Expression and Regulation of liver regeneration associated protein ALR under patho-physiological conditions

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“Life is not about finding yourself. Life is about creating yourself.”

George Bernard Shaw (1856-1950)
Irish dramatist and nobel prize winner for literature in 1925.

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I. INTRODUCTION

1. The liver

The liver is an important and life-saving organ, which is one of the biggest glands in the human body. It plays a central role in regulating the metabolic homeostasis which includes synthesis, storage and secretion of different molecules such as carbohydrates, fats and vitamins. It fulfils a main task in detoxification and removes toxic substances by metabolic conversion and biliary excretion. Furthermore, the liver is responsible for the biotransformation of endogenous substances such as bilirubin, bile acids and hormones, as well as exogenous substances like xenobiotics and alcohol. The liver is composed of different cell types, which are summarized as parenchymal and non-parenchymal cells (NPC). Hepatocytes as well as cholangiocytes belong to parenchymal cells and hepatocytes, which are the main cell type compose up to 80% of the liver cells and carry out most of the liver functions. Non-parenchymal cells include endothelial cells, Kupffer cells, stellate cells and cells from the blood compartment. Endothelial cells compose the circulatory blood vessels and form the walls of the hepatic sinusoids. Kupffer cells, liver-specific macrophages, are essential for the phagocytosis of foreign organisms in the liver and represent beside the stellate cells, the main source of cytokines and inflammatory factors [1]. Lymphocytes play an important role for innate immunity and defense against infection in the liver. Stellate cells -also termed Ito cells- have different functions e.g. they serve as fat and vitamin A-storage cells and produce most of the extracellular matrix (ECM) proteins [1].

2. Liver regeneration

The liver has an extraordinary capacity to regenerate after injury and this ability protects animals and humans from liver failure caused by infections or toxins. The ancient Greeks were among the first who described the hepatic regeneration as “ever-regenerating liver” in the myth of Prometheus’ [2]. It was told that Zeus punished the Titan Prometheus for stealing the fire and giving it to man. He was chained on the top of Mt. Caucasus, where an eagle ate daily from his liver. As an immortal Titan, Prometheus’ liver constantly regrew during the night, making the torment endless [3] (Fig. 1).

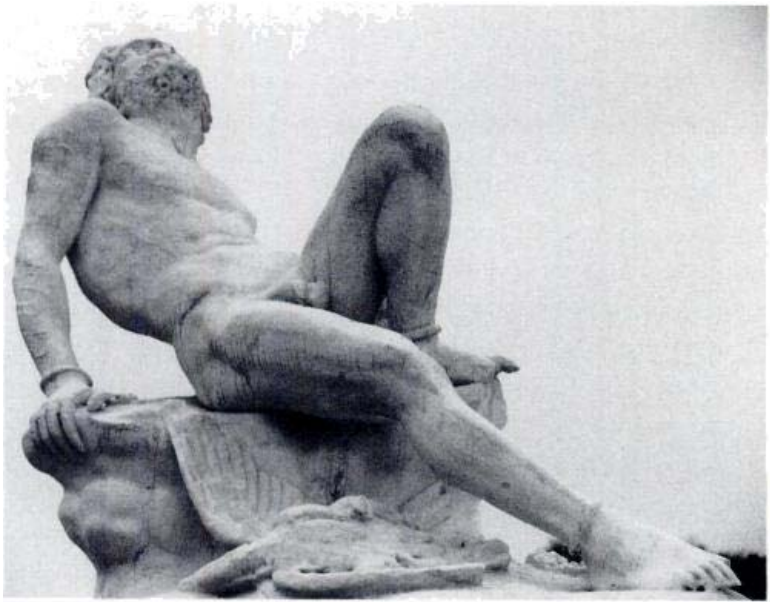


Fig. 1: A statue representing the torment of Prometheus. The picture was taken at the Jardin des Tuileries, Paris (S.P.) [3].

As mythical as the story is, but this describtion of an observation is based on the ability of the liver to renew. To date, the explanation of this phenomen has been in the focus of many researchers [4]. The word “regeneration” means the ability of an organ to replace tissue mass after partial removal or injury [5,6]. The process of liver regeneration is a multistep and well-orchestrated process which is initiated by liver injury such as physical damage (hepatectomy), infectious or toxic injury. This regenerative capacity is based on the ability of hepatocytes to proliferate in a differentiated state. Under normal conditions, hepatocytes are quiescent and rarely replicate in normal healthy livers [2,6-8]. But after damage, the resulting metabolic overload activates hepatic cells to initiate signals towards hepatocytes for entering the cell cycle. This process begins with DNA replication in the parenchymal cells reaching a peak after 24 hours, followed by proliferation of the non-parenchymal cells which usually starts with a delay of one day [1,9,10].

The hepatic regeneration consists of two major steps. First, priming which represents the transition of G0 to G1 and second, progression represented by the transition of G1 to S [7] (Fig. 2). After partial hepatectomy, released cytokines and/or circulating endotoxin (LPS) activate the non-parenchymal cells especially Kupffer cells stimulating them to produce tumor necrosis factor (TNF-α), interleukin IL-1 and IL-6. These factors stimulate hepatocytes enabling them for entering into G1-phase (Fig. 2). While hepatocytes undergo transition from G0 to G1, a large number of genes are either enhanced or *de novo* expressed. The first state of gene expression,

also termed immediate early phase, occurs within 4 h after injury and is identified by increased expression of protooncogenes such as c-fos, c-jun and c-myc (Fig. 2).

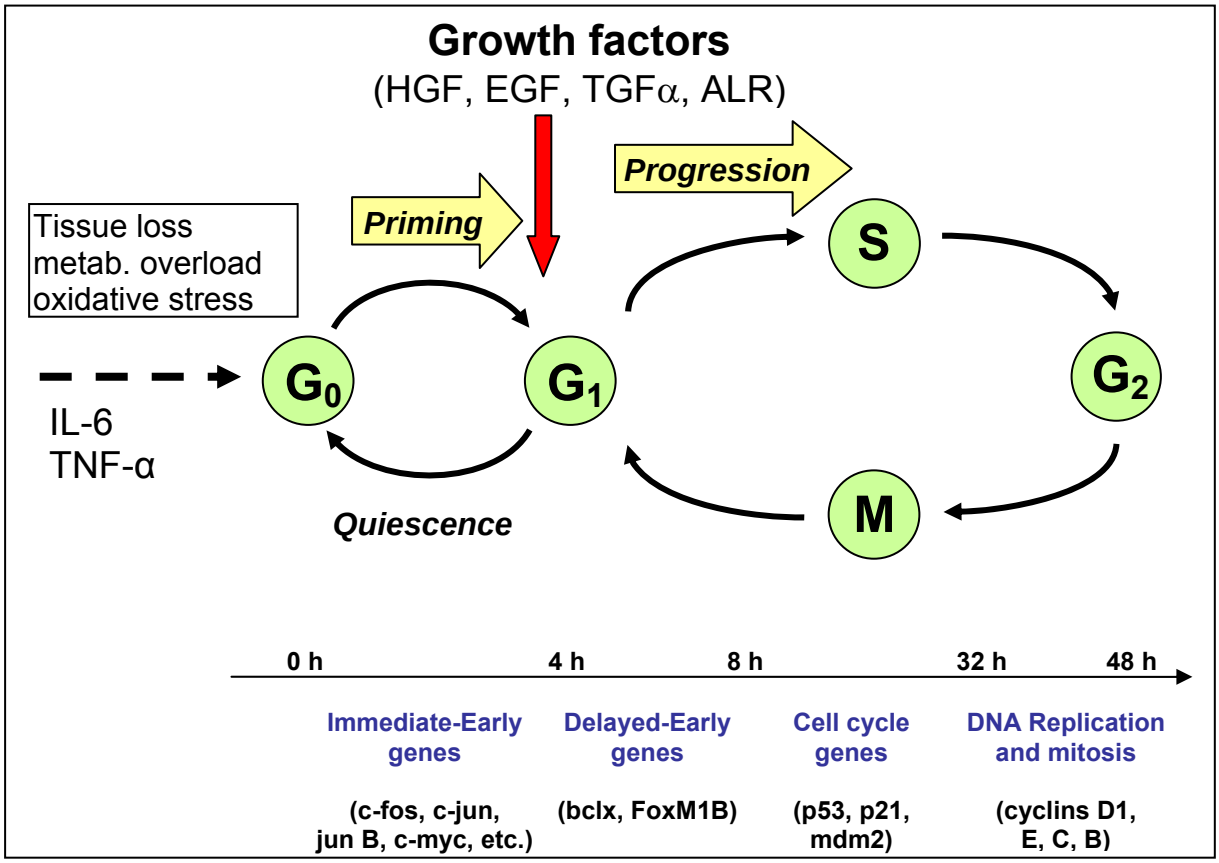


Fig. 2: Liver regeneration is triggered by liver damage such as partial hepatectomy [11].

After reaching the G1-phase, hepatocytes are able to respond to a wide variety of mitogens such as HGF, EGF, TGF-α and ALR, or to co-mitogens such as insulin [11,12]. The mitogenic factors have been shown to stimulate hepatocytes to pass the restriction point -also termed cell cycle checkpoint- and to enter the cell cycle followed by DNA synthesis [13-15]. This progression phase is accompanied by increased expression of the so-called “delayed early genes”. The transcription of these genes such as bcl-x and FoxM1B is followed by cell cycle progression which is associated with enhanced expression of the cell cycle genes e.g. p53, p21, mdm-2, cyclines and cyclin dependent kinases. The p53 and p21 are cell cycle inhibitors, while mdm-2 and cyclin D1 stimulate DNA replication [11] (Fig. 2). Once the liver reaches its requisite original mass, hepatocytes stop to divide and return to the non-proliferative state. The termination of this process requires different factors, and the most well-known hepatocyte anti-proliferative factor is TGF-β, which is released from stellate cells. TGF-β is blocked during the proliferative phase but is restored when

regeneration is completed, and this restoration of TGF-β inhibits DNA synthesis and supports hepatocytes to re-enter the quiescent state [1].

Growth factors implicated in regulating liver regeneration could be divided into two main classes namely complete mitogens and incomplete mitogens, also named co-mitogens. Complete mitogens are growth factors which can stimulate hepatocyte replication *in vitro* in the absence of other factors. These include HGF, EGF, TGF-α and ALR which are released from stellate cells, duodenum and hepatocytes, respectively (Fig. 3).

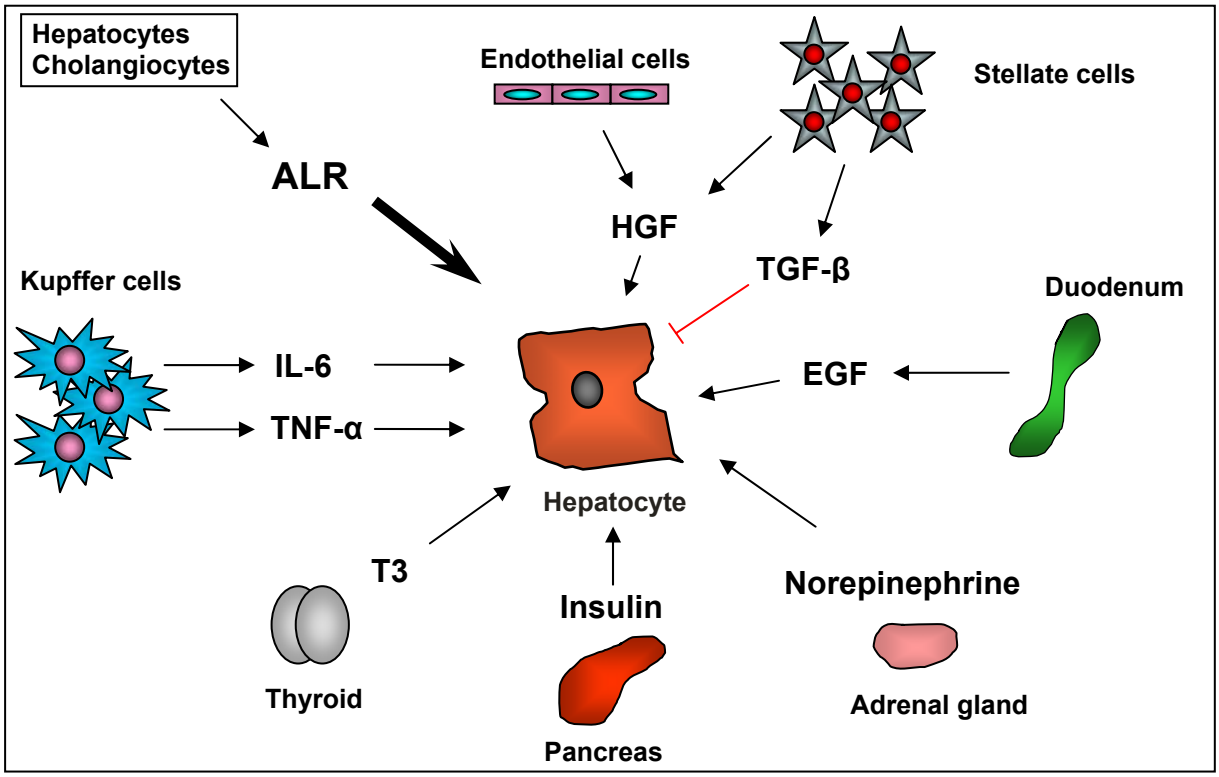


Fig. 3: Cytokines, growth factors and co-mitogens involved in liver regeneration [2,16].

In contrast, co-mitogens secreted from the pancreas (insulin), adrenal gland (norepinephrine) and thyroid gland (triiodothronine; T3) are factors which augment the effect of complete mitogens *in vitro*, but have no effect alone on hepatocyte replication [11,12,17] (Fig. 3).

3. Augmenter of liver regeneration (ALR)

3.1. ALR and mitogenic growth

Early studies have focused on the quest of factors involved in liver regeneration and possibly exerting a pro-proliferative effect on hepatocytes. 1975, LaBrecque and Pesch first isolated an extract called hepatic stimulator substance (HSS) from weanling and regenerating rat livers [18]. This factor was first discovered in the cytosol of parenchymal cells in the liver [18-20], and was reported to stimulate the cellular proliferation in hepatectomized livers of rats [18] and dogs [19,20]. Later, the purified peptide from the extract HSS has been sequenced, the isolated protein renamed ALR [21,22], and the rat, mouse and human ALR gene was cloned [23,24]. Augmenter of liver regeneration (ALR, also known as Hepatopoietin, HPO), a heat-stable hepatotrophic growth factor, different from other growth factors is organ specific, but species non-specific [25].

Unlike other common well-known growth factors such as EGF, HGF and TGF-α, which can stimulate the proliferation of different cell types [7], ALR has a specific proliferative action on the liver [26]. This specific hepatic effect of ALR is due to the existence of a high affinity ALR receptor on the surface of primary hepatocytes and hepatoma cells [27]. This receptor could not be detected on the membrane of other cell types like kidney, lung, ovary, larynx and hematopoietic tissue cell lines [27]. Although ALR has been originally described as a factor which exerts its effect only on hepatic cells, it is expressed in a wide variety of different tissues. It has been found in kidney, brain, cerebellum and the highest expression was observed in testis and liver [23,24,28].

3.2. ERV1/ALR family

ALR belongs to a new protein family named ERV1/ALR (Family ID: F00004892). This family includes members which are represented in different organisms, including plants [29], prokaryocytes (except in bacteria) as well as in lower and higher eukaryotes.

1996 Giorda *et al*. could isolate the human ALR cDNA (Gene ID: 2671) and reported the amino acid sequence of human ALR and its alignment with the rat and mouse

sequences [24]. Human ALR gene has been mapped next to the polycystic kidney disease gene (PKD1) on chromosome 16 [30]. Analysis of human ALR genomic DNA revealed that it consists of three exons and two introns. The longest hALR transcript encodes for a 205 amino acid protein corresponding to a molecular weight of 23 kDa [31], whereas the smallest hALR transcript encodes a protein of 125 amino acid (15 kDa) [32,33], which lacked the N-terminal 80 amino acids [31]. Since the 15 kDa isoform of hALR protein does not contain any known leader-sequence, it is mainly located in the cytosol and also found in the nucleus [34]. In contrary and under normal conditions, the full-length hALR is mainly located in the mitochondrial intermembrane space (IMS) [35]. Both forms of the human ALR protein were found to be present in hepatocytes, and these two spliced forms of ALR are able to dimerize which allows hALR protein to exist as a dimer. In addition, it has been shown that hALR gene could be alternatively spliced at the transcriptional level, that means that both isoforms are synthesized from the same mRNA using different initiation codes [34]. Figure 4 shows the sequence alignment and conserved amino acids of the full length rat, mouse and human ALR.

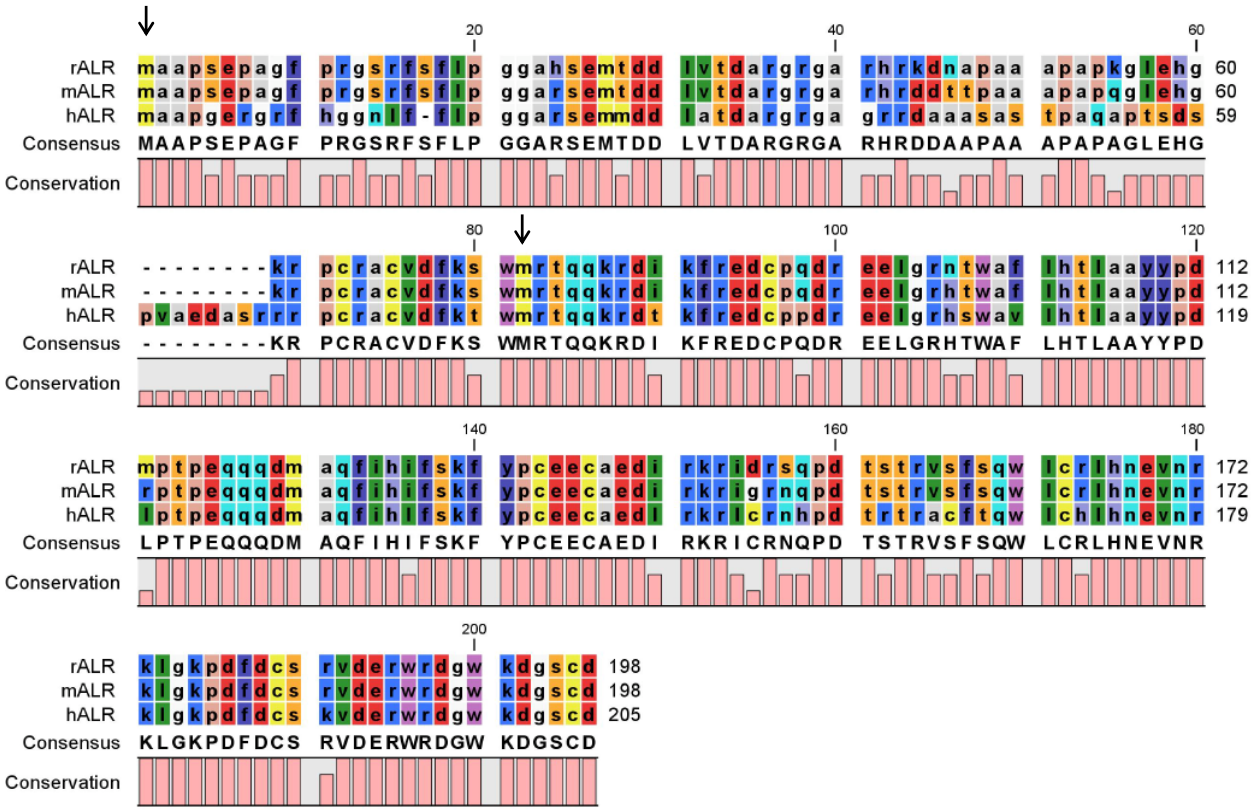


Fig. 4: Sequence alignment of the full length rat (r), mouse (m) and human (h) ALR. Identical amino acids found at the same position in all three proteins are shown in the conservation line. Dashes indicate absence of the base. The first 70 amino acids show significantly less conserved amino acid residues than the rest of the protein sequences. Arrows mark the

methionine residues of both long and short form of ALR. The sequences of rALR (EDM03859), mALR (NP_075527) and hALR (NP_005253) were obtained from Pubmed search tool.

The three-dimensional structure of human ALR protein has been examined by Farrell *et al.*, and is shown in Fig. 5B. Subunit A is shown in blue and subunit B in gray (with N- and C-termini highlighted in green). Cysteine residues are numbered and shown in yellow, and the FAD is in orange. The second disulfide bridge (C91-C108), shown in Fig. 5A, is conserved between ERV1, ERV2 and mammalian ALR [36]. The third disulfide bond forms intersubunit links (C15-C124) between the N- and C- termini of ALR [36]. These two intersubunit disulfide bonds are unique to ALR, they have not been found in ERV1 and ERV2.

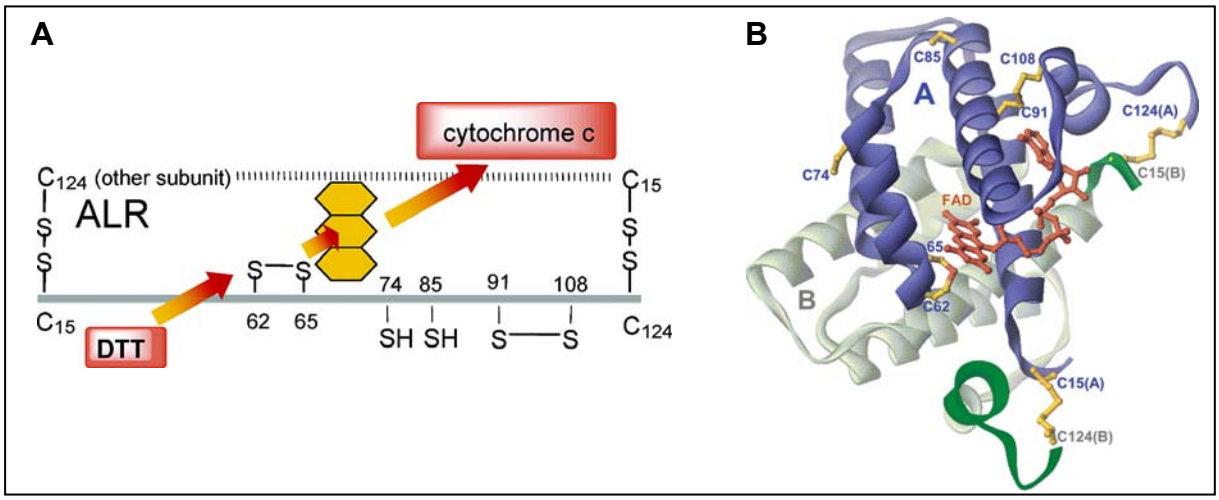


Fig. 5: A) Proposed thiol/disulfide pattern in human ALR. ALR is a disulfide bridged dimer (linked *via* C15−C124) with two free cysteine residues (C74 and C85) per monomer, and these free cysteines are not essential for enzymatic activity toward DTT, consistent with their lack of conservation between rat and human sequences. The C15−C124 disulfides are not critical for dimer formation and have insignificant impact on the dithiothreitol (DTT) oxidase activity of ALR. Cytochrome c is about a 100-fold better electron acceptor for ALR than oxygen when DTT is the reducing substrate. B) Homology model of human short-form ALR (125 amino acids) based on the crystal structure of rat ALR. The FAD in subunit B is omitted, and only C15 (B) and C124 (B) are depicted [37].

Each 15 kDa isoform of human ALR contains two additional nonconserved free cysteine residues (C74 and C85) depicted in Fig. 5A [37]. Farrell *et al.* proposed that ALR may not necessarily function as a sulfhydryl oxidase in the mitochondrial intermembrane space but it may communicate with the respiratory chain *via* the mediation of cytochrome c [37] (Fig. 5).

Giorda *et al*. in 1996, first cloned and characterized the mouse ALR gene (Gene ID: 11692). They showed that mALR gene is comprised of three exons, and is mapped to the mouse chromosome 17. Furthermore, they have found that the function of mALR protein is not limited to liver regeneration, but it is also involved in the synthesis or stability of the nuclear and mitochondrial transcripts. In addition, mouse ALR has been found to be highly expressed in actively regenerating cells, particularly in hepatocytes of hepatectomized livers and in germ cells of the testis [24]. The long form of mouse ALR protein shares extensive amino acid homology (79%) with the long form of human ALR protein (Fig. 4).

Rat ALR (Gene ID: 27100) was first discovered by Hagiya *et al*. in 1994. They cloned and sequenced the rat ALR gene, which was considered to encode rat HSS [23]. Furthermore, analysis of the rat genomic DNA of ALR revealed that the gene is composed of 3 exons and 2 introns and is 1508 nt long [38]. The rat ALR protein, which consists of 125 amino acids (15 kDa), acts as a homodimer [23] and the long form of rat ALR protein (22 kDa) exhibits 77% homology to human ALR (Fig. 4).

The ERV1/ALR family also includes ERV1 (*E*ssential for *R*espiration and *V*egetative growth) protein (scERV1) from Saccharomyces cerevisiae. The yeast scERV1 (Gene ID: 416547) was found to be essential for the viability of the cell, the cell cycle and the maintenance of intact mitochondrial genomes [39,40]. It is involved in mitochondrial biogenesis, and this function lies on its enzymatic flavin-linked sulfhydryl oxidase activity [41-43]. The most recent studies have focused on the function of ERV1 in the biogenesis of mitochondrial intermembrane space (IMS) proteins i.e. small Translocases of the Inner Membrane (TIMs). Translocation of these small proteins is performed *via* Translocase of Outer Membrane, the TOM complex. The TOM complex forms a channel which allows unfolded proteins to pass freely into the IMS, in both directions [44]. This import is mediated primarily by the essential IMS protein Mia40, which plays an essential role in the folding of imported cysteine-rich precursors [45]. It has been found that ERV1 is required for the import of the TIM proteins and it plays an important role for their biogenesis *via* interactions with Mia40. The latter oxidizes the newly imported proteins resulting in their final folded conformation. Subsequently, Mia40 is re-oxidized by direct interaction with ERV1 *via* thiol-disulfide exchange reactions [46,47]. The fact that scERV1 is oxidized by cytochrome c *in vivo* prompted Allen *et al*. to conclude that scERV1 functionally links

the Mia40-dependent import pathway to the respiratory chain transferring electrons from incoming polypeptides to cytochrome c [48]. The scERV1 has been found to exhibit 50% homology to the short form of rat ALR protein (15 kDa) [23]. Analysis of the complete genome sequence from S. cerevisiae revealed a second gene with 30% similarity and structural homologies to ERV1. In addition, the newly discovered gene ERV2 is the first member of the ERV1/ALR protein family located in the microsomes. Different from the mitochondrial ERV1, ERV2 is located in endoplasmatic reticulum (ER) in yeast, contains a CXXC motif and exhibits a sulfhydryl oxidase activity [49-51].

Recent studies in Drosophila melanogaster have shown that a gene (CG12534), orthologous to mammalian ALR may have a function during regeneration. Mutations in this gene affected the regeneration by either delaying, reducing or positioning the regeneration blastema. Based on these results, McClure *et al*. have proposed that CG12534 functions early in the regenerative proliferation and growth in drosophila [52,53].

The vaccinia virus protein E10R belongs also to this family and plays a central role in the virus life cycle. It has been shown that E10R protein is associated with the intracellular mature virions and is required for virion morphogenesis [54,55]. Another viral member of this family was found in African Swine Fever Virus (ASFV). ASFV encodes a protein termed 9GL which plays an essential role in viral growth in macrophages and viral virulence in swine [56].

3.3. ALR and protein biogenesis

Iron/sulfur (Fe/S) cluster-containing proteins are key players for electron transfer, metabolic and regulatory processes. Mitochondria in eukaryotes are one of the organelles responsible for protein biogenesis such as formation of Fe/S cluster proteins [51,57-59]. Functional cytosolic and nuclear Fe/S proteins act as sensors of oxygen or reactive oxygen species and therefore important for regulation of oxygen-responsive genes [60] and in the process of sperm development [28].

In addition, the mitochondrial isoform of ALR protein (23 kDa) was found to be involved in the export of Fe/S clusters from the mitochondrial matrix [35]. Furthermore, Lange *et al*. have found that ALR in the IMS is important for the assembly and biogenesis of cytosolic Fe/S proteins. Additionally, they have investigated which part

of the ALR protein is important for Fe/S protein assembly. Interestingly, it was found that the entire ALR protein (23 kDa) including the well-conserved C-terminus and the poorly conserved N-terminus is crucial for the assembly function.

In addition, in the nervous system ALR might be of importance in metal ion homeostasis whose dysregulation can induce neurodegenerative disorders. The involvement of ALR in the function of brain could be explained by the fact that ALR plays an important role in the iron homeostasis [35] and might interact with metallothioneins (MTs) [61], which bind metal ions such as zinc and copper [62].

A further reason for the involvement of ALR in the iron homeostasis is that mitochondrial iron overload can be a direct consequence of impaired iron/sulfur cluster assembly. Iron/sulfur proteins may serve as sensors of mitochondrial iron status and an inadequate iron/sulfur clusters impair the sensor function. A dysregulation of mitochondrial iron uptake as well as export may lead to iron overload [63].

