UCP-3 Uncoupling Protein Confers Hypoxia Resistance to Renal Epithelial Cells and is Upregulated in Renal Cell Carcinoma

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von

Braun, Norbert

2016

Dekan:	Professor Dr. I. B. Autenrieth
1. Berichterstatter:	Professor Dr. S. Huber
2. Berichterstatter:	Professor Dr. B. Schittek
3. Berichterstatter:	Professor Dr. U. Gaipl
Tag der Disputation	21.12.2016

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Abbreviations

ADP	adenosine diphosphate
Akt	protein kinase B
ANOVA	analysis of variance
ATF-1	cyclic AMP-dependent transcription factor
ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma 2
caspase 9	cysteine-aspartic protease 9
CCCP	carbonyl cyanide-3-chlorophenylhydrazone
CD	cluster of differentiation
cDNA	complementary DNA
CM-H_2DCFDA	5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein-diacetate acetyl ester
c-myc	cellular myelocytomatosis oncogene
CXCR4	C-X-C chemokine receptor type 4; CD184
DMEM	Dulbecco’s modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
G418	Geneticin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSK-3	glycogen synthase kinase 3
H/R	hypoxia/reoxygenation
HEPES	32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HIF-1	hypoxia-inducible factor 1
IAP-2	inhibitor of apoptosis protein 2

IgG	immunoglobulin G
IR	ionizing radiation
LOX	lysyl oxidase
MAP	mitogen-activated protein
mitoK$_{ATP}$	mitochondrial ATP-sensitive potassium channel
mitoK$_{Ca}$	mitochondrial Ca-sensitive potassium channel
mmHg	millimeter of mercury
MMP2	matrix metalloproteinase-2; type IV collagenase
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotid
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
p53	tumor protein p53
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI	propidiumiodide
PI3K	phosphatidylinositide 3-kinases
pO_2	partial pressure of oxygen
PT	proximal convoluted tubule
PVDF	polyvinylidene fluoride
RCC	renal cell carcinoma
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
siRNA	small interfering RNA
SOD	superoxide dismutase
SV40	simian vacuolating virus 40
TBS	tris-buffered saline
TCGA	The Cancer Genome Atlas

TGF-β	transforming growth factor beta
TLR4	toll-like receptor 4; CD284
TMRE	tetramethylrhodamine ethyl ester perchlorate
UCP	uncoupling protein
VEGF	vascular endothelial growth factor
$\Delta\Psi_m$	inner mitochondrial membrane potential

This thesis is based on the paper “Braun, N. *et al.* UCP-3 uncoupling protein confers hypoxia resistance to renal epithelial cells and is upregulated in renal cell carcinoma. *Sci. Rep.* **5,** 13450 (2015).”. To indicate quotes taken from this paper, they are marked with “ “.

1 Introduction

1.1 Tumor Hypoxia

Hypoxia is a common feature in advanced solid tumors. Hypoxic tissue areas have been found in a variety of different tumors such as breast cancer, head and neck cancer and soft tissue sarcomas[1–4]. Hypoxia is not only detected in locally advanced or metastatic tumors but also in premalignant lesions or small tumors[5] and plays an important role in the evolutionary process of carcinogenesis[6,7]. Mean pO_2 in tumors is generally reduced compared to normal tissue and most tumors show areas where pO_2 is lower than 5 mmHg[8,9].

Tumor hypoxia results from an imbalance between oxygen consumption and oxygen supply, mainly caused by morphologically abnormal vessels[8]. Besides an abnormal vessel wall[10], they often show twisting and bending, arteriovenous shunts and sinusoidal parts[11]. Together with a heterogeneous distribution, these abnormalities lead to functional problems resulting in an unstable direction and speed of flow, microthrombosis and arteriovenous shunt perfusion, leading to insufficient oxygen supply[8]. This perfusion-related or ischemic hypoxia is called acute hypoxia. It mainly is a transient effect and can lead to reoxygenation injury[12,13].

Besides perfusion-related hypoxia, diffusion-related hypoxia also contributes to a reduced supply with oxygen and other nutrients in cells more than 70 µm distant from vessels[14]. Hypoxic conditions (pO_2 < 5 mmHg) can be found at distances around 70-80 µm from a vessel, near anoxic conditions (pO_2 < 0.5 mmHg) are normally found more than 150 µm away from vessels[9]. This is consistent with findings that there are no viable tumor cells at distances greater than 160 µm from vessels and there are heterogeneous distributed areas with chronic hypoxia in tumors[15,16].

Additionally, a limited O_2 transportation capacity in cancer patients can aggravate hypoxic conditions in tumor tissues[17]. Anemia is a common condition in patients suffering from cancer and is often seen as a side effect of cytostatic treatment. Furthermore, numerous other factors like chronic inflammation, hypersplenism,

tumor-associated bleeding and nutritional deficiencies[18] contribute to a further reduction in hemoglobin levels in cancer patients.

In normal tissue, hypoxia normally leads to cell cycle arrest and inhibition of mRNA translation to prevent the cells from further damage[19,20]. Prolonged hypoxic states or anoxic conditions often lead to cell death via apoptosis or necrosis[21]. Though tumor cells are normally more resistant to hypoxic stress[22], they are not completely immune to hypoxic damage[23]. Additionally, reoxygenation can contribute to cell death after hypoxic episodes by several mechanisms such as activation of FAS-dependent death pathways, mitochondrial damage[13] or formation of reactive oxygen species (ROS)[24].

To evade cell death and cell cycle arrest, some cells have achieved a number of mechanisms to survive in a hypoxic environment. Generally, hypoxia activates several survival pathways including HIF-1- and NF-κB-signalling[25], which enable cells to evade apoptosis and which are often altered in tumor cells[26]. Among others, tolerance to chronic or intermittent hypoxia has been associated with loss of the tumor suppressor p53, upregulation of free radical scavenging systems, and deregulated expression of antiapoptotic proteins of the Bcl-2 family[6,27].

1.2 Hypoxia and Tumorigenesis

Today, tumorigenesis is seen as a multistep development based on spontaneous mutations which enables cells to grow with tumor specific characteristics. In the year 2000, D. Hanahan defined six characteristic biological capabilities, the “Hallmarks of Cancer”, which can be seen in almost any kind of cancer[7] (Figure 1). They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis.

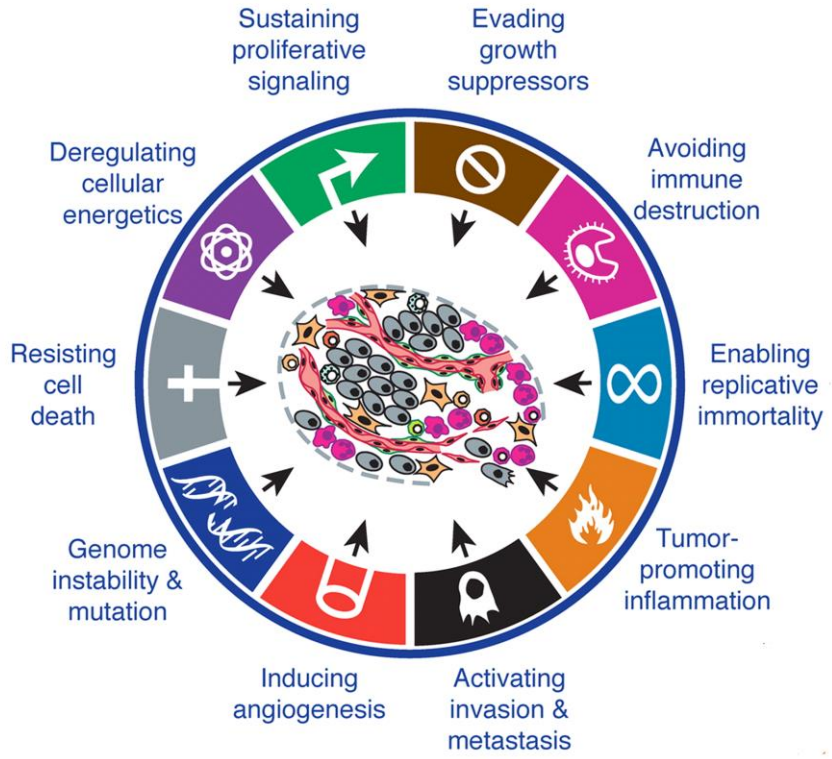

Figure 1 - The Hallmarks of Cancer *Typical characteristics of tumors are shown in this widely accepted model of tumorigenesis; adapted from Hanahan, D. & Weinberg, R. a. Hallmarks of cancer: the next generation. Cell 144, 646–74 (2011), Copyright © 2011 Elsevier Inc*

In 2011, based on further development in cancer research, he added reprogramming of energy metabolism, evading immune destruction, tumor promoting inflammation and genome instability as further characteristics to his widely accepted model[28].

Hypoxia has an influence on several of these hallmarks and tumor hypoxia is associated with poor prognosis as seen in several neoplastic entities like head and neck cancer[29] and soft tissue sarcomas[30,31]. Referring to the underlying hallmarks, hypoxia has been shown to promote resistance to cell death by selection for tumor cells that can evade hypoxia driven apoptosis occurring in the early stage of tumor development. This, for example, includes cells with loss of the tumor suppressor p53[6] or upregulation of the antiapoptotic protein IAP-2[27] followed by a resistance to HIF-1 mediated cell death[26]. On the other hand, HIF-1 is also a driver of proliferation as a second hallmark and is often expressed on a high level in tumor cells[32]. HIF-1 is known to induce the production of growth factors like TGF-β[33,34] or PDGF[35] as seen in human glioblastoma cells. Besides

HIF-1, there are other hypoxia induced pathways that promote proliferation like the PI3K/Akt/GSK-3 pathway[36,37].

According to Hanahan, the induction of angiogenesis is another hallmark of neoplastic disease. As tumors are fast growing tissues, there is a need for sufficient supply with nutrients and oxygen and vascularization as described above. There are several pathways upregulated in a variety of tumors that promote the angiogenetic switch either in a hypoxia dependent or hypoxia independent way[38–40]. One of the most important factors regarding angiogenesis is VEGF, which is secreted in a HIF-dependent manner and is known to be upregulated in different tumors[41]. Even though VEGF has been shown to be an effective target for cancer therapy with antiangiogenetic drugs like bevacizumab[42], VEGF-induced angiogenesis is not a crucial pathway for tumor development[43].

Invasion and metastasis are further steps that are prompted by hypoxia. Hypoxic tumors are more likely to show distant metastasis[3,44]. This process, among other steps, includes epithelial to mesenchymal transition (EMT) characterized by the hypoxia-induced loss of E-cadherin[45,46]. Further steps are an increased tumor cell migration and metastatic homing resulting from the hypoxia-dependent overexpression of lysyl oxidase (LOX)[47] and chemokine receptor CXCR4[48,49].

The deregulated growth in neoplastic disease additionally involves adjustments of energy metabolism. This effect was initially described by Otto Warburg[50,51] who found tumor cells to limit their energy production to glycolysis and lactic acid fermentation even under aerobic conditions. These adjustments are driven on the one hand by the tumor microenvironment that selects for cells capable of handling varying oxygen supply and on the other hand by oncogene activation that cause changes in metabolism[28,52]. The reprogramming of metabolism provides cells with substrates for biosynthetic pathways[53,54] that are more efficient than oxidative phosphorylation regarding the needs for active cell proliferation. Similar adaptions of cell metabolism can be seen during embryogenesis[55]. Additionally, changes in metabolism affect apoptosis pathways[56] and might be involved in local

signaling supporting tumor proliferation[57] or leading to increased angiogenesis[58,59].

1.3 Hypoxia – Reoxygenation Injury

Whereas short or moderate hypoxia is known to result mainly in cell cycle arrest[19,25], severe hypoxia might also induce necrosis or apoptosis via cytochrome C release, caspase 9 activation and the following activation of effector caspases resulting in cell death[23,60]. However, not only oxygen deprivation but also reoxygenation can cause cellular damage. This so called reperfusion injury is a widely seen phenomenon in myocardial infarction or stroke, and also after reperfusion of transplanted organs[61]. Besides sterile inflammation mediated for example by TLR4[62], reactive oxygen species (ROS) significantly contribute to reoxygenation injuries. Oxidative stress may be triggered by Ca^{2+} overload of the mitochondria, concomitant hyperpolarization of the voltage ($\Delta\Psi_m$) across the inner mitochondrial membrane and and may result in superoxide formation[63,64]. Under physiological conditions, these oxygen radicals are eliminated by superoxide dismutase (SOD). ROS generation exceeding detoxification generates excessive hydroxyl radicals, which have a high potential of damaging cellular structures, enzymes or channel proteins on the cellular membrane, finally leading to cell death[65]. To cope with oxidative stress, cells/organs have developed a variety of antioxidant defense mechanisms, including preventative mechanisms, repair mechanisms, physical defenses and antioxidant defenses[66]. These mechanisms are responsible for an effect called preconditioning, which describes the phenomenon that cells/organs that are repetitively exposed to cycles of non-lethal hypoxia/reoxygenation (H/R) are less affected by a subsequent phase of severe hypoxia/reoxygenation. This effect has first been described as “warm-up phenomenon” in angina pectoris[67] and been further evaluated in a variety of models[68]. It involves for example HIF-1 dependent mechanisms[69], adenosine signalling[70] and also ROS induced mechanisms[71,72].

