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Genetics and epigenetics of direct and indirect radiation responses in normal mammary and breast cancer cells

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GENETICS AND EPIGENETICS OF DIRECT AND INDIRECT RADIATION RESPONSES IN NORMAL MAMMARY AND BREAST CANCER CELLS

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DOCTOR OF PHILOSOPHY

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GENETICS AND EPIGENETICS OF DIRECT AND INDIRECT RADIATION RESPONSES IN NORMAL MAMMARY AND BREAST CANCER CELLS

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DEDICATION

I dedicate this thesis to my supportive and caring partner, Danny Christie, my supportive parents, Oleksandra Luzhna and Ivan Luzhnyy, and my helpful and encouraging sister, Nina Luzhna.
ABSTRACT

The successful approach for decreasing breast cancer fatalities depends upon reliable diagnostic screening and optimal treatment modalities. Ionizing radiation is widely used for both screening and therapeutic procedures.

The main aim of this study was to analyze the effect of low (diagnostic) and high (treatment) doses of ionizing radiation on healthy breast cells, breast cancer cells, and cancer cells resistant to common drug therapies. The results presented here show that ionizing radiation initiates immune and apoptotic response in normal cells, and causes epigenetic alterations that may lead to genomic instability. In addition, our results demonstrate differential dose response to radiation in MCF-7 cells, and decreased sensitivity to radiation in tamoxifen-resistant MCF-7 cells.

Our results may serve as foundation for the future analysis of the mechanisms of radiation responses of mammary gland tissues, and as an additional step for future analysis of effectiveness of combination therapy.
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AGO2 – argonaute 2
APAF – apoptotic peptidase activating factor 1
APC – adenomatous poliposis coli
ATM – ataxia telangiectasia mutated
BAX – Bcl2-associated X protein
BCL2 – B-cell lymphoma 2
BER – base excision repair
BORIS – brother of the regulator of imprinted sites
BRCA – breast cancer gene
CCNA/B – cyclin A/B
CDK – cyclin-dependent kinase
CHK/CHEK – checkpoint kinase
COBRA – combined bisulfite restriction analysis
CTSK – cathepsin K
DDR – DNA-damage response
DNMT – DNA methyltransferase
DSB – double strand break
EGF – epidermal growth factor
ER – estrogen receptor
GADD45G – growth arrest and DNA-damage-inducible, gamma
GSTK – glutathione S-transferase kappa
HDAC – histone deacetylase
HER2 – human epidermal growth factor receptor 2
HR – homologous recombination
IGF-1R – insulin-like growth factor 1 receptor
IR – ionizing radiation
LCN2 – lipocalin 2
LINE1 – long interspersed element
LNT – linear-non-threshold theory
LOH – loss of heterozygosity
MAPK – mitogen-activated protein kinase
MBD – methyl-binding domain
MCF-7 – Michigan Cancer Foundation-7 (institute where the cell line was established)
MCM – minichromosome complex
MeCP2 – methyl CpG binding protein
MLH1 – mutL homolog 1
MMR – mismatch repair
NER – nucleotide excision repair
NHEJ – non-homologous end-joining
ORF – open reading frame
PCR – polymerase chain reaction
PI3K – phosphoinositide 3-kinase
Pla2G2 – phospholipase
PS – phosphatidylyserine
PTTG1 – pituitary tumor transforming 1
RAS – rat sarcoma
PTEN – phosphatase and tensin homolog
Rb – retinoblastoma
RFC – replication factor C
RIGI – radiation-induced genome instability
RISC – RNA-induced silencing complex
RNP – ribonucleoprotein
RT-PCR – real-time polymerase chain reaction
SERD – selective estrogen receptor down-regulator
SERM – selective estrogen receptor regulator
SESN – sestrin
SINE B1 – short interspersed element, B1 subfamily
SIPS – stress-induced senescence
TGFB – transforming growth factor beta
TOR – target of rapamycin
TPRT – target-site-primed-transcription
TSPAN1 – tetraspanin 1
VDAC – voltage-dependent anion channel
VEGF – vascular endothelial growth factor
CHAPTER 1: GENERAL INTRODUCTION

BREAST CANCER

Breast cancer originates within breast cells that start to behave abnormally. It is generally accepted that the initiation of breast cancer is a result of uncontrolled cellular proliferation and aberrant apoptosis (programmed cell death) due to genetic alterations that involve the activation of proto-oncogenes and the inactivation of tumor suppressor genes (Black 1994; Russo, Hu et al. 2000). Breast cancer ranks highly in all countries, but in North America and Europe its incidence is twice as high as that in Asian countries (Saika and Sobue 2013). According to DeSantis and colleagues, one in eight women in the United States will develop breast cancer in her lifetime (DeSantis, Ma et al. 2014). The Canadian Cancer Society recognizes breast cancer as the most common cancer and the second leading cause of death among Canadian women. It is estimated that in 2014, approximately 24,400 Canadian women will be diagnosed with breast cancer, and around 5,000 women will die from breast cancer; on average, 67 women will be diagnosed with breast cancer every day, and 14 women will die from it every day (www.cancer.ca).

Genetic alterations that lead to breast cancer can be either inherited as germline mutations (5-10%) or acquired as somatic mutations. Inherited mutations in BRCA1 and BRCA2 genes (normally they prevent uncontrolled growth) define breast cancer susceptibility and contribute to hereditary breast cancer (Balmana, Diez et al. 2011). Similarly, other genetic risk factors associated with breast cancer are: mutations in the DNA repair gene, ATM (Ahmed and Rahman 2006), the tumor suppressor gene, p53 (Gasco, Shami et al. 2002), the cell growth regulator, PTEN (Gonzalez-Angulo, Ferrer-
Lozano et al. 2011), the human epidermal growth factor HER2 (Cooke, Reeves et al. 2001), etc. Somatic mutations result from exposure to environmental carcinogens of physical (ionizing radiation), chemical (nitrosoureas, polycyclic hydrocarbons) and biological (viruses) nature (Russo, Hu et al. 2000). Multiple studies on rodents reveal many common chemicals that cause mammary tumors, a variety of hormone disruptors that target the estrogen receptor and promote cellular proliferation, and various toxic elements that affect lactation and breast cancer predisposition; all of these agents are widespread in air, water, consumer products, and human tissues (Brody, Kripke et al. 2014).

Breast cancer is a hormone-dependent disease. The ovarian hormones estrogen and progesterone contribute to all stages of breast cancer (Bernstein and Ross 1993; Pike, Spicer et al. 1993). The risk of breast cancer has been traditionally linked to estrogen exposure due to the fact that breast cancers often express the estrogen receptors. There are at least three mechanisms of estrogen carcinogenicity: (a) the receptor-mediated hormonal activity which stimulates cellular proliferation; (b) the cytochrome P450-mediated metabolic activation that causes genotoxic damage and the increased mutation rates; and (c) the accumulation of genomic lesions and compromised DNA repair due to estrogen-induced tumorigenesis (Russo, Hu et al. 2000).

Despite the high incidence of breast cancer, breast cancer mortality has been declining in the majority of developed countries due to better education, widespread screening programs and more effective treatment methods (Guarneri and Conte 2004). The effective screening programs allow for the detection of the disease at early stages
when chances of successful therapy are higher. The early detection followed by effective therapy prevents cancer cells from spreading (metastasizing) and reduces death rates. In the “Breast cancer guide to diagnosis and treatment”, the authors describe the following types of breast cancer diagnosis: a high quality mammogram (low-dose x-ray of the breast when both breasts are compressed between two panels and examined top-down and side-to-side), breast ultrasound (a test that uses sound waves to examine a limited area of breast and shows whether a lump is a fluid-filled cyst or a solid lump), breast MRI (the use of strong magnetic field to create detailed images of part of the body), and breast biopsy (taking samples of an abnormal lump with a core needle biopsy under x-ray guidance). At the time of diagnosis, cancer cells should be screened for the presence of two proteins (hormone receptors for estrogen/progesterone and HER2) that are important for the selection of medical treatment (Laronga, Hayes et al. 2014). The curability of breast cancer is largely dependent on the effectiveness of therapy strategies (Guarneri and Conte 2004). Early-stage cancer (stages I, II) is removed surgically by mastectomy (breast removal) or lumpectomy (breast-conserving surgery). Adjuvant systemic therapy (body-wide) is recommended for stage II breast cancers and during metastases formations, and it involves chemotherapy (for hormone receptor-negative invasive breast cancers), endocrine therapy (anti-estrogen treatment for receptor-positive breast cancers), and molecularly targeted therapy (for HER2-positive breast cancer) (Laronga, Hayes et al. 2014). Many novel targeted therapies are under exploration, especially for triple-negative breast cancer, an aggressive disease that affects the young population and for which effective therapy is not yet available (Schmadeka, Harmon et al. 2014).
Radiation therapy is used after breast-conserving surgery, mastectomy, in combination with chemotherapy, and to treat cancers that have spread to other parts of the body. The effect of radiation on cancer development and cancer treatment as well as the problems of radioresistance are discussed further on.

IONIZING RADIATION: NATURE, SOURCES, AND USES

Background information

Radiation, whether harmful or beneficial, has a profound effect on human life. The effects of radiation exposure are usually limited to the source of radiation, its type, a dose received, duration of exposure (time) and radiation sensitivity of body organs. When living amongst all types of radiation, it becomes necessary to understand what radiation is, and how it interacts with matter. Radiation consists of particles or waves that have enough energy to travel through and interact with matter. Radiation that has enough energy to change the chemical structure of atoms and molecules and has the ability to ionize atoms and molecules of matter is widely known as ionizing radiation which, in contrast to non-ionizing radiation, is considered to be bioactive and therefore more harmful (Gerner, Meyn et al. 1974; Wrixon, Barraclough et al. 2004). The spectrum of ionizing radiation includes alpha and beta particles, neutrons and cosmic protons, while the electromagnetic spectrum includes gamma-rays, X-rays and ultraviolet light. The ultraviolet spectrum is in the range of non-ionizing radiation, it causes damage by either breaking or altering chemical bonds. α and β particles can directly interact with the outer electrons via the coulomb electric force by knocking the electrons out of the atoms and producing an ion and a free electron (Wrixon, Barraclough et al. 2004).
When a DNA molecule is directly hit by ionizing radiation, the DNA replication process is prevented, which affects cell division and cell survival. Considering that the area in a cell occupied by DNA is very small, it is fair to believe that most of the radiation interacts with water which makes up most of the volume in the cell. Energy absorption by water breaks molecular bonds creating free radicals: \( \text{H}^+ \) (proton) and \( \text{OH} \) (hydroxyl radical) (Goldman 1982). Through a series of reactions, toxic \( \text{H}_2\text{O}_2 \) (hydrogen peroxide) is formed which can subsequently damage DNA. This mechanism is an example of an indirect effect of radiation on a cell (Freeman and Crapo 1982; Hutchinson 1985). Certainly, cells are able to recognize and repair DNA damage caused by ionizing radiation. The inability to detect or cope with the damage leads to cell death and consequently to tissue and organ failure. DNA lesions caused by low-energy radiation may be passed on to subsequent cell divisions in the form of mutations, thus contributing to cancer formation.

The level of DNA packaging varies amongst different cell types. Obviously, the densely packaged and protected DNA would be less susceptible to ionizing radiation, and so would be a cell. In general, radiation sensitivity is proportional to the rate of cell division and inversely proportional to the level of cell differentiation. As a result, living cells and the organs that they build are classified according to their radiation sensitivity. The most sensitive to radiation are blood-forming cells and lymphocytes that are constantly regenerating. Gastrointestinal, reproductive and skin cells are less sensitive. The highly differentiated muscle and nerve cells are the least sensitive. It is necessary to emphasize that the developing embryo is also very sensitive to radiation, especially during early development stages. Similarly, the fast-growing tumor cells are very, if not
the most, sensitive to ionizing radiation. This feature of cancer cells has made ionizing radiation one of the most powerful tools in a fight against solid tumors.

**Sources of ionizing radiation**

Ionizing radiation has always been around us, and people are constantly exposed to its various sources. Natural sources make the greatest contribution to an average effective dose, and among such sources radon and radon decay products are the largest contributors (Hall 1989). The other natural sources are cosmic radiation, terrestrial sources (rocks and soil), and internal radiation that occurs in a human body. Amongst man-made sources of radiation, medical exposure contributes the largest dose. Occupational radiation exposure includes nuclear fuel cycles, while consumer products include smoking cigarettes, using phosphate fertilizers, watching color television, using smoke detectors, and using natural gas for cooking, etc. (Hall 1989). Contributions from nuclear power and consumer products (excluding tobacco) are minor. There is no difference in radiation effects between natural and man-made sources of radiation. But there can be no denying that natural radiation has always existed and will always exist, while man-made radiation sources are being continuously added to the background radiation that we receive naturally. This causes a great concern for the society and urges people to make proper lifestyle choices.

An immediate effect of ionizing radiation is cell death which can be observed after doses of several grays. The late effects are due to DNA damage to cells that manage to survive after radiation: if it is a germ cell, the mutation may be expressed in the next generation, whereas if it is a somatic cell, the mutation may lead to cancer development.
Another type of radiation-induced biological effects is bystander effects. Bystander effects are expressed by cells whose nuclei have not been directly exposed to radiation. When significant doses accumulate out-of-field due to photon scattering, they impact cellular response in these regions, and such response includes DNA damage, chromosomal instability, mutations, and apoptosis (Marin, Martin et al. 2015).

**Ionizing radiation in diagnostic and therapeutic medicine**

Since W. C. Roentgen discovered that X-rays can penetrate soft tissues and bones to different degrees, the use of X-rays for determining broken bones or cancers became a ubiquitous procedure throughout the world. The use of ionizing radiation such as X-rays in diagnostic radiology is represented by multiple chest examinations (fluoroscopy, mammography), determining blood-vessel structure (angiography), whole-body examination (CT), and all sorts of X-ray tests on multiple organs (bones, teeth, etc.). Radiation can also be swallowed or inhaled during nuclear medical exams. Radionuclides are distributed to the organs where they emit gamma rays which are subsequently caught by gamma cameras, thus showing the structure of tissues and organs (Wrixon, Barraclough et al. 2004). Radiation doses used in diagnostic imaging procedures are usually low, although the collective dose could be much higher. Therefore, such exposure should be kept at as low as possible level, and unnecessary procedures should be avoided. High doses of ionizing radiation are used in therapy for treatment of malignant diseases. High dose “ablative” radiation therapy approach has shown considerable clinical promise despite initial concerns about excessive tissue complications (Murray, McBride et al. 2014). Radiation therapy is the use of ionizing radiation to destroy cancer cells; it has
been practiced in cancer therapy for more than hundred years (Camphausen and Lawrence 2008). Radiotherapy has become a recognized treatment modality for cancer patients. Radiotherapy works by damaging DNA of cells (Cuzick 2005). There are two types of radiation therapy applied in cancer treatment: external and internal radiotherapy. External beam radiation is the most common radiation therapy given after surgery. In this technique, a special X-ray machine delivers a beam of high-energy radiation to the area of cancer. Internal radiation or brachytherapy is a less common form of radiation treatment. During brachytherapy, small pieces of radioactive material called seeds are placed at the tumor site, and they emit radiation into the surrounding tissue (Wrixon, Barraclough et al. 2004).

As the use of medical radiation increases, so does the public concern about potential health risks, and many studies address the issues and controversies of low dose radiation (Morgan and Bair 2013). It is important to mention that low doses are defined as those ones that are less than 100 mSv, and low dose rates are those below 0.1 mSv min⁻¹ (Mullenders, Atkinson et al. 2009). Diagnostic uses of radiation become more prominent when some CT (computerized tomography) scans deliver up to 100 mSv, thus putting the risk/benefit ratio under question, especially for asymptomatic patients (Brenner and Hall 2007). Ionizing radiation is considered a non-threshold carcinogen. The Linear-No-Threshold (LNT) model, states that there is no dose level below which radiation exposure is safe, and there is a finite probability that even the lowest possible dose may be responsible for cancer initiation (Mullenders, Atkinson et al. 2009). The LNT model is regularly challenged by hormesis or the hormetic effect theory according to which exposure of cells to low doses of radiation may make them less susceptible to
later high dose exposure and may have health benefits (Calabrese and Baldwin 2003; Feinendegen 2005; Mullenders, Atkinson et al. 2009). If the hormetic theory is indeed correct, then the conventional LNT model may create an unnecessary concern and unjustified avoidance of diagnostic and screening procedures. Therefore, the current understanding of the effects of low dose radiation is unclear and is divided between overprotective (LNT) and under protective (hormesis) views (Fig. 1.1). For instance, thirty-four of the thirty-six studies searched on PubMed and Cochrane Library databases by Oh and Koea showed a positive association between medical imaging radiation and an increased risk of cancer (Oh and Koea 2013). The potential increase of a child’s lifetime risk of malignancy from CT scans was reported to be known by many pediatric physicians (Boutis, Fischer et al. 2014).

Significant dose-response relationships were found with breast cancer risk in patients with tuberculosis who received fluoroscopy frequently (Boice, Preston et al. 1991; Howe and McLaughlin 1996). On the other hand, there is experimental evidence that low-level exposures to ionizing radiation modulate anti-tumor activity by stimulating immune mechanisms mediated by natural killer (NK) cells (Nowosielska, Wrembel-Wargocka et al. 2005).
**Figure 1.1. Possible relationships between radiation dose and cancer risk.**
Epidemiological data provide direct measurements down to \( \sim 100 \) mSv (the solid black line). Below this level, a linear non-threshold projection is often made (the dashed black line); however, some responses may indicate that this approach is either underprotective (the green dashed line) or overprotective (the red dashed line). Adopted with permission from Mullenders et al., 2009 (Mullenders, Atkinson et al. 2009).

**RADIORESISTANCE**

The role of radiotherapy in breast cancer treatment is profound; it is used for treatment of very-good-prognosis invasive tumors as well as for *in situ* breast cancers (Cuzick 2005). However, patients often develop recurrences of breast cancer due to radioresistance, and at least half of such patients will have invasive cancer with a high risk of metastasis and death (Frykberg and Bland 1994; Boyages, Delaney et al. 1999). Each type of cancer has different radiosensitivity, and breast cancers are ranked as ranging from moderately radiosensitive to radioresistant, therefore they require higher
doses of radiation (45-60 Gy) to achieve a radical cure than other cancer types. The total
dose is divided into 1.8-2 Gy fractions per day for several weeks (Tutt and Yarnold
2006). Undifferentiated breast cancer cells usually reproduce faster and have a lower
capacity to repair sub-lethal damage caused by radiation in comparison to healthy
differentiated cells. The main limitation of radiotherapy is that cancer cells of solid
tumors become deficient in oxygen after radiation treatment. Such tumors outgrow their
blood supply causing hypoxia (Harrison, Chadha et al. 2002). The presence of oxygen is
crucial for radiation to form DNA-damaging free radicals, and therefore under hypoxic
conditions, cancer cells can be 2 to 3 times more resistant. It has been shown that normal
mammary epithelial stem cells contain a lower concentration of reactive oxygen species
(ROS) than their more mature progeny cells. Cancers originating in these stem cells
develop less DNA damage after irradiation, and they have an increased expression of free
radical scavenging systems in comparison to cancers developed from more mature cells
(Diehn, Cho et al. 2009). The mechanisms of radioresistance are not clearly elucidated,
but more studies have appeared that contribute to understanding of radioresistance. For
instance, the activation of Akt and Wnt/β-catenin signaling pathways within tumor
initiating cells has been shown to play a critical role in radiation resistance (Zhang,
Atkinson et al. 2010). Radioresistance can also be promoted by preferential activation of
the DNA damage response (Bao, Wu et al. 2006). Apoptosis resistance of MCF-7 breast
adenocarcinoma cells to ionizing radiation has been shown to be caused by the lack of
caspase-3 that is essential for the fully functional caspase pathway (Essmann, Engels et
al. 2004). According to Jameel and colleagues, there are various extranuclear and
intranuclear factors implicated in radioresistance of breast cancer (Jameel, Rao et al.
The following table discusses these factors and their role in mediating radiation response.

Table 1.1. Factors implicated to influence radiation response of a breast cancer cell.

<table>
<thead>
<tr>
<th>Extracellular</th>
<th>Factors implicated in radioresistance</th>
<th>References</th>
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<tr>
<td><strong>Extranuclear</strong></td>
<td><strong>Insulin-like growth factor-1 receptor (IGF-IR)</strong> – a tyrosine kinase that regulates cell growth, transformation and apoptosis. Its expression is elevated in ER-positive breast cancers and linked to increased radioresistance and cancer relapse.</td>
<td>(Turner, Haffty et al. 1997; Bartucci, Morelli et al. 2001; Peretz, Jensen et al. 2001).</td>
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<td><strong>Epidermal growth factor (EGF)</strong> – controls cell growth and proliferation. It has been shown to promote radioresistance of MCF-7 cells by increasing the fraction of S-phase cells and glutathione levels.</td>
<td>(Wollman, Yahalom et al. 1994).</td>
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<td><strong>Human epidermal growth factor receptor (HER)</strong> – plays a role in mammalian growth and development. Its overexpression is widely correlated with resistance to radiotherapy. HER inhibitors induce apoptosis and cell cycle arrest after radiation. Trastuzumab, an antibody against HER2, sensitizes breast cells to radiotherapy.</td>
<td>(Earp, Calvo et al. 2003; Liang, Lu et al. 2003; Sartor 2003).</td>
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<td><strong>Vascular endothelial growth factor (VEGF)</strong> – is a mediator of endothelial cell proliferation, survival and angiogenesis. It is upregulated under hypoxic conditions and is believed to contribute to radioresistance.</td>
<td>(Gupta, Jaskowiak et al. 2002; Manders, Sweep et al. 2003; Wachsberger, Burd et al. 2003).</td>
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Intranuclear P53 mutations – associated with pure prognosis and chemo/radioresistance due to absence of p53-dependant apoptosis, loss of control over cell cycle and DNA repair.

BRCA1 and BRCA2 genes - responsible for 80-90% of hereditary breast cancers. Tumor suppressor genes play a role in DNA repair. Wild-type BRCA2 induces homologous recombination and increases radioresistance. The mutated BRCA1 sensitizes cancer cells to ionizing radiation.

HER2/neu – the proto-oncogene that codes for HER2 protein, plays a role in cell survival and proliferation after ionizing radiation by initiating signal transduction pathways. It is overexpressed in 30% of breast cancers.

Telomeres – functional elements of eukaryotic chromosomes, play a role in cellular response to DNA damage. Telomere length has been proposed to be used as a marker of radiosensitivity.

Radiotherapy is often combined with other types of treatment such as chemo- and hormonal therapy. There is evidence that chemoresistant cells acquire radioresistance and re-grow after irradiation. MCF-7 breast cancer cells resistant to paclitaxel and docetaxel have been shown to be radioresistant to γ-radiation, while cells of doxorubicin-resistant breast cancer MCF-7 and human fibrosarcoma HT1080/DR4 show resistance to X-ray radiation (Miller, Hill et al. 1992; Kars, Iseri et al. 2009; Luzhna, Golubov et al. 2013).

Cell lines with acquired resistance to melphalan and cisplatin were shown to have increased levels of glutathione and to be cross-resistant to radiation (Ozols, Masuda et al. 1988). Similarly, multidrug resistant human leukemia cells were reported to have an increased capacity to repair radiation-induced DNA damage (Shimm, Olson et al. 1988).
MCF-7 cells resistant to the most common hormonal therapy drug (tamoxifen) were also reported to be more resistant to ionizing radiation than their sensitive counterparts (Wazer, Tercilla et al. 1989; Paulsen, Strickert et al. 1996). Multi-drug resistance involves a myriad of mechanisms, such as an increased DNA repair capacity, resistance to apoptosis, an increased capacity of the glutathione pathway (detoxifies reactive oxygen species), and its replicative adaptation to high levels of DNA damage. Such mechanisms are thought to predispose cancer cells to radiation resistance (Luzhna, Golubov et al. 2013).

Many studies focus on improving the beneficial effects of radiotherapy through molecular targets. Amongst the possible strategies to improve clinical outcome of radiotherapy are transduction signaling pathways alterations, growth factor receptor blockages, DNA damage enhancement, apoptosis stimulation (Feofanova, Geraldo et al. 2014).

THE EFFECTS OF IONIZING RADIATION ON THE GENOME, CELL SURVIVAL, AND CELL TRANSFORMATION

Radiation-induced DNA damage and DNA repair

The cytotoxic effect of ionizing radiation relies on the ability to damage DNA. Radiation induces a variety of DNA lesions, such as damage to nucleotide bases, cross-linking, DNA single- and double-strand breaks (Little 2000). DNA damage by ionizing radiation arises from two processes: direct and indirect effects. The direct effects result from the direct ionization of DNA, while the indirect effects are due to water ionization
and production of the hydroxyl radical (HO·) which then interacts either with 2’-deoxyribose by H-abstraction or with nucleobases by addition to an unsaturated bond (Close, Nelson et al. 2013). The most common forms of radiation-induced damage that are difficult to repair are double-strand breaks (DSBs) in DNA. About 40 DNA double-strand breaks are induced in a cell for each 1 Gy (Kanaar, Hoeijmakers et al. 1998; Rich, Allen et al. 2000). There are several features that make DSBs difficult to repair. When both strands of DNA are broken, the broken ends can dissociate and interact with breaks at other sites that might lead to translocations and deletions. The ends of breaks have sustained damage to bases that need to be replaced. In a single-strand break, such bases can be easily replaced using the opposite complementary strand as a template, while in DSB, such a template is unavailable (Tutt and Yarnold 2006).

