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Adenovirus vectors with modified tropism for the treatment of colorectal cancer

Sergio Lavilla-Alonso

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in Haartman Institute, Lecture Hall 1, Haartmaninkatu 3, Helsinki, on May 4th 2012, at 12.00.

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To Noa

If the grape is made of wine, then perhaps we are the words that tell who we are

Eduardo Galeano

TABLE OF CONTENTS

PART A

i. LIST OF ORIGINAL PUBLICATIONS 1
ii. ABBREVIATIONS 2
iii. ABSTRACT 7

PART B

1. REVIEW OF THE LITERATURE 9
1.1. Introduction 9
1.2. Adenovirus biology 11
1.2.1. Viral structure 11
1.2.2. Capsid proteins 11
1.2.2.1. Hexon 11
1.2.2.2. Penton base 13
1.2.2.3. Fiber 15
1.2.2.4. Minor capsid proteins 15
1.2.2.5. Virion core proteins 16
1.2.2.6. Adenovirus genome 17
1.2.3. Life cycle 18
1.2.3.1. Interaction with host cell 18
1.2.3.2. Adenovirus internalization 20
1.2.3.3. Adenovirus uncoating and genome delivery to the cell nucleus 21
1.2.3.4. Genome transcription and replication 22
1.2.3.5. Assembly 25
1.2.3.6. Viral release 25
1.2.4. Immune response to adenovirus infection 25
1.2.4.1. Innate immune response 26
1.2.4.2. Adapted immune response 27
1.3. Human diseases caused by adenovirus 28
1.4. Adenovirus as biological drugs for the treatment of cancer 31
1.4.1. Adenovirus vectors according to the type of modification 33
1.4.1.1. Transcriptional targeting 33
1.4.1.2. Transductional targeting 35
1.4.1.3. Translational targeting 35
1.4.2. Adenovirus vectors according to the final effect on host cells 36
1.4.2.1. Oncolytic adenovirus 36
1.4.2.2. Armed (transgene-expressing) adenovirus 36
1.5. Colorectal cancer 38
1.6. Murine models of cancer 41
1.6.1. Syngeneic models 41
1.6.2. Xenogeneic models 41

1.6.2.1. Metastatic xenogeneic models of colorectal cancer 42
1.6.3. Genetically engineered models (GEM) 43
1.7. Tumor microenvironment as a barrier for adenoviral vector delivery 44
1.7.1. Use of proteases to increase viral spreading 45
2. AIMS OF THE THESIS 47
3. MATERIALS AND METHODS 48
3.1. Cell lines 48
3.2. Adenovirus 48
3.3. Animals 51
3.4. *In vitro* studies 51
3.4.1. Adenovirus transduction assay (I, II, IV) 51
3.4.2. Oncolytic potency in human colorectal cancer cells (I, II, IV) 51
3.5. Mouse models 52
3.5.1. Subcutaneous tumor xenograft models (I, IV) 52
3.5.2. Intra-hepatic tumor model of colorectal cancer (II) 52
3.5.3. Metastatic human colorectal cancer model (III, IV) 52
3.6. *In vivo* studies 52
3.6.1. Biodistribution in a tumor xenograft model of mice (II) 52
3.6.2. Antitumor efficacy in a intra-hepatic tumor model of colorectal cancer (II) 52
3.6.3. Viral replication of adenovirus in intra-hepatic colorectal cancer tumors: in mice (II) 53
3.6.4. Effect of protease on the oncolytic potency of an adenovirus in a *subcutaneous murine* model of colorectal cancer (III, IV) 54
3.6.5. Oncolytic adenovirus efficacy and influence on tumor progression in a metastatic murine model of colorectal cancer (III, IV) 54
3.7. Magnetic resonance imaging 54
3.8. Statistics 54
4. RESULTS AND DISCUSSION 55
4.1. RGD insertion into fiber enhances Ad5 *in vitro* transduction to cancer cell lines (II) 55
4.2. Biodistribution of adenoviral vectors with RGD insertions in the capsid (I, II) 56
4.3. Oncolytic potency of RGD-modified viruses *in vitro* (III) 57
4.4. Antitumor efficacy/efficiency of RGD-modified viruses in metastatic model of human colorectal cancer (II) 57
4.5. Analysis of transgene expression and oncolytic potency of RGD-modified viruses co-administered with proteases *in vitro* (II) 58
4.6. Construction and validation of a new murine model of metastatic colorectal cancer (IV) 59
4.7. Use of the optimized mouse model to study the effects of an experimental treatment (IV) 61
4.8. Effect of protease pre-treatment on adenoviral oncolytic potency in subcutaneous tumors (IV) 62
4.9. Effect of MME on oncolytic efficacy of adenovirus in splenic primary tumors and liver metastases (IV) 62
5. SUMMARY AND CONCLUSIONS 64
6. ACKNOWLEDGEMENTS 66
7. REFERENCES 70

PART C – ORIGINAL PUBLICATIONS 92

PART A

i. **LIST OF ORIGINAL PUBLICATIONS**

I. Neus Bayo-Puxan, Marta Gimenez-Alejandre, Sergio Lavilla-Alonso, Alea Gros, Manel Cascallo, Akseli Hemminki, Ramon Alemany. *Replacement of Ad5 fiber shaft HSP-binding domain with RGD for improved tumor infectivity and targeting*. Hum Gene Ther. 20(10):1214-21. 2009

II. Sergio Lavilla-Alonso, Gerd Bauerschmitz, Usama Abo-Ramadan, Juha Halavaara, Sophie Escutenaire, Iulia Diaconu, Turgut Tatlisumak, Anna Kanerva, Akseli Hemminki, Sari Pesonen. *Adenoviruses with an $\alpha v\beta$ integrin targeting moiety in the fiber shaft or the HI-loop increase tumor specificity without compromising antitumor efficacy in magnetic resonance imaging of colorectal cancer metastases.* J Transl Med. 23;8:80. 2010

III. Sergio Lavilla-Alonso, Usama Abo-Ramadan, Juha Halavaara, Sophie Escutenaire, Turgut Tatlisumak, Kalle Saksela, Anna Kanerva, Akseli Hemminki and Sari Pesonen. *Optimized mouse model for the imaging of tumor metastasis upon experimental therapy.* PLoS ONE. 6(11):e26810, 2011

IV. Sergio Lavilla-Alonso, Margit Bauer, Usama Abo-Ramadan, Ari Ristimäki, Juha Halavaara, Renee A. Desmond, Deli Wang, Sophie Escutenaire, Laura Ahtiainen, Kalle Saksela, Turgut Tatlisumak, Akseli Hemminki and Sari Pesonen. *Macrophage Metalloelastase (MME) as adjuvant for intratumoral injection of oncolytic adenovirus and its influence on metastases development*. Cancer Gene Therapy. 19(2):126-34, 2011

ii. ABBREVIATIONS

5-FC	5-fluorocytosine
AD	adenoid degeneration
Ad1-51	adenovirus serotype 1-51
ADP	adenovirus death protein
AJCC	American Joint Committee on Cancer
ANOVA	analysis of variance
AP	adenoviral protease
Apc	adenoma polyposis coli
APC	adenomatous polyposis gene
ARD	acute respiratory disease
ATCC	American type culture collection
ATP	adenosine triphosphate
C4BP	C4-binding protein
CAR	coxsackie and adenovirus receptor
CD	cytosine deaminase
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
cDNA	complementary DNA
CoCa	colorectal cancer
CRAD	Conditional replicative adenovirus
CREB	cAMP response element binding protein
CT	computerized tomography
CtBP1	C-terminal binding protein 1

CTL	cytotoxic T lymphocyte
Da	Dalton
DAI	DNA-dependent activator of IFN-regulatory factors
DBP	DNA-binding protein
ECM	extra-cellular matrix
EGFR	epithelial growth factor receptor
ER	endoplasmic reticulum
F	phenylalanine
FCS	fetal calf serum
FIX	factor IX
FX	factor X
GAGA	glycine-alanine-glycine-alanine
GATK	glycine-alanine-threonine-lysine
GCV	ganciclovir
GEM	genetically engineered models
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GON	groups of nine
HEW	histidine-glutamic acid-tryptophan
HSPG	heparan sulfate proteoglycans
HSV-TK	herpes simplex virus thymidine kinase
HVR	hypervariable region
IFN	interferon
Ig	immunoglobulin

IL-1	interleukin 1
IL-1R	interleukin 1 receptor
ITR	internal terminal repeat
KKTK	lysine-lysine-threonine-lysine
LRP	lipoprotein receptor-related protein
luc	luciferase
MHC	major histocompatibility complex
miRNA	micro-RNA
MLP	major late promoter
mm^3	cubic millimeter
MMP	macrophage metalloprotease
MOI	multiplicity of infection
mT	millitesla
mTOR	mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl) -2-(4-sulfophenyl)-2H- tetrazolium
N	asparagine
NK-κβ	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NOD	nucleotide oligomerization domain
NPC	nuclear pore complex
P	proline
PBS	phosphate buffer solution
pDC	plasmacytoid dendritic cell
pfu	plaque forming unit

PML	promyelocytic leukemia
Pol	DNA polymerase
pTP	pre-terminal protein
QPE	glutamine-proline-glutamic acid
RARE	rapid acquisition with relaxation enhancement
Rb	retinoblastoma protein
RES	reticuloentothelial system
RF	radiofrequency
RGD	arginine-glycine-aspartic acid
RLU	relative luminescence units
shRNA	short hairpin RNA
siRNA	small-interfering RNA
T2	transverse relaxation time
TEeff	effective echo time
TLR	toll-like receptor
TNF	tumor necrosis factor
TNM	tumor / nodes / metastases
TP	terminal protein
TR	repetition time
TRAIL	TNF-related apoptosis-inducing ligand
V	valine
VEGF	vascular endothelial growth factor
VP	viral particles
WT	wild-type

x g	times gravity
Y	tyrosine
Å	Ångström
Ψ	packing sequence

iii. ABSTRACT

Despite the rapid advance of cancer research and improvement of conventional therapeutic regimes like chemotherapy, surgery, immunotherapy or radiotherapy, cancer remains a leading cause of death worldwide. Metastatic disease normally represents the most deadly stage and leads to the poorest prognosis. The disseminated location of metastases makes it even more difficult nowadays for existing drugs to target tumors without damaging healthy tissues. The work presented in this Doctoral Thesis is aimed at developing targeted adenoviral vectors and regimes that can be applied to the treatment of cancer, especially colorectal cancer.

Gene therapists have explored widely interactions of the viruses to cancer and normal cells and have proved that molecular modifications in the capsid can unleash striking differences in viral tropism. In this Doctoral Thesis, the utility of arginine-glycine-aspartic acid (RGD) targeting $\alpha_v\beta$ integrins substituted for the lysine-lysine-threonine-lysine (KKTK) domain of the fiber shaft or inserted in the HI-loop of adenovirus serotype 5 (Ad5) was evaluated for increased tumor targeting and antitumor efficacy. Both modifications increased gene transfer efficacy in colorectal cancer cell lines and improved the tumor to-normal ratio after systemic administration of the vector. Furthermore, antitumor potency was not compromised with RGD modified viruses suggesting that an increased safety profile did not involve any loss of therapeutic effect.

Treatments based on adenovirus vectors should not have negative effects on tumor progression or metastases. In order to evaluate this possibility, we designed a novel murine model of human colorectal cancer (CoCa) to test our treatments. To this end, we have developed a readily imageable mouse model of colorectal cancer featuring highly reproducible formation of spontaneous liver metastases derived from intrasplenic primary tumors. We optimized several experimental variables, and found that the correct choice of cell line and genetic background of the recipient mice as well as their age, were critical for the establishment of a useful animal model. A magnetic resonance imaging (MRI) protocol was optimized for use with this mouse model, and demonstrated to be a powerful method for analyzing the antitumor effects of an experimental therapy.

Poor spreading of the virus through tumor tissue is one of the major issues limiting efficacy of oncolytic adenoviruses, even after local administration by intratumoral injection. In this study, ECM-degrading proteases relaxin, hyaluronidase, elastase, and macrophage metalloelastase (MME) were used to increase oncolytic adenovirus spreading. Moreover, MME improved the overall antitumor/antitumour efficacy of oncolytic adenovirus in subcutaneous HCT116 xenografts. In a liver metastatic colorectal cancer model, intratumoral treatment of HT29 primary tumors with MME monotherapy or with oncolytic adenovirus inhibited tumor growth. Combination therapy showed no increased mortality in comparison to monotherapies. In addition,

our work demonstrated for the first time in a metastatic animal model that MME, as a monotherapy or in combination with an oncolytic virus, does not increase tumor invasiveness. Co-administration of MME and oncolytic adenovirus may be a suitable approach for further optimization of metastatic colorectal cancer treatment.

To summarise, we described how RGD moieties inserted in the fiber protein are capable of improving tumor targeting of wild-type or capsid-modified adenovirus vectors. We also showed that MME is a safe coadjuvant to be used in combination with oncolytic adenoviruses for intratumoral administration and we presented a highly optimized mouse model for liver metastasis of colorectal cancer.

PART B

1. REVIEW OF THE LITERATURE

1.1. INTRODUCTION

In 1953, Dr. Wallace Rowe was trying to find a causal agent for common flu in children adenoid tissue samples and isolated an unknown etiologic agent, probably a virus (Rowe et al. 1953). Subsequently, Dr. Harold S. Ginsberg succeeded in maintaining it in a established cell culture (HeLa cells) demonstrating its capability to make important morphological changes in cells, which were thought to represent viral replication (Ginsberg, 1999). Soon, it was confirmed that the new virus corresponded with the etiological agent for large number of cases of an acute respiratory disease among army recruits from different forts in the United States.

The structure was proposed in 1959 (Horne, R.W., Brenner, S., Waterson, A.P., Wildy, P. 1959): the virion having DNA genome was described as an icosahedral particle with one fiber protein: at each apex that produced infections by interaction with host cells through its fibers. Several names were given to the new agent: adenoid degeneration (AD), adenoid-pharyngeal conjunctival or acute respiratory disease (ARD), but finally consensus was established and the new virus was called Adenovirus (Enders et al. 1956).

Adenoviruses are classified within the *Adenoviridae* family, that includes four *genera*: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus* and *Siadenovirus*. *Mastadenovirus* is found in mammals, while *Aviadenovirus* has been isolated from birds. The last two *genera* include a wide range of host species. The only adenovirus found in fish is classified in a fifth clade (see figure 1) (Benko, Harrach 1998, Kovacs et al. 2003, Benko et al. 2002). The host most frequently infected by each virus species is indicated with a letter at the beginning of the name, while the last letter distinguishes the species within each genus. Sometimes, a number can be added at the end to indicate the serotype. For example, BAdV-B mostly infects bovine hosts and belongs to genus Mastadenovirus species B(Benkő, M., Harrach, B., Russell, W. C. 2000).

Human adenoviruses include 6 species: A, B, C, D, E and F according to the capability to agglutinate erythrocytes of different species and the oncogenicity they present in rodents. Species B can be subdivided into B1 and B2. Although species are not classified according to their tissue tropism, for example, species B1, C and E mainly invade the respiratory system, while species B, D and E can induce ocular diseases. In parallel, human adenoviruses can be divided into 51 different serotypes according to how they are neutralized by a specific antisera (Russell, 2009). Species A includes Ad12, 18 and 31; species B: Ad 3, 7, 11, 14, 16, 34, 35 and 50; species C:

Ad1, 2, 5 and 6; species D: Ad8-10, 13, 15, 17, 19, 20, 22-30, 33, 36-39, 42-49 and 51; species E: Ad4; species F: Ad40 and 41(Berk AJ 2007).

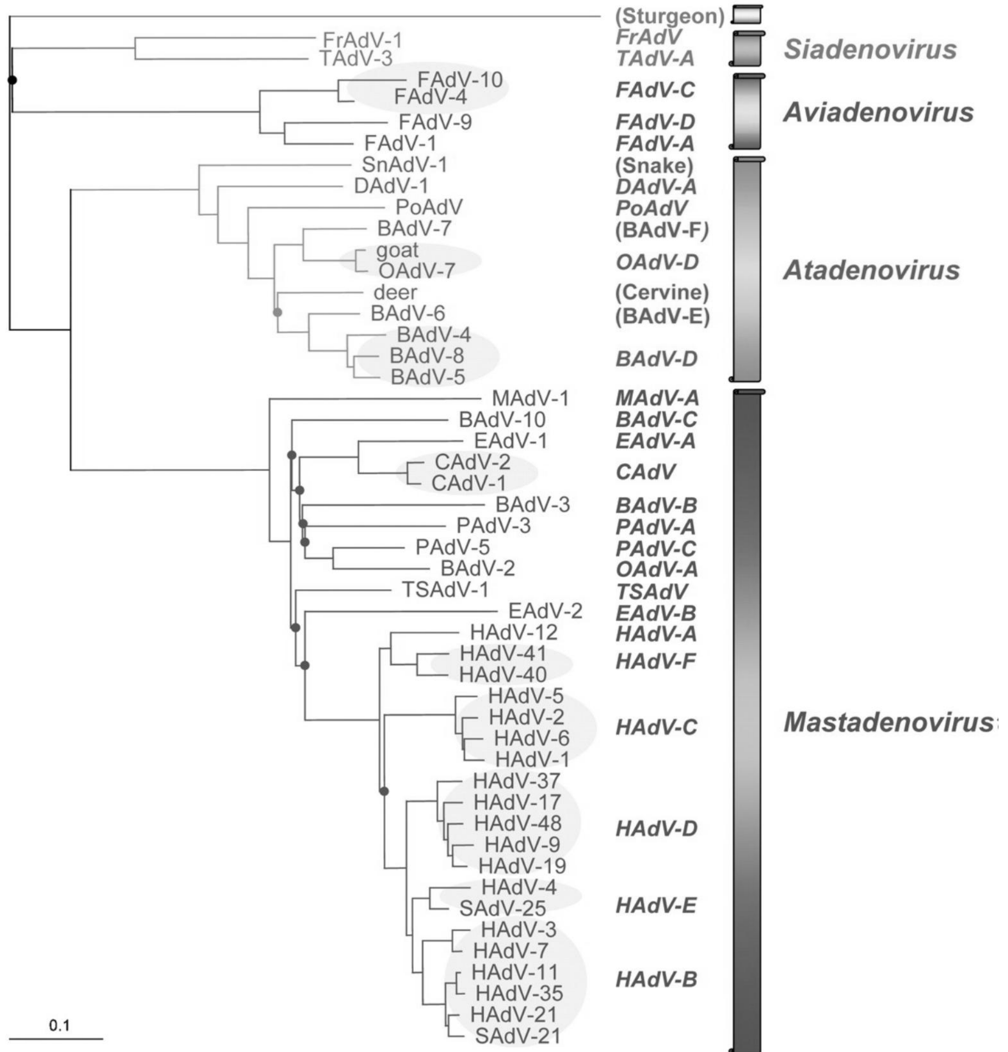

Figure 1: Phylogenetic tree of *Adenoviridae* family

Adapted from Davison et al., 2003 (Davison, Benko & Harrach 2003)

1.2. ADENOVIRUS BIOLOGY

1.2.1. Viral structure

Adenoviruses are non-enveloped viruses with double-stranded lineal DNA genome. They measure around 90 nm in diameter and have an approximate mass of 150 x 10^6 Da. Viral capsid proteins comprise 87% of the mass, while the rest (1%) comes from the genome. Adenovirus capsid is composed of three major proteins: hexon, penton base and fiber proteins. These proteins are assembled together with four minor structural proteins: proteins IIIa, VI, VIII and IX (Berk AJ 2007). See figure 2 for a complete understanding of location and organization of the structural proteins. The lineal DNA genome measures around 36 kb and is tightly packed inside the capsid by four proteins: V, VII, μ, IVa and the terminal protein (TP). It also contains several copies of adenoviral protease (AP) (Russell, 2009).

