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Adenovirus infection is dependent on regulation and accessibility of the receptor CAR.

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Miracle Fair

The commonplace miracle:
that so many common miracles takes place.

The usual miracle:
invisible dogs barking
in the dead of night.

One of the many miracles:
a small and airy cloud
is able to upstage the massive moon.

Several miracles in one:
an elder is reflected in the water
and is reversed from left to right
and grows from crown to root
and never hits bottom
though the water isn't deep.

A run-of-the-mill miracle:
winds mild to moderate
turning gusty in storm.

A miracle in the first place:
cows will be cows.

Next but not the least:
just this cherry orchard
from just this cherry pit.

A miracle minus top hat and tails:
fluttering with white doves.

A miracle (what else can you call it):
the sun rose today at three fourteen a.m.
and will set tonight at one past eight.

A miracle that's lost on us:
The hand actually has fewer than six fingers
but still it's got more than four.

A miracle, just take a look around:
the inescapable earth.

An extra miracle, extra and ordinary:
the unthinkable can be thought

by Wislawa Szymborska

List of original publications

This thesis is based on the following papers that will be referred to in the text by their roman numbers

I. Vincent T, Harvey BG, Hogan SM, Bailey CJ, Crystal RG, Leopold PL. (2001). Rapid assessment of adenovirus serum neutralizing antibody titer based on quantitative, morphometric evaluation of capsid binding and intracellular trafficking: population analysis of adenovirus capsid association with cells is predictive of adenovirus infectivity. *J Virol* **75**: 1516-21.

II. Leopold PL, Wendland RL, **Vincent T,** Crystal RG. (2006). Neutralized adenovirus-immune complexes can mediate effective gene transfer via an Fc receptor-dependent infection pathway. *J Virol* **80**: 10237-47.

III. Vincent T, Pettersson RF, Crystal RG, Leopold PL. (2004). Cytokine-mediated downregulation of coxsackievirus-adenovirus receptor in endothelial cells. *J Virol* **78:** 8047-58.

IV. Vincent T, Neve EPA., Kukalev A, Virtanen I, Philipson L, Leopold PL Crystal RG, Moustakas A, Pettersson RF, Fuxe J. (2007). A Snail-Smad transcriptional repressor complex promotes TGFβ-mediated epithelial mesenchymal transition. Manuscript.

Other publications not included in this thesis:

Bezdicek P, Worgall S, Kovesdi I, Kim MK, Park JG, **Vincent T,** Leopold PL, Schreiber AD, Crystal RG. (1999). Enhanced liver uptake of opsonized red blood cells after in vivo transfer of FcgammaRIIA cDNA to the liver. *Blood* **15**: 3448-3455.

Bailey CJ, **Vincent T,** Crystal RG, Leopold PL. (2007). Cell type-specific intracellular trafficking of adenovirus capsid and genome. Manuscript

Vincent T, Kukalev A, Pettersson RF, Percipalle P. (2007). The glycogen synthase kinase (GSK) 3β inhibits RNA polymerase I transcription in H-RAS transformed cells. Manuscript.

POPULAR SCIENTIFIC SUMMARY

A decade ago, scientists, excited by the undertaking of the human genome project, were drawn to what was heralded to be the 'killer application' in biomedicine: gene therapy. The therapy involves taking a common virus, deleting the virus's genetic material, replacing it with a human gene and trafficking that virus into the body. The success of gene therapy could have far reaching consequences ranging from generating blood vessels to providing groundbreaking treatments for genetic diseases like cystic fibrosis as well as cancer. Two critical elements play an integral role in insuring a successful treatment. First, the ideal viral candidate for trafficking of said material needs to be identified followed by discovering a docking site that will act as a conduit assisting the viral payload into the host's cell. Adenovirus appeared to fit the bill perfectly due to its proficient delivery of genetic material to a host and its reasonably innocuous properties. Having discovered an ideal candidate, the next step was to discover an available docking site. In 1997, three different groups discovered the Coxsackie and Adenovirus Receptor (CAR) and four years later, CAR's precise position in the body was identified. CAR occupied a unique position in the cellular make up as it acted as an adhesive helping to bind cells to one another. As a result of this finding, CAR provoked the interest of many in and outside of the field. These were the compelling circumstances that initiated and drove this thesis study.

The objective of the study was to ascertain the capability of adenovirus to deliver genes in three different scenarios. The first objective was to study the virus's behavior in the presence of antibodies that act as a natural opponent to adenovirus infection. The second and third studies sought to evaluate the virus and the receptors behavior in the context of two pathological conditions: inflammation and cancer.

The results obtained from the first study revealed that only a small fraction of adenoviruses were able to deliver genes to the host cell due to the presence of antibodies binding to the virus. The binding of antibodies prevented the virus from reaching its primary docking site, CAR. The fraction of viruses that successfully delivered genes met up with a secondary docking site known as Fc-gamma. Hence, understanding the importance and behavior of a viral docking site became the critical motivating factor for further studies.

Viral gene delivery was hindered after subjecting the virus to pathological conditions such as inflammation and cancer. The data extracted from the inflammation and cancer studies provided a simple explanation: CAR was no longer detectable. The absence of CAR in two pathological conditions where one detects a decrease in cell adhesion implies that CAR may play a role in binding cells together. The cancer study sought to provide a more detailed explanation as to why CAR disappears under these conditions. Observation centered on two DNA binding cancer proteins: Snail and Smad. Whenever Snail and Smad were present, sitting next to one another

on a DNA strand, CAR could not be detected. Was this a chance event or did these proteins perform other functions in addition to regulating CAR? The question led to investigating the behaviors of these proteins in conjunction with another adhesive protein called E-cadherin. The outcome of these experiments yielded the same results as found in the CAR studies: E-cadherin was not detectable when Snail and Smad where sitting on the DNA strand.

As a result of studying adenovirus in normal as well as pathological conditions, we have garnered significant knowledge regarding successful gene delivery and the function of CAR as a cell adhesion protein. CAR represents a promising example of the merging of two major fields of research today: virology and cell biology.

ABSTRACT

Coxsackie and Adenovirus Receptor (CAR) displays dual biological functions since it acts both as a viral receptor for the two unrelated viruses, coxsackie and adenovirus (Ad), and as a cell-adhesion molecule in mammalian cells. The number and accessibility of CAR receptors expressed at the cell surface is a major determinant for successful infection. The aim of this thesis is to evaluate how CAR and Ad infection vary depending on the physiological milieu surrounding the cell. To address the aim of the thesis, CAR-mediated Ad infection was studied under three different conditions, namely in the presence of anti-Ad neutralizing antibodies that constitute a humoral immune response to the viral capsid, in the presence of cytokines that constitute secreted pro-inflammatory mediators, and in the presence of altered intracellular signaling pathways that constitute hallmarks of cancer progression.

To examine the impact of neutralizing antibodies on CAR-mediated Ad infection, quantitative methods were developed to measure cell-associated virus and successful viral infection (gene expression). In the presence of neutralizing antibodies, Ad was hindered from interaction with CAR and infection was prevented. The impact of neutralizing antibodies was further characterized by determining the extent to which the decrease in infection resulted from hindered receptor binding versus actual viral inactivation. To examine this question, target cells were modified by introducing the expression of an Fcγ receptor that was capable of binding and internalizing Ad-antibody complexes in a CAR-independent manner. These experiments showed that infectious virus was present in Ad-antibody complexes and that hindrance of binding to CAR likely constituted a major factor in neutralizing Ad.

To examine the impact of inflammation on Ad infection, CAR expression and Ad infection of human endothelial cells were studied in the presence and absence of the pro-inflammatory cytokines, tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ). The data showed that these cytokines suppressed CAR protein and mRNA levels in endothelial cells and inhibited Ad infection in a time and dose-dependent manner, demon-strating that cytokine-mediated changes in cell physiology had the potential to affect CAR-dependent Ad infection by changing the availability of CAR.

Finally, to examine the impact of cancer-related intracellular signaling pathways on CAR-mediated Ad infection, several *in vitro* models were established to recreate progression of tumor cells from low to high-grade malignancy, a process known as epithelial to mesenchymal transition (EMT). CAR was suppressed both at the transcriptional and translational level in these models, and a novel transcriptional repressor complex involving Snail and Smads was identified. This complex mediated effective suppression of CAR as well as another cell adhesion protein E-cadherin reflecting the fact that CAR expression and Ad infection can be modulated as part of larger, long term changes in cell physiology.

In summary, these thesis studies shed new light on mechanisms involved in adenovirus interaction with host cells and on regulation and accessibility of CAR during normal and pathological conditions. As such this thesis has, in part, contributed to a better understanding of the intimate interplay between virology and cell biology.

Abbreviations

aa	amino acid
Ad	adenovirus
ADE	antibody-dependent enhancement
ALK5	activin receptor-like kinase 5
AP-1	activator protein 1
APC	antigen presenting cell
β-gal	β-galactosidase
BT-IgSF	brain and testis immunoglobulin superfamily
CAR	coxsackie and adenovirus receptor
CBP	CREB-binding protein
ChIP	chromatin immunoprecipitation
cIAPs	cellular inhibitors of apoptosis
CLMP	CAR like membrane protein
CMV	cytomegalovirus
co-Smad	cofactor Smad
CPE	cytopatic effect
CR3	complement receptor 3
CTL	cytotoxic T-lymphocyte
CTX	Cortical Thymocyte in Xenopus
DC	dendritic cells
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
ERK	extracellular signal-regulated kinase
ESAM	endothelial cell selective adhesion molecule
E-selectin	endothelial-selectin
FADD	fas-associated death domain protein
FAK	focal adhesion kinase
FasL	Fas Ligand
FcR	Fc receptor
FGF	fibroblast growth factor
FGFR	FGF receptor
GAS	IFNγ activating sites
GSK3β	glycogen synthase β
H-cadherin	heart-cadherin
HGF	hepatocyte growth factor
HIV	human immunodeficiency virus
H-Ras	Harvey-Ras
HUVEC	human umbilical vein cells
ICAM	intercellular adhesion molecule
IFNGR1	interferon gamma receptor 1
Ig	immunogloubulin
IgSF	immunoglobulin superfamily
Iκκ	inhibitor of kappa β kinase
IL-2	Interleukin-2
ILK	integrin-linked kinase
INFγ	interferon gamma
I-Smad	inhibitory Smad
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
JAK1	janus activated kinase 1
JAM	junctional adhesion molecule
JAM-L	JAM-like

K-cadherin	kidney-cadherin
kDa	kilodaton
K-Ras	Kirsten-Ras
LNX	ligand-of-numb-protein X
LOXL2	lysyl oxidase like protein 2
L-selectin	leukocyte selectin
MAG1-1β	membrane–associated guanlyate kinase 1β
MAPK	Mitogen-Activated Protein Kinase
MEK1	mitogen-activated protein kinase and ERK kinase 1
MET	mesenchymal epithelial transition
MHC	major histocompatibitity complex
MIP-1α	macrophage inflammatory protein-1α
MLCK	myosin light chain kinase
MMP	matrix metalloproteinase
MTOC	microtubule organizing center
MTs	microtubules
MUPP-1	multi-PDZ domain protein-1
N-cadherin	neural-cadherin
NFκβ	nuclear factor kappa beta
NK cells	natural killer cells
NLS	nuclear localization signal
N-Ras	Neuroblastoma-Ras
P-cadherin	placental cadherin
PDK1	phosphoinositide-dependent kinase 1
PDZ	PSD95/DLG/ZO-1
PECAM	platelet/endothelial cell adhesion molecule 1
PI3K	phosphatidyl inositol phosphatase kinase
PICK1	protein interacting with protein C kinase
PIP3	phosphatidylinositol 3, 4, 5 triphosphate
PKC	protein kinase C
P-selectin	platlet-selectin
PTEN	phosphatase and tensin homolog
R-cadherin	retinal-cadherin
RGD	Arg-Gly-Asp
RIP1	receptor interacting protein 1
R-Smad	receptor Smad
SARS	severe acute respiratory syndrome
SBEs	Smad binding elements
SEAP	secreted alkaline phosphatase
SODD	silencer of death domains
STAT1	signal transducers and activators of transcription
TER	transepithelial electrical resistance
TGFβ	tumor growth factor beta
TNFα	tumor necrosis factor alpha
TNFR1	tumor necrosis factor receptor 1
TRADD	TNFR1 associated death domain protein
TRAIL	TNF related apoptosis inducing ligand
TRAF2	TNF-receptor-associated factor 2
VCAM	vascular cell adhesion molecule 1
VE-cadherin	vascular endothelial cadherin
WT	wild-type
ZO	zona occludens
ZONAB	ZO-1-associated nucleic acid-binding protein

To my family and Damisi.

PREFACE

During evolution viruses have acquired the ability to use cell surface proteins as their receptors and main port of entry to cells. In particular, viruses use cell surface proteins that play a role in cell adhesion and migration. The initial interaction between the virus and its cellular receptor is a major determinant for successful infection and will be dependent on the number of these receptors expressed as well as their accessibility. These surface proteins are often modulated in response to changes in the environment surrounding the cells, which can alter viral receptor expression and accessibility. The local milieu will therefore affect the receptor-dependent uptake of virus either from the apical or basolateral side and, as a result, will enhance or suppress viral infection. This thesis will focus on one virus, namely adenovirus, that utilizes the cell adhesion protein, the coxsackie and adenovirus receptor (CAR), as its receptor. Adenovirus binding and infection has been extensively studied in recent years due to its utility as a vector for gene therapy. Despite the fact that CAR was identified a decade ago as the receptor for adenovirus, little is known about the biological function of this protein, but a role for CAR as a cell-adhesion protein has been proposed. The dual role of CAR as a viral receptor and a cell-adhesion protein created the foundation for this thesis. We examined how the efficiency of adenovirus infection can be altered under normal and pathological conditions by modulating the expression and accessibility of CAR. In the following sections, adenovirus biology and its usefulness as a vector in gene therapy protocols will be discussed. This will be followed by a summary of the current understanding of CAR biology. Finally, relevant pathological conditions such as inflammation and cancer that affect adenovirus infection and CAR expression will be addressed to provide a more comprehensive understanding of the studies that were conducted and described in this thesis.

ADENOVIRUS

Adenovirus biology

Adenoviridae

Adenovirus (Ad) was first identified in 1953 and its name is derived from the adenoid tissue from which the virus was first isolated (Rowe et al., 1953). The family *Adenoviridae* is divided into mammalian adenovirus (mastadenovirus) and avian adenovirus (avianadenovirus) (Shenk, 2001). Adenoviruses most commonly cause respiratory illness, however, depending on the infecting serotype, they may also cause gastroenteritis, conjunctivitis or cystitis. Presently, 51 serologically distinct types of human Ad have been identified, based on their resistance to neutralization to other known adenovirus serotypes. These serotypes are further subdivided into six subgroups, A-F based on their hemagglutination activity, i.e. the ability of each serotype to agglutinate red blood cells. Hemagglutination is the result of crosslinking between fibers, monomeric or aggregated pentons on the surface of red blood cells. The serotypes have also been confirmed by restriction and sequence analysis.

Adenoviral structure

The adenovirus is a non-enveloped, double-stranded DNA virus with a 30-38 kb genome, depending on the serotype, contained within an icosahedral capsid (Shenk, 2001). The capsid is 70-100 nm in diameter and consists mainly of the three proteins hexon (130 kilodalton (kDa)), penton (82 kDa) and fiber (62 kDa). The penton capsomere is composed of five penton subunits that are non-covalently linked to the trimeric fibers. Two hundred forty hexons form the 20 faces of the capsid while 12 vertices contain 12 pentons each carry a protruding fiber. The penton capsomere harbours five identical penton base subunits, each containing an Arg-Gly-Asp (RGD) peptide motif. The RGD motif is conserved in serotypes A, B, C and E and is also found in cellular adhesion molecules. The Ad capsid is further stabilized through three minor proteins IIIa, VIII and IX, that link the capsid components to the core proteins and are important in assembly of the virions. In addition, protein VI tethers the Ad genome to the capsid wall.

The Ad genome is composed of double stranded DNA that is tightly coiled around histone-like proteins V and VII thereby forming a chromatin-like structure (Shenk, 2001). At each end of the linear genome the terminal protein is covalently attached to the free 5' ends. The core particle contains the Mu (μ) protein, which is thought to be involved in condensing the Ad genome and about 10 copies of the cysteine protease L3/p23. The Ad genome is comprised of early (E1-E4), intermediate and late genes (L1-L5).

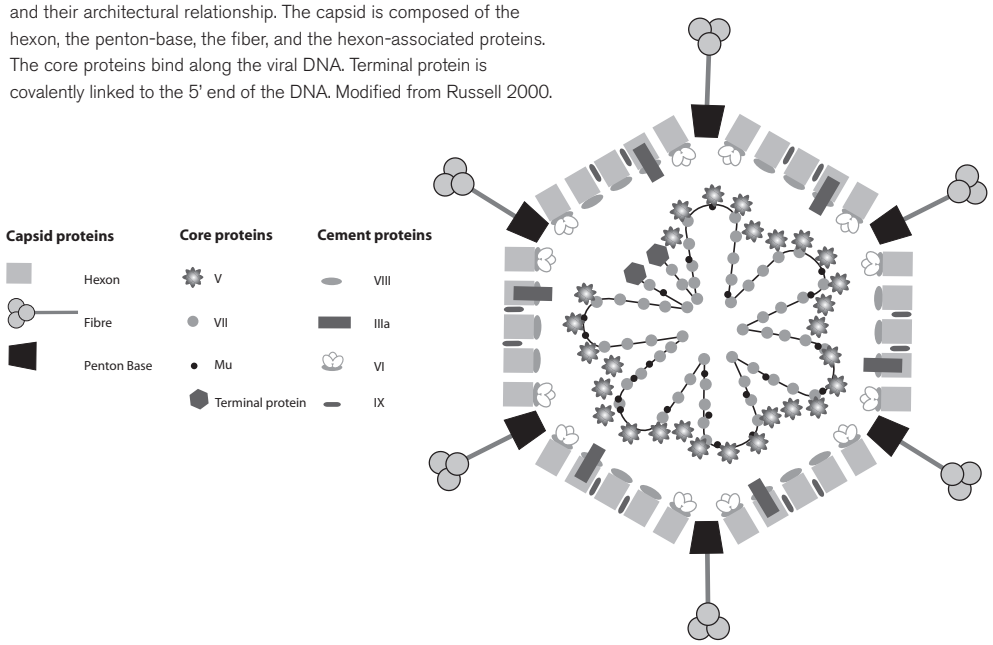

Fig1. Adenoviral particle. Schematic model of an adenoviral particle showing structural proteins, the nucleoprotein core within the virion, and their architectural relationship. The capsid is composed of the hexon, the penton-base, the fiber, and the hexon-associated proteins. The core proteins bind along the viral DNA. Terminal protein is covalently linked to the 5' end of the DNA. Modified from Russell 2000.

Adenoviral infection

The adenoviral infection pathway has been extensively studied and is well documented. It is, however, important to keep in mind that the majority of the studies performed used subgroup C, and that the infection pathway may vary depending on the virus subgroup.

Binding of adenovirus

For many cell types, Ad infectivity depends on the interaction of Ad with its high affinity receptor, the coxsackie and adenovirus receptor (CAR) (Bergelson et al., 1997; Carson et al., 1997; Tomko et al., 1997). All subgroups of Ad, except B have been shown to bind CAR (Roelvink et al., 1998). Recently, CD46 was identified as the cellular receptor for subgroup B viruses (Gaggar et al., 2003; Gaggar et al., 2005; Marttila et al., 2005; Segerman et al., 2003; Sirena et al., 2004). The viral particles attach to the target cell surface through an interaction between the knob of the protruding fiber protein with the extracellular domain of CAR (Philipson et al., 1968). The binding to CAR tethers the Ad capsid to the cell surface enabling the penton base of the capsid to interact, via its RGD motif, with integrins on the cell surface (Bai et al., 1994; Mathias et al., 1994; Nemerow et al., 1994; Wickham et al., 1994; Wickham et al., 1993). CAR, solely functions as a docking site for the viral

capsid and only the extracellular domain (but not the tail) is required for virus internalization and trafficking (van't Hof and Crystal, 2001; Walters et al., 2001; Wang and Bergelson, 1999).

The most important integrins involved in Ad viral infection are thought to be $\alpha_v\beta_3$ or $\alpha_v\beta_5$ since no virus internalization could be detected when soluble synthetic RGD peptides or monoclonal antibodies raised against the functional domain of these integrins were used (Wickham et al., 1993). However, depending on the cell type Ad may use different integrins (Davison et al., 2001; Mathias et al., 1994; Nemerow and Stewart, 1999). It has been suggested that the binding of the virus to the target cell leads to a rearrangement in the plasma membrane, eventually resulting in cooperative binding of the viral particle. In other words, binding of one fiber protein on the viral particle promotes binding of a second fiber protein on the same viral particle to CAR (Persson et al., 1983; Persson et al., 1985). The initial attachment of the virus and the internalization through binding of the integrins are two distinct and independent events. Soluble fiber or anti-fiber antibodies have previously been shown to inhibit virus attachment whereas soluble penton base or anti-penton antibodies did not affect attachment (Philipson et al., 1968). In addition, an integrin deficient cell line bound equal amounts of virus but failed in the uptake of virus (Wickham et al., 1993).

