The role of liver regeneration-associated protein ALR, Augmenter of Liver Regeneration, in cholestatic liver diseases

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Dedicated to the memory of my Mother, Emtithal Saleh, and my Father, Mohammad Ibrahim.

"Do the best you can until you know better. Then when you know better, do better."

Maya Angelou (1928-2014)
American poet and civil rights activist.

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Chapter 1

Introduction

1. Introduction - Augmenter of Liver Regeneration (ALR)

Liver regeneration is a well-orchestrated process that is triggered by hepatocellular death and can be induced by toxins, viral infections or by tissue loss due to trauma or surgical resection [1]. The phenomenon of liver regeneration has been known since the myth of Prometheus who stole fire from Zeus and gave it to mankind. As a punishment, Zeus's eagle would feast on Prometheus's liver each day, and his liver would regrow at night until the eagle's return [2].

In the early twentieth century, scientists became interested in identifying the factors that regulate hepatic growth and regeneration. The first scientific evidence of the liver's ability to regenerate was confirmed by performing partial hepatectomy in rats and showing the restoration of the liver mass [3]. In 1975 LaBrecque and Pesch [4] prepared an extract from weanling rat livers and demonstrated that injecting this substance into partially hepatectomized rats stimulates liver regeneration and therefore named it Hepatic Stimulatory Substance (HSS). Several attempts were applied to further identify and characterize the molecule responsible for the stimulation of liver regeneration [5-7]. After progressive purification of HSS, a fraction with augmenting effect following a 40% hepatectomy in rats was obtained containing three protein bands (14, 30 and 35 kDa) [8]. The 30 kDa band was chosen for further sequencing and was published in 1994 by by Hagiya *et al.*, who first coined the term "Augmenter of liver Regeneration, ALR" [9]. The sequenced rat protein consisted of 125 amino acids with a molecular weight of 15 kDa under reducing conditions and 30 kDa under non-reducing conditions [9]. The published rat sequence was then corrected in 1995 [10] and proposed the presence of three ATGs and therefore opened the possibility of multiple isoforms of ALR. Afterwards, the sequence of human and mouse ALR was published in 1996 [11]. Moreover, Hepatopoietin (HPO) was cloned and sequenced from human fetal liver tissue in 1997 and was proved to be identical with ALR, therefore HPO is also referred to as ALR [12].

ALR protein is encoded by GFER (growth factor erv1 homolog of *s. cerevisiae*) gene that consists of 3 exons and 2 introns [10, 13] and can be regulated by different stimuli. In this review, we aimed to systemically review the literature discussing ALR structure, regulation, function and clinical importance. We included original articles, published in English, investigating ALR, HPO or GFER and presenting a clear indication of ALR isoform and molecular size. This is of note and critical, since there are some publications using the term "ALR" as a synonym for HSS, but using, in their

studies, a purified protein fraction (HSS) instead of a single sequenced protein (ALR) (e.g. [14-16]). Furthermore, a few reports indicated that HSS has a molecular size of 15 kDa but then claimed that ALR is different from HSS [17], without presenting a sequence for HSS. Due to the ambiguity about HSS protein/fraction, articles on HSS were only taken into consideration, if a clear indication of gene name and molecular size are given.

Although a considerable size of work has been devoted to summarizing the knowledge on ALR (see previous reviews [1, 18, 19]), an investigation of ALR expression in different organs as well as delineation between the ALR isoforms and the function of endogenous and exogenous ALR is still missing. Therefore, we aspired to revise the knowledge about ALR and gather evidence of the expression of different ALR isoforms and the functional role of the endogenous or exogenously applied ALR as well as to highlight the clinical importance of this protein.

2. ALR isoforms and structure

The first gene sequence of ALR was reported in 1994 [9] and was later corrected in 1995, where an additional G was added at position 266. This generated two additional in-frame ATG initiation sites and raised the possibility of different ALR isoforms [10]. Indeed, the expression of different ALR isoforms in mouse, rat and human organs has been thoroughly investigated and the existence of three isoforms of ALR has been shown (Table I). Furthermore, the sequence of full length ALR shows 74% homology to mouse ALR and 73% homology to rat ALR (Fig. 1A). The longest transcript of ALR encodes a 205 amino acid protein corresponding to a molecular weight of 23 kDa [20]. Moreover, the smallest transcript of ALR encodes a 125 amino acid protein corresponding to a molecular weight of 15 kDa (short form ALR, sfALR) [13]. The sfALR is 80 amino acids shorter than lfALR at the N terminus [20] (Fig. 1B). Those 80 amino acids are also referred to as “N80”. N80 includes (i) N-terminal (N-N80) domain which is described as an IDD (intrinsically disordered domain) and acts as a crucial targeting signal from the cytosol into the mitochondria [21]. (ii) C-terminal of N80 (C-N80) contains the CRAC motif (71-74 aa) (Fig. 1B). CRAC motif acts as a recognition site in the disulfide relay system of mitochondrial inter-membrane space (IMS). CRAC mediates the “recycling” of Mia40, an oxido-reductase, in charge of oxidative protein folding in the IMS [21-23]. Moreover, it was previously shown that mutating the cysteine residues (C71 or C74) in ALR’s

CRAC motif leads to preventing the reaction with Mia40 [23]. Nevertheless, Mia40 can still exchange electrons with ALR through the CEEC motif (142-145 aa) (corresponds to CXXC motif in Erv1, yeast homologue of ALR) (Fig. 1B) but this exchange is less efficient due to the “buried” structure of the CEEC motif. This argues for a specific hydrophobicity near the CRAC motif of long form ALR that drives the reaction between Mia40 and the N terminus of ALR [23]. In addition, Lu *et al.* have demonstrated that interaction between ALR and JAB1 occurs *via* amino acids 81-143 (corresponds to 1 to 63 aa in sfALR) [24] (Fig. 1B). Furthermore, the CEEC motif (Fig. 1B) neighbors the FAD binding domain and is affected by the reaction between Mia40 and FAD. Moreover, the “CEEC-FAD” region of ALR is referred to as “redox active center” that donates electrons to Mia40 [23].

Additionally, disulfide bonds formation in ALR (Fig. 1B) is of importance for ALR functionality. C95 and C204 are responsible of forming head-to-tail dimers of all isoforms of ALR and therefore stabilizing the structure [25]. The dimerization also facilitates the binding to the FAD molecule by non-covalent bonds [26, 27]. Other cysteins, C171 and C188 form “intramolecular” disulfide bonds that flank the FAD molecule. It is worth to mention that C154 and C165 (non-conserved cysteins) in human ALR (Fig. 1A) may account for oligomers formation ofter seen during the preparation of recombinant human ALR and mutating those cysteins prevented the formation of oligomers in recombinant human ALR [23].

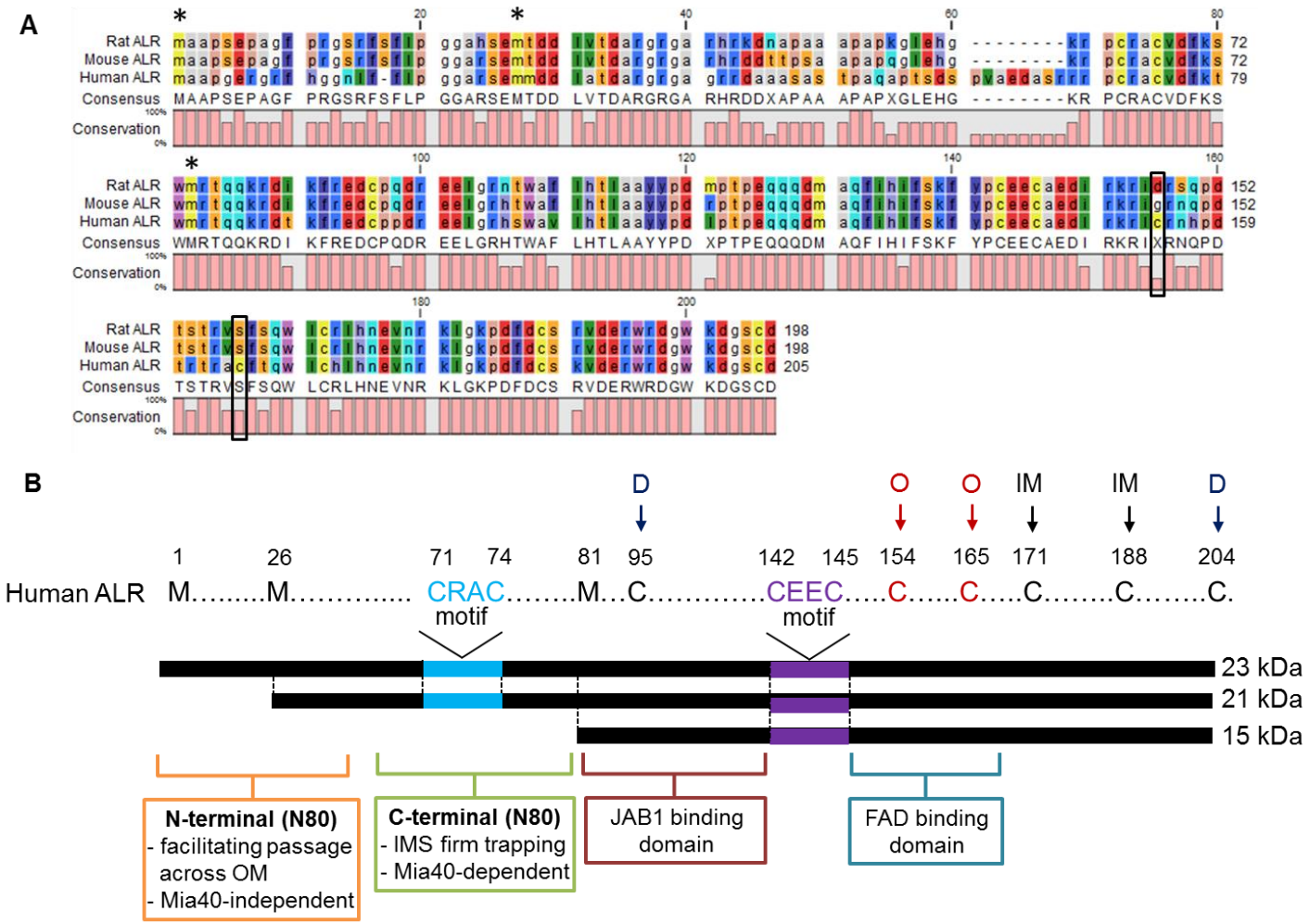

Fig. 1. (**A**) An illustration of ALR sequence homology in human, mouse and rat is shown. Sequences of rat, mouse and human ALR were obtained from Pubmed search tool. Dashes indicate absence of the amino acid, black frames the non-conserved cysteins and * the initial methionines. (**B**) The structure of human ALR isoforms. Long forms (23 and 21 kDa) of ALR posess additional 80 amino acids (N80) at the N terminus of the short form ALR (15 kDa). N80 harbors the IDD and CRAC motif, which are responsible for mitochondrial localization and Mia40 recognition. The CEEC motif (corresponds to CXXC in yeast homologue Erv1) and the neighboring FAD binding domain, which “recycle” Mia40 by catalyzing the electron transfer from ALR to Mia40. Disulfide bonds formed by cysteins are indicated by arrows; D: functional dimerization; O: Oligomerization (non-conserved cysteins) and IM: intramoleclular bonds. IDD: intrinsically disordered domain.

3. Regulation of ALR

The human ALR gene (GFER) (growth factor *erv1*-like) has been mapped on chromosome 16 next to the polycystic kidney disease gene (PKD1) [28]. Analysis of ALR gene (Fig. 2) has shown that it consists of 3 exons and 2 introns [10, 13]. ALR gene promoter has been shown to be TATA-less and to have some characteristics of an oncogene and a growth factor [29]. It further showed an initiator-like element with three tandem repeats (-66, -1) [29]. It was shown that regulatory elements of ALR promoter might exist in the region between -447 and -49bp. interestingly, it was found that a putative AP1/AP4 binding site (-375, -369) is responsible for negative transcriptional activity of ALR by AP1 (Activator Protein 1) in HepG2 cells and AP4 (Activator Protein 4) in COS-7 cells [30]. Additionally, it was reported that ALR expression could be attenuated by EGF (epidermal growth Factor) *via* C/EBPβ (CCAAT/enhancer binding proteins) binding to its binding site (-292, -279) within ALR promoter [31]. Analyzing a selected promoter region (-252, -49) from the transcriptional start site has shown that HNF4α (hepatocyte nuclear factor 4 alpha) binds to a binding site (-209, -204) and reduces ALR expression [32]. In addition, SP1 (specific protein 1) positively regulates ALR expression by binding to its binding site at (-152, -145) [32]. Interestingly, a recent study including the first intron of GFER gene (-733 to +527 bp) [33] investigated the potential regulatory elements in the introns that may affect the expression of different genes [34, 35]. It was demonstrated that HNF4α binds at (+421, +432) to ALR promoter and induces ALR expression [33] despite the presence of an upstream HNF4α binding site with repressing activity. However, the inducing effect of HNF4α is diminished upon SHP (Small Heterodimer Partner) activation e.g. by bile acids [33]. Exposure of hepatocytes to oxidative stress induces Nrf2 (nuclear factor erythroid 2-related factor 2), which in turn, by binding to an anti-oxidant response element (ARE) located between (-27, -19), induces ALR expression [36]. Interestingly, involvement of Nrf2 in ALR regulation was further confirmed by studies showing ALR induction upon treatment with known Nrf2 inducers such as tBHQ (tertiary butylhydroquinone), HBV (hepatitis B virus) infection [36], phenethyl isothiocyanate and sulforaphane [37, 38]. In addition, FOXA2 (Forkhead Box A2, also known as HNF3β) binds to a binding site (+276, +282) within ALR promoter inducing ALR expression and this binding is enhanced by IL6 [39]. Moreover, ALR is also regulated by Egr1 (early growth

response protein-1) that binds to its binding site (+304, +314) and induces ALR expression [33].

Furthermore, phenylbutyrate, a HDAC1 (histone deacetylase 1) inhibitor and demethylation reagent, was shown to induce the expression of ALR in the brain of a Huntington's disease mouse model [40]. This proposes the possibility that ALR could be regulated by histone acetylation and/or methylation [40] and therefore by epigenetic mechanisms [41, 42]. Additionally, MIF (macrophage Migration Inhibitory Factor), a pluripotent cytokine involved in cell cycle, cell proliferation and liver regeneration, has been demonstrated to increase the promoter activity of ALR [43]. Interestingly, a recent report has suggested that ALR may be regulated by miRNAs and showed that Toluene exposure up-regulated. miR7109-5p, which may regulate ALR [44].

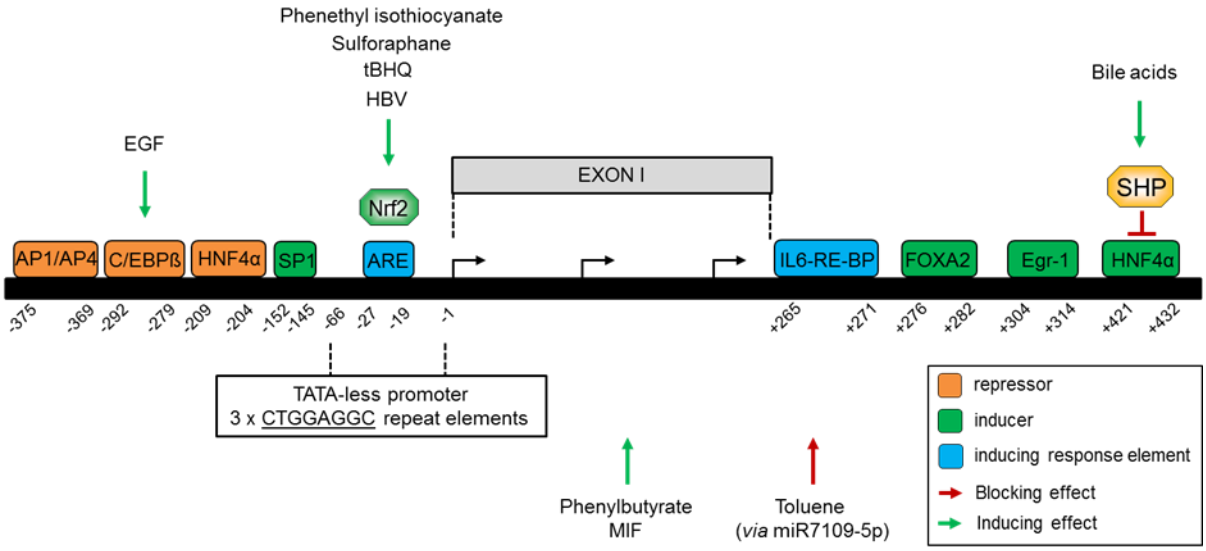

Fig. 2. Schematic illustration of ALR promoter region, transcription factors and response elements involved in ALR regulation. SP1, FOXA2, Egr-1 and HNF4α (+421, +432) are inducers of ALR expression, however, the induction by HNF4α (+421, +432) is reversed by SHP which is activated by bile acids. Furthermore, AP1/AP4, HNF4α (-209, -204) and C/EBPβ (induced by EGF) are repressing factors of ALR expression. IL6-RE-BP increases the activating effect of FOXA2 whereas ARE (-27, -19) induces ALR expression by binding of Nrf2 to this response element. The latter could be activated by HBV infection, tBHQ, sulforaphane and phenethyl isothiocyanate. Moreover, HDAC1-inhibitor phenylbutyrate and MIF activate the expression of ALR whereas Toluene exposure represses ALR expression *via* miR7109-5p. MIF: macrophage migration Inhibitory Factor, HDAC1: histone deacetylase 1, C/EBPβ: CCAAT/enhancer binding proteins.

4. Location of ALR

Expression of ALR mRNA was detected in several tissues and was found to be highly expressed in the liver, testis, brain and kidneys. Moreover, low levels of ALR mRNA were detected in the heart, muscles, spleen and lungs [10, 11]. Interestingly, it

was shown that mRNA expression of ALR in muscle tissues differs between males and females between 18 and 35 years of age [45]. A variety of studies were published analyzing the expression of ALR protein expression, including its isoforms and their respective cellular localization. Table (I) summarizes reports with detailed information about ALR isoform (molecular weight), organ and organelle affiliation. In the rat brain, ALR was found to expressed in its long forms (23 and 21 kDa) [46, 47] in neurons and glial cells located in the nucleus and mitochondria [47]. Furthermore, this was partly confirmed in a study demonstrating cytosolic expression of these isoforms in a human glioma cell line [48]. In addition, cytosolic expression of 15 kDa ALR (short form ALR, sfALR) was detected in human neuroblastoma cell lines [49]. All ALR isoforms (23, 21 and 15 kDa) have been identified in rat muscle tissue [46] and other studies performing electron microscopy demonstrated ALR localization in the mitochondrial inter-membrane space or associated to mitochondrial cristae in human muscle fibers [45, 50]. Moreover, all three ALR isoforms were detected in rat kidneys [46] and the short form ALR (15 kDa) expression was reported in the renal cortex as well as medulla of rat kidneys after injury [51, 52]. In addition, testis of mouse [11, 46] and rat [46] also showed positive expression of long form isoforms (23 and 21 kDa). However, Since ALR was first detected in the livers of weanling rats, the hepatic expression of ALR isoforms has been thoroughly investigated. The first report demonstrating ALR protein expression by Giorda *et al.* [11] showed western blots with ALR bands of 40, and 23 kDa under non-reduced conditions in rat and mouse liver tissue, and a 28 kDa band was also detected in rat liver. In the meantime, the expression of ALR under reducing conditions showed 2 bands (~23 and ~21 kDa) in mouse [36, 53, 54] and rat [46, 54-56]. Using non-reducing conditions revealed expression of 3 bands (40, 38 and 36 kDa) of ALR in rats [57] and in normal as well as disease-mouse models (NASH and ALD) [53, 57]. Interestingly, in a study that used liver-specific knock-out of ALR (by an albumin-Cre/LoxP system) it was shown that ALR protein expression, after being repressed at birth, reappeared in the livers of the KO-mice after 1 year. The same study indicated the presence of oval cells (hepatic progenitor cells, HPC) in the KO-mice [57] which might explain the recurred expression of ALR by activation of HPCs and consequently giving rise to cholangiocytes or hepatocytes [58]. Gandhi and his colleagues did not describe ALR expression in cholangiocytes [53, 57, 59], but based on the findings that ALR is expressed in epithelial cells of the human liver,

both in hepatocytes and cholangiocytes [36, 60, 61], a potential expression of ALR in HPC might be proposed. So far, expression of ALR in non-parenchymal liver cells could not be detected [36, 61] except noted in one study [59] with low expression of ALR in stellate, Kupffer and hepatic endothelial cells, which was not detectable after 2 days of cell culture. It is worth to mention that in human liver tissue, hepatocytes and hepatoma cell lines under reducing conditions ALR is expressed up to all three isoforms (23, 21, 15 kDa) and in several bands (40, 38, 36 kDa) under non-reducing conditions [57]. Furthermore, it was demonstrated that hepatoma cell lines (HepG2, Huh7 and Hep3B) express the 23 and 21 kDa ALR [33, 36, 54, 62] in the mitochondria as well as cytosol [54]. Moreover, primary human hepatocytes (PHH) were analyzed and showed expression of 3 isoforms of ALR in the cytosol, whereas 23 and 21 kDa were additionally found in the mitochondria [54]. Another study by Li *et al.* using human fetal, adult normal and tumorous liver tissue reported 23 kDa ALR in the cytosol and its 15 kDa isoform in the nucleus [63]. In addition, expression of ALR was investigated in normal [54, 61, 62], HCC (hepatocellular carcinoma) [61-64], CCC (cholangiocellular carcinoma) [61], and HBV positive [36] liver tissue and demonstrated reduced ALR expression in normal liver compared to diseased tissues. The expression of three isoforms of ALR (23, 21, 15 kDa) is attributed most likely to the presence of 3 initial in-frame ATG initiation sites in ALR gene [10]. Therefore it is of interest to find out why more or less isoforms of ALR are expressed in different tissues and under conditions of disease. It was hypothesized that in rats the 22 kDa isoform is post-translationally modified by dimerization to form the observed 40, 38 and 36 kDa bands under non-reducing conditions [59]. Recently published data by the same group showed two isoforms of ALR (22 and 20 kDa) under reducing conditions [53]. Considering the estimated molecular sizes on SDS-PAGE gells and the detection of 22 and 20 kDa bands of ALR under reducing conditions, the three bands (40, 38, 36 kDa), detected under non-reducing conditions, may be due to the formation of homo- and hetero-dimers of the 22 and 20 kDa ALR.

Finally, contradictory evidence about the expression of ALR isoforms may be due to technical limitations like resolution of gels used in SDS-PAGE or the specificity and sensitivity of anti ALR antibodies for immune detection. For example, our group demonstrated the expression of a 23 kDa ALR in normal, cirrhosis, HCC and CCC samples under reduced conditions, but also showed multiple unresolved bands under non-reducing conditions [61]. Nevertheless, more recent studies of our group using

various new developed anti-ALR antibodies have shown that ALR is expressed in human liver in 3 different isoforms: 23, 21 and 15 kDa [54, 62]. Therefore, the variable findings in detecting ALR isoforms might in part be attributed to reduction of protein lysates, the quality of gel resolution and the quality of antibodies.

Table (I): Summary of studies investigating expression of ALR protein isoforms in different organs

Organ	Species	Remarks	Cell type	Sub-cellular localization	Isoform (kDa)	References
Brain	Rat	diencephalon, cerebellum, brainstem	neurons, glial cells	nucleus, external envelope of mitochondria	23, 21	[47]
		brain, cerebellum	-	-	23, 21	[46](1)
	Human	-	neuroblastoma cell line SH-5YSY	Cytosol	15	[49](2)
		-	glioma cell line (T98G)	Cytosol	23, 21	[48]
Skeletal Muscle	Rat	-	-	-	23, 21, 15	[46]
	Human	mitochondrial myopathy patients	muscle fibers	mitochondrial innermembrane and cristae, cytosol	-	[45, 50]
Kidney	Rat	-	-	-	23, 21, 15	[46]
		induced expression of ALR in renal cortex and outer strip of outer medulla upon acute kidney injury (AKI) (Gentamicin- or I/R-induced injury)	renal tubular epithelial cells	after AKI ALR was detected in the cytosol and apical plasma membrane	15	[51, 52]
		induced expression of ALR after obstructive nephropathy			15	[65]
Testis	Mouse	-	-	soluble, insoluble fractions	40, 38, 23 23 (n.r.)(3)	[11](4)

		seminiferous tubules	spermatogonia , elongated sperm cells	mitochondrial inner membrane	23, 21	[46]
	Rat	-	-	-	23, 21	[46]
Blood	Mouse	blood plasma after hemorrhagic shock	-	-	ELISA[(5)]	[66]
	Rat	blood from liver (negative in venous and arterial blood)	-	-	23, 21	[46]
		serum after PCS (portacaval shunt), endotoxemia, gram-negative sepsis	-	-	ELISA[(5)]	[66]
		serum after partial hepatectomy	-	-	21	[56]
		serum after partial hepatectomy	-	-	ELISA[(5)]	[59]
		culture medium of hepatocytes upon TNFα, LPS, Actinomycin D or MMS treatment	-	-	ELISA[(5)]	[66]
	Human	serum of patients	-	-	ELISA[(5)]	[67]
		serum of patients	-	-	ELISA[(5)]	[60]
		-	leukemic T cells	-	23	[68]
		serum of patients with various liver diseases	-	-	ELISA[(5)]	[69]
		-	multiple Myloma cell line (U226)		23, 21	[70]

Liver	Mouse	-	-	-	40, 23 (n.r.)[3]	[11]
		-	-	-	23, 21	[36]
		normal, ALD, NASH liver tissue	hepatocytes	-	40, 38, 36 (n.r.)[3]	[57]
		normal and after EtOH feeding	-		40, 38, 36 (n.r.) and 22, 20 (r.)[3]	[53]
		-	-	-	22, 20	[54]
Liver	Rat	-	-	-	40, 28, 23 (n.r.)[3]	[11]
		-	-	-	23, 21	[46]
		-	hepatocytes, sparely in non-paranchymal cells	cytosol	40, 38, 36 (n.r.)[3]	[59]
		-	-	cytosol	23, 21	[56]
		-	-	-	23, 21	[55]
		-	-	-	22, 20	[54]
	Human		hepG2 cells	cytosol, perinucleus region	-	[24]
		human liver (fetal, adult and tumor)	-	cytosol, nucleus	23 (cytosol), 15 (nucleus)	[63]
		-	hepatocytes, cholangiocytes	cytosolic, perinuclear immunostaining	23	[61]
		-	cell line SMMC-7721	mitochondria	23 (mitochondria), 15	[71][6]

		-	hepatocytes, rarely in cholangiocytes and endothelial cells	cytosol, negative in nucleus	-	[60]
			HepG2	mitochondria, cytosol	23, 21	[33, 36, 54, 62]
		human liver samples (normal and HCC)	-	-	23, 21, 15	[62]
		human liver sections (normal, ALD, HBV postive)	hepatocytes and cholangiocytes	-	-	[36]
		human liver sections	hepatocytes	cytosol, barely in nucleus	-	[64]
Liver		human liver samples (normal, ALD, NASH, HCV)	-	-	40, 38, 36 (n.r.)[(3)]	[57]
			PHH	lfALR in mitochondria, cytosol, sfALR in cytosol	23, 21, 15	[54]
			Hep3B	-	23, 21	[54]
		normal liver tissue	-	-	23, 21, 15	[54]
		-	Huh7	-	23, 21	[54]
Cervix	Human	-	Hela cells	mitochondria	23	[72]

(1) Klissenbauer *et al.* have investigated the expression of ALR protein in different rat tissues, 23 kDa ALR was detected in heart. Moreover, 23 and 21 kDa ALR were detected in fat tissue, spleen and lungs.

(2) Plomino *et al*. (2009) have pointed out the possibility of ALR up-take upon treatment with recombinant ALR (15 kDa).

(3) (n.r.) stands for non-reduced conditions and demonstrated analysis of ALR dimers, whereas (r.) stands for analysis of ALR under reduced conditions.

(4) Giorda *et al.* (1996) investigated the expression of ALR in mouse spleen and demonstrated the expression of 23 kDa ALR in soluble and insoluble fractions.

(5) ALR detection using ELISA includes all isoforms of ALR.

(6) Cao *et al.* (2009) investigated the expression of ALR in SMMC-7721 cell line and detected 15 and 23 kDa ALR, the latter was localized in the mitochondria. Nevertheless, a recent report has suggested not considering the studies including BEL7402, SMMC7721 or SKHEP1 cell lines due to the highly possible contamination with Hela cells which was reported in different laboratories in Asia and Europe. [73].

5. Clinical importance of ALR

5.1. Mutations in GFER gene

Over the last years, the clinical importance of ALR has been underlined by studies that identified several mutations in ALR gene (GFER) (Fig. 3) leading to severe mitochondrial disorders. The first described mutation in ALR gene (GFER) (R194H) was reported to cause an infantile mitochondrial disorder [74]. Di Fonzo *et al.* reported three children who suffered from, among others, progressive myopathy, partial combined respiratory chain deficiency and development delays. Later on, the mutation (R194H) was characterized structurally and enzymologically in both lfALR (23 kDa) and sfALR (15 kDa) and was demonstrated to adversely affect the stability of both isoforms of ALR (characterized by weaker FAD binding and lower thermal stability) with minimal effects on its enzyme activity (characterized by minor changes to interaction with Mia40 and cytochrome C) [75]. However, in a more recent study it was emphasized that the mutated site (R194H in human ALR that corresponds to R182H in yeast Erv1) causes lower thermal stability and the quick release of the FAD cofactor but also pointed out the loss of enzyme activity (interaction with Mia40) [76]. Furthermore, North *et al.* [77] described a patient at the age of 18 months with neonatal onset of lactic acidosis, poor feeding, irritability, hepatomegaly, bilateral cataracts and adrenal insufficiency. This patient was diagnosed with a mitochondrial disorder, but the genetic etiology was unknown. At the age of 19 years old, the patient was presented with significant muscle weakness in the face, neck, trunk and proximal portions of all extremities [78]. Using “Next generation sequencing” (NGS) the R194H mutation as well as another mutation in GFER gene (Q125X; C.373C>T) (Fig. 3) were reported. This mutation (Q125X) was predicted to cause loss of normal function of ALR protein either by premature protein truncation or nonsense mediated mRNA decay. In addition, three further GFER mutations (C.259-25_259-24delCA, C.219delC, C.217delG) (Fig. 3) were identified in a study and were suggested to cause frame shift variations or truncation of ALR [79]. Interestingly, in a recent report, the authors suggested using rapid targeted genomics to analyze genetic defects in GFER gene in critically ill infants, since they identified R194H mutation in a 5 day old infant suffering from lactic acidosis and dysmorphic features [80]. This might open the possibility that investigating ALR mutation may contribute to adjustment of treatment plans and improvement in pre-symptomatic and prenatal testing.

