Anti-tumor activity of baculoviruses displaying and expressing the cancer-specific apoptosis inducing cytokine, Mda-7

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Master's Thesis

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PREFACE

This study was conducted in the University of Jyväskylä, Department of Biological and Environmental Science, Division of Molecular Recognition.

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Riikka Kapanen

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Abstract

Recently, it has been widely proposed that cancer could be treated by transferring therapeutic genes or proteins into the cancer tissue by using different viral or non-viral systems. Among the vectors under development is the insect baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), which represents a versatile tool for both mammalian gene delivery and protein display. Due to its low cytotoxicity and innate inability to replicate in mammalian cells, *Ac*MNPV holds potential for *in vivo* applications. The melanoma differentiation associated gene 7 (Mda-7), in turn, is a novel multifunctional tumor-specific cytokine that suppresses growth and induces apoptosis in numerous human cancer cells. Furthermore, Mda-7 possesses immunomodulatory and anti-angiogenic features, as well as a robust bystander activity. The objectives of this study were to generate and characterize recombinant baculovirus vectors expressing and/or displaying Mda-7, as well as to analyze the anti-tumor activity of these viruses. Two mammalian expression vectors, *Ac*-Mda-7 and the control virus *Ac*-Luc, were constructed, expressing Mda-7 or firefly luciferase, respectively. Additionally, by utilizing these vectors as backbones, two surface-modified viruses, *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7, displaying Mda-7 by fusion to the transmembrane anchor of vesicular stomatitis virus G-protein (Mda-7-VSVg) on their surface, were developed. The Mda-7-VSVg fusion protein was successfully expressed in insect cells and incorporated into the budded virions of *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7 as demonstrated by confocal microscopy and Western blotting. Importantly, analysis by Western blotting and flow cytometry showed no significant differences between the infectivities of the unmodified and the Mda-7-VSVg-displaying viruses. Furthermore, Annexin V-staining and subsequent analysis by flow cytometry revealed clear induction of apoptosis by *Ac*-Mda-7, *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7, but not by the control virus *Ac*-Luc in human prostate cancer PC-3 cells at 5 days post transduction. These results demonstrate the functionality of baculovirus mediated gene delivery and protein display, highlighting the potential of baculovirus vectors in cancer therapy.

Keywords: cancer, baculovirus, *Ac*MNPV, Mda-7, gene therapy, protein display

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Tiivistelmä

Viimeaikoina on laajalti esitetty, että syövän eteneminen voitaisiin pysäyttää siirtämällä terapeuttisia geenejä tai proteiineja syöpäkudokseen esimerkiksi virusten tai muiden kuljettimien eli vektoreiden avulla. Yksi tällä hetkellä kehiteltävistä virusvektoreista on hyönteisiä infektoiva bakulovirus *(Autographa californica* multiple nucleopolyhedrovirus, *AcMNPV*), joka mahdollistaa sekä nisäkässoluihin kohdistuvan geeninsiirron, että vieraiden proteiinien ilmentämisen viruspartikkelin pinnalla. Koska *AcMNPV* on luontaisesti kykenemätön lisääntymään nisäkässoluissa ja sillä on vähän soluille myrkyllisiä vaikutuksia, on se lupaava työväline myös *in vivo* -sovelluksiin. Melanooman erilaistumiseen osallistuva geeni 7 (Melanoma differentation associated gene 7, Mda-7), puolestaan on hiljattain löydetty monipuolinen, syöpäspesifinen sytokiini, joka tukahduttaa syöpäsolujen kasvua ja saa aikaan ohjelmoitua solukuolemaa eli apoptoosia useissa eri ihmisen syöpäsoluissa. Mda-7:llä on lisäksi immuniteettiä muokkaava ja verisuonten kasvua estävä vaikutus, sekä kyky vaikuttaa myös geeninsiirron kohdesolua ympäröiviin soluihin. Tämän tutkimuksen tavoitteena oli kehittää ja luonnehtia muokattuja bakulovirusvektoreita, jotka ilmentävät ja/tai pintailmentävät Mda-7:ää, sekä analysoida näiden virusten syövänvastaista aktiivisuutta. Kaksi vektoria, *Ac*-Mda-7 ja *Ac*-Luc kehitettiin ilmentämään nisäkässoluissa Mda-7:ää ja tulikärpäsen lusiferaasi-entsyymiä, vastaavasti. Lisäksi, käyttämällä näitä vektoreita lähtökohtana, kehitettiin kaksi pinnaltaan muokattua virusta, *Ac*Mda-7-Mda-7 ja *Ac*Mda-7-Luc, jotka ilmentävät pinnallaan Mda-7 proteiinia liitettynä VSV-viruksen (vesicular stomatitis virus) g-proteiiniin (VSVg). Konfokaalimikroskooppi- sekä Western Blotting-tutkimukset havainnollistivat Mda-7-VSVg-yhdistelmäproteiinin ilmentyvän hyönteissoluissa ja kiinnittyvän silmikoituvien *Ac*Mda-7-Mda-7 ja *Ac*Mda-7-Luc -virusten pinnalle onnistuneesti. Mikä tärkeää, analyysit Western Blotting-tekniikalla ja virtaussytometrillä todistivat, ettei kyseinen pintamuokkaus vaikuttanut virusten infektiokykyyn. Lisäksi, Annexin-V-värjäys ja sitä seurannut virtaussytometrianalyysi osoittivat *Ac*-Mda-7, *Ac*Mda-7-Mda-7 ja *Ac*Mda-7-Luc -virusten aikaansaavan ohjelmoitua solukuolemaa ihmisen PC-3 prostatasyöpäsoluissa. Nämä tulokset havainnollistavat bakulovirusvälitteistä geeninsiirtoa ja pintailmennystekniikkaa, sekä korostavat bakulovirusvektoreiden mahdollisuuksia syövän hoidossa.

Avainsanat: syöpä, bakulovirus, *Ac*MNPV, Mda-7, geeniterapia, proteiinien pintailmennys

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ABBREVIATIONS

AAV	adeno-associated virus
*Ac*MNPV	*Autographa californica* multiple nucleopolyhedrovirus
Ad	adenovirus
BSA	bovine serum albumin
BV	budded virus
CAR	coxsackie- and adenovirus receptor
CRF2	class II cytokine receptor
CT	cytoplasmic
DIC	differential interference contrast
E. coli	*Escherichia coli*
eIF-2α	alpha subunit of eukaryotic translation initiation factor 2
ER	endoplasmic reticulum
HA	hemaglutinin
IL	interleukin
INF	interferon
JAK	Janus kinase
MAPK	mitogen activated protein kinase
Mda-7	melanoma differentiation associated gene 7
MNPV	multiple nucleopolyhedrovirus
NPV	nucleopolyhedrovirus
NSCLC	non-small cell lung carcinoma
ODV	occlusion-derived virus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PFU	plaque forming unit
PI3K	phosphatidylinositol 3-kinase
PKR	double-stranded RNA-activated protein kinase
p.i.	post infection
p.t.	post transduction
SARS	severe acute respiratory syndrome
SDS	sodium dodecyl sulphate
*Sf*9	*Spodoptera frugiperda* 9
SNPV	single nucleopolyhedrovirus
STAT	signal transducer and activator of transcription
SV40	simian virus 40
TBS	Tris-buffered saline
TM	transmembrane
TNF-α	tumor necrosis factor alpha
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
VEGF	vascular endothelial growth factor
VSVg	vesicular stomatitis virus g-protein

1 INTRODUCTION

1.1 Cancer gene therapy

Cancer is a complex disease involving genetic alterations in cell signaling pathways that normally control cell proliferation and cell death. The two main classes of genes generally mutated during cancer development are proto-oncogenes and tumor suppressor genes. The protein products of proto-oncogenes normally control cell growth allowing cell division under suitable conditions and limiting the growth when not appropriate. Mutation or over-expression of a proto-oncogene transforms it to an oncogene, allowing unregulated growth even in the absence of growth signals. Instead, tumor suppressor genes normally restrict unusual cell growth by inducing cell cycle arrest and programmed cell death, apoptosis. Hence, mutations in tumor suppressor genes increase the possibility of further mutations. The consequences of alterations in oncogenes and tumor suppressor genes are uncontrolled proliferation of large amounts of cells and/or decreased cell death, which may eventually result in the development of cancer. (For review, see El-Aneed, 2004; Wilson, 2002).

During the past decade, it has been generally proposed that cancer could be treated by transferring therapeutic genes or proteins into the cancer tissue to reinstate the normal functions of the cells. Numerous studies have suggested that restoration or augmentation of the normal growth and death pathways of the cells or elicitation of anti-tumor immune responses could lead to limitation of cancer growth. The restorative changes could be achieved by transferring tumor suppressor genes, pro-apoptotic genes or genes that inhibit the activity of oncogenes into the tumor cells to mediate direct cytotoxic or cytolytic effects. Additional approaches include the transfer of anti-angiogenic genes that inhibit the formation of tumor blood vessels, or immunomodulatory genes, such as cytokines, which stimulate the innate immune system to directly attack the cancer cells. Moreover, transgenes for tumor antigens could be used as cancer “vaccines” to induce immune system to recognize the tumor antigen and eradicate the cells expressing it. (For review, see El-Aneed, 2004; Wilson, 2002).

To date, many different strategies involving viral or non-viral gene delivery systems have been developed to transfer therapeutic genes into tumor cells (for review, see El-Aneed, 2004; Wilson, 2002). Non-viral applications range from direct injection of DNA to combining DNA with targeting molecules such as polymers or cationic lipids that enable the penetration through the plasma membrane (for review, see Verma and Somia, 1997). Recently, *in vivo* tumor imaging as well as targeted delivery of genes and anti-tumor agents has been achieved by using nanoparticles (Sanvicens and Marco, 2008). However, the utilization of non-viral vectors is often limited by the inadequate efficiency of gene delivery and transgene expression (for review, see Verma and Somia, 1997). Hence, most of the current gene therapy approaches apply modified viruses for gene delivery. These viral systems make use of the potent machinery of the viruses to deliver genes into cells, but have been reformed to decrease the pathogenicity and improve the safety of these vectors (for review, see El-Aneed, 2004; Wilson, 2002; Verma and Somia, 1997). Viral vectors used for gene delivery applications range from human retroviruses (for review, see Wilson, 2002; Verma and Somia, 1997), adenoviruses (see section 1.2.4.1) and adeno-associated viruses (see section 1.2.4.2) to an insect baculovirus (see section 1.3.3).

1.2 Melanoma differentiation associated gene-7 (Mda-7)

Melanoma differentiation associated gene-7, Mda-7, is a novel cytokine that has shown promise in both preclinical and clinical studies against cancer. Mda-7 suppresses growth and induces apoptosis in numerous human cancer cells without harming normal cells (Jiang et al., 1995; Su et al., 1998; Madireddi et al., 2000; Mhashilkar et al., 2003; Tong et al., 2005; Gopalan et al., 2007; Tahara et al., 2007). Mda-7 also possesses immunomodulatory (Miyahara et al., 2006; Tahara et al., 2007) and anti-angiogenic (Saeki et al., 2002; Ramesh et al., 2003; Tong et al., 2005) properties as well as a potent bystander activity (Chada et al., 2004a; Su et al., 2005; Sauane et al., 2006), which is mediated by the secreted Mda-7 protein.

The *mda-7* was first discovered when human melanoma HO-1 cells were induced to terminally differentiate by treatment with human interferon beta and a protein kinase C activator mezerein (Jiang et al., 1995). The *mda-7*, composed of seven exons and six

introns (Huang et al., 2001), has later been mapped to an interleukin-10 (IL-10) related gene cluster in the chromosome 1q32 (Blumberg et al., 2001). The major open reading frame of the 1718 nucleotide full-length *mda-7* cDNA encodes a highly conserved protein of 206 amino acids with a molecular weight of 23,800 (Jiang et al., 1995), which is a precursor form of the mature cleaved, post-translationally modified and secreted product. Three asparagine residues at amino acid positions 85, 99 and 126 are N-glycosylated (Sauane et al., 2003b), and a 49-amino acid signal peptide allows the mature Mda-7 protein to be cleaved and secreted (Gupta et al., 2006). The Mda-7 is expressed in certain human cells and tissues associated with immune system including peripheral blood leukocytes (PBMC), melanocytes, spleen and thymus. Instead, Mda-7 is not expressed without induction at the mRNA level in most other normal or cancer cell types (Huang et al., 2001).

Rat and mouse orthologs of Mda-7 have been identified. The rat ortholog, c49a/mob-5, is involved in wound healing (designated as c49a; Soo et al., 1999) and in transformation of the oncogenic Ras-protein (designated as mob-5; Zhang et al., 2000). The mouse ortholog, designated as IL-4 induced secreted protein, is selectively expressed and secreted by type 2 helper T cells (Schaefer et al., 2001). The rat and mouse proteins share 93% identity with each other (Schaefer et al., 2001) as well as 60% (Soo et al., 1999) and 69% (Schaefer et al., 2001) identity with the human Mda-7, respectively.

1.2.1 Mda-7 as a member of the interleukin-10 cytokine family

Based on characteristics of the DNA and amino acid sequences, Mda-7 has been reclassified as IL-24 by the Human Gene Organization (Caudell et al., 2002) and designated as a member of the IL-10 cytokine subfamily (Chada et al., 2004b). In addition to IL-10 and Mda-7, this cytokine family includes IL-19 (Gallagher et al., 2000), IL-20 (Blumberg et al., 2001), IL-22 (Dumoutier et al., 2000a), IL-26 (Knappe et al., 2000), IL-28A, IL-28B as well as IL-29 (Kotenko et al., 2003; Sheppard et al., 2003). The member genes of the IL-10 family compose three clusters in the human genome. The genes encoding IL-10, IL-19, IL-20 as well as Mda-7 are located on the chromosome 1q32 (Blumberg et al., 2001), while the genes for IL-22 and IL-26 are mapped to the

chromosome 12q15 (Knappe et al., 2000; Dumoutier et al., 2000b), and for IL-28A, IL-28B and IL-29 to the chromosome 19q13 (Sheppard et al., 2003).

IL-10 is a versatile immunoregulatory cytokine that was identified in 1989 by its ability to inhibit the synthesis of several cytokines produced by type 1 T helper cells. Therefore, IL-10 was first designated as cytokine synthesis inhibitory factor (Fiorentino et al., 1989). IL-10 is produced by various human cell populations: type 2 T helper cells, monocytes, macrophages, and B cells, for example. The main biological function of IL-10 seems to be the regulation of proliferation and differentiation of immune cells as well as limitation of inflammatory responses. Recent results, however, have suggested an additional impact of IL-10 in inflammatory, malignant and autoimmune diseases (for review, see Asadullah et al., 2003). During the last few years, the functions of the other members of the IL-10 cytokine family have also been elucidated more comprehensively. IL-19 was identified in 2000 by exploring sequence databases for possible IL-10 homologs (Gallagher et al., 2000). Although IL-19 appears to have a role in the pathogenesis of an inflammatory skin and joint disease, psoriasis (Li et al., 2005b; Otkjaer et al., 2005), the main features and functions of this cytokine remain to be determined. IL-20 and IL-22 are suggested to be involved in the pathogenesis of psoriasis as well (Blumberg et al., 2001; Otkjaer et al., 2005; Wolk et al., 2006; Sa et al., 2007). IL-20 is expressed in a variety of human tissues and specific cell types, namely squamous epithelial cells, endothelial cells, macrophages, and lymphocytes. IL-20 and its receptors are associated with inflammatory diseases including rheumatoid arthritis and atherosclerosis (for review, see Wei et al., 2006). IL-22, instead, is produced by activated Th1- and natural killer cells and affects tissues that have permanent contact to the exterior world. The receptor for IL-22 is expressed in kidneys, skin and tissues of the digestive and respiratory tract. In these cells, IL-22 is suggested to function by participating in pathogen defense, wound healing and tissue re-organization (for review, see Wolk and Sabat, 2006). The functions of the newest members of the IL-10 cytokine family, IL-26, IL-28A, IL-28B and IL-29, are just beginning to be explored. IL-26 was originally identified when the cellular features of T lymphocytes were characterized after herpes virus saimiri-induced transformation. One of the novel cDNAs cloned during the experiment, *ak155*, shared some nucleotide homology with cellular IL-10 (Knappe et al., 2000) and was later reclassified as IL-26 (Fickenscher et al., 2002). IL-

28A, IL-28B and IL-29 were identified in 2003, in several cell lines upon viral infection. These cytokines have also been designated as interferon lambdas (Kotenko et al., 2003; Sheppard et al., 2003). Thus far, IL-28A, -B, as well as IL-29 are known to possess antiviral activity against herpes simplex virus 2, for example, (Ank et al., 2006), and anti-tumor activity against certain cancer types (Zitzmann et al., 2006; Numasaki et al., 2007).

