Purification and Characterization of the Mda-7 Tumor Suppressor Protein Expressed in Insect Cells

Paula Turkki

Master's Thesis

University of Jyväskylä

Department of Biological and Environmental Science

Molecular Biology

May 2008

PREFACE

This study was carried out at the University of Jyväskylä, Department of Biological and Environmental Science, Division of Biotechnology.

I would like to take this opportunity and thank several people who have helped and supported me during the course of this study. First, I would like to thank Professor Christian Oker-Blom for the opportunity to work in the biotechnology group. I would like to express my sincere gratitude to my supervisor, assistant Anna Mäkelä, for her guidance and support during my study. Further I would like to thank all the other members of the biotechnology group, especially laboratory technician Eila Korhonen for her advice and assistance. Specifically, I would like to thank my co-graduate student Riikka Kapanen with whom we shared both trouble and success in and out of the lab.

I would also like to thank my family and friends for support and encouragement throughout my studies. I am more than grateful to Miika for his love, support and patience throughout this work.

Finally, I would like to acknowledge the Finnish Cultural Foundation for a personal grant.

Jyväskylä, May 2008

Paula Turkki

University of Jyväskylä
Faculty of Mathematics and Science

Abstract of Master's Thesis

Author: Paula Turkki
Title of thesis: Purification and Characterization of the Mda-7 Tumor Suppressor Protein Expressed in Insect Cells
Finnish title: Hyönteissolussa tuotetun mda-7 kasvunestoproteiinin puhdistus ja karakterisointi
Date: 9.5.2008 **Pages:** 57 pages

Department: Department of Biological and Environmental Science
Chair: Molecular biology
Supervisors: Prof. Christian Oker-Blom, MSc Anna R. Mäkelä

Abstract:

The melanoma differentiation associated gene-7 (*mda-7*), also known as interleukin-24 (IL-24) is a novel cytokine, which exhibits both tumor suppressing and immunostimulatory activities. Mda-7 has the ability to restrain growth and induce programmed cell death (apoptosis) in a wide variety of human cancer cells without having similar effects on normal cells. This study was performed to investigate the proteochemical and tumor suppressing properties of soluble human mda-7 produced in baculovirus-insect cell expression vector system. The effects of the post-translational modifications, especially glycosylation, on the activity of mda-7, are controversial. To investigate the role of glycosylation, a polyhistidine tagged mda-7 (mda-7-His) fusion protein was produced in two different insect cell lines: in *Spodoptera frugiperda* 9 (*Sf*9) and in Mimic™ cells, the latter cell line has been genetically engineered to stably express a variety of mammalian glycosyltransferases allowing processing of complex N-glycans to a similar extent with mammalian cells. Furthermore, the production and purification protocols of the proteins were optimized and the proteochemical properties of the fusion proteins were characterized by SDS-PAGE and Western blotting. Finally, the induction of apoptosis by the mda-7-His fusion protein was tested in human MDA-MB-435 breast carcinoma and PC-3 prostate adenocarcinoma cells by Trypan blue staining and Annexin V labeling. The fusion proteins were successfully produced and purified from insect cells. Analyzed by Western blotting, the mda-7-His expressed in *Sf*9 cells was approximately 23 kDa in size, representing the non-glycosylated form of the protein. The protein sample from the Mimic™ cells had two main bands, about 25 kDa and 45 kDa in size, representing the non-glycosylated or partially glycosylated and fully glycosylated forms of the protein, respectively. Despite the differences in glycosylation between the proteins, both of the proteins retained their apoptosis inducing functions. Accordingly, with PC-3 cell line the percentage of apoptotic cells was up to 26% with mda-7-His produced in *Sf*9 cells and 25% with the mda-7-His produced in Mimic™ cells. From these result it can be concluded that both glycosylated and non-glycosylated soluble mda-7-His produced in baculovirus-insect cell expression system are able to induce apoptosis in human cancer cells. These results demonstrate that this expression system can be employed to easily produce large amounts of biologically active mda-7, rendering it useful for further studies on the structure and function of mda-7.

Keywords: apoptosis; cancer cell; cancer therapy; IL-24; mda-7

Jyväskylän yliopisto **Pro gradu –tutkielman tiivistelmä**
Matemaattis-luonnontieteellinen tiedekunta

Tekijä	Paula Turkki
Tutkielman nimi:	Hyönteissolussa tuotetun mda-7 kasvunestoproteiinin puhdistus ja karakterisointi
English title:	Purification and Characterization of the Mda-7 Tumor Suppressor Protein Expressed in Insect Cells
Päivämäärä:	9.5.2008 **Sivumäärä:** 57 sivua
Laitos:	Bio- ja ympäristötieteiden laitos
Oppiaine:	Molekyylibiologia
Tutkielman ohjaajat:	Prof. Christian Oker-Blom, FM Anna R. Mäkelä

Abstrakti:

Mda-7 (engl. *melanoma differentiation associated gene-7*), joka tunnetaan myös nimellä interleukiini 24 (IL-24), on sytokiini, jolla on syövän kasvua rajoittavia sekä immuunivastetta aktivoivia ominaisuuksia. Sen on osoitettu rajoittavan syövän kasvua sekä aiheuttavan ohjelmoitua solukuolemaa (apoptoosi) useissa ihmisen syöpäsolutyypeissä. Tämän tutkimuksen tavoitteena oli tuottaa ihmisen mda-7 proteiinia muokattujen bakuloviruksien avulla hyönteisoluissa sekä tutkia tuotettujen proteiinien proteiinikemiallisia ja syöpäsolun kasvua estäviä vaikutuksia. Glykosylaation vaikutuksesta proteiinin toimintaa on ollut ristiriitaisia tutkimustuloksia. Jotta voitaisiin tutkia glykosylaation roolia proteiinin toiminnassa, proteiini tuotettiin käyttäen kahta eri hyönteissolulinjaa: *Spodoptera frugiperda* 9 (*Sf*9) ja Mimic^{TM} soluja. Mimic^{TM} solut ovat geneettisesti muokattu ilmentämään useita nisäkässolujen glykosyylitransferaaseja, joiden ansiosta tämä hyönteissolulinja kykenee tuottamaan nisäkässolujen kaltaisia proteiineja. Tuotettujen proteiinien tuotto- ja puhdistusprotokollat optimoitiin sekä proteiinien proteiinikemialliset ominaisuudet karakterisoitiin. Tämän jälkeen proteiinien kyky aiheuttaa apoptoosia määriteltiin sekä ihmisen MDA-MB-435 rintasyöpä- että PC-3 eturauhasyöpäsoluissa. Apoptoosikokeet toteutettiin Trypan blue-värjäyksellä sekä Annexin V-leimauksella. Proteiinien molekyylipainot määriteltiin Western blot-analyysin avulla. *Sf*9 soluissa tuotetun proteiinin molekyylipaino havaittiin olevan 23 kDa, joka on proteiinin glykosyloimaton muoto. Mimic^{TM} soluissa tuotetusta proteiinista oli havaittavissa kahta eri muotoa, 25 kDa ja 45 kDa kooltaan, joista ensimmäinen on glykosyloimaton tai osittain glykosyloitu muoto ja jälkimmäinen on proteiinin glykosyloitu muoto. Erilaisesta glykosylaatiosta huolimatta, molemmat proteiinit kykenivät aiheuttamaan apoptoosia ihmisen syöpäsoluilla. PC-3 soluilla apoptoottisten solujen osuus oli 26% kokonaissolumäärästä inkuboidessa soluja *Sf*9 soluissa tuotetulla mda-7-his proteiinilla. Vastaavaksi luvuksi Mimic^{TM} soluissa tuotetulla proteiinilla saatiin 25%. Näistä tuloksista voidaan päätellä että sekä glykosyloitu että glykosyloimaton hyönteisolussa tuotettu mda-7-His proteiini kykenee indusoimaan apoptoosia ihmisen syöpäsoluilla. Tämä tutkimus osoittaa että bakuloviruksen avulla hyönteisoluissa kyetään vaivattomasti tuottamaan suuria määriä biologisesti aktiivista mda-7 proteiinia tarjoten mahdollisuuksia mda-7:n rakenteen ja toiminnan tutkimukselle.

Keywords: apoptoosi; IL-24; syöpäsolu; syöpäterapia; mda-7

TABLE OF CONTENTS

ABBREVIATIONS

AAV	adeno-associated virus
Ad	adenovirus
AP	alkaline phosphatase
APA	alkaline phosphatase assay buffer
BEVS	baculovirus expression vector system
CAR	coxsackie–Ad receptor
ER	endoplasmic reticulum
FCS	fetal calf serum
GADD	growth arrest and DNA damage inducible
GST	glutathione *S*-transferase
Had	human Ad
IL	interleukin
JAK-STAT	Janus-activated kinase/signal transducers and activators of transcription
MAPK	mitogen-activated protein kinase
mda-7	melanoma differentiation associated gene 7
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PS	phospholipid phosphatidylserine
RB	retinoblastoma
RCC	renal cell carcinoma
ROS	reactive oxygen species
SDS-PAGE	sodium-dodecyl sulphate electorophoresis
*Sf*9	*Spodoptera frugiperda* 9
STAT	signal transducer and activator of transcription
TBS	Tris-buffered saline

1. INTRODUCTION

1.1 Melanoma differentiation associated gene 7 (*mda-7*)

The melanoma differentiation associated gene-7 (*mda-7*) is a tumor suppressor gene and a promising novel candidate for cancer therapy. For review see (Fisher, P.B., 2005). On the basis of its gene location, structure and cytokine-like properties, it has been classified as a member of the interleukin-10 (IL-10) family of cytokines and it is also known as interleukin 24 (IL-24) (Huang, E.Y. et al., 2001). Mda-7 has the ability to selectively restrain growth and induce programmed cell death (apoptosis) in a wide spectrum of human cancers including carcinomas, without harming normal cells (Allen, M. et al., 2004, Gopalan, B. et al., 2007, Ramesh, R. et al., 2004, Shi, H. et al., 2007, Su, Z.Z. et al., 1998). *Mda-7* was identified in 1995 by subtraction hybridization analysis from a human melanoma cell line HO-1. It is normally expressed in human melanocytes, its expression is decreased in advanced melanomas and is nearly undetectable in metastatic cancers (Jiang, H. et al., 1995, Jiang, H. et al., 1996). In addition to inhibition of the progression of melanoma, mda-7 has similar effects on a variety of different cancer cells (Allen, M. et al., 2004, Gopalan, B. et al., 2007, Ramesh, R. et al., 2004, Shi, H. et al., 2007, Su, Z.Z. et al., 1998). *Mda-7* is mainly expressed in the cells of the immune system, while its transient expression can be induced by treatment with fibroblast interferon and protein kinase C activator mezerein in certain other cells also (Huang, E.Y. et al., 2001).

Currently, phase II clinical studies are being conducted with melanoma and other solid tumors by using a replication incompetent adenovirus (Ad) expressing mda-7 (Ad.*Mda-7*; INGN 241; Introgen Therapeutics, Inc.). Ad.*Mda-7* was administered by intratumoral injections to patients with advanced carcinomas and melanomas, and was found to be well tolerated, active and efficient. In addition, mda-7 can be combined with other therapeutic agents to increase its activity. These include radiation, chemotherapy as well as monoclonal antibodies (Gupta, P. et al., 2006a). Accordingly, Ad.*Mda-7* combined with radiation therapy has entered the phase III studies (Introgen Therapeutics, Inc.).

1.1.1 Structure and function of mda-7 protein

Mda-7 is an actively secreted protein, and it was first found to be secreted from the Human Peripheral Blood Mononuclear Cells (PBMC) (Caudell, E.G. et al., 2002). Mda-7 has an unusually long hydrophobic signal sequence consisting of 49 amino acids and three predicted N-glycosylation sites at the amino acid residues 95, 109, and 126 (Caudell, E.G. et al., 2002). There are species-specific differences in the post-translational modifications of the secreted form of the protein, and also some discrepancies according to the size and the active form of the protein produced in the human cells (Chada, S. et al., 2004b). The predicted molecular weight of the human mda-7 is 23.8 kDa (Chada, S. et al., 2004b), but the mda-7 secreted by BPMCs and a His-tagged mda-7 (mda-7-His) secreted by stably transfected 293T appears to be closer to 35 kDa. After deglycolysylation of the secreted proteins, the molecular mass was about 23 kDa and all the extra weight seemed to be from N-linked glycosylations (Wang, M. et al., 2002). After transduction of human cancer cells with Ad.*Mda-7*, the secreted form was reported to be about 40 kDa (Chada, S. et al., 2004b). The calculated molecular weight of the intracellular form of mda-7 is about 23/30 kDa (Chada, S. et al., 2004b). Recently, it has been reported that the secreted form of mda-7 expressed by stably transfected human cells has multiple forms with different molecular weights (Mumm, J.B. et al., 2006). These are probably because of alternative splicing and different glycosylation patterns (Chada, S. et al., 2004b, Mumm, J.B. et al., 2006). In addition, it has been suggested that the active form of the protein exists as an N-glycosylated dimer. Accordingly, two main forms of the purified protein exist; the native covalently associated dimer (55 kDa) and a tetramer, a dimer of two dimers (90 kDa) (Mumm, J.B. et al., 2006). In both of these studies, the characterized proteins were found to be biologically active, suggesting that these different modifications and folding patterns do not affect the activity of the protein (Chada, S. et al., 2004b, Mumm, J.B. et al., 2006).

The effects of the post-translational modifications, especially glycosylation, on the activity of mda-7, are controversial. It has been postulated that the mda-7 produced in bacteria or in baculovirus-insect cell expression system is unable to activate the signal transducer and

activator of transcription 3 (STAT-3) and thus induce the release of secondary cytokines (Chada, S. et al., 2004b). Because of both bacteria and insect cells cannot perform complex glycosylation, the cytokine activity of the protein has been thought to be dependent on proper glycosylation (Chada, S. et al., 2004b). However, in another study, it was shown that a completely deglycosylated dimeric form of the protein exhibited only slightly reduced cytokine activity, indicating that the glycosylation of the protein is not required for its cytokine function (Mumm, J.B. et al., 2006). It has also been shown that the glycosylation of the protein is not necessary for growth inhibition, apoptosis induction or antitumor bystander activity in cancer cells (Sauane, M. et al., 2006). An adenovirus vector expressing a nonsecreted and nonglycosylated version of mda-7 was generated via deletion of its signal peptide and point mutations of its three N-glycosylated sites. In this study, it was demonstrated that both constructs were similarly transported to the same location in the endoplasmic reticulum (ER) and mediated apoptosis through JAK/STAT-independent and p38 MAPK-dependent pathways (Sauane, M. et al., 2006). Both constructs also led to the interaction with BiP/GRP78 (Sauane, M. et al., 2006), which has been shown to be critical to the apoptosis inducing function of mda-7 (Gupta, P. et al., 2006b). This intracellular nonglycosylated mda-7 was shown to be as effective as wild-type mda-7 in inducing apoptosis in multiple tumor cell lines (Sauane, M. et al., 2006).

Similar to the other members of the IL-10 family of cytokines, mda-7 binds and signals through two heterodimeric receptors; IL-20R1/IL-20R2 and IL-22R1/IL-20R2 (Wang, M. et al., 2002). Mda-7 has equal affinity to both of these receptor complexes, and its binding to its receptor primarily leads to activation of STAT-3 and to a lesser extent STAT-1 (Wang, M. et al., 2002). Shortly after the receptor binding, the expression of STAT-3 is notably increased and the phosphorylated form of STAT-3 is translocated to the nucleus (Chada, S. et al., 2004b). Mda-7 induced STAT-3 activation is followed by elaboration of secondary T-helper type 1 cytokines (Chada, S. et al., 2004b).

1.1.2 Immunoregulatory activities of mda-7

The genomic locus of *mda-7* is in 1q32 within an IL-10 related gene cluster containing four genes including *IL-10, IL-19,* and *IL-20* (Huang, E.Y. et al., 2001). Mda-7 is normally expressed in tissues associated with the immune system such as spleen, thymus, peripheral blood leukocytes, and in certain cell types, including melanocytes (Huang, E.Y. et al., 2001).

Mda-7 has the ability to induce several secondary cytokines and the induction is inhibited by administrating interleukin 10 (IL-10) (Caudell, E.G. et al., 2002). This has lead to the idea that mda-7 could be a part of a previously unknown immune-regulatory group (Chada, S. et al., 2004b). Mda-7 has the ability to induce the secretion of IFN-γ and IL-6 from melanoma cells but not from lung and breast cancer cells. It activates the IFN-γ and NF-κB immune regulatory signaling pathways, and it has been shown to have these immunostimulatory functions also *in vivo.* For review see (Chada, S. et al., 2004b). After Ad.*Mda-7* administration, an apparent induction of secondary cytokines was detected in phase I/II clinical studies. For review see (Fisher, P.B. et al., 2003).