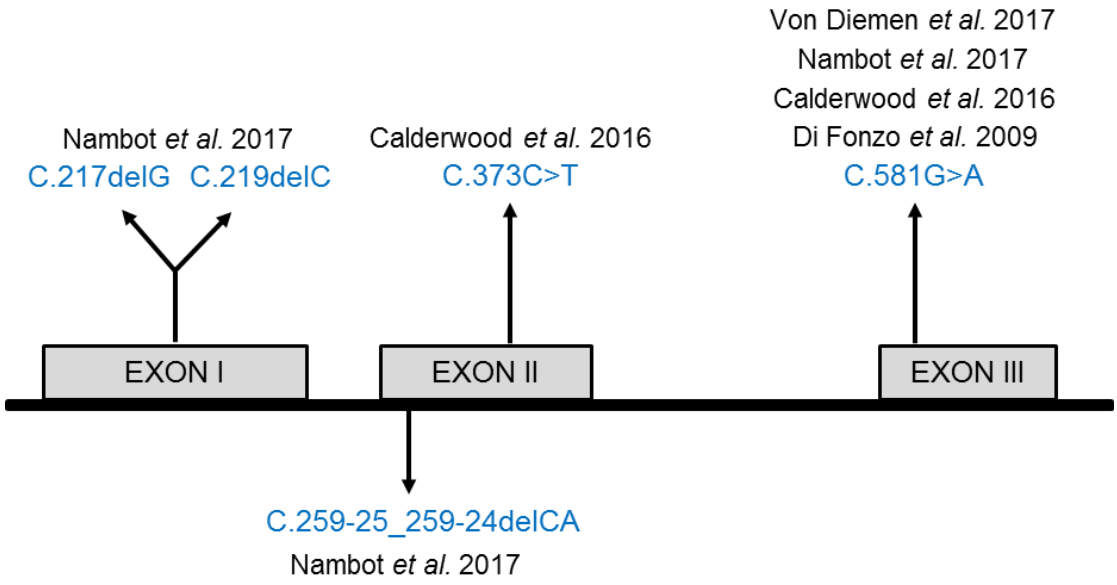

Fig. 3. Schematic illustration demonstrating the localization of reported mutations in patients. C.217delG and C.219delC (p.(Ala73Profs*77) and p.(Cys74Alafs*76) respectively) are of truncating nature, C.373C>T (p.(Gln125X)) causes either premature protein truncation or non-sense mediated mRNA decay. C.259-25_259-24delCA causes frame shift variations and C581G>A (p.(Arg194His)) causes loss of stability or enzyme inactivation. Adapted from Nambots *et al.* 2017 [79].

5.2. ALR and liver diseases

The mRNA and protein expression of ALR have been investigated in specific organs linked to various diseases and using different injury models. For example, performing an ischemia/reperfusion injury (IRI) in mice and a hypoxia/re-oxygenation (H/R) model induced expression of 15 kDa of ALR in the kidneys [51, 52, 65, 81]. Acute kidney injury (AKI) also induced the expression of ALR [51, 52]. Moreover, the expression of ALR was reported to be induced in muscle biopsies from patients with mitochondrial myopathies [50] and was shown to be inversely correlated with the tumor grading in samples from patients with colorectal carcinoma and colon cancer cell lines [82].

ALR expression was thoroughly analyzed in samples from patients with various liver diseases (see summary in Table II) and was found to be strongly increased in diseases associated with normal or abnormal regeneration like cirrhosis or HCC. Several reports have shown that the expression of ALR is increased in liver tissue from patients with HCC [60-62, 83], CCC [61] and cirrhosis [61, 69, 84]. Interestingly, ALR protein expression in HCC correlates inversely with tumor grading and its angio-invasion [62, 83] and this was found presumably to be attributed to expression of the cytosolic 15 kDa ALR isoform (sfALR) [62]. Furthermore, ALR mRNA [54] and protein [57] expression were shown to be reduced in the liver of patients with non-alcoholic steatohepatitis (NASH). In hepatic samples from patients with alcoholic

liver diseases (ALD) ALR expression was either reduced [57] or not altered [36] compared to normal liver tissue. Moreover, ALR mRNA expression has been investigated in liver tissues from patients with cholestatic liver diseases and was found to be significantly reduced (chapter 5).

Infection with hepatitis B virus leads to induction of several genes among them target genes of Nrf2 such as ALR [36]. Hence increased ALR mRNA and protein expression was found in HBV positive liver tissues [36]. Furthermore, ALR serum levels were found to be induced in patients with HBV-related acute, chronic and severe hepatitis or cirrhosis [69]. Serum levels of ALR might be considered a candidate for prognosis of HBV related-liver failure since it was found to be induced in patients with HBV-related ACLF (acute-on chronic liver failure) compared to normal patients and to be also induced in the surviving ACLF patients compared to dead patients [85]. In addition, patients with improved hepatic failure (HF) show increased serum levels of ALR protein compared to patients with deteriorating HF [60]. On the other hand, HCV infection impairs induction of Nrf2 [86] and therefore as expected, ALR expression is not altered in HCV positive liver tissue [36, 57]. Interestingly, hepatic ALR expression is low or even unchanged after insulting the liver by fat, alcohol or HCV infection, respectively. But as a consequence of these insults progression to cirrhosis or even tumorigenesis occurs with induction of ALR expression as part of the regenerative response that occurs regardless of the underlying etiology [61].

5.3. ALR and *in vivo* models

Multiple studies have reported the use of mouse and rat models that mimic diseases conditions in order to explore the effect of ALR in those diseases. It was reported that ALR serum levels are elevated in rats after PCS (portacaval shunt) surgery, endotoxemia or sepsis, and in a mouse model of hemorrhagic shock [66]. Furthermore, ALR serum levels are increased in rats after tissue loss by partial hepatectomy (PH) [56, 59] and return to normal levels after 12 hours [59] or 36 hours [56]. On the contrary, hepatic level of ALR after PH remains unchanged (after 40% PH) or decreases (after 70% PH) [59]. ALR liver-specific knock-out mice have been shown to endure accelerated steatosis and to develop liver tumors after 1 year [57]. The same mouse model was used to investigate the alcohol-induced injury and was shown to accelerate injury [53]. Interestingly, using hepatocytes transfected with ALR (23 kDa) expression plasmid have shown that endogenous ALR enhances the effect

of anti-tumor agents [87] and reduces IRI injury in steatotic liver [88]. Further, the subcutaneous-injected hepatoma cells expressing sfALR (15 kDa, [62]) or lfALR (23 kDa, [83]) showed reduced tumor growth in mice due to their reduced metastatic ability. Injecting ALR (23 kDa) expression plasmid into mice with CCl_4-induced injury suppresses apoptosis and attenuates the acute injury [84] which is underlined with a study that reported increased ALR serum levels after CCl_4-injury in rats [85]. Interestingly, ALR-knock-in and knock-out models in Zebrafish have been established and demonstrated the functional role of ALR in vertebrate hepatogenesis [89]. It is worth to mention that the physiological effects of ALR in spermatogenesis were investigated by an ALR transgenic mouse model driven by the human TSPY (testis-specific protein, Y-encoded) promoter that allows the transgene to be specifically activated in the testes [90]. This study highlighted that temporal expression of ALR is required for normal testicular development and spermatogenesis.

Table (II): Summary of studies investigating hepatic expression of ALR in various liver diseases with the methods used in the investigation.

Liver disease	Study subject	Target	Methods	Reference
Hepatocellular carcinoma (HCC)	HCC *vs.* corresponding surrounding non-tumorous tissue (n=4), HCC *vs.* surrounding cirrhotic tissue (n=5)	mRNA	PCR	[61]
	normal *vs.* HCC (n=5)	protein	IHC, wb	
	normal (n=10) *vs.* HCC (n=20)	serum	ELISA	[60]
	HCC *vs.* HF and normal	mRNA	PCR	
	HCC *vs.* HF	protein	IHC	
	normal (n=1) *vs.* HCC (w/o angioinvasion n=2 and with angioinvasion n=2)	protein	Wb	[62]
	HCC liver sections (n=53) (high vs. Low expression of ALR)	protein	IHC	
	non-tumorous (w/o HBV nor alcohol, n=5) *vs.* tumorous (w/o HBV nor alcohol, n=12)	protein	IHC	[36]
	HCC (n=22) *vs.* para-cancerous liver tissue (n=22)	mRNA	PCR	[64]
	HCC *vs.* para-cancerous liver tissue	protein	IHC	
	HCC with low ALR (n=19) *vs.* HCC with high ALR (n=25)	mRNA	PCR	[83]
	HCC w/o angioinvasion (n=29) *vs.* HCC w angioinvasion (n=14)	mRNA	PCR	
	HCC w/o angioinvasion (n=4) *vs.* HCC w angioinvasion (n=4)	protein	IHC, wb	
Cholangiocyte carcinoma (CCC)	CCC *vs.* corresponding non-tumorous tissue (n=5)	mRNA	PCR	[61]
	normal *vs.* CCC (n=3)	protein	IHC, wb	
Hepatitis B virus (HBV) infection	normal (n=10) *vs.* Chronic HBV infection (n=20)	serum	ELISA	[85]
	HCV (n=10) *vs.* HBV (n=10)	mRNA	PCR	[36]
	non-tumorous with HBV (n=8) *vs.* tumorous (with HBV, n=7)	protein	IHC	
	non-tumorous w/o HBV (n=5) *vs.* non-tumorous with HBV (n=8)	protein	IHC	
	normal (n=10) *vs.* Acute (n=5), chronic (n=30) and severe (n=15) hepatitis B infection	serum	ELISA	[69]

Hepatitis C virus (HCV) infection	normal (n=5) *vs.* HCV infection (n=7)	protein	Wb	[57]
Non-alcoholic fatty liver diseases (NAFLD)	normal (n=5) *vs.* NASH (n=5)	protein	Wb	[57]
	normal (n=17) *vs.* Steatosis (n=27) and NASH (n=29)	mRNA	PCR	[54]
Alcoholic liver diseases (ALD), alcoholic steatohepatisis (ASH)	non-tumorous with alcohol (n=4) *vs.* HCC with alcohol (n=14)	protein	IHC(1)	[36]
	normal (n=5) *vs.* ALD (n=5)	protein	Wb	[57]
Cholestatic liver diseases (CLD)	normal (n=13) *vs.* Cholestasis (n=45)	mRNA	PCR	chapter 5
Hepatic failure (HF) and Acute liver diseases (ALD)	improved HF (n=6) *vs.* deteriorating HF (n=12)	serum	ELISA	[60]
	normal (n=10) *vs.* HBV related ACLF (n=40)	serum	ELISA	[85]
	survival ACLF patients (n=10) *vs.* Dead ACLF patients (n=10)	serum	ELISA	
	normal (n=2) *vs.* Acute liver failure (n=2)	protien	Wb	[84]
Cirrhosis	normal (n=18) *vs.* Cirrhosis (n=25, with n=14 alcohol-induced cirrhosis, n=7 HCV-induced cirrhosis and n=4 autoimmune diseases induced cirrhosis)	mRNA	PCR	[61]
	normal *vs.* Alcohol-induced cirrhosis (n=5)	protein	IHC, wb	
	normal (n=10) *vs.* Cirrhosis (n=30)	serum	ELISA	[69]
	normal (n=2) *vs.* Liver cirrhosis (n=2)	protein	Wb	[84]

Wb = western blot; PCR = qRT-PCR; IHC = immunohistochemistry

(1) Dayoub *et al.* (2013) investigated ALR protein expression in liver sections from patients with HCC and non-tumorous tissue (with or without HBV or alcohol) by IHC and quantitatively evaluated the intensity of anti-ALR staining (from 0 to 3).

6. Function of ALR

Different isoforms of ALR have been associated with different subcellular localizations and therefore specific functions. Several studies have investigated the role of the 23 kDa ALR (long form, lfALR) and the 15 kDa ALR (short form, sfALR) related to different diseases and pointed out the beneficial effects of the individual ALR isoforms. In the next paragraph we summarize the studies that investigated the over-expression of lfALR and sfALR as well as the studies that explored the impact of ALR silencing (effects all isoforms) on cellular functions.

6.1. The functional role of mitochondrial lfALR (23 kDa)

As implied before, the 23 kDa of ALR is located in the mitochondria (see the location of ALR) and the mitochondrial targeting of ALR is attributed to the IDD domain in the initial 80 amino acids (N80) at the N terminal of lfALR (see the structure of ALR). In mammalian cells, lfALR is a part of the disulfide relay system that recycles Mia40 (mitochondrial import and assembly 40) in the inter-mitochondrial space (IMS) while transporting proteins into the IMS. Mia40 interacts with its substrates by its CPC motif [91, 92] and oxidizes those substrates which leads to substrate folding and trapping in the IMS. Furthermore, reduced Mia40 is re-oxidized -“recycled”- by the CRAC and CXXC motif in ALR, which itself obtains electrons from cytochrome C [22, 23, 93]. For more detailed information about the role of ALR in mitochondrial biogenesis we would like to refer to the recent review article of Kallegri *et al.* 2014 [94] and Erdogan *et al.* 2017 [95].

Besides its role in mitochondrial protein import, there has been contradictory evidence about the role of ALR in the maturation and export of cytosolic Fe-S cluster proteins. Fe-S proteins were reported to be involved in enzymatic catalysis, DNA synthesis and repair, iron homeostasis and heme synthesis [96]. Lange *et al.* [97] have suggested that ALR, may be involved in the maturation of cytosolic Fe-S proteins. The authors claimed that ALR interacts with Atm1 (an ABC transporter in the inner membrane of the mitochondria) and facilitates the export of Fe-S proteins to the cytosol [97]. However, later it was suggested that, in yeast cells, ALR plays neither a direct nor an indirect role in the maturation of Fe-S cluster [98]. Nevertheless, a recent study in mammalian cells has pointed out the role of ALR in exporting MitoNEEt to the outer mitochondrial membrane (OMM). MitoNEEt is a

Fe-S protein that is synthesized in the mitochondrial matrix. Upon synthesis, MitoNEEt translocates through the inner membrane (IM) of the mitochondria by ABCb7 and then through the IMS by ALR to the OMM where it contributes to cell proliferation [72]. However, whether ALR is involved in the maturation and export of other Fe-S protein still requires further investigations.

Furthermore, endogenous lfALR (23 kDa) has been investigated in various disease models and Table (III) summarizes the studies about over-expression of lfALR and its role in different diseases. Expression of lfALR promotes liver growth during hepatogenesis [89], reduces fibrotic injury [99, 100], protects against radiation-induced oxidative stress [71] and attenuates acute liver injury by acetaminophen or CCl_4 [84, 101]. The latter has been attributed to autophagy activation (by promoting p62 degradation and LC3II conversion) and apoptosis repression [84, 101]. In addition, lfALR has been reported to reduce ER stress [102] and promote the anti-tumor effects of doxorubicin by increasing its cellular retention [87]. Furthermore, lfALR has been shown to increase the pluripotency of embryonic stem cells (ESC) by preserving the mitochondrial integrity and function in ESCs [103] On the other hand, it was shown that lfALR does not contribute to reducing the bile acid-induced apoptosis [104].

It is worth to mention that over-expression of lfALR in the testis of the mice (transgenic mouse) was reported to cause abnormal spermatogenesis and reduced fertility in those mice [90]. Interestingly, it was suggested that continuous over-expression of lfALR in mammalian cells leads to the accumulation of lfALR not only in the mitochondria but also in the cytosol [105]. Since no exclusive mitochondrial localization of lfALR is demonstrated in previously mentioned studies, the question rises whether the previously reported effects e.g. altering gene expression by endogenous lfALR may be due to its cytosolic accumulation rather than its mitochondrial localization.

Table (III): Summary of the studies investigating the over-expression of lfALR (23 kDa)

Vehicle	Host	Disease / Model	Function	Molecular explanation	Reference
pcDNA3	injection into rat caudal vein	liver fibrosis (induced by colchicine)	protects against fibrosis, enhances regeneration	reduces the expression of TIMP1, collagen I and collagen III	[99]
pcDNA3.1/ Myc-HisB vector in Lentivirus	cell lines (HepG2, SMMC-7721, BEL-7402, 293FT, L02)	radiation (oxidative stress)	protects against radiation-induced oxidative stress, improves cell viability	reduces ROS production, mitochondrial dysfunction, apoptosis; reduction of cytochrome C release and caspase 3/7 activity	[71]
pcDNA3.1/ Myc-HisB	transfection into Huh7-NTCP cells	cholestasis (GCDCA)	no reduction of caspase 3/7 activity	-	[104]
MSCV-IHRES-GFP retrovirus	transfection into mouse ESC (Embryonic stem cells)	-	promotes ESC pluripotency, preserves mitochondrial structural integrity and function	reduces expression of Drp1 (Dynamine-related protein 1)	[103]
mRNA of ALR	injection into 1-2 cell stage Zebrafish embryos	hepatogenesis	promotes liver growth	-	[89]
Minicircle vector or pcDNA3.1	transfection into SMMC-7721, HSC-T6 (rat hepatic stellate cells), injection into rat tail	liver fibrosis	reduces the fibrotic injury, increases the survival rate *via* deactivation of HSC	-	[100]
Lentivirus	injection into mouse tail	acute liver injury by acetamiophen (APAP) over-dose (oxidative stress)	protects liver by activation of autophagy and reduction of apoptosis	increases p62 degradation and LC3II conversion, reduces release of AIF, cytochrome C and caspase 3 activation	[101]

pcDNA3.0	injection in mouse tail or transfection into AML12 cells	CCl_4-induced acute liver injury	protects against acute liver injury by autophagy induction, suppression of apoptosis, promoting proliferation	induces p62 degradation, LC3II conversion and levels of ATG5, ATG7 and Beclin-1.	[84]
p-Adxsi-Flag tagged vector	transfection into HepG2 cells (xenografts in mice)	HCC and anti-tumor agents (doxorubicin)	sensitizes hepatocytes to anti-tumor effects of doxorubicin	reduces expression of ABCB1 and ABCG2 (MDR proteins) partially by blocking Snail/Akt pathway	[87]
pcDNA 3.0	transfection into HepG2 cells	lipotoxicity (palmitic acid treatment)	reduces ER stress by reducing Ca^{+2} release to the cytosol	reduces IP3R expression, induces IP3R and Bcl2 interaction, reduces mitochondrial Bax expression	[102]
flag tagged pAdxsi into Adenovirus	transfection into mice or HepG2 cells	IRI liver injury and steatosis	reduces inflammation and necrosis caused by IRI	enhances oxygen consumption and ATP production	[88]
-	transfection into MHCC97H and xenografts in mice	HCC and metastasis	reduces HCC metastasis	blocks ERK pathway	[83]

6.2. The functional role of cytosolic sfALR (15 kDa)

Short form ALR (sfALR, 15 kDa) is located in the cytosol [54] and presumably in the nucleus [63], due to the lack of the IDD (responsible for mitochondrial targeting) in the initial 80 amino acids (N80) found only in lfALR (see ALR structure). However, the potential effects (summarized in Table IV) and molecular functions (Fig. 4) of sfALR have been investigated in different models. sfALR was shown to induce the response of hepatocytes to IL6 signaling by induced STAT3 (signal transducer and activator of transcription 3*)* phosphorylation, which increases the expression of e.g. acute phase proteins (APP) like fibrinogen β (FGB) and haptoglobin (HP) [106]. In addition, the activation of STAT3 might account for the anti-apoptotic effect of sfALR by reduced DR5 (death receptor 5) expression and caspase 3/7 activity during cholestasis (chapter 5) and free fatty acid (FFA)-induced toxicity [54]. Interestingly, the reduced DR5 expression by sfALR may also be due to reduced CHOP (C/EBP-homologous protein, an inducer of DR5 expression [107]) which is repressed upon attenuation of FFA-induced ER-stress by sfALR [54].

In addition, sfALR was attributed to have anti-metastatic effects by reducing the migratory and invasion activity of hepatoma cells and as well as maintaining an epithelial like state with less signs of aggressive proliferation. sfALR mediates this by reducing Snail expression which induces the expression of epithelial cell markers like CDH1 (E-Cadherin) and ZO-1 (Zona Occludens-1) [62]. Moreover, the hepatic metabolic capacity is affected by endogenous sfALR by reducing cholesterol catabolism into bile acids due to reduced CYP7A1 (rate-limiting enzyme in bile acid synthesis) expression and reduced HNF4α expression (chapter 5). Additionally, lipid metabolism genes (CPT1α, FABP1, ELOVL6) were reported to have altered expression in sfALR over-expressing cells under steatotic conditions [54], which could be due to repressed activation of JNK [54, 108] or the activation of miRNA expression e.g. miR122 [108].

The interactions of sfALR protein with other molecules have been also reported in multiple studies (Fig. 4). Endogenous sfALR interacts with JAB1 (c-Jun-activating domain binding protein 1) [24, 109-111] in the nucleus and might include the whole CSN (COP9 signalosome, consisting of COP9 and JAB1) [111]. This interaction results in AP1 activation [109, 111] in a MAPK- and JNK-independent manner [24]. Furthermore, it was shown that ALR activation of AP1 is dependent on the CXXC motif in ALR [109]. Interestingly, a study in hematopoietic stem cells (HSC) demon-

strated that binding of ALR to JAB1 blocks JAB1 interaction with $p27^{kip1}$, which promotes cell cycle arrest and restricts the abnormal proliferation of HSC and the subsequent exhaustion [110]. This was confirmed by ALR knock-down that caused reduced $p27^{kip1}$ nuclear retention and therefore a hyper-proliferative response of the HSC [110]. Moreover, cytosolic sfALR interacts with MIF (macrophages migration inhibitory factor) [43], which also results in AP1 activation due to MIF's ability to block AP1 [43], this interaction might as well block MIF effects on p53 and results in tumor suppression [112] which may contribute to the described anti-metastatic effects of ALR [62]. Furthermore, ALR interacts with thioredoxin (TRX) [113] by its CXXC motif (C142-C145) and contributes to the maturation of viral proteins [113]. This interaction results in oxidized TRX which therefore induces AP1, c-Jun and NFκB activity [26]. Additionally, ALR interacts with the pro-apoptotic protein BNIPL (Bcl-2/adenovirus E1B) and leads to growth inhibition in hepatoma cells (BEL-7402) [114].

Table (IV): Summary of the studies investigating over-expression of sfALR (15 kDa)

Host, traget cell	Vehicle	Disease/ model	Function of sfALR	Molecular explanation	Reference
infection of BS-C-1 cells (Kidney)	pTargeT vector controlled by T7 promoter in vaccinia virus	-	interacts with thioredoxin	interacts with thioredoxin (TRX) by the CXXC motif	[113]
transfection into COS-7 and HepG2 cells	pAS vector	-	increases c-Jun phosphorylation and AP-1 activation	interacts with JAB1 (c-Jun activation domain-binding protein 1) in the nucleus, activates AP1 independent of MAPK, activates c-Jun independent of ERK1/2 and JNK pathways	[24]
transfection into COS-7 cells	pCMV-Myc vector		activates AP1	interacts with JAB1 and activates AP1 *via* the CXXC motif	[109]
transfection into BEL-7402 cells	pGEX-5x-1 vector	-	interacts with BNIPL (apoptotic protein)	-	[114]
transfection into yeast cells and HepG2 cells	pAS2-1, pACT2 or pFlag-CMV-2 vector	-	activates AP1	interacts with MIF in the cytosol	[43]
transfection into COS-7 and HepG2 cells	pCMV-Myc vector	-	activates AP1	interacts with JAB1 which activates AP1 in a MAPK independent manner	[111]
transfection into COS-7 cells and Yeast Y190 cells	pcDNA3.1A, pEGFP, pGEX-4T, pFlag-CMV or pAS vectors	-	activates AP1, NFκB and c-Jun phosphorylation	interacts with thioredoxin	[26]
Intravenous injection into rats after CCl_4-induced liver injury	pcDNA3.1 vector	acute hepatic injury, hepatic failure	increases cell proliferation and survival rates	-	[115]

transfection into HSC (hematopoietic stem cells)	murine stem cell virus (MSCV)	-	restricts abnormal proliferation of HSC	interacts with JAB1 and inhibits the JAB1-$p27^{kip1}$ interaction	[110]
transfection into HepG2 cells	pcDNA3.1 vector	HCC and metastasis	reduces cell motility and EMT (epithelial mesenchymal transition)	reduces Snail expression, induces E-Cadherin and ZO-1 expression	[62]
mice on MCD diet	pcDNA3.1 vector	NAFLD	reduces the severity of fatty acid injury	suppresses JNK phosphorylation and fatty acid synthesis genes, induces miR122 levels	[108]
transfection into HepG2 cells	pcDNA3.1 vector	acute phase response (APR)	induces expression of APP: fibrinogen β and haptoglobin	induces STAT3 phosphorylation	[106]
transfection into HepG2 and Huh7 cells	pcDNA3.1 vector	NAFLD	attenuates ER stress and lipoapoptsis, alters lipid metabolism genes, reduces TAG levels	reduces DR5, Bax and CHOP expression and JNK phosphrylation, increases ATP synthesis, alters expression of CPT1α, FABP1 and ELOVL6	[54]
transfection into HepG2 cells	pcDNA3.1 vector	cholestasis	reduces bile acid synthesis and bile acid-induced apoptosis	preserves STAT3 activation, reduces HNF4α and DR5 expression	chapter 5

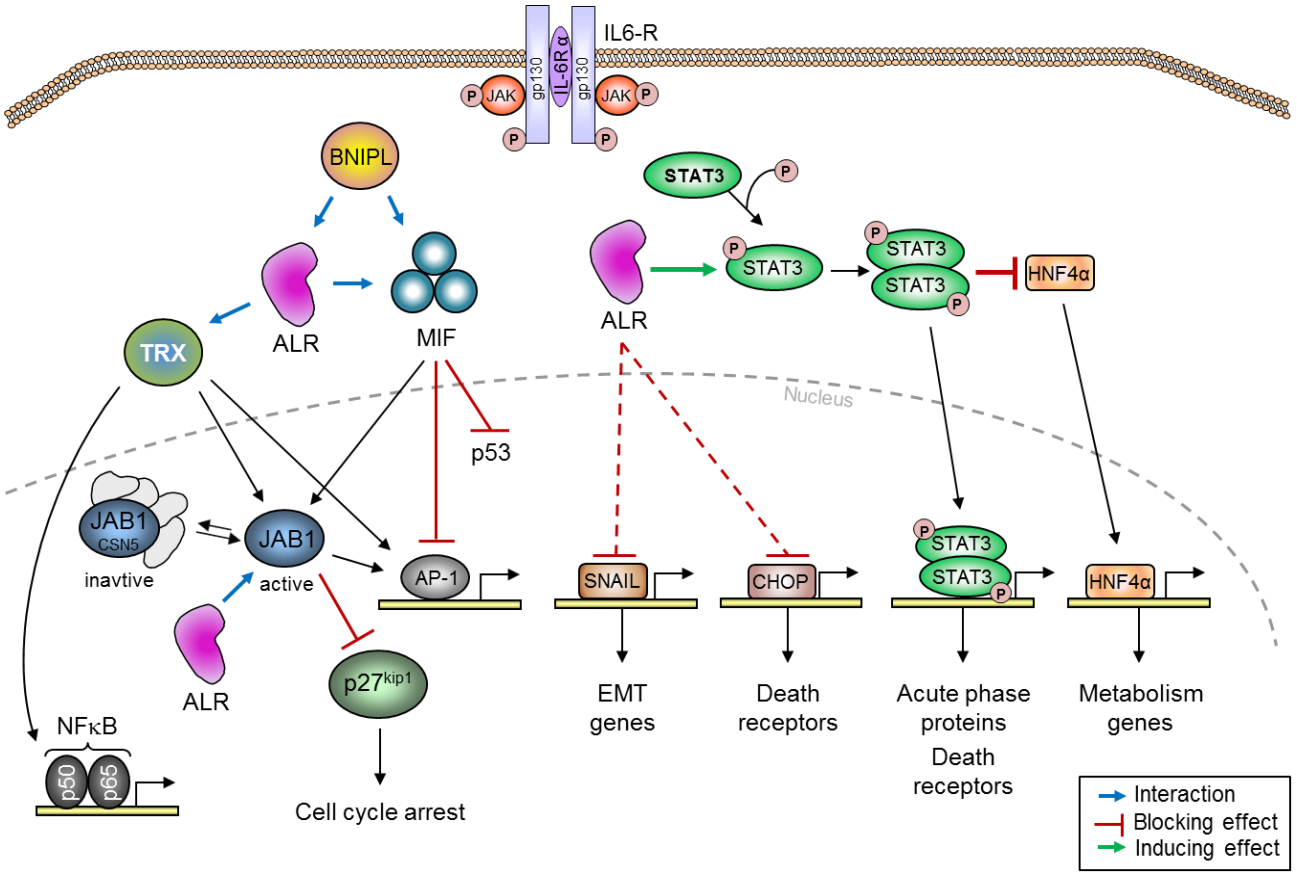

Fig. 4. An illustration of how endogenously expressed sfALR affects hepatocytes. ALR promotes IL6 signaling by increasing STAT3 phosphorylation and thereby regulating STAT3 target genes. Furthermore, STAT3 activation by sfALR blocks HNF4α and reduces its trans-activating effects. sfALR also reduces the expression of SNAIL and CHOP (by reducing ER stress) and thereby their target genes. ALR interacts with MIF and blocks its inhibitory effects on p53 and AP1, which results their activation and tumor suppression by p53. ALR also interacts with thioredoxin and promotes AP1 and NFκB activation. Moreover, ALR interacts with JAB1 in the nucleus and thereby activates AP1. MIF and thioredoxin bind to JAB1 to prevent JAB1 interaction with $p27^{kip1}$ which increases the nuclear retention of $p23^{kip1}$ and therefore induces cell cycle arrest at G1 phase. In addition, the pro-apoptotic protein BNIPL interacts with ALR and MIF and regulates cell survival. BNIPL: Bcl-2/adenovirus E1B, MIF: macrophage migration inhibitory factor, CHOP: C/EBP-homologous protein, STAT3: signal transducer and activator of transcription 3, TRX: thioredoxin, JAB1: c-Jun-activating domain binding protein 1, CSN5: COP9 signalosome subunit 5, HNF4α: hepatocyte nuclear factor 4 alpha, AP1: Activator protein 1.

6.3. ALR silencing

In order to further clarify the role of ALR for cellular functions, many studies have explored the effect of ALR silencing (all isoforms) and monitored the outcome of its absence on the development of various diseases. Table (V) summarizes the main finding of the studies that inspected the effect of silencing ALR.

In the kidney, it was shown that ALR knock-down had no effect on HK-2 cells viability [81, 116] but aggravated the H/R injury by increased ROS production and apoptosis due to the activation on AMPK/mTOR pathway [116]. Morevoer, ALR silencing inhibited cytokine production and the inflammatory response after H/R injury by blocking MAPK pathway [81]. Furthermore, it was suggested that ALR silencing decreases NKκB nuclear translocation in kidney cells [81]. On the contrary, it was reported that over-expression of sfALR (15 kDa isoform) does not affect NFκB's nuclear translocation in hepatocytes during cholestasis (chapter 5), which might argue for organ specific effects of ALR or more likely for a specific role of different ALR isoforms due to their location and function.

