Effect of MMR status and growth rate of colon carcinoma cells in response to 5-fluorouracil

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by

Bhavesh Choudhary

(Darbhanga, India)

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2. Summary

Mismatch repair (MMR) system plays an important role in the repair of damaged DNA. 10-15% of sporadic colorectal cancers and more than 40% of hereditary nonpolyposis colorectal cancers (HNPCC) are MMR-deficient, most frequently as a result of a mutation in one of the MMR genes: hMLH1 or, much less frequently, hMSH2 or hMSH6. Whether the MMR deficiency increases or decreases the sensitivity of colon carcinomas to 5-fluorouracil (5FU) is a matter of controversy. Clarification of the effect of MMR status on colon carcinoma susceptibility to 5FU is necessary to establish criteria for selecting the population of patients who will benefit from treatment.

Apart from the DNA repair function, MMR has been reported to affect cell proliferation (57-60). Whether MMR increases or decreases cell proliferation is not clarified.

The main aim of my work was to investigate the cellular mechanism of 5FU cytotoxicity and how MMR status and growth speed influence it. I investigated the effect of MMR on cell proliferation.

The investigation was carried out in isogenic (mock 1-transfectants and clone 43), semiisogenic (HCT116, HCT116+chr2 and HCT116+chr3) colon carcinoma cell lines and tumour xenografts differing in their MMR status.

The present data showed that MMR does not affect cell proliferation and demonstrate that the MMR affects clonogenic survival after 5FU treatment. The lesser clonogenic survival in MMR-proficient cells was associated with more DNA-double strand breaks and more short-term apoptosis than MMR-deficient cells. The data showed for the first time that MMR mediated senescence and not apoptosis determines low clonogenic survival in response to 5FU treatment.

The data showed that the cell density affects the response to 5FU treatment. I investigated the long-term response of cells grown at a high density which may give

more information about the *in vivo* response than the cells grown at a low density i.e. grown in clonogenic assay.

The data showed for the first time that the long-term cell survival in response to 5FU is determined by programmed necrosis which is regulated by the growth speed and not by the MMR. The necrosis was dependent on the presence of caspase protease and of Bax protein.

The data in mouse model *in vivo* showed for the first time that tumour growth speed is a much more important determinant of response to 5-fluouracil than the MMR status.

In sum, the present data indicate that MMR does not affect cell proliferation. The growth speed and not MMR determines long-term cell response to 5FU treatment. Cell growth inhibition and not the cell death is the main effect of 5FU cytotoxicity.

3. Zusammenfassung

Das *mismatch* Reparatur System (MMR) spielt bei der Reparatur der DNA Schäden eine wichtige Rolle. 10-15% von sporadischen Kolonkarzinomen und alle HNPCC Kolonkarzinome weisen MMR-Defekte auf. Diese entstehen auf Grund einer Mutation in einem der MMR-Gene, wie hMLH1 oder, weit weniger häufig, hMSH2 oder hMSH6. Ob ein MMR-Defekt die Sensitivität des Kolonkarzinoms auf 5-Fluorouracil (5FU) steigert oder vermindert, wird kontrovers diskutiert. Ebenfalls ungeklärt ist der Einfluss von MMR auf die Zellproliferationsrate. Die Klärung des Einflusses des MMR-Status auf die Sensitivität der Zellen auf 5FU würde erlauben die Patienten zu identifizieren, die von der Behandlung profitieren.

Das Hauptziel meiner Arbeit war es zu untersuchen, inwieweit MMR und die Zellproliferationsrate die Mechanismen der Zytotoxizität von 5FU beeinflussen.

Die Analysen wurden in isogenen und semiisogenen Paaren von Kolonkarzinomzelllinien und in xenotransplantierten Tumoren, die sich hinsichtlich ihres MMR-Status unterschieden, durchgeführt.

Die Daten zeigten, dass MMR die Zellproliferationsrate nicht beeinflusst. Die geringere klonogene Überlebensrate der MMR-kompetenten im Gegensatz zu den MMR-defizienten Zellen war nicht durch Apoptose sondern durch einen langfristigen Arrest und anschliessende Seneszenz der MMR-kompetenten Zellen verbunden.

Die Untersuchung der Langzeitwirkung von 5FU zeigte, dass sie nicht durch den MMR Status sondern hauptsächlich duch die Proliferationsrate der Zellen bestimmt ist. Auf der zellulären Ebene bestimmt die Nekrose das Ausmaß des langfristigen Zelltodes. Die Nekrose *in vitro* ist von den Caspasen und von dem bisher nur als proapoptotisch charakterisierten Protein Bax abhängig. Die gesamte antiproliferative Wirkung von 5FU wird jedoch nur in geringem Maße von dem nekrotischen Zelltod bestimmt, vielmehr macht die Inhibition der Proliferation den Hauptanteil der Zytotoxizität aus.

Im Mausmodell konnte gezeigt werden, dass der Unterschied in der Proliferationsrate der Zellen bei weitem stärkeren Einfluß auf die Suszeptibilität gegenüber 5FU hat als der Unterschied im MMR Status.

Zusammenfassend zeigen die Daten, dass die Wachstumsgeschwindigkeit und nicht das MMR-System die Langzeitantwort der Zellen auf eine 5FU Behandlung bestimmt. Die Hauptwirkung von 5FU ist die Proliferationsinhibition und nicht die Induktion des Zelltodes.

4. Abbreviation

APC	Adenomatous Polyposis Coli
APS	Ammonium Peroxydisulphate
Apf-1	Apoptotic protease activating factor
ATP	Adenosine Triphosphate
Bax	Bcl-2 associated protein X
Bcl2	B-cell lymphoma/leukemia
bp	Base Pair
BSA	Bovine Serum Albumin
CDC	Cell-Divison Cycle kinase
CDK	Cyclin Dependent kinase
Chr2/chr3	human Chromosome 2/3
CIP1	Cyclin Inhibitory Protein 1
CPT	Camptothecin
DMEM	Dulbecco's MEM
DMSO	Dimethylsufoxide
DNA	DeoxyriboNucleic Acid
dNTP	Deoxy Nucleotide Triphosphate
DTT	Dithothreitol
dUTP	Deoxy Uridine Triphosphate
EDTA	Ethylenediaminetetraacetic acid
hMutS	Human Mut-S Homologue
hMutL	Human Mut-L Homologue
hMSH2	Human Mut-S Homolgue-2

hMSH3	Human Mut-S Homolgue-3
hMLH1	Human Mut-L Homolgue-1
kDa	KiloDalton
KIP1	Kinase Inhibitor Protein 1
LB	Lysogeny-Bertani
Mdm2	Murine Double Minute-2
mut	Mutated
OD	Optical Density
PVDF	Polyvinylidene Fluoride
PCR	Ploymerase Chain Reaction
PBS	Phosphate Buffer Saline
PMS1	Postmeiotic Segregation 1
PAGE	Polyacrylamide Gel Electrophoresis
rpm	Revolutions Per Minute
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
TAE	Tris-Acetate EDTA
TBS	Tris Buffer Saline
UV	Ultra Violet
wt	Wild Type

5. Introduction

5.1. Etiology of colorectal Cancer

Colorectal cancer (CRC) is one of the most commonly diagnosed cancer and second leading cause of cancer deaths in the western world (1-3). In Europe, CRC represents 12.9% of total number of cancers and ranks second in cancer deaths (4). The risk factors associated with CRC include a positive family history, age, meat and alcohol consumption, fat intake and smoking (5-7). The physical activity and the use of non-steroidal anti-inflammatory drugs (8 -10) reduce the risk of CRC.

CRC can be sporadic or hereditary.

5.1.1. Sporadic colon cancer

Sporadic colon carcinoma is a result of accumulation of somatic mutations, which block or enhance essential biochemical pathways (13). 75% - 80% of CRC tumours are of sporadic origin. A colorectal carcinogenesis model was proposed by Fearon and Vogelstein (14). As it is shown in Figure.1, every step from the normal mucosa towards the carcinoma involves specific and well-defined genetic alterations. The most frequent mutation at the early stage occurs in the APC gene (a component of the *wnt*-pathway), Ki-ras gene (MAP-kinase signalling pathway) and at the later stage in the p53-gene (responsible for cell arrest and apoptosis).

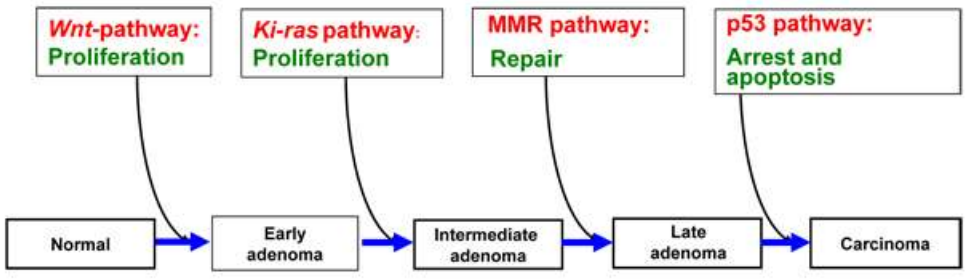

Figure 1. Molecular alterations in sporadic colon carcinogenesis. Modified after Fearon and Vogelstein ()

5.1.2. Hereditary colon cancer

15-20% CRC is hereditary. Hereditary CRC is associated with the germ-line mutations in a tumour-suppressor gene or oncogene or DNA-repair gene. The two most common inherited colorectal cancer syndromes are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC).

5.1.2.1. Familial adenomatous polyposis (FAP)

Familial adenomatous polyposis is a rare condition characterized by the development of multiple colonic adenomas which later develop to adenocarcinomas. FAP is caused by germ-line mutation in adenomatous polyposis coli (APC) gene which is located at chromosome 5q21 and inhibits Wnt signaling pathway (17). Germ-line mutations are located throughout the entire *APC* gene and more than 90% of mutations introduce a premature stop codon that results in a truncated protein (18).

5.1.2.2. Hereditary non-polyposis colorectal cancer (HNPCC)

Hereditary non-polyposis colorectal cancer (HNPCC) also known as Lynch syndrome is the most common type of hereditary CRC characterized by microsatellite instability (MSI) (11-12, 15-16)). HNPCC accounts for nearly 3% of all CRC in the western world (19-20) and is caused by the germ-line mutation in one of the DNA mismatch repair genes MLH1, MSH2, MSH6 and PMS2 (21-22). MMR defect or MSI is a hallmark of HNPCC.

5.2. DNA repair pathways

The integrity of DNA can be affected by error in DNA replication or by internal or external DNA damaging agents. In order to maintain DNA integrity, cell recognizes and repairs damaged DNA by activating multiple DNA repair pathways. The major DNA repair pathways as shown in Fig. 2 include Base Excision Repair (BER), Mismatch Repair (MMR), Nucleotide Excision Repair (NER) and Homologous Recombination (HR).

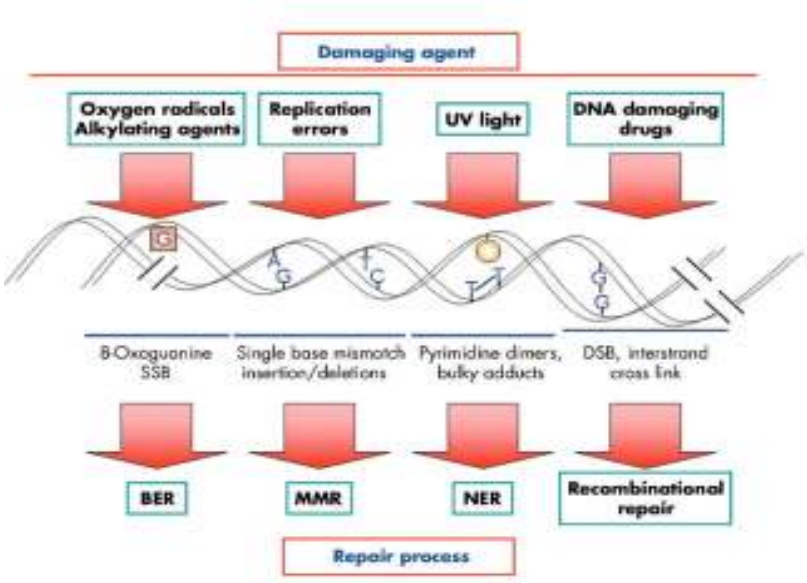

Fig.2. Major DNA repair pathways

5.2.1. Base excision repair (BER)

BER pathway is a multistep process by which eukaryotic cells repair damaged bases arising from alkylation or deamination or oxidation by various DNA damaging agents. BER is mediated by several DNA glycosylases which are often specific for a particular type of base damage (23-24). As it is shown in Fig. 3A, DNA damage caused by mispairing of uracil base with guanine is repaired by BER pathway. The uracil DNA glycosylase (UNG) catalyzes the excision of the damaged uracil base, creating an abasic (AP) site. The APEX endonuclease 1 (APEX1) recognizes the AP site and catalyzes the incision of the DNA backbone. After the incision of DNA, DNA polymerase β (POLB) polymerizes DNA to fill in the gap. Finally, DNA ligase III (LIG3) enzyme catalyzes the formation of a phosphodiester bond and completes the repair pathway.

BER functions via two sub-pathways which are based on the length of the repair patch: short-patch and long-patch BER (25-26). As the name indicates, short-patch BER replaces the lesion with a single nucleotide whereas the long-patch BER replaces the lesion with approximately 2 to 10 nucleotides.

5.2.2. Mismatch repair (MMR)

The MMR pathway plays an essential role in the correction of replication errors such as base-base mismatches and insertion/deletion loops (IDLs) that result from misincorporation of nucleotides and template slippage respectively by DNA polymerase. MMR is a tightly regulated process which includes several proteins like MSH2, MSH3, MSH6, MLH1, PMS1 etc. and is initiated by heterodimeric complexes of MSH2-MSH6 (MutSα) and MSH2-MSH3 (MutSβ). As is shown in Fig.3.B, the MutSα complex recognizes and binds to base-base mispairs followed by ATP-dependent recruitment of a heterodimeric complex of MLH1 and PMS2 (MutLα). The interaction between the MutS and the MutL complexes triggers the activation of subsequent MMR steps by recruiting exonuclease 1 (Exo1) which catalyses the excision of DNA containing the mispair. Finally, the DNA gap of approx. 150 bp is filled in by DNA polymerase and the nick is sealed by DNA ligase (27- 28).

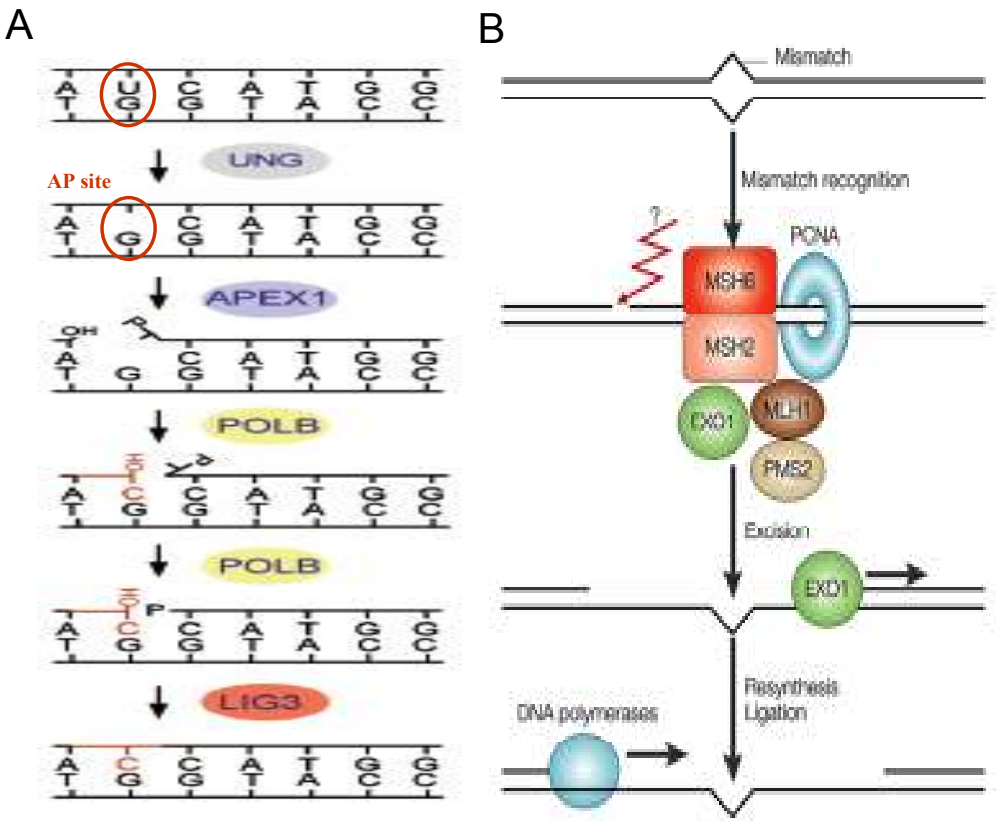

Fig. 3. A.: Short-patch Base Excision Repair pathway; **B.:** Mismatch Repair pathway

5.3. Cellular and Molecular response to DNA damage

In response to DNA damage by several endogenous or exogenous DNA damaging agents, eukaryotic cells activate multiple processes which either repair the damage or cause cell arrest or cell death, if the damage is unrepairable. These processes are collectively called DNA damage response (29-30). The most common DNA damage responses are discussed below:

5.3.1. Cell cycle check point activation

Eukaryotic cell cycle has four phases, G1 (gap before DNA replication), S (DNA synthesis), G2 (gap after DNA synthesis) and M (mitosis). Cell-cycle checkpoints are regulatory mechanisms by which cell actively halts progression through the cell cycle phases until it can ensure that an earlier process is complete.

As it is shown in Fig. 4, cells responds to DNA damage by activating G1/S, S and G2/M check points. The entire process is tightly regulated and require a specific set of proteins and enzymes such as Ataxia telangiectasia mutated (ATM) Ataxia telangiectasia and Rad3-related protein (ATR) kinase, p53, NBS1 (Nijmegen breakage syndrome 1), BRCA1 (breast cancer 1), SMC1 (structural maintenance of chromosome 1) and check point kinase 1 and 2 (Chk1 and 2) (31).

5.3.1.1. G1/S check point

The dominant checkpoint response to DNA damage in mammalian cells traversing through G1/S is the ATM/ATR - Chk2 /Chk1 - p53/MDM2 - p21 pathway (32). As it is shown in Fig. 4, ATM/ATR kinase directly or indirectly via Chk1/Chk2 phosphorylates and activates p53 tumour suppressor protein (32-33). Additionally, ATM/ATR kinases phosphorylate and inhibit the ubiquitin ligase MDM2 which causes p53 degradation (34). Both these processes stabilise p53 protein and enhance its transcriptional activity. p53 regulates several target genes such as $p21^{CIP1WAF1}$ which leads to G1 or G1/S arrest. Another pathway which is independent of p53 and regulates G1/S checkpoint is Chk1/Chk2 - Cdc25A pathway. DNA damage results in an increase

activity of Chk1 and Chk2 kinases which further downregulate Cdc25A phosphatases required for the activation of Cdk2 (35).

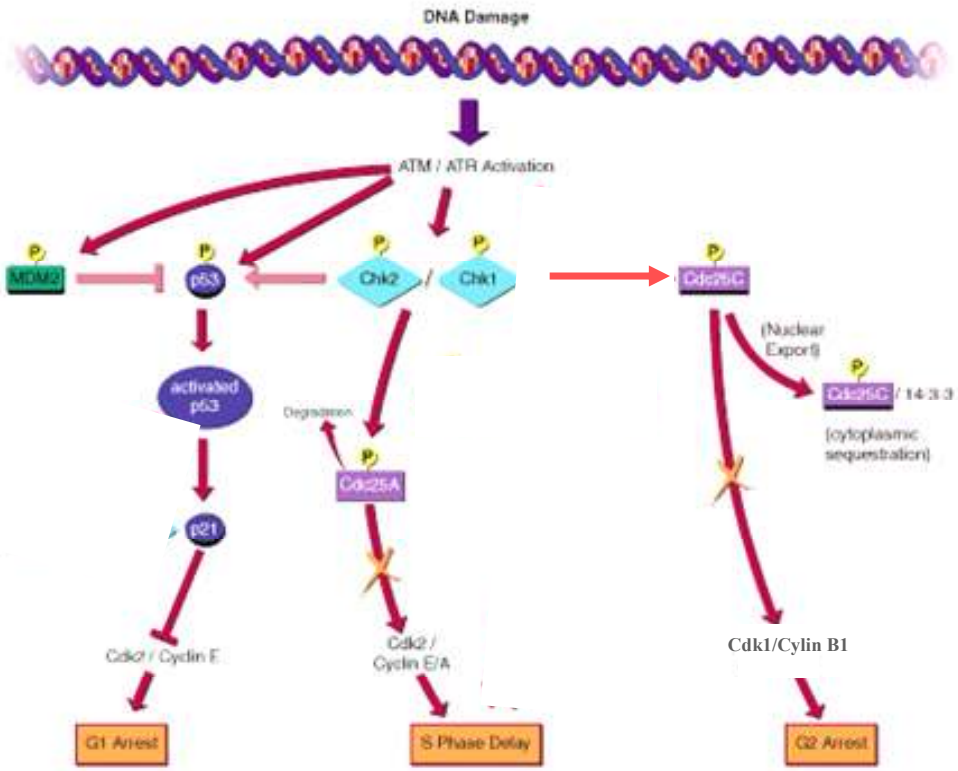

Figure.4. ATM/ATR mediated activation of G1- or S- or G2- checkpoints after DNA damage.