1.2.2 Receptors of Mda-7

The members of the IL-10 cytokine family transmit signals from the site of the release to the effector cells through different combinations of class II cytokine receptors (CRF2s) on the cell surface. The CRF2s are tripartite transmembrane proteins composed of an extracellular domain containing the ligand-binding residues, the membrane spanning segment, and the cytoplasmic tail domain. The functional receptors of the IL-10 family members are heterodimeric, consisting of two CRF2 subunits, designated R1 and R2. The R1 subunit generally has a larger intracellular domain and higher affinity to its ligand than those of the R2 subunit. Seven different receptor subunits of the IL-10 family have been identified; IL-10 receptor 1 (IL-10R1), IL-10 receptor 2 (IL-10R2), IL-20 receptor 1 (IL-20R1), IL-20 receptor 2 (IL-20R2), IL-22 receptor 1 (IL-22R1), IL-22 binding protein (IL-22BP) as well as IL-28 receptor alpha (IL-28Rα). Although some of the cytokines of the IL-10 family share receptors (Table 1.1), receptor activation is ligand-specific, thus resulting in ligand-dependent responses in the target cell (for review, see Langer et al., 2004).

The Mda-7 signals through two cytokine receptor complexes, IL-20R1/IL-20R2 and IL-22R1/IL-20R2. The binding of Mda-7 to either of the receptor complexes may activate the JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathway (Dumoutier et al., 2001; Wang et al., 2002). Upon ligand binding, the receptor activates JAK thyrosine kinases that, subsequently, recruit signal transducing STATs by phosphorylation. These activated gene regulatory proteins then dissociate from the receptor complex and translocate to the nucleus where they join the other transcriptional factors to stimulate the transcription of cytokine-responsive genes (for review, see Kotenko and Pestka, 2000; Hennighausen and Robinson, 2008).

Table 1.1. The IL-10 cytokine family members and the class II cytokine receptor subunits. The members of the IL-10 cytokine family recognize and bind to their specific two-subunit receptor complexes on the target cell surface. The table presents the two distinct receptor subunits required for signaling for each cytokine. Abbreviations: IL-10R1=IL-10 receptor 1; IL-10R2=IL-10 receptor 2; IL-20R1=IL-20 receptor 1, IL-20R2=IL-20 receptor 2; IL-22R1=IL-22 receptor 2, IL-22BP=IL-22 binding protein; IL-28Rα=IL-28 receptor alpha. Modified from (Langer et al., 2004).

Ligand	Receptor subunits	
	R1	R2
IL-10	IL-10R1	IL-10R2
IL-19	IL-20R1	IL-20R2
IL-20	IL-20R1 , IL-22R1	IL-20R2 , IL-20R2
IL-22	IL-22R1 , IL-22BP	IL-10R2 , -
IL-24	IL-20R1 , IL-22R1	IL-20R2 , IL-20R2
IL-26	IL-20R1	IL-10R2
IL-28A and -B	IL-28Rα	IL-10R2
IL-29	IL-28Rα	IL-10R2

STAT3 (Dumoutier et al., 2001; Wang et al., 2002; Parrish-Novak et al., 2002) and STAT1 (Wang et al., 2002; Parrish-Novak et al., 2002) first seemed to be the major transcription factors mediating the effects of Mda-7. More recent results, however, have demonstrated that Mda-7 is able to exert its actions through multiple JAK/STAT-independent signaling pathways (see section 1.2.3.1 and Figure 1.1) (Mhashilkar et al., 2001; Pataer et al., 2002; Ekmekcioglu et al., 2003; Tong et al., 2005; Chada et al., 2006a; Gopalan et al., 2007).

Although Mda-7 belongs to the IL-10 cytokine family, only few studies have been conducted to examine Mda-7 as a cytokine compared to the large amount of studies on anti-tumor activity of Mda-7 (for review, see Inoue et al., 2006). An *in vitro* study by Caudell and coworkers (2002) first suggested that Mda-7 functions as a type 1 helper cell cytokine thus having pro-immune activities. It was postulated that cytokines induced by Mda-7 may activate antigen presenting cells to display tumor antigens hence eliciting an anti-tumor immune response (Caudell et al., 2002). In a more recent *in vivo* study with murine fibrosarcoma, adenovirus-mediated Mda-7 (Ad-Mda-7) was observed to promote immune activation in syngeneic tumors leading to anticancer immunity (Miyahara et al., 2006). Furthermore, studies in a phase I clinical trial involving intratumoral injection of Ad-Mda-7 into patients with solid tumors propose that these *in vitro* effects are repeated in patient settings, supporting the immune-modulating properties of this cytokine (see section 1.2.3.5).

1.2.3 Mda-7 as an anti-cancer agent

1.2.3.1 Anti-tumor effect of exogenously expressed Mda-7

As described above, the Mda-7 was first identified when the genes involved in terminal differentiation of human HO-1 melanoma cells were characterized. The mRNA level of Mda-7 was elevated in actively proliferating normal human melanocytes in comparison to primary or metastatic human melanomas. Transfection of Mda-7 expression constructs into human melanoma cell cultures resulted in reduced cell growth and colony formation, suggesting tumor suppressor properties of Mda-7 (Jiang et al., 1995). Since this initial study by Jiang and colleagues (1995), many laboratories have tested the anti-tumor activity of Mda-7 both *in vitro* and *in vivo*. Using either secreted Mda-7 protein (Su et al., 2003; Chada et al., 2004a; Sauane et al., 2004; Sauane et al., 2006), replication-defective adenoviruses (Kawabe et al., 2002; Sarkar et al., 2002; Nishikawa et al., 2004) or adeno-associated viruse (AAVs) (Tahara et al., 2007), suppression of cell growth and/or induction of apoptosis have been detected upon over-expression of Mda-7 in a variety of cancer cells including melanoma (Jiang et al., 1995), osteosarcoma, glioblastoma (Su et al., 2003; Jiang et al., 1996), hepatocellular carcinoma (Wang et al., 2006a) and cancers of colon (Jiang et al., 1996; Mhashilkar et al., 2001), lung (Saeki et al., 2002; Pataer et al., 2002; Pataer et al., 2005), nasopharynx, cervix (Jiang et al., 1996), breast (Su et al., 1998; Mhashilkar et al., 2001), ovary (Gopalan et al., 2005) as well as prostate (Jiang et al., 1996; Lebedeva et al., 2003; Tahara et al., 2007). In contrast, Mda-7 appears to be harmless to a broad range of normal cells including melanocytes (Mhashilkar et al., 2001), lung fibroblasts (Saeki et al., 2000), hepatocytes (Wang et al., 2006a) and neuronal cells (Su et al., 2003) as well as epithelial cells of mammary (Mhashilkar et al., 2001), prostate (Gopalan et al., 2005; Lebedeva et al., 2003) and bronchus (Saeki et al., 2002).

Albeit the apoptosis inducing activity of Mda-7 was observed already in 1995, the molecular mechanisms underlying this anti-tumor activity are largely unidentified. To date, Mda-7-mediated tumor suppression has been shown to affect the regulation of various molecules in cancer cells, many of which regulate numerous cellular processes and responses including cell signaling, gene regulation and apoptosis. Although JAK/STAT signaling pathways first seemed to be the main mediators of the activity of Mda-7

(Dumoutier et al., 2001; Caudell et al., 2002; Parrish-Novak et al., 2002 Wang et al., 2002), further studies have proved otherwise (Mhashilkar et al., 2001; Pataer et al., 2002; Ekmekcioglu et al., 2003; Sauane et al., 2003a; Tong et al., 2005; Chada et al., 2006a; Gopalan et al., 2007). By utilizing tyrosine kinase inhibitors and cancer cells defective in the JAK/STAT pathway it has been shown that induction of apoptosis by Mda-7 occurs separately from the activation of JAK/STAT. Treatment with tyrosine kinase inhibitors had no effect on the percentage of apoptotic cells and, moreover, cell lines lacking several components of the signal transduction pathway were found to be susceptible to Mda-7-mediated killing (Sauane et al., 2003a).

The endoplasmic reticulum (ER) is the primary organelle for protein synthesis and folding, as well as calcium signaling and storage in eukaryotic cells. Conditions interfering with the functions of ER, such as accumulation of a misfolded protein in the ER, cause ER stress that triggers the activation of stress responses. Prolonged activation of ER-stress-associated signaling pathways ultimately results in the induction of apoptosis (for review, see Inoue et al., 2006). External growth factors provided by the surrounding cells play an important role in a complex process of regulation of cell proliferation in mammalian cells. Mitogen activated protein kinase (MAPK) pathways with their complex signal cascades are significant contributors in this process. One such kinase is p38 MAPK, which, upon activation by ER stress, participates in responses such as differentiation, proliferation, cell cycle arrest and apoptosis (for review, see Zhang and Liu, 2002). Mda-7 is known to exploit the p38 MAPK pathway to mediate its anti-tumor activity in human cancer cells. Ad-Mda-7-mediated expression of Mda-7 has been shown to increase the phosphorylation of p38 MAPK in human melanoma cells but not in normal immortal melanocytes, resulting in induction of cancer cell specific apoptosis (Sarkar et al., 2002). In breast and lung tumor cells, instead, transduction of Ad-Mda-7 resulted in decreased expression of proto-oncogenes involved in phosphatidylinositol 3-kinase (PI3K) signaling. PI3K is an important regulator of various cell functions including survival, growth and malignant transformation. In addition, PI3K is able to activate multiple signaling molecules such as oncogenes and itself possesses oncogenic activity. Mda-7 has been shown to inhibit PI3K activity and the functions of other proto-oncogenes regulated by PI3K (Mhashilkar et al., 2003).

Cells are also able to undergo apoptosis through mitochondrial apoptotic pathway, which is a highly regulated cascade of events proceeding from an apoptotic stimulus to the mitochondrial outer membrane permeabilization and cell death. The permeabilization of the mitochondrial membrane is in control of the Bcl-2 family of proteins including the pro-apoptotic protein BAX. As a result of an apoptotic stimulus, a conformational change of BAX occurs, followed by translocation of BAX from the cell cytoplasm into the mitochondrial membrane. Membrane permeabilization by BAX stimulates the release of cytochrome C from mitochondria, thus triggering apoptosis (for review, see Lalier et al., 2007). Mda-7 has been shown to up-regulate the expression of BAX in cancer cells of diverse histological features including prostate cancer (Lebedeva et al., 2003) mesothelioma, breast and colorectal cancer as well as non-small cell lung cancer (NSCLC) cells, thus promoting the mitochondrial apoptotic pathway (Mhashilkar et al., 2001; Cao et al., 2002; Chada et al., 2006a).

Mda-7 also kills lung tumor cells via activation of double-stranded RNA-activated protein kinase (PKR) (Pataer et al., 2002; Pataer et al., 2005). PKR is a serine/threonine kinase with two activities: auto-phosphorylation and phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF-2α). PKR is able to phosphorylate eIF-2α in response to stress signals (e.g. ER stress) leading to general inhibition of protein synthesis. In addition to translational regulation, PKR is involved in signal transduction. Upon activation by auto-phosphorylation, PKR is able to phosphorylate various target molecules that are important in growth control and induction of apoptosis (for review, see Garcia et al., 2006). In a study conducted by Pataer and coworkers (Pataer et al., 2002), expression by Ad-Mda-7 lead to activation of PKR, subsequent phosphorylation of both eIF-2α, p38MAPK and other PKR target molecules and subsequent induction of apoptosis.

The over-expression of Mda-7 also up-regulates the expression of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Saeki et al., 2002). TRAIL is an apoptosis inducing cytokine that exerts its actions by binding to death receptors on its target cell surface. Upon TRAIL binding, activated receptors recruit adapter proteins, which subsequently recruit a group of cysteine proteases, called caspases (for review, see Thorburn, 2007). Caspases are key components of evolutionally conserved cell death

machinery and linked to complex signal pathways. Activation of initiator caspase precursors by activator molecules leads to activation of effector caspases. Subsequently, effector caspases mediate the cleavage of apoptosis regulators and other proteins leading to cell death. Some of the caspases activate the mitochondrial apoptosis pathway as well (for review, see Nunez et al., 1998). In a study conducted by Saeki and colleagues (2002) inhibition of cell proliferation, up-regulation of TRAIL as well as induction of apoptosis was observed in Ad-Mda-7 transduced lung cancer cells *in vitro*. The subsequent *in vivo* studies with subcutaneous lung cancer cell derived tumors further demonstrated the Mda-7 mediated anti-tumor activity, as significant inhibition of tumor growth was observed in Ad-Mda-7 treated nude mice (Saeki et al., 2002).

Moreover, Mda-7 has been observed to induce apoptosis through the Fas/Fas ligand pathway in ovarian cancer cells (Gopalan et al., 2005). Fas ligand belongs to the tumor necrosis factor (TNF) family of proteins and is able to initiate apoptosis by binding to its receptor molecule Fas on the cell surface. Receptor binding triggers the formation of the death inducing signaling complex and subsequent activation of the proteolytic caspase cascade (for review, see Kavurma and Khachigian, 2003). In a study by Gopalan and colleagues, (Gopalan et al., 2005), transduction of Ad-Mda-7 resulted in increased death receptor Fas and Fas ligand expression as well as increased surface expression of Fas in ovarian cancer cells.

Importantly, the pro-apoptotic activity of Mda-7 has been shown to be independent of the status of tumor suppressor mutations such as p53 (Jiang et al., 1996; Su et al., 1998; Lebedeva et al., 2003; Su et al., 2003) and retinoblastoma (Jiang et al., 1996; Mhashilkar et al., 2001) in the target cell. Mda-7 exerts its anti-tumor activity in both mutant and wild type cases (Jiang et al., 1996; Su et al., 1998; Mhashilkar et al., 2001; Lebedeva et al., 2003; Su et al., 2003), which is important from a gene therapy point of view, since heterogeneity of p53 status is common in clinical settings of certain cancer types (for review, see Lebedeva et al., 2005).

Genetic defects in tumor cells affect multiple signaling pathways by activating or inhibiting them. Thus, the unique ability of Mda-7 to act on several signaling pathways in

cancer cells and regulate both pro-apoptotic and anti-apoptotic proteins in different cell types is extremely advantageous. Unlike several other gene-based drugs, Mda-7 does not have to depend on a single molecule to mediate its anti-tumor activity (Inoue et al., 2006). It appears that Mda-7 is able to find the weaknesses in defense of most cancer cells and take advantage of intrinsic flaws to eradicate these tumor cells (for review, see Gupta et al., 2006). The numerous signaling pathways regulated by Mda-7 in cancer cells are summarized in Figure 1.1.