The anti-oxidative role of ALR was further evidenced in human derived glioma cells (T98G) which showed that silencing of ALR reduces the expression of anti-oxidative protein clusterin and induces the activity of caspase 3 and 9 [48]. Additionally, ALR silencing sensitizes HepG2 cells to radiation-induced oxidative stress [71]. ALR silencing suppresses liver growth [89], reduces cell viability and induces apoptosis in rat hepatocytes [117] as well as HepG2 cells [118]. In the liver, after partial hepatectomy ALR silencing resulted in reduced compensatory hepatocellular proliferation, increased pro-apoptotic proteins and caspase activity [55]. Furthermore, liver specific ALR knock-out (KO) mice developed steatosis accompanied by altered expression of lipid metabolism genes, enhanced ROS production, mitochondrial dysfunction, increased Bax expression and NK as well as $CD8^+$ cell recruitment to the liver [57]. Interestingly, it was shown that ALR-KO mice develop liver tumors within 6 months of age and therefore ALR silencing links non-alcoholic liver disease to hepato-carcinogenesis [57]. The same group showed later that ALR-KO mice suffer accelerated alcohol injury, which they attributed to changes in lipid metabolism genes, altered alcohol metabolism by ADH1 (alcohol dehydrogenase 1), ALDH1 (aldehyde dehydrogenase 1) and CYP2E1 (cytochrome P450 2E1) and augmented oxidative stress upon injury [53]. In addition, it was shown that diminishing ALR expression enhances free fatty acid-induced ER stress and lipoapoptosis [102] and

reduces the anti-tumor effects of doxorubicin due to enhanced expression of export transporters and therefore reduced cellular retention of doxorubicin [87]. In contrast to Tang *et al.* [118], who showed reduced tumor growth of HepG2 in nude mice after ALR silencing, others reported promoted cell growth and EMT (epithelial mesenchymal transition) [83]. It is worth to mention that silencing ALR in HSC (Hematopoietic stem cells) caused a hyper-proliferative response due to reduced JAB1 binding and therefore reduced nuclear retention of $p27^{kip1}$ [110]. In addition, ALR silencing in mouse embryonic stem cells (ESC) increases Drp1 (Dynamine-related protein 1) expression and activates mitochondrial autophagy, which results in enhanced apoptosis of ESC [119]. Interestingly, a chemical screen identified a molecule that is capable of inhibiting ALR activity, MitoBloCK-6 [120]. MitoBloCK-6 was shown to induce apoptosis by cytochrome C release in ESCs but not in differentiated cells which suggests a vital role of ALR in the ESC homeostasis [120].

Table (V): Summary of studies investigating ALR silencing (all ALR isoforms)

Organ	Host	Vehicle	disease/ model	Effect of ALR silencing	molecular explaination	Reference
Liver	rat heptocytes	antisense oligo-nucleotide	hepatocytes survival	reduced cell viability and induced apoptosis	induced rounding, detachment of cells, increased LDH and cytochrome C re-lease, Caspase 3 activity, reduced ATP	[117]
	HepG2, L02 cells	lentivirus with shRNA	radiation (oxidative stress)	inhibited cell viability, induced sensibility to radiation (HepG2), minimal effects on normal cell line	-	[71]
	HepG2 cells, xenografts in nude mice	siRNA in pSIALR-A plasmid	HCC growth	inhibited growth in HepG2 and xeno-grafted HCC tumors in nude mice	-	[118]
	Zebrafish embryos	Morpholino antisense oligonucleotides	hepatogenesi s	suppressed liver growth	reduced hepatocyte proliferation without affecting apoptosis	[89]
	HepG2 cells (and xenografts in mice)	shRNA	doxorubicin treatment	reduced caspase 3 activity due to reduced cellular retention of doxorubicin	induced snail and therefore ABCB1 and ABCG2 (export pump) expression	[87]
	HepG2, Huh7, Huh7-sfALR cells	siRNA	-	reduced expression of the three isoforms of ALR	-	[54]
	HepG2 cells	PLVX- shRNA-plasmid	lipotoxicity (palmitic acid treatment)	induced ER stress by increasing Ca^{+2} release into the cytosol	induced expression of IP3R and Bax, reduced expression of Bcl2	[102]
	HepG2, MHCC-97H cells xeno-grafts in mice	shRNA	HCC and metastasis	promoted cell growth and migration *in vivo* and *in vitro*	induced EMT in hepatoma cells due to activated ERK pathway	[83]
	rat	adenovirus with	partial	reduced hepatocyte proliferation and	-	[55]

		shRNA	hepatectomy	polyamine synthesis, increased pro-apoptotic proteins and caspase activity		
Liver	mice	liver-specific ALR knock out (Albumin Cre-lox)	Steatosis	ALR-KO in mice results in accelerated hepatic steatosis after 2 weeks, development of liver tumors after 6 months of age	increased ratio of Bax /Bcl2, induced IL1β, TNFα and IL6, increased recruitment of NK cells and $CD8^+$ cells, increased ROS, mtDNA damage, reduced ATP levels and expression of ACACA, SREBP1c, CPT1α and PPARα	[57]
	mice	liver-specific ALR knock out (Albumin Cre-lox)	alcohol induced injury	accelerated alcohol induced liver injury in ALR-KO mice	reduced expression of FASN, ACACA, SREBP1c, CPT1α (lipid metabolism genes), and activity of ADH1, ALDH1 and CYP2E1 (alcohol metabolism genes), augmented oxidative stress, fibrosis, inflammation by EtOH feeding	[53]
Stem cells	murine ESC (Embryonic stem cells)	FG12-Lentivirus–GFP–shRNA	-	loss of mitochondrial function in ESC, reduced proliferation and enhanced apoptosis	triggered mitochondrial autophagy or mitophagy by increased Drp1 (Dynamine-related protein 1)	[119]
	hematopoietic stem cells, bone marrow transplantation in mice	FG12-Lentivirus–GFP–shRNA	-	hyper-proliferative response and exhaustion in hematopoietic stem cells (HSC)	reduced p27kip1 expression through its binding to JAB1	[110]
	ESC, Hela cells, and Zebrafish embryos	MitoBloCK-6 („ALR inhibitor“)	-	induced apoptosis by cytochrome C release and impaired cardiac development in Zebrafish embryos	Inhibition of ALR activity	[120]
	HK-2 cells	lentivirus with shRNA/ALR	H/R injury	no effect on viability, inhibited inflammatory response and cytokines production after H/R injury	blocked MAPK pathway and decreased nuclear translocation of NFκB	[81]

	HK-2 cells	lentivirus with shRNA/ALR	I/R kidney injury	no effect on cell viability, increased autophgy and ROS production *in vitro*	activated AMPK/mTOR pathway	[116]
Glioma cells	T98G cells (human)	siRNA	H_2O_2 (oxidative stress)	reduced expression of clusterin (anti-oxidative protein) and Bcl2, induced Caspase 3 and 9 activity	-	[48]
Myeloma cells	U266 cells	shRNA	-	promoted apoptosis	induced Bax expression, reduced Bcl2 expression and IL6 synthesis	[70]

6.4. The functional role of exogenous ALR (recombinant ALR, rALR)

Although the current emphasis is placed on the development of “gene therapy” for human diseases, the production and use of recombinant proteins have a great impact on genetic diseases due to improvements in production of these biomolecules as well as their availability [121]. Therefore in this paragraph we will summarize the effects and action of recombinant ALR (rALR) application in different injury models (Table VI).

Studies in the kidneys showed that application of rALR attenuates the injury after IR (ischemia reperfusion) [51, 81, 122-124] and after gentamicin application [52]. It was suggested that the activation of PI3k/Akt (phosphoinositide 3-kinase/ Akt) by ALR reduces p53 phosphorylation and increases Bcl2/Bax ratio and therefore reduces apoptosis [123]. Furthermore, ALR signaling represses the activity of NFκB in the kidneys [122, 124, 125] which reduces the production of pro-inflammatory cytokines like IL6 and IL1β [122, 124]. ALR was also reported to reduce renal fibrosis by modulating the TGFβ1 signaling and reducing EMT in renal tubular cells [125]. Moreover, in human derived glioma [48] and neuroblastoma cells [49] it was shown that rALR attenuates H_2O_2-induced injury by reducing reactive oxygen species (ROS) and cytochrome C release, which leads to reduced apoptosis. In addition a similar outcome of ALR is seen on concanavalin A-treated human lymphocytes [126] with reduced apoptosis. Interestingly, rALR could also contribute to survival of transplanted hepatocytes [127] and liver after transplantation [128] by modulating the inflammatory response as well as reducing the production of pro-inflammatory cytokines [127, 128]. It is worth to mention that application of rALR upon liver transplantation reduces the renal tubular injury by repressing the expression of the pro-inflammatory TNFα [129]. In addition, Adam *et al.* have shown that application of rALR reverses diabetes in the pancreas of fetal rats upon transplantation [130].

Previous reports have focused on analyzing the impact of rALR on the liver and how rALR transduces its signals in liver cells (Fig. 5). The augmenting effect of rALR in liver regeneration was proposed by reduced activity (killing ability) of natural killer (NK) cells after rALR treatment [67, 131] and subsequently enhanced survival of hepatocytes after injury [67]. Further, rALR treatment of Kupffer cells has been shown to activate NFκB signaling and to induce the synthesis of IL6 and TNFα [132]. The authors found an ALR specific- and cholera toxin sensitive- receptor on Kupffer cells may be responsible for rALR effect on liver regeneration after 40% partial

hepatectomy [132]. On the other hand, there have been contradictory reports about the expression of an ALR receptor (ALR-R) on hepatocytes [59, 133]. Whereas on rat hepatocytes, no ALR receptor could be found so far [59], on human hepatoma cells it was shown that ALR binds to its receptor (ALR-R, not sequenced yet) and activates the EGF receptor on the cytoplasmatic side [133]. This results in the activation of EGF-R pathways *via* MAPK activation [134] and subsequently AP1 activation [109]. Upon activation of the MAPK pathway, rALR increases polyamines synthesis, a prerequisite for liver regeneration, by increasing the expression of c-myc and ODC (ornithine-decarboxylase), the rate-limiting enzyme of polyamine synthesis [135]. Moreover, after rALR treatment, primary human hepatocytes show increased proliferation, but reduced expression and activity of various cytochrome P450 [136], which might be mediated by activated NFκB ([136], chapter 6). Later on, it was reported that rALR activates the PI3k/Akt pathway and was shown to induce different kinetics from EGF [137]. Interestingly, a recent study have investigated the role of rALR in modulating the IL6 signaling pathway and the acute phase response (APR) [106] by reducing the activation of STAT3 and subsequently the expression of acute phase response proteins (APP) [106].

The anti-apoptotic effect of rALR has been demonstrated in different injury models in the liver. It was shown that rALR reduces caspase 3/7 activity after free fatty acid- [54] and bile acid-induced injury (chapter 6), which may be due to reduced expression of Bax and therefore preserving mitochondrial integrity [54]. Furthermore, rALR reduces EtOH- and death ligand-induced apoptosis by reducing cytochrome C release [138]. This was further confirmed in a study showing that rALR reduces cell damage after partial hepatectomy by inducing the expression of anti-oxidative clusterin and anti-apoptotic Bcl2 and suggested that rALR boosts liver regeneration by attenuating apoptosis rather that inducing proliferation [56]. In addition, besides its immune-modulating effect, anti-apoptotic effects of rALR may account for the reduced liver injury upon IRI [139] and bile duct ligation (BDL), a common model of cholestatis [140].

Table (VI) Summary of the studies investigating the role of recombinant ALR (15 kDa)

Organ	Effect of rALR	Remarks	Reference
Liver	reduces NK ability of direct and indirect killing	-	[131]
	no receptors of ALR on rat hepatocytes	-	[59]
	ALR has a receptor on human hepatocytes (ALR-R)	detection of a 75 kDa band interacting with rALR	[133]
	ALR-R binds to ALR-R, activates EGF-R signaling and subsequently MAPK pathway	-	[134]
	protects against liver injury by inhibition of hepatic natural killer cell activity	-	[67]
	activates AP1	activates MAPK pathway	[109]
	induces polyamines synthesis	activates EGF-R signaling pathway and c-myc expression	[135]
	reduces expression, activity of cytochrome P450 enzymes	activates NFκB, reduces CAR expression	[136]
	does not induce DNA synthesis in isolated rat hepatocytes	-	[117]
	reduces apoptosis (extrinsic induced, EtOH).	-	[138]
	affects its receptor and activates PI3k/Akt signaling pathway	-	[137]
	promotes liver regeneration in rats after 40% partial hepatectomy (PH) (not in 70% PH rats)	binds to its cholera toxin-sensetive receptor on Kupffer cells, activates NFκB, induces the expression and release of TNFα, IL6 and iNOS	[132]
	anti-apoptotic effects, reduced cell damage after PH	reduces Bax expression, induces Bcl2, clusterin expression	[56]
	promotes hepatocytes regeneration, attenuates acute injury, suppresses immunological responses, improves survival after	reduces pro-inflammatory cytokines (IL1β and TNFα),	[127]

Liver	hepatocytes transplantation	reduces apoptosis and $CD4^+$ recruitment	
	Reduces hepatic ischemia reperfusion injury (IRI)	reduces the serum transaminases, reduces apoptosis and infiltration of inflammatory cells (neutrophiles)	[139]
	attenuates the injury after obstructive cholestasis (BDL)	reduces hepatic transaminase, preserves mitochondrail DNA	[140]
	induces survival and reduced organ rejection after liver transplantation in rats	reduces IL2 and IFNγ expression, promotes T cell apoptosis, reduces TNFα expression, induces IL10 expression	[128]
	reduces acute phase response and the expression of acute phase proteins	reduces STAT3 phoshorylation	[106]
	reduces lipoapoptosis	reduces expression of pro-apoptotic protein Bax, reduces caspase 3/7 activity, no effect on DR5 expression	[54]
	reduced bile acid synthesis and bile acid-induced apoptosis	activates NFκB, reduces CAR expression, reduces CYP7A1 expression, reduces caspase 3/7 activity	chapter 6
Kidney	attenuates renal tubular epithelial cell injury after liver transplantation in rats	reduces TNFα production, increases regenerating cells	[129]
	reduces gentamicin induced injury, improves renal dysfunction	-	[52]
	reduces tubular injury after I/R injury in rats	-	[51]
	attenuated renal dysfunction and injury after IRI in rats	activates PI3k/Akt pathway, increases Bcl2/Bax ratio, represses p53 activation	[123]
	attenuates the inflammatory response after hypoxia / reperfusion (H/R) injury	reduces expression of IL1β and IL6 by down-regulation of TLR4 and NFκB expression	[122]
	reduces renal fibrosis	represses TGFβ-receptor II, alleviates TGFβ1 signaling, reduces Smad2 and NFκB phosphorylation, reduces EMT (epithelial mesenchymal transition) in renal tubular cells	[125]
	attenuates I/R injury in rats	reduces apoptosis and cytokine production, inhibits NFκB	[124]

	improves renal dysfunction after acute kidney injury (AKI)	-	[81]
Glioma cells (human)	reduces H_2O_2-induced oxidative stress in human glioma cells	reduces ROS production	[48]
Neuro-blastoma	induces cell viability after H_2O_2 injury and preserves mitochondrial integrity	reduces cytochrome C release	[49]
Pancreas	reverses diabetes in fetal rodent upon pancreas transplantation	-	[130]
Human Lymphocytes	represses concanavalin A-induced apoptosis in activated lymphocytes	reduces PARP cleavage, decreases Bax/Bcl2 ratio, reduces cytochrome C release	[126]

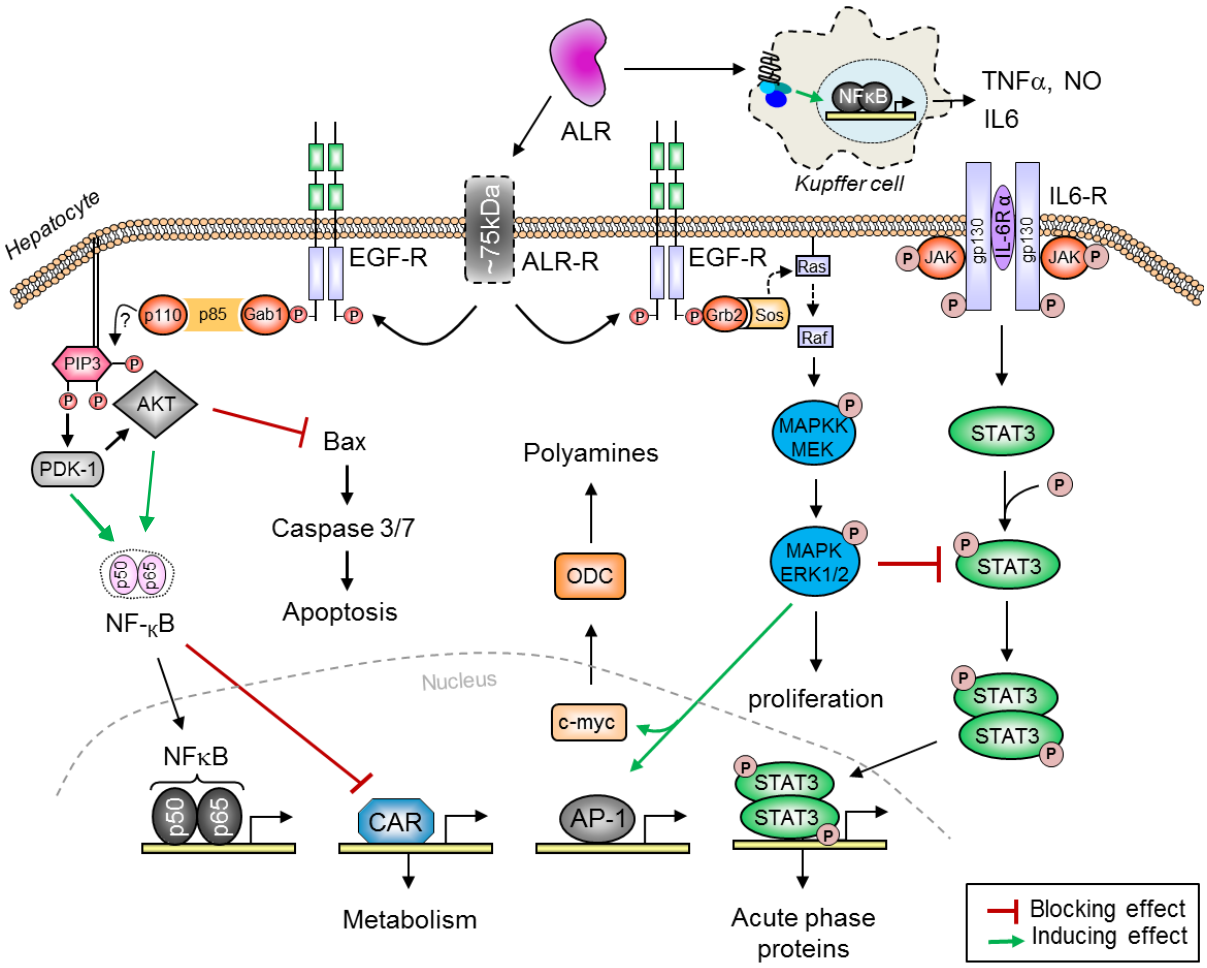

Fig. 5. An illustration of how exogenous ALR affects the liver. ALR binds to its cholera toxin-sensitive G-protein-coupled high affinity receptor on Kupffer cells to activate NFκB and increases the expression and release of NO, TNFα and IL6. Furthermore, ALR binds to its receptor (approx. 75 kDa) on hepatocytes and after phosphorylation of EGF-R on cytoplasmatic Tyrosin sites, EGF-R signaling pathways PI3k/Akt and MAPK are activated. Activation of PI3k/Akt induces NFκB, attenuates Bax expression leading to reduced apoptosis and represses CAR expression and subsequently less target gene expression. Moreover, phosphorylated MAPK reduces STAT3 activation, activates AP1 and c-myc expression, which leads to increased ODC expression and therefore enhanced polyamine concentrations. NO: Nitric oxide, TNFα: Tumor necrosis factor alpha, IL6: Interleukin 6, ODC: Ornithine decarboxylase, PI3K/Akt: phosphoinositide 3-kinase/ Akt, CAR: Constitutive androstane receptor, AP1: Activator Protein 1, PDK1: phosphoinositide dependent kinase-1.

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Chapter 2

Aims of the thesis

Cholestatic liver injury is initiated with the hepatic accumulation of bile acids and leads to hepatocyte injury that manifests in ER-stress, oxidative stress and apoptosis. Furthermore, the inflammatory response during cholestasis is involved in modulating the injury. If cholestasis persists, it might result in liver fibrosis, cirrhosis and eventually liver failure. Augmenter of Liver Regeneration (ALR) has been proved to have beneficial effects on cells under oxidative stress or apoptosis. Moreover, ALR is a liver-specific proliferation-augmenting factor. Therefore, the targeting of such an organ-specific hepatoprotective factor could have great clinical importance in reducing the detrimental effects of bile acids on hepatocytes during cholestasis.

The aim of this thesis was to shed some light on the regulation of ALR expression under circumstances of cholestasis in an *in vitro* model notably by bile acids as well as cholestasis-related cytokines and to identify the factors controlling ALR expression on a transcriptional level. In addition, a cohort of liver samples collected from patients with cholestasis was analyzed to determine the expression of ALR under cholestatic conditions.

Furthermore, we aimed to elucidate the effect of ALR on the causes and outcomes of cholestatic injury and to explain the mechanism through which ALR attenuates bile acid-induced injury.

The goals of this study could be summarized in the following points:

Analysis of ALR regulation by bile acids, bile acid-induced transcription factors and cholestasis-related cytokines such as IL1β:

- Regulation of ALR expression by bile acids through binding motive “Bile Acid Response Element” (BARE).
- Regulation of ALR expression by bile acid-induced transcription factor Egr-1.
- Regulation of ALR expression by HNF4α1 and SHP interplay.
- Regulation of ALR expression by IL1β *via* transcription factor SP1.
- Role of Egr-1 in regulating ALR expression in the presence of IL1β.

Analysis of how over-expressed sfALR (short form ALR) affects cholestatic liver injury:

- Impact of sfALR on *de novo* synthesis of bile acids by classical and acidic pathway: regulation of CYP7A1 and CYP27A1 expression and activity by cytosolic sfALR.

- Role of cytosolic sfALR on expression of transcription factor HNF4α.
- Analysis of sfALR impact on bile acid-induced apoptosis and effector caspases in liver cells.
- Regulation of death receptor expression by endogenous sfALR: DR4, DR5 and FASR.
- Analysis of ALR expression in liver tissue from cholestasis patients.

Analysis of how exogenously-applied rhALR (recombinant human ALR, 15 kDa) impacts cholestatic liver injury:

- Impact of rhALR on *de novo* synthesis of bile acids by classical and acidic pathway: regulation of CYP7A1 and CYP27A1 by rhALR.
- Regulation of transcription factors FXR, SHP and HNF4α by rhALR.
- Regulation of the nuclear receptor CAR (CYP7A1 inducer and mediator of apoptosis) by rhALR.
- Effect of rhALR on bile acid-induced apoptosis and expression of DR5.

Chapter 3

Regulation of ALR by bile acids

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Abstract

Bile acids (BA) are signaling molecules that activate nuclear factors and g-protein coupled receptors signaling to maintain metabolic homeostasis. However, accumulation of toxic BA promotes liver injury by initiating inflammation, inducing apoptosis and causing oxidative stress leading to cirrhosis and liver failure. Augmenter of Liver Regeneration (ALR) is a hepatotrophic growth factor with anti-apoptotic and anti-oxidative properties that has been shown to improve mitochondrial and hepatic functions in rats after bile duct ligation. In the current study we aimed to analyze the regulation of the pro-survival protein, ALR, under conditions of cytotoxic concentrations of BA. Promoter studies of ALR (−733/+527 bp) revealed potential binding sites for various transcription factors like Egr-1, HNF4α and two bile acid response elements (BARE). Using a full-length and several truncated promoter constructs for ALR we analyzed promoter activity and showed that BA reduce ALR promoter activity whereas Egr-1 transfection induces it. EMSA and supershift analysis confirmed the specific binding of Egr-1 to its response element within ALR promoter and this binding was reduced upon simultaneous stimulation with BA. We also showed that ALR promoter activity and protein expression are induced by HNF4α1 and repressed by SHP. In conclusion, these results indicate that BA negatively regulate ALR expression by SHP activation.

1. Introduction

Bile acids are synthesized and excreted from hepatocytes into the bile and then into the duodenum where they act as amphiphilic agents to facilitate lipids absorption [1]. Bile acids have also gained great attention over the last two decades as important signaling molecules that regulate different metabolic process [2].

The synthesis of bile acids is the main catabolism pathway for cholesterol and is tightly controlled by regulating the expression of the synthesis enzymes [3]. CYP7A1, also known as cholesterol 7α-hydroxylase, is the rate-limiting enzyme of Bile acids *de novo* synthesis, and is regulated by bile acids through “Bile Acid Response Element I” (BARE I) and BARE II [4, 5]. Several transcription factors bind to BAREs, for instance, HNF4α (hepatocyte nuclear factor 4α) binds to BARE II activating CYP7A1 expression. Upon accumulation, bile acids bind to the nuclear receptor FXR (Farnesoid X Receptor) which activates SHP (Small Heterodimer Partner). Subsequently, SHP interacts with HNF4α and represses its transactivating action [6, 7].

Gallstones, local tumors and genetic deficiencies in bile acids transport proteins amongst others are common causes of cholestatic liver diseases [8, 9]. By definition, cholestasis represents pathophysiologic syndromes defined as an impaired bile flow from the liver. As an outcome, toxic bile acids accumulate and promote hepatocytes injury followed by the development of liver cirrhosis and liver failure [10]. The molecular mechanism of injury by bile acids has been thoroughly analyzed. bile acids activate the expression of Egr-1 (early growth response protein-1), a zinc finger transcription factor, that activates the expression of chemotactic agents leading to accumulation of inflammatory cells and subsequently liver injury [11]. Furthermore, bile acids induce apoptosis in a death receptor-dependent mechanism [12, 13] and manipulate the mitochondrial membrane stability by oxidative stress and ROS (reactive oxygen species) production [14]. Moreover, *in vitro* and *in vivo* studies have demonstrated that toxic bile acids induce the Nuclear factor erythroid-2 like factor 2 (Nrf2) target genes to enhance cell survival [15].

Augmenter of Liver Regeneration (ALR) is a heptotrophic growth factor that was found to be expressed in parenchymal liver cells (hepatocytes and cholangiocytes) [16]. Moreover, ALR is a member of the ALR/Erv1 protein family with a flavin adenine dinucleotide (FAD)-linked sulfhydryl oxidase activity [17]. It was reported that the promoter region of ALR is TATA-less and has some characteristics of an oncogene

and growth factor [18]. ALR possess proliferation-augmenting properties which are attributed to its ability to activate the epidermal growth factor receptor (EGF-R) and the subsequent MAPK (mitogen-activated protein kinase) /Erk (extracellular signal-regulated Kinase) [19] and PI3K/Akt pathways [20]. Furthermore, ALR reduces lipoapoptosis caused by free fatty acids [21] and acts as an anti-apoptotic/ cell survival factor in hepatoma cells after oxidative stress injuries [22, 23]. A recent study by our group have shown that exposure of hepatic cells to oxidative stress activates Nrf2 which in turn activates ALR expression [24]. On the other hand, FOXA2 (Forkhead Box A2) binds to its response element within ALR promoter and activates ALR expression [25]. Since ALR was shown to reduce serum transaminases and preserve mitochondrial DNA in bile duct-ligated rats [26], we aimed to analyze the potential regulation of ALR under conditions of toxic concentrations of bile acids and to identify the transcription factors involved in the regulation of ALR expression.

2. Materials and methods

2.1. Cell culture

Human hepatoma cell lines HepG2 and Huh7 [24] cells were grown at 37°C, 5% CO_2 in DMEM (BioWhittaker, Verviers, Belgium) supplemented with penicillin (100 units/ml), streptomycin (10 µg/ml) and 10% fetal calf serum (Biochrom, Berlin, Germany). HepG2-NTCP cells were previously described [27] and kindly provided by Prof. Dr. Dieter Häussinger and Verena Keitel. For Luciferase and expression experiments, cells were cultured at a density of $5x10^4$ cells/cm^2 and treated with 100 or 200 µM deoxycholic acid (DCA) (Sigma-Aldrich, St. Louis, MO) for 24 hours.

2.2. Promoter constructs

Two constructs of the human ALR promoter (NC_000016.9) (-733/+240, -733/+527) were previously generated [25]. For ALR promoter constructs (-733/-58, -733/+493), the genomic sequence of ALR published earlier [28] was used and a forward primer containing the restriction site for BglII was designed (5'-atagatctcgcaaggaggcacaggaatccc-3'). Reverse primers were designed containing HindIII restriction site (5'-ataagctttccaggccagcgcgcggccacag-3' and 5'-ataagctttccgaacaaaggcgcccaaagct-3'). Human genomic DNA isolated from leukocytes using the Qiamp blood kit (Qiagen, Hilden, Germany) served as a template. For the amplification of the promoter sequence, Taq PCR Core Kit (Qiagen,

Hilden, Germany) was used according to manufacturer's instructions. Reporter gene constructs were cloned by ligation of PCR fragments into the BglII and HindIII restriction sites of the pGL_2-basic vector. The identity of the subcloned DNA fragments was confirmed by DNA sequencing.

2.3. Transient transfection and reporter gene assays

HepG2-NTCP, HepG2 and Huh7 cells were transfected using Lipofectamine® 3000 (ThermoFisher Scientific, Darmstadt, Germany) with an ALR promoter construct and either pcDNA3.1, pcDNA3.1-Egr-1 expression plasmid, pCMV-3xflag-dnEgr-1 expression plasmid (a generous gift from Xiaojia Chen, China), or pcDNA3.1-SHP, pcDNA3.1-HNF4α1 or pcDNA3.1-HNF4α7 expression plasmids (received thankfully from Dr. Oliver Burk, Stuttgart). Dual luciferase assays were carried out 24 hours after transfection using dual luciferase reporter assay system (Promega, Mannheim, Germany). pRL-TK Renilla-vector was co-transfected to determine transfection efficiency and the promoterless vector pGL_2-basic served as negative control. Short form ALR (sfALR) expression plasmid was previously generated [21]. Each experiment was repeated at least three times.

2.4. RNA isolation and qRT-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). One µg of total RNA was reverse-transcribed using the Reverse-Transcription System (Qiagen, Hilden, Germany). Transcript levels of ALR and HPRT were quantified using real time PCR technology (Roche, Penzberg, Germany). Following primers were used (Metabion, Martinsried, Germany): ALR Fwd: 5'-gaagcgggacaccaagttta-3', ALR Rev: 5'-ttcagcacactcctcacagg-3'. HPRT Fwd: 5'-tgacactggcaaaacaatgca-3' and HPRT Rev: 5'-ggtccttttcaccagcaagct-3'.

2.5. SDS-PAGE and immunoblotting

Briefly, 30 µg total proteins per lane were separated by 12% SDS-PAGE (Biorad, Hercules, CA, USA), transferred onto PVDF membranes (Biorad, Hercules, CA, USA), incubated with specific antibodies and developed using enhanced chemiluminescence reagent (ThermoFisher Scientific, Darmstadt, Germany). Anti-ALR polyclonal antibody was prepared by Davids Biotechnology (Regensburg,

Germany), anti-β-actin antibody (#4970) was purchased from Cell Signaling (Danvers, MA).

2.6. Electrophoretic mobility shift assay (EMSA)

HepG2 cells were transfected with 500 ng Egr-1 expression plasmid. Nuclear extracts were prepared using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific, Darmstadt, Germany) according to manufacturer's description. Complementary synthetic biotin-labeled oligonucleotides corresponding to the Egr-1c-RE within ALR promoter were obtained from Metabion (Martinsried, Germany): Egr-1c-RE Fwd: 5'-cctgtccccgcccccgcccaggta-3'; Egr-1c-RE Rev: 5'-tacctgggcgggggcggggacagg-3'. Supershifting was performed using anti-Egr-1 antibodies (Santa Cruz, CA, USA) after incubation with 2,5 µg nuclear extract for 20 min at 4°C before adding the reaction. The biotin-labeled DNA was detected using enhanced chemiluminescence reagent (ThermoFisher Scientific, Darmstadt, Germany). Competition experiments were performed using a 200 fold excess of the same unlabeled binding site 20 min prior to electrophoresis using 6% DNA retardation gels (Biorad, Hercules, CA, USA).

2.7. Statistical analysis

All data are presented as mean plus/minus standard deviation of the mean. Data were compared between groups using the student t-test. p value of $< 0,05$ was considered significant.