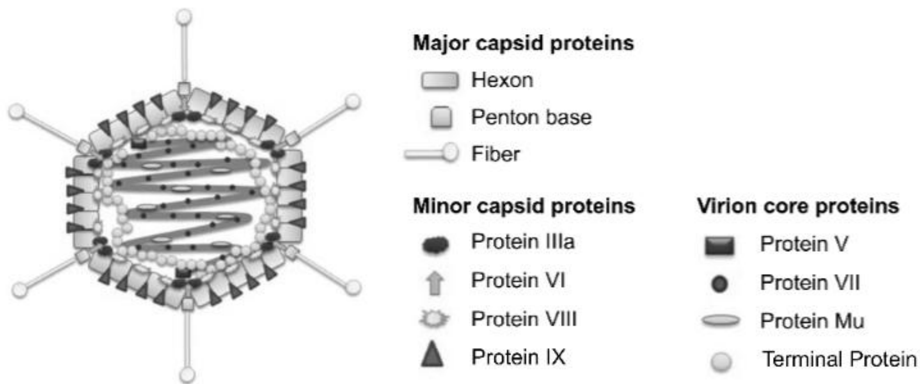

Figure 2: Structure of *Adenovirus sp.*

Structural proteins in a *Adenovirus sp.* particle. Modified from Chailertvanitkul et al, 2010 (Chailertvanitkul, Pouton 2010)

1.2.2. Capsid proteins

1.2.2.1. Hexon

The Hexon protein or polypeptide II is located at the 20 facets of the icosahedral capsid and represents 63% of the adenovirus molecular mass. The size of the hexon varies between serotypes, the biggest hexon capsomers are 967 aa and are found in Ad2 (Russell, 2009). 240 hexon capsomers are present in every adenovirus particle and present a pseudo-hexagonal conformation that is surrounded by six other elements, either hexon or penton capsomers (Berk AJ 2007, Rux, Burnett 2004).

The hexon is a trimeric protein with three repetitions of two similar domains (V_1 and V_2) assembled in a characteristic structure known as β-barrels (see figure 3A).

Such a conformation is found in many spherical viruses and provides important resistance to proteolysis. While the base of the protein is highly conserved, the top part ends in three "tower" domains that contain up to nine hypervariable regions (HVR) in total. These regions are related to the antigenicity of the hexon and are most responsible for the raise of neutralizing antibody activity over the adenovirus, thus determining the viral serotype (Rux, Burnett, 2004; Berk AJ, 2007; Russell, 2009).

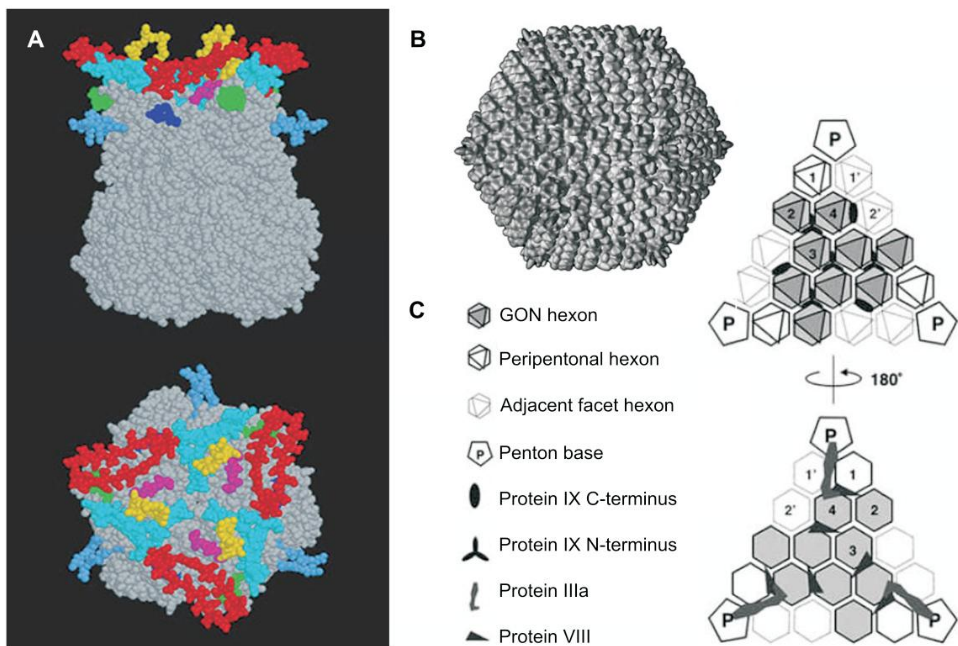

Figure 3: Hexon protein

A: Trimeric hexon protein structure from the side (upper) and from the top, i.e. external side of the virion (lower). Equivalent monomers are depiced in the same color modified from Roberts et al., 2006. B: External view of the particle showing the hexon proteins organization (adapted from Rux et al., 2004). C: Organization of the different types of hexons with other structural proteins in the viral particle facet (modified from Campos et al., 2007.

There are four types of hexons, located in specific parts of each facet and subjected to very different environments. A single adenoviral particle contains six H1 hexon capsomers that are associated with the twelve pentons located at the apices and H2, H3 and H4 commonly named "groups of nine (GON)" that are situated at the

center of the facets and only interact with other hexons and structural proteins, but never with penton capsomers (figure 3C) (Russell, 2009).

Owing to high level of conservation of the hexon base, it is possible to create chimeric viruses by the replacement of hexons for other serotypes, with the aim of avoiding serotype-specific neutralization by antibodies and gaining tolerance to repeated administration of adenovirus-based vectors (Wu et al. 2002a; Tian et al., 2011). For the same purposes, specific loops in the variable region can be substituted for different serotypes (Gall, Crystal & Falck-Pedersen 1998, Roy et al. 1998).

1.2.2.2. Penton base

Penton is a covalent complex of two proteins: the homopentameric penton base (polypeptide III) and the homotrimeric fiber (polypeptide IV). Its name is due to its capability to bind to five different capsomers at a time. Penton base proteins are located on the twelve vertices of the icosahedrons and are responsible for the attachment and internalization of the virus to the host cell (Berk AJ, 2007; Nemerow, Stewart 1999).

The penton base monomer is comprised of 471 aminoacids for Ad2. Its structure was resolved by Stewart et al. (Stewart et al. 1991) as a complex of two domains: a basal jelly-roll domain and an upper unit with irregular folds as well as two arising loops. The first loop contains the RGD (Arginine – Glycine – Aspartic Acid) motif loop and the second loop, also called the variable loop, differs in its length and characteristics depending on the serotype (figure 4B). The RGD motif is necessary for fiber interaction with host cell surface α_v integrins, an essential step for the internalization of adenovirus subgroups A, B, C and E (Russell 2009; Rux, Burnett 2004; Zubieta et al. 2005). See figures 4A, 4B and 4C.

Pentamerization of the penton base provides stability, since it hides hydrophobic surfaces, and creates a central hole of around 30 Å diameter that is filled by the fiber protein. The binding of fiber and penton base occurs through the fiber motif FNPVYPY, located in five equivalent fiber-binding sites in the penton base (Rux, Burnett 2004; Zubieta et al. 2005).

In the absence of other virion components, some pentons can assemble by themselves and create dodecahedral complexes. Interestingly, in many serotypes, penton base proteins are produced in excess, so dodecahedral complexes efficiently enter the cells and accumulate at the nuclear membrane (Schoehn et al. 1996, Fender et al. 2005, Zubieta et al. 2005).

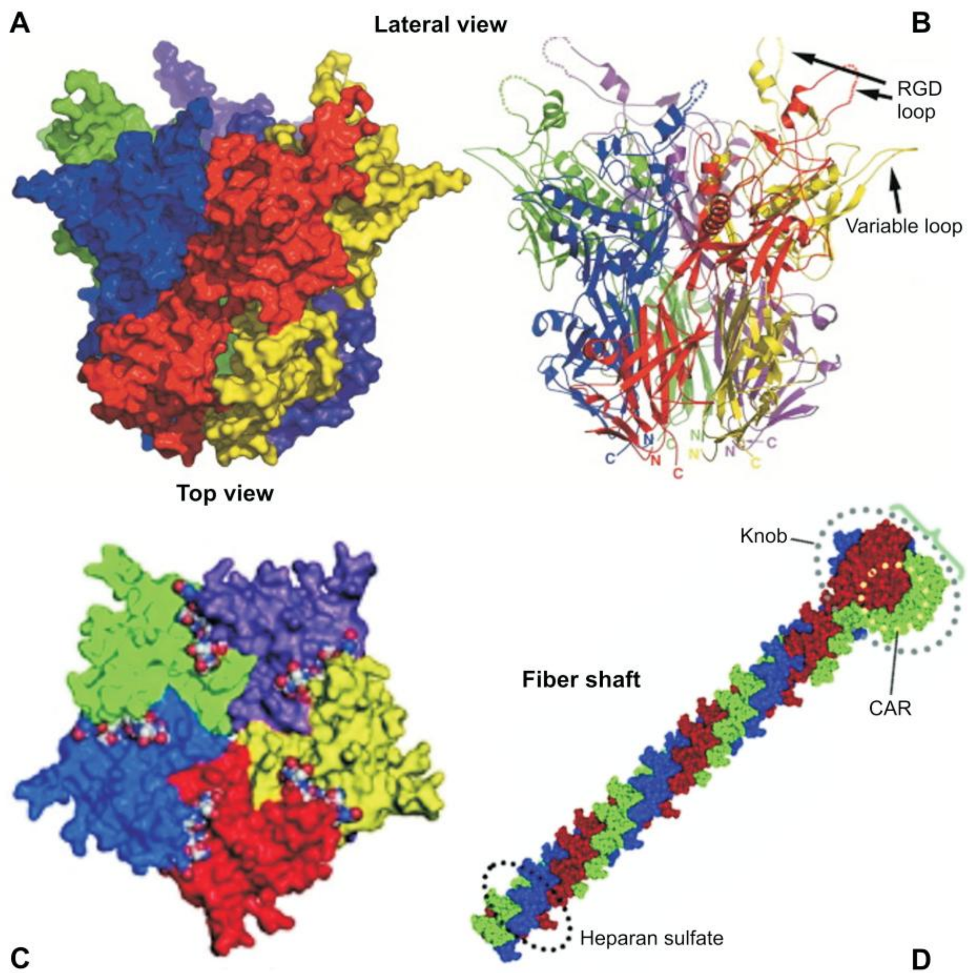

Figure 4: Penton and fiber proteins

Lateral (A) and top (C) view of the penton protein and the location of the RGD and variable loops (B). Fiber protein, its two domains: knob and shaft and binding *loci* to CAR and heparan sulfates (D). A and B have been adapted from Zubieta et al., 2005. C and D were modified from Zhang and Bergelson, 2005

1.2.2.3. Fiber

The fiber protein consists of 582 residues. This protein plays a significant role on virion structure stabilization, hence virions lacking fibers are less stable and leak DNA out of the capsid (Zubieta et al. 2005; Von Seggern et al. 1999).

The fiber protein is trimeric and divided in three different domains: a tail that attaches to the penton base in the N-terminus, a fiber shaft with around 15 repeated motives and a globular knob domain in the C-terminus that plays a central role in cell surface protein recognition (Rux, Burnett 2004).

The N-terminus of the trimeric protein binds to the pentameric penton base. This symmetry mismatch caused much controversy in the scientific community until the discovery that the binding of the fiber triggers conformational changes in the penton base that make the binding to exactly three fiber proteins exclusive. This binding is mediated by the FNPVYPY sequence, with the complementation of several hydrogen bonds and a salt bridge (Zubieta et al. 2005; Russell 2009).

The fiber shaft consists of six (in Ad3) to 22 (in Ad2 and Ad5) sequence repetitions (Berk AJ 2007) arranged in a unusual triple β-spiral fold topology. The fiber knob structure contains eight-stranded β-barrels that form the core of each subunit, with a central depression and three valleys in a conformation that differs importantly from the hexon and penton. The binding to cell membrane components depends on several loops named DG, HI and AB, all emanating from the knob domain (figure 4 D). The Binding of Ad5 to different cell receptors will be further discussed in the section 1.2.3.1 (Russell 2009).

1.2.2.4. Minor capsid proteins

Minor capsid proteins (IIIa, VI, VIII and IX) are also called cement proteins because of their role in assembling and keeping the rest of structural proteins together. In addition to that, they are involved in the efficient disassembly of the virion during infection. Unfortunately, their structure is not yet well-known, for only a few crystallographic data are available (Rux, Burnett 2004).

Protein IIIa is a 570 aa (for Ad2) polypeptide positioned below the penton base, on the exterior of the capsid. Its helical structure-based N-terminal binds at the same time to the penton base, hexons and protein VI. By doing so, it stabilizes the interface between facets (Rux, Burnett 2004; Russell 2009).

Polypeptide VI (500 aa for Ad2) contains two long α-helices, one of them binds to the cavity in the base of the hexon and to protein III at the apices. Its distribution in

the capsid is not clear: the low number of VI per viral particle suggests a non homogenous distribution within the facet but a exclusive binding to peripentonal hexons (Russell 2009; Rux, Burnett 2004; Fabry et al. 2005). By this way, protein VI plays a cementing role during viron assembly. Its N-terminus is basic and interacts with the nucleic acid inside the capsid, while the C-terminus is an activator of the adenovirus protease (AP). This mechanism ensures that hexon, DNA and cement protein are spatially and temporally close before the assembly of the capsid vertexes (Mangel, Baniecki & McGrath 2003).

The 140 aa (for Ad2) protein VIII is probably located in the inner side of the capsid in two different positions: five copies surround the peripentonal (H1) hexons connecting them to the GONs, while three copies are located around the axes and stabilize the GONs (Russell 2009, Rux, Burnett 2004; Fabry et al. 2005).

Polypeptide IX (140 residues for Ad2) is located in the center of central hexon interfaces in the exterior surface of the capsid. Its structure is based on the high propensity to arrange itself in coiled coils. The N-terminus of protein IX is responsible for the attachment to the capsid and its stabilization (Russell 2009). Meanwhile, the C-teminus interacts with the HVR-4 loop of the hexon and is involved in other roles related to viral replication. For example, it binds to TATA-containing promoters, acting as a transcriptional activator of adenovirus late genes. Another property of IX is to form amorphous inclusions, which are structures that block interferon-mediated antiviral activity of the host cell (Rux, Burnett 2004, Parks 2005). Protein IX has been extensively modified to generate fusion proteins for gene therapy applications (Meulenbroek et al. 2004; Parks 2005).

1.2.2.5. Virion core proteins

The virus core includes the viral genome plus structural proteins V, VII, μ and terminal protein (TP). Some authors also include polypeptide IVa2 and the adenovirus protease (AP) in this group (Russell 2009, Berk AJ 2007).

Polypeptides V, VII and μ are basic proteins (rich in arginine residues) so they can attach to DNA and condense it within the core. Polypeptide VII is the major core protein (800 copies per virion) and mainly responsible for DNA organization into a condensed nucleoprotein. Protein V is strongly associated with the capsid protein VI, polypeptide VII and viral DNA, thus providing stabilization of the core nucleoproteins and the capsid. μ is an arginine-rich DNA interacting protein, but its disposition in the core is not clear. Protein IVa2 binds to a specific region of the viral DNA and has a critical role in its packaging process. Similarly, terminal protein (TP) interacts selectively with the 5′terminus of the DNA strand (Russell 2009, Berk AJ 2007).

Adenoviral protease (AP) or adenain contains two different domains and a polar interface (McGrath et al. 2003). It is non-specifically activated by binding to viral DNA, but requires binding to protein VI C-terminus to achieve its optimal activity. This protease is necessary to cleave capsid precursor to mature structural proteins IIIa, VI, VII VIII, TP and μ in order to complete the encapsidation process (Russell 2009, Mangel, Baniecki & McGrath 2003; McGrath et al. 2003).

1.2.2.6. Adenovirus genome

Adenovirus 2 was the first adenovirus to be completely sequenced. Some years later, Adenovirus 5 sequence was also described (Chroboczek, Bieber & Jacrot 1992). Adenovirus genome consists of a linear double-stranded DNA of around 36 kb. It is flanked at both ends by two identical inverted terminal repeats (ITR) ranging in size from 36 to more than 200 bp depending on the adenovirus species (Berk AJ 2007). At the 5′-end of each ITR a 55 kDa terminal protein (TP) is covalently bound and acts as replication initiator, being the ITRs origins of replication. Other elements necessary for replication are DNA polymerase (pol) and the DNA binding protein (DBP) (de Jong, van der Vliet & Brenkman 2003, King, van der Vliet 1994). A *cis*-acting packaging sequence, named Ψ, is essential for viral encapsidation. It is located between the left terminal repeat and the first protein coding region (E1A) (Hearing et al. 1987).

