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ON THE MECHANISMS AND CONSEQUENCES OF CELL TO CELL DNA TRANSFER

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Cover Images; hybrid endothelial cells stained with CD31, SV40LT and DAPI (front), FISH with mouse and rat specific probes on hybrid endothelial cells, MEF cell phagocytosing DNA from apoptotic cell stained with LaminB1, BrdU and DAPI. Photo; M. Kost-Alimova and J. Ehnfors

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To my family

ABSTRACT

Development of cancer is a multistep process where an accumulation of genetic alterations drives normal cells to malignancy. In most cancer cells genetic changes can be observed both at the level of point mutations and as gains and losses of chromosomes. The eukaryotic cell has a complex protection system to recognize genetic alterations and, in the case of oncogene activation or severe DNA damage, direct the cell to undergo apoptosis. One of the morphological attributes initially used to define apoptosis is the fragmentation of DNA and the formation of apoptotic bodies. In healthy tissues apoptotic cells are rapidly phagocytosed by neighboring cells. Dying cells can thereby be cleared without leakage of harmful enzymes or eliciting an inflammatory response. The fragmentation of DNA that occurs in apoptotic cells is complemented by DNases in the phagocytosing cell, which ensure the destruction of potentially harmful DNA. However, it has been recently demonstrated that genes from apoptotic cells can avoid fragmentation and be salvaged and reutilized by the phagocytosing cell. This newly recognized mechanism of introducing foreign DNA is efficient since 15 percent of bovine aortic endothelial cells have been demonstrated to display uptake of apoptotic DNA and express this DNA. Furthermore, normal cells have been reported to be protected from propagation of DNA recovered from dying cells by the p53-p21 signaling pathway.

This thesis demonstrates that DNA from apoptotic cells can enter the nucleus of phagocytosing cells by a previously undescribed mechanism of nuclear fusion named *Pirinosis* as an acronym of the Greek words *Pirinas* (nucleus) and *Enosis* (union). The degree of DNA fragmentation after phagocytosis is demonstrated to be crucial for signaling via the Chk2-p53-p21 pathway. Furthermore, DNA fragments within the nucleus of phagocytes are demonstrated to co-localize with early markers of the DNA damage pathway.

Others have shown that endothelial cells in the tumor microenvironment display genetic alterations and aneuploidy and hence the dogma that diploid cells of the blood vessel wall are stable is challenged. This turned our focus to horizontal gene transfer between tumor cells and the surrounding stroma. Tumor associated endothelial cells isolated from rat tumors grown in mice displayed the same karyotype and inter-species chromosome fusions as detected after uptake of DNA from apoptotic cells *in vitro*. The hybrid tumor associated endothelial cells are shown to be functional in forming blood vessels that anastamose with the host circulatory system. We argue that phagocytosis of dying tumor cells by endothelial cells may create viable hybrid cells that are capable of creating functional vessels *in vivo*.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Bergsmedh A, **Ehnfors J**, Kawane K, Motoyama N, Nagata S, and Holmgren L
DNase II and the Chk2 DNA damage pathway form a genetic barrier blocking replication of horizontally transferred DNA
Mol Cancer Res., 2006, 4(3):187–95

II. Bergsmedh A*, **Ehnfors J***, Spetz A, and Holmgren L
A Cre-loxP based system for studying horizontal gene transfer
FEBS Letters, 2007, 581(16): 2943–2946

III. **Ehnfors J**, Kost-Alimova M, Bergsmedh A, Castro J, Levchenko-Tegnebratt T, Luna Persson N, and Holmgren L
Horizontal gene transfer in the tumor microenvironment
Submitted

IV. **Ehnfors J**, Bergsmedh A, Kost-Alimova M, and Holmgren L
Pirinosis: mechanism of horizontal transfer of DNA by uptake of apoptotic cells
Submitted

* Both authors contributed equally

CONTENTS

LIST OF ABBREVIATIONS

APC	adenomatosis polyposis coli
APC	antigen presenting cells
ATM	ataxia-telangiectasia mutated
ATR	ATM and rad3 related
BAE	bovine aortic endothelial
bFGF	basic fibroblast growth factor
BMD-EC	bone marrow-derived endothelial cells
BrdU	5-bromo-2-deoxyuridine
CAD	caspase-activated DNase
CD31	cluster of differentiation molecule 31
CDK	cyclin dependent kinase
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
CIN	chromosomal instability
C-myc	cellular myc
DC	dendritic cells
DNA	deoxyribonucleic acid
DNaseII	DNA nuclease II
DNA-PK	DNA-dependent protein kinase
DSB	double strand break
EBV	Epstein-Barr Virus
EC	endothelial cells
EndoG	Endonuclease G
EPC	endothelial precursor cells
FACS	fluorescence activated cell sorting
FasL	Fas-ligand
FasR	FAS-receptor (CD95, Apo-1, TNFRSF6)
FGF-2	fibroblast growth factor-2
FISH	fluorescence *in situ* hybridization
HEK-293	human embryonic kidney cells 293
HIV	human immunodeficiency virus
HPV	human papillomavirus
H-ras	Harvey ras
H-ras^{v12}	Harvey ras, glycine to valine mutation at residue 12
ICAD	Inhibitor of CAD
ICAD-L	Inhibitor of CAD-Long
ICAD-Ldm	Inhibitor of CAD-Long double mutated
ICAD-S	Inhibitor of CAD-Short
LOH	loss of heterozygosity
MAX	myc associated x
MEF	mouse embryonic fibroblasts
Mdm2	transformed mouse 3T3 cell double minute 2 p53 binding protein
MIN	microsatellite instability
MRN	Mre11-Rad50-Nbs1

NER	nucleotide-excision repair
NP	influenza A nucleoprotein
PECAM	platelet/endothelial cell adhesion molecule
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI3K	phosphatidylinositol-3-kinase
PIKKs	phosphoinositide 3-kinase related kinases
pRB	retinoblastoma protein
PS	phosphatidylserine
REF	rat embryonic fibroblasts
RSV	Rous sarcoma virus
RT	reverse transcriptase
SAPK	stress-activated protein kinase
SCID	severe combined immunodeficiency
SV40LT	Simian virus 40 large T antigen
TEC	tumor derived endothelial cells
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial cell growth factor
WHO	World Health Organization
Wt	Wild type
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

1 INTRODUCTION

DNA is the molecule that carries the instructions used for the development and function of all known living organisms. This deoxyribonucleic acid, consisting of only four different bases in a long polymer, has the capacity to be the blueprint and long-time storage of all information needed to form a human being. Although the coding variation of four bases is limited, the length of the polymer makes the possibilities enormous. For instance, the largest human chromosome, chromosome number 1, is an incredible 250 million base pairs long. The human genome, i.e. the complete set of DNA, is made up of some 3 billion base pairs divided on 23 distinct chromosomes that can be found in all the cells within the body, except for mature red blood cells. Dispersed within this huge number of base pairs resides approximately 25 000 genes, which comprise only 2% of the genome (Venter *et al*., 2001). The proteins encoded by the genes execute the control within the cell, from proliferation to death. The DNA molecule is constantly assaulted by extracellular agents, such as chemicals and radiation, but also from within the cell by natural by-products of cellular metabolism. Although the cell has complex repair machinery, sometimes genetic changes occur that persist. Cancer is the name of a group of diseases where genetic alterations have lead to the possibility of cells that violate the rules that are hardwired into cells and tissues. Although there are several different types of cancers they all seem to manifest six essential alterations in cell physiology, namely: self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). These hallmarks of cancer are not necessarily a consequence of the same genetic or epigenetic alterations in different cancers but, as the neoplasia turns into a malignant tumor, they will be shared by all tumors.

One of the hallmarks of cancer is the possibility of a tumor to sustain angiogenesis, i.e. the formation of new blood vessels. If a tumor is not in the vicinity of an adequate vasculature network it will only reach the size of approximately 0.4 mm in diameter (Gimbrone *et al*., 1972) due to an absence of angiogenesis, which restrains the size by balancing cell proliferation with programmed cell death (Holmgren *et al*., 1995; Parangi *et al*., 1996). Programmed cell death, i.e. apoptosis, is a controlled way for organisms to eliminate unwanted cells without eliciting an inflammatory response. Apoptosis consists of a series of precisely designed steps that results in the corpse being rapidly engulfed, i.e. phagocytosed, by neighboring cells (Wyllie *et al*., 1980). DNA from these apoptotic cells has been shown to be able to transfer into the phagocytosing cell and thereby be incorporated into the genome and reutilized (Bergsmedh *et al*., 2001; Bergsmedh *et al*., 2002; de la Taille *et al*., 1999; Holmgren *et al*., 1999). This thesis is focused on the still largely unknown underlying mechanisms of horizontal cell to cell DNA transfer in eukaryotic cells, and the consequences *in vitro* and in the tumor microenvironment *in vivo*.

2 BACKGROUND

2.1 HORIZONTAL GENE TRANSFER

The fact that DNA is the molecule responsible for the program of life is well established. Less well known is that the DNA content of different organisms can be remarkably fluid. DNA is transferred from one organism to another and in this new organism it can be incorporated, permanently changing the genetic composition. This process is in sharp contrast to the inheritance of DNA that descends from one's parents.

2.1.1 Bacteria

Bacteria are classified as prokaryotic unicellular microorganisms that typically are a few micrometers in length. Transfer of DNA between bacteria is an established and well-studied mechanism as a vehicle to spread resistance to antibiotics and adaptation to new environments (Summers, 2006). The dogma that diseases caused by bacteria can be cured with antibiotics is challenged by the fact that genes conferring resistance to antibiotics are widespread in nature and that these genes can be transferred between pathogens. There are several different mechanisms of horizontal transfer of DNA in bacteria but to me the conjugation pathway is the most intriguing. The "fertility" plasmid of *Escherichia coli* serves here as an example (Fig. 1). Conjugation is a unidirectional transfer of DNA from a donor to a recipient cell that requires about 40 genes and is initiated by the protrusion of the conjugative pilus. When contact is made the conjugative pilus retracts so that the cells are brought into contact with each other and DNA can transfer via the mating pore (Lanka and Wilkins, 1995). DNA transfer between bacteria and yeasts has been reported to occur in a conjugation-like manner and is named transkingdom conjugation (Heinemann and Sprague, 1989; Inomata *et al*., 1994). Furthermore, the transfer of eukaryotic expression plasmids has been demonstrated to occur from bacteria to mammalian cells, although the mechanisms in these cases are unknown (Courvalin *et al*., 1995; Darji *et al*., 1997; Sizemore *et al*., 1995)

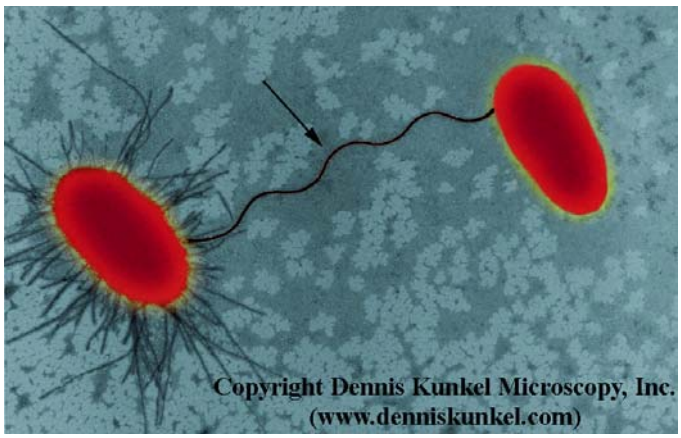

Fig. 1 *Electron microscopy photo of* Escherichia coli *strains undergoing conjugation. One strain has fimbriae. Arrow indicates pilus (published with permission from Dennis Kunkel).*

2.1.2 Yeast

Yeast is a eukaryotic unicellular microorganism in the same physical size range as bacteria. As many as 1 500 different species of yeast have been described and most of these reproduce asexually by budding. *Saccharomyces cerevisiae*, which has been used for baking for thousands of years, exists both as haploid and diploid cells that reproduce by budding. Haploid cells are also capable of mating between the two different mating types, MAT**a** and MAT**α.** The intra- and inter-species exchange of DNA has been shown to create novel combinations of genetic material and has been proposed as a way of spreading virulence factors from pathogenic species to nonpathogenic species of yeast (Marinoni *et al*., 1999; Mentel *et al*., 2006). Mating is initiated by exchange of pheromone signals between cells of opposite mating type. This pheromone signaling results in several consequences, namely cell cycle arrest to ensure that both cells have only one copy of each chromosome, polarization and formation of a mating projection and the induction of mating genes. The two cells then follow a highly controlled series of events that leads to fusion of the cell membranes (Chen *et al*., 2007). The fusion of the cell membranes results in a single cell comprising two separate nuclei. The mating of yeast is completed by the fusion of these two nuclei in a process called karyogamy (Rose, 1996). The nuclear envelope consists of two lipid bilayers where the outer layer continues with the endoplasmic reticulum (ER). In contrast to the nuclear envelope of human cells, the nuclear envelope of yeast remains intact throughout mitosis, meiosis and mating (Kurihara *et al*., 1994).