By investigating the different forms of secreted mda-7, it has been noticed that the two activities of this protein, the cytokine function and the tumor suppressing function are both mediated by the same N-glycosylated and dimeric form of the protein (Mumm, J.B. et al., 2006). These functions are probably mediated by different pathways, since the IL-20R1/IL-20R2 and IL-22R1/IL-20R2 receptors are not expressed in most of the immune cells (Wolk, K. et al., 2004). Although these functions are normally mediated by the same N-glycosylated form of the protein, complete enzymatic deglycosylation of the protein has been shown to only partially reduce its cytokine functions and its ability to induce melanoma cell death (Mumm, J.B. et al., 2006).

1.1.3 Apoptosis inducing properties of mda-7

After its characterization from the melanoma cells, mda-7 was observed to have similar growth inhibiting and apoptosis inducing properties in a broad spectrum of other human tumors (Allen, M. et al., 2004, Gopalan, B. et al., 2007, Ramesh, R. et al., 2004, Shi, H. et al., 2007, Su, Z.Z. et al., 1998). Its role in inducing apoptosis has been actively studied since it was identified. Despite the intense investigations, the exact mechanism for this protein to act as a tumor suppressor is still unknown. Whether there even is only one main mechanism to all the cancer cells is still unsure (Fisher, P.B., 2005). Multiple molecules and pathways depending on the tumor cell line and the receptors and signaling pathways in question, seem to be involved (Chada, S. et al., 2004a, Ekmekcioglu, S. et al., 2003, Lebedeva, I.V. et al., 2003, Sauane, M. et al., 2003, Su, Z.Z. et al., 1998, Wang, M. et al., 2002, Yacoub, A. et al., 2003). Both the secreted and the intracellular mda-7 are able to induce apoptosis and inhibit tumor cell invasion (Sauane, M. et al., 2004). But only the secreted form has been reported to have the antitumor bystander activity (Sauane, M. et al., 2006).

The apoptosis inducing properties of mda-7 were first tested using replication incompetent adenoviral vector (Ad.*Mda-7*) in breast cancer cell lines (Su, Z.Z. et al., 1998). Ad.*Mda-7* inhibited growth and decreased the survival of the tumor cells *in vitro* and *in vivo* without having the same effects on normal cells (Su, Z.Z. et al., 1998). This apoptosis inducing ability of Ad.*Mda-7* was demonstrated to be independent from the p53 or retinoblastoma (RB) tumor suppressor status of the target cells (Su, Z.Z. et al., 1998). Additionally, these studies showed that mda-7 up-regulates the proapoptotic protein BAX (Su, Z.Z. et al., 1998). This up-regulation alters the ratios of BAX and the anti-apoptotic protein BCL-2, thereby modulating the cell to choose apoptosis before survival (Su, Z.Z. et al., 1998). Later, its role in inducing apoptosis by regulating the *bcl-2* family proteins has been verified in various human cancers. For review see (Fisher, P.B., 2005).

Although it has been shown that mda-7 can activate the Janus-activated kinase/signal transducers and activators of transcription (JAK-STAT) signaling pathway leading to

activation of STAT-1 and STAT-3 (Wang, M. et al., 2002), varying results exist whether the JAK-STAT signaling pathway is required for apoptosis induction (Chada, S. et al., 2004b, Sauane, M. et al., 2003). It has been shown for a variety of tumor cells that the induction of apoptosis can occur independent of IL-20R1/IL-20R2 and IL-22R1/IL-20R2 receptors or functional JAK-STAT signaling pathway (Sauane, M. et al., 2003, Wang, M. et al., 2002). Using breast cancer cell lines, which have functional IL-20R1/IL-20R2 and IL-22R1/IL-20R2 receptors, mda-7 was observed to be able to bind to these receptors, leading to the activation of STAT-3 (Zheng, M. et al., 2007). In this study, it was the IL-20 receptor complex rather than the IL-22 receptor complex, which mediated the apoptosis induction. This could be because of the greater abundance of these receptors on the cell surface (Zheng, M. et al., 2007). No correlation has been identified so far between the expression of IL-20R1/IL-20R2 and IL-22R1/IL-20R2 receptors and the cellular susceptibility to mda-7 (Sauane, M. et al., 2003).

Interestingly, in prostate cancer cells, it has been demonstrated that free radicals are involved in mda-7-induced apoptosis (Lebedeva, I.V. et al., 2003). Ad.*Mda-7* selectively induces apoptosis by mitochondrial dysfunction and reactive oxygen species (ROS) production (Lebedeva, I.V. et al., 2003), which is known to induce apoptosis in response to different stimuli (Jacobson, M.D., 1996). Specifically, mda-7 increased the ROS production by 3-5 fold in cancer cells without having the same effect on normal cells (Lebedeva, I.V. et al., 2003).

Recent studies have indicated that the apoptosis induction in multiple different cancers results from the interaction of mda-7 with the ER resident chaperone BiP/GRP78 and from its downstream targets, the p38 mitogen-activated protein kinase (MAPK) and the family of growth arrest and DNA damage inducible (*GADD*) genes (Gupta, P. et al., 2006b, Sarkar, D. et al., 2002). As mentioned earlier, in many cancer cells it has been noticed that mda-7 has the ability to down-regulate bcl-2 protein (Lebedeva, I.V. et al., 2003, Su, Z.Z. et al., 1998). This down-regulation is caused by this p38 MAPK and GADD153 signaling pathway mediated induction (Gupta, P. et al., 2006b, Sarkar, D. et al., 2002). The interaction of mda-7 with the BiP/GRP78 was found to be critical to its apoptosis inducing activity (Gupta, P. et al., 2006b). In this particular study, a truncated form consisting only of amino acids 104 to 206 of the full length protein was identified, retaining the same cancer-specific growth-suppressive and

apoptosis-inducing properties as the full-length protein. It was noticed that both the C and F helixes of the protein were crucial for maintaining its activity. These results suggest that a small mimetic peptide/protein with mda-7 activity to induce apoptosis could be developed, further increasing the possible therapeutic applications of mda-7 (Gupta, P. et al., 2006b). Mda-7 lacking both the signal peptide and the N-glycosylations can also induce the up-regulation of p38MAPK and PKR and interact with the ER resident chaperone BiP/GRP78 similar to the wild-type protein (Sauane, M. et al., 2006).

A number of molecules involved in apoptotic signaling of mda-7 in tumor cells have been identified (Chada, S. et al., 2004a, Ekmekcioglu, S. et al., 2003, Lebedeva, I.V. et al., 2003, Sauane, M. et al., 2003, Su, Z.Z. et al., 1998, Wang, M. et al., 2002, Yacoub, A. et al., 2003). It is not clear, however, which are the critical molecules or signaling pathways for tumor cell apoptosis. Further investigations are clearly required to have a precise understanding of the mechanism of action of mda-7 in cancer cells.

1.1.4 Antitumor bystander activity

It has been shown that the secreted form of mda-7 has "antitumor bystander activity" meaning that it can exert its apoptosis inducing and growth suppressing activity to tumor cells located at distant site from the administration or secretion of the protein (Sauane, M. et al., 2004). The exact mechanism of this activity is not yet known but it is thought to involve direct cytotoxicity, antiangiogenic activity or it can be result of its immunoregulatory activities. For review see (Fisher, P.B., 2005).

To test the antitumor bystander activity of mda-7 many groups have produced and purified different mda-7 fusion proteins. A bacterially synthesized glutathione *S*-transferase (GST)-mda-7 fusion protein was constructed with the ability to enhance the growth inhibitory and cytotoxic effect of ionizing radiation (Yacoub, A. et al., 2004). In addition, a plasmid-derived fusion protein, rhMda-7 has been expressed in HEK 293 cells (Ekmekcioglu, S. et al., 2003). Mda-7 has also been produced by a C-terminal fusion to the human placental alkaline phospatase (mda-7-AP) or a His-tag (mda-7-His), which were both active (Wang, M. et al.,

2002). Studies with an adenovirus vector expressing a nonglycosylated version of mda-7 showed that the glycosylation of mda-7 is not essential for antitumor bystander activities in cancer cells (Sauane, M. et al., 2006).

The antitumor bystander activity is dependent on the existence of the IL-20R1/IL-20R2 and IL-22R1/IL-20R2 receptors on the tumor cells (Su, Z. et al., 2005b). These receptors are present in a vast majority of normal and tumor cell lines (Blumberg, H. et al., 2001). The secreted form of mda-7 is able to modify the anchorage-independent growth, invasiveness, sensitivity to radiation as well as the survival of cancer cells that express functional IL-20/IL-22 cell surface receptors (Su, Z. et al., 2005b). In melanoma cells, the secreted form has been shown to induce the activation and nuclear translocation of STAT-3 which leads to up-regulation of BAX and induction of apoptosis. Other members of the IL-10 family are also able to activate STAT-3 but this activation does not lead to induction of apoptosis. Mda-7 is also able to activate the STAT-3 in normal cells without leading to apoptosis, indicating that the antitumor bystander activity is selective to cancer cells (Chada, S. et al., 2004a).

In cancer gene therapy, one of the greatest limitations is the challenge of transducing the whole tumor mass and possible metastatic lesions with the wanted suppressor gene (Fisher, P.B. et al., 2007). It has been shown that mda-7 is secreted from both normal and cancer cells after administration of Ad.*Mda-7* (Su, Z. et al., 2005b). Both of these secreted proteins possess the antitumor bystander activity and are able to suppress growth, induce apoptosis and enhance radiation lethality in human cancer cells (Su, Z. et al., 2005b). This ability of normal cells to produce, reservoir and secrete this cancer-cell specific apoptosis inducing cytokine could be a way to overcome many of the limitations in the cancer gene therapy (Su, Z. et al., 2005b).

1.1.5 Antiangiogenic activity

Angiogenesis is a complex process that involves the formation of new blood vessels. It is a fundamental event for the tumor growth and metastasis. For review see (Dear, R. et al., 2008). A number of angiogenesis inhibitors have been found and proved to be effective in preclinical

studies, but very few have had therapeutic effects in clinical trials. For review see (Cristofanilli, M. et al., 2002). It has been demonstrated that the mda-7 secreted from transfected 293 cells inhibits endothelial cell differentiation and tumor growth (Saeki, T. et al., 2002). Inhibition of tumor growth was observed to be associated with reduced tumor vascularization (Saeki, T. et al., 2002). In another study using also mda-7 secreted from transfected 293 cells, mda-7 was detected to be more potent in inhibiting endothelial cell differentiation than endostatin. Mda-7 also inhibited the endothelial cell migration (Ramesh, R. et al., 2003).

1.1.6 Combinatorial therapy with mda-7

Although mda-7 is able to efficiently induce apoptosis in a wide variety of human cancers (Allen, M. et al., 2004, Gopalan, B. et al., 2007, Ramesh, R. et al., 2004, Shi, H. et al., 2007, Su, Z.Z. et al., 1998), there are some exceptions including pancreatic (Su, Z. et al., 2001) and ovarian (Emdad, L. et al., 2006) cancer. Mda-7 does not significantly alter growth or induce apoptosis in human pancreatic cancer cells (Su, Z. et al., 2001). This is due to the expression of oncogenic K-*ras*, which is a common genetic alteration in pancreatic cancers (Almoguera, C. et al., 1988). This resistance for mda-7 can be overcome by combinatorial treatment with mda-7 and an antisense K-*ras* expression vector (Su, Z. et al., 2001). Another way to overcome the problem is to use mda-7 in combination with non-toxic doses of a chemical compound from the endoperoxide class (Lebedeva, I.V. et al., 2007). It has been reported that mda-7 is able to induce apoptosis in ovarian cancer cells (Leath, C.A.,3rd et al., 2004) although with a low efficiency (Emdad, L. et al., 2006). Induction of apoptosis by mda-7 can be enhanced by treating ovarian cells with ionizing radiation in combination with Ad.*Mda-7* (Emdad, L. et al., 2006). The studies with these cancer cells have provided evidence for the use of combinatorial therapy with mda-7.

1.1.7 Heterologous expression of recombinant mda-7

The soluble mda-7 has already been produced in bacterial (Sauane, M. et al., 2004, Yacoub, A. et al., 2004) and mammalian cell expression systems (Ekmekcioglu, S. et al., 2003). No published data exist of the production of mda-7 in yeast or baculovirus-insect cell expression systems.

Mammalian cell expression system is still one of the leading systems for the production of recombinant proteins for clinical applications, because of their capacity to perform proper folding and assembly of the protein and post-translational modifications. For review see (Wurm, F.M., 2004). Although the mammalian cells produce properly modified and folded proteins, compared to the other systems they require more work and are more expensive to maintain because they require a medium rich in nutrients and growth factors. For reviews see (Sudbery, P.E., 1996, Wurm, F.M., 2004). The *E. coli* bacterial expression system is favoured because of its many properties; it is easy and quick to grow, the cloning protocols are easy and well developed and its molecular genetics are well understood. It has some disadvantages such as lack of post-translational modifications and improper folding of the proteins, which can affect the functionality of the protein. For review see (Baneyx, F., 1999). Both the insect and yeast cell expression systems have many advantages as recombinant protein production systems; high production level, versatility, speed and low cost. For reviews see (Cereghino, J.L., and Cregg, J.M., 2000, Patterson, R.M. et al., 1995, Sudbery, P.E., 1996, Summers, M.D., 2006). One of the advantages over bacterial production system is that the proteins expressed using insect and yeast cells are often post-translationally modified similar to mammalian cells, resulting in biologically active proteins (Celik, E. et al., 2007, Kubelka, V. et al., 1994, Lorent, E. et al., 2008, Williams, P.J. et al., 1991).

The baculovirus expression vector system (BEVS) was developed during the 1980s and since then several hundreds of recombinant proteins have been successfully produced using BEVS. For review see (Summers, M.D., 2006). The advantages of BEVS compared to mammalian cell expression systems are high insertion capacity for foreign genes, a very high yield of

heterologous proteins, biosafety due to the inability of baculovirus to infect mammalian cells, insect cells are also easy and inexpensive to maintain. The extremely high yield of recombinant protein has made it a useful tool for pharmaceutical protein production, in addition more and more proteins in the preclinical stage are produced in insect cell systems. For reviews see (Hu, Y.C., 2005, Kost, T.A. et al., 2005a). The main disadvantage of BEVS is its inability to perform complex glycosylations. Although insect cells are able to perform most of the post-translational modifications, there are some differences in the N-glycosylations between the mammalian and insect cells (Kubelka, V. et al., 1994, Williams, P.J. et al., 1991). Many mature mammalian N-glycosylated proteins terminate in sialic acid residues while insect cells mostly produce N-glycosylated proteins which terminate in mannose residues (Butters, T.D., and Hughes, R.C., 1981, Kubelka, V. et al., 1994, Williams, P.J. et al., 1991). To overcome the deficiencies in insect cell glycosylation, transgenic insect cell lines have been engineered which express mammalian glycosyltransferases and are able to produce mammalian-like proteins (Hollister, J. et al., 2002).

So far, mda-7 has been produced in mammalian cell expression system using HEK-293 cell line (Caudell, E.G. et al., 2002, Ekmekcioglu, S. et al., 2003). A bacterially synthesized GST-mda-7 fusion protein has been studied by many groups (Sauane, M. et al., 2004, Su, Z.Z. et al., 2003, Yacoub, A. et al., 2004). In these studies the fusion proteins have been found to be active and able to inhibit the growth of cancer cells (Sauane, M. et al., 2004, Su, Z.Z. et al., 2003, Yacoub, A. et al., 2004). Treatment with GST-mda-7 has also potentiated radiation-induced apoptosis qualitatively similar to that resulting from Ad.*mda-7* (Su, Z.Z. et al., 2003). In addition, rhMda-7 (Ekmekcioglu, S. et al., 2003), mda-7-AP and mda-7-His (Wang, M. et al., 2002) has been found to be active. In all of these studies with mda-7 fusion proteins the proteins have been found to be as active as the *mda-7* gene.

1.1.8 *Mda-7* gene delivery by viral expression vectors

In the majority of the studies the vector used to express *mda-7* has been an adenovirus vector (Sauane, M. et al., 2006, Wang, C.J. et al., 2006, Xue, X.B. et al., 2006). *Mda-7* has also been expressed by using an adeno-associated virus (AAV) type 1 vector (Tahara, I. et al., 2007).

Ad vectors have been the center of attention during the last few years for their potential applications for cancer gene therapy. Ad vectors have several advantages over other vectors such as the ability to transduce both the dividing and non-dividing cells, ease of manipulation and capability to produce high vector titers. The main disadvantages of the Ad vectors are the pre-existing immunity in the majority of human population and a rapid development of immune response against the administrated vector. For reviews see (Bangari, D.S., and Mittal, S.K., 2006, Rein, D.T. et al., 2006). There are over 50 known serotypes of human Ads (HAds) and the majority of human population is exposed to it leading to the development of Ad specific immune response (Harvey, B.G. et al., 1999). This problem can be partly overcome by modifying the capsid proteins of the virus (Roy, S. et al., 1998). An Ad vector, which encodes sequences from a different adenovirus serotype has been shown to be capable of transducing livers of mice that were previously immunized by the original adenovirus serotype (Roy, S. et al., 1998). Another limiting step for Ad vectors is the availability of coxsackie–Ad receptors (CARs), which are the primary receptors for Ad in cancer cells. The expression of CAR greatly enhances the efficiency of Ad-mediated gene delivery and the manipulation of the receptor expression might be useful in achieving efficient Ad transduction (Bergelson, J.M. et al., 1997). Several groups have reported that the availability of CAR in the primary human tumor cells is highly variable (Cripe, T.P. et al., 2001, Fechner, H. et al., 2000, Li, Y. et al., 1999).

