RADIATION INDUCED EPIGENETIC DYSREGULATION IN RAT MAMMARY GLAND TISSUE

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In loving memory of Betty J. McRae.

Thank-you for teaching us to always follow the 4 L's of Life:

Live, Love, Laugh, and Learn.

ABSTRACT

Most breast cancer patients undergo radiation diagnostics and are also treated with radiotherapy. In addition to being an important treatment modality, ionizing radiation (IR) is a potent tumour-causing agent that has been linked to breast cancer development. However, the exact molecular etiology of IR-induced mammary gland carcinogenesis remains unknown.

We set out to analyze the role of DNA methylation in mammary gland responses to low dose IR using a well-established rat model. We also studied low dose IR effects on global gene expression and microRNAome. We found that exposure to low, mammography-like dose of IR led to a significant loss of global DNA methylation in rat mammary gland tissue. Furthermore, low dose IR significantly affected rat mammary gland transcriptome and microRNAome.

The datasets generated within the scope of this thesis may be used to identify novel predictive biomarkers for assessment of the magnitude of IR effects on mammary gland tissue.

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IR – ionizing radiation

LNT – linear no-threshold

HER2 / ERRB2 – human epidermal growth receptor 2

kVp – peak kilovoltage (unit)

DNMT3b – DNA methyltransferase 3b

DNMT3a – DNA methyltransferase 3a

DNMT1 – DNA methyltransferase

stRNA – small-temporal RNA

miRNA – microRNA

kB – kilobases

mRNA – messenger RNA

pri-miRNA – primary RNA

dsRNA – double stranded RNA

RanGTP – Ras-related nuclear protein bound to GTP (guanosine triphosphate)

TRBP – thyroid hormone receptor-binding protein

PACT – protein activator of the interferon-induced protein kinase

AGO2 – Argonaute 2 (gene/protein)

MRE – putative DNA G:T-mismatch repair endonuclease

UTR – untranslated region

PTEN – phosphatase and tensin homolog

p27/Kip – cyclin-dependent kinase inhibitor 1B

ERK5 – extracellular signal-regulated kinase 5

VCAM – vascular cell adhesion molecule

HDAC – histone deacetylase

LET – linear energy transfer

p16 – tumor suppressor gene/protein

H2AX – histone H2A variant

Ser – serine

H4K20 – histone H4 variant

H3K9 – histone H3 variant

LD – lethal dose

NCTR – National Center for Toxicological Research (colleges)

HpaII – (restriction enzyme)

MspI – (restriction enzyme)

µg – microgram (unit)

PCR – polymerase chain reaction

mM – millimolar (unit)

$MgCl_2$ – magnesium dichloride

^oC – degrees celcius

h –hour

µl – microliter

M – molar

Grb7 – growth factor receptor-bound protein 7

SH2-domain – src homology 2 domain

PKB – protein kinase B

PI3-K – phosphatidylinositol – 3 – kinase

KIT (CD117) – cytokine receptor

ESR1 – estrogen receptor alpha

CT – computed tomography

ANOVA – analysis of variance

NF-kappaB – nuclear factor kappa B

SOCS1 – suppressor of cytokine signalling 1

ER – estrogen receptor

PR – progesterone receptor

Rbl2 – Retinoblastoma-like protein 2

Mapk6 – mitogen-activated protein kinase 6

PU-box – purine-rich box

FxR – fragile X mental retardation protein

JNK1 – mitogen-activated protein kinase 8

RISC – RNA-induced silencing complex protein

INTRODUCTION

RADIATION EFFECTS - A HISTORIC PERSPECTIVE

Radiation exposure to humans occurs to everyone on a daily basis; this form is known as cosmic radiation, which originates from outside of our solar system. Humans are also exposed to other types of radiation such as specific isotopes of potassium, uranium, and thorium (that are naturally located within the earth). One of the largest sources of radiation exposure to humans comes from man-made sources such as medical diagnostic tools, nuclear power, weapons of mass destruction, food sterilization, and safety measures such as x-ray scanners located in airports. There is much debate over the risk associated with exposure to various doses of ionizing radiation.

In 1991, the International Commission on Radiological Protection adopted a linear no-threshold (LNT) hypothesis as a fundamental basis for predicting the risk associated with exposure to ionizing radiation (Protection) 1991). The LNT phenomenon for humans basically means that not only is the risk directly proportional to the total dose, but that there is no threshold below which exposure does NOT pose a risk. Because of this “no-threshold,” it can be postulated that multiple doses of any level can create some sort of risk, thus can be treated as additive. Yet, based on observational data, and not the LNT hypothesis, it has previously been assumed that the risk associated with low

dose exposure is much less than that of higher dose exposures (Mitchel 2007). In the last 5-10 years this line of thought has come into serious question (Tubiana, Aurengo et al. 2006). Recent studies have provided compelling evidence that low energy x-rays (similar to those used for mammography breast screening) are 2 – 6 times more effective in causing mutational damage to breast tissue than higher energy x-rays (Frankenberg, Kelnhofer et al. 2002; Frankenberg, Kelnhofer et al. 2002; Heyes, Mill et al. 2009).

Cells respond to ionizing radiation in a few different manners and may even undergo a form of an adaptive response. Cellular adaptive response has been described as a mechanism by which cells protect themselves against the detrimental effects of subsequent harmful events (Mitchel 2007). One form of cellular adaptive response has been termed the “bystander effect” (Mitchel, Jackson et al. 2004; Asur, Balasubramaniam et al. 2010; Asur, Balasubramaniam et al. 2010; Tsukimoto, Homma et al. 2010; Wright 2010). The bystander effect occurs when only some of an organisms cells receive a low dose of ionizing radiation and subsequently pass along the “protective message” to surrounding cells through chemical signalling.

Therefore, some researchers feel that radiation at low levels actually promotes such a type of “protectiveness” for the cell or organism (Ahmed, Fan et al. 2008; Mishra, Ahmed et al. 2008; Tsai, Stuart et al. 2009), while their opponents hold to the linear no-threshold (LNT) limit theory.

Due to the compelling data obtained by researchers who are investigating the effects of low-dose ionizing radiation on various organisms' systems, one may predict that the LNT limit theory is the better choice for assessing the actual risk with exposure to any dose level of IR, especially when it comes to human exposure. Notwithstanding, it has been a very difficult to substantiate the LNT theory based on epidemiological studies. Because cancer is the disease feared and associated the most with exposure to any level of IR, when scientists and statisticians try to associate low level IR exposure with cancer, many other factors must be considered because of their known (or suspected) contribution to the initiation and propagation of cancer (such as smoking, alcohol consumption, diet, occupation, age, sex, etc.) which adds a great deal of complexity to the situation.

We have progressed a great deal with respect to our knowledge of ionizing radiation and it's direct, indirect, short term, and/or long term effects that are induced in radiation-exposed living organisms and most importantly, humans. For instance, in the early 1900's, the "shoe-fitting fluoroscope," designed by the Adrian X-ray Company, was used to assess how a shoe fit your foot before you bought it. This practice went on for approximately 25 years, until doctors and researchers started to become more concerned with exposure to radiation (Lewis and Caplan 1950). Due to accumulation of a substantial amount of data on radiation effects, today an idea like this would be considered ludicrous and would never be allowed to be put into public use.

Notwithstanding, a lot has to be learned about the molecular and cellular effects of radiation exposure in general and low dose radiation exposure in particular.

RADIATION AND CANCER

Ionizing radiation (IR) is an important diagnostic and treatment modality. Notwithstanding, it is a potent DNA damaging agent that can lead cause serious health effects including cancer (Little 1999). The first IR-induced cancer was reported in 1902 (Little 2000). Yet, IR still remains the key diagnostic and treatment tool for the majority of cancers (Pollack, Zagars et al. 2000; Roof, Fidias et al. 2003; Potter 2006; Erven and Van Limbergen 2007). However, while modern cancer IR-based diagnostics and IR therapy have led to increased patient survival rates, the risk of treatment-related deleterious effects, including secondary cancers, is becoming a growing problem (Boice, Harvey et al. 1992; Leone, Mele et al. 1999; Brenner, Curtis et al. 2000; Rund and Ben-Yehuda 2004; Brenner, Hall et al. 2005; Hall 2006).

Furthermore, data suggest that even relatively low doses of IR such as those being used in X-ray diagnostic procedures or Computer Tomography can underlie the development of IR-induced cancers (Preston-Martin, Thomas et al. 1989; Brenner and Hall 2004). A large pool of knowledge about IR-induced effects stems from data of atomic bomb survivors and individuals that were exposed to radiation as a result of industrial accidents. The atomic bomb survivor studies show that IR exposures lead to significantly increased cancer

rates, especially leukemia (Folley, Borges et al. 1952), breast cancer (Watanabe, Shimosato et al. 1972; Wakabayashi, Kato et al. 1983; Carmichael, Sami et al. 2003), thyroid carcinoma (Watanabe, Shimosato et al. 1972; Wakabayashi, Kato et al. 1983), and lung and stomach cancers (Wakabayashi, Kato et al. 1983). The significantly elevated cancer rates were also reported in human populations exposed to IR from nuclear power accidents and at various nuclear test sites (Kossenko 1996; Shilnikova, Preston et al. 2003).

Amongst the accidents, the Chernobyl disaster in April 1986 was the most devastating. It led to significant increases in the incidence of thyroid carcinomas (Bogdanova, Zurnadzhy et al. 2006; Likhtarov, Kovgan et al. 2006; Williams 2006) , leukaemia and lymphoma (Gluzman, Imamura et al. 2005; Balonov 2007), breast cancer (Pukkala, Kesminiene et al. 2006; Prysyazhnyuk, Gristchenko et al. 2007), bladder cancer (Morimura, Romanenko et al. 2004), and renal-cell carcinomas (Romanenko, Morimura et al. 2000; Baverstock and Williams 2006; Williams and Baverstock 2006). Elevated cancer rates were also documented as being reported in the population of the Semipalatinsk nuclear test site (Salomaa, Lindholm et al. 2002; Tanaka, Iida et al. 2006).

Ionizing radiation influences a wide variety of processes in exposed cells. It can lead to changes in gene expression, disruption of mitochondrial processes, cell cycle arrest and apoptotic cell death (Amundson, Bittner et al. 2003; Amundson and Fornace 2003; Criswell, Klokov et al. 2003; Fei and El-Deiry 2003; Iliakis, Wang et al. 2003; Powell and Kachnic 2003; Jeggo and

Lobrich 2006; Rodemann, Dittmann et al. 2007; Valerie, Yacoub et al. 2007) Most importantly, IR is a powerful DNA damaging agent capable of inducing cross linking, nucleotide base damage and single and double strand breaks (Ward 1995; Little 2000; Huang, Fang et al. 2003). Accumulation of DNA damage caused by IR in conjunction with disrupted cellular regulation processes can lead to carcinogenesis (Little and Muirhead 2000; Barcellos-Hoff 2005; Sowa, Arthurs et al. 2006).

BREAST CANCER - INCIDENCE AND MOLECULAR AETIOLOGY

Breast cancer is the most common type of malignancy in women (Parkin 2001; Parkin, Bray et al. 2005) with worldwide incidence continuing to rise (Ellsworth, Ellsworth et al. 2004). Breast cancer has become the second leading cause of cancer-related deaths among North American women and the leading cause of death among women aged 35 to 55 years (Widschwendter and Jones 2002; Schairer, Mink et al. 2004). According to the Canadian Cancer Society, approximately 22, 700 women will be diagnosed with breast cancer in one year and 5400 will die from it. That is to say that on average, 437 Canadian women will be diagnosed with breast cancer every week and 104 will die from breast cancer every week.

In order to understand how breast cancer starts and propagates (with the possibility leading to new methods of treatment and possible cure) one must

first understand the parts of the breast and their function and/or purpose. The human breast is made up of glands, ducts and fatty tissue.

Breast cancer progresses similarly to the other cancer types, and the stages of breast carcinogenesis include initiation, propagation and later-metastasis. There are many types of breast cancer including phyllodes tumors, angiosarcomas (both beginning in the connective tissue of the breast) (Bjurstam, Bjorneld et al. 1997; Dalberg, Mattsson et al. 1997; Reis-Filho, Simpson et al. 2005; Li, Daling et al. 2006), ductal carcinoma and lobular carcinoma (Daling, Malone et al. 2002; Li, Malone et al. 2003; Li, Daling et al. 2006), which are primarily defined by the location of the tumor within the mammary gland. Not only are breast cancers defined by where they start, but also whether or not they are invasive (i.e. spreading outside of the membrane that lines the duct or lobule, thus associated with the stage/level of cancer) or non-invasive (meaning they remain within the tissue of origin).

When a patient is diagnosed with breast cancer, the method(s) of treatment utilized by the physician will be based on a number of criteria, including the stage and grade of the tumor, the hormone receptor status, HER2 receptor status, the type of breast cancer, age and overall health of the patient, menopausal status and the personal preference and/or situation of the patient. Breast cancer treatments can include one or a combination of the following: surgery, radiation therapy, chemotherapy, hormonal therapy and biological therapy (enhancing the individuals' own immune system to aid in the fighting of

the cancer) (Li, Daling et al. 2006). The sequential accumulation of various genetic changes in the genesis of breast cancer has been studied (Shackney and Silverman 2003; Ellsworth, Ellsworth et al. 2004; Simpson, Reis-Filho et al. 2005), whereas the contribution of epigenetic alterations to the early molecular aetiology of breast cancer must still be analyzed.

The cellular and biological mechanisms that are implicated in the predisposition, initiation and progression of human breast cancer are still poorly understood, however it is an area of great interest. If we can deepen our understanding of these mechanisms, it can provide a great deal of aid in the prevention and treatment of breast cancer.

RADIATION EXPOSURE AND BREAST CANCER

Only 5% of breast cancer cases are due to abnormal genetic function (Ronckers, Erdmann et al. 2005). Factors contributing to the remaining 95% of breast cancer cases remain unclear; however, the role of environmental mutagens in breast cancer etiology has recently received a lot of attention (Ronckers, Erdmann et al. 2005). Amongst these, ionizing radiation (IR) has been shown to strongly induce breast cancer in exposed individuals (Boice, Preston et al. 1991; Storm, Andersson et al. 1992; Mattsson, Ruden et al. 1993; Howe and McLaughlin 1996; Mattsson, Hall et al. 1997; Brenner, Sawant et al. 2002; Land, Tokunaga et al. 2003; Constine, Tarbell et al. 2008). Awareness of IR-induced breast cancer is derived from epidemiological studies of atomic bomb survivors and women exposed to diagnostic and therapeutic irradiation

(Boice, Preston et al. 1991; Storm, Andersson et al. 1992; Mattsson, Ruden et al. 1993; Howe and McLaughlin 1996; Land, Tokunaga et al. 2003). IR-induced breast cancer in the medically-exposed population is a growing clinical problem (Ronckers, Erdmann et al. 2005). Elevated breast cancer risks have been reported in patients with scoliosis and tuberculosis (Boice, Preston et al. 1991; Howe and McLaughlin 1996), women treated for benign breast disease and post-partum mastitis (Mattsson, Ruden et al. 1993; Land, Tokunaga et al. 2003; Ronckers, Erdmann et al. 2005) and in cancer survivors, all of whom have received radiation therapy (Storm, Andersson et al. 1992; Ronckers, Erdmann et al. 2005). Average IR-exposure doses linked to the development of breast cancer range between 0.2 and 20 Gy (Ronckers, Erdmann et al. 2005). IR-exposure is especially dangerous in young women. Women irradiated for Hodgkin's disease when <30 years of age have a much higher risk of breast cancer (Constine, Tarbell et al. 2008).

There has been much debate about benefits and risks of diagnostic mammography in the detection of breast cancer (Brenner, Sawant et al. 2002; Berrington de Gonzalez and Reeves 2005; Nekolla, Griebel et al. 2008). Specifically, risks of mammography-related IR-exposure in conjunction with risks of IR-induced carcinogenesis (Brenner, Sawant et al. 2002; Berrington de Gonzalez and Reeves 2005; Constine, Tarbell et al. 2008; Nekolla, Griebel et al. 2008) have been questioned. Glandular doses from mammography are low, typically around 3 mGy of 26-30kVp X-rays (Kruger and Schueler 2001;

Brenner, Sawant et al. 2002); however the main concern is that these low energy rays are more hazardous, per unit dose, than high-energy X- or γ-rays (Brenner, Sawant et al. 2002). Low-energy X-rays exhibited a higher oncotransformation potential than 200kVp X-rays, suggesting that low-energy X-rays used in mammography are considerably more biologically active than previously thought (Frankenberg, Kelnhofer et al. 2002; Frankenberg, Kelnhofer et al. 2002). This finding is consistent with the data on chromosome aberration induction by low energy X-rays (Brenner and Amols 1989).

In vitro studies (Shellabarger 1976; van Bekkum and Broerse 1991; Russo and Russo 1996; Ronckers, Erdmann et al. 2005) have also shown that IR can alter tissue function by promoting neoplastic transformation of normal breast cells (Calaf and Hei 2000; Calaf and Hei 2001; Calaf, Alvarado et al. 2005), but specific mechanisms have not been determined as of this point in time. Molecular mechanisms contributing to the etiology of IR-induced breast carcinogenesis consist of prominent genetic and epigenetic changes and have been the subject of extensive research in recent years.

The sequential accumulation of various genetic changes in the genesis of breast cancer has been studied, whereas the contribution of epigenetic alterations to the early molecular etiology of breast cancer still must be analyzed.

EPIGENETICS AND CANCER

The term “epigenetics” has become a very common term used in the various fields of molecular biology. Basically, it has been defined as changes in gene expression based on heritable factors that do not involve changes to the DNA sequence. Epigenetic processes encompass meiotically heritable and mitotically stable alterations in gene expression that include DNA methylation, histone modification, and RNA-associated silencing (Jaenisch and Bird 2003).