3.4. ALR and liver regeneration

In the liver, ALR has been found to be constitutively expressed and stored exclusively in hepatic parenchymal cells [18-20]. Tanigawa *et al*. have determined the ALR mRNA expression in hepatic tissues and ALR protein levels in blood samples. Interestingly, they found that hepatic ALR mRNA expression was higher in patients with liver disease than in healthy controls, and a correlation was observed between serum ALR values and hepatic levels of ALR mRNA. In addition, several studies [26,64-67] have been published reporting that ALR augments liver regeneration and this is mediated by different mechanisms. The following conceivable mechanisms have been suggested (Fig. 6):

(i) activation of mitogen-activated protein kinase (MAPK) cascade through the induction of tyrosine phosphorylation of (EGF-R)
(ii) regulation of AP-1 activity
(iii) suppression of liver-resident natural killer (NK) cells.

Ad (i), ALR augments liver regeneration by activation of MAPK in hepatic cells followed by activation of various transcription factors e.g. AP-1 and NF-κB. ALR binds

to its own specific surface receptor on the hepatocytes [27] and this binding results in phosphorylation of MAPK cascades [64]. Therefore, ALR seems to resemble other growth factors e.g. EGF, HGF, TGF- α, which have similar mitogenic effect on hepatocytes *via* activation of MAPK pathway in a rapid and transient manner [7]. Further, it has been shown that both MAPK activation and the mitogenic effect of ALR on hepatoma cells were completely blocked by a specific inhibitor of EGF-R tyrosine kinase activity but not by blocking EGF-R suggesting that tyrosine phosphorylation of EGF-R may be essential for ALR signal transduction [26] (Fig. 6 and 9). In the same study, it was demonstrated that ALR has no effect on HGF receptor (c-met) [26].

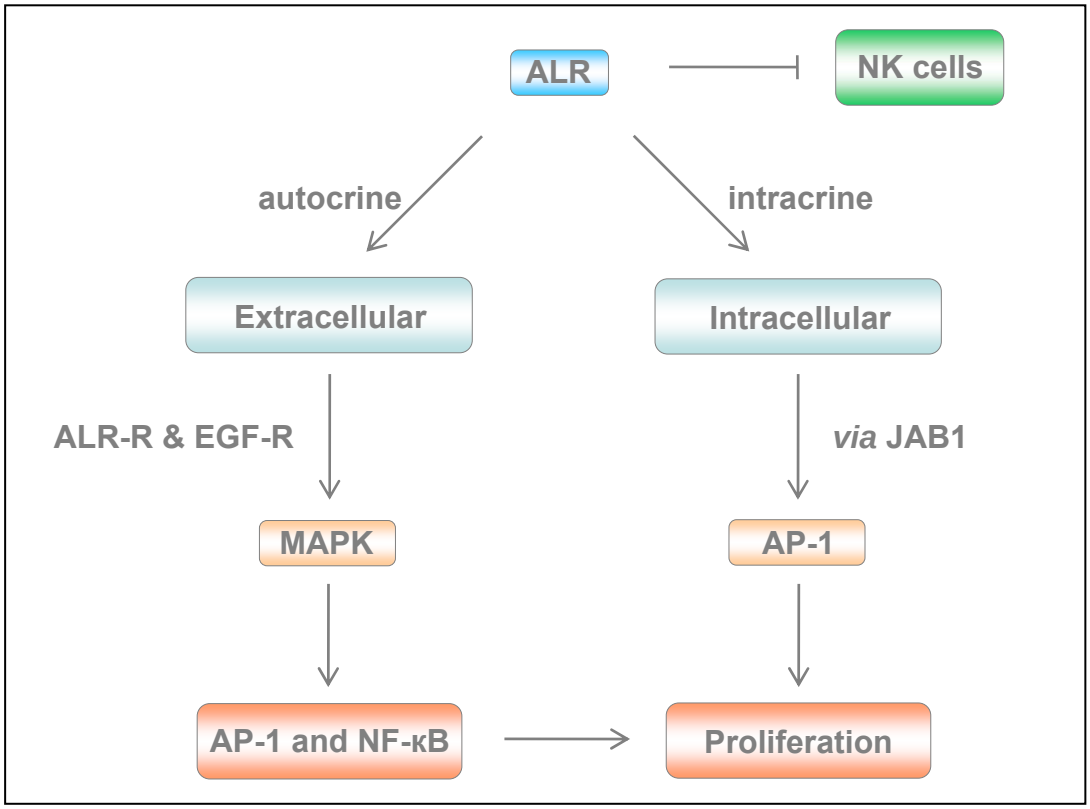


Fig. 6: Schematic drawing demonstrating signaling pathways of ALR. While extracellular ALR activates the MAPK signaling pathway by binding to its specific receptor on hepatocytes and hepatoma cells, intracellular ALR activates AP-1 *via* JAB1 in MAPK-independent fashion [65]. In addition, ALR augments the liver regeneration by supressing the NK cells [67,68].

Ad (ii), ALR exerts its hepatotrophic effect on the liver by regulation of the activating protein-1 (AP-1). Intracellular ALR interacts with c-Jun activation domain-binding protein (JAB1) *in vitro* and *in vivo* triggering the transcriptional activity of AP-1 in a MAPK-independent manner [65] (Fig. 6). JAB1, the fifth subunit (CSN5, S5) of mammalian COP9 signalosome (CSN), is highly conserved from yeast to human [69].

JAB1, a co-activator of c-jun/AP-1 transcription factor, enhances binding of c-jun-containing AP-1 complex to their DNA consensus sites and increases the transactivation of an AP-1 dependent promoter [70]. Further, it was found that ALR colocalizes with JAB1 in the nucleus of hepatic cells [65]. Interestingly, it has been hypothesized that the increased activation of AP-1 by ALR and JAB1 in COP9 signalosome is due to the enhanced c-jun phosphorylation [65,71] (Fig. 9). ALR is the first intracrine hepatotrophic factor identified to trigger AP-1 cascade *via* intracellular interaction with a multicomponent protein regulator complex. This interaction suggests a possible linkage between CSN and liver regeneration [71]. This might be a molecular link between the enzymatic redox function of ALR and its role as a factor with extracellular growth-promoting effect. It was shown that the cysteine residues (CXXC) in the C-terminus of ALR protein are necessary for the intracellular potentiation of AP-1 activity but not for the extracellular mitogenic effect of ALR [72]. Based on this finding, they concluded that ALR induced AP-1 activity depends on sulfhydryl oxidase activity and therefore they hypothesized a redox regulation of COP9 signalosome by ALR. Taken together, hepatic parenchymal cells synthesize ALR which exerts its pro-proliferative effect as intracrine (*via* JAB1) as well as paracrine (*via* MAPK) factor.

Ad (iii), ALR may augment liver regeneration by modulation of the lytic activity of natural killer (NK) cells. NK cells have been described as the major effector cells of nonadaptive immunity. Without prior antigen recognition, NK cells are able to mediate cytotoxicity against selected tumors and virus-infected cells [73,74]. In addition, NK cells secret a wide variety of cytokines regulating the adaptive immune response against microbial infections [73-75].

It has been shown that *in vivo* administration of ALR supress the hepatic NK cytotoxic activities in rats but do not affect the mononuclear leukocytes derived from spleen or peripheral blood [66] suggesting that ALR may be at least partially responsible for the local suppression of liver-resident NK cells [66]. Tanigawa *et al.* have made the observation that in humans increased ALR blood levels parallels decreased NK cell activity [67]. These data suggest that ALR may augment liver regeneration by local regulation of NK cells. Further, it was found that administration of ALR in healthy rats reduced the level of Interferon-γ (IFN-γ) in hepatic NK cells by suppressing the activity of NK cells. On the other hand, administration of IFN-γ in

70% hepatectomized rats led to significant reduction of both expression of mitochondrial transcription factor A (mt-TFA) and hepatic regeneration [68] (Fig. 7).
Figure 7 summarizes results from Polimeno *et al.* [68] and shows that ALR exerts two effects, the first reduction of IFN-γ in hepatic NK cells leading to reduced cytotoxic activity of NK cells, whereas second induction of mt-TFA followed by upregulation of mitochondrial DNA (mt-DNA) expression and mitochondrial metabolism. Taken together, IFN-γ is one of the main mediators of ALR biological activity both as growth factor and immunoregulator [68].

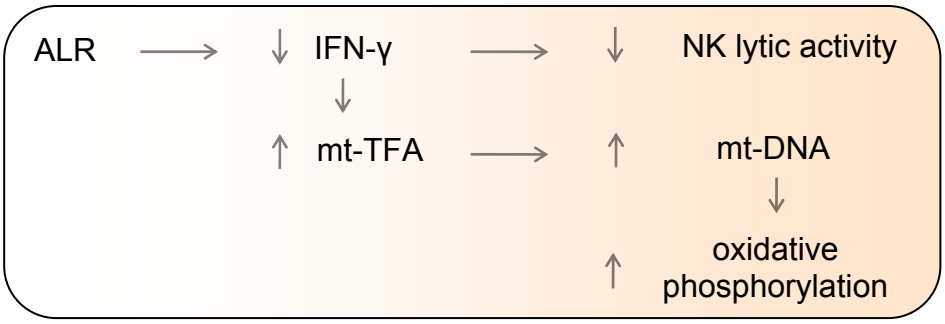


Fig. 7: ALR as immunoregulator and growth factor. The immunoregulatory effect of ALR is mediated by inhibition of IFN-γ expression followed by a strong decrease in NK cell lytic activities and by increase of hepatic mt-TFA which results in upregulation of mt-DNA and oxidative phosphorylation [68].

In addition to the mitogenic effect of the hepatotrophic factor ALR, it was shown that ALR modulates the regulation of hepatic metabolism by repression of phase I reactions (Cytochrome P450) in human hepatocytes. In contrast, ALR had no effect on phase II reactions such as UGT activity and the GSH/GSSG ratio. Treatment of human hepatocytes with ALR downregulated basal and induced P450 activities, and this was due to increased activity of NF-κB and decreased expression of constitutive androstane receptor (CAR) [76]. Based on these findings, it was suggested that ALR may have an effect which cross-links growth signals to regulation of hepatic metabolism [76] (Fig. 8).

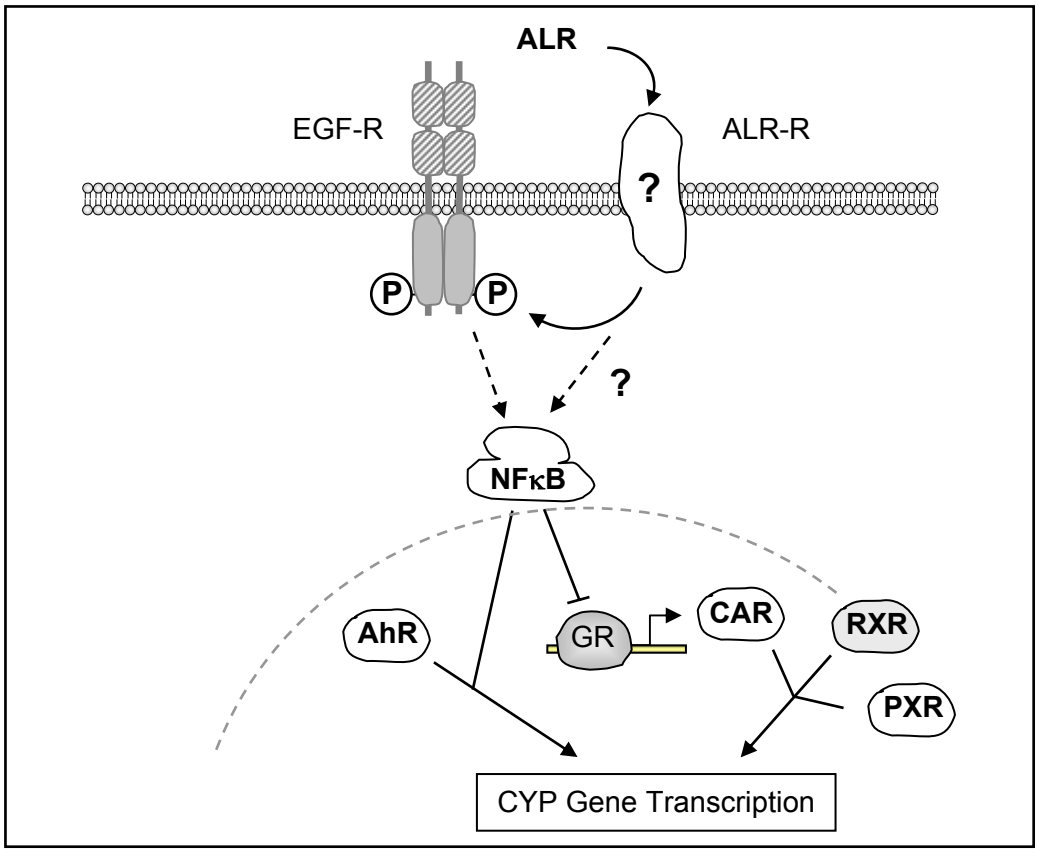


Fig. 8: Repression of cytochrome P450 enzyme activity by ALR. ALR stimulates its receptor, and this stimulation leads to EGF receptor activation followed by induction of NF-κB activity. NF-κB activation is known to interact with glucocorticoid receptor (GR) resulting in reduction of CAR expression and altered P450 expression. In contrast, ALR has no effect on the expression of other orphan nuclear receptors like aryl hydrocarbon receptor (AhR) and pregnane X receptor (PXR) [76].

Another hepatotrophic effect of ALR on the hepatic regeneration has been demonstrated by our group. We demonstrated that exogenously administered ALR was able to induce c-myc mRNA expression in primary human hepatocytes, resulting in elevated levels of ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase, both key enzymes of polyamine biosynthesis [77]. Polyamines, mainly putrescine, spermidine and spermine were considered essential molecules in several biological pathways like cell growth and differentiation [78,79]. Treatment with ALR induced the expression of putrescine, spermidine and spermine by regulation of ODC induction *via* NF-κB as well as *via* c-myc [77]. Since the polyamine metabolism plays a central role in hepatic regeneration [80], we conclude that ALR exerts its liver regeneration augmenting effect at least in part *via* activation of its signaling pathway leading to enhanced polyamine synthesis (Fig. 9).

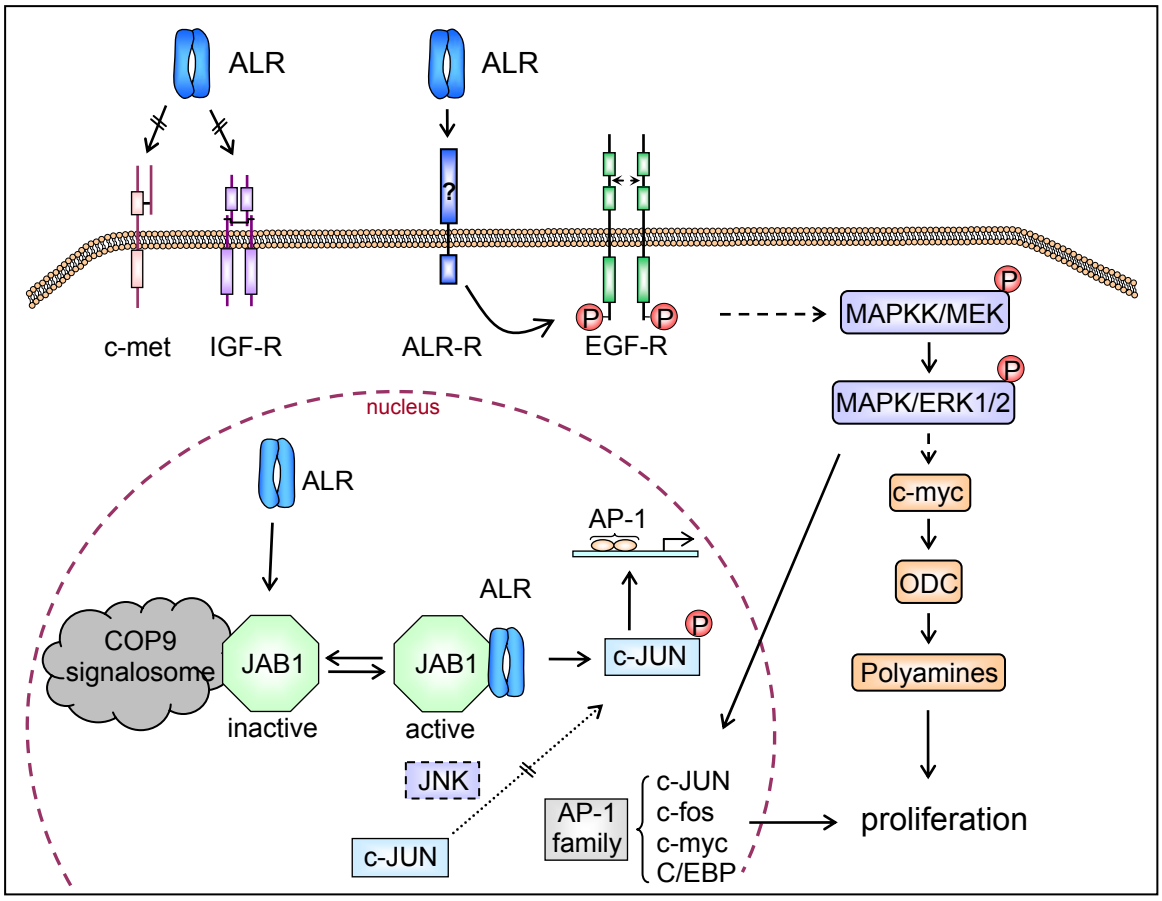


Fig. 9: Signal transduction of ALR. ALR binds to its receptor (ALR-R) resulting in activation of mitogen-activated protein kinase MAPK cascade *via* phosphorylation of EGF-R [26]. In addition, the activating protein-1 (AP-1) transcription factor family get activated after treatment with ALR in MAPK-dependent manner leading to cell growth and proliferation. Activated MAPK pathway leads also to elevated levels of polyamines *via* c-myc activation and ODC regulation [77]. Intracellular ALR interacts with c-Jun activation domain-binding protein (JAB1) triggering AP-1 transcriptional activity [65]. Dashed lines indicate that interaction/downstream effect might include several steps.

We also have demonstrated that ALR protein expression were elevated in livers from patients with cirrhosis, hepatocellular and cholangiocellular carcinoma [81]. Interestingly, the expression of ALR was inversely correlated with histological grading and angioinvasion of HCC suggesting that ALR may affect the invasiveness of HCC (manuskript in preparation).

Recently, researchers aimed to investigate the potential therapeutic application of ALR expression in a model of rat hepatic fibrosis. Liver fibrosis is the process of accumulative deposition of collagen and other extracellular matrix (ECM) components resulting from enhanced ECM synthesis and decreased ECM degradation [82]. In two studies an ALR recombinant plasmid was constructed and after application to rats with hepatic fibrosis reduced expression of TIMP-1 (tissue inhibitor of metalloproteinase), collagen type 1 and 3 was observed. Furthermore, a

marked reduction of ECM deposition in fibrotic liver tissue could be detected [83,84]. Taken together, ALR has beneficial effects on rat hepatic fibrosis by i) ameliorating the regeneration capacity of injured liver cells and ii) reversing liver fibrosis through reduction of ECM deposition.

3.5. ALR promoter and its regulation

ALR has been shown to be involved in constitutive cellular processes such as protein folding [85] and mitochondrial maintenance [39,43], which are indispensable in all cell types. On the other hand, there might be inducible functions of ALR [26,27] under patho-physiological circumstances such as acute liver diseases, liver cirrhosis and hepatocellular carcinoma [67,81]. Therefore, it would be of interest to investigate the regulation of ALR expression under physiological as well as patho-physiological conditions. However, up to now, there is little known about the regulation of ALR and the transcriptional control of ALR expression.

It was reported that the promoter region of ALR is TATA-less and has some characteristics of a housekeeping gene, oncogene, growth factor and transcription factors [86]. They hypothesized that a functional initiator (Inr)-like element and its flanking repeat elements together comprise a core promoter controling the transcriptional initiation of ALR gene. This core promoter, located between -54 bp and +42 bp, could maintain constitutive expression of at least one transcript of ALR [86]. On the other hand, it was shown that the major regulatory elements of the promoter of human ALR gene might exist in the region between -447 and -49 bp, and that a putative AP1/AP4 binding site (-375/-369 bp) may be crucial for basal and cell-specific promoter activity. Interestingly, they found that this region was responsible for the AP1 binding which exhibited a negative transcriptional activity in HepG2 cells, while the transcription factor AP4 was involved in the positive regulation in Cos7 cells. This dual regulation of AP1/AP4 *cis*-acting elements may account for differential expression of ALR gene in different tissues [87].

A recent study revealed a transcriptional regulation of the -252/-49 bp region from the transcriptional start site of the human ALR gene in hepatocytes [88]. They have demonstrated that SP1 functions as an essential transcriptional activator of basal expression of human ALR binding to the SP1 site (-152/-145) [88]. SP1 was found to be constitutively expressed in almost all tissues expressing human ALR [24]. In

addition, they have identified HNF-4α as another ALR regulating transcription factor and showed that HNF-4α could bind to its binding site (-209/-204), repress the hALR transcription and supress hALR gene expression in HepG2 cells [88].

4. Liver-Enriched Transcription Factors/LETF

Liver regeneration is a process which requires a wide variety of factors to achieve a successful tissue restoration. These factors include cytokines, growth factors, and transcription factors. In the liver, Liver-Enriched Transcription Factors (LETFs) play a central role in the regenerative process by maintaining the hepatocyte differentiation as well as controling the liver-specific gene expression [89]. They bind different hepatocyte-specific DNA regulatory regions encoding essential proteins for liver functions such as plasma proteins, clotting factors and enzymes involved in hepatic metabolism [90-92]. LETFs are classified by their ability to recognize specific DNA binding motifs and are divided into five major families: HNF1, HNF3, HNF4, HNF6, C/EBP and their corresponding isoforms [92-94].

The HNF3 family, also termed Forkhead box (Fox) familiy [95], composes of three winged helix proteins: HNF3α, HNF3β and HNF3γ, also known as FOXA1, FOXA2 and FOXA3, respectively. These proteins bind to DNA as monomer [96] and share strong homology (greater than 90%) in their DNA binding domain and therefore recognize the same DNA sequence [97]. HNF3β plays an essential role in gene regulation in hepatocytes and in gastric, pancreatic, intestinal and bronchiolar epithelium [92]. Based on cDNA microarrays technology, a wide variety of genes were found to be regulated during hepatic regeneration in livers from mice, who underwent partial hepatectomy. Interestingly, one of these genes is HNF3β (FOXA2), which was significantly upregulated (4,26 FC) two hours after hepatectomy, and declined after 6 hours [98].

Analysis of FOXA2 expression revealed not only a differential expression in HCC samples compared to normal liver tissue, but also demonstrated a constant upregulation of ALR in liver samples of patients with HCC [99]. Similar to ALR expression, FOXA2 was also found to be regulated during liver regeneration as well as in hepatocellular carcinoma. Furthermore, overexpression of FOXA2 in hepatocytes led to elevated levels of ALR in FOXA2-transgenic mice [100]. Taken together,

i) expression profiles of both ALR and FOXA2 are similar during liver regeneration as well as in liver samples of patients with HCC.

ii) expression of ALR is enhanced in the FOXA2-overexpressing liver tissue.

iii) promoter sequence of ALR exhibits putative binding sites for FOXA2.

These findings hint at a potential role of FOXA2 in regulating ALR expression.

II. AIM OF THE THESIS

Aims of the study

Liver regeneration is triggered and regulated by a variety of different factors, among them, ALR seems to play a major role due to its hepatotrophic and pro-proliferative effects. An organ-directed support/mediation of the regenerative process is of major clinical interest and might be a potential therapeutic application in the future.

The goal of this thesis was to increase the understanding of how ALR expression is regulated and what impact an altered expression of ALR might have on the liver.

In particular we aimed to investigate the expression profile of ALR during liver regeneration, the potential regulatory effect of regeneration-induced factors on the expression of ALR and the effect of intracellularly over-expressed ALR on hepatoma cells. The goals of this study are summarized as follows:

- Investigation of ALR expression in distinct models of hepatic injury, as well as in hepatoma cells and primary human hepatocytes.
- Analysis of the impact of liver associated factors, known to play a central role in liver regeneration, on the expression of ALR.
- Analysis of ALR promoter and putative transcription factor binding sites.
- Investigation of a putative regulation of ALR by the transcription factor FOXA2.
- Analysis of ALR overexpression in hepatoma cells and its impact on cellular growth and transformation.

III. MATERIALS & METHODS

1. Materials

1.1. Reagents, Chemicals, Kits, Solutions

Reagents and chemicals

Agarose	Biozym, Hameln, Ger.
Albumin Standard	Pierce, Rockford, USA
Ammonium persulfate (APS)	Sigma, Steinheim, Ger.
Ampicillin	Roche, Mannheim, Ger.
AMV-Reverse Transcriptase	Promega, Madison, USA
APAAP	Sigma, Missouri, USA
Aquatex	Merck, Darmstadt, Ger.
Bacto-Agar	Difco-Laboratories, Detroit, USA
Bacto-Trypton	Difco-Laboratories, Detroit, USA
β-mercaptoethanol	Roth, Karlsruhe, Ger.
Bis-Acrylamide	BIO-RAD, München, Ger.
Bromphenol blue	Serva, Heidelberg, Ger.
BSA (Lipid-free)	Sigma, Steinheim, Ger.
Complete Mini Protease Inhibitor Cocktail	Roche, Mannheim, Ger.
DL-Norleucin	Sigma, Steinheim, Ger.
DMEM	BioWhittaker, Verviers, Belgium
EDTA (Di-sodium)	Pharmacia Biotech, Freiburg, Ger.
ECL Super Signal® West Pico	Pierce, Rockford, USA
ECL Super Signal® West Femto	Pierce, Rockford, USA
Epidermal growth factor (EGF)	R&D Systems, Wiesbaden, Ger.
Ethanol	Merck, Darmstadt, Ger.
Ethidium bromide	Serva, Heidelberg, Ger.
Fast Red Chromogen	Roche, Penzberg, Ger.
FCS	Biochrom, Berlin, Ger.
G-418	GIBCO BRL, Karlsruhe, Ger.
GelPilot Mid Range Ladder	Qiagen, Hilden, Ger.
GlucaGen®	Novo Nordisk Pharma, Mainz, Ger.
Haematoxylin (Vektor H3404)	Buringame, Canada
Halt™ Protease Inhibitor Cocktail Kit	Pierce, Rockford, USA
HGF	R&D Systems, Wiesbaden, Ger.

Hydrocortison100	Rotexmedica, Trittau, Ger.
H_2O Nuclease-free	Promega, Madison, USA
IFN-γ	Tebu-Bio, Offenbach, Ger.
IL-1	R&D Systems, Wiesbaden, Ger.
IL-6	PeproTech GmbH, Hamburg, Ger.
Insulin	Lilly, Bad Homburg, Ger.
Kanamycin	Roche, Ingelheim, Ger.
KGF	R&D Systems, Wiesbaden, Ger.
L-glutamine	Biochrom, Berlin, Ger.
LPS	Sigma, Steinheim, Ger.
Mark12™ Protein Standard	Invitrogen, Karlsruhe, Ger.
MEM (Non-essential amino acid)	Invitrogen, Scotland
Methanol	Merck, Darmstadt, Ger.
Natrium chloride	Merck, Darmstadt, Ger.
Nucleic acid sample loading buffer (5x)	BIO-RAD, München, Ger.
O'GeneRuler™, DNA Ladder Mix	Fermentas, St. Leon-Rot, Ger.
PDGF	R&D Systems, Wiesbaden, Ger.
Penicillin/Streptomycin	Biochrom, Berlin, Ger.
Ponceau S	Sigma, Steinheim, Ger.
Powdered Milk	Carl Roth, Karlsruhe, Ger.
Potassium chloride	Merck, Darmstadt, Ger.
Potassium dihydrogenphosphate	Merck, Darmstadt, Ger.
Phosphate Buffered Saline w/o Ca^{2+}/Mg^{2+}	PAA, Pasching, Austria
Proteinase K	Qiagen, Hilden, Ger.
Ready-Load™ 1kb DNA Ladder	GIBCO BRL, Karlsruhe, Ger.
Restrictionendonucleases	NEB, Frankfurt, Ger.
SDS	Sigma, Steinheim, Ger.
siPort™ *XP-1*	Ambion, Austin, USA
T4-DNA-Ligase	GIBCO BRL, Berlin, Ger.
Taq-DNA-Polymerase	Roche, Mannheim, Ger.
TEMED	Merck, Darmstadt, Ger.
TNF-α	PeproTech GmbH, Hamburg, Ger.
TRIS Ultrapure	USB, Cleveland, USA.
Triton X-100	Boehringer, Mannheim, Ger.
Trypan blue solution (0.4%)	Sigma, Steinheim, Ger.

Trypsin/EDTA	Sigma, Deisenhofen, Ger.
Tween®20	Merck, Darmstadt, Ger.
1,4-Dithiothreit (DTT)	Roth, Karlsruhe, Ger.
100 bp Ladder, DNA	Pharmacia, Freiburg, Ger.
3% human serum (block solution)	BIO-RAD, München, Ger.