The other potential virus for cancer gene therapy is AAV, which was discovered first as a contaminant of Ad preparations. AAV is unable to replicate on its own and a co-infecting helper virus, Ad or herpesvirus, is needed for a productive infection (Atchison, R.W. et al., 1965). AAV belongs to the family of *parvoviridae* and to a separate genus designated *dependovirus*. Despite the many serotypes of AAV in human population, the virus has not been linked to any human illness. For review see (Goncalves, M.A., 2005). Recombinant AAV vector can efficiently transduce and express reporter gene to immunocompetent mice muscle tissue with no evidence of immune response (Xiao, X. et al., 1996). One of the main limitations of the AAV-based gene therapy is the small packaging capacity (about 4.7 kb) of the virus (Dong, J.Y. et al., 1996). A dual-vector heterodimerization, which joins two

independently transduced AAV genomes both encompassing a portion of a transcriptional unit, can be used to overcome this limitation (Yan, Z. et al., 2002).

In addition to being an important insect cell based recombinant protein expression system, recombinant baculoviruses are proving to be promising gene delivery vectors for gene therapy. Baculoviruses are able to transduce mammalian cells but they are incapable of replicating in them, rendering them safer vectors for gene therapy compared to pathogenic vector counterparts. For review see (Hu, Y.C., 2005, Kost, T.A. et al., 2005b). Baculoviruses are also able to transduce both dividing and non-dividing cells (van Loo, N.D. et al., 2001). The virus has been used for *in vivo* gene transfer with an efficiency comparable to Ads (Airenne, K.J. et al., 2000). In this study, some signs of inflammation were detected but these were also comparable to those of Ads (Airenne, K.J. et al., 2000). A number of studies have shown that the baculovirus with an appropriate eukaryotic promoter is able transfer and express target genes efficiently in several mammalian cell types (Boyce, F.M., and Bucher, N.L., 1996, Condreay, J.P. et al., 1999). The main limitations with baculovirus are its ability to induce immune responses (Abe, T. et al., 2003) and the inactivation of the virus by the complement system (Hofmann, C., and Strauss, M., 1998). Baculovirus could be one alternative to the *mda-7* based gene therapy, it possess many advantages as a gene therapy vector; the genome is large, thus the virus can carry multiple genes or large inserts, baculoviruses are easy to construct and produce to high-titers (Hu, Y.C., 2005).

1.2 Mda-7 cancer therapy

The ultimate goal of cancer therapy is the long-term elimination of tumor cells, without toxic effects on healthy tissues. The most common treatments used for cancer are surgery, radiotherapy and chemotherapy. These are all relatively non-specific and toxic to all cells in their mode of action. For review see (Rosenberg, S.A., 2001). New, so called targeted therapies has now become as an option. These biological targeted therapies are drugs, of which effects are targeted to a specific molecule or molecules involved in tumor growth and progression. For review see (Widakowich, C. et al., 2007). Gene therapy is one of the

promising new candidates for cancer treatment. For review see (Cross, D., and Burmester, J.K., 2006). The first approved clinical trial using gene therapy started in 1990. In that study, adenosine deaminase gene was transferred into the T cells by using retrovirus-mediated delivery (Blaese, R.M. et al., 1995). Cancer gene therapy has two main basic approaches: oncolytic (viro)therapy and immunotherapy. For review see (Cross, D., and Burmester, J.K., 2006).

Oncolytic vectors are used to infect cancer cells and to induce cell death through the propagation of the virus or expression of cytotoxic protein or by cell lysis. For reviews see (Cross, D., and Burmester, J.K., 2006, Donahue, J.M. et al., 2002). After viral infections and live virus vaccinations, anticancer effects have been reported with the patients. In 1950s there was an example of varicella-induced remission of acute leukemia (Bierman, H.R. et al., 1953). Similar responses occurred following vaccination of cancer patients when a smallpox vaccination induced remission of chronic leukemia (Hansen, R.M., and Libnoch, J.A., 1978). These observations have led to the use of viruses in cancer therapy. There are at least six different species of oncolytic viruses that have entered clinical trials. These include genetically engineered vaccinia, herpes simplex virus, adenovirus and measles. Non-engineered viruses include Newcastle disease virus and reovirus. For review see (Liu, T.C., and Kirn, D., 2007). The problem with the use of oncolytic viruses, is the activation of the complement system. It has been shown that the efficiency of delivering viruses into tumors is limited by innate antiviral activities present in the serum (Ikeda, K. et al., 1999). Another problem with the use of non-engineered oncolytic viruses as a single therapy has been the inefficacy of the treatment (Liu, T.C., and Kirn, D., 2007). For example in a clinical trial with Newcastle disease virus, most patients did not experience any measurable regression (Pecora, A.L. et al., 2002). As the non-engineered oncolytic viruses were unable to induce a complete and durable tumor eradication, the next step was to engineer the viruses to express therapeutic genes. This approach takes advantage of the viruses' ability to selectively induce the tumor mass and deliver therapeutic genes to target tissues. These non-replicating viral gene-delivery vehicles are usually used for delivery of tumorsuppressor genes, antiangiogenic genes or genes associated with the immune system. For review see (Hermiston, T.W., and Kuhn, I., 2002).

Immunotherapy belongs to the targeted therapies. It is a mode of therapy, which is used to increase the anti-cancer immunity (Mazzolini, G. et al., 2007). A major motivator in the tumor immunology studies was the recovery that administration of IL-2 can mediate the regression of human cancer (Lotze, M.T. et al., 1986). Studies performed with immunodeficient and immunocompetent mice have strongly supported the idea of a tumor suppressor role of the immune response. In those studies, it was shown that lymphocytes and IFNγ work together against development of sarcomas and spontaneous epithelial carcinomas (Shankaran, V. et al., 2001). It has also been suggested that this tumor suppressor role leads to the immunoselection of tumor cells that are more capable of surviving in an immunocompetent host (Shankaran, V. et al., 2001). The most potent immunity-mediated cancer therapies include genetically modifying T cells and blocking tumors inhibitory signals against the immune system. For review see (Xue, S.A., and Stauss, H.J., 2007).

Several studies demonstrating growth inhibition and apoptosis induction in various tumors suggest that *mda-7* has clinical potential as an anti-cancer drug (Sauane, M. et al., 2003, Sauane, M. et al., 2004, Wang, C.J. et al., 2006, Xue, X.B. et al., 2006). Mda-7 has many advantages that render it a potential cancer therapeutic compared to existing cancer therapeutics, these include the activity against broad range of tumor types, the lack of cytotoxicity against normal human cells (Allen, M. et al., 2004, Gopalan, B. et al., 2007, Ramesh, R. et al., 2004, Shi, H. et al., 2007, Su, Z.Z. et al., 1998), the ability to induce antitumor bystander activity (Chada, S. et al., 2004a, Sauane, M. et al., 2006, Su, Z. et al., 2005a), the ability to inhibit tumor angiogenesis (Ramesh, R. et al., 2003, Saeki, T. et al., 2002) and synergy with radiation, chemotherapy and monoclonal antibody therapies and immune modulatory activity. These anti-cancer effects have been confirmed with mda-7 using viral vector or as a fusion protein. For reviews see (Fisher, P.B. et al., 2007, Gupta, P. et al., 2006a).

1.3 Other interleukins in cancer therapy

There are over thirty interleukins identified so far. They play a major role in the immune system and many of them have been linked to autoimmune diseases and immune deficiencies

(Araujo, M.I. et al., 2004, Rott, O. et al., 1994, Sadlack, B. et al., 1995, Stern, A.S. et al., 1996). Some of them has also been shown to regulate cancer and are used in cancer therapy (Atkins, M.B. et al., 1999, Duvic, M. et al., 2006). Despite their immune system inducing functions, most of these cancer-regulating cytokines can also aid the growth of the tumor cells, instead of restraining it. They can enhance the growth of tumor cells directly by functioning as a growth factor or by increasing cell adhesiveness and enhancing tumor angiogenesis. They can also modulate the immune system by blocking cell mediated mechanisms for destroying the tumor cells. For review see (Nash, M.A. et al., 1999).

IL-2 has been the most successful therapy for metastatic renal cell carcinoma (RCC). It is the only therapy that has consistently induced complete durable remission in patients with RCC (Atzpodien, J. et al., 2004, McDermott, D.F. et al., 2005). Also in studies with patients with metastatic melanomas, the best results have been observed by using IL-2 therapy. In that study, high dose IL-2 treatment produced durable complete or partial response (Atkins, M.B. et al., 1999). IL-2 acts as an antitumor agent by increasing the cytolytic activity of cytotoxic T lymphocytes and natural killer cells. It also increases the gene expression responsible for encoding the lytic component of cytotoxic granules (Lotze, M.T. et al., 1981). IL-2 has been used in clinical applications but there has been a variety of adverse effects. Therefore very careful patient selection is needed before administering IL-2 therapy. For review see (Reang, P. et al., 2006).

IL-6 has both anti-cancer and pro-cancerous properties. Depending on the cell type and the presence of its receptors, it can either inhibit or stimulate cancer cell proliferation. For review see (Trikha, M. et al., 2003). IL-6 expression level is elevated during tumor growth. This has been shown in multiple studies (Nakashima, J. et al., 2000, Okada, S. et al., 1998, Preti, H.A. et al., 1997). Elevated levels of serum IL-6 are significant indicators of poor prognosis for patients with prostate cancer (Nakashima, J. et al., 2000). It has been demonstrated that anti-IL-6 antibody induces prostate tumor apoptosis and regression of xenografted human cancer cells in a nude mouse model (Smith, P.C., and Keller, E.T., 2001).

IL-12 is a highly effective cytokine, which induces antitumor immunity. IL-12 is able to restrain tumors growth and its production by macrophages is inhibited by tumor cell derived

soluble factors e.g. prostaglandin E_2 (Mitsuhashi, M. et al., 2004). There have been several studies according to the antitumor activities of IL-12. For review see (DeGroot, L.J., and Zhang, R., 2001). In one of the studies, an adenoviral vector carrying *IL-12* was directly injected into neuroblastoma tumors in mice, most of the injected mice had tumor regression and almost half of the mice completely rejected their tumors (Davidoff, A.M. et al., 1999). In another study, the most potent antitumor effect achieved with IL-12 was encountered by using a combination of IL-12, IL-18 and IL-1. Different combinations of these interleukins were tested to cause regression of implanted adenocarcinoma in mice (Oshikawa, K. et al., 1999). Phase I and phase II clinical trials with IL-12 have shown clear antitumor activity and it was found to be relatively well tolerated (Duvic, M. et al., 2006).

Similar to IL-6, IL-18 is also a cytokine with dual effects on cancer. It can activate the cells of the immune system by binding to the AP-1 site in the IFN-γ promoter region (Barbulescu, K. et al., 1998). It is also expressed and secreted in common skin tumors including squamos cell carcinoma, melanoma and skin cancer cell line, and the enhanced expression of IL-18 is positively correlated with malignancy of skin tumors (Park, H. et al., 2001). Similarly in gastric cancer patients, IL-18 expression has found to be higher in tumor region than it was in non-tumor region (Ye, Z.B. et al., 2007). IL-18 has also been postulated to stimulate angiogenesis in gastric cancer by stimulating trombosponding-1 production, which is a pro-angiogenic factor (Kim, J. et al., 2006). Despite of its ability to activate the immune system it is also able to give melanoma cells resistance to the immune system by inducing Fas ligand expression (Cho, D. et al., 2000). Therefore, although IL-18 can activate the immune system of the host, therapy using IL-18 must be considered carefully because of its ability to also stimulate aggressiveness of cancer. For review see (Park, S. et al., 2007).

2. AIMS OF THE STUDY

The aims of the study were:

1. To produce mda-7-His fusion protein in *Sf*9 and Mimic^{TM} insect cells
2. To optimize the production and purification protocols
3. To characterize the proteochemical properties of the purified fusion proteins
4. To test the induction of apoptosis by the mda-7-His fusion proteins in human cancer cells

3. MATERIALS AND METHODS

3.1 Production of the recombinant baculovirus

The recombinant baculovirus *Ac*mda-7-His used in this study was kindly provided by Anna R. Mäkelä (University of Jyväskylä, Finland). Briefly, the gene encoding the C-terminally His-tagged mda-7-fusion protein (mda-7-His) was cloned under the polyhedrin promoter in the pFastBac™Dual transfer vector (Invitrogen, Carlsbad, CA) using *Not*I and *Xba*I restriction enzymes (Fig. 1). The recombinant virus was generated with the Bac-to-Bac® Baculovirus Expression System (Invitrogen).

The recombinant baculoviruses and proteins were produced in *Spodoptera frugiperda* 9 insect cells (*Sf*9, Gibco-BRL, Grand Island, NY). The cells were grown in a suspension culture (2 x 10^6 cells/ml) in a shaking incubator at 28°C using Insect-XPRESS™ medium (Bio-Whittaker, Lonza, MD), and infected with a multiplicity of infection (MOI) 0.1-1 to produce high-titer virus. The supernatant was harvested and cleared at 72 h post infection (p.i.) by removing the cells and large debris by centrifugation 1000 x g, 10min, +4°C and 2800 x g, 20 min, +4°C (Hermle Z 513 K, Hermle Labortechnik GmbH, Wehingen, Germany). The virus-containing medium was supplemented with fetal calf serum (FCS; PAA Laboratories GmbH, Austria) to a final concentration of 2.5% and stored at +4ºC. Infectious viral titers were determined by end-point dilution assay using standards protocols (O´reilly, 1994). The titer of *Ac*mda-7-His was 2.65 plaque forming units/ml (pfu/ml).

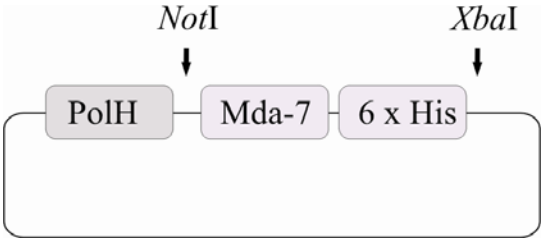


Figure 1. The pFastBac™Dual transfer vector construct for *Ac*mda-7-His. The C-terminally His-tagged mda-7-fusion protein (mda-7-His) was cloned under the polyhedrin promoter using *Not*I and *Xba*I restriction enzymes.

3.2 Production and purification of the mda-7-His fusion protein

3.2.1 Optimization of mda-7-His protein production in *Sf*9 cells

The *Sf*9 cells (2 x 10^6 cells/ml) were infected with *Ac*mda-7-His with a MOI of 0.1-10, to determine the optimal virus concentration for achieving the highest protein production efficiency. Additionally, the supernatant was harvested at 24-120 h p.i to determine the optimal production time. The harvesting was conducted by removing the cells and large debris by centrifugation first at 1000 x g for 10 min at +4°C and subsequently at 2800 x g for 20 min at +4°C (Hermle Z 513 K, Hermle Labortechnik GmbH, Wehingen, Germany). Subsequently, the protein-containing medium was kept on ice at +4°C. To remove the virus, the medium was centrifuged at 11953 x g for 6 h, +4°C (Sorwall RC-5, SS-34 rotor, Sorwall Products, DuPont Co., Wilmington, DE). For analysis by sodium-dodecyl sulphate electorophoresis (SDS-PAGE) and Western blotting, samples of infected cells, budded virus, and mda-7-His fusion protein-containing medium were acquired from every step of the production.

3.2.2 Production of mda-7-His protein in *Sf*9 and Mimic™ cells

The mda-7-His fusion protein was produced in *Sf*9 and Mimic™ cells (Invitrogen). The cells were infected with a MOI of 1 and the supernatants were harvested at 72 h p.i. These conditions were defined by optimization of the infection time and viral amount. Protein production was conducted using the same procedure as in section 3.2.1 for the exception that the Mimic™ cells were grown and infected in a monolayer culture without agitation at a density of 1 x 10^6 cells/ml.

