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The p53 Family of Transcription Factors Represses the Alpha-fetoprotein Gene Expression in Hepatocellular Carcinoma

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ABSTRACT

Background: p53 deletion and mutation as well as upregulation of alpha-fetoprotein (AFP) are hallmarks of hepatocarcinogenesis. p63 and p73 belong to the family of p53-related transcription factors expressing a variety of isoforms. The expression of dominant negative (ΔN) p73 is related to the reduced survival of patients with hepatocellular carcinoma (HCC). In this study, we characterized the interaction between p53 family-dependent signaling pathways and the regulation of AFP at the gene and protein levels as essential determinants of therapeutic response and prognosis in HCC.
Methods: Putative p53-, p63- and p73-binding sites within the *AFP* gene were identified *in silico*. Hep3B cells were transfected with plasmids encoding for p53, p63 and p73 to analyze the interplay of the p53 family with AFP. AFP transcription was determined by RT-qPCR. Protein levels of AFP, p53, p63 and p73 were analyzed by Western blot.
Results: Underlining the importance of the crosstalk between the p53 family-dependent pathways and AFP regulation we identified eight novel putative binding sites for the members of the p53 family within the introns 1, 2, 3, 4, 7, 8, 11, and 12 of the *AFP* gene. Accordingly, full-length isoforms of p53, p63 and p73 efficiently downregulated AFP both on mRNA and protein level. Thus, the p53 family members were identified to be major regulators of AFP repression. Of note, p63 was characterized as a novel and p73 as the most efficient repressor of AFP.
Conclusion: p53 mutation and upregulation of AFP are essential oncogenic events in the development of HCC. Here we show that AFP gene regulation occurs via a combined action of the p53 family members p53, p63 and p73. All three tumor suppressors reduce AFP gene and protein expression. Thus, our findings reveal a novel interaction of p53 family-dependent signaling pathways and AFP regulation at the gene and protein levels in HCC.

Key words: alpha-fetoprotein – hepatocellular carcinoma – p53 family – gene repression – p53 – p63 – p73.

Abbreviations: ATBF1: zinc finger homeobox protein 3; AFP: alpha-fetoprotein; C/EBP: CCAAT-enhancer binding protein; cIAP2: the inhibitor of apoptosis 2; CUX: Cut homeobox1; HCC: hepatocellular carcinoma; HNF1: hepatocyte nuclear factor 1; NF1: neurofibromin 1; TA: transactivation; VEGF: vascular endothelial growth factor; VEGFR: VEGF receptor; XIAP: X-linked inhibitor of apoptosis; ZBTB20: BTB domain containing 20; ZHX2: zinc fingers and homeoboxes 2; ΔN: amino-terminally truncated.

INTRODUCTION

Physiologically, oncogenic factors are supposed to be repressed by tumor suppressor proteins. p53, as "guardian of the genome" and, thus, one of the most important tumor suppressor proteins, exerts pro-apoptotic and anti-proliferative functions in response to oncogenic stress [1, 2]. All members of the p53 family – p53, p63 and p73 – are involved in differentiation, proliferation, and regulation of cell death. p63 and p73 play a pivotal role in embryonic development and differentiation of epidermal and neuronal structures, but are also involved in the induction of extrinsic and intrinsic apoptotic pathways in cancers like hepatocellular carcinoma (HCC) [3-7].

Therefore, the p53 proteins are relevant for prognosis and therapeutic response of HCC [4, 8-10]. By usage of multiple promoters and alternative splicing cells can generate multiple protein isoforms from a single p53 family gene. Full-length

p63 and p73 isoforms contain a transactivation (TA) domain homologous to that of full-length p53. Amino-terminally truncated (ΔN) isoforms lack this TA domain. We previously demonstrated that ΔN-isoforms have opposing functions compared to their corresponding full-length isoforms and display oncogenic capacities [3, 5, 6]. N-terminally truncated isoforms are upregulated in different human cancers including HCC but not in normal tissues [5, 11-13]. Of clinical relevance, the network of the p53 family members p53, p63 and p73 is essential for hepatocarcinogenesis, and response to therapy. We have previously shown that not only the mutational status of p53 but also the expression of ΔN-isoforms of p63 and p73 is of prognostic relevance in HCC. Overexpression of ΔNp73 in HCC correlated with reduced patient survival [5].

Alpha-fetoprotein (AFP) is the only biomarker which is currently validated for detection of early HCC, assessment of prognosis, and prediction of response to systemic therapies, and as a marker to identify patients who will benefit from ramucirumab [14, 15]. Ramucirumab, a monoclonal antibody against vascular endothelial growth factor (VEGF) receptor (VEGFR)-2, has demonstrated overall survival benefit against placebo as a second line therapy for patients with AFP > 400 ng/ml in the REACH-2 trial. This is the first positive trial in a biomarker-selected subgroup of patients and is a milestone in achieving a personalized HCC treatment approach. Furthermore, emerging data suggest crosstalk of AFP and VEGF signaling cascades and silencing of AFP has been shown to inhibit VEGF production in HCC cells *in vitro* [16].

