AUS DEM LEHRSTUHL
FÜR INNERE MEDIZIN I
PROF. DR. MARTINA MÜLLER-SCHILLING
DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

p63 dependent regulation of microRNAs in Hepatocellular Carcinoma

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Medizin

der
Fakultät für Medizin
der Universität Regensburg

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Tag der mündlichen Prüfung: 15.03.2022

List of contents

Abbreviations

Ad-	Adenovirus
AFP	Alpha Fetoprotein
BAX	Bcl-2 associated X protein
BCLC	Barcelona Clinic Liver Cancer (group)
CDH	N-cadherin, Cadherin 2
CDK	Cyclin-dependent kinase
cm^2	Square centimeter
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
EASL	European Association for the study of the liver
EMT	Epithelial mesenchymal transition
EORTC	European Organization for the Research and Treatment of Cancer
g	gram
h	hours
HCC	Hepatocellular carcinoma
HNF4α	Hepatocyte Nuclear Factor 4 Alpha
IGFBP3	Insulin-like-growth-factor-binding-protein 3
k	Kilo (1000)
kb	Kilobase
mg	Milligram
miR	microRNA
miRNA	microRNA
ml	Milliliter
NASH	non-alcoholic steato hepatits
OD	oligomerization domain
PDGFR	platelet-derived growth factor receptor
RAC	Ras-related C3 botulinum toxin substrate
RISC	RNA-induced silencing complex
RFP	radiofrequency ablation
RNA	ribonucleic acid
rpm	rounds per minute
SAM	sterile alpha motif

SCC	squamous cell carcinoma
SV40	Simian virus 40
TACE	transarterial chemoembolization
TAD	Transactivation domain
TAp63	p63 isoform containing a transactivation domain
UTR	Untranslated region
UV	Ultraviolet
VEGFR	vascular endothelial growth factor receptor
x g	times earth gravitation
ZEB	zinc finger E-box-binding homeobox
µg	Microgram
µM	Micro molar

0. Deutschsprachige Zusammenfassung

Hintergrund: Transkriptionsfaktoren der p53-Familie (p53, p63 und p73) führen bei zellulärem Stress zur Expression bestimmter Gene. In Tumoren wie dem hepatozellulären Karzinom (HCC) wirken sie tumorsuppressiv. p63 ist ein Tumorsupressor, der auch auf die Entwicklung von Epithelien und die sog. Epithelial-mesenchymale Transition wirkt, ein Prozess, der zur Metastasierung von Tumoren führen kann. Es ist jedoch nicht bekannt, welche Rolle p63 in der Entstehung des hepatozellulären Karzinoms spielt. microRNAs (miRNAs, miR) sind kleine, nicht kodierende RNA Moleküle, die die Genexpression beeinflussen, indem sie an mRNAs binden und so die Translation verhindern. Die Expression von miRNAs kann durch Mitglieder der p53-Familie reguliert werden. In vorangegangenen Studien wurden auch von unserer Arbeitsgruppe miRNAs identifiziert, die durch p53 im HCC reguliert werden (miR34a, miR149, miR192und miR194). Es ist bereits erwiesen, dass miR34a durch p53 induziert werden kann und so, indem es p53-abhängige Reaktionen auf zellulären Stress stabilisiert, tumorsuppressiv wirken kann. Im Plattenepithelkarzinom (Squamous cell carcinoma, SCC) wurde bereits nachgewiesen, dass p63 miRNA-level beeinflussen kann. Es ist jedoch nicht bekannt, welche Rolle p63 in der miRNA-Expression beim HCC spielt.

Material und Methoden: Das Ziel der Studie war herauszufinden, ob die miRNA-Expression in HCC durch p63 beeinflusst werden kann. Hep3B-Zellen, die kein p53 exprimieren, wurden mit adenoviralen Vektoren (rAd-p63 und rAd-GFP) transduziert. Anschließend wurde die Expression von miR34a, miR149, miR192 und miR194 via qPCR gemessen. Die transfizierten Zellen wurden zudem über bis zu 72 Stunden mit Substanzen und in Konzentrationen, die zur Behandlung von HCC – lokal (Doxorubicin, Bleomycin) und systemisch (Sorafenib, Tivantinib und Regorafenib) – eingesetzt werden, behandelt und die miRNA-Expression wurde gemessen.

Ergebnisse: Die Überexpression von p63 führte zu einer Hochregulation von miR34a (2,18-fach) und miR149 (2,309-fach) nach 48 Stunden. Zudem führte sie zu einer Hochregulation von miR192 (2,77-fach) und miR194 (2,35-fach) nach 24 Stunden. In nicht transduzierten Zellen hatte eine *in vitro* Behandlung mit HCC-relevanten Therapeutika keinen Einfluss auf die miRNA-level. In Kombination mit einer Überexpression von p63 konnten die miRNAs jedoch durch die Therapeutika hochreguliert werden. Nach 72-stündiger in vitro Behandlung mit Doxorubicin kam es in p63-überexprimierenden Zellen zu einer 4,72-fach erhöhten Expression von miR34a, einer 10,29-fach erhöhten Expression von miR149 und einer 3,29-fach erhöhten Expression von miR192, als Kontrolle dienten rAd-GFP-transduzierte Zellen. Durch eine Behandlung mit Bleomycin kann es zu einer 4,12-fach erhöhten Expression von miR34a und einer 15,65-fach erhöhten Expression von miR149 in p63-überexprimierenden Zellen.

Sorafenib hatte ebenfalls einen Einfluss auf die p63-abhängige Expression vom miR34a (3,64-fach), miR192 (2,57-fach) und miR194 (2,29-fach). Tivantinib führte zu einer 2,77-fach erhöhten Expression von miR192 und einer 4,50-fach erhöhten Expression von miR194 in p63-überexprimierenden Zellen.

Es ist wichtig und klinisch relevant zu erwähnen, dass eine Überexpression von p63 in Kombination mit systemischen Therapeutika (Sorafenib, Tivantinib) vor allem zu einer erhöhten Expression von miR192 und miR194 führte, während eine Behandlung von p63-überexprimierenden Zellen mit lokalen, zytotoxischen Therapeutika zu einer erhöhten Expression von miR34a und miR149 führte.

Zusammenfassung: Es konnte erstmalig ein Effekt von p63-Überexpression auf miRNAs in HCC-Zelllinien gezeigt werden. Die Ergebnisse weisen darauf hin, dass p63 mit relevanten Therapeutika interagiert und so die Expression von miRNAs im HCC beeinflusst. Durch die Ergebnisse der Studie ergeben sich weitere Hinweise auf die Art, wie das komplexe, tumorsuppressive Netzwerk der p53-Familie im HCC wirkt. Diese Studie charakterisiert p63 erstmals als einen klinisch relevanten Regulator von miRNAs im HCC und als relevanten Faktor für das Therapie-Ansprechen des HCC.

1. Introduction

1.1 Hepatocellular carcinoma

1.1.1 Overview

Hepatocellular carcinoma (HCC) is the most frequent primary cancer of the liver and is derived from hepatocytes (1). HCC is the 7th most common cancer worldwide, due to its complexity and its challenging and often late diagnosis the 3rd highest ranked cancer in mortality (2,3). The overall survival rate after 5 years is in between 3 to 5% (4). In Germany HCC incidence is a bit below the international level, making it the 12th most common cancer in men and 18th most common cancer in women. In Germany HCC is ranked as cancer with the the 5th highest mortality rate in men and 10th highest mortality rate in women (5).

HCC can be triggered by various factors. Over 90% of HCCs occurin livers affected by chronic liver disease (6). In up to 78% of the cases, it is caused by an underlying infection with either hepatitis B or C virus that results in cirrhosis (7). HCC is also linked to an unhealthy lifestyle and excessive alcohol consumption. It is estimated that the number of HCC cases linked to unhealthy lifestyle are to increase in the future (8). Other etiologies are known but make up only a small part of all HCC cases (9–11).

There are only a few specific tumor markers for HCC. One of them is the alpha fetoprotein (AFP), though in numerous cases of HCC the AFP-serum-level is not elevated. Thus, it is no reliable screening parameter (12). Screening and diagnosis of HCC follow the guidelines by the European Association for the study of the liver (EASL) and the European Organization for the Research and Treatment of Cancer (EORTC) (13). Treatment and staging of HCC follow the Barcelona Clinic Liver Cancer (BCLC) Guidelines in Europe (13,14).

One of the challenges for both, treatment and research, on HCC is that HCC is not the result of a single mutation but of a variety of cell transformations that lead to malignant cells. Different pathogenic etiologies can damage the liver, thus leading to an increased cell turnover and therefore, an enhanced likelihood of mutations. Common genetic alterations in HCC include activation of oncogenes, inactivation of tumor suppressor genes and defects in DNA repair or replication (15).

Most frequent mutations in HCC are found in the genes for p53, a tumor suppressor, cyclin D1, a regulator of the cell cycle, and ß-catenin, which promotes cellular proliferation

(16). However, countless other mutations in HCC are known and many more are discovered every year, making it one of the most complex carcinomas of the human body.

1.1.2 Treatment of HCC

Treatment of HCC in Europe follows the EASL-EORTC guidelines, which are constantly reviewed and revised (13,17). In Germany, after diagnosis of HCC, the tumor is staged by and treated according to the "S3 Leitlinie", the German guidelines concerning HCC treatment (18). Staging includes staging the tumor according to the Milan and UCSF criteria. The Milan criteria include single tumors under 5 cm in diameter and no more than three tumors under 3 cm in diameter. The UCSF criteria include single tumors under 6.5 cm in diameter and no more than three tumors under 4.5 cm in diameter with the sum of the diameters not exceeding 8 cm (18). Stage A1 includes patients with a single tumor smaller than 5 cm in diameter or no more than three tumors < 3 cm, which show no other symptoms or signs of liver dysfunction. Stage A2 includes patients with single tumors bigger than 5 cm in diameter or not more than three tumors smaller than 5 cm in diameter without any symptoms or abnormal liver function. In stage B, the liver contains multiple lesions but the patient shows no other symptoms or liver dysfunction (Child-Pugh A-B). Stage C includes patients with tumor spreading to the surrounding anatomical structures (blood vessels, lymph nodes, other organs), impaired performance status (ECOG 1-2), yet the liver function remains good (Child-Pugh A-B). Stage D includes patients, who are severely compromised in their everyday life (ECOG 3-4) or who show a high level of liver dysfunction (Child-Pugh C) (19,20). Following the algorithm shown below (Figure 1), patients are treated either with curative or palliative aim. The most effective treatment in tumors limited to the liver is transplantation. As curative treatment the tumor can be resected or the tumor can be treated using ablation (RFA or MWA) with or without combination with transarterial chemoembolization (TACE). If not resectable, the tumor can be treated with transarterial chemoembolization (TACE). From stage C on the patient will receive immuntherapy atezolizumab/bevacizumab or in case of contraindications lenvatinib or sorafenib as a first line treatment. The drugs sorafenib, regorafenib, cabozanitinib, or ramucirumab (if AFP is above 400 ng/ml) are used as second and third line systemic targeted therapy (details on these drugs are discussed below). At terminal stage the patient is submitted to best supportive care (13). Treatment with immunotherapy in Germany is shown in figure 1b.

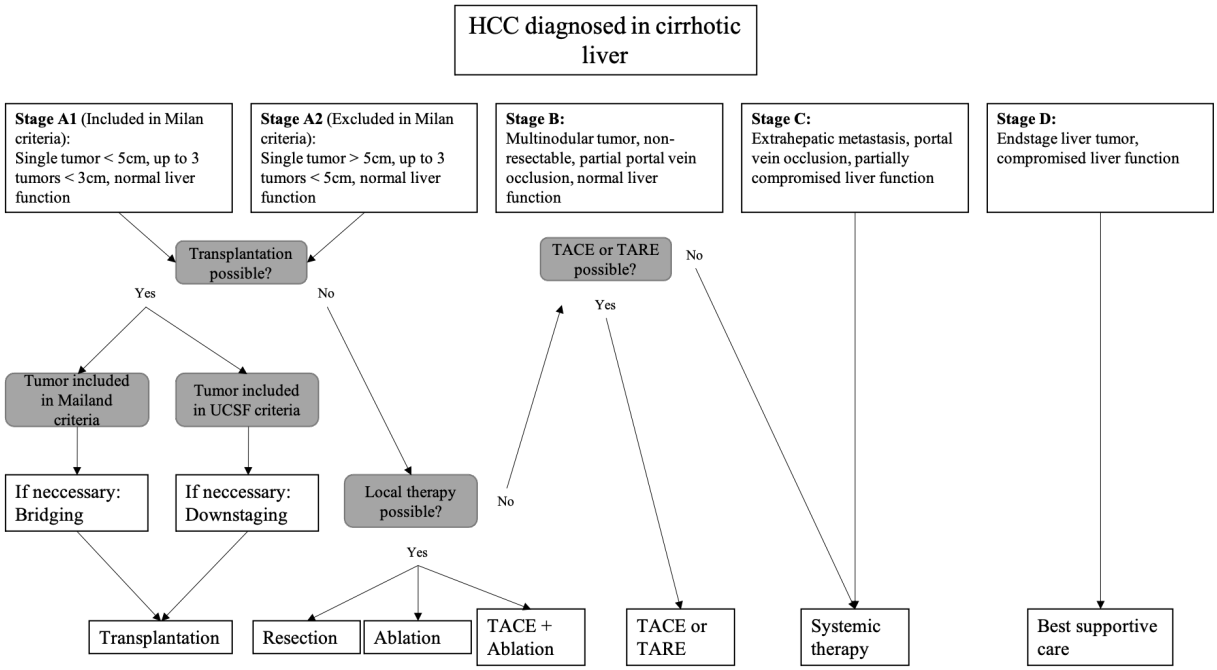

Figure 1a: Treatment of HCC diagnosed in cirrhotic liver. Patients are classified depending on the size of their tumor and their liver function. Adapted from (18).

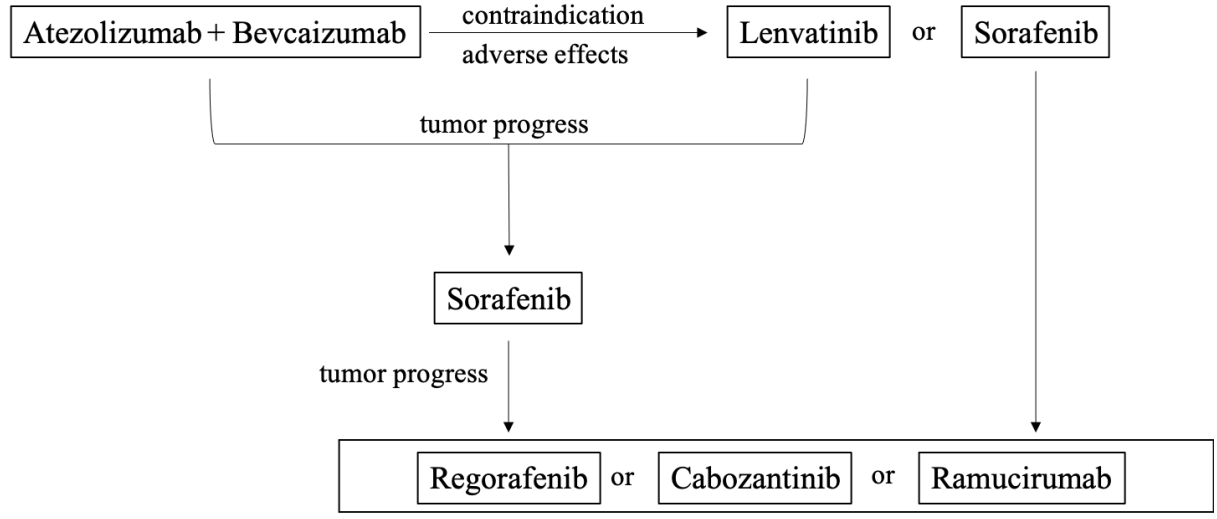

Figure 1b: Immunotherapy treatment according to the German guidelines (2021). Adapted from (18).

1.1.3 Common substances used in HCC treatment

By the start of research for this thesis in 2017, the new EASL-EORTC guidelines (13,21) as well as the updated German guidelines (18) were not yet published. We therefore focused on substances commonly used in HCC treatment at that time as well as promising new substances such as regorafenib and tivantinib.

1.1.3.1 Doxorubicin

Doxorubicin is the most common chemotherapeutic agent used in TACE in intermediate stage HCC (13). Doxorubicin is an anthracycline that intercalates DNA, inhibits topoisomerase II, and leads to generation of reactive oxygen species, resulting in a higher apoptosis rate and a general cytotoxic effect (22). Recent studies have shown that tumor cells surviving doxorubicin treatment generally emerge more resistant to second doses and result in poorer outcome in repeatedly treated tumors (23). Doxorubicin treatment activates TAp63, a protein which is part of the p53 family (described below), in osteosarcoma cells (U2OS) and non-small lung cancer cells (H1299) (24).

1.1.3.2 Sorafenib

Sorafenib is an orally given substance that inhibits serine/threonine kinases and thus results in reduced cell proliferation. It also inhibits vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR) and suppresses vascularization of tumors by suppressing angiogenesis (25). Sorafenib was over more than ten years the most common therapy and until approval of Atezolizumab/Bevacizumab the first line therapy in unresectable tumors. Various trials have shown its efficacy, though sorafenib merely prolongs survival by about 3 months (26). Patients are commonly treated with 400 mg sorafenib, which results in serum levels of about 4.5 µM sorafenib (27).

1.1.3.3 Tivantinib

Tivantinib is an orally given therapeutic agent selectively targeting receptor tyrosine kinase MET (mesenchymal-epithelial transition factor). In phase II trials patients with HCC with high

MET levels showed an improved median progression time after treatment with tivantinib. However, these results could not be reproduced by a randomized, placebo-controlled phase III trial (28). As this study was published in 2018, tivantinib was included in our studies due to the phase II trial results.

1.1.3.4 Bleomycin

Bleomycin is a tumor suppressive metallo-glycopeptide antibiotic and one of the oldest chemotherapeutic agents, which has been used in treatment of testicular cancer, squamous cell carcinoma (SCC) and lymphatic carcinomas. Bleomycin inhibits DNA synthesis and induces single or double strand breaks (29). The optimal serum concentration of bleomycin for cancer treatment is 1.5-3 µg/ml (30). Bleomycin is not commonly used in HCC treatment anymore, but is one of the oldest chemotherapeutic agents. Therefore, we decided to include bleomycin in this study to be able to compare its effects with those of novel therapeutic drugs.