The response to radiation-induced DNA damage includes recognizing DSBs and further recruiting of a highly regulated signal transduction cascade that regulates changes in cell cycle progression and chromatin modifications around sites of DNA DSBs. Unrepaired DSBs could lead to apoptosis (Rich, Allen et al. 2000). Therefore, the recognition of DSB is a sensitive and rapid mechanism. Some data suggest that the sensing mechanisms may distinguish between DNA damage that could be repaired and those which require a wider response such as cell cycle checkpoint activation (Bradbury and Jackson 2003). The checkpoint pathways are broadly divided into damage sensing, signal transduction, and effector components (Fig. 1.2). The initial sensing molecules are unknown, but the main model of DNA damage response suggests the recruitment of proteins of the PI3-kinase-like family to the damaged sites and phosphorylation of histones around DSBs. The second phase includes signal transducers which recruit repair
proteins to the site of damage, and the third phase directly targets components of cell cycle machinery (Tutt and Yarnold 2006). Upon radiation exposure, the specific DNA damage sensors, such as MRN complex (MRE11-RAD50-NBS1), transmit a signal to transducers, such as ATM, and then to CHK1, CHK2. ATM (ataxia telangiectasia) kinase is activated and promotes phosphorylation of H2AX, and transduction of the signal to effector agents (p53, BRCA1, BRCA2) that are involved in the global DNA damage response (DDR) including DNA repair, apoptosis and cell cycle arrest. Mutations in ATM confer an increased cancer susceptibility to affected individuals (Taylor and Byrd 2005). There are two main DSB repair mechanisms: homologous recombination (HR) and non-homologous end-joining (NHEJ) (Fig. 1.2). Homologous recombination (HR) involves RAD51-dependent processing of DSBs to single-stranded DNA structures followed by DNA strand invasion. HR occurs without a loss of genetic information. In NHEJ, the Ku70/Ku80 dimer recruits repair proteins that join two broken ends together, therefore, NHEJ does not restore any information that is lost during DSB formation (Mullenders, Atkinson et al. 2009).
**Radiation-induced apoptosis and senescence**

Cells unable to repair radiation-induced damage undergo cell death (apoptosis) and/or stress-induced senescence (Fig. 1.2). The apoptotic pathway is known to be initiated through p53. Mutations in p53 are frequently associated with reduced radiosensitivity (Chiarugi, Magnelli et al. 1998). P53 influences the levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins. Pro-apoptotic factors induce a reduction in mitochondrial membrane potential, alterations in membrane permeability, and a release of cytochrome c. Cytochrome c binds to apoptotic protein activating factor (APAF) that activates the family of proteolytic caspases, such as caspase 9 and 3. Caspases cause genome degradation into nucleosomal fragments and cell death (Gewirtz 2000).
Many cells enter a state of permanent cell-cycle arrest instead of apoptosis upon ionizing radiation and various other DNA-damaging stressors. Senescent cells are not able to divide and form colonies. Stress-induced senescence (SIPS) is also mediated through an ATM-p53-p21 mechanism. Cells in SIPS contain high levels of γH2AX foci which represent irreparable DSBs. P53 induces p21 levels and cell-cycle arrest. The key protein in cell-cycle arrest during SIPS is Rb. Unphosphorylated Rb negatively regulates the transcription factor E2F which is necessary for cell-cycle progression, and initiates senescence (Vavrova and Rezacova 2011). Cells enter an irreversible G1 arrest which isolates the potentially mutated cells from the population of normal cells.

**Radiation-induced mutations and carcinogenesis**

Misrepaired DSBs are the principle lesions for the induction of gene mutations and chromosomal aberrations (Ward 1995). The mutagenic potential of radiation predisposes cells to the initiation of carcinogenesis. Radiation induces both point mutations and deletions, but deletions predominate, which differs the radiation-induced mutation spectrum from the spontaneous mutation spectrum where point mutations are more common. The predominant mutational changes are large-scale events that involve the loss of heterozygosity (LOH). LOH is mainly a result of deletion or recombination processes and can extend on the chromosome to affect other loci and many other genes. The majority of such large-scale changes arise as a consequence of DSBs. The inactivation of tumor suppressor genes by LOH rather than the activation of oncogenes is considered to be the main event of carcinogenesis initiation (Little 2000). For instance, LOH of Rb tumor suppressor gene resulted in the induction of secondary cancers upon
radiotherapy in retinoblastoma patients (Wong, Boice et al. 1997). P53 mutations also occur in radiation-induced cancer transformation, however, LOH at this gene may not be the initiating event (Bouffler, Kemp et al. 1995). Overall, there are no specific unique genetic alterations in radiation-induced tumors. There are multiple pathways with a variety of mutated tumor suppressor genes and oncogenes involved in cancer transformation, but they are non-specific. One of the early events in radiation-induced carcinogenesis is a loss of G1 checkpoint control. Cells with extensive DNA damage progress through cell cycle and continue proliferating rather than become senescent or apoptotic. The loss of the G1 checkpoint is often a result of the loss of p53 function (Hartwell and Kastan 1994; Murnane 1995).

The initiation of carcinogenesis is generally associated with mutation. When DNA repair is ineffective or cells are not able to eliminate unrepaired and misrepaired DNAs, an increased risk of carcinogenesis arises. The key phase in carcinogenesis is the promotion phase during which the initiated cells acquire new properties, such as resistance to hypoxia, a release of angiogenic factors, immortalization, etc. (Tubiana 2009). Usually, another common mutation or an epigenetic event in at least one cell from the clone of initiated cells gives rise to a subclone of precancerous cells. There is a somewhat Darwinian competition between subclones for a more rapid growth. Dominant subclones acquire new genomic and/or epigenetic events that give rise to new subclones with even a more rapid growth and higher autonomy. Gradually, precancerous cells proliferate regularly without any stimulation and enter the third phase of carcinogenesis, which is progression. The cells acquire the capacity to invade surrounding tissues and metastasize (Tubiana 2009).
RADIATION AND EPIGENETICS

A role of epigenetics in response to ionizing radiation was drawn out of studies on radiation-induced chromosomal instability in irradiated cells and its transduction to the progeny of irradiated cells and moreover, to the cells adjacent to or distant from radiation-targeted cells. A series of alpha particle radiation experiments has shown the induction of sister chromatid exchanges (SCE) in targeted and bystander cells (Nagasawa and Little 1992; Deshpande, Goodwin et al. 1996; Lehnert, Goodwin et al. 1997). Chromosomal aberrations in the progeny of irradiated bone marrow stem cells have allowed the authors to propose a possible radiation-induced genomic instability (RIGI) (Kadhim, Macdonald et al. 1992). Subsequent work by Morgan’s group described factors of genomic instability induced by ionizing radiation (Moran, Holmes et al. 1996; Morgan, Corcoran et al. 1998; Morgan 2003; Morgan 2003). Today, RIGI and radiation-induced carcinogenesis cannot be studied without taking epigenetics into consideration.

The initial understanding of epigenetics proposed by Waddington reflects a model of gene interactions with the surroundings. Depending on a gene and its surroundings, such interactions produce a specific phenotype. Nowadays, epigenetics is rather defined as a memory of stable changes in gene expression without changes in gene sequence that can be passed on to progeny (Jiang, Langley et al. 2008). Such memory explains differences between genetically identical cells in a multicellular organism. Cells in different tissues selectively choose genes for expression. The ability of cells to change gene expression without altering the gene sequence not only allows for maintaining tissue identity, but also provides a possibility for adaptation to the changing environment
should such changes occur (Jirtle and Skinner 2007). Because transcription requires chromatin proteins to modify chromatin structure and transcription factors, the objective of epigenetics is to find how the genetic code in DNA sequence and the way DNA is packaged together control gene expression (Bock and Lengauer 2008). Epigenetic regulation includes at least four outlined mechanisms: DNA methylation, histone modifications, chromatin remodeling and non-coding RNA expression (Gibney and Nolan 2010).

**DNA methylation**

DNA methylation was discovered first; it is the most extensively studied. It is the only epigenetic mechanism that directly targets DNA. A methyl group replaces a hydrogen atom at the cytosine base of DNA, creating a new covalent bond. Such modification happens predominantly in cytosine-phosphate-guanine (CpG)-dinucleotides (Bird 2009). However, non-CpG methylation has also been observed (Woodcock, Crowther et al. 1987). The addition of a methyl group does not affect the transcription of cytosine, but it alters chromatin in a way that lowers the binding capacity of transcription factors to DNA (Weber and Schubeler 2007). Methyl-CpG-binding proteins (MBPs) recruit transcriptional suppressors to modify chromatin (Fujita, Watanabe et al. 2003; Kondo, Gu et al. 2005). The enzymes conducting DNA methylation are DNA methyltransferases: DNMT1, DNMT2, DNMT3a and DNMT3b. DNMT1 can maintain a DNA methylation pattern by reading and faithfully copying it from the old DNA strand to a newly synthesized strand during replication (Bestor 2005). DNMT3a and b target the unmethylated CpG sites for *de novo* methylation in embryonic stem cells and cancer cells
The highest expression of DNMT3a and b is found in embryonic cells, stem cells and germ line. Such methylation activity is important for the establishment of parental imprints (Kato, Kaneda et al. 2007). The role of DNA methylation is critical for normal development, proliferation and genome stability. The distribution of CpG-dinucleotides is not random in the genome. Most of the CpG sites are clustered in the promoter regions of genes, creating so-called CpG islands (Bird 2009). DNA methylation controls gene expression that is important for tissue specificity (Nagase and Ghosh 2008). Usually, the promoters of tumor suppressor genes are hypomethylated, which allows their expression for the normal functioning of a cell (Herman and Baylin 2003); whereas oncogenes and some repeat elements are silenced through hypermethylation, thus maintaining genomic integrity (Huang, Fan et al. 2004). Reanimated transposons can lead to translocations, gene disruption and chromosomal instability (Bestor 2005). Methylation that causes genomic imprinting allows suppression one of parental alleles and establishment of heterozygous expression (Feinberg and Vogelstein 1983). X-chromosome inactivation is also a result of hypermethylation (Reik and Lewis 2005).

Profound changes in DNA methylation are often associated with cancers. In fact, some cancer cells exhibit global genomic DNA hypomethylation that induces oncogene expression (Wolff, Byun et al. 2010) and is associated with chromosomal instability (Gaudet, Hodgson et al. 2003). In parallel with global genomic hypomethylation, local hypermethylation at the gene promoters of tumor suppressor genes has been observed (Rhee, Bachman et al. 2002). The main tumor suppressor genes that are usually hypermethylated in sporadic tumors are: cyclin-dependent kinase inhibitor 2A (p16),
retinoblastoma (RB1), breast cancer 1 (BRCA1), von Hippel-Lindau (VHL), MLH1 and adenomatous polyposis coli (APC) (Aypar, Morgan et al. 2011). Transposon-mediated tumorigenesis has been found to be associated with a loss of methylation in repeat elements (Hedges and Deininger 2007). Methylated cytosine is prone to spontaneous deamination to thymine, which can result in the induction of point mutations (Ketterling, Vielhaber et al. 1994).

Ionizing radiation can alter DNA methylation. Hamster and human cells experienced global hypomethylation after gamma-ray irradiation (Kalinich, Catravas et al. 1989). Exposure to X-rays led to profound DNA hypomethylation in the liver but not the spleen and brain of mice (Tawa, Kimura et al. 1998). In rodents, radiation exposure led to dose-dependent and sex- and tissue-specific global genome hypomethylation. When C57/BI mice were irradiated with X-rays in the dose range of 0.5-5 Gy, a dose-dependent loss of global methylation was detected in the spleen of male and the liver and spleen of female mice (Pogribny, Raiche et al. 2004). Similar results were found in the thymus of mice exposed to fractionated whole-body X-ray exposure. Global hypomethylation in the thymus was coupled with decreased levels of DNMT1, DNMT3a and b, methyl-CpG-binding protein 2 (MeCP2), and methyl-CpG-binding domain protein 2 (MBD2) (Pogribny, Koturbash et al. 2005). Similar molecular changes were found in irradiated mammary tissue of rats and caused genomic instability (Loree, Koturbash et al. 2006). Modulation of global DNA hypomethylation in MCF-7/DOX cells with methylation agent SAM sensitized cells to radiation-induced apoptosis (Luzhna and Kovalchuk 2010). Besides global DNA hypomethylation, irradiation causes promoter hypermethylation in tumor suppressor genes. An example of this is methylation in the
promoter of p16 tumor suppressor after low-dose x-ray exposure (Kovalchuk, Burke et al. 2004). Increased DNA methylation was also observed at repeat elements SAT2 and MLT1A in the progeny of irradiated human keratinocytes, and the correlation with RIGI was described (Kaup, Grandjean et al. 2006).

In summary, ionizing radiation causes DNA methylation changes similar to those found in early stages of carcinogenesis. Such changes are: global DNA hypomethylation, specific promoter hypermethylation, and hypermethylation of CpG-dinucleotides in repeat elements of the genome. Because altered DNA methylation was linked to RIGI, it is hypothesized that epigenetically-induced RIGI is a middle link between radiation and cancer (Ilnytskyy and Kovalchuk 2011).

**Histone modifications**

DNA in eukaryotes is not naked; it is combined with histone proteins into chromatin. The relaxed chromatin state facilitates numerous biological processes such as replication, transcription and repair. The condensation of chromatin can become an obstacle for these processes. Dynamic changes in chromatin structure provide for balanced cellular activities such as proliferation, cell cycle progression, apoptosis, etc. Uncontrolled chromatin remodeling can result in dysregulated gene expression and cancer initiation. The unit of chromatin, nucleosome, consists of 4 histones: H2A, H2B, H3 and H4. DNA is wrapped around histones, and a linker histone H1 stabilizes the octamer structure (Ma, Liu et al. 2010). The amino-terminal tails of core histones (25-40 residues) are not wrapped around DNA but extend into the surrounding space and therefore can be targeted by specific histone modifiers. The main histone modification
events known to date are: methylation, acetylation, phosphorylation and ubiquitination. Histone modifications do not cause changes in the DNA sequence, but they change the chromatin state and alter gene expression (Ma, Liu et al. 2010). Histone tails are rich in lysine amino acid residues that provide a positive charge to histones. Positively charged histones interact with negatively charged DNA, and a tight connection between DNA and histones is achieved. The acetylation of lysines in the histone tails lowers the positive charge, thus leading to the relaxed chromatin state. An opposite event, deacetylation, represses gene expression (Jenuwein and Allis 2001). Frequently targeted histones are H3 and H4 at positions 9, 14, 18, 23 and 5, 8, 12, 16, respectively (Roth, Denu et al. 2001). The acetylation/deacetylation switches are provided through the action of histone acetyltransferases (HAT) and histone deacetylases (HDAC) (Verdone, Agricola et al. 2006).

Methylation events in lysine residues cause various chromatin states. Thus, methylation of lysines 4 and 79 of H3 always correlates with gene expression, while methylation of H4 at position 20 and H3 at positions 9 and 27 causes transcriptional repression (van Leeuwen and van Steensel 2005). There can be mono-, di-, or tri-methylation of histones H3 and H4 (Bannister, Zegerman et al. 2001). DNA methylation can affect histone modifications. In tumors, a massive loss of trimethylation was observed at lysine 20 of H4 (Tryndyak, Kovalchuk et al. 2006). Another important form of histone modification is histone phosphorylation that mainly happens at serine residues of histones H2 and H3. Phosphorylation events happen at Ser 139 of H2AX during DNA damage (Rogakou, Pilch et al. 1998). Histone ubiquitination is not well understood yet, but it is known to mark proteins for degradation (Swedlow, Schuster et al. 1990).
Ubiquitination of H2A and H2B was reported to cause damage checkpoint response. H2B ubiquitination was found to be coupled with methylation of lysines 4 and 79 in histone H3, suggesting a possible role in the activation of other histone modification events (Briggs, Xiao et al. 2002).

Radiation exposure causes the decreased methylation of H4, which results in the relaxed chromatin state and possibly leads to genomic instability (Pogribny, Koturbash et al. 2005). Fractionated whole-body X-ray irradiation causes a decrease in trimethylation of Lysine 20 of H4 together with global hypomethylation (Bostelman, Keller et al. 2007). Less condensed chromatin is more sensitive to radiation and the formation of double-strand breaks. Phosphorylation of H2AX targets the repair machinery to the sites of DSBs. 40 minutes post irradiation, these changes are replaced by deacetylation of H4 lysine 5 and increased dimethylation of H3 lysine 9 and a more condensed chromatin state (Falk, Lukasova et al. 2008). DNA breaks recruit heterochromatin protein 1(HP1) to H3K9me and promote chromatin changes that initiate a DNA damage response. Histone H2AX phosphorylation happens after the formation of radiation-induced DSBs, and gamma-H2AX is a well-recognised biomarker of DSBs (Kuo and Yang 2008). Ultraviolet radiation has been shown to cause ubiquitination of H2A (Bergink, Salomons et al. 2006). Main alterations in chromatin structure following ionizing radiation are: histone modifications, the incorporation of histone variants into nucleosomes, and ATP-dependent chromatin remodeling (Vaquero, Loyola et al. 2003).
MicroRNAs

MicroRNAs (miRNAs) were first discovered and identified in *Caenorhabditis elegans* as post-transcriptional gene silencing modulators (Lee et al., 1993). Today, they are known to regulate gene silencing in mammals, fish, frogs, insects, worms, flowers and viruses. Approximately 2% - 3% of the human genome encode for microRNAs (Alvarez-Garcia and Miska, 2005). MicroRNAs are important for cellular proliferation, apoptosis, differentiation, tissue and organ development. It is now well known that aberrant expression of microRNAs is associated with cancer development and progression. The first evidence about the involvement of microRNAs in cancer came from chronic lymphocytic leukemia (CLL), which is the most common adult leukemia. Two miRNAs, miR-15 and miR-16 were deleted or down-regulated in CLL (Calin et al., 2002).

The use of microarray technologies for the analysis of miRNAs helps rapidly identify miRNAs that are either up- or down-regulated in different types of human cancer, including prostate, breast, ovarian, colorectal, kidney, bladder and cervical. The term microRNome is often used in analogy to the genome and transcriptome to describe a set of miRNAs produced by specific tissues (Cummins et al., 2006). Similarly to genes that encode mRNA, genes coding for miRNAs are classified as oncogenes (oncomirs) and tumor suppressors based on their expression levels and mRNA targets (Esquela-Kerscher and Slack, 2009). Therefore, if to define the functions of miRNAs in a simplified way, then we can say that oncomirs target tumor suppressor mRNAs, and miRNAs with tumor suppressor functions target oncoproteins. The fact that one miRNA
can cause silencing of multiple genes and any gene can be down-regulated by several different miRNAs allowed to assume the possible use of miRNAs for cancer identification and prediction.

Although biogenesis of miRNA was elucidated only a few years ago, it has already been well studied and understood. MiRNA genes are encoded in cellular DNA and transcribed by RNA polymerase II into large RNA precursors called pri-miRNAs (500 - 3000 bases) that are 5’ 7-methylguanosine-capped and polyadenylated (Liu, Fortin et al. 2008). In the nucleus, pri-miRNAs are microprocessed by Drosha and Pasha (also known as DiGeorge-syndrome critical region protein 8 - DGCR8). Both are endonucleases (double-stranded RNA-binding proteins) of the RNase III family. The products of Drosha and Pasha are ~ 70-nucleotide pre-miRNAs fold into stem-loop structures, with a 3’ overhang of 2 nt (Lee, Feinbaum et al. 1993; Landthaler, Yalcin et al. 2004). Pre-miRNAs are exported from the nucleus to the cytoplasm by RAN GTP-dependent exportin 5 of the karyopherin nucleocytoplasmic transport factor. In the cytoplasm, pre-miRNAs undergo further processing by another RNaseIII endonuclease called Dicer (Lee, Jeon et al. 2002). Dicer excises a miRNA duplex from a pre-miRNA hairpin, creating a double-stranded RNA approximately 22 nucleotides in length. Such miRNA duplex can incorporate into and form a RNA-induced silencing complex (RISC). Dicer along with the trans-activation response RNA-binding protein (TRBP), protein activator of interferon-induced protein kinase (PACT), the nuclease Tudor-SN and the Argonaute protein (AGO) contribute to the formation of RISC (Kim, Han et al. 2009). In the RISC complex, the miRNA duplex is unwound by specific helicases to form a single-stranded mature miRNA that is capable of negatively regulating its target mRNAs. The
second strand miRNA is degraded. In humans, there are four known AGO proteins (AGO 1, 2, 3, 4). AGO 2 is a catalytic component of RISC that belongs to the RNase H family and is often referred to as a slicer. AGO 2 has been shown to interact with the eIF4E factor that binds to m7G cap sites and repress the translation of mRNAs (Kiriakidou, Tan et al. 2007). There are at least three known ways of negative regulation of target genes by miRNAs, depending on the target itself and its degree of complementarity to miRNA (Esquela-Kerscher and Slack 2006).

MiRNAs with nearly perfect complementarity to a mRNA sequence induce the RNA-mediated interference (RNAi) pathway. miRISC binds within the open reading frame (ORF), and its specific ribonucleases, mainly Argonaute 2, cause cleavage of mRNA, resulting in mRNA degradation. This mechanism is believed to be predominant in plants, but it has also been proven to happen in mammals. However, most animal mRNAs are thought to be down-regulated rather than cleaved. By this mechanism, miRNAs bind to imperfect complementary sequences within the 3’ untranslated regions (UTRs) of target mRNAs and repress mRNA gene expression (Stark, Brennecke et al. 2005). In such a way protein levels are reduced, but the levels of mRNAs remain stable. There are two types of translational repression. During post-initiation translational repression, the target mRNA is repressed in polysomes, and protein synthesis is blocked. Pre-initiation repression involves the sequestration of target mRNA into distinct sites, such as processing bodies (P-bodies) in the cytoplasm, away from the translational machinery: the initiation step of translation is blocked, and protein synthesis is never started. The RISC complex is known to bind the active chromatin sites in yeast and plants, causing histone methylation and transcriptional inactivation.
MiRNA expression varies in different tumors, and genes coding for miRNAs can function as both tumor suppressors and oncogenes. The reduction or deletion of miRNAs that function as tumor suppressors leads to cancer development. Such reduction can occur because of defects at any stage of miRNA biogenesis, and it promotes the overexpression of miRNA target oncoproteins. The amplification or overexpression of miRNAs with oncogenic functions would also result in tumor formation. Inappropriate amounts of such miRNAs can be produced at the wrong time and in wrong tissues, leading to the inhibition of target tumor suppressor proteins (Esquela-Kerscher and Slack 2006).

Changes in miRNA expression as a response to ionizing radiation were detected in several cells and tissues (Czochor and Glazer 2014; Mao, Liu et al. 2014). The first evidence about radiation influence on miRNA came from Drosophila studies (Jaklevic, Uyetake et al. 2008). The bantam miRNA was up-regulated after the irradiation of fly larvae. Bantam miRNA was shown to down-regulate the pro-apoptotic gene hid by binding to the 3’-UTR of the gene transcript. This led to a decrease in apoptosis and larvae survival (Jaklevic, Uyetake et al. 2008). The effect of radiation on miRNA seems to vary according to radiation dose, the time after exposure and cell type. Thus, 2.5 Gy of X-rays caused the up-regulation of miR-34a and the down-regulation of miR-7 in hematopoietic tissues (Ilnytskyy, Zemp et al. 2008), whereas 2.5 Gy of γ-rays caused little changes in miRNA expression in lymphoblasts (Marsit, Eddy et al. 2006). The targets for miR-34a are oncogenes myc, notch1, e2f3 and cyclin D1, and miR-7 targets a regulator of DNA methylation, the lymphoid-specific helicase (LSH). An acute response (6h after exposure) to 0.5 Gy X-ray and 0.1 Gy per day for 5 days involved changes in miRNome of skin tissues, but all the changes disappeared past a 6h time point (Ilnytskyy,
The sex-specific deregulation of miRNAs 34a and 7 was shown in spleen and thymus tissues of whole-body irradiated mice (Ilnytskyy, Zemp et al. 2008). Similar results were detected in murine brains. Tissue-, time- and sex-specific radiation-induced changes in microRNome were shown in the hippocampus, cerebellum and frontal cortex of irradiated mice (Koturbash, Zemp et al. 2011). Radiation-induced DNA damage in murine testes results in a significant increase of miR-709 which targets the Brother of the Regulator of Imprinted Sites (BORIS). BORIS is an important regulator of DNA methylation and imprinting, and its decrease prevents a massive erasure of DNA methylation (Tamminga, Kathiria et al. 2008). Real-time PCR analysis of microRNA expression in irradiated cells has shown an increase in let-7 that targets the oncogene cMyc (Chaudhry 2009). In response to high and low doses of γ-radiation, significant changes in miRNome were observed in human B lymphoblastic (IM9) cells. All targets were involved in apoptosis, cell cycle and DNA damage/repair processes. Low dose (0.5 Gy) irradiated cells have shown a decrease in onco-miRNAs - miR-20 and 21, while high doses (10 Gy) cause the up-regulation of miR-197 which can stimulate carcinogenesis (Cha, Shin et al. 2009). It has been hypothesised that low doses of irradiation suppress carcinogenesis, while high doses can promote it, and these effects will be miRNA-mediated. The up-regulation of miR-24 in the irradiated and terminally differentiated blood cells, directly targets γ-H2AX that is needed at the sites of DSBs. This has led to the suggestion about a connection between radiation, chromatin structure and miRNAs. The miRNA-mediated response to ionizing radiation is very similar to one caused by oxidative radical exposure. miRNAs of the let-7 family were similarly regulated after both ionizing radiation and peroxide. This means that it is very possible that the
radiation-induced oxidative stress is the cause of miRNome response after radiation exposure (Simone, Soule et al. 2009).

All these studies show that radiation exposure affects the expression of miRNAs through initiating of DNA damage, oxidative stress and DNA repair that alter cell cycle, apoptosis and might play a role in the initiation of genomic instability and cancer development.

**In sum, from the existing literature we have learned that:**

- Ionizing radiation is ubiquitously used in medical diagnostic and treatment procedures, and although its role in cancer identification and treatment is profoundly beneficial, a controversy exists about causing unnecessary harm to overexposed individuals.
- Ionizing radiation may cause severe damage to the DNA molecule, and the cellular response to radiation varies from efficient DNA repair processes to cell death or senescence as well as to the propagation of mutations and cancer initiation. The type of radiation response underlies the risk/benefit outcome of low dose medical manipulation and the effectiveness of high dose radiotherapy.
- There is a controversy between the LNT model of low-dose radiation effect according to which any radiation dose is unsafe and the hormesis theory stating that the initial low dose radiation could offer protection and promote cellular adaptation to subsequent higher doses.
Radioresistance is a multifactorial characteristic of cancer cells that is often a result of drug resistance. The data on radiation responses of chemoresistant and especially hormone therapy-resistant tumors are scarce and contradictory. Some studies suggest that a combination of chemo-/hormone therapy with radiation therapy is significantly beneficial, whereas others present evidence that such combination may cause no effect on drug-resistant cancer cells.

Cell response to radiation depends on genetic and epigenetic mechanisms that promote gene expression alterations, thus proper investigation of radiation-induced gene expression changes requires both genetics and epigenetics to be considered.

OBJECTIVES AND HYPOTHESIS

The main goal of the current thesis is to investigate the role of genetic and epigenetic effectors in dose-dependent radiation responses of normal breast cells, breast cancer cells, and hormone therapy-resistant breast cancer cells.

Guiding Hypothesis:

Based on evidence from the literature and our preliminary studies, we hypothesize that genome and epigenome dysregulation in the mammary gland upon ionizing radiation may be involved in the initiation of breast carcinogenesis, and such IR effects are dose and energy level dependent. Moreover, we predict that breast cancer cells are more sensitive to medium and high doses of ionizing radiation rather than low doses and that
breast cancer cells resistant to common hormonal therapy drugs are much more resistant to radiation exposure than their drug-sensitive analogues.

The following experiments were conducted to achieve the goal and test the proposed hypotheses:

Experiments 1 and 2 (Chapters 2 and 3): To analyze gene expression changes and epigenetic dysregulation in the mammary gland exposed to low, medium, and high doses of X-rays in combination with low and high radiation energy levels.