Alpha-fetoprotein is the most important and most accepted serum biomarker in HCC management and its expression is induced in 60 to 80 % of all HCCs [10]. Alpha-fetoprotein is one of the four members of the albumin gene family localized in a tandem arrangement forming a multi-gene cluster [17, 18]. As a member of the albumin plasma protein family, AFP is a transporter protein for copper, nickel, fatty acids and bilirubin, which is produced by the yolk sac and fetal liver and exerts essential functions during fetal development. There are three major isoforms (AFP-L1, AFP-L2 and AFP-L3) that are differentially expressed in diverse physiological or pathophysiological conditions [19]. Immediately after birth, AFP levels decline and remain low throughout adulthood [17, 20]. However, AFP synthesis is reactivated to a low level in liver regeneration after viral or chemical liver injury and to a higher level in most HCCs [21, 22]. Alpha-fetoprotein is mainly regulated at the transcriptional level. Its gene has an upstream regulatory region consisting of a tissue-specific promotor, three independent enhancers and two silencer regions [22]. The latter may be involved in the decrease in *AFP* gene expression in adult livers [23]. Genetic regulation of *AFP* has not been fully characterized. The *AFP* promoter has a variety of binding sites for transcription factors, enhancers, and repressors such as CCAAT-enhancer binding protein (C/EBP), zinc finger homeobox protein 3 (ATBF1), and neurofibromin 1 (NF1). These factors ensure *AFP* expression after birth and during hepatic development. One of the crucial binding sites in the *AFP* promoter, which plays an important role after birth but also in carcinogenesis, is for the hepatocyte nuclear factor 1 (HNF1). Not only is HNF1 expression increased immediately after birth, but it also correlates with AFP levels in patients with HCC [22, 24]. In addition, two important transcriptional repressors correlating with increased *AFP* expression in HCC bind to the *AFP* gene: ZHX2 (zinc fingers and homeoboxes 2) and BTB domain containing 20 (ZBTB20) [25, 26]. The *ZHX2* promoter is frequently hypermethylated in HCC. This epigenetic alteration leads to a significant downregulation of ZHX2 and thus to an induction of *AFP* expression [26, 27]. ZBTB20 is specifically targeted and downregulated by microRNA122. This microRNA is regulated in a complex manner, involving Cut homeobox1 (CUX1) protein as the key factor. CUX1 mainly regulates the motility and invasiveness of tumor cells. Increased expression of CUX1 is a sign of increased aggressiveness in HCC and is linked to microRNA122 induction and ZBTB20 repression [26, 27]. Moreover, AFP repression mediated by various cytokines including TGF-β signaling is discussed [28]. Interestingly, two binding sites for p53 family members have been identified within the silencer regions of the *AFP* gene [22, 29] suggesting that the p53 family may play a role in AFP regulation.

Due to its function in fetal development AFP has predominantly pro-proliferative effects: it promotes cell growth and migration and is involved in cell cycle regulation [30]. Furthermore, AFP expression is linked to sensitivity towards apoptosis in HCC. AFP interacts with the X-linked inhibitor of apoptosis (XIAP) and the inhibitor of apoptosis 2 (cIAP2) and, therefore, blocks activation of caspase 3 and induction of cell death [31]. Accordingly, knockdown of *AFP* leads to enhanced induction of apoptosis and cell cycle arrest in G0/G1 in HCC cell lines [32].

In addition to these direct growth-supporting effects, elevated AFP levels counteract tumor immune responses, e.g. by dampening phagocytic activities of macrophages, by inhibition of antigen-presenting cells, inactivation of NK cells and general downregulation of the adaptive immune system [33-35]. Thus, AFP enhances tumor growth by promoting tumor immune escape [17].

In the study presented here, we characterized the crosstalk of the members of the p53 family with AFP expression. To evaluate whether transcriptional control can occur via binding to specific domains within the *AFP* gene, potential p53 family binding sites in the *AFP* locus were identified. Furthermore, we characterized the specific effects of p53-, p63- and p73-induction on *AFP* gene and protein expression. Thus, our data connects downregulation of p53 family members and upregulation of AFP expression. Our data shows the essential role of p53, p63 and p73 as *AFP* repressors and suggests pharmacological upregulation of p53 proteins to repress *AFP* and counteract its tumorigenic effects.

METHODS

Cell Culture

Hep3B cells (human liver carcinoma, deficient in p53) were cultured in Minimum Essential Medium Eagle (Sigma Aldrich, Saint Louis, USA) supplemented with 10% fetal calf serum (Sigma Aldrich, Saint Louis, USA) at 37°C in humidified atmosphere of 5% CO_2.

Plasmid Transfection

Full length sequences encoding p53, p63 and p73 were cloned into the expression vector pcDNA3.1 (Thermo Fisher V79020). To express specific p53 family members, Hep3B cells were transfected with the plasmids pcDNA3.1 p53, pcDNA3.1 p63 and pcDNA3.1 p73, respectively, using Lipofectamine (Invitrogen GmbH, Karlsruhe, Germany). Controls were transfected with pcDNA GFP. 24-72h after transfection cells were harvested and prepared for RNA and/or protein isolation.