2.1.3 Virus

The transfer of DNA between human eukaryotic cells has not been shown to occur in the same way as in bacteria or yeast, although gene transfer by viruses occurs both in prokaryotic and eukaryotic cells. A broad division of viruses are those containing DNA or RNA. Retroviruses are made up of RNA that is enclosed by a lipid bilayer. Contained within the core are three virus-encoded enzymes that are common to all retroviruses. These enzymes are 1) the reverse transcriptase enzyme (RT), which is responsible for the synthesis of a cDNA copy of the viral RNA genome, 2) the integrase protein (IN), which is required for the integration of the viral cDNA into the host cell DNA, and 3) the protease enzyme (PR), which cleaves synthesized polymers of viral proteins into individual proteins. The fusion of retroviruses and cells either occurs at the cell membrane or in the endosome if the virus first has been engulfed, i.e. endocytosed. Once the viral RNA is within the cell cytoplasm the RT enzyme makes a cDNA copy. This retroviral replication is quite error prone and allows retroviruses to evolve extremely rapidly. The viral cDNA is then integrated into the genome of the host cell by the IN protein. This process results in a stable integration that means that the viral genes are duplicated and transmitted to the daughter cell if the infected cell proliferates (Coffin J.M., 1997). Viruses made up of DNA can also have their genetic information integrated into the host genome but this is a random event.

Viruses can promote tumor formation in several different ways either by introduction of cancer-causing genes, i.e. oncogenes, insertional activation of oncogenes or gene inactivation by retroviral integration. Uninfected cells often carry a cellular version of viral oncogenes that functions normally in the cell but that has the potential to induce cell transformation and thus cancer. These inactive oncogenes are called proto-oncogenes.

2.1.4 Cell fusion

Cell fusion is a process in which two or more cells merge their plasma membranes and thereby become one. This new cell, consisting of two or more cells is known as a hybrid or syncytium. Fusion of cells is quite easily done in the laboratory setting with chemicals like polyethylene glycol (PEG) or the virus envelope of Sendai virus, but how this occurs in live multicellular organisms is a poorly understood multistep process that involves cell-cell recognition, cell adhesion and finally membrane fusion (Chen *et al*., 2007). Cell fusion plays an important role throughout the life of humans, starting already where life begins with the fusion of our parents' gametes. The muscle fibers in our bodies are multinucleated syncytia that are the result of fusions between hundreds, or even thousands, of myoblasts during our development (Horsley and Pavlath, 2004). Furthermore, the maintenance of bones in adults is partly performed by osteoclasts, which are multinuclear cells. The fusion of normal somatic cells is tightly controlled and restricted to a few cell types and the multinuclear hybrids that are formed are terminally differentiated and non-proliferating cells.

2.1.4.1 Cell fusion and cancer

Already in the beginning of the 20th century Aichel proposed that the hybridization between leukocytes and somatic cells could lead to malignancy and that the differences between cells seen within tumors were due to unequal chromosome distribution after fusion (Rachkovsky *et al*., 1998). In the beginning of the 1970s Mekler stated that the hybridization of transformed cells with lymphocytes was the probable cause of tumor progression to metastatic malignancy (Mekler, 1971). The concept of hybridization of tumor cells with somatic cells is a possible mechanism of generating metastasis. The features of leukocytes, e.g. homing, extravasation, motility etc, would be combined with the tumor cells uncontrolled proliferation and capacity to avoid apoptosis. Fusion of normal cells with different cancer cells has been shown, at least in experimental systems, to form hybrid cells with enhanced metastatic potential (De Baetselier *et al*., 1984; Kerbel *et al*., 1983; Lagarde and Kerbel, 1985; Larizza and Schirrmacher, 1984; Rachkovsky *et al*., 1998). Even though these reports exist, the general view is that the traits needed for metastasis are generated by a series of mutations solely within the tumor cell itself. In fact hybridization experiments performed by Harris *et al*., also in the early 1970s, showed that fusions between tumor cells and fibroblasts suppressed tumorigenic features rather than accelerating them (Harris, 1971; Harris *et al*., 1971). These experiments actually lead to the notion of tumor suppressors, which was later confirmed by the cloning of pRB and p53 (Harris, 1996).

2.1.5 Cell to cell DNA transfer via uptake of apoptotic cells

The first reports of DNA being transferred from one eukaryotic cell to another by phagocytosis of apoptotic cells were published in 1999 (de la Taille *et al*., 1999; Holmgren *et al*., 1999; Spetz *et al*., 1999). By following the fate of specific genes it was demonstrated that the DNA within dying cells can be salvaged and reutilized in phagocytosing cells.

2.1.5.1 Horizontal transfer of virus DNA

Holmgren *et al*. showed that horizontal transfer of DNA coding for the Epstein-Barr virus (EBV) into human fibroblasts, macrophages and aortic endothelial cells occurred after uptake of apoptotic cells. This transfer was dependent on the integration of DNA into the dying cells since episomal DNA was not transferred. The EBV-coding DNA was not only transferred to the nucleus of the phagocytosing cell but was functional and expressed. Furthermore, it was shown that the expression of different genes was dependent on the new host. The expression of EBV-encoded proteins was analyzed after horizontal transfer experiments using phagocytosing macrophages and endothelial cells and a high efficiency was reported, i.e. 51% and 20%, respectively. Horizontal transfer of DNA was reproducible when apoptosis was induced by either irradiation or Ectoposide but not when the cells were subjected to hypo-osmotic shock. The authors proposed that the presence of EBV-DNA and its expression, which has been reported in cells that lack the complement receptor2 (CR2), may be due to horizontal transfer from dying EBV infected cells *in vivo*. In addition, it was stated that the uptake of DNA from dying cells into antigen presenting cells, such as macrophages, could in part elicit an immune response to viral antigens. The authors further speculated that in conditions with high levels of apoptosis, such as tumors treated with irradiation or chemotherapy, this novel mechanism may be of importance.

In the paper by Spetz *et al*., HIV-DNA was shown to transfer via apoptotic bodies to cells with phagocytosing capacity, such as human fibroblasts, endothelial cells and dendritic cells. In the same study the transfer was shown to be dependent on the induction of apoptosis since co-cultivation without induction of apoptosis did not lead to transfer of DNA. Furthermore, the expression of the transferred genes was verified by use of immunofluorescent labeling. The authors claimed that this could affect virus persistence in infected individuals and the transfer of HIV-1-DNA to receptor negative cells since HIV-1 infection is characterized by an increased frequency of apoptosis. In addition to this, the prolonged capacity of antigen presentation in antigen presenting cells was speculated to be a result of horizontal transfer of DNA (Spetz *et al*., 1999).

2.1.5.2 Apoptosis dependent transfer of drug resistance

The transfer of genes coding for drug resistance from dying cells to phagocytosing cells was reported by de la Taille and coworkers (de la Taille *et al*., 1999). By co-cultivating tumor cell lines resistant to different drugs and selectively inducing apoptosis in one of them the researchers could show that resistance could accumulate within the same cell. This was not detected if no apoptosis was induced or if the cell lines were grown separately, clearly showing that the induction of apoptosis was crucial and that no spontaneous resistance developed. Resistant clones were verified by use of PCR and Southern blotting, showing the presence of DNA encoding for both the resistance genes. The gene from the dying cell was further reported to be integrated into the phagocytosing cells genome. The authors named the event of genetic transfer via phagocytosis "apoptotic conversion" and stated that although the process is likely to occur *in vivo*, normal cells would not be affected since they do not undergo the proliferation that is needed for the appropriate incorporation and transmission of DNA following standard transfection. They further argued that treatment of cancer with a regimen that preferentially induces apoptosis in only one of the genetic variants in a

heterogenetic tumor could lead to the development of more aggressive cancer cell types by accumulating genetic alterations in one cell (de la Taille *et al*., 1999).

2.1.5.3 Transformation via uptake of apoptotic bodies

Two years after the first reports on the transfer of DNA via apoptotic bodies, a paper by Bergsmedh and co-workers was published that investigated the role of horizontal gene transfer on cell transformation and tumor progression (Bergsmedh *et al*., 2001). Rat embryonic fibroblasts (REF) were stably transfected with the oncogenes H-ras^{v12} and human c-myc and the resultant rat fibrosarcoma cell line (REFrm) used to determine whether oncogenes could transfer via apoptotic bodies into, and transform, normal cells. By inducing apoptosis in the rat fibrosarcoma cell line and co-cultivating with mouse embryonic fibroblasts the authors were able to show that the functionality of the tumor suppressor gene p53 is crucial for focus formation. No foci were formed if normal mouse embryonic fibroblast (MEF) cells with functional p53 were used as recipient cells or if the donor cell line lacked the introduced oncogenes. PCR analysis showed that the oncogenes were amplified in the clones picked from foci. However the signals were gradually lost during propagation. This was not the case when resistance to drugs were transferred and then selected for by cultivation in media that contained a particular drug. Hence, the transferred DNA is propagated if selected for. In the same study the foci that were formed after co-cultivation between REFrm- and p53-deficient MEF cells were inoculated into immunodeficient mice to see whether a tumorigenic potential had developed. Tumors were formed after 3 weeks and these were subjected to fluorescence *in situ* hybridization (FISH) analysis to investigate how the transferred DNA had been propagated. Since different species had been used, FISH probes that could distinguish between donor and recipient cells could be employed. Analyses of metaphase spreads from cells derived from the tumors revealed the presence of rat chromosomes as well as hybrid rat/mouse chromosomes. The lack of transformation in normal fibroblasts by apoptotic bodies containing oncogenes was proposed to depend on the known accumulation of p53 after introduction of oncogenes and DNA damage. The authors argued that the uptake of DNA via apoptotic bodies may be the mechanism by which genetic instability and genetic diversity is generated within tumors since p53 has been shown to commonly be inactivated (Bergsmedh *et al*., 2001).