1.1.3.5 Regorafenib

Regorafenib is an oral multi-kinase inhibitor and structural analog to sorafenib that targets receptor tyrosine kinases (RTK) and shows anti-angiogenic activity by targeting VEGFR2-TIE2 tyrosine kinase. It was developed by Bayer® and is used in treatment of metastatic colorectal cancer and advanced gastrointestinal stroma tumors. In a phase III trial (RESORCE trial) regorafenib has proven its efficacy as a second line treatment in HCC (31). Since 2017 it is approved in the US as a second line systemic treatment in patients who have been treated with sorafenib but were not able to tolerate the treatment or had progressive disease under treatment with sorafenib (32). Recent EASL-EORTC guidelines recommend regorafenib as a second line treatment for advanced HCC (13).

1.2 The p53-family

1.2.1 Overview

In our research, we focused on p53 and members of the p53 family, genes important for the regulation of apoptosis and cell death.

p53, a phosphoprotein, is probably the most important tumor suppressor known so far. It is known as the "guardian of the genome" and plays an important role in the regulation of various cell functions in almost all tissues (33,34). Loss of function mutations of p53 promote carcinogenesis (35–37). p53 was first discovered independently by David P. Lane and Lionel Crawford (38) and Daniel Linzer and Arnold Levine (39) by observing tumorigenic effects of SV40-virus on cells (40). Other members of the p53-family have been discovered shortly thereafter. p63 and p73 are proteins homologous to p53 (41). All three proteins are able to activate molecular pathways by acting as transcription factors that induce cell cycle arrest and apoptosis subsequently (42).

1.2.2 Structure

All members of the p53-family have a similar structure and contain a DNA-binding domain (DBD), an oligomerization domain (OD), and a transactivation domain (TAD) (42). Various isoforms of all members of the p53 family are known. These isoforms are a result of different promoters as well as of alternative splicing. p63 contains 3 different promoters and, depending on the promoter, TAp63 isoforms or ΔNp63 isoforms can be expressed (43). TAp63 contains an N-terminal transactivation domain, a DBD, an OD, a sterile alpha motif (SAM), which cannot be found in the p53 protein, as well as a transcription inhibiting domain (TID). While promoter 1 and 2 are specific for TAp63, the tumor suppressive form, promoter 3 is specific for ΔNp63, an oncogenic isoform. Following promoter 2 TAp63 contains a TAD followed by a DBD, an OD and a sterile alpha motif (SAM). Alternative splicing on the 3' end leads to five different types, α, β, γ, δ and ε (compare Figure 2). In contrast to p63, p53 does not contain a SAM. It has a TAD, a DBD, an OD and a CTD responsible for DNA binding and transcriptional activity. So far 12 isoforms of p53 are known. p73 shows some similarities to p53 with the TAD being 30% identical with p53, the DBD being 63% identical with p53, and the OD being 38% identical with p53 (30). Similar to p63, p73 contains a SAM. While the role of p63 and p73 for cancer development remains uncertain, mutant p53 isoforms have been shown to interact with either p63 or p73 (44).

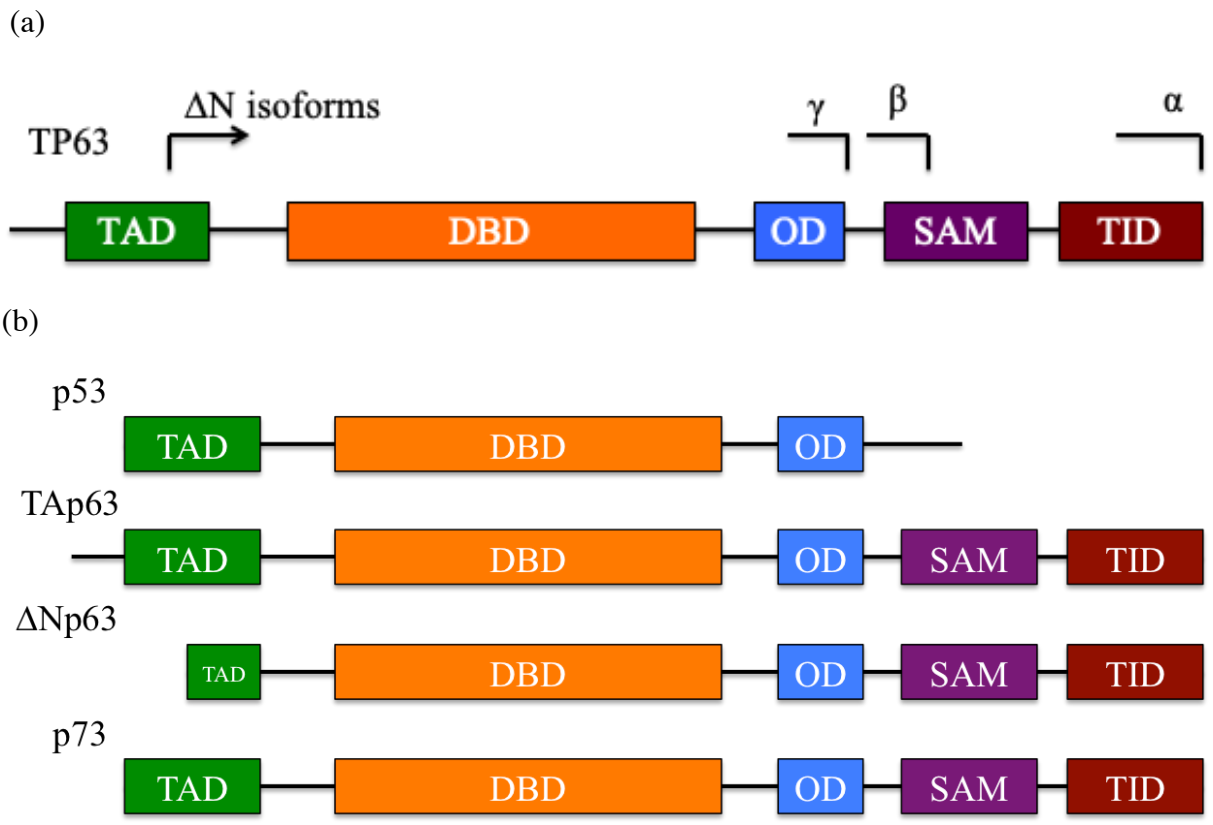

Figure 2: (a) Architecture of the human *TP63* gene structure: alternative splicing (shown: α, β, γ), transactivation domain (TAD), DNA-binding domain (DBD), oligomerization domain (OD), sterile alpha motif domain (SAM) and transcription inhibition domain (TID) are indicated. ΔN isoforms start with a different promoter and depending on alternative splicing α, β, γ isoforms result. (b) Architecture of the p53 family members gene structure: p53 does not contain a SAM and a TID, ΔNp63 is truncated at the TAD, TAp63 and p73 contain a TAD, a DBD, an OD, a SAM and a TID. Adapted from (44)

1.2.3 Function

The most important function of p53 is its role as a transcription factor. Its activity is highly influenced by cell-toxic stress such as ionizing radiation, UV rays, chemotherapy and other toxic agents. As a consequence of cellular stress, several post-translational DNA modifications occur. The N-terminal part of the p53 can be phosphorylated after exposure to ionizing radiation, the C-terminal regulatory domain can be acetylated, sumoylated and phosphorylated after genotoxic stress. As a result, p53 is stabilized and can fulfill its function by promoting apoptosis and cell cycle arrest. Modification of p53 additionally supports binding to different target genes and thus regulating their transcription and translation (45,46). Due to its function

as a promoter of cell death the concentration of p53 is kept low in healthy cells. Numerous genes are regulated by p53, such as *IGFBP3*, *PUMA, Bax, Bid, CD95 and TRAIL-R2* (42,47–49). Furthermore, p53 has a great influence on the expression of microRNAs (miRNAs), which are known to control transcription and thus protein synthesis in cells (50). Loss of function of p53 can result in carcinogenesis and rapid tumor growth (51,52). By losing its function, p53 is no longer capable of inducing apoptosis and cell death in damaged cells (53). Thus, oncogenic cells can proliferate in an uncontrolled fashion, leading to tumorgenesis (35).

Both p63 and p73 are also known to induce apoptosis but differ in certain functions from p53. p63 influences ectodermal structures such as epithelial, craniofacial and limb development (54). p73 was shown to play a role in neurogenesis (55).

1.2.4 DNA binding

All described effects of the three p53 family members are based on their capability to bind to DNA and thus to influence the expression of specific target genes. All members contain a DNA binding domain (DBD). DNA binding can either occur via DNA-binding polypeptides, non-specifically to DNA, or in a sequence-specific manner. For sequence-specific binding, p53 specifically recognizes TGCCT-repeats. These repeats are found mainly at replication origins of the genes targeted by p53 (56). The DBD of p63 is 60% identical with p53s DBD (42).

1.2.5 p63

p63 is known for its role in the development of epithelia (54,57,58) as well as developmental neuronal death (59) and plays a role in epithelial mesenchymal transition (EMT), a process that results in metastasis of primary cancers (60). There are various known isoforms, the most important being TAp63 and ΔNp63. TAp63 contains a full-length transactivation domain (TAD) at the N-terminus whereas ΔNp63 is lacking the TAD (see Figure 2). TAp63 acts as a tumor suppressor. In contrast, ΔNp63 antagonizes p53 and therefore has oncogenic potential (61,62). However, ΔNp63α is also associated with lower metastasis rates, suppressed EMT, higher levels of epithelial integrity and tumor aggressiveness in squamous cell carcinoma (SCC) (63). ΔNp63 can target and repress p73 in SCC, resulting in a higher tumor rate (64). In contrast, little is known about the role of p63 in HCC development and progression. Mutant p53 can interact with p63 isoforms, thus resulting in higher invasion and metastasis rates as well as

lower apoptosis rates (44). Various genes such as EGFR and integrins have been proven to be targeted by p63 and its isoforms (65).

1.2.6 Expression of p63 isoforms after treatment with drugs commonly used for HCC treatment

Interestingly, ΔNp63 is only expressed in p53-depleted cells. Treatment with DNA-damaging agents such as doxorubicin had no impact on the expression of ΔNp63 in HCC cells. On the other hand, treatment with doxorubicin led to an upregulation of TAp63, independent of the p53 status in HCC cells (66). p63 has been proposed to take over functions of p53 in HCC (67). Treatment of cells with bleomycin also led to an accumulation of TAp63 (30). Studies on how sorafenib, tivantinib or regorafenib influence p63-expression have not been conducted so far.

1.3 microRNA

1.3.1 Structure and function

As focus of this thesis, we chose microRNAs (miRNAs) and asked how they react to altered p63-expression as well as different drug treatments commonly used in HCC.
miRNAs are small non-coding RNAs with a length of 20-22 nucleotides. They play an important role in post-translational gene regulation by targeting the 3´UTR of mRNAs (68) thus blocking the ribosome and leading to translation inhibition and a faster de-adenylation. This results in a lower concentration of the corresponding protein (see figure 3).

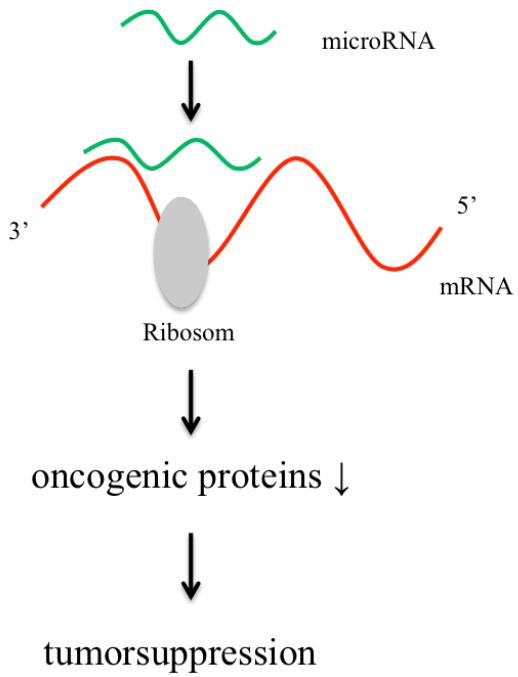

Figure 3: Mechanism of tumor-suppressive miRNA action. By targeting specific mRNA miRNAs inhibit translation, leading to a lower level of the target protein.

miRNA synthesis follows a number of steps from gene to the final miRNA (Figure 4). First, a pri-miRNA of 1-4kb is transcribed in the nucleus by RNA polymerase III. This pri-miRNA contains both, the miRNA-strand and the passenger-strand. DROSHA-DGCR8, a nuclease, then crops it to precursor-microRNA (pre-miRNA), a hairpin loop of 70 nt length. The pre-miRNA is then transported from the nucleus to the cytoplasm by Exportin 5. There, the nuclease DICER crops the pre-miRNA to a miRNA-miRNA duplex made from the miRNA-strand and the passenger-strand. The miRNA-strand is then incorporated in RNA-induced silencing complex (RISC), resulting in the final product, mature miRNA (69).

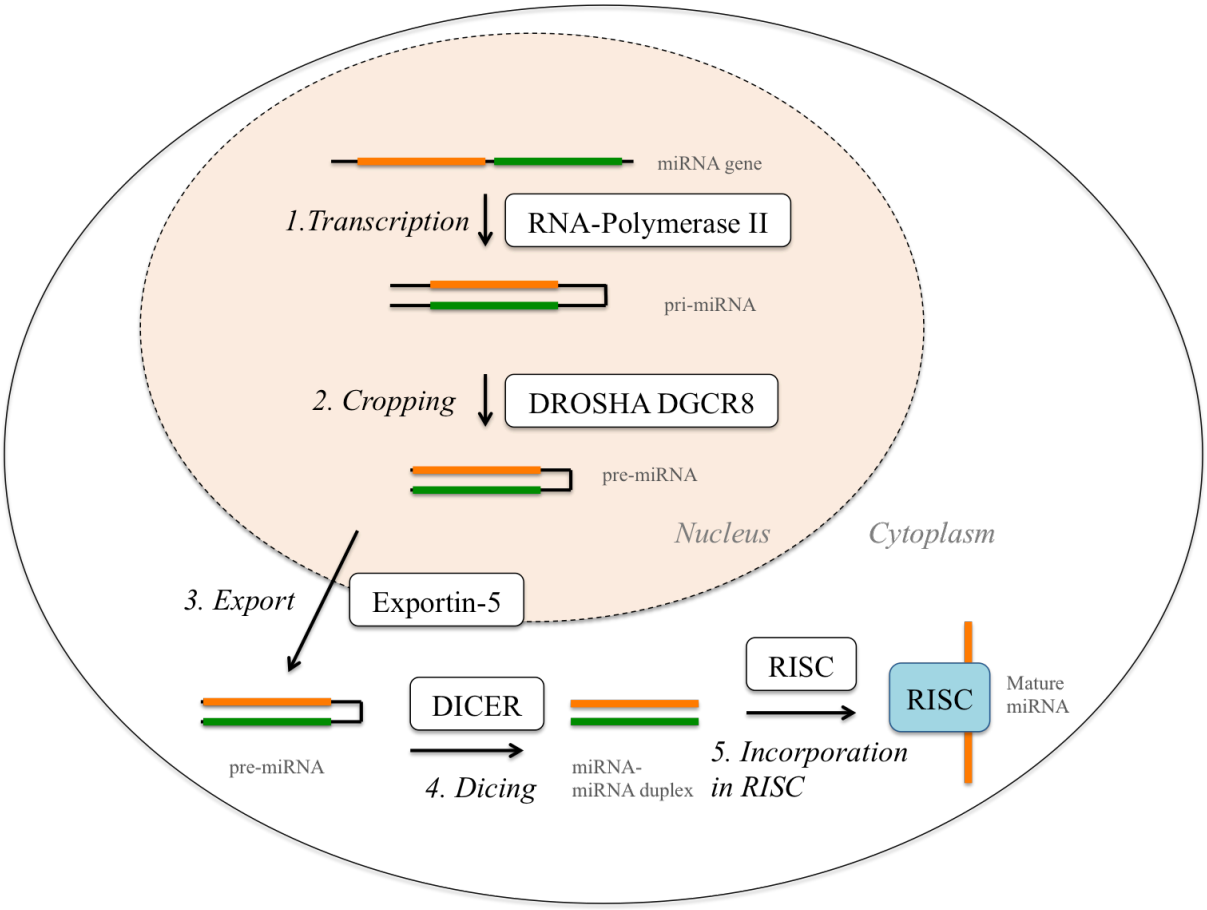

Figure 4: miRNA-Synthesis: miRNA biogenesis involves multiple steps (1.) Transcription of the miRNA gene requiring RNA Pol II for transcription of the 1–4 kb primary transcript called pri-miRNA, (2.) Cropping by nuclease Drosha-DGCR8 of the single-stranded sequences flanking the double-stranded stem–loop structure of the pre-miRNA precursor (length approximately 70nt) (3.) Export of the pre-miRNA from the nucleus to the cytoplasm by Exportin-5 and (4.) dicing by nuclease Dicer, cutting out the loop to generate the mature miRNA of about 22-nt that will be (5.) incorporated into the RISC. The miRNA guide strand is shown in orange, the passenger strand is shown in green. Adapted from (69)

Regulation of miRNAs is a complex process. It can affect either miRNA transcription or the mature miRNA. There are various targets for genes to interfere with miRNA-maturation in the synthesis process. Transcription can be compromised by transcription factors binding directly to the promoter region of the miRNA gene. Recent studies have also shown that DICER can be compromised in HCC (69). DICER is a transcriptional target of TAp63. As described above mutant p53 can interfere with p63 functions, resulting in a higher invasion rate (44,69).

In this thesis, we focused on four miRNAs, which have distinct functions in regulating tumor suppression and metastasis. The miRNAs were chosen based on previous data of our group concerning miRNAs regulated by p53 in HCC. Details on each miRNA are described below.