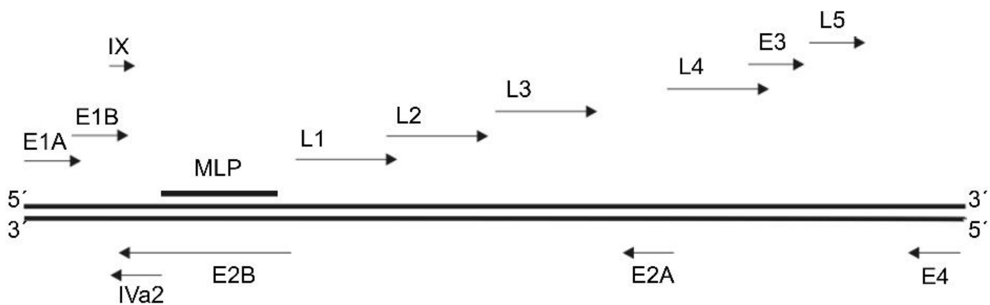

Figure 5: *Adenovirus sp.* transcription map

Adenovirus genome includes eight transcription units grouped in five early transcription units, two intermediate transcription units and one late transcription unit, under the control of the Major Late Promoter (MLP). The early transcription unit is depiced, by consensus, on the left of the transcription map.

The adenoviral genome is divided into transcription units: five early transcription units (i.e. E1A, E1B, E2, E3 and E4); three delayed early transcription units (i.e. IX, IVa, E2 late) and one late transcription unit, under control of the major late promoter (MLP) that is processed to five families of late mRNA: L1-L5 (see figure 5). This disposition of the different genes along the genome is responsible for the development of the adenovirus infectious cycle in two subsequent phases, as will be further discussed in the section 1.2.3.4. (Berk AJ 2007; Russell 2000). While DNA sequences encoding most structural proteins and proteins involved in viral replication and assembly are largely conserved between all adenovirus species, other DNA sequences are *genus*-exclusive (Davison, Benko & Harrach 2003). By consensus, the genome map is drawn with the E1A gene at the left end (figure 5).

1.2.3. Life cycle

Adenovirus infects eukaryotic cells through interaction with cell surface receptors. The viral genome is internalized to the cell by an endocytic process and delivered to the nucleus by active transportation through microtubules (figure 6). Viral replication occurs in two subsequent phases. Release of new virions is mediated exclusively by cell lysis. The complete life cycle of Adenovirus serotype 5 will be described in the following sections 1.2.3.1 – 1.2.3.6.

1.2.3.1. Interaction with host cell

Cell attachment by Ad5 is mainly mediated by its interaction with Coxsackie and Adenovirus Receptor (CAR). CAR is a member of the immunoglobulin (Ig) superfamily with two Ig-like domains, a transmembrane anchor and a cytoplasmic tail of 107 aminoacids (Bergelson et al. 1998) (figure 6A). CAR expression is mainly associated with tight junctions in the basolateral surface of polarized epithelial cells. This is why it is unlikely that natural adenovirus infections start by binding to CAR in tight junctions; it is more likely that infection starts either in subpopulations of non-polarized cells that expose CAR to the luminal membrane or through lesions present before the adenovirus infection that will expose the basolateral membrane to adenovirus (Meier, Greber 2004). In any case, after viral infection, viable particles, together with an excess of fiber proteins, are released to the extracellular space where they interact with CAR and produce the disruption of tight junctions (figure 6A). Consecutively, the morphology of epithelial cells changes to a discontinuous and abnormally permeable layer, through which thradenovirus can easily reach the apical compartment (Walters et al. 2002).

The first interaction of the viral particle with the cell occurs through CAR binding to the fiber knob. Interestingly, CAR does not interact with the distal extreme of the fiber knob but with several loops located on the sides. Specifically, the Ig-like

domain of CAR binds to the AB loop that is highly conserved among the adenovirus species that use CAR (i.e. species A and C) (Nemerow et al. 2009). Given the location of the AB loop in the lateral part of the knob, fiber flexibility is essential for optimal CAR binding, as well as the length of the fiber shaft that depends on the number of β-repeat elements it possesses. Several studies estimate that the fiber should contain between 5.5 and 22.5 elements to permit optimal binding (Shayakhmetov, Lieber 2000).

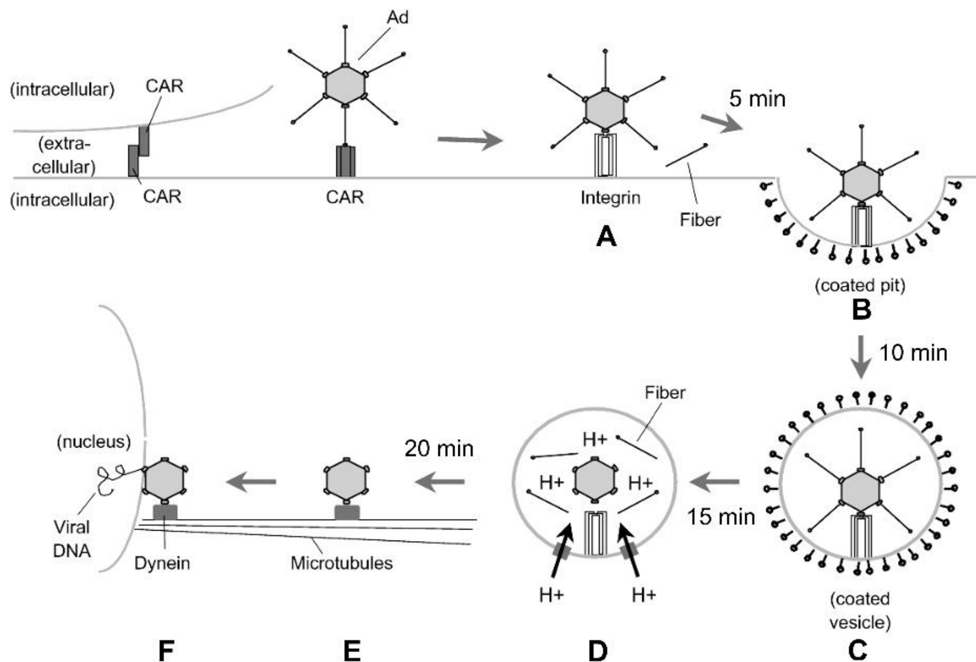

Figure 6: Infection and DNA transfection by Adenovirus particles

Interaction of fiber knob with CAR receptor followed by binding of penton base to cell surface integrins (A). Viral internalization through clathin-coated vesicles (B) and formation of an endosome (C). Degradation of the viral capsid occurs mainly in the endosome and is accompanied by a pH decrease (D). Viral particles are translocated to the perinuclear compartment by the dynein protein through the microtubules (E-F). Adapted from Medina-Kauwe et al., 2003

Immediately after CAR binding, a second interaction occurs: α_v integrins on the host cell external surface bind to the RGD domain that is located in one of the variable loops of the penton base. Once again, the fiber needs to be flexible enough to guarantee a correct binding (Russell 2009, Nemerow et al. 2009). Rather than

enhancing virus attachment, penton-integrin binding promotes virus infection by activating internalization through clathrin-coated vesicles (Russell 2009, Nemerow et al. 2009) (figure 6B).

Unexpectedly, studies done with adenovirus with mutated CAR-binding domains in the AB loop showed significant decrease for *in vitro* infectivity, but a similar rate of *in vivo* transfection to hepatocytes (Alemany, Curiel 2001, Smith et al. 2002).This casted many doubts on the theory of a unique role of CAR-integrin receptors in the adenovirus infection and suggested that an alternative route for adenovirus entry existed. Indeed, Ad5 can also infect cells by the interaction of its KKTK domain, located in the third β-repeat of the fiber shaft, with cell heparan sulfate proteoglycans (HSPG) (Dechecchi et al. 2001, Dechecchi et al. 2000). It has been shown that ablation of the KKTK domain, by mutation to GAGA, dramatically reduces *in vivo* delivery to hepatocytes (Smith et al. 2003, Rittner et al. 2007, Bayo-Puxan et al. 2006). However, the data concerning the role of the KKTK motif in liver cell infection is controversial and some studies reveal that the direct interaction of KKTK domain with HSPG is irrelevant for *in vivo* liver cell infection after systemic administration (Di Paolo, Kalyuzhniy & Shayakhmetov 2007, Rogee et al. 2008). Other alternative explanations for these results with KKTK-ablated chimeric virus are related to the fiber flexibility loss due to structural changes caused by the mutation or alterations in post-internalization (Kritz et al. 2007).

In recent years, data has proved that blood factors,especially factor X, is the main determinant of Ad5 biodistribution *in vivo*, leaving CAR with little or no role. In this case, access to the host cell occurs by non-fiber mediated entry mechanisms, but through binding of factor X to the hexon protein and subsequent binding to HSPG on liver hepatocyes (Alba et al. 2009). Although hexon protein plays a major role on Ad5 initial attachment and transduction at the host cell, binding of integrins to penton base RGD motif is necessary for correct internalization and intracellular transportation to perinuclear compartment. This process is necessary for effective replication of the virus (Bradshaw et al. 2010). Other blood components, like complement system's proteins C3 or C4Bp, lactoferrin and factor IX, have also shown to participate and enhance liver transfection (Shayakhmetov et al. 2005b).

1.2.3.2. Adenovirus internalization

Independently of the receptor used for a first interaction of the viral capsid with the host cell, penton base-integrin binding is necessary for a rapid uptake of the virus into the cell in a process called internalization (figure 6B-D). The integrins that mediate in this binding are subtyes $\alpha_v\beta1$, $\alpha_v\beta3$ and $\alpha_v\beta5$ (Nemerow et al. 2009, Smith et al. 2010, Shayakhmetov et al. 2005a, Nemerow, Stewart 1999, Bradshaw et al. 2010). Integrins are cell membrane proteins that participate in many important

cell biological processes, like cell adhesion, migration, differentiation and proliferation. They are heterodimeric proteins comprised of two non-covalently bound subunits: α and β (Ruoslahti, Pierschbacher 1987). Integrins bind to RGD sequences present in components of the extracellular matrix (ECM) like vitronectin (Ruoslahti, Pierschbacher 1987). RGD motives are conserved in the penton base in most adenovirus serotypes. The spacing of the RGD loops in the penton base permits to maximize the binding properties and allow up to 4-5 integrins to be bound to each pentamer (Nemerow et al. 2009).

Viral internalization triggers several cell responses, such as activation of cell signaling that leads to the polymerization of actin filaments, a process needed for the rapid internalization of the virus into clathrin-coated pits and vesicles (Wang et al. 2000). Adenovirus infection also promotes formation of post-translationally modified more stable microtubules, utilized by adenovirus for their efficient translocation to the nucleus and rapid onset of viral gene replication (Warren, Cassimeris 2007) (figure 6E-F).

1.2.3.3. Adenovirus uncoating and genome delivery to the cell nucleus

After integrin-mediated endocytosis, the virus is still topologically out of the cell. Before being delivered to the nucleus, the viral genome still needs to cross three barriers: the capsid of the virus particle, the endosomal membrane and nuclear envelope. Uncoating is described as the dissolution of the first barrier, the viral capsid, and occurs in several subsequent stages. The first step consists of vertex dissociation. Vertices are structurally weak parts of the capsid. Adenovirus particles are assembled in such a manner that the association between penton and peripentonal hexons is labile. The vertex removal mechanism is not fully known, but it is suspected to be dependent on integrin engagement. It is not known either whether the fiber or the entire penton-fiber complex is removed together (Smith et al. 2010).

After vertex removal, the virus goes through several uncoating steps inside the endosome. This process progresses with loss of peripentonal hexons and proteins IIIa, VIII, IX and VI. The interaction between the peripentonal hexons and the rest of the facet is different to the interphase between hexons within the facets. This explains why central hexon complexes can remain in the endosome after complete uncoating.

The endosomal membrane represents the second barrier for genome delivery. Not until this membrane is permeable, can the genome escape the endosome and make its way to the nucleus. However, how the virus turns this membrane permeable is another point of controversy between scientists. While former studies point to the

penton base as responsible for the unstabilization of endosomal membranes, more modern studies claim that such a hypothesis is not valid (Greber et al. 1996). Protein VI is another candidate to be the architect of endosomal membrane permeabilization. First because it shows lytic activity over cell membranes, and second because its presence is essential for proper genome delivery to the cell through endosomes (Wiethoff et al. 2005). Integrin binding exposes sites in protein VI that are vulnerable to adenovirus protease (AP) cleavage, once AP becomes activated by the reducing environment of the endosomes (Smith et al. 2010). Hence, AP that is responsible for the cleavage and maturation of several capsid proteins, also plays a role in adenovirus entry.

Once the endosome is disrupted and most of the capsid proteins are uncoated and released to the cytoplasm, the hexons interact with dynein, a cytoplasmic protein that permits cytoplasmic transportation of virions along the microtubules towards the nucleus. By the time the subviral capsid reaches the nuclear pore complex, it is mainly composed of hexon proteins and viral DNA: hexon facets are estabilized by protein IX and the viral DNA is condensed by proteins VII, V and X. At this point, protein IX on the hexons of the subviral particle attach to the kinesin-1 light chain, which remains bound to the microtubules, thus releasing the GON trimers. In parallel, some kinesin-1 proteins are removed from the nuclear pore complex (NPC), causing its disruption. This allows the viral DNA import to the nuclear compartment (Strunze et al. 2011). The virus undergoes further uncoating at the nucleus pore and the viral genome is transferred to the nucleus (Leopold et al. 2000, Leopold, Crystal 2007, Strunze et al. 2005). Inside the nucleus, the terminal protein (TP) binds to the nuclear membrane through an interaction with lamin B and the genome is ready to start its replication (Russell 2000).

1.2.3.4. Genome transcription and replication

Adenovirus transcription occurs in two-phases: early and late. As explained in the section 1.2.2.6., the genome is divided into early, intermediate and late transcripted genes.

Early transcripted genes encode for five transcription units: E1A, E1B, E2, E3 and E4. The first viral transcription unit to be expressed is E1A that includes two transcripts and translates proteins 289R and 243R. E1A proteins act as *trans*-activators for other early transcription units, i.e.: E1B, E2, E3 and E4. In general terms, they force the cell to S-phase in order to permit viral replication. They do so by interacting with many cellular proteins, the effects on the Retinoblastoma (Rb) pathway being of special importance for viral vector design. Rb is a tumor suppressor protein whose mechanism of action involves binding and blockage of

E2F, a transcriptional activator required for the onset of S-phase. (McConnell, Imperiale 2004; Russell 2000; Nemajerova et al. 2008).

Table 1: Interactions between adenovirus and host cell proteins that induce activation of the host cell (Berk AJ 2007)

Gene / transcription unit	Adenoviral gene product	Host cell target	Function	Effect
E1A	243 / 12S / small E1A protein	p300 histone acetylase	Activation of E1B promoter	Entry into S-phase
		CREB-binding protein		
	E1A (12S and/or 289/13S/large E1A)	pRb	Release of E2F	
		p107	Release/activation of E2F	
		p130		
		p400 complex	Transcription repression	
		CtBP1		
		MLP	Activation of MLP	Expression of late mRNAs
E1B	E1B-55K	p53	Degradation of p53	Inhibition of cell cycle arrest and apoptosis
E1B	E1B-19K	Bak / Bax	Inhibition of mitochondrial membrane pores formation	Inhibition of apoptosis
E4	E4orf1	PDZ-containing proteins in plasma membrane	mTOR activation	High rate of protein synthesis in absence of mitogens and nutrients
	E4orf4	phosphatase PP2A		
	E4orf6	p73	Transcription activation	Inhibition of cell cycle arrest and apoptosis
		p53	Degradation of p53	
		(association with E1B-55K)	Degradation of MRN	Inhibition of DNA damage response
	E4orf3	PML nuclear bodies	Inactivation of MRN	

Many other gene products co-operate with E1A in different ways. For example, E4orf6 or E1B promote oncogenesis and transformation by inhibiting apoptosis. In fact E1B-19K is an analogue of Bcl-2, a potent inhibitor of the Bax pro-apoptotic protein family. Eventually, cell cycle deregulation by E1A results in accumulation of tumor suppressor p53. In normal circumstances activation of p53 would lead to apoptosis but in adenovirus infected cells E1B-55K blocks p53-dependent apoptosis by directly binding p53 and impedes expression of pro-apoptotic genes. This viral protein also facilitates viral mRNA transportation to the cytoplasm (McConnell, Imperiale 2004). A summary of the most important interactions between virus and host cell proteins mediating host cell activation to replicate viral DNA massively are summarized in table 1 (Berk AJ 2007).

Proteins encoded by the E2 genes are subdivided into those expressed by E2A (DNA-binding Protein (DBP) and E2B (pre-terminal protein (pTP) and viral DNA polymerase (Pol)). They provide the machinery for viral DNA replication and the effective transcription of late genes (Russell 2000).

E3 genes are dispensable for viral replication but play an important role in avoiding host defense mechanisms and enhance persistence in infected cells. One of the E3 gene products is the Adenovirus Death Protein (ADP) that promotes cell cytolysis of the infected cell to force the release of the progeny. Another E3 gene product is E3gp19K that prevents loading of peptides onto the MHC class I molecules to be presented on the cell surface, where they would be recognized by Cytotoxic T Lymphocytes (CTLs) (McConnell, Imperiale 2004).

The E4 transcription unit encodes for proteins that play a role in cell cycle control and transformation by many different mechanisms. For example, E4orf4 estimulates p53-dependant apoptosis, while E4orf6 inhibits p53 biding to transcription factors and, thus, prevents p53 of inhibiting cell transformation. At the same time, it collaborates with E1B-55K to target p53 for degradation. Proteins originated from the E4 transcription unit also facilitate virus mRNA metabolism and promote virus DNA replication and blockage of host protein synthesis. They also contribute to increase resistance to lysis by CTLs (Berk AJ 2007, McConnell, Imperiale 2004, Kaplan et al. 1999). Adenovirus also transcribes a set of RNAs, the VA RNAs, that are not translated but play a significant role in combating cellular defense mechanisms.