Experiment 3 (Chapter 4): To analyze the molecular mechanisms of radiation resistance in breast adenocarcinoma cells resistant to hormonal drugs, tamoxifen and faslodex.

Experiment 4 (Chapter 5): To analyze the mechanisms of radiation response of breast adenocarcinoma cells to low, medium, and high doses of X-rays.
CHAPTER 2: LOW DOSE IRRADIATION PROFOUNDLY AFFECTS TRANSCRIPTOME AND MicroRNAme IN RAT MAMMARY GLAND TISSUE: POSSIBLE MECHANISMS FOR ADAPTIVE RESPONSE

Chapter 2 has been accepted for publication in its entirety:

ABSTRACT

Ionizing radiation has been successfully used in medical tests and treatment therapies for a variety of medical conditions. However, patients and health-care workers are greatly concerned about overexposure to medical ionizing radiation and possible cancer induction due to frequent mammographies and/or CT scans. Diagnostic imaging involves the use of low doses of ionizing radiation, and its potential carcinogenic role creates a cancer risk concern for exposed individuals. In this study, the effects of X-ray exposure of different doses on the gene expression patterns and the micro-RNA expression patterns in normal breast tissue were investigated in rats. Our results revealed the activation of immune response pathways upon low dose of radiation exposure. Low dose of radiation has led to the activation of the following pathways: natural killer mediated cytotoxicity pathway, antigen processing and presentation pathways, chemokine signaling, and T- and B-cell receptor signaling pathways. Both high and low doses of radiation led to miRNA expression alterations. Increased expression of miR-34a may be linked to cell cycle arrest and apoptosis. Up-regulation of miR-34a was correlated with down-regulation of its target E2F3 and up-regulation of p53. This data suggests that ionizing radiation at specific high and low doses leads to cell cycle arrest and a possible initiation of apoptosis.
INTRODUCTION

Ionizing radiation is a powerful tool in medical diagnostics and the most successful cancer treatment after surgery. The major difference in the use of ionizing radiation between diagnostic procedures and radiation therapy is the applied radiation dose. High doses of radiation possess cytotoxic properties required to kill tumor cells (Camphausen and Lawrence 2008). Diagnostic imaging, on the other hand, involves the use of low doses of ionizing radiation to gather the necessary information about a disease without harmful side effects (Brenner and Hall 2007). However, a potential carcinogenic role of ionizing radiation creates a cancer risk concern for exposed individuals. The biological effects of low doses and dose rates of radiation on normal tissues have been the subject of intense research and discussion (Schuler, Parris et al. 2011). According to the Linear-Non-Threshold (LNT) model, low-dose and low-dose-rate exposure results in a similar cancer risk as high-dose exposure (Mullenders, Atkinson et al. 2009). On the other hand, the LNT model is frequently challenged by the hormetic effect theory according to which low doses of radiation may make the exposed cells less susceptible to later high-dose exposure and may have health benefits (Calabrese and Baldwin 2003).

Microarray technology for gene expression analysis may provide a better understanding of biological effects of low doses of ionizing radiation. The radiation response at the gene expression level can help reveal the mechanisms of cellular response and identify key genes responsible for specific endpoints (Kruse, te Poele et al. 2004). There are only a few published in vivo studies focusing on gene expression analysis in tissues exposed to low doses of ionizing radiation. A clear distinction between high and low doses of gamma radiation has been shown in the liver tissue of mice (Uehara, Ito et
Similar effects of low and high doses of radiation have been found in the thymus tissue of mice, with 2421 and 608 genes being affected after high and low doses, respectively (Shin, Lee et al. 2011). A different response has been shown for internal low-dose radiation from $^{131}$I. The response of transcripts has been found to be independent of a dose but rather tissue dependent (Schuler, Parris et al. 2011). Overall, there is no clear evidence of an exact mechanism of radiation response at the gene expression level, especially in *in vivo* models. Some reasons might be tedious animal handling, the heterogeneity of the absorbed dose, and a mixture of cell types within a tissue.

Gene expression is strongly regulated by epigenetic modifications, including negative regulation of protein synthesis by microRNAs. Ionizing radiation causes alterations in miRNA expression and subsequently, in protein levels of key regulators of the cell cycle. For instance, 2.5 Gy of X-rays caused upregulation of miR-34a and downregulation of miR-7 in hematopoietic tissues (Ilnytskyy, Zemp et al. 2008). Targets for miR-34a are oncogenes myc, notch1, e2f3, and cyclinD1; miR-7 targets a regulator of DNA methylation, a lymphoid-specific helicase (LSH). The differential expression of miRNAs in response to different doses of gamma radiation was observed previously in human B lymphoblastic (IM9) cells. Low-dose (0.5 Gy) irradiated cells showed a decrease in onco-miRNAs - miR-20 and 21, while high-dose irradiation (10 Gy) caused upregulation of miR-197 that can stimulate carcinogenesis (Cha, Shin et al. 2009). It was hypothesized that low doses of radiation suppressed carcinogenesis, while high doses could promote it, and these effects would be miRNA-mediated.
The aim of this study was to investigate the effects of different doses of X-ray exposure on gene expression patterns and micro-RNA expression patterns in normal rat breast tissues.

MATERIALS AND METHODS

Animal models and irradiation conditions

Six-week-old intact female Long-Evans rats were obtained from Charles River (Wilmington, MA). The animals were housed two per cage in a temperature-controlled (24 °C) room in a 12-hour light-dark cycle and given ad libitum access to water and an NIH-31 pelleted diet. Six rats were randomly assigned to one of the following X-ray radiation treatment groups: 80kVp/0.1 Gy, 80kVp/1 Gy, 80kVp/2.5 Gy, 30kVp/0.1 Gy, and sham treated controls. Each group of animals was humanely sacrificed 6, 96 hours, and 4, 12, and 24 weeks after radiation treatment. The paired caudal inguinal mammary glands were excised. Tissue was frozen immediately in liquid nitrogen and stored at -80°C for subsequent analyses.

RNA isolation

Total RNA was isolated using the Illustra RNASpin Mini kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Approximately 50–70 mg of mammary gland tissue was processed following the manufacturer's instructions. The samples were eluted in Ultrapure DNase/RNase-free distilled water provided in the kit. RNA samples were quantified by ultraviolet spectroscopy (NanoDrop, Wilmington, DE) and were further assessed for RNA integrity (RIN) on the Aglient 2100 Bioanalyzer (Santa Clara, CA)
using the RNA Nano-chip Kit. RNA samples with RIN values of seven or better were followed through to analysis.

**Whole-genome gene expression profiling**

**Library preparation**

For this study, cRNA was created using the Ambion Illumina TotalPrep RNA Amplification Kit (Applied Biosystems, Carlsbad, CA), with an input of 500 ng of total RNA per sample. Briefly, oligo-dT primers were used to synthesize first-strand cDNA containing a phage T7 promoter sequence. The single-stranded cDNA was converted into a double-stranded DNA template via DNA polymerase. RNase H acted simultaneously to degrade RNA, and cDNA samples were purified in filter cartridges to remove excess RNA, primers, enzymes, and salts. The recovered cDNA was subjected to *in vitro* transcription using biotinylated UTPs. This step created the labeled and amplified cRNA. A final purification step removed unincorporated NTPs, salts, inorganic phosphates, and enzymes to prepare samples for hybridization.

**Hybridization and detection**

The Illumina's direct hybridization assay kit was used to process samples according to the manufacturer's protocol (Illumina, San Diego, CA). Briefly, 750 ng from each cRNA sample was hybridized to the Illumina Rat-Ref-12 Whole Genome Expression BeadChip arrays overnight. Afterward, a 10-minute incubation with the supplied wash buffer at 55°C preceded a 5-minute room-temperature wash. The arrays were incubated in 100% ethanol for 10 minutes. A second room temperature wash for
two minutes with gentle shaking completed this high stringency wash step. The arrays were blocked with buffer for 10 minutes and washed before a 10-minute probing with streptavidin-Cy3 (1:1000). After a five-minute wash at room temperature, BeadChips were dried and imaged. Six controls were also built into the Whole-Genome Gene Expression Direct Hybridization Assay system to cover the aspects of array experiments. These included controls for a biological specimen (14 probes for housekeeping controls), three controls for hybridization (six probes for Cy3-labeled hybridization, four probes for low stringency hybridization, one probe for high stringency hybridization), signal generation (two probes for biotin control) and ~800 probes for negative controls on an eight-sample BeadChip. The arrays were scanned on the iScan platform (Illumina), and the data were normalized and scrutinized using Illumina BeadStudio software.

**BeadChip statistical analysis and data processing**

The false discovery rate (FDR) was controlled by the Benjamini-Hochberg method. The Illumina Custom Model took FDR into account and was used to analyze the data. Differential gene expression (at least a 0.5-fold change) from sham-treated animals was determined to be statistically significant if the p-value after the adjustment with the Benjamini-Hochberg method was less than 0.05. The values were transformed to show a log2 scale.

Lists of regulated transcripts were put into the web-based DAVID Bioinformatics Resources 6.7 (NIAID/NIH) Functional Annotation Tool (Huang da, Sherman et al. 2009; Huang da, Sherman et al. 2009). This program was used to group genes into functionally relevant categories and pathways for further analysis of the association of
genetic profiles with breast cancer susceptibility. The minimum number of genes in each altered pathway was set to three. The pathways were deemed significantly altered if at least 80% of the genes were shifting the pathway in the same direction (Kars, Iseri et al. 2009).

**Real-time polymerase chain reaction (qRT-PCR)**

Quantitative real-time PCR was performed to confirm the Whole-Genome Gene Expression results for the regulation and direction (either up or down) of the selected genes. Four genes (Cathepsin K, Lipocalin 2, Phospholipase 2, and Tetraspanin 1) were selected from the gene list of significantly differentially expressed transcripts that represented a preliminary review of the acquired gene expression data. β-Actin was used as a reference gene. All reactions were performed using cDNA synthesized from 500 ng of RNA sample using the Bio-Rad iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). The samples were stored at -20°C for long-term storage and at 4°C until they were used for subsequent qRT-PCR reactions.

The primers were designed using the NCBI database and PrimerQuest (Integrated DNA Technologies, Inc., Coralville, IA). The primers were as follows: **CTSK** forward primer 5'-ATG TGC AGC AGA ATG GAG GCA TTG-3' and reverse primer 5'-TGC TCT CTT CAG GGC TTT CGT GTT-3'; **LCN2** forward primer 5' -ACA ACG TCA CTT CCA TCC TCG TCA- 3’ and reverse primer 5' -TGG CAA ACT GGT CGT AGT CAG TGT- 3’; **PLA2G2A** forward primer 5' -CAT GGC CTT TGG CTC AAT TCA GG-3' and reverse primer 5' -TGG CAA ACT GGT CGT AGT CAG TGT- 3'; **TSPAN** forward primer 5' -TTG TCA ACG TGG GCT ACT GCC TTG-3’ and reverse primer 5' -
AGC ACA CAC TTG TTC TCG GAG TGA- 3'; and beta-Actin reference gene forward primer 5'-CCT CTG AAC CCT AAG GCC AA-3' and reverse primer 5'-AGC CTG GAT GGC TAC GTA CA-3'. Reactions were prepared using 1 μL of diluted cDNA, 10 pmol/μL of each forward and reverse primer and Ssofast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Samples were prepared in triplicate and were run on the Bio-Rad C1000 Thermal Cycler equipped with the CFX96 Real-Time System. The qRT-PCR protocol consisted of denaturation at 95°C for two minutes; 43 cycles of denaturation (95°C, five seconds) and annealing/extension (55°C, five seconds); and the final extension at 65°C for five seconds. For every set of primers, annealing temperature optimization, melting curve analysis, and gel analysis of amplicon were performed. To evaluate PCR efficiency, the standard curve was established using series of cDNA dilutions. The data were captured and organized by the Bio-Rad CFX Manager 2.1 software (Bio-Rad Laboratories, Hercules, CA).

The quantification data from the Bio-Rad CFX Manager software were analyzed in Microsoft Excel using the Pfaffl method (Pfaffl 2001). The graphs showing fold change from the sham group were created showing transcript regulation directions (up- or down regulation).

miRNA microarray expression analysis

Total RNA from mammary gland frozen tissues was isolated using Trizol reagent (Invitrogen, Burlington, ON) according to the manufacturer's instructions. One ug of the total extracted RNA represented as two repeats per experimental group was sent to LC Sciences (Austin, TX) for miRNA microarray analysis.
Western immunoblotting

For protein isolation, 30–50 mg of mammary gland tissue were washed in PBS, lysed, and sonicated in 0.25 mL of 1% sodium dodecyl sulfate (SDS) containing protein inhibitors. The lysates were cleared using centrifugation. The protein content was determined using the Bradford protein determination assay (BioRad, Hercules, CA). Equal amounts of lysate protein were subsequently run on 10–12% SDS-polyacrylamide gels and transferred to PVDF membranes (GE Healthcare, Baied'Urfé, Québec).

Western immunoblotting was conducted using the well-established protocols (Ertel, Verghese et al. 2006; Tryndyak, Kovalchuk et al. 2006). The membranes were incubated with antibodies against mouse anti-TP53, rabbit anti-transgelin, rabbit anti-E2F3 (1:100 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and mouse anti-Actin (1:1000 dilution, Abcam Inc., Cambridge, MA). Antibody binding was revealed through the incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ) and the ECL Plus immunoblotting detection system (GE Healthcare, Piscataway, NJ). Chemiluminescence was detected using BioMax MR films (Eastman Kodak, New Haven, CT). The unaltered PVDF membranes were stained with Coomassie Blue (BioRad, Hercules, CA) to prove equal protein loading. Signals were quantified using NIH ImageJ 1.63 software and normalized to loading controls. The images are representative of two independent immunoblots. The results are presented as mean ± S.E.M. The statistical analyses were conducted using the student's t-test. P-values less than 0.05 were considered significant.
RESULTS

The effects of low, intermediate, and high doses of radiation on whole-genome gene expression in the mammary gland

Isolated RNA from the mammary gland was used for gene expression profiling. A drastic difference in the radiation-induced gene expression changes was discovered between the doses/energy levels applied. Ninety-six hours after radiation, only high energy level/low doses of X-ray exposure (80kVp/0.1 Gy) led to significant alterations in the expression level of 567 genes (Table 2.1). Other doses did not affect gene expression, and only a few genes were altered. Interestingly, the alterations noticed at an early time point disappeared by 24 weeks, while a slight (51 genes) delay in gene expression alterations was noticed for the high level/high doses (80kVp/2.5 Gy) of radiation (Table 2.1). Most of the altered genes were unique in their experimental groups, and there were not many genes common to all the treatment groups (Fig. 2.1).

Further, we evaluated 567 genes that changed their expression level 96 hours after 80kVp/0.1 Gy of X-rays: 295 genes were upregulated, and 272 genes were downregulated. With the help of the DAVID functional annotation array analysis tools, we were able to identify and group the evaluated genes according to their function and possible role in certain pathways. Subsequently, genes with a similar or identical function were grouped together; and based on their expression changes, the role of certain pathways in radiation response was evaluated (Table 2.2). Most of the changed genes contributed to certain immunological pathways (Table 2.2). Some examples of such elevated pathways are as follows: antigen processing and presentation (16 genes altered),
B- and T-cell receptor signaling (13 and 15 genes, respectively), chemokine signaling (14 genes), Fc gamma R-mediated phagocytosis (11 genes), natural killer cell-mediated cytotoxicity (18 genes), etc. Upregulation of immunological pathways reveals the activation of immune defense against possible damage caused by either ionizing radiation or other forms of potential stressors. The visual representation of one of such pathways (natural killer cell-mediated cytotoxicity) is presented in Figure 2.2. Most downregulated genes contributed to metabolic pathways: citrate cycle (8 genes), fatty acid metabolism (6 genes), glutathione metabolism (7 genes), pyruvate and tryptophan metabolism (7 and 6 genes, respectively) (Table 2.2). The number of altered genes in the 24-week/80kVp/2.5 Gy group was too small to group in pathways; therefore, we analyzed singular genes of interest.

The validity of gene expression profiling was confirmed by qRT-PCR for genes with the most change and the greatest radiation response in both the 96 hours/80kVp/0.1 Gy and 24 weeks/80kVp/2.5 Gy groups. Therefore, the primary targets for qRT-PCR were cathepsin K (CTSK), lipocalin 2 (LCN2), phospholipase 2 (Pla2G2), and tetraspanin 1 (TSPAN1) (Fig. 2.3). Cathepsin K, a lysosomal cysteine proteinase that was known to be overexpressed in breast cancers, was significantly elevated at 4 and 24 weeks after high-dose radiation (80kVp/2.5 Gy). Lipocalin 2, an oncogene that may function as a growth factor, was also upregulated at 24 weeks after the highest dose of X-rays. Both phospholipase 2 and tetraspanin 1 play a role in cell growth, signaling and motility. Similarly to the gene expression analysis, qRT-PCR showed that these genes were downregulated in most experimental groups at 24 weeks after radiation exposure (Fig. 2.3).
miRNA expression in the irradiated mammary gland

miRNAs regulate gene expression epigenetically; therefore, we proceeded to analyze the role of miRNAs in response to low, intermediate, and high doses of radiation in mammary gland tissue at 96 hours after treatment. miRNAs involve the epigenetic control of gene expression regulation through the RNA interference pathway. miRNAs negatively affect the levels of their target transcripts and proteins encoded by these transcripts. In this way, miRNAs contribute to gene silencing, and changes in miRNA expression are common in cancers and in response to radiation.

Interestingly, we identified the alterations in miRNA expression after high dose/energy level (80 kVp/2.5 Gy) and low dose/low energy level (30 kVp/0.1 Gy) radiation (Table 2.3). Upregulation of miR-34a has been found to be common for both doses, and the expression level has been increased 1.55- and 1.08-fold after 80 kVp/2.5 Gy and 30 kVp/0.1 Gy, respectively. MiR-34a directly inhibits the expression of transcription factor E2F3 that is necessary for cell progression through the cell cycle and the expression of transgelin, an actin cross-linking protein, which may contribute to replicative senescence. The MiR-34 family is known to be activated by the p53-dependant pathway in response to DNA damage.

Tp53, E2F3, and transgelin expression in the irradiated mammary gland

The elevated expression of miR-34a was interesting to us, and we decided to proceed with identifying protein levels of its targets E2F3 and transgelin as well as p53, the key protein in DNA damage response. Western analysis was performed for tissues
exposed to 80 kVp/2.5 Gy and 30 kVp/0.1 Gy radiation, at 96 hours and 24 weeks after exposure. The level of Tp53 was shown to be significantly elevated at 24 weeks after low-dose exposure (Fig. 2.4). The increased levels of the phosphorylated p53 protein stimulate radiation response and DNA damage repair.

The level of E2F3 protein was shown to be decreased in response to both 80 kVp/2.5 Gy and 30 kVp/0.1 Gy radiation treatments at the early time point (96 hours) (Fig. 2.4). The downregulation of E2F3 is known to stimulate G1 arrest, senescence, and/or apoptosis. There were no significant differences in the expression of transgelin in the irradiated tissues in comparison to non-irradiated controls (Fig. 2.4).

**DISCUSSION**

Ionizing radiation has been successfully used in medical tests and treatment modalities for a variety of medical conditions, including breast cancer screening and therapy. Nevertheless, a strong concern about overexposure to medical ionizing radiation and possible cancer induction due to continuous mammography procedures and/or CT scans exists amongst patients and individuals who provide patient care (Mullenders, Atkinson et al. 2009). The concern raised is based on the ability of low doses of ionizing radiation used for diagnostic procedures to cause DNA damage that is not extensive enough to induce cell death, but may result in mutations, genomic rearrangements and cancer initiation (Ward 1995). Ionizing radiation is considered to be a non-threshold carcinogen. The Linear-No-Threshold (LNT) model states that there is no dose level below which radiation exposure is safe, and there is a finite probability that even the lowest possible dose may be responsible for cancer initiation (Mullenders, Atkinson et al.
It is evident that choosing the right dose of radiation as well as the amount of radiation used during screening and therapy is vital for any medical procedure to minimize any potential risk of harm. Overall, the data on the response of healthy mammary tissues to low versus high doses and energy levels of radiation are scarce and indeed need more experimental evidence.

In the present study, the immediate (96 hours) and prolonged (24 weeks) radiation-induced changes in mammary gland gene expression were investigated and compared between different radiation doses and energy levels. Unexpectedly, the large-scale gene expression alterations were only noticed after the application of high energy/low dose (80kVp/0.1 Gy) X-rays at 96 hours after treatment (Table 2.1). Neither high-dose nor low-dose exposures combined with low-energy radiation caused significant modifications in gene expression at the transcription level. The altered genes mainly constituted the immunological pathways that were shown to be activated upon radiation (Table 2.2). Radiation is generally considered to be an immunosuppressive agent that kills radiosensitive cells, and this makes radiotherapy one of the most successful cancer therapies. However, under certain circumstances, especially exposure to low-dose radiation may enhance immunity. Our study has shown an increase in antigen processing and presentation, a process by which antigen-presenting cells digest foreign proteins and display antigenic peptide fragments on MHC molecules for the recognition by T cells during infections and abnormal cell growth. Among the genes that were upregulated and contribute to this pathway were the following: CD74 (the major histocompatibility complex class two that plays a role in MHCII antigen processing), CD8a (involved in T cell-mediated killing by identifying cytotoxic T cells that interact with MHC class I),
Ifi30 (the interferon gamma inducible protein that facilitates MHC class I and II recognition of antigens containing disulfide bonds), and other genes with similar functions. A similar effect of radiation on antigen presentation by MHC class I was reported previously in murine colon adenocarcinoma cells (Reits, Hodge et al. 2006). Similarly, non-cytotoxic effects of ionizing radiation on MHC class I antigen presentation were demonstrated in bone marrow-derived dendritic cells (Liao, Wang et al. 2004). The modulation of antigen presentation pathways provides protective anti-tumor immunity to the irradiated cells and tissues. Eighteen genes constituting the natural killer (NK) cell-mediated cytotoxicity pathway were also upregulated (Table 2.2, Fig. 2.2). NK cells play a role in immune surveillance for cancer by providing anticancer immunity to cells (Schmitt, Ghazi et al. 2008). The activated genes were CD247 (it plays a role in signal transduction upon antigen triggering), Icam 1 and 2 (they are ligands for leukocyte adhesion), Lat (a linker for T activation), among others. The enhancement of NK cell-mediated cytotoxicity after radiation in combination with HDAC inhibitor was recently reported in lung cancer cells (Son, Keum et al. 2014). B- and T-cell receptor signaling pathways were also upregulated upon low dose/high energy radiation (Table 2.2). Both pathways stimulate immune response to cancer initiation and are the prime targets for the treatment of many malignancies. Various chemoattractants for blood monocytes and memory T-helper cells as well as chemokine receptor genes were activated; this activation upregulated the chemokine signaling pathway (Table 2.2). Similar CXC chemokines were shown to be upregulated by low doses of ionizing radiation in normal human fibroblasts (Fujimori, Okayasu et al. 2005). The upregulation of the phagocytosis pathway was due to an increased expression of 11 genes. Phagocytosis activation has
been known to be induced after radiation exposure as a consequence of recognition and clearance of radiation-induced apoptotic cells. Such inflammatory-type response to radiation exhibits the bystander effect of radiation rather than the direct effect of radiation (Lorimore, Coates et al. 2001). Overall, the activation of immune response pathways upon radiation exposure may indicate anti-tumor protection and eradication of damaged cells. Similar effects of internal low-dose irradiation on gene expression and activation of immune response in normal tissues in mice were reported previously (Schuler, Parris et al. 2011). Interestingly, immune response was the only common biological process affected by irradiation in all tissues studied (the liver, lung, spleen, kidney medulla, and kidney cortex), while alterations in other biological processes were tissue-specific (Schuler, Parris et al. 2011).

Radiation response has also shown metabolic changes, mainly downregulation of citrate cycle, pyruvate, and fatty acid metabolism pathways (Table 2.2). A metabolic response to radiotherapy is very important. A progressive decrease in glucose metabolism in cancer has been shown to be useful for the prediction of a radiotherapy response (Giovacchini, Picchio et al. 2009). Metabolic properties of pre-cancerous and cancer cells depend on glycolisis, increased rate of fatty acids synthesis, and increased rates of glutamine metabolism. These properties often result in therapeutic resistance (Zhao, Butler et al. 2013). Our results on gene expression have demonstrated radiation-induced metabolic inhibition that may lead to cell death rather than cancer initiation.

Gene expression analysis was confirmed by qRT-PCR for four genes with the highest changes in gene expression. Tetraspanin 1 RNA expression was proved to be
decreased upon exposure to ionizing radiation (Fig. 2.3). This protein mediates signal transduction events that play a role in the regulation of cell development, activation, growth, and motility. Phospholipase 2 was down-regulated at the early and late time points, and was thought to participate in the regulation of phospholipid metabolism in biomembranes, including eicosanoid biosynthesis. Phospholipases are ubiquitously expressed and have diverse biological functions, including role in inflammation, cell growth, signaling and death, and the maintenance of membrane phospholipids. Interestingly, both gene expression and qRT-PCR analyses have shown an increased expression of lipocalin 2 and cathepsin K 24 weeks after being exposed to the highest dose (80kVp/2.5 Gy). Both proteins are known to be oncogenes and are ubiquitously expressed in breast cancers. It is important to note that high expression of these genes was not accompanied by the upregulation of particular pathways to which these genes belong.