1.4 Irradiation Effects

Irradiation is besides chemotherapy and surgery the third basis of cancer treatment and is included in about 50% of all cancer treatment concepts[73,74]. It is used for example to support chemotherapy, prevent local recurrence or pretreat tumors before surgery. Irradiation works by inducing cell death via a number of different ways. The main mechanisms are direct and indirect induction of DNA strand lesions that lead, if not repaired, to cell death via apoptosis or necrosis[75–77] and generation of reactive oxygen species (ROS) causing DNA and cell damage[66,78]. Long term effects of irradiation include genomic instability and bystander effects[79,80], that could lead to cell death, as well as modulation of the immune system and the tumor microenvironment[81]. Regarding tumor hypoxia, there is a clinically relevant oxygen enhancement effect[82,83] affecting the susceptibility of tumors to irradiation. This is based on a higher rate of ROS induction and a higher rate of interaction with DNA in the presence of oxygen[78,84]. Furthermore, DNA-damage repair mechanisms work more efficient in the absence of oxygen[82]. Additionally, tolerance to chronic intermittent hypoxia has been associated with loss of the tumor suppressor p53, upregulation of free radical scavenging systems, and deregulated expression of antiapoptotic proteins of the Bcl-2 family, promoting a crossresistance to irradiation[6,85,86]. The increase in radical scavenging systems is of particular therapeutical interest, because low cellular ROS-levels have been associated with radiation resistance, e.g. in glioma stem cells[87].

1.5 Reactive Oxygen Species

Reactive oxygen species (ROS) are chemically highly reactive molecules containing oxygen[88,89]. The most important forms are superoxide and besides that hydroxyl and peroxyl radicals, which are inductors of secondary ROS[90]. The majority of ROS are produced during mitochondrial respiration. Complex I (NADH oxidase) and III (cytochrome C reductase) of the mitochondrial electron transport chain may leak single electrons to molecular oxygen. By that electron leakage, 1-2% of total molecular oxygen consumed by normal respiration is converted into the relatively stable superoxide anion (O_2^-). Dismutation of O_2^- by superoxide

dismutase produces H_2O_2. Subsequent interaction of H_2O_2 and O_2^- or Fe^{2+}- or Cu^{2+}-driven cleavage of H_2O_2 can generate the highly reactive hydroxyl radical ($OH^{-}\cdot$)[91,92].

Both the rate and the efficiency of the oxidative phosphorylation are regulated by the substrates of the mitochondrial respiratory chain and by the efficacy and activity of the proton transport complexes I, II, IV and V of the respiratory chain. Additionally, extrinsic uncoupling, i.e. transport processes which lower the proton motive force across the inner mitochondrial membrane but do not belong to the respiratory chain, has a significant influence on oxidative phosphorylation. Activity and efficacy of these proton pumps are mainly fine-tuned by the dependence of the ATP synthase (complex V) on the membrane potential $\Delta\Psi_m$, the ATP/ADP ratio, as well as by reversible phosphorylation of the complexes I and IV[93]. It is suggested that under physiological conditions, i.e. high ATP/ADP ratios, the membrane potential $\Delta\Psi_m$ is kept low through the potential dependence of the ATP synthase and the allosteric inhibition of phosphorylated cytochrome C oxidase (complex IV). The efficacy of the respiratory chain at low $\Delta\Psi_m$ is high. At higher ATP demand or decreasing cellular ATP levels, cytochrome C oxidase is relieved from ATP blockade and $\Delta\Psi_m$ increases. High $\Delta\Psi_m$ values, however, lower the efficacy of cytochrome C oxidase and increase the probability of single electron leakage at complex I and III to molecular oxygen resulting in an increased O^{2-} production[93–95].

Besides mitochondria, there are also other sources of ROS. Certain enzymes produce ROS for physiological uses for example in peroxisomes for degradation[89] of macromolecules. It is further known that under certain metabolic conditions the production of ROS is increased[96]. Hyperglycemia for example can cause oxidative stress by increased ROS formation, which results in vascular damage and an increased risk for cardiovascular disease[97]. Furthermore, as mentioned above, hypoxia/reoxygenation injury is mainly mediated by increased ROS production during the hypoxic period and especially after reoxygenation[12,98,99].

Cancer therapy is another source of increased oxidative stress. As described above, irradiation is a direct and indirect source of ROS causing DNA damage and cell death[78]. Chemotherapeutics like actinomycin D and cisplatin are also known to increase ROS generation leading to additional cell damage besides their primary mode of action[100].

Reactive oxygen species have different physiological and pathophysiological functions in cell signaling and metabolism. Physiologically, they are mainly involved in signaling pathways regarding cell growth and differentiation. This includes for example oxygen homeostasis via the induction of HIF-1, VEGF[101,102] and cell adhesion[103]. Inflammation is another important process ROS are involved in. The so-called oxidative burst is a massive production of ROS by activated neutrophils and macrophages and plays a key role in defence against environmental pathogens. These cells produce large quantities of superoxide radicals and other ROS via the NADPH oxidase[104] and myeloperoxidase[105]. Aside from that ROS, are important for the activation of the immune response via T-cell activation following long term immunity[106].

Furthermore, reactive oxygen species are involved in the process of apoptosis, not only as an inductor but also during the activation phase[107].

Negative effects of reactive oxygen species can be summarized under the term oxidative stress. It results from the secondary oxidation of DNA, proteins and lipids[89,90]. Oxidative stress is known to be involved in aging and numerous diseases like cancer and cardiovascular disease[66] and can be quantified by validated biomarkers[108]. In cardiovascular disease for example, oxidative stress plays a role in hypoxia/reoxygenation injury as mentioned above but also in ROS mediated Ca^{2+}-overload and subsequent cell injury[109]. Compared to their normal counterparts, cancer cells produce higher levels of ROS[110,111] and are known to be adapted to the resulting oxidative stress to a certain degree[112]. The level of oxidative stress determines the consequences regarding tumor promotion, mutagenesis and cell death[66] as presented in the following diagram (Figure 2).

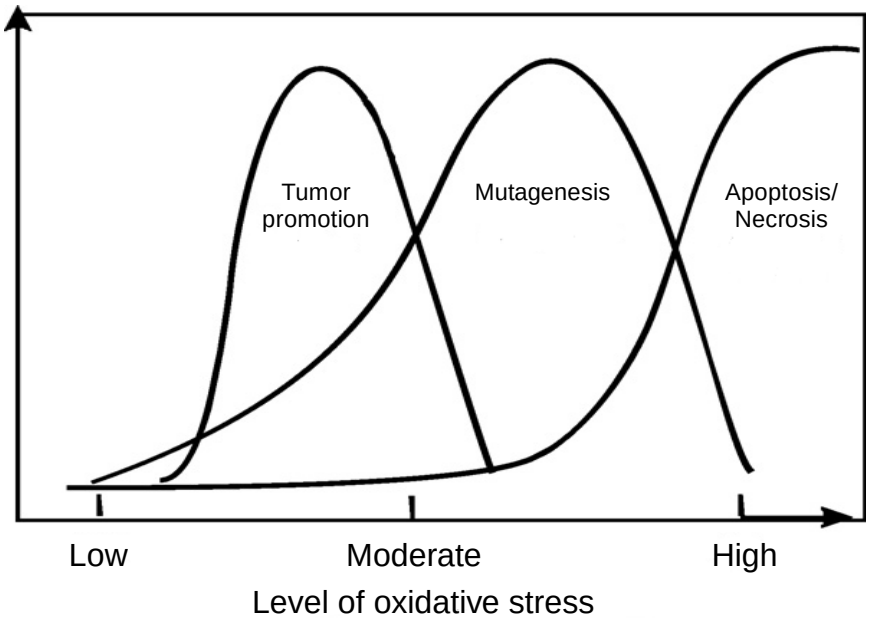

***Figure 2 - Effect of Oxidative Stress on Tumorigenesis**; adapted from Valko et al. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39, 44–84 (2007), Copyright © 2007 Elsevier Inc.*

As shown in the diagram, low or moderate oxidative stress is involved in mutagenesis leading to cancer. This thesis is supported by the fact that agents like cadmium[113], arsenic[114] or tobacco smoke[115] are known to increase free radical formation leading to DNA damage, higher mutation rate and cancer[116].

With regards to cancer progression, there is a certain imbalance in redox homeostasis in cancer cells compared to normal tissue[66] which promotes some of the hallmark characteristics described above. Growth signaling is enhanced by ROS via NFκB-signaling[117] or MAP-kinase[118,119] and angiogenesis can be promoted by ROS dependent stabilization of HIF-1 and VEGF[102]. Invasion and metastasis as another hallmark of cancer is stimulated by free radicals via an increased expression of MMP2[120] and EGFR[121] and resulting epithelial to mesenchymal transition [122].

As in normal cells, levels of ROS exceeding a certain amount lead to cell death in tumor cells. Considering the higher intrinsic oxidative stress, tumor cells have developed a number of adaptive responses to prevent cellular damage. Many tumor cells exhibit increased levels of antioxidants like thioredoxin peroxidase[123] or gluthation that are partially under the control of signaling pathways related to proliferation like c-myc[124]. Another way of evading cell damage are alterations of cell death pathways like ROS induced changes in the PI3K/Akt signaling[125]. Adaptions are promoted by ROS induced genetic instability and clonal selection of more resistant cells supporting a vicious circle leading to tumor cells that are adapted to high levels of oxidative stress as shown in the following scheme (Figure 3).

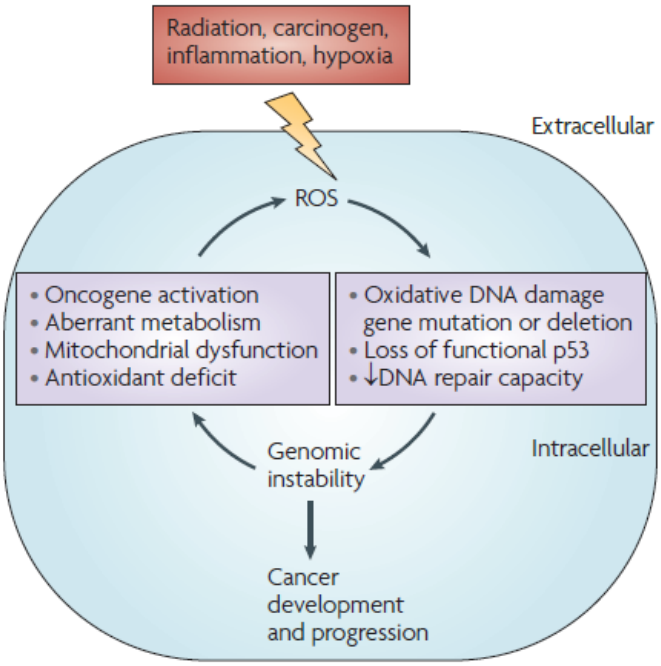

Figure 3 - The Vicious Circle of ROS Stress in Cancer *from Trachootham et al. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nature reviews. Drug discovery. 8, 579-91 (2009), Copyright © 2009 Macmillan Publishers Limited*[112].

Another way of adaption to high levels of oxidative stress could be to establish ways of reducing the formation of free radicals in situations where high amounts of ROS are generated like hypoxia/reoxygenation or irradiation.

Since mitochondrial ROS formation increases with increasing $\Delta\Psi m$ [93–95], lowering of the mitochondrial $\Delta\Psi_m$ is proposed to be a key adaptation event in ischemic

preconditioning[126]. Lowering of $\Delta\Psi_m$ reduces not only mitochondrial O_2^- production but also the mitochondrial Ca^{2+}-overload during reoxygenation[127,128]. The hypoxic preconditioning-associated reduction of $\Delta\Psi_m$ is partially achieved by up-regulation of ATP-sensitive (mitoK_{ATP}) and Ca^{2+}-activated (mitoK_{Ca}) K^+ channels in the inner mitochondrial membrane which short-circuit $\Delta\Psi_m$[127–129].

1.6 Uncoupling Proteins

Further proteins that contribute to depolarization of $\Delta\Psi_m$ are a family of proteins called uncoupling proteins (UCP). This class of proteins was first described when a leak of protons was detected in isolated mitochondria from brown adipose tissue[130]. UCP-1, the first described protein[131] of this family was identified as a 32 kDa proton carrier that was responsible for uncoupling in brown adipose tissue promoting thermogenesis. Following this, more uncoupling proteins were discovered. Up to now, there were five UCPs described in mammals, with UCP 1, 2 and 3 showing high sequence identity and a molecular weight between 31-34 kDa whereas UCP 4 and 5 being quite different[132]. In contrast to UCP-1 which is highly expressed in brown adipose tissue, UCP-2 is expressed in various human tissues, whereas UCP-3 is mainly expressed in skeletal muscle and heart tissue[132,133]. As RNA expression does not necessarily predict protein expression of UCP-2/3, it is crucial that changes in the cellular rate of RNA-expression are confirmed at the protein level if a biochemical or physiological role for either of these proteins is to be claimed[134,135]. Regulation and activation of UCP-2 and -3 are a matter of ongoing debates. Both proteins have been shown to be activated by superoxide with the exact mechanism still unknown. Theoretically, superoxide could either directly activate UCPs or via intermediates like hydroxynonenal[136,137]. Expression control of UCP-2 and -3 is known to be influenced by fatty acids[138] indicating a contribution of UCPs in fatty acid metabolism. High expression of UCP-3 has been demonstrated in skeletal muscle where it suppresses mitochondrial oxidant emission during fatty acid-supported respiration[139] and after anaerobic muscle contraction[140,141]. Accordingly, overexpression of UCP-3 in cultured human muscle cells lowers $\Delta\Psi_m$, raises the ATP/ADP ratio, and

favours fatty acid instead of glucose oxidation[142]. Hypoxia is another known inductor of UCP-3 expression in a p38 MAP kinase/ATF-1 dependent way[143,144].