3.2.3 Purification of mda-7-His

Virus-free supernatant was dialyzed against 100 x volume of phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.8 mM KH2PO4; pH 7.4) overnight at +4°C. The mda-His fusion protein was purified by using nickel affinity chromatography. The Streamline™ chelating resin (GE healthcare, Buckingshamshire, UK) was first washed with 3 column volumes of sterile deionised water, followed by activation with 5 column volumes of charge-buffer (50 mM $NiSO_4$) and balanced with 3 column volumes of binding-buffer (Binding Buffer; 5 mM Imidazole, 300 mM NaCl, 20 Mm Tris pH 7.4). The resin and the mda-7-His-containing medium were coincubated overnight in a shaker at +4°C. The medium was allowed to flow through the column and the resin was washed with 6 column volumes of binding buffer and with 6 column volumes of washing buffer (Wash buffer; 20 mM Imidazole, 300 mM NaCl, 20 Mm Tris pH 7.4). The recombinant mda-7-His was eluted with the elution buffer (Elution Buffer; 500 mM Imidazole, 300 mM NaCl, 20 Mm Tris pH 7.4) and the fractions were collected. From each purification step, SDS-PAGE samples were taken. The collected fractions and the samples of the purification steps were analyzed by SDS-PAGE and Western blotting. The fractions containing the purified protein were pooled and concentrated by centrifugation first at 3992 x g for 1 h 15 min at +4°C (megafuge 1.0R Heraeus) and then after resuspension to PBS again at 3992 x g for 45 min at +4°C using Amicon® Ultra-15 Centrifugal Filter Devices 10,000 NM/WL (Amicon millipore, Cork, Ireland). Total protein concentrations of the samples were measured with Nanodrop ND-1000 Spectometer (NanoDrop Technologies Inc., Wilmington, DE). The concentration of mda-7-His protein was visually estimated from gels stained for total protein with Fermentas Pageblue™ (Fermentas; Burlington, ON, Canada) to be approximately half of the total protein concentration.

3.3 Characterization of mda-7-His

3.3.1 SDS-PAGE and Western blotting

SDS-PAGE samples were collected during protein production, purification and characterization. The infected cells from the protein productions were centrifuged at 400 x g for 3 min (Eppendorf centrifuge 5415D; Eppendorf, Hamburg, Germany) and the pellets were suspended in 2 x laemmli buffer + 5% β-mercaptoethanol (2x laemmli buffer + β-mercaptoethanol; 63 mM Tris-HCl, 25% [v/v] glycerol, 2% SDS, 0.01% bromophenol blue [Sigma-Aldrich], 5% β-mercaptoethanol [Merck]) to a final concentration of 10^4 cells/µl. The protein-containing medium samples were suspended in 5 x laemmli buffer + β-mercaptoethanol to obtain 1 x concentration. The samples were then boiled for 5 min at 95ºC. An unstained marker representing molecular weights of 14.4 kDa to 116 kDa was used (MBI Fermentas cat. no #SM0431, Vilnius, Lithuania). The estimated protein concentrations for the characterization of the fusion proteins were 2.3 µg/ well for mda-7-His produced in *Sf*9 cells and 40.5 µg/ well for mda-7-His produced in Mimic^{TM} cells

The proteins were separated in electrode buffer (Electrode buffer; 25 mM Trizma base, 0.2 M glycine [Riedel-deHaën], 3.5 mM SDS) using vertical (1.5 mm) 5% stacking (5% stacking gel ; 5% acrylamide/Bis solution [29:1] [Bio-Rad Laboratories], 0.125 M Tris-HCl [pH 6.8], 0.1% SDS, 0.1% [w/v] ammoniumpersulfate [Bio-Rad Laboratories], 0.1% [v/v] TEMED [N,N,N',N'-tetramethylene diamine; Bio-Rad Laboratories]) and 15% resolving gels (15% resolving gel ; 15% acrylamide/Bis solution [29:1] [Bio-Rad Laboratories], 0.375 M Tris-HCl [pH 8.8], 0.1% SDS, 0.1% [w/v] ammoniumpersulfate [Bio-Rad Laboratories], 0.04% [v/v] TEMED [N,N,N',N'-tetramethylene diamine; Bio-Rad Laboratories]) and Mini 2-D electrophoresis Cell apparatus (Bio-Rad Laboratories). The current of 100 V was used to run the samples through the stacking gel, the run was continued with 180V current for 60 minutes.

The SDS-PAGE gels were stained with Fermentas Pageblue to visualize the total protein amount of the samples. Gels were stained at RT overnight with rocking. After the staining, the gels were washed with sterile deionised water and dried with BIO-RAD 583 gel dryer (Bio-Rad Laboratories) in 80ºC for 90 min.

Western blotting was used to transfer the proteins from the gel to the nitrocellulose paper (pore size 0.45µm). The Western blotting was performed using Mini-PROTEAN 3 cell electroblotting apparatus (Bio-Rad Laboratories), the apparatus was kept in cold using ice and cold transfer buffer (Transfer buffer; 25 mM Trizma base, 0.2 M glycine, 20% [v/v] methanol). The blotting was conducted with current of 100V and was continued for 60 minutes. To visualise the molecular weight marker and total proteins the blots were stained with 0.2 Ponceau S (0.2% Ponceau S ; 0.2% [w/v] Ponceau S [Sigma], 5% [v/v] acetic acid) for 5 minutes at RT and washed with sterile deionised water. After marking the markers, the blots were blocked overnight or for 120 minutes with rocking in 5 % milk in Tris-buffered saline (TBS; Tris-buffered saline; 20 mM Trizma base, 0.5 M NaCl)supplemented with 0.05% Tween® 20 (Fluka Chemie GmbH, Buchs, Switzerland) (milk-TBS-Tween).

Immunolabeling was used to detect the protein from the nitrocellulose paper. All the antibodies used were diluted in milk-TBS-Tween. The blots were incubated with primary antibodies for 60 minutes at RT with rocking. Primary antibodies used were; rabbit polyclonal anti-His diluted in 1:3000 (1 mg/ml; Immunology consultant laboratory, lake Oswego, Oregon) and rabbit polyclonal anti-mda-7 (0.5 mg/ml; Abcam, Cambridge, USA) diluted in 1:2000. After the incubation the blots were washed 3 x 5 min with TBS-Tween. As a secondary antibody an alkaline phosphatase-conjugated goat anti-rabbit IgG (1 mg/ml; Promega, Madison, WI) diluted in 1:5000 was used. The blots were incubated with secondary antibodies for 60 min at RT with rocking and after the incubation, the blots were washed again for 3 x 5 min with TBS-Tween. Before the immunodetection, the blots were equilibriated in alkaline phosphatase assay buffer (APA; alkaline phospatase assay buffer ; 0.1M Trizma base , 0.1 M NaCl, 5 mM MgCl2) for 5 min. The protein bands were detected using APA buffer with 0.33 mg/ml nitro blue tetrazolium (NBT; Sigma-Aldrich; in 70% dimethylformamide [Merck]) and 0.165 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate

p-toluidine salt, Sigma-Aldrich; in 100% dimethylformamide [Merck]). After the detection, the blots were washed with sterile deionised water and air dried.

3.3.2 Immunolabeling of infected *sf*9 cells

To analyze whether the recombinant baculoviruses and mda-7-His fusion protein was correctly produced and secreted, *sf*9 cells were immunolabeled and analyzed by using confocal microscopy. The *sf*9 cells were allowed to attach to glass coverslips for 1 h at 28ºC followed by infection with *Ac*mda-7-His. At different times p.i., the cells were fixed with 4% paraformaldehyde (PFA; Merck) in PBS for 20 min at RT.

The cells were doublelabeled to detect the gp64 viral envelope protein and the mda-7-His fusion protein. Prior to the labelling, the cells were permeabilized with 0.1% Triton X (Sigma-Aldrich) diluted in 1.5% BSA-PBS (BSA; Roche diagnostics corporation, Indianapolis, IN). The antibody dilutions were also prepared in the same permeabilization buffer. Primary antibodies used were mouse monoclonal anti-gp64 (B12D5; kindly provided by Dr. Loy Volkman from the university of California, Berkeley, CA) diluted in 1:50 and rabbit polyclonal anti-mda-7 diluted in 1:100. The cells were incubated with the primary antibodies for 60 minutes at RT, after the incubation cells were washed 3 x 15 min with 1.5% BSA-PBS. Secondary antibodies used were goat anti-mouse IgG conjugate Alexa Fluor 488 (green; 2 mg/ml; Invitrogen Molecular Probes; 1:200) and goat anti-rabbit IgG conjugate Alexa Fluor 546 (red; 2 mg/ml; Invitrogen Molecular Probes; 1:200). Cells were incubated with secondary antibodies for 30 min at RT in the dark. After the incubation, the coverslips were washed 3 x 15 min with 1.5% BSA-PBS and mounted to microscope slides using Mowiol (Mowiol 4-88 in glycerol and Tris-HCl; Calbiochem, Darmstadt, Germany) supplemented with 25 mg/ml Dabco (1,4-diazobicyllo-[2.2.2]-octane; Aldrich, Steinheim, Germany).

3.4 Apoptosis assays

3.4.1 Mammalian cell culture

To test the fusion proteins ability to induce apoptosis and restrain cell growth two human cancer cell lines were used; Breast cancer cell line MDA-MB-435 (Pirjo Laakkonen, University of Helsinki, Finland) and prostate adenocarcinoma cell line PC-3 (American Type Culture Collection, Manassas, VA; number CRL-1435™). The cells were maintained as monolayer cultures in Dulbecco's Modified Eagle's Medium (D-MEM; Gibco® Invitrogen Life technologies, Paisley, UK) supplemented with 10% (v/v) heat inactivated FCS and 1% (v/v) penicillin- 1% (v/v) streptomycin mixture (Gibco®). The D-MEM used with PC-3 was additionally supplemented with 10 mM Hepes pH 7.3 (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid, MP Biomedicals LLC Eschwege, Germany) and 2mM L-glutamine (Gibco®). The cells were grown in 37ºC in a humidified incubator with atmosphere of 5% of CO_2.

3.4.2 Trypan blue staining

Trypan blue staining was conducted with MDA-MB-435 and PC-3 human cancer cell lines to determine the ratio of dead and viable cells. Trypan blue solution 0.4% (Sigma) is able interact only with the dead cells because of their damaged cell membrane. To test the proteins ability to induce cell death in human cancer cells, the MDA-MB-435 and PC-3 cells were allowed to attach overnight or for 48h, respectively, at 37ºC. The cells were then transduced with fusion proteins using different concentrations (100 ng/ml, 250 ng/ml, 500 ng/ml, 1000 ng/ml). The cells were stained and counted at 24-120 h post transduction (p.t.). The cells were detached by scraping into the medium and stained according to the manufacturer´s instructions and the viable and non-viable cells were counted under the light microscope. Three samples from each time point or concentration were counted three times.

3.4.3 Annexin V staining

The Annexin V staining was conducted with MDA-MB-435 and PC-3 human cancer cell lines to define the ability of the fusion proteins to induce apoptosis in cancer cells. Annexin V has high affinity to the membrane phospholipid phosphatidylserine (PS), which in apoptotic cells is exposed to the extracellular environment. Thus the apototic cells can be identified using fluorochrome conjugated, in this case FITC conjugated Annexin V (Annexin V-FITC; BD PharMingen, UK).

MDA-MB-435 and PC-3 cells were allowed to attach on coverslips overnight or for 48h, respectively, at 37ºC. The MDA-MB-435 cells were then transduced with the mda-7-His fusion protein produced in *Sf*9 cells (500 ng/ml). The cells were fixed with 4% PFA-PBS for 20 minutes at RT at different time points p.i.. The cells were incubated in the dark for 20 minutes at RT with 5% Annexin V in Annexin V Binding buffer (BD PharMingen, UK). After the incubation, the coverslips were washed 3 x 15 min with 1.5% BSA-PBS and mounted to microscope slides using Mowiol-Dabco.

PC-3 cells were transduced with both fusion proteins using different protein concentrations (100 ng/ml, 500 ng/ml, 1000 ng/ml) for 120h. After the transduction the cells were scraped and washed twice with PBS. The cells were then stained with Annexin V-FITC according to the manufacturers instructions. The flow cytometry was conducted immediately after staining. The Annexin samples were analyzed with FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany). The percentages of the apoptotic cells were calculated using Cellquest software. From every sample 3x 10 000 cells were analyzed.

3.4.4 Confocal microscopy

The samples were visualized with a confocal microscope (Carl Zeiss Axiovert 100 M, Carl Zeiss Inc., Jena, Germany) equipped with an LSM 510 58 laser-scanning confocal module. The microscope contains a 488 nm argon laser and 543 nm and 633 nm helium-neon lasers.

Plan-apochromat 63 x /1.40 oil objective was used. The fluorescence and differential interference contrats (DIC) images were acquired and processed using LSM 510 software. Fluorochromes were detected through different channels to avoid false colocalization.

4. RESULTS

4.1 Protein production, purification and characterization

4.1.1 Production of mda-7-His fusion protein

The recombinant baculovirus *Ac*mda-7-His was cloned under the polyhedrin promoter in the pFastBac™Dual transfer vector. High-titer viruses were produced in *Sf*9 insect cells.

To analyze whether the mda-7-His fusion protein was correctly expressed and secreted from the *Sf*9 cells, the *Ac*mda-7-His infected cells were immunolabeled and analyzed by confocal microscopy (Fig. 2A). The cells were doublelabeled with antibodies raised against the baculovirus major envelope protein gp64 (green) and mda-7 (red). At 72 h p.i., gp64 was detected to normally localize on the plasma membrane whereas the mda-7-His fusion protein localized in the cytoplasm, likely in the endoplasmic reticulum, on its way to be secreted out of the cell.

4.1.2 Optimization of the protein production

The optimization of the protein production was conducted using *Sf*9 cells. The cells were infected with *Ac*mda-7-His with a MOI of 0.1-10 and the supernatant was harvested at different points in time p.i. (from 24 h to 120 h). The cell-free medium and the infected cells were analyzed by SDS-PAGE and Western blotting. Within the first 24 h p.i., no mda-7-His was detectable in the medium. The protein production was the most efficient between 48 h and 72 h p.i. (Fig. 2B), after which the level of protein expression started to decrease. Based on these results, the optimal virus concentration was determined at 72 h p.i. Surprisingly, the viral amount appeared to have a less significant impact on the efficiency of mda-7-His production. The expression level of mda-7-His seemed to be equally efficient with all virus

concentrations tested (Fig. 2C). Consequently, the mda-7-His was constantly produced by infecting *Sf*9 cells with *Ac*mda-7-His at a MOI of 1 for 72 h.

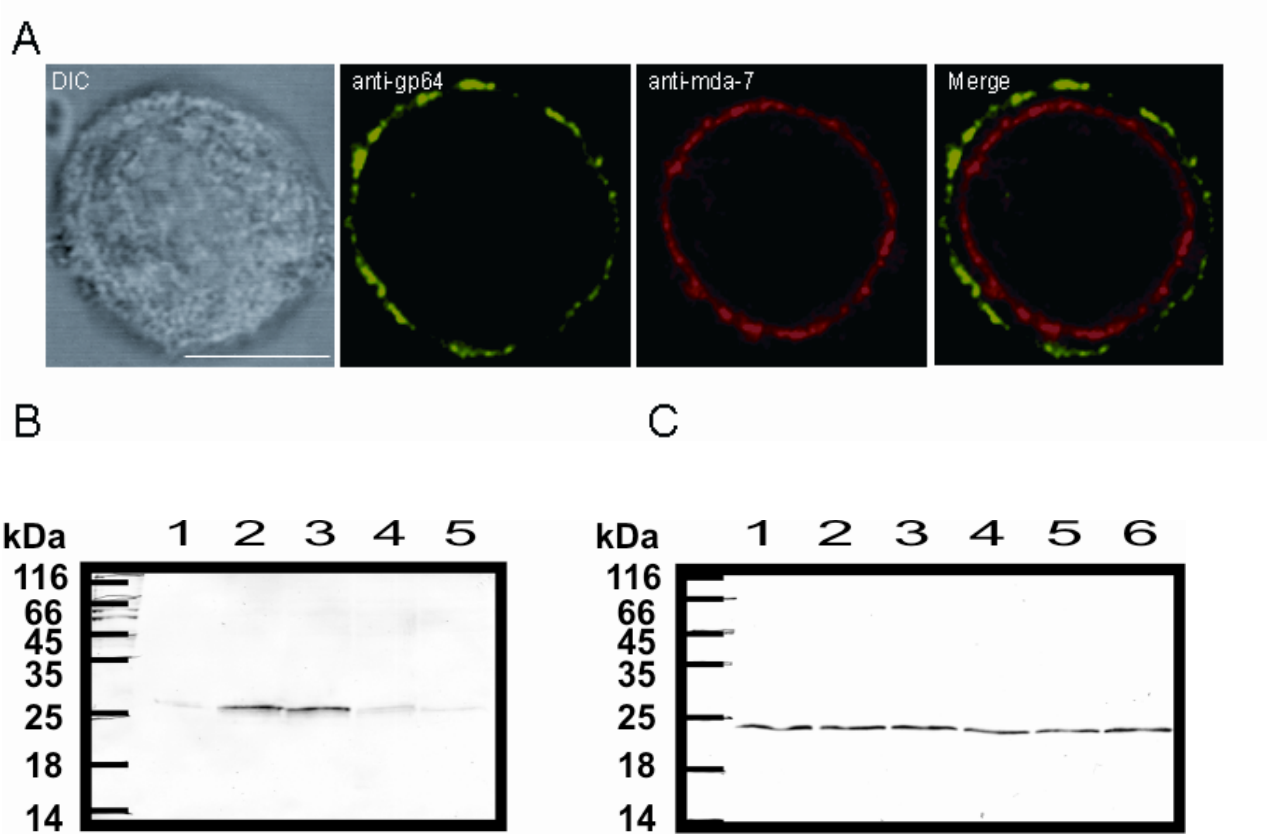


Figure 2. Expression and subcellular localization of the mda-7-His fusion protein in *Sf*9 cells. A) The *Sf*9 cells were infected at a MOI of 3 with *Ac*mda-7-His and analyzed by confocal microscopy at 72 h p.i. The *Ac*MNPV gp64 was detected with the mouse monoclonal B12D5 gp64-specific primary and Alexa Fluor® 488 secondary antibodies (green), and the mda-7-His fusion protein with the rabbit polyclonal mda-7 primary and Alexa Fluor® 546 secondary antibodies (red). The images are single confocal midsections of approximately 0.8 µm in thickness (DIC, differential interference contrast). Scale bars 10 µm. B) Western blot analysis of the optimal infection time for mda-7-His production in *Sf*9 cells with *Ac*mda-7-His at a MOI of 1 at 24 h (lane 1), 48 h (lane 2), 72 h (lane 3), 96 h (lane 4), and 120 h (lane 5) p.i. (C) Western blot analysis of the optimal virus concentration for mda-7-His production in *Sf*9 cells with *Ac*mda-7-His at a MOI of 0.1 (lane 1), 0.5 (lane 2), 1 (lane 3), 2.5 (lane 4), 5 (lane 5), and 10 (lane 6).

