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**CANCER VACCINE STRATEGIES AND STUDIES OF
HUMAN THIOREDOXIN REDUCTASE SPLICE VARIANTS**

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Front Cover: This image displays surface (v3 isoform of TrxR1 in red) and volume (actin in green) rendering of confocal z-stacks obtained from a DFNA25 patient-derived cell, which was serum-starved for 7 days to induce the formation of membrane protrusions.

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ABSTRACT

Cancer involves abnormal, uncontrolled proliferation of cells, and evasion from the immune system. Immune evasion can be caused by defects in one or more of the components of the major histocompatibility complex class I antigen machinery, e.g. the β_2 -microglobulin (β_2m) molecule. Here, it was demonstrated that vaccination of mice with β_2m -deficient dendritic cells, prior to a challenge with a tumorigenic dose of β_2m -free tumor cells of syngeneic origin, protected the vaccinated mice from tumor development. Antitumor immune reactions depend upon the cytokine composition in the tumor microenvironment. An efficient antitumor immune response correlates with local high-level expression of the granulocyte-macrophage colony-stimulating factor (GM-CSF) cytokine. A heat-inducible DNA vector for amplified GM-CSF expression was herein constructed, which showed almost three-fold greater expression levels upon induction by hyperthermia than a prototypic CMV promoter construct, while both constructs had similar basal levels.

Thioredoxin reductase (TrxR) is an essential redox-active selenoenzyme important for human health and disease. For example, TrxR is involved in many steps of tumorigenesis, with TrxR expression often upregulated in tumors. Moreover, TrxR is known to be a target of platinum-based drugs, which are used in anticancer therapy. Data presented here show that in organotypic cochlear cultures, direct exposure of equimolar concentrations of the frequently ototoxic platinum-based drug cisplatin and the rarely ototoxic oxaliplatin both targeted cochlear TrxR and caused hair loss to similar degrees. These findings emphasize the importance of understanding pharmacodynamics (oxaliplatin does not reach the inner ear *in vivo*) and may add to improve therapeutic strategies in order to lower patient toxicity caused by platinum-based drugs.

TrxR1 is the most abundant TrxR isoenzyme, which is predominantly found in the cytosol. The gene encoding TrxR1 – *TXNRD1* – harbors a complex genomic structure, leading to numerous splice variants. Peculiarly, the v3 splice variant encompasses a unique glutaredoxin domain, transcription of which is guided by an alternative promoter, which is located upstream of the TrxR1 core promoter. Expression of v3 was herein found in the developing human cochlea and the Leydig cells of the testis. Transcripts encoding v3 were detected in human heart, liver, spleen, ovary, kidney and pancreas, as well as several cancer cell lines. Several cell stressors (including starvation, hypoxia, etoposide, rapamycin, nocodazole) increased v3 promoter activity and v3 expression, while simultaneously repressing the TrxR1 core promoter activity and expression of the classical form of TrxR1. Translation of v3 was found to be IRES-dependent. In particular, prolonged starvation induced expression of v3, dynamic formation of membrane protrusions – to which endogenous v3 was localized – and an increase in cell motility, all of which correlated in time. Recombinant overexpression of v3 in transfected cell lines induced a similar phenotype, with the dynamic formation of membrane protrusions. The data presented herein indicate that the formation of membrane protrusions and increased cell motility are linked with each other and with the induction of v3 expression.

In conclusion, cell-based and DNA vector-based cancer vaccine strategies were studied with the potential to be used in therapeutic cancer vaccine approaches. The splice variant v3 may possibly be targeted in anticancer therapy to interfere with cancer cell motility and ultimately thus, the formation of metastases.

LIST OF PUBLICATIONS

- I. **Dammeyer P**, Mwakigonja AR, Rethi B, Chiodi F, Wolpert EZ. Vaccination with beta(2)-microglobulin-deficient dendritic cells protects against growth of beta(2)-microglobulin-deficient tumours.
Scand. J. Immunol. **70**, 44-52 (2009).
- II. **Dammeyer P**, Jaramillo MC, Pipes BL, Badowski MS, Tsang TC, Harris DT. Heat-inducible amplifier vector for high-level expression of granulocyte-macrophage colony-stimulating factor.
Int. J. Hyperthermia **22**, 407-419 (2006).
The journal's web site is <http://www.tandf.co.uk> (quoted in accordance with the journal's requirements for article reproduction)
- III. **Dammeyer P**, Damdimopoulos AE, Nordman T, Jiménez A, Miranda-Vizuete A, Arnér ESJ. Induction of cell membrane protrusions by the N-terminal glutaredoxin domain of a rare splice variant of human thioredoxin reductase 1.
J. Biol. Chem. **283**, 2814-2821 (2008).
- IV. **Dammeyer P**, Jacob S, Kostyszyn B, Sundström E, Kirkegaard M, Petek E, Arnér ESJ. A cell stress-induced IRES-dependent expressional switch of human thioredoxin reductase 1 to v3 correlates with modulation of cellular morphology.
Manuscript
- V. **Dammeyer P**, Hellberg V, Wallin I, Laurell G, Shoshan M, Ehrsson H, Arnér ESJ, Kirkegaard M. Both cisplatin and oxaliplatin target thioredoxin reductase in organ of Corti cultures whereas their ototoxicity profiles differ *in vivo*.
Manuscript

The above articles will be referred to by the Roman numerals throughout the dissertation.

The following article is included as **Appendix** in the dissertation:

Dammeyer P, Arnér ESJ. Human Protein Atlas of Redox Systems – What can be learnt?

Submitted Manuscript

Related article not included in this dissertation:

Wolpert EZ, **Dammeyer P**. T cells with potential to target metastatic cells.

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LIST OF ABBREVIATIONS AND ACRONYMS

A549	Human lung carcinoma cell line
AIDS	Acquired immune deficiency syndrome
β_2m	β_2 -microglobulin
B6	C57Bl/6
Cisplatin	<i>Cis</i> -diamminedichloroplatinum(II)
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
EBV	Epstein-Barr virus
GFP	Green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
Grx	Glutaredoxin
GSH	Glutathione
<i>H. pylori</i>	<i>Helicobacter pylori</i>
HC	Heavy chain
HEK293	Human embryonic kidney cell line 293
HeLa	Human cervical carcinoma cell line
HIV	Human immunodeficiency virus
HMGB	High-mobility group box
HSP70B	Heat shock protein 70B
IL-2	Interleukin 2
IFN γ	Interferon γ
IRES	Internal ribosome entry site
MHC	major histocompatibility complex
NADPH	β -nicotinamide adenine dinucleotide phosphate
NK	Natural killer
Oxaliplatin	R,R-1,2-diaminocyclohexane-platinum(II)
s.c.	Subcutaneous(ly)
SecTRAP	Selenium-compromised thioredoxin reductase-derived apoptotic protein
siRNA	Small interfering RNA
TAP	Transporters associated with antigen processing
Tat	Transactivator of transcription
TCR	T-cell receptor

TGF- β	Transforming growth factor β
Trx	Thioredoxin
TrxR	Thioredoxin reductase
TXNRD1_v1	Variant 1 isoform of thioredoxin reductase 1
TXNRD1_v3	Variant 3 isoform of thioredoxin reductase 1
TIL	Tumor-infiltrating lymphocyte
v1	<i>see</i> TXNRD1_v1
v3	<i>see</i> TXNRD1_v3

1 INTRODUCTION

1.1 Cancer Biology

Cancer is a disease of great complexity, where a variety of factors are likely to be involved in both the cause and the progression of the disease, and are likely to influence each other. Generally speaking, cancer is the result of abnormal and uncontrolled proliferation of dedifferentiated cells, and develops as a consequence of an imbalance in homeostasis. Accumulated cellular defects – due to genetic mutations – play a major role in the development of neoplasm. Such mutations can result from normal homeostatic or metabolic processes, and also numerous factors of physical (e.g. radiation), chemical and biological nature, including toxins and infective agents. Eventually these mutations can lead to imbalanced cellular processes, which in turn can further skew the ratio between the occurrence of mutational events and DNA repair. In most cases the causal elements are difficult to directly identify, since causes are usually multiple, and cause and effect are often not closely linked in time. Additionally, a potential genetic predisposition in an individual can influence the cause and progression of this disease.