The function of UCPs has controversially been discussed. Initially, a role in mitochondrial proton leak was questioned since UCP-2/3 knock-out mice in contrast to UCP-1 knockout mice do not show a significant lack in thermogenesis[138,145]. Later on, it has been shown that under physiological conditions UCP-2/3 only cause a depolarization of $\Delta\Psi_m$ of 15 mV described as mild uncoupling[146,147] whereas under unphysiological conditions UCP-3 can also contribute to thermogenesis[148]. A second thesis is that UCP-2/3 are mainly involved in ROS metabolism, which is supported by many studies. It has been shown that a moderate decrease of $\Delta\Psi_m$ can significantly lower ROS production[93–95]. As superoxide activates UCPs, they could be effectors of a feedback loop which restricts overshooting ROS production[136] both in isolated mitochondria and in intact cells[149,150]. This is supported by findings of elevated ROS in UCP-2/3-deficient mice[145,151] and an increase in oxidative damage in UCP-3-deficient mice[152]. Based on the function of reducing oxidative stress, UCP-2/3 have been shown to be involved in a number of processes that are related to ROS production like aging[153] and diabetes[154,155]. As described above, hypoxia/reperfusion injury is mainly caused by increased production of ROS. Besides other factors, UCPs contribute to ischemic tolerance both in stroke[156] and myocardial infarction[157] and are involved in ischemic preconditioning[126,158,159]. Regarding oxidative stress in cancer, UCP-2 has been identified as an antiapoptotic protein preventing cancer cells from ROS-induced cell death after exposure to hypoxia or menadione[160,161]. It has also been shown to reduce the effect of many antineoplastic drugs including doxorubicin, gemcitabine and cisplatin[162–164] and its expression is increased in colon cancers correlating with the degree of neoplastic changes[165]. Concerning the influence of UCP-3 on cell death and cancer, similar studies have not been published yet.

1.7 Aim of the Study

“The present study aimed to define mechanisms of hypoxia/reoxygenation adaptation in vitro by comparing H/R-adapted with highly hypoxia-sensitive parental cells. For H/R adaptation, immortalized primary cultures of mouse proximal convoluted tubule cells (PT) which are highly dependent on oxidative respiration and therefore highly hypoxia-sensitive were subjected to repeated cycles of hypoxia and reoxygenation. That way H/R-adapted PT cultures were then compared with the continuously normoxic-grown parental control cells in terms of H/R- and radiotherapy-induced impairment of mitochondrial function, formation of reactive oxygen species (ROS), cell death and gene expression with a focus on UCP-3-mediated effects.

To estimate whether this in vitro findings might be translated to the in vivo situation, the present study in a second step analyzed UCP-3 expression in PT-derived clear cell renal cell carcinoma.”

2 Material and Methods

2.1 Selection of Partial Hypoxia/Reoxygenation (H/R) - resistant Proximal Convoluted Tubules (PT) Cells.

"PT were microdissected from newborn mice as described elsewhere[166]. PT cells were immortalized by SV40 large T-antigen transformation[167] as accomplished by transfection with SV3 neo, selected in geneticin-containing medium and cultured on collagenated surfaces in equal quantities of DMEM and Ham's F-12 (GIBCO) medium containing 1 mM $NaHCO_3$ (GIBCO), 5% FCS (Biochrom), 2.5 mM glutamine, 2.5 mg/l insulin, 2.5 mg/l transferrin, 15 nM sodiumselenite (ITSS Supplement, Roche Diagnostics), 25 nM dexamethasone (Sigma Aldrich), 5 ng/l epidermal growth factor (Calbiochem) and G418-BC (60U/ml, Biochrom) at 37°C with 5% CO_2. As shown below (Figure 4), four parallel cultures of PT cells were passaged (once per week) for 12 weeks. In this period of time, cells where weekly subjected to hypoxia (0.1% oxygen for 48 h starting 2-3 d after passaging the cells applied by the BD GasPak EZ Pouch System (Becton and Dickinson). For control, further four PT cultures were grown under continuous normoxia, passaged twice weekly for 12 weeks. Thereafter, all cultures were passaged twice to increase cell number, aliquoted and frozen." Three cultures were then selected for further testing[168].

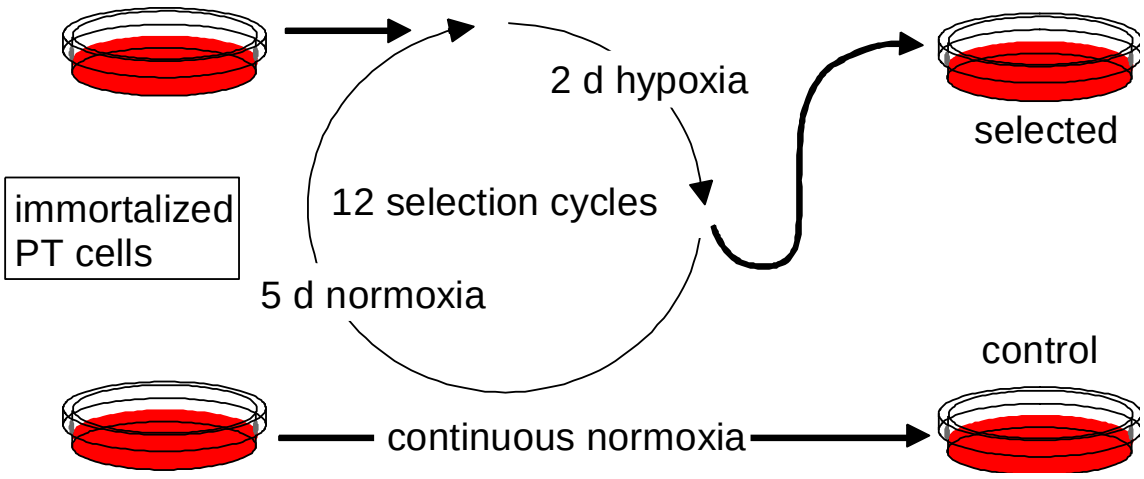

Figure 4 - Selection Protocol for H/R-resistant Cells *Repetitive exposure to hypoxia/reoxygenation (H/R) selected partial H/R-resistent proximal convoluted tubule (PT) cells.* [168]

2.2 Freezing and Thawing

Long-time storage of cells was done at -196°C in liquid nitrogen. For freezing 1 x 10^6 – 3 x 10^6 cells were resuspended in normal growth medium supplemented with 10% FCS and 10% DMSO (Sigma), aliquoted and frozen using a Nalgene® Mr. Frosty at –80°C overnight and then stored in liquid nitrogen.

After thawing cells were washed with medium to remove DMSO and then cultivated in normal growth medium. Cells were passaged at least once before using them for further testing.

2.3 H/R- and Radiation-induced Cell Death

“To test for an acquired hypoxia resistance, subconfluent H/R-adapted and control cultures were grown for 48 h under normoxia or hypoxia (0.1% oxygen) followed by 0.5, 24 or 48 h of reoxygenation. To test for radiation resistance, in further experiments H/R-adapted and control cultures were irradiated with single doses of 0, 5, or 10 Gy photons by a linear accelerator (LINAC SL25 Philips) at a dose rate of 4 Gy/min at room temperature under normoxic conditions. Following irradiation, cells were postincubated in supplemented medium for 24 h or 48 h. H/R-treated or irradiated cells were trypsinated and then permeabilized and stained (30 min at room temperature) with propidiumiodide (PI) solution (containing 0.1% Na-citrate, 0.1% triton X-100, 10 µg/ml PI in phosphate-buffered saline, (PBS)) and the DNA amount was analyzed by flow cytometry (FACS Calibur, Becton Dickinson, Heidelberg, Germany, 488 nm excitation wavelength) in fluorescence channel FL-2 (logarithmic scale, 564-606 nm emission wavelength)[169]. The subG1 population in the PI histogram defined dead cells with degraded DNA. Data was analyzed with the FCS Express 3 software (De Novo Software, Los Angeles, CA, USA).[168]”

Furthermore, adherent cells were stained (10 min, 37°C) with Hoechst 33342 (Calbiochem; 1.5 nM) and PI (Sigma-Aldrich, 0.5 nM) after exposure to hypoxia (48 h) and reoxygenation (24h) and cell death was evaluated by fluorescence microscopy (Axiovert 25, Carl Zeis, Jena, Germany). Pictures of three areas in every culture dish were taken (Canon EOS 500D) and vital and dead cells were

counted. Vital cells are only stained with Hoechst 33342 as the PI is excluded by the intact cell membrane and have an oval-shaped blue nucleus. Apoptotic cells typically contain several fragmented blue or in late apoptosis red bodies. Necrotic cells appear as cells with a red nucleus that is round in shape[170]. Examples are shown in the pictures below.

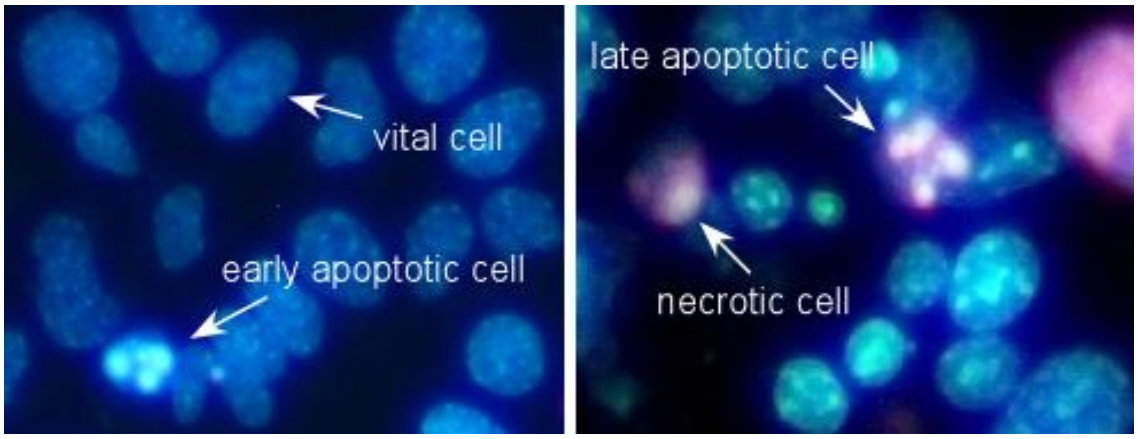

Figure 5 - Hoechst/PI Staining *Representative examples showing vital and dead (apoptotic and necrotic) cells after Hoechst 33342/PI staining (Axiovert 25, Carl Zeiss; 20x; Canon EOS 500D; Canon EOS Utility)*

2.4 Inner Mitochondrial Membrane Potential ($\Delta\Psi_m$).

"To determine $\Delta\Psi_m$, hypoxia (48 h)/reoxygenation (24 h)-subjected as well as normoxic grown H/R-adapted and control PT cultures were trypsinated, washed and incubated for 30 min at room temperature in a NaCl solution (in mM: 125 NaCl, 5 D-glucose, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) titrated with NaOH to pH 7.4) containing the $\Delta\Psi_m$ specific dye tetramethylrhodamine ethyl ester perchlorate (TMRE, 25 nM, Invitrogen). $\Delta\Psi_m$ was analyzed by flow cytometry in FL-2 in the absence or presence of the proton ionophore carbonyl cyanide-3-chlorophenylhydrazone (CCCP, 1 µM) as a control for dissipated $\Delta\Psi_m$. Data was analyzed with the FCS Express 3 software (De Novo Software, Los Angeles, CA, USA).[168]"

2.5 Formation of Reactive Oxygen Species (ROS) and Mitochondrial ROS.

To test for production of ROS, control or H/R-adapted PT cells were pretreated (normoxia or hypoxia (48 h)/ reoxygenation (24 h)), detached, incubated for 30 min at room temperature in NaCl solution (see above) containing 10 µM of the redox-sensitive dye 5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein-diacetate acetyl ester (CM-H_2DCFDA, Invitrogen) and ROS-specific fluorescence was recorded by flow cytometry in Fl-1 (515-545 nm emission wavelength)[171,172]. To test for fluorescence dye loading, control samples were oxidized (1 mM *tert*-butylhydroperoxide) for 15 min and recorded.

“To test for mitochondrial production of superoxide anion as the most common mitochondrial ROS, control or H/R-adapted PT cells were pretreated (normoxia or hypoxia (48 h)/ reoxygenation (24 h)), detached, incubated for 10 min at 37°C in NaCl solution (see above) containing 5 µM of an superoxide anion-sensitive dye (MitoSOX, Invitrogen)[173], and specific fluorescence was recorded by flow cytometry in Fl-2. To test for fluorescence dye loading, control samples were oxidized (1 mM *tert*-butylhydroperoxide) for 30 min and recorded[168].”

2.6 Quantitative RT-PCR

“Messenger RNAs of H/R-adapted and control PT cultures (normoxic and after hypoxia (48 h)/ reoxygenation (24 h)) were isolated (Qiagen RNA extraction kit, Hilden, Germany) and reversely transcribed in cDNA (RT² First Strand Kit, SABiosciences, Qiagen)” according to the manufacturers protocols. Quality control and DNA content was evaluated photometrically using a Nanodrop1000[174] (Thermo Fisher). Fragments of interest were amplified by the use of PCR arrays (RT ProfilerPCR ArrayPAMM-012 (Apoptosis) and PAMM-065 (Oxidative Stress), SABiosciences, Qiagen, Hilden, Germany) and a Roche Light Cycler 480 according to the manufacturer’s instructions and using the following PCR protocoll.”