3. Results

3.1. Bile acids reduce the expression of ALR

To analyze the role of bile acids in the regulation of ALR expression, HepG2 cells were incubated with increasing concentrations of deoxycholic acid (DCA). DCA is a secondary bile acid that is formed in the intestine and driven back to the liver by the portal vein where it activates several transduction pathways in hepatocytes to initiate apoptosis [29] and inflammatory response *via* Egr-1 activation [30]. mRNA and protein levels of ALR were evaluated by qRT-PCR and western blot, respectively. As shown in Fig. 1A, ALR transcription is significantly reduced after treatment with 200 μM of DCA. Furthermore, long forms of ALR (21 and 23 kDa), were reduced upon treatment with DCA (Fig. 1B). In addition, transfected HepG2 cells transiently expressing sfALR (15 kDa) were treated with DCA to explore the effect of bile acids on the endogenously expressed sfALR. The expression of sfALR was reduced in HepG2-sfALR cells after treatment with DCA (Fig. 1C). These data suggest that bile acids treatment down-regulated ALR mRNA and protein expression in liver cells. The same experiments were performed in HepG2 cells over-expressing sodium taurocholate co-transporting polypeptide (NTCP, responsible for bile acid uptake into hepatocytes) (HepG2-NTCP) and showed similar results to HepG2 wild-type cells (data not shown). It is worth to mention that other bile acids like glycochenodeoxycholic acid (GCDCA) and glycocholic acid (GCA) also attenuated the protein expression of ALR (data not shown), which suggests that the molecular mechanism of ALR regulation by bile acids is not limited to a specific bile acid.

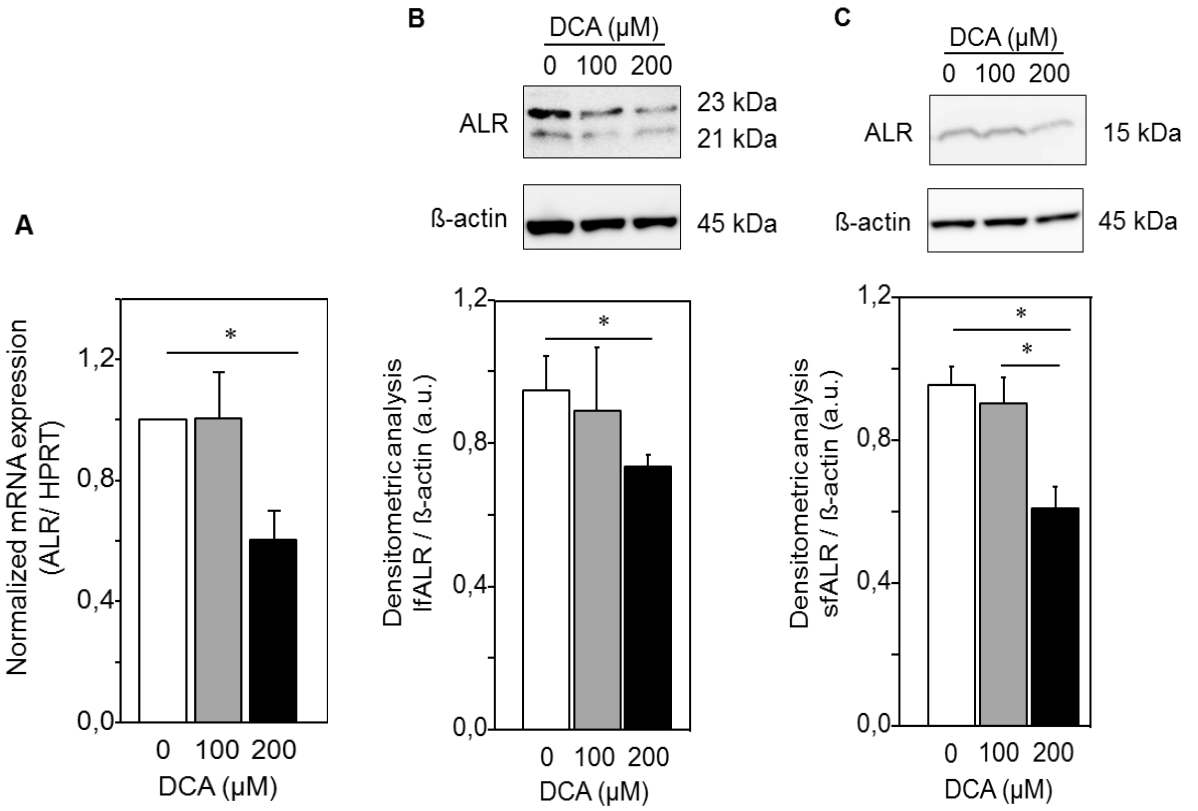

Fig. 1. Bile acids inhibit the expression of ALR. HepG2 cells were treated with 100 or 200 μM DCA for 24 hours, followed by analyzing mRNA expression of ALR by qRT-PCR (**A**), and lfALR protein expression by western blot (**B**). (**C**) Western blot analysis demonstrated decreased sfALR protein expression (15 kDa) after transient transfection of sfALR expression plasmid into HepG2 cells and treatment with 100 or 200 μM of DCA for 24 hours. Densitometric analysis was performed by analyzing 3 different blots and a representative blot is illustrated (Three independent experiments, Mean±SD).

3.2. ALR promoter harbors several bile acid-related response elements and its activity is reduced by bile acids

Analysis of the human ALR 5'-flanking sequence (ALR GeneID 2671) revealed multiple response elements and binding site, a schematic illustration in Fig. 2A points out the response element for different transcription factors known to be regulated by bile acids and are therefore included in this study. ALR promoter study revealed two putative bile acid response elements (BARE I and BARE II) (AGGTCA and AGTTCA) respectively. Those BAREs [AG(T/G)TCA] were reported to be located at the promoter CYP7A1, and to regulate CYP7A1 expression [4, 5]. ALR promoter study also revealed three putative response elements for Egr-1 [GCCCCCG] at -149 to -140 (Egr-1a-RE), -114 to -107 (Egr-1b-RE) and +304 to +314 (Egr-1c-RE). Furthermore, a response element for HNF4α [AGGACTTTGGC] was identified at +421 to +432. Four truncated ALR promoter constructs were generated (Fig. 2A) to identify the response element responsible for ALR repression by bile acids. Construct 1 represents the full length of ALR promoter, construct 2 a truncated ALR

promoter lacking BARE II, construct 3 lacking Egr-1c-RE, FOXA2-RE, HNF4α-RE and BARE II and construct 4 that includes Egr-1a-RE and Egr-1b-RE. HepG2-NTCP cells were transfected with ALR promoter constructs and treated with DCA for 24 hours. The luciferase activity assay revealed a significant reduction in ALR promoter activity upon DCA treatment in the full length promoter (construct 1) and construct 2 (lacking BARE II). On the other hand, the promoter activity remained unchanged in construct 3 and construct 4 (Fig. 2B). This suggests that ALR repression by DCA is mediated by a response element, such as FOXA2-RE, Egr-1c-RE as well as a HNF4α-RE. Moreover, BARE I and BARE II seem to be not involved in ALR repression by DCA.

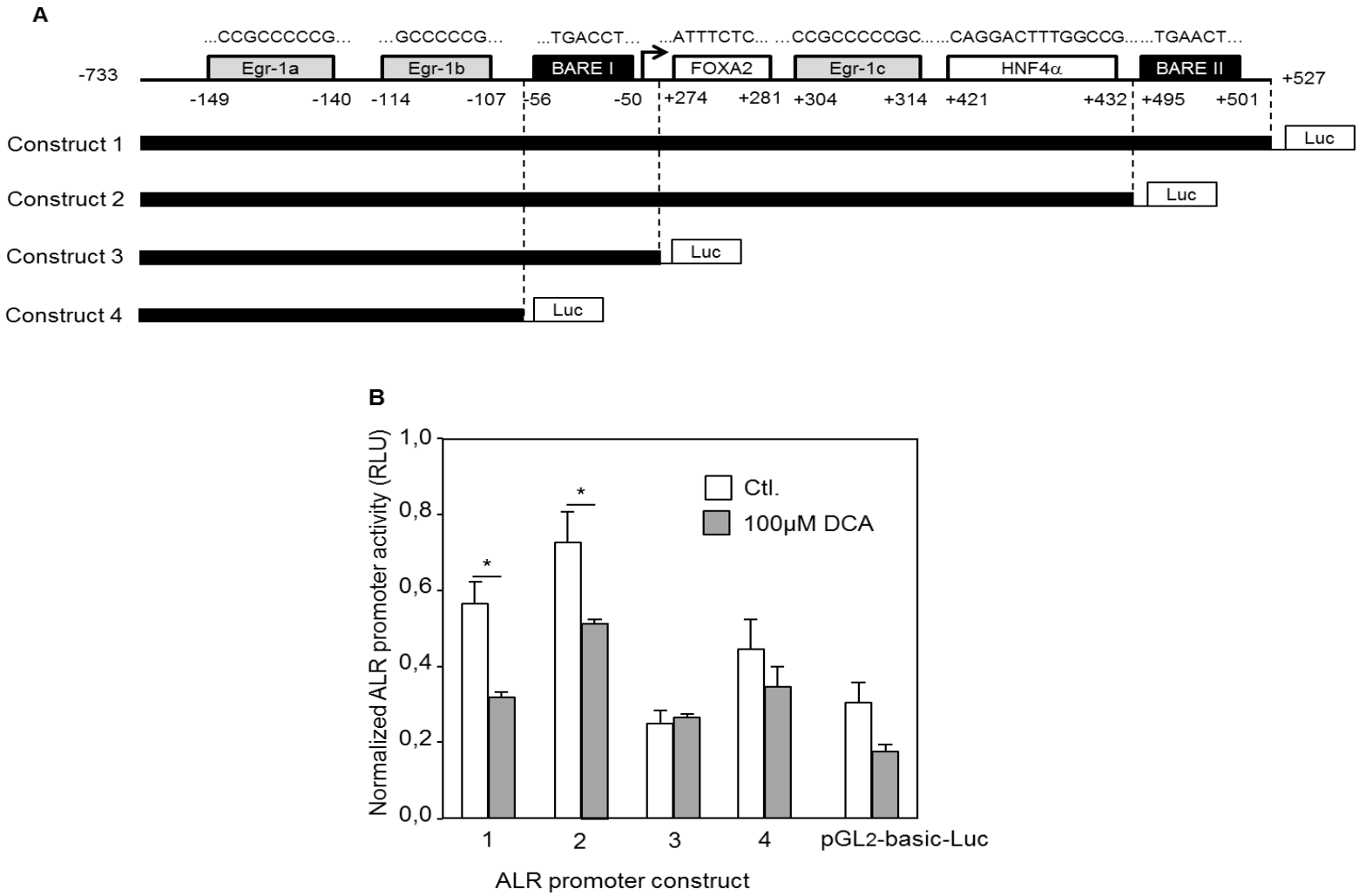

Fig. 2. Bile acids reduce the activity of ALR promoter. (**A**) Schematic structure of the human ALR gene showing the location of three potential Egr-1 response elements (Egr-1-a,b,c-RE), two bile acid response elements (BARE), a potential HNF4α response element (HNF4α-RE) and a verified FOXA2-RE. Construct 1 (full length promoter) spans the region from -733 to +527 bp, construct 2 spans the region from -733 to +493 bp (lacking BARE II), construct 3 from -733 to +240 bp lacking BARE II, HNF4α, Egr-1c-RE and FOXA2-RE and construct 4 spans the region from -733 to -58 bp containing Egr-1a-RE and Egr-1b-RE. (**B**) Reporter gene assays using HepG2-NTCP transfected with full length and truncated ALR promoter constructs. Cells were treated with 100 μM DCA for 24 hours and lysed to measure luciferase expression (five independent experiments, Mean±SD).

3.3. Egr-1-mediated activation of ALR expression is attenuated by bile acids

Since ALR promoter harbors many putative Egr-1 response elements and bile acids were reported previously to activate Egr-1, HepG2 and Huh7 cells were transfected with Egr-1 to evaluate its impact on ALR promoter. As shown in Fig. 3A, the activity of the full length promoter (construct 1) was induced whereas the promoter lacking Egr-1c-RE (construct 3) remained unchanged after Egr-1 transfection. The induction in construct 1 activity was reversed by the addition of dominant negative Egr-1 (dnEgr-1) which suggests that the Egr-1c-RE (+304 to +314) is responsible for Egr-1-mediated activity. This was confirmed by performing EMSA, oligonucleotides representing Egr-1c-RE were incubated with nuclear extracts of HepG2 over-expressing Egr-1 and we analyzed the binding of Egr-1 to its response element. The protein-DNA complex in Fig. 3B is specific for Egr-1 because incubation with anti-Egr-1 antibody significantly reduced the complex formation (Fig. 3C). ALR protein expression was also induced by Egr-1 transfection (Fig. 3D). These data suggest that Egr-1 binds to (Egr-1c-RE) within ALR promoter and activates ALR expression.

Since Egr-1 is activated under cholestatic conditions, we investigated the ability of bile acids to regulate ALR expression in the presence of Egr-1. Therefore, HepG2-NTCP cells were transfected with Egr-1 expression plasmid and the full length ALR promoter and treated with 100 µM DCA. We found that ALR promoter activity is reduced after DCA treatment despite the presence of Egr-1 (Fig. 3E). This was confirmed by performing EMSA assays demonstrating that Egr-1 binding to its response element is significantly reduced upon incubation with bile acids (Fig. 3F, lane 4 and Fig. 3G). Our data suggest that ALR induction by Egr-1 is attenuated by bile acids due to reduced Egr-1 binding to its response element.

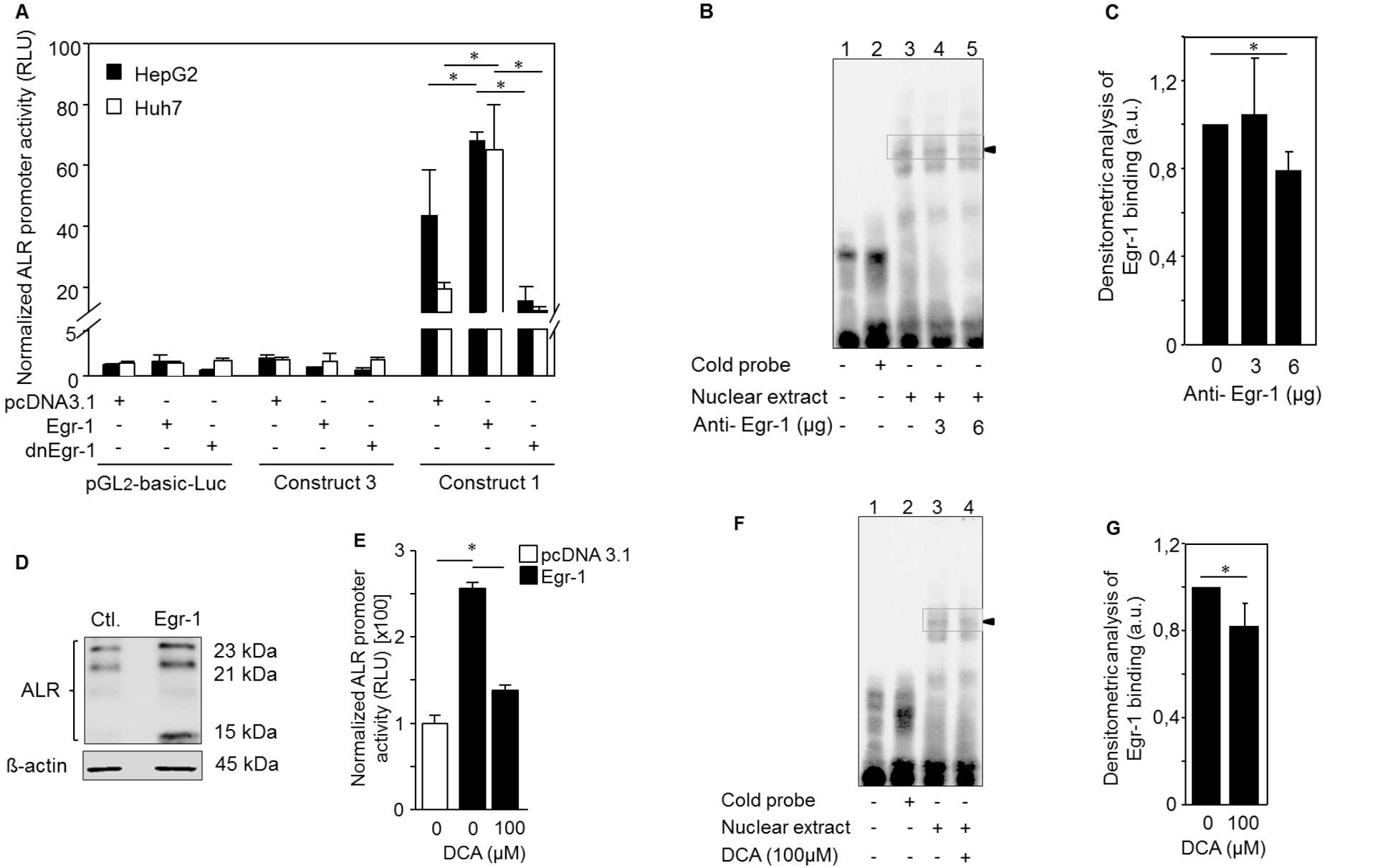
A
Normalized ALR promoter activity (RLU)
HepG2
Huh7
pcDNA3.1
Egr-1
dnEgr-1
pGL2-basic-Luc
Construct 3
Construct 1
B
Cold probe
Nuclear extract
Anti- Egr-1 (µg)
C
Densitometric analysis of Egr-1 binding (a.u.)
Anti- Egr-1 (µg)
D
Ctl.
Egr-1
ALR
23 kDa
21 kDa
15 kDa
ß-actin
45 kDa
E
pcDNA 3.1
Egr-1
Normalized ALR promoter activity (RLU) [x100]
DCA (µM)
F
Cold probe
Nuclear extract
DCA (100µM)
G
Densitometric analysis of Egr-1 binding (a.u.)
DCA (µM)

Fig. 3. Induction of ALR expression by Egr-1 is attenuated by bile acids. (**A**) Reporter gene assays using HepG2 and Huh7 cells transfected with ALR-promoter constructs with/without Egr-1 expression plasmid or dnEgr-1 expression plasmid. Afterwards, cells were lysed to measure luciferase expression (three independent experiments, Mean±SD). (**B**) Labeled oligonucleotides representing the Egr-1C-RE (+304 to +314) were incubated in the absence or presence of nuclear extracts derived from HepG2 cells. For competition experiments a 200 fold excess of unlabeled Egr-1 consensus oligonucleotides were added (lane 2). Egr-1 specifically binds to Egr-1 binding site in ALR promoter (lane 3). Addition of anti-Egr-1 antibody reduced the formation of Egr-1-DNA complex (lanes 4 and 5). (**C**) Densitometric analysis of Egr-1 binding to its response element within ALR promoter after addition of anti-Egr-1 antibodies (three different experiments were analyzed). (**D**) Western blot analysis demonstrated an increased ALR protein expression (23, 21 and 15 kDa) upon transfection with Egr-1 expression plasmid in HepG2 cells. (**E**) Reporter gene assays. HepG2-NTCP cells were transfected with full length ALR promoter construct with/without Egr-1 expression plasmid, treated with 100 µM DCA for 24 hours and lysed to measure luciferase expression (three independent experiments, Mean±SD). (**F**) Labeled oligonucleotides representing the Egr-1c-RE (+304 to +314) were incubated in the absence or presence of nuclear extracts derived from HepG2 cells with/without treatment with 100 µM DCA for 15 minutes. For competition experiments a 200 fold excess of unlabeled Egr-1 consensus oligonucleotides were added (lane 2). Egr-1 specifically binds to Egr-1 binding site within ALR promoter (lane 3). Treatment of the cells with DCA decreased the binding of Egr-1 to its response element (lane 4). (**G**) Densitometric analysis of Egr-1 binding to its response element within ALR promoter after treatment with 100 µM DCA for 24 hours (three different experiments were analyzed).

3.4. Bile acids repress ALR activity through HNF4α1/SHP crosstalk

We have shown so far that bile acids negatively regulate ALR expression (Fig. 1) independently from BARE I, BARE II (Fig. 2) and Egr-1 (Fig. 3). Therefore, we analyzed the role of HNF4α1 and SHP known to be regulated by bile acids by performing luciferase assays. The activity of the full length promoter is induced by co-transfection with HNF4α1 (Fig. 4A). Additionally, transfection with SHP reduces ALR promoter activity (Fig. 4A). However, transfection with HNF4α7 showed no increase in ALR expression (Fig. 4A). Interestingly, HNF4α1 increased ALR protein expression and adding SHP reversed this increase (Fig. 4B). Moreover, due to its known repression by bile acids through SHP, the effect of HNF4α on ALR expression was investigated. We found that bile acids reduce the protein expression of different isoforms of ALR (Fig. 4C) despite HNF4α transfection in both HepG2 and in sfALR transfected HepG2 cells. This suggests that HNF4α1 activates ALR expression and this activation is diminished by bile acid-activated SHP.

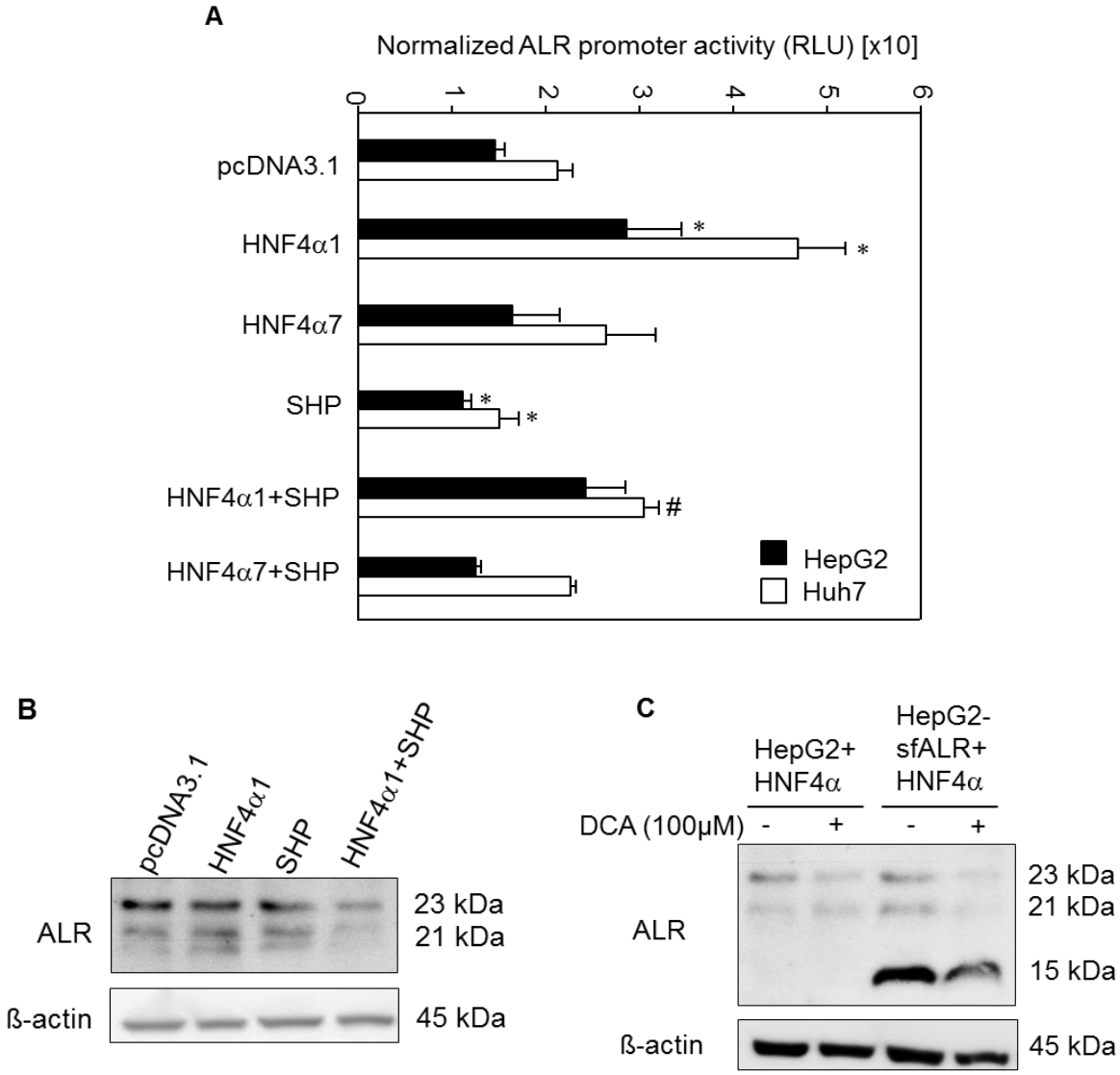

Fig. 4. Bile acid-regulated transcription factors (HNF4α1 and SHP) regulate ALR promoter activity and expression. (**A**) Reporter gene assays using HepG2 and Huh7 cells transfected with ALR-promoter construct 1 and HNF4α1, HNF4α7 or SHP expression plasmids. Three independent experiments, Mean±SD. p<0,05 * compared to transfection with pcDNA3.1, # compared to transfection with HNF4α1 expression plasmid. (**B**) ALR protein expression in HepG2 cells after transfection with mentioned expression plasmids. (**C**) Western blot analysis demonstrated decreased ALR protein expression after HNF4α1 transfection and treatment with 100 μM of DCA for 24 hours in both HepG2 and sfALR transfected HepG2 cells (HepG2-sfALR).

4. Discussion

Over the last two decades, bile acids role as signaling molecules has gained growing attention. Bile acids regulate their synthesis by a negative feedback loop that involves FXR/SHP/HNF4α. Furthermore, bile acids accumulation causes liver injury by inducing apoptosis [12, 13], increasing mitochondrial instability [14] and activating the expression of chemotactic agents to attract inflammatory cells to the site of injury

[11]. Nevertheless, little is known about the regulation of pro-survival/anti-apoptotic factors by bile acids.

ALR (Augmenter of liver regeneration) is a hepatoprotective factor that has been shown to be involved in several cellular processes like protein folding [31] and mitochondrial maintenance [32]. In addition, ALR is an anti-apoptotic factor that reduces death receptors expression and decreases ER-stress [21].Further, ALR expression has been shown to be activated by FOXA2 [25]. Additionally, oxidative stress was also shown to activate ALR expression *via* Nrf2 which suggested the anti-oxidative properties of ALR [24]. Since bile acids accumulation causes oxidative stress and activation of Nrf2-target genes [15], we hypothesized that ALR expression might be regulated by bile acids. To assess the regulation of ALR by bile acids, we performed a promoter study including the first intron to include possible regulatory elements within introns which were proved to have an important role in the regulation of different target genes [33]. Interestingly, sequence analysis of ALR promoter revealed several putative “bile acid-regulated response elements”: BARE I, BARE II, Egr-1-a,b,c-RE and HNF4α-RE. qRT-PCR and western blot results showed that treatment with DCA represses the expression of ALR in its 3 isoforms. BAREs are known to be responsible for the negative feedback of CYP7A1 by bile acids [4, 5]. Nevertheless, deleting those response elements from the ALR promoter constructs did not affect the repression of ALR by DCA which suggests that BARE I and BARE II may not be involved in the regulation of ALR by DCA. Moreover, HNF4α1, but not HNF4α7, have shown an increase in ALR promoter activity as well as protein expression and this induction is reversed by SHP. SHP is an orphan nuclear factor that lacks a DNA binding domain [34] and is activated by bile acids’ nuclear receptor FXR [6]. Interestingly, SHP binds to other transcription factors and blocks their transcriptional activates [7]. Thus we could postulate that the negative regulation of ALR by bile acids is mediated by HNF4α1/SHP crosstalk.

It was previously shown that liver regeneration is impaired in Egr-1 knock-out mice [35]. Furthermore, Egr-1 luciferase transgenic mice showed that Egr-1 is activated at the site of wound healing after partial hepatectomy [36]. interestingly, ALR was shown to have an augmenting effect on the process of hepatic healing after resection [37]. Moreover, ALR accumulates *in vivo* after partial hepatectomy and during liver regeneration [38, 39]. In our study we show that Egr-1 binds to its response element

within ALR promoter and increases its expression. Taken all together, this suggests Egr-1 as a novel inducer of ALR expression.

It is worthwhile to mention that Egr-1 is also regulated by HNF4α/SHP [40]. Therefore we suggest that bile acids repress ALR expression by, at least, three possible mechanisms. First, the direct binding of bile acid-activated SHP to HNF4α1 blocks HNF4α1-activation of ALR. Second, SHP activation by bile acids represses Egr-1 prolonged activation and therefore ALR expression is reduced. Third, the absence of ALR activating transcription factors like FOXA2 that translocates to the cytosol under cholestatic conditions [41] where it remains inactive.

In the light of these data, we hypothesize that attenuated expression of anti-apoptotic and anti-oxidative ALR by bile acids contributes to the cholestatic liver injury, which suggests that boosting ALR expression under cholestatic conditions may be of benefit for hepatocytes by promoting pro-survival and anti-apoptotic effects.

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Chapter 4

Regulation of ALR by cholestasis-related cytokines

Abstract

During cholestatic liver diseases, bile acids accumulate and therefore cause liver injury. The mechanism of this injury includes signaling pathways that initiate oxidative stress, ER-stress and apoptosis in hepatocytes and cholangiocytes. Interestingly, innate immune cells in the liver have been shown to contribute to liver injury during cholestasis. As an outcome of hepatocyte injury, T helper cells are activated and release IL17. IL17 binds to its receptor and particularly activates Kupffer cells. When activated, Kupffer cells increase the expression and release of IL6, IL1β and TNFα that were shown to influence cholestatic liver injury e.g. by altering bile acid synthesis and transport. Augmenter of liver regeneration (ALR) is a hepatotrophic growth factor that was found to have anti-oxidative and anti-apoptotic properties. Interestingly, application of recombinant ALR protein in bile duct ligated-rats has been demonstrated to attenuate liver injury. Moreover, ALR is positively regulated by IL6 *via* increased recruitment of FOXA2 (Forkhead Box A2, an inducer of ALR) to its binding site within ALR promoter. However, little is known about ALR regulation by Kupffer cell-released IL1β. Therefore, we aimed to investigate the expression of ALR by IL1β and to elucidate the molecular mechanism of this regulation. We found that ALR promoter activity as well as mRNA and protein expression are reduced upon treatment with IL1β. Furthermore, expression of SP1 (Specificity Protein 1), an inducer of ALR, was reduced upon IL1β treatment. Moreover, Egr-1 (early growth response protein-1) was induced by IL1β but could not activate the expression of ALR which could be attributed to reduced Egr-1 binding to ALR promoter upon treatment with IL1β. In conclusion, this study offers evidence about the regulation of anti-apoptotic and anti-oxidative ALR by IL1β and suggests a further role of Kupffer cells in cholstatic liver injury.