The effects caused by early transcription genes start immediately after infection and occur before DNA replication. DNA replication starts from both DNA termini and requires both ITRs as origins of replication. After DNA replication, late transcription results in a set of five transcription units that express structural components of the virus. They are named late transcription genes L1-L5 and

operate under the control of the Major Late Promoter (MLP) (Berk AJ 2007). There is also a peak of IVa2 and IX gene expression and a specific activation of the MLP. Although the encapsidation process is not completely defined, it is clear that late proteins play a crucial role (McConnell, Imperiale 2004).

1.2.3.5. Assembly

Although the encapsidation process is not up to date, fully understood, it seems clear that formation of a pro-capsid, containing structural and non-structural (scaffolding) proteins, is needed, to fully complete DNA packaging. Unfortunately, little is known about the structure of this pro-capsid, which has not even been yet isolated. The packing sequence Ψ is responsible for the recognition and incorporation of the DNA in the pro-capsid in a ATP-dependant process through an opening in the pro-capsid known as the portal. The portal is sealed as soon as the DNA is inserted and maturation of the capsid starts.

A panel of at least twelve viral proteins and the viral DNA are involved in viral assembly. Most of the structural proteins are synthesized as longer precursors and undergo processing by AP before they are ready to participate in viral encapsidation. There are several non-structural proteins associated with adenovirus assembly and maturation of the capsid. Except IVa2, most of those proteins are expressed by the late transcription unit (Ostapchuk, Hearing 2005).

1.2.3.6. Viral release

While the capsid undergoes maturation process, the nuclear membrane turns more permeable and eases the escape of viral particles to the cytoplasm. Finally, the Adenoviral Death Protein (ADP), transcribed from the E3 region, forces cell rupture and permits viral particle release. As an average, every infected cell releases 10.000 viral particles. The exact mechanism how adenovirus can lyse the cell is not clear but most evidence points to the important participation of autophagy, a process that involves the formation of an autophagosome that fuses with lysosomes which triggers caspase activation leading to the destruction of cell structures and cell lysis (Jiang et al. 2011).

1.2.4. Immune response to adenovirus infection

In natural conditions, adenovirus infections would occur from the exterior through the epithelial tissue along the respiratory channels or through other epithelia, e.g. conjunctiva. Nevertheless, this first barrier is sometimes omitted when adenovirus are administered as vectors for gene therapy. In both cases, the immune reaction is complex and occurs through several groups of events. Nevertheless, some

responses are more important than others depending on the route the virus uses to access the body. This section will be focused on the most influential immune reactions caused by adenovirus.

1.2.4.1. Innate immune response

The innate immune response constitutes the first defense of the immune system against adenovirus infection. It is initiated by interaction between the host cell and the virus and is independent of gene transcription. The innate immune system involves a highly complex network, with high levels of redundancy but also cell-level specificity. In addition, many external factors increase its diversity of effects. Special attention will be paid to two of the most influential mechanisms of the innate immunity to adenovirus: the interferon α receptor (IFN-α receptor) and interleukin-1 (IL-1) receptor pathways (Thaci et al. 2011).

The interleukin-inflammatory pathway

The interleukin-inflamatory pathway leads to the recruitment of pro-inflammatory infiltrate aimed to eliminate the pathogen. Even if an early recognition of the virus can already activate an immature form of IL-1, the maximum inflammatory response is based on a fully-functional IL-1R. Downstream events of IL-1R activation lead to the induction of NF-κB, a transcription factor that triggers the expression of chemokines in the nucleus, including IL-1(Thaci et al. 2011).
The earliest cell sensor that gets activated upon viral infection is the interaction of CAR with the fiber protein (Tamanini et al. 2006). However, opinions are divided and some authors claim that RGD interaction with αβ-integrins is indeed the first event inducing innate immune response. In any case, both interaction coincide with the NF-κB mediated expression of chemokines (Russell 2009, Thaci et al. 2011).

The intensity of later events in the innate immune response depends on the efficiency of the virus to escape from the endosome and, consecutively, the amount of viral DNA in the cytosol. But prior to that, double-stranded DNA present inside the endosome is already detected by several cell receptors: toll-like receptors (TLR), especially TLR9, DNA-dependent activators of IFN-regulatory factors (DAI) and/or nucleotide oligomerization domain (NOD)-like receptors (NLRs). Downstream effectors of the immune response will depend on which pathway becomes activated (Russell 2009, Thaci et al. 2011). For example, TLR9 activation induces maturation of pro-IL-1β in macrophages that is dependent on a cytosolic innate molecular complex known as the "inflammasome". Activation of caspase-1 is needed for the formation of this complex (Barlan, Danthi & Wiethoff 2011, Muruve et al. 2008).

Interferon response

Three groups of IFNs have been identified according to the receptor they recognize. Type I includes IFN-α and β that signal through the IFN-AR receptor. Type II IFNs are secreted by lymphocytes in response to pathogen antigens during adaptive immune response and type III IFNs are not well characterized (Meyer 2009, Levraud et al. 2007). Induction of type I IFNs is responsible for NK cell activation and regulation of the innate immune response against adenovirus (Zhu, Huang & Yang 2008). Adenovirus-mediated IFN responses are partly induced by recognition of foreign nucleic acid. Interaction of adenovirus with cell surface receptors does not cause induction of IFNs (Thaci et al. 2011). Endosomal TLR9 recognizes CpG-rich viral DNA and activates plasmacytoid dendritic cells (pDC) that lead to the secretion of IFN-α through the MyD88-dependent pathway. In reality, a complex interplay between the IFN and inflammatory pathways are needed to clear adenovirus infections completely (Thaci et al. 2011).

1.2.4.2. Adapted immune response

The innate immune response is responsible for initiation of the adaptive response and modulates its progression. In order to start the adaptive response, adenoviral antigens must be presented to cytotoxic T-cells by dendritic cells and macrophages. In contrast to the innate response, the adaptive immune response requires B and T cell maturation and function, and takes approximately one week to become effective. The mechanism of elimination of adenovirus through the adapted immune response is similar to the removal of other antigens (Zaiss, Machado & Herschman 2009; Russell 2000).

Intracellular antigens are presented to CD8+ cytotoxic cells (CTL) through the MHC class I. On the other hand, antigens in the viral capsid are presented through a MHC class II to CD4+ helper cells. While CTL produce lysis of the infected cell, helper cells trigger a B cell proliferative response against the infection. This provides the amount of immunoglobulines needed for the humoral response (Zaiss, Machado & Herschman 2009; Russell 2000).

Adenoviruses combat the CTL response with E319K protein that retains the MHC class I in the ER and impedes its translocation to the cell surface to complete antigen presentation (Fu, Li & Bouvier 2011). In parallel, E4 gene products inhibit cytolysis by T cells (Kaplan et al. 1999).

The humoral response

The humoral response is the major component for the host cell defense towards adenoviral infections and is based on the production of surface immunoglobulins by B cells that recognize a certain adenoviral antigen. This first recognition of the antigen initiates an industrial proliferation of T helper cells (CD4+) accompanied by

the release of immunoglobulins against the antigen. We will refer to those immunoglobulins as adenovirus neutralizing antibodies (Russell 2000). Neutralizing antibodies are directed against the hexon and other capsid proteins, fiber and penton. In the hexon, the epitopes are located in the hyper-variable region (HVR) that is the least conserved part of the protein. The recognition of the capsid epitopes by different neutralizing antibodies permitted the classification of adenovirus in their 51 different serotypes (Russell 2000).

1.3. HUMAN DISEASES CAUSED BY ADENOVIRUS

Adenoviruses are, in general, species specific. Excluding very few exceptions, humans and animals are not susceptible to pathogenicity by the same adenovirus serotypes. However, some adenoviruses cause non-symptomatic infections in humans and in animals that can be detected by presence of antibodies. Among children, the most usual route of transmission is feco-oral which is facilitated by adenovirus accumulation into faces due to the prolonged carriage of the virus in the intestines. However, the spread can also occur through the respiratory track. The epidemiologic significance of the long latency in tonsil tissue is still unknown (Wold S.M. 2007).

Although symptomatic adenovirus infections can occur many times during the life of a human being, they rarely become persistent. It is estimated that adenoviruses are present only in 3% of the asymptomatic civilian population, and in 7% of the cases of patients that present febrile symptoms. Among children, prevalence is 5 %, and 10 % in the case when the children are presenting febrile symptoms (Wold S.M. 2007). A recent study detected adenovirus in fecal samples of 3,6 % of children hospitalized with symptoms of gastroenteritis (Andreasi et al. 2008) and another study found adenovirus particles in 2 – 9 % (depending on the screening method) of autopsies from pediatric patients of fatal pneumonia (Ou et al. 2008). Still, serologic surveys show that antibodies to Ad1, 2 and 5 are present in 40-60 % of children. Although adenovirus infections are not related to particular habits, during the outbreaks of adenoviral infection in military recruits during World War II, factors such as fatigue due to everyday training, the winter season and congregation of people sleeping together were considered to increase the infection (Wold S.M. 2007). Similarly, a recent study showed that adenoviral conjunctivitis in children clustered around environments with congregation of children, like daycare centers (Adlhoch et al. 2010).

Table 2: Diseases associated with adenovirus infections (Wold S.M. 2007)

Disease	Main serotypes	Individuals at higher risk	Symptoms	Prognosis
Acute febrile pharyngitis	1, 2, 5, 6, (3, 7)	Children < 5 years old	Local: Nasal congestion, coryza, cough. Systemic: malaise, fever, chills, myalgia, headache	
Pharyngoconjunctival fever	3, 7, 14	Children	Same as above plus conjunctivitis	
Acute respiratory disease (ARD)	4, 7, (3)	Military recruits. Risk factor: fatigue and crowding	Fever, respiratory symptoms, cough, sore throat, pneumonia (Kajon et al. 2010)	Some cases: death due to pneumonitis
Pneumonia (children)	1-3, 7	Infants, young children		
Pneumonia (adults)	4, 7	Military recruits		
Pertussis-like syndrome	5	Infants, young children	Clinical whooping cough (together with other causal agent)	
Eye infections	1-4, 6,7,9-11,15-17, 20, 22		Mild symptoms of conjunctivitis	Complete recovery is most usual
Epidemic keratoconjunctivitis	8, 11, 19, 37	Children and adults	Follicular conjunctivitis, eyelid edema, pain, lacrimation, photophobia, (corneal opacity)	Some cases: corneal opacities lasting for years; or progression to hemorrhagic conjunctivitis
Acute hemorrhagic cystitis	11, (21)	Young children, mostly males	Hematuria	Self-limited disease
Meningoencephalitis	7, 12, 32	Children, immunocompromised hosts		
Gastroenteritis	40, 41	Infants, young children		
Hepatitis	1, 2, 5	Infants and children with liver transplants		

The most common sites of Adenovirus infection and replication are the respiratory track, eye and gastrointestinal track. Less frequent sites are the urinary bladder and the liver. Very seldom, infections of other organs have been reported, such as the pancreas, myocardium or central nervous system.

Normally the association between adenovirus and disease is attributed to virus or antibodies detection in blood or in a specific tissue. However, the sole presence of the virus or viral DNA in a tissue is not enough to confirm association with the symptoms, since adenovirus can persist at very low levels in humans for a long time (Wold S.M. 2007). Table 2 summarized the most relevant diseases caused directly by adenoviruses (Wold S.M. 2007).

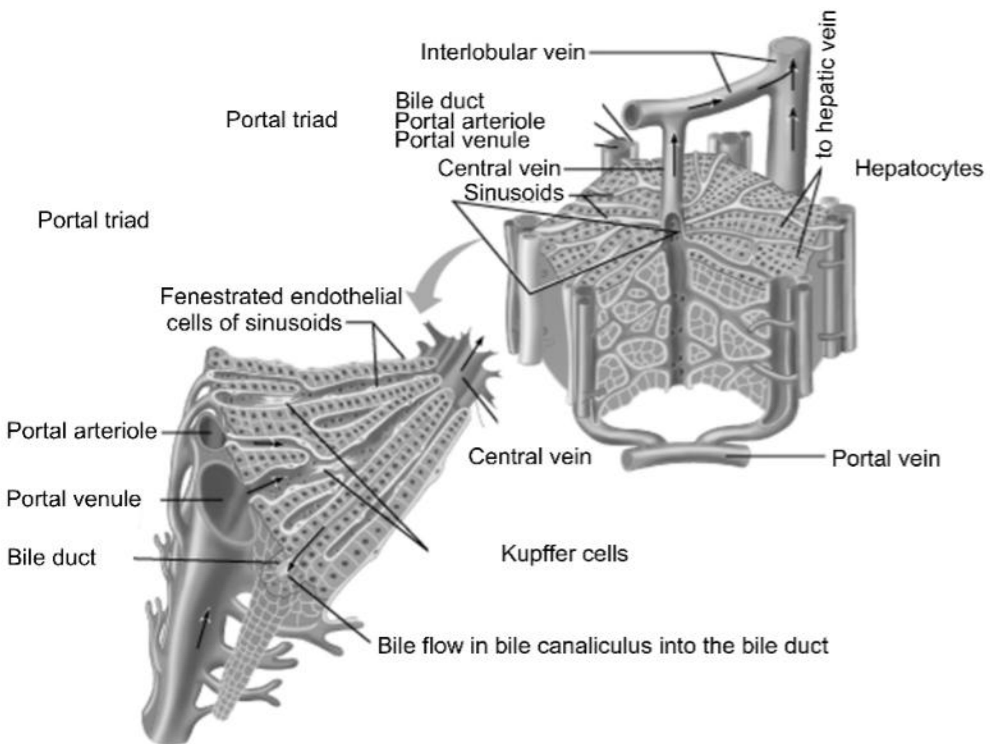

Figure 7: Anatomy of the hexagonal lobules in the liver

The liver is divided into hexagonal functional units called *lobuli*, which are separated from each other by portal triads that consist of branches of the bile duct, portal vein and hepatic artery. The portal triad and the interlobular veins are connected are in the center of the hexagonal *lobules*. They carry blood to the hepatic vein, through the sinusoids. Adapted from Benjamin Cummings (2001)

1.4. ADENOVIRUSES AS BIOLOGICAL DRUGS FOR THE TREATMENT OF CANCER

Adenovirus vectors are normally delivered to the organism through completely different routes than in naturally occurring infections. In animal models, the persistence of adenoviral particles in the blood after intravenous administration is lower than 2 minutes (Alemany, Suzuki & Curiel 2000) due to rapid sequestration to multiple organs including the spleen, heart, lung and specially the liver. However, transgene expression occurs mainly in hepatocytes. Clinical human data on adenovirus pharmacokinetics / pharmacodynamics is rather limited. However, a biodistribution study of one patient who died due to cancer progression after adenoviral treatment, established that after 56 hours, nearly all adenovirus particles had disappeared from the blood and were retained in spleen and liver hepatocytes (Alemany 2007). This retention of viral particles by liver tissue represents two major obstacles for vector delivery: the decrease in the fraction of dose effectively delivered to target tissue (e.g. tumor) and the potential toxic effects in the liver (Zaiss, Machado & Herschman 2009). Liver toxicity by adenovirus starts with an acute inflammatory response caused by a rapid induction of pro-inflammatory cytokine and chemokine expression. This leads to massive leukocyte infiltration that results in necrosis and tissue damage. Hepatotoxicity caused by adenovirus is dose-dependent but non-related to viral gene expression which means that the viral capsid by itself is capable of inducing an innate immune reaction (Muruve et al. 1999)

The liver is divided into hexagonal functional units called lobuli (figure 7). A lobul is separated from its neighbor by portal triads that consist of branches of the bile duct, portal vein and hepatic artery. The portal triad is connected to the interlobular veins that are located in the center of the hexagonal lobulus and carry blood to the hepatic vein, through the sinusoids. Cells in the sinusoids are a compilation of endothelial cells (70% of sinusoid cells), Kupffer cells (20%), fat-storing cells (or stellate cells; 10%)) and pit cells (NK cells; <1%). All these constitute 33 % of the number of cells resident in the liver, the rest is occupied by liver parenchymal cells, i.e. hepatocytes (Jacobs, Wisse & De Geest 2010). After systemic administration, adenoviral vectors enter the liver through the sinusoids. Precisely, fenestrae in the liver sinusoid endothelial cells, provide direct access for the adenovirus to the liver parenchyma, that occurs through the space of Disse, located between the sinusoid cells and hepatocytes. Since Kupffer cells are also present, it is at this point when the adenovirus gets in contact with them. (Jacobs, Wisse & De Geest 2010; Shayakhmetov et al. 2004) (Figure 8). Indeed, the diameter of fenestrae is the main determinant of adenovirus sequestration by the liver. Gene therapy vectors based on other viruses with larger particle size (e.g. lentiviral vectors) feature lower transduction to hepatocytes (Follenzi et al. 2002). At the same time, liver infection with adenovirus vectors is significantly more effective in animal species with larger

fenestrae (Lievens et al. 2004, Wisse et al. 2008, Follenzi et al. 2002, Follenzi et al. 2002).

Kupffer cells are specialized macrophages, located in the liver, that belong to the reticuloendothelial system (RES) that phagocityze adenoviral particles and activate innate immune response against them. Adenovirus uptake by Kupffer cells, which is not dependant on CAR, presents a threshold dose. Under this dose, almost all adenovirus particles are sequestered by Kupffer cells whereas above it, Kupffer cells get saturated and viral particles are available to target other cell types (Shayakhmetov et al. 2004). In mice, the threshold dose is $3x10^{10}$ viral particles. This dose is very important to design experimental protocols for *in vivo* experimentation (Tao et al. 2001). According to some studies, sinusoid epithelial cells would be the next cell type to interact directly with adenovirus (Liu et al. 2003) .

The biodistribution pattern of Ad5 urges the need to obtain vectors with modified tropism in order to increase their persistence in circulation and achieve a reasonable distribution to the target organ. Gene therapy for cancer faces also the problem of tumor cells presenting a differential pattern of surface membrane proteins, for example lower amount of CAR than normal cells. Designing viral particles with increased affinity to those proteins expressed exclusively in tumor cells (tumor targeting) and modifying the adenovirus particle to avoid infection of liver cells (liver de-targeting) are the two major goals of cancer gene therapy. Most of the work presented in this thesis explores these issues.

