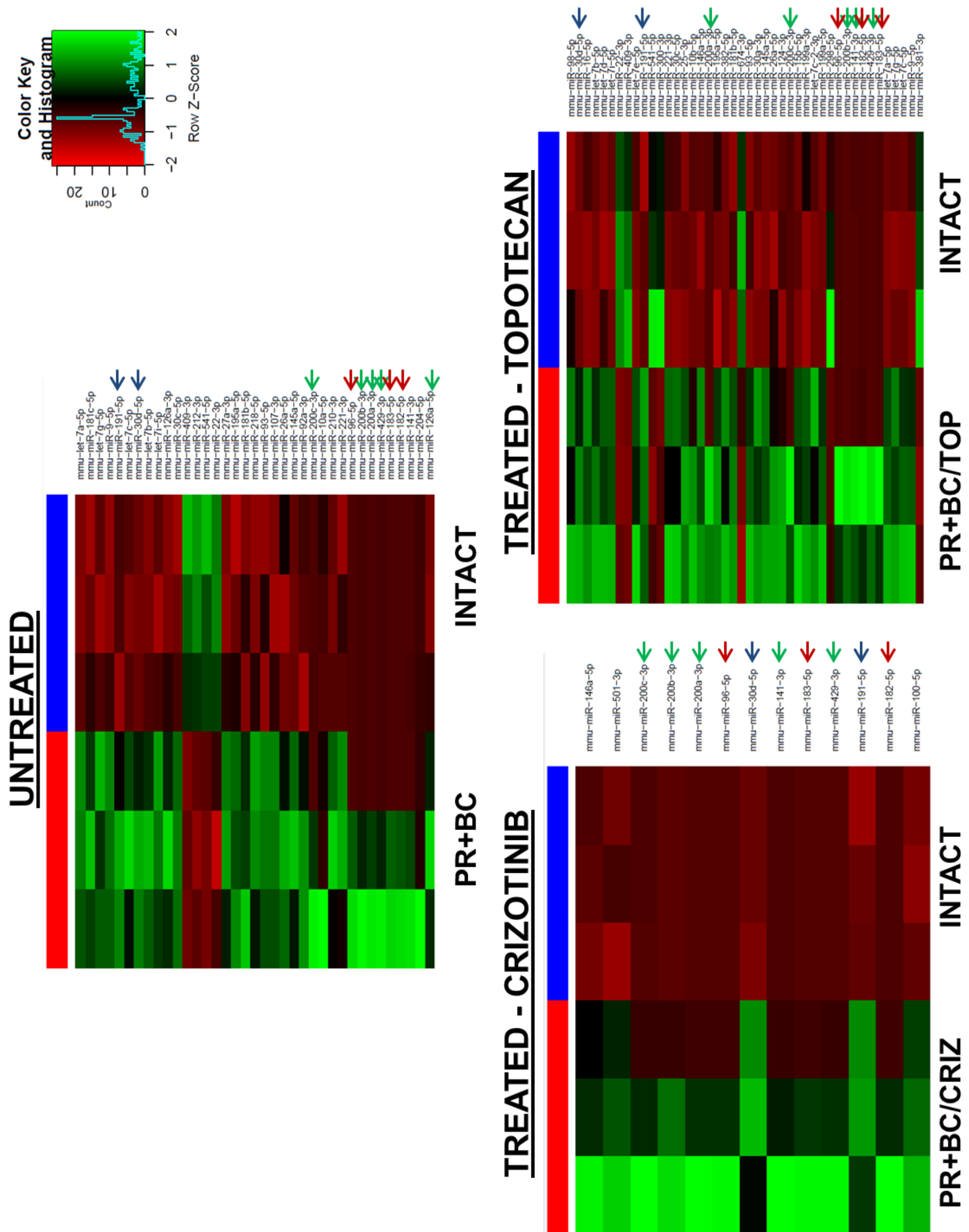


Figure 3.5: Heatmaps of microRNAs differentially expressed in the PFC tissues of the PR+BC untreated, PR+BC/CRIZ and PR+BC/TOP mice as compared to intact controls. Arrows indicate miRNAs commonly regulated in both groups. Green arrow – miR-200 family; blue arrow - miR-183/96/182 cluster; red arrow – other common miRNAs.

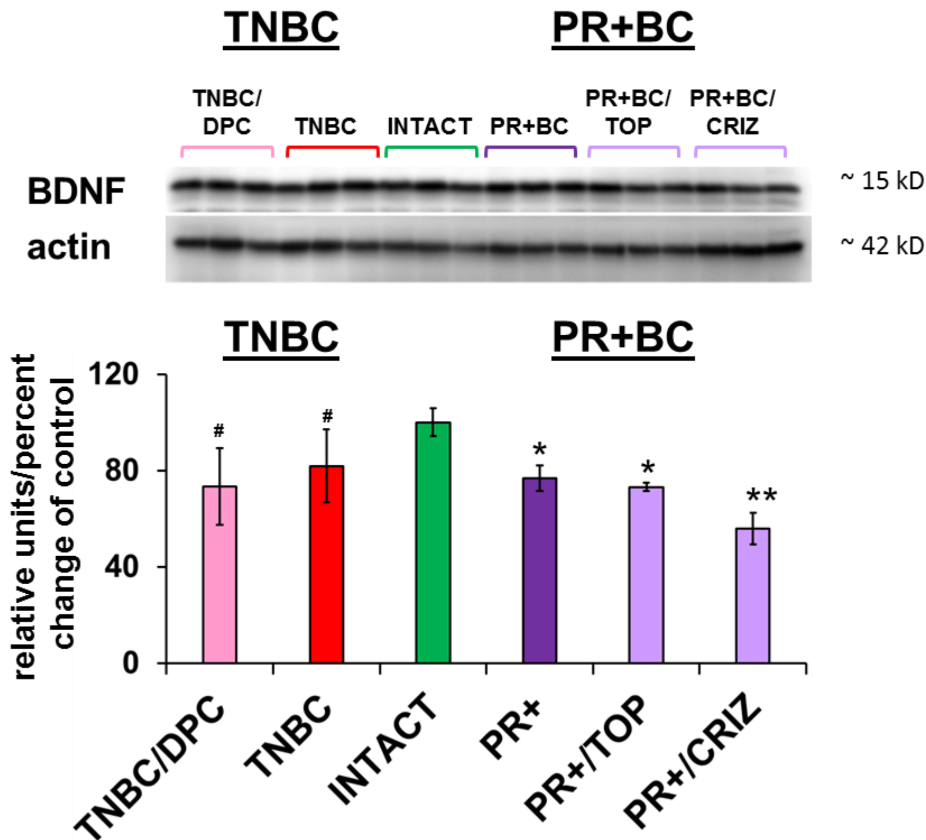


Figure 3.6: Levels of BDNF in the PFC tissues of intact and TNBC and PR+BC-bearing chemotherapy treated and untreated TumorGraft mice. Data are shown as relative units/percent change of control. Each line represents an individual animal sample, with 3 samples/group. Due to protein size differences and scarcity of tissue membranes were re-used several times in context of a large scale study. #p<0.10; * p<0.05; ** p<0.01, Student's *t*-test.

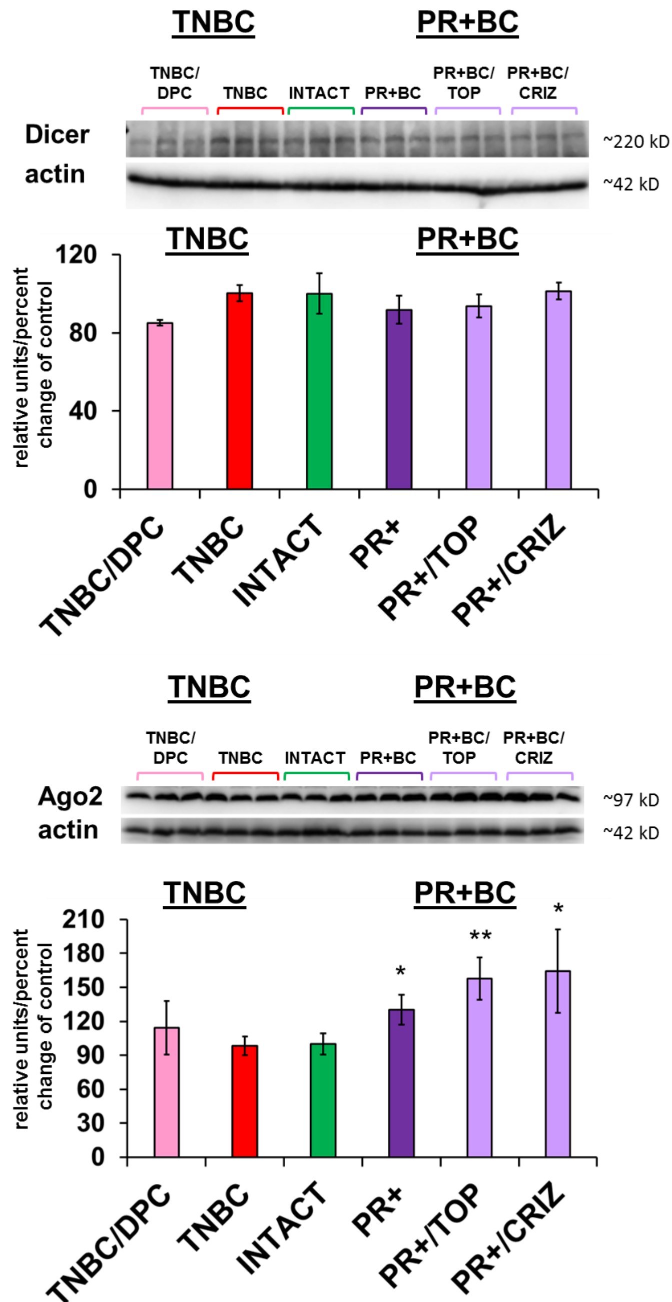


Figure 3.7: Levels of Dicer and Ago2 in the PFC tissues of intact and TNBC and PR+BC-bearing chemotherapy treated and untreated TumorGraft mice. Data are shown as relative units/percent change of control. Each line represents an individual animal sample, with 3 samples/group. Due to protein size differences and scarcity of tissue membranes were re-used several times in context of a large scale study. * $p<0.05$; ** $p<0.01$, Student's *t*-test.

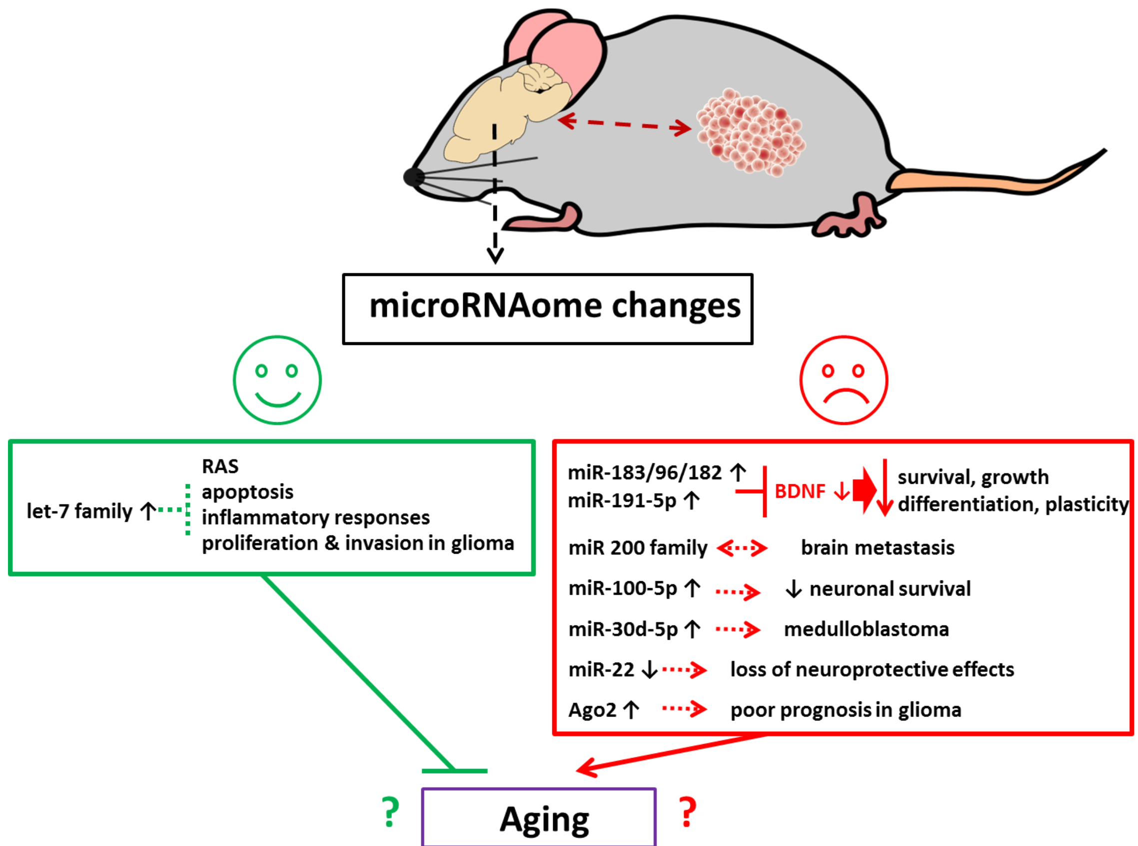


Figure 3.8: Schematic representation of possible biological effects of tumor brain and chemo brain-induced miRNAome changes in the brain.

Table 3.1: Total small RNA library composition

Absolute composition

CATEGORY	INTACT	TNBC	TNBC/DPC	PR+BC	PR+BC/TOP	PR+BC/CRIZ
number of samples	3	4	4	3	3	3
total	3853355	3061761	5573723	4534609	4291129	3808590
miRNA	3148510	2420296	4457646	3537230	3108049	3125959
snoRNA	32438	20672	37850	30798	26334	24089
snRNA	1422	1516	2806	2314	2524	1720
rRNA	21921	16848	29983	22662	21086	18516
tRNA	108118	176533	246617	276325	497035	145168
piRNA	30690	20876	38425	32886	30772	28125
exons	196436	160890	322187	265234	247267	180506
repats	42549	27304	50582	36629	35009	32587
introns	30529	25988	47328	40121	39528	28231
unclassified	22427	15676	30110	24959	22658	19392
no_match	218315	175162	310189	265451	260867	204297

Relative composition

CATEGORY	INTACT	TNBC	TNBC/DPC	PR+BC	PR+BC/TOP	PR+BC/CRIZ
Total %	100.0	100.0	100.0	100.0	100.0	100.0
miRNA	81.7	77.8	79.8	77.9	72.4	82.1
snoRNA	0.8	0.6	0.7	0.7	0.6	0.6
snRNA	0.0	0.1	0.1	0.1	0.1	0.1
rRNA	0.6	0.6	0.5	0.5	0.5	0.5
tRNA	2.8	6.5	4.5	6.2	11.5	3.6
piRNA	0.8	0.7	0.7	0.7	0.7	0.8
exons	5.1	5.5	5.8	5.9	5.8	4.7
repats	1.1	0.9	0.9	0.8	0.8	0.9
introns	0.8	0.9	0.9	0.9	0.9	0.8
unclassified	0.6	0.5	0.5	0.6	0.5	0.5
no_match	5.7	5.8	5.6	5.9	6.1	5.4

CHAPTER 4: GROWTH OF TRIPLE NEGATIVE AND PROGESTERONE POSITIVE BREAST CANCER CAUSES OXIDATIVE STRESS AND DOWN-REGULATES NEUROPROTECTIVE TRANSCRIPTION FACTOR NPAS4 AND NPAS4-REGULATED GENES IN HIPPOCAMPAL TISSUES OF TUMORGRAFT MICE

Chapter 4 submitted being submitted for publication in its entirety:

Kovalchuk, A. Ilnytskyy, Y., Rodriguez-Juarez, R., Katz,A., Sidransky, D., Kolb, B., Kovalchuk, O. Growth of triple negative and progesterone positive breast cancer causes oxidative stress and down-regulates neuroprotective transcription factor NPAS4 and NPAS4-regulated genes in hippocampal tissues of TumorGraft mice. *Frontiers in Genetics* (in submission).