RNA Isolation and Reverse Transcription

Cells were harvested in RLT buffer (Qiagen, Hilden, Germany). RNA isolation and reverse transcription were performed according to the manufacturer's protocol of RNeasy Mini Kit and QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany).

Quantitative Real-Time PCR

Expression of *AFP* and *GAPDH* was analyzed by qRT-PCR using a Light Cycler 480 RT-PCR System (Roche Diagnostics, Mannheim, Germany). Primers detecting *AFP* (QT00085183) and *GAPDH* (QT00079247) were purchased from Qiagen GmbH (Hilden, Germany). *GAPDH* served as internal standard. qPCR data were analyzed based on the double cycle threshold method using the average cycle threshold values of duplicates or triplicates.

Protein Isolation and Western Blot

Cells were washed with PBS, lysed with RIPA lysis buffer containing protease and phosphatase inhibitor cocktail (cOmplete, phosSTOP, Roche Diagnostics GmbH, Mannheim, Germany). Samples were incubated on ice for 1h, spun at 14,000 rpm for 1h at 4°C and supernatant was collected. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes. Anti-AFP (Thermo Fisher Scientific Inc., Waltham, USA), anti-p53 (sc-126; Santa Cruz Biotechnology, Dallas, USA), anti-p63 (ab179874; Abcam, Cambridge, UK), anti-p73 (ab40648; Abcam, Cambridge, UK), anti-β-actin (A3854; Sigma Aldrich, Missouri, USA), anti-mouse IgG (A9044; Sigma Aldrich, Missouri, USA) and anti-rabbit IgG (A0545; Sigma-Aldrich, Missouri, USA) were used for detection. Densitometric analysis was performed using Image Lab Software (Bio-Rad Laboratories Inc., Munich, Germany), Fusion Pulse TS (Vilber Lourmat, Eberhardzell, Germany) and Image Studio Lite Version 5.2 (Lincoln, NE, USA).

Binding Site Analysis

The full intronic sequence of the *AFP* gene as well as the upstream promoter sequence (ENSG00000081051) were depicted from ensembl.org. Putative p53-, p63- and p73-binding sites were identified by Transfac (MATCH) and Jaspar 2020 transcription factor binding profile database. Aforementioned transcription factors are capable of binding certain consensus sequences with an associated nucleotide frequency matrix that are specified and validated in both, Transfac and Jaspar 2020. In case of p53, three consensus sequences (ID: MA0106.1, MA0106.2, MA0106.3) are available. For p63 two consensus sequences (ID: MA0525.1, MA0525.2) have been applied and for p73 one consensus sequence (ID: MA0861.1) is available and has been used. For concurrent analyses, all sequence IDs have been selected to be aligned to the *AFP* gene sequence, simultaneously. Putative binding sites were ranked according to "relative profile score thresholds". Only sequences exceeding 75% score threshold were considered as putative binding sites. For our analyses, we considered a 75% score threshold as most suitable to investigate the sequential intersections between the related p53-, p63-, and p73-transtription factors.

Statistical Analysis

Results are depicted as box plots or bar charts. Data was analyzed using the Welch's t-test. Sigma Plot V.14.0 software (Systat, Erkrath, Germany) was used for graphics and statistical analysis.

RESULTS

Identification of Novel p53 Family Binding Sites within the *AFP* Gene

To analyze a potential interplay of p53 family-dependent signaling and *AFP* gene regulation we first performed data base analyses to search for binding sites of the p53 family members p53, p63 and p73 in the *AFP* locus, (Fig. 1). *In silico* binding site analysis using Jaspar and Transfac confirmed the two p53 family binding sites (position -1873 to -1781 (silencer 1); position -295 to -215 (silencer 2)) within the promotor of the

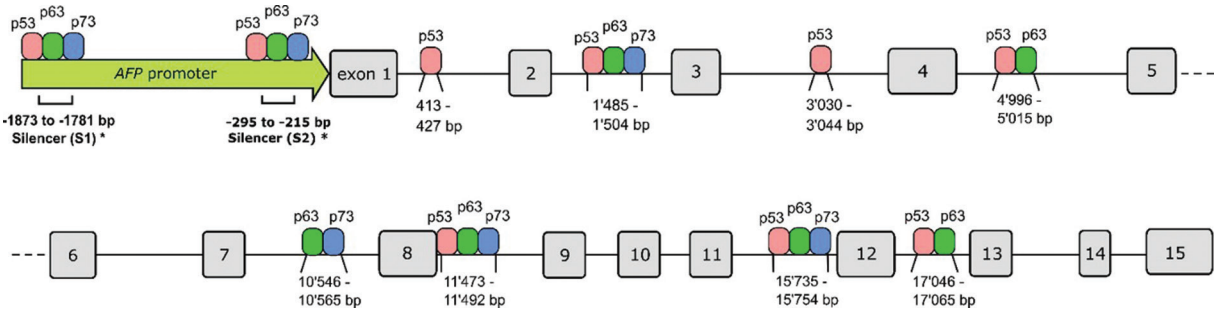

Fig. 1. Identification of eight novel putative p53 family binding sites within the human *AFP* gene locus. Known matrix sequences of p53, p63 and p73 were compared to the 5'-promoter enhancer region and intronic/exonic sequences of the human *AFP* gene. Novel putative binding sites were identified in introns 1-12 for all members of the p53 family. Previously described silencer regions [22] are indicated by *.

