Charles University in Prague

Faculty of Science

Program: Molecular and Cell Biology, Genetics and Virology

LENKA KYJACOVÁ, MSc

Radiation-induced plasticity of prostate cancer cells

PhD Thesis

Supervisor: Zdeněk Hodný, MD, PhD

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„Success is nothing more than going from failure to failure with undiminished enthusiasm."

Jacques Bernier

„Good things come to those who believe, better things come those who are patient and the best things come to those who don´t give up."

(unknown author)

(picture from (Almeida-Souza and Baets, 2012))

Unpublished data authorship statement

To maintain the consistency of the content, following yet-unpublished data from my colleagues from the Laboratory of Genome Integrity, Institute of Molecular Genetics (Prague) were used in this Thesis: data shown in Fig. 4.5a and c was provided by Kateřina Krejčíková. Data in Fig. 4.5b and 5-azacytidine-treated HeLa cell populations for microarray analysis were delivered by Soňa Hubáčková. Data in Fig. 4.6d and 4.11d were obtained by Terezie Imrichová. Microarray data analysis was performed in cooperation with Hynek Strnad (Genomics and Bioinformatics Unit, IMG, Prague).

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Declaration of honour

This study was elaborated at the Laboratory of Genome Integrity at the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic (Prague) under the supervision of Zdeněk Hodný, MD, PhD.

I hereby confirm on my honour that I personally prepared the presented academic work. I also confirm that I have used no resources other than those declared. Furthermore, I confirm that the entire content of this thesis or its major part was not previously used for obtaining of the same or other academic degree.

Prague, 19th June, 2015

Signature

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Foreword

Ionizing radiation (IR) is a commonly used treatment strategy to manage cancer with up to 60% of all cancer patients receiving the radiation therapy (RT) during the course of their disease. Radiation is typically delivered to patient in multiple 2-Gy fractions for 5 – 7 weeks in total dose 60 – 79 Gy. Despite the initial advantage in local tumor control, small fraction of cancer cells does not die immediately following radiation. Such radiation-surviving cells and even the cells undergoing cell death shape the tumor microenvironment by production of various cytokines, hormones and growth factors. More importantly, such survivors possess multiple genome alterations predisposing them to gain agressive behaviour. In this context, repeated fractions of irradiation, as administered also to prostate cancer (PCa) patients, cause surviving cells with altered phenotype that may affect the responsiveness to subsequent targeted therapies. In this Thesis, I wanted to analyze the phenotype of radiation-surviving cells with the purpose to identify potentional molecular therapeutic targets with the radiosensitizing effect.

To my knowledge, this is the first study using clinically relevant fractionated irradiation (35 doses of 2 Gy) to study radiation-surviving prostate cancer cells *in vitro*. Importantly, along with commonly studied irradiation-surviving adherent cells, we identified yet-unexplored surviving non-adherent fraction (derived from adherent cell layer) containing viable stem-like but proliferatively dormant cells with the potential to readhere and reinitiate growth after the end of genotoxic stress and recovery phase mimicking the metastatic invasion cascade.

In the Introduction, I summarize the current knowledge of the PCa in general and its treatment strategies including RT. I continue to provide the information about formation of metastases and I finish with the effects of genotoxic stress to cancer cells at the molecular level. In the following sections, I present and discuss the most important aspects resulting from the experimental part of my doctoral studies concerning characterisation of radiation-surviving PCa populations. Importantly, a part of the Result section (sub-section 4.5) is dedicated to yet unpublished follow-up data obtained from the whole-genome expression analysis of radiotherapy and/or chemotherapy-treated surviving cancer cells, which extends the first part (sub-sections 4.1 - 4.4). In the section Significance of Results and Future Prospects, I aimed to put the presented results into a broader context and highlight their importance for the clinical radiobiology and oncology.

Taking together, I hope this thesis represents a comprehensive study providing deeper understanding of the resistance of cancer cells to genotoxic treatment.

Lenka Kyjacová, Prague, 2015

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Abbreviations

AKTi	Akt inhibitor;
APC	allophycocyanin;
ATM	ataxia-telangiectasia mutated;
Aza	5-azacytidine;
BM	basement membrane;
BMP	bone morphogenetic protein;
BMDC	bone marrow-derived cells;
BrdU	5-bromo-2'-deoxyuridine;
CAM	cell adhesion molecule;
CAF	cancer-associated fibroblast;
CDK	cyclin-dependent kinase;
Chk1/2	checkpoint kinase 1/2;
CKIα	casein kinase I alpha;
COX	cyclooxygenase;
CSC	cancer stem cell;
CSF	colony-stimulating factor;
CTC	circulating tumor cell;
CTM	circulating tumor microemboli;
d	day/s;
DAMP	damage-associated molecular pattern;
DAPI	4',6-diamidino-2-phenylindole;
DC	dendritic cell;
DDR	DNA damage response;
dox	doxycycline;
doxo	doxorubicine;
DSB	double-strand break;
DTC	disseminating tumor cell;
ECM	extracellular matrix;
EdU	5-ethynyl-2'-deoxyuridine;
EGF	epidermal growth factor;
EMT	epithelial-to-mesenchymal transition;
ERβ	estrogene receptor-beta;
ERKi	Erk inhibitor;
FACS	fluorescence activated cell sorting;
FDR	fold discovery rate;
FGF	fibroblast growth factor;
fIR	fractionated irradiation;
fRT	fractionated radiotherapy;
GF	growth factor;
GOBP	gene ontology biological process;
GOCC	gene ontology cellular component;
GOMF	gene ontology molecular function;
GS	Gleason score;
Gy	Gray;
h	hour/s;
HCA	hierarchical clustering analysis;
HDAC	histone deacetylases;
HGPIN	high-grade prostatic intra-epithelial neoplasia;
HIF	hypoxia-inducible factor;
HPEC	human prostate epithelial cells;
HR	homologous recombination;
HTS	high throughput sampler;
IL	interleukin;
IFN	interferon;
IR	ionizing radiation;
IRDS	interferon-related DNA signature;
IRF	interferon regulatory factor;
JNK	Jun N-terminal kinase;
LIF	leukemia inhibitory factor;
LFC	log fold change, base-2 logarithm;
lnRNA	long non-coding RNA;
LSD	lysine-specific demethylase;
MACS	magnetic-activated cell sorting;
mCRPC	metastatic castrate-resistant prostate cancer;
MDSC	myeloid-derived supressor cells;
me	methyl;
MEF	mouse embryonic fibroblasts;
MET	mesenchymal-to-epithelial transition;
min	minute/s;
miRNA	microRNA;
MMP	matrix metalloproteinase;
NC	negative control;
NHEJ	non-homologous end joining;
NLR	Nod-like receptors;
NSCLC	non-small cell lung cancer;
NuRD	nucleosome remodeling and deacetylase;
PAMP	pathogen-associated molecular pattern;
PCa	prostate carcinoma;
PCA	principal component analysis;
PcG	Polycomb group;
PDGF	plateled derived growth factor;
PGCC	polyploid giant cancer cell;
PI	propidium iodide;
PKC	protein kinase C;
PMA	phorbol 12-myristate 13-acetate;
PML NBs	promyelocytic leukemia nuclear bodies;
PRC	polycomb repressive complex;
PRR	pattern recognition receptors;
PSIS	p53-suppressed invasiveness signature;
PSA	prostate-specific antigen;
PMA	phorbol 12-myristate 13-acetate;
RLR	Rig1-like receptor;
ROS	reactive oxygen species;
RT	radiation therapy / room temperature (in Materials and Methods section);
qRT-PCR	quantitative real time polymerase chain reaction;
s	second/s;
SA-β-gal	senescence-associated beta-galactosidase;
SASP	senescence-associated secretory phenotype;
SBRT	stereotactic body radiotherapy;
scaRNA	small Cajal body RNA;
SCID	severe combined immunodeficiency;
S.D.	standard deviation;
SHP	Src homology phosphotyrosine phosphatase;
SIPS	stress-induced premature senescence;
snRNA	small nuclear RNA;
snoRNA	small nucleolar RNA;
SSB	single-strand break;
TAM	tumor-associated macrophages;
Tet	tetracycline;
TF	transcription factor;
TIC	tumor initiating cell;
TLR	Toll-like receptor;
wt	wild type.

1 ABSTRACT

Resistance of various cancers to conventional therapies including radio- and chemo-therapy is one of the most investigated phenomena in the molecular and clinical oncology. Recurrent disease is characterized by the presence of metastases, which are responsible for 90% of cancer-related mortality. Fractionated ionizing radiation (fIR) combined with surgery or hormone therapy represent the first-choice treatment for medium to high risk localized prostate carcinoma (PCa). In PCa, the failure of radiotherapy (RT) is often caused by radioresistance and further dissemination of escaping (surviving) cells.

To investigate the radioresistance-associated phenotype, we exposed four metastasis-derived human PCa cell lines (DU145, PC-3, LNCaP, and 22RV1) to clinically relevant daily fractions of ionizing radiation (fIR; 35 doses of 2 Gy) resulting in generation of two surviving populations: adherent senescent-like cells expressing common senescence-associated markers and non-adherent anoikis-resistant stem cell-like cells with active Notch signaling and expression of stem cell markers CD133, Oct-4, Sox2, and Nanog. While the radioresistant adherent cells were capable to resume proliferation shortly after the end of irradiation, the non-adherent cells started to proliferate only after their reattachment occurring several days after the irradiation-driven loss of adhesion. Like the parental non-irradiated cells, radioresistant readherent DU145 cells retained tumorigenic potential after injection to immunocompromised mice. We showed that fIR-induced phenotypic plasticity in PCa cells was accompanied with the epithelial-to-mesenchymal transition (EMT) as well as its reverse process mesenchymal-to-epithelial transition (MET). The radiation-induced loss of adhesion was dependent on expression of EMT-driver Snail (SNAI1), as transient siRNA or permanent shRNA-mediated knockdown of Snail prevented loss of protein of adherent junctions E-cadherin (CDH1) and cell detachment. On the other hand, survival of the non-adherent cells required active Erk signaling, as chemical inhibition of Erk1/2 by a Mek selective inhibitor or Erk1/2 downregulation by siRNAs resulted in anoikis-mediated death in the non-adherent cell fraction. Notably, whereas combined inhibition of Erk and PI3K/Akt signaling triggered cell death in the non-adherent cell fraction and blocked proliferation of the adherent population of the prostate cancer cells, such combined treatment had only marginal if any impact on growth of control normal human diploid cells. Importantly, irradiated re-adherent cells exhibited less senescent-like colonies in clonogenic cell survival assay and enhanced anoikis-resistant survival upon reirradiation pointing to the acquired radioresistance.

Since dormant fIR-surviving non-adherent PCa cells shared common features with metastases-related disseminated tumor cells (DTCs) such as low proliferation potential, expression of stemness markers and capability to resume adherent growth after the end of genotoxic stress and as these characteristics are known to contribute to therapy resistance, development of metastases and tumor recurrence, we investigated the stress-induced 'floatation' phenomenon in more detail. We observed the same phenotypic plasticity in both breast adenocarcinoma cells (MCF-7) treated with fIR (10 x 2 Gy) and cervical cancer cells (HeLa) treated with chemotherapeutic drug 5-azacytidine (4 μM / 24 hours for 7 days), showing that occurrence of viable non-adherent cells is not restricted to cancer cell origin or to the type of genotoxic insult. As a next step, we performed high-throughput whole genome transcriptional profiling of radio- and/or chemoresistant cancer cell populations. Data analysis revealed the exclusive expression pattern in radio- and chemo-therapy-surviving non-adherent cancer cells including active cytokine signaling and induction of interferon-responsive genes.

Taking together, these results contribute to better understanding of radiation-induced heterogeneous molecular response of human metastatic PCa cells, document treatment-induced phenotypic plasticity of stress-surviving cells, decipher a key molecular mechanisms of radio- and chemo-resistance, and finally, provide options to overcome the therapy resistance.

ABSTRAKT

Rezistence různých typů nádorů ke konvenční léčbě radiací nebo chemoterapeutiky patří mezi nejčastěji zkoumané fenomény v oblasti molekulární a klinické onkologie. Pro rekurentní onemocnění je charakteristická přítomnost metastáz, které jsou v 90% odpovědné za úmrtí pacientů. Frakcionované ionizující záření (fIR; *fractionated ionizing radiation*) v kombinaci s chirurgickým odstraněním nádoru nebo hormonální terapií představuje nejčastější typ léčby v případě lokalizovaného nebo lokálně pokročilého karcinomu prostaty (KP). Hlavní příčinou neúspěchu radioterapie KP je radiorezistence s následnou diseminací přeživších buněk do okolních tkání.

Ve snaze lépe pochopit fenotyp buněk asociovaný s radiorezistencí jsme vystavili čtyři buněčné linie KP derivované z metastáz (DU145, PC-3, LNCaP a 22RV1) klinicky relevantním, denně se opakujícím dávkám ionizujícího záření (fIR; 35 x 2 Gy), což mělo za následek vznik dvou přeživších buněčných populací - adherentních buněk vykazujících známky senescence včetně exprese markerů asociovaných se senescencí a neadherentních buněk rezistentních k anoikis, s rysy kmenových buněk, včetně aktivní sinalizace Notch a exprese kmenových znaků CD133, Oct-4, Sox2 a Nanog. Zatímco radiorezistentní adherentní buňky byly schopné obnovit proliferaci krátce po skončení ozařování, neadherentní buňky začaly proliferovat pouze po readhezi, ke které docházelo několik dnů po ztrátě adheze indukované iradiací. Podobně jako neozářené parentální buňky si radiorezistentní readheretní buňky DU145 zachovaly tumorigenní potenciál po transplantaci do imunokompromitovaných myší. Ukázali jsme, že pozorovaná fenotypová plasticita buněk KP indukovaná fIR byla doprovázena epiteliálně-mezenchymální tranzicí (EMT; *epithelial-to-mesenchymal transition*), stejně jako opačným procesem - mezenchymálně-epiteliální tranzicí (MET; *mesenchymal-to-epithelial transition*). Ztráta adheze indukovaná pomocí fIR byla závislá na expresi faktoru regulujícího EMT - transkripčního faktoru Snail (SNAI1), protože krátkodobé (siRNA) nebo dlouhodobé (shRNA) snížení hladiny Snailu zabránilo inaktivaci mezibuněčného adhezivního proteinu E-kadherinu (CDH1) a ztrátě adheze. Na druhé straně přežití neadherentních buněk vyžadovalo aktivní signalizaci Erk, protože chemická inhibice Erk1/2 prostřednictvím selektivního inhibitoru kinázy Mek nebo tranzientní snížení kináz Erk1/2 prostřednictvím siRNA mělo za následek buněčnou smrt (anoikis). Zatímco kombinovaná inhibice signalizace Erk a PI3K/Akt u neadherentních buněk indukovala buněčnou smrt a měla za následek zablokování proliferace u adherentních buněk KP, toto

kombinované ošetření mělo jen marginální efekt na růst normálních lidských diploidních buněk. Důležité je, že ozářené readherentní buňky tvořily v klonogenním testu méně kolonií se senescentním fenotypem a vykazovaly zvýšenou odolnost vůči anoikis po opětovném ozáření, což poukazuje na rozvoj získané radiorezistence.

Vzhledem k tomu, že dormantní neadherentní buňky, které přežily fIR, sdílejí společné rysy s buňkami dávajícími vznik vzdáleným metastázam, jako například sníženou schopnost proliferace, expresi znaků kmenových buněk a schopnost obnovit adherentní růst po ukončení působení genotoxického stresu, a protože o těchto vlastnostech je známo, že přispívají k rezistenci vůči terapii, k rozvoji metastáz a recidivy nádorového onemocnění, rozhodli jsme se studovat fenomén ztráty adheze po fIR podrobněji. Obdobnou fenotypovou plasticitu jsme pozorovali u ozářených (10 x 2 Gy) buněk adenokarcinomu prsu (MCF-7) a nádorových buněk děložního čípku (HeLa) po ošetření chemoterapeutikem 5-azacytidinem (4 μM/24 hodin po dobu 7 dní), což naznačuje, že výskyt životaschopných neadherentních buněk není vázan na původ rakovinných buněk ani na typ aplikovaného genotoxického stresu. Jako další krok jsme provedli celogenomovou expresní analýzu několika radio-/chemo-rezistentních rakovinných buněčných populací. Analýza dat poukázala na exkluzivní expresní profil u neadherentních buněk rezistentních vůči radio-/chemo-terapii s aktivní cytokinovou signalizací a indukcí genů stimulovaných interferony.

Souhrnně vzato, tyto výsledky přispívají k lepšímu pochopení heterogenity lidských metastatických nádorových buněk prostaty přežívajících ozáření, dokumentují plasticitu přeživších buněk indukovanou radiací, popisují molekulární odpovědi na ozáření a naznačují možnosti, jak v rámci protinádorové terapie překonat radiorezistenci využitím cílených radiosenzitizujících látek.

2 INTRODUCTION

2.1 PROSTATE CANCER

In general, cancer represents a leading cause of deaths in economically developed countries (Siegel et al., 2014), most likely due to chronic unsatisfactory life-style choices. PCa is the most frequent diagnosed cancer among men of advanced age worldwide (see **Fig. 2.1** for USA) and it is 6th leading cause of cancer deaths in men (Jemal et al., 2011b). According to International Agency for Research on Cancer (EUCAN; http://eco.iarc.fr/eucan/Country.aspx?ISOCountryCd=203) and The Czech National Cancer Registry (CNCR), incidence of PCa in Czech Republic was 22.29% (2012) which makes the PCa again the leading oncologic diagnosis among men (Dusek et al., 2010). Systematic screening for specific PCa markers like serum prostate-specific antigen (PSA) levels over the last two decades resulted in the detection of more PCa cases and contributed to the lower mortality rate relative to the incidence rate. On the other hand, it was shown that prevention and early treatment of PCa has no effect on disease-related mortality and additionally can have severe side effects resulting in the treatment-resistant disease (Andriole et al., 2009).

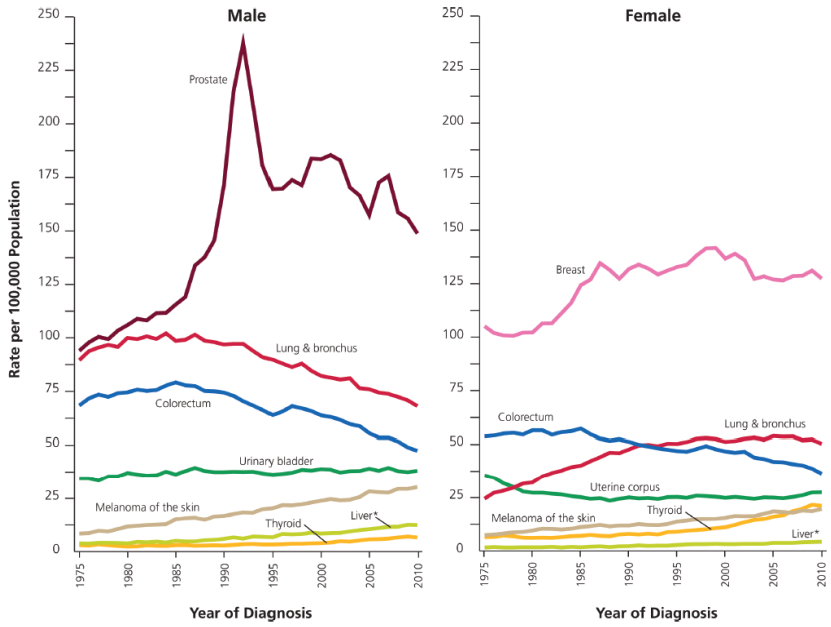

Figure 2.1. Trends in incidence rates of various cancers in both sexes, United States, 1975 – 2010. Prostate and breast cancer are the most frequently diagnosed cancers according to sex (Siegel et al., 2014).

2.1.1 Prostate cancer progression

Precursors for PCa are (i) proliferative inflammatory atrophy (PIA), characterized by the presence of atrophic cells, low apoptosis and high proliferation of epithelial cells; (ii) prostatic intraepithelial neoplasia (PIN) or high-grade prostatic intraepithelial neoplasia (HGPIN) containing genotypically and architecturaly altered cells expressing either markers of early invasive carcinoma or markers of senescence; and (iii) atypical small acinar proliferation (ASAP) which represents precancerous lesion most related to PCa (Felgueiras et al., 2014). PCa is a very heterogeneous disease which usually arises from prostate epithelial cells positive for androgen receptor (AR) (Bostwick, 1989). Factors that contribute to the development of PCa are genomic alterations (loss of heterozygosity, activation of oncogenes, inactivation of tumor-suppressor genes, gene fusions), epigenetic modifications, inflammation, oxidative stress, DNA damage and many others (Felgueiras et al., 2014). Although localized PCa tumors are initially androgen-dependent, the disease could become hormone refractory and metastatic with metastases in bone, lung, brain or liver (Bubendorf et al., 2000) (**Fig. 2.2**).

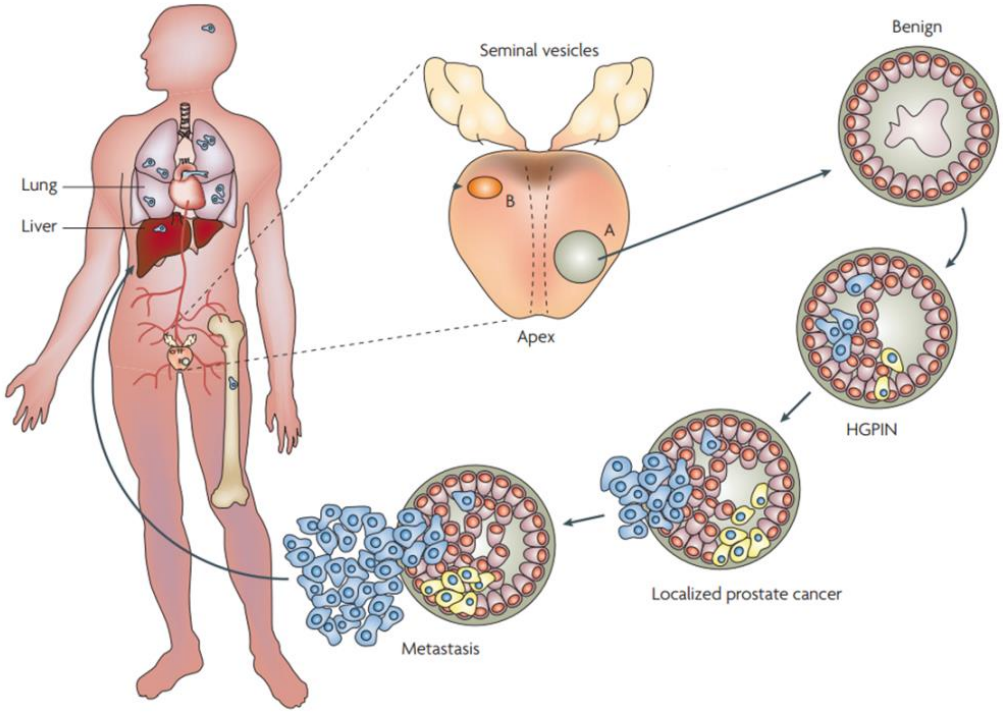

Figure 2.2. Prostate cancer progression. In prostate, multiple genetically distinct histological foci ('A' and 'B') with benign nature can represent precursors for the development of high-grade prostatic intra-epithelial neoplasia (HGPIN). Localised tumor often contains highly invasive cells which can establish secondary tumors (metastasis) at distant organs including brain, lungs, liver, and bones (adapted from (Kumar-Sinha et al., 2008)).

2.1.2 Prognostic markers for prostate cancer

Besides the increased age, the most common risk factors for PCa represent combination of genetic (family history of the disease, race/ethnicity) and environmental influences like age, inappropriate eating habits, early start with sexual life and sexually transmitted diseases (Nelson et al., 2003). Currently, the most common prognostic markers for PCa diagnosis is the prostatic hypertrophy detected by initial digital rectal examination (DRE), serum PSA levels and transrectal ultrasonography (TRUS). For definite diagnosis of PCa, prostate biopsy is important followed with the histopathological analysis, which serves as a basis for estimation of disease grade by so called Gleason score (GS; 5 grade system). Another grading system, TNM (Tumor, Node, Metastases), evaluates a size of the tumor, an extent of lymph nodes affliction and presence of metastases in distant organs. TNM categorizes the disease into four stages (I-IV) (Edge and Compton, 2010). The advances in genomic technologies led to development of new biomarkers such as urine-detected PCA3 and TMPRSS2-ERG gene fusion (Prensner et al., 2012). The biomarker research utilizes also new tissue and imaging techniques (see **Fig. 2.3**). The presence of circulating tumor cells (CTCs) in the bloodstream usually signalizes aggressive or metastatic PCa and predicts the worst overall survival (de Bono et al., 2008). A completely new area is a detection of prostate-derived exosomes (prostatosomes) in the blood. It was demonstrated that elevated levels of blood exosomes in PCa patients correlate with increasing GS (Prensner et al., 2012; Tavoosidana et al., 2011).

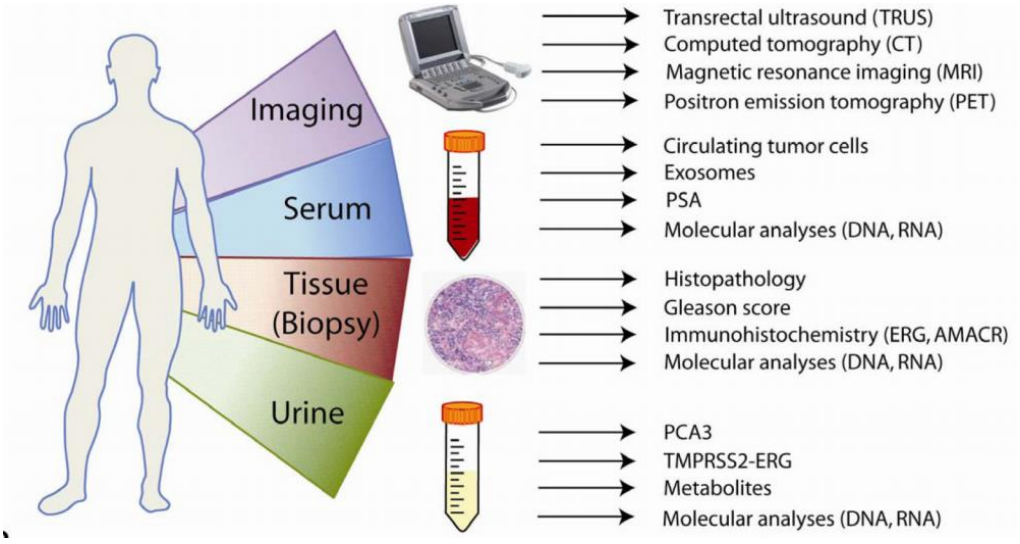

Figure 2.3. Prostate cancer biomarkers. The list of imaging biomarkers and biomarkers present in biological samples such as serum, tissue and urine (Prensner et al., 2012)**.**

2.1.3 Therapeutic approaches for treating prostate cancer

Treatment of PCa should be adjusted according to (i) tumor grade and stage, (ii) serum PSA levels, and (iii) estimated patient life expectancies. Therapy should be chosen also according to stages typical for tumor progression, which include (i) hormone sensitivity, (ii) androgen-independency, (iii) presence of oligometastases, and (iv) presence of advanced metastases (Oudard, 2013). The most common therapy for localized PCa is surgery (radical prostatectomy) followed by fractionated radiotherapy (fRT) (Wilt et al., 2008). After the dissemination, but retained hormone sensitivity of PCa cells, androgen deprivation therapy achieved by surgical or chemical castration is applied. However, tumor often becomes castration-independent as disease progresses, also as a result of applied treatment. Castration-resistant PCa, usually accompanied by the presence of metastases (metastatic castration-resistant prostate cancer, mCRCP), is largely incurable due to the high heterogeneity and acquired resistance to current therapeutics (Ewald et al., 2013). Approved in 2004, the first-choice therapy for mCRCP was docetaxel combined with prednisone; however, treatment strategies of relapsing disease were missing. Recent advances in PCa treatment led to approval of promising novel agents with overall survival benefit including immunotherapeuticals, radiopharmaceuticals, AR signaling-suppressors, and non-hormonal small molecule inhibitors (Suzman and Antonarakis, 2014).

2.1.3.1 Radiation therapy

RT is broadly used to manage many cancerous diseases as a (i) neoadjuvant therapy before the main treatment (e.g. surgery); (ii) adjuvant therapy after the main treatment (e.g. surgery); (iii) curative therapeutic modality; (iv) salvage treatment for managing recurrent disease (both local and distant - metastasis) in patients who failed to respond to conventional treatment or (v) as a palliative care (Boyer et al., 2014; Mohiuddin et al., 2015). Currently, about 50% of cancer patients are treated by RT during the course of their disease (Baskar et al., 2012). Low-dose-rate/high-dose-rate brachytherapy (LDRB/HDBT, see below) and high-dose external-beam RT (EBRT) currently represent major modalities for localized PCa. Radical prostatectomy is often combined with the post-operative salvage RT in high-risk localized PCa in order to eliminate residual transformed cells (Hayden et al., 2010). However, 6-year biochemical relapse-free survival after salvage RT was only 37% (Sia et al., 2008). Brachytherapy involves a permanent insertion of multiple radioactive sources into the prostate with the half-life of 2 months (Law and McLaren, 2010). Typical 8-week-long treatment with

EBRT consists of several fractions of IR with the respect to minimization of IR-related toxicity to surrounding tissue. The optimal dose for EBRT has not been yet established but some randomized trials showed advantage of escalating IR doses (74 – 79 Gy) over conventional IR doses (64 – 70 Gy) (Zietman et al., 2010). On the other hand, Pinkawa suggested that the optimal dose for preventing PCa metastases and increase overall survival is 60 – 66 Gy (Pinkawa, 2010).

The death-inducing effect of IR is based on X-ray ionization and release of electrons which cause DNA damage as well as generation of reactive oxygen species (ROS) by mitochondria, endoplasmatic reticulum and peroxisomes (Finkel and Holbrook, 2000). DNA lesions include oxidized bases, abasic sites, single-strand breaks (SSBs), and most deleterious double-strand breaks (DSBs) leading to the activation of DNA damage response (DDR) (Widdrington et al.) and usually to transient cell cycle arrest (Ward, 1995). DNA damage is either repaired by repair mechanisms, or if unrepaired, cells die through mitotic catastrophe or apoptosis (Pfeiffer et al., 2000). Importantly, it was demonstrated both *in vitro* and *in vivo* that occasional IR-surviving cells exhibit so called radiation-induced genomic instability which can be transmitted through the progeny (Niwa, 2006; Suzuki et al., 2003). Interestingly, it is suggested that IR can, besides mutations and chromosome alterations, cause also heritable epigenetic changes (Ilnytskyy and Kovalchuk, 2011; Mothersill and Seymour, 2012) and deregulated oxidative metabolism (Limoli and Giedzinski, 2003; Limoli et al., 2003), i.e. processes which can collectively stand behind radiation-induced carcinogenesis. Among non-apoptotic responses, permanent growth arrest, or premature senescence is commonly observed in response to IR. Senescent cells, despite their non-proliferative nature, remain metabolically active and produce various cytokines, chemokines, proteases, and growth factors which can grossly affect normal conditions of the adjacent microenvironment (Sabin and Anderson, 2011). Most importantly, it was demonstrated *in vivo* that stress-induced premature senescence (SIPS) is an important mediator of the transformation of pre-neoplastic cells (Krtolica et al., 2001; Liu and Hornsby, 2007) and enhances proliferation of yet transformed cells *in vitro,* as was shown for breast carcinoma (Tsai et al., 2009).

Due to a high heterogeneity of PCa and low reliability of current prognostic markers based on biopsy material, systemically expressed PSA is used as a main prognostic marker for relapse of the disease upon the therapy (Brawer, 2002). Importantly, the 5-year relapse rates in EBRT treated PCa patients divided into 4 PSA-based prognostic groups ranged between 12% - 81% making serum PSA the most relevant predictive marker for the disease outcome and responsiveness to RT (Zagars et al., 1995). Relapse of PCa after the RT was defined by

Kuban *et al.* as a PSA greater than 4.0 ng/ml (Kuban et al., 1995). On the other hand, Zietman *et al.* defined failure as PSA greater than 1.0 ng/ml 2 years or more after the RT or 10% increase of PSA in the first two years following RT (Zietman et al., 1994). Recurrent PCa is usually aggressive and is associated with the presence of metastases, as the risk of metastases 10 years after the surgery was 6%, 48%, and 81% for GS 6, 7, and 8 – 10 (Antonarakis et al., 2012). After EBRT, the recurrence and metastasis-free 10-year survival following RT was 81% and 100% for the low-risk group, 78% and 94% for the intermediate group, and 62% and 90% for the high-risk group, respectively (Alicikus et al., 2011).

IR has the potential to negatively influence both intracellular signaling and tumor microenvironment, therefore it is tempting to assume that there can be a direct or indirect link between RT and development of post-therapy cancer metastases as was already suggested (Ruegg et al., 2011; Su et al., 2012).

2.2 TUMOR METASTASES IN GENERAL

Metastases represent the end stage of multistep process of tumor progression termed the invasion-metastasis cascade (Valastyan and Weinberg, 2011). Metastases have become of great interest since more than 90% of cancer-related mortality is caused by metastatic cancer. Once developed, metastatic disease is largely incurable according to its systemic nature and high heterogeneity of disseminated tumor cells with acquired resistance to current therapeutics (Gupta and Massague, 2006; Weigelt et al., 2005).

The invasion-metastasis cascade is characterized by several step-by-step processes including (i) local invasion of primary tumor cells with tumor-initiating capacity (tumor-initiating cells; TICs) into surrounding tumor-associated stroma; (ii) intravasation into lumina of lymphatic or blood vessels; (iii) survival of malignant CTCs in circulation; (iv) extravasation out of endothelial vasculature by penetrating the endothelial layers that separate vessel lumina from stromal microenvironment as single cells or small number of clustered cells (disseminated tumor cells; DTCs); (v) surviving of extravasated carcinoma cells in the foreign microenvironment and subsequent formation of micrometastases; and (vi) formation of large macroscopic metastases in the process of metastatic colonization (Gupta and Massague, 2006; Valastyan and Weinberg, 2011) (**Fig. 2.4**).

The invasion-metastasis cascade is known to be a highly inefficient multistep process. Despite the fact that high numbers of CTCs are present in the bloodstream of the majority of carcinoma patients (Nagrath et al., 2007), only as few as 0.01% of tumor cells that enter into the circulation are able to form detectable metastasis at anatomically distant places of the body (Chambers et al., 2002; Zhe et al., 2011). However, solitary tumor cells or micrometastases can persist in the body in dormant state for many years or even decades post anti-cancer treatment and might eventually outgrowth to clinically detectable macrometastases as was documented for many cancer types including breast cancer (Ossowski and Aguirre-Ghiso, 2010; Willis et al., 2010; Yamashita et al., 2009).

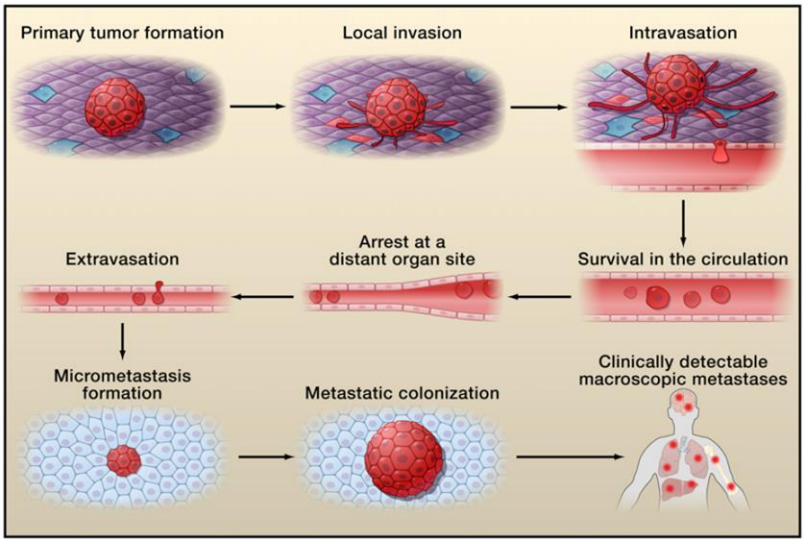

Figure 2.4. The invasion-metastasis cascade. The invasion-metastasis cascade encompasses several rate-limiting processes including local invasion of tumor cells followed by intravasation, arrest in distant organs after short circulation in the bloodstream and eventually extravasation to form micro- or macrometastasis in the process called metastatic colonization (Valastyan and Weinberg, 2011).

Importantly, such period of latency is not common for all cancer types, since lung and pancreatic adenocarcinomas display rather rapid progression to macrometastases (Nguyen et al., 2009). The most rate-limiting step of the invasion-metastasis cascade is the metastatic colonization with rate of attrition exceeding 99% cells, which initially survive in a foreign microenvironment to form oligometastasis (Valastyan and Weinberg, 2011).

Until 2009 it was thought that metastasis is exclusively an unidirectional process. Then Massagué and colleagues provided evidence that CTCs can also colonize their tumors of origin, in a process termed ‘tumor self-seeding’ (Kim et al., 2009). They showed that in case of breast, colon and melanoma tumors, tumor-derived cytokines IL6 and IL8 serve as CTCs attractants whereas both matrix metalloproteinase 1 (MMP-1/collagenase-1) and actin bundling protein fascin-1 (FSCN1) act as mediators of CTCs infiltration into mammary tumors. They also provided evidence that such self-seeding enhances tumor growth, angiogenesis and stromal recruitment, including leukocytes, macrophages, neutrophils, and myeloid cells. This recruitment is mediated by cytokine CXCL1 produced by seeder cells (Kim et al., 2009).

It is not clear whether dissemination of tumor cells with metastatic potential is an early or late event in tumor progression. According to the ‘linear progression model’, tumor dissemination occurs after the substantial expansion of primary tumors. In contrast, ‘parallel progression model’ suggests early tumor dissemination from even early premalignant lesions

as was documented for human breast carcinoma patients and ERBB2 (HER2/neu) mutant breast cancer (Husemann et al., 2008; Klein, 2009).

2.2.1 Origin of metastatic precursors

Two major models concerning the origins of TICs potentially leading to metastasis are the 'cancer stem cell' and the 'clonal evolution' model.

2.2.1.1 The cancer stem cell model

The cancer stem cell (CSC) hypothesis proposes the hierarchical organization of the normal tissues where the ability to initiate tumors and to give rise to heterogeneous cell populations is attributed exclusively to CSC population with the self-renewal ability (Hamburger and Salmon, 1977; Nguyen et al., 2012). These stem cells generate progeny with diverse differentiation status and thus represent a source of overall tumor heterogeneity (Alison and Islam, 2009). CSC model was strongly supported by the identification of CSCs in various human cancers including malignant germ cell cancers, leukemias (Lapidot et al., 1994), melanomas (Schatton et al., 2008) and cancers of the brain (Reya et al., 2001), breast (Al-Hajj et al., 2003), prostate (Collins et al., 2005), lung (Kim et al., 2005), pancreas (Hermann et al., 2007; Li et al., 2007), colon (Dalerba et al., 2007), and several others.

CSCs represent a quiescent/slow cycling pool of cells which make them less responsive to conventional treatment strategies (Li et al., 2008). Moreover, potential reactivation of quiescent CSCs might induce a tumor relapse even decades after the therapy (Pantel et al., 2009a). In head and neck, pancreatic, and non-small cell lung cancers the frequency of CSCs represent a very small subpopulation of cells (<0.02%) (Ishizawa et al., 2010). CSCs in serous ovarian cancer seem to be also infrequent (<0.04%) (Stewart et al., 2011). Interestingly, it was shown that cancers can harbor biologically distinct CSC populations with the ability to shuttle between particular phenotypes (Schober and Fuchs, 2011). It is believed that within CSC population, CSCs subsets with the tumor-propagating and/or metastatic potential exist. $CD44^+$ CSCs population of breast primary tumor cancer is directly involved in metastasis (Liu et al., 2010). Similarly, the presence of $CD26^+$ cells in the primary tumors of colorectal cancer predicts metastasis formation in patients (Pang et al., 2010).

2.2.1.2 The clonal evolution model

Alternative model for initiation and propagation of cancer is the clonal evolution model. This concept also assumes a clonal origin of cancers; however, it does not propose hierarchical organization for tumors. In this concept each cell within a tumor has equal potential to acquire stochastic genetic and/or epigenetic changes conferring proliferative and/or survival advantage and to undergo clonal selection in the terms of Darwinian evolution model (Greaves and Maley, 2012; Nowell, 1976). However, Darwinian model seems to be in conflict with the fact that molecular requirements for carcinoma cells which reside in primary tumors are different from those in metastatic lesions and that metastatic colonization is the rate-limiting event, therefore it is not clear how cells that are able to metastasize to distant organs arise within the primary tumors (Valastyan and Weinberg, 2011). Despite discrepancies, clonal evolution model is followed by some cancers, however, a growing body of scientific evidence supports a hierarchical model for the majority of solid tumors (Shackleton et al., 2009).

2.2.1.3 Cancer cell plasticity

Deregulation of genes governing stemness by genetic or epigenetic mutations in differentiated normal or cancer cells can induce plastic changes (dedifferentiation) leading to acquiring the stem cell properties (Marjanovic et al., 2013). Some authors suggest the important role of EMT in achieving the CSC-like state. Differentiated epithelial mammary cancer cells ectopically expressing EMT drivers (Snail, Twist1) express also CSCs markers, have self-renewal capability, and exhibit enhanced tumorigenicity (Mani et al., 2008; Morel et al., 2008). Moreover, Chaffer *et al*. showed that differentiated mammary epithelial cells (non-CSC) can revert to a stem-like state in a stochastic manner *in vitro* which brings the clonal and CSC models closer together (Chaffer et al., 2011). Overall, we shoud consider two sources of intratumor heterogeneity: (i) clonal heterogeneity resulting from changes in genome and epigenome and (ii) non-CSC-to-CSC plasticity influencing the tumor cell hierarchy (Marjanovic et al., 2013).