The subcellular localization of CAR at tight junctions (Cohen et al., 2001b) revealed why infection of polarized epithelial cells was limited (Pickles et al., 2000; Walters et al., 1999). Because of its location, CAR is often not accessible for Ad infection raising the question of whether there are additional unidentified receptors that may bind Ad prior to its binding to CAR. These receptors might bring Ad in close proximity to CAR and subsequently mediate binding (Philpsson and Wadell 2006, personal communications). Other possibilities are that infection is achieved through binding to non-polarized cells expressing CAR on the luminal side or that injury to the epithelium allows the virus access to the tight junctions (Meier and Greber, 2003; Walters et al., 2002). In addition to CAR, major histocompatibitity complex I (MHC class I) (Hong et al., 1999) and heparan sulfate glycosaminoglycans (Dechecchi et al., 2001; Smith et al., 2003a; Smith et al., 2003b) have been reported to mediate Ad binding. However, the MHC class I mediated binding of Ad is uncertain since subsequent studies did not confirm this finding (Davison et al., 1999; McDonald et al., 1999).

Uptake of adenovirus

Ad entry into cells occurs through receptor-dependent mediated endocytosis, whereby the virus enters clathrin coated pits after binding to the cell surface. To verify receptor-mediated internalization of Ad, studies were performed using

substances that are known to enter the cell via receptor mediated endocytosis such as epidermal growth factor (EGF). These studies demonstrated that Ad and EGF were recovered in the same vesicles (FitzGerald et al., 1983). Similarly, ligands known to traffic via endosomes to lysosomes, have been found to co-localize with subgroup B adenovirus (Miyazawa et al., 1999). The receptor mediated endocytosis of Ad into clathrin coated pits was studied in more detail by several groups and is estimated to have a half-time of < 5 min (Greber et al., 1993; Leopold et al., 1998; Wickham et al., 1994). Because of the specific regulation of clathrin mediated endocytosis, the role of dynamin, a GTPase, was studied in the internalization of Ad. Dynamin was previously suggested to mediate constriction of coated pits resulting in the budding of vesicles from the plasma membrane (Chen et al., 1991; van der Bliek and Meyerowitz, 1991). When using a dominant negative form of dynamin, adenovirus entry and infection were markedly reduced, supporting an important role for clathrin mediated endocytosis (Wang et al.,1998). However, this study, as well as others, showed that viral infection is not completely abolished when clathrin mediated endocytosis is inhibited, suggesting the prescence of a slower, and less efficient, clathrin independent uptake of Ad.

Co-incident with the uptake and internalization of Ad, the penton-base integrin interaction induces several signaling cascades including the phosphatidyl inositol phosphatase kinase (PI3K) pathway, which together with induction of the actin remodelling Rho family of small GTP binding proteins, Rac1 and Cdc42, is necessary for viral entry (Li et al., 1998a; Li et al., 1998b). In addition, activation of the Raf/Mitogen-Activated Protein Kinase (MAPK) signaling pathway and the Rab5 GTPase have been shown to promote Ad endocytosis (Bruder and Kovesdi, 1997; Rauma et al., 1999).

Endosomal escape of adenovirus

Following endocytosis Ad escapes from the early endosome to the cytoplasm, an event initiated immediately after internalization. Ad escapes from endosomes at, or prior to, fusion of the endocytic vesicles with the sorting endosomes, within minutes after internalization (Greber et al., 1993; Leopold et al., 1998). When the virus enters the endosomal compartment, it encounters a different physiological milieu. The extracellular pH is 7, whereas the pH of the endosome ranges from pH 6.2 to 6.5 in early endosomes, to pH 5.0 to 5.5 in late endosomes and lysosomes (Mukherjee et al., 1997; Seth et al., 1987). It is thought that the low pH within the vesicle mediates a conformational change of the viral capsid exposing new epitopes of the hexon and penton-base. This conformational change is thought to give the capsid a more hydrophobic character resulting in a better interaction between the capsid and the lipids in the vesicle membrane (Blumenthal et al., 1986; Seth et al., 1984a;

Seth et al., 1985; Svensson, 1985). This enhanced interaction could potentially lead to a local weakening of the fragile vesicle thus altering the membrane permeability. Treatment of cells with weak bases that inhibit endosomal acidification, inhibited Ad infection in some reports (Greber et al., 1993; Seth et al., 1984b; Svensson, 1985) but not in others (Rodriguez and Everitt, 1996). These conflicting results could be caused by the different protocols used. The importance of the penton-base in the endosomal escape was confirmed to be essential not only for subgroup C but also for subgroup B that uses CD46 as their main cellular receptor (Shayakhmetov et al., 2005a). The contribution of the fiber in the viral escape was demonstrated in a study, where chimeric Ad vectors expressing fiber protein from Ad7 and caspsid from Ad5, were retained inside the late endosomes and lysosomes far longer than the parental vectors. This suggested that the fiber might play a role in dictating the conditions under which lysis of the endosomes can occur (Miyazawa et al., 1999).

Upon internalization of the virus the viral protease, L3/p23 cysteine protease within the capsid structure, becomes activated. This activation occurs in two separate steps, first at the cell surface through integrin binding and secondly by the reducing environment within the endosome. Proper activation of the protease is necessary for correct uncoating and trafficking of the virus. Viruses lacking a functional protease failed to penetrate the membrane and was not able to escape the endosome (Cotten and Weber, 1995; Greber, 1998; Greber et al., 1996).

In conclusion, binding and uptake are crucial and rate-limiting events in virus infection. However, later steps such as proper uncoating, trafficking and delivery of the viral genome to the nucleus as will be discussed below, are also important for successful infection.

Uncoating of adenovirus

Dismantling of the viral capsid occurs stepwise starting at the cell surface and is not completed until the virus DNA reaches its final destination, the cell nucleus. The viral uncoating process was initially described morphologically and later confirmed biochemically. Prior to entry, Ad appears in the transmission electron microscope as a thin electron dense circle (capsid) around an electron dense core (genome). After infection, the shape of the capsid changes losing the vertices and becoming more rounded (Morgan et al., 1969). The penton-base interaction with integrins in combination with the reducing milieu within the endosomes, activates the dormant protease L3/p23 located inside the capsid resulting in degradation of the capsid stablizing protein IV (Greber et al., 1996). Triggering this proteolytic activity appears to initiate the dismantling of the capsid vertices including the loss of fiber and penton-base. Once in the cytoplasm proteins IIIa, VIII and IX are removed from the surface of the virus followed by the other capsid proteins. The

uncoating process is correlated with the time and rate at which the viral genome becomes sensitive to deoxyribonuclease. When the capsid finally reaches the nucleus and binds the nuclear envelope, the electron dense core exits from the capsid leaving empty capsids associated with the nuclear envelope (Dales and Chardonnet, 1973).

Trafficking of adenovirus

Once in the cytoplasm, the viral capsid has been shown to be associated with microtubules (MTs) and the microtubule organizing center (MTOC) during the translocation to the nucleus (Chardonnet and Dales, 1972). More recent studies in living cells have demonstrated that Ad, after exit from the endosomes, moves through the cytosol with a speed of 2 μm/sec and that this movement is dependent on intact MTs and the MT associated protein dynein (Leopold et al., 1998; Leopold et al., 2000; Suomalainen et al., 1999). Cytoplasmic dynein is the major motor protein responsible for movement toward the MTOC and the interaction of dynein with a cargo, such as Ad, requires the protein complex dynactin (Kelkar et al., 2004; Leopold et al., 2000; Suomalainen et al., 1999). The plus ended microtubule motor protein kinesin has also been suggested to interact with Ad during transit to the nucleus (Suomalainen et al., 1999).

Nuclear delivery of adenoviral genome

The mechanism by which Ad particles disassociate from MTs was addressed in a recent study (Strunze et al., 2005). By using specific drugs as well as siRNA it was shown that an export factor CRM1 directs incoming Ad particles to the nucleus. In the absence of CRM1, Ad was trapped at the MTOC, a phenomenon previously observed in enucleated cells, and binding to the nuclear membrane was prevented (Bailey et al., 2003).

The final destination for Ad in the cell is the nuclear envelope where the nuclear pore complex serves as a docking site and gateway for the import of the Ad genome. Here, the final step of uncoating occurs which requires enzymatic activity (Chardonnet and Dales, 1972; Greber et al., 1997). Ad genome enters the nucleus by binding to the cytoplasmic fibril protein CAN/nup214 of the nuclear pore complex (Trotman et al., 2001). This nuclear import is independent of additional cytosolic factors as well as Ran-GTP, which commonly are involved in export and import of proteins to or from the nucleus. Once the virus is bound, the proximity of the capsid to histone H1 enables a hexon-histon H1 binding and the H1-import factors, Impβ and Imp7, then initiate the final capsid disassembly (Trotman et al., 2001).

Hexons and protein XI remain outside the nucleus and the viral DNA is separated from the capsid. The viral DNA and protein VII are both imported into the nucleus although it is not known whether they enter as a complex or individually

(Greber et al., 1997). The covalently bound terminal protein remains associated with the DNA and initiates viral replication by binding to the nuclear matrix (Shenk, 2001). The terminal protein contains a nuclear localization signal (NLS) motif and has therefore been postulated to thread the DNA through the nuclear pore.

Expression of adenoviral genes

In the nucleoplasm the cellular machinery is utilized to transcribe the Ad genome into a large number of virus-specific mRNAs (Shenk, 2001). The expression can be divided in three different phases; pre-early, early and late. Only a small portion of the viral genome (4% including the regulatory protein E1A) will initiate the early phase and proteins necessary for viral DNA replication and eventually for the onset of the late genes. The late genes initiate viral particle assembly. The viral replication cycle ends with host cell lysis and release of new viral particles to the extracellular environment.

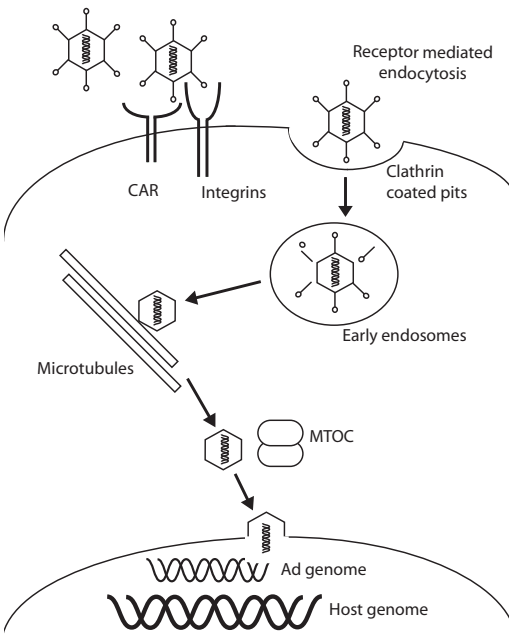

Fig2. Adenoviral infection. Schematic model of adenoviral infection showing binding, internalization, endosomal escape, uncoating, trafficking and delivery of the viral DNA to the nucleus. Modified from Greber 1993.

Adenovirus as a gene therapy vector

During the last 10 to 15 years, Ad has been widely studied because of its utility as a vector for gene therapy. Gene therapy is a technique for introducing a gene to target cells in order to correct or treat a human disease or injury. The popularity of Ad as a gene transfer vector is based on the efficiency with which it delivers its genome to the nucleus, infects a variety of proliferating and quiescent cells, and the

ease of propagating the virus to high titres with high purity. In addition, it has a capacity for large cDNA transgene cassettes, and is safe since the virus does not insert its genome into the host DNA (Russell, 2000). The main disadvantage is that long-term transgene expression is not possible and that Ad vectors elicit strong humoral and cellular immune responses (Schagen et al., 2004). The first generation recombinant Ad vectors were based on Ad serotype 5 or 2 with deletions in E1, which renders the virus unable to replicate, and E3, which allows space for the insertion of the desired transgene cassette. The vector is typically propagated in a cell line that expresses E1 genes such as the human embryonic kidney cell line 293 in order to accomplish efficient vector expression (Crystal, 1995; Graham, 1987).

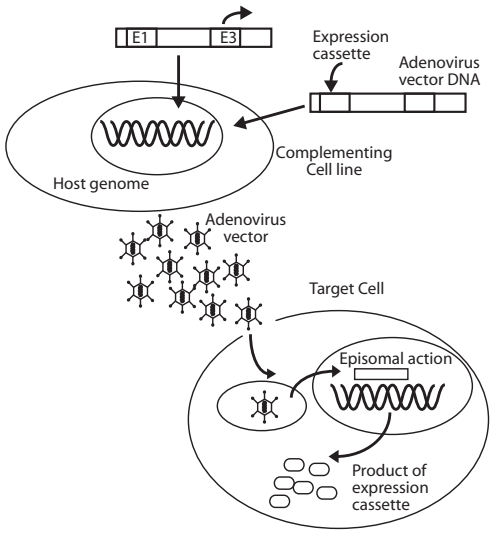

Fig 3. Adenovirus vector design, production and transfer. Schematic picture illustrating the design, production and transfer of first generation recombinant Ad vectors. To produce an E1-, E3- vector, viral E1 and E3 genes are removed, and an expression cassette is inserted in the E3 position. The vector is transfected to a complementing cell line with E1 sequence in its genome. The vectors produced are replication deficient since they contain no E1 region. The vector infects the target cell in a similar way as a wild type virus. Modified from Crystal 1995.

A variety of Ad vectors with different deletions and serotypes have been developed in recent years, mainly to evade the immune system and specifically target the vectors (Bangari and Mittal, 2006; Russell, 2000; Wickham, 2000). When using Ad vectors one must take into account their brief duration of expression, which has been attributed to the epichromosomal location and the immune response against the vector, in addition to other phenomena such as silencing of the cytomegalovirus (CMV) promoter which is used to drive expression of the transgenes in many Ad based vectors. Generally, vector expression lasts up to four weeks reaching a peak within two to three days post administration. As such, Ad is most suited for applications that require transient expression. To date there are over 200 clinical trials where Ad gene therapy vectors are being utilized, representing 26% of all vector-based protocols (Thomas et al., 2003). These protocols include treatment for cancer,

cystic fibrosis, hemophilia A and B, anti-trypsin deficiency and coronary diseases (Bangari and Mittal, 2006; Thomas et al., 2003).

Adenoviral vector mediated immunity

One obstacle in successful long-term transgene expression using Ad based vectors has been the humoral and cellular immune response elicited by the virus. This has been observed in several animal models as well as in humans and consists mainly of three different responses: the innate immune response induced by neutrophils, macrophages and natural killer (NK) cells, the cytotoxic T-cell response mediated by CD8+ T-lymphocytes and finally the humoral, neutralizing anti-viral response i.e. the production of anti-Ad neutralizing antibodies which are produced after CD4+ T-lymphocyte activation of B-lymphocytes (Schagen et al., 2004).

Innate immune response

The innate, or non-adaptive response to Ad is rapid and non-specific. It keeps the pathogen under control while the adaptive response is being initialized, and influences the adaptive response by inducing the production and secretion of different chemokines. The innate response also includes the activation of complement by the alternative pathway and the engulfment of pathogens by phagocytosis.

After the death of a patient enrolled in an Ad gene therapy trial in 1988, the importance of the innate inflammatory response against Ad vectors was highlighted (Marshall, 1999; Thomas et al., 2003). Previous rodent and rhesus-monkey studies, as well as human trials, revealed that induction of different cytokines including, tumor necrosis factor α (TNFα), interleukin-6 (IL-6), IL-8, IL-10, macrophage inflammatory protein (MIP)-1α and MIP2 can occur as a direct response to the viral vectors (Crystal et al., 2002; Harvey et al., 2002). Macrophages and dendritic cells (DC) have been reported to be the major source of these cytokines (Chirmule et al., 1999). These cells are also responsible for the clearance of Ad as shown in a study in mice, where 90% of the recombinant viral genome was eliminated from the liver 24 hrs after intravenous injection of vector (Schiedner et al., 2003; Wolff et al., 1997; Worgall et al., 1997). The rapid elimination of vector was attributed to the liver macrophages i.e. the Kupffer cells, since depletion of theses cells increased the persistence of the vector. Depletion of alveolar macrophages from the lung also prolonged the survival of the Ad genome following intratracheal administration of the vector (Worgall et al., 1997). In a later study the half-time for clearance of Ad in mice was determined to be less than two minutes and colocalization of injected Ad with Kupffer cells was observed (Alemany et al., 2000). Depletion of the Kupffer cells resulted in prolonged blood persistence of the vector supporting previous studies.

Both CAR dependent and independent clearance mechanisms have been pro-

posed (Awasthi et al., 2004; Chirmule et al., 1999; Chirmule et al., 2000a; Harrod et al., 1999; Shayakhmetov et al., 2005b; Shayakhmetov et al., 2004; Smith et al., 2003a; Smith et al., 2003b; Zinn et al., 1998; Zinn et al., 2004). One possible mechanism by which the clearance is mediated was observed when clearance of Ad was enhanced in the presence of a surfactant protein (SPA). As part of the innate response, SPA is primarily expressed on epithelial cells, which use it to opsonize micro-organisms. Serum factors such as C3, C4 and blood factor IX can mediate CAR independent uptake and clearance of Ad (Shayakhmetov et al., 2005b; Zinn et al., 2004). In addition, it has also been shown that heparan sulfate glycosaminoglycans aid in the clearance of Ad (Smith et al., 2003a; Smith et al., 2003b). Other studies have pointed to a CAR dependent clearance of Ad. When labeled Ad5 knob was coinjected with an excess of unlabeled Ad5 knob, clearance was inhibited whereas with an excess of unlabeled Ad3 knob, labeled Ad5 could no longer be detected in the circulation, verifying the importance of the CAR-fiber interaction in the clearance of Ad particles (Zinn et al., 1998). Another study showed that fiber knob which was unable to bind CAR was cleared more slowly from the blood in comparison to fiber knob capable of CAR binding (Awasthi et al., 2004).

Cellular immune response

The cellular immune response against Ad is mainly mediated by the antigen presenting cells (APCs). After the virus is taken up and internalized, the viral proteins and transgene are processed into small peptides, which are presented at the cell-surface by major histocompatibility complex (MHC) class I or II molecules. Binding of CD8+ lymphocytes to the MHC I complex initiates the formation of specific, class I-restricted cytotoxic T-lymphoctyes (CTL), which eliminates the infected cells. The activated CD4+ lymphocytes (Th1 CD4+) will aid in this process by secreting cytokines such as IL-2 and interferon gamma (IFNγ), which induces the maturation of the CD8+ lymphocytes to CTLs. For proper activation of the CD4+ lymphocytes to occur, binding to MHC II-peptide complex is necessary. IFNγ has also been shown to upregulate MHC I thus reinforcing the CTL response.

The humoral response is initiated by the cellular response via binding of Ad to B-lympho-cytes. The MHC II on the B-lymphocyte will present the foreign antigen to CD4+ lymphocytes (Th2 CD4+), which in response secrete cytokines like IL-6, IL-4 and IL-10. These cytokines induce the maturation of the B-lymphocyte to a plasma cell capable of secreting antibodies directed against Ad. As such the antibodies are not responsible for the elimination of Ad infected cells and inhibition of prolonged transgene expression but rather hinder Ad reaching the cell or promote phagocytosis of Ad by the macrophages. Neutralizing antibodies have therefore represented an obstacle in gene transfer by prohibiting readministration of Ad vectors.

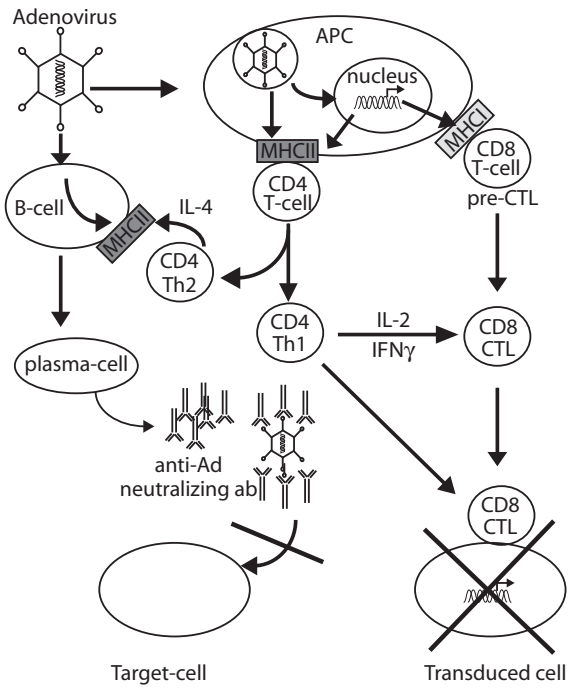

Fig 4. Adenoviral vector induced immunity. Schematic model of the activation of the immune system upon delivery of adenoviral based vectors. Antigen presenting cells (APCs) engulf Ad and viral capsid proteins will be presented to the immune system by MHC class II and newly synthesized viral proteins by MHC I. The MHC class I interaction with CD8+ T-cells triggers the formation of CTL which will eliminate virally transduced cells. MHC II interacts with CD4+ T-cells that both stimulates the proliferation of CTLs but also induces B-cells to mature into plasma cells capable of secreting Ad-specific neutralizing antibodies. The Ad-specific antibodies will block gene transfer upon readministration. Modified from Schagen 2004.