Abstract

While the refinement of existing and the development of new chemotherapeutic regimens has significantly improved cancer treatment outcomes and patient survival, chemotherapy still causes many persistent side effects. Central nervous system (CNS) toxicity is of particular concern, as cancer patients experience significant deficits in memory, learning, cognition, and decision-making. These chemotherapy-induced cognitive changes are termed chemo brain, and manifest in more than half of cancer survivors. Moreover, recent studies have emerged suggesting that neurocognitive deficits manifest prior to cancer diagnosis and treatment, and thus may be associated with tumor presence, or 'tumor brain'. To dissect the molecular mechanisms of tumor brain, we used TumorGraft™ models, wherein part of a patient's tumor is grafted into immune-deficient mice. Here, we analyzed molecular changes in the hippocampal tissues of mice carrying triple negative (TNBC) or progesterone receptor positive (PR+BC) xenografts. TNBC growth led to increased oxidative damage, as detected by elevated levels of 4-hydroxy-2-nonenal, a product of lipid peroxidation. The growth of TNBC and PR+BC tumors altered global gene expression in the murine hippocampus and affected multiple pathways implicated in PI3K-Akt and MAPK signalling, as well as other pathways crucial for the proper functioning of hippocampal neurons. TNBC and PR+BC tumor growth also led to a significant decrease in the levels of neuronal transcription factor NPAS4, a regulator that governs the expression of brain-derived neurotrophic factor (BDNF), and several other brain neurotrophic factors and pro-survival molecules. The decreased expression of ERK1/2, NPAS4, and BDNF are also seen in neurodegenerative conditions and aging, and may constitute an important tumor brain mechanism.

Introduction

The development of new chemotherapeutic agents and amelioration of existing protocols have significantly increased patient survival and improved treatment outcomes. Nevertheless, chemotherapy still has many long-term side effects that negatively influence the quality of life of cancer patients. Among these side effects, manifestations of central nervous system (CNS) toxicity are of particular concern (Soffietti et al., 2014, Ahles et al., 2012). Chemotherapy causes significant declines in processing and long-term memory, learning, and cognition. It interferes with sleep and decision-making and, as noted by many patients, with the very way they think. These effects are widespread across all cancer patients, but are most pronounced and were first noted by breast cancer survivors, manifesting in up to 75% of all breast cancer cases. It was breast cancer patients who first coined the term "chemo brain" to explain their condition; the term is now widely used to refer to chemotherapy-induced cognitive changes (Wefel and Schagen, 2012).

Several studies that used both pre- and post-treatment patient cohorts revealed that in 30% of patients, neurocognitive deficits manifested prior to chemotherapy. Even though these studies are scarce, they suggest that chemo brain has to be extended to include cancer-induced cognitive impairments, a concept that we have recently termed 'tumor brain'. Tumor brain remains much less investigated than chemo brain. Meanwhile, chemo brain has recently gained widespread attention and has been extensively analyzed using animal models where animals were exposed to various chemotherapy agents.

The mechanisms behind chemo brain include increased oxidative stress, altered levels of neuronal proliferation and apoptosis, inhibition of neuronal differentiation, disruption of hippocampal neurogenesis, increased inflammation, disruption of the blood-brain barrier, alterations in brain blood flow, and changes in metabolism (Raffa, 2011, Lyons et al., 2011, Briones and Woods, 2014, Briones et al., 2015, Christie et al., 2012, Han et al., 2008, Joshi et al., 2010, Seigers and Fardell, 2011). Chemo brain is epigenetically regulated and linked to aberrant histone modification levels. In our recent study, we showed that exposure to mitomycin C and cyclophosphamide alters DNA methylation and global gene expression, and causes oxidative DNA damage in the prefrontal cortex and hippocampus of exposed mice.

To date, the vast majority of chemo brain studies have used animals without tumors, and the mechanisms of tumor brain are therefore under-investigated. In another set of studies, we used TumorGraft animal models that are used in precision oncology approaches to analyze the mechanisms behind tumor brain and chemo brain in tumor-bearing chemotherapy treated and untreated mice. We found that the presence of a tumor caused pronounced changes in the levels of gene and small RNA expression, global DNA methylation and hydroxymethylation, as well as in oxidative DNA damage and the levels of several neurotrophic factors (Chapters 2 and 3). Tumor presence played a governing role in the framework of the observed effects, while chemotherapy treatments further exacerbated tumor-induced changes. Within the scope of that study, we observed pronounced molecular effects of extracranial malignant tumor growth on the prefrontal cortex of non-CNS tumor-bearing animals. While our studies looked at the prefrontal cortex, in the past, the majority of chemo brain analysis has focused on the hippocampus.

However, while the effects of chemotherapy on the hippocampus have been demonstrated, changes caused by non-CNS tumor growth have never been analyzed in the hippocampal domain.

Here, we analyzed molecular changes in the hippocampal tissues of TNBC and PR+BC bearing mice. This is the first study to show that the growth of TNBC and PR+BC tumors alters global gene expression in the murine hippocampus and affects multiple pathways implicated in mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K)-Aktsignalling, as well as other pathways that are crucial for the proper functioning of hippocampal neurons. MAPK and PI3K-Akt pathways were previously shown to regulate expression of Neuronal PAS Domain Protein 4, an important neuron-specific transcription factor implicated in synaptic plasticity. Here we show that tumor growth leads to downregulation of NPAS4, as well as its target, brain-derived neurotrophic factor, and several other important neurotrophic factors.

Results

Impact of non-central nervous system (CNS) tumor growth on the levels of 4-hydroxynonenal in the murine hippocampus

Oxidative stress is a feature of cancer, and has been implicated in chemo brain and in numerous other neurological diseases and conditions. In cells and tissues, oxidative stress leads to the generation of several lipid peroxidation end-products, one of which is 4-hydroxynonenal (4-HNE), a highly reactive aldehyde produced from the peroxidation of omega-6-polyunsaturated fatty acids. Levels of 4-HNE are biomarkers of oxidative stress. To analyze whether or not growth of triple-negative breast cancer (TNBC) or

progesterone receptor-positive breast cancer (PR+BC) patient-derived xenografts (PDXs) causes oxidative stress, we evaluated the levels of 4-HNE in the hippocampal tissues of tumor-bearing mice (Vila et al., 2008, Zheng et al., 2014). Our analysis revealed that growth of malignant TNBC tumors significantly ($p<0.05$) upregulated levels of 4-HNE in the hippocampus of tumor-bearing mice (Fig. 4.1). On the contrary, growth of PR+BC tumors resulted in decreased levels of 4-HNE.

Effect of triple negative and progesterone positive breast cancer growth on global gene expression in the hippocampal tissues of tumor-bearing mice

Profiling global gene expression reveals all molecular changes, both mutational and non-mutational; hence, global transcriptome analysis is the best method for understanding the entirety of molecular and cellular processes. Analyzing global gene expression provides a full picture of normal tissue development and functioning, as well as the underlying causes of diseases and conditions. To explore the effects of TNBC and PR+BC tumors on the murine brain, we used the Illumina next-generation sequencing platform to carry out a global gene expression analysis of the hippocampal tissues of PDX mice. Global gene expression profiling revealed pronounced changes in the hippocampal tissues of TNBC and PR+BC animals. Principal component analysis based on the entire gene expression dataset showed clustering of groups and differences between transcriptome profiles in the hippocampal tissues of control and TNBC and PR+BC tumor-bearing mice (Fig. 4.2).

We noted that in the hippocampal tissues of TNBC animals, 61 genes were upregulated and 130 genes were downregulated, as compared to control mice. In PR+BC animals, 150 genes were upregulated and 579 were downregulated (Fig. 4.3). Of those,

23 genes were commonly upregulated and 94 genes were downregulated in the hippocampal tissues of both TNBC and PR+BC animals (the adjusted p-value was <0.10; the log fold change was 0.58). Commonly changed genes, both up- and downregulated, were mapped to KEGG biological pathways using the DAVID Bioinformatics and Paintomics platform. Commonly deregulated pathways included the PI3K-Akt signalling pathway, Protein digestion and absorption, ECM-receptor interactions, and Neuroactive ligand-receptor interactions.

In the ECM-receptor interactions pathways, the GABA A receptor gene was downregulated in the hippocampal tissues of PR+BC animals, and upregulated in TNBC animals. The expression of this gene was further analyzed at the protein level, whereby GABA A Receptor protein was downregulated in both animal groups (Fig. 4.4). Another altered pathway included PI3K-AKT signalling. Protein analysis further revealed statistically significant downregulation of AKT1 and ERK1/2 in the hippocampal tissues of TNBC and PR+BC animals (Fig. 4.5).

Effect of non-CNS tumor growth on the expression of Neuronal PAS Domain Protein 4 (NPAS) and its targets

ERK1/2 signalling was reported to regulate NPAS4, which is an important transcription factor. In-depth gene expression analysis revealed that Npas4 was one of the most downregulated genes in the hippocampi of both TNBC and PR+BC tumor-bearing animals, as compared to controls (log fold -1.74 and -1.38, respectively). Along with Npas4, several Npas4 target genes were downregulated, including natriuretic peptide receptor 3 (Npr3), proprotein convertase subtilisin/kexin type 1 (Pcsk1), and FBJ osteosarcoma oncogene (Fos) genes (Fig. 4.6). Further analysis revealed that NPAS4

protein levels were also significantly downregulated in the hippocampi of tumor-bearing mice. Additionally, levels of NPAS4 target proteins – brain-derived neurotrophic factor (BDNF) and FBJ murine osteosarcoma viral oncogene homolog B (FOS B) – were significantly downregulated in the hippocampi of TNBC animals, but not PR+BC animals. Levels of proliferating cell nuclear antigen (PCNA) protein were also downregulated in both TNBC and PR+BC animals (Fig. 4.6).

Discussion

This study is the first in-depth analysis of the molecular mechanisms of TNBC and PR+BC growth-induced tumor brain manifestations in the hippocampal tissues of TumorGraft mice. The growth of malignant non-CNS tumors had a profound impact on molecular processes in the murine hippocampus. The major findings of our study are as follows: (i) the growth of TNBC and PR+BC tumors significantly altered gene expression in the murine hippocampus; (ii) TNBC tumor growth caused oxidative stress that manifested as significantly elevated levels of 4-HNE; (iii) tumor growth negatively affected the levels of neuronal transcription regulator NPAS4 and its target genes, among them, one of the members of the neurotrophin family of growth factors – BDNF; (iv) tumor growth was associated with significant downregulation of PCNA, AKT 1, and ERK1/2, proteins that are central for the control of neuronal proliferation and survival; and (v) observed molecular changes strongly resembled those associated with neurodegenerative diseases and brain aging. Overall, gene expression changes were more prominent in PR+BC mice than in TNBC mice.

We discovered that TNBC growth causes upregulation of 4-HNE levels in the hippocampus. 4-HNE is a highly reactive, neurotoxic product of lipid peroxidation.

Likewise, it is both genotoxic and cytotoxic, and is involved in the pathogenesis of Alzheimer's and Parkinson's diseases, bipolar disorder, and other neurodegenerative and psychiatric diseases (Tsirulnikov et al., 2012, Newton et al., 2017). Increased levels of 4-HNE have been associated with neurodegeneration and Alzheimer's protofibril formation (Siegel et al., 2007). Levels of 4-HNE in the brain are known to be elevated by exposure to ionizing radiation (Mao et al., 2016), alcohol (Tian et al., 2016), low-frequency electric fields (Akpinar et al., 2016a), blast injury to the brain (Du et al., 2013), as well as cerebral ischemia (Katayama et al., 2017) and cancer (Zhong and Yin, 2015). Overall, elevated levels of 4-HNE are a sign of brain aging (Benedetti et al., 2014) and neurodegeneration (Farooqui and Horrocks, 2006). In addition, we found a downregulation of ERK1/2 in the hippocampi of tumor-bearing mice. Given recent reports showing the effects of 4-HNE on the levels of MAPK activity in lung cancer cells, as well as the effects of 4-HNE on AKT-mediated regulation of proliferation and apoptosis, analyzing the interplay between MAPK signalling and 4-HNE is of interest.

Another important finding of this study is the downregulation of the neuronal PAS domain protein 4 (Npas4) gene in the hippocampal tissues of TNBC and PR+BC mice, as compared to controls. NPAS4 is a neuron-specific transcription factor that is involved in synaptic plasticity and provides an important link between neuronal activity and memory (Klaric et al., 2017). NPAS4 is important in long-term memory formation in multiple regions of the brain, including the hippocampus, and Npas4 knockout mice fail to form contextual fear memories (Ramamoorthi et al., 2011). The gene may also be implicated in neural circuit plasticity (Sun and Lin, 2016). Moreover, recent studies suggest

that NPAS4 may exert neuroprotective effects in ischemic stroke via regulation of cell death and of the inflammatory response (Choy et al., 2015).

NPAS4 modulates activity-dependent synaptic connections in both GABAergic and glutamatergic synapses by regulating numerous downstream genes (Sun and Lin, 2016). Among those, NPAS4 has been shown to regulate Bdnf, Nrp3, Fos, and many other genes (Bloodgood et al., 2013, Maya-Vetencourt, 2013). Of those, one of the main NPAS4 targets is BDNF, which belongs to the neurotrophin family and governs and facilitates neuronal differentiation, maturation, growth and survival, and plays a role in neural plasticity (Takami et al., 2005, Park and Poo, 2013). BDNF protein levels were shown to be downregulated by various stressors (Lee and Kim, 2010) and by adverse prenatal environments (Kundakovic and Jaric, 2017). Decreased BDNF levels were reported in Alzheimer's disease, neurodegenerative and psychiatric disorders, stroke, and brain aging, just to name a few (Lee et al., 2005, Park and Poo, 2013) (Fig. 4.7).

NPAS4 also regulates c-Fos and FosB, immediate early response genes that govern cell proliferation and differentiation in response to extracellular stimuli. c-FOS is a marker of neuronal activity, and levels of c-FOS are reduced during brain aging and are associated with age-related decreases in neuronal function and plasticity in various brain regions. c-Fos knockout mice exhibit significant learning impairment and hyperactivity (Velazquez et al., 2015). Meanwhile, downregulated FosB levels have been reported in the hippocampi of both depressed and addicted individuals (Gajewski et al., 2016).

We noted a significant downregulation of Npas4 and its target genes in the hippocampal tissues of TNBC- and PR+BC tumor-bearing animals, as compared to controls. This downregulation of neurotrophins and pro-survival genes may lead to

alterations in synaptic plasticity, neuronal survival and maturation, and may in turn underlie memory impairments associated with tumor brain. In the future, it will be important to understand region and cell-type specificity of the observed changes, as well as the time frame for downregulation. The mechanisms of NPAS4 downregulation also need to be analyzed in greater detail. These may be epigenetically regulated, and may involve altered methylation of the NPAS4 promoter (Furukawa-Hibi et al., 2015), or the function of small non-coding RNAs.

Mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways were shown to partake in the induction of Npas4 expression (Sun and Lin, 2016). Here, we observed downregulation of ERK1/2 and AKT 1, important constituents of these pro-survival pathways. The observed concomitant downregulation of AKT1, ERK1/2, NPAS4, BDNF, and other NPAS4 targets strongly suggests the importance of these pathways in tumor brain, as it manifests in the hippocampus. Further studies are needed to investigate the other components of these pathways, and the roles of upstream and downstream signalling.

Likewise, it will also be important to discern the mechanisms of transcriptional regulation in tumor brain, as well as the roles of epigenetic changes – such as those in DNA methylation, histone modifications, and small RNAs – in tumor brain's manifestation in the hippocampus. The timing and interplay between various levels of regulation of gene expression may lead to a better understanding of the initial series of events underlying tumor brain, allowing for the development of novel strategies for diagnosis, prevention, and mitigation.

The hippocampus has been reported as one of the targets of chemo brain. Furthermore, reports of memory impairment in cancer patients prior to diagnosis strongly suggest hippocampal involvement. All of the observed molecular changes – such as increased levels of 4-HNE and decreased levels of AKT1, ERK1/2, NPAS4, PCNA, and BNDF – have negative effects, especially since all of these proteins partake in the regulation of neuronal differentiation and survival. Oxidative stress and the loss of expression of important regulators of neuronal survival and functioning proteins may underlie tumor brain. Some of the TNBC and PR+BC growth-induced changes in the hippocampus were similar to those previously observed in the prefrontal cortex (PFC) of these animals, whereby we noted increased oxidative stress and decreased levels of BDNF. Hence, those mechanisms may be pivotal for tumor brain manifestations in various brain regions. In the future, research should expand to analyze tumor brain in other brain regions.

The analysis of gene expression changes in the hippocampi of TNBC and PR+BC tumor-bearing animals revealed changes in many metabolic pathways, such as galactose metabolism, arachidonic acid metabolism, alpha linolenic acid metabolism, amino acid and sugar metabolism, fatty acid biosynthesis, and many others (Table 4.1). This suggests that metabolic alterations are taking place in the hippocampi. These findings may warrant future metabolomics analysis of tumor brain.