2.3 STAGES OF METASTATIC PROCESS

2.3.1 Initial neoplastic transformation

In accordance to the clonal evolution model, tumor cell heterogeneity is indispensable for the effective selection for advantageous traits in terms of invasion-metastatic cascade. Such aggressive phenotype includes self-renewal capacity, increased invasiveness, enhanced motility, survival after the detachment, and many other characteristics (prerequisites). It is believed that cellular heterogeneity is supplied by the intrinsic instability of cancer genomes, chromosomal rearrangements, and epigenetic alterations (Fidler, 2003).

Cells face several intrinsic and extrinsic pressures that can select for aggressive-hyperproliferative phenotype. The intrinsic barriers against tumorigenesis are genotoxic stress induced by oncogenesis, activation of senescence, apoptotic and growth inhibitory pathways or telomere shortening/telomerase inhibition. Extrinsic pressure comes from tumor microenvironment and includes extracellular matrix (ECM) components, basement membranes (BM), reactive oxygen species (ROS), hypoxia, nutrients or oxygen limitations, and the action of immune system cells. Importantly, the higher heterogeneity is present within the primary tumor, the higher possibility of cancerous cells to bypass barriers against the metastatic progression (Gupta and Massague, 2006).

It was shown that DNA damage is an early step in tumor initiation present even in normally appearing cells and becomes more prominent as tumor progresses (Bartkova et al., 2005; Gorgoulis et al., 2005). Dysregulation of DNA repair machinery can promote accumulation of DNA mutations and generate genomic instability, thus potentiate the tumor initiation, progression and even metastasis (Broustas and Lieberman, 2014). Genomic instability may be driven by mutations in the DNA and can result in various outcomes. For example, inactivation of cell cycle suppressor Rb affects mitotic checkpoint and finally results in aneuploidy (Michel et al., 2004). Hyperphosphorylated (activated) protein-kinase Akt can attenuate DNA damage checkpoint machinery through the inactivation of checkpoint kinase 1 protein (Chk1) (Puc et al., 2005).

Epigenetic genome modulation is also supposed to be an important source of tumor cell heterogeneity. For example, ectopic overexpression of EZH2 (the polycomb group protein) resulted in chromatin remodeling which correlated with metastasis and poor prognosis in PCa patients (Varambally et al., 2002). Moreover, there is strong evidence that

epigenetic regulation is closely linked to CSC state and epithelial-mesenchymal plasticity, traits typical for TICs (Tam and Weinberg, 2013).

2.3.2 Local invasion

The first steps of invasion-metastases cascade encompass processes like (i) changes of initial cancer cell plasticity; (ii) tumor cell invasion and degradation of local stroma; and finally (iii) intravasation.

2.3.2.1 Epithelial-mesenchymal plasticity

Cancer cells evolve different strategies how to invade other tissues. During the cancer progression, a variety of tumor cells can exhibit different changes in terms of morphological and phenotypical plasticity including (i) the collective-to-amoeboid transition (CAT) (Hegerfeldt et al., 2002); (ii) the mesenchymal-to-amoeboid transition (MAT) (Wolf et al., 2003); and finally, the most common and also the most studied (iii) epithelial-to-mesenchymal transition (EMT) (Tsai and Yang, 2013).

Carcinoma cells residing in primary tumors exhibit predominantly epithelial characteristics with typical E-cadherin-containing cell-to-cell contacts. In order to invade and disseminate to distant organs, neoplastic epithelial cells must shift, at least transiently, to a mesenchymal state. This transition is achieved by a complex biological program termed the EMT. The EMT and the reverse process, termed the mesenchymal-to-epithelial transition (MET), was originally recognized as a highly conserved developmental program crucial for metazoan embryogenesis, chronic inflammation and fibrosis (Kalluri and Weinberg, 2009). During the tumor progression, carcinoma cells often gain the ability to reactivate the EMT program and use it for their own purpose. Over the last two decades is EMT considered as a crucial event in cancer progression and metastatic process including breast, prostate, colon, head and neck, ovary, and lung cancer (Thiery et al., 2009). Phenotypically, EMT is characterized by the loss of epithelial polarity and cell-to-cell contacts followed by gain of mesenchymal traits including the increased migratory and invasive capabilities (Kalluri and Weinberg, 2009). At a molecular level, this transition is orchestrated by various signals that come from surrounding microenvironment acting in autocrine and paracrine manner (Moustakas and Heldin, 2007; Scheel et al., 2011) and activating the EMT-inducing transcription factors (EMT-TFs), which regulate their downstream target genes required to execute the most steps of the invasion-metastasis cascade (Polyak and Weinberg, 2009).

Distant metastases to many organs including liver, lung, bone, and brain often exhibit epithelial phenotype of primary tumors suggesting transient and reversible nature of the EMT (Chaffer et al., 2006; Prudkin et al., 2009). Moreover, EMT is coupled to CSCs-related traits involving a low proliferative potential. Growth arrest is also characteristic for many normal tissue stem cells, as well as for CTCs and DTCs (Brabletz et al., 2001; Jung et al., 2001). This led to the hypothesis that differentiated tumor cells should be reverted in the MET process in order to overcome EMT-related proliferative block (Chaffer et al., 2006; Chaffer et al., 2007) (**Fig. 2.5**). For example, in isogenic system of breast cancer cell lines, transfection of mesenchymal clone 4T07 with MET-inducing microRNA (miRNA) finally enabled macrometastatic growth (Dykxhoorn et al., 2009). Using transforming growth factor-β (TGF-β) reporter genes and mouse model, it was shown that TGF-β-induced EMT was necessary for lung cancer cells dissemination but on the other hand, constitutive TGF-β signaling prevented metastatic growth in lungs (Giampieri et al., 2009). Consistent with this findings, differentiated metastases have been observed in lung, prostate, breast, colorectal and hepatic carcinoma models (Tsuji et al., 2009).

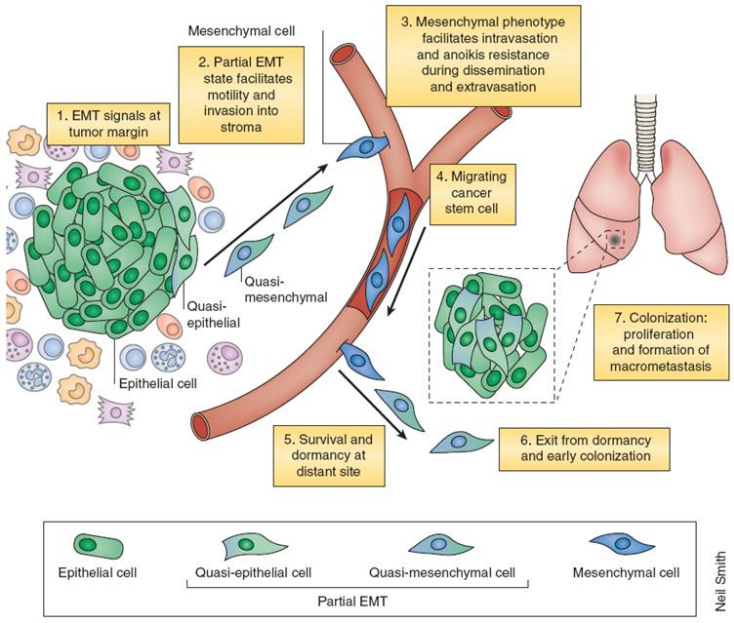

Figure 2.5. Epithelial-mesenchymal plasticity during the invasion-metastasis cascade. Transformation towards mesenchymal phenotype is important for gaining invasive properties. In contrast, epithelial features are necessary for successful colonization of DTCs (Tam and Weinberg, 2013).

2.3.2.1.1 Role of microenvironmental signals in promoting epithelial-mesenchymal plasticity

Tumor microenvironment comprises different cell types including cells of the blood and lymphatic system, pericytes, stromal myofibroblasts (or cancer-associated fibroblasts,

CAFs), bone marrow-derived cells (BMDCs), namely mast cells, macrophages, neutrophils, myeloid cell-derived suppressor cells (MDSCs), and mesenchymal stem cells that can support cells to increase metastatic potential (Coussens and Werb, 2002; Joyce and Pollard, 2009) (**Fig. 2.5**). Various extracellular signals including TGF-β, Wnt, Notch, and growth factors (GFs) acting through tyrosine kinase receptors were documented to induce EMT (Gao et al., 2012b). Labelle *et al.* showed that platelet-derived TGF-β activates EMT in CTCs leading to the activation of TGF-β/Smad and NF-κB downstream pathways and enhancing metastasis *in vivo* (Labelle et al., 2011). Tumor-associated macrophages (TAMs) were shown to promote tumor progression via enhanced angiogenesis, immune suppression and inducing EMT via TGF-β (Bonde et al., 2012). TAMs in F9-teratocarcinoma-bearing mice promoted EMT via TGF-β and activation of β-catenin pathway (Bonde et al., 2012). Similar effect in promotion of the EMT was described in bone marrow-derived mesenchymal cells (Martin et al., 2010). Besides the role of CAFs in ECM dynamic remodeling, they are also implicated in induction of EMT. CAFs derived from lung tumors produce hepatocyte GF thus activate EMT-related c-MET pathway (Wang et al., 2009). CAFs isolated from prostate tumors activate EMT program by producing metalloproteinases (Giannoni et al., 2010).

2.3.2.1.2 EMT-inducing transcriptional factors and miRNAs

Downregulation of adhesion between malignant cells in order to gain invasive behavior is a key step in local cell invasion. Lost of cell cohesion is driven by lower expression of cell adhesion molecules (CAMs). Among most studied CAMs belong (i) cadherins (including E-cadherin) which represent cell-to-cell adhesion molecules involved in formation of adherens junction; (ii) their accessory cytoplasmic proteins catenins needed for full cell-to-cell adhesion activity (Vasioukhin, 2012); and (iii) integrins, important for the process of local stromal invasion (Eke and Cordes, 2014).

Loss of E-cadherin is an important hallmark of EMT. Extracellularly, E-cadherin directly interacts homotypically with E-cadherin molecules on surrounding cells, thereby stabilizes the cell-to-cell contacts. Intracellularly, E-cadherin binds to β-catenin, α-catenin and p120-catenin which mediates the further signaling and links adherent junctions to the actin cytoskeleton (Harris and Tepass, 2010). Mechanisms of E-cadherin loss in epithelial tumors include various genetic (inactivating mutations) and epigenetic alterations (promoter hypermethylation) (Strathdee, 2002), proteolytic cleavage (Johnson et al., 2007), proteolytic degradation (Fujita et al., 2002), and transcriptional regulation via EMT-TFs (Zheng and Kang, 2014).

EMT-TFs include Snail (SNAI1) (Battle et al., 2000; Cano et al., 2000), Slug (SNAI2) (Hajra et al., 2002), Twist1 (Yang et al., 2004), Twist2 (Fang et al., 2011), Zeb1 (Eger et al., 2005), Zeb2 (Comijn et al., 2001), KLF8 (Wang et al., 2007), TCF3 (Perez-Moreno et al., 2001), and others. Expression of individual EMT-TFs has been found to activate EMT program leading to mesenchymal phenotype and invasive behavior in cancer (Peinado et al., 2007). Intracellular level of the EMT driver Snail is regulated through various signals from tumor microenvironment including components of TGF-β, Notch, and Wnt signaling, ROS and hypoxia, affecting its transcription (Cichon and Radisky, 2014; Radisky et al., 2005; Zhang et al., 2013; Zheng and Kang, 2014). For example, intracellular domain of Notch binds to Snail promoter under hypoxic conditions. Notch also facilitates binding of HIF1-α (hypoxia-inducible factor 1-alpha) to LOX gene promoter to upregulate lysyl oxidase (LOX) which stabilizes Snail protein (Sahlgren et al., 2008). Snail stability is also regulated through various post-translational modifications. Lats2-mediated phosphorylation of Snail at threonine 203 holds Snail in the nucleus and increases its stability. PAK1-mediated Snail phosphorylation at serine 246 promotes its repressive function and retains it in the nucleus. PDK1-mediated phosphorylation of Snail at serine 11 targets Snail from nucleus to cytoplasm for proteasomal degradation. Snail stability is regulated also by the Wnt/GSK3β/β-Trcp signaling pathway (Zheng and Kang, 2014).

Recently, numerous miRNAs have been demonstrated to regulate EMT, including Zeb1/miR-200 and Snail/miR-34 reciprocal feedback loops. EMT-TFs Zeb1 and Snail directly inhibit the transcription of corresponding miRNAs and, conversely, the miRNAs block their inhibitory EMT inducers at translation level. Zeb1 and Snail dominancy results in EMT-associated cell motility, stemness, growth arrest, and cell survival, whereas active miRNAs drive cells toward MET-associated differentiation, proliferation and drug sensitivity (Kim et al., 2011a; Park et al., 2008). Interestingly, both miRNA families are activated by p53 tumor suppressor, thereby shifting both feedback loops towards MET-associated phenotype (Chang et al., 2011; Kim et al., 2011b; Yamakuchi and Lowenstein, 2009).

2.3.2.1.3 The role of epigenetics in regulation of epithelial-mesenchymal plasticity

Recent studies have revealed important an connection between EMT-TFs and the control of the chromatin state resulting from histone modifications (Tam and Weinberg, 2013). Polycomb group (PcG) proteins compose polycomb repressive complexes (PRCs) which repress transcription of genes by histone modification and recruitment of various additional repressors. For example, Snail binds to the CDH1 (E-cadherin) promoter and

recruits components of PRC2 complex which catalyzes the trimethylation of H3K27 in neighbor nucleosomes resulting in CDH1 gene silencing (Herranz et al., 2008). Both Snail and Twist1 recruit the Mi-2-nucleosome remodeling and deacetylase (NuRD) repressive complex, containing histone deacetylases (HDACs), to the CDH1 promoter and contribute to its silencing (Fu et al., 2011; Peinado et al., 2004). Snail also mediates a recruitment of lysine-specific demethylase (LSD1) resulting in silencing epithelial genes including E-cadherin, claudins and cytokeratins (Lin et al., 2010). Moreover, Snail was shown to interact with histone methyltransferases to create H3K9me3 mark in promoter-associated chromatin which leads to long-term gene silencing of particular genes (Dong et al., 2013; Dong et al., 2012).

2.3.2.1.4 The role of EMT in the induction of stemness program

Recently, EMT has been linked to the CSCs phenotype in breast, pancreatic and colorectal tumors. CSCs were found to express EMT-TFs such as Twist1, Snail and Slug and, *vice versa*, EMT-undergoing cells were found enriched for CSC markers (Thiery et al., 2009). Untransformed immortalized human mammary epithelial cells undergo EMT upon expression of Snail or Twist1, or in the presence of TGF-β. Transformation of immortalized mammary epithelial cells by Ras or Her2/neu led to enrichment of $CD44^{high}/CD24^{low}$ cell subpopulation with concomitant induction of EMT phenotype (Mani et al., 2008; Morel et al., 2008). In pancreatic cancer, EMT-TF Zeb1 links EMT to stemness-maintenance by suppressing stemness-inhibiting miRNAs like miR-203, miR-200c, and miR183 (Wellner et al., 2009). Importantly, the Zeb1/miR-200 feedback loop mechanism is controlled by tumor suppressor p53 (Schubert and Brabletz, 2011). The Wnt signaling and myofibroblast-secreted HGF, both inducers of EMT, have been implicated in reprogramming of non-CSCs towards the CSCs-like phenotype in colon cancer (Vermeulen et al., 2010). Taken together, cancer stemness can be partially defined by environmental signals and EMT transformation further supported by mutations in tumor supressor genes like p53.

2.3.2.2 Cell invasion strategies and degradation of local stroma

Disruption of a BM and invasion into the stromal compartment is one of the first steps in invasion-metastasis cascade. Integrins, an important group of CAMs, mediate cell-to-matrix interactions during the cell invasion and migration by binding components of the BM and interstitial stroma including fibronectin, laminin, tenascin, thrombospondin, vitronectin, and collagen (Eke and Cordes, 2014). Degradation of ECM is performed by several proteases, including serine, cysteine, aspartyl proteinases, and metalloproteinases (MMPs). Besides BM

and ECM degradation, MMPs-expressing cells could also increase a concentration of GFs that are sequestered there, which potentiates the cancer cell proliferation (Kessenbrock et al., 2010). As the primary tumor progression proceedes, the stroma becomes 'reactive' and acquires characteristics of the wound healed, chronically inflamed stroma.

Cancer cells use several strategies also to invade surrounding tissue. They penetrate either in clusters by (i) collective cell invasion, or as single cells by (ii) mesenchymal cell invasion or (iii) amoeboid cell invasion (Friedl and Wolf, 2003). The group-of-cells invasion occurs in epithelial cancers such as breast, endometrial, colorectal cancers, and in melanoma (van Zijl et al., 2011). Collectively invading cells retain their cell-to-cell contacts, while the tip cells exhibit rather mesenchymal phenotype. Collective migration can be induced by the stromal cell-derived factor (SDF1/CXCL12) or signaling molecules from fibroblast growth factor (FGF) and TGF-β families. Traction force for the movement is provided by integrin-mediated binding to the ECM. For example, the leading cells express β_1 and β_3 integrins to connect to ECM protein fibronectin (Friedl and Gilmour, 2009), $\alpha_2\beta_1$ and $\alpha_V\beta_3$ integrins to attach to collagen and fibrin-rich surfaces (Farooqui and Fenteany, 2005), and the $\alpha_6\beta_1$ integrin to bind to BM protein laminin (Torimura et al., 1999). It was shown that tip cells produce a rate-limiting factor of collective invasion, metalloproteinase MT1-MMP, which enables cells to degrade ECM (Artym et al., 2006). The invasion of single mesenchymal (fibrosarcoma and glioblastoma) and epithelial cells (melanoma) was observed. However, in most carcinomas it is a case of epithelial cells which have undergone EMT (van Zijl et al., 2011). Activation of TGF-β and subsequent nuclear localization of Smad2 is responsible for single cell detachment from collectively moving cells (Giampieri et al., 2009). The invasion of single amoeboid cell was documented in breast cancer, lymphoma, sarcoma, melanoma, small-cell lung, and prostate carcinoma (van Zijl et al., 2011). Amoeboid cell migration is a faster migratory phenotype which is protease-independent. Treating cancer cell clusters with β_1 integrin or MMP inhibitors resulted in the morphological switch and amoeboid strategy for cell invasion indicating that invasion styles are not exclusive but rather act simultaneously or cooperatively (Hegerfeldt et al., 2002; Sabeh et al., 2009).

2.3.3 Intravasation

Process of intravasation involves tumor cells entering into systemic blood and lymphatic circulation. It is still not well understood whether intravasation is a passive or active process. Butler and Gullino and also Liotta *et al*. noted that millions of tumor cells are daily shed from a tumor, even though only few detectable metastatic colonies are formed

(Butler and Gullino, 1975; Liotta et al., 1974). Interestingly, Mehes *et al*. and Larson *et al*. referred that only few events isolated from peripheral blood from breast and prostate cancer patients were intact CTCs. The rest represented damaged cells positive for apoptotic markers or cell fragments (Larson et al., 2004; Mehes et al., 2001). Tumor larger than 1 mm in diameter needs to attract new blood vessels to supply oxygen and nutrients. These newly formed tumor-associated blood vessels are immature, poorly structured (leaky), and absent for precise pericyte coverage (Alitalo et al., 2005). Passive intravasation is also supported by the fact that trauma or massage of primary tumor increase number of CTCs in the vasculature and that for tumor cell intravasation is limiting the blood vessel size rather than the capacity of tumor cells to enter the circulation (Liotta et al., 1976). On the other hand there is increasing evidence that intravasation can be an active process relying on the secretion of growth factors and cytokines. For example, TGF-β type II receptor/Smad4 activation is implicated in dissemination of breast carcinoma (Giampieri et al., 2009). The intravasation of breast carcinoma can be also enhanced by secretion of epidermal growth factor (EGF) and colony stimulating factor (CSF-1) by perivascular TAMs via a positive-feedback loop with carcinoma cells (Wyckoff et al., 2007).

2.3.4 Survival in the vasculature and lymphatics

2.3.4.1 Circulating tumor cells

Despite the fact that only minority of CTCs finally forms detectable metastases, they undergo sequential genetic and/or epigenetic changes thus represent a highly heterogeneous cell population (Powell et al., 2012). Scatena *et al*. distinguished in their review three types of CTCs; (i) bystander CTCs with no EMT and/or CSCs characteristics, which enter into circulation passively by trauma and/or inflammation in the site of primary tumor and represent "non-tumorigenic" cancer cells, (ii) pathologically active CTCs with EMT and/or CSCs traits and (iii) migrating or circulating CSCs (Scatena et al., 2013). CTCs represent the route between primary tumors and metastasis, therefore are often termed as 'metastatic intermediates'. Yu *et al*. reported that CTCs isolated from breast cancer patients were enriched for mesenchymal clusters attached to TGF-β secreting platelets. Long-term CTCs monitoring enabled to uncover reversible nature of EMT/MET phenotype mapping the applied therapy (Yu et al., 2013). They also adopted microfluidic device for isolation of CTCs from mouse pancreatic cancer model and identified WNT2 as a candidate gene enriched in CTCs. WNT2 suppresses anoikis (see below), supports anchorage-independent growth and increases metastatic potential *in vivo* (Yu et al., 2012). It was also shown that CTCs can be

used to monitor the changes in epithelial tumors during the particular treatment (Maheswaran et al., 2008; Miyamoto et al., 2012).

CTCs exhibit a resistance to cell detachment-induced cell death – anoikis (Berezovskaya et al., 2005), mechanistically linked to EMT (Frisch et al., 2013; Howe et al., 2011). EMT-TF Twist1 confers anoikis resistance through the activation of tumor suppressor $p14^{ARF}$ (Frisch et al., 2013). Downregulation of E-cadherin in CTCs *per se* serves as a mechanism for anoikis resistance (Berezovskaya et al., 2005) and promotes metastases (Onder et al., 2008). Interestingly, Charpentier and Martin with their teams highlighted the role of EMT and deformable cytoskeleton of breast anoikis-resistant CTCs since mesenchymal protein vimentin promotes production of tubulin-based microtentacles (McTNs) potent to penetrate endothelial cells and support CTC survival and reattachment at distant organs (Charpentier and Martin, 2013; Willipinski-Stapelfeldt et al., 2005).

2.3.5 Extravasation

CTCs are transported to distant organs of the body in relatively short time after dissemination. It was demonstrated that the half-life of the disseminated breast cancer cell in the circulation is 1 – 2.4 hour (Meng et al., 2004). CTCs are relatively quickly either mechanically trapped in the microvessels or adhere to the vascular endothelium. In the circulation, CTCs face several stresses including physical damage, immune-mediated attacks and activation of cell death mechanism (anoikis) as a consequence of loss of adhesive support (Shibue and Weinberg, 2011). The tumor cell extravasation is associated with the retraction of endothelial cells from one another or results in their death (Miles et al., 2008). Gupta *et al*. have identified epiregulin (EREG), cyclooxygenase 2 (COX-2), MMP-1, and MMP-2 as factors involved in the disruption of pulmonary vascular endothelial cells-to-cell junctions during the extravasation of breast carcinoma cells in lungs (Gupta et al., 2007). Angiopoietin-2 (Angpt2), MMP-3, MMP-10, placental growth factor, and VEGF secreted by various types of tumors are also able to increase pulmonary permeability prior to the CTCs extravasation. Besides these factors, P-, L- and E-selectins, β_2- and β_3-containing integrins and splicing variants of CD44 were also shown to promote CTCs extravasation (Sleeman et al., 2011; Valastyan and Weinberg, 2011).

The deposition of fibrin and platelets around the CTCs serve as a barrier against mechanical stress as well as attacks of immunocytes such as natural killer (NK) cells (Hejna et al., 1999). Moreover, fibrin and platelets can facilitate the arrest of tumor cells in capillaries. Interestingly, a depletion of platelets in animal models suppresses metastasis

formation, and *vice versa*, platelet infusion promotes metastasis (Camerer et al., 2004; Karpatkin et al., 1988). In circulation, platelets can support EMT of CTCs by releasing TGF-β and plateled derived growth factor (PDGF) (Labelle et al., 2011). Platelets not only protect CTCs, but also provide pro-angiogenic and other growth factors that support growth of extravasated tumor cells. Proteases provided by recruited macrophages promote efficient extravasation and subsequent survival and outgrowth of the extravasated tumor cells (Qian et al., 2009).

2.3.6 Micrometastasis formation

Stephen Paget proposed his 'seed and soil' hypothesis concerning the organ-specific pattern of metastasis already in 1889 (Paget, 1889). He suggested that the competence of cancer cells to growth in distant places of the body (the 'seed') is dependent on the compatibility of the distal organ (the 'soil') for their growth. For example, breast cancer usually metastasizes to the lungs, liver, and brain. Advanced PCa predominantly metastatizes to bones whereas lung or liver metastases are less common. Uveal melanomas specifically metastasize to liver and sarcomas to the lungs (Gupta and Massague, 2006). Extravasated tumor cells are subjected to cell death, dormancy (survival without increase in cell number) or colony formation (survival with increase in cell number) (Chambers et al., 2002). The destiny they will follow depends on microenvironmental conditions at the site of future metastasis termed 'premetastatic niche'. Premetastatic induction of MMP-9 by endothelial cells and macrophages conditions the microenvironment to tumor cell outgrowth by stimulation of various integrins and liberation of molecules sequestered in ECM (Hiratsuka et al., 2002; Psaila and Lyden, 2009). Importantly, soluble factors produced by primary tumor appear to determine the organ site of future metastasis formation as well (Kaplan et al., 2005). For example, breast cancer expresses chemokine receptors CXCR4 and CCR7, while their ligands are expressed by cells in lung, liver and bone marrow, which are all sites of breast cancer metastasis (Muller et al., 2001).

2.3.6.1 Tumor cell dormancy

Tumor cells that have undergone extravasation are frequently kept in so called dormant state. Dormant tumor cells are supposed to be in quiescent state characterized by the high expression of cyclin inhibitors $p21^{waf1/cip1}$ and $p27^{kip1}$ which enable cell cycle arrest (Ossowski and Aguirre-Ghiso, 2010). Dormant cells that exist as micrometastases with lack of sufficient blood supply can balance between proliferation and cell death (Holmgren et al.,

1995). It was documented that single tumor cells can be detected in the blood of prostate or colorectal cancer patients in early stages of tumor progression (Melchior et al., 1997; Weitz et al., 1998). Single tumor cells were detected also in the lymph nodes and bone marrow of healthy woman with history of early-stage breast cancer (Pantel et al., 2009b). Dormant tumor cells are relatively stable. In PCa patients, the percentage of dormant DTCs in the bone marrow remains unchanged 5 years after the radical prostatectomy (Morgan et al., 2009). In the animal model, dormant tumor cells remain viable for 6 months (Goodison et al., 2003). As dormant tumor cells are resistant to the therapies preferentially targeting proliferating cells, they can persist in quiescent state for many years, therefore represent increased risk factor for metastasis formation even several years or decades post-therapy.

It is believed that formation of neovasculature ('angiogenic switch') at the site of micrometastasis is a critical step for escape from tumor dormancy. Supplementation of melanoma cells with only one angiogenic factor (VEGF) or simultaneous injection of tumor and angiogenic cells was able to induce the escape from the dormancy (Bayko et al., 1998; Indraccolo et al., 2006). Spontaneous escape from angiogenesis-dependent dormancy was described also in animal models of human breast cancer, glioblastoma, osteosarcoma, and liposarcoma. Switch to fast-growing phenotype was accompanied by stable genetic reprogramming (Almog et al., 2006; Naumov et al., 2006).

2.3.7 Metastatic colonization

Invasion and survival of tumor cells at distant organs do not guarantee the further proliferation and formation of macroscopic metastases. The gene expression analyses of breast cancer cells which metastasize to different secondary organs uncover organ-specific patterns indicating that distinct tissue microenvironments impose different requirements for the metastatic colonization (Bos et al., 2009; Kang et al., 2003; Minn et al., 2005; Tabaries et al., 2011). For example, breast carcinoma colonizing the lungs will use different transcriptional and/or epigenetic programs than the same breast carcinoma colonizing the bone or brain. Additionally, breast carcinoma colonizing the bone will use different programs than prostate cancer colonizing the same tissue (Valastyan and Weinberg, 2011).

In conclusion, development of cancer metastasis represents the final step of the invasion-metastasis cascade tightly controlled by both intracellular and environmental signals.

2.4 THE ROLE OF GENOTOXIC STRESS IN CANCER CELL PLASTICITY

2.4.1 Genotoxic stress-induced DNA damage and repair

One dose of 1 Gy generates more than 1000 SSBs, 40 DSBs and 30 DNA cross-links per one cell (Hennequin et al., 2008). The clinically used IR dose of 2 Gy produces about 3000 DNA lesions per cell with the increase in DSBs with the growing dose (Lomax et al., 2013; Rothkamm and Lobrich, 2003). DSBs and lesions that prevent the replication and transcription of DNA lead to the activation of DDR resulting in activation of downstream repair mechanisms (Bartek et al., 2007). Besides solitary DSBs, fractionated doses of IR used in clinical oncology produce so called clustered DNA damage (two or more lesions within one or two turns of the DNA), including complex DSBs which are very difficult to repair (Lomax et al., 2013; Nikjoo et al., 2001). Importantly, low non-cytotoxic doses of IR can induce non-DSB clustered damage in normal tissue in the vicinity of the tumor. Since these lesions are poorly repaired they can lead to mutations and eventually to cancerogenesis (Das and Sutherland, 2011).

The main IR-induced sensor, serine/threonine kinase from phosphatidyl inositol 3-kinase-like kinases family, ATM (ataxia-telangiectasia mutated; (Shiloh, 2001) is activated by autophosphorylation and subsequent monomerization upon recruitment to the DSB with pre-bound heterotrimetic MRN complex (Mre11-Rad50-Nbs1). After binding to the DSB-MRN complex, ATM phosphorylates, besides a plethora of other substrates, histone H2AX at serine 139 (termed γH2AX; (Rogakou et al., 1998)) in the neighboring chromatin which facilitates the subsequent DNA repair (Kurz and Lees-Miller, 2004). DDR signaling activates crucial cell cycle checkpoints in order to repair incurred DNA lesions during halted cell cycle progression. Progression through cell cycle is tightly regulated via cyclin-dependent kinases (CDKs) with specific function in each phase of cell cycle and inhibitors of CDK-cyclin complexes from INK4 ($p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$), CIP ($p21^{WAF1/CIP1}$), and KIP families ($p27^{KIP1}$ and $p57^{KIP2}$) (Malumbres and Barbacid, 2009). ATM-mediated phosphorylation of checkpoint kinase 2 (Chk2) leads to the inhibition of CDC25A phosphatase resulting in the inhibitory phosphorylation of CDK-cyclin complexes and finally in cell cycle arrest. Similarly, ATM-mediated phosphorylation of the tumor suppressor p53 leads to the activation of CDK inhibitor $p21^{waf1/cip1}$ which arrests cell in G1 phase of the cell cycle (Zou and Elledge, 2003).

Once the cell is arrested, IR-induced DSBs are repaired via homology-based mechanism (HR; homologous recombination) or via non-homologous end joining process (NHEJ) (Hartlerode and Scully, 2009). Mediator of DNA damage repair pathways 53BP1 privileges NHEJ-mediated DSBs repair thus affects the choice between homology-based and non-homology-based repair mechanisms in the damaged cells (Zimmermann and de Lange, 2014). In case the DNA damage is repaired properly, cell survives and can proceed to the next cell division. However, unrepaired or persistent DNA damage can either result in cell death (apoptosis, autophagy, necrosis, mitotic cell death) or can contribute to the development of premature senescence, the non-proliferative but metabolicaly active state (Eriksson and Stigbrand, 2010; Hanahan and Weinberg, 2011).

2.4.2 Genotoxic stress-induced cell death mechanisms

The usefulness of fIR as an anti-tumor modality is due to its tumor cell death-inducing effects. Two major cell death mechanisms triggered by IR are apoptosis and mitotic catastrophe. p53-dependent apoptosis occurs within hours after radiation exposure of interphase cells and does not require cell division (Eriksson and Stigbrand, 2010). Apoptosis is executed via activation of caspase cascade by intrinsic or extrinsic pathways (Riedl and Shi, 2004). Targets of activated effector caspases include various mediators and regulators of apoptosis as well as DNA repair and cell cycle regulators (Jin and El-Deiry, 2005). For the intrinsic pathway, mitochondrial outer membrane permeabilisation and release of cytochrome c to the cytoplasm regulated via pro-apoptotic Bax-like (Bax, Bak, Bok) and BH3-only (Bid, Bad, Bim, Noxa, Puma) and anti-apoptotic members (Bcl-2, Bcl-XL, Mcl1) of Bcl-2 family (Jin and El-Deiry, 2005) is typical. The extrinsic pathway of apoptosis is mediated via ligand (TNFα, FasL, TRAIL)-dependent activation of the membrane receptors from TNF superfamily (Johnstone et al., 2008; Wang, 2008; Yu and Shi, 2008).

TP53 gene, coding p53, is inactivated in more than half of human cancers (Hollstein et al., 1991; Soussi and Beroud, 2001), however, tumors frequently acquire resistance to apoptosis even in the presence of functional p53 (Igney and Krammer, 2002). IR-related cell death is executed by the mitotic catastrophe which seems to be promoted in the presence of p53 (Ianzini et al., 2006). However, in contrast to apoptosis and senescence, presence of p53 is not required for mitotic catastrophe (Roninson et al., 2001). Mitotic catastrophe is a type of cell death that occurs during or as a result of improper mitosis and is typically executed days after the IR (Jin and El-Deiry, 2005). Furthermore, problems in mitosis can result in altered cytokinesis causing multinucleation and aberrant chromosome segregation, multiplication of

nuclei, and polyploidy (see **Fig. 2.6**) (Erenpreisa et al., 2005; Eriksson et al., 2007; Roninson et al., 2001). Mechanistically, in p53-deficient tumors, cells can enter mitosis prematurely with unrepaired DNA damage as a consequence of compromised G2/M checkpoint (Eriksson et al., 2007; Kawamura et al., 2004). Some cells are able to enter next G1 phase without cytokinesis resulting in giant cancer cells with polyploid genomes (PGCCs; polyploid giant cancer cells). Importantly, in contrast to p53-positive cells, p53-negative status allows to undergo several cycles of DNA amplification and related accumulation of genome aberrations (Weaver and Cleveland, 2005). Another model suggests the role of multiplicated centrosomes in promoting mitotic catastrophe via multipolar mitotic spindle formation or polyploidisation (**Fig. 2.6c**) (Dodson et al., 2007).

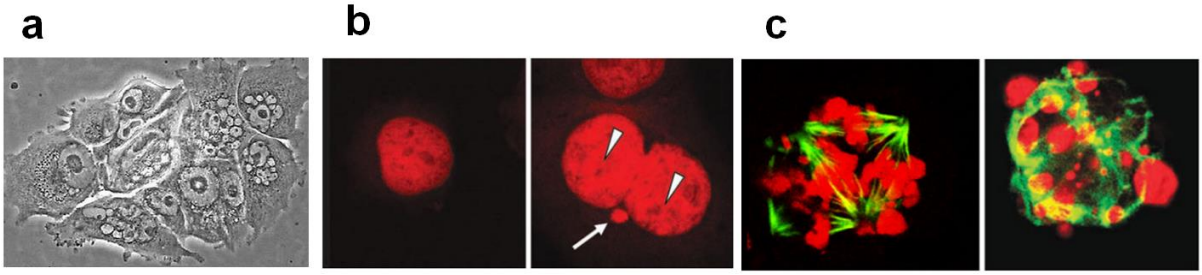

Figure 2.6. Mitotic catastrophe following IR. (**a**) Morphological features of cells undergoing mitotic catastrophe (Portugal et al., 2009). (**b**) Irradiated cells (right) display increased frequencies of multiple nuclei (arrowheads) and micronuclei (arrow) (adapted from (Castedo and Kroemer, 2004)). (**c**) Irradiated cells undergoing mitotic catastrophe as a result of hyperamplified centrosomes and formation of multipolar mitotic spindles (green, left) and subsequent induction of multiple micronuclei (red, right) (adapted from (Eriksson and Stigbrand, 2010)).

Necrosis and autophagy are less understood cell death mechanisms in response to DNA damage-inducing agents. For necrosis, increased vacuolisation, organelle degeneration with cell swelling and activation of pro-inflammatory pathways is typical as a result of release of cellular components from necrotic cells (Verheij, 2008). Such inflammatory response can, on one hand, recruit components of immune system to the sites of the tumor but, on the other hand, can induce various mitogens and pro-survival signals or increase motility of tumor cells leading to metastases (Ricci and Zong, 2006). Although it was thought that necrosis is non-programmed process, some data indicate that it can be partially regulated, therefore called in particular context necroptosis (Zong et al., 2004). Moreover, necrosis is, along with apoptosis, a final step in IR-induced mitotic catastrophe (Jonathan et al., 1999).

Autophagy is a physiological mechanism for degradation of unnecessary or damaged cellular components (Cuervo, 2004). Appearance of acidic vesicular organelles and autophagic vesicles upon exposure to IR indicate its role in DNA damage-mediated cell death (Paglin and Yahalom, 2006), however, the proper mechanism remains to be investigated.

2.4.3 Genotoxic stress-induced premature senescence

Cellular senescence is a physiological process often characterized as an irreversible growth arrest of serially cultured cells whose telomeres are damaged (‘replicative senescence’) (Munoz-Espin and Serrano, 2014; Shay and Wright, 2005). It is believed that senescence acts as a natural tumor-suppressive mechanism in normal cells and along with various cell death mechanisms represents a common response of tumor cells to the radio- and/or chemo-therapy (Schmitt, 2007). Therapy-induced senescence in cancer cells shares multiple traits with the replicative senescence such as altered gene expression (e.g. increase in p53, $p16^{INK4A}$, $p19^{INK4D}$ and $p21^{waf1/cip1}$) (Shelton et al., 1999), increased senescence-associated beta-galactosidase (SA-β-Gal) activity (Dimri et al., 1995), increased expression of PML and multiplication of PML nuclear bodies (PML NBs) (Janderova-Rossmeislova et al., 2007), presence of persistent DNA damage foci (Sedelnikova et al., 2004), senescence-associated heterochromatin foci (SAHF) (Kosar et al., 2011; Narita et al., 2003), and senescence-associated secretory phenotype (SASP, see below) (Coppe et al., 2010a). Senescence is accompanied also by the presence of enlarged, flattened morphology of cells, remodeled nuclear architecture and changed genome ploidity (Bridger et al., 2000; Mosieniak and Sikora, 2010).

SIPS occurs in response to various intrinsic or extrinsic damaging insults including IR, oxidative stress, cytotoxic agents (‘drug-induced senescence’), cytokines ('cytokine-induced senescence'), bacterial toxins and oncogenic activation (‘oncogene-induced senescence’) (Serrano and Blasco, 2001). The initial event in the induction of SIPS is the initialization of DDR followed by the activation of p53/$p21^{waf1/cip1}$ and $p16^{INK4A}$/Rb pathways (Roninson, 2003; Schmitt, 2007). DNA damage-mediated activation of p53 leads to the upregulation of CDK inhibitor $p21^{waf1/cip1}$ and the subsequent inhibition of cyclin E-CDK2 complex resulting in the activation (hypophosphorylation) of pRb and G1 arrest (Jeyapalan et al., 2007; Sabin and Anderson, 2011). Since SIPS is characteristic by the increased level of CDK inhibitor $p16^{INK4A}$ activated upstream of pRb, $p16^{INK4A}$ is used as a SISP marker in situations where Rb is not mutated (Krishnamurthty et al., 2004). p53 and $p21^{waf1/cip1}$ are common for activation of both apoptosis and senescence, however, the decision mechanism which destiny to follow, whether apoptosis or senescence, remains unclear.

It is well documented *in vivo* that senescence provides a barrier to tumorigenesis in response to tumor-inducing agents (Braig et al., 2005; Chen et al., 2005). However, if senescence cells are not effectively cleared by immune system (immuno-surveillance), they can promote tissue aging and impair tissue regeneration (Krishnamurthy et al., 2006;

Krizhanovsky et al., 2008; Molofsky et al., 2006). Direct link between the presence of senesent cells in various tissues and age-related diseases was provided by the study of Baker *et al*. in which targeted elimination of p16-expressing cells in Bub-R1 progeroid mouse background resulted in delay of the onset or attenuation of yet developed age-related pathologies (Baker et al., 2011).

As already mentioned, senescent cells remain metabolically active and produce various signaling molecules such as interleukins, chemokines, proteases, and GFs (Coppe et al., 2010b; Coppe et al., 2008). Besides physiological role of SASP e.g. to attract various immune cells (Freund et al., 2010; Krizhanovsky et al., 2008), secretome of senescent cells can alter surrounding microenvironment in the way that supports the tumor growth via persistent inflammation, DNA damage (Davalos et al., 2010), and angiogenesis (Coppe et al., 2006).

Acosta *et al*. and Kuilman *et al*. showed that molecular components of SASP can be regulated by NF-κB and C/EBPβ pathways (Acosta et al., 2008; Kuilman et al., 2008). DNA damage *per se* is important for induction and maintenance of SASP. Cells exhibiting SIPS phenotype possess irreparable DNA damage foci thus have persistently activated DDR. It was shown that downregulation of key DDR factors like ATM, NBS1 or Chk2 prevented an increase of several SASP components including pro-inflammatory cytokines IL6 and IL8 (di Fagagna, 2008; Rodier et al., 2009). Freund and Campisi with theit teams showed that stress-induced p38 MAPK pathway is important for development of senescent phenotype at later stages after initial DNA damage (Freund et al., 2011). In addition, the main components of SASP, IL6 and IL8, were shown to be negatively regulated via miRNA-146a and miRNA-146b/NF-κB feedback loop (Bhaumik et al., 2009; Taganov et al., 2006) and also by other components of SASP as IL1α (Orjalo et al., 2009).

In addition, despite the proliferative inactivity of senescent cells (i.e. normal/non-transformed) it was shown that senescent polyploid cancer cells can give rise to aneuploid cells (termed Raju cells; (Rajaraman et al., 2006; Sundaram et al., 2004)) with transient stem-like features in the process of anomalous nuclear division (nuclear budding) called neosis. Raju cells can contribute to tumor heterogeneity and could represent a basis for tumor post-therapy resistance and recurrence (Rajaraman et al., 2006; Sundaram et al., 2004).