Humoral immune response

Since early vector development mainly used the Ad serotypes 2 and 5, it is common to find pre-existing immunity among potential recipients of Ad-based therapeutics (Aste-Amezaga et al., 2004; Chirmule et al., 1999; Sumida et al., 2005). The prevalence of antibodies against Ad1, Ad2 and Ad5 are the most common and are present in 40 to 60% of children (Horwitz, 2001). This is in contrast to antibodies against Ad3, Ad4 and Ad7, which are low in children of that age. Adults are rarely infected with Ad from the first group but show higher susceptibility to the latter group (Horwitz, 2001). Furthermore, repeated administration of therapeutic Ad vectors in a single patient is not feasible due to the generation of high amounts of anti-Ad neutralizing antibodies (Chen et al., 2000; Mastrangeli et al., 1996; Yang et al., 1995).

Upon viral infection the antigenic epitopes on the viral capsid, namely fiber, penton and hexon proteins will evoke a humoral immune response leading to the production of antibodies (Bessis et al., 2004; Schagen et al., 2004). The antigenic epitopes of the different capsid components are either group-specific that is common to all human and non-human adenovirus except for the avian virus, or serotype-specific and unique for each serotype (Horwitz, 2001). Since crossreactions to Ads from different serotypes can occur, certain epitopes are shared within and between Ad from different subgroups (Horwitz, 2001; Norrby, 1969a; Norrby et al., 1969). All together, neutralization is not an all or nothing event, which suggests that there may be a range of responses from heterotypic to homotypic neutralization.

Two different and distinct mechanisms of neutralization of Ad have been suggested, extracellular and intracellular neutralization (Wohlfart, 1988; Wohlfart et al., 1985). Extracellular neutralization of Ad means that the virus is physically hindered from binding to the cell surface thereby preventing entry into the cell. Anti-fiber neutralizing antibodies are thought to be responsible for this type of neutralization. Anti-penton and anti-hexon neutralizing antibodies have been reported to neutralize Ad via an intracellular mechanism, trapping the virus in the endosome and preventing virus trafficking to the nucleus (Wohlfart, 1988; Wohlfart et al., 1985). The following sections will review in further detail how neutralization of Ad is achieved by the different capsid antibodies.

Neutralization by anti-fiber antibodies

The first insight into the neutralization capability of the anti-fiber neutralizing antibodies was gained from the early studies by Norrby (Norrby, 1969b). These showed that fibers of different lengths, representing different serotypes have different neutralizing activities. Serotypes carrying fibers of similar or shorter lengths than immunoglobulin IgG antibodies (15-20nm) are more easily neutralized than serotypes with longer fiber, which cannot be completely shielded by the antibody. Others showed that the neutralizing activity of the fiber depended on the part of the fiber to which the antibody was raised (Wadell, 1972). Antibodies raised against the entire fiber had less neutralizing activity compared to the antibodies raised against the fiber knob region, which would directly hinder the virus from interacting with CAR. Serogroups with longer fibers belonging to subgroup C can neutralize Ad via another mechanism, namely by aggregation and crosslinking of viruses to each other thus creating complexes that are too large to bind and internalize (Norrby, 1969a; Norrby, 1969b). That fiber antibodies act primarily to inhibit target cell binding was supported by the studies of Philipson that showed that only non-bound viruses were affected by anti-fiber antibodies (Philipson et al., 1968). Another study however, showed that not all, but about 15% of Ad was still internalized even in the presence

anti-fiber antibodies, and that these aggregated virions could be recovered in intracellular vesicles (Wohlfart et al., 1985). The aggregated virions were not able to replicate, either due to their aggregation status or the lack of conformational change in the viral capsid. The importance of anti-fiber in neutralizing Ad was supported by in an *in vivo* study of lung cancer patients receiving one dose of Ad vector (Gahery-Segard et al., 1998).

Neutralization by anti-hexon and anti-penton antibodies

The neutralization mechanism referred to as intracellular neutralization is the mechanism by which both hexon and penton-base antibodies function, with retention of virus in the endosomes as the end result. This retention of Ad is thought to be mediated via steric hindrance of the hexon and penton antibodies and thereby preventing the interaction with the endosomal membrane (Wohlfart, 1988; Wohlfart et al., 1985). This theory is supported by a study where anti-hexon and anti-penton treated virus bound to cells and was endocytosed to the same extent as untreated virus but was retained in the endosomes (Wohlfart, 1988; Wohlfart et al., 1985). However, the neutralization by anti-penton antibodies never exceeded 50% suggesting that neutralization attributed to anti-penton antibodies is important but not crucial for endosomal escape (Wohlfart, 1988). The importance of hexon anti-bodies was later verified *in vivo*, in large study of individuals from United States and sub-Saharan Africa (Sumida et al., 2005). Furthermore, studies using hexon-chimeric viruses have supported the contribution of hexon-specific immunity against Ad vectors *in vivo* (Gall et al., 1998; Roy et al., 1998; Youil et al., 2002). A study with sera from liver cancer patients verified that the neutralizing effect of penton antibodies was attributed to events occurring after binding of Ad to target cells (Hong et al., 2003). This study together with a previous one showed that the antibodies against the epitopes of the RGD domain of the penton base were not prevalent and had poor neutralizing activity as depletion of these antibodies did not significantly change the neutralizing activity (Stewart et al., 1997).

There has been an ongoing debate during the last decades regarding which of the capsid epitopes trigger the main neutralization response. Some *in vitro* studies and *in vivo* studies have indicated that it is mainly the hexon antibodies (Sumida et al., 2005; Toogood et al., 1992; Wohlfart, 1988) but some recent clinical data contradict this and show that the fiber and penton-base neutralizing antibodies are in excess and act synergistically (Gahery-Segard et al., 1998; Hong et al., 2003; Stallwood et al., 2000). It is hard to explain the differences in results from these studies but it is important to bear in mind that the early studies were performed with purified antibodies directed toward the major capsid proteins and not obtained from patients. As such they may not give an accurate depiction of how neutralizing antibodies

will neutralize a virus *in vivo* where the virus will encounter a heterologous mixture of antibodies. In addition, different studies have employed different techniques determining the neutralizing titer of antibodies making a comparison difficult.

Classification of neutralizing antibodies

Neutralizing antibodies are classified via various assays and the main differences between assays are input virus, cell-type and readout in neutralization. Viruses used in these assays have either been wild-type (WT) virus or replication-deficient Ad that will affect the choice of cell-type i.e. a cell-line that supports uptake and replication of Ad. It is however in the readout that the major differences among these assays arise. Classically the readout was performed microscopically by scoring the Ad-mediated cytopathic effect (CPE), the formation of plaques, or cell viability. The major disadvantage of these types of readouts is that they are dependent on the formation of a visible plaque or a CPE which is a complex and time-dependent event which usually takes four to eight days. It is also time-consuming, subjected to variations in protocol procedures and dependent on subjective scoring methods.

Based on these limitations there has been a need for new technologies to determine neutralizing antibodies and during recent years several new assays have been developed. Common to all of these new techniques is their improved time-efficiency because they use transgene expression as a readout, which can be assessed as early as 24 hrs post-infection. Several of these studies have shown to be robust, simple, sensitive and can be subjected to automation. These studies include the use of a recombinant Ad expressing different reporter expression cassettes including AdLaZ (Kuriyama et al., 1998) AdGFP (Stallwood et al., 2000), AdLuciferase (Sprangers et al., 2003) and secreted alkaline phosphatase (SEAP) (Aste-Amezaga et al., 2004). The major conclusion of these studies is that they all have stated the importance of standardization of parameters that affect the outcome of the neutralization, such as concentration of infectious and non-infectious viral particles, time of infection and the pre-incubation time of sera with the virus. Standardization of a neutralization assay will help in the interpretation and comparison of results obtained from different groups.

Strategies to circumvent immunity

Many strategies have been developed during recent years to overcome the hurdle of both the pre-existing immunity as well as the immunity acquired upon repetitive administration of Ad vectors. These strategies include modulation of the immune response elicited by the Ad vector and modification of the Ad vector itself (Schagen et al., 2004).

Immunosuppression or immunomodulation will reduce both the innate and the acquired immunity elicited by the Ad vectors. Immunosupression with anti-inflammatory reagents such as corticosteroids and cyclophosphamide were shown to prolong transgene expression in animal models (Dai et al., 1995; Kolb et al., 2001). Other possible approaches include depletion of macrophages and DC (Alemany et al., 2000; Worgall et al., 1997). Immunomodulation strategies also include the inhibition of the activities of the CD8+/CD4+ T-lymphocytes and co-stimulatory factors such as CD40 or CD40 ligands, by using antibodies raised against these lympho-cytes or factors respectively (Poller et al., 1996; Sawchuk et al., 1996; Yang et al., 1996). Both strategies will decrease the cellular and humoral immune response and transgene expression will be prolonged by the inhibition of T-cell activation and the CTL response against the Ad vector. The major disadvantage of both immunosuppression and immunomodulation is that the effect is non-specific, associated with side-effects and most likely not preferably to use in patients that already are immunoincompetent (Schagen et al., 2004).

Another strategy for avoiding immunity is based on Ad capsid modification since the capsid is a major immune stimulus. For example, increased duration of transgene expression can be achieved by attaching different polymers to the Ad capsid shielding them from CTLs and neutralizing antibodies (Chillon et al., 1998; Croyle et al., 2001). A different approach is to change the native tropism of the virus so that uptake of the Ad is CAR-independent (Wickham, 2000). Yet another approach has been the serotype switch, using vectors derived from different serotypes to accomplish sequential, *in vivo* administration of Ad. This enables readministration by circumventing the humoral immune defense and cross-reactivity with pre-existing neutralizing antibodies is avoided (Mastrangeli et al., 1996).

Numerous studies have shown that the viral backbone of the vector is responsible for the induction of cellular immune response, which has led to the development of a new, less immunogenic generation of Ad vectors including the second and third generation of vectors and finally the "gutless vectors" (Bangari and Mittal, 2006; Schagen et al., 2004). In the "gutless vectors" most of the Ad genome is deleted and these vectors therefore require a helper virus for propagation. These vectors have been shown to induce low immunity, low toxicity and transgene expression up to 10 months *in vivo* (Schiedner et al., 1998). Lately, an approach that has gained more popularity is the usage of non-human Ad vectors such as canine, chimpanzee and porcine Ad vectors. These vectors have the advantage that they do not crossreact with pre-existing neutralizing antibodies and are infecting a variety of human cells (Bangari and Mittal, 2006).

Adenovirus based vaccines

Whereas the avid immune response to Ad vectors represented a pitfall to the use of Ad to correct metabolic defects, a new and exciting field has emerged; the field of Ad based genetic vaccines. The logic for using Ad as a vaccine delivery vehicle takes advantage of the inflammatory response elicited by the virus to create a protective and potent immunity. Animal models show very promising results and vaccines tested include pathogens like human immunodeficiency virus (HIV), severe acute respiratory syndrome (SARS) corona virus, anthrax, pseudomonas, Ebola virus, Dengue virus and Herpes virus (Basak et al., 2004; Boyer et al., 2005; Tatsis and Ertl, 2004). However, the pre-existing immunity is a limiting factor just as in regular gene therapy as indicated by several *in vivo* studies in mice, rhesus monkeys and human clinical trial (phase I) (Basak et al., 2004; Boyer et al., 2005; Tatsis and Ertl, 2004).

In summary, the use of Ad vectors has gone through many stages from the very encouraging results in animal models in the beginning of the 1990's to the realization of dose-limiting toxicity and immune related hurdles later in that decade. From this history the promise of recombinant Ad based vaccines has developed. Looking forward, the extensive basic research focusing on the mechanisms behind Ad-mediated gene transfer will continue to aid in the pre-clinical and clinical development of Ad as a therapeutic.

COXSACKIE AND ADENOVIRUS RECEPTOR

CAR is not only a viral receptor but it is also a cell adhesion molecule localized to tight junctions in epithelial and endothelial cells. In this section, the structure, expression, localization, regulation and function of CAR will be discussed. Understanding the biology of CAR will be important in elucidating its role as an adhesion molecule in normal and pathological conditions including inflammation and cancer. This knowledge may then form the basis for a rational approach to the use of Ad vectors in pathophysiological processes where the efficacy of the vector, as discussed previously, correlates with successful CAR-dependent infection. As such a greater understanding of the biological role of CAR may be of value for elucidating the mechanisms of inflammatory and malignant disease, as well as developing Ad-mediated treatments.

Structure and genomic organization

CAR or CXADR (coxsackie adenovirus receptor) was first identified in 1997 by three different groups (Bergelson et al., 1997; Carson et al., 1997; Tomko et al., 1997). It was given its name because it was shown to mediate binding of two unrelated viruses, i.e. coxsackie and adenovirus to the plasma membrane. CAR is a cell surface glycoprotein, which belongs to the immunoglobulin superfamily (IgSF) of proteins. It consists of two IgG domains (IG1/IG2) that make up the extracellular domain, a single membrane-spanning domain, and a cytoplasmic domain (Bergelson et al., 1997; Tomko et al., 1997). Based on its structure, CAR was later classified as being a member of the Cortical Thymocyte Xenopus (CTX) subfamily of proteins. The hallmarks of the CTX subfamily was defined after the identification of the founding member the Cortical Thymocyte in Xenopus (CTX) protein (Chretien et al., 1998; DuPasquier and Chretien, 1996). Three structural and genomic features are shared by most members namely, the conserved exon-intron structure, the protein structure consisting of two Ig domains (preferentially one V and one C2 type), a transmembrane domain, an intracellular tail and the C2 domain which encompasses an extra disulfide bridge between the two cysteines. Additionally most CTX members have a PSD95/DLG/ZO-1 (PDZ) binding domain in their extreme C terminal part. The PDZ binding domain enables them to bind intracellular PDZ-domain containing partners, and to be posttranslationally modified such as glycosylated and phosphorylated. Today more than ten members have been identified, with CAR being one of them. Other members are CTX, junctional adhesion molecule (JAM)-A/B/C, JAM4, JAM-like (JAM-L), endothelial cell selective adhesion molecule (ESAM), CAR like membrane protein (CLMP), A33, A34 and brain and testis immunoglobulin superfamily (BT-IgSF) (DuPasquier and Chretien, 1996; Heath et al., 1997; Hirata et al., 2001; Mandell and Parkos, 2005; Raschperger et al., 2004; Scanlan et al., 2006; Suzu et al.,2002).

The gene encoding human CAR (hCAR) is localized on chromosome 21 and mouse CAR (mCAR) is on chromosome 16. The CAR gene is highly conserved between different species and has also been identified in rat, dog, cow, pig, frog and zebrafish (Andersson et al., 2000; Bergelson et al., 1997; Bowles et al., 1999; Chen et al., 2003; Fechner et al., 1999; Thoelen et al., 2001a; Tomko et al., 1997). The CAR protein is encoded from eight exons. Exon one to five encodes the signal peptide (19 aa) and part of the extracellular domain (216aa). The remainder of the extracellular domain and the transmembrane domain (23 aa) are encoded by exon six. The last exons encode the intracellular tail of CAR, which may have different lengths depending on alternative splicing (CAR-1 (TVV isoform); 92 aa and CAR-2 (SIV isoform); 107 aa) (Chen et al., 2003; Thoelen et al., 2001b). Both isoforms of CAR are found in mice and humans (Andersson et al., 2000). In the mouse, an additional heart mRNA specific splice variant, CAR-3, has been identified (Andersson et al., 2000). Three other CAR mRNA splice variants have been documented that lack the transmembrane domain and are likely to represent soluble forms of CAR. These splice variants have never been identified as proteins *in vivo* (Chen et al., 2003; Dorner et al., 2004).

The mature CAR protein has a molecular weight of 46 kDa, due to posttranslational modifications including phosphorylation, N-glycosylation and acetylation (Beausoleil et al., 2004; Honda et al., 2000). Proper palmitylation of the two S-acetylation motifs in the intracellular domain has been shown to be important for correct targeting of the protein to the basolateral side of the plasma membrane (Cohen et al., 2001a; van't Hof and Crystal, 2002).

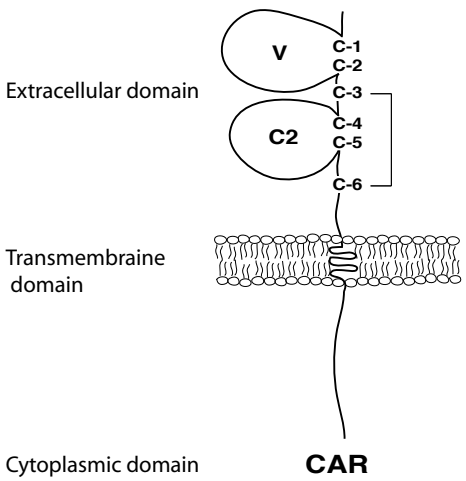

Fig 5. CAR protein. Schematic illustration of the CAR protein. CAR is composed of an extracellular domain with two immunoglobulin-like domains, one transmembrane domain and a cytoplasmic tail. Modified from Raschperger 2006.

Expression and localization

The expression pattern of CAR has been extensively studied in humans and rodents. The first studies of both human and mouse CAR evaluated the mRNA expression pattern due to the lack of available antibodies. These studies showed CAR to be expressed in human kidney, pancreas, brain, heart lung, liver, small intestine, colon and prostate. In contrast, the placenta, ovary, thymus, peripheral blood lymphocytes and spleen were all CAR negative (Bergelson et al., 1997; Fechner et al., 1999; Tomko et al., 1997). The expression pattern in the adult mouse is almost identical to human with a few exceptions (Tomko et al., 1997). CAR expression is not limited to epithelial cells, but is also expressed in skeletal muscle and mature sperm (Mirza et al., 2006; Nalbantoglu et al., 1999).

It was not clear from the early reports where CAR was localized but gene therapy studies indicated that the protein must have a restricted expression pattern, since Ad vectors were unable to infect a polarized epithelium (Grubb et al., 1994; Knowles et al., 1995). Two studies demonstrated that CAR was localized to the basolateral side of the plasma membrane (Pickles et al., 2000; Walters et al., 1999), but it was not until 2001, that the protein was shown to be localized to the tight junctions (Cohen et al., 2001b). In these initial *in vitro* studies it was shown that CAR colocalized and interacted via its PDZ-domain binding motif with the classical tight junctional protein Zona occludens (ZO-1). This localization to tight junctions was later confirmed *in vivo* (Nagai et al., 2003; Raschperger et al., 2006). Recently, cytosolic proteins other than ZO-1 have also been shown to interact with CAR, with or without dependence on interaction with the CAR PDZ-domain binding motif, including, membrane–associated guanlyate kinase 1β (MAG1-1β), β-catenin, post-synaptic density 95 (PSD-95), multi-PDZ domain protein-1 (MUPP-1) as well as ligand-of-numb-protein X (LNX) and LNX-2 (Excoffon et al., 2004; Mirza et al., 2005; Sollerbrant et al., 2003; Walters et al., 2001).

Regulation

To date very little is known about the regulation of CAR at the transcriptional, translational or posttranslational level. However, the available literature indicates multiple levels of regulation during development, inflammation and cancer. These studies mainly investigated expression levels *in vivo* during different stages of development, regeneration, inflammation or cancer progression; and they have been supported by several *in vitro* studies.

That CAR is highly regulated during development has been verified by various expression studies in rodents. Of particular interest is the rapid decrease in expression level of CAR in the brain and in the cardiac and skeletal muscle upon birth (Fechner et al., 2003; Honda et al., 2000; Hotta et al., 2003; Kashimura et al., 2004; Nalbantoglu

et al., 1999; Raschperger et al., 2006; Tomko et al., 1997). The exact mechanism through which this regulation may occur is currently unknown. CAR expression is induced in models mimicking normal and pathological regeneration processes of cardiac and skeletal muscle. Possible mechanisms for this induction were postulated to be mediated via cell-cell contact (Fechner et al., 2003) and by inflammatory mechanisms, potentially induced by soluble factors released by the surrounding milieu in these disease models (Ito et al., 2000; Kawashima et al., 2003).

Due to use of Ad as a vector in many cancer protocols the expression levels of CAR have been extensively studied in many different types of cancer. In the majority of cancers, including prostate, bladder, head and neck, and glioma tissues or cell cultures derived from these cancers, an inverse relationship between the expression levels of CAR and tumor progression has been shown - i.e. low expression of CAR correlated with high tumorigenicity (Fuxe et al., 2003; Jee et al., 2002; Li et al., 1999; Miller et al., 1998; Okegawa et al., 2000; Okegawa et al., 2001; Sachs et al., 2002). In many cancers the Raf/MEK/ERK signaling pathway is activated and by using small molecule inhibitors the participation of this signaling pathway in the regulation of CAR expression was investigated. In low CAR expressing epithelial cancer cell lines, CAR expression was induced by MEK inhibitors (Anders et al., 2003a; Anders et al., 2003b). In contrast, upon treatment with a PI3K inhibitor CAR expression was reduced. In light of these findings, loss of CAR expression has been proposed to be an indicator of cancer progression and may represent a possible diagnostic marker. However, recently published data suggests this conclusion might represent an oversimplified view, since an increase in CAR expression levels in cancer cell lines and tissues have been documented (Anders et al., 2003b; Bruning et al., 2005; Korn et al., 2006; Martin et al., 2005; Qin et al., 2004). The *in vivo* data combined with the *in vitro* data indicate complex regulatory mechanisms of CAR expression during cancer progression.