DNA Methylation

Mammalian DNA methylation has been described as a covalent addition of a methyl group at the 5-carbon position of the cytosine residues found within cytosine-guanine dinucleotides (CpG). Methylation plays a key part in controlling gene expression, genetic imprinting, and tissue- or temporal-specific gene expression (Gopalakrishnan, Van Emburgh et al. 2008). DNA methylation is a stable and heritable yet reversible epigenetic trait of mammalian genomes (Jaenisch and Bird 2003; Baylin 2005; Baylin and Ohm 2006; Jirtle and Skinner 2007; Weber and Schubeler 2007; Weidman, Dolinoy et al. 2007; Gopalakrishnan, Van Emburgh et al. 2008; Ooi and Bestor 2008). CpG dinucleotides are found throughout any eukaryotic organism’s genome. The CpG dinucleotides that tend to cluster into islands containing the GC content of >55% in a 500-bp region (Turek-Plewa and Jagodzinski 2005; Weber and Schubeler 2007). The status and maintenance of the methylation of CpG

islands is a critical factor that affects gene transcription, as CpG islands are present in approximately 70% of human gene promoters and/or the first exon of many genes (Saxonov, Berg et al. 2006). It has been known for some time now that methylation of CpG islands is common and essential for silencing and regulation of many types of DNA sequences (Ooi, O'Donnell et al. 2009).

It is well accepted that the methylation patterns of a gene promoter are crucial to the regulation of the expression of the gene, and a correlation between methylation status and gene expression is apparent (Mohn and Schubeler 2009). The general rule of thumb is that if the gene promoter is highly methylated, then that gene is silenced.

Since CpG islands are observed within promoter regions of about ~72% of human genes, methylation of CpG-rich promoters frequently coincides with reduced gene activity (Saxonov, Berg et al. 2006). Reduced gene expression is achieved either directly through disruption of the transcription factor and RNA polymerase binding or indirectly through the recruitment of methyl-CpG binding domain proteins as subsequent chromatin remodeling (Klose and Bird 2006).

This silencing method, in which the cell is capable of turning genes on and off when needed either in the maintenance of the cell itself or during development of the organism, is essential to the survival of the organism. Also, this principle allows for mechanisms within the cell to methylate foreign DNA thus not allowing transcription of the foreign DNA to interfere with the normal

workings of the cell (Jahner, Stuhlmann et al. 1982). Thus, besides controlling gene expression, DNA methylation suppresses parasitic DNA sequences such as transposons and endogenous retroviruses (Esteller 2005). Interestingly, global hypomethylation is a hallmark of all stages of tumor cells with a 20%-60% decrease in methylated cytosines. This decrease in methylated DNA coincides with the reactivation of transposable elements, mitotic recombination (leading to loss of heterozygosity) and aneuploidy (Robertson and Wolffe 2000; Robertson 2002; Weber and Schubeler 2007; Weidman, Dolinoy et al. 2007). Furthermore, cells lacking the activity of DNMT3b display high levels of chromosome aberrations (Xu, Bestor et al. 1999). Therefore, in a hypomethylated environment chromosomal instability increases and genome integrity is challenged.

Under normal conditions (i.e. no introduction of foreign DNA, influence of environmental factors, etc.) DNA methylation patterns are very dynamic during development of the organism. This is due to the cell's need to turn genes on and off based on their need during developmental growth of the organism. However, once cells have become differentiated, changes in DNA methylation patterns become stagnant and are actually inherited across the generations of the cells. Once a cell has become differentiated, it's DNA methylation patterns are inherited by its progeny in order to conserve the appropriate expression of genes required for homeostasis of the tissue, organ and the organism overall (Ooi and Bestor 2008; Ooi, O'Donnell et al. 2009).

Changes in methylation resulting in hypermethylation or hypomethylation of various regions of an organisms' genome can have a drastic effect not only on the cells directly affected, but it can contribute to the development of malignant or autoimmune diseases within the organism. Even though the exact mechanism by which DNA methylation changes may contribute to carcinogenesis still needs to be precisely defined, it can be deduced that not only can DNA methylation changes contribute to genome instability, but they may also directly affect the expression of oncogenes and other important regulatory genes within the cell.

Three main groups of proteins partake in establishing and maintaining DNA methylation patterns within mammalian cells. These are DNA methyltransferase (DNMT) 1, DNMT3a, and DNMT3b (Goll and Bestor 2005; Brenner and Fuks 2006; Gopalakrishnan, Van Emburgh et al. 2008). DNMT3a and DNMT3b are responsible for *de novo* methylation of sequences. Mutant mice lacking either of these genes die within weeks of birth (DNMT3a mutants) or are not viable through embryonic stages (DNMT3b mutants) (Gopalakrishnan, Van Emburgh et al. 2008). Contrarily, DNMT1, an enzyme that co-localizes to the replication forks, is responsible for maintaining methylation patterns of hemi-methylated DNA following replication (Goll and Bestor 2005; Jirtle and Skinner 2007; Weber and Schubeler 2007). DMNT1-/- mice are embryonic lethal (Li, Bestor et al. 1992).

DNA methylation in cancer and breast cancer

Numerous studies revealed that abnormal DNA methylation is not just a phenomenon that is frequently observed in cancers, but it has become a well-accepted hallmark of cancer (Cheung, Lee et al. 2009). Early studies conducted in 1983 by Feinberg and Vogelstein (Kovalchuk, Tryndyak et al. 2007; Cheung, Lee et al. 2009) showed that the human cancer genome (i.e. the genomes of various types of cancers from various individuals) is hypomethylated on a global basis. It is currently well-accepted that global DNA hypomethylation occurs early in tumorigenesis. DNA hypomethylation makes affected cells susceptible not only to genomic instability but also to further genetic changes and cellular changes. Widschwendter's group found a correlation between hypomethylation and tumor progression and cancer metastasis in ovarian tumors (Widschwendter, Jiang et al. 2004). By comparing two specific areas of DNA on Chromosome 1 of a variety of ovarian tumors at various stages and comparing them to non-neoplastic samples, they determined that there was a significant difference in the levels of methylation (i.e. the tumorous tissues were hypomethylated) in these areas of the chromosome. They also found that the level of hypomethylation of these areas (Sat2 and Sat-alpha) correlates significantly with the tumor metastasis and relapse.

DNA methylation occurs predominantly in the context of CG dinucleotides. It is crucially important for normal development, cell proliferation,

and proper maintenance of genome stability (Rountree, Bachman et al. 2001; Jaenisch and Bird 2003; Shames, Minna et al. 2007). DNA methylation is associated with an inactive chromatin state and repressed gene expression activity (Robertson and Wolffe 2000; Robertson 2002; Klose and Bird 2006). Aberrant global DNA methylation is a well-known feature of cancer cells (Mattsson, Ruden et al. 1993). It is frequently characterized by global genome hypomethylation, as well as concurrent hypermethylation of selected CpG islands within gene promoters (Baylin 2005; Baylin and Ohm 2006; Weidman, Dolinoy et al. 2007). Altered DNA methylation has been linked to the activation of transposable elements, elevated chromosome breakage, aneuploidy, increased mutation rates and, thus to the phenomena of global genomic instability and carcinogenesis (Robertson and Wolffe 2000; Rountree, Bachman et al. 2001; Jaenisch and Bird 2003; Weber and Schubeler 2007).

Altered genome DNA methylation patterns are very important in the etiology and pathogenesis of breast cancer (Szyf, Pakneshan et al. 2004; Tryndyak, Kovalchuk et al. 2006; Chekhun, Lukyanova et al. 2007). Two types of changes in the DNA methylation pattern occur in breast cancer, global genome hypomethylation and regional hypo- and hypermethylation of specific genes (Yang, Yan et al. 2001; Szyf, Pakneshan et al. 2004; Ronckers, Erdmann et al. 2005). Until now, most of the research in the field of cancer epigenetics, including the epigenetics of breast cancer, has been focused on the role of hypermethylation of the promoters of tumor suppressor genes (Fackler, McVeigh et al. 2004; Widschwendter, Siegmund et al. 2004; Jones 2005). In

contrast, global DNA hypomethylation, although it was the first epigenetic abnormality identified in cancer, has received much less attention (Bernardino, Roux et al. 1997; Szyf, Pakneshan et al. 2004). Not only is DNA hypomethylation in cancer correlated withbreast tumor initiation and progression, but it was also found to have a significant correlation with histologic grading, disease staging, and tumor size of breast carcinoma samples (Soares, Pinto et al. 1999). It has been well established that both types of methylation patterns occur in breast cancer, in that regional hypermethylation of certain genes occurs as well as global hypomethylation (Szyf, Pakneshan et al. 2004).

Overall, the role of epigenetic changes in the etiology of radiation-induced breast cancer is not fully understood.

MicroRNAome

In October of 2001, three papers addressing the role of small-temporal RNAs (stRNAs) on *Caenorhabditis elegans* development appeared in Science magazine (Lagos-Quintana, Rauhut et al. 2001; Lau, Lim et al. 2001; Lee and Ambros 2001). While stRNAs were known to be involved in the negative regulation of protein-coding genes, the finding of over 100 new tiny RNAs sequences from three different species, including humans, demonstrated that this mechanism may be more conserved and more complex than initially perceived. These newly discovered small RNAs were named microRNAs

(miRNAs), and have since become a hotspot for discovery and study of developmental processes and disease pathogenesis. Currently, there are more than 700 human miRNAs listed in the miRNA database, miRBase (http://microrna.sanger.ac.uk/sequences/), representing >1 % of all genes in the human genome. Furthermore, these miRNAs can potentially target up to one-third of human coding genes making their role in cellular biology even more apparent (Fujita and Iba 2008; Griffiths-Jones, Saini et al. 2008).

Functional (mature) miRNAs are derived either from three types of loci with annotated transcripts (the introns of protein coding genes, the exons of non-coding genes, and the introns of non-coding genes) or from intergenic regions within the genome (Rodriguez, Griffiths-Jones et al. 2004; Liu, Calin et al. 2008; Liu, Spizzo et al. 2008). MiRNAs within known annotated transcripts are under the direct transcriptional control of their host genes. Intergenic miRNAs, however, are under their own control and in most cases are transcribed via the action of RNA polymerase II (Fujita and Iba 2008; Liu, Calin et al. 2008; Liu, Spizzo et al. 2008). Approximately 36% of miRNAs in the human genome are organized into clusters ≤10kB apart (Griffiths-Jones, Saini et al. 2008), and many of these clusters are only 100-1000kB from each other. This has led to the discovery that many miRNAs are transcribed together as a single transcriptional unit, or polycistron (Fujita and Iba 2008; Griffiths-Jones, Saini et al. 2008). The function of these multi-miRNA polycistrons are thought to be for the efficient targeting of a single mRNA transcript, or to target multiple transcripts in a signal molecular pathway. Some of these polycistrons play

important roles in cellular proliferation and apoptosis, and dysregulation of these miRNAs can perpetuate a cancer phenotype.

Following transcription, a primary miRNA (pri-miRNA) forms a stem-loop structure with a double-stranded RNA (dsRNA) stem of ~33 nucleotides (Liu, Calin et al. 2008; Liu, Spizzo et al. 2008; Winter, Jung et al. 2009). This dsRNA intermediate is then recognized by the RNaseIII-type enzyme Drosha and its dsRNA-binding partner DGCR8/Pasha (Han, Lee et al. 2004; Kim 2005; Kim 2005; Liu, Calin et al. 2008; Liu, Spizzo et al. 2008). This microprocessor than excises the dsRNA stem from the pri-miRNA stem loop, creating a precursor miRNA (pre-miRNA) (Kim 2005; Kim 2005; Liu, Calin et al. 2008; Liu, Spizzo et al. 2008). The pri- to pre-miRNA cropping also utilizes a number of accessory proteins to produce different types, or subsets of miRNAs, allowing additional levels of miRNA regulation (Fukuda, Yamagata et al. 2007; Guil and Caceres 2007; Winter, Jung et al. 2009). After excision, the pre-miRNA is bound by the nuclear export factors Exportin-5 and RanGTP. This binding functions to stabilize the duplex, protect it from degradation, and transport the pre-miRNA to the cytoplasm (Kim 2005; Liu, Calin et al. 2008; Liu, Spizzo et al. 2008).

In the cytoplasm, the RNase-III endonuclease Dicer, in association with the proteins TRBP, PACT, and AGO2, recognize and bind the pre-miRNA. This stimulates the cleavage of the pre-miRNA ~22-nucleotides from the 3'-OH, and produces the mature miRNA duplex with 3' 2-nucleotide overhangs (Kim 2005;

Kim 2005; Liu, Calin et al. 2008; Liu, Spizzo et al. 2008; Winter, Jung et al. 2009). The Argonaut (AGO) protein then stimulates the dissociation of one of the strands, which is subsequently degraded, leaving a single-stranded functional miRNA; however, the exact mechanism of strand-selection in mammals has yet to be uncovered. The Dicer protein then dissociates from the complex, creating the active machinery for silencing, termed the miRNA/AGO ribonucleoprotein (miRNP), also known as the RNA-induced silencing complex (RISC) (Winter, Jung et al. 2009).

The miRNP use the mature miRNAs as guides to direct silencing in a sequence specific manner by binding to target mRNAs at miRNA recognition elements (MREs). These MREs are usually found in the 3' untranslated region (UTR) of the mRNA; however, recent evidence for 5' UTR binding has also been presented (Lytle, Yario et al. 2007). The exact mechanism of miRNP-mediated translation inhibition still eludes silencing investigators, but several working hypothesis have been suggested. The most widely accepted putative mechanism in mammals occurs at the initiation step of translation (Humphreys, Westman et al. 2005; Wakiyama, Takimoto et al. 2007).

MicroRNAome in cancer and breast cancer

Regulatory miRNAs and their processing machinery have a great impact on cellular differentiation, proliferation, apoptosis, and possibly even on the predisposition to cancer (Esquela-Kerscher and Slack 2006; Fabbri, Ivan et

al. 2007; Mack 2007). Extensive studies have documented profound alterations of miRNA expression in all major human cancers.

In cancer microRNAs can act as tumor suppressors or oncogens (oncomiRs). Overexpression and amplification are the main criteria used for defining a miRNA as an oncomiR. The most abundant cancer-related oncomiR is miR-21. This miRNA is up-regulated in over 15 different cancers, including some of the most aggressive cancers such as glioblastoma, lymphoma, pancreatic, and lung cancers (Ciafre, Galardi et al. 2005; Yanaihara, Caplen et al. 2006; Lee, Gusev et al. 2007; Lawrie, Gal et al. 2008). miR-21 is located on chromosome 17 and can act as an oncogene by regulating the tumor-suppressor genes PTEN (Meng, Henson et al. 2007) and PDCD (Frankel, Christoffersen et al. 2008). Inactivation of miR-21 in several cell lines resulted in increased cell death by reactivating caspases (Chan, Krichevsky et al. 2005; Si, Zhu et al. 2007), and activation of PTEN (Meng, Henson et al. 2006).

The miR-17-92 cluster was among the first miRNA groups discovered to be deregulated in a number of human tumors, including lymphomas (O'Donnell, Wentzel et al. 2005), leukemias (Venturini, Battmer et al. 2007), lung (Hayashita, Osada et al. 2005), breast (Hossain, Kuo et al. 2006), and testicular (Novotny, Nielsen et al. 2007; Novotny, Sonne et al. 2007) cancers, among others. The miR-17-92 cluster contains six miRNAs that are transcribed together as a single polycistron. Up-regulation of these miRNAs is correlated with increased levels of cellular proliferation and decreased levels of apoptosis.

The miR-221/222 tandem, located less that 1Kb from each other on chromosome X, is an example of an oncomiR cluster. The aberrant expression of this cluster has significance in thyroid carcinoma (He, Jazdzewski et al. 2005; Pallante, Visone et al. 2006; Visone, Russo et al. 2007), hepatocellular carcinoma (Wong, Lung et al. 2008), pancreatic adenocarcinoma (Bloomston, Frankel et al. 2007), non-small lung cancer (Garofalo, Quintavalle et al. 2008), and prostate cancer (Mercatelli, Coppola et al. 2008). The possible oncomiR mechanism of miR-221/222 is through the suppression of p27/Kip, a key mediator of cell cycle progression inhibitors (Visone, Russo et al. 2007; Mercatelli, Coppola et al. 2008; Mayoral, Pipkin et al. 2009).

Among numerous tumor-suppressor miRNA genes, the most interesting are members of the let-7-family, the miR-15a-16-1 cluster, the miR-34 family, and the miR-143-145 cluster. Special characteristics and the mechanisms of tumor-suppressor activity of the let-7-family, the miR-15a-16-1 cluster and the miR-34 family were recently described in several excellent reviews (Lee and Dutta 2009; Ventura and Jacks 2009).

The lesser known miR-143-145 cluster consists of two miRNAs, miR-143 and miR-145, that are located ~1500Kb apart within the fragile site 5q33. Many cancers exhibit down-regulation of these miRNAs such as colorectal, ovarian, breast and lung cancers, chronic lymphocytic leukemia, cervical, bladder and prostate cancers (Iorio, Ferracin et al. 2005; Yanaihara, Caplen et al. 2006; Akao, Nakagawa et al. 2007; Akao, Nakagawa et al. 2007; Iorio,

Visone et al. 2007; Ichimi, Enokida et al. 2009). The only confirmed target for miR-143 is ERK5, which is involved in cell growth promotion and proliferation (Wang and Tournier 2006), and overexpression of ERK5 has already been detected in several cancers (Mehta, Jenkins et al. 2003; Carvajal-Vergara, Tabera et al. 2005). Other potential tumor-suppressor miRNAs include miR-99, miR-100, miR-125a and 125b, miR-126, miR-139 and miR-140. These miRNAs are down-regulated in three or more cancers; and their experimentally confirmed targets include ERRB2, ERBB3, vascular cell adhesion molecule VCAM1, and histone deacetylase HDAC4(Datta, Kutay et al. 2008; Nasser, Datta et al. 2008; Silber, Lim et al. 2008).

Additionally, several studies have pointed towards a putative role for the miRNA processing machinery in tumor cells. It has recently been shown that levels of Ago2 were elevated in breast tumors (Adams, Claffey et al. 2009), and that levels of Dicer expression correlated with the clinical stage, lymph node status and survival in prostate adenocarcinoma, Burkitt lymphoma and non-small cell lung carcinomas (Kaul and Sikand 2004; Karube, Tanaka et al. 2005; Chiosea, Jelezcova et al. 2006; Chiosea, Jelezcova et al. 2007). Also, aberrant levels of miRNAs have been reported in a variety of human cancers (Fabbri, Garzon et al. 2007), including breast cancer (Iorio, Ferracin et al. 2005; Foekens, Sieuwerts et al. 2008). However, the role of microRNAs in genotoxic stress response in general and in ionizing radiation-induced mammary gland carcinogenesis in particular has yet to be studied in detail.