Programm	Cycles	Temperature (°C)	Time (hh:mm:ss)	Ramp Rate (°C/s)
Start	1	95	00:10:00	4,4
Quantification	40	95 65	00:00:15 00:01:00	4,4 2,2
Melting Curve	1	95 65 95	00:00:10 00:01:00	4,4 2,2 0,11
Cooling	1	40	00:00:10	1,5

"C_t (threshold cycle) values of the PCR amplifications were normalized to those of the housekeeper genes. Mouse UCP-3 and GAPDH specific cDNAs were additionally amplified by QuantiTect Primer Assays (#QT00115339 and QT01658692, respectively, Qiagen)" and the Roche Light Cycler 480 for further evaluation[168].

2.7 Transfection with siRNA

"Adherent PT cells were grown in normal medium and transfected at 60% confluence with a transfection reagent (TransIT-TKO, Mirus Bio, Madison, WI, USA) according to the manufacturer's protocol. UCP-3 siRNA and non-targeting siRNA (ONTARGETplusSMARTpool, ON-TARGET Non-targeting siRNA, ThermoScientificDharmacon, Chicago, IL, USA) were used at a final concentration of 50 nM. Transfection efficiency and viability was determined by transfecting the cells with 400 nM green fluorescence siGLO siRNA (ThermoScientificDharmacon) followed by propidium iodide exclusion dye and flow cytometric analysis. Immediately after transfection, cells were cultured either under normoxia or hypoxia (48 h)." Irradiation after downregulation with siRNA was done 24 h after transfection. "Downregulation of UCP-3 was controlled by immunoblotting." Cell death and ROS formation was evaluated with flow-cytometry as described above after 24 h of reoxygenation or 24 h post irradiation[168].

2.8 Immunoblotting

“Following normoxia or hypoxia (48 h)/ reoxygenation (24 h) H/R-adapted and control PT cultures as well as freshly frozen specimens from dissected human renal cell carcinoma with adjacent normal renal tissue (patients gave informed consent and the experiments were approved by the ethical committee) were lysed in a buffer (containing in mM: 50 HEPES pH 7.5, 150 NaCl, 1 EDTA, 10 sodium pyrophosphate, 10 NaF, 2 Na_3VO_4, 1 phenylmethylsulfonylfluorid (PMSF) additionally containing 1% triton X-100, 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 3 µg/ml pepstatin) and separated by SDS-PAGE under reducing condition. Segregated proteins were electro-transferred onto PVDF membranes (Roth, Karlsruhe, Germany). Blots were blocked in TBS buffer containing 0.05% tween 20 and 5% non-fat dry milk for 1 h at room temperature. The membrane was incubated overnight at 4°C with rabbit anti-UCP antibody 1:1000 (anti UCP-3 #ab3477, anti-UCP-1 #ab57687 or anti UCP-2 #ab67241, Abcam, Cambridge, UK). Equal gel loading was verified by an antibody against β-actin (mouse anti-β-actin antibody, clone AC-74, Sigma #A22281:20,000) or GAPDH (mouse anti GAPDH, #ab8245 clone 6C5, Abcam, 1:20,000)” as well as PageBlue™ Protein Staining Solution (ThermoFisher). “Antibody binding was detected with a horseradish peroxidase-linked goat anti-rabbit or horse anti-mouse IgG antibody (Cell Signaling # 7074 and #7076, New EnglandBiolabs, 1:1000 and 1:2000 dilution in TBS-Tween/5% milk, respectively) incubated for 1 h at room temperature and enhanced chemoluminescence (ECL Western blotting analysis system, GE Healthcare/Amersham-Biosciences, Freiburg, Germany). Where indicated, protein levels were quantified by densitometry using ImageJ software (ImageJ 1.40g NIH, USA). UCP-3 expression of the tumor samples was semi-quantified using an arbitrary score from 0 (not expressed) to 4 (highest expression)[168].”

2.9 Statistics

“Given data are means ± standard error (SE). Differences between experimental groups were assessed by (Welch-corrected) two-tailed student t-test or ANOVA where appropriate. P values of ≤ 0.05 were defined as significant.”

3 Results

3.1 Selection of Partial H/R-resistant Proximal Convoluted Tubule (PT) Cells

“Four parallel cultures of PT cells were passaged (once per week) for 12 weeks. During this period, cells where subjected to weekly cycles of hypoxia (0.1% oxygen for 48 h) and reoxygenation (5 days). Each cycle started 2-3 d after passaging the cells. As a control, further four PT cultures were grown under continuous normoxia and passaged twice weekly for 12 weeks. Thereafter, all cultures were passaged twice to increase the cell number, aliquoted and frozen. To test for an acquired H/R resistance, sub-confluent H/R-adapted and control cultures were grown for 48 h under normoxia or hypoxia (0.1% oxygen) followed by 0.5, 24 or 48 h of reoxygenation. Thereafter, the DNA of the cells was stained with propidium iodide (Nicoletti protocol). H/R induced a G_2/M cell cycle arrest in both control and H/R-adapted PT cells, suggestive of H/R-causing genotoxic stress. In addition, H/R resulted in cell death as defined by the subG_1 population of the propidiumiodide histogram (Figure 6)[168].”

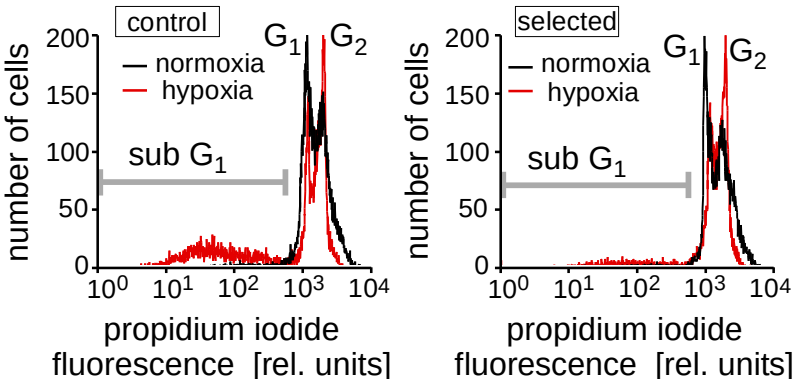

Figure 6 - PI Staining after Hypoxia/Reoxygenation *Histograms showing the propidium iodide fluorescence intensity of permeabilized control and H/R-adapted PT cells. Cells were recorded by flow cytometry either under control conditions (72 h of normoxia, black line) or after 48 h of hypoxia (0.1% oxygen) followed by 24 h of reoxygenation (red line). The marker indicates the dead cells (sub G1 population).*

"Cell death induction was dependent on reoxygenation time (Figure 7). Most importantly, cell death was significantly reduced in the H/R-adapted cells as compared to the control cultures indicating acquisition of a partial H/R resistance during the selection time."

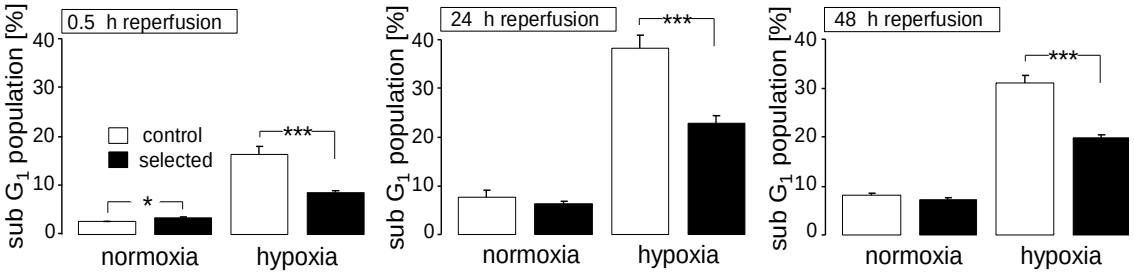

Figure 7 - Influence of Reperfusion Time on Cell Death *Mean percentage (± SE, n = 24 from 4 cultures each measured in hexaduplicates) of dead cells (sub G_1 population) in control (open bars) and H/R-adapted cultures (closed bars) grown under normoxia (left) or under hypoxia (48 h of 0.1% oxygen) followed by 0.5 h (left), 24 h (middle) and 48 h (right) of reoxygenation. * and *** indicate p ≤ 0.05 and p ≤ 0.001, respectively (ANOVA).*

Significant difference in the percentage of cell death after 48 h was confirmed by staining with Hoechst 33342/PI (Figure 8). There was also seen a cycle-dependent increase in the partial H/R resistance during the selection process.

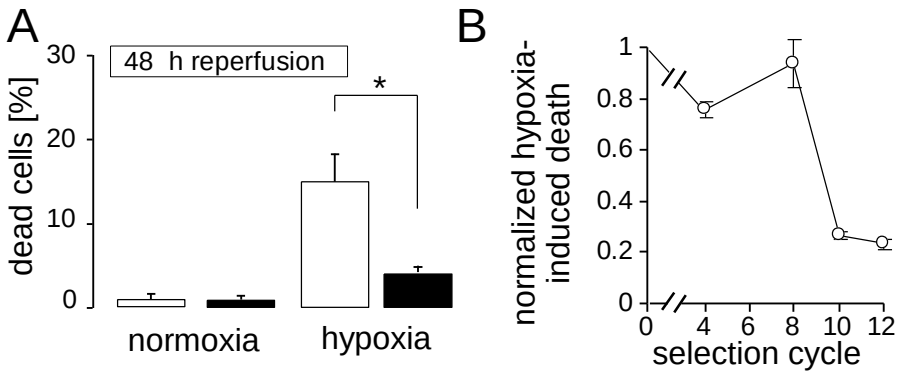

Figure 8 - Hoechst/PI Staining and Time Course of Resistance Acquisition A *Mean percentage (+/- SE, n = 27 from 3 cultures each measured in triplicates) of dead cells (apoptotic or necrotic in Hoechst/PI staining) in control (open bars) and H/R-adapted cultures (closed bars) grown under normoxia (left) or hypoxia (48h of 0.1% oxygen, 48h reoxygenation);* indicates p ≤ 0.05* ***B*** *Time course of resistance acquisition. Shown is the selection-cycle-dependent H/R-induced cell death of the H/R-adapted cultures normalized to that of the particular control cultures (means ± SE, n = 4; * indicates p ≤ 0.05 (ANOVA))).*

3.2 H/R-induced Hyperpolarization of the Inner Mitochondrial Membrane Potential ($\Delta\Psi_m$)

"Reportedly, reoxygenation may be associated with hyperpolarization of $\Delta\Psi_m$.[63] We therefore tested for the effect of hypoxia (48 h)/reoxygenation (24 h) on $\Delta\Psi_m$ in control and H/R-adapted PT-cultures by flow cytometry applying the voltage-sensitive fluorescence dye TMRE. H/R induced break-down of $\Delta\Psi_m$ was seen in a significant cell fraction confirming H/R-induced cell death (Figure 9 A). In the surviving cells, on the other hand, H/R hyperpolarized $\Delta\Psi_m$ (Figure 9, B and D). Markedly, H/R-adapted PT cultures exhibited both significantly less H/R-induced $\Delta\Psi_m$ break-down (Figure 9, C) and significantly less $\Delta\Psi_m$ hyperpolarization in the surviving cell population (Figure 9, D)[168]."

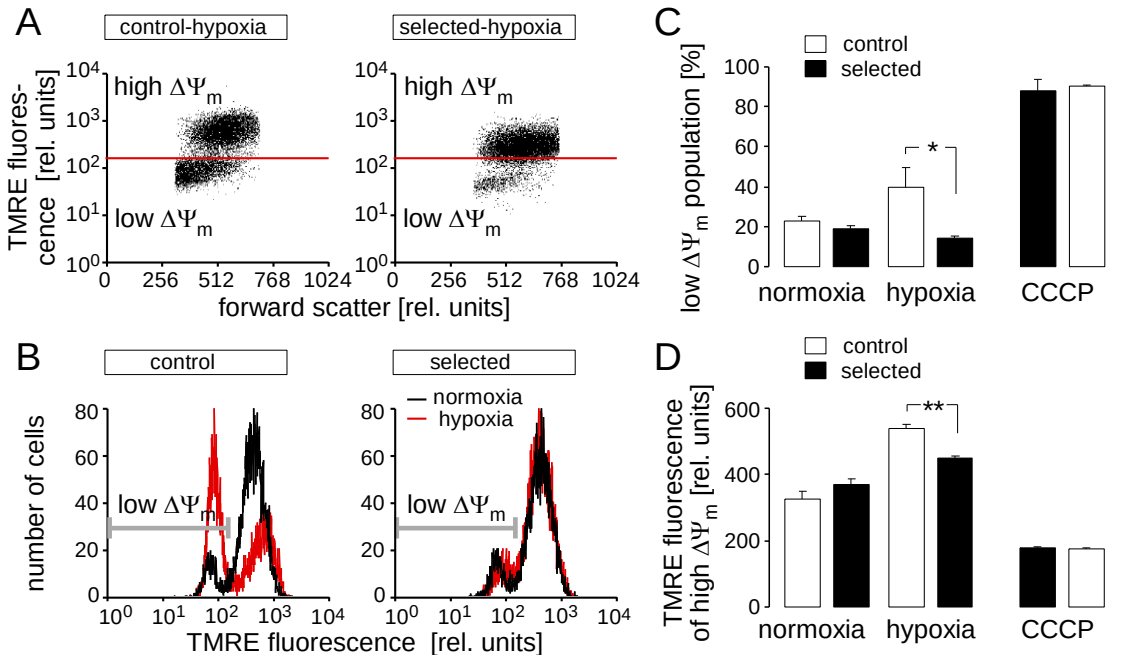

Figure 9 - $\Delta\Psi_m$ Analysis in Flow Cytometry (TMRE staining) *After H/R stress, H/R-adapted cultures exhibit less hyperpolarization of the inner mitochondrial membrane potential ($\Delta\Psi_m$) than control cultures and less break down of $\Delta\Psi_m$.* ***A, B****. Dot plots (A) and histograms (B) showing forward scatter and tetramethylrhodamine-ethyl-ester-perchlorate (TMRE) fluorescence as a measure of cell size and $\Delta\Psi_m$, respectively. Depicted are a control (left) and a H/R-adapted PT culture (right) recorded by flow cytometry under normoxic conditions (black lines in B) and after H/R stress (48 h hypoxia / 24 h reoxygenation); (A and red histograms in B). Cell populations with dissipated $\Delta\Psi_m$ (low $\Delta\Psi_m$) are indicated by gate and marker in A and B, respectively. (n = 9 from 3 cultures, each determined in triplicates)* ***C, D****. Mean percentage of control (open bars) and H/R-adapted cells (closed bars) with broken-down $\Delta\Psi_m$ (C) and mean TMRE fluorescence intensity of the cell population with high $\Delta\Psi_m$ (D) recorded as in (B) under normoxic conditions (left), after H/R stress (48 h hypoxia / 24 h reoxygenation, middle), or after pharmacological break-down of $\Delta\Psi_m$ by the proton ionophore carbonyl cyanide-3-chlorophenylhydrazone (CCCP, 1 µM)(right). (± SE, n = 9 from 3 cultures each determined in triplicate) * and ** indicate p ≤ 0.05 and p ≤ 0.01, respectively (ANOVA)*

3.3 H/R-induced Formation of Reactive Oxygen Species (ROS)

ROS formation is known to increase with hyperpolarization of $\Delta\Psi_m$ and can be induced by hypoxia/reoxygenation as described elsewhere. We therefore analyzed cumulative ROS formation of the control and selected cultures during normoxia and after hypoxia (48 h)/reoxygenation (24h) by flow cytometry using the redox-sensitive fluorescence dye CM-H2DCFDA. As expected and shown below (Figure 10), H/R-induced ROS formation was significantly lower in H/R-adapted than in control PT cultures.