1.3.2 miR34a

miR34a is one of the most important tumor suppressive miRNAs known so far. It is downregulated in various carcinomas including HCC (70). miR34a has been shown to be downregulated in HCC (71,72). miR34a downregulation has been linked to higher rates of metastasis (73). In diabetic patients miR34a is significantly upregulated in HCC (74). miR34a is a direct target of p53 (75,76). It targets various tumor pathways such as the Bcl-2, Cyclin D1 and CDK6 pathway, resulting in tumor suppression by preventing cell proliferation and inducing apoptosis (77,78). It is commonly downregulated in HCC (79). In epidermal cells, miR34a is directly targeted and downregulated by p63 (78).

1.3.3 miR149

miR149 functions as tumor suppressor and is commonly repressed in HCC cells. Low miR149 levels result in low five-year survival rates and are poor prognosis factors (80). miR149 inhibits migration and invasion in HCC. This miRNA is often downregulated in HCC tissue, resulting in worse clinical prognosis (81).

1.3.4 miR192

miR192 is a tumor suppressive miRNA. It influences EMT by targeting zinc finger E-box-binding homeobox 1 and 2 (*ZEB1* and *ZEB2*), genes that are important for EMT (63). Recent studies have shown that ZEB1 plays an important role in invasiveness of lung cancer cells and metastasis formation (82). Changes in miR192 levels have been shown in non-alcoholic steatohepatitis (NASH) as well as HCC (83).

1.3.5 miR194

miR194 is another known tumor suppressive miRNA that targets various oncogenes in HCC. Overexpression of miR194 in HCC cells correlates with a lower migration rate und fewer metastatic lesions. It has been shown that miR194 can be downregulated in HCC (72,84). miR194 suppresses the expression of genes important for EMT and cancer metastasis such as N-cadherin (Cadherin 2, CDH2), type 1 insulin-like growth factor receptor (IGF1R), Ras-related C3 botulin toxin substrate 1 (RAC1) and heparin-binding epidermal growth factor-like growth factor (HBEGF) (83,84).

1.4 Aims of this thesis

Earlier studies from our group revealed that p53 and p73 influence specific miRNAs in HCC, yet little to nothing is known about the role of p63 in the regulation of miRNAs in HCC. Based on a microarray that identified target miRNAs of p53 in HCC, five different microRNAs, miR34a, miR145, miR149, miR192, and miR194, were selected for further analysis (85). It was known that a truncation of p63, ΔNp63, influences miR194 in squamous cell carcinoma (86). *In silico* analysis of gene sequences identified response elements for p63 in the DNA of miR145 and miR192 (87). Yet no other study has so far focused on the relationship between p63 and miRNA expression in HCC.

The main aim of this work was therefore to answer the question whether higher expression of p63 had an impact on the expression profiles of the above-mentioned miRNAs in HCC cells. In a second step, the effects of HCC relevant therapeutic agents (both systemic (targeted therapies: sorafenib, tivantinib, regorafenib) and agents acting locally (doxorubicin, bleomycin)) on miRNA levels in cells overexpressing p63 were analyzed.

These analyses aimed to evaluate the relations between p63 overexpression and levels of tumor-suppressive miRNAs to be able to aim for a better therapeutic approach in treatment of HCC depending on the expression of p63.

2. Materials and Methods

2.1 Materials

2.1.1 Devices

Biometra® T3 Thermocycler	Biometra GmbH, Göttingen, Germany
Heraeus Safety cabinet HS12	Kendro Laboratory Products GmbH, Hanau, Germany
Heraeus BB6220 CO_2 Incubator	Thermo Scientific, Waltham, USA
Heraeus Pico Centrifuge	Thermo Fisher Scientific, Langenselbold, Germany
Heraeus Megafuge 16R	Thermo Fisher Scientific, Langenselbold, Germany
LightCycler® 480 System	Roche Diagnostics GmbH, Mannheim, Germany
NanoPhotometer	Implen GmbH, Munich, Germany

2.1.2 Laboratory Equipment

BD Falcon 15ml/50ml	BD, Franklin Lakes, USA
Corning® costar® Stripette®	Corning Incorporated, Corning, USA
Corning® 125/75 cm^2 Cell Culture Flask	Merck KGaA, Darmstadt, Germany
costar® Cell Culture Plate Non Pyrogenic, 96 Wells	Corning Incorporated, Corning, USA
Counting Slides, Dual Chamber for Cell Counter	Bio-Rad Laboratories GmbH, Munich, Germany
Falcon™ Aspirating Pipets	Fisher Scientific, Hapmton, USA
Light Cycler® 480 Multiwell Plate 384, white	Roche Diagnostics GmbH, Mannheim, Germany
PCR Soft Tubes, 0,2ml	Biozym Scientific GmbH, Oldendorf, Germany
Safe Lock Tubes 1,5ml/2ml/5ml	Eppendorf AG, Hamburg, Germany

2.1.3 Reagents

Bleomycin	Hölzel Diagnostika Handels GmbH, Cologne, Germany
DMSO Hybri-Max®	Sigma-Aldrich Chemie GmbH, Munich, Germany
Doxorubicin	Hölzel Diagnostika Handels GmbH, Cologne, Germany
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich Chemie GmbH, Munich, Germany
GENTAMICIN	ratiopharm GmbH, Ulm, Germany
Minimum Essential Medium Eagle	Sigma-Aldrich Chemie GmbH, Munich, Germany
Regorafenib	Hölzel Diagnostika Handels GmbH, Cologne, Germany
Sorafenib	Hölzel Diagnostika Handels GmbH, Cologne, Germany
Tivantinib	Hölzel Diagnostika Handels GmbH, Cologne, Germany

2.1.4 Kits

Qiagen miRNEasy Mini Kit	Qiagen N.V., Venlo, Netherlands
ReliaPrep™ miRNA Cell and Tissue Miniprep System	Promega Corporation, Madison, USA
TaqMan® MicroRNA Reverse Transcription Kit	Applied Biosystems, Foster City, USA
TaqMan® Universal PCR Master Mix	Applied Biosystems, Foster City, USA
TaqMan® MicroRNA Assays	Applied Biosystems, Foster City, USA

2.1.5 Software

LightCycler® Software 1.5	Roche Diagnostics GmbH, Mannheim, Germany
Microsoft Excel	Microsoft Corporation, Redmond, USA

2.1.6 Cells

Hep3B

Hep3B cells have originally been derived from HCC tissue of an 8-year-old boy. They are p53-negative and p63- and p73- positive cells. The cells contain an integrated Hepatitis B virus genome and underlie S2 bio safety standards in Germany.

Medium:

Minimal Essential Medium (MEM)	500ml
Fetal calf serum (FCS)	50ml

2.2 Methods

2.2.1 Cell culture

Hep3B cells were cultured in 125 cm^2 or 75 cm^2 flasks containing 20 ml medium as described above. They were split at about 90% confluence, usually twice a week. For this, cells were washed with 10 ml PBS buffer to inactivate FCS. They were washed with 5 ml Trypsin and incubated at 37°C for 3-5 minutes. Fresh medium was used to bring the cells back into solution and the new cell suspension was distributed in 3-5 new flasks.

For the experiments, cells were seeded into 6 well plates with a concentration of 150.000 cells per 2 ml of medium. They were then cultured for 24 h before transducing them with adenoviruses.

2.2.2 Virus transfection

To infect cells with adenoviruses (rAd-GFP and rAdp63) 10 infectious units per cell were needed. The required amount of virus solution was suspended in medium. The old medium was replaced with the virus solution. After 4h virus-containing medium was removed and fresh virus-free medium supplemented with 50 µg/ml Gentamycin was added.

2.2.3 Treatment with HCC-relevant therapeutics

Transfected cells were treated with HCC-relevant chemotherapeutics and targeted therapies, respectively: doxorubicin 0.04 µM, sorafenib 4.5 µM, bleomycin 3µg/ml (2.12 µM) , tivantinib 5.4 µM, regorafenib 5.1 µM, and DMSO 0.02%. Concentrations used were based on the average plasma concentration in humans treated with the respective drugs.

2.2.4 Cell harvesting

For harvesting the whole medium was transferred to 2 ml or 15 ml tubes and cells were removed from the wells with cell scrapers. Wells were rinsed with 500 µl PBS to bring the scraped cells into solution. Tubes were then centrifuged at 1500 rpm for 5 minutes. The aqueous phase was discarded and the remaining pellet was washed with 500µl PBS. After centrifugation for 5min at 21.100 x g supernatants were discarded and cell pellets were dissolved in lysis buffer provided with the ReliaPrep^{TM} miRNA cell and tissue miniPrep system.

2.2.5 miRNA isolation

For miRNA isolation the ReliaPrep^{TM} miRNA cell and tissue miniPrep system were used. miRNA isolation was performed according to the manual provided by the manufacturers.

2.2.6 Reverse transcription

For reverse transcription the TaqMan® MicroRNA Reverse Transcription Kit was used. Reverse transcription was performed according to the manual provided by the manufacturer. Finally, a Biometra® T3 Thermocycler was used with the settings given by the manual of the Reverse Transcription Kit.

2.2.7 qPCR

Using TaqMan® Universal PCR Master Mix and TaqMan® MicroRNA Assays qPCR, reaction plates were pipetted following the instructions provided with the kit. Final measuring and qPCR were performed using the LightCycler® 480 System. For qPCR we used Thermo Fisher Primers (hsa-miR34a, hsa-miR149, hsa-miR192 and hsa-miR194). As a housekeeping gene we used RNU6B. qPCR was performed in replicates of three to eliminate errors in measurement. The

results were analyzed by calculating the means of the three replicates as well as the standard deviations. We then calculated ΔCt values and the mean of these values. From this mean, we calculated a single ΔΔCt value.

3. Results

3.1 miRNA expression after p63 overexpression

In the first set of experiments, our aim was to evaluate whether overexpression of TAp63 affected miRNA levels in Hep3B cells. To address this question, Hep3B cells were transduced with adenoviruses rAd-GFP used as control or rAd-p63 to overexpress p63. Control transfections with rAd-GFP were performed to evaluate, whether changes in miRNA levels were a result of TAp63 overexpression or due to the virus infection itself. As negative control un-transduced Hep3B cells were analyzed. To verify whether the infection was successful, both adenoviruses carried the *GFP* gene. Thus, all successfully transfected cells expressed the GFP protein and could be detected by fluorescence microscopy (see figure 5).

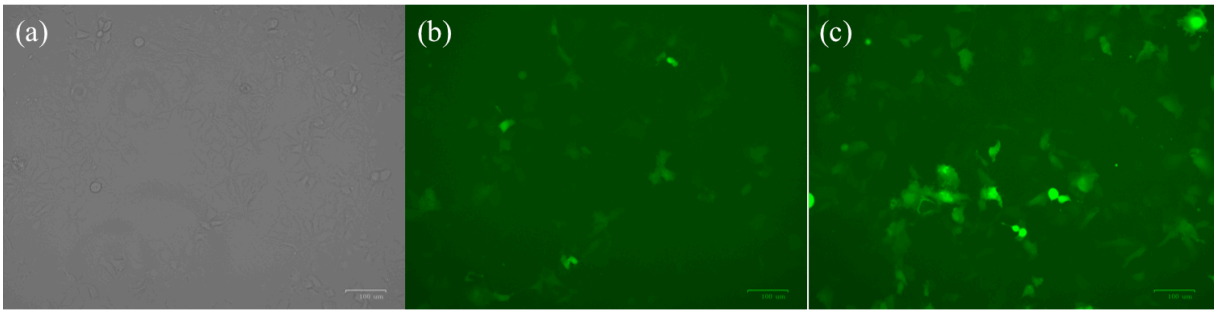

Figure 5: Fluorescence microscopy of (a) untransfected cells, (b) rAd-GFP transfected cells, (c) rAd-p63 transfected cells. GFP-dependent fluorescence 48h after transfection.

In a second set of experiments, expression level of *TP63* was measured in transfected cells using qPCR. In Figure 6 relative *TP63*-expression is shown. 48h after transfection with the overexpression vector rAd-p63, *TP63* mRNA levels increased by 11- and 24 fold in two p63-transfections in comparison to cells transduced with the corresponding control vector, rAd-GFP.

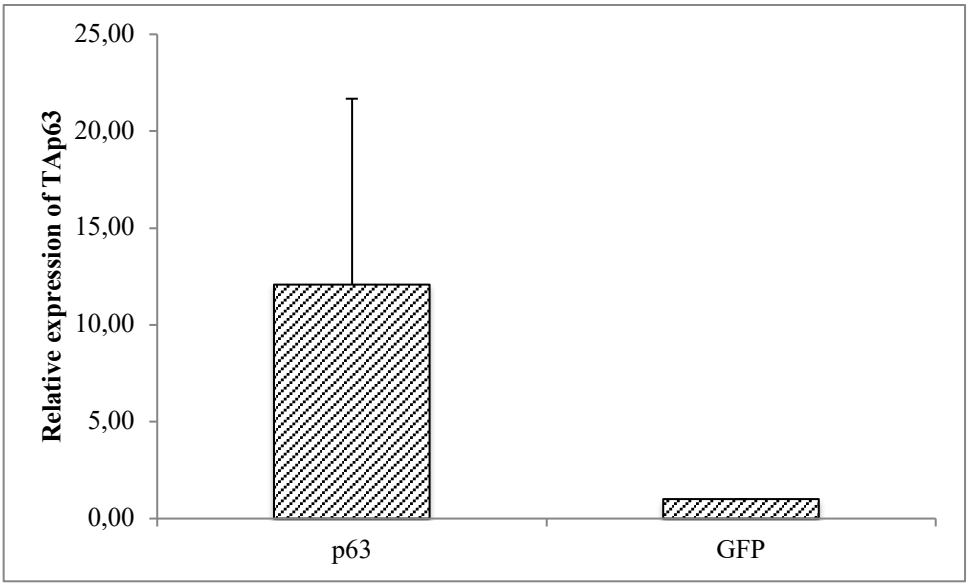

Figure 6: Relative expression (ΔΔCt value) of *TP63* in rAd-GFP- and rAd-p63- transduced cells measured by qPCR (n=3).

After determining successful transfection by detecting equal GFP fluorescence by microscopy, cells were harvested after 24 h, 48 h and 72 h after transfection, and miRNA levels were analyzed. Corresponding levels of p63-induced miRNAs were then compared to those in uninfected cells. Noteworthy, overexpression of TAp63 resulted in an increase in relative expression of all tested miRNAs.

3.1.1 Overexpression of TAp63 induces miR34a

The first miRNA examined was miR34a, a classical tumour suppressive miRNA that is also known to be influenced by p53.

In comparison to un-transded cells, in GFP transfected cells miR34a levels increased to 1.3-fold at 24h, decreased to 0.8-fold at 48h and increased again to 1.8-fold after 72h. Compared to this, miR34a levels in p63-overexpressing cells increased to almost 2-fold after 24h, dropped to 1.3-fold at 48h and increased again to 1.7-fold after 72h compared to untransfected cells (Table 1, Figure 7).

Table 1: Relative expression of miR34a after transfection with rAd-GFP and rAd-p63

Time after transfection	Relative expression of miR34a in untransfected cells	Relative expression of miR34a in rAd-GFP-transfected cells	Relative expression of miR34a in p63-overexpressing cells
24h	1	1.295	1.917
48h	1	0.76	1.332
72h	1	1.77	1.747

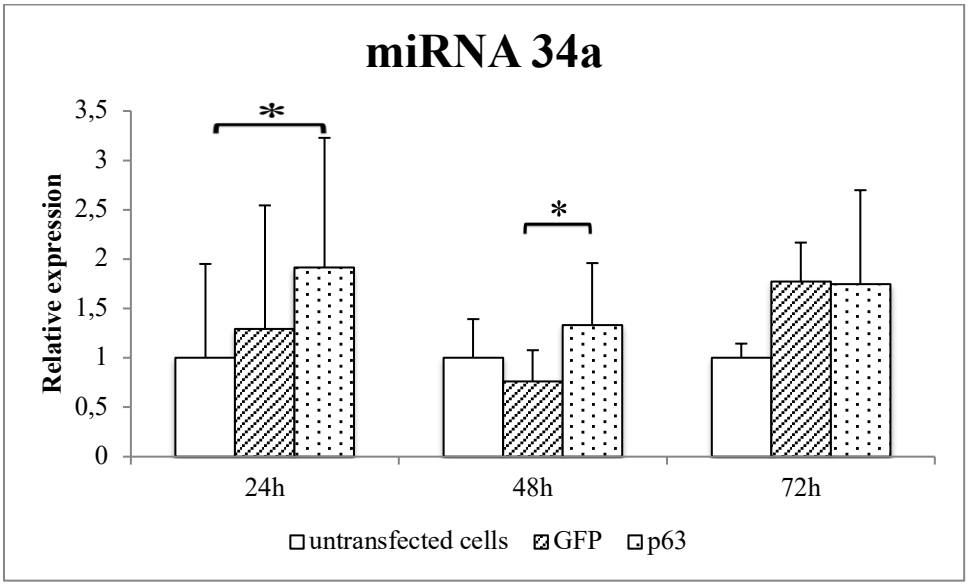

Figure 7: p63-overexpression induces miR34a**.** qRT-PCR analysis of relative expression of miR34a after transfection with vectors encoding rAd-GFP and rAd-p63 in relation to uninfected cells. Transfection of Hep3B cells with rAd-GFP (control) and rAd-p63, analysis of RNA-lysates 24h, 48h and 72h after transfection. These RNA lysates were examined for the expression of miR34a. p63 overexpression had the biggest impact on miR34a-expression 48h after transfection. Data are presented a means +standard deviation, n=3.

To show whether the effects witnessed were due to transfection with an adenovirus or due to p63 overexpression we used rAd-GFP transfected cells as an additional control. To analyze the effect p63 overexpression on miRNA levels regardless of viral effects, the relative expression of miR34a in cells overexpressing p63 was divided by relative expression of miR34a in cells transfected with rAd-GFP .

By deducting the virus effect, I could show that miR34a levels in p63-overexpressing cells rose to 1.75-fold at 24h, increased further to 2.179-fold at 48h and dropped back to normal levels after 72h (Table 2, Figure 8). Compared to miR34a levels in GFP-transfected cells, the increase in miR34a levels in p63 overexpressing cells has been proven to be significant. Thus, miR34a is a miRNA influenced by p63 expression in HCC.