4.1.3 Purification and characterization of the mda-7-His fusion protein

The fusion proteins were produced in two different insect cell lines, *Sf*9 and Mimic™. Insect cells are usually unable to process complicated glycosylation, which can affect the structure and function of the expressed protein. Mimic™ cells are a derivative of the parental *Sf*9 cell line. The cells have been genetically engineered to stably express a variety of mammalian glycosyltransferases, which allows the insect cells to process complex N-glycans to a similar extent with mammalian cells (Hollister, J. et al., 2002). The mda-7-His fusion protein was produced in these two cell lines to investigate the role of glycosylation in the activity and function of the protein.

The mda-7-His fusion protein was purified by using nickel affinity chromatography. The samples obtained during the purification and from the purified fractions were characterized by SDS-PAGE and Western blotting (Fig. 3A and 3B). In the samples obtained during the purification steps, mda-7-His was detected from the infected cells and also from the virus pellet sample in the *Sf9* production. The eluted fractions contained some impurities, especially the fractions obtained from the Mimic™ production. The protein concentrations of the pooled and concentrated proteins were 0.5 mg/ml and 0.9 mg/ml for the mda-7-His produced in *Sf*9 and Mimic™ cells, respectively. For Western blotting, approximately 2.3 µg of the mda-7-His produced in *Sf*9 cells (Fig. 3C, lane 1) and 40.5 µg of the mda-7-His produced in Mimic™ cells (Fig. 3C, lane 2) were applied per well. However, visual estimation of the blots revealed the protein quantities to be quite the opposite, the amount of mda-7-His fusion protein produced in *Sf*9 cells was 5-fold higher than that of mda-7-His produced in Mimic™ cells. This is probably due to the impurities in the samples, contributing to difficulties to determine the actual concentration of mda-7-His.

There were some differences between the molecular weights of the protein samples produced in different cells, probably due to differences in post-translational modifications especially in glycosylation of the proteins. The protein sample from the *Sf*9 cells had only one band which was about 23 kDa in size (Fig. 3C, lane 1). The protein sample from the Mimic™ cells had

two main bands, the other one was about 25 kDa and the other one was around 45 kDa in size and there were also some other lighter bands (Fig. 3C, lane 2).

The infected cell samples showed three different bands around 25 kDa (Fig. 3C). For Western blotting, 100 000 *Sf*9 cells and 400 000 Mimic^{TM} cells were applied per well. Visual estimation of the blots shows that the protein production with *Sf*9 cells was far more efficient than the production with Mimic^{TM} cells. The proteins were detected with polyclonal antibodies raised against the His-tag (Fig. 3) and mda-7 (not shown).

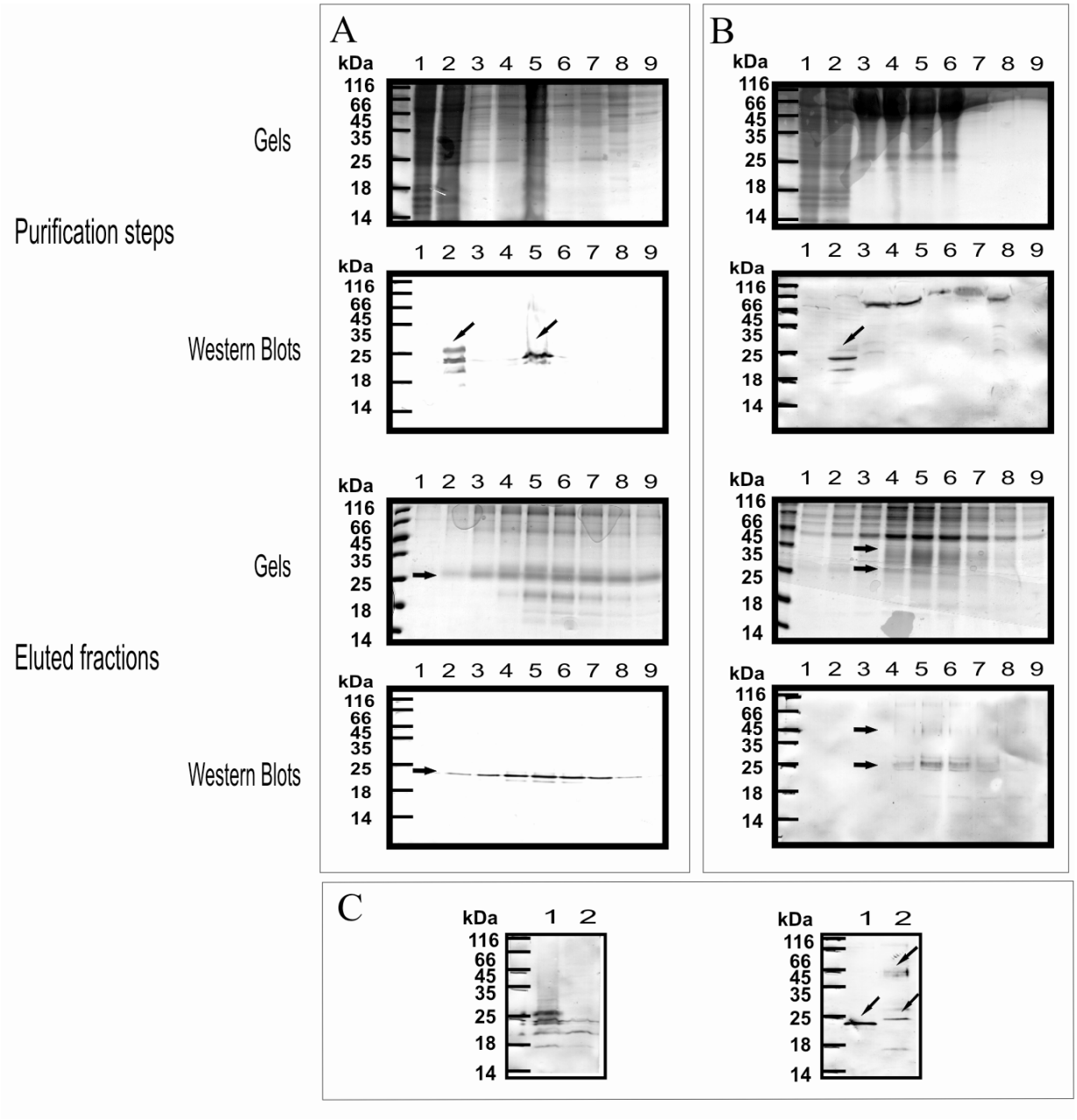


Figure 3. Purification and characterization of mda-7-His fusion protein. Coomassie stained gels and Western blots from A) the *Sf*9cell production and from B) the Mimic^{TM} cell production. Samples in the purification steps; non-infected *Sf*9 cells (lane 1), infected *Sf*9 or Mimic^{TM} cells (lane 2), virus containing supernatant (lane 3), virus-free supernatant (lane 4), virus pellet (lane 5), flow through (lane 6), wash with binding buffer (lane 7), washes with wash buffer (lanes 8 and 9). C) Characterization of the purified proteins. Infected cell samples (left) and protein samples (right). Amount of infected cells were 100 000 cells/well and 400 000 cells/well for the mda-7-His produced in *Sf*9 (lane 1) and Mimic^{TM} cells (lane 2), respectively. Protein produced in the *Sf*9 cells (lane 1) showed only one band around 23 kDa and the Mimic^{TM} produced protein (lane 2) showed two main bands, around 25 kDa and 45 kDa. The protein concentrations per well were 2.3 µg and 40.5 µg for the mda-7-His produced in *Sf*9 and Mimic^{TM} cells, respectively. The proteins were detected with anti-His antibody.

4.2 Apoptosis assays

4.2.1 Apoptosis assay with MDA-MB-435 human cancer cell line

The ability of mda-7-His to induce apoptosis was first tested in MDA-MB-435 cell line with the fusion protein produced in *Sf*9 cells. To visualize apoptotic cells, the samples were labeled with FITC-conjugated Annexin V and analyzed by confocal microscopy. After 96 h of incubation, there were significantly more apoptotic cells in the samples transduced with mda-7-His than there were in the control samples treated with PBS (Fig. 4A).

To further characterize the apoptotic activity of mda-7-His, a live/dead cell assay using Trypan blue staining was performed at 24 - 120 h p.t. At 24 h p.t., no difference was detectable in the percentage of dead cells between the samples treated with mda-7-His or PBS. At 72 h p.t., the percentage of dead cells was 32% with mda-7-His and 17% with the control, and at 120 h p.t. the corresponding percentages were 60% and 30% (Fig. 4B). From these results it can be concluded that the mda-7-His produced in *Sf*9 cells is able to induce significant cell death in MDA-MB-435 cells.

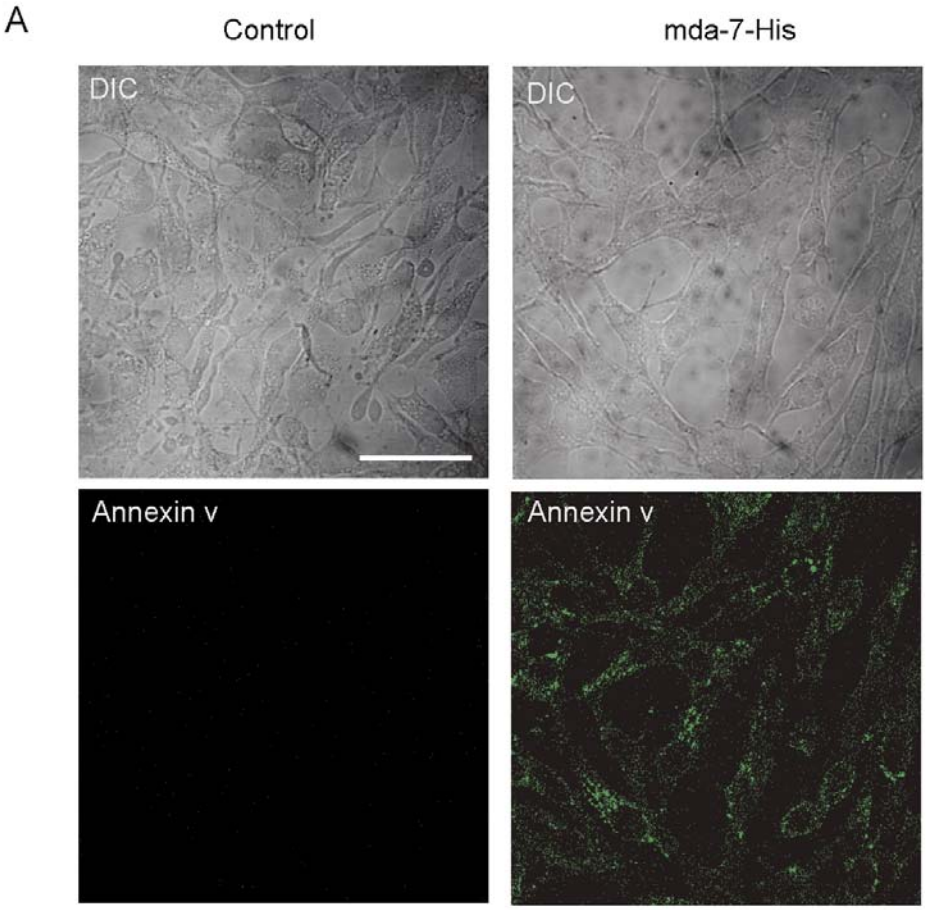


Figure 4. Apoptosis assay with mda-7-His fusion protein produced in *Sf*9 insect cells using MDA-MB-435 human cancer cells. A) Confocal microscopy images of tumor cells cultured with either mda-7-His or PBS (control). The images were acquired at 96 h p.t. and the cells were labeled with Annexin V-FITC (green). DIC, differential interference contrast. Scale bar, 40 μm. B) The cells were treated with PBS (control) or transduced with mda-7-His for different times after which the cells were stained with Trypan blue and the portion of the dead cells was calculated. The percentages of dead cells (± standard deviation) are indicated. Results from an independent representative experiment are shown.

4.2.2 Apoptosis assays with PC-3 human cancer cell line

After first defining the ability of mda-7-His to induce apoptosis with the protein produced in *Sf*9 cells, tests were conducted with human PC-3 prostate cancer cells to define and compare the proapoptotic activity of mda-7-His produced in *Sf*9 and Mimic^{TM} cells. These tests were conducted with different concentrations of the recombinant proteins and analyzed by Trypan blue staining or Annexin V labeling followed by flow cytometry.

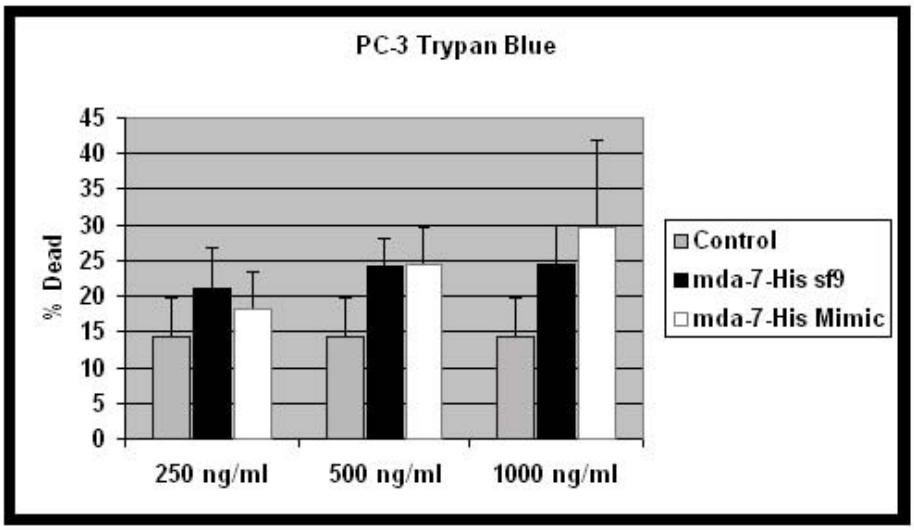
Trypan blue staining was performed using different concentrations of the proteins (250 ng/ml, 500 ng/ml and 1000 ng/ml) with 96 h transduction (Fig. 5A). Both proteins appeared to induce PC-3 cells cell death and the effect seemed to be concentration related. With protein concentrations of 100 ng/ml, the percentage of dead cells were 21% with mda-7-His produced in *Sf*9 cells and 18% with the mda-7-His produced in Mimic^{TM} cells and 14% with the control. At concentrations of 1000 ng/ml the corresponding percentages were 25%, 29% and 14%, respectively.

Annexin V-FITC labeling and flow cytometric analysis was conducted with the PC-3 cells. The cells were incubated with different concentration (100 ng/ml, 500 ng/ml and 1000 ng/ml) of the fusion proteins or with the corresponding amount of PBS for 120 h. The cells were then analyzed with flow cytometer (Fig. 5B). Both proteins appeared induce apoptosis to PC-3 cells and the effect seemed to be concentration related. With protein concentrations of 1000 ng/ml the percentage of apoptotic cells was 26% with mda-7-His produced in *Sf*9 cells and 25% with the mda-7-His produced in Mimic^{TM} cells higher than the controls. The incubation times were chosen on the basis of the results from tests conducted with MDA-MB-435 cell line.