5.3.1.2. S-phase (intra-S-phase) check point

Cells experiencing genotoxic stress during DNA synthesis, delay S-phase progression in a transient manner. If the damage is not repaired during this delay, they exit S-phase and arrest in G2 or G1 checkpoint (31). As it is shown in Fig.4, S-phase checkpoint is regulated most commonly by ATM/ATR kinase. One of the pathways operates through ATM/ATR - Chk2 /Chk1 - Cdc25A –Cdk2 network. This pathway blocks the loading of Cdc45 protein which is required for DNA polymerase recruitment into assembled pre-replication complexes, and thereby prevents the initiation of new replication origin firing (35-36).

5.3.1.3. G2/M check point

The G2/M also known as the G2 checkpoint prevents cells from initiating mitosis when they experience DNA damage during G2 or with some unrepaired damage inflicted during previous S-or G1- phase (37)

As it is shown in Fig. 4, the main target of the G2/M checkpoint is the activity of the cyclin B/Cdk1 kinase complex whose activation is inhibited by ATM/ATR, Chk1/Chk2 and/or p38-kinase mediated sub-cellular sequestration, degradation and/or inhibition of the Cdc25C phosphatases that normally activate Cdk1(38).

5.3.2. Apoptosis

Apoptosis is a genetic program of cell death. It is characterised by several morphological features such as reduction of cellular volume, chromatin condensation, nuclear fragmentation, plasma membrane blebbing. The biochemical features include the presence of active caspases and cleaved caspase substrates (PARP), presence of DNA ladder and exposition of phosphatidylserine to the outer surface of the plasma membrane.

As it is shown in Fig. 5, two major apoptotic pathways are operative in mammalian cells: extrinsic and intrinsic. The extrinsic or death-receptor pathway is triggered by ligand binding to cell-surface receptors (CD95, DR4/5) resulting in the recruitment of various proteins to form the death-inducing signalling complex (DISC). This complex triggers the activation of caspase-8, which in turn activates caspase-3. Caspase 3 protease finally induces the cellular changes that characterise apoptosis.

The intrinsic or mitochondrial pathway is triggered by cytotoxic stress which induces the translocation of pro-apoptotic Bcl-2 family members (Bid, Bak, Bax etc.,) to the mitochondrial membrane. This leads to the release of mitochondrial cytochrome c into the cytosol where it promotes the oligomerisation of the pro-apoptotic factor Apaf-1 into a complex called the apoptosome. The aptoptosome recruits and activates caspase-9 which in turn promotes the activation of death executioner caspase-3. The

crosstalk between extrinsic and intrinsic pathways is mediated by a proapoptotic protein Bid.

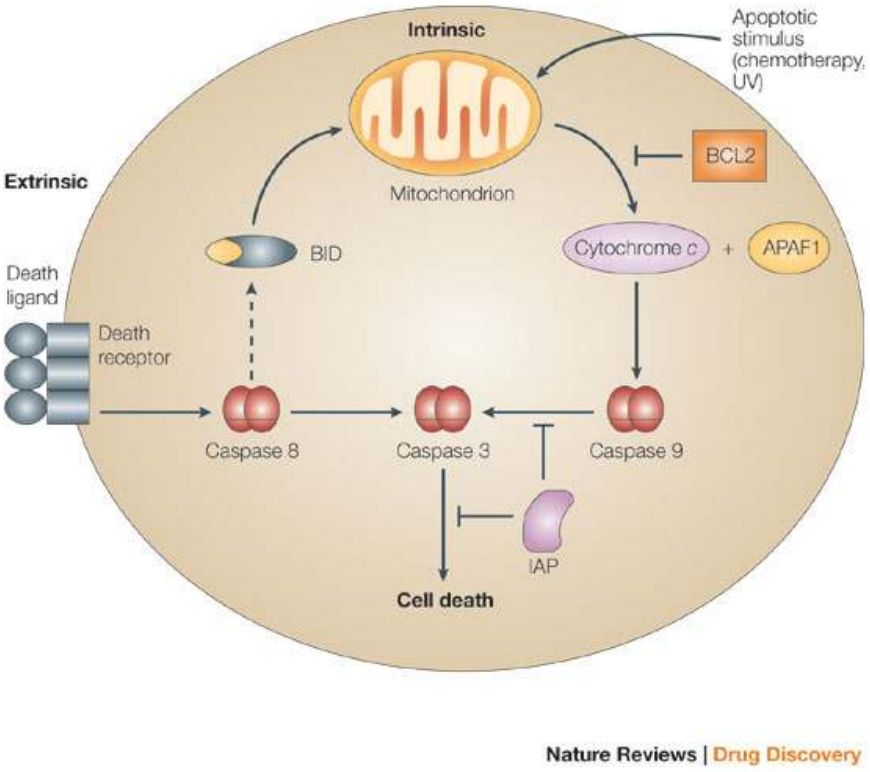

Fig.5. Extrinsic and intrinsic pathways for apoptosis.

5.3.3. Necrosis

Necrosis is a form of cell death caused by intolerable stresses such as lack of oxygen or nutrients, high temperature, toxic compounds and mechanical strain. Necrosis is characterized by mitochondrial swelling, dilatation of endoplasmic reticulum and extensive vacuolization of cytoplasm (Fig. 6). Unlike during apoptosis, during necrosis cell swells and eventually lyses without the formation of vesicles (41). For a long time, necrosis has been considered merely as an accidental uncontrolled form of cell death but the growing number of evidence indicates that the necrosis follows defined signalling pathways. Several studies have suggested that cells undergo programmed necrosis in response to DNA damage (42-44). It has been reported that the caspases

(45-46), receptor interacting serine-threonine protein (RIP) kinases (47), PARP1, calpains and bax (48) proteins are involved in programmed necrotic cell death.

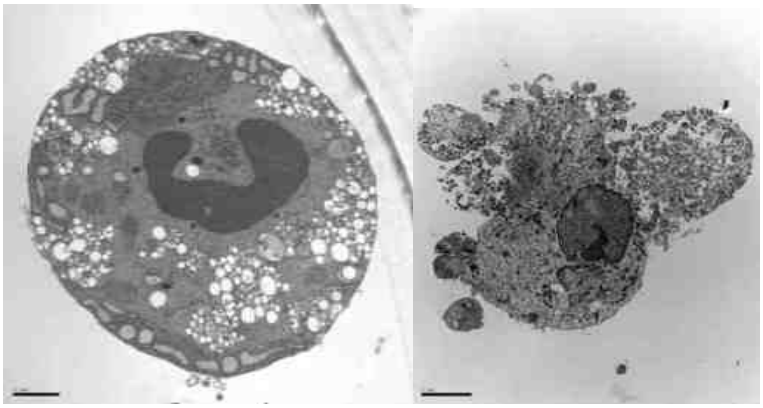

Figure.6. Electron micrograph of cells undergoing programmed necrosis. **A.:** Extensive intracellular vacuolation and mitochondrial swelling, **B.:** Extensive loss of plasma membrane integrity. (Dr. F. Chan, Department of Pathology, University of Massachusetts Medical School, USA)

5.3.4. Mitotic Catastrophe

Mitotic catastrophe is a cell death mechanism occurring either during or shortly after dysregulated or aberrant mitosis (49). Mitotic catastrophe finally leads to apoptotic or necrotic cell death and is characterised by the formation of giant cell with micronucleation and multinucleation (presence of two or more nuclei in a cell) (50). Mitotic catastrophe is caused by various physical or chemical stresses. DNA damage induced mitotic catastrophe results from the premature induction of mitosis before the completion of S and G2-phase (50). Several S- or G2-checkpoint regulator proteins such ATM, ATR, Chk1, Chk2 (49), p53 and p21 (51) have been shown to regulate DNA damage-induced mitotic catastrophe.

5.3.5. Senescence

Senescence is a regulated process which leads to the irreversible cell arrest. The permanently arrested cells remain metabolically active and viable. Cells undergoing

senescence are enlarged, flattened and granular. These cells express high level of β-galactosidase (49). Senescence is classified as replicative or accelerated (52).

Replicative senescence is induced by the shortening of telomere at the chromosomal ends and is associated with aging whereas accelerated senescence is triggered by DNA damage or increased oncogenic signaling or oxidative stress (49, 52). DNA damage-induced senescence is triggered by regulated pathways comprising several tumour suppressor proteins such as p53, p21, p16, retinoblastoma (Rb) and p27 (49, 52).

5.4. 5FU

5-fluorouracil (5FU) is the most frequently used drug in the treatment of colon, breast, head and neck cancers. 5FU treatment is given alone or in combination with other drugs such as leucovorin, irinotecan and oxaliplatin.

5.5. Mechanisms of 5FU mediated cytotoxicity

5-fluorouracil (5FU) is a uracil analogue. It enters the cell using the same facilitated transport mechanism as uracil and is converted intracellularly to several active metabolites such as fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). Following possible mechanisms by which 5FU exerts its anticancer effects have been described (56):

5.5.1. Thymidylate synthase (TS) inhibition

Thymidylate synthase enzyme catalyses the formation of deoxythymidine monophosphate (dTMP) form deoxyuridine monophosphate (dUMP) using 5, 10 methylenetetrahydrofolate as a methyl group donor. This reaction provides the sole *de novo* source of thymidylate which is necessary for DNA replication. The 5FU metabolite FdUMP inhibits TS enzyme activity by binding and forming a stable ternary complex and thereby inhibits dTMP synthesis (53). The exact molecular mechanisms that mediate events downstream of TS enzyme inhibition have not been fully elucidated. However, depletion of dTMP leads to the depletion of deoxythymidine

triphosphate (dTTP) and thereby causes deoxynucleotide pool imbalance. The whole process leads to the inhibition of DNA synthesis (53).

5.5.2. RNA directed cytotoxicity

The 5FU metabolite FUTP is extensively incorporated into RNA and disrupts normal RNA processing and functions. Significant correlation between 5-FU incorporation into RNA and loss of clonogenic survival has been reported in a breast carcinoma cell line (54). Several data report that FUTP incorporation in RNA inhibits the processing of pre-rRNA into mature rRNA (55), post-transcriptional modification of tRNAs and splicing of pre mRNA ((56). These evidences confirm that 5FU incorporation into RNA can potentially disrupt several aspects of RNA processing and may eventually affect the cell metabolism and viability (54-55).

5.5.3. DNA directed cytotoxicity

DNA directed effects of 5FU arise from the direct incorporation of the FdUTP into DNA (56, 70). The two most important consequences of these events are the potential mutagenic effects of base analogues in DNA and triggering of DNA damage response in the cell's attempts to repair these lesions.

5.6. Effect of MMR on cell proliferation

Apart from the DNA repair function, MMR has been reported to affect the cell proliferation (57-60). It has been reported that the overexpression of hMLH1 decreases (57-58) or increases (59) or does not affect (60) cell proliferation. However, a report indicates that the silencing of hMLH1 protein in colon cancer cell does not affect proliferation (59).

5.7. Response of $p53^{mut}$ cells to 5-FU

p53 is a tumour suppressor protein which is located in the nucleus and affects in a number of molecular pathways regulating processes like autophagy, DNA damage responses, differentiation, senescence, cell-cell interactions and apoptosis. Inactivating mutations in p53 gene are the most common genetic alterations in human

cancers (61). It is estimated that more than 50% of all human cancers express either a mutant p53 (62).

One of the cytotoxic effects of 5FU drug has been attributed to its ability to induce p53 protein (51). Several cell culture and animal studies have shown that the mutations or deletions of p53 gene result in the resistance of cells to 5FU treatment (51, 53, 63). A group of clinical data, in agreement with the *in vitro* data, have reported that the patients with p53 mutant tumour respond less to 5FU based therapy (64). The strategy focusing on restoring the function of mutant p53 in cancer cell to enhance sensitivity towards chemotherapy is an active area of cancer research (63-65).

5.8. Role of MMR in response to 5-FU

15% to 20% of colorectal cancers are MMR-deficient. They are characterized by microsatellite instability (MSI). Many studies have advocated the association of MSI with better prognosis (66-67) of patients with CRC but others have not found this relationship (68-69). Numerous investigations have been attempted to correlate MMR-defect or MSI status with the response of cancer cells or tumours to 5FU treatment. A group of data indicates that the MMR-deficient colon carcinoma cells are more resistant to 5FU treatment than MMR-proficient cells (71-74). In agreement with the *in vitro* data, a set of clinical data shows that the patients with MMR-deficient tumours show less overall survival than patients with the MMR-proficient tumours after 5-FU treatment (75-77). On the contrary, several reports suggest that the patients with MMR-deficient tumours show better survival than patients with MMR-proficient tumours following 5-FU therapy (78-80).

Thus, whether the MMR-defect increases or decreases the sensitivity of colon carcinomas in patients to 5FU therapy is a matter of controversy. Clarification of the effect of MMR status on colon carcinoma susceptibility to 5FU is necessary to establish criteria for selecting the population of patients who will benefit from the treatment.

6. Objectives

The main objectives of my work were to answer the following questions:

A.: Does MMR affect proliferation speed of colon cancer cells?

B.: How MMR and growth speed affect the response of colon carcinoma cells to 5FU treatment?

C.: What are the mechanisms of 5FU cytotoxicity and how MMR affects them?

D.: How MMR affects the tumour response to 5FU *in vivo?*

7. Materials and Methods (10-12 pages)

7.1. Molecular biology

7.1.1. Transformation of bacterial cells with plasmid DNA

Top 10 bacterial competent cells were taken out from -70^{o}c deep freezer and kept on ice for 10 min. 1-2 µl (10-20 ng, diluted in sterile water or TE) of plasmid DNA or sterile water or TE (negative control) was added to the competent cells and incubated on ice for 20 min. After 40 seconds of heat shock at 42^{o}C in water bath followed by 2 min incubation on ice, 125 µl of SOC medium (Invitrogen GmbH, Darmstadt, Germany) was added to cell suspension and incubated at 37^{o}C for 1 h with gentle shaking. 25 µl cell suspension was used for spreading on LB agar plate containing appropriate antibiotic. After overnight incubation at 37^{o}C, several colonies were picked for further processing.

7.1.2. Isolation of plasmid DNA from bacterial cells

Qiagen kit (Qiagen GmbH, Hilden, Germany) was used for the isolation of plasmid DNA. Single bacterial colony picked from a freshly streaked LB agar plate was inoculated into 3 ml LB medium containing appropriate antibiotic. After 8 h of incubation at 37^{o}C in a water bath shaker (New Brunswick Scientific, New Jersey, USA), whole bacterial culture was inoculated into 100 ml LB medium containing appropriate antibiotic. Plasmid DNA was purified and eluted in sterile water or TE by using standard Qiagen protocol. The concentration of plasmid DNA was determined by measuring absorbance at 260 nm wavelength using Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).The integrity and correctness of plasmid DNA was checked by agarose gel electrophoresis (6.1.4). Plasmids were stored at -20^{o}C until further use.

7.1.3. Preparation of bacterial glycerol stock

1 ml of overnight grown bacterial culture (in exponential phase) was added to 1 ml of sterile LB: glycerol (1:1) solution, in 2 ml cryotubes (Nunc GmbH, Langenselbold,

Germany). After gentle mixing of glycerol and bacterial cell suspension, cryotubes were stored at -70^{o}C.

7.1.4. Agarose gel electrophoresis

1% agarose (Qbiogene, Heidelberg, Germany) gel was made in 1 x TAE buffer to which ethidium bromide (Sigma, Taufkirchen, Germany) was added at a final concentration of 0.2 µg/ml. Isolated plasmid DNA was loaded in the gel with 1 x TAE loading buffer (Fermentas GmbH, St. Leon-Rot, Germany). The gel was run at 100 V in 1 x TAE buffer for 30-60 min. DNA fragments were visualized under UV transilluminator (Konrad Benda, Weisloch, Germany).

7.1.5. Transient transfection of cell lines

7.1.5.1. Overexpression of hMLH1 gene using adenoviral vector

0.2 x 10^6 HCT116 cells were seeded in 22 cm^2 dish in medium containing 10 % FCS. 24 h after seeding, cells were washed with PBS, then incubated with medium without FCS containing AxCAhMLH1WT (wild type hMLH1 protein expressing adenovirus, provided by Dr.Yokoyama, BioResource Center, Japan) or AdCMV-Lac Z (negative control, provided by Dr. P. Daniel, Max-Delbruck-Center for Molecular Medicine, Berlin, Germany) at the desired multiplicity of infection (MOI). After 90 min incubation at 37^{0}C, 10% FCS was added. After 48 h, transduced cells were used for lysate preparation or for further assay.

7.1.5.2. Suppression of p53 gene using shRNA (short hairpin RNA)

2.4 x 10^6 cells were trypsinized and washed with PBS and resuspended in 500 µl RPMI without FCS. 20 µg of pSuper-EGFP (a kind gift from Dr. M. Truss, Charité Campus Virchow, Berlin, Germany) or pSuper-p53 (p53 shRNA-expressing plasmid, Oligoengine, Seattle, USA) plasmid was added to cells and incubated at RT for 5 min. Cells were electroporated at 290 V and 1050 µF in 0,4 cm electroporation cuvette using Gene Pulsar Xcell^{TM} (Biorad Laboratories, München, Germany). Electroporated cells were seeded in prewarmed culture medium with FCS and incubated at 37^{o}C. After 24-48 h, cells were used for lysate preparation or further assay.

7.1.5.3. Suppression of hMLH1 expression using siRNA (small interfering RNA)

0.2 x 10^6 cells of clone 43 were seeded in 9.6 cm^2 dish in medium with 10% FCS. 24 h after seeding, the medium was removed and fresh medium was added.100 µl transfection solution, containing 25 nM P1-siRNA (negative control, directed against a mouse gene) - or hMLH1-siRNA (seq: GCCAUGUGGCUCAUGUUACTT), (both from Invitrogen GmbH, Darmstadt, Germany) and 3 µl HiPerfect transfection reagent (Qiagen GmbH, Hilden, Germany), was prepared in medium without FCS. After 10 min incubation at RT, transfection solution was added to cells. 24 h after transfection, cells were used for lysate preparation or MTT assay.

7.1.6. Preparation of cell lysate from cell lines

Adherent cells were trypsinized with 0.25 % trypsin (Sigma-Aldrich Chemie, Taufkirchen, Germany) at different time points and pooled with the floating cells. After washing with PBS, cell pellets were resuspended in lysis buffer (0.2% NP-40, 137 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM sodium orthovanadate, 12 mM β-glycerophosphate, 1 mM EDTA, 1.5 mM $MgCl_2$, 50 mM NaF, 10% glycerol, 1 mM PMSF and 10 µg/ml aprotinin). After 20 min of incubation on ice, lysates were centrifuged at 15,000 x g for 20 min at 4^0C. Supernatant was collected and stored at – 70^oC for further use.

7.1.7. Western Blotting

Protein concentration in lysate was determined by Bradford assay (Biorad Laboratories, München, Germany). 20 or 40 µg of protein per lane were separated on SDS polyacrylamide gel and electroblotted onto Immobilon-P membrane (Millipore, Eschborn, Germany). The membranes were blocked with 5% nonfat dry milk in 0.1% Tween 20 in TBS for 1h, and then incubated overnight at 4°C with the corresponding first antibody (Table.1). After thorough washing with PBS, membrane was incubated with appropriate second antibody (8 or 9 in Table 1) for 1 h at RT. After PBS wash, detection was carried out using Super Signal West Pico chemiluminescent substrate

Antigen	MW (kDa)	Antibody type and name	Source	Concentration used (in TBST)
PARP	115 or 85	mouse IgG1	BD Transduction Laboratories, Lexington, KY, USA)	Ascites fluid 1:4000
hMLH1	80 kDa	mouse IgG1	BD pharmingen, Heidelberg, Germany	0.1 µg/ml
Phospho-histone H2AX Ser139	17 kDa	rabbit IgG	Cell Signaling, Frankfurt, Germany	1:1000 + 5% BSA
gamma tubulin	48 kDa	mouse IgG1	Sigma-Aldrich	Ascites fluid 1:10000
p53	53kDa	mouse IgG	Dako, Hamburg, Germany	Ascites fluid 1:3000
BrdU		mouse IgG-FITC	BD Pharmingen	1:3
mouse IgG1		mouse IgG-Biotin	Dianova, Hamburg, Germany	Ascites fluid 1:3000
mouse (IgG+IgM)		goat IgG1-POX	Dianova	Ascites fluid 1:3000
rabbit IgG		goat IgG-POX	Dianova	Ascites fluid 1:1500

Table 1: Antibodies used in the work.