Kit systems

BCA Protein Assay Kit	Pierce, Rockford, IL, USA
CellTiter 96® AQ_{ueous} Proliferation Assay	Promega, Madison, USA
DNeasy Tissue Kit	Qiagen, Hilden, Ger.
Dual-Luciferase® Reporter Assay System	Promega, Madison, USA
ECL™ Western Blotting Analysis System	Amersham, Braunschweig, Ger.
FastStart DNA Master SYBR Green I	Roche, Basel, Switzerland
Halt™ Protease Inhibitor Cocktail Kit	Pierce, Rockford, USA
LightShift® Chemiluminescent EMSA Kit	Pierce, Rockford, USA
MycoAlert® Mycoplasma Detection Kit	Cambrex, Rockland, USA
NE-PER™ Extraction Reagents	Pierce, Rockford, USA
Plasmid Maxi Kit	Qiagen, Hilden, Ger.
QIAprep Spin Miniprep Kit	Qiagen, Hilden, Ger.
QIAquick Gel Extraction Kit	Qiagen, Hilden, Ger.
QIAquick PCR Purification Kit	Qiagen, Hilden, Ger.
Reverse Transcription System	Promega, Madison, USA
RNeasy Mini Kit	Qiagen, Hilden, Ger.
Migration assay	BD Bioscience, Heidelberg, Ger.
Invasion assay	BD Bioscience, Heidelberg, Ger.

Antibodies

Antibody	**Host**	**Provider**
Polyclonal anti-human ALR	Rabbit	Davids Biotech., Rgbg, Ger.
Polyclonal anti-human FOXA2	Goat	Santa Cruz, CA, USA
Polyclonal anti-human β-actin	Rabbit	Santa Cruz, CA, USA
Polyclonal Anti-rabbit	Mouse	Dako, Hamburg, Ger.

Polyclonal Ig-mouse	Rabbit	Dako, Hamburg, Ger.
Polyclonal IgG	Rabbit	Sigma, München, Ger.
Polyclonal anti-rabbit	Goat	DAKO, Hamburg, Ger.
APAAP complex	mouse	DAKO, Hamburg, Ger.
Polyclonal anti-human CDH1	Rabbit	Cell Signaling, Danvers, USA
Polyclonal anti-human HIF-1α	Rabbit	Novus Biologicals, CO, USA
Poylclonal anti-human SNAIL	Rabbit	Santa Cruz, CA, USA

Films and membranes

Biodyne® Nylon Transfer Membranes	PALL, Dreieich, Ger.
Chromatography paper, 3 mm	Whatman, Dassel, Ger.
ECL Hyperfilm	Amersham, Freiburg, Ger.
PVDF-Membrane, 0,45 µm	PALL, Dreieich, Ger.

Organisms and plasmids

Organisms	**Characteristics**	**Reference/Number**
Caco-2	Human colorectal cancer cells	ATCC/HTB-37
E. coli GC 10	Competent cells for cloning	Biomol, Hamburg, Ger.
HepG2	Human hepatoma cells	ATCC/HB-8065
HepG2.2.15	Human hepatoma cells	ATCC/CRL-11997
Hep3B	Human hepatoma cells	ATCC/HB-8064
Huh-7D12	Human hepatocellular cells	ECACC/01042712
H1299	Human cell lung cancer cells	ATCC/CRL-5803
PHH	Primary human hepatocytes	this work
PLC	Human hepatoma cells	ATCC/CRL-8024

Plasmids	**Marker**	**Reference**
pcDNA3.1 (see appendix)	Amp^r	Invitrogen, Karlsruhe, Ger.
Constructs based on pcDNA3.1	Amp^r	this work
pGL2-Basic (see appendix)	Amp^r	Promega, Madison, USA
Constructs based on pGL2-Basic	Amp^r	this work

Preparation of solutions

PBS, 20×

NaCl	320 g (2,74 M)
KCl	8 g (53,7 mM)
Na_2HPO_4 x $2H_2O$	56,8 g (159,5 mM)
KH_2PO_4	8 g (29,4 mM)
Aqua dest.	Ad to 2L

PBS, 10× + 1% Tween

20x PBS	500 ml
Tween®20	10 ml
Aqua dest.	Ad to 1L

PBS, 1× + 0,1% Tween

10x PBS	100 ml
Aqua dest.	Ad to 1L

Anode Buffer A

Methanol	100 ml (20%)
Tris Cl (1 M)	12,5 ml (25 mM)
Aqua dest.	Ad to 500 ml

Anode Buffer B

Methanol	100 ml (20%)
Tris Cl (1 M)	150 ml (0,3 M)
Aqua dest.	Ad to 500 ml

Cathode Buffer

DL-Norleucin	2,63 g (40 mM)
Tris Cl (1 M)	12,5 ml (25 mM)
Aqua dest.	Ad to 500 ml

6× SDS loading Buffer (Laemmli dye)

Tris Cl (0,5 M, pH 6,8)	6 ml (300 mM)
SDS (10 %)	2 ml (2 %)
Bromphenolblau	10 mg (0,1 %)
Glycerin	6 ml (10 %)
Aqua dest.	Ad to 10 ml

10× SDS –Gel running Buffer

Tris Base	30,2 g (250 mM)
Glycin	144 g (1,9 M)
SDS	10 g (1 %)
Aqua dest.	Ad to 1L

For reducing conditions add 100 mM DTT or 25 µl/ml β-mercaptoethanol

5× TBE-buffer (DNA)

Tris	54 g (0,445 M)
Boric acid	27,5g (0,445 M)
EDTA (pH 8,0)	20 ml (0,5 M)
Aqua dest.	Ad to 1L

Ethidium bromide stock solution (DNA)

10 mg/ml ethidium bromide in H_2O

1.2. Oligonucleotides

All sequences are denoted from 5' to 3'.

For Cloning

Oligonucleotides were ordered from Sigma/Genosys (Steinheim, Germany).

Primer	Sequence
hALR-Pro-Bgl2-Fwd	ata gat ctc gca agg agg cac agg aat ccc
hALR-Pro-HindIII-Exon1-Rev	ata agc ttc cac gtc ttg aag tcg acg cag g
hALR-Pro-HindIII-Exon2-Rev	ata agc ttg cgg aag cag gcc gac cga gag

For Sequencing

Sequencing analysis were performed by GENEART (Regensburg, Germany) using free of charge oligonucleotides (T7-promoter and BGH reverse primer).

For Gene expression

The mRNA sequences for ALR, FOXA2 and 18S ribosomal RNA were derived from GenBank and the primers were designed using OligoPerfect™ Designer tool (Invitrogen, Karlsruhe, Germany). If not otherwise noted, the oligonucleotides were obtained from Sigma/Genosys (Steinheim, Germany).

Primer	Sequence
hALR-Fwd.	cac aat gaa gtg aac cgc aag
hALR-Rev.	cac cca act gag aca caa cag
mALR-Fwd.	cac agg atc ggg aag aat tg
mALR-Rev.	att cct cgc agg ggt aaa ac
rALR-Fwd.	agc ggg aca tca agt tta gg
rALR-Rev.	atg aac tgg gcc ata tcc tg
hMMP-1-Fwd.	tca cca agg tct ctg agg gtc aag c
hMMP-1-Rev.	gga tgc cat caa tgt cat cct gag c
hMMP-3-Fwd.	obtained from Tib molbiol (Berlin, Germany)

hMMP-3-Rev.	obtained from Tib molbiol (Berlin, Germany)
18S Ribosomal RNA-Fwd.	gta acc cgt tga acc cca tt
18S Ribosomal RNA-Rev.	cca tcc aat cgg tag tag cg

For EMSA

Oligonucleotides were ordered from Metabion (Martinsried, Germany)

Oligonucleotide	**Sequence**
wt FOXA2-Fwd.	tgc ccg att tct ccc agc ccc gcg c
wt FOXA2-Rev.	gcg cgg ggc tgg gcg aaa tcg ggc a
wt FOXA2-Biotin-Fwd.	Biotin-tgc ccg att tct ccc agc ccc gcg c
wt FOXA2-Biotin-Rev.	Biotin-gcg cgg ggc tgg gcg aaa tcg ggc a
wt IL6-RE–Fwd.	acc ccg gca gag ctt ccc agg gtt g
wt IL6-RE-Rev.	caa ccc tgg gaa gct ctg ccg ggg t
wt IL6-RE–Biotin-Fwd.	Biotin-acc ccg gca gag ctt ccc agg gtt g
wt IL6-RE–Biotin-Rev.	Biotin-caa ccc tgg gaa gct ctg ccg ggg t
wt C/EBPβ-Fwd.	cca ggg ttg cct gtc cct gaa cct t
wt C/EBPβ-Rev.	aag gtt cag gga cag gca acc ctg g
wt C/EBPβ-Biotin-Fwd.	Biotin-cca ggg ttg cct gtc cct gaa cct t
wt C/EBPβ-Biotin-Rev.	Biotin-aag gtt cag gga cag gca acc ctg g

1.3. Technical equipment

Balance ScalteC-SBA53	Denver Instrument, Göttingen, Ger.
Biofuge 15R	Heraeus, Hanau, Ger.
BioPhotometer 6131	Eppendorf, Hamburg, Ger.
Blue Blot Wet/100	Serva Electrophosesis,Heidelberg, Ger.
Cell culture Incubator BB6220	Heraeus, Hanau, Ger.
Centrifuge 5417C	Eppendorf, Hamburg, Ger.
Centrifuge 5417R	Eppendorf, Hamburg, Ger.
Digital camera colorview 12	Olympus, Hamburg, Ger.
ELISA-reader GENios plus	Tecan, Stuttgart, Ger.
Freezing container	Nalgene, Rochester, USA
Horizontal Shaker KS250 basic	IKA® Labortechnik, Staufen, Ger.
Hypercassette Neutral	Amersham, Buckinghamshire, UK

Kodak Film Processor M35 X-Omat	Kodak, Stuttgart, Ger.
LaminAir® Hood HB2474	Heraeus, Hanau, Ger.
LightCycler® 1.2 Instrument	Roche, Basel, Switzerland
LUMAT LB9501	Berthold, München, Ger.
Magnetrührer RCT basic B	IKA® Labortechnik, Staufen, Ger.
Megafuge 1.0R	Heraeus, Hanau, Ger.
Microscope Leica DMIL	Leica microsystems, Wetzlar, Ger.
Microwave Inverter NN-SD456W	Panasonic, Hamburg, Ger.
Mini centrifuge 5415D	Eppendorf, Hamburg, Ger.
Milli-Q Biocel	Millipore, Bradford, USA
Mini Transblot Cell	Bio-Rad Laboratories, München, Ger.
Peltier Thermal Cycler PTC-200	Biozym, Oldendorf, Ger.
PH-Meter 766 calimatic	Knick, Berlin, Ger.
Powerpack 200	Bio-Rad, München, Ger.
Semi Dry Transfer Cell Trans-Blot SD	Bio-Rad, München, Ger.
Shaker Certomat R	Vitaris, Baar, Switzerland
Shaking water Bath 1092	GFL, Burgwedel, Ger.
SONOPULS HD 2070	Bandelin, Berlin, Ger.
Sub-Cell® GT	Bio-Rad Laboratories, München, Ger.
Thermomixer	Eppendorf, Hamburg, Ger.
Varioklav®	Thermo Electron, Oberschleißheim, Ger.
Vortex mixer Genie 2	Scientific Industries, NY, USA
Wide Mini-Sub® Cell GT	Bio-Rad Laboratories, München, Ger.

1.4. Databases research

BLAST	http://www.ncbi.nlm.nih.gov/BLAST
PubMed	http://www.ncbi.nlm.nih.gov/entrez
TESS	http://www.cbil.upenn.edu/cgi-bin/tess
TFsearch	http://www.cbrc.jp/research/db/TFSEARCH.html
Transfac	http://www.gene-regulation.com/pub/databases.html

2. Methods

2.1. Primary cells and cell lines

2.1.1. Isolation of primary human hepatocytes

Tissue samples from human liver resections were obtained from patients undergoing partial hepatectomy for metastatic liver tumors of colorectal cancer. Experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR (Human Tissue and Cell Research), with the informed patient's consent [101] approved by the local Ethical Committee of the University of Regensburg. Human hepatocytes were isolated using a modified two-step EGTA/collagenase perfusion procedure and maintained in culture as described previously [76,102]. Viability of isolated hepatocytes was determined by trypan blue exclusion method and cells with a viability > 85% were used for cell culture experiments.

2.1.2. Cultivation of hepatocytes and cell lines

Hepatocytes were plated in collagen-coated 6-well plates (BD BioCoat™ Collagen I) at a density of 1,0 x 10^5 cells/cm^2 in appropriate volume of culture media. The medium consisted of Dulbecco's modified Eagle's medium with 5% fetal calf serum, 2 mM L-glutamine, and supplements as follows: 1,7 mU/ml insulin, 3,75 ng/ml hydrocortisone, 100 µg/ml streptomycin, 100 U/ml penicillin. 24 h after plating, medium was replaced by medium with 0,5% FCS. Cells were incubated at 37°C in a humidified incubator with 5% CO_2. Viability of hepatocytes during culture period was monitored by cell morphology (light microscopy, image analysis) and determination of enzyme release into culture medium (aspartate aminotransferase activity).

Cell lines were cultured in DMEM supplemented with 10% inactivated FCS, L-glutamine (2 mM), antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and 0,1 mM non-essential amino acids (MEM). FCS was incubated at 56°C for 30 min in order to inactivate the complements. Cells were grown in monolayer T-75 flasks with approximately 10 ml of medium until confluency.

Cell passaging

Cell cultures were split at a ratio of 1:3 to 1:5 in fresh medium every 3-4 days. Adherent cells were washed once with PBS and either scrapped or disaggregated by incubation with 0,05% Trypsin/0,02% EDTA/PBS (2-3 ml per 75 cm^2 culture vessel area) at 37°C for 5 min until cells detached. Trypsin was then inactivated by adding 6 ml medium with 10% FCS. After centrifugation at 30 g for 7 min, the supernatant was discarded and the cells were resuspended and plated.

Assessing cell vitality

Number of viable cells was determined by trypan blue exclusion method. The cell suspension was diluted with 0,2% (w/v) trypan blue solution and the cells were counted in a Neubauer haemocytometer.

Freezing and thawing cells

Confluent cells were harvested, washed with PBS and pellet obtained at 30 g was frozen in DMSO/DMEM (1:10) including 10% FCS. To allow gradual freezing at a rate of 1°C/min, the cryo-vials were placed in isopropanol-filled cryo-containers and frozen at -20°C for 2 h and then transferred to -80°C for 48 h. For long-term storage, samples were stored in liquid nitrogen.

In contrast, frozen cells were rapidly thawed by resuspending them in 5 ml warm medium. The cells were then centrifuged at 30 g for 7 min, the supernatant was discarded, and the cells were washed and plated with standard medium including 10% FCS. Next day, medium was replaced to remove all traces of DMSO.

2.1.3. Stimulation and treatment of cells

For stimulation experiments, 10,5 x 10^3 cells/cm^2 were seeded into each well in 6-well plate with medium containing 10% FCS. 24 hours after plating, the medium was replaced by serum-free medium for the next 24 hours. Cells were then incubated in fresh serum-free medium in the absence or presence of different growth factors and cytokines for another 24 hours until they were harvested. Treatment was performed in triplicates at least 3 times.

Stimulating agents were used at following concentrations:

Stimulator	Final concentration
HGF	10 ng/ml
EGF	20 ng/ml
KGF	100 ng/ml
PDGF	10 ng/ml
IFN-γ	5000 U/ml
LPS	1 µg/ml
Et-OH	0,1%
Insulin	0,5 U/ml
IL-1	250 U/ml
IL-6	30 ng/ml
TNF-α	10 ng/ml

2.1.4. Transfection of cell lines

Transient transfection of luciferase-reporter plasmids

For transfections, HepG2 cells were cultured as mentioned above. The passage number of the cells ranged from P10- P20 from the ATCC stock culture. 10,5 x 10^{3-} 21 x 10^3 cells/cm^2 were seeded in 6-well plate over night. After 24 hours, 0,5 µg of hALR promoter constructs (construct 1: -733 to +240 bp, and construct 2: -733 bp to + 502 bp) were transiently co-transfected with 0,1 or 0,5 µg of the expression plasmid pcDNA3.1-FoxA2 (gift from R. H. Costa) using the siPORT™ *XP-1* Transfection Agent following manufacturer's instructions. To determine the transfection efficiency, pRL-TK Renilla-vector was co-transfected. The promoterless vector pGL2-Basic served as negativ control.

Luciferase reporter assays

24 hours after transfection, cells were washed twice with PBS then lysed in 500 µl Reporter lysis Buffer (Promega) using a shaker. In order to perform the DLR™ Assay, 100 µl of LAR II were predispensed into a luminometer tube into which 20 µl of cell lysate were transferred. After mixing the firefly luciferase activity was measured using

the luminometer LB9507 (Berthold). Afterwards, 100 µl of Stop & Glo® Reagent were added into the tube, briefly mixed and the Renilla luciferase activity was immediately measured. Each experiment was repeated three times and all measurements were performed in triplicates. Results are expressed in comparison to promoterless pGL2-Basic vector activity.

Stable transfection of HepG2 cells

For transfection of expression plasmid pcDNA3.1-hALR into the HepG2 cells, siPORT™ *XP-1* Transfection Agent, a polyamine-based DNA transfection reagent, was used following Ambion instructions. Briefly, one day prior to transfection 21 x 10^3 HepG2 cells/cm^2 were seeded per well into a 6-well plate in normal growth medium, until 30-60% confluency after 24 hours. Next day, 2 µl transfection reagent was added to 98 µl medium, shortly vortexed and incubated at RT for 20 min. Afterwards, 0,5 µg DNA was added, mixed gently by flicking, and further incubated for 20 min at RT to allow the formation of the transfection-complex. In the meantime, cells were washed and incubated with 1,9 ml normal growth medium. Finally, 100 µl of the transfection-complex were applied dropwise onto the cells. The empty vector pcDNA3.1 was transfected into HepG2 cells and served as mock-control.

Antibiotic selection of stable-transfected HepG2 cells

48 hours after transfection, cells were cultured based on positive selection with G-418. A concentration of 1 mg/ml G-418 was used for the selection process. The normal growth medium supplemented with G-418 was renewed every 2 days. The selection time varied from 12 to 14 days and ended after all cells of the non-transfected control were dead. The picking of clones was started when round clones with a shiny, well-defined border appeared. The concentration of G-418 during cultivation was 0,4 mg/ml.

Picking, expansion and freezing of HepG2 clones

Each individual drug-resistant clone was picked from the selection plate using a pipette with a yellow tip adjusted to a volume of 20 µl. 24 clones were picked, transferred into a 48-well plate and further cultured under normal conditions. Once clones reached confluency, they were split into 24-well plate. The cells were then expanded in T-75 culture flasks and when they reached 80% confluency, they were frozen and stored in liquid nitrogen.

2.1.5. Migration and invasion assay

To determine the effect of over-expressed ALR on cell motility, migration assays were performed using modified Boyden chambers. Briefly, 0,5 x 10^5 of both wt- and stably ALR expressing HepG2 cells were resuspended in 1% FCS-DMEM and seeded into inserts with 8 µm filter pores, which were either uncoated (migration assay) or matrigel-coated (invasion assay). 10% FCS-DMEM served as a chemo-attractant. After 48 hours, cells were fixed and stained with Diff-Quick and all migrated or invaded cells were counted.

2.2. Protein biochemical methods

2.2.1. Preparation of cell protein extracts from mammalian cells

Isolation of whole cellular proteins

The cells were washed twice with cold PBS to remove residual proteins of the FCS which cause high background signals by unspecific antibody binding. Cells were harvested by scrapping in cold RIPA-Buffer containing protease inhibitors. Afterwards, the samples were homogenized by sonification and the homogenate was centrifuged at 20,000 g for 20 min at 4°C. The supernatant was transferred into 1,5 ml Eppendorf cups and the proteins were used immediately for protein determination or stored at -20°C.

Isolation of nuclear proteins

In order to isolate the nuclear proteins, NE-PER™ Nuclear and cytoplasmic Extraction Reagents was used following PIERCE instructions. 2 x 10^6 HepG2 cells were washed with cold PBS and centrifuged at 500 g for 3 min. The supernatant was carefully removed, and the pellet was resuspended with 200 µl of ice-cold CER I. After vortexing for 5 sec and incubating on ice for 1 min, the sample was centrifuged at 16,000 g for 5 min, and the supernatant containing the cytoplasmic extract was transferred to a clean pre-chilled tube. The insoluble pellet-fraction was then resuspended with 100 µl of ice-cold NER and vortexed at maximum speed for 15 sec. Afterwards, the sample was incubated on ice and vortexed for 15 sec every 10 min, for a total of 40 min. Subsequently, the sample was centrifuged at full speed 16,000 g for

10 min, and the supernatant containing the nuclear fraction was transferred to a clean pre-chilled tube. All extracts were then used to determine the protein concentration and immediately stored at -80°C until use in many downstream assays.

Determination of protein concentration

For protein quantification, the BCA Assay protein Quantitation Kit was used. The method is based on the reduction of Cu^{2+}-ions to Cu^{1+}-ions in alkaline environment (Biuret reaction). The Cu^{1+}-ions form a complex with bicinchonic acid (BCA) a purpled colored complex with a peak absorption at 562 nm. The absorption is linear in a range of 20-2000 µg/ml and is proportional to the protein concentration. BCA reagent was freshly prepared by adding 4% $CuSO_4$ to the protein solution at a ratio of 1:50. 10 µl of the probe or the standard were added to a microtiter 96-well plate and mixed with 200 µl of the prepared BCA solution. After incubation at 37°C for 30 min the extinction was measured in the TECAN's microplate reader GENios Plus and the protein concentration was calculated.

2.2.2. SDS-Polyacrylamid-Gel Electrophorese (SDS-PAGE)

Protein samples were separated according to their size by using a discontinuous gel system, which is composed of stacking (5%) and separating gel (14%) layers which differ in salt and acrylamide concentration.

For gel electrophoresis, the stacking and separating gels were prepared as follows:

	Stacking gel	Separating gel
40% acrylamid	312,5 µl	1750 µl
0,5 M Tris.Cl (pH 6,8)/1 M Tris.Cl (pH 8,8)	312,5 µl/-	-/1250 µl
10% SDS	25 µl	50 µl
H_2O	1825 µl	1900 µl
Tetramethylethylenediamine (TEMED)	2,5 µl	2 µl
10% Ammonium persulfate (APS)	25 µl	50 µl

Next day, the separating gel was cast and overlaid with water-saturated isobutanol until polymerization is finished. After 1 hour isobutanol was discarded and the stacking

gel was poured on top of the separating gel, and the comb inserted immediately. After polymerization, the stacking gel was mounted in the electro-phoresis tank, which was filled with running buffer. 20 µg of protein lysate were denatured by adding 3 x SDS sample buffer ± 100 mM DTT incubating at 95°C for 10 min. The gel was run with 125 volt until the sample reached the surface of the stacking gel, then the current was increased to 160 volt and the gel was run for 2-4 h.

2.2.3. Western Blot analysis

After separation by SDS-PAGE, proteins were transferred electrophoretically onto a Polyvinylidene Fluoride (PVDF) microporous membrane using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The transfer conditions were 43 mA for 45 min at RT. Next steps were then performed as follows:

	Diluation/Solution	Duration
Blocking	5% non-fat dry milk/PBST (0,1%)	2 hours
Primary antibody	Variable/5% non-fat dry milk	Over night
Washing	PBST (0,1%)	5 x 5 minutes
Second antibody	1:10000/5% non-fat dry milk	1 hour
Washing	PBST (0,1%)	5 x 5 minutes

Signal detection was performed using SuperSignal West Pico Chemiluminescent Substrate according to Thermo-Scientific's instruction and the blots were exposed to an autoradiography film (Hyperfilm ECL^{TM}, Amersham) for 5 sec to 30 min depending on the signal intensity.

Stripping of membranes

This method was perfomed as described in Restore™ Western Blot Stripping Buffer booklet. Briefly, the membrane was incubated with stripping buffer for 15 min at RT, then washed twice using large volumes of wash buffer at RT. Afterwards, immunodetection of other proteins was performed.

2.2.4. Immunohistochemistry (IHC)

Immunostaining was performed using Alkaline Phosphatase-Anti-Alkaline Phosphatase (APAAP) complex method.

Preparation, fixation and permeabilization of slides

2 µm sections were cut from paraffin-embedded tissue and transferred to silanized glass slides (Super Frost™). The slides were deparaffinized in xylene for 20 min at RT and rehydrated in graded concentrations of ethyl alcohol (100%, 96%, 90%, 80%, 70%) then water. To enhance the antigen retrieval, sections were treated with Proteinase K diluted in 50 mM Tris-Puffer (PH 7,5) (1:4) at RT for 10 min and then washed in distilled water.

Blocking and staining

The sections were placed into PBS buffer containing 3% human serum (block solution) for 30 min. Primary polyclonal anti-ALR antibody diluted 1:600 (0,16 mg/ml) in block solution was pipetted onto the slides and incubated overnight at 4°C. The slides were washed three times with PBS buffer and incubated with 1:50 diluted secondary mouse anti-rabbit antibody for 1 h. Slides were then washed with PBS and incubated for one additional hour with Ig-mouse-antibody. After washing with PBS, slides were incubated with the mouse APAAP complex at RT for 30 min. Staining was completed with a 20 min incubation using Fast Red Chromogen in 0,1 M TRIS buffer pH 8,2. Counter-staining was performed with haematoxylin and rabbit IgG was used as isotype control.

2.3. Nucleic acid methods

2.3.1. RNA-related methods

2.3.1.1. RNA isolation, reverse transcription and cDNA synthesis

The cells were washed twice with PBS, and harvested using RLT Buffer containing β-mercaptoethanol and proteinase K. The cells were collected in clean RNase-free 1,5 ml Eppendorf tubes and were either stored at -80°C or used immediately for RNA extraction. Total RNA was isolated using the RNeasy Mini Kit according to Qiagen's instructions. The cell lysate was sucked at least 5 times through a blunt 20-gauge

needle (0,9 mm diameter) fitted to a RNase-free syringe. After homogenization, the cell lysate was mixed with 1 volume of 70% ethanol, applied onto RNeasy Mini Spin Columns and centrifuged at 8,000 g for 15 sec, the columns were washed by adding RW1 buffer and centrifuged at 8,000 g for 15 sec, then re-washed twice with RPE buffer. Afterwards, the silica-gel membranes were dried by centrifugation the columns at maximum speed for 1 min followed by elution of total RNA with 20-30 µl RNase-free water centrifuging for 1 min at maximum speed. The concentration of eluted total RNA was determined by a UV spectrophotometer. Purified total RNA was either stored at -80°C or was used immediately to prepare cDNA. AMV Reverse Transcription System from Promega was used to synthesize single-stranded cDNA from total RNA. 1 µg of total RNA was reverse transcribed in a 20 µl reaction mixture containing 4 µl $MgCl_2$ (25 mM), 2 µl Reverse Transcription Buffer (10x), 2 µl dNTP mixture (10 mM), 0,5 µl RNasin® Ribonuclease Inhibitor, 0,75 µl AMV Reverse Transcriptase and 1 µl Oligo (dT_{15}) Primer, in a total volume of 20 µl. he 1 µg RNA is placed in a RNase-free cup and incubated at 70°C for 10 min, then the mix is added and the cup is incubated at 42°C for 60 min. The sample was then heated at 95°C for 5 min to inactivate the AMV Reverse Transcriptase and to prevent it from binding to the cDNA and the reaction was immediately cooled on ice for at least 5 min. The synthesized cDNA was immediately used for PCR or stored at -20°C until usage.

2.3.1.2. Polymerase chain reaction (PCR)

Amplifying of hALR-cDNA

In order to generate an expression plasmid of hALR, PCR was performed to amplify hALR-cDNA (378 bp) in 50 µl containing:

	Volume (µl)
cDNA	1
Forward Primer (10 µM)	0,5
Reverse Primer (10 µM)	0,5
10x PCR Buffer	5
dNTP (10 mM)	1
$MgCl_2$ (50 mM)	1,5
Taq Polymerase (5 U/µl)	0,5
H_2O	31

The PCR was started by heating the sample at 94°C for 5 min, followed by 35 cycles of following program:

	Denaturation	Annealing	Extention
Temperature	94°C	60°C	72°C
Time	1 min	1 min	1,5 min

After loading the PCR product on a 1,5% agarose gel, the specific amplicon was gel-extracted and purified using QIAquick Gel Extraction Kit according to Qiagen's Instruction.

Amplifying of genomic DNA

To generate the luciferase-reporter constructs, PCR was used to amplify specific regions of the hALR promoter sequence using Taq PCR Core Kit according to Qiagen's instructions. Briefly, 70 ng/µl human genomic DNA was amplified in 25 µl reaction containing 1x PCR-Buffer, 1x Q-Solution, 10 mM dNTP, 1,5 mM $MgCl_2$, 10 µM of both forward and reverse primer and 0,025 Unit Taq DNA Polymerase. The template was denatured at 94°C for 5 min followed by 35 cycles of:

	Denaturation	Annealing	Extention
Temperature	94°C	64°C	72°C
Time	1 min	1,5 min	1,5 min

The elongation step was completed by incubation for 5 min at 72°C and the reaction was cooled to 4°C. Afterwards, the samples (Amplicon 1: 995 bp and Amplicon 2: 1281 bp) were loaded on an agarose gel (1%) and following gel electrophoresis. The PCR products were verified by sequencing (GENEART). Due to the terminal transferase activity of the Taq polymerase, a single deoxyadenosine was added to the 3´end of PCR products, making them usable for TA-cloning. The amplicons were then cloned into pBAD-TOPO-TA vector.