AFP gene (Fig. 1) [22, 29]. Of major importance, we identified eight novel putative binding sites within the introns of the *AFP* gene (Fig. 1): in intron 1 a p63 binding site (413-417), in intron 2 a combined binding site for p53, p63 and p73 (1485-1504), in intron 3 a binding site for p63 (p3030-3044), in intron 4 a combined binding site for p53 and p63 (4996-5015), in intron 7 a combined binding site for p63 and p73 (10546-10565), in intron 8 (11473-11492) and intron 11 (15735-15754) a combined binding site for p53, p63 and p73 and in intron 12 a combined binding site for p53 and p63 (17046-17065). Thus, the *AFP* gene contains a plethora of potential and previously unidentified binding sites for the p53 family, pointing at a significant role of this gene family in *AFP* regulation. For the first time, these data provide evidence, that not only p53 but also p63 and p73 are involved in transcriptional regulation of the *AFP* gene.

Hep3B Cells Are a Suitable Model System to Analyze p53 Family-Dependent Regulation of the *AFP* Gene

Since the *AFP* gene contains putative binding sites for all three members of the p53 family (Fig. 1), we aimed to confirm the regulatory potential of p53, p63 and p73 regarding AFP transcription and protein level. We chose Hep3B cells as particularly appropriate for these studies because they lack p53 [36-38]. Hep3B cells were transfected with expression vectors encoding for the transactivation domain-containing (TA) isoforms of p53, p63 and p73 to induce protein production of the respective transcription factors. Cells transfected with a GFP encoding plasmid were used as corresponding controls.

According to the nine identified putative binding sites for p53 in the *AFP* gene (Fig. 2A) 72h after p53 induction *AFP* transcription was repressed by 48% (Fig. 2B). Consistently, expression of p53 also resulted in a reduction of AFP protein levels (Fig. 2C). Densitometric analyses confirmed that AFP was downregulated by up to 27% after p53 induction (Figure 2D). Thus, repressive properties of p53 could be verified [29, 39] and, therefore, Hep3B cells are a valid model system to analyze the crosstalk between p53 family members and AFP.

p63 - A Novel Repressor of AFP Transcription and Protein Synthesis

So far less is known about the regulatory role of p63 in *AFP* expression. Experiments using myc-tagged p63 overexpression revealed no effect on *AFP* transcription [29]. However, it could not be excluded that the protein tag interfered with p63 folding, translocation or activity. In our study, all p53 family members were expressed without modification to exclude a possible impact on protein function. Remarkably and in concordance with the eight identified putative p63 binding sites within the gene (Fig. 3A), *AFP* transcription continuously decreased after expression of unmodified p63 and was significantly reduced 72h after transfection (Fig. 3B). Thus, we show for the first time that p63 is a potent repressor of *AFP*, downregulating its expression by up to 25%. Furthermore, this repressive effect was confirmed on protein level. Western blot analysis after transfection with p63 revealed that AFP protein levels were reduced by 36% after 48h and even further decreased, resulting in a reduction of 55% after 72h (Figs. 3C, D). Thus,