Due to the difficulties to determine the effective concentration of mda-7-His, the protein concentrations used in these tests are only rough estimates and the comparisons between the proteins cannot be accurately made. As a result, it can be concluded that both of the proteins are able to induce apoptosis in PC-3 cancer cells and it is probable that the protein produced in Mimic^{TM} cells is more efficient. This assumption can be derived from the fact

that the concentration of the protein produced in Mimic™ cells has been heavily overestimated (see section 4.1.3).

A



B

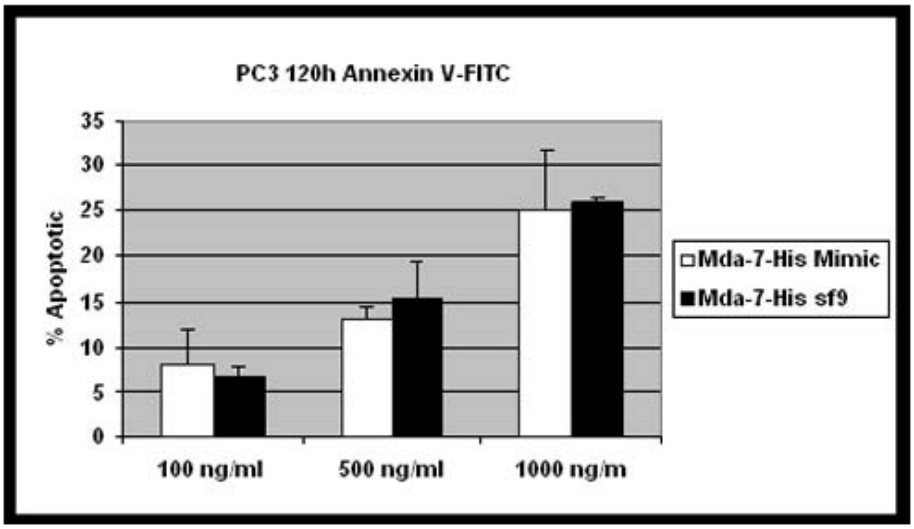


Figure 5. Apoptosis assay with mda-7-His fusion proteins using PC-3 human cancer cell line. The cells were treated with PBS (control) or transduced with different concentration of the fusion proteins A) for 96 h after which the cells were trypan blue stained and the portion of the dead cells was calculated. B) cells were transduced for 120 h after which the cells were Annexin V labeled and analyzed with flow cytometer. The expression percents were conducted by reducing the expression percents of the control cells. The percentages of apoptotic cells (± standard deviation) are indicated. Results from independent representative experiments are shown.

5. DISCUSSION

Mda-7 displays selective apoptosis-inducing activity in a wide variety of tumors without damaging normal cells. In this study, mda-7-His fusion protein was produced in *Sf*9 and Mimic^{TM} insect cells. Mimic^{TM} cells are genetically engineered to stably express a variety of mammalian glycosyltransferases, allowing the insect cells to process complex N-glycans to a similar extent with mammalian cells (Hollister, J. et al., 2002). Furthermore, the production and purification protocols of the proteins were optimized and the proteochemical properties were characterized. The protein products from these insect cell lines were found to have different forms likely due to the different glycosylation pattern of the fusion proteins. According to the molecular sizes of the proteins and on the basis of previous studies, it was concluded that the protein produced in *Sf*9 cells lack glycosylation and the protein produced in Mimic^{TM} cells was properly glycosylated. The fusion proteins were then tested for their ability to induce apoptosis in human cancer cells. Despite of the differencies in the glycosylation of the proteins, both of the fusion proteins were found active and were able to effectively induce apoptosis in human cancer cells.

5.1 Protein production, purification and characterization

Mda-7 is a tumor suppressor gene, which belongs to the IL-10 family of cytokines and it is also known as IL-24 (Huang, E.Y. et al., 2001). It has the ability to selectively restrain growth and induce apoptosis in a wide spectrum of human cancer cells (Allen, M. et al., 2004, Gopalan, B. et al., 2007, Ramesh, R. et al., 2004, Shi, H. et al., 2007, Su, Z.Z. et al., 1998). Mda-7 is an actively secreted and heavily N-glycosylated protein (Caudell, E.G. et al., 2002). There are many forms detected of the secreted protein, it can exist as a monomer (Chada, S. et al., 2004b), a dimer or as a tetramer (Mumm, J.B. et al., 2006). The effects of post-translational modifications, especially glycosylation, on the function of the protein have been actively studied with controversial results (Chada, S. et al., 2004b, Mumm, J.B. et al., 2006, Sauane, M. et al., 2006).

In this study, mda-7-His was produced in baculovirus-insect cell expression system (BEVS). Similarly, a functional C-terminally His-tagged mda-7 has previously been constructed by another group (Wang, M. et al., 2002). This fusion protein was produced in a stable 293T cell line constitutively secreting mda-7-His. The ability of the protein to induce apoptosis was not tested, but the protein was found to be active, as it was able to bind to its receptors and induce STAT activation. The same group has also reported the C-terminal fusion of mda-7 to the human placental alkaline phospatase (mda-7-AP) possessing activity comparative to mda-7-His (Wang, M. et al., 2002). In addition, rhMda-7 (Ekmekcioglu, S. et al., 2003) and GST-mda-7 fusion proteins has been constructed (Sauane, M. et al., 2004, Su, Z.Z. et al., 2003, Yacoub, A. et al., 2004), and all of these fusion proteins have been found active (see section 1.1.7). Mda-7 has already been produced in bacterial (Sauane, M. et al., 2004, Yacoub, A. et al., 2004) and mammalian expression systems (Ekmekcioglu, S. et al., 2003), but no published data exist on the production of mda-7 with BEVS. BEVS was used for its many advantages, including the high yield of heterologous proteins and simplicity of the system. The ability of insect cells to perform many of the post-translational modifications found in eukaryotic cells is a major advantage over prokaryotic expression systems (Hu, Y.C., 2005, Kost, T.A. et al., 2005a). Here the mda-7-His fusion protein was correctly produced and secreted from the insect cells. The protein production was most efficient between 48 h and 72 h p.i (Fig. 2B), and the production was equally efficient with all virus concentrations tested (Fig. 2C). This might be due to the fact that mda-7 as a human protein could be toxic to the insect cell. Another reason might be the overexpression of mda-7 because of the strong polyhedrin promoter used, which is why the protein production had reached the maximum expression efficiency even with the lowest MOI tested.

The fusion proteins were purified using nickel affinity chromatography and the eluted fractions were pooled and concentrated. Both proteins contained some impurities, these impurities were all 60 kDa and over in size (Fig. 3). These impurities might be some protein components secreted by the insect cells. Due to the impurities, especially in the protein produced in Mimic^{TM} cells, there were some difficulties in determining the actual concentration of mda-7-His and the concentration of the Mimic^{TM} produced protein has

been heavily overestimated. Gel filtration/size exclusion chromatography could be used to further optimize the purification protocol.

The Western blot from the infected cells showed multiple bands (Fig. 3C), reflecting the presence or absence of the signal sequence and to the different stages of modifications. As visually estimated from the blots of infected cells, the protein production with *Sf*9 cells was far more efficient than the production with Mimic^{TM} cells. This was probably due to the high passage of the Mimic^{TM} cells and due to the differential growth conditions of the cell lines. The Mimic^{TM} cells were grown in monolayer and in far smaller scale than the *Sf*9 cells which were grown in suspension. In future studies, the growth conditions for the cell lines will be optimized in order to accurately compare the characteristics of the fusion proteins.

Characterization of the proteins was conducted by Western blot analysis (Fig. 3C). There are some differential results according to the size of the secreted mda-7 (Chada, S. et al., 2004b). The predicted molecular weight of the human mda-7 is 23.8 kDa (Chada, S. et al., 2004b), but the secreted mda-7 appears to exist in multiple forms with different molecular weights (Mumm, J.B. et al., 2006). There are multiple reported sizes of the secreted mda-7 from different groups, 35 kDa (Wang, M. et al., 2002) and 40 kDa (Chada, S. et al., 2004b) where the extra weight seems to be from N-linked glycosylations (Chada, S. et al., 2004b, Wang, M. et al., 2002) and 55 kDa and 90 kDa where the extra weight comes from the different multimeric forms of the glycosylated protein (Mumm, J.B. et al., 2006). In addition, variation may exist because of alternative splicing (Chada, S. et al., 2004b, Mumm, J.B. et al., 2006). It has also been suggested that mda-7 may be differentially processed prior to secretion depending on the cell type in question, this could reflect to variations in the extent of phosporylation and glycosylation of the protein (Lebedeva, I.V. et al., 2002).

The Western blot analysis of protein sample produced in *Sf*9 cells revealed one band approximately 23 kDa in size (calculated molecular weight of non-processed mda-7-His is 23.8 kDa (Chada, S. et al., 2004b)), which has previously been reported to be the size of the deglycosylated form of the secreted protein (Wang, M. et al., 2002). This was assumed

to be the secreted non-glycosylated form of the protein, were the signal peptide has been cleaved. The protein sample from the Mimic™ cells had two main bands, the other one was about 25 kDa and the other was around 45 kDa in size and were probably due to the non-glycosylated or partially glycosylated and fully glycosylated form of the protein, respectively. These results indicate that only the protein produced in Mimic™ cells was glycosylated.

5.2 Apoptosis assays

Mda-7 has been shown to induce apoptosis in a wide spectrum of human cancer cells (Allen, M. et al., 2004, Gopalan, B. et al., 2007, Ramesh, R. et al., 2004, Shi, H. et al., 2007, Su, Z.Z. et al., 1998). Although there have been many reported forms and sizes of mda-7, most of them have been found to be biologically active, suggesting that these different modifications and forms do not necessarily affect the activity of the protein (Chada, S. et al., 2004b, Mumm, J.B. et al., 2006, Sauane, M. et al., 2006). Interestingly, a truncated form consisting only of amino acids 104 to 206 of the full length protein retained its cancer-specific growth-suppressive and apoptosis-inducing properties similar to the full-length protein (Gupta, P. et al., 2006b). Despite the differences in the glycosylation of the produced proteins, both proteins appeared to induce apoptosis in MDA-MB-453 and PC-3 human cancer cell lines in a concentration-dependent manner.

With MDA-MB-435 cells the percentages of dead cells were up to 60% for mda-7-His produced in *Sf*9 cells and 30% with the control (Fig. 4B). Although not directly comparable, similar efficiencies have been achieved with an AAV type 1 vector expressing mda-7 where the percentage of apoptotic cells was about 20% with MDA-MB-435 cell line (Zheng, M. et al., 2007). With PC-3 cells the percentage of apoptotic cells was up to 26% with mda-7-His produced in *Sf*9 cells and 25% with the mda-7-His produced in Mimic™ cells. The corresponding percentages of dead cells were 25% and 29% (Fig. 5B). These percentages are consistent with the results from previous studies, where the percentage of apoptotic cells was about 30% with PC-3 cell line using bacterially expressed GST-mda-7 fusion protein (Sauane, M. et al., 2004). In contrast, with Ad. vector expressing mda-7 the

corresponding percentages were under 20% in PC-3 cell line, although in other prostate cancer cell lines (DU-145 and LNCaP) the percentages were around 30% (Lebedeva, I.V. et al., 2003). With melanoma cell lines the reported percentages of apoptotic cells are also around 20%-25%. With Ad. vector carrying *mda-7* the percentages for melanoma cell lines MeWo, HO-1 and FO-1 were 22%, 20% and 25%, respectively (Lebedeva, I.V. et al., 2002).

Although the percentages of apoptotic cells obtained with mda-7-His are consistent with previous studies using GST-mda-7 fusion protein, the protein concentrations used in these studies are quite different. In the study with the GST-mda-7 fusion protein, the protein concentration used was 50 ng/µl (Sauane, M. et al., 2004). The protein concentrations used in this study are only estimates and thus any accurate comparisons between these studies can not be properly conducted.

According to these results both insect cell derived proteins seem to be equally efficient in inducing apoptosis, but it must be taken into consideration that the concentration of the protein produced in *Sf*9 cells might be up to 5-fold higher than the protein produced in Mimic™ cells. Because of this, comparisons between the efficiency of the proteins cannot be accurately made, but it is highly probable that the protein produced in Mimic™ cells is more efficient, suggesting that glycosylation contributes to the efficiency. Similar results have been previously obtained as it was noticed that complete enzymatic deglycosylation of the mda-7 partially reduces its ability to induce melanoma cell death (Mumm, J.B. et al., 2006). The impurities in the protein samples might have contributed to some level of apoptosis but it probably has no significant effect on it. The percentages obtained from these tests are consistent with the maximum percentages reported from other groups (Lebedeva, I.V. et al., 2002, Lebedeva, I.V. et al., 2003, Sauane, M. et al., 2004).

In previous studies, the growth inhibiting and apoptosis inducing functions of mda-7 has been shown to be selective to cancer cells only. In the majority of cancer cell lines, mda-7 has had no effects on the growth or viability of the corresponding normal cells (Gopalan, B. et al., 2007, Lebedeva, I.V. et al., 2002, Wang, C.J. et al., 2006, Xue, X.B. et al., 2006). In future studies, further optimization of the purification protocol and comparisons with

normal cells will be conducted in order to more precisely define the efficiency and to demonstrate the selectivity of the mda-7-His fusion proteins.

5.3 Conclusions

It can be concluded that the active mda-7 protein can be produced with baculovirus insect cell expression system and with engineered insect cell lines we are able to produce complex N-glycans to a similar extent with mammalian cells (Hollister, J. et al., 2002). Although the mda-7-His produced in *Sf*9 cells lacked apparent glycosylation, it is still able to induce apoptosis in human cancer cells, suggesting that the glycosylation is not essential for the proper function of the protein. This is consistent with the results from other groups (Mumm, J.B. et al., 2006, Sauane, M. et al., 2006). Although both of the proteins were able to induce apoptosis, the glycosylated form is probably more efficient. In this study, it was shown that the insect cell derived soluble mda-7 is able to induce apoptosis to a comparable extent as the Ad. and AAV vectors carrying *mda-7* as has been reported in previous studies (Lebedeva, I.V. et al., 2003, Zheng, M. et al., 2007).

The present study gives additional proof that the mda-7 protein can induce apoptosis in human cancer cells and that the induction is independent on the glycosylation state of the protein. Induction of apoptosis with mda-7-His is consistent with the previously reported results obtained with other mda-7 fusion proteins and with Ad. and AAV vectors expressing mda-7. Considering the tumor selectivity and apparent safety of mda-7, these findings provide support for the therapeutic applications of mda-7. These results indicate that BEVS can be used to easily produce active mda-7 protein to further study the function and structure of mda-7.

6. REFERENCES

Abe, T., H. Takahashi, H. Hamazaki, N. Miyano-Kurosaki, Y. Matsuura, and H. Takaku. 2003. Baculovirus induces an innate immune response and confers protection from lethal influenza virus infection in mice. *J.Immunol.* 171:1133-1139.

Airenne, K.J., M.O. Hiltunen, M.P. Turunen, A.M. Turunen, O.H. Laitinen, M.S. Kulomaa, and S. Yla-Herttuala. 2000. Baculovirus-mediated periadventitial gene transfer to rabbit carotid artery. *Gene Ther.* 7:1499-1504.

Allen, M., B. Pratscher, F. Roka, C. Krepler, V. Wacheck, C. Schofer, H. Pehamberger, M. Muller, and T. Lucas. 2004. Loss of novel mda-7 splice variant (mda-7s) expression is associated with metastatic melanoma. *J.Invest.Dermatol.* 123:583-588.

Almoguera, C., D. Shibata, K. Forrester, J. Martin, N. Arnheim, and M. Perucho. 1988. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. *Cell.* 53:549-554.

Araujo, M.I., B.S. Hoppe, M. Medeiros Jr, and E.M. Carvalho. 2004. Schistosoma mansoni infection modulates the immune response against allergic and auto-immune diseases. *Mem.Inst.Oswaldo Cruz.* 99:27-32.

ATCHISON, R.W., B.C. CASTO, and W.M. HAMMON. 1965. Adenovirus-Associated Defective Virus Particles. *Science.* 149:754-756.

Atkins, M.B., M.T. Lotze, J.P. Dutcher, R.I. Fisher, G. Weiss, K. Margolin, J. Abrams, M. Sznol, D. Parkinson, M. Hawkins, C. Paradise, L. Kunkel, and S.A. Rosenberg. 1999. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *J.Clin.Oncol.* 17:2105-2116.

Atzpodien, J., H. Kirchner, U. Jonas, L. Bergmann, H. Schott, H. Heynemann, P. Fornara, S.A. Loening, J. Roigas, S.C. Muller, H. Bodenstein, S. Pomer, B. Metzner, U. Rebmann, R. Oberneder, M. Siebels, T. Wandert, T. Puchberger, M. Reitz, and Prospectively Randomized Trial of the German Cooperative Renal Carcinoma Chemoimmunotherapy Group (DGCIN). 2004. Interleukin-2- and interferon alfa-2a-based immunochemotherapy in advanced renal cell carcinoma: a Prospectively Randomized Trial of the German Cooperative Renal Carcinoma Chemoimmunotherapy Group (DGCIN). *J.Clin.Oncol.* 22:1188-1194.