(Pierce, Rockford, IL, USA). The membranes were exposed in Image Reader LAS 4000 instrument. To asses the relative band intensity, the bands were scanned and evaluated by Multi Gauge v3.2 programme (both from Fujifilm Corporation, Tokyo, Japan).

7.2. Cell biology

7.2.1. Cell lines and culture conditions

The cell lines used and their genotype are listed in Table. 2. An isogenic cell pair 5 and 6 was established by M.L.Hanski by stably transfecting HCT116 cells with pcDNA3.1 empty vector or pcDNA3.1-hMLH1 respectively. A semiisogenic cell pair 3 and 4 carries human chromosome 2 or chromosome 3 (harboring a gene coding for hMLH1 protein) respectively.

No	Cell lines	Genotype	Medium	Antibiotic
1	HCT116	$p53^{-/-}$ $hMLH1^{-}$	DMEM + 10 % FCS	G418 (400 µg/ml) Hygromycin (0.1 mg/ml)
2	HCT116	$p53^{+/+}$, $hMLH1^{-}$	DMEM + 10 % FCS	not used
3	HCT116+chr2	$p53^{+/+}$, $hMLH1^{-}$	DMEM + 10 % FCS	G418 (400 µg/ml)
4	HCT116+chr3	$p53^{+/+}$, $hMLH1^{+}$	DMEM + 10 % FCS	G418 (400 µg/ml)
5	Mock	$p53^{+/+}$, $hMLH1^{-}$	DMEM + 10 % FCS	G418 (1mg/ml)
6	Clone 43	$p53^{+/+}$, $hMLH1^{+}$	DMEM + 10 % FCS	G418 (1mg/ml)
7	A2780/CP70	$p53^{+/+}$, $hMLH1^{-}$	RPMI + 10 % FCS	not used
8	A2780	$p53^{+/+}$, $hMLH1^{+}$	RPMI + 10 % FCS	not used

Table 2: Cell lines, their genotype, medium and antibiotics used.

The cells were used within 8-10 weeks from the day of thawing from frozen stock.

All the experiments were carried out in antibiotic-free medium.

7.2.2. Clonogenic survival assay

Cells were sub-cultured one day before the start of experiment. 200-600 cells were seeded as a single cell suspension in 3 ml medium with FCS in 22 cm^2 tissue culture dishes (0.009 to 0.03 x 10^3 cell / cm^2). 24 h after seeding, cells were treated with 5-fluorouracil (5FU) at different concentrations. After 48 h of treatment, cells were washed with PBS and 5FU-free medium was added. After 12 - 14 days, medium was removed and the colonies were washed once with PBS and then fixed in fixing solution (2% formamide, 0.2% glutaraldehyde in PBS) for 10 min at RT. After thorough PBS washing, colonies were stained in 0.2% crystal violet solution in PBS for 20 min. Stained colonies were thoroughly washed with PBS and colonies of at least 50 cells were counted. The experiment was performed at least three times using triplicate cultures for each drug concentration and the mean value ± SD was taken.

7.2.3. Long-term cell survival assay

1x10^6 cells were seeded in medium with FCS in 25 cm^2 flasks (40 x 10^3 cell / cm^2). 24 h after seeding, cells were treated with 30 μM 5-FU. After 48 h treatment, cells were washed with PBS and fresh medium without 5-FU was added. At different time intervals after the start of treatment, adherent cells were trypsinized and pooled together with the floating cell. Cells were stained in 0.4% trypan blue (Sigma-Aldrich Chemie, Taufkirchen, Germany) in PBS and then total white cells were counted using haemocytometer. Every experiment was performed at least three times and the mean value ± SD was taken.

7.2.4. MTT assay

2000 - 4000 cells were seeded in 100 µl medium per well in a flat bottom 96 well plate. At different time points, 25 µl 3-(4, 5-Dimethylthiazol-2yl) - 2, 5 diphenyltetrazoliumbromide solution (MTT, 5mg/ml in PBS, Sigma-Aldrich Chemie, Taufkirchen, Germany) was added per well. After 2 h incubation at 37°C, 100 µl extraction buffer (20% SDS in 50% dimethylformamide in H_2O + 80% acetic acid + 1M HCl, pH 4.7) was added. After proper mixing of cell lysates, the plate was further

incubated for 1-2 h at 37°C followed by measuring the absorbance at 550 nm using an ELISA reader (Dynatech, Guernsey Channel Islands, United Kingdom). Alternatively, total cell number at a particular MTT extinction was empirically determined by trypsinizing and counting total cells in corresponding wells in other 96 well plate kept at same condition.

7.2.5. Determination of doubling time

0.1 x 10^6 or 0.25 x 10^6 cells were seeded in 10 ml medium containing 10% FCS or 3 - 4% FCS respectively in 56 cm^2 dishes. At different time points, adherent cells were trypsynized, stained in 0.4% trypan blue and counted using haemocytometer. Doubling time was calculated from the growth curve of cells growing in exponential phase. The experiment was repeated three times and the mean value ± SD was taken.

7.2.6. Detection of apoptosis

7.2.6.1. DAPI staining

At different time points, treated or non-treated adherent cells were trypsynized and pooled together with floating cells in PBS. 10 000 cells were immobilized on microscope glass slides by cytospin. Slides were dried for 1 h or overnight and then fixed in acetone for 10 min. Slides were stored at –70°C for further use. Cells which were used for immediate staining did not require acetone fixation. Prior to staining, the cells were fixed in ice-cold methanol for 6 min followed by PBS wash and then stained in 0.5 μg/ml 4', 6-Diamidino-2-Phenylindole (DAPI) solution. After 10 min, cells were washed with PBS and then coated with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL, USA), covered with glass coverslips and stored at +4°C for further evaluation. Stained cells were evaluated under fluorescent microscope (Olympus BX60, Japan). % condensed nuclei was determined by counting 300-400 nuclei in each group of cells.

7.2.6.2. PARP cleavage

At different time points after treatment, adherent and floating cells together were

collected and cell lysates were prepared (7.1.6). 20-40 μg total protein was separated in 7.5% polyacrylamide gel followed by western blotting (7.1.7) and scanning. % PARP (Poly ADP (Adenosine Diphosphate)-Ribose Polymerase) cleavage was determined by dividing the intensity of 85 kDa band (cleaved PARP) by the sum of intensity of total PARP (115 kDa + 85 kDa bands).

7.2.7. Detection of DNA-double strand breaks (DSB)

At different time points after treatment, adherent and floating cells were collected and cell lysates were prepared. 20 μg of the protein were separated in 12.5% polyacrylamide gel followed by western blotting. Phospho-histone H2AX (phosphorylated at Ser 139) band of ~17 kDa was detected.

7.2.8. Detection of necrosis

7.2.8.1. LDH assay

CytoTox-One TM Homogenous Membrane Integrity Assay kit (Promega Corporation, Madison, WI, USA) was used to detect lactate dehydrogenase (LDH) released into the medium. At different time points after treatment, 100 µl medium was removed from the flask and mixed with 100 µl substrate mix in a flat bottom black 96 well plate (Greiner Bio-One GmbH, Frickenhausen, Germany). After 10 min of incubation at RT in the dark, 50 µl stop solution was added and Relative Fluorescence (RF) was measured at 560 nm (excitation) and 590 nm (emission) wavelength in GeminiEM spectrofluorometer (Molecular Devices, California, USA). Total RFU were calculated for 5 ml medium in the flask and expressed per 100 cells.

7.2.8.2. Detection of senescence associated β galactosidase expression

Cells were seeded at a desired density in 22 cm^2 dishes. At different time points after 5FU treatment (30 µM, 2 d), cells were washed with PBS and fixed in 2 ml fixing solution (2% formamide, 0.2% glutaraldehyde in PBS) for 5 min at RT. After PBS wash, 2.5 ml freshly prepared staining solution (40 mM citric acid-phosphate buffer, pH-6, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl2, 1 mg/ml 5-Bromo-4-chloro-3-indolyl-β-D- galactopyranoside (X-gal)) was

added to the cells. After overnight incubation at 37°C, cells were washes with PBS and observed under the microscope for the presence of blue cells.

7.2.9. Analysis of cell cycle by FACS

At different time points after treatment, adherent cells were trypsinized and pooled with the floating cells. 1 x 10^6 cells were washed with cold PBS and fixed in 1 ml 70% ice-cold ethanol in PBS. Cells were stored at -20°C for 2 h or until further use. Ethanol fixed cells were washed with PBS and stained in 800 µl propidium iodide (PI) solution (20 µg/ml in 0.1% Triton X-100, 20 µg/ml RNase A in PBS) for 30 min. Stained cells were analysed by using FACSCalibur instrument (BD Biosciences, California, USA), ModFit LT2 software (Verity software House, Topsham, ME, USA) was used to analyse the cell cycle distribution.

7.2.10. Immunofluorescence staining

Cells were seeded at a desired density in 9.6 cm^2 dishes in 2 ml medium with FCS. At different time points after treatment, cells were labelled with 10 µM BrdU (Bromodeoxyuridine) for 30 min at 37°C. Cells were washed with PBS and fixed in 4% formaldehyde solution (in 0.5 % Triton X-100, 1 mM $MgCl_2$ in PBS) for 15 min at RT. After PBS wash, cells were treated with 2 M HCl for 20 min at RT followed by washing with TBS. Cells were permeabilised with 0.5% Triton X-100 for 15 min and then washed with TBS. Cells were blocked in blocking solution (5% donkey serum, 0.1% Triton-X 100 in TBS) for 20 min and then incubated with anti-BrdU-FITC antibody for 1 h. After TBS wash, cells were incubated with anti-mouse IgG-Biotin antibody for 40 min followed by TBS washing. The cells were incubated with Streptavidin-Cy3 solution for 25 min and then washed with TBS. After staining with 1 µg/ml DAPI for 10 min followed by TBS washing, cells were covered with coverslips by using Fluoromount G and evaluated under fluorescent microscope. % of cells incorporating BrdU was determined by dividing total anti-BrdU stained nuclei by total nuclei. 300-400 nuclei in each group of cells were counted. The experiment was repeated two times.

7.2.11. FACS analysis of BrdU incorporation

1 x 10^6 cells were seeded in 56 cm^2 dishes in 10 ml medium with FCS. 20-24 h after seeding, cells were labelled with 10 µM BrdU for 10 min at 37°C. After washing with PBS, fresh medium was added into the dishes and cells were incubated at 37°C. At different time points, cells were trypsinized, fixed in 70% ethanol and stored at -20°C as for FACS analysis (6.2.7). After washing with PBS and PBS + 0.5% BSA, cell pellet was resuspended in 100 µl 2M HCl and incubated for 20 min at RT. Cells were washed with PBS + 0.5% BSA, cell pellet was resuspended in 500 µl 0.1M sodium tetraborate ($Na_2B_4O_7$, pH 8.5) and incubated at RT for 2 min. After washing with PBS + 0.5% BSA, cell pellet was resuspended in 60 µl anti-BrdU-FITC antibody solution (in 0.25% Triton-X 100, 0.5% BSA in PBS) and incubated for 1 h at RT. After thorough washing with PBS + 0.5% BSA, the cells were stained in 800 µl PI solution. Stained cells were analysed by using FACSCalibur instrument.

7.3. Mice experiment

Female athymic Balb/c mice were purchased from Charles River, Sulzfeld, Germany. Human colon carcinoma cells growing in exponential phase were trypsinized and resuspended in PBS. To generate HCT116 or HCT116+chr2 or HCT116+chr3 xenograft, 5 x 10^6 cells suspended in 200 µl PBS were injected subcutaneously into the flank of mice. When the tumour volume reached 50 -100 mm^3, mice were injected intraperitoneally with 5-FU (50 mg/kg) dissolved in PBS or PBS alone. 5-FU was injected once weekly for three consecutive weeks. The tumour volume was determined by measuring 3 orthogonal diameters (D) and calculating the volume was estimated as D1xD2xD3 / 2. Relative tumour volume at different time points was determined as a ratio of tumour volume at the time point to the tumour volume at the start of treatment. Mean relative tumour volume ± SD was plotted vs. time from the start of treatment.

8. Results

8.1. Effect of MMR status on cell proliferation

The data on the effect of MMR system on proliferation are contradictory: certain authors claim that MMR decreases proliferation (57-58), others found the opposite i.e. MMR increases (59) or does not affect (59, 60) proliferation. I investigated the effect of MMR on cell proliferation in the present cell system.

8.1.1. MMR-defect does not affect cell proliferation

Clone 43 stably expressing hMLH1 protein was transiently transfected with hMLH1-siRNA or, as a control with P1-siRNA (within mouse gene). 24 h later, the transfectants were reseeded in 96 well plates. Cell proliferation was determined by MTT assay after 3 days.

hMLH1-suppressed and non-suppressed cells showed no difference in cell number (Fig.7).

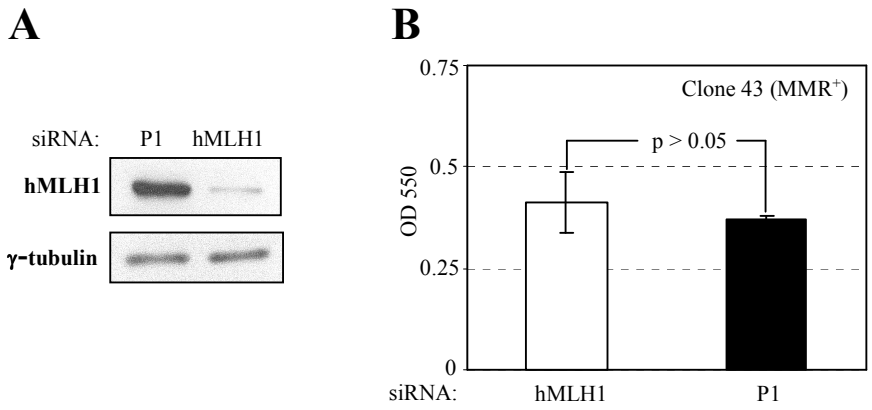

Fig. 7. hMLH1-suppression does not affect cell proliferation. Clone 43 was transiently transfected with hMLH1-siRNA or P1-siRNA. **A.:** hMLH1 protein is suppressed after 24 h of transfection; **B.:** hMLH1-suppressed and non-suppressed clone 43 shows the same extinction in MTT assay. Mean values ± SD of three experiments.

The result indicates that the depletion of hMLH1 does not affect cell proliferation.

8.1.2. MMR-defect does not affect DNA synthesis

I examined further the effect of MMR status on DNA synthesis by measuring the incorporation of bromodeoxyuridine (BrdU) or ^{3}H-thymidine in MMR-proficient or -deficient cells. HCT116 and clone 43 cells were incubated with BrdU for 30 min. and percentage BrdU incorporating cell was determined by FACS.

Alternatively, HCT116+chr2 and HCT116+chr3 cells were incubated with ^{3}H-thymidine. DNA was extracted and the DNA-bound radioactivity was determined by scintillation counting.

HCT116 and clone 43 showed no difference in BrdU incorporation. Also, the amount of incorporated ^{3}H-thymidine was similar in HCT116+chr2 and HCT116+chr3 cells (Fig. 8).

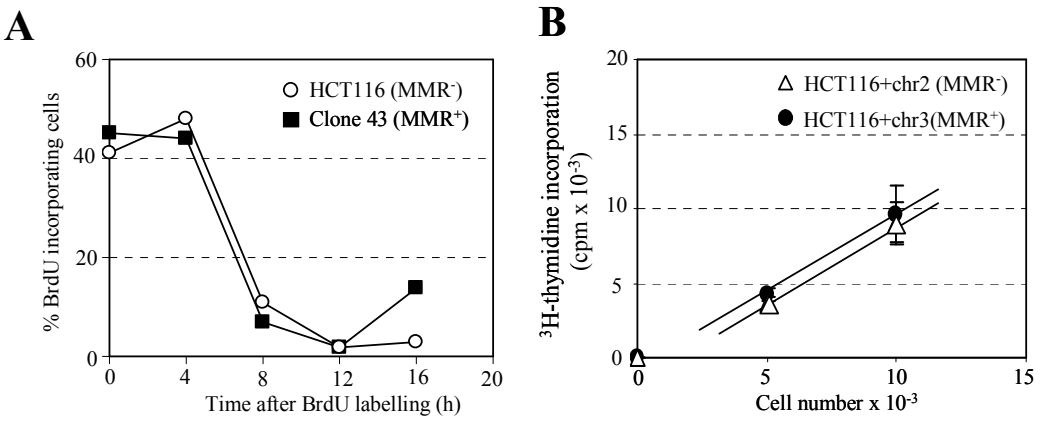

Fig. 8. MMR status does not affect BrdU or ^{3}H-thymidine incorporation into DNA. A.: HCT116 cells and clone 43 show the same incorporation and time course of removal of BrdU; **B.:** HCT116+chr2 and HCT116+chr3 cells show the same incorporation of ^{3}H-thymidine into DNA. A.: Representative for two experiments; B.: Mean values ± SD of three experiments. FACS measurement done by M. L. Hanski.

Both results demonstrate that MMR-proficient and -deficient cells synthesize DNA at the same rate.

8.1.3. MMR-defect does not affect the doubling time

I further investigated the effect of MMR status on growth speed by determining the

doubling time of each cell line (Table 3).

Cell line	Doubling time (h)
Mock 1-transfectants	17.79 ± 0.8
Mock 1-transfectants – 3% FCS	23.94 ± 1.3
HCT116	17.5 ± 1.08
HCT116p53 $^{-/-}$	19.24 ± 1.2
HCT116Bax$^{-/-}$	18.8 ± 0.86
HCT116+chr2	23.4 ± 0.95
Clone 43	18.4 ± 0.2
Clone 43 – 3% FCS	25.1 ± 1.1
HCT116+chr3	24.8 ± 1.18

Table 3: Doubling time of cell lines. Mean values ± SD of three experiments

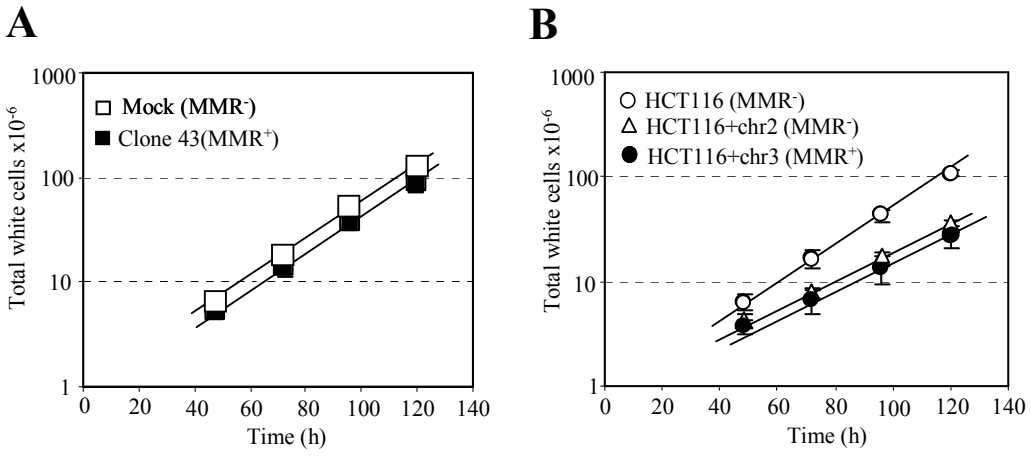

Fig. 9. MMR status does not affect the growth speed. A.: Mock 1-transfectants and clone 43 grow at the same speed; **B.:** HCT116, HCT116+chr2 and HCT116+chr3 cells grow at the same speed while HCT116 grows faster. Mean values ± SD of three experiments.

Mock 1-transfectants and clone 43 showed similar doubling times of 17.8 h and 18.4 h, respectively. Similarly, HCT116+chr2 cells and HCT116+chr3 cells showed similar doubling times of 23.4 h and 24.8 h, respectively (Table 3, Fig. 9). The results of points 8.1.1 - 8.1.3 clearly indicate that in the present system the MMR status does not affect the doubling time.

8.2. Effect of MMR status on colon carcinoma cell survival after 5FU treatment

8.2.1. MMR-deficiency is associated with a better clonogenic survival

Several authors using a semiisogenic cell system (HCT116, HCT116+chr2 and HCT116+chr3) showed that MMR-deficient cells show a better clonogenic survival than -proficient cells (71-73). In the present work the isogenic cell pair was investigated and the semiisogenic cell lines were used as a control. Cell lines were treated with 5FU and clonogenic survival was determined. Mock 1-transfectants showed a better clonogenic survival than clone 43 (Fig. 10A). As expected, HCT116 cells or HCT116+chr2 cells showed higher percentage of surviving colonies relative to non-treated than HCT116+chr3 cells (Fig. 10B). Also the ovarian cancer cell line pair A2780/CP70 (MMR^-) and A2780 (MMR^+), showed a better clonogenic survival of the MMR-deficient cells (Fig. 10C).