Table 2: Relative Expression of miR34a after overexpression of p63 deducting the virus effect

Time after transfection	-fold induction	p-value
24h	1.75	0.229
48h	2.179	0.043*
72h	1.01	0.796

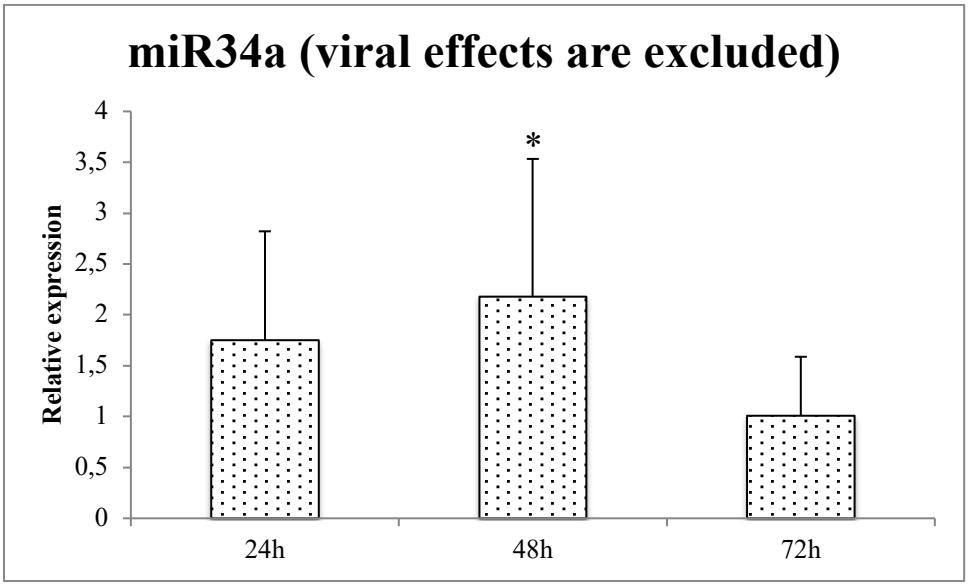

Figure 8: Relative Expression of miR34a after overexpression of p63 deducting the virus effect. Expression of miR34a after deducting the virus effect by dividing relative expression of miR34a after p63 overexpression by relative miR34a expression after rAd-GFP transfection. Highest relative expression of miR34a was observed 48h after p63-overexpression. Data are presented a means + standard deviation, n=3.

3.1.2 Overexpression of p63 induces miR149

The second miRNA examined was miR149, a tumor suppressive miRNA commonly repressed in HCC.

miR149 levels increased by 1.5-fold in GFP transfected cells after 24h, then decreased to 0.99-fold at 48h and increased again to 1.46-fold after 72h compared to un-transfected cells. p63-overexpressing cells showed an increase in miR149 levels of 2-fold after 24h compared to un-transfected cells. The levels further increased to 2.2-fold after 48h and then decreased to 1. 8-fold after 72h (Table 3, Figure 9). The increase in miR149 levels in p63-overexpressing cells compared to un-transfected cells after 24h was proven to be significant with a p value <0.05. miR149 levels were higher in all cells overexpressing p63.

Table 3: Relative expression of miR149 after transfection with rAd-GFP and rAd-p63

Time after ttransductio n	Relative expression of miR149 in un-transduced cells	Relative expression of miR149 in rAd-GFP-transduced cells	Relative expression of miR149 in p63-overexpressing cells
24h	1	1.489	1.965*
48h	1	0.99	2.198
72h	1	1.46	1.78

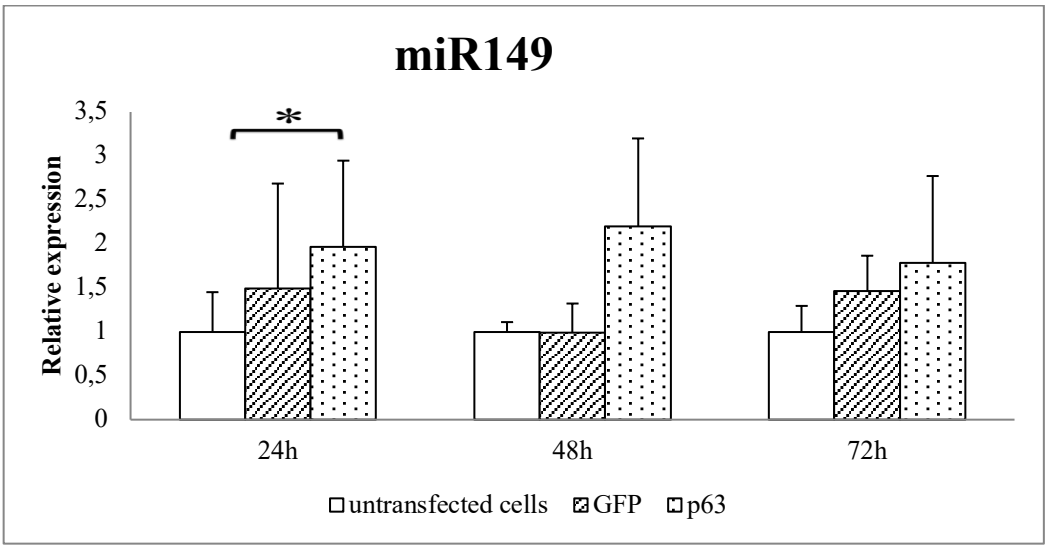

Figure 9: p63-overexpression induces miR149. qRT-PCR analysis of relative expression of miR149 after transfection with rAd-GFP and rAd-p63 in relation to uninfected cells. Transfection of Hep3B cells with rAd-GFP (control) and rAd-p63, analysis of RNA-lysates 24h, 48h and 72h after transfection. These RNA lysates were examined for the expression of miR149. p63-overexpression has the highest impact on miR149 expression 48h after transfection. Data are presented a means + standard deviation, n=3.

miR149 levels in p63-overexpressing cells increased by 1.614-fold 24h after transfection after subtraction of viral effects. miR149 expression further increased to 2.309-fold after 48h and decreased to 1.17-after 72h. The most prominent effect of p63-overexpression on miR149 levels was visible 48h after transfection (Table 4, Figure 10), demonstrating that p63-overexpression influences miR149 levels in HCC.

Table 4: Relative expression of miR149 after p63 overexpression deducting the virus effect

Time after transduction	-fold induction	p-value
24h	1.614	0.433
48h	2.309	0.096
72h	1.17	0.539

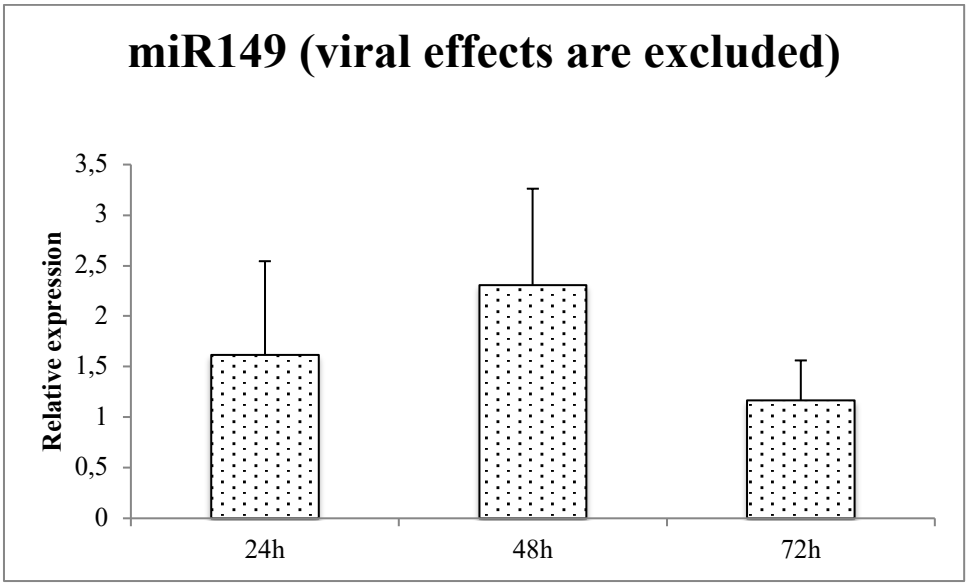

Figure 10: Relative expression of miR149 after p63 overexpression deducting the virus effect. Expression of miR149 after deducting the virus effect by dividing relative expression of miR149 after p63-overexpression by relative miR149 expression after rAd-GFP transfection. Highest relative expression of miR149 after 48h. Data are presented a means + standard deviation, n=3.

3.1.3 Overexpression of p63 induces miR192

As a third miRNA I examined miR192, a miRNA that plays a role in EMT. It has also been shown not to be influenced by p53 in HCC.

Compared to un-transfected cells, miR192 levels in GFP-transfected cells increased by 2-fold after 24h, decreased to 1.1-fold after 48h and stayed low at 1.2-fold after 72h. In p63-overexpressing cells miR192 levels increased by 3.9-fold 24h after transfection, decreased to 1.5-fold after 48h and increased again to 2.1-fold expression after 72h compared to un-transfected cells. The increase in miR192 expression after 24h in p63-overexpressing cells compared to un-transfected cells was significant (Table 5, Figure 11).

Table 5: Relative expression of miR192 after transfection with rAd-GFP and rAd-p63

Time after transduction	Relative expression of miR192 in untransduced cells	Relative expression of miR192 in rAd-GFP-transduced cells	Relative expression of miR192 in p63-overexpressing cells
24h	1	1.965	3.86*
48h	1	1.073	1.54
72h	1	1.2	2.07

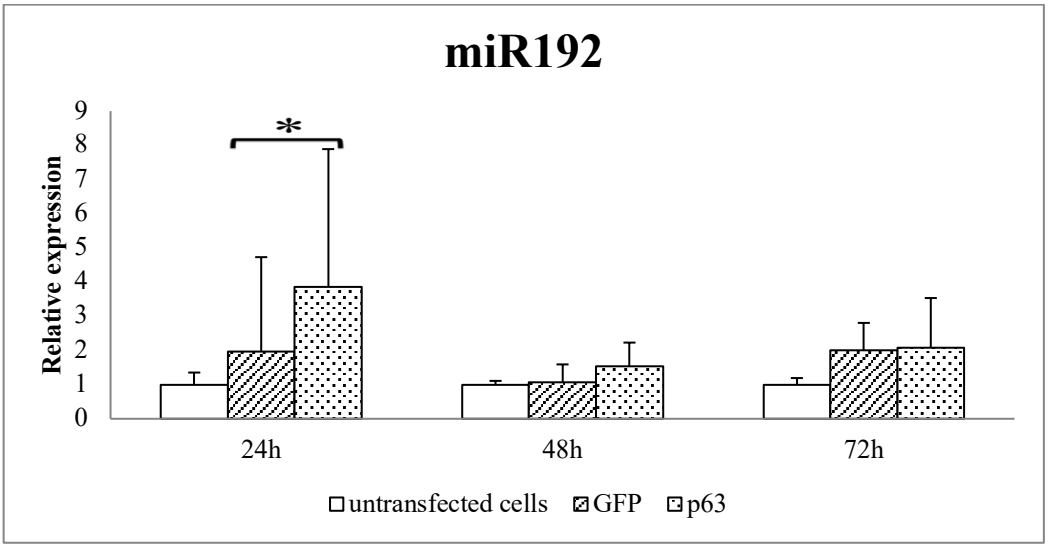

Figure 11: p63-overexpression induces miR192. Relative expression of miR192 after infection with rAdGFP and rAdp63 in relation to uninfected cells. qRT-PCR analysis of relative expression of miR192 after transfection with rAd-GFP and rAd-p63 in relation to uninfected cells. Transfection of Hep3B cells with rAd-GFP (control) and rAd-p63, analysis of RNA-lysates 24h, 48h and 72h after transfection. These RNA lysates were examined for the expression of miR192. p63-overexpression had the highest impact on miR192 expression 24h after transfection. Data a presented as means + standard deviation, n=3.

By deducting the virus effect, we could show that miR192 levels increase by 2.77-fold in p63-overexpressing cells 24h after transfection, slightly decrease to 2.21-fold after 48h and then decrease further to 0.94-fold after 72h (Table 6, Figure 12). The most prominent effect of p63-overexpression on miR192 levels was detected after 24h. I conclude that p63-overexpression induces miR192 in HCC.

Table 6: Relative expression of miR192 after p63 overexpression deducting the virus effect

Time after transduction	-fold induction	p-value
24h	2.77	0.259
48h	2.21	0.13
72h	0.94	0.98

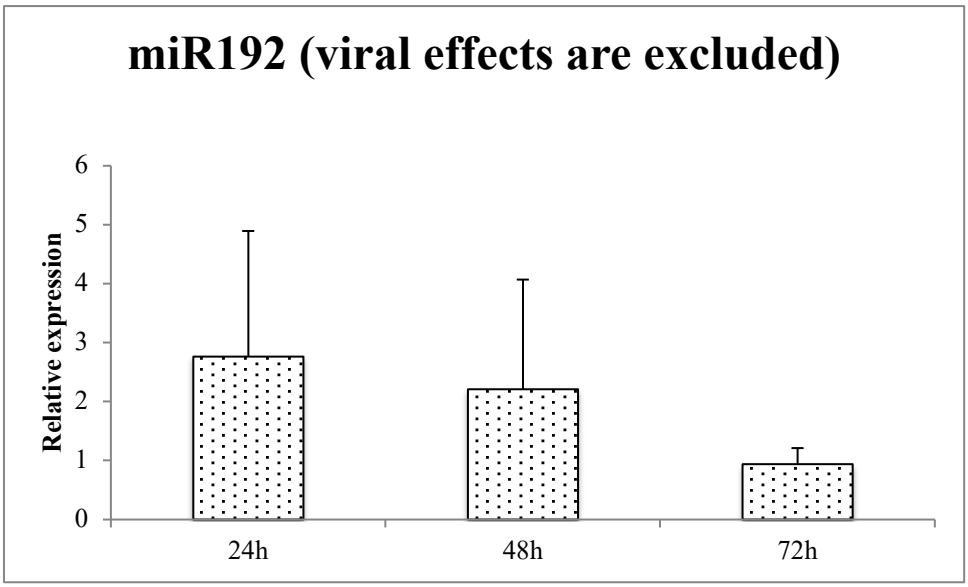

Figure 12: Relative expression of miR192 after p63 overexpression deducting the virus effect. Expression of miR192 after deducting the virus effect by dividing relative expression of miR192 after p63-overexpression by relative miR192 expression after rAd-GFP transfection. Highest relative expression of miR192 24h after p63-overexpression. Data are presented as means + standard deviation, n=3.

3.1.4 Overexpression of p63 induces miR194

As a fourth miRNA we examined miR194, a tumor suppressive miRNA that targets various oncogenes in HCC. It also suppresses genes responsible for EMT and metastasis. Like miR192 miR194 levels are not influenced by p53 in HCC.

miR194 levels in GFP-transfected cells rose to 2.86-fold 24h after transfection, dropped back to 1.01-fold 48h after transfection and increased again to 1.90-fold 72h after transfection compared to un-transfected cells. miR194 levels in p63-overexpressing cells increased by 5.26-fold after 24h, decreased to 1.55-fold after 48h and stayed the same at 1.51-fold after 72h compared to un-transfected cells (Table 7, Figure 13).

Table 7: Relative expression of miR194 after transfection with rAd-GFP and rAd-p63

Time after transduction	Relative expression of miR194 in untransduced cells	Relative expression of miR194 in rAd-GFP-transfected cells	Relative expression of miR194 in p63-overexpressing cells
24h	1	2.86	5.26
48h	1	1.01	1.55
72h	1	1.90	1.51

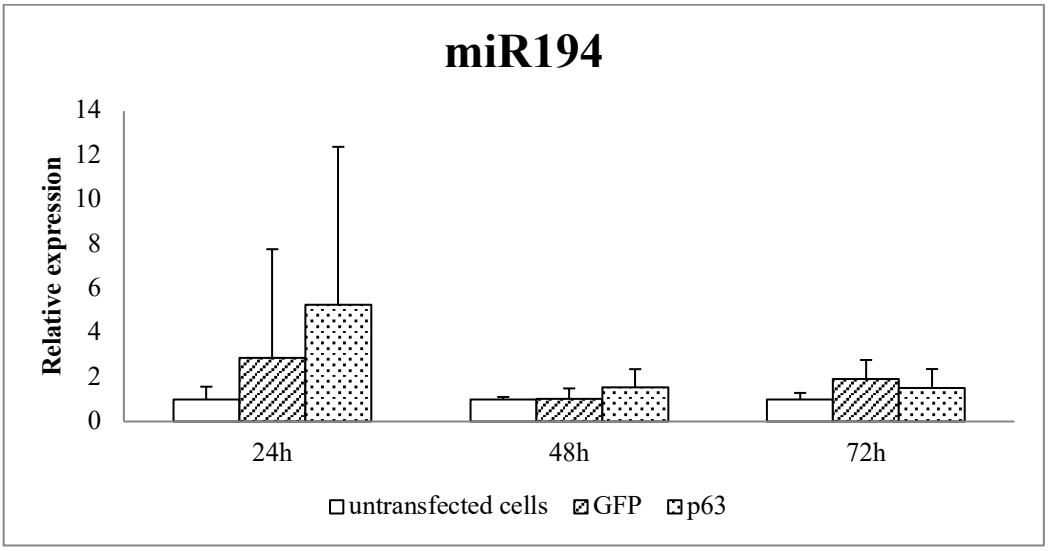

Figure 13: p63-overexpression induces miR194. qRT-PCR analysis of relative expression of miR194 after transfection with rAd-GFP and rAd-p63 in relation to uninfected cells. Transfection of Hep3B cells with rAd-GFP (control) and rAd-p63, analysis of RNA-lysates 24h, 48h and 72h after transfection. These RNA lysates were examined for the expression of

miR194. p63-overexpression had the highest impact on miR194 expression 24h after transfection. Data are presented as means + standard deviation, n=3.

By deducting the virus effect, we could show that miR194 levels in p63-overexpressing cells rose to 2.35-fold at 24h, decreased to 1.83-fold after 48h and further decreased to 0.768-fold 72h after transfection (Table 8, Figure 14). The most prominent effect of p63-overexpression on miR194 levels was visible 24h after transfection.