Several *in vitro* studies have been performed with the objective of elucidating the mechanisms behind the regulation of CAR. These studies have mainly involved treatment of cultured cells with different stimuli and subsequent analysis of the expression level of CAR and the susceptibility to Ad infection. Two reports as well as unpublished observations from our lab have shown that cell density affects CAR expression levels (Carson et al., 1999; Fechner et al., 2003). As confluence of HUVEC cells increased, CAR expression was induced (Carson et al., 1999). In contrast, CAR levels decreased as cultured cardiomyocytes approached confluence (Fechner et al., 2003). Others have investigated the potential role of inflammatory cytokines in regulating CAR expression. Bruning and Runnebaum 2003 have shown, for example, that TNFα can either suppress or induce CAR depending on cell type. In contrast, tumor growth factor β (TGFβ) only had inhibitory effects on CAR expression in-

dependently of cell-type (Bruning and Runnebaum, 2003). The TGFβ response was investigated in more detail in an article by Lacher 2006, where CAR was shown to be downregulated during TGFβ-mediated epithelial-mesenchymal transition (EMT) (Lacher et al., 2006). The same study showed that the downregulation of CAR could be abrogated by addition of a TGFβ-receptor inhibitor. A screen for CAR modulating agents identified several factors inducing CAR both *in vitro* and *in vivo* (Hakkarainen et al., 2003; Hemminki et al., 2003). Many of these compounds were agents that are involved in epigenetic gene regulation. Several other reports corroborated the importance of epigenetic regulation of CAR and particularly histone acetylation (Goldsmith et al., 2003; Kitazono et al., 2001; Kitazono et al., 2002; Okegawa et al., 2005; Pong et al., 2003; Sachs et al., 2004; Taura et al., 2004).

One method to gain insight in CAR gene regulation is by analyzing its promoter, which has recently been cloned and partly characterized (Pong et al., 2003). The human CAR promoter contains no classical TATA box and the transcription initiation site was identified to be located around 150 bp upstream of the ATG. By using a promoter search program, the predicted promoter sequence was determined to be between -470 and -791. Based on these findings the authors made truncated versions of the upstream region of CAR and were able to map a core promoter region of 186 bp located between -400 and -585 bp upstream of the translation initiation site. The activity of this core promoter correlated with the endogenous expression of CAR in all cell lines examined. Furthermore they also showed that the CAR promoter was positively regulated through histone acetylation. This was verified by inhibiting de-acetylation and by chromatin immunoprecipitation (ChIP) showing that expression of CAR was associated with binding of acetylated histone H4. Binding of acetylated histone H4 is linked to an open chromatin structure and was correlated with high CAR expression. In addition, some predicted transcription factor binding sites were identified upstream of the promoter region including E2F and Sp1.

Biological function as an adhesion protein

A rapidly increasing number of reports describing CAR expression levels and patterns, localization, regulation and effects of gene deletions, have contributed to elucidating the biological function of CAR. From the comprehensive expression studies of CAR, both *in vitro* and *in vivo*, the proposed role for CAR as an adhesion molecule is generally accepted (Philipson and Pettersson, 2004). In this context, CAR may exert many *in vivo* cellular functions, such as barrier maintenance or formation, tissue stability and homeostasis, as well as anchoring properties to other cells and to the cytoskeleton inside the cell. However, localization of CAR to the tight junctions, which is a major signaling platform, indicates that CAR may possess signaling properties.

CAR's role in barrier function has been suggested by the observations that overexpression of CAR in epithelial cells leads to an increase in the transepithelial electrical resistance (TER), an effect that is reversed with addition of CAR specific antibodies or soluble CAR protein (Cohen et al., 2001b). Moreover, the TER in primary epithelial cells correlates well with CAR expression levels (Cohen et al., 2001b; Walters et al., 2002). This is supported by a recent report from our lab, demonstrating that in the permeable part of the renal tube no CAR was expressed, whereas the more impermeable regions, such as the collecting ducts and distal tubules, expressed very high levels of CAR (Raschperger et al., 2006). This restricted expression pattern of CAR, which coincides with permeability of the renal tubules supports the involvement of CAR in barrier maintenance. Barrier function is mediated either through homophilic binding between CAR molecules or heterophilic interaction to other CTX members such as JAM-C or JAM-L on adjacent cells. Both the V and C domain have been implicated to be important in these interactions (Mirza et al., 2005; Zen et al., 2005). Stabilization, flexibility and formation of the epithelial barrier are dependent on a link between the junctional and the cytosolic proteins. CAR provides such a link by binding cytosolic protein as was addressed previously (Excoffon et al., 2004; Mirza et al., 2005; Sollerbrant et al., 2003; Walters et al., 2001). CAR is also responsible for recruitment of proteins including ZO-1, MAGI-1β, PSD-95, MUPP-1 and protein interacting with protein C kinase (PICK1) to newly formed cell-cell contacts (Cohen et al., 2001b; Coyne et al., 2004; Excoffon et al., 2004).

The ability of CAR to mediate cell-adhesion together with the *in vivo* data indicating suppressed levels of CAR in tumors suggests that CAR may possess a tumor suppressor function in a similar fashion as the adherens junction protein, E-cadherin. Some *in vivo* and *in vitro* studies have been performed where this hypothesis has been addressed. The main findings suggest that overexpression of CAR resulted in reduced cell migration, increased cell aggregation (Bruning and Runnebaum, 2004) and growth inhibition (Okegawa et al., 2000; Okegawa et al., 2001). Moreover, it was demonstrated that the transmembrane and intracellular domains of the protein were responsible for mediating this effect (Excoffon et al., 2005; Kim et al., 2003; Okegawa et al., 2000). A very recent report has shown a physical interaction between the cytoplasmic domain of CAR and the microtubules via tubulin. Based on this observation, the authors proposed that CAR may affect migration directly by modulating the dynamics of microtubules (Fok et al., 2007).

As previously discussed, CAR is highly regulated during embryogenesis, although the mechanisms underlying this remains to be elucidated. The importance of CAR expression in heart development has clearly been demonstrated by three different knock-out studies of CAR (Asher et al., 2005; Chen et al., 2006; Chretien et al., 1998;

Dorner et al., 2005). The three studies all showed similar phenotypes as indicated by embryonic death due to malformation of the heart. The phenotype and interpretation of the results however, varied somewhat among the studies. No other gross abnormalities could be detected suggesting that the dramatic phenotype is due to the heart-specific phenotype and that CAR must display a heart-specific function. The exact mechanism giving rise to this phenotype needs to be investigated further preferentially by additional conditional knock-out studies supplemented with *in vitro* studies. It is interesting to note that only CAR out of all the CTX family members has a lethal phenotype.

In conclusion, due to its specific localization at tight junctions, importance in embryogenesis and migration we are only in the beginning of understanding the biological function of CAR in normal and pathoglogical processes.

LOSS OF CELL ADHESION DURING PATHOLOGICAL PROCESSES

Inflammation

The main function of inflammation is to eliminate and remove pathogens or noxious agents from the body and subsequently initiate repair of any damage that has been caused. This multi-step process involves the innate and the adaptive immune system. The innate system is the first line of defense and is pathogen-independent. Later, the immune system mounts an adaptive, pathogen-specific response that includes the production of antibodies and an immunological memory. These two arms of the immune system are intimately linked through the phagocytic cells (macrophages, dendritic cells and neutrophils) that become activated during the innate phase. The phagocytic cells express certain cell surface receptors known as Fc receptors that enable engulfment of pathogens by phagocytosis, the first step in antigen presentation. During antigen presentation, B-cells become activated and start producing antibodies specific for the infectious pathogen. In addition, the phagocytic cells will also produce and secrete a broad spectrum of cytokines reinforcing the inflammatory response. These cytokines will induce migration of different immune cells, including the APC, out of the circulation and allow them to extravasate to the site of injury. This is a multi-step process and involves physical interaction between the immune cell and the endothelium and subsequent disassembly of the endothelial cellular junctions to assist cell migration into the connective tissue. In this section the Fc receptors will be discussed because of their importance in engulfing antibody-coated pathogens such as viruses, as well as transmigration of immune cells to the site of injury. In addition, two major cytokines, tumor necrosis factor alpha and interferon gamma, involved in controlling both immune processes will be described.

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The Fc Receptors and clearance of pathogens

The cellular receptors for the Fc domain of immunogloubulin (Ig) are expressed on the surface of all cells of the immune system. These receptors are ultimately responsible for bridging the innate and the adaptive immune response. Each subclass of immunogloubulins has their specific Fc receptor (FcR) including IgA (FcαR), IgD (FcδR), IgE (FcεR), IgM (FcμR) and IgG (FcγR) (Gessner et al., 1998). In this section FcγR will be described in more detail.

FcγRs are expressed on the surface on most cells of the immune system including B-lymphocytes, dendritic cells, macrophages/monocytes, NK cells, neutrophils and mastcells. However some FcγRs are also expressed on endothelial cells, Langerhans cells, platlets, eosinophils and melanoma cells (Cohen-Solal et al., 2004). To date three different classes in humans have been identified: FcγRI (CD64), FcγRII (CD32)

and FcγRIII (CD16). The FcγRI displays high affinity for monomeric IgG whereas FcγRII and FcγRIII have a lower affinity but a broader isotype binding pattern (Nimmerjahn and Ravetch, 2006).

Based on the broad and important functions FcγR plays in the immune response, a tight regulation of these receptors is needed. This specificity is achieved through the existence of two classes of FcγR, the activating and the inhibitory receptors, differing in their cytoplasmic tail and/or their associated chains. These domains are referred to as the Immunoreceptor Tyrosine-based Activation Motif (ITAM) and Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM), respectively. FcγRI, FcγRIIA, FcγRIIC and FcγRIIIA are all activating receptors while FcγRIIB is an inhibitory receptor. Most cells express both classes of receptors resulting in a balanced immune response, with the exception of NK cells and B-cells which only express FcγRIIIA and FcγRIIB (Cohen-Solal et al., 2004; Ravetch and Bolland, 2001).

The main function of the inhibitory FcγR is to take up IgG-coated particles and transport them to endosomes where the particles are degraded and coupled to major histocompatibility complex (MHC) II. The MHC II will present the foreign antigen at the plasma membrane to the immune system and the humoral immunity will be triggered and production of antibodies initiated. This is in contrast to the activating FcγR, which are involved in phagocytosis of large particles both during the innate and adaptive immune response resulting in clearing the pathogen from the tissue. In addition they are responsible for inducing a cytotoxic T-lymphocyte (CTL) response as well as inducing the transcription of cytokine genes and subsequent release of inflammatory mediators (Alberts, 2002; Nimmerjahn and Ravetch, 2006; Ravetch and Bolland, 2001). The outcome of an immune response will therefore be influenced by the relative expression of the inhibitory and activating FcγRs, a property that is determined, in part, by the microenvironment. For example, the tumor necrosis factor alpha (TNFα), and interferon gamma (INFγ) also known as Th1 cytokines, will increase the expression of the activating receptors and subsequently induce cell-mediated immunity. In contrast, the IL-4 and IL-10 or the Th2 cytokines, will upregulate the inhibitory receptors, and propel the immune response in the direction of humoral immunity (Cohen-Solal et al., 2004; Nimmerjahn and Ravetch, 2006).

In this thesis, the focus will be on the activating, low affinity FcγRs, especially FcγRIIA receptors because they are responsible for clearing immune complexes from tissue and serum and for enhancing the immune response to foreign antigens contained in antibody-antigen complexes. This function of FcγR has been verified by several knock-out analyses (Ravetch and Bolland, 2001) transgenetic studies (McKenzie et al., 1999) and also by expressing FcγRIIA on non-phagocytic cells rendering them capable of engulfing and clearing immune complexes (Bezdicek et

al., 1999). FcR also take up virus coupled to immunoglobulins. This mechanism of uptake is in the literature referred to as antibody-dependent enhancement (ADE) of viral infection (Takada and Kawaoka, 2003). ADE was first described in the 1960s but was not generally accepted until studies with Dengue virus were performed (Halstead and O'Rourke, 1977). The most common mechanism for ADE is FcR dependent and was first shown for West-Nile virus by using monoclonal antibodies against FcγRII which resulted in decreased infection (Peiris et al., 1981). Another type of ADE mechanism described, is the complement receptor dependent uptake (Cardosa et al., 1983). This was verified by addition of monoclonal antibody raised against complement receptor 3 (CR3), which in a similar manner to FcRII antibodies, resulted in decreased infection of West-Nile virus. Several viruses display ADE including ebola, HIV, herpes, coxsackie, influenza as well as adenovirus (Mercier et al., 2004; Takada and Kawaoka, 2003). The importance of ADE and to what extent it occurs *in vivo* varies between viruses and will require additional studies (Takada and Kawaoka, 2003).

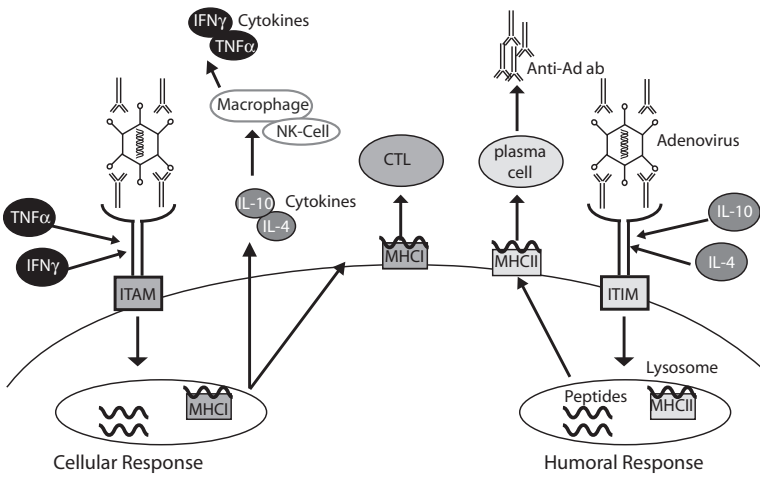

Fig 6. Fcγ receptors. Schematic model showing the two classes of Fcγ*R*, the activating (ITAM) and inhibitory (ITIM) Fcγ*R*. Upon binding of an IgG-coated pathogen, such as Ad, to the activating Fcγ*R*, a cellular immune response is triggered resulting in the production of CTLs and secretion of cytokines. A humoral response is induced and neutralizing antibodies are produced if the IgG-coated pathogen binds to an inhibitory Fcγ*R*.

In summary, presence of the FcR on immune cells provides the organism with an instant clearing mechanism of pathogens in addition to creating a memory for the pathogen in order to be better prepared for future challenges. It also provides an alternate method of entry for some viruses to select target cells.

Transmigration and disassembly of endothelial cellular junctions

Leukocytes are transported through the body as part of fluid movement within the circulatory system. In order to respond to injury or inflammatory stimuli, leukocytes must halt their movement within the circulatory system, a process that is initiated by cell-cell contact between leukocytes and endothelial cells that have been activated by the inflammatory stimulus. The interaction slows the flow of the leukocyte and gradually leads to a "rolling" phenotype of the lymphocyte along the activated endothelium. The initial contact of the leukocyte with the endothelium is initiated by interaction with the selectin family of proteins which is induced in response to injury (Carlos and Harlan, 1994).

The initial interaction or tethering of the leukocyte to the endothelium is mediated via three different selectins, L(leukocyte)-selectin on the leukocyte and P(platlet)- or E(endothelial)-selectin on the endothelium (Patel et al., 2002; Sperandio, 2006). The selectins are transmembrane glycoproteins that bind sialylated carbohydrate moieties in a calcium-dependent manner. L-selectin is constitutively expressed at the surface of circulating leukocytes, in contrast to E- and P-selectin that are induced by inflammatory mediators. It is believed that P-selectin mediates the early leukocyte recruitment and tethering, since the protein is stored in intra cellular organelles, or so called Wiebel-Palade bodies in endothelial cells and α-granules in platelets, and can be expressed at the cell surface within minutes. This rapid response contrasts with E-selectin, that needs to be synthesized and expression is usually induced within 4 hrs and declines after 24hrs (Patel et al., 2002). E-selectin together with the chemotactic stimuli generated from surrounding tissue cells, leukocytes and endothelium, aid in the next step of leukocyte recruitment. The chemokines activate integrins and induce the expression of endothelial cell surface adhesion molecules and mediates the transition from the initial slow rolling to firm adhesion and leukocyte arrest. This arrest is mediated via leukocyte integrins interacting with the endothelial cell adhesion molecules intracellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) (Johnston and Butcher, 2002; Sperandio, 2006). Once firm adhesion is established, diapedesis, or transmigration, of the leukocytes to the site of injury can be initiated. The leukocyte will now cross the borders of two endothelial cells by acquiring an amoeboid shape, and squeezing through the barrier with a minimal disruption of the vascular lining (Carlos and Harlan, 1994).

Some studies suggest that leukocytes preferentially target junctions where three endothelial cells meet (tricellular junctions) since the tight junctions at this location are discontinuous (Burns et al., 1997). Regardless of the exact position of diapedesis, it is a rapid process that leukocytes *in vitro* complete in less than 90 seconds (Muller, 2003). Diapedesis mainly involves homophilc interactions between the leukocyte and the endothelium and requires only transient disassembly of cellular

junctions to allow transmigration of the leukocyte.

Six different molecules have been implicated in the process of transmigration including platelet endothelial cell adhesion molecule-1 (PECAM), CD99, VE-(vascular endothelial)-cadherin and JAM-A-B-C (Cook-Mills and Deem, 2005; Muller, 2003). They are all located at the lateral borders of endothelial cells and appear to be involved at distinct steps of transmigration. PECAM and the different members of the JAM family all belong to the Ig gene superfamily, VE-cadherin is a member of the cadherin family and CD99 is a transmembrane glycoprotein (Muller, 2003; Vestweber, 2002).

PECAM is expressed on the surface of leukocytes and concentrated at the borders of endothelial cells. Homophilic interaction between the Ig domains 1 and 2 of the PECAM molecule, on the leukocyte and endothelial cell respectively, are required for efficient transmigration. If this interaction is blocked, the leukocyte cannot extend pseudopods into the intracellular junction and will remain at the apical surface of the endothelial cell (Liao et al., 1995; Muller et al., 1993). However, studies in PECAM null mice have shown that PECAM is not the only molecule required to recruit leukocytes to the site of inflammation (Duncan et al., 1999). In addition, PECAM is also thought to aid diapedesis via heterophilic interaction with the extracellular matrix (ECM) and integrins (Liao et al., 1995; Thompson et al., 2001).

Like PECAM, CD99, a small glycoprotein, is expressed on the surface of most leukocytes and concentrated at endothelial cell borders. CD99 has been shown to act at a later step in diapedesis compared to PECAM. CD99-null cells have been observed to be arrested during transmigration with the leading edge of the leukocyte located below the endothelial cell while the majority of the cell body is retained in the cellular junctions (Schenkel et al., 2002).

VE-cadherin is concentrated at the adherens junctions of the endothelium and is connected to the actin cytoskeleton via molecules of the catenin family. It is part of cadherin superfamily of transmembrane proteins that mediate Ca^{2+}-dependent, homophilic cell-cell interactions and is a type II cadherin. The importance of VE-cadherin in diapedesis was shown by blocking the homophilic interaction between the endothelial cells with VE-cadherin specific antibodies. Interfering with the barrier function of VE-cadherin led to an increased rate of leukocyte infiltration at the site of injury (Gotsch et al., 1997). Whether the disappearance of VE-cadherin is the cause or effect of the transmigration of neutrophils remains unclear.

The final group of proteins involved in diapedisis is the members of the JAM family. They bind directly to the leukocytes and facilitates transmigration. The JAM A has been identified to bind to integrin $\alpha L\beta 2$ (LFA-1), JAM-B to $\alpha 4\beta 1$ (VLA-4) and JAM-C to Mb2 (Mac-1) through heterophilic interactions (Mandell and Parkos, 2005; Maschler et al., 2005; Muller, 2003).

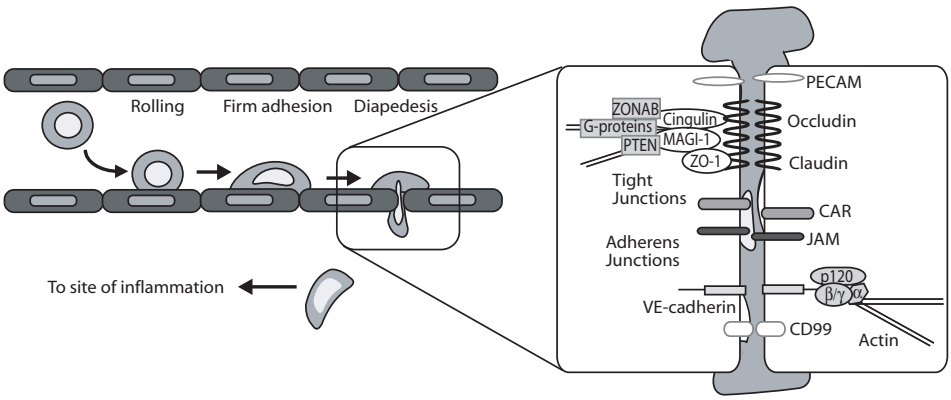

Fig7. Leukocyte recruitment. Schematic model of the leukocyte recruitment to site of inflammation. Leukocyte recruitment starts with activation of the endothelium, which initiates the rolling of leukocytes along the epithelium. Subsequently, leukocytes adhere firmly and eventually transmigrate between the endothelial cells (diapedesis). Diapedesis requires a transient disassembly of endothelial intercellular junctions and involves several adhesion components. Modified from Dejana 2004 and Carlos 1994.