These results confirmed in the isogenic cell pair, in agreement with previous reports, that the clonogenic survival following 5FU treatment is higher in MMR-deficient cells than MMR-proficient cells.

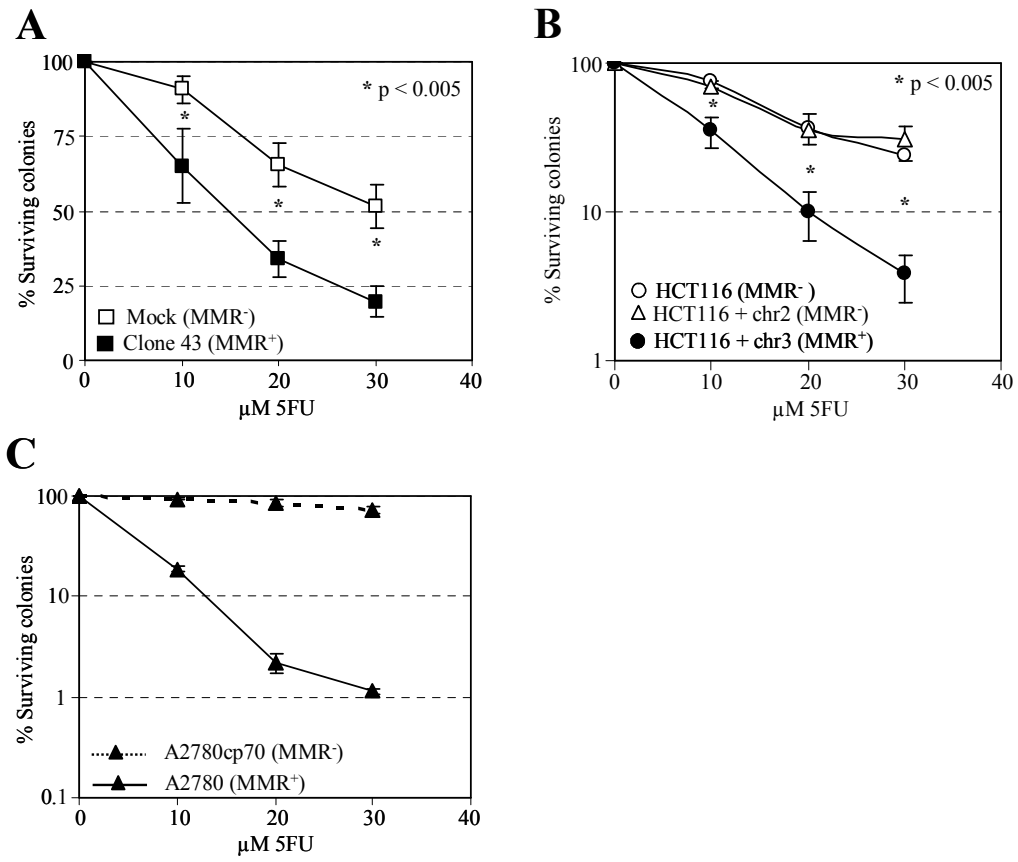

Fig. 10. MMR-deficient cells show a better clonogenic survival than -proficient cells after 5FU treatment (2 d). **A.:** Mock 1-transfectants vs. clone 43; **B.:** HCT116 or HCT116+chr2 vs. HCT116+chr3; **C.:** A2780/CP70 vs. A2780 cells. A.: Mean values ± SD of three experiments; B.: Mean values ± SD of five experiments; C: Results of two experiments, deviation showed by bars.

8.2.2. MMR-deficiency is associated with less DNA-double strand breaks and less short-term apoptosis

The time course of the cellular response underlying the different clonogenic survival of MMR-deficient and -proficient cells was further investigated. The effect of MMR on DNA-double strand breaks (DSB) and apoptosis was checked in the isogenic and semiisogenic cell lines after 5FU treatment. For the time course of apoptosis in mock 1-transfectants and clone 43, cell lysates on day 1, 2, 3, 4 and 5 after start of treatment were checked in western blotting.

There was more PARP cleavage (Fig. 11B) and condensed nuclei (Fig. 11C) in clone 43 than in mock 1-transfectants after 2 days from the start of treatment. MMR dependent PARP cleavage showed a maximum at day 2 and then decreased to a hardly detectable level at day 5 (Fig. 11A). There was more histone H2AX phosphorylation in clone 43 than mock 1-transfectants. Similarly, HCT116+chr3 cells showed more PARP cleavage or degradation and more histone H2AX phosphorylation than HCT116 or HCT116+chr2 cells (Fig. 11B). The condensed nuclei in HCT16+chr3 cells were more than HCT116+chr2 cells (Fig. 11D). However, the condensed nuclei in HCT116+chr3 and HCT116 cells were similar.

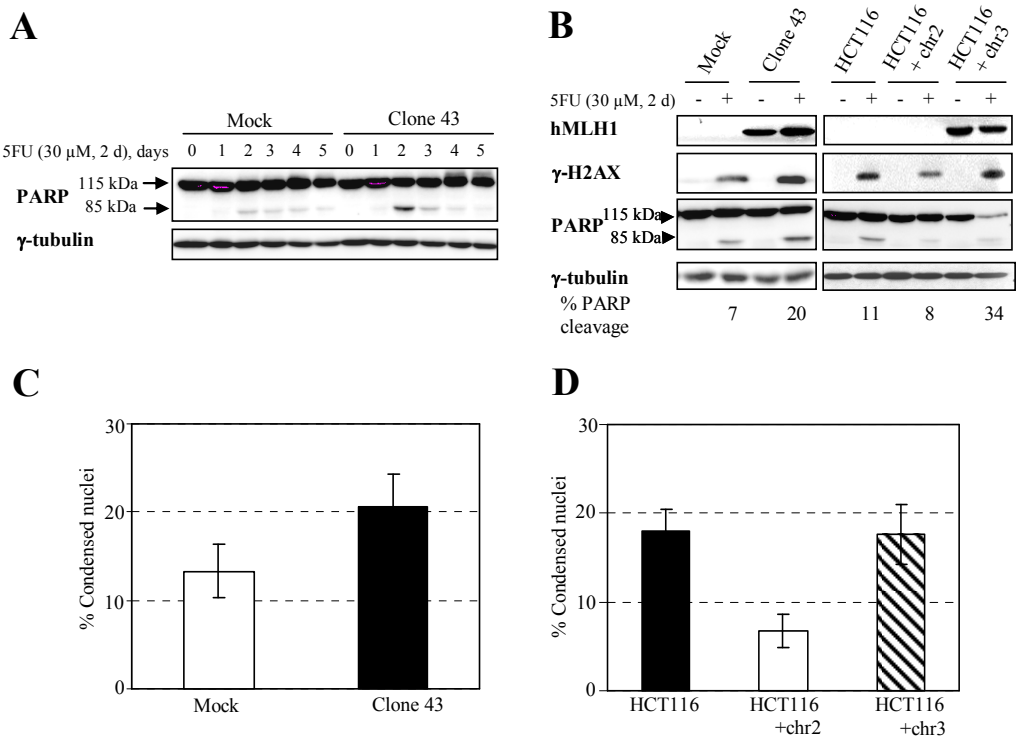

Fig. 11. MMR-deficient cells show less histone H2AX phosphorylation, PARP cleavage and less condensed nuclei than -proficient cells after 5FU treatment (30 µM, 2 d). **A.:** Clone 43 shows more PARP cleavage than mock 1-transfectants after 2 days treatment. PARP cleavage in clone 43 decreased to a very low level at day 5; **B.:** Clone 43 shows more PARP cleavage and histone H2AX phosphorylation than mock 1-transfectants. HCT116+chr3 cells show more PARP cleavage and histone H2AX phosphorylation than HCT116 cells or HCT116+chr2 cells; **C.:** Clone 43 shows a higher percentage of condensed nuclei than mock 1-transfectants; **D.:** HCT116+chr3 cells show

higher percentage of condensed nuclei than HCT116+chr2. HCT116+chr3 and HCT116 cells show similar percentage of condensed nuclei. A, B: Representative for three experiments; C, D: Mean values ± SD of four experiments.

These results clearly indicated that DNA-double strand breaks (DSB) and apoptosis were mediated by MMR in response to 5FU treatment but the apoptosis process was of short duration and rapidly decreased after removal of the drug.

8.2.3. Fast growth increases short-term apoptosis

The HCT116 cells which are fast-growing (Fig. 9B) showed more DNA-double strand breaks and more apoptosis (Fig. 11B, D) than HCT116+chr2 cells which are slow-growing. To further test if the growth speed has an effect on apoptosis, growth speed of mock 1-transfectants or of clone 43 was slowed down by reducing FCS concentration to 3% in the medium. After one week of adaptation, the doubling time was determined.

Mock 1-transfectants or clone 43 growing in medium with 3% FCS showed a doubling time of 24.3 h or 25.3 h respectively, while the same cells growing in medium with 10% FCS showed doubling time of 16.5 h and 17.3 h respectively (Table 3, Fig. 12A, B).

Fast- and slow-growing cells were treated with 30 µM 5FU for 2 days. Cell lysates were analysed in western blotting.

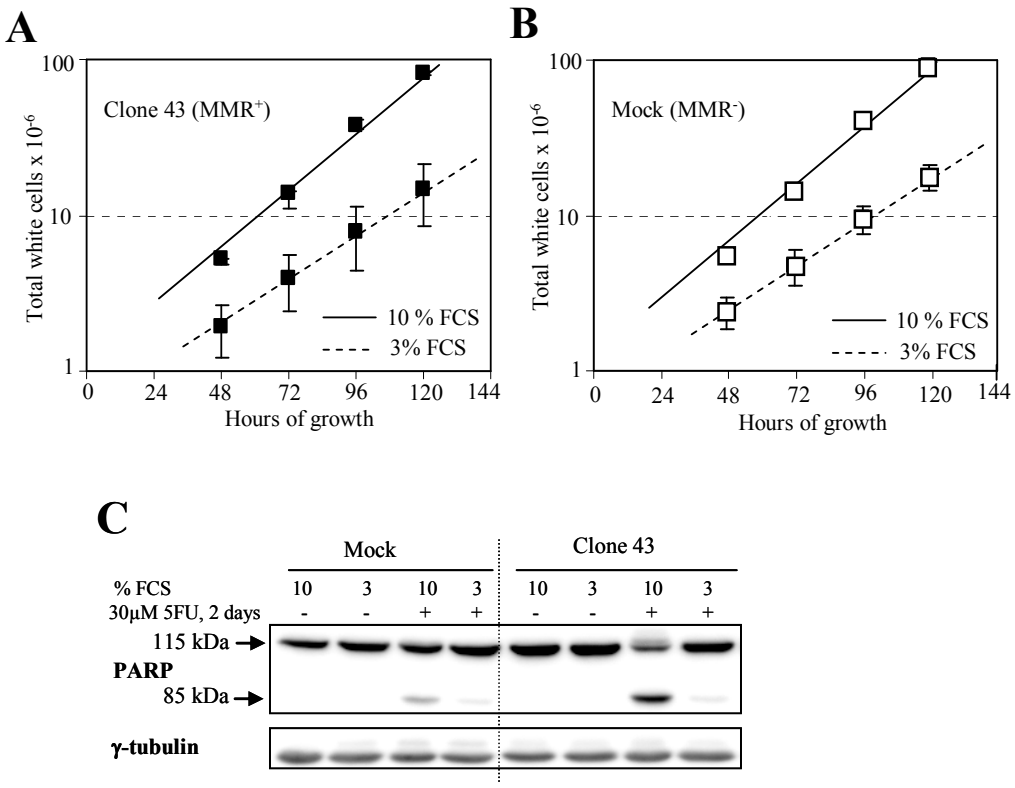

Fig. 12. Fast-growing cells show more PARP cleavage than slow-growing cells after 2 days of 5FU treatment (30 µM). **A.:** Growth curve of clone 43 growing in medium containing 10% or 3% FCS; **B.:** Growth curve of mock 1-transfectants growing in medium containing 10% or 3% FCS; **C.:** Fast-growing mock 1-transfectants or clone 43 shows more PARP cleavage than slow-growing cells after treatment. A, B: Mean values ± SD of three experiments; C: Representative for three experiments.

More PARP cleavage was found in fast-growing mock 1-transfectants or clone 43 than in the same cells growing at a lower rate (Fig. 12C).

The result confirms the observation made in HCT116 / HCT116+chr2 cell line pair that the fast-growing cells make more apoptosis in response to 5FU treatment than the slow-growing ones.

8.2.4. DNA-double strand breaks, apoptosis and clonogenic survival are dependent on MMR status

The clonogenic survival assay result indicated that MMR proficient cells exhibit more DNA-double strand breaks, more apoptosis and less clonogenic survival after

treatment with 5FU than MMR-deficient ones. By restoring hMLH1 expression in HCT116 cells, I examined if there is a causal relationship between (a) MMR status and DNA-double strand breaks, (b) MMR status and apoptosis and (c) MMR status and clonogenic survival.

HCT116 cells were transduced with adenovirus expressing hMLH1 or LacZ gene (negative control) at MOI of 10 virus particles per cell. Two days after transduction, cells were re-seeded and the clonogenic survival after 2 days of 5-FU treatment was tested. Also the effect of MMR restoration on DSB and apoptosis was examined after 5FU treatment. The restoration of MMR led to more histone H2AX phosphorylation, more PARP cleavage (Fig. 13A) and less clonogenic survival after treatment (Fig. 13B).

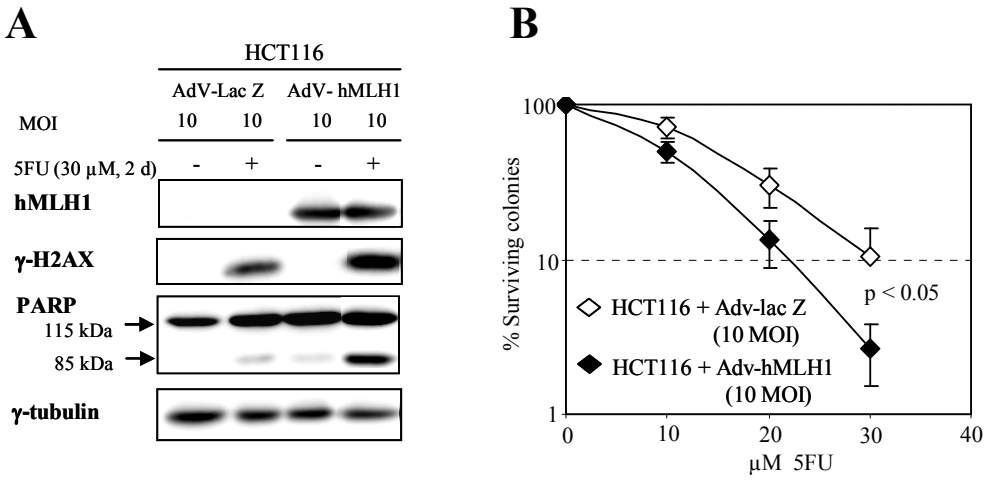

Fig. 13. MMR restoration causes more histone H2AX phosphorylation, more PARP cleavage and less clonogenic survival after 5FU (2 d) treatment. HCT116 cells were transduced with adenovirus expressing hMLH1 or LacZ protein. **A.:** HCT116-Adv-hMLH1 shows more histone H2AX phosphorylation and more PARP cleavage than HCT116-Adv-LacZ after 30 µM 5FU treatment; **B.:** HCT116-Adv-hMLH1 shows less clonogenic survival than HCT116-Adv-LacZ after 5FU treatment. A: Representative for three experiments; B: Mean values ± SD of four experiments.

These experiments demonstrated that DSB, apoptosis and clonogenic survival are dependent on MMR status.

8.2.5. Apoptosis and clonogenic survival are dependent on p53

Tumour suppressor protein p53, apart from its other functions, induces cell cycle arrest and apoptosis in response to genotoxic stress. Since the cell lines used in the present study contain wild type p53 protein and show apoptosis after 5FU treatment, I examined the link between (a) p53 status and apoptosis (b) p53 status and clonogenic survival. To test the effect of p53 status on apoptosis, p53 gene was silenced by transient transfection of mock-transfectants or clone 43 with pSuper-p53 plasmid expressing p53 shRNA (7.1.5.2). 24 h after transfection, the transfectants were treated with 5FU. Additionally, an isogenic cell pair HCT116 and HCT116 $p53^{-/-}$ was used. After treatment with 5FU (30 µM, 2 d), whole cell lysate was prepared and checked for PARP cleavage by western blotting. To test the effect of p53 status on clonogenic survival, HCT116 and HCT116$p53^{-/-}$ cells were treated with 10 - 30 µM 5FU for 2 days and clonogenic survival was determined.

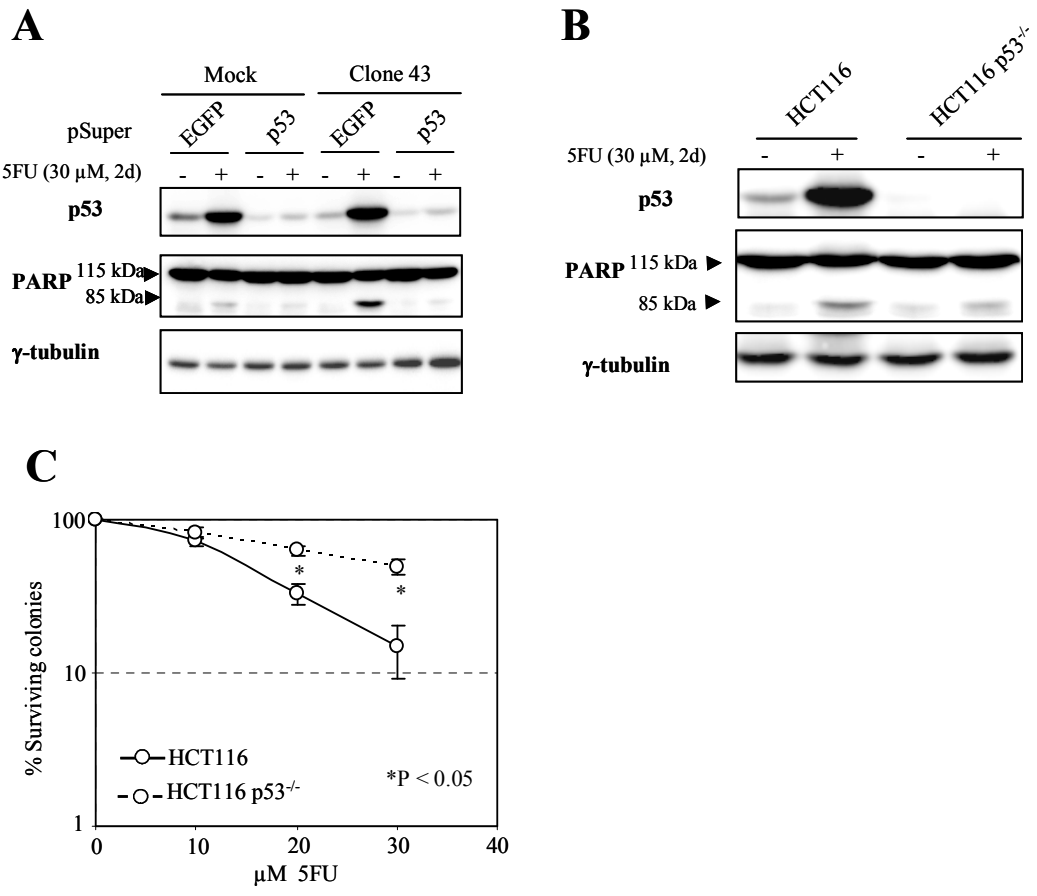

Fig. 14. $p53^{-/-}$ cells show less PARP cleavage and a better clonogenic survival than $p53^{wt}$ cells after 5FU treatment (30 µM, 2 d). A.: p53-suppressed mock 1-transfectants or clone 43 shows less PARP cleavage than the non-suppressed; **B.:** HCT116$p53^{-/-}$ cells show less PARP cleavage than HCT116 cells; **C.:** HCT116$p53^{-/-}$ cells show a better clonogenic survival than HCT116 cells. A, B: Representative for two to three experiments; C: Mean values ± SD of three experiments.

p53 suppression in mock 1-transfectants and in clone 43 led to a decrease of PARP cleavage after treatment in comparison to non-suppressed cells (Fig. 14A). Similarly, HCT116$p53^{-/-}$ cells showed a very low PARP cleavage in comparison to $p53^{wt}$ cells (Fig. 14B). $p53^{-/-}$ cells showed a better clonogenic survival than $p53^{wt}$ cells (Fig. 14C). The results obtained here confirm the previous reports that the apoptosis (81-83) and clonogenic survival (83) are dependent on p53 status

8.2.6. Clonogenic survival is not dependent on caspase or Bax

The better clonogenic survival of MMR-deficient cells is associated with less apoptosis after treatment (8.2.1.2, 8.2.1.4 and 8.2.1.5). I tested if there is a causal relationship between apoptosis and clonogenic survival by using broad spectrum caspase inhibitor Q-VD-OPh (here abbreviated as CI) or $Bax^{-/-}$ cells.