In this study, we analyzed tumor brain in animals with breast cancer patient-derived tumor xenografts. In future research, we will seek to expand this analysis and dissect the changes induced by other types of tumors, as well as evaluate tumor-induced brain changes as a function of tumor stage and grade. Furthermore, molecular changes caused

by malignant non-CNS tumor growth on the brain need to be analyzed over the course of tumor development, and to be correlated with the neuroanatomical and behavioural manifestations of tumor brain. The mechanisms of tumor brain signalling remain obscure, but these signals may be transmitted through the blood. Therefore, it is important to analyze and compare changes in the brain and blood. This will allow us to establish the nature of the tumor brain signal and propose novel blood-based tumor brain diagnostic and prognostic biomarkers.

Materials and methods

Animal model

To analyze the effects of non-CNS tumor growth on the brain, we used TumorGraft technology developed by precision medicine company Champions Oncology, Inc. (Baltimore, MD), who provided frozen brain tissues of TumorGraft mice carrying TNBC and PR+BC patient-derived xenografts (PDX). Patients diagnosed with TNBC and PR+BC had their tumors surgically removed and engrafted into mice to generate personalized TumorGraft mouse models for the development of precision oncology applications. All patients provided their informed consent for the use of tumor material for research purposes. The TumorGraft models were generated as previously described (Bertotti et al., 2011, DeRose et al., 2011, Hidalgo et al., 2011, Morelli et al., 2012, Stebbing et al., 2014). Tumor samples were obtained during surgery, and small tumor fragments containing both malignant cells and supportive stromal components were subcutaneously implanted into the flanks of six-week-old immunodeficient female mice (female *nu*/*nu* athymic mice; Harlan Laboratories, Indianapolis, IND). Animal experiments were approved by Institutional Animal Care and Use Committee protocols.

Tumor dimensions were regularly measured and tumor volumes were calculated as previously described (Stebbing et al., 2014). Upon propagation, when TumorGrafts reached more than 200 mm^3, the animals were divided into groups of three to five. This study focused on the effects in TNBC (n=4), and PR+BC (n=3) animals. Intact animals of the same strain (no tumor, no treatment, n=3) served as baseline controls. Upon completion of the treatment, animals were euthanized using Euthansol overdose; the brains were removed and immediately frozen in liquid nitrogen and stored in −80 °C for molecular analysis. The tissues were split to accommodate RNA and protein analysis.

Gene Expression Analysis

Hippocampal tissues of three–four animals per group were used for the analysis of gene expression profiles. RNA was extracted from hippocampal tissues using TRIzol® Reagent (Invitrogen, Carlsbad, CA), further purified using an RNeasy kit (Qiagen), and quantified using Nanodrop2000c (ThermoScientific). Afterwards, RNA integrity and concentration were analyzed using 2100 BioAnalyzer (Agilent). Sequencing libraries were prepared using Illumina's TruSeq RNA library preparation kits, and global gene expression profiles were determined using the Next 500 Illumina deep-sequencing platform at the University of Lethbridge Facility. Statistical comparisons between the control and PDX-bearing groups were performed using the DESeq Bioconductor package (version 1.8.3) and the baySeq Bioconductor package (version 1.10.0). Clustering of the samples was analyzed with multidimensional scaling (MDS) plots, built using the plotMDS function from the edgeR Bioconductor package. MA plots showing the relationship between the average level of expression and the log2 fold change were created for each of the comparisons. The MA-plot is a plot of the distribution of the

red/green intensity ratio (“M”), plotted by the average intensity (“A”). Features with a false discovery rate (FDR) < 0.1 (10% false positive rate) were considered differentially expressed between conditions.

The functional annotations of differentially expressed genes were performed using DAVID, GO (Gene Ontology) Elite, and GO-TermFinder (Boyle et al., 2004). Pathways were visualized using Pathview/KEGG and DAVID Bioinformatics Resources 6.7/KEGG Pathway platforms(Huang da et al., 2009b, Huang da et al., 2009a, Huang et al., 2007).

Western Immunoblotting

Western immunoblotting was carried out as previously described (Silasi et al., 2004, Kovalchuk et al., 2016a, Kovalchuk et al., 2016b, Kovalchuk et al., 2016c). In brief, hippocampal tissues (n=3 per group) were sonicated in ice-cold 1% SDS and immediately boiled. Protein concentrations were determined using the Bradford assay (BioRad, Hercules, CA). Equal amounts of protein (10-30 μg) were separated by SDS-PAGE into slab gels of 10-15% polyacrylamide and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Baie d’Urfé, Quebec). Altogether, eight membranes were prepared. The membranes were incubated with primary antibodies against 4-HNE, AKT 1, NPAS4 (1:1000, Abcam), ERK1/2, FOSB, PCNA (1:1000, Cell Signalling), and actin (1:2000, Abcam) overnight at 4° C. Primary antibody binding was detected using horseradish peroxidase-conjugated secondary antibodies and the Enhanced Chemiluminescence Plus System (Amersham Biosciences, Baie d’Urfé, Quebec). Chemiluminescence was detected using a FluorChem HD2 camera with FluorChem software (Cell Biosciences). The membranes were stained with Coomassie blue (BioRad,

Hercules, CA) to confirm equal protein loading. Signals were quantified using NIH Image J64 software and normalised relative to actin or Coomassie staining.

Statistical analysis

Statistical analysis (Student's *t*-test) for DNA methylation, oxidative stress, and protein levels was carried out using Microsoft Excel.

Figures

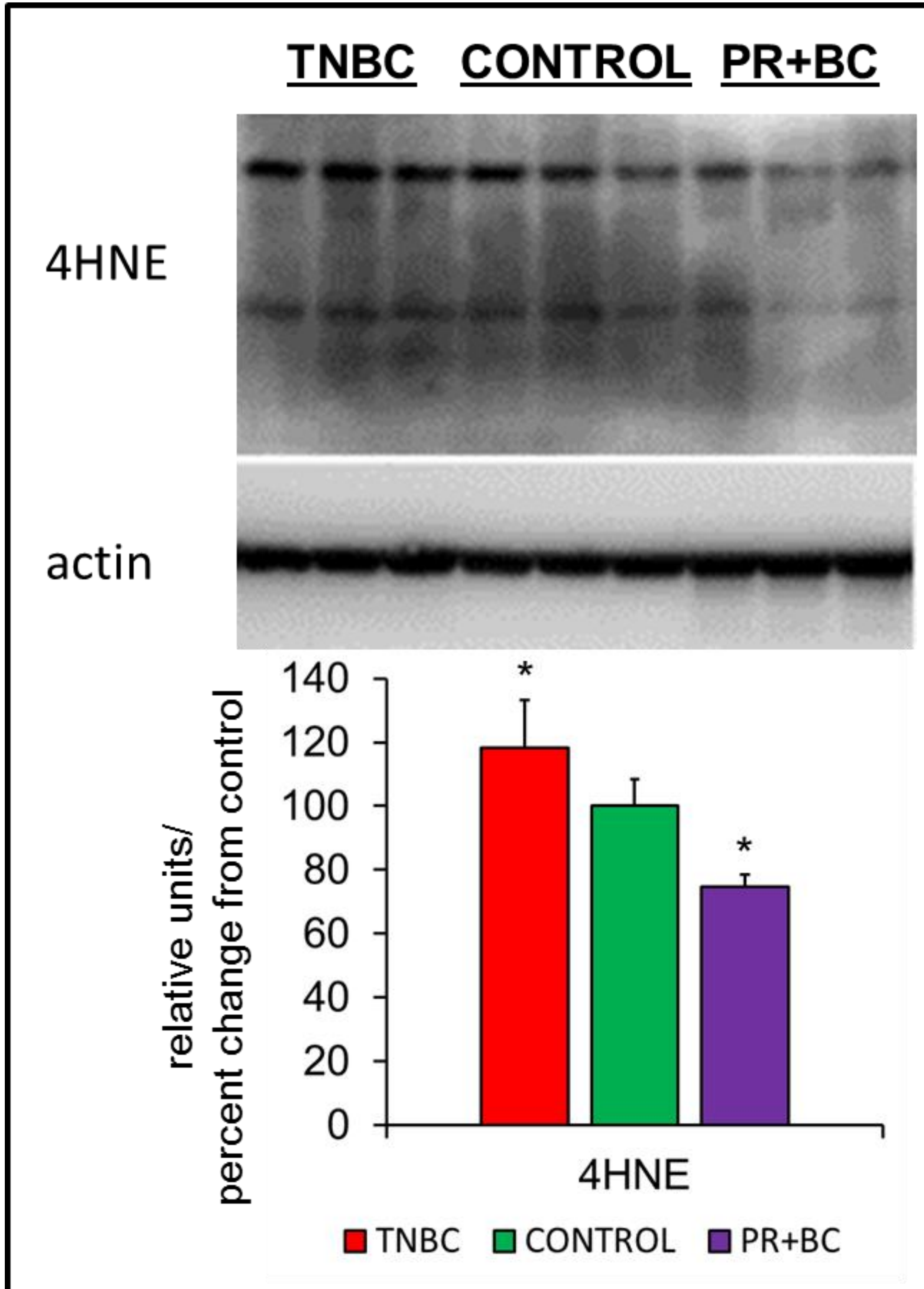


Figure 4.1: Levels of 4-hydroxy-2-nonenal (4-HNE) in the hippocampal tissues of control and TNBC and PR+BC TumorGraft mice. Western immunoblotting analysis of the levels of 4-HNE; data are shown as relative units/percent change of control; * $p<0.05$, Student's *t*-test. Each line represents an individual sample, 3 samples/group.

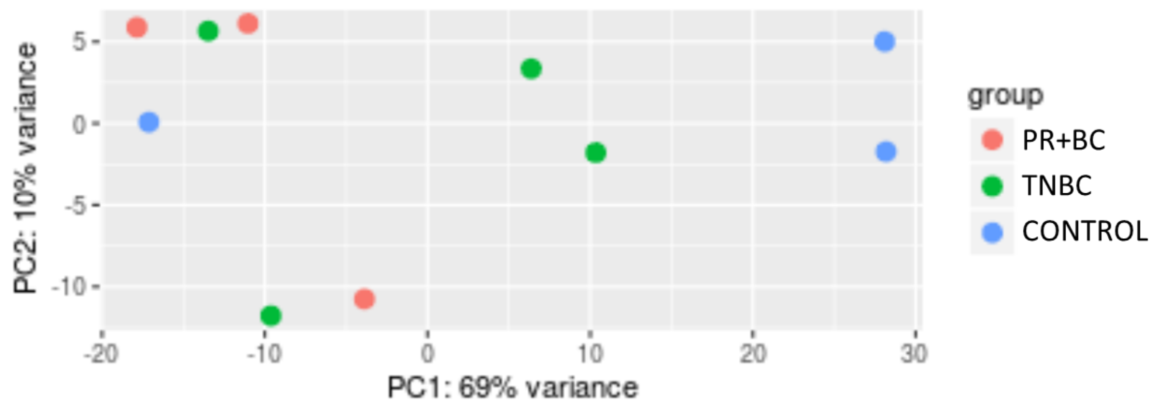


Figure 4.2: Principal component analysis based on the entire gene expression dataset showed clustering of groups and differences between transcriptome profiles in the hippocampal tissues of control and TNBC and PR+BC tumor-bearing mice. PCs represent % variance.

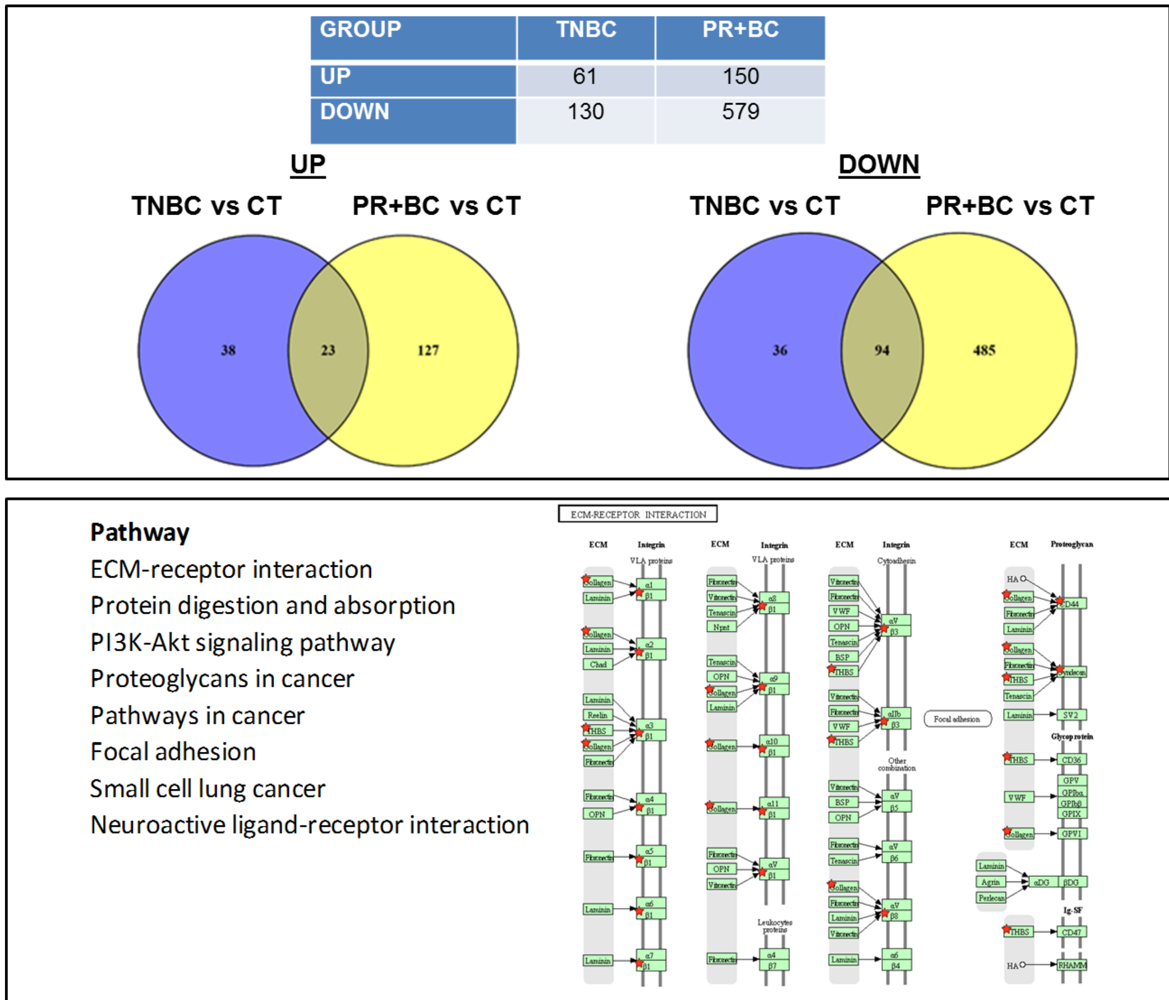


Figure 4.3: Next generation sequencing-based analysis of gene expression in the hippocampal tissues of control and TNBC and PR+BC TumorGraft mice. Table and Venn diagrams show numbers of genes that were significantly different between TNBC and PR+BC mice, as compared to controls. Below is a list of molecular pathways that were commonly down-regulated in the hippocampal tissues of TNBC and PR+BC mice. Pathview/KEGG analysis was used to determine differentially affected pathways. Figure displays the KEGG ECM-Receptor Interaction pathway.