The mechanism by which the induction of transmigration occurs is complex and involves the migrating leukocyte, the endothelium, and inflammatory mediators released in the microenvironment (Hordijk, 2006; van Buul and Hordijk, 2004). Binding of the leukocyte to the endothelium induces several signaling cascades that lead to a reorganization of the cytoskeleton and increased permeability. In addition, leukocytes may release proteases that break down junctions by cleaving the extracellular domains of junctional proteins such as VE-cadherin (Dejana, 2004). Endothelial cells respond to inflammatory stimuli by disassembling the cellular junctions resulting in increased permeability. The disassembly of junctions in the presence of inflammatory mediators has mainly been studied *in vitro* using human umbilical vein endothelial cells (HUVEC). Culturing HUVEC or other types of endothelial cells in the presence of inflammatory cytokines TNFα and IFNγ causes redistribution and/or down-regulation of junctional proteins including PECAM, VE-cadherin and JAM A (Hofmann et al., 2002; Ozaki et al., 1999; Rival et al., 1996; Romer et al., 1995; Shaw et al., 2001; Stewart et al., 1996; Wojciak-Stothard et al., 1998; Wong et al., 1999). Both translational as well as transcriptional mechanisms contribute to the downregulation of these cell adhesion proteins. The acute response to inflammatory mediators usually occurs within hours and involves predominantly post-translational modifications and the long-term response observed after 24-48 hrs also involves transcriptional mechanisms. Tyrosine phosphorylation is one mechanism

for downregulating VE-cadherin as well as PECAM and is a posttranslational mechanism associated with loss of cell-adhesion after short time exposure of cytokines (Nwariaku et al., 2002). Other studies have indicated that the downregulation of VE-cadherin is transcriptional after longer exposure time to cytokines (Hofmann et al., 2002). Contradicting results are observed for PECAM with one group reporting only redistribution of PECAM (Romer et al., 1995) and two others reporting transcriptional suppression (Rival et al., 1996; Shaw et al., 2001). The same contradicting results was also observed for JAM A by two different groups (Ozaki et al., 1999; Shaw et al., 2001). The explanation for these differences is not clear but could be due to differences in experimental procedures.

Several different cytokines have been implicated in the process of transmigration and disassembly of cellular junctions, two of them will be described in more detail, namely tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ).

Interferon gamma

The interferons (IFNs) are classified as type I or type II based on their sequence homology and receptor specificity. To date, several IFNs belong to the type I family including IFN-α, β, ε, κ and ω, but only one IFN belongs to the type II family, IFNγ (Schroder et al., 2004). Here, only IFNγ will be described in more detail.

IFNγ is structurally unrelated to the type I IFNs, binds to a different receptor and is encoded at a separate chromosomal locus but was classified as an interferon because of its capability of interfering with viral infection (Ikeda et al., 2002). IFNγ is produced by T-cells, NK cells, B-cells and APCs (DC and macrophages). IFNγ produced by NK cells and APC is thought to be important for the innate immune response as a first line of defense, whereas IFNγ secreted by T-cells is important for the adaptive response (Schroder et al., 2004). The production of IFNγ is positively regulated by other cytokines secreted by APCs particularly IL-12 and IL-8. Inhibition of IFNγ production is mediated by IL-4, IL-10 and TGFβ (Schroder et al., 2004). In addition to its crucial role in immunity, IFNγ has also been suggested to prevent tumor formation but the mechanism is still not clear (Ikeda et al., 2002).

IFNγ signaling is initiated after the binding of IFNγ to the type II IFNγ receptor, which consists of two subunits, IFNGR1 and IFNGR2 (Pestka et al., 1997). This binding will cause a rearrangement and dimerization of the receptor subunits. IFNGR1 will interact with janus activated kinase 1 (JAK1) and IFNGR2 with JAK2 and resulting in autophosphorylation and activation of the JAKs. The activated JAKs will in turn phosphorylate signal transducers and activators of transcription 1 (STAT1) causing STAT1 to dimerize, and rapidly translocate to the nucleus, bind DNA, and regulate gene expression. The STAT1 homodimers will bind specific elements also known as IFNγ activating sites (GAS) present in certain gene promoters. STATs are

also known to interact with other co-activators such as p300 and CREB-binding protein (CBP) to regulate gene expression (Zhang et al., 1996). In addition to the JAK-STAT pathway IFNγ also stimulates other signaling path-ways including PI3K, and protein kinase C (PKC). In this way, IFNγ can induce several different effects on target cells and tissues (Platanias, 2003; Schroder et al., 2004).

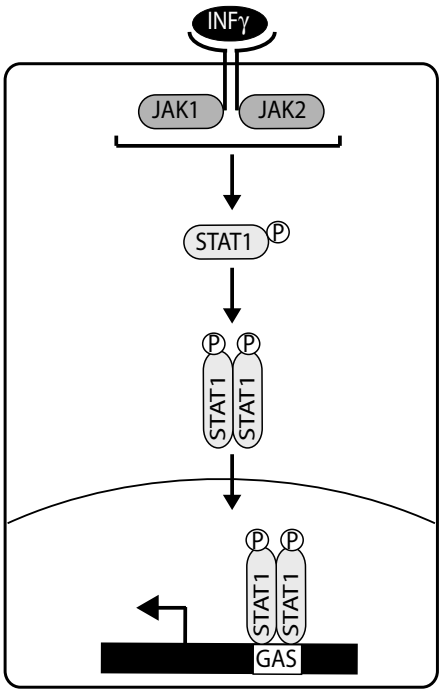

Fig8. IFNγ signaling pathway. IFNγ binds to IFNγ receptors, IFNGR1 and IFNGR2 which activates janus activated kinase 1 (JAK1) and JAK2. JAKs will phosphorylate signal transducers and activators of transcription (STAT1) causing STAT1 to dimerize and translocate to the nucleus and bind to DNA via specific IFNγ activating sites (GAS). Modified Platanias 2005.

Tumor necrosis factor alpha

Tumor necrosis factor alpha, (TNFα), belongs to the large TNF superfamily which includes at least 19 different ligands such as lymphotoxin α, CD40 ligand (CD40L), Fas Ligand (FasL) and TRAIL (TNF related apoptosis inducing ligand) (Aggarwal, 2003). TNFα induces a wide spectrum of biological responses such as lymphocyte and leukocyte activation and migration, fever, acute phase response, cell proliferation, differentiation and apoptosis. From this point of view, it is interesting that the other ligands of the family such as CD40L exert a very narrow biological response upon activation. This might be explained, at least partly, by the fact that the other members of the TNFα superfamily ligands do not activate as many signaling pathways and

have a more restricted expression pattern (Baud and Karin, 2001). TNFα is mainly produced by macrophages, monocytes and to a lesser extent B-cells, T-cells, NK-cells, Kupffer and glial cells. The most potent and best characterized inducer of TNFα is bacterial endotoxin (LPS), but IFNγ and bradykinin also increase TNFα, while glucocorticoids and cyclosporine A decrease its production (Tracey and Cerami, 1993). TNFα was identified and given the name tumor necrotizing factor in the 1960s after a study showing that this serum factor could cause tumor regression after LPS injection. About a decade later it was renamed tumor necrosis factor (Aggarwal, 2003).

TNFα binds as a trimer to two distinct receptors, tumor necrosis factor receptor 1 (TNFR1) and TNFR2, and this binding induces receptor trimerization and subsequently recruitment of different signaling proteins (Baud and Karin, 2001). After ligand binding to the TNFR1, inhibitory proteins of the silencer of death domains (SODD) are released leaving the intracellular domain of TNFR1 unoccupied. TNFR1 associated death domain protein (TRADD) is recruited to this domain forming a platform to which three additional proteins will be recruited including receptor interacting protein 1 (RIP1), fas-associated death domain protein (FADD) and TNF-receptor-associated factor 2 (TRAF2). Occupancy of the second receptor TNFR2 will recruit TRAF2 directly, which in turn will recruit TRAF1. These signaling proteins will induce different signaling pathways. TRAF2 is a very potent activator of MAPK pathway, a pathway that operates through JNK and p38. In contrast, RIP protein has been shown to be crucial in activating the nuclear factor kappa beta (NFκβ) pathway. These signaling pathways will ultimately induce the transcription factor, activator protein 1 (AP-1), and NFκβ-mediated gene responses, respectively (Baud and Karin, 2001).

NFκβ is a transcription factor that is kept inactive in the cytoplasm via binding to an inhibitory complex called inhibitor of kappa β (IKβ). TNFα induces activation of NFκβ by a multi-step process that initially involves activation of inhibitor of kappa β kinase (Iκκ) complex. This complex phosphorylates Iκβ and subsequently degrades IKβ resulting in a release of NFκβ. NFκβ then translocates to the nucleus, binds DNA and induces or represses gene expression (Baud and Karin, 2001).

Another process induced by TNFα is apoptosis, and this is mainly mediated through the FADD domain. TRADD, in complex with FADD, recruits caspase 8 to TNFR1 where it is activated, presumably by self-cleavage. Active caspase 8 eventually cleaves additional caspases (effector caspases) and induces apoptosis. The NFκβ signaling can inhibit apoptosis through induction of cellular inhibitors of apoptosis (cIAPs). This shows that the different signaling pathways merge and determine the cellular response to TNFα, providing the cytokine with apoptotic as well as anti-apoptotic activities (Baud and Karin, 2001).

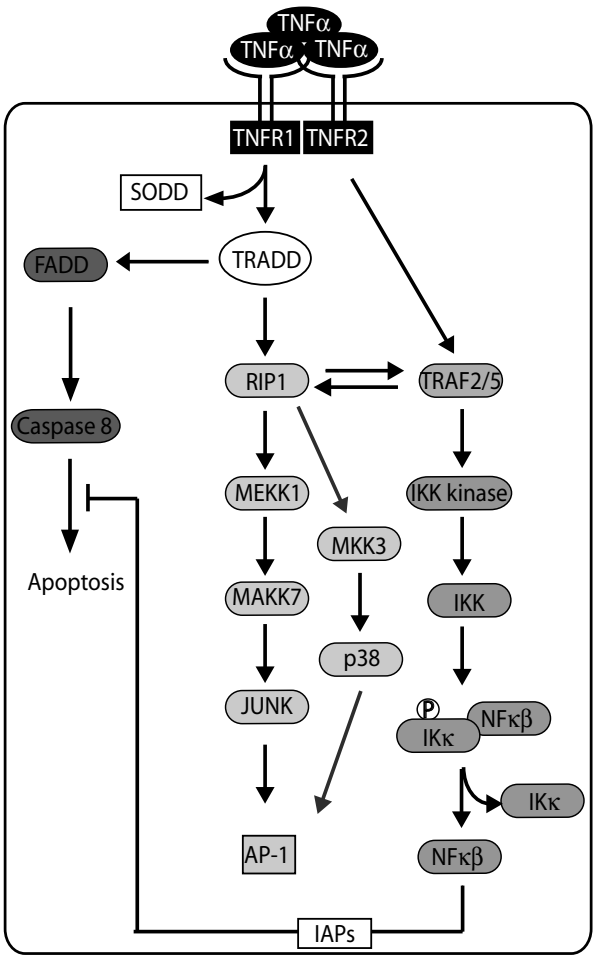

Fig 9. TNFα signaling pathway. TNFα binds as trimer to the tumor necrosis factor receptor 1 (TNFR1) and TNFR2. This results in release of silencer of death domains (SODD) and recruitment of TNF-receptor-associated factor 2/5 (TRAF2/5) and receptor interacting protein 1 (RIP1) through the adaptor protein TNFR1 associated death domain protein (TRADD). TRAF2/5, RIP1 induces the activation of transcription factors AP-1 and nuclear factor kappa beta (NF-κβ) through activation of JNK and inhibitor of kappa β kinase (IKK) respectively. Fas-associated death domain protein (FADD) recruits and activates caspases 8 which induces apoptosis. Apoptosis is inhibited by some of the NF-κβ target genes encoding the cellular inhibitors of apoptosis (cIAPs). Modified from Baud 2001.

Cancer

Cancer progression is another pathological process involving cell migration and disassembly of cellular junctions. Today cancer is the second most common cause of mortality in the world, and ninety percent of all cancer deaths arise from tumor metastasis (Christofori, 2006). For a normal cell to transform into a cancer cell, six phenotypic changes in the physiology of the cell are required. These changes are also referred to as the six "hallmarks of cancer" and include self-sufficiency in growth signals, insensitivity to anti-growth signals, resistance to apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2000). The final hallmark, tissue invasion and metastasis, occurs by mechanisms that are still not completely understood. It is therefore important to study the basic molecular mechanisms underlying how a tumor cell can leave its site of origin, break through the basal membrane, migrate into the blood stream, reattach to another site and form a metastasis (Christofori, 2003). The majority of cancers are of epithelial origin and in order to understand how a cell can gain a malignant and motile phenotype an understanding of the architecture of the epithelial cell under normal conditions is needed (Hanahan and Weinberg, 2000; Nowell, 2002; Vogelstein and Kinzler, 2004). In this section a description of the architecture of the epithelium, its main cellular components and regulation of some these components in cancer will be discussed. Finally some of the mechanisms behind loss of cellular junctions, a common event in cancer progression, will be addressed.

Integrity of epithelial cell layers

The immobile structure of the epithelium is important for maintenance of the barrier structure between tissues and different body compartments. The epithelium is supported by the basal membrane, which is attached to the underlying connective tissue. Cellular junctions enable the preservation of this architecture via both cell-cell and cell-matrix interactions. The different junctions include the tight and adherens junctions, desmosomes as well the communicative gap junctions. The junctions do not only provide adhesive properties, but also function as important signaling platforms controlling cell proliferation, polarization and differentiation. The different compartments will be described individually and discussed in the context of normal physiology as well as during cancer progression.

Organization of the epithelium

Tight Junctions

Tight junctions localize to the most apical side of the plasma membrane and are visualized by electron microscopy as several fusion points (kissing points) between

two adjacent epithelial cells. This is in contrast to the adherens junctions and desmosomes, which are separated by 15-20 nm. As such, tight junctions form a tight seal between cells while adherens junctions and desmosomes link cells together and anchor them to the extracellular matrix (ECM) (Aijaz et al., 2006; Tsukita et al., 2001). Tight junctions have three major functions. First, they are crucial for maintenance of the barrier in both epithelial and endothelial cells and restrict the diffusion of solutes. This restriction will depend on the size of the solute, as well as the tightness of the tight junction. However, the paracellular permeability is dynamic and can be changed as a response to physiological and pathological stimuli (Aijaz et al., 2006; Tsukita et al., 2001). Second, tight junctions contribute to forming and maintaining cell polarity by holding adjacent cells together and creating a fence restricting the movement of proteins between the apical and basolateral side (Aijaz et al., 2006; Tsukita et al., 2001). Third, tight junctions function as a signaling platform, which controls junction assembly and polarization but also cell proliferation, differentiation and gene expression (Matter and Balda, 2003).

Today more than 50 proteins are localized and expressed in the tight junctions (Aijaz et al., 2006; Gonzalez-Mariscal et al., 2003). These proteins belong mainly to two groups, the transmembrane proteins and the cytosolic proteins. The transmembrane group of proteins includes, occludin the first junctional protein to be identified, the claudin family and the CTX family of proteins, including CAR. These are the proteins that form the tight junctional strands visualized by electron microscopy. The transmembrane proteins are linked to the cytoskeleton via their C-terminal domain, mainly through specific PDZ domain binding motifs. PDZ is an acronym of the first three PDZ-containing proteins that were identified, the postsynaptic protein PSD-95/SAP90, the Drosophila septate junction protein Discs-large, and the tight junction protein ZO-1 (Hung and Sheng, 2002). Proteins containing PDZ domains are able to form large complexes via protein-protein interactions, have an important role in clustering and anchoring proteins to the plasma membrane as well as recruiting cytoskeletal and signaling proteins. ZO-1, as well as ZO-2 and ZO-3 belong to the family of membrane-associated guanylate kinase proteins (MAGUK), which in addition to PDZ domains, contain SH3 (src homology 3) and GK (guanylate kinase) domains. These domains are also responsible for protein-protein interactions and recruitment of proteins to subcellular domains (Gonzalez-Mariscal et al., 2000a). Aside from ZO-1/2/3, ZO-1-associated nucleic acid-binding protein (ZONAB), cingulin, PTEN and G-proteins, which lack PDZdomains, are also examples of important cytosolic proteins associated with cell-cell junctions (Aijaz et al., 2006; Gonzalez-Mariscal et al., 2003; Tsukita et al., 2001).

Tight junctions from different tissues can differ in their tightness and this is exemplified by the expression pattern of tight junction proteins in the kidney.

In the renal tubular segments, where reabsorption of water and ions occurs, the tight junctions are leaky and the number of tight junctional strands are few (Gonzalez-Mariscal et al., 2000b; Zhang et al., 1996). This in contrast to the presence of multiple numbers of strands in segments where urine is transported out of the kidney, where the tight junctions are almost impermeable. The tightness of tight junctions can be measured as TER and a study comparing tight junctions of different epithelia has shown that an increase in the number of strands is correlated with a logarithmic increase in the TER (Saitou et al., 2000). In addition to the number of strands, the composition of proteins making up the tight junctions also affects the tightness. This has been shown by gene expression pattern studies of several tight junction proteins in different tissues as well as studies involving gain and loss of function with different tight junction proteins including occludin, claudin and CAR (Aijaz et al., 2006; Raschperger et al., 2006; Tsukita et al., 2001).

Adherens Junctions

Adherens junctions are located just beneath the tight junctions and, in concert with desmosomes, constitute the anchoring junctions. They are linked to the cytoskeleton via actin filaments and stabilize the cell via a belt-like structure that surrounds the cell. Adherens junctions are mainly composed of proteins from the cadherin family, the catenin family, and the nectin family (Gumbiner, 1996; Wheelock and Johnson, 2003). Adherens junctions possess multiple functions including junction polarization and formation, stabilization and signaling.

The cadherin family is a superfamily of transmembrane proteins that mediate Ca^{2+}-dependent homophilic cell-cell interactions and function in cell recognition, tissue morphogenesis and tumor suppression (Angst et al., 2001). Today five different subfamilies are distinguished, the classical cadherins type I, which are mainly localized to adherens junctions, the highly related type II cadherins, desmosomal cadherins (desmocollins and desmogleins), which form desmosomal junctions, protocadherins important in neural development and cadherin-related protein like Flamingo and Fat-like cadherins. Within the classical type I cadherins, E(epithelial)-cadherin is considered being the prototypic cadherin and is the best characterized, followed by N(neural)-, P(placental) and R(retinal)-cadherins (Angst et al., 2001; Wheelock and Johnson, 2003). The type II cadherins are less characterized and includes VE-cadherin, K-(kidney) and H-(heart)-cadherin. Of the type II cadherins, VE-cadherin has gained the most attention due to its important role in vasculogenesis and angiogenesis (Dejana, 2004). Different expression patterns of the different cadherins during development and in the adult organism have gained insight to their different functions as well as their adhesive properties. For example, E- and P-cadherin are found in adherens junctions where they promote tight cell-associations

while N-cadherin is primarily found in neural tissues and fibroblast structures with less stable and confined cell contacts (Wheelock and Johnson, 2003).

The structure between the different subfamilies of cadherins is conserved, especially in the extracellular domain (Angst et al., 2001; Wheelock and Johnson, 2003). The type I cadherin have five cadherin domains and these domains are responsible for the homophilic interactions. The strong adhesive property of the cadherin-cadherin interaction is thought to be mediated by dimerization of two cadherins on the same cell surface creating a zipper-like structure. The cytoplasmic domain differs between different subfamilies and is responsible for linking the classical cadherins to the actin cytoskeleton via the intracellular proteins, the catenins. This binding is important in relaying signals that mediate growth and differentiation. Today three different catenins have been described: α, β, γ catenins. A complex of α together with either β or γ catenins binds to carboxy-terminal cytoplasmic domain of a cadherin molecule. The interaction between β-catenin with E-cadherin has been intensively studied both during development and in cancer progression (Brembeck et al., 2006; Huber et al., 1996). Loss of E-cadherin or β-catenin is lethal during embryonic development and loss of the linkage between E-cadherin and β-catenin in the adult induces tumor progress and invasion (Brembeck et al., 2006; Huber et al., 1996; Wheelock and Johnson, 2003).

Another important interaction partner of the classical type I and non-classical type II cadherin is the protein p120. p120 directly affects the adhesiveness of the adherens junctions by controlling the turn-over rate of the cadherins at the cell-surface. In the absence of p120, cadherin is internalized and degraded and can only be recycled if reassociation with p120 occurs (Reynolds and Roczniak-Ferguson, 2004). In this thesis, E-cadherin and N-cadherin will be discussed in further detail.

Desmosomes and Hemidesmosomes

Desmosomes and hemidesmosomes are the second group of anchoring junctions and are located on the lateral side of basal membrane (Gumbiner, 1996; Wheelock and Johnson, 2003). They tie cells together via the transmembrane protein, desmosomal cadherin in a spot like manner. Desmosomal cadherin binds desmoplakin, plakoglobins and the intermediate filaments and this complex stabilizes and protects the cell against shear forces. Hemidesmosomes are located at the very basal part of the plasma membrane where they mediate binding to the ECM. This binding is mediated through the integrins anchoring the desmoplakin, plakoglobins to the intermediate filaments and plays an important role in signaling.