In the 1970's, it was suggested that tumorigenesis is a multistage process, usually involving 3-7 mutagenic events (1). Some of these mutations can be inherited, thus potentially fewer mutagenic events in the lifetime of the genetically predisposed offspring can already lead to disease. For example, in a familiar form of retinoblastoma only two mutagenic insults were required in a carrier of the inherited predisposing mutation (1). This model was therefore termed the “two-hit” model of carcinogenesis. In the following decade the concept of oncogenes and tumor suppressor genes were proposed, along with the model of multistep carcinogenesis (2,3). The multistep carcinogenesis model explains that in the progressive conversion of normal cells into cancerous cells, a succession of genetic changes leads to acquired capabilities of autonomous and uncontrolled neoplasia, which are the defining characteristics of cancer. At the turn of this century, the seminal ‘hallmarks of cancer’ were proposed as self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion from apoptosis, limitless replicative potentials, sustained angiogenesis, and tissue invasion and metastasis (4). These original six hallmarks are paramount to the onset and progression of cancer, but have been expanded and refined in the last ten years to

include cancer-related inflammation and interaction with the immune system (5,6), interaction with the tumor stroma (7) and the transformation of cellular metabolism in the cancer cells (8,9) can be viewed as additional hallmarks.

Table 1. A timeline of selected milestones in cancer research and therapy from the 1940' to present day. (Adapted from Corbellini G & Preti C, The Evolution of the Biomedical Paradigm in Oncology: Implications for Cancer Therapy, 2008 (10), with personal modifications and additions for the purpose of relevance to this dissertation.)

Year	Milestones in cancer research and therapy
1946 to 1950	Serendipitous discovery of anticancer activity of nitrogen mustard derivates and of synthetic antifolates (MTX). Cancer chemotherapy can be pursued.
1950 to 1960	Development of <i>in vitro</i> and <i>in vivo</i> models. Methodological foundation of cancer chemotherapy. Discovery of new anticancer drugs, mainly by empirically testing natural and artificial products. Vincristine Cyclophosphamide
1960 to 1970	The first successes for treatment of Hodgkin's lymphoma and acute lymphoblastic leukemia, resulted from clinical experiences supported by new ideas on chemotherapy kinetics. Taxanes Cisplatin Adriamycin "Remission induction therapy" or "total therapy" of acute lymphoblastic leukemia
1970 to 1980	"Two-hit" model of tumorigenesis was suggested Adjuvant chemotherapy shows efficacy and endocrine treatment comes of age, but cancer cells hold genetic mechanism to acquire resistance to anticancer drugs. Tamoxifen Resistance of cancer cells to MTX
1980 to 1990	Concepts of oncogenes and tumor suppressor genes were proposed Setting molecular biotechnology to fighting cancer: genes for target proteins are mapped and cloned, monoclonal antibodies and recombinant vaccines are developed (immunology enters the game). Interferon therapy Recombinant hepatitis B vaccines
1990 to 2000	The rise of targeted therapies (azacytidine, trastuzumab, imitinib mesylate, bevacizumab and gefitinib) and the bright hopes of tailored/personalized therapies Colony stimulating factors Interleukin-2 Rituximab Trastuzumab - Herceptin® Imitinib mesylate - Gleevec® Bevacizumab (Avastin®)
2000 to 2010	"Hallmarks of cancer" were proposed HPV vaccines

The broad range of strategies and targets for anticancer therapies that have been developed, or are in development, reflects the diversity of cellular processes that are rendered aberrant and/or utilized by cancer cells for their autonomous and uncontrolled growth. It is beyond the scope of this thesis to provide an encyclopedic overview of this vast and complex field of anticancer strategies. However, a few selected milestones of cancer research and anticancer strategies are presented in **Table 1**.

Hanahan *et al.* proposed that the evolutionary paradigm of the multistep tumorigenesis and cancer progression model required heterogeneity in the cell population undergoing conversion in order for selection to occur (4), thus genetic variation forms the basis that makes mutagenesis an intrinsic feature of cancer. Moreover, heterogeneity can exist between different tumor deposits in the same individual, as observed in prostate cancer (11). Furthermore, this heterogeneity is even more drastically represented in cells of advanced stage cancers, where the cancer cells show a great degree of genetic instability, which often leads to aneuploidy (12).

1.2 Cancer Vaccine Strategies and the Immune System

It is now being recognized that the immune system plays an influential role in the regulation of each stage of cancer development (6). An altered immune status or altered immune responses, particularly a state of chronic inflammation, can create an environment that promotes cancer development (5,6). It has been estimated that approximately 15% of all cancers are due to infections, some of which can cause a state of chronic inflammation (6). For instance, a chronic infection with *Helicobacter pylori* (*H. pylori*) can cause stomach ulcers and an environment of chronic inflammation, which promotes carcinogenesis (13). The correlation of *H. pylori* infection with the development of cancer was awarded the Nobel Prize in Physiology or Medicine in 2005. Another more recent Nobel Prize (2008) honored the discovery that viral infection can actually lead directly to cancer in an immunocompetent host. The most prominent example is infection with the human papillomavirus (HPV), which can lead to cervical cancer (14). These advances have led to the recent development of commercial vaccines that can prevent the development of cancer by interfering with HPV infections.

1.2.1 Immunosurveillance and Immune Evasion

It is widely accepted that an uncompromised immune system, in a process termed immunosurveillance, eliminates aberrant cells before they turn cancerous (15,16). The idea of immunosurveillance is supported by observations in immunocompromised patients. For example, different malignancies can occur in association with viral infections in immunosuppressed patients following transplantation, or in patients with acquired immune deficiency syndrome (AIDS) (17).

In relation to immunosurveillance, immune evasion describes a passive selection process for traits, which are beneficial to the survival of the tumors, by evading detection or elimination by the immune system, or by rendering the immune system anergic (18). Thus, immune evasion is concordant with the evolutionary paradigm of tumor development. However, when considering immune evasion, it should be borne in mind that the tumor evolves from self-tissue and thus, can also benefit from immune tolerance (6,15,16,19). Yet, as a tumor grows, necrotic or apoptotic cell death occurs within the tumor mass and/or in the surrounding tissue, and reactive oxygen species can be generated, pH imbalance can occur in the tumor microenvironment, as well as stress-responsive factors can be upregulated (19). As a consequence, growth of the tumor can result in more cellular processes turning aberrant, which can actually render the tumor cells more immune-activating.