It is well accepted that gene expression is strongly regulated by epigenetic factors (Jaenisch and Bird 2003). A number of studies have indicated substantial alterations of epigenetic elements, including changes in DNA methylation, histone modification, and short RNA patterns as a result of radiation exposure (Aypar, Morgan et al. 2011). Radiation-induced changes in miRNA expression usually lead to changes in the synthesis of proteins involved in the main cellular biological pathways. As per Table 2.3, the validated targets of misregulated miRNAs fall in cell cycle and apoptosis categories (Table 2.3). Interestingly, a low radiation dose causes similar miRNA expression changes to the highest dose. The increased expression of miR-34a may be linked to cell cycle arrest and apoptosis. The ectopic expression of miR-34 genes is known to cause a G1
phase arrest (Tarasov, Jung et al. 2007). Furthermore, the high expression of miR-34a has been shown to induce apoptosis (Raver-Shapira, Marciano et al. 2007). The main targets of miR-34a are E2F3 transcription factor, transgelin, and possibly CDK4/6, cyclin E2, c-myc (Hermeking 2010). Bommer et al. showed that Bcl-2 was targeted by miR-34a (Bommer, Gerin et al. 2007). Interestingly, several reports have shown that the miR-34 family is a direct target of p53, and its activation induces apoptosis and cell cycle arrest (Bommer, Gerin et al. 2007; Corney, Flesken-Nikitin et al. 2007). In addition, the activation of miR34-a by p53 feeds back to p53, and such positive feedback leads to further activation of p53 (Hermeking 2010). We further decided to conduct Western blot analysis to identify protein levels of E2F3 and transgelin that are targets miR-34a. The expression level of E2F3 protein was indeed downregulated at 96 hours after radiation treatment with both low and high doses (Fig. 2.4). E2F3 binds specifically to RB1 and is involved in the control of cell cycle progression from G1 to S phase. Low levels of E2F3 lead to cell cycle arrest in response to DNA damages that result from ionizing radiation. We did not notice any significant changes in the protein level of transgelin. However, an elevated level of p53 protein was detected after exposure to a low dose of ionizing radiation. Such correlation between upregulated miR-34a, the downregulation of its target E2F3, and the upregulation of p53 allows us to suggest that ionizing radiation at specific high and low doses leads to cell cycle arrest and a possible initiation of apoptosis. The induction of cell cycle arrest and promotion of apoptosis when the damage is too severe to be repaired are considered to be important for tumor suppression (Vousden and Lane 2007). In his report, Hermeking described the role of p53 as a mediator of tumor suppression through the activation of miR-34 family members (Fig. 2.5).
Overall, both post radiation gene expression and miRNA expression analyses have demonstrated an increased immunological response and cell cycle response directed to prevent cancer initiation. However, these characteristics were not detected for every dose applied. Further investigation of the cellular response may shed more light on the correlation between differential radiation doses and their effects on apoptosis/cancer.
**Table 2.1 Gene expression profiling in mammary gland tissue exposed to low and high doses of ionizing radiation.** The number of significantly changed genes in the rat mammary gland upon low energy level/low dose (30kVp/0.1 Gy), high energy level/low dose (80kVp/0.1 Gy), high energy level/medium dose (80kVp/1 Gy) and high energy level/high dose (80 kVp/2.5 Gy) of radiation in comparison to their corresponding un-irradiated controls at 96 hours and 24 weeks time points, as identified by gene expression profiling analysis.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>96 hours</th>
<th></th>
<th>24 weeks</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total number of genes changed</td>
<td>Number of up-regulated genes</td>
<td>Number of down-regulated genes</td>
<td>Total number of genes changed</td>
</tr>
<tr>
<td>30 kVp/0.1 Gy</td>
<td>14</td>
<td>8</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td>80 kVp/0.1 Gy</td>
<td>567</td>
<td>295</td>
<td>272</td>
<td>37</td>
</tr>
<tr>
<td>80 kVp/1 Gy</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>80 kVp/2.5 Gy</td>
<td>32</td>
<td>13</td>
<td>19</td>
<td>51</td>
</tr>
</tbody>
</table>


Figure 2.1 Differentially expressed genes commonly shared between treatment groups. The Venn diagram groups the common altered genes between experimental groups.
Table 2.2 Significantly altered KEGG pathways in mammary gland upon 96h of 80kVp/0.1 Gy in comparison to the corresponding untreated controls. In this table, the pathway significance (%) is defined as the ratio of gene alterations that similarly affect a certain pathway (either up- or down-regulate) to the total number of altered genes in the pathway. “+” – the pathway is up-regulated; “-” – the pathway is down-regulated. N/S – non-significant.

<table>
<thead>
<tr>
<th>Pathways</th>
<th>Pathway Significance, % (total number of genes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen processing and presentation</td>
<td>+ 93.8% (16)</td>
</tr>
<tr>
<td>B cell receptor signaling</td>
<td>+ 100% (13)</td>
</tr>
<tr>
<td>Cell adhesion molecules (CAM)</td>
<td>+ N/S (20)</td>
</tr>
<tr>
<td>Chemokine signaling</td>
<td>+ 100% (14)</td>
</tr>
<tr>
<td>Citrate cycle (TCA)</td>
<td>- 100% (8)</td>
</tr>
<tr>
<td>Cytosolic DNA-sensing pathway</td>
<td>+ 100% (6)</td>
</tr>
<tr>
<td>ECM-receptor interaction</td>
<td>- 88.9% (9)</td>
</tr>
<tr>
<td>Fatty acid metabolism</td>
<td>- 100% (6)</td>
</tr>
<tr>
<td>Fc epsilon RI signaling</td>
<td>+ 100% (10)</td>
</tr>
<tr>
<td>Fc gamma R-mediated phagocytosis</td>
<td>+ 100% (11)</td>
</tr>
<tr>
<td>Glutathione metabolism</td>
<td>- 85.7% (7)</td>
</tr>
<tr>
<td>Graft-vs-host disease</td>
<td>+ 100% (7)</td>
</tr>
<tr>
<td>Hematopoietic cell lineage</td>
<td>+ N/S (9)</td>
</tr>
<tr>
<td>Intestinal immune network for IgA production</td>
<td>+ 100% (6)</td>
</tr>
<tr>
<td>Leukocyte transendothelial migration</td>
<td>+ 89.5% (19)</td>
</tr>
<tr>
<td>Lysosome</td>
<td>+ N/S (13)</td>
</tr>
<tr>
<td>Natural killer cell mediated cytotoxicity</td>
<td>+ 100% (18)</td>
</tr>
<tr>
<td>PPAR signaling</td>
<td>- 100% (10)</td>
</tr>
<tr>
<td>Primary immunodeficiency</td>
<td>+ 100% (8)</td>
</tr>
<tr>
<td>Pyruvate metabolism</td>
<td>- 100% (7)</td>
</tr>
<tr>
<td>T cell receptor signaling</td>
<td>+ 100% (15)</td>
</tr>
<tr>
<td>Tryptophan metabolism</td>
<td>- 83.3 % (6)</td>
</tr>
</tbody>
</table>
Figure 2.2 The natural killer cell mediated cytotoxicity pathway. Red stars represent genes that were up-regulated.
Figure 2.3 Altered levels of gene transcripts of cathepsin K, lipocalin 2, phospholipase 2, and tetraspanin 1, as detected by RT-PCR. Data are shown as fold changes to respective controls. Each treatment group was compared to its corresponding control; B-actin was used as a reference gene (calculated by Pfaffl). P-values (in a table below the graphs) were calculated by student’s t-test.
Table 2.3 Radiation-induced microRNA expression changes in rat mammary gland. Relative miR expression values are represented in folds in the irradiated cells in comparison to non-irradiated control cells as analyzed by miRNA microarray. Significance of differences was analyzed by the Student’s t-test.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>MiRNA changed</th>
<th>Log2 (G/CT)</th>
<th>Validated targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 kVp/0.1 Gy</td>
<td>2</td>
<td>Low fold change</td>
<td>-</td>
</tr>
<tr>
<td>80 kVp/1 Gy</td>
<td>Low signals</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>80 kVp/2.5 Gy</td>
<td>miR-34a</td>
<td>1.55</td>
<td>E2F3, Tagln, INHBB</td>
</tr>
<tr>
<td></td>
<td>miR-29c</td>
<td>-1.02</td>
<td>Tpm1</td>
</tr>
<tr>
<td></td>
<td>miR-20b-5p</td>
<td>-1.65</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>miR-204</td>
<td>-1.39</td>
<td>-</td>
</tr>
<tr>
<td>30 kVp/0.1 Gy</td>
<td>miR-34a</td>
<td>1.08</td>
<td>E2F3, Tagln, INHBB</td>
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<tr>
<td></td>
<td>miR-20b-5p</td>
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<td>-</td>
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<td>miR-98</td>
<td>-1.16</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>miR-127</td>
<td>2.08</td>
<td>-</td>
</tr>
</tbody>
</table>
A.

Figure 2.4 Tp53, E2F3, and transgelin protein levels in rat mammary gland upon whole body irradiation. (A) Protein levels relative to those of control non-irradiated animals are shown as Mean ± StEr. (B) Representative blots from two independent experiments. * - p<0.05, student’s t-test.
Figure 2.5 The miR-34 family as mediator of tumor suppression by p53. Adapted with permission from Hermeking, 2010 (Hermeking 2010).
Main findings described in chapter 2:

1. Radiation exposure leads to early (96 hours) changes in gene expression. Most profound effect has been shown for the 80 kVp/0.1 Gy dose exposure.

2. Most genetic changes have shown an immunological pathway response to radiation. But certain oncogenes were activated 24 weeks after highest dose of radiation.

3. MiRNA profile has been profoundly changed after lowest 30 kVp/0.1 Gy and highest 80 kVp/2.5 Gy doses of X-ray. The alterations affected cell cycle and apoptosis processes.
CHAPTER 3: MOBILIZATION OF LINE-1 IN IRRADIATED MAMMARY GLAND TISSUE MAY POTENTIALLY CONTRIBUTE TO LOW DOSE RADIATION-INDUCED GENOMIC INSTABILITY

Chapter 3 has been submitted in its entirety:

ABSTRACT

It is known that cellular stresses such as ionizing radiation activate LINE-1 (long interspersed nuclear element type 1, L1), but the molecular mechanisms of LINE-1 activation have not been fully elucidated. There is a possibility that DNA methylation changes induced by genotoxic stresses might contribute to LINE-1 activation in mammalian cells. L1 insertions usually cause major genomic rearrangements, such as deletions, transductions, intrachromosomal homologous recombination between L1s, and generation of pseudogenes, which could lead to genomic instability. The purpose of this study was to evaluate the effects of low and high doses of ionizing radiation on the DNA methylation status of LINE-1 transposable elements in rat mammary glands. Here we describe radiation-induced hypomethylation and activation of LINE-1 ORF1 in rat mammary gland tissues. We show that radiation exposure has also led to the translation of the LINE-1 element, whereby the 148 kDa LINE-1 protein level was increased 96 hours after treatment with a low dose and low energy level radiation and remained elevated for 24 weeks after treatment. The mobilization of LINE-1 in irradiated tissue may potentially contribute to genomic instability. The observed activation of mobile elements in response to radiation exposure is consistently discussed as a plausible mechanism of cancer etiology and development.
INTRODUCTION

LINE-1 (long interspersed nuclear element type 1, L1) belongs to the family of non-long terminal repeat retrotransposons. With over 500,000 copies, L1 comprises 17-18% of the human genome and is capable of its own expansion and also mobilization of other non-L1 elements that may dramatically shape the genome (Lander, Linton et al. 2001; Ergun, Buschmann et al. 2004). It is well known that most of the L1 elements are mutated, rearranged, and/or truncated (at the 5’ end) and therefore, are not capable of further retrotransposition. Nevertheless, a small subset (80-100) of the full-length L1 elements is active, functional and potentially capable of self-expansion (Brouha, Schustak et al. 2003; Belgnaoui, Gosden et al. 2006). A retrotransposition-competent L1 element (RC-L1) is 6-7 kb in length and consists of 5’-untranslated region (5’-UTR) with its internal CpG-rich promoter, two non-overlapping open reading frames (1kb ORF1 and 3.8 kb ORF2) separated by a 63bp intergenic spacer, and a 206nt 3’-UTR terminator with a poly(A) tail (Goodier, Zhang et al. 2007). The internal or “minimal promoter” is generally believed to be the most important region for successful transcription of L1. However, in their study Alexandrova and colleagues have shown that the promoter strength is mostly dependant on the 390-526 bp region within the human L1 5’-UTR. Deletion of this fragment resulted in the significant decrease of promoter activity (Alexandrova, Olovnikov et al. 2012). In the same study the authors proposed a model in which an internal enhancer region (390-526) of L1 5’-UTR is responsible for recruitment of the transcription initiation complex and might serve as a basis for enhanceosome formation. This internal enhancer overlaps with the region of L1 5’-UTR that drives transcription in opposite direction suggesting the existence of bidirectional transcription.
of L1 (Alexandrova, Olovnikov et al. 2012). ORF1 encodes a 40 kDa (p40) protein with the RNA-binding activity, while ORF2 encodes a 150 kDa protein with N-terminal endonuclease and C-terminal reverse transcriptase enzymatic activities (Ostertag and Kazazian 2001). ORF1 proteins (ORF1p) are predominantly cytoplasmic and form large ribonucleoprotein (RNP) complexes with L1 RNA and DNA (Hohjoh and Singer 1996). ORF1p is reported to possess chaperone activity and possibly be significant in reverse transcription reaction (Moran, Holmes et al. 1996). The function of ORF2p in retrotransposition is much well-determined, as the endonuclease nicks the target DNA strand exposing a 3’-hydroxyl group that primes reverse transcription of L1 RNA by reverse transcriptase. Such mechanism of retrotransposon replication is termed as target-site-primed reverse transcription (TPRT) (Luan and Eickbush 1995).

L1 insertions usually cause major genomic rearrangements such as deletions, transductions, intrachromosomal homologous recombination between L1s, and the generation of pseudogenes that can lead to genomic instability (Gilbert, Lutz-Prigge et al. 2002; Symer, Connelly et al. 2002). L1 transcription and integration into the genome leads to recombination events that harvest chimeric retrotranscripts or pseudogenes that consist of the fused DNA copies of various RNAs. One study reported at least 81 of such chimeric pseudogenes which were classified in nine families (Buzdin, Gogvadze et al. 2003). Later it was confirmed that chimeric retrotranscripts are generally composed of the copies of transcripts of mRNAs, ribosomal RNAs, or snRNAs fused to the 3’ site of L1 and of 5’ sites derived from nucleolar RNAs (Buzdin, Gogvadze et al. 2007). Most of RC-L1s are strongly methylated (Hata and Sakaki 1997) or silenced by the RNA interfering pathway (Soifer, Zaragoza et al. 2005), but the loss of methylation or silencing
can activate LINE-1, and such activity was shown to be linked to several diseases including cancers.

Several studies provide solid evidence that the insertion of LINE-1 into structural genes may play a role in the origin and/or progression of cancers. L1 retrotransposons were previously detected in significant amounts in breast cancer: in 7 out of 8 malignant cell lines and in 9 of 12 primary infiltrating ductal carcinomas (Bratthauer, Cardiff et al. 1994). Line-1 retrotransposons and ORF1p were also isolated and characterized in rat chloroleukemia cells (Kirilyuk, Tolstonog et al. 2008). Some of the early studies have shown L1 expression and ORF1p in human germ cell cancers (teratocarcinoma and choriocarcinoma cell lines) (Leibold, Swergold et al. 1990). A recent study demonstrated the up-regulation of LINE-1 together with another retrotransposon, SINE B1, at a very early stage of murine mammary tumorigenesis. Moreover, they reported that these retrotransposons were rapidly amplified during cancer progression (Gualtieri, Andreola et al. 2013). Similarly, LINE-1 quantification in sera of breast cancer patients was shown to be useful for detecting early-stage breast cancer, and the copy number was correlated with tumor size (Sunami, Vu et al. 2008). The insertion of a LINE-1 element into the c-Myc gene and the APC gene was shown in primary breast cancer and colorectal cancer, respectively (Morse, Rotherg et al. 1988; Miki, Nishisho et al. 1992).

Epigenetic alterations are well known to cause gene expression changes and affect genome stability. The loss of DNA methylation is usually associated with gene activation, while hypermethylation silences genes. Both events are associated with a cancer phenotype. Not surprisingly, the evidence of aberrant DNA methylation in LINE-
1 retroelements exists and correlates with the activity of transposons. CpG-rich L1 promoter hypomethylation leads to the activation of ORF1 sense transcription in chronic myeloid leukemia (CML) and is associated with a poorer prognosis for the cytogenic response to interferon and imatinib (Roman-Gomez, Jimenez-Velasco et al. 2005). Furthermore, a decrease in methylation levels of L1 has been shown to be associated with breast cancer risk in a dose-dependent manner (Deroo, Bolick et al. 2013). Similarly, the hypomethylation-induced activation of L1 has been reported in testicular tumor, prostate and hepatocellular carcinomas, and chronic lymphocytic leukemia (Bratthauer and Fanning 1992; Dante, Dante-Paire et al. 1992; Santourlidis, Florl et al. 1999; Lin, Hsieh et al. 2001).

Genomic instability that results from genetic and/or epigenetic changes and leads to carcinogenesis, is often associated with environmental factors. One example is radiation-induced genomic instability (RIGI). The cytotoxic effect of ionizing radiation relies on the ability to damage DNA. Radiation induces a variety of DNA lesions including damage to nucleotide bases, cross-linking, DNA single- and double-strand breaks (Little, 2000). Ionizing radiation can also alter DNA methylation. In rodents, radiation exposure was shown to cause dose-dependent and sex- and tissue-specific global genome hypomethylation. When C57/Bl mice were irradiated with X-rays in the dose range of 0.5-5 Gy, a dose-dependent loss of global methylation was detected in male spleen and female liver and spleen (Pogribny et al. 2004). Radiation-induced global hypomethylation in mice was correlated with a lower expression of both maintenance and de novo methyltransferases (Raiche et al. 2004). Similar results were found in the thymus of mice exposed to fractionated whole-body X-ray exposure. Global hypomethylation in
the thymus was coupled with decreased levels of DNMT1, DNMT3a and b, methyl CpG binding protein 2 (MeCP2) and methyl CpG binding domain protein 2 (MBD2) (Pogribny et al. 2005). DNA hypomethylation in mouse bone marrow was linked to radiation-induced leukemia (Giotopoulos et al. 2006). Similar molecular changes were found in the irradiated rat mammary tissues and were pronounced to cause genomic instability (Loree et al. 2006). When modulating global DNA hypomethylation in MCF-7/DOX cells with methylation agent SAM, cells were sensitized to radiation-induced apoptosis (Luzhna and Kovalchuk 2010). Ionizing radiation is also known to contribute to the mobilization of transposable elements. Retrotransposition of L1 was shown to be increased up to 4-fold in cultured cells subjected to gamma irradiation. The frequency of such retrotransposition was proportional to the level of phosphorylated H2AX foci (Farkash, Kao et al. 2006). Similarly, gamma irradiation induced a moderate increase in the Ty 1 element in S. cerevisiae (Sacerdot, Mercier et al. 2005). L1 retrotransposition events were shown to regulate gene expression after 5 Gy of X-ray exposure in EA.hy926 LINE-1 cell clones (Banaz-Yasar, Gedik et al. 2012).

All the evidence of the radiation-induced DNA methylation changes and LINE-1 retrotransposition suggests a possible effect of ionizing radiation on methylation status and/or activation of L1. Here, we describe the radiation-induced hypomethylation and activation of LINE-1 ORF1 in the rat mammary gland. The mobilization of LINE-1 in the irradiated tissues potentially contributes to genomic instability and cancer initiation.

MATERIALS AND METHODS

Animal models and irradiation conditions
Six-week-old intact female Long-Evans rats were obtained from Charles River (Wilmington, MA). The animals were housed two per cage in a temperature-controlled (24 °C) room in a 12-hour light-dark cycle and given *ad libitum* access to water and an NIH-31 pelleted diet. Six rats were randomly assigned to one of the following X-ray radiation treatment groups: 80kVp/0.1 Gy, 80kVp/1 Gy, 80kVp/2.5 Gy, 30kVp/0.1 Gy, and sham treated controls. Each group of animals was humanely sacrificed 6, 96 hours, and 4, 12 and 24 weeks after radiation treatment. The paired caudal inguinal mammary glands were excised. Tissue was frozen immediately in liquid nitrogen and stored at -80°C for subsequent analyses.

**Analysis of LINE-1 ORF1 methylation status by the COBRA assay**

The combined bisulfite restriction analysis (COBRA) assay consisted of bisulfate modification of genomic DNA, the subsequent polymerase chain reaction (PCR) amplification and digestion of PCR product with specific restriction endonucleases (Xiong and Laird 1997; Koturbash, Boyko et al. 2007). Genomic DNA was extracted using QiagenDNAeasy kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer’s protocol. Bisulfite conversion of genomic DNA was performed using EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) according to the manufacturer’s protocol. Further, the bisulfite-modified DNA was PCR amplified with primers corresponding to the regulatory region of rat LINE-1 ORF1 sequence (Tryndyak, Kovalchuk et al. 2007). The sense primer was 5’-TTT GGT GAG TTT GGG ATA- 3’ and the anti-sense primer was 5’-CTC AAA AAT ACC CAC CTA AC- 3’. PCR products were digested with Rsal and BstUI restriction endonucleases (New England Biolabs,
Beverly, MA) separated on 3% high resolution agarose gels (Sigma, St Louis, MO) and stained with ethidium bromide. The banding pattern analysis and the estimation of the ratio of intensities in digestion products and undigested bands were performed using both AlphaView SA 3.2.2. EXE and NIH ImageJ 1.63 Softwares.

Before conducting the above-mentioned assay, a methylation standard was made to check the untested primers. Briefly, fully methylated and unmethylated DNA was obtained and mixed creating a methylation gradient: 0%, 5%, 10%, 25%, 50%, 75% and 100% methylation. To obtain fully methylated DNA, genomic DNA was treated with SAM and SssI methylase (NEB, Ipswich, MA). The non-methylated DNA was obtained by amplifying genomic DNA using the WGA amplification kit (Sigma St Louis, MO) according to the manufacturer’s protocol. The COBRA assay was conducted on methylation gradient as described above.

**RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was isolated using the Illustra RNAspin mini kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Approximately 50–70 mg of mammary gland tissue was processed following the manufacturer’s instructions. Samples were eluted in Ultrapure DNase/RNase-free distilled water provided in the kit. RNA samples were quantified by ultraviolet spectroscopy (NanoDrop, Wilmington, DE).

Quantitative real-time PCR was performed to detect the expression level of LINE-1 ORF1 transcript. $\beta$-Actin was used as a reference gene. All reactions were performed using cDNA synthesized from 500 ng of RNA sample using the Bio-Rad iScript Select
cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Samples were stored at -20 °C for long-term storage and at 4 °C until used for the subsequent qRT-PCR reactions.

Primers were designed using the NCBI database and PrimerQuest (Integrated DNA Technologies, Inc., Coralville, IA). Primers were as follows: LINE-1 ORF1 forward primer 5’-AAG AAA CAC CTC CCG TCA CA-3’ and reverse primer 5’-CCT CCT TAT GTT GGG CTT TAC C-3’; beta-Actin reference gene forward primer 5’-CCT CTG AAC CCT AAG GCC AA-3’ and reverse primer 5’-AGC CTG GAT GGC TAC GTA CA-3’. Reactions were prepared using 1 μL of diluted cDNA, 10 pmol/μL of each forward and reverse primer and SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions. Samples were prepared in triplicate and were run on the Bio-Rad C1000 Thermal Cycler equipped with the CFX96 Real-Time System. The qRT-PCR protocol consisted of denaturation at 95 °C for 2 min; forty-three cycles of denaturation (95 °C, 5 sec) and annealing/extension (55 °C, 5 sec); and the final extension at 65 °C for 5 sec. For every set of primers, annealing temperature optimization, melting curve analysis and gel analysis of amplicon were performed. To evaluate PCR efficiency, the standard curve was established using series of cDNA dilutions. The data were captured and organized by the Bio-Rad CFX Manager 2.1 software (Bio-Rad Laboratories, Hercules, CA).

Quantification data from the Bio-Rad CFX Manager software were analyzed in Microsoft Excel using the Pfaffl method (Pfaffl 2001). Graphs showing a fold change from the sham group were created showing transcript regulation directions (up- or down-regulation).
Western immunoblotting

For protein isolation, 30-50 mg of mammary gland tissue were washed in PBS, lysed, and sonicated in 0.25 mL of 1% sodium dodecyl sulphate (SDS) containing protein inhibitors. The lysates were cleared using centrifugation. The protein content was determined using the Bradford protein determination assay (BioRad, Hercules, CA). Equal amounts of lysate protein were subsequently run on 10-12% SDS-polyacrylamide gels and transferred to PVDF membranes (GE Healthcare, Baied’Urfé, Québec).

Western immunoblotting was conducted using well-established protocols (Ertel, Verghese et al. 2006; Tryndyak, Kovalchuk et al. 2006). The membranes were incubated with antibodies against rabbit anti-Line-1 and mouse anti-c-Myc (1:100 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antibody binding was revealed through the incubation with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ) and the ECL Plus immunoblotting detection system (GE Healthcare, Piscataway, NJ). Chemiluminescence was detected using BioMax MR films (Eastman Kodak, New Haven, CT). The unaltered PVDF membranes were stained with Coomassie Blue (BioRad, Hercules, CA) to prove equal protein loading. Signals were quantified using NIH ImageJ 1.63 software and normalized to loading controls. Images are representative of two independent immunoblots. The results are presented as mean ± S.E.M. Statistical analyses were conducted using the student’s t-test and p-values less than 0.05 were considered significant.
RESULTS

The purpose of this study was to evaluate the effect of low and high doses of ionizing radiation on DNA methylation status of the LINE-1 transposable element in the rat mammary gland. The animals received a whole body exposure of different combinations of radiation energy levels and doses: low energy levels and low doses of X-rays (30kVp, 0.1 Gy), high energy levels and low doses (80kVp, 0.1 Gy), high energy levels and intermediate doses (80kVp, 1 Gy), and high energy levels and high doses (80kVp and 2.5 Gy). We examined the role of DNA methylation in the activation of LINE-1 transposon following radiation exposure. An increased expression of retroelements may lead to genomic instability and cancer initiation in the breast tissue that is often exposed to radiation for diagnostic and therapeutic procedures.

DNA methylation levels of LINE-1 ORF1 in the irradiated mammary gland

Methylation status of the LINE-1 regulatory region was determined by the COBRA assay (Koturbash, Boyko et al. 2007). This method is based on bisulfite modification of DNA - treatment of genomic DNA with bisulfite that converts unmethylated cytosines into uracils, while the methylated cytosines remain unchanged. The subsequent polymerase chain reaction (PCR) with primers corresponding to the regulatory region of rat LINE-1 results in the 163-nt fragment (Fig. 3.1, 3.2A). This fragment contains two sequences that are recognized by two endonucleases, BstUI and RsaI. BstUI digestion occurs at the recognition sequence CGCG only if both cytosines are methylated and thus protected from bisulfite conversion. Complete cleavage (in the case of complete methylation) results in two bands of 80 and 83 nt in length.
Unmethylated DNA (converted) would resist cleavage and contribute to the 163-nt band (Fig. 3.1, 3.2A). An RsaI recognition site, GTAC, can be formed from the GGCACG sequence when non-CpG cytosine is unmethylated (therefore, converted), while CpG cytosine is methylated. Non-CpG cytosine methylation is very rare, and the RsaI recognition site is influenced mainly by the methylation status of CpG cytosine. Cleavage of the 163-nt fragment generates 48- and 115-nt bands, while the loss of CpG cytosine methylation prevents the cleavage and contributes to the 163-nt band (Fig. 3.1, 3.2A).

In order to ensure the validity of the assay and check the untested primers, a DNA methylation gradient and a methylation standard were prepared (0 - 100 % methylation), and PCR products were digested by BstUI and RsaI enzymes. Figure 1 represents the methylation standard curves and the sizes of digested fragments. Both BstUI and RsaI restriction reactions show methylation-dependent digestion: if unmethylated (0 % methylation), it resulted only in the 163-nt bands, whereas an increase in methylation led to the appearance of the 80/83-nt (for BstUI) and 48-nt (for RsaI) fragments (Fig. 3.1). The intensity of the 163-nt bands gradually decreased with an increase in DNA methylation. According to the methylation standard, the method is plausible and valuable in determining methylation levels of LINE-1 before and post radiation treatment.