8.2.6.1. Optimisation of CI concentration

Optimal concentration of caspase inhibitor was determined by pretreating HCT116 cells with CI at a concentration of 5 - 40 µM for 1 h, followed by 5FU treatment (30 µM). After 2 days, lysates were analysed in western blotting.

I found that 5 µM CI completely inhibited PARP cleavage in HCT116 (Fig. 15A).

8.2.6.2. Caspase inhibition does not increase clonogenic survival

To test the effect of apoptosis on clonogenic survival, mock 1-transfectants or HCT116 or HCT116+chr2 (all MMR^-) or clone 43 or HCT116+chr3 (both MMR^+) cells were pretreated with 5 µM CI for 1 h and then 5FU (30 µM) was added. CI was maintained in the medium for 2 weeks until the end of the experiment.

Mock 1-transfectants or clone 43 (Fig. 15B) or HCT116 or HCT116+chr2 or HCT116+chr3 cells (Fig. 15C) in presence of CI showed the same percentage of surviving colonies as cells in the absence of CI.

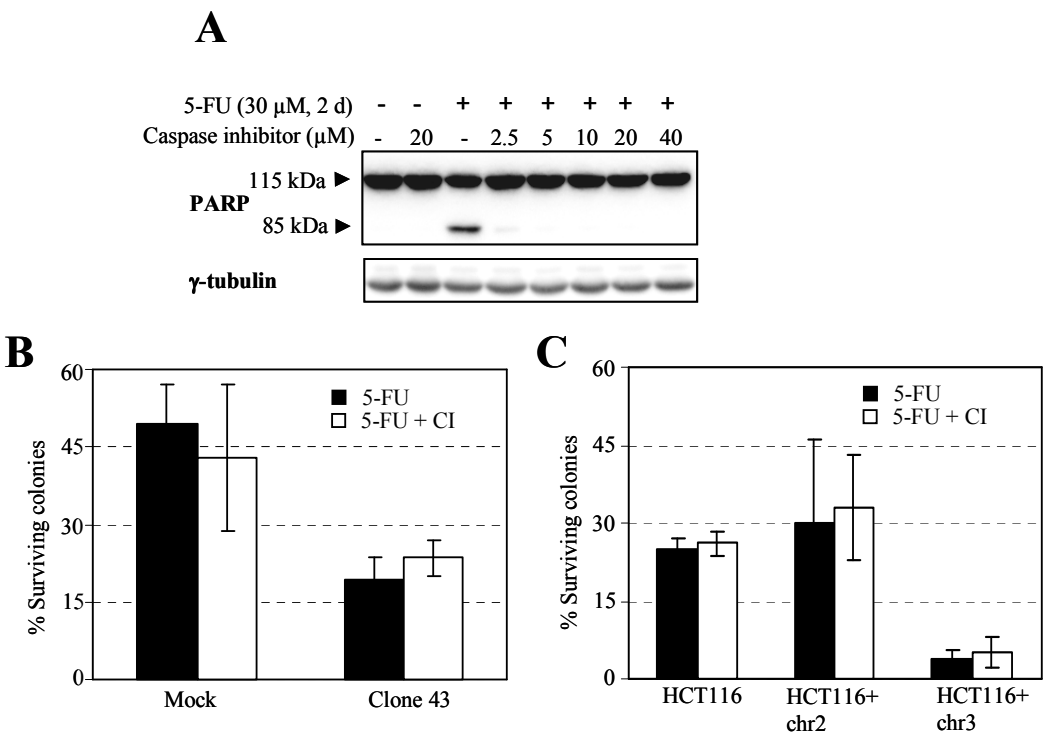

Fig. 15. Caspase inhibition does not affect the clonogenic survival after 5-FU treatment (30 µM 5FU, 2 d). A.: Treatment with CI at a concentration of 5µM completely inhibits PARP cleavage in HCT116 cells; **B.:** Clonogenic survival is similar in mock 1- transfectants or clone 43 in the presence and absence of CI; **C.:** Clonogenic survival is similar in HCT116 or HCT116+chr2 or HCT116+chr3 cells in the presence or absence of CI. A.: Representative for two experiments; B.: Results of two experiments, deviation showed by bars; C.: Mean values ± SD of three experiments.

8.2.6.3. Bax elimination does not increase clonogenic survival

To additionally test the effect of apoptosis on clonogenic survival, HCT116 and HCT116Bax$^{-/-}$ cells were treated with 5FU and the clonogenic survival was determined.

Expectedly, HCT116Bax$^{-/-}$ cells showed less PARP cleavage than HCT116 (Fig. 16A). Interestingly, HCT116Bax$^{-/-}$ cells did not show an increase in the clonogenic survival

in comparison to HCT116 cells, indeed the survival decreased from 25% to 17% (Fig. 16B).

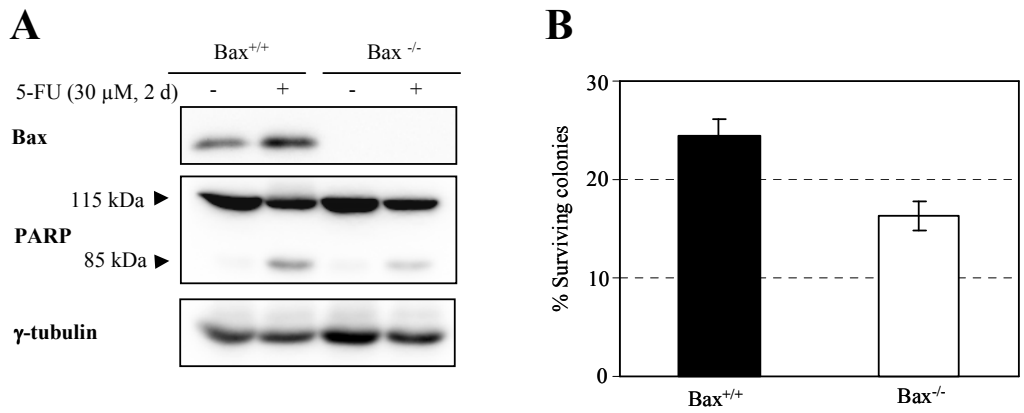

Fig. 16. Elimination of Bax does not increase clonogenic survival after 5-FU treatment (30 µM, 2 d). A.: HCT116Bax$^{-/-}$ cells show less PARP cleavage than HCT116 cells; **B.:** HCT116Bax$^{-/-}$ cells do not show increase in clonogenic survival in comparison to HCT116 cells. **A:** Representative for three experiments; **B:** Mean values ± SD of five experiments.

It is concluded that the clonogenic survival after 5-FU treatment is neither dependent on caspase nor Bax i.e. it is not dependent on apoptosis.

8.2.7. Cells growing at a low density show a very low level of apoptosis in response to 5FU

The present data indicated that the reduction or inhibition of apoptosis did not affect the clonogenic survival of MMR-proficient or -deficient cells (8.2.6.2 and 8.2.6.3). However, we noticed in these experiments that the cells which were used to check apoptosis were grown at a much higher density (30 - 40 x 10^3 cells/cm^2) than the cells grown (0.009 - 0.03 x 10^3 cells/cm^2) in clonogenic assay. To check if the cell density affects apoptosis, HCT116, HCT116+chr2 and HCT116+chr3 cells were seeded at 0.5 x 10^3 cells/cm^2 (low density) or 30 x 10^3 cells/cm^2 (high density). After treatment with 5FU (30 µM, 2 d), apoptosis was determined by the analysis of condensed nuclei.

The result indicated that the cells seeded at a low density showed after 5FU treatment a lower percentage of condensed nuclei than the cells seeded at a high density (Fig. 17).

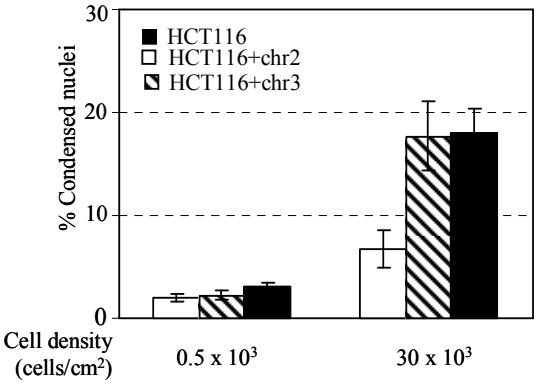

Fig. 17. Cells seeded at a low density show a lower percentage of condensed nuclei after 5-FU treatment (30 µM, 2 d) than cells seeded at a high density. HCT116 or HCT116+chr2 or HCT116+chr3 cells seeded at a low density show a very low percentage of condensed nuclei in comparison to cells at a high density. Mean values ± SD of three experiments.

It is concluded that the cells growing at a low density show lower apoptosis than cells at a high density following 5FU treatment. The result supports the previous finding (8.2.6.2 and 8.2.6.3) that the difference in clonogenic survival after 5FU treatment is independent from apoptosis.

8.2.8. Cells seeded at a low density grow slower after 5FU treatment than the cells seeded at a high density

Since the previous result (8.2.7) indicated that the cell density affects apoptosis, the question was asked if the cell density affects proliferation after 5FU treatment as well. In order to answer this question, HCT116, HCT116+chr2 and HCT116+chr3 cells were seeded at 0.5 or 30 x 10^3 cells/cm^2 and treated with 5FU (30 µM, 2 d). Cells were incubated with 10 µM BrdU for 1 h at the end of 5FU treatment. BrdU incorporating cells were detected by immunohistochemistry (7.2.10).

After 5FU treatment, HCT116 or HCT16+chr2 or HCT116+chr3 seeded at low density showed approx. four times less BrdU-incorporation than cells grown at a high density (Fig. 18B).

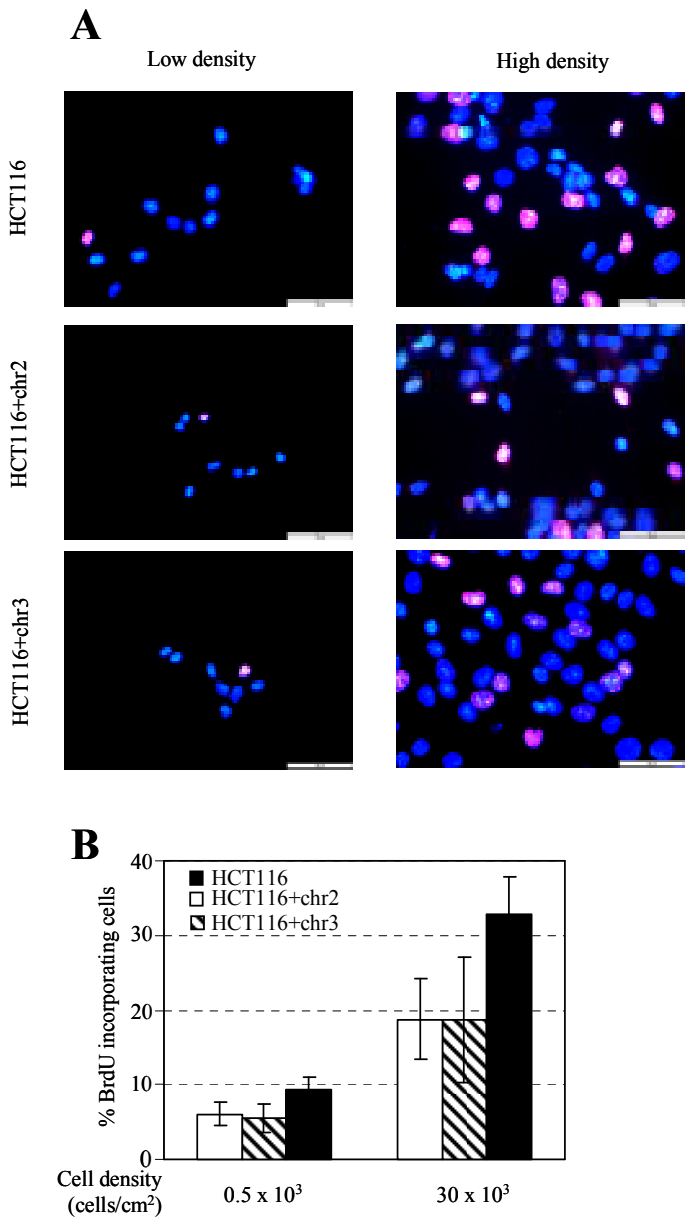

Fig. 18. Cells seeded at a low density show less BrdU incorporation after 5-FU treatment (30 µM, 2 d) than cells seeded at a high density. A.: The nuclei of cells seeded at a low and at a high density detected with DAPI (blue) and with anti-BrdU-antibody (pink); **B.:** Quantitative estimation of the percentage of BrdU incorporating cells. A.: Bar: 50 µm; B.: Mean values ± SD of three experiments.

It is concluded that in response to 5FU, cells growing at a low density proliferate less than the cells growing at a high density i.e. cells at a low and at a high density react differently to 5FU treatment.

8.2.9. Lower clonogenic survival of MMR-proficient cells is associated with the presence of more tiny colonies

The present data indicated that apoptosis does not cause clonogenic survival (8.2.6.2 and 8.2.6.3) after 5FU treatment. It was therefore hypothesised that cell arrest determines clonogenic survival and a fraction of seeded cells remains arrested after the treatment as a single cells or tiny colonies. To test this hypothesis, I examined the frequency of normal, large (> 50 cells) and tiny colonies (1-10 cells) in MMR-proficient and -deficient cells.

I counted the total number of large and tiny colonies of mock 1-transfectants and clone 43 or HCT116, HCT116+chr2 and HCT116+chr3 cells after treatment.

HCT116+chr3 cells showed significantly more tiny colonies than HCT116 or HCT116+chr2 cells (Fig. 19, Table 4). The latter two showed similar number of tiny colonies. The sum of large and tiny colonies in all the three cell lines was similar. Clone 43 showed also more tiny colonies than mock 1-transfectants and the sum of large and tiny colonies between them was similar (Table 4).

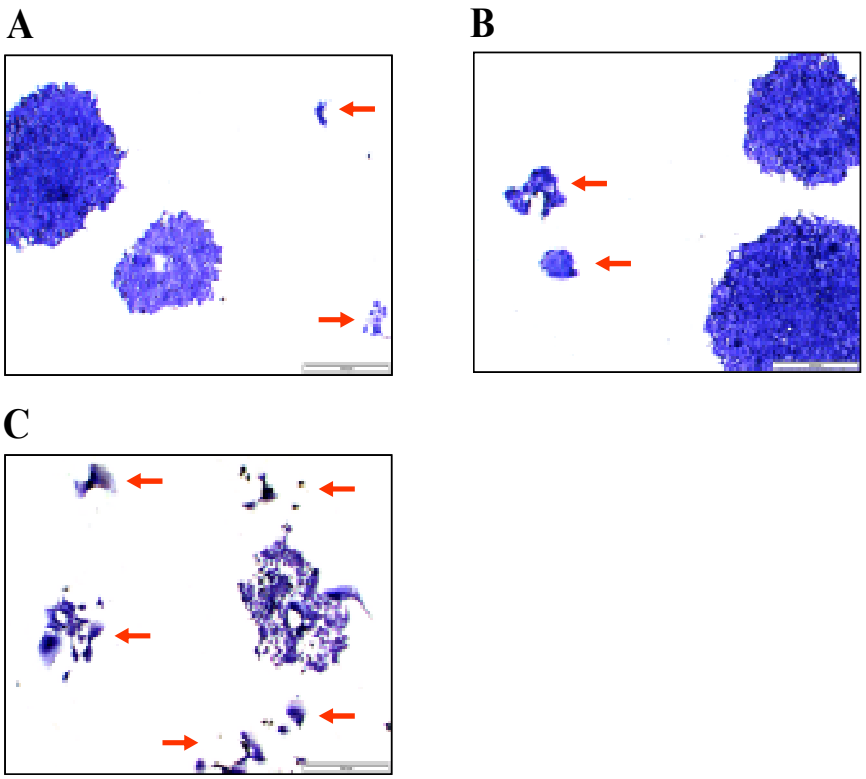

Fig. 19. MMR-proficient cells show more tiny colonies than -deficient cells in clonogenic assay after 5FU treatment (30 µM, 2 d). A.: HCT116 cells show similar frequency of large and tiny colonies; **B.:** HCT116+chr2 cells show similar frequency of large and tiny colonies; **C.:** HCT116+chr3 cells show more frequently tiny colonies than large colonies. Photographs representative for experiments evaluated in table 2. Bar: 500 µm.

Cells	Large colonies	Tiny colonies	Total colonies	***Surviving colonies related to nontreated
Mock 1*	85 ± 6.3 (77%)	24 ± 2.8 (33%)	109 ± 3.5 (100%)	49.6%
HCT116**	45.8 ± 15.4 (52%)	43.4 ± 20.3 (48%)	89.2 ± 28.1 (100%)	25%
HCT116+ chr2**	56.3 ± 19.7 (50%)	55.3 ± 10.1 (50%)	111.4 ± 24.6 (100%)	31%
Clone 43*	33 ± 4.2 (36%)	59 ± 6.4 (64%)	92 ± 10,6 (100%)	19.4%
HCT116+ chr3**	7 ± 2 (6%)	106 ± 21.3 (94%)	113 ± 23.3 (100%)	3.8%

Table 4: Distribution between large and tiny colonies in clonogenic assay after 5FU treatment (30 µM, 2 d). * Representative for two experiments, deviation showed by bars. **Mean values ± SD of three experiments; *** Values are taken from Fig. 10A, B.

From these data, it is concluded that clonogenic survival is associated with the long-term cell arrest.

8.2.10. Single cells or tiny colonies in clonogenic assay are associated with senescence

Since the previous results indicated that the clonogenic survival is determined by long-term arrest, it was checked if arrested single cells or the cells present in the tiny colonies are senescent. The colonies of HCT116 or HCT116+chr2 or HCT116+chr3

were incubated with X-gal solution to check the presence of β-galactosidase (β-gal) activity, used as a marker of

senescence.

Interestingly, single cell or cells in tiny colonies expressed β-gal. The β-gal expressing cells were flattened and vacuolated.

HCT116+chr3 showed more senescent cells than HCT116+chr2 or HCT116 cells (Fig. 20).

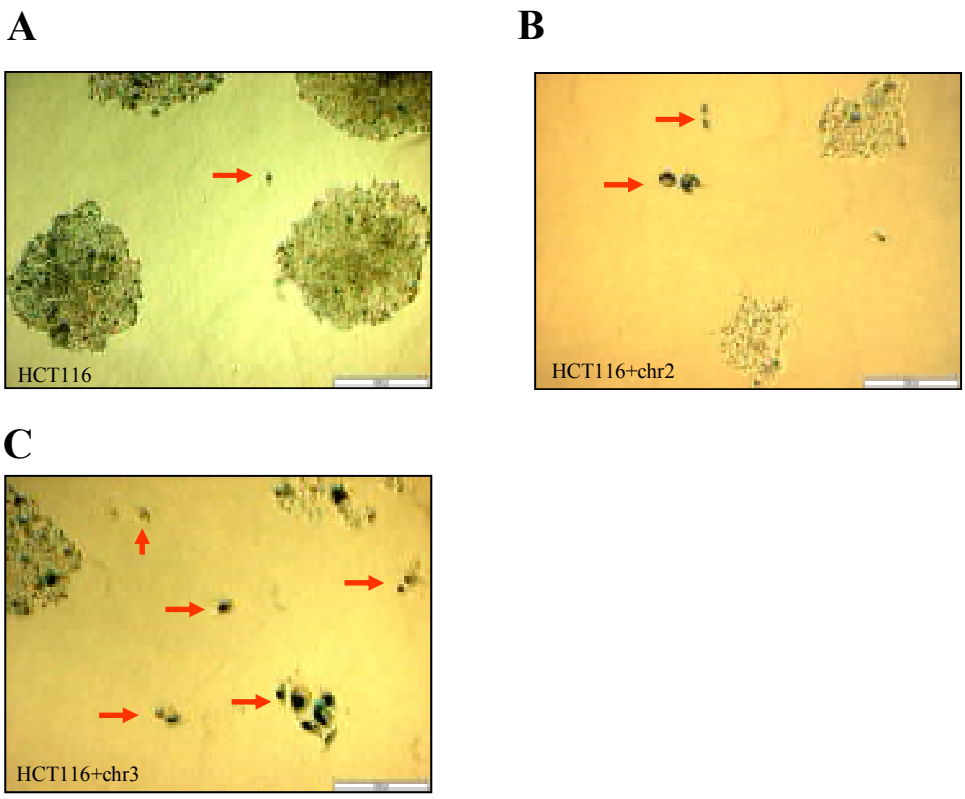

Fig. 20. Cells in large colonies are white, single cells or cells in tiny colonies express β-gal after 5FU treatment (30 μM, 2 d). Arrow: blue cells. Representative for two experiments. Bar: 500 μm.