1. Introduction

Cholestatic liver diseases occur when excretion of bile acids is impaired due to either a direct inhibition of bile flow or genetic defects impairing bile acid transporters [1, 2]. Whatever the cause, hepatic bile acids accumulation causes liver injury by inducing apoptosis [3, 4], manipulating the mitochondrial membrane stability by oxidative stress and ROS (reactive oxygen species) production [5] as well as attracting inflammatory cells [6]. Furthermore, cholestatic liver injury may lead to liver fibrosis, cirrhosis and possibly to liver failure if it is left untreated [7]. The inflammatory response during cholestasis is initiated by hepatocytes which release several cytokines upon injury [8]. It was previously reported that exposure of cultured mouse hepatocytes to bile acids leads to a pathologically relevant increase in the expression of ICAM1 (Intercellular Adhesion Molecule-1), MIP2 (Macrophage Inflammatory Protein-2) and MCP1 (Monocyte Chemoattractant Protein 1) [6, 9]. Interestingly, this induction was attributed to increased Egr-1 (early growth response protein-1) expression [6, 10]. This was further confirmed in isolated human hepatocytes that showed increased release of cytokines into culture medium upon treatment with bile acids [8]. Furthermore, the hepatocyte-released cytokines enhanced neutrophils chemotaxis to the site of injury which initiate the inflammatory response [8]. Moreover, cholestatic liver injury is affected by hepatic macrophages which include liver resident Kupffer cells and bone marrow-derived monocytes/macrophages [11, 12]. Recent studies have shown that activation of Kupffer cells by IL17 (released from T helper cells and neutrophils during cholestasis) [13, 14] leads to the induction of TNFα and IL1β (pro-inflammatory cytokines) [15]. Moreover, exposure of macrophages to bile acids activates TGR5 (G-protein coupled bile acid receptor) and reduces pro-inflammatory cytokines while maintaining anti-inflammatory cytokines (IL4, IL10 and IL13) expression thus promoting the development of an anti-inflammatory macrophage phenotype [16]. In addition, it was shown that IL1β and TNFα are involved in the downregulation of hepatic organic anion transporters in cholestasis like NTCP (Na^+-Taurocholate Co-transporting Polypeptide) and BSEP (Bile Salt Export Pump) [17, 18]. Therefore, these findings indicate that Kupffer cells may contribute to cholestatic liver injury.

Augmenter of Liver Regeneration (ALR), a hepatotrophic growth factor [19], is constitutively expressed and stored in the hepatic parenchymal cells [20-22]. ALR was previously shown to attenuate free fatty acid induced-apoptosis [23]. Further,

ALR acts as an anti-apoptotic/ cell survival factor in hepatoma cells after oxidative stress injuries [24, 25]. Moreover, ALR was shown to modulate the reaction of hepatocytes to IL6 treatment by altering the activation of STAT3 (signal transducer and activator of transcription 3) [26]. It was previously demonstrated that exposure of hepatic cells to oxidative stress activates Nrf2 (Nuclear factor erythroid 2–related factor 2) which in turn activates ALR expression [27]. In addition, FOXA2 (Forkhead Box A2) binds to ALR promoter and increases its expression and this binding is enhanced by IL6 [28]. However, the regulation of the heptoprotective factor, ALR, by cholestasis-induced cytokine IL1β has not been investigated, therefore we aimed to analyze the regulation of ALR expression by IL1β and to elucidate which transcription factors are involved in this regulation.

2. Materials and methods

2.1. Cell culture

The human hepatoma cell line HepG2 was obtained from American Type Culture Collection (HB-8065, ATCC, Manassas, VA) and grown at 37°C, 5% CO2 in DMEM (BioWhittaker, Verviers, Belgium) supplemented with penicillin (100 units/ml), streptomycin (10 µg/ml) and 10% fetal calf serum (Biochrom, Berlin, Germany). IL1β (50 ng/ml) (PeproTech GmbH, Hamburg, Germany) was added after 24 hours starvation for indicated times.

2.2. Transient transfection and reporter gene assays

HepG2 cells were cultured at the density of $5x10^4$ cell/cm^2, cells were then transfected using Lipofectamine® 3000 (ThermoFisher Scientific, Darmstadt, Germany) with 200 ng of human ALR promoter construct (-733/+527) (reported in [28]) and 200 ng of either pcDNA3.1or pcDNA3.1-Egr-1 expression plasmid (a generous gift from Xiaojia Chen, China). After 24 hours, IL1β was added (50 ng/ml) for indicated times. Dual luciferase assays were carried out 24 hours after transfection using dual luciferase reporter assay system (Promega, Mannheim, Germany). pRL-TK Renilla-vector was co-transfected to determine transfection efficiency and the promoterless vector pGL_2-basic served as negative control. Each experiment was repeated at least three times.

2.3. RNA isolation and qRT-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). One µg of total RNA was reverse-transcribed using the Reverse-Transcription System (Qiagen, Hilden, Germany). Transcript levels of ALR, SP1, Egr-1 and HPRT were quantified using real-time PCR technology (Roche, Penzberg, Germany). PCR reaction products were verified by sequence analysis and each quantitative PCR was performed in triplicates. Table (I) includes the used primers for qRT-PCR.

2.4. SDS-PAGE and immunoblotting

Total proteins were isolated, prepared and subjected to western blot analysis. Briefly, 30 µg per lane were separated by 12% SDS-PAGE (Biorad, Hercules, CA, USA) and proteins were transferred onto PVDF membranes (Biorad, Hercules, CA, USA), incubated with specific antibodies and developed using enhanced chemiluminescence reagent (ThermoFisher Scientific, Darmstadt, Germany). Anti-ALR polyclonal antibody was prepared against 15 kDa ALR (short form ALR) prepared by Davids Biotechnology (Regensburg, Germany), anti-β-actin antibody was purchased from Cell signaling (Danvers, MA) and anti-Egr1 purchased from Santa Cruz (CA, USA).

Table (I)*:* Primers used in qRT-PCR expreiments

ALR, sense	5'-gaagcgggacaccaagttta-3'
ALR, antisense	5'-ttcagcacactcctcacagg-3'
SP1, sense	5'-ttgaaaaaggagttggtggc-3'
SP1, antisense	5'-tgctggttctgtaagttggg-3'
Egr-1, sense	5'-tactcctctgttccccctgctt-3'
Egr-1, antisense	5'-gaaaaggttgctgtcatgtccg-3'
HPRT, sense	5'-tgacactggcaaaacaatgca-3'
HPRT, antisense	5'-ggtccttttcaccagcaagct-3'

2.5. Electrophoretic mobility shift assay (EMSA)

HepG2 cells were transfected with 500 ng of Egr-1 expression plasmid and nuclear extracts were prepared using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific, Darmstadt, Germany) according to the manufacturer's description. Complementary synthetic biotin-labeled oligonucleotides corresponding to the Egr-1 binding site (+304/+314) within ALR promoter were obtained from Metabion (Martinsried, Germany): Egr-1-RE Fwd: 5'-cctgtccccgcccccgcccaggta-3'; Egr-1-RE Rev: 5'-tacctgggcgggggcggggacagg-3', for mutated potential overlapping SP1 binding site following oligonucleotides were used (mutated nucleotides are underlined): Egr-1-ΔSp1-Fwd: 5'-cctgtcc<u>tt</u>gcccccgcccaggta-3' and Egr-1-ΔSp1-Rev:5'-tacctgggcgggggc<u>aa</u>ggacagg-3'. The biotin-labeled DNA was then detected using enhanced chemiluminescence reagent (ThermoFisher Scientific, Darmstadt, Germany). Competition experiments were performed using a 200 fold excess of the same unlabeled binding site 20 min prior to electrophoresis using 6% DNA retardation gels (Biorad, Hercules, CA, USA) at room temperature.

2.6. Statistical analysis

All data are presented as mean plus/minus standard deviation of the mean. Data were compared between groups using the student *t*-test. *p* value of $< 0,05$ was considered significant.

3. Results

3.1. IL1β treatment reduces ALR promoter activity and expression

To analyze the activity of ALR promoter upon IL1β treatment, HepG2 cells were transfected with an ALR promoter construct (-733/+523) and then treated with 50 ng/ml IL1β for 2, 4, 8, 16 and 24 hours. As shown in Fig. 1A. ALR promoter activity is significantly reduced after 4 hours of IL1β treatment. Furthermore, mRNA expression of ALR was reduced upon treatment with IL1β (Fig. 1B). In addition, mRNA levels of SP1 (specificity protein 1, an inducer of ALR expression [29]) were accordingly reduced upon IL1β treatment (Fig. 1B). This was further confirmed by performing western blot to investigate ALR protein expression after IL1β treatment. ALR expression was reduced after 16 and 24 hours of IL1β treatment (Fig.1C). These results suggest that IL1β reduces the expression of ALR and this reduction might be mediated, at least partially, by the reduced expression of SP1.

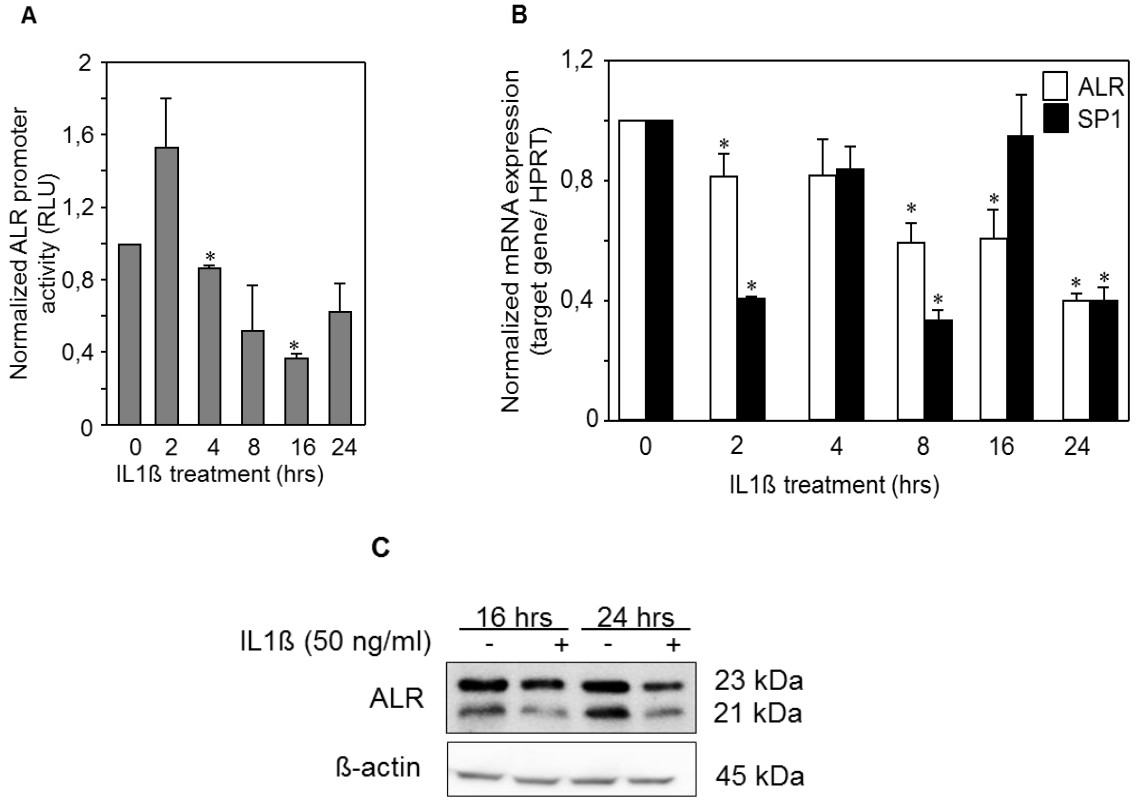

Fig. 1. Effect of IL1β on ALR promoter activity and expression. (**A**) HepG2 cells were transfected with ALR promoter construct (-733 to +527 bp), starved for 24 hours and then treated with 50 ng/ml IL1β for indicated time points, cells were then lysed to measure luciferase expression (three independent experiments, Mean±SD). (**B**) HepG2 cells were starved for 24 hours and then incubated with 50 ng/ml of IL1β for indicated times. mRNA levels of ALR and SP1 were analyzed and normalized to HPRT. (C) Western blot analysis using specific anti-ALR antibodies demonstrated decreased ALR protein expression in HepG2 cells upon treatment with 50 ng/ml IL1β for 16 or 24 hours (β-actin served as loading control).

3.2. IL1β reduces the Egr-1-mediated ALR induction

Egr-1, a zinc finger transcription factor, induces ALR expression by binding to its response element within ALR promoter [30]. On the other hand IL1β was shown to increase the expression of Egr-1 [31, 32]. Therefore the effect of Egr-1 on ALR expression under conditions of high IL1β concentrations was analyzed. ALR promoter construct was co-transfected with an Egr-1-expression plasmid and promoter activity was analyzed upon treatment with 50ng/ml of IL1β for 2, 4, 8, 16 and 24 hours. As shown in Fig. 2A, Egr-1 activates ALR promoter activity. This activity is then significantly reduced after IL1β application for 4, 8 and 16 hours. Moreover, the mRNA expression of Egr-1 (Fig. 2B) is increased 16 and 24 hours after IL1β treatment. Egr-1 protein expression was analyzed and showed an increase 24 hours after IL1β application (Fig. 2C). This was further confirmed by performing EMSA to analyze Egr-1 binding to its response element within ALR promoter after IL1β treatment. We found that Egr-1 binds to its response element within ALR promoter and this binding is reduced after the application of IL1β (Fig. 2D). Furthermore, mutating the potential overlapping SP1 response element next to Egr-1 response element [31, 33] increases the DNA-protein binding of Egr-1. The binding is nevertheless reduced upon IL1β treatment (Fig. 2D). Based on these data, we propose that ALR suppression by IL1β might be attributed to reduced Egr-1 binding to its binding site within ALR promoter (+304/+314). However, this reduced binding of Egr-1 to ALR promoter seems to be SP1 independent.

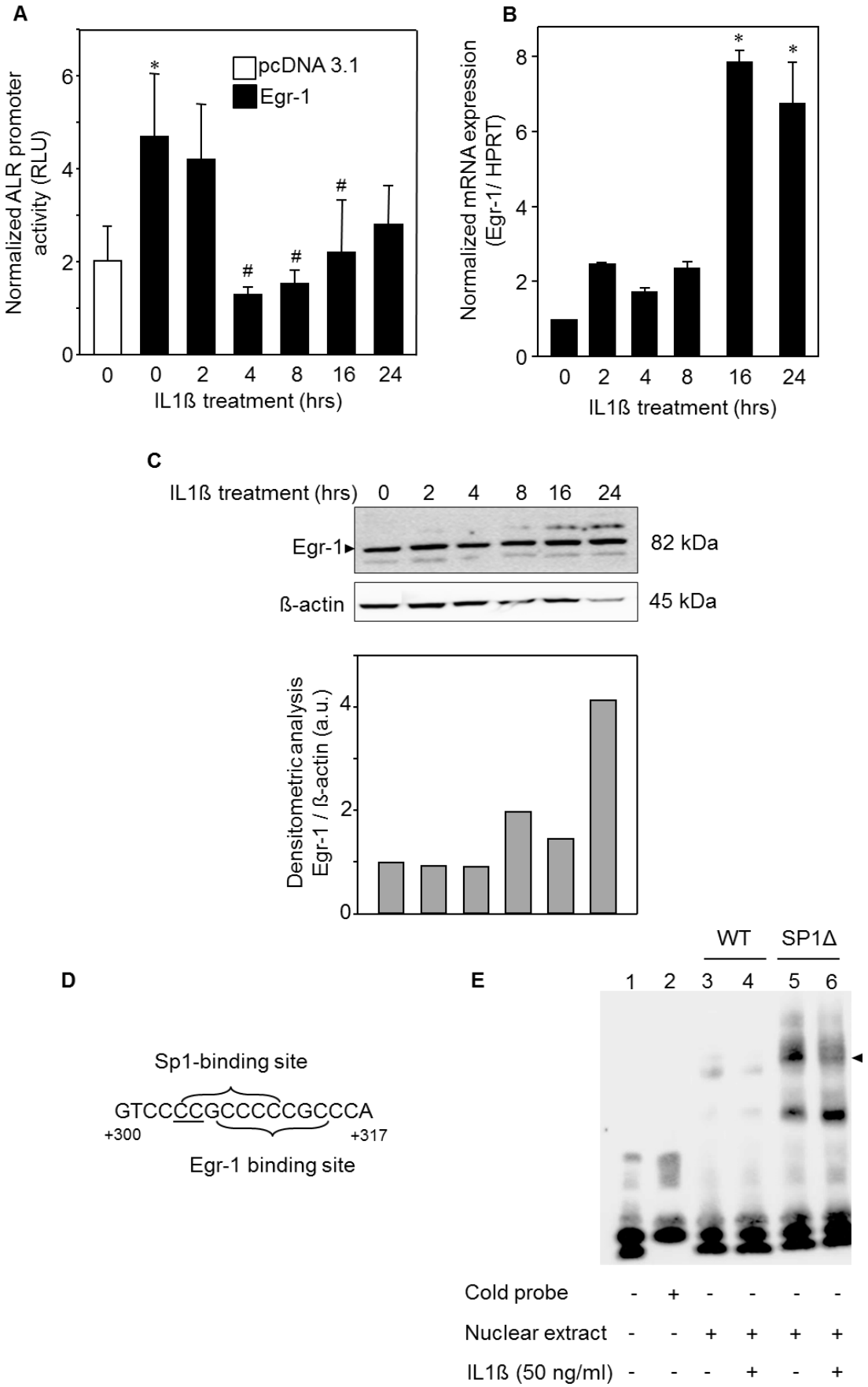

Fig. 2. Effect of IL1β on the expression of Egr-1. HepG2 cells were starved for 24 hours and then incubated with 50 g/ml of IL1β for indicated times (**A**) ALR promoter activity was measured after transfection with Egr-1 expression plasmid and then addition of IL1β for indicated time points (three independent experiments, Mean±SD). (**B**) mRNA levels and (**C**) protein expression of Egr-1 upon treatment with IL1β with the corresponding densitometric analysis. (**D**) Schematic illustration of the

overlapping binding sites of Egr-1 and SP1 within ALR promoter (+300, +317) and the mutations (C→T) generated in the potential SP1 binding site are underlined. (**E**) Labeled oligonucleotides representing Egr-1 binding site (+304/+314) with wild type SP1 binding site (WT) or mutated SP1 binding site (ΔSP1). Oligonucleotides were incubated in the absence or presence of nuclear extracts derived from HepG2 cells with or without treatment with 50 ng/ml IL1β for 15 minutes. For competition experiments a 200 fold molar excess of unlabeled Egr-1 consensus oligonucleotides were added (lane 2). Egr-1 specifically binds to Egr-1 binding site within ALR promoter (lane 3). Treatment of the cells with IL1β decreased the binding of Egr-1 to its response element (lane 4). Lane 5 and 6 represent the ΔSP1 oligonucleotides. Increased Egr-1 binding to DNA upon SP1 mutation (lane 5), this binding is nevertheless reduced after IL1β addition (lane 6).

4. Discussion

Cholestasis is a pathological syndrome caused by impaired or disrupted bile flow from the liver [34]. As an outcome, bile acids (main component of the bile) accumulate and cause apoptosis [3, 4], oxidative stress [5] and attract inflammatory cells [6]. Upon exposure to toxic bile acids, hepatocytes activate the expression of chemokines like ICAM1 and MIP2 that attract neutrophils [6]. Furthermore, IL17, released from T helper cells, activates intrahepatic macrophages (Kupffer cells) [13, 14] which in turn release several pro-inflammatory cytokines like IL1β and TNFα [15]. Interestingly, accumulating evidence have emphasized the role of IL1β and TNFα in modulating cholestatic injury, like altering expression of bile acid transporters [17, 18] and inhibiting the expression of bile acid synthesis enzymes [35, 36]. Furthermore, IL1β receptor knockout mice has demonstrated that these mice show insusceptibility against LPS challenge after BDL, secreting fewer cytokines and showing lower mortality compared with wild-type mice [37]. Another study in TNF-α knockout mice showed improved survival rate and attenuated BDL-induced liver damage and fibrosis [38]. These studies indicate that Kupffer cells and their cytokines are associated with mortality in cholestatic liver injury [39]. Nevertheless, the molecular mechanism of cytokine-induced injury is not fully clear.

Augmenter of liver regeneration (ALR) is an anti-apoptotic and an ER-stress reducing factor [23]. Furthermore, ALR is activated by Nrf2 upon exposure to oxidative stress and is therefore considered anti-oxidative [27]. Additionally, application of recombinant ALR to bile duct-ligated rats was demonstrated to reduce serum transaminases and preserve mitochondrial DNA [40]. Moreover, it has been shown that endogenous short form ALR (15 kDa) increases that activation of STAT3 (signal transducer and activator of transcription 3) [26] and thereby alters the reaction of hepatocytes to IL6 signaling. Interestingly, it was previously demonstrated that STAT3 is a negative regulator of the bile acid synthesis and protects from bile acid-

induced apoptosis [41]. Additionally, STAT3 activation reduces the expression of death receptor 5 (DR5) [42]. Therefore, enhancing the expression of ALR in hepatocytes might protect hepatocytes against the toxicity of bile acids during cholestasis.

It has been previously reported that ALR expression is enhanced by IL6 [28]. Furthermore, TNFα was demonstrated to increase the release of ALR in an *in vitro* model [43] which could be attributed to increased nuclear localization of ALR's inducer FOXA2 [44] or induction of Egr-1 [45], another inducer of ALR expression [30]. Nevertheless, little is known about the regulation of ALR expression by Kupffer cell-released IL1β. We assessed the regulation of ALR by IL1β by luciferase assays, qRT-PCR and western blot techniques and found that ALR expression and promoter activity are reduced by IL1β. Interestingly, expression of SP1 (an activator of ALR expression [29]) was reduced by IL1β. This is in line with previous reports showing that SP1 expression [46] as well as promoter occupancy [31] in chondrocytes are decreased by IL1β. Therefore, we hypothesis that reduced SP1 expression might be, in part, responsible for reduced ALR expression. Furthermore, Egr-1 expression, an inducer for ALR [30], is induced by IL1β [31, 32]. We analyzed the ability of Egr-1 to regulate ALR promoter activity in the presence of IL1β and found that Egr-1-induced ALR promoter activity is reversed by IL1β due to reduced Egr-1 binding to ALR promoter. It is worth to mention that the negative regulation of ALR by IL1β could be due to induced AP1 expression [47]. AP1 was previously reported to bind to its binding site (-375/-369) within ALR promoter and repress its activity [48]. Moreover, the repressed expression of HNF4α by IL1β [49] may contribute to ALR repression by the absence of ALR activator, HNF4α [30]. In conclusion, the decreased expression of anti-apoptotic and anti-oxidative ALR by IL1β might be considered a further contribution of Kupffer cells to cholestatic liver injury and suggests that boosting the expression of ALR during cholestasis could be of clinical interest due to its heptoprotective properties in reducing bile acids synthesis and reducing bile acid-induced apoptosis.

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Chapter 5

Role of cytosolic sfALR in altering cholestatic injury

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Abstract

Cholestasis represents pathophysiologic syndromes defined as an impaired bile flow from the liver. As an outcome, bile acids accumulate and promote hepatocytes injury followed by liver cirrhosis and liver failure. Bile acids induce apoptosis, ER stress and mitochondrial membrane instability. In this study we aimed to investigate the role of cytosolic short form of ALR (Augmenter of Liver Regeneration) in the synthesis of bile acids and bile acid-induced apoptosis. Human hepatoma cells over-expressing the short form of ALR (sfALR, 15 kDa) were incubated with glycochenodeoxycholic acid (GCDCA), and then primary bile acids' production and apoptosis were analyzed. High levels of cytosolic sfALR reduced CYP7A1 mRNA expression and activity, the rate-limiting enzyme in the classic pathway of bile acid synthesis. This reduction was attributed to the STAT3 (signal transducer and activator of transcription 3) activation and reduction of HNF4α (Hepatocyte nuclear factor 4α). Furthermore, apoptosis induction by GCDCA and TRAIL was reduced in cells over-expressing sfALR which was attributed to reduced expression of death receptor 5 (DR5). We found decreased hepatic mRNA levels of ALR and FOXA2 (Forkhead Box A2), an inducer of ALR expression, in human cholestatic liver samples which might explain the increased accumulation of bile acids and bile acid-induced apoptosis in cholestasis patients.

1. Introduction

Gallstones, local tumors and the genetic deficiencies in bile acids exporters amongst other different etiologies are common causes of cholestatic liver diseases [1, 2]. By definition, cholestasis represents pathophysiologic syndromes defined as an impaired bile flow from the liver. As an outcome, bile acids accumulate and promote hepatocytes injury followed by the development of liver cirrhosis and liver failure [3].

Considered to be both lipids emulsifiers and signaling molecules, bile acids are preserved in normal biological levels by a tight regulation feedback-loop. The *de novo* synthesis of bile acids proceeds *via* two different pathways: the classical pathway and the alternative pathway. First, the classical pathway accounts for 90% of the total bile acid pool, thereby the regulation of its rate-limiting enzyme cytochrome P450 7A1 (CYP7A1) has received great attention. CYP7A1 initiates the catabolism of cholesterol into primary bile acids (cholic acid: CA and chenodeoxycholic acid: CDCA) [4, 5]. Second, the alternative pathway of bile acid synthesis (also known as the acidic pathway) accounts for 10% of the total bile acid pool and is rate-limited by CYP27A1 (mitochondrial 27-hydroxylase). The main product of this pathway is CDCA [6, 7]. The promoter of CYP7A1 gene harbors several response elements to which different transcription factors can bind and further activate or repress its activity. Hepatocyte nuclear factor 4α (HNF4α) and Liver-related homolog-1 (LRH-1) bind to the “bile acid response element II” (BARE II) within CYP7A1 promoter and activate its transcription. Upon accumulation, bile acids bind to their nuclear receptor, farnesoid x receptor (FXR) [8], which in turn activates small heterodimer partner (SHP), SHP then blocks HNF4α and LRH-1-mediated activation of CYP7A1 [9]. MicroRNAs could also regulate CYP7A1 indirectly by controlling transcription factors known to regulate its expression. It was reported that STAT3 (signal transducer and activator of transcription 3) phosphorylation activates miRNA24 and miRNA629 leading to downregulation of HNF4α [10].

Under cholestatic conditions, hepatocytes are exposed to elevated concentrations of bile acids, which alters gene expression in different pathways. Moreover, bile acids were proved to induce apoptosis by increasing the expression of TRAIL-receptor 2, also known as death receptor 5 (DR5), through activation of JNK pathway, thereby sensitizing hepatocytes to TRAIL-mediated apoptosis [11, 12]. Bile acid-induced apoptosis can also be mediated by Fas receptor (FasR or CD95) [13]. Upon micromolar exposure of glycochenodeoxycholate (GCDCA), hepatocytes induce

FasR aggregation on the plasma membrane [13, 14] which promotes its oligomerization and initiates a death-signaling pathway [15].

Augmenter of liver regeneration (ALR), a hepatotrophic growth factor [16], is constitutively expressed and stored in the hepatic parenchymal cells [17-19]. Subcellular fractionation of mitochondria and cytosol of primary human hepatocytes (PHH) revealed the expression of mainly 3 isoforms of ALR, a 21 and a 23 kDa isoform, both located in mitochondria and cytosol and a 15 kDa isoform (the short form, hereafter sfALR) detected solely in the cytosol of hepatocytes [20]. sfALR reduces ER-stress, lipoapoptosis and cellular steatosis following treatment with free fatty acids [20]. In addition, we have previously shown the ALR is induced by Nrf2 (nuclear factor erythroid-2 like factor 2) [21] which is known to regulate anti-oxidative genes like GCLC (glutamate cysteine ligase catalytic subunit) and NQO1 (NAD(P)H quinone oxidoreductase 1) [22, 23]. Interestingly, high levels of cytosolic sfALR induce the reaction of hepatocytes to IL6 treatment by increasing the tyrosin phosphorylation of STAT3, without affecting STAT3 expression, leading to altered gene expression in liver cells [24].

The aim of the current study was to analyze the expression of ALR under cholestatic conditions and to elucidate a potential protective effect of sfALR in cholestatic liver injuries, in regards to bile acid synthesis and accumulation as well as bile acid-induced apoptosis.

2. Materials and methods

2.1. Human liver samples and primary human hepatocytes

Experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR (Human Tissue and Cell Research, Regensburg, Germany), with the written informed patient's consent. The study and the consent form were approved by the local ethical committee of the University of Regensburg (ethics statement 12-101-0048, University of Regensburg, Germany). All experiments involving human tissues and cells have been carried out in accordance to The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Human liver tissues from 58 patients were collected, including 45 liver samples for the “cholestasis” group and 13 samples of “normal” liver tissue. Cholestatic livers (n=13 obstructive extrahepatic cholestasis, n=3 obstructive intrahepatic cholestasis, n=24 non-obstructive cholestasis) were chosen based on histopathological

examination of liver sections with positivity for proliferating bile ducts. Normal liver samples were collected from patients with liver metastasis due to colorectal carcinoma. Liver sections collected from those patients showed no signs of histopathological cholestasis (for clinico-pathological characteristics of the cohort see Table 1). Primary human hepatocytes (PHH) were isolated from normal (n=3) and cholestatic (n=8) liver tissues and analyzed by qRT-PCR [21, 25].

Table (I): Clinico-pathological parameters of patients in the study cohort (serum parameters: Bilirubin, Alkaline phosphatase (ALP), Alanine transaminase (ALT), Gamma-glutamyltransferase (GGT). Data are showed as mean and range of values) and p values between normal and cholestatic patients.

Parameter	**Normal liver** (n=13)	**Cholestatic liver** (n=45)	***P* value**
Gender (female / male)	(7/6)	(28/17)	-
Age [years] (mean/range)	55,92 (30-66)	52,62 (19-78)	0,502
Bilirubin [mg/dl]	0,60 (0,30-0,90)	9,06 (1,00-34,43)	**<0,001**[a]
ALP [U/L]	83,20 (46-137)	222,60 (23-540)	**<0,001**
ALT [U/L]	21,98 (8-33)	169,54 (5-1959)	**<0,001**
GGT [U/L]	n.d.	364,88 (15-1558)	-

[a] Boldfaces indicate significant differences.

2.2. Cell culture and treatments

The human hepatoma cell line HepG2 was obtained from American Type Culture Collection (HB-8065, ATCC, Manassas, VA) and grown at 37°C, 5% CO2 in DMEM (BioWhittaker, Verviers, Belgium) supplemented with penicillin (100 units/ml), streptomycin (10 µg/ml) and 10% fetal calf serum (Biochrom, Berlin, Germany). Cells were seeded at a density of 5x104 cells/cm2 and treated for 24 hours with 100 µM of each bile acid (glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid

(TCDCA), taurolithocholic acid (TLCA) and glycocholic acid (GCA) all obtained from Sigma-Aldrich, St. Louis, MO). For caspase 3/7 assays, 100 µM of AG490 (Merck, Darmstadt, Germany) and 25 ng/ml TRAIL (Abcam, Cambridge, UK) were applied to cells for 24 and 6 hours respectively. Supernatants were collected and mRNA and protein samples were isolated and appropriately stored for upcoming analyses. Stable transfection has been previously reported [26]. Transfected HepG2 cells with sfALR are referred to as HepG2-sfALR cells.

2.3. RNA isolation, reverse transcription and qRT-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). One µg of total RNA was reverse-transcribed using the Reverse Transcription System (Qiagen, Hilden, Germany). Transcript levels of CYP7A1, HNF4α, HPRT, ALR, FOXA2 and YWHAZ were quantified using real-time PCR technology (Roche, Penzberg, Germany). Amplicons were verified by sequence analysis and each quantitative PCR was performed in duplicates for 3 sets of RNA preparations. The following primers were used: for CYP7A1, sense 5'-gaatgctggtcaaaaagtc-3' and anti-sense 5'-tgaaatcctccttagctgt-3', for HNF4α, sense 5'-tgtcccgacagatcacctc-3' and anti-sense 5'-cactcaacgagaaccagcag-3', for HPRT, sense 5'-tgacactggcaaaacaatgca-3' and anti-sense 5'-ggtccttttcaccagcaagct-3', for ALR, sense 5'-gaagcgggacaccaagttta-3' and anti-sense 5’-ttcagcacactcctcacagg-3’, for YWHAZ, sense 5'-gcaattactgagagacaactt gaca-3' and anti-sense 5'-tggaaggccggttaatttt-3' and for FOXA2 sense 5’-ccgttctccatc aacaacct-3’ and anti-sense 5’-ggggtagtgcatcacctgtt-3’.