2.1.5.4 Propagation of horizontally transferred DNA

Earlier reports suggest that DNA derived from apoptotic bodies triggers cell cycle arrest or senescence in a p53-dependent pathway. Bergsmedh and collaborators published in 2002 an investigation on the upstream activator p19 ARF and one of the downstream targets of p53, i.e. p21 (Cip1/Waf1). By using the same rat fibrosarcoma, as described above, in focus formation assays it was shown that transfer of oncogenes via apoptotic cells could not induce loss of contact inhibition in the p19ARF-negative MEF cells. However when the same apoptotic cells were added to p21-deficient MEF cells foci were detected that had lost contact inhibition. The presence of H-ras^{v12} and c-myc was verified by PCR but was gradually lost in the same way as in p53-negative cells as earlier studies had shown. Inoculation of the foci formed after co-cultivating apoptotic REFrm cells with p21^{-}/-MEF cells in severe combined immunodeficiency (SCID) mice resulted in tumor formation. Cells from these tumors were subjected to FISH analysis to visualize their genetic content and revealed that, similarly to the

experiments with $p53^{-/-}$-MEF cells, chromosomes of both species and fusion chromosomes were present. The results in Bergsmedh *et al.*'s paper in 2002, in combination with the aforementioned paper by the same group in 2001, indicate that the activation of p21 by p53 protects normal cells from propagating DNA salvaged from apoptotic bodies. The authors speculated that since knocking out p19 ARF, which is a known sensor of activated oncogenes, did not result in propagation of transferred DNA, p53 activation is probably via the DNA damage pathway (Bergsmedh *et al.*, 2002).

To summarize the field of horizontal gene transfer by uptake of apoptotic cells. The following can be stated:

- DNA from dying cells can be salvaged and reutilized by phagocytosing cells.
- Horizontally transferred DNA is expressed in the recipient cell.
- Induction of apoptosis is crucial since use of necrotic cells or living cells in the experimental setup show no signs of gene transfer.
- Selection pressure is needed for phagocytes to propagate the transferred DNA.
- Protection from propagating transferred DNA is dependent on p53 and p21 in normal cells.

In the following sections I will briefly detail a number of areas that are of relevance to the process of horizontal transfer of DNA via uptake of apoptotic cells. To start with, the DNA damage signaling pathway will be discussed since it has been implied to protect normal cells from propagation of transferred DNA.

2.2 DNA DAMAGE SIGNALING PATHWAY

Keeping the genetic information intact is crucial for organisms to survive since genetic alterations may lead to, for example, tumor formation. This requires accuracy during DNA replication and chromosomal segregation but also controlling the DNA integrity. Extracellular agents, such as radiation and chemicals, and endogenous agents, such as reactive species of oxygen which are a natural by-product of cellular metabolism, constantly damage the DNA sequence. Eukaryotic cells avoid the deleterious consequences of DNA damage by rapid activation of a signaling network that not only recognizes DNA lesions but also delays cell cycle progression and activates the repair machinery and, in severe cases of DNA damage, induces apoptosis. Among the many different ways that DNA can be damaged the double strand break (DSB) is probably the most detrimental. DSBs are difficult to resolve because of the risk of erroneous rejoining leading to, for example, deletion of tumor suppressors, amplification of oncogenes, or translocations creating protein fusions. These mentioned genetic alterations are all common in cancer cells. The DNA damage pathway can be summarized to consist of damage sensors, signal transducers and various effector pathways. A multifunctional complex of Mre11-Rad50-Nbs1 (MRN) has been proposed to recognize DSB lesions (Lukas *et al.*, 2004; Moreno-Herrero *et al.*, 2005; Petrini and Stracker, 2003). This redistribution and accumulation of the MRN damage sensor complex at DSB recruits the phosphoinositide 3-kinase related kinases (PIKKs), i.e. the proteins ataxia-telangiectasia mutated (ATM), ATM-Rad3-related (ATR) and

DNA-dependent protein kinase (DNA-PK). The phosphorylated ATM in turn phosphorylates a series of downstream targets, among them the C-terminal tail of Histone 2AX (H2AX). The phosphorylated form of H2AX is called gamma-H2AX (γ-H2AX) and appears within seconds after DSBs are introduced (Burma *et al*., 2001; Rogakou *et al*., 1998). Another substrate for ATM phosphorylation is the check point kinase Chk2 that leads to delayed cell-cycle progression through both CDC25 and p53, providing time for the cell to repair the damaged DNA (Ahn *et al*., 2000; Matsuoka *et al*., 2000; Melchionna *et al*., 2000). The signaling network involved in DNA damage is complex and involves a large number of different proteins that in many cases are redundant and also capable of activating each other. Fig. 2 illustrates an approximate scheme of the signaling that takes place after the introduction of DSBs. In cases where a temporary halt of the cell cycle is not enough for the cell to recover after DNA damage, apoptosis can be induced to avoid the consequences of genetic alterations and thus risk of the life for the whole organism.

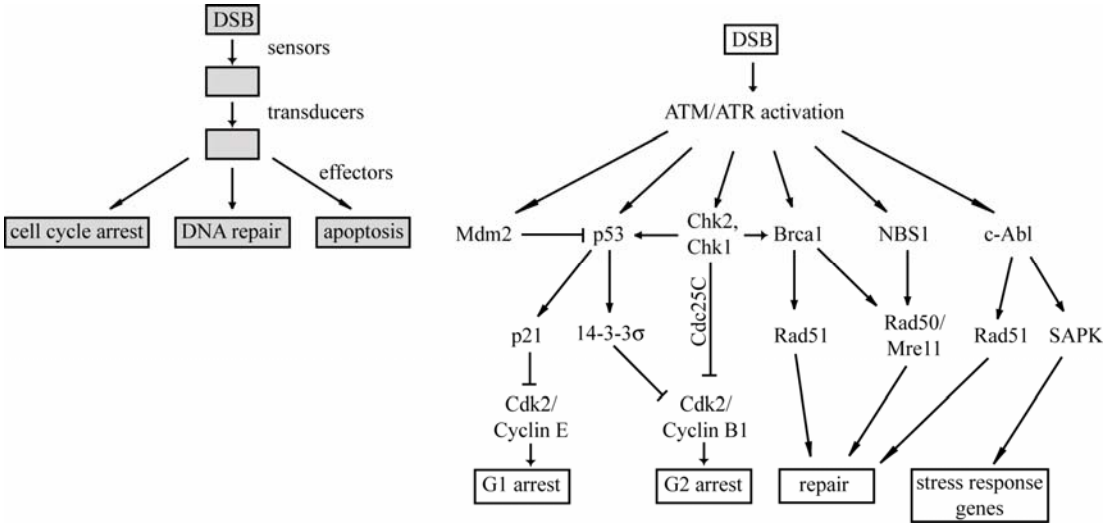

Fig. 2 *General organization of the response pathway after the introduction of a DNA double strand break (DSB). Adopted from (Khanna and Jackson, 2001).*

2.3 CELL DEATH AND THE REMOVAL OF DYING CELLS

2.3.1 Cell death

Cell death and the way that dying cells are removed in multicellular organisms are crucial for the horizontal transfer of DNA to occur. In the following section these fundamental biological mechanisms will be introduced.

Induction of cell death, i.e. apoptosis, can be divided into the intrinsic and the extrinsic pathways, indicating from where the signals triggering apoptosis originate, i.e. from within the cell or from outside the cell. The intrinsic pathway, also called the mitochondrial pathway, is induced by, for example, DNA damage originating from cytotoxic drugs or UV- or γ-irradiation. As mentioned above DNA damage induces re-

localization and accumulation of several sensor proteins that in turn activate a cascade of proteins. One of these proteins is the tumor suppressor p53. The accumulation of the transcription factor p53 in the nucleus of cells leads either to a transient arrest of the cell cycle, senescence or the induction of apoptosis. One of the target genes for p53 is BAX, which translocates to the membrane of the mitochondria and thereby triggers the release of cytochrome c (Gross *et al*., 1999). Sequestered in the space between the outer and inner membrane of the mitochondria the normal function of cytochrome c is in electron transfer in the respiratory chain. The permeabilization of the mitochondrial membrane permits cytochrome c and other proteins to leak out into the cytosol. Once in the cytosol, cytochrome c together with apoptotic protease activating factor-1 (Apaf-1) binds and activates procaspase-9 (cysteine aspartyl-specific protease-9) to form the apoptosome (Cain *et al*., 1999). This multimere in turn activates the procaspase-3, which is one of the executioner caspases together with caspase-6 and caspase-7. These caspases are responsible for a broad spectrum of functions that cause many of the morphological changes that characterize apoptosis (Budihardjo *et al*., 1999; Earnshaw *et al*., 1999). One of the substrates for caspase-3 is the protein ICAD (inhibitor of CAD), which is a chaperone for and normally binds CAD (caspase activated DNase) (Sakahira *et al*., 2000; Uegaki *et al*., 2000). The cleavage of ICAD by caspase-3 makes CAD free to perform the function that it is specialized to do, i.e. DNA fragmentation (Enari *et al*., 1998; Sakahira *et al*., 1998). Caspases are also responsible for the proteolysis of several cytoskeletal proteins that change the overall cell shape and degradation of lamins that result in nuclear shrinking (Degterev *et al*., 2003).

The extrinsic or receptor-activated apoptotic pathway is called so due to the fact that the signal that induces apoptosis originates from outside the cell. The death receptors are transmembrane proteins that have the capacity to activate the apoptotic program. Two members of the tumor necrosis factor (TNF) receptor superfamily are CD95 (APO-1/Fas) and TNF-related apoptosis-inducing ligand (TRAIL) receptors (Walczak and Krammer, 2000). Stimulation of these receptors by ligand binding, results in the activation of the initiator caspase-8, which in turn directly activates the procaspase-3. This activation of caspase-3, in combination with the truncation of BID, thereby leads to cytochrome c release from the mitochondria and the convergence of the intrinsic and extrinsic pathways of inducing apoptosis. One of the proteins involved in sensing oncogene activation is ARF (an alternative reading frame protein expressed from the INK4a locus, that also express the $p16^{Ink4a}$ an inhibitor of cyclin D) (Kamijo *et al*., 1997). ARF is activated by over expression of oncogenes such as c-myc, ras, v-abl, E1a, and E2F (Bates *et al*., 1998; de Stanchina *et al*., 1998; Palmero *et al*., 1998; Radfar *et al*., 1998; Zindy *et al*., 1998). The activation of ARF leads to binding to Mdm2 and this inhibits Mdm2s normal function, which is binding to p53. The binding of p53 by Mdm2 not only stops the transcriptional activity of p53 but also exports p53 from the nucleus and functions as an ubiquitin ligase target that degrades the p53 protein. The sequestering of Mdm2 by ARF thereby leads to accumulation of p53 that is free to perform its transcriptional activity. The apoptotic signaling network is complex and tightly controlled and the proteins mentioned above represent only a partial description of how a cell undergoes apoptosis. The key events are summarized in Fig. 3.