Taking together, SIPS has an anti-tumor effect in response to therapy, however senescent cells, if not removed by immune system, can modulate tumor microenvironment in the paracrine way that can promote post-therapy relapse.

2.4.4 Genotoxic stress-induced pro-survival signaling

It was shown that low doses of IR (≥0.8 Gy) transiently activate pro-survival factors Mek/Erk and PI3K/Akt promoting cell viability and endothelial cell migration *in vitro* and tumor growth and metastases *in vivo* in a VEGF receptor-dependent manner (Sofia Vala et al., 2010). Main DNA damage sensor kinase ATM together with kinases Erk1 and Erk2 form a regulatory feedback loop implicated in HR repair (Amundson et al., 2003). Treatment of glioblastoma U87 cell line with bromodeoxyuridine (BrdU) causing DNA damage corresponding with low doses of IR (≤2 Gy) resulted in ATM-dependent Erk1/2 phosphorylation via Akt, whereas higher concentration (≥2 Gy equivalents) led to the Erk1/2 dephosphorylation (Hawkins et al., 2011; Khalil et al., 2011). Pro-survival kinase Akt has a direct role in promoting the repair of IR-induced DSBs by NHEJ. Akt directly interacts with the catalytic subunit of DNA-PK (DNA-PKcs) and promotes its binding to DSBs with pre-bound Ku dimers. Moreover, Akt promotes kinase activity of DNA-PK by its autophosphorylation during the repair process and mediates a release of DNA-PK from the damaged site when the lesion is repaired (Toulany et al., 2012).

Cells composing solid tumors often grow under hypoxic conditions due to abnormal blood supply and microcirculation (Vaupel et al., 1989). HIF1-α is considered as a major mediator of oxygen homeostasis in tumors and the factor responsible for hypoxia-mediated cancer progression (Vaupel, 2004). Kim *et al*. showed that although IR led to the activation of Erk, JNK, and p38 MAPK, only p38 MAPK promoted mesenchymal transformation and angiogenesis via stabilization of HIF1-α in glioma cells (Kim et al., 2014).

IR-mediated activation of PI3K/Akt and Mek/Erk signaling can increase the expression of various anti-apoptotic proteins (e.g. Bcl-XL and Mcl-1) as well as inactivate signaling from pro-apoptotic members from Bcl-2 family (e.g. Bad and Bim) (Pardo et al., 2002; Reed et al., 2004). Moreover, IR-induced Erk1/2 activity is associated with the expression of DNA repair proteins such as ERCC1, XRCC1 and XPC (Shvartsman et al., 2002). In human colorectal cancer cells, the active Erk1/2 increases a protein level of p53-negative regulator Mdm2 thus contributes to resistance to stress-induced apoptosis (Caron et al., 2005). Interesting pro-survival mechanism was suggested by Candas *et al*. who showed that cyclin B1-Cdk1-mediated phosphorylation of mitochondrial manganese superoxid dismutase (MnSOD) resulted in cellular resistance to radiation-induced apoptosis (Candas et al., 2013).

To conclude, IR can induce pro-survival signaling in cancer cells in the dose- and cancer cell origin-dependent manner.

2.4.5 The role of genotoxic stress in EMT/stem cell-like phenotypic reprogramming and resistance of cancer cells

There is increasing evidence that drug and/or radiation treatment itself could induce reprogramming in various cancers in terms of EMT and stemness. Such cells exhibit various features making them more resistant to particular treatment. For example, breast $CD24^{-/low}/CD44^{+}$ CSCs show greater clonogenic survival after IR and capability to grow as mammospheres when compared to total cell population (Phillips et al., 2006). In another study, IR of breast adenocarcinoma cell line MCF-7 with 2 and 6 Gy led to the enrichment of cell subpopulations with stem and progenitor characteristics (Woodward et al., 2007). Cho *et al*. reported that PCa cell lines showed an increase in stem cell properties with a long-term recovery indicating that despite initial damage caused by IR cells have the capacity to repair such DNA damage (Cho et al., 2012). In case of non-small cell lung cancer (NSCLC), radiation-surviving sphere cells were positive for CSC markers CD24 and CD44, had nuclear β-catenin and expressed EMT markers Snail, vimentin, N-cadherin, and PDFGR-β (Gomez-Casal et al., 2013a). Achuthan *et al.* demonstrated that in breast cancer model treatment with doxorubicin (doxo) generates chemoresistant cells with expression of CD133 and Oct-4 (Achuthan et al., 2011). Ghisolfi *et al.* showed that exposure of non-stem hepatocellular carcinoma cells to IR increase expression of pluripotency genes Sox2 and Oct3/4 and led to enhanced spherogenesis in non-CSCs. Conversely, knockdown of Sox2 and Oct3/4 inhibited radiation-induced spherogenesis and sensitized cancer cells to radiation (Ghisolfi et al., 2012).

The higher radioresistance correlated with the activation of cell cycle checkpoint leading to enhanced DNA damage repair capacity and reduced sensitivity to radiation-induced apoptosis in $CD133^{+}$ glioblastoma cells (Bao et al., 2006). Sensitivity to radiation and stemness could be also modified by microenvironmental factors. It was shown that hypoxic tumor cells are more radioresistant than oxygenated ones (Gray et al., 1953; Wright and Howard-Flanders, 1957). For example, Daoy medulloblastoma cells naturally expressing CD133 exhibited an increase in CD133 expression after the exposure to reduced oxygen levels (2% vs. 20%) (Blazek et al., 2007). Drug-derived $CD133^{+}$ and $Oct\text{-}4^{+}$ breast cancer cells exhibit low levels of ROS and an increase in antioxidant enzymes (Achuthan et al., 2011). Hypoxia modulates stem cell generation by HIFs. For example, HIF2-α may induce expression of stemness factor Oct-4 and HIF1-α interacts with stemness-related Notch signaling pathway (Covello et al., 2006; Gustafsson et al., 2005).

It was shown that IR-induced mesenchymal phenotype in cancer cells confers resistance to the following treatment. In ovarian cancer, cisplatin-resistant cells expressed

EMT-TFs like Snail, Slug, Twist2, and Zeb2 with downregulation of epithelial marker E-cadherin and upregulation of mesenchymal marker vimentin. Importantly, knockdown of Snail and Slug had re-sensitization effect to cisplatine (Haslehurst et al., 2012). Snail was able to confer chemo- and radio-resistance even in non-treated pancreatic cancer cells (Zhang et al., 2010). Non-invasive human mammary MCF10A cells with stable expression of Snail exhibited enhanced resistance to chemotherapeutic drugs and had altered various signaling cascades related to the cell death regulation including TGF-β, Notch, JAK/STAT, IL1R, Wnt, and MAPK (Lim et al., 2013a).

Collectively, genotoxic stress can induce reprogramming towards the mesenchymal phenotype and induce stem-like characteristics in cancer cells.

2.5.6 The role of genotoxic stress in cancer metastases

It was demonstrated by many researchers that IR-surviving tumor cells have enhanced motility and invasiveness (Moncharmont et al., 2014). RT improves local tumor control but tumor recurrence within yet irradiated field is associated with the higher risk of metastases in breast and head and neck cancers (Vicini et al., 2003; Vikram et al., 1984). Von Essen first highlighted the presence of metastatic cells at the site of primary tumor as well as in the normal tissue after IR (von Essen, 1991). Recently, Martin *et al*. stressed the role of RT in tumor cell dissemination. They showed that patients with NSCLC treated either with palliative or curative-intent RT had increased numbers of single or clumped CTCs, termed circulating tumor microemboli (CTM) in peripheral circulation. CTMs were composed also of non-tumor (stromal) cells which could have tumor growth-promoting potential (Martin et al., 2014). Tumor-bearing mice treated with RT had increased rates of lung metastasis compared with non-irradiated controls (Kaplan and Murphy, 1949). Similar result of increased lung metastases formation was observed in mice with tumors that were irradiated before surgical removal (Sheldon and Fowler, 1976). The link between the type of fRT and frequency of metastases was also documented. NSCLC patients treated with Continous Hyperfractionated Accelerated Radiotherapy Trial (CHART) consisting of 36 doses of 1.5 Gy, three times daily to 54 Gy in 12 days showed 24% reduction in the relative risk of metastases when compared to patients treated with conventional RT (60 Gy in 30 fractions in 6 weeks) (Saunders et al., 1997). Recently, Vala *et al*. reported that 'residual' IR of endothelial cells in the close vicinity of the tumor induces VEGF production under hypoxia-mimicking conditions. Using mouse experimental models of leukemia and breast cancer authors showed that low doses of IR (≤0.8 Gy) promote tumor growth and metastases by VEGF receptor-2-mediated cell migration and

resistance to cell death (Sofia Vala et al., 2010). Moreover, low single dose of IR (3 Gy) promotes cell survival and Akt signaling-dependent capillary formation (Tan et al., 2006). Interestingly, breast cancer tumors grown in pre-irradiated mammary glands had reduced microvasculature and were more necrotic and hypoxic indicating that pre-irradiation reduces local tumor growth. On the other hand, the pre-irradiation promotes tumor cell dissemination and metastases. Monnier *et al*. identified matricellular protein CYR61 and $\alpha_V\beta_5$ integrin as the proteins cooperating to mediate these effects (Monnier et al., 2008). Finally, ionizing radiation generates two waves of ROS in cells which can have effect on lipids, proteins and DNA. Such damage generates pro-inflammatory signals like NF-κB, TNFα and IFNγ supporting tumor cell dissemination (Chiang et al., 1997).

Taken together, genotoxic stress-based anti-cancer therapies have not only cell death and premature senescence-inducing effect but can also promote pro-survival signaling, epithelial-mesenchymal and stem-like transitions in the therapy-resistant clones which are responsible for tumor relapse and occurrence of metastases.

3. AIMS OF THE STUDY

The main purpose of this study was to uncover molecular pathways leading to therapy resistance of prostate cancer cells. This general aim can be further specified, as follows:

- to characterize prostate cancer cells that survive a clinical dose of fractionated ionizing radiation at phenotypic and molecular level including the quantitative high throughput screening approach;

- to decipher molecular mechanisms of radioresistance;

- to identify potent radio- and/or chemo-sensitizing drug(s) effective in the elimination of therapy-resistant cancer cells.

4. RESULTS

4.1 GENERATION AND CHARACTERISATION OF FRACTIONATED IONIZING RADIATION (fIR)-SURVIVING PROSTATE CANCER CELL POPULATIONS

4.1.1 Fractionated ionizing radiation generates phenotypically different adherent and non-adherent surviving prostate cancer cell populations

In high-risk PCa patients, regional and distant recurrences are relatively frequent after the curative treatment. The patients with post-treatment oligometastatic disease can be, besides the androgen-deprivation therapy (Heidenreich et al., 2014), indicated to so called metastasis-directed therapy like surgery or again X-ray-based therapy – stereotactic body RT (SBRT) (Tree et al., 2013). Frequent distant relapses after the primary RT and 2-year local control only in 27 – 78% oligometastatic SBRT-treated patients points to the fact that some primary tumor/metastatic cancer cells can resist the radiation-based therapies (for review, see (Tree et al., 2013)).

To address the radioresistance of metastatic PCa cells, we decided to follow the clinically used RT regimen (Heidenreich et al., 2011; Mottet et al., 2011) and exposed four human PCa cell lines derived from various metastatic sites (DU145 – brain metastasis, PC-3 – bone metastasis, LNCaP – lymph node metastasis, and 22RV1 – PCa xenograft line) to cumulative dose of 70 Gy (2 Gy applied every 24 hours for 35 days). Characteristic *in vitro* phenotypic changes observed during the fIR course are schematically depicted in **Fig. 4.1a** and shown in **Fig. 4.1b**. Approximately after the third dose of fIR, a continuing loss of adhesion, mostly as a result of cell death, was observed in all four cell lines. Intriguingly, besides the dead cells, apoptotic bodies and cell debris, non-adherent fraction contained low abundant population of viable cells (*non-adherent cells*), which were able to reattach within 2 – 3 weeks after the last irradiation dose (*re-adherent cells*). Moreover, upon 35 doses of irradiation, a small subset of the initial cell population with senescent-like morphology remained vital and attached (*adherent cells*). Interestingly, the same effect of IR-induced loss of adhesion was reproduced in the human breast adenocarcinoma cell line MCF-7 after

10 x 2 Gy (**Fig. 4.2a**, **b**) indicating that this phenomenon is not restricted to prostate cancer cells only.

In the context of relevance of *in vitro* models to the RT of tumors in the clinics, daily fractions of irradiation are a more useful approach than a single-dose exposure, as suggested also by Tsai *et al*. (Tsai et al., 2007). However, we found that some features, such as initial loss of adhesion and subsequent readhesion could be reproduced *in vitro* with irradiation regimens consisting of either ten consecutive doses of 2 Gy or one single dose of 10 Gy. The main difference between a single dose versus repeated doses was the percentage of viable cells present in the total non-adherent fraction. The more doses cells get the less viable non-adherent cells we were able to detect. In order to perform an extensive RNA and protein analysis of viable non-adherent fraction, shortened (10 x 2 Gy) irradiation regimens were used. A single dose of 10 Gy was used only in experiments where we tested the effect of specific siRNA-mediated knock down on irradiation-mediated loss of adhesion and anchorage-independent survival.

Regarding the non-adherent cell population, small subsets of the non-adherent cells assessed as Hoechst33258$^-$ and AnnexinV-FITC$^-$ cells by fluorescence-activated cell sorting (FACS) analysis were resistant to anoikis, since they were capable of long-term survival and a resumed adherent growth after a period of 10 – 21 days (**Fig. 4.1b**, **c**; **Fig. 4.2a**). Once initiated, such readhesion event was a sudden process lasting few days and included the majority of non-adherent cells, many of them utilizing the already attached cells as adhesion substrate (**Fig. 4.1c**, **Fig. 4.2c**). Reattachement was accompanied with the formation of rosette-like membrane blebbing structures (**Fig. 4.2c**) as well as with the ability to grow as spheres (**Fig. 4.1c**).

All PCa cell lines used form tumors after the subcutaneous injection into immunocompromised mice. Notably, the tumorigenic potential of the irradiated re-adherent cells (only DU145 tested) in immunodeficient (SCID) mice was preserved (**Fig. 4.2d**).

To conclude, fractionated irradiation of tumor cell lines led to development of two phenotypically distinct adherent and non-adherent fIR-surviving cell populations, both partially capable of the renewal of proliferation after the termination of radiation stress.

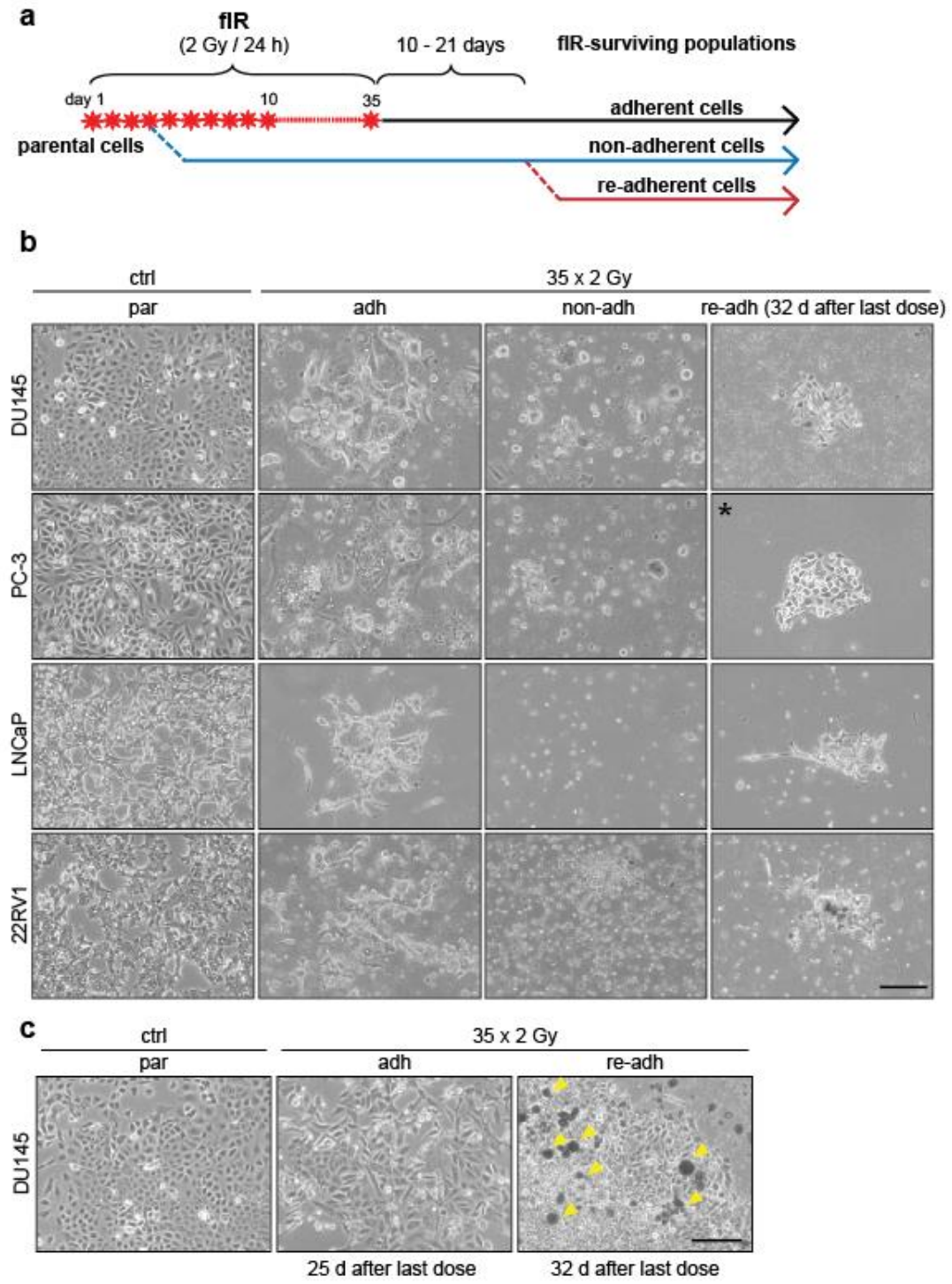

Figure 4.1. Generation of adherent and non-adherent radiation-surviving subpopulations in human prostate carcinoma cells by fractionated ionizing radiation. (**a**) Schematic representation of the irradiation protocol using 2 Gy every 24 hours resulting in generation of radiation-surviving populations (adherent, non-adherent and re-adherent) in human prostate cancer cell lines DU145, PC-3, LNCaP and 22RV1 (see Material and Methods for details). (**b**) Phase contrast microscopic images of non-irradiated parental cells (par) and fIR-surviving DU145, PC-3, LNCaP and 22RV1 cell populations after 35 x 2 Gy fIR (adh and non-adh) or 32 days after the last dose of fIR (re-adh). Representative phase contrast images of one from three independent experiments are shown. The image with asterisk represents the re-adherent colony of PC-3 cells obtained after 10 days of fIR. Bar, 100 μm. (**c**) Phase contrast images of control (parental) and irradiated adherent and re-adherent DU145 single-cell colonies captured at indicated time points after 35 x 2 Gy of fIR. Note, the 'black' cells (arrowheads) are non-adherent cells starting to attach to the layer of adherent cells. Bar, 100 μm. Par – parental, adh – adherent; non-adh – non-adherent; readh – re-adherent.

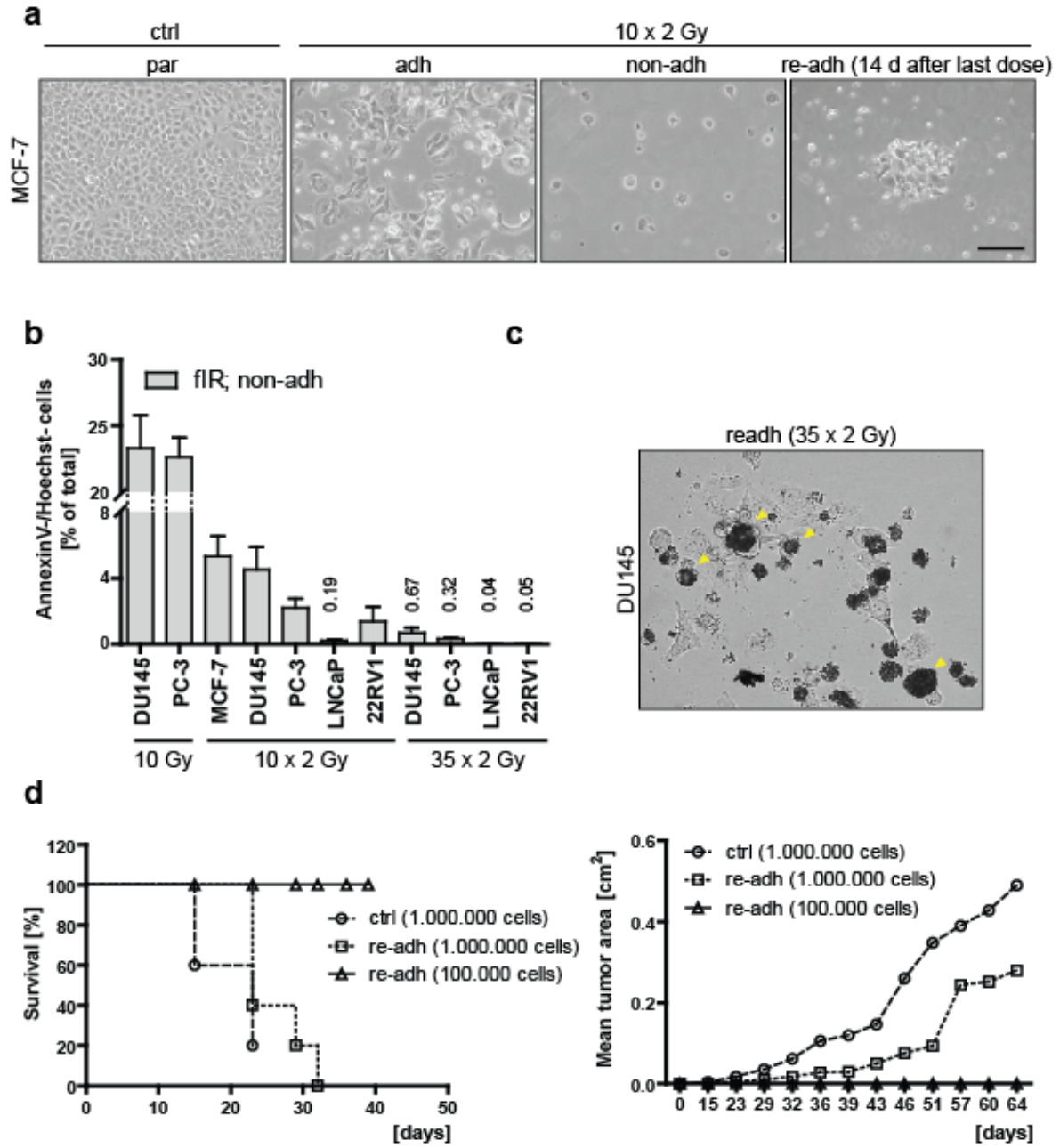

Figure 4.2. fIR generates surviving subpopulations with preserved tumorigenic potential. (**a**) Phase contrast microscopic images of non-irradiated parental cells (par) and radiation-surviving MCF-7 cell populations after 10 x 2 Gy fIR (adh and non-adh) or 14 days after fIR (re-adh). Representative phase contrast images of one of two independent experiments are shown. Bar, 100 µm. (**b**) Flow cytometry viability analysis of DU145, PC-3, LNCaP, 22RV1 and MCF-7 non-adherent cells exposed to different irradiation regimens (1 x 10 Gy, 10 x 2 Gy and 35 x 2 Gy) expressed as relative amount (in percentage, numbers above bars) of viable AnnexinV-FITC$^-$/Hoechst33258$^-$ cells in total non-adherent fraction. Values represent means ±S.D. from two independent experiments. (**c**) Phase contrast microscopic images of radiation surviving re-adherent DU145 cell populations after 35 x 2 Gy fIR. Non-adherent cells with membrane blebbing (arrowheads) attaching to the layer of adherent cells are shown. (**d**) Kaplan-Meier survival plot (left) and tumor growth curve (right) reflecting percentage of tumor-free SCID mice and mean tumor area, respectively, at indicated times after subcutaneous injection of either control or 10 x 2 Gy-irradiated re-adherent DU145 cells. $p<0.05$ estimated by the Newman-Keuls multiple-comparison test (N = 5).

4.1.2 Fractionated ionizing radiation induces persistent DNA damage response in fIR-surviving adherent prostate cancer cell population

Next, we characterized the fIR-surviving adherent fraction. Since ionizing radiation can cause various types of DNA damage including SSBs and DSBs leading either to cell cycle arrest and DNA repair or, if not repaired, to cell death, we investigated whether PCa cells can effectively repair resulted damage leading to their survival. Active DNA damage signaling was assayed as phosphorylation of Chk2 and p53 and elevated expression of the p53 target CDK inhibitor $p21^{waf1/cip1}$ (**Fig. 4.3a**) and DNA damage foci positive for 53BP1 and γH2AX (**Fig. 4.3c**). ATM/p53/p21 signaling exhibited cumulative pattern with increasing numbers of irradiation fractions even in p53-negative PC-3 cells (**Fig. 4.3c**). Formation of micronuclei as an effect of problems to repair numerous DSBs was observed (**Fig. 4.3d**) in both p53-mutated DU145 and p53-null PC-3 cells. Despite gradual increase of CDK inhibitors $p21^{waf1/cip1}$ and $p27^{kip1}$ (DU145, PC-3 and LNCaP) or $p16^{INK4a}$ (22RV1) during fIR (**Fig. 4.2b**), proliferation in the adherent fraction was not grossly affected until the 10^{th} dose of irradiation, as detected by an eFluor670 proliferation assay. The proliferation of irradiated adherent cells ceased between doses 11 – 15 (**Fig. 4.3e**). Importantly, DU145 and PC-3 cells were positive for DNA-damage associated molecular markers even 72 hours after the last dose of 10 x 2 Gy indicating their low sensitivity to DNA damage-mediated cell cycle arrest and ability to complete division with damaged DNA (**Fig. 4.3c**).

In conclusion, despite fIR induces DNA damage response and activation of CDK inhibitors; proliferation of PCa cell lines was not grossly affected until the 15^{th} dose of 2 Gy.

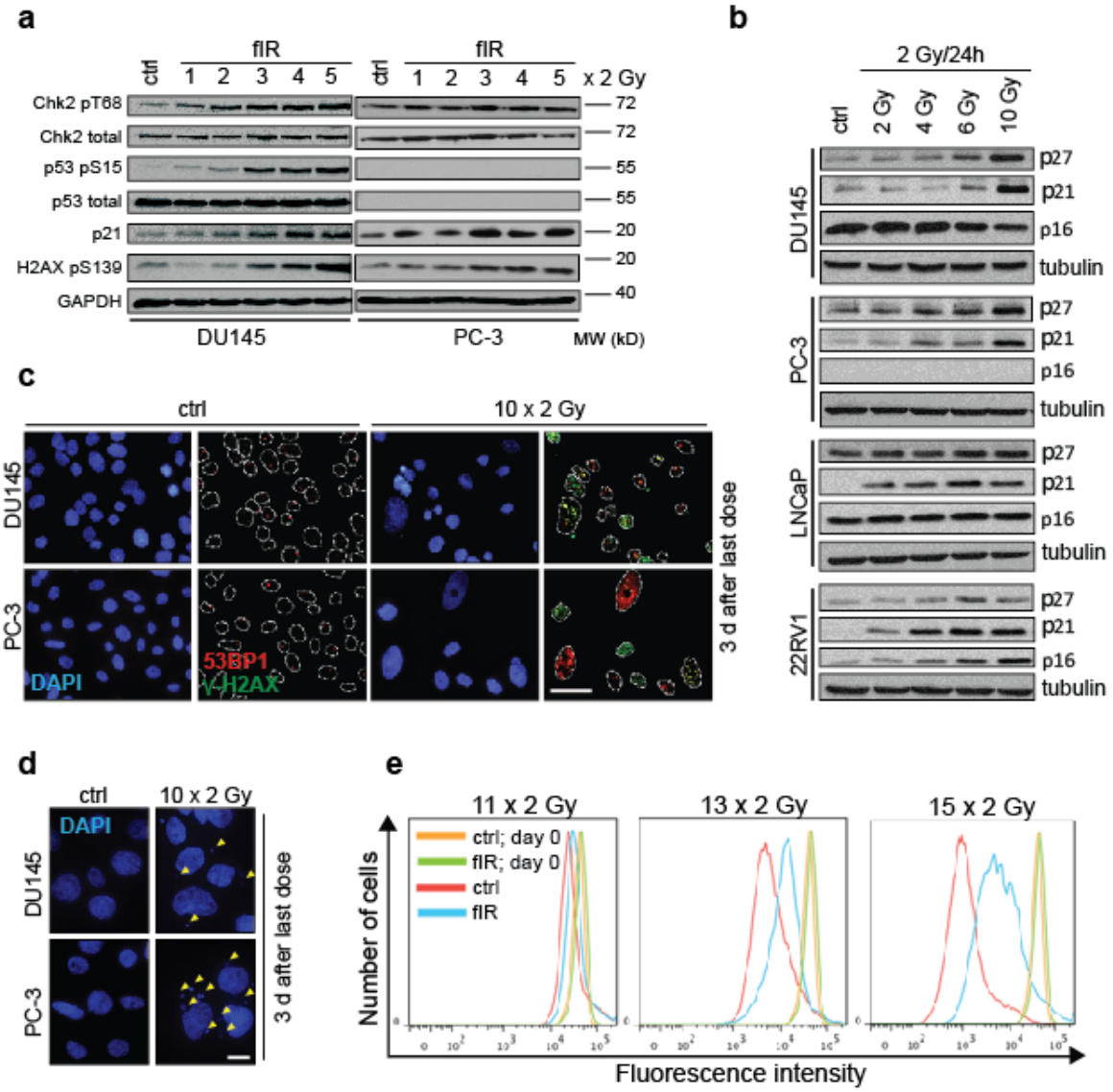

Figure 4.3. fIR induces DNA damage response in prostate cancer cell lines. (**a**) Immunoblotting detection of total Chk2, phosphothreonine 68 of Chk2, total p53, phosphoserine 15 of p53, phosphoserine 139 of H2AX, and $p21^{waf/cip}$ (p21) in control and irradiated (2 – 10 x 2 Gy) DU145 and PC-3 cells. GAPDH was used as a loading control. (**b**) Immunoblotting detection of $p27^{kip1}$ (p27), p21, and $p16^{INK4A}$ (p16) in control and irradiated (2 – 10 x 2 Gy) DU145, PC-3, LNCaP, and 22RV1 cells. γ-tubulin was used as a loading control. (**c**) Immunofluorescence detection of 53BP1 (red) and γH2AX (green) foci in DU145 and PC-3 cells three days after irradiation (10 x 2 Gy). Nuclei were visualized by DAPI (blue). Bar, 50 µm. (**d**) Fluorescence detection of micronuclei formation (arrowheads) using DAPI (blue) in DU145 and PC-3 cells estimated three days after 10 x 2 Gy. Bar, 15 µm. (**e**) Flow cytometric estimation of cell proliferation by eFluor670 dye dilution in adherent control (red line) and irradiated (blue line) DU145 cells (11, 13 and 15 x 2 Gy daily; see Material and Methods for details) expressed as histogram of fluorescence intensity. Note, control (orange line) and irradiated (10 x 2 Gy; green line) were labeled and analyzed immediately after the staining (day 0).

4.1.3 Fractionated ionizing radiation induces a development of premature senescence in fIR-surviving adherent prostate cancer cell subpopulation

fIR-surviving adherent PCa cells exhibited stress-induced premature senescence with typical morphological features (**Fig. 4.1b**) and affected replication as shown by decreased EdU incorporation in DU145 (29.5% ± 5.5 labeled, compared to 39.1% ± 11.7 control cells) and PC-3 (22.5 ± 5.9 labeled, compared to 36.0% ± 10.6 control cells) (**Fig. 4.4a**). The senescence phenotype has been progressively developed in the course of continuing irradiation with typical spreading and vacuolisation (**Fig. 4.1b**, **Fig. 4.4f**), frequent bi- to poly-nucleation, the presence of lobed or fragmented nuclei (**Fig. 4.4d, e**), and occurrence of chromatin bridges between incompletely divided nuclei (**Fig. 4.4d**). Presence of senescent cells was confirmed by positivity for SA-β-gal (**Fig. 4.4b**), an increase of PML NBs detected by indirect immunofluorescence (**Fig. 4.4c**), and SASP (**Fig. 4.5a**, **b**, **c**). To measure SASP, we estimated RNA and protein levels of selected cytokine species in irradiated adherent DU145 cells. We estimated protein levels of IL6 and IL8 in cultivation media which we found elevated after irradiation (**Fig. 4.5a**). RNA levels of several cytokines such as colony stimulating factor 2 (CSF2), bone morphogenetic protein 2 (BMP2), interleukine 6 (IL6) and interleukine 1F8 (IL1F8) were elevated 3 days after single dose of 10 Gy (**Fig. 4.5b**). Moreover, RNA levels of all cytokines tested (INHBA, BMP2, IL8, IL1F8 and CSF2) exhibited cumulative pattern after the end of fIR (**Fig. 4.5c**). Notably, senescent DU145 cells exposed to an 24-hour BrdU pulse showed asynchronous labeling of nuclei (**Fig. 4.4d**) indicating ongoing DNA reduplication in surviving adherent cells likely due to lack of functional pRb (Talluri et al., 2010). After the end of fIR (35 x 2 Gy) all surviving cells exhibited senescence-associated cell cycle arrest. However, after few days such cells were able to re-enter the cell cycle (see **Fig. 4.1c** for day 18 and 25 after the last dose). This proliferative switch was accompanied with the presence of PGCCs and rare occurrence of small cells – *senescence escapers* (see **Fig. 4.4f** for PC-3 cells).

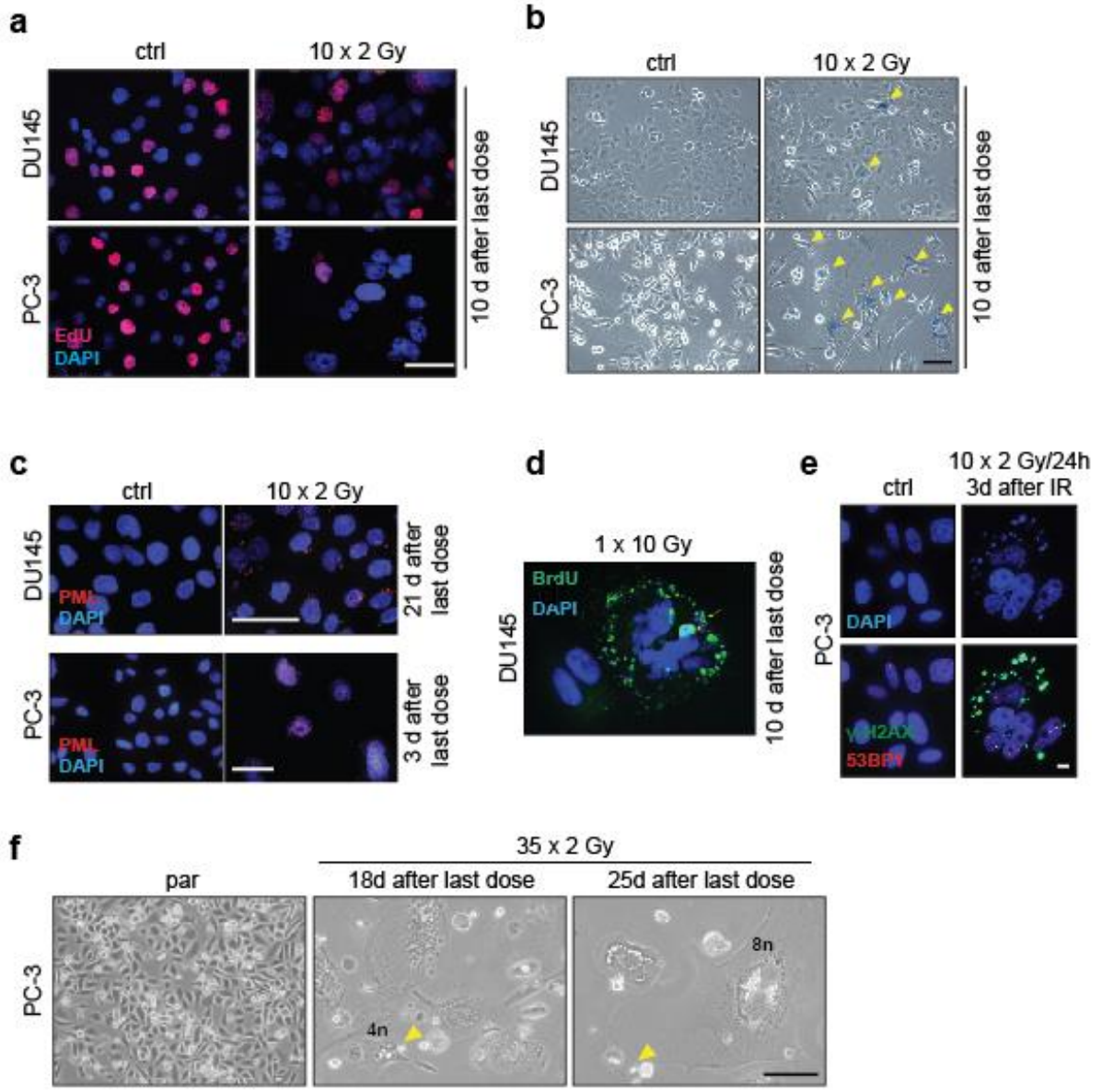

Figure 4.4. fIR induces accelerated cellular senescence in prostate cancer cell lines. (**a**) Indirect immunofluorescence detection of EdU incorporation (red) after 10 x 2 Gy in DU145 and PC-3 cells. Nuclei were visualized by DAPI (blue). Bar, 50 µm (**b**) SA-β-gal activity in DU145 and PC-3 cells estimated 10 days after 10 x 2 Gy. Yellow arrowheads indicate senescent cells. Bar, 100 µm. (**c**) Indirect immunofluorescence detection of PML (red) in irradiated (10 x 2 Gy) DU145 and PC-3 three weeks and three days after irradiation, respectively. Nuclei were visualized by DAPI (blue). Bars, 50 µm. (**d**) indirect immunofluorescence detection of BrdU incorporation (green) after 1 x 10 Gy in DU145 cells. Nuclei were visualized by DAPI (blue). Yellow arrows illustrate sites of active replication. (**e**) Indirect immunofluorescence detection of DNA damage markers γH2AX (green) and 53BP1 (red) after 10 x 2 Gy in PC-3 cells. Nuclei were visualized by DAPI (blue). Bar, 10 µm. (**f**) Phase contrast microscopic images of non-irradiated parental cells (par) and irradiated (35 x 2 Gy) adherent polyploid (4N/8N) giant senescence PC-3 cells 18 or 25 days after the last dose. Yellow arrowheads indicate senescence escapers. Bar, 100 µm.

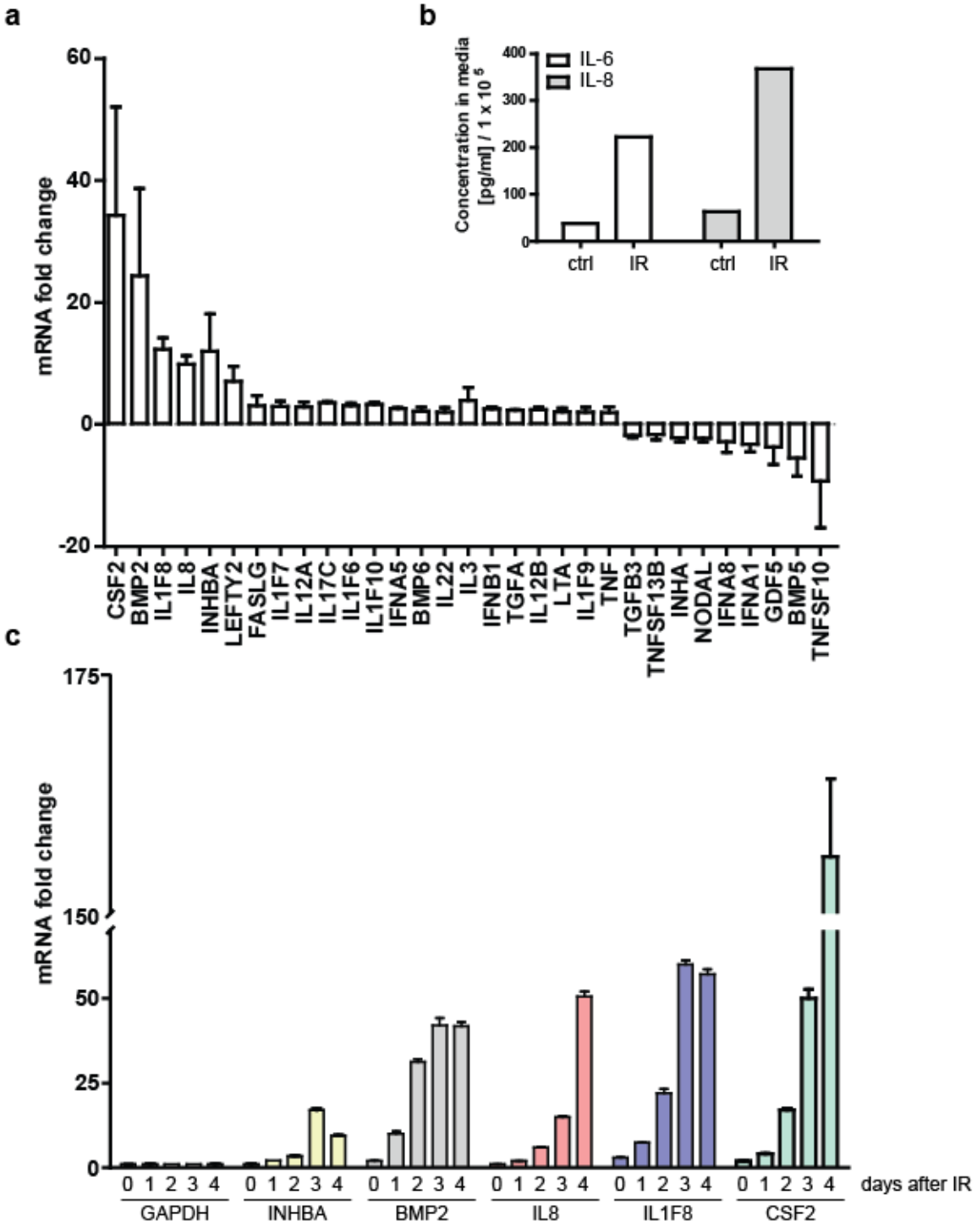

Figure 4.5. IR induces expression of various cytokines in adherent prostate cancer cells. (**a**) mRNA expression profile of selected cytokines in irradiated (1 x 10 Gy) adherent DU145 cells measured by qRT-PCR. Data were normalized to B2M, HPRT1, RPL13a, GAPDH, and ACTB. (**b**) Concentration of secreted IL6 and IL8 proteins in cultivation media in control and irradiated (1 x 10 Gy) DU145 cells estimated by flow cytometry based bead array (see Material and methods) 3 days after IR. (**c**) qRT-PCR detection of INHBA, BMP2, IL8, IL1F8 and CSF2 in control (day 0) and irradiated DU145 cells (1 - 4 days after 1 x 10 Gy). GAPDH was used as a reference gene. Data in (a) and (c) represent means ± S.D. from two independent experiments performed in triplicates. Data in (b) are from one experiment.