It is concluded that the decrease of clonogenic survival in MMR-proficient cells after 5FU treatment is caused by long-term arrest, associated with senescence.

8.2.11. Effect of MMR status and of the growth speed on the long-term response of colon carcinoma cells to 5-FU treatment

Since the long-term response of cells grown at a high density may give more information about the *in vivo* response than the cells grown at a low density i.e. grown in clonogenic assay, I investigated the effect of MMR on the long-term cell survival (7.2.3) after 5FU treatment.

8.2.12. Long-term cell response is affected by 5-FU concentration

I examined the effect of 5FU on cell proliferation and long-term cell survival. HCT116, HCT116+chr2 and HCT116+chr3 cells were treated with 2.5, 10 and 30 μM 5FU for 2 days and live cells were counted at different time points.

After treatment with 2.5 μM 5FU, the fast-growing HCT116 cells continued to grow faster than the slow-growing HCT116+chr2 or HCT116+chr3 cells (Fig. 21A). At a concentration of 10 or 30 μM 5FU, HCT116 cells were affected more and showed a slower proliferation and less live cells than HCT116+chr2 and HCT116+chr3 cells (Fig. 21B, C).

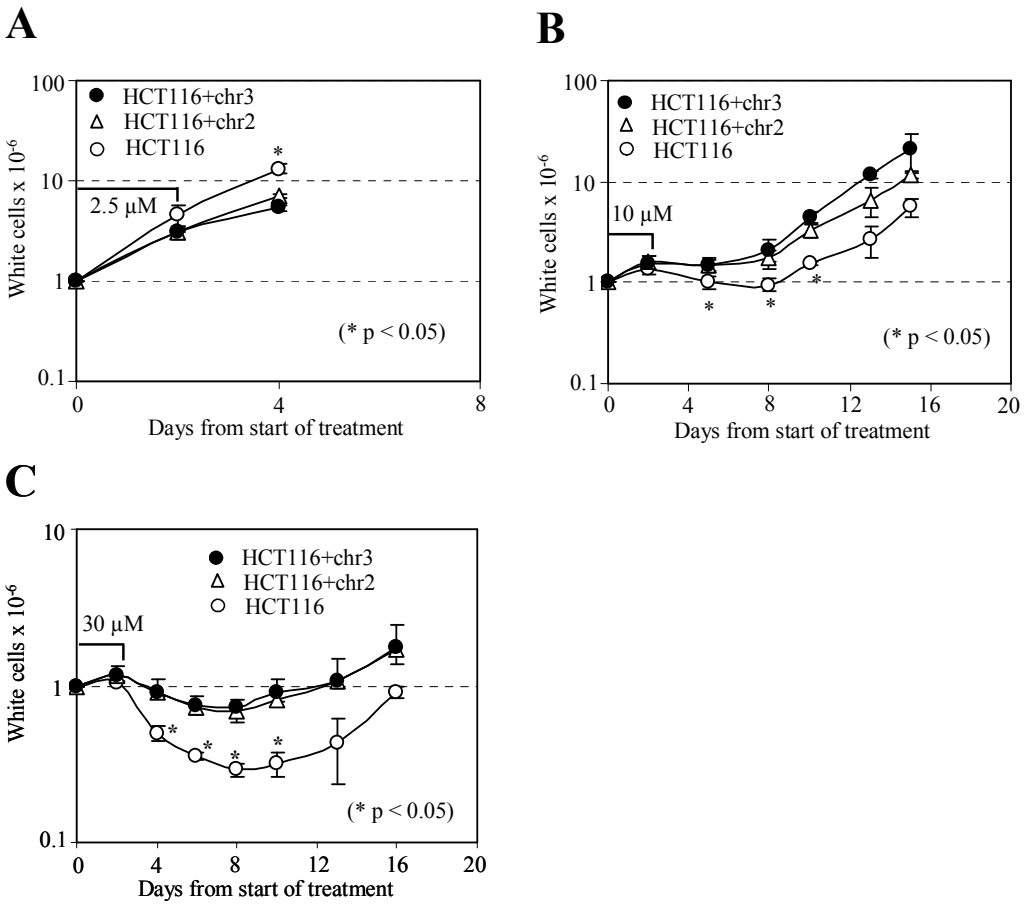

Fig. 21. Long-term response of slow- and fast-growing cells depends on 5-FU concentration. A.: HCT116 cells proliferate faster than HCT116+chr2 and HCT116+chr3 cells at concentration of 2.5 µM; **B.:** HCT116 cells proliferate slower than HCT116+chr2 and HCT116+chr3 cells at concentration of 10 µM; **C.:** HCT116 cells show less number of live cells than HCT116+chr2 and HCT116+chr3 cells at concentration of 30 µM. A.: Results of two experiments, deviation showed by bars; B, C.**:** Mean values ± SD of three experiments.

These results demonstrate that at a 5-FU concentration of 2.5 µM, the fast-growing cells continue to grow faster and their survival is only slightly affected but at a 5FU concentration of 10 or 30 µM, the fast-growing cells show more growth inhibition and less survival than the slow-growing cells.

8.2.13. Fast-growing cells show more necrosis and less long-term survival than slow-growing, irrespectively from their MMR status

The previous results indicated that the growth speed affects cellular responses i.e. it affects apoptosis (Fig. 11D, 12C) and proliferation (Fig. 18) after 5FU treatment. In view of these results, it was hypothesised that the growth speed may influence long-term cell survival after 5FU treatment. In order to test this hypothesis and the role of MMR in long-term cell survival, HCT116, HCT116+chr2 and HCT116+chr3 cells were treated with 5FU (30 µM, 2 d). Since the previous data indicated that apoptosis is a short-term process (Fig. 11A), I tested the possible role of necrosis in long-term cell survival. The growth was monitored by cell counting and the LDH release was determined in the time intervals 1-2 days after start and 3-6 days after start, termed here time windows.

HCT116 cells showed more LDH release (Fig. 22A) and less live cells (Fig. 22B) than HCT116+chr2 and HCT116+chr3 cells after 5FU treatment. HCT116+chr2 and HCT116+chr3 cells showed the same LDH release and the same live cells after treatment.

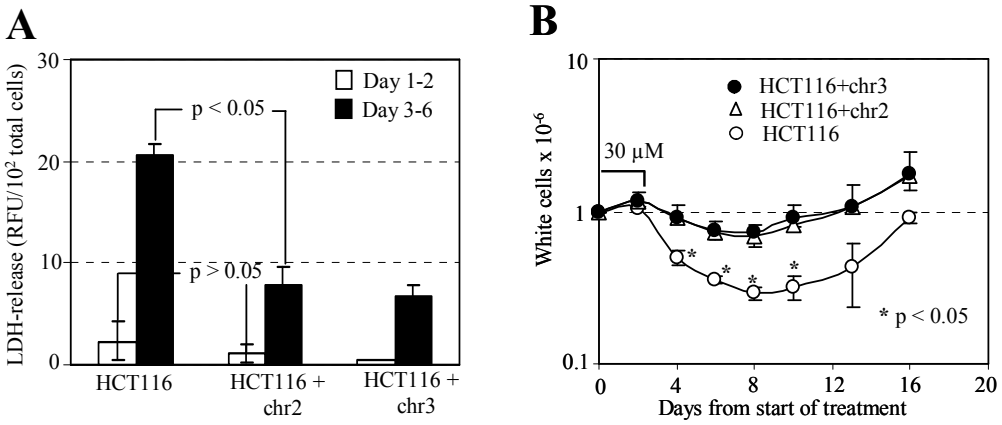

Fig. 22. Fast-growing cells show more LDH release and less live cells than the slow-growing cells after 5-FU treatment (30 µM, 2 d). A.: HCT116 cells show more LDH release than the slow-growing HCT116+chr2 and HCT116+chr3 cells at day 3 to 6 after treatment. The latter two show the same LDH release; **B.:** HCT116 cells show less live cells than HCT116+chr2 and

HCT116+chr3 cells after treatment. The latter two show the same number of live cells. Mean values ± SD of three experiments.

It is concluded that the necrosis and the long-term cell survival are associated with the growth speed, irrespectively from the MMR status.

8.2.14. Effect of growth speed on necrosis and long-term cell survival after 5-FU treatment

The present data indicated that the growth speed plays a more important role than MMR status in determining necrosis and long-term cell survival after 5FU treatment. To further strengthen this statement, I examined the effect of growth speed on necrosis and long-term cell survival using mock-1-transfectants growing at a normal speed (in the regular medium) or at a low speed (in medium with 3% FCS). Further, clone 43 growing at a normal speed or at a low speed (in medium with 3% FCS) was used.

There was more LDH release after the treatment in fast-growing than in slow-growing mock 1-transfectants or clone 43 (Fig. 23A). Fast-growing mock 1-transfectants or clone 43 showed less live cells than slow-growing ones (Fig. 23B, C).

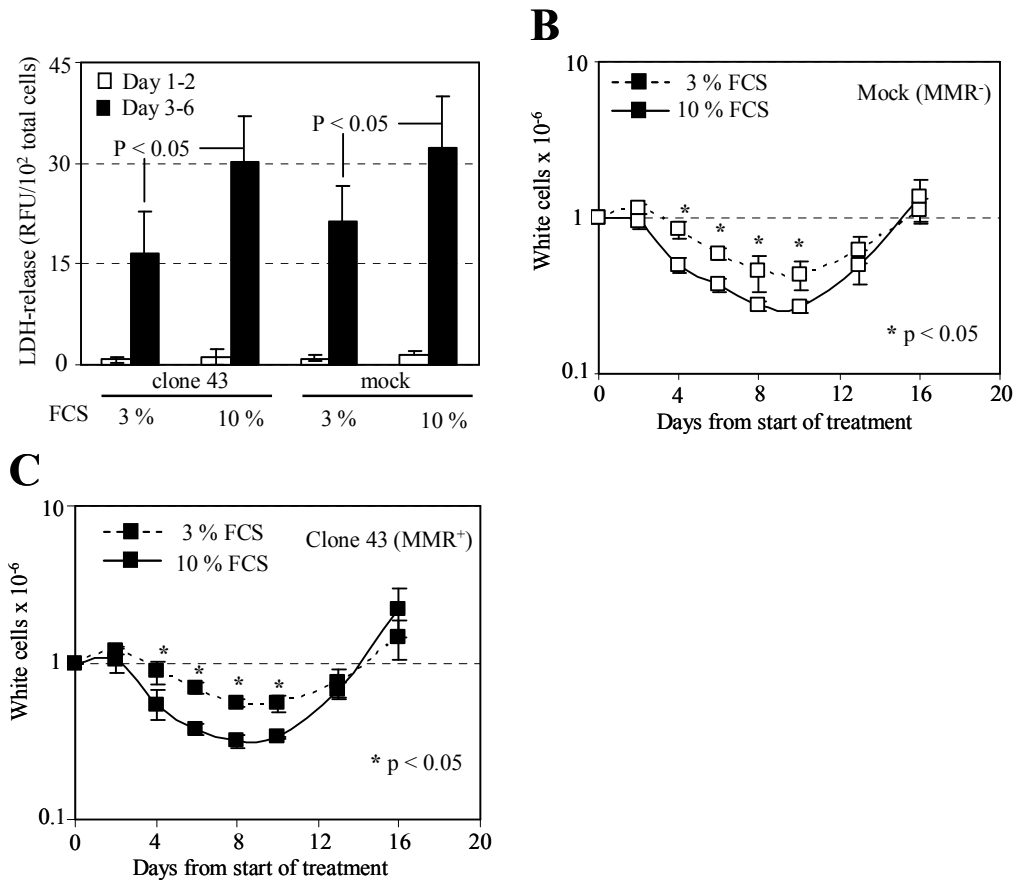

Fig. 23. Growth speed but not the MMR status determines LDH release and long-term cell survival after 5-FU treatment (30 µM, 2 d). A.: Fast-growing mock 1-transfectants or clone 43 show more LDH release than slow-growing ones at day 3 to 6 after treatment; **B.:** Fast-growing mock 1-transfectants show less live cells than slow-growing ones; C**.:** Clone 43 under the conditions of fast growth show less live cells than under the conditions of slow growth. Mean values ± SD of three experiments.

In agreement with the previous result (8.2.13), this corroborates that the growth speed but not the MMR status determines long-term cell survival which is associated with necrosis.

8.2.15. The relationship between apoptosis and necrosis

The present data indicate that the apoptosis is a short-term and necrosis is a long-term response to 5FU treatment. In order to test if necrosis is the result of apoptosis, I

examined the onset of necrosis in the absence of apoptosis in HCT116 cells. The apoptosis was blocked by using caspase inhibitor or by using $Bax^{-/-}$ cells.

8.2.15.1. CI or Bax-elimination inhibits apoptosis

The presence of CI or Bax-deficiency reduced apoptosis shown by PARP cleavage, in HCT116 cells after 2 days 5FU treatment (Fig. 15A, 16A). Since PARP cleavage alone was used as a marker for apoptosis in this experiment, the question was asked if CI presence or Bax-deficiency reduces condensed nuclei formation at different time window after 5FU treatment. To answer this question, HCT116 cells in the presence or absence of CI or HCT116$Bax^{-/-}$ cells were treated with 5FU (30 μM, 2 days).

Expectedly, HCT116 cells showed maximum PARP cleavage and high percentage of condensed nuclei at the time window 1 to 2 days and then decreased to a low level at 3 to 6 days or at 7 to 10 days after treatment. The presence of CI or Bax-deficiency inhibited the PARP cleavage or condensed nuclei formation at all the time windows after treatment (Fig. 24).

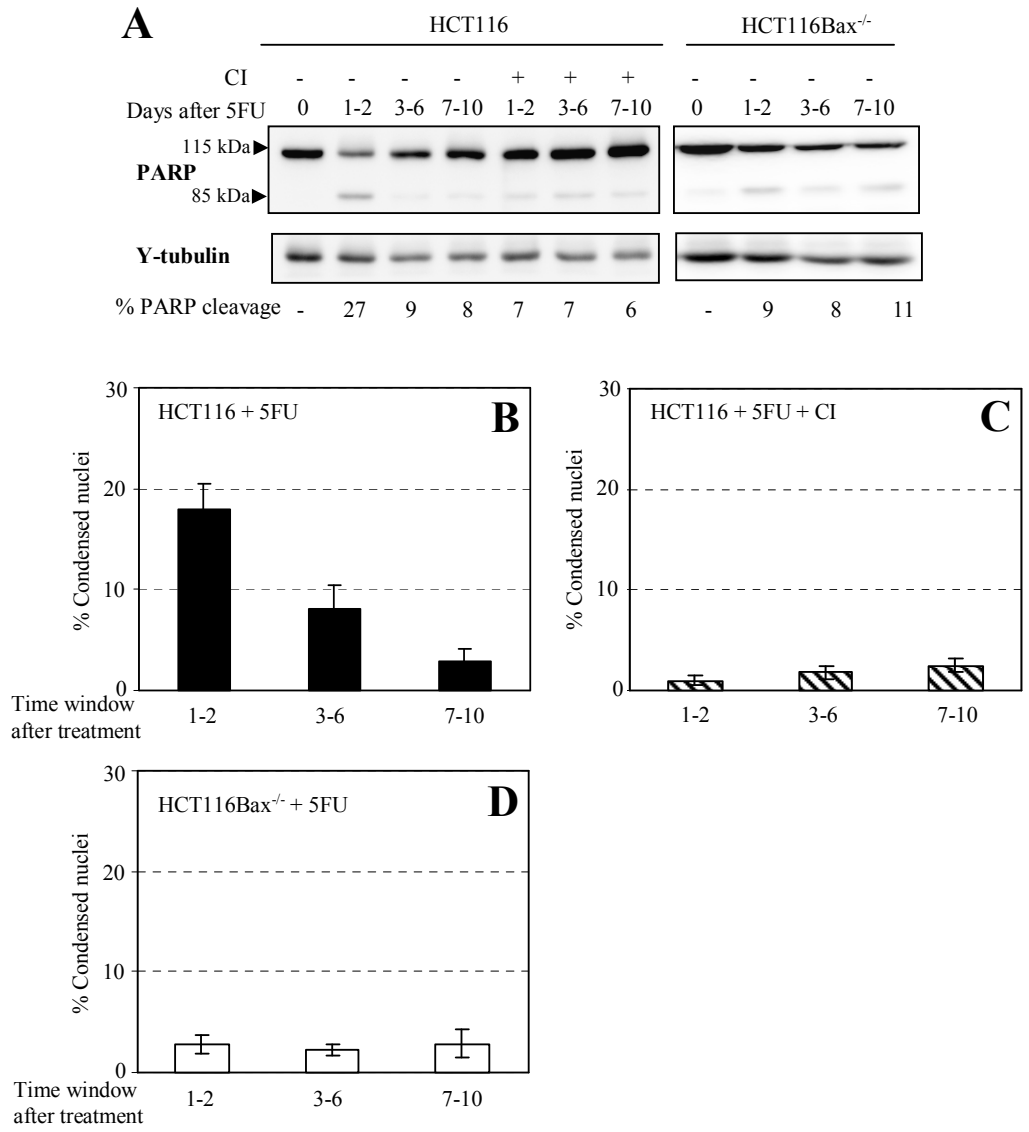

Fig. 24. Short-term apoptosis after 5FU treatment (30 µM, 2 d) is dependent on caspase or on Bax protein. A.: CI presence or Bax-deficiency decreases the PARP cleavage to a very low level; **B.:** HCT116 cells show higher percentage of condensed nuclei at day 1 to 2 than at day 3 to 6 or at day 7 to 10 after treatment; **C.:** CI presence causes a very low percentage of condensed nuclei at all time windows after treatment; **D.:** HCT116Bax$^{-/-}$ cells show a very low percentage of condensed nuclei at all time windows after treatment. A.: Representative for three experiments; B, C, D.: Mean values ± SD of three experiments.

This data confirmed the earlier results (Fig. 15A, 16A) that short-term apoptosis is reduced by caspase inhibition or Bax-elimination.

8.2.15.2. Necrosis is caspase or Bax dependent

I tested the time course of LDH release, as a measure of necrosis *in vitro*, in the absence of apoptosis in HCT116 cells after 5FU treatment.

In the presence of CI the LDH release was reduced 4-fold in the time window 3 to 6 days after 5FU treatment (Fig. 25B). Similarly, HCT116Bax$^{-/-}$ cells showed 4-fold decrease of LDH release in the same time window (Fig. 25C).

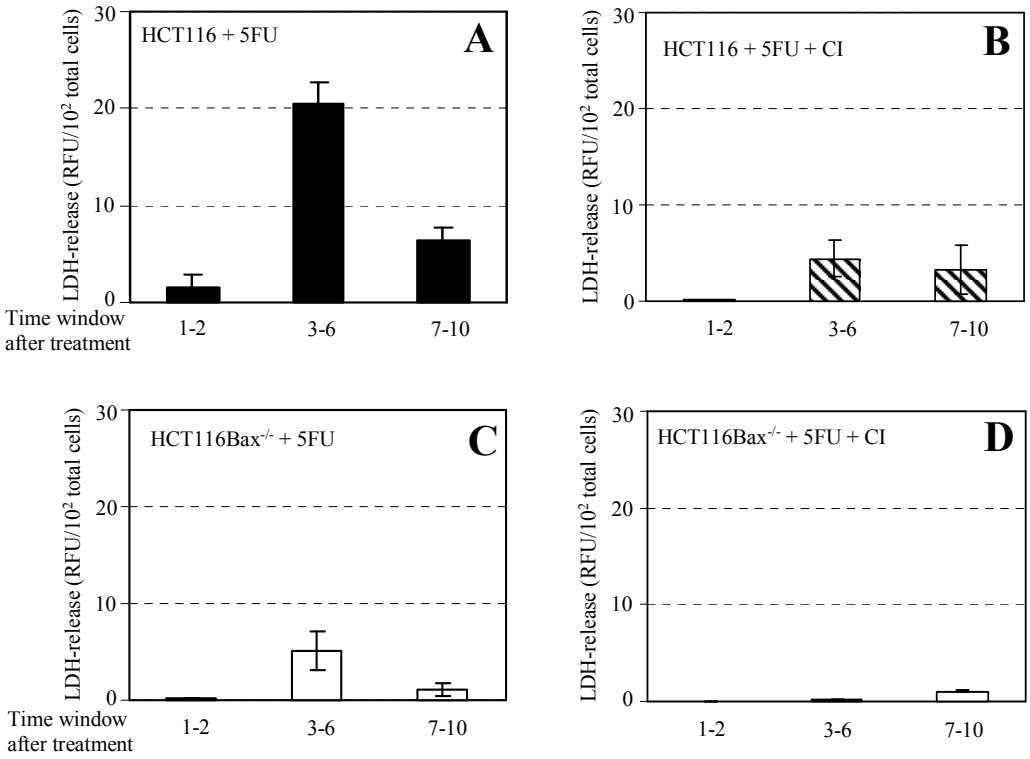

Fig. 25. LDH release in HCT116 cells is dependent on caspase or Bax after 5-FU treatment (30 µM, 2 d). A.: HCT116 cells show the maximal LDH release in the time window 3 to 6 days after treatment; **B.:** CI presence decreases LDH release to a very low level at all time windows after treatment; **C.:** HCT116Bax$^{-/-}$ cells show a very low level of LDH release at all time windows after treatment; **D.:** CI presence completely inhibits the LDH release in HCT116Bax$^{-/-}$ cells in the time window 3 to 6 days after treatment. A, B, C.: Mean values ± SD of three experiments, D.: Results of two experiments, deviation showed by bars.