2.3.1.3. Real-Time Polymerase Chain Reaction (RT-PCR)

Gene expression monitoring with SYBR-Green I dye

The LightCycler experiments were performed with the hot-start DNA Master SYBRGreen I kit and using the LightCycler® apparatus (Roche, Germany) following the manufacturer's instructions. The primers used in the PCR reaction were generated by Metabion.

The amplification was carried out in a total volume of 20 µl containing 1 µl non-deluted cDNA, 2,4 µl $MgCl_2$ (50 mM), 0,5 µl of each of the sense and antisense primers (10 µM) and 2 µl of Fast-Start Mix (containing buffer, dNTPs, SYBR-Green dye and Taq polymerase). A standard curve was generated for the housekeeping gene pair by amplifying three dilutions (1; 1:10; 1:100) of the most concentrated sample. The 18S ribosomal-RNA housekeeping gene served as an internal control and was used to normalize for differences in the amount of cDNA.

The amplification program consisted of 1 cycle of 95°C with a 10 min hold (hot start) followed by 50 cycles of 95°C with a 15 sec hold, 62°C annealing temperature with a 5 sec hold and 72°C with a 10 sec hold. Amplification was followed by melting curve analysis to verify the correctness of the amplicon. A negative control without cDNA was run with every PCR to assess the specificity of the reaction. Analysis of data was performed using LightCycler software version 3.5.

The slope of the standard curve was an indicator of the amplification efficiency. Standard curves were subsequently used to calculate the relative abundance of each transcript in each sample. The measurements were performed in triplicates. All results are represented as mean ± SD.

2.3.2. DNA-related methods

2.3.2.1. Molecular cloning

Restriction endonuclease digestion

To verify the presence and orientation of plasmid-inserts, or to clone insert DNA into a plasmid, DNA was digested with appropriate restriction enzymes. The restriction digest can either be performed analytically to analyze the DNA or preparative to prepare DNA for further cloning. Enzymes and their buffers were purchased from Roche or New England Biolabs (Germany). The digestion of plasmid DNA or PCR

products was carried out using 5 U enzyme/1 µg DNA in 20 µl at 37°C for 2 h. Afterwards, the reaction was stopped by incubating for 15 min at 65°C.

Dephosphorylation of plasmid-DNA with alkaline phosphatase

To prevent re-ligation of cohesive ends dephosphorylation was carried out. Digested vectors were treated with shrimp alcaline phosphatase (SAP) to remove the 5´-phosphate at 37°C for 10 min followed by heat–inactivation of the SAP for 15 min at 65°C. The sample can directly be used for ligation.

Purification of plasmid-DNA by gel extraction

After dephosphorylation, the DNA vector was purified by running on an ethidium bromide-containing agarose gel, excising the band containing the fragment under UV illumination and subsequent gel extraction using QIAquick Gel Extraction Kit following Qiagen's instructions.

Ligation

In the presence of ATP and Mg^{2+} ions T4-DNA-ligase is able to covalently join blunt or cohesive ends by a phosphodiester bridge between a 5´-phosphate and a 3´-OH group. After gel extraction, the dephosphorylated vector was used as follows:

Insert-DNA	3 µg
T4 DNA ligase	1 µl
5x T4 DNA ligase-buffer	4 µl
H_2O	variable
Total	20 µl

The mixture was incubated at 25°C for 1,5 h then at 37°C for 30 min followed by overnight incubation at 13°C and subsequently used for transformation of E. coli.

Transformation of competent E. coli

50 µl of GC5-competent cells were thawed on ice, mixed by pipetting with 5 µl ligation mixture and immediately placed on ice for 30 min. Then, the mixture was heat-shocked for 30 sec in a 42°C water bath followed by incubation on ice for 2 min. Afterwards, 250 µl LB-medium were added to the reaction and the mixture was then

incubated at 37°C for 1 h with shaking at 300 rpm. In case of ampicillin selection, cells were then directly plated on pre-warmed LB-selection plates (37°C) and placed overnight in an incubator at 37°C.

Plasmid isolation from E. coli

Single E. coli colonies were picked and cultured with LB-selection medium at 37°C over night. Then plasmids were isolated using QIAGEN Plasmid Midi, Maxi or Mega Kits following the supplier's instructions.

2.3.2.2. DNA sequencing and sequence analysis

Sequencing was performed by GENEART (Regensburg, Germany). Database searches in GenBank were performed using web based sources of NCBI (URL: http://www.ncbi.nlm.nih.gov).

2.3.2.3. Electrophoretic Mobility Shift Assay (EMSA)

To verify the binding of FOXA2 to hALR promoter EMSAs were conducted. Biotin-labeled probes were purchased from Metabion. First, 10 µl of complementary pairs of oligonucleotides (100 pmol) were annealed by incubation at 95°C for 10 min. Afterwards, the heat was gradually reduced overnight or until the oligonucleotides have reached room temperature. The assays were performed using LightShift® Chemiluminescent EMSA Kit following Pierce's instructions. Briefly, 20 fmol Biotin-labeled target duplexes ranged in size from 21-25 bp were incubated with 2 µg HepG2 NE-PER Nuclear Extract. The binding reactions were supplemented with 2,5% glycerol, 50 ng/µl poly (dI•dC) and 0,05% NP-40. For supershift assays, 5 and 10 µg anti-FOXA2 antibodies were added to the reaction and incubated for 20 min at room temperature. After adding 5 µl of 5x Loading Buffer to each 20 µl binding reaction, probes were loaded onto 6% Polyacrylamide gel. After electrophoresis, the binding reactions were transferred onto a nylon membrane and cross-linked using transilluminator 312 nm for 15 min. The biotin end-labeled DNA is detected using the streptavidin-horseradish peroxidase conjugate and the chemiluminescent substrate.

2.4. Bacterial culture

Bacterial growth medium

Liquid cultures were grown in LB medium with the appropriate antibiotics:

LB medium		**LB-agar plates**	
NaCl	10 g	Agar	15 g
Bacto tryptone	10 g	NaCl	10 g
Yeast extract	5 g	Bacto tryptone	10 g
H_2O	to 1 L	Yeast extract	5 g
		H_2O	to 1L

autoclave, cool to 50°C and add the appropriate antibiotics. Pour the LB-agar solution into 10 cm Petri dishes and store at 4°C.

Cultivation of E. coli

E. coli cultures were cultivated either on LB-agar plates or in liquid LB-medium overnight at 37°C in an incubator. Bacterial cultures were obtained by inoculating a single colony or suspension of bacteria from a glycerol culture into LB-medium. Liquid cultures were incubated in a shaker at 37°C and 240 rpm overnight. It is necessary to add an appropriate antibiotic (Ampicillin; 100 µg/ml or kannamycin 30 µg/ml) to select the bacteria containing the specific plasmid.

Glycerol stocks

For long-term storage at -80°C, E. coli cultures were permanently stored in glycerol stocks. To achieve this, 5 ml from the bacterial culture were centrifuged at 300 g for 5 min. Afterwards, the supernatant was discarded, and the pellet was resuspended with 1 ml mixture of LB-medium and sterile glycerol (1:1), filled in a cryo-tube, shock frozen in liquid nitrogen and stored at -80°C.

2.5. Animal models

Experimental procedures were approved by the institutional Animal Care and Use Committee of the University of Regensburg and the regional authorities. Ten-week-old female athymic nude mice NMRI (nu/nu) mice with a mean body weight of 30 g were used, 6 of each for mock and ALR expressing HepG2 cells. In brief, 1 x 10^6 cells were harvested, washed twice with PBS and injected subcutaneously into the peritoneal cavity of nude mice. After 21 days the formed tumours were taken out and stored at -80°C for subsequent analysis.

IV. RESULTS

1. Analysis of ALR expression

1.1. Expression of ALR in regenerating livers

Liver regeneration after partial hepatectomy involves initiation of proliferation of remaining parenchymal cells and is an excellent *in vivo* model depicting controlled hepatocellular growth. In addition, there are other injury models including liver fibrosis and hepatic fat accumulation which affect the liver and might trigger the hepatic regeneration. All these models represent useful tools for studying signaling molecules and factors that are involved in the process of liver regeneration. Therefore, distinct experimental injury models were used to analyze the expression levels of ALR in the liver and to find out whether ALR expression is regulated during liver regeneration. Thus, total RNA was isolated from livers after hepatectomy and other models including hepatic fibrosis and liver fat accumulation. The results of the quantitative real-time reverse transcription PCR (RT-PCR) showed that ALR expression in rats rose shortly after the injury (3,64 ± 1,04 FC) 12 h post-operation over control (Fig. 10A). In contrast, the level of ALR mRNA in mouse liver continuously increased to about 4,99 ± 0,85 fold over control after 72 hours post-hepatectomy (Fig. 10B). We further investigated the expression of ALR in additional models of liver injury like bile duct ligation (BDL) or after CCl_4 treatment. Both models have been shown to induce liver fibrosis in animal models [103,104]. Figure 10C shows enhanced expression of ALR comparing to the control group in response to the injury induced by BDL. Further, the CCl_4 model demonstrated moderate increase of ALR expression which began to rise up to 24 hours after treatment and returned to the near-basal expression at 96 h after treatment (Fig. 10D). Another hepatic injury model like fatty liver was also used to investigate whether the expression of ALR is regulated. Therefore we used two mouse models in which two different diets were applied. In particular, “methionine-choline deficient” diet (MCD) was applied 3 weeks to mice and “Paigen” diet was applied 12 weeks. Liver tissues were collected, total RNA was isolated and the expression of ALR mRNA was measured using qRT-PCR (Fig. 10E and F). Both diets caused hepatic lipid accumulation in mice associated with different stages of inflammation and revealed higher expression of ALR compared to the control group. Taken together, the hepatic tissue damage in all

investigated models increased the expression of ALR indicating that ALR could be potentially involved in the process of hepatic regeneration.

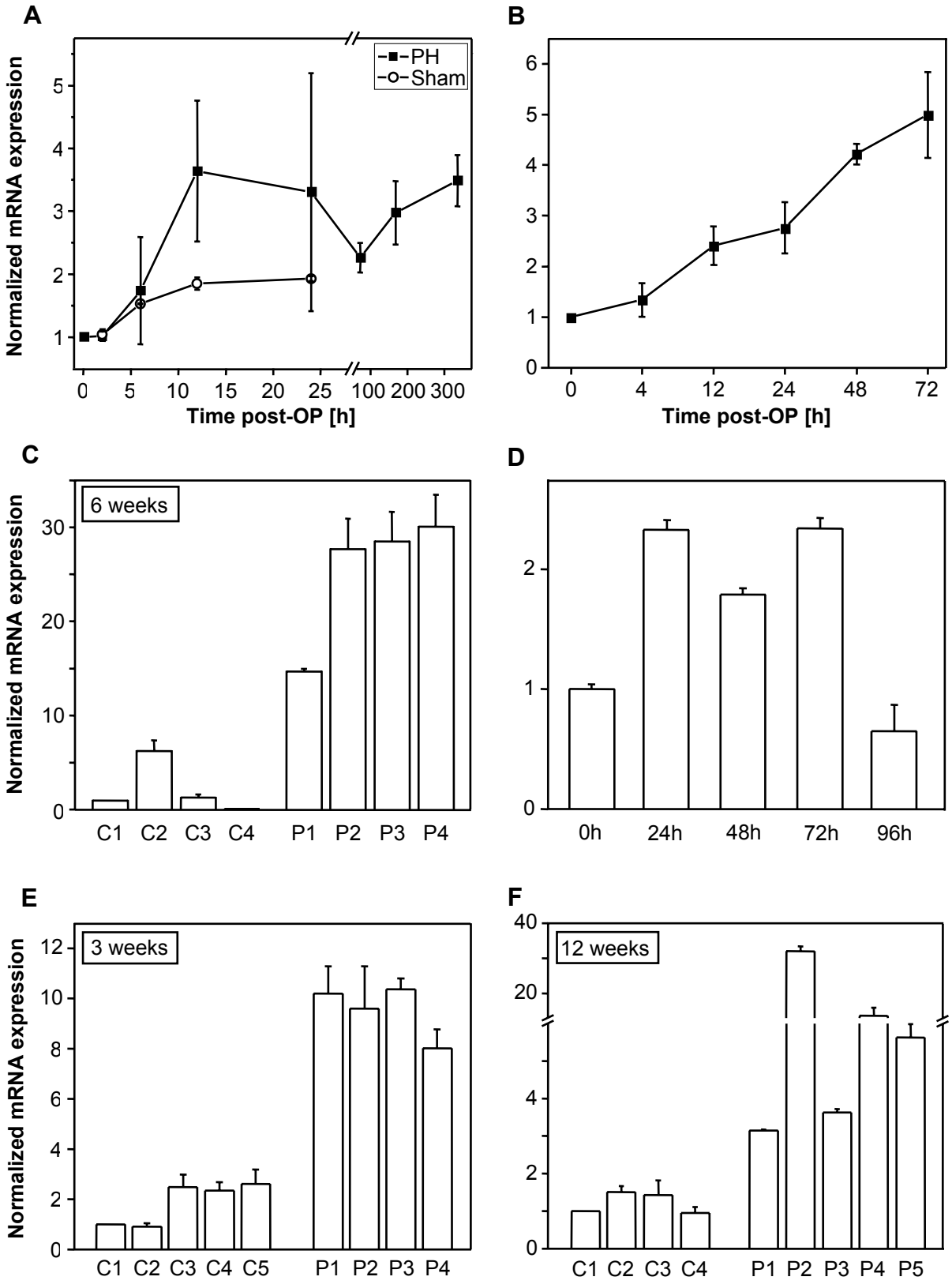


Fig. 10: Quantification of ALR mRNA levels in hepatic tissue after liver injury using qRT-PCR. A) Expression of ALR in rat livers undergoing 70% hepatectomy (n=4 animals per time point) compared to sham operation (n=2 animals per time point) or B) ALR expression

over time postoperation in hemihepatectomized mice. Expression of ALR in response to the injury induced by BDL (C) or with CCl_4 (D). Expression of ALR in fatty livers of mice fed with MCD-diet (for 3 weeks) (E) or paigen-diet (for 12 weeks) (F). Endogenous 18S ribosomal RNA was amplified and used as internal control.

1.2. Expression of ALR in parenchymal liver cells

To monitor ALR transcription in parenchymal mammalian liver cells, expression levels of ALR in primary human hepatocytes (PHH) and hepatoma cells were examined using qRT-PCR. Interestingly, ALR expression in proliferative parenchymal hepatic cell lines (hepatoma cells) was approximately 3-4 fold higher, peaking in HepG2 cells with 15 fold increase over ALR expression in quiescent primary human hepatocytes (Fig. 11). As shown in Fig. 11, we found that ALR expression is lower in PHH than in hepatocellular cell lines.

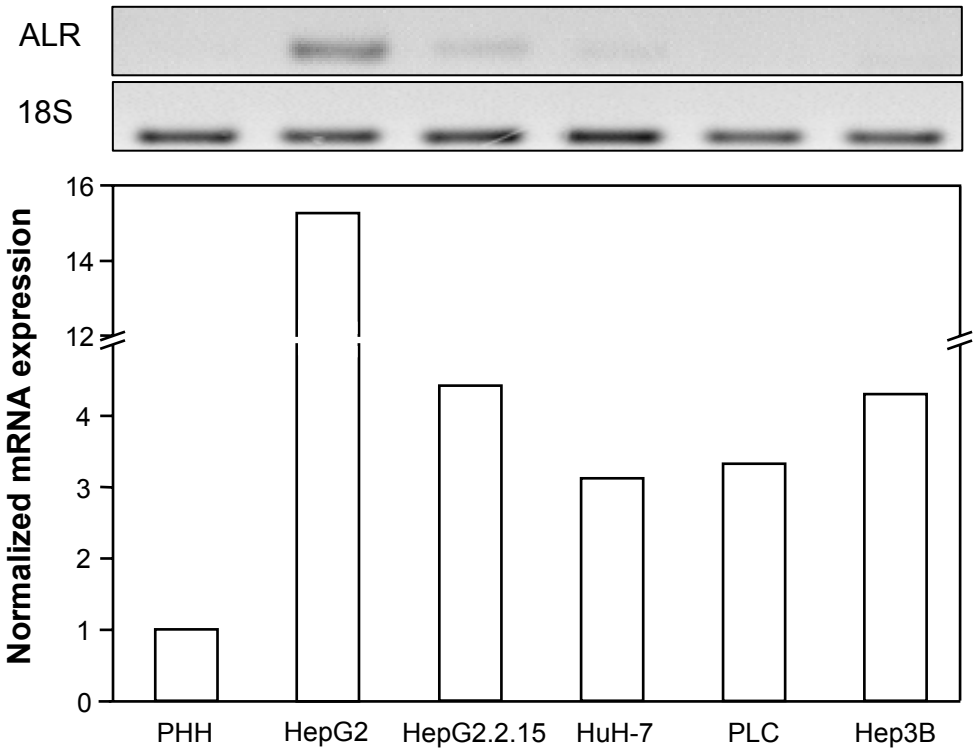


Fig. 11: Total mRNA levels of ALR in hepatic parenchymal cells. ALR mRNA expression in hepatoma cell lines and PHH are quantified by densitometric analysis. Endogenous 18S ribosomal RNA was amplified to normalize the amount of ALR mRNA.

1.3. Expression of ALR in human HCC tissues

Next we aimed to investigate the expression pattern of ALR in samples of malignant hepatocellular carcinoma compared to normal liver tissue. Therefore, total RNA was extracted from eight paired samples of HCC tissues and the corresponding normal liver tissue and analyzed by qRT-PCR. In five out of 8 cases, ALR mRNA expression was higher in HCC tissues than in the corresponding non-malignant liver tissue adjacent to the tumour (samples number 2-4 and 6-7) (Fig. 12). In two samples, ALR expression in HCC tissues was nearly same as those in the adjacent normal tissues (samples number 5 and 8), but in one case, ALR expression in HCC tissue was lower than that in adjacent healthy tissue (sample number 1). The expression of ALR was considered as increased when the expression ratio in tumor/normal tissue was greater than 1,5. These findings are consistent with our recent results of an immunohistochemical study which demonstrates that malignant liver tissues exhibit enhanced ALR expression compared to non-malignant adjacent tissue (manuscript in preparation).

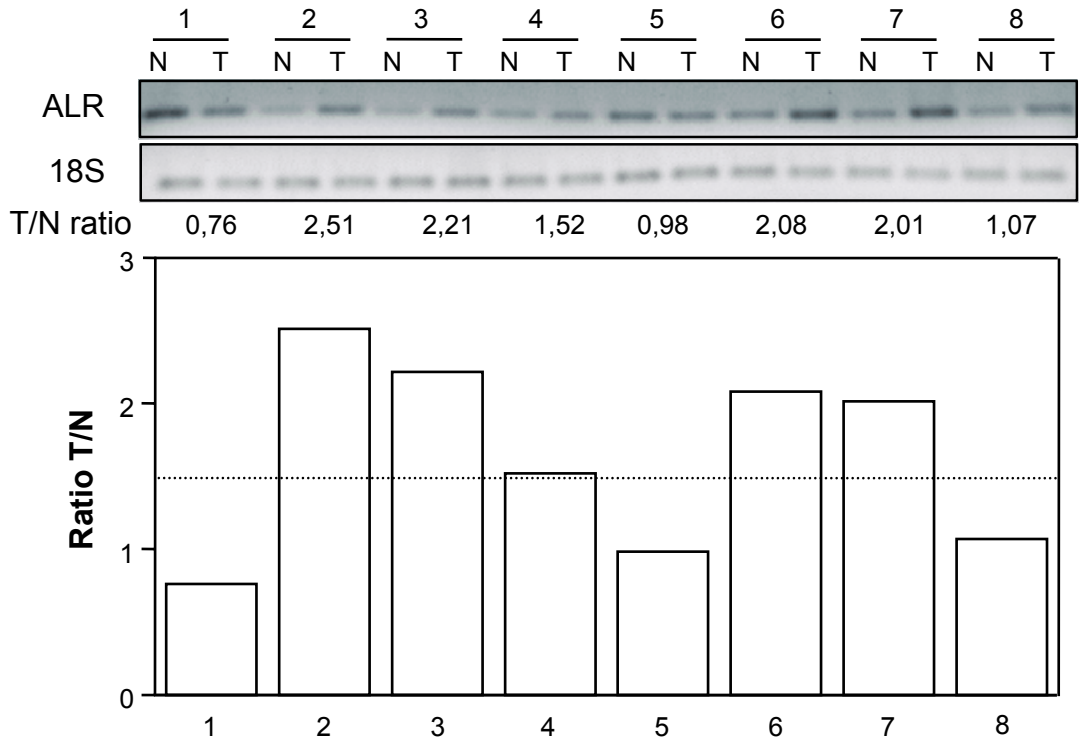
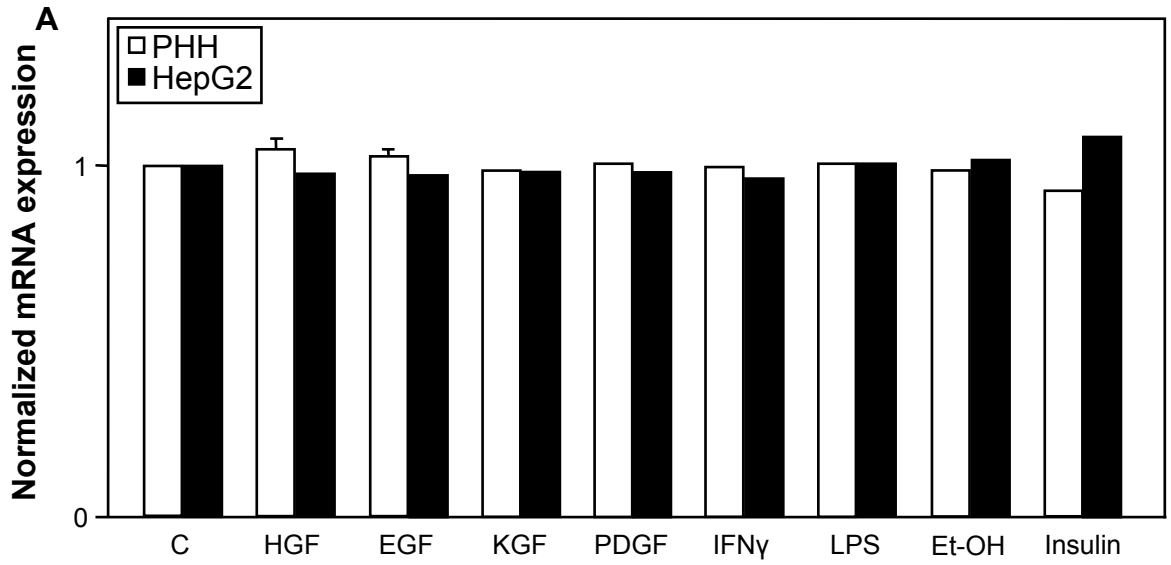


Fig. 12: ALR expression in tissue samples from patients with HCC compared to the surrounding non-affected tissue. Eight paired samples of HCC tissues and the corresponding normal tissues adjacent to the tumor were analyzed by qRT-PCR and densitometric analysis. Annotation T designate the HCC tissues and N corresponds to the paired adjacent healthy tissues. Expression of the housekeeping gene 18S ribosomal RNA serves as an internal control.

2. Regulation of ALR expression

2.1. Liver specific cytokines and growth factors

Hepatocytes rarely divide and are normally in a resting G0 state. After liver injury, all hepatic cells undergo transition into G1 phase (initiation state) under the control of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6. Once the cells have been initiated, progression through the cell cycle requires continued stimulation by growth factors such as HGF, EGF, KGF and PDGF. As described in section 1.1 ALR is upregulated in different models of liver injury and therefore we investigated if factors known to be involved in responses to liver injury affect ALR expression. More specifically, we wanted to assess the involvement of different cytokines and growth factors, related as responses in diseased livers, in regulating ALR expression. Therefore, primary human hepatocytes and hepatoma cells were treated with cytokines (TNF-α, IL-1 and IL-6) and growth factors (HGF, EGF, KGF and PDGF). Cells were harvested 24 h after stimulation, and total RNA was isolated and analyzed by qRT-PCR. We found that growth factors and other co-mitogens had no significant effect on ALR mRNA expression (Fig. 13A). Interestingly, stimulation of PHH with cytokines IL-1 or IL-6 for 24 h led to enhanced expression of ALR (Fig. 13B). ALR levels were induced after stimulation with IL-1 (2,23 ± 0,09 FC) or with IL-6 (2,37 ± 0,14 FC) over control (Fig. 13B). In contrast, TNF-α could not significantly regulate the expression of ALR.



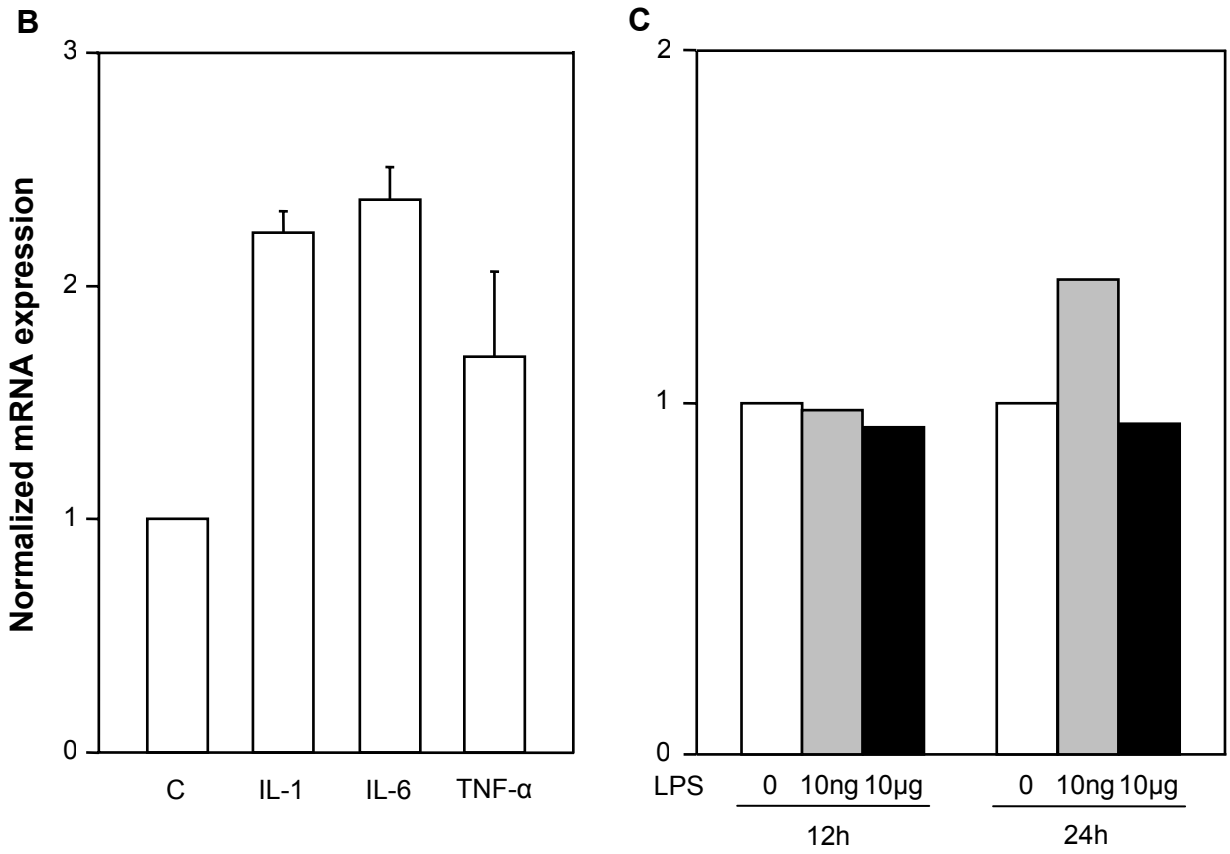


Fig. 13: Regulation of ALR expression by cytokines and growth factors. A) Real-time RT-PCR determinations of ALR mRNA expression after stimulation with growth factors in PHH (open bar) and HepG2 cells (filled bar) for 24 h. B) The expression of ALR in PHH after exposure to IL-1, IL-6 and TNF-α for 24 h. C) The effect of Kupffer cell conditioned-medium with or without stimulation with LPS for 12 h and 24 h.

Since non-parenchymal cells (NPC) and particularly Kupffer cells (liver-resident macrophages) are the main source of cytokines (IL-1 and IL-6) in the liver, we performed an experiment in which Kupffer cells were stimulated to secrete cytokines. We cultured Kupffer cells in the absence and presence of LPS (10 ng/ml or 10 µg/ml) which has been shown to stimulate Kupffer cells for 12 and 24 hours. Afterwards, the supernatants were collected and used as conditioned medium to stimulate PHH. Total RNA was analyzed for ALR expression and results are shown in Fig. 13C. Interestingly, we found that Kupffer cell conditioned-medium could not affect the expression of ALR, assuming that Kupffer cells in addition may release other factors which may inhibit the expression of ALR.