RADIATION EFFECTS ON EPIGENETIC PARAMETERS

Direct radiation exposure strongly influences epigenetic effectors. DNA damaging agents including IR have been reported to affect DNA methylation patterns (Kalinich, Catravas et al. 1989; Tawa, Kimura et al. 1998; Minamoto, Mai et al. 1999; Kovalchuk, Burke et al. 2004). Acute exposures to low LET X-rays or g-rays were noted to result in global hypomethylation (Kalinich, Catravas et al. 1989; Tawa, Kimura et al. 1998). It was recently shown that the IR exposure leads to the profound dose-dependent and sex- and tissue specific global DNA hypomethylation (Pogribny, Raiche et al. 2004; Raiche, Rodriguez-Juarez et al. 2004; Koturbash, Pogribny et al. 2005; Pogribny, Koturbash et al. 2005; Loree, Koturbash et al. 2006). Exposure to IR also affects methylation of the promoter of the p16 tumor suppressor in a sex- and tissue-specific manner (Kovalchuk, Burke et al. 2004). The DNA hypomethylation observed after irradiation was found to be related to DNA repair (Pogribny, Raiche et al. 2004). It also correlated with the radiation-induced alterations in the expression of DNA methyltransferases, especially de novo methyltransferases DNMT3a and DNMT3b (Raiche, Rodriguez-Juarez et al. 2004; Pogribny, Koturbash et al. 2005). Most importantly, the radiation-induced global genome DNA hypomethylation appeared to be linked to genome instability in the exposed tissue (Pogribny, Raiche et al. 2004; Raiche, Rodriguez-Juarez et al. 2004; Pogribny, Koturbash et al. 2005; Loree, Koturbash et al. 2006).

DNA methylation is closely connected to other components of chromatin structure. Although much attention has been given to the radiation-induced changes in DNA methylation, histones have been largely overlooked. Among the histone modifications that change upon radiation exposure, phosphorylation of histone H2AX is being studied most intensively. Histone H2AX, a variant of histone H2A, is rapidly phosphorylated at Ser139 upon the induction of DNA strand breaks by irradiation, and it can be effectively visualized within repair foci using phosphor-specific antibodies (Sedelnikova, Pilch et al. 2003). Recent studies have also indicated that radiation-induced global loss of DNA methylation may correlate with the changes in histone methylation, specifically with the loss of histone H4 lysine trimethylation (Pogribny, Koturbash et al. 2005).

The data on the IR effects of microRNAome are in their infancy (Ishii and Saito 2006; Marsit, Eddy et al. 2006). However, it has been determined that exposure to IR results in significant changes to the microRNAome. These changes can be detected as early as several hours after exposure to IR (Ilnytskyy, Zemp et al. 2008; Koturbash, Zemp et al. 2008; Ilnytskyy, Koturbash et al. 2009), and can persist for days, weeks (Koturbash, Zemp et al. 2008; Tamminga, Kathiria et al. 2008; Ilnytskyy, Koturbash et al. 2009), and even months (Koturbash, Boyko et al. 2007) post exposure. Analysis of miRNA profiles from different tissues after treatment with IR has shown tissue-dependent and sex-specific mechanisms of radiation-induced miRNA regulation (Koturbash, Boyko et al. 2007; Ilnytskyy, Zemp et al. 2008; Koturbash, Zemp et

al. 2008; Tamminga, Kathiria et al. 2008). Some miRNAs have shown species-, time-, and tissue-independent regulation of expression. miR-194, for example, was found up-regulated at early and late post exposure time points in both the spleen and blood of rats and mice (Koturbash, Boyko et al. 2007; Ilnytskyy, Koturbash et al. 2009). Interestingly, miR-194 was also up-regulated in non-exposed bystander tissue after low (0.5 Gy) and high (20 Gy) doses of radiation (Koturbash, Boyko et al. 2007; Ilnytskyy, Koturbash et al. 2009). This finding might suggest that despite variability of miRNA response to IR, there are also some common mechanisms that are not limited to sex, dose or tissue specificity. The exact roles of miRNAs in radiation-induced carcinogenesis still need to be delineated.

BACKGROUND INFORMATION FOR THE PROJECT

RADIATION-INDUCED EPIGENETIC CHANGES

Most of studies published on IR-induced epigenetic changes have been conducted by Dr. Olga Kovalchuk's group (Kovalchuk, Burke et al. 2004; Kovalchuk, Hendricks et al. 2004; Pogribny, Raiche et al. 2004; Raiche, Rodriguez-Juarez et al. 2004; Koturbash, Pogribny et al. 2005; Pogribny, Koturbash et al. 2005; Koturbash, Baker et al. 2006; Koturbash, Rugo et al. 2006; Loree, Koturbash et al. 2006; Koturbash, Boyko et al. 2007; Koturbash, Kutanzi et al. 2008; Koturbash, Zemp et al. 2008). They have shown that single dose and fractionated direct IR exposure leads to profound, persistent, dose-dependent, and sex- and tissue-specific DNA hypomethylation (Pogribny,

Raiche et al. 2004; Pogribny, Koturbash et al. 2005) which is related to DNA repair (Pogribny, Raiche et al. 2004) and is linked to IR-induced alterations in the expression of DNA methyltransferases (Raiche, Rodriguez-Juarez et al. 2004). Furthermore, they reported that whole-body IR-exposure leads to a profound loss of histone H4K20 trimethylation (Pogribny, Koturbash et al. 2005). They have also proven that IR-induced bystander effects in distant naïve tissues, as well as changes in the progeny of exposed parents are epigenetically regulated (Koturbash, Baker et al. 2006). Kovalchuk's group has demonstrated that direct and bystander IR triggers a significant and sex-specific deregulation of the microRNAome and alters levels of the miRNA-processing enzyme Dicer and components of the RNA-induced silencing complex (RISC) (Koturbash, Zemp et al. 2008). Furthermore, Kovalchuk's lab has shown that direct and localized IR exposure results in altered levels of miRNAs in the blood of exposed animals (Koturbash, Boyko et al. 2007).

EPIGENETIC DYSREGULATION IN ESTROGEN–INDUCED MAMMARY GLAND CARCINOGENESIS

In collaboration with Dr. Pogribny's group, the Kovalchuk laboratory has conducted several studies to address the role of epigenetic changes in breast carcinogenesis. They have analyzed the nature and role of epigenetic changes in estrogen-induced breast carcinogenesis using a rat model. During their study, they found that the development of estrogen-induced mammary gland tumors was associated with substantial alterations in global and locus-specific

DNA methylation levels, the loss of histone H3K9 and H4K20 trimethylation, the changed expression of DNA and histone methyltransferases and significantly altered miRNA expression profiles. MicroRNA changes observed in rat mammary tumors were similar to those reported in human breast cancer. Importantly, microRNAome deregulation preceded pathological changes (Kovalchuk, Tryndyak et al. 2007).

EPIGENETIC AND GENETIC CHANGES IN RAT MAMMARY GLAND EXPOSED TO X-RAYS

The Kovalchuk group also explored the effects of IR on rat mammary gland and was the first to show that IR exposure resulted in notable epigenetic changes. Specifically, they found that a single application of 5 Gy of X-rays resulted in a strong and significant loss of DNA methylation in mammary tissue of rats 6 and 96 hours after exposure. Global DNA hypomethylation was paralleled with significant reductions in levels of maintenance methyltransferase DNMT1, *de novo* DNA methyltransferases DNMT3a and 3b, and methyl-binding proteins 6 and 96 hours after exposure (Loree, Koturbash et al. 2006). Having seen profound changes in DNA methylation after IR exposure, researchers decided to further substantiate their analysis and study the long-term effects of irradiation on the rat mammary gland. In a preliminary set of experiments conducted by the Kovalchuk laboratory, juvenile female rats were randomly assigned to one of the following treatment groups (n=6 per group): sham treated controls and IR-exposed treated group. IR treated animals were

exposed to 2.5 Gy of X-rays (LD50 for rat 7.5 Gy). Morphological analysis revealed that IR exposure resulted in an increased vascularization of rat mammary gland tissue in all animals 6 weeks after exposure. By 18 weeks post exposure, all animals developed fibroadenomas, and one out of 6 animals exhibited adenocarcinoma of the breast. Irradiation also led to significant global DNA hypomethylation 6 weeks after IR exposure that was paralleled by a decrease in cellular levels of DNMT3a and a decrease in trimethylation of lysine 20 of histone H4. Epigenetic changes were also paralleled by increased levels of proliferation and apoptosis. In addition, the group found that radiation exposure caused significant alterations in miRNA levels. Irradiation resulted in up-regulation of 11 and down-regulation of 5 miRNAs in rat mammary tissue 6 weeks after exposure.

HYPOTHESES

The literature and preliminary research conducted by Dr. Kovalchuk's laboratory have shown that: (i) ionizing radiation is a mammary gland carcinogen, yet mechanisms of IR-induced mammary gland carcinogenesis are unknown; (ii) high-dose IR exposure exerts profound global epigenetic DNA methylation and miRNA changes in exposed mammary gland tissue, and these changes may be important in breast carcinogenesis.

While this research has provided extremely pertinent information in the area of mammary gland radiation responses and carcinogenesis, there are still mysteries that remain unsolved. It is important to analyze the effects of low radiation doses on mammary gland. The role of the microRNAome in low dose IR-induced breast carcinogenesis needs to be delineated. The exact contribution of DNA methylation to the generation and maintenance of IR-induced genome instability and carcinogenesis in mammary gland also needs to be established.

We hypothesized that the methylome and microRNAome dysregulation that occurs in mammary gland upon IR exposure may be a mechanism involved in mammary gland IR responses. **We suggest that IR effects will be dose and X-ray energy level specific**.

EXPERIMENTAL MODEL

Rodent, specifically rat models of mammary gland carcinogenesis are well-established and well-accepted as an initial point to begin the understanding of breast carcinoma in humans. The rat model provides a unique opportunity for the study of breast cancer initiation and progression (Shull, Spady et al. 1997; Harvell, Strecker et al. 2000; Shull, Pennington et al. 2001; Li, Weroha et al. 2004; Ronckers, Erdmann et al. 2005). Rat models have been widely used to address the incidence and mechanisms of chemical- or IR-associated breast carcinogenesis, because their lifespan (100–150 weeks) is relatively short, and they develop mammary tumors (Bartstra, Bentvelzen et al. 1998; Bartstra, Bentvelzen et al. 1998; Bartstra, Bentvelzen et al. 2000; Rudel, Attfield et al. 2007). ACI, Long Evans and WAG/Rij rat strains are the most widely used in experiments. These strains exhibit a spontaneous incidence of mammary carcinoma ranging from 10-20% (Bartstra, Bentvelzen et al. 1998; Bartstra, Bentvelzen et al. 1998; Bartstra, Bentvelzen et al. 2000). This baseline incidence is somewhat higher than the reported incidence of breast cancer in Canadian women, which is approximately 8-10% (Bartstra, Bentvelzen et al. 2000). However, in subpopulations of women who are at an increased risk of breast cancer the incidence may be as high as 50–80% (Bartstra, Bentvelzen et al. 2000). Therefore, the spontaneous incidence of mammary cancer in rats may be considered realistic, in view of the situation that occurs in humans.

Importantly, rats are physiologically relevant models for studying human

breast cancer, since mammary gland carcinogenesis in rats is remarkably similar to human breast cancer (Shull, Spady et al. 1997; Bartstra, Bentvelzen et al. 2000; Harvell, Strecker et al. 2000; Shull, Pennington et al. 2001). Using rat models provides an excellent opportunity to dissect the process of carcinogenesis, identify the exact sequence of carcinogenic events, and to define the role of epithelial and stromal cells in IR responses and IR-induced carcinogenesis. Therefore, in this study we used the Long Evans (LE) rat strain. LE rats exhibit a low spontaneous incidence of mammary carcinoma and are susceptible to radiation carcinogenesis.

DOSE RANGE

Doses were determined based on epidemiologic literature and our data. Specifically, studies of atomic bomb survivors (Tokunaga, Land et al. 1994), patients repeatedly subjected to fluoroscopy (Boice, Preston et al. 1991; Howe and McLaughlin 1996), women irradiated for mastitis or other benign breast diseases (Shore, Hildreth et al. 1986; Mattsson, Ruden et al. 1993), and Hodgkin's disease patients treated with radiation therapy (Hancock, Tucker et al. 1993; Bhatia, Robison et al. 1996) clearly show a carcinogenic risk with IR doses between 1 and 5 Gy. These populations received either a single dose or fractionated doses of IR. Breast cancer screening involves much lower doses, typically 1–4 mGy per mammogram. Mammography screening yields a total cumulative dose of approximately 0.1 Gy over a period of 20 years (Bartstra, Bentvelzen et al. 2000). As the effects of such small doses are unknown, breast cancer risk estimates due to mammography are extrapolated from observations on risks from higher doses, assuming that these risks would decrease linearly with decreasing a total dose. However, it has been proven that linearity does not apply to very low doses (Brenner, Sawant et al. 2002). Low dose exposure may be much more dangerous than previously thought. Therefore, it is very important to compare the effects of high, intermediate and low doses of IR on mammary gland carcinogenesis.

MATERIALS AND METHODS

ANIMAL EXPOSURE

In this study we used six-week-old female LE rats. Handling and care of animals was in strict accordance with the recommendations of the Canadian Council for Animal Care and Use. The procedures were approved by the University of Lethbridge Animal Welfare Committee prior to the commencement of the experiment. Animals were housed in a virus-free facility and given food and water *ad libitum*.

For the study, six-week-old female LE rats were randomly assigned to one of the following treatment groups and received either intermediate–high (1 Gy) or low (0.1 Gy) IR doses (n=30 per group; 5 animals per each dose/time point). An intermediate-high dose is close to the dose received by healthy breasts during radiotherapy and/or CT diagnostics. A low dose is slightly higher than the cumulative dose from multiple mammography screens. Furthermore, the low dose groups will be split into an intermediate-high (80kVp) or low (30kVp) energy X-ray groups. High energy rays are used for therapy and CT scans, while low energy rays are used for mammography.

Group 1: 80kVp X-rays, 1 Gy – Intermediate-high dose/high energy;

Group 2: 80kVp X rays, 0.1 Gy – Low dose/high energy;

Group 3: 30kVp X rays, 0.1 Gy – Low dose/low energy; and

Group 4: Sham treated controls.

Five animals per group were sacrificed by Euthansol overdose at 3 hours, 1 week, 2 weeks, 6, 20 and 26 weeks after irradiation to study early and delayed effects. Statistical analysis of the experimental design conducted by our NCTR colleagues indicates that this design has an 80% probability of detecting differences at $p<0.05$ level.

TISSUE SAMPLING AND PROCESSING

Paired inguinal mammary glands were quickly excised upon sacrifice. One gland was frozen immediately and stored at -80°C for subsequent molecular analysis. The contralateral gland was observed with transluminescence, which allowed the orientation of ducts and alveolar lobules to be visualized (Adams, Claffey et al. 2009). Specimens were fixed in 10% neutral buffered formalin for 48 hours, processed, embedded in paraffin, sectioned at 4 microns, and mounted on glass slides. Sections were stained with hematoxylin and eosin (H&E) for histopathological examination.

DNA METHYLATION

Total DNA was prepared from rat mammary gland tissue of exposed and control animals using Qiagen DNAeasy Kit (Qiagen, Mississauga, Ontario) according to the manufacturer's protocol. Levels of the global genome DNA methylation in mammary gland tissue were measured by the HpaII /MspI cytosine extension assay. HpaII that cleaves CCGG sequences when internal cytosine residues are unmethylated on both strands. MspI is an isoschizomer

of HpaII which cleaves CCGG sites in DNA regardless of CpG methylation status. The cytosine extension assay and the determination of the absolute percent of double-stranded unmethylated CCGG sites was conducted as previously described (Pogribny, Raiche et al. 2004; Pogribny, Koturbash et al. 2005; Koturbash, Baker et al. 2006).

In brief, total DNA was prepared from mammary gland tissues using Qiagen DNAeasy Kit (Qiagen, Mississauga, Ontario) according to the manufacturer's protocol. DNA (1 µg) was digested overnight with a 10-fold excess of HpaII endonuclease according to manufacturers protocol (New England Biolabs, Beverly, MA). A second DNA aliquot (1 µg) was digested with methylation-insensitive isoschizomer MspI, which cleaves CCGG sites in DNA regardless of CpG methylation status, to serve as a control for the digestion efficiency. Undigested DNA served as a background control.

The single nucleotide extension reaction was performed in 25 µg of DNA, 1X PCR bufferII, 1.0 mM MgCl2, 0.25 units of Taq DNA polymerase (Fisher Scientific, Ottawa, ON), [3H]dCTP (57.4 Ci/mmol) (Perkin Elmer, Boston, MA) and incubated at 55°C for 1 h, then immediately placed on ice. Duplicate aliquots (25µl) from each reaction were placed on Whatman DE-81 ion-exchange filters and washed three times 10 minutes with gentle agitation with sodium phosphate buffer (0.5 M, pH 7.0) at room temperature. The filters were dried and processed by scintillation counting (Beckman Counter). Background label incorporation was subtracted from enzyme-digested samples and results were expressed as relative [3H]-dCTP incorporation/1µg of DNA or

as percent change from control (Pogribny, Raiche et al. 2004; Raiche, Rodriguez-Juarez et al. 2004; Pogribny, Koturbash et al. 2005).

The absolute percent of double-stranded unmethylated CCGG sites was calculated by relating the data of HpaII and MspI digests. DNA methylation changes in the exposed cohorts were related to the age-matched controls.

MicroRNA Analysis

Total RNA was extracted from rat mammary gland tissues using TRIzol Reagent (Invitrogen, Burlington, Ontario) according to the manufacturer's instructions. Tissue from 3 animals per group was used for the analysis. The miRNA microarray analysis was performed by LC Sciences (Houston, TX).