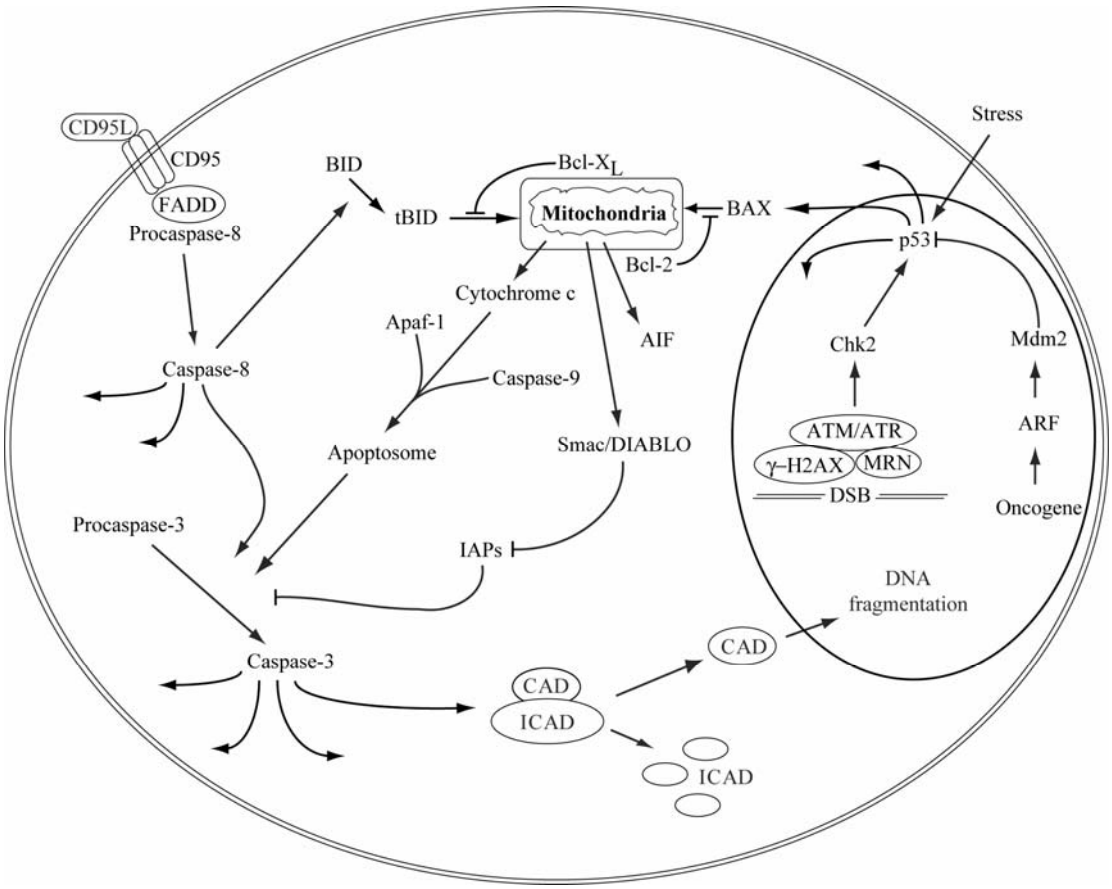

Fig. 3 *The map of death: outline and key players in the intrinsic and extrinsic apoptotic pathways.*

Apoptosis plays a key role during both embryonic development and maintenance of homeostasis in adults. The sculpting of digits in some higher vertebrates is a well-studied example where apoptosis eliminates the cells between the forming digits in the developing embryo (Jacobson *et al*., 1997). In adults apoptosis is involved in many processes among them tissue homeostasis where cell proliferation is balanced with cell death, clearly visualized in the intestine where massive numbers of cells die by apoptosis (Potten, 1992). This controlled way of cell death may also function as an anti-cancer mechanism by eliminating cells that have required unleashed proliferative potential or that harbor severely damaged DNA. Apoptosis is a term adapted from Greek and describes something similar to the falling of leaves from trees in the autumn. It was first described as the morphological characteristics of chromatin condensation, nuclear fragmentation, cell shrinkage and ultimately the formation of membrane-enclosed apoptotic bodies (Kerr *et al*., 1972). The apoptotic bodies are rapidly phagocytosed in healthy tissues by neighboring cells (Bursch *et al*., 1990). Several ligands, receptors and serum factors have been reported to be involved in the recognition of apoptotic cells. Among the cell surface changes, the loss of plasma

membrane phospholipid asymmetry by the externalization of phosphatidylserine (PS) is probably the most well-studied. The translocation of PS to the outer cell membrane appears to be a critical step for recognition and uptake of apoptotic cells (Fadok *et al*., 2001; Fadok *et al*., 2000; Savill and Fadok, 2000). Transient exposure of PS has been reported in non-apoptotic cells (Dillon *et al*., 2000; van den Eijnde *et al*., 2001) indicating that additional eat-me signals are required for selective engulfment of apoptotic cells. Other changes in the apoptotic cell that have been proposed to be involved in the recognition are ICAM3 (Moffatt *et al*., 1999), milk-fat-globule 8 (Hanayama *et al*., 2002), thrombospondin-1 (Savill *et al*., 1992) and serum protein S (Anderson *et al*., 2003).The surface changes that occur on the surface of apoptotic cells are recognized by phagocytosing cells and the receptors that have been reported to be involved in this process are the vitronectin receptor $\alpha_v\beta_3$ (Savill *et al*., 1990), $\alpha_v\beta_5$ (Albert *et al*., 1998a), PS receptor (PSR) (Fadok *et al*., 2000) and the scavenger receptors (Platt *et al*., 1996) among many others. Once the apoptotic cell is recognized by a phagocyte it will be engulfed and ingested via large endocytic vesicles called phagosomes. The phagosome fuses with lysosomes inside the cell and the ingested material is degraded. In the acidic environment of phagosomes the protein DNaseII is active and degrades DNA (McIlroy *et al*., 2000; Nagata *et al*., 2003).This way unwanted cells can be eliminated without an inflammatory response. In fact, the uptake of apoptotic cells stimulates the release of anti-inflammatory mediators, such as TGF-β1 and IL-10, and can inhibit the secretion of proinflammatory mediators, such as TNF-α, from phagocytes (Fadok *et al*., 1998; Huynh *et al*., 2002; Voll *et al*., 1997). The controlled mechanism of apoptosis (Fig. 4) is in contrast to cells dying by necrosis. Necrosis is the accidental death of cells with irreversible swelling of the cytoplasm and organelles, and the rupture of the plasma membrane, followed by tissue scaring and inflammation, although debris from necrotic cells eventually are degraded by phagocytes as well (Wyllie *et al*., 1980).

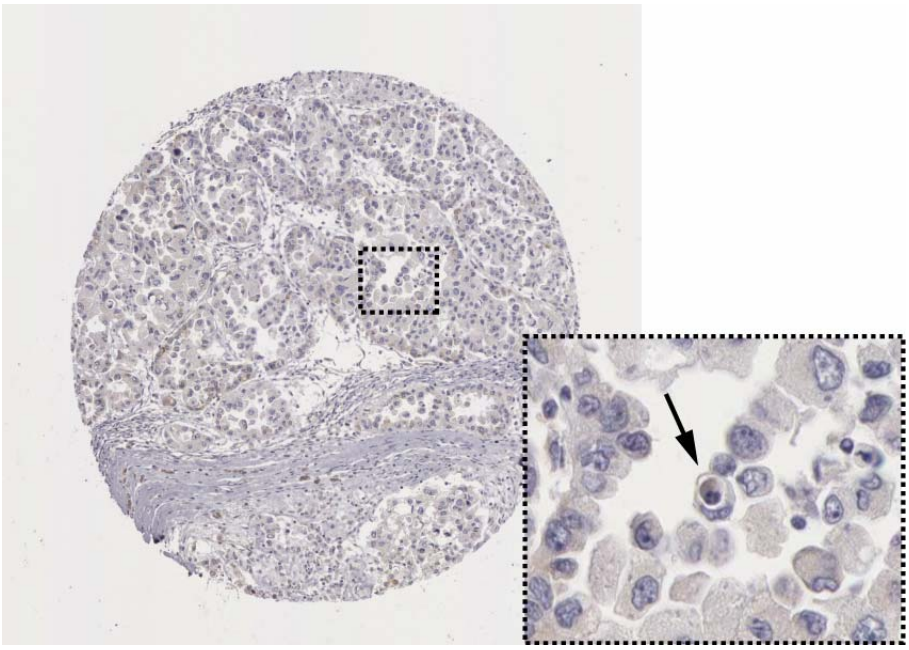

Fig. 4 *Hematoxylin and eosin staining of a section from an ovarian cancer. Arrow indicates a phagocytosed cell. Adopted from the Protein Atlas (www.proteinatlas.org).*

2.4 CELL TRANSFORMATION

Primary rodent cells can be transformed into tumorigenic cells by the introduction of as few as two oncogenes (Land *et al*., 1983; Ruley, 1983). However, this is not the case with human cells, which have been shown to be much harder to transform by introduction of certain oncogenes (Hahn *et al*., 1999). The functions of oncogenes are complex and interactions between different pathways keep emerging (Hahn and Weinberg, 2002). With this in mind I would like to briefly go through the functions of the two cellular oncogenes, i.e. H-ras and c-myc, and one viral oncogene, i.e. Simian virus 40 large T antigen (SV40LT), which has been used in this thesis to study horizontal gene transfer and transformation.

2.4.1 Ras

Ras proteins belong to a family of G proteins that are involved in the regulation of cell growth. H-ras is inserted in the cytoplasmic membrane and functions as a regulated GDP/GTP switch that cycles between the inactive GDP-bound and active GTP-bound state (Bourne *et al*., 1990). In quiescent cells the GDP-bound inactive state of Ras is predominant but when cells are stimulated by a diverse group of extracellular stimuli, Ras is converted to the active GTP-bound state. This cycle between the inactive and active states of Ras are controlled by two classes of regulatory proteins, i.e. guanine nucleotide exchange factors (GEFs), which stimulate the formation of Ras-GTP, and GTPase activating proteins (GAPs), which cause GTP to hydrolyze back to GDP (Lowy *et al*., 1993). Mutated forms of ras are often found in cancers. These mutations are missense and make the Ras proteins constitutively active in the absence of extracellular signals. The H-ras^{val12} that is used in this thesis is mutated at residue 12, which renders the protein insensitive to GAPs and thereby keeps the protein in the active state (Lowy and Willumsen, 1993) . The best characterized downstream targets of Ras are the serine/threonine kinase Raf that in turn starts the Raf/MEK/ERK kinase cascade, and the phosphatidylinositol-3-kinase (PI3K) pathway. Ras mutations are found in approximately 30% of all human cancers (Bos, 1989). Introduction of mutated forms of ras in rodent cells is not enough to result in transformation into a tumorigenic state (Land *et al*., 1986) and, unless p53 function is impaired, the cells become senescent (Lowe, 1999; Serrano *et al*., 1997).

2.4.2 Myc

The Myc protein is a tightly controlled transcription factor that is activated in various processes such as cell proliferation, differentiation, and apoptosis. External signals including growth factors activate Myc to form heterodimers with the protein Max. These heterodimers activate transcription of wide variety of target genes. Among the proteins from these target genes many have a potent effect on the cell cycle, supporting cell proliferation but also activation of, for example, telomerase (Marcu *et al*., 1992; Wang *et al*., 1998). The myc gene has been shown to exhibit a deregulated expression pattern in a wide variety of cancers due to over expression, point mutations, enhanced translation, amplification and chromosomal translocations (Popescu and Zimonjic, 2002; Vita and Henriksson, 2006). The deregulated expression of myc makes it no

longer dependent on external signals and is often associated with poorly differentiated, aggressive tumors (Schlagbauer-Wadl *et al*., 1999). Over expression of the myc gene in combination with mutated ras or Bcl-XL, or in combination with loss of p53 or p19ARF, transforms rodent cells but not human cells (Evan *et al*., 2005; Land *et al*., 1986).