To proof whether stimulation of PHH with cytokines IL-1 and IL-6 leads to enhanced protein expression of ALR and to confirm the qRT-PCR, Western Blot analyses were performed. Cells were treated for 24 h with cytokines (IL-1α, IL-1β and IL-6), and results are presented in Fig. 14. IL-1α and IL-1β are pleiotropic cytokines which are

mainly produced by Kupffer cells [105,106] and affect inflammation and immune responses [107,108]. IL-1α slightly induced ALR protein expression while IL-6 treatment resulted in a significant higher expression of ALR protein compared to control. In contrast, culturing of the cells in the presence of IL-1β results in reduction of ALR protein levels (Fig. 14). Therefore we summarize that liver specific growth factors have no impact on ALR expression, but cytokines involved in initiating liver regeneration such as IL-6 induce ALR expression. On the other hand, it seems that IL-1β resembles an inhibitory factor in ALR regulation.

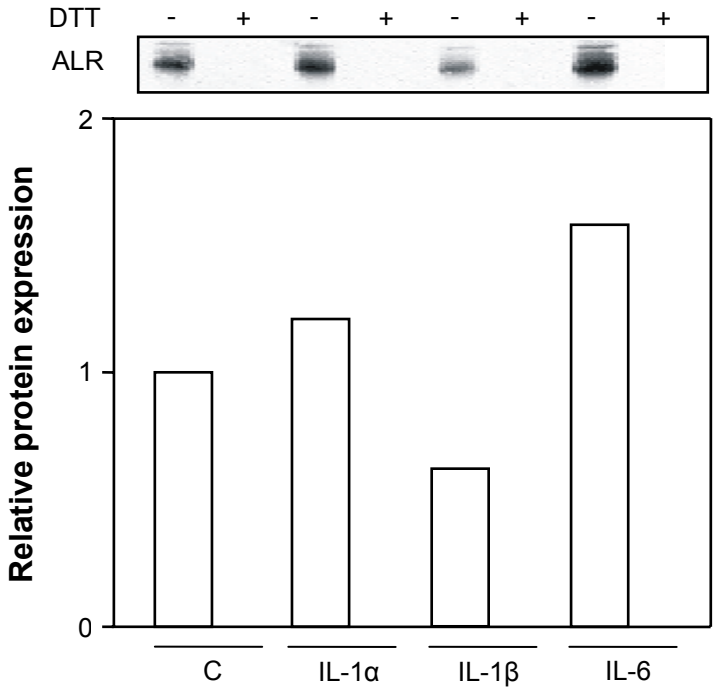


Fig. 14: ALR protein expression after stimulation with IL-1α, IL-1β and IL-6 in PHH. Primary human hepatocytes were stimulated with IL-1α (250 U/ml), IL-1β (250 U/ml) and IL-6 (30 ng/ml) for 24 h. Western Blot analysis using specific anti-ALR antibodies showing the expression of ALR-homodimers under non-reducing conditions. The protein expression of ALR was measured by densitometric analysis.

2.2. Analysis of ALR promoter:
Putative transcription factor binding sites

Besides cytokines and growth factors, differentially expressed transcription factors during liver regeneration play a pivotal role in the regulation of the regenerative process. As described in section 2.1, hepatocytes stimulated with IL-6 revealed an increase of mRNA and protein expression of ALR compared to resting hepatocytes.

The next step was to find out what kind of transcription factors may regulate the expression of ALR. The full-length cDNA of ALR consists of around 2,5 kb, located on chromosome 16, and is organized in 3 exons with three potential ATG start codons in exon 1. Preliminary insights into putative regulatory elements of the ALR promoter were obtained by data bank searches of the genomic 5'-flanking region. In order to investigate potential transcription factors involved in the regulation of ALR expression, we performed promoter studies analyzing transcriptional regulation of ALR. Sequence analysis of a fragment (-733 to +502 bp from start codon) of ALR promoter by software search tools (TRANSFAC, Tfsearch and TESS) revealed that 24 putative responsive elements are located in the regulating sequence of ALR (Table 1).

Table 1. Putative transcription factors which may transactivate ALR promoter.

Transcription factor	**Program**		
	Transfac	Tfsearch	TESS
FOXA2	x 4	-	-
IL-6 RE-BP	-	-	x 2
C/EBPβ	x 4	-	-
NF-κB	-	-	x 2
SP1	-	x 3	x 53
HNF-4α1	-	-	x 2
YY1	x 1	-	x 1
AP-1	x 7	-	x 1
AP-2	-	x 2	x 6
AP-4	-	-	x 2
CACCC-binding factor	-	-	x 2
C/EBPα	-	-	x 1
GATA-1	-	x 2	x 1
GCF	-	-	x 10
GCMα	-	-	x 1
HSF	-	x 14	-
NF-1	x 16	-	x 1
Oct-1	-	-	x 1
RAR-α	-	-	x 1
RAR-β	-	-	x 1
RXR-α	-	-	x 1
RXR-β	-	-	x 1
TFIID	-	-	x 1
USF	x 6	-	-

Although promoter analysis revealed a putative NF-κB binding site, our previous studies have demonstrated that stimulation with TNF-α, an inductor of NF-κB activation, has no effect on the expression of ALR. Therefore, we assumed that NF-κB, might not be involved in the regulation of ALR. As shown in table 1, hepatocyte nuclear factor-3 β, also termed Forkhead box A2 (FOXA2), has 4 putative binding sites. This finding is in agreement with the observation [100] that increased levels of FOXA2 are accompanied by induced hepatic ALR expression in mice. We further identified other transcription factor binding sites located in the promoter, like IL-6 response element binding protein (IL-6 RE-BP) and CCAAT/Enhancer binding protein beta (C/EBPβ) which might be involved in mediating the signal transduction of IL-6 (Fig. 15).

ACATTTATGAGTGTGAGTCCTTGTTTTTTTTTCCTGTCTTGACCACTGGATTATGAACAGAGAAGGTCAG

GGAACAGAATTGTGTTCCCTGCTCTGCCCAGCACTTGGAACAGCGCTTGGAACAGAGCGGTCACTGCG

CAAATAATAGCGGAATGGATAAATGAACGCAAGGAGGCACAGGAATCCCAGAGGCCGCTCCAACCGG

GAGCCCGACCCCTCATCCCAGGCTCTAGGATCCGCGACAGCCGGCGAGGGGCGCCAGGGAGCCCAA

GGCACGCGCCAACCCTCTGCGGCCGCGCCGCGCCCTGCGTTGCCAGACGCCCGTTGCCATGGCGCC

AGGGAGGGGACAAGAGCCCAGGACCCTAGCGCGCGCCGCAATCTGTGCGGTCACGCCCGGCCCGCG

TTGAGAGAGCGCTTGGCAGCCAGCGGGTGGACGCGCCCCTCAGCGGAGGGCACAAAGCCTGGCCGC

AGGCACGCAGGACACTCAGAACGGAGCGATGGGGACGTCGCGGGCCGCAACACGGACTTTGCCTGG

GTGTCAGCTCGGGTCCACAGCGCGGCCGAGGCGCACCTTGCGCCACACACTGCTCTTTTACTGGAGA

AAGCGGGACGGCCGCCACGGACGGCGCAACCCCTGCGTGCGCCGCGGATCGACGCCTGAGGGCGC

CAGCAGGGTCCGACCCTCCTGCTCCGTCCCCGCCCCTGTCCTCGGGCCCGGCCAGCGCGCGGCCTC

TGGCTCCGCCTCCACACGGGCCCGCAAGCAGGCACCGCCCCCGACTCTGCCCCCAGCCCCGGCTCG

GGCCCGGCCCCCGCGAGCACGGCGCGCGCCTCCGGCTCCTGTGGCCGCGCGCTGGCCTGGAGGCT

GACCTGGAGGCTCATCTGGAGGCCGAGCTGACCCGGCAGGCCTTGCGCGGGCAACATGGCGGCGCC

CGGCGAGCGGGGCCGCTTCCACGGCGGGAACCTCTTCTTCCTGCCGGGGGGCGCGCGCTCCGAGA

TGATGGACGACCTGGCGACCGACGCGCGGGGCCGGGGCGCGGGGCGGAGAGACGCGGCCGCCTC

GGCCTCGACGCCAGCCCAGGCGCCGACCTCCGATTCTCCTGTCGCCGAGGACGCCTCCCGGAGGCG

GCCGTGCCGGGCCTGCGTCGACTTCAAGACGTGGATGCGGACGCAGCAGAAGGTGCAGTTCCCTGC

CCGATTTCTCCCAGCCCCGCGCAGCCCCTGTCCCCGCCCCCGCCCAGGTACCCCGGCAGAGCTTCCC

AGGGTTGCCTGTCCCTGAACCTTGCCCCCCGGGTAGGCCCGGCCTTACAGCCTTCATCCGCGCGTGG

GTTGGATCGTCTGCAGGACTTTGGCCGGAGTCCAGTGGGCCACCGGCTGGGCCGTACAGTGGGGAG

CTTTGGGCGCCTTTGTTCGGAGAATGAACTCACTCTCGGTCGGCCTGCTTCCGC**AGCGGGACACCAA**

GTTTAGGGAGG.....

Fig. 15: Promoter sequence of human ALR. Consensus binding sites are underlined. The transcribed sequence is printed in bold, and the ATG transcription start codons are shown in red and italics. The red arrows indicate the primers used to amplify the genomic DNA. ARE,

antioxidant response element; SP1, stimulating protein 1; FOXA2, Forkhead box A2; IL-6 RE-BP, IL-6 response element binding protein; HNF-4α, hepatocyte nuclear factor 4α; YY1, ying yang 1.

A lot of prominent transcription factors have been found by our promoter analyses, which may play an important role in regulating ALR. Hepatocyte nuclear factor-4 alpha (HNF-4α) has different potential binding sites in the promoter sequence of ALR. It has been shown [88] that HNF-4α regulates the expression of ALR and that overexpression of HNF-4α in HepG2 cells led to a dramatic repression of the promoter activity of ALR. The same authors demonstrated that SP1 (Stimulating Protein-1) activates the basal expression of ALR in hepatoma cells. On the other hand, Zhao *et al.* [86] reported that the TATA-box less core promoter of ALR could not be regulated by AP-1, and that YY1 and RNA polymerase might be involved in the regulation of ALR promoter.

Based on this prediction, promoter regions of 938 bp and 1236 bp (Fig. 16) were subcloned into pGL2-Basic vector, a promoterless luciferase reporter plasmid, and used for further investigations.

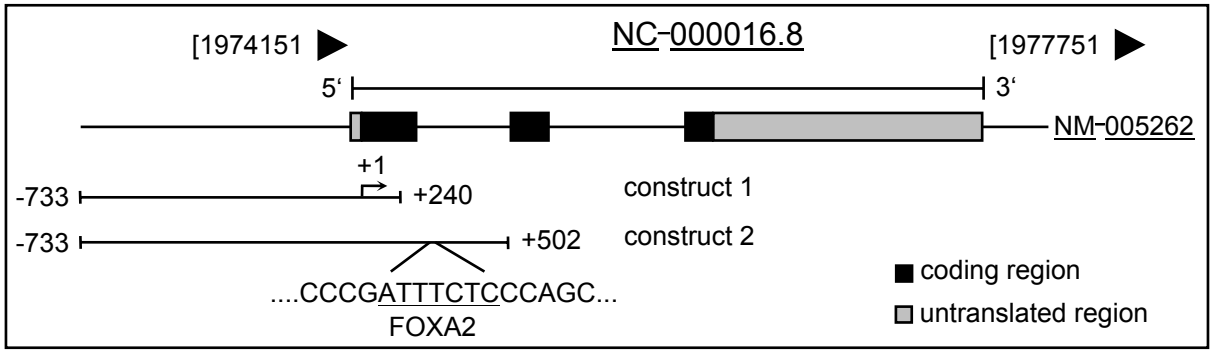


Fig. 16: Schematic diagram showing the structure of the hALR gene and the construction of hALR promoter constructs. ALR promoter constructs (construct 1: -733 to +240 bp, and construct 2: -733 to +502 bp) were subcloned into the promoterless pGL2-Basic vector.

2.3. Impact of FOXA2 on ALR expression

Both promoter constructs introduced in Fig. 16 have been used to investigate the promoter activity using luciferase assays. We have found that the basal activity of promoter construct 1 (-733 to +240 bp), Pro 1, was 8 fold and promoter activity of construct 2 (-733 to +502 bp), Pro 2, was 14 fold higher over the control in HepG2 cells (Fig. 17). This finding suggests the presence of regulatory elements located in

these promoter regions. The Pro 2 construct reveals higher promoter activity than Pro 1 construct indicating the presence of important response elements in the region between +241 and +502 bp. Figure 15 displays potential binding sites for FOXA2, IL-6 RE-BP, CEBP/β and HNF-4α in the sequence of Pro 2. In a recent study, Hughes *et al*. have shown that in a transgenic mouse model for FOXA2 increased expression levels of ALR were observed [100]. Furthermore, a nearly perfect FOXA2 consensus site was identified in the Pro 2 construct. Therefore, we intented to investigate the potential regulatory role of FOXA2 on the expression of ALR, and tested both reporter constructs Pro 1 (-733 to +240 bp) and Pro 2 (-733 to +502 bp) for their response to FOXA2. Both constructs were transfected to HepG2 cells (human liver cancer cells), H1299 cells (human lung cancer cells) and Caco-2 cells (human colorectal cancer cells). After 24 hours the cells were lysed and the luminescence signal was measured. Firefly luciferase activities were normalized to the Renilla luciferase activity of pRL-TK co-transfected vector as internal control. Reporter gene assays demonstrated strong activity of the promoter construct Pro 2 (2,69 ± 0,10) x 10^4 RLU, and this activity could be further induced in HepG2 cells by co-transfection with a FOXA2 expression plasmid (8,29 ± 0,45) x 10^4 RLU (Fig. 17).

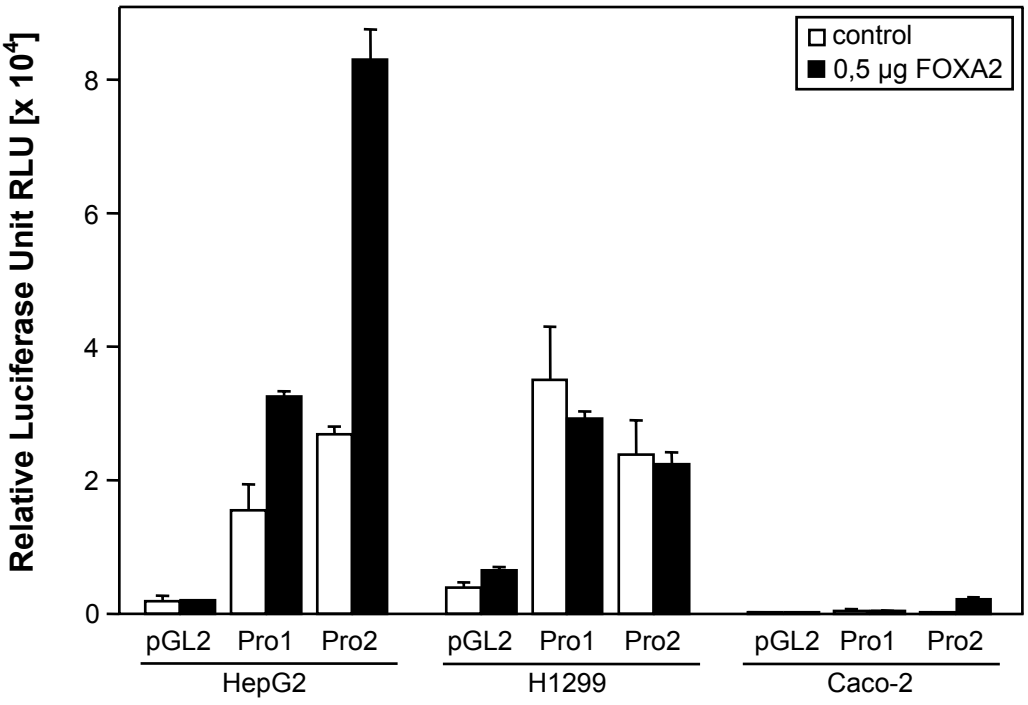


Fig. 17: Transactivation of ALR promoter by FOXA2. ALR constructs Pro 1 and Pro 2 were transfected wih 0,5 µg (filled bar) or without (open bar) FOXA2 expression plasmid in three cell lines of different origin. In contrast to H1299 cells (lung cancer cells) and Caco-2 cells (colorectal cancer cells), HepG2 cells (liver cancer cells) revealed higher activity of ALR promoter in the presence of FOXA2. All results are represented as mean ± SD.

Interestingly, FOXA2 was able to enhance the activity of ALR in hepatoma cells e.g. HepG2, compared to H1299 cells and Caco-2 cells. Subsequently, we investigated the effect of different amounts (0,1 and 0,5 µg) of FOXA2-pcDNA3.1 expression plasmid on the activity of ALR promoter in HepG2 cells. While no change in the activity of the construct Pro 2 (0,45 ± 0,00) could be found after using 0,1 µg FOXA2, 0,5 µg FOXA2 transactivates the promoter of ALR in HepG2 cells resulting in increased activity of the construct Pro 2 (2,54 ± 0,29) x 10^4 RLU over the control (0,78 ± 0,00) x 10^4 RLU (Fig. 18). Based on these observations we assume that FOXA2 needs a hepatic environment to specifically regulate ALR expression in the liver.

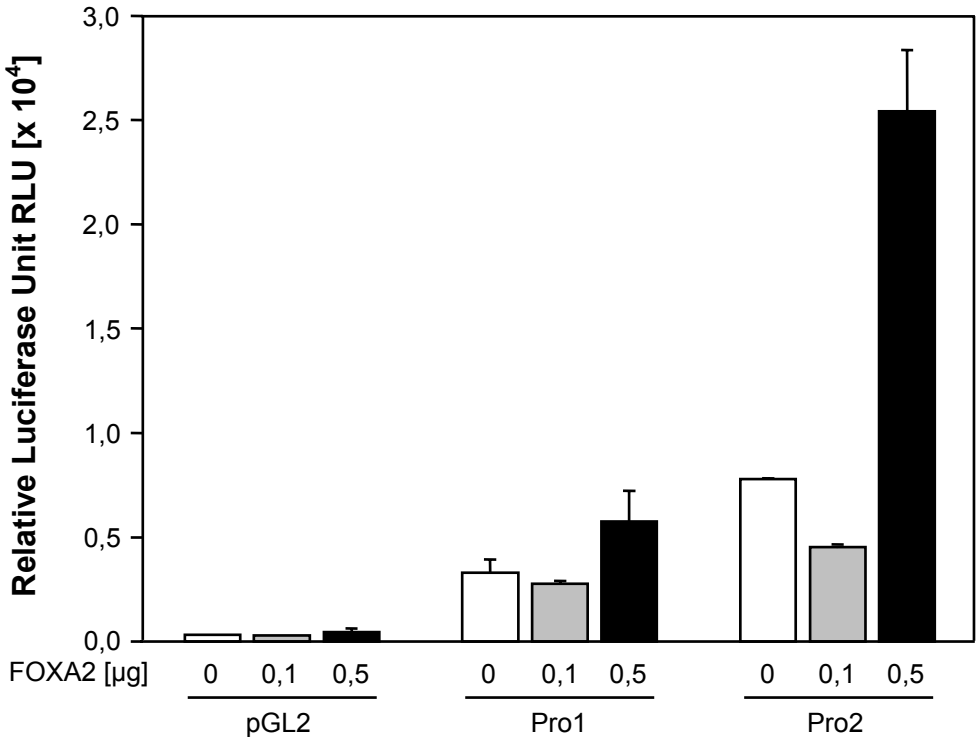


Fig. 18: Transactivation of ALR promoter using different amounts of FOXA2. Reporter-gene constructs were transfected into HepG2 cells with different amounts of FOXA2 expression vector. In contrast to 0,1 µg, 0,5 µg FOXA2 enhanced the luciferase activity of Pro 2 nearly 3,5 fold over the control indicating the presence of FOXA2 response element in the Pro 2 sequence.

Based on the finding that FOXA2 exclusively activates construct Pro 2, we summarize that the responsiveness of ALR promoter to FOXA2 is due to the FOXA2 response element and this binding site is located within the first intron of ALR gene. To gain more insight whether this putative FOXA2 response element could bind FOXA2 we performed Electrophoresis Mobility Shift Assays (EMSA).

Oligonucleotides representing the FOXA2 element were incubated with nuclear extract of HepG2 cells and binding of the nuclear complex to the FOXA2 consensus oligonucleotide was analyzed (Fig. 19A, lane 3). The protein-DNA complex is specific for the FOXA2 binding site, because co-incubation of 200 fold excess of unlabeled oligonucleotides completely abrogated its formation indicating that nuclear proteins binding FOXA2 oligonucleotide also recognize the unlabeled FOXA2 consensus sequence (Fig. 19A, lane 2). Furthermore, incubation with 5 µg specific anti-FOXA2 antibody significantly reduced the formation of the FOXA2-DNA complex (Fig. 19A, lane 4). When the nuclear extracts were incubated with 10 µg anti-FOXA2 antibody (Fig. 19A, lane 5), the complex disappeared confirming the specific binding of FOXA2 to ALR promoter. FOXA2-DNA complex was quantified by densitometric analysis (Fig. 19B).

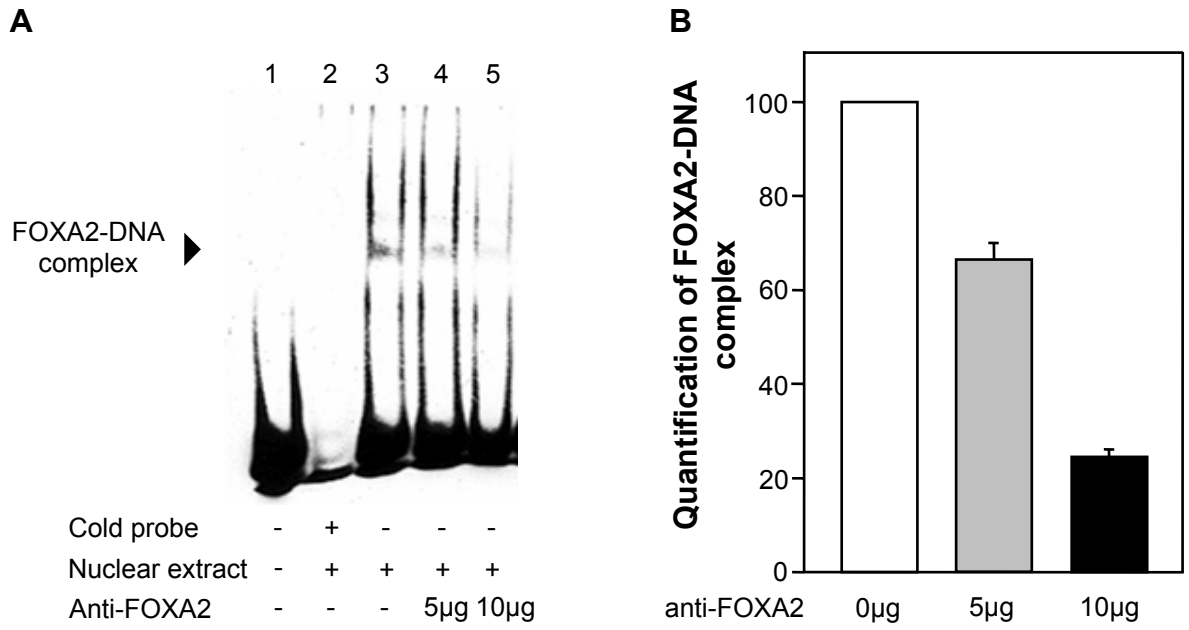


Fig. 19: Binding of FOXA2 to FOXA2 response element in the ALR promoter. A) Labeled oligonucleotides representing the FOXA2 site in the wild type ALR promoter (lanes 2-5) were incubated with 2 µg of nuclear extracts derived from HepG2 cells cultured in the absence (lanes 2, 3) or presence of (5 µg and 10 µg) anti-FOXA2 antibodies (lanes 4, 5), respectively. 200 fold molar excess of cold FOXA2 consensus oligonucleotides were added in lane 2. Lane 1 represents the free probe. The protein-DNA complex is indicated by an arrow. B) FOXA2-DNA complex (from A) was quantified by densitometric analysis.

Normally, when an antibody recognizes and binds to its target protein, the DNA-protein complex becomes larger and migrates more slowly than DNA-protein complex without antibody. This phenomenon is referred to as a supershift and indicates the specific binding of the antibody to its target protein. In addition, the

disappearance of the DNA-protein complex exhibits further form of supershift. In our study, the disappearance of the specific EMSA band may be due to the fact that the specific anti-FOXA2 antibody masks the epitope in the FOXA2 protein structure which is required for binding to its recognition sequence at the ALR promoter. This may explain the abrogation of the formation of protein-DNA complex and therefore the disappearance of the specific EMSA-complex by using specific antibodies. The disappearance of EMSA-complex is a form of supershift which has been described in several publications [109,110]. We showed that FOXA2 specifically binds to its binding site and therefore may regulate the expression of ALR.

2.4. Effect of IL-6 RE-BP and C/EBPβ on ALR promoter activity

As described in section 2.3, the first intron of ALR gene shows high ability to get activated suggesting the presence of regulatory elements located in this promoter region. Therefore, we analyzed the sequence of the first intron of ALR gene using different transcription factor prediction softwares as shown in Fig. 15 and table 1. In addition to the binding site for FOXA2, we found two putative response elements for IL-6 and two binding sites for C/EBPβ (Fig. 20).

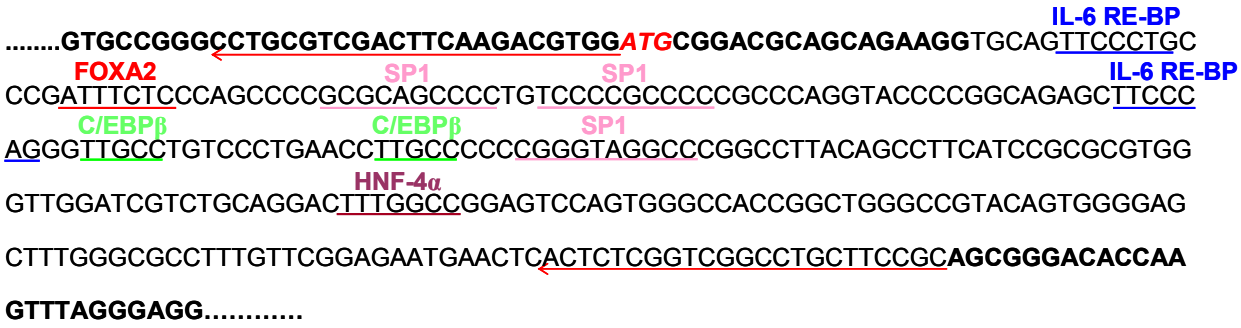


Fig. 20: Promoter sequence of human ALR between exon 1 and exon 2. Consensus binding sites are underlined. The transcribed sequence is printed in bold and the ATG transcription start codon is shown in red and italics. The red arrows indicate the primers used to amplify the genomic DNA.

We wanted to know whether the observed regulation of ALR by IL-6 (see Fig. 13B, and Fig. 14) is mediated by IL-6 RE-BP, and therefore we performed EMSA assays. HepG2 cells were treated with 30 ng/ml IL-6 for 15 min and the subsequently nuclear proteins were incubated with IL-6 RE oligonucleotides. No specific binding of nuclear proteins to the IL-6 RE consensus was observed (Fig. 21A). This indicates that IL-6 RE located next to C/EBPβ binding site could not directly bind to the IL-6 RE

sequences (5'-TTCCCAG-3'). Further, it seems that IL-6 may not regulate the expression of ALR *via* the IL-6 response element binding protein (IL-6 RE-BP) located in the vicinity of C/EBPβ binding site in the first intron of ALR gene. Using EMSA technique, no transactivation of the promoter was observed when using the potential C/EBPβ site demonstrating that C/EBPβ is not able to bind to its potential binding site (5'-TTGCC-3') located in the first intron (Fig. 21B, lane 3). Additionally, we could not observe any C/EBPβ-DNA complex when the HepG2 cells were simultaneously treated with IL-6 (Fig. 21B, lane 4). Further, we performed luciferase assays investigating the effect of C/EBPβ expression plasmid on the activity of ALR promoter in the absence and presence of 30 ng/ml IL-6. No changes in the promoter activity could be shown after transfection of C/EBPβ (data not shown) indicating that C/EBPβ could not transactivate the promoter of ALR. Based on this finding, we hypothesize that C/EBPβ plays no direct regulatory effect on the expression of ALR and that C/EBPβ is not involved in mediating the IL-6 regulation of ALR expression.