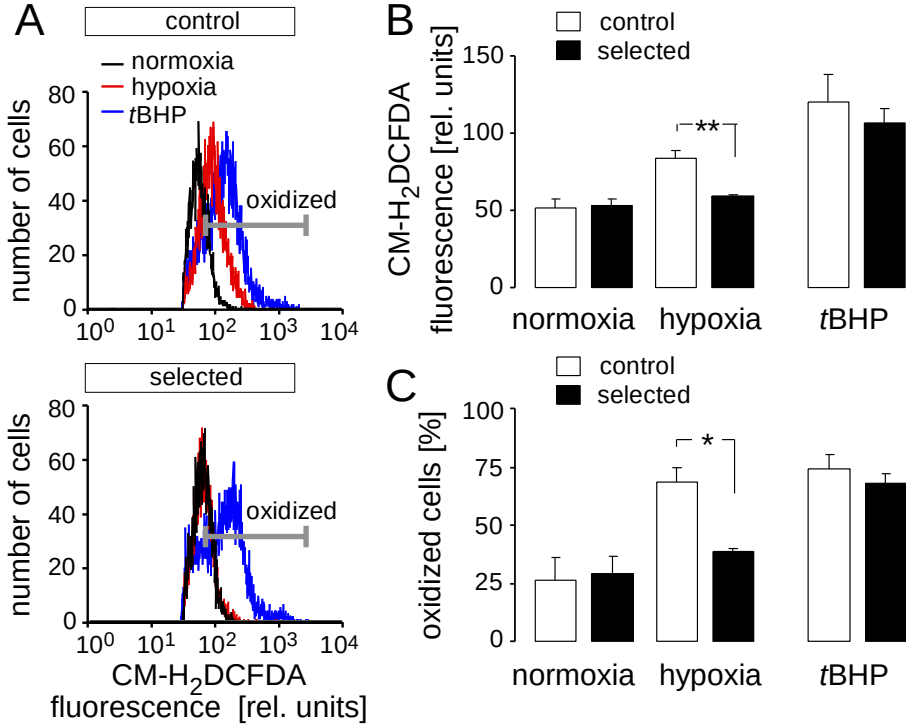

Figure 10 - Total ROS Production after H/R *H/R produces less reactive oxygen species (ROS) in H/R-adapted than in control cultures.* ***A.*** *Histograms showing the 5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA) fluorescence as a measure of ROS production. PT cells were recorded by flow cytometry from a control (top) and a H/R-adapted culture under normoxic conditions (black lines), after H/R stress (48 h hypoxia/24 h reoxygenation, red lines) or after oxidation with tertbutylhydroperoxide (tBHP, 1 mM, blue lines). The marker indicates the oxidized cell population.* ***B, C.*** *Mean CM-H2DCFDA fluorescence (B) and mean percentage (C) of oxidized cells (± SE, n = 6 from 3 cultures each determined in duplicates) recorded as in (A) under normoxic conditions (left), after H/R stress (48 h hypoxia / 24 h reoxygenation, middle), or after oxidation with tBHP(right). * and ** indicate p ≤ 0.05 and p ≤ 0.01, respectively (ANOVA).*

As we were especially interested in mitochondrial ROS formation, “we analyzed mitochondrial superoxide, the most important mitochondrial ROS agent, of the control and selected cultures during normoxia and after hypoxia (48

h)/reoxygenation (24 h) by flow cytometry using the redox-sensitive fluorescence dye MitoSOX. As shown in below (Figure 11), H/R-induced superoxide formation was, like total ROS formation; significantly lower in H/R-adapted than in control PT cultures[168]".

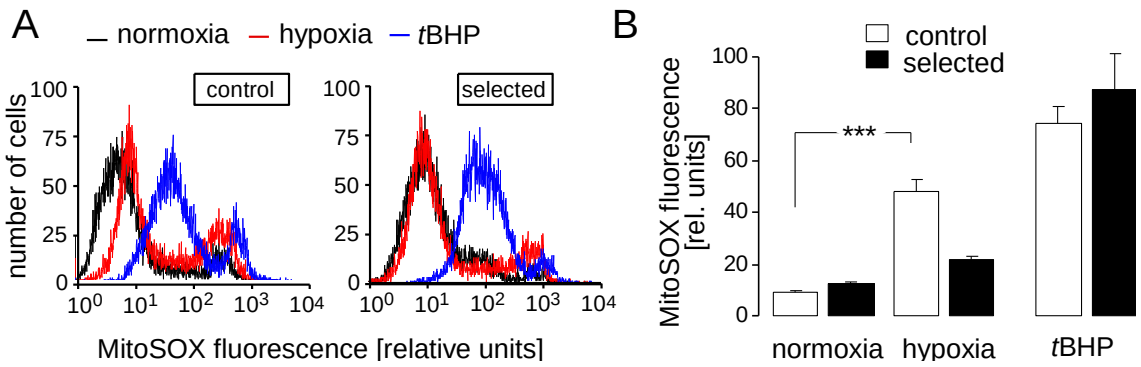

Figure 11 - Mitochondrial ROS Production after H/R *H/R induces less mitochondrial ROS in H/R-adapted than in control cultures.* ***A.*** *Histograms showing the MitoSOX fluorescence as a measure of mitochondrial superoxide production. PT cells were recorded by flow cytometry from a control and a H/R-adapted culture under normoxic conditions (black lines), after H/R stress (48 h hypoxia / 24 h reoxygenation, red lines) or after oxidation with tertbutylhydroperoxide (tBHP, 1 mM; blue lines).* ***B*** *Mean MitoSOX fluorescence intensities recorded as in (A) under normoxic conditions (left), after H/R stress (48 h hypoxia / 24 h reoxygenation, middle, data are means ± SE, n = 10-12 from 4 cultures each determined in duplicate or triplicate), or after oxidation with tBHP (means ± SE, n = 4 from 4 cultures recorded under normoxia) *** indicates p ≤ 0.001 (ANOVA).*

3.4 Gene Expression and Function of Uncoupling Protein-3 (UCP-3)

"To define candidate genes that might confer H/R resistance, mRNA abundances (quantitative RT-PCR microarrays) were compared between control and H/R-adapted PT cultures after normoxic culture conditions and after hypoxia (48h)/ reoxygenation (24 h). Several gene transcripts involved in apoptosis and antioxidative defense differed between the control and H/R-adapted PT cultures. This suggests that long-term H/R adaptation was accompanied by upregulation of oxidative defense, DNA-repair and apoptosis inhibition on the one hand, and by an enhancement of the apoptotic cell death machinery on the other.

H/R-adapted PT cultures constitutively upregulated the anti-apoptotic survivin, the caspase-recruitment domain-containing proteins PYCARD and Bcl-10, the antioxidative-defense associated thioredoxin reductase and superoxide dismutases 2 and 3, the DNA repair enzyme Ataxia telangiectasia and rad3

related (ATR), as well as the actin filament-stabilizing tropomodulin-1. In addition, the H/R adaptation induced the constitutive downregulation of the presumably antiapoptotic cytoglobin.

Acute H/R induced higher mRNA abundances in the H/R-adapted as compared to the control PT cultures of the pro-apoptotic FADD (Fas-associated via death domain) and caspase-6, the stress-inducible Trp53inp1 (tumor protein p53-inducible nuclear protein 1), and the uncoupling protein UCP-3. Glutathione peroxidase 2, in contrast, was more induced during acute H/R in control than in H/R-adapted PT cultures[168].”

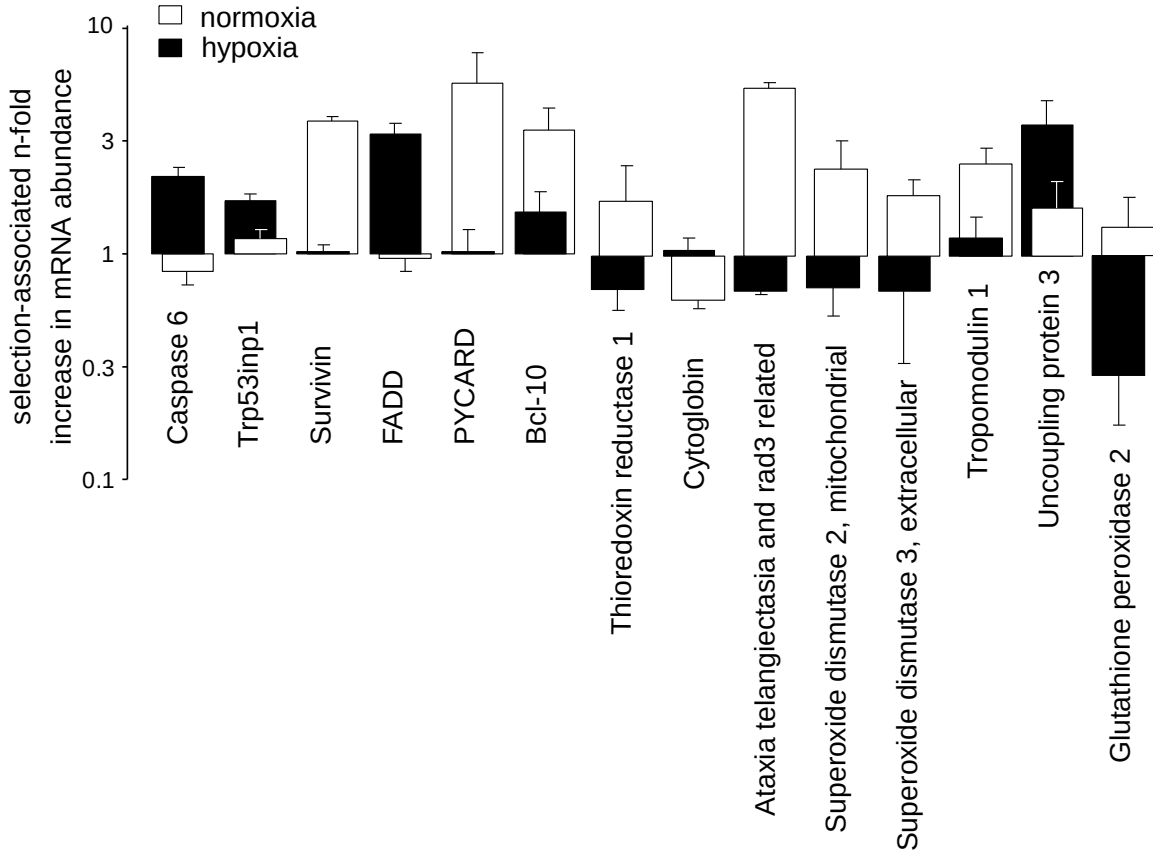

Figure 12 - qPCR Analysis of Gene Expression *H/R adaptation is associated with upregulation of mRNAs involved in oxidative defense, DNA-repair, apoptosis, and mitochondrial uncoupling. Comparison of mRNA abundances (quantitative RT-PCR microarrays) in control and H/R-adapted PT cultures after normoxic culture conditions (open bars) and after hypoxia (48 h)/ reoxygenation (24 h) (closed bars) by quantitative RT-PCR microarrays.*

“In addition, H/R-adapted PT cultures upregulated uncoupling protein-3 (UCP-3) mRNA, in particular, after acute H/R (Figure 13, A).” UCP-3 upregulation on mRNA basis was confirmed by qPCR. “Western blotting experiments confirmed that H/R induced also a significant upregulation of UCP-3 protein expression (~ 4-fold increase) in the H/R-adapted but not in the control cultures (Figure 13, B and C)[168].”