The COBRA assay revealed hypomethylation of BstUI recognition sequences 96 hours after radiation treatment (Fig. 3.2). A significantly lower cleavage of PCR products digested by BstUI enzyme was observed in the case of intermediate and high doses at a high energy level (80kVp, 1 Gy and 80kVp, 2.5 Gy) and low doses at a low energy level (30kVp, 0.1 Gy). Hypomethylation was also observed at low doses with high energy.
exposure (80kVp, 0.1 Gy) at 6-hour time point, but it did not persist with time. Although, such hypomethylation did not persist for longer than 4 weeks, it was indicative of immediate short-term LINE-1 reactivation which may contribute to genomic rearrangements and instability. Cytosine methylation at the Rsal site was not significant, and therefore data are not shown.

**LINE-1 ORF1 gene expression in the irradiated mammary gland**

The RT-PCR analysis was conducted in order to test a hypothesis that the loss of DNA methylation may be correlated with the expression level of LINE-1. The expression of LINE-1 ORF1 in the irradiated mammary gland tissue was shown to be increased compared to controls (Fig. 3.3). Interestingly, initially (at 6 hours after exposure), the transcription level of ORF1 in the irradiated tissues was decreased in comparison to controls. Starting at 96 hours, the expression level returned back to the control point and was significantly elevated in the 30kVp/ 0.1 Gy treatment group. The high expression level of ORF1 was noticed 12 and 24 weeks after exposure in the 80kVp/ 1 and 2.5 Gy and 30kVp/ 0.1 Gy treatment groups (Fig. 3.3). These data suggest that the short-term hypomethylation of LINE-1 could possibly lead to a later more prolonged increase in LINE-1 ORF1 gene expression.

**LINE-1 and c-myc protein levels in the irradiated mammary gland**

Having seen a pronounced and persistent LINE-1 gene expression, we proceeded with the detection of LINE-1 protein. A LINE-1 protein is an RNA-binding protein that has a high affinity to LINE-1 RNA, possesses endonuclease and reverse transcriptase activities, and forms ribonucleoprotein required for LINE-1 retrotransposition (Mathias,
We noted that the levels of LINE-1 protein were statistically significantly up-regulated 96 hours, 4 and 24 weeks after treatment with 30kVp and 0.1 Gy of X-ray and 24 weeks after treatment with 80kVp and 2.5 Gy of X-ray (Fig. 3.4). Such protein up-regulation is in agreement with RT-PCR results for the corresponding radiation doses and time points. Several studies demonstrated that hypomethylation-induced LINE-1 retrotransposition could activate certain oncogenes such as c-MYC (Morse, Rotherg et al. 1988). We found that c-MYC protein expression was elevated 96 hours and 24 weeks after treatment with 30kVp and 0.1 Gy of X-ray and 24 weeks after treatment with 80kVp and 2.5 Gy of X-ray (Fig. 3.4). The elevated c-MYC protein level in the rat mammary gland tissue has resulted from radiation exposure and is possibly linked to LINE-1 hypomethylation and reactivation.

DISCUSSION

A wide source of ionizing radiation exposure is delivered by medical diagnostic and therapeutic procedures (Cuzick 2005). Damage to DNA imposed by low and intermediate doses of ionizing radiation may initiate neoplastic development in a healthy mammary gland. The most critical types of damage caused by ionizing radiation are double-strand breaks (DSBs) in the DNA helix that can either force a damaged cell to programmed cell death (apoptosis) or can be repaired (Little 2000). However, if repair mechanisms fail, cancer induction can start. There is a wide spectrum of mechanisms of radiation-induced cancer initiation. The activation of mobile elements in response to radiation exposure is consistently discussed as a plausible mechanism of cancer etiology.
and development. For instance, it has been shown that gamma radiation and chemotherapeutic drugs are associated with the induction of SINE expression (Hagan and Rudin 2002). Because of their mutagenic activity, transposons pose a threat to genome integrity (Kidwell and Lisch 2000). LINE-1 expression is associated with DSB formation through L1-encoded endonuclease activity and may be a source of genotoxic stress in irradiated cells (Farkash, Kao et al. 2006; Wallace, Belancio et al. 2010). Extrachromosomal accumulation of L1 DNA was reported in HIV-1 infected primary CD4(+) cells, while an increased retrotransposition of L1 was shown for HIV-1 infected Jurkat cells which could lead to HIV-1-induced genomic instability (Jones, Song et al. 2013). Line-1 endonuclease creates DSBs allowing new Line-1 copies to integrate into DNA (Belgnaoui, Gosden et al. 2006). Using tagged RC-L1 clones in cultured cells, it has been shown that approximately 10% of LINE-1 insertions cause vast genomic deletions and chromosomal rearrangements that are a source of genomic instability (Gilbert, Lutz-Prigge et al. 2002; Symer, Connelly et al. 2002). Although it has been known that cellular stresses such as ionizing radiation activate LINE-1, the molecular mechanisms of LINE-1 activation are not fully elucidated. There is a possibility that DNA methylation changes induced by genotoxic stresses might contribute to LINE-1 activation in mammalian cells. Stribinskis and Ramos discussed that the epigenetic dysregulation of retroelements in the BaP-treated cells may contribute to carcinogen-induced mutations and genomic instability (Stribinskis and Ramos 2006). Similarly, hypomethylation of LINE-1 has been reported in many cancers, and it has been suggested to promote genomic instability and facilitate tumor progression (Kazazian and Goodier 2002; Roman-Gomez, Jimenez-Velasco et al. 2005).
Evidence presented in this report suggests that exposure to genotoxic ionizing radiation may involve the epigenetic activation of LINE-1 mobile elements in mammary tissue. The results of this study show the loss of CpG methylation in promoter region of LINE-1 in the rat mammary gland exposed to radiation. Interestingly, it was an early response (96 hours) to both high energy levels and intermediate-high doses and to low-energy levels and low doses. The methylation status of LINE-1 promoter returned to the control level and was not observed 12 and 24 weeks after treatment (Fig. 3.2). Several adaptive possibilities, such as changes in methyl group metabolism and the inactivation of DNA methyltransferases, could return the methylation status to its original level.

Nevertheless, short-term hypomethylation was associated with a long-term reactivation of LINE-1. According to the qRT-PCR analysis, the high elevation of LINE-1 ORF1 gene expression was observed and persisted for 12 and 24 weeks, regardless the fact that the methylation level of LINE-1 was restored at these time points (Fig. 3.3). Such a phenomenon is interesting on its own, but it is difficult to explain at the moment without further investigation. Considering that LINE-1 retrotransposon activation may lead to its migration and insertion into the genome, we assume that one or few copies could be inserted in a very active genome region and remain unmethylated there retaining a very high copying potential. To prove this suggestion, a further analysis of copying ability of LINE-1 has to be performed.

Radiation exposure has also led to the translation of LINE-1 element. The level of a 148-kDa LINE-1 protein was increased at 96 hours after treatment with low-energy-level and low dose radiation and remained high at 24 weeks after treatment (Fig. 3.4). Meanwhile, a significant elevation of LINE-1 protein was also detected at 24 weeks after
treatment with high energy/high dose radiation. The LINE-1 protein possesses an endonuclease and reverse transcriptase activities (Mathias, Scott et al. 1991; Feng, Moran et al. 1996). The ORF1 and ORF2 proteins associate with their encoding transcript, form a retrotransposition unit that allows for the retrotransposition and integration of LINE-1 into the genome (Belgnaoui, Gosden et al. 2006). The observed radiation-induced activation of LINE-1 protein may contribute to further insertion and activation of the LINE-1 element. The active Line-1 element can and does insert into genes (very often into proto-oncogenes) thus, changing their expression. Roman-Gomez and colleagues have demonstrated that epigenetic changes in the LINE-1 promoter alter the expression of c-MET oncogene that is highly expressed in CML patients (Roman-Gomez, Jimenez-Velasco et al. 2005). c-MYC activation that depends on LINE-1 insertion has been reported in breast cancer (Morse, Rotherg et al. 1988). Our results show an increased level of c-MYC protein in rat mammary tissue after radiation exposure (Fig. 3.4). As in the LINE-1 protein, it is the highest and lowest doses of radiation that cause c-MYC protein synthesis.

In conclusion, our results present the evidence that ionizing radiation decreases CpG methylation in the LINE-1 promoter following the up-regulation of LINE-1 RNA levels and increases the synthesis of LINE-1 protein. There was no specific radiation dose-dependent response. Both low and high doses/energy levels had a similar effect on LINE-1 activation. Such LINE-1 activation may be related to the activation of the c-MYC oncogene. These findings suggest that mammary tissue exposed to genotoxic radiation may develop genomic instability due to the epigenetic activation of mobile elements and initiate cancer development.
1. Created methylated genomic DNA by treating DNA with SAM and Sss1 methylase.

2. Created non-methylated DNA by amplifying DNA with WGA DNA polymerase.

3. Methylation gradient:
   - 0%  5%  10%  25%  50%  75%  100%

4. Bisulfite conversion and subsequent PCR
   - 163 nt

5. PCR product was cleaned and digested with BstUI and RsaI

Figure 3.1 A. Preparation of methylation gradient for testing primers for the COBRA assay. Steps of preparation of methylation gradient (0% - 100% methylation) from mixing fully unmethylated and fully methylated DNA. B. Methylation standard after digestion of bisulfite treated DNA with BstUI and RsaI restriction endonucleases. The higher the percentage of cut 163 bp fragment by BstUI, the higher the methylation status of DNA. The higher the percentage of uncut 163 bp fragment, the lower the methylation status of DNA.
Figure 3.2 CpG methylation of LINE-1 promoter in the mammary gland of irradiated rats determined by COBRA assay. A. PCR amplification of 163 bp from LINE-1 promoter. B. Methylation dependent retention of pre-existing BstU1 restriction sites. Unmethylated CpG cytosines (highlighted) at CGCG recognition sequence can be lost by bisulfite conversion, resulting in uncut 163 bp fragments. Methylation at both sites allows cleavage, resulting in 80/83 bp bands. C. Methylation dependent retention of cytosine (highlighted) at GGCACG sequence forms RsaI recognition site, leading to cleavage of 163 bp fragment into 48 and 115 bp fragments. Loss of methylation at CpG cytosine will prevent cleavage. D. Quantification of BSTU1 cut fragments by AlphaView, presented as mean values ± SD, n=4-6. * - significantly different from the respective control, p<0.05; ** - significantly different from the respective control, p<0.01, student’s t-test.
Figure 3.3 Fold change in levels of Line-1 ORF1 transcript detected by qRT-PCR. Each treatment group was compared to its corresponding control. β-actin was used as a reference gene (calculated by Pfaffl). * - significant, p<0.01; ** - significant, p<0.05; *** - significant, p<0.05 (Student’s t-test).
Figure 3.4 LINE-1 and c-MYC levels in rat mammary gland upon whole body irradiation. Protein levels relative to those of control non-irradiated animals are shown as Mean ± SE. * - p<0.05, student's t-test.
Main findings described in chapter 3

1. X-ray exposure has caused hypomethylation of ORF1 promoter in the rat mammary tissue, mainly 96 hours after 30 kVp/0.1 Gy, 80 kVp/1 Gy and 80 kVp/2.5 Gy treatments.

2. ORF1 gene expression has increased 96 hours after 30 kVp/0.1 Gy radiation treatment and 12-24 weeks after 30 kVp/0.1 Gy, 80 kVp/1 Gy and 80 kVp/2.5 Gy treatments.

3. Line-1 protein level has increased after 30 kVp/0.1 Gy and 80 kVp/2.5 Gy treatments.

4. C-myc protein level has increased after 30 kVp/0.1 Gy and 80 kVp/2.5 Gy treatments.
CHAPTER 4: HIGH AND LOW DOSE RADIATION EFFECTS ON MAMMARY
ADENOCARCINOMA CELLS – AN EPIGENETIC CONNECTION

Chapter 4 has been submitted in its entirety:

Luzhna L. and Kovalchuk O. High and low dose radiation effects on mammary
adenocarcinoma cells – an epigenetic connection. Oncoscience (in review)
ABSTRACT

The successful treatment of cancer, including breast cancer, depends largely on radiation therapy and proper diagnostics. The effect of ionizing radiation on cells and tissues depends on the radiation dose and energy, but there is insufficient evidence concerning how tumor cells respond to the low and high doses of radiation that are often used in medical diagnostic and treatment modalities. The purpose of this study was to investigate radiation-induced gene expression changes in a MCF-7 breast adenocarcinoma cell line. Using microarray technology tools, we were able to screen the differential gene expressions between various radiation doses applied to MCF-7 cells. Here, we report the substantial alteration in the expression level of genes after high dose treatment. In contrast, no dramatic gene expression alterations were noticed after the application of low and medium doses of radiation. In response to a high radiation dose, MCF-7 cells exhibited down-regulation of biological pathways such as cell cycle, DNA replication, and DNA repair and activation of the p53 pathway. Similar dose-dependent responses were seen on the epigenetic level, tested by a microRNA expression analysis. MicroRNA analysis showed dose-dependent radiation-induced microRNA expression alterations that were associated with cell cycle arrest and cell death. An increased rate of apoptosis was determined by an AnnexinV assay. The results of this study showed that high doses of radiation affect gene expression genetically and epigenetically, leading to alterations in cell cycle, DNA replication, and apoptosis.
INTRODUCTION

Ionizing radiation kills cells by damaging their DNA. Radiation induces a variety of DNA lesions, such as damage to nucleotide bases, cross-linking, and DNA single- and double-strand breaks (Little 2000). Radiation can damage normal cells as well as cancer cells and is often used in diagnostic and treatment medical procedures. Any use of ionizing radiation, therefore, must be carefully planned to minimize side effects and deliver optimal results. Diagnostic imaging procedures use low doses of radiation, whereas radiation therapy uses high energy radiation to shrink tumors. About half of all cancer patients receive radiotherapy during the course of their treatment, and all cancer patients are exposed to diagnostic-related radiation. Although benefits from the medical procedures greatly outweigh any potential low risk of harm, more evidence has been found to prove that harm from diagnostic X-rays is linked to an increased risk of cancer. This harm is correlated to the radiation dose absorbed (Linet, Slovis et al. 2012).

Radiation dose is the amount of energy absorbed by the body in radiation interactions. Different types of radiation may produce different biological effects, and the magnitude of the effect varies according to the dose rate (Linet, Slovis et al. 2012). Stochastic effects of radiation, such as cancer and hereditary effects are caused by mutations and other permanent changes in which a cell remains viable. The probability of such stochastic effects increases with dose (no threshold), but the severity of the outcome is not related to the dose (Hall and Giaccia 2006). Nevertheless, epidemiologic studies continue to reveal cancer risks associated with diagnostic radiologic procedures (Linet, Slovis et al. 2012). Oh and Koea provide an overview of radiation-related cancer risk.
associated with multiple computed tomographic scans required for follow up in colorectal patients. Of 36 studies analyzed in their review, 34 showed a positive association between medical imaging radiation and increased cancer risk, albeit radiation risk from low doses was uncertain (Oh and Koea 2013). Significant dose-response relationships were found for breast cancer risk for patients with tuberculosis who received fluoroscopy frequently (Boice, Preston et al. 1991; Howe and McLaughlin 1996). Furthermore, there is a statistical association between radiation doses and types of diagnostic X-ray examinations and chromosome translocation frequencies (Sigurdson, Bhatti et al. 2008; Bhatti, Doody et al. 2010), whereby high doses of radiation are more successful in killing cells, whereas low doses contribute to mutational events that lead to carcinogenesis.

Moreover, the vast majority of low dose radiation effect and radiation-induced cancer studies have been conducted on non-cancerous tissues. Very little is known about the effects of low-dose diagnostic radiation exposure in actual cancer cells and tissues. It is possible that low doses of radiation can contribute to the genomic instability of cancer cells, leading to an increase in malignancy and potentially making cancer cells resistant to further radiation with higher doses.

One of the major obstacles to successful cancer management is acquired resistance to radiation therapy. The mechanisms of such resistance have considerable clinical significance but are poorly defined. The limitation of radiotherapy is that solid tumor cells often become deficient in oxygen after radiation exposure. Such tumors can outgrow their blood supply, causing hypoxia (Harrison, Chadha et al. 2002). Under hypoxic conditions, cancer cells can become two to three times more resistant to
radiation. There are several extra-nuclear factors that cause resistance to radiation. The levels of IGF-IR and its substrate are elevated in ER-positive breast tumors and can be linked to with increased radio-resistance and cancer relapse (Bartucci, Morelli et al. 2001).

MCF-7 breast carcinoma cells are known to be resistant to radiation-induced apoptosis due to the lack of caspase-3, and apoptosis which is independent of cell cycle control (Essmann, Engels et al. 2004). Radioresistance is also common in chemoresistant cancer cells. For example, MCF-7/Pac and MCF-7/Doc were found to be radioresistant to γ-radiation, and MCF-7/DOX cells showed increased resistance to X-rays (Kars, Iseri et al. 2009; Luzhna, Golubov et al. 2013). According to Zhang and colleagues, lower doses of ionizing radiation led to inhibition of HIF-1 (transcription factor involved in the process of gene related hypoxic adaptation of neoplasm), whereas high doses increased HIF-1α, HPSE-1, EEGF, and CD31 levels in irradiated mice (Zhang, Jiang et al. 2014). Because response to ionizing radiation correlates with existence of oxygen that forms DNA-damaging free radicals, hypoxic regions in tumor require higher radiation doses to obtain the same damage as normoxic regions. Certain factors, including HIF-1α, improve tumor adaptation to hypoxia and are involved in radioresistance (Zhang, Jiang et al. 2014).

Based on the information in the literature, we concluded that the effect of ionizing radiation on cells and tissue is dependent on radiation dose and energy, but there is not enough evidence on how the tumor cells respond to low and high doses of radiation, which are often used in medical diagnostic and treatment modalities. Therefore, the aim
of this study was to investigate the response of MCF-7 breast carcinoma cells to low, medium, and high doses of X-rays and to define any radiation-associated changes in gene expression and apoptosis levels.

MATERIALS AND METHODS

Cell line and cell culture conditions

The MCF-7 human breast adenocarcinoma cell line was previously developed and described elsewhere (Chekhun, Lukyanova et al. 2007; Kovalchuk, Filkowski et al. 2008). Cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM /F-12) with 2.5 mM L-Glutamine, without HEPES and Phenol Red (HyClone, Logan, UT), supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), in the presence of antibiotics 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich Chemical Co., St. Louis, MO), and in a 5% CO₂ atmosphere at 37°C. Cells were harvested for analyses by trypsination (Chekhun, Lukyanova et al. 2007; Kovalchuk, Filkowski et al. 2008).

Irradiation conditions

Cells were irradiated at 60% confluency in Dulbecco's Modified Eagle's Medium (DMEM). Three radiation doses (0.05, 0.5, and 5 Gy, 90 kVp, 5 mA) were applied to check the cellular radiation responses. Unirradiated cells served as the control. Cells were harvested 24 hours and 48 hours after irradiation. All the cells were tested in triplicate. The experiments were independently reproduced twice.
Whole-genome gene expression profiling

RNA isolation

Total RNA was isolated using the Illustra RNAspin Mini kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Approximately $5 \times 10^6$ cultured cells were processed following the manufacturer's instructions. Samples were eluted in Ultrapure DNase/RNase-free distilled water, which was provided in the kit. RNA samples were quantified using ultraviolet spectroscopy (NanoDrop, Wilmington, DE) and were further assessed for RNA integrity (RIN) on the Aglient 2100 Bioanalyzer (Santa Clara, CA) using the RNA Nano-chip Kit. RNA samples with RIN values of seven or better were used for further analysis.

Library preparation

cRNA was created using the Ambion Illumina TotalPrep RNA Amplification Kit (Applied Biosystems, Carlsbad, CA) with an input of 500 ng of total RNA per sample. Briefly, oligo-dT primers were used to synthesize first strand cDNA containing a phage T7 promoter sequence. Single-stranded cDNA was converted into a double-stranded DNA template via DNA polymerase. RNase H simultaneously acted to degrade the RNA. Samples of cDNA were purified in filter cartridges to remove excess RNA, primers, enzymes, and salts. The recovered cDNA was subjected to in vitro transcription using biotinylated UTPs. This step created, labeled, and amplified cRNA. A final purification step removed unincorporated NTPs, salts, inorganic phosphates, and enzymes, which prepared the samples for hybridization.
Hybridization and detection

Illumina's direct hybridization assay kit was used to process samples according to the manufacturer's protocol (Illumina, San Diego, CA). Overnight, 750 ng from each cRNA sample was hybridized into the Illumina HumanHT-12_v4 Whole Genome Expression BeadChip arrays. Afterward, a 10-minute incubation with a supplied wash buffer at 55°C preceded a 5-minute room-temperature wash. The arrays were incubated in 100% ethanol for 10 minutes. A second room-temperature wash lasted two minutes with gentle shaking, which completed this high stringency wash step. The arrays were blocked with a buffer for 10 minutes and washed before a 10-minute steptavidin-Cy3 (1:1000) probing. After a five-minute wash at room temperature, the BeadChips were dried and imaged. Six controls were also built into the Whole-Genome Gene Expression Direct Hybridization Assay system to cover aspects of the array experiments, including controls for the biological specimen (14 probes for housekeeping controls), three controls for hybridization (six probes for Cy3-labeled hybridization, four probes for low stringency hybridization, and one probe for high stringency hybridization), signal generation (two probes for biotin control), and approximately 800 probes for negative controls on an eight-sample BeadChip. The arrays were scanned on the iScan platform (Illumina), and data were normalized and scrutinized using Illumina BeadStudio Software.

BeadChip statistical analysis and data processing

The false discovery rate (FDR) was controlled using the Benjamini-Hochberg method. The Illumina Custom Model took the FDR into account and was used to analyze
the data. Differential gene expression (at least a 0.6-fold change) from control non-radiated cells was determined to be statistically significant if the $p$ value after the Benjamini-Hochberg method adjustment was lower than 0.05. The values were transformed to show a log2 scale.

Lists of regulated transcripts were inserted into the web-based DAVID Bioinformatics Resources 6.7 (NIAID/NIH) Functional Annotation Tool (Huang da, Sherman et al. 2009; Huang da, Sherman et al. 2009). This program was used to group genes into functionally relevant categories: metabolic processes, response to stimulus/stress, immune response, apoptosis, and cell cycle processes.

**Quantitative real-time PCR**

Quantitative real-time PCR was performed to confirm the Whole-Genome Gene Expression results for the regulation direction (either up or down) of select genes. Six genes (aurora B, cyclin A, GADD45G, polymerases A, D, and E) were selected from the gene list of significantly differentially expressed transcripts, representing a preliminary review of the acquired gene expression data. 18SrRNA was used as a reference gene. All the reactions were performed using cDNA synthesized from the same RNA extraction as the BeadChip experiments, and 500 ng of the sample was used for the Bio-Rad iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Samples were stored at -20°C for long-term storage and at 4°C until they were used for subsequent qRT-PCR reactions.
Primers were designed using the NCBI database and PrimerQuest (Integrated DNA Technologies, Inc, Coralville, IA). The following primers were designed: hAURKB forward primer (5’-TGA GGA GGA AGA CAA TGT GTG GCA-3’) and reverse primer (5’-AGG TCT CGT TGT GTG ATG AAC TCT TCT-3’); 18SrRNA reference gene primers (5’-GTC AAG TTC GAC CGT CTT CT-3’ and 5’-AGC TTG CGT TGA TTA AGT CC-3’); CCNA2 forward primer (5’-ATG AGC ATG TCA CCG TTC CTC CTT-3’) and reverse primer (5’-TCA GCT GGC TTC TTC TGA GCT TCT-3’); hGADD45G forward primer (5’-TGC TGC GAG AAC GAC ATC GAC ATA-3’) and reverse primer (5’-TCG AAA TGA GGA TGC AGT GCA GGT-3’); hPOLA1 forward primer (5’-GGC AAT GGC TTT GAA ACC AGA CCT-3’) and reverse primer (5’-ATG CTG AAA GCC ATC ACA CTC TT-3’); hPOLD1 forward primer (5’-AAC CTG TGT TAC ACC ACG CTC CTT-3’) and reverse primer (5’-TCC GCA CTG AGG TCT TCA CAA ACT-3’); hPOLE forward primer (5’-AGA TTG TGC AGA TCA GCG AGA CCA-3’) and reverse primer (5’-TTA CCT TGC GAT ACG AAG CAC CCT-3’). Reactions were prepared using 1 μL of diluted cDNA, 10 pmol/μL of each forward and reverse primer, and Ssofast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA), prepared according to the manufacturer's instructions. Samples were prepared in triplicate and were run on the Bio-Rad C1000 Thermal Cycler equipped with the CFX96 Real-Time System. The qRT-PCR protocol consisted of denaturation at 95°C for two minutes; 43 cycles of denaturation (95°C, 5 seconds) and annealing/extension (55°C, 5 seconds); and a final extension at 65°C for five seconds. For every set of primers, annealing temperature optimization, melting curve analysis, and a gel analysis of the amplicon were performed. To evaluate PCR efficiency, a standard curve was established using a series of cDNA dilutions. Data
was captured and organized using Bio-Rad CFX Manager 2.1 software (Bio-Rad Laboratories, Hercules, CA).

qRT-PCR statistical analysis

Quantification data from the Bio-Rad CFX Manager software was analyzed using the Pfaffl method in Microsoft Excel (Pfaffl 2001). Graphs showing a fold change from the control group were created, and transcript regulation directions (up- or downregulation) were matched to the Whole-Genome Gene Expression results.

miRNA microarray expression analysis

Total RNA from MCF-7 cells was isolated using Trizol reagent (Invitrogen, Burlington, ON) according to the manufacturer's instructions. One ug of total extracted RNA represented as two repeats per experimental group was sent to LC Sciences (Austin, TX) for miRNA microarray analysis.