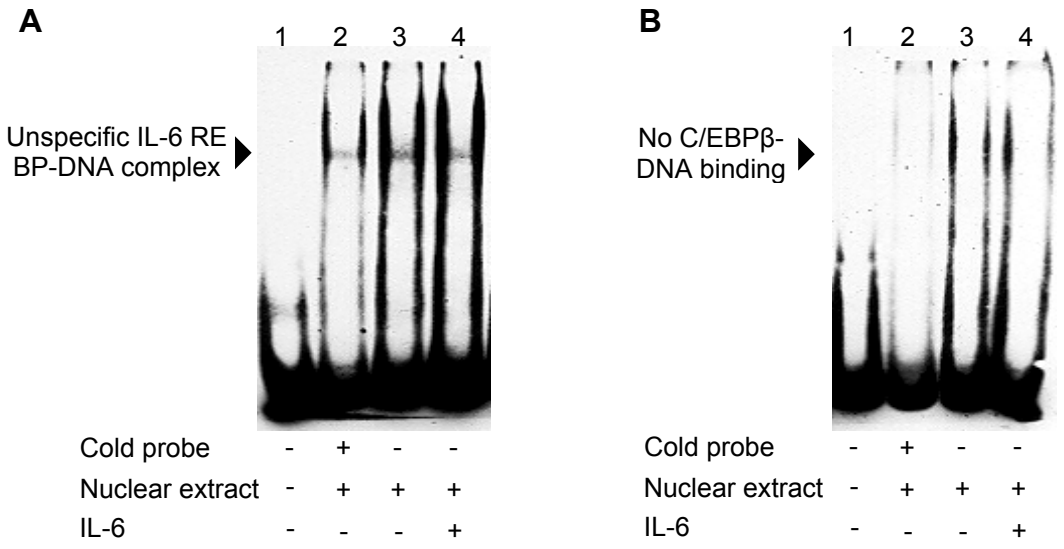


Fig. 21: EMSA experiments with either IL-6 RE (A) or C/EBPβ (B) consensus. Assay was performed with 2 µg nuclear extracts from cultured HepG2 cells in the absence (lane 3) or presence of IL-6 (30 ng/ml) (lane 4). 200 fold molar excess of cold consensus oligo-nucleotides were added in lane 2. Lane 1 represents the free probe. IL-6 RE-BP, IL-6 response element binding protein; C/EBPβ, CCAAT/Enhancer Binding Protein beta.

2.5. Synergistic effect of FOXA2 and IL-6 on ALR promoter

Since the FOXA2 binding site is located next to IL-6 responsive element, we investigated whether the FOXA2 binding site (5'-ATTTCTC-3') and the IL-6 RE (5'-TTCCCTG-3') may interact, or whether this potential interaction is necessary for

the transactivation of ALR promoter through IL-6. To elucidate the putative combined effect of FOXA2 and IL-6 on the activity of ALR promoter, we performed luciferase assays using 1 µg of the ALR promoter construct 2 and 0,5 µg of the expression plasmid of FOXA2 in the absence or presence of 30 ng/ml of IL-6. Promoter activity of ALR was induced after IL-6 stimulation and this induction was able to be further enhanced when the cells were co-transfected with FOXA2 (Fig. 22).

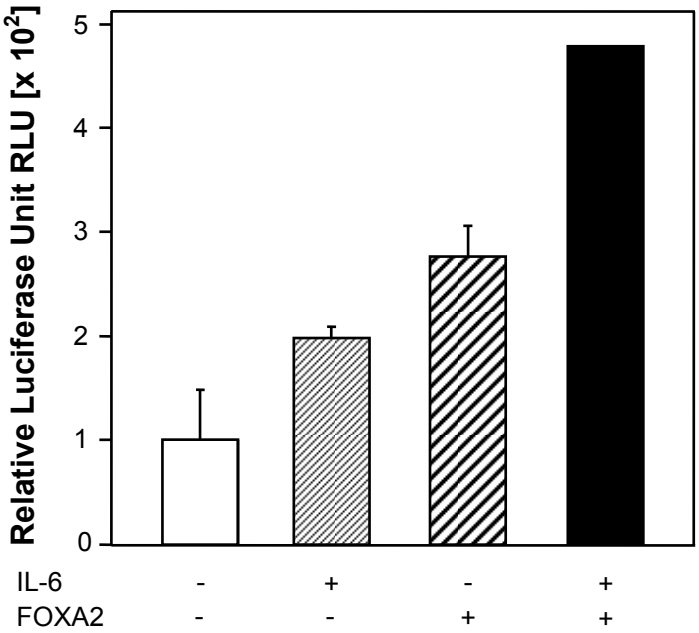


Fig. 22: Enhancement of ALR promoter activity by IL-6 and FOXA2. 1 µg ALR-pGL2 was transfected with or without 0,5 µg FOXA2-expression plasmid into HepG2 cells using siPORT-*XP*1. 30-36 hours after transfection, cells were serum-starved overnight and IL-6 (30 ng/ml) was added for 15 min. After stimulation, cells were harvested and cellular lysates were analyzed for luciferase expression using a luminometer.

To confirm the luciferase results and to verify the synergistic effect of IL-6 and FOXA2, EMSA was performed. Oligonucleotides contained either FOXA2 or IL-6 binding elements and nuclear extracts isolated from HepG2 cells were incubated for 40 min in the absence or presence of IL-6 (30 ng/ml). We observed that the binding of FOXA2 to the DNA was enhanced after stimulation with IL-6 (Fig. 23, lane 4) compared to the non-stimulated HepG2 nuclear extracts (Fig. 23, lane 3). These results underline an important effect of IL-6 transactivation for the binding of FOXA2 to ALR promoter. On the other hand, a binding of transcription factors to the IL-6 RE is not involved in FOXA2 interaction (Fig. 23, lanes 5-8). Thus, it seems that IL-6 RE is not required for a correct positioning of FOXA2 to its recognition site. Based on these findings, we suppose that IL-6 activates some other transcription factors which may act as co-activators for FOXA2.

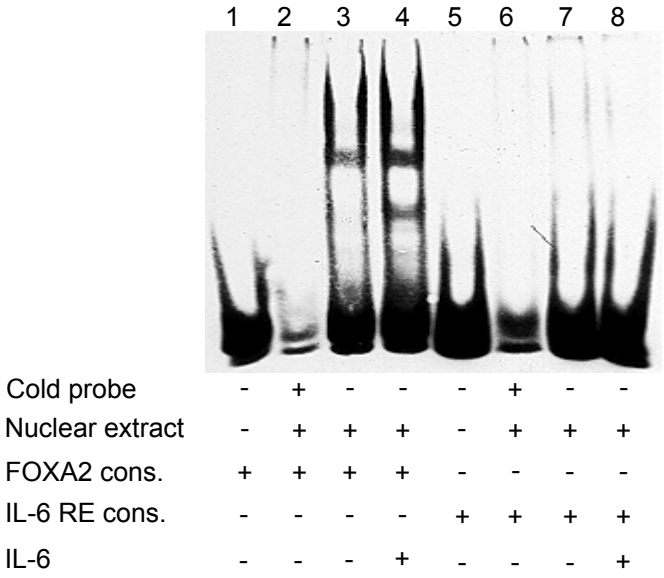


Fig. 23: Enhancement of FOXA2 binding to ALR promoter upon stimulation with IL-6. The oligonucleotide which contains FOXA2-RE binds FOXA2 (lanes 3, 4), whereas the oligonucleotide which contains IL-6 RE does not (lanes 7, 8). 2 µg nuclear extract isolated from HepG2 cells in the absence (lanes 3, 7) or presence of IL-6 stimulation (30 ng/ml for 40 min) (lanes 4, 8) were used. Cons., consensus.

Additionally, we investigated the expression of ALR protein after stimulation with IL-6 and FOXA2 (Fig. 24) by Western Blot and densitometric analysis. As shown in Fig. 24, stimulation with IL-6 (30 ng/ml) as well as with FOXA2 (0,5 µg) enhanced levels of ALR protein. Taken together, both IL-6 and FOXA2 could induce the protein expression of ALR.

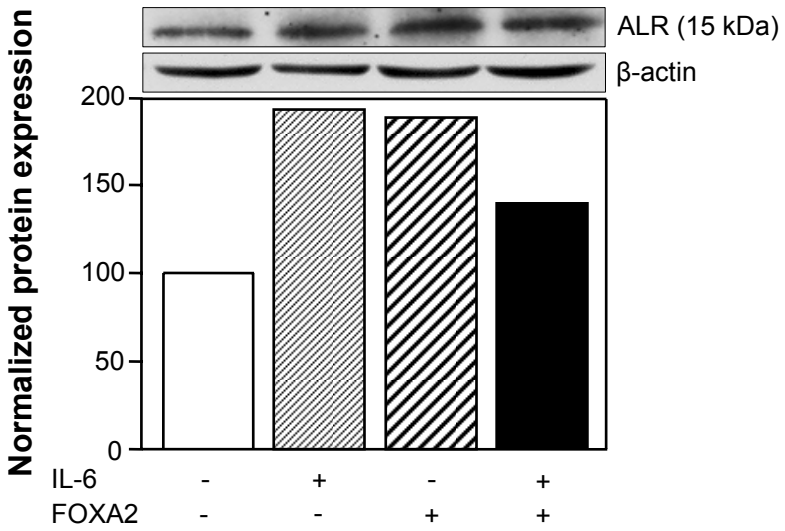


Fig. 24: ALR protein expression after stimulation with IL-6 and FOXA2. Western Blot analysis using specific anti-ALR antibodies showing the expression of ALR-monomers (15 kDa) and quantification of the protein bands by densitometric analysis.

3. Overexpression of ALR

3.1. Generation of ALR-overexpressing HepG2 cells

Liver regeneration is a process which involves various cell biological processes as cell activation and proliferation of hepatic cells [11]. Some of these processes including cell growth and proliferation are common to hepatocancerogenesis. Furthermore, we have demonstrated an up-regulation of ALR expression in regenerative processes as well as in hepatocellular carcinoma [81]. To gain more insight into the impact of ALR on cellular growth and potential tumorigenicity, we stably overexpressed ALR in HepG2 cells. We designed an ALR-expression plasmid and subcloned the full-length cDNA of human ALR (375 bp) which encodes 125 amino acid protein (15 kDa) (Fig. 25) into an expression vector pcDNA3.1 under the control of the cytomegalovirus (CMV) promoter. The ligated plasmid was then sequenced to proof the presence and the direction of the insert.

T7 Promotor NheI HindIII
TAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGCGTTTAAACTTAAGCTTGGTACCGAGCTCGGA
hALR
TCCC*ATG*CGGACGCAGCAGAAGCGGGACACCAAGTTTAGGGAGGACTGCCCGCCGGATCGCGAGGA
ACTGGGCCGCCACAGCTGGGCTGTCCTCCACACCCTGGCCGCCTACTACCCCGACCTGCCCACCCCA
GAACAGCAGCAAGACATGGCCCAGTTCATACATTTATTTTCTAAGTTTTACCCCTGTGAGGAGTGTGCT
GAAGACCTAAGAAAAAGGCTGTGCAGGAACCACCCAGACACCCGCACCCGGGCATGCTTCACACAG
TGGCTGTGCCACCTGCACAATGAAGTGAACCGCAAGCTGGGCAAGCCTGACTTCGACTGCTCAAAAG
EcoRV
TGGATGAGCGCTGGCGCGACGGCTGGAAGGATGGCTCCTGTGAC*TAG*AATTCTGCAGATATCCAGCA
NotI pcDNA3.1/BGH reverse priming site
CAGTGGCGGCCGCTCGAGTCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAG
TTGC

Fig. 25: Design of an ALR-expression plasmid. The cDNA sequence of ALR (375 bp) is printed in bold and restriction sites are underlined. Both ATG transcription start codon and TAG stop codon are shown in red and italics.

After successful transfection of HepG2 cells with the expression vector, ALR-overexpressing cells were selected due to G-418 resistance over a period of two weeks. Cells bearing a vector without insert served as negativ control (mock). To verify whether the stably transfected HepG2 cells do overexpress ALR, real-time RT-PCR analysis was performed for all 24 selected clones (data not shown). Among all screened clones, two clones (named clone 1 and clone 2) have been choosen to

be used in all following experiments. Figure 26A shows ALR mRNA expression in clone 1 and clone 2 and demonstrated a significantly increased level compared to wild type cells and mock-transfected cells. To verify whether the increased mRNA levels of ALR lead to enhanced protein expression, ALR protein was analyzed performing Western Blot analysis (Fig. 26B). Both ALR-overexpressing HepG2 cell clones demonstrated significant increase of ALR protein levels compared to wild type and mock cells (Fig. 26B).

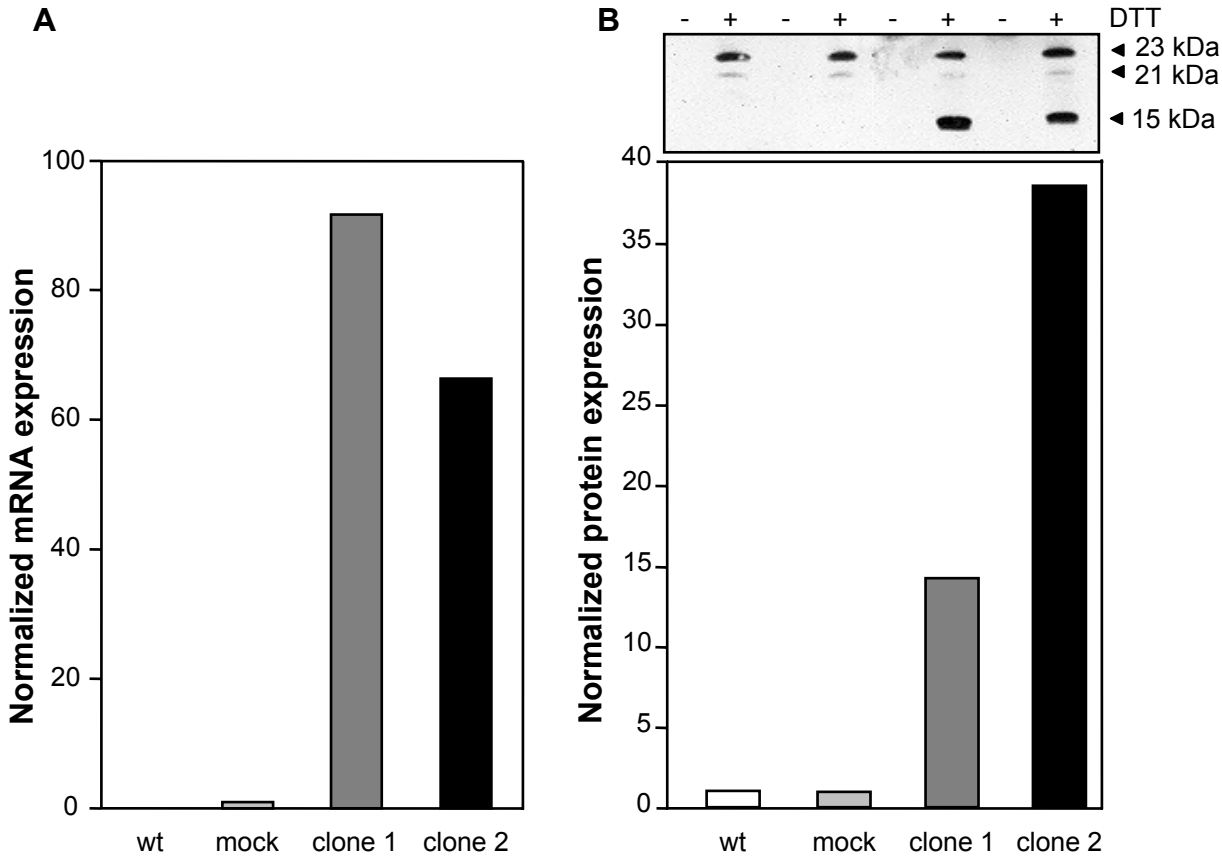


Fig. 26: Determination of ALR expression in ALR-overexpressing HepG2 cells compared to wild type and mock cells. A) Quantitative RT-PCR analysis revealed a strong induction of ALR mRNA expression in both cell clones (clone 1 and clone 2), whereas no significant change of ALR expression was observed in mock transfected cells. B) Expression of ALR protein using Western Blot analysis in non-transfected HepG2 cells (wt), mock-transfected HepG2 cells (mock) and stable-transfected ALR-overexpressing HepG2 cells (clone 1 and clone 2). The protein expression was quantified by densitometric analysis.

Further, we attempted to investigate whether overexpression of ALR has an impact on the proliferation of hepatoma cells. As shown in Fig. 27, we could not find any change in the proliferation curve between ALR-overexpressing HepG2 cells compared to mock cells.

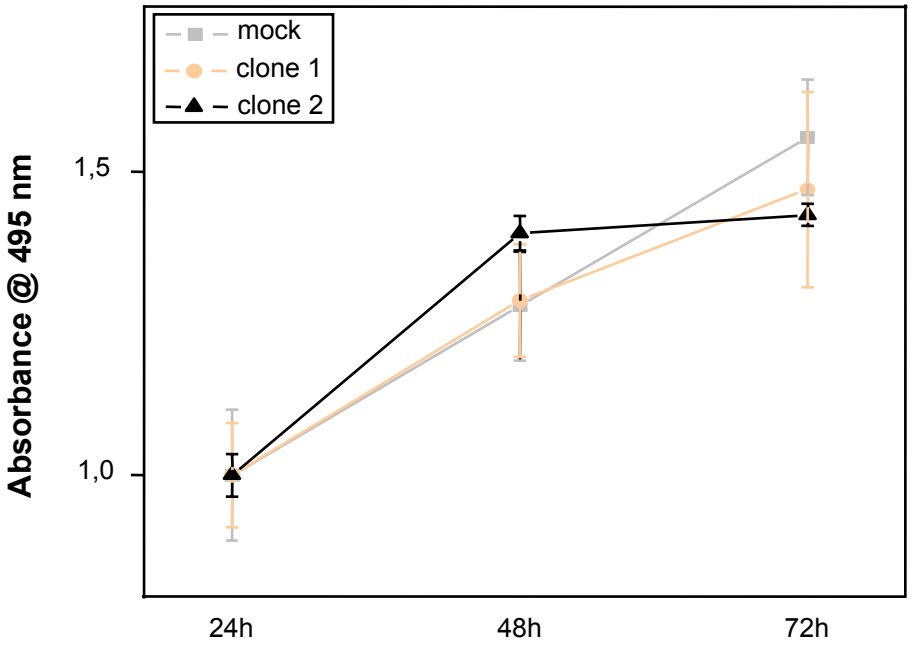


Fig. 27: Proliferation rate of HepG2 cells upon overexpression of ALR. Cellular growth was determined after 24 h, 48 h and 72 h using MTT assay. The value of cell growth of each cell type at 24 h was set at 1. Mock, mock-transfected HepG2 cells; clone 1 and clone 2, stable-transfected ALR-overexpressing HepG2 cells. Assay was performed in triplicate.

3.2. Tumorigenicity of ALR-overexpressing HepG2 cells

After we had generated ALR-overexpressing HepG2 cells and in addition to the above *in vitro* data, we investigated the cellular behavior of wild type (wt-HepG2) cells and ALR-overexpressing (ALR-HepG2) cells *in vivo*. We injected 1 x 10^6 cells of clone 1, clone 2 and mock-control subcutaneously into the peritoneal cavity of immune incompetent nude mice. One week after injection of hepatoma cells, all mice from all groups (n=6) developed tumors. The body weight and tumor size were measured for all mice every week. After three weeks mice were sacrificed and resulting tumor tissue was snap-frozen and stored for further analysis. In figure 28 the average of body weight of all groups is shown demonstrating that mice xenografted with clone 2 cells have less weight than mice xenografted with clone 1 cells or mock cells.

Further, we determined the size of the resulting xenograft tumors after 21 days and found smaller tumors derived from clone 2 cells compared to tumors derived from clone 1 cells or mock cells (Fig. 29).

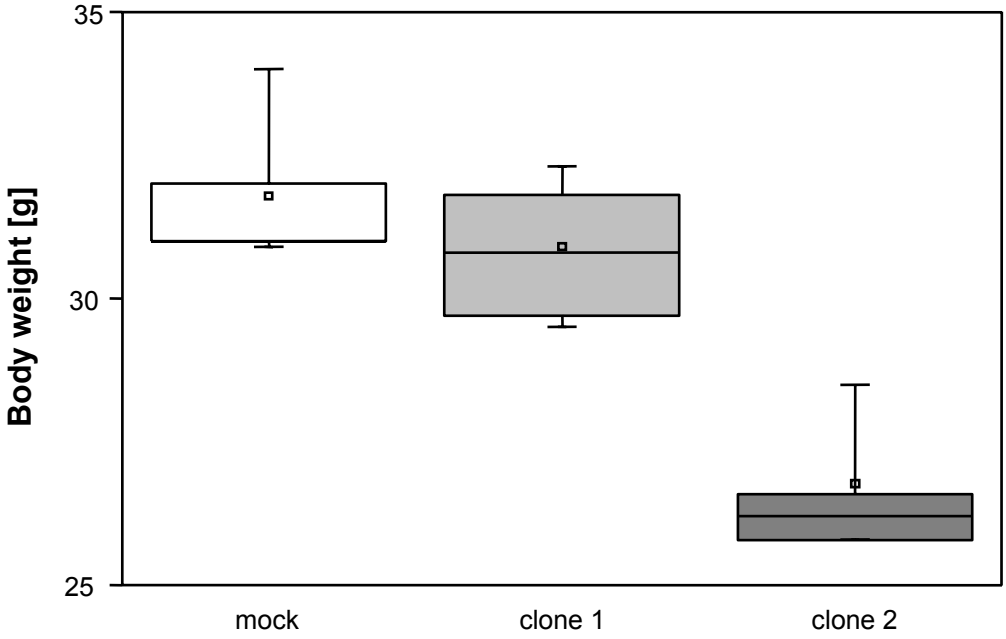


Fig. 28: Determination of final body weight (after 3 weeks) of mice injected with ALR-overexpressing HepG2 cells or mock cells. Average body weight of mice xenografted with ALR-overexpressing cells (clone 2) was lower than that of mice xenografted with clone 1 cells or mock cells, (n=6/group). Data were analyzed using boxplot method. The box contains 50% of the data. The upper edge of the box indicates the 75th percentile of the data set and the lower edge indicates the 25th percentile. The end of the vertical lines indicates the minimum and maximum data values. Small rectangles represent the mean value of the data and the line in the box indicates the median value of the data.

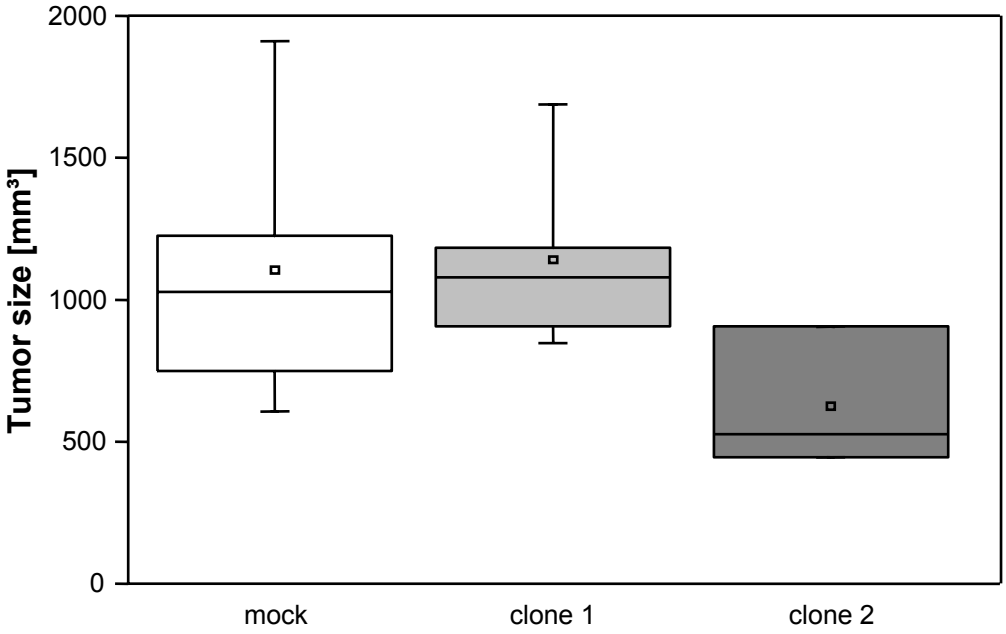


Fig. 29: Size of tumors from ALR-overexpressing hepatoma cells *in vivo*. Tumour size was determined 21 days after injection of 1 x 10^6 ALR-overexpressing HepG2 cells (clone 1 and clone 2) and mock transfected cells (control) into immuno incompetent nude mice (n=6/group). Data were analyzed using boxplot method. The box contains 50% of the data. The upper edge of the box indicates the 75th percentile of the data set and the lower edge indicates the 25th percentile. The end of the vertical lines indicates the minimum and

maximum data values. Small rectangles represent the mean value of the data and the line in the box indicates the median value of the data.

The observed differences of mouse total body weight or tumor size might be due to different expression levels of ALR (see Fig. 26B). Therefore we determined the ALR mRNA levels of the xenograft tumors by qRT-PCR and found very high levels of ALR in tumors derived from clone 2 cells but low expression in tumors derived from clone 1 cells, where the latter is comparable to ALR levels in tumors derived from mock cells (Fig 30).

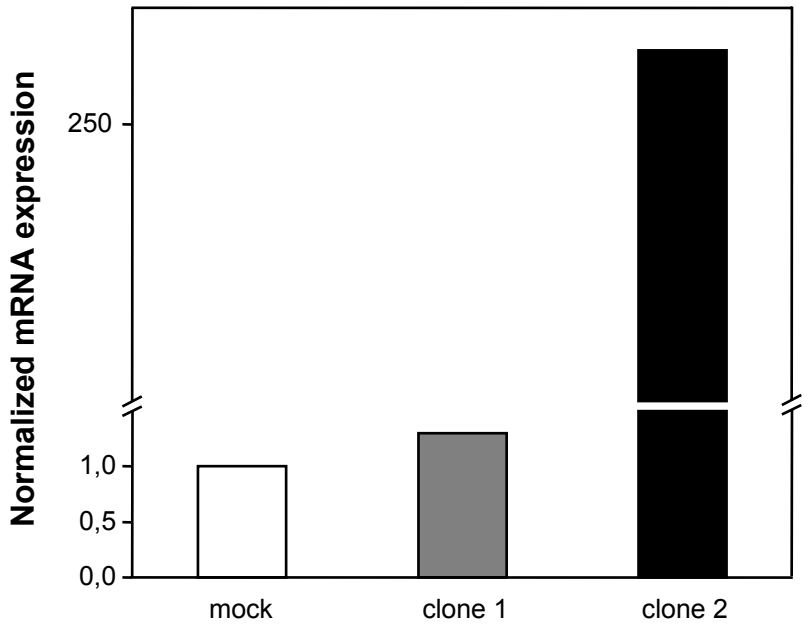


Fig. 30: Quantification of ALR mRNA in xenograft tumor tissue. Total RNA was isolated from tumor tissues, reverse-transcribed and determined using LightCycler technology. The results were normalized to 18S rRNA.

Further, immunohistochemical analysis were performed to investigate the protein expression of ALR in tumors derived from mock, clone 1 and clone 2 cells (Fig. 31) and to confirm the results of qRT-PCR. Results of immunohistochemical analysis are shown in Fig. 31 and confirm the finding of the qRT-PCR analysis which showed that clone 2 cells overexpress ALR (Fig. 31E, F). In contrast, tumors derived from mock cells (Fig. 31A, B) and clone 1 cells (Fig. 31C, D) showed no immunoreactivity for ALR. Based on this finding, clone 2 cells were further used in upcoming experiments and were renamed as “ALR-HepG2 cells”.

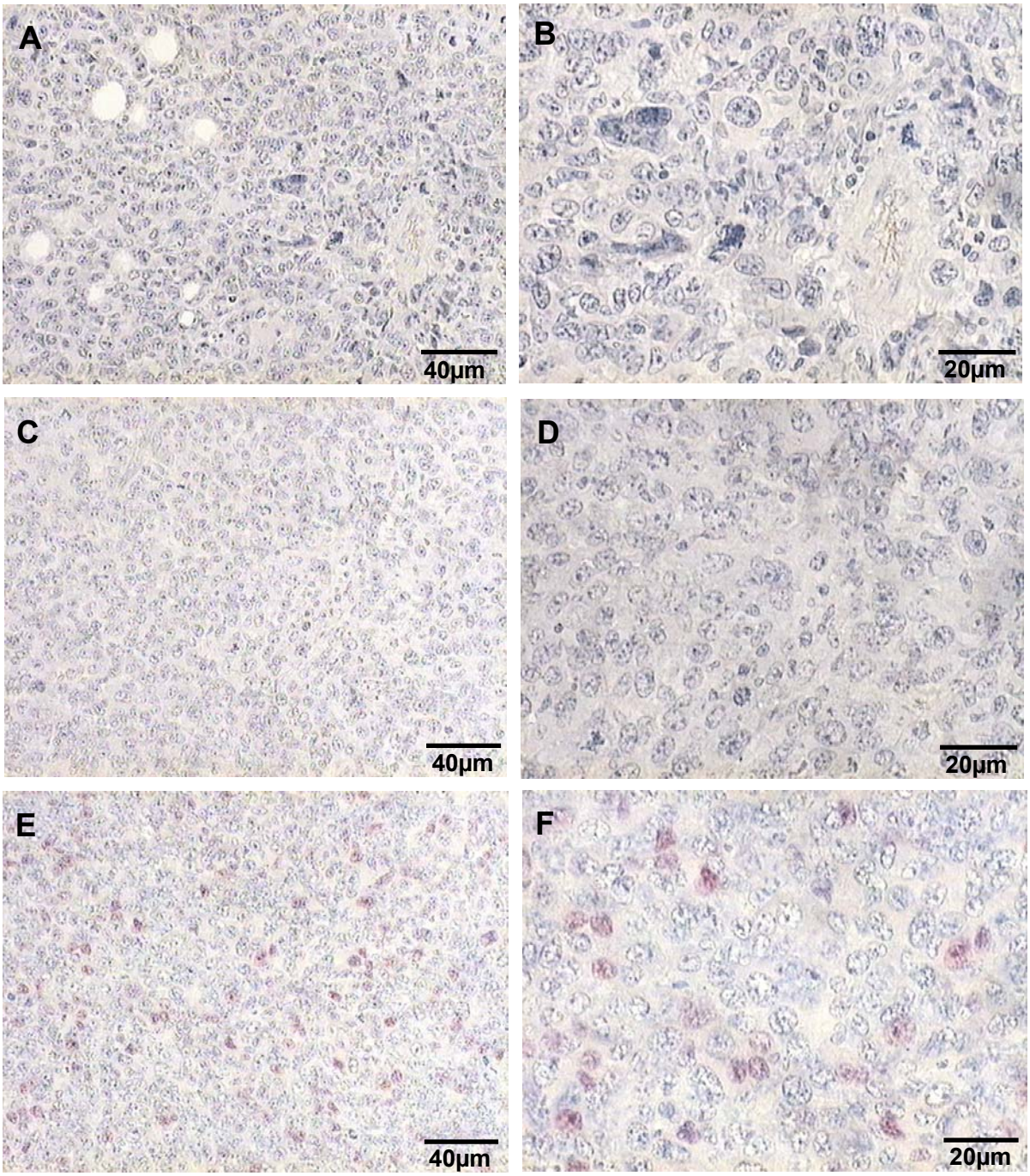


Fig. 31: Analysis of ALR expression by immunohistochemistry in tumor tissues. Tumors derived from mock cells (A, B) and clone 1 cells (C, D) show comparable low immunoreactivity for ALR, whereas clone 2 cells (E, F) revealed more intense immunosignal for ALR.