About 1/4th of the total LDH release at the time window 3 - 6 days was caspase- or Bax -independent. Interestingly, the presence of CI completely inhibited the LDH release in HCT116Bax$^{-/-}$ cells at the same time window (Fig. 25D).

It is concluded that necrosis is a late process which depends on caspase or Bax. The data indicated that necrosis completely depends on caspase in the absence of Bax.

8.2.15.3. Necrosis is independent from apoptosis

The present data indicated that the inhibition of necrosis by caspase inhibition or by Bax-elimination was associated with the inhibition of short-term apoptosis. The question was still open if necrosis which has a different time course than apoptosis is dependent on it. I examined the cellular morphology of HCT116 cells at the time window 1 to 2 days and 3 to 6 days after 5FU treatment, when apoptosis and necrosis, respectively, were expected to be high.

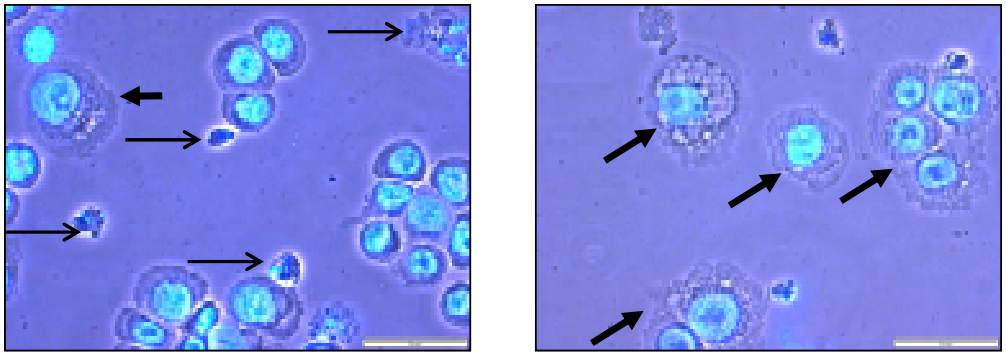

Fig. 26. HCT116 cells show more necrotic features at the time window of 3 to 6 days than 1 to 2 days after 5-FU treatment (30 µM, 2 d). Floating and adherent cells were collected at the time window of 1-2 or 3 - 6 days after treatment. Cytospins were made and stained with DAPI. Picture of DAPI stained nuclei is merged with the phase contrast picture of cells. Thin arrows: condensed nuclei, thick arrows: cells with necrotic morphology. Representative for three experiments. Bar 50µm.

HCT116 cells showed more condensed nuclei at the time window 1 to 2 days than 3 to 6 days (Fig. 26) as expected from Fig. 24B. The frequency of big, swollen and

vacuolated cells i.e. with morphology associated with necrosis was low at day 1 to 2. The cells with the described morphology increased at time window 3 to 6 days after treatment (Fig. 26)

The result indicates that the high frequency of necrotic but not the apoptotic cellular morphology is associated with high LDH release at the time window 3 to 6 days after treatment. It is concluded that the apoptosis and necrosis are two independent processes which appear at different time after 5FU treatment.

8.2.16. Long-term cell survival is affected by caspase or Bax

The present data indicated that the long-term cell survival is associated with the necrosis (8.2.13 and 8.2.14) which depends on caspase or Bax (8.2.15.2). To test if there is a relationship between necrosis and the long-term cell survival, I inhibited necrosis by CI treatment or using HCT116Bax$^{-/-}$cells and counted the live cells after 5FU treatment.

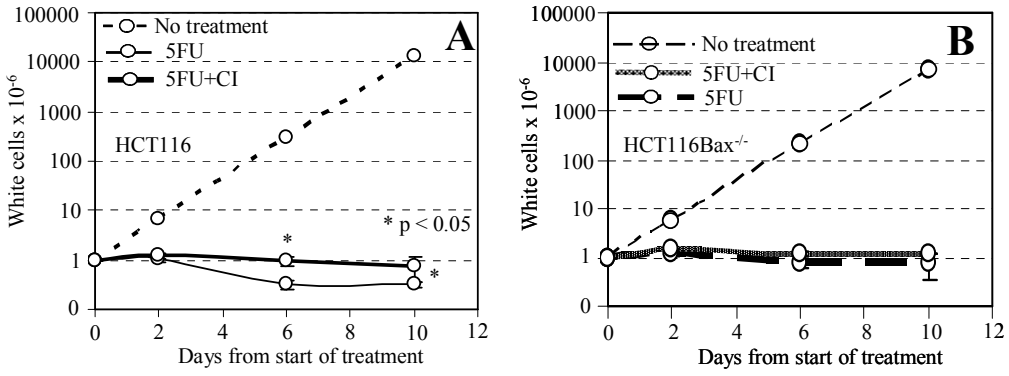

Fig. 27. Long-term survival of HCT116 cells after 5-FU treatment (30 µM, 2 d) is affected by caspase or Bax. A.: The presence of CI increases the number of live HCT116 cells; **B.:** More live HCT116Bax$^{-/-}$ cells than HCT116 (A) after treatment. CI presence caused a minor increase of the number of live HCT116Bax$^{-/-}$ cells. Mean values ± SD of three experiments. The growth curve of the nontreated cells in A and B is theoretical and the cell numbers are calculated from the doubling time (Table 1).

Caspase inhibition increased the long-term survival of HCT116 cells after 5FU treatment (Fig. 27A). Similarly, Bax-deficiency increased the live cells after treatment

(Fig. 27B). Further, caspase inhibition increased the long-term survival of $Bax^{-/-}$ cells after treatment (Fig. 27B).

$HCT116Bax^{-/-}$ and HCT116 cells treated with CI showed approx.10 000 fold decrease in cell number related to non-treated cells at day 10 after treatment. HCT116 cells showed 2 to 3 fold decrease in cell number related to CI treated or $Bax^{-/-}$ cells at day 10 after 5FU treatment.

It is concluded from these experiments that the abrogation of necrosis inhibits cell death in response to 5FU treatment. However, inhibition of cell death does not allow cell growth. Proliferation inhibition is the major effect of 5FU than the necrotic cell death.

8.3. p53 status affects long-term response of colon carcinoma cells to 5-FU treatment

8.3.1. $HCT116p53^{-/-}$ cells show late apoptosis and less long-term survival than $p53^{wt}$ cells after 5-FU treatment

$HCT116p53^{-/-}$ cell showed a very low short-term apoptosis in comparison to HCT116 cells after 5-FU treatment (Fig. 14B). I used these cells as an apoptosis-deficient model. The intention was to investigate necrosis in the absence of apoptosis in this cell model by checking necrosis at different time window after 5FU treatment.

$HCT116p53^{-/-}$ cells showed very little PARP cleavage at day 1 to 2 after treatment but it was higher than HCT116 cells at day 7 to 10 after treatment (Fig. 28A).

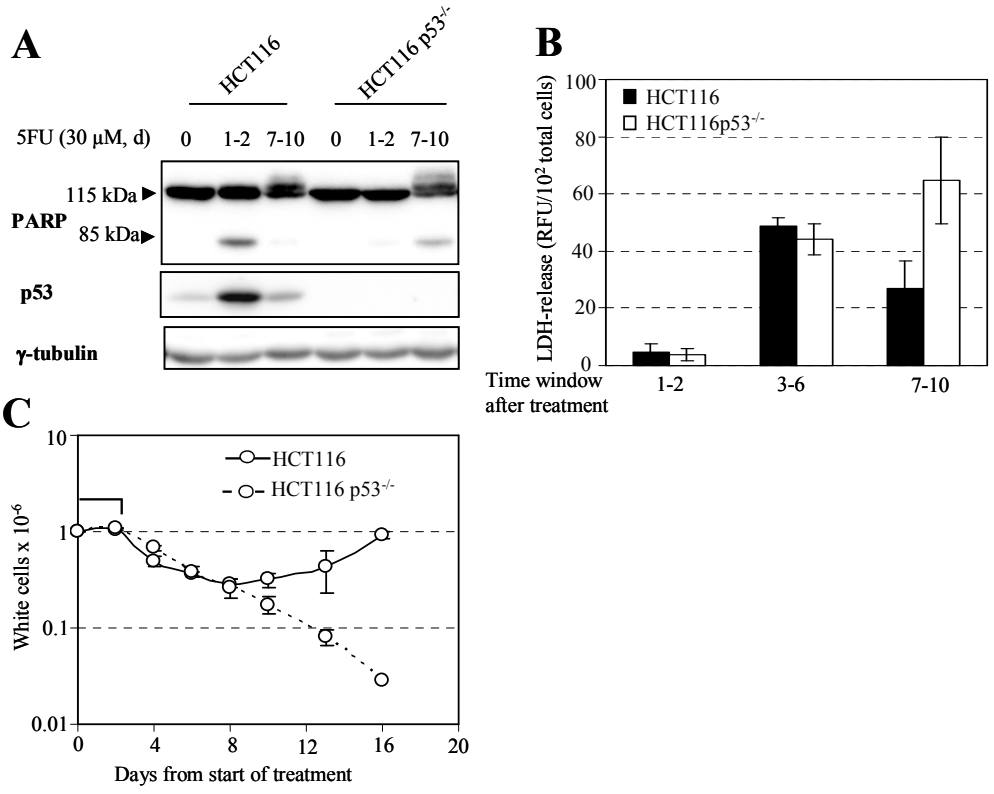

Fig. 28. HCT116p53$^{-/-}$ cells show late PARP cleavage, more LDH release and less long-term survival than HCT116 after 5-FU treatment (30 µM, 2 d). A.: HCT116p53$^{-/-}$ cells show no PARP cleavage at day 1 to 2 after treatment. At day 7 to 10, HCT116p53$^{-/-}$ cells show PARP cleavage; **B.:** HCT116p53$^{-/-}$ cells show more LDH release than HCT116 at day 7 to10 after treatment; **C.:** HCT116p53$^{-/-}$ cell show less long-term survival than HCT116 after treatment. A.: Representative for three experiments; B.: Mean values ± SD of three experiments; C.: Results of two experiments, deviation showed by bars.

The increase of PARP cleavage in HCT116p53$^{-/-}$ cells was associated with more LDH release at day 7 to 10 (Fig. 28B). The HCT116p53$^{-/-}$ cells showed less live cells than HCT116 and they did not recover from the damage within 16 days (Fig. 28C).

Thus p53-deficient cells show late apoptosis which was associated with necrosis and less long-term cell survival. Since HCT116p53$^{-/-}$ cells show apoptosis, the investigation of necrosis alone in this model was not possible.

8.3.2. Late apoptosis in p53-deficient cells is associated with mitotic catastrophe

HCT116p53$^{-/-}$ cells have been reported to undergo mitotic catastrophe associated apoptotic cell death (84). I examined whether the late apoptosis in HCT116p53$^{-/-}$ cells was associated with mitotic catastrophe by analyzing nuclear morphology.

HCT116 cells showed higher percentage of condensed nuclei at day 1 to 2 than at day 6 to 8 and at 10 to 12 after treatment. On the contrary, HCT116p53$^{-/-}$ cells did not show condensed nuclei at day 1 to 2 (Fig. 29B) but showed at day 6 to 8 (Fig. 29C) and at day 10 to 12 (Fig. 29D) after treatment. At the latter time intervals, HCT116p53$^{-/-}$ but not HCT116 cells showed big multinucleated cells, a hallmark of mitotic catastrophe.

It is concluded that the late apoptosis in p53-deficient cells is preceded by mitotic catastrophe.

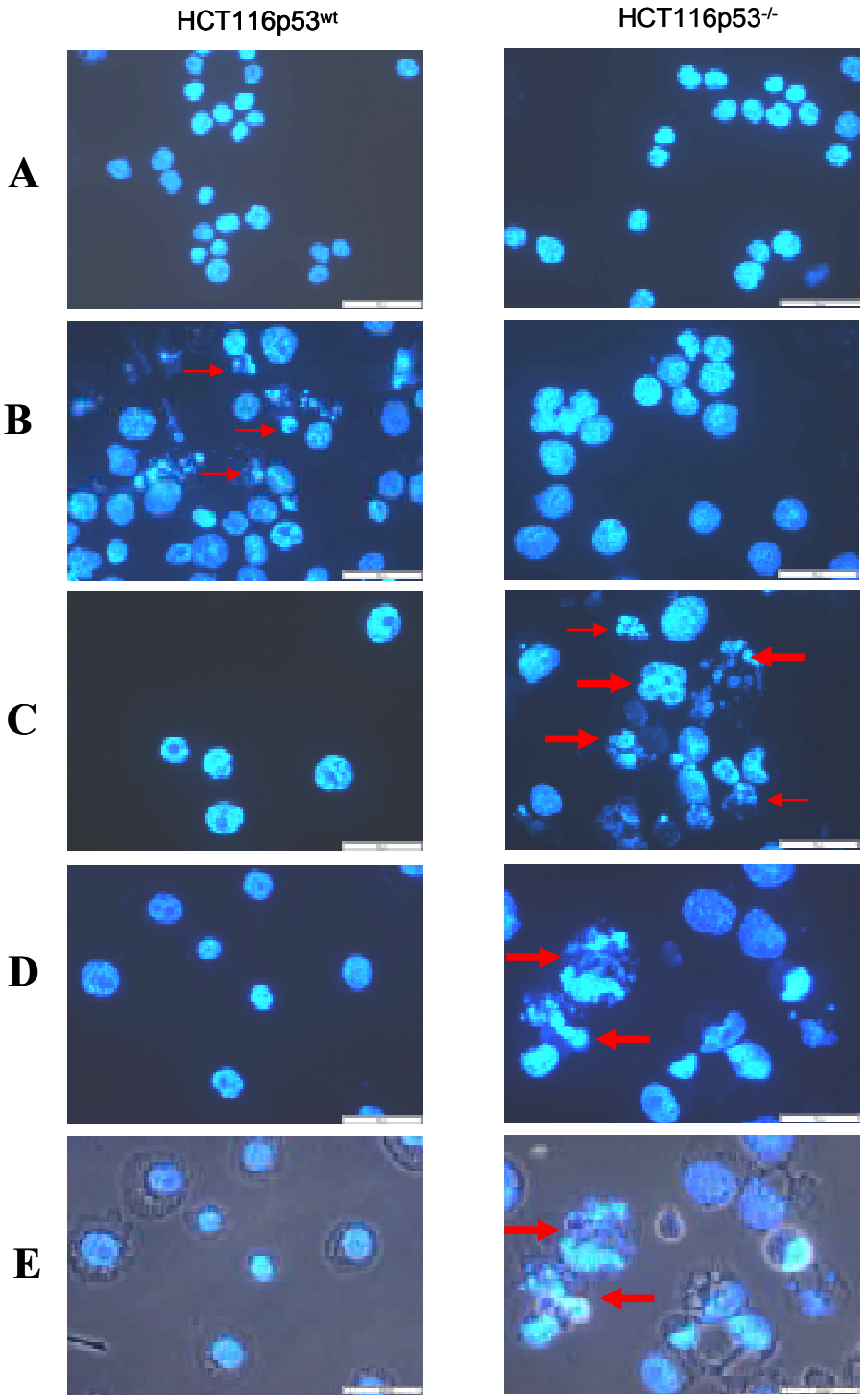

Fig. 29. HCT116p53$^{-/-}$ cells show big micronucletaed cell after 5-FU treatment (30 µM, 2 d). Thick arrows: big multinucleated nuclei, thin arrows: condensed nuclei**. A.:** No treatment; **B.:** HCT116 but not HCT116p53$^{-/-}$ cells show condensed nuclei at day 1 to 2; **C.:** HCT116p53$^{-/-}$ but not HCT116 cells show big multinucleated and high condensed nuclei at day 6 to 8 and; **D.:** At day 10 to 12

after treatment; **E.:** D merged with phase-contrast picture. Representative for two experiments. Bar: 50 µm.

8.3.3. $p53^{mut}$ colon carcinoma cell are more susceptible to 5FU than $p53^{wt}$ cell at higher 5FU concentration

Since it was observed that HCT116$p53^{-/-}$ cells are more susceptible to 5FU than HCT116$p53^{wt}$ cells, the question was asked if colon carcinoma cells with p53 mutation show similar susceptibility to 5FU as shown by p53-dificient cells. To answer this question, I examined the effect of 5FU on long-term cell survival of DLD1 ($p53^{mut}$) and LS174T ($p53^{wt}$) cells. Both cell lines have been reported to have similar doubling time (74).

At 5FU concentration of 30 µM, DLD1 cells were affected less than LS174T cells (Fig. 30A). Both the cell lines did not show cell death (blue cells) at this concentration of 5FU.

In previous result, the high susceptibility of HCT116$p53^{-/-}$ cells to 5-FU was associated with the cell death (Fig. 28). I found that a 5FU concentration of 100 µM triggered cell death in DLD1 and LS174T cells after treatment. The cell death was estimated qualitatively by observing percentage floating dead cells at day 3 to 6 after treatment.

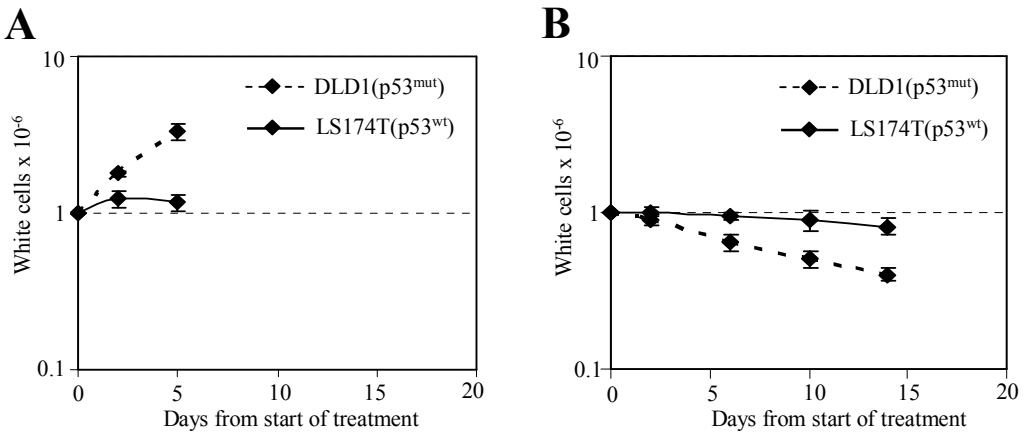

Fig. 30. $p53^{mut}$ cells show less long-term survival than $p53^{wt}$ cells at higher 5-FU concentration (2 d). A.: At 5FU concentration of 30 µM, DLD1 cells show more proliferation than

LS174T cells; **B.:** At 100 µM 5FU, DLD1 cells show less long-term survival than LS174T. Results of two experiments, deviation showed by bars.

Further, at 5FU concentration of 100 µM (2 d), DLD1 cells showed less long-term survival than LS174T cells (Fig. 30B). However, none of the cell lines showed recovery within 15 days after treatment.

These results indicate that at lower concentration of 5FU, the $p53^{mut}$ cancer cells are less susceptible than $p53^{wt}$ cells. At higher 5FU concentration which triggers cell death, the effect of 5FU is reversed i.e. $p53^{mut}$ colon cancer cells are more susceptible than $p53^{wt}$ cells. The present data is the result obtained in two cell lines and need to be confirmed in a set of cancer cell lines differing in p53 mutation status.

8.4. Fast-growing tumor xenograft shows more growth delay after 5FU treatment than slow-growing, independently from their MMR status

In vitro data indicates that the growth speed but not the MMR status determines the long-term cell survival in response to 5-FU treatment. To proof if this is the case *in vivo*, the effect of MMR status and of growth speed on tumour xenograft progression was investigated in the mouse model after 5-FU treatment.