Tumor immune evasion can be mediated both by the tumor microenvironment and the immune system. For instance, regulatory T cells can suppress potential responder T cells in function, therefore inhibiting an antitumor immune response (18,20). The tumor environment itself can exert immunosuppressive mechanisms, which can inhibit an antitumor immune response. For example, many human tumors express the tryptophan-degrading enzyme, indoleamine 2,3-dioxygenase, which impairs function of T cells in the tumor microenvironment by decreasing tryptophan levels (16,20). Another mechanism involves the expression of the B7-H1 surface molecule, which binds to the PD-1 receptor on T cells, thereby eliciting an increased in apoptosis in the interacting T cells (21). B7-H1 has been found to be expressed on human lung, ovary and colon carcinomas as well as melanomas, and normal cells of the macrophage lineage, but absent on most other normal human tissues.

Table 2. Frequencies of aberrations in MHC class I antigen presentation in cancer.

Frequency	Type of aberration	Type of cancer observed	Reference
85-95%	Loss of expression of at least one human leukocyte antigen allele	Breast, prostate, colon and cervical carcinoma	(22)
52%	MHC class I antigen loss	Primary breast carcinomas	(23)
49%	MHC class I antigen loss	Primary cervical carcinoma	(24)
38%	MHC class I antigen loss	Primary non-small cell lung carcinomas	(25)
34%	MHC class I antigen loss	Primary prostate carcinomas	(23)
n/d	Loss of β 2m expression	Patient-derived tumor cells from melanoma	(26-28)
n/d	Loss of β 2m expression	Primary non-small cell lung carcinomas	(25)
n/d	Loss of β 2m expression	Primary cervical carcinoma	(29)

n/d – not determined

On one hand, genetic mutations and epigenetic dysregulation can create new tumor antigens. On the other hand, these events add to the pool of new traits, which can be selected for when providing growth or survival advantages for the tumor cell. In terms of antigen recognition, these genetic and epigenetic events can directly affect a gene encoding the antigenic peptide and thereby cause antigen loss, which the effector T cells previously targeted. However, even when the tumor cells produce the relevant antigens, downregulated expression of major histocompatibility complex (MHC) class I antigen presenting molecules on the cell surface hinders the T-cell receptor (TCR) of cytotoxic T lymphocytes (CTLs) to recognize target cells (30-32). Thus, a more comprehensive type of antigen loss can be achieved by impaired function or expression of components of the MHC class I antigen presentation, including MHC class I heavy chains (HCs), the β_2 -microglobulin (β_2m) light chain, the transporters associated with antigen processing (TAP), ER-resident chaperones such as tapasin, and the proteasome among others (22,24,25,33-35). **Table 2** gives an overview of the frequencies with which MHC class I antigen aberrations have been observed in different types of cancer.

1.2.2 Tumor Infiltration with Immune Cells

The infiltration of a tumor with immune cells of the innate arm of the immune system – except natural killer (NK) cells – is rather associated with a worsened disease

prognosis, whereas the infiltration with cells of the adaptive arm of the immune system and NK cells correlates with an improved prognosis (6). T cells infiltrating the inside of tumor cell areas has been associated with a favorable prognosis of disease in patients with primary melanoma, prostatic adenocarcinomas, and colorectal cancer (36-38). In contrast, tumor-specific T cells infiltrating only the margin of the tumor or the tumor stroma did not show this correlation, as observed in patients with colorectal cancer or esophageal carcinomas (38,39). T cells that are located in a tumor microenvironment are generally referred to as tumor-infiltrating lymphocytes (TILs). This T-cell population is usually heterogeneous in phenotype, antigen specificity, avidities and functional characteristics (18).

1.2.3 T Cells and Cancer

CD8⁺ T cells are potent effector cells in antitumor immune responses (18,20). Autologous T cells are able to respond to antigens expressed on human tumor cells. Antigens that are displayed on the cell surface by MHC class I molecules allow screening by the TCR of CD8⁺ T cells (40). Therefore, CD8⁺ T cells are able to scan the vast pool of self-peptides for foreign, altered or pathogenic peptides. Cell-surface MHC class I molecules are trimeric complexes of a highly polymorphic membrane-anchored glycoprotein, termed the MHC class I HC, a small soluble protein called β_2m , and the antigen, a short peptide of 8-10 amino acids (34,40). Two homologous polymorphic domains of the MHC class I HC form the peptide-binding groove. Some antigens displayed by MHC class I molecules are uniquely expressed on tumors, while others can also be found on normal tissues. Mutational events, or epigenetic dysregulation of an otherwise silent gene, can lead to the expression of tumor-specific antigens. Cancer-testis antigens, which are often expressed in human melanoma, mutated shared antigens, and differentiation antigens represent other forms of partly “tumor-specific” antigens, i.e. antigens that are expressed by other specific normal tissue, to a limited extend or in a closely related form.

Despite the expected natural immune tolerance, differentiation antigens – derived from proteins that are part of the differentiation pathway in normal melanocytes – are recognized by T cells in melanoma patients (20,41). In most melanoma patients, the frequencies of CD8⁺ T cells that recognize a specific peptide of the Melan-A/Mart-1 differentiation antigen can reach numbers of 10^{-4} to 10^{-3} in blood, and even above 10^{-3} among CD8⁺ T cells infiltrating cutaneous metastases or lymph nodes (20). Despite the several fold increase in frequency of antitumor CTLs, and the capability of the

antitumor CTLs to infiltrate tumor tissue, most melanoma patients show disease progression. Thus, the tumor cells “interfere” in the immune attack, or the antitumor immune response becomes impaired, both of which describe consequences of immune evasion. In cancer patients where tumors have grown to a clinically detectable size, spontaneous tumor regression, mediated by the immune system, becomes rare (15,19).

Expansion of human T lymphocytes can be achieved *ex vivo* by using single cell suspensions of human TILs together with an undefined number of tumor cells extracted from freshly resected melanomas, in a short-term culture in the presence of IL-2 (42). Objective tumor responses were observed in a higher proportion of patients upon adoptive transfer of these lymphocytes in combination with application of high-dose IL-2 to the patients, compared with IL-2 treatment alone (42). T lymphocytes are capable of inducing regression of metastatic growth, as shown by the infusion of activated tumor-specific T cells in patients suffering from progressed metastatic disease (43). Advanced metastatic melanoma is usually refractory to standard therapies, including aggressive chemotherapy and treatment with high-dose IL-2. In patients suffering from this disease, adoptive transfer of highly selected tumor-reactive T cells achieved persistent clonal repopulation of functional active T cells, which were proliferating and migrating to the tumor sites (18,43).

1.2.4 Cytokines

Antitumor immune responses appear to be critically influenced by the composition of cytokines in the tumor microenvironment (44). As a mechanism of immune evasion some tumor cells secrete cytokines such as TGF- β and IL-10, which can add to the inhibition of antitumor immune responses (16,20). Conversely, in experimental models mice often quickly reject injected or xenografted tumor cells, which have been genetically engineered to secrete cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) or IL-2 (45). In addition, these mice can develop immune memory to protect against subsequent tumor challenges, even if the tumors are poorly immunogenic. In a study comparing the potency of vaccination with tumor cells transduced to express different cytokines, tumor cells expressing GM-CSF resulted in the most potent systemic immune protection against challenge with wild-type tumor cells (46). The cytokine GM-CSF is a growth factor for proliferation, differentiation, and maturation of myeloid cells (41,47). Vaccination of animals with tumor cells, transduced to secrete GM-CSF, induced complete rejection of tumors, or a significant decrease or inhibition of tumor growth, compared with control animals (48-50). In

some cases this vaccination strategy stimulated additional antitumor immunity that protected against subsequent challenges of tumor outgrowth in mouse models for head and neck squamous cell carcinoma (51) and for metastatic melanoma (52).