2.4.3 Simian Virus 40 Large T antigen (SV40LT)

The Simian virus 40 (SV40) early region encodes both the SV40 large T (SV40LT) antigen and the SV40 small t antigen (ST). These viral oncoproteins have been shown to bind several proteins but the ability of SV40LT to interact and disable the p53 and retinoblastoma (RB) tumor suppressor proteins is probably their most important function in transformation (Boehm and Hahn, 2005). This is highlighted by the fact that the role of SV40LT in cell transformation can be performed by other proteins that inactivate p53 and RB in ways distinct from SV40LT, e.g. either the human papillomavirus proteins E6 and E7 (Hahn *et al*., 2002) or E1A and MDM2 (Seger *et al*., 2002). Stably expressed short interfering hairpin RNA specific for the p53 and RB pathways has also been shown to substitute the role of SV40LT (Voorhoeve and Agami, 2003). ST shares several amino acids with SV40LT at the amino-terminal domain, but has a unique carboxy-terminal domain that binds the heterotrimeric serine-threonine phosphatase PP2A (Hahn *et al*., 2002; Mungre *et al*., 1994). The function of PP2A is not fully understood but the protein is known to increase cell proliferation (Sontag *et al*., 1993). Between 1955 and 1963 approximately 10 to 30 million American people were exposed to the SV40 virus since polio vaccines were administered containing the SV40 virus as a contaminant (Shah and Nathanson, 1976). However, epidemiological studies conducted over the following decades indicated no increased risk of cancer among those exposed to the vaccines (Shah, 2007).

2.5 TUMOR PROGRESSION

The development of cancers in humans is the result of accumulated genetic alterations within one cell. This multistep process usually occurs over a long period of time and the underlying mechanism is not fully understood. The normal mutation rate, as a result of errors in DNA replication in cells, has been reported to not be sufficient to generate the large amount of genetic mutations found in cancers (Loeb *et al*., 1974). It has therefore been proposed that specific mutations in genes that control DNA repair or the genetic integrity as a whole, such as the tumor suppressor p53, results in genomic instability and increased mutability (Hollstein *et al*., 1991; Lengauer *et al*., 1998; Loeb *et al*., 2003). The tumor suppressor p53, which has been called “The guardian of the genome”, is reported to be mutated in over 50% of all human cancers (Lane, 1992). The fact that p53 is so commonly mutated in cancers raises the possibility that mutations are accumulated by horizontal gene transfer. Activation of an oncogene in a normal cell will likely induce apoptosis via p19 ARF. The phagocytosis and salvage of this activated oncogene by a $p53^{-/-}$ cell would be tolerated and the oncogene propagated. This way, two genetic alterations can be accumulated within one cell although they originated from mutations in different cells. In the subsequent three sections I will detail the theory behind the multistep progression of cancer, the

described genomic instability in cancer and clonal expansion. These areas would clearly be affected by DNA transfer from dying cells but this has not yet been shown.

2.5.1 The Vogelgram

Attempts to explain the multistep progression of cancer is most commonly illustrated by colorectal cancers (Kinzler and Vogelstein, 1996; Vogelstein *et al*., 1988). Colon carcinoma typically develops over decades and requires a number of genetic alterations within one colonic epithelial cell. Adenomatosis polyposis coli is a heritable colon cancer syndrome, usually called familial adenomatous polyposis (FAP), which results in an inherited susceptibility to develop hundreds of nonmalignant adenomatous polyps in the colon. Although these polyps are nonmalignant the large number infer an increased risk that some will progress to the development of carcinomas. Causing this cell proliferation within the colon is a tumor suppressor gene denoted adenomatosis polyposis coli (APC). In normal colonic crypts stem cells are protected at the bottom and, as they migrate upwards towards the intestinal lumen, they lose their stem cell features and instead function as the epithelial lining of the gut before dying by apoptosis and being shed into the colonic lumen. This highly effective defense mechanism results in the death of cells that acquire genetic damage before they can develop into cancer. When the APC protein is defective the stem cells replicating in the bottom of colonic crypts fail to migrate upward and thereby accumulate and ultimately generate an adenomatous polyp. The majority of colorectal cancers have an inactive tumor suppressor APC gene and the remaining minority harbor other genetic changes that mimic the outcome observed when APC is missing (Fearnhead *et al*., 2001; Weinberg, 2007). Although people with FAP develop hundreds of polyps in their colon, this represents only a small fraction of all the epithelial stem cells. It has therefore been suggested that somatic mutation of the wild type APC allele inherited from the unaffected parent is required (Ichii *et al*., 1992; Levy *et al*., 1994; Luongo *et al*., 1994). This mechanism supports the classical “two-hit” hypothesis put forward to explain childhood tumor retinoblastoma, which proposes that both of the alleles of tumor suppressors must be mutated to form tumors (Knudson, 1993). In the progression of colorectal cancers activation of K-ras and loss of p53 are other events that are needed for malignant growth. This model of a series of genetic alterations that step by step paves the way to carcinoma has been nicknamed the “Vogelgram” in honor of Bert Vogelstein (Fig. 5). Evidence for the progression from adenoma to carcinoma is, for example, the clinical observation that carcinomas sometimes grow directly out of adenomas, and from clinical studies in large cohorts of patients where surgical removal of polyps shows a clear reduction of colorectal cancer incidence (Winawer *et al*., 1993). Although the loss of APC seems to be a common first event towards malignancy, the road after this is varied in different colon cancers.

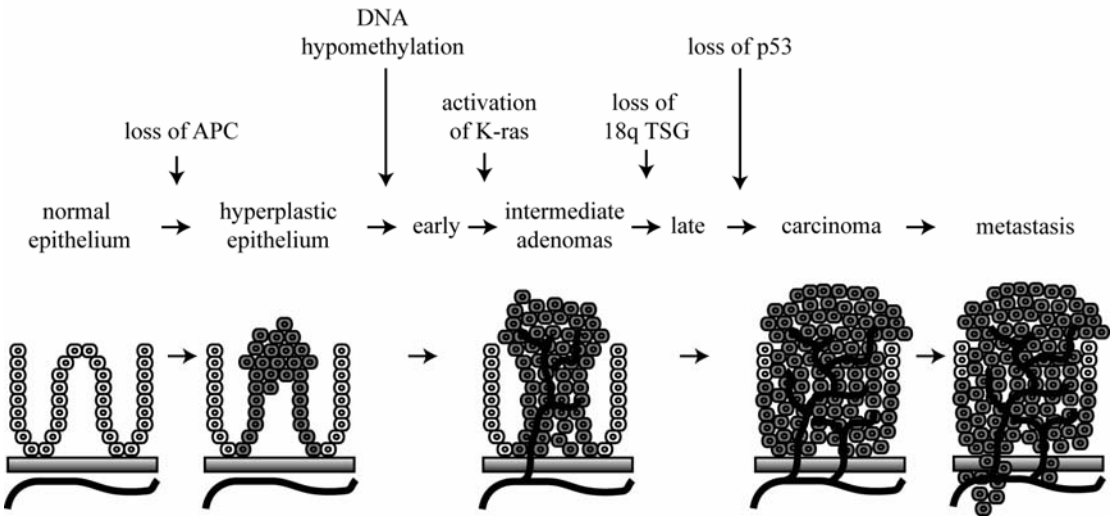

Fig. 5 *A step-wise model of the genetic changes associated with colorectal tumor progression. Adapted from (Rajagopalan* et al.*, 2003; Weinberg, 2007).*

2.5.2 Genomic instability

Genomic alterations occur commonly in tumors and include minute changes in the DNA sequence as well as cytogenetically visible changes, such as chromosome losses, gains and translocations. The genetic alterations can be subdivided into four categories, namely 1) subtle sequence changes that involve deletions, insertions or substitutions of few nucleotides, 2) alterations in chromosomal number, 3) chromosome translocations and 4) gene amplifications (Kinzler and Vogelstein, 1996; Lengauer *et al*., 1998). Instabilities due to a lack of fidelity during DNA polymerization or DNA repair giving rise to subtle sequence changes are not very common in human cancers, although the types where they do occur are very aggressive. Patients with xeroderma pigmentosum, exhibit a malfunctioning nucleotide-excision repair (NER) system that makes them develop skin tumors if exposed to the sun (Cleaver, 1968). Another example is patients with hereditary non-polyposis coli (HNPCC) that are predisposed to colon cancer. These patients have mutations in mismatch repair genes that lead to widespread alterations of microsatellites of poly(A) and poly(CA) repeats, giving rise to the name "microsatellite instability" (MIN) (Fishel *et al*., 1993; Ionov *et al*., 1993; Thibodeau *et al*., 1993). MIN can be found in 15% of colorectal cancers and renders tumor cells susceptible to the acquisition of point mutations in genes affecting the balance between cell proliferation and apoptosis. Alterations at the chromosomal level in the form of gains or losses of whole chromosomes are termed chromosomal instability (CIN) or aneuploidy. Karyotypic data show that the majority of cancers are subjected to losses or gains of chromosomes (Beheshti *et al*., 2001; Lengauer *et al*., 1998; Miyoshi *et al*., 2000; Nomoto *et al*., 1998). The molecular basis of aneuploidy is in most cases unknown in human cancers although involvement of abnormal centrosomes and checkpoint genes that monitor proper progression of the cell cycle have been proposed (Elledge, 1996; Pihan *et al*., 1998). Studies in yeast have shown that a large number of different genetic alterations can give rise to aneuploidy and among these are genes

involved in chromosome condensation, cell-cycle regulation, checkpoint control and spindle-assembly (Kolodner *et al*., 2002; Nasmyth, 2002; Spencer *et al*., 1990). The presence of genetic alterations either at the sequence level or at the chromosomal level in cells does not necessarily mean that the cell is genetically instable, since instability is a matter of rate. It is possible that cancer cells develop genetic alterations at the same rate as normal cells but since many of these events are lethal in normal cells we do not see them, especially as one of the hallmarks of cancer cells is the ability to avoid apoptosis. Given that the presence of aneuploidy is so common in cancers it is likely that this contributes to tumor progression. This could be through inactivation of tumor suppressor genes via the loss of chromosomes or fragments thereof. As mentioned above both the alleles of tumor suppressors must be inactivated for cells to acquire a growth advantage (Knudson, 2001). Loss of heterozygosity (LOH) is defined as the loss of the remaining tumor suppressor allele via chromosomal loss or other structural changes and aneuploidy is a potential mechanism by which LOH is achieved. Another aspect of aneuploidy is the change in expression levels of genes regulating cell proliferation or cell death. As little as a 1% increase in the ratio between proliferation and death will result in clonal overgrowth over time. For instance, if a cell divides once every 24 h the affected cell will increase from 0.001% of the population to 99.9% of the population in approximately 5 years (Rajagopalan and Lengauer, 2004; Rajagopalan *et al*., 2003). A plausible way of acquiring a proliferative advantage is via the uptake of an activated oncogene from a dying cell.