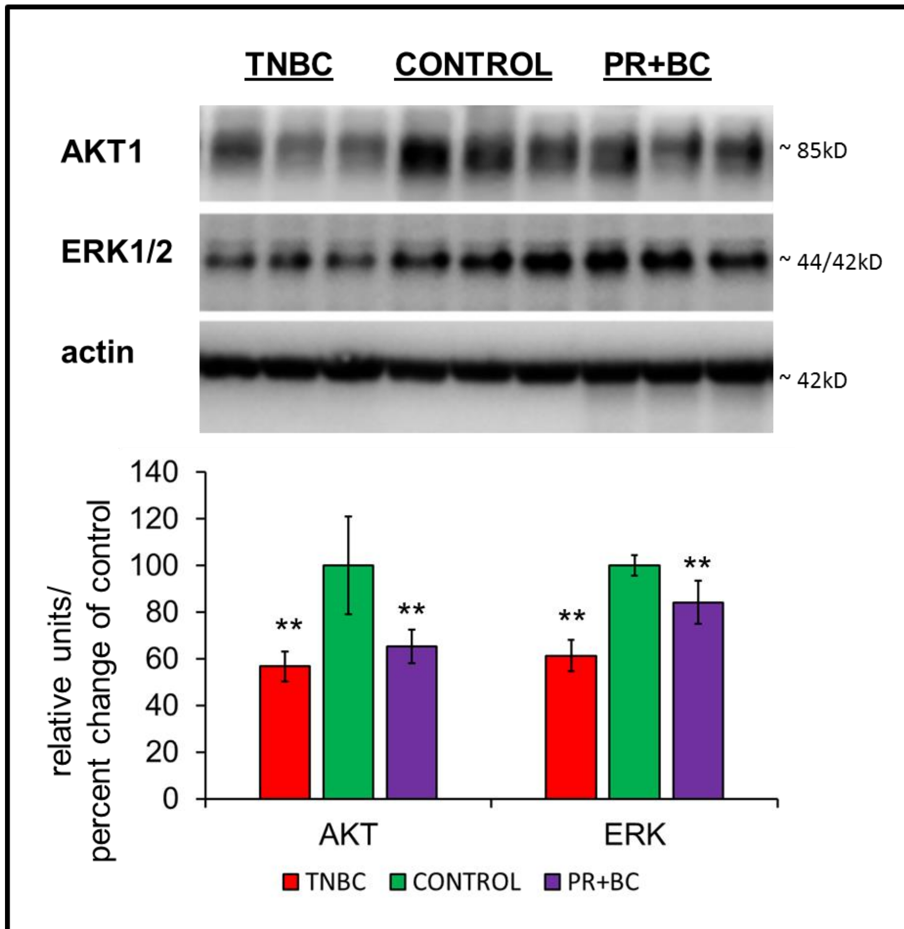


Figure 4.4: Levels of AKT1 and ERK1/2 in the hippocampal tissues of control and TNBC and PR+BC TumorGraft mice. Data are shown as relative units/percent change of control. Due to protein size differences and scarcity of tissue, membranes were re-used several times. ** $p<0.01$, Student's *t*-test. Each line represents an individual sample, with 3 samples/group.

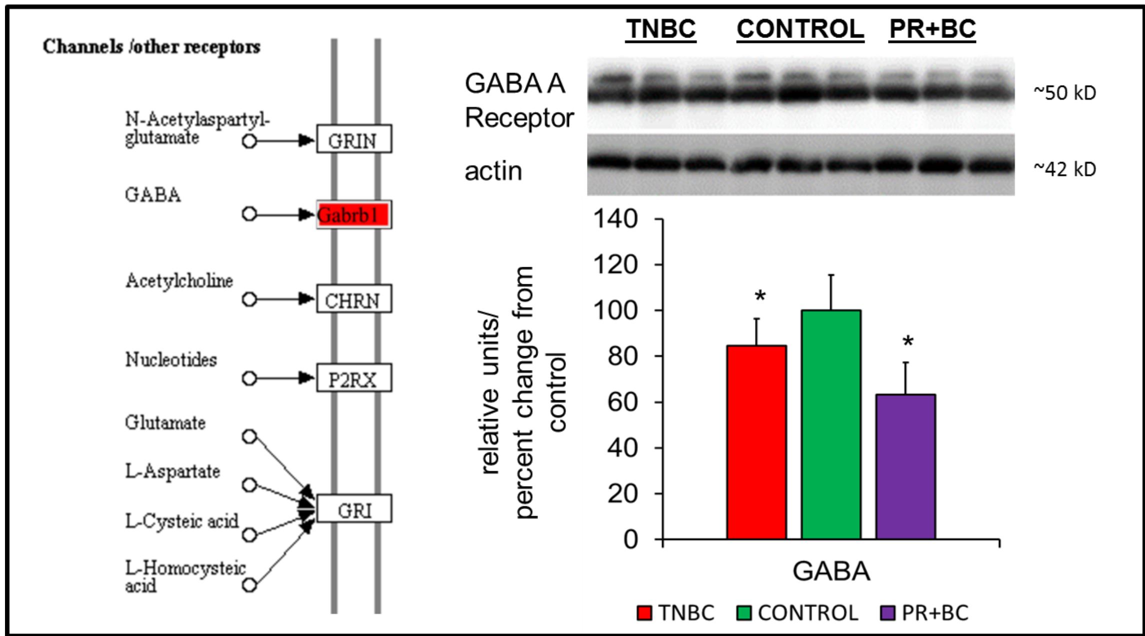


Figure 4.5: Levels of GABA A Receptor in the hippocampal tissues of control and TNBC and PR+BC TumorGraft mice. The diagram shows the Channels/Other Receptors pathways commonly altered in both TNBC and PR+BC animals, as compared to controls. The graph represents western immunoblotting results. Intensity of both bands was taken into consideration. Data are shown as relative units/percent change of control. Due to protein size differences and scarcity of tissue, membranes were re-used several times. * $p<0.05$, Student's *t*-test. Each line represents an individual sample, with 3 samples/group.

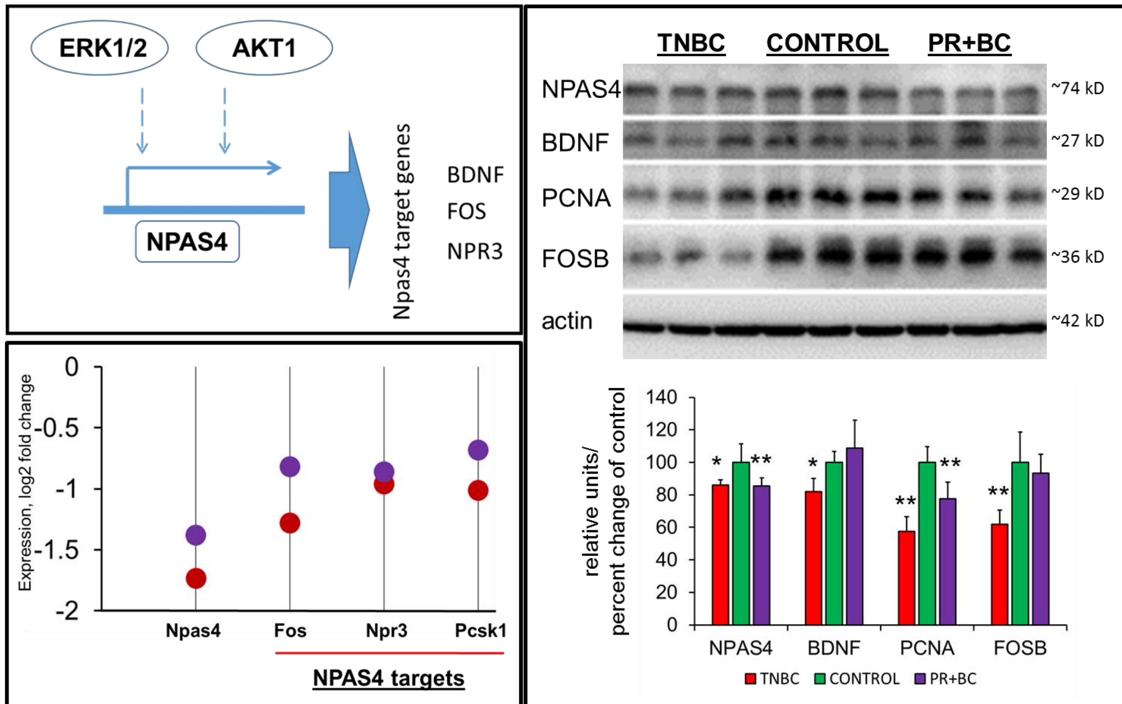


Figure 4.6: Levels of NPAS4, NPAS4 targets and PCNA in the hippocampal tissues of control and TNBC and PR+BC TumorGraft mice. Schematic representation of the control of NPAS4 protein by AKT1 and ERK1/2 pathways. Expression levels of Npas4 gene and its target genes 9Fos, Npr3, and Pcsk1 in TNBC (red) and PR+BC (purple) animals are given in log2 fold change, as compared to controls. Western immunoblotting was performed to determime the levels of NPAS4, BDNF, FOSB, and PCNA. The graph represents Western immunoblotting results. Data are shown as relative units/percent change of control. Each line represents an individual sample, with 3 samples/group. Due to protein size differences and scarcity of tissue, membranes were re-used several times. * $p<0.05$; ** $p<0.01$, Student's *t*-test.

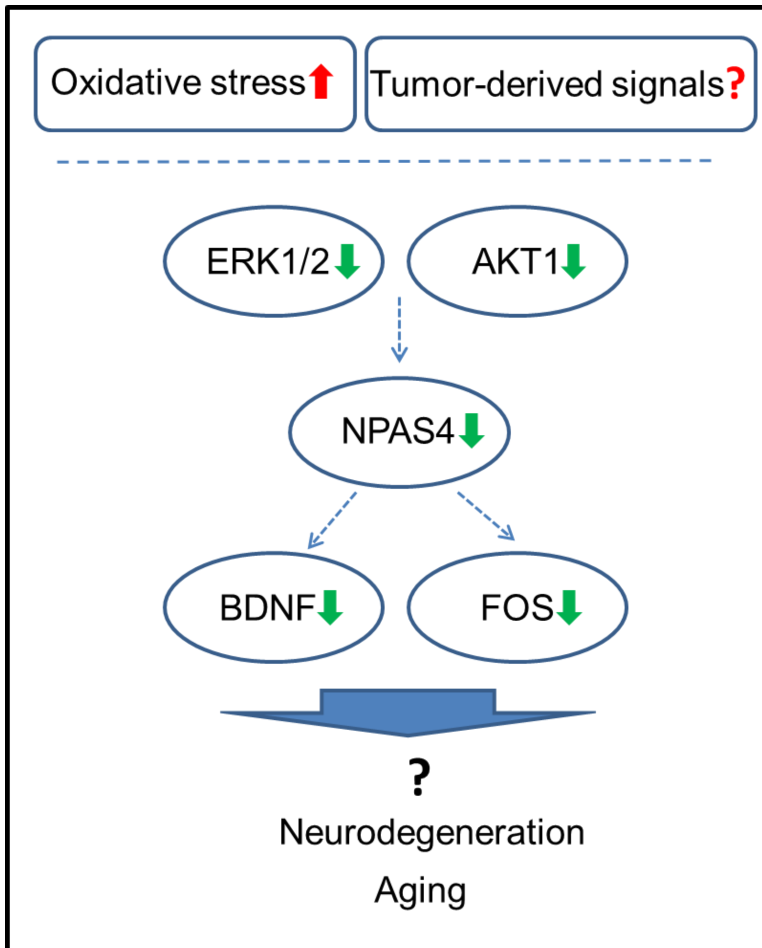


Figure 4.7: Schematic representation of the roles of NPAS4 and its regulatory network in tumor brain manifestation in the hippocampus.

Table 4.1: Differentially regulated pathways

TNBC vs INTACT			PR+BC vs INTACT		
KEGGID	*P value*	*Term*	*KEGGID*	*P value*	*Term*
UP			**UP**		
52	0.01	Galactose metabolism	591	0.00	Linoleic acid metabolism
4975	0.01	Fat digestion and absorption	590	0.00	Arachidonic acid metabolism
524	0.02	Butirosin and neomycin biosynthesis	4670	0.00	Leukocyte transendothelial migration
520	0.03	Amino sugar and nucleotide sugar metab	4514	0.00	Cell adhesion molecules (CAMs)
590	0.04	Arachidonic acid metabolism	592	0.01	alpha-Linolenic acid metabolism
4512	0.07	ECM-receptor interaction	4622	0.01	RIG-I-like receptor signaling pathway
1100	0.07	Metabolic pathways	5340	0.03	Primary immunodeficiency
4950	0.08	Maturity onset diabetes of the young	564	0.03	Glycerophospholipid metabolism
592	0.08	alpha-Linolenic acid metabolism	4975	0.03	Fat digestion and absorption
			565	0.03	Ether lipid metabolism
			524	0.03	Butirosin and neomycin biosynthesis
			4666	0.04	Fc gamma R-mediated phagocytosis
			4650	0.04	Natural killer cell mediated cytotoxicity
			4062	0.05	Chemokine signaling pathway
			10	0.07	Glycolysis / Gluconeogenesis
			4610	0.08	Complement and coagulation cascades
DOWN			**DOWN**		
4512	0.00	ECM-receptor interaction	910	0.00	Nitrogen metabolism
4974	0.00	Protein digestion and absorption	4974	0.00	Protein digestion and absorption
640	0.04	Propanoate metabolism	4512	0.00	ECM-receptor interaction
565	0.04	Ether lipid metabolism	4514	0.00	Cell adhesion molecules (CAMs)
4350	0.04	TGF-beta signaling pathway	4530	0.00	Tight junction
4510	0.05	Focal adhesion	600	0.01	Sphingolipid metabolism
61	0.05	Fatty acid biosynthesis	280	0.02	Valine, leucine and isoleucine degradation
4620	0.05	Toll-like receptor signaling pathway	565	0.02	Ether lipid metabolism
510	0.09	N-Glycan biosynthesis	5217	0.03	Basal cell carcinoma
5200	0.09	Pathways in cancer	780	0.07	Biotin metabolism
			4670	0.07	Leukocyte transendothelial migration

CHAPTER 5: GROWTH OF MALIGNANT NON-CNS TUMORS ALTERS BRAIN METABOLOME

Abstract

Cancer survivors experience numerous treatment side effects that negatively affect their quality of life. Cognitive side effects are especially insidious, as they affect memory, cognition, and learning. Neurocognitive deficits occur prior to cancer treatment, arising even before cancer diagnosis, and we refer to them as ‘tumor brain’.

Metabolomics is a new area of research that focuses on metabolome profiles and provides important mechanistic insights into various human diseases, including cancer, neurodegenerative diseases, and aging. Many neurological diseases and conditions affect metabolic processes in the brain. However, the tumor brain metabolome has never been analyzed.

In our study we used direct flow injection/mass spectrometry (DI-MS) analysis to establish the effects of the growth of lung cancer, pancreatic cancer, and sarcoma on the brain metabolome of TumorGraft™ mice. We found that the growth of malignant non-CNS tumors impacted metabolic processes in the brain, affecting protein biosynthesis, and amino acid and sphingolipid metabolism. The observed metabolic changes were similar to those reported for neurodegenerative diseases and brain aging, and may have significant mechanistic and diagnostic value for future tumor brain research.

Introduction

Recent successes in the development of cancer treatments have changed cancer from being a deadly disease to a chronic condition, thereby bringing cancer survivorship and quality of life to the forefront of healthcare. Cancer survivors suffer numerous side effects from treatments, including fatigue and gastrointestinal, haematological, and skin issues. Moreover, they experience chemotherapy-associated cognitive changes spanning across various domains such, as working memory, cognition, executive function, and processing speed. These chemotherapy-induced cognitive changes result in 'chemo brain' and affect up to 75% of patients, persisting for years or even decades in one-third of individuals (Ahles, 2012, Andreotti et al., 2015, Janelsins et al., 2011).

Several studies conducted over the past decade have indicated that cognitive impairment occurs long before cancer treatment begins and even before cancer diagnosis (Ahles, 2012, Hurria et al., 2007b). These findings suggest that cancer alone (independent of any therapy or treatment) exerts a negative impact on the central nervous system (CNS) (Hurria et al., 2007b). However, the mechanisms of cancer-induced cognitive impairment, or 'tumor brain,' still need further study.

In our recent studies, we established that the growth of malignant non-CNS tumors resulted in noticeable changes to global gene expression patterns, affecting numerous signalling and metabolic pathways. These alterations in gene expression patterns may in turn impact cellular metabolite levels (Brink-Jensen et al., 2013).

The cellular metabolome is comprised of all the low molecular weight molecules, called metabolites, which are the end products of biochemical and gene expression pathways in cells and tissues. Metabolomics is a relatively new area of research and seeks

to analyze metabolome profiles and provide biologically relevant insights into metabolic processes. This is valuable for the analysis of various human diseases, including cancer, neurodegenerative diseases, and aging, yielding both mechanistic insights and new disease biomarkers (Armitage and Barbas, 2014, Ivanisevic and Siuzdak, 2015, Zhang et al., 2016a, Wishart et al., 2016, Zhang et al., 2016b, Shao et al., 2016, Botas et al., 2015, Jove et al., 2014, Jones and C.A.B. International, 2014). Metabolomics facilitates the understanding of an organism's physiology and its responses to nutrition and various environmental conditions and stimuli. It has also been proposed as a new tool for exposure studies (Lenox, 2015, Wild, 2005, National Academies of Sciences, 2015, Golla et al., 2017, Cheung et al., 2017). Cancer significantly alters the metabolic profiles the blood, urine and saliva (Armitage and Barbas, 2014, Shao et al., 2017, Shao et al., 2016, Wishart et al., 2016, Zhang et al., 2016b, Zhou et al., 2017, Falegan et al., 2015, Mal, 2016). However, the tumor brain metabolome has never been analyzed.