2.4. Measurement of primary bile acids in supernatant (effeciency of bile acid synthesis enzymes)

HepG2 and HepG2-sfALR cells were cultured at a density of 5x104 cells/cm2, after 24 hours, 800 µL of serum free medium was applied to the cells and 4 hours later the supernatant was collected to determine the levels of primary bile acids (free and conjugated) by LC-MS/MS. The measured concentrations were later normalized to protein concentration in each sample. This method of measurement has been previously reported [27] and recently updated [28].

2.5. SDS-PAGE and immunoblotting

Total protein homogenates (30 µg per lane) were separated by 12% SDS-PAGE (Biorad, Hercules, CA, USA) under reducing conditions using 100 mM DTT. Proteins were transferred onto PVDF membranes (Biorad, Hercules, CA, USA), incubated with specific antibodies and developed with ECL reactions (Pierce, Rockford, IL, USA). Nuclear fractions were prepared using NE-PERTM Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific, Darmstadt, Germany). Following antibodies were used: anti-HNF4α (#3113), anti-death receptor 4 (#42533), anti-FasR (C1812,), anti-STAT3 (#9139), anti-ß-actin (#4970), anti-GAPDH (#5174), anti pNFκB (p-p65) (#8242) and anti-NFκB (p65) (#3033) all obtained from Cell Signaling (Danvers, MA). Anti-HDAC1 (#05-100) purchased from Merck (Darmstadt, Germany), anti-death receptor 5 (DR5) (#ab8416) and anti-pSTAT3 (Tyr705) (#ab76315) were obtained from Abcam (Cambridge, UK). Secondary goat HPR-conjugated antibodies (anti-rabbit # P0448 and anti-mouse #P0447) were obtained from Dako (Hamburg, Germany).

2.6. Caspase 3/7 activity

Following treatments, cells were subjected to caspase 3/7 activity measurement with Caspase-Glo 3/7 Assay (Promega, Madison, USA) according to the manufacturer's instructions.

2.7. Statistical analysis

All data are presented as mean plus/minus standard deviation of the mean. Data were compared between groups using Mann-Whitney test. p value of <0,05 was considered significant. Correlation coefficients were calculated using Spearman’s correlation test.

3. Results

3.1. Over-expressed cytosolic short form ALR (sfALR) diminishes the expression of CYP7A1 and bile acid synthesis

Initially, to investigate the potential effect of over-expressed cytosolic sfALR on the *de novo* synthesis of bile acids we used HepG2 (expressing the long forms of ALR, 23 and 21 kDa) and HepG2-sfALR cells (additionally over-expressing short form of ALR, 15 kDa) (Fig. 1A). mRNA expression of CYP7A1 was analyzed by qRT-PCR in HepG2 and sfALR-expressing HepG2 cells with or without incubation with different bile acids. Since TCDCA, GCDCA, GCA (primary bile acids) and TLCA (secondary bile acid) were shown to be elevated in cholestatic patients [29] we chose those bile acids to compare their potential effect in the sfALR-mediated regulation of CYP7A1. HepG2-sfALR cells showed a significantly less CYP7A1 expression (60% reduction) compared with wild type HepG2 cells without treatment (Fig. 1B). Interestingly, treatment with TCDCA and TLCA almost abolished CYP7A1 mRNA expression in HepG2-sfALR cells when compared with similarly treated HepG2 cells (Fig. 1B). In addition, the mRNA expression of CYP27A1, the rate-limiting enzyme for the alternative pathway of bile acids synthesis, was analyzed and showed no significant change in HepG2-sfALR cells (data not shown). Moreover, CYP8B1 (involved in subsequent steps after CYP7A1 in the classical pathway of CA synthesis) was analyzed and showed no significant change in HepG2-sfALR cells (data not shown).

To confirm the mRNA data, we used LC-MS/MS to assess the efficiency of bile acid synthesis enzyme by determining the levels of taurine- and glycine-conjugated primary bile acids (CA and CDCA), products of the classic pathway of bile acid synthesis. HepG2-sfALR cells, compared to HepG2 cells, showed a 30% reduction in total primary bile acid pool (free and conjugated) (Fig. 1C). This reduction in primary bile acids in HepG2-sfALR was attributed mainly to a reduction in CDCA concentration and partially to reduction in TCA concentration (Fig. 1D).

These data indicate that over-expressed cytosolic sfALR reduces the *de novo* synthesis of bile acids by the classical pathway through modulating the expression of its rate limiting enzyme CYP7A1 without affecting the alternative pathway of bile acid synthesis. On the other hand, the proportions of free and conjugated bile acids were not altered in HepG2-sfALR cells which suggests that sfALR reduces the overall pool of primary bile acids without affecting its composition (data not shown).

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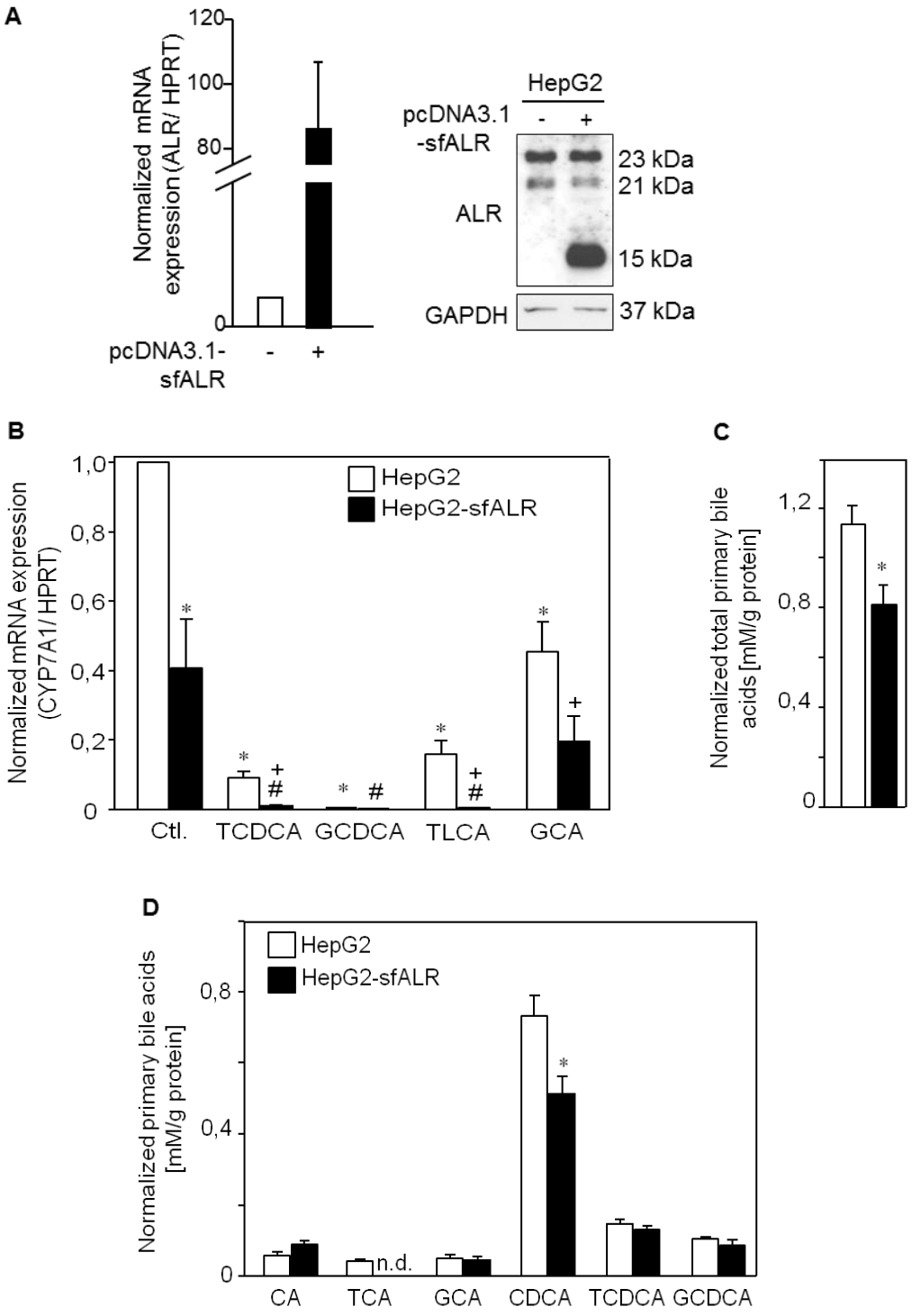


Fig. 1. Over-expressed cytosolic sfALR diminishes the expression and the activity of CYP7A1. (**A**) HepG2 and HepG2 over-expressing sfALR (HepG2-sfALR) cells were analyzed for ALR mRNA levels by qRT-PCR with HPRT as housekeeping gene and normalized to HepG2 cells (three independent experiments, mean ± SD). Total protein was isolated and isoforms of ALR and GAPDH were analyzed by western blot techniques (GAPDH served as a loading control). (**B**) HepG2 and HepG2 expressing sfALR (HepG2-sfALR) cells were incubated for 24 hours with or without 100 µM of each bile acid. mRNA levels of CYP7A1 were analyzed by qRT-PCR with HPRT as housekeeping gene and normalized to untreated HepG2. The supernatants from untreated HepG2 and HepG2-sfALR cells were collected and the concentration of total (**C**) and specific (**D**) primary bile acids was determined using LC-MS/MS and normalized to protein concentration. p<0,05 * compared to untreated HepG2 cells, # compared to untreated HepG2-sfALR cells, + compared to the corresponding treated HepG2 cells.

3.2. sfALR represses CYP7A1 *via* activating STAT3 and thereby inhibiting HNF4α

The expression and therefore activity of CYP7A1 undergo tight regulation by various transcription factors. Since HNF4α is known to activate CYP7A1 expression, we aimed to analyze its expression in HepG2 and HepG2-sfALR cells using qRT-PCR. As shown in Fig. 2A, a significant decrease in the expression of HNF4α in HepG2-sfALR cells was observed (approx. 67%). This reduction was confirmed by western blot showing a reduction in HNF4α protein in HepG2-sfALR compared to HepG2 cells (Fig. 2B). It has been previously shown that HNF4α is downregulated upon phosphorylation of STAT3 on tyrosine 705 [10]. Therefore, we aimed to assess the phosphorylation of STAT3 in HepG2-sfALR cells after treatment with GCDCA. As shown in Fig. 2B, STAT3 phosphorylation is higher in HepG2-sfALR compared to HepG2 cells. Our data suggest that sfALR modulates CYP7A1 expression, at least partially, by activating STAT3 and thereby downregulating HNF4α.

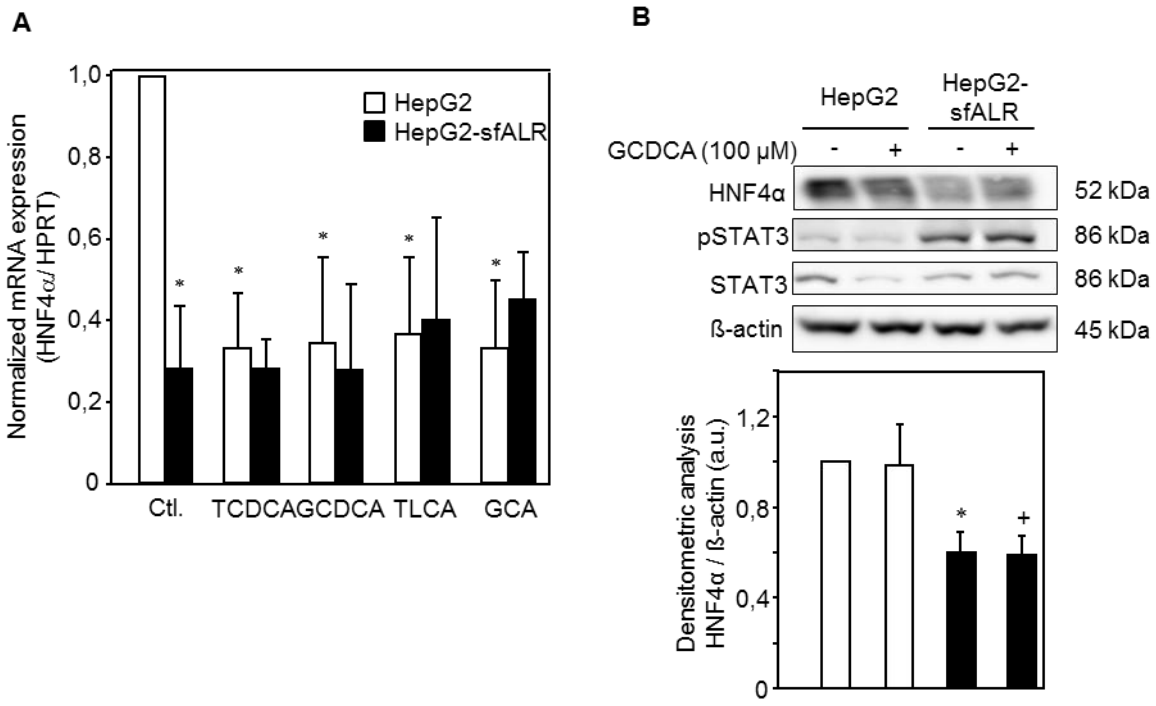


Fig. 2. Diminished CYP7A1 expression in HepG2-sfALR is attributed to the activation of STAT3 and absence of HNF4α. HepG2 and HepG2-sfALR cells were incubated for 24 hours with or without 100 µM of the respective bile acid, (**A**) mRNA levels of HNF4α were analyzed by qRT-PCR with HPRT as housekeeping gene and normalized to untreated HepG2. (**B**) Total protein was isolated and protein expression of HNF4α, pSTAT3, STAT3 and β-actin were analyzed by western blot techniques. Immunoblots from 3 different experiments were analyzed by densitometric analysis and one representative is shown. $p<0,05$ * compared to untreated HepG2 cells, + compared to GCDCA-treated HepG2 cells.

3.3. Over-expressed sfALR decreases the bile acid-induced apoptosis

Several toxic bile acids were shown to contribute to liver injury by inducing apoptosis. Therefore the activity of caspase 3/7 was investigated in HepG2 and HepG2-sfALR cells after treatment with GCDCA and/or apoptosis-inducer TRAIL (TNFα-related apoptosis inducing ligand). GCDCA was chosen for the assays due to its known toxicity and elevated concentrations in patients with cholestasis [30]. As shown in Fig. 3A, HepG2-sfALR cells showed lower caspase 3/7 activity (approx. 70%) before and after treatment with GCDCA or TRAIL compared to their corresponding HepG2 cells. Furthermore, the combination of GCDCA and TRAIL induced caspase 3/7 activity in both HepG2 and in HepG2-sfALR cells by 3 folds. However, GCDCA and TRAIL-treated HepG2-sfALR show a 30% reduction in caspase 3/7 activity when compared to the similarly treated HepG2 cells. This finding suggests that over-expressed cytosolic sfALR has a protective effect against apoptosis under cholestatic conditions.

To elucidate the mechanism of the anti-apoptotic effect of sfALR, the expression of the TRAIL death receptors, DR5 and DR4, was determined in HepG2-sfALR and HepG2 cells in absence of presence of GCDCA. A decreased expression of DR5 in HepG2-sfALR cells was noted after treatment with GCDCA compared to HepG2 cells (Fig. 3B). On the other hand, no different expression in DR4 was seen after treatment with GCDCA in both cell lines (Fig. 3C). Furthermore, FASR expression, known to be involved in bile acid-induced apoptosis, was not altered in HepG2-sfALR cells after GCDCA treatment compared to HepG2 cells (Fig. 3C). Our data suggest that sfALR protects hepatocytes from bile acid- and TRAIL-induced apoptosis at least in part by downregulating DR5 expression.

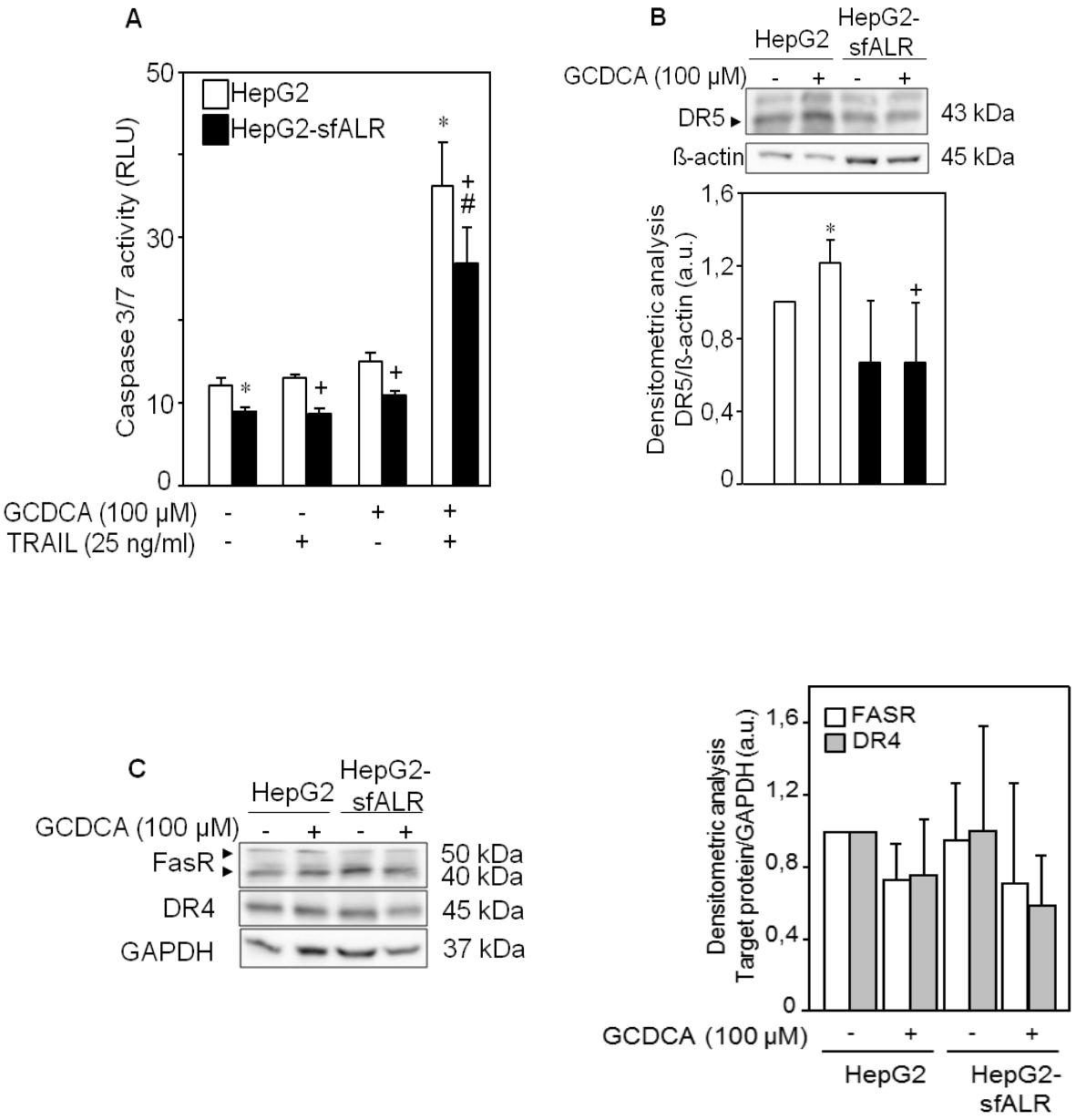


Fig. 3. Over-expressed sfALR protects the cells from TRAIL-induced apoptosis by downregulating DR5 expression. (**A**) HepG2 and HepG2-sfALR cells were incubated with or without 100 µM GCDCA for 24 hours. After 6 hours treatment with death receptor ligand TRAIL (25 ng/ml), caspase 3/7 activity was determined. HepG2 and HepG2-sfALR cells were incubated with GCDCA for 24 hours and total protein was isolated to analyze protein expression of DR5 (**B**), DR4 and FASR (**C**) by western blot (β-actin and GAPDH served as loading controls). Immunoblots from 3 different experiments were analyzed by densitometric analysis and one representative is shown. p<0,05 * compared to untreated HepG2 cells, # compared to untreated HepG2-sfALR cells,+ compared to corresponding treated HepG2 cells.

3.4. ALR exhibits its anti-apoptotic effects *via* activation of STAT3

Known to regulate the expression of DR5, the activation of both NFκB and STAT3 was assessed in HepG2 and HepG2-sfALR cells. As shown in Fig. 4A (see also Fig. 2B), the tyrosin phosphorylation of STAT3 (a repressor for DR5 expression), but not expression, was increased with or without GCDCA treatment in HepG2-sfALR compared to HepG2 cells. The importance of pSTAT3 for the protective effects of

sfALR was also investigated by the use of the tyrphostin tyrosine kinase inhibitor AG490 that has been extensively used for inhibiting JAK2 (Janus Kinase 2) and thereby the phosphorylation of STAT3 [31] without affecting STAT3 expression. A measurement of caspase 3/7 activity in HepG2-sfALR cells showed an overall increase in the caspase 3/7 activity in the AG490-treated cells with or without adding TRAIL and/or GCDCA (Fig. 4B). In addition, the expression of DR5 was increased in HepG2-sfALR after treatment with AG490 and GCDCA compared to HepG2-sfALR treated only with GCDCA (Fig. 4C). Additionally, the activation of NFκB (a DR5-inducer) was analyzed in HepG2-sfALR cells. Fig. 4D shows that NFκB activation is not altered in HepG2-sfALR. Taken together, these results demonstrate that the protective effects of sfALR are attributed to the activation of STAT3 which leads to a decreased DR5 expression and therefore protecting the liver from DR5-mediated apoptosis.

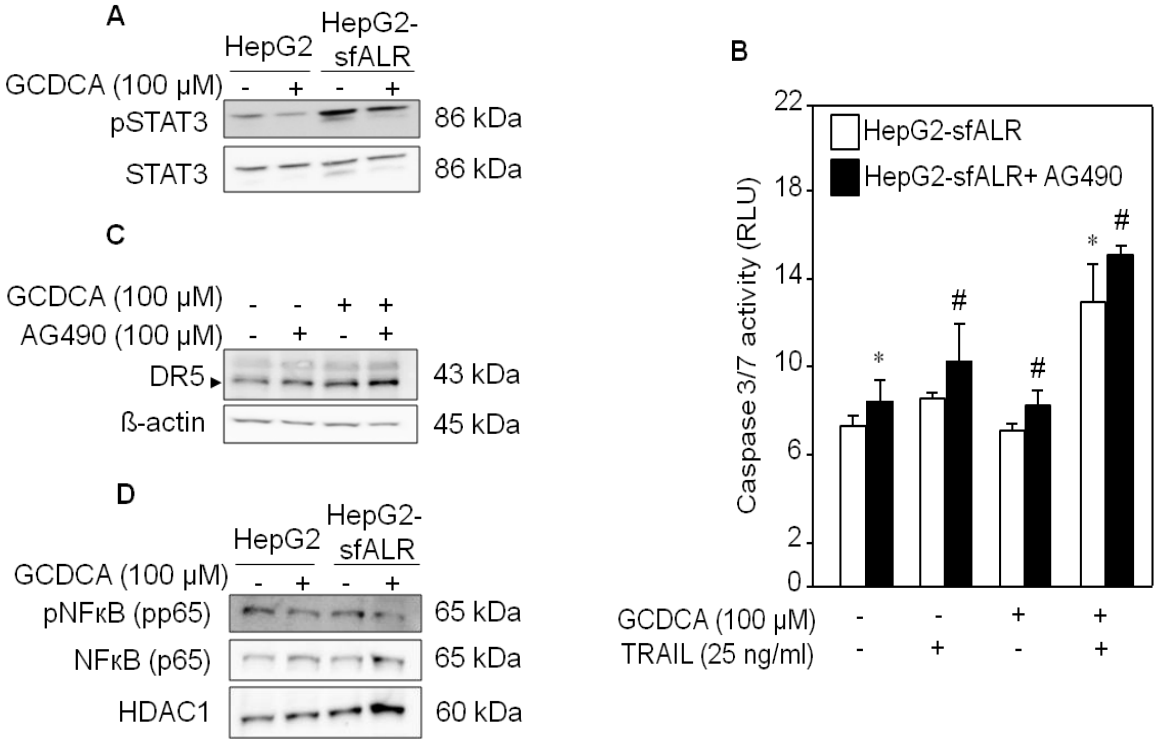
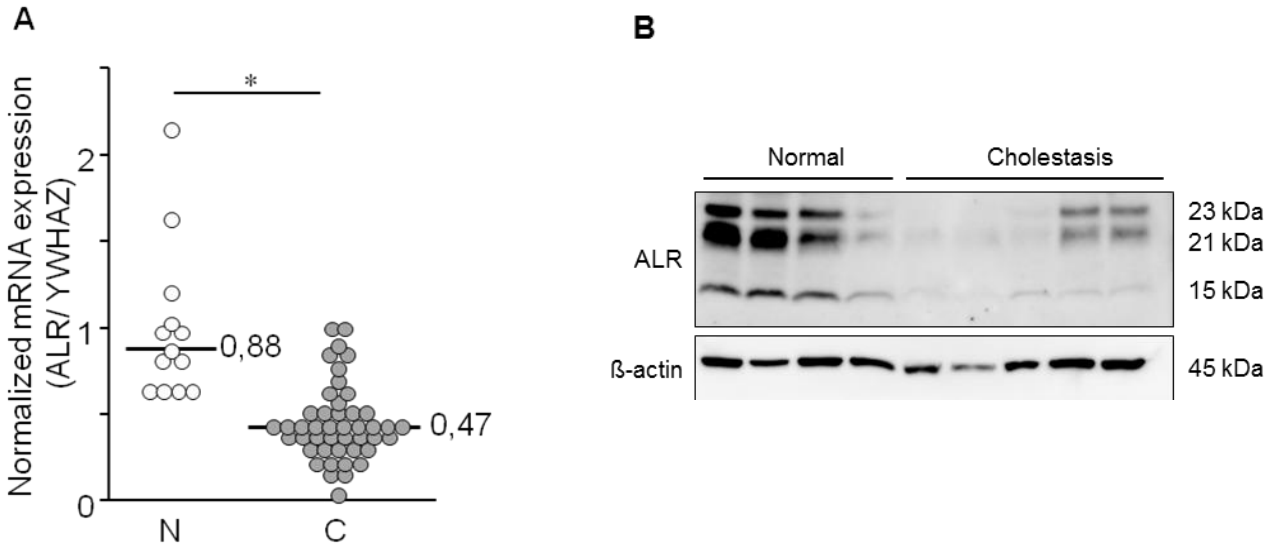


Fig. 4. Over-expressed sfALR exhibits its anti-apoptotic effects *via* activation of STAT3. (**A**) HepG2 and HepG2-sfALR cells were incubated with GCDCA for 24 hours and total protein was isolated to analyze protein levels of pSTAT3 and STAT3 (ß-actin served as a loading control). (**B**) HepG2-sfALR cells were incubated with or without 100 µM GCDCA and/or AG490 for 24 hours. After treatment with 25 ng/ml TRAIL for 6 hours caspase 3/7 activity was analyzed. $p<0,05$ * compared to untreated HepG2-sfALR cells, # compared to corresponding HepG2-sfALR cells. (**C**) HepG2-sfALR cells were incubated with 100 µM GCDCA and/or AG490 for 24 hours and total protein was isolated to analyze protein expression of DR5 and ß-actin by western blot techniques. (**D**) After treatment of HepG2 and HepG2-sfALR cells with GCDCA for 24 hours, nuclear extracts were prepared and total as well as phosphorylated NFκB (p65) were analyzed by western blotting (HDAC1 served as a loading control). One representative immunoblot out of 3 different experiments is shown.

3.5. Reduced ALR expression in liver tissue from patients with cholestasis

Previous results indicate that enhanced levels of cytosolic sfALR lead to reduced apoptosis and reduced bile acid pool, therefore we aimed to investigate the expression of ALR in hepatic tissues under cholestatic conditions. mRNA expression of ALR in 13 normal liver tissues were compared to 45 samples collected from patients with histopathological proven cholestasis. ALR expression is significantly reduced in cholestatic (0,46±0,22) compared to normal liver samples (1,01±0,44) (Fig. 5A). Furthermore, ALR is inversely expressed compared to bilirubin serum levels (ALR expression in patients with bilirubin <1mg/dL (n=14): 1,01±0,44 vs. patients with bilirubin ≥1 mg/dL (n=44) 0,45±0,22) with a $p<0,05$. However, no correlation was detectable between ALR expression and serum ALP (Alkaline phosphatase), ALT (Alanine transaminase) or GGT (Gamma-glutamyltransferase). Moreover, ALR protein expression was investigated in normal liver tissue (n=4) and liver tissue collected from patients with cholestasis (n=5) (Fig. 5B). Western blot analysis confirms our mRNA study demonstrating a reduction of primarily sfALR (15 kDa) protein expression in cholestatic liver tissue. Furthermore, we analyzed the mRNA expression of ALR and FOXA2 (Forkhead Box A2, an inducer of ALR expression [32]) in primary human hepatocytes isolated from 3 normal and 8 cholestatic liver tissues. Interestingly, the expression of ALR positively correlates with FOXA2 expression (r=0,900) (Fig. 5C).



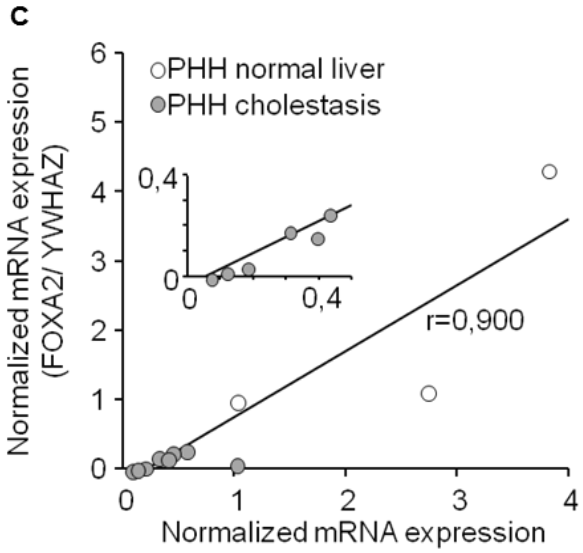


Fig. 5. Reduced ALR mRNA expression (ALR/ YWHAZ) in liver tissues collected from patients with cholestasis. (**A**) Expression of ALR mRNA was analyzed by qRT-PCR in liver tissue samples from patients with histopathological proven cholestasis (n=45) and normal liver tissue (n=13), median is indicated with a black bar. * p<0,05 compared to normal liver tissues. (**B**) Protein expression of ALR was analyzed by western blotting in liver tissue samples from patients with histopathological proven cholestasis (n=5) and normal liver tissue (n=4). (**C**) Primary human hepatocytes (PHH) were isolated from normal (n=3) and cholestatic (n=8) liver tissues and mRNA expression of ALR and FOXA2 was analyzed by qRT-PCR technique and normalized to YWHAZ. A correlation plot of ALR and FOXA2 mRNA expression is shown with best fit line for normal (n=3) and cholestatic (n=8) liver tissue samples (r=0,900). Insert demonstrates a magnification of the samples with an mRNA expression below 0,5 [gene expression/YWHAZ].