Table 8: Relative expression of miR194 after p63 overexpression deducting the virus effect

Time after transduction	-fold increase	p value
24h	2.35	0.36
48h	1.83	0.116
72h	0.768	0.417

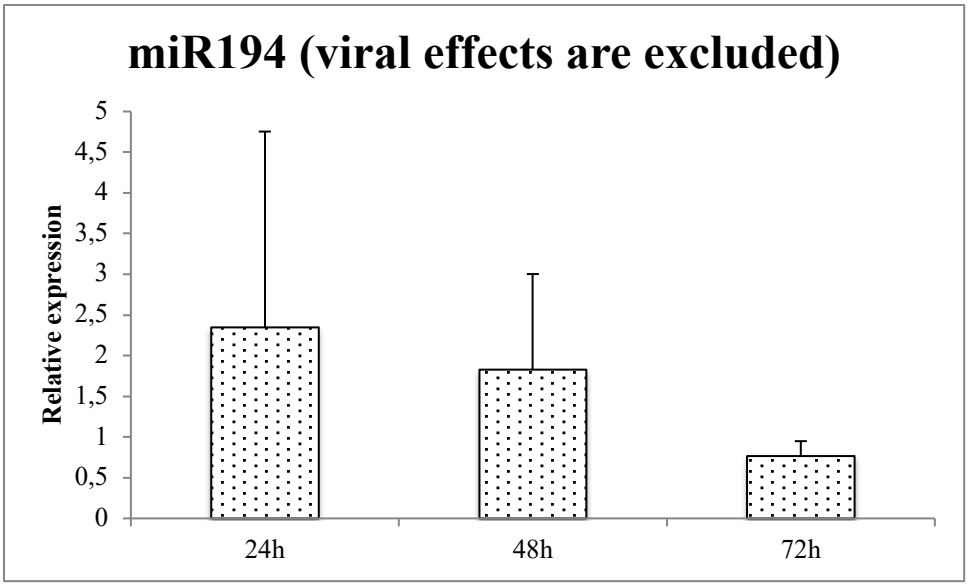

Figure 14: Relative expression of miR194 after p63 overexpression deducting the virus effect. Expression of miR194 after deducting the virus effect by dividing relative expression of miR194 after p63 overexpression by relative miR34a expression after rAd-GFP transfection. Highest relative expression of miR194 was observed 24h after p63-overexpression. Data are presented as means + standard deviation, n=3.

Taken together overexpression of p63 influences the expression profiles of miR34a, miR149, miR192 and miR194 in Hep3B cells. In cells overexpressing p63 regardless of viral effects on

miRNA levels, miR34a and miR149 showed the greatest increase in expression after 48h whilst miR192 and miR194 showed the greatest increase in expression after 24h. Thus, I could show that p63-overexpression has an influence on the expression of all miRNAs examined.

3.2 Effect of HCC-relevant drugs on miRNA levels in p63 overexpressing cells

Next, I asked whether p63 overexpression affected miRNA levels after treatment with chemotherapeutic agents and targeted therapies used for treating HCC in patients. To clarify this topic, we treated Hep3B cells transfected with either rAd-GFP (as a control) or rAd-p63 and thus overexpressing p63 with HCC relevant drugs. We used doxorubicin, sorafenib, bleomycin, tivantinib and regorafenib that are commonly applied as local or systemic therapy in HCC. The doses used were based on the average plasma concentrations of these drugs measured in adults after HCC treatment (27,30). These were 0.04 µM of doxorubicin, 4.5 µM of sorafenib, 3 µg/ml of bleomycin, 5.4 µM of tivantinib and 5.1 µM of regorafenib.
Cells were transfected with rAd-GFP or rAd-p63 24h after splitting the cells to enable prior cell growth and were treated with doxorubicin, sorafenib, bleomycin, tivantinib and regorafenib after additional 24h. Cells were then harvested 24h, 48h or 72h after the treatment. As a control, we used Hep3B cells infected with rAd-GFP and treated with DMSO, the vehicle of the drugs used here. By means of these controls we were able to evaluate whether the observed effects were a result of the combination of HCC relevant drugs and overexpression of p63 or mediated by transfection or the solvent.

3.2.1 Effect of HCC-relevant drugs on miR34a expression in p63-overexpressing cells

We used rAd-GFP transfected cells treated with DMSO as a control. All changes in miRNA levels were compared to these controls.
Doxorubicin. Treatment with doxorubicin in p63-overexpressing cells led to a 5.13-fold increase in miR34a expression after 72h. In comparison, cells transfected with rAd-GFP and treated with doxorubicin showed an increase in miR34a expression of 1.38-fold after 24h and 1.39-fold after 72h (Table 9, Figure 15). Deducting the virus effect, doxorubicin lead to a 0.54-fold decrease after 24h in p63-overexpressing cells and a 4.72-fold increase after 72h. DMSO-treated controls overexpressing p63 show an increase in miR34a levels by 2.08-fold

after 72h. In p63-overexpressing cells treated with doxorubicin miR34a levels increase by 4.72-fold, displaying a synergistic effect doxorubicin and p63 have on miR34a-expression (Table 10, Figure 16).

Sorafenib. This drug had a similar effect on miR34a in p63-overexpressing cells as doxorubicin. After 24h the expression of miR34a increased by 1.74-fold in rAd-p63 transfected cells compared to a 0.61-fold decrease in rAd-GFP transfected cells. After 72h the expression of miR34a increased about 4-fold in p63 overexpressing cells, while the levels rAd-GFP transfected cells remained unchanged (Table 9, Figure 15). Deducting the viral effect on miRNA levels, miR34a increased by 3.14-fold after 24h and 3.65-fold after 72h in p63-overexpressing cells. In DMSO treated cells overexpressing p63 miR34a levels increased by 2.08-fold after 72h. In sorafenib treated cells overexpressing p63 miR34a levels increased by 3.65-fold. This suggests a synergistic effect p63-overexpression and treatment with sorafenib have on miR34a expression (Table 10, Figure 16).

Bleomycin increased miR34a expression in p63-overexpressing cells significantly to almost 2-fold after 24h and 4-fold at 72h, compared to a decrease to only marginal changes in rAd-GFP transfected cells (Table 9, Figure 15). Minus the viral effect miR34a levels in p63-overexpressing cells showed an increase by 4.66-fold after 24h and 4.1-fold after 72h. Taking into account, that miR34a levels increase by 2.08-fold in DMSO treated cells overexpressing p63 this suggests that p63-overexpression and treatment with bleomycin exert a synergistic effect on miR34a levels.

Tivantinib. Treatment with tivantinib led to a 2.33-fold increase of miR34a levels after 24h in rAd-p63 transfected cells compared to a 1.38-fold increase in rAd-GFP transfected cells. After 72h the levels of miR34a were increased by 3.11-fold in p63 overexpressing cells while they slightly increased to 1.43-fold in rAd-GFP transfected cells (Table 9, Figure 15). Without the virus effect this equals an increase in miR34a levels in p63-overexpressing cells by 1.71-fold after 24h and 2.13-fold after 72h. The increase in miR34a levels 72h after treatment is about the same to DMSO-treated p63-overexpressing cells. Tivantinib had no additional effect on miR34a levels (Table 10, Figure 16).

Regorafenib showed a slightly smaller effect on the expression of miR34a. The levels increased by 1.39 -old after 24h and 2.06-fold after 72h in rAd-p63 transfected cells. In comparison the miR34a levels decreased by factor 0.77 after 24h in rAd-GFP transfected cells and increased to 1.18-fold after 72h (Table 9, Figure 15). Deducting the viral effect this equals an increase of miR34a levels in p63-overexpressing cells by 1.69-fold after 24h and 1.98-fold after 72h. Compared to DMSO-treated p63-overexpressing cells the effect on

miR34a expression remains the same, suggesting that regorafenib itself does not affect miR34a expression (Table 10, Figure 16).

Based on these observations, it can be concluded that HCC therapeutics enhance the p63-dependent induction of miR34a expression. The most pronounced effects were observed 72h after treatment. After 24h except from doxorubicin no drugs showed an induction greater than in the control. In cells treated with doxorubicin, sorafenib and bleomycin we could show synergistic effects to p63 overexpression on miR34a levels.

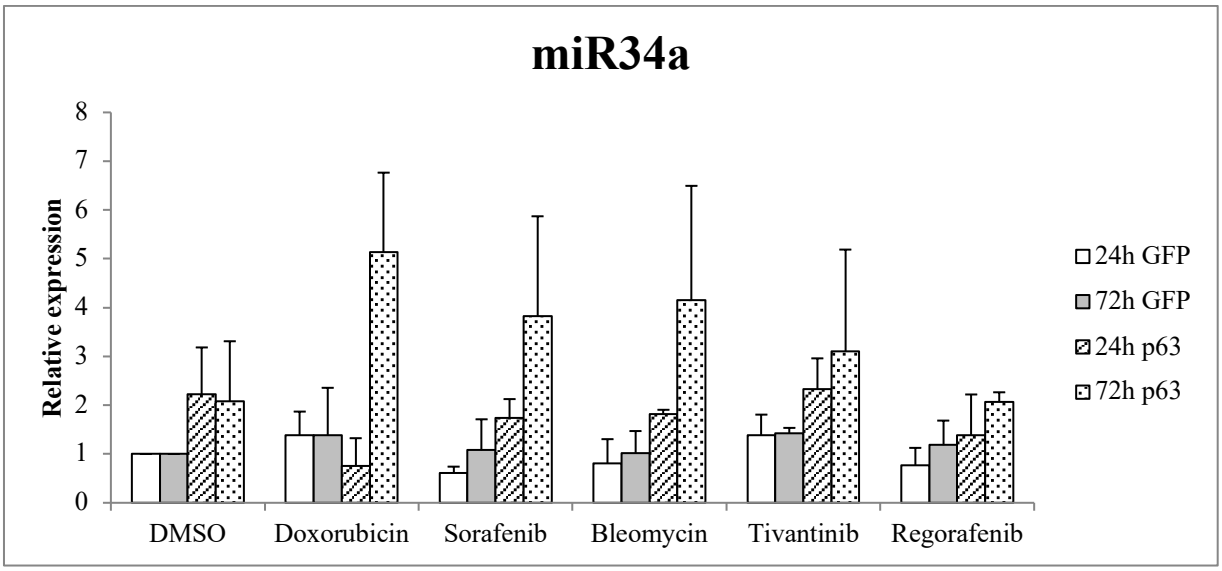

Figure 15: Treatment of Hep3B cells with HCC relevant therapeutics increases miR34a expression in p63-overexpressing cells. Relative expression of miR34a in rAdGFP- and rAdp63-transfected cells after treatment with HCC relevant drugs (doxorubicin, sorafenib, bleomycin, tivantinib and regorafenib) compared to DMSO-treated cells with normal p63 expression at the respective times. Data are presented as means + standard deviation, n=3.

Table 9: Relative expression of miR34a after treatment with HCC-relevant drugs and transduction with rAd-GFP and rAd-p63

Induction rate of miR34a				
	24h rAd-GFP	72h rAd-GFP	24h rAd-p63	72h rAd-p63
DMSO	1	1	2.22	2.08

doxorubicin	1.38	1.387	0.749	5.13
sorafenib	0.61	1.079	1.74	3.82
bleomycin	0.81	1.01	1.81	4.15
tivantinib	1.38	1.43	2.33	3.11
regorafenib	0.77	1.18	1.385	2.065

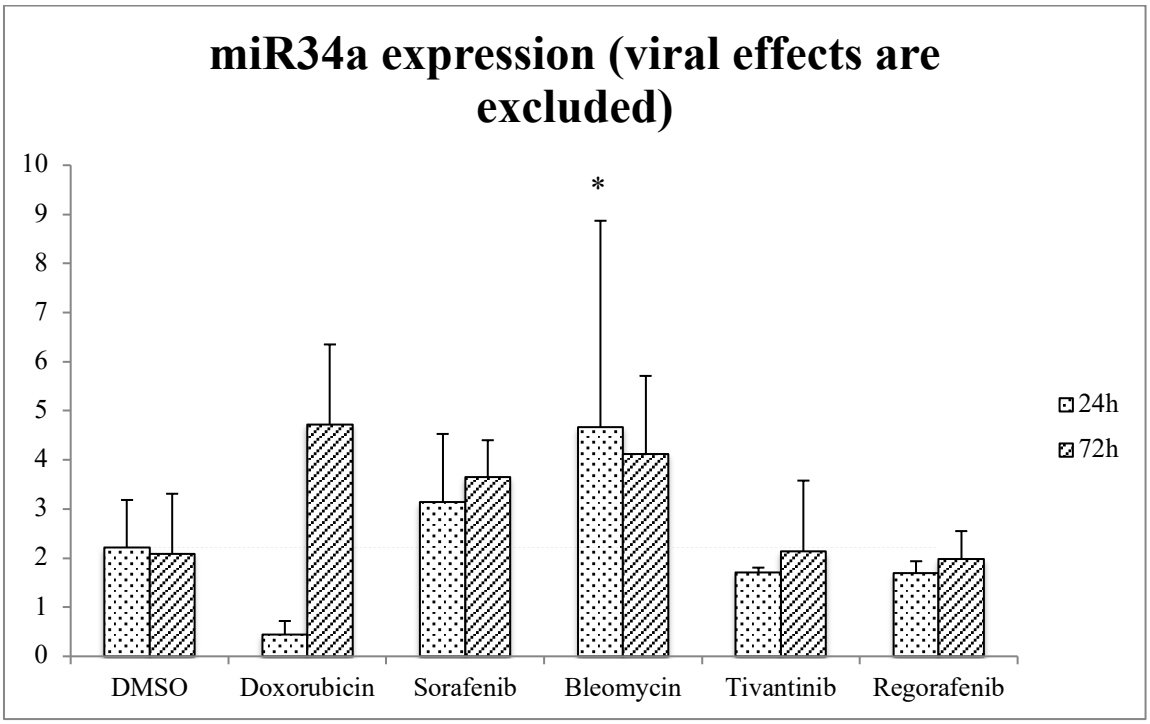

Figure 16: Expression of miR34a after treatment with HCC-relevant drugs in p63-overexpressing cells deducting the viral effect. Data shown is relative expression of miR34a in p63-overexpressing cells divided by relative expression of rAd-GFP transfected cells, line drawn at relative expression in DMSO treated cells after 24h. Data are presented as means + standard deviation, n=3.

Table 10: Expression of miR34a after treatment with HCC-relevant drugs in p63-overexpressing cells deducting the viral effect

Induction rate of miR34a				
	24h	p value 24h	72h	p value 72h
DMSO	2.22	0.148	2.08	0.282

doxorubicin	0.445	0.3	4.72	0.05
sorafenib	3.14	0.1	3.64	0.145
bleomycin	4.66	0.048*	4.12	0.137
tivantinib	1.71	0.4	2.13	0.7
regorafenib	1.69	0.392	1.98	0.082

3.2.2 Effect of HCC-relevant drugs on miR149 levels in p63-overexpressing cells

Doxorubicin. miR149 expression showed a considerable increase in p63-overexpressing cells 72h after treatment with doxorubicin (8.09-fold). 24h after treatment with doxorubicin miR34a levels increased by 3.48-fold in p63-overexpressing cells in comparison to 1.47-fold in rAd-GFP transfected controls. miR149 levels in the controls didn't change after 72h (Table 11, Figure 17). Deducting the viral effect doxorubicin thus led to a 2.37-fold increase in p63 overexpressing cells after 24h and a significant increase by 10.3-fold after 72h. In DMSO-treated cells overexpressing p63 miR149 levels increased by 2.01-fold after 72h. They increased by 10.3-fold in p63-overexpressing cells treated with doxorubicin, showing a synergistic effect of p63-overexpression and treatment with doxorubicin on miR149 levels (Table 12, Figure 18).
Sorafenib. 24h after treatment with sorafenib the expression of miR149 in p63-overexpressing cells by increased by 2.18-fold. 72h after treatment miR149 levels increased to 3.03-fold in rAdp63-transfected cells compared to 1.66-fold in rAd-GFP transfected cells (Table 11, Figure 17). Deducting the viral effects sorafenib lead to a 2.61-fold increase of miR149 levels after 24h and 2.11-fold after 72h. These effects are minor compared with cells overexpressing p63 treated with DMSO only, suggesting that the effect on miR149 expression is mainly a result of p63-overexpression (Table 12, Figure 18).
Bleomycin. Treatment with bleomycin increased the expression of miR149 in p63-overexpressing cells by 2.58-fold 24h after treatment compared to rAd-GFP transfected cells where the levels remained at 1.08-fold relative expression. After 72h miR149 levels in p63-overexpressing cells increased by 3.91-fold and decreased to 0.25-fold in the controls (Table 11, Figure 17). Deducting the viral effect this equals a 4.24-fold increase after 24h and a significant 15.64-fold increase after 72h in p63 overexpressing cells treated with bleomycin. miR149 expression in p63-overexpressing cells treated with DMSO increased by 2.01-fold in comparison to cells with normal p63 levels after 72h. In p63-overexpressing cells the increase

in miR149 levels after 72h is even higher, suggesting a synergistic effect of p63 overexpression and treatment with bleomycin on miR149 levels (Table 12, Figure 18).

Tivantinib. 24h after treatment with tivantinib miR149 levels increased by 1.22-fold in rAd-GFP transfected cells and by 3.37-fold in p63-overexpressing cells. Deducting the viral effect treatment with tivantinib in p63-overexpressing cells increased relative expression of miR149 by 3.34-fold. 72h after treatment miR149 levels in the controls increased to 1.63-fold and decreased to 2-fold in p63-overexpressing cells (Table 11, Figure 17). Without the virus effect this equals a significant increase in miR149 expression in p63-overexpressing cells of 1.06-fold 72h after treatment with tivantinib (Table 12, Figure 18).

Regorafenib. 24h after treatment with regorafenib miR149 levels decreased to 0.69-fold in the controls and increased to 1.44-fold in p63-overexpressing cells. This equals an increase by 3.56-fold deducting the viral effect in p63-overexpressing cells 24h after treatment with regorafenib. 72h after treatment miR149 levels in p63-overexpressing cells increased to 1.64-fold and in the controls to 0.84-fold (Table 11, Figure 17). Deducting the viral effect miR149 levels 72h after treatment with regorafenib increased by almost 2-fold in p63 overexpressing cells. The effect after 72h in p63-overexpressing cells is about the same in cells only treated with DMSO, suggesting it is mainly due to p63-overexpression rather than treatment with regorafenib (Table 12, Figure 18).