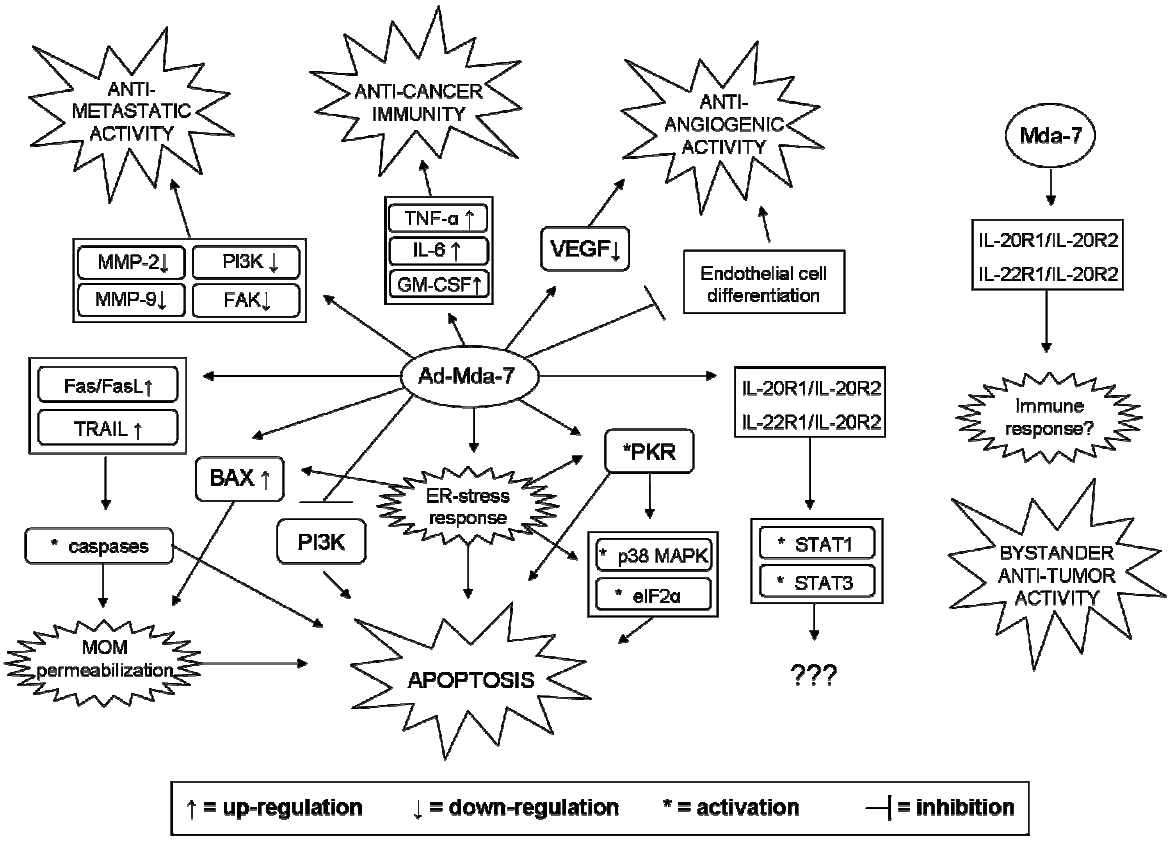

Figure 1.1 A schematic representation of the diverse cellular signaling pathways involved in cancer specific anti-tumor activity of Ad-Mda-7 and Mda-7. Mda-7 induces apoptosis in a variety of human cancer cells via multiple different signaling pathways and possess anti-metastatic as well as anti-angiogenic activity and promotes anti-cancer immunity. The functions of Mda-7 are exerted by both intracellular and extracellular Mda-7 protein. Abbreviations: Ad-Mda-7= adenovirus vector carrying the *melanoma differentiation associated gene-7* (*mda-7*) gene; Mda-7= secreted Mda-7 protein; MMP-2; matrix metalloproteinase-2; MMP-9; matrix metalloproteinase-9; PI3K= phosphatidylinositol 3-kinase; FAK= focal adhesion kinase; TNF-α= tumor necrosis factor α; IL-6= interleukin-6; GM-CSF= granulocyte-macrophage colony stimulating factor; VEGF= vascular endothelial growth factor; Fas= Fas protein; FasL= Fas ligand; TRAIL= tumor necrosis factor-related apoptosis-inducing ligand; BAX= BAX protein; MOM= mitochondrial outer membrane; PKR= double-stranded RNA-activated protein kinase; p38 MAPK= p38 mitogen activated kinase; elF-2α= alpha subunit of eukaryotic initiation factor 2; IL-20R1= Interleukin 20 receptor 1; IL-20R2= Interleukin 20 receptor 2; IL-22R1= Interleukin 22 receptor 1; STAT= signal transducer and activator of transcription. Modified from Fisher, 2005; Gupta et al., 2006; and Inoue et al., 2006.

1.2.3.2 Anti-tumor bystander activity

In addition to vector-mediated cancer cell killing, Mda-7 has been found to exert its anti-tumor effects through bystander apoptosis-inducing activity in adjacent tumor cells not initially receiving the *mda-7* gene. Mhashilkar and coworkers (2001) first proposed the anti-tumor activity of the secreted Mda-7. Since then, purified human Mda-7 protein produced in eukaryotic (Chada et al., 2004a; Chada et al., 2005; Su et al., 2005; Zheng et al., 2007) or prokaryotic (Su et al., 2003; Sauane et al., 2004) expression systems have been shown to exert direct cytotoxic effects selectively in tumor cells. In addition to several human cancer cell lines (Mhashilkar et al., 2001; Wang et al., 2002; Sauane et al., 2004; Su et al., 2005), Human Embryonic Kidney 239 cells (Chada et al., 2004a; Chada et al., 2005; Zheng et al., 2007; Mumm et al., 2006) as well as immortal normal P69 prostate epithelial cells (Su et al., 2005), inter alia, have been utilized to secrete the Mda-7 protein.

The mechanism by which the secreted Mda-7 elicits its anti-cancer activity is unclear. It seems, however, that unlike the anti-tumor effect mediated by the intracellular Mda-7, the bystander activity of the secreted Mda-7 is dependent on the presence of the IL-20R1/IL-20R2 and IL-22R1/IL-20R2 receptor complexes on the target cell surface (see Figure 1.1). In pancreatic cancer cells, the blocking of IL-22R1 or IL20-R1 with receptor-specific antibodies abrogated the tumor cell killing activity only partially, suggesting that both receptors are involved in anti-tumor bystander activity in pancreatic tumors (Chada et al., 2005). In the MeWo melanoma cell line expressing all three receptor subunits, only IL-20R1 and IL-20R2 were found to be involved in the induction of apoptosis. Treatment of cells with the IL-22R2-specific antibody did not affect the Mda-7 bystander activity, whereas blocking of IL-20R1 with the specific antibody resulted in the elimination of the Mda-7 protein mediated tumor cell killing (Chada et al., 2004a). In a study applying normal cells to secrete Mda-7, tumor cells lacking a complete set of the canonical Mda-7 receptors did not respond to the Mda-7 mediated bystander suppression of growth or induction of apoptosis (Su et al., 2005). Anti-tumor activity mediated by the secreted Mda-7 is schematically illustrated in Figure 1.1.

The exact molecular events following the receptor binding by the Mda-7 protein are not known. In melanoma cells, Mda-7 seems to exert some of its actions via the JAK-STAT

signaling pathway, since the treatment with secreted Mda-7 results in rapid phosphorylation and activation of STAT3. However, although both cytotoxicity and STAT3 activation were observed in the Mda-7 treated melanoma cells, they seemed to be independent effects (Chada et al., 2004a). In a study by Sauane and colleagues (2004) cellular localization of the secreted Mda-7 protein in normal and prostate cancer cells was investigated. Since confocal microcopy studies revealed that considerable proportion of Mda-7 entered ER and Golgi apparatus, it was suggested that Mda-7 may induce ER-stress responses, which in turn induce the pro-apoptotic events. However, because localization of Mda-7 was similar in both normal and cancer cells, it seems that cellular positioning of the protein is not a direct mechanism of selective apoptosis induction in cancer cells (Sauane et al., 2004).

1.2.3.3 Anti-metastatic and anti-angiogenic activity of Mda-7

Tumor development and metastasis are complicated processes involving multiple changes in cancer cell biochemistry and physiology. The main components of tumor progression promoting the metastatic phenotype are tumor cell invasion and migration (for review, see Gupta et al., 2006). The idea of Mda-7 possessing anti-metastatic activity stemmed from a study by Ellenhorst and colleagues (2002), who observed an inverse correlation between the expression of Mda-7 and invasion of melanoma tumors (Ellerhorst et al., 2002). Since then, the effects of Mda-7 on tumor cell invasion have been studied by utilizing both Ad-Mda-7 (Ramesh et al., 2004; Sauane et al., 2004) and the secreted Mda-7 protein (Su et al., 2005). In a study by Sauane and coworkers (2004), Ad-Mda-7 was found to inhibit migration of metastatic human melanoma cells through matrigel, without affecting cell viability (Sauane et al., 2004). Furthermore, by utilizing human NSCLC and large cell lung carcinoma cells, Ad-Mda-7 was shown to inhibit tumor cell invasion and migration, independent of the growth inhibitory activity (Ramesh et al., 2004). Mda-7 thus seems to exert its anti-metastatic action by inhibiting the expression of proteins associated with invasion and migration of tumor cells (see Figure 1.1) (Mhashilkar et al., 2003; Ramesh et al., 2004). These proteins negatively-regulated by Mda-7 include PI3K (Mhashilkar et al., 2003), focal adhesion kinase (Mhashilkar et al., 2003; Ramesh et al., 2004), as well as matrix metalloproteinase-2 and -9 (Ramesh et al., 2004). Anti-metastatic activity of Mda-7 has been further investigated *in vivo*, resulting in reduced number of tumor metastases in

nude mice injected with Ad-Mda-7 infected NSCLC cells (Ramesh et al., 2004). Importantly, in a recent phase I clinical study with patients with solid tumors, intratumoral injection of Ad-Mda-7 led to distant biological activity outside the injection site, thus suggesting therapeutic potential of Mda-7 in treating metastatic disease (see section 1.2.3.5).

A crucial factor in cancer development and tumor progression is the presence of sufficient blood supply to assure the persistence of the primary and secondary (metastatic) tumors. This process relies on the development of new blood vessels, angiogenesis, which, in turn, is regulated by growth factors secreted by the surrounding cells. To date, one of the main focuses for anti-cancer therapy is on identifying methods to inhibit angiogenesis (for review, see (Gupta et al., 2006). To define additional mechanisms for the anti-tumor activity of Mda-7 Saeki and colleagues (2002) assessed the anti-angiogenic properties of Ad-Mda-7. *In vitro* experiments with human umbilical vein endothelial cells revealed Ad-Mda-7 mediated inhibition of differentiation of endothelial cells into tube-like structures, suggesting that Mda-7 might possess anti-angiogenic activity *in vivo*. Subsequent analysis was conducted on the expression of the tumor neo-vasculature marker CD31 in NSCLC tumors in nude mice. In Ad-Mda-7 treated tumors, reduced CD31 expression and tumor vascularization was observed, strongly proposing the anti-angiogenic activity of Mda-7 (Saeki et al., 2002).

Indirect inhibitory action of Ad-Mda-7 on angiogenesis both *in* vitro and *in vivo* has been further demonstrated. When expressed at supraphysiological levels Mda-7 was shown to suppress NSCLC tumor xenograft vascularization indirectly via downregulation of expression of pro-angiogenic growth factors, such as vascular endothelial growth factor (VEGF), in mice (Nishikawa et al., 2004). In prostate cancer cells, Ad-Mda-7 was also observed to inhibit angiogenesis by inhibiting VEGF production by tumor cells (Inoue et al., 2005). Anti-angiogenic activity of Mda-7 is schematically represented in Figure 1.1.

Further studies have showed inhibitory effects of the secreted Mda-7 protein on tumor angiogenesis as well. Affinity purified Mda-7 was observed to inhibit endothelial cell differentiation in a dose-dependent manner without affecting cell proliferation. Moreover,

Mda-7 protein mediated anti-angiogenic activity was shown to be receptor-dependent *in vitro*, as the inhibition of tube formation was abrogated upon IL-22R1 receptor blocking. *In vivo* inhibition if angiogenesis by secreted Mda-7 protein was subsequently investigated, and Mda-7 was detected to systemically inhibit lung tumor xenograft growth and decrease micro vessel density in nude mice (Ramesh et al., 2003).

1.2.3.4 Combination therapy

Although the potential of *mda-7*-based gene therapy for the treatment of human cancers have been shown by numerous laboratories, as a monotherapy, gene therapy does have its limitations. Previous preclinical and clinical experiments have shown that gene therapy combined with chemotherapy or radiotherapy has an improved therapeutical effect. Several studies have been conducted to evaluate the possibilities to utilize Mda-7 in combination therapy against cancer (for review, see Inoue et al., 2006).

Ad-Mda-7 have been found to radiosensitize NSCLC cells thus enhancing the therapeutical effect *in vitro* (Kawabe et al., 2002) and *in vivo* (Nishikawa et al., 2004). Similarly, Ad-Mda-7 sensitizes breast carcinoma cells to radiotherapy, biological therapies and chemotherapy (Chada et al., 2006b), and together with radiotherapy results in better therapeutical effect in gliomas (Yacoub et al., 2003). Furthermore, combined with the non-steroidal anti-inflammatory drug *sulindac*, Ad-Mda-7 has been shown to effectively restrain human lung cancer growth both *in vitro* and *in vivo* (Oida et al., 2005). Although several studies demonstrate that combining Mda-7 with other therapeutic agents or treatment modes can further augment its anti-tumor activity, further studies are needed to determine if these and other agents will also lead to enhanced therapeutic activity in patients (for review, see Gupta et al., 2006).

1.2.3.5 Clinical studies with Mda-7

A phase I clinical trial has recently been conducted to characterize the safety and biological activity of the *mda-7* gene transfer. In a phase I clinical study patients with solid resectable tumors were treated with intratumoral injections of a non-replicating Ad containing the *mda-7* construct (INGN 241; Introgen Therapeutics Inc.). Advanced cancer patients with histologically versatile tumors such as breast or colon carcinoma, squamous cell carcinoma

of the head and neck, melanoma, hepatoma or large cell lymphoma, were divided in cohorts according to viral dose, time of post-treatment biopsy and mode of treatment. Following injections of Ad-Mda-7, tumor biopsies were performed and examined for vector penetration, Mda-7 protein expression and apoptosis induction. In addition, blood samples from the patients were analyzed for vector DNA, cytokines, T-cell subsets and blood chemistry. (Cunningham et al., 2005; Tong et al., 2005).

The Ad-Mda-7 treatment was shown to be well-tolerated as the intratumoral injection lead to minimal or no toxicity in all patients (Cunningham et al., 2005; Tong et al., 2005). Gene transfer and subsequent Mda-7 expression was shown to be successful in all injected lesions and signal intensity was observed to be reduced distance away from the injection site. Furthermore, distinct apoptotic activity was observed in Ad-Mda-7-treated tumors corresponding to areas of Mda-7 protein expression (Tong et al., 2005). In addition, increased amounts of proinflammatory cytokines IL-6, tumor necrosis factor-α (TNF-α) and granulocyte-macrophage colony stimulation factor as well as $CD3^{+}CD8^{+}$ T-cells were observed in treated patients (see Figure 1.1), proposing that Ad-Mda-7 up-regulated the systemic production of type I T helper cell cytokines and activated $CD8^{+}$ T-cells (Cunningham et al., 2005; Tong et al., 2005). Moreover, evidence of clinical activity was found in 44% of lesions with repeated Ad-Mda-7 injections, including complete and partial responses in two melanoma patients. Although Ad-Mda-7 was administered directly to tumors, evidence of distant biologic activity was observed, suggesting that the therapy may have utility in treating primary tumors as well as metastatic disease (Cunningham et al., 2005). Thus, the findings of the first clinical trial are consistent with previous preclinical studies and suggest therapeutical potential of Mda-7 in treating human cancers (Cunningham et al., 2005; Tong et al., 2005).

At the moment INGN 241 is being tested in a phase II clinical trial with patients suffering from advanced melanoma and in a phase III trial in combination with radiation therapy in solid tumors with patients with head and neck cancer (Press Release; Introgen Therapeutics Inc. 2007).

1.2.4 Expression of Mda-7 by viral vectors

1.2.4.1 Adenoviral (Ad) vectors

A majority of the studies exploring the anti-tumor activity of Mda-7 have utilized human Ad vectors. Since their discovery in 1953 from human adenoid tissue, Ads have been extensively studied (for review, see Campos and Barry, 2007). Ads are non-enveloped icosahedral particles, composed of a nucleocapsid and a 26-40 kb linear double-stranded (ds) DNA genome (for review, see Volpers and Kochanek, 2004). In addition to 50 serotypes of human Ads identified so far, numerous other mammalian, avian, amphibian, reptilian and fish serotypes of adenoviruses are known (for review, see Campos and Barry, 2007). Human Ads can cause infections ranging from acute respiratory infections and conjunctivitis to gastroenteritis (for review, see Volpers and Kochanek, 2004).