4.2 INVOLVEMENT OF EMT/MET IN FRACTIONATED IONIZING RADIATION-INDUCED CELLULAR PLASTICITY OF CANCER CELLS

4.2.1 Fractionated ionizing radiation induces EMT/MET-related changes in prostate cancer cells

We observed that the phenotypic switch (the cycle of irradiation-induced loss of adhesion and subsequent readhesion) was repeatable at least three times indicating the stress-induced reversible cell plasticity. During the fIR, the morphology of cells substantially changed. Epithelial-like morphology in parental population shifted towards the mesenchymal-like morphology during fIR (**Fig. 4.1c**, **4.2a**). Several population doublings of the re-adherent cells were necessary to revert the mesenchymal phenotype (detected as the expression of vimentin) back to the epithelial phenotype (detected as the expression of E-cadherin; MET, see **Fig. 4.6a** for changes in morphology and **Fig. 4.6b** for changes in vimentin and E-cadherin expression after period of readherent growth). The reprogramming towards the epithelial phenotype after the reattachment of DU145 and PC-3 cells was confirmed also by scratch wound healing assay where re-adherent cells exhibited slower migration into the scratch in both cases (**Fig. 4.6c**). Mesenchymal phenotype of early re-adherent cells could be pre-determined already in non-adherent stage in which cells lack the expression of E-cadherin (see below) and have increased levels of vimentin when compared to parental cells (**Fig. 4.6d**).

4.2.2 Fractionated ionizing radiation induces Snail-dependent loss of cellular adhesion in prostate cancer cells

As the loss of adhesion was accompanied by resistance to anoikis and long-term non-adherent survival indicating the cell reprogramming including, we compared both the non-adherent and adherent fIR-surviving PCa cell fractions for selected factors known to regulate the cell adhesive properties. We analyzed mRNA levels of selected TFs involved in the regulation of cell adhesion (Snail, Slug, Twist1, Twist2, Zeb1, and Zeb2) by qRT-PCR during fIR in all four populations: parental (non-irradiated), irradiated adherent, non-adherent, and readherent.

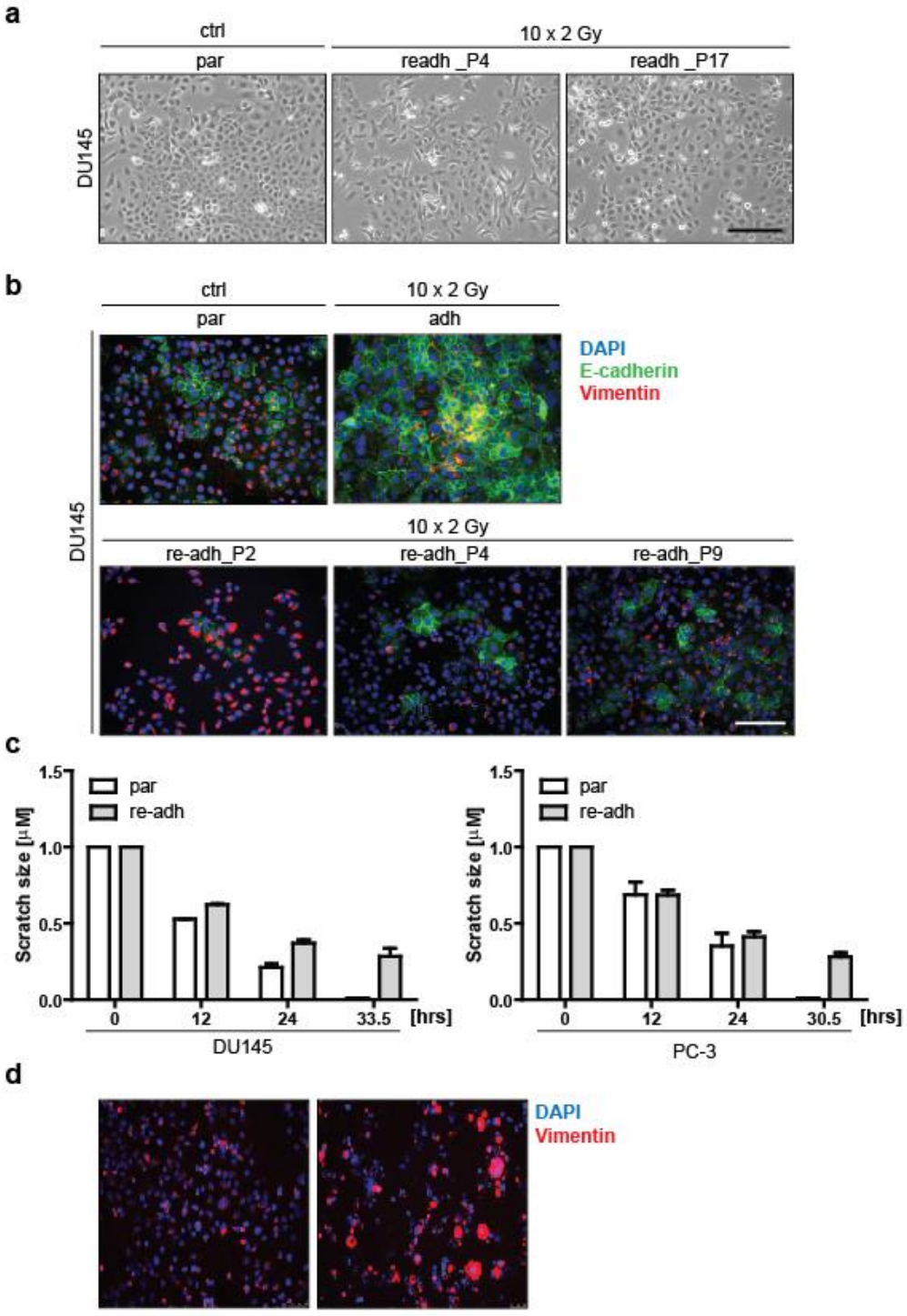

Figure 4.6. fIR induces EMT/MET in prostate cancer cells. (**a**) Phase contrast microscopic images of non-irradiated parental cells (par) and irradiated (10 x 2 Gy) re-aderent DU145 cells (passage 4 and 17). Bar, 100 µm. (**b**) Indirect immunofluorescence detection of the epithelial marker E-cadherin (green) and the mesenchymal marker vimentin (red) in control (parental) and irradiated (10 x 2 Gy) adherent and re-adherent (passages 2, 4 and 9) DU145 cells. Nuclei were visualized by DAPI (blue). Bar, 100 µm. Representative images from two independent experiments are shown. (**c**) The scratch wound healing assay showing migration of parental and fIR (10 x 2 Gy) re-adherent DU145 (left) and PC-3 (right) cells measured at indicated time points. Bars represent relative values of scratch size (µm) normalized to parental cells ('1') at the time point 0. Data represent means ± S.D from two independent experiments performed in triplicates. (**d**) Indirect immunofluorescence detection of the mesenchymal marker vimentin (red) in control (parental) and irradiated (10 x 2 Gy) non-adherent DU145 cells. Nuclei were visualized by DAPI (blue). Bar, 50 µm.

mRNA levels of Twist2 and Zeb1 were not significantly changed among fIR-surviving populations therefore only data for those TFs which mRNA levels were altered, e.g. Snail (SNAI1), Slug (SNAI2), Twist1, Zeb2 are shown (**Fig. 4.7a**, **b**). Although we observed an increase of mRNA levels of all four TFs assayed in the non-adherent fraction, the most consistent changes were observed for the TF Snail, known to regulate stress-resistance, stem cell-like phenotype, and EMT in various cancer types (Lim et al., 2013a). Snail was induced by all irradiation regimens used (e.g. 10 x 2 Gy, 1 x 10 Gy, 35 x 2 Gy) in the non-adherent cell fraction in both PCa cell lines DU145 and PC-3 compared to parental and irradiated adherent cells at both mRNA (**Fig. 4.7b**, **Fig. 4.8a**, **e**) and protein (**Fig. 4.8b**, **c**) levels (for basal expression of Twist1 in DU145 and PC-3 cells, see **Fig. 4.7c**). Notably, analogous Snail induction of both mRNA and protein levels was observed in breast cancer cell lines MCF-7 exposed to 10 x 2 Gy (**Fig. 4.8a**, **b**). Twist1 can functionally overlap with Snail in terms of negative regulation of E-cadherin (Yang et al., 2004). To test whether the loss of cell adhesion depends on Snail or Twist1, siRNA-mediated knockdown of Snail and Twist was performed in DU145 and PC-3 cells before a single dose of irradiation (10 Gy; **Fig. 4.8d**). As shown in **Fig. 4.8e**, knockdown of Snail but not Twist1 reverted the enhanced loss of adhesion in irradiated cells while having no effect in control cells. Importantly, a similar suppressive impact of Snail downregulation on loss of adhesion was obtained using a tetracycline (tet)-inducible Snail shRNA system in DU145 cells exposed to 10 x 2 Gy fIR (**Fig. 4.8f**, **g**).

Furthermore, genes known to be positively regulated by Snail, such as MMP-7, or negatively regulated, e.g. integrin alpha2 (ITGA2; (Neal et al., 2011)), laminin alpha3 (LMNA3), laminin gamma2 (LAMC2; (Haraguchi et al., 2008)), and E-cadherin (CDH1; (Batlle et al., 2000; Cano et al., 2000)) were consistently altered in irradiated non-adherent DU145 cells (**Fig. 4.7d**), indicating the Snail activity. Indeed, knockdown of Snail led to decreased MMP-7 and increased ITGA2, LMNA3, LAMC2, and E-cadherin (**Fig. 4.7e**), indicating that these genes are under the control of Snail and underscoring the increased transcriptional activity of Snail in irradiated non-adherent cells.

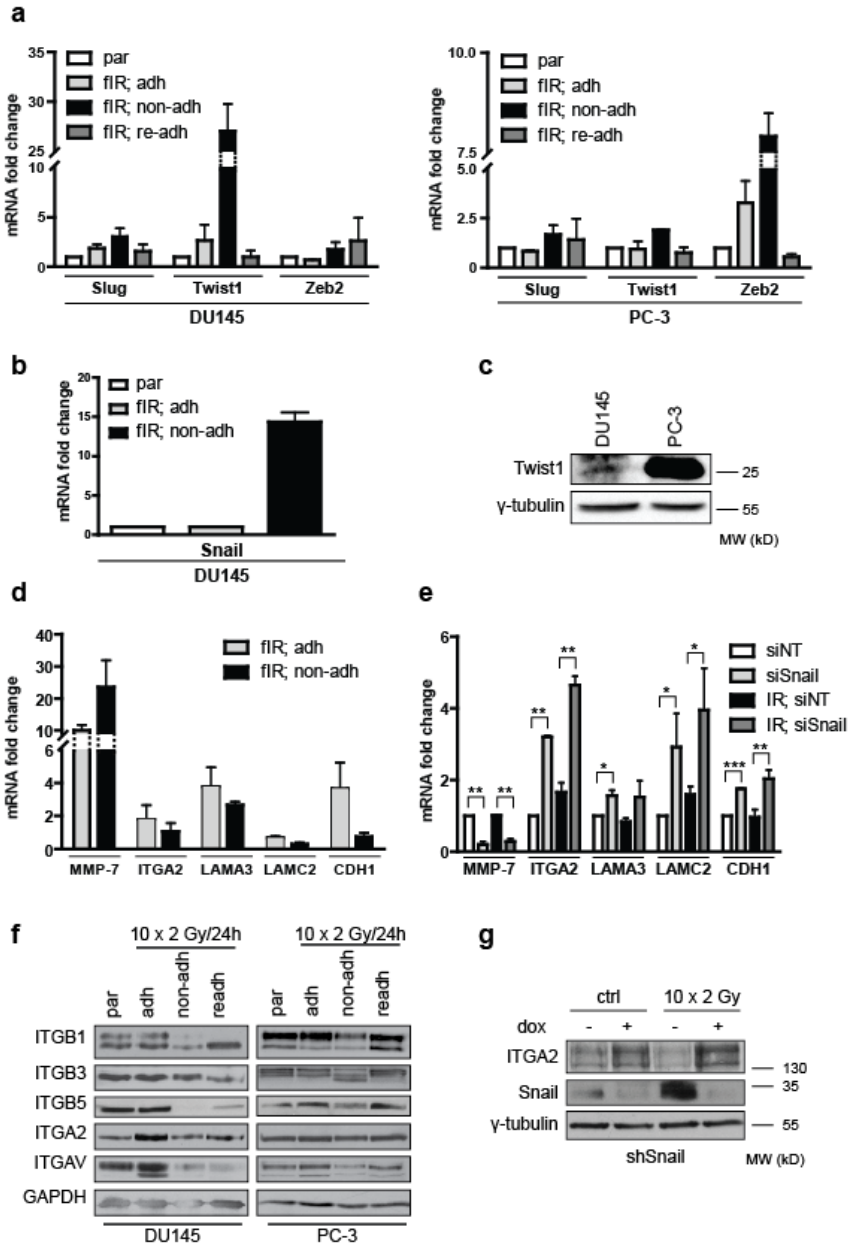

Figure 4.7. fIR induces expression of EMT drivers and modulates expresion of cell adhesive molecules in prostate cancer cells. (**a**) qRT-PCR quantification of Slug, Twist1 and Zeb2 in control (parental) and irradiated (10 x 2 Gy) adherent, non-adherent and re-adherent DU145 (left) and PC-3 (right) cells. RPL37a was used as a reference gene. (**b**) qRT-PCR estimation of Snail mRNA level detected in control (parental) and irradiated (35 x 2 Gy) adherent and non-adherent DU145 cells. RPL37a was used as a reference gene. (**c**) Immunoblotting detection of Twist1 in non-treated DU145 and PC-3 cells. γ-tubulin was used as a loading control. (**d**) qRT-PCR quantification of mRNA levels of Snail-regulated genes MMP-7, ITGA2, LAMA3, LAMC2 and CDH1 (E-cadherin) in radiation-surviving adherent and non-adherent DU145 cells after irradiation (10 x 2 Gy). β-actin was used as a reference gene. (**e**) Effect of siRNA knockdown of Snail (siSnail) on Snail-activated (MMP-7) or -repressed genes (ITGA2, LAMA3, LAMC2, and CDH1) estimated by qRT-PCR in control and irradiated (1 x 10 Gy) DU145 cells. β-actin was used as a reference gene. (**f**) Immunoblotting detection of integrins beta1 (ITGB1), beta3 (ITGB3), beta5 (ITGB), alpha2 (ITGA2), and alphaV (ITGAV) in control and irradiated (10 x 2 Gy) adherent, non-adherent and re-adherent DU145 and PC-3 cells. GAPDH was used as a loading control. (**g**) Effect of doxycycline-induced (dox, 0.7 μg/ml) shRNA-mediated Snail knockdown in lentivirally transduced DU145 (shSnail) cells on integrin alpha2 (ITGA2) protein level with or without irradiation (10 x 2 Gy) detected by immunoblotting. γ-tubulin was used as a loading control. qRT-PCR data represent at least two (**d** and **e**) or three (**a** and **b**) independent experiments executed in triplicates. Data in **a**, **b**, **d** and **e** represent means ± S.D. *p<0.05; **p<0.01; ***p<0.001.

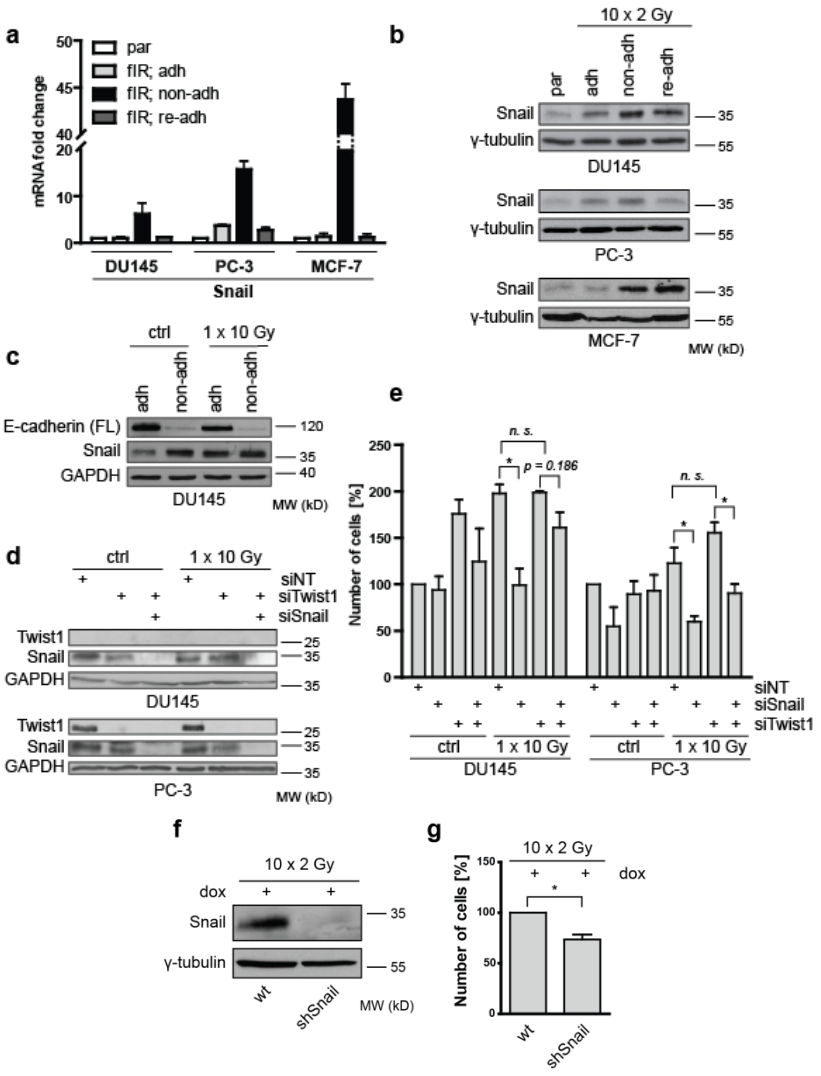

Figure 4.8. Snail is expressed in fIR-surviving non-adherent prostate and breast cancer cells and is indispensable for irradiation-mediated loss of adhesion. qRT-PCR quantification of Snail mRNA levels (**a**) and Snail immunoblotting detection (**b**) in DU145, PC-3 and MCF-7 control (parental) or irradiated (10 x 2 Gy) adherent, non-adherent and re-adherent cell populations. RPL37a (DU145 and PC-3) and GAPDH (MCF-7) were used as reference genes in **a**; γ-tubulin was used as a loading control in **b**. (**c**) E-cadherin and Snail immunoblotting detection in adherent and non-adherent control and irradiated (1 x 10 Gy) DU145 cells. GAPDH was used as a loading control. (**d**) Efficiency of siRNA-mediated knockdown of Snail (siSnail) and Twist1 (siTwist1) estimated by immunoblotting detection of Snail and Twist1 in control or irradiated (1 x 10 Gy) DU145 and PC-3 cells. GAPDH was used as a loading control. (**e**) Effect of siRNA knockdown of Snail (siSnail) and Twist1 (siTwist1) on loss of adhesion expressed as relative number of detached cells assessed by FACS in control or irradiated (1 x 10 Gy) non-adherent DU145 and PC-3 cells. Non-targeting siRNA (siNT) was used as a control. Cells were irradiated 24 hours after transfection and FACS-analyzed 48 hours after IR. Non-irradiated cells transfected with non-targeting siRNA were set as 100% in both cell lines. (**f**) Effectiveness of doxycycline-induced (dox, 0.7 μg/ml) shRNA-mediated Snail knockdown in lentivirally transduced DU145 cells during fIR (10 x 2 Gy) verified by immunoblotting. γ-tubulin was used as a loading control. Non-transduced cells were used as a control (wt). (**g**) Effect of dox-induced shRNA-mediated Snail knockdown on loss of adhesion during fIR (10 x 2 Gy) expressed as the relative number of detached cells (40 μl/sample) assessed by flow cytometry in control non-transduced DU145 cells (wt) and stable DU145 cell line generated by lentiviral transduction with the pLKO-Tet-On-shRNA-Snail (shSnail) vector. Non-transduced control cells (wt) were set as '1'. Panels concerning qRT-PCR represent data from three independent experiments executed in triplicates. Panel **e** represents three independent experiments. Data in **a**, **e** and **g** represent means ± S.D. *p<0.05; **p<0.01; ***p<0.001.

Furthermore, integrin alpha2 protein level was elevated in the non-adherent fraction of DU145 cells with a stably integrated tet-inducible Snail shRNA after doxycycline (dox) induction, consistent with Snail operating as a transcriptional repressor of this gene (**Fig. 4.7g**). Importantly, protein levels of cell adhesion factors integrin beta1 (ITGB1), integrin beta3 (ITGB3), integrin beta5 (ITGB5), integrin alpha2 (ITGA2), and integrin alphaV (ITGAV) were decreased in fIR-induced non-adherent population of both DU145 and PC-3 cells (**Fig. 4.7f**). Moreover, protein levels of the major epithelial adhesion molecule E-cadherin were markedly decreased in non-adherent fraction of both irradiated and control cells (**Fig. 4.8c**), while mesenchymal marker vimentin was elevated in irradiated non-adherent cells in comparison to control cells (**Fig. 4.6d**), confirming that EMT events accompanied the loss of cellular adhesion.

We concluded that irradiated non-adherent PCa cells lose adhesive properties due to changes of expression of several adhesive molecules controlled by elevated expression of Snail.

4.2.3 fIR-surviving non-adherent prostate cancer cells exhibit stem cell traits

Resistance to anoikis seen in irradiated non-adherent prostate and breast cancer cells is an important prerequisite for CTCs to survive in circulation after dissemination. Since anoikis-resistant CTCs exhibit stem-cell progenitor phenotype (Theodoropoulos et al., 2010) we presumed that the anoikis-resistant survival of non-adherent PCa cells can be associated with acquisition of stem cell properties induced during IR. Indeed, compared to all other fractions of control and irradiated cells, the non-adherent DU145 and PC-3 cells showed higher mRNA levels of stem cell-associated genes such as CD133, Sox2, Oct-4 and Nanog (**Fig. 4.9a**, **b**; (Richardson et al., 2004; Yu et al., 2007)). Shortly after cell readhesion, these transcripts returned almost to the pre-irradiation levels observed in the parental cells, indicating transiently mobilized stem-like cell stage during the non-adherent phase. The elevation of both Nanog and Oct-4 proteins was confirmed by immunoblotting (**Fig. 4.9c**).

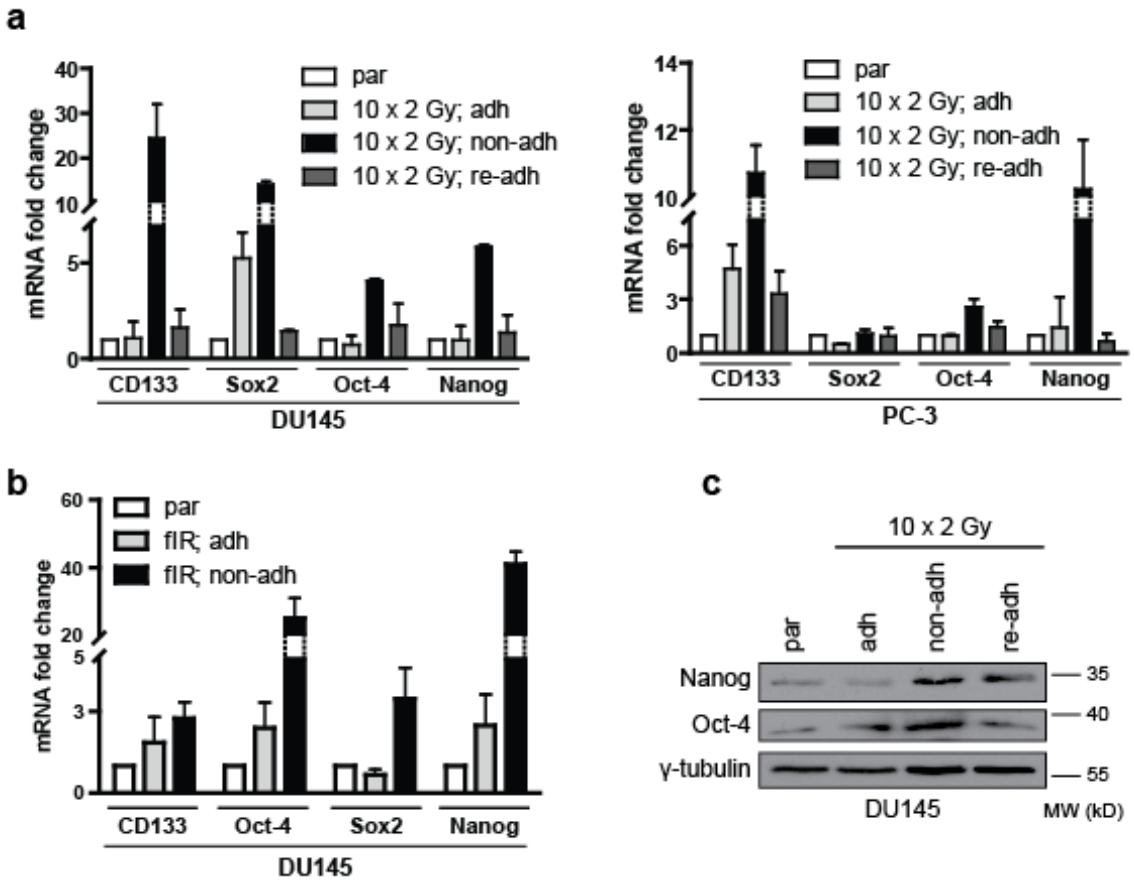

Figure 4.9. fIR non-adherent prostate cancer cells express stem cell-like markers. (**a**) qRT-PCR detection of CD133, Sox2, Oct-4 and Nanog in control (parental) and irradiated (10 x 2 Gy) radiation-surviving DU145 (left) and PC-3 (right) adherent, non-adherent and re-adherent cell populations. RPL37a was used as a reference gene. Data represent means ± S.D. from two independent experiments performed in triplicates. (**b**) qRT-PCR quantification of stem cell-related genes CD133, Sox2, Oct-4 and Nanog in control and irradiated (35 x 2 Gy) adherent and non-adherent DU145 cells. (**c**) Immunoblotting detection of Nanog and Oct-4 in control (parental) and irradiated (10 x 2 Gy) adherent, non-adherent and re-adherent DU145 cells. γ-tubulin was used as a loading control.

Notably, the increased activity of the Notch signaling pathway characteristic for several types of progenitor cells (Dontu et al., 2004; Fre et al., 2005) was detected as elevated mRNA of the Notch pathway-regulated genes Hes1 and Hey1 in the non-adherent irradiated fractions of both DU145 and PC-3 cells after 10 x 2 Gy (**Fig. 4.10a**) and in DU145 non-adherent cells after 35 x 2 Gy (**Fig. 4.10b**). The induction of Hes1 and Hey1 (Jarriault et al., 1995; Maier and Gessler, 2000) transcription factors was accompanied by elevated Notch ligands DLL1 and DLL4 in irradiated cells (see mRNA levels in **Fig. 4.10a** and DLL1 protein level in **Fig. 4.10c**). The elevated mRNA of DLL1, DLL4, Jag1, Hes1, and Hey1 in irradiated cells reverted nearly to parental cell levels after cell readhesion (**Fig. 4.10a**).

Altogether, the non-adherent irradiated cells differ from the adherent irradiated cell population by the enhanced expression of stem-like cell specific markers and progenitor cell-

associated Notch signaling. These irradiation-induced changes were deregulated in transient fashion as the expression of all genes tested was reverted upon cell readhesion.

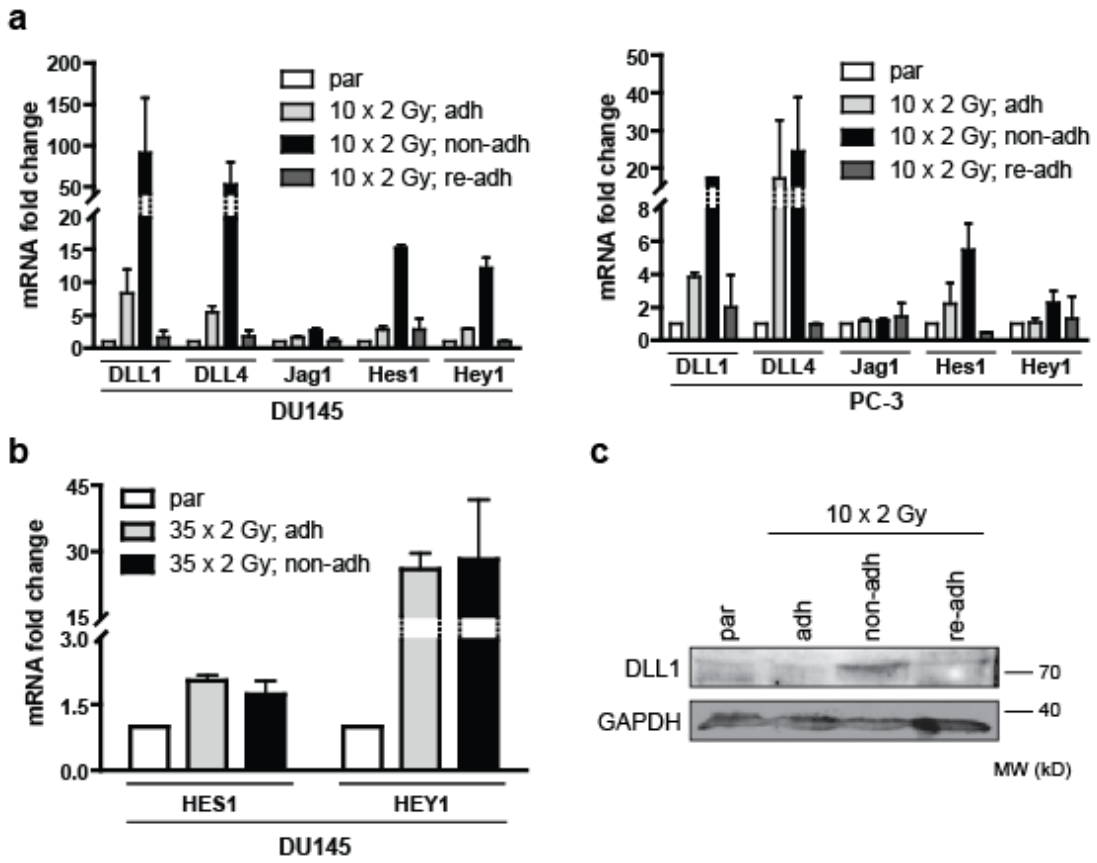

Figure 4.10. fIR non-adherent prostate cancer cells have active Notch signaling. (**a**) qRT-PCR quantification of Notch signaling ligands DLL1, DLL4, Jag1 and Notch signaling target genes Hes1 and Hey1 by qRT-PCR in control (parental) and irradiated (10 x 2 Gy) DU145 (left) and PC-3 (right) radiation-surviving adherent, non-adherent and re-adherent cell populations. RPL37a was used as a reference gene. Data in graphs represent means ± S.D. from two independent experiments performed in triplicates. (**b**) qRT-PCR quantification of Notch signaling-responsive genes Hes1 and Hey1 by qRT-PCR in control (parental) and irradiated (35 x 2 Gy) DU145 radiation-surviving adherent and non-adherent cell populations. RPL37a was used as a reference gene. (**c**) Immunoblotting detection of DLL1 in control (parental) and irradiated (10 x 2 Gy) adherent, non-adherent and re-adherent DU145 cells. GAPDH was used as a loading control. Data represent means ± S.D. from two (**a**) or three (**b**) independent experiments performed in triplicates.

4.2.4 fIR-surviving non-adherent fraction represents a proliferatively dormant population

To assess proliferation of the radiation-surviving non-adherent cells, we labeled cells with the cell proliferation dye eFluor670 that binds prevalently to membrane proteins and is diluted to half with each cell division as a consequence of equal redistribution of cell membranes among daughter cells (Quah and Parish, 2012). The non-irradiated non-adherent cells readhered rapidly during 24 hours and upon readhesion diluted the dye almost completely within 28 days consistent with the ongoing cell proliferation. On the other hand,

the non-adherent irradiated cells retained almost the same eFluor670 fluorescence intensity for the 28-day period (**Fig. 4.11a**, **b**) indicating the lack of cell proliferation. This was confirmed by very low level of EdU incorporation after a short pulse of EdU (3 hours) in non-adherent cell fraction (less than 2% EdU-positive cells at day 3 after the end of 10 x 2 Gy fIR), suggesting very low if any mitotic activity of the non-adherent cells (**Fig. 4.11c**). Cells from irradiated adherent fraction started to proliferative between days 10 – 15 after the last dose of fIR (10 x 2 Gy) indicating termination of the proliferative block (**Fig. 4.11c**). Propidium iodide (PI) staining of control and non-adherent populations indicated activation of G2/M cell cycle checkpoint upon fIR (10 x 2 Gy) (**Fig. 4.10d**). Cell cycle arrest in irradiated non-adherent cells was further supported by decreased levels of cell cycle regulators Plk1, Cdc25c, cyclin B1, and cyclin A (**Fig. 4.11c**).

Normal stem or progenitor cells derived from breast tissue can be enriched by culturing in non-adherent conditions as spheres (Dontu et al., 2003). It was also shown that human cancer cell lines are able to form (tumor) spheres enriched for stem-like cells (Cao et al., 2011; Fillmore and Kuperwasser, 2008). The tumorsphere forming assay therefore represents useful method for the assessment of tumor-initiating potential of cancer cells (Cao et al., 2011). As formation of CSCs-containing spheres was reported also for PCa, we adopted cultivation protocol from Duhagon *et al*. (Duhagon et al., 2010) and Liu *et al*. (Liu et al., 2012) to test sphere-formation capacity of fIR non-adherent cells. Despite our efforts we were not able to induce spheroid growth further supporting proliferatively dormancy of irradiated non-adherent cells.

Considering that the non-adherent irradiated cells restarted proliferation shortly after readhesion, the non-adherent survivors represent a pool of transiently non-proliferating cells capable of proliferation after reestablishment of the adherent phenotype.

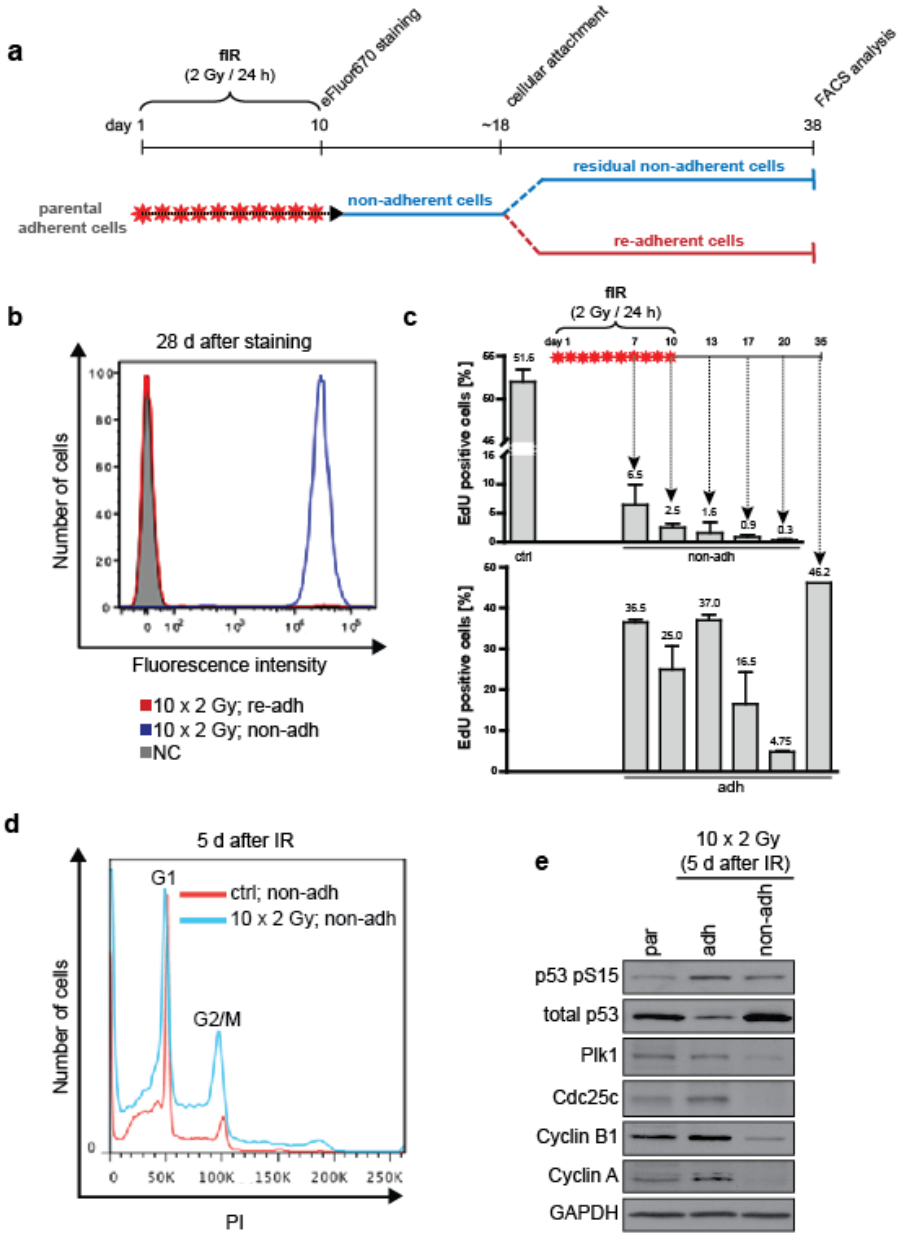

Figure 4.11. Proliferation of fIR-surviving adherent and non-adherent DU145 cells. (**a**) Schematic representation of the irradiation protocol using 2 Gy every 24 hours for 10 days. Generation of radiation surviving non-adherent population of DU145 cell line is depicted. Non-adherent cells were stained with eFluor670 immediately after the last dose of irradiation. Approximately one week after staining, a small fraction of non-adherent cells became re-adherent. Both non-adherent and re-adherent cells were analyzed by FACS at day 28 after staining. (**b**) Flow cytometric estimation of proliferation of irradiated (10 x 2 Gy) non-adherent and re-adherent DU145 cells using the eFluor670 dye dilution assay at day 28 after staining. Note, the eFluor670 dye was diluted with progress of cell divisions, see the left side 'shift' of the peak in the histogram in case of proliferation. Fraction of irradiated non-adherent DU145 cells which reattached was FACS-analyzed simultaneously with the residual non-adherent cells. Non-stained cells were used as a negative control (NC). (**c**) Flow cytometry analysis of EdU incorporation using Click-iT EdU proliferation assay in irradiated (10 x 2 Gy) non-adherent and adherent DU145 cells, during and after fIR, expressed as EdU positive cells (from viable fraction in percentage, numbers above bars) of total cells analyzed. Non-irradiated adherent cells were used as a control (ctrl). Data (except adherent cells collected at day 35) represent means ± S.D. from two independent experiments. (**d**) Flow cytometric analysis of cell cycle of control and irradiated (10 x 2 Gy) non-adherent DU145 cells 5 days after the last dose using propidium iodide (PI) DNA staining. (**e**) Immunoblotting detection of phosphoserine 15 of p53, total p53, Plk1, Cdc25c, cyclin B1 and cyclin A in control (parental) and irradiated (10 x 2 Gy) adherent and non-adherent DU145cells harvested 5 days after the last dose. GAPDH was used as a loading control.

4.3 SENSITIZING fIR-SURVIVING PROSTATE CANCER CELL POPULATIONS TO IONIZING RADIATION

4.3.1 Inhibition of Erk1/2 signaling supresses Snail expression and impairs survival and fIR-mediated loss of adhesion of non-adherent prostate cancer cells

Besides other mechanisms, Snail levels are regulated by the Erk1/2 and PI3K/Akt pathways (Barbera et al., 2004; Vega et al., 2004). Moreover, both Erk1/2 and PI3K/Akt mediate pro-survival signaling and enhance DSBs repair (Golding et al., 2009). Therefore, we investigated Erk1/2 and Akt activity during the fIR. Both Akt and Erk1/2 activities were highest between the doses 2 and 3 of fIR (**Fig. 4.12a**). This coincided with the highest levels of Snail and preceded the loss of adhesion. The ensuing loss of cell adhesion correlated with loss of active Akt and partial loss of Erk1/2 activity in the remaining adherent cells (**Fig. 4.12b**). Furthermore, in agreement with a previous report (Barbera et al., 2004), inhibition of Erk1/2 with the Mek/Erk unhibitor U0126 (ERKi) supressed the levels of Snail in adherent fraction (**Fig. 4.12c**), while treatment of the non-adherent cells with the ERKi resulted in cell death and also prevented cell detachment after a single dose of IR (10 Gy; **Fig. 4.12d**, **e**).