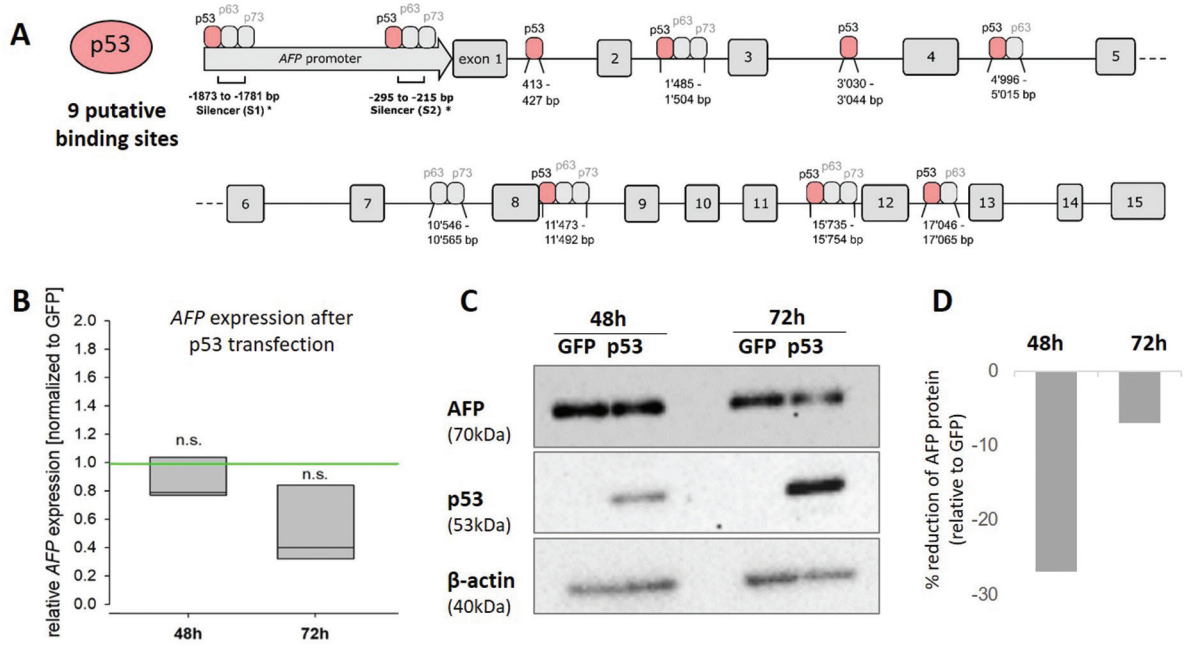

Fig. 2. p53-dependent repression of AFP gene and protein expression. (A) Scheme depicting two known and seven novel putative p53 binding sites within the *AFP* locus. Previously described silencer regions [22] are indicated by *. (B) Quantitative PCR analysis of *AFP* gene expression in Hep3B cells expressing p53 is shown. *AFP* mRNA levels were measured 48h and 72h after transfection with p53 and normalized to *AFP* values of *GFP*-transfected control cells (n=3). Statistical analysis was performed using Welch's t-test comparing p53-transfected cells to GFP-transfected controls at each time point (n.s.= no significant difference). (C) Western blot analyses of AFP protein in Hep3B cells expressing p53. Cells were analyzed 48h and 72h after transfection with p53. One exemplary blot with (D) densitometric analysis is shown. AFP levels were normalized to β-actin and are depicted as reduction compared to GFP-transfected control.

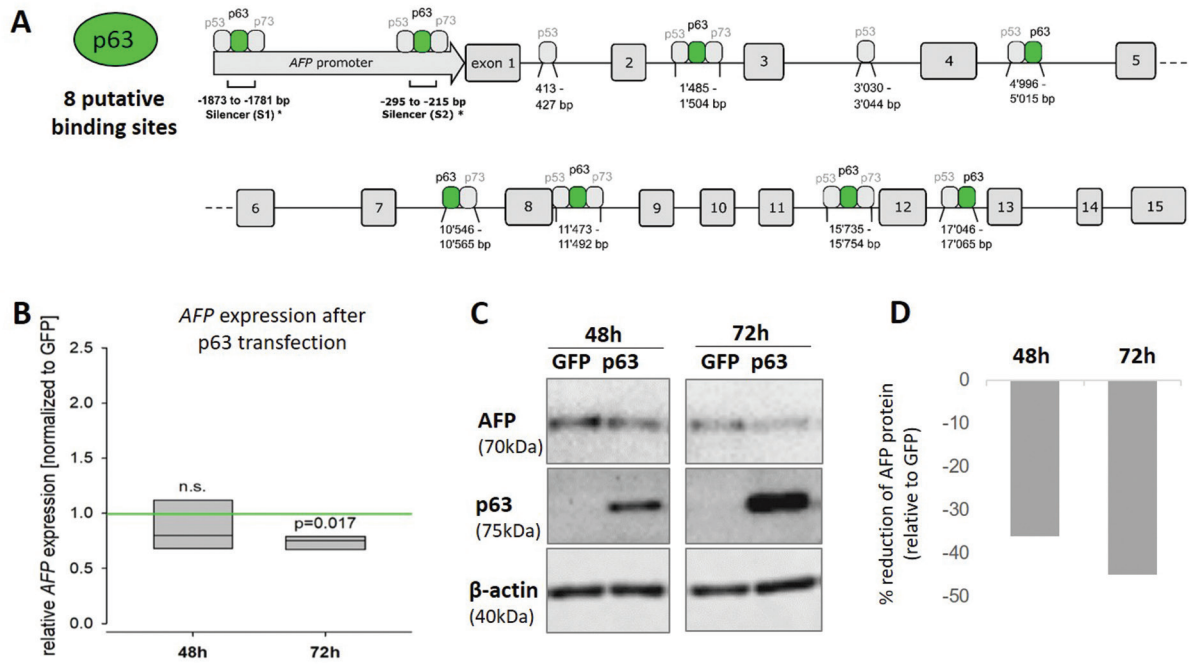

Fig. 3. p63 is a novel negative regulator of AFP gene and protein expression. (A) Scheme depicting two known and six novel putative p63 binding sites within the *AFP* locus. Previously described silencer regions [22] are indicated by *. (B) Quantitative PCR analysis of *AFP* expression in Hep3B cells expressing p63 is shown. *AFP* mRNA levels were measured 48h and 72h after transfection with p63 and normalized to *AFP* values of GFP-transfected control cells (n=3). Statistical analysis was performed using Welch's t-test comparing p63-transfected cells to GFP-transfected controls at each time point (n.s.= no significant difference). (C) Western blot analyses of AFP protein in Hep3B cells expressing p63. Cells were analyzed 48h and 72h after transfection. One exemplary blot with (D) densitometric analysis is shown. AFP levels were normalized to β-actin and are depicted as reduction compared to the GFP-transfected control.

p63-dependent repressive effects on AFP protein expression surpass those of p53.