Ads have become popular vectors for gene therapy research because of their high nuclear transfer efficiency, broad tissue tropism, and rather low pathogenicity. Moreover, Ads can be produced in high titers, they can transduce cells *in vivo* with transgenes of more than 30 kb and they are unable to integrate into the human genome (Volpers and Kochanek, 2004). Most human Ads utilize the cellular coxsackie- and Ad receptor (CAR) to enter the target cells. However, some cell types in the human body including hematopoietic and endothelial cells, skeletal and smooth muscle cells, as well as many tumor cells totally lack or express only low levels of CAR receptors. Thus, different targeting strategies must be employed to enable the Ad-mediated gene delivery into therapeutically relevant tissues lacking CAR receptors (for review, see Volpers and Kochanek, 2004).

Because Ad is a human pathogen, systemic delivery of Ad vectors results in activation of innate host responses in humans, which represents a barrier to safe clinical gene therapy (for review, see Campos and Barry, 2007). Elicitation of strong humoral and cellular immunity upon Ad-mediated gene delivery has even resulted in the death of a patient (Marshall, 1999). Rapid physiological responses such as induction of cytokines, activation of innate immunity, inflammation, transient liver toxicity and thrombocytopenia upon systemic delivery of Ad vectors are suggested to result from the uptake of the viruses by

Kupffer cells, resident liver macrophages (for review, see Campos and Barry, 2007). In the previously mentioned phase I clinical gene therapy trial utilizing replication incompetent Ad-Mda-7, strong induction of anti-Ad antibodies was observed after single injection of Ad-Mda-7. However, this did not block either viral transduction upon single injection or anti-tumor activity after repeat administration (Cunningham et al., 2005; Tong et al., 2005).

The majority of the Ad vectors utilized in pre-clinical Mda-7 studies have been replication-defective (Pataer et al., 2002; Nishikawa et al., 2004; Oida et al., 2005; Tong et al., 2005; Wang et al., 2006a) to exclude the uncontrollable viral propagation. Recently, conditionally replication competent Ads have also been constructed and studied for augmentation of Mda-7 gene expression (Sarkar et al., 2007). However, utilization of replication competent viruses in gene delivery purposes raises serious safety concerns. Thus, precise restriction of viral replication both spatially, confining replication to certain cell types, and temporally, must be achieved to enable responsible use of these viruses in gene therapy (Chong et al., 2002). Recently, a novel strategy for regulation of Ad replication has been developed. In a study by Ylösmäki and colleagues (2008), a liver specific microRNA was used to reduce Ad replication in cells of hepatic origin. It was suggested that by utilizing this approach, liver toxicity following the systemic delivery of oncolytic Ads could be bypassed, and furthermore, that by tissue specific expression of other microRNAs targeted replication of Ads could be achieved (Ylosmaki et al., 2008).

1.2.4.2 Adeno-associated virus (AAV) vectors

As mentioned previously, Tahara and colleagues (2007) have studied the potential of Mda-7 in cancer gene therapy by using an adeno-associated virus (AAV) type 1 vector. AAVs are small, non-enveloped, icosahedral viruses consisting of a capsid and a linear single stranded 4.7 kb DNA genome. AAVs belong to the Dependovirus genus in the parvovirus family thus requiring a helper virus to enable productive infection and replication. In the absence of a helper virus a latent infection is established, during which AAVs incorporate into the host genome by site-specific integration or persist in episomal forms (for review, see Coura Rdos and Nardi, 2007). Whereas the wild-type AAV integrates into the chromosome 19 in human genome, the recombinant vectors stay mostly episomal,

although random integration may occur. To date, nine primate serotypes of AAV have been identified, AAV2 being the most studied and currently used in gene therapy trials for inherited diseases (Li et al., 2005a) such as cystic fibrosis and hemophilia as well as prostate cancer (for review, see Coura Rdos and Nardi, 2007).

Tahara and coworkers selected an AAV1 vector for their systemic cancer gene therapy study because AAVs are non-pathogenic to humans. Furthermore, because AAV1 has been isolated from rhesus monkeys, it was suggested to be less likely to cause immunological side effects compared to Ad vectors (Tahara et al., 2007). Other advantages of AAVs as gene delivery vectors in general include the ability to transduce both dividing and non-dividing cells and a wide host and cell type tropism range, which vary between the AAV serotypes (for review, see Coura Rdos and Nardi, 2007). However, some drawbacks in utilizing AAV vectors for gene delivery applications also occur. The packaging capacity of AAVs for foreign DNA is restricted (for review, see Hu, 2006) and the onset of gene expression occurs slowly (for review, see Coura Rdos and Nardi, 2007). Large scale virus production is rather complicated (for review, see Hu, 2006), but high amounts of AAV vectors can simply be produced in insect cell culture by utilizing baculovirus expression vector system (Urabe et al., 2002). Importantly, due to the capacity of AAVs to incorporate into the host genome, there is a risk of mutagenesis as well as germ line transmission upon systemic administration of AAV vectors (Tahara et al., 2007).

1.3 Baculoviruses

Baculoviruses belong to the large and diverse family of *Baculoviridae*, occluded double-stranded DNA viruses that mainly infect the larval stages of insects of the orders Lepidoptera (butterflies and moths), Diptera (mosquitoes) and Hymenoptera (sawflies) (for review, see (Okano et al., 2006). To date, more than 500 different types of baculoviruses have been identified (for review, see Bonning, 2005) from more than 600 host insect species (Slack and Arif, 2007). Baculovirus particles consist of an enveloped, rod-shaped nucleocapsid enclosing the covalently closed circular genome ranging from 82 to 180 kb in size (Jehle et al., 2006). Based on the occlusion body morphology, baculoviruses have been divided into two genera; *Nucleopolyhedrovirus* (NPV) and *Granulovirus*. NPVs form

large occlusion bodies containing multiple virions in the nuclei of the infected cells, whereas granuloviruses form small granular occlusion bodies each enclosing a single virion. NPVs can be further designated as single or multiple NPVs (SNPV or MNPV, respectively) based on the number of nucleocapsids within the viral envelope (for review, see Blissard and Rohrmann, 1990; Summers, 2006; Bonning, 2005).

Initially, baculoviruses were studied mainly for agricultural applications due to their natural ability to control certain insect pest populations (Blissard and Rohrmann, 1990; Summers, 2006). Later, the discoveries of the unique structure of baculoviruses and their natural process of infection have led to widespread research utilizing baculoviruses for the study of fundamental biological processes, for recombinant protein expression, and for gene therapy (for review, see Bonning, 2005; Summers, 2006).

The eukaryote-based baculovirus expression vector system was developed during the 1980s, pioneered by Smith and colleagues (1983), who first explored baculoviruses as vectors for the expression of human interferon beta (INF-β) in insect cells (Smith et al., 1983). Since then, the potential of the baculovirus-insect cell system has been exploited in a wide variety of applications (for review, see Summers, 2006). Over the past two decades hundreds of complex human, animal and viral proteins requiring folding, subunit assembly and extensive post-translational modifications have been successfully produced in insect cells (for review, see Kost et al., 2005). Important attributes and advantages of protein production in baculovirus-insect cell system include large insertion capacity for foreign genes, ease and high yield of heterologous protein production driven by the strong baculoviral promoters, and biosafety due the innate inability of baculoviruses to infect mammalian cells (for review, see Hu, 2005; Kost et al., 2005; Hu, 2006). However, there are some drawbacks associated with the baculoviral expression system, such as inadequate glycolysation in some cases. Furthermore, progressing baculovirus infection interferes with the post-translational machinery and the secretory pathway of the cell thus affecting the processing and production of proteins and, ultimately, results in cell death and lysis (for review, see Hu, 2005). However, to overcome the weaknesses of insufficient insect glycosylation, stable insect cell lines expressing mammalian glycosyltransferases have been developed, thus allowing production of “mammalianized” recombinant glycoproteins

in baculovirus-insect cell system. Correspondingly, the genes needed for adequate protein processing can be inserted into a baculovirus vector, to be expressed during infection (for review, see Harrison and Jarvis, 2006).

Since the highly efficient gene delivery into many cell types, baculoviruses have recently gained increasing interest as vectors for gene therapy and other *in vivo* applications. Baculoviruses appear to be promising candidates for gene therapy due to their large insertion capacity, low cytotoxicity and incapability to replicate in mammalian cells. However, some challenges for baculovirus-mediated *in vivo* gene therapy occur, including elicitation of innate immune responses and inactivation of the virus by the serum complement system (for review, see Hu, 2005; Kost et al., 2005; Hu, 2006). Recently, a novel strategy enabling presentation of foreign peptides or proteins on the baculovirus surface has been developed. This surface display technology has several auspicious applications, rendering baculoviruses as versatile tools for functional genomics, antigen presentation (for review, see Grabherr et al., 2001; Oker-Blom et al., 2003), and targeted gene delivery (Mäkelä et al., 2006; Mäkelä et al., 2008).

1.3.1 ***Autographa californica*** multiple nucleopolyhedrovirus (***Ac***MNPV)

Among the various baculoviruses, the *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) is the most extensively studied. *Ac*MNPV was originally isolated from *Autographa californica* alphalpha looper, but is able to infect more than 400 species within the Lepidoptera (for review, see Bonning, 2005).

1.3.1.1 Baculovirus phenotypes

During infection *Ac*MNPV occurs in two phenotypes; budded virus (BV) and occlusion-derived virus (ODV) both having identical nucleocapsids and carrying the same genetic information (Summers and Volkman, 1976; Blissard and Rohrmann, 1990). *Ac*MNPV has a genome of 133 894 kb, which has been fully sequenced (Ayres et al., 1994). The nucleocapsids are rod-shaped, 30-60nm in diameter and 250-300nm in length and surrounded by an envelope (Jehle et al., 2006). The two virus phenotypes differ in their envelope structure due to their different origins; ODV acquires its envelope from the host

cell nucleus, whereas BV is surrounded by a membrane obtained by budding through the plasma membrane (Volkman and Summers, 1977; Braunagel and Summers, 1994). As a result, the phenotypes have distinct lipid and protein compositions, tissue specificities and mechanisms of entry into the host cell (for review, see Blissard and Rohrmann, 1990; Braunagel and Summers, 1994). BV virions occur predominantly as single nucleocapsids enclosed in loose envelopes, whereas ODVs may have one or several nucleocapsids surrounded by a single tight-fitting envelope (Summers and Volkman, 1976, for review, see Bonning, 2005). A major component of the BV envelope, absent from ODVs, is the virus-encoded membrane glycoprotein gp64, which has many important functions in viral entry and budding. ODV virions, instead, are further embedded within proteinaceous occlusion bodies surrounded by an additional envelope (for review, see Bonning, 2005). The structural differences between the two phenotypes are reflected in their separate roles in the virus life cycle; ODV is responsible of the primary infection of the host larvae, whereas BV transmits the infection within the host (Volkman et al., 1976; Braunagel and Summers, 1994, for review, see Blissard and Rohrmann, 1990; Bonning, 2005). The two baculovirus phenotypes are schematically represented in Figure 1.2.

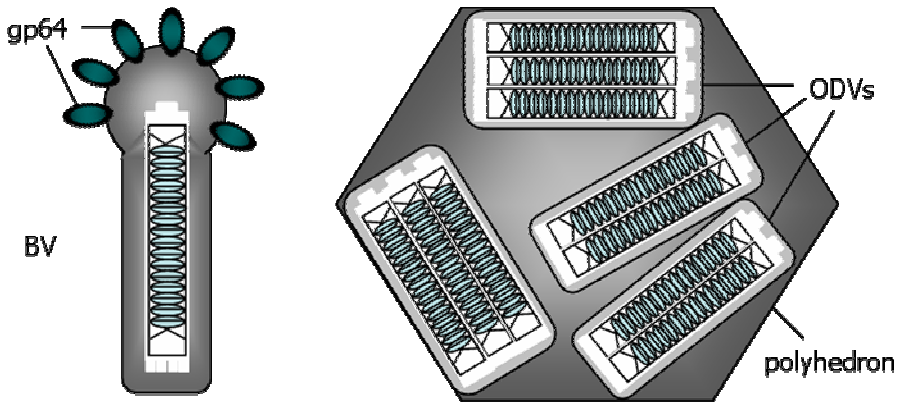

Figure 1.2 Baculovirus virion phenotypes. Schematic representation of *Ac*MNPV budded virion (BV) and occlusion-derived virion (ODV) surrounded by an occlusion body (polyhedron). (Modified from Mäkelä et al., 2008)

1.3.1.2 Life cycle

The first round of natural baculovirus infection is established when a feeding larval host ingests the ODV-containing viral occlusions called polyhedra. The protein matrix of the polyhedra dissolves in the basic environment of the larval midgut releasing the infectious

ODVs, which successively fuse with microvilli of the midgut epithelium thus entering the host cells. The primary round of viral replication occurs early during the infection producing progeny BV nucleocapsids, which subsequently bud from the epithelial cell nucleus. The primary envelope acquired from the nuclear envelope is lost in the cytoplasm during the nucleocapsid transport to the plasma membrane. Finally, BVs obtain their distinctive envelopes with virus-encoded proteins by budding through the plasma membrane of the basal side of the midgut epithelial cells. The released viruses are able to infect numerous cell types within the host to produce the second round of infection (for review, see Blissard and Rohrmann, 1990; Okano et al., 2006).

BV is known to enter insect cells via receptor mediated endocytosis (Volkman and Goldsmith, 1984; Zhou and Blissard, 2008) presumably through clathrin coated pits (Long et al., 2006). The virus-encoded envelope fusion protein gp64, a major component of the BV envelope (Volkman et al., 1984), has been shown to be essential for virus entry into insect cells. Upon virus entry, gp64 mediates the low pH-triggered envelope fusion with host endosomal membranes thus allowing nucleocapsid transport into the cytoplasm and nucleus (Blissard and Wenz, 1992). Gp64 accumulates at the surface of the infected cells and at the poles of the BVs as homotrimers (Volkman and Goldsmith, 1984) forming typical peplomer structures (Summers and Volkman, 1976). Each peplomer consists of three identical gp64 proteins, stabilized by intermolecular disulfide bonds (Volkman and Goldsmith, 1984; Oomens et al., 1995; Markovic et al., 1998). Gp64 has, furthermore, been shown to be crucial for viral budding from insect cells (Oomens and Blissard, 1999) and spreading the infection through cell-to-cell transmission (Monsma et al., 1996).

During the very late phase of the infection, at approximately 24 h post infection (p.i.), virus production switches primarily from BVs to ODVs. Viral-induced membrane projections in the nucleus envelope the nucleocapsids to produce ODVs which, subsequently, are embedded in the occlusion matrix composed of the viral protein polyhedrin. Each occluded polyhedron is additionally surrounded by envelope or calyx. Polyhedra are resistant to dryness and freezing and thus extremely stable in the environment. However, when exposed to direct sunlight ODVs rapidly lose most of their activity due to the DNA damage caused by UV radiation. While ODVs have an essential role in spreading the

infection from host to host, BVs are, instead, responsible for spreading the infection within the host as well as for viral propagation in cell culture (for review, see Bonning, 2005; Ghosh et al., 2002) and the primary form used in the laboratory as an expression vector (Hu, 2005).

1.3.2 Baculovirus and mammalian cells

1.3.2.1 Baculovirus transduction of mammalian cells

The safety concerns regarding the use of baculoviruses as pesticides led investigators to seek information on biological interactions between baculoviruses and non-target cells. In 1983, Tjia and coworkers first demonstrated that *Ac*MNPV can be internalized by mammalian cells. Although some of the viral DNA entered the nucleus, the nuclear DNA did not persist and no transcription of the viral DNA was observed (Tjia et al., 1983). Later studies by others (Volkman and Goldsmith, 1983; Groner et al., 1984; Carbonell et al., 1985) confirmed the consequences of baculoviral entry into mammalian cells. In the 1990s, two groups reported that recombinant baculoviruses containing a cytomegalovirus promoter-*luciferace* gene cassette (Hofmann et al., 1995) or a Rous sarcoma virus long terminal repeat promoter-*β-galactosidase* cassette (Boyce and Bucher, 1996) efficiently transduced hepatocytes and an array of non-hepatic cell lines. Efficient transduction and high-level reporter gene expression was observed in primary hepatocytes and hepatoma cells, whereas in several other cell lines little to no expression was observed. Boyce and Bucher (1996) suggested that in less receptive cells the reporter gene expression was arrested subsequent to viral entry, because high- and low-expressing cells internalized comparable amounts of virus. More recently, it has been suggested that block to transduction in some mammalian cells may reside in the cytoplasmic trafficking or nuclear import of the nucleocapsids (Kukkonen et al., 2003). The significance of promoter strength in baculovirus transduction efficiency was demonstrated by Shoji and co-workers (1997), who showed that by using a strong CAG promoter, which is a combination of chicken beta-actin promoter and cytomegalovirus immediate-early enhancer, efficient transduction of non-permissive cell lines could be achieved (Shoji et al., 1997).