1.3 The Anticancer Drug Target Thioredoxin Reductase

1.3.1 Platinum-Based Drugs Commonly Used in Chemotherapy

The platinum-based compound cisplatin, and its next-generation analogues carboplatin and oxaliplatin, are commonly used as chemotherapeutic agents (53,54). Cisplatin is regularly used in the clinic to treat head and neck, and germ cell cancer, such as the vast majority of testicular cancer. Carboplatin is used in the treatment of most ovarian cancer and non-small cell lung carcinoma. Oxaliplatin is used to treat colorectal cancer, and shows efficacy in several other types of cancer. Each of these platinum compounds is associated with dose-limiting toxicity, or inherent or acquired resistance. However, the clinical toxicity profiles of these agents differ. Ototoxicity, nephrotoxicity, peripheral neuropathy and myelosuppression are the most common adverse effects associated with cisplatin in clinical use (53). In contrast, oxaliplatin is commonly associated with neurotoxicity, but rarely with ototoxicity.

1.3.2 Cytotoxicity of Cisplatin

Cytotoxicity of platinum-based drugs occurs primarily through the formation of adducts on DNA (53,55). There are several types of DNA lesions that can form on purine bases (guanine and adenine), the majority of these being 1,2-d(GpG) crosslinks (56). Although they occur at similar sites, DNA adducts of cisplatin and oxaliplatin differ both in the DNA contortion and structure. As a consequence, these distinct structures are identified differently by the various proteins involved in DNA damage recognition or mismatch repair, which may give rise to the differences in the spectrum of anticancer activities and cytotoxicities exhibited by cisplatin and oxaliplatin (57). Moreover, cisplatin and oxaliplatin differ in their pharmacokinetic and pharmacodynamic profiles, which have been suggested to be responsible for the different frequencies of ototoxicity; cisplatin is frequently associated with dose-limiting ototoxicity, yet oxaliplatin rarely is because it does not reach the cochlea (58).

The proteins that recognize the adducts of platinating compound and DNA include the family of high-mobility group box (HMGB) proteins, among others (57,59). The extraordinary sensitivity to cisplatin in testis, which expresses several HMGB proteins,

emphasizes the important role of HMGB proteins in cisplatin-induced cytotoxicity (53). Chemotherapy using cisplatin in combination with other drugs leads to an exceptional overall cure rate of over 90% in testicular cancer and of almost 100% in the early stages of this disease (54). It has been shown that exposure to estrogen increases the expression of HMGB1 in breast tumor cells, which renders them as more sensitive to cisplatin treatment (60). A complex of DNA, platinum compound and binding proteins, such as the HMGB proteins, has been shown to interfere with DNA transcription, replication and repair (59). This interference may lead to signaling of DNA damage, and ultimately to the initiation of apoptosis (61). Interestingly, the affinity of HMGB proteins to DNA-oxaliplatin adducts is lower than to DNA-cisplatin adducts (59).

In addition to the primary cytotoxic mechanism via DNA damage, cisplatin can also act on a number of secondary cellular targets. One such target is the endoplasmic reticulum stress pathway, which is based on defective folding or transport of proteins, the activation of which can lead to activation of caspases (62). Other important secondary mechanisms include the targeting of endogenous cellular antioxidant molecules such as glutathione (GSH), glutaredoxins and thioredoxin reductase (TrxR) (63,64).

1.3.3 Thioredoxin Reductase – Anticancer Drug Target of Cisplatin

Thioredoxin reductase (TrxR) is involved in several steps during tumorigenesis, and is often found to be overexpressed in tumors and cancer cell lines (65,66). TrxR is a known target of platinum-based and alkylating anticancer agents (67). Cisplatin can elicit nucleus-independent cytotoxicity, which has been shown in enucleated cytoplasts (68,62). Both cisplatin and oxaliplatin efficiently inhibit TrxR (63,64). Cisplatin can either inhibit TrxR activity directly, or as an adduct with GSH (63). When derivatized by cisplatin, TrxR gains pro-oxidant activity in the form of so-called selenium-compromised thioredoxin reductase-derived apoptotic proteins (SecTRAPs), which can provoke cell death (69). However, the inhibition of normal TrxR enzyme activity may itself cause cytotoxicity (66,67).

1.3.4 Biochemical Roles of Thioredoxin Reductase

TrxR is a selenoprotein that contains selenium in the form of the naturally occurring selenocysteine as the penultimate amino acid residue in the carboxyterminal active site of the enzyme (70). The selenolate moiety in reduced TrxR is highly reactive and thus, very susceptible to targeting by electrophilic compounds, such as cisplatin.

TrxR is a key component of the thioredoxin system, which as a whole is involved in cell proliferation and differentiation, antioxidant defense, redox regulation of protein function, and maintenance of deoxyribonucleotide synthesis (71,65,66,72-75). TrxR isoenzymes have broad substrate specificity, but are principally responsible for maintaining the enzymatically active state of the thioredoxin system by catalyzing the reduction of its prime substrate thioredoxin (Trx) using NADPH as the electron donor. Trx in turn exerts most of the physiological functions of the Trx system. Together, TrxR isoenzymes, Trx isoenzymes and NADPH compose the thioredoxin system. TrxR1 and TrxR2, as well as Trx and Trx2, are essential since the knockout of any of these in mice is embryonically lethal (76-79).

1.3.5 Genomic Organization of Thioredoxin Reductase

Three genes encode the TrxR isoenzymes in human (65,71,72). The *TXNRD1* gene is located on chromosome 12 (12q23-q24.1) and encodes the classical and most abundant TrxR1 enzyme, which occurs predominantly in the cytosol (72,74,80,81). *TXNRD1* is organized in a complex genomic structure and gives rise to numerous transcripts, which are subject to extensive splicing, particularly at the 5' end (80-84). As a consequence, this results in several protein isoforms.

The peculiar *TXNRD1_v3* isoform – hereafter simply denoted as “v3” – contains an additional aminoterminal glutaredoxin (Grx) domain, which is unique to v3 and encoded by three 5' exons (termed β_{VIII} , β_{VI} and β_{V}), which are located upstream of the TrxR1 core promoter. Thus, transcription of v3 starts upstream of the well-characterized TrxR1 core promoter (80,83-86), and must be guided by an alternative, as yet uncharacterized, promoter. Transcription of v3 potentially continues passed the core promoter to share coding sequences with the classical TrxR1 module. The Grx domain of v3 has an atypical dithiol active site, which does not exhibit classical activity in any of the classical Grx assays (65,82). Interestingly, v3 is expressed in human, chimpanzee and dog, but not in mouse or rat (82).