The Annexin V assay

For the early detection of apoptosis, an Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) was used according to the manufacturer's protocol. Cells were grown and irradiated as previously described (Section 2.2). The analysis was performed 24 and 48 hours after radiation exposure. Cells were harvested, washed with PBS, resuspended in a 1X binding buffer, stained with Annexin V and propidium iodide for 15 minutes at 25 °C in the dark, and analyzed using flow cytometry within one hour at the Flow Cytometry Core Facility (University of Calgary, Calgary, AB). The results were represented as a percentage of gated Annexin V positive cells.
RESULTS

Effect of low, medium, and high doses of radiation on whole genome gene expression in MCF-7 cells

Isolated RNA from MCF-7 breast adenocarcinoma cells was used for gene expression profiling. A drastic difference in radiation-induced gene expression changes was discovered between the doses applied. Only high doses of X-ray exposure led to dramatic alterations in gene expression, whereas low and medium doses did not affect gene expression. A total of 2, 10, and 777 genes were affected by the 0.05, 0.5, and 5 Gy of radiation, respectively (Fig. 4.1). Further, we evaluated the 777 genes that changed their expression level: 437 genes were upregulated and 340 genes were downregulated. With the help of the DAVID functional annotation array analysis tools, we were able to identify and group the evaluated genes according to their function and possible role in certain pathways. Subsequently, genes with similar or identical functions were grouped together and, based on their expression changes, the role of certain pathways in radiation response was evaluated (Table 4.1). Twenty-nine cell cycle genes and twenty-one genes responsible for DNA replication were downregulated (Table 4.1, Fig. 4.2). The primary repair processes were shut down by the decreased expression of key genes. MCF-7 cells lost their MMR, NER, BER, and HR due to the downregulation of the 9, 12, 8, and 6 pathway genes, respectively (Table 4.1). These changes usually lead to cell death. Moreover, the genes responsible for cell death from the p53 signaling pathway were upregulated (Table 4.1).
The validity of gene expression profiling was confirmed by qRT-PCR for the genes with the most change and the greatest radiation response. Therefore, the primary targets for qRT-PCR were DNA polymerases A, D, and E, which are the key components in DNA replication and DNA repair pathways, and cyclin A, GADD45G, and aurora B, which play an important role in cell cycle and p53 signaling pathways.

Aurora B is a protein kinase that functions through the attachment of the mitotic spindle to the centromere and provides equal chromosome movement and segregation during mitosis. The level of \textit{AURKB} transcript levels gradually and significantly decreased in the MCF-7 cells after X-ray treatment with 0.5 and 5 Gy (Fig. 4.3). Similar to \textit{AURKB}, cyclin A (\textit{CCNA}) was downregulated in MCF-7 cells after a high dose of X-ray exposure (Fig. 4.3). Because cyclin A binds to S phase Cdk2 and is required for the cell to progress through the S phase, the deficit of cyclin A may contribute to cell cycle arrest. GADD45G is a growth arrest and DNA-damage-inducible protein which levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents. The protein encoded with \textit{GADD45G} responds to environmental stresses by mediating the activation of the p38/JNK pathway. Both 0.5 and 5 Gy of X-rays caused an increase in \textit{GADD45G} transcript levels in MCF-7 cells, which is in contrast to levels in control cells (Fig. 4.3). All three polymerases (A, D, and E) were significantly downregulated in response to a 5 Gy radiation treatment, disabling the polymerization of deoxyribonucleotides into a DNA strand.
miRNA expression in irradiated MCF-7 breast adenocarcinoma cells

In search of possible regulators of gene expression, we proceeded to analyze the role of miRNAs in the radiation response of MCF-7 cells. miRNAs involve epigenetic control of gene expression regulation through an RNA interference pathway. miRNAs negatively affect the levels of their target transcripts and the levels of proteins encoded by these transcripts. In this way, miRNAs contribute to gene silencing, and changes in miRNA expression are common in cancers and in response to radiation.

We identified that one, three, and six miRNAs were significantly changed after exposure to 0.05, 0.5, and 5 Gy of X-rays, respectively (Table 4.2). miR-106a was significantly downregulated in a dose-dependent manner after all three radiation doses. Its putative target is RB1 protein that regulates cell cycle and promotes cell cycle arrest. Five Gy of radiation led to downregulation of miR-17 and miR-106b, which target BIM and p21 apoptosis inducing factors, whereas miR-23b and miR-149, targeting NOTCH (cell signaling pathway) and AKT (promotes proliferation), were upregulated (Table 4.2). Thus, changes in miRNAs expression seem to contribute to cell cycle arrest and initiation of apoptosis in MCF-7 cells exposed to ionizing radiation, influencing cellular stress response, and this response is dose dependent.

Radiation-induced apoptosis in MCF-7 breast adenocarcinoma cells

IR exposure is known to induce apoptotic cell death in many irradiated cells. Therefore, we analyzed the levels of IR-induced apoptosis in MCF-7 cells. Early apoptosis is characterized by various changes in the cellular plasma membrane; the
primary change is the translocation of phosphatidylserine (PS) from the inner layer to the surface of the membrane. Annexin V possesses a high affinity to PS, and this allows for the early detection of apoptotic changes (Vermes, Haanen et al. 1995). Here, we analyzed IR-induced apoptosis using an Annexin V assay.

Figure 4.4 shows that MCF-7 cells began to undergo early apoptosis 48 hours after irradiation with 5 Gy. Low and medium doses did not cause apoptosis levels different from the control level (Fig. 4.4). In contrast, we found a 1.81-fold increase in Annexin V positive cells 48 hours after exposure to the high dose. These data indicate that MCF-7 breast adenocarcinoma cells can withstand low and medium doses of ionizing radiation and only exhibit apoptotic response to high doses.

DISCUSSION

Successful treatment of cancer, including breast cancer, is largely dependent on radiation therapy and proper diagnostics. Radiation therapy is widely used in combination with other treatment modalities, such as surgery, chemotherapy, and hormonal therapy, for treatment of initial and advanced cancers (Koukourakis, Koukouraki et al. 1999; Chakravarthy, Nicholson et al. 2000). Choosing the appropriate radiation dose and timing for radiotherapy is vital for receiving the optimal result. Each type of cancer has different radiosensitivity (Nunez, McMillan et al. 1996). Breast cancers are ranked as moderately radiosensitive to radioresistant, therefore requiring higher doses of radiation (45–60 Gy) to achieve radical cure than many other tumor types. The total dose is divided into 1.8–2 Gy fractions per day for several weeks (Tutt and Yarnold 2006). There is no data on the effect of low and medium diagnostic doses that might potentially contribute to the
severity of malignancy. Overall, data on the molecular mechanisms of radiation response to different doses of ionizing radiation on tumor cells are scarce.

The purpose of this study was to investigate the radiation-induced gene expression changes in the MCF-7 breast adenocarcinoma cell line. Using microarray technology tools, we were able to screen the differential gene expressions between various doses applied to MCF-7 cells. Here, we report the substantial alteration in the expression level of genes after high-dose treatment. In contrast, no dramatic gene expression alterations were noticed after low and medium doses of radiation application. We believe that the ability of the cancer cells to retain their gene expression potential at a constant level after applying low and medium doses of DNA-damaging radiation insults mean that these doses of ionizing radiation neither contribute to further genomic instability that might result in more severe malignancies nor cause cell death. Gene expression profiling showed that the expression level of more than 700 genes was changed in the MCF-7 cell line due to 5 Gy X-rays (Fig. 4.1). MCF-7 cells exhibited the expected downregulation of biological pathways, such as cell cycle, DNA replication, DNA repair, and the activation of the p53 pathway (Table 4.1). Twenty-nine cell cycle regulators were downregulated, which led to cell cycle shutdown. These genes were encoded for cyclins (A2, B1, B2), cyclin-dependant kinases (CDK2, CDK4), cell division cycle proteins (CDC20, CDC25A, CDC7), E2F transcription factors (E2F2, E2F4), mitotic polo-like kinase PLK1, checkpoint kinase CHEK1, mini-chromosome maintenance complex components (MCM 2,3,4,5,6,7), and other cell cycle-associated proteins.
The upregulation of the transforming growth factor beta (TGFB) and growth arrest and DNA damage-inducible factors (GADD45A and GADD45G) also contributed to cell cycle deactivation. Obviously, cell cycle deactivation paralleled inhibited DNA replication. Twenty-one genes involved in replication were downregulated: DNA polymerases (A1, A2, D1, D2, E, E2, E3 (except for D4, which was upregulated)), replication factors (RFC2,3,4,5), replication protein (RPA2), mini-chromosome maintenance complex components (MCM 2,3,4,5,6,7), ligase 1, endonuclease FEN, and ribonuclease H2 (RNASEH2A) (Fig. 4.2).

A specialized DNA damage response was initiated through the activation of the p53 pathway due to the overexpression of BCL2-associated X protein (BAX), damage-specific DNA-binding protein (DDB2), sestrin1 (SESN1), and growth arrest and DNA damage-inducible factors (GADD45A and GADD45G). DNA repair processes were downregulated primarily due to the decrease in the expression of specific repair polymerases and replication factors. For instance, base excision repair downregulation was caused by a low expression of polymerases (D1, D2, E, E2, E3), uracil-DNA glycosylase (UNG), ligase 1 (LIG1), and endonuclease (FEN1); NER deficiency was due to the same polymerases and ligase 1, as well as replication factors (RFC2,3,4,5) and RPA2; MMR deactivation was caused by a low level of MSH6, polymerases D1 and D2, LIG1, RPA2, RFC2,3,4,5, and exonuclease 1 (EXO1); and decreased homologous recombination was caused by low expression levels of RAD54L, XRCC3, polymerases D1 and D2, RPA2, Bloom syndrome, RecQ helicase-like (BLM), and topoisomerase (TOP3A).
Gene expression profiling data were confirmed through the qRT-PCR analysis of six genes that were changed into MCF-7 cells after radiation treatment. Polymerases A, D, and E were involved in most of the biological processes that were affected in MCF-7 cells after radiation exposure (Fig. 4.2, 4.3). As GADD45G, cyclin A, and aurora B are involved in DNA damage responses, cell cycle, and cell division, their expression levels were of great interest to us.

Members of the aurora kinases family have been actively studied as mitotic progression targets in cancer studies. Mutations associated with aurora gene amplification were reported in human cancers (Cahill, Lengauer et al. 1998). Tumor development and progression due to aberrant chromosomal segregation and aneuploidy is a common outcome of the misregulation of the aurora B function (Nguyen, Makitalo et al. 2009).

Inhibition of aurora B during the fractionated radiation treatment suppressed the repopulation of human cancer cells (Sak, Stuschke et al. 2012). Similarly, 5-Gy X-rays caused a significant downregulation of aurora B in drug-sensitive cell lines, which was correlated with slower mitotic progression and the suppressed repopulation of the cells. Cyclin A expression was also decreased, which may be associated with a lower DNA replication status and suppressed cell cycle progression. In addition, GADD45G, which is a member of growth arrest and DNA-damage inducible genes, was over-expressed after both 0.5 and 5 Gy of irradiation (Fig. 4.3). This indicates the existence of radiation stress in the cells, which can result in cell cycle arrest, senescence, and apoptosis (Liebermann, Tront et al. 2011).
Significant downregulation of polymerases A, D, and E confirms the suppression of DNA replication and DNA repair processes. Overall, gene expression profiling and qRT-PCR analysis showed a strong response in MCF-7 cells to high dose of ionizing radiation, allowing us to conclude that these cells were high dose radiation-sensitive. In contrast, cells did not respond to low and medium doses of X-rays on the gene expression level, which signifies that they are low-dose radioresistant.

Similar dose-dependent response was seen on the epigenetic level tested by the microRNA expression analysis. Radiation-induced changes in miRNA expression usually lead to changes in the synthesis of proteins involved in main cellular biological pathways. As per Table 4.2, validated targets of misregulated miRNAs fall in cell cycle and apoptosis categories (Table 4.2). For instance, downregulation of miR-106a may inhibit cell proliferation by activation of RB1 tumor suppressor. RB1 is a transcriptional repressor of E2F1 and, when active, leads to cell cycle arrest. Activated transcription of RB1, together with p21 and p16, was shown to suppress tumor cell growth (Chano, Ikebuchi et al. 2010). Another study has reported that inactive RB1 pathway, a hallmark of cancer, is associated with accumulation of Akt oncogene (El-Naggar, Liu et al. 2009). As we can see from Table 4.2, Akt is a validated target of the miR-149, which was upregulated after 5 Gy of X-rays. Akt kinase regulates multiple biological processes such as proliferation, cell survival, growth, and angiogenesis; therefore, its potential inactivation by epigenetic miRNA mechanism might lead to cell death after high dose radiation treatment. Similarly, p21, a cyclin-dependent kinase inhibitor, is a target of miR-17 and miR-106b (Table 4.2). P21 blocks cell cycle progression in response to DNA damage and was shown to be activated after radiation exposure (Kim, Cho et al. 2004).
Another target of miR-17 is pro-apoptotic factor BIM of Bcl-2 family. BIM induces anoikis through a caspase-mediated pathway and is known to be activated after ionizing radiation exposure (Yang, Xia et al. 2006). Overall, miRNA analysis has shown dose-dependent radiation-induced miR expression alterations that are associated with cell cycle arrest and cell death. An increased rate of apoptosis was determined by Annexin V assay (Fig. 4.4). Only a high dose (5 Gy) of radiation led to early apoptosis 48 hours after radiation treatment.

The results of this study show that high doses of radiation affect gene expression genetically and epigenetically, leading to alterations in cell cycle, DNA replication, and apoptosis. Further investigation is required to reveal exact molecular mechanisms of such alterations, which would enable the improvement of cancer treatment methods and radiosensitivity.
Figure 4.1 Gene expression profiling of MCF-7 breast adenocarcinoma cells. The Venn diagram shows the number of significantly changed genes in the MCF-7 cell line upon low (0.05 Gy), medium (0.5 Gy) and high (5 Gy) doses of radiation in comparison to their corresponding un-irradiated controls, as identified by the gene expression profiling analysis.
Table 4.1 The significantly altered KEGG pathways in MCF-7 cells after 5 Gy of X-ray treatment in comparison to the corresponding untreated controls. In this table, the pathway significance (%) is defined as the ratio of gene alterations that similarly affect a certain pathway (either up- or down-regulate) to the total number of altered genes in the pathway. “↑” – the pathway is up-regulated; “↓” – the pathway is down-regulated.

<table>
<thead>
<tr>
<th>Pathways</th>
<th># Genes changed</th>
<th>Pathway direction and confidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA replication</td>
<td>21</td>
<td>↓95.2%</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>29</td>
<td>↓96.6%</td>
</tr>
<tr>
<td>Nucleotide excision repair</td>
<td>12</td>
<td>↓66.7%</td>
</tr>
<tr>
<td>Mismatch repair</td>
<td>9</td>
<td>↓88.9%</td>
</tr>
<tr>
<td>p53 signaling pathway</td>
<td>12</td>
<td>↑83.3%</td>
</tr>
<tr>
<td>Glutathione metabolism</td>
<td>10</td>
<td>↑88.9%</td>
</tr>
<tr>
<td>Base excision repair</td>
<td>8</td>
<td>↓87.5%</td>
</tr>
<tr>
<td>Oocyte meiosis</td>
<td>13</td>
<td>↓84.6%</td>
</tr>
<tr>
<td>Homologous recombination</td>
<td>6</td>
<td>83.3%</td>
</tr>
<tr>
<td>Arginine and proline metabolism</td>
<td>8</td>
<td>↑87.5%</td>
</tr>
<tr>
<td>Pyrimidine metabolism</td>
<td>10</td>
<td>↓80.0%</td>
</tr>
<tr>
<td>Other glycan degradation</td>
<td>4</td>
<td>↑100%</td>
</tr>
<tr>
<td>Progesterone-mediated oocyte maturation</td>
<td>8</td>
<td>↓85.7%</td>
</tr>
</tbody>
</table>
Figure 4.2 The KEGG DNA replication pathway. All encircled genes were down-regulated.
Figure 4.3 Altered levels of gene transcripts of aurora B, cyclin A, Gad45G and polymerases A, D, E as detected by RT-PCR. Data are shown as fold changes to respective controls. Each treatment group was compared to its corresponding control; 18SrRNA was used as a reference gene (calculated by Pfaffl). P-values (in tables below the graphs) were calculated by Student’s t-test.
Table 4.2 Radiation-induced microRNA expression changes in MCF-7 cells. Relative miR expression values are represented in folds in the irradiated cells in comparison to non-irradiated control cells as analyzed by miRNA microarray. Significance of differences was analyzed by the student’s t-test.

<table>
<thead>
<tr>
<th>miRNA changed</th>
<th>Fold, 0.05 Gy</th>
<th>Fold, 0.5 Gy</th>
<th>Fold, 5 Gy</th>
<th>Validated targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>23b</td>
<td></td>
<td>0.48</td>
<td></td>
<td>Notch</td>
</tr>
<tr>
<td>149</td>
<td></td>
<td>1.77</td>
<td></td>
<td>AKT</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>-0.85</td>
<td></td>
<td>BIM, p21, VEGF</td>
</tr>
<tr>
<td>106b</td>
<td></td>
<td>-0.72</td>
<td>-0.93</td>
<td>VEGF, RB1</td>
</tr>
<tr>
<td>106a</td>
<td>-0.25</td>
<td>-0.42</td>
<td>-0.93</td>
<td>VEGF, RB1</td>
</tr>
<tr>
<td>20a</td>
<td></td>
<td>-0.91</td>
<td></td>
<td>VEGF</td>
</tr>
<tr>
<td>let7a</td>
<td></td>
<td>1.14</td>
<td></td>
<td>Dicer</td>
</tr>
<tr>
<td>let7b</td>
<td></td>
<td>0.66</td>
<td></td>
<td>CDK6</td>
</tr>
</tbody>
</table>
Figure 4.4 Radiation-induced apoptosis in MCF-7 breast adenocarcinoma cells. The number of cells in early apoptosis was measured using Annexin V-FITC assay for control cells (CT) and cells irradiated with 0.05 Gy, 0.5 Gy and 5 Gy of X-rays 24 and 48 hours post exposure. The results are presented as mean values ±S.E.M., n=3. * - significantly different from respective control, p<0.05, student’s t-test.
Main findings described in chapter 4:

1. Gene expression profile has been altered in response to the high dose of radiation.

2. Most of the gene expression changes affected cell cycle, DNA replication and repair and apoptosis pathways.

3. Similar to gene expression changes, miRNA profile has been mainly altered after the highest dose of radiation and affected cell cycle and apoptosis processes.

4. MCF-7 cells showed highest rate of early apoptosis 48 hours upon 5 Gy of radiation.
CHAPTER 5: ALTERED RADIATION RESPONSES OF BREAST CANCER
CELLS RESISTANT TO HORMONAL THERAPY

Chapter 5 has been accepted for publication in its entirety:

ABSTRACT

Endocrine therapy agents (the selective estrogen receptor (ER) modulators such as tamoxifen or selective ER down-regulators such as ICI 182,780) are key treatment regimens for hormone receptor-positive breast cancers. While these drugs are very effective in controlling ER-positive breast cancer, many tumors that initially respond well to treatment often acquire drug resistance, which is a major clinical problem. In clinical practice, hormonal therapy agents are commonly used in combination or sequence with radiation therapy. Tamoxifen treatment and radiotherapy improve both local tumor control and patient survival. However, tamoxifen treatment may render cancer cells less responsive to radiation therapy.

Only a handful of data exist on the effects of radiation on cells resistant to hormonal therapy agents. These scarce data show that cells that were resistant to tamoxifen were also resistant to radiation. Yet, the existence and mechanisms of endocrine therapy and radiation therapy cross-resistance need to be established.

Here, we for the first time examined and compared radiation responses of MCF-7 breast adenocarcinoma cells (MCF-7/S0.5) and two antiestrogen resistant cell lines derived from MCF-7/S0.5: the tamoxifen resistant MCF-7/TAM\textsuperscript{R}-1 and ICI 182,780 resistant MCF-7/182R\textsuperscript{-6} cell lines. Specifically, we analyzed the radiation-induced changes in the expression of genes involved in DNA damage, apoptosis, and cell cycle regulation. We found that the tamoxifen resistant cell line in contrast to the parental and ICI 182,780-resistant cell lines displayed a blunted radiation-induced decrease in the expression of genes involved in DNA repair. Furthermore, we show that MCF-7/TAM\textsuperscript{R}-1 cells were less susceptible to irradiation than MCF-7/S0.5. These data indicate that
tamoxifen resistant breast cancer cells have a reduced sensitivity to radiation treatment. The current study may therefore serve as a roadmap to the future analysis of the mechanisms of cross-resistance between hormonal therapy and radiation.

INTRODUCTION

Endocrine therapy is a widely accepted treatment of choice for hormone receptor-positive breast cancers in early stages and during advanced metastasis (Moy and Goss 2006). Women with estrogen receptor- (ER) and/or progesterone receptor-positive breast cancers are the best candidates for hormone therapy (Gonzalez-Malerva, Park et al. 2011). The ERα-positive normal breast cells may produce growth factors that stimulate the proliferation of neighboring cells leading to breast cancer development. In contrast, ERβ is essential for breast tissue differentiation, and its loss is associated with breast carcinogenesis (Riggins, Schrecengost et al. 2007). The selective estrogen receptor modulators (SERMs) such as Tamoxifen bind to the ligand-binding domain (LBD) of ER preventing its stimulation by estrogen, while the selective estrogen receptor down-regulators (SERDs) such as ICI 182,780 (Fulvestrant, Faslodex) bind, block and increase the degradation of ER (Riggins, Schrecengost et al. 2007; Robertson 2007). Both drugs are currently established as effective treatment therapy with beneficial outcomes. Unfortunately, in the case of advanced disease, acquired resistance to both drugs inevitably develops, which is a major clinical problem (Hutcheson, Knowlden et al. 2003; Sommer, Hoffmann et al. 2003; Ring and Dowsett 2004; Nicholson, Hutcheson et al. 2005). Drug resistance is usually accompanied with an aggressive cell behavior and invasiveness. The evidence exists that the main mechanism of hormone therapy
resistance is the deregulation of growth factor-signaling cascades. The over-expression of
growth factors, their receptors and downstream signaling elements promotes hormone
Thrane, Lykkesfeldt et al. 2013). Also, a long-term estrogen-deprived tumor cells may
adapt to low levels of estrogen by increasing their sensitivity to it (Masamura, Santner et
al. 1995). Such enhanced sensitivity to estrogen may result from the activation of several
signaling pathways such as RAS, RAF, MEK and MAPK (Song, McPherson et al. 2002;
Martin, Farmer et al. 2003). Moreover, it has been shown that tamoxifen- and
fulvestrant- resistant MCF-7 cells overexpress receptors in the HER family, e.g., EGFR
and HER2 (Hutcheson, Knowlden et al. 2003; Sommer, Hoffmann et al. 2003;
Nicholson, Hutcheson et al. 2004; Ring and Dowsett 2004; Frogne, Benjaminsen et al.
2009; Thrane, Lykkesfeldt et al. 2013). The overexpressed EGFR and HER2 are well
known to recruit MAPK, AKT and PKC signaling cascades (Bonni, Brunet et al. 1999;
Gibson, Tu et al. 1999; Campbell, Bhat-Nakshatri et al. 2001).

The combination of hormone therapy and radiation is widely used in clinical
practice. The application of tamoxifen and radiotherapy is believed to improve both local
control and patient survival (Azria, Lemanski et al. 2004; Fodor 2006). Nevertheless, a
suspicion also exists that tamoxifen may render cancer cells less responsive to
radiotherapy by providing a protective effect against radiation. Early studies on cell
culture have shown that tamoxifen causes an arrest of cells in the radioresistant G0/G1
phase of the cell cycle reducing the radiosensitivity of tumor cells pretreated with
tamoxifen (Osborne, Boldt et al. 1983; Lykkesfeldt, Larsen et al. 1984; Wazer, Tercilla et
al. 1989; Paulsen, Strickert et al. 1996). Today, the most important clinical concern is the
optimal scheduling (either concurrent or sequential) of radiation and hormonal therapy administration (Harris, Christensen et al. 2005; Whelan and Levine 2005). Even less data and evidence exist on the radiation response of cells resistant to hormonal therapy, which we believe is important considering the high incidence of resistance to systemic therapy in patients with breast cancer. In their study, Paulsen and colleagues investigated the influence of radiation on different breast cancer cell lines including cells resistant to tamoxifen (MCF-7/TAM\(^R\)-1). The results of the study showed that the MCF-7/TAM\(^R\)-1 cells were more resistant to ionizing radiation than MCF-7 and MDA-MB-231 cell lines (Paulsen, Strickert et al. 1996).

In this study, we analyzed gene expression changes during radiation responses in MCF-7 breast adenocarcinoma cells (MCF-7/S0.5) and in the tamoxifen resistant cell line MCF-7/TAM\(^R\)-1 and the ICI 182,780 resistant cell line, MCF-7/182\(^R\)-6, derived from the MCF-7/S0.5 cell line. For the first time, we have shown that MCF-7/TAM\(^R\)-1 cells have an elevated potential to withstand radiation-induced DNA damage and display a decreased sensitivity to radiation than MCF-7/S0.5 cells.

MATERIALS AND METHODS

Cell lines and cell culture conditions

The MCF-7/S0.5 (MCF-7), MCF-7/TAM\(^R\)-1 (TAM\(^R\)-1) and MCF-7/182\(^R\)-6 (182\(^R\)-6) cell sublines were a kind gift from Anne Lykkesfeldt (Breast Cancer Group, Cell Death and Metabolism, Danish Cancer Society Research Center, DK-2100 Copenhagen, Denmark). Subline 0.5 derived from MCF-7 cells was originally adapted to
grow on 0.5% fetal calf serum (Briand and Lykkesfeldt 1984). Tamoxifen and ICI 182,780 (fulvestrant, Faslodex) resistant sublines were derived from MCF-7/S0.5 as described previously (Lykkesfeldt and Briand 1986; Lykkesfeldt, Madsen et al. 1994; Lykkesfeldt, Larsen et al. 1995). MCF-7/S0.5 cells were grown and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM /F-12) with 2.5 mM L-Glutamine, without HEPES and Phenol Red (HyClone, Logan, UT), supplemented with 2% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 6 ng/ml of insulin (Sigma-Aldrich Chemical Co., St. Louis, MO) at 37 ºC in a 5% CO₂ atmosphere. The MCF-7/TAM²⁻¹ and MCF-7/ICI²⁻⁶ cell lines were grown in the medium described above and supplemented with either 1 μM tamoxifen (Sigma-Aldrich) or 0.1 μM ICI 182,70 (Tocris Bioscience), respectively. Cells were harvested for analyses by trypsinization.

**Irradiation conditions**

Cells were irradiated at 80% confluency in Dulbecco's Modified Eagle Medium (DMEM). Two radiation doses (0.5 Gy and 5 Gy, 90 kVp, 5 mA) were applied to check cellular radiation responses. Unirradiated cells served as controls. Cells were harvested 30 minutes and 24 hours after irradiation. All treatments were tested in triplicate. The experiments were independently reproduced twice.

**Whole-genome gene expression profiling**

**RNA isolation**

Total RNA was isolated using the Illustra RNAspin mini kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Approximately 5 x 10⁶ cultured cells were processed
following the manufacturer’s instructions. Samples were eluted in Ultrapure DNase/RNase-free distilled water provided in the kit. RNA samples were quantified using ultraviolet spectroscopy (NanoDrop, Wilmington, DE) and were further assessed for RNA integrity (RIN) on the Aglient 2100 Bioanalyzer (Santa Clara, CA) using the RNA Nano-chip Kit. RNA samples with RIN values of seven or higher were used for further analysis.