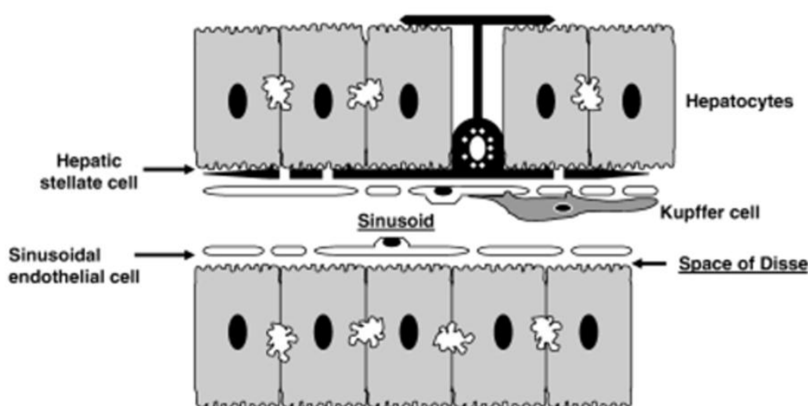

Figure 8: Anatomy of the liver sinusoid

The sinusoid is surrounded by a fenestrated layer of endothelial cells. This layer is separated from the hepatocytes by the space of Disse. The Kupffer cells are located on the external surface of the endothelial cells (Follenzi et al. 2002). Adapted from *Liver Int. © 2005. Blackwell Publishing*

During the last decades, many novel modifications have been applied to adenovirus in order to control their tropism and maximize the selective delivery of viral DNA to the target cells. Owing to the large amount and complexity of adenovirus vector types, any attempt to arrange them in a simple classification would run the risk of becoming over-simplistic. Nevertheless, general trends or groups of vectors will be classified and described according to two different criteria: the stage of viral infection / replication aimed to modify and the final effect the vector is designed to achieve.

1.4.1. Adenovirus vectors according to the type of modification

A common requirement for all adenovirus vectors is the need to control their tropism. In the case of cancer, the ideal vector should only be capable of infecting and/or replicating in tumor cells, leaving normal cells unharmed. Modifications can be made in the protein capsid or viral genome in order to control some of the steps of infection. Depending on which stage is altered, adenoviral vectors can be classified as described in this section.

1.4.1.1. Transcriptional targeting

Transcriptional modifications interfere with the normal expression of adenoviral genes at the level of transcription. Suppression of certain transcripts can be conditional or constitutive. Most common methods to alter normal adenovirus transcription are the following:

Genetic deletions

Deletions of the adenoviral genome have been extensively used in order to restrict viral replication or expression of an artificially-inserted transcript to some specific context (e.g. tumor cells). An important impediment to develop replication competent vectors that conditionally replicate in cancer cells is that effective adenovirus replication requires the participation of most adenoviral genes. For that reason, genetic deletions must be very selective and precise to preserve the replication machinery. Therefore, instead of removing entire regions of the genome, deletions of small genes or of particular exomic sequences in the genome have been commonly utilized. A well-known example is the mutant vector dl1520 or Onyx-015. It was used in clinical trials and consists of an adenovirus serotype 5 vector where E1B-55 kDa gene is deleted. This gene confers conditional replication in p53-deficient cells (Nemunaitis et al. 2001, Ganly et al. 2000). Another sequence scission commonly found in the literature is the 24-bp deletion (Δ24) in region CR2 of E1A that impairs E1A protein to bind Rb protein and, hence, the release of E2F transcription factor. As a consequence, the virus can only replicate in cells with a defective p16/Rb pathway (Fueyo et al. 2000, Heise et al. 2000).

On the contrary, when viral replication is to be ablated in any cell type, larger regions of the genome can be omitted, giving room to additional benefits, such as the capacity of inserting larger transgenes. The development of new replication-incompetent adenoviral vectors evidenced how blurry the idea of "essential regions for viral replication" truly is. Even the so-called "first generation vectors" where E1 and/or E3 regions have been totally or partially removed, still show minimal production of replication-competent particles *in vivo*, especially at high MOIs (Russell 2000, McConnell, Imperiale 2004, Stone et al. 2000, Hoff, Margetts 2006). Therefore, "second generation vectors" featured partial or total deletion of genes in the E2 region. Those regions express genes important for viral replication such as polymerase or the pre-terminal protein (Berk AJ 2007). The "third generation vectors" consist of adenoviruses where additional genes have been deleted, and this group includes the "gutless" vectors (see figure 9 for a schematic representation of the different groups of vectors with impaired replication due to gene deletions) (Russell 2000, Hoff, Margetts 2006; McConnell, Imperiale 2004; Stone et al. 2000). These vectors go to the extreme in including only the ITRs and the packing sequence. Replication is only possible with aid of a helper virus (Alba, Bosch & Chillon 2005) (See figure 9).

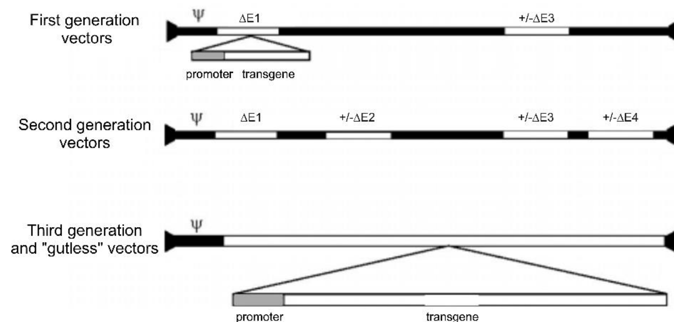

Figure 9: Classification of transcriptionally modified non-replicating vectors using gene deletions into so-called "vector generations"

First generation vectors comprise those with total or partial deletion of E1 and/or E3 genes. Second generation vectors add a partial or total deletion of genes in the E2 region that express important genes for viral replication as polymerase or the pre-terminal protein. The third generation vectors consist on adenoviruses where additional genes have been deleted. Modified from Hoff et al., 2006

Specific promoters or control on promoter expression

Specific promoters can be used to drive the expression of genes under specific conditions. Those selectively-expressed genes would include artificially inserted

transgenes, one example is the expression of thymidin kinase driven by the hypoxia-inducible promoter in cancer cells (Harvey et al. 2011) or the expression of luciferase transgene driven by survivin promoter (Zhu et al. 2004). Essential genes for replication can also be conditioned to a specific context, like in the case of the conditional replication adenovirus where the E1A gene is driven by the wnt promoter (Liu et al. 2011).

1.4.1.2. Transductional targeting

Transductional targeting comprises of all those modifications based on promoting or ablating certain viron-cell interactions in order to change viral tropism. This includes strategies such as molecular mutation of hexon, fiber or penton proteins, substitution of capsid proteins for other serotypes or the incorporation of ligands to the capsid proteins. Capsids or certain proteins of the capsid can also be coated with polymers in order to change tropism directly or by helping the virus to escape from the humoral immune response (Hedley et al. 2006, Kreppel, Kochanek 2008).

1.4.1.3. Translational targeting

Translational targeting is focused on modifying the viral transcriptome in order to achieve selective translation of genes according to the cell type. This means that, even if the transcriptome is similar as with wild type virus, certain genes are selectively translated under defined conditions. This can be achieved by the use of RNA technology, and particularly micro RNA (miRNA) systems (Sakurai, Katayama & Mizuguchi 2011; Edge et al. 2008, Barnes et al. 2008; Kelly et al. 2008). miRNA are single-stranded RNA molecules of 21-23 nucleotides in length, that regulate gene expression. Although miRNAs are transcribed from genomic DNA, they are not translated into protein. Mature miRNA molecules are partially complementary to one or more messenger RNA (mRNA) sequences, and their main function is to down-regulate gene expression (Ambros 2001, Moss 2002).

The most used method of translational targeting is the insertion of miRNA complementary binding sequences to the 3'UTR of the gene or transcript that is to be selectively expressed. This results in the silencing of the target gene when the miRNA is present in the infected cell. One example of this is the insertion of liver specific miRNA binding sequences in order to ablate replication (Ylosmaki et al. 2008) or transcript expression (Suzuki et al. 2008) in liver cells.

1.4.2. Adenovirus vectors according to the final effect on host cells

1.4.2.1. Oncolytic adenovirus

Oncolytic adenoviruses complete their lytic life cycle in the host cell. As a consequence, oncolytic adenoviruses replicate as well as destroy the host cell and release the progenies to the extracellular compartment. The release of new viral particles (10.000 viral particles per cell infected on average) is the basis of the self-amplifying effect of oncolytic adenovirus-based treatments (Kay, Glorioso & Naldini 2001). Oncolytic adenoviruses are designed to selectively replicate in cancer cells while leaving normal cells unharmed.

1.4.2.2. Armed (transgene-expressing) adenovirus

Adenoviruses are transient expression vectors that allow efficient delivery of genetic material but, unfortunately, cannot accommodate large transgenes. It is estimated that they cannot increase their genome more than 5%, so they can only incorporate a maximum of 2 kb of "extra" DNA (Cody, Douglas 2009). A larger increase in genome size rises the likelihood of genome packaging mistakes and impedes proper viral assembly. Therefore, non-essential regions must be eliminated before insertion of large DNA trangenes. What regions are essential depends on the used vector, and especially on whether a replicating or non-replicating vector is expected. For the design of non-replicating vectors, most frequent regions to be complete or partially substituted are E1 and E3 (Russell 2000, McConnell, Imperiale 2004; Stone et al. 2000). The most frequently used groups of transgenes used for the treatment of cancer are those that directly enhance cancer cell killing and those that indirectly enhance cell killing by modulating the tumor microenvironment. A third group would be those transgenes that stimulate an immune response against the tumor(Cody, Douglas 2009).

Transgenes that enhance cell killing

Use of certain transgenes can ultimately enhance cell killing by the adenovirus by improving selectivity towards cancer cells. These type of transgenes act at three different levels: within the infected cell, through a bystander effect or by increasing viral spread (Cody, Douglas 2009). The first group improves the destruction of the host cell by a mechanism independent of direct viral oncolysis but restricted to the cell the virus is infecting. Such an oncolytic effect can be obtained by silencing of particular cell essential genes using antisense cDNAs (Gao et al. 2006, Zhou et al. 2005), small-interfering RNA (siRNA) (Zhang et al. 2006) or genes that stimulate apoptosis of the infected cell (Gao et al. 2006, Zhou et al. 2005, Cui et al. 2008). However, restricting the killing of the infected cell, as safe as it might seem in principle, implies an important limitation to reach a generalized elimination of the tumor tissue. To avoid this, the so-called suicide genes are expressed only in

infected cells but are also lethal for neighboring, non-infected cells through a collateral effect. Gene products of suicide genes are pro-drug converting enzymes that turn non-toxic pro-drugs into toxic metabolites. Two of the earliest enzyme/pro-drugs combinations that were used were herpes simplex virus thymidine kinase / gancyclovir (HSV-TK / GCV) and the cytosine deaminase / 5-fluorocytosine (CD / 5-FC). Other options for cell killing are strategies that enhance viral spread. This can be achieved by reinforcing the lytic phase of the virus by over-expression of ADP. In practice, ADP is inserted into an "extra" region (e.g. E3 region (Tollefson et al. 1996, Doronin et al. 2000)) or under the control of a foreign promoter, more potent than MLP (e.g. CMV(Yun et al. 2005)). Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is another promising transgene that has shown to enhance viral spread without toxicity in vivo (Sova et al. 2004).

Transgenes that modulate the tumor microenvironment

The tumor microenvironment has emerged as an important factor in cancer development and as a key determinant of the disease outcome. Most transgenes aimed to modulate the tumor microenvironment are divided into those that target the ECM and those that inhibit angiogenesis (Cody, Douglas 2009). Within the first group, contradictory results have been presented. Several authors have expressed metalloproteinase (MMP) inhibitors, such as TIMP3 (Lamfers et al. 2005), in order to reduce proteolysis of the ECM and basement membrane components to achieve superior tumor invasiveness suppression. However, other authors showed how expressing ECM-degrading proteases bear the benefit of increasing tumor spreading of the gene therapy vector (Ganesh et al. 2007, Kim et al. 2006). This second approach is more broadly discussed in the section 1.7.1.

A second group of tumor microenvironment modulating transgenes would consist of those that are aimed to inhibit angiogenesis. Examples include endostatin (Fang et al. 2010) or Flt1 that blocks angiongenesis by inhibition of vascular endothelial growth factor (VEGF) (Zhang et al. 2005), short-hairpin RNAs (shRNA) directed against VEGF (Yoo et al. 2007) and expression of a zinc-finger protein targeted against VEGF promoter (Kang et al. 2008).

Immunomodulatory transgenes

A third group of transgenes are used to stimulate the immune system, generally by recruitment of immune cells to the site of infection and subsequent induction of their proliferation and activation. Recruitment of immune cells to the primary tumor not only has the potential to directly destroy it but also to impede metastatic progression and recurrence (Cody, Douglas 2009). In this regard, GM-CSF encoding adenoviruses have been shown to improve oncolytic potency of intratumorally injected replicating vectors (Burke 2010). This effect was multiplied when the virus expressed the T-cell co-stimularory molecule B7-1 (Choi et al. 2006). Other

immunity mediators that have been co-expressed by adenovirus, are TNF-α that increases MHC class I expression, IFNs or interleukins like IL-4 and IL-12. Of special mention is the strategy of co-expressing the so-called heat shock proteins. These proteins act as chaperones for antigen-presenting cells, so they trigger antitumor response and activate several immune cells (Guo, Fang 2008).

1.5. COLORECTAL CANCER

Colorectal cancer (CoCa) is driven by the development of a primary tumor in the colon, rectum or vermiform appendix. While tumors confined within the colon wall are often curable with surgery, cancers that have spread widely around the body are normally not curable and lead to a survival of less than 5 years (Coleman et al. 2008, Center, Jemal & Ward 2009). Colorectal cancer is the fourth most common type of cancer in men and the third most common in women worldwide. More than one million people are diagnosed with colorectal cancer every year. Prognosis rates have improved in the last decades and vary among countries. In Europe, 5-year survival accounts for 70 – 79% while in Finland, Sweden, North America, Australia and Japan it goes up to 80% of the patients (Coleman et al. 2008, Center, Jemal & Ward 2009). Early diagnostic is essential for a good prognosis. Metastatic disease represents the most aggressive form of the disease, in fact even lesions that undergo curative resection lead to a survival higher than 5 years in 50% of patients. Most common locations for metastases are the liver, followed by the lungs and peritoneal wall (Simmonds et al. 2006).

Typical risk factors for CoCa are advanced age (Patel et al. 2009), personal and family history of cancer (Gala, Chung 2011), polyps in the colon, smoking (Office of the Surgeon General (US), Office on Smoking and Health (US) 2004), a diet rich in red meat and poor in vegetables and fresh fruit (Corpet 2011, Aune et al. 2011), obesity and/or physical inactivity (Calle et al. 2003), excessive alcohol consumption (Cho et al. 2004) and low vitamin B6 intake (Le Marchand et al. 2011). CoCa may cause several symptoms that are not specific of the disease, such as change in bowel habits, blood (either red or dark) in the stool, diarrhea, constipation, feeling that the bowel does not empty completely, narrower stools than usual, frequent gas pains, bloating, fullness, abdominal cramps, weight loss for no known reason, asthenia or vomiting.

Diagnostic of CoCa is based on the results from colonoscopy or sigmoidoscopy with tumor biopsy. In order to decide a treatment strategy, it is necessary to assess the stage of the cancer. This is done by physical examination, a complete colonoscopy and computerized tomography (CT) of the chest, abdomen and pelvis to identify possible metastatic disease. In particular, CT is very useful for establishing the specific localization of the tumors. In the case of rectal cancer, it is crucial to define

the tumor extension and depth of invasion in the bowel wall and, for that, high-resolution MRI and ultrasounds are particularly useful. The presence and extent of the possible metastatic disease is determined by ultrasound, CT and MRI (Cunningham et al. 2010).

The most common colon cancer cell type is adenocarcinoma (95% of cases), followed by lymphoma and squamous cell carcinoma. Since adenocarcinomas are malignant tumors, they eventually invade the colorectal wall. Thereby, tumors are classified according to the level of penetration and dissemination/invasion to other tissues. The most common classification is the TNM (tumors/nodes/metastases) system from the American Joint Committee of Cancer (AJCC). Other classifications are the Dukes system or Astler-Coller system.

The staging of the tumors and the physical condition of the patient are the variables used to decide a treatment modality. Surgery is a common management option and can be curative when the tumor is localized and has not invaded other tissues. It is applied to colon tumors by total resection of the tumor and its margins. In parallel, lymphadenectomy is performed in order to establish nodal staging by histological analyses of the explants. In the case of rectal cancer, complete local excision or total excision of the mesorectum is performed together with lymphadenectomy. Risk of lymph-node involvement is associated with the depth of tumor invasion in the rectal wall. Laparoscopic colectomy is used for colon cancer, especially for left-sided cancers, and shows similar long-term recurrence rates. Surgery is also used with some lung and liver metastases in combination with chemotherapy (oxaliplatin) given before and after tissue removal. Some unresectable liver metastases can become resectable after chemotherapy treatment if the response to the drug is good. Radiation therapy is commonly used, as an adjuvant to surgery, to treat highly invasive tumors. Chemotherapy can be used with two different goals: as an adjuvant to surgery or as the primary therapy. When used as an adjuvant, drugs selected are normally 5-fluorouracil, leucovorin, irinotecan or oxaliplatin. On the other hand, as a treatment for the metastatic disease, combination of several chemotherapeutics are most frequent, e.g. 5-fluorouracil, leucovorin and oxaliplatin, (FOLFOX). Other therapeutical strategies are also possible, like radiation therapy or monoclonal antibodies (especially bevacizumab or anti-EGFR monoclonal antibodies) (Cunningham et al. 2010).

At the molecular level, colorectal cancer is driven by the transformation and progression of normal colonic epithelial cells to cancer cells through a mechanism based on the accumulation of genetic and epigenetic alterations. This process,that takes from 8 to 12 years, is caused by the activation of oncogenes and inactivation of tumor suppressor genes by specific mutations. It is estimated that for the entire process to be completed, at least 4-5 genes have to be mutated, but the order how

those mutations occur is not important. When the mutation affects a tumor suppressor gene, a biological effect is produced even if the mutation is present in the heterozygote state, (Fearon, Vogelstein 1990; Saif, Chu 2010). Most important steps in this genetic model for CoCa are represented in figure 10.