During the past decade, the array of cells permissive to baculovirus transduction has rapidly expanded, to date including cell lines of human, rodent, porcine, bovine and even fish origin (for review, see Hu, 2006). Furthermore, baculoviruses have been shown to be able to transduce non-dividing cells (van Loo et al., 2001), primary cells, such as human pancreatic islet cells (Ma et al., 2000) and neural cells (Sarkis et al., 2000) as well as mesenchymal stem cells obtained from human umbilical cord blood and bone marrow (Ho et al., 2005). Instead, baculovirus transduction of cell lines of hematopoietic origin seems to be inefficient (for review, see Hu, 2006). The transduction efficiencies vary significantly among the permissive cell types, but can be markedly enhanced by the addition of histone deacetylace inhibitors, agents that induce hyperacetylation of the chromatin and enhance the transcription in transduced cells (Hu, 2005). However, the use of such compounds including sodium butyrate, trichostatin A (Condreay et al., 1999) and valproic acid (Hu et al., 2003), is often associated with cytotoxicity and cell cycle arrest (Hu et al., 2003). Another approach to improve transduction efficiency is merely adjusting the transduction protocol (e.g. temperature, culture medium, or surrounding solution) (for review, see Hu, 2005). In 1997, Barsoum and coworkers demonstrated that a pseudotyped baculovirus expressing the vesicular stomatitis virus g-protein (VSVg) on its surface exerts enhanced transduction and a wider host range than the wild-type virus. The VSVg was speculated to increase the baculovirus escape from endosomes thus improving the transduction efficiency (Barsoum et al., 1997).

1.3.2.2 The mechanism of baculovirus entry and intracellular trafficking in mammalian cells

BV enters insect cells via receptor mediated endocytosis (Volkman and Goldsmith, 1984; Zhou and Blissard, 2008). Although the exact mechanism of baculovirus entry into mammalian cells has not been fully determined, baculovirus has generally been suggested to enter mammalian cells via a low pH dependent endocytic pathway, since gene expression is restrained by lysosomotropic agent which block the endosomal maturation (Hofmann et al., 1995; Boyce and Bucher, 1996; van Loo et al., 2001). More recently, baculovirus has been proposed to utilize clathrin-mediated endocytosis (Matilainen et al., 2005; Long et al., 2006) and macropinocytosis (Matilainen et al., 2005) to enter mammalian cells. Furthermore, in a recent study by Laakkonen and colleagues (2009)

baculovirus was shown to utilize a clathrin-independent entry mechanism that resembles phagocytosis. This pathway was found to require actin rearrangement as well as regulation by dynamin and the actin mediators Arf6 and RhoA (Laakkonen et al., 2009). Calveolae, in addition, have been suggested to play a part in baculovirus entry into mammalian cells, since transduction is considerably augmented in the presence of a calveolar endocytosis inhibitor, genistein (Long et al., 2006). Similar to its role in insect cell entry, the baculovirus major envelope protein gp64 seems to be vital for virus entry into mammalian cells as well. The significance of gp64 in virus transduction has been concluded from studies showing that monoclonal antibodies specific for gp64 prevent the baculoviral transduction of mammalian cells (Hofmann and Strauss, 1998; Hefferon et al., 1999; van Loo et al., 2001). Moreover, a mutant virus lacking gp64 on the viral envelope was shown to fail transducing mammalian cells (Abe et al., 2005) and, in turn, baculovirus vectors over-expressing gp64 on virion surface have been found to exhibit enhanced levels of transduction (Tani et al., 2001).

Although the importance of gp64 for baculovirus entry has been documented, little is known about of the mechanism involved in cell recognition by the virus. Initially, baculovirus transduction was suggested to be liver-specific and asialoglycoprotein was presumed to be involved in virus binding (Hofmann et al., 1995; Boyce and Bucher, 1996). It was later shown, however, that baculovirus transduction is not restricted to liver-originating cells (Shoji et al., 1997; Condreay et al., 1999) and that transduction is not dependent on asialoglycoprotein receptor, since cells not expressing asialoglycoprotein could be successfully transduced (van Loo et al., 2001). Furthermore, it has been proposed that electrostatic interactions and heparin sulphate moieties may be required for baculovirus binding to the mammalian cell surface and, that the mechanism of virus uptake is non-specific, rather than involving the use of a high affinity receptor (Duisit et al., 1999). Moreover, phospholipids on the cell surface have been suggested to facilitate the viral entry into mammalian cells by serving as an important docking point for gp64 (Tani et al., 2001). In a recent study, Mäkelä and colleagues (2008) showed that the kinetics of baculoviral binding and internalization into human cancer cells can be escalated by viral surface modification. By displaying a lymphatic homing peptide LyP-1 on the baculovirus envelope, enhanced affinity of viral binding to human breast carcinoma (MDA-MB-435)

and hepatocellular liver carcinoma (HepG2) cells was observed. Furthermore, the surface display of LyP-1 peptide resulted in increased viral uptake, earlier nuclear accumulation as well as higher transgene expression compared to the control virus with wild-type surface phenotype (Mäkelä et al., 2008).

Having lost their envelopes during endosomal escape, viral nucleocapsids are transported into different subcellular compartments depending on the target cell (Abe et al., 2005). In the cytoplasm, the nucleocapsids appear to induce the formation of actin filaments, which presumably enable the nucleocapsid transport into the nucleus. An agent causing reversible depolymerization of actin filaments, Cytochalasin D, was found to strongly inhibit the reporter gene expression without preventing the uptake of the viruses into the endosomes or their escape into the cytoplasm (van Loo et al., 2001). Furthermore, in addition to the viral genome, both major capsid protein and electron-dense capsids were detected inside the nucleus, proposing that the nucleocapsids are transported through the nuclear pores (van Loo et al., 2001; Laakkonen et al., 2008).

While baculovirus is unable to replicate in mammalian cells, low levels of transcription and translation of some viral genes have been shown to occur in certain mammalian cells (Fujita et al., 2006; Kitajima et al., 2006a; Kitajima et al., 2006b; Liu et al., 2007; Laakkonen et al., 2008). Fujita and colleagues (2006) detected the transcripts of 43 viral genes in HeLa human cervical cancer cells as well as 14 transcripts in baby hamster kidney cells following baculoviral transduction (Fujita et al., 2006). Furthermore, in a recent study by Laakkonen and coworkers, both transcription of baculoviral immediate-early transregulator genes (*ie-1, ie-2*) and translation of IE-2 protein were observed in human cells upon baculovirus treatment (Laakkonen et al., 2008). Whether and how expression of baculoviral proteins affects mammalian cells and their functions remain to be ascertained. Moreover, further investigations are required to determine the precise process of cell recognition, binding and entry of BV into mammalian cells as well as pathways of intracellular trafficking and detailed mechanisms of nuclear transport.

1.3.3 Baculovirus in gene therapy

Since the highly efficient transduction of variety of mammalian cells *in vitro*, BV has gained increasing attention as a vector for *in vivo* gene delivery (Hu, 2006). To date, an array of mammalian tissues of diverse origins have been successfully transduced *in vivo* (Airenne et al., 2000; Sarkis et al., 2000; Huser et al., 2001; Lehtolainen et al., 2002; Tani et al., 2003; Hoare et al., 2005). Such tissues include rat brain (Sarkis et al., 2000; Lehtolainen et al., 2002), mouse brain (Sarkis et al., 2000), rabbit carotid artery (Airenne et al., 2000), rat liver (Huser et al., 2001), mouse skeletal muscle (Pieroni et al., 2001) and mouse liver (Hoare et al., 2005), for example. Baculoviruses appear to be attractive vector candidates for *in vivo* applications as they readily deliver genes into many cell types without being able to replicate in these cells (Tjia et al., 1983; Volkman and Goldsmith, 1983; Groner et al., 1984; Hofmann et al., 1995; Shoji et al., 1997). Furthermore, baculovirus transduction is nontoxic to mammalian cells even at high multiplicity of infection (MOI) (Hofmann et al., 1995; Shoji et al., 1997; Gao et al., 2002; Tani et al., 2003), and baculovirus has the capacity to accommodate large inserts of foreign DNA (Cheshenko et al., 2001). Further beneficial features of baculovirus include the rather simple construction and propagation of recombinant vectors (Hu, 2006) as well as capability to transduce both dividing and non-dividing cells (van Loo et al., 2001). Baculovirus-mediated transgene expression is generally transient (Airenne et al., 2000; Hu, 2005; Wang et al., 2005), but under selective pressure *in vitro* partial integration of viral DNA into the host genome has been detected (Condreay et al., 1999; Merrihew et al., 2001). Furthermore, integration-competent baculovirus hybrid vectors have been constructed to attain prolonged or sustained transgene expression (Palombo et al., 1998; Zeng et al., 2007) and recently, stable baculovirus-mediated gene expression was observed in primary myoblasts differentiating into myotubes (Shen et al., 2008).

Despite the various advantages described above, there are a number of limitations concerning the utilization of baculoviruses for systemic gene delivery. One major challenge in the use of baculoviruses for *in vivo* applications is the elicitation of innate immune responses (Gronowski et al., 1999; Airenne et al., 2000; Abe et al., 2003) and subsequent viral inactivation by the serum complement system (Sandig et al., 1996;

Hofmann and Strauss, 1998). One approach to avoid vector inactivation is to circumvent direct viral contact with the complement components. This has been achieved by Airenne and colleagues (2000) who applied baculovirus particles in adventitial cells of rabbit carotic artery by utilizing a silastic collar that prevented the viral contact with blood (Airenne et al., 2000). Furthermore, exploiting the absence of the complement system from the brain seems to be another auspicious approach. Direct injection of baculovirus vectors into brains of mice (Sarkis et al., 2000) and rats (Sarkis et al., 2000; Lehtolainen et al., 2002) has resulted in efficient transduction and subsequent transgene expression without inactivation of the viruses. Moreover, some applications designed to avoid the inactivation of baculovirus vectors take advantage of agents that inhibit the complement system. Viral inactivation has been prevented *in vitro* by treating human serum with antibodies against the complement factor 5 as well as applying cobra venom factor to human blood and serum (Hofmann and Strauss, 1998). In addition, successful *in vivo* gene transfer without viral inactivation has been achieved by utilizing a baculovirus displaying human decay-accelerating factor, a complement system inhibitor, on the viral envelope (Huser et al., 2001).

Albeit baculoviruses are non-pathogenic to humans, several studies suggest that they may elicit various immune responses when introduced to human body. *In vitro*, baculoviruses have been observed to induce production of pro-inflammatory cytokines such as TNF-α , IL-1α, IL-1β (Beck et al., 2000), IL-6 (Abe et al., 2003) as well as INF-α and INF- β (Gronowski et al., 1999). Furthermore, baculoviruses hold potent adjuvant properties in mice stimulating strong humoral as well as adaptive immune responses against co-administered antigens (Hervas-Stubbs et al., 2007). Recently, elicitation of adaptive anti-tumor immune responses by wild-type baculovirus has been observed (Kitajima and Takaku, 2008) rendering baculovirus a promising tool for vaccination and immunotherapy as well (see section 1.3.4).

1.3.3.1 Cancer gene therapy

The potential of baculovirus as a cancer gene therapy vector was first investigated in 2001, by Song and Boyce who constructed a recombinant baculovirus containing wild-type p53 gene under the control of the cytomegalovirus immediate-early promoter. Upon

baculovirus transduction, expression of the tumor suppressor protein p53 and subsequent induction of apoptosis were observed in p53-deficient human Saos-2 osteosarcoma cells (Song and Boyce, 2001). Half a decade later, Wang and coworkers (2006) transduced malignant glioma cells both *in vitro* and *in vivo* with a baculovirus carrying a bacterial *Diphteria toxin A* gene (Wang et al., 2006b). By utilizing a transcriptional regulatory sequence of the glial fibrillary acidic protein, the expression of the highly toxic transgene was restricted to glioma cells to limit the side effects in non-target cells. *In vitro*, enhanced transduction of rat glioma cells was achieved, followed by inhibition of protein synthesis as well as cell growth. Furthermore, upon injection of the virus into the glioma xenografts into rat brain, apparent inhibition of tumor cell growth was observed (Wang et al., 2006b). More recently, anti-tumor activity of baculovirus-mediated expression of a tumor suppressor protein named normal epithelial cell specific-1 was explored in gastric cancer models of mice. Intratumoral injections of the recombinant baculovirus resulted in significant inhibition of tumor growth, further highlighting the potential of baculovirus as a gene therapy vector (Huang et al., 2008).

1.3.4 Baculovirus in immunotherapy

Vaccination is a common method to fight against infectious diseases (Tabi and Man, 2006). Since baculovirus has the ability to elicit immune responses in humans, it could be exploited to develop vaccine vectors. To date, baculovirus-mediated immune system activation has been studied in several animal models. Intranasal injection of recombinant baculovirus encoding the *hemagglutinin* (*HA*) gene of the influenza virus has been found to elicit a potent innate immune response in mice, thus protecting them from lethal influenza virus infection (Abe et al., 2003). Similarly, effective immunization of mice against severe acute respiratory syndrome (SARS) coronavirus has been achieved by utilizing recombinant baculoviruses expressing the nucleocapsid or spike protein of bat SARS-like coronavirus. Upon subcutaneous or intraperitoneal injections of the vector, elicitation of both humoral and cellular immune responses against the SARS-like virus was detected (Bai et al., 2008).

1.3.4.1 Baculovirus surface display

The baculovirus surface display technology with several different strategies allows incorporation of a foreign protein onto the virus particle, thus offering further possibilities for antigen presentation and immunotherapy. The most common and the best characterized baculovirus-based display platform is the BV membrane protein gp64 (Mäkelä and Oker-Blom, 2008), which can be utilized by fusing the foreign gene between the N-terminal signal sequence and the C-terminal transmembrane anchor of a second copy of *gp64* (Mottershead et al., 1997). Alternatively, short peptides can be directly cloned within the native *gp64* sequence, but in such case the insertion site must be carefully chosen to assure correct presentation of the peptide. Furthermore, the accurate conformation of gp64 must be preserved to avoid disturbances in the function of the protein during the membrane fusion and budding (Spenger et al., 2002). Hence, the importance of gp64 in baculovirus infection and functionality has resulted in creation of alternative display platforms (Mäkelä and Oker-Blom, 2008). One strategy is to use a combination of the gp64 signal peptide and the transmembrane anchor domain of VSVg (Mäkelä et al., 2006) or signal and membrane anchoring sequences from influenza virus HA (Ernst et al., 1998). Whereas gp64 fusion proteins accumulate at one pole of the rod-shaped BV virion, utilization of heterologous membrane anchors permits uniform scattering and more efficient expression of the fusion proteins on the viral envelope (Chapple and Jones, 2002 ; Mäkelä and Oker-Blom, 2008). Furthermore, Kukkonen and coworkers (2003) have developed an approach to display foreign protein moieties on the viral capsid both *in vitro* and *in vivo*. By fusing the target peptide either to the N-terminus or C-terminus of the baculoviral major capsid protein vp39 efficient fusion protein expression has been detected without affecting the viral titer or functionality (Kukkonen et al., 2003).

To date, baculovirus surface display technology has also been exploited in vaccine research. In 2007, Strauss and coworkers constructed a baculovirus both expressing and displaying the *Plasmodium falciparum* circumsporozoite protein to study the potential of baculovirus vectors for malaria vaccination. Upon intramuscular injection into mice, the virus was able to transduce dendritic cells, the professional antigen presenting cells, and trigger their maturation. Moreover, vector induced high titers of antibodies against the

expressed and displayed protein and elicited a strong T-cell mediated immune response (Strauss et al., 2007). More recently, baculovirus surface display technology was utilized in the development of an influenza vaccine. In a study by Prabakaran and colleagues (2008) a recombinant baculovirus displaying a HA fragment of H5N1 influenza strain was found to act as an effective vaccine in mice, when a recombinant cholera toxin B subunit was used as a mucosal adjuvant. Intranasal injection of the HA-displaying baculovirus resulted in complete protection against both homologous and heterologous H5N1 strains. Furthermore, when compared with inactivated H5N1 influenza vaccine the recombinant baculovirus was more efficient, thus holding promise as treatment against H5N1 with fewer biosafety concerns (Prabakaran et al., 2008).