In our study, we applied a direct flow injection/mass spectrometry (DI-MS) analysis to determine whether the growth of malignant stage IV non-small cell lung carcinoma (NSCLC), pancreatic cancer, and sarcoma caused alterations in the metabolome of the left hemibrain of TumorGraft^{TM} mice. We discovered that the growth of malignant non-CNS tumors affected metabolic processes in the brain and identified the metabolic fingerprints for tumor brain. The observed metabolic changes were similar to those reported for neurodegenerative diseases and brain aging, and may have significant mechanistic and diagnostic value for future tumor brain research.

Results and discussion

We analyzed the levels of various metabolites belonging to acylcarnitines, glycerophospholipids, sphingolipids, hexose, amino acids, and biogenic amines in the left hemibrains of lung cancer-, pancreatic cancer-, and sarcoma-bearing tumor graft mice. We identified the metabolic changes and established the metabolic profiles of the brains of tumor-bearing mice. Initially we used principle component analysis (PCA), a statistical tool to help analyze sample differences and ascertain the main variables within a multidimensional data set. PCA was based on all the analysed metabolites. Although there was no distinct clustering observed by the first and second principal components, the control group clearly separated from the samples of pancreatic tumor –bearing mice across the second component (Fig. 5.1A). The heat map of various analyzed metabolites revealed differences between individual samples, and metabolite patterns across the three groups of tumor-bearing mice and control animals (Fig. 5.1B), and showed that non-CNS tumor growth led to noticeable changes in the metabolic activity in the brain of tumor-bearing animals. Similarly to PCA analysis, in the heat map analysis, samples from pancreatic cancer-bearing mice formed separate clusters compared to the other tumor bearing and intact mice.

Due to small sample sizes, we haven't observed statistical significance in metabolite changes. However, metabolites exist and function as part of complex metabolic pathways and networks; thus, focusing on individual, or even groups of, metabolites is not informative. To gain an in-depth understanding of the magnitude and functional significance of the observed changes, we analyzed the metabolites in the context of metabolic pathways and performed the metabolite set enrichment analysis

(MSEA) by using MetaboAnalyst software (Xia et al., 2015, Xia and Wishart, 2016). MSEA allowed us to establish which pathways were affected in the brains of tumor-bearing mice as compared to controls. We also compared the metabolic pathway patterns between the groups. The analysis revealed several interesting patterns: 17 pathways were deregulated in the brains of the pancreatic cancer-bearing mice, 15 in the brains of the lung cancer-bearing mice, and 14 in the brains of the sarcoma-bearing animals (Figs. 5.2 & 5.3). Five pathways were affected in all groups. To understand if the observed enrichment patterns were caused by up- or down- regulated metabolites, we also performed enrichment analysis on up- and down-regulated metabolites separately. Some pathways were identified only in the lists of upregulated, some only in the lists of downregulated metabolites, while several exhibited bi-directional alterations in the pathway metabolites (Fig. 5.4 & 5.5).

Of the five pathways that were affected in all three experimental groups, the process of protein biosynthesis was consistently upregulated (with Holm p value less than 0.05 in all three cancers). Furthermore, in all groups, amino acid metabolism was affected, as evidenced by the changes in phenylalanine and tyrosine metabolism, as well as valine, leucine, and isoleucine degradation (Figs. 5.3 & 5,5, 5.6). The fold changes of all the amino acids, as compared to controls, are represented in Table 5.1. In the brain, excess amino acids are usually used for energy production; and oftentimes, when neurons cannot catabolize glucose, they oxidize amino acids as alternative energy sources.

These results suggest that the tumor–brain phenomenon may be similar to neurodegeneration and aging (Griffin and Bradshaw, 2017). Several previous studies have shown altered amino acid levels in the brains of Alzheimer’s disease (AD) patients

and AD mouse models, even though the functional and mechanistic significance of these changes has not yet been established (Griffin and Bradshaw, 2017). The changes in these AD studies were similar to the ones noted in tumor brain.

Decreases in the levels of amino acid in the brain, or deregulation of the machinery that metabolises them, may cause neuronal death. Likewise, amino acid oxidation and catabolism that leads to the release of ammonia may also cause neuronal apoptosis. This is due to much lower levels of several urea cycle enzymes that are needed for ammonia detoxification in neurons and glia. One of these enzymes is glutamine synthetase, which sequesters ammonia into glutamine and is expressed at very low levels in neurons. Changes in protein synthesis may cause neuronal cell death and thereby contribute to neurodegeneration. Altered amino acid metabolism was previously linked to neurological deficits in dementia patients (Griffin and Bradshaw, 2017, Liu et al., 2014), and aromatic amino acids (phenylalanine and tryptophan) increased in the AD brain(Xu et al., 2016). Furthermore, alterations in protein biosynthesis pathways were previously reported in the brains of transgenerationally stressed animals (Kiss et al., 2016), and were suggested to be related to neurological deficits.

Amino acids play pivotal roles in neural cells as neurotransmitters and their precursors. We observed small increases in the levels of glutamate, an excitatory neurotransmitter and precursor of inhibitory neurotransmitter gamma-aminobutyric acid (GABA)(Xu et al., 2016). Glutamate is involved in the pathophysiology of Alzheimer's disease, and altered glutamate levels were previously reported in AD patients (Xu et al., 2016). Aromatic amino acids are precursors of cerebral neurotransmitters, monoamine (serotonin) and catecholamine (dopamine, norepinephrine and epinephrine) (Xu et al.,

2016). We observed significant changes (according to Holm-Bonferroni *p* values) in catecholamine metabolism in the brain tissues of pancreatic cancer-, lung cancer-, and sarcoma-bearing mice (Fig. 5.7). Catecholamine biosynthesis had differential regulation due to dopamine down-regulation in pancreatic and lung cancer-bearing mice and to dopamine up-regulation in sarcoma-bearing animals.

The observed alterations in catecholamine biosynthesis and the increases in levels of aromatic amino acids (phenylalanine and tryptophan) may cause neurotransmitter imbalances analogous to those previously exemplified by lowered levels of serotonin, dopamine, and norepinephrine in the AD brain (Matthews et al., 2002, Garcia-Alloza et al., 2005, Storga et al., 1996, Xu et al., 2016), again suggesting a potential link between tumor–brain and neurodegeneration. The causes of protein and amino acid metabolism deregulation need to be further analyzed. Altered protein biosynthesis may occur as part of a compensatory or repair mechanism in response to oxidative stress and oxidative DNA damage. In addition, more studies are needed to further analyze the roles of deregulated protein and amino acid metabolisms in tumor brain and the mechanisms leading to this deregulation.

Along with protein and amino acid metabolism and amino acid degradation, we observed changes in the urea cycle of the brains of the tumor-bearing mice. The urea cycle was enriched in the brains of the lung cancer- and sarcoma-bearing animals, but not in the pancreatic cancer-bearing animals. One of the constitutive compounds of the urea cycle—citrulline—which is also a member of the amino acid pathway, was downregulated in the brains of the sarcoma animals, but upregulated in the brains of the lung and pancreatic cancer animals (Table 5.2). This deregulation may be the

consequence of altered protein metabolism, and its roles remain elusive; however, it is yet another pathway implicated in neurodegeneration (Xu et al., 2016). We also noted changes in methionine metabolism in the brains of all experimental groups (Table 5.3). Previously, altered methionine metabolism was reported to occur upon traumatic brain injury (Dash et al., 2016).

Overall, a lot of the observed changes are similar to those seen in neurodegeneration and aging and thus may have implications for chemotherapy patients as they age. This study is the first to analyse the effects of non-CNS tumor growth on the brain metabolome. In the future it would be important to correlate metabolome levels with the levels of gene expression, as well as with epigenome alterations. It would also be important to analyze and compare left versus right hemibrain metabolome, along with brain-region specific metabolic changes. Multi-level integration of various molecular domains may shed light on the molecular mechanisms and outcomes of tumor brain. It may also help develop tumor brain diagnostic and prognostic biomarkers, and guide the development of appropriate mitigation and prevention strategies.

Materials and Methods

Animal Model

To study the effects of non-CNS tumor growth on the brain metabolome, we used the mouse TumorGraft models developed and provided by the precision medicine company Champions Oncology, Inc. (Baltimore, MD). We obtained frozen brain tissues of TumorGraft mice carrying pancreatic cancer, sarcoma and lung (NSCLC) cancer patient-derived xenografts (PDX). Patients diagnosed with sarcoma, pancreatic, and lung cancer had their tumors surgically removed and small pieces of the tumor were implanted

in mice. This allowed the production of personalized TumorGraft mouse models for the development of precision oncology strategies. All patients gave their full informed consent for the use of their tumor tissues for research purposes.

The animal experiments were approved by Institutional Animal Care and Use Committee protocols. To generate mouse TumorGrafts, small tumor tissue fragments with both malignant cells and supportive stroma were implanted into the flanks of six-week-old immunodeficient female mice (female *nu*/*nu* athymic mice; Harlan Laboratories, Indianapolis, IND) and propagated as previously described(Bertotti et al., 2011, DeRose et al., 2011, Hidalgo et al., 2011, Morelli et al., 2012, Stebbing et al., 2014). When the TumorGrafts reached more than 200 mm^3, the animals were divided into groups of three. Tumor growth was monitored; tumor dimensions were regularly measured and tumor volumes were calculated as previously described (Stebbing et al., 2014). Intact animals (no tumor, no treatment, n=3) served as baseline controls. Animals were euthanized by Euthansol overdose. The brains of the animals were removed from their skulls and split in half. They were then frozen in liquid nitrogen and stored at −80 °C until further metabolomics analysis.

Tissue Sample Extraction

Metabolomic profiling was carried out at The Metabolomics Innovation Center, Edmonton, AB using mouse left hemibrains. Each tissue sample was weighed and its mass was recorded, and a tissue exaction buffer was prepared (85 mL MeOH + 15 mL phosphate buffer solution [10 mM]). Next, each tissue sample was homogenized in the tissue extraction buffer at a volume three times that of the tissue. For example, 90 uL of tissue extraction buffer was used for 30 mg of tissue. Then, the homogenized samples

were centrifuged at 14,000 rpm and the supernatant was transferred into a new vial. The resultant supernatant was stored at -20°C until further analysis by liquid chromatography tandem-mass spectrometry (LC-MS/MS).

Direct Flow Injection Mass Spectrometric Compound Identification and Quantification

We applied a targeted quantitative metabolomics approach to analyze the samples by using direct flow injection mass spectrometry (AbsoluteIDQ™ Kit). This kit assay, in combination with a 4000 QTrap (Applied Biosystems/MDS Sciex) mass spectrometer, was used for the targeted identification and quantification of a large number of endogenous metabolites, including amino acids, acylcarnitines, glycerophospholipids, sphingolipids, and sugars. This method combines the derivatization and extraction of analytes with selective mass-spectrometric detection using multiple reaction monitoring (MRM) pairs. Isotope-labeled internal standards are integrated in a Kit plate filter for metabolite quantification.

The AbsoluteIDQ™ kit contains a 96-deep well plate with a filter plate attached by sealing tape, as well as reagents and solvents used to prepare the plate assay. Fourteen wells in the kit were used for the following: one blank, three zero samples, seven standards, and three quality control samples that were provided with each kit. The samples were left to thaw on ice. Once thawed, they were vortexed and then centrifuged at 13,000 x g. Next, 10 µL of supernatant for each sample were loaded on a filter paper of the kit plate and dried in a stream of nitrogen. Next, 20 µL of a 5% solution of phenyl-isothiocyanate was added for derivatization. After incubation, the filter spots were dried again using an evaporator. Extraction of the metabolites was then achieved by adding 300

μL of methanol containing 5 mM ammonium acetate. The extracts were obtained by centrifugation in the lower 96-deep well plate, followed by a dilution step with a kit MS running solvent. A mass spectrometric analysis was performed on an API4000 Qtrap® tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) equipped with a solvent delivery system. The samples were delivered to the mass spectrometer by liquid chromatography, followed by a direct injection (DI) method. Biocrates MetIQ software was used to control the entire assay workflow, from sample registration and the automated calculation of metabolite concentrations and to the exporting of the data into other data analysis programs. A targeted profiling scheme was used to screen for known small molecule metabolites using multiple reaction monitoring, neutral loss, and precursor ion scans.

In-depth Analysis of Brain Metabolome

For each cancer-bearing animal sample, we obtained the mean metabolite level value and divided it by that of the control samples to get the fold change (FC) values. Then, we submitted the up- regulated genes (FC > 1.5) or down-regulated genes (FC < 0.8), or both, to MetaboAnalyst (Xia et al., 2012, Xia et al., 2015, Xia and Wishart, 2016). The principal component analysis (PCA) (Raychaudhuri et al., 2000), statistical analysis, and hierarchical clustering were performed using the *R* platform with the '*stats*' package. Metabolite set enrichment analysis (MSEA) was performed using the MetaboAnalyst software platform (Xia et al., 2012, Xia et al., 2015, Xia and Wishart, 2016, Chagoyen and Pazos, 2013). Pathway analysis was performed in Cytoscape, and pathway diagrams were obtained from the KEGG

(http://www.kegg.jp/kegg/pathway.html) and the SMPDB (http://smpdb.ca/) pathway databases.

Figures

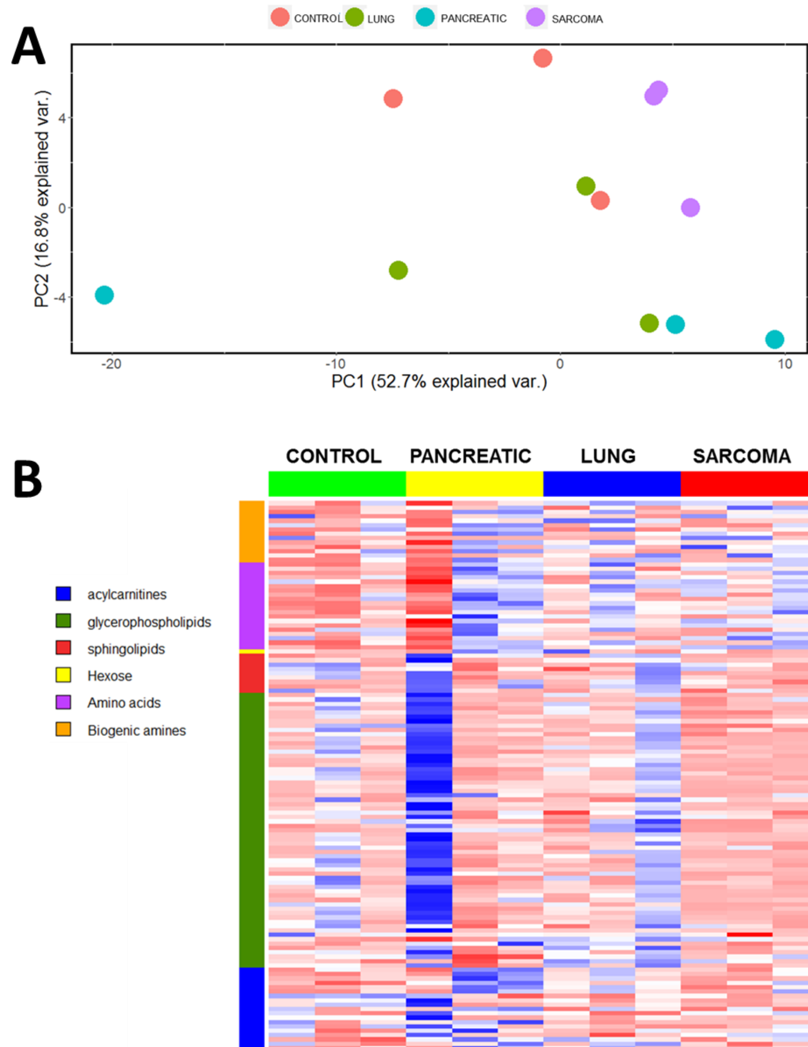


Figure 5.1: Effects of non-CNS tumor growth on the brain metabolite profiles. A. PCA analysis score plot based on the analysis of all metabolites. PCA revealed the separation between distinct groups within the analyzed dataset. B. Metabolite profile heat map of brain tissues of pancreatic cancer-, lung cancer-, and sarcoma-bearing mice, as compared to controls. Each line represent an individual sample, 3 samples/group. The heat map visually represents a metabolic signature of each individual sample, as well as reveals either the up- or down-regulation of metabolites in samples belonging to various groups, whereby red denoted increased levels, and blue denotes decreased levels. X axis shows sample group; Y axis depicts individual metabolites in metabolic groups.