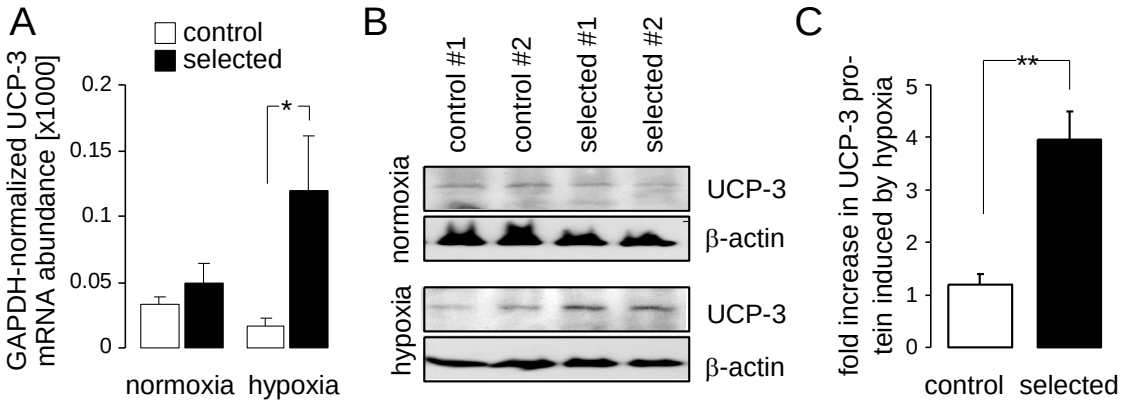

Figure 13 - UCP-3 Expression *H/R induces an up-regulation of the mitochondrial uncoupling protein-3 (UCP-3) in H/R-adapted but not in control PT cultures.* ***A.*** *Mean (± SE, n = 3) GAPDH-normalized UCP-3 mRNA abundance of control (open bars) and H/R-adapted PT cultures (closed bars) under normoxia (left) or after hypoxia (48 h)/ reoxygenation (24 h) as determined by quantitative RT-PCR.* ***B.*** *representative Immunoblot of PAGE-separated proteins from control (1st and 2nd lane) H/R-adapted PT cultures (3rd and 4th lane) probed against UCP-3 and β-actin. Cell lysates were prepared from normoxic (upper blot) and cultures which underwent H/R stress (lower blot).* ***C.*** *Densitometrically semi-quantified increase in UCP-3 protein of control (open bar) and H/R-adapted PT-cultures induced by hypoxia(48 h)/ reoxygenation (24 h; means ± SE, n = 3 cultures each; * indicates p ≤ 0.05, two-tailed Welch-corrected t-test)*

“Since UCP-3 has been demonstrated to reduce mitochondrial ROS formation, to lower ischemia/reperfusion insults and to become upregulated by anaerobic muscle exercise (see discussion), upregulation of UCP-3 might directly contribute to the observed partial H/R resistance. Therefore, we knocked-down UCP-3 in control and H/R-adapted cultures by RNA interference (Figure 14, A) and determined mitochondrial superoxide formation and the subG1 fraction of propidium iodide-stained cells after normoxia or hypoxia (48 h)/ reoxygenation (24 h). A knockdown-mediated decrease by ~ 40% of UCP-3 protein abundance

significantly increased mitochondrial superoxide formation (Figure 14 B, C) in control and H/R-adapted PT cultures[168].”

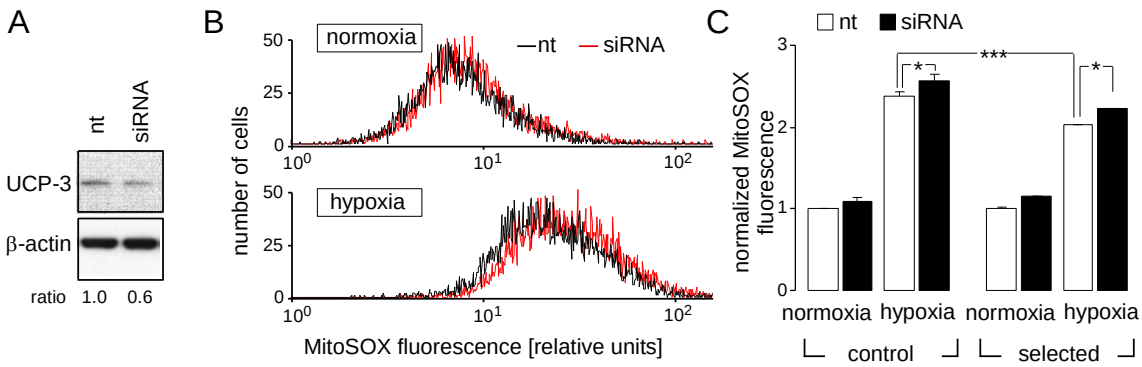

Figure 14 - Influence of UCP-3 Knock-Down on ROS Formation *UCP-3 knock-down increases H/R-induced superoxide production and cell death.* ***A.*** *Immunoblot showing the UCP-3 (upper gel) and - for loading control - the β-actin (lower gel) protein abundance in PT culture transfected with non-targeting (nt) RNA (left lane) or with UCP-3 siRNA (right lane). The ratio indicates the densitometrically semi-quantified β-actin-normalized relative UCP-3 protein abundance.* ***B.*** *Histograms showing the MitoSOX fluorescence of nt- (black lines) and UCP-3 siRNA-transfected (red lines) PT cells after normoxia (top) or H/R stress (48 h hypoxia / 24 h reoxygenation, bottom), recorded as in Fig. 3.* ***C.*** *Mean normalized MitoSOX fluorescence intensities of nt- (open bars) and UCP-3 siRNA-transfected (closed bars) control and H/R-adapted PT cultures PT cells recorded in as in (B) under normoxic conditions or after H/R stress. Data are means ± SE, n = 6 from 2 cultures each determined in triplicate.*

“Moreover UCP-3 knock-down significantly lowered survival after H/R of the H/R-adapted but not of the control PT cultures (Figure 15 A, B). This suggests that UCP-3 upregulation contributes to the H/R adaptation[168].”

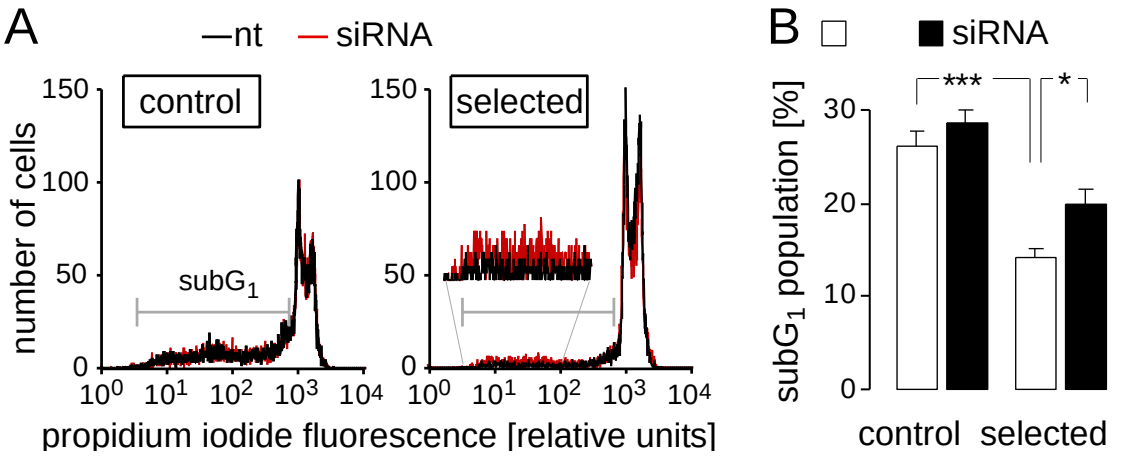

Figure 15 - H/R-induced Cell Death after UCP-3 Knock-Down A. *Histograms showing the propidium iodide fluorescence of permeabilized nt- (black line) and UCP-3 siRNA-transfected (red line) control (left) and H/R-adapted PT cultures (right) after hypoxia (48 h)/ reoxygenation (24 h, recorded as in Fig. 1).* ***B.*** *Mean percentage (± SE, n = 7-9 from 3 cultures each recorded in duplicate or triplicate) of dead cells (sub G1 population) in nt- (open bars) or UCP-3 siRNA-transfected control and H/R-adapted PT cultures after H/R stress (48 h hypoxia / 24 h reoxygenation. * and *** indicate p ≤ 0.05 and p ≤ 0.001, respectively (ANOVA).*

3.5 UCP Expression in Renal Cell Carcinoma

"To test whether UCP-3 might fulfill similar function *in vivo*, UCP-3 protein abundance was determined by immunoblotting in frozen specimens from human renal cell carcinoma (RCC) and normal renal tissue. Twentyseven tumor specimens (18 clear cell RCCs (ccRCCs), 6 papillary RCCs (pRCCs) and 3 mixed clear cell/papipllary RCCs, median histological grading G = 2) as well as 15 non-cancerous renal specimens were selected form 21 patients that underwent (partial) nephrectomy. The patients (11 men, 10 women, mean age = 67 ± 2 years) developed large tumors ranging from 3 cm to 21 cm in diameter (mean diameter = 8 ± 1 cm). These tumors had large necrotic areas pointing to insufficient vascularization (Figure 16)[168]."

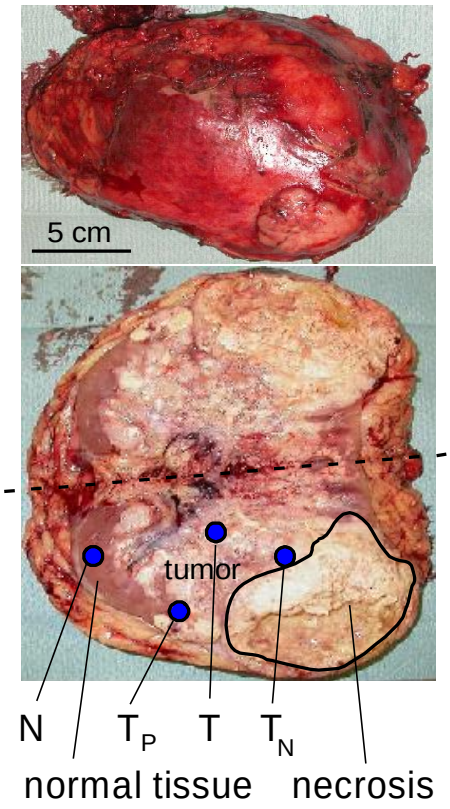

Figure 16 - RCC Specimen *Human nephrectomy specimen top view (upper panel) and opened cut halves (lower panel) with renal cell carcinoma (RCC). Areas of necrosis and residual normal tissue, as well as locations of the sample taking are indicated (N: normal renal tissue, T: tumor without defined origin. T_N: tumor close to necrotic area, T_P: tumor at the periphery).*

"The majority of tumor specimens showed various levels of UCP-3 protein abundance (Figure 17 A and B) that was on average significantly higher than in the non-cancerous renal samples indicating upregulation of UCP-3 during tumor development. Although some tumor specimens close to necrotic tumor areas (i.e., from presumed hypoxic regions, T_N in Figure 17 A) showed extremely high UCP-3 protein abundance while peripheral tumor (T_P in Figure 17 A) exhibited very low expression, the average UCP-3 abundance of T_N specimens was not significantly different from that of the tumor samples of non-defined origin. No difference in UCP-3 protein abundance was found between ccRCS (Figure 17 B, red triangles) and pRCCs (Figure 17 B, blue triangles). In addition, univariate testing of the data suggested that UCP-3 abundance was not associated with patients' age, gender, tumor size, occurrence of lymph node or distant metastasis, or histological tumor grading[168]."

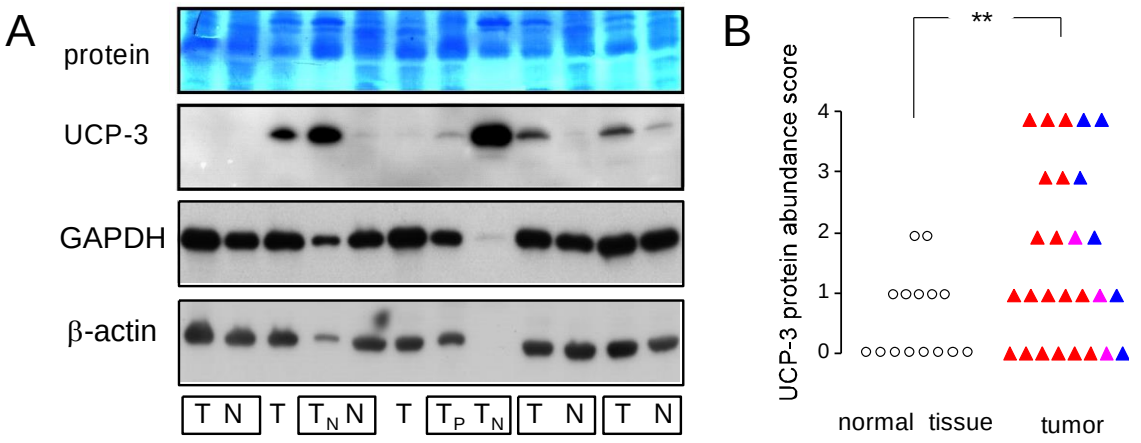

Figure 17 - UCP-3 Expression in RCC and Renal Tissue A. *Immunoblot of renal cell carcinoma and normal renal tissue (as shown in A) probed against UCP-3 (2nd panel), GAPDH (3rd panel) and β-actin (4th panel). The 1st panel depicts the corresponding protein stain (PageBlue). The low or even missing housekeeper bands of the 4th and 8th sample (from left) might be explained by strong overexpression of other proteins by these two tumors. Since electrophoresis loading volume was adjusted to total protein concentration strong overexpression of some proteins dilutes the housekeeper proteins in these samples. Boxes indicate samples which originated from the same kidney.* ***B.*** *Scoring of the UCP-3 protein abundance in non-cancerous renal tissue (open circles) and clear cell RCCs (closed red triangles), papillary RCCs (closed blue triangles) or mixed RCCs (closed violet triangles). ** indicates p ≤ 0.01, Welch-corrected, two-tailed t-test.*

“To test whether RCCs similarly to UCP-3 upregulate other mitochondrial uncoupling proteins, UCP-1 and UCP-2 protein abundance was determined by immunoblotting in the RCC resection material (Figure 18). The results suggested downregulation of UCP-1 by all and upregulation of UCP-2 by some of the tested RCCs[168].”