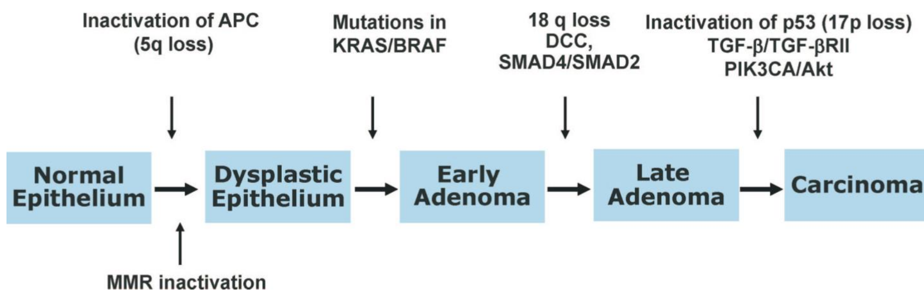

Figure 10: Genetic model for colorectal cancer

The genetic model defines the progression of the normal colon epithelium to carcinoma cells as a multistep process that five levels of cell differentiation. The progression from one level to the next depends on several genetic and epigenetic events, some of the most important are included in the figure (Fearon, Vogelstein 1990, Saif, Chu 2010).

According to their etiology, CoCa can be divided into sporadic or hereditary. Sporadic or non-hereditary CoCa accounts for 80 to 85% of the cases, while hereditary CoCa occurs in 8 to 15% of the patients. The two major forms of hereditary CoCa are familiar adenomatous polyposis (1-2%), caused by genetic mutations in the adenomatous polyposis coli (APC) gene, and hereditary nonpolyposis CoCa (around 5%), which arises from genetic mutations in the family of mismatch repair genes. Interestingly, similar genetic alterations as those found in hereditary CoCa have been implicated in the development of sporadic CoCa. These alterations include the Wnt/β-catenin, TGF-β receptor, Notch and hedgehog signaling pathways. Besides, other signaling pathways, crucial for colonic epithelial cell growth mediation, include the epidermal growth factor receptor (EGFR), the RAS/RAF/MAPK cascade and the phosphoinositide 3′-kinase (PI3K)/Akt pathway. All these altered signalling pathways offer many possible targets for rational drug design (Saif, Chu 2010).

1.6. MURINE MODELS OF CANCER

The optimal animal model for assessing activity of a potential new anti-tumor drug is usually a cause of controversy among researchers. The reason for that is the scarce predictive power of the traditional *in vivo* tumor models to foretell how humans will respond to the treatment in the future. This is not common in some other pharmacological areas, where a positive response to the drug in animal models is followed by a positive response in humans (e.g. antifungal drugs). In any case, *in vivo* models generally represent a more relevant platform for drug testing compared to many other systems like *in vitro* models (Cespedes et al. 2007). A brief introduction to the most common types of *in vivo* models will be further described. Xenograft models of colorectal cancer will be emphasized in order to introduce the research topics described later in this Doctoral Thesis.

1.6.1. Syngeneic models

In syngeneic models, mice bear tumors originated from individuals belonging to their own species or strains. The tumors are induced initially by chemical or surgical intervention and material from the primary tumor (tissue explants or cells) is introduced to naïve members of the same mouse strain. Syngeneic models have a relative low cost and are reproducible regarding tumor histology and tumor growth rate. Besides, the stroma-cancer cell interactions are well preserved. Furthermore, tumor implantation is not immunogenic, so fully immunologically competent mice can be used. Unfortunately, in many cases, target cells or tissues present significant differences to human ones, which represents an important limitation (de Jong, Maina 2010).

1.6.2. Xenogeneic models

Xenogeneic models are constructed by implantation of material from a human tumor into an immunosupressed host. Indeed most xenograft models are not compatible with a fully functional immune system that would reject implanted cells. For that reason, most used experimental xenogeneic models are based on genetically manipulated athymic mice (nu/nu) (i.e. they lack the thymus and, hence, are unable to produce T cells) or severe combined immunodeficiency mice (SCID) that lack both humoral and cellular immune components. In any case, tumors of human origin in xenogeneic models are better predictors of drug efficacy in humans than murine tumors in syngeneic models. Also, they count with a broad pool of well-characterized cell lines, therapeutic targets are homolog and they are highly reproducible. The main drawback is that the tumor mass consists of cells derived from two species: cancer cells of human origin in murine-derived stroma. This may lead to discrepancies in tumor histology and intra- and peritumoral vasculature as a result of altered interaction patterns between those elements (de Jong, Maina 2010).

Xenogeneic and syngeneic models can be divided according to the site of tumor implantation. Thus, we define orthotopic models as those where the tumor material is implanted at the site of the primary tumor source, while in non-orthotopic models tumor material is implanted in a different location, normally subcutaneously (de Jong, Maina 2010; Sausville, Burger 2006).

1.6.2.1. Metastatic xenogeneic models of colorectal cancer

Metastatic progression, normally to the liver but also to lungs or peritoneum, represents the most aggressive consequence of colorectal cancer. The metastatic process includes primary tumor implantation, cell migration, release to blood flow, survival in blood stream / lymphatic vessels, invasion of vessel wall, migration to target organ, colonization and tumor tissue development (Peeters et al. 2008). Because metastases is such a complex event, few *in vitro* or *in vivo* models can mimic all the processes and normally they just reproduce one of few stages (Makale 2007, de Jong et al. 2009).

For the *in vivo* study of human metastatic colorectal cancer, xenograft models are broadly in use. The most straight forward xenograft models are based on systemic cell injection giving rise to disseminated tumors. For such models, location of the tumor is dependent on the injection site and influenced by the cancer cell type (Nguyen, Bos & Massague 2009). For example, after intracardial cell injection most tumors are found in several sites including bone marrow, but for prostate and breast cancers there is marked tropism to bone. In this type of model, the metastasis process avoids its early stages which leads to different profiles of tumors derived from experimental metastases and those from spontaneous metastases (Khanna, Hunter 2005).

Subcutaneous tumors have very limited progression and very rarely induce metastases. However, some authors have reported metastatic spread of human cancer cells in lymph nodes and / or lungs, detected by imaging methods (Yamamoto et al. 2003, Hansen, Khanna 2004; Koike et al. 2009). These models are easy to develop and offer good reproducibility but they face the problem of being far from reality since the primary tumor grows in a complete different location and environment than in a, for example, real colorectal cancer which makes their morphology differ very markedly from tumor biopsies, especially in the amount of stromal elements. All in all, the metastatic process develops in a completely different scenario and as a consequence, these cells released from the tumor very rarely turn into a mature tumor tissue. Notably, metastatic cells are not metastases; specific conditions are needed for metastatic cells to develop to a mature tumor tissue. Only a few amount of cells (1:100) released to blood flow successfully

develop into metastatic tumor tissue (Bouvet et al. 2006, Fukumura, Jain 2007; Nakagawa et al. 2004). Even when metastatic cells have reached a target organ, they can remain as single cells or micro-metastases until certain conditions permit their development to metastases (Bockhorn, Jain & Munn 2007).

In humans, the liver is the most common receptor organ for metastases originating from colorectal tumors (Joyce, Pollard 2009). Therefore, three types of mechanisms have been used for human cell engraftment in murine liver: 1) direct implantation in liver parenchyma (Kollmar et al. 2006, Chiappa et al. 2009), 2) injection to the portal vein (Ni et al. 2006, Li et al. 2004) and 3) implantation of a primary tumor that releases metastatic cells through the portal system (Hiraoka et al. 2006, Ishizu et al. 2007, Lee et al. 2006, Bouvet et al. 2006). The first and second type avoid selection of cells with metastatic potential, so the hepatic tumors have similar phenotype to that of a primary tumor. This would partially explain the variability in the number of mice that develop intrahepatic tumors after cell administration (Chiappa et al. 2009, Thalheimer et al. 2009). Interestingly, when cells are implanted in a location with vascular access to the liver through the portal system, like the cecum wall or spleen, tumorigenicity in the liver is more uniform than when cells are injected in the portal vein (Bouvet et al. 2006).

1.6.3. Genetically engineered models (GEM)

Mice can be genetically engineered to express molecular targets of interest or to express a foreign reporter protein with the aim of monitoring tumor induction and propagation in the host. Nowadays, transgenic mice can develop cancers in many different organs with their own controlled progression closely resembling human carcinogenesis. Nonetheless, use of such models has been limited in translationalresearch because of their high cost, limited availability and other logistic and practical problems (de Jong, Maina 2010).

The most common ways to produce GEM are the activation of oncogenes, the inactivation of tumor suppression genes or both. This is mostly done with the use of knock-out models (i.e. a certain allele is suppressed in the mouse by genetic manipulation of the germ line by deletion or mutation of a certain gene) and knock-in models (i.e. an allele is added to the genome). The presence or lack of the gene can be restricted to a certain tissue or to a certain time, such models are defined as conditional knock-in/out models (Cheon, Orsulic 2011).

The study of human colorectal cancer carcinogenesis has described a multi-step chain of mutations which involve genes in the WTN (APC), RAS, TP53, DCC and TGFβ signaling pathways and trigger transformation to cancer cells (Vogelstein, Kinzler 2004). These mutations have been utilized in mice to develop GEM of

colorectal cancer. The first of such models to be established was the *Apc*$^{min/+}$ mouse, where the adenoma polyposis coli (*Apc*) gene was mutated through random chemical carcinogenesis (Moser, Pitot & Dove 1990). Although this model has been widely utilized in research, it presents several limitations. For example, while mice develop clearly adenomas, they do rarely produce adenocarcinoma, which are very frequent in human patients, and consequently do not invade other tissues. The location of the tumors in *Apc*$^{min/+}$ does not exactly correspond with clinical situations since most of them are located in the small intestines rather than in the colon. Still, despite these limitations, the *Apc*$^{min/+}$ model has been the most widely used GEM for cancer prevention studies that involve the gastro-intestinal tract (Green, Hudson 2005).

1.7. TUMOR MICROENVIRONMENT AS A BARRIER FOR ADENOVIRAL VECTORS DELIVERY

Although different types of tumors feature their own particularities, certain characteristics, not found in normal tissues, are common in several or all tumor types (L Addison 2006, Kuppen et al. 2001). Due to particular morphological characteristics, the access of drugs to all areas of the tumor is normally compromised. Oncolytic adenovirus drugs are not an exception: poor spreading throughout the tumor mass and the difficulty to reach other cells by viral particles produced *in situ* are strongly linked to poor efficacy of the treatment (Sauthoff et al. 2003). In normal tissues, interstitial pressure is slightly lower than intracapillary pressure, thus creating a transcapillary gradient between these compartments (Heldin et al. 2004). This outward transcapillary flow permits tissue homeostasis (i.e. tissue nutrition and oxygenation) and also facilitates drug availability. In contrast, hydrostatic and osmotic pressures are often elevated in solid tumors, which result in increased interstitial fluid pressure, creating a barrier for drug delivery (Heldin et al. 2004, Jain 1987a, Jain 1987b). In the case of virotherapeutic drugs, the acidic and hypoxic tumor environment, that rises rapidly as the distance with the nearest blood vessel increases, are limiting factors for adenovirus replication (figure 11). This is particularly true for colorectal carcinoma (Less et al. 1992). The reasons for increased interstitial fluid pressure are not fully understood but are thought to involve an increased blood vessel permeability and leakiness due to tumor associated growth factors, lymph vessel abnormalities, interstitial fibrosis, abnormal contraction of the interstitial space by stromal fibroblasts and overexpression of tumor-related extracellular matrix (ECM) components (Heldin et al. 2004). ECM, as a physical barrier, may also directly limit the spreading of the therapeutics (Beyer et al. 2011).

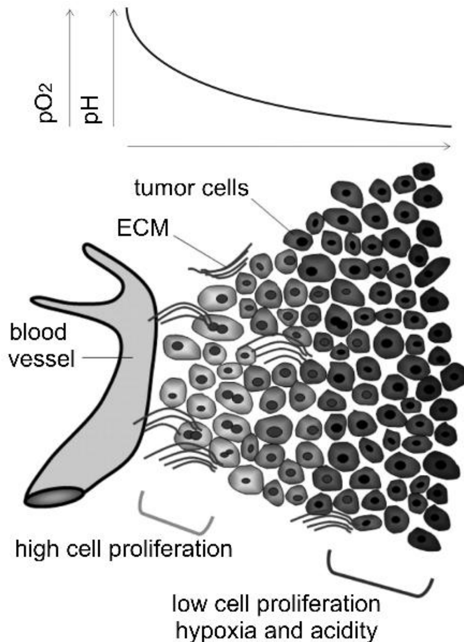

Figure 11: Particularities of tumor microenvironment that impede access and replication of adenoviral particles
Oxygen partial pressure (pO_2) and pH decrease dramatically as the distance to a blood vessel increases, thus creating hypoxic and acidic environment in the tumor that prevents adenovirus replication.
Adapted from Trédan et al., 2007

1.7.1. Use of proteases to increase viral spreading

In order to confront the problems derived from the poor distribution of drugs in general, and more precisely of adenovirus, ECM-degrading proteases can be administered with the treatment. In fact, some proteases have been given in order to enhance tumor spreading of chemical (Frost 2007) and biological (Eikenes et al. 2004) drugs. Opposite to chemical drugs, that are not susceptible to degradation by proteases, viral vectors could get damaged by the action of proteases. Particularly, alterations in the proteic capsid can disturb viral infection of cells by degrading proteins needed for cell receptor binding (Kim et al. 2006). In any case, proteases have been shown to increase viral spread of oncolytic adenovirus and, therefore, improve the antitumor effect of the virus therapy (Kim et al. 2006, Ganesh et al. 2007, Ganesh et al. 2008). However, the presence of high levels of proteases in tumors is an indicator of poor prognosis (Ruppert et al. 1997, Nastase et al. 2011) and their influence on tumor invasiveness and metastases rises many concerns. In fact, some proteases have been explicitly reported to increase invasiveness in experimental models (Silvertown, Geddes & Summerlee 2003). For that reason, treatments for solid tumors involving proteases or protease activators are regarded as potentially risky. Therefore, the tendency of the treated tumor to metastases should be assessed when studying new therapeutic options. In the case of new

drugs or coadjuvant candidates, the relative gain in treatment efficacy should be evaluated together with the impact on metastases development.

As a matter of fact, some treatments with adenovirus co-expressing proteases have shown to improve their efficacy and avoid negative effects on tumor progression. This has been shown in studies with non-replicating and replicating adenoviruses coding for relaxin, where mice treated with these vectors developed less metastases than untreated animals (Ganesh et al. 2007, Kim et al. 2006). Similarly, the metastatic potential of xenograft tumors in murine models did not increase after intravenous administration of hyaluronidase combined or not with the oncolytic adenovirus compared to non-treated animals (Ganesh et al. 2008).

2. AIMS OF THE THESIS

- To utilize new capsid modified adenoviral vectors with reduced liver tropism and increased efficacy to transductionally target cancer cells *in vivo*.
- To set up a new xenogeneic murine model of human metastatic colorectal cancer which can be followed-up by MRI in order to measure primary tumor volume and development of liver metastases during and after treatment
- To evaluate the use of proteases as coadjuvants of oncolytic adenovirus to increase their intratumoral spreading and, consequently, enhance antitumor activity.

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3. MATERIALS AND METHODS

3.1. Cell lines

Cell lines used in this Doctoral Thesis are summarized in Table 3. All cell lines were propagated in the conditions recommended by the manufacturer.

Table 3: Summary of the cell lines used in the studies

Cell line	Origin	Provider	Used in
HEK-293 (293)	Human transformed embryonic kidney	ATCC	I, II
293-6his	Human transformed embryonic kidney	Joanne T. Douglas*	II
211B	Human transformed embryonic kidney	Dan J. Von Seggern**	II
A549	Human lung adenocarcinoma	ATCC	I, II
HCT116	Human colorectal carcinoma	ATCC	I, II, III, IV
HT29	Human colorectal adenocarcinoma	ATCC	I, II, III, IV
Co115	Human colon carcinoma	ATCC	I, II, IV
SW480	Human colorectal adenocarcinoma	ATCC	I, II
SW620	Human colorectal adenocarcinoma	ATCC	I, II, IV
CaCo-2	Human colorectal adenocarcinoma	ATCC	I, II

* Joanne T. Douglas (Department of Pathology, Division of Human Gene Therapy, University of Alabama at Birmingham, USA)
*** Dan J. Von Seggern (Department of Immunology, IMM19, The Scripps Research Institute, La Jolla, CA, USA)

3.2. Adenovirus

A description of all the adenoviruses used in the studies is to be found in table 4 (replication uncompetent) and 5 (replication competent). Replication deficient viruses are deleted for E1A and have both luciferase (Luc) and green fluorescent protein (GFP) marker genes.

Table 4: Non-replicating viruses used in the study (all viruses are deleted for E1A and have both marker genes).