After 5FU treatment, HCT116 xenograft showed a delay of tumour growth by 15 days (Fig. 31A). HCT116+chr2 (Fig. 31B) and HCT116+chr3 (Fig. 31C) xenografts did not respond to 5FU treatment.

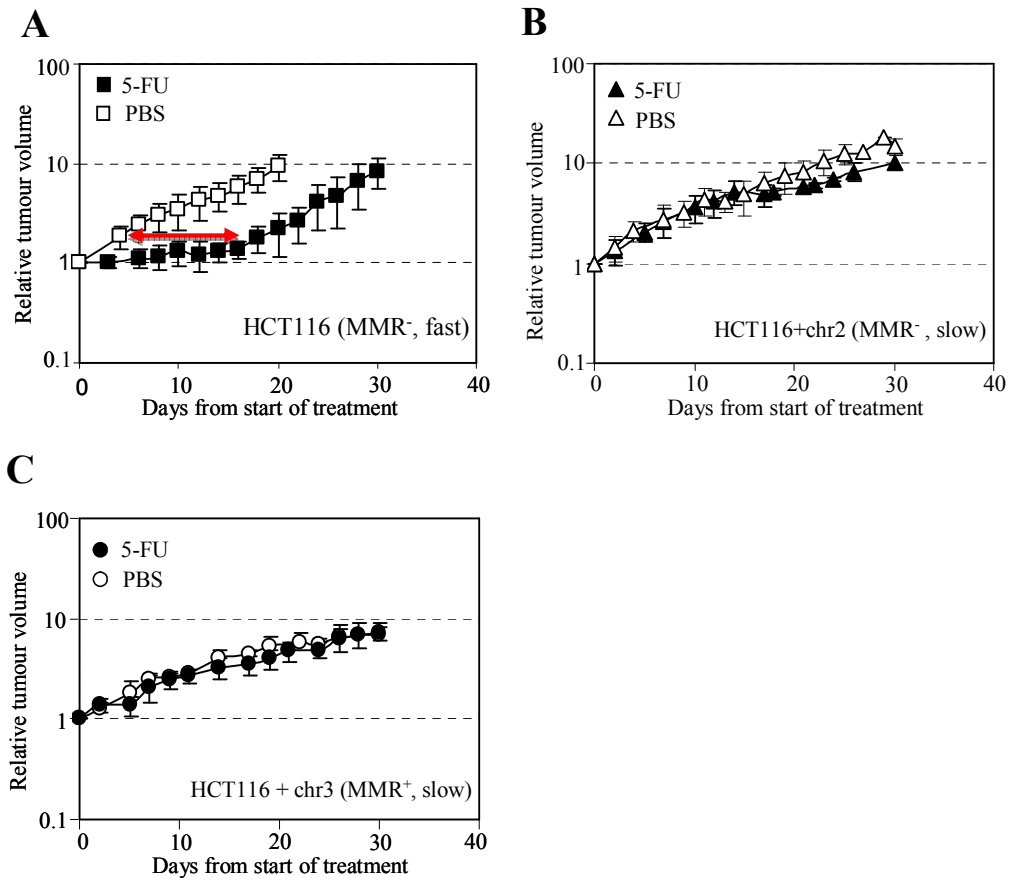

Fig. 31. Fast-growing tumour xenograft showed more delay of tumour progression than slow-growing xenograft after 5-FU treatment (50 mg/kg). A.: HCT116 xenograft show s15 days of delay of tumour progression; **B.:** HCT116+chr2 xenograft shows no delay of tumour progression; **C.:** HCT116+chr3 xenograft shows no delay of tumour progression. Experiments A and C were carried out by Dr. Hanski with the help of Dr. Bhonde.

It is concluded that the slow growing-tumours are less susceptible to 5FU than fast-growing tumour. This result does not allow to draw any conclusion as to the role of MMR.

9. Discussion

9.1. MMR status does not affect the growth speed

In the present work, we have demonstrated that the change of MMR status by elimination of hMLH1 or by exogenous expression of hMLH1 does not affect cell proliferation (Fig.7), BrdU or ^{3}H-thymidine incorporation (Fig. 8) and the doubling time (Table 3, Fig. 9). The present result is in agreement with previous reports (59-60). Our result contradicts the earlier findings made by Brieger et. Al. (57) and Shin et. al. (58), showing that MMR restoration by exogenous expression of hMLH1 in HCT116 cell decreases proliferation. In the work of Brieger et al, experimenter has used a readout which is similar to MTT assay, to check the proliferation of HCT116 cells transiently transfected with hMLH1. The use of MTT-like readout alone and transient transfection which does not ensure the uniform expression of hMLH1 with time, are insufficient to reach to the conclusion made by the author. However, our data completely contradict the data showed by Shin et. al.

9.2. MMR status determines clonogenic survival

Using isogenic (Fig. 10A) or semiisogenic (Fig. 10B) colon or ovarian cancer cell model (Fig. 10C), we have demonstrated that MMR-deficient cells show a better clonogenic survival than MMR-proficient cells after 5FU treatment. Several authors (71-73) using the same cell model have previously reported that MMR-proficient cell showed less clonogenic survival than MMR-deficient ones after 5FU treatment. Carethers et al. reported that HCT116+chr3 cell line showed less clonogenic survival than HCT116 or HCT116+chr2 after continuous treatment with 5 μM 5FU for 10 days (71). Similarly, Mayers et. al. reported that HCT116+chr3 cell line showed less clonogenic survival than HCT116 after continuous treatment with 7.5 -10 μM 5FU or 5FdUrd for 10 days (72).

In the present work, we have used the concentration of 30 μM 5FU for 2 days treatment and then the drug was removed. Even though there was a difference in

5FU concentration and in the treatment period, our data are in complete agreement with the data showed by other authors.

9.3. MMR induces DNA-double strand breaks and short-term apoptosis after 5FU treatment

In the present work, we have shown that MMR-proficient cells make more DNA-double stand breaks (DSB) and more apoptosis than MMR-deficient cells after 5FU treatment (Fig 11). MMR mediated apoptosis was short-term i.e. it was maximal at day 2 and it decreased to a low level at day 5 from the start of treatment (Fig. 11A).

We observed that the MMR-deficient cells show less PARP cleavage than MMR-proficient cells (Fig. 11B), however, we found that the percentage of the condensed nuclei was similar in these two cell lines (Fig. 11D). Since the PARP cleavage is an earlier event than condensed nuclei formation in apoptosis, we relied on the percent condensed nuclei as a more accurate marker for apoptosis.

Our present data support the observation made by Mayers et. al. (72) who showed that HCT116+chr3 cells show more DSB than HCT116 cell after continuous treatment with 10 μM 5FdUrd or 5FU for 3 days. They found that there was no difference in apoptosis between HCT116 and HCT116+chr3 cells after the treatment. Our data suggest that HCT116 was not an appropriate control for HCT116+chr3 in the previous study because apart from MMR status both the cell lines differ in the growth speed.

In the present work we further investigated the role of MMR in apoptosis using HCT116+chr2 cells as a control which differ in only MMR status and not in the growth speed from HCT116+chr3 cells. The present work is the first report which shows that MMR determines apoptosis in response to 5FU treatment using HCT116+chr2 and HCT116+chr3 cell model.

9.4. Growth speed affects apoptosis

We investigated the effect of MMR status on apoptosis after 5FU treatment (8.2.2). Surprisingly, the fast-growing HCT116 cells showed more apoptosis than the slow-growing HCT116+chr2 cells with the same MMR status (Fig. 11D). This was the initial

indication that along with MMR, the growth speed might play a role in triggering apoptosis after 5FU treatment. We tested this hypothesis in another cell model and demonstrated that the growth speed indeed determines apoptosis after 5FU treatment, independently from the MMR status (Fig. 12C).

The present work is the first investigation to show that the growth speed influences apoptosis after 5FU treatment *in vitro*.

9.5. Apoptosis and clonogenic survival are dependent on MMR

We observed that the MMR proficiency was associated with more apoptosis and less clonogenic survival after 5FU treatment (8.2.1, 8.2.2). We further strengthen the statement by demonstrating a causal relationship between MMR and apoptosis or between MMR and clonogenic survival. We showed in another cell model that after the restoration of MMR status, apoptosis increases and clonogenic survival decreases after 5FU treatment (8.2.4).

Thus, the present work systematically demonstrates that the apoptosis is increased and the clonogenic survival is decreased by MMR after 5FU treatment.

9.6. Apoptosis and clonogenic survival are influenced by p53 status

In the present work we have demonstrated that the apoptosis (Fig. 14A, B) and clonogenic survival (Fig. 14C) are dependent on p53 after 5FU treatment (8.2.5). It is widely accepted that p53 protein regulates cell cycle arrest and apoptosis in response to genotoxic stress. However, there are several reports indicating that apoptosis may not require p53 (85-86). In agreement with the previous reports (81-83), we have demonstrated in HCT116 cell model that 5FU-triggered apoptosis is p53-dependent. Wu et al. reported that p53-deficient ovarian cancer cell line shows less clonogenic survival than p53-proficient cells after Etoposide or Adriamycin treatment (83). Our present data, in agreement with the observation of Wu et. al., clearly demonstrate that the clonogenic survival of colon cancer cell in response to 5FU is determined by p53 status.

9.7. Clonogenic survival is not dependent on apoptosis

We observed the association between apoptosis and clonogenic survival (8.2.1, 8.2.2) after 5FU treatment. After showing the association, we investigated the causal relationship between the two by using broad-range caspase inhibitor or $Bax^{-/-}$ cells as an apoptosis deficient model.

Our results have demonstrated that in the presence of caspase inhibitor, 5FU-induced apoptosis was completely inhibited (Fig. 15A) but the clonogenic survival was not altered (Fig. 15B, C) neither in MMR-proficient nor in -deficient cell lines. Similarly, we observed that the apoptosis was Bax dependent (Fig. 16A). Bax-deficiency did not, however, improve the clonogenic survival (Fig. 16B). In these experiments, however, the cells which showed apoptosis were grown at a higher density than cells seeded for clonogenic assay.

The present data suggest that the apoptosis but not the clonogenic survival is dependent on caspase or Bax after 5FU treatment. On the other hand, they indicate that the cell density may influence the cell response to 5FU.

9.8. The reaction of cells to 5-FU is influenced by the growth density

During the investigation of the involvement of caspase or Bax in clonogenic survival (8.2.6), we found that the cell density affects the response to 5FU. We found that the cells grown at a low density proliferate slower (Fig. 18) and show much less apoptosis (Fig. 17) after 5FU treatment than cells grown at a high density.

The present work shows that the cell density affects the response to 5FU treatment and that apoptosis does not determine the clonogenic survival. The exact mechanism behind the different cell response caused by the different cell density is not clearly understood.

9.9. The difference in clonogenic survival of MMR-proficient and - deficient cells is caused by senescence

Since apoptosis was not the reason for the difference in clonogenic survival, we investigated cell arrest and senescence. We demonstrated that MMR-proficient cells

show more tiny colonies than -deficient cells after 5FU treatment (Fig. 19, Table 4). We further showed the presence of similar number of total colonies in MMR-proficient and -deficient cells (Table 4). We found that the single cells or cells in tiny colonies had the properties of senescent cells (Fig. 20).

Several authors have previously reported MMR-dependent clonogenic survival after 5FU treatment (71-73) without elucidating the cellular mechanism behind the difference in clonogenic survival.

The present work is the first report which demonstrates that the MMR is associated with senescence which determines the clonogenic survival after 5FU treatment. Our report has clearly demonstrated that the cell death is not involved in the determination of clonogenic survival.

9.10. Long-term cell response depends on 5FU concentration

We have demonstrated that at lower 5-FU concentration, the fast-growing cells were affected less and thus continue to grow faster (Fig. 21A) but at a higher 5-FU concentration, the effect was reversed i.e. the fast-growing cells were affected more. They show more growth inhibition and less survival than the slow-growing cells (Fig. 21B, C).

It has been previously shown that after intravenous continuous infusion of standard doses of 5FU, patient plasma levels are in the range of 0.06–11.3 μM (87-88). Our data suggest that the lower concentration (2.5 and 10 μM) which lie in the reported range and the higher concentration (30 μM) of 5FU trigger different long-term responses in colon carcinoma cell. Thus are in agreement with the published reports (89-91) which advocate that 5-FU may act via two different pathways depending on its dose.

9.11. Growth speed and not the MMR status determines the long-term cell response to 5FU

We have demonstrated that cells growing at similar speed show similar long-term

survival after 5FU treatment, and the fast-growing cells show less long-term survival than slow-growing ones, independently from their MMR status (Fig. 22B, 23B, C).

This observation indicates that the growth speed and not the MMR status determines the long-term response of cells to 5FU treatment.

Bracht et al (74) have recently shown that after the treatment of different colon carcinoma cells with 5FU for a span of two doubling-times, the MMR-proficient cells were more growth-inhibited than MMR-deficient ones. In this work authors determined the short-term effect of 5FU. In contrast to the above mentioned work, our data demonstrate the long-term effect of 5FU by determining the cell growth in the span of several doubling-times.

The present work is the first report which demonstrates that the growth speed and not the MMR plays a role in long-term response to 5FU treatment.

9.12. Long-term cell survival is determined by necrosis which is regulated by the growth speed and not by the MMR

We have shown that after 5FU treatment, cells grown at a similar speed show similar necrosis and the fast-growing cells show more necrosis than slow-growing ones, independently from their MMR status (Fig. 22A, 23A).

Our data suggest that the apoptosis is the short-term effect of 5FU i.e. more pronounced at day 2 and decreasing later (Fig. 11A, 24A, B). We have concluded from our data that the apoptosis is regulated by both MMR (Fig. 11B, D) and growth speed (Fig. 11D, 12C). However, our data suggest that the cell viability is not affected at day 2 where we observed the maximum apoptosis after the treatment. We concluded from the observation that the short-term apoptosis is not sufficient to affect the cell viability; thus not playing any role in determining cell survival.

On the other hand, our data clearly indicate that unlike apoptosis, necrosis is the long-term effect of 5FU i.e. more pronounced at day 6 from the start of treatment (Fig. 22A, 23A, 25A, 26) which is associated with the less cell survival.

In the present work, we have demonstrated the role of apoptosis and necrosis in the long-term cell survival after 5FU treatment. Our data suggest that the apoptosis and necrosis are two independent processes which are induced in response to 5FU treatment and demonstrate that the necrosis and not the apoptosis is the main mechanism which determines the long-term cell survival.

9.13. Necrosis is regulated by caspase and Bax

We further demonstrated in HCT116 cell line that the short-term apoptosis depends on caspase or Bax (Fig. 24). The major proportion of necrosis was dependent on caspase (Fig. 25B) or Bax (Fig. 25C). However, we found that the minor proportion of necrosis was caspase- or Bax-independent. Our results indicated that necrosis was completely dependent on caspase in the absence of Bax (Fig. 25D). We found that the inhibition of necrosis improved the long-term cell survival after 5FU treatment (Fig.27).

Several authors have previously reported that necrosis is not a passive process in response to some specific signals (42-44) e.g. after DNA damage. Caspase (45-46) and Bax (48) were shown to regulate necrotic cell death. In agreement with the published reports (45, 46, 48), our present data indicate that 5FU triggered necrosis is regulated by Bax and caspase.

The present work is the first report on the involvement of programmed necrosis regulated by Bax and caspase in response to 5FU.

9.14. Growth inhibition and not cell death is the major effect of 5FU

Our data show that after 5FU treatment, proliferation inhibition caused at day 10 after treatment aprox.10 000-fold decrease in cell number related to non-treated cells. By contrast, the necrotic cell death contributed to approx. 2 to 3 fold decrease in cell number related to necrosis inhibited cells (Fig. 27A, B).

It is widely known that the necrosis is unfavourable response in cancer chemotherapy because it evokes undesirable inflammatory response *in vivo*. Our present data

suggest that the proliferation inhibition but not the necrotic cell death is the major cellular mechanism of response to 5FU treatment.

9.15. 5FU dose can affect the chemosensitivity of p53-deficient cells

Our results indicate that HCT116p53$^{-/-}$ colon carcinoma cells are more susceptible to 5FU than p53^{wt} cells. They show more apoptosis, necrosis and less long-term survival than p53^{wt} cells after treatment (Fig. 28). Our data showed that p53-deficiency triggers mitotic catastrophe induced apoptosis in response to 5FU (Fig. 29). It has been shown previously in our group that CPT-11 treatment of HCT116p53$^{-/-}$ cells leads to the mitotic catastrophe and eventually to apoptosis, while the p53^{wt} cells remained arrested (84). Our present result is in agreement with previous report (84). Our data showed that at higher but not al lower concentration of 5FU, p53^{mut} cell lines are more susceptible than p53^{wt} cell (Fig. 30).

More than 60% of colon carcinomas are p53-mutated. Several reports (51-52, 63) suggest that p53^{mut} cells are resistant to 5FU in comparison to p53^{wt} cells. The present data are in agreement with the previous reports (51-52, 63) at lower 5FU concentration. On the contrary, the increasing concentration of 5FU can reverse its effect i.e. p53^{mut} cells can show more susceptibility than p53^{wt} cells. However, our data need to be confirmed in a large panel of cancer cell lines differing in p53 mutation status to strengthen the statement.

9.16. Growth speed and not the MMR status determines the tumour xenograft progression in response to 5FU treatment

Our results in agreement with the previous report (71) demonstrated that the HCT116 xenograft grows faster than HCT116+chr2 or HCT116+chr3. The latter two grow at a similar speed. After 5FU treatment, fast-growing xenograft showed delay of tumour growth by 15 days (Fig. 31A). Whereas the slow-growing HCT116+chr2 (Fig. 31B) or HCT116+chr3 (Fig. 31C) xenograft showed minimum late response or no response, respectively.

In agreement with the *in vitro* data (8.2.13 and 8.2.14), *in vivo* data indicate that the slow-growing cells are less susceptible to 5FU than fast-growing ones. The present work shows for the first time that the clonogenic assay is not a suitable readout to test the effect of a drug on long-term cell response *in vitro*. It is the first report on the effect of growth speed and MMR status on tumour growth in response to 5FU using HCT116, HCT116+chr2 and HCT116+chr3 xenograft model. This experiment, however, does not allow to draw any conclusion about the role of MMR.

10. Conclusion

The major findings of this work can be summarised as follows:

- MMR does not affect cell proliferation.
- MMR mediated senescence and not apoptosis determines low clonogenic survival in response to 5FU
- Cell density affects the response to 5FU treatment, *in vitro.*
- Growth speed and not MMR determines long-term cell survival in response to 5FU.
- Long-term cell survival is regulated by necrosis and not apoptosis.
- Necrosis is regulated by caspase protease or Bax protein.
- Growth speed affects tumour response *in vivo* after 5FU treatment.
- Clonogenic assay is not a suitable readout to test the effect of a drug on long-term cell response *in vitro.*

11. Perspective

The present data indicate that the MMR is not playing any role in cell response to 5FU treatment and suggest that MMR status of tumours in patients with CRC is not a suitable predictive marker to 5FU therapy.

Our finding suggests that patients with fast-growing tumour may respond better than patients with slow-growing tumours to 5FU therapy. The present work emphasizes that the proliferation speed of tumour must be considered in predicting response to 5FU therapy.

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13. Publications and Posters

- **B. Choudhary,** M.L. Hanski, M. Zeitz, C. Hanski. Role of MMR and proliferation rate in the long-term response of colon carcinoma cells to 5FU treatment. (Manuscript is submitted)
- S. Krishna-Subramanian, M.L. Hanski, C. Loddenkemper, **B. Choudhary,** B. Jebautzke, M. Zeitz, C. Hanski. UDCA slows down intestinal cell proliferation by inducing high and sustained ERK phosphorylation. (Manuscript is submitted)
- **B. Choudhary**, M.L. Hanski, M. Zeitz, C. Hanski. MMR status determines short-term apoptosis while growth speed determines necrosis and long- term survival of colon carcinoma cells after 5-FU treatment. German Journal of Gastroenterology 2010; 48: P167 **Poster**.
- **B. Choudhary**, M.L. Hanski, M. Zeitz, C. Hanski. Growth speed plays a greater role than MMR status in determining the long-term survival of colon carcinoma cells treated with 5-FU. German Journal of Gastroenterology 2009; 47: P356 **Poster**.
- **B. Choudhary,** M. L. Hanski, M. Bhonde, M. Zeitz, C. Hanski. Effects of MMR status on colon carcinoma cell survival after 5-FU treatment in vitro and invivo. EJC Supplements 2008; 6 (12); 38 **Poster**.
- S. Krishna-Subramanian, **B.Choudhary**, M.L. Hanski, M. Zeitz, C. Hanski. EGF and IGF-1 pathways are targeted by the chemopreventive agent ursodeoxycholic acid. Onkologie 2010; 33(suppl.2): 39 **Poster**