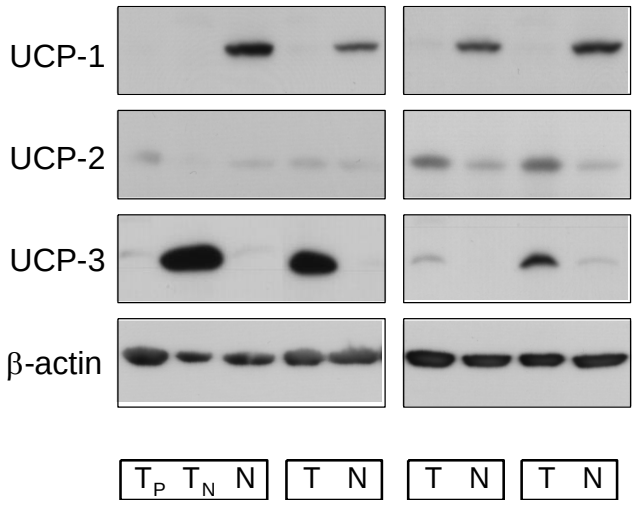

Figure 18 - UCP Expression in RCC and Renal Tissue *Immunoblot of protein lysates from human RCC and adjacent normal tissue probed against UCP-1, UCP-2, UCP-3 and for loading control against β-actin (N: normal renal tissue, T: tumor without defined origin. T_N: tumor close to necrotic area, T_P: tumor at the periphery. Boxes indicate samples which originated from the same kidney).*

“In addition, the ccRCC database of The Cancer Genome Atlas (TCGA) was queried for UCP-1, 2, and 3 mRNA expression of the tumor and survival of the ccRCC patients (Figure 19). The TCGA data suggest co-occurrence of high abundant UCP-2 and UCP-3 mRNA in the ccRCC specimens as well as shorter survival of ccRCC patients with high UCP-2 or UCP-3 mRNA abundance in the tumors as compared to the patients with “middle-rate” UCP expressions (Figure 19). High abundance of UCP-1 mRNA, by contrast, was not associated with altered survival of the ccRCC patients. Since subgroup analysis concerning tumor staging, treatment regimes, etc. could not be performed, the conclusions drawn from the TCGA data is constrained. Nevertheless, the observed associations might point to a prognostic value of the UCP-3 or UCP-2 expression by the ccRCC. In addition, the data might hint to a functional redundancy of UCP-2 and UCP-3[168].”

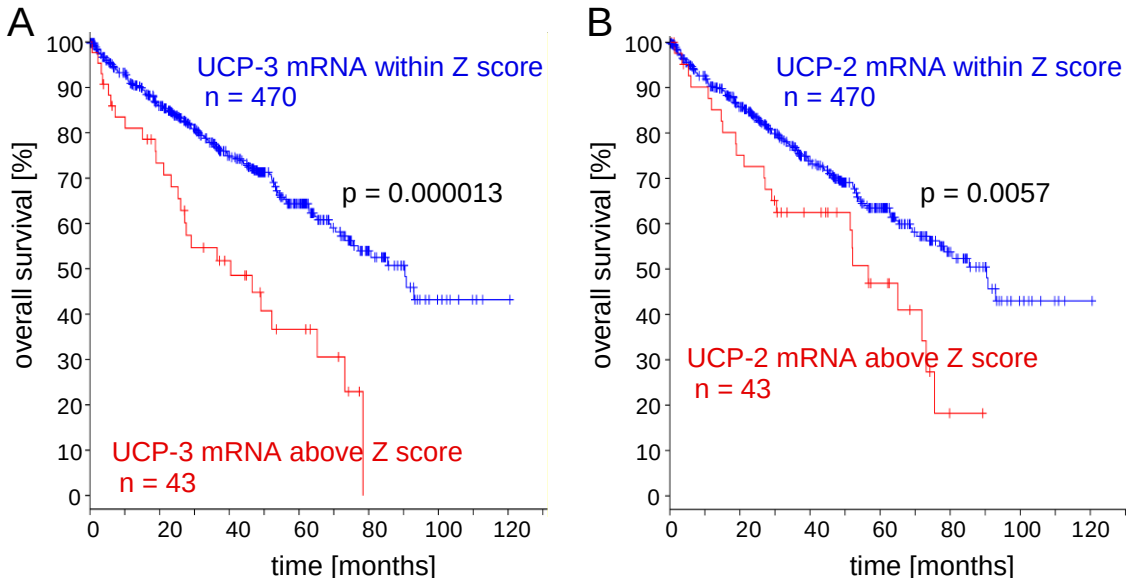

Figure 19 - Correlation Between Survival and UCP Expression *Kaplan Meier plots showing the overall survival of ccRCC patients with high UCP-3 (A, red) or high UCP-2 mRNA-expressing tumors (C, red) in comparison with the respective control cohorts (blue). Patient numbers and p-values (log rank test) are indicated.*

3.6 H/R-adapted Cultures Exhibit Higher Resistance Against Ionizing Radiation

Hypoxia resistance is associated with chemotherapy and irradiation resistance as shown before. “We therefore finally tested the possibility that increased UCP-3 expression might be accompanied by increased resistance against anticancer therapies such as irradiation. To this end, control and H/R-adapted cultures were irradiated under normoxia with a single dose of 0, 5, or 10 Gy, and subG$_1$ population was recorded by flow cytometry (DNA staining of propidium iodide-permeabilized cells applying the Nicoletti protocol) as a measure of cell death 24 h and 48 h thereafter. As shown below (Figure 20), irradiated (10 Gy) H/R-adapted cells died significantly less than irradiated control cells 48 h after irradiation. This suggests that adaptation to H/R leads to partial crossresistance against ionizing radiation possibly due to upregulation of DNA repair and oxidative defense (see Figure 12). The latter might mitigate the radiation-caused cellular lesions[168].”

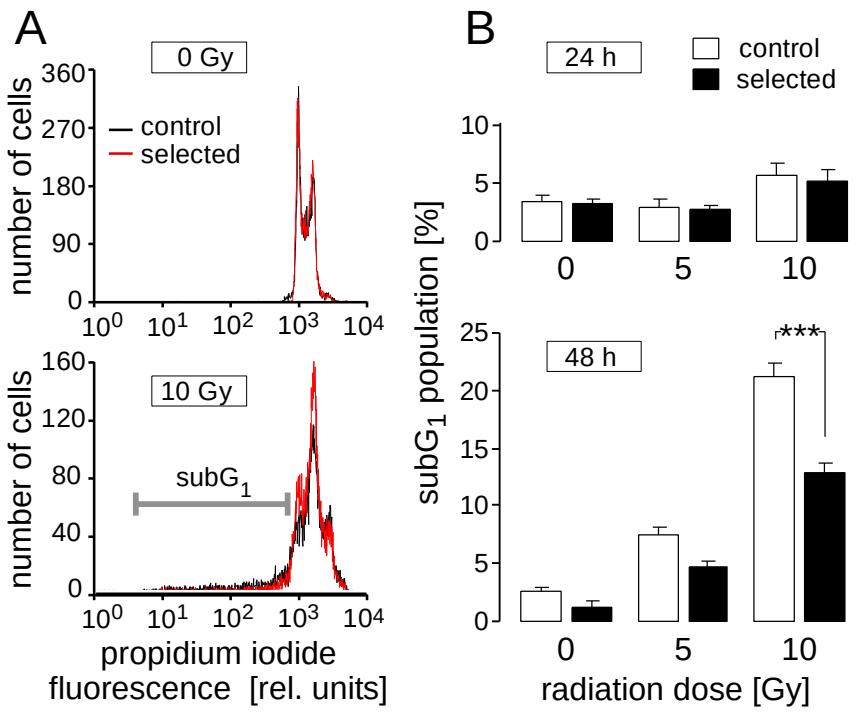

Figure 20 - Cross Resistance of H/R-adapted Cultures Against Ionizing Radiation A. *Histograms showing the propidium iodide fluorescence of permeabilized control (black) and H/R-adapted (red) PT cells. Cells were recorded by flow cytometry 48 h after irradiation (under normoxic conditions) with 0 Gy (top) or 10 Gy (botom). The marker indicates the dead cells (sub G_1 population).* ***B.*** *Percentage of dead cells (sub G_1 population) in control (open bars) and H/R-adapted cultures (closed bars) 24 h (top) and 48 h (bottom) after irradiation (under normoxic conditions) with 0, 5, or 10 Gy. Data are means ± SE, n = 9 from 3 cultures each determined in triplicates. *** indicates $p \leq 0.001$ (ANOVA).*

“To directly test for an involvement of UCP-3 in the acquired radioresitance of the H/R-adapted PT cultures, the effect of UCP-3 knockdown on radiogenic cell death was determined in control and H/R-adapted PT cultures 48 h after irradiation (0 or 10 Gy) and 96 h after transfection with nt or UCP-3 siRNA, subG1 population of propidium iodide-stained cells was determined by flow cytometry (Figure 21 A, B). While having no effect on control cultures, UCP-3 knockdown increased slightly but significantly the fraction of dead cells caused by radiation (Figure 21 B, C) showing an involvement of UCP-3 in radioresistance[168].”

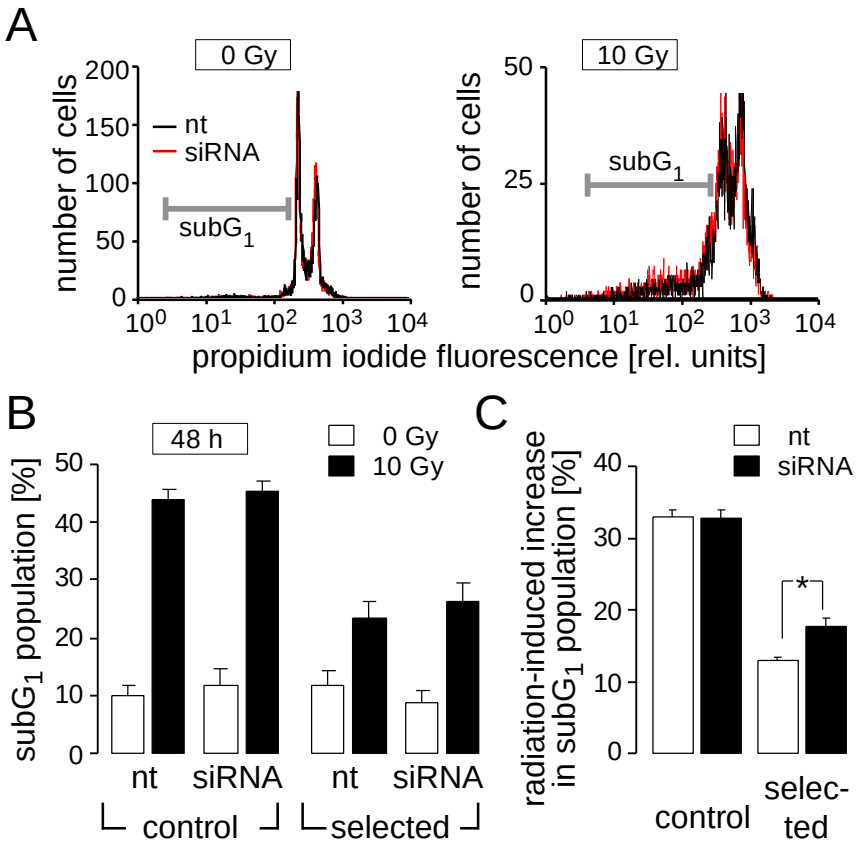

***Figure 21 - Effect of UCP-3 Downregulation on IR-induced Cell Death A**. Propidium iodide histograms of nt RNA- (black) and UCP-3 siRNA (red) transfected H/R-adapted PT cells 48 h after irradiation with 0 Gy (left) or 10 Gy (right). **B.** Sub G_1 population control (left) and H/R-adapted PT cultures (right) 96 h after transfection with nt or UCP-3 siRNA (as indicated) and 48 h after irradiation with 0 Gy (open bars) and 10 Gy (closed bars). **C.** Radiation (10Gy) induced increase in Sub G1 population with nt (open bars) and UCP-3 siRNA. Data are means ± SE, n = 6 from 2 cultures each determined in triplicates. * indicates $p \leq 0.05$ (ANOVA).*

4 Discussion

“UCP-3 uncoupling protein belongs to the mitochondrial anion transporter superfamily and is highly expressed in the mitochondrial inner membrane of brown adipose tissue, skeletal muscle and heart[132,133,175]. In the present study, selected, partially HR-resistant PT cultures differed from continuously normoxic grown control cultures by the expression of genes involved in DNA repair, apoptosis and oxidative defense and by the ability to up-regulate mitochondrial UCP-3 uncoupling protein during H/R stress.” This resulted in decreased oxidative stress and lower cell death in the selected cells. “Knockdown of UCP-3 by RNA interference significantly attenuated the H/R resistance in the selected cultures while having no effect in the control cultures, showing a functional significance of this UCP-3 upregulation for the partial H/R resistance in the selected cultures”

“As described above, an increasingly growing number of reports demonstrate that UCP-3 may lower mitochondrial ROS production by decreasing $\Delta\Psi_m$ as a “mild uncoupler”. This mild uncoupling function of UCP-3 is in particular evident from the observation that UCP-3 knockout results in increased mitochondrial ROS formation and oxidative damage of isolated skeletal muscle mitochondria or permeabilized muscle cells[139,145,146,176]. Importantly, chemical uncoupling in UCP-3 knockout mitochondria partially mimics the function of UCP-3[146]. Moreover, UCP-3 knockout lowers fatty acid- or reactive aldehyde-stimulated increase in proton leak of the inner mitochondrial membrane[136,177]. Accordingly, overexpression of UCP-3 in myotubes decreases both $\Delta\Psi_m$[178–180] and mitochondrial ROS formation[179].”