In brief, ten micrograms of total RNA were size-fractionated (<200 nucleotides) by using a mirVana kit (Ambion, Austin, TX). Poly-A tails were added to the RNA sequences at the 3′ ends using a poly(A) polymerase, and nucleotide tags were then ligated to the poly-A tails. The tagged RNAs were then hybridized to the dual-channel microarray µParaFlo microfluidics chips (LC Sciences) containing 439 miRNA probes to rat and mouse miRNAs and then labeled with tag-specific dendrimer Cy3 and Cy5 fluorescent dyes. Dye switching was performed to eliminate the dye bias. The detection probes melting temperature was balanced by incorporating varying numbers of modified nucleotides with the increased binding affinities. Hybridization images were collected using a GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, CA), and then digitized using the Array-Pro image analysis software

(Media Cybernetics, Silver Spring, MD). The maximum signal level of background probes was 180. A miRNA detection signal threshold was defined as twice the maximum background signal.

Normalization was performed with a cyclic LOWESS (locally weighted regression) method to remove system-related variations, as previously described (Bolstad, Irizarry et al. 2003; Pogribny, Tryndyak et al. 2007). Data adjustments included data filtering, log 2 transformation, and gene centering and normalization. The *t*-test analysis was conducted the different irradiated groups (IR dose or energy level) groups and their respective age-matched control groups. MicroRNAs with p-values < 0.05 were selected for cluster analysis.

WESTERN IMMUNOBLOTTING

Western immunoblotting for PACT and beta-actin was conducted using rat mammary gland tissue of exposed and control animals as described before (Koturbash *et al*, 2006b; Pogribny *et al*, 2005) using the anti-PACT (1:500, Santa Cruz Biotechnology, CA) and anti-actin (1:2000, Santa Cruz Biotechnology) antibodies. Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and the ECL Plus immunoblotting detection system (Amersham, Baie d'Urfé, Québec). Chemiluminescence was detected by Biomax MR films (Eastman Kodak, New Haven, CT). Signals were quantified using NIH ImageJ 1.63 Software and normalized to both GAPDH and the Mr 50,000 protein which

gave consistent results. Protein levels in the exposed cohorts were related to the age-matched controls.

RNA ISOLATION

In this study, the tissues were homogenized in Trizol Reagent (Invitrogen, CA, USA). RNA isolation was performed according to the Qiagen RNeasy Mini Kit column (Qiagen, CA, USA) protocol. The RNA was quantified and the quality was checked by electrophoresis (Ding, Xie et al. 2008).

RNA LABELING AND MICROARRAY HYBRIDIZATION

RNA labeling and microarray hybridization were performed by Genome Quebec and McGill University Innovation Centre. Illumina Rat Ref-12 Expression BeadChip (Illumina, Inc.) rat whole-genome expression arrays were used in this study. Three biological replicates were used per each experimental group. In brief, each RNA sample was amplified using the Ambion Illumina RNA amplification kit with biotin UTP (Enzo) labeling. The Ambion Illumina RNA amplification kit uses T7 oligo(dT) primer to generate single stranded cDNA followed by a second strand synthesis to generate double-stranded cDNA, which is then column purified. *In vitro* transcription was conducted to synthesize biotin-labeled cRNA using T7 RNA polymerase. The cRNA was column purified and checked for size and yield. cRNA was hybridized using standard Illumina protocols with streptavidin-Cy3 (Amersham, Piscataway, NJ, USA). Slides were scanned on an Illumina Beadstation and analyzed using BeadStudio (Illumina, Inc) (Ding, Xie et al. 2008).

Normalization, clustering and significance analysis were done by Genome Quebec and McGill University Innovation Centre as previously described (Ding, Xie et al. 2008).

STATISTICAL ANALYSIS

Statistical analysis was performed using MS Excel 2007 and JMP5 software packages.

RESULTS AND DISCUSSION

EXPERIMENT 1 - ANALYSIS OF GLOBAL DNA METHYLATION IN RADIATION-EXPOSED RAT MAMMARY GLAND TISSUES

Tumour cells harbour numerous genomic as well as epigenomic alterations (Feinberg and Tycko 2004). Furthermore, epigenetic changes are known to play a central role in tumorigenesis. Epigenetic changes seen in cancer cells include global loss of DNA methylation paralleled by a pronounced regional hypo- and hypermethylation (Feinberg and Vogelstein 1983; Flatau, Bogenmann et al. 1983; Gama-Sosa, Slagel et al. 1983; Jones and Baylin 2002; Feinberg 2004; Feinberg and Tycko 2004; Tryndyak, Kovalchuk et al. 2006).

Global DNA hypomethylation was the first epigenetic abnormality that was reported in cancer cells. Overall, DNA hypomethylation is known to be a hallmark of cancer (Feinberg and Vogelstein 1983; Feinberg and Vogelstein 1983; Flatau, Bogenmann et al. 1983; Gama-Sosa, Midgett et al. 1983; Gama-Sosa, Slagel et al. 1983; Feinberg 2004; Tryndyak, Kovalchuk et al. 2006). A very pronounced global DNA hypomethylation was reported in human breast, liver, skin, colorectal and many other cancers. Furthermore, global DNA hypomethylation occurred at very early premalignant stages of carcinogenesis (Fearon and Vogelstein 1990; Bernardino, Roux et al. 1997; Soares, Pinto et al. 1999; Lin, Hsieh et al. 2001; Fraga, Herranz et al. 2004; Szyf, Pakneshan et al.

2004; Hu, Yao et al. 2005; Karpinets and Foy 2005). Therefore, global genome and locus-specific hypomethylation was proposed to be a key step in carcinogenesis, and recent works by the groups of professor Jaenisch has reported that DNA hypomethylation plays a causative role in tumorigenesis (Gaudet, Hodgson et al. 2003; Yamada, Jackson-Grusby et al. 2005). Global DNA hypomethylation has been associated with chromosomal and genomic instability (Lengauer, Kinzler et al. 1997; Chen, Pettersson et al. 1998; Vilain, Vogt et al. 1999; Ehrlich 2002). Since genome instability and DNA hypomethylation occur very early during cancer predisposition and development, DNA hypomethylation may in turn cause genome instability by promoting loss of heterozygosity in some key regions that regions contain tumour suppressor and DNA repair genes (Ehrlich 2002; Feinberg and Tycko 2004; Tryndyak, Kovalchuk et al. 2006). Additionally, it has been proposed that DNA hypomethylation may facilitate aberrant gene expression, lead to activation of oncogenes and thus, promote carcinogenesis.

Exposure to DNA damaging agents, including IR, was reported to cause global DNA hypomethylation is various tissues in vivo (Koturbash, Pogribny et al. 2005; Pogribny, Koturbash et al. 2005; Ilnytskyy, Koturbash et al. 2009). Furthermore, exposure to high therapeutic doses of IR led to induction of significant DNA hypomethylation in the rat mammary gland tissue. Effects of low doses of IR on global DNA methylation in the mammary gland tissue remained unexplored. With this in mind we set out to analyse the role of low

and intermediate –level IR doses on the DNA methylation in rat mammary gland tissue.

In the course of this study, six-week-old female LE rats were randomly assigned to one of the following treatment groups and received either intermediate–high (1 Gy) or low (0.1 Gy) IR doses. An intermediate-high dose is close to the dose received by healthy breasts during radiotherapy and/or CT diagnostics. A low dose is slightly higher than the cumulative dose from multiple mammography screens. Furthermore, the low dose groups were split into a high (80kVp) or low (30kVp) energy X-ray groups. High energy rays are used for therapy and CT scans, while low energy rays are used for mammography. Animals were sacrificed at 3 hours, 1 week, 2 weeks, 6 weeks, 20 weeks and 26 weeks after exposure to radiation to study early and delayed effects.

We employed a well-established and sensitive HpaII/MspI-based cytosine extension assay that measures the proportion of CCGG that had lost methyl groups on both strands. HpaII is a methylation-sensitive restriction endonuclease that cleaves CCGG sequences when internal cytosine residues are unmethylated on both strands and leaves a 5'-guanine overhang after cleavage that can be used for subsequent single nucleotide extension with [3H]dCTP. While HpaII cleaves CCGG sequences when internal cytosine residues are unmethylated on both strands, its isoschizomer MspI cleaves

CCGG sites in DNA regardless of CpG methylation status. The absolute percent of double-stranded unmethylated CCGG sites can be calculated by relating the data of HpaII and MspI digests (Pogribny, James et al. 2004). Because the vast majority of the frequently occurring HpaII tetranucleotide recognition sequences are constitutively methylated in vivo, an increase in cleavage at these sites is an indicator of genome-wide hypomethylation (Pogribny, James et al. 2004; Koturbash, Pogribny et al. 2005; Tryndyak, Kovalchuk et al. 2006; Tryndyak, Kovalchuk et al. 2007).

We noted that whole body low- and intermediate doses of X-ray exposure resulted in a statistically significant increase in the absolute percent of unmethylated CCGG sites in rat mammary gland tissues 3 hours after irradiation. Application of 1 Gy of X-rays resulted in the most pronounced DNA methylation loss seen as a significant decrease in the level of methylated CCGG sites in the genome (Figure 1). Interestingly, the loss of global DNA methylation that was observed 3 hours after exposure returned to normal 1 week after exposure and further stayed at the same levels up to 2, 20 and 26 weeks after irradiation. IR-induced global loss of DNA methylation seen 3 hours after exposure was a very interesting finding.

Several mechanisms may contribute to DNA hypomethylation. These may include a reduction of cellular methylation capacity, altered expression and activity of DNA methyltransferases, inability of mammalian maintenance DNA

methyltransferase DNMT1 to methylate double-stranded unmethylated CpG sites. Furthermore, it may be linked to DNA damage and presence of unrepaired lesions in DNA. These lesions interfere with methylation ability of DNA methyltransferases (Koturbash, Pogribny et al. 2005).

Indeed, IR is a well-documented potent DNA damaging agent that can induce formation of a variety of DNA lesions and activate DNA repair mechanisms. DNA damage was previously reported to interfere with methylation ability of DNA methyltransferases (Turk, Laayoun et al. 1995; Panayiotidis, Rancourt et al. 2004). Moreover, during repair DNA synthesis cellular DNA polymerases incorporate cytidine, but not methyl-cytidine. Consequently, the presence and repair of radiation-induced DNA lesions may result in DNA hypomethylation. Therefore, the observed DNA hypomethylation may be associated with IR-induced DNA damage. By 1 week after exposure damage was repaired and DNA methylation levels were restored.

Interestingly, we have previously seen that exposure to 5 Gy of X rays, a high therapeutic-range dose, caused global DNA hypomethylation 6 hours and 96 hours after exposure. Our current data correlate with these previous findings. One can predict that it takes longer than 96 hours, but less than a week to fully repair DNA damage and to restore DNA methylation. Additionally, the IR-induced DNA methylation changes seen in this study were less pronounced than those seen before (Loree, Koturbash et al. 2006). Indeed,

exposure to 5 Gy of X-rays caused much more significant changes in DNA methylation (Loree, Koturbash et al. 2006). The differences may be due to the different doses, and consequently, different DNA damaging potential of the applied IR doses.

Yet, the most interesting finding is an observation of significant DNA hypomethylation promoted by exposure to very low doses of IR – 0.1 Gy, both at high (80kVp) and low energy (30kVp) levels. The changes induced by the 30kVp rays were the most intriguing, since this energy range is widely used in mammography. It was previously reported that the low energy mammography rays were more hazardous, per unit dose, than high-energy X- or γ-rays (Brenner, Sawant et al. 2002). Low-energy X-rays exhibited a higher oncotransformation potential than 200kVp X-rays, suggesting that low-energy X-rays used in mammography are considerably more biologically active than previously thought (Frankenberg, Kelnhofer et al. 2002; Frankenberg, Kelnhofer et al. 2002). The latter finding is consistent with the data on significant increases in chromosome aberrations induced by low energy X-rays (Brenner and Amols 1989). All the aforementioned biological processes may be affected by the altered DNA methylation levels. Yet, future studies are needed to establish the mechanistic links between low-dose and low energy radiation-induced DNA hypomethylation and chromosome aberrations and onco-transformation of the cells.

EXPERIMENT 2 - ANALYSIS OF IR-INDUCED GENE EXPRESSION IN RAT MAMMARY GLAND TISSUES

Having seen significant changes in DNA methylation 3 hours after exposure to IR and the restoration of DNA methylation levels seen 1 week after exposure, we decided to further analyse the IR-induced changes in gene expression. Indeed, DNA hypomethylation is known to cause gene expression changes.

To analyze gene expression, we employed a highly sensitive Illumina microarray featuring Bead Array technology (Fan, Gunderson et al. 2006; Kennerly, Ballmann et al. 2008; Anantamongkol, Charoenphandhu et al. 2010). Our analysis revealed that exposure to 0.1 Gy/30kVp x-rays altered expression of 401 genes 3 hours after x-ray irradiation. Exposure to 0.1Gy/80kVp of X-rays affected the expression of 135 genes, and exposure to 1 Gy/80kVp x-rays – of 389 genes (Figure 2).

The fact that low dose and low energy X-ray exposure was a stronger inducer of gene expression changes agrees well with the previous finding of the pronounced biological effectiveness of low energy mammography-type X-rays (Frankenberg, Kelnhofer et al. 2002; Frankenberg, Kelnhofer et al. 2002). The exact roles of the observed gene expression changes in the mammary gland responses to mammography-type X-rays need to be further elucidated. By 2 weeks after exposure, only 21, 9 and 90 genes were affected by exposure to

0,1 Gy/30kVp, 0.1 Gy/80kVp and 1 Gy/8-kVp X-rays, respectively (Figure 2). The lists of identified genes are presented in tables 1-6.

Interestingly, amongst the genes that changed their expression 3 hours after exposure to 0.1 Gy/30kVp, 0.1 Gy/80kVp and 1.0 Gy/80kVp X-rays 43 genes were commonly affected (Table 7). These genes were involved in a wide variety of cellular processes, such as regulation of apoptosis, cell cycle control, DNA repair and metabolism, cell-cell signalling, oxidative stress responses and many others.

One of the interesting genes affected by all the studied doses was growth factor receptor-bound protein 7 (Grb7). The product of this gene belongs to a small family of adaptor proteins that are known to interact with a number of receptor tyrosine kinases and signalling molecules, including HER2/neu (Shen and Guan 2004; Holt and Daly 2005; Bai and Luoh 2008; Lucas-Fernandez, Garcia-Palmero et al. 2008; Nadler, Gonzalez et al. 2010; Wang, Chan et al. 2010). Specifically, GRB7 is an SH2-domain adaptor protein that binds to receptor tyrosine kinases and provides the intra-cellular direct link to the Ras proto-oncogene (Depetris, Wu et al. 2009).

Aberrant over-expression of Grb7 has been found in numerous human cancers, including ovarian, pancreatic, liver and breast cancer, lymphoma, osteosarcoma and testicular germ cell tumours (Eppert, Wunder et al. 2005;

Maqani, Belkhiri et al. 2006; Goddard, McIntyre et al. 2007; Itoh, Taketomi et al. 2007; Kishimoto, Kitamura et al. 2007; Myllykangas, Junnila et al. 2008; Nadler, Gonzalez et al. 2010; Wang, Chan et al. 2010). Its role in breast cancer is very important. Human GRB7 is located on the long arm of chromosome 17, next to the ERBB2 (alias HER2/neu) proto-oncogene. *GRB-7* is amplified concurrently with *HER-2/Neu* in most of breast cancer with chromosome 17q11–21 amplification. *GRB-7* gene amplification is associated with its over-expression (Stein, Wu et al. 1994; Tanaka, Mori et al. 1997; Vinatzer, Dampier et al. 2005). In breast cancer, over expression of Grb7 defines a subset of breast cancer patients with decreased survival, indicating that Grb7 might be a valuable prognostic marker and drug target (Nadler, Gonzalez et al. 2010).

We have seen a profound decrease in the levels of GRB7 mRNA after irradiation. Given a strong oncogenic potential of Grb7, its down-regulation after radiation exposure may be viewed as a protective strategy. In the future, the role of this gene in mammary gland radiation responses still has to be defined. The role of this gene in mammary gland radiation responses still has to be defined. We have seen a profound decrease in the levels of GRB7 mRNA after irradiation. Given a strong oncogenic potential of Grb7, its down-regulation after radiation exposure may be viewed as a protective strategy.

Another gene that exhibited altered expression after irradiation was AKT1 or protein kinase B (PKB). Activation of AKT1 plays a pivotal role in

fundamental cellular functions such as cell proliferation and survival by phosphorylating a variety of substrates. Akt1 is implicated in regulation of cellular survival pathways by suppressing apoptotic processes (Franke, Hornik et al. 2003; Song, Ouyang et al. 2005). It also regulated protein synthesis (Yang, Tschopp et al. 2004). As such, Akt1 has been implicated as a major factor in many types of cancer, including breast cancer (Toker and Yoeli-Lerner 2006; Arendt and Schuler 2008; Gonzalez and McGraw 2009; Jiang, Enomoto et al. 2009).

Interestingly, the phosphatidylinositol-3-kinase (PI3-K)/ AKT pathway is associated with radiation resistance. Activation of PI3-K/AKT is associated with intrinsic radiosensitivity, tumour cell proliferation, and hypoxia (Zhan and Han 2004; Valerie, Yacoub et al. 2007; Bussink, van der Kogel et al. 2008; Schuurbiers, Kaanders et al. 2009). Overall, mounting evidence suggests that the AKT pathway is a major contributor to radioresistance (Zhan and Han 2004). Interestingly, AKT1 expression and phosphorylation was up-regulated in the rat mammary gland tissue after exposure to 5 Gy of X-rays (Loree, Koturbash et al. 2006).

We also noted that low dose-low energy radiation exposure altered the levels of KIT, FOS and JUNB oncogenes. KIT, or CD117 is a receptor tyrosine kinase involved in cell signal transduction in several cell types (Roussidis, Theocharis et al. 2007). The role of KIT, its expression and function

in breast cancer is a very controversial subject (Roussidis, Theocharis et al. 2007). Some researchers propose that the loss of KIT expression is linked with tumor progress, whereas other reports indicate not only its expression but also the key role of KIT in breast cancer progression (Roussidis, Theocharis et al. 2007). Interestingly, recent studies reported differential expression of c-Kit in human breast tissue in response to therapeutic irradiation (Westbury, Reis-Filho et al. 2009). Specifically, a reduction in c-Kit and an increase in ESR1 (oestrogen receptor-alpha) mRNA and protein levels were observed in irradiated samples as compared to non-irradiated ones (Westbury, Reis-Filho et al. 2009). More analysis is clearly needed to define the role(s) of this gene in mammary gland responses to therapeutic and diagnostic irradiation and overall to mammary gland IR-induced carcinogenesis.