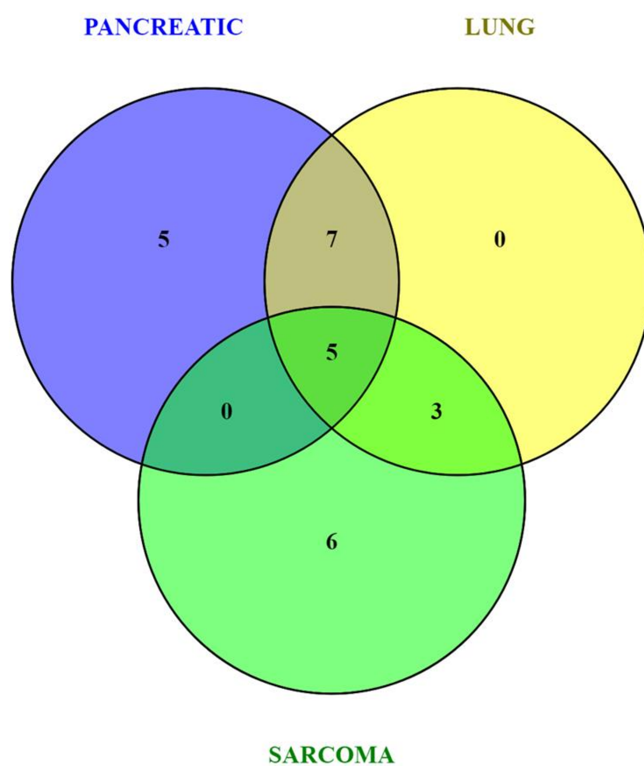


Figure 5.2: Venn diagram of metabolic pathways altered in the brains of pancreatic cancer-, lung cancer-, and sarcoma-bearing mice, as compared to controls.

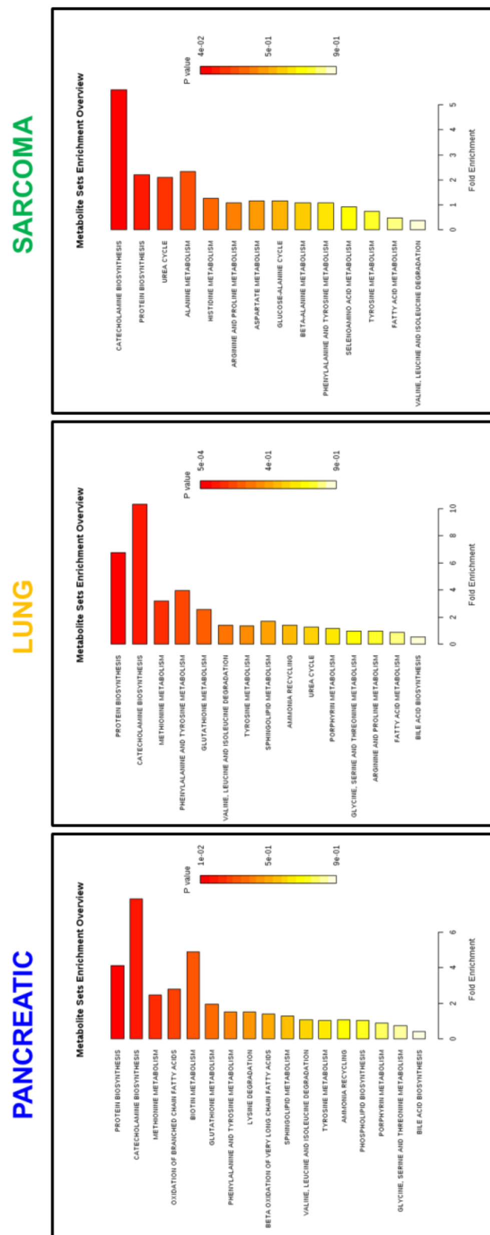


Figure 5.3: Analysis of metabolic pathways bi-directionally altered in the brains of pancreatic cancer-, lung cancer-, and sarcoma-bearing mice, as compared to controls. Metabolite set enrichment analysis allowed to establish which pathways were affected in the brains of tumor-bearing mice as compared to controls. Plots show the results of over representation analysis of various metabolic pathways based on individual metabolites that were identified in each sample within experimental groups. Only one pathway (protein [amino acid] biosynthesis) was consistently up-regulated in all three cancers. Color coding represents the p values for metabolic pathways (dark red showing the lowest p value and white showing the highest p value). The length of the bars represents the fold enrichment. For pancreatic cancer the Holm-Bonferroni adjusted p value range was 0.01 (dark red) to 0.9 (white). For lung cancer p values ranged from 0.0005 (dark red) to 0.9 (white), and for sarcoma p values ranged from 0.04 to 0.9.

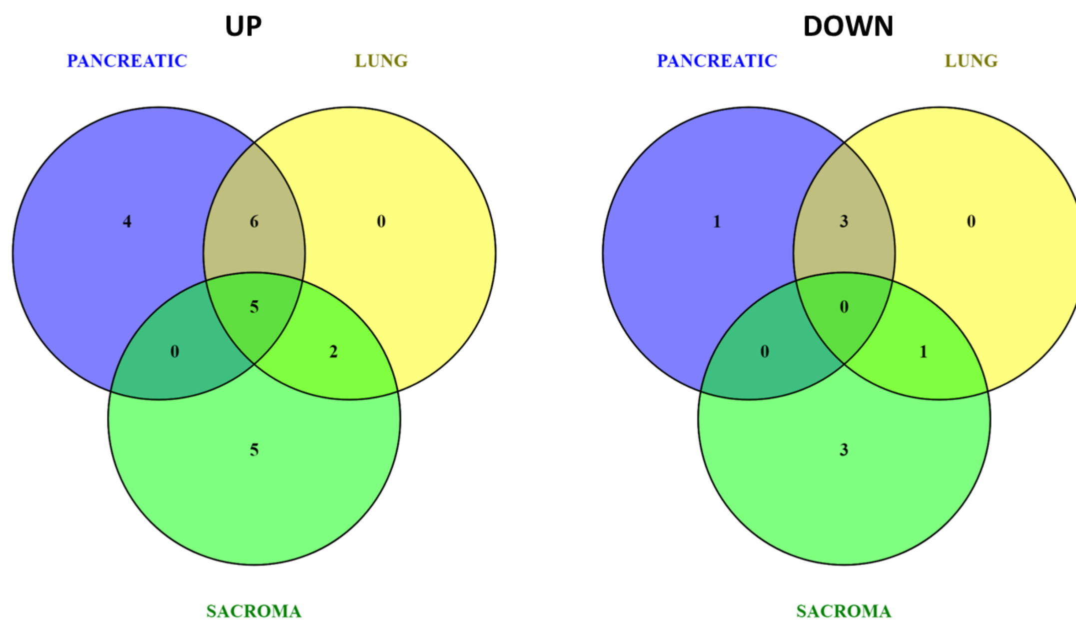


Figure 5.4: Venn diagrams of up- and down-regulated metabolic pathways in the brains of pancreatic cancer-, lung cancer-, and sarcoma-bearing mice, as compared to controls.

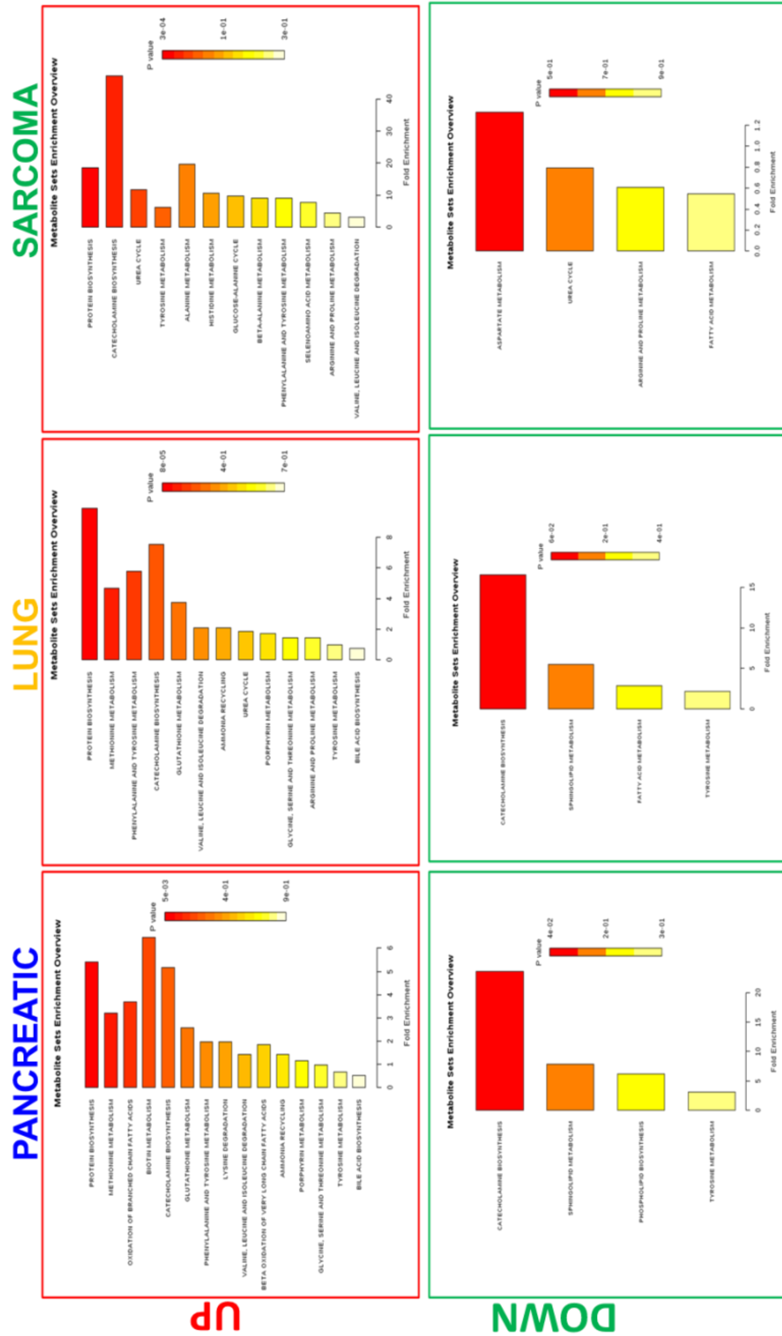
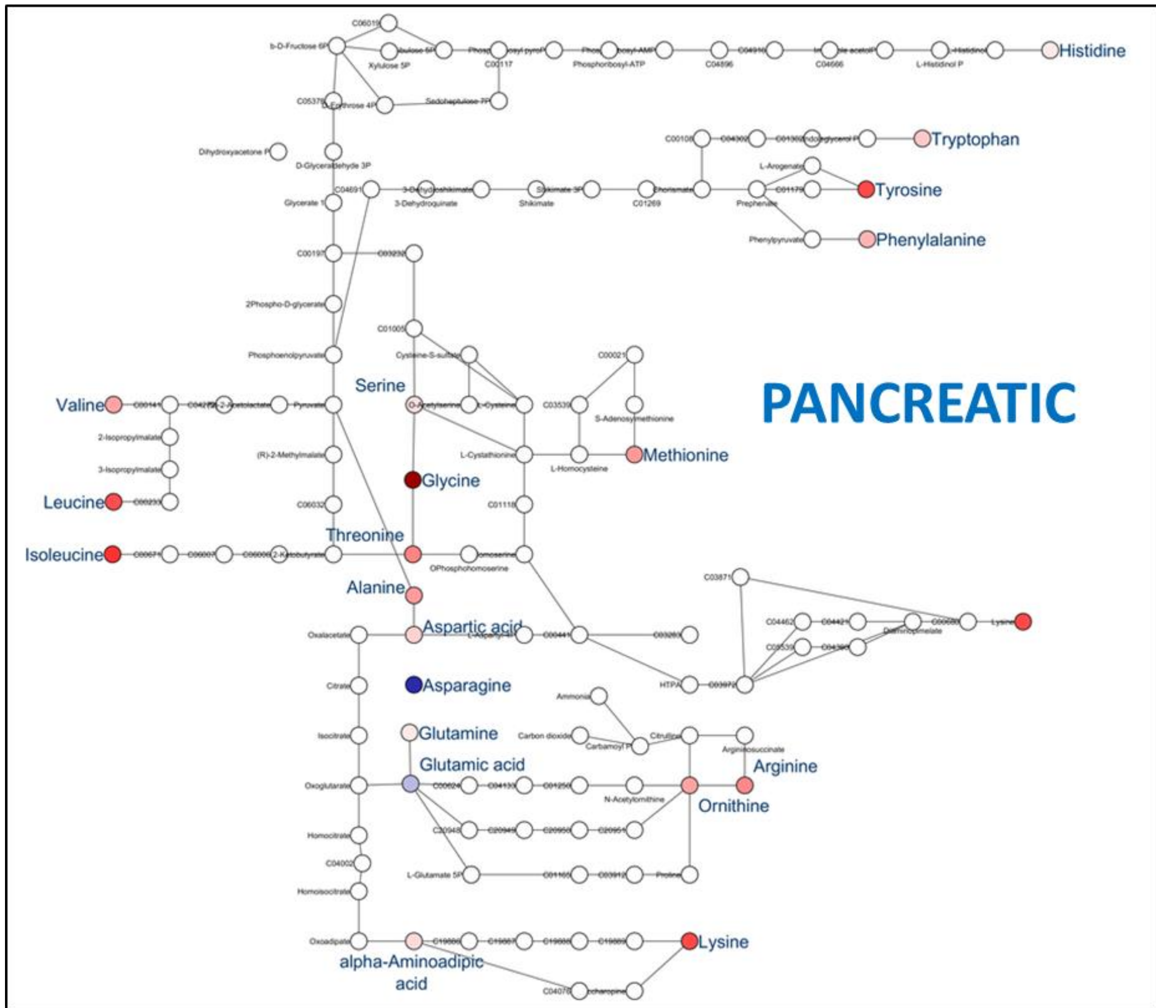
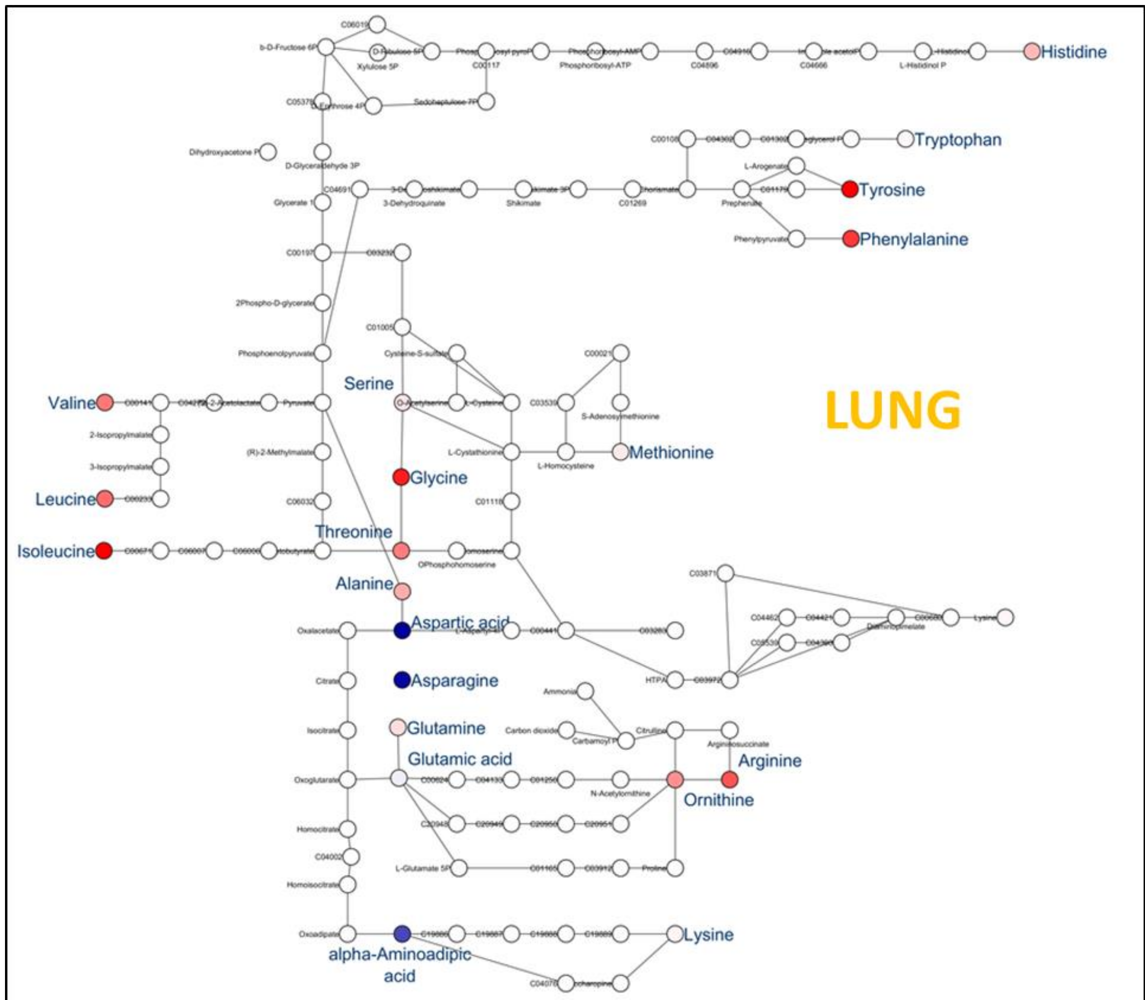


Figure 5.5: Analysis of metabolic pathways up- and down-regulated in the brains of pancreatic cancer-, lung cancer-, and sarcoma-bearing mice, as compared to controls. Metabolite set overrepresentation analysis allowed to establish which pathways were affected in the brains of tumor-bearing mice, as compared to controls. Two separate lists of up- and down-regulated metabolites were supplied for the analysis (indicated in the left part of the figure as "UP" or "DOWN"). Color coding represents the p values for metabolic pathways (dark red showing the greatest p value and white showing the highest p value). The length of the bars represents the fold enrichment.