“”In addition to these in vitro data, animal studies utilizing UCP-3 knockout or UCP-3 overexpressing mice demonstrated that UCP-3 decreases mitochondrial ROS-formation in fasted mice[152,176] and protects from diet-induced obesity[181] and insulin resistance[155], two diseases associated with oxidative stress[66,179]. Moreover, downregulation of UCP-3 in rats with doxorubicin chemotherapy-induced heart failure improves efficiency of cardial ATP synthesis at an expense of increased mitochondrial ROS formation[182]. Finally, UCP-3 knockdown

increased ischemia/reperfusion-induced mitochondrial ROS formation in the heart[159]. Again, chemical mitochondrial uncoupling partially compensated for UCP-3 function in the UCP-3 knock-out heart[159]. Combined, these data provide overwhelming evidence that UCP-3 lowers stress-induced mitochondrial ROS formation by preventing excessive hyperpolarization of $\Delta\Psi_m$."

"However, the UCP-3-generated transports that may short-circuit the inner mitochondrial membrane are still ill defined. It has been suggested that UCP-3 may export pyruvate[183] and fatty acids including lipid radicals[177,184] from the mitochondrial matrix along their electrochemical gradients. Besides lowering $\Delta\Psi_m$ and directly decreasing the concentration of lipid radicals in the matrix, pyruvate and fatty acids transports are thought to ensure an equilibrium between glycolysis and oxidative phosphorylation and to prevent Co-enzyme A shortage in the matrix and consecutive lipid-induced mitochondrial damage, respectively[185,186]. As a matter of fact, UCP-3 upregulation increases the efficiency of fatty acid oxidation in exercising muscle[139]. In accordance with that, in animal models and in human beings, fastening[187,188], high fat diet[189], or direct infusion of fatty acids[188] have been demonstrated to upregulate UCP-3 expression, which is consistent with a specific function of UCP-3 in switching the metabolism from glucose to fatty acid respiration. A recent meta-analysis demonstrates an association between the 55C/T polymorphism in the UCP-3 gene and obesity further suggesting such a UCP-3 function[190]."

"Other triggers of UCP-3 expression include (anaerobic) exercise of skeletal muscle[139,140] and ischemia/reperfusion of the heart[159,191]. Likewise, oxidative stress following inhibition of glutathione reductase has been demonstrated to upregulate UCP-3 expression in rat myocardium[192] where UCP-3 has been shown to be indispensable for ischemic preconditioning[159]. Moreover, reversible glutathionylation during oxidative stress is reportedly required to activate/inhibit UCP-3[193]. Together, these reported data indicate a function of oxidative-stress induced up-regulation of UCP-3 in mitigating ischemic/hypoxia-reperfusion/reoxygenation injury of heart and skeletal muscle."

“In the present study, UCP-3 upregulation was paralleled by an attenuated H/R-mediated $\Delta\Psi_m$ hyperpolarization and mitochondrial superoxide formation of the PT cultures hinting to a UCP-3 function similar to that proposed in ischemic heart cells or anaerobic muscle contraction. UCP-3 protein was also upregulated in the majority of specimens from human renal cell carcinoma (RCC) as compared to the protein abundance of co-resected normal kidney tissue. Since clear cell RCC originates from neoplastic transformation of proximal tubular cells, it might be possible to speculate that UCP-3 in RCC might also confer resistance to H/R. One might further speculate that UCP-3-mediated hypoxia tolerance and survival of the tumor cells in hypoxic areas confers resistance to chemo- and radiation therapy: the wash-in of chemotherapeutics is hampered by the malperfusion and the efficacy of ionizing radiation is lowered by the low oxygen pressure of hypoxic tumors. Furthermore, the TGCA query of the present study hint to the possibility that high UCP-3 expression by the RCC might be associated with a poor prognosis.”

“Similar to the upregulation of UCP-3 by RCC in the present study, other UCPs such as UCP-1, UCP-2, UCP-4 or UCP-5 are reportedly upregulated in a number of aggressive human tumors (leukemia, breast, colorectal, ovarian, bladder, esophagus, testicular, kidney, pancreatic, lung, and prostate cancer) where they are proposed to contribute to the malignant progression[63]. Moreover, UCP-2 expression has been associated with paclitaxel resistance of p53 wildtype lung cancer[162], CPT-11 resistance of colon cancer[164], and gemcitabine resistance of pancreatic adenocarcinoma, non-small cell lung adenocarcinoma, or bladder carcinoma[163]. Accordingly, experimental targeting of UCPs has been demonstrated to sensitize tumor cells to chemotherapy in vitro[63].”

“In the present study, some RCC specimens exhibited elevated UCP-2 protein amounts compared to normal renal tissue. UCP-1 protein, in contrast, was downregulated in the RCCs. UCP-2 might exert a function in RCC similar to that proposed for UCP-3. This might be deduced from the TCGA database query of the present study which suggested that - likewise UCP-3 - high RCC UCP-2 expression might be associated with bad prognosis.”

“In the present study, adaptation to H/R was accompanied by partial radioresistance. Knockdown of UCP-3 however, did only slightly increase radiation-induced cell death of the H/R-adapted PT cultures. Upon UCP-3 knockdown the radiation-induced cell death of the control cultures was still much higher than in the selected cultures suggesting that radioresistance in the H/R-adapted PT cultures was substantially mediated by upregulation of DNA-repair and other oxidative defense mechanisms as seen in the PCR array.”

“Ionizing radiation has been reported to upregulate UCP-2 expression in colon carcinoma cells[194] and in a radiosensitive subclone of B cell lymphoma[195], as well UCP-3 expression in rat retina[196]. Moreover, multi-resistant subclones of leukemia cells reportedly show higher UCP-2 protein expression, lower $\Delta\Psi_m$, lower radiation induced formation of reactive oxygen species and decreased DNA damage as compared to their parental sensitive cells[197]. Combined, these reported data hint to a possible function of UCPs in the development of radioresistance also in other tumor entities.”

“In conclusion, hypoxia/reoxygenation-tolerant PT cultures as well as renal cell carcinoma upregulate UCP-3 uncoupling protein in the inner mitochondrial membrane. UCP-3 upregulation attenuates at least in the in vitro PT model mitochondria-born oxidative stress during hypoxia/reoxygenation, and thus, confers partial hypoxia/reoxygenation resistance. Since tumor hypoxia promotes both, malignancy and therapy resistance, UCP-3 protein expression might be a prognostic and predictive marker in human neoplasms[168].”

5 Summary

“Tumor cells can adapt to a hostile environment with reduced oxygen supply. The present study aimed to identify mechanisms that confer hypoxia resistance. Partially hypoxia/reoxygenation (H/R)-resistant proximal tubular (PT) cells were selected by exposing PT cultures to repetitive cycles of H/R. Thereafter, H/R-induced changes in mRNA and protein expression, inner mitochondrial membrane potential ($\Delta\Psi_m$), formation of superoxide, and cell death were compared between H/R-adapted and control PT cultures. As a result, H/R-adapted PT cells exhibited lower H/R-induced hyperpolarization of $\Delta\Psi_m$ and produced less superoxide than the control cultures. As a consequence, H/R triggered $\Delta\Psi_m$ break-down and DNA degradation in a lower percentage of H/R-adapted than control PT cells. Moreover, H/R induced upregulation of mitochondrial uncoupling protein 3 (UCP-3) in H/R-adapted PT but not in control cultures. In addition, ionizing radiation killed a lower percentage of H/R-adapted compared to control cells suggestive of an H/R-radiation cross-resistance developed by the selection procedure. Knockdown of UCP-3 decreased H/R- and radioresistance of the H/R-adapted cells. Finally, UCP-3 protein abundance of PT-derived clear cell renal cell carcinoma and normal renal tissue was compared in human specimens indicating upregulation of UCP-3 during tumor development. Combined, our data suggest functional significance of UCP-3 for H/R resistance[168].”

6 Zusammenfassung

Tumorzellen können sich an ein hypoxisches Milieu anpassen. Die vorliegende Studie untersucht Mechanismen, die zu einer Resistenz gegenüber Hypoxie beitragen können. Partiell gegenüber Hypoxie/Reoxigenierung (H/R) unempfindliche proximale Tubuluszellen (PT) wurden durch repetitive Zyklen von Hypoxie und Reoxigenierung aus ursprünglichen PT-Kulturen herausselektioniert. Im Anschluss erfolgte eine Untersuchung der H/R induzierten Veränderungen hinsichtlich der mRNA- und Proteinexpression, des mitochondrialen Membranpotentials, der Bildung freier O_2-Radikale und des induzierten Zelltodes verglichen mit den unselektionierten Kontrollkulturen. H/R-angepasste Zellen zeigten eine geringere H/R bedingte Hyperpolarisierung des mitochondrialen Membranpotentials und eine geringere Bildung freier O_2-Radikale. In der Folge verursachte H/R bei den angepassten Zellen in einem geringeren Prozentsatz einen Zusammenbruch des Membranpotentials und der DNA-Degradierung. Zudem wurde durch H/R in den selektionierten Zellen das Uncoupling Protein 3 (UCP-3) im Vergleich zu den Kontrollzellen stärker exprimiert. Des Weiteren hatte Bestrahlung in den selektionierten Zellen eine geringere Wirkung als in den Kontrollzellen, was auf eine Kreuzresistenz durch den Selektionierungsprozess hindeutet. Eine Herunterregulation der UCP-3 Expression führte zu einer Verringerung der Resistenz gegenüber H/R und Bestrahlung. Zuletzt erfolgte ein Vergleich der UCP-3 Expression in aus Tubuluszellen enstehenden klarzelligen Nierenzellkarzinomen und normalem Nierengewebe menschlichen Ursprungs. Zusammenfassend weisen die vorliegenden Daten auf die Bedeutung von UCP-3 für die H/R Resistenz hin.

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8 Declaration

Die experimentellen Arbeiten wurde an der Klinik für Radioonkologie im Labor für Experimentelle Radioonkologie, Hoppe-Seyler-Straße 3, Tübingen unter der Betreuung durch Prof. Dr. Stephan Huber durchgeführt.

Die Konzeption der Studie erfolgte durch Norbert Braun in Zusammenarbeit mit Prof. Dr. Huber und Prof. Dr. M. Bleif (Klinik für Radioonkologie Tübingen, jetzt Klinikum Göppingen)

Die Versuche wurden nach Einarbeitung durch die Mitglieder der Arbeitsgruppe von mir eigenständig durchgeführt. Die immortalisierten Zellen wurden durch Ch. Duranton zur Verfügung gestellt. Die Entnahme der Tumorpräparate sowie die daraus durchführte Analyse der UCP-3 Expression erfolgte durch J. Hennenlotter und J. Bedke, Klinik für Urologie Tübingen. Die Versuche zur Abbildung 21 wurden zusammen mit D. Klumpp durchgeführt.

Die statistische Auswertung erfolgte nach Anleitung durch Prof. Huber durch mich.

Ich versichere, das Manuskript mit Beratung durch Prof. Huber selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Tübingen, den 11.04.2016

9 Publications

Parts of this thesis have been published under the following titles:

Braun, N., Klumpp, D., Hennenlotter, J., Bedke, J., Duranton, C., Bleif, M., & Huber, S. M. (2015). UCP-3 uncoupling protein confers hypoxia resistance to renal epithelial cells and is upregulated in renal cell carcinoma. Scientific Reports, 5, 13450.

Huber, S. M., Butz, L., Stegen, B., Klumpp, D., **Braun, N**., Ruth, P., & Eckert, F. (2013). Ionizing radiation, ion transports, and radioresistance of cancer cells. Frontiers in Physiology, 4, 212.

10 Acknowledgements

“Humor and patience are camels guiding through any desert.”

Arab Proverb

Besides humor and patience, there are several people who contributed in various ways to this work and who I would like to thank.

First of all, special thanks to my supervisor Stephan Huber for giving me the opportunity to write this thesis and giving me an insight into medical research. Thank you for your ideas, the serious discussions and for being there, when I needed professional advice or support.

Additionally, I would like to thank:

- Martin Bleif for your ideas and giving me the chance to get an insight into the wide field of radiation oncology
- Justine Rudner for assisting my first steps of experimental research, for all kinds of technical assistance and patiently answering my questions.
- Heidrun Faltin for helping with all kinds of assistance.
- Dominik Klumpp for support with the final experiments and all the other postgraduates working with me

Furthermore, I would like to thank my family and my friends for their ongoing support and their confidence during my years of studies and afterwards. Cordial thanks to my parents for their love and support. Without you, I would not stand where I am today.

Finally, special thanks to the love of my life Helene. Thank you for being at my side anytime I need you, for encouraging me to proceed, for your patience and inspiration, and for everything words cannot describe