Consistently, the opposite manipulation to activate Erk1/2 pathway by GFs (a mixture of FGF and EGF) resulted in increased total numbers of detached cells (not shown) and also higher number of surviving non-adherent cells in both the control and irradiated samples of DU145 cells (**Fig. 4.12e**). To exclude potential nonspecific effects of the Erk inhibitor, siRNA-mediated knockdown of Erk1 and Erk2 was performed. Knockdown of both kinases impaired the loss of adhesion (not shown) and also cell survival (**Fig. 4.12g**) both in non-adherent control as well as in irradiated DU145 cells, thereby supporting the role of Erk1/2 signaling in the emerging anoikis-resistant cell survival after irradiation.

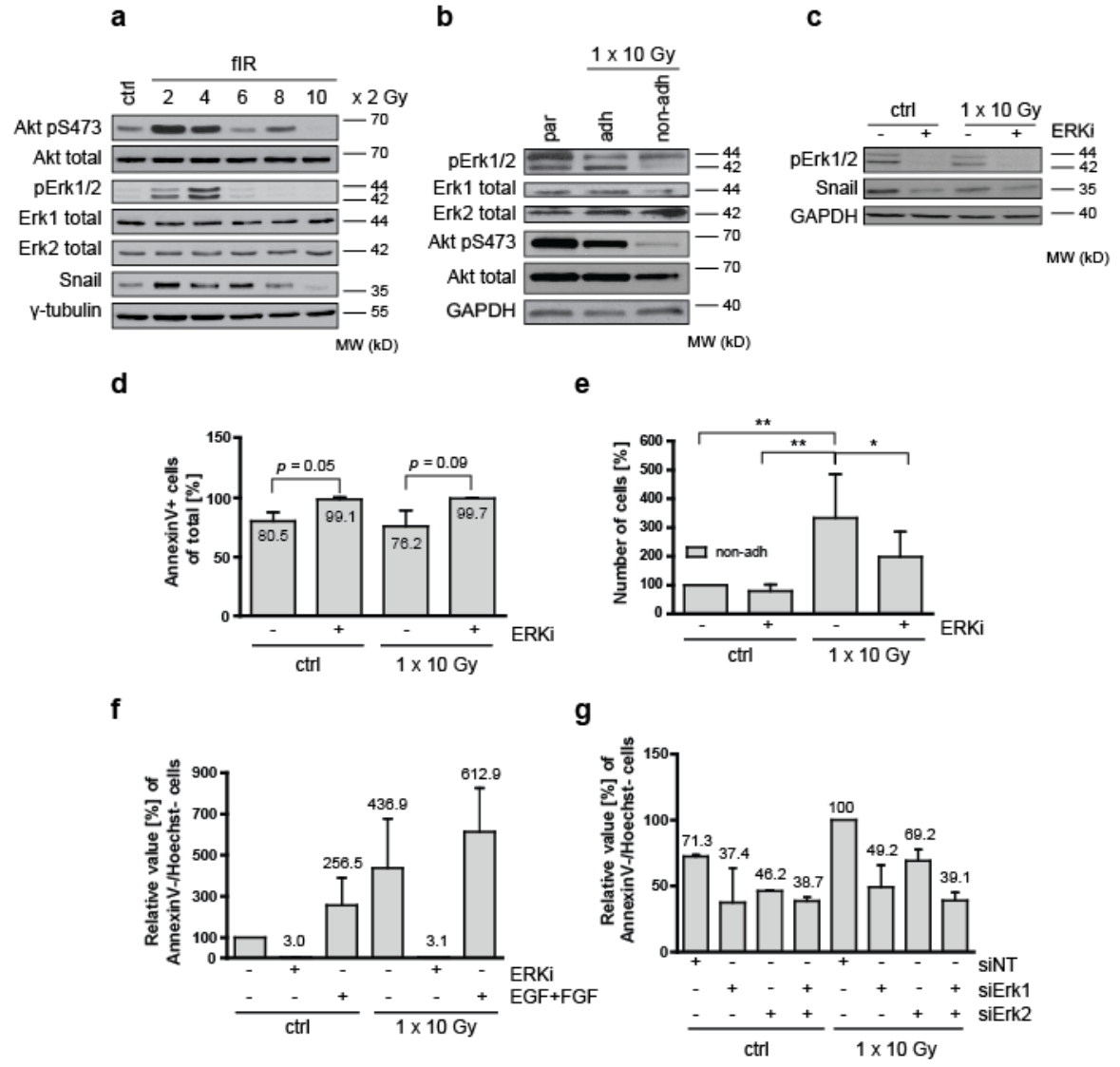

Figure 4.12. Effect of Erk1/2 inhibition on viability and loss of adhesion of fIR-surviving non-adherent DU145 cells. (**a**) Effect of Erk1/2 inhibition by ERKi on Snail in control and irradiated (1 x 10 Gy) DU145 cells detected by immunobloting. GAPDH was used as a loading control. (**b**) Immunobloting detection of total Akt, phosphoserine 473 of Akt, total Erk1 and Erk2, phosphothreonine 204/phosphotyrosine 204 of Erk (pErk1/2), and Snail in control and irradiated (2 - 10 x 2 Gy) DU145 cells. γ-tubulin was used as a loading control. (**c**) Immunoblotting detection of total Erk1 and Erk2, phosphothreonine 204/phosphotyrosine 204 of Erk1/2 (pErk1/2), total Akt and phosphoserine 473 of Akt in control (parental) and irradiated (1 x 10 Gy) adherent and non-adherent DU145 cells. GAPDH was used as a loading control. (**d**) Effect of Erk1/2 inhibition by ERKi on cell survival in control or irradiated (1 x 10 Gy) non-adherent DU145 cells assessed by flow cytometry 48 h after IR. Bars represent relative amount (in percentage, numbers above bars) of apoptotic cells (AnnexinV-FITC$^+$/Hoechst33258$^+$ plus AnnexinV-FITC$^+$/Hoechst33258$^-$) in total non-adherent fraction. (**e**) Effect of Erk1/2 inhibition by ERKi (49 h) on irradiation-induced (1 x 10 Gy) loss of adhesion expressed as the relative number of detached cells in control or irradiated non-adherent DU145 cells assessed by flow cytometry 48 h after IR. Bars represent relative amount (in percentage) of total cells. Non-irradiated control cells were set as 100%. (**f**) Effect of Erk1/2 activation (mixture of 10 ng/ml EGF and 50 ng/ml FGF) or chemical inhibition (ERKi) on cell survival assessed by flow cytometry in control or irradiated (1 x 10 Gy) non-adherent DU145 cells analyzed 48 h after IR by flow cytometry. Bars represent relative amount (in percentage, numbers above bars) of viable AnnexinV-FITC$^-$/Hoechst33258$^-$ cells in non-adherent fraction. Non-treated cells were set as 100%. (**g**) Effect of siRNA-mediated knockdown of Erk1 (siErk1) and Erk2 (siErk2) on cell survival in control or irradiated (1 x 10 Gy) non-adherent DU145 cells analyzed 48 h after IR and 72 h after transfection by flow cytometry. Nontargeting siRNA (siNT) was used as a negative control. The bars represent relative amount (in percentage, numbers above bars) of viable AnnexinV-FITC$^-$/Hoechst33258$^-$ cells in non-adherent fraction. Irradiated cells transfected with control siRNA (siNT) were set as 100%.

4.3.2 Erk1/2 regulates anoikis-resistant survival of prostate cancer cells by Bcl-2 family proteins

As anoikis is Bim-mediated apoptosis (Reginato et al., 2003) and the level (and activity) of pro-apoptotic Bim is regulated by the Erk1/2 pathway (Marani et al., 2004; Reginato et al., 2003; Weston et al., 2003), we assessed the levels of Bim in irradiated DU145 cells. Consistent with resistance to anoikis, the level of Bim in irradiated non-adherent cells was substantially lower than in the irradiated adherent cells (**Fig. 4.13a**). Chemical inhibition of Erk1/2 led to increased levels of Bim both in control and irradiated cells (**Fig. 4.13b**). In contrast to pro-apoptotic Bim, the anti-apoptotic Bcl-XL protein (Cheng et al., 1996) was increased in non-adherent cells after both 1 x 10 Gy and 10 x 2 Gy (**Fig. 4.13a**). Chemical inhibition of Erk1/2 led to a decrease of Bcl-XL (**Fig. 4.13b**) indicating that during fIR Bcl-XL is controlled by Erk1/2 signaling. Ectopic expression of Bcl-XL in DU145 cells (**Fig. 4.13c**) resulted in a statistically significant increase of survival of the non-adherent cell fraction after fIR by 10 x 2 Gy, whereas chemical inhibition of Erk1/2 signaling suppressed survival among the Bcl-XL-overexpressing cells (**Fig. 4.13d**), supporting the role of the Erk1/2/Bcl-XL axis in radiation-induced anoikis resistance. Interestingly, transient knockdown of Snail by siRNA (siSnail) resulted in the decreased Erk1/2 activity indicating a mutual regulatory loop between Snail and Erk1/2 in response to genotoxic stress (**Fig. 4.13e**).

In conclusion, loss of cell adhesion, Snail expression, levels of the apoptotic modulators Bim and Bcl-XL, as well as anoikis resistance of the radiation-surviving non-adherent cell fraction is controlled by the Erk1/2 activity.

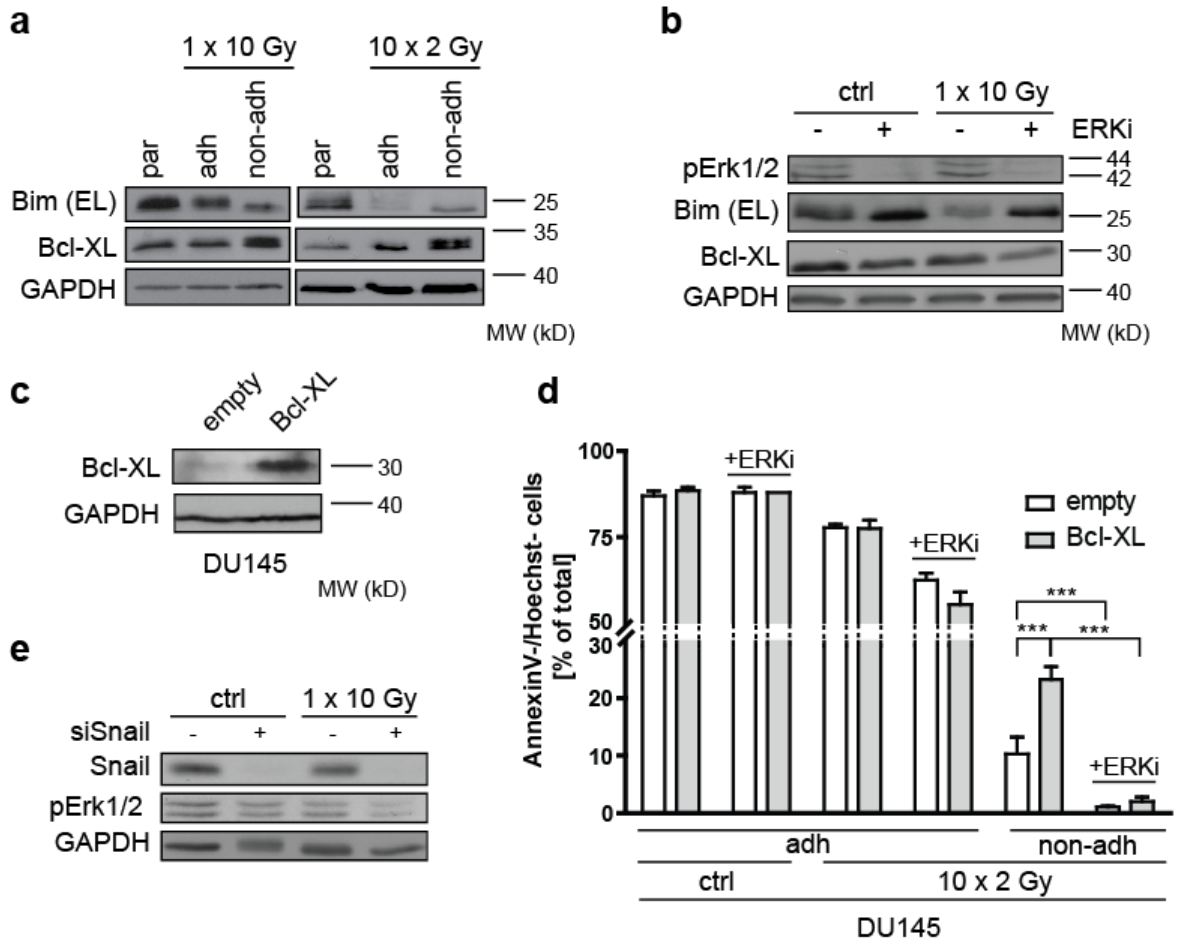

Figure 4.13. Erk1/2 regulates anoikis resistant survival in prostate cancer cells after IR. (**a**) Effect of Erk1/2 inhibition (ERKi) on Bim (EL, extra large form) and Bcl-XL levels in control or irradiated (1 x 10 Gy) DU145 cells assessed by immunoblotting. GAPDH was used as a loading control. (**b**) Immunoblotting detection of Bim (EL) and Bcl-XL levels in control or irradiated (1 x 10 Gy or 10 x 2 Gy) adherent or non-adherent DU145 cells. GAPDH was used as a loading control. (**c**) Immunobloting detection of Bcl-XL in DU145 stable cell lines generated by infection with lentiviruses possesing pCDH-CMV-MCS-EFI-Neo-empty (empty) or pCDH-CMV-MCS-EFI-Neo-Bcl-XL (Bcl-XL) vector. GAPDH was used as a loading control. (**d**) Effect of ERKi (10 μM for 16 h, added after the last dose of fIR) on cell survival in control or irradiated (10 x 2 Gy) adherent and non-adherent stable DU145 cells constitutively expressing Bcl-XL (Bcl-XL) analyzed by flow cytometry. The bars represent relative amount (in percentage) of viable (AnnexinV-FITC$^-$/Hoechst33258$^-$) cells in non-adherent fraction. Stable DU145 cell line transduced with pCDH-CMV-MCS-EFI-Neo-empty vector (empty) was used as a control. Data in (**d**) represent means ± S.D from four (w/o ERKi) or two (+ERKi) independent experiments performed in duplicates. ***p<0.001. (**e**) Effect of Snail siRNA-mediated knockdown (siSnail) on Erk1/2 activity (phosphorylation on phosphothreonine 204/phosphotyrosine 204, pErk1/2) assessed by immunoblotting. GAPDH was used as a loading control.

4.3.3 Simultaneous inhibition of Erk1/2 and Akt pathways in prostate cancer cells has an additive radiosensitizing effect

Enhanced Mek/Erk and PI3K/Akt signaling contribute to chemo- and radio-resistance of PCa (Kajanne et al., 2009; Kumar et al., 2005; Skvortsova et al., 2008). Therefore, we exposed DU145 cells either to a single dose of 10 Gy or 10 daily fractions of 2 Gy in the presence or absence of chemical inhibitors of Erk1/2, Akt or their combination and followed survival and proliferation of cells in both non-adherent and adherent fractions. Inhibition of Erk1/2 alone eliminated majority of live non-adherent cells while the effect of Akt inhibition alone on survival of non-adherent cell fraction was weaker (**Fig. 4.14b**) correlating with low Akt activity in the non-adherent cells (**Fig. 4.12b**). The combined inhibitors eliminated survival of IR-exposed non-adherent cell fraction and, more importantly, showed a greater anti-proliferative effect than the treatment with either inhibitor alone on the IR-resistant adherent PCa cells (**Fig. 4.14a**, **c**, **d**).

Complementary to the radiosensitization experiments, we showed that the combined inhibition of Akt and Erk1/2 completely suppressed also chemoresistance of PCa cells, otherwise manifested as outhgrowth of senescence-resistant colonies of DU145 cells exposed to a subapoptotic, senescence-inducing treatment with doxorubicin (0.75 μM; 4-hour exposure; **Fig. 4.14e**).

Overall, the combined inhibition of Akt and Erk1/2 signaling impaired survival of the anoikis-resistant non-adherent PCa cells and prevented escape from senescence in the adherent PCa population after IR and doxorubicine treatment.

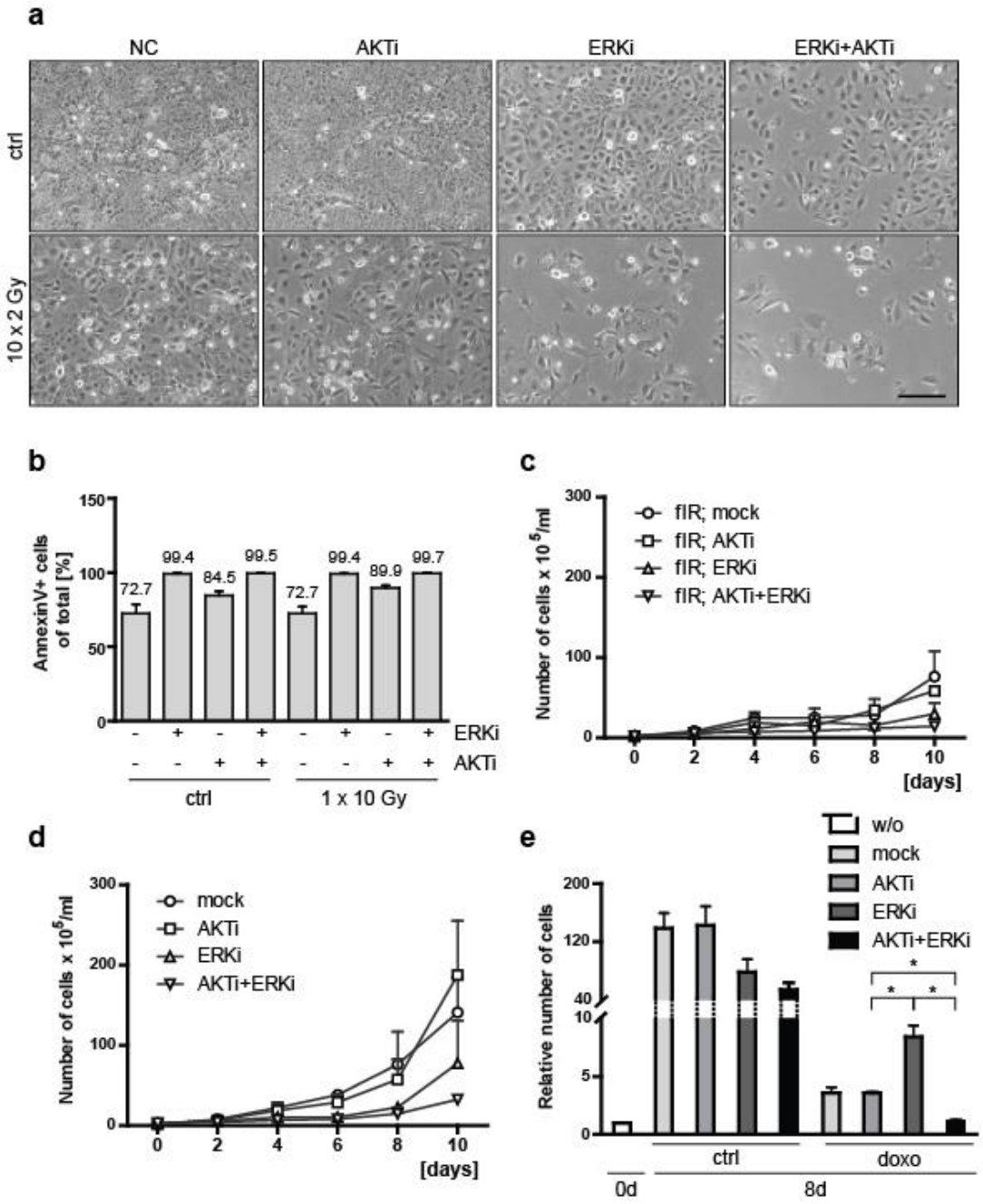

Figure 4.14. Differential sensitivity of fIR-surviving adherent and non-adherent DU145 cells to inhibitors of Erk and Akt signaling. (**a**) Phase contrast microscopic images of control or irradiated adherent (10 x 2 Gy) DU145 cells after Erk (ERKi) and/or Akt (AKTi) inhibition captured at day 10 after IR. Bar, 100 µm. (**b**) Effect of Erk1/2 inhibition (ERKi) and/or Akt inhibition (AKTi) on cell survival in control or irradiated (1 x 10 Gy) non-adherent DU145 cells analyzed by flow cytometry 48 h after IR. The bars represent relative amount (in percentage, numbers above bars) of apoptotic cells (AnnexinV-FITC$^+$/Hoechst33258$^+$ plus AnnexinV-FITC$^+$/Hoechst33258$^-$) in total non-adherent fraction. (**c**) Effect of Erk1/2 inhibition (ERKi) and/or Akt inhibition (AKTi) on proliferation of irradiated (10 x 2 Gy) DU145 cells. Trypan blue negative cells were counted at time points as indicated. (**d**) Effect of Erk1/2 inhibition (ERKi) and/or Akt inhibition (AKTi) on proliferation of control non-irradiated DU145 cells. Trypan blue negative cells were counted at time points as indicated. (**e**) Effect of Erk1/2 inhibition (ERKi) and/or Akt inhibition (AKTi) on proliferation of control or doxorubicine-treated (0.75 µM) DU145 cells. Relative numbers of cells were assessed at day 0 (w/o; without any additives) and day 8. Data in all bars are normalized to control cells (i.e. to doxorubicin-untreated cells) at day 8. Data in panels **a** – **d** represent mean values ± S.D. from two independent experiments. *p<0.05. In **b** – **d**, control cells were treated with DMSO diluent (mock).

4.4 ACQUIRED RADIORESISTANCE OF PROSTATE CANCER CELLS

4.4.1 fIR-surviving re-adherent prostate cancer cells exhibit acquired radioresistance

Conceptually as well as therapeutically relevant issue is whether the adherent or the non-adherent cell fractions feature 'radioresistance memory', e.g. enhanced survival upon repeated round(s) of irradiation. To assess whether the fIR-surviving cells are more radioresistant than the parental cells, we exposed DU145 cells that survived 35 doses of 2 Gy as either adherent (adherent survivors) or non-adherent fraction after its readhesion (re-adherent survivors) to a single dose of 10 or 40 Gy. Although activation of DDR (**Fig. 4.15a**) and the overall survival of both adherent and re-adherent survivors did not differ markedly from the parental cells (**Fig. 4.15b**) and the colony-forming ability was increased only for the adherent survivors compared to the parental cells (**Fig. 4.15c**; in agreement with previous study (Skvortsova et al., 2008)), the colonies of the re-adherent survivors (although equally numerous as colonies of the parental cells) featured significant fewer colonies composed of senescent-like cells (**Fig. 4.15c**; red dots). Importantly, the anoikis-resistant survival of the re-adherent survivors of dose 10 x 2 Gy was enhanced after re-irradiation with 40 Gy compared to both the parental cells and adherent survivors (**Fig. 4.15d**).

Taken together, the radiation-induced phenotypes likely reflect contributions from both adaptive (partly transient) responses due to the plasticity of the metastatic human PCa cell populations, and more durable (heritable), selection-acquired resistance to radiation-evoked cellular senescence and anoikis.

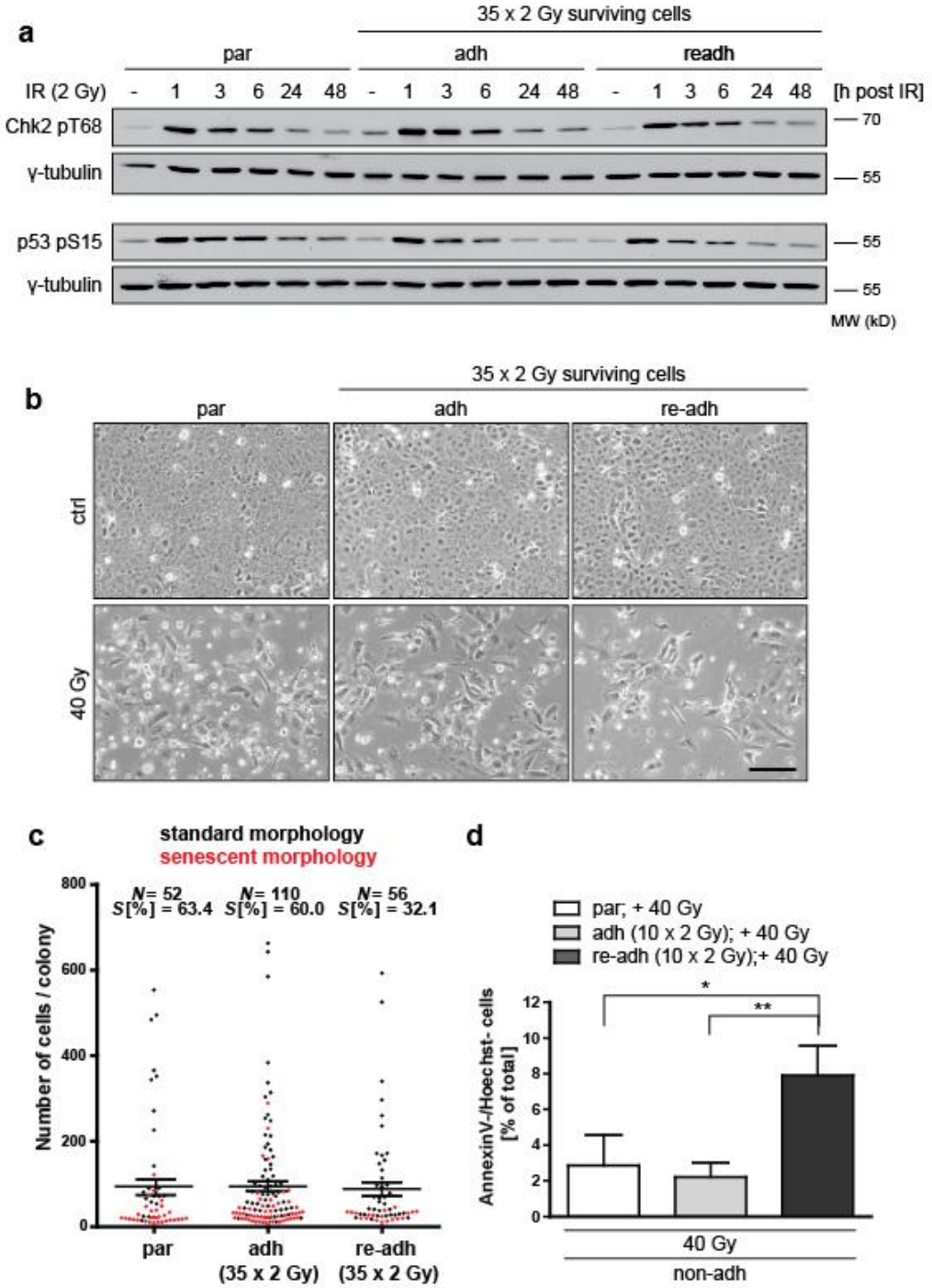

Figure 4.15. **Evaluation of radioresistance of fIR-surviving cells.** (**a**) Phase contrast microscopic images of parental (par) and irradiated (35 x 2 Gy) adherent (adh) and re-adherent (readh) DU145 survivors 3 days after a single dose (40 Gy) or without re-irradiation (ctrl). Bar, 100 µm. (**b**) Clonogenic cell survival assay. Parental or irradiated (35 x 2 Gy) adherent and re-adherent DU145 survivors were counted and 1 x 10^3 cells seeded into 6 well plates in triplicates. Number of cells from all colonies (>10 cells) formed at day 13 following re-irradiation (10 Gy) were counted and plotted. N - number of total colonies formed. S - number of senescent colonies formed/number of total colonies formed x 100. Each dot in the plot represents one colony characterized by number of cells and presence (red dot) or absence (black dot) of senescent cells according to cell morphology. (**c**) Anoikis-resistant survival of parental (par) and previously irradiated (10 x 2 Gy) adherent (adh) and re-adherent (readh) DU145 survivors after re-irradiation (40 Gy) assessed by flow cytometry 72 h after IR. Bars represent relative amount (in percentage) of viable cells (AnnexinV-FITC$^-$/Hoechst33258$^-$) in total non-adherent fraction. Data represent mean values ± S.D. from three independent experiments performed in duplicates. *p<0.05; **p<0.01.

4.5 HIGH-THROUGHPUT GENE EXPRESSION ANALYSIS OF fIR-SURVIVING CANCER CELL POPULATIONS

4.5.1 Irradiated non-adherent prostate cancer cells represent the most variable fIR-surviving population

As we showed above, fIR (even therapeutical doses 35 x 2 Gy) generated phenotypically distinct surviving PCa populations *in vitro* that can be relevant as a model of post-therapy cancer recurrence *in vivo* (senescence escapers, anoikis-resistant and re-adherent survivors). Moreover, the observed cellular plasticity of PCa cells (e.g. EMT/MET changes, anoikis-resistant survival and stem-like characteristics of dormant non-adherent cells) can resemble the invasion-metastases cascade (see **Fig. 7.1**) indicating that this *in vitro* model could be potentionally relevant to cancer post-therapy recurrence and development of metastases. The thorough analysis of resistant cells can highlight not only the nature of these cell populations but also unravel the specific molecular determinants potentially utilizable as diagnostic/prognostic factors or therapeutic targets.

With this in mind, we performed high-throughput whole genome expression analysis of parental and irradiated adherent, non-adherent and re-adherent DU145 cells. Since 35 doses of 2 Gy resulted only in very low percentage of viable non-adherent cells and satisfactory reproducibility of key data (expression of Snail, stemness markers, Notch pathway-related genes) using shortened regimen (10 doses of 2 Gy), we decided to use this shortened regimen also for the microarray analysis. As shown in dendrogram resulted from hierarchical clustering analysis (HCA), irradiated non-adherent cells manifested the greatest inter-cluster distance when compared to the resulting populations (**Fig. 4.16a**). Irradiated re-adherent DU145 cells showed a greater inter-cluster distance than irradiated adherent cells which is in agreement with their origin from non-adherent cells (**Fig. 4.16a**). We obtained the same clustering pattern using principal component analysis (PCA; **Fig. 4.16b**). Next, compared expression patterns of fIR-surviving populations and parental cells plotted resulting significantly up or downregulated genes ($p<0.05$, $|LFC\ (\log_2\ fold\ change)|>1$). By this approach we detected commonly or exclusively expressed genes in fIR-surviving PCa fractions. Again, we identified fIR-non-adherent cells as the most disimilar population (2769 exclusive genes) in comparison to fIR-adherent (60 exclusive genes) and re-adherent (201 exclusive genes) cells. Only 19 genes were commonly deregulated in all three irradiated populations compared to control cells (**Fig. 4.16c**).

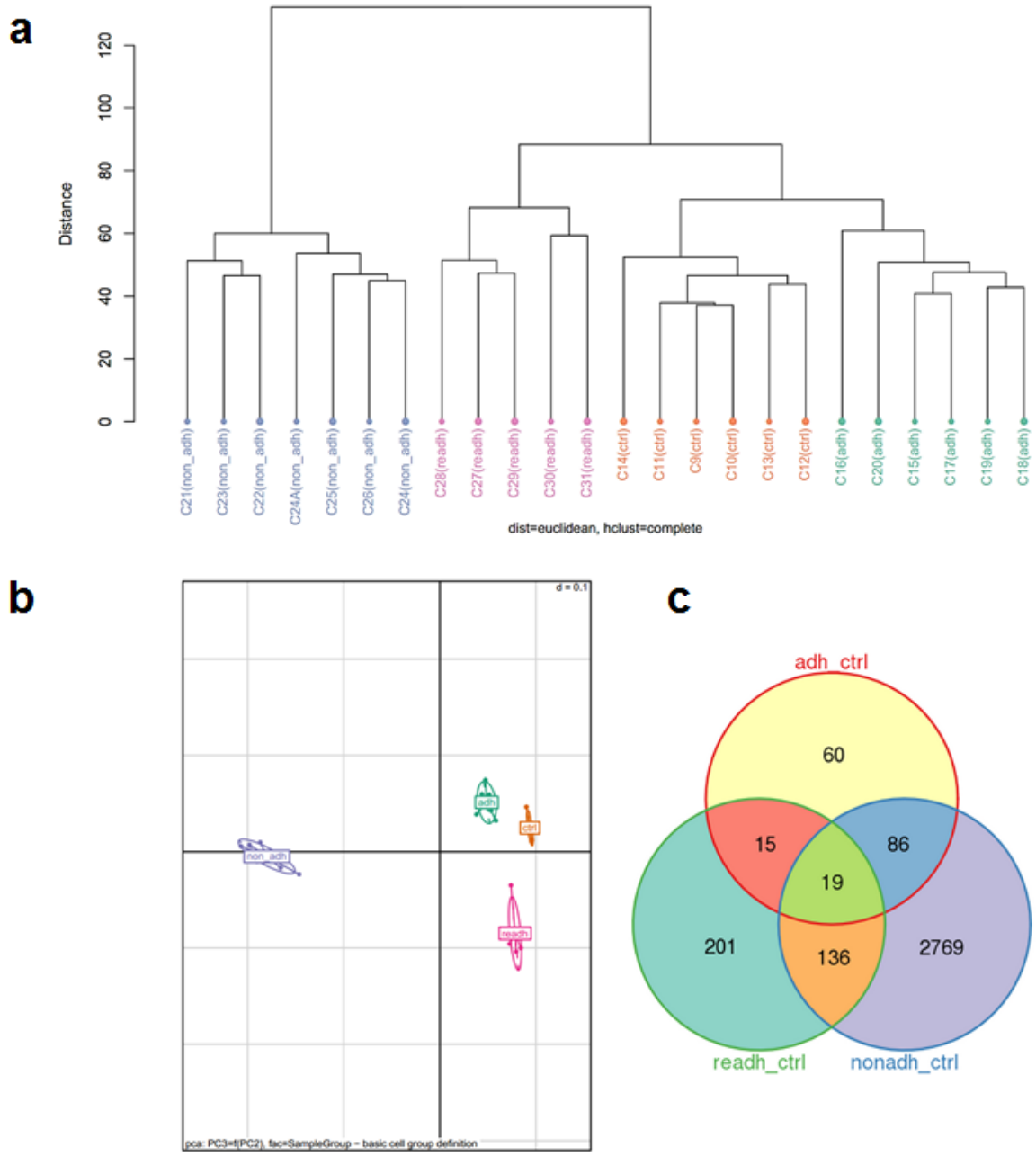

Figure 4.16. Hierarchival clustering analysis of microarray expression data of irradiated DU145 cells. (**a**) Hierarchical clustering analysis and principal component analysis (**b**) of control (orange) and irradiated (10 x 2 Gy) adherent (green), non-adherent (blue) and re-adherent (purple) DU145 cells. Data from each sample represent two biological replicates irradiated as three technical replicates (except re-adherent cells which represented two technical replicates). (**c**) Venn diagram representing gene expression overlap between fIR-surviving adherent (red), non-adherent (blue) and re-adherent (green) DU145 subpopulations. Numbers represent single and common significantly up or downregulated genes over control non-irradiated sample ($p<0.05$, $|LFC|>1$).

Heatmap containing the top 100 most variable genes is depicted in the **Fig. 4.17**. Of these genes, approximately one third represented specific RNA-coding genes including the top scoring long non-coding RNA (lnRNA; RN7SK), small nucleolar RNAs (snoRNAs) containing C/D box (e.g. SNORD12B, SNORD43, SNORD64, SNORD66 and SNORD95), small nuclear RNAs (snRNAs) involved in mRNA splicing (e.g. RNU1-5, RNU1-3, RNU1G2, RNU143, RNU4-2, RNU4-1RNU6-1 and RNU6-15), small Cajal body RNA (scaRNA; SCARNA2) and microRNAs (miRNAs; e.g. MIR425 and MIR320A). Protein coding genes included genes participated in cell cycle regulation like CDK inhibitor 1A ($p21^{waf1/Cip1}$, CDKN1A) or cyclin B1 interacting protein 1 (CCNB1IP1), and genes expressed in a response to oxidative stress like oxidative stress responsive serine-rich 1 (OSER1) and superoxide dismutase 2 (SOD2). One of the best scoring protein coding gene was presenilin 1 (PSEN1) implicated in intramembrane cleavage of integral membrane proteins such as Notch receptors or E-cadherin (De Strooper et al., 1999; Marambaud et al., 2002). Interestingly, among 100 significantly affected genes, 10% represented inflammation-associated genes including tumor necrosis factor (TNFα), jun proto-oncogene (JUN), interleukin 23A (IL23A), interferon α1 (IFNL1), interferon β1 (IFNB1), FBJ murine osteosarcoma viral oncogene homolog B (FOSB), chemokine (C-C motif) ligand 3-like 1 (CCL3L1), chemokine (C-C motif) ligand 20 (CCL20), lymphotoxin alpha (LTA), and activating transcription factor 3 (ATF3).

To distinguish whether irradiation-induced changes are IR-related and are not the artifacts caused by the loss of adhesion *per se*, we performed additional clustering analysis to compare fIR-non-adherent DU145 cells with the non-adherent fraction naturally present in asynchronous cell culture. As shown in the heatmap containing 1000 out of 2556 probes (**Fig. 4.18**), expression profile of the irradiated non-adherent cells differed markedly from their non-irradiated counterparts.

In conclusion, microarray data analysis revealed fIR-induced exclussive expression pattern of irradiated non-adherent PCa fraction.

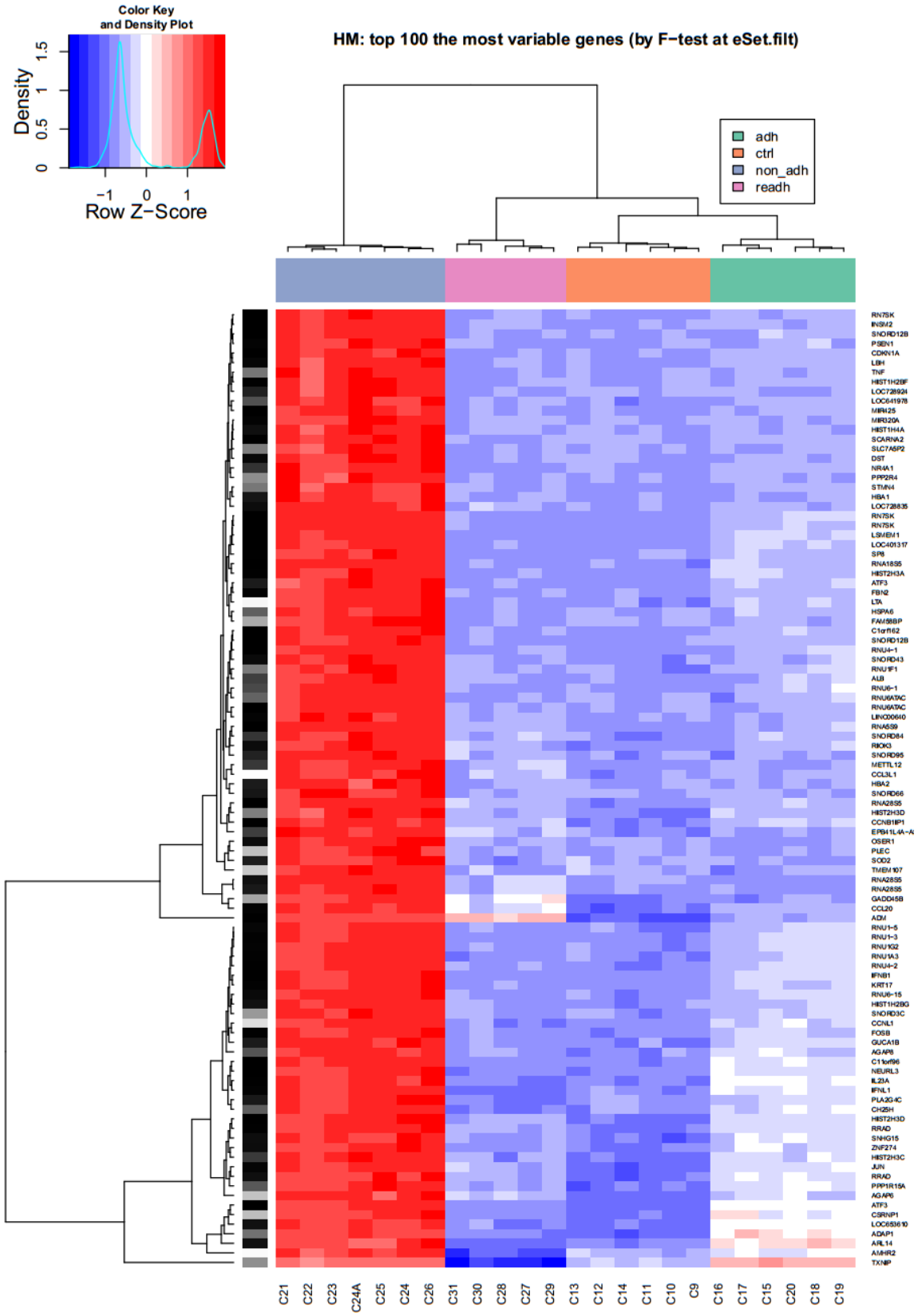

Figure 4.17. Hierarchical clustering of changes in gene expression in fIR-surviving DU145 subpopulations. Heatmap generated from microarray data by hierarchical clustering represents the contrast between non-irradiated control (orange line) and irradiated (10 x 2 Gy) adherent (green cluster), non-adherent (blue cluster) and re-adherent (purple cluster) DU145 cells. Inside the heatmap, blue color represents downregulated and red color represents upregulated genes ($q<0.05$). Array was performed with cells derived from two independent batches of DU145 cells, both cultured and irradiated as three technical replicates.