Baneyx, F. 1999. Recombinant protein expression in Escherichia coli. *Curr.Opin.Biotechnol.* 10:411-421.

Bangari, D.S., and S.K. Mittal. 2006. Current strategies and future directions for eluding adenoviral vector immunity. *Curr.Gene Ther.* 6:215-226.

Barbulescu, K., C. Becker, J.F. Schlaak, E. Schmitt, K.H. Meyer zum Buschenfelde, and M.F. Neurath. 1998. IL-12 and IL-18 differentially regulate the transcriptional activity of the human IFN-gamma promoter in primary CD4+ T lymphocytes. *J.Immunol.* 160:3642-3647.

Bergelson, J.M., J.A. Cunningham, G. Droguett, E.A. Kurt-Jones, A. Krithivas, J.S. Hong, M.S. Horwitz, R.L. Crowell, and R.W. Finberg. 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science.* 275:1320-1323.

BIERMAN, H.R., D.M. CRILE, K.S. DOD, K.H. KELLY, N.L. PETRAKIS, L.P. WHITE, and M.B. SHIMKIN. 1953. Remissions in leukemia of childhood following acute infectious disease: staphylococcus and streptococcus, varicella, and feline panleukopenia. *Cancer.* 6:591-605.

Blaese, R.M., K.W. Culver, A.D. Miller, C.S. Carter, T. Fleisher, M. Clerici, G. Shearer, L. Chang, Y. Chiang, P. Tolstoshev, J.J. Greenblatt, S.A. Rosenberg, H. Klein, M. Berger, C.A. Mullen, W.J. Ramsey, L. Muul, R.A. Morgan, and W.F. Anderson. 1995. T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science.* 270:475-480.

Blumberg, H., D. Conklin, W.F. Xu, A. Grossmann, T. Brender, S. Carollo, M. Eagan, D. Foster, B.A. Haldeman, A. Hammond, H. Haugen, L. Jelinek, J.D. Kelly, K. Madden, M.F. Maurer, J. Parrish-Novak, D. Prunkard, S. Sexson, C. Sprecher, K. Waggie, J. West, T.E. Whitmore, L. Yao, M.K. Kuechle, B.A. Dale, and Y.A. Chandrasekher. 2001. Interleukin 20: discovery, receptor identification, and role in epidermal function. *Cell.* 104:9-19.

Boyce, F.M., and N.L. Bucher. 1996. Baculovirus-mediated gene transfer into mammalian cells. *Proc.Natl.Acad.Sci.U.S.A.* 93:2348-2352.

Butters, T.D., and R.C. Hughes. 1981. Isolation and characterization of mosquito cell membrane glycoproteins. *Biochim.Biophys.Acta.* 640:655-671.

Caudell, E.G., J.B. Mumm, N. Poindexter, S. Ekmekcioglu, A.M. Mhashilkar, X.H. Yang, M.W. Retter, P. Hill, S. Chada, and E.A. Grimm. 2002. The protein product of the tumor suppressor gene, melanoma differentiation-associated gene 7, exhibits immunostimulatory activity and is designated IL-24. *J.Immunol.* 168:6041-6046.

Celik, E., P. Calik, S.M. Halloran, and S.G. Oliver. 2007. Production of recombinant human erythropoietin from Pichia pastoris and its structural analysis. *J.Appl.Microbiol.* 103:2084-2094.

Cereghino, J.L., and J.M. Cregg. 2000. Heterologous protein expression in the methylotrophic yeast Pichia pastoris. *FEMS Microbiol.Rev.* 24:45-66.

Chada, S., A.M. Mhashilkar, R. Ramesh, J.B. Mumm, R.B. Sutton, D. Bocangel, M. Zheng, E.A. Grimm, and S. Ekmekcioglu. 2004a. Bystander activity of Ad-mda7: human MDA-7 protein kills melanoma cells via an IL-20 receptor-dependent but STAT3-independent mechanism. *Mol.Ther.* 10:1085-1095.

Chada, S., R.B. Sutton, S. Ekmekcioglu, J. Ellerhorst, J.B. Mumm, W.W. Leitner, H.Y. Yang, A.A. Sahin, K.K. Hunt, K.L. Fuson, N. Poindexter, J.A. Roth, R. Ramesh, E.A. Grimm, and A.M. Mhashilkar. 2004b. MDA-7/IL-24 is a unique cytokine--tumor suppressor in the IL-10 family. *Int.Immunopharmacol.* 4:649-667.

Cho, D., H. Song, Y.M. Kim, D. Houh, D.Y. Hur, H. Park, D. Yoon, K.H. Pyun, W.J. Lee, M. Kurimoto, Y.B. Kim, Y.S. Kim, and I. Choi. 2000. Endogenous interleukin-18 modulates immune escape of murine melanoma cells by regulating the expression of Fas ligand and reactive oxygen intermediates. *Cancer Res.* 60:2703-2709.

Condreay, J.P., S.M. Witherspoon, W.C. Clay, and T.A. Kost. 1999. Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. *Proc.Natl.Acad.Sci.U.S.A.* 96:127-132.

Cripe, T.P., E.J. Dunphy, A.D. Holub, A. Saini, N.H. Vasi, Y.Y. Mahller, M.H. Collins, J.D. Snyder, V. Krasnykh, D.T. Curiel, T.J. Wickham, J. DeGregori, J.M. Bergelson, and M.A. Currier. 2001. Fiber knob modifications overcome low, heterogeneous expression of the coxsackievirus-adenovirus receptor that limits adenovirus gene transfer and oncolysis for human rhabdomyosarcoma cells. *Cancer Res.* 61:2953-2960.

Cristofanilli, M., C. Charnsangavej, and G.N. Hortobagyi. 2002. Angiogenesis modulation in cancer research: novel clinical approaches. *Nat.Rev.Drug Discov.* 1:415-426.

Cross, D., and J.K. Burmester. 2006. Gene therapy for cancer treatment: past, present and future. *Clin.Med.Res.* 4:218-227.

Davidoff, A.M., S.A. Kimbrough, C.Y. Ng, S.J. Shochat, and E.F. Vanin. 1999. Neuroblastoma regression and immunity induced by transgenic expression of interleukin-12. *J.Pediatr.Surg.* 34:902-6; discussion 906-7.

Dear, R., N. Wilcken, and J. Shannon. 2008. Beyond chemotherapy--demystifying the new 'targeted' cancer treatments. *Aust.Fam.Physician.* 37:45-49.

DeGroot, L.J., and R. Zhang. 2001. Clinical review 131: Gene therapy for thyroid cancer: where do we stand? *J.Clin.Endocrinol.Metab.* 86:2923-2928.

Donahue, J.M., J.T. Mullen, and K.K. Tanabe. 2002. Viral oncolysis. *Surg.Oncol.Clin.N.Am.* 11:661-680.

Dong, J.Y., P.D. Fan, and R.A. Frizzell. 1996. Quantitative analysis of the packaging capacity of recombinant adeno-associated virus. *Hum.Gene Ther.* 7:2101-2112.

Duvic, M., M.L. Sherman, G.S. Wood, T.M. Kuzel, E. Olsen, F. Foss, R.J. Laliberte, J.L. Ryan, K. Zonno, and A.H. Rook. 2006. A phase II open-label study of recombinant human interleukin-12 in patients with stage IA, IB, or IIA mycosis fungoides. *J.Am.Acad.Dermatol.* 55:807-813.

Ekmekcioglu, S., J.A. Ellerhorst, J.B. Mumm, M. Zheng, L. Broemeling, V.G. Prieto, A.L. Stewart, A.M. Mhashilkar, S. Chada, and E.A. Grimm. 2003. Negative association of melanoma differentiation-associated gene (mda-7) and inducible nitric oxide synthase (iNOS) in human melanoma: MDA-7 regulates iNOS expression in melanoma cells. *Mol.Cancer.Ther.* 2:9-17.

Emdad, L., D. Sarkar, I.V. Lebedeva, Z.Z. Su, P. Gupta, P.J. Mahasreshti, P. Dent, D.T. Curiel, and P.B. Fisher. 2006. Ionizing radiation enhances adenoviral vector expressing mda-7/IL-24-mediated apoptosis in human ovarian cancer. *J.Cell.Physiol.* 208:298-306.

Fechner, H., X. Wang, H. Wang, A. Jansen, M. Pauschinger, H. Scherubl, J.M. Bergelson, H.P. Schultheiss, and W. Poller. 2000. Trans-complementation of vector replication versus Coxsackie-adenovirus-receptor overexpression to improve transgene expression in poorly permissive cancer cells. *Gene Ther.* 7:1954-1968.

Fisher, P.B. 2005. Is mda-7/IL-24 a "magic bullet" for cancer? *Cancer Res.* 65:10128-10138.

Fisher, P.B., R.V. Gopalkrishnan, S. Chada, R. Ramesh, E.A. Grimm, M.R. Rosenfeld, D.T. Curiel, and P. Dent. 2003. mda-7/IL-24, a novel cancer selective apoptosis inducing cytokine gene: from the laboratory into the clinic. *Cancer.Biol.Ther.* 2:S23-37.

Fisher, P.B., D. Sarkar, I.V. Lebedeva, L. Emdad, P. Gupta, M. Sauane, Z.Z. Su, S. Grant, P. Dent, D.T. Curiel, N. Senzer, and J. Nemunaitis. 2007. Melanoma differentiation associated gene-7/interleukin-24 (mda-7/IL-24): novel gene therapeutic for metastatic melanoma. *Toxicol.Appl.Pharmacol.* 224:300-307.

Goncalves, M.A. 2005. Adeno-associated virus: from defective virus to effective vector. *Virol.J.* 2:43.

Gopalan, B., M. Shanker, S. Chada, and R. Ramesh. 2007. MDA-7/IL-24 suppresses human ovarian carcinoma growth in vitro and in vivo. *Mol.Cancer.* 6:11.

Gupta, P., Z.Z. Su, I.V. Lebedeva, D. Sarkar, M. Sauane, L. Emdad, M.A. Bachelor, S. Grant, D.T. Curiel, P. Dent, and P.B. Fisher. 2006a. mda-7/IL-24: multifunctional cancer-specific apoptosis-inducing cytokine. *Pharmacol.Ther.* 111:596-628.

Gupta, P., M.R. Walter, Z.Z. Su, I.V. Lebedeva, L. Emdad, A. Randolph, K. Valerie, D. Sarkar, and P.B. Fisher. 2006b. BiP/GRP78 is an intracellular target for MDA-7/IL-24 induction of cancer-specific apoptosis. *Cancer Res.* 66:8182-8191.

Hansen, R.M., and J.A. Libnoch. 1978. Remission of chronic lymphocytic leukemia after smallpox vaccination. *Arch.Intern.Med.* 138:1137-1138.

Harvey, B.G., S. Worgall, S. Ely, P.L. Leopold, and R.G. Crystal. 1999. Cellular immune responses of healthy individuals to intradermal administration of an E1-E3- adenovirus gene transfer vector. *Hum.Gene Ther.* 10:2823-2837.

Hermiston, T.W., and I. Kuhn. 2002. Armed therapeutic viruses: strategies and challenges to arming oncolytic viruses with therapeutic genes. *Cancer Gene Ther.* 9:1022-1035.

Hofmann, C., and M. Strauss. 1998. Baculovirus-mediated gene transfer in the presence of human serum or blood facilitated by inhibition of the complement system. *Gene Ther.* 5:531-536.

Hollister, J., E. Grabenhorst, M. Nimtz, H. Conradt, and D.L. Jarvis. 2002. Engineering the protein N-glycosylation pathway in insect cells for production of biantennary, complex N-glycans. *Biochemistry.* 41:15093-15104.

Hu, Y.C. 2005. Baculovirus as a highly efficient expression vector in insect and mammalian cells. *Acta Pharmacol.Sin.* 26:405-416.

Huang, E.Y., M.T. Madireddi, R.V. Gopalkrishnan, M. Leszczyniecka, Z. Su, I.V. Lebedeva, D. Kang, H. Jiang, J.J. Lin, D. Alexandre, Y. Chen, N. Vozhilla, M.X. Mei, K.A. Christiansen, F. Sivo, N.I. Goldstein, A.B. Mhashilkar, S. Chada, E. Huberman, S. Pestka, and P.B. Fisher. 2001. Genomic structure, chromosomal localization and expression profile of a novel melanoma differentiation associated (mda-7) gene with cancer specific growth suppressing and apoptosis inducing properties. *Oncogene.* 20:7051-7063.

Ikeda, K., T. Ichikawa, H. Wakimoto, J.S. Silver, T.S. Deisboeck, D. Finkelstein, G.R. Harsh 4th, D.N. Louis, R.T. Bartus, F.H. Hochberg, and E.A. Chiocca. 1999. Oncolytic virus therapy of multiple tumors in the brain requires suppression of innate and elicited antiviral responses. *Nat.Med.* 5:881-887.

Jacobson, M.D. 1996. Reactive oxygen species and programmed cell death. *Trends Biochem.Sci.* 21:83-86.

Jiang, H., J.J. Lin, Z.Z. Su, N.I. Goldstein, and P.B. Fisher. 1995. Subtraction hybridization identifies a novel melanoma differentiation associated gene, mda-7, modulated during human melanoma differentiation, growth and progression. *Oncogene.* 11:2477-2486.

Jiang, H., Z.Z. Su, J.J. Lin, N.I. Goldstein, C.S. Young, and P.B. Fisher. 1996. The melanoma differentiation associated gene mda-7 suppresses cancer cell growth. *Proc.Natl.Acad.Sci.U.S.A.* 93:9160-9165.

Kim, J., C. Kim, T.S. Kim, S.I. Bang, Y. Yang, H. Park, and D. Cho. 2006. IL-18 enhances thrombospondin-1 production in human gastric cancer via JNK pathway. *Biochem.Biophys.Res.Commun.* 344:1284-1289.

Kost, T.A., J.P. Condreay, and D.L. Jarvis. 2005a. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat.Biotechnol.* 23:567-575.

Kubelka, V., F. Altmann, G. Kornfeld, and L. Marz. 1994. Structures of the N-linked oligosaccharides of the membrane glycoproteins from three lepidopteran cell lines (Sf-21, IZD-Mb-0503, Bm-N). *Arch.Biochem.Biophys.* 308:148-157.

Leath, C.A.,3rd, M. Kataram, P. Bhagavatula, R.V. Gopalkrishnan, P. Dent, P.B. Fisher, A. Pereboev, D. Carey, I.V. Lebedeva, H.J. Haisma, R.D. Alvarez, D.T. Curiel, and P.J. Mahasreshti. 2004. Infectivity enhanced adenoviral-mediated mda-7/IL-24 gene therapy for ovarian carcinoma. *Gynecol.Oncol.* 94:352-362.

Lebedeva, I.V., D. Sarkar, Z.Z. Su, S. Kitada, P. Dent, C.A. Stein, J.C. Reed, and P.B. Fisher. 2003. Bcl-2 and Bcl-x(L) differentially protect human prostate cancer cells from induction of apoptosis by melanoma differentiation associated gene-7, mda-7/IL-24. *Oncogene.* 22:8758-8773.

Lebedeva, I.V., Z.Z. Su, Y. Chang, S. Kitada, J.C. Reed, and P.B. Fisher. 2002. The cancer growth suppressing gene mda-7 induces apoptosis selectively in human melanoma cells. *Oncogene.* 21:708-718.

Lebedeva, I.V., Z.Z. Su, D. Sarkar, S. Kitada, P. Dent, S. Waxman, J.C. Reed, and P.B. Fisher. 2003. Melanoma differentiation associated gene-7, mda-7/interleukin-24, induces apoptosis in prostate cancer cells by promoting mitochondrial dysfunction and inducing reactive oxygen species. *Cancer Res.* 63:8138-8144.

Lebedeva, I.V., I. Washington, D. Sarkar, J.A. Clark, R.L. Fine, P. Dent, D.T. Curiel, N.J. Turro, and P.B. Fisher. 2007. Strategy for reversing resistance to a single anticancer agent in human prostate and pancreatic carcinomas. *Proc.Natl.Acad.Sci.U.S.A.* 104:3484-3489.

Li, Y., R.C. Pong, J.M. Bergelson, M.C. Hall, A.I. Sagalowsky, C.P. Tseng, Z. Wang, and J.T. Hsieh. 1999. Loss of adenoviral receptor expression in human bladder cancer cells: a potential impact on the efficacy of gene therapy. *Cancer Res.* 59:325-330.

Liu, T.C., and D. Kirn. 2007. Systemic efficacy with oncolytic virus therapeutics: clinical proof-of-concept and future directions. *Cancer Res.* 67:429-432.