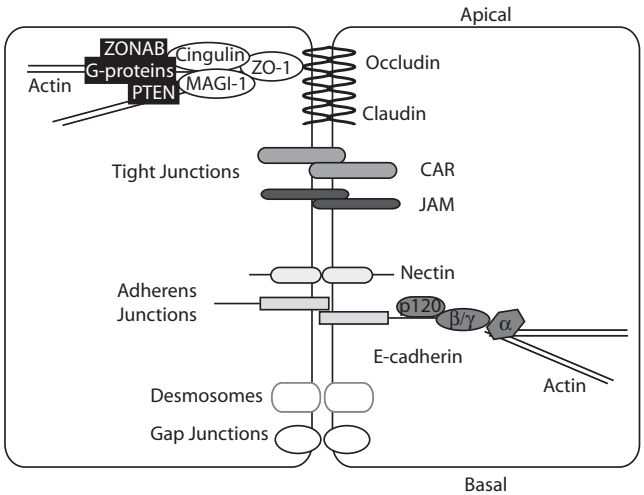

Fig10. Organization of the epithelium. Schematic model illustrating the organization of the epithelium. The different junctions are the tight and adherens junctions, desmosomes and the communicative gap junctions. Tight junctions are composed of transmembrane proteins; occludin, claudin, junctional adhesion molecule (JAM), coxsackie- and adenovirus receptor (CAR) and cytosolic proteins; zona occludens (ZO-1), ZO-1-associated nucleic acid-binding protein (ZONAB), membrane–associated guanlyate kinase 1β (MAGI-1β) cingulin, phosphatase and tensin homolog (PTEN) and G-proteins. Adherens junctions are composed of epithelial-cadherin (E-cadherin), the α/β/γ catenins, nectin, and p120. The desomosomes and gap junctions are located below tight and adherens junctions. Modified from Tsukita 2001.

Deregulation of cellular junctions in cancer

E-cadherin

The importance of E-cadherin during tumor progression has been well established (Peinado et al., 2004b; Perl et al., 1998; Semb and Christofori, 1998). Many epithelial cancers display loss of E-cadherin function based on inactivation of the E-cadherin gene, transcriptional repression or proteolysis of the extra-cellular domain. Over-expression of E-cadherin in cultured cancer cells or in transgenic mouse models has demonstrated that it inhibits the invasiveness of tumor cells. By studying the mechanisms regulating E-cadherin a better understanding of the importance of adhesion and signaling in tumorogenesis, has been obtained.

E-cadherin gene expression is regulated by epigenetic and transcriptional mechanisms or a combination of both (Peinado et al., 2004b). A relationship between cancer and silencing of the E-cadherin promoter due to methylation has been observed in many different types of tumors including prostate and breast (Graff et al., 1995; Yoshiura et al., 1995). Treatment of cancer cells with 5-azacytidine, a methylation inhibitor, showed that E-cadherin expression could be reactivated

(Graff et al., 1995; Yoshiura et al., 1995). However, epigenetic alterations are early events in tumor progression and occur prior to invasion so detection of a heterogenous methylation pattern of E-cadherin together with re-expression of E-cadherin at metastatic lesions may be somewhat puzzling. Since DNA methylation is considered to be an irreversible mechanism, other epigenetic mechanisms and/or gene regulatory mechanisms might dominate over this early silencing of E-cadherin and cause re-expression. This is supported by the finding that the E-cadherin promoter often continues to be expressed while methylated (Graff et al., 2000). Re-expression of E-cadherin in cells present in the blood stream and at secondary sites was later suggested to be one mechanism that would allow cancer cells to survive and form a metastasis (Graff et al., 2000; Peinado et al., 2004b; Strathdee, 2002). Seemingly, expression of E-cadherin is dynamic during cancer progression.

E-cadherin is also regulated by direct transcriptional mechanisms. The mouse E-cadherin promoter is a TATA-less promoter containing several regulatory elements including a CCAAT box, a GC rich domain and a palindromic element which is made up from two adjacent E-boxes and flanked by four inverted nucleotides referred to as "E-pal"(Behrens et al., 1991). For basal expression, the CCAAT and GC-rich regions are necessary and the constitutive transcription factors known to bind these regions are AP-2, SP-1 and CAAT binding proteins (Behrens et al., 1991; Faraldo et al., 1997; Hennig et al., 1996). The E-pal element is an epithelial-specific regulator and active repressor site. Moreover, an epithelial-specific enhancer, binding AP-2 has been located in the first intron. An additional repressor element containing Ets binding sites has also been identified in the mouse E-cadherin promoter region (Hennig et al., 1996; Rodrigo et al., 1999). The regulatory elements of the CCAT, GC and E-box-1/E-box-3 in the mouse E-cadherin promoter are conserved between species such as human and dog (Comijn et al., 2001). However the E-box2 is only present in the mouse promoter while the E-box4 downstream of the initiation site is present solely in the human promoter (Comijn et al., 2001; Peinado et al., 2004b).

Several transcription factors have been identified which bind to the E-boxes of the mouse and human E-cadherin promoter including Snail, Slug, E47, ZEB, SIP1 and Twist (Batlle et al., 2000; Blanco et al., 2002; Bolos et al., 2003; Cano et al., 2000; Cheng et al., 2001; Grooteclaes and Frisch, 2000; Hajra et al., 2002; Perez-Moreno et al., 2001; Poser et al., 2001; Sugimachi et al., 2003; Yang et al., 2004). The extent to which each of these repressors inhibits E-cadherin is currently being investigated as the different repressors binds with different affinities (Bolos et al., 2003). Snail, may be the dominant E-cadherin repressor since it binds with the highest affinity to the promoter and overexpression induces a process known as epithelial mesenchymal transition (EMT)(Batlle et al., 2000; Bolos et al., 2003; Cano et al., 2000). EMT is a phenotypic change of the epithelial cells to acquire a more migratory and invasive state.

N-cadherin

Whereas E-cadherin expression is suppressed during tumorogenesis, N-cadherin expression increases in invasive forms of cancer. When overexpressed in epithelial cells, N-cadherin induces a scattered, more motile and invasive phenotype both in cell culture and in nude mice models (Hazan et al., 2000). Increased N-cadherin expression in human cancers is associated with a decrease in E-cadherin levels. However, this invasive state is present even in the presence of E-cadherin, implying that the effect of N-cadherin expression is dominant over the tumor suppressor activity of E-cadherin (Hazan et al., 2000; Nieman et al., 1999). Based on these studies a new theory evolved and is referred to as the "cadherin-switch" from an E-cadherin-dependent adhesive and stabile phenotype to an N-cadherin-dependent mesenchymal and motile phenotype (Cavallaro and Christofori, 2001; Cavallaro and Christofori, 2004; Christofori, 2003). Studies in patients with melanoma and prostate cancer have further supported this theory. It is not clear why this switch occurs but one explanation could be that the tumor cells need to acquire new and other adhesive properties for maintenance and survival (Cavallaro and Christofori, 2004; Christofori, 2003). The invasive effect N-cadherin has on cells can partly be explained by the interaction with the fibroblast growth factor receptor 1 (FGFR1) at the plasma membrane. It is thought that N-cadherin forms a complex with the FGFR1 via its IgG domain. This interaction enables a stabilization of the FGFR1 leading to a sustained MAPK-ERK activation after fibroblast growth factor 2 (FGF2) binding (Hazan et al., 2004; Suyama et al., 2002). As a result an activation of genes which are linked to increased invasiveness such as matrix metalloproteases (MMPs) occurs.

Mechanisms behind loss of cellular junctions in cancer

Today, numerous studies indicate that loss of both the adherens junctions as well as the tight junctions is a prerequisite for invasion and metastasis, representing the final hallmark of cancer. The tumor cell needs to lose its adhesive property to other cells, break down and transverse the basal membrane, survive in the circulation and finally reattach at a secondary site to form a metastasis. The first step of cancer progression includes the downregulation of several of the members of the cadherin family, E-cadherin being the most important one, occludin, claudin and members of the CTX-family including CAR and A33. One mechanism by which some of these molecules are downregulated is through a mechanism known as EMT.

Epithelial Mesenchymal Transition

Epithelial mesenchymal transition (EMT) is an evolutionarily conserved embryonic process in vertebrates and invertebrates whereby epithelial cells are transformed to

mesenchymal cells. During this process the typical epithelial traits are lost resulting in a mesenchymal phenotype. EMT was initially a morphological description, depicting a change in cell shape during embryonic development. Today however, EMT is typically characterized as loss of epithelial markers such as E-cadherin and gain of mesenchymal features such as vimentin, fibronectin and N-cadherin. In addition, cells undergoing EMT change shape, lose polarity and gain motility due to the drastic remodelling of the cytoskeleton (Bakin et al., 2000; Zavadil and Bottinger, 2005). The process of EMT is thought to play a role in metastasis as the transformation to the mesenchymal phenotype enables cells to move and tranverse through the basal membrane, reach the blood stream and are able to attach to a secondary site. Here, the reverse process, mesenchymal epithelial transition (MET), has been suggested to occur so that the malignant cells can attach properly and de novo form epithelial junctions. This could in part explain the somewhat contra-dictory data that metastatic tissues express high levels of cell adhesion proteins.

Disassembly of cell-cell junctions is the first sign of EMT. Numerous studies have shown downregulation of E-cadherin, ZO-1, claudins, occludin, MUC1 and desmoplakins through transcriptional repression by EMT related transcription factors (Guaita et al., 2002; Jechlinger et al., 2003). In addition to downregulation of adhesion proteins, an upregulation or "switch" to molecules with migratory and mesenchymal properties occurs as previously described. Examples are a switch from E-cadherin to N-cadherin and integrin $\alpha_6\beta_4$ to the mescenchymal integrin $\alpha_5\beta_1$ (Cavallaro et al., 2002; Maschler et al., 2005).

Increased motility is the next step in EMT and is mediated by changes in the cytoskeleton mostly induced by activation of members of the Ras superfamily, the Rho family. Another property associated with EMT is the induction of expression of matrix metalloproteases (MMPs) (Illman et al., 2006; Lochter et al., 1997). The MMPs comprise a large family of about 24 endopeptidases, that can degrade most ECM components as well as cell surface and pericellular proteins (Lemaitre and D'Armiento, 2006). Through MMP actions, ECM can be degraded and remodelled upon endogenous stimuli such as TGFβ or FGF from the surrounding tissues. MMPs also induce the release and activation of several bioactive molecules from the ECM such as TGFβ, which in their latent form are bound to ECM molecules. The importance of MMPs in EMT has been shown by studies reporting irreversible EMT as a result of MMP overexpression (Illman et al., 2006; Lochter et al., 1997). Induction of MMPs may also result in the degradation of adhesion molecules such as E-cadherin. In the presence of MMPs the extracellular domain of E-cadherin is proteolytically cleaved resulting in degradation (McGuire et al., 2003).

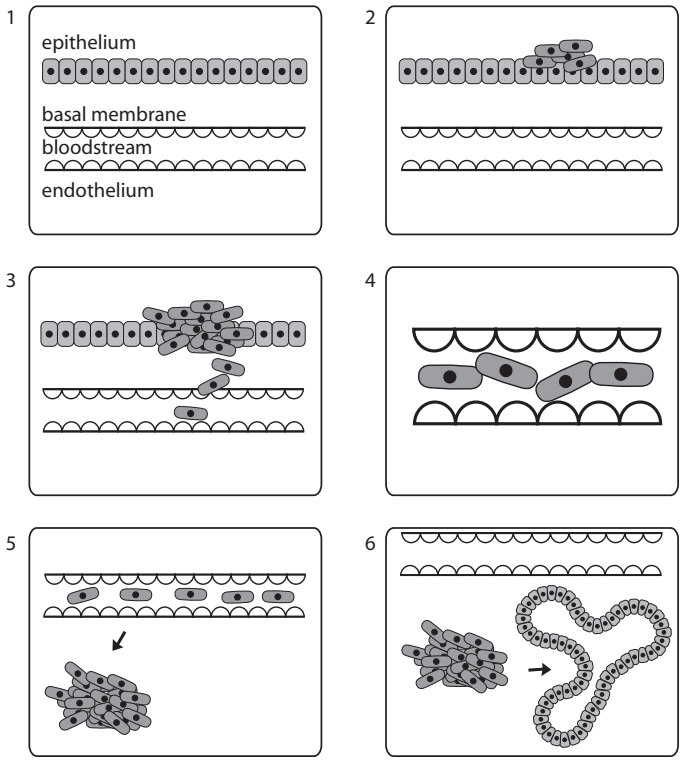

Fig 11. Epithelial mesenchymal transition. Schematic pictures illustrating EMT and cancer progression *in vivo*. 1. Normal epithelium. 2. Growth of primary tumor 3. Invasive growth by transmigration through the basal membrane 4. Survival and transport of cells in the bloodstream. 5. Re-establishment and attachment of cells to secondary site. 6. Organization and formation of a metastasis. Possible induction of MET. Modified from Huber 2005.

Signaling Pathways involved in EMT

EMT is induced by different stimuli such as TGFβ, FGF2, EGF, hepatocyte growth factor (HGF) and Wnts. These factors will in turn activate downstream signaling cascades, including Ras/Raf, PI3K, Smad, Notch, NF-κβ, p38 MAPK and JNK (Bakin et al., 2000; Grego-Bessa et al., 2004; Grille et al., 2003; Huber et al., 2004; Janda et al., 2002; Larue and Bellacosa, 2005; Santibanez, 2006; Timmerman et al., 2004). According to most studies cooperation between different signaling pathways is essential for induction and maintenance of the EMT process. In this section two major signaling cascades and their components will be described, namely Ras and TGFβ.

Ras signaling

The Ras proteins belong to a large family of membrane associated monomeric GTPases that continuously cycle between an active GTP-bound state and an inactive GDP-bound state. This cycle is controlled by the guanine-nucleotide exchange factor (GEFs) and the GTPase activating proteins (GAPs). GEFs induces the release of GDP and binding of GTP, whereas GAPs promotes the hydrolysis of GTP and brings Ras back to an inactive form (Giehl, 2005; Mor and Philips, 2006). The Ras superfamily is divided into six subfamiles including Ras, Rho, Arf, Rab, Ran and Rad. The classical Harvey (H)-Ras, Neuroblastoma (N)-Ras and two splice variants of Kirsten (K)-Ras are all members of the Ras subfamily. These proteins share 85% aa identity but differ in their hypervariable region. This suggests that the classical Ras members might display functional differences. Indeed knock-out analysis of both H-Ras and N-Ras are viable whereas K-Ras is not. In addition, the different isoforms have been shown to preferentially activate specific signaling cascades (Giehl, 2005). Ras induces several signaling proteins mainly localized at the plasma membrane. The best characterized are the Raf kinase, activating the MAPK signaling cascade, and the PI3K activating the Akt signaling cascade (Downward, 2003).

Growth factors including EGF induce Ras signaling, by activating Ras, which enables Ras to target Raf kinase to the plasma membrane and subsequently activate Raf. Active Raf phosphorylates mitogen-activated protein kinase and ERK kinase 1 (MEK1) and MEK2, which phosphorylate extracellular signal-regulated kinase (ERK), that translocates to the nucleus and interacts with different transcription factors. The major functions attributed to the Raf-MEK-ERK pathway are altered gene expression leading to cell proliferation, differentiation and migration. Overactive Raf-MEK-ERK has been reported in several cancers, leading to transformation of fibroblasts, inducing angiogenesis and activating migratory proteins such as the myosin light chain kinase (MLCK) and focal adhesion kinase (FAK).

PI3K/Akt signaling pathway is mainly responsible for cell survival, proliferation and growth (Downward, 2004). In addition, recent data have also proposed a role in actin reorganization and motility. Upon activation PI3K, induces the production of phosphatidylinositol 3,4, 5 triphosphate (PIP_3). PIP_3 recruits Akt to the plasma membrane whereby Akt is phosphorylated presumably involving phosphoinositide-dependent kinase 1 (PDK1). Several studies have indicated the importance of Akt in cancer including its overexpression in tumor cells, its localization to the leading edge in migrating cells, enhanced production of MMPs, downregulation of E-cadherin, changes in cytoskeleton and inhibition of apoptosis via inactivating components in the apoptotic pathway such as caspases. In addition to Raf and PI3 K active Ras also induces Nf-κβ and stabilization of hypoxia inducible factor alpha (HIF-α) both important for tumor formation, maintenance and progression (Giehl, 2005).

Other members of the Ras superfamily are the Rho GTPases including Rac1, RhoA and Cdc42, which have been reported to participate together with PI3K and the Raf-MEK-ERK pathway in transformation of cells (Ridley, 2004). The Rho family is induced by extracellular stimuli and cell-cell and cell-matrix interactions as well as mechanical stress. The major function of this family of proteins is to regulate the actin cytoskeleton, cell-cycle progression and gene transcription. The precise mechanism for the cross-talk between Ras and Rho is still under investigation (Giehl, 2005). Studies have shown that Ras both inhibits and activates the Rho family indicating that the cross-talk is most likely context and cell dependent. This lead to the conclusion that activation of these members is not a general response to Ras activation but rather specific for different types of cancers and much more complex than originally anticipated.

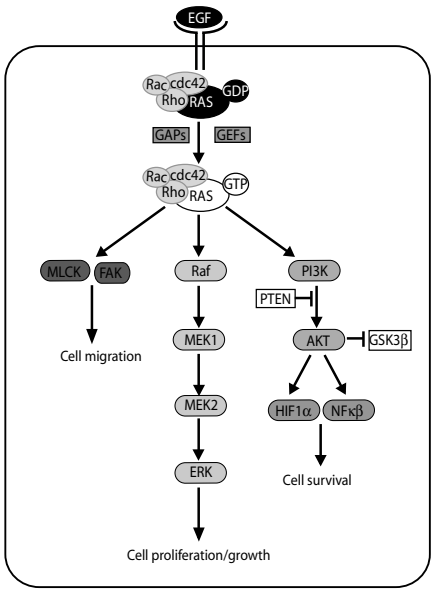

Fig 12. Ras signaling. Epidermal growth factor (EGF) activates Ras and Ras subsequently activates Raf kinase. Active Raf phosphorylates mitogen-activated protein kinase and ERK kinase 1 (MEK1) and MEK2, which phosphorylate extracellular signal-regulated kinase (ERK). Phosphorylated ERK translocates to the nucleus and induces cell growth/proliferation. Active Ras induces migratory proteins myosin light chain (MLCK) and focal adhesion kinase (FAK). Phosphoinositide-3 kinase (PI3K)/Akt signaling is induced by active Ras and promotes cell survival. Phosphatase and tensin homolog (PTEN) is an inhibitor of PI3 K. Active Ras also induces nuclear factor kappa beta (NF-kβ) and stabilization of hypoxia-inducible factor 1 alpha (HIF1α). Modified from Downward 2002.

Transforming growth factor signaling

TGFβ was originally identified as a secreted polypetide from transformed fibroblasts and its name was derived from its capacity to transform fibroblasts *in vitro*. Shortly thereafter TGFβ was found to also possess anti-proliferative properties (Akhurst and Derynck, 2001). This dual role of TGFβ acting as tumor suppressor in early stages of tumor development but promoting invasiveness and metastasis in advanced carcinoma has been a key question to answer in the cancer research area.

TGFβ is produced by several cells including stromal cells, macrophages and platelets and the family includes three isoforms: TGFβ1, TGFβ2 and TGFβ3 (Bachman and Park, 2005). TGFβ is secreted as an inactive disulfide linked homodimeric poly-

peptide, which is activated upon proteolytic cleavage. The active TGFβ forms a complex with the signaling tyrosine/serine kinases receptors TGFβ type II (TβRII) and TGFβ type I (TβRI)(also known as activin receptor-like kinase 5, ALK5) (Pardali and Moustakas, 2007). TGFβ binds as a dimer to TβRII and TβRI heterodimers and results in a transphosphorylation of the serine/theronine kinases of TβRI by TβRII kinase. This induces a conformational change and exposes the catalyticdomain of TβRI thereby activating the receptor. Active TβRI binds and phosphorylates the transcription factors, receptor-Smads (R-Smads) and initiates the Smad signaling cascade.

The Smads are composed of a N-terminal Mad homology I (MH1) domain, which is responsible for nuclear localization, protein-protein interaction as well as DNA binding, an intermediate linker, which contains the regulatory element by which several kinases such as MAPK and CDKs can control the half-life of the protein by recruiting ubiquitin ligases, and a C-terminal MH2 domain which mediates protein-protein interactions and contains the phosphorylation motif (Massague et al., 2005).

When R-Smads, Smad2 and 3, are phosphorylated by the activated TβRI receptor they form trimeric complexes with the cofactor Smad, Smad4 which is not phosphorylated (Massague et al., 2005; Pardali and Moustakas, 2007). Monomeric Smad proteins are continuously shuttled between the nucleus and the cytoplasm but the trimeric complex of Smad2/Smad3/Smad4 favors nuclear retention. Once inside the nucleus, the Smad complex binds directly to DNA via specific Smad binding elements (SBEs), 5'- GTCT- 3'. The Smad complex can associate with numerous transcription factors, co-activators and co-repressors to achieve its transcriptional effect. This association of Smads with other transcription factors is important for mediating high affinity DNA binding since the SBE only provides low affinity binding (Akhurst and Derynck, 2001; Massague et al., 2005; Pardali and Moustakas, 2007). Depending on which complexes are formed, the outcome of the TGFβ response will vary thus giving rise to an array of complex responses of TGFβ signaling.