**Library preparation**

cRNA was created using the Ambion’s Illumina TotalPrep RNA Amplification Kit (Applied Biosystems, Carlsbad, CA) with an input of 500 ng of total RNA per sample. Briefly, oligo-dT primers were used to synthesize first-strand cDNA containing the phage T7 promoter sequence. Single-stranded cDNA was converted into a double-stranded DNA template via DNA polymerase. Simultaneously, RNase H degraded the RNA. Samples of cDNA were purified in the Filter Cartridge to remove excess RNA, primers, enzymes, and salts. The recovered cDNA was subjected to *in vitro* transcription using biotinylated UTPs. In this step, cRNA was created, labeled, and amplified. A final purification step removed unincorporated NTPs, salts, inorganic phosphates and enzymes, thus preparing samples for hybridization.

**Hybridization and detection**

The Illumina’s direct hybridization assay kit was used to process samples according to the manufacturer’s protocol (Illumina, San Diego, CA). Overnight 750 ng of each cRNA sample were hybridized into the Illumina HumanHT-12_v4 Whole Genome
Expression BeadChip arrays. A 10-minute incubation in the supplied wash buffer at 55°C preceded a 5-minute room temperature wash. The arrays were incubated in 100% ethanol for 10 minutes. A second room temperature wash lasted two minutes with gentle shaking, thus completing this high-stringency wash. The arrays were blocked with a buffer for 10 minutes and washed before a streptavidin-Cy3 (1:1000) probe for 10 minutes. After a five-minute wash at room temperature, the BeadChips were dried and imaged. Six controls were also built into the Whole-Genome Gene Expression Direct Hybridization Assay system to cover the aspects of array experiments, including controls for: the biological specimen (14 probes for housekeeping controls), three controls for hybridization (six probes for Cy3-labeled hybridization, four probes for low-stringency hybridization, and one probe for high-stringency hybridization), signal generation (two probes for biotin control), and approximately 800 probes for negative controls on an eight-sample BeadChip. The arrays were scanned on the iScan platform (Illumina), and the data were normalized and scrutinized using Illumina BeadStudio Software.

**BeadChip statistical analysis and data processing**

The false discovery rate (FDR) was controlled using the Benjamini-Hochberg method. The Illumina Custom Model took FDR into account and was used to analyze the data. Differential gene expression (at least a 0.5-fold change) from non-radiated cells was determined to be statistically significant if the \( p \) value after the adjustment using the Benjamini-Hochberg method was lower than 0.05. The values were transformed to show a log2 scale.
Lists of regulated transcripts were inserted into the web-based DAVID Bioinformatics Resources 6.7 (NIAID/NIH) Functional Annotation Tool (Huang da, Sherman et al. 2009; Huang da, Sherman et al. 2009). This program was used to group genes into functionally relevant categories: metabolic processes, responses to stimulus/stress, DNA repair processes, apoptosis, and cell cycle processes. The minimum number of genes in each altered pathway has been set to three. The pathways were deemed significantly altered if at least 80% of genes were shifting the pathway in the same direction (Ertel, Verghese et al. 2006).

**Quantitative real-time PCR**

Quantitative real-time PCR was performed to confirm the results of the Whole-Genome Gene Expression analysis for the regulation of the direction (either up or down) of selected genes. Five genes (CCNA2, CCNB2 CDC20, PTTG1 and BAX) were selected from the gene list of significantly differentially expressed transcripts representing a preliminary review of the acquired gene expression data. Actin was used as a reference gene. All reactions were performed using cDNA synthesized from the same RNA extraction as the BeadChip experiments, and 500 ng of the sample was used for the Bio-Rad iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). The samples were stored at -20°C for long-term storage and at 4°C until they were used for the subsequent qRT-PCR reactions.

Primers were designed using the NCBI database and PrimerQuest (Integrated DNA Technologies, Inc, Coralville, IA). The following primers were designed: the forward primer for the ACTA2 reference gene (5’-TAG CAC CCA GCA CCA TGA
AGA TCA-3’); and the reverse primer (5’-GAA GCA TTT GCG GTG GAC AAT GGA-3’); CCNA2 forward primer (5’-ATG AGC ATG TCA CCG TTC CTC CTT-3’) and the reverse primer (5’-TCA GCT GGC TTC TTC TGA GCT TCT-3’); CCNB forward primer (5’-TGC TTC CTG CTT GTC TCA GAA GGT-3’) and the reverse primer (5’-CAT TCT TGG CCA TGT GCT GCA TGA-3’); CDC20 forward primer (5’-ATG CGC CAG AGG GTT ATC AGA ACA-3’); and the reverse primer (5’-CAT TTC GGA TTT CAG GCG CAT CCA-3’); PTTG1 forward primer (5’-AGT GGA GTG CCT CTC ATG ATC CTT-3’); and the reverse primer (5’-TCC AGG GTC GAC AGA ATG CTT GAA-3’); BAX forward primer (5’-TTT CTG ACG GCA ACT TCA ACT GGG-3’) and the reverse primer (5’-TGT CCA GCC CAT GAT GGT TCT GAT-3’). The reactions were prepared using 1 μL of diluted cDNA, 10 pmol/μL of each forward and reverse primer, and SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA) prepared according to the manufacturer’s instructions. The samples were prepared in triplicate and were run on the Bio-Rad C1000 Thermal Cycler equipped with the CFX96 Real-Time System. The qRT-PCR protocol consisted of denaturation at 95°C for 2 minutes; 43 cycles of denaturation (95°C, 5 seconds) and annealing/extension (55°C, 5 seconds); and a final extension at 65°C for 5 seconds. Annealing temperature optimization, melting curve analysis, and gel analysis of the amplicon were performed for every set of primers. To evaluate PCR efficiency, a standard curve was established using a series of cDNA dilutions. The data was captured and organized using Bio-Rad CFX Manager 2.1 software (Bio-Rad Laboratories, Hercules, CA).
**QRT-PCR statistical analysis**

The quantification data from the Bio-Rad CFX Manager software were analyzed using the Pfaffl method in Microsoft Excel (Watters 1999). Graphs showing a fold change from the untreated cells were created, and transcript regulation directions (up- or down-regulation) were matched to the results of the Whole-Genome Gene Expression analysis.

**Immunofluorescence**

For immunocytochemical analysis, cells were grown on two-well Lab-Tek chamber slides (Nulge Nunc International Corp., Naperville, IL) and irradiated. After irradiation, the cells were fixed in 4% paraformaldehyde in PBS, permeabilized with 70% ethanol, and washed in PBS containing 0.1% TRITON-X100. Blocking was done in 8% BSA in PBS. For immunocytochemical detection, the cells were incubated for two hours at room temperature using an anti-γH2AX (Ser 139) rabbit antibody (1:100, Cell Signaling Technology Inc., Danvers, MA). Afterwards, the cells were rinsed and incubated with a 1:500 diluted secondary antibody - goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen Molecular Probes, Eugene, OR). Cell nuclei were counterstained with 0.1 mg/mL 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich Chemical Co., St. Louis, MO). The slides were mounted with an anti-fade fluorescence medium prepared from 1,4-diazabicyclo[2.2.2]octane (DABCO), polyvinyl alcohol and glycerol and analyzed using a Zeiss epifluorescent microscope.
The number of γH2AX foci per cell was counted in at least 200 cells from each cell group, as previously described (Sedelnikova and Bonner 2006). The levels of γH2AX were represented as the mean ± SE; P ≤ 0.05.

The Alkaline Comet Assay

The alkaline comet assay protocol was based on Olive and Bannath (2006) and Tice and Vasques (1995) at cometassay.com (Tice and Strauss 1995; Olive and Banath 2006). The cells that were grown in cultures were trypsinised, collected in 15-ml tubes, and centrifuged for three min at 1000 g to form a pellet. Next, the pellet was washed three times with ice-cold phosphate-buffered saline (PBS) without -Ca$^{2+}$ and -Mg$^{2+}$. Finally, the cells were resuspended in their final concentration of 1000 cells per 1 µL of cell suspension in ice-cold PBS. The cell suspension was stored on ice during the course of subsequent procedures.

Ten microliters of cell suspension were mixed with 75 ul of 1% low melting point (LMP) agarose pre-heated to 40 °C, mixed gently through pipetting up and down, and applied to a fully frosted microscope slide (VWR) that was pre-coated with normal melting point agarose. The agarose was overlaid with a cover slip and allowed to solidify for two to three minutes on ice. The removal of the cover slip was followed by an application of 85 ul of 1% LMP agarose pre-heated to 40 °C in order to form a protective layer on the top of the layer containing the cell suspension. The cover slip was repositioned, and the slides were placed on ice to allow the agarose to solidify.

The cover slips were removed, and the slides were placed in a freshly prepared alkaline lysis solution (2.5 M NaCl, 100 mM Na$_2$EDTA, 10 mM Tris base, 1% Triton,
and 0.1% Sodium Lauroyl Sarcosine (pH 10.0) adjusted to 4 °C), left overnight at 4 °C, and protected from light. Following the lysis step, the slides were rinsed with a freshly prepared electrophoresis solution (300 mM, 2mM EDTA (pH>14)). Next, the slides were placed in an electrophoresis tank, covered with a thin layer (1-2 mm) of electrophoresis buffer, and left for 30 min to permit alkaline DNA unwinding. Electrophoresis was performed for 25 minutes at 0.7 V/cm. Each electrophoresis included slides that belonged to the same experimental time-point.

After the completion of electrophoresis, the slides were washed three times for five minutes in a neutralization buffer (0.4 M Tris (pH=7.5)). The slides were stained with SYBR gold dye (Invitrogen), the comets were viewed using a epifluorescence microscope (Zeiss), and the image information was collected using a Comet Assay IV system (Perceptive Instruments).

The statistical analysis was performed to obtain the tail intensity data using SPSS software (IBM) and according to recommendations on the statistical analysis of the Comet assay (Bright, Aylott et al. 2011). The data was collected from three replicate cell culture flasks, at two slides per flask, and 50 cells were examined on each slide. The median of the log tail intensity from 50 cells was evaluated per each slide followed by the calculation of the mean of two medians from two slides derived from one cell culture flask. Finally, the mean values were compared between three flasks representing each treatment point using a one-way ANOVA. The levels of tail intensity were represented as mean ± SD; P ≤ 0.05.
The Annexin V assay

For the early detection of apoptosis, an Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) was used according to the manufacturer’s protocol. Cells were grown and irradiated as previously described (Section 2.2). The analysis was performed 24 hours after exposure to radiation. Cells were harvested, washed with PBS, resuspended in a 1X binding buffer, stained with Annexin V and propidium iodide for 15 min at 25 °C in the dark, and analyzed using flow cytometry within one hour at the Flow Cytometry Core Facility (University of Calgary, Calgary, AB). The results were represented as a percentage of gated Annexin V positive cells.

RESULTS

The effects of radiation on whole-genome gene expression in antiestrogen-sensitive and antiestrogen-resistant MCF-7 cells

The gene expression analysis was conducted for MCF-7/S0.5 and the antiestrogen-resistant derivatives, MCF-7/TAM<sup>R</sup>-1 and MCF-7/182<sup>R</sup>-6, with the purpose to evaluate and compare the radiation response between cell lines. Differential gene expression in the MCF-7 cell lines was found upon exposure to radiation. In fact, the expression level of 402, 371, and 187 genes was significantly altered due to X-ray exposure in MCF-7/S0.5, MCF-7/182<sup>R</sup>-6 and MCF-7/TAM<sup>R</sup>-1, respectively (Fig. 5.1). Interestingly, most of the altered genes were down-regulated. Amongst 134 genes that were common for all three cell lines, 27 genes were up-regulated and 107 genes were down-regulated. The majority of gene expression changes observed in the antiestrogen
resistant cell lines was also seen in the parental MCF-7/S0.5, (73.6 and 73.8% of the
genes in MCF-7/182\(^R\)-6 and in MCF-7/TAM\(^R\)-1, respectively). The least gene expression
changes were found in the MCF-7/TAM\(^R\)-1 cell line which had only half as many gene
changes as the parental and ICI 182,780 resistant cells, and only 30 unique genes changes
in response to radiation treatment (Fig. 5.1).

Further, we uploaded the gene lists consisting of 402, 371 and 187 genes from the
MCF-7/S0.5, MCF-7/182\(^R\)-6 and MCF-7/TAM\(^R\)-1 lines, respectively, through the
DAVID pathway-specific enrichment analysis in order to identify casual relationships
between the genes and organize them into specific pathways according to their functions.
Subsequently, the genes with similar or identical functions were grouped together and
organized by the KEGG database into pathways. The least number of genes that could
constitute a pathway was three; therefore, only 83 genes and the 12 pathways they belong
to were further studied (Table 5.1). Mainly, those were the genes that play a role in cell
cycle, DNA replication, base excision repair (BER), nucleotide excision repair (NER),
mismatch repair (MMR), homologous recombination (HR), p53 signaling, gap junction,
drug metabolism, purine and pyrimidine metabolism and spliceosome. Based on each
gene’s function and its expression trend, the roles of the above-mentioned pathways were
evaluated and compared between cell lines (Tables 5.1 and 5.2). For this, the pathways
were deemed significantly altered if at least 80% of the genes from the pathway were
shifting the pathway in the same direction (Table 5.2) (Ertel, Verghese et al. 2006). For
instance, in the MCF-7/S0.5 line, eight out of ten genes from the p53 signaling pathway
represented in Table 5.1 were changed in a way that functionally shifted the pathway to
overall up-regulation. These eight genes represented 80% of pathway significance in the
MCF-7/S0.5 line, which allowed us to conclude that the p53 signaling pathway was significantly up-regulated in the MCF-7/S0.5 cells upon exposure to radiation (Table 5.2). An identical analysis approach was applied for the remaining 11 pathways in each cell line.

Table 5.2 demonstrates the pathways’ specific differences between MCF-7/S0.5, MCF-7/182R-6 and MCF-7/TAMR-1 in response to X-ray radiation (Table 5.2). As expected, 5 Gy of X-ray caused cell cycle deregulation in all three MCF-7 cell lines (Fig. 5.2). The down-regulation in the expression level of 18 genes involved in cell cycle was common for MCF-7/S0.5, MCF-7/TAMR-1 and MCF-7/182R-6. These genes constituted the components of the mitotic checkpoint CHEK, MAD2L1, BUB1 and BUB1B, E2F transcription factor 2, CCNA2 and CCNB2 encoding cyclins A2 and B2, cyclin-dependent kinase CDC20, the components of the minichromosome maintenance (MCM) complex, protein-kinase TTK, protease ESPL11 and a regulator of chromosome stability PTTG1. In addition, MCF-7/S0.5 and MCF-7/182R-6 shared the down-regulation of RAD2, CDC25C, CDC7, CDK2 and a negative regulator of entry into mitosis PKMYT. Both antiestrogen-resistant cell lines overexpressed growth arrest and GADD45A, a DNA-damage-inducible factor, upon radiation treatment (Table 5.1). The second pathway that like the cell cycle was mostly affected by ionizing radiation in all cell lines was DNA replication. 20, 16 and 9 genes involved in the process of DNA replication were down-regulated in MCF-7/S0.5, MCF-7/182R-6 and MCF-7/TAMR-1, respectively (Table 5.2). Specifically, they were components of the minichromosome complex (MCM 2-7), DNA polymerases A, D and E, replication factors RFC 2, 3, 4, and 5, the replication protein RPA3 and others (Table 5.1). Moreover, the main DNA repair pathways were also
downregulated in MCF-7/S0.5 and MCF-7/182R-6 in response to 5 Gy of X-rays. Base excision repair, mismatch repair, and homologous recombination were down-regulated in MCF-7/S0.5 and MCF-7/182R-6; and nucleotide excision repair (NER) was significantly down-regulated in MCF-7/S0.5 (Tables 5.1 and 5.2). Moreover, the purine and pyrimidine metabolism pathways that could contribute to DNA replication and DNA repair by providing the necessary deoxyribonucleotides were also down-regulated in response to X-ray radiation. An inability of cells to ultimately replicate and repair their DNA leads to cell death. The P53 signaling pathway was functionally up-regulated in MCF-7 sensitive and antiestrogen-resistant cell lines in response to exposure to radiation (Table 5.2).

The decreased expression of tubulins, the main components of microtubules, resulted in the overall down-regulation of the gap junction pathway in MCF-7/S0.5 and MCF-7/182R-6 cells which could contribute to the apoptotic response; the down-regulation of spliceosome in MCF-7/182R-6 is translated into the absence of RNA processing that is necessary for protein synthesis and cell proliferation. Interestingly, an increase in the expression state of genes that contribute to drug metabolism was observed in the MCF-7/TAMR-1 cell line after radiation treatment. These genes were: flavin-containing monooxygenase (FMO5), glutathione S-transferase kappa 1 (GSTK1) and monoamine oxidase A (MAOA) that could potentially increase drug-resistance of MCF-7/TAMR-1 cells.

Overall, although the radiation response of the three MCF-7 cell lines was similar in the way that all cells showed down-regulation of cell cycle, DNA replication, DNA
repair and activation of the apoptotic pathway, the most dramatic response was found in the antiestrogen sensitive MCF-7/S0.5 cell line. The cells resistant to ICI 182,780 were also very sensitive to radiation, while tamoxifen-resistant cells showed the least dramatic response. Moreover, the up-regulation of the drug metabolism pathway post-radiation exposure suggests a possible strengthening of drug resistance by ionizing radiation in MCF-7/TAMR-1 cells. The gene expression data have been confirmed by the qRT-PCR analysis on the five genes that play a role in the cell cycle and apoptosis: CCNA2 and CCNB2, CDC20, PTTG1 and BAX. Similarly to the gene expression data, qRT-PCR showed a significant down-regulation of CCNA2, CCNB2, CDC20, PTTG1 and up-regulation of BAX in the three MCF/7 cell lines 24 hours after radiation exposure (Fig. 5.3).

**Radiation-induced DNA damage in MCF-7/S0.5, MCF-7/182R-6 and MCF-7/TAMR-1 cells**

The gene expression changes found in the three MCF-7 lines, MCF-7/S0.5, MCF-7/182R-6 and MCF-7/TAMR-1, were accompanied with the extensive DNA damage caused by radiation. Ionizing radiation (IR) is a potent DNA-damaging agent capable of inducing cross-linking, nucleotide base damage, and most importantly, single- and double-strand breaks (DSBs) which are well-known inducers of apoptosis (Little 2000; Huang 2003). Therefore, we analyzed and compared the levels of IR-induced DNA damage in MCF-7/S0.5, MCF-7/182R-6 and MCF-7/TAMR-1 cells by detecting γH2AX foci, a well accepted indicator of DNA double-strand breaks (Bonner, Redon et al. 2008) and by the Comet assay. To better study the dynamics of the appearance of γH2AX foci
in MCF-7 breast cancer cells, we added another time point (30 minutes) and a lower IR
dose (0.5 Gy) to the already existing experimental conditions. As expected, the
appearance of γH2AX foci in all three cell lines was dose-, and time-dependant. Both the
intermediate (0.5 Gy) and high (5 Gy) doses of X-rays caused a significant elevation in
the level of γH2AX foci in antiestrogen-sensitive and antiestrogen-resistant cells (Fig.
5.4). The highest γH2AX level was observed at the 30-minute time point. Specifically,
12.1-, 7.84-, and 6.07-fold changes compared to controls were caused by 0.5 Gy; and
27.3-, 20.5-, and 14.8-fold changes were caused by 5 Gy of X-rays 30 minutes after
exposure in MCF-7/S0.5, MCF-7/TAMR-1 and MCF-7/182R-6, respectively (Fig. 5.4).
Here, it is important to note that at 30-min time point both antiestrogen-resistant cell lines
accumulated significantly less DSBs than their sensitive MCF-7/S0.5 cell line after both
0.5 and 5 Gy. Approximately a halfway decrease in the level of γH2AX foci was
achieved from the 30-min to 24-h time point in all three cell lines indicating DNA repair
and/or damage-induced apoptosis during this period. Therefore, at the 24-hour time point,
the level of foci was different from that in the control non-radiated cells by 4.12-, 3.03-,
and 3.11-fold for the 0.5 Gy dose and by 8.71-, 5.11-, and 8.73-fold for the 5 Gy dose of
X-rays in MCF-7/S0.5, MCF-7/TAMR-1 and MCF-7/182R-6, respectively (Fig. 5.4).
Interestingly, MCF-7/TAMR-1 cells displayed a more complete repair than other two
lines at 24-h time point after 5 Gy. Overall, the immunofluorescent analysis showed that
the background level of γH2AX foci was similar for the three cell lines, and the induction
of foci by radiation had a similar trend between the MCF-7/S0.5 cell line and the two
antiestrogen-resistant cell lines, MCF-7/TAMR-1 and MCF-7/182R-6. Nevertheless,
MCF-7/S0.5 cells displayed higher level of DNA DSBs after each applied dose. MCF-7/TAM$^R$-1 cells were able to repair damages at 24-h time point.

In the comet assay, the super coiled duplex DNA underwent unwinding and denaturation under strong alkaline conditions (Olive 1999). This led to the reduction of DNA fragment size and the expression of alkali labile sites as single-strand breaks which are stretched out by electrophoresis. A comet tail consisting of the broken or damaged DNA fragments was analyzed through the intensity in MCF-7/S0.5, MCF-7/TAM$^R$-1 and MCF-7/182$^R$-6 cells after radiation treatment (Fig. 5.5). A 5 Gy X-ray treatment led to significant damage in MCF-7 parental and both drug resistant cells immediately (30 min) after the application. These damages are believed to represent DSBs, SSBs, alkali labile sites, and breaks from replication events. But the persistence of damages was only observed in MCF-7/S0.5, and MCF-7/182$^R$-6 cells at the 6- and 24-hour time points, and no significant damages were observed in the drug-resistant line MCF-7/TAM$^R$-1 (Fig. 5.5). Such difference could be associated with a higher potential for DNA repair in cells resistant to tamoxifen.

Radiation-induced apoptosis in MCF-7/S0.5, MCF-7/TAM$^R$-1 and MCF-7/182$^R$-6 cells

IR exposure is known to induce apoptotic cell death. Therefore, we analyzed the levels of IR-induced apoptosis in MCF-7/S0.5 and two antiestrogen-resistant lines, MCF-7/182$^R$-6 and MCF-7/TAM$^R$-1. Early apoptosis is characterized by various changes in the cellular plasma membrane; the primary change is the translocation of phosphatidylserine (PS) from the inner layer to the surface of the membrane. Annexin V possesses a high
affinity to PS, and this allows for the early detection of apoptotic changes (Vermes, Haanen et al. 1995). Here, we analyzed IR-induced apoptosis using an Annexin V assay for MCF-7 breast adenocarcinoma cells 24 h post radiation exposure. Interestingly, 0.5 Gy of X-rays did not cause any significant changes in the level of early apoptosis in either of cell lines. In contrast, 5 Gy X-rays led to a significant apoptosis in all three cell lines (Fig. 5.6). The percentage of annexin V-positive cells increased from 4.96 % to 30.0 % in MCF-7/S0.5; from 7.98% to 14.1 % in MCF-7/182R-6; and from 1.7 % to 6.04 % in MCF-7/TAMR-1 at 5 Gy of irradiation at 24 hours post radiation (Fig. 5.6). Overall, the annexin V assay showed that the antiestrogen-sensitive MCF-7/S0.5 line is more sensitive to radiation-induced apoptosis than the antiestrogen-resistant MCF-7/182R-6 and MCF-7/TAMR-1 lines.

DISCUSSION

The effect of systemic therapy on patients with breast cancer has been widely debated. A variety of alternatives for breast cancer treatment are constantly expanding but the combination of chemotherapy, radiation therapy, surgery and hormone therapy for the appropriate treatment plan is still complex (Gonzalez-Angulo, Morales-Vasquez et al. 2007). Although these therapies have proven to be beneficial, a large number of patients acquire resistance to treatments.

The purpose of this study was to investigate radiation-induced gene expression changes in three cell lines of breast adenocarcinoma: the parental MCF-7/S0.5 and the antiestrogen- resistant MCF-7/TAMR-1 and MCF-7/182R-6. Using microarray technology tools, we were able to screen differences in gene expression in response to radiation
between MCF-7/S0.5, MCF-7/TAM\textsuperscript{R}-1 and MCF-7/182\textsuperscript{R}-6. Here, we show that these three cell lines respond differently to radiation at the gene expression level. Gene expression profiling showed that the expression level of at least 402 and 371 genes changed in the antiestrogen-sensitive MCF-7/S0.5 cell line and in cells resistant to ICI 182,780, respectively, due to 5 Gy X-rays. However, in MCF-7/TAM\textsuperscript{R}-1 cells, only 187 genes changed (Fig. 5.1). We believe that the ability of cells to retain their gene expression potential at a close to constant level regardless of DNA-damaging insults may be due to some features acquired by antiestrogen-resistant cells and shared in other forms of resistance, such as radiation resistance. Interestingly, most of the changed genes were down-regulated in all three cell lines. Using David software, we have revealed that these genes belong mainly to the cell cycle, DNA replication and DNA repair pathways (Table 5.1). The most profound down-regulation of gene expression was observed in genes involved in the cell cycle pathway (Table 5.2, Fig. 5.2). The reduced expression of the S and M cyclins, cyclin A2 and B2 (Fig. 5.3), and their cyclin-dependant kinase CDK2 indicate cell cycle arrest in S or G2/M phases of the cell cycle (Table 5.1, Fig. 5.2). Moreover, the similar down-regulation of the E2F transcription factor may prevent cells from entering the S-phase. In addition, the lower expression of PTTG1, the TTK protein kinase that is usually present in rapidly proliferating cells, (Fig. 5.3) that peaks in the M phase, ORC3L that binds to origins of replication, CDC7, one of the regulators of the G1/S transition, CDC25C, an inducer of mitotic control that is necessary for cell cycle progression, and CDC20 (Fig. 5.3), an activator of APC and a major regulator of cell division, reflects cell cycle disturbance in all three cell lines. One would expect that the cells were arrested at the cell cycle checkpoints, but surprisingly, most of the mitotic
checkpoint regulators were also down-regulated. Among them were: CHEK1 that phosphorylates the components of CDC25 for cell cycle arrest; MAD2 that interacts with CDC20 and is a component of the spindle-assembly checkpoint that prevents anaphase until chromosomes are correctly aligned, and BUB1 that is involved in cell cycle checkpoint enforcement (Table 5.1). These gene expression data represent the total cell-cycle shutdown and checkpoint failure which are most probably due to extensive DNA damages caused by ionizing radiation. Cell cycle checkpoints usually contribute to cell survival allowing for DNA damage repair; and the lack of checkpoints makes cells more sensitive to killing by ionizing radiation (Jaklevic, Uyetake et al. 2006).