Lorent, E., H. Bierau, Y. Engelborghs, G. Verheyden, and F. Bosman. 2008. Structural characterisation of the hepatitis C envelope glycoprotein E1 ectodomain derived from a mammalian and a yeast expression system. *Vaccine.* 26:399-410.

Lotze, M.T., A.E. Chang, C.A. Seipp, C. Simpson, J.T. Vetto, and S.A. Rosenberg. 1986. High-dose recombinant interleukin 2 in the treatment of patients with disseminated cancer. Responses, treatment-related morbidity, and histologic findings. *JAMA.* 256:3117-3124.

Lotze, M.T., E.A. Grimm, A. Mazumder, J.L. Strausser, and S.A. Rosenberg. 1981. Lysis of fresh and cultured autologous tumor by human lymphocytes cultured in T-cell growth factor. *Cancer Res.* 41:4420-4425.

Mazzolini, G., O. Murillo, C. Atorrasagasti, J. Dubrot, I. Tirapu, M. Rizzo, A. Arina, C. Alfaro, A. Azpilicueta, C. Berasain, J.L. Perez-Gracia, A. Gonzalez, and I. Melero. 2007. Immunotherapy and immunoescape in colorectal cancer. *World J.Gastroenterol.* 13:5822-5831.

McDermott, D.F., M.M. Regan, J.I. Clark, L.E. Flaherty, G.R. Weiss, T.F. Logan, J.M. Kirkwood, M.S. Gordon, J.A. Sosman, M.S. Ernstoff, C.P. Tretter, W.J. Urba, J.W. Smith, K.A. Margolin, J.W. Mier, J.A. Gollob, J.P. Dutcher, and M.B. Atkins. 2005. Randomized phase III trial of high-dose interleukin-2 versus subcutaneous interleukin-2 and interferon in patients with metastatic renal cell carcinoma. *J.Clin.Oncol.* 23:133-141.

Mitsuhashi, M., J. Liu, S. Cao, X. Shi, and X. Ma. 2004. Regulation of interleukin-12 gene expression and its anti-tumor activities by prostaglandin E2 derived from mammary carcinomas. *J.Leukoc.Biol.* 76:322-332.

Mumm, J.B., S. Ekmekcioglu, N.J. Poindexter, S. Chada, and E.A. Grimm. 2006. Soluble human MDA-7/IL-24: characterization of the molecular form(s) inhibiting tumor growth and stimulating monocytes. *J.Interferon Cytokine Res.* 26:877-886.

Nakashima, J., M. Tachibana, Y. Horiguchi, M. Oya, T. Ohigashi, H. Asakura, and M. Murai. 2000. Serum interleukin 6 as a prognostic factor in patients with prostate cancer. *Clin.Cancer Res.* 6:2702-2706.

Nash, M.A., G. Ferrandina, M. Gordinier, A. Loercher, and R.S. Freedman. 1999. The role of cytokines in both the normal and malignant ovary. *Endocr.Relat.Cancer.* 6:93-107.

Okada, S., T. Okusaka, H. Ishii, A. Kyogoku, M. Yoshimori, N. Kajimura, K. Yamaguchi, and T. Kakizoe. 1998. Elevated serum interleukin-6 levels in patients with pancreatic cancer. *Jpn.J.Clin.Oncol.* 28:12-15.

Oshikawa, K., F. Shi, A.L. Rakhmilevich, P.M. Sondel, D.M. Mahvi, and N.S. Yang. 1999. Synergistic inhibition of tumor growth in a murine mammary adenocarcinoma model by combinational gene therapy using IL-12, pro-IL-18, and IL-1beta converting enzyme cDNA. *Proc.Natl.Acad.Sci.U.S.A.* 96:13351-13356.

Park, H., D. Byun, T.S. Kim, Y.I. Kim, J.S. Kang, E.S. Hahm, S.H. Kim, W.J. Lee, H.K. Song, D.Y. Yoon, C.J. Kang, C. Lee, D. Houh, H. Kim, B. Cho, Y. Kim, Y.H. Yang, K.H. Min, and D.H. Cho. 2001. Enhanced IL-18 expression in common skin tumors. *Immunol.Lett.* 79:215-219.

Park, S., S. Cheon, and D. Cho. 2007. The dual effects of interleukin-18 in tumor progression. *Cell.Mol.Immunol.* 4:329-335.

Patterson, R.M., J.K. Selkirk, and B.A. Merrick. 1995. Baculovirus and insect cell gene expression: review of baculovirus biotechnology. *Environ.Health Perspect.* 103:756-759.

Pecora, A.L., N. Rizvi, G.I. Cohen, N.J. Meropol, D. Sterman, J.L. Marshall, S. Goldberg, P. Gross, J.D. O'Neil, W.S. Groene, M.S. Roberts, H. Rabin, M.K. Bamat, and R.M. Lorence. 2002. Phase I trial of intravenous administration of PV701, an oncolytic virus, in patients with advanced solid cancers. *J.Clin.Oncol.* 20:2251-2266.

Preti, H.A., F. Cabanillas, M. Talpaz, S.L. Tucker, J.F. Seymour, and R. Kurzrock. 1997. Prognostic value of serum interleukin-6 in diffuse large-cell lymphoma. *Ann.Intern.Med.* 127:186-194.

Ramesh, R., I. Ito, B. Gopalan, Y. Saito, A.M. Mhashilkar, and S. Chada. 2004. Ectopic production of MDA-7/IL-24 inhibits invasion and migration of human lung cancer cells. *Mol.Ther.* 9:510-518.

Ramesh, R., A.M. Mhashilkar, F. Tanaka, Y. Saito, C.D. Branch, K. Sieger, J.B. Mumm, A.L. Stewart, A. Boquoi, L. Dumoutier, E.A. Grimm, J.C. Renauld, S. Kotenko, and S. Chada. 2003. Melanoma differentiation-associated gene 7/interleukin (IL)-24 is a novel ligand that regulates angiogenesis via the IL-22 receptor. *Cancer Res.* 63:5105-5113.

Reang, P., M. Gupta, and K. Kohli. 2006. Biological response modifiers in cancer. *MedGenMed.* 8:33.

Rein, D.T., M. Breidenbach, and D.T. Curiel. 2006. Current developments in adenovirus-based cancer gene therapy. *Future Oncol.* 2:137-143.

Rosenberg, S.A. 2001. Progress in human tumour immunology and immunotherapy. *Nature.* 411:380-384.

Rott, O., B. Fleischer, and E. Cash. 1994. Interleukin-10 prevents experimental allergic encephalomyelitis in rats. *Eur.J.Immunol.* 24:1434-1440.

Roy, S., P.S. Shirley, A. McClelland, and M. Kaleko. 1998. Circumvention of immunity to the adenovirus major coat protein hexon. *J.Virol.* 72:6875-6879.

Sadlack, B., J. Lohler, H. Schorle, G. Klebb, H. Haber, E. Sickel, R.J. Noelle, and I. Horak. 1995. Generalized autoimmune disease in interleukin-2-deficient mice is triggered by an uncontrolled activation and proliferation of CD4+ T cells. *Eur.J.Immunol.* 25:3053-3059.

Saeki, T., A. Mhashilkar, X. Swanson, X.H. Zou-Yang, K. Sieger, S. Kawabe, C.D. Branch, L. Zumstein, R.E. Meyn, J.A. Roth, S. Chada, and R. Ramesh. 2002. Inhibition of human lung cancer growth following adenovirus-mediated mda-7 gene expression in vivo. *Oncogene.* 21:4558-4566.

Sarkar, D., Z.Z. Su, I.V. Lebedeva, M. Sauane, R.V. Gopalkrishnan, K. Valerie, P. Dent, and P.B. Fisher. 2002. mda-7 (IL-24) Mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. *Proc.Natl.Acad.Sci.U.S.A.* 99:10054-10059.

Sauane, M., R.V. Gopalkrishnan, H.T. Choo, P. Gupta, I.V. Lebedeva, A. Yacoub, P. Dent, and P.B. Fisher. 2004. Mechanistic aspects of mda-7/IL-24 cancer cell selectivity analysed via a bacterial fusion protein. *Oncogene.* 23:7679-7690.

Sauane, M., R.V. Gopalkrishnan, I. Lebedeva, M.X. Mei, D. Sarkar, Z.Z. Su, D.C. Kang, P. Dent, S. Pestka, and P.B. Fisher. 2003. Mda-7/IL-24 induces apoptosis of diverse cancer cell lines through JAK/STAT-independent pathways. *J.Cell.Physiol.* 196:334-345.

Sauane, M., P. Gupta, I.V. Lebedeva, Z.Z. Su, D. Sarkar, A. Randolph, K. Valerie, R.V. Gopalkrishnan, and P.B. Fisher. 2006. N-glycosylation of MDA-7/IL-24 is dispensable for tumor cell-specific apoptosis and "bystander" antitumor activity. *Cancer Res.* 66:11869-11877.

Sauane, M., I.V. Lebedeva, Z.Z. Su, H.T. Choo, A. Randolph, K. Valerie, P. Dent, R.V. Gopalkrishnan, and P.B. Fisher. 2004. Melanoma differentiation associated gene-7/interleukin-24 promotes tumor cell-specific apoptosis through both secretory and nonsecretory pathways. *Cancer Res.* 64:2988-2993.

Shankaran, V., H. Ikeda, A.T. Bruce, J.M. White, P.E. Swanson, L.J. Old, and R.D. Schreiber. 2001. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature.* 410:1107-1111.

Shi, H., L.L. Wei, C.F. Yuan, J.X. Yang, F.P. Yi, Y.P. Ma, and F.Z. Song. 2007. Melanoma differentiation-associated gene-7/interleukin 24 inhibits invasion and migration of human cervical cancer cells in vitro. *Saudi Med.J.* 28:1671-1675.

Smith, P.C., and E.T. Keller. 2001. Anti-interleukin-6 monoclonal antibody induces regression of human prostate cancer xenografts in nude mice. *Prostate.* 48:47-53.

Stern, A.S., J. Magram, and D.H. Presky. 1996. Interleukin-12 an integral cytokine in the immune response. *Life Sci.* 58:639-654.

Su, Z., L. Emdad, M. Sauane, I.V. Lebedeva, D. Sarkar, P. Gupta, C.D. James, A. Randolph, K. Valerie, M.R. Walter, P. Dent, and P.B. Fisher. 2005a. Unique aspects of mda-7/IL-24 antitumor bystander activity: establishing a role for secretion of MDA-7/IL-24 protein by normal cells. *Oncogene.* 24:7552-7566.

Su, Z., L. Emdad, M. Sauane, I.V. Lebedeva, D. Sarkar, P. Gupta, C.D. James, A. Randolph, K. Valerie, M.R. Walter, P. Dent, and P.B. Fisher. 2005b. Unique aspects of mda-7/IL-24 antitumor bystander activity: establishing a role for secretion of MDA-7/IL-24 protein by normal cells. *Oncogene.* 24:7552-7566.

Su, Z., I.V. Lebedeva, R.V. Gopalkrishnan, N.I. Goldstein, C.A. Stein, J.C. Reed, P. Dent, and P.B. Fisher. 2001. A combinatorial approach for selectively inducing programmed cell death in human pancreatic cancer cells. *Proc.Natl.Acad.Sci.U.S.A.* 98:10332-10337.

Su, Z.Z., I.V. Lebedeva, D. Sarkar, R.V. Gopalkrishnan, M. Sauane, C. Sigmon, A. Yacoub, K. Valerie, P. Dent, and P.B. Fisher. 2003. Melanoma differentiation associated gene-7, mda-7/IL-24, selectively induces growth suppression, apoptosis and radiosensitization in malignant gliomas in a p53-independent manner. *Oncogene.* 22:1164-1180.

Su, Z.Z., M.T. Madireddi, J.J. Lin, C.S. Young, S. Kitada, J.C. Reed, N.I. Goldstein, and P.B. Fisher. 1998. The cancer growth suppressor gene mda-7 selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice. *Proc.Natl.Acad.Sci.U.S.A.* 95:14400-14405.

Sudbery, P.E. 1996. The expression of recombinant proteins in yeasts. *Curr.Opin.Biotechnol.* 7:517-524.

Summers, M.D. 2006. Milestones leading to the genetic engineering of baculoviruses as expression vector systems and viral pesticides. *Adv.Virus Res.* 68:3-73.

Tahara, I., K. Miyake, H. Hanawa, T. Kurai, Y. Hirai, M. Ishizaki, E. Uchida, T. Tajiri, and T. Shimada. 2007. Systemic cancer gene therapy using adeno-associated virus type 1 vector expressing MDA-7/IL24. *Mol.Ther.* 15:1805-1811.

Trikha, M., R. Corringham, B. Klein, and J.F. Rossi. 2003. Targeted anti-interleukin-6 monoclonal antibody therapy for cancer: a review of the rationale and clinical evidence. *Clin.Cancer Res.* 9:4653-4665.

van Loo, N.D., E. Fortunati, E. Ehlert, M. Rabelink, F. Grosveld, and B.J. Scholte. 2001. Baculovirus infection of nondividing mammalian cells: mechanisms of entry and nuclear transport of capsids. *J.Virol.* 75:961-970.

Wang, C.J., X.B. Xue, J.L. Yi, K. Chen, J.W. Zheng, J. Wang, J.P. Zeng, and R.H. Xu. 2006. Melanoma differentiation-associated gene-7, MDA-7/IL-24, selectively induces growth suppression, apoptosis in human hepatocellular carcinoma cell line HepG2 by replication-incompetent adenovirus vector. *World J.Gastroenterol.* 12:1774-1779.

Wang, M., Z. Tan, R. Zhang, S.V. Kotenko, and P. Liang. 2002. Interleukin 24 (MDA-7/MOB-5) signals through two heterodimeric receptors, IL-22R1/IL-20R2 and IL-20R1/IL-20R2. *J.Biol.Chem.* 277:7341-7347.

Widakowich, C., G. de Castro Jr, E. de Azambuja, P. Dinh, and A. Awada. 2007. Review: side effects of approved molecular targeted therapies in solid cancers. *Oncologist.* 12:1443-1455.

Williams, P.J., M.R. Wormald, R.A. Dwek, T.W. Rademacher, G.F. Parker, and D.R. Roberts. 1991. Characterisation of oligosaccharides from Drosophila melanogaster glycoproteins. *Biochim.Biophys.Acta.* 1075:146-153.

Wolk, K., S. Kunz, E. Witte, M. Friedrich, K. Asadullah, and R. Sabat. 2004. IL-22 increases the innate immunity of tissues. *Immunity.* 21:241-254.

Wurm, F.M. 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat.Biotechnol.* 22:1393-1398.

Xiao, X., J. Li, and R.J. Samulski. 1996. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J.Virol.* 70:8098-8108.

Xue, S.A., and H.J. Stauss. 2007. Enhancing immune responses for cancer therapy. *Cell.Mol.Immunol.* 4:173-184.

Xue, X.B., J.W. Zheng, C.J. Wang, K. Chen, H.Y. Hu, H. Hu, Y. Yu, and Z.D. Wu. 2006. Adenovirus vector expressing MDA-7/IL-24 selectively induces growth arrests and apoptosis in human hepatocellular carcinoma cell lines independent of the state of p53 gene. *Zhonghua Gan Zang Bing Za Zhi.* 14:670-675.

Yacoub, A., C. Mitchell, Y. Hong, R.V. Gopalkrishnan, Z.Z. Su, P. Gupta, M. Sauane, I.V. Lebedeva, D.T. Curiel, P.J. Mahasreshti, M.R. Rosenfeld, W.C. Broaddus, C.D. James, S. Grant, P.B. Fisher, and P. Dent. 2004. MDA-7 regulates cell growth and radiosensitivity in vitro of primary (non-established) human glioma cells. *Cancer.Biol.Ther.* 3:739-751.

Yacoub, A., C. Mitchell, I.V. Lebedeva, D. Sarkar, Z.Z. Su, R. McKinstry, R.V. Gopalkrishnan, S. Grant, P.B. Fisher, and P. Dent. 2003. mda-7 (IL-24) Inhibits growth and enhances radiosensitivity of glioma cells in vitro via JNK signaling. *Cancer.Biol.Ther.* 2:347-353.

Yan, Z., T.C. Ritchie, D. Duan, and J.F. Engelhardt. 2002. Recombinant AAV-mediated gene delivery using dual vector heterodimerization. *Methods Enzymol.* 346:334-357.

Ye, Z.B., T. Ma, H. Li, X.L. Jin, and H.M. Xu. 2007. Expression and significance of intratumoral interleukin-12 and interleukin-18 in human gastric carcinoma. *World J.Gastroenterol.* 13:1747-1751.

Zheng, M., D. Bocangel, B. Doneske, A. Mhashilkar, R. Ramesh, K.K. Hunt, S. Ekmekcioglu, R.B. Sutton, N. Poindexter, E.A. Grimm, and S. Chada. 2007. Human interleukin 24 (MDA-7/IL-24) protein kills breast cancer cells via the IL-20 receptor and is antagonized by IL-10. *Cancer Immunol.Immunother.* 56:205-215.