Adenovirus	Modification / s	Used in	Reference
AdTL	Wild type 5 capsid.	I, II	
DATL	Y477A substitution in DE loop of fiber knob for CAR ablation. Penton base's RGD domain mutated to RGE for $\alpha_v\beta$ integrin ablation. 6xhistidine carboxy-terminal tag for the propagation in 293-6his cells	I, II	(Nettelbeck et al. 2004)
AdTLG	Fiber shaft's KKTK domain mutated to GATK for HSPG ablation.	I, II	(Bayo-Puxan et al. 2006)
AdTLGR	RGD insertion to HI loop of fiber knob for $\alpha_v\beta$ integrin targeting. Fiber shaft's KKTK domain mutated to GATK for HSPG ablation.	I, II	(Bayo-Puxan et al. 2006)
AdTLYG	Y477A substitution in DE loop of fiber knob for CAR ablation. Fiber shaft's KKTK domain mutated to GATK for HSPG ablation.	I, II	(Alemany, Curiel 2001; Bayo-Puxan et al. 2006)
AdTLYGR	Y477A substitution in DE loop of fiber knob for CAR ablation. RGD insertion to HI loop of fiber knob for $\alpha_v\beta$ integrin targeting. Fiber shaft's KKTK domain mutated to GATK for HSPG ablation.	I, II	(Alemany, Curiel 2001; Bayo-Puxan et al. 2006)
AdTLY	Y477A substitution in DE loop of fiber knob for CAR ablation.	II	(Alemany, Curiel 2001)
Ad5luc1RGD	RGD insertion to HI loop of fiber knob for $\alpha_v\beta$ integrin targeting.	II, III	(Wu et al. 2002b)
AdTLRGDK	Fiber shaft's KKTK domain mutated to RGDK for αvβ integrin targeting. HSPG ablation via mutated KKTK.	I, II	I

Propagation of AdTLG, AdTLYG, AdTLGR and AdTLYGR performed done in 211B cells for 3 rounds followed by a last round of amplification in 293 cells as described previously(Bayo-Puxan et al. 2006). DATL was propagated in 293-6his as reported before elsewhere (Bayo-Puxan et al. 2006). The other non-replicating viruses were propagated in 293 cells, while replication competent viruses were amplified in A549 cells. All viruses were purified on cesium chloride gradients. The particle concentration was measured at 260 nanometers (nm) by spectrometry and by a standard 50% tissue culture infective dose ($TCID_{50}$) test to determine functional units. In a nutshell, this test estimates the dose of virus required to produce cytopathic effect in 50% of inoculated cell culture samples. The test is performed by infecting 293 cells with a series of dilutions of the virus stock with unknown concentration. After 10 days, the relative number of wells with cells presenting cytopathic effect is counted and results are given in $TCID_{50}$ units. The rationale behind the conversion of $TCID_{50}$ units into plaque forming units (pfu) is that 1 ml of viral stock is expected to produce double the amount of plaques on cell monolayers than of $TCID_{50}$ units, provided the test was done under similar conditions. A precise estimate of the pfu/ml titer is obtained mathematically from the results of the $TCID_{50}$ test, in $TCID_{50}$ units, using the Poisson distribution (Reed, L.J., & Muench, H. 1938).

Table 5. Replication competent viruses used in the study.

Adenovirus	Modification / s	Used in	Reference
WT	Replicating wild type 5 virus.	II	
WT-RGD	RGD insertion in HI loop of fiber knob for $\alpha_v\beta$ integrin targeting.	II	(Cascallo et al. 2007)
WT-RGDK	Fiber shaft's KKTK domain mutated to RGDK for $\alpha_v\beta$ integrin targeting. HSPG ablation via mutated KKTK	II	I
Ad5-Δ24-RGD	24-bp deletion in the CR2 region of E1A. RGD insertion in HI loop of fiber knob for $\alpha_v\beta$ integrin targeting.	III, IV	(Dmitriev et al. 1998, Bauerschmitz et al. 2002)

3.3. Animals

All animal experiments were conducted according to the rules set by the Federation of European Laboratory Animal Science Associations and the Provincial Government of Southern Finland. Pathogen-free, 3-4-week-old female NMRI nude mice were purchased from Taconic (Ejby, Denmark), Charles River (Sulzfeld, Germany) and Scanbur (Sollentuna, Sweden), while 10-11-week-old female SCID (severe combined immunodeficiency) mice were obtained from Taconic, Charles River, and Harlan (Horst, Holland). Animals were quarantined for 2 weeks. They were fed *ad libitum* and maintained in a HEPA-filtered environment with cages, food, and bedding sterilized by autoclaving.

3.4. *In vitro* studies

3.4.1. Adenovirus transduction assay (I, II, IV)

One day before infection, cells were seeded in 24 or 96-well plates. Viruses in growth medium were added at the desired dose of viral particles (VP) per cell and incubated for 20 hours for 293 cells and for 48 hours for the other cell types. In IV, some of the samples were incubated at the same time with isomolar doses of different proteases. 30 minutes after the growth medium was renewed. To quantify luciferase activity and protein content, luciferase assay reagent (Luciferase Assay System, Promega, Madison, WI, USA) was used and measured in a luminometer (Berthold Junior; Berthold). Protein concentration of supernatant was quantified using Bio-Rad protein assay. Results are expressed in relative light units (RLU) per µg of protein.

3.4.2. Oncolytic potency in human colorectal cancer cells (I, II, IV)

Cells were infected with replication competent viruses or a non-replicating control virus and, after 1 or 2 hours, infection medium was replaced with another containing 5% FCS. In the experiments reported in IV, growth medium was replaced again after 12 hours and equimolar doses of different proteases were added in medium with 5 % FCS. Growth medium was renewed every other day. Eight to 11 days later (at the optimal time point for each cell line), cell viability was analyzed with the mitochondrial activity-based 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl) -2-(4-sulfophenyl)-2H- tetrazolium (MTS) assay (Cell Titer 96 AQueous One Solution Cell Proliferation Assay; Promega, Stockholm, Sweden).

All statistical analyses were carried out with SPSS 15.0 for Windows. One-way analysis of variance (ANOVA) followed by Dunnett´s Pairwise Multiple Comparison t-test was applied.

3.5. Mouse models

3.5.1. Subcutaneous tumor xenograft models (I, IV)

Subcutaneous tumors were implanted in both flanks of NMRI nude mice by injection of the corresponding human cancer cells in a given volume of DMEM without supplements.

3.5.2. Intra-hepatic tumor model of colorectal cancer (II)

The surgical procedure was similar to what has been previously described (Yu et al. 2004). Mice were anesthetized with ketamine (Ketaminol® 75 mg/kg; Intervet, Boxmeer, Netherlands) / dexmedetomidine (Dexdormitor® 1 mg/kg; Orion Pharm, Espoo, Finland) mixture and the spleen was exteriorized through a left lateral flank incision. Tumors were established by intrasplenic injection of 2×10^6 HCT116 cells suspended in 50 µl of serum-free growth media using a 27-gauge needle. The injection site of the spleen was pressed with a cotton stick wet in iodine-polividone solution (Betadine®; Leiras, Helsinki, Finland) in order to remove extravasated cells and ensure hemostasis. The peritoneum and skin were closed in a single layer using surgical thread. Finally, atipamezole (Antisedan® 1 mg/kg; Orion Pharm, Espoo, Finland) was injected subcutaneously to reverse anesthesia.

3.5.3. Metastatic human colorectal cancer model (III, IV)

Tumors were established by intrasplenic injection of 1×10^6 HT29 cells as described in the section 3.5.2.

3.6. *In vivo* studies

3.6.1. Biodistribution in a tumor xenograft model of mice (II)

The mouse model described in 3.5.2 was used. Tumors were left to grow and 21 days after intrasplenic injection of HCT116 cells, 3×10^{10} VP of AdTL, AdTLGR, or AdTLRGDK in 150 µl of PBS were injected through the tail vein of NMRI nude mice. After 48 hours, mice (n= 5 in each group) were sacrificed and organs and tumors were harvested for luciferase analysis. Data was normalized for protein concentration by Pierce BCA Protein Assay Kit® (Thermo Scientific, Rockford, IL, USA).

3.6.2. Antitumor efficacy in a intra-hepatic tumor model of colorectal cancer (II)

Tumors were implanted as described in 3.5.2. On day 23 and 24 after cell injection, mice were treated with two intravenous injections of 3×10^{10} VP of WT, WT-RGD, or WT-RGDK in 100 µl volume of PBS (n= 4, 11, and 9, respectively). Mock animals (n=9) were treated with PBS only. Tumor volume was followed up in the abdomen by MRI. Mice were imaged under isoflurane (Baxter, Helsinki, Finland) anesthesia.

30 minutes before imaging, 1 mg/kg of contrast agent Endorem® (Guerbet, Roissy CdG Cedex, France) in 100 µl volume was administered intravenously.

Tumor tissue areas in the liver were measured in every slice and a total tumor volume was calculated using the formula: ∑ (Area*slice height) or ∑ (Area*0.7). In order to distinguish hepatic tumor tissue from vessels or other structures present in the liver, all images were compared to a baseline image of each mouse taken before tumor implantation. Daily volumes of hepatic tumor tissue were normalized to tumor volume a day before treatment. Animals were examined daily for behavioral or physical signs of pain or distress and survival data was collected.

3.6.3. Viral replication of adenovirus in intra-hepatic colorectal cancer tumors in mice (II)

The xenograft model described in 3.5.2. was used: mice were treated 29 days after cell injection with 3 x 10^{10} VP of WT, WT-RGD, or WT-RGDK in 100 µl of PBS, or PBS alone (mock) (5 in all groups, except for WT-RGDK 6). Three days after treatment, mice were sacrificed and hepatic tumors were harvested, homogenized and diluted in growth media. After three freeze and thaw cycles (-80C / room temperature), tumor lysates were centrifuged, supernatant was collected and added to 293 cells to perform $TCID_{50}$ test. Plaque forming units per ml (pfu/ml) values were normalized for total hepatic tumor volume and the final values were given as amount of pfu/tumor.

3.6.4. Effect of proteases on the oncolytic potency of an adenovirus in a subcutaneous murine model of colorectal cancer (III, IV)

The subcutaneous murine model described in 3.5.2. was used. Tumors were allowed to grow for 14 days. Thereafter, 10^{-9} mols of each protease in an injection volume of 25 µl of PBS were injected intratumorally. Control animals received PBS only. The number of animals in each group was 4. Half of the animals from each protease group received 1.0 x 10^7 pfu of Ad5-Δ24-RGD intratumorally 24 hours later in a 50 µl volume of DMEM without FCS or antibiotics. Tumor volumes were calculated according to the formula V = ½ * length * width ^ 2 (Euhus et al. 1986; Tomayko, Reynolds 1989) at day 5 and data was expressed as relative volumes normalized to tumor volume a day before adenoviral treatment.

3.6.5. Oncolytic adenovirus efficacy and influence on tumor progression in a metastatic murine model of colorectal cancer (III, IV)

The mouse model described in 3.5.3. was used for these tests. Ad5-Δ24-RGD and/or MME were administered 21 days after tumor cell implantation by intratumoral injection to splenic primary tumors. Solution containing 2.3×10^{-10} mols of MME in 2 µl PBS and adenoviral suspension containing 1.1×10^{7} VP in 8 µl PBS were prepared extemporally and a single microinjection of 10 µl volume was given to each animal. Mock animals received PBS only. Tumor growth (both in the spleen and liver) was followed weekly with MRI for 4 weeks. Tumor volumes were estimated using the formula $V = \Sigma$ (Area x slice thickness). For intrahepatic tumors, the total area occupied by tumors in each slice was included in the formula. The number of animals in each group was 12 (mock), 10 (Ad5-Δ24-RGD), 8 (MME), and 12 (Ad5-Δ24-RGD + MME).

3.7. Magnetic resonance imaging

MRI studies were performed with a 4.7 T scanner (PharmaScan, Bruker BioSpin, Ettlingen, Germany) using a 90-mm shielded gradient capable of producing a maximum gradient amplitude of 300 mT / m with an 80-µs rise time. A linear birdcage RF coil with an inner diameter of 38 mm was used. T2-weighted images were acquired using rapid acquisition with relaxation enhancement (RARE) sequence (TR / TEeff = 3767/ 36 ms, matrix size = 256 x 256, Rare Factor = 8, field-of-view = 33 x 33 mm2, 32 slices, slice thickness = 0.7 mm, number of averages = 8).

3.8. Statistics

All statistical analyses were done with SPSS 15.0 or PASW Statistics 17 for Windows. One-way analysis of variance (ANOVA) followed by Dunnett´s Pairwise Multiple Comparison t-test was used to analyze the differences in the cell killing potency of viruses *in vitro* (studies II, III and IV) and tumor growth and virus replication *in vivo*. The Mann-Whitney test was used to analyze the differences in the biodistribution and tumor-to-organ ratios. Survival data was plotted into a Kaplan–Meier curve and groups were compared pair-wise with a log-rank test. A value for $p < 0.05$ was considered statistically significant.

4. RESULTS AND DISCUSSION

4.1. RGD insertion into fiber enhances Ad5 *in vitro* transduction to cancer cell lines (II)

Many capsid modifications have already proven to be effective as tools for ablating *in vitro* viral transfection to liver cells, either by impeding binding to CAR, HSPG or $\alpha_v\beta$-integrins. Unfortunately, those receptors also play a role in the viral entry mechanism to cancer cells. In order to establish which fiber modifications aimed to decrease liver tropism, still permit sufficient tumor targeting, a panel of several human cancer cell lines were transduced with non-replicating luciferase-expressing adenoviral vectors.

Tyrosine-to-alanine substitution (Y447A) in the DE-loop of the fiber knob hinders binding to CAR (AdTLY). This modification is enough to eliminate gene delivery to cancer cells, thus confirming the crucial role of CAR for *in vitro* infection of cancer cells. Obviously, no better results were obtained with DATL, a vector which adds to AdTLY the mutation of the integrin binding sequence RGD to RGE. Similarly, vectors modified to interfere with HSPG binding (AdTLG) showed reduced gene transfer compared to Ad5. As expected, double ablations for CAR and HSPG (AdTLYG) binding reduced gene expression levels as well. On the contrary, vectors where a RGD tripeptide motif had been inserted into the HI loop of the fiber knob (Ad5TLRGD) or substituted for the fiber shaft KKT domain (AdTLRGDK) proved to be clearly superior to transduce cancer cells (up to 800-fold and up to 145-fold respectively). Differences between gene delivery of AdTLRGDK and AdTLRGD depended on the cell line studied. In general, AdTLRGD infected all cell lines more efficiently than the vector with the RGD insertion at the HI-loop when the shaft had also been mutated (AdTLGR). AdTLRGDK transduced SW480 and SW620 cells as well as AdTLRGD did, but AdTLRGDK transduced Co115 and CaCo-2 cells better than AdTLRGD. Nevertheless, in a shaft-mutated background, RGD generally mediated better transduction when located at the KKTK site than at the HI-loop (AdTLRGDK vs. AdTLGR). This indicates that the shaft mutation GATK impedes proper exposure of RGD into the HI-loop, in agreement with other authors (Bayo-Puxan et al. 2006), and points out the potential of the KKTK domain of the Ad5 shaft as an alternative locus to insert tumor-selective peptides.

These results justify the use of RGD insertions either in knob or shaft domain to rescue vector infectivity to cancer cells after having been detargeted from CAR/HSPG-binding by other modifications in the capsid. Thereby, this first screening in several *in vitro* models represents a starting point to define the characteristics of vectors displaying additional RGD moieties in the fiber protein.

4.2. Biodistribution of adenoviral vectors with RGD insertions in the capsid (II)

Removal of the KKTK putative domain, by mutation of KKTK to GATK, as a unique capsid modification has proven to be effective to reduce liver transduction *in vivo* (Bayo-Puxan et al. 2006). Unfortunately, cell detargeting is not specific to normal cells and, as discussed in section 4.1, *in vitro* results showed that transduction levels with this vector in different cancer cells were lower compared to a similar virus with wild-type capsid. As those *in vitro* results also pointed out, insertions of the $\alpha v\beta$ integrin-binding moiety RGD in KKTK-mutated vectors were effective to rescue their infectivity towards cancer cells.

In order to define how these RGD insertions in KKTK-mutated vectors influence their biodistribution *in vivo*, NMRI nude mice bearing intrasplenic and intrahepatic HCT116 tumors were systemically injected with 3×10^{10} VP of AdTL (Ad5 control), AdTLGR (RGD in the HI loop; KKTK mutated to GATK), or AdTLRGDK (KKTK mutated to RGDK). At 48 hours, luciferase activity and protein concentration of organs and tumors (in spleen and liver) were measured.

The vector AdTLRGDK displayed the highest transgene expression in both types of tumors, but only in splenic tumors the result was statistically significant. Specifically, in comparison with unmodified virus, AdTLRGDK increased spleen and liver tumor transduction six- and five-fold while increase with AdTLGR was six and four-fold respectively. Both RGD-modified viruses showed increased hepatic tumor-to-liver and splenic tumor-to-spleen ratios in transgene expression.

Interestingly, AdTLRGDK and AdTLGR viruses presented very different biodistribution profiles. In normal liver tissue, AdTLGR displayed 10-fold lower and AdTLRGDK 23-fold higher transgene expression if compared to AdTL. A similar trend was seen in the spleen, but the difference between AdTLRGDK and AdTL was not statistically significant. For the kidneys and lungs, the only statistically significant difference was enhanced gene transfer of AdTLGR in comparison with AdTL. In the heart, no significant differences in the efficacy of gene transfer was seen between viruses.

All in all, AdTLRGDK showed an apparent tendency to present higher transduction rates than the other viruses in all organs. However, when the values were analyzed as tumor-to-liver ratios, relative targeting of tumors was improved but independent of the RGD insertion site. Ratios were compared to un-modified viruses. The results for these modified vectors show a more favorable profile in tumor targeting with a lower liver-to-tumor ratio than the wild-type.

4.3. Oncolytic potency of RGD-modified viruses *in vitro* (I, II)

Capsid modifications studied the previous sections with non-replicating vectors were brought also to the context of replication competent (oncolytic) adenoviruses. WT-RGD and WT-RGDK were used. The first of them has the RGD moiety in the fiber knob while the second presents the KKTK → RGDK substitution in the fiber shaft. Oncolytic potency of replication competent viruses and WT control virus was analyzed in six colorectal cancer cell lines by MTS assay. At the lowest viral dose (0.1 VP/cell), RGD modified viruses killed cells more effectively in comparison with WT in three out of six cell lines. At higher viral doses, however, RGD insertion in the HI loop of the fiber (WT-RGD) or in the shaft (WT-RGDK) did not increase the oncolytic potency and all three replication competent viruses showed an equal cell killing capability in all six established colorectal cancer cell lines. The E1-deleted Ad5 control virus did not cause oncolytic cell death.

Even if at the low doses RGD insertion plays a meaningful role in oncolytic efficacy in some cell lines, this effect is overcome by the rapid cell killing seen at doses of 1 VP/cell or higher. This effect is somehow surprising compared with the results obtained from non-replicating viruses (section 4.1). Nonetheless, the fact that replication competent viruses exponentially accumulate in the culture medium, while luciferase expression of non-replicating vectors relies on the one-time dose transduction of the target cells, might explain these results. In any case, the possibility of a decreased cancer cell lysis potential of the KKTK-mutated virus WT-RGDK is discarded with this experiment. However, for the reasons explained above, further research should clarify whether the influence of such modifications is undetected in the context of this particular experimental method or if it is simply irrelevant when applied to oncolytic adenoviruses.