Based on these observations it can be concluded that treatment with HCC therapeutics leads to higher miR149 expression in p63-overexpressing cells. Especially the chemotherapeutic agents doxorubicin and bleomycin led to a major increase in miR149 expression 72h after treatment, suggesting a synergistic effect of these therapeutics and p63-overexpression on miR149 expression. A 2-fold increase of p63-dependent miR149 expression was noted after 24h treatment with all used HCC drugs. It has to be noted that these changes after 24h also occurred in p63-overexpressing cells only treated with the solvent DMSO. Only miR149 levels 72h after treatment with doxorubicin and bleomycin in p63-overexpressing cells exceeded miR149 expression in p63-overexpressing cells after treatment with the solvent.

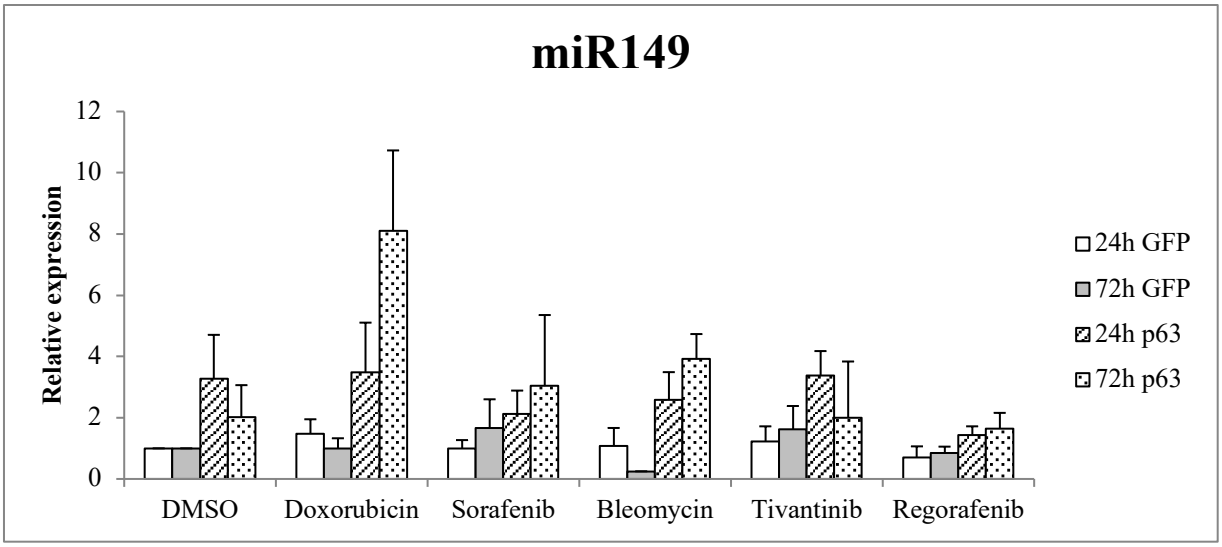

Figure 17: Treatment with local HCC relevant therapeutics induces miR149 expression in p63-overexpressing cells.

Relative expression of miR149 in rAd-GFP- and rAd-p63-transfected cells after treatment with HCC relevant drugs (doxorubicin, sorafenib, bleomycin, tivantinib and regorafenib). Data are presented as means + standard deviation; n=3.

Table 11: Relative expression of miR149 after treatment with HCC-relevant drugs and transfection with rAd-GFP and rAd-p63

Induction of miR149				
	24h GFP	72h GFP	24h p63	72h p63
DMSO	1	1	3.274	2.01
Doxorubicin	1.47	0.985	3.48	8.095
Sorafenib	0.99	1.657	2.128	3.034
Bleomycin	1.08	0.25	2.578	3.91
Tivantinib	1.217	1.63	3.37	2.003
Regorafenib	0.694	0.839	1.44	1.64

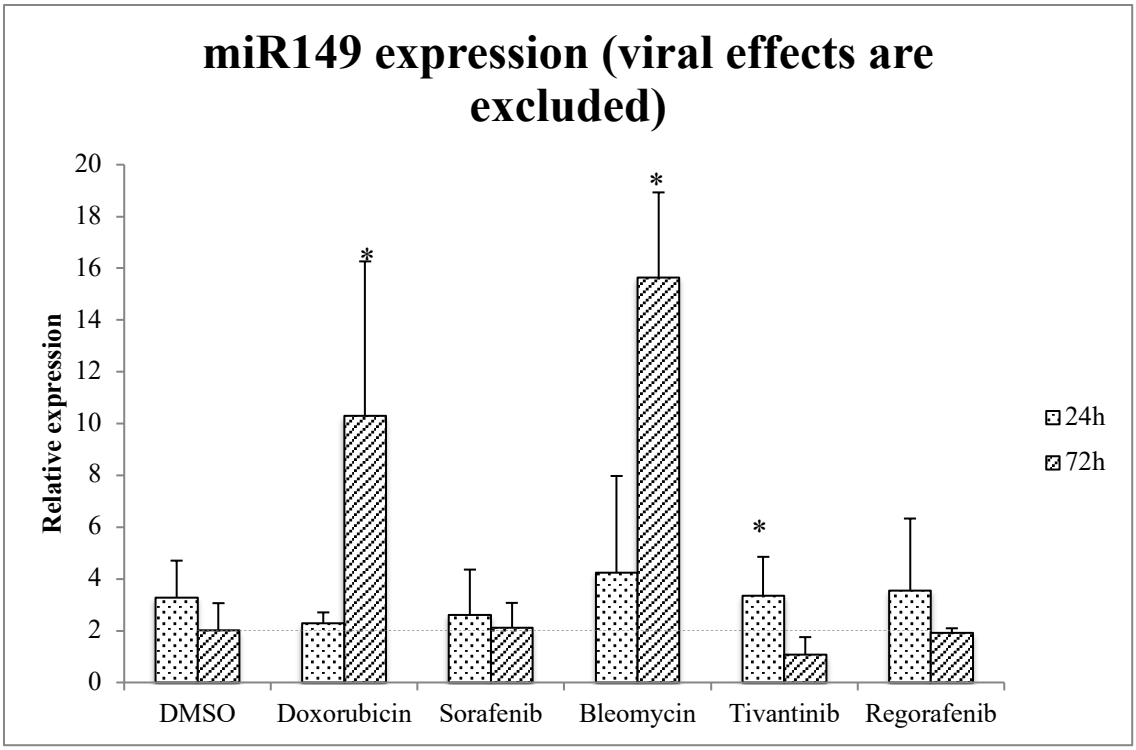

Figure 18: Expression of miR149 after treatment with HCC-relevant drugs in p63 overexpressing cells deducting the viral effect. Data shown is relative expression of miR149 in p63-overexpressing cells divided by relative expression of rAd-GFP transfected cells, line drawn at relative expression of miR149 in DMSO treated cells after 72h, Data are presented as means + standard deviation, n=3, significant values with $p<0.05$ are marked with an asterisk.

Table 12: Expression of miR149 after treatment with HCC-relevant drugs in p63-overexpressing cells deducting the viral effect, significant values with $p<0.05$ are marked with an asterisk

	24h	p value 24h	72h	p value 72h
DMSO	3.274	0.088	2.01	0.248
doxorubicin	2.27	0.169	10.29	0.019*
sorafenib	2.60	0.117	2.11	0.481
bleomycin	4.24	0.122	15.65	0.003*
tivantinib	3.34	0.033*	1.06	0.803
regorafenib	3.56	0.083	1.91	0.114

3.2.3 Effect of HCC-relevant drugs on miR192 expression in p63-overexpressing cells

Doxorubicin. Treatment with doxorubicin led to 3.31-fold increased levels of miR192 in p63-overexpressing cells after 24h in comparison to rAd-GFP transfected cells, where miR192 levels increased by 2.84-fold. After 72h miR192 levels dropped slightly to 2.86-fold while they dropped to 1.07-fold in the controls (Table 13, Figure 19). Deducting the viral effect this equals an increase of miR192 expression by 0.90-fold after 24h and by 3.29-fold after 72h in p63-overexpressing cells treated with doxorubicin. With only a slight increase in miR192 expression in DMSO treated cells overexpressing p63 after 72h, the effect after 72h in p63-overexpressing cells treated with doxorubicin was mainly due to treatment rather than p63-overexpression (Table 14, Figure 20).

Sorafenib. 24h after treatment with sorafenib, miR192 levels in p63-overexpressing cells increased by 4.23-fold and decreased to 0.89-fold in rAd-GFP transfected cells (Table 13, Figure 19). Without the viral effect this equals an increase by factor 3.81 after 24h. 72h after treatment with sorafenib miR192 levels dropped slightly to 3.01-fold. At 24h p63-overexpressing cells treated with DMSO showed an increase in miR192 levels of 3.341-fold. After treatment with sorafenib miR192 levels increase by 3.81-fold in p63-overexpressing cells. This suggests a synergistic effect p63-overexpression and treatment with sorafenib on miR192 levels 24h after treatment (Table 14, Figure 20).

Bleomycin. Treatment with bleomycin led to an increase in miR192 expression by 1.72-fold in p63-overexpressing cells and to a decrease to 0.77-fold in rAd-GFP transfected cells after 24h. After 72h miR192 levels dropped to 1.03-fold expression in p63-overexpressing cells and remained about the same in the control (Table 13, Figure 19). Without the viral effect this equals an increase in miR192 expression by 2.28-fold after 24h and a slight decrease back to 1.4-fold after 72h. miR192 expression in cells treated with bleomycin is about the same to miR192 expression in cells treated with DMSO, suggesting that the effect on miR192 expression is mainly due to p63-overexpression (Table 14, Figure 20).

Tivantinib. 24h after treatment with tivantinib miR192 levels increased by 1.48-fold in p63-overexpressing cells and decreased to 0.56-fold in the controls. 72h after treatment miR192 levels rose to a 2.72-fold increase in p63-overexpressing cells while remaining unchanged in the controls (Table 13, Figure 19). Deducting the viral effect this means an increase in miR192 expression by 1.84-fold 24h after treatment with tivantinib and a further increase by 2.77-fold after 72h. At 72h miR192 expression does not increase in DMSO treated cells overexpressing

p63 while it increases by 2.77-fold in p63-overexpressing cells treated with tivantinib. The effect on miR192 is supposedly mainly due to treatment with tivantinib (Table 14, Figure 20).
Regorafenib. Treatment with regorafenib led to an increase by 1.31-fold in p63-overexpressing cells after 24h and a decrease to 0.83-fold in rAd-GFP transfected cells. After 72h miR192 levels remained at 1.23-fold in p63-overexpressing cells and rose slightly in the controls (Table 13, Figure 19). Without the viral effect this equals in an increase of miR192 expression by 1.85-fold after 24h and 1.27-fold after 72h in p63-overexpressing cells (Table 14, Figure 20). Thus, treatment with regorafenib had no impact on miR192 expression.

From these observations we conclude that doxorubicin, sorafenib and tivantinib, therapeutics commonly used in HCC treatment have an inductive effect on miR192 expression in p63 overexpressing cells. Treatment with doxorubicin and sorafenib has a slight effect on miR192 levels. This effect is about the same in DMSO treated cells, thus it is more likely to be an effect of p63-overexpression rather than the treatment itself. 72h after treatment with doxorubicin, sorafenib and tivantinib miR192 levels were higher in p63-overexpressing cells in comparison to DMSO treated cells. Sorafenib and p63-overexpression seem to have synergistic effects on miR192 expression. Treatment with doxorubicin, sorafenib and tivantinib showed an increase in miR192 expression 72h after treatment, while p63-overexpression had no impact.

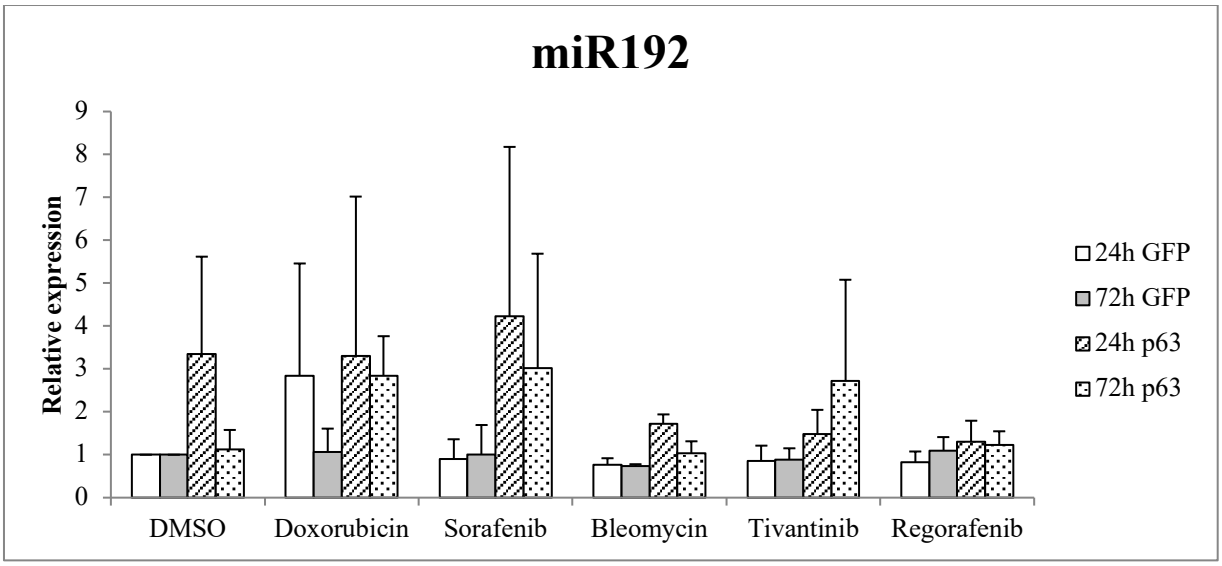

Figure 19: Treatment with HCC relevant therapeutics induces miR192 expression in p63-overexpressing cells. Relative expression of miR192 in rAd-GFP- and rAd-p63-transfected cells after treatment with HCC relevant drugs (doxorubicin, sorafenib, bleomycin, tivantinib and regorafenib). Data are presented as means + standard deviation; n=3.

Table 13: Relative expression of miR192 after treatment with HCC-relevant drugs and transfection with rAd-GFP and rAd-p63

	24h GFP	72h GFP	24h p63	72h p63
DMSO	1	1	3.341	1.122
doxorubicin	2.836	1.067	3.31	2.837
sorafenib	0.895	1.005	4.234	3.012
bleomycin	0.772	0.74	1.718	1.035
tivantinib	0.858	0.883	1.482	2.716
regorafenib	0.83	1.102	1.306	1.227

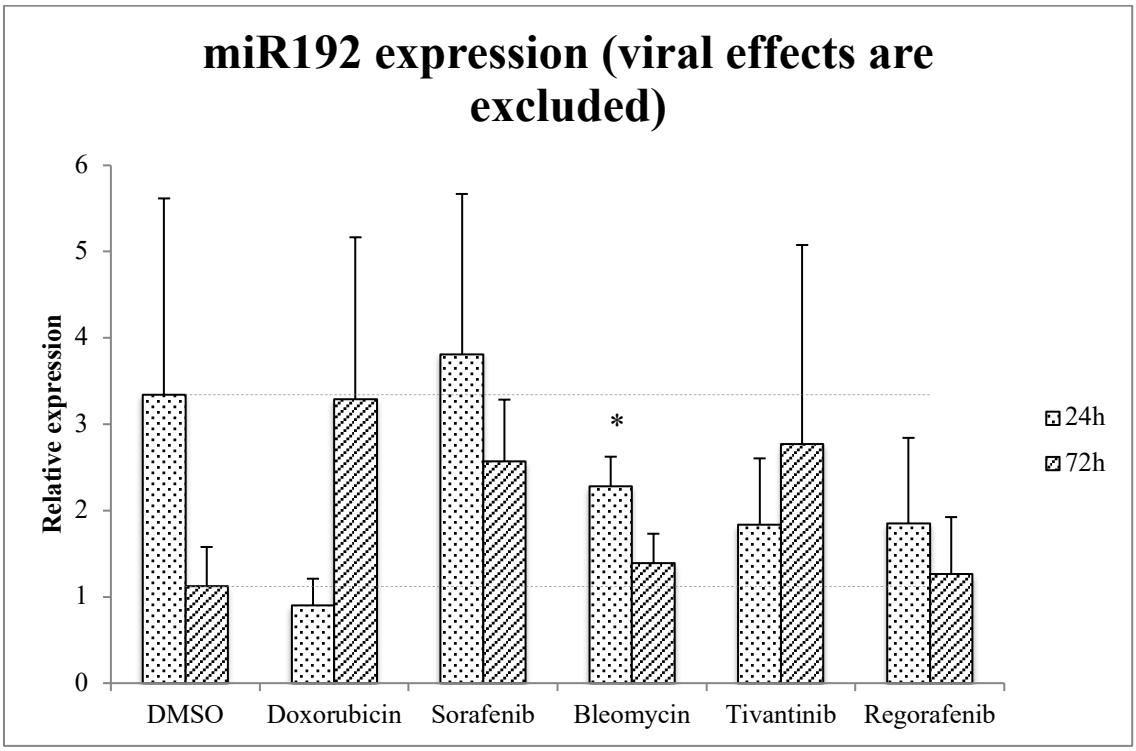

Figure 20: Expression of miR192 after treatment with HCC-relevant drugs in p63-overexpressing cells deducting the viral effect. Data shown is relative expression of miR192 in p63-overexpressing cells divided by relative expression of rAd-GFP transfected cells, lines drawn at miR192 levels in cells treated with DMSO, Data are presented as means + standard deviation, n=3

Table 14: Expression of miR192 after treatment with HCC-relevant drugs in p63-overexpressing cells deducing the viral effect

	24h	p value 24h	72h	p value 72h
DMSO	3.341	0.219	1.122	0.725
Doxorubicin	0.90	0.7	3.291	0.08
Sorafenib	3.81	0.3	2.57	0.362
Bleomycin	2.27	0.007*	1.39	0.212
Tivantinib	1.84	0.256	2.77	0.314
Regorafenib	1.85	0.285	1.27	0.712

3.2.4 Effect of HCC-relevant drugs on miR194 expression in p63 overexpressing cells

Doxorubicin. Doxorubicin did not have a great impact on miR194 expression in p63-overexpressing cells after 24h with no increase of miR194 levels at all. 72h after treatment miR194 levels increased slightly by 1.63-fold compared to 0.9-fold in the controls (Table 15, Figure 21). Deducting the viral effect this equals an increase by 1.86-fold 72h after treatment with doxorubicin (Table 16, Figure 22).