4. Discussion

Augmenter of liver regeneration (ALR), a hepatotrophic co-mitogen, was shown to play a pivotal role in liver regeneration *in vivo* [33, 34]. After partial hepatectomy, ALR was found to be upregulated in the liver [35]. Additionally, it was previously demonstrated that ALR can modulate hepatic metabolism [36]. Furthermore, it was shown that over-expression of sfALR alters IL6-signaling by inducing STAT3 phosphorylation [24], counteracts palmitic acid-induced apoptosis and reduces both ER stress and PA-induced DR5 expression [20].

Cholestatic liver diseases are pathophysiologic syndromes, caused by different etiologies [1, 2] and defined as an impaired bile flow from the liver. The accumulation of cytotoxic bile acids activates different signaling pathways and interrupts multiple metabolic processes [37] thereby the transcriptional regulation of CYP7A1, the rate-limiting enzyme of the classical pathway in bile acids synthesis, has received great attention. Furthermore, many studies were dedicated to understanding the molecular mechanism of cholestatic injury [38]. Bile acid-induced apoptosis is considered the main cause of injury [13, 15, 39]. It has been shown that both Fas

receptor (FasR) and TRAIL-Receptor 2 (DR5) are involved in bile acid-induced apoptosis [11, 13, 15, 40, 41].

The regulation of CYP7A1 by bile acids has been well described [9, 42, 43]. The FXR/SHP/HNF4α feedback loop is considered the main pathway of bile acid-mediated negative feedback [9, 42, 44, 45]. We found that the expression and activity of CYP7A1, but not CYP27A1, are reduced under conditions of high cytosolic sfALR. Interestingly, the expression of HNF4α, an activator of CYP7A1, was also decreased in HepG2-sfALR cells. A previous study has shown that activation of STAT3 results in a reduced CYP7A1 and CYP27A1 expression [46]. Moreover, STAT3Δhc mice (specific ablation of STAT3 in mouse hepatocytes and cholangiocytes) show an increased bile acid pool [47]. Interestingly, over-expression of cytosolic sfALR was shown to increase the activation of STAT3 by increasing the tyrosine phosphorylation upon IL6 treatment [24]. On the other hand, STAT3 phosphorylation was shown to activate miRNA24 and miRNA629 which in turn reduce the expression of HNF4α, a main activator of CYP7A1 [10]. Thereby we postulate that high levels of cytosolic sfALR, through activating STAT3 phosphorylation, reduce the expression of HNF4α and thus repress the expression of CYP7A1 and bile acids production.

Bile acids induce liver injury by apoptosis in a death receptor-dependent and -independent mechanisms [11, 12]. Upon treatment with deoxycholic acid (DCA), DR5 expression is increased, whereas DR4 remains unchanged, due to the activation of JNK pathway and the activation of SP1 binding to its response element within DR5-promoter [12]. Higuchi et al. have also shown that GCDCA treatment *in vitro* increased the DR5 expression and thus increased apoptosis [11]. In addition, sfALR expression reduces the activation of JNK upon treatment with free fatty acid [20]. Although HepG2-sfALR cells showed a 30% reduction in caspase 3/7 activity compared to HepG2 cells, the combination of TRAIL and GCDCA treatment lead to a 3 fold increase in caspase 3/7 activity in both cells lines, which suggests that sfALR anti-apoptotic effects are independent from the activation of JNK pathway by GCDCA treatment. Nevertheless, over-expressed sfALR preserved the reduced apoptosis even under extreme cholestatic conditions (high concentrations of GCDCA and TRAIL). It is worthwhile to mention that the expression of NTCP (sodium taurocholate co-transporting polypeptide, responsible for the uptake of bile acids into hepatocytes) is induced by HNF4α [48], which might explain the reduced caspase 3/7 activity in

GCDCA treated HepG2-sfALR cells due to reduced HNFα4 levels. Furthermore, Denk and colleagues have shown that transfection of the 23 kDa isoform of ALR (located in the intermembrane space of mitochondria) into Huh7 cells over-expressing NTCP did not reduce the GCDCA-induced apoptosis [49] which may confirm the different roles of ALR isoforms. In the current study we used HepG2 cells that possess 40% of normal liver's content of OATP (organic anion transporting polypeptide) which mediates hepatocellular uptake of bile acids [50]. Interestingly, using HepG2-NTCP cells diminished the protective effect of ALR (data not shown) which suggests that endogenous sfALR exerts its anti-apoptotic effects by regulating death receptors and is not able to reduce the toxicity induced by accumulation of intracellular bile acids. We identified DR5 as the death receptor responsible for the reduced apoptosis in HepG2-sfALR. The expression of DR5 is reduced by the activation of STAT3 [51]. STAT3 activation, characterized by tyrosine (705) phosphorylation, is increased by over-expression of sfALR. Interestingly, blocking STAT3 activation by JAK2 inhibition increased DR5 expression and caspase 3/7 activity in HepG2-sfALR cells. Furthermore, NFκB activation was shown to induce DR5 expression [52], but we did not find any altered NFκB activation in HepG2-sfALR cells compared to HepG2 cells. Taken together, we hypothesize that sfALR's anti-apoptotic effects are attributed, at least in part, to its ability to maintain the activation of STAT3 and thereby reducing DR5 expression and cell sensitivity towards extrinsic death signals like TRAIL.

It is worth to mention that GCDCA reduces the phosphorylation of STAT3 by diminishing gp130 (IL6 receptor) expression. This contributes to the hepatotoxic effects of bile acids by eliminating the positive effects of IL6 signaling on liver regeneration under cholestatic conditions [53]. Furthermore, liver specific gp130 knock-out mice that underwent bile duct ligation showed impaired acute-phase response leading to reduced survival and an increase of bacterial growth in the bile and the liver [54]. Therefore we postulate that the induction of sfALR expression in hepatocytes during cholestasis might possibly serve in a preserved STAT3 activation and thereby a better protection against secondary biliary infections. However, the molecules involved in STAT3 activation by sfALR are still unknown. It was previously shown that sfALR-HepG2 cells show increased STAT3 phosphorylation at tyrosin (705) whereas STAT3 phosphorylation at serine (727) as well as total STAT3 remain unchanged [24]. In addition, it was shown that repressors of STAT3 signaling like

PIAS3 (protein inhibitors of STATs 3) as well as SOCS3 (suppressor of cytokines signaling 3) remain unchanged in HepG2-sfALR cells [24]. Therefore, investigating the molecules that link over-expressed sfALR with STAT3 activation is of great importance.

Moreover, in this study we found reduced hepatic ALR mRNA and protein expression in patients with cholestasis compared to normal liver tissues. Furthermore, ALR expression correlated strongly with FOXA2 expression in primary human hepatocytes (an inducer of ALR expression).This is in line with a report showing that the expression of FOXA2 is reduced in patients with cholestasis and in rodent models of cholestasis [55, 56]. Taken together, we may postulate that FOXA2 deficiency leads to reduced cytosolic ALR expression resulting in aggravating cholestatic injury by increased accumulation of bile acids, increased DR5 expression and therefore susceptibility of hepatocytes to apoptosis. The detrimental effects of bile acids might be thereby reversed by elevating ALR levels in hepatocytes which suggests ALR as a possible target for therapy in cholestatic liver diseases.

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Chapter 6

Role of recombinant ALR in reducing bile acids toxicity during liver regeneration

Abstract

Bile acids are both amphiphilic agents that facilitate lipid absorption and signaling molecules that regulate different cellular process. Bile acid synthesis is restricted to hepatocytes and is rate-limited by CYP7A1 (cholesterol 7α hydroxylase). CYP7A1 undergoes tight regulation through different transcription factors and is repressed after partial hepatectomy in rodents to prevent the accumulation of toxic bile acids in the remaining hepatocytes. Augmenter of Liver Regeneration (ALR) is a hepatotrophic factor that was shown to be increased after partial hepatectomy. Nevertheless, little is known about the role of ALR in regulating CYP7A1 expression and therefore potentially protecting hepatocytes from bile acid accumulation and bile acid-induced apoptosis. Therefore, human hepatocytes were incubated with recombinant human ALR (rhALR) and the expression of CYP7A1 as well as bile acid-induced apoptosis were analyzed. We found that application of rhALR reduces CYP7A1 expression by reducing the expression of its transcriptional activator CAR (Constitutive Androstane Receptor). Moreover, rhALR reduced apoptosis induction by toxic concentrations of bile acids (intrinsic apoptosis) but showed no effect on the expression of death receptor 5 (DR5) or the extrinsic apoptosis (ligand-dependent apoptosis) under cholestatic conditions. Taken all together, rhALR might contribute to protecting hepatocytes from toxic concentrations of bile acids and thereby this suggests a new role of ALR in augmenting the process of liver regeneration.

1. Introduction

Bile acids are physiological detergents that facilitate the absorption and transport of sterols and lipid-soluble vitamins as well as the disposal of toxic metabolites and xenobiotics [1]. The synthesis of bile acids is restricted to the liver and proceeds *via* either neutral or acidic pathway [2]. The first and rate-limiting enzyme of the classical pathway is cholesterol 7α hydroxylase (CYP7A1, cytochrome P450 7A1) [3]. CYP7A1 expression is inhibited by bile acids returning to the liver *via* enterohepatic circulation [3]. Due to their amphiphilic nature, bile acids concentrations in hepatocytes are tightly regulated to prevent their toxic effects [1]. bile acids activate their nuclear receptor FXR (Farnesoid X Receptor) that induces the expression of Small Heterodimer Partner (SHP) which then represses the expression of CYP7A1 [4]. Moreover, it has been reported that CYP7A1 is repressed upon partial hepatectomy and after liver damage in both animals and patients [5-7]. This repression lowers bile acids concentration and protects remaining hepatocytes from accumulating toxic bile acids [8]. Earlier studies have shown that Hepatocyte Growth Factor (HGF) is involved in the repression of CYP7A1 in hepatocytes [8]. Additionally CYP7A1 over-expression in mice impaired liver regeneration after partial hepatectomy and caused increased hepatocyte death [9], probably due to induced apoptosis in hepatocytes through different mechanisms [10-14]. Augmenter of liver regeneration (ALR) is a hepatotrophic factor [15] that has recently gained grown interest, ALR is found in hepatocytes in 3 isoforms: 23, 21 and 15 kDa [16]. Its 15 kDa accumulates in the liver upon partial hepatectomy and liver injury [17] and during liver regeneration [17, 18]. A previous study by our group also showed increased ALR expression in livers from patients with HCC (hepatocellular carcinoma) and CCC (cholangiocyte carcinoma) compared to normal liver tissue [19]. Furthermore, recombinant ALR (rALR) alters different P450 isoenzyme activities [20] and exerts liver-specific anti-apoptotic effects [21]. Nevertheless, it is not clear whether this highly liver-specific protein is involved in the protection of hepatocytes from cytotoxic bile acids by repression of CYP7A1 or reducing bile acid-induced apoptosis upon liver damage. In this study we show that rhALR represses the expression of CYP7A1 through the activation of NFκB (Nuclear Factor 'kappa-light-chain-enhancer' of activated B-cells) and also reduces the activity of caspase 3/7 in hepatoma cells upon treatment with toxic bile acids.

2. Materials and methods

2.1. Cell culture and treatments

The human hepatoma cell line HepG2 was obtained from American Type Culture Collection (HB-8065, ATCC, Manassas, VA) and grown at 37°C, 5% CO_2 in DMEM (BioWhittaker, Verviers, Belgium) supplemented with penicillin (100 units/ml), streptomycin (10 µg/ml) and 10% fetal calf serum (Biochrom, Berlin, Germany). The recombinant human short form (15 kDa) ALR (rhALR) was prepared as described previously [19]. Cells were seeded at a density of $5x10^4$ cells/cm^2, starved for 24 hours and treated with indicated concentrations of rhALR for 24 hours. Primary human hepatocytes (PHH) were isolated and cultured as reported previously [22], starved for 24 hours and treated with indicated concentrations of rhALR for 24 hours. For Caspase 3/7 assays, 100 µM glycodeoxycholic acids (GCDCA) (Sigma-Aldrich, St-Louis, MO) and/or 25 ng/ml TRAIL (Abcam, Cambridge, UK) were applied to cells for 24 and 6 hours respectively. mRNA and protein samples were isolated for further analysis.

2.2. RNA isolation, reverse transcription and qRT-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). One µg of total RNA was reverse-transcribed using the Reverse-Transcription System (Qiagen, Hilden, Germany). Transcript levels of CYP7A1, CYP27A1, FXR, SHP, HNF4α, CAR and HPRT were quantified using real-time PCR technology (Roche, Penzberg, Germany). Amplicons were verified by sequence analysis and each quantitative PCR was performed in duplicates for 3 sets of RNA preparations. Primers used for qRT-PCR are listed in Table (I).

2.3. SDS-PAGE and immunoblotting

Total protein fractions (30 µg per lane) were separated by 12% SDS-PAGE (Biorad, Hercules, CA, USA) under reducing conditions using 100 mM DTT. Proteins were transferred onto PVDF membranes (Biorad, Hercules, CA, USA), incubated with specific antibodies and developed with ECL reactions (Pierce, Rockford, IL, USA). Following antibodies were used: anti pNFκB (p-p65), (#8242), anti-NFκB (p65) (#3033) and anti-β-actin (#4970) all obtained from Cell Signaling (Danvers, MA). Anti-HDAC1 (#05-100) purchased from Millipore, Merck, Darmstadt, Germany, anti-death receptor 5 (DR5) (#ab8416) was obtained from Abcam (Cambridge, UK). Secondary

goat HPR-conjugated antibodies were obtained from Dako (Hamburg, Germany) (anti-rabbit # P0448 and anti-mouse #P0447).

Table (I): Primers used for qRT-PCR

CYP7A1, sense	5'-gaatgctggtcaaaaagtc-3'
CYP7A1, antisense	5'-tgaaatcctccttagctgt-3'
CYP27A1, sense	5'-cacaaactcccggatcat-3'
CYP27A1, antisense	5'-aggctcagagaaggcagt-3'
FXR, sense	5'-tacatgcgaagaaagtgtcaaga-3'
FXR, antisense	5'-actgtcttcattcacggtctgat-3'
SHP, sense	5'-ggtgcccagcatactcaagaa-3'
SHP, antisense	5'-ggacttcacacagcacccagt-3'
HNF4α, sense	5'-tgtcccgacagatcacctc-3'
HNF4α, antisense	5'-cactcaacgagaaccagcag-3'
CAR, sense	5'-tgatcagctgcaagaggaga-3'
CAR, antisense	5'-aggcctagcaacttcgcata-3'
HPRT, sense	5'-tgacactggcaaaacaatgca-3'
HPRT, antisense	5'-ggtccttttcaccagcaagct-3'

2.4. Caspase 3/7 activity

Following treatments, cells were subjected to Caspase 3/7 activity measurement using Caspase-Glo 3/7 Assay (Promega, Madison, USA) according to the manufacturer's instructions.

2.5. Statistical analysis

All data are presented as mean plus/minus standard deviation of the mean. Data were compared between groups using the student *t*-test. *p* value of $< 0,05$ was considered significant.

3. Results

3.1. Exogenous rhALR reduces the expression of CYP7A1 but not CYP27A1

Initially, we analyzed the potential effect of exogenous rhALR (15 kDa) on the classical and alternative pathway of bile acid *de novo* synthesis. Therefore mRNA expression of CYP7A1 and CYP27A1 (mitochondrial 27-hydroxylase), the rate-limiting enzymes of the classic and alternative pathways of bile acid synthesis respectively, was analyzed by qRT-PCR in HepG2 cells with or without rhALR. rhALR treated-HepG2 cells showed a significantly reduced CYP7A1 expression compared with untreated HepG2 cells after 12 and 24 hours of incubation (Fig. 1A). In addition, the mRNA expression of CYP27A1 showed no significant change in HepG2-sfALR cells (Fig. 1B) which suggests that rhALR reduces only the expression of CYP7A1.

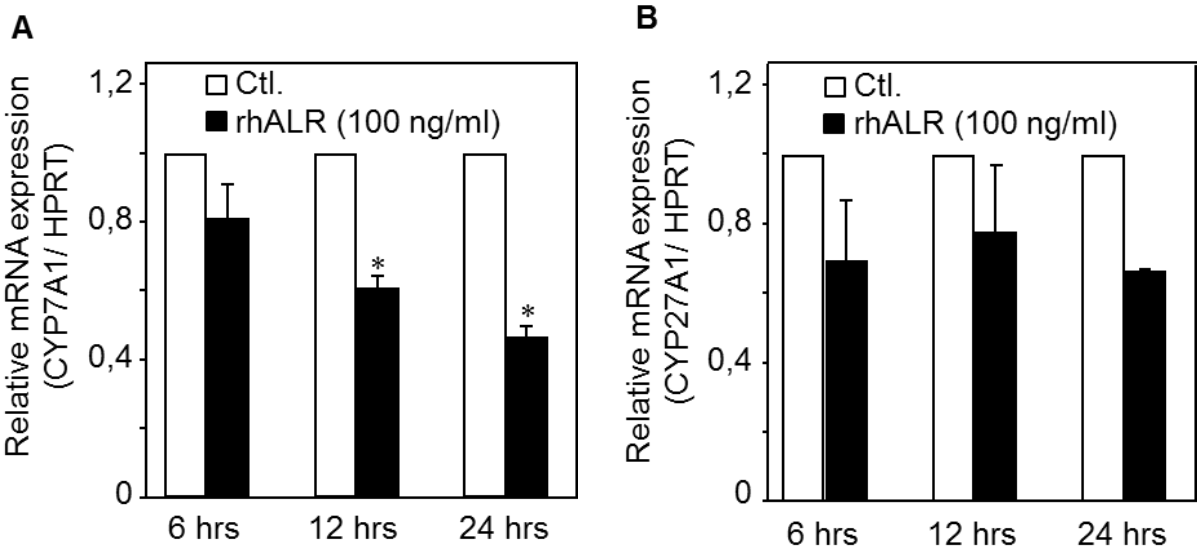

Fig. 1. Effect of exogenous rhALR on the expression of *de novo* synthesis enzymes. HepG2 cells were starved for 24 hours and then incubated with 100 ng/ml of rhALR for 6, 12 and 24 hours. mRNA levels of (**A**) CYP7A1 and (**B**) CYP27A1 were analyzed by qRT-PCR and normalized to HPRT. * p<0,05 compared to the corresponding untreated HepG2 (three independent experiments, Mean±SD).

3.2. Exogenous rhALR reduces the expression of CYP7A1 repressor, FXR

In order to elucidate which transcription factors mediate CYP7A1 repression, mRNA expression of FXR, SHP and HNF4α (hepatocyte nuclear factor 4α) was analyzed. The activation of FXR leads to inducing SHP that blocks the HNF4α-mediated activation of CYP7A1. As shown in Fig. 2A, mRNA levels of FXR were significantly reduced after 24 hours of ALR incubation. Moreover, SHP (Fig. 2B) and HNF4α (Fig. 2C) showed no change in expression. Therefore, the reduction of CYP7A1 by rhALR seems to be independent of FXR, SHP or HNF4α.

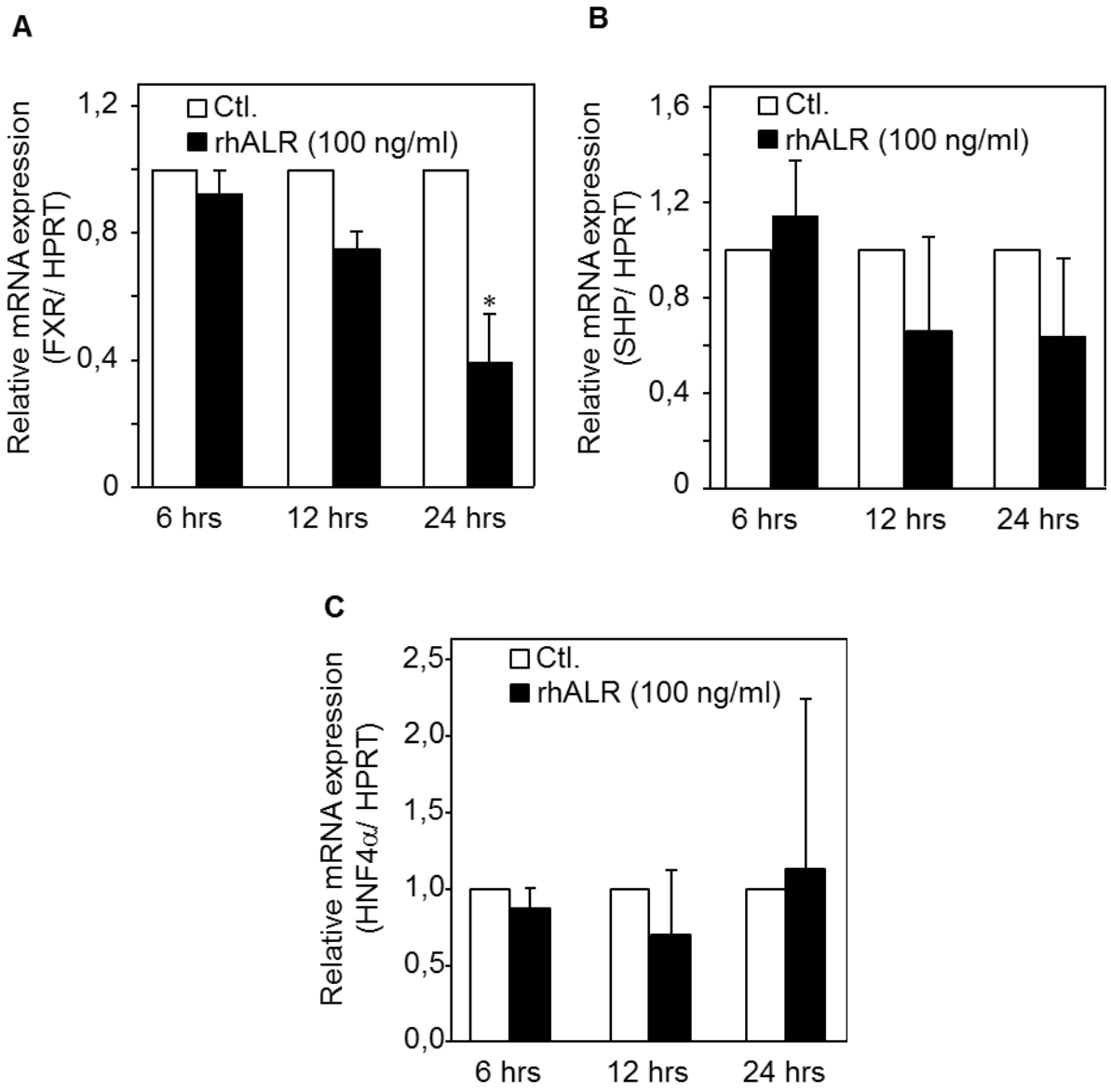

Fig. 2. rhALR reduces the expression of FXR without reducing the expression of SHP and HNF4α. HepG2 cells were incubated after 24 hours starvation with 100 ng/ml rhALR for 6, 12 and 24 hours. mRNA levels of (**A**) FXR, (**B**) SHP and (**C**) HNF4α were analyzed by qRT-PCR and normalized to HPRT. * p<0,05 compared to the corresponding untreated HepG2 (three independent experiments, Mean±SD).

3.3. Exogenous rhALR reduces the expression of CAR *via* NFκB activation

CAR activation was proved to activate bile acid synthesis and prevent the formation of cholesterol gallstones [23]. Additionally, rhALR negatively regulates the expression of CAR by activating NFκB [20]. Therefore, CAR expression was analyzed upon rhALR treatment. As shown in Fig. 3A, CAR is repressed by rhALR after 12 and 24 hours of incubation. NFκB activation and translocation into the nucleus is increased in the nuclear fractions of rhALR treated-HepG2 cells (Fig. 3B). These results indicate that reduction of CYP7A1 by rhALR may be mediated by repressing CAR expression *via* NFκB activation.

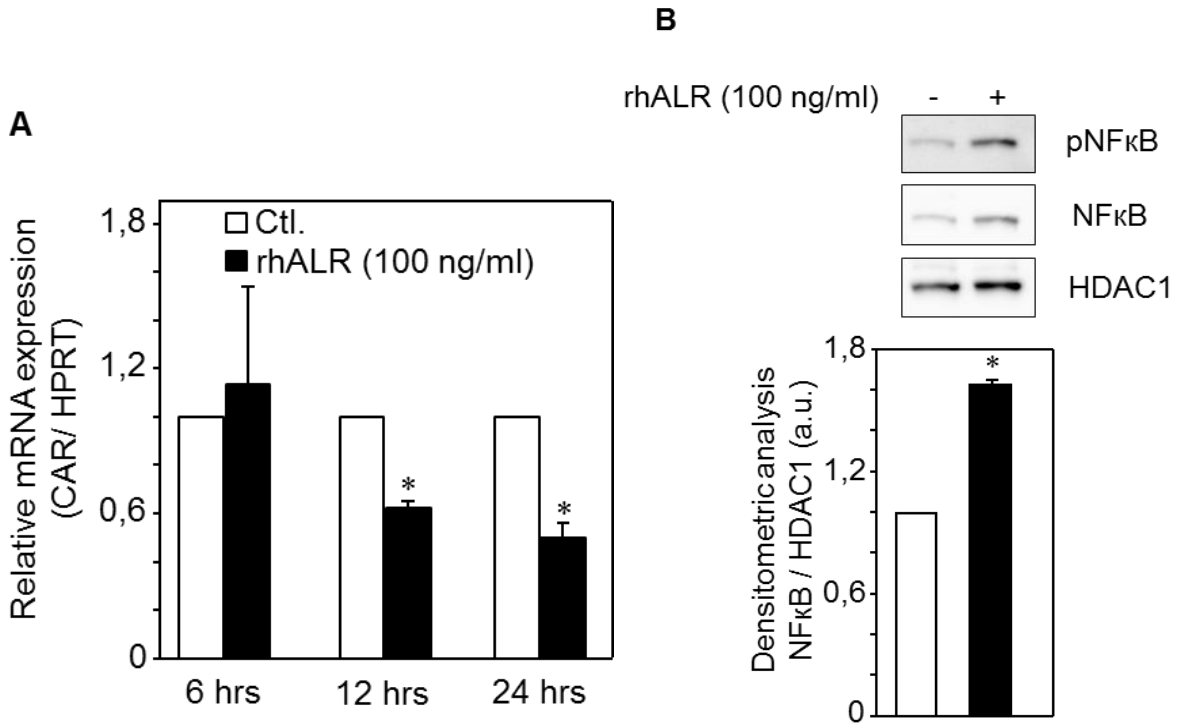

Fig. 3. rhALR reduces the expression of CYP7A1-activator CAR by activating NFκB. HepG2 cells were starved for 24 hours and then treated with 100 ng/ml rhALR for 6, 12 and 24 hours. (**A**) mRNA expression of CAR was analyzed by qRT-PCR and normalized to HPRT. * p<0,05 compared to the corresponding untreated HepG2 (three independent experiments, Mean±SD). (**B**) Nuclear extracts of HepG2 cells were prepared after 24 hours treatment with rhALR (100 ng/ml), the phosphorylation of NFκB was assessed using western blot technique. Three different experiments were analyzed by densitometric analysis and a representative immunoblot is illustrated. * p<0,05 compared to the corresponding untreated HepG2 cells.

3.4. Exogenous rhALR reduces caspase 3/7 activity upon treatment with high concentrations of toxic GCDCA independent from DR5 regulation

Several bile acids were shown to contribute to apoptosis in a death receptor-dependent mechanism or by activating intrinsic apoptotic pathways. In order to assess rhALR effect on the apoptotic signals induced by bile acids, caspase 3/7 assay was performed under conditions of cytotoxic concentrations of GCDCA and/or extrinsic apoptosis-inducer (TRAIL). As shown in Fig. 4A, rhALR treated-HepG2 cells show a decreased caspase 3/7 activity prior to any treatment. Whereas rhALR significantly reduced apoptosis caused by toxic GCDCA, it showed no efficacy in reducing extrinsic apoptosis induced by TRAIL. Furthermore, the combination of TRAIL and GCDCA induced caspase 3/7 activity in rhALR-treated HepG2 cells significantly more than their corresponding HepG2 cells without rhALR treatment. Since TRAIL-induced apoptosis is mediated by death receptor 5 (DR5), the expression of DR5 was analyzed in primary human hepatocytes and human hepatoma cells (HepG2) upon treatment with different concentrations of rhALR. Figures 4B and 4C demonstrate that DR5 expression remains unchanged upon

rhALR treatment. These data suggest the rhALR contributes to the protection of hepatocytes against bile acid-induced apoptosis in a DR5-independent mechanism.

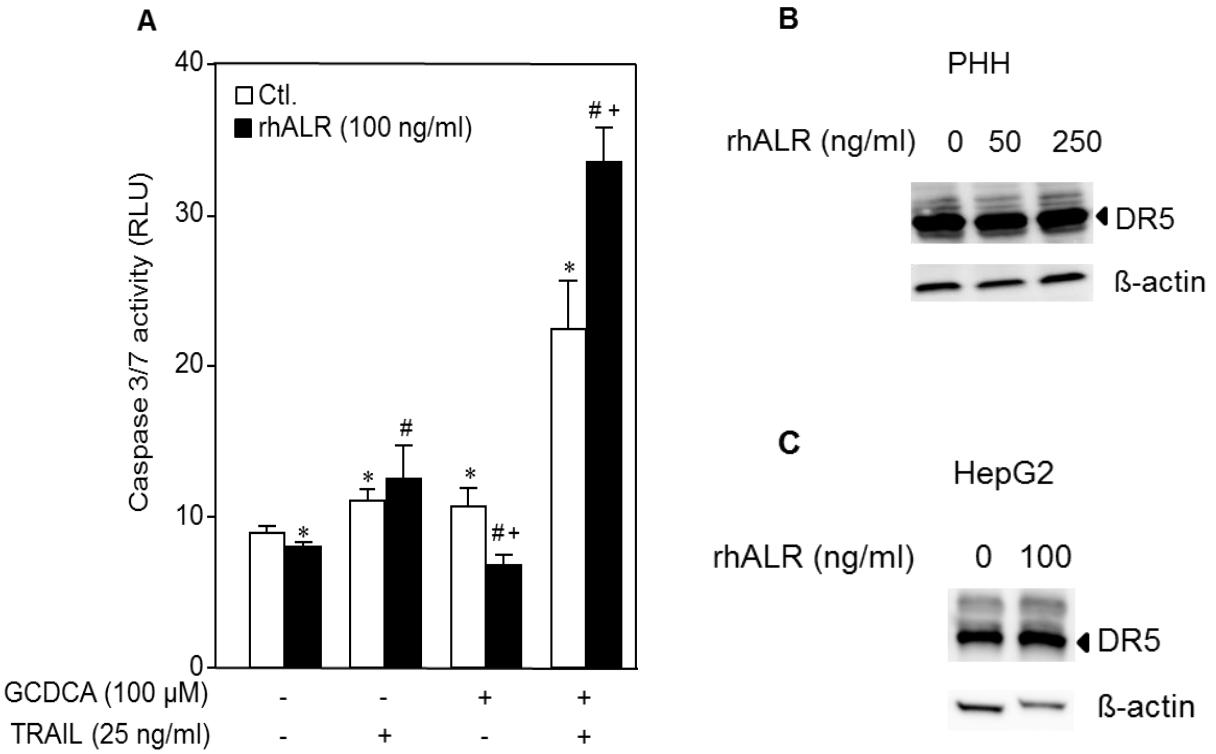

Fig. 4. Exogenous rhALR reduces caspase 3/7 activity upon treatment with high concentrations of toxic GCDCA independent from DR5 regulation. (**A**) HepG2 cells were starved for 24 hours and pre-treated with 100 ng/ml rhALR. Later 100 µM GCDCA and/or apoptosis-inducer TRAIL were added for 24 and 6 hours respectively, followed by Caspases 3/7 activity assay.* $p<0,05$ compared to untreated HepG2, # compared to rhALR treated-HepG2, + compared to corresponding HepG2 without rhALR treatment (three independent experiments, Mean±SD). (**B**) Primary human hepatocytes were starved for 24 hours and then treated with 50 or 250 ng/ml rhALR, total protein was then isolated to measure protein levels of DR5 and β-actin by western blot techniques. (**C**) HepG2 cells were starved for 24 hours and then treated with 100 ng/ml of rhALR, total protein was isolated to measure the protein levels of DR5 and β-actin by western blot techniques.