JunB is an important member of the AP-1 (activator protein-1) family of transcription factors. Strikingly, JUNB plays a dual role in the regulation of the cell cycle. On the one hand it acts as a cell proliferation inhibitor, a senescence inducer and a tumour suppressor. It exerts its actions through its positive influence on the p16INK4alpha cyclin-dependent kinase inhibitor and its negative effect on cyclin D1 in G1-phase. On the other hand, JUNB can promote cell division via stimulation of cyclin A2 expression in the S-phase (Piechaczyk and Farras 2008). JUNB was shown to be increased in murine spleen tissue after exposure to IR (Wan and Ishihara 2004). It was also activated in irradiated normal skin fibroblasts (Martin, Vozenin et al. 1997).

Exposure to gamma irradiation dramatically increased the levels of Fos and JunB in rat fetal brain (Borovitskaya, Evtushenko et al. 1996). Yet, its precise roles in radiation responses in general and mammary gland radiation responses in particular need to be further deduced.

c-Fos is a cellular proto-oncogene and a member of the immediate early gene family of transcription factors (van Straaten, Muller et al. 1983; Abate, Baker et al. 1993; Kerppola, Luk et al. 1993). Its transcription is up-regulated in response to many extracellular signals and factors, including ionizing radiation (Weichselbaum, Hallahan et al. 1994). FOS dimerizes with the JUN proteins and forms the AP-1 transcription factor which regulates expression of a wide variety of genes involved in control of cellular proliferation and differentiation. The AP-1 complex plays a key part in transformation and progression of cancer (Milde-Langosch 2005; Matthews, Colburn et al. 2007; Verde, Casalino et al. 2007; Durchdewald, Angel et al. 2009).

Recent studies have demonstrated that the early response genes such as c-jun and c-fos are induced following exposure of mammalian cells to ionizing radiation. The products of these genes may regulate downstream genes that are important in the adaptation of cells and tissues to radiation-induced stress (Weichselbaum, Hallahan et al. 1994). The exact roles of AP-1 transcription complex members in low dose radiation responses of mammary gland tissue need to be further discerned.

EXPERIMENT 3 - EFFECTS OF RADIATION EXPOSURE ON RAT MAMMARY GLAND MICRORNAOME

Ionizing radiation (IR) is an invaluable diagnostic and treatment tool, yet it is also a well-documented cytotoxic agent and a potential carcinogen (Trosko 1996; Little 2000). IR exposure causes a number of alterations in the irradiated cells and tissues, including induction of DNA strand breaks (Little 2000; Barcellos-Hoff and Nguyen 2009; Cwikel, Gidron et al. 2010; Mah, El-Osta et al. 2010), altered gene expression (Little 2000; Barcellos-Hoff 2008), cell cycle arrest, apoptosis (Nagar, Smith et al. 2003; Ahmed 2004; Gupta and Ahmed 2004) as well as a number of epigenetic changes resulting in genomic instability (Morgan, Day et al. 1996; Trosko 1996; Barcellos-Hoff 2001; Bourguignon, Gisone et al. 2005; Kovalchuk 2008; Kovalchuk and Baulch 2008). Epigenetic changes have been increasingly recognized as key elements of radiation responses (Pogribny, Raiche et al. 2004; Raiche, Rodriguez-Juarez et al. 2004; Pogribny, Koturbash et al. 2005; Loree, Koturbash et al. 2006; Kovalchuk 2008; Kovalchuk and Baulch 2008; Tamminga, Koturbash et al. 2008).

Amongst those, the least studied is the newly emerged mechanism of epigenetic control mediated through the involvement of small regulatory RNAs (Niwa and Slack 2007; Filipowicz, Bhattacharyya et al. 2008; Grosshans and Filipowicz 2008). There are a number of functional classifications of small regulatory RNAs, and among them, microRNAs (miRNAs) are of a particular interest (Niwa and Slack 2007). MiRNAs are evolutionally conserved, small,

single-stranded, non-protein-coding RNA molecules which are presently recognized as major regulators of gene expression (Niwa and Slack 2007; Filipowicz, Bhattacharyya et al. 2008; Grosshans and Filipowicz 2008).

By regulating gene expression miRNAs impact numerous cellular processes, such as differentiation, proliferation, apoptosis, and even predisposition to cancer (Esquela-Kerscher and Slack 2006; Garzon, Fabbri et al. 2006; Slack and Weidhaas 2006). Indeed, aberrant levels of miRNAs have been reported in a variety of human cancers, including breast cancer (Iorio, Ferracin et al. 2005; Esquela-Kerscher and Slack 2006; Slack and Weidhaas 2006; Calin, Liu et al. 2007; Fabbri, Garzon et al. 2007; Fabbri, Ivan et al. 2007). However, less is knows about the roles of miRNAs in response to genotoxic stress in general, and to IR, in particular (Cha, Seong et al. 2009; Ilnytskyy, Koturbash et al. 2009; Shin, Cha et al. 2009; Shin, Cha et al. 2009; Simone, Soule et al. 2009).

With this in mind we analyzed the effects of low and intermediate doses of IR on microRNA expression in rat mammary gland tissue. As in the previous experiment, six-week-old female LE rats were randomly assigned to one of the following treatment groups and received either intermediate–high (1 Gy) or low (0.1 Gy) IR doses of radiation. An intermediate-high dose is close to the dose received by healthy breasts during radiotherapy and/or CT diagnostics. A low dose is slightly higher than the cumulative dose from multiple mammography

screens. Furthermore, the low dose groups were split into a high (80kVp) or low (30kVp) energy X-ray groups. High energy rays are used for therapy and CT scans, while low energy rays are used for mammography.

Analysis of the rat mammary gland microRNAome revealed a number of intriguing patterns. We found that exposure to ionizing radiation resulted in gross microRNAome perturbations in the rat mammary gland tissue. Seven microRNAs exhibited altered expression 3 hours after exposure, as defined by ANOVA analysis (Figure 3). These were miR-155, miR-184, miR-30c-2*, miR-324-3p, miR-690 and miR-146b. Amongst these microRNAs, the most interesting one was miR-155. The level of this microRNA was significantly increased in rat mammary gland tissue 3 hours after exposure to 0.1 Gy/80kVp and 1 Gy/80kVp (Figure 3).

MiR-155 is a well-known oncogenic miRNA. Elevated expression of miR-155 is a predictor of a poor survival in pancreatic tumors (Greither, Grochola et al. 2010). Over-expression of miR-155 was shown to significantly down-regulate the core mismatch repair proteins, hMSH2, hMSH6 and hMLH1 leading to the induction of a mutator phenotype and microsatellite instability in colon cancer (Valeri, Gasparini et al. 2010). More importantly, Valeri and colleagues have clearly shown that MSI colorectal tumors with unknown cause of mismatch repair inactivation exhibited a pronounced over-expression of miR-

155 (Valeri, Gasparini et al. 2010). Therefore, miR-155 is indispensible for regulation of mismatch repair in tumor cells (Valeri, Gasparini et al. 2010).

Recent data published by Rai and co-authors have provided a proof that miR-155 directly targets the bone morphogenetic protein (BMP)-responsive transcriptional factor SMAD5 and thus partakes in TGF-beta pathway and lymphomagenesis (Rai, Kim et al. 2010). MiR-155 also targets inositol phosphatase SHIP1 to promote TNF alpha-dependent growth of B cell lymphomas (Pedersen, Otero et al. 2009). Interestingly, miR-155 is also crucial for B-cell maturation and its activation appears to be controlled through the extracellular signalling-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways but not the p38 pathway (Yin, Wang et al. 2008). Additionally, miR-155 is thought to provide a crucial link between inflammation and cancer (Tili, Croce et al. 2009; Banerjee, Schambach et al. 2010). Recent data also implicated miR-155 in hepatocarcinogenesis. MiR-155 was shown to be significantly up-regulated at early stages of hepatocarcinogenesis induced by choline-deficient and amino acid-defined diet in C57BL/6 mice. This up-regulation was trans-activated by nuclear factor kappa B (NF-kappaB) (Wang, Majumder et al. 2009).

MiR-155 is very important in breast cancer (O'Day and Lal 2010; Zhu, Hu et al. 2010). It was shown to function as an oncomiR by targeting the suppressor of cytokine signaling 1 gene (SOCS1) (Jiang, Zhang et al. 2010).

MiR-155 regulates cell survival, growth and chemosensitivity of breast cancer cells by targeting FOXO3a (Kong, He et al. 2010).

Kong and colleagues reported that TGF-beta induced miR-155 expression and promoter activity through the function of Smad4. Additionally, they have shown that the knockdown of miR-155 inhibited the TGF-beta-induced epithelial-mesenchymal transition, cell migration and invasion. The role of miR-155 in the TGF-beta-induced EMT may position it as an important target for future therapeutic breast cancer interventions (Kong, Yang et al. 2008).

Ectopic expression of miR-155 was proven to induce the proliferation of breast cancer cells, and, more importantly, to promote development of tumors in nude mice (Jiang, Zhang et al. 2010). Moreover, the expression of miR-155 is strongly up-regulated in primary breast cancer, especially in patients with ER and PR+ tumors (Zhu, Hu et al. 2010). Not only was this miRNA identified in breast cancers, it was also found in the serum samples of women affected by this disease (Zhu, Qin et al. 2009). Yet, most importantly, this oncogenic miRNA was shown to be upregulated in MO59K cells after radiation exposure (Chaudhry, Sachdeva et al. 2010).

As the next step of our analysis we set out to identify novel predicted targets of miR-155 that may be pertinent for radiation responses and mammary gland carcinogenesis. To further discern the mode of action of miR-155 and to

gain more insight to its role in mammary gland radiation responses, we set out to identify novel yet unknown targets of this miRNA. A number of computer programs are used to analyze potential regulation and biological functions of miRNAs. Assaying putative targets in the rodent model is achieved using TARGETSCAN 4.0. This program uses the 5' ends of miRNAs, known as seed sites, to look for perfect to near-perfect alignment with the 3'UTR of genes (Lewis, Shih et al. 2003; Lewis, Burge et al. 2005). Additionally, computer-predicted target genes for differentially expressed miRNAs will be determined using miRNA target databases MiRanda (Memorial Sloan-Kettering Cancer Center) and Sanger (Welcome Trust Sanger Institute) (http://cbio.mskcc.org and http://microrna.sanger.ac.uk/sequences/).

Amongst the novel targets, we were especially interested in those genes that regulate transcription, apoptosis, cell cycle and DNA repair, chromatin structure and epigenetic regulation. Analysis identified Transcription factor PU.1 (Sfpi1), Ras-related protein Rab-9 (Rab-9A), RAB30, member RAS oncogene family (Rab30), Retinoblastoma-like protein 2 (Rbl2) and Mitogen-activated protein kinase 6 (Mapk6).

To confirm targeting and translational repression of selected mRNAs by miR-155, we tested the ability of miR-155 to target 3'UTRs of selected genes in a well-established luciferase reporter assay. To examine whether the selected genes were indeed functionally targeted by miR-155, the segments of Sfpi1 and

Rab9a 3′-UTRs containing the miR-155 complementary sites were cloned into the 3′-UTR of a luciferase reporter system. The resulting reporter vectors were transfected into the MCF-7 cells together with the transfection controls and miR-155.

Figure 4 shows that miR-155 inhibited the luciferase activity from the construct with the Sfpi1 and Rab9a 3′-UTR segments in a concentration-dependent manner. There was no change in the luciferase reporter activity when the cells were co-transfected with the negative control (scrambled oligonucleotides). In the future, it will be important to define the role of these miR-155 targets in the radiation responses of mammary gland tissue.

Rab proteins play key roles in intracellular transport(Simons and Zerial 1993). Specifically, Rab9, a Ras-like GTPase, is involved in protein transport from late endosomes to the Golgi network (Shapiro, Riederer et al. 1993). Its roles in radiation responses, cancer and breast cancer are still to be defined. The precise targets of Rab9 signaling have yet to be defined.

PU.1 (Sfpi1), a pleiotropic regulator, is expressed from the first embryonic stages (Olive, Wagner et al. 2007). Sfpi1 gene encodes an ETS-domain transcription factor that activates gene expression during myeloid and B-lymphoid cell development. It encodes a nuclear protein that binds to a purine-rich sequence known as the PU-box found near the promoters of target

genes. Spfi1 is a putative proto-oncogene involved in murine virus-induced acute erythroleukemias (Levesque, Mattei et al. 1991). Graded repression of PU.1/Sfpi1 gene transcription by GATA factors regulates hematopoietic cell fate and is important for hematopoiesis (DeKoter, Kamath et al. 2007; Chou, Khandros et al. 2009). Interestingly, it was recently reported that the loss of PU.1/Sfpi1 leads to the induction of acute myeloid leukemia (Rosenbauer, Wagner et al. 2004). Induction of miR-155 observed in this experiment may in turn lead to the loss of its target PU.1/Sfpi1. The contribution of this miR-155-PU.1/Sfpi1interaction in breast cancer and mammary gland radiation responses still have to be further analyzed.

Amongst other miRNAs that exhibited altered expression in radiation-exposed mammary gland tissue, we noted miR-184. This miRNA was upregulated after exposure to 0.1 Gy/80kVp and 1 Gy/80kVp. miR-184 was down-regulated in prostate cancer(Schaefer, Jung et al. 2010). Yet, it acts as a potential oncogenic microRNA in squamous cell carcinoma of tongue (Wong, Liu et al. 2008). Its roles it breast cancer and radiation responses still have to be defined.

One week after exposure only 3 microRNAs exhibited differential expression. These were miR-101a, miR-28* and miR-30a* (Figure 5). Amongst those miR-101a deserves a special mention (Tanaka, Haneda et al. 2009). This microRNA controls mammary gland development by regulating

cyclooxygenase-2 expression (Tanaka, Haneda et al. 2009). Tanaka and colleagues have recently reported a significant increase of miR-101a expression throughout differentiation and involution of mammary gland tissue (Tanaka, Haneda et al. 2009). Over expression of this miRNA also inhibited HC11 cell proliferation and influenced the cellular levels of cyclooxygenase 2 (Chakrabarty, Tranguch et al. 2007; Daikoku, Hirota et al. 2008; Tanaka, Haneda et al. 2009). IR is known to induce inflammation and increase Cox2 levels (Ning, Chen et al. 2007; Nandi, Ulasov et al. 2008). Therefore, an increase of miR-101a after irradiation may be viewed as an anti-inflammatory strategy. Future studies are needed to dissect the roles of miR-101a and Cox2 in the mammary gland radiation responses.

Interestingly, 6 weeks after exposure to 0.1 Gy/30kVp and 1 Gy/80kVp of X-rays we noted a significant down-regulation of miR-434-3p and miR-214 (Figure 6). miR-214 is an important oncomiR. High expression of miR-214 was associated with unfavourable outcome in gastric cancer (Ueda, Volinia et al. 2010). This miRNA induces cell survival and cisplatin resistance in ovarian tumors by targeting PTEN (Yang, Kong et al. 2008). miR-214 regulates the expression of MEK3 and JNK1 (Yang, Chen et al. 2009). PTEN, MEK and JNK1 are important regulators of radiation responses (Dent, Yacoub et al. 2003; Caron, Yacoub et al. 2005) therefore, in the future it would be very important to dissect the role of miR-214 in radiation effects.

Most interestingly, we have seen microRNAome changes 6 weeks after exposure to a very low dose of low energy X-rays. The mechanisms of these changes will have to be established and their cellular and organismal repercussions need to be further established.

To control translation of target mRNAs, miRNAs must associate with RNA-induced silencing complex (RISC) proteins such as Argonaute (Ago), PACT (a protein activator of the interferon-induced protein kinase), fragile X mental retardation protein (FxR), TudorSN or other proteins (Jin, Zarnescu et al. 2004; Chendrimada, Gregory et al. 2005; Scadden 2005; Lee, Hur et al. 2006; Liang, Ridzon et al. 2007). Our analysis revealed that radiation exposure altered PACT levels in rat mammary gland tissue 1 week after irradiation (Figure 7).

PACT is a vital member of the RISC complex, which interacts directly with Dicer and contributes to its stabilization (Lee, Hur et al. 2006). Data on the cellular effects of PACT are relatively scarce. The up-regulation of PACT was reported in bronchoalveolar carcinoma, and higher levels of PACT were considered a poor prognostic factor (Roh, Kwak et al. 2005). Our previous studies have shown that radiation exposure altered PACT levels in the directly irradiated and distant bystander tissue (Koturbash, Zemp et al. 2008). The biological repercussions of these changes still need to be delineated. Overall,

the role of PACT in genotoxic stress responses, carcinogenesis and radiation-induced breast carcinogenesis has yet to be established.

GENERAL DISCUSSION AND CONCLUSIONS

The possible adverse health effects of low-dose IR exposure constitute a growing concern. IR is a well-accepted breast carcinogen. Average IR-exposure doses linked to the development of breast cancer range between 0.2 and 20 Gy (Ronckers, Erdmann et al. 2005). Therefore, there has been much debate about benefits and risks of diagnostic mammography (Brenner, Sawant et al. 2002; Berrington de Gonzalez and Reeves 2005; Nekolla, Griebel et al. 2008). Specifically, patients are often concerned about the potential risks of mammography-related IR-exposure and carcinogenesis (Brenner, Sawant et al. 2002; Berrington de Gonzalez and Reeves 2005; Constine, Tarbell et al. 2008; Nekolla, Griebel et al. 2008). The doses from mammography are low, typically around 3 mGy of 26-30kVp X-rays (Kruger and Schueler 2001; Brenner, Sawant et al. 2002). Overall, mammography screening yields a total cumulative dose of approximately 0.1 Gy over a period of 20 years (Bartstra, Bentvelzen et al. 2000). The effects of such small doses are unknown; however the main concern is that these low energy rays are more hazardous, per unit dose, than high-energy X- or γ-rays (Brenner, Sawant et al. 2002).

Therefore, we set out to investigate the effects of either intermediate–high (1 Gy) or low (0.1 Gy) IR doses on rat mammary gland tissue. An intermediate-high dose is close to the dose received by healthy breasts during radiotherapy and/or CT diagnostics. A low dose is slightly higher than the

cumulative dose from multiple mammography screens. Furthermore, we compared the effects of intermediate-high (80kVp) or low (30kVp) energy X-ray groups. High energy rays are used for therapy and CT scans, while low energy rays are used for mammography.