p73 – The Most Efficient AFP Repressor of the p53 Family

p73 has been shown to predominantly work in combination with p53 to enhance repressive properties on *AFP* expression [29]. Of note, Hep3B cells lack p53 [36-38]. Thus, we analyzed the effects of p73 on AFP without any combinatorial or enhancing effects of p53. The *AFP* gene contains two binding sites for p73 in the promotor region and, in addition, we have identified novel putative binding sites in introns 2, 7, 8 and 11 (Fig. 4A). Of relevance, in comparison to p53 and p63, the most prominent suppressive effects on *AFP* expression were exerted by p73 (Fig. 4B). Transfection with the TA-isoform of this protein resulted in a significant reduction of *AFP* transcription by 70% and 85% after 48h and 72h, respectively. Concordantly with these mRNA analyses, the most effective suppression of AFP protein levels was observed in cells transfected with p73. p73 time-dependently downregulated AFP protein levels to 17% after 72h (Figs. 4C, D). Therefore, among all p53 family members, we identified p73 to be the most efficient repressor of AFP gene expression. These data point out that, in addition to a combined action with p53 [29], p73-mediated repression can also occur independent from p53.

p53 mutation and the expression of dominant negative isoforms of the p53 family members p63 and p73 as well as upregulation of AFP are hallmarks in development, progression, therapeutic response, and prognosis of HCC. Here, we characterized the interconnection of both signaling pathways in HCC: (i) We identified novel putative binding sites for p53 family members in the intronic regions of the *AFP* gene. (ii) We demonstrated suppressive effects of the p53 family on AFP synthesis. (iv) We show that p63 is a novel suppressor of AFP expression and (v) that p73 is the most powerful inhibitor of AFP.

DISCUSSION

Identification of novel therapeutic targets for HCC treatment requires a detailed molecular understanding of hepatocarcinogenesis. High-throughput analyses provided evidence that HCC is associated with genetic alterations, which result in tumor development, progression of the disease and resistance towards therapy [17]. Next Generation Sequencing (NGS) techniques identified the main driver genes involved in hepatocarcinogenesis. These impact six key biological signaling pathways: 1. telomere maintenance, 2. Wnt/ß-catenin, 3. cell cycle regulation, 4. epigenetic dysregulation, 5. oxidative stress and activation of RAS/RAF/MAP kinase, and 6. PI3K/AKT/MTOR pathways [40-42]. Of clinical relevance, hepatitis B virus (HBV) has additional properties to induce liver carcinogenesis, including the action of a viral oncoprotein and the integration of the virus in the human genome [43]. Mutations of p53 are observed in 20-50% of HCC [44, 45]. Based on genomic, transcriptomic, and epigenetic data, a classification of HCC correlating with clinical characteristics, risk factors and