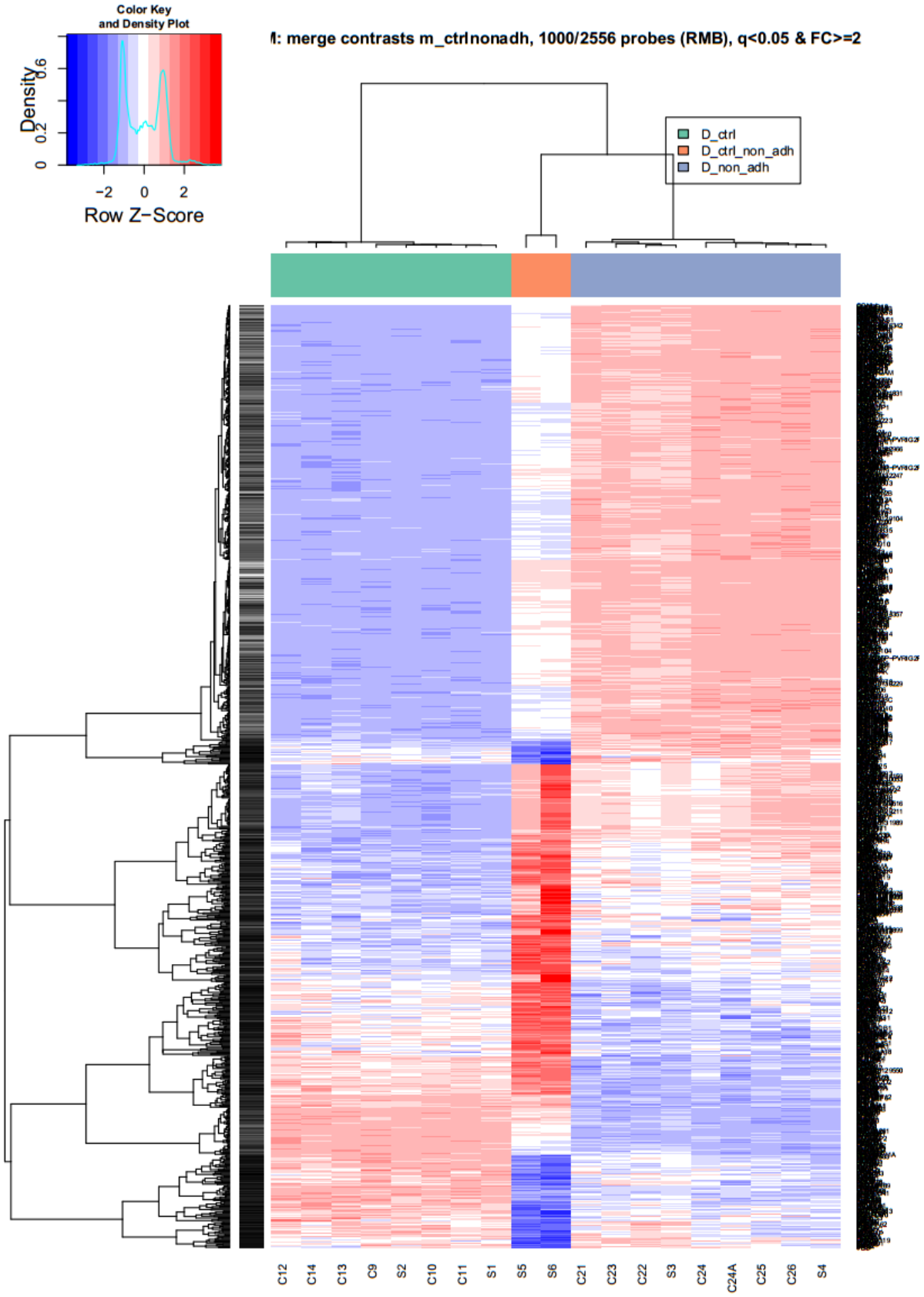

Figure 4.18. Hierarchical clustering of changes in gene expression of control non-adherent and irradiated non-adherent DU145 cells. Heatmap generated from microarray data by hierarchical clustering represents the contrast between control adherent (green cluster), control non-adherent (orange cluster) and irradiated (10 x 2 Gy) non-adherent (blue line) DU145 cells. Inside the heat map, blue color represents downregulated and red color represents upregulated genes ($q<0.05$, $|LFC|\geq 2$).

4.5.2 Comparative analysis of expression profiles of irradiated or 5-azacytidine-treated surviving cancer cell populations

As mentioned above, we observed fIR-induced EMT/MET-related phenotypic plasticity not only in PCa cell lines but also in irradiated breast adenocarcinoma cell line MCF-7 (see **Fig. 4.2a**, **b**; **Fig. 4.8.a**, **b**). Importantly, we were able also to evoke Snail-dependent loss of adhesion and subsequent readhesion by treating cervical cancer cell line HeLa with 5-azacytidine (4 μM; data not shown), indicating that observed phenotypic plasticity is not restricted to the cell origin or type of genotoxic stress but represents a more general mechanism how cancer cells can escape the genotoxic stress.

To uncover a subset of genes specifically expressed in response to genotoxic stress but not dependent on the cell type and the kind of genotoxic insult, we performed a whole genome microarray with irradiated (10 x 2 Gy) MCF-7 and 5-azacytidine-treated HeLa cells included. The three cell lines (DU145, MCF-7 and HeLa) responded to irradiation and/or drug treatment by considerable transcriptome deregulation at the 2-fold threshold levels (**Fig. 4.19a**). The highest changes in gene expression were observed in 5-azacytidine-treated HeLa cells compared to control cells (**Fig. 4.19a**). Treated non-adherent surviving fraction was the most dissimilar population in contrast to parental cells in all three cases with 1612 altered genes in DU145, 1763 altered genes in MCF-7, and 2076 altered genes in HeLa cells (**Fig. 4.19a**). Differentially expressed genes in particular therapy surviving fractions from DU145, MCF-7 and HeLa cells compared to the control cells were merged resulting in expression signatures typical for each surviving fraction (**Fig. 4.19a**).

Most importantly, by this approach we identified 463 differentially expressed genes ($p<0.05$, $|LFC|>1$) in surviving non-adherent DU145, MCF-7, and HeLa cells when compared to parental populations. The most upregulated genes shared among all three non-adherent populations are shown in **Fig. 4.19b**.

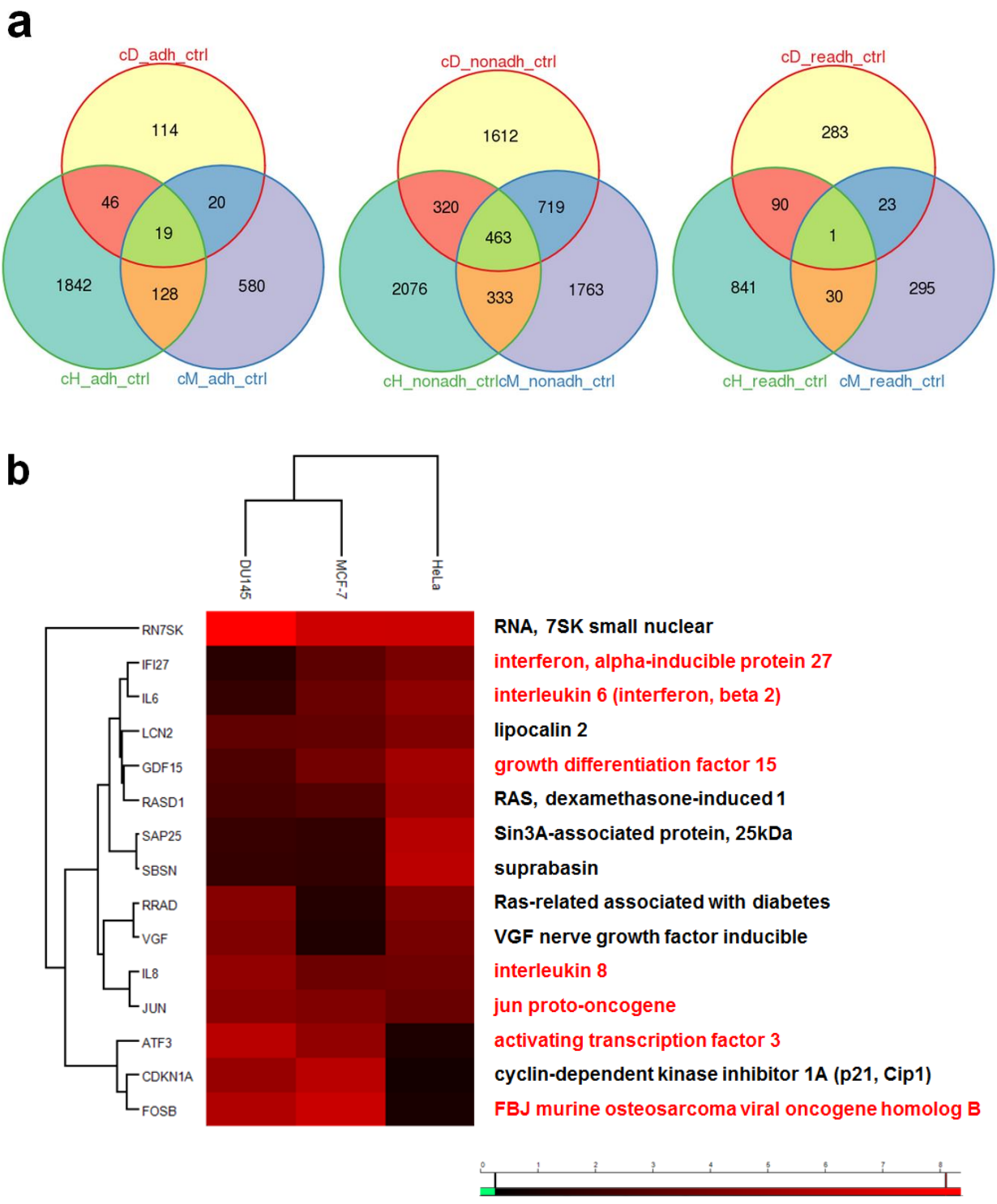

Figure 4.19. Expression patterns in *treated* non-adherent cancer cells. (**a**) Venn diagrams representing the gene expression overlap between fIR-surviving adherent (left), non-adherent (middle) and re-adherent (right) populations derived from cancer cells lines exposed either to 10 x 2 Gy (DU145, 'cD'; MCF-7, 'cM') or treatment with 4 μM 5-azacytidine added daily for 7 d with fresh media (HeLa, 'cH'). Numbers represent cell line-specific and common significantly up or downregulated genes over control non-irradiated sample ($p<0.05$, $|LFC|>1$). (**b**) Dendrogram showing the most upregulated ($p<0.05$, $|LFC|>1$) genes common for *treated* non-adherent DU145, MCF-7 and HeLa cells over controls. Genes implicated in IFN-mediated signaling are highlighted in red. Data were processed by Perseus software.

4.5.3 Genotoxic stress induces cytokine signaling and immune/interferon response-related genes in non-adherent cancer cell population

To uncover commonly deregulated pathways in the non-adherent populations, we classified all differentially expressed genes into functional categories using Z-score that is based on t-statistics. A complete list of significant functional categories according to KEGG database for each cell line that was differentially expressed with q<0.05 (orange) is depicted in **Fig. 4.20**. Gene ontology classification (KEGG) of genes differentialy expressed in *treated* non-adherent cells revealed that genes implicated in regulation of metabolism were the most deregulated in all three cancer cell lines (DU145: $p=1.03 \times 10^{-5}$; MCF-7: $p=6.67 \times 10^{-3}$; HeLa: p=0.136). Interestingly, as indicated by the first microarray analysis of DU145, cytokine-cytokine receptor interaction pathway was strongly affected also in fIR non-adherent MCF-7 and 5-azacytidine-treated HeLa cells (DU145: $p=6.34 \times 10^{-3}$; MCF-7: $p=6.67 \times 10^{-3}$; HeLa: p=0.292) (**Fig. 4.20**).

Besides cytokine-cytokine receptor interaction pathways (see **Fig. 4.21** for individual genes; only those genes that fit the pathway according to KEGG database are shown), in treated non-adherent cancer cells was also upregulated Jak/STAT and Toll-like, NOD-like and RIF-I-like receptor signaling pathways, all containing numerous interferon (IFNα, IFNβ and IFNγ)-stimulated genes (**Fig. 4.20**).

KEGG	Name	gDET	cD_nonadh_ctrl	cM_nonadh_ctrl	cH_nonadh_ctrl
hsa01100	Metabolic pathways	651	1.03E-05	0.00667	0.136
hsa05322	Systemic lupus erythematosus	52	0.00263	0.12	0.136
hsa04060	Cytokine-cytokine receptor interaction	87	0.00634	0.00667	0.292
hsa05323	Rheumatoid arthritis	48	0.0571	0.12	0.415
hsa00640	Propanoate metabolism	25	0.207	0.416	0.315
hsa04630	Jak-STAT signaling pathway	71	0.207	0.138	0.217
hsa04623	Cytosolic DNA-sensing pathway	28	0.207	0.347	0.493
hsa00280	Valine, leucine and isoleucine degradation	36	0.207	0.357	0.312
hsa05010	Alzheimer's disease	92	0.215	0.217	0.292
hsa04620	Toll-like receptor signaling pathway	52	0.23	0.217	0.478
hsa00010	Glycolysis / Gluconeogenesis	36	0.248	0.493	0.45
hsa04621	NOD-like receptor signaling pathway	33	0.254	0.303	0.478
hsa00620	Pyruvate metabolism	27	0.254	0.422	0.359
hsa00982	Drug metabolism - cytochrome P450	21	0.254	0.491	0.493
hsa04622	RIG-I-like receptor signaling pathway	40	0.254	0.303	0.492
hsa00071	Fatty acid metabolism	28	0.266	0.387	0.478
hsa04380	Osteoclast differentiation	67	0.266	0.12	0.445
hsa00020	Citrate cycle (TCA cycle)	25	0.266	0.245	0.305
hsa04146	Peroxisome	51	0.27	0.467	0.478
hsa05012	Parkinson's disease	73	0.271	0.12	0.35
hsa00380	Tryptophan metabolism	23	0.294	0.469	0.492
hsa05016	Huntington's disease	112	0.363	0.166	0.359
hsa04010	MAPK signaling pathway	148	0.38	0.146	0.384

Figure 4.20. Functional gene categories deregulated in *treated* non-adherent cancer cells. Enrichment of individual functional gene categories in populations derived from cancer cells lines exposed either to 10 x 2 Gy (DU145, 'cD'; MCF-7, 'cM') or treatment with 4 μM 5-azacytidine added daily for 7 d with fresh media (HeLa, 'cH') in comparison to parental (control) cells assessed by gene ontology that was differentially expressed with

q<0.05 (orange). Values with p<0.4 (even statistically non-significant) are highlighted in yellow. gDET represents a number of individual genes significantly altered in the particular pathway grouped according to KEGG pathway database (see KEGG number on the left).

a

DU145				
SYMBOL	GENENAME	logFC	p-value	FDR
IL8	interleukin 8	4.74	2.5E-16	1.1E-14
IFNB1	interferon, beta 1, fibroblast	4.49	1.3E-31	3.2E-28
IFNL1	interferon, lambda 1	4.35	4.2E-32	1.3E-28
CCL20	chemokine (C-C motif) ligand 20	4.33	3.9E-22	6.5E-20
IL23A	interleukin 23, alpha subunit p19	3.9	6.2E-30	1E-26
CXCL2	chemokine (C-X-C motif) ligand 2	3.41	1.6E-15	5.8E-14
CCL5	chemokine (C-C motif) ligand 5	3.35	3.6E-17	1.9E-15
CCL5	chemokine (C-C motif) ligand 5	2.98	1.4E-16	6.6E-15
IFNL1	interferon, lambda 1	2.96	2.5E-25	1.1E-22
IFNL2	interferon, lambda 2	2.92	5.3E-27	4.6E-24
CCL3L3	chemokine (C-C motif) ligand 3-like 3	2.9	1.9E-25	8.9E-23
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	2.79	1.3E-10	1.6E-09
LTA	lymphotoxin alpha	2.78	4.7E-26	2.7E-23
CSF2	colony stimulating factor 2 (granulocyte-macrophage)	2.51	4.8E-22	7.8E-20
CXCL10	chemokine (C-X-C motif) ligand 10	2.31	7.8E-19	6.2E-17
TNF	tumor necrosis factor	2.29	7E-20	6.9E-18
IL11	interleukin 11	2.1	4.4E-15	1.4E-13
CCL3	chemokine (C-C motif) ligand 3	2.07	8.6E-24	2.3E-21
IFNL3	interferon, lambda 3	1.95	1.7E-23	4.3E-21
TNFRSF9	tumor necrosis factor receptor superfamily, member 9	1.93	1.1E-17	6.7E-16
LEP	leptin	1.9	5.2E-13	1E-11
IL6	interleukin 6 (interferon, beta 2)	1.87	2.9E-06	1.6E-05
TNFRSF9	tumor necrosis factor receptor superfamily, member 9	1.77	4.2E-12	6.9E-11
CCL3L1	chemokine (C-C motif) ligand 3-like 1	1.59	4.8E-20	4.9E-18
CCR6	chemokine (C-C motif) receptor 6	1.55	5.5E-08	4.1E-07
IL12A	interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	1.53	1.2E-14	3.6E-13
TNFRSF10B	tumor necrosis factor receptor superfamily, member 10b	1.5	4.7E-19	3.9E-17

b

MCF-7				
SYMBOL	GENENAME	logFC	p-value	FDR
IFNB1	interferon, beta 1, fibroblast	5.77	1.1E-24	1.2E-20
IFNL2	interferon, lambda 2	5.74	2.4E-26	5.7E-22
IFNL1	interferon, lambda 1	5.38	1.4E-24	1.2E-20
CCL5	chemokine (C-C motif) ligand 5	4.94	6E-13	9.4E-11
CCL22	chemokine (C-C motif) ligand 22	4.65	1.1E-35	5E-31
CCL5	chemokine (C-C motif) ligand 5	4.53	8.7E-13	1.3E-10
IFNL3	interferon, lambda 3	4.31	1.5E-24	1.2E-20
IL8	interleukin 8	3.57	6.5E-06	0.00011
TNF	tumor necrosis factor	3.57	3.6E-16	1.4E-13
IFNL1	interferon, lambda 1	3.57	8.7E-18	5.4E-15
IL6	interleukin 6 (interferon, beta 2)	3.51	1.2E-05	0.00019
CCL20	chemokine (C-C motif) ligand 20	2.82	4.1E-08	1.4E-06
LEP	leptin	2.56	1.7E-08	6.9E-07
CCR6	chemokine (C-C motif) receptor 6	2.44	7E-06	0.00012
TNFRSF9	tumor necrosis factor receptor superfamily, member 9	2.24	3.1E-07	8.1E-06
AMHR2	anti-Mullerian hormone receptor, type II	2.24	2.1E-10	1.5E-08
IL10	interleukin 10	2.01	1E-06	2.3E-05
CXCR4	chemokine (C-X-C motif) receptor 4	1.8	0.00024	0.0023
CLCF1	cardiotrophin-like cytokine factor 1	1.78	5E-09	2.4E-07
TNFSF14	tumor necrosis factor (ligand) superfamily, member 14	1.78	1.9E-05	0.00027
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b	1.73	3E-10	2.1E-08
IL18	interleukin 18 (interferon-gamma-inducing factor)	1.71	7.3E-10	4.5E-08
CXCR4	chemokine (C-X-C motif) receptor 4	1.69	3.2E-06	0.00006
IL23A	interleukin 23, alpha subunit p19	1.67	3.4E-09	1.7E-07
TNFSF15	tumor necrosis factor (ligand) superfamily, member 15	1.62	2.5E-05	0.00034

c

HeLa				
SYMBOL	GENENAME	logFC	p-value	FDR
IL24	interleukin 24	5.48	1.7E-16	3.4E-14
IL24	interleukin 24	3.64	2.5E-18	7.2E-16
IL24	interleukin 24	2.85	2.2E-16	4.3E-14
IL11	interleukin 11	2.82	4.7E-11	3.3E-09
TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	2.09	4.6E-11	3.2E-09
TNFRSF9	tumor necrosis factor receptor superfamily, member 9	1.65	1.3E-05	0.00021
INHBE	inhibin, beta E	1.59	1.4E-06	3.1E-05

Figure 4.21. Cytokine-cytokine receptor signaling (KEGG) in *treated* non-adherent cancer cells. The most commonly upregulated genes from cytokine-cytokine receptor interaction pathway according to KEGG database common for *treated* non-adherent DU145 (**a**), MCF-7 (**b**) and HeLa (**c**) cells compared to parental (control) population. See p-values and fold discovery rate (FDR) values for statistical significance. Only genes with LFC≥1.5 are shown.

As the next step, we performed additional 1-D annotation enrichment analysis applying GOBP (Gene Ontology Biological Process), GOMF (Gene Ontology Molecular Function) and GOCC (Gene Ontology Cellular Component) annotations using Perseus software package (Cox and Mann, 2012). As a result we identified 'immune system process' ($p=1.137 \times 10^{-6}$), 'immune response' ($p=1.508 \times 10^{-5}$) and 'regulation of system process' ($p=2.897 \times 10^{-5}$) pathways enriched according to GOBP annotations in irradiated (10 x 2 Gy) non-adherent MCF-7 population. Interestingly, despite these pathways did not reach significant enrichment in the non-adherent DU145 and HeLa cells, several genes implicated in these pathways were significantly upregulated in all three cell lines when compared to non-treated controls (**Fig. 4.22**). Importantly, a number of genes related to these pathways represented the most upregulated genes at the same time (e.g., IL8, JUN; see **Fig. 4.19**). By this approach, we detected other IFN-related genes such as transcription regulators AIRE and NFKB2 (a key component of NF-κB signaling), IRAK2 (a known inducer of NF-κB via IL1), or IFN-responsive gene ISG15 with possible role in the stress-response of cancer cells.

In conclusion, treatment-resistant non-adherent cancer cells express various cytokines including IL6 and IL8 and have upregulated Jak/STAT, NF-κB and IFN/immune response-related genes suggesting the role of these pathways in stress-induced phenotypic plasticity and the therapy resistance.

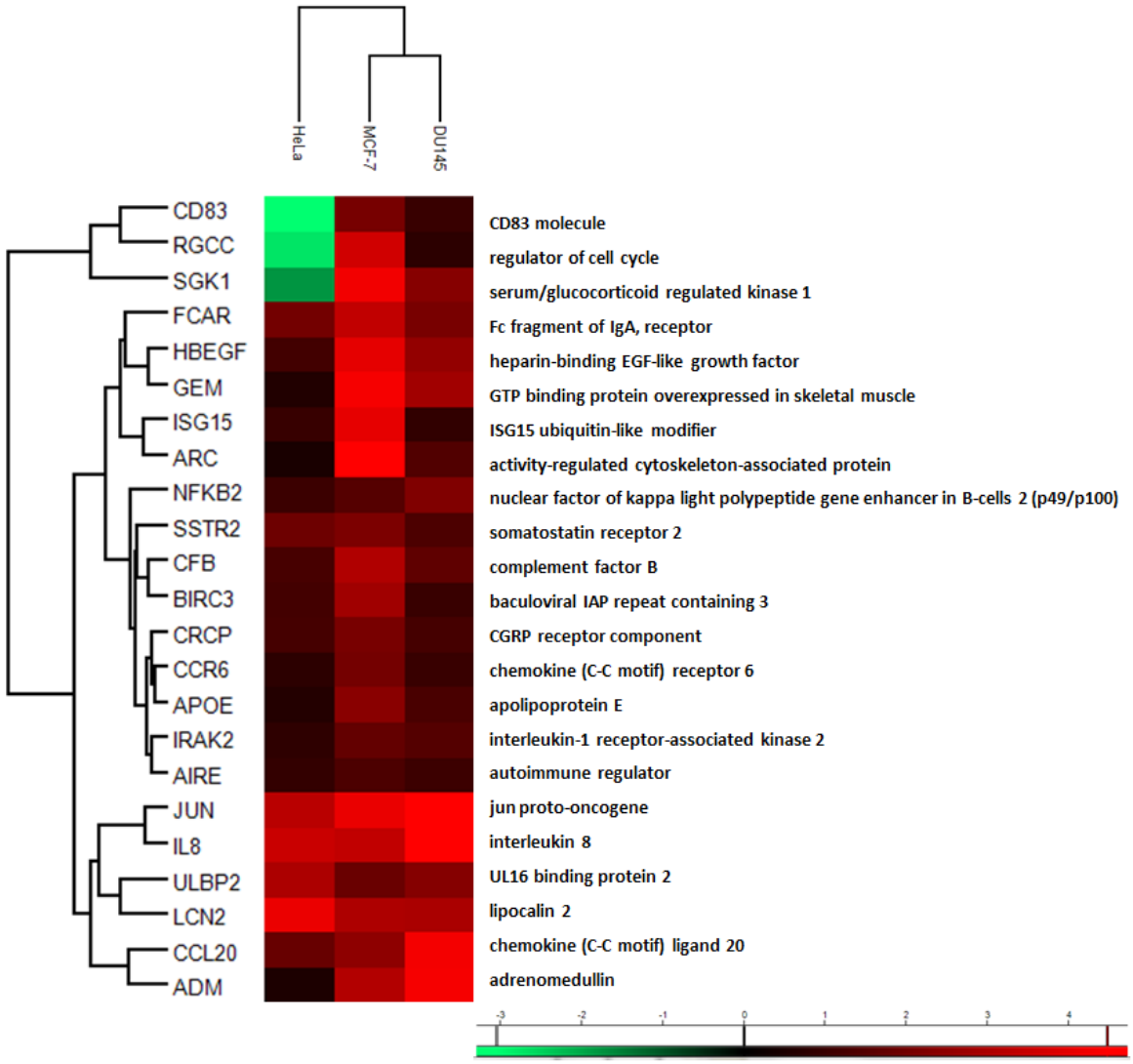

Figure 4.22. Signaling pathways enriched (GOBP) in irradiated (10 x 2 Gy) MCF-7 non-adherent cells. 1-D annotation enrichment analysis (GOBP, GOMF, GOCC) of treated non-adherent cancer populations revealed 'immune system process' ($p=1.137 \times 10^{-6}$), 'immune response' ($p=1.508 \times 10^{-5}$) and 'regulation of system process' ($p=2.897 \times 10^{-5}$) enriched according to GOBP names in irradiated (10 x 2 Gy) non-adherent MCF-7 cells when compared to non-irradiated (control) population. Dendrogram showing expression of genes implicated in particular pathways in treated non-adherent DU145, MCF-7 and HeLa cells. Only genes with the $p<0.05$, $|LFC|>1$ were used in the analysis. Data were processed by Perseus software.

5 MATERIAL AND METHODS

5.1 Chemicals and antibodies

Mek/Erk inhibitor U0126 (ERKi; 10 μM), Akt1/2 kinase inhibitor (AKTi; 1 μM; Cat. No. A6730), doxycycline hydrochloride (dox), doxorubicine hydrochloride (doxo), and 5-azacytidine (aza; 4 μM) were purchased from Sigma (St. Louis, MO, USA). Recombinant EGF and FGF were obtained from Preprotech (Rocky Hill, NJ, USA).

The following antibodies were used for immunoblotting: mouse monoclonal antibody against Erk1 (ECM Bioscience, Versailles, KY, USA), rabbit polyclonal antibody against Cdc25c, goat polyclonal antibody against DLL1 and mouse monoclonal antibody against Twist1 and cyclin A from Santa Cruz Biotechnology (Dallas, TX, USA), rabbit monoclonal antibodies against Snail, Akt, Nanog XP, Oct-4A, Bcl-XL and phosphoserine 473 of Akt, rabbit polyclonal antibodies against Chk2, p53, Bim, ITGB1, ITGB3, ITGB5 and ITGAV and mouse monoclonal antibodies against cyclin B1, $p21^{waf1/cip1}$, phosphoserine 15 of p53, phosphothreonine 68 of Chk2, and rabbit monoclonal antibody against Snail (SNAI1), all from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibody against GAPDH was purchased from GeneTEX (Irvine, CA, USA), mouse monoclonal antibody against phosphoserine 139 of histone H2AX from Millipore (Billerica, MA, USA), mouse monoclonal antibody against E-cadherin from BD Biosciences (San Jose, CA, USA) and rabbit polyclonal antibody against phosphothreonine 202/phosphotyrosine 204 of Erk1/2 from Promega (San Luis Obispo, CA, USA). Mouse monoclonal antibody against ITGA2 was purchased from Abcam (Cambridge, UK). Rabbit polyclonal antibody against Plk1 was provided by Libor Macurek (Institute of Molecular Genetics, Prague, Czech Republic). Mouse monoclonal antibody against γ-tubulin was provided by Pavel Draber (Institute of Molecular Genetics, Prague, Czech Republic). Mouse monoclonal antibody against Erk2 (B3B9) was prepared by Mike Weber and provided by Tomas Vomastek (Institute of Microbiology, Prague, Czech Republic).

The following antibodies were used for indirect immunofluorescence: mouse monoclonal antibody PG-M3 against PML and rabbit polyclonal antibody against 53BP1 from Santa Cruz Biotechnology (Dallas, TX, USA), mouse monoclonal antibody against phosphoserine 139 of histone H2AX (Millipore, Billerica, MA, USA), mouse monoclonal antibody against E-cadherin from BD Biosciences (San Jose, CA, USA), rabbit polyclonal antibodies against vimentin from Cell Signaling Technology (Danvers, MA, USA).

Secondary antibodies were anti-mouse IgG conjugated with Cy3 from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and anti-rabbit IgG antibody Alexa Fluor 568 and anti-mouse IgG antibody Alexa Fluor 488 from Invitrogen (Carlsbad, CA, USA).

5.2 Cell cultures

Human prostatic carcinoma cell lines DU145, PC-3, LNCaP, 22RV1, human breast carcinoma cell line MCF-7, human cervical cancer cell line HeLa, human embryonic kidney cells HEK293T stably expressing the SV40 large T antigen, and human fibroblast cells BJ were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). DU145, PC-3, MCF-7, HeLa, and HEK293T cells were cultured in DMEM (glucose 4.5 g/l), LNCaP and 22RV1 in RPMI-1640 (both media from Biochrom), and BJ in DMEM (glucose 1 g/l; Invitrogen, Carlsbad, CA, USA), all supplemented with 10% foetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin sulfate (Sigma, St. Louis, MO, USA). Cells were kept at 37°C under 5% CO_2 atmosphere and 95% humidity.

Cells were irradiated with orthovoltage X-ray instrument T-200 (Wolf-Medizintechnik) using 0.5 Gy/min dose rate and thorium filter daily with 10 or 35 doses of 2 Gy (fIR) or with a single dose of 10 Gy. Non-adherent cells generated during the fIR were collected during fIR (35 x 2 Gy) or 24 hours (h) after the last dose (10 or 35 x 2 Gy). There was no difference between these two modes of cell collection. However, to avoid possible contamination of non-adherent fraction by mitotic cells of adherent fraction irradiated with 10 doses of 2 Gy, non-adherent cells were transferred through another cultivation flask to enable attachment of mitotic cells (6 – 8 h), then moved to a new flask and carefully checked for 'prematurely' attached cells on daily basis. Using such procedure, potential contamination by non-adherent mitotic cells was practically eliminated.

To obtain re-adherent colonies from non-adherent cells, irradiated non-adherent prostate cancer cells were separated from adherent fraction as stated above and followed for initiation of adherent growth. After the readhesion, cells were cultured as described above. As a non-adherent control, DU145 cells were kept in confluence for 3 to 5 days (d) and then non-adherent cells were collected. Images of live cells were captured by inverted tissue culture microscope Nikon Eclipse TE300 (Nikon, Tokyo, Japan) equipped with Leica DFC490 camera and LAS AF software (Leica Microsystems, Wetzlar, Germany).

5.3 Preparation of lentiviral constructs and stable cell lines

Lentiviral vector pCDH-EFI-Neo-Bcl-XL constitutively expressing Bcl-XL, was generated by subcloning the EcoRI fragment from pSFFV-Neo-Bcl-XL (#8749 Addgene, Cambridge, MA, USA) into pCDH-CMV-MCS-EFI-Neo (Systems Biosciences, CA, USA). pLKO-Tet-On-shRNA-Snail was generated by ligation of the double-stranded oligo 5´-CCGGCCAGGCTCGAAAGGCCTTCAACTCGAGTTGAAGGCCTTTCGAGCCTGG TTTTT-3´ between the AgeI and EcoRI sites in the pLKO-Tet-On vector as described (Wiederschain et al., 2009). Recombinant lentiviruses were prepared using the 2nd generation packaging system (Barde et al., 2010). To produce DU145-empty and DU145-Bcl-XL cell lines, DU145 cells were transduced either with pCDH-CMV-MCS-EFI-Neo or with pCDH-EFI-Neo-Bcl-XL viral particles (Velimezi et al., 2013). For inducible expression of shRNA against Snail mRNA, DU145 were transduced with pLKO-Tet-On-shRNA-Snail particles. To obtain stable expression, cells were plated into media containing geneticin (G418; 800 μg/ml) or puromycin (2 μg/ml) 72 h post-transduction and selected for further 10 or 3 d, respectively. Snail shRNA expression was induced with 0.7 μg/ml dox, supplied every 48 h to the culture media. Non-transduced DU145 cells (wt, wild type) treated with corresponding amounts of dox were used as a control.

5.4 Magnetic-activated cell sorting (MACS)

AnnexinV-negative fraction of irradiated non-adherent DU145, PC-3 and MCF-7 cells was obtained by incubation with Dead Cell Removal MicroBeads (Dead Cell Removal Kit, Miltenyi Biotec, Germany) for 15 minutes (min) and separation in magnetic field of AutoMACS Pro magnetic separator (Miltenyi Biotec, Germany). The separated cells were harvested for immunoblotting or qRT-PCR analysis.

5.5 Fluorescence-activated cell sorting (FACS)

5.5.1 AnnexinV-FITC/Hoechst33258 staining and analysis

Cell survival of non-adherent DU145, PC-3, LNCaP, and 22RV1 cells after single-dose or multiple-doses of IR was assessed by staining with 1 – 5 μg/ml Hoechst33258 (Invitrogen, Carlsbad, CA, USA) in combination with AnnexinV-FITC (AnnexinV: FITC Apoptosis Detection Kit, BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. Cells were collected in PBS (300 x g at 4°C for 10 min), stained in AnnexinV Binding Buffer (BD Biosciences, San Jose, CA, USA) for 15 min at RT and then analyzed using the LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). Cells from

following quadrants were analyzed: viable AnnexinV-FITC$^-$/Hoechst33258$^-$ cells (A^-/H^-), early apoptotic AnnexinV-FITC$^+$/Hoechst33258$^-$ cells (A^+/H^-) and late apoptotic AnnexinV$^+$/Hoechst33258$^+$ cells (A^+/H^+). To assess the radiation-induced loss of adhesion, non-adherent control and irradiated cells (1 x 10 Gy) were washed twice with PBS and collected (300 x g at 4°C for 5 min) in 300 µl of AnnexinV Binding Buffer (BD Biosciences, San Jose, CA, USA). Defined volume of cell suspension was analyzed to assess the total number of cells. The precision of volume intake by High throughput sampler (HTS) unit of LSRII flow cytometer was checked using fluorescent AlignFlo Flow Cytometry Alignment Beads, 2.5 µm (Invitrogen, Carlsbad, CA, USA) excitable at 488 nm. Volume intake-error was estimated as ± 5.13%.

5.5.2 eFluor670 staining and analysis

Proliferation of irradiated adherent DU145 cells was estimated with Cell Proliferation Dye eFluor670 (eBioscience, San Diego, CA, USA), which binds unspecifically membrane proteins and is distributed equally between daughter cells during cell division. Dye fluorescence intensity in APC channel was measured by LSRII flow cytometer. Control and irradiated adherent cells were detached by Versene (Gibco, Carlsbad, CA, USA) after 10th dose of irradiation, stained with eFluor670 and either immediately analyzed by FACS or further irradiated (5 daily doses of 2 Gy) and analyzed after doses11, 13 and 15. To assess the proliferation of irradiated (10 x 2 Gy) non-adherent cells, cells were collected in PBS after the last dose of irradiation (300 x g at 4°C for 10 min), stained with eFluor670 and further cultured in new Petri dish for 28 d. At day 28 after the staining, non-adherent and newly established re-adherent cells were analyzed by FACS as described above.

5.3.3 Click-iT EdU proliferation assay and analysis

Proliferation of non-adherent DU145 cells was assessed during fIR (after 7 x 2 Gy and 10 x 2 Gy) and at several post-fIR time points (3, 7 and 10 d after fIR). First, cells were pulse-labeled with 10 µM EdU for 3 h, washed with PBS and stained with fixable viability fluorescent dye eFluor780 (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol. Second, cells were fixed (4% formaldehyde, 15 min at RT), washed twice with PBS and stored at 4°C. At the post-fIR time points, medium with non-adherent cells was transferred to a new Petri dish one d before EdU pulse to prevent contamination with mitotic cells from adherent layer. DNA replication was measured using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen, Carlsbad, CA, USA), customized for flow

cytometry. EdU was detected after permeabilization (0.2% Triton X-100, 5 min at RT) by staining the cells with Click-iT chemistry for 25 min at RT (azide labeled by Alexa Fluor 488, $CuSO_4$ and EdU buffer additive in 100 mM Tris, pH 8.5). Samples were analyzed using LSRII flow cytometer with 488 nm excitation. Background values were estimated by measuring non-EdU pulsed, but Click-iT chemistry stained cells.

5.5.4 Propidium iodide staining and analysis

Cells were stained with Fixable Viability Dye eFluor780 (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol to exclude dead cells from the analysis. After several washing steps with PBS, cells were fixed with 100% ethanol (2 h at -20°C), incubated in PBS containing 200 μg/ml RNaseA (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at RT, and stained with 35 μg/ml PI Fluoro Pure Grade (Invitrogen, Carlsbad, CA, USA) in PBS containing 0.1% Nonidet P-40 (AppliChem, Darmstadt, Germany) and 200 μg/ml of RNaseA. Cell cycle analysis (estimated as PI-stained DNA) was performed by LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). All flow cytometry data were processed in FlowJo 9.5.2 software (TreeStar, Ashland, OR, USA).

5.6 Indirect immunofluorescence

Cells grown on glass coverslips were fixed by methanol:acetone (1:1) at 4°C or by 4% formaldehyde at RT and permeabilized by 0.2% Triton X-100 15 min at RT. After washing with PBS, cells were incubated in 10% FBS diluted in PBS to block unspecific signal. Cells were subsequently incubated with diluted primary antibodies for 1 h at RT and then extensively washed with PBS. The incubation with secondary antibodies was performed for 1 h at RT. Nuclei were counterstained with 4',6-diamidino-2-phenylindole staining (DAPI; Sigma, St. Louis, MO, USA) followed by mounting in Mowiol (Sigma, St. Louis, MO, USA) or VECTASHIELD HardSet Mounting Medium containing DAPI was used (Vector Laboratories, Burlingame, CA, USA). Images were captured by fluorescent microscope Leica CTR6000 (Leica Microsystems, Germany) equipped with monochrome digital camera DFC350 FX and Leica LAS AF Lite software or Zeiss Axio Imager.A2 equipped with Zeiss AxioCam HR camera and Metamorph software.

5.7 SDS-PAGE and immunoblotting

Cells were washed with PBS, harvested into Laemmli SDS sample lysis buffer (2% SDS, 50 mM Tris-Cl, 10% glycerol in double distilled H_2O) and sonicated (3 x 15 seconds (s)

at 4 micron amplitude with 15 s cooling intervals) on Soniprep 150 (MSE, London, UK). Concentration of proteins was estimated by the BCA method according to manufacturer's protocol (Pierce Biotechnology, IL, Rockford, USA). 100 mM DTT and 0.01% bromphenol blue were added to lysates before separation by SDS-PAGE (10 and 12% gels were used). The same protein amount (25 or 30 μg) was loaded into each well. Separated proteins were electrotransferred onto a nitrocellulose membrane using wet transfer and detected by specific antibodies combined with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit, goat anti-mouse, Bio-Rad, Hercules, CA, USA). Peroxidase activity was detected by ECL (Pierce Biotechnology, Rockford, IL, USA) or SuperSignal West Femto Substrate (Thermo Fisher Scientific, Waltham, MA, USA). GAPDH or γ-tubulin was used as a marker of equal loading.

5.8 Quantitative real time PCR

Total RNA samples were isolated using RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA) as described (Vlasakova et al., 2007). Briefly, first strand cDNA was synthesized from 200 ng of total RNA with random hexamer primers using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster city, CA, USA).

qRT-PCR was performed in ABI Prism 7300 (Applied Biosystems, Foster City, CA, USA) using SYBR Select Master Mix containing SYBR GreenE dye (Applied Biosystems, Foster City, CA, USA). The relative quantity of cDNA was estimated by ΔΔCT (Livak and Schmittgen, 2001) and data were normalized to RPL37a, GAPDH or β-actin. Following primers were purchased from East Port (Prague, Czech Republic): **Snail**: 5´-TGC CCT CAA GAT GCA CAT CCG A-3´, 5´-GGG ACA GGA GAA GGG CTT CTC-3´; **Slug**: 5´- ATC TGC GGC AAG GCG TTT TCC A-3´; 5´- GAG CCC TCA GAT TTG ACC TGT C-3´; **Twist1**: 5´- GCC AGG TAC ATC GAC TTC CTC T-3´, 5´- TCC ATC CTC CAG ACC GAG AAG G-3´; **Zeb2**: 5´- AAT GCA CAG AGT GTG GCA AGG C-3´, 5´- CTG CTGA TGT GCG AAC TGT AGG-3´; **CDH1**: 5´- TGA AGG TGA CAG AGC CTC TGG AT-3´, 5´- TGA AGG TGA CAG AGC CTC TGG AT-3´; **MMP-7**: 5´- TCG GAG GAG ATG CTC ACT TCG A-3´, 5´- GGA TCA GAG GAA TGT CCC ATA CC-3´; **ITGA2**: 5´- TTG CGT GTG GAC ATC AGT CTG G-3´, 5´- GCT GGT ATT TGT CGG ACA TCT AG-3´; **LAMA3**: 5´- CCG ATA GTA TCC AGG GCT ACA AC-3´, 5´- AAC CAG ATG AGC ATC ACA TTC CTG-3´; **LAMC2**: 5´- ACC TGT GAA GCG GTG ACA CTG-3´, 5´- TAC AGA GCT GGA AGG CAG GAT G-3´; **CD133**: 5´-TTT TGC GGT AAA ACT GGC TAA-3´, 5´-CCA TTT TCC ATA TTT TTC ATG G-3´; **Sox2**: 5´-CAA GAT GCA CAA CTC GGA GA-

3′, 5′-GCT TAG CCT CGT CGA TGA AC-3′; **Oct-4**: 5′-CAG CTT GGG CTC GAG AAG-3′, 5′-CCT CTC GTT GTG CAT AGT CG-3′; **Nanog**: 5′-CTC CAA CAT CCT GAA CCT CAG C-3′, 5′-CGT CAC ACC ATT GCT ATT CTT CG-3′; **DLL1**: 5′-TGC CTG GAT GTG ATG AGC AGC A-3′, 5′-ACA GCC TGG ATA GCG GAT ACA C-3′; **DLL4**: 5′-CTG CGA GAA GAA AGT GGA CAG G-3′, 5′--3′; **Jag1**: 5′-TGC TAC AAC CGT GCC AGT GAC T-3′, 5′-TCA GGT GTG TCG TTG GAA GCC A-3′; **Hes1**: 5′-GGA AAT GAC AGT GAA GCA CCT CC-3′, 5′-GAA GCG GGT CAC CTC GTT CAT G-3′; **Hey1**: 5′-TGT CTG AGC TGA GAA GGC TGG T-3′, 5′-TTC AGG TGA TCC ACG GTC ATC TG-3′ **RPL37a**: 5′- AGG AAC CAC AGT GCC AGA TCC-3′, 5′--3′; GAPDH: 5′-GTC GGA GTC AAC GGA TTT GG-3′; **β-actin**: 5′-CCA ACC GCG AGA AGA TGA-3′, 5′-CCA GAG GCG TAC AGG GAT AG-3′. The data are expressed as the means ± S.D. of a minimum of two independent experiments performed in triplicates. The p-values were estimated using two-tailed Student's t-test. $p<0.05$ were considered statistically significant.