2.5.3 Clonal expansion

Random genetic mutations are assumed to create genetic variation within cell populations. The selection pressure from the microenvironment may then favor the outgrowth of individual cells that have acquired mutations that support proliferation and survival in clonal expansion (Nowell, 1976). The generation of multiple mutations within one cell that preside cancer has been argued to occur through waves of these clonal expansions (Tomlinson *et al*., 1996). The scheme is that random mutations create a cell with advantages in growth or survival properties that proliferates more effectively than the rest of the population, eventually forming a clonal population that in turn is large enough to acquire a second advantageous mutation that will make this new cell proliferate more effectively than the rest of the population and so forth. Clonal expansion is triggered by an infrequently occurring favorable mutation and therefore these expansions are expected to be far apart in time. The clonal expansion theory proposes that all the cells within a tumor mass are genetically identical to each other and that malignancy is due to a linear series of events (Fig. 6). However, tumor progression is complicated by increased genetic instability as cells progress towards malignancy. Mutations affecting, for example, DNA repair genes will increase the genome mutability and speed up the process of genetic diversification and, as a consequence of this, tumor progression will be much more complex. It is also a common feature of tumor cells to show morphological and metabolic alterations that can be interpreted as loss of differentiation as the cell progresses more towards malignancy.

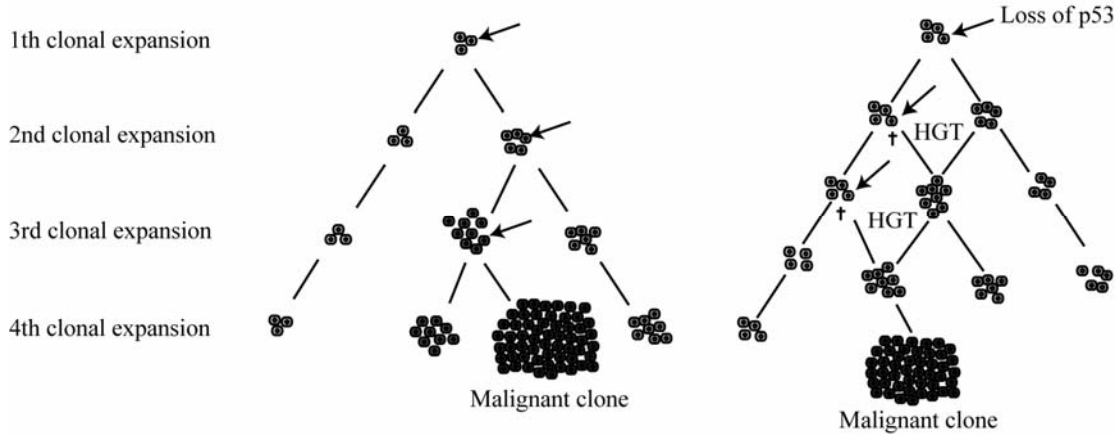

Fig. 6 *Left: Schematic illustration of clonal expansion. Cells with growth or survival advantages will form large clonal populations that are likely to receive further genetic hits. Arrows indicate mutations. Right: Horizontal gene transfer (HGT) enables accumulation of mutations and genetic diversification.*

2.6 TUMOR MICROENVIRONMENT

2.6.1 Angiogenesis

One of the hallmarks of cancer, as stated by Hanahan and Weinberg in 2000, is the ability of malignant cells to sustain angiogenesis (Hanahan and Weinberg, 2000). Angiogenesis is a physiological normal process in growth and development that makes new blood vessels. Although not perfectly strict, the term sprouting angiogenesis refers to the formation of blood vessels from preexisting vessels and the term vasculogenesis is applied to blood-vessel formation occurring by *in situ* differentiating endothelial cells and intussusception when blood vessels are formed by the splitting of already existing vessels (Risau, 1995). In adults, angiogenesis is normally only activated in wound-healing and in the female reproductive cycle. However, there are several diseases associated with increased blood vessel formation, such as macular degeneration, rheumatoid arthritis and cancer (Carmeliet and Jain, 2000; Folkman, 1995). Tumors, like normal tissues, require a close proximity to blood vessels for a steady supply of oxygen and nutrients, but also an effective way of removing waste products. In 1971 Folkman stated that tumor growth is angiogenesis-dependent (Folkman, 1971). This publication proposed that angiogenesis inhibitors could be used as therapy against cancer and that tumor dormancy could be based on the absence of angiogenesis. Since then, the field of angiogenesis has developed rapidly and the use of anti-angiogenic therapy has been approved for cancer in over 25 countries (Folkman, 2006). The anti-angiogenic treatment is based on the concepts that 1) growth of neoplastic tissue is angiogenesis dependent, 2) microscopic cancers *in situ* must switch to an angiogenic phenotype to grow beyond the range of oxygen diffusion and 3) the microvascular

endothelial cells that are recruited by tumors are genetically stable and are possible targets for treatment. Proliferation of tumor cells in the absence of the formation of blood vessels can give rise to dormant microscopic tumors that remain *in situ* and are harmless to the host (Gimbrone *et al*., 1972; Holmgren *et al*., 1995). In dormant metastasis it has been shown that proliferation is not significantly different from that of growing metastasis. However, in dormant metastasis a much higher incidence of apoptosis is exhibited, suggesting that inhibition of metastatic growth by angiogenesis inhibitors occurs indirectly by increasing apoptosis (Holmgren *et al*., 1995). There are reports that tumor growth can occur without the induction of angiogenesis, i.e. instead of inducing the formation of new blood vessels, the tumor cells were shown to cooperate with the host vasculature by growing along existing vessels (Holash *et al*., 1999; Pezzella *et al*., 1997; Sakariassen *et al*., 2006; Wesseling *et al*., 1994). Vasculogenic mimicry has also been described as an alternative way to form functional vessel networks in aggressively growing tumors (Hammersen *et al*., 1985; Maniotis *et al*., 1999). Liver metastases of uveal melanomas have been shown to form networks of interconnected loops that are lined by tumor cells, have anastamosed with the normal vascular network and are associated with worse patient outcome (Folberg *et al*., 1993).

2.6.1.1 Sprouting angiogenesis

Sprouting angiogenesis is, as described above, the formation of new capillary vessels out of pre-existing vessels. This form of angiogenesis involves several sequential steps that start with the activation of endothelial cells by the binding of growth factors to their receptors. One model often used to depict the regulation of tumor angiogenesis is the illustration of a scale with anti-angiogenic molecules on one side and pro-angiogenic molecules on the other. Induction of angiogenesis is then dependent on how heavily the balance shifts to pro-angiogenesis. There are several angiogenic growth factors such as basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and vascular endothelial cell growth factor (VEGF) (Hillen and Griffioen, 2007). The expression of pro-angiogenic factors is dependent on physiological stimuli, such as hypoxia due to increased tissue mass and oncogene activation. The pro-angiogenic factors are balanced by a number of anti-angiogenic factors, such as angiostatin, endostatin and thrombospondin-1 (Folkman, 2006). Besides these angiogenic factors tumor cells can produce other factors that promote tumor angiogenesis and stabilize newly formed vessels. The activation of endothelial cells results in the surrounding extracellular matrix and basement membrane being degraded by activated proteases (Moses, 1997). The degraded matrix in turn enables the proliferating endothelial cells to migrate through the matrix. As a result of polarization and lumen formation by the migrating endothelial cells, a new vessel is formed.

2.6.1.2 Vasculogenesis

The *de novo* formation of blood vessels from endothelial precursor cells (EPC) is called vasculogenesis. This process was until recently believed to occur only in the developing embryo, but in 1997 Asahara *et al*. isolated a subpopulation of mononuclear blood cells that were able to differentiate into endothelial cells *in vitro* (Asahara *et al*., 1997). The incorporation of EPCs into blood vessels *in vivo* has been shown by bone marrow transplantations in animal models (Asahara *et al*., 1997; Kalka *et al*., 2000; Lin *et al*., 2000). The bone marrow harbors organ-specific stem cells for endothelium but

also for muscle, brain, pancreas and liver cells (Krause *et al*., 2001). Stem cells detach from their quiescent niche to proliferate and differentiate into endothelial precursor cells via promotion by growth factors, chemokines, and cytokines (Heissig *et al*., 2002). These EPCs then move to the vascular zone of the bone marrow and are released into the circulation. A complex process that includes chemoattractants makes the EPCs able to home and incorporate at the site of microvasculature were angiogenesis is needed. The neovascularization in tumors has been shown to be a site of incorporation of EPCs. Transplantation of bone marrow with endothelium specific expression of β-galactosidase has been shown to result in incorporation of EPCs into both tumor blood vessels and the tumor stroma (Asahara *et al*., 1999). The involvement of EPCs in the actual vessel growth has been shown to differ from being the leading process and comprise only a minimal contribution. This tenet is supported by similar results reported from studies in cancer patients (Gothert *et al*., 2004; Larrivee *et al*., 2005; Lyden *et al*., 2001; Machein *et al*., 2003; Peters *et al*., 2005; Rajantie *et al*., 2004; Ruzinova *et al*., 2003; Sussman *et al*., 2003).

2.6.2 Tumor stroma

Human cancers evolve in a complex 3-dimensional microenvironment that includes extracellular matrix, vasculature, fibroblasts and inflammatory cells. The dynamic interactions between tumor cells and tumor stroma has been suggested to select for subsets of stromal cells that promote tumor growth and that differ from normal stroma (Schor and Schor, 2001; Shekhar *et al*., 2001). Recent data has not only reported the presence of mutations and epigenetic changes in the stroma of human carcinomas but suggested that this probably has an influence on cancer progression (Hu *et al*., 2005; Kurose *et al*., 2001; Moinfar *et al*., 2000; Paterson *et al*., 2003; Tuhkanen *et al*., 2004; Wernert *et al*., 2001). Studies in mice that spontaneously form prostate carcinomas have revealed the selective loss of p53 in the tumor stroma as a result of oncogenic stress (Hill *et al*., 2005). In addition to the aforementioned changes in the tumor stroma, the genetic stability of tumor-associated endothelial cells has been challenged. In 2004, Streubel and coworkers studied the endothelial cells from the tumors of 27 lymphomas (Streubel *et al*., 2004). In all of these lymphomas 15 to 85 percent of the tumor-associated endothelial cells harbored the same chromosomal rearrangement as the respective lymphoma cells. Furthermore, it was reported that the endothelial cells that lined the micro vessels in these lymphomas carried the same secondary aberrations of chromosomal gains as the lymphoma cells. In the same year, Hida and coworkers reported that endothelial cells from human highly metastatic melanoma and human liposarcoma grown subcutaneously in mice display abnormal karyotypes (Hida *et al*., 2004). Aneuploidy was detected in 75 to 80 percent of the isolated endothelial cells compared to skin and adipose endothelial cells, which were strictly diploid with few precisely tetraploid exceptions. Heterogeneous aneuploid karyotypes and chromosomal rearrangements in endothelial cells from the same tumor suggested that the cytogenetic alterations detected were not of clonal origin but had arisen within different tumor-associated endothelial cells. Centrosome abnormalities in the tumor-associated endothelial cells were also reported. In these two reports of genetic alterations within the endothelial cells of human tumors grown in mice, the underlying mechanism was not shown.

3 THE PRESENT INVESTIGATION

3.1 AIMS OF THE PRESENT THESIS

The propagation of horizontally transferred DNA has been determined to be dependent on the functions of the tumor suppressor p53 and its downstream target the cyclin kinase inhibitor p21 (see section 1.1.5). Although the concept of DNA being salvaged from dying cells and reutilized in phagocytosing cells has been shown, there are still several steps in this process that are unknown. For example, the signaling pathways underlying the protection in normal cells, the molecular mechanism behind the entrance of DNA into the nucleus, whether this actually occurs *in vivo* and the implications for tumor progression *in vivo* have not been described.