Smads have been reported to regulate transcription via two distinct mechanisms (Massague et al., 2005). One is direct activation or repression by recruitment of different transcription factors together with the Smads, and is referred to as primary activation or suppression. The other mechanism is a multi-step process referred to as self-enabled gene response whereby Smads induces a gene response that enables other Smad dependent responses.

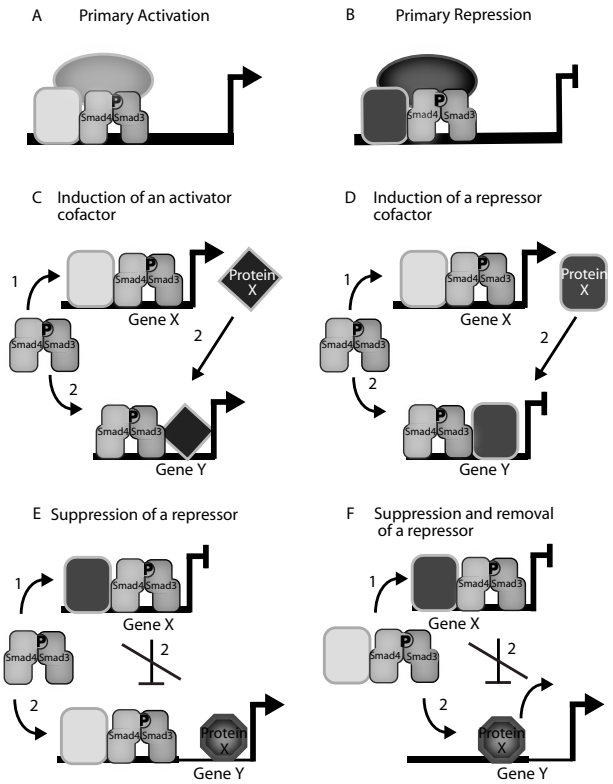

Fig 13. Smad-mediated transcriptional regulation. Recruitment of multiple transcription factors together with the Smads results in primary activation or suppression (A, B). Self-enabled gene responses. Smads induce a gene response that enables another Smad dependent gene response (C-F). Smads can either induce the transcription of an activator or a repressor co-factor, and subsequently form a complex with this co-factor and bind DNA to exert its function (C, D). Alternatively, Smads can downregulate gene X which mediates suppression of gene Y and by doing so alleviating the suppression of gene Y and giving the Smads an opportunity to positively regulate gene Y, instead (E). Smads induce downregulation of gene X and not just alleviating its response on gene Y but also competitively displacing gene X from gene Y (F). Modified Massague 2005.

To balance the TGFβ response there is also an inhibitory loop mainly mediated by three different proteins; the inhibitory Smads (I-Smads), ubiquitin ligases of the Smurf family and phosphatases (Pardali and Moustakas, 2007). In short, at the same time as the R-Smads are phosphorylated the I-Smad (Smad7) is recruited to the TβRI/TβRII complex and competitively inhibits further R-Smad phosphorylation. Smad7 also recruits phosphatases, which deactivate the entire receptor complex by dephosphorylation. The receptor complex is targeted for endocytosis and lysosomal degradation via ubiquination initiated by Smad7 by binding to the Smurf ubiquitin ligases.

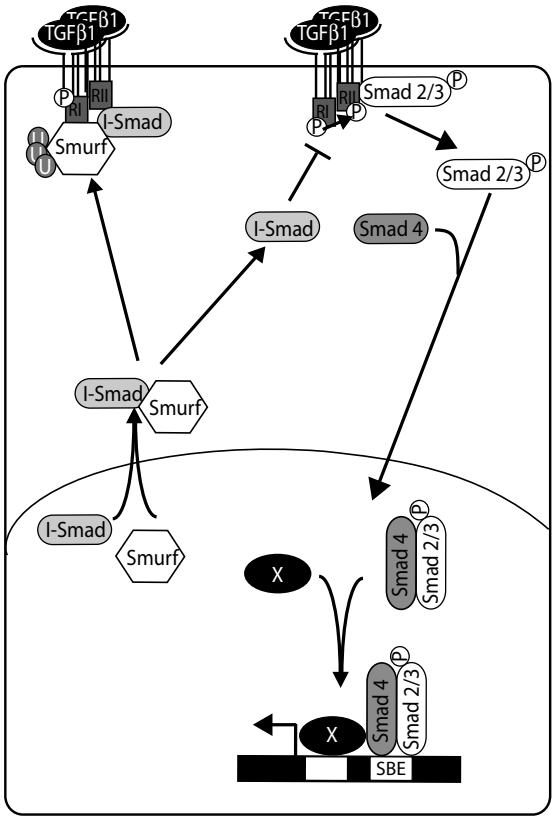

Fig 14. TGFβ Signaling. Tumor growth factor β (TGFβ) binds as a dimer to TGFβ receptor II (TβRII) and TβRI heterodimers and induces a transphosphorylation and activation of TβRI by TβRII kinase. Active TβRI binds and phosphorylates Smad2/3. Phosphorylated Smad2/3 form a trimeric complex with Smad4. The Smad2/3/4 complex translocates to the nucleus and binds co-factor X to DNA leading to regulated transcription. Upon TGFβ signaling inhibitory Smad (I-Smad) and Smurf are transported out of the nucleus, forms a complex and binds the TβRI-TβRII receptor complex. This binding targets the receptor complex for degradation. The I-Smad-Smurf complex also competitively inhibits further Smad2/3 phosphorylation. Modified Pardali 2007.

Transcription factors important in EMT

Different co-operative signaling pathways upregulate or stabilize several transcription factors including Snail, Slug (Snail2), Twist, SIP1, LEF-1/β-catenin, HoxB7 and Hey/Hes (Batlle et al., 2000; Bolos et al., 2003; Cano et al., 2000; Comijn et al., 2001; Kim et al., 2002; Wu et al., 2006; Yang et al., 2004; Zavadil and Bottinger, 2005; Zavadil et al., 2004). The best characterized and perhaps most important one, is the Snail family of zinc finger transcription factors (Batlle et al., 2000).

The Snail family consists of three different members Snail (Snai1), Slug

(Snai2) and Smuc. Snail has been shown to act as a repressor via binding to E-box elements in the promoters of E-cadherin, occludin and claudins (Batlle et al., 2000; Cano et al., 2000; Carrozzino et al., 2005; Cheng et al., 2001; Ikenouchi et al., 2003). The importance of Snail in cancer has been extensively investigated in several *in vitro* and *in vivo* studies. For example, MDCK cells stably expressing Snail display tumorogenic properties when injected into nude mice, verifying the transforming capacity of Snail (Cano et al., 2000). Furthermore, invasive tumors in humans, including breast, gastric cancer and hepatocellular carcinoma, all express high levels of Snail (Blanco et al., 2002; Cheng et al., 2001; Rosivatz et al., 2002; Sugimachi et al., 2003). In addition, silencing of Snail suppresses tumor growth and invasiveness *in vivo* (Olmeda et al., 2006). One mechanism by which Snail operates is by blocking the cell-cycle through a direct repression of cyclin D2 resulting in decreased proliferation. Accordingly, Snail expression is highest in the invasive front of carcinomas, which is a site of low proliferation. It is possible that decreased proliferation may be required for the reorganization occurring during EMT to proceed. Snail expression also protects cells against apoptotic stimuli (Vega et al., 2004).

Snail is induced by several factors including TGFβ, Wnt, Ras, Notch, integrin-linked kinase (ILK), Akt, and NF-κβ all of which are also important for EMT (Barbera et al., 2004; Yook et al., 2005). The Snail protein is a highly unstable protein and is posttranslationally regulated by glycogen synthase β (GSK3β) via phosphorylation on two motifs. One motif is responsible for shuttling Snail out of the nucleus and the other phosphorylation motif targets the protein for degradation by the β-trp destruction complex (Zhou et al., 2004). In addition, GSK3β also inhibits the transcription of Snail. Two positive regulators of Snail have been described, lysyl oxidase like protein2 (LOXL2) and Axin2 (Peinado et al., 2005; Yook et al., 2006). LOXL2 masks the phosphorylation motif recognized by GSK3β and Axin2 binds GSK3β, resulting in the stabilization of Snail.

The loss of cell-adhesion during two pathologcial processes have been described in this section. Clearly the dynamic properties of the endothelial and epithelial barrier is crucial for the clearance of pathogens from the tissue and initiation of repair. However the maintenance of this barrier is also of importance in preventing normal cells to become migratory and invasive an initial process associated with tumorogenesis.

AIM OF THE THESIS WORK

Successful viral infection depends on several factors: binding of the virus to the target cell, uptake and transport of the virus, and the final delivery of the viral genome to the nucleus. The initial interaction of Ad with the cell is a major determinant for successful viral infection. This interaction is dependent on the accessibility as well as the number of viral receptors.

The major aim of the current thesis research was to determine how virus attachment and receptor accessibility is regulated in normal and pathological conditions and how this may affect the efficiency of Ad infection. To address this, three different approaches were taken. The first investigation focused on the effect of humoral immunity on the interaction of Ad with the target cells via the native fiber-CAR interaction as well as the adopted CAR-independent antibody-Fc receptor interaction (Paper I + II). Second, the effect of inflammatory mediators, such as cytokines, on CAR expression and subsequent Ad infection was studied (Paper III). Third, regulation of CAR expression during the progression from low to high-grade malignancy was investigated based on the knowledge that decreased expression of many cell adhesion molecules may contribute to invasive tumor growth (Paper IV). Altogether, these studies have shed light on the general topic of viral entry and the normal physiological function and regulation of CAR, and may in addition, have implications for the use of Ad as a gene therapy vector.

RESULTS AND DISCUSSION

Paper I. Quantification of adenovirus receptor binding and infectivity

"Rapid assessment of adenovirus serum neutralizing antibody titer based on quantitative, morphometric evaluation of capsid binding and intracellular trafficking: population analysis of adenovirus capsid association with cells is predictive of adenovirus infectivity."

To deliver therapeutic genes successfully to target cells in a controlled manner, we need to understand the basic science behind each step of virus infection, i.e. binding, internalization, trafficking and delivery of the genome to the nucleus. Anti-Ad neutralizing antibodies have been characterized through their ability to inhibit viral infection (Shenk, 2001). In the field of gene transfer these neutralizing antibodies represent an obstacle to success, because of the possibility that anti-Ad humoral immunity may prevent gene transfer in individuals with pre-existing immunity, or following repeated Ad vector administration (Mack et al., 1997; Mastrangeli et al., 1996; Yang et al., 1995). A wealth of research regarding Ad tropism, in recent years has suggested that virus receptor binding is a rate-limiting step in successful viral infection and gene transfer (Hidaka et al., 1999; Miller et al., 1998; Russell, 2000). Therefore the focus of our first studies was to analyze the binding and trafficking of Ad in the presence of neutralizing antibodies.

We hypothesised that the number of Ad particles bound to cells or associated with the nucleus, would be directly proportional to Ad infection and subsequent transgene expression. To test this, digital image analysis was used to measure the extent to which the presence of anti-Ad serum inhibited cell association and intracellular trafficking of fluorophore-conjugated virions. The human sera used in these studies were obtained from patients undergoing gene therapy clinical trials, and contained different levels of anti-Ad neutralizing antibodies (Harvey et al., 1999). Antibody titers were quantified by a viral replication assay (plaque reduction assay) and antibodies against the viral capsid proteins were determined by Western blot analysis. By the plaque assay, the four sera analyzed had anti-Ad neutralizing antibody titers of <10 (non-neutralizing), 640, 2,560 and 49,000 (all neutralizing). Western blot analysis of these human sera confirmed the presence of anti-Ad antibodies in all four sera, including the one that had no neutralizing activity by plaque assay.

To confirm our hypothesis that the number of Ad particles that enter the cell and traffic to the nucleus correlated with the infectious titer of the virus, epithelial cells were infected with a high concentration of fluorophore-conjugated Ad to saturate Ad cell surface receptors. Infections were performed in the presence of a serial dilution of human sera and after a 10-minute incubation, unbound virus was washed away, and bound virus was permitted to traffic into the cell for an additional 60 minutes before fixation. We applied two separate morphometric read-outs for the subsequent analysis, total cell-associated Ad and percentage of Ad associated with

the cell nucleus. Analysis by fluorescence microscopy showed that, as expected, no virus was able to bind to cells in the presence of high concentrations of serum containing Ad-neutralizing antibodies ("extracellular neutralization"). As the neutralizing serum was diluted, an increasing association of Ad with cells was observed. However, subsequent trafficking to the nucleus was still inhibited as compared to the no serum control indicating the presence of "intracellular neutralization". Further dilution resulted in the same number of Ad becoming associated with the nucleus as the control in the absence of neutralizing serum. The non-neutralizing serum (titer <10) had no effect on viral binding, or trafficking to the nucleus, indicating that the amount or specificity of antibodies present was insufficient to inhibit the virus.

Total cell-associated fluorescence and the percentage nucleus-localized fluorescence in the morphological assay were quantitatively analyzed using digital image analysis. The results from the morphometric assays of Ad infectivity were then compared to a functional assay, i.e. a transgene expression assay. As with the previous experiment, the non-neutralizing serum (titer <10) did not change the infectivity, as indicated by reporter gene β-galactosidase (β-gal) expression relative to control. In contrast, sera with neutralizing titers (640, 2,560 and 49,000) blocked transgene expression at high serum concentrations and permitted transgene expression at low serum concentrations. We concluded that both the results from the morphometric and transgene expression assays were comparable and that an analysis of the particles that trafficked to the nucleus of infected cells correlated well with the level of infection. In other words, any inhibition in the infectivity observed by gene expression was matched by an inhibition of viral binding to cells and intracellular trafficking. The correlation between morphological and gene expression data indicates that nearly all Ad particles that bind to cells are infectious and that a morphological assessment of a population of Ad inside cells accurately reflects infectivity. These data also provide additional evidence supporting the importance of Ad binding to its cell surface receptors. As such, the biological mechanisms that regulate the availability of cell surface proteins for viral binding becomes an important topic for consideration and investigation.

Paper II. CAR independent uptake of adenovirus

"Neutralized adenovirus-immune complexes can mediate effective gene transfer via an Fc receptor-dependent infection pathway."

The observation in our previous study suggested that neutralization was mainly extracellular at high titers of anti-Ad neutralizing antibodies. We therefore hypothesised that Ad-immune complexes may still contain viable and infectious virus but that they were unable to infect cells because of an inability to bind CAR.

The capacity of phagocytic cells to take up IgG-bound virus through the Fc gamma receptor (FcγR) has previously been shown (Takada and Kawaoka, 2003). Based on this knowledge, we hypothesised that Ad in the presence of neutralizing antibodies could be taken up through a CAR-independent FcγR pathway, and successful transgene expression can be achieved. This question was addressed by expressing FcγR in target cells and subsequently infecting the same cells with Ad in combination with different ratios of Ad neutralizing antibodies. This study aimed to mimic the viral neutralization by serum that would occur *in vivo*, where Ad is cleared from the blood with a half-time of less than two minutes (Alemany et al., 2000). Therefore, anti-Ad sera and Ad was mixed five minutes prior to infection of a monolayer of A549 epithelial cells.

Fluorophore Cy3-labeled Ad was used for studying binding and trafficking of the virus in presence of anti-Ad antibodies. In parallel, Adβgal was used to evaluate the effect of anti-Ad antibodies on Ad mediated gene expression of the β-galactosidase gene. Three target cells were chosen for these studies; naïve A549 (which normally do not express the FcγR), A549 infected with an Ad encoding the FcγR (AdFcγRIIA), or as a control, A549 infected with an Ad vector containing no insert (AdNull) in the presence of various concentrations of human sera ranging from 0.01% to 10% anti-Ad neutralizing sera. In presence of 0.1% anti-Ad serum (titer 2,560) cell-associated Cy3Ad was decreased compared to control infection without serum, and at 1% almost all Cy3Ad was prevented from reaching the target cells. As expected the same was seen for the AdNull infected A549. In contrast, A549 cells expressing the FcγR sustained the capability to bind Ad-immune complexes at all serum dilutions. The majority of the Cy3Ad was detected in the cytoplasm but a distinct fraction of internalized Cy3Ad was able to traffic to the nucleus. AdFcγR-transfected cells were also analyzed for co-localization of Ad-immune complexes and FcγR via indirect immunofluorescence. In FcγR negative cells no colocalization of the Cy3Ad and FcγR was detected, however in the FcγR expressing cells co-localization of Cy3Ad and FcγR was observed, indicating that Cy3Ad had successfully been internalized. The trafficking studies predicted that FcγR-bearing cells would show Ad mediated gene expression in the presence of neutralizing antibodies. The persistence of β-gal expression, despite the presence of anti-Ad serum in FcγR expressing cells, indicated that a significant fraction of "extracellularly neutralized" Ad was viable and could deliver genes in a CAR-independent, but FcγR-dependent manner.

To confirm that the FcγR receptor was indeed responsible for the uptake of Ad-immune complexes, cells were infected with either AdNull or AdFcγR and exposed in a similar manner to Adβgal pre-mixed with 0.1% sera in the presence or absence of purified Fc domain. In the presence of Fc domain the gene transfer in

the FcγR bearing cells was decreased by 75% demonstrating the importance of the Fc-FcγR interaction in the uptake of Ad-immune complexes. To evaluate whether the cytoplasmic tail of FcγR was essential to the uptake of Ad, as was previously described for FcγR-mediated phagocytosis of large ligands, another set of experiments were performed (Indik et al., 1991). A549 cells were infected with an Ad vector encoding a tail-less FcγR (Adtailess FcγR) or with an AdFcγR in which the cytoplasmic tail was intact, followed by infection with Adβgal and evaluated for transgene expression. No difference could be observed in the kinetics of inhibition with different concentrations of human sera between cells expressing these two constructs leading to the conclusion that the tail had no impact on the uptake of Ad-immune complexes.

To evaluate whether CAR or integrin expression would aid in the process of Ad uptake, similar experiments were performed in CAR-deficient fibroblast. The importance of CAR was evaluated by expressing AdNull or FcγR in fibroblasts. Equal amounts of virus in the presence of neutralizing antibodies were taken up by FcγR in the CAR null fibroblasts as the CAR expressing A549 epithelial cells indicating that the presence of CAR is not necessary for the FcγR dependent uptake of Ad. The requirement for integrin interaction was evaluated by comparing infection of FcγR cells with βgal-expressing Ad vectors constructed with either a wild type capsid or a genetically-modified capsid in which the RGD motif responsible for interacting with the integrins was removed (AdΔRGD). When comparing the kinetics of the inhibition of the two vectors, the AdΔRGD had a lower IC_{50}, demonstrating the importance of the integrin interaction for mediating successful infection of Ad contained within Ad-immune complexes.

In summary, we have addressed the question raised in our previous study (Paper I) and shown that the extracellularly neutralized Ad-immune complexes still contain infectious virus, which can transfer genes in a CAR-independent way. In addition, we have tried to mimic the *in vivo* situation when Ad is exposed to sera containing a mixture of anti-Ad antibodies instead of using monoclonal antibodies against the different capsid proteins, to gain knowledge about the neutralization mechanism of Ad. We have also determined that the mechanism responsible for the uptake of Ad is dependent on an Fc-FcγR interaction. This demonstration was important, not only for understanding mechanisms of neutralization, but also for identifying a potential *in vivo* retargeting of viable Ad to FcγR-bearing cells (e.g. dendritic cells and macrophages). This mechanism of Ad uptake, is referred to as antibody dependent enhancement (ADE), and was recently also described for Ad by others (Mercier et al., 2004). Mercier showed that Ad could interact with DC very efficiently in the presence but not in the absence of Ad-specific antibodies and that this interaction was FcγRII/FcγRIII dependent. However, targeting of Ad to FcγR was inhibited in

presence of high levels of antibodies against the entire Ad virion, and successful infection was only achieved after dilution of the sera. Our findings together with Mecier's suggest that ADE of Ad is a dose-dependent mechanism. Mercier also proposes that anti-Ad sera contain a mixture of neutralizing and non-neutralizing antibodies together with enhancing and non-enhancing antibodies. The ratio of these antibodies will determine whether Ad is neutralized or taken up through a CAR-dependent or independent FcγR-dependent manner.

The FcγR mediated uptake of Ad has implications in the field of Ad mediated gene transfer but also, and perhaps more importantly, in the emerging field of Ad-based vaccines. Our observations may provide a mechanism by which a host with pre-existing Ad immunity can achieve efficient antigen presentation. The majority of the Ad-immune complexes will be phagocytosed and the viral protein will be presented by MHC class II but some viruses may infect APC via the FcγR, leading to presentation of proteins via MHC class I as well. This will result in a stronger inflammatory response and consequently an induction of a more potent immunity against the Ad-based vaccine. In addition, the finding that integrin binding is important in the uptake of Ad-immune complexes might indicate a novel application of the AdΔRGD. The use of these vectors will limit the MHC class I response and as a result, reduced cell-mediated immunity against the Ad vector may occur. More *in vitro* and *in vivo* studies are required to confirm the importance of ADE in the context of Ad uptake and induction of the immune response.

Paper III. CAR regulation during inflammation

"Cytokine-mediated downregulation of coxsackievirus-adenovirus receptor in endothelial cells."

Endothelial cells form a barrier between the circulatory system and the tissue, and have the ability to change the permeability of the barrier in response to injury or inflammation (Leopold, 2003). Physiological changes in endothelial cells involve changes in intracellular proteins as well as cell surface proteins, especially proteins that mediate cell-cell interactions (Carlos and Harlan, 1994). As a putative cell-adhesion molecule, we hypothesized that CAR might exhibit physiological regulation during inflammation.