PANCREATIC
Histidine
Tryptophan
Tyrosine
Phenylalanine
Serine
Valine
Methionine
Glycine
Leucine
Threonine
Isoleucine
Alanine
Aspartic acid
Asparagine
Glutamine
Glutamic acid
Arginine
Ornithine
Lysine
alpha-Aminoadipic
acid


LUNG
Histidine
Tryptophan
Tyrosine
Phenylalanine
Serine
Valine
Methionine
Glycine
Leucine
Threonine
Isoleucine
Alanine
Aspartic acid
Asparagine
Glutamine
Glutamic acid
Arginine
Ornithine
Lysine
alpha-Aminoadipic
acid

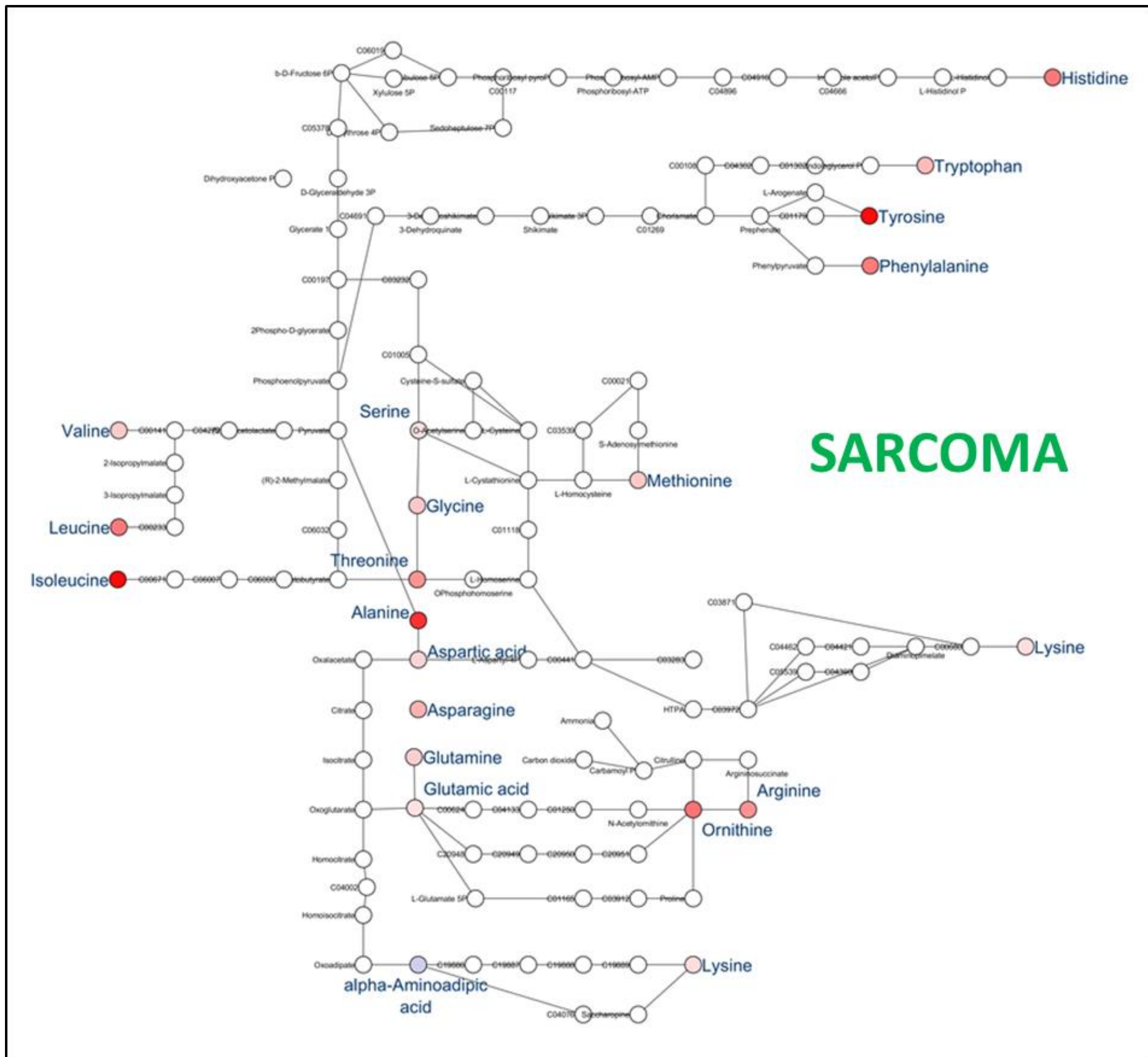
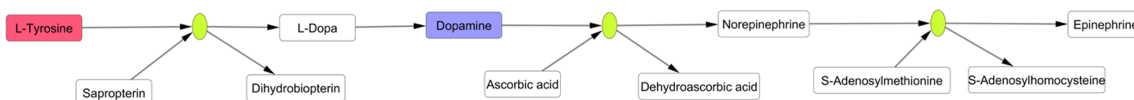
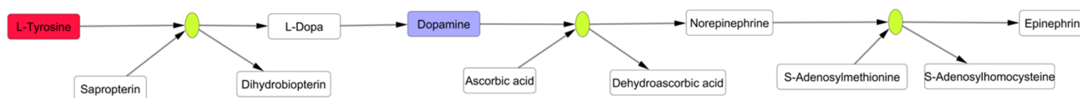


Figure 5.6: Schematic representation of the KEGG Biosynthesis of amino acids pathway in the brains of pancreatic cancer-, lung cancer-, and sarcoma-bearing mice, as compared to controls. Red denotes upregulation, as compared to control; blue denotes downregulation, as compared to control (see Table 5.1 for numeric values).

PANCREATIC



LUNG



SARCOMA

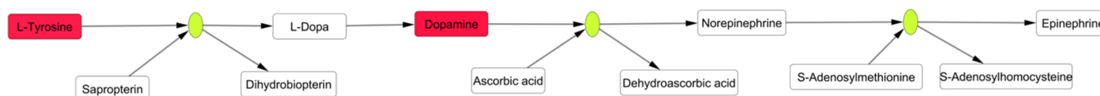


Figure 5.7: Schematic representation of SMPDB Catecholamine biosynthesis pathway. Two compounds belonging to this pathway were measured: L-Tyrosine and dopamine. L-Tyrosine was up-regulated in all three cancer-bearing groups, while dopamine was only up-regulated in sarcoma-bearing groups. Red denotes upregulation, as compared to control; blue denotes downregulation, as compared to control. Green nodes represent enzymes.

Table 5.1: Levels of amino acids in the brains of tumor-bearing animals (fold changes as compared to control animals). Red denotes upregulation as compared to control; blue denotes downregulation to as compared to control, albeit the observed changes were not statistically significant after multiple corrections. Color intensity reflects magnitude of change.

Compound	PANCREATIC	NSCLC	SARCOMA
Alanine	1.36	1.30	1.75
Isoleucine	1.75	1.93	1.89
Leucine	1.64	1.53	1.48
Lysine	1.66	1.03	1.12
Methionine	1.37	1.07	1.19
Ornithine	1.34	1.40	1.51
Phenylalanine	1.27	1.72	1.48
Proline	0.89	0.98	1.13
Serine	1.12	1.10	1.10
Threonine	1.45	1.47	1.39
Tryptophan	1.20	1.02	1.25
Arginine	1.43	1.62	1.39
Tyrosine	1.66	1.93	1.89
Asparagine	0.86	0.83	1.28
Aspartic acid	1.17	0.83	1.15
Citruline	1.15	1.08	0.66
Glutamine	1.08	1.12	1.17
Glutamic acid	0.95	0.99	1.10
Glycine	2.47	1.82	1.20
Histidine	1.08	1.25	1.49

Table 5.2: Levels of urea cycle components in the brains of tumor-bearing animals (fold changes as compared to control animals). Red denotes upregulation as compared to control; blue denotes downregulation to as compared to control, albeit the observed changes were not statistically significant after multiple corrections. Color intensity reflects magnitude of change.

Compound name	PANCREATIC	LUNG	SARCOMA
Citrulline	1.15	1.08	0.66
L-Glutamic acid	0.95	0.99	1.10
L-Aspartic acid	1.17	0.83	1.15
L-Glutamine	1.08	1.12	1.17
L-Arginine	1.43	1.62	1.39
Ornithine	1.34	1.40	1.51
L-Alanine	1.36	1.30	1.75

Table 5.3: Methionine metabolism (fold changes as compared to control animals). Red denotes upregulation as compared to control; blue denotes downregulation to as compared to control, albeit the observed changes were not statistically significant after multiple corrections. Color intensity reflects magnitude of change.

Compound name	**PANCREATIC**	**LUNG**	**SARCOMA**
Putrescine	0.97	2.14	0.93
L-Serine	1.12	1.10	1.10
L-Methionine	1.37	1.07	1.19
Glycine	2.47	1.82	1.20
Spermidine	1.76	1.60	1.42

CHAPTER 6: DISCUSSION AND OUTLOOK

Elevated cancer rates have led to increased awareness and to an outpouring of research seeking new ways to improve cancer prevention, achieve effective early detection and precise diagnostics, and develop better treatment options. Significant advances in development and implementation of new anti-cancer treatments, drugs, and regiments have turned cancer from a deadly disease to a chronic condition. In Canada, cancer survival rates for all cancers combined increased from 53% in 1992-1994 to 60% in 2006–2008, and continue to rise. In breast cancer, successes were the most pronounced, leading to 95 percent 5-year survival. While previous anti-cancer treatment and management strategies have been focused on extending life expectancy, more and more effort is being put into increasing and maintaining quality of life and reducing side effects. Ensuring that cancer patients have the best possible quality of life and suffer minimal side effects from their treatments is of great importance (Kovalchuk and Kolb, 2017).

Cancer treatment side effects manifest as CNS toxicity (Soffietti et al., 2014), and chemotherapy regimens can be more toxic to healthy brain cells than to the cancer cells they were devised to treat (Kovalchuk and Kolb, 2017),(Han et al., 2008). Chemotherapy impairs the cognitive domains of memory, attention, and executive function (Seigers and Fardell, 2011, Seigers et al., 2015, Seigers et al., 2010a, Seigers et al., 2008, Seigers et al., 2009, Seigers et al., 2013, Seigers et al., 2010b, Christie et al., 2012), causing a condition called 'chemo brain' that persists for as long as 5 to 10 years after the cessation of treatment (Mitchell and Turton, 2011a, Ahles et al., 2002, Ahles et al., 2005, Ahles et al., 1998, Vardy et al., 2008b). Chemo brain manifestations impact employability,

interactions with family and colleagues, and even patients' sense of self-worth (Wang et al., 2015a, Soffietti et al., 2014).

Until recently the mechanisms of chemo brain were not well-understood(Kaiser et al., 2014). Evidence also suggested that cognitive dysfunction occurs prior to cancer treatments and even prior to cancer diagnosis, pointing to the existence of cancer-related cognitive dysfunction (Andreotti et al., 2015, Hurria et al., 2007b), or as we've termed it, tumor brain. The mechanisms behind tumor brain, that is, of the effects on the CNS imposed solely by the growth and presence of a non-CNS tumor, also remain obscure.

Within the scope of this thesis we conducted a large-scale multifaceted analysis of the molecular mechanisms of chemo brain and tumor brain. We used non-CNS tumor-bearing mice that did not receive any chemotherapy treatment as models of tumor brain. For chemo brain analysis, we used tumor-bearing mice that were treated with different chemotherapy agents. These models account for both the presence of the tumor and, in the latter, the added effects of chemotherapy.

This is the first study to show that malignant non-CNS tumor growth affects many cellular domains, such as the transcriptome, the small RNAome, the metabolome, and global patterns of 5mC and 5hmC. In addition, tumor brain manifests as oxidative stress and an accumulation of oxidative DNA damage in the PFC and hippocampus of TumorGraft mice.

We noted that non-CNS malignant tumor growth affected global gene expression profiles in the PFC and hippocampus tissues of tumor-bearing mice (Fig. 6.1). Of those, four genes were commonly upregulated in the PFC and hippocampus tissues of both TNBC- and PR+BC-bearing mice. These were the Pleckstrin Homology and FYVE

Domain Containing 1 gene (Plekhf1), the sterol regulatory element binding transcription factor 2 genes (Srebf2), gamma-aminobutyric acid receptor subunit beta-1 gene (Gabrb1), and the high temperature requirement of a serine peptidase 1 gene (Htra1).

The Plekhf1 gene encodes for the PLEKHF1 lysosome-associated protein that takes part in caspase-independent apoptosis via the lysosomal-mitochondrial pathway (Chen et al., 2005). A recent study reported the Plekhf1 gene as an RNA biomarker of graft versus host disease (Pidala et al., 2017). Srebf2 was also identified as one of the genetic risk factors in schizophrenia (Steen et al., 2016). Its upregulation was associated with the induction of microglial apoptosis (Yoshino et al., 2011).

Gabrb1 gene is upregulated in the prefrontal cortex of rats that were exposed to methamphetamine (Wearne et al., 2016). HtrA1 encodes for a serine protease that is important in a variety of cellular processes, and its deregulation is associated with cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL), which is a severe cerebral small vessel disease that often underlies stroke and dementia (Beaufort et al., 2014).

The observed upregulation of Plekhf1 and Srebf2 in tumor brain might therefore be associated with apoptosis. Future studies are needed to uncover their precise roles in tumor brain and their contribution to neuronal apoptosis and neurodegeneration. Another intriguing outcome is the downregulation of 13 genes in the PFC and hippocampus of TNBC- and PR+BC-bearing animals. Of those, the neuronal PAS domain protein 4 (Npas4) gene was strongly downregulated in both PFC and hippocampus tissues. NPAS4 is one of the factors associated with neuronal activity and neuroprotection. It is also involved in the development of GABA synapses (Guidotti et al., 2012). Through

regulation of a number of its targets, NPAS4 coordinates the excitatory-inhibitory balance of neural circuits (Spiegel et al., 2014) and is pivotal for memory formation (Lin et al., 2008). One NPAS4 target (the neuronal activity associated transcription factor Fos), as well as two other transcription factors (Egr2 and Egr4), was also downregulated in all studied tissues. Upregulation of these factors usually mediates neuroprotection. The observed downregulation of these genes may inhibit neurogenesis and may underlie neuronal death and degeneration in tumor brain (Lai et al., 2015, Yun et al., 2010). Future studies are needed to dissect the roles of NPAS4 and its targets in tumor brain.

One of the NPAS4 targets is BDNF, a neurotrophic factor important for neural development, neuroprotection, and regeneration following injury (Zhao et al., 2017). BDNF participates in the induction and control of neurogenesis and neuronal differentiation, facilitates synaptic plasticity, and promotes neuronal survival (Behl and Kotwani, 2017). Hence, its downregulation may negatively impact those processes and thus serve as a foundation for the cognitive deficits observed in tumor brain and chemo brain. We saw that NPAS4 and BDNF were downregulated in both tumor-bearing chemotherapy treated and untreated mice, in both the hippocampus and the PFC. We noted elevated levels of oxidative stress and oxidative DNA damage in the hippocampus and PFC of tumor brain and chemo brain mouse models. Moreover, we observed significant upregulation of several miRNAs – most notably, the miR-200 family, the miR-183/96/182 cluster, and several others that regulate BDNF. BDNF may be a protein whose downregulation is crucial to the development of tumor brain and chemo brain. Its regulation in context of chemo brain and tumor brain may be achieved through either the functions of NPAS4 or miRNAs.