1.3.4.2 Baculovirus in cancer immunotherapy

In recent years, cancer has become another target for vaccine development (Tabi and Man, 2006). The aim of cancer immunotherapy is to instruct immune system to identify the antigens expressed only in tumor cells, to be able to eradicate these malformed cells while leaving the normal cells intact (Guinn et al., 2007). In 2007, a VSVg-pseudotyped baculovirus expressing murine telomerase reverse transcriptase was found to elicit an anti-tumor immune response in a murine glioma model. Upon vaccination with the recombinant baculovirus escalated amounts of IFN-γ-secreting T cells and augmented natural killer cell activity were detected in the splenocytes of the vaccinated mice (Kim et al., 2007). Furthermore, wild type baculovirus has been recently shown to induce anti-cancer immunity in mice. In a study by Kitajima and Takaku (2008), baculovirus was detected to elicit acquired anti-tumor immunity against challenged tumor cells in a liver metastasis as well as subcutaneous tumor model. Intravenous injection of the wild-type virus led to increased tumor-specific cytotoxic T-cell activity as well as tumor-specific antibody production, indicating the potential of baculovirus in cancer immunotherapy (Kitajima and Takaku, 2008).

2 AIM OF THE STUDY

The aims of the present study were:

1. To generate recombinant baculovirus vectors that either express, display or both express and display the novel cancer specific apoptosis inducing cytokine, Mda-7

2. To characterize the ability of the surface modified viruses to display Mda-7 protein on both baculovirus and insect cell surface

3. To compare the apoptosis inducing abilities of the recombinant viruses in human prostate cancer PC-3 cells

3 MATERIALS AND METHODS

3.1 Construction of the recombinant viruses

The recombinant baculoviruses used in this study, namely *Ac*-Luc, *Ac*-Mda-7, *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7, were previously generated (Mäkelä et al., manuscript). *Ac*-Mda-7 was designed to express the cancer specific apoptosis inducing cytokine, melanoma associated gene 7 (Mda-7) under the simian virus 40 (SV40) promoter in mammalian cells. *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7 expressed *luciferase* and *Mda-7*, respectively, and displayed an Mda-7-containing fusion protein on their surface. (Mäkelä et al., manuscript). *Ac*-Luc, expressing the North American firefly (*Photinus pyralis) luciferase* reporter gene in transduced mammalian cells, served as a control virus.

3.1.1 Construction of the recombinant plasmids

The recombinant plasmids were previously generated (Mäkelä et al., manuscript) using the pFastBac™Dual transfer vector (Invitrogen Life technologies, Carlsbad, CA) as a vector backbone. Briefly, the expression plasmids pSV40-Luc and pSV40-Mda-7 contained *luciferase* reporter gene and *Mda-7*, respectively, under the mammalian cell specific simian virus 40 (SV40) promoter. The baculoviral *polyhedrin* promoter (*polh*) was deleted from the vector backbone by *Acc*III restriction enzyme digestion followed by cloning of the SV40 promoter into the vector between the *Bam*HI and *Sal*I restriction sites. *Luciferase* and *Mda-7* (from pORF9-hlIL24, InvivoGen, San Diego, CA, USA) were inserted between the *Not*I and *Xba*I restriction sites resulting in plasmids pSV40-luc and pSV40-Mda-7, respectively. These expression plasmids were utilized as backbones for the generation of the display vectors pMda-7-SV40-Luc and pMda-7-SV40-Mda-7. The *Mda-7* cDNA, and a membrane anchoring domain composed of a linker region encoding 20 alanine residues (Ala; Mäkelä et al., 2006), as well as the transmembrane (TM) and cytoplasmic (CT) domains of vesicular stomatitis virus g-protein (VSVg) (Mäkelä et al., 2006) were cloned in frame into the expression vectors downstream the p10 promoter using the *Nco*I/*Nhe*I and *Nhe*I/*Kpn*I restriction enzymes, respectively (Mäkelä et al., manuscript).

3.1.2 Production and purification of the recombinant baculoviruses

The recombinant viruses were produced using the Bac-to-Bac™ baculovirus expression system principally according to the manufacturer´s instructions (Invitrogen, Carlsbad, CA). Briefly, the recombinant plasmids pSV40-Luc, pSV40-Mda-7, pMda-7-SV40-Luc and pMda-7-SV40-Mda-7 were transformed into electrocompetent DHC10Bac™ *Escherichia coli* (*E. coli)* cells to facilitate site-specific transposition into the baculoviral genome and subsequent formation of the recombinant bacmids. The recombinant viruses were produced in *Spodoptera frugiperda* 9 insect cells (*Sf*9; Gibco-BRL, Grand Island, NY) grown in monolayer or suspension cultures using Insect-Xpress Protein-Free medium supplemented with L-Glutamine (BioWhittaker, Lonza, MD) at 28^{o}C (Mäkelä et al., manuscript). To obtain higher virus titers for apoptosis experiments, tertiary virus stocks were concentrated. Cell debris was first pelleted by centrifugation at 5000 x g for 20 min at 4^{o}C (Hermle Z 513 K, Hermle Labortechnik GmbH, Wehingen, Germany) followed by concentration of the viruses (11 953 x g, 8 h, 4^{o}C; Sorvall RC5-C centrifuge, rotor SS34). Concentrated viruses were dissolved in ice-cold phosphate-buffered saline (PBS) and stored at 4^{o}C. The infectious viral titers were determined from non-concentrated or concentrated tertiary virus stocks by end point dilution assay using standard protocols (O´reilly 1994).

3.2 Characterization of the recombinant viruses

3.2.1 Western blot analysis

To characterize *Ac*-Luc, *Ac*-Mda-7, *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7, infected *Sf*9 cells and concentrated viruses were exposed to analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The cells were collected at 72 h post infection (p.i.) by centrifugation (380 x g, 3 min, RT; Biofuge Pico, rotor #3325, W. C. Heraeus GmbH, Hanau, Germany) and solubilized in reducing 2 x Laemmli sample buffer containing β-mercaptoethanol (2 x Laemmli buffer + β-mercaptoethanol: 63 mM Tris-HCl, 25 % [v/v] glycerol [J. T.Baker, Deventer, Holland], 2 % SDS [BDH, Laboratory Supplies Poole, England], 0.01 % bromophenol blue [Sigma-Aldrich, MO, USA] and 5 % β-mercaptoethanol [Merck, Darmstadt, Germany]) to obtain a

final cell concentration of 1 x 10^4 cells/µl. Non-infected *Sf*9 cells served as a negative control.

Equal plaque forming units (PFUs) of each recombinant virus from tertiary virus stocks were concentrated as follows. Cell debris was first removed by centrifugation (5000 x g, 20 min, 4^{o}C; Hermle Z 513 K, Hermle Labortechnik GmbH, Wehingen, Germany) followed by concentration of the viruses (11 953 x g, 11 h, 4^{o}C; Sorvall RC5-C centrifuge, rotor SS34). The viral pellets were solubilized in reducing 4 x Laemmli sample buffer containing β-mercaptoethanol (4 x Laemmli buffer + β-mercaptoethanol: 125mM Tris-HCl, 50 % [v/v] glycerol [J. T.Baker], 4 % SDS [BDH], 0.01 % bromophenol blue [Sigma-Aldrich], and 5 % β-mercaptoethanol [Merck]) to obtain a final virus concentration of 5 x 10^6 pfu/µl. The samples were denatured at 100^{o}C for 5 min and kept on ice before separation by SDS-PAGE.

The protein separation was conducted using vertical 1.5 mm thick gels consisting of 5 % stacking gel (5% acrylamide/Bis solution [29:1] [Bio-Rad Laboratories GmbH, Munich, Germany], 0.125 M Tris-HCl [pH 6.8], 0.1% SDS [BDH], 0.1% [w/v] ammoniumpersulfate [Bio-Rad Laboratories], and 0.1% [v/v] TEMED [N,N,N',N'-tetramethylene diamine; Bio-Rad Laboratories]) and 12% resolving gel (12% acrylamide/Bis solution [29:1], 0.375 M Tris-HCl [pH 8.8], 0.1% SDS, 0.1% [w/v] ammoniumpersulfate, and 0.04% [v/v] TEMED). Unstained protein molecular weight marker (10 µl; MBI Fermentas, Vilnius, Lithuania) was used to estimate the molecular weights of the sample proteins. Electrophoresis was performed using a Mini 2-D Electrophoresis Cell apparatus (Bio-Rad Laboratories) with electrode running buffer (25 mM Trizma base, 0.2 M glycine [Riedel-deHaën, Seezle, Germany] and 3.5 mM SDS). The samples were concentrated at the boundary of stacking and separating gels using 100 V current for approximately 15 min. The current was raised to 180 V and the samples were run for 60 to 70 min until the dye front reached the bottom of the resolving gel.

Separated proteins were transferred onto nitrocellulose sheets (pore size 0.45 mm) by electroblotting with a Mini-PROTEAN 3 Cell electroblotting apparatus (Bio-RadLaboratories) in presence of ice-cold transfer buffer (25 mM Trizma base, 0.2 M

glycine, and 20% [v/v] methanol). The blotting was performed with 100 V current for 1 h. Subsequently, the nitrocellulose sheets were stained with Ponceau S (0.2% [w/v] Ponceau S [Sigma, St. Louis, MO] and 5% acetic acid [Riedel deHaen, Seelze, Germany]) for 5 min at RT and washed with sterile distilled H_2O to visualize the total protein amount as well as the molecular weight marker. The sheets were blocked in 5% dried fat free milk powder in Tris-buffered saline (TBS: 20 mM Trizma base [Sigma], and 0.5 M NaCl) supplemented with 0.2% Tween® 20 (Fluka Chemie GmbH, Buchs, Switzerland) (milk-TBS-Tween) for 1 h at RT.

The blots were immunolabeled to detect specific protein bands. Rabbit polyclonal VSVg tag antibody (5×10^{-4} mg/ml; Sigma-Aldrich, St. Louis MO) as well as mouse monoclonal gp64 (Whitt and Manning, 1988; B12D5,1:1000) and vp39 (Keddie et al., 1989; p10 C 6 α-capsid, 1:1000) antibodies (both kindly provided by Dr. Loy Volkman from the University of California, Berkeley, CA) were used as primary antibodies. All the antibodies were diluted in milk-TBS-Tween and incubations were performed for 1 h at RT with rocking. After washes (3 x 5 min) with TBS-Tween, the primary antibodies were detected with either a goat anti-rabbit IgG or a goat anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (Promega, Madison, WI). The secondary antibodies were diluted 1:5,000 in milk-TBS-Tween and incubated for 1 h at RT with rocking, followed by washes (3 x 5 min) with TBS-Tween. Subsequently, the blots were equilibrated in alkaline phosphatase assay buffer (0.1 M Trizma base [Sigma], 0.1 M NaCl, and 5 mM $MgCl_2$ [Merck]) for 5 min. Detection of the proteins was accomplished at RT by incubating the blots in alkaline phosphatase substrate solution containing 50mg/ml Nitro-Blue Tetrazolium (NBT) (NBT [Sigma] in 70 % dimethylformamide [Merck]) and 50mg/ml 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) (BCIP [Sigma] in 100 % dimethylfomamide) diluted in alkaline phosphatase assay buffer. The blots were washed with sterile distilled H_2O to arrest the reaction and air-dried.

3.2.2 Immunolabeling of infected *Sf*9 cells

To characterise *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7 and incorporation of the display construct into the insect cell membrane, *Sf*9 cells, grown in a suspension culture, were infected with a multiplicity of infection (MOI) of 10 for each recombinant virus. *Ac*-Luc

and *Ac*-Lyp-1-luc (Mäkelä et al., 2006) were used as negative and positive control viruses, respectively. Cells (2 x 10^6 cells per sample) were harvested at 44 h p.i. by centrifugation at 380 x g for 3 min and washed once with cold PBS. Double-immunolabeling was performed to identify the surface proteins on infected insect cells: the baculovirus major envelope protein gp64 and the displayed Mda-7-VSVg fusion protein. The primary antibodies used were mouse monoclonal gp64 antibody diluted 1:50, 1:100 and rabbit polyclonal VSVg tag antibody (0,01mg/ml; Sigma-Aldrich). The antibodies were diluted in 1.5% bovine serum albumin in PBS (BSA-PBS) and incubated for 1 hour at 4^{o}C with rotation, followed by washes (2 x 15 min) with cold BSA-PBS at 4^{o}C with rotation. For confocal microscopy, the cells were subsequently labeled with fluorescent secondary antibodies Alexa Fluor® 488 goat anti-mouse IgG conjugate (green) and Alexa Fluor® 546 goat anti-rabbit IgG conjugate (red) (Invitrogen Molecular Probes, Eugene, OR, USA) diluted 1:200 in BSA-PBS for 30 min at 4^{o}C with rotation, followed by washes (2 x 15 min) with cold BSA-PBS at 4^{o}C with rotation. Subsequently, cells were rinsed once with PBS, pelleted at 380 x g for 3 min at 4^{o}C, resuspended to Mowiol (Mowiol 4-88 [Calbiochem, Darmstadt, Germany] in glycerol and Tris-HCl) supplemented with Dabco (25 mg/ml; 1,4- diazobicyllo-[2.2.2]-octane [Aldrich, Steinheim, Germany] and mounted on microscope slides using glass cover slips. All centrifugations were conducted using Biofuge Pico, rotor #3325 (W. C. Heraeus GmbH).

3.2.3 Confocal microscopy

The immunolabeled samples were observed with a confocal laser scanning microscope (LSM510, Zeiss Axiovert 100 M, Carl Zeiss Inc., Jena, Germany) equipped with a 488 nm argon laser and 543 nm helium-neon laser. Plan-Apochromat 63x/1.40 oil objective as well as appropriate excitation and emission settings were used. The differential interference contrast (DIC) and fluorescence images were acquired using LSM510 software.

3.2.4 Flow cytometric analysis

To analyze the infectivities of the surface-modified viruses *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7, *Ac*-Luc was used as a negative and *Ac*-Lyp-1-luc (Mäkelä et al., 2006) as a positive control virus. For analysis by flow cytometry, *Sf*9 cells were grown, infected, harvested

and immunolabeled using the same procedures as described in section 3.2.2., this time using mouse monoclonal gp64 antibody and Alexa Fluor® 488 goat anti-mouse IgG conjugate (green) (Invitrogen Molecular Probes, Eugene, OR, USA) as primary and secondary antibodies, respectively. Subsequently, the cells were suspended to cold PBS and filtered through a nylon mesh. Samples were stored on ice in the dark until analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany) using CellQuest software to detect the gp64 expression levels by fluorescence measurements.

3.3 Apoptosis experiments with human prostate cancer cells

3.3.1 Human prostate cancer PC-3 cell culture

Human prostate cancer PC-3 cells (American Type Culture Collection, Manassas, VA; number CRL-1435™) were maintained as a monolayer culture using Dulbecco's Modified Eagle's Medium (Gibco® Invitrogen Life technologies, Paisley, UK) supplemented with L-glutamine (Gibco®), 10 mM Hepes pH 7.3 (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid, MP Biomedicals LLC Eschwege, Germany), 10% (v/v) heat-inactivated fetal calf serum (FCS), 1% (v/v) penicillin -1% (v/v) streptomycin mixture (Gibco®) and 0.1 mM non-essential amino acids (Gibco®). The cells were grown in a humidified atmosphere of 5% CO_2 at 37^{o}C.