One well-characterized and studied model system for the regulation of cell surface proteins is the response of endothelial cells to inflammatory cytokines. Exposure of human umbilical vein endothelial cells (HUVEC) to inflammatory cytokines leads to a coordinated series of well-described changes in protein composition at the cell surface that promotes endothelial cell-leukocyte interaction including the upregulation of ICAM, VCAM, E-selectin, and P-selectin (Bevilacqua, 1993; Carlos and Harlan, 1994). At the same time, interactions between endothelial

cells are diminished through the downregulation and/or redistribution of tight and adherens junction proteins including PECAM, VE-cadherin and JAM A (Hofmann et al., 2002; Ozaki et al., 1999; Rival et al., 1996; Romer et al., 1995; Shaw et al., 2001; Stewart et al., 1996; Wojciak-Stothard et al., 1998; Wong et al., 1999). Given the characteristics of CAR as cell adhesion protein expressed at tight junctions we postulated that CAR might be downregulated following treatment with inflammatory cytokines in endothelial cells. To test this, we utilized HUVEC treated with the inflammatory cytokines tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), common agents used in *in vitro* model systems for the study of inflammation (Bradley and Pober, 1996; Pober et al., 1986; Stolpen et al., 1986). Treated HUVECs were then assessed for CAR expression, Ad binding and Ad gene expression.

Both TNFα and IFNγ were found to downregulate CAR expression at the mRNA and protein levels. In addition, reduced CAR protein resulted in decreased Ad binding and transgene expression. The effects of the cytokines were synergistic, since treatment with both cytokines produced a larger effect than treatment with either cytokine alone, for each parameter measured including cell surface CAR expression level (flow cytometry), total cellular CAR protein (Western blot analysis), CAR mRNA level (semi-quantitative PCR), and Ad-mediated gene expression (β-gal transgene assay). This synergistic effect of the cytokines correlated with the conversion of HUVEC from cobblestone to spindle-shaped morphology and with an upregulation of the cell surface protein ICAM.

The decrease in CAR mRNA and protein showed time dependence with a progressive decrease in CAR levels observed after 24 to 48 hrs of cytokine treatment. The decrease in CAR message and protein showed that the amount of CAR protein and mRNA in HUVEC decreased approximately 4-fold compared to normal levels following a 24 hr treatment with a combination of TNFα and IFNγ. After a 48 hr treatment, the effect was even more pronounced and CAR mRNA and protein were decreased approximately 20-fold. The correlation in the decrease in CAR protein and mRNA levels suggests that cytokine-induced downregulation of CAR is principally controlled at the level of transcription of CAR mRNA. To determine whether the cytokine-dependent downregulation of CAR was specific for macrovascular cells such as HUVEC, we treated microvascular endothelial cells derived from lung with TNFα and IFNγ and evaluated CAR protein levels and susceptibility to Ad infection. Similar to HUVEC, CAR levels were decreased as well as susceptibility to viral infection in microvascular endothelial cells. We further wanted to investigate whether the downregulation in response to inflammatory cytokines was cell-type dependent and we therefore analyzed both primary and transformed epithelial cells treated with TNFα and IFNγ. The results obtained from the primary epithelial cells differed from those of the endothelial cells. We could detect a downregulation of CAR protein

levels corresponding to decreased susceptibility to Ad infection in the presence of IFNγ alone or the combination of both cytokines. However, TNFα had no effect on CAR expression and Ad mediated gene transfer. In A549 cells, a transformed lung epithelial cell line, the combined treatment of cytokines induced a moderate increase in transgene expression. The strong suppression of CAR protein to almost undetectable levels occurred only in endothelial cells derived from the macro and microvasculature.

Currently, very little is known about posttranslational modifications and transcriptional regulation of CAR in both endothelial and epithelial cells. It can be postulated that downregulation of CAR might occur by similar mechanisms as other junctional proteins such as VE-cadherin and JAM since their response to inflammatory cytokines is similar to that observed for CAR. The first response to inflammatory cytokines occurs within the first hours of stimulation and has been suggested to involve mainly posttranslational modifications such as tyrosine phosphorylation (Dejana, 2004). Treatment with TNFα will lead to an increase in tyrosine phosphorylation of VE-cadherin, which causes it to be sequestered away from the adherens junctions (Angelini et al., 2006; Nwariaku et al., 2002; Wong et al., 1999). This results in a disassembly of adherens junctions, rearrangement of the cytoskeleton, and increased permeability. In addition, several signaling pathways are induced including JAK-STAT, p38 and NF-κβ (Angelini et al., 2006; Nwariaku et al., 2002). We believe that these signaling pathways are not only responsible for the rapid response initiated by the cytokines but also for downregulation of junctional proteins at a transcriptional level. For our purpose, we performed an *in silico* analysis of the upstream region of the CAR gene and interestingly, the CAR gene contains several NF-κβ and STAT binding sites, suggesting that the CAR promoter may be responsive to these transcription factors. The difference in effect on CAR expression, observed between the epithelial and endothelial cells in response to inflammatory cytokines, raises the question if there are cell-type specific mechanisms regulating CAR.

In addition, this study may carry important implications for patients receiving gene therapy. A variety of patients with afflictions including rheumatoid arthritis, adult respiratory distress syndrome, and skin or gastrointestinal ulcers, have been documented to have locally elevated levels of TNFα and/or IFNγ ranging from 0.5 to 10 ng/ml (Carty et al., 2000; Grayson et al., 1993; Saxne et al., 1988; Suter et al., 1992; Wallace and Stacey, 1998). Based on our study, the levels of cytokine expression in these clinical settings may be sufficient to alter CAR-mediated gene transfer to endothelial cells at the site of inflammation.

Paper IV. CAR regulation in cancer

*"A Snail-Smad transcriptional repressor complex promotes TGF*β*-mediated epithelial mesenchymal transition."*

Cancer is one of the major causes of death in the western world and about ninety percent of these deaths are as result of metastasis (Christofori, 2006). For the majority of cancers that are of epithelial origin, invasion and subsequent metastasis requires disassembly of junctions between epithelial cells. This capacity for disassembly is linked to an altered cellular phenotype with increased migratory and invasive properties, and is referred to as epithelial mescenchymal transition (EMT) (Thiery, 2003). E-cadherin was the first junctional protein shown to be suppressed during EMT. Several *in vitro* and *in vivo* studies demonstrated that the presence of E-cadherin is crucial for the maintenance of epithelial architecture (Batlle et al., 2000; Cano et al., 2000) and conversely, a loss of E-cadherin has been associated with tumor progression. In the last few years, other junctional proteins such as claudin and occludin have also been shown to be repressed during EMT.

Based on its localization to tight junctions and its downregulation in several types of cancers, CAR may be functioning as a tumor repressor (Fuxe et al., 2003; Jee et al., 2002; Li et al., 1999; Miller et al., 1998; Okegawa et al., 2000; Okegawa et al., 2001; Sachs et al., 2002). Also some studies have implicated that CAR is involved in regulating migration and growth of tumor cells (Bruning and Runnebaum, 2004; Fok et al., 2007; Okegawa et al., 2000)). The aim of our study was to investigate the regulation of CAR during EMT and to elucidate possible regulatory mechanisms responsible for the repression of CAR observed in many cancers.

EMT can be induced by many different signaling cascades including Ras, TGFβ and Wnt (Thiery, 2003). We utilized two established model systems of stable and transient TGFβ-induced EMT to study CAR regulation. The stable EMT model system used was the EpH4-EpRas-EpXT, where EMT is induced by cooperative actions of TGFβ and Ras (Janda et al., 2002). The non-tumourigenic and well-polarized mouse mammary epithelial EpH4 cells, were transformed with oncogenic H-Ras, creating EpRas cells. The EpRas cells formed rapidly growing tumors in mice and underwent EMT *in vivo* in response to the TGFβ autocrine loop and giving rise to mesenchymal-like EpXT cells. The inducible and transient cell system used in our study was mammary epithelial cells, NMuMG cells which upon TGFβ stimulation undergo EMT (Brown et al., 2004; Piek et al., 1999).

We first analyzed CAR expression in the stable EMT system and we observed that CAR was repressed at the translational and transcriptional levels as assessed by Western blot analysis and quantitative RT-PCR, respectively. During EMT, a switch from the E-cadherin to N-cadherin has been shown both *in vitro* and *in vivo*. The function of this switch is still unknown, but it has been proposed to provide the

tumor with different adhesive properties and improved survival in the microenvironment (Cavallaro and Christofori, 2004; Christofori, 2003). We therefore analyzed E-cadherin and N-cadherin expression in EpH4 and EpXT cells. Indeed, a switch had occurred in the EpXT cells as no E-cadherin could be detected, and this was paralleled by a dramatic increase in N-cadherin as visualized by immunofluorcence microscopy. The E-cadherin expression was completely abolished in EpXT cells in comparison to CAR that displayed low, but detectable expression levels. This indicates that there are some differences between the regulation of CAR and E-cadherin. In addition, infection of EpH4 and EpXT cells with an AdGFP vector revealed that the reduction in CAR expression in EpXT cells correlated with a lower sensitivity to Ad infection. In contrast, EpH4 cells were permissible to Ad infection and showed clear GFP expression. We treated NMuMG cells with TGFβ and observed a downregulation of CAR and E-cadherin as well an elongation of the cells suggestive of EMT. Similar to the stable EMT system, the distribution of CAR had shifted from a continuous to a discontinuous pattern at cell-cell contacts in NMuMG cells after TGFβ treatment. At the same time, E-cadherin staining was greatly diminished at cell-cell contacts.

We next sought to determine the transcription factors that may be involved in the repression of CAR and E-cadherin during EMT. EMT is, as mentioned before, induced by many signals, however, in our stable model system it is the combination of oncogenic Ras signaling with the autocrine TGFβ loop that ultimately induces EMT (Janda et al., 2006). Several transcription factors are induced by these signaling cascades, and of these we chose to focus on Snail and Smads. Snail was chosen because it is stabilized by Ras signaling and is known to be one of the most important transcription factors in inducing EMT (Nieto, 2002). The Smad proteins, including Smad3 and Smad4, are direct targets of TGFβ-induced signaling and have also been shown to be important for EMT in several studies (Nawshad et al., 2005). In the stable EMT system as well as in the TGFβ-treated NMuMG cells, we observed nuclear accumulation of all three transcription factors: Snail, Smad3 and Smad4. To test if these transcription factors indeed could bind to the promoters for our genes of interest, we performed chromatin immunoprecipitation (ChIP) analysis on both EpH4 and EpXT cells. We observed in EpH4 cells that Snail and to a lesser extent Smad4, were associated with the CAR promoter. This was in contrast to the EpXT cells where Snail, high amounts of Smad4, and additionally Smad3, were associated with the CAR promoter. We also performed similar studies on the E-cadherin promoter as this is the classical EMT marker. Indeed, in the EpH4 cells none of these transcription factors were found to be associated with the E-cadherin promoter, but in the EpXT cells Snail, Smad4 and Smad3 were all present. The fact that Snail, Smad3 and Smad4 exhibited physiologically regulated interactions with the CAR and

E-cadherin promoters that correlated with changes in EMT supported a model in which these transcription factors were intimately involved in the EMT process.

Next we performed an *in silico* analysis by searching the promoter regions of both CAR and E-cadherin for putative Snail and Smad binding elements (SBE). In close proximity of predicated Snail sites we found in both promoters, putative SBE sites. This finding prompted us to investigate whether these transciption factors could interact with each other. We performed co-immunoprecipitation experiments and detected an interaction between endogenously expressed Snail and Smad4 in EpXT cells. To further investigate the functional importance of such complexes we performed the following set of experiments. We verified that Snail is transcriptionally repressing CAR, and for these studies we used MCF-7 cells overexpressing Snail. As indicated by both Western blot analysis and immunofluorescence microscopy, CAR protein in these Snail overexpressing cells was undetectable in comparison to the strong signal observed in parental cells. Interestingly, in this study, we also detected increased nuclear accumulation of Smad4 in the Snail overexpressing clones. To confirm the effect of the transcription factors with the CAR and E-cadherin promoters, we took advantage of a reporter assay using the luciferase gene driven by the predicted promoter regions of CAR and E-cadherin. These reporter constructs were co-transfected with Snail, Smad3 and Smad4, separately or in combination and analyzed for luciferase activity. Snail alone could repress both CAR and E-cadherin, while Smad3 and Smad4 by themselves had no effect. The effect of both Smad3 and Smad4 together was moderate. Combination of Smad3 or Smad4 with Snail led to a more extensive repression of luciferase expression. However, the greatest effect was observed when all three transcription factors were present simultaneously and resulted in the most effective repression of the CAR and E-cadherin promoters. The additative effect of combining the three transcription factors suggests the need for TGFβ to cooperate with Ras signaling.

A downstream target of Ras is GSK3β and GSK3β is an important regulator of Snail activity (Thiery, 2003). When Ras is active, GSK3β is inhibited resulting in Snail stabilization. We hypothesized that Snail could function as a molecular switch that renders cells to be more sensitive to TGFβ-induced EMT through its ability to form complexes with Smad 3 and 4. In order to investigate this we treated NMuMG cells with TGFβ in the presence or absence of a specific inhibitor of GSK3β and analyzed the CAR and E-cadherin expression levels. TGFβ-treated NMuMG suppressed CAR and E-cadherin, as was also previously shown. GSK3β inhibitor alone had no effect on either CAR or E-cadherin levels. This was surprising, as inhibition of GSK3β has previously been reported to be sufficient for induction of EMT (Bachelder et al., 2005). However, we did observe, that in the presence of TGFβ, stabilization of Snail by inhibition of GSK3β, the Smad-Snail complex was formed and

provided a more effective suppression of both CAR and E-cadherin.

This study provides important insights into not only the biology of CAR, but also the regulatory mechanisms eliciting EMT. CAR has been proposed to function as a tumor suppressor and is downregulated in several types of cancers. Here, we provide a molecular mechanism that contributes to this repression. Through the identification of three transcription factors; Snail, Smad3 and Smad4 that are able to directly repress CAR transcription, we have shown that CAR expression is regulated, in similar way to E-cadherin and may be necessary for maintenance of an epithelial phenotype. Once disassembly occurs, an event intimately linked to EMT, both tight and adherens junctions are lost resulting in a more migratory and invasive phentype. This finding also provides a molecular explanation for the lack of CAR expression and subsequently poor Ad mediated gene transfer to mesenchymal cells (Kawashima et al., 2003) emphasizing the benefit of retargeting Ad and enabling non-CAR dependent uptake (Tsuda et al., 2003).

In summary, we have found that Snail and Smad transcription factors form a transcriptional repressor complex, which acts to repress junction proteins in TGFβ-induced EMT. This represents a new mechanism of gene repression and may be important for the ability of TGFβ to induce EMT both in development and in carcinoma progression into a more invasive state.

SUMMARY AND FUTURE PERSPECTIVES

This thesis aimed to elucidate how normal and pathological conditions can alter the CAR-dependent and -independent uptake of Ad and subsequently influence the outcome of infection. We found that anti-Ad neutralizing antibodies could not only hinder CAR-dependent Ad infection, but also when bound to the virus, allow Ad to infect cells in a CAR-independent manner. The Ad-antibody complex appeared to enable infection by binding to an alternative receptor namely, the FcγR. Thereby a significant fraction of "extracellularly neutralized" Ad was rescued and could sucessfully deliver genes. Consequently we together with others, have shown that the rate-limiting step in infection is determined by the initial interaction of the virus with a receptor. As such it will be interesting to understand what other means of uptake exist for Ad. We still cannot explain some of the *in vivo* observations concerning the uptake of Ad. For example, ablating CAR-dependent uptake in the liver does not substantially increase the half-life of Ad in the circulation, implying that Ad is able to bind other cellular receptors. Indeed, some recent studies suggest that serum factors such as C4, C3 and blood factor IX can bind Ad and allowing the virus to enter the cell after interaction with an alternative binding partner (Shayakhmetov et al., 2005b; Zinn et al., 2004). It will be intriguing to identify additional factors and the receptors that are responsible for this CAR-indpendent uptake of Ad. Such interactions are not only important for understanding viral infection but also for understanding the immune response elicited by the Ad vectors. Little is known about how Ad infects professional APC such as macrophages and DC and even less with regard to the uptake by neutrophils. The uptake via receptors such as FcγR, which is expressed by most immune cells including lymphocytes, macrophages and NK-cells, might be similar, but the immune response elicited by these different cells will most likely differ. We know that the uptake of Ad triggers several signaling pathways leading to activation of both the innate and the adaptive immune response, but the exact mechanism is still under investigation (Muruve, 2004; Schagen et al., 2004). By gaining a greater understanding of CAR-dependent and -independent uptake of Ad we will not only learn about how to optimize gene therapy protocols and vaccine strategies, but also gain insight into the basic virology of Ad infection.

CAR was originally identified as the receptor for coxsackie and adenovirus. Later the protein was shown to be localized to the tight junctions leading to the hypothesis that it has a physiologic role in mediating cell-adhesion (Cohen et al., 2001b; Philipson and Pettersson, 2004). Cell adhesion is crucial for the maintenance of both the epithelial and the endothelial architecture and during pathological as well as developmental processes, this organization is sometimes lost. Inflammation and cancer are two processes characterized by the disassembly of cellular junctions, and

in our studies we observed that both of these processes coincided with a suppression of CAR and subsequently, limited the uptake of virus. These findings are important as they support the role of CAR as a cell-adhesion molecule and places CAR as a member of the family of cell adhesion molecules, together with the more characterized cadherins, claudin, occludin as well as other members of the CTX family such as JAM. In this context, it is worth mentioning that CAR differs from the other members of the CTX family in that its knock-out results in an embryonic lethal phenotype (Asher et al., 2005; Chen et al., 2006; Chretien et al., 1998; Dorner et al., 2005). Further studies are needed to elucidate the true function of CAR and the generation of conditional CAR knock-out mice will be a useful tool. Also, elucidating the mechanisms that regulate CAR at the transcriptional, translational and posttranslational level are needed to further understand the biological role of CAR.

Today the role of CAR during cancer progression is debated. Expression studies of human cancers have shown both decreases and increases in CAR levels, depending on the type and grade of cancer (Miller et al., 1998; Okegawa et al., 2000, Korn et al., 2006; Martin et al., 2005). In addition, *in vitro* studies have shown that CAR participates in the migration process via binding to tubulin, thereby suppressing tumor growth (Bruning and Runnebaum, 2004; Fok et al., 2007). Our studies provide one molecular mechanism how of CAR is regulationed during cancer pro-gression from low to high-grade malignancies, a process known as EMT. The loss of CAR facilitates the migration and invasion of tumors in a similar manner as loss of other junctional proteins, such as E-cadherin. The regulation of CAR is similar to that of E-cadherin, and an interesting observation in this work was the finding that CAR and E-cadherin are both regulated during EMT by a transcriptional complex composed of both Snail and Smads. This is further corroborated by similarities in the CAR and E-cadherin promoter regions. They are both TATA box-less promoters and several transciption factor sites including sites for Smads, Snail and SP1 are present in both promoters. In addition, CAR and E-cadherin are postulated to be regulated by epigenetic mechanisms such as acetylation. The heterologous expression pattern of CAR in different types and grades of cancers is also observed for E-cadherin, which indicates that the expression of these proteins is dynamic during cancer progression. We speculate that these differences in expression patterns may reflect the prevalence of various signaling cascades in different types and grades of cancers.

Our finding of that Snail and Smads act via the same transcriptional complex raises interesting questions not only concerning the regulation of cell-adhesion molecules such as E-cadherin and CAR but also the general importance of this novel repression complex in cancer progression. Further studies are needed to establish how these transcription factors specifically interact with each other and to explore the possibility that additional factors are part of this transcriptional complex.

This finding would not be surprising since it is well known that Snail interacts and collaborates with Sin3A, HDAC1 and HDAC2 to fully repress E-cadherin (Peinado et al., 2004a). It would also be intriguing to identify other mechanisms and or signals that enable this transcription factor complex to form and also to disassemble.

Both Snail and Smad4 are essential during development in processes such as gastrulation and delamination of neural crest cells (Carver et al., 2001; Nieto, 2002; Sirard et al., 1998; Yang et al., 1998). Knock-out of both transcription factors in mice results in embryonic lethality because the mesoderm cannot form. It is not known whether Smads and Snail work together or independently of each other or whether these transcription factors regulate CAR and E-cadherin during these events. For these questions to be answered, several *in vivo* studies need to be performed. The chick embryo represents an attractive model system for this kind of studies as it provides a rapid read-out and genetic manipulations can rather easily be conducted. By overexpressing or silencing Snail and Smads and evaluating E-caherin and CAR expression it will be possible to determine if the Snail-Smad complex is also repressing these proteins during developmental processes *in vivo*.

CAR's dual function as a viral receptor and cell-adhesion molecule, is a beautiful example of the complex interplay between virology and cellbiology. This thesis work has in part, contributed to a better understanding of this interesting field of research.

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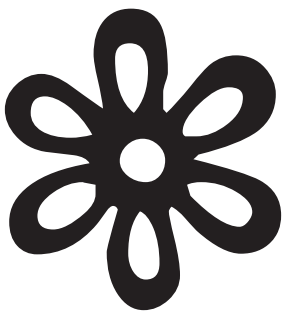

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