There are two additional TrxR isoenzymes, which are encoded on different chromosomes. The TrxR2 isoenzyme is encoded by the *TXNRD2* gene on chromosome 22, which localizes mainly to the mitochondria where it reduces Trx2 (87-89). The TGR isoenzyme is encoded by the *TXNRD3* gene on chromosome 3, and is mainly expressed in testis. TGR contains an additional monothiol glutaredoxin domain, which is aminoterminally fused to the TrxR module (90).

1.3.6 *TXNRD1* is Part of the DFNA25 Locus

Intriguingly, the *TXNRD1* gene is part of a genetic locus, termed DFNA25, which has been associated with a hereditary autosomal dominant form of high-frequency sensorineural hearing loss (91). The DFNA25 locus was mapped to 12q21-24 by linkage analysis (91). A similar DFNA25-related hearing loss has been observed in a patient with a *de novo* deletion in the paternal allele of the DFNA25 locus, which was then confined to 12q22-q24.1 (92). As a consequence of the deletion, the DFNA25 patient is monoallelic for that particular locus, including the *TXNRD1* gene, among several other genes.

2 AIMS

The overall aim of this dissertation was to study cancer vaccine strategies, *in vitro* by constructing and characterizing a DNA vector, as well as *in vivo* using cell-based vaccination. Another approach in cancer studies was to characterize the splice variants of human thioredoxin reductase 1, particularly the regulation of expression, expression pattern and function.

The specific aims were:

Cancer Vaccine Strategies

- Vaccination of mice with matured β_2m -deficient dendritic cells (DCs) to protect against subsequent challenges of growth of tumor cells, showing an immune evasive loss of MHC class I antigen phenotype (loss of β_2m).
- Construction and *in vitro* characterization of a two-promoter two-gene DNA vector for amplified expression of the cytokine GM-CSF, following induction by heat.

Studies of Human Thioredoxin Reductase Splice Variants

- Cloning and characterization of the alternative promoter, which guides v3 expression and is located upstream of the TrxR1 core promoter of the *TXNRD1* gene.
- Investigation of regulation of expression, expression pattern and function of the v3 splice variant in relation to classical variants of TrxR1.
- Analysis of v3 splice variant expression and its genetic integrity in cells and DNA derived from a patient, who suffers from a DFNA25-related hearing loss, carrying a paternal deletion in the gene encoding TrxR1 among other genes in the DFNA25 locus.
- Study of the direct effects of two chemotherapeutic platinum compounds – the frequently ototoxic cisplatin and the rarely ototoxic oxaliplatin – on cochlear TrxR activity and hair cells, *in vivo* and *in vitro* by using organotypic cultures.

3 RESULTS

3.1 Article I

Dammeyer P, Mwakigonja AR, Rethi B, Chiodi F, Wolpert EZ.

Vaccination with beta(2)-microglobulin-deficient dendritic cells protects against growth of beta(2)-microglobulin-deficient tumours.

Scand. J. Immunol. **70**, 44-52 (2009).

Here a strategy was designed and investigated *in vivo* for a cancer vaccine to protect against the outgrowth of tumors, which had lost functional MHC class I antigen expression. This tumor phenotype results from mutations in the gene encoding the light chain, β_2m , of MHC class I antigen molecules. The tumor cells investigated were from a β_2m -free cell line, C4.4-25⁻, of syngeneic origin to the C57Bl/6 (B6) mice studied.

Firstly, the tumorigenic dose of tumor cells was investigated, which was then used throughout this study. Mice were injected subcutaneously (s.c.) into one flank with different tumor cell doses. A dose of 10^5 tumor cells per injection did not cause tumor formation in the injected mice, whereas injection with 10^6 tumor resulted in s.c. tumor formation in all mice injected. A dose of 7×10^5 tumor cells per injection (i.e. per mouse) was selected for tumor challenge of vaccinated mice. B6 mice were injected with DCs extracted from the bones of the hind legs (femur and tibia) of β_2m -deficient mice, which were of syngeneic origin to the B6 mice. β_2m -deficient DCs were cultured and matured from single cell suspension, extracted from the bone marrow, and activated with CpG oligonucleotides 24 h prior to injection. B6 mice received these *in vitro* matured and activated DCs in three consecutive weekly injections. Control mice were either kept unvaccinated or were injected with DCs cultured from β_2m -intact B6 mice of syngeneic origin. One week after the third immunization, the mice were challenged with β_2m -free tumor cells.

Two vaccination experiments were performed in B6 mice. Both of these investigations showed with statistical significance that a smaller proportion of vaccinated mice developed tumors compared to unvaccinated control mice. In the first experiment, 10/12 of the unvaccinated control mice showed s.c. tumor formation whereas only 6/12 of the mice vaccinated with β_2m -deficient DCs developed a s.c. tumor. In the second experiment using the same experimental conditions, the difference in frequency of mice that formed a s.c. tumor was even greater between unvaccinated

and vaccinated mice with 8/10 versus 3/12, respectively. Vaccination with β_2m -intact DCs of syngeneic origin in the second experiment resulted in tumor formation in 7/12 of the mice and did not significantly protect the mice from tumor cell growth.

Furthermore, *in vitro* analysis of effector cells generated by the vaccinated mice suggested that the effector cell population contained CD3⁺ cells. Target cell analysis revealed that cells from this effector cell population were able to kill β_2m -deficient tumor cells (C4.4-25⁻) and β_2m -deficient non-malignant cells *in vitro*. In addition, tumor cells (EC7.1) lacking functional TAP and classical MHC class I HC expression were killed in the *in vitro* cytotoxicity assay. Moreover, cells of non-malignant origin with intact MHC class I antigen expression were also recognized and killed *in vitro*.

Vaccinated mice developed proteinuria, and histopathological analysis indicated signs of a mild inflammatory kidney reaction (glomerulonephritis) in the immunized mice. However, no histopathological changes were observed in the other organs investigated, i.e. liver, gut, thymus lymph and nodes. Vaccinated mice were kept for over one year without any overt signs of disease.

3.2 Article II

Dammeyer P, Jaramillo MC, Pipes BL, Badowski MS, Tsang TC, Harris DT.
Heat-inducible amplifier vector for high-level expression of granulocyte-macrophage colony-stimulating factor.
Int. J. Hyperthermia **22**, 407-419 (2006).

In this article a DNA vector, termed pAD-HotAmp-GM-CSF, was constructed to contain two promoters and two genes of interest. The first promoter consisted of a fragment of the heat shock protein 70B (HSP70B) promoter, which contains the minimal promoter elements for inducible promoter activity. The HSP70B promoter controlled the transcription of the first gene, which was the human immunodeficiency virus (HIV) transactivator of transcription (Tat). The second promoter in the construct was a fragment derived from the HIV2 promoter, which contains Tat-responsive elements. The HIV2 promoter guided transcription of the second gene of interest, the gene encoding GM-CSF.

The idea behind this was to make a single construct with pAD-HotAmp-GM-CSF, which combined features for inducible and amplified GM-CSF expression. Thus, the HSP70B promoter fragment provided the heat-inducible component to the construct,

which controlled expression of Tat. Binding of Tat to the Tat-responsive elements in the HIV2 promoter fragment stimulates amplification of the GM-CSF expression.