Therefore, more research is needed to determine the precise regulation of NPAS4 and BDNF in tumor and chemo brain, as well as the contributions of various mechanisms of their expression control. Moreover, it would be important to examine the timing and sequence of molecular events in chemo brain and tumor brain in order to determine their molecular causes and consequences. An analysis of the cross-talk and co-regulation of various pathways may also shed light on tumor brain and chemo brain mechanisms.

Within the scope of this thesis we focused on the roles of miRNAs in tumor and chemo brain and identified the roles of miR-200 family and the miR-183/96/182 cluster; the latter was recently reported as taking part in the regulation of long-term memory formation (Woldemichael et al., 2016). We also noted changes in the levels of other small RNAs, such as tRNAs, piRNAs, and snoRNAs, in the PFC tissues of TNBC- and PR+BC-bearing mice. miRNAs, as well as piRNAs, tRNAs, tRNA fragments, and other small RNA molecules, regulate neuronal development and function. They are also implicated in neurodegeneration and brain aging, including a wide array of neurodegenerative diseases, such as Parkinson's, Huntington's and Alzheimer's disease, stroke, amyotrophic lateral sclerosis, and brain tumors (Lardenoije et al., 2015, Szafranski et al., 2015, Iyengar et al., 2014, Kovalchuk and Kovalchuk, 2012, Koch et al., 2013, Baulina et al., 2016). However, neither tumor brain nor chemo brain have been explored in the small ncRNA domain further than miRNAs. Understanding the roles of various other small RNAs in cancer and cancer-associated cognitive impairment may shed light on the mechanisms of these CNS side effects.

Along with small RNAs, we also analyzed alterations in the levels of DNA methylation and DNA hydroxymethylation in the PFC tissues of tumor-bearing

chemotherapy treated and untreated mice. These epigenetic mechanisms are fundamental regulators of gene expression and genome stability (Gibb et al., 2011, Iorio and Croce, 2012, Koturbash et al., 2011b) (Mattick and Makunin, 2006), partaking in the regulation of neuronal survival, plasticity, cognitive regulation, and memory, as well as neurodegeneration and aging (Lardenoije et al., 2015, Szafranski et al., 2015, Iyengar et al., 2014, Kovalchuk and Kovalchuk, 2012, Koch et al., 2013, Baulina et al., 2016). We observed changes in the global levels of 5-mC and 5-hmC in the PFC tissues of tumor-brain and chemo-brain mice. To gain a better picture of the role of DNA methylation and hydroxymethylation in tumor brain and chemo brain, there would need to be an analysis of the levels of 5mC and 5hmC in other brain regions, such as the hippocampus. Previous studies have shown that changes in DNA methylation occur in defined regions of the genome (Weber and Schubeler, 2007). Determining the precise distribution, locus-specificity, and plasticity of the observed DNA methylation and hydroxymethylation changes would allow for the correlation of locus-specific DNA methylation and hydroxymethylation patterns with gene expression (Weber et al., 2007, Wilson et al., 2006, Wen and Tang, 2014).

Along with altered gene and miRNA expression, we noted metabolome deregulation. Gene expression pathways control metabolic pathways and, as such, the cellular metabolome constitutes the outcome of global gene expression. We saw altered regulation of protein synthesis, amino acid metabolism and degradation, sphingolipid metabolism, and several other metabolic pathways in the brains of tumor-bearing animals. Metabolic changes, as well as the vast majority of other molecular effects observed in this study, were previously implicated in aging and neurodegenerative

diseases. One way to better understand the functional significance and interconnection between mechanisms and pathways in tumor brain and chemo brain, is through integrating multi-omics data. Such a large amount of data could only be handled using special approaches – e.g. sophisticated machine learning techniques – that have shown greater promise in handling large amounts of complex, nonlinear, and multidimensional datasets than traditional approaches. Machine learning, and deep learning in particular, provides a tremendous opportunity to identify novel biomarkers of diseases and conditions (Putin et al., 2016, Mamoshina et al., 2016).

We analyzed the gene expression and epigenetic changes caused by the growth of TNBC and PR+BC breast cancer, as well as the metabolomics effects of lung cancer, pancreatic cancer, and sarcoma growth. Future studies ought to look at gene expression changes in the brains of lung cancer–, pancreatic cancer–, and sarcoma-bearing animals, and analyze the brain metabolome of TNBC- and PR+BC-bearing mice. This will allow for better comparison of tumor brain patterns as a function of tumor type, which, in addition, will require analysis of tumor brain as a function of tumor stage, grade, invasiveness, and metastatic potential.

Tumor brain must also be analyzed in context of aging. While cognitive impairments are important for adult cancer survivors, they are more critical for pediatric cancer patients and young adults. In Canada, 1,500 new pediatric cancer cases are diagnosed yearly, and approximately 10,000 Canadian children today live with cancer. Leukemia is the predominant type of malignancy among pediatric cancers, constituting close to 1/3 of all cases (http://childhoodcancer.ca/education/facts_figures). With the development of new therapeutic regiments, 80–85% of pediatric leukemia patients

survive. Nevertheless, many young cancer survivors suffer severe cognitive dysfunction (Follin et al., 2016, Hearps et al., 2016, Kunin-Batson et al., 2014). Since a developing brain is more sensitive to stress and to various toxic exposures, tumor brain manifestation may thus be more pronounced in young individuals. On the other hand, a developing brain is highly plastic and may harbor higher repair capacities. Cognitive impairment is a growing concern in cancers survivors older than 65 years of age (Mandelblatt et al., 2014). Hence, an in-depth analysis of tumor brain age-dependency will shed important light on the age-specificity of these phenomena.

Overall, the majority of molecular changes in the PFC and hippocampus of tumor-bearing animals – such as altered gene expression and miRNA expression, decreased levels of NPAS4 and BDNF, deregulated levels of DNA methylation and hydroxymethylation, oxidative stress and oxidative DNA damage, as well as altered metabolome – were previously associated with aging and neurodegeneration (Fig. 6.2). Thus, cancer and cancer therapies may accelerate brain aging.

Furthermore, it would be important to put the tumor brain results in context of the health and wellbeing of each individual patient whose tumors were studied using the TumorGraft precision oncology approach.

Here we used the TumorGraft PDX models to analyze molecular underpinnings of tumor brain and chemo brain. TumorGraft PDX models provide an excellent opportunity to discern the effects of tumor growth and chemotherapy on the brain. Over the past years they have become one of the best precision oncology tools, leading to successful treatments and durable remissions in many patients (CO, 2017). In the TumorGraft model both tumor and stromal cells are implanted, representing the entire

tumor microenvironment, thereby making the TumorGraft model superior to the xenograft model where only tumor cells are implanted into animals. Nonetheless, while TumorGraft models are excellent precision oncology tools, they may harbor certain limitations for the analysis of tumor brain and chemo brain in the organismal context. Tumor growth and the associated health deterioration in mice may cause behavioral changes that, in turn, may affect epigenetic processes in the brain. Tumor growth and chemotherapy treatments may impact levels of discomfort and pain, affect appetite, impair general activity and fitness levels, and thus may contribute to the molecular and epigenetic manifestations of tumor brain and chemo brain. Therefore, in the future it would be important to establish a precise timeline of tumor growth and any changes in animals' health and fitness, behaviour, and the molecular epigenetic characteristics of tumor brain and chemo brain, and establish careful correlations between them. On the one hand, TumorGraft models may have some yet unexplored behavioral shortfalls due to cancer growth, on the other hand, similar concerns are valid when analysisng tumor brain in human cancer patients. Cancer patients experience fatigue, pain, distress, and a wide array of psychological issues, all of which can also contribute to tumor brain as well as chemo brain. Therefore, regardless of possible limitations, TumorGraft models are excellent tumor brain and chemo brain models that may provide a lot of mechanistic, anatomical, and behavioral insights into this severe CNS complication.

Given that non-CNS tumor growth and chemotherapy exposure lead to molecular epigenetic changes, analyzing neuroanatomical and behavioral post-chemotherapy outcomes is an interesting area for future study.

Other potential limitation of the TumorGraft models in their price and labor intensiveness, leading to rather small group sizes. While in the future it would be interesting to increase the sample size and further reconfirm the data, animals TumorGraft numbers can be a rate-limiting factor, as the numbers of animals with tumor grafts depends upon each individual tumor propagation and implantation rates. Albeit, based on the long-term experience of the Champions Oncology, analysis of 3 to 5 animals per group leads to robust precision oncology outcomes.

While this research sheds some light on the mechanisms of tumor brain and chemo brain, signalling between the brain and the tumor remains enigmatic. Reactive oxygen species may be potential messengers – though they are very short-lived. Alternately, inflammatory cytokines or cell-free DNA or RNA might carry out messaging, albeit it is unclear whether those can cross the blood-brain barrier.

Cells release a wide array of micro-vesicles into the extracellular space. Among these, exosomes (the 30–100 nm nano-vesicles released by cells and present in all biological fluids) have emerged as potential regulators of cellular function. Previously thought to be waste carriers, exosomes have recently become the centres of attention for understanding the etiology of many conditions (Mathivanan et al., 2010, Thery et al., 2002). Secreted by all cell types, exosomes can transfer molecules from one cell to another via the membrane vesicle traffic regulating function of distant cells and organs, such as the regulation of immune responses (Thery et al., 2002). Exosomes are implicated in numerous diseases, such as cancer, in which they are involved in the initiation, growth, progression, and drug-resistance of tumors (Weidle et al., 2017). They also contribute to cell-cell communication, and recent studies have shown that exosomes derived from

cancer cells are important mediators of cell migration, invasion, and metastasis. Exosomes may be involved in long-distance radiation effects – that is, effects that occur in cells and tissue that have not been irradiated and receive signals from irradiated cells instead (Al-Mayah et al., 2012, Jella et al., 2014, Al-Mayah et al., 2015, Xu et al., 2015). Therefore, exosomes may be candidates for tumor brain signalling.

One important yet under-investigated aspect of tumor brain and chemo brain is the development of precision health biomarkers associated with predisposition to this negative side effect, as well as the development of novel strategies to mitigate CNS toxicity manifestations. Mitigation of tumor brain and chemo brain may relieve cancer patients' suffering and significantly increase their quality of life. Such interventions could be developed based on approaches studied and formulated for recovery after brain injuries in animal models. These approaches are environmental enrichment, complex housing, psychomotor stimulants, tactile stimulation, and exercise. Corresponding strategies may include lifestyle and nutritional approaches, vitamin and nutrient supplementation, and diet and exercise that have all been shown to promote brain plasticity and neurogenesis. These strategies have been successfully applied to facilitate recovery after stroke and to prevent the progression of Alzheimer's and Parkinson's diseases. Moreover, given a link between tumor brain and aging, some novel geroprotectors can be explored as potential anti-tumor brain regimens (Moskalev et al., 2015).

Preclinical animal model–based data can serve as a foundation for clinical research and for the development of cancer- and cancer therapy–related cognitive impairment biomarkers. For that, molecular changes observed in the brain ought to be

correlated with those in the blood. This may allow for the development of informative liquid biopsy biomarkers of tumor brain and chemo brain. This study lays a foundation for future translational research approaches and for the development of novel research-based strategies and interventions that can help prevent and mitigate tumor brain and chemo brain.

Figures

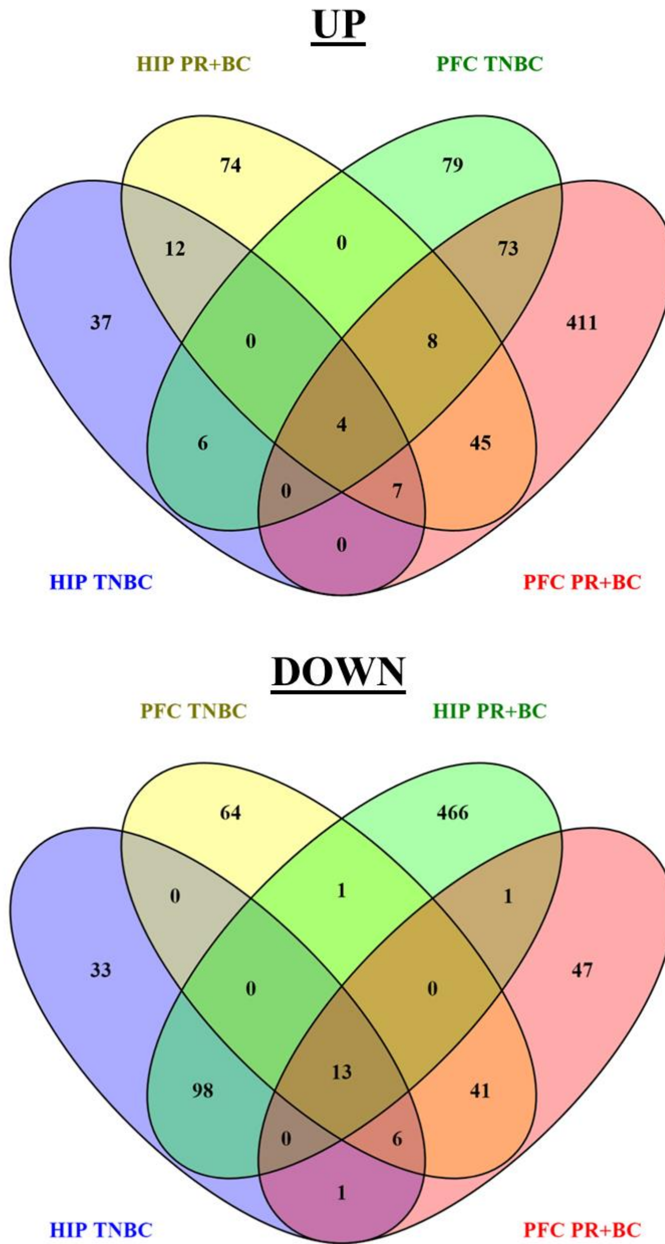


Figure 6.1: Global gene expression in the PFC and hippocampus tissues of tumor-bearing mice, as compared to controls.

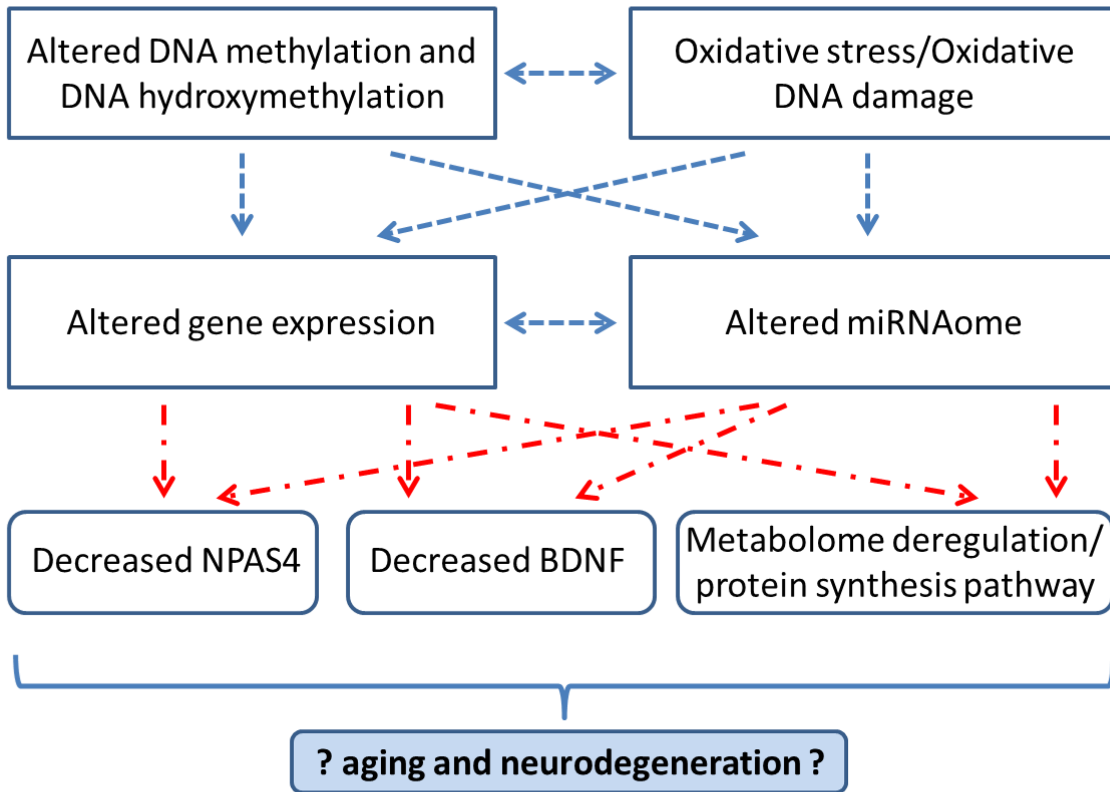


Figure 6.2: Molecular changes in tumor brain and aging: schematic representation.

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