The specific aims for the four papers in this thesis are:

- To determine the implications of DNA degradation for the propagation of horizontally transferred DNA and protection via the Chk2/p53/p21 DNA damage pathway (paper I).
- To establish a model-system based on cre-loxP recombination showing functional DNA transfer via the uptake of apoptotic cells, and activation and expression in professional antigen–presenting cells (paper II).
- To demonstrate that DNA transfer via engulfment of apoptotic cells occurs *in vivo* and the potential implications for the vascular network (paper III).
- To determine the mechanism of DNA translocation from apoptotic cells to the nucleus of phagocytosing cells and to link this DNA to the early events of DNA damage signaling (paper IV).

3.2 RESULTS AND DISCUSSION

3.2.1 DNA degradation is the key to protection (paper I)

The propagation of DNA transferred via the uptake of apoptotic cells has been demonstrated to be dependent on p53 and p21 (Bergsmedh *et al*., 2001; Bergsmedh *et al*., 2002). This defense mechanism protects normal cells from propagating potentially harmful DNA acquired from dying cells. How the p53 protein is activated after the introduction of DNA from apoptotic cells is not known. The p19 ARF protein that activates p53 after uncontrolled oncogene expression has been analyzed but the ablation of the gene was not enough to enable propagation of foreign DNA (Bergsmedh *et al*., 2002). We hypothesized that DNA from apoptotic cells is recognized by the Chk2/p53/p21 DNA damage pathway due to the degradation of DNA during apoptosis and phagocytosis.

A hallmark of apoptosis is the fragmentation of DNA (Kerr *et al*., 1972). This DNA degradation is performed by caspase activated DNase (CAD) after caspase 3 cleavage of the inhibitor of CAD (Sakahira *et al*., 1998). In addition to the DNA fragmentation that occurs in dying cells, DNaseII is active in lysosomes after phagocytosis (McIlroy *et al*., 2000; Nagata *et al*., 2003).

In this study phagocytosis of apoptotic cells was shown to activate p53 and p21 in MEF cells. To analyze the effect of DNA degradation on this activation we used a mutated form of the CAD inhibitor protein that is not cleavable by caspase 3 and hence CAD remains inactive. The inactivation of CAD resulted in loss of internucleosomal DNA fragmentation during apoptosis and delay in the p53 response after phagocytosis.

Since DNA is also degraded in lysosomes after phagocytosis the DNaseII protein was inactivated either by a chemical drug, Bafilomycin A1, or by use of genetic ablation (Bowman *et al*., 1988). Knocking out the DNaseII gene resulted in a delayed p53 response in phagocytosing cells compared to when CAD was inhibited. A combination of inhibiting the degradation in both the apoptotic and the phagocytosing cells resulted in a complete block of p53 accumulation. We then proceeded to show that eliminating DNaseII is sufficient to enable propagation of horizontally transferred DNA. This was verified by re-transfecting a functional DNaseII gene.

To investigate the involvement of the DNA damage signaling pathway we turned our attention to Chk2, which is a known upstream activator of p53. Chk2 has been reported to phosphorylate p53 at serine 23 in response to DNA double strand breaks. This phosphorylation is known to interfere with Mdm2 binding and in turn prevent the ubiquitination and degradation of p53 (Hirao *et al*., 2000). No accumulation of p53 was detected after phagocytosis of apoptotic cells by MEF Chk2-/- and horizontally transferred DNA was shown to be propagated. Analyses by PCR revealed the transfer and propagation of oncogenes in the MEF Chk2-/- cells. However, although oncogenes were detected, no sign of transformation was observed. The lack of transformation is likely due to the presence of a functional p19 ARF protein in the MEF Chk2-/- cells that activates p53 in response to unrestrained oncogene expression.

Conclusions from paper I

- Accumulation of p53 is dependent on the fragmentation of DNA both in the apoptotic cell by CAD and in the phagocytosing cell by DNaseII.
- Chk2 is crucial for p53 accumulation in phagocytosing cells after uptake of apoptotic cells.
- The Chk2/p53/p21 signaling pathway together with DNaseII protects cells from propagation of horizontally transferred DNA (Fig. 7).

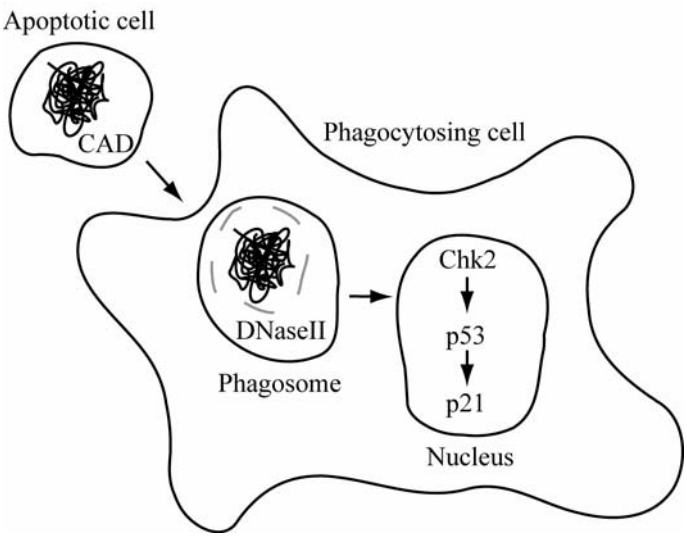

Fig. 7 *DNA degradation during apoptosis and phagocytosis and the hypothesized Chk2/p53/p21 DNA damage pathway that together with DNaseII protects cells from replicating horizontally transferred DNA.*

3.3 ESTABLISHING A REPORTER SYSTEM FOR DNA TRANSFER (PAPER II)

In this study we used the cre-loxP recombination system to establish a reporter system for DNA transfer via apoptotic cells. The underlying hypothesis was that silent DNA would be activated if translocated to the nucleus after phagocytosis. The cre-loxP recombination system is based on the site-specific DNA recombinase Cre, originally derived from the bacteriophage P1. The Cre protein recognizes loxP sites and efficiently catalyses DNA recombination between pairs of these sites (Sauer, 1996). The constructs used in this study was designed so that a reporter gene was activated only in the presence of Cre.

By inducing apoptosis in cell lines carrying a silent reporter gene and subsequently feeding the apoptotic cells to phagocytes expressing Cre, the activation of the reporter gene was shown. This clearly shows that DNA from apoptotic cells can be translocated to the nucleus after phagocytosis.

Earlier studies have shown that viral DNA from Epstein-Barr virus and the human immunodeficiency virus can be transferred via apoptotic cells into antigen presenting cells such as macrophages and dendritic cells (Holmgren *et al*., 1999; Spetz *et al*., 1999). The mechanisms behind antigen presentation of apoptotic-derived proteins are not fully understood although antigen presenting cells have been shown to present proteins from apoptotic bodies and thereby activate an immune response (Albert *et al*., 1998a; Albert *et al*., 1998b; Kokhaei *et al*., 2003; Spetz *et al*., 2002).

This study clearly illustrated that DNA encoding the viral antigen NP can be transferred from a dying cell to an antigen presenting cell via phagocytosis (Fig. 8). The silent NP construct was strictly activated by cre since there was no activation when the phagocytosing cells lacked the cre expression. Furthermore, co-cultivation experiments performed without inducing apoptosis in the donor cell line were negative for activation of the reporter gene. This lack of activation when living cells were mixed is evidence

that apoptosis is required for horizontal gene transfer. The observation that a silent viral gene could be transferred into an antigen presenting cell, such as a macrophage, enables us to exclude the possibility of protein transfer in antigen presentation.

Conclusions from paper II

- Cre-dependent activation of silent reporter genes in apoptotic cells occurs only after phagocytosis by cre-expressing cells.
- The cre-loxP recombination system can be used to study the horizontal transfer and expression of genes via the uptake of apoptotic cells.
- DNA encoding viral antigens can be transferred and expressed from dying cells into phagocytosing antigen presenting macrophages.

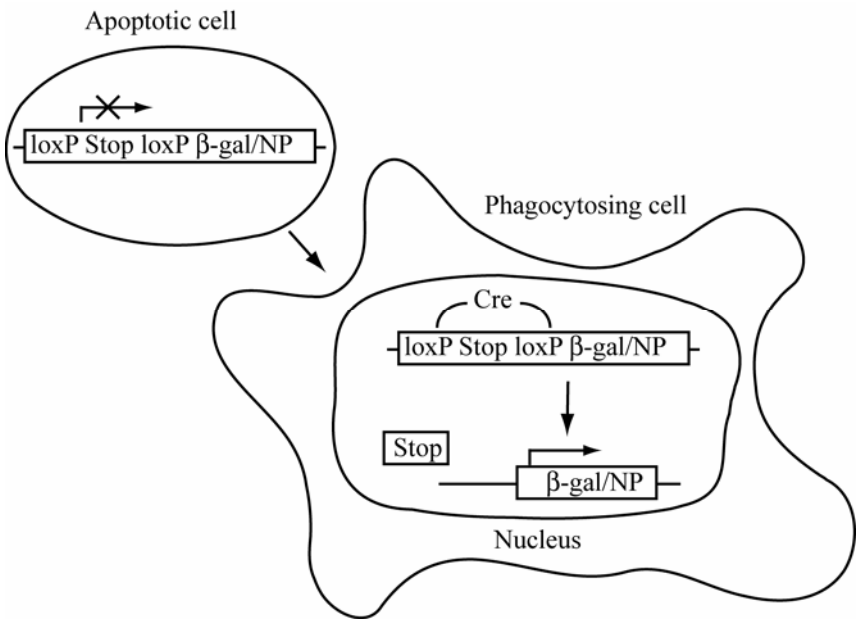

Fig. 8 *Schematic overview of the experimental set up for the activation of silent reporter genes by Cre recombinase.*

3.4 THE TUMOR MICROENVIRONMENT ENABLES TRANSFER OF DNA BETWEEN TUMOR CELLS AND STROMAL CELLS (PAPER III)

It is tempting to speculate that DNA is transferred between cells in developing tumors, especially since the tumor suppressor gene p53, or the p53 pathway, is inactivated in the majority of tumor cells and tumor cells frequently die by apoptosis. It has been suggested that transfer of DNA could explain the accumulation of genetic alterations needed to generate malignant tumors and the genetic havoc displayed by tumor cells (de la Taille *et al*., 1999; Holmgren *et al*., 1999). So far this has only been speculative as this has never been shown to occur *in vivo*.

The p53 pathway has been reported to be critical for the propagation of DNA transferred via engulfment of apoptotic cells. We therefore speculated that transfer of oncogenes dominantly inactivating p53 would enable replication of transferred genes in

normal cells. The SV40LT oncogene was chosen due to its ability to bind and inactivate p53 and the potential to easily detect its expression in cells by immunostaining.

The presence of SV40LT in dying cells allowed propagation of DNA recovered from apoptotic cells by normal cells *in vitro*. Not only was propagation of DNA coding for drug resistance shown but also transfer of oncogenes. Analyses of the DNA content of the transformed cells showed transfer of whole chromosomes and pieces thereof from the dying cells. Fusion of chromosomes between the dying cells and the phagocytosing cells was also detected.

The expansion of tumors is dependent on sufficient supply of oxygen and nutrients (Folkman, 1995). As tumors grow the formation of new functional capillaries is needed. This requirement is met by secretion of angiogenic factors from tumor cells that induce sprouting angiogenesis and the differentiation of hemapoietic stem cells to endothelial cells, which contribute to vessel formation (see section 1.6).