Of the different temperatures and durations tested, hyperthermia at 42°C for 30 min gave the best outcome regarding balancing a compromise between inducing the HSP70B promoter fragment and maintaining cell viability following heat-shock treatment. Accumulated secreted GM-CSF levels peaked for all transfected constructs 24 h after hyperthermia. GM-CSF expression obtained with the cytomegalovirus (CMV) promoter plasmid and the heat-inducible, but non-amplifying, construct pAD-Hot-GM-CSF reached similar levels following heat treatment. Interestingly, basal GM-CSF expression was not detectable with the inducible pAD-Hot-GM-CSF vector. Thus, pAD-Hot-GM-CSF showed the most remarkable inducibility of all constructs transfected. The highest GM-CSF expression (13,634 pg/ml) was obtained following heat-induction of the inducible and amplifying pAD-HotAmp-GM-CSF vector, which was over 2.5-fold higher than that obtained with the CMV promoter plasmid after the same treatment. Basal expression of GM-CSF with pAD-HotAmp-GM-CSF, which was at approximately 15% of its induced and amplified levels, was of similar levels as the basal CMV promoter-controlled GM-CSF expression. These results demonstrate the successful construction of a DNA vector for GM-CSF expression, inducible by mild to moderate hyperthermia and amplified approximately 2.5-fold over CMV promoter-controlled levels.

3.3 Article III

Dammeyer P, Damdimopoulos AE, Nordman T, Jiménez A, Miranda-Vizueté A, Arnér ESJ.

Induction of cell membrane protrusions by the N-terminal glutaredoxin domain of a rare splice variant of human thioredoxin reductase 1.

J. Biol. Chem. **283**, 2814-2821 (2008).

Here the v3 splice variant of TrxR1 was studied in relation to expression, cellular localization and function. The strongest expression of v3 was detected in testis using a probe specific for the three exons unique to v3 in Northern blot analysis, and using first-strand cDNA analysis. Furthermore, immunohistochemical analysis of testis – using polyclonal antibodies raised against a peptide contained in the Grx domain unique to v3 – gave a strong signal in the interstitial Leydig cells. The v3 splice variant was also expressed in heart, liver, spleen, ovary, pancreas and kidney, but these were to lesser extents compared to the expression in testis, as identified by analysis of first-

strand cDNA. This type of analysis also showed that several cancer cell lines expressed the v3 transcript. However, transcript levels of variant 1 of TrxR1 were expressed at much higher levels compared to v3 in all cancer cell lines analyzed. Treatment of HeLa cells with testosterone or estradiol upregulated v3 transcription.

To investigate the function of v3, HeLa and HEK293 cells were transfected with constructs to recombinantly overexpress v3 fused with the green fluorescent protein (GFP), and appropriate control constructs. This overexpression of v3 resulted in an intriguing dynamic change in shape of the transfected cells and the induction of dynamic and rapid formation of cell-membrane protrusions. In order to induce this phenotype, it was sufficient to overexpress the Grx domain of v3, fused with GFP, even without the TrxR1 module. This Grx domain is unique to the v3 splice variant. Immunofluorescent microscopy revealed localization of v3 in the cytoplasm, particularly to the cell membrane and in the membrane protrusions. In these protrusions, v3 was expressed in close proximity to actin and seemed to guide actin in the formation of the protrusions, since v3 expression was detected throughout the protrusions until the edges and the tip. In contrast, TrxR1-GFP showed stronger distribution throughout the cytoplasm and around the nucleus in transfected cells.

3.4 Article IV

Dammeyer P, Jacob S, Kostyszyn B, Sundström E, Kirkegaard M, Petek E, Arnér ESJ. A cell stress-induced IRES-dependent expressional switch of human thioredoxin reductase 1 to v3 correlates with modulation of cellular morphology.

Manuscript

Here the expression pattern, regulation of expression and function of the endogenously expressed v3 were studied. Moreover, a potential role for v3 in a DFNA25-related hearing loss was investigated. Immunohistochemical analysis of human embryonic/fetal cochlear specimens revealed positive immunostaining in the developing sensory epithelium and nerve fibers, and in both structures v3 expression coincided with actin staining. β III-tubulin expression was found in the spiral ganglion and cochlear nerve fiber in close proximity to v3.

Following the analysis of v3 expression in the developing human cochlea, the genomic integrity of selected regions of the DFNA25 locus from a patient suffering from DFNA25-related hearing loss was investigated. The patient harbors a *de novo* deletion of the paternal allele of the DFNA25 locus and is thus, monoallelic for this

locus, which encompasses the *TXNRD1* gene, among others. The three 5' exons unique to v3 (exons β -VIII, β -VI, β -V) and the downstream exons important for TrxR1 function (exons II, III and V), as well as the corresponding intron-exon boundaries of patient DNA, were sequenced and analyzed for mutations in comparison with sequences from the parental DNA and public reference databases. Sequence analysis revealed no obvious mutations in these v3-relevant exons or the intron-exon boundaries. The DFNA25 locus also contains the *SLC17A8* gene, which encodes the VGLUT3 glutamate transporter. A mutation in *SLC17A8* had been previously associated with DFNA25-related hearing loss, but no mutations were found in the DFNA25 patient studied here.

Following this sequence integrity analysis for the *TXNRD1* gene in the DFNA25 patient, cells derived from this patient were used as a model system to study the expression regulation of TrxR1 splice variants from a monoallelic *TXNRD1* gene. In anti-TrxR1 immunoblots both patient-derived and control cells (both Epstein-Barr virus (EBV)-immortalized lymphoblasts) showed a single band at 55 kDa, which corresponds to the classical and most abundant variant 1 of TrxR1 (v1). However, when these cells were starved for 5 days, a second band appeared at approximately 57-60 kDa in the anti-TrxR1 blots. In this study, the term starvation refers to culturing cells without replacing the initially serum-containing complete medium, which is otherwise changed every three days. Importantly, this heavier band at approximately 57-60 kDa was also immunopositive in anti-v3 immunoblots, using polyclonal rabbit anti-human v3 antibodies, which were described in Article III. Thus, it was concluded that this variant of TrxR1 corresponded to the v3 protein isoform, the expression of which was induced upon starvation. It is noteworthy that prolonging starvation for 9 consecutive days induced an exclusive “switch” in TrxR1 protein isoform expression from the classical v1 (55 kDa) to the v3 isoform (~57-60 kDa) in the patient-derived cells. Only one band was present in these long-term starved cells, which was immunopositive for both anti-TrxR1 and anti-v3 antibodies. This starvation-induced switch in protein isoform expression was also associated with loss of TrxR activity, as measured with the endpoint-insulin assay. Interestingly, the A549 cancer cell line, which was known to express high levels of the classical form of TrxR1, showed expression of the v3 isoform even when being well nourished, as seen with immunoblot analyses presented in Article IV.

Prolonged starvation of DFNA25 patient-derived cells induced a change in phenotype, which was associated with, dynamic change in cell shape and the rapid and

dynamic formation of membrane protrusions. These protruding cells also became loosely adherent to the culture surface rather than their usual growth in suspension. Immunofluorescence confocal microscopy showed expression of endogenous v3 in the long, thin protrusions of starved DFNA25 patient-derived cells. In addition to induction by starvation, immunocytochemistry showed that endogenous v3 expression could also be induced by testosterone or nocodazole treatment in A549 cells. Nocodazole caused cell cycle arrest at the G2/M phase by interfering with the polymerization of microtubules inside a cell. Prolonged starvation of A549 cells for 7 days induced a similar phenotype, with the rapid formation of membrane protrusions. Importantly, starvation in culture up to 18 days gradually increased the motility of A549 cells.