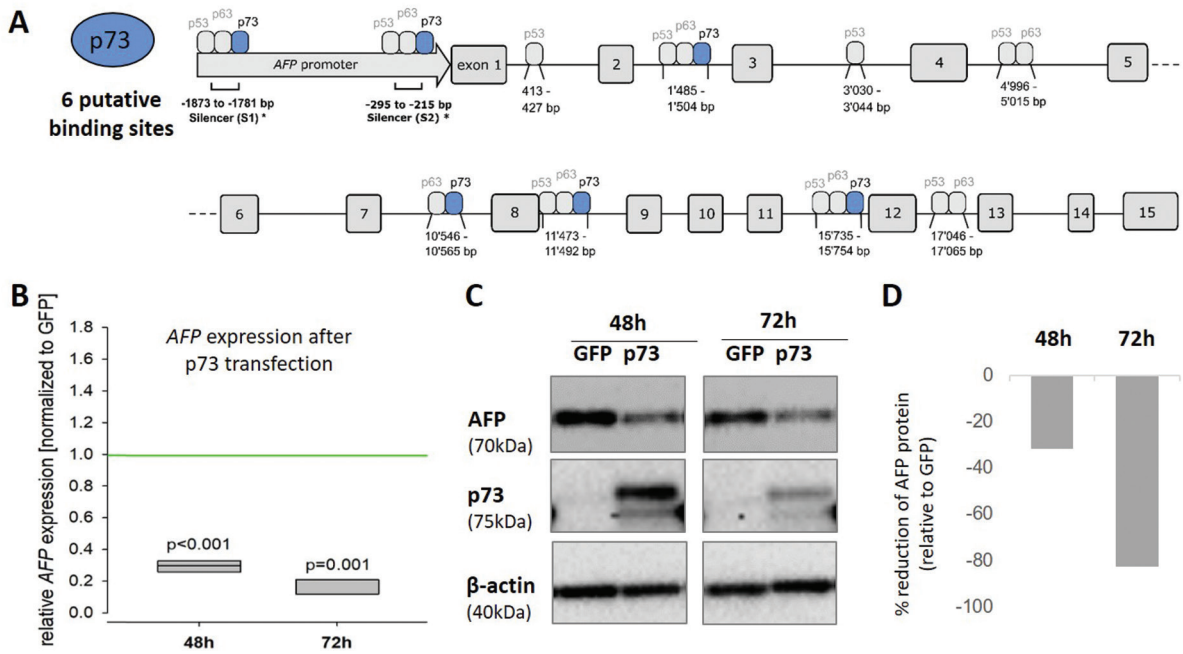

Fig. 4. Most efficient repression of AFP gene and protein expression by p73. (A) Scheme of two known and four putative novel p73 binding sites within the AFP locus. Previously described silencer regions [22] are indicated by *. (B) Quantitative PCR analysis of AFP expression in Hep3B cells expressing p73 is shown. AFP mRNA levels were measured 48h and 72h after transfection with p73 and normalized to AFP values of GFP-transfected control cells (n=3). Statistical analysis was performed using Welch's t-test comparing p73-transfected cells to GFP-transfected controls at each time point. (C) Western blot analyses of AFP protein in Hep3B cells expressing p73. Cells were analyzed 48h and 72h after transfection. One exemplary blot with (D) densitometric analysis is shown. AFP levels were normalized to β-actin and are depicted as reduction compared to the GFP-transfected control.

histopathology was suggested [44]. This classification includes a "proliferative" class and a "non-proliferation" class. The "proliferative" class comprises poorly differentiated tumors with a high serum AFP level. In addition, these tumors are characterized by chromosomal instability, *TP53* inactivating mutations, as well as the activation of pathways involved in cell proliferation and survival, namely RAS/RAF/MAP kinase, PI3K/AKT/TOR pathways. The "non-proliferation" class comprises well-differentiated and chromosomally stable tumors which are associated with chronic hepatitis C virus (HCV) infection or chronic alcohol consumption. In summary, the most common mutations in HCC include *TERT* promoter, *CTNNB1*, and *TP53* mutations.

p53 is mutated in more than 50% of aflatoxin B1-induced HCC, in up to 45% of HBV-related HCC and in about 13% of HCV-related HCC [8, 46]. Activation of the p53 family is a central event in the DNA damage response, sensitivity towards therapy, and prognosis of HCC. Inactivation or loss of p53 expression results in increased proliferation, resistance towards cell death and accumulation of further mutations [2, 7, 47, 48]. We have demonstrated that the p53 family members p63 and p73 play an essential role in regulation of cell death, proliferation and response to treatment in HCC [4-6]. Thus, mutations of p53 family members and alterations in the ratio of TA- versus dominant negative (ΔN)-isoforms of p63 and p73 leads to a poor prognosis of patients with HCC [49-51].

In addition to AFP repression by ZXH2 and ZBTB20, our findings presented here show a role for p53, p63 and p73 in the regulation of AFP expression and, therefore, underline the prominent role of the p53 family as tumor suppressors in HCC. We demonstrate that regulation of the *AFP* gene occurs in a combined action of p53, p63 and p73. Our i*n silico* analysis confirmed two known binding sites for p53 family members within silencer regions located upstream of the promoter [22, 29] and, in addition, identified 8 novel binding sites within intronic regions of the *AFP* gene for all three p53 family members. This is in line with Riley et al. [52], showing that only ~50% of all binding sites are located in the 5'-promotor enhancer region of a p53-controlled gene, while ~50% are located in intronic and extronic sequences. Thus, as a major difference to the described regulation mechanisms of *AFP* transcription that are supposed to work upstream of the start codon [22, 29, 53], our database analysis indicates that especially p53-family-dependent *AFP* regulation can also occur via binding to intronic regulatory elements in the *AFP* gene locus. In addition, we show that p63 and p73 are novel negative regulators of *AFP* expression. So far one other study examined possible effects of p63 on *AFP* expression [29]. This study, using Myc-tagged p63, did not observe any p63-dependent inhibitory effects. Of note, protein modification such as protein tags can interfere with protein folding, protein activity and/or protein transport into the nucleus [54-56]. In contrast to this study, we used unmodified/untagged p63 for expression analyses and, therefore, assume correct protein function. Thus, our data for the first time provide evidence that p63 exerts even higher suppressive functions regarding *AFP* regulation when compared to p53. Further, we demonstrate that p73 exhibits the most prominent suppressive effect regarding *AFP* expression, resulting in an efficient downregulation by up to 85% on mRNA level. This is in line with data from Cui et al.

[29], describing TAp73 predominantly in combination with p53 as a direct repressor of *AFP* transcription. Of note, in our model we used Hep3B lacking endogenous p53 expression [36-38]. Thus, repressive effects in our model are not based on a cooperative interaction of p53 and p73 [29]. It is p73 alone or in combination with p63 that leads to effective downregulation of AFP. Clinically, our findings are in line with studies correlating p53 mutations with enhanced AFP expression [57-60]. Transcriptional inactivation of p73 in tumors occurs rather via overexpression of the ΔNp73 isoform than by mutation or deletion [61]. However, this enhanced expression of transcriptionally inactive ΔN isoforms in HCC is associated with reduced apoptosis, chemosensitivity and poor prognosis [5, 11, 12, 62].