It is worth to mention that tumors derived from ALR-overexpressing cells displayed less-necrotic areas and showed a more epithelial-like cell growth (Fig. 32A) compared to wt-HepG2 cells which revealed signs of necrosis (Fig. 32B) and a more agressive growth (Fig. 32D). Additionally, less polymorphisms were observed in

tumors derived from ALR-HepG2 cells (Fig. 32C), whereas tumors derived from wt-HepG2 cells demonstrated atypical mitotic figures, as shown in Fig. 32D.

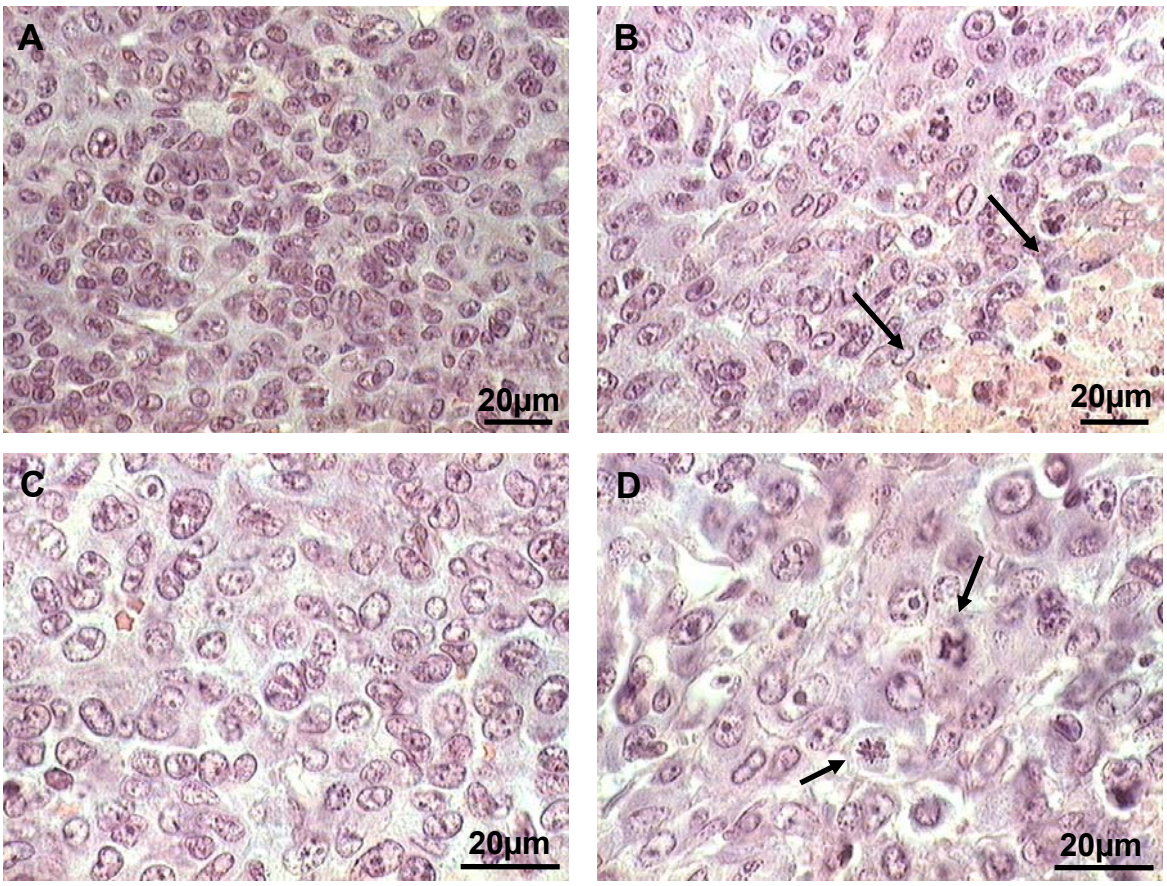


Fig. 32: Analysis of growth behavior of xenograft tumors. Samples of mouse *in vivo* tumors were analyzed by HE-staining and showed for ALR-HepG2 cells (A) a more epithelial-like growth compared to ALR low expressing cells indicating necrotic areas (B, arrows). Furthermore tumors from wt-HepG2 cells showed atypical mitotic figures (D, arrows), whereas ALR-overexpressing tumors demonstrated typical cellular growth (C).

Based on the observation of different tumor sizes and the cellular growth *in vivo* we may speculate that overexpression of ALR in HepG2 cells attenuate tumorigenic growth.

3.3. Impact of ALR on migration and invasiveness of hepatoma cells

The finding of a potential influence of ALR on tumorgenicity of hepatoma cells *in vivo* encouraged us to analyze the impact of ALR on motility and potential metastasis. Metastasis is a critical step in tumor progression and is a main cause of human cancer deaths [111,112]. It occurs in a series of multiple steps including tumor cell migration, invasion and proliferation [113,114]. To elucidate the effect of ALR on the

motility of hepatoma cells, migration assays were performed using modified Boyden chambers. Briefly, 5 x 10^4 cells (ALR-HepG2 or wt-HepG2) were seeded with 1% FCS-DMEM into inserts with 8 µm filter pores which were either uncoated (migration assay) or matrigel-coated (invasion assay). As a chemoattractant 10% FCS were used and after 24, 48, 72 h cells were fixed, stained and counted in each field. Interestingly, ALR-HepG2 cells reduced cell motility (63,67 ± 16,74 cells/field) compared to the wt-HepG2 cells (622,00 ± 74,10 cells/field) after 48 hours (Fig. 33). A similar result was observed after performing the invasion assay. ALR-overexpressing HepG2 cells showed a significantly reduced potential to invade (10,97 ± 2,65 cells/field) than wt-HepG2 cells (49,30 ± 6,00 cells/field) (Fig. 33). These data suggest that migration and invasion properties of hepatoma cells was markedly reduced after expression of ALR.

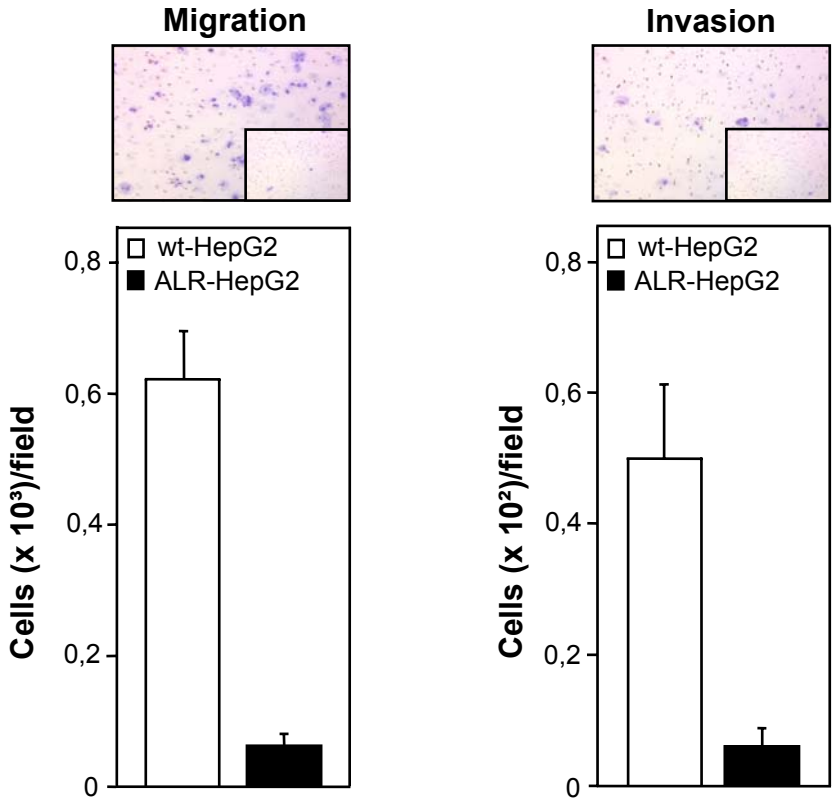


Fig. 33: Impact of ALR on the migration and invasiveness of hepatoma cells. Migration and invasion assays using Boyden chambers were performed for 48 hours. The membranes were fixed and stained and cells were counted. ALR-HepG2 cells, ALR-overexpressing HepG2 cells; wt-HepG2 cells, wild type HepG2 cells.

These results are in agreement with other data from our laboratory which showed that high levels of ALR in HCC tissues are inversely correlated with the invasive activities of hepatocellular carcinoma (manuscript in preparation). Based on these

findings we hypothesize that ALR might interfere with migration- and invasiveness-related pathways in hepatocellular carcinoma cells.

The change in migratory and invasion properties of ALR-overexpressing HepG2 cells prompted us to investigate whether ALR influences the cell-cell contact or cell adhesion molecules. Therefore, we performed attachment assays 15 min after replating of wt-HepG2 cells or ALR-HepG2 cells in 96-well plate. We found that ALR-overexpressing HepG2 cells showed significantly induced attachment rates (7812,50 ± 2809,02 cells) over those of wild type HepG2 cells (3812,50 ± 625,00 cells) (data not shown). Further, to confirm the ability of high cellular levels of ALR to modulate cell migration, we performed wound healing assays using wt-HepG2 cells and ALR-HepG2 cells over a period of 72 hours (Fig. 34). Wild type HepG2 cells showed a significantly faster “wound” closure compared to ALR-overexpressing HepG2 cells. Taken together, we assume that ALR may regulate adherence and cell-cell contact of the hepatoma cells.

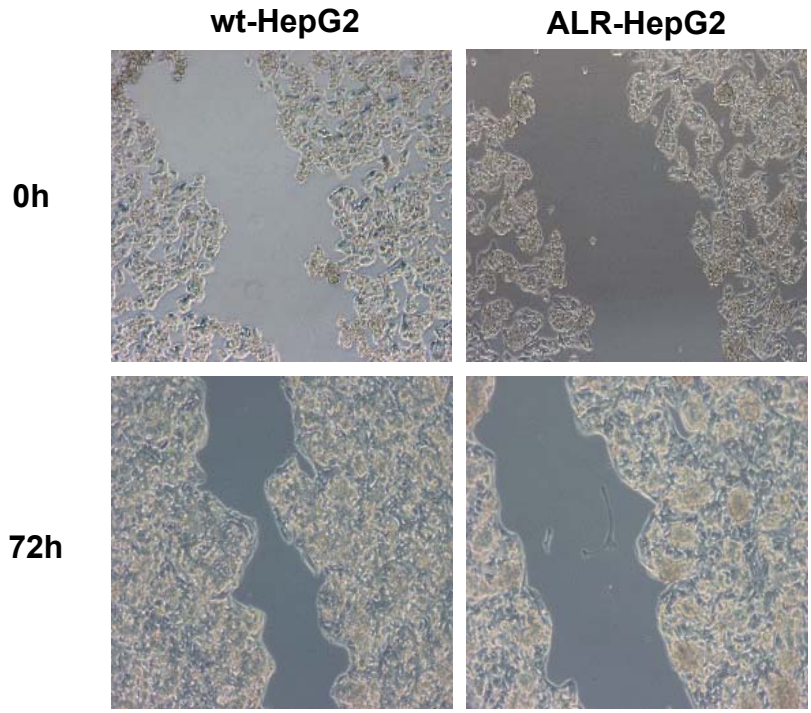


Fig. 34: Impact of ALR on the migratory potential of hepatoma cells. Monolayer scratch assays were performed with ALR-overexpressing HepG2 cells (ALR-HepG2) and wild type HepG2 cells (wt-HepG2) and representative pictures of the areas between scratch fronts after 3 days are shown.

3.4. Effect of ALR on EMT markers

Migration and invasion are mainly mediated by specific cell-adhesion molecules. Therefore to analyze the molecular background of the observed altered ability of migration and invasion we aimed to assess the expression of prominent cell-adhesion molecules like the epithelial cadherin (E-cadherin). The family of cadherins are Ca^{2+}-dependent proteins which mediate homotypic interaction between adjacent epithelial cells, of which E-cadherin is a typical factor. It is known that down-regulation or mutation of E-cadherin is frequent in human tumors and often occurs during cancer progression [115]. In order to investigate the potential regulation of E-cadherin by ALR, we seeded wt-HepG2 cells and ALR-HepG2 cells on chamber slides and cultured them for 24 hours. Cells were fixed and stained using anti-E-cadherin antibody. Interestingly, we observed that wt-HepG2 cells showed no immunoreactivity for E-cadherin staining whereas ALR-HepG2 cells demonstrated a clear plasma membrane staining (Fig. 35, arrows).

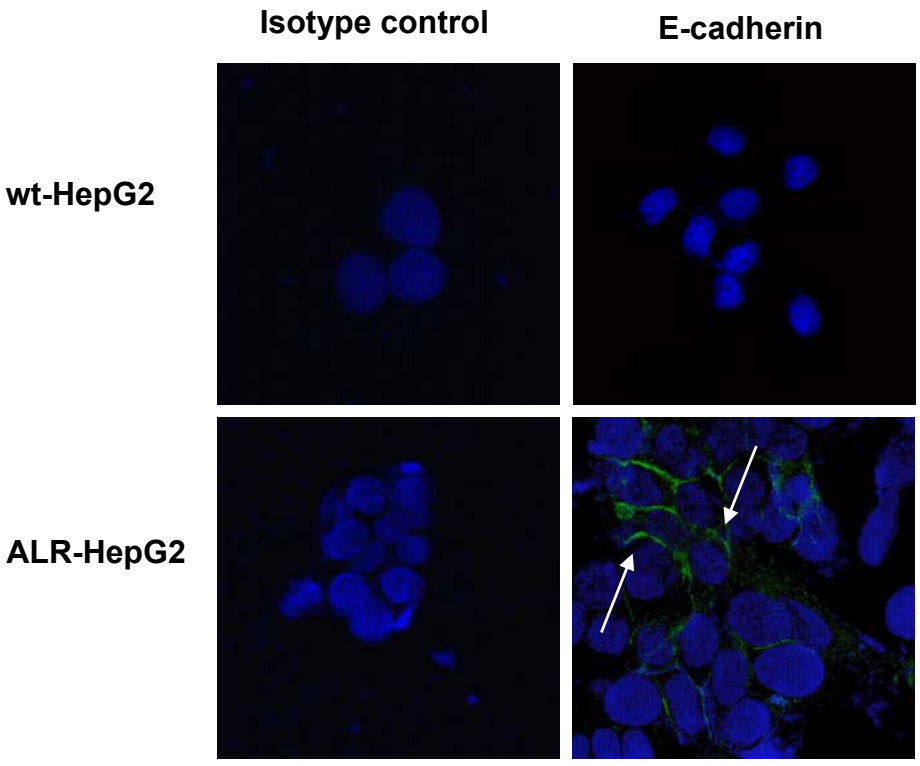


Fig. 35: E-cadherin expression in ALR-overexpressing HepG2 cells and in wild type HepG2 cells. Immunofluorescence analysis shows induced expression of E-cadherin protein on the cell membrane in ALR-HepG2 cells (white arrows) compared to wt-HepG2 cells. The rabbit IgG isotype control served as a negative control for immunofluorescent staining.

In addition, performing Western Blot analysis we could confirm our immuno-cytochemical result, showing that only ALR-overexpressing HepG2 cells express E-cadherin (Fig. 36).

To analyze the mechanism by which ALR regulates E-cadherin, we first investigated the impact of ALR on the expression of the transcription factor SNAIL because E-cadherin was found to be directly regulated/repressed by SNAIL. It has been shown by others that SNAIL converts epithelial cells into mesenchymal cells by directly repressing the expression of E-cadherin [116]. In our study we found that ALR expression in hepatoma cells down-regulates the expression of SNAIL. On the other hand, we investigated that effect of ALR expression on the stability of HIF-1α protein. Stability of the hypoxia-inducible factor (HIF-1α) has been reported to be necessary and sufficient to suppress E-cadherin expression by inducing the expression of transcriptional repressors of E-cadherin [117,118]. No changes were observed in the levels of HIF-1α protein indicating that elevated intracellular levels of ALR did not affect the stability of (HIF-1α) (Fig. 36).

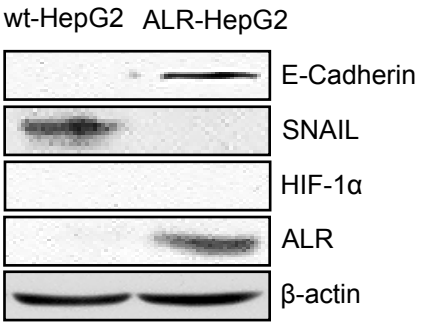


Fig. 36: Impact of ALR on the protein expression of both E-cadherin and SNAIL and on HIF-1α stability. ALR-HepG2 cells exhibit induced expression of E-cadherin and with reduced expression of SNAIL. Whereas ALR expression could not influence the stability of HIF-1α. β-actin served as intern control.

Based on these data, we speculate that ALR up-regulates the expression of E-cadherin by reducing the expression of SNAIL. Furthermore, HIF-1α seems to be not involved in E-cadherin upregulation by ALR.

One more feature of EMT is the overexpression and/or activation of the matrix metalloproteinases (MMPs), which are main features of tumor progression and are associated with invasion, angiogenesis and metastasis. Among them, MMP-3 (stromelysin-1, Str-1), exerts oncogenic effect and plays an important role by triggering EMT *in vitro* [119] and *in vivo* [120]. In addition, it was reported that MMP-3 can activate other MMPs in ECM like MMP-1 which is known to play a critical role in invasion and tumor grwoth in the metastatic process [121]. Therefore, we additionally

investigated the impact of ALR on the expression of both MMP-3 and MMP-1 and found that ALR reduces the mRNA expression of both MMP-3 and MMP-1. The transcriptional expression of both was inhibited up to 80% in ALR-HepG2 cells compared to wt-HepG2 cells (Fig. 37).

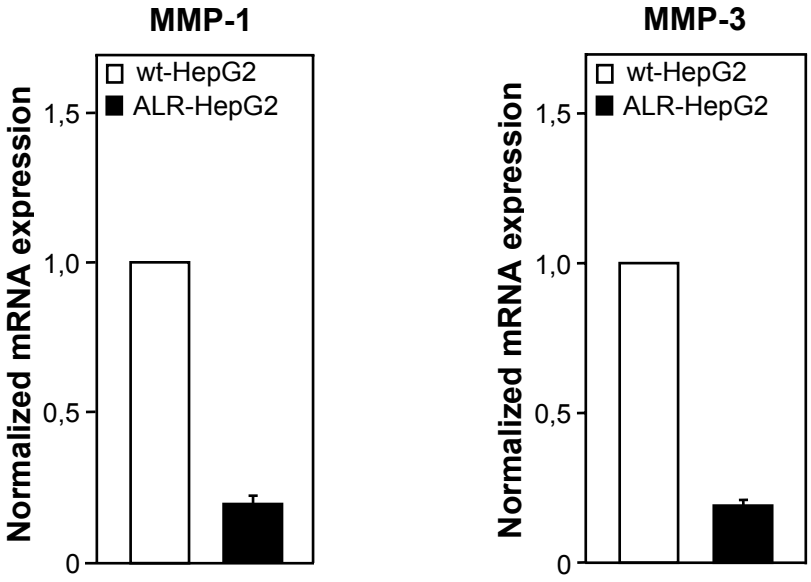


Fig. 37: mRNA expression of MMP-1 and MMP-3 in wt-HepG2 cells and ALR-HepG2 cells by qRT-PCR. ALR-overexpressing HepG2 cells show reduced expression of both MMP1 and MMP-3 compared to wt-HepG2 cells. Expression of the housekeeping gene 18S ribosomal RNA serves as an internal control.

Since MMP-3 has been shown to transcriptionally induce the expression of SNAIL [122], we hypothesize that ALR attenuates the expression of MMP-3 followed by SNAIL inhibition which in turn up-regulates the expression of E-cadherin.

V. DISCUSSION

The liver possesses a remarkable capacity to regenerate after physical damage or injury caused by infections or toxins [5,123]. The fascinating ability of the liver to regenerate is the effect of cross-talk between cytokines, growth factors and hormones which are required for cellular growth and proliferation. The impact of many cytokines (IL-1, IL-6 and TNF-α) and growth factors (EGF, HGF and TGF-α) on human hepatocytes has been described by several authors [2,11,13,14]. These factors have been found to be highly expressed upon liver damage and were shown to play an important role within the process of liver regeneration [2,124]. Recently, the liver regeneration associated protein ALR, has received great attention and its role as a hepatotrophic factor has been partially clarified. Its has been found that ALR is an ubiquitous protein and its hepatotrophic effect as extracellular factor seems to be restricted to the liver. This liver-specific effect might be due to a specific receptor, so far only found on the surface of hepatocytes and hepatoma cells [27]. In addition, ALR was found to be a pivotal intracellular survival factor in hepatocytes [125]. It was shown that transfection of primary rat hepatocytes with antisense oligonucleotides for ALR mRNA (long form) resulted in mitochondrial and cellular depletion of ALR, accompanied by profound loss of ATP, release of cytochrome c from mitochondria, cellular release of LDH and apoptotic/necrotic death of hepatocytes [125].

Previous ALR expression studies in rat liver revealed a specific detection of ALR in the cytosol of hepatocytes and non-parenchymal liver cells [126]. However, non-parenchymal liver cell immunostaining varied and could not be verified in cultured cells [126]. Furthermore, the authors could not observe increased ALR expression in liver tissue after hepatectomy [126]. They described rather a decrease in ALR expression in hepatic tissue levels accompanied with an increase of ALR serum levels 12 h after 70% hepatectomy [126]. A controversial finding concerning the latter report has been published by others regarding increased ALR mRNA expression levels in the remnant liver of a partially hepatectomized rat 12 h post-operation [67,127-129]. In this study, we investigated the expression of ALR in distinct experimental models of liver injury induced by different causes. We observed an increase in ALR mRNA content in livers with injury caused not only by tissue loss (hepatectomy), but also by bile duct ligation (BDL), CCl_4 treatment and hepatic lipid accumulation (NASH model). Regarding these results we conclude that ALR is part of the regeneration process in general, independent of the initial injury, and therefore we additionally suggest an important role of ALR in cirrhosis as well as in liver

carcinoma [81]. This hypothesis is based on the fact that the regeneration process and carcinogenesis show many similarities including cellular growth. Repeated hepatocyte proliferation during liver regeneration may cause disorder of genes which regulate the cell cycle in hepatocytes, therefore favouring HCC development [130]. Growth factors such as HGF, EGF and TGF-α have been shown to be increased in cirrhotic tissues [131-134] and associated with hepato-cancerogenesis [134-137], even though the HGF expression in HCC development is controversial [138,139]. Our previous finding on induced ALR protein expression in cirrhosis as well as in HCC parallel these reports [81], since ALR was originally discovered as a hepatotrophic factor with mitogenic effect in liver regeneration [140].

The observation that ALR is differentially expressed during regenerative processes raises many questions on its potential regulation by factors known to be elevated within these processes. More specifically, we wanted to assess the involvement of different cytokines, growth factors and transcription factors, known to be differentially expressed/activated in liver regeneration as well as in HCC, in regulating the gene expression of ALR. In particular, we aimed to define which *cis*-acting elements are required for transcription of human ALR gene. Thus, promoter analyses were performed using different transcription factor prediction softwares. Computer analysis of the 5'-regulatory sequences (-733 to +502 bp) of ALR predicts the presence of potential binding sites for IL-6 RE-BP, NF-κB, FOXA2, C/EBPβ, AP1, SP1, HNF4α and Nrf2. In contrast to previous studies analyzing the promoter sequence only to +205 bp (-1222 to +205 bp) [86] we included the first intron of ALR gene in our promoter analysis to adress the potential specific regulation of the short form (15 kDa) of ALR (initiating ATG, +241 bp). In addition, the regulatory elements and potential transcription factor binding sites located within introns have been found to play an important role in regulating the expression of target genes [141-146]. Interestingly, sequence analysis of ALR promoter revealed that the first intron of ALR gene exhibits putative binding sites for SP1, FOXA2, IL-6 RE-BP, C/EBPβ and HNF-4α (see sections 2.2 and 2.4).

IL-6 RE-BP, FOXA2 and C/EBPβ are known to be regulated/activated after stimulation with IL-6 [147,148], while NF-κB has been shown to get activated by TNF-α [149]. Therefore, we performed several stimulation experiments using various cytokines and growth factors. We have found that ALR is regulated neither by growth factors which have been used in our experiments nor by TNF-α demonstrating that

these factors may not act upstream of ALR. In contrast, we observed that IL-6 induced ALR gene expression 24 hours after stimulation. Based on the fact that Kupffer cells, besides hepatic stellate cells are the main source of IL-6 in the hepatic environment, we investigated the expression pattern of ALR in hepatocytes stimulated with Kupffer cell conditioned-medium. No change could be observed regarding the expression profile of ALR although high amounts of IL-6 were detected in the Kupffer cell conditioned-medium. This unexpected result may be explained by the fact that in addition to IL-6, Kupffer cells produce several other factors [2,11,16,150,151] which may negatively modulate the expression of ALR or at least reverse the stimulatory effect of IL-6. One of these factors might be IL-1β which is known to have anti-proliferative effects on hepatocytes [150,152,153]. It has been shown that IL-1β exerts a transient inhibitory effect to the IL-6 stimulatory signal [154]. The inhibitory action of IL-1β on IL-6-inducible gene expression depends on the interruption of the IL-6 signal transduction pathway from the cell surface to the nucleus [154,155]. The inhibition of IL-6 cascade might be explained at least by two mechanisms, the dephosphorylation of STAT1 and the attenuation of STAT1/STAT3 binding to promoter of target gene [154,155]. To confirm this hypothesis, we analyzed the protein expression of ALR after stimulation with IL-1β and found slightly reduced ALR levels while incubation with IL-6 revealed increased ALR protein expression. Taken together, we conclude that both IL-6 and IL-1β are involved in ALR gene regulation by enhancing and reducing ALR expression, respectively. A similar gene regulation by IL-6 was reported for HGF demonstrating several IL-6 RE located in the HGF promoter [156,157]. These IL-6 response elements have been shown to mediate the stimulatory effect of IL-6 on the gene expression of HGF [158]. These data underline the mitogenic character of ALR similar to HGF, because ALR does not only have pro-regenerative hepatotrophic effects but also seems to be regulated by similar cytokines. Furthermore, ALR is regulated only by factors such as cytokines which are known to initiate the process of regeneration. Therefore besides mitogens, ALR is regulated by some upstream factors of the process of liver regeneration.

ALR promoter analysis revealed binding sites for several transcription factors of which four of them, one in the first intron, have been found for FOXA2. FOXA2, a winged helix protein which belongs to the Forkhead box (FOX) class of monomeric transcription factor [159], was shown to be mainly expressed in liver and gut regulating a number of hepatocyte-specific genes [93,160]. Furthermore, using cDNA array

expression analysis, FOXA2 has been identified as one of several genes which are consistently upregulated in livers from patients with HCC [99] and during liver regeneration after hepatectomy [98]. In addition, using a transgenic mouse model for FOXA2 high expression levels of ALR were found in the liver [100]. Therefore, it is likely that FOXA2 may be involved in the regulation of ALR expression which could be confirmed in this thesis. We found a strong response to FOXA2 specifically in intronic promoter region suggesting the presence of FOXA2 response element within the first intron of ALR gene. The presence of potential binding sites for FOXA2 within introns have been described by several authors [142] and in case of ALR promoter we could show a functional binding of FOXA2 [141] within the first intron (+276/+282 bp) of ALR gene. As reported above ALR promoter was shown to be sensitive to IL-6 treatment and the underlying IL-6 response element is also located in the first intron (+255/+261 bp) in the proximity of FOXA2 and C/EBPβ binding sites. IL-6 RE was demonstrated to be required for a correct positioning of FOXA2 and C/EBPβ [161], another transcription factor which has been found to mediate IL-6 response of many hepatic genes [147,161,162]. Therefore, analyzing ALR gene response to IL-6 we have focused on IL-6 RE and the binding sites of FOXA2 and C/EBPβ located in the first intron of ALR gene.