Activity of the v3 promoter was induced in a hypoxic environment (2% O₂), and maintained in a hypoxic environment together with etoposide treatment, as seen by luciferase reporter expression. In contrast, TrxR core promoter activity was repressed under both these conditions, i.e. hypoxia alone or together with etoposide treatment. Moreover, treatment with testosterone or rapamycin induced reporter gene expression. Rapamycin blocks cap-dependent translation and thus, indicates a translation mechanism of v3, which is mediated by an internal ribosome entry site (IRES). Using dicistronic constructs in cell-based transfection and *in vitro* transcription and translation experiments, resulted in increased expression of the second cistron, when the intercistronic sequence contained the 5' leader sequence of v3, as compared to an irrelevant mock sequence. This increased translation of the second cistron also indicates an IRES-driven translation mechanism for the v3 isoform of TrxR1.

3.5 Article V

Dammeyer P, Hellberg V, Wallin I, Laurell G, Shoshan M, Ehrsson H, Arnér ESJ, Kirkegaard M.

Both cisplatin and oxaliplatin target thioredoxin reductase in organ of Corti cultures whereas their ototoxicity profiles differ *in vivo*.

Manuscript

The targeting of TrxR in the cochlea by clinically used platinum drugs, the frequently ototoxic compound cisplatin and the rarely ototoxic compound oxaliplatin, were investigated. To analyze the direct effects of the drugs, organotypic cultures of organ of Corti and lateral wall from rat were directly exposed to either cisplatin or oxaliplatin a number of equimolar concentrations. Both drugs induced a similar extend of hair cell death in the basal turn of the cochlea in a dose-dependent manner.

Immunohistochemistry confirmed previously described immunostaining of TrxR in the inner and outer hair cells, the stria vascularis and the spiral ganglion.

Direct exposure to equimolar concentrations (20 μ M) of either cisplatin or oxaliplatin significantly inhibited TrxR activity in the organ of Corti and lateral wall cultures, compared to untreated controls. Moreover, these results indicate that in the Organ of Corti cultures, oxaliplatin inhibits TrxR activity more than cisplatin does, whereas in cultures of lateral wall TrxR activity was inhibited to similar levels. Guinea pigs were treated with equimolar concentrations of either cisplatin or oxaliplatin to investigate *in vivo* effects on cochlear TrxR activity. Neither of these compounds had significant effects on TrxR activity in lateral wall or liver tissue. In contrast, both drugs reduced TrxR activity in kidney, although only oxaliplatin treatment gave a statistically significant decrease. The data for organ of Corti resulted from a pilot experiment where only cisplatin was used with a limited number of mice (2 treated and 1 untreated). Therefore, the data cannot be analyzed for statistical significance of any difference. However, the result does indicate that cisplatin treatment could substantially inhibit TrxR activity in the organ of Corti.

4 DISCUSSION AND CONCLUSION

In a cancer vaccine approach in mice, vaccination with syngeneic β_2m -deficient DCs, prior to a challenge with β_2m -deficient tumor cells, protects the vaccinated mice from developing tumors (**Article I**). This study design uses the cancer vaccine as a prophylactic measure before challenge with a set number of tumor cells as a tumorigenic dose, which apparently resembles an artificial system to control experiment modalities. Obviously one limitation of disease models used in research, is the physiological relevance of the organism suddenly receiving many cancerous cells s.c. However, the strength of experimental science is in controlling confounding variables and manipulating one variable at a time, which has to be balanced against limited physiological relevance in order to achieve progress of knowledge in a research field. In patients who respond to vaccination with tumor antigens, antivaccine T cells are thought to rather interact with the tumor than kill the mass of the tumor. It is hypothesized that this interaction focally reverses the existing immunosuppression and causes an activation of other antitumor T cells, which then in turn lead to advanced tumor destruction (20,93). Thus, it would be very interesting to investigate whether the vaccination with β_2m -deficient DCs, applied in **Article I** also shows efficacy as a therapeutic cancer vaccine.

Instead of subjecting the mice to a tumor challenge after immunization, a tumorigenic dose of tumor cells could be s.c. injected before vaccination to cause the growth of a tumor at the site of the injection. When the tumor has grown to a predefined size or grown over a predefined period, the tumor-bearing mice could be administered with β_2m -deficient DCs into the site of the tumor. This would allow the investigation of what size of tumor the mouse immune system is capable of combating, and also how many applications of the treatment would be needed for an efficient antitumor response. In patients who respond to vaccination with tumor antigens, antivaccine T cells are thought to rather interact with the tumor than kill the mass of the tumor. It is hypothesized that this interaction focally reverses the existing immunosuppression and triggers an activation of other antitumor T cells. These antitumor T cells then lead to advanced tumor destruction (20,93).

It needs to be kept in mind that such a cancer vaccine approach bears the risk of inducing autoimmunity, since the vaccine is based on cells that originated from the same genetic background (syngeneic/autologous) to induce an immune response

against cells with an MHC class I antigen loss phenotype. It is not yet known what the effector cells, isolated from vaccinated animals, recognize on the target cells. However, non-malignant cells were recognized *in vitro* by these effector cells, independently of intact or defect MHC class I antigen presentation on their cell surface (**Article I**). Moreover, the experimental approach was associated with signs of mild inflammation in the kidneys, although this difference in inflammation was not statistically significant when compared to unvaccinated control mice (**Article I**). In addition, the vaccinated mice had developed proteinuria, which was observed 10 days after the third vaccination and usually indicates kidney dysfunction. All other tissues investigated, including spleen and thymus, showed no signs of damage under histopathological examination.

A two-promoter two-gene DNA vector was constructed for inducible and amplified expression of the cytokine GM-CSF (**Article II**). Mild to moderate hyperthermia induces GM-CSF expression from this construct to levels almost three-fold greater than those obtained with a prototypic CMV promoter plasmid, yet both plasmids have similar basal expression levels. Such constructs can be tested in animal models, however, the expression of Tat is probably not suitable for clinical application even if it is shown to be a successful strategy in mice. Despite the putative favorable effects for recombinant gene expression, it may not be appropriate to express the Tat transcription factor in patients since. The reason is that – in addition to its transcriptional activities – Tat can be released extracellularly, where it interacts with different cell membrane-associated receptors. Moreover, extracellular Tat can be internalized into other cells through active endocytosis (94).

The heat-inducible but non-amplifying pAD-Hot-GM-CSF construct is free from these concerns, since transcription of GM-CSF in that construct is directly guided by the heat-inducible HSP70B promoter fragment (**Article II**). However, regarding the pAD-HotAmp-GM-CSF vector there is the possibility to exchange the two elements, the gene encoding Tat and the Tat-responsive elements, with other elements that can take over the role of amplification of GM-CSF expression. The current design of the pAD-HotAmp-GM-CSF vector could still be used in further experiments to study theoretical use in a therapeutic cancer vaccine. In the vaccine approach envisioned, the heat-inducible amplifying pAD-HotAmp-GM-CSF or the heat-inducible pAD-Hot-GM-CSF vector, can be locally transfected into the tumor of a tumor-bearing mouse. Following injection, mild to moderate hyperthermia can be applied, potentially using microwaves, to induce local expression of high levels of GM-CSF. The GM-CSF expression is thought to contribute towards a focal reverse of potential

immunosuppression in the tumor microenvironment, and to induction of a more efficient antitumor immune response. GM-CSF expression resulting from the transfected vectors could also be used as an adjuvant in other cell-based or cell-free vaccinations.