4.4. Antitumor efficacy of RGD-modified viruses in metastatic model of human colorectal cancer (II)

The benefits of RGD modified vectors in oncolytic adenovirus therapies were evaluated with the following study. Human colorectal cancer cells (HCT116) were injected into the spleen of NMRI nude mice and intrasplenic and hepatic tumors were allowed to grow for 23 days. Two intravenous injections of viruses were given on consecutive days, and hepatic tumor volumes were followed by MRI thereafter. By day 21, tumors in all treated groups were already three times bigger than non-treated tumors. However, no differences could be observed between capsid modified and unmodified vectors. Nevertheless, on day 35 (end of the experiment), only WT-RGD and WT-RGDK treated animals showed statistically significant reduction in tumor growth in comparison with non-treated animals, while lower differences were observed between WT and mock groups. Median survival of mice

treated with WT, WT-RGD and WT-RGDK was of 44.5, 41, and 46 days, respectively, while it dropped down to 28 days for untreated animals. In any case, the differences were statistically significant.

It is interesting to see how the strong influence of capsid modifications in non-replicating vectors (see section 4.1) to increase cancer cell infectivity does not produce major effects when applied to replication competent vectors. One reason for that is that the effects on infectivity could be overcome by the potent replication rate of oncolytic viruses. In any case, if changes in liver targeting really influence toxicity of the treatment, viruses with RGD insertions would be more safe and, therefore, could be administered with higher doses, which would improve treatment efficacy.

Modified particles did not impede a long persistence of the virus in target tissue as was seen after assessing the amount of actively replicating viruses in tumor lysates by $TCID_{50}$. All treated tumors presented measurable titers for replicating virus, showing that modified capsids are capable of sustained active replication in a tumor target tissue. However, no statistically significant differences in the functional titers were observed between different viruses.

4.5. Analysis of transgene expression and oncolytic potency of RGD-modified adenovirus vectors co-administered with proteases *in vitro* (IV)

Broad spectrum proteases can degrade many components of tumor ECM and ease oncolytic adenovirus distribution throughout the tissue. Therefore, we hypothesized that co-administration of proteases with oncolytic adenovirus therapies can improve viral spreading in order to maximize tumor volume reduction.
As a first step to validate proteases as good coadjuvants for oncolytic administration, we aimed to study whether proteases have any effect on cancer cells that would alter transduction efficiency of adenovirus vectors. Thereby, human colorectal cancer cell lines HCT116 and HT29 were infected with Ad5lucRGD alone or in combination with equimolar doses of either elastase, hyaluronidase, relaxin, or MME. In general, proteases had no major effect on the transduction efficiency of Ad5lucRGD. However, for HCT116 cells, hyaluronidase produced a 2-fold decrease of transgene expression at the lowest viral dose. Relaxin also decreased transgene expression in less than 2-fold in combination with a viral dose of 1000 VP/cell, while MME increased transgene expression at viral doses of 200 and 1000 VP/cell (2 and 1-fold). In HT29 cells, all proteases slightly increased transgene expression. Relaxin and hyaluronidase in combination with lowest dose of the oncolytic virus gave the most relevant increase in transduction (6 and 9-fold). Importantly, no incompatibilities were detected between any of the proteases or the cells used for the study that would have an important decrease on viral infectivity.

The possible effect of proteases on the viability of human colorectal cancer cell lines and their capability to permit adenovirus replication was also tested. To this end, cancer cell monolayers were infected with 4500 VP/cell of the oncolytic virus Ad-Δ24-RGD and incubated for 12 hours. This period of time was long enough to permit maximum adenovirus transduction but not complete replication. After this 12-hour period, growth media was renewed with media containing equimolar doses the proteases. Cell viability was assessed regularly by the MTS assay.

First cells were treated with the proteases only. Of note, the MTS test detected some decrease in viability with cells incubated with MME. In contrast, hyaluronidase, relaxin, and elastase either slightly increased or had no effect on cell viability. The only exception for this was in the case of HT29 cells incubated with hyaluronidase, where cell viability decreased moderately after 7 days of incubation. The effect of proteases in combination with oncolytic adenovirus on cell viability was assessed similarly and, in this case, none of the proteases showed any difference in the cell killing in comparison to virus treatment alone.

Although this assay does not take into account the effect of ECM on intratumoral viral penetration, its reveals no major negative effect of proteases on the cells, like inhibition of viral replication. There is, however, no reason to reject any of the proteases for further studies.

4.6. Construction and validation of a new murine model of metastatic colorectal cancer (III)

A new animal model was designed to globally understand the effects of anticancer drugs in the context of a binary system, where both the treated primary tumor and metastatic tumors can be followed accurately during the lifespan of the animal. This new murine model features an intrasplenic primary tumor that spontaneously metastases to the liver through the portal system. There is at least one week of margin between the time the primary tumor is big enough to be intratumorally treated (i.e. presents a diameter of 2 mm or larger) and the detection of the first liver metastases, thus providing a good platform to test the influence of experimental treatments on tumor progression and metastases.

The injection of a certain volume of liquid and subsequent development of a fast-growing tissue, like a xenograft tumor tissue, requires a recipient organ that is solid and resistant enough to sustain it. We previously saw that mature NMRI-NUDE mice spleens gathered the necessary characteristics to do so. Unfortunately, given that mice have to remain under isoflurane anesthesia for 10 minutes or more during MRI, hairless NMRI-NUDE mice rarely survived the process. This is why SCID mice

were used as no death related to hypothermia was ever seen thanks to their thick fur layer and all the mice imaged survived the session. As a drawback, mutations used to generate deficiencies of the immune system in SCID mice delay normal spleen development (Seymour, Sundberg & Hogenesch 2006) and lead to morphologically immature spleens in adult mice. Those spleens were more small, soft, and pale than in wild type (NMRI) mice and too fragile to even sustain a small volume injection. In fact, these properties were so striking that they could even be observed with MRI, where the spleens of SCID mice appear squeezed between neighboring organs due to their malleable consistency. Interestingly, important differences in spleen morphology were seen not only between mice strains, but also among mice from a different provider. Therefore, the animals had to be of at least 125 days of age for the splenic tumors to be implanted. At that time, all mice obtained from Taconic presented mature spleens at the time of imaging, so they were selected for further tests.

A crucial prerequisite for our model is that a clear distribution in time exists between the formation of the primary tumor and the subsequent spreading and detection of liver metastases. It has been shown that injection of many cancer cells in the spleen leads to hepatic tumors caused by direct implantation and not by a metastatic process in the host organism (Bouvet et al. 2006). In order to avoid this, we followed splenic and hepatic tumor growth after intrasplenic injection of different human colorectal cancer cell lines: Co115, HCT116, SW620, HT29. While Co115 cells produced intrasplenic and intrahepatic tumors between 14 and 43 days after cell injection in 5/8 and 3/8 animals respectively, HCT116 cells, produced intrasplenic tumors after 7 days in 5/7 and after 32 days in 6/7 animals. SW620 did not induce tumors neither in the spleen nor liver in any of the animals (0/4). For HT29, all animals (8/8) developed both primary tumors and metastases. In all cases, intrasplenic tumor growth was detectable by 21 days after cell injection while tumors in the liver were first observed 1 to 4 weeks later, starting from day 28 after cell implantation. According to these data, HT29 was the cell line that showed less intra-individual variability. Indeed, the portal system allows easy spread of metastatic cells from splenic tumors to the liver but may also mediate direct implantation of the cells in hepatic tissue after intrasplenic injection. Therefore, it is crucial to distinguish between these events. Direct implantation is something we observed with HCT116, where 2/7 mice presented hepatic tumors but no intrasplenic ones and 1 mouse presented a hepatic tumor several days before the splenic tumor grew. In opposition to this, with HT29, all mice developed liver tumors 7 to 21 days after the appearance of primary intrasplenic tumors, strongly supporting the metastatic origin of these tumors. This delay in intrahepatic tumor development and the fact that in all mice it occurs at least one week after splenic tumor detection, demonstrates a metastatic origin for the liver tumors.

For its optimal utility, the imaging method should allow fast and real time identification of tumor development in the spleen and the liver. A general difficulty with MRI is to differ between tumors and stromal elements such as vessels, the gall bladder, hepatic lymph nodes or ligaments. Therefore it was important to compare our MRI results with laparotomy findings. Some mice were thus inspected through laparotomy to compare the location, size and shape of the tumors to their MRI images. Also, the entire organ and a section of the organ, where the tumor was engrafted, gave an accurate idea of the location and shape of the tumor, which corresponded to its predicted location and shape by MRI. This demonstrates that MRI is a reliable method for splenic and liver tumors diagnosis in mice.

4.7. Use of the optimized mouse model to study the effects of an experimental treatment (IV)

Animals were implanted with HT29 cells and imaged with MRI weekly for 6 weeks. Following previous model validation results, mice developed both intrasplenic (primary) tumors and liver metastases which were detected in each case 1 to 3 weeks after intrasplenic tumor formation. At least one week before metastases detection, primary tumors were considered suitable for a 8 µl microinjection of the therapeutic agent. Of note, this time window of at least one week makes the model useful for the evaluation of possible pro- and anti-metastatic properties of the treatment.

Murine cancer models for drug design should be sensitive enough to antitumor drugs' effects and, thus, produce a clear differential response. In the case of this murine model, changes on splenic and metastatic tumor growth and number of metastases should be both easily detectable to permit good quality of the data collected. Oncolytic adenoviruses have been widely tested in mouse xenograft models of human cancers and shown to effectively reduce tumor masses (Liu 2006). Therefore, oncolytic virus Ad5-Δ24-RGD was used as an example of an antitumor agent to assess model responsiveness. When a moderate dose of this drug was used as a single intratumoral administration , significant intrasplenic tumor growth reduction, detected after a latency period of two weeks (i.e. days 21 to 35 after tumor implantation) was seen. This response was clearly different to untreated mice, whose tumors grew steadily until the end of the experiment. Follow up of liver metastases did not reveal any pro-metastatic effect linked to the oncolytic virus administration. On the contrary, a reduced number of metastatic lesions as well as significantly a smaller amount of total tumor mass in the liver were observed.

In conclusion, this murine model is capable of clear, accurate and sensitive detection of changes in tumor volume. It also permits repeated and frequent measurements without the need of sacrificing the animal, thus reducing importantly the amount of

mice required for the experiment. Last but not least, it detects the possible effects of the administered drug or intervention on the amount and growth rate of metastases originated from the primary tumor.

4.8. Effect of protease pre-treatment on adenoviral oncolytic potency in subcutaneous tumors (IV)

Given the lack of incompatibilities seen with relaxin, hyaluronidase, elastase or MME in conjunction with oncolytic adenovirus to treat solid tumors, all four proteases were included in an *in vivo* screening to test the effects of the protease on the efficacy of cancer treatment. Subcutaneous tumors were implanted by HCT116 cell injection in NMRI nude mice (four mice per group; two tumors per animal). Mice were treated intratumorally with one of the proteases and, 24 hours later, half of the animals were infected with the oncolytic adenovirus Ad5-Δ24-RGD. Control animals (four animals per group) received PBS intratumorally on two consecutive days, or PBS followed by intratumoral injection of the oncolytic virus. Tumor volumes were measured after 5 days and normalized to their volume one day before treatment with oncolytic adenovirus.

The dose used of the oncolytic virus treatment alone did not reduce tumor growth significantly. On the contrary, a pronounced inhibition was seen with the combination treatment of oncolytic virus and each protease, showing how some of the proteases (i.e. relaxin, elastase and MME) improved significantly the efficacy of the treatment. No statistically significant difference between oncolytic virus therapy and combination therapies was detected. None of these proteases reduced tumor growth significantly alone, except hyaluronidase. However, important reductions (over 2-fold) were observed between untreated tumors and those treated with MME. This could be related to some effect of the protease on the tumor microenvironment, for example on the vascularization of the tissue and hence the amount of liquid retained. Also some effect of MME on the tumor cells could be expected, considering that MME, decreased cell viability *in vitro*, as described in section 4.5. Animals did not show signs of post-treatment distress in any treatment groups.

4.9. Effect of MME on oncolytic efficacy of adenovirus in splenic primary tumors and liver metastases (IV)

The protease MME was selected among other proteases for its inhibitory effect on tumor growth seen *in vitro* and for its positive effects on tumor microenvironment described in the scientific literature (Acuff et al. 2006, Cornelius et al. 1998, Dong et al. 1997, Gorrin-Rivas et al. 2001, Houghton et al. 2006, Kerkela et al. 2001, Shi et al.

2006, Yang et al. 2001). Human cancer cell line HT29 was used to establish intrasplenic tumors in SCID mice. 21 days after tumor implantation, all intrasplenic tumors sizes were at least of 2 mm in diameter and the liver did not present any metastases. Animals (8 to 12 animals per group) were injected intratumorally with Ad5-Δ24-RGD, MME, or combination of both. Tumor volumes in the spleen and liver, and the number of liver metastases were followed weekly with MRI. Importantly, metastases appeared at least one week after treatment injection, being their development directly influenced by the treatment.

Untreated and MME-treated splenic tumors grew steadily until the end of the experiment. However, MME-treated tumors grew slightly more slow, possibly due to the effects of MME on tumor cells or tumor microenvironment suggested in section 4.8. Equal reduction in tumors treated with oncolytic adenovirus or in combination with MME was observed. Both treatments showed statistically significant difference in comparison to untreated tumors at three weeks after treatments and thereafter. No differences were observed between oncolytic adenovirus with or without MME, unlike it happened in the subcutaneous model. In order to improve the effect of MME as coadjuvant, it could be useful to increase its dose, since no toxic effects were seen with the doses of protease used for the present study.

In general, liver tumors could be first detected by MRI four weeks after injection of cancer cells to the spleen. All mock treated (PBS) animals (n=12) showed development of liver tumors by day 14. 88% (7/8), 70% (7/10), and 92% (11/12) of animals receiving intratumoral treatment of their primary tumors with MME alone, oncolytic virus alone, and combination treatment developed liver tumors, respectively. All treatments seemed to decrease the growth of liver metastases equally in comparison to untreated mock animals, resulting in smaller tumor size. However, in comparison to untreated animals, statistical significance was achieved only in groups treated with oncolytic virus alone and protease alone. No statistically significant differences in the numbers of liver tumors were seen among the groups. The present experiment did not show that the treatments would lead to an important increase of metastases compared to the control, probably because of the big variability within every group.This points out that variability of the number and, especially, total volume of metastases could be a limitation for the animal model, since it could demand bigger number of animals for drugs that do not present a strong anti- or pro-metastatic effect. Animals did not show signs of post-treatment distress in any treatment groups.

5. SUMMARY AND CONCLUSIONS

The work reported in this Doctoral Thesis seeks to improve adenovirus-based viral vectors for the treatment of cancer. While the first part focused on modifying vector biodistribution towards an improved cancer targeting / toxicity balance, in the second part we propose the use of proteolytic adjuvants in order to increase viral distribution after local injection into the tumor mass. In addition, we validated a new murine model of colorectal cancer that permits to detect possible increases in primary tumor metastasis potential as an adverse effect of proteolytic treatment.

The RGD-motif located in the penton base is responsible for viral internalization and, when artificially displayed in the knob domain, can increase transduction of cancer cells, whose cellular membranes are rich in integrins. At the same time, KKTK-domain in the shaft has a major role in liver transfection *in vivo*. Simultaneous ablation of liver targeting by KKTK-domain removal and tumor targeting by RGD display in different parts of the fiber was thus considered. The importance of the KKTK motif *in vivo* was corroborated since a 142-fold reduction on liver tropism with AdTLG, whose KKTK-motif was substituted for GATK, was detected in comparison with a wild type capsid virus (AdTL). Unfortunately, the striking decrease in liver targeting was accompanied by a substantial decrease in tumor transduction. In order to correct this drawback, we included a RGD motif in the fiber shaft by substitution of the KKTK-domain for RGDK. While the new virus could only decrease liver targeting 6-fold compared to wild-type virus, the infectivity of cancer cells *in vitro* was in general higher than with AdTLG and the *in vivo* transduction to subcutaneous tumors increased 3-fold. These improvements were associated with an improved capability of the virus to reduce tumor volumes and increase the survival of the mice in comparison with wild-type capsid vectors.

Once the adenovirus vector reaches the target, an efficient spreading throughout the tumor mass is important. A panel of proteases was used for co-injection with the oncolytic virus Ad5-Δ24-RGD: relaxin, hyaluronidase, elastase and macrophage metalloelastase (MME). Among them, MME increased tumor volume reduction after viral treatment of a subcutaneous model of colorectal cancer made by implantation of HCT116 cells. Further tests in a metastatic colorectal cancer model not only proved the utility of MME in mono- or in combination therapy, but proved to be safe too for it did not induce a significant increase in the metastatic potential of the cancer.

The work performed here with tumor stroma degrading proteases demands from accurate models to study tumor microenvironmental changes after experimental therapies. In this regard, a novel murine model of human colorectal cancer was constructed by intrasplenic injection of the carcinoma cell line HT29. Primary tumor growth showed small variability among subjects when using the correct mouse

strain and provider. Besides, an optimized MRI protocol permitted us to follow up the primary tumor growth and the development of liver metastases over a detection limit as small as 0,7 mm (tumor diameter). The primary tumor growth and subsequent progression to metastases was clear and well defined: for every mouse, between one to three weeks, passed between the detection of the primary tumor until the first liver metastases. This offers a broad window to administer the study treatment before metastases start and confirms the metastatic origin of the hepatic tumors. The model was indeed capable of evaluating the antitumor potential and influence of an oncolytic adenovirus on the number and total volume of metastases.

All in all, the development of novel oncolytic viruses by capsid modifications, which can be applied to non-replicating vectors for therapeutic gene transfer, offers the possibility to construct stable vectors with a more beneficial tropism for therapeutic purposes. This approach can be combined with the use of proteases. Particularly, the use of MME is suggested for improved intratumoral spreading of the virus. Further investigations should assess whether a stronger degradation of the tumor microenvironment by higher doses of MME would significantly improve tumor rejection by the oncolytic virus and also if, still, no influence on tumor progression and metastases would be observed. In addition, more research on the effects of MME *per se* on tumor cells and tumor tissue in general would be desirable. The new murine model presented here can assist the development of this type of work, and be useful for the study of new factors influencing tumor progression to metastases.

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PART C - ORIGINAL PUBLICATIONS