First, we found no binding of IL-6 RE binding protein and C/EBPβ to their binding sites suggesting that these regulatory elements may not be involved in the IL-6 response of ALR promoter. Second, we could demonstrate that FOXA2 binding to ALR promoter is stronger after treatment with IL-6 indicating a putative positive involvement of IL-6 RE-BP on FOXA2 binding to the promoter. FOXA transcription factors are regulators of genes important in inflammation [161,163-165] and development [166-170], and are regulated at the transcriptional level [148]. It is worthwhile to mention that both C/EBPβ and C/EBPγ proteins have been shown to get activated by both cytokines IL-1 and IL-6 and further to induce the gene expression of FOXA2 [148,171]. Therefore we suggest that IL-6 induces the activity of ALR promoter by two possible mechanisms. First, IL-6 activates C/EBPβ increasing the gene expression of FOXA2 which in turn transactivates ALR promoter. Second, IL-6 activates IL-6 RE-BP which may interacts with FOXA2 augmenting its binding to ALR promoter. In both cases, activation of ALR promoter leads to enhanced protein expression of ALR upon IL-6 treatment (Fig. 38).

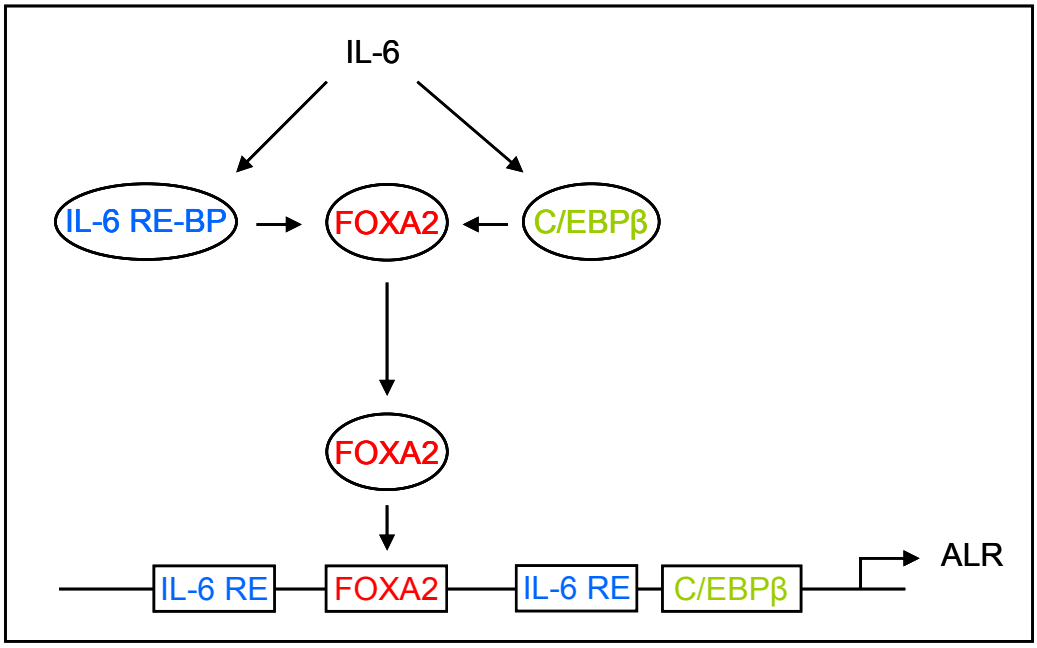


Fig. 38: Schematic drawing of ALR promoter regulation. The first intron of ALR gene exhibits potential binding sites for IL-6 RE-BP, FOXA2 and C/EBPβ. Our studies showed that C/EBPβ could not bind to ALR promoter and therefore is not able to transactivate ALR promoter. However, activated C/EBPβ by IL-6 may induce the expression of FOXA2 resulting in transactivation of ALR promoter. Another mechanism by which IL-6 induces ALR expression may be mediated by IL-6 RE-BP. We have demonstrated that upon treatment with IL-6, IL-6 RE-BP could not bind to IL-6 RE located in the proximity of FOXA2 response element. This finding suggests that IL-6 RE-BP may interact with FOXA2 augmenting its binding to ALR promoter. Transcription factors are shown in oval and transcription factor binding sites are shown in frames.

In addition to FOXA2, IL-6 RE-BP and C/EBPβ, we also identified potential binding sites for SP1 and HNF-4α. SP1 is a member of a huge family which binds to and acts through the GC box (GC-rich sequence) [172] and has been shown to be involved in the basal expression of TATA-less promoters [173]. It was found that SP1 functions as an essential transcriptional activator of basal expression of ALR [88]. Further, they found an HNF-4α binding site (DR-1) within the human ALR promoter and reported that HNF-4α could bind to this region and suppress the gene expression of human ALR [88].

In our promoter study we further found other regulatory elements which could be involved in the regulation of ALR expression. We identified a perfect and nearly conserved antioxidant response element (ARE) in the promoter sequence of human ALR. ARE is a *cis*-acting sequence and has been found in the 5'-flanking region of many genes encoding phase II detoxifying enzymes including NAD(P)H: quinone reductase (NQO1), glutathione transferases (GSTs), heme oxygenase-1 (HO-1), aflatoxin B1 dehydrogenase and ferretin [174]. Nuclear E2-factor related factor 2 (Nrf2),

a member of Cap'n'Collar family of basic region-leucine zipper transcription factors, was described to be an essential component of the ARE-binding transcriptional machinery [175]. It has been demonstrated that Nrf2 translocates into the nucleus and binds cooperatively to the ARE with small MAF proteins after activation by redox/oxidative stress. The Nrf2 and target genes have been shown to be essential for protection against oxidative stress caused by chemical-induced cellular damage to the liver [176,177]. Based on these findings we may speculate about a putative role of ALR as a hepatoprotective factor against oxidative stress caused by different insults such as chemical-induced liver injury. This assumption is underlined by reports which demonstrated several Nrf2 regulated protective proteins including ALR by microarray analysis [178,179]. Taken together, the existence of an ARE within ALR promoter and the Nrf2 dependent-increase in the gene expression of ALR transcripts show evidence that Nrf2 may be involved in the regulation of ALR expression.

ALR was shown to be upregulated under circumstances of liver regeneration, but less is known about its impact on cellular growth and potential tumorigenicity. Using an ALR-overexpressing hepatoma cell line we could not detect an altered proliferation rate *in vitro*, but rather an enhanced ability to adherence compared to wild type hepatoma cells. This was confirmed by *in vivo* growth studies of these cells demonstrating a more epithelial-like growth of ALR-expressing xenografts compared to a more tumorigenic and necrotic growth of wt-HepG2 xenografts. These findings prompted us to elucidate the molecular link between ALR overexpression and tumorgenetic features like altered adherence and cell motility. Metastasis (Greek: The change in the state) occurs in a series of multiple steps including tumor cell migration, invasion and proliferation [113,114], and is a main cause of human cancer deaths [111,112]. Performing migration and invasion assays we found that increased levels of ALR reduced hepatoma cell motility diminishing migratory and invasive potential of hepatoma cells. This observation is underlined by an immunohistological study on human HCC samples which showed that high levels of ALR in HCC tissues are inversely correlated with the angioinvasion of hepatocellular carcinoma. Both migration and invasion are necessary for tumor growth and metastasis, and are part of a process called EMT [180]. Epithelial-mesenchymal transition (EMT) is an orchestrated series of events in which cell-cell and cell-extracellular matrix (ECM) interactions are altered, the cytoskeleton is reorganized to confer the ability to move through a three-dimensional ECM, and a new transcriptional program is induced to

gain a mesenchymal phenotype [181]. A prominent marker of EMT is the cell adhesion molecule E-cadherin, member of the family of Ca^{2+}-dependent cadherins which mediate homotypic interaction between adjacent epithelial cells. Downregulation or a complete shutdown of E-cadherin expression have been observed in carcinoma cells [115,182] and in human tumors, while loss of E-cadherin-mediated cell adhesion correlates with the loss of epithelial morphology and with the acquisition of metastatic potential [183]. Importantly, we demonstrate in this thesis that ALR-overexpressing hepatoma cells exhibit induced protein expression of E-cadherin which could not be detecetd in wild type hepatoma cells. Therefore, we conclude that increased levels of ALR recovers the expression of E-cadherin allowing for greater cell–cell adhesion and decreased cell motility. In order to analyze the mechanism by which ALR regulates E-cadherin, we analyzed if ALR may regulate factors upstream of E-cadherin such as SNAIL and HIF-1α or proteases known to cleave E-cadherin like MMP-3. SNAIL, a member of the SNAIL superfamily of zinc-finger transcriptional factors, has been shown to promote key steps in tumorigenesis [116,184] converting epithelial cells into mesenchymal cells by repressing E-cadherin expression [116] (Fig. 39) [185]. The hypoxia-inducible factor (HIF-1α) has been reported to be necessary and sufficient to suppress E-cadherin expression by inducing the expression of transcriptional repressors of E-cadherin [117,118]. Another feature of EMT is the over-expression and/or activation of the matrix metalloproteinases (MMPs), which are involved in tumor invasion, angiogenesis and metastasis. Among them, MMP-3 (stromelysin-1, Str-1), exerts oncogenic effects and plays an important role by triggering EMT *in vitro* [119] and *in vivo* [120]. It was reported further that MMP-3 can degrade the extracellular matrix (ECM) and activate other MMPs like MMP-1, MMP-9 and MMP-13 [186,187]. MMP-1 was shown to be involved in the progression of human malignancies by promoting invasion and tumor growth in the metastatic process [121]. It was shown that MMP-3 reduces E-cadherin by cleaving the E-cadherin protein on the cell membrane [119] and transcriptionally by inducing SNAIL expression *via* smad 2,3/4 [122,188-191]. In our study we found that ALR expression in hepatoma cells down-regulates SNAIL expression, while no effect on HIF-1α stability was observed, the second important regulating transcription factor of E-cadherin. In addition we found decreased levels of MMP-1 and MMP-3 in ALR expressing cells compared to wild type hepatoma cells. Therefore, the observed upregulation of E-cadherin may be explained either by modulating Smad/SNAIL or by MMP-3 pathways and include a

possible crosstalk/interaction of these cascades (Fig. 39). ZO-1 (Zona Occludens-1), a tight junction associated protein has been found to play an important role in cell-cell contact. ZO-1 shuttles between the plasma membrane and the nucleus or the cytosol and this cytoplasmic/nuclear relocalization has been shown to be involved in EMT and tumor invasion [192]. We observed that ALR-overexpressing hepatoma cells exhibit high levels of ZO-1 which was mainly localized in the cytosol increasing cell-cell contact and decreasing cell motility (data not shown). Results presented in this thesis underline a suggested role of ALR in regulating EMT.

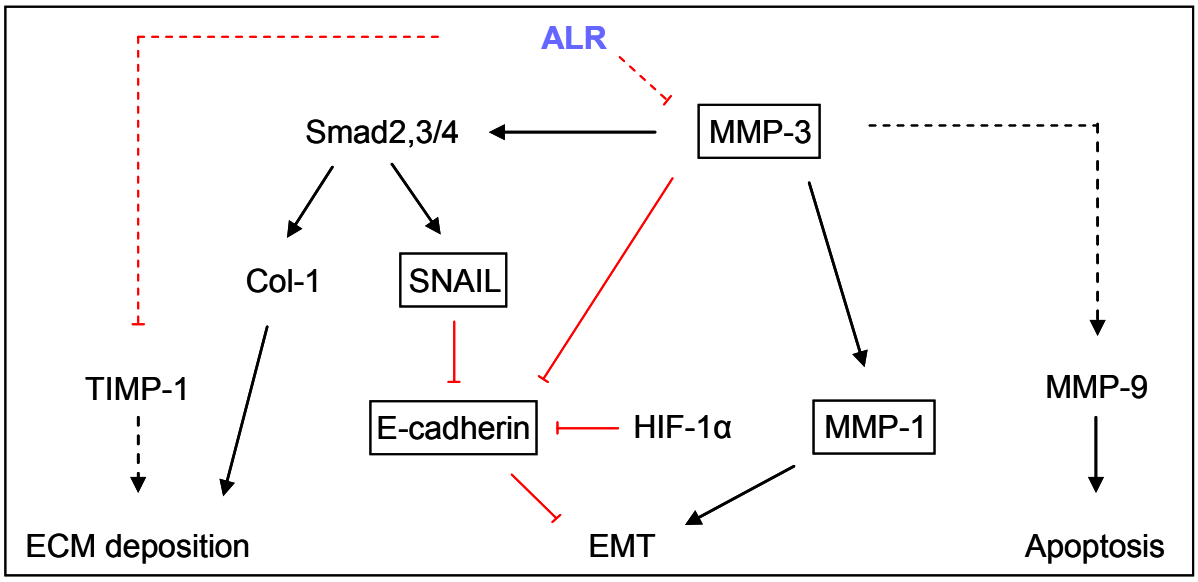


Fig. 39: Schematic drawing demonstrating the influence of ALR on EMT. ALR reduces the ECM deposition by decreasing TIMP-1 expression [83]. In addition, ALR supports the epithelial phenotype by reducing MMP-3 and MMP-1 expression resulting in enhanced E-cadherin levels. Furthermore, ALR enhances the expression of MMP-9 triggering the myofibroblast cell death. Results demonstrated in this thesis are shown in frames. Dashed lines include several steps.

Previous studies have provided evidence that ALR has a hepatoprotective effect on fibrogenesis because exogenously applied ALR ameliorated hepatic damage and decreased the degree of hepatic fibrosis induced by toxins such as CCl_4/Ethanol, thioacetamide or porcine serum [84,193,194]. While two studies have described the anti-fibrotic effect of ALR to inhibit collagen deposition [193] or to decrease gene and protein expression of TIMP-1 (inhibitor of MMPs) [84], little is known regarding the underlying mechanism. Additionally, we have found that treatment of primary human hepatocytes with ALR decreased pro-fibrogenic gene expression of collagen type 1, IL-8, TIMP-1 and TGF-β (data not shown). Our preliminary *in vitro* data might parallel *in vivo* results [83,84] demonstrating that ALR ameliorates the hepatic fibrosis by

decreasing markers which are known to be involved in the initiation/progression of liver fibrosis such as TGF-β, collagen type 1 and TIMP-1 [195-199]. The repression of pro-fibrogenic gene expression as well as reversion of an EMT process by ALR renders this protein as an interesting candidate for anti-fibrotic therapy.

Hepatic fibrosis is a wound-healing process in the liver caused by acute and chronic injury and characterized by an excess production and deposition of extracellular matrix components. Recently, in many models of experimental fibrosis, it has been reported that administration of HGF resulted in reduced tissue damage and fibrosis in the liver [200-205]. HGF abrogates the expression of collagen type 1 as well as fibronectin and upregulates cell adhesion proteins such as E-cadherin and ZO-1 resulting in a reversion of EMT [206]. Further, HGF has been shown to induce MMP-9-dependent myofibroblast cell death and thereby to reduce pulmonary fibrosis [207]. In addition, HGF blocks bile duct EMT in a model of bile duct ligation and ameliorates the hepatic fibrotic lesions by reducing the expression of α-smooth muscle actin, attenuation of collagen type 1, 3 deposition and supressing the expression of TGF-β [200]. Recent publications have demonstrated that HGF and ALR share several similarities like activating proliferative pathways such as MAPK cascade [11,12,14,26,64,77], high expression rate in liver diseases e.g. cirrhosis and hepatocellular carcinoma [67,81,134-136,208] and modulation of hepatic metabolism [76,209]. The new data in this thesis may add another common feature of HGF and ALR acting as an anti-fibrotic proteins attenuating fibrogenesis or even reversing fibrosis. Despite the possible application of HGF as a potent therapeutic agent with anti-cirrhotic [204,205], and anti-neoplastic [210] effects as well as supporting of liver regeneration [211,212], HGF remains an almost unspecific growth factor. In contrast, ALR seems to be a promising hepatotrophic drug with anti-cirrhotic, anti-fibrotic [83] and anti-metastatic effects restricted to the liver due to its high organ specifity. In this regard, our findings underscore that the blockade of EMT by ALR presents a novel strategy for prevention/reversion of hepatic fibrosis protecting the liver tissues from developing HCC.

After analyzing the expression levels of ALR in regenerating livers as well as in human liver tumors, there are still some unanswered questions such as whether ALR expression is also regulated in other organs with regenerative capacity like muscle and intestine. At the protein level, the finding that the transcript of ALR is rapidly

induced by FOXA2 and IL-6, paves the way to future to determine whether anti-proliferative factors like TGF-β or IL-10 may reduce the expression of ALR not only in hepatocytes but also in non-parenchymal cells such as hepatic stellate cells and Kupffer cells.

The cellular mechanism underlying the ALR-mediated prevention/reversion of hepatic fibrosis is still not completely understood. However, our findings presented in this thesis that ALR attenuates EMT progression *in vitro*, encouraged us to establish an Adv5/ALR virus which can be further used in different experimental fibrosis models. This tool would help to uncover the molecular mechanism underlying the anti-fibrotic impact of ALR by investigating the expression of several proteins known to be involved in both fibrolysis and fibrogenesis. More specifically, we would investigate whether ALR application influences the expression of TGF-β, collagen type 1 and 3, α-smooth muscle actin and the activation of Smads. Furthermore, application of ALR in experimental tumor models would help us to clarify the molecular background of the anti-metastatic effect of ALR. In details, it would be very interesting to analyze the expression and/or localization of various proteins which are involved in the process of metastasis such as cell-surface markers (E-cadherin, ZO-1), cytoskeletal markers (β-catenin, cytokeratin), ECM proteins (fibronectin, laminin) and transcription factors (SNAIL, SLUG and Twist). Additionally this approach, when coupled to microarray gene expression analysis, may help to gain more insight into ALR association in other cellualr processes such as inflammation, redox homeostasis as well as lipid metabolism and to identify genes which could be potentially regulated by ALR.

VI. SUMMARY

Liver regeneration is a multistep and well-orchestrated process which is initiated by injuries like tissue loss, infectious or toxic insults. It has been shown that expression of the hepatotrophic factor “Augmenter of Liver Regeneration” (ALR) is increased within liver regeneration as well as in liver diseases like cirrhosis and liver carcinoma. The aim of this thesis was to analyze how ALR is expressed, regulated and what impact an altered expression of ALR might have on the liver. Therefore, we investigated the expression of ALR in experimental models of liver injury which resemble hepatic patho-physiological disorders such as liver fibrosis and non-alcoholic steatohepatitis. ALR mRNA expression could be shown to be upregulated independent of different insults and is increased in HCC tissues compared to normal tissues. Furthermore, our results showed that IL-6 rapidly induces the mRNA expression and protein level of ALR in human hepatocytes and hepatoma cells, whereas IL-1β reduces the expression levels of ALR. No changes in ALR levels could be observed after stimulation with growth factors suggesting that ALR is regulated only by factors which are known to initiate the process of regeneration. Furthermore, we analyzed the promoter sequence of ALR and identified putative regulatory elements for FOXA2, IL-6 RE-BP and C/EBPβ. Using luciferase assays and EMSA, we demonstrated that both IL-6 RE-BP and C/EBPβ could not transactivate/bind to the ALR promoter. In contrast, FOXA2 has been found to regulate the expression of ALR, and this regulation was amplified by simultaneously activation using IL-6. Additionally, ALR promoter exhibits a nearly conserved Nrf2 binding site underlining a hepatoprotective function of ALR against oxidative stress. Using ALR-overexpressing HepG2 cells we could not detect an altered proliferation rate neither *in vitro* nor *in vivo*. ALR-overexpressing cells displayed an enhanced acquirement to adhere and a significant lower ability to migrate and invade compared to wild type cells. This finding is underlined by an immunohistochemical study which revealed that ALR expression in HCC is inversely correlated with tumor grading and angioinvasion. Furthermore, we demonstrated that ALR expression induces the expression of the adhesion molecule E-cadherin and reduces the expression of MMP-3 and SNAIL explaining the modulation of E-cadherin by ALR. In conclusion, this thesis was shown that ALR plays a role in hepatic regeneration independent of its cause and is regulated by upstream factors of liver regeneration like cytokines. Addionally, it was demonstrated that ALR expression counteracts the epithelial

mesenchymal transition (EMT) suggesting that application of ALR represents a promising therapeutical strategy for diseases in which EMT is involved.

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ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
ALR	Augmenter of liver regeneration
ALR-R	Augmenter of liver regeneration receptor
AP-1	Activator protein-1
APAAP	Alkaline Phosphatase-Anti-Alkaline Phosphatase
BDL	Bile duct ligation
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CAR	Constitutive androstane receptor
CCl_4	Carbon tetrachloride
CDH1	E-cadherin
cDNA	Complementary DNA
cis-acting	acting from the same molecule
Col-1	Collagen type I
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxyde
DNA	Deoxyribonucleic Acid
dNTP	Deoxinucleotide Triphosphate
DTT	Dithiothreitol
E-cadherin	Epithelial cadherin
ECL	Enhanced Chemiluminescence
ECM	Extracellular matrix
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic Acid
e.g.	"for example"
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal regulated protein kinase
ERV1	Essential for respiration and vegetative growth 1

et al.	“and others”
FAD	Flavin adenin dinucleotide
FC	Fold change
FOXA2	Forkhead box A2
g	Gram; Gravitational force
G-418	Geneticin
h	Hour
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HNF	Hepatocyte nuclear factor
HPO	Hepatopoietin
HSS	Hepatic stimulator substance
i.e.	“that is”
IFN-γ	Interferon γ
IGF	Insulin-like growth factor
IL	Interleukin
IL-6 RE-BP	Interleukin-6 response element binding protein
in vitro	lat.: „within the glass”
in vivo	lat.: „within the living”
JAB	c-Jun activation domain-binding protein
k	Kilo
kb	Kilo base
KC	Kupffer cell
kDa	Kilo-Dalton
KGF	Keratinocyte growth factor
L	Liter
LETF	Liver-enriched transcription factor
LPS	Lipo polysaccharide
LR	Liver regeneration
m	Milli (10^{-3})

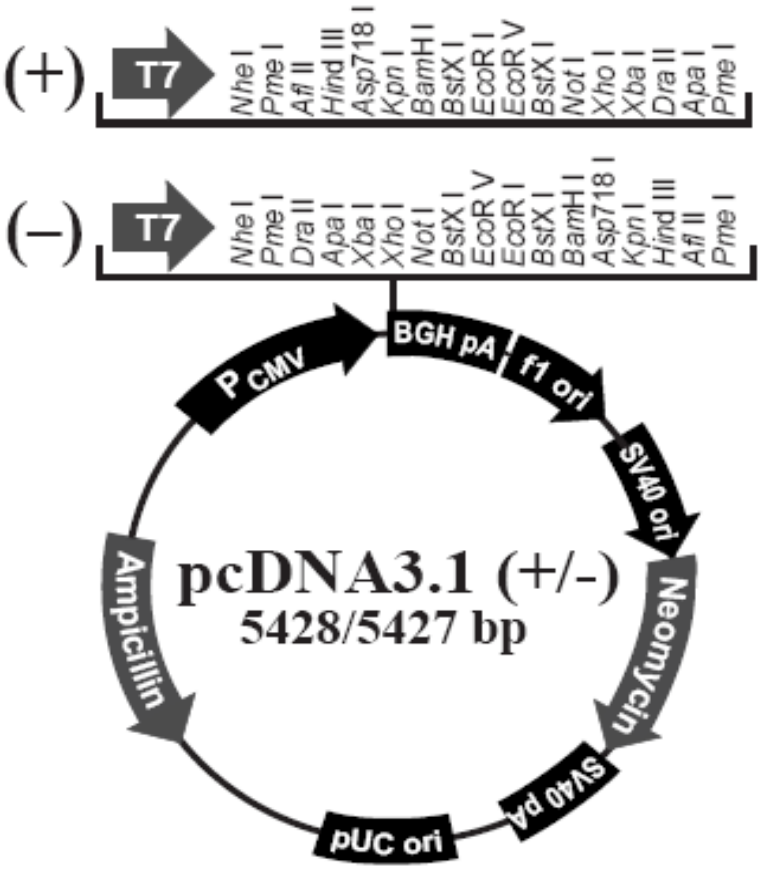
M	Molar
µ	Mikro (10^{-6})
mA	Milliampere
MCD	Methionine-choline deficient
MEK Kinase	MAP Kinase Kinase
MEKK1/2	MAP Kinase Kinase 1/2
min	Minute
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
mt-DNA	Mitochondrial DNA
mt-TFA	Mitochondrial Transcription Factor A
n	Nano (10^{-9})
NASH	Non-alcoholic steatohepatitis
Neo	Neomycin
NF-κB	Nuclear factor of kappa light chain gene enhancer in B-cells
nm	Nanometer
NASH	Non-alcoholic steatohepatitis
NP-40	Nonidet P-40
NPC	Non-parenchymal cell
nt	Nucleotide
OD	Optical density
ODC	Ornithine decarboxylase
p	Pico (10^{-12})
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Plateled-derived growth factor
PH	Partial hepatectomy
PVDF	Polyvinylidenfluorid
PXR	Pregnane X receptor
qRT-PCR	Quantitative Real-Time PCR
RLU	Relative luciferase unit

RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Rounds per minute
rRNA	Ribosomal RNA
RT	Room Temperature
SD	Standard deviation
SDS	Sodium Dodecyl Sulfate
sec	Second
siRNA	Small interference RNA
SP1	Stimulating protein 1
STAT	Signal transducers and activators of transcription
TEMED	N,N,N',N',-Tetramethylenediamine
TF	Transcription factor
TFIIB	Transcription factor B of Pol II
TGF-α	Transforming growth factor α
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumor necrosis factor α
trans-acting	acting from a different molecule
U	Unit
via	lat.: „By way of"
WB	Western Blot
wt	Wild type

APPENDIX

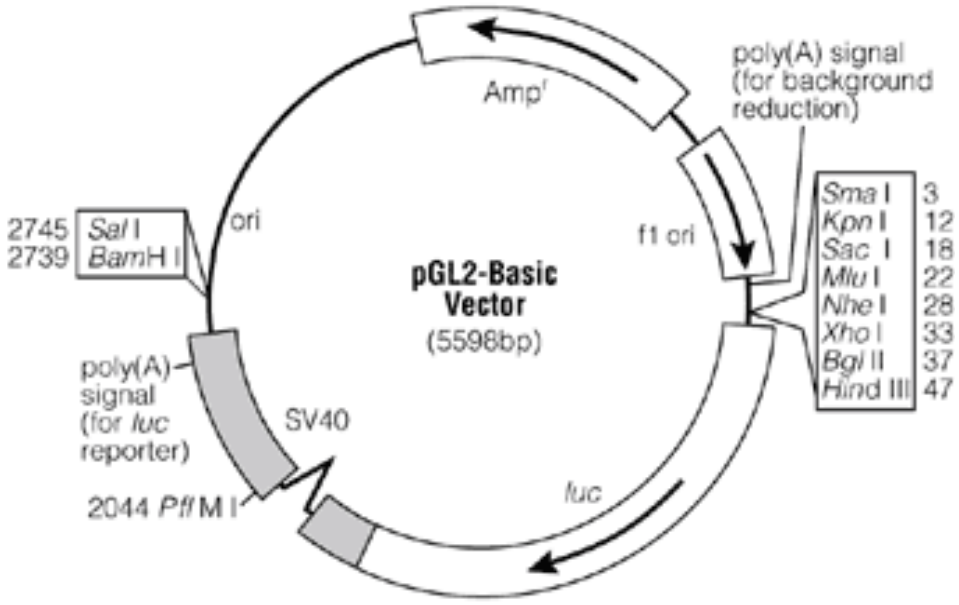
Map of pcDNA3.1 vector (Invitrogen)

The figure below summarizes the features of the pcDNA3.1 (+) and pcDNA3.1 (-) vectors. Into pcDNA3.1(+) vector ALR cDNA was subcloned to generate the stable ALR-overexpressing HepG2 cells.



Map of pGL2-Basic vector (Promega)

The figure below summarizes the features of the pGL2-Basic vector. The promoterless pGL2-Basic vector was used to perform the Luciferase Reporter gene Assays.



PUBLICATIONS & PRESENTATIONS

1. Publications

Dayoub R*, Thasler WE*, Bosserhoff AK, Singer T, Jauch KW, Schlitt HJ, Weiß TS. Regulation of polyamine synthesis in human hepatocytes by hepatotrophic factor augmenter of liver regeneration. *BBRC*. 2006 Jun; 345(1): 181-7.

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Thasler WE, **Dayoub R**, Mühlbauer M, Hellerbrand C, Singer T, Gräbe A, Jauch KW, Schlitt HJ, Weiß TS. Repression of cytochrome P450 activity in human hepatocytes *in vitro* by a novel hepatotrophic factor, augmenter of liver regeneration. *J. Pharmacol Exp Ther*. 2006 Feb; 316(2): 822-9.

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2. Posters and presentations

Wagner H, Thasler WE, **Dayoub R**, Bataille F, Hartmann A, Schlitt HJ, Weiß TS (2006). The hepatotrophic factor ALR is highly expressed in cirrhosis and hepatocellular carcinoma. *Eur Surg Res* 38 (S1), 43 O81; 17.-20.05.2006: 41st Congress of the European Society for Surgical Research, Rostock, Germany.

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