5.9 Cytokine expression array

Total RNA samples were isolated using RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA). RT-PCR was performed as described above. The mRNA levels 84 cytokine genes were measured by the RT^2 Profiler PCR Array System ('Common Cytokines' cat. no. APHS-021, SuperArray Bioscience Corp., Frederic, MD, USA) in in ABI Prism 7300 (Applied Biosystems, Foster City, CA, USA) using SYBR Select Master Mix containing SYBR GreenE dye (Applied Biosystems, Foster City, CA, USA). The relative quantity of cDNA was estimated by ΔΔCT (Livak and Schmittgen, 2001) and data were normalized to B2M, HPRT1, RPL13a, GAPDH, and ACTB. The data are expressed as the means ± S.D. of two independent experiments performed in triplicates. The p-values were estimated using two-tailed Student's t-test. $p<0.05$ were considered statistically significant.

5.10 Whole-genome expression array

5.10.1 RNA amplification, labeling and hybridization

Total RNA was isolated by using RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA). RNA integrity was assessed on Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip (Agilent Technologies, Santa Clara, CA, USA). Total RNA was amplified using Illumina TotalPrep RNA Amplification Kit from Applied Biosystems (Foster City, CA, USA), according to the standard protocol, from a starting amount of 200 ng. cDNA quality and quantity were assessed on Agilent 2100 Bioanalyzer and RNA 6000 Nano LabChip.

cDNA (750 ng) was hybridized, washed, and scanned according to the manufacturer's instructions. All analyses were done at least in three biological replicates.

5.10.2 Data analysis

The raw expression data resulting from microarray were analysed using the BeadArray package (Dunning et al., 2007) of the bioconductor within the R environment (R Development Core Team 2007). All hybridizations passed the quality control. The data were background-corrected and normalized with the probe level quantile method. Differential expression analysis was performed with the Limma package (Smyth et al., 2005) on intensities that were variance-stabilized by logarithmic transformation. Annotation provided by bioconductor was used (illuminaHumanv4BeadID.db). To identify significantly perturbed pathways, we performed SPIA (Tarca et al., 2009) analysis on KEGG pathways: genes with $p<0.05$ were considered as differentially transcribed.

5.11 Senescence-associated β-galactosidase assay

Staining for SA-β-Gal activity was performed by Senescence β-Galactosidase Staining Kit from Cell Signaling Technology (Danvers, MA, USA) according to manufacturer's protocol. Coverslips were mounted in Mowiol containing 4',6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO, USA) and images were captured by fluorescent microscope Leica DM6000 (Leica Microsystems, Germany) equipped with color camera DFC490 and Leica LAS AF Lite software.

5.12 EdU incorporation assay

Cells grown on glass coverslips were pulse-labeled with 10 μM EdU for 1 h, fixed by 4% formaldehyde at RT and permeabilized by 0.2% Triton X-100 15 min at RT. EdU incorporation to DNA was detected by Click-iT EdU Alexa Fluor647 Imaging Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Images were captured by fluorescent microscope Leica DM6000.

5.13 BrdU incorporation assay

Cells cultured on glass coverslips were pulse-labeled with 10 μM BrdU (Sigma, St. Louis, MO, USA) for 24 h before fixation with 4% formaldehyde for 15 min at RT. After DNA denaturation in 2 M HCl (30 min), cells were washed in PBS, incubated with mouse monoclonal antibody against BrdU (GeneTex, Irvine, CA, USA). Images of cells with DAPI-

counterstained nuclei were captured by fluorescence microscope Axio Imager.A2 (Zeiss, Germany) using Metamorph software (version 6.2r6; Molecular Devices, Sunnyvale, CA, USA).

5.14 Determination of IL6 and IL8 proteins in cultivation media

The culture medium was collected 24 h after change of fresh medium (and IR) and the number of cells per each dish was counted. The concentrations of secreted IL6 and IL8 proteins were estimated by 'FACS bead array' using FlowCytomix Human Simplex Kit (IL6, BMS8213FF; IL8, BMS8204FF; Bender MedSystems, Wien, Austria) on flow cytometer LSRII (BD Biosciences, San Jose, USA) according to the manufacturer's protocol.

5.15 siRNA-mediated gene knockdown

Specific siRNAs were introduced into cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA). All siRNAs were purchased from Applied Biosystems (Foster City, CA, USA). Non-targeting siRNA sequences (siNT) were used as a negative control siRNA. Sense sequences of used siRNAs are listed below: siErk1: 5´-GGA CCG GAU GUU AAC CUU Utt-3´, siErk2: 5´-CAA CCA UCG AGC AAA UGA tt-3´ siSnail: 5´-GAA UGU CCC UGC UCC ACA Att-3´, siTwist1: 5´-AGA ACA CCU UUA GAA AUA Att-3´.

5.16 Cell proliferation measurement

DU145 and BJ cell proliferation curves were estimated by counting trypan blue-negative cells with Countess automated cell counter (Invitrogen, Carlsbad, CA, USA) every two days during 8 – 10 d time course. ERKi (10 μM) and AKTi (1 μM) were changed with fresh culture medium every two days 30 - 45 min before irradiation. Data represent the means ± S.D. of two independent experiments.

5.17 The scratch wound healing assay

After 24 h of cell culture, a sterile yellow pipette tip was used to make a straight scratch on the monolayer of confluent cell culture seeded in 6 well plate in triplicate. Time-lapse images were taken at 5 min intervals for 34 h with a light microscope (Leica DMI 6000B, Leica Microsystems, Germany) equipped with a digital camera (Leica DFC 360 FX). Wound repopulation was analyzed using Leica LAS AF Lite software. Assay was performed in two independent experiments.

5.18 Clonogenic cell survival assay

Clonogenic assay of control (parental) and irradiated 'radioresistant' (adherent and re-adherent) DU145 cells was performed as described (Franken et al., 2006). Briefly, cells were seeded on 6-well plates in triplicates and subsequently (6 h post-seeding) irradiated with 10 or 40 Gy. Cells were fixed with ice-cold 100% methanol (10 min at -20°C) and stained with 0.5% crystal violet in 20% methanol (10 min at RT) at day 13, and groups with more than 10 cells were counted as colonies. In some experiments, the size of the colonies above the threshold, and presence of senescence-like cells in colonies, was examined.

5.19 Estimation of tumorigenicity

Immunodeficient male SCID mice (6 week-old) purchased from AnLab, s.r.o. (Prague, Czech Republic) were acclimated for two weeks. Experimental protocols were approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics AS CR, Prague. 1 x 10^5 or 1 x 10^6 control (parental) or irradiated (10 x 2 Gy) re-adherent DU145 cells in 300 µl of DMEM were injected subcutaneously into the right flank of the mice. Before injection, cells were stained with trypan blue and counted for viability using Countess automated cell counter (Invitrogen, Carlsbad, CA, USA). Tumor onset and tumor volume (mm^3) were measured weekly with the use of calipers and calculated following formula (length × $width^2$) divided by 2. N = 5. Statistical significance was estimated by Newman-Keuls multiple-comparison test.

5.20 Data processing and statistical analysis

FACS data were analyzed using FlowJo 9.6.4 cytometric analytical software (Tree Star, Stanford University, USA). Graphs were generated using Prism 5 (GraphPad Software, La Jolla, CA USA). P-values were calculated using Student's t-test for two samples assuming unequal variances (Microsoft Excel 2010, Microsoft, Redmond, WA, USA). Microarray data were processed as described above. 1-D annotation enrichment analysis was performed using Perseus software package (Cox and Mann, 2012).

6 LIST OF JOURNAL PUBLICATIONS

Research article I

Kyjacova L., Hubackova S., Krejcikova K., Strauss R., Hanzlikova H., Dzijak R., Imrichova T., Simova J., Reinis M., Bartek J., Hodny Z. (2015) Radiotherapy-induced plasticity of prostate cancer mobilizes stem-like non-adherent, Erk signaling-dependent cells. *Cell Death and Differentiation*, 22(6): 898-911. IF (2013): **8.385**

The author of this thesis as the first autor elabored major part of experiments, participated on project coordination, manuscript preparation and submission. Data presented in this publication represent a substantial part of the Results section.

Research article II

Hubackova S., Kucerova A., Michlits G., Kyjacova L., Reinis M., Bartek J. and Hodny Z. (2015) Cytokine senescence: IFNγ induces oxidative stress, DNA damage and tumor cell senescence via TGFβ/SMAD signaling-dependent induction of Nox4 and suppression of ANT2. *Oncogene*. IF (2013): **8.559**

The author of this thesis as a co-autor elabored experiments concerning Nox4 protein analysis. The data presented in this publication are not included in this thesis.

Research article III *(manuscript in preparation)*

Hubackova S., Moudra A., Novakova G., Kyjacova L., Dzijak R., Tambor V., Strnad H., Machalova V., Benada O., Jonasova A., Bartek J. and Hodny Z. (2015)* Interferon gamma and 5-azacytidine reprogram to stem cell state.

The author of this thesis participated on preparation, verification and analysis of the microarray data. Data which are part of this publication represent part 4.5 of the Result section.

**Order of the names and title may change.*

Review paper I *(manuscript in preparation)*

Kyjacova L., Bartek J. and Hodny Z. (2015) Breaking dormancy and therapeutic resistance of tumor cells.

The author of this thesis prepared the manuscript. Parts of this review represent part of the Discussion section.

7 DISCUSSION

The majority of PCa patients receive RT either as primary therapy or salvage therapy for local recurrence. RT is also recommended as adjuvat therapy after prostatectomy. However, 10% of low-risk and 30 – 60% of high-risk PCa patients suffer for biochemical recurrence within 5 years after RT. Importantly, 20 – 30% of PCa patients with recurrent disease die within 10 years (Boorjian et al., 2011; D'Amico et al., 2008; Kuban et al., 2003; Zietman et al., 2005). It is therefore important to understand molecular pathways underlying intrinsic but also acquired radioresistance of PCa cells. With this aim, we investigated an effect of clinically used fIR on survival of prostate (and breast) cancer cells *in vitro*. We identified irradiation-induced loss of adhesion and subsequent anchorage-independent survival as a novel cell death-resistant mechanism in response to genotoxic stimuli. Particular aspects of this phenomenon are discussed bellow.

7.1 INTRINSIC RESISTANCE OF PROSTATE CANCER CELLS TO IONIZING RADIATION

The main determinant of tumor response to radiotherapy is the intrinsic radiosensitivity defined by detection and repair of IR-induced DNA damage (Gerweck et al., 2006). ATM-mediated signaling from irradiation-induced DSBs stabilizes and activates tumor suppressor p53, which controls the response to IR either by induction of cell death mechanisms or cell cycle arrest and senescence (see Chapters 2.4.2 and 2.4.3).

7.1.1 Intrinsic resistance to radiation-induced cell death

It is important to realize that cells of normal prostate epithelium have attenuated cell cycle checkpoints as has been shown by several studies from Laiho's laboratory (Jaamaa et al., 2012; Zhang et al., 2011). They showed that in normal prostate tissue and primary cultures (HPECs, human prostate epithelial cells), p53 and Wee1A-mediated DNA damage-induced checkpoints are attenuated after IR in comparison to HSVECs (human seminal vesicle epithelial cells) which are less prone to tumorigenesis (Hallstrom and Laiho, 2008) (Kiviharju-af Hallstrom et al., 2007). This indicates that PCa cells are intrinsically predisposed to skip cell cycle checkpoints after DNA damage which could be one of the

prerequisites for quick disease progression and development of genomic instability. In mice, the gut-specific knockout of casein kinase I alpha (CKIα) which causes hyperactivation of Wnt signaling (but is not sufficient for tumorigenesis) correlated with activation of p53 tumor supressor. Gut-specific knockdown of both CKIα and p53 resulted in increased invasiveness and led to identification of the p53-suppressed invasiveness signature (PSIS) (Elyada et al., 2011). In accordance to weak IR-mediated checkpoints in normal prostate epithelium and, link of p53 to invasiveness, mutations of the *TP53* gene are predominantly associated with locally advanced, androgen-refractory PCa and metastases (Hamdy et al., 1994; Navone et al., 1993) rather than with initial stages of the disease (Visakorpi et al., 1992).

The increase of p53 levels is observed in response to a variety of stimuli including irradiation and is followed by transactivation of p53 targets as $p21^{waf/cip1}$ or Bax implicated in cell cycle regulation and apoptosis (Vogelstein et al., 2000). Cell models used in this study are well-established cell lines derived from metastatic PCa either with wt alleles of the *TP53* gene (LNCaP), a missense mutation in the tetramerization domain (Q331R) of one allele (22RV1), inactivating point mutations in both alleles (P223L and V274F) or loss of the *TP53* gene (PC-3) (Lehmann et al., 2007). In fact, altered p53 function *per se* is considered a radioresistance mechanism as cancer cells can bypass stress-induced apoptosis (Lee and Bernstein, 1993; Yount et al., 1996). In PCa, p53 status is an important determinant of sensitivity to docetaxel, the first line treatment for mCRPC (Liu et al., 2013). In LNCaP, docetaxel stabilizes $p21^{waf/cip1}$ in a p38/p53-dependent manner, and a single p53, $p21^{waf/cip1}$ or p38 siRNA-mediated knockdown leads to sensitisation to apoptosis (Gan et al., 2011). In our setting, fIR led to ATM-mediated phosphorylation of histone H2AX at serine 139 (γH2AX) that triggered recruitment and activation of DDR machinery in both DU145 and PC-3. Interestingly, although the p53 DNA-binding activity is abolished in DU145 and *TP53* gene is lost in PC-3 cells, $p21^{waf/cip1}$ protein level was increased in a dose-dependent manner during fIR indicating p53-independent regulation. This result differs from the response of the same cell lines to docetaxel where such treatment did not influence $p21^{waf/cip1}$ levels (Gan et al., 2011). In another study, isoflavone genistein resulted in p53-independent, but estrogene receptor-beta (Erβ)-dependent $p21^{waf/cip1}$ expression and increased $p21^{waf/cip1}$ binding to Cdk2 resulting in cell cycle arrest in PC-3 cells (Choi et al., 2000; Matsumura et al., 2008). Chk2 has an important role in checkpoint control in response to DNA damage, apoptosis and replicative senescence (Bartek et al., 2001; di Fagagna et al., 2003). The ectopic overexpression of activated (threonine 68-phosphorylated) Chk2 in SK-BR-3 (human breast cancer cells) and HaCat cells (immortalized human keratinocytes) lacking normal p53 function led to the cell

cycle arrest and $p21^{waf/cip1}$-dependent senescence. In this study it was also shown that Chk2 can positively activate *CDKN1A* transcription, particularly in situation when p53 in not transactivated (Aliouat-Denis et al., 2005). Since the function of Chk2 is not altered in neither PCa cell line with mutated or lost *TP53* we used, it is possible that $p21^{waf/cip1}$ is regulated in a Chk2-dependent manner in these cells. However, whether $p21^{waf/cip1}$ is regulated via Chk2 or by other mechanism in p53-defective PCa cells shoud be further investigated.

7.1.2 Intrinsic resistance to radiation-induced senescence

DNA-damaging agents including IR can induce a senescence-like phenotype in various tumor cell lines (Chang et al., 1999). Vulnerability to senescence-associated terminal growth arrest can be also the factor determining the intrinsic radioresistance of cancer cells (Bromfield et al., 2003). Besides p53-target genes $p21^{waf/cip1}$ and $p15^{INK4B}$, $p16^{INK4A}$ is often accumulated in senescent cells (Campisi, 2005), as was shown also for senescent mouse prostate tumors (Chen et al., 2005). In our experiments, fIR of 10 doses of 2 Gy were not sufficient for developement of cell cycle arrest and senescence-like phenotype in DU145 and PC-3 cells as we detected proliferating cells even after 20 doses of 2 Gy, and only few cells were positive for SA-β-Gal. Moreover, we detected EdU-positive cells 10 days after fIR 10 x 2 Gy despite presence of permanent/unrepaired DNA lessions. PC-3 cells have retained normal Rb function, however, p16 promoter was found to be hypermethylated (Jarrard et al., 1997). In case of DU145, Rb is mutated (Ikediobi et al., 2006), and a coding sequence of p16 gene bears a missense mutation (Itoh et al., 1997) corresponding with our data where p16 protein was not detected in PC-3 cells and was not elevated during fIR in DU145. The only cell line with a dose-dependent elevation of p16 during fIR was 22RV1 cell line. Importantly, shRNA-mediated knockdown of Rb sensitized PC-3 and MDA-MB-231 cells to paclitaxel-mediated cell death showing that initial Rb status can determine the intrinsic resistance to treatment (Zhao et al., 2014). Relativelly late (after cummulative dose of 10 Gy) activation of CDK inhibitors ($p21^{waf/cip1}$ and $p27^{kip1}$) resulted in almost unaffected proliferation of DU145 cells after 10 doses of 2 Gy. It was shown that DU145 exhibit an increase in G2/M-arrested population 24 h after 6 Gy, whereas have impaired G1 cell cycle arrest was not recovered even after ectopic expression of Rb. Moreover, DU145 cells are unable to mantain the G2/M checkpoint which can lead to genetic instability and accumulation of radioresistance-underlying mutations (Lehmann et al., 2007).

As suggested by others (Leith et al., 1994) and also by our data, DU145 cells represent one of the most radioresistant cancer cell lines. Besides the mutation in tumor-supressor

genes, this could be given also by a high threshold for ATM activation (Collis et al., 2004) or compaction of chromatin since significantly less γH2AX foci were detected after IR in comparison to 22RV1 and LNCaP (Bohnke et al., 2004) (Lehmann et al., 2007). Despite the fact that an increasing number of irradiation cycles finally induced cell death or senescence-associated cell cycle arrest in all four PCa cell lines, the latter was not permanent as some cells were able to recover proliferative potential (see below).

It is believed that besides cell type-specific intrinsic resistance to genotoxic insults tumor cells can be reprogrammed towards the resistant phenotype by therapy *per se*. Particular aspects of acquired resistance are discussed below.

7.2 ACQUIRED RESISTANCE OF PROSTATE CANCER CELLS TO IONIZING RADIATION

It is suggested that local PCa recurrence after fractionated irradiation is an attribute of a small cell population within a tumor with acquired radioresistance. It is suggested and was shown also for PCa (Skvortsova et al., 2008) that irradiation-surviving cells possess altered signaling which provides them survival advantage when they are re-exposed to radiation. Several signaling cascades implicated in resistance to RT, metastasis, and disease recurrence are activated in PCa including those leading to EMT, gaining a CSCs phenotype or PI3K/Akt and Mek/Erk1/2 signaling pathways. It is well documented that such programmes do not operate alone but rather intense cross-talks exist between them, resulting in an agressive cancer phenotype.

7.2.1 Epithelial-to-mesenchymal transition and radioresistance

One of our key findings is the involvement of EMT in the response to fIR in PCa as well as breast cancer MCF-7 cells. fIR potentiated expression of several drivers of mesenchymal phenotype in fIR-surviving non-adherent PCa cells. The phenotypic switch was strongly dependent on the Erk1/2-mediated expression of Snail. Chemical inhibition of Erk1/2 signaling and siRNA-mediated knockdown of Erk1/2 and Snail affected viability as well as loss of adhesion of PCa cells upon fIR. The result was further supported by observed modulation of known Snail target genes that are implicated in regulation of cell adhesion and EMT in irradiated non-adherent cells. fIR-induced crosstalk between Erk1/2 and Snail was associated with the repression of Snail transcriptional target and EMT-related cell adhesion

molecule E-cadherin (Haraguchi et al., 2008), consistent with its role in the maintenance of epithelial phenotype (Imhof et al., 1983), anchorage-independent growth, and anoikis resistance (Kumar et al., 2011).

Downregulation or mutation of E-cadherin as the main component of epithelial adherent junctions is common in many carcinomas and leads to increased invasive behavior of tumor cells (Derksen et al., 2006; Thiery et al., 2009). In non-malignant breast cancer cells, the loss of E-cadherin resulted in disrupted actin and microtubule cytoskeletons and altered adherence and migration characteristics, but was not sufficient to induce *typical* EMT (Chen et al., 2014; Derksen et al., 2006). However, the absence of E-cadherin *per se* can support expression of EMT-related molecules by modulating the growth factor response, therefore, it represents an additive factor in tumor progression (Andersen et al., 2005; Mohamet et al., 2011). Moreover, the loss of this gene was shown to be associated with a higher metastasis frequency *in vivo* (Perl et al., 1998) suggesting its role in tumor cell dissemination. In another study, E-cadherin conditional mutation in the epithelium-specific knockout of p53 resulted in accelerated invasive and metastatic behavior in murine model of breast cancer. In addition, E-cadherin loss resulted in anoikis-resistance and enhanced angiogenesis in invasive lobular breast carcinoma, supporting again the development of metastasis (Derksen et al., 2006).

The mechanistic link between genotoxic stress and cellular reprogramming is unclear. The findings of several recent reports show a direct association between DDR and activation of EMT drivers (Sun et al., 2012; Zhang et al., 2014a). Indeed, Snail was shown to be a direct substrate of ATM kinase in breast cancer cell lines exposed to topoisomerase I inhibitor camptothecin or IR. ATM activation-mediated Snail phosphorylation on serine 100 (pS100Snail) resulted in inhibition of its GSK3β-mediated proteosomal degradation. Elevated Snail levels were found in breast cancer tissues from patients with lymph-node metastases. Transient or stable expression of phosphomimetic Snail ($Snail^{S100E}$) in MDA-MB-231 cells resulted in both increased migratory and invasive capabilities *in vitro* and enhanced metastatic potential *in vivo* (Sun et al., 2012). Interestingly, it was demonstrated by the same group that ATM-mediated Snail phosphorylation is implicated in tumor cell radioresistance and promotes the cell invasion evoked by IR (Boohaker et al., 2013). In analogy to Snail, Zhang *et al*. recently showed that Zeb1 is another EMT-related phosphorylation target of ATM kinase in response to IR. ATM-mediated phosphorylation of Zeb1 on serine 585 resulted in its stabilization followed by its direct interaction with deubiquitinase USP7 which in turn deubiquitylates and stabilizes Chk1 which enhance DNA repair and tumor radioresistance (Zhang et al., 2014a). Whether the loss of adhesion and EMT observed after fIR of PCa cells

is a consequence of ATM-driven phosphorylation of Snail, Zeb1 or other EMT driver, remains to be explored.

Besides its role in EMT, Snail is also implicated in the resistance to apoptosis by activation of the PI3K/Akt and Erk1/2 cascades (Vega et al., 2004), as supported also by our observation that inhibition of above mentioned signaling pathways sensitized PCa cells to cell death (see below). Increased levels of EMT-TF Snail are associated with therapy resistance in many cancer types including breast (Lim et al., 2013b), colorectal (Han et al., 2013b), pancreatic (Izumiya et al., 2012), and lung cancer (Gomez-Casal et al., 2013b) which corresponds also with our data where siRNA-mediated knockdown of Snail led to a decreased survival in fIR non-adherent DU145 cells and dephosphorylation of pro-survival kinase Erk1/2. Not only Snail but also other members of EMT-TFs were shown to be linked to therapy resistance. Snail together with Slug is involved in radio- and chemo-resistance in ovarian cancer (Kurrey et al., 2009). Expression of Twist1 is implicated in resistance to taxol and vincristine (Wang et al., 2004) and its depletion partially reverses multidrug resistance in breast cancer (Li et al., 2009). Last but not least, Zeb1 confers resistance to both IR (Zhang et al., 2014a) and chemotherapy (Ren et al., 2013). EMT-related therapy-resistance is linked to the stemness phenotype as epithelial cancers undergoing EMT often show characteristics of CSCs (Scheel and Weinberg, 2011).

7.2.2 Cancer stem cell-like phenotype and radioresistance

The increasing evidence suggests that therapy resistance of EMT-undergoing cells is due to the acquired stem-like phenotype. EMT is coupled to activation of stemness program in both normal (Mani et al., 2008) and transformed cells (Han et al., 2013b). CSCs express EMT-transcription factors such as Twist1, Snail, and Slug and, *vice versa*, EMT-undergoing cells were found enriched for CSCs markers (Thiery et al., 2009). Our data indicate that fIR non-adherent cells are not only more mesenchymal-like but they also express multiple stemness-related genes and have active Notch signaling pathway important for maintaining various progenitor cells in the undifferentiated state (Koch et al., 2013).

Radioresistant non-adherent PCa cells exhibit enhanced expression of stem cell-associated gene CD133, a reported marker of PCa ‘stem cells’ (Richardson et al., 2004). Cell subpopulation expressing CD133 along with others stemness-related markers ($CD44^+/\alpha2\beta1^{high}/CD133^+$) isolated from prostate tumors exhibit self-renewal capacity (Collins et al., 2005). Dubrovska *et al*. showed that $CD133^+/CD44^+$ cells derived from PC-3 and DU145 cells have tumor-initiating potential and are able to grow under sphere-forming

conditions (Dubrovska et al., 2009). Besides CD133, we detected increased expression of Oct-4, Sox2, and Nanog which were referred to reprogram various differentiated cells towards stemness (Takahashi and Yamanaka, 2006; Yu et al., 2007). Moreover, fIR-surviving non-adherent PCa cells showed enhanced activity of Notch signaling resulting in the expression of transcription factors Hes1 and Hey1 executing Notch signaling functions such as encompassing stem cell maintenance, cell fate determination, and regulation of proliferation and apoptosis (Radtke and Raj, 2003). This situation is very similar to breast cancer, where IR also induces Notch signaling (Lagadec et al., 2013) and stem-like phenotype, including Notch-dependent upregulation of Oct-4 (Lagadec et al., 2012). In the same line, Notch signaling promotes radioresistance in glioma $CD133^+$ stem cells (Wang et al., 2010) and is also implicated in regulation of EMT (Sahlgren et al., 2008). Importantly, analysis of prostate tumor samples revealed association between presence of Notch ligand JAG1 and increased metastases and tumor recurrence (Santagata et al., 2004) implicating that in some cases, radiotherapy might have metastases-promoting effects in surviving PCa cells.

Since their discovery, CSCs were associated to drug and/or radiotherapy resistance, metastasis, and cancer recurrence (reviewed in (Baumann et al., 2008)). In relation to non-responsiveness of CSCs to current treatment modalities, some authors have successfully detected putative CSCs following therapy as the only surviving cell population (Levina et al., 2008). The enrichment of CSCs after IR was demonstrated in breast (Phillips et al., 2006; Woodward et al., 2007), NSCLC (Gomez-Casal et al., 2013a), prostate cancer (Cho et al., 2012), and others (see Chapter 2.4.5). This points to the activation of signaling ensuing radioresistance in CSCs which is mainly associated with enhanced DDR. In breast cancer, Sca^+ progenitors in murine mammary epithelial culture as well as Sca^+ subpopulation of MCF-7 cells was enriched after IR and exhibited significantly less γH2AX foci 2 hours after 2 Gy than their Sca^- counterparts. Moreover, Sca^+ subpopulation had selectively activated Wnt/β-catenin pathway and elevated its transcriptional target survivin implicated in the resistance to apoposis and escape from replicative senescence by enhancing telomerase activity (Woodward et al., 2007). In another study, analysis of biomarkers in $LIN^-/CD29^{high}/CD24^{high}$ tumor-initiating cell subpopulation derived from a p53-null mammary gland tumor involved genes implicated in DDR, DNA repair, and self-renewal (Zhang et al., 2008). $CD133^+$ human glioma xenografts as well as primary glioblastoma cells activate DNA damage checkpoints and repair DNA damage with higher efficiency than $CD133^-$ cells. Importantly, in was shown that such radioresistance can be attenuated by inhibiting Chk1 and Chk2 kinases (Bao et al., 2006). The role of adhesion molecule and putative marker of PCa

progenitor cells, CD44, in PCa progression remains controversial (Patrawala et al., 2006). Xiao *et al*. investigated CD44 in PCa and showed that siRNA-mediated knockdown of CD44 sensitize PC-3, PC-3M-luc and LNCaP to radiation. Cells with low CD44 levels had ineffective cell cycle arrest, delayed phosphorylation of main ATM downstream kinases Chk1 and Chk2 implicated in DNA repair, and significant accumulation of γH2AX foci 24 hours after 2 Gy in comparisom to cells with normal CD44 levels (Xiao et al., 2012). However, in our hands, there was no significant difference in CD44 expression in fIR-surviving non-adherent PCa cells populations when compared to either fIR adherent population or control parental cells. Such absence of CD44 in irradiated non-adherent cells could be explained by the work from Patrawala *et al*.. They showed that highly purified $CD44^+$ PCa cells were enriched in metastatic progenitor cells on the one hand but on the other hand had increased proliferative and clonogenic potential when compared to the isogenic $CD44^-$ subset, which is not in accordance with the dormant character of fIR non-adherent cells (Patrawala et al., 2006).

In our experiments, all fIR-non-adherent PCa cells cell lines (DU145, PC-3, LNCaP, and 22RV1) were proliferatively dormant for extended period of time with decreased levels of cyclin A and B1 (CDK1), Plk1, and Cdc25c and enrichement in G2 phase of cell cycle which is consistent with the prolonged dormancy seen in DU145 and breast tumor cells SUM159 after clinicaly relevant chemotherapy (Li et al., 2014). This is supported also by a significantly decreased mRNA level of CDK1 which is an important factor for entry of cell into mitosis (Nurse, 1990). We were not able to induce spheroid growth indicating that irradiated non-adherent cells represent a proliferatively dormant subpopulation which is not in conflict with stemness characteristics, as reported also for neurospheres (Pastrana et al., 2009). EMT/MET can be coupled to radio- and/or chemo-resistance in terms of exit from cell cycle, proliferative dormancy (Chaffer and Weinberg, 2011). Whether the increased expression of cyclin-dependent kinase inhibitors $p15^{INK4B}$, $p19^{INK4D}$, $p21^{waf/cip1}$ and $p57^{kip2}$, which we have found upregulated at RNA level in fIR non-adherent cells, and/or decreased expression of cyclin-dependent kinases involved in regulation of cell cycle progression are causal for the observed cell cycle arrest of non-adherent fIR survivors, should be explored. We observed that the duration of non-adherent state of irradiated survivors before re-adhesion is not constant and differs for independent experimental replicates. We proposed several possible scenarios involved in the escape from proliferative dormancy of PCa cells including (i) successful repair of massive DNA damage followed by exit from fIR-induced cell cycle arrest, (ii) cell reprogramming towards the epithelial phenotype in the process of MET, (iii)

modifications of cell surface adhesive properties facilitating return to adhesive growth. However, the exact factor or molecular mechanism controlling the persistence of the non-adherent state or the re-establishment of readhesion remains to be determined. A coordinated action between processes mentioned above also cannot be excluded.

Tumor cells that have undergone the EMT express low levels of proliferation marker Ki67 but express a cell cycle inhibitor and senescence marker $p16^{INK4A}$ (Brabletz et al., 2001; Jung et al., 2001). It is therefore suggested that MET is a prerequisite for resuming proliferative potential of EMT/stem-like CTCs. Consistently with this idea, Tsai *et al*. recently reported that reversion of EMT by downregulation of Twist1 promotes proliferation of disseminated tumor cells and metastases formation in spontaneous squamous cell carcinoma mouse model (Tsai et al., 2012).

Thus it is tempting to speculate that the phenotypic changes observed in our study might resemble EMT/MET switches during carcinoma progression *in vivo*, including the change from the non-proliferative non-adherent state to the proliferative adherent one, the latter coupled to re-expression of E-cadherin and repression of vimentin. The quiescence of fIR-surviving non-adherent PCa cells might be consistent with proliferative dormancy of disseminated tumor cells during tumor progression (Aguirre-Ghiso, 2007), providing them potential selective advantage in response to conventional treatment strategies which are designed to target proliferating cells (Li et al., 2008). Subsequent reactivation of dormant cells might induce tumor relapse even decades after therapy by mechanism which is so far poorly understood (Pantel et al., 2009a; Willis et al., 2010). Using single cell-derived lines from primary human colorectal cancers, Kreso *et al*. showed that not only genetic heterogeneity but also non-genetic (functional) heterogeneity within a genetically uniform lineage of tumor cells could contribute to the relapse after chemotherapy. Importantly, they identified dormant/slowly proliferating cell population that retained tumor-propagating potential and, in contrast to actively proliferating population, became dominant after the round of chemotherapy, thus directly contributing to the therapy resistance (Kreso et al., 2013).

7.2.3 Resistance to genotoxic stress-induced cell death

Resistance to cell death is a major hallmark of cancer therapy resistance. Radiation-induced plasticity of PCa is caused not only by adaptive transient responses but also by acquired resistance to radiation-mediated cell death mechanisms. We noticed that fIR-surviving re-adherent cells exhibited less senescent-like colonies in clonogenic assay and more viable non-adherent cells after re-irradiation, pointing to the acquired radioresistance.

Similarly, Skvortsova *et al*. showed that radioresistant adherent PCa cells (LNCaP-IR, PC3-IR and DU145-IR) surviving 5 doses of 2 Gy were less sensitive to re-irradiation in clonogenic assays and had an increased motility compared to parental population. Besides a proposed role of activated Ras/MAPK, PI3K/Akt, and Jak/STAT pathways in overcoming the cytotoxic effect of IR, they also identified several proteins specifically expressed in all radioresistant PCa populations involved in cell survival, growth, proliferation invasion, motility, and DNA repair. In addition, downregulation of APEX1, DNA damage repair protein, enhanced the radiosensitivity in all three resistant subsets (Skvortsova et al., 2008). In the recent study, Chang *et al*. developed three radioresistant PCa cell lines (DU145RR, PC-3RR and LNCaPRR) with increased clonogenicity, sphere formation capacity, and EMT and CSC-like phenotype. Basal levels of anti-apoptotic proteis Bcl-2 and Bcl-XL and DDR-related proteins including Chk2 were higher whereas the γH2AX levels were significantly reduced in PCa radioresistant cells. Moreover, using mass spectrometry proteomic approach, they identified PI3K/Akt/mTOR as general signaling cascade responsible for radioresistance in PCa cells. Importantly, they showed that single or dual PI3K/Akt/mTOR inhibitors radiosensitized radioresistant PCa cells to cell death (Chang et al., 2014).

The most important part of our study is that we described new radioresistant subpopulation in PCa which was non-adherent and therefore resistant to anchorage-dependent cell death, anoikis. fIR-mediated loss of adhesion as well as resistance to cell death was controlled by MAPK activity via regulation of Snail expression and modulation of pro- (Bim) and anti-apoptotic (Bcl-XL) proteins from Bcl-2 family. Simultaneous inhibition of MAPK (Mek) and Akt activities by specific inhibitors suppressed the resistance to anoikis and prevented regrowth of adherent PCa cells in response to fIR and doxorubicine, consistent with the role of MAPK/Erk and PI3K/Akt pathways in promoting cell growth, regulation of apoptosis and resistance to chemotherapy (McCubrey et al., 2011; Steelman et al., 2011). As already suggested (Steelman et al., 2010) and based on our data, the combined use of Erk1/2 and Akt inhibitors as adjuvants to systemic therapies might have a sensitizing effect by abrogating radio-/chemo-therapy-induced tumor cell heterogeneity, resulting in decreased recurrence and, therefore, could provide overall beneficial effects for patients with epithelial cancer.

Lehman *et al*. showed that important determinant of the response of PCa cell lines to IR is p53 status. According to their *in vitro* study and also according to frequent mutation of p53 in advanced PCa, it can be suggested that other, non-apoptotic, models can contribute to the reduced clonogenic survival in PCa after IR. Lehman *et al*. showed that apoptosis is not

dominant cell death mechanism in PCa after IR and rather necrosis and therapy-induced senescence eliminate PCa cells after IR. Programmed necrosis (necroptosis) was also shown to contribute to IR-mediated cell death in thyroid and adenocortical cancers (Nehs et al., 2011). Importantly, in necrosis, cellular components are released to the extracellular environment where they can act as damage-associated molecular patterns (DAMPs) and trigger the pro-inflammatory signaling (see below; (Hou et al., 2013)). Notably, normal and cancer-associated fibroblasts (CAFs) can be forced by non-adherent culture conditions to a specific, internally induced, necrosis/necroptosis-like mechanism called nemosis, associated with activation of proinflammatory, proteolytic and GF response (Bizik et al., 2004). We noticed that fIR-surviving mesenchymal-like non-adherent cells share some features with cells undergoing nemotic cell death, forming light-diffracting black-appearing structures associated with presence of cellular debris or autophagic vacuoles (Bizik et al., 2004) and production of various cytokines such as IL1β, IL6, IL8, IL11 and leukemia inhibitory factor (LIF) (Kankuri et al., 2008). Thus it is likely that also necroptosis/nemosis, resistance to which might also contributes to generation of fIR-surviving non-adherent cells, however, proper mechanism is not known.

The role of autophagy in the field of therapy resistance of cancer cells remains controversial. In PCa, low LC3A (marker of autophagosomal membranes) and high LAMP2 expression (marker of lysosomal membranes) was detected in comparison to normal prostate glands which indicate high autophagy flux. Recently was shown that high autophagic activity can determine resistance to radiotherapy in PCa, as blocking of LC3A and LAMP2 resulted in cell death in DU145 and PC-3 cells after IR (Koukourakis et al., 2015). Moreover, PI3K/Akt/mTOR inhibitors reduce autophagic activity in radioresistant PCa cells which can contribute to their radiosensitizing effect (Chang et al., 2014). Whether intensified autophagy flux contributes to radioresistance also in non-adherent fraction of PCa cells remains to be investigated.

7.2.4 Resistance to genotoxic stress-induced premature senescence

Besides non-adherent cells, another pool of fIR-survivors of PCa cell lines stemmed from surviving adherent cells, most of which acquired senescent-like features during the course of fIR but the proliferation of some survivors was resumed after the end of irradiation. Analogous *in vitro* model for dormancy and local recurrence of adherent breast and prostate cancer cell populations following short-term chemotherapy has been recently proposed by Li *et al*. (Li et al., 2014).

It was thought for a long time that cellular senescence represents the irreversible growth arrest induced in proliferating normal (and tumor) cells by various stress agents, providing a barrier against malignant transformation and tumor progression (Campisi and d'Adda di Fagagna, 2007). However, if not removed from tissues, persistent pre-malignant senescent cells can pose a considerable cancer risk as was demonstrated using the oncogenic model of hepatocellular carcinoma (Kang et al., 2011). There is emerging evidence that especially in case of tumor cells the senescence-associated cell cycle arrest is not always permanent (Sabisz and Skladanowski, 2009; Walen, 2008). As was shown recently, the escape from genotoxic stress-induced senescence is possible mainly under conditions of polyploidy and reprogramming for totipotency (Erenpreisa and Cragg, 2013). Notably, this switch is associated with resistance of tumor cells to chemo- and/or radio-therapy (Elmore et al., 2005; Illidge et al., 2000; Puig et al., 2008) and corresponds with the decreased overall survival in NSCL cancer patients with the expression of senescence marker SA-β-gal in tumors after non-adjuvant therapy (chemoradiotherapy or chemotherapy; (Wang et al., 2013)).

7.2.4.1 Escape from genotoxic stress-induced premature senescence through polyploidisation

During the fIR, some prematurely senescent PCa cells exhibited polyploid nuclei (4N/8N). Different mechanisms are responsible for establishment of tetraploid phenotype in cancer cells including (i) cell-to-cell fusions; (ii) cytokinesis failure or metaphase/anaphase problems; and (iii) endoreduplication of the genome (reviewed in (Davoli and de Lange, 2011)). The latter together with failure in mitosis was found as a cause of tetraploidy in cancer cells with persistent activation of DNA damage (Davoli et al., 2010). Repeated genotoxic insults, as is the case of fIR, cause irreparable DNA damage and cell cycle arrest in G2/M phase. G2/M-arrested tumor cells lack Cdk1/CycB, therefore tend to skip mitosis and enter into a following S phase with 4N genome. Since APC/Cdc20 is not activated in such situation, linked duplicated chromatids or diplochromosomes are frequently observed during the next mitosis (Davoli et al., 2010). Persistent DNA damage can result also in the generation of dicentric chromosomes leading to uncompleted mitosis followed by tetraploidy (Davoli et al., 2010). Special case of multiplicated genome is polyploidy with the general occurrence of diplochromosomes, 46 pairs of tetrachromatid chromosomes instead of typical 96 bichromatid chromosomes. Diplochromosomal polyploidy can be reversed by bipolar mitosis and generation of genome-reduced cells (Walen, 2007). Polyploid state can provide survival

advantage for tumor cells with damaged DNA, since bypass of mitotic catastrophe is not followed by spindle checkpoint-mediated apoptosis (Vakifahmetoglu et al., 2008).