Samples donated from a patient were studied, who suffers from DFNA25-related hearing loss and harbors a paternal deletion in the DFNA25 locus (92). It was found that the patient carries neither a previously described mutation in the VGLUT3 glutamate transporter – which has been previously associated with DFNA25-related hearing loss (95) – nor any mutations in v3-relevant exons and intron-exon boundaries in the remaining allele of the DFNA25 locus (**Article IV**). Hence, the patient's DFNA25-related hearing loss may have its cause in another potentially dysfunctional gene. Alternatively, the DFNA25 locus could encode potential proteins required for hearing function and/or development, and thus the haploinsufficiency itself in the locus may be the cause of the hearing loss by affecting gene dosage (92). However, the haploinsufficiency of the DFNA25 patient for the *SLC17A8* gene, encoding VGLUT3, is unlikely to be the cause for the hearing loss, since mice with a haploinsufficiency in the *Slc17a8* gene, showed normal hearing responses and anatomy (95).

With no obvious mutations found in v3-relevant sequences, immortalized cells derived from the DFNA25-patient were used as a model system to study the regulation of v3 expression from a monoallelic *TXNRD1* gene (**Article IV**). These cells offer the advantage of studying the regulation of two alternative promoters – the TrxR1 core promoter guiding transcription of the most abundant splice variants of TrxR1 (**Article III** and Ref. (80)), and the alternative upstream promoter guiding v3 transcription – in a single allele for *TXNRD1*, whilst excluding any potential allelic imbalance in expression. Several cell stressors (starvation, hypoxia, etoposide, rapamycin, nocodazole) increased v3 promoter activity and v3 expression, while at the same time repressing the TrxR1 core promoter activity and expression of the classical form of TrxR1 (**Article IV**). Moreover, the v3 transcription and promoter activity were induced by testosterone treatment (**Articles III and IV**). Translation of v3 was found to be IRES-dependent (**Article IV**).

In particular, prolonged starvation of the DFNA25 patient-derived cells induced an endogenous switch in protein isoforms expression from the classical variant 1 of TrxR1 to the v3 isoform (**Article IV**). This switch to v3 isoform expression was accompanied by the loss of TrxR activity. Moreover, prolonged starvation also induced changes in cell morphology, along with rapid and dynamic formation of membrane protrusions,

both in the DFNA25-derived patient cells and A549 cancer cells (**Article IV**). Intriguingly, endogenous v3 was expressed in the long and thin membrane protrusions, which were usually several times the width of the cell's length.

A similar type of rapid and dynamic change in cell shape, with dynamic formation of membrane protrusions, was induced by recombinant overexpression of v3 fused with GFP (**Article III**). The induction of this phenotype was independent of the expression of the TrxR1 module in v3. The overexpressed v3 localizes to the cell membrane and to the protrusions, similarly to the endogenous v3, where it appeared to induce actin polymerization. The membrane protrusions were further characterized as filopodia-like structures (96). Furthermore, starvation of the cultured cells also induced an increase in motility of the cultured cells (**Article IV**).

Transcripts encoding v3 were detected in heart, liver, spleen, ovary, kidney and pancreas, as well as several cancer cell lines (**Article III**). Strong expression of the v3 splice variant was found in the Leydig cells of the testis and in distinct structures of the developing cochlea (**Articles III and IV**).

TrxR is a known target of platinum drugs, such as cisplatin (63,64) that is known to be frequently ototoxic leading to high-frequency hearing loss in clinical use (53,97). Results presented in this thesis (**Article V**) show that cisplatin and oxaliplatin target TrxR in the cochlea where they inhibit TrxR activity and cause hair cell loss. **Article IV** shows that v3 is also expressed in the developing human embryonic/fetal cochlea in distinct structures, such as the developing sensory epithelium and the connecting nerve fibres. Further studies are needed to reveal the role of v3 in the cochlea, to elucidate whether v3 is required for cochlear development, and whether it is important for structural and functional components beyond development. Thus, it would be beneficial to investigate in future studies whether platinum drugs can also target the v3 splice variant, and specifically whether cochlear v3 is targeted. However, one limitation of these proposed future studies is that rodents cannot be used, since v3 is not expressed in mice or rats, and it is yet unknown whether v3 is expressed in guinea pigs.

Expression of v3, increased cell motility and formation of membrane protrusions all correlated in time and were induced in cells upon cell stress in the form of starvation (**Article IV**). Importantly, endogenous v3 was found in the protrusions. Moreover, recombinant overexpression of v3 directly induced the dynamic formation of and localize in the membrane protrusions (**Article III**), and starved protruding cells were highly motile – as can be seen in the movies supplementary to **Articles III and IV**. This already links two pair-wise combinations – v3 expression with the formation of

protrusions, as well as formation of protrusions with cell motility – of these three features. Therefore, it is highly likely that both of these cellular features – increase in motility and formation of protrusions – are linked with each other and linked with the induction of endogenous v3 expression. Explicit evidence of these links is still to be provided, yet, the findings presented in this dissertation clearly indicate a potential role of v3 in cell motility, and thus, ultimately in the formation of metastases. Therefore, this implies potential implications for v3 as a target in anticancer therapy or as an antigen in cancer vaccination.

5 FUTURE PERSPECTIVES

Based on the discussions in the previous section, new studies for the nearest 5- to 10-year period could include the following:

- Develop a therapeutic cancer vaccine based upon β_2m -deficient DCs.
- Rule out indications of a possible triggered autoimmunity based upon vaccination with β_2m -deficient DCs.
- Develop a therapeutic cancer vaccine based upon heat-inducible and amplified expression of GM-CSF to generate local high-level expression of GM-CSF.
- Rule out the possibility of provoking leukemic cell growth with local expression of high-level GM-CSF.
- Rule out the risk of evoking systemic expression or distribution of high levels of GM-CSF.
- Develop the heat-inducible and amplified expression of GM-CSF as an adjuvant in combination with vaccination with β_2m -deficient DCs, potentially by transfecting the DCs with the GM-CSF expression vector.
- Establish a functional correlation between v3 and tumorigenesis, formation of metastases and expression of v3. In a feasible way, this could be investigated in an approach using siRNA to knock down v3 expression, for instance in tumor xenograft models.
- Investigate whether function of v3 is associated with redox activity, potentially by mutation of cysteine residues in the Grx.
- Investigate whether v3 is directly or indirectly involved in actin polymerization and identify interacting factors.
- Explore a potential functional correlation between v3 and cell motility, tumorigenesis, and the formation of metastases, potentially by using siRNA directed against v3.

The ultimate aims of these studies include:

- Development of new clinical protocols for a cure of cancer based upon immunotherapy or cancer vaccines.
- Improve therapeutic strategies of existing platinum-based compounds with high clinical efficacy by reducing or counteracting toxicities in order to

overcome current dose limitations and thus, to utilize the full potential of the drugs.

- Development of new v3 as a target in anticancer therapies, in particular for metastatic disease, based upon v3 as an anticancer drug target or v3 as an antigen in cancer vaccination, in particular in metastatic disease.

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