3.3.2 Annexin V staining and flow cytometry analysis of infected PC-3 cells

Induction of apoptosis by the recombinant baculoviruses was examined using PC-3 cells. A wild-type baculovirus, *Ac*WT (kindly provided by Anna Mäkelä from the University of Jyväskylä), and *Ac*-Luc served as control viruses for apoptosis induction. PC-3 cells were allowed to attach on 12-well cell culture plates at 37^{o}C for 48 h and subsequently transduced with a MOI of 500 and 1000 with each virus. Non-transduced PC-3 cells, used as negative control cells, were treated with PBS, instead. The viruses were allowed to bind to cells for 1 h on ice with rocking in a minimal volume of PBS followed by addition of warm growth medium and transduction for 5 days at 37^{o}C. Subsequently, the cells were detached by scraping, pelleted by centrifugation (400 x g, 5 min, Centrifuge 5415 D, Eppendorf AG, Hamburg, Germany), washed once with cold PBS and suspended to 1 x

Annexin V binding buffer (BD Biosciences Pharmingen). Cells undergoing apoptosis were stained by treating the samples with fluorescein isothiocyanate (FITC) conjugated Annexin V (Annexin V-FITC, BD Biosciences Pharmingen) according to the manufacturer's instructions. Samples were gently vortexed and incubated in the dark for 20 min at RT, followed by suspension to ice-cold 1 x Annexin V binding buffer. Samples were filtered through a nylon mesh and stored on ice in the dark until the fluorescence of cells was analyzed with FACSCalibur flow cytometer using CellQuest software.

4 RESULTS

4.1 Construction of the recombinant viruses

The four recombinant baculoviruses used in this study were previously generated (Mäkelä et al., manuscript). Two mammalian expression vectors, *Ac*-Mda-7 and the control virus *Ac*-Luc, were developed to express the cancer specific apoptosis inducing cytokine, melanoma associated gene 7 (Mda-7) and the reporter protein luciferase, respectively, under the mammalian cell-specific simian virus 40 (SV40) promoter (Fig. 4.1A). Additionally, by utilizing these expression vectors as backbones, two surface modified viruses, namely *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7, displaying the full-length Mda-7 by fusion to the transmembrane and cytoplasmic domains of VSVg through a polyalanine linker on their envelope, were developed. Expression of the Mda-7-VSVg fusion protein was controlled by the strong baculoviral p10 promoter (Fig. 4.1A and 4.1B; Mäkelä et al., manuscript).

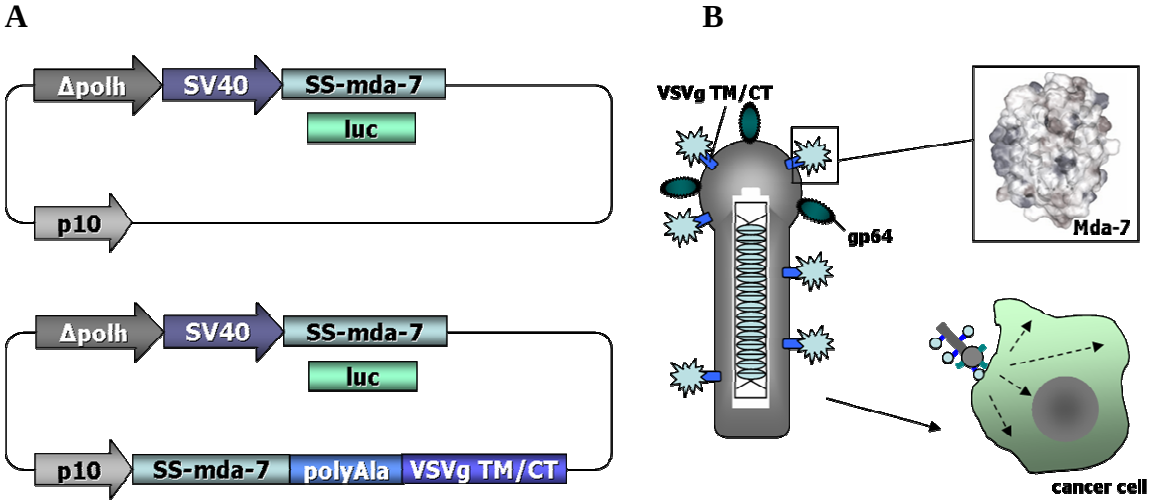

Figure 4.1 A schematic representation of the recombinant baculovirus constructs and of a recombinant baculovirus displaying the Mda-7-VSVg fusion protein on the viral envelope. A) Above: Ac-Mda-7 and Ac-Luc, below: AcMda-7- Mda-7 and AcMda-7-Luc. B) A recombinant baculovirus (e.g. AcMda-7-Luc or AcMda-7-Mda-7) displaying the Mda-7-VSVg fusion protein on the viral envelope. Abbreviations: luc = luciferase gene, p10 = p10 promoter, Δpolh = deleted polyhedrin promoter, polyAla = a linker sequence encoding 20 alanine residues, SS-mda-7 = melanoma associated gene 7 signal sequence and mda-7 gene, SV40 = simian virus 40 promoter, VSVgTM/CT = the transmembrane and cytoplasmic domains of vesicular stomatitis virus G protein. (Predicted Mda-7 protein structure; modified from Chada et al, 2004.)

4.2 Characterization of the surface modified baculoviruses

4.2.1 Mda-7-VSVg fusion protein was successfully incorporated into both infected insect cells and budded virions

The Mda-7-VSVg fusion protein was directed to the surface of *Sf*9 insect cells infected with the recombinant baculoviruses by using the endogenous signal sequence of Mda-7. The transmembrane and cytoplasmic domains of VSVg were designed to anchor the fusion protein into the insect cell and baculoviral membrane (Mäkelä et al., 2006). To determine whether the surface modified baculoviruses were able to display the Mda-7-VSVg fusion protein on the insect cell surface, *Sf*9 cells infected with *Ac*Mda-7-Luc, *Ac*Mda-7-Mda-7, *Ac*Lyp-1-luc (Mäkelä et al, 2006) and *Ac*-Luc were characterized by confocal microscopy. The cells were double-immunolabeled with antibodies raised against the baculovirus major envelope protein gp64 and the C-terminus of the VSVg cytoplasmic tail. To confirm the success of the VSVg labeling, *Ac*Lyp-1-luc, displaying the LyP-1 tumor-homing peptide by fusion to the VSVg TM/CT (Mäkelä et al., 2006) was used as a positive control, and *Ac*-Luc, possessing the wild-type baculovirus surface phenotype, served as a negative control. As a result, both gp64 and the Mda-7-VSVg fusion protein were distinctly detectable on the plasma membranes of the *Ac*Mda-7-Luc-, *Ac*Mda-7-Mda-7- and *Ac*Lyp-1-luc-infected *Sf*9 cells at 44 h p.i. (Fig. 4.2). The distribution of the Mda-7-VSVg fusion protein on the cell surface appeared to be rather clustered and co-localization with gp64 was detectable to some extent (Fig 4.2, yellow areas). Clear labeling of gp64 was observed in the control cells infected with *Ac*-Luc, whereas these cells showed no reactivity with the VSVg-tag antibody.

To evaluate the incorporation of the Mda-7-VSVg fusion protein into budded virions, Western blot analysis was performed with mock-, *Ac*-Luc-, *Ac*-Mda-7-, *Ac*Mda-7-Luc- and *Ac*Mda-7-Mda-7-infected *Sf*9 cells as well as with the corresponding concentrated viruses. As shown in Figure 4.3A, labeling with the VSVg antibody identified several positive bands (approximately 23-30 kDa) in *Ac*Mda-7-Luc- and *Ac*Mda-7-Mda-7-infected *Sf*9 cells as well as in the corresponding virus samples (Fig 4.3A, lanes 5, 6, 9 and 10, respectively),

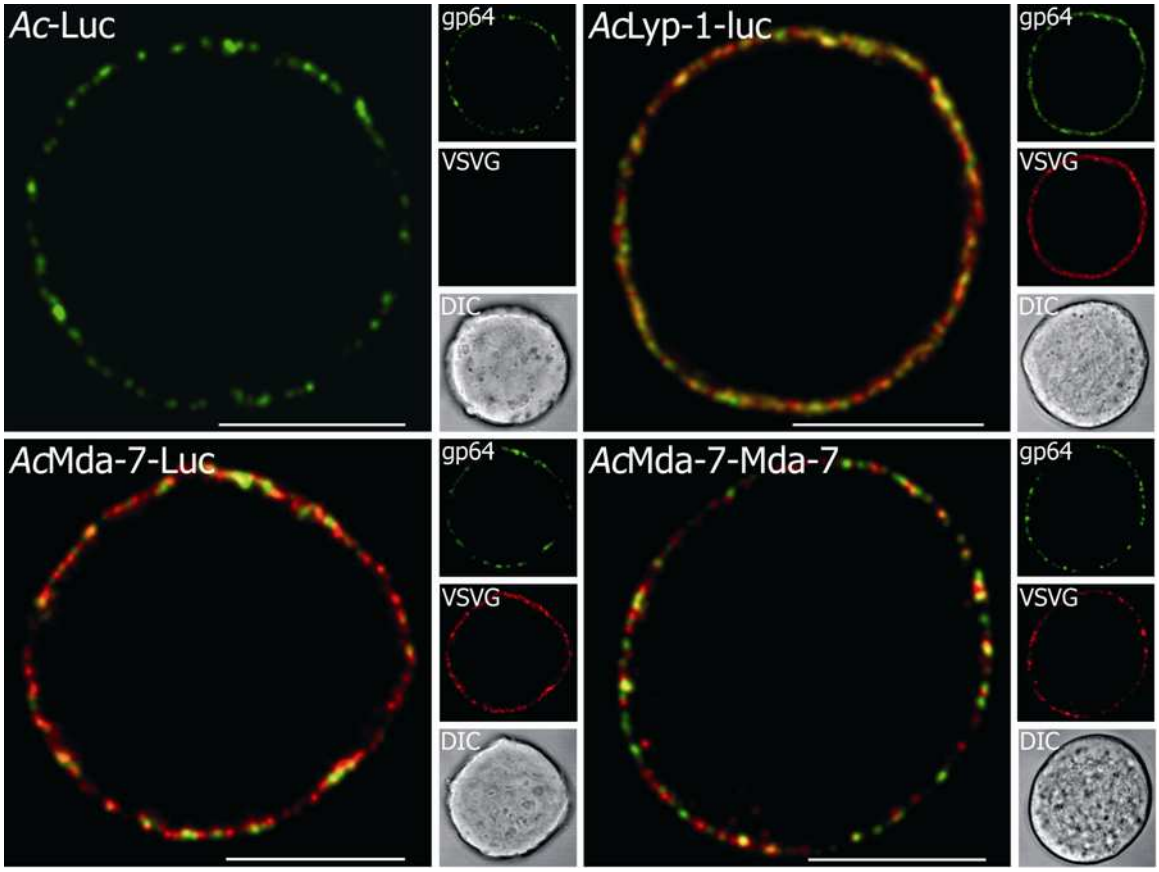

Figure 4.2 *Sf*9 cells infected with *Ac*-Luc, *Ac*Lyp-1-luc, *Ac*Mda-7-Luc or *Ac*Mda-7-Mda-7. Expression of the baculovirus major envelope protein gp64 and the Mda-7-VSVg fusion protein were detected at 44 h p.i. with mouse monoclonal gp64 and rabbit polyclonal VSVg-tag antibodies, as well as with Alexa Fluor® 488 (green) and Alexa Fluor® 546 (red) conjugated secondary antibodies, respectively. The images are single confocal optical sections of z-stacks from the middle of the cell. Scale bar 10 µm. DIC= differential interference contrast**.**

representing different post-translationally modified forms of the Mda-7-VSVg fusion protein. Mock-infected *Sf*9 cells, cells infected with *Ac*-Luc or *Ac*-Mda-7, as well as *Ac*-Luc and *Ac*-Mda-7 viruses possessing the wild-type baculovirus phenotype (Fig 4.3A, lanes 2, 3, 4, 7 and 8, respectively), showed no reactivity with the VSVg-antibody. These results indicate that the Mda-7-VSVg fusion protein is appropriately expressed, successfully transported to the surface of the infected *Sf*9 cells and as a result, incorporated into the recombinant baculovirus particles.

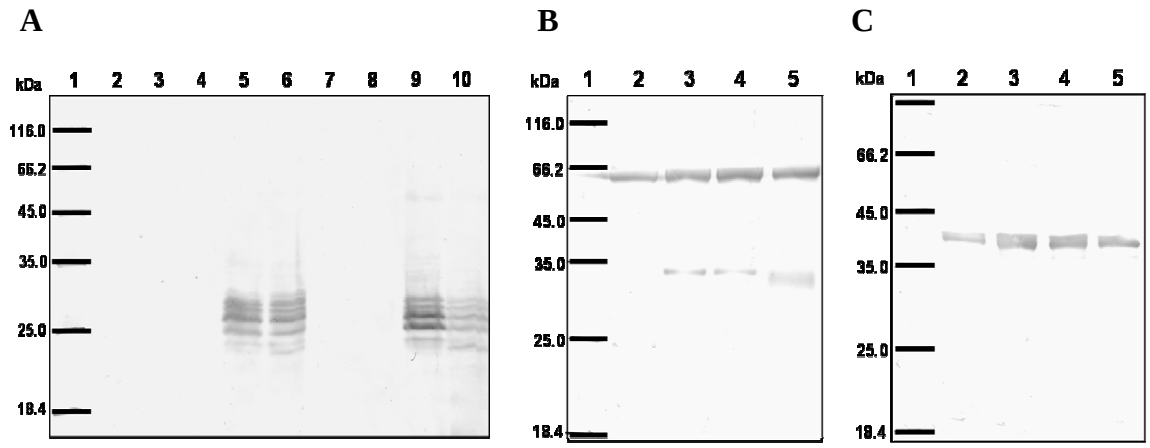

Figure 4.3 Characterization of Mda-7-displaying baculovirus vectors. A) Western blot analysis of the recombinant baculovirus infected *Sf*9 cells and corresponding viruses for the detection of Mda-7-VSVg fusion protein using rabbit polyclonal VSVg-tag antibody and alkaline phosphatase-conjugated secondary antibody. The blot contains protein molecular weight marker (lane 1); non-infected *Sf*9 cells (5 x 10^4 cells; lane 2) as a control, as well as 5 x 10^4 *Ac*-Luc- (lane 3), *Ac*-Mda-7- (lane 4), *Ac*Mda-7-Luc- (lane 5) and *Ac*Mda-7-Mda-7-infected *Sf*9 cells (lane 6); 1 x 10^8 infectious particles (pfu) of *Ac*-Luc (lane 7); *Ac*-Mda-7 (lane 8); *Ac*Mda-7-Luc (lane 9); and *Ac*Mda-7-Mda-7 (lane 10). **B)** and **C)** Determination of the ratio of total particle number vs. the amount of infectious virus by immunoblot analysis using mouse monoclonal antibodies raised against baculoviral structural proteins gp64 **(B)** or vp39 **(C)**, as well as alkaline phosphatase conjugated secondary antibody. The blots contain protein molecular weight marker (lane 1); 2.5 x 10^7 pfu of *Ac*-Luc (lane 2); *Ac*-Mda-7 (lane 3); *Ac*Mda-7-Luc (lane 4); and *Ac*Mda-7-Mda-7 (lane 5).