We investigated the IR-related DNA methylation, gene expression and microRNA expression patterns mammary gland tissue of rats. The rat model provides a unique opportunity for the study of breast cancer initiation and progression and for the analysis of mammary gland radiation effects (Shull, Spady et al. 1997; Harvell, Strecker et al. 2000; Shull, Pennington et al. 2001; Li, Weroha et al. 2004; Ronckers, Erdmann et al. 2005). Importantly, rats are physiologically relevant models for studying human breast cancer, since mammary gland carcinogenesis in rats is remarkably similar to human breast cancer (Shull, Spady et al. 1997; Bartstra, Bentvelzen et al. 2000; Harvell, Strecker et al. 2000; Shull, Pennington et al. 2001).

We have demonstrated that exposure to 0.1 Gy/30kVp, 0.1 Gy/80 kVp and 1Gy/80kVp X-rays leads to noticeable DNA methylation changes, gene expression changes and microRNAome alterations in the rat mammary gland.

The main conclusions of the present study are:

(i) Radiation exposure led to the global genome hypomethylation that occred 3 hours after exposure and diminished by 1 week after exposure;

(ii) Radiation exposure resulted in significant changes in radt transcriptome, where 401 genes were differentially expressed in the mammary gland tissue of rat exposed to low mammaography-like X-rays 90.1 Gy/30kVp);

(iii) Radiation exposure altered microRNAome of mammary gland tissue.

Radiation-induced mammary gland carcinogenesis is a poorly understood and multifaceted process. The precise mechanisms leading to predisposition and development of IR-induced breast cancer remain obscure. Furthermore, the evidence for the carcinogenic potential of low-dose and low-energy X0rays still has to be confirmed. Importantly, our study proves that exposure to intermediate–high (therapy-like) or low (mammography-like) IR doses alters several pivotal cellular processes and pathways (DNA methylation, transcriptome and microRNAome), each of which can potentially contribute to cancer predisposition.

This thesis is the first to show the loss of DNA methylation in the mammary gland tissue exposed to low dose and low energy X-rays. Importantly, the DNA methylation changes were seen 3 hours after irradiation,

and the patterns restored by 1 week after exposure. We suggest that low dose IR-induced DNA methylation changes are related to IR-induced DNA damage.

According to the epigenetic hypothesis of cancer predisposition and initiation, genetic and epigenetic changes may play complementary rather than contradictory parts in carcinogenesis and interact in such a manner that epigenetic alterations may significantly influence effect of initial genetic insults and DNA damage (Feinberg 2004; Loree, Koturbash et al. 2006).

Our current results showing radiation-induced DNA hypomethylation in exposed mammary gland provide additional experimental support for this hypothesis.

Another important finding of our study is the occurrence of profound gene expression changes in the IR-exposed mammary gland tissues. We noted that low dose – low energy X-rays induced a very profound alteration in gene expression. Changes induced by 0.1Gy/30kVp X-rays were more drastic that those induced by intermediate-high X-rays. Interestingly, many genes were induced in all three exposure groups. We noted that low and intermediate doses of X-rays changes he expression of several important proto-oncogenes implicated in breast carcinogenesis. The roles of genes such as Grb7, Akt 1,

JunB and others in radiation responses of mammary gland tissue need to be further investigated.

Overall, the observed transcriptome changes may be further explored for their potential usability as low dose IR biomarkers.

The changes in miRNAs expression in the rat mammary glands induced by IR exposure constitute another novel and important outcome of this thesis. miRNAs are small non-coding RNAs that function as key negative regulators of gene expression (Taft, Pang et al. ; Voorhoeve). Aberrant levels of miRNAs have been shown in a variety of human cancers including breast cancer. This observation suggested that deregulation of miRNAs expression may play an important role in the pathogenesis of human tumours. However the dynamics of miRNA changes during pre-malignant and early predisposition stages have not been studied. Our analysis has defined several miRNAs that changes heir expression in response to mammary glands radiation exposure. One of them is an important oncogenic miRNA – miR155. We have identified two novel targets for miR-155: Spfi1 and Rab 9. Future studies are required for the analysis of the roles of the identified radiation responsive miRNAs and their targets in radiation-induced effects in mammary glands.

In the future our results may serve as a road map for the analysis of rat mammary gland radiation responses. Furthermore, the list of low dose radiation-responsive genes and miRNAs may be used as source for potential biomarkers of low dose radiation exposure.

FIGURES

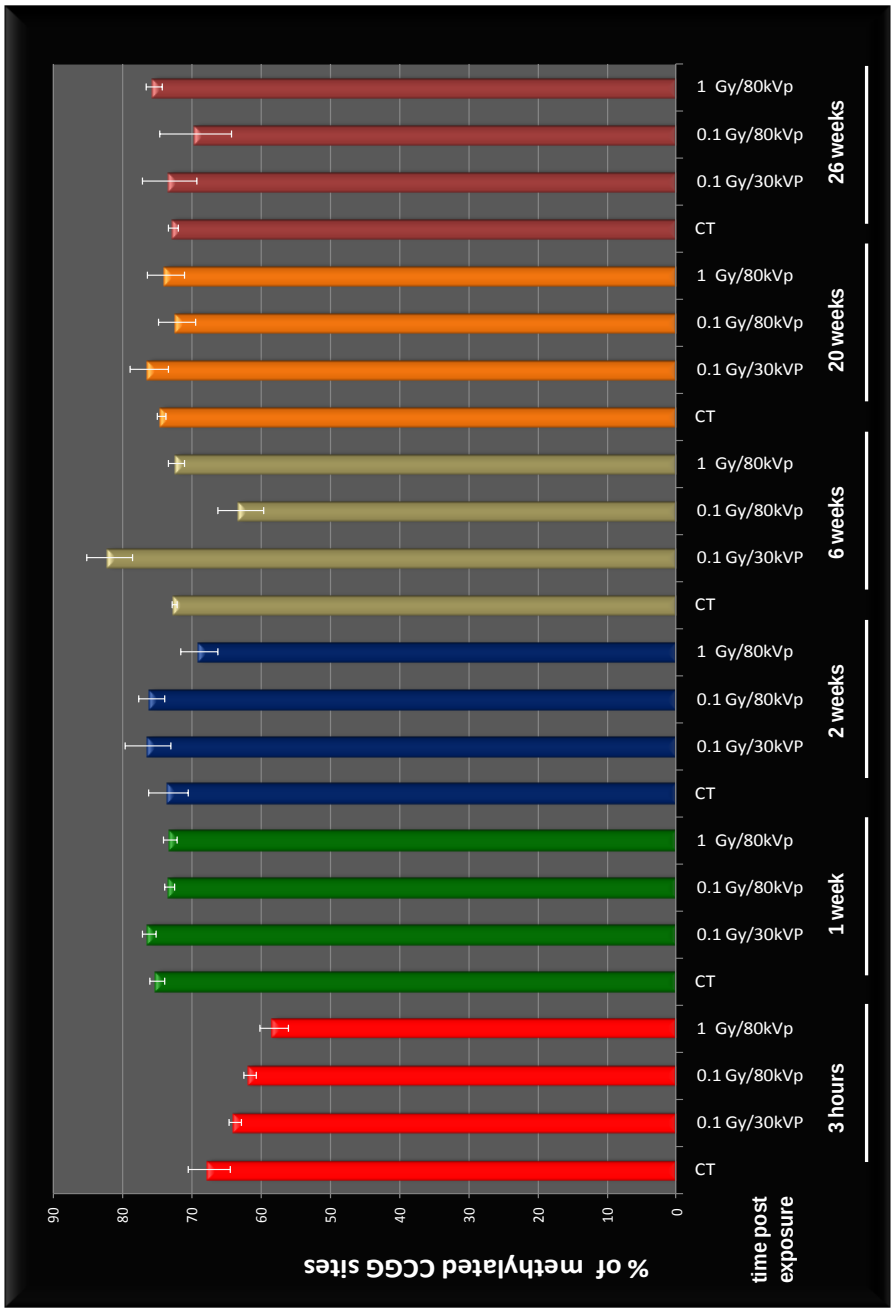
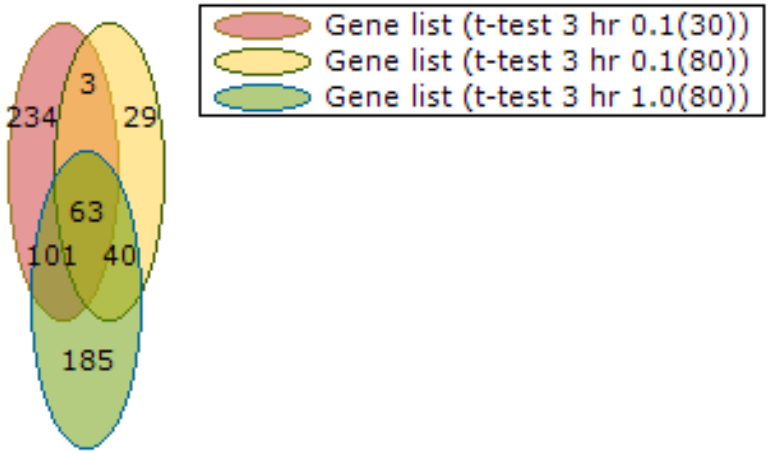


Figure 1. Radiation-induced DNA methylation changes in the rat mammary gland tissue.

The levels of global genome DNA methylation in mammary glands of irradiated rats were measured by the HpaII/MspI cytosine extension assay. HpaII that cleaves CCGG sequences when internal cytosine residues are unmethylated on both strands. MspI is an isoschizomer of HpaII that cleaves CCGG sites in DNA regardless of CpG methylation status. The absolute percent of double-stranded unmethylated CCGG sites was calculated by relating the data of HpaII and MspI digests. Data are presented as mean values ± SD, $p<0.05$, Student's s t-test.

3 hours after exposure



2 weeks after exposure

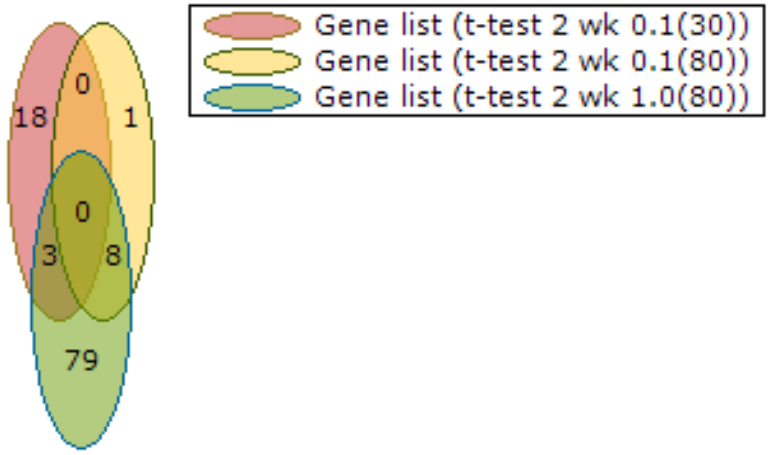


Figure 2. Venn diagrams of genes differentially expressed after radiation treatment.

mRNA gene lists were generated from array data obtained from Illumina. Genes shown were differentially expressed in mammary tissue when compared to age-matched controls at 3 hours and 2 weeks after exposure to ionizing radiation. Genes represented met the following statistical criteria: $-1 \leq \log 2 \text{ Fold Change} \geq 1$, $p \leq 0.05$.

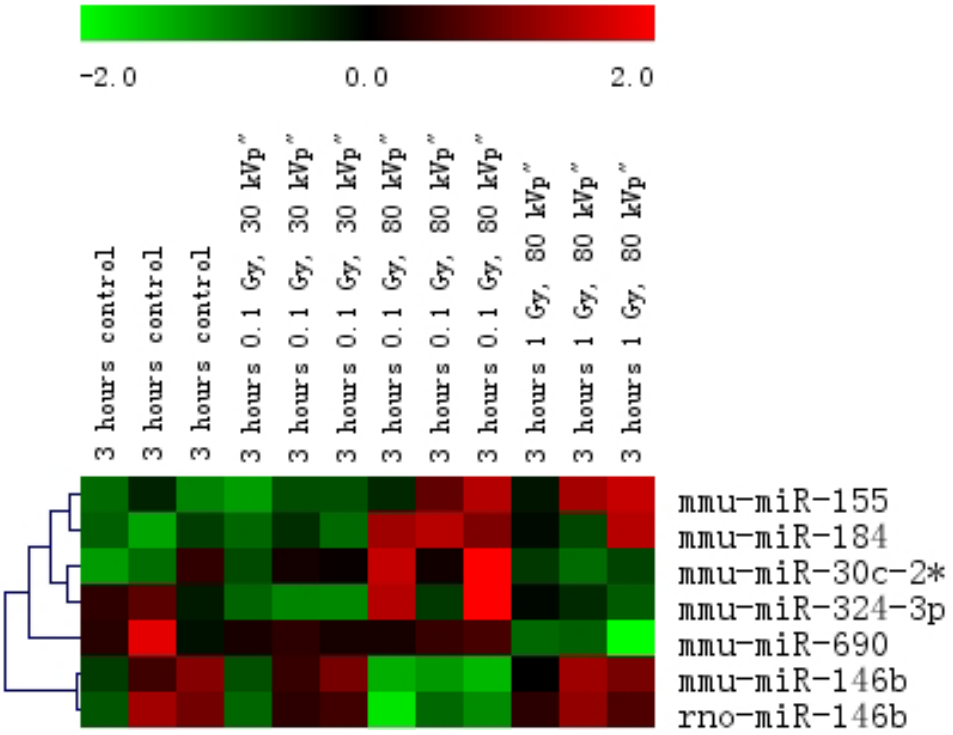


Figure 3. MiRNAs that were differentially expressed in the mammary gland tissues of rats 3 hours after exposure to X-rays.

Hierarchical clusters of differentially expressed miRNA genes in mammary glands of rats exposed to 0.1 Gy/30kVp, 0.1 Gy/80kVp and 1 Gy/80kVp X-rays (as determined by ANOVA). Each miRNA listed is differentially expressed between control and exposure groups ($P < 0.05$).

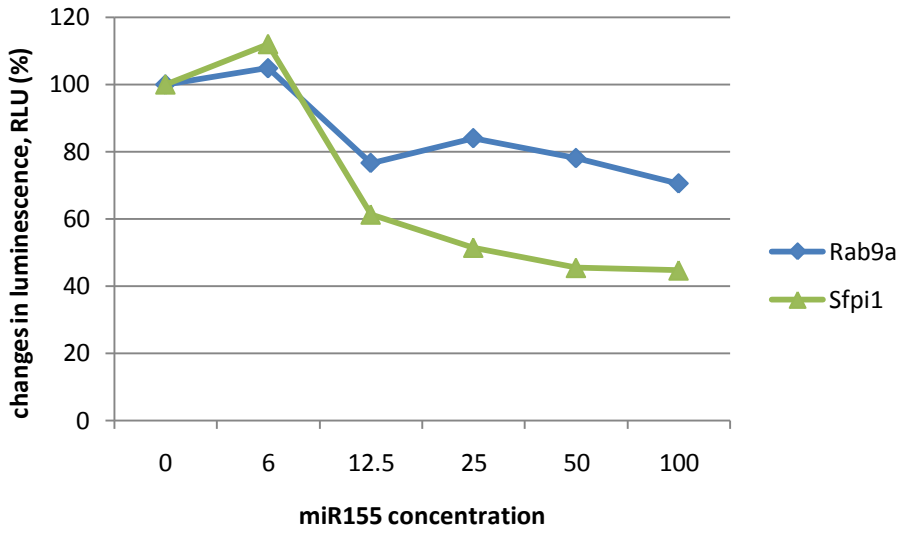


Figure 4. mir-155 directly targets PU.1 (Sfpi1), Rab 9.

Graph shows a dose-dependent inhibition of Sfpi1, Rab 9 expression in the luciferase assay after transfection of the HEK293 cells with miR-155 or a negative control.

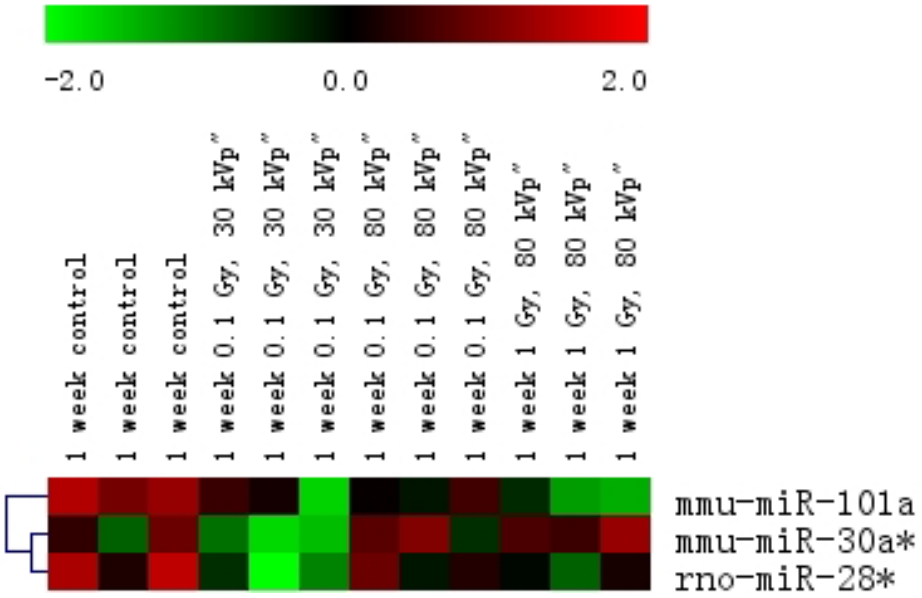


Figure 5. MiRNAs that were differentially expressed in the mammary gland tissues of rats 1 week after exposure to X-rays.

Hierarchical clusters of differentially expressed miRNA genes in mammary glands of rats exposed to 0.1 Gy/30kVp, 0.1 Gy/80kVp and 1 Gy/80kVp X-rays (as determined by ANOVA). Each miRNA listed is differentially expressed between control and exposure groups ($P < 0.05$).