Both the cell cycle and DNA replication pathways shared the common down-regulation of six components of the minichromosome maintenance complex (MCMs: 2, 3, 4, 5, 6, 7) in all three cell lines (Table 5.1, Fig. 5.2). The MCM 2-7 helicase complex is important for the replication fork formation and elongation during DNA replication (Labib, Tercero et al. 2000). In fact, it is required for the assembly of pre-replication complexes (pre-RCs) at replication origins at the end of mitosis and during late G1 (Diffley 2001; Braun and Breeden 2007). It is evident that mammalian cells decrease the rate of ongoing DNA synthesis in response to DNA damage at the level of origin initiation and fork progression (Larner, Lee et al. 1994). Obviously, the inactivation of the MCM complex inhibits DNA replication and cell proliferation and can be the mechanism of cell cycle arrest. Indeed, the down-regulation of MCM2 and MCM6 was associated with Notch-dependant cell cycle arrest in endothelial cells and human fibroblasts (Noseda, Niessen et al. 2005).
In response to genotoxic stress such as ionizing radiation, the ATM/ATR checkpoint pathways are activated and target stalled replication forks. The MCM complex is also a target of checkpoint signaling (Cortez, Glick et al. 2004). Stalled replication forks must retain MCM proteins in order to resume replication. Otherwise, replication licensing cannot be reassembled as origins fire only once in each cell cycle (Diffley 2001). The down-regulation of MCM 2-7 in MCF-7/S0.5, MCF-7/TAM\textsuperscript{R}-1 and MCF-7/182\textsuperscript{R}-6 (Table 5.1) in response to X-ray radiation indicates aberrant DNA replication or its absence and cell cycle arrest. In addition, reduced expression levels of DNA polymerases add up to the disruption of DNA replication and/or repair. Here, it is important to emphasize that mainly DNA polymerases from MCF-7/S0.5 and MCF-7/182\textsuperscript{R}-6 are inhibited, and only polymerase PolE2 is also effected in MCF-7/TAM\textsuperscript{R}-1 (Table 5.1). The other necessary components of the DNA replication/repair pathway which were down-regulated in response to ionizing radiation were: LIG1 (a ligase that seals nicks in double-stranded DNA during replication, recombination and repair), PRIM1 (a primase that synthesizes short RNA primers for Okazaki fragments during discontinuous replication), FEN1 (an endonuclease that cleaves the 5'-overhanging flap structure that is generated by displacement synthesis when DNA polymerase encounters the 5'-end of a downstream Okazaki fragment), RNASEH2A (a ribonuclease that removes RNA primers from lagging-strand Okazaki fragments), RFC 2-5(replication factors that play a role of a clamp loader for loading PCNA on DNA during replication), and RPA3 (the replication protein that binds ssDNA and keeps it unwound for DNA replication or repair). Overall, 20, 16 and 9 DNA replication genes were down-regulated in MCF-7/S0.5, MCF-7/182\textsuperscript{R}-6 and MCF-7/TAM\textsuperscript{R}-1, respectively. Furthermore, the
detected down-regulation of purine and pyrimidine metabolism mainly in MCF-7/S0.5 and MCF-7/182R-6 contributes to the decreased DNA replication/repair. The importance of sufficient nucleotide pools in the S phase is reflected by the G1 arrest when the pools are inadequate (Linke, Clarkin et al. 1996).

Further evaluation of genes by functional relationships with pathways showed the similarity in the radiation response between MCF-7/S0.5 and MCF-7/182R-6 (Table 5.2). Both cell lines exhibited a lower expression of DNA repair genes following radiation exposure. Specifically, the down-regulation of base excision repair, nucleotide excision repair, mismatch repair and homologous recombination was observed. In addition to the previously mentioned genes (DNA polymerases, RFCs, RPAs, FEN1 and LIG1) that clearly participate in DNA repair, some specific repair genes were also down-regulated (Table 5.1). These genes were the following: uracil-DNA glycosylase (UNG that excises uracil residues from DNA that can arise as a result of misincorporation of dUMP residues by DNA polymerase or due to the deamination of cytosine); poly(ADP-ribose) polymerase 2 (PARP2 that catalyzes the poly (ADP-ribosylation of a limited number of acceptor proteins involved in chromatin architecture and DNA metabolism) and high-mobility group box1-like1 (HMGB1L1 that binds preferentially single-stranded DNA and unwinds double-stranded DNA) in BER; mutS homolog 6 (MSH6 that heterodimerizes with MSH2 to form MutS alpha that binds to DNA mismatches, thereby initiating DNA repair) in MMR; Bloom syndrome , REcQ helicase-like (BLM that unwinds single- and double-stranded DNA in a 3'-5' direction); RAD51 homolog C (RAD51C that is involved in the homologous recombination repair pathway of double-stranded DNA breaks arising during DNA replication or induced by DNA-damaging
agents); RAD54-like (RAD54L that is involved in DNA repair and mitotic recombination) and X-ray repair complementing defective repair in Chinese hamster cells 3 (XRCC3 that is thought to repair chromosomal fragmentation, translocations and deletions) in HR. Interestingly, two genes involved in NER, damage-specific DNA binding protein (DDB2) and xeroderma pigmentosum complementation group C (XPC) involved in DNA damage recognition and initiation of DNA repair were up-regulated in MCF-7/S0.5 and MCF-7/182R-6. This might mean that DNA damages are initially recognized, but the actual repair failed due to the lack of downstream components of the pathway. Such results demonstrate that radiation-induced DNA damages (especially in MCF-7/S0.5 and MCF-7/182R-6) are too great for cell survival and lead to DNA repair failure and possibly to cell death. In contrast, there were no significant changes in the expression level of DNA repair genes in MCF-7/TAMR-1 cells.

The immunocytochemical staining of cells for γH2AX proved the radiation-induced formation of DNA damages, specifically DSBs, and the initiation of DNA repair in all three cell lines. The induction of the DSBs was dose- and time-dependant (Fig. 5.4). Although many DSBs were repaired in 24 hours, the level of γH2AX never returned to the initial one. At the 24-hour time point, a lot of DSBs caused by both low and high doses remained unrepaired in all three cell lines. Interestingly, MCF-7/TAMR-1 cells displayed significantly lower levels of 5 Gy-induced γH2AX foci at 24 hours in comparison to the other two cell lines that were shown to be DNA repair defective in gene expression analysis.
Considering, that γH2AX staining only detects DSB damages in DNA, we performed the Comet assay to evaluate the broader types of damages. These damages are believed to represent DSBs, SSBs, alkali labile sites, and breaks from replication events. Although, all three cell lines displayed a rapid increase (30 minutes) in the levels of radiation-induced DNA damage, MCF-7/TAM\textsuperscript{R}-1 cells showed no significant persistence of DNA damages (Fig. 5.5). 6 and 24 hours after radiation exposure, the level of DNA damages represented by the comet tail intensity was similar to the control level in MCF-7/TAM\textsuperscript{R}-1 cells. In contrast, the level of DNA damages in MCF-7/S0.5 and MCF-7/182\textsuperscript{R}-6 cells remained high even at 24 hours post radiation. These data suggest that MCF-7/TAM\textsuperscript{R}-1 cells have a higher DNA repair activity after radiation in comparison to MCF-7/S0.5 and MCF-7/182\textsuperscript{R}-6 cells. The ability to withstand and repair DNA damages may result in reduced sensitivity to radiation and possibly demands other types of cancer treatment.

The majority of DNA damage signaling proteins may be inactivated by caspases during the execution phase of apoptosis (Watters 1999). P53 is one of the main executioners of cellular response to ionizing radiation and apoptosis. Its levels are elevated in response to ionizing radiation affecting a number of downstream effector genes, such as Bax, p21, GADD45G and Mdm2 (Watters 1999). Radiation-induced p53 activation causes cell cycle arrest allowing for DNA repair and in the case of repair failure, p53 triggers apoptosis (Verheij and Bartelink 2000). In agreement with the above, p53 signaling was activated in all three cell lines in response to radiation. Up-regulated BAX (Table 5.1, Fig. 5.3) is known to accelerate programmed cell death by binding and inhibiting an apoptosis repressor Bcl-2. The activation of sestrin 1 (Table 5.1) was
previously shown upon genotoxic exposure, and its cytoprotective function based on regeneration of overoxidized peroxiredoxins was described (Budanov, Shoshani et al. 2002). A few years ago, Budanov and Karin showed that sestrin is a target of p53 and an inhibitor of TOR (target of rapamycin). mTOR is a phosphatidylinositol kinase-related kinase that positively regulates cell growth. P53-mediated activation of sestrin upon genotoxic stress inhibits mTOR through the AMP-responsive protein kinase (AMPK) (Budanov and Karin 2008). Gene activated by p53, the ribonucleotide reductase (RRM2B), was up-regulated in MCF-7/S0.5 and MCF-7/ICI. RRM2B plays a role in DNA repair of arrested cells by supplying deoxyribonucleotides during cell cycle arrest in a p53-dependent manner. Although it is not clear whether this gene actually affected DNA repair, considering the fact that its homolog RRM2 that also provided precursors for DNA synthesis was down-regulated in all three cell lines). Finally, the increased expression of Gadd45A and TP53I3 in antiestrogen-resistant cells also indicate cell cycle arrest after X-ray treatment (Table 5.1).

The gene expression data correlate with the results of the annexin V assay on early apoptosis. Exposure to 5 Gy of X-rays initiated apoptotic cell death in all three cell lines. However, the degree of apoptosis was different among the cell lines. The highest apoptosis level was detected in MCF-7/S0.5 cells (30%), while in cells resistant to tamoxifen, only 6% of the cells were undergoing apoptosis (Fig. 5.6). Such differences can be attributed to the radio-resistance of MCF-7/TAMR-1 cells. In fact, although the response to X-rays (such as an increase in DNA damages and cell cycle arrest) in MCF-7/TAMR-1 cells and the other two cell lines was similar, MCF-7/TAMR-1 cells did not
lose their DNA repair capacity and exhibited lower fraction of apoptotic MCF-7/TAM\textsuperscript{R}-1 cells compared to parental and ICI 182,780 resistant cells.

According to the gene expression profiling and the data of pathway enrichment analysis, a strong down-regulation of the gap junction pathway was caused by the ionizing radiation in MCF-7/S0.5 and MCF-7/182\textsuperscript{R}-6 but not in MCF-7/TAM\textsuperscript{R}-1 (Table 5.2). All down-regulated genes that constituted the pathway were members of cytoskeletal elements, tubulins alpha and beta. An altered level of expression of cytoskeletal elements plays a considerable role in radiation-mediated transformation. The differential modulation of genes encoding cytoskeletal elements upon radiation exposure was previously documented, where actin and tubulin mRNA accumulation was reported to be similar to that in transformed cells (Woloschak, Shearin-Jones et al. 1990). Cancer cells are characterized by a complicated ultrastructural organization. Breast cancer cells resistant to doxorubicin and cisplatin display an increase in the number of microtubules and varying widths of microfilaments (Huang da, Sherman et al. 2009). Tubulins are critical for cell division, which made them a target for several anti-cancer drugs. An elevated expression of tubulin correlates with a lack of response to chemotherapy. In fact, βIII-tubulin expression has been acknowledged as a predictor of the docetaxel resistance in metastatic prostate cancer (Sanchez, Gupta et al. 2012). Another study claimed that βII-tubulin is a strong predictor of outcome in patients treated with the platinum-based induction chemotherapy for locally advanced squamous carcinoma of the head and neck (Cullen, Schumaker et al. 2009). A possible explanation for such observation was based on the fact that tubulin binds to the voltage-dependent anion channel, VDAC, and regulates the permeability of the mitochondrial outer membrane. Binding of tubulin to
VDAC inhibits the binding of proapoptotic drugs which induce a rapid cytochrome c release (Cullen, Schumaker et al. 2009). In the present study, a decrease in tubulin expression in the MCF-7/S0.5 and MCF-7/182R-6 cell lines indicates the inability of the formation of a microtubular apparatus necessary for cell division, and it also supports the data on early apoptosis. In contrast, MCF-7/TAM\(^R\)-1 cells did not show any expression changes in a single tubulin gene, which at least partly may contribute to the reduced sensitivity to radiation.

In addition, three genes involved in drug metabolism were up-regulated in MCF-7/TAM\(^R\)-1 cells. One of these genes was glutathione S-transferase kappa 1 (GSTK), a radical scavenger that is involved in the metabolism of xenobiotics. It was previously found that GST plays an important role in the acquisition of drug resistance through the decreased intracellular drug accumulation and the stimulation of drug-induced DNA damage repair (Pfaffl 2001; Sedelnikova and Bonner 2006). Using an \textit{in vivo} mouse model, it has been shown that tamoxifen-resistant tumors had a statistically significant increase in GST activity, the increased levels of other antioxidant enzymes such as SOD, and the reduced glutathione levels (Schiff, Reddy et al. 2000). The authors discussed the effects of tamoxifen on the intracellular redox status of breast cancers, the induction of lipid peroxidation and the activation of antioxidant enzymes. Such oxidative changes appeared to be tamoxifen-specific as they were not found in ICI-resistant tumors (Schiff, Reddy et al. 2000). In a recent study, a quantitative proteomic analysis revealed up-regulation of GST in breast cancer cells during the transition to acquired tamoxifen resistance (Zhou, Zhong et al. 2012). Taking into consideration that ionizing radiation may also influence the redox status of cells, we believe that GST may be involved in the resistance of cancer cells to
radiation, and therefore, may be considered one of the common molecular indicators for chemo- and radio-resistance. The up-regulation of the drug metabolism pathway in MCF-7/TAM^R^-1 cells after radiation treatment indicates that ionizing radiation may potentially decrease the sensitivity of tamoxifen resistant cells to other treatment modalities.

This study provides the analysis of the roles of DNA repair and apoptosis in response to radiation, in antiestrogen-sensitive and resistant cells. The ability of tamoxifen-resistant cells to retain their DNA repair capacity upon radiation treatment allows us to suggest that DNA repair genes could possibly be considered as putative targets of future anti-cancer therapy. Further detailed studies are needed to determine the cellular and molecular processes that are altered in resistant cells that allow them to survive genotoxic treatments such as irradiation.
Figure 5.1 Gene expression profiling of MCF-7/S0.5, MCF-7/TAM\textsuperscript{R}-1 and MCF-7/182\textsuperscript{R}-6. The Venn diagram shows the number of significantly changed genes in the MCF-7/S0.5, MCF-7/TAM\textsuperscript{R}-1 and MCF-7/182\textsuperscript{R}-6 cell lines upon radiation in comparison to their corresponding un-irradiated controls, as identified by the gene expression profiling analysis. The arrows beside the numbers in brackets represent the direction of genes alteration (up- or down-regulation).
Table 5.1 The list of differentially expressed genes involved in critical biological pathways in MCF-7/S0.5, MCF-7/TAMR-1 and MCF-7/182R-6 cells. “+” – the gene is up-regulated; “-” – the gene is down-regulated.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Cell line(s) where the gene expression is changed</th>
<th>KEGG Pathway(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEN1</td>
<td>flap structure-specific endonuclease 1</td>
<td>MCF-7/S0.5, MCF-7/182R-6</td>
<td>DNA replication, BER</td>
</tr>
<tr>
<td>LIG1</td>
<td>ligase I, DNA, ATP-dependent</td>
<td>MCF-7/S0.5</td>
<td>DNA replication, NER, BER, MMR</td>
</tr>
<tr>
<td>MCM2</td>
<td>minichromosome maintenance complex component 2</td>
<td>MCF-7/S0.5, MCF-7/182R-6, MCF-7/TAMR-1</td>
<td>DNA replication, Cell cycle</td>
</tr>
<tr>
<td>MCM3</td>
<td>minichromosome maintenance complex component 3</td>
<td>MCF-7/S0.5, MCF-7/182R-6, MCF-7/TAMR-1</td>
<td>DNA replication, Cell cycle</td>
</tr>
<tr>
<td>MCM4</td>
<td>minichromosome maintenance complex component 4</td>
<td>MCF-7/S0.5, MCF-7/182R-6, MCF-7/TAMR-1</td>
<td>DNA replication, Cell cycle</td>
</tr>
<tr>
<td>MCM5</td>
<td>minichromosome maintenance complex component 5</td>
<td>MCF-7/S0.5, MCF-7/182R-6, MCF-7/TAMR-1</td>
<td>DNA replication, Cell cycle</td>
</tr>
<tr>
<td>MCM6</td>
<td>minichromosome maintenance complex component 6</td>
<td>MCF-7/S0.5, MCF-7/182R-6, MCF-7/TAMR-1</td>
<td>DNA replication, Cell cycle</td>
</tr>
<tr>
<td>MCM7</td>
<td>minichromosome maintenance complex component 7</td>
<td>MCF-7/S0.5, MCF-7/182R-6, MCF-7/TAMR-1</td>
<td>DNA replication, Cell cycle</td>
</tr>
<tr>
<td>POLA1</td>
<td>polymerase (DNA directed), alpha 1, catalytic subunit</td>
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</tr>
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<tr>
<td>POLE</td>
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<td>MSH6-</td>
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<td>RAD51C-</td>
<td>RAD51 homolog C (S. cerevisiae)</td>
<td>MCF-7/S0.5</td>
<td>HR</td>
</tr>
<tr>
<td>RAD54L-</td>
<td>RAD54-like (S. cerevisiae)</td>
<td>MCF-7/S0.5, MCF-7/182R-6</td>
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<td>deoxyuridine triphosphatase</td>
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<td>RRM1-</td>
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<tr>
<td>Gene</td>
<td>Description</td>
<td>Cell Lines</td>
<td>Function</td>
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<td>Gap junction</td>
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<td>Gap junction</td>
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<td>Gap junction</td>
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<td>cyclin G2</td>
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<td>monoamine oxidase A</td>
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Table 5.2 The significantly altered KEGG pathways in MCF-7/S0.5, MCF-7/TAM$^{R}$-1 and MCF-7/182$^{R}$-6 cells after X-ray treatment in comparison to the corresponding un-treated controls. In this table, the pathway significance (%) is defined as the ratio of gene alterations that similarly affect a certain pathway (either up- or down-regulate) to the total number of altered genes in the pathway. “+” – the pathway is up-regulated; “-” – the pathway is down-regulated. Numbers in brackets represent the total number of altered genes in the pathways. “N/S” – not significant, which could be due to either less than 80% significance or less than 3 of the total number of genes altered in the pathway.

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<th>Pathway</th>
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<td>-100% (25)</td>
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<td>-</td>
<td>+100% (3)</td>
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<tr>
<td>Gap junction</td>
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<td>-100% (7)</td>
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<td>Gene</td>
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</table>

**Figure 5.2 The KEGG cell cycle pathway.** The red stars indicate genes that were downregulated in all three cell lines.
Figure 5.3 Fold change in the levels of CCNA2, CCNB2, CDC20, PTTG1 and BAX transcripts detected by qRT-PCR. Each treatment group was compared to its corresponding control. Actin was used as a reference gene (calculated by Pfaffl). * - significant, p<0.001; ** - significant, p<0.01. (Student’s t-test).
Figure 5.4 Radiation-induced H2AX phosphorylation in MCF-7/S0.5, MCF-7/TAMR-1 and MCF-7/182R-6 cells. The results are presented as an average number of γH2AX foci per cell ± SE, n = 200. * - significantly different from the respective control; p < 0.05; Magnification, × 100. Blue – DAPI, green – γH2AX.
Figure 5.5 Radiation-induced DNA damage in MCF-7/S0.5, MCF-7/TAM$^R$-1 and MCF-7/182$^R$-6 cells as determined by the Alkaline Comet assay. The graphs represent the percentage of DNA in the comet tails (tail intensity) obtained by the Alkaline Comet assay performed on MCF-7 cells 30 minutes, 6 and 24 hours after X-ray irradiation. Tail intensity levels are represented as mean ± SD; * - significantly different from the respective control, p < 0.01; ** - significantly different from the respective control, p<0.05. (Student’s t-test). Comet representative pictures of tail intensity are located beside the charts.
Figure 5.6 Radiation-induced apoptosis in MCF-7/S0.5, MCF-7/TAM<sup>R</sup>-1 and MCF-7/182<sup>R</sup>-6 cells. The number of cells in early apoptosis was measured using the Annexin V-FITC assay for control cells (CT) and cells irradiated with 0.5 Gy and 5 Gy of X-rays. M1 – AnnexinV-positive cells; Viable cells - AnnexinV- and PI-negative (the lower left quadrants); Cells in the early apoptosis state - AnnexinV-positive and PI-negative (the lower right quadrants); Cells in the late apoptosis state or already dead cells - both Annexin V- and PI-positive (the upper right quadrants).
Main findings described in chapter 5:

1. Tamoxifen resistant cell line in contrast to parental and Faslodex resistant cell lines displayed significantly less radiation-induced decrease in expression of genes involved in DNA repair and differed by up-regulation of drug metabolic pathways.

2. Tamoxifen resistant cells developed significantly less DNA damages and were least sensitive to radiation-induced apoptosis.
GENERAL DISCUSSION AND CONCLUSIONS

The most common type of malignancy in women, breast cancer, has worldwide incidence that continues to rise (Ellsworth, Ellsworth et al. 2004). A positive breast cancer treatment outcome strongly depends on advances in early detection and an understanding of the molecular mechanisms of cancer response to treatment procedures.

The role of ionizing radiation in medical diagnostics and cancer therapy has a long, controversial history. Radiotherapy as a treatment for breast cancer has clearly demonstrated reduced local relapse (Cuzick 2005). Nevertheless, numerous studies continue in their attempt to understand well the mechanisms involved in mediating radioresistance (Jameel, Rao et al. 2004). Ionizing radiation-dependent diagnostic screening (CT scanning, mammography) provides accurate, individualized decision making about the most beneficial treatment approach (Walter and Schonberg 2014). However, epidemiological studies provide evidence of increased cancer risk after prolonged exposure to low doses of ionizing radiation (Mullenders, Atkinson et al. 2009).

The radiation response between normal and cancer cells differs and is believed to be dependent on the radiation dose and energy level involved (Tutt and Yarnold 2006). The major difference is on the ability of cells to withstand radiation-induced DNA damage and the subsequent effect on gene expression. On the one hand, extensive DNA damage naturally leads to apoptosis which eliminates genetically non-stable cells from the population (valuable in eradicating cancer cells during radiotherapy). On the other hand, cells may develop the ability to propagate damage through the cell cycle and, thus,
avoid cell death. Such cells give rise to cancer initiation, which is the main concern in the use of a low radiation dose.

A number of studies have indicated substantial alterations of epigenetic mechanisms in response to ionizing radiation, including changes in DNA methylation and microRNA expression. DNA methylation is crucial in the regulation of gene expression and chromatin state and thus, in the normal development, cell proliferation, and proper maintenance of genome stability in an organism (Jaenisch and Bird 2003). microRNAs can cause silencing of multiple genes and are important in cellular proliferation, apoptosis, differentiation, and tissue and organ development (Alvarez-Garcia and Miska 2005).

This study aims to analyze and compare the radiation responses of normal mammary gland tissue to various doses of ionizing radiation, as well as, the low- versus high- dose radiation response of MCF-7 breast adenocarcinoma cells and their counterparts that are resistant to tamoxifen and faslodex.

We discovered that low and intermediate doses of ionizing radiation stimulate the expression of immune system-related genes and promote apoptosis-directed alterations of the miRNAome in normal mammary tissue. On the other hand, low doses of ionizing radiation may lead to epigenetic activation of mobile elements, such as transposons. We found that breast cancer cells are sensitive to high doses of radiation and that resistance to ionizing radiation may possibly be related to an acquired hormone therapy resistance.
The major findings of this thesis are as follows:

1. Rat mammary gland cells activate the expression of the genes that boost the immune system to fight foreign cytotoxic agents in response to low and intermediate doses of radiation. This activation is accompanied by the altered regulation of specific microRNAs, such as activation of miR-34a, which may be responsible for radiation-induced cell death.

2. Low doses of ionizing radiation contribute to hypomethylation and the subsequent activation of Line-1, a transposable mobile element.

3. Breast adenocarcinoma MCF-7 cells differentially respond to high, medium, and low doses of ionizing radiation, and they show a strong apoptotic response only after a high dose of X-rays.

4. Tamoxifen-resistant cells accumulate less DNA damage and are less sensitive to radiation-induced apoptosis than their parental cell line, MCF-7/S0.5.

Our findings are important in the following:

1. Selecting the optimal number of diagnostic screening procedures

2. Selecting the appropriate modality for breast cancer treatment

3. Predicting the development of resistance to already existing treatment methods

4. Developing novel epigenetic therapy strategies
FUTURE RESEARCH PROSPECTS:

The findings of this study only partially describe the radiation response of mammary gland tissue and cells. Cellular response to ionizing radiation involves a variety of defensive pathways that require further investigation. Moreover, the common mechanisms of drug- and radioresistance should be elucidated in depth. Our suggestions for future studies are as follows:

1. The role of the genes that change their expression upon ionizing radiation in mammary gland tissue should be investigated further. Specifically, the targeted up- or downregulation of these genes in a breast cell culture would allow us to understand the role of these genes in promoting or preventing carcinogenesis. Identifying the molecular mechanisms of changes in gene expression and defining which molecular process (e.g., mutation, epigenetic alteration, and transcription factors) within the gene or the gene promoter is affected by ionizing radiation are important.

2. The phenomenon of radiation-induced transposon activation was observed while studying only one (Line-1) out of many existing transposable elements. Various other transposons (SINEs, class II transposons) could be explored to make a precise conclusion about the role of mobile elements in radiation-induced carcinogenesis. Determining the exact promoter sites within the retrotransposon gene that were hypomethylated after the application of ionizing radiation is especially important.
3. In this thesis, we evaluated the response of MCF-7 cells to low, medium, and high doses of ionizing radiation. The MCF-7 cell line represents breast adenocarcinoma cells with an ER-positive tumor phenotype and low invasive potential. Numerous breast cancer phenotypes with strong metastatic potential exist. Studying the response of several other breast cancer lines to ionizing radiation is necessary.

4. Drug resistance was explained with the use of only two hormonal therapy drugs as examples. An entire cohort of other hormonal agents and anthracyclines could be explored to make precise conclusions about radioresistance in breast cancer cells that display resistance to most common drugs used for breast cancer treatment.

5. The radiation response of normal breast cells, as well as breast cancer cells, was shown to be mediated by microRNA expression. Short non-coding RNAs are of special interest in cancer biology nowadays. MicroRNAs are epigenetic regulators of gene expression, inhibitors of translation of a variety of proteins, and important contributors to radiation-induced apoptosis, tumor development, and chemoresistance. In the future, further investigation of the role of microRNAs and their putative targets in the radiation response of breast cancer cells, as well as the establishment of the role of microRNAs as the potential biomarkers for the prediction of treatment responses, would be necessary.

Overall, the studies presented in this thesis indicate the emerging notion that radiation use in diagnostic imaging and radiation treatment has much more to offer
than just being a powerful cytotoxic agent. Nevertheless, further evidence is required to establish the definitive role of tumor radiation.
REFERENCES