In general, the escape from polyploidy encompasses the processes resembling meiotic prophase including nuclei reconstruction, chromosome double-loop formation and telomere clustering (Erenpreisa et al., 2000; Ianzini et al., 2009). Mechanistically, it was shown that polyploidization and its reversibility is regulated through aberrant levels of cyclin-dependent kinase Cdk1/Cdc2 as a minor fraction of senescence cells possessing high levels of Cdk1/Cdc2 are enriched for polyploid cells and overexpression of Cdk1/Cdc2 promotes further polyploidization and cell cycle reversibility. It was demonstrated that Cdk1/Cdc2 activity and polyploid formation after induction of DNA damage is negatively regulated by $p27^{kip1}$ (Roberson et al., 2005; Wang et al., 2013).

Stress-induced polyploidy is coupled to stemness properties and self-renewal pattern in breast cancer (Lagadec et al., 2012), lymphoma and cervical cancer after IR (Salmina et al., 2010). Achuthan *et al*. showed that the emergence of actively proliferating drug-resistant aggressive tumor cells associated with the presence of non-cycling senescent cells expressed stem cell markers CD133 and Oct-4 (Achuthan et al., 2011). Escape from drug-induced premature senescence in *in vitro* lung tumor model was associated with emergence of side population cells enriched for $CD34^{+}/CD117^{+}$ CSCs (Sabisz and Skladanowski, 2009). Polyploid but not diploid ovarian cancer cells are both resistant to hypoxia and positive for sphere formation in sphere-induction assay (Zhang et al., 2014b).

As already mentioned, the EMT is coupled with stem cell phenotype (Han et al., 2013a; Kurrey et al., 2009) and also with survival advantage for tumor growth in hypoxic conditions (Jiang et al., 2011). It can be suggested that it has also a role in the regulation of poly/de-polyploidization and escaping from senescence-related growth arrest. Ansieu *et al*. showed that EMT-drivers Twist1 and Twist2 mediate escape from premature senescence and apoptosis by blocking expression of key regulatory molecules $p21^{waf/cip}$ and $p16^{INK4A}$ of p53 and Rb signaling pathways, respectively, and this inactivation of cell safeguard programs led to complete EMT and acquisition of invasive potential in human mammary epithelial cells (Ansieau et al., 2008). Importance of Kras/Twist1/$p16^{INK4A}$ senescence bypass pathway in tumor initiation was documented also in pancreatic duct epithelial cells (Lee and Bar-Sagi, 2010). Consistently, mouse embryonic fibroblasts (MEFs) derived from Zeb1 gene mutant mice underwent $p15^{INK4B}/p21^{waf1/cip1}$-dependent premature senescence *in vitro* (Liu et al., 2008). Although EMT inducers neutralize oncosuppressive pathways, their direct role in poly/de-polyploidization of yet senescent cells remains to be investigated.

7.2.4.2 Escape from stress-induced premature senescence through neosis

In our experiments, low abundant small cells with reduced cytoplasm were observed in the vicinity of senescent cells. Sundaram *et al*. described a specific type of cell division termed neosis (Sundaram et al., 2004). In neosis, post-treatment mitotic-catastrophe-escapers or PGCCs give rise to several small viable Raju cells via nuclear budding and asymmetric cytokinesis. Bypass of senescence/polyploidy and emergence of Raju cells with transient stem cell-like properties were seen in different tumor types including hematological malignancies, carcinoma and sarcoma and represent mitotic active, aneuploid pool with different gene expression profile than parental cells (Rajaraman et al., 2006). Taking into account the described genetic and epigenetic differences among individual Raju cells, neosis can provide a basis for the rapid selection for advantageous traits in the process of neoplastic transformation as well as in the development of treatment-resistance and cancer recurrence (Rajaraman et al., 2006; Sundaram et al., 2004). Whether the presence of rare Raju-like cells in our experiments could be relevant to re-gaining proliferative capacity of senescent fraction after the end of fIR cannot be excluded and remains to be investigated.

7.2.4.3 Role of senescent cells in the generation of fIR-surviving non-adherent population

During the course of fIR, most of PCa adherent cells undergo typical senescent-like changes including accumulation of persistent DNA damage, and polyploidization. Inactivation of p53 and Rb and subsequent re-activation of telomerase is positively correlated with the occurrence of tetraploidy in various cancers which is consistent with frequent mutations of these molecules in aggressive PCa (Bast et al., 2009; Davoli and de Lange, 2011; Meeker, 2006; Ruijter et al., 1999). Notably, some polyploid senescent-like cells were actively replicating their DNA, though in asynchronous manner consistent with the study of Puig *et al*. (Puig et al., 2008). Importantly, we observed that even senescent-like cells underwent the cycle of detachment/readhesion contributing to the pool of non-adherent cell death-resistant fraction. Using normal human fibroblast model of stress-induced senescence, Huna *et al*. showed that presenescent cells that escape 4N-G1 checkpoint were simultaneously positive for markers of self-renewal (Nanog), persistent DNA damage (γH2AX), whereas fully developed senescent cells possess attenuated protein level of Nanog associated with the increased expression of cell cycle inhibitors ($p21^{waf1/cip1}$, $p16^{INK4A}$) suggesting the link between senescence and self-renewal program (Huna et al., 2011). Salmina *et al*. reported that irradiated p53-mutated lymphoma cell lines with endopolyploid genome express stem cell markers Oct4, Sox2 and Nanog with the accumulation of the senescence-associated nuclear

structures – PML NBs (Janderova-Rossmeislova et al., 2007). Bi- and/or multipolar mitoses of these polyploid tumor cells generate de-polyploidized progeny with preserved stemness characteristics (Salmina et al., 2010). Thus we speculate that not only senescent cells but also senescent cell-derived descendants can contribute to the fIR-cell death-resistant non-adherent population. Whether this is the case and whether such cells also resume proliferation after the reattachment and thus contribute to the re-adherent population remains to be investigated. Both DU145 and PC-3 cells exhibit abnormal p53 function (p53 mutated at codons 223 and 274 in DU145 and p53 deletion in PC-3) which can permit mitosis or replication to proceed even without successful repair of the IR-induced DNA damage (Simone et al., 2013). Observed resistance to IR and subsequent escape from senescence-related growth arrest in fIR-adherent population of DU145 and PC-3 cells can be a result of successful de-polyploidization of PGCCs, the mechanism seen also by others in p53-deficient tumor cells (Erenpreisa et al., 2008; Illidge et al., 2000). Such reversal of senescence after IR was recently linked with downregulation of mTOR, activation of autophagy, attenuation of DDR signaling and induction of stem cell markers (Chitikova et al., 2014) which are all characteristics of fIR-non-adherent PCa cells (Kyjacova et al., 2015); and our unpublished data).

Whether stress-induced premature senescence and SASP contribute in any aspect to the observed cellular plasticity and/or therapy-resistant phenotype will be a subject of our next studies.

7.2.5 Inflammation and cancer radioresistance

The whole genome expression analysis of radio- and/or chemo-resistant DU145, MCF-7 and HeLa populations (adherent, non-adherent, re-adherent) revealed the unique expression signature for each fraction. The most divergent treatment-resistant population was represented by surviving non-adherent cells in all three cell lines with more than 1.500 significantly deregulated genes in comparison to non-treated (control) parental cells. The most commonly affected signaling were the pathways associated with cellular metabolism and cytokine signaling, including the increase of various IFN-related genes (e.g. IFNB1, IFNL1, IFNL2, IFNL3, IFIT1, IFIT3, IFITM1, IFI27, OASL, AIRE, IRAK2, NFKB2, ISG15), interleukins (e.g. IL6, IL8, IL23A, IL11, IL12A, IL24, IL11), chemokines (e.g. CCL5, CCL20, CCL22, CXCL2, CXCL10), and/or tumor necrosis factor alpha (TNFα) with its receptors (TNFRSP9, TNFRSP10B).

Khodarev *et al.* described the IFN-related DNA signature (IRDS), specific gene-expression profile of cancer cells related to IFN signaling and resistance to IR-induced DNA

damage (Khodarev et al., 2004). Out of 36 IRDS genes enriched in 2 Gy-surviving fractions of 34 various cancer cell lines (Weichselbaum et al., 2008), almost 70% were also found deregulated in irradiated non-adherent DU145 cells out of which 72% were significantly upregulated ($q<0.05$; 18 out of 25 genes). Immune response genes IFI27, OASL, IFIT1 and IFIT3 were found to be upregulated following fIR (10 x 1 Gy) in PCa cell lines DU145, PC-3 and LNCaP (Simone et al., 2013) correlating with the upregulation of all four genes in irradiated non-adherent DU145 cells and even more upregulated in fIR-non-adherent MCF-7 cells. However, only IFI27 was upregulated in 5-azacytidine-treated non-adherent HeLa cells. Khodarev *et al*. also showed that IR-resistant tumor xenograft cells selected from sensitive head and neck squamous cell carcinoma line SCC-61 overexpress genes associated with IFN signaling pathway. The most upregulated genes were signal transducer and activator of transcription genes - STAT1α and STAT1β, both able to ensure experimental radioresistance (upon radiation exposure 3 Gy) when overexpressed in radiosensitive cells (Khodarev et al., 2004) correlating with the upregulation of STAT1 in radiation-surviving adherent DU145 cells. Hovewer, STAT1 as well as STAT3 and STAT4 were not significantly upregulated in fIR non-adherent cells; instead STAT5A was increased in this fraction. STAT5 is implicated in resistance to apoptosis and confers chemoresistance to cisplatine and 5-fluorouracil in colorectal cancer (Hong et al., 2012). STAT5A is important also in self-renewal of hematopoetic cells and resistance to oxidative stress and DNA damage in chronic myeloid leukemia cells (Casetti et al., 2013). Most importantly, it was demonstrated that different cancers including breast and prostate can be segregated according to their IRDS profiles to $IRDS^-$ and $IRDS^+$ groups. The $IRDS^-$ patients exhibited fewer distant relapses upon adjuvant chemotherapy as well as lower local-regional failure after adjuvant RT which indicate that IRDS status can be used as the therapy predictive marker and marker of tumor recurrence (Weichselbaum et al., 2008). In another study, the induction of IFN-related genes in DU145, MCF-7 and SF539 (gliosarcoma) was selective, as it was evident only after fIR and not after a single dose of IR and changed continuously throughout the time supporting the fact that these genes are really influenced by repeated fractions of IR. Authors identified genes commonly increased in all three cell lines 24 hours after radiation exposure (1 x 10 Gy or 5 x 2 Gy). 7 out of 13 genes represented IFN-responsive genes (Tsai et al., 2007). Of them, IFIT2, LGALS3BP and IFITM1 were also altered in fIR-surviving non-adherent DU145 fraction. Interestingly, non-adherent MCF-7 cells had not only upregulated IFIT2, LGALS3BP and IFITM1 but also OAS3 and BST2 genes while non-adherent HeLa cells exhibited only upregulation of OAS3 indicating that irradiated MCF-7 cells exhibited the strongest IFN

response among tested lines. Interestingly, different signaling pathways were altered upon IR in PCa cell lines with standard (LNCaP) or abnormal (DU145, PC-3) p53 function. Genes implicated in DNA replication and DNA repair were most prominently altered in LNCaP cells whereas apoptosis, immune and IFN response genes were most exclusively expressed in cell lines with abnormal p53 function placing the p53 tumor supressor to the position of major regulator of cellular responses to DNA damage (Simone et al., 2013) further suporting by older study where IFN-related genes IFITM2 and IFITM were upregulated in p53-deficient leukemic KG1a cells after 3 Gy IR (Clave et al., 1997).

The very upstream regulators of IFN signaling, type I (IFNβ) and type III IFNs (IFNλ) but not type II IFN (IFNγ) were upregulated in fIR-non-adherent cancer cells according to our expression data. Despite expression of IFNγ itself is not increased as a response to radiation (Sreekumar et al., 2001), treatment with IFNγ alone led to development of non-adherent phenotype and dormancy in HeLa cells *in vitro* (our unpublished results) suggesting that IFN response could be implicated in phenotypic switch after genotoxic stress. However, proper molecular mechanisms need to be revealed.

We identified interleukin 6 (IL6), and 8 (IL8) as the most upregulated cytokines in stress-induced non-adherent cancer cells. Increasing evidence suggests that both cytokines are implicated in regulation of EMT in cancer cells, and IL8 is associated with acquiring CSCs features, formation of tumorspheres and chemo-/radio-resistance (Hwang et al., 2011; Sullivan et al., 2009). In general, various components of tumor microenviroment are potential sources of soluble factors capable of inducing EMT in tumor cells and *vice versa* cancer cells themselves can secrete cytokines, growth factors and other mediators which influence surrounding stroma and may also act as inducers of EMT in neighboring cancer cells via paracrine signaling (Nannuru and Singh, 2010; Sansone et al., 2007). Some studies indicate that autocrine cytokine signaling loops executed also via IL6 and IL8 are important not only for the induction but also for the maintenance of mesenchymal status of cancer cells (Gregory et al., 2011; Palena et al., 2012; Scheel et al., 2011). IL6 inflammatory feedback loop was important for enrichment of CSCs with mesenchymal phenotype in response to chemotherapy treatment in breast cancer (Korkaya et al., 2012; Yang et al., 2014). Moreover, IL6 promotes metastases by inducing EMT via JAK/STAT3/Snail axis in head and neck cancer (Yadav et al., 2011). Importantly, Hwang *et al*. showed that IL8 represents direct trancriptional target of Snail in $CD44^{+}/CD166^{+}$ chemo-/radio-resistant colonospheres. The inhibition of IL8, disrupted Snail-mediated expression of stem cell-related genes and affected colonospheres

formation indicating cross-talk between cytokines and EMT drivers in the maintenance of stemness phenotype of cancer cells (Hwang et al., 2011).

Recently, Makinde *et al.* studied expression changes in PCa cell lines in response to both single dose (5 or 10 Gy) or multiple doses of IR (0.5, 1 or 2 Gy fractions to total dose 10 Gy). Besides the typical expression changes related to DNA damage, cell cycle arrest and apoptotic signal transduction they found also activation of immune response, TGF-β signaling and survival pathways while p53 status played a major role in this response (Makinde et al., 2013). Since TGF-β is also implicated in the EMT regulation it would be interesting to investigate its role along with IL6 and IL8 in irradiation-induced loss of adhesion.

The importance of inflammation in tumorigenesis is widely accepted (Grivennikov et al., 2010). Inflammation originates intrinsicaly from genetic events leading to tumor transformation as well extrinsicaly from signals from 'reactive stroma' (Aguirre et al., 2003; Clark et al., 2007). Inflammatory components such as NF-κB, TGF-β, Jak/STAT, and TNFα signaling are implicated in the induction of epithelial-mesenchymal plasticity (reviewed in (Zhou et al., 2012)). In turn, EMT-TFs (e.g. Snail) are involved in signaling feedback loops via pro-inflammatory mediators such as IL1, IL6, and IL8 (Lopez-Novoa and Nieto, 2009). Interestingly, breast carcinoma cells cultivated in CAF-derived media resulted in E-cadherin loss followed by anchorage-independent growth and increased motility similarly to our model (Lebret et al., 2007). Moreover, inflammation confers EMT and a stem-cell phenotype via cyclooxygenase-2 (COX2), NF-κB, and HIF1 in PCa (Giannoni et al., 2011). Complex picture regarding the interplay between inflammation, EMT, stemness, and tumor initiation/ progression was provided by Rhim *et al*. using various murine models of pancreatic cancer. Essentially, they showed that both treatment- and surgery-induced inflammation resulted in the formation of pancreatic premalignant lessions, increased EMT, and early dissemination of circulating pancreatic cells expressing stemness markers CD24 and CD44 even in the absence of a detectable tumor. However, despite their increased total numbers, their clonal growth in comparison to tumor-derived CTCs was significantly diminished (Rhim et al., 2012). The importance to study pro-inflammatory signaling in fIR-surviving PCa cells is further supported by the recent study where cells collected from blood of PCa patients treated by intensity-modulated RT had activated pro-inflammatory signaling as well as positive regulators of MAPK/Erk and NF-κB signaling, corresponding with our *in vitro* data (El-Saghire et al., 2014).

The important question is: what is the source/trigger of inflammatory signature in fIR non-adherent cells *in vitro* where no pathogenes or immune-response cells are present. Toll-

like (TLRs), Nod-like (NLRs), and Rig1-like (RLRs) receptors are transmembrane pattern recognition receptors (PRRs) that play a key role in the innate immune system (Kumagai et al., 2008). They recognize pathogen-associated molecular patterns (PAMPs) to defend cells against bacteria, viruses, fungi, and parasites. However, besides PAPMs, PRRs also sense endogenous damage-associated molecular patterns (DAMPs) resulting from non-physiological cell death, damage, or stress (see **Fig. 7.1**; (Bianchi, 2007; Lotze et al., 2007)). In the context of fIR, resulting molecules (debris) released from necrotic cells or apoptotic cells dying for secondary necrosis can represent a source of DAMPs that can trigger early innate immune responses mediated through PRRs. Stimulation of TLRs leads to the activation of MAPKs, Jun N-terminal kinases (JNKs), p38, Erk, PI3K, as well as interferon regulatory factors (IRF3, IRF5, and IRF7) pathways which result in pro-inflammatory cytokines secretion (Lee and Kim, 2007). DAMPs ligated to its PRRs also lead to the activation of NF-κB which promotes further expression of pro-inflammatory cytokines, chemokines, angiogenic factors, MMPs, and anti-apoptotic genes (Karin and Greten, 2005). In our experiments, cellular debris was not effectively cleared from the culture media during fIR. Moreover, the most enriched signaling pathways in treated non-adherent fraction were TLR-, NLR-, RLR-mediated signaling pathways; cytokine-cytokine receptor interaction; cytosolic DNA-sensing pathway; Jak/STAT signaling pathway, and MAPK signaling pathway (see **Fig. 4.20**), strongly pointing to the activation of a complex innate immune response.

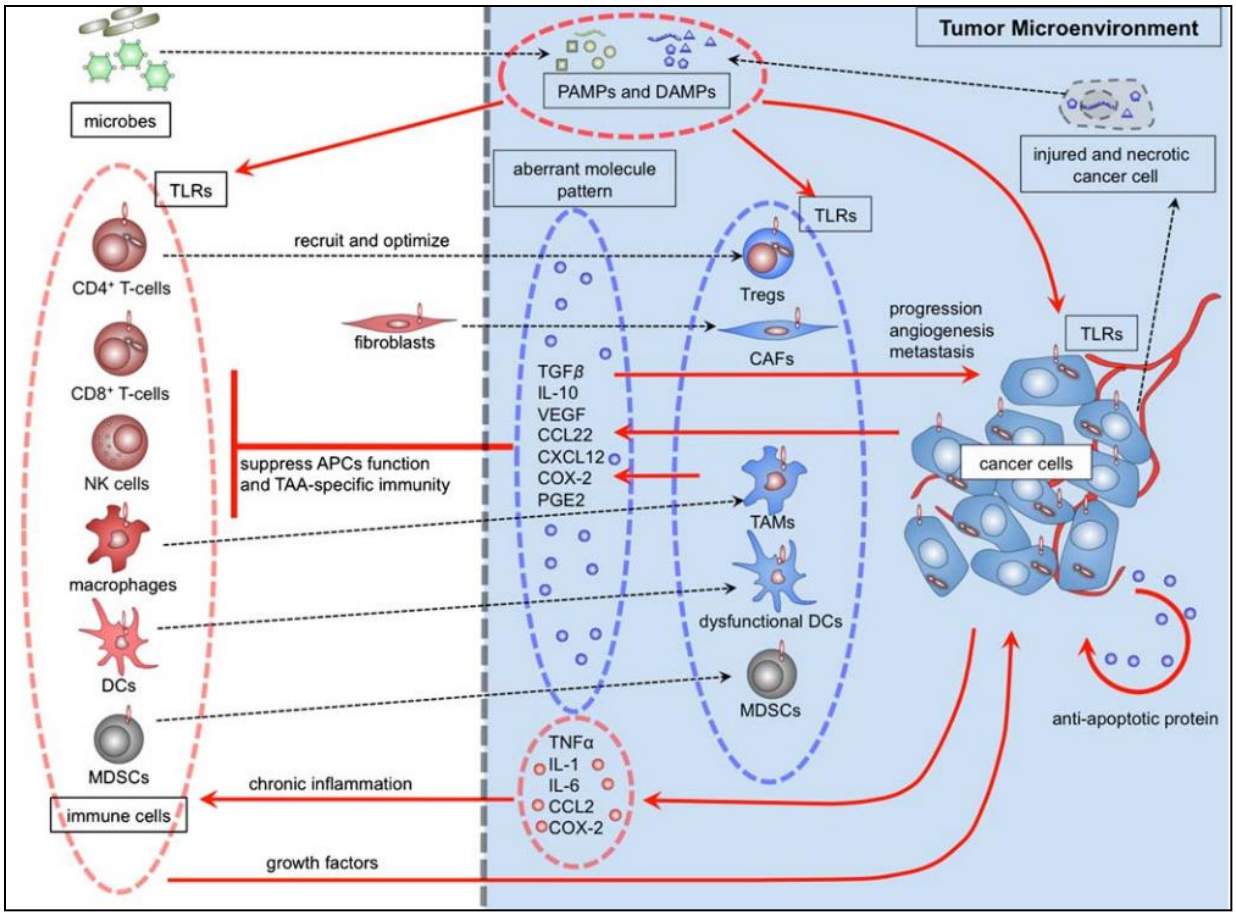

Figure 7.1. Cross-talk between alarmins (PAMPs/DAPMs) and Toll-like receptors (TLRs) in the tumor microenvironment. Activation of TLRs in the surface of immune cells *and* cancer cells by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) result in release of pro-inflammatory cytokines and chemokines which can aberrantly affect the tumor microenviroment. Tregs: regulatory T-cells; TAMs: tumor-associated macrophages; DCs: dendritic cells; CAFs: cancer-associated fibroblasts; MDSCs: myeloid-derived supressor cells (adapted from (Sato, 2009 #13322)).

DAMP molecules were shown to have both protumor and antitumor effects (see (Campana et al., 2008)) and were associated with resistance to chemotherapy via PI3KC3/Mek/Erk-dependent activation of autophagy (Liu et al., 2011). In PCa patients, 85 out of 112 samples were positive for TLR3, TLR4, and TLR9. Moreover, patient samples with high TLR3 and TLR9 expression were significantly associated with a higher probability of biochemical relapse (Gonzalez-Reyes et al., 2011). TLR3 was shown to regulate angiogenesis and apoptosis under hypoxic conditions via HIF1-α and protein kinase C (PKC) in PCa (Paone et al., 2010; Paone et al., 2008). On the other hand, expression of TLR5 in PCa cells recruits cytotoxic immune cells to the tumor site resulting in the inhibition of tumor growth (Galli et al., 2010).

To conclude, genotoxic stress-induced inflammation/IFN-related signaling in cancer cells could have a relevant role in the treatment-induced EMT/stemness phenotype and

therefore in therapeutic resistance *per se*. Observed inflammatory signature in treated non-adherent cells could be caused by DAMPs released from necrotic cells which have a pottential to trigger innate immune responses via transmembrane PRRs.

7.2.6 Radioresistance and cancer metastasis

Viable non-adherent cells surviving the fIR are similar to CTCs which were also shown to have features of CSCs (Allard et al., 2004; Baccelli et al., 2013; Tinhofer et al., 2014). Recently, Yu *et al*. reported that the number of epithelial- or mesenchymal-like CTCs isolated from patients cured for metastatic breast cancer correlated with the type of therapy applied (Yu et al., 2013), suggesting that the EMT/MET switch might be therapy-dependent. Importantly, a positive therapeutic response was accompanied with less CTCs featuring prominent epithelial phenotype, whereas patients suffering from disease unresponsive to chemotherapy possessed higher numbers and mostly mesenchymal CTCs in post-treatment samples. Moreover, NSCLC patients treated with either palliative or curative RT had increased numbers of single or clumped mesenchymal-like vimentin-positive CTCs in peripheral circulation (Martin et al., 2014), corresponding with our data and further stressing the role of therapy in the induction or support of EMT.

There is an increasing evidence that EMT and its reverse process MET are linked to the formation of metastases via so called 'migrating CSCs', concept combining EMT with the activation of self-renewal program in DTCs (Brabletz, 2012; Brabletz et al., 2001; Brabletz et al., 2005; Thiery, 2002), both characteristics of fIR-surviving non-adherent cells. In the invasion-metastasis cascade, the EMT has not only a role in the initial malignant transformation (Husemann et al., 2008) but also in the single-cell invasion as was shown using mouse tumor models with tissue-specific deregulation of E-cadherin (Derksen et al., 2006; Perl et al., 1998). EMT-TFs upregulate the expression of various proteins facilitating degradation of underlying ECM and a BM in order to enter circulation (Huang et al., 2009; Olmeda et al., 2007; Ota et al., 2009). Moreover, Twist1 regulates the formation of metastasis-associated protrusions serving as protease-scaffolds named invadipodia (Eckert et al., 2011). In addition, Zeb1 expression facilitates transendothelial migration and metastasis formation in PCa cells (Drake et al., 2009). Interestingly, Shibue *et al*. showed that extravasated breast tumor cells use Twist1/Snail-dependent integrin β filopodium-like protrusions for the interaction with components of ECM at distant tissues essential for successful metastatic colonization (Shibue et al., 2012).

An interesting aspect of our study is the reprogramming of fIR-surviving cells in terms of mesenchymal and stem-like traits upon the reattachment. A readhesion of E-cadherin-negative/low fIR non-adherent PCa cells was coupled to a restored proliferation and gradual E-cadherin re-expression accompanied with gradual vimentin loss during the first passages suggesting the reprogramming towards the epithelial phenotype in the process resembling MET. There is increasing evidence that prior establishment of metastatic foci, the MET as the reverse process of EMT is important for the successful colonization of CTCs (Brabletz, 2012). It was reported that some metastases express equal or higher levels of E-cadherin than original primary tumors (Kowalski et al., 2003). E-cadherin re-expression was observed even in metastases derived from originally E-cadherin-low/negative primary tumors (Chao et al., 2010). The importance of redifferentiation for macrometastatic growth was first highlighted by Chaffer *et al*., who showed that although the mesenchymal subclones of bladder cancer cells had a high capacity to disseminate and form micrometastases, they failed to progress to macrometastases. In contrast, epithelial subclones successfully formed macrometastases after the injection into the circulation (Chaffer et al., 2006; Chaffer et al., 2007). Analogously, constitutive stimulation of primary tumor cells with EMT inducer TGF-β resulted in the increased cell dissemination but prevented the metastatic outgrowth in lungs (Giampieri et al., 2009). Notably, Valiente *et al*. recently showed that plasmin from reactive brain stroma serves as a defense against infiltrating lung and breast cancer cells and that a successful breaking blood-brain barrier and brain colonization by cancer cells requires expression of serpins, inhibitors of plasminogen activation, serving as protecting shield against this defense. Mechanistically, serpins promoted survival of cancer cell by inhibiting FasL-mediated apoptosis produced by astrocytes and also supported the spread of metastatic cells on the surface of the brain capillaries by preserving L1CAM protein levels (Valiente et al., 2014). Expression of L1CAM is dependent on EMT-inducer TGF-β (Geismann et al., 2011) suggesting that MET undergoing cells during colonization of brain by metastases colonization may retain a part of the mesenchymal signature to overcome the blood-brain barrier.

As mentioned before, dormant CTCs are oligometastasis precursors contributing to the therapy recurrence. Therefore it is important to answer the question which molecular mechanisms are implicated in the exit from CSCs-associated cell cycle arrest, and in analogy to our *in vitro* system, which molecular pathways determine the exit from non-adherent state and cause the readhesion in the absence of stress insult. As the metastasis recapitulates the pathology of primary tumor, MET seems to be a crucial process in the initial stages of metastasis formation. However, regulatory mechanisms controlling MET of carcinoma cells

are not well resolved. So far, it was shown that Fox-2 binds sequence-specific element present in the FGFR2 pre-mRNA and regulates its preferential exon IIIb maintenance. Fox-2-dependent switch between FGFR2(IIIb) to FGFR2(IIIc) variants was associated with MET in T Rex-293 cells (Baraniak et al., 2006). Inhibition of Src homology phosphotyrosine phosphatase 2 (SHP2) attenuated Erk and PI3K/Akt signaling and led to the conversion to epithelial phenotype in breast cancer cells with upregulation of E-cadherin and downregulation of vimentin and fibronectin (Zhou and Agazie, 2008). The TGF-β superfamily member, bone morphogenetic protein 7 (BMP7) was also associated with MET in PCa (Buijs et al., 2007). Whether the above mentioned factors are implicated in the proliferative switch observed during fIR in prostate and breast cancer cell lines needs to be addressed.

Besides MET-associated cytokines or its receptors (mentioned above and reviewed in (Yao et al., 2011)), particular EMT-TFs and miRNAs have been implicated in the MET (Bullock et al., 2012). For example, transfection of miR-200 into mesenchymal clone 4T07 promoted MET and finally enabled the macrometastatic growth (Dykxhoorn et al., 2009). The expression of some EMT drivers is negatively regulated by specific miRNAs (see Chapter 2.3.2.1.2). Similarly to miR200-Zeb1 negative feedback loop (Korpal et al., 2008; Park et al., 2008), also miR34-Snail reciprocal regulation contributes to EMT/MET switch (Kim et al., 2011a; Siemens et al., 2011). However, besides the intrinsic EMT/MET regulation, also external factors originating from pre-metastatic niche are important regulators of MET (Buijs et al., 2007; Gao et al., 2012a). During the characterization of non-adherent cells, we identified several miRNAs significantly deregulated in fIR-non-adherent DU145 cells including decreased levels of Snail negative regulator miR34 (Kim et al., 2011a; Siemens et al., 2011); elevated levels of miR21 implicated in chemoresistance (Deng et al., 2014) and induction of EMT and cancer stem cell phenotype *in vitro* (Han et al., 2012) or cell cycle negative regulator miR221 (Fornari et al., 2008; Sun et al., 2011). In addition, significant portion of MET regulation signals comes from the tumor microenvironment itself. For example, co-culturing of DU145 and PC-3 cells with hepatocytes led to the downregulation of EGFR, re-expression of E-cadherin, and formation of heterotypic cell-cell adhesions mimicking *de novo* metastasis formation.

In this study, we showed that fIR generates two resistant (adherent and non-adherent) PCa populations with the sensitivity to Erk and/or Akt inhibitors when combined with IR. About 70% of prostate high-stage samples exhibit *PTEN* loss or PI3K activation (Taylor et al., 2010). Despite the activation of RAS is rare in PCa (Gumerlock et al., 1991), it was

shown that activation of RAS in DU145 cells promotes metastasis to the brain and bone *in vivo* (Yin et al., 2007). Moreover, mice bearing prostate-specific hyperactivation mutations of both Akt and Erk pathways (C^{+}; $Pten^{L/W}$; $K\text{-}ras^{L/W}$ or C^{+}; $Pten^{L/L}$; $K\text{-}ras^{L/W}$) developed invasive carcinoma and macrometastatic lessions (and maintained morphology similar to primary cancers) in the lung and liver with 100% penetrance and exhibit premature lethality when compared to mice bearing only single mutation in Akt or Erk signaling. Ras activation in *PTEN*-null prostate epithelium resulted in EMT and expression of Snail, Twist1, Zeb1, vimentin, fibronectin, MMP2 and Foxc2 and generation of C^{+}; $Pten^{L/L}$; $K\text{-}ras^{L/W}$ sphere cells showing high metastatic potential in orthotropic transplantation murine models. Most importantly, and corresponding to our *in vitro* data, mutant mice receiving rapamycin (mTOR inhibitor) and PD325901 (Mek inhibitor) had a reduced primary tumor mass, inhibited EMT, and a weaker metastatic potential of C^{+}; $Pten^{L/L}$; $K\text{-}ras^{L/W}$ (Mulholland et al., 2012). Interestingly, treatment with only PD325901 alone reduced metastasis, however, the effect on primary tumor was less significant compared with the combined treatment (Mulholland et al., 2012), further supporting our data showing a greater radiosensitizing effect on adherent fraction when both Erk and Akt inhibitors were used. Study by Mulholland *et al.* provides *in vivo* evidence of the direct role of Erk and Akt signaling in promoting PCa metastasis. It would be exciting to test the effect of mTOR and Mek inhibitors on metastatic potential of C^{+}; $Pten^{L/L}$; $K\text{-}ras^{L/W}$ cells in combination with fIR in order to answer the questions regarding radioresistance and radiation-induced invasiveness in PCa.

In conclusion, the irradiation-induced plasticity described in this study is analogical to the invasion-metastatic cascade (see **Fig. 7.2**) and as such could serve as a simple *in vitro* platform for elucidating important therapeutically-relevant questions in the field of prostate cancer molecular oncology, in particular in post-therapy cancer recurrence.

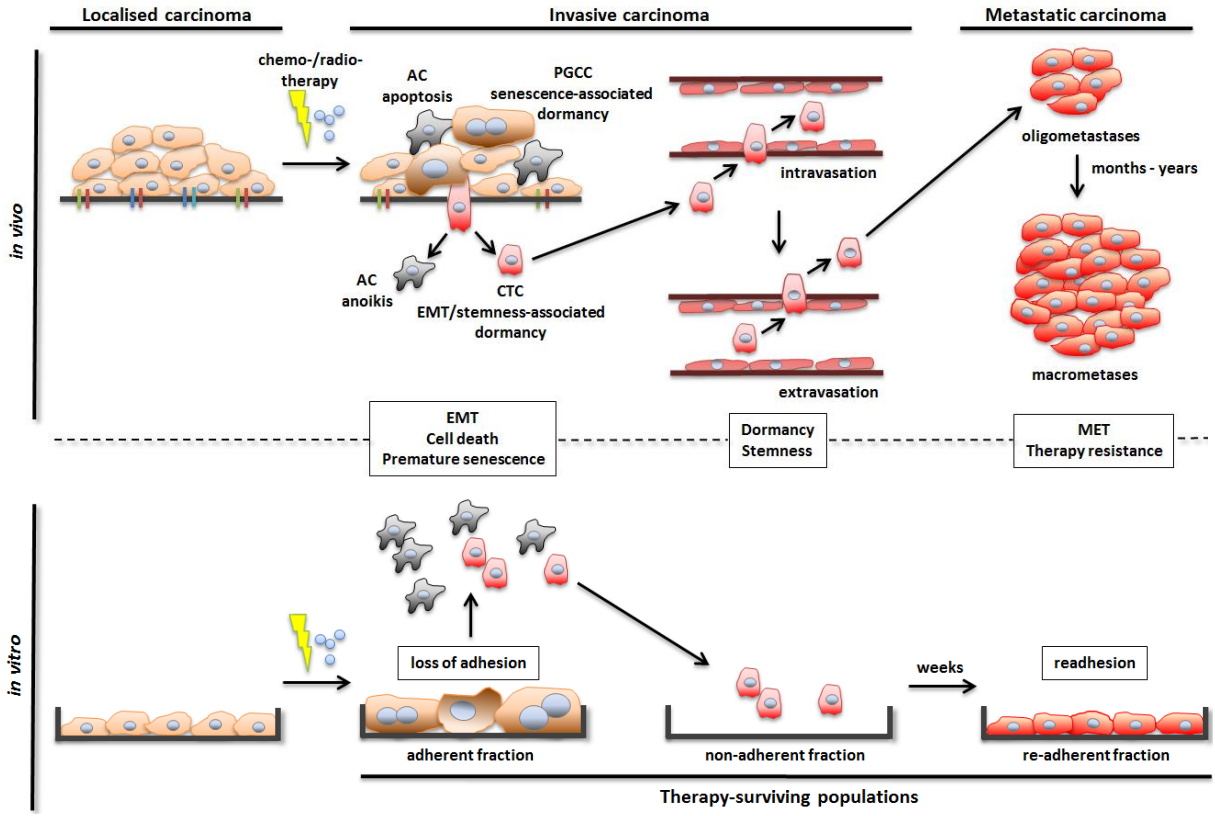

Figure 7.2. Genotoxic stress-induced changes *in vitro* and *in vivo*. Chemo- and/or radio-therapy in cancinomas lead primarily to the cell death (AC, apoptosis/anoikis-undergoing cell) and/or premature senescence with the presence of polyploid giant cancer cells (PGCCs). However, genotoxic-stress can induce the epithelial-to-mesenchymal transition (EMT), a key molecular program which together with its opposing process mesenchymal-to-epithelial transition (MET) leads to increased invasivity and metastases by generating stem-like circulating tumor cells (CTCs). Formation of metastases encompasses several rate-limiting processes such as intravasation of CTCs into circulation, persistance in the circulatory system, extravasation from blood vessels and either formation of oligometastases or large secondary tumors (macrometastases) in the process called colonization. Genotoxic stress *in vitro* induces processes resembling the invasion-metastases cascade at both cellular and molecular level including induction of EMT/MET changes, activation of stem-like program, acquiring anoikis resistance, and resistance to the subsequent therapy (Kyjacova et al., 2015).

8 CONCLUSIONS

PCa patients treated with RT relatively often experience a relapse of the disease not only as a result of the local control failure (primary tumor relaps) but also in the form of distant metastasis suggesting the existence of radiation-surviving clones with the enhanced motility and invasivity. In this thesis, we tested the assumption that fIR *per se* could induce series of changes colectivelly leading to the resistance and agressive behaviour of escaping cells.

The key message of this thesis is that RT regimen used in clinical oncology as a therapy for PCa patients (fIR; 35 x 2 Gy; 2 Gy/24 hours) is not sufficient to eradicate all metastasis-derived PCa cells *in vitro* as we identified viable fIR-surviving 'adherent' and 'non-adherent' populations with the capacity to either escape from premature senescence or from dormancy-like state. To our knowledge, this is the first report showing that fIR generate viable dormant non-adherent cells expressing stem cell markers with the potential to recapitulate adherent growth (fIR-surviving 're-adherent' cells), strongly resembling the invasion-metastasis cascade. Therefore, as soon as we identified fIR-surviving PCa populations we proceeded a thorough analysis in order to better understand the basis for the radioresistant phenotype. Main findings specified in this thesis can be summarized as follows:

- fIR generates (i) surviving adherent and (ii) surviving non-adherent population with the capacity to recapitulate adherent growth (re-adherent population) in prostate and breast cancer cells;
- fIR-surviving adherent cells exhibit features of premature senescence (flattened morphology, altered ploidity, expression of CDK inhibitors, presence of persistent DNA damage foci, PML NBs, micronuclei, and multiple vacuoles, and others) and have the potential to overcome senescence-associated growth arrest in order to renew regular proliferation;
- fIR-surviving non-adherent cells (originating from the adherent fraction) represent a proliferative dormant, anoikis-resistant pool with active Notch signaling and expression of stem cell markers;
- fIR-surviving re-adherent cells (originating from the non-adherent fraction) proliferate comparably to non-irradiated parental cells and have a retained tumorigenic potential in immunocompromised mice;

- fIR induces plasticity related to EMT in prostate and breast cancer cells with the increase of mesenchymal features (loss of E-cadherin, expression of EMT-TFs) in non-adherent fraction and gradual reprogramming for epithelial phenotype after the readhession (decreased expression of vimentin, re-expression of E-cadherin);
- fIR-induced EMT-TF Snail is crucial for fIR-dependent loss of cellular adhesion and formation of surviving anoikis-resistant non-adherent cells in prostate and breast cancer cells;
- fIR-surviving non-adherent cells have active Mek/Erk1/2 pro-survival signaling;
- inhibition of Mek/Erk1/2 signaling suppresses Snail expression and impairs both fIR-mediated loss of adhesion and anoikis-resistant survival of non-adherent PCa cells;
- Erk1/2 regulates anoikis-resistant survival via proteins from the Bcl-2 family, pro-apoptotic Bim and anti-apoptotic Bcl-XL;
- inhibition of Erk1/2 has a radiosensitising effect on fIR-surviving non-adherent PCa cells with the elimination of almost all (>90%) viable cells;
- simultaneous inhibition of Erk1/2 and Akt pathways has an additive radiosensitizing effect on fIR-surviving adherent PCa cells;
- fIR-surviving re-adherent cells exhibit acquired resistance to IR-mediated premature senescence and have enhanced IR-induced anchorage independent survival;
- stress-induced plasticity driven by EMT/MET-related changes is not restricted to the type of genotoxic insult (physical/chemical) or cancer cells origin;
- stress-induced non-adherent cancer cells have markedly altered expression profile with the main changes in cellular metabolism, cytokine signaling and inflammation/IFN-mediated signaling associated with innate immune response;
- stress-induced phenotypic plasticity of prostate cancer cells resembles the invasion-metastatic cascade and therefore can be exploitable as a simple *in vitro* model for the study of therapy resistance.

Taken together, the results presented in this Thesis help to better understand the irradiation-induced cellular plasticity with the unforeseen contribution of non-adherent CSC/CTC-like fIR-surviving population. Described *in vitro* model of genotoxic stress-induced resistance of tumor cells can reflect the metastatic cycle and as such is exploitable for better understanding the phenomenon of cancer cell metastases, therapeutic resistance and disease recurrence.

9 SIGNIFICANCE OF RESULTS AND FUTURE PROSPECTS

Knowledge of the effects evoqued by fIR at cellular and molecular levels *in vitro* is an important prerequisite for successful cancer treatment in clinics. Escape from premature senescence can represent the underlying mechanism for the post-therapy relapse, and analogically, involvement of non-adherent stem-like cells in fIR-surviving pool can explain the post-therapy occurence of distant metastases. From the therapeutical point of view, we showed that treatment either with Erk or Akt inhibitor alone or with their combination effectivelly radiosensitizes both populations to cell death indicating their potentionally beneficial effects in the prevention of the post-radiotherapy relapse.

As a next step, we would like to examine altered signaling in genotoxic stress-surviving non-adherent cells like Jak/STAT, NF-κB, and IFN-mediated signaling pathways as well as on pro-inflammatory cytokines with the emphasis on the non-adherent fraction and the role of premature senescence in this phenotype. Moreover, we would like to investigate the fIR-induced tumor cell plasticity *in vivo* using immunodeficient mice models to take into account paracrine signals from adjacent microenvironment in this process. Finally, our intent will be also to explore the metastasizing potential of fIR-surviving non-adherent cells to make the current *in vitro* model of genotoxic stress-induced resistance more relevant to the events occuring in patients undergoing radiation therapy.

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