Sorafenib. 24h after treatment with sorafenib miR194 levels in p63-overexpressing cells increased by 1.7-fold in comparison to a decrease to 0.55-fold in rAd-GFP transfected cells. 72h after treatment miR194 levels continued to increase to 2.33-fold in p63 overexpressing cells and increased slightly to 0.86-fold in the controls (Table 15, Figure 21). Without the viral effect this equals an increase by 3.32-fold after 24h and an increase in miR194 levels by 2.28-fold 72h after treatment with sorafenib. miR194 levels increased slightly in p63-overexpressing cells treated with DMSO to 1.57-fold after 24h. Thus, p63-overexpression and treatment with sorafenib have synergistic effects on miR194 levels after 24h (Table 16, Figure 22).

Bleomycin. 24h after treatment with bleomycin, miR194 levels increased by 1.49-fold in p63-overexpressing cells and decreased to 0.65-fold in rAd-GFP transfected cells. After 72h miR194 levels decreased to 1.14-fold in p63-overexpressing cells and increased to 0.8-fold in the controls (Table 15, Figure 21). Deducting the viral effect this equals an increase in miR194 expression in p63-overexpressing cells by 3.09-fold after 24h and 1.36-fold after 72h. miR194 levels increase slightly by 1.57-fold in cells overexpressing p63 treated with DMSO after 24h.

Treatment with bleomycin and p63-overexpression seem to have synergistic effects on miR194 levels 24h after treatment (Table 12, Figure 22).

Tivantinib. Regarding all therapeutics used in this experiment tivantinib had the strongest impact on miR194 expression. 24h after treatment miR194 levels increased slightly by 1.61-fold in p63-overexpressing cells. 72h after treatment miR194 levels increased by 3.38-fold in p63-overexpressing cells and decreased to 0.74-fold in the controls (Table 15, Figure 21). Without the viral effect this equals an increase by 4.50-fold in p63-overexpressing cells after 72h. miR194 expression remained unchanged in p63-overexpressing cells treated with DMSO at 72h. Thus, the effect seen at 72h is likely due to treatment with tivantinib rather than p63-overexpression (Table 16, Figure 22).

Regorafenib. 24h after treatment with regorafenib miR194 levels increased slightly by 1.43-fold in p63-overexpressing cells compared to rAd-GFP transfected cells where miR194 levels decreased to 0.57-fold (Table 15, Figure 21). Deducting the viral effect this equals an increase by 2.69-fold in p63-overexpressing cells 24h after treatment with regorafenib. After 72h these effects were not detectable anymore. At 24h miR194 levels increase slightly in p63-overexpressing cells treated with DMSO. Regorafenib and p63-overexpression seem to have synergistic effects on miR194 levels 24h after treatment (Table 16, Figure 22).

Taken together, only targeted therapies (sorafenib, tivantinib, regorafenib) had an impact on miR194 levels in p63-overexpressing cells. The effect tivantinib had on miR194 expression at 72h was mainly due to tivantinib rather than p63-overexpression. Sorafenib and regorafenib showed synergistic effects with p63-overexpression on miR194 levels 24h after treatment. Cytotoxic drugs (doxorubicin, bleomycin) had no impact on miR194 expression in p63-overexpressing cells. Effects of tivantinib and regorafenib on miR194 expression 24h after treatment could be proven to be significant.

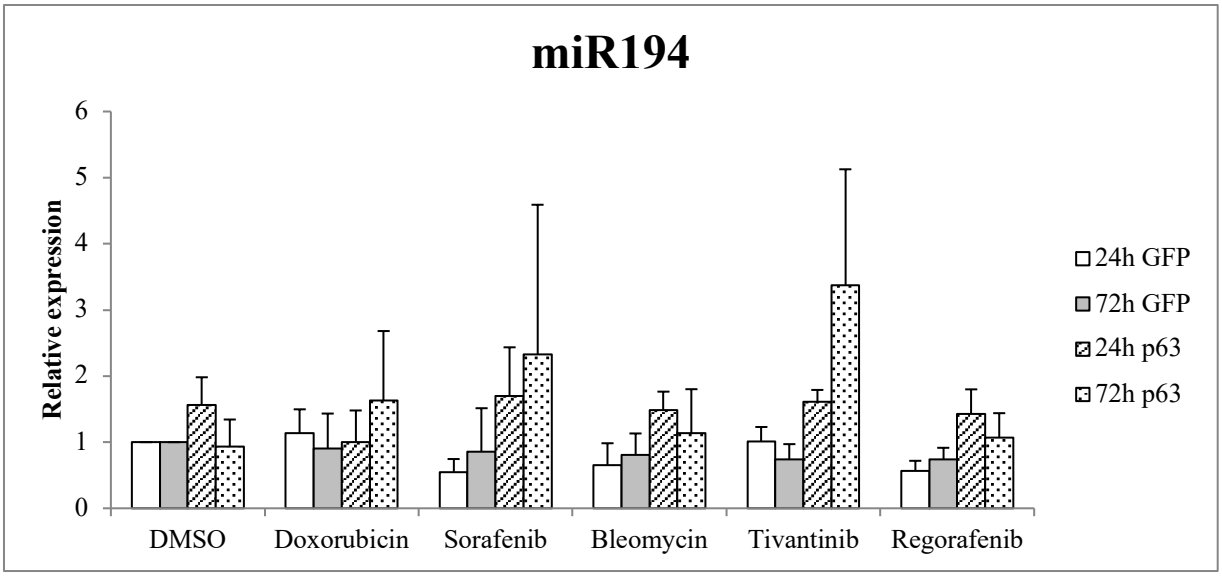

Figure 21: Treatment with systemic HCC relevant therapeutics induces miR194 expression in p63-overexpressing cells. Relative expression of miR194 in rAdGFP- and rAdp63-transfected cells after treatment with HCC relevant drugs (doxorubicin, sorafenib, bleomycin, tivantinib and regorafenib). Data are presented as means + standard deviation; n=3.

Table 15: Relative expression of miR194 after treatment with HCC-relevant drugs and transfection with rAd-GFP and rAd-p63

	24h GFP	72h GFP	24h p63	72h p63
DMSO	1	1	1.57	0.94
Doxorubicin	1.138	0.9	1.00	1.63
Sorafenib	0.55	0.859	1.697	2.326
Bleomycin	0.653	0.807	1.489	1.137
Tivantinib	1.009	0.74	1.611	3.375
Regorafenib	0.568	0.742	1.43	1.066

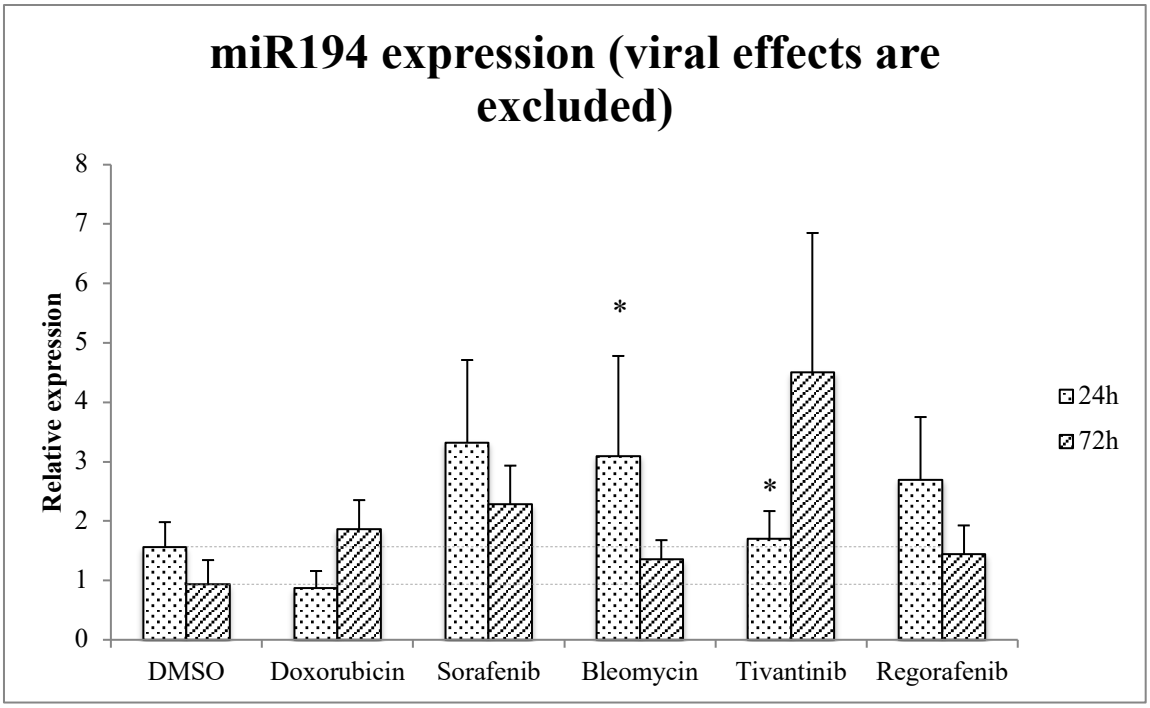

Figure 22: Expression of miR194 after treatment with HCC-relevant drugs in p63-overexpressing cells deducting the viral effect. Data shown is relative expression of miR194 in p63 overexpressing cells divided by relative expression of rAd-GFP transfected cells, lines drawn at miR194 levels in cells treated with DMSO. Data are presented as means + standard deviation, n=3.

Table 16: Expression of miR194 after treatment with HCC-relevant drugs in p63-overexpressing cells deducting the viral effect

	24h	p value 24h	72h	p value 72h
DMSO	1.57	0.127	0.937	0.836
doxorubicin	0.88	0.761	1.859	0.433
sorafenib	3.32	0.101	2.29	0.428
bleomycin	3.09	0.051	1.36	0.562
tivantinib	1.698	0.04*	4.50	0.103
regorafenib	2.69	0.038*	1.44	0.326

3.2.5 Conclusion

As previously shown in 3.1 all miRNAs examined were affected by p63-overexpression 24h after treatment (equaling 48h after transfection). The effect p63-overexpression had on the

miRNAs examined was visible in miR34a and miR149 expression 72h after treatment (86h after transfection). In miR192 and miR194 sorafenib and tivantinib were responsible for the increase in miRNA expression 72h after treatment, while p63-overexpression did not have an effect at that time. We observed synergistic effects of p63-overexpression and treatment on miRNA levels in miR34a with doxorubicin, sorafenib and bleomycin 72h after treatment. p63-overexpression and treatment with doxorubicin and bleomycin, both cytotoxic chemotherapeutics, had synergistic effects on miR149 levels 72h after treatment. p63-overexpression and treatment with sorafenib, a targeted therapy, had synergistic effects on miR192 and miR194 expression 24h after treatment. Treatment with bleomycin and regorafenib and p63-overexpression had synergistic effects on miR192 expression 24h after treatment.

In conclusion, chemotherapeutics such as doxorubicin and bleomycin affected miR34a and miR149, classical tumor suppressive miRNAs, while targeted therapies such as sorafenib and tivantinib had a bigger impact on miR192 and miR194 expression.

Table 17: Overview of miRNAs influenced by p63-overexpression, miRNAs influenced mainly by therapeutics and miRNAs influenced by synergistic effects of p63-overexpression and treatment

miRNA	influenced by p63-overexpression	influenced mainly by treatment	synergistic effects of treatment and p63-overexpression
miR34a	24h and 72h after treatment	-	72h after treatment with doxorubicin, sorafenib, bleomycin
miR149	24h and 72h after treatment	-	72h after treatment with doxorubicin, bleomycin
miR192	24h after treatment	72h after treatment with doxorubicin, sorafenib, tivantinib	24h after treatment with sorafenib
miR194	24h after treatment	72h after treatment with sorafenib, tivantinib	24h after treatment with bleomycin, sorafenib, regorafenib

4. Discussion

Although the function of p63 and its isoforms in cancer has been examined in a range of studies, it is still not very well understood. Here I evaluated the impact of p63 overexpression on the expression of four miRNAs, miR34a, miR149, miR192 and miR194 in the HCC cell line Hep3B. Furthermore, I showed that several commonly used chemotherapeutics (doxorubicin, bleomycin) and targeted therapies (sorafenib, tivantinib, regorafenib) alter miRNA expression independent of expression of p53 family members p53 and p63.

I showed that p63-overexpression in Hep3B cells also changes the levels of tumor suppressive miRNAs miR34a, miR149, miR192 and miR194. Drug treatment directly affects expression of selected miRNA levels. The expression of miR34a increased after treatment with doxorubicin, sorafenib and bleomycin. It further increased in cells overexpressing p63, suggesting a synergistic effect both p63 overexpression and treatment have on miR34a levels.

Levels of miR149 increased after treatment with doxorubicin and bleomycin and as miR34a miR149 levels increased even further in cells overexpressing p63, suggesting additive and synergistic effects treatment and p63-expression have on miR149 levels. miR192 expression increased after treatment with doxorubicin, sorafenib and tivantinib, independent of p63 levels, and the expression of miR194 increases after treatment with sorafenib and tivantinib, independent of p63-expression. Synergistic effects of sorafenib and p63-overexpression on miR192 levels could be seen 24h after treatment. Sorafenib, bleomycin and tivantinib seemed to have additive effects on miR194 levels in p63-overexpressing cells. These data provide an improved foundation for future treatment of HCCs overexpressing p63. As miRNA levels change depending on both p63 levels as well as therapeutic agents, it is possible that cancers showing a change in p63-expression react different to a specific treatment compared to those with normal p63 expression.

By choosing p53-deficient Hep3B cells we eliminated the effect p53 has on the miRNAs examined, as our previous studies showed that p53 increases the expression of miR34a and miR149 (85).

4.1 p63-expression has an impact on miRNA expression in HCC

4.1.1 miR34a and miR149 are classic tumor suppressive miRNAs influenced by both p63-overexpression and chemotherapeutic substances

Both miR34a and miR149 were increased after overexpressing p63 in Hep3B cells. Previous studies by our group could also show this effect after overexpression of p53 (85).
miR34a inhibits HCC growth *in vitro* and *in vivo* by inducing apoptosis (79). Low miR34a levels in HCC lead to higher rates of migration and invasion. Whereas a regulation loop between miR34a and p53 seems to exist in HCC (88) and miR34a is a direct target of p53, the relation between miR34a and p63 in HCC has not been examined yet. TAp63-deficient mice show suppressed miR34a levels. The same study found p63 binding sites in the promoter region of miR34a (60). miR34a has been found to influence the proto-oncogene MET in gastric cancer cells. It suppresses MET's functions and thus leads to reduced migration and invasion rates in gastric cancer (89). Furthermore, a loss of function of miR34a leads to a higher rate of EMT in gastric cancer (90). These data indicate that also p63 targets miR34a, thus leading to decreased migration and invasion rates of cells, as well as EMT.
miR149 influences tumorigenesis in HCC. Suppressed levels of miR149 in HCC lead to higher tumor masses, higher invasion rates and increased malignancy, correlating with a low 5-year survival rate (80), and ΔNp63α leads to a downregulation of miR149 in keratinocytes (91). As mentioned above ΔNp63α suppresses EMT and thus metastasis despite being the oncogenic isoform of p63.
It still remains to be shown that these effects occur in patient cells, however my *in vitro* studies suggest that also p63 plays a role in regulation of miR34a and miR149 in HCC cells. Further investigations need to clarify whether miR34a and miR149 are direct targets of p63 in HCC cells and which effects p63 overexpression and overexpression of miR34a and miR149 exert on migration, invasion and apoptosis in HCC cells *in vivo*.

Clinically relevant HCC therapeutics (doxorubicin, sorafenib, bleomycin and tivantinib) had a stronger influence on miR34a in p63-overexpressing cells. miR149 is mostly affected by only classic chemotherapeutic drugs (doxorubicin, bleomycin). Whereas the effects of doxorubicin on miR34a expression were not tested so far, overexpression of miR34a in diffuse large B-cell lymphoma leads to a better response to treatment with doxorubicin as well as a lower chemoresistance against doxorubicin (92). It has also been shown that overexpression of

miR34a enhanced the inhibitory effects of doxorubicin on HepG2 cells (71). My observations nicely agree with these previous findings.

Doxorubicin affects the expression of miR149 in triple negative breast cancer cells. High miR149 levels lead to a better response to treatment (93). Low miR149 expression is linked to chemoresistance to doxorubicin in breast cancer (94). It is possible that similar effects occur in HCC, though this has not been examined yet.

Like observed for p63, p53 overexpression increases miR34a and miR149 levels in cells treated with doxorubicin (studies conducted by our group), suggesting that both, p53 and p63, operate in similar ways.

miR34a expression in p63-overexpressing cells treated with bleomycin is about 2-fold increased (Table 9, Figure 15). Previous studies of our group have shown an influence of bleomycin treatment on p53-dependent miR34a-expression (studies conducted by our group), but no other studies have shown a link between miRNA expression and treatment with bleomycin in HCC so far. In fibrotic lung tissue, Shetty *et al.* showed that bleomycin treatment of p53-depleted cells did not influence the levels of miR34a, suggesting that expression of p53 is important for bleomycin-dependent upregulation of miR34a (95). We show that miR34a is influenced by bleomycin in p53-depleted cells in HCC, suggesting that other p53 family members play a role in bleomycin-induced upregulation of miR34a. It therefore can be important to assess the role other p53 family members play in bleomycin-dependent upregulation of miR34a. Bleomycin itself can lead to accumulation of p53, p63 and p73, thus having an impact on the p63-dependent expression of miRNAs (30). It would therefore be important to analyze p63-levels after bleomycin treatment to determine if the effect on the miRNAs tested is related to overexpression of p63 induced by bleomycin itself.

The inductive effects of sorafenib on miR34a expression were stronger in p63 overexpressing cells than in cells with regular p63 levels (Table 10, Figure 16), indicating that p63 in combination with sorafenib additively affect miRNA34a levels and thus the effectiveness of this treatment. Indeed, treatment with sorafenib is more effective in cells with higher miR34a levels (77).