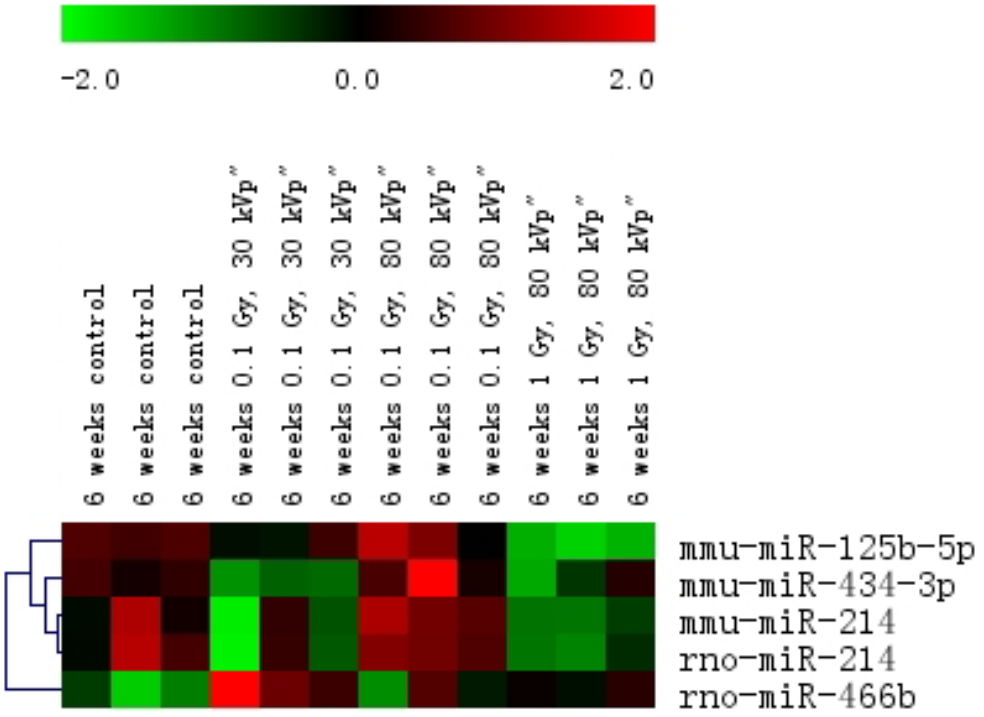


Figure 6. MiRNAs that were differentially expressed in the mammary gland tissues of rats 6 weeks after exposure to X-rays.

Hierarchical clusters of differentially expressed miRNA genes in mammary glands of rats exposed to 0.1 Gy/30kVp, 0.1 Gy/80kVp and 1 Gy/80kVp X-rays (as determined by ANOVA). Each miRNA listed is differentially expressed between control and exposure groups ($P < 0.05$).

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THESIS SUPPLEMENT - TABLES

Table 1. List of genes differentially expressed 3 hours after exposure to 0.1 Gy of 30kVp X – rays

Symbol	Entrez Gene Name	Fold Change (log_2)
AADAT	aminoadipate aminotransferase	-1.32306
ABCD2	ATP-binding cassette, sub-family D (ALD), member 2	1.465818
ACBD4	acyl-Coenzyme A binding domain containing 4	1.01609
ACSL1	acyl-CoA synthetase long-chain family member 1	1.333228
ACTN4	actinin, alpha 4	-1.88544
AKT1	v-akt murine thymoma viral oncogene homolog 1	-2.00113
ALCAM	activated leukocyte cell adhesion molecule	-1.44256
ALDH9A1	aldehyde dehydrogenase 9 family, member A1	-1.40758
ANXA8	annexin A8	-2.3041
AOC3	amine oxidase, copper containing 3 (vascular adhesion protein 1)	1.213536
ASNS	asparagine synthetase	1.534154
ASRGL1	asparaginase like 1	1.37079
ATAD1	ATPase family, AAA domain containing 1	-1.09237
AXL	AXL receptor tyrosine kinase	-1.04192
BASP1	brain abundant, membrane attached signal protein 1	-1.43783
BAT2	HLA-B associated transcript 2	-1.34704
BCKDHA	branched chain keto acid dehydrogenase E1, alpha polypeptide	-1.45539
BCL2L14	BCL2-like 14 (apoptosis facilitator)	-2.3221
C6	complement component 6	1.307153

CACNB3	calcium channel, voltage-dependent, beta 3 subunit	-1.36291
CAP1	CAP, adenylate cyclase-associated protein 1 (yeast)	-3.18319
CAPNS1	calpain, small subunit 1	-1.46997
CAPZB	capping protein (actin filament) muscle Z-line, beta	-1.74502
CAV1	caveolin 1, caveolae protein, 22kDa	-2.38332
CD14	CD14 molecule	-1.44376
CD24	CD24 molecule	-3.39208
CD99 (includes EG:4267)	CD99 molecule	-2.04683
CD99 (includes EG:652929)	CD99 antigen	-2.04683
CD99 (includes EG:673094)	CD99 antigen	-2.04683
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	-1.85582
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	-1.5754
CES2 (includes EG:234671)	carboxylesterase 2	1.574427
CES2 (includes EG:8824)	carboxylesterase 2 (intestine, liver)	1.574427
CFI	complement factor I	-2.60558
CGREF1	cell growth regulator with EF-hand domain 1	1.637981
CHDH	choline dehydrogenase	-1.28076
CHST1	carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	-1.97386
CITED1	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 1	-2.73422
CLCA2 (includes EG:362052)	chloride channel calcium activated 2	-1.77928
CLCA2 (includes EG:80797)	chloride channel calcium activated 2	-1.77928
CLCA2 (includes EG:9635)	chloride channel accessory 2	-1.77928
CLDN3	claudin 3	-3.61524
CLDN4	claudin 4	-1.9394

CLDN8	claudin 8	-1.27788
CLSTN1	calsyntenin 1	-1.09001
CLU	clusterin	-1.0997
CMTM8	CKLF-like MARVEL transmembrane domain containing 8	-2.07703
COL15A1	collagen, type XV, alpha 1	1.41071
COL1A1	collagen, type I, alpha 1	1.335132
CPA1	carboxypeptidase A1 (pancreatic)	1.468828
CRABP2	cellular retinoic acid binding protein 2	-1.43875
CRB3	crumbs homolog 3 (Drosophila)	-1.83786
CREB3L4	cAMP responsive element binding protein 3-like 4	-1.58633
CSN1S2B (includes EG:12992)	casein alpha s2-like B	-4.25836
CSN1S2B (includes EG:317712)	casein alpha s2-like B	-4.25836
CSNK1G2	casein kinase 1, gamma 2	-1.11246
CSPG4	chondroitin sulfate proteoglycan 4	1.660326
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	-2.04875
CX3CL1	chemokine (C-X3-C motif) ligand 1	-1.80034
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	-1.07518
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	-3.41509
DDB1	damage-specific DNA binding protein 1, 127kDa	-1.65652
DUOX1	dual oxidase 1	-1.34404
EGR1	early growth response 1	-1.38779
EHD2	EH-domain containing 2	-1.52507

EIF5A	eukaryotic translation initiation factor 5A	-3.66073
EMP3	epithelial membrane protein 3	1.078284
EPN1	epsin 1	-1.11735
EZR	ezrin	-2.05972
FAH	fumarylacetoacetate hydrolase (fumarylacetoacetase)	1.688517
FAM63A	family with sequence similarity 63, member A	-1.15296
FBLN2	fibulin 2	-3.23629
FGG	fibrinogen gamma chain	-2.53046
FOS	FBJ murine osteosarcoma viral oncogene homolog	-1.60993
GALNT3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3)	-2.45352
GATA3	GATA binding protein 3	-2.32743
GDI1	GDP dissociation inhibitor 1	-2.05166
GJA1	gap junction protein, alpha 1, 43kDa	-1.22583
GJB2	gap junction protein, beta 2, 26kDa	-2.56185
GLG1	golgi apparatus protein 1	-1.84073
GPD1	glycerol-3-phosphate dehydrogenase 1 (soluble)	1.293394
GPX1	glutathione peroxidase 1	-1.47802
GRB7	growth factor receptor-bound protein 7	-1.40115
GSR	glutathione reductase	-1.4085
GSTT1	glutathione S-transferase theta 1	1.448635
GYS2	glycogen synthase 2 (liver)	1.08806
HMGB2	high-mobility group box 2	-1.91899

HMGCS2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)	-1.81203
HOXC10	homeobox C10	1.486303
IL18	interleukin 18 (interferon-gamma-inducing factor)	-2.00545
IL1R2	interleukin 1 receptor, type II	1.610613
IRX2	iroquois homeobox 2	-2.86173
ITGA7	integrin, alpha 7	1.998909
JUNB	jun B proto-oncogene	-1.35211
JUP	junction plakoglobin	-1.32729
KCNK1	potassium channel, subfamily K, member 1	-2.68387
KCNK3	potassium channel, subfamily K, member 3	1.521259
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	-1.20026
KLC3	kinesin light chain 3	-1.10721
KRT19	keratin 19	-4.20861
LBP	lipopolysaccharide binding protein	-3.2013
LEMD2	LEM domain containing 2	-1.09221
LOC290704	similar to palladin	-1.46407
LOC310926	hypothetical protein LOC310926	-3.70489
LOC498525	Bm403207	-1.51811
LSR	lipolysis stimulated lipoprotein receptor	-2.15209
LTBP2	latent transforming growth factor beta binding protein 2	-1.44674
LYPLA2	lysophospholipase II	-1.13178
MAPK3	mitogen-activated protein kinase 3	-2.44177
MAPRE1	microtubule-associated protein, RP/EB family, member 1	-1.51116

MCART1	mitochondrial carrier triple repeat 1	-1.45733
MDK	midkine (neurite growth-promoting factor 2)	-2.17035
MFGE8	milk fat globule-EGF factor 8 protein	-2.24151
MMP12	matrix metallopeptidase 12 (macrophage elastase)	-1.8696
MSN	moesin	-1.3974
MST1	macrophage stimulating 1 (hepatocyte growth factor-like)	1.531732
MUC4	mucin 4, cell surface associated	-1.78116
MYH14	myosin, heavy chain 14, non-muscle	-1.68825
NCALD	neurocalcin delta	-1.58757
NFIX	nuclear factor I/X (CCAAT-binding transcription factor)	-1.71811
NID1	nidogen 1	-2.26977
NID2	nidogen 2 (osteonidogen)	1.154947
NISCH	nischarin	-1.62492
NPM1 (includes EG:18148)	nucleophosmin 1	-1.51976
NPM1 (includes EG:4869)	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	-1.51976
NPR3	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	1.399811
NRTN	neurturin	-1.24893
NS5ATP4	NS5A (hepatitis C virus) transactivated protein 4	-1.48577
OBFC2A	oligonucleotide/oligosaccharide-binding fold containing 2A	1.527597
OLAH	oleoyl-ACP hydrolase	-4.41303
PFN1	profilin 1	-1.2034
PIAS4	protein inhibitor of activated STAT, 4	-1.51279
PKP2	plakophilin 2	-1.927

PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid)	2.557423
PLAGL1	pleiomorphic adenoma gene-like 1	1.392866
PLSCR2	phospholipid scramblase 2	-1.81892
PLXNB2	plexin B2	-1.90056
PPIF	peptidylprolyl isomerase F	-1.48118
PPP1CA	protein phosphatase 1, catalytic subunit, alpha isoform	-1.59264
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta	1.334147
PROM1	prominin 1	-1.40398
PTMA (includes EG:29222)	prothymosin alpha	-1.7989
PTMA (includes EG:5757)	prothymosin, alpha	-1.7989
PTMS	parathymosin	-1.81651
PXMP2	peroxisomal membrane protein 2, 22kDa	1.501408
QARS	glutaminyl-tRNA synthetase	-1.53811
RAP1GAP (includes EG:5909)	RAP1 GTPase activating protein	-1.6353
RELN	reelin	-1.61663
RESP18	regulated endocrine-specific protein 18	1.215714
RNF10	ring finger protein 10	-1.19204
RPL18A	ribosomal protein L18a	-1.54901
RPL30	ribosomal protein L30	4.012354
RPS2	ribosomal protein S2	-4.42427
RPS2 PREDICTED	ribosomal protein S2, pseudogene 6	-4.42427
RPS7	ribosomal protein S7	-1.71474
SCNN1A	sodium channel, nonvoltage-gated 1 alpha	-1.22163

SCNN1B	sodium channel, nonvoltage-gated 1, beta	-2.31668
SECTM1	secreted and transmembrane 1	-3.12136
SERPINA3	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	1.412449
SLC44A4	solute carrier family 44, member 4	-1.49615
SOD3	superoxide dismutase 3, extracellular	1.131305
SOX10	SRY (sex determining region Y)-box 10	-1.9759
SPINT2	serine peptidase inhibitor, Kunitz type, 2	-1.9472
ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	-2.14853
SUCNR1	succinate receptor 1	1.319822
TACR1	tachykinin receptor 1	-1.21528
TACSTD2	tumor-associated calcium signal transducer 2	-1.62702
TAGLN2	transgelin 2	-3.72198
TGFB3	transforming growth factor, beta 3	-1.33287
TIMP2	TIMP metallopeptidase inhibitor 2	-2.78586
TMC4	transmembrane channel-like 4	-2.61694
TMEM158	transmembrane protein 158	-1.99916
TMEM184A	transmembrane protein 184A	-1.77765
TOMM22	translocase of outer mitochondrial membrane 22 homolog (yeast)	-1.47965
TOMM40	translocase of outer mitochondrial membrane 40 homolog (yeast)	-1.13672
TSHR	thyroid stimulating hormone receptor	1.742799
TSN	translin	-2.1849
TSPAN4	tetraspanin 4	-1.30166

UCP2	uncoupling protein 2 (mitochondrial, proton carrier)	-1.71848
USP19	ubiquitin specific peptidase 19	-1.44886
VTN	vitronectin	1.391231
LYPLA2P1	lysophospholipase II pseudogene 1	-1.13178
MCART1L	mitochondrial carrier triple repeat 1-like	-1.45733
TOMM40B	translocase of outer mitochondrial membrane 40 homolog B (yeast)	-1.13672
TOMM40L	translocase of outer mitochondrial membrane 40 homolog (yeast)-like	-1.18674
BAT2D1	BAT2 domain containing 1	-1.34704
BAT2L	HLA-B associated transcript 2-like	-1.26540
CD24B	CD24b antigen	-3.39208
CD24C	CD24c antigen	-2.67546
CD99L2	CD99 molecule-like 2	-2.04683
GPD1L	glycerol-3-phosphate dehydrogenase 1-like	1.293394
Il18r		-2.00545
MST1R	macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	1.531732
LSR1	listeria resistance	-2.15209
TSPAN2	tetraspanin 2	-1.30166
CD14/TLR4/LY96		-1.44376
JUN/JUNB/JUND		-1.35211
SMOC2	SPARC related modular calcium binding 2	-1.47165

Table 2. List of genes differentially expressed 3 hours after exposure to 0.1 Gy of 80kVp X-rays

Symbol	Entrez Gene Name	Fold Change (log_2)
ACOT1 (includes EG:26897)	acyl-CoA thioesterase 1	-1.28326
ACOT1 (includes EG:641371)	acyl-CoA thioesterase 1	-1.28326
ALDOC	aldolase C, fructose-bisphosphate	-2.35395
ANXA8 (includes EG:11752)	annexin A8	-1.66973
ANXA8 (includes EG:653145)	annexin A8	-1.66973
AOC3	amine oxidase, copper containing 3 (vascular adhesion protein 1)	1.077522
AQP7	aquaporin 7	1.331518
ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	1.174741
CD53	CD53 molecule	1.927602
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	1.503892
CLCA2 (includes EG:362052)	chloride channel calcium activated 2	-1.31796
CLCA2 (includes EG:80797)	chloride channel calcium activated 2	-1.31796
CLCA2 (includes EG:9635)	chloride channel accessory 2	-1.31796
CPA1	carboxypeptidase A1 (pancreatic)	1.440268
CRB3	crumbs homolog 3 (Drosophila)	-1.50732
CSN1S1 (includes EG:12990)	casein alpha s1	-3.87554
CSN1S1 (includes EG:1446)	casein alpha s1	-3.87554
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	-3.04088
DUOX1	dual oxidase 1	-1.51006
DYNLT3	dynein, light chain, Tctex-type 3	1.734937

GJB2	gap junction protein, beta 2, 26kDa	-2.16071
GRB7	growth factor receptor-bound protein 7	-1.27412
HMGCS2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)	-1.75843
ICA1	islet cell autoantigen 1, 69kDa	-1.27921
ITGA7	integrin, alpha 7	1.340622
ITGB6	integrin, beta 6	-1.43413
LPXN	leupaxin	1.151434
LYPD3	LY6/PLAUR domain containing 3	-1.62516
MIA	melanoma inhibitory activity	-2.3021
MSLN	mesothelin	-1.57986
MUC1	mucin 1, cell surface associated	-1.04742
MUC4	mucin 4, cell surface associated	-1.5575
NCALD	neurocalcin delta	-1.30339
NPR3	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	1.197441
OBFC2A	oligonucleotide/oligosaccharide-binding fold containing 2A	1.782079
PLEK	pleckstrin	1.593182
PPAP2C	phosphatidic acid phosphatase type 2C	-1.49294
PRKCB	protein kinase C, beta	1.701982
PTPN6	protein tyrosine phosphatase, non-receptor type 6	1.33895
PYGL	phosphorylase, glycogen, liver	1.127892
RAP1GAP (includes EG:5909)	RAP1 GTPase activating protein	-1.31293
RESP18	regulated endocrine-specific protein 18	1.251276
S100B	S100 calcium binding protein B	1.375019

SCNN1A	sodium channel, nonvoltage-gated 1 alpha	-1.14654
SERINC2	serine incorporator 2	-1.31475
SERPINA12	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 12	1.472555
SLC44A4	solute carrier family 44, member 4	-1.2813
SOX10	SRY (sex determining region Y)-box 10	-1.78786
ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	-1.6303
TGFB3	transforming growth factor, beta 3	-1.33962
TMEM184A	transmembrane protein 184A	-1.34347
WAP	whey acidic protein	-2.7355
ICA1L	islet cell autoantigen 1,69kDa-like	-1.27921
PLEK2	pleckstrin 2	-1.13757
SERPINA1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	1.472555

Table 3. List of genes differentially expressed 3 hours after exposure to 1 Gy of 80kVp X-rays