Endothelial cells of the tumor microvasculature have traditionally been considered to be stable diploid cells and suitable targets for anti-tumor treatment, especially since tumor cells are known to be genetically unstable. Recent data has challenged the notion of genetic stability of the tumor-associated microvasculature. The formation of vasculature networks made up by tumor cells (Maniotis *et al*., 1999), the presence of tumor specific chromosomal translocations in endothelial cells (Streubel *et al*., 2004), and the genetically unstable endothelial cells of human tumors grown in mice (Hida *et al*., 2004) demonstrate the close relationship between tumor cells and the tumor microvasculature (Fig. 9).

The experimental setup using rat tumor cells that express SV40LT enabled us to analyze whether DNA transfer occurred between tumor cells and tumor stroma *in vivo*. Stromal cells that harbored the SV40LT gene were detected in SV40LT-expressing tumors grown in mice. Endothelial cells isolated from these tumors expressed SV40LT and could be propagated *in vitro*. DNA analysis demonstrated the same karyotype with whole chromosomes and pieces of chromosomes transferred from the tumor cells, as well as similar inter-species fusion chromosomes between rat and mouse as when horizontal gene transfer had occurred *in vitro*. The underlying mechanism of hybrid formation in tumors may therefore be similar to the uptake of apoptotic cells *in vitro*.

Hybrid cells of tumor and endothelial cells were shown to be negative for CD133 but express the endothelial cell surface markers PECAM, sialomucin, VE-cadherin and VEGF-R2 and were further shown to be functional in forming blood vessels that anastamosed with the host circulatory system *in vivo*.

These results indicate that transfer of genetic material between tumor cells and the tumor associated endothelium enable endothelial cells to proliferate and establish functional vessels in an as yet undescribed way.

Conclusion from paper III

- The SV40LT oncogene enables propagation of horizontally transferred DNA in normal MEF and bovine aortic endothelial (BAE) cells *in vitro*.
- DNA transfer between tumor and stromal cells occurs spontaneously *in vivo*.
- The hybrid cells from tumor stroma demonstrate the same karyotype, with gain of tumor specific chromosomes and chromosome fusions, as when horizontal gene transfer experiments are performed *in vitro*.

- Hybrids between tumor cells and tumor associated endothelial cells display endothelial specific markers, contain a mix of tumor and endothelial DNA, and are capable of forming functional vessels.

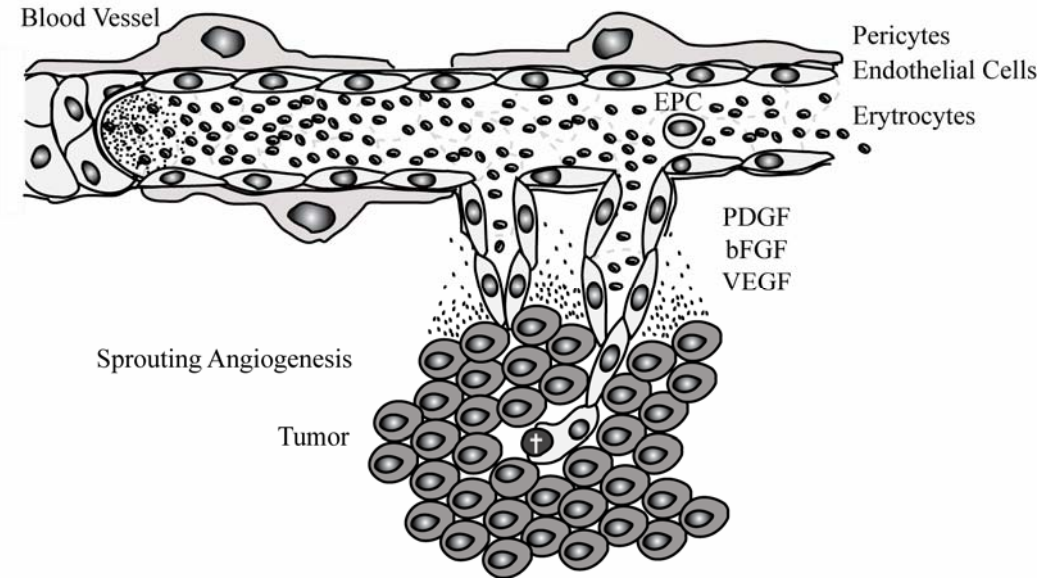

Fig. 9 *The induction of angiogenesis is crucial for tumors to expand in size. Hybrid formation between tumor and endothelial cells has the potential to be a new mechanism of inducing blood vessel formation.*

3.5 MECHANISM OF DNA TRANSLOCATION FROM APOPTOTIC CELL TO PHAGOCYTE NUCLEUS (PAPER IV)

We hypothesized that DNA from apoptotic cells escapes from the endosome and is transported to the nucleus of phagocytosing cells. Once inside the nucleus cell division is arrested via Chk2/p53/p21 signaling, hence normal cells are protected from propagation of potentially harmful foreign DNA.

In this study we used the thymidine analog 5-bromo-2-deoxyuridine (BrdU) to follow the fate of DNA from apoptotic cells after phagocytosis by MEF cells. Use of this method allowed the DNA from apoptotic cells to be discriminated from the DNA from phagocytosing cells. In complement to this, the nuclear compartment of the phagocytosing cell was visualized by staining for the fibrous LaminB1, which is a structural protein that spans just beneath the inner nuclear membrane.

Several dense BrdU positive entities were detected within the same MEF cells, demonstrating that fibroblasts are capable of phagocytosis. Phagocytosing MEF cells were detected comprising apoptotic DNA that clearly deformed the nuclear cage. In other cells the nuclear membrane was intact but surrounded by the BrdU positive DNA. After eight hours of co-cultivation, BrdU positive DNA fragments were detected within the nuclear cage of approximately 2% of the phagocytosing cells. Furthermore, no visible traces of LaminB1 surrounding the nuclear cage was observed and the nuclear cage had regained its normal spherical structure. By creating 3-dimensional

images the position of the apoptotic DNA was verified to be within the nuclear cage of the phagocytosing cell.

The fragments of DNA from apoptotic cells within the nucleus of the MEF cells co-localized with both MRE11 and γ-H2AX. This not only links the DNA damage signaling pathway to horizontally transferred DNA but also demonstrates that the transferred DNA is truly within the nucleus.

Since this mechanism of DNA from apoptotic cells fusing with the nucleus of phagocytosing cells, to our knowledge, has never been reported we suggest the name *Pirinosis* as an acronym of the Greek words *Pirinas* meaning nucleus and *Enosis* meaning union (Fig. 10).

Nuclear fusion explains the presence of large fragments of DNA and even whole chromosomes, which have been reported after horizontal transfer of DNA via uptake of apoptotic cells. It further argues that the translocation of DNA from the endosome does not follow the normal mode of transport to and from the nucleus via nuclear pores.

Conclusions from paper IV

- DNA from apoptotic cells enters the nucleus of phagocytosing MEF cells by an as yet undescribed mechanism of fusion.
- DNA fragments from apoptotic cells co-localize with markers of activated DNA damage response in the nucleus of phagocytosing MEF cells.

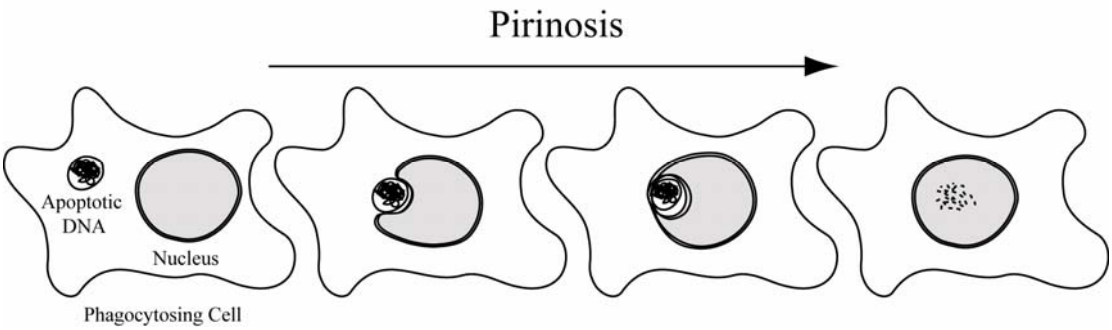

Fig. 10 *The process of Pirinosis. DNA from apoptotic cells is transferred to the nucleus of phagocytes by fusion with the nucleus.*

3.6 CONCLUDING REMARKS

In this thesis I have further investigated the field of cell to cell DNA transfer via the engulfment of apoptotic cells. When I started this work it was known that DNA from dying cells could be salvaged by phagocytosing cells, but the mechanism of how DNA ended up in the nucleus was unknown. Furthermore, lateral gene transfer in tumors with a high degree of apoptosis had been speculated to occur, particularly after irradiation or chemotherapy, but never shown.

This thesis demonstrates a totally new mechanism of introducing large fragments of DNA and whole chromosomes into the nucleus of eukaryotic cells. The mechanism is named Pirinosis since it appears as if dense entities of apoptotic DNA can fuse with the nucleus of phagocytes. Although previous studies have reported expression of genes transferred from apoptotic cells, the transfer of protein or mRNA has never been possible to exclude. With the cre-loxP model system, transfer of protein or mRNA was clearly ruled out since silent genes were only activated when the cre protein was present in the phagocyte. The cre-loxP system further demonstrated the transfer and expression of viral DNA into macrophages. This implies that the uptake of viral DNA and subsequent expression by antigen presenting cells is a potential mechanism of eliciting the immune system. In fact, a therapeutic vaccine against HIV, which is based on the uptake of apoptotic cells by antigen presenting cells, is tested. This method has been shown to be successful in mice and is planned to be tested in a phase I clinical trial starting 2008 by Avaris (Feldreich, 2007).

Normal cells are protected from propagation of DNA recovered from dying cells via the p53-p21 pathway. The reason why these two genes were critical for propagation has been speculated to be dependent on the introduction of DNA breaks after uptake of dying cells. This speculation was confirmed by our results that demonstrate the importance of DNaseII and Chk2 in the phagocyte as well as the accumulation of early markers of the DNA damage pathway.

Tumor cells are notorious for harboring p53 mutations or an impaired p53 signaling pathway. This in combination with a high frequency of apoptosis has led to the speculation that DNA could transfer between tumor cells. It has been demonstrated *in vitro* that oncogenes can be transferred via uptake of apoptotic bodies. But, so far, transfer of genetic material between tumor cells *in vivo* has never been reported. Due to the methodological problems of determining the origin of DNA within tumors the focus was turned to the tumor stroma. The endothelial compartment of the tumor stroma was especially interesting since recent reports have described genetic instability in these cells. The advantage of inoculating a rat tumor in mice made it possible to isolate hybrid immortalized endothelial cells from SV40LT positive tumors. Interestingly, the endothelial cell phenotype was maintained although the DNA content was a mixture of rat and mice chromosomes. When tested for functionality the hybrid endothelial cells were able to form blood vessels *in vivo* that anastamosed with the host animal's circulatory system. The uptake of apoptotic cells is therefore a potential mechanism for tumors to stimulate proliferation of endothelial cells and may even be critical for inducing angiogenesis.

Even though the actual uptake of apoptotic cells by tumors remains to be shown, the similarities in the karyotypes between spontaneously formed hybrids *in vivo* and after horizontal gene transfer *in vitro* makes it appealing to believe that Pirinosis is the mechanism.

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