Therefore, repression of *AFP* expression by upregulation of p53 proteins is a possible therapeutic option to treat HCC. Thus, restoring a physiological p53 level and function will not only sensitize tumor cells towards apoptosis but also restore physiological AFP repression. There are several ways to restore p53 expression and function [63]: (i) p53 can be upregulated by reducing its degradation. p53 is targeted for degradation by the E3 ubiquitin ligase MDM2 [64, 65]. Several MDM2 inhibitors against the p53-binding pockets of MDM2 have been developed [66, 67] and can be used to stabilize p53 protein levels. These inhibitors include nutlins, spiro-oxindole derivatives, piperidinone-containing compounds and competitive peptides [66]. However, tumors harboring mutations of p53 do not respond to these drugs. (ii) To target cells expressing mutant p53, restoring its function is another potential therapeutic approach [68]. In general, mutations in p53 lead to misfolding of the protein and loss of activity. The misfolding often induces pocket formation in the core domain of p53 affecting several cysteine residues. Consequently, molecules reacting covalently with the thiols in these cysteine residues can induce conformational changes leading to refolding and reactivation of p53. Various small molecules such as derivates of quinuclidinone, maleimide, pyrimidine, quinazoline, quinoline, pyrol and pyrazole as well as α/β- unsaturated carbonyl compounds and zinc metallo-chaperones are under investigation and show promising results. The most intensive characterized small molecules restoring p53 function are the quinuclidinones PRIMA-1 (APR-017) and PRIMA-1MET (APR-246), the maleimide MIRA-1 and the quinazoline STIMA-1 [68]. (iii) In the case of deleted p53 the only therapeutic option is to reintroduce p53 expression e.g. by transfection with adenoviral vectors. A promising study described the combination of transarterial chemoembolization (TACE) with injection of adenoviral vectors containing recombinant wild-type p53. In this study patients with unresectable HCC show an improved overall survival and progression-free survival in comparison to patients with TACE monotherapy [69]. Thus, restoring p53 by stabilization, reactivation or transduction is a future therapeutic option in HCC therapy and leads to induction of apoptosis and via restoration of physiological repression of AFP to inhibition of proliferation of HCC tumor cells.

Our study demonstrated that the p53 family of transcription factors – p53, p63 and p73 - were direct regulators of AFP at the gene and protein level. All three tumor suppressors reduced AFP gene and protein expression. Of clinical relevance, almost 50% of all patients with HCC display an impaired p53 signaling pathway [70, 71] and mutations of p53 correlate with enhanced circulating AFP values [57-60]. HCCs with *TP53* inactivating mutations and high serum AFP levels are classified as "proliferative", are poorly differentiated and show an aggressive clinical course with poor prognosis of the patients. Furthermore, this "proliferative" class of HCCs includes a subgroup of so-called "progenitor" HCCs defined by the overexpression of hepatic progenitor markers, AFP, insulin-like growth factor (IGF) 2 and inactivating mutations of *RPS6KA3* and BRCA1 associated protein-1 (*BAP1*). The definition of the "proliferative" class and the "progenitor" subgroup of HCCs includes mutations in *TP53* and high AFP expression as essential oncogenic events in the development of HCC.

Our data elucidate the interconnection of both signaling pathways in HCC. We identified novel putative binding sites for all p53 family members in the intronic regions of the *AFP* gene and demonstrated suppressive effects of the TA (tumor-suppressive) isoforms of the p53 family on AFP synthesis. Consequently, mutations in p53 lead to either loss of transcriptional repression of the *AFP* gene or in case of a gain-of-function mutation to enhanced transcriptional activation of the *AFP* gene. Of note, not solely p53 but the complex network of the p53 family including p63 and p73 determines initiation, progression, and prognosis of HCC. Here, we show for the first time that p63 is a suppressor of AFP expression and that – among the p53 family members – p73 is the most efficient inhibitor of AFP. Furthermore, gain-of-function mutants of p53 can physically interact with the TA isoforms of p63 and p73, inhibiting their interaction with specific binding sites on the DNA and thus interfering with p63/p73 target gene repression or activation.

We provide evidence of the direct interaction of p53 family-dependent signaling and *AFP* gene regulation in HCC. Thus, the recent emergence of p53-based therapeutic approaches targeting p53-dysfunctional cancers using small molecules that restore wild-type p53 activity, as well as p53-based immunotherapy strategies would have the potential to restore p53, the TA isoforms of p63 and p73 and to concomitantly target *AFP* gene expression and *AFP*-mediated proliferation and immune escape.

CONCLUSIONS

In summary, our data show crosstalk of AFP and p53 family signaling cascades. Wild type p53 and the TA isoforms of p63 and p73 are repressors of *AFP* gene transactivation via binding to response elements in the promoter and intronic regions of the *AFP* gene.

Conflicts of interest: None to declare.

Authors' contribution: M.M.S. initiated the project and designed the work. M.M.S., S. Schlosser and E.O. were responsible for experimental design. G.W., E.O. and A.S. performed the majority of the experiments. G.W., K.G. and C.K. drafted the manuscript. E.O., A.S., S. Schlosser, S. Schmid, P.H., K.G., C.K. analyzed the results.

E.O., D.T. and K.G. performed data base analysis. D.T., C.K., G.W. prepared the figures. G.W., C.K., K.G. and M.M.S. wrote and edited the manuscript. All authors approved the final version of the manuscript.

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