Symbol	Entrez Gene Name	Fold Change (log_2)
ABCD2	ATP-binding cassette, sub-family D (ALD), member 2	1.291568
ACOT1 (includes EG:26897)	acyl-CoA thioesterase 1	-1.48748
ACOT1 (includes EG:641371)	acyl-CoA thioesterase 1	-1.48748
ACVR1C	activin A receptor, type IC	1.762574
ADD3	adducin 3 (gamma)	1.604066
ADIPOQ	adiponectin, C1Q and collagen domain containing	1.41913
AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	1.241295
ANXA8 (includes EG:11752)	annexin A8	-2.13406
ANXA8 (includes EG:653145)	annexin A8	-2.13406
AOC3	amine oxidase, copper containing 3 (vascular adhesion protein 1)	1.167846
AQP11	aquaporin 11	1.15638
AQP7	aquaporin 7	1.608711
AREG	amphiregulin	-1.62227
ASAM	adipocyte-specific adhesion molecule	1.456262
ASRGL1	asparaginase like 1	1.473672
BCL2L14	BCL2-like 14 (apoptosis facilitator)	-2.34847
BZW2	basic leucine zipper and W2 domains 2	-1.46222
CA5B	carbonic anhydrase VB, mitochondrial	1.346396
CACNB3	calcium channel, voltage-dependent, beta 3 subunit	-1.53745
CCL21	chemokine (C-C motif) ligand 21	-1.60737

CCL7	chemokine (C-C motif) ligand 7	2.064533
CCR1	chemokine (C-C motif) receptor 1	1.233851
CD24	CD24 molecule	-3.47291
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	-1.71472
CES1 (includes EG:1066)	carboxylesterase 1 (monocyte/macrophage serine esterase 1)	2.016765
CES2 (includes EG:234671)	carboxylesterase 2	1.189263
CES2 (includes EG:8824)	carboxylesterase 2 (intestine, liver)	1.189263
CES3	carboxylesterase 3	1.277086
CFB	complement factor B	-1.14669
CFI	complement factor I	-2.38297
CGREF1	cell growth regulator with EF-hand domain 1	1.997955
CHDH	choline dehydrogenase	-1.14864
CHST1	carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	-1.3066
CITED1	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 1	-2.38727
CLCA2 (includes EG:362052)	chloride channel calcium activated 2	-1.68055
CLCA2 (includes EG:80797)	chloride channel calcium activated 2	-1.68055
CLCA2 (includes EG:9635)	chloride channel accessory 2	-1.68055
CLDN3	claudin 3	-3.1061
CLDN4	claudin 4	-1.8386
CLDN8	claudin 8	-1.2458
CLU	clusterin	1.07743
CMTM8	CKLF-like MARVEL transmembrane domain containing 8	-2.24685
COL15A1	collagen, type XV, alpha 1	1.343638

COL1A1	collagen, type I, alpha 1	1.142441
CPA1	carboxypeptidase A1 (pancreatic)	1.942779
CPZ	carboxypeptidase Z	1.475224
CRB3	crumbs homolog 3 (Drosophila)	-1.96205
CSAD	cysteine sulfinic acid decarboxylase	1.042063
CSF1	colony stimulating factor 1 (macrophage)	1.443845
CSN1S1 (includes EG:12990)	casein alpha s1	-4.60327
CSN1S1 (includes EG:1446)	casein alpha s1	-4.60327
CSN1S2B (includes EG:12992)	casein alpha s2-like B	-3.51592
CSN1S2B (includes EG:317712)	casein alpha s2-like B	-3.51592
CSPG4	chondroitin sulfate proteoglycan 4	1.680827
CX3CL1	chemokine (C-X3-C motif) ligand 1	-1.3985
CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1	1.443982
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	-2.54179
DGAT2	diacylglycerol O-acyltransferase homolog 2 (mouse)	1.676773
DLGAP4	discs, large (Drosophila) homolog-associated protein 4	1.422329
DPYSL3	dihydropyrimidinase-like 3	1.464703
DUOX1	dual oxidase 1	-1.35902
DYNLT3	dynein, light chain, Tctex-type 3	1.582418
EMP3	epithelial membrane protein 3	1.11399
EZR	ezrin	-2.0603
FGF7	fibroblast growth factor 7 (keratinocyte growth factor)	1.359386
FGG	fibrinogen gamma chain	-1.86742

FXYD3	FXYD domain containing ion transport regulator 3	-2.54401
GALNT3	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3)	-2.2771
GATA3	GATA binding protein 3	-2.29378
GJB2	gap junction protein, beta 2, 26kDa	-2.49704
GRB7	growth factor receptor-bound protein 7	-1.51715
HMGCS2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)	-2.50989
IGFBP2	insulin-like growth factor binding protein 2, 36kDa	-2.85254
IL18	interleukin 18 (interferon-gamma-inducing factor)	-1.73363
IL1R2	interleukin 1 receptor, type II	1.422606
IRX2	iroquois homeobox 2	-2.24454
ITGA1	integrin, alpha 1	1.556625
ITGA7	integrin, alpha 7	1.269478
ITGB6	integrin, beta 6	-1.49668
KCNB1	potassium voltage-gated channel, Shab-related subfamily, member 1	1.59028
KCNK1	potassium channel, subfamily K, member 1	-2.68838
KLC3	kinesin light chain 3	-1.0499
KRT14	keratin 14	-2.86507
KRT15	keratin 15	-2.93569
KRT17	keratin 17	-1.18915
KRT19	keratin 19	-3.75348
KRT8	keratin 8	-2.9519
LBP	lipopolysaccharide binding protein	-2.26107
LGALS7	lectin, galactoside-binding, soluble, 7	-1.45534

LSAMP	limbic system-associated membrane protein	1.679468
LSR	lipolysis stimulated lipoprotein receptor	-2.28006
LTBP2	latent transforming growth factor beta binding protein 2	-1.54146
LYPD3	LY6/PLAUR domain containing 3	-2.14915
MDK	midkine (neurite growth-promoting factor 2)	-1.87434
MFGE8	milk fat globule-EGF factor 8 protein	-1.52772
MGLL	monoglyceride lipase	2.326522
MGMT	O-6-methylguanine-DNA methyltransferase	1.05849
MIA	melanoma inhibitory activity	-3.21813
MLPH	melanophilin	-1.1281
MMP12	matrix metallopeptidase 12 (macrophage elastase)	-1.70063
MMP23B	matrix metallopeptidase 23B	1.35272
MSLN	mesothelin	-1.6473
MT1A	metallothionein 1A	-1.88259
MUC1	mucin 1, cell surface associated	-1.10772
MUC4	mucin 4, cell surface associated	-1.76392
MYH14	myosin, heavy chain 14, non-muscle	-1.65216
NCALD	neurocalcin delta	-1.793
NPR3	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	1.227653
NRTN	neurturin	-1.22351
NUP210	nucleoporin 210kDa	-1.31999
OBFC2A	oligonucleotide/oligosaccharide-binding fold containing 2A	1.590383
OLAH	oleoyl-ACP hydrolase	-3.54133

PALMD	palmdelphin	1.283961
PC	pyruvate carboxylase	1.526366
PCOLCE	procollagen C-endopeptidase enhancer	1.279318
PCP4	Purkinje cell protein 4	-2.21855
PENK	proenkephalin	-1.73748
PKP2	plakophilin 2	-2.03685
PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid)	1.950802
PLIN2	perilipin 2	1.128144
PLSCR2	phospholipid scramblase 2	-1.80613
PMM1	phosphomannomutase 1	1.264668
PPAP2C	phosphatidic acid phosphatase type 2C	-1.70115
PPIF	peptidylprolyl isomerase F	-1.87262
PPP1CB	protein phosphatase 1, catalytic subunit, beta isoform	-1.15238
PPP1R1B	protein phosphatase 1, regulatory (inhibitor) subunit 1B	-1.04281
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta	1.099812
PROM1	prominin 1	-1.87177
PRRX1	paired related homeobox 1	2.093468
PYGL	phosphorylase, glycogen, liver	1.234931
RAP1GAP (includes EG:5909)	RAP1 GTPase activating protein	-1.55694
RELN	reelin	-1.65086
RESP18	regulated endocrine-specific protein 18	1.480874
RGS7	regulator of G-protein signaling 7	1.525065
RHBG	Rh family, B glycoprotein (gene/pseudogene)	1.562568

RPL30	ribosomal protein L30	3.235673
S100B	S100 calcium binding protein B	1.88612
SAT1	spermidine/spermine N1-acetyltransferase 1	-1.09281
SCD	stearoyl-CoA desaturase (delta-9-desaturase)	1.054327
SCNN1A	sodium channel, nonvoltage-gated 1 alpha	-1.29534
SCNN1B	sodium channel, nonvoltage-gated 1, beta	-2.26947
SDPR	serum deprivation response	1.584154
SECTM1	secreted and transmembrane 1	-2.771
SERINC2	serine incorporator 2	-1.38035
SERPINA12	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 12	1.842721
SHOX2	short stature homeobox 2	1.162329
SLC22A3	solute carrier family 22 (extraneuronal monoamine transporter), member 3	1.481995
SLC44A4	solute carrier family 44, member 4	-1.4608
SLC7A10	solute carrier family 7, (neutral amino acid transporter, y+ system) member 10	1.794097
SOX10	SRY (sex determining region Y)-box 10	-1.98022
SPINT1	serine peptidase inhibitor, Kunitz type 1	-1.11954
SPINT2	serine peptidase inhibitor, Kunitz type, 2	-1.98792
ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	-1.90031
SV2B	synaptic vesicle glycoprotein 2B	2.307462
TACSTD2	tumor-associated calcium signal transducer 2	-1.74162
TC2N	tandem C2 domains, nuclear	-1.48321
TEX264	testis expressed 264	1.280726
TGFB3	transforming growth factor, beta 3	-1.30275

TMC4	transmembrane channel-like 4	-2.56555
TMEM184A	transmembrane protein 184A	-1.67004
TSHR	thyroid stimulating hormone receptor	1.390339
UCP2	uncoupling protein 2 (mitochondrial, proton carrier)	-1.33222
WAP	whey acidic protein	-2.81514
WDFY1	WD repeat and FYVE domain containing 1	2.068261
WFDC2	WAP four-disulfide core domain 2	-2.0347
NUP210L	nucleoporin 210kDa-like	-1.31999
PCOLCE2 (includes EG:26577)	procollagen C-endopeptidase enhancer 2	1.279318
PCOLCE2 (includes EG:684050)	procollagen C-endopeptidase enhancer 2	1.279318
MMP23A	matrix metallopeptidase 23A (pseudogene)	1.35272
CCR1L1	chemokine (C-C motif) receptor 1-like 1	1.233851
CSF1R	colony stimulating factor 1 receptor	1.443845
Il18r		-1.73363
SMOC2	SPARC related modular calcium binding 2	-2.64634

Table 4. List of genes differentially expressed 2 weeks after exposure to 0.1 Gy of 30kVp X-rays

Symbol	Entrez Gene Name	Fold Change (log_2)
ELN	elastin	-1.5452
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	-1.05207
NNAT	neuronatin	-1.08815

Table 5. List of genes differentially expressed 2 weeks after exposure to 0.1 Gy of 80kVp X-rays

Symbol	Entrez Gene Name	Fold Change (log_2)
ACE	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	-2.24477
FAP	fibroblast activation protein, alpha	-1.31783
Fcgr3		-1.0876

Table 6. List of genes differentially expressed 2 weeks after exposure to 1 Gy of 80kVp X-rays

Symbol	Entrez Gene Name	Fold Change (log_2)
HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	1.230511
COL5A1	collagen, type V, alpha 1	-1.12193
ACE	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	-2.15796
CSF1R	colony stimulating factor 1 receptor	-1.4715
WNT2B	wingless-type MMTV integration site family, member 2B	-1.42072
DNM1L	dynamin 1-like	-1.34629
LAMB2L	laminin, beta 2-like	-1.28102
PCOLCE2 (includes EG:684050)	procollagen C-endopeptidase enhancer 2	-1.32221
PCOLCE2 (includes EG:26577)	procollagen C-endopeptidase enhancer 2	-1.32221
WNT2	wingless-type MMTV integration site family member 2	-1.42072
THBD	thrombomodulin	-1.56622
RAMP2	receptor (G protein-coupled) activity modifying protein 2	-1.18181
QPRT	quinolinate phosphoribosyltransferase	-1.26425
PTGIS	prostaglandin I2 (prostacyclin) synthase	-1.53013
PROCR	protein C receptor, endothelial (EPCR)	-1.6477
PPAP2B	phosphatidic acid phosphatase type 2B	-1.11399
PLAT	plasminogen activator, tissue	-1.24503
PDPN	podoplanin	-1.23253
PCOLCE	procollagen C-endopeptidase enhancer	-1.32221
NUCB2	nucleobindin 2	-1.32061
NT5E	5'-nucleotidase, ecto (CD73)	-1.40654
NOVA1	neuro-oncological ventral antigen 1	-1.41956

NBL1	neuroblastoma, suppression of tumorigenicity 1	-1.57246
METRNL	meteorin, glial cell differentiation regulator-like	-1.30896
LTBP1	latent transforming growth factor beta binding protein 1	-1.24871
LRRC17	leucine rich repeat containing 17	-1.7696
LOXL1	lysyl oxidase-like 1	-1.265
LOC310926	hypothetical protein LOC310926	1.973774
LOC305633	similar to Antxr2 protein	-1.14967
LAMB2	laminin, beta 2 (laminin S)	-1.28102
KLF4	Kruppel-like factor 4 (gut)	-1.12312
ITGBL1	integrin, beta-like 1 (with EGF-like repeat domains)	-1.27348
GSTM2	glutathione S-transferase mu 2 (muscle)	-1.41369
GAS7	growth arrest-specific 7	-1.66603
GAP43	growth associated protein 43	-2.26783
Fcgr3		-1.08019
FEZ1	fasciculation and elongation protein zeta 1 (zygin I)	-1.65734
FBN1	fibrillin 1	-1.38892
FAP	fibroblast activation protein, alpha	-1.38291
ENTPD2	ectonucleoside triphosphate diphosphohydrolase 2	-1.18712
ELN	elastin	-1.98489
ECM1	extracellular matrix protein 1	-1.83312
DNM1	dynamin 1	-1.34629
CSF1	colony stimulating factor 1 (macrophage)	-1.4715
C1QC	complement component 1, q subcomponent, C chain	-1.26776
C1QA	complement component 1, q subcomponent, A chain	-1.19117
BMP7	bone morphogenetic protein 7	-1.45355

ANPEP	alanyl (membrane) aminopeptidase	-1.55912
ADCY2	adenylate cyclase 2 (brain)	-2.09126

Table 7. List of selected genes that were similarly regulated by all exposure types.

		Fold change		
Target ID	**Gene name**	**0.1(30)**	**0.1(80)**	**1.0(80)**
ALDOC	aldolase C, fructose-bisphosphate	-2.8	-2.4	-3.0
ANXA8	annexin A8	-2.3	-1.7	-2.1
AOC3	amine oxidase, copper containing 3 (vascular adhesion protein 1)	1.2	1.1	1.2
ASRGL1	asparaginase like 1	1.4	1.1	1.5
BCL2L14	BCL2-like 14 (apoptosis facilitator)	-2.3	-1.6	-2.3
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	-1.9	-1.3	-1.7
CGREF1	cell growth regulator with EF-hand domain 1	1.6	1.2	2.0
CLCA2	chloride channel calcium activated 2	-1.8	-1.3	-1.7
CLDN4	claudin 4	-1.9	-1.4	-1.8
CPA1	carboxypeptidase A1 (pancreatic)	1.5	1.4	1.9
CRB3	crumbs homolog 3 (Drosophila)	-1.8	-1.5	-2.0
CREB3L4	cAMP responsive element binding protein 3-like 4	-1.6	-1.1	-1.6
CSPG4	chondroitin sulfate proteoglycan 4	1.7	1.2	1.7
CYP24A1	cytochrome P450, family 24, subfamily A, polypeptide 1	-3.4	-3.0	-2.5
DUOX1	dual oxidase 1	-1.3	-1.5	-1.4
GRB7	growth factor receptor-bound protein 7	-1.4	-1.3	-1.5
HMGCS2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)	-1.8	-1.8	-2.5
ICA1	islet cell autoantigen 1, 69kDa	-1.2	-1.3	-1.2
IL1R2	interleukin 1 receptor, type II	1.6	1.2	1.4
ITGA7	integrin, alpha 7	2.0	1.3	1.3
LYPD3	LY6/PLAUR domain containing 3	-1.8	-1.6	-2.1
MIA1	melanoma inhibitory activity	-2.8	-2.3	-3.2
MUC4	mucin 4, cell surface associated	-1.8	-1.6	-1.8
MYH14	myosin, heavy chain 14, non-muscle	-1.7	-1.3	-1.7
NCALD	neurocalcin delta	-1.6	-1.3	-1.8
NPR3	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	1.4	1.2	1.2
NRTN	neurturin	-1.2	-1.1	-1.2

OBFC2A	oligonucleotide/oligosaccharide-binding fold containing 2A	1.5	1.8	1.6
PCOLCE	procollagen C-endopeptidase enhancer	1.2	1.0	1.3
PKP2	plakophilin 2	-1.9	-1.4	-2.0
PPAP2C	phosphatidic acid phosphatase type 2C	-2.0	-1.5	-1.7
RAMP1	receptor (G protein-coupled) activity modifying protein 1	1.4	1.1	1.2
RAP1GA1	RAP1 GTPase activating protein	-1.6	-1.3	-1.6
RESP18	regulated endocrine-specific protein 18	1.2	1.3	1.5
RTKN	rhotekin	-1.1	-1.3	-1.3
SCNN1A	sodium channel, nonvoltage-gated 1 alpha	-1.2	-1.1	-1.3
SLC44A4	solute carrier family 44, member 4	-1.5	-1.3	-1.5
SOX10	SRY (sex determining region Y)-box 10	-2.0	-1.8	-2.0
ST6GALNAC2	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 2	-2.1	-1.6	-1.3
TCFAP2C	transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)	-2.2	-1.4	-2.0
TGFB3	transforming growth factor, beta 3	-1.3	-1.3	-1.3
TMEM184A	transmembrane protein 184A	-1.8	-1.3	-1.7