4. Discussion

Liver regeneration is a well-orchestrated process, driven by the action of several cytokines and growth factors e.g. HGF and EGF [24]. The regeneration-promoting effects of those molecules have been demonstrated and characterized using different *in vivo* and *in vitro* models [25, 26]. Among the co-mitogens is ALR which was shown to accumulate in regenerating livers and to augment liver regeneration in response to tissue loss or damage [19, 27]. Compared to other growth factors, ALR has proven to exert its actions primarily on hepatocytes and hepatoma cells [17, 28]. Furthermore, ALR has been previously shown to alter hepatic P450-metabolizing enzyme activities in a dose dependent manner [20].

Huang *et al* have demonstrated that partial hepatectomy (PH) increases bile acids flux and causes metabolic stress [29]. Two parallel mechanisms cooperate to insure the protection of hepatocytes from toxic bile acids after PH, first: FXR activates the liver regeneration and second: CYP7A1 expression is strongly repressed. The importance of CYP7A1 repression during the acute phase of liver regeneration has been further confirmed by infecting mice with a CYP7A1-expressing adenovirus followed by 70% PH. Indeed, liver regeneration was repressed in mice overexpressing CYP7A1 [9] which suggests that CYP7A1 repression is essential during early stages of liver regeneration to protect hepatocytes from cell death and damage. Furthermore, in *vitro* work of Song *et al.* [8] and Kakizaki *et al.* [30] indicated that HGF represses CYP7A1 expression in an SHP-dependent and SHP-independent pathways. Nevertheless, it is not clear whether the liver-specific growth factor ALR contributes to CYP7A1 repression upon PH. Therefore, we aimed in this study to analyze the effect of ALR on CYP7A1 expression. In this study we show that treatment of HepG2 cells with rhALR leads to reduced CYP7A1 expression. On the other hand, CYP27A1, the rate-limiting enzyme in the alternative pathway of bile acids synthesis, remained without significant change. The repression of CYP7A1 by FXR/SHP has been extensively analyzed in several studies [4, 31] therefore the expression of FXR and SHP was analyzed upon rhALR treatment. Unlike SHP, FXR expression was reduced after rhALR-treatment which suggests that ALR-mediated repression of CYP7A1 is FXR/SHP independent.

Interestingly, treatment of primary mouse hepatocytes with TNFα, a typical NFκB inducer, suppressed CYP7A1 expression while leaving FXR-target genes unchanged [32]. Moreover, rhALR was shown to modulate the hepatic P450-metabolizing enzymes activities by activating NFκB [20]. Therefore, we investigated the activation and translocation of the p65 subunit of NFκB into the nucleus after rhALR treatment and found increased p65 in the nuclear fractions of rhALR-treated HepG2 cells. In addition, CAR activation was shown to induce CYP7A1 expression and thereby reduce the formation of cholesterol gallstones [23]. Furthermore, IL6 treatment was confirmed to repress P450 genes by down-regulation of CAR and PXR, nuclear receptor family members that regulate P450 gene expression [33]. Additionally, NFκB activation by TNFα was shown to reduce the transactivation of HNF4α by downregulating its AF1 and AF2 domains [34]. Therefore, we analyzed the expression of CYP7A1 inducers: HNF4α and CAR in rhALR-treated HepG2 cells.

While HNF4α remains without significant change, CAR is significantly reduced after 12 and 24 hours of rhALR treatment. This suggests that rhALR exerts its repression on CYP7A1 by activating NFκB and reducing the transactivation of CYP7A1 by CAR. Bile acid-induced apoptosis contributes to cholestatic liver injury. Bile acids induce apoptosis in a death-receptor dependent mechanism [10, 13, 14], in particular TRAIL-R2 (or death receptor 5, DR5) and FAS receptors (FASR). Moreover, Micromolar concentrations of GCDCA induce mitochondrial release of cytochrome C by FASR-dependent and -independent pathways [35]. Mitochondrial release of cytochrome C is dependent on Bax, a pro-apoptotic protein. Rodrigues *et al.* [36] demonstrated that Bax-translocation into mitochondria initiates the mitochondrial permeability transition (MPT) and cytochrome C release during deoxycholate-induced apoptosis. Interestingly, we have shown previously that upon free fatty acid treatment, rhALR reduces apoptosis accompanied by reducing Bax protein expression. On the other hand, NFκB activation by cytokines protected rat hepatocytes against bile acid-induced apoptosis. Furthermore, NFκB inhibition by genetic or pharmacological approaches rendered Hela cells more susceptible to TNFα-induced apoptosis [37]. Interestingly, rhALR reduces cytochrome C release from mitochondria caused by ethanol treatment [21]. Therefore we investigated the ability of rhALR to reduce bile acid-induced apoptosis and found that rhALR treated cells show lower caspase 3/7 activity. In addition, treatment with toxic concentrations of GCDCA induced apoptosis but this induction was reduced upon rhALR treatment. On the other hand, TRAIL-induced apoptosis was not reduced by rhALR addition and DR5 expression was not altered by rhALR treatment. Therefore, we hypothesize that rhALR could contribute to protecting cells against bile acid-induced apoptosis by activating NFκB and reducing Bax protein levels and therefore reducing cytochrome C release without effecting the expression of DR5. In conclusion, rhALR induction upon liver injury may contribute to protecting hepatocytes from toxic bile acids by (i) reducing the synthesis of bile acids by repressing CYP7A1 expression and (ii) by reducing bile acid-induced apoptosis. Therefore, ALR protects hepatocytes from developing intra-hepatic cholestasis in acute stages of liver regeneration.

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Chapter 7

Conclusion and Summary

1. Summary

It was in 1994 when the term “Augmenter of Liver Regeneration (ALR)” was first coined to describe a protein that augments the proliferative response of the liver after partial hepatectomy in rats and dogs. Since then extensive work has been attributed to identify the expression of ALR’s different isoforms and exploring their localization-dependent functions. In the human liver, ALR is expressed in hepatocytes and cholangiocytes. Specifically, the long forms of ALR (lfALR, 23 and 21 kDa) have been identified in the cytosol and mitochondria where it acts as a disulfide relay system coupled to the oxidoreductase Mia40 for facilitating protein import into mitochondria. Additionally, the short form of ALR (sfALR, 15 kDa) was detected solely in the cytosol with some reports suggesting its release into serum upon hepatic injury. Upon release, ALR binds to its receptor and activates different signaling pathways. Depending on its localization, the dual signaling pathways of sfALR have been investigated and were found to be differential in various pathological models for various liver injuries (chapter 1).

Cholestasis represents pathophysiologic syndromes defined as an impaired bile flow from the liver due to, among others, gallstones, local tumors and the genetic deficiencies in bile acids exporters. Regardless of the cause, the accumulation of toxic bile acids leads to liver injury by initiating apoptosis, ER-stress, mitochondrial instability and inflammatory response due to activation of neutrophils and Kupffer cells. Therefore, the regulation of ALR expression by bile acids (chapter 3) and cholestasis-related cytokines (IL1β) (chapter 4) have been investigated and found that under cholestatic conditions, ALR expression is attenuated due to the activation of SHP (Small Heterodimer Partner) by bile acids (chapter 3). Furthermore, IL1β release from activated Kupffer cells also leads to reduced ALR expression that could be attributed to reduced SP1 expression and diminished Egr-1 binding to its binding site within ALR promoter and therefore eliminating its transactivation action (chapter 4).

Furthermore, due to the different etiologies of cholestasis and the possible progression into liver cirrhosis and liver failure, exploring potential therapeutic options for cholestasis might be of great clinical importance. Interestingly, high levels of cytosolic endogenous sfALR (15 kDa) protect hepatocytes from cholestatic injury by reducing bile acid synthesis as well as desensitizing hepatocytes to extrinsic-induced apoptosis by activating STAT3 (signal transducer and activator of transcription 3).

Moreover, ALR expression is reduced in patients with cholestasis due to presumably high concentrations of bile acids and inflammatory cytokines (chapter 5). In addition, application of recombinant human ALR (rhALR, 15 kDa) contributes to liver protection through reducing bile acid synthesis and intrinsic apoptosis (caused by accumulation of bile acids in hepatocytes) *via* activation of NFκB (Nuclear Factor 'kappa-light-chain-enhancer' of activated B-cells) (chapter 6).
A schematic illustration of bile acid-induced injury and the protective effects of ALR found in this thesis are depicted in Fig. 1.

2. Conclusion

ALR expression is reduced in the liver during cholestatic liver diseases. Furthermore, over-expression of sfALR (15 kDa) or application of recombinant human ALR (rhALR, 15 kDa) alleviate the detrimental effects of bile acids by reducing bile acids synthesis as well as reducing bile acid-induced apoptosis. This suggests that attenuated ALR expression in cholestasis could contribute to aggravating cholestatic injury. Moreover, it proposes the possibility of using ALR as a potential therapeutic agent for attenuating the progression of cholestatic liver injury.

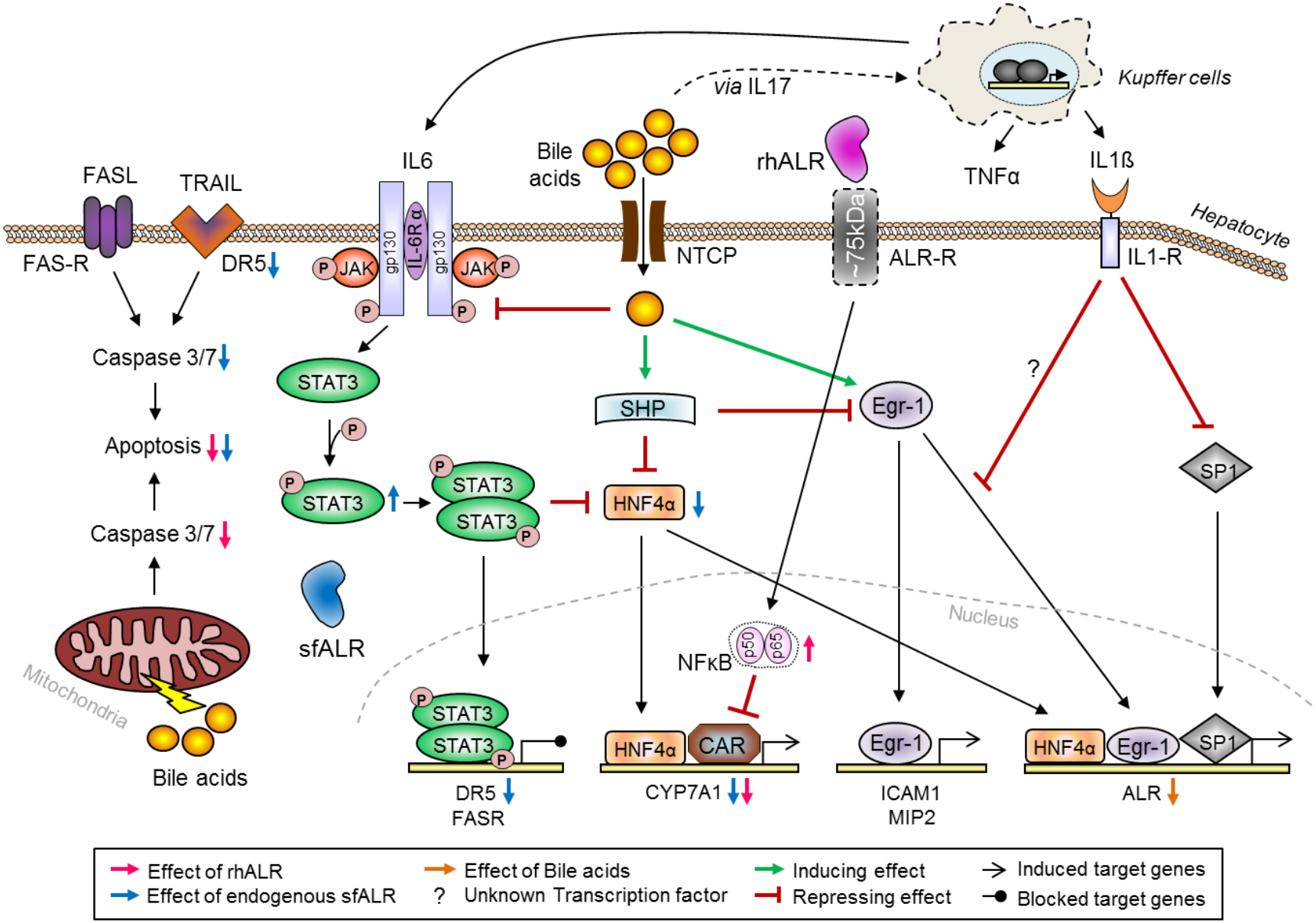

Kupffer cells
via IL17
TNFα
IL1ß
IL6
Bile acids
rhALR
FASL
TRAIL
FAS-R
DR5
JAK
gp130
IL-6R α
gp130
JAK
NTCP
~75kDa
ALR-R
IL1-R
Hepatocyte
Caspase 3/7
Apoptosis
Caspase 3/7
STAT3
STAT3
STAT3
STAT3
SHP
Egr-1
?
SP1
HNF4α
sfALR
Mitochondria
Bile acids
Nucleus
NFκB
p50
p65
STAT3
STAT3
DR5
FASR
HNF4α
CAR
CYP7A1
Egr-1
ICAM1
MIP2
HNF4α
Egr-1
SP1
ALR
Effect of rhALR
Effect of endogenous sfALR
Effect of Bile acids
? Unknown Transcription factor
Inducing effect
Repressing effect
Induced target genes
Blocked target genes

Fig. 1. Schematic illustration for bile acid-induced injury and the contribution of ALR in attenuating this injury. Bile acids accumulate during cholestasis and are imported into hepatocytes by NTCP (Na+-taurocholate co-transporting polypeptide). Bile acids de-activate IL6 signaling pathway by reducing gp130 phosphorylation and the subsequent STAT3 (signal transducer and activator of transcription 3) activation. This results in activation of DR5 (death receptor 5) and FASR (FAS receptor) expression and increases the number of death receptors on cell surface. Therefore, the cells are rendered more susceptible to external death ligands like TRAIL (TNF-Related Apoptosis Inducing Ligand) and FASL (FAS ligand) that bind to their receptors (DR5 and FASR respectively) and cause apoptosis. Furthermore, bile acids activate SHP which blocks the trans-activation activity of HNF4α and the expression of its target genes. Moreover, bile acids activate Egr-1 and induce the expression of its target genes ICAM1 (Intercellular Adhesion Molecule 1) and MIP2 (Macrophage Inflammatory Protein 2) that initiate inflammation. In addition, bile acids indirectly activate Kupffer cells (by IL17 release from activated T Helper cells). IL17 activates Kupffer cells and induces the expression and release of pro-inflammatory cytokines like IL1β, IL6 and TNFα. In **chapter 3** we show that bile acids reduce the expression of ALR (hepato-protective factor) (orange arrow) by (i) reduced HNF4α (blocked by SHP) and (ii) reduced binding of Egr-1 to its activating binding site within ALR promoter. Moreover, we demonstrate the IL1β (released from Kupffer cells) (i) reduces SP1 expression (an activator of ALR expression) and (ii) reduces the binding of Egr-1 to ALR promoter which results in reduced ALR expression (**chapter 4**). In addition, the potential protective effects of endogenous short form ALR (sfALR) are investigated in **chapter 5** (blue arrows). Endogenous sfALR reduces DR5 expression *via* STAT3 activation, and thereby reduces caspase 3/7 activity and apoptosis under cholestatic conditions. Endogenous sfALR might also reduce bile acids accumulation by reducing HNF4α expression and thereby the expression of CYP7A1 (the rate-limiting enzyme of bile acids synthesis) and therefore the bile acid pool. Furthermore, recombinant human ALR (rhALR) (pink arrows) binds to its receptor and activates the phosphorylation and nuclear translocation of NFκB which therefore reduces the expression of CAR and reduces the expression of CYP7A1. Moreover, rhALR reduces the caspase 3/7 activity and apoptosis initiated by cellular accumulation of bile acids and mitochondrial instability without affecting the expression of DR5 (**chapter 6**).

Appendix

Abbreviations

µ	Micro (10^{-6})
Aa	Amino acid
ACLF	Acute on chronic liver failure
ADH1	Alcohol dehydrogenase 1
AIF	Apoptosis inducing factor
AKI	Acute kidney injury
ALD	Alcoholic liver diseases
ALDH1	Aldehyde dehydrogenase 1
ALR	Augmenter of Liver Regeneration
ALR-R	ALR receptor
APP	Acute phase proteins
APR	Aucte phase responce
ARE	Anti-oxidant response element
BA	Bile acids
BARE	Bile acid response element
BDL	Bile duct ligation
BNIPL	Bcl-2/adenovirus E1B
Bp	Base pair
BSEP	Bile salt export pump
C/EBPβ	CCAAT/enhancer binding protein β
CA	Cholic acid
CAR	Constitutive androstane receptor
CCC	Cholangiocellular carcinoma
CCl_4	Carbon tetrachloride
CDCA	Chenodeoxycholic acid
CDH1	E-Cadherin
cDNA	Complementary DNA
CHOP	C/EBP-homologous protein
CNS	COP9 signalosome
CPT1α	Carnitine palmitoyltransferase 1alpha
CYP2E1	Cytochrome P450 2E1
CYP7A1	Cytochrome P450 7A1
CYP27A1	Cytochrome P450 27A1
DCA	Deoxycholic acid
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
dnEgr-1	Dominant negative Egr-1
DR	Death receptor
DTT	Dithiothreitol
e.g.	"for example"
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
Egr-1	Early growth response protein-1
ELISA	Enzyme linked immunosorbent assay
ELOVL1	Elongation of very long chain fatty acids protein 6
EMSA	Electrophoretic mobility shift assay

EMT	Epithelial mesenchymal transition
ERK	Extracellular signal regulated protein kinase
ER-stress	Endoplasmic reticulum stress
Erv1	Essential for respiration and vegetative growth 1
ESC	Embryonic stem cells
et al.	“and others”
EtOH	Ethanol
FABP1	Fatty acid binding protein 1
FAD	Flavin adenin dinucleotide
FASR	FAS receptor
FFA	Free fatty acid
FGB	Fibrinogen β
FOXA2	Forkhead Box A2
FXR	Farnesoid X Receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GDCA	Glycodeoxycholic acid
GFER	Growth factor *erv1*-like
h	Hour
H/R	Hypoxia/reoxygenation
HP	Haptoglobin
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDAC1	Histone deacetylase 1
HF	Hepatic failure
HGF	Hepatocyte growth factor
HNF4α	Hepatocyte nuclear factor 4 alpha
HPC	Hepatic progenitor cells
HPO	Hepatopoietin
HPRT	Hypoxanthin guanin phosphoribosyl transferase
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cells
HSS	Hepatic stimulator substance
HTCR	Human tissue and cell research
i.e.	“that is”
ICAM1	Intercellular adhesion molecule 1
IDD	Intrinsically disordered domain
IHC	Immunohistochemistry
IL	Interleukin
IM	Inner membrane
IMS	Intermembrane space
in vitro	lat.: „within the glass”
in vivo	lat.: „within the living”
IRI	Ischemia/reperfusion injury
JAB1	C-Jun-activating domain binding protein 1

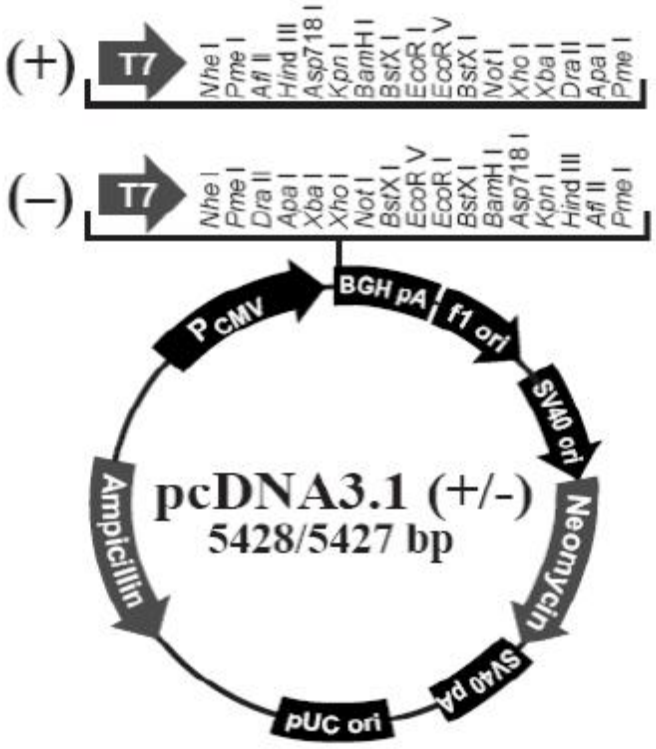
JNK	C-Jun N-terminal kinase
Kb	Kilo base
kDa	Kilo Dalton
KO	Knock-out
L	Liter
LC-MS/MS	Liquid chromatography-mass spectrometry
lfALR	Long form of Augmenter of Liver Regeneration
LPS	Lipopolysaccharide
LRH-1	Liver-related homolog-1
m	Milli (10^{-3})
M	Molar
MAPK	Mitogen-activated protein kinase
MCP1	Monocyte chemoattractant protein 1
Mia40	Mitochondrial import and assembly 40
MIF	Macrophage migration inhibitory factor
min	Minute
MIP2	Macrophage inflammatory protein 2
miRNA	micro RNA
MPT	Mitochondrial permeability transition
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
NAFLD	Non-alcoholic fatty liver diseases
NASH	Non-alcoholic steatohepatitis
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NGS	Next generation sequencing
NK	natural killer cells
Nrf2	Nuclear factor erythroid 2-related factor 2
nt	Nucleotide
NTCP	Na^+-taurocholate co-transporting polypeptide
ODC	Ornithine decarboxylase
OMM	Outer mitochondrial membrane
PBS	Phosphate-buffered saline
PCS	Portacaval shunt
PH	Partial hepatectomy
PHH	Primary human hepatocytes
PI3K/Akt	Phosphoinositide 3-kinase/AKT
PKD1	Polycystic kidney disease gene 1
pSTAT3	Phosphorylated signal transducer and activator of transcription 3
PTM	Post translational modifications
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
RE	Response element
rhALR	Recombinant human ALR
RLU	Relative luciferase unit
ROS	Reactive oxygen species
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

sfALR	Short from Augmenter of Liver Regeneration
SHP	Small Heterodimer Partner
SP1	Specificity protein 1
STAT3	Signal transducer and activator of transcription 3
t test	Student's test
tBHQ	Tertiary butylhydroquinone
TCDCA	Taurochenodeoxycholic acid
TGR5	G protein-coupled bile acid receptor
TLCA	Taurolithocholic acid
TNFα	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
TRX	Thioredoxin
TSPY	Testis-specific protein, Y-encoded
Via	lat.: „By way of"
WB	Western blot
Wt	Wild type
ZO-1	Zona occludens-1

Expression plasmids: Backbone maps

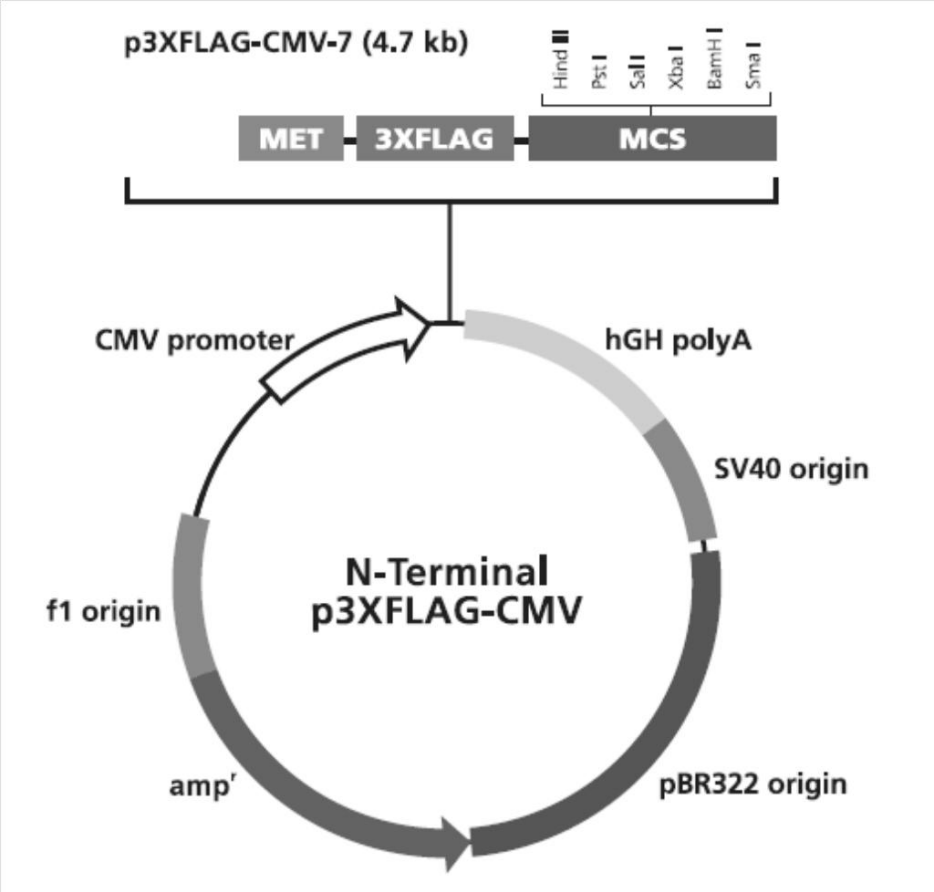
Map of pcDNA3.1 vector

The figure below summarizes the features of the pcDNA3.1 (+) and pcDNA3.1 (-) vectors. cDNA sequence of ALR, Egr-1, HNF4α1, HNF4α7 and SHP were subcloned into pcDNA3.1(+) vectors to generate overexpression vectors (chapter 3 and chapter 4).



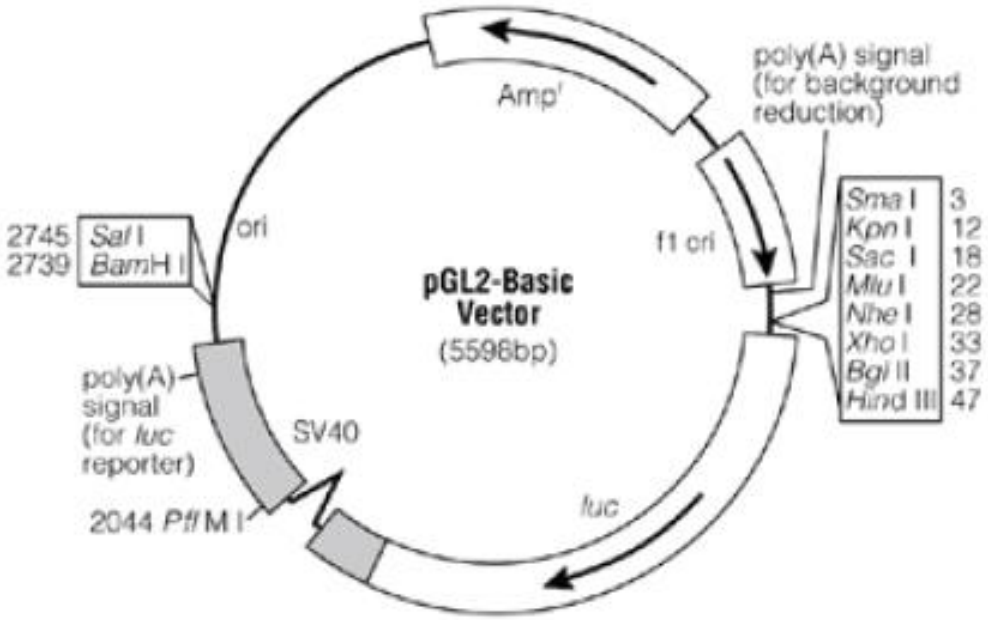
Map of p3XFLAG-CMV (Sigma Aldrich)

The figure below summarizes the features of the p3XFLAG-CMV vector. dnEgr-1 sequence was subcloned into p3XFLAG-CMV vector to generate overexpression vectors (chapter 3).



Map of pGL_2-basic vector (Promega)

The figure below summarizes the features of the pGL_2-basic vector. The sequence of truncated human ALR promoter were cloned into a pGL_2-basic vector.The promoterless pGL_2-basic vector was used to perform the Luciferase Reporter gene Assays (chapter 3 and chapter 4).



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Publications and Presentations

1- Publications

1. **Ibrahim S.**, Dayoub R., Melter M., Weiss TS. (2018): Bile acids down-regulate the expression of Augmenter of Liver Regeneration (ALR) *via* SHP/HNF4α1 and independent of Egr-1. *Experimental and Molecular Pathology.* 105(3):236-242.

2. **Ibrahim S**., Dayoub R., Krautbauer S., Liebisch G., Wege A., Melter M., Weiss TS. (2018): Bile acid-induced apoptosis and bile acid synthesis are reduced by over-expression of Augmenter of Liver Regeneration (ALR) in a STAT3-dependent mechanism. *Experimental cell Research.* DOI: 10.1016/j.yexcr.2018.11.023

3. Weiss TS., Lupke M., **Ibrahim S**., Buechler C., Lorenz J., Ruemmele P., Hofmann U., Melter M., Dayoub R. (2017): Attenuated lipotoxicity and apoptosis is linked to exogenous and endogenous Augmenter of Liver Regeneration by different pathways. *PLoS ONE*. 12(9):e0184282.

4. Dayoub R., Buerger L., **Ibrahim S**., Melter M., Weiss TS. (2017): Augmenter of Liver Regeneration (ALR) exhibits a dual signaling impact on hepatic acute-phase response. *Experimental and Molecular Pathology*. 102(3):428-433.

2- Presentations

1. Augmenter of Liver Regeneration (ALR) regulates the proinflammatory marker intracellular adhesion molecule-1 (ICAM-1, CD54) in liver cells.
Z Gastroenterol 2018; 56(01): E2-E89
DOI: 10.1055/s-0037-1612754

2. The liver regeneration associated factor ALR attenuates IL-6 induced acute-phase reactants during hepatic inflammation.
Z Gastroenterol 2016; 54(12): 1343-1404
DOI: 10.1055/s-0036-1597450

3. Activation of inflammation and cholestasis by Egr-1 is linked to liver regeneration by regulating ALR expression.
Z Gastroenterol 2016; 54(12): 1343-1404
DOI: 10.1055/s-0036-1597428

4. Egr-1 mediates inflammation and regeneration in cholestatic liver diseases
Journal of Hepatology 2016; 64: S213-424
DOI: 10.1016/S0168-8278(16)00510-9

5. Antiapoptotic and antioxidative protein ALR in Cholestatic Liver Diseases – Do bile acids regulate ALR expression *via* Egr1?
Z Gastroenterol 2015; 53 - A3_7
DOI: 10.1055/s-0035-1568027

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Geburtsdatum: 01.09.1991

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