It is possible that chemotherapeutic drugs induce not only miRNA expression, but also boost p63 expression and therefore the expression of miRNAs controlled by p63. To clarify this, p63 levels in cells treated with the specific substances have to be examined. While we do not know the detailed pathway yet, treatment of p63-overexpressing cells with doxorubicin and bleomycin leads to an upregulation of miR34a and miR149 *in vitro* and thus might result in higher apoptotic rates as well as decelerated tumor growth *in vivo* by the mechanisms described

above. Further studies on p63-depleted, or -overexpressing cancer cells will be important to evaluate whether chemosensitivity is due to overexpression of miRNAs caused by the treatment rather than a side effect of increased p63 levels.

4.1.2 miR192 and miR194 are part of a genetic network in cells

p53 and p63 are both proapoptotic members of the p53 family, however show significant differences in their expression profiles. In our studies we could show that both miR192 and miR194 expression increase 24h after overexpression of p63, but not p53 (studies conducted by our group). To understand the relevance of p63 dependent regulation of miR192 and miR194 it is important to see their function in a network of genes interacting in cancer cells.

Previous studies from our group suggested that ΔNp63 acts as an oncogene rather than a tumor suppressor (66). TAp63 instead influences tumor metastasis by targeting DICER and miR130b (60). Low TAp63 levels and those of the miRNA-processing enzyme DICER correlate with aggressive tumors and higher rates of metastasis. miR130b like miR192 is another tumorsuppressive miRNA that interferes with ZEB1 synthesis and thus leads to lower migration and invasion rates (96). For further understanding of the effects the miRNAs examined have on EMT it would be important to show whether high levels of miR192 and miR194 also lead to lower migration and invasion rates. In recent studies it has been shown that miR192 and miR194 can be downregulated in HCC cells lacking HNF4α, a nuclear factor controlling tissue differentiation in livers (97).

The role of p63 isoforms has been examined more closely in context of EMT. High ΔNp63 levels in cervical cancer led to increased ß-catenin and E-cadherin levels together with lower vimentin and ZEB1 levels. ß-catenin is a transcription factor that promotes angiogenesis and cell proliferation and can have both pro- and antiapoptotic functions (98). E-cadherin is a single-pass transmembrane protein that mediates cell-cell interaction and adhesion. A loss of E-cadherin function leads to an increased rate of tumor invasion and metastasis (99). Vimentin is a type III intermediate filament protein needed for stable cell adhesion. A loss of function leads to a lower level of tissue-integrity (100). ZEB1 is a zinc finger protein acting as a transcription factor and influencing mesenchymal factors. ZEB1 also influences epithelial integrity and represses genes encoding for tight junctions and other adhesive factors. ZEB1 represses epithelial factors, thus an increase in ZEB1 has reportedly led to higher rates of migration, invasion and metastasis (82). ΔNp63 influences various miRNAs in SCC and various other epithelial carcinomas, preventing EMT and leading to overall lower rates of metastasis (86).

ZEB1 could be one of the key factors for EMT and thus might influence the decision whether p63 isoforms repress or increase EMT. While these effects have been shown in other types of cancer, they have not yet been examined in HCC. So far, we can only speculate that they also occur in HCC.

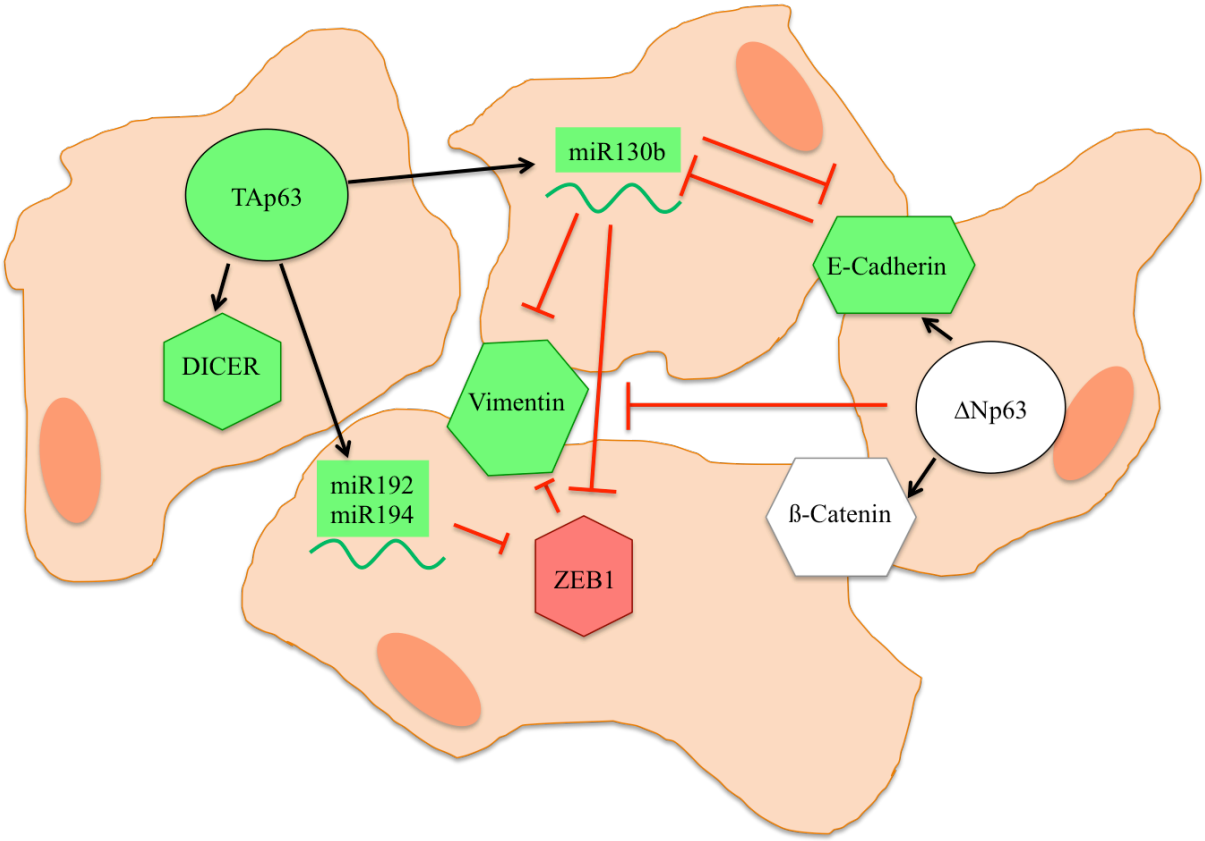

Figure 23: Network of genes and miRNAs influencing each other in tumor tissue (as shown in SCC and cervical cancer). Arrows indicate that the gene upregulates its target, an arrow with a crossed out tip indicates a downregulation of its target. Genes suppressing EMT are marked green, genes that increase EMT are marked red. Genes marked white can do both. In this network TAp63 surpresses EMT while ΔNp63 promotes tissue integrity.

Both miR192 and miR194 also target ZEB1 and ZEB2 (86). Since both miRNAs, miR192 and miR194, are influenced by p63 in HCC, it would be important to find out whether there is a link between p63, higher levels of miR192 and miR194 and a higher metastasis rate in HCC or other carcinomas. It would be also of interest to find out how exactly –directly or indirectly- p63 influences miR192 and miR194. By including miR192 and miR194 in the network of genes influencing each other in EMT as shown in figure 23 we expand our understanding how genes

influence each other in EMT. By now, we can only speculate that these miRNAs also have an impact on EMT as it has been demonstrated for miR130b (96).

4.1.3 miR192 and miR194 expression is influenced by p63 and targeted therapies

In comparison to miR34a and miR149, which are mainly influenced by classical chemotherapeutics, treatment with targeted therapies had a greater impact on expression of miR192 and miR194.

In the presented study, we show that treatment of p63-overexpressing cells with doxorubicin, sorafenib and tivantinib leads to upregulation of miR192 while treatment with bleomycin and regorafenib had no big impact on the expression of miR192 and miR194.

Treatment with doxorubicin lead to an upregulation of miR192 by 2.84-fold after 72h in p63-overexpressing cells. It has been shown by Zhang *et al.* that breast cancer cells overexpressing miR192 were more sensitive to doxorubicin (101). In a similar manner, it would be interesting to find out if this is also true for HCC cells in the *in vivo* situation. Our studies revealed an upregulation of miR192 by 3-fold 72h after treatment with sorafenib in p63-overexpressing cells. Whereas so far no studies on the influence of sorafenib on miR192 in HCC have been conducted yet, it was shown that miR192 is deregulated in sorafenib-resistant CB17 SCID mouse cells (102). Moreover, no data on the effects of tivantinib on miRNA profiles in HCC are available. Our study therefore is the first one to show an influence of sorafenib and tivantinib on p63-dependent miR192 expression.

While treatment with chemotherapeutics did not influence p63-dependent expression of miR194, sorafenib and tivantinib led to an upregulation of miR194 after 72h. It could be shown that miR194 is deregulated in sorafenib-resistant HepG2 and CB17 SCID cells (102). Thus, our study is the first to indicate a p63-mediated regulation of miR194 expression after treatment with targeted therapies in HCC.

4.2 Targeted therapies influence miRNAs suppressing EMT

p63 overexpression led to a higher expression of all examined miRNAs. It is interesting to note that p53 also regulates miR34a and miR149, however expression of miR192 and miR194 remain unchanged after overexpression of p53 (81, as well as other studies conducted by our

group). With p53 being a classic tumorsuppressor, p63 has a higher impact on tissue integrity. In the clinic, targeted therapies are used to treat advanced, metastasized, inoperable HCCs. Interestingly, miR192 and miR194 were upregulated by sorafenib and tivantinib treatment after 72h. These miRNAs seem to play a role in EMT as does p63. These miRNAs are not influenced by overexpression of p53 (Figure 23). This also supports the theory that p63 and the interaction of p63 with tumorsuppressive miRNAs might play an essential role in EMT rather than in carcinogenesis.

miR34a and miR149 on the other hand are strongly influenced by the local therapeutic agents doxorubicin and bleomycin. While miR34a and miR149 seem to play a more conventional tumor suppressive role (70,104), miR192 and miR194 seem to be important for tumor metastasis (63,83,84). It is very intriguing that those miRNAs that are linked to metastasis display an enhanced response to systemic treatment, while classic tumor suppressing miRNAs are highly responsive to local, cytotoxic treatment.

Therefore, classical chemotherapeutics might have a wide cytotoxic and apoptosis-inducing effect, whereas targeted therapies influence specific proteins or sequences in cells. However, it is not yet known if these therapeutics only exert an effect on the specific proteins or also on other proteins or genes. Therefore, we do not know yet if the targeted therapies also influence either p63 expression or miRNA expression.

As previously demonstrated, there is a network of influences on p63, p63-dependent miRNA expression, chemosensitivity and treatment-associated upregulation of p63 or the miRNAs examined. It is therefore challenging to differentiate between effects of the treatment itself and possible treatment-induced overexpression of p63 might have on miRNA levels. Also, upregulation of certain miRNAs has been shown to improve the sensitivity of cells to treatment. The genetic profile of a HCC cell can therefore have an impact on the efficacy of treatment as well as on the choice of treatment to which the cancer might respond best. This suggests that miRNAs could be a target of different therapies and that understanding of the miRNA expression profile in HCC as well as the expression of p53 family members could lead to a better personalized approach in chemotherapy or targeted therapy in patients with HCC.

4.3 Future directions

In my work, I could show the impact p63 expression has on the level of miRNA 34a, 149, 192 and 194 in HCC as well as how common therapies effect the expression in p63 overexpressing cells. I could not clarify whether p63 directly or indirectly regulates miRNAs. It will thus be informative to analyze the respective miRNA levels in p63-depleted HCC cells. While our studies focused on *in vitro* analysis and the question whether p63 overexpression has an influence on the expression of tumorsuppressive miRNAs, the detailed mechanisms have yet to be examined. To determine whether the miRNAs are direct targets of p63 it is useful to follow the criteria by Riley *et al.* for p53-responsive genes (105). As p63 shares a couple of similarities with p53 concerning structure and mechanism of action, these criteria could also be used for examining p63 and its possible targets. A response element (RE) for p63 in the gene or close to it has to be identified. The expression of the target RNA (in this case the target miRNA) has to change after overexpression of p63. This we showed in the set of experiments performed. A cloned p63 response element has to be placed near the test gene in a luciferase assay and shown that it regulates the tested gene. In a last step the presence of p63 on the RE site has to be shown by chromatin-immunoprecipitation analysis (ChIP assay). A p63-binding site has been identified within the promoter region of miR34a, yet it remains to be determined whether this is also the case for miR149, miR192 and miR194. p63 overexpression had an effect on the expression of miRNAs as shown here, and gene reporter assays could be conducted to validate possible response elements. Here, chromatin-immunoprecipitation analysis (ChIP assay) with p63 could be helpful to determine if direct binding occurs.

By this, one could show whether miR149, miR192 and miR194 are direct targets of p63 in HCC. As an alternative to overexpression, p63 knockdown by siRNA will be useful to determine if the effects on miRNA expression are in fact p63-dependent.

While our study suggests that p63 targets miR34a, miR149, miR192 and miR194 and thus has an influence on tumorigenesis and EMT, the clinical relevance of this regulatory mechanism in HCC remains to be determined. Also, it should be examined which genes are targeted by the abovementioned miRNAs in HCC. In a further step, *ex vivo* analysis of p63-depleted HCC tissue will be useful, focussing on the expression of the abovementioned miRNAs to determine whether the effect shown *in vitro* plays a role in HCC. As Hep3B cells are p53-deficient, the *in vivo* effects of p63 will need to be evaluated also in the presence of p53. Nevertheless, a deeper understanding of p63 and its targeted miRNAs in cancer could become important in personalized cancer therapy as miRNA expression can essentially influence therapy responses.

By understanding the effect of p63 on miRNA expression it is possible to find an optimal therapeutic intervention for each individual HCC patient.

5. Summary

Background: Transcription factors of the p53 family (p53, p63, p73) respond to cellular stress signals by inducing a defined set of target genes. In tumors such as hepatocellular carcinoma (HCC) they exert tumorsuppressive functions. p63 is known as a tumor suppressor with an important role in the development of epithelia and in epithelial mesenchymal transition, a process that results in metastasis of a primary cancer. However, the role of p63 in the development of HCC is yet unknown. microRNAs (miRNAs, miR) are small non coding RNA molecules that control gene expression by binding to target mRNAs and inhibit their translation. Expression profiles of miRNAs can be regulated by p53-family members. In previous studies we identified a set of p53-regulated miRNAs in HCC (miR34a, miR149, miR192 and miR194). It is known that miRNA 34a can be induced by p53 and – in turn - exerts its tumorsuppressive functions by stabilizing the robustness of p53 responses to genotoxic stress. p63 has been shown to influence miRNA levels in squamous cell carcinoma (SCC) and in a number of other epithelial carcinomas, but little is known about the role of p63 in miRNA regulation in HCC.

Aims and Methods: The aim of this study was to evaluate whether miRNA profiles in HCC cells are regulated by p63. p53-deficient Hep3B cells were transduced with rAd-p63 or rAd-GFP. Expression of miR34a, mir145, miR149, miR192 and miR194 was determined by qPCR. To analyse effects of HCC-relevant therapeutics on p63-dependent microRNA regulation transduced Hep3B cells were treated with HCC-relevant local and systemic therapeutics (doxorubicin, sorafenib, bleomycin, tivantinib and regorafenib) for up to 72 hours in concentrations corresponding to the serum levels of patients undergoing therapy.

Results: Overexpression of p63 resulted in anincrease of miR34a (2.18-fold) and miR149 (2.309-fold) after 48h and a stronger induction of miR192 (2.77-fold) and miR194 (2.35 fold) after 24h. In untransduced cells, *in vitro* incubation with HCC-relevant therapeutics had no effect on expression levels of the analysed miRNAs. However, in combination with p63 expression of miRNAs was strongly induced by the used drugs. 72h *in vitro* incubation with doxorubicin resulted in an increase of p63-dependent expression of miR34a (4.72-fold), miR149 (10.29-fold) and miR192 (3.29-fold) compared to rAd-transduced controls. Bleomycin treatment increased p63-dependent miR34a expression by 4.12-fold and miR149 expression by 15.65-fold. Sorafenib had an augmenting effect on p63-dependent miR34a expression (3.64-fold), miR192 expression (2.57-fold) and miR194 expression (2.29-fold). Treatment with Tivantinib increased p63-dependent expression of miR192 by 2.77-fold and miR194 by 4.50-fold. Interestingly, in combination with p63 overexpression systemic drugs such as sorafenib

and tivantinib showed a stronger impact on miR192 and miR194, while local treatment such as doxorubicin and bleomycin predominantly increased levels of miR34a and miR149.

Conclusion: This study demonstrates for the first time a p63-dependent induction of tumorsuppressive miRNAs in HCC. Furthermore, these results indicate that p63 interacts with HCC-relevant therapeutics to control expression profiles of specific microRNAs in HCC. This finding provides further understanding of the complex regulatory network by which p53 family members exert their tumorsuppressive functions in HCC and establishes p63 as an important mediator of therapeutic response.

6. List of figures

6.1 Figures

6.2 Tables

7. Literature

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8. Acknowledgements

First of all, I wish to express my deepest gratitude to my academic supervisor, Professor Dr. med. Müller-Schilling for offering me the opportunity to work on this highly interesting project and supporting me in finding my own point of focus and interest in my research. I would also like to express my sincere appreciation for all the opportunities she gave me in presenting my project at national and international congresses and to scientists and researchers from various nations. Her support went well beyond what is expected of academic supervisors, also supporting me in my academic career and placement as an intern abroad. Without her support this thesis would not have been possible and for that I am extremely grateful.

Secondly, I'd like to thank PD Dr. Claudia Kunst for her great advice and help in finding my directions in the thesis as well as supporting me when facing difficulties. I am grateful for all her help in writing up this thesis as well as her support in my applications to speak at various congresses.

I am indebted to the academic crew and technical staff working with me in the laboratory, guiding me in my research and offering me their support and experience. For this I would like to thank Karsten Gülow, Sophie Schlosser, Elisabeth Aschenbrenner, Kirstin Pollinger, Manuela Gunckel, Heidi Gschwendtner and Marika Haderer.

I would also like to thank Lisa Kaser and Rafael Hermelink who worked on p73-mediated miRNA regulation and often offered their advice and made a smooth cooperation among the various project possible.

Finally, I'd like to pay my special regards to my parents who with their utter support and confidence in me and my work made it possible for me to complete this thesis.