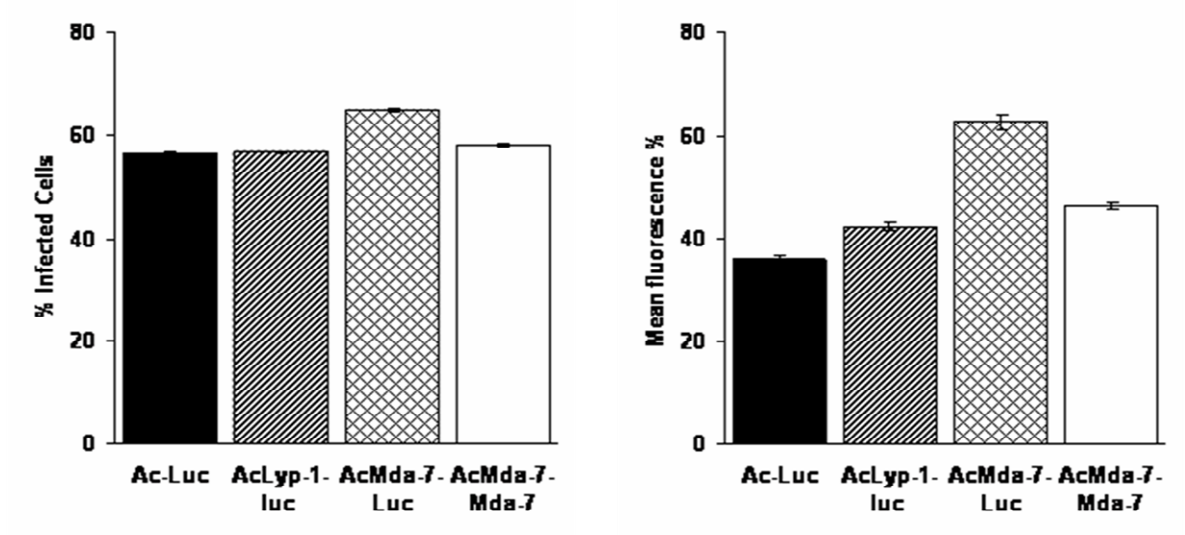

Figure 4.4 Analysis of the infectivity of the surface modified baculoviruses *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7 in *Sf*9 cells. Expression of gp64 and thus the viral infectivity was analyzed in *Sf*9 cells at 44 h p.i. by flow cytometry using anti-gp64 and Alexa Fluor® 488 antibodies. *Ac*-Luc, possessing the wild-type baculovirus surface phenotype, as well as the surface modified *Ac*Lyp-1-luc (Mäkelä et al., 2006) served as control viruses. The percentage of infected cells (± standard deviation) as well as mean fluorescence intensity (± standard deviation) reflecting the gp64 expression level, are indicated.

4.2.2 The surface display of Mda-7 did not alter viral infectivity

To study the infectivities of the surface modified baculoviruses, the ratio of total particle number (TP) versus the amount of infectious virus particles (IP) was determined. The TP/IP ratio was similar with the control and modified viruses as determined by immunoblotting of equal PFUs with anti-gp64 and anti-vp39 antibodies (Fig 4.3B and 4.3C). Additional smaller bands presumably representing proteolytically cleaved forms of gp64 were also detected (Fig. 4.3B). To further study the infectivity of the modified viruses, immunofluorescence labeling with anti-gp64 and Alexa Fluor® 488 antibodies followed by analysis by flow cytometry was performed by using *Ac*-Luc and *Ac*Lyp-1-luc (Mäkelä et al., 2006) as control viruses. As shown in Figure 4.4, no significant differences were detected in the infectivities between *Ac*-Luc and the surface modified viruses *Ac*Lyp-1-luc, *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7. These results indicate that the surface modifications do not significantly alter the infectivities of the recombinant baculoviruses.

4.3 Apoptosis experiments with human prostate cancer cells

4.3.1 *Ac*Mda-7, *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7 induced apoptosis in PC-3 cells

To analyze the apoptosis inducing abilities of the recombinant baculoviruses in human cancer cells, PC-3 prostate cancer cells were transduced with *Ac*-Mda-7, *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7. A wild-type baculovirus, *Ac*WT, as well as *Ac*-Luc served as negative control viruses, and non-transduced PC-3 cells were used as control cells to define the basal level of apoptotic cells in the PC-3 population. The induction of apoptosis was monitored by AnnexinV staining and subsequent analysis by flow cytometry at 5 days p.t. As shown in Figure 4.4, *Ac*-Mda-7, *Ac*Mda-7-Luc as well as *Ac*Mda-7-Mda-7 induced apoptosis in PC-3 cells. With 500 pfu/cell of *Ac*Mda-7, *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7, 17.70±7.14%, 13.07±4.35% and 17.97±2.19% of the cells, respectively, were positive for AnnexinV. With 1000 pfu/cell, the corresponding percentages were 16.70±1.31%, 9.88±5.66 % and 13.15±5.95%. Thus, the induction of apoptosis in PC-3 cells appeared to be somewhat dependent on viral dose, 500 pfu/cell being more efficient than 1000 pfu/cell.

In cells transduced with wild-type baculovirus, the percentages of AnnexinV positive cells were 0.17±0.33% and 0.99±1.5% with 500 and 1000 pfu/cell, respectively. In cells transduced with *Ac*-Luc, the corresponding percentages were 3.14±3.36% and 5.13±1.98%.

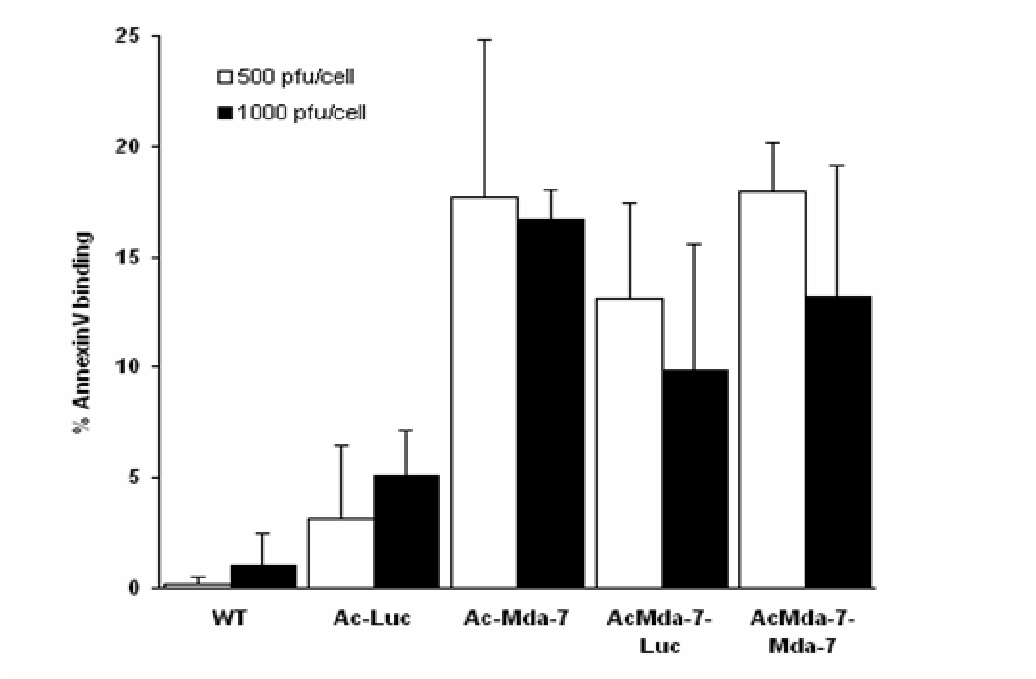

Figure 4.5 Induction of apoptosis in PC-3 cells by *Ac*Mda-7, *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7. Apoptosis induction by the recombinant baculoviruses were examined by transducing human prostate cancer PC-3 cells with 500 and 1000 plaque forming units (PFU) per cell of each virus. Wild-type baculovirus, WT, and *Ac*-Luc were used as control viruses. The cells undergoing apoptosis were identified by labeling with FITC-conjugated AnnexinV at 5 days p.t, followed by flow cytometry analysis. The percentages of AnnexinV-positive cells ± standard deviation are indicated.

5 DISCUSSION

Cancer is a complicated disease involving mutations in both oncogenes and tumor suppressor genes. Several preclinical studies have implied that cancer growth could be arrested by transferring a gene or genetic element into cancer cells to restore the normal cell growth and death pathways or by elicitation of immune system to recognize and eradicate the tumor cells (for review, see Wilson, 2002; El-Aneed, 2004). Mda-7 is a novel cytokine demonstrating significant anticancer potential by regulating multiple signaling pathways involved in cancer development and metastasis (Saeki et al., 2002; Sarkar et al., 2002; Pataer et al., 2005; Ramesh et al., 2003; Tong et al., 2005; Chada et al., 2006a; Miyahara et al., 2006; Tahara et al., 2007). Since its finding in 1995, Mda-7 has fast proceeded from a laboratory tool to a potential therapeutic agent for cancer (Inoue et al., 2006). Thus far, studies have shown the anti-cancer activity of Mda-7 mediated by Ad (Jiang et al., 1996; Kawabe et al., 2002; Sarkar et al., 2002; Nishikawa et al., 2004) and AAV (Tahara et al., 2007) vectors. Among various viral gene delivery vectors based on human pathogens, the insect baculovirus, *Ac*MNPV, represents a fascinating alternative. By utilizing mammalian cell specific promoters, the BV phenotype of *Ac*MNPV has been used to transiently and stably transduce a large array of mammalian cells of diverse origins, both *in vitro* and *in vivo* (Condreay et al., 1999; Airenne et al., 2000; Sarkis et al., 2000; Huser et al., 2001; Merrihew et al., 2001; Lehtolainen et al., 2002; Tani et al., 2003; Hoare et al., 2005). Low cytotoxicity (Hofmann et al., 1995; Shoji et al., 1997; Gao et al., 2002; Tani et al., 2003) and innate inability to replicate in mammalian cells (Tjia et al., 1983; Volkman and Goldsmith, 1983; Groner et al., 1984; Hofmann et al., 1995; Shoji et al., 1997) make baculovirus a safe, appealing candidate for biomedical applications. BV surface display technology further highlights the beneficial features of baculovirus by rendering it an auspicious tool for applications such as antigen presentation (Strauss et al., 2007; Prabakaran et al., 2008) and targeted gene delivery (Mäkelä et al., 2006; Mäkelä et al., 2008). In this study, the apoptosis inducing ability of recombinant BV vectors that either express, display or both express and display Mda-7 was characterized in human prostate cancer PC-3 cells.

Baculoviral vector was chosen to mediate the activity of Mda-7 because of its nonpathogenic nature. In addition, the unique structure of the BV phenotype of *Ac*MNPV enables the construction of dual-acting viruses both expressing foreign proteins and displaying them on the viral surface. Two mammalian expression vectors, *Ac*-Mda-7 and *Ac*-Luc, were developed to express Mda-7 and firefly luciferase, respectively. Additionally, two display viruses, *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7, both displaying a fusion protein consisting of Mda-7 conjugated to the transmembrane anchor of VSVg through a polyalanine linker, were developed. Expression of the Mda-7-VSVg fusion protein was controlled by the strong baculoviral p10 promoter to enable fusion protein expression during virus production in insect cells. The fusion protein was directed to the insect cell surface by utilizing the endogenous signal sequence of Mda-7. A truncated form of VSVg consisting of the TM and CT domains has been shown to allow uniform scattering and enhanced expression of the fusion proteins on the baculoviral envelope (Chapple and Jones, 2002). Furthermore, by using VSVg-based fusion strategy the native gp64 is left intact to promote viral infection and transduction. The polyalanine linker was expected to provide a flexible arm for the Mda-7-VSVg fusion protein to be presented properly without risking viral binding or transduction.

Mäkelä and coworkers (2006) constructed recombinant baculoviruses displaying tumor-targeting peptides by means of a membrane anchor corresponding to the VSVg-construct used in the present study. Appropriate expression and transfer of the fusion proteins led to successful display of the Lyp-1, F3 and CGKRK tumor-homing peptides on the viral envelope and, subsequently, increased binding and transduction of human cancer cells (Mäkelä et al., 2006). In this study, characterization of the recombinant viruses by immunolabeling and confocal microscopy showed that Mda-7-VSVg fusion protein was successfully incorporated into *Sf*9 insect cell surface upon infection with the envelope modified *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7 viruses. Further characterization of the surface modified viruses demonstrated that the fusion protein was successfully incorporated into the budded *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7 virions. Importantly, as shown by western blotting analysis and flow cytometry, no significant differences were detected in the infectivities between *Ac*-Luc, possessing the wild-type baculovirus surface

phenotype, and the envelope modified viruses, thus demonstrating that the surface modifications did not alter the infectivities of the recombinant viruses.

In 2001, Song and Boyce studied the possibility of developing a baculoviral vector for cancer gene therapy. Treatment of human osteosarcoma Saos-2 cells with a recombinant baculovirus carrying the *p53* tumor suppressor gene lead to dose-dependent apoptosis induction with a maximum of 91% apoptotic cells with 480 pfu/cell of the virus (Song and Boyce, 2001). Later, Wang and coworkers (2006) eradicated malignant glioma cells both *in vitro* and *in vivo* by using a baculovirus vector expressing bacterial *Diphteria toxin A* gene. In this study, *Ac*-Mda-7 was developed to induce apoptosis in mammalian tumor cells by expression of Mda-7. Based on the knowledge of anti-tumor activity of the Mda-7 protein (Chada et al., 2004a; Chada et al., 2005; Su et al., 2005; Zheng et al., 2007), the present study further aimed at developing a dual-acting baculovirus, *Ac*Mda-7-Mda-7, hypothesized to induce apoptosis both by binding of the displayed Mda-7 on the tumor cell surface and by expressing Mda-7 inside the cell.

Indeed, considerable apoptosis induction was detected in PC-3 cells transduced with 500 or 1000 pfu/cell of *Ac*Mda-7-Mda-7, 500 pfu being more efficient, presumably due to saturation of transduction with 1000 pfu/cell of the virus. However, only slightly lower levels of apoptotic cells were obtained by using *Ac*-Mda-7 or *Ac*Mda-7-Luc indicating that both expression and surface display of Mda-7 alone were sufficient to promote apoptosis in prostate cancer cells. Thus, it seems that the effects of simultaneously expressed and displayed Mda-7 protein are non-cumulative since *Ac*Mda-7-Mda-7 did not induce apoptosis as efficiently as *Ac*-Mda-7 and *Ac*Mda-7-Luc in total. The control virus *Ac*-Luc showed some cytotoxicity in PC-3 cells, presumably due to high luciferase expression. Studies have shown that the anti-tumor activity of Ad-Mda-7 (Pataer et al., 2002; Sarkar et al., 2002; Mhashilkar et al., 2003; Chada et al., 2006b) and AVV-Mda-7 (Tahara et al., 2007) is mediated through various intracellular signaling pathways. In PC-3 prostate cancer cells, Ad-Mda-7 has been found to increase the percentage of cells in the G2/M phase of the cell cycle (Lebedeva et al., 2003; Saito et al., 2005), up-regulate the expression of pro-apoptotic proteins such as BAX and BAK and decrease the expression of the anti-apoptotic Bcl-X_L protein (Lebedeva et al., 2003). Furthermore, Ad-Mda-7

mediated PC-3 cell killing has been shown to involve induction of the caspase cascade (Saito et al., 2005) as well as activation of the extracellular signal-regulated kinase (ERK) 1/2, which is a MAP kinase presumed to play a part in cancer cell-specific apoptosis induction (Sauane et al., 2004). Due to being an expression vector as Ad-Mda-7, *Ac*-Mda-7 most likely exerts its actions by utilizing similar intracellular apoptosis promoting routes. In a study by Lebedeva and coworkers (2003), secreted Mda-7 protein was found in the supernatants of Ad-Mda-7 infected PC-3 cells (Lebedeva et al., 2003). Thus, due to the endogenous signal sequence of *mda-7*, *Ac*-Mda-7 and *Ac*Mda-7-Mda-7 might, moreover, mediate the secretion of Mda-7 from the transduced cells and thus spread the apoptotic effect to adjacent tumor cells not initially receiving the *mda-7* gene. Instead, *Ac*Mda-7-Luc induces apoptosis in via the displayed Mda-7 protein, which presumably binds to the IL-20R1/IL-20R2 or IL-22R1/IL-20R2 receptor complexes on the target cell surface thus eliciting responses similar to the bystander effects mediated by the secreted Mda-7 protein (Chada et al., 2004a; Chada et al., 2005; Su et al., 2005). Furthermore, since *Ac*Mda-7-Mda-7 is able to both express and display Mda-7, it is likely to promote apoptosis both intracellularly and extracellularly. Moreover, in clinical settings, due to the displayed Mda-7 protein, *Ac*Mda-7-Luc and *Ac*Mda-7-Mda-7 might serve as cancer vaccines by stimulating the immune system to fight cancer.

In summary, this study demonstrates the functionality of baculovirus mediated gene delivery and protein display, highlighting the potential of baculovirus vectors in cancer gene and immunotherapy. Albeit baculovirus has several attractive features making it a promising candidate for *in vivo* applications, the virus has some limitations. Innate immune responses and inflammatory reactions elicited by baculovirus raise safety concerns and viral inactivation by the serum complement system makes vector development challenging. Thus, further studies will show whether these obstacles may be overcome and baculovirus be used for